Am J Physiol Regul Integr Comp Physiol 302: R1101–R1110, 2012.
First published March 7, 2012; doi:10.1152/ajpregu.00030.2012.

Normal lactational environment restores cardiomyocyte number after uteroplacental insufficiency: implications for the preterm neonate

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Submitted 20 January 2012; accepted in final form 5 March 2012

Black MJ, Siebel AL, Gezmish O, Moritz KM, Wlodek ME. Normal lactational environment restores cardiomyocyte number after uteroplacental insufficiency: implications for the preterm neonate. *Am J Physiol Regul Integr Comp Physiol* 302: R1101–R1110, 2012. First published March 7, 2012; doi:10.1152/ajpregu.00030.2012.—A reduced complement of cardiomyocytes in early life can adversely affect life-long cardiac functional reserve. In the present study, using a cross-fostering approach in rats, we examined the contributions of the prenatal and postnatal environments in the programming of cardiomyocyte growth. Rat dams underwent either bilateral uterine vessel ligation (Restricted) or sham surgery (Control) on day 18 of gestation. One day after birth, Control and Restricted pups were cross-fostered onto Control (normal lactation) or Restricted (impaired lactation due to impaired mammary gland formation) mothers. In male offspring, genes involved in cardiomyocyte differentiation, proliferation, hypertrophy and apoptosis were examined at gestational day 20 and postnatal days 1 and 7 to assess effects on cardiomyocyte growth. At postnatal day 7 cardiomyocyte number was determined stereologically. Offspring were examined at age 6 mo for evidence of hypertension and pathological cardiac gene expression. There was an increase in *Igf1* and *Igf2* mRNA expression in hearts of Restricted pups at gestational day 20. At postnatal day 7, *Agtr1a* and *Agtr1b* mRNA expression as well as *Bcl2* and *Cmyc* were elevated in all hearts from offspring that were prenatally or postnatally growth restricted. There was a significant reduction (−29%) in cardiomyocyte number in the Restricted-on-Restricted group. Importantly, this deficit was prevented by optimization of postnatal nutrition (in the Restricted-on-Control group). At 6 mo, blood pressure was significantly elevated in the Restricted-on-Restricted group, but there was no difference in expression of the cardiac hypertrophy, remodeling or angiogenic genes across groups. In conclusion, the findings reveal a critical developmental window, when cardiomyocytes are still proliferating, whereby improved neonatal nutrition has the capacity to restore cardiomyocyte number to normal levels. These findings are of particular relevance to the preterm infant who is born at a time when cardiomyocytes are immature and still dividing.

intrauterine growth restriction; early life growth restriction; heart; developmental origins of health and disease; early life programming

CARDIOVASCULAR DISEASE IS the leading cause of death and disability worldwide. Over the past two decades, large epidemiological studies in many countries have linked low birth weight, due to intrauterine growth restriction, with cardiac and coronary disease in both men and women (2, 20, 34). Importantly this association is strongest when there is accelerated postnatal growth after birth (16). Intrauterine growth restriction is defined as birth weight below the 10th percentile for gestational age and results from factors that limit the intrinsic growth potential of the fetus (33). In most developed countries, being born small occurs as a result of a reduction in the delivery of nutrients and/or oxygen to the fetus due to placental insufficiency, thus restricting fetal growth. Intrauterine growth restriction is often a co-morbidity of preterm birth.

During fetal development, myocardial growth is regulated by controlled proliferation and apoptosis of cardiomyocytes (36). In the human heart, the proliferative capacity of cardiomyocytes is markedly decreased during late gestation when cardiomyocytes undergo a process of maturation and become terminally differentiated (36). In the rat heart the cardiomyocytes continue to proliferate in the early neonatal period and commence the maturation process at around postnatal day 3/day 4 when they cease proliferation and the cardiomyocytes become differentiated (sarcomeric striations in the cytoplasm) (22). From about postnatal day 4 the rat cardiomyocytes undergo a process of binucleation, whereby the majority of cardiomyocytes are binucleated by 2 wk after birth (22). Although there is some evidence in the adult heart that cardiomyocytes can divide in response to injury (4, 19), with recruitment of myocardial stem cells (18), it is generally accepted that proliferation of adult cardiomyocytes is rare (both in vivo and in vitro). Hence, the majority of cardiac growth postnatally is due to hypertrophy of existing cardiomyocytes (36) and extracellular matrix deposition (45). Given the limited proliferative potential of the cardiomyocytes after birth, a reduced complement of cardiomyocytes is likely to adversely impact on the functional capacity and adaptive capabilities of the adult heart when exposed to challenges such as hypertrophy or ischemia, thus programming for cardiac vulnerability later in life.

In experimental studies, placental insufficiency during late gestation has been shown not only to adversely affect fetal growth trajectory (leading to asymmetric growth restriction) but also promote abnormal changes to cardiac development (50–51). In rat studies the combined and differential contributions of hypoxia and undernutrition to placental insufficiency on subsequent long-term cardiovascular outcomes have been examined. It has been reported that there is structural remodeling of the aorta following fetal hypoxia and changes to cardiac morphology in cases of undernourished pregnancies (8). Importantly, in human studies, fetal growth restriction has been shown to lead to structural alterations in the myocardium both in utero (27) and in childhood (12). In addition, in sheep, where cardiomyocyte maturation closely resembles that of the human, there is decreased cell cycle activity (25) and altered maturation of cardiomyocytes in growth-restricted fetal lamb

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hearts (7, 25, 31), with the total number of cardiomyocytes in the early postnatal period being directly proportional to heart size (40). Fetal growth restriction is also associated with increased insulin-like growth factor 2 (*Igf2*), *Igf1 receptor 1* (*Igf1r*) and *Igf2r* mRNA expression which is likely involved in cardiomyocyte growth and maturation. In a maternal protein restriction model of intrauterine growth restriction in rats there is a reduction in the complement of cardiomyocytes at birth (11), followed by accelerated cardiac growth in the lactational period (24). At weaning, the number of cardiomyocytes remains directly proportional to heart size (24). Taken together, the ovine and rodent studies highlight the potential for intervention during the early postnatal lactational period, when a proportion of cardiomyocytes are still dividing, to impact on the final complement of cardiomyocytes within the heart. In the case of the preterm infant, where a high proportion of the cardiomyocytes still express an immature proliferative phenotype at birth, the postnatal nutritional environment is likely to be critically important in determining final cardiomyocyte number.

Bilateral uterine vessel ligation is a well-established model of uteroplacental insufficiency in rats (28, 30, 32, 39, 43, 46–48). We have shown using this model, that uteroplacental insufficiency leading to growth restriction of the fetus also impairs mammary development and lactation, thus reducing postnatal nutrition and growth of the offspring after birth, impacting on later life cardiovascular and metabolic health (28, 30, 38–39, 46–47). In the present study, using a cross-fostering approach in this model, we have examined the separate contributions of the prenatal and postnatal environments in the programming of cardiomyocyte number; as well, genes associated with cardiomyocyte proliferation and maturation have been examined.

