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Programming Heart Disease: Does poor maternal nutrition alter expression of cardiac markers of proliferation, hypertrophy, and fibrosis in offspring?

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Abstract

Maternal malnutrition can affect fetal organogenesis, metabolic processes, and factors involved in developmental regulation. Of the many physiological effects poor maternal nutrition can induce in offspring, one of the most important organs affected is the heart. Cardiovascular disease has been associated with poor maternal diet. It also been suggested that hypertension can originate during impaired intrauterine growth and development. Hypertension can trigger hypertensive heart disease and is associated with numerous heart complications. We hypothesized that poor maternal nutrition would alter critical growth factors associated with normal heart development, specifically, insulin-like growth factor (IGF)-1, IGF-2, transforming growth factor (TGF)β, and connective tissue growth factor (CTGF). Ewes (n = 82) were fed control (100% of NRC), over-fed (140% of NRC), or restricted-fed (60% of NRC) diets. The left ventricles of fetuses were collected at d 90 and 135 of gestation and gene expression was analyzed by real-time PCR. Data were analyzed using a MIXED model with treatment, gender, time point, and offspring as main effects and all interactions. Statistical significance was considered at $P \leq 0.05$ and a tendency at $P > 0.05$ and ≤ 0.10. IGF-2 mRNA expression was increased in OVER singletons when compared with OVER triplets ($P = 0.04$), in CON twins when compared with CON triplets ($P = 0.038$), in RES triplets when compared with CON ($P = 0.031$) and OVER triplets ($P = 0.014$), and in RES triplets when compared with RES singletons ($P = 0.048$). TGFβ mRNA expression was increased in RES females when compared with OVER females ($P = 0.029$). CTGF mRNA expression was increased in OVER twins when compared with RES twins ($P = 0.0079$), in RES triplets when compared with CON triplets ($P = 0.017$), in RES triplets when compared with RES singletons ($P = 0.0028$) and twins ($P = 0.0009$), in RES males when compared with CON ($P = 0.05$) and OVER males ($P = 0.018$), and

was decreased in RES males when compared with RES females ($P = 0.034$). There were no effects of offspring number by treatment ($P = 0.5806$), gender by time point ($P = 0.6349$), treatment by gender ($P = 0.6233$), or offspring by time point ($P = 0.6470$) on IGF-1 mRNA expression. These results suggest that poor maternal nutrition affects expression of cardiac markers important for normal heart development in a manner specific to treatment, gender, and litter size.

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Literature Review

Introduction

In response to challenges that occur during critical developmental periods, the fetus undergoes a process known as developmental programming (Godfrey et al., 2001). Developmental programming refers to the reorganization of numerous bodily processes in offspring due to maternal stressors such as nutritional and environmental related events during pregnancy. This phenomenon often leads to a multitude of problems related to metabolism, reproductive dysfunction, body composition, and growth (Godfrey et al., 2001). The Developmental Origins of Health and Disease Hypothesis describes the process by which a stimulus or insult to a growing offspring impose predispositions to unpredicted changes in organogenesis, metabolism, and tissue development during critical developmental periods that may persist into later life (Godfrey et al., 2001). Some causes of poor maternal nutrition are due to unfavorable environmental conditions and unregulated management (Wu et al., 2006). Additionally, flushing, a management practice for enhancing reproductive performance in livestock, also disrupts an animal's normal nutritional balance (Wu et al., 2006; Schoonmaker 2004). Of the many physiological effects poor maternal nutrition can induce in offspring, one of the most important organs affected is the heart, which is responsible for pumping blood throughout the body, providing the necessary nutrients and oxygen to tissues, and assisting in the removal of metabolic wastes.

Normal heart development during gestation

During gestation, cardiac stem cells, or early cardiac progenitor cells, express cardiac-specific transcription factors such as GATA binding protein 4 (GATA-4), Homeobox protein

Nkx-2 (Csx/Nkx-2.5), and T-box 5 protein (TBX5) which mediate cardiogenesis (Cecchetto et al., 2010). These progenitors give rise to either cardiomyocytes or their supporting cells. A population of cardiac stem cells reside in the cardiac crescent (Cecchetto et al., 2010). In humans, between days 15 and 22 of gestation, progenitors residing in the cardiac crescent give rise to the atria and ventricles (Martinsen et al., 2005). Early progenitors can also originate from the second heart field (Martinsen et al., 2005), where between days 22 and 28 of gestation, these cells are responsible for giving rise to the heart's outflow tracts (Martinsen et al., 2005). Cardiac progenitors from this region are also required for chamber maturation which contribute to normal development of the right ventricle (Martinsen et al., 2005; Roche et al., 2012). Proper development of normal outflow tracts and the right ventricle for a single heart requires proper migration and proliferation of cardiac progenitors from the second heart fields (Martinsen et al., 2005). The proepicardium is another contributor to cardiac development, and this portion of the heart is responsible for giving rise to the coronary vasculature between days 22 and 28 of human development (Martinsen et al., 2005). Additionally, the proepicardium also gives rise to the epicardium, or the outer epithelial layer of the heart, and to epicardium-derived cells, which populate the myocardial wall (Schlueter1 & Brand, 2011). These epicardium-derived cells eventually differentiate into smooth muscle cells and fibroblasts (Schlueter1 & Brand, 2011). An additional cell population from the cardiac neural crest is responsible for forming the venous pole conduction system of the developing heart as well as generating cell populations responsible for cardiac function and contractility between weeks 4 to 8 of gestation in humans (Cecchetto et al., 2010; Martinsen et al., 2005). Together, this sequence of events, which involves fusion of progenitor cells, allows for the formation of the cardiac tube, looping of the tube, separation and

further fusion of cellular processes, eventually giving rise to the fully formed and functional four-chambered heart by 11th week of human pregnancy (Martinsen et al., 2005).

After initial heart development, the majority of cardiac growth occurs through proliferation and hypertrophy of cardiomyocytes (Burrell et al., 2003). Located around the periphery of immature myocytes, myofibrils increase in volume and physically inhibit complete cell division (Burrell et al., 2003). As a result, cells become binucleated as there is an absence of cytoplasmic separation. Binucleated cells are also an indication of terminally differentiated cells (Burrell et al., 2003). Burrell et al. (2003), collected right and left ventricular heart samples from a cohort of sheep to observe gestational changes in uni- and binucleated cardiomyocytes and cell volumes. Sheep are a well-documented model for studying cardiac anatomy and development as their morphometry and function is similar to that of humans (Burrell et al., 2003). Although 2% of myocytes were binucleated at d 77 of gestation, this number increased by 50% at d 135 of gestation and 90% between 4 to 6 weeks of postnatal life (Burrell et al., 2003). Before 110 days of gestation, fetal cardiac growth and development appeared to result from hyperplasia of cardiomyocytes as myocyte numbers and ventricular weight increased at the same rate (Burrell et al., 2003). After 110 days of gestation, both hypertrophy and hyperplasia were simultaneously occurring. The myocyte number per gram of ventricular weight decreased indicating an increase in cell volume rather than number, and there was a greater proportion of binucleated cells indicating proliferation of myocytes (Burrell et al., 2003). As with any cellular processes involving cell proliferation and differentiation, a vast array of interactions between organs, cytokines, growth factors and hormones play important roles in proper development of the offspring.

