# Transcriptional Origins of Adult Cardiac Disease in Response to Maternal Obesity

by

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## Abstract

The transcriptional networks that maintain adult cardiac homeostasis are established during early stages of cardiac formation. Obesity during pregnancy poses a significant deviation from embryonic homeostasis which negatively impacts heart function, as early as the first trimester, and increases the risk for adult-onset cardiovascular disease. The phenotypic trajectory of these offspring that ultimately results in adult cardiomyopathy, and the transcriptional pathways altered in the embryonic heart and conferring increased risk of adult-onset heart disease are unknown. Here, I examined the functional and molecular consequences of maternal obesity on the offspring's hearts during fetal and adult life. I found that adult mice born to obese mothers are phenotypically silent in young adulthood but develop mild heart dysfunction as they age. Hearts of these mice dysregulated genes controlling extracellular matrix remodeling, metabolism, and TGF-β signaling, known to control heart disease progression. Moreover, in response to cardiovascular stress, the offspring of obese mice developed exacerbated myocardial remodeling and excessively upregulated the expression of genes that regulate cell-extracellular matrix interactions but failed to upregulate metabolic regulators. These pathways were already dysregulated in cardiac progenitors in embryos of obese mice and human fetal hearts from obese donors. I found that the expression of these “obesity-responsive” genes was developmentally regulated. Accordingly, the expression levels of *Nkx2-5*, a key regulator of heart development, inversely correlated with maternal body weight in mice. Nkx2-5 target genes were dysregulated in cardiac progenitors, adult hearts born to obese mice, and in human fetal hearts from pregnancies affected by obesity. Altogether, I have discovered that obesity during pregnancy alters transcription in differentiating cardiac progenitors, throughout gestation, and in the adult heart, poising the adult heart to dysregulated stress responses. My findings bridge a gap in

knowledge regarding the transcriptional responses to maternal obesity, its impact on the heart, and the developmental time frame when cardiac disease risk is programmed. The genes and pathways identified in this thesis as well as the early functional consequences of maternal obesity on the offspring heart could have broad ramifications in screening and treatment strategies to help reduce the potential risk of cardiovascular disease in future generations.

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# *1. Introduction*

## 1.1. Origins and proposed theoretical basis of fetal programming

### 1.1.1. Developmental origins of health and disease (DOHaD)

Embryonic development is a critical forming period in life that can determine the long-term health of the organism. Stress during embryonic development can have immediate and long-term effects that impact health during subsequent stages of life. The seminal discovery by Barker and Osmond in 1986, associated the increased prevalence of cardiovascular disease (CVD) mortality with poor early life nutrition in Wales and England[1]. Since then, numerous studies have found similar increases in CVDs and other non-communicable diseases (NCDs) such as type 2 diabetes (T2D), asthma, cancer, osteoporosis and neuropsychiatric disorders in response to poor nutrition in Netherlands, South Africa, Finland, the United States, Brazil, and China[2]. The number of embryonic stressors that determine long-term risk has also increased. It is now established that obesity, under-nutrition, hypoxia, drug use and emotional stress during pregnancy can increase the risk of several NCDs – including metabolic and cardiovascular disease. Thus, although NCDs manifest mainly in adulthood, the mounting evidence from human and animal studies demonstrates that risk factors for several NCDs, including CVDs, are established in response to adverse conditions during fetal development. This is referred to as "fetal programming" or the "developmental origins of health and disease" (DOHaD) hypothesis.

### 1.1.2. Adaptive and mal-adaptive fetal responses to the *in utero* environment

Several models have been proposed to explain the associations between *in utero* exposure to stress and the increased risk of adult-onset NCDs. Hales and Barker first proposed the "thrifty phenotype" hypothesis in 1992[3]. This hypothesis postulates that organisms mount an adaptive response to intrauterine stress to optimize resources in favor of critical organs, such as the brain and heart, at the expense of others, such as the pancreas, which increases immediate survival but leads to long-term deficits[3]. This hypothesis provided a framework to link suboptimal fetal development, caused by maternal malnourishment or placental insufficiency, with long-term consequences to adult health, such as T2D[4–8]. In this example, poor pancreatic growth would be

the cost for the immediate adaptive advantage that ensures fetal survival but results in poor insulin secretion in adult life[9,10]. Similar developmental tradeoffs were known to occur in many species. For example, the spadefoot toad undergoes early metamorphosis during drought conditions at the expense of growth, which improves reproductive success at the cost of increased risk of predation due to a smaller body size[11]. However, the thrifty phenotype hypothesis fails to address several persistent physiological alterations present in both fetal and adult life, such as cardiac deficits[12–16], that have no immediate adaptive advantages. In addition, insulin resistance, which was proposed by Barker and Hales to limit fetal growth and increase survival to birth, was shown to develop postnatally in human and mouse models of DOHaD, not during fetal development[17]. Furthermore, the thrifty phenotype hypothesis assumed that developmental changes were induced only by deprivation, in the form of severe insult or stress. Thus, another model of developmental plasticity was put forward to address these limitations. It proposed that embryos adjust developmental trajectories in response to environmental cues to either gain an immediate benefit for the embryo or to achieve better fitness in later life; the latter termed "predictive adaptive responses"[18–20]. A predictive adaptive response does not confer immediate adaptive advantage and was proposed to follow a continuous trajectory, with more severe insults resulting in more severe phenotypes. Importantly, it relied on constant environments from conception and throughout adulthood. This hypothesis was supported by animal models of stress exposure. For example, female mice exposed to the stress of predation during gestation had greater activation of the hypothalamic-pituitary-adrenal (HPA) axis which resulted in offspring with greater alertness and risk assessment behaviours[21,22]. If the postnatal environment matches the prediction (i.e. activated HPA and predators), the offspring will have a survival advantage. However, when there was a mismatch between the environment and the prediction (i.e. activated HPA and no predators) the offspring will develop the same anxiety-like behavior and the outcome is detrimental. This hypothesis appears to be validated in specific stress situations, such as HPA activation. However, it fails to explain several adaptations that offer no immediate or predictive adaptive advantage. Tobacco, medication, nutrient insufficiency, and obesity during pregnancy increase the risk for several diseases during fetal and adult life (discussed below). The "thrifty phenotype hypothesis" and the "predictive adaptive responses" model explain several fetal programming events that confer an immediate or long-

term adaptive advantage in a constant environment. However, these models are lacking in cases of mal-adaptative responses, where the fetal stressors are induced by pathological stimuli that are unrepresentative of the contemporary environment. In these situations, the fetal response may induce both immediate and long-term damage.

There are several examples of mal-adaptive fetal responses that have immediate and long-term negative impacts on the offspring's health, such as the response to tobacco, micronutrient deficiencies (e.g. folate), and obesity. Smoking during pregnancy causes a chronic hypoxic environment in the fetus mediated by increased placental resistance, decreased uterine blood flow, and increased carboxyhemoglobin. This has immediate effects on the fetus such as premature birth, increased rate of congenital defects and intrauterine growth restriction (IUGR), but it also leads to increased obesity, neurodevelopmental and behavioral disorders. Deficiency in folic acid increases the risk for neural tube defects and preterm births[23] and also increases the risk for T2D later in life in surviving offspring[24]. Obesity negatively impacts blood flow dynamics in the heart, increases ventricular thickness, increases placental defects, and also causes long-term increases in the risk of several NCDs including CVDs[25] (discussed in depth later), and metabolic disease. These responses are detrimental adaptations of the fetus that cause immediate harm and also increase the risk of long-term disease. Responses to such stressors represent an important group of fetal programming “mal-adaptations” which confer an underlying predisposition to disease in the fetus and adult[26]. Understanding the processes mediating mal-adaptive responses is important for early detection of at-risk fetuses as well development of preventative and treatment strategies for the adult affected offspring[25]. The knowledge gained about the immediate and long-term implications of these pathological stressors has led to effective global policies that led to the global decline in fetal exposure to several stressors such as tobacco, folate deficiency and undernutrition during pregnancy. However, one stressor that remains a global epidemic and continues to rise to this date, is obesity.

## 1.2. Obesity and fetal programming

Obesity is a global epidemic with major detrimental impacts on the health of the affected individual and their offspring. It is estimated that ~23% of women of childbearing age in Canada

are obese[27]. Since the work by Barker and Osmond in 1986, where infant mortality rates in the early 1920s were associated with increased rates of adult CVDs 60-70 years later, numerous studies have validated the link between fetal stress and CVDs. CVD is currently the leading cause of death worldwide[28], and due to the increasing rates of obesity amongst women of childbearing age (~25% and ~30% in the UK[29] and US[30] respectively), the prevalence of CVD is expected to rise in future generations as offspring of obese women reach adulthood. This predicts a major health problem in the future. While Barker and Osmond pointed to undernutrition during early life as a cause for increased CVD mortality in adulthood[1], almost 40 years after their seminal publication, undernutrition in western countries has declined but obesity rates continue to grow. Maternal obesity or increased maternal BMI increases the risk of CVDs, metabolic syndrome, cancer, and all-cause mortality in the adult middle-aged offspring. Notably, the extent of obesity also determines the magnitude of disease and mortality risk; every unit increase in BMI is associated with an increase in risk for mortality and CVD the offspring. Cohorts which determined this late onset disease susceptibility were born in the 1960s, when obesity rates were as little as 6%. The rate of morbid obesity was 1.4% in 1960-62 (when the current published cohorts were born), compared to 10.5% in 2013-2014 in the United States, and continues to rise today[31]. Thus, increased risks of CVDs in the offspring in the next generations will have even more severe impacts on global health, especially given the rise in both obesity rates and the severity of obesity in women. Understanding how fetal exposure to an obesogenic environment determines disease risk trajectory in the developing heart is currently lacking.

### 1.2.1. Clinical studies on the effects of maternal obesity on the offspring heart

#### 1.2.1.1. Fetal heart dysfunction in response to maternal obesity

Maternal obesity is associated with fetal cardiac dysfunction beginning at the first trimester and persisting throughout gestation **(Fig. 1)**. Obese women in the studies discussed below, except 6 out of 54 in one study [12], do not exhibit any form of diabetes, clearly distinguishing between the well-established effects of diabetes on fetal cardiac health[32,33] and those of obesity during pregnancy. Women presenting complications associated with obesity including pre-eclampsia,

hypertension, IUGR, congenital heart disease, or other fetal anomalies were also excluded from these studies, supporting the primary effects of maternal obesity on fetal cardiac function.