Recent experimental studies have demonstrated that males born small tend to exhibit a more severe cardiovascular phenotype than females (28, 30, 37, 46–47), and this can be linked to programming of the renin-angiotensin system (29). Importantly in this regard, we have previously shown that exposure of male pups to a nutritionally restricted environment prenatally and postnatally leads to impaired postnatal growth, a nephron deficit and hypertension in adulthood (46–47); hypertension was not observed in female offspring (28, 30). Interestingly, cross-fostering a male pup from a restricted dam onto a control dam after birth, improved postnatal growth and prevented the nephron deficit and hypertension (46).

In the present study we hypothesized that male offspring born to a restricted dam and cross-fostered onto a restricted dam after birth would exhibit a significant cardiomyocyte deficit. Furthermore, we hypothesized that improving the postnatal lactational environment by cross-fostering a male growth restricted pup onto a control mother at birth would lead to catch-up cardiomyocyte hyperplasia and thus overcome the congenital cardiomyocyte deficit by restoring early postnatal nutrition and growth. To address this, we stereologically measured total cardiomyocyte number at postnatal day 7 using an optical disector/fractionator technique. Messenger RNA expression of the cardiomyocyte growth factors, *Igf1* and *Igf2*, the proliferation marker (myelocytomatosis oncogene, *Cmyc*) and the anti-apoptotic factor (*Bcl2*) were examined in hearts at gestational day 20 and postnatal day 1. Key genes associated with cardiomyocyte differentiation and maturation (GATA binding protein 4, *Gata4*; natriuretic peptide A, *Nppa*; myosin light chain 2, *Myl2* and myosin heavy chain 7, *Myh7*), proliferation (*Cmyc*) and anti-apoptosis (*Bcl2*) were examined at postnatal day 7, as well as stimuli of cardiomyocyte growth (*Igf1* and *Igf2* and angiotensin II type 1 receptor A, *Agtr1a* and *Agtr1b*). In addition, a cohort of cross-fostered males were examined at 6 mo of age for evidence of hypertension and altered cardiac expression of gene markers relating to hypertrophy (*Nppa* and *Myh7*) remodelling (collagen type 1 alpha, *Collα1;* collagen type III, *Col3;* transforming growth factor β, *Tgfβ1;* matrix metalloproteinase 2, *Mmp2* and tissue inhibitor of metalloproteinase 2, *Timp2*) and angiogenesis (vascular endothelial growth factor A, *Vegfa*).

MATERIALS AND METHODS

Animals and cross-fostering groups. All experiments were approved by The University of Melbourne Pharmacology, Physiology, Biochemistry & Molecular Biology and Bio21 Institute Animal Ethics Committee prior to commencement. Wistar Kyoto rats (9–13 wk of age) were obtained from the Australian Resource Centre (Murdoch, WA, Australia), housed with a 12-h light/dark cycle and had access to food and water ad libitum. On day 18 of gestation, pregnant rats were randomly allocated to Restricted (bilateral uterine vessel ligation) or Control (sham surgery) groups. The Restricted group underwent bilateral uterine artery and vein ligation to induce uteroplacental insufficiency and fetal growth restriction as described previously (28, 30, 32, 39, 43, 46–48). Sham surgery was performed in the same manner except that the uterine vessels were not ligated. Only male offspring were examined in this study as we have previously shown that males exhibit a more severe cardiovascular phenotype in adulthood (28, 30, 46–47). One cohort of animals were studied on gestational day 20 (Gestational Study, $n = 9$ per group). All mothers in the other experimental cohorts delivered pups naturally at term (22 days). Rats for the Postnatal Study cohort were studied on postnatal day 1 ($n = 8$ per group). Male offspring from the 2 cross-fostering cohorts were studied at postnatal day 7 ($n = 8$ per group) and at 6 mo after cross-fostering ($n = 8$–11 per group). In all cohorts, only one pup per litter (selected randomly) was included in the experimental groups. Pup body weights and litter size were recorded on postnatal day 1. In the cross-fostering studies pups from each of the 2 groups, Control and Restricted, were cross-fostered 1 day after birth onto a Control (sham surgery) or Restricted dam (uteroplacental insufficiency surgery) (28, 30, 39, 46). We have previously shown that following bilateral uterine artery ligation, rat dams exhibit impaired mammary gland formation and reduced milk production (32). All pups in the Control and Restricted groups were cross-fostered regardless of litter size. This resulted in 4 experimental groups (Pup-on-Mother): Control-on-Control, Control-on-Restricted, Restricted-on-Control and Restricted-on-Restricted ($n = 8$–11 mothers per group). For those males studied at 6 mo of age, one male per litter was studied and they were weaned at postnatal day 35, as in previous studies (28, 30, 39, 46).

Gestational and postnatal studies. On gestational day 20 and postnatal days 1 and 7 mothers and pups were anaesthetized with an intraperitoneal injection of a mixed solution containing ketamine (Parnell Laboratories, Pty., Alexandria, NSW, Australia, 50 mg/kg of body wt) and Ilium Xylazil - 20 (Troy Laboratories, Pty., Smithfield, NSW, Australia, 10 mg/kg of body wt). Fetal (gestational day 20) or pup (postnatal day 1) body and heart weights and body dimensions were measured and litter averages presented. Fetal and postnatal day 1 male pup hearts were pooled within a litter and snap frozen in liquid nitrogen and stored at −80°C. For the postnatal day 7 cross-foster cohort, body and heart weights are litter averages, and one male heart from each litter was snap frozen in liquid nitrogen and stored at

−80°C and another male heart was fixed in 10% neutral-buffered formalin for subsequent analysis of cardiomyocyte number.

Adult blood pressure. Systolic blood pressure was measured in preconditioned conscious rats at 6 mo of age by a tail-cuff method, prior to necropsy (46, 49). Rats were anesthetized with an intraperitoneal injection of a mixed solution containing ketamine (Parnell Laboratories, Pty., Alexandria, NSW, Australia, 50 mg/kg of body wt) and Ilium Xylazil - 20 (Troy Laboratories, Pty., Smithfield, NSW, Australia, 10 mg/kg of body wt). Body and heart weights were measured. The left ventricle was frozen in liquid nitrogen and stored at −80°C for subsequent gene expression analyses.