The fetal hypothalamic-pituitary-adrenal axis is an essential component of proper cardiac development as it has a major role in fetal response to intra-uterine stress and also plays a role in organogenesis, growth, and cardiovascular regulation (Tappia et al., 2006). Two growth factors involved in the hypothalamic-pituitary-adrenal axis are insulin-like growth factor (IGF)-1 and IGF-2 (Dong et al., 2005). Through circulating and local pericardial concentrations, these two growth factors are responsible for cardiac proliferation and hypertrophy during pre- and postnatal life (Dong et al., 2005). Both IGF-1 and IGF-2 can bind to either IGF-1 receptor (IGF-1R) or IGF-2R to stimulate cardiac growth and development (Dong et al., 2005). The expression of both growth factors (IGF-1 and IGF-2) and their receptors (IGF-1R and IGF-2R) have been observed in both the left and right ventricles as early as d 80 of gestation in sheep (Wang et al., 2012). These growth factors act through two primary intracellular signaling pathways — the phosphatidylinositol-3 kinase (PI3K)/Akt pathway and the extracellular signal-regulated kinase (ERK) pathway (Dong et al., 2005; Beyar & Landesberg, 2010; Troncoso et al., 2014).

When IGF-1 or IGF-2 binds to its tyrosine kinase receptors (IGF-1R or IGF-2R), the binding of the SH2 domain of the regulatory subunit of PI3K to the tyrosine kinase receptor activates PI3K (Dong et al., 2005; Beyar & Landesberg, 2010). Of the various classes of PI3Ks, class I PI3K, is the main effector in which isoform PI3Kα is activated by IGF-1 or IGF-2 (Dong et al., 2005; Beyar & Landesberg, 2010). PI3Kα activates secondary messengers such as phosphatidylinositol-3 4 5-trisphosphate (PIP3; Dong et al., 2005; Beyar & Landesberg, 2010). PIP3 can induce expression of protein kinase B (AKT) by further activating proteins such as mammalian target of rapamycin (mTOR) which is involved in cell proliferation and hypertrophy of the heart (Dong et al., 2005; Beyar & Landesberg, 2010).

Binding of IGF-1 or IGF-2 to its receptors (IGF-1R or IGF-2R), activates adaptor proteins such as growth factor receptor-bound protein 2 (Grb-2) which in turn phosphorylates ERK through the mitogen-activated protein kinase (MEK) axis (Troncoso et al., 2014). Phosphorylated ERK can then translocate to the cell's nucleus and initiate gene expression leading to cardiomyocyte proliferation and hypertrophy (Troncoso et al., 2014).

Additionally during cardiac development, formation of non-myocytes (fibroblasts, endothelial cells, mast cells, vascular smooth muscle cells) also occurs (Mcmullen et al., 2007). Cardiac fibroblasts, one of the other cellular components of the heart, contribute to the extracellular matrix in several specific structures of the organ, including the valves, the atrioventricular node, and the cardiac skeleton (Souders et al., 2009). Transforming growth factor (TGF)β and connective tissue growth factor (CTGF) control myocyte proliferation, extracellular matrix deposition, cardiac remodeling, myofibroblast activation, and heart contractility (Dong et al., 2005, Dobaczewski et al., 2011, Fan et al., 2012). While both TGFβ and CTGF are important growth factors for normal heart development, they are also associated with myocardial fibrosis. In the adult heart, cardiac fibrosis is triggered when pathological conditions such as disease causes pressure or volume overload of the heart by increasing wall stress on the left ventricle (Mcmullen et al., 2007). To counterbalance the increase in wall stress, the heart triggers cardiac remodeling through a pathological hypertrophic response (Souders et al., 2009). TGFβ is one of the key mediators that triggers cardiomyocyte hypertrophy as well as fibroblast hyperplasia (Bujak & Frangogiannis, 2007; Souders et al., 2009). Furthermore, increased proliferation of fibroblasts upregulates collagen and fibronectin synthesis and decreases extracellular matrix degradation through activation of protease inhibitors (Bujak & Frangogiannis, 2007; Souders et al., 2009). This results in the accumulation of excessive extracellular matrix (Bujak &

Frangogiannis, 2007; Souders et al., 2009). Ultimately, this pathological response can impair myocardial contractility and cause cardiovascular dysfunction (Bujak & Frangogiannis, 2007; Souders et al., 2009).

Three isoforms of TGFβ exist: TGFβ-1, TGFβ-2, and TGFβ-3 (Takeda & Manabe, 2011). Once TGFβ becomes activated, the growth factor binds to TGFβ-type 1 receptor (TGF-βR1) and type 2 receptor (TGF-β2R) on cardiomyocytes and non-myocytes (Takeda & Manabe, 2011). The binding of the growth factor to TGF-βR1 phosphorylates downstream intracellular intermediates such as receptor-regulated SMAD family proteins which then activate common-mediator SMAD proteins (Takeda & Manabe, 2011). These common-mediator SMAD proteins act as transcription factors. Specifically, SMAD3 is required for TGFβ to induce expression of genes required for myocardial fibrosis (Takeda & Manabe, 2011). Besides SMAD-mediated transcription, TGFβ activates SMAD-independent signaling pathways, including extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), TGF-β-activated kinase 1 (TAK1), abelson nonreceptor tyrosine kinase (c-Abl) and p38 Mitogen-activated protein kinase (p38 MAPK) pathways (Bujak & Frangogiannis, 2007).

The other growth factor involved in triggering many of the cellular processes underlying fibrosis which involves cell proliferation and synthesis of extracellular matrix is CTGF (Takeda & Manabe, 2011). This growth factor is expressed in cardiomyocytes as well as non-myocytes such as fibroblasts (Takeda & Manabe, 2011). Expression of CTGF can be induced by growth factors such as TGFβ (Takeda & Manabe, 2011). While the expression of CTGF weakly promotes fibrosis and hypertrophy of cardiomyocytes, when acting as a cofactor through induction of TGFβ, there is a more robust response (Takeda & Manabe, 2011).

While heart growth in early gestation occurs mainly through cardiomyocyte proliferation, heart growth in late gestation is predominately a result of hypertrophy (Porrello et al., 2008). Therefore, at birth, the heart contains most of the cardiomyocytes that it will have for life; thus, insults during gestation may have long lasting consequences.

Causes of poor maternal nutrition

Research in several species has revealed that alterations in maternal nutrition have long-term effects on postnatal development of offspring, which may predispose the offspring to a variety of diseases. Poor maternal nutrition is defined as inappropriate levels of nourishment to the mother, which is often passed on to the offspring in utero. Nutrient depletion, abnormally high-energy or high-fat diets, or macro- or micro-nutrient imbalance are all examples of poor maternal diet (Wu et al., 2006).

Maternal undernutrition can result from environmental or physiological extremes such as high milk output, metabolic disorders, multiple births, or continued growth of the dam (Schoonmaker 2014). Restricted feed intake during dry and winter seasons or under high environmental temperatures have been observed in pregnant dams due to poor quality of roughages or because of unfavorable temperature conditions (Wu et al., 2006).

In contrast, maternal overnutrition involves excessive feed intake, which can be defined as high-energy, high-fat, or high protein diets (Wu et al., 2006). In the food animal industry, one of the causes of maternal overnutrition is due to traditional management practices. For example, the common practice of flushing involves increasing feed intake for a short period of time around conception to maximize the numbers of oocytes for ovulation in livestock. This practice can increase the number of fetuses per animal which will increase profits (Wu et al., 2006). Additionally, management of livestock which allows extensive grazing or situations where

animals are grouped and fed based on average body weight may cause below average body weight animals to be overfed (Schoonmaker 2014).