Ventricular strain and strain rates describe ventricular deformation, and their alteration is indicative of cardiac dysfunction. This is because the outer and inner cardiac muscle layers are arranged longitudinally and the middle muscle layers are circumferential, leading to complex contraction patterns[34]. A reduced strain is one of the earliest and most sensitive markers of cardiac injury that manifests prior to functional impairment of the ventricle[35,36]. At 14 weeks of gestation, the global strain rate (GSR) of the left ventricle (LV), right ventricle (RV), and interventricular septum (IVS) is reduced in fetuses of obese women[12]. Although GSR is also decreased at mid and late gestation, it has the highest fold decrease at early gestation, indicating that early cardiac development is highly susceptible to an obesity insult[12]. In contrast, morphological and cardiac hemodynamic changes in the fetal heart of obese women manifest at later stages of gestation. The wall velocities of the LV and IVS during contraction are reduced at mid and late gestation[12], indicating impaired contractile function[37]. The peak late filling velocity of the LV (A′), an indicator of contractile function, is significantly reduced in fetuses of obese women in the third, but not first or second, trimester[12]. The IVS is 26% thicker and the width of both ventricular chambers is reduced in fetuses of obese women, as compared to lean women, in the third trimester. These dimensions are not different in the first and second trimesters[12]. Thus, fetuses of obese women present early signs of cardiac stress beginning at the first trimester, with a progressive reduction in cardiac function throughout gestation.

Diastolic function is also significantly reduced in fetuses of obese women. Diastolic functional decline is characterized by slower pressure decay in the ventricle, which causes diastolic relaxation to proceed more slowly [38]. This increases the isovolumetric relaxation time (IVRT), which is the time interval between the end of systolic ejection and the beginning of diastolic filling[38]. IVRT in fetuses of obese, as compared to lean women, is prolonged in both the LV and RV by 14% and 19.7%, respectively, at the third trimester [13]. The increase in IVRT indicates slower pressure decay in the ventricles and a reduced ability to relax. Additional signs of reduced diastolic function in these fetuses include a significant increase in tissue velocities during relaxation (Ea and Aa velocities) of the interventricular septum, left ventricle posterior wall, and right ventricle free wall, and reduced Ea/Aa ratios [13]. In contrast, mitral and tricuspid

E/A ratios, which represent blood flow velocities through the valves, are not altered, suggesting that the overt diastolic function is not affected. Reduced diastolic function is considered an early predictor of cardiac disease[39,40]. Diastolic function has not been thoroughly examined during the first and second trimesters and it is unclear when these phenotypes manifest. Overall, obesity during pregnancy induces early signs of cardiac disease in the fetal heart that might predispose to further functional decline later in life.

In summary, maternal obesity leads to significant impairment in fetal cardiac form and function, affecting both the left and right ventricles in the first trimester, persistently and progressively leading to functional decline during the second and third trimesters **(Fig. 1)**. Thickening of the ventricular walls begins during the third trimester when diastolic function is also impaired **(Fig. 1)**. However, it is important to note that these alterations remain subclinical and likely represent early markers of heart disease susceptibility[35,36,39,40]. The cause of these subclinical alterations in the fetal heart, their significance, their persistence after birth, and their long-term effects are still unclear. Follow-up studies during early neonatal stages, childhood, and adulthood examining cardiac function would be crucial to determine whether alterations of the fetal heart persist or exacerbate after birth. In addition, the effects of maternal obesity on the human fetal heart at the cellular and molecular levels remain unexplored.

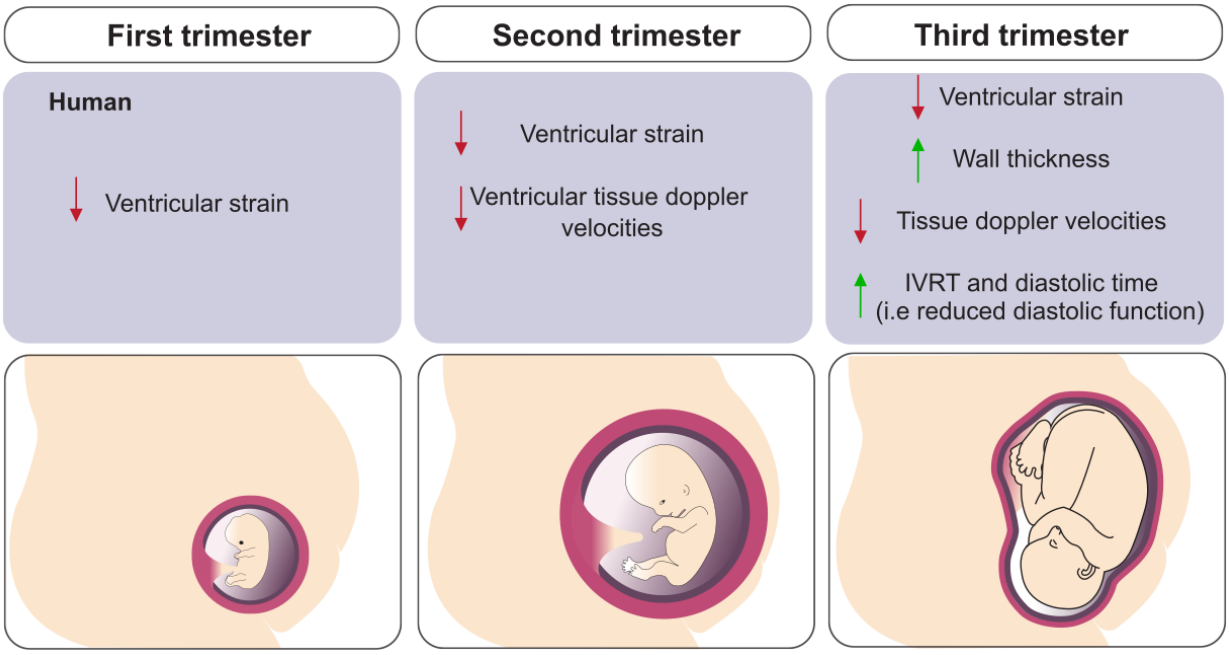



**Figure 1. Maternal obesity negatively impacts human fetal cardiac function.** Human fetuses of obese women exhibit progressive impairment in heart function beginning at the first trimester and progressing throughout pregnancy. Ventricular strain rate is reduced during the first trimester and throughout gestation, which is an early indicator of cardiac dysfunction. As gestation progresses, strain is not increased. However, more parameters of cardiac dysfunction are altered. Ventricular tissue velocity is decreased beginning at the second trimester and persists at the third. Finally, during the third trimester, ventricular wall thickness increases, which is characteristic of hypertrophy, and diastolic function is reduced (although diastolic function has not been measured at earlier stages). Figure is reproduced from [25].

#### 1.2.1.2. Post-natal CVD in response to maternal obesity

Although the specific long-term effects of the subclinical fetal cardiac dysfunction on the neonatal and postnatal heart have not been directly addressed in the context of maternal obesity, numerous studies have associated maternal obesity with increased predisposition to CVD in the offspring. Children (age 6) of obese mothers have a higher incidence of adverse cardiometabolic risk factors, such as high systolic blood pressure and lower HDL[41]. Cardiometabolic health refers to the risk of having cardiovascular disease and metabolic disorders. Cardiometabolic risk factors tend to track from childhood to adulthood and are associated with cardiovascular disease in later

life[41]. Consistently, CVD incidence and mortality are increased in adult offspring of obese mothers. In 1997, Forsen *et al* published the first study demonstrating a significant correlation between increased maternal BMI and mortality due to coronary heart disease (CHD) in the offspring. Every standard deviation increase in maternal BMI had a hazard ratio of 1.24 (1.10 to 1.39) for CHD in offspring [15]. This was only significant in mothers below average height, possibly due to the limited sample size. Since then, two large-scale studies further confirmed the association between maternal BMI and risk of CVD in the offspring. The largest study to date included 37,709 people and despite a very low obesity rate in their population (4% maternal obesity), maternal overweight or obesity categorically correlated with significantly increased mortality in the offspring with a hazard ratio of 1.11 (1.03 to 1.19) and 1.35 (1.17 to 1.55), respectively [14]. The mortality rate also showed a continuous upward trend for risk above maternal BMI of 28; the higher the maternal BMI, the higher the risk for mortality in the offspring [14]. Importantly, maternal overweight or obesity increased hospitalizations due to cardiovascular events in offspring with hazard ratios of 1.15 (1.04 to 1.26) or 1.29 (1.06 to 1.57), respectively [14]. This study did not find significant increases in the risk of myocardial infarction, stroke, angina, cerebrovascular disease, or peripheral arterial disease individually [14]. This is likely because the population studied was too young, with an age range between 34 and 61 years old, which is below the mean age to develop disease [42]. However, maternal obesity was associated with an increased risk of hospitalization due to all other combined CVDs including hypertension, hypertrophic cardiomyopathy, etc. [14]. Increased maternal BMI was associated with increased rates of CHD and stroke in an older population of 13,345 men and women[16]. This association was sex-specific as males were at increased risk for coronary heart disease (1.031 (1.009 to 1.054) per $kg/m^2$) but not stroke, whereas females had an increased risk of stroke (1.059 (1.019 to 1.101) per $kg/m^2$), but not CHD. These gender differences may be due to the lower incidence rates and later onset of CHD in females [42]. Indeed, it is likely all studies to date have grossly underestimated the risk as the mean ages of offspring are below the mean age to develop disease, which is a major limitation [42].

In summary, high maternal BMI during pregnancy is associated with a modest increase in the risk of CVD and mortality in the offspring. The magnitude of this risk is directly proportional to the maternal BMI. Still, it is unclear why some offspring born to obese mothers develop disease

and others do not. Dissociating between the primary effects of maternal obesity on the heart and the secondary effects of metabolic abnormalities in the offspring [43–46] has not been possible in human studies.