Real-time PCR analysis. Total RNA was extracted from hearts and reverse transcription and real-time polymerase chain reaction (PCR) was performed as previously described using the Rotor-Gene v6 (Corbett Research, Mortlake, Australia) (28, 30, 39, 46–48). Selected genes involved in growth, proliferation, differentiation/maturation, apoptosis and hypertrophy of cardiomyocytes were examined in hearts at gestational day 20 and postnatal day 1 (*Igf1*, *Igf2*, *Bcl2* and *Cmyc*) and postnatal day 7 (*Igf1, Igf2, Agtr1a, Agtr1b, Gata4, Nppa, Myl2* and *Myh7*). At 6 mo of age, key genes involved in cardiac hypertrophy, remodeling and angiogenesis (*Nppa, Myh7, Col*α1, *Col3, Tgf*β*1, Mmp2, Timp2* and *Vegfa*) were examined. Real-time PCR primers and TaqMan probes were designed using the real-time software by Biosearch Technologies (Biosearch Technologies, Novaro, CA). Primer and probe sequences are shown in Table 1 or have been reported previously (30, 38, 46–47). Optimal concentrations for primers and probes were 300 and 100 nM, respectively. Relative quantification of gene expression was performed by the comparative CT ($\Delta\Delta$CT) method with ribosomal 18S as the endogenous control.

Assessment of cardiac tissue volume. Fixed hearts from the 7-day-old offspring were weighed, embedded in glycolmethacrylate resin (Technovit 7100, Kulzers, Wehrhem/Ts, Germany), exhaustively sectioned at 20 μm, every 10th section collected and stained with hematoxylin (11, 24). Heart wall volume was then estimated using the Cavalieri principle (17).

Stereological estimation of the total number of cardiomyocytes. We chose to measure cardiomyocyte number at postnatal day 7, because previous studies have shown that by postnatal day 7 the cardiomyocytes have ceased proliferating in the rat heart and are differentiated; by day 12 the majority of cardiomyocytes in the rat heart are binucleated (21). Using the 20 μm glycolmethacrylate sections, we used an optical disector/fractionator approach to determine the total number of cardiomyocyte nuclei within the heart. Using an unbiased counting frame, we counted cardiomyocyte nuclei in a systematic uniform random sample of fields. The total number of cardiomyocyte nuclei within the heart was then determined by multiplying the number of cardiomyocyte nuclei counted by the reciprocal of the sampling fractions. This method for counting cardiomyocytes in rat hearts is described in detail in our previous study (11). The total number of cardiomyocytes within the heart was then calculated based on the proportion of mononuclear/binuclear cardiomyocytes (see below).

Assessment of cardiomyocyte nuclearity. To ensure that growth restriction did not affect the maturation of cardiomyocytes, the nuclearity of cardiomyocytes was assessed in hearts from Control-on-Control and Restricted-on-Restricted male offspring at postnatal day 7. Hearts from male offspring were embedded in paraffin, and 20-μm sections were cut and stained with fluorescently labeled wheat germ agglutinin-Alexa Fluor 488 conjugate (to stain the cell membranes) and DAPI (to stain nuclei). Using confocal microscopy and subsequent three-dimensional image analysis (NIS-Elements, Nikon, Japan) we examined the number of nuclei within individual cardiomyocytes (5). At least 100 cardiomyocytes (cut in longitudinal or oblique section) per heart were randomly sampled and nuclearity analysed.

Statistical analysis. For group comparisons, data were analyzed by an unpaired Student's *t*-test (gestational study and postnatal day 1 study) or one-way analysis of variance (ANOVA, postnatal day 7 and 6 mo cross-foster studies) followed by a Student-Newman-Keul's test for post-hoc comparisons (SPSS-X, SPSS Encinitas, CA). Data are presented as means ± SE and $P < 0.05$ was taken as statistically significant.

Table 1. *Real-time PCR primer and Taqman probe sequences*

Gene	Sequence (5′ to 3′)	GenBank Accession
Cmyc		
forward	CCCGACAGTCACGACGATG	NM_012603
reverse	CGAGTCGTAGTCGAGGTCATAG	
probe	TCAACGTGAGCTTCGCTAACAGGA	
Gata4		
forward	GCGGCCTCTACATGAAGCTC	NM_144730
reverse	GAGGACCTGCTGGTGTCTTAG	
probe	TTCCCAGGCCTCTTGCAATGCG	
Igf2		
forward	ACTCGGCGGGAAGCATGT	NM_031511
reverse	TTTGCCGCATCCTTGGATATG	
probe	ACATGCCCCTATTGTAAAGACAGTGAGG	
Myl2		
forward	GACCCAGATCCAGGAGTTCA	NM_001035252
reverse	GGCAGCAAACGTGTCCCTTAG	
probe	AGGAGGCCTTCACAATCATGGACCA	
Myh7		
forward	CATGCTGACAGATCGGGAGAA	NM_017240
reverse	CCCTCTTGGTGTTGACAGTCTTAC	
probe	CCAGTCCATCCTCATCACCGGA	

All primers/probes concentrations are 300 nM/100 nM, respectively. *Cmyc*, myelocytomatosis oncogene; *Gata4*, GATA binding protein 4; *Igf2*, insulin-like growth factor 2; *Myl2*, myosin light chain 2; *Myh7*, myosin heavy chain 7.

RESULTS

Litter size, body weight and dimensions. Litter size in the Restricted (bilateral uterine vessel ligation) groups was significantly lower than in the sham Controls for all cohorts (Table 2: gestational day 20, postnatal days 1 and 7 and 6 mo) as reported previously (30, 32, 39, 46, 48). There was no difference in the number of males and females born in litters from Control and Restricted dams.

Body weights of offspring in the Restricted bilateral uterine vessel ligation groups were significantly reduced (Fig. 1*A*, $P < 0.01$) compared with the sham controls on gestational day 20 and postnatal day 1. At postnatal day 7, body weights of the Restricted-on-Restricted offspring were significantly reduced compared with Control-on-Control and this persisted to 6 mo of age (Figs. 1*C* and 2*A*, $P < 0.05$). Cross-fostering Restricted offspring onto a mother with normal lactation (Restricted-on-Control) did not influence body weight at postnatal day 7 (Fig. 1*C*). By 6 mo of age, body weights in the Restricted-on-Control group were not different to Control-on-Controls (Fig. 2*A*). Cross-fostering a normally grown Control pup onto a Restricted mother did not influence growth in the immediate postnatal period (Fig. 1*C*) or long-term (Fig. 2*A*).