Effects of maternal malnutrition on offspring

Maternal malnutrition can affect fetal organogenesis, metabolic processes, and factors involved in developmental regulation (Godfrey et al., 2001). For example, in a study conducted by George et al. (2010), offspring from overfed ewes had increased fetal crown-rump lengths, thoracic and abdominal girths, and fetal perirenal fat compared with control offspring at mid-gestation. Furthermore, from the same study, fetal heart, pancreas, and liver weights, as well as the lipid content of fetal liver were also increased compared with controls at mid-gestation (George et al., 2010). Insulin resistance, disruption of glucose homeostasis, and increased levels of lipid biosynthesis have all been observed in offspring of overfed mothers (Borengasser et al., 2013).

Changes in maternal diet can also predispose offspring to developing cardiovascular disease (CVD), which may be a result of compromised cardiac function and structure (Godfrey et al., 2001). The Dutch Famine is a well-documented case that established a link between maternal undernutrition and an increased predisposition to develop cardiovascular disease in later life (Godfrey et al., 2001). Some observed effects of both over- and restricted-fed models include alternations in ventricular weights, cardiac morphology, and growth and fibrotic gene expression in fetuses (Vonnahme, 2003; Dong et al., 2005; Ge et al., 2013). While CVD has been associated with poor maternal diet, it also been suggested that hypertension, or high blood pressure, can originate during impaired intrauterine growth and development, and this condition can trigger hypertensive heart disease which is associated with numerous heart complications

(Godfrey et al., 2000). Hypertensive heart disease causes heart disorders such as heart failure, thickening and enlargement of the heart, and coronary artery disease. If cellular signals contributing to cardiac hypertrophy are maintained or excessive, left ventricular hypertrophy becomes pathological leading to poor prognosis (Wang et al., 2012). Additionally, reduced growth before birth is a risk factor for left ventricular hypertrophy (Wang et al., 2012). Left ventricular hypertrophy can also induce problems related to coronary arteries as an enlarged heart can compress these vital structures which pump blood away from the heart. Other issues that may arise due to coronary heart disease and left ventricular hypertrophy include ischemic heart disease, heart failure, and arrhythmia.

Effects of maternal nutrient restriction on fetal hearts

Maternal nutrient restriction during gestation alters fetal heart size, weight, and morphology. Specifically, at d 78 of gestation, greater right and left ventricular weights per unit fetal weight were observed in offspring of ewes fed a restricted diet from d 28 to 78 of gestation (Vonnahme, 2003). This may be due to increased heart wall thickness and size, as increased ventricular size and thickness was observed at d 78 and d 135, respectively, in fetal offspring of ewes fed a restricted diet between d 28 and 78 of gestation (Dong et al., 2005). Importantly, this effect may be multigenerational. In a study by Bertram et al. (2008), male offspring obtained from the second generation of guinea pigs fed a restricted-fed diet from d 1 to d 35 exhibited increased left ventricular thickness and mass as well as elevated blood pressure. Because an increase in fetal cardiac thickness, weight, and size could be indications of a pathological response to nutritional stress, myocardial fibrosis may very likely be initiated. In fact, myocardial fibrosis occurs during maternal undernutrition as researchers confirmed presence of myocardial

fibrosis through histological staining in both left and right ventricles of adult sheep offspring from ewes fed a restricted-fed diet between d 28 to d 78 of gestation when compared with controls (Ge et al., 2013). As alternations in fetal heart weight, size, thickness as well as myocardial fibrosis have been detected, it is likely that changes in growth factor expression may be the underlying factor to these observances.

Variances in critical growth factors related to cardiac hypertrophy and proliferation and fibrosis have been observed. For instance, differences in protein expression of cardiac genes involved in heart development were observed by Dong et al., (2005). In their study, ewes were placed on a restricted fed-diet from d 28 to d 78 of gestation and some were euthanized at d 78, while the rest were placed back on a normal diet from d 79 to d 135 of gestation (Dong et al., 2005). There was increased protein expression of IGF-1R and IGF-2R in the left ventricle as well an increased protein expression of IGF-1R in the right ventricle of offspring obtained from restricted-fed ewes fed at d 78 of gestation (Dong et al., 2005). Furthermore, fetuses from ewes that were placed back on a normal diet until d 135 only had elevated IGF-2R. Additionally, in a study conducted by Dong et al. (2008), ewes placed on a restricted-fed diet from d 28 to d 78 of gestation had fetuses with lower concentrations of plasma IGF-1. As growth factors such as IGF-1 and IGF-2 and its receptors (IGF-1R and IGF-2R) are critical in proper heart development, the increased protein levels of IGF-1R and IGF-2R as well as decreased levels of plasma IGF-1 could provide an explanation to why there was increased fetal heart weight, size, and thickness in maternal restricted-fed models. Furthermore, as fibrosis may be accompanying changes to fetal cardiac morphology in models of maternal undernutrition, it is also important to examine expression of growth factors involved in myocardial fibrosis. Indeed, Menendez-Castro et al. (2011), confirmed increased mRNA expression of profibrotic marker CTGF by almost 3 fold in

aortas newborn rat pups from restricted-fed protein mothers when compared with controls. Additionally, histological staining of fetal hearts obtained from restricted-fed ewes also confirmed that maternal nutrient restriction does indeed cause myocardial fibrosis (Fan et al., 2012). Together, when growth factors responsible in extracellular matrix proteins are increased, this indicates fibrotic remodeling (Fan et al., 2012). As an increase in extracellular matrix material ultimately contributes to stiffness of the heart, this not only distorts the organizational structure of the organ, but it also affects its function by affecting ventricular contraction and relaxation (Fan et al., 2012).

Effects of maternal overnutrition on fetal hearts

Similar to the observances seen in fetal hearts of maternal undernutrition models, maternal overnutrition alters fetal heart weight and thickness. For example, fetal offspring from ewes fed an obesogenic diet from d 28 to d 78 of gestation exhibited increased heart and ventricular weights at d 78 (Dong et al., 2008). This may be due to increased heart wall thickness as increased right ventricular wall thickness was observed in fetal hearts obtained from ewes fed an overfed diet 60 days prior to mating to d 75 of gestation (Kandadi et al., 2013). As increased fetal heart weight and thickness could be indications of a pathological response to nutritional stress, myocardial fibrosis may also be initiated. Indeed, confirmation of myocardial fibrosis in fetal hearts was verified in fetuses obtained from ewes fed an over-fed diet between d 28 to d 75 of gestation as there was increased collage deposition in fetal myocardium at d 135 of gestation as evidenced through histological staining (Huang et al, 2010). As alternations in fetal heart weight and thickness as well as myocardial fibrosis have been examined, it may be likely that changes in growth factor expression is the underlying factor to these observations.

Indeed, differences in growth factor expression involved in cardiac development in maternal overnutrition models were observed by Dong et al., 2005. Their study observed increased plasma IGF-1 levels in fetal hearts obtained from ewes on an overfed diet from d 28 to d 78 of gestation (Dong et al., 2005). This change in circulating concentrations of IGF-1 may perhaps have contributed to the increase in fetal ventricular weight and thickness in previous studies examining models of maternal overnutrition. Furthermore, as modifications to fetal cardiac morphology due to maternal overnutrition can be categorized as pathological response due to nutritional stress, expression of pro-fibrotic markers such as TGFβ could be affected. Indeed, in a study by Huang et al. (2010), there was increased protein expression of TGFβ at d 75 and d 135 in fetal hearts of ewes fed an obesogenic diet from d 28 to d 75 of gestation. Furthermore, there was also an observance of increased in collagen deposition in fetal hearts obtained from the same study as a result of increased protein expression of TGFβ (Huang et al, 2010). Ultimately, like fetal hearts obtained from maternal restricted-fed models, increased collagen deposition and expression of profibrotic markers such as TGFβ indicates fibrotic remodeling which can adversely affect the heart by altering its normal structure and function (Fan et al., 2012).