### 1.2.2. Fetal cardiac dysfunction leads to the improper development of the cardiovascular system and sensitizes the heart to adult-onset disease

The impact of fetal cardiac stress and subclinical dysfunction, and whether such changes also persist to adulthood in the context of maternal obesity, has not been directly addressed. Nonetheless, several lines of evidence from other intrauterine stressors demonstrate that fetal cardiac dysfunction similar to that reported in obese pregnancies can persist in the postnatal life and can increase susceptibility to adult-onset cardiovascular disease.

Cardiovascular consequences to intrauterine stressors, such as the stress of fetal growth restriction (FGR), can manifest as subclinical fetal cardiac dysfunction similar to those observed in fetuses of obese mothers[47]. These subclinical changes persist into childhood and adolescence leading to increased susceptibility to adult-onset cardiovascular disease[47]. Like fetuses of obese mothers, the ventricular walls of growth-restricted fetuses are moderately thickened and their IVRT is also increased[48,49]. These alterations represent subclinical changes in fetal cardiac function (discussed in-depth in [47]), they persist after birth. Neonatal hearts of growth-restricted fetuses continue to show signs of subclinical cardiac dysfunction such as IVS hypertrophy, increased IVRT, and reduced contractility. Diastolic function is also reduced in children 3-6 years of age[50] and adolescents 8-12 years of age[51]. Adult hearts also present subclinical changes in cardiac form and function. Indeed, most cases of cardiovascular disease are preceded by a prolonged period of sub-clinical disease that may last decades before symptoms appear[52]. Subclinical cardiac dysfunction may progress with age or may be exacerbated by secondary stressors. Ultimately people who underwent FGR have higher cardiovascular mortality in later life[47]. Overall, subclinical cardiac dysfunction in growth-restricted fetuses can persist and progress in postnatal life, and is associated with an increased incidence of CVD.

While obesity and FGR are distinct stressors and likely have different impacts at the cellular and molecular level, long-term analysis of the effects of FGR in the fetal and postnatal heart highlights that the developing heart responds to *in utero* stress. Cardiac responses to *in utero*

stress manifest as subclinical reductions in fetal cardiac function and persist postnatally for decades, ultimately increasing disease risk and mortality in offspring. The persistence or progression of cardiac dysfunction during postnatal life has not been thoroughly investigated in the offspring of obese mothers. Key questions remain regarding how and why the fetal cardiac responses to stress lead to immediate and long-term cardiac dysfunction, and whether additional postnatal stimuli favor disease progression.

### 1.2.3. Early stages of cardiac development are most susceptible to stress

The heart is particularly vulnerable to environmental stressors during early stages of heart development. Exposure to famine during early, but not mid or late gestation increases the risk for CVD in offspring. Fetal growth restriction that occurs at early gestation is also associated with worse outcomes. Fetuses with early-onset FGR are more highly predisposed to develop hypertrophic phenotypes at late gestation, compared to fetuses with late-onset FGR. Fetuses with early-onset FGR also have high postnatal blood pressures and have the poorest perinatal outcomes[49], indicating that early and persistent exposure to stress is associated with the most severe impact on the cardiovascular system. In mouse models, transient hypoxia (for 8hrs) during development perturbs cardiac development and increases heart defects observed at embryonic day (E) 17.5 with a statistically significant peak (maximal effect) when embryos were exposed at E9.5[53]. This risk for heart defects is also dependent on the intensity of the stressor. For example, severe depletion of oxygen (5.5%) is associated with defects in ~50% of embryos. In contrast, mild hypoxia (8%) causes heart defects in ~0-12.5% of embryos; while exposure to >9% oxygen does not cause heart defects[53,54]. Thus, the heart is most susceptible to severe stress during the early stages of heart development. Identifying how the early fetal heart responds to maternal obesity and the association between the severity of obesity and early cardiac responses may help elucidate how long-term disease risk trajectory is established in the progeny.

## 1.3.Overview of cardiac development

Cardiac development is a tightly regulated multistep process that is highly conserved across species. Misregulation of this process can lead to immediate detrimental outcomes, such as embryonic lethality, or can have long-term consequences on adult health. Understanding the

events that occur during early development and their outcomes can help elucidate potential implications of maternal obesity on the offspring's immediate and long-term cardiovascular health.

The heart is one of the first organs to form and function in the developing embryo. Cardiac progenitor cells (CPCs), which are the precursors of cardiomyocytes, endothelial cells, and smooth muscle cells[55], originate from the anterior part of the primitive streak[56]. They migrate anterior-laterally during gastrulation to form bilateral mesodermal cardiogenic plates at E7, in mouse development, located in the lateral plate mesoderm (known as the cardiogenic mesoderm)[56] (**Fig. 2a, b**). CPCs are subdivided into two populations: the first and second heart field (FHF and SHF, respectively). The heart fields are adjacent along the anterior-posterior axis and represent early and late migrating cells from the primitive streak[55,57]. These cells migrate medially and converge in the midline. Cells from the FHF form the cardiac crescent at E7.5 which fold ventrally, concomitant with foregut closure, and fuse on the anterior-posterior axis to create the heart tube at E8.0[57] **(Fig. 2c, d)**. The SHF occupies a medial-posterior territory to the cardiac crescent and is delayed in differentiation. Cells of the SHF are added to the heart tube later, at the inflow and outflow poles, to elongate the heart tube (**Fig. 2c, d**)[55,57]. By E8.5 the beating heart tube establishes the primitive circulation with blood flow in the embryonic body restricted to the developing vasculature of the head, aorta and the forming cardinal vein[58]. Chorioallantoic attachment also occurs at E8.5 establishing the connection between the fetus and placenta[59,60]. Blood flow to the placenta (via the umbilical artery) and to the yolk sac (via the vitelline artery) is detected as soon as these connections are established at E8.5[61], coinciding with the initiation of the heartbeat. Thereafter the heart tube begins looping with a right-side rotation from the midline **(Fig. 2e)**. By E9.5 the primitive atria, LV and RV are formed, consisting of immature, beating cardiomyocytes and endothelial cells[57]. The LV is derived largely from the FHF, the majority of the RV and the outflow tract from the SHF, and the atria are derived from both lineages[57,62] **(Fig. 2f)**. The cardiac progenitors of the SHF continue to migrate from the second pharyngeal arch (also known as the second branchial arch) into the looping heart tube accounting for a second wave of cardiac cell addition into the heart **(Fig. 2e)**[63]. Fibroblasts, epicardium and coronary smooth muscle cell precursors migrate from the proepicardial organ at E9.5 **(Fig. 2e)**. Proepicardial cells migrate over the heart in a single monolayer forming the

epicardium[64,65]. Over the next two days, a subset of epicardial cells undergo epithelial-mesenchymal transition (EMT) and migrate into the heart where they differentiate into fibroblasts. At E11.5, the first cardiac fibroblasts apear[64,65].The conduction system begins to form concurrently, with proepicardial cell migrations. The sinoatrial node (SAN) is the first conduction system component that forms in the developing heart[66]. The SAN primordium is formed between E9.5-12.5 at the venous pole of the heart tube where it adopts the function as the primary pacemaker site. The ventricular conduction system (specifically, the bundle branches and Purkinje fibers) originate from cardiomyocytes of the trabeculae located just beneath the endocardium, at E10.5, coinciding with the initiation of atrial and ventricular septation[66]. The atrioventricular node (AVN) and the bundle of His form later, at ~E12.5. The primordia of AVN and bundle of His are distinct but both are found in the AVC at E9[66]. After septation is complete (E13.5), the heart continues to grow by both proliferation as well as hypertrophy of the cardiomyocytes[67,68] **(Fig. 2g)**. In humans, the heart develops through a similar chain of events. However, formation of the 4 chambers is completed at early-gestation (within the first 9 weeks of a 40 week gestation period) in humans as opposed to mid-gestation (within the first 13.5 days of a 21 day gestation period) in mice[69] **(Table 1)**.