Absolute and relative heart growth. Absolute fetal heart weight and relative fetal heart weight at gestational day 20 were not different between groups (Fig. 1*B* and Table 2). On postnatal day 1, absolute, but not relative heart weight was significantly reduced ($P < 0.05$) in the Restricted group compared with Controls (Fig. 1*B* and Table 2). Improving postnatal nutrition (Restricted-on-Control) resulted in an absolute heart

Table 2. *Litter size and heart weight*

	Total Litter Size	Male Litter Size	Heart Weight: Body Weight Ratio
Gestational day 20			
Control	11.0 ± 0.4	5.9 ± 0.6	0.47 ± 0.04
Restricted	9.0 ± 0.6*	5.4 ± 0.4	0.53 ± 0.04
Postnatal day 1			
Control	7.7 ± 0.5	3.4 ± 0.7	0.49 ± 0.04
Restricted	5.8 ± 0.7*	3.1 ± 0.3	0.47 ± 0.04
Postnatal day 7 (pup-on-mother)			
Control-on-Control	9.3 ± 0.6^{b}	4.9 ± 0.2^{c}	0.58 ± 0.03^{a}
Control-on-Restricted	8.3 ± 0.5^{b}	3.5 ± 0.4^{b}	0.63 ± 0.02^{ab}
Restricted-on-Control	4.5 ± 0.7^{a}	2.3 ± 0.3^{a}	0.69 ± 0.02^{bc}
Restricted-on-Restricted	4.3 ± 0.4^{a}	1.9 ± 0.4^{a}	0.72 ± 0.03^{c}
Adult 6 mo (pup-on-mother)			
Control-on-Control	10.5 ± 0.4^{d}	4.1 ± 0.4^{b}	0.37 ± 0.005
Control-on-Restricted	8.6 ± 0.3^{c}	4.4 ± 0.4^{b}	0.35 ± 0.015
Restricted-on-Control	6.5 ± 0.5^{b}	3.9 ± 0.4^{b}	0.36 ± 0.010
Restricted-on-Restricted	4.6 ± 0.4^{a}	2.1 ± 0.3^{a}	0.37 ± 0.005

Data are expressed as means ± SE (n = 8–11 per group). Litter size at birth (for whole litter and for males only) and male offspring relative heart weight at *gestational day 20* and *postnatal day 1* and in the four cross-foster groups for the 7-day and 6-mo cohorts. Significant differences ($P < 0.05$) between the two groups (Control vs. Restricted) at *gestational day 20* and *postnatal day 1* are indicated by * and across the four cross-foster groups at *postnatal day 7* and 6 mo are indicated by superscript letters that differ (a, b, c, d); for example, "a" is different from "b", but not different from "ab".

weight that was not significantly different to Control-on-Controls on postnatal day 7 (Fig. 1*D*). Restricted-on-Restricted offspring at postnatal day 7 had a larger relative heart weight compared with the Control-on-Control and Control-on-Restricted groups, but was not different to the Restricted-on-Control group (Table 2). At 6 mo of age, there were no differences in absolute or relative heart weights (Fig. 2*B* and Table 2) or left ventricular weights (data not shown) across the cross-foster groups.

Blood pressure. Rats in the Restricted-on-Restricted group had significantly elevated blood pressure relative to Control-on-Controls at 5 mo of age; blood pressures were on average 16 mmHg higher than Control-on-Controls (Fig. 2*C*). The Restricted-on-Control group and Control-on-Restricted group exhibited levels of blood pressure intermediate between the two groups; blood pressures in the Restricted-on-Control group were not significantly different to the Restricted-on-Restricted group (Fig. 2*C*).

Cardiomyocyte number and nuclearity. The number of cardiomyocytes was examined at an early postnatal time point (postnatal day 7) when cardiomyocytes have stopped proliferating (22). At this time, about 75 to 80% of the cardiomyocytes were binuclear and 20 to 25% of the cardiomyocytes were mononuclear. There was no significant difference in the proportion of mononuclear and binuclear cardiomyocytes between

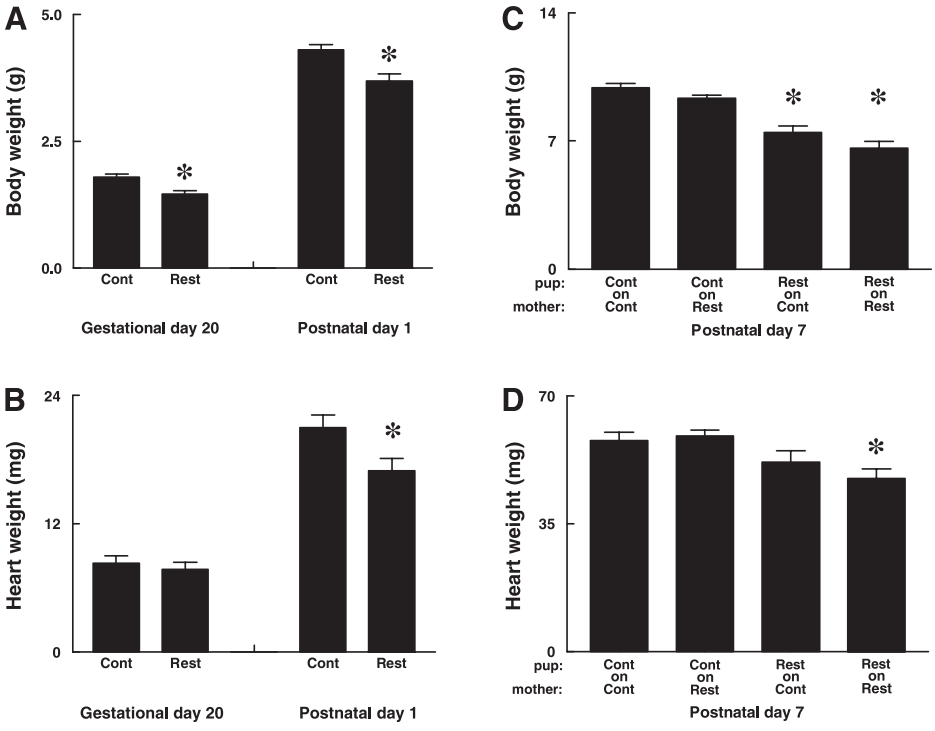

Fig. 1. Body weight (*A*, *C*) and absolute heart weight (*B*, *D*) for offspring at *gestational day 20* and *postnatal day 1* and in the four cross-foster groups for the *postnatal day 7* cohort. Significant differences (*$P < 0.05$) between the two groups (control vs. restricted) at *gestational day 20* and *postnatal day 1* are shown, and significant differences (*$P < 0.05$) across the cross-foster groups relative to control (Cont-on-Cont) at *postnatal day 7* are shown. Data are expressed as means ± SE (n = 8 or 9 per group).