Objective & Hypothesis

Alterations in maternal nutrition can have long-term effects that increase the risk of cardiovascular disease, thus, understanding changes in gene expression related to cardiomyocyte proliferation, hypertrophy, and fibrosis may improve our ability to determine appropriate interventions. My objective for this project was to determine whether poor maternal nutrition during gestation alters fetal cardiac mRNA expression of IGF-1, IGF-2, TGFβ, and CTGF at d

90 and d 135 of gestation. Based on results in previous studies, I hypothesized that poor maternal nutrition (over- or restricted-feeding) would increase mRNA expression of IGF-1, IGF-2, TGFβ, and CTGF in the fetal hearts of over and restricted-fed ewes compared with controls at d 90 and d 135 of gestation.

Materials and Methods

All procedures were approved by the University of Connecticut's Institutional Animal Care and Use Committee (Protocol # A13-059). Multiparous Western Whiteface ewes (n=99) were estrus synchronized using an intravaginal progesterone insert (CIDR; Easi-Breed CIDR Sheep Insert, Zoetis, Florham, NJ) for twelve days. Subsequently, ewes received a single intramuscular injection of $PGF_{2\alpha}$ (Lutalyse, 5 mg/mL; Zoetis; Knights et al., 2001a, b) after CIDR removal and were randomly bred to 1 of 4 genetically related Dorset rams wearing marking harnesses. Day 0 of pregnancy was recorded when a rump mark was evident. Ewes were placed into individual pens twenty days after mating. All animals transitioned to a complete pelleted feed for a duration of ten days. Confirmation of pregnancy for ewes (n=82) was detected using trans-abdominal ultrasound (Jones et al., 2015). After confirmation of pregnancy, ewes were randomly assigned to one of three diets: restricted-fed (RES: 60% of National Research Council [NRC] requirements for total digestible nutrients), over-fed (OVER; 140% of NRC requirements for total digestible nutrients), or control (CON; 100% of NRC requirements for total digestible nutrients) at d 30 ± 0.2 of gestation. Fresh water and salt licks were readily available to the animals at all times. On a weekly basis, each ewe was weighed and body condition scored by two trained handlers. Once body weight and body condition scores were recorded, rations were adjusted according to the ewe's

body weight. For each diet group, samples from the offspring were collected at d 45, 90, and 135 of gestation, and within 24 h of birth.

Ewes (n = 5 to 7 per diet group/time point) were weighed and euthanized at d 45, 90 or 135 of gestation (gestation length 145 days) with an intravenous injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ). The uterus and fetuses were removed for necropsy and tissue collection. Lambs were weighed and necropsied within 24 h of birth.

Cardiac Sample Collection and Preparation

Hearts (n = 11 to 13 per diet group/time point) were collected, measured, weighed, snap frozen in liquid nitrogen and stored at -80°C. At d 45 of gestation, the entire fetal heart was collected. For samples taken at d 90 of gestation, two samples of left ventricle and one for the right were taken from each fetus. At d 135 and birth, three samples were taken from the left and right ventricles from each fetus. For this study, RNA was isolated from the left ventricle (~100 mg) of samples collected at d 90 and d 135 using a standard phenol/chloroform extraction protocol. Genomic DNA contamination was removed using Ambion Turbo DNase (Ambion Inc., Austin, TX) and cDNA was synthesized from 1 μg purified RNA with Superscript 3 reverse transcriptase (Life Technologies, Inc., Grand Island, NY) and random hexamers.

Real-time PCR

Quantitative real-time RT-PCR was used to assess the relative abundance of IGF-1, IGF-2, TGFβ, and CTFG using primer sequences from Table 1. All real-time RT-PCR was carried out using the SYBR green fluorescence method in triplicate using an ABI Prism 7300 sequence detection system (PE Applied Biosystems/Life Technologies, Inc.). The specificity of the

products was determined by gel electrophoresis and analysis of the melting temperatures. Data are expressed as relative expression of target gene to acidic ribosomal-protein large subunit-P0 (RpP0) mRNA expression (Duffield et al. 2009; Gentili et al. 2009).

Statistical analysis

Data were analyzed using the MIXED procedure in SAS. Differences between diet groups was identified with the LSMEANS statement. Differences between treatments were deemed significant at $P \leq 0.05$ and was considered a tendency at $P > 0.05$ and ≤ 0.10.

Results

IGF-1 mRNA expression

There were no effects of offspring number by treatment ($P = 0.5806$), gender by time point ($P = 0.6349$), treatment by gender ($P = 0.6233$), or offspring by time point ($P = 0.6470$) on IGF-1 mRNA expression.

IGF-2 mRNA expression

There was an effect of offspring number by treatment ($P = 0.013$) on IGF-2 mRNA expression. IGF-2 mRNA expression was increased in OVER singletons when compared with OVER triplets (Fig 1; $P = 0.04$). There were no differences in IGF-2 mRNA expression between OVER twins and OVER triplets ($P = 0.11$) or between OVER singletons and OVER twins (Fig 1; $P = 0.37$). IGF-2 mRNA expression was significantly increased in CON twins when compared with CON triplets (Fig 1; $P = 0.038$). There was a tendency for IGF-2 mRNA expression to be increased in CON twins when compared with CON singletons ($P = 0.073$). There was no

difference in CON singletons when compared with CON triplets (Fig 1; $P = 0.51$). RES triplets had increased expression of IGF-2 when compared with CON triplets (Fig 1; $P = 0.031$) and OVER triplets (Fig 1; $P = 0.014$). There was no difference in IGF-2 mRNA expression between CON and OVER triplets (Fig 1; $P = 0.17$). IGF-2 mRNA expression was increased in RES triplets when compared with RES singletons (Fig 1; $P = 0.048$) and tended to be increased in RES triplets when compared with RES twins ($P = 0.097$). There was no difference in IGF-2 mRNA expression between RES singletons and twins (Fig 1; $P = 0.45$).

There was an effect of offspring number by time point ($P = 0.042$) on IGF-2 mRNA expression. IGF-2 mRNA expression was significantly higher in d 135 twins when compared with d 135 singletons (Fig 2; $P = 0.007$) and d 135 triplets (Fig 2; $P = 0.039$). There was no difference in IGF-2 mRNA expression between d 135 singletons and triplets (Fig 2; $P = 0.83$). There was a significant increase in IGF-2 mRNA expression in d 135 twins when compared with d 90 twins (Fig 2; $P = 0.0072$).

There were no effects of treatment by time point ($P = 0.11$), gender by time point ($P = 0.39$), or treatment by gender ($P = 0.087$) on IGF-2 mRNA expression.