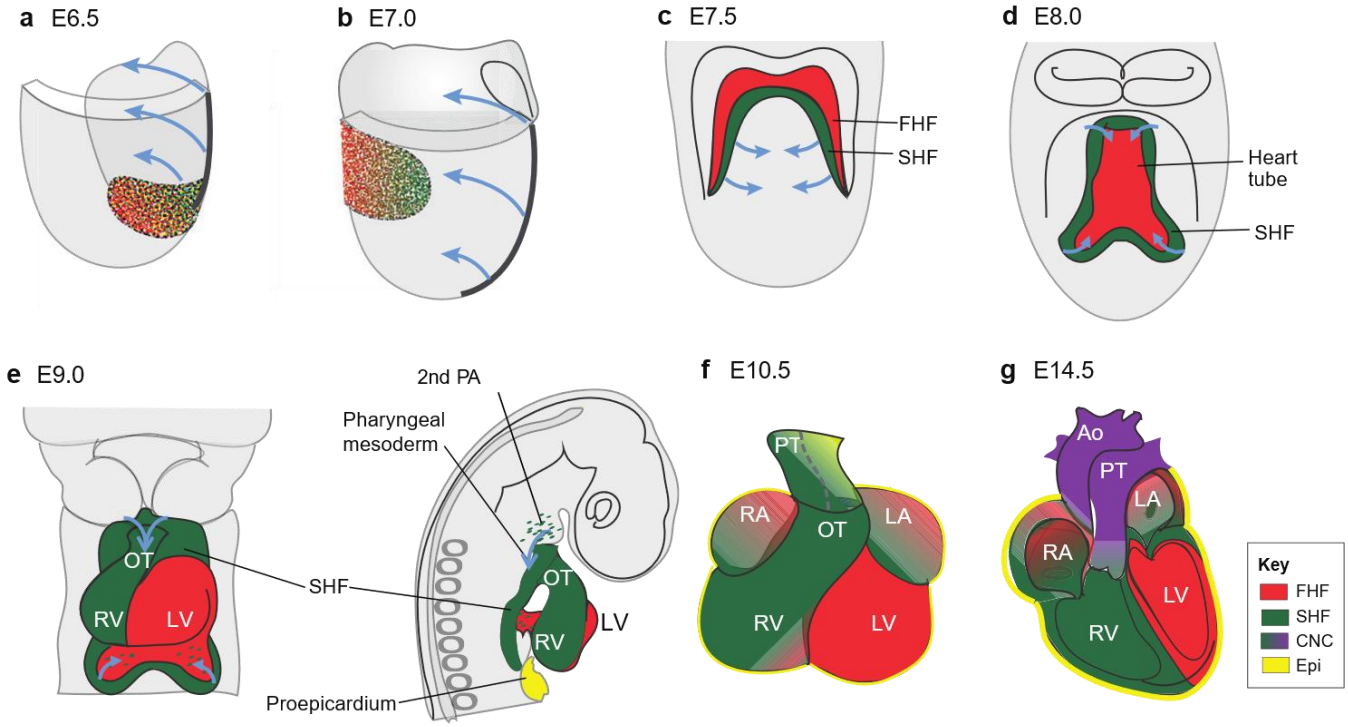



**Figure 2. Early cardiac development in the mouse embryo.** a, b) During gastrulation cardiogenic mesoderm cells migrate laterally (arrows) away from the primitive streak (thick black line) c) The cardiac crescent is formed at E7.5 from the FHF. The SHF is posterior and medial to the cardiac crescent. d) Medial migration of cardiac progenitors forms the early heart tube at E8.0 e) Frontal and lateral views of the heart region at E9.0 showing rightward looping of the heart tube. SHF progenitors continue to migrate from the pharyngeal mesoderm and the second pharyngeal arches (Solid arrows). f) The heart chambers at E10.5 and g) E14.5 are shown. Cell lineage contributions of the FHF, SHF, cardiac neural crest (CNC), and epicardium (Epi) are denoted by red, green, purple and yellow, respectively. Ao, aorta; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; OT, outflow tract; PT, pulmonary trunk. Modified from [67].

Table 1. Parallel stages of cardiovascular development in mouse and human.

| Mouse developmental stage (E) | Human Carnegie stage | Human EGA (weeks) | Major morphogenetic events |
|---|---|---|---|
| 9.5–10.5 | 13–17 | 6 4/7–7 5/7 | Cardiac looping |
| 11.5 | 15–16 | 7 1/7–7 3/7 | Atrial septation, muscular interventricular septum formation, early outflow septation |
| 12.5 | 17–18 | 7 5/7–8 | Atrial septation, outlet ventricular septum formation, beginning of semilunar valve formation |
| 13.5 | 19–21 | 8 2/7–8 6/7 | Completion of membranous/inlet ventricular septum formation |
| 14.5 | 22 | 9 1/7 | All definitive major cardiac structures identifiable |
| 15.5–17.5 | 23 | 23 | Progressive myocardial compaction, AV and semilunar valve refinement |
| 17.5 to birth | No comparable stage | No comparable stage | Continued myocardial compaction, semilunar valve refinement |

Table 1 was reproduced from [69]. AV; atrioventricular; E, days post-conception; EGA, estimated gestational age.

## 1.4. Cardiac kernel transcription factors regulate gene networks during heart development

Cardiac progenitor differentiation, heart morphogenesis and maintenance of cardiac homeostasis throughout life are fine-tuned processes that require complex and strict regulation of transcriptional programs. These processes are orchestrated by a core set of conserved cardiac transcription factors that regulate development and also adult cardiac homeostasis[70] (discussed later). The earliest committed cardiac progenitors are marked by Eomesodermin (*Eomes*), which is expressed in the gastrulating embryo at ~E6. Eomes is a T-box transcription factor required for the formation of cardiovascular progenitors. Activation of the *Eomes* is triggered by high levels of TGF-β ligands from the adjacent epiblast and visceral endoderm tissue[71]. Eomes directly binds to and activates *Mesp1* transcription factor in the primitive cardiogenic mesoderm[72–75]. At E7.5, the *Mesp1* expressing mesoderm contributes to the heart as well as blood vessels, trunk and limb mesenchyme[76]. The cardiac precursors are further specified by the expression of a core set of cardiac transcription factors. This core set of transcription factors includes NK2 Homeobox 5 (Nkx2-5), GATA Binding Protein 4 (Gata4), myocyte enhancer factor-2 (Mef2) and T-Box Transcription Factor 5 (Tbx5), all of which are indispensable for proper development of the heart. They directly regulate the gene expression network that controls cardiac developmental processes, including terminal differentiation of cardiomyocytes, chamber identity, patterning boundaries and maturation of the heart. They form a highly conserved sub-circuit within the gene

regulatory network known as the cardiac "kernel"[77] and perform essential upstream functions **(Fig. 3)**. In the cardiogenic mesoderm, *Gata4* marks a subset of *Mesp1* expressing cells in the early embryo that contribute to the heart[2]. As a pioneer factor, Gata4 binds condensed chromatin regions associated with inactive transcription and displaces histones, thus "opening" heterochromatic regions. Through its activity as a pioneer transcription factor (TF), Gata4 can initiate the formation of regulatory complexes and promote the activation of cardiomyocyte-specific genes[78–80]. Loss of a *Gata4* (and its closely related *Gata6*) results in loss of expression of other kernel TFs, such as Nkx2-5, leading to acardia in mice[81,82]. Nkx2-5 is a key node in cardiac kernel regulates the function of other kernel TFs throughout cardiac development **(Fig. 3)**. It is essential for the proper assembly of kernel TF complexes[83,84]. It mediates the cooperative binding of kernel TFs to form complexes that mediate proper cardiac development and function[83,84]. Indeed, NKX, TBX, and GATA homologs in *drosophila* converge on the same set of mesodermal enhancers by cooperative co-recruitment[83]. Their functions in regulating the expression of cardiac gene networks is dependent on their ability to co-recruit each other[83]. In summary, development of the heart is coordinated by key transcription factors that engage in Nkx2-5-dependent physical and functional interactions to establish transcriptional programs controlling developmental and postnatal homeostasis in the heart.

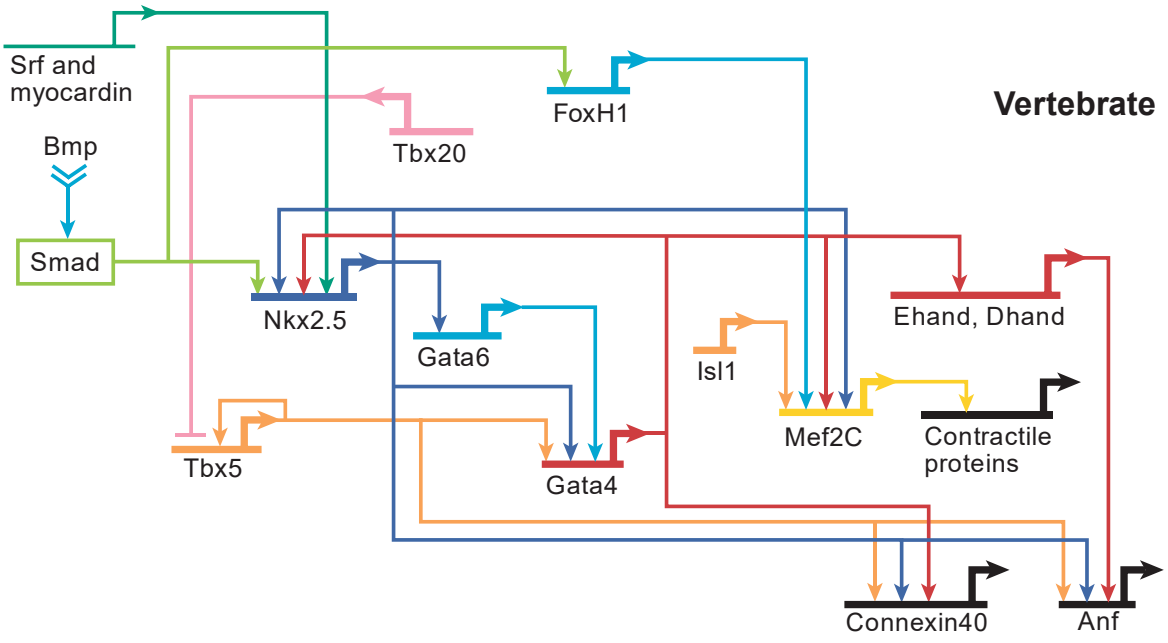



**Figure 3. Putative cardiac gene regulatory network kernel.** The network is obtained from [77] and was assembled from several literature sources (refer to [77] for a detailed description of how

this network was generated). The GRN that underlies heart-field specification in vertebrates is shown. A core set of regulatory genes are used and linked in a similar way across species.

## 1.5. Function of Nkx2-5 in cardiac development and disease

*NKX2-5* is the most commonly mutated single gene in congenital heart disease accounting for ~4% of cases[85]. It is also associated with onset and susceptibly of adult cardiomyopathy. Over 40 mutations of the *NKX2-5* gene have been identified in patients with cardiovascular disease and various ages of onset. NKX2-5 is mutated in patients with congenital and adult-onset disease with diverse clinical phenotypes such as septal defects, tetralogy of Fallot (TOF), patent ductus arteriosus (PDA), hypoplastic left heart, and dilated cardiomyopathy[85,86]. *Nkx2-5* is expressed in the developing heart and throughout adulthood, but its functions are dynamic and temporally regulated. It is essential for cardiac morphogenesis, cardiomyocyte growth during development, conduction and contraction in fetal life, and its function also determines adult cardiac homeostasis and cardiac disease predisposition.

### 1.5.1. Nkx2-5 protein domains and their functions

Nkx2-5 belongs to the NK homeobox gene family of transcription factors which is highly conserved from *drosophila* to mammals. Nkx2-5 contains three protein domains: a homeodomain, an NK-2 domain and a TN domain[87,88] **(Fig. 4)**. The homeodomain mediates interaction with specific DNA motifs. It is also the target for phosphorylation, which alters its DNA binding affinity[89]. It is worth noting that the protein sequence of the NK-2 homeodomain is highly conserved amongst the NK-2 family of proteins and the consensus DNA-binding sites within the NK-2 family are only subtly different[90]. Binding of NK-2 factors to DNA targets is further specified by protein binding partners[90]. Although Nkx2-5 can bind target DNA as a monomer, it preferentially binds as a homo- or heterodimer[91]. The interaction with binding partners is mediated by both the homeodomain as well as the NK-2 domain. The NK-2 domain also has an auto-repressor function. Interactions with other binding partners such as Gata4 are thought to induce a conformational change that displaces this auto-inhibitory domain[92,93]. The TN-domain interacts with the Groucho family co-repressors to repress genes[94]. Thus, the function of Nkx2-5 as a repressor or activator, and the specificity of its DNA binding are

determined by its autonomous DNA binding motif specificity, phosphorylation of its homeodomain, and its interaction with different partners.