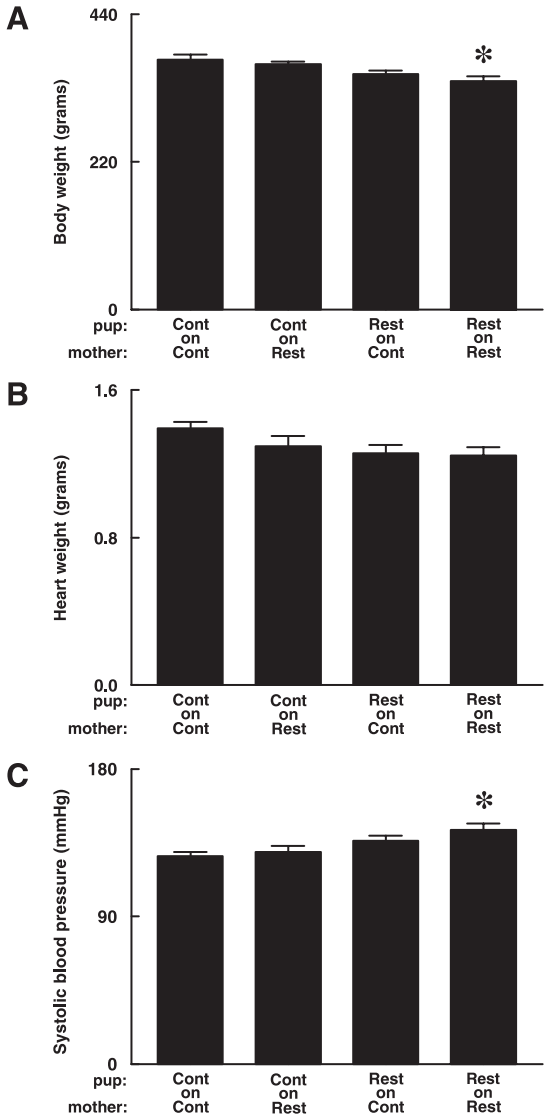

Fig. 2. Body weight (*A*) and absolute heart weight (*B*) at 6 mo of age and blood pressure at 5 mo of age (*C*) for the cross-foster groups. Significant differences (*$P < 0.05$) across the cross-foster groups relative to control (Cont-on-Cont) are shown. Data are expressed as mean ± SE (n = 8–10 per group).

groups (Control-on-Control: 76.4 ± 6.7% binucleates vs Restricted-on-Restricted: 80.4 ± 3.2% binucleates).

The male Restricted-on-Restricted offspring had significantly fewer cardiomyocytes (−29%) compared with Control-on-Controls (Fig. 3, $P < 0.05$). When restricted offspring were cross-fostered onto a mother with normal lactation (Restricted-on-Control) the total number of cardiomyocytes was 28% higher than in the Restricted-on-Restricted group and was not different to Control-on-Controls (Fig. 3). When Control offspring were cross-fostered onto dams with Restricted lactation there was no significant difference in the number of cardiomyocytes compared with Control-on-Controls (Fig. 3).

Expression of genes in early life associated with cardiac growth. At gestational day 20 there was a significant increase in *Igf1* and *Igf2* mRNA expression (Fig. 4, *A* and *B*). This was accompanied by a marked upregulation of *Bcl2* and *Cmyc* mRNA expression (Fig. 5, *A* and *B*). At postnatal day 1 there was no significant difference in the mRNA expression of any of the genes examined (Figs. 4 and 5).

At postnatal day 7 there was no significant difference in *Igf1* and *Igf2* mRNA expression in any of the cross-foster groups compared with the Control-on-Controls (Fig. 4, *C* and *D*), whereas *Agtr1a*, *Agtr1b* and *Bcl2* mRNA expression were significantly upregulated in the hearts of all cross-foster groups relative to the Control-on-Control group (Table 3 and Fig. 5*C*). Likewise, relative *Cmyc* mRNA expression was upregulated in the hearts of all cross-foster groups relative to the Control-on-Control groups, and this was statistically significant in the Control-on-Restricted and Restricted-on-Restricted groups (Fig. 5*D*). There was no difference in the mRNA expression of any of the other cardiomyocyte differentiation/maturation markers examined.

At 6 mo of age there was no significant difference in the cardiac mRNA expression of any of the cardiac hypertrophy, angiogenesis and remodelling genes that were examined, across the four cross-foster groups (Table 4).

DISCUSSION

The findings of this study clearly demonstrate that uteroplacental insufficiency followed by impaired early postnatal nutrition leads to a marked reduction in body size, absolute heart size, and the number of cardiomyocytes within the heart of offspring. Importantly, the cardiomyocyte deficit observed in the offspring with prenatal and postnatal growth restriction was prevented when postnatal lactation was optimized. Our findings show that both the prenatal in utero environment and the early postnatal lactational environment can directly influence the number of cardiomyocytes within the developing rat heart. These findings have particular clinical relevance to the immature heart of the preterm infant, whereby early postnatal nutrition is likely to directly influence the final complement of cardiomyocytes and therefore long-term functional reserve.

In this study, we showed that intrauterine growth restriction (due to uteroplacental insufficiency) followed by early postnatal growth restriction (due to impaired lactation) leads to a 28% reduction in total cardiomyocyte number. These findings are in contrast to our earlier findings in the same rat strain using a different model of prenatal and postnatal growth restriction (24). We have previously examined in WKY rats the growth

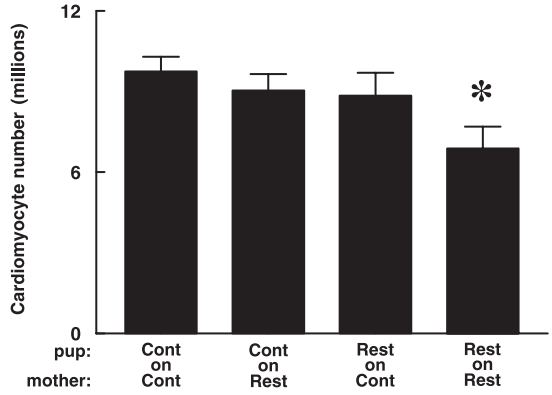

Fig. 3. Cardiomyocyte nuclei number at *postnatal day 7* in the four cross-foster groups. Significant differences (*$P < 0.05$) across the cross-foster groups relative to control (Cont-on-Cont) are shown. Data are expressed as means ± SE (n = 8 or 9 per group).

