In utero exposure to diesel exhaust is associated with alterations in neonatal cardiomyocyte transcription, DNA methylation and metabolic perturbation

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Abstract

Background: Developmental exposure to particulate matter air pollution is harmful to cardiovascular health, but the mechanisms by which this exposure mediates susceptibility to heart disease is poorly understood. We have previously shown, in a mouse model, that gestational exposure to diesel exhaust (DE) results in increased cardiac hypertrophy, fibrosis and susceptibility to heart failure in the adult offspring following transverse aortic constriction.

Results: In this study, we have analyzed gene expression in neonatal cardiomyocytes after gestational exposure by RNA-sequencing and have identified 300 genes that are dysregulated, including many involved in cardiac metabolism. We subsequently determined that these cardiomyocytes exhibit reduced metabolic activity as measured by Seahorse extracellular flux analysis. We also surveyed for modifications in DNA methylation at global regulatory regions using reduced representation bisulfite sequencing and found hypomethylation of DNA in neonatal cardiomyocytes isolated from in utero DE exposed neonates.

Conclusion: We have demonstrated that in utero exposure to diesel exhaust alters the neonatal cardiomyocyte transcriptional and epigenetic landscapes, as well as the metabolic capability of these cells. Understanding how exposure alters the developing heart through dysregulation of gene expression, metabolism and DNA methylation is vital for identifying therapeutic interventions for air pollution-related heart failure.

Keywords: Diesel, PM₂.₅, Transcription, DNA methylation, Metabolism

Background

Exposure to particulate matter air pollution has been associated with increased incidence of cardiovascular disease [1–3], including arrhythmias [4], myocardial infarction [3] and heart failure [5]. Prenatal and early-life exposure to PM₂.₅ and PM₁₀ is associated with deleterious effects, such as decreased placental mitochondrial DNA [6], decreased fetal growth [7], reduced birth weight [8] and increased newborn systolic blood pressure [9]. Currently, understanding the effects of in utero exposure to DE on newborn and early life health has become a public health concern, as multiple studies link maternal exposure to air pollution with increased risk of congenital heart defects [10, 11], childhood cancers such as retinoblastoma and acute lymphoblastic leukemia [12], and increased incidence of maternal gestational type 2 diabetes mellitus [13], which can in turn increase infant risk of metabolic and cardiac disorders [14]. Our lab has shown that in utero exposure to DE in mice predisposes the male offspring to heart failure after transverse aortic constriction (TAC) surgery [15].

There is growing evidence of alterations in epigenetic control due to PM₂.₅ exposure. PM₂.₅ and PM₁₀ exposure have been correlated with significantly decreased global DNA methylation in blood cells as indicated by LINE1 and Alu methylation [16–18], as well as changes in targeted loci such as decreased methylation in the...
iNOS promoter [17] and increased methylation at the p16 promoter [19]. Diesel exhaust exposure specifically has also been shown to cause dysregulation of epigenetic signatures. Human bronchial epithelial cells exposed to diesel exhaust particulates via culture showed dysregulation of > 60% of microRNAs [20], and BALB/c mice exposed to diesel exhaust and given allergic stimuli showed hypermethylation of the interferon-gamma promoter and hypomethylation of interleukin-4 in T-cells [21]. We have also previously shown hypomethylation in exon 1 of GM6307 in neonatal cardiomyocytes (NCMs) resulting from in utero exposure to DE, which correlates with dysregulation of the contiguous miR133a-2 gene [22].

We have previously identified transcriptional dysregulation in the hearts of male adult mice exposed in utero to DE [22]. To hone in on the early changes that might underlie the previously observed later life susceptibility to heart failure, we have performed RNA-sequencing on NCMs isolated from postnatal day 0 (PND0) offspring exposed to either filtered air (FA) or DE in utero. We observed a large number of transcriptional changes associated with in utero exposure to DE, many of which affect genes involved in normal cardiac metabolism. We have also determined the baseline metabolic phenotype of neonatal cardiomyocytes after gestational exposure and have found that DE exposure is associated with metabolic dysregulation. Additionally, we have performed reduced representation bisulfite sequencing (RRBS) on NCMs, and have found diminished methylation at CpG regions in DE-exposed NCMs. This study focuses on male mice based on our previous findings of in utero DE exposure affecting male adult cardiac outcomes [15, 23], and our observation that in utero DE exposure did not result in any change in female adult cardiac outcomes (C. Weldy, Y. Liu and M.T. Chin, unpublished data). This study is the first of its kind to show widespread cardiomyocyte changes in DNA methylation, gene transcription and metabolism as a result of in utero exposure to DE.