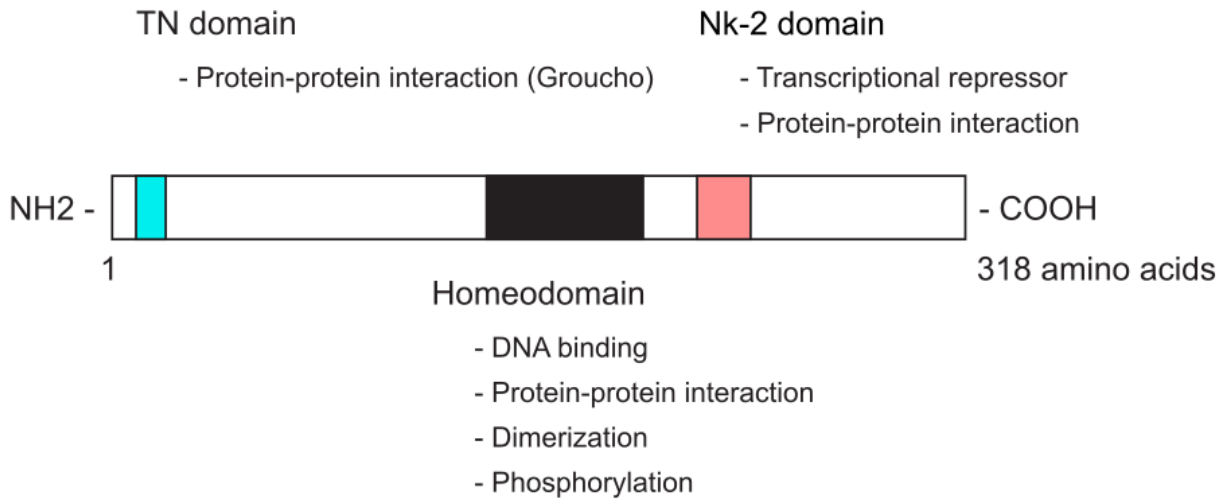



**Figure 4. General domain structure and function of Nkx2-5.** The homeodomain is indicated by a black box, TN domain with a blue box, and NK-2domain with a red box. Figure modified and updated from [87].

### 1.5.2. Function of Nkx2-5 in cardiac development

Nkx2-5 is expressed in the heart throughout development, but it has distinct temporally defined functions **(Fig. 5)**. Conditional knockout models have elucidated the effects of the complete loss of *Nkx2-5* at discrete stages of development, highlighting a requirement of Nkx2-5 throughout gestation for proper development of the heart.

The essential function of Nkx2-5 in specification of mesodermal progenitors towards the cardiac lineage was first reported in *drosophila*. Mutants for the *Nkx2-5* homolog "tinman" completely lack the cardiac primordium in the embryo[95]. In contrast, cardiac progenitors are specified, and the heart tube forms in *Nkx2-5* mutant mice. However, proliferation of SHF progenitors is significantly decreased. This reduces the contribution of SHF to the heart tube resulting in a grossly truncated OFT, an underdeveloped RV and lethality at E9-10[96]. Nkx2-5 has a dual function as an activator and repressor during these early stages of CPC specification and differentiation. Nkx2-5 activates several regulators of cardiomyocyte differentiation and proliferation such as homeodomain only protein (*HOP)*[97] as well as cardiomyocyte structural

genes such as ventricle specific Myosin light chain 2 (*MLC2V)*[98]. It also mediates the establishing of left/right (L/R) asymmetry by activating Heart And Neural Crest Derivatives Expressed 1 (Hand1)[99]. However, such functions are not required for looping morphogenesis of the heart. Its functions as a repressor are causally implicated in the defective cardiac looping of mutant embryos. Nkx2-5 represses several genes controlling cardiac specification and the maintenance of CPCs in the progenitor state, such as *Isl1*, *Mef2c, Tgfβ2, Bmp2* and *Smad1*[96]. Loss of *Nkx2-5* increases expression of these genes within the cardiac crescent and heart tube at E7.5-8.5, resulting in over-specification of mesodermal cells towards the cardiac lineage and an initial increase in CPCs[96]. Overspecification increases BMP2/SMAD1 signaling in the SHF which subsequently reduces proliferation of SHF. Reduced SHF contribution to the developing heart results in truncation of the OFT and impairs looping of the heart tube at E9-10[96]. Misregulation of the Nkx2-5-BMP2-SMAD signaling axis in CPCs can also cause long-term changes in the heart which results in disease and lethality at later stages of development. Nkx2-5 hypomorphs die postnatally due to cardiac malformations. Notably, CPCs of hypomorphic embryos upregulate genes controlling cardiac specification such as *Bmp2,* at E7.75. Despite this early misregulation in the gene expression of CPCs, these hypomorphic embryos develop mild phenotypes at E8.5 such as a slight OFT truncation but severe phenotypes manifest at later developmental stages, such as septal defects at E14.5. Inhibition of the BMP2/SMAD1 signaling in CPCs reduces the severity of late-onset cardiac malformations, such as septal defects. Thus, reduced levels of *Nkx2-5* during early development leads to a failure to repress genes controlling cardiac specification which can have immediate or late consequences during cardiac development.

After cardiac looping morphogenesis, Nkx2-5 is essential for formation, maturation, and maintenance of the conduction system[100]. Conditional deletion of *Nkx2-5* at later developmental stages, in committed ventricular cardiomyocytes (after E8)[101] or at mid-gestation[102], causes conduction defects. The functions of *Nkx2-5* in the conduction system are multifaceted. *Nkx2-5* dosage determines the number of myocytes that form the conduction system[100]. Heterozygous deletion of Nkx2-5 causes reduction in AVN, the bundle of His and Purkinje fibers in mid-gestation hearts[100]. Complete loss of Nkx2-5 completely abolishes the AVN primordium. Nkx2-5 is also essential in maintenance of the cells in the conduction system after it is established, in the

postnatal heart[100,101]. Targeted homozygous deletion of Nkx2-5 in the ventricular myocardium and conduction system, at E8.75, causes gradual postnatal degeneration of the AVN, cell dropout and fibrosis in the conduction system[101]. Nkx2-5 also has a direct role in activating genes encoding ion channels in cardiomyocytes[102]. Loss of Nkx2-5 reduces expression of ryanodine receptor 2 (RyR2) and sarco-endoplasmic reticulum $Ca^{2+}$ ATPase 2a (Serca2a), Nav1.5-α subunit (Scn5a), Kcne1/minK, Connexin 40 (Cx40), T-type $Ca^{2+}$ channels (α1H, α1G), and several others necessary for electric polarization of cardiomycytes[102]. Thus, once CPC specification and differentiation has begun, embryonic expression of Nkx2-5 is required for activating ion channel gene expression, determining the cell numbers of the conduction system, and maintenance of the conduction system.

Conditional mouse models have also enabled precise temporal assessment of Nkx2-5 function in the postnatal heart. Inactivation of Nkx2-5 in the perinatal heart (E19-P2) using tamoxifen-inducible recombination to knockout *Nkx2-5* in mice results in prolonged PR interval and QRS duration, occasional AV blocks, reduced cardiac function and increased cardiac weight and dilation. These phenotypes are detected within 4 days after Nkx2-5 deletion and ultimately cause 50% lethality in the first 3 weeks of life. Within these 3 weeks, the expression of several ion channels including RyR2, Scn5a, KcneI/minK, T-type Ca2+ channels (α1H, α1G) and Cx40, decreases rapidly and progressively with age in the knockout mice. Nkx2-5 directly activates genes encoding cardiac ion channels, such as Scn5a, by direct binding at the proximal promoter[103]. Loss of *Nkx2-5* at P14 also results in the same conduction and functional defects and mild hypertrophy. Notably, deletion at P14 does not cause lethality and is associated with a delay in disease onset and reduced severity. Phenotypic abnormalities emerge over a span of 7 weeks in the P14 knockouts, compared to only 4 days when *Nkx2-5* is lost perinatally. The decrease in ion channel gene expression is also less severe in magnitude and occurred over 7 weeks. It is hypothesized that the delayed transcriptional reprogramming of the Nkx2-5 targets is due to the more compact chromatin structure in older mice, which may delay transcriptional reprogramming. Overall, complete loss of *Nkx2-5* at postnatal stages alters gene expression of critical downstream targets, such as ion channels, leading to progressive conduction and contractile defects.

### 1.5.3. Reduced function of Nkx2-5 during development leads to misregulation of gene expression and the onset of disease in the adult heart

Maintenance of proper control of transcriptional gene networks during cardiac development is essential for adult cardiovascular health and homeostasis. *Nkx2-5* mutations do not affect the morphology or function of the heart in a temporally isolated event but have continuous and progressive negative effects. Disrupting the functions of Nkx2-5 and its resulting developmental outcomes compound to its consequences in the adult heart. The importance of proper cardiac development for adult cardiac health is highlighted by the increased risk for cardiac dysfunction in individuals with congenital heart disease despite the success of corrective interventions[104]. Postnatal inactivation of Nkx2-5 bypasses its requirement for cardiogenesis, and the resulting phenotypes do not account for the long-term effects of disrupting Nkx2-5 function in the fetal heart. Therefore, this approach provides limited insights into the mechanisms of action of Nkx2-5 in adult-onset disease predisposition. Several hypomorphic Nkx2-5 mutations have been used to study the long-term effects of Nkx2-5 dysfunction on the heart. These studies have demonstrated that reduced Nkx2-5 function throughout development causes mild impairment in adult cardiac function but severe cardiomyopathy in response to stress.