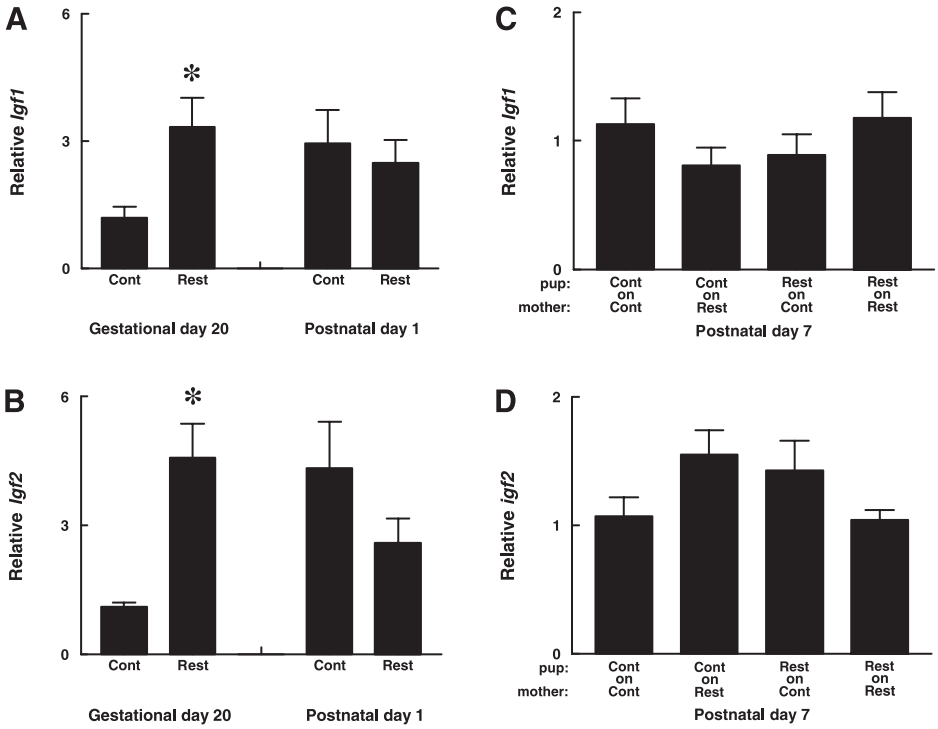

Fig. 4. Relative cardiac mRNA expression of *Igf1* (*A*, *C*) and *Igf2* (*B*, *D*) in offspring at *gestational day 20* and *postnatal day 1* and in the four cross-foster groups for the *postnatal day 7* cohort. Significant differences (*$P < 0.05$) between the two groups (Control vs. Restricted) are shown. Data are expressed as means ± SE ($n = 8$ or 9 per group).

response of the heart in growth-restricted offspring exposed to maternal protein restriction throughout both pregnancy and lactation. Interestingly, in contrast to the current findings, we previously observed accelerated growth of the heart postnatally in the maternal protein restriction model; at birth there was a congenital cardiomyocyte deficit and 18% reduction in body weight (11), and this was followed by an increase in relative heart size and apparent restoration of cardiomyocyte number at the time of weaning (24). In rats, at the time of birth all cardiomyocytes are immature and express a proliferative phenotype. In the first few days after birth (around postnatal day 3/day 4) the cardiomyocytes undergo a process of maturation/differentiation and cease dividing. In the present study we chose to examine the growth of cardiomyocytes at postnatal day 7, when the cardiomyocytes have ceased proliferating and the final complement of cardiomyocytes is formed. In this way,

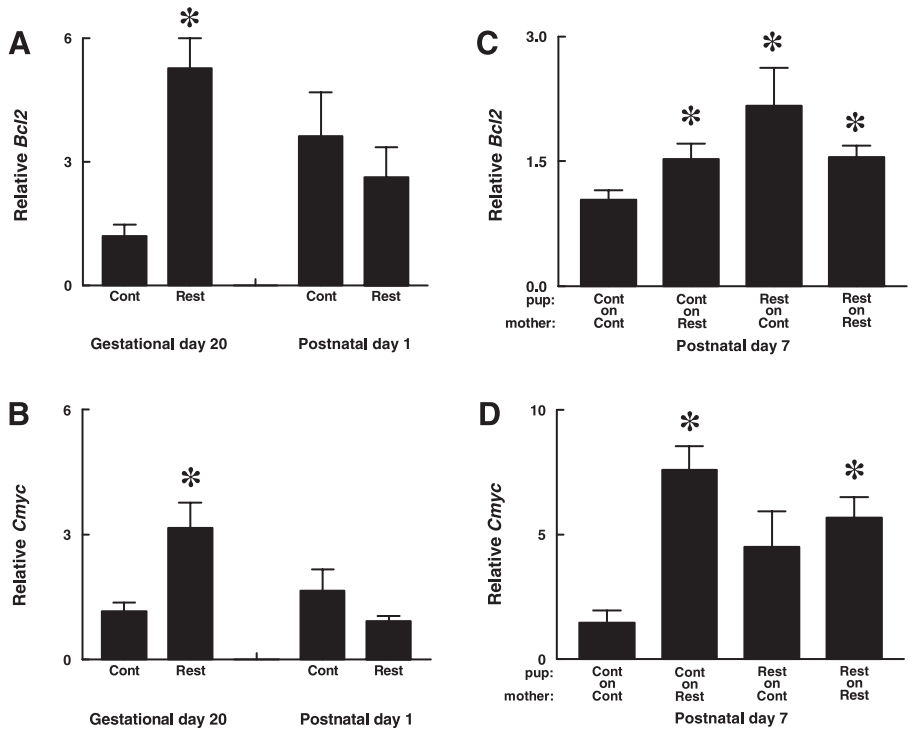

Fig. 5. Relative cardiac mRNA expression of *Bcl2* (*A*, *C*) and *Cmyc* (*B*, *D*) in offspring at *gestational day 20* and *postnatal day 1* and in the four cross-foster groups for the *postnatal day 7* cohort. Significant differences (*$P < 0.05$) between the two groups (Control vs. Restricted) are shown, and significant differences (*$P < 0.05$) across the *postnatal day 7* cross-foster groups relative to control (Cont-on-Cont) are shown. Data are expressed as means ± SE ($n = 8$ or 9 per group).

Table 3. *Cardiac gene expression at postnatal day 7*

Gene	Control-on-Control	Control-on-Restricted	Restricted-on-Control	Restricted-on-Restricted
	$n = 8$	$n = 10$	$n = 8$	$n = 10$
		Angiotensin II Receptors		
Agtr1a	1.05 ± 0.13[a]	2.30 ± 0.53[b]	1.58 ± 0.04[b]	1.73 ± 0.15[b]
Agtr1b	1.08 ± 0.17[a]	1.94 ± 0.27[b]	2.96 ± 0.63[b]	1.81 ± 0.13[b]
		Differentiation/Maturation Markers		
Gata4	1.03 ± 0.09	1.40 ± 0.23	1.61 ± 0.29	1.38 ± 0.18
Nppa	1.08 ± 0.15	1.50 ± 0.14	1.35 ± 0.30	1.32 ± 0.19
Myl2	1.07 ± 0.15	1.59 ± 0.25	1.06 ± 0.16	0.91 ± 0.07
Myh7	1.01 ± 0.06	1.15 ± 0.14	1.07 ± 0.17	0.79 ± 0.10

Data are expressed as means ± SE ($n = 8$–10 per group). Cardiac gene expression in male offspring of the four cross-foster groups in the 7-day cohort. Significant differences ($P < 0.05$) across the cross-foster groups at a given age are indicated by superscript letters that differ (a,b), for example "a" is different from "b". *Agtr1a*, angiotensin II receptor, type 1a; *Agtr1b*, angiotensin II receptor, type 1b; *Gata4*, *Nppa*, natriuretic peptide type A; *Myl2*, myosin light chain 2; *Myh7*, myosin heavy chain 7.