**Results**

In utero exposure to DE results in altered gene expression

To assess alterations in transcription due to in utero exposure to DE (dams were exposed to a time weighted hourly average of 53 μg/m³ diesel exhaust-created PM₂.₅, 5 days a week from embryonic day 0.5 (E0.5) to E17.5), RNA-seq was performed on isolated NCMs from FA and DE PND0 offspring. No effects on birth weight, litter size or sex were observed (data not shown). We identified 300 transcripts that were significantly differentially expressed between FA and DE exposure. Table 1 shows the top ten genes with differential expression between exposure groups. Table 2 shows the top ten pathway hits using Qiagen's Ingenuity Pathway Analysis, with arrows indicating the direction of expression change for genes identified as altered. In both, we can see a clear link between exposure and altered transcription of genes that affect cellular metabolism, showing a consistent decrease in expression in the DE NCMs. Additionally, we observe that many genes involving cell cycle control and cardiomyocyte function were affected. Gene expression differences were validated by qPCR for a select number of top scoring genes, shown in Fig. 1.

In utero exposure to DE-induced changes in gene expression mimic changes observed in hypertrophic NCMs

Given our observations of increased cardiac hypertrophy in adult mice following in utero exposure to DE and TAC surgery [15, 23], we wanted to compare our observed changes in NCM gene expression to other experiments examining the changes in NCM gene expression due to other hypertrophic stressors. As shown in Table 3,
using a dataset examining changes in hypertrophic NCM gene expression following transgenic overexpression of CHF1/Hey2 and serum stimulation [24] (GSE14288), we can observe significant changes in the top ten altered pathways identified in Table 2 in the GSE14288 dataset. When accounting for differential expression following the direction of majority gene change noted in Table 2, we can see that the most of pathways we identified share significant changes, including the mitochondrial L-carnitine shuttle pathway, fatty acid β-oxidation and AMPK signaling. When removing the bias of directional change, we observe that all of our top ten pathways show a significant difference in the GSE14288 dataset. This demonstrates that the gene expression changes we observe in our in utero DE NCMs are also present in NCMs that undergo hypertrophic stimuli.

**In utero exposure to DE alters neonatal cardiomyocyte oxygen consumption**

To determine whether the transcriptional changes identified in Tables 1 and 2 correlated to a functional change in the metabolic capacity of NCMs, we tested the rate of oxygen consumption of DE and FA NCMs using the Seahorse XFe24 Analyzer. However, since the switch from glucose oxidation to fatty acid oxidation in the neonatal heart doesn’t occur until sometime between postnatal day 1–7 [25, 26], we tested the response of NCMs using pyruvate/glutamate to fuel respiration. Figure 2a shows the average

**Table 2** Top ten pathways showing significantly altered transcription as identified by Ingenuity Pathway Analysis (Qiagen). Arrows indicate expression in DE samples compared to FA

| Ingenuity Canonical Pathway                                      | p-value  | Molecules                                      |
|-----------------------------------------------------------------|----------|------------------------------------------------|
| Mitochondrial L-carnitine Shuttle Pathway                       | 2.14E-06 | ↓Slc27a1, Acs1, Cpt1A, Cpt2, Cpt1B             |
| Fatty Acid β-oxidation                                          | 3.47E-06 | ↓Slc27a1, Acaa2, Acs1, Hadha, Ec2, Hadhb       |
| AMPK Signaling                                                  | 4.68E-06 | ↓Mlycd, Adra1B, Pik3r3, Cpt1A, Acacb, Lipe, Cpt2, Cpt1B |
| Cell Cycle Control of Chromosomal Replication                  | 2.51E-05 | ↑Adra2a, Cnd1, PKI, Slc2a1                     |
| LPS/IL-1 Mediated Inhibition of RNR Function                   | 4.17E-05 | ↑Slc27a1, Hmgcs2, Aldh9a1, Acs1, Ppargc1b, Fabp3, Cat, Cpt1A, Cpt2, Cpt1B |
| GADD45 Signaling                                               | 1.00E-04 | ↑Cnc1, Cnd1, Pcn, Cdk1                        |
| Hepatic Fibrosis / Hepatic Stellate Cell Activation             | 1.70E-04 | ↑A2m, Igfbp4, Pgf, Col27a1, Col5a3, Igfbp3, Col1a1, Ccl21 |
| Ketogenesis                                                    | 2.57E-04 | ↓Hmgcs2, Hadha, Hadhb                          |
| Estrogen-mediated S-phase Entry                                 | 2.57E-04 | ↑Cnc1, Cnd1, E2f1, Cdk1                       |
| Oleate Biosynthesis II (Animals)                               | 5.89E-04 | ↑Scd4                                         |