#### 1.5.3.1. Mouse models of *Nkx2-5* hypomorphic mutations

Due to the early lethality of embryonic knockout models of Nkx2-5, hypomorphic and heterozygous mutations have been used to better understand the adult consequences of long-term disruption of *Nkx2-5* function. Mouse models recapitulating familial, heterozygous *NKX2-5* patient mutations[104,105] or overexpressing a dominant-negative NKX2-5 (DN-NKX2-5)[106] have been used to elucidate the mechanisms underlying adult-onset cardiomyopathy. The hypomorphic patient mutation and DN-NKX2-5 have reduced DNA binding activity but can still bind interacting proteins. Therefore, both mutations reduce the binding of NKX2-5-containing complexes to target genes and sequester wildtype Nkx2-5 and interacting proteins[106,107]. The expression of NKX2-5 target genes is decreased in the heart throughout the fetal and adult life in both models[106,107].

### 1.5.3.2. Long-term disruption of Nkx2-5 disrupts expression of metabolic regulators and ion channels in the adult heart

In both Nkx2-5 hypomorphic models discussed above, the LV function is mildly affected[106], or is functionally compensated in early adulthood[104,105]. However, fibrosis is increased and there is predominant impairment of the structure, number and function of mitochondria[104–106]. Adult Nkx2-5 hypomorphic hearts dysregulate the mRNA expression of genes associated with ion handling and mitochondrial function, as assessed using unbiased transcriptomic approaches[104,105]. Nkx2-5 directly binds and regulates the expression of genes encoding ion channels and metabolic regulators, including fatty acid metabolism, insulin signaling and carbohydrate metabolism, in the adult heart[104]. It is important to note that despite misregulation of metabolic pathways, basal and maximal respiration capacities are reduced dramatically, while ATP production is not. The respiration levels of *Nkx2-5* hypomorphic cardiomyocytes are close to maximum capacity under baseline conditions, which reduces respiratory reserve[105]. Respiratory reserve allows the heart to increase ATP production in response to a sudden increase in energy demand[108,109]. Reduction in respiratory reserve sensitizes the heart to sudden increases in energy demand and increases the risk of cardiomyopathy[108,109]. Altogether, this evidence suggests that Nkx2-5 regulates cardiomyocyte metabolism, and that reducing its function limits the respiratory reserve, thus potentially compromising the response of the heart to stress.

### 1.5.3.3. Cardioprotective function of Nkx2-5

Nkx2-5 increases susceptibility to stress in the fetal and adult heart. Exposure of *Nkx2-5* heterozygous mice to transient (8 hr) severe hypoxia at E9.5 is associated with worse cardiac outcomes compared to wildtype mice including lower heart rates and higher frequency of severe heart defects[110]. Notably, increased susceptibility to stress is not common to heterozygous mutations of other cardiac kernel TFs. Loss of one *Tbx5* allele does not increase susceptibility to cardiac defects during gestational hypoxia[110]. This suggests a specific function of Nkx2-5 in modulating the response of the fetal heart to stress. Adult hearts of Nkx2-5 hypomorphs are also more severely impacted by various stressors including obesity or cardiotoxic agents, compared to wild-type mice. Long-term feeding with a high fat diet (24 weeks) reduces LV function and augments hypertrophy in mice bearing hypomorphic *Nkx2-5* patient mutations, compared to

wildtype mice[104]. Genes regulating cardiac hypertrophy and several TFs known to interact with Nkx2-5 such as Gata4, Hand2 and Mef2, are upregulated after only 9 weeks of high fat diet consumption and prior to the cardiac functional decline in Nkx2-5 hypomorphs. Metabolic pathways, including regulators of fatty acid and glucose metabolism are also dysregulated, leading to an altered cardiac metabolome[104]. This indicates that misregulation of Nkx2-5 complex components and downstream metabolic targets precedes functional decline in obese mice bearing the Nkx2-5 mutation. Nkx2-5 hypomorphs are also sensitized to doxorubicin-induced cardiotoxicity. Decrease in cardiac function is augmented in DN-NKX2-5 after treatment with doxorubicin compared to treated controls[106]. This augmented response is associated with increased damage to the myofibrillar structure, higher cell death, and an exacerbated deregulation of several doxorubicin-responsive genes[106]. This indicates that altering *Nkx2-5* function during cardiac development and throughout adult life increases cardiac susceptibility to adult stressors.

In summary, NKX2-5 is required for mounting the appropriate cardiac response to fetal and adult stressors. Reduced Nkx2-5 function exacerbates pathological consequences to the heart in response to environmental insults. Importantly, whether increased adult-onset disease susceptibility in hypomorphic mice is due to altered function of Nkx2-5 in the adult heart or has embryonic origins is not known.

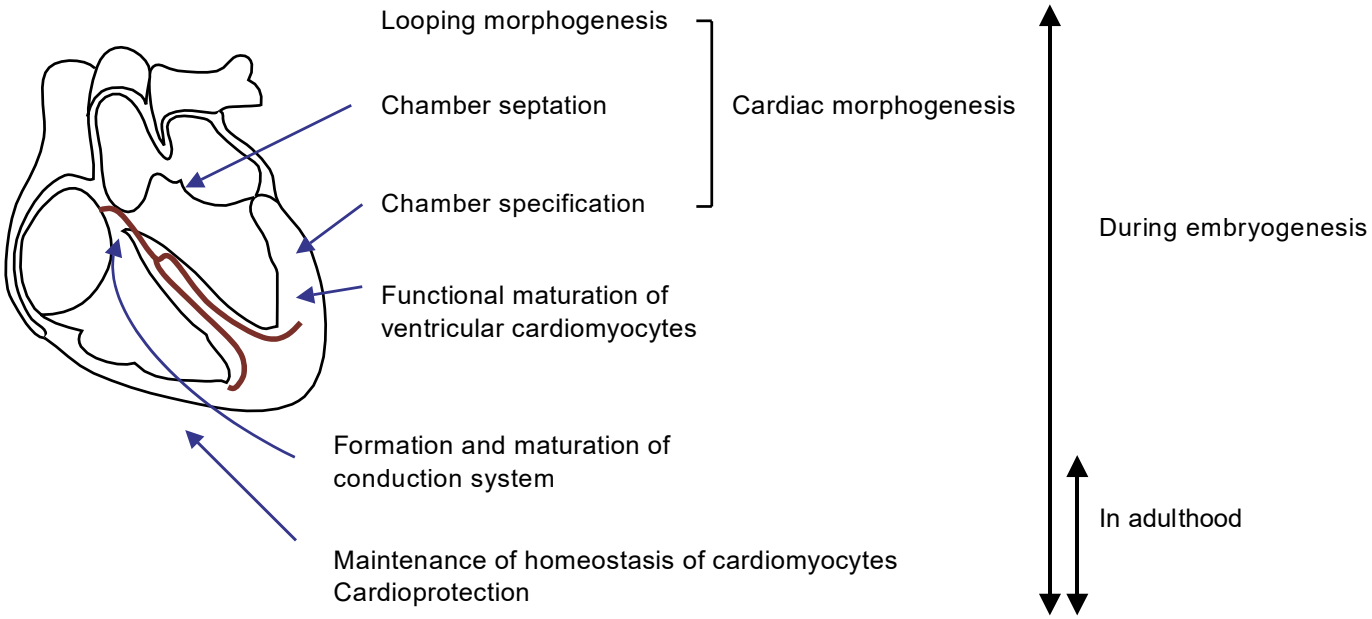



**Figure 5. Functions of Nkx2-5 in the fetal and adult heart. N**kx2-5 regulates gene expression in the heart throughout life and is essential for proper cardiac developmental and postnatal homeostasis. Figure is adapted and modified from [87].

### 1.5.4. Environmental stress alters Nkx2-5 function which increases cardiac disease incidence

Environmental-genetic interactions account for ~90% of the etiological basis for developmental abnormalities of the heart[111]. Mutations in *NKX2-5* predispose carriers to variable clinical phenotypes (mentioned in **section 1.5**) even within the same family. The same nucleotide mutation can also lead to variable cardiac malformations and severity in backcrossed mice with nearly identical genetic background[112] suggesting that non-genetic disease modifiers influence disease trajectory. Recent evidence in mouse and tissue culture models indicates that the expression and functions of Nkx2-5 are responsive to environmental stimuli.

The response of Nkx2-5 to stress can determine the disease outcome for the heart. Exposure of *Nkx2-5* heterozygous mice to transient (8 hr) severe hypoxia at E9.5 leads to a reduction in *Nkx2-5* mRNA and protein expression at E10.5 and increased incidence of cardiac

malformation[110]. Mild persistent gestational hypoxia beginning at mid-gestation (E10.5) in wild-type mice also leads to a 50% reduction in Nkx2-5 protein levels, and a reduction in its total protein phosphorylation[113]. These changes in Nkx2-5 are associated with misregulation of Nkx2-5 targets. 60% of misregulated genes in E12.5 wildtype hypoxic hearts overlap with those in *Nkx2-5* mutant normoxic hearts[113]. In addition, the downstream effects of fetal hypoxia overlap with fetal outcomes of *Nkx2-5* mutations. Fetal hypoxia increases the incidence of cardiac malformations such as VSD and ASD, which are also present in heterozygous *Nkx2-5* mutant mice[113]. This indicates that Nkx2-5 and its downstream targets are misregulated in the stressed fetal heart and may mediate subsequent cardiac phenotypic consequences. Notably, hypoxia is also associated with a progressive increase in collagen deposition with age, increased heart size, and reduced functional recovery from ischemic-reperfusion injury[114]. This highlights the age-dependent progression of pathological responses and deregulated stress responses that occur when Nkx2-5 downstream targets are misregulated.

Nkx2-5 also responds to and regulates stress responses in the adult heart. Hyperglycemia in adult zebrafish increases the expression of *Nkx2-5* in the heart. *Nkx2-5* activates downstream pro-hypertrophic and pro-apoptotic genes such as *Calreticulin* and *P53* which increase cardiomyocyte hypertrophy and apoptosis[115]. Diabetes in mice increases O-GlcNAcylation of Nkx2-5. This excessive O-GlcNAcylation decreases Nkx2-5 protein levels in the heart[116]. *In vitro* experiments, using neonatal rat cardiomyocytes, have also demonstrated that *Nkx2-5* is downregulated in response to several stressors including doxorubicin, $H_2O_2$, paraquat, and etoposid[117]. Upregulation of Nkx2-5 in these stress conditions, using adenoviruses containing Nkx2-5 over-expression vectors, can protect against cell death whereas downregulation exacerbates cell death[117]. Thus, environmental stressors in the postnatal heart alter the expression of Nkx2-5 and its post-translational modifications which can lead to dysregulation of genes and pathways that control cardiac stress responses.