we could assess the effects of lactation on total cardiomyocyte number and determine whether early postnatal nutrition can alter the total complement of cardiomyocytes within the heart (which is of clear relevance to the preterm neonate). Given our present findings, the mechanisms of the cardiac growth response appear to be quite different between the uterine artery and vein ligation and maternal protein restriction models of intrauterine growth restriction. In this regard, the growth restriction model used in the current study leads to late-gestation uteroplacental insufficiency, which more closely reflects the main cause of intrauterine growth restriction in developed countries. In contrast, the maternal protein restriction model results in offspring that are not necessarily undernourished, but instead malnourished (i.e., dietary protein is substantially reduced but starch content is increased such that the diets are close to isocaloric) (24). This then results in a more severe degree of growth restriction, which may account for the differences in the cardiac growth response. Furthermore, the insult was throughout pregnancy and lactation, influencing maternal physiology and embryonic, fetal and postnatal development.

Interestingly, the cardiomyocyte deficit observed in prenatally and postnatally growth-restricted male offspring resembles the adverse effects on nephrogenesis we previously reported (46). Similar to the findings of this study, we found that Restricted-on-Restricted male offspring exhibited a significant reduction in nephron number in the kidney compared with Control-on-Controls; however, the reduction in nephron number was corrected by suckling a pup that was born small onto a control dam, thus providing a normal lactational environment and improvement in postnatal nutrition (46).

There have been many epidemiological studies demonstrating an association between low birth weight and the development of hypertension later in life (1, 13, 15). This suggests that the uteroplacental environment, in controlling fetal growth and ultimately birth weight, is an important factor in the prenatal programming of blood pressure. In support of this concept, we have consistently observed an increase in blood pressure later in life in male Restricted-on-Restricted offspring (46–47), suggesting that their elevation in blood pressure has been programmed in early life. We have previously reported a significant 16 mmHg increase in resting mean arterial blood pressure in male Restricted-on-Restricted offspring at 20 wk of age (46) and in the present study blood pressure was raised by 16 mmHg in the Restricted-on-Restricted offspring at 24 wk of age compared with the Control-on-Control offspring. To date, we have only measured blood pressure using a tail-cuff method, which has technical limitations, due to stress related with the procedure. Future studies using telemetry and echocardiography would enable validation of the degree of high

Table 4. *Cardiac gene expression at 6 mo*

Gene	Control-on-Control	Control-on-Restricted	Restricted-on-Control	Restricted-on-Restricted
	$n = 8$	$n = 10$	$n = 8$	$n = 10$
		Cardiac Hypertrophy and Angiogenesis Markers		
Nppa	1.13 ± 0.21	1.06 ± 0.21	1.35 ± 0.24	1.53 ± 0.50
Myh7	1.03 ± 0.09	0.91 ± 0.08	0.80 ± 0.11	0.70 ± 0.16
Vegfa	1.38 ± 0.35	1.92 ± 0.50	1.82 ± 0.40	1.81 ± 0.27
		Remodeling/Extracellular Matrix Genes		
Col1α1	1.26 ± 0.38	1.09 ± 0.17	0.71 ± 0.11	1.10 ± 0.22
Col3	1.06 ± 0.15	1.20 ± 0.16	1.06 ± 0.27	1.26 ± 0.28
Tgfβ1	1.05 ± 0.12	0.89 ± 0.06	0.75 ± 0.09	1.06 ± 0.22
Mmp2	1.04 ± 0.12	0.72 ± 0.04	0.76 ± 0.10	0.95 ± 0.18
Timp2	1.11 ± 0.20	0.95 ± 0.12	0.68 ± 0.14	1.13 ± 0.21

Data are expressed as means ± SE ($n = 8$–10 per group). Cardiac gene expression for male offspring in the four cross-foster groups of the 6-mo cohort. There were no significant differences across the cross-foster groups for any of the genes analyzed. *Nppa*, natriuretic peptide type A; *Myh7*, myosin heavy chain 7; *Vegfa*, vascular endothelial growth factor A; *Col1α1*, collagen, type I, alpha 1; *Col3*, collagen, type III; *Tgfβ1*, transforming growth factor, beta 1; *Mmp2*, matrix metalloproteinase 2; *Timp2*, tissue inhibitor of metalloproteinase 2.

blood pressure experienced by the growth-restricted offspring and the consequences to in vivo heart function. Additional analyses of cardiac function and cardiac structure would also help to elucidate whether a cardiomyocyte deficit in early life predisposes to cardiac dysfunction and/or adverse remodelling in adulthood.

Altered renin-angiotensin activity has been previously implicated in the pathogenesis of hypertension in growth-restricted rats (3). For instance, modulated expression patterns of Angiotensin II (Ang II) receptor genes have been documented following adverse intrauterine environments such as fetal hypoxia (50). Xue et al. (50) demonstrated the programming of increased type 2 Ang II receptor gene expression and the subsequent increased susceptibility to ischemic injury in the hearts of rats exposed to hypoxia in utero (50). Relevant to this, our study demonstrated increased expression of the renin-angiotensin receptors *Agtr1a* and *Agtr1b* in both prenatally and postnatally growth-restricted rat offspring, suggesting that the renin-angiotensin system may be upregulated in the heart as a compensatory response to suboptimal prenatal and/or postnatal environments. However, the observation that only the intrauterine growth-restricted offspring with subsequent restricted early postnatal growth progressed to develop high blood pressure suggests that impaired postnatal growth, in addition to the deficit in nephron endowment in these offspring, may be contributing factors in the development of the observed elevated blood pressures in the Restricted-on-Restricted offspring and highlights the importance of the lactational period in the programming of hypertension. In turn, the deficit in cardiomyocyte number in the Restricted-on-Restricted offspring is likely to impair the adaptive and functional capabilities of the heart when it is challenged by the induction of hypertension in adulthood. Future studies in which an additional stressor is added, such as a high-fat or high-salt diet, or indeed ageing, may allow exploration into the possible emergence of overt disease and/or pathophysiology.

In addition to its pressor actions, angiotensin II is a well-known trophic factor for cardiomyocytes, and it has been shown to stimulate hyperplasia of immature cardiomyocytes in vitro (42). Hence, in this study the *Agtr1a* and *Agtr1b* genes may have been "switched on" in an attempt to stimulate cardiac growth in offspring experiencing body growth restriction. Along with an increase in *Agtr1a* and *Agtr1b* expression in all growth-restricted hearts, there was a significant increase in the expression of the anti-apoptotic gene *Bcl2* and proliferative gene *Cmyc* in the hearts of offspring that had been prenatally and/or postnatally growth restricted. Overall, the greatest increase in *Bcl2* and *Agtr1a* gene expression was observed in the hearts of Restricted-on-Control offspring, and these may potentially be linked to the increased cardiomyocyte growth in this group compared with the Restricted-on-Restricted group. This is supported by a recent study, which showed that angiotensin II treatment of neonatal and adult rat cardiomyocytes resulted in a significant induction of Bcl2 protein expression during hypertrophy (10).