**Fig. 1** qPCR validation of select top scoring genes. qPCR was performed in the RNA samples used in sequencing plus 2 additional unsequenced samples in each group for the genes Acot1, Acot2, Cpt1a, Cpt1b, and Scd4. The fold change in expression is represented as 2^-deltadeltaCT (ddCT). * indicates p-value < 0.05, ** < 0.01
In utero exposure to DE significantly decreases DNA methylation in regulatory regions

The discovery of 62 DMRs showing decreased methylation and only 1 showing an increase in the DE NCMs suggests a significant loss of methylation in the DE NCMs. This general reduction in overall DNA methylation is further demonstrated in Fig. 3a, where we examine the frequency of each CpG showing a specific percent methylation. We observe a clear decrease in the amount of methylation present in the DE samples, both through a higher proportion of 0–5% methylated CpGs and the lower amount of CpGs that were found to be methylated in >25% of the sequences. Additionally, the genomic location of the identified DMRs is graphed in Fig. 3b, with the majority of DMRs located in intergenic and intronic regions. The genes Gnas, Gng12 and Pde6H were heavily implicated in many of the altered pathways, and the DMRs associated with these genes were found in the intronic (Gnas) and intergenic (Gng12, Pde6H) regions.

When evaluating the individual CpGs that were identified to have significantly different methylation status between the FA and DE NCMs given a false discovery rate (FDR) of 0.1, including those that were incorporated into DMRs, we identified 12,629 differentially methylated CpGs (564 of which were included in DMRs). Of those, only 293 showed increased methylation in the DE NCMs. Assuming a null hypothesis of differential methylation affecting the samples equally, under the binomial distribution we would reject the null hypothesis with a p-value of 2.2e-16. Taken together, these indicate that in utero exposure to DE significantly decreases DNA methylation in CpG-rich regions.

Comparison between transcriptional and DNA methylation alterations

In order to determine how the observed change in DNA methylation may link to the transcriptional alterations, the datasets were compared by identifying any genes within 1 Mb of a DMR that showed significant changes in expression. Surprisingly, only 14 genes were found to have significantly altered transcription near identified DMRs: Acab, Cde45, Chst12, Cox4i2, Cys1, Etv4, Fkbp5, Glipr2, Gng11, Melk, Pibd1, Sirt12, Tubb3 and Vwa8. However, only Etv4 and
Fig. 2 Baseline and maximal oxygen consumption rates are lower in DE NCMs. a shows the injection scheme and average oxygen consumption rate after injections for all FA and DE NCMs, normalized to total μg DNA. FCCP - Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, AMA/Rot – Antimycin A and Rotenone. b-f shows measurements for basal and maximal respiration, ATP production, spare respiratory capacity and coupling efficiency. * indicates p-value < 0.05, ** < 0.01

Table 4 Top ten significant DMRs in NCMs associated with in utero DE exposure

| Gene                | Chr. | Start        | End          | #CpGs | Location       | AreaStat |
|---------------------|------|--------------|--------------|-------|----------------|----------|
| 3110070M22Rik       | chr13| 119,488,228  | 119,488,562  | 90    | Intron         | −1875.26 |
| Grb10               | chr11| 12,026,282   | 12,026,409   | 49    | Intron         | −715.82  |
| 1700030C10Rik       | chr12| 20,232,946   | 20,234,272   | 36    | Intergenic     | −465.28  |
| Mid1                | chrX | 169,994,141  | 169,994,213  | 16    | Intron         | −332.46  |
| Zip444              | chr7 | 6,189,544    | 6,189,675    | 24    | Exon           | −218.94  |
| Erdr1               | chrY | 90,807,240   | 90,807,512   | 13    | Intron         | −214.27  |
| Unc45b              | chr11| 82,933,209   | 82,933,329   | 22    | Intron         | −196.00  |
| Gm13152             | chr4 | 147,219,459  | 147,219,685  | 17    | Intron         | −138.56  |
| H13                 | chr2 | 152,686,786  | 152,687,008  | 13    | Intron         | −118.63  |
| Gnas                | chr2 | 174,297,352  | 174,297,431  | 10    | Intron         | −95.56   |
Glipr2 were identified to have associated DMRs in the more stringent assessment of altered DNA methylation discussed above. Of these genes, Acacb and Cox4i2 are involved in cellular metabolism, and Gng11 is involved in GPCR signaling. Acetyl-CoA Carboxylase Beta (Acacb) is interesting due to its role in catalyzing the conversion of acetyl-CoA to malonyl-CoA in the fatty acid cycle, as well as being a key regulator of fatty acid oxidation through the ability of malonyl-CoA to block carnitine-palmitoyl-CoA transferase [27]. Cytochrome c oxidase subunit 4 isoform 2 (Cox4i2) is a subunit of the cytochrome c oxidase complex within the electron transport chain [28]. G protein subunit gamma 11 (Gng11) is a subunit of the GPCR signaling complex, which has also been described as a controller of cellular senescence [29]. While these genes are biologically interesting, we would expect to see a stronger link between changes in DNA methylation and changes in gene expression. It is possible that the altered DNA methylation would not manifest in transcriptional changes until the cells are stressed, or at a later age.