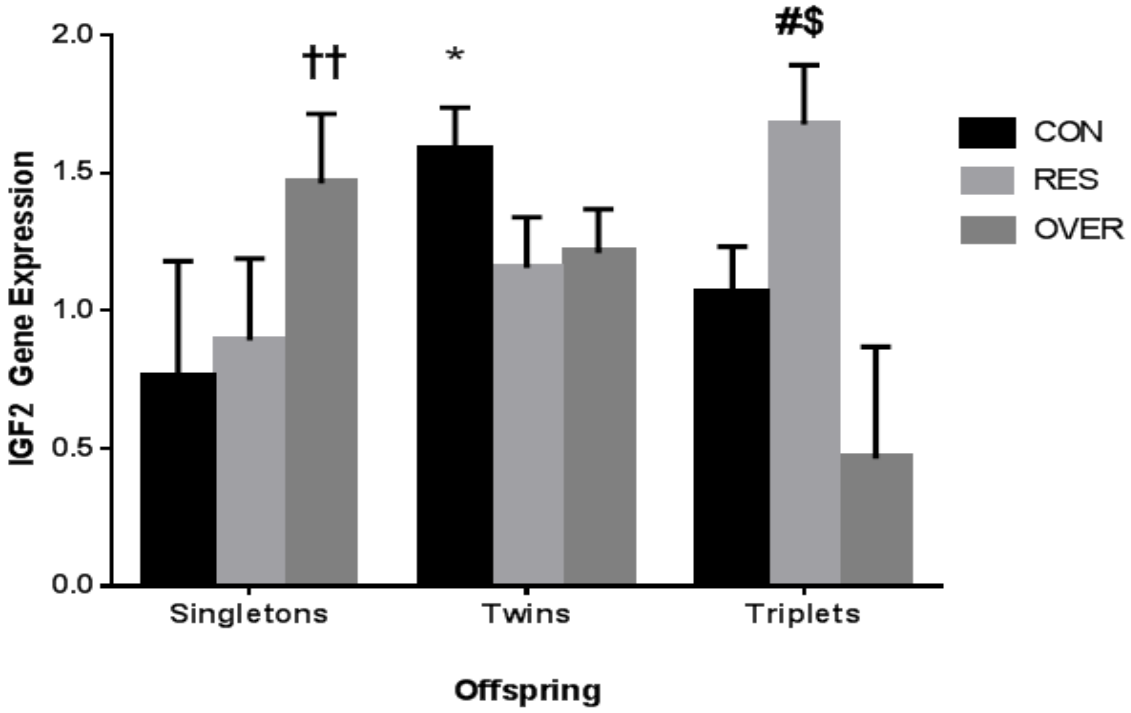

Figure 1. Effects of maternal nutrition over- and undernutrition during mid-to-late gestation on fetal cardiac IGF-2 mRNA expression in singletons, twins, and triplets. IGF-2 expression was increased in OVER singletons when compared with OVER triplets, in CON twins when compared with CON triplets, in RES triplets when compared with CON and OVER triplets, and in RES triplets when compared with RES singletons. ††$P \leq 0.05$ compared with OVER triplets. *$P \leq 0.05$ compared with CON triplets. #$P \leq 0.05$ compared with CON and OVER triplets. \$$P \leq 0.05$ compared with RES singletons.

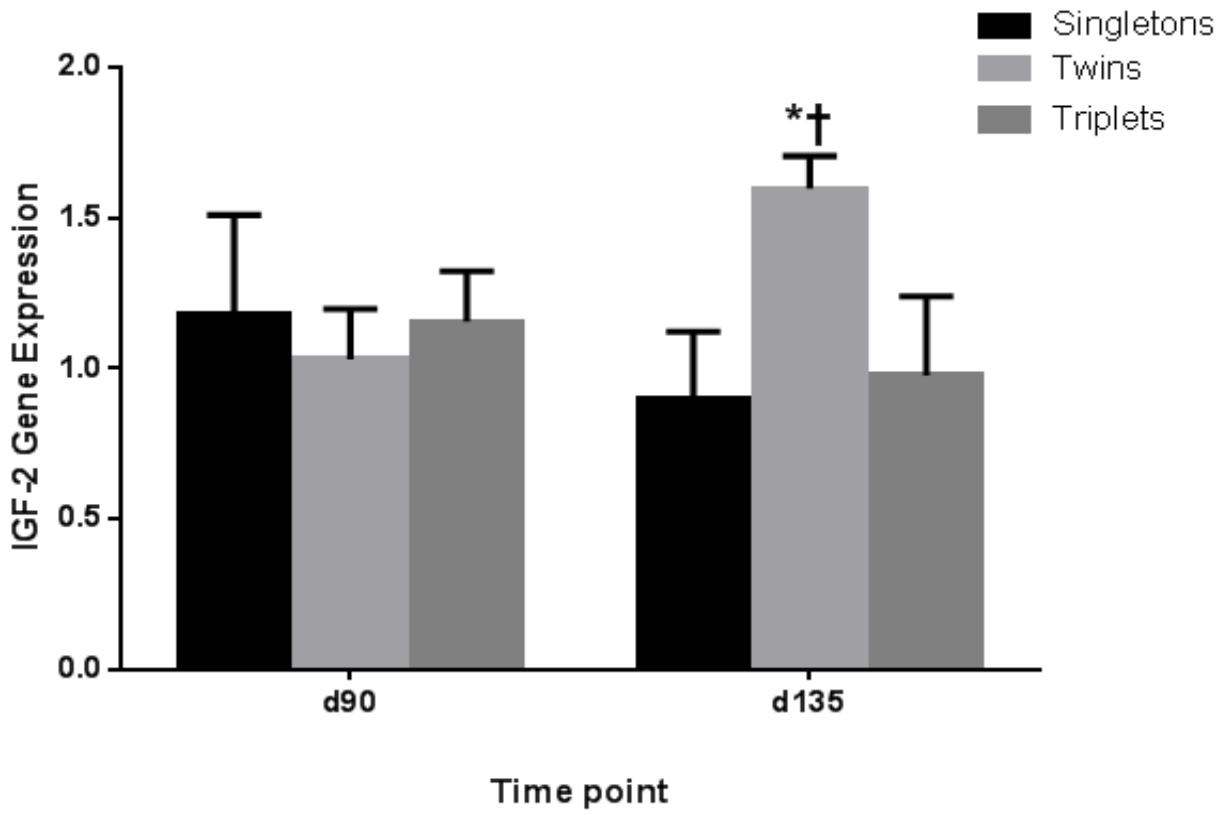

Figure 2. Fetal cardiac IGF-2 mRNA expression at days 90 and 135 of gestation in singletons, twins, and triplets. IGF-2 expression was increased in d 135 twins when compared with d 135 singletons and triplets and also when compared with d 90 twins. *$P \leq 0.05$ compared with singletons and triplets within the same time point. †$P \leq 0.05$ compared with d 90 twins.

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TGFβ mRNA expression

There was an effect of treatment by gender ($P = 0.046$) on TGFβ mRNA expression. Transforming growth factor β mRNA expression was increased in RES females when compared with OVER females (Fig 3; $P = 0.029$). There were no differences in TGFβ mRNA expression between CON and RES females (Fig 3; $P = 0.51$) or between CON and OVER females (Fig 3; $P = 0.12$), but tended to be increased in RES females when compared with RES males ($P = 0.071$).

There was an effect of offspring number by time point ($P = 0.032$) on TGFβ mRNA expression. TGFβ mRNA expression was increased in d 135 twins when compared with d 135 singletons (Fig 4; $P = 0.02$) and tended to be increased in d 135 twins when compared with triplets ($P = 0.064$). There was no difference in TGFβ mRNA expression between d 135 singletons and triplets (Fig 4; $P = 0.91$). There was a significant increase in TGFβ mRNA expression in d 135 twins compared with d 90 twins (Fig 4; $P < 0.0001$).

There were no effects of offspring number by treatment ($P = 0.24$), treatment by time point ($P = 0.38$), and gender by time point ($P = 0.86$) on TGFβ mRNA expression.