It is well established that insulin-like growth factors also play a key role in regulating cardiomyocyte growth during cardiac development (9, 26, 41), and recent studies in ovine cardiomyocytes suggest that the IGFs, in particular IGF-2, play a role in the induction of cardiac hypertrophy in offspring exposed to fetal growth restriction (44). In support of these findings, we observed a marked increase in *Igf1* and *Igf2* gene expression at gestational day 20 as a result of growth restriction, with a concomitant increase in cardiac *Cmyc* mRNA expression. There is evidence that variable expression of *Igf1* can influence expression of downstream *Cmyc* in murine skin cells, pancreatic beta-cells (35) and human preadipocytes (14). However, the increased cardiac *Bcl2* gene expression we observed at gestational day 20 is contradictory to previous literature, which showed overexpression of *Igf1* in mice can attenuate aging-induced increases in cardiac *Bcl2* gene expression (23). This discrepancy is most likely due to different mechanisms acting before and after birth. Overall, it appears that there may be compensatory mechanisms acting in the hearts of growth-restricted fetuses, with an *in utero* increase in the expression of key growth factors (*Igf1* and *Igf2*), the cell proliferative marker (*Cmyc*), and anti-apoptotic marker (*Bcl2*). Indeed, the reactive rise in *Igf1* and *Igf2* expression may account for the fact that heart weight is not different in the Restricted and Control groups at gestational day 20, even though body weight is significantly reduced. In this regard, a reactive increase in *Igf1* gene expression has been previously described in tissues undergoing restricted growth due to apoptosis (6).

Collectively, our mRNA analyses suggest that there is initiation of a heart-sparing response in the developing heart when it is exposed to a suboptimal prenatal and/or postnatal environment to enhance/preserve myocardial growth. There was upregulation of a number of key growth-promoting genes in all experimental groups that had been exposed to prenatal, postnatal or both prenatal and postnatal growth restriction, compared with Controls-on-Controls on postnatal day 7. As a follow-up to our early gene analyses, it is important in future studies to confirm that protein expression of these genes is also differentially regulated. From our findings, there is no clear evidence from the genes that we examined of particular genes being linked to the cardiomyocyte deficit in the Restricted-on-Restricted offspring. Likewise, we did not clearly identify the mediators for the restoration of cardiomyocyte number in the offspring that were growth restricted in utero, but were well nourished after birth. In this regard, we did observe the highest levels of mRNA expression for the *Agtr1b*, *Bcl2* and *Cmyc* genes in the hearts of the Restricted-on-Control group, which suggest that they may play a role at this early postnatal age.

Since cardiomyocytes lose their proliferative potential soon after birth, it was conceivable that ventricular remodelling in the form of cardiac/cardiomyocyte hypertrophy and deposition of extracellular matrix would take place as a means of long-term adaptive growth in the rats with a cardiomyocyte deficit. Hence, we predicted upregulation of a number of key genes involved in cardiac remodelling by 6 mo of age. Unexpectedly, contrary to our initial hypothesis, there was no difference in the mRNA expression of any of the remodelling genes that we quantified across all four cross-foster groups. However, before definitive conclusions can be made in relation to the long-term effects of a congenital deficit on the adaptive capabilities of the adult heart, it is imperative in follow-up studies to fully examine the spatial and temporal mRNA and protein expression within the heart as it ages, as well as comprehensively examine the biochemical structure of the heart. Certainly, we would expect that the adaptive capabilities of the hearts in the Restricted-on-Restricted group will be compromised when

the heart is challenged by the induction of hypertension. In future studies it would be of interest to compare the structural composition of the hearts in the cross-foster groups in adulthood when blood pressure has been elevated in the Restricted-on-Restricted group for a prolonged period of time and left ventricular hypertrophy has ensued.

In the present study, although there was no evidence of cardiac hypertrophy at 6 mo of age in the Restricted-on-Restricted group, it is expected that left ventricular hypertrophy will develop over the long-term with ageing in response to the increased load on the left ventricle.

Given the relevance of the present study to the preterm infant, it is encouraging that strategies have been identified for the potential restoration of cardiomyocyte number after birth; these potentially can be exploited therapeutically. Of particular clinical importance is the observation that improved postnatal nutrition can stimulate postnatal cardiomyocyte growth, such that the complement of cardiomyocytes is restored to normal in the hearts of offspring that had been growth-restricted in utero. As cardiomyocytes cease proliferation when they become terminally differentiated early in life, it is important to maximize the absolute number of cardiomyocytes in the heart before the cells irreversibly exit the cell cycle. In this regard, the findings highlight the importance of postnatal nutrition on the growth of the heart in the preterm infant and clearly demonstrate a developmental window whereby cardiomyocyte proliferation can be stimulated by improved nutrition in the early neonatal period prior to the maturational switch from proliferation to maturation; this, in turn, will directly impact on long-term cardiac health.

Perspectives and Significance

In conclusion, suboptimal prenatal and early postnatal growth is associated with a smaller heart size and a concomitant cardiomyocyte deficit, which, importantly, can be restored when postnatal nutrition is improved. As such, the present study reveals a critical window early in the postnatal period whereby the growth restricted heart has the capacity to restore cardiomyocyte number to the norm; this is particularly relevant to the immature heart of the preterm infant and may be explored therapeutically. Future studies, including a "second hit" of aging or high-fat or high-salt diets may reveal the long-term pathophysiological consequences of a cardiomyocyte deficit and/or high blood pressure in offspring born small.

ACKNOWLEDGMENTS

The authors wish to thank Kerryn Westcott for professional assistance with animal surgery and handling and Kom Yin for analysis of cardiomyocyte nuclearity and assistance in manuscript preparation.

GRANTS

This research was supported by a grant from the National Health and Medical Research Council of Australia to MEW and KMM (NHMRC; no. 400004). ALS was supported by a NHMRC Peter Doherty Biomedical Research Fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.J.B., K.M.M., and M.E.W. conception and design of research; M.J.B., A.L.S., O.G., K.M.M., and M.E.W. interpreted results of experiments; M.J.B. drafted manuscript; M.J.B., A.L.S., O.G., K.M.M., and M.E.W. edited and revised manuscript; M.J.B., A.L.S., K.M.M., and M.E.W. approved final version of manuscript; A.L.S. and O.G. performed experiments; A.L.S. and O.G. analyzed data; O.G. and M.E.W. prepared figures.

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