### Discussion

We have shown that the transcriptional profile of NCMs is significantly altered as a result of in utero exposure to DE, in large part affecting genes associated with metabolic pathways (Tables 1, 2). Metabolic dysregulation is a hallmark feature of heart failure [30], with decreased fatty acid oxidation and increased glycolysis playing an important role in late-stage heart failure [31, 32]. While it is unclear how these findings might affect adult susceptibility to heart failure, multiple studies have discovered a link between exposure to air pollution and metabolic dysregulation [33–35]. Combined with our prior findings of increased expression of Ptprf [22], a transcriptional change associated with decreased sensitivity to insulin signaling [36], this suggests a potential role for metabolic dysregulation as a causal link between in utero DE exposure and adult susceptibility to heart failure. Additionally, we have shown that respiratory capabilities of NCMs from in utero DE exposed neonates are hindered at both baseline and maximal respiration (Fig. 2b and c). NCMs from in utero

### Table 5

| Ingenuity Canonical Pathway | p-value | Molecules |
|----------------------------|---------|-----------|
| Cardiac Hypertrophy Signaling | 0.002 | Map3k9, Gnas, Adra2c, Gng12 |
| Gαs Signaling | 0.002 | Rapgef2, Gnas, Gng12 |
| G Protein Signaling Mediated by Tubby | 0.003 | Gnas, Gng12 |
| Gαi Signaling | 0.003 | Gnas, Adra2c, Gng12 |
| Cardiac β-adrenergic Signaling | 0.004 | Gnas, Gng12, Pde6h |
| Relaxin Signaling | 0.004 | Gnas, Gng12, Pde6h |
| Ephrin Receptor Signaling | 0.008 | Epha7, Gnas, Gng12 |
| CCR5 Signaling in Macrophages | 0.012 | Gnas, Gng12 |
| Ephrin B Signaling | 0.013 | Gnas, Gng12 |
| Protein Kinase A Signaling | 0.014 | Ptprf, Gnas, Gng12, Pde6h |

### Fig. 3

**A** Histogram of % sequences methylated within sequenced CpGs. Individual CpGs were analyzed and placed into 5% incremental bins displaying the % of CpGs from all reads methylated for a given bin. The count of CpG sites from all sequences of a given sample that show methylation in 0–5% of a samples reads are put in the 0–5% bin, and so on. The counts for each bin are averaged between samples (n=4, FA and DE). Error bars are shown as standard deviation between n, which are biological replicates. **B** Genomic location of DMRs. The genomic locations of the identified DMRs were labeled as promoter (<3000 kb upstream of TSS), intronic, exonic, or intergenic, and plotted in a pie chart showing the percentage of DMRs for a given designation.
DE exposed neonates also demonstrate decreased ATP production and coupling efficiency (Fig. 2d and f), indicative of higher energy loss and ROS production [37]. Given the known role of impaired metabolic activity on heart failure [38], these findings suggest that metabolic dysregulation during development may increase the susceptibility to heart failure at adulthood in our model. However, the persistence of the observed transcriptional alterations and the observed metabolic defects through adulthood and the causal relationships with the development of heart failure remain to be elucidated. Overall, our findings of transcriptional dysregulation in NCMs as a result of in utero DE exposure will aid in guiding the experimental focus for determining modifications in cardiac function caused by exposure.