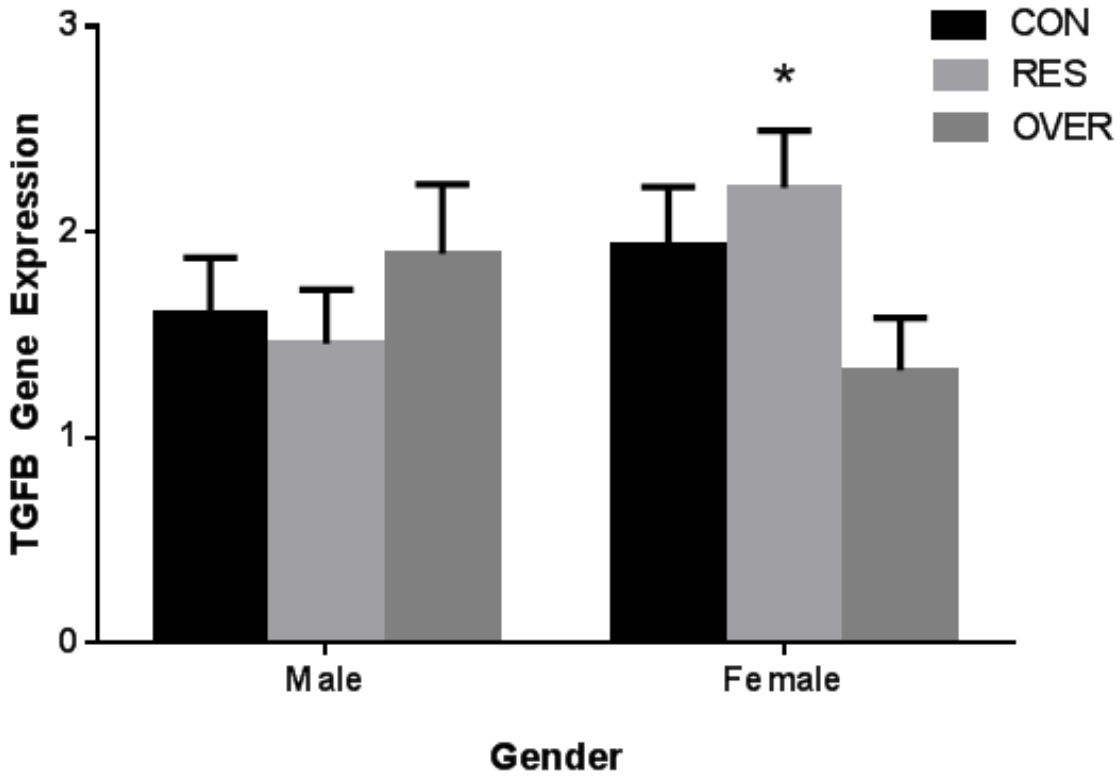

Figure 3. Effects of maternal nutrition over- and undernutrition during mid-to-late gestation on fetal cardiac TGFβ mRNA expression. TGFβ mRNA expression was increased in RES females when compared with OVER females. *$P \leq 0.05$ compared with OVER females.

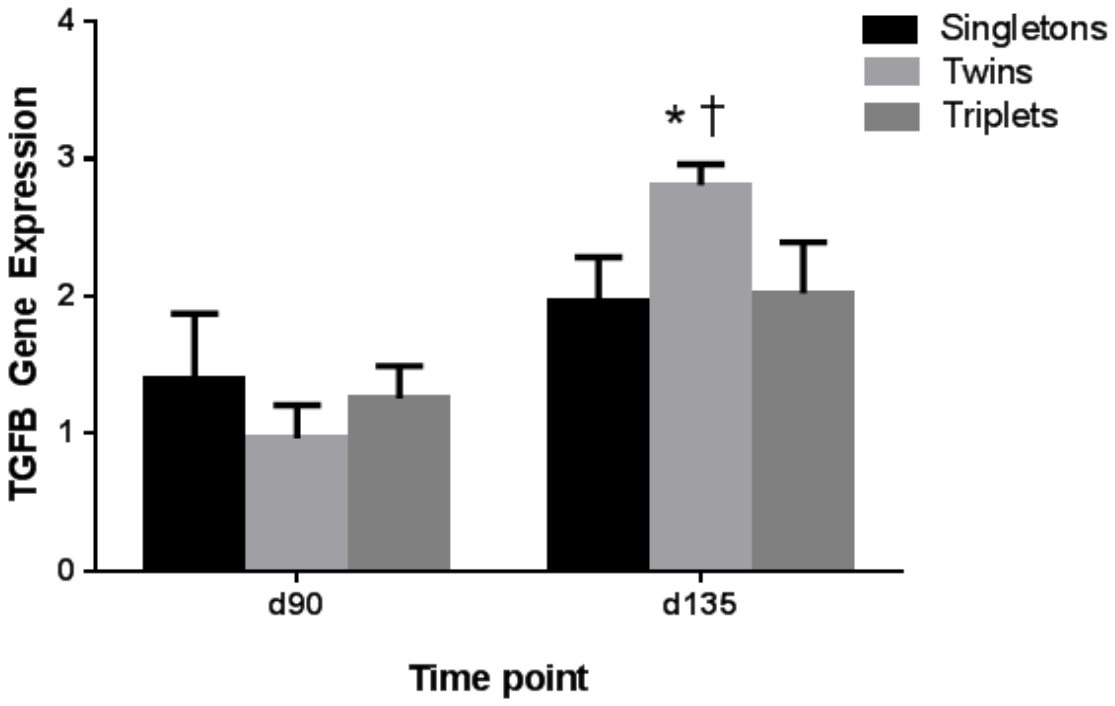

Figure 4. Fetal cardiac TGFβ mRNA expression at d 90 and d 135 of gestation in singletons, twins, and triplets. TGFβ expression was increased in d 135 twins when compared with d 135 singletons and d 90 twins. *$P \leq 0.05$ compared with d 135 singletons. †$P \leq 0.05$ was considered significant when compared with d 90 twins.

CTGF mRNA expression

There was an effect of offspring number by treatment ($P = 0.034$) on CTGF mRNA expression. CTGF mRNA expression was significantly increased in OVER twins when compared with RES twins (Fig 5; $P = 0.0079$), but only tended to be decreased in RES twins when compared with CON twins (Fig 5; $P = 0.085$). There was no difference in CTGF mRNA expression between CON and OVER twins (Fig 5; $P = 0.30$). There was an increased CTGF expression in RES triplets when compared with CON triplets (Fig 5; $P = 0.017$), but only tended to be increased in RES triplets when compared with OVER triplets ($P = 0.079$). There was no difference in CTGF mRNA expression between CON and OVER triplets (Fig 5; $P = 0.95$). CTGF mRNA expression was increased in RES triplets when compared with RES singletons (Fig 5; $P = 0.0028$) and twins (Fig 5; $P = 0.0009$), but there was no difference in CTGF mRNA expression in RES singletons when compared with RES twins (Fig 5; $P = 0.87$).

There was an effect of treatment by gender ($P = 0.032$) on CTGF mRNA expression. CTGF mRNA expression was decreased in RES males when compared with CON males (Fig 6; $P = 0.05$) and OVER males (Fig 6; $P = 0.018$), but there was no difference in CTGF mRNA expression between CON and OVER males (Fig 6; $P = 0.49$). There was an increase in CTGF expression in RES females when compared with RES males (Fig 6; $P = 0.034$).

There were no effects of offspring number by time point ($P = 0.65$), treatment by time point ($P = 0.46$), and gender by time point ($P = 0.13$) on CTGF mRNA expression.