In summary, Nkx2-5 is responsive to environmental stimuli including diabetes, hyperglycemia, hypoxia and several others. Altered function of Nkx2-5 mediates the stress response in the fetal and adult heart. However, whether Nkx2-5 function is altered during maternal obesity and how altering Nkx2-5 in the fetal heart could mediate a long-term predisposition to adult-onset heart disease is not known.

### 1.5.5. Nkx2-5 target selection and function are determined by its interacting partners

Like many cardiac TFs, the function of Nkx2-5 is dependent on combinatorial interactions with activators or repressors[118]. Studies in *drosophila* have found that NKX, TBX, and GATA homologs converge on the same set of mesodermal enhancers by cooperative co-recruitment **(Fig. 6)**. Activation of mesodermal gene targets does not require the consensus motifs of all TFs to be present, instead, high-affinity motifs for a subset of these factors can initiate recruitment of all the other factors likely through protein-protein interactions[83]. In mouse CPCs, Nkx2-5 physically interacts with Tbx5 and Gata4 to regulate cardiac gene expression. Homozygous deletion of Tbx5 in CPCs induces a redistribution of Nkx2-5 across the genome[84]. Nkx2-5 is also essential for proper gene targeting of heterotypic complexes. Loss of Nkx2-5 alters the binding of both Tbx5 and Gata4 to their targets resulting in failure to activate ion channels and other genes involved in cardiac differentiation[84]. In addition to this loss of ability to bind known targets, Gata4 and Tbx5 also aberrantly target and ectopically activate several genes in CPCs when Nkx2-5 is lost[84]. Gata4 targets genes encoding ECM components while Tbx5 targets genes regulating heart development and neuronal development in Nkx2-5 KO cardiomyocytes[84]. Thus, Nkx2-5 is crucial for proper targeting of heterotypic TF complexes during heart development. In addition, Nkx2-5 also indirectly regulates several genes by regulating the expression of other transcription factors. For example, Nkx2-5 can indirectly repress genes by turning on transcriptional repressors Carp and Hop[119]. Thus, gene misregulation induced by Nkx2-5 mutations is not only due to the lack of Nkx2-5 binding to its direct targets genes or altered interaction with coregulators, but also to the redistribution of other transcription factors across the genome.

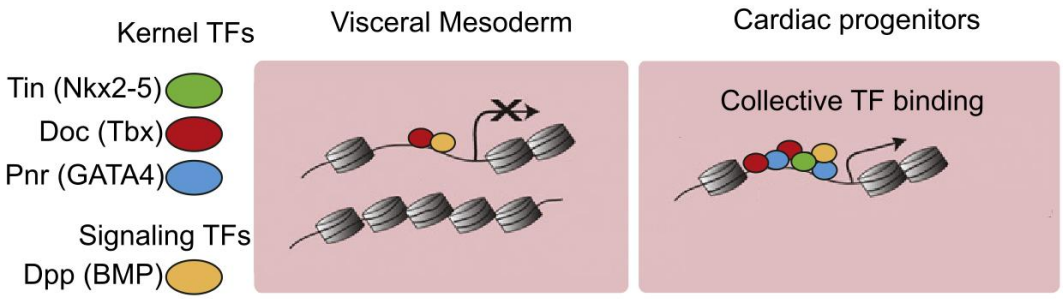



**Figure 6. Model of co-operative TF regulation during cardiac development.** Schematic representation of gene expression regulation in visceral mesoderm and cardiac progenitors. Visceral mesoderm enhancers/promoters (left) contain motifs that recruit a subset of the kernel and signaling TFs to establish mesodermal identity. In contrast, all the cardiac kernel TFs cooperatively occupy enhancers/promoters of cardiac-specific genes in cardiac progenitors. Names of TFs in drosophila and their mouse orthologues are shown on the left. Figure is modified from [83].

The precise regulation of Nkx2-5 function depends on the spatial and temporal expression of other transcription factors and ubiquitous factors[118]. T-Box Transcription Factor 2 (Tbx2) is a transcriptional repressor expressed exclusively in the atrioventricular canal, outflow tract, and inflow tract. Tbx2 physically interacts with Nkx2-5, displacing the transcriptional activator Tbx5 from the protein complex **(Fig. 7)**. This interaction between Nkx2-5 and Tbx2 mediates tissue-specific repression of *ANP*[120] **(Fig. 7)**. Similarly, by interacting with the Groucho family of co-repressors via the TN-domain, Nkx2 proteins can also repress their target genes[94,121]. In the adult heart, Nkx2-5 mediates repression of ion channels when interacting with Tbx3 (another transcriptional repressor) in the conduction system, but activates the same target genes when interacting with Tbx5[122]. Thus, the function of Nkx2-5 as an activator or repressor is determined by interaction with binding partners whose expression is spatially and temporally restricted.

In summary, decreasing the levels or function of Nkx2-5 alters the composition, target recognition and transcriptional output of heterotypic complexes and it also alters the expression of other TFs. The transcriptional consequences can vary broadly across developmental times and in different cardiac structures, depending on the spatial and temporal expression of NKX2-5 interacting proteins.

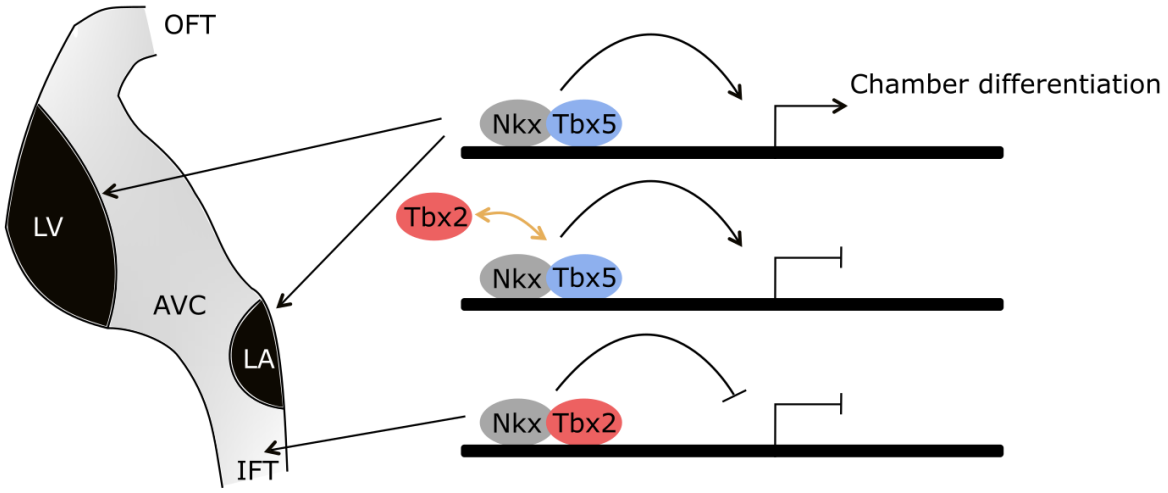



**Figure 7. Mechanism for differential regulation of Nkx2-5 function by Tbx factors in the cardiac chambers.** Tbx5 and Nkx2-5 form an activator complex that stimulates cardiac gene expression in chamber wall myocardium. Tissue-specific expression of Tbx2 in the inflow tract, atrioventricular canal, and outflow tract spatially restricts formation of a repressor complex that suppresses chamber-specific gene expression. IFT: Inflow tract. LA: left atrium. AVC: atrioventricular canal; LV: left ventricle; OFT: outflow tract. Figure is modified from [120].

## 1.6. Transcriptional dysregulation during embryonic development can alter long-term gene expression in the heart, leading to adult-onset cardiac disease

Misregulation of core developmental gene networks can have immediate and long-term consequences on cardiac health. Gene misregulation during fetal development can cause cardiac malformation which can lead to congenital heart disease or embryonic lethality. In addition, several studies have shown that failure to establish cardiac gene expression networks during development can have long-term effects on the transcriptome of the adult heart leading to adult-onset cardiac disease. Gene misregulation in the adult heart that originates in response to developmental programming can be caused by two mechanisms.

First, aberrant activation or repression of genes regulating specific pathways in the developing heart can persist into adulthood. For example, reduction of repressive histone

methylation in CPCs at E7.5, due to deletion of the histone methyltransferase *Ezh2*, causes a failure to repress "early" cardiac gene networks. Activation of the "early" cardiac gene network does not impair cardiac development. However, these genes are persistently activated in the adult heart leading to the adult-onset of hypertrophic cardiomyopathy[123]. Similarly, loss of PITX2 transcription factor at E8.5 causes persistent misregulation of sodium and potassium channels in the embryonic and adult atria, leading to adult arrhythmias in the hearts[124,125]. Thus, aberrant expression of a common gene set in the fetal and adult heart can have long-term consequences on adult cardiac health.

Second, misregulation of upstream regulators of gene expression can cause distinct transcriptional responses at different developmental stages and in different cell types. Feuer *et al* analyzed the effect of several forms of stress on gene expression in mouse zygotes cultured *in vitro* and then implanted into surrogate mothers[126]. Although ~300, ~400, and ~1300 genes were misregulated in the blastocytes, inner cell mass(ICM), and adult hearts, respectively, in response to zygotic stress, less than 2% of the genes misregulated in adult hearts overlapped with those misregulated ICM or blastocytes. Over half of those shared genes between stages were altered in opposite directions. Genes misregulated in the adult heart were highly enriched for disease-related pathways, particularly inflammatory pathways, and were associated with subtle left ventricular hypertrophy[127]. Genes misregulated in early embryos were enriched for cell cycle, growth and pluripotency functions. However, the upstream regulators of the misregulated genes in the blastocysts, ICM and adult hearts were shared, including genes such as *TGFβ1* and *TGFβR2*, implicated in development and adult cardiac disease. Similarly, the long-term transcriptional responses in other tissues including skeletal muscle, liver, pancreas and fat shared very little overlap when compared to the transcriptional responses of early embryos or amongst each other. However, upstream regulators of the misexpressed genes were shared in both adult and embryonic stages, despite this limited overlap in the identity of misregulated genes. This indicates that altering the function of a common upstream regulator can impart a unique transcriptional signature that is tissue-specific and time-dependent.