While the observed changes in metabolic transcription and function are potentially important mediators of in utero exposure-induced susceptibility to heart failure, other mechanisms involving cardiac remodeling and fetal gene expression may be involved. One gene that showed differential expression was the alpha myosin heavy gene, Myh6. The RNA-seq profile showed a significant reduction in expression of Myh6 in DE NCMs compared to FA. This gene is essential in cardiac development, with ablation experiments showing that Myh6+/− is embryonic lethal, and Myh6−/− mice showing impaired contractility and relaxation, as well as irregular sarcomere structure [39]. MYH6 mutations in humans have been associated with congenital heart defects such as atrial septal defect [40], as well as in hypertrophic and dilated cardiomyopathy [41, 42]. Further exploration into the dysregulation of Myh6 in this model would aid in understanding the potential developmental defects associated with in utero DE exposure.

Our prior findings of adult cardiovascular disease following in utero exposure suggest a temporally durable mechanism of action. Alterations of epigenetics through DNA hypomethylation have been implicated in the development and progression of cardiovascular disease [43, 44], and due to the double-stranded nature of CpG methylation these changes are incredibly stable [45]. Changes in DNA methylation both at the targeted level and genome-wide have been associated with exposure to air pollution [22, 46]. We are the first to demonstrate a change in DNA methylation at CpG-rich regions of cardiomyocytes as a result of in utero exposure to DE. The discovery of decreased methylation in DMRs associated with genes involved in GPCR and cardiac hypertrophic signaling suggests a predisposition to dysregulation in these pathways. However, there was no observed increase in transcription of Gnas, Gng12, Adra2c or Pdeth in the transcriptional profile of DE-exposed NCMs, though Gng11 was identified to have increased expression. It is possible that the changes in transcription affected by DNA methylation would be stimulated through stress, or will manifest themselves sometime beyond the neonatal period. GPCR signaling also plays a role in the autonomic nervous system control of cardiac regulation. Considering our prior findings of decreased blood pressure at baseline in in utero DE exposed 10 week old mice [15], it is important to further understand how these molecular changes are affecting physiological outcomes in both the neonatal and adult hearts. Future studies targeting autonomic features such as heart rate variability would likely be informative.

It is unclear whether this change in DNA methylation is occurring through a directed or undirected pathway. Exposure to PM2.5 has been shown to have an associated increase in oxidative stress [47–49]. Passive removal of DNA methylation can occur due to oxidative damage from ROS by damaging the DNA to the extent that DNA methyltransferases (DNMTs) are unable to bind to the lesion area, thereby averting the maintenance of methylation in daughter cells [50, 51]. If this process is occurring early in development during the repatterning of methylation, these changes would be extremely heritable, and likely occurring in multiple cell and organ types [45]. It is also possible that cellular damage due to oxidative stress prevents DNA methylation through sequestration of glutathione [52]. Targeted changes in DNA methylation in response to air pollution have been described previously. Hypermethylation of the p16 promoter in alveolar epithelial cells of mice exposed to PM2.5 in conjunction with increased DNMT1 expression has been reported [19] and decreased methylation on the first exon of Gm6307, which drives mir133a-2 expression, has also been reported in association with in utero DE exposure [22]. Our studies of transcriptional dysregulation in NCMs did not identify any DNA methyltransferase or TET family enzyme genes as being differentially expressed, though this does not preclude the possibility of altered expression earlier in development leading to decreased methylation that was maintained until birth.

For future studies, refining the contributions of in utero DE exposure and later life cardiac outcomes will be important for focusing future experiments. Meta-analysis of epidemiological studies have demonstrated the increased risk of congenital heart defects associated with maternal exposure to nitrogen dioxide, sulfur dioxide, and particulate matter [53, 54]. Although we favor particulates as being likely responsible for the observed effects, contributions from other components cannot be ruled out. Additionally, it is not clear whether maternal effects, such as placental insufficiency brought on from inflammatory response to exposure, are the main influencers on the offspring cardiac outcomes, or if particulate and/or gaseous matter is able to cross the placental barrier and directly interact with the cardiovascular system of the developing embryos.
Conclusions
Our study is the first to systematically analyze and demonstrate the profound effects of in utero DE exposure on cardiomyocyte gene transcription, DNA methylation and metabolism. It is unclear whether these effects are due to a direct effect of DE exposure or an indirect mechanism via the maternal response to exposure, although our previous observation that maternal exposure leads to placental inflammation [15] is consistent with an indirect mechanism. A limitation of our study is that the correlation between mRNA expression, DNA methylation and metabolic activity is imperfect. Nevertheless, these findings provide important datasets for mining the potential pathways that mediate the deleterious effects of diesel exhaust on the developing heart that predispose to heart disease. Identifying pathological pathways that promote the development of air pollution-related heart disease is a first step in developing potential therapies.