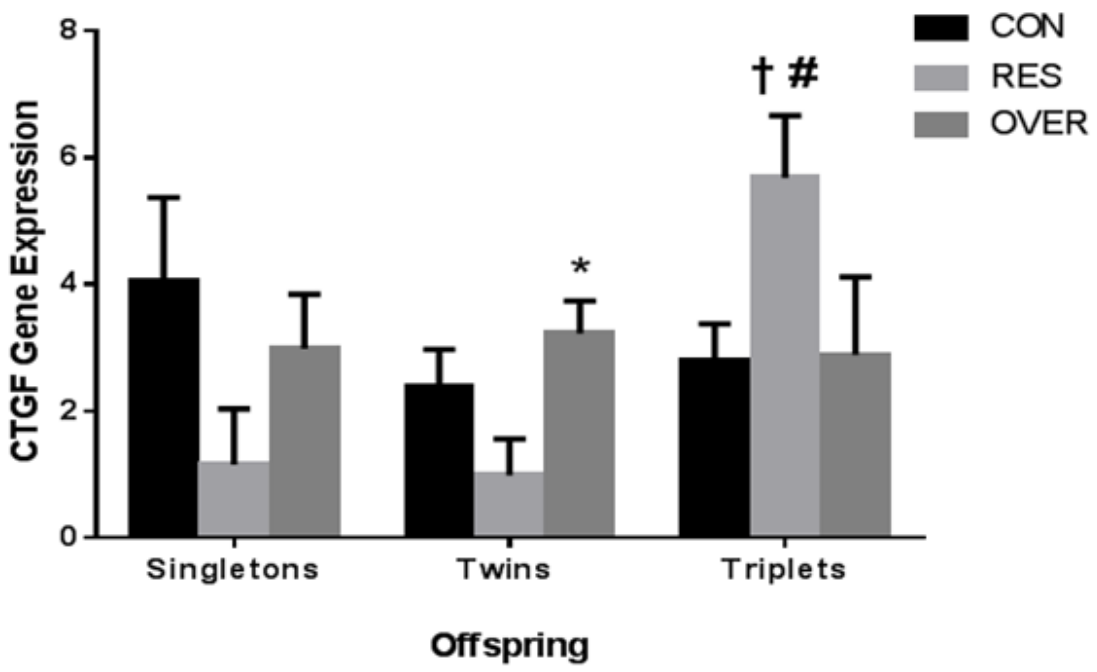

Figure 5. Effects of maternal nutrition over- and undernutrition during mid-to-late gestation on fetal cardiac CTGF mRNA expression in singletons, twins, and triplets. CTGF expression was increased in OVER twins when compared with RES twins, in RES triplets when compared with CON triplets, and in RES triplets when compared with RES singletons and twins. *$P < 0.05$ compared with RES twins. †$P < 0.05$ compared with CON triplets. #$P < 0.05$ compared with RES singletons and twins.

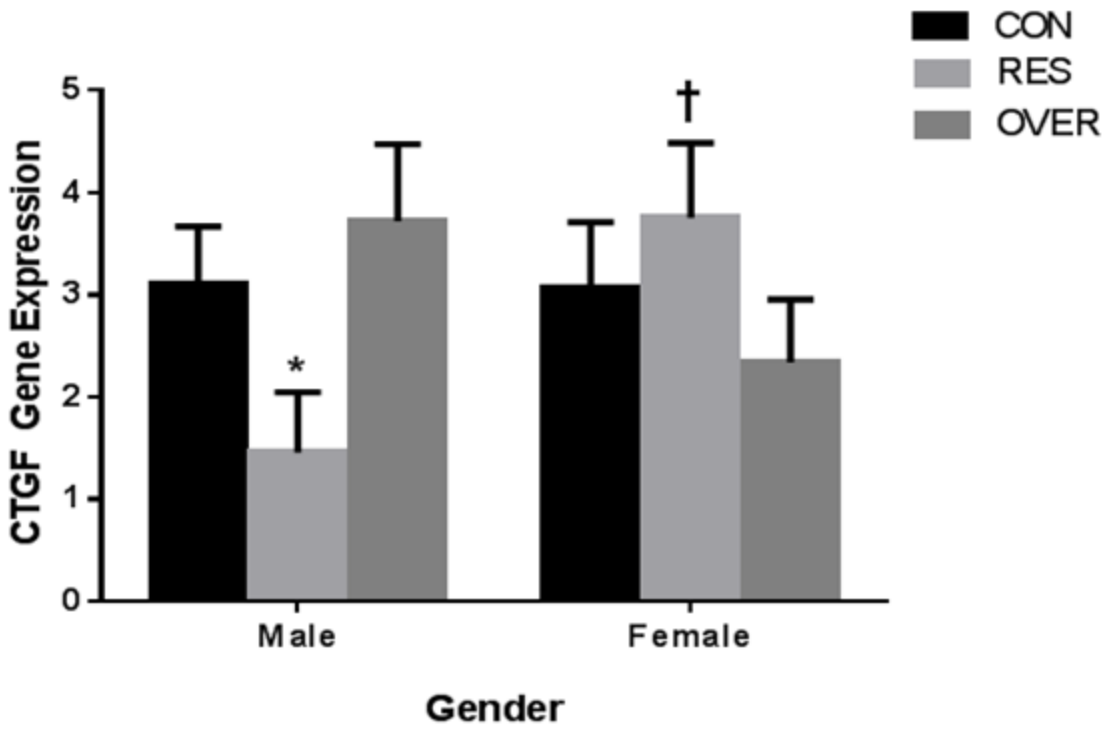

Figure 6: Effects of maternal nutrition over- and undernutrition during mid-to-late gestation on fetal cardiac mRNA expression for CTGF relative to RPLP0. CTGF expression was decreased in RES males when compared with CON and OVER males while its expression was increased in RES females when compared with RES males. $^{*}P \leq 0.05$ was considered significant when compared with males. $^{\dagger}P \leq 0.05$ was considered significant when compared with RES males.

Discussion

The aim of this study was to elucidate possible mechanisms behind the effects of poor maternal nutrition on fetal heart development. Fetal cardiac mRNA expression of IGF-1, IGF-2, TGFβ, and CTGF at d 90 and d 135 of gestation was therefore examined as they are important growth factors during normal and pathological heart development. Results from our experiment indicated that aside from IGF-1 mRNA expression, poor maternal nutrition affects expression of IGF-2, TGFβ, and CTGF in a manner specific to treatment, gender, and litter size.

Changes in IGF-2 & CTGF mRNA expression due to maternal dietary treatment and offspring number

We detected a difference in IGF-2 mRNA expression among singletons, twins, and triplets depending on maternal dietary treatment. IGF-2 mRNA expression was increased in CON twins when compared with CON triplets. It is likely that IGF-2 mRNA expression was increased in singletons when compared with triplets as the latter tend to have lower birth weights and size when compared with smaller sized litters (Yokoyama et al., 2011). Smaller fetal size and lower birth weight may lead to reduced heart formation due to a decrease of growth factors responsible in cardiac proliferation and hypertrophy, one being IGF-2 (Dong et al., 2005). IGF-2 mRNA expression was also increased in OVER singletons when compared with OVER triplets and in RES triplets when compared with RES singletons. This indicates that maternal overnutrition increases IGF-2 mRNA expression in smaller litters when compared with larger litters, while maternal undernutrition increases IGF-2 mRNA expression in larger litters when compared with smaller litters. An explanation for this phenomenon could be that maternal overnutrition may be providing sufficient or excessive fetal cardiac growth in smaller litters as

opposed to larger litters by maintaining or increasing secretions of growth factors such as IGF-2. Maternal undernutrition may be increasing IGF-2 mRNA expression in larger litters as this will ensure adequate cardiac growth to maximize the chances of all of the offspring's' survival (Dong et al., 2005). In a similar study by Lie et al. (2013), there was no change in an abundance of key factors involved in cardiac proliferation and hypertrophy such as protein kinase C alpha (PKCα), ERK or mTOR in singletons obtained from restricted-fed ewes, but there was an increase in cardiac abundance of IGF-2R and PKCα in twins. These results along with our findings indicate that increasing growth factors important for cardiac development such as IGF-2 may be an important modification to protect heart growth in larger litters when there is a predicted decrease in fetal nutrition. Furthermore, we also observed an increase in IGF-2 mRNA expression in RES triplets when compared with CON and OVER triplets. Maternal undernutrition rather than maternal overnutrition induces maternal stress during pregnancy as confirmed by Amdi et al., (2013). Their study indicated that gilts fed a restricted-fed diet at d 25 to d 90 of gestation had higher cortisol levels when compared with control and over-fed gilts (Amdi et al., 2013). Additionally, it is likely that maternal undernutrition affects fetal development by altering fetal cardiac morphology. This has been confirmed by Dong et al. (2008), who observed increased heart and ventricular weights in fetuses obtained from restricted-fed ewes.