Methods
Diesel exhaust exposure and mice
Housing and exposure facility conditions were carried out as previously described [15, 22, 23]. C57Bl/6 mice were used in these experiments, housed in specific pathogen free (SPF) conditions, on a 12/12-light/dark cycle. Female and male mice between the ages of 12 to 16 weeks were transferred to our Northlake Diesel Exposure Facility located near the University of Washington (UW) and housed under SPF conditions in Allentown caging systems. Female mice were paired with male mice for timed mating in FA. After observation of a vaginal plug, pregnant mice were put into FA or DE with exposures beginning at E0.5 and lasting until E17.5, at which point pregnant mice were transferred from the Northlake facility to the UW Medicine South Lake Union SPF vivarium for neonatal sample collection.

Diesel exhaust was generated from a single cylinder Yanmar diesel engine (Model YDG5500EV-6EI) operating on 82% load. A detailed analysis of DE particulate components in this system has been previously reported [55]. DE exposures were conducted for 6 h per day (9 am – 3 pm) 5 days a week (Monday – Friday) and DE concentrations were regulated to 300 μg/m³ of PM_{2.5}. A 300 μg/m³ concentration of PM_{2.5} 6 hours a day, 5 days a week equates to a time weighted hourly average of 53 μg/m³. The exposure characteristics detailing gas, particle-bound polycyclic aromatic hydrocarbons (PAH), and particle diameter of this system have been previously reported [55, 56]. In previous measurements, oxides of nitrogen concentrations were 1800 ppb NO_{x} and 60 ppb NO_{2}, carbon monoxide was 2 ppm, and carbon dioxide was 1000 ppm. The mass fraction of particle-bound PAH was 20 ng/mg PM_{2.5} and the ratio of the organic carbon to elemental carbon mass concentration was 0.10. The mass median aerodynamic diameter of particles was 85 nm (GSD 1.2) and the count median thermodynamic equivalent diameter was 87 nm (GSD 3.0). The exposure approximates real world ambient exposure in an urban setting.

Isolation of NCMs
Upon birth, PND0 hearts were harvested, trimmed of surrounding vascular and atrial tissue, and dissociated as previously described [57, 58]. Males were detected through post-mortem dissection and identification of testes. Dissociation was performed using 1 mg/mL Liberase TH (Roche; Pleasanton, CA, USA) in 1X HBSS by incubating hearts at 37 degrees for 5 min in solution, with pipetting to release cells after incubation. Media containing released NCMs was adjusted to 20% FBS-DMEM, and cellular dissociation was continued until the majority of cells were released. Cells were then filtered using a 70 μm sieve, re-eluted in 20% FBS-DMEM with 20 μm Ara-C and incubated at 37 degrees for 1 h to allow fibroblasts to attach onto the plate. After incubation, media with suspended cardiomyocytes was carefully removed, spun and purified cardiomyocyte pellets were collected and frozen at –80 °C.

RNA sequencing
RNA was purified from frozen PND0 isolated NCMs using Trizol (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer’s protocol. For each sequencing sample, NCMs from one male neonatal heart were isolated and used for each n (n = 3 for FA, DE). Ribosomal RNA was depleted by poly-A enrichment, and sample libraries were created using the TruSeq Stranded mRNA kit (Illumina, San Diego, CA). Each library was barcoded using the Illumina adapters, and amplified with 13 cycles of PCR. Library concentrations were quantified using the Quant-it dsDNA Assay (Life Technologies, Carlsbad, CA). Libraries were normalized and pooled based on Agilent 2100 Bioanalyzer results (Agilent Technologies, Santa Clara, CA), and the pools were sequenced on an Illumina HiSeq 2500.

To process sequences, Illumina RTA-generated BCL files were converted to FASTQ files and sequence read and base quality were checked using the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences were aligned to mm10 with reference transcriptome Ensembl v67 using TopHat [59]. Lane level bam data files were merged using the Picard MergeSamFiles tool and suspected PCR duplicates were marked, not removed, in the alignment files using the Picard MarkDuplicates tool (http://broadinstitute.github.io/picard/). Local realignment was performed around indels, and base quality score recalibration was run using GATK tools [60]. Variant detection was performed with
the GATK Unified Genotyper version 2.6.5 [61], and aligned data were used for isoform assembly and quantitation with Cufflinks [59, 62]. Fragment counts were generated using the featureCounts function in the Bioconductor Rsubread package [63]. Comparisons were performed using the Bioconductor edgeR package. Genes were identified as significantly different between groups if they had a 25% or greater difference in expression between the two groups at an FDR adjusted p-value of 0.05 or less. To assess consistency between our data and previous results (GSE14288) we performed self-contained gene set tests using the eleven pathways we identified using IPA (Table 2), testing for evidence that those gene sets were differentially expressed in the previous results as well. We used the limma fry function to perform the gene set tests [64].

Select genes were validated by qPCR using iTaq Universal Sybr Green Supermix (BioRad). Primers used for validation were as follows:

| Primer       | Sequence                        |
|--------------|---------------------------------|
| Acot1-For    | 5′-TACGTAGACCTCCTCCTAGAA-A-3′   |
| Acot1-Rev    | 5′-AGCCCAATTCCAGGTCTT-3′        |
| Acot2-For    | 5′-ACAACTACGAGCCCTCCCTC-3′      |
| Acot2-Rev    | 5′-AGCCCAATTCCAGGTCTT-3′        |
| Cpt1a-For    | 5′-TCCA TGCAATCCAAATGGGAC-3′    |
| Cpt1a-Rev    | 5′-CGAT GTTCTTGCTGTCGGCTT-3′    |
| Cpt1b-For    | 5′-AGCCCACTCATTAGTGGAC-3′      |
| Cpt1b-Rev    | 5′-AGTTTGCGGGCGATA CATGA-3′    |
| Scd4-For     | 5′-TGGAGCCACCGAAACCTATA A-3′   |
| Scd4-Rev     | 5′-GGCCCAATTCATACACGTTCAT-3′   |
| 18S-For      | 5′-GGGACAGGTACGGACAGATGATG-3′   |
| 18S-Rev      | 5′-ATCGCTCCCAAACACTAAGA-3′     |

Expression was normalized to ribosomal RNA 18S. Samples were run on the 7900HT Fast Real-Time PCR System (ABI). Statistical analysis was carried out using a t-test for comparison of ddCT values.

**Metabolic capacity analysis**

Cellular respiration was performed on NCMs using the mitochondrial stress test (Agilent; Santa Clara, CA, USA), measured using a Seahorse XFe24 extracellular flux analyzer (Agilent). NCMs were plated on provided 24 well plate coated in fibronectin, plating 3–5 replicates per sample, and cultured for 3 days in 20% FBS-DMEM with 20 μM Ara-C. This time period allowed for the non cardiomyocyte cells to become metabolically inactive after the isolation procedure. On the day of measurement, cells were washed with XF assay medium (Agilent) supplemented with 25 mM glucose, 1 mM sodium pyruvate and 2 mM L-glutamine, and replenished with the same media and incubated at 37 °C without CO₂ an hour prior to measurement. Injection scheme is represented in Fig. 1a, with concentrations at 1.5 μM oligomycin, 1 μM FCCP, 1 μM Rotenone and 1 μM antimycin A. Normalization to μg DNA/well was performed by phenol/chloroform extracting DNA from each well, and measuring DNA concentration using the Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific), following the manufacturers protocol.

Rates were averaged between the 3–5 replicate plated wells per sample, giving one averaged read per sample n. Calculations for basal and maximal respiration, ATP production, spare respiratory capacity, and coupling efficiency were performed as recommended by Agilent:

- **Basal respiration** = [last rate measured before first (oligomycin) injection] - NMOC.
- **Maximal respiration** = [max. Rate measured after FCCP injection] – NMOC.
- **ATP production** = [last rate measured before oligomycin] – [min. Rate measurement after oligomycin injection].
- **Spare respiratory capacity** = [maximal respiration] – [basal respiration].
- **Coupling efficiency** = [ATP production rate] / [basal respiration] × 100.

Statistical comparisons were performed using a t-test between the measurements made in male DE vs. FA NCMs (n = 7, n = 8 respectively).