Unlike IGF-2, our results for IGF-1 mRNA expression indicated that there were no effects of poor maternal nutrition on fetal hearts in a manner specific to treatment, gender, or litter size. While production of IGF-2 is essential for normal embryonic growth, IGF-1 is considered to be more important for postnatal growth and development (Griffeth et al., 2014). This may explain why there were no differences in IGF-1 mRNA expression in our study. Future

research evaluating other critical genes involved in embryonic development for the fetal heart should thus be examined.

For CTGF, OVER twins had increased CTGF mRNA expression when compared with RES twins. In a similar study by Huang et al. (2010), myocardial fibrosis in fetal hearts was verified in fetuses obtained from ewes fed an over-fed diet between d 28 to d 75 of gestation as there was increased collage concentrations in fetal myocardium at d 135 of gestation. As myocardial fibrosis is induced by an increase in growth factors which are responsible for extracellular matrix deposition, Huang et al., (2010) and our findings indicate that fibrotic remodeling in fetuses of overfed ewes may have been caused by an increase in expression of profibrotic markers such as CTGF (Fan et al., 2012). Future research examining whether increased CTGF mRNA expression due to maternal overnutrition elevates an individual's risk to CVD in later life should therefore be evaluated. Additionally in our study, there was increased CTGF expression in RES triplets when compared with CON triplets and also when compared with RES singletons and twins. Since there were no significant differences between RES singletons and CON singletons or between RES twins and CON twins, these results indicate that maternal undernutrition in triplets may be inducing increased expression of growth factors such as CTGF which is responsible for cardiomyocyte and fibroblast proliferation.

Changes in TGFβ & CTGF mRNA expression due to maternal dietary treatment and gender

Female offspring of restricted-fed ewes had increased TGFβ mRNA expression when compared with OVER females, but was no significant difference in RES males when compared with OVER males. This indicates that females from restricted-fed mothers are more inclined to secrete profibrotic growth factors such as TGFβ than those from overfed mothers. Similar to our

experiment, in a study conducted by Ge et al., (2013), researchers confirmed presence of myocardial fibrosis in both left and right ventricles of adult sheep offspring obtained from ewes fed a restricted-fed diet between d 28 to d 78 of gestation. These results along with our findings indicate that maternal undernutrition induces fibrotic processes in fetal hearts in a gender specific manner possibly through activation and increased mRNA expression of TGFβ. It would be interesting to examine if increased TGFβ mRNA expression in females from malnourished mothers would increase the likelihood of women developing CVD in later life.

We also identified an effect of maternal dietary treatment in a gender specific manner on CTGF mRNA expression. CTGF mRNA expression was decreased in RES males compared with CON and OVER males. Additionally, CTGF mRNA expression was increased in RES females when compared with RES males. Because CTGF is involved in both normal and pathological development of the heart, excessive or insufficient amounts may alter cardiac morphology and function (Dong et al., 2005, Dobaczewski et al., 2011, Fan et al., 2012). Firstly, our results indicate that males from restricted-fed mothers may be less prone to activating pathways involved in preserving the heart than those from overfed mothers. Secondly, it may be that females from nutrient restricted gestational conditions are far more inclined than males exposed to the same in-utero condition to secrete greater amounts of growth factors such as CTGF to preserve the heart. This may also be a fibrotic response as a result of maternal dietary stress. Maternal undernutrition induces myocardial fibrosis Ge et al., (2013) so it is possible that profibrotic growth factors such as CTGF may be increased in a gender specific manner as a result of maternal nutrient restriction. It would be interesting to examine whether the decrease in CTGF mRNA expression in males or the increase in its expression in females from malnourished mothers would make individuals more susceptible to CVD in later life.

Changes in IGF-2 & TGFβ mRNA expression due to offspring number and time point

In addition to the effects of maternal nutrition on offspring, there were interactions of offspring number by time point for IGF-2 and TGFβ mRNA expression. IGF-2 expression was increased in d 135 twins when compared with d 135 singletons and triplets and also when compared with d 90 twins. Similarly, TGFβ mRNA expression was increased in d 135 twins when compared with d 135 singletons and d 90 twins. Both IGF-2 and TGFβ mRNA expression was most likely increased in d 135 twins when compared with d 90 twins as it is during later stages of gestation where cardiac growth factors important for normal heart development. During this time, IGF-2 and TGFβ are upregulated to support the differentiation of cardiomyocytes towards the later end of gestation (Burrell et al., 2003; Dong et al., 2005, Dobaczewski et al., 2011, Fan et al., 2012).

IGF-2 and TGFβ mRNA expression may have been increased in fetal hearts of d 135 twins when compared with d 135 singletons as a mechanism to ensure guaranteed survival of offspring from larger litters. As nutrients have to be allocated to more than one organism for twins, fetal programming of the heart for twins may occur to maximize their survival. Increasing growth factors important for cardiac development such as IGF-2 and TGFβ may therefore result in adequate cardiac growth. Increased expression of IGF-2 and TGFβ in fetal hearts however, could also alter cardiac morphometry as excessive amounts of both of these growth factors could result in abnormal thickening and excessive collagen deposition of the heart (Bujak & Frangogiannis, 2007; Souders et al., 2009). Ultimately, this is detrimental as unwarranted extracellular material and thickening of the heart can impair myocardial contractility and cause cardiovascular dysfunction (Bujak & Frangogiannis, 2007; Souders et al., 2009). Furthermore, IGF-2 mRNA expression was also increased in d 135 twins when compared

with triplets. One might assume d 135 triplets to display similar results in IGF-2 mRNA expression to d 135 twins as both groups are multiples. As we only have a limited number of triplet litters, this could have contributed to unpredicted results. It would be interesting to examine if the increase in IGF-2 mRNA expression of offspring from larger litters would result in an enhanced vulnerability to CVD in later life.

Conclusion

In this study, left ventricular samples of fetuses were collected at d 90 and 135 of gestation, and gene expression was analyzed by real-time PCR. Fetal cardiac mRNA expression of IGF-1, IGF-2, TGFβ, and CTGF were quantified to study the effects of poor maternal nutrition. There were no observed effects of poor maternal nutrition on time point, offspring, or gender for IGF-1 mRNA expression. These results indicate that IGF-1 mRNA expression is not critical in terms of fetal cardiac development. Furthermore, the results of the experiment also indicate that maternal malnutrition affects mRNA expression of IGF-2, TGFβ, and CTGF in a manner specific to gender and litter size. Differences observed in mRNA expression of IGF-2 and TGFβ is also dependent on a time point by offspring number interaction. Alternations of IGF-2, TGFβ, and CTGF mRNA expression in offspring may modify cardiac morphometry and function thereby predisposing them to CVD in later life. Future studies examining this claim should thus be extensively investigated.

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