**Reduced representation bisulfite sequencing**

DNA was isolated from frozen PND0 isolated cardiomyocytes using Qia-gen (Germantown; MD, USA) DNeasy Blood and Tissue kit, according to the manufacturer’s protocol. For each sequencing sample, NCMs from one male neonatal heart were isolated and used for each n (n = 4 for FA, DE). 500 ng of DNA was digested overnight with Mspl (New England Biolabs Inc., Ipswich, MA, USA) and then directly prepared with the KAPA Hyper Library prep protocol (KAPA Biosystems, Wilmington, MA, USA). Samples were then adapter-ligated using SeqCap Adapters (Roche-Nimblegen, Pleasanton, CA, USA), and cleaned using a 2.5X post-ligation Agencourt AMPure XP bead cleanup (Beckman Coulter, Indianapolis, IN). Size selection was performed to a size range of 160 bp–340 bp on a 2% Pippin Prep gel (Sage Science, Beverly, MA, USA), followed by bisulfite conversion using the Zymo EZ DNA Methylation Lightning kit (Zymo Research, Irvine, CA, USA). The converted DNA was then put back into the KAPA Hyper Library prep protocol at the library amplification step, and amplification was performed with 19 PCR cycles using 2X KAPA HiFi HotStart Uracil+ ReadyMix (KAPA Biosystems, Wilmington, MA, USA). Post-amplification cleanup was performed with 0.8X Agencourt AMPure XP beads (Beckman Coulter). Library size distributions were validated using the Agilent High Sensitivity D1000 ScreenTape run on an Agilent 2200 TapeStation (Agilent Technologies, Inc., Santa Clara, CA, USA). Additional library QC, blending of pooled indexed libraries, and cluster optimization was performed using Life Technologies-Invitrogen Qubit® 2.0 Fluorometer (Life Technologies-
Invitrogen, Carlsbad, CA, USA). Sample libraries were sequenced on the Illumina HiSeq 2500 (Illumina; San Diego, CA, USA).

Sequencing reads were processed by discarding the low quality reads using Illumina’s base call quality filter, and raw reads were trimmed of adapter sequences and flanking low quality base reads using the trimmomatic read trimmer [65], discarding any reads less than 25 nt, after which there were between 13.6–31.4 million reads per sample. Methylation status for each CpG was estimated by aligning reads to the mm10 genome using the Bismark aligner [66], read into R using the Bioconductor bsseq package [67], and differential methylation was called using the Bioconductor DSS package [68]. There were between 2.85 and 4.47 million CpGs per sample. Low read coverage CpGs were filtered, based on the criteria that a CpG had to have read coverage > 5 in at least half of the samples, reducing the total number of CpGs to ~646 thousand. After this filtering, median read depth per CpG was 8–25. We then selected a set of differentially methylated regions, based on a set of criteria; to be considered a differentially methylated region (DMR), a) the region had to be at least 50 bp in length with at least 3 CpGs present, and b) at least 50% of the CpGs in the region had to be significant, based on a p < 0.001 and show a minimum 10% difference in methylation (e.g., genes that increase expression as methylation decreases). The authors declare that they have no competing interests.

Abbreviations
- Acacb: acetyl-CoA Carboxylase Beta;
- Cox4i2: cytochrome c oxidase subunit 4 isoform 2;
- CpG: 5′-Cytosine-phosphate-Guanine-3′;
- DE: diesel exhaust;
- DMR: differentially methylated region;
- DNMT: DNA methyltransferase;
- FA: filtered air;
- FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone;
- Gng11: G protein subunit gamma 11;
- NCM: neonatal cardiomyocyte;
- PAH: polycyclic aromatic hydrocarbon;
- PM<sub>2.5</sub>: particulate matter with a diameter of 10 μm or less;
- PM<sub>10</sub>: particulate matter with a diameter of 2.5 μm or less;
- PND: post-natal day;
- RRBS: reduced representation bisulfite sequencing;
- TAC: transverse aortic constriction

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Availability of data and materials
The datasets generated for RNA-seq and RNBS are available in the NCBI GEO Database under the SuperSeries accession number GSE110793.

Authors’ contributions
JMG, contributed to generating hypotheses and experimental design, conducted timed matings and exposures, isolated neonatal myocytes for Seahorse analysis and prepared samples for RNA-seq and RRBS, interpreted data, and is the primary author of the manuscript; JWM, performed key bioinformatics analyses for RNA-seq and RRBS and contributed to preparation of the manuscript; TKB, contributed to bioinformatics analyses and contributed to preparation of manuscript; WMC, assisted in molecular biology experiments and contributed to preparation of manuscript; MTC, contributed to generating hypotheses and experimental design, contributed to preparation of manuscript and was responsible for overall coordination of the project. All authors read and approved of the final copy of the manuscript.

Ethics approval and consent to participate
All animal studies were approved by the University of Washington Institutional Animal Care and Use Committee under protocol 4134–01. All other methods and experimental protocols were carried out under Institutional, State and Federal guidelines with approval from the appropriate University of Washington Environmental Health and Safety officials overseeing lab safety, chemical safety and biohazards.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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