In summary, misregulation of gene expression networks during early embryonic development can have a long-term impact on the transcriptome in the adult heart. Altering the function of a few upstream regulators of gene expression may result in unique transcriptional

signatures in each developing tissue in accordance with unique, tissue-specific, and temporally regulated developmental cues. Alternatively, broad misexpression of genes regulating disease-related pathways can persist in fetal and adult life. Whether obesity during pregnancy alters embryonic gene expression broadly and persistently or if specific upstream regulators of gene expression are responsive to maternal obesity is not known.

## 1.7. Signaling pathways disrupted in the fetal and adult heart in response to maternal obesity in animal models

The effects of maternal obesity on the gene expression of fetal and adult hearts have not been thoroughly investigated using global unbiased "omics" approaches. However, several studies have provided evidence for both persistent as well as temporally defined changes in the expression of genes regulating disease-related pathways. I have reviewed all these pathways and their consequences in [25]. Here, I will focus on three specific pathways: TGF-beta signaling, inflammation, and metabolic pathways - summarized in **(Fig. 9)**.

### 1.7.1. Transforming growth factor beta (TGFβ) signaling in the heart

#### 1.7.1.1. TGFβ signaling in the developing and adult heart

Transforming growth factor beta (TGFβ) signaling regulates multiple processes during cardiac development, including specification, differentiation, proliferation, and hypertrophy of cardiomyocytes[128]. TGFβ signaling is required for proper formation of the heart[128], and it also maintains adult cardiac hemostasis[128–131]. Recent evidence from animal models has highlighted altered TGFβ signaling in fetal and adult hearts in response to maternal obesity.

TGFβ signaling regulates specification and proliferation of CPCs in the gastrulating embryo. At E5.5-6.5, epiblast and visceral endoderm tissue secrete the TGF-β ligand Nodal which induces the cardiac mesoderm[72]. From E7.5-9.5, TGFβ signaling acts in a negative feedback loop with Nkx2-5 to regulate the expression of genes controlling cardiac specification[96]. At E7.5, TGFβ signaling activates genes regulating CPC specification and determines the number of CPCs in the cardiac crescent and SHF[96]. Increased BMP2 ligand in CPCs activates SMAD1 which increases mesodermal specification towards the cardiac lineage. At E8.5-9.5, BMP2/SMAD1 inhibits proliferation of the SHF[96]. Expression of TGFβ ligand BMP10 at E9.5-

12.5 in the trabeculae mediates the hypertrophy and trabeculation of the ventricle. Aberrant upregulation of BMP10 mediates pathological hypertrophic cardiomyopathy and hypertrabeculation phenotypes in postnatal viable models of ventricular restricted loss of Nkx2-5[101]. These examples highlight the heterogeneous expression of various TGFβ ligands and their downstream functions that vary at different stages in development and in different tissues. In adult hearts, over-activation of TGFβ is key for disease pathogenesis as it promotes cardiac fibrosis [132], hypertrophy [133] and cardiomyocyte cell death [134]. Indeed, it is considered a "master of all trades" due to its different functions in adult cardiac disease pathogenesis (reviewed in [131]).

#### 1.7.1.2. TGFβ signaling is activated in the heart of offspring in response to maternal obesity

In animal models of maternal obesity, TGFβ signaling is aberrantly activated in the fetal and adult heart. TGFβ protein levels are increased at mid and late gestation in the heart of fetuses of ewes fed an obesogenic *vs* a control diet[135]. The levels of phosphorylation of the Smad3 transcription factor, a downstream effector of TGFβ, increase during late but not mid-gestation [135]. Connective tissue growth factor (CTGF), a downstream TGFβ target that promotes fibrosis[136,137], is also increased in the hearts of fetuses of obese baboons, at late gestation[138]. This is consistent with increased fibrosis in the hearts of fetus of obese dams at late gestation[135,138], but when fibrous tissue deposition begins has not been explored. In the adult heart of the offspring of obese ewes, *TGFβ* mRNA levels are also increased[139]. However, whether increased *TGFβ* mRNA expression leads to increased signaling in the adult heart has not been investigated. Overall, despite increased expression of the TGFβ ligand throughout mid to late gestation and during adulthood, activation of some downstream signaling components, such as Smad3, is age-dependent, but largely unexplored. The temporal origins of TGFβ upregulation, and their downstream effects during early development and adulthood in the context of maternal obesity are not known.

### 1.7.2. Inflammatory signaling in the heart

#### 1.7.2.1. Inflammatory signaling in the developing heart

The onset of *in utero* inflammation due to pathological stimuli alters cardiac function and disrupts gene networks controlling cardiac development and disease[140,141]. Several TFs that mediate the inflammatory response, such as RelA, NEMO and IKKB (members of the NF-κB signaling pathway)[142] and their downstream proinflammatory cytokines such as TNFα[143], IL-1[144], IL-2[145]v and IL-6[146], are not required for cardiac development. Conversely, overactivation of such factors is detrimental to cardiac gene expression and function during development and in adulthood. Constitutive activation of the NF-κB pro-inflammatory signaling at ~E9.5 in the heart causes embryonic lethality, with about ~15% immediate lethality at E9.5[147]. Notably, lethality continuously increases throughout embryonic life in response to cardiac inflammation, with ~50% lethality by E12.5, and ~98% lethality at pregnancy term[147]. This indicates that inflammation does not interfere strictly with a single chronologically defined event, but rather with general conditions required for normal heart development throughout embryonic life. Inflammation also impacts fetal cardiac function. Induction of intrauterine inflammation by injection of LPS or bacteria in mouse or macaque compromises myocardial performance index and increases IVRT[140,141]. Inflammation also directly impacts cardiac gene expression. Increased cardiac inflammation disrupts the expression of genes regulating cardiac morphogenesis as well as several regulators of cell cycle, apoptosis and cardiac muscle contraction[141,147]. Although the long-term effects of fetal cardiac inflammation are not known, several conditions associated with fetal inflammation, including maternal obesity, cause long-term activation of inflammatory signaling in the heart and are associated with increased susceptibility to adult-onset cardiovascular disease.

#### 1.7.2.2. Inflammatory signaling in the adult heart

Cardiac inflammation is a common hallmark of adult-onset heart disease. Acute inflammation serves an adaptive function in early stages of cardiac injury, such as ischemic injury or pressure overload[148]. Inhibiting pro-inflammatory transcription factors, such as NF-κB, that bind and activate transcription of pro-inflammatory cytokines exacerbates cardiac dysfunction and increases apoptosis in the heart in acute models of cardiac injury[148]. This indicates an important role for acute inflammation in limiting cell death and thereby preserving cardiomyocyte number and funciton[148]. In contrast, chronic inflammation is detrimental to the

heart. Inhibition of inflammatory signaling in chronic models of cardiac injury increases cardiac function and organism survival by reducing cytokine expression, apoptosis and cell death-associated fibrosis[148,149]. Thus, regulation of inflammatory responses of the heart is crucial for maintenance of cardiac homeostasis.

#### 1.7.2.3. Inflammation is increased in response to maternal obesity

Maternal obesity is a chronic inflammatory condition that increases systemic and placental inflammation [150–152]. Although many inflammatory cytokines cannot cross the placenta [153,154], IL-6 levels are significantly higher in umbilical blood of obese women [155], suggesting that maternal obesity activates pro-inflammatory cytokine release from the fetus. Indeed, NF-κB is progressively activated in the fetal heart of obese ewes. Phosphorylation of inhibitor of nuclear factor kappa B (IκB), which mediates its degradation and subsequent activation of NF-κB, is significantly increased at late gestation in fetal hearts of obese compared to lean ewes [156] **(Fig. 9)**. Consistently, both mRNA and protein levels of several NF-κB targets and pro-inflammatory cytokines, such as IL1-a and IL-6, are increased in the heart at mid and late gestation, respectively [156]. However, while some pro-inflammatory cytokines increase, it is important to note that protein levels of other proinflammatory cytokines, such as IL-18, are reduced[156] **(Fig. 9)**. This suggests compensatory mechanisms might attenuate some inflammatory signaling during development. It is unknown if inflammatory pathways are persistently activated in the adult offspring of obese mothers if offspring are maintained on a healthy diet. However, in response to a short-term increase in caloric intake by the adult offspring, the mRNA and protein levels of TNFα and the proinflammatory marker CD68 are increased in the hearts of offspring of obese, compared to lean ewes[139] **(Fig. 9)**. However, the levels of several cytokines such as IL6, IL18 or TLR4 are not altered [139], which contrasts with their activation in fetal hearts [156]. This highlights that despite the activation of several pro-inflammatory cytokines in fetal and adult hearts of offspring of obese females, the identity of the activated cytokines differs at each stage. The regulation of inflammatory pathways, their temporal onset, and their impact on cardiovascular development and adult disease in offspring of obese mothers has not been investigated.

### 1.7.3. Metabolic signaling pathways in the heart

The heart undergoes drastic changes in energy metabolism during development, maturation, and disease. Cardiac metabolism is largely determined by two key factors: energy substrate availability and the activity of metabolic enzymes[157]. Both of these determining factors are altered during development and disease and are highly dependent on environmental factors. Maternal obesity alters substrate availability in the heart during development as well as the activity of key metabolic regulators. Cardiac metabolism is also persistently affected in adult hearts of offspring of obese mothers and is associated with increased cardiac disease susceptibility. However, the temporal onset of metabolic misregulation during development, the specific gene regulatory networks driving these changes in developing and adult hearts, and their impact on the adult heart are not known.

#### 1.7.3.1. Cardiac metabolism during development, adulthood, and disease

Cardiac metabolism is highly dynamic during fetal and adult life. The developing heart undergoes sequential maturation of its metabolic program beginning in early migrating cardiac progenitors **(Fig. 8)**. During early cardiac progenitor specification at E6.5 until E11.5, the heart mitochondria are immature[158]. Although the complexes that compose the electron transport chain (ETC) are present in CPCs, there is no coupling between the ETC and ATP generation[158]. Thus, while CMs are differentiating the early developing heart is completely reliant on glycolysis **(Fig. 8)**. At E11.5, the ETC and ATP begin coupling and by E13.5 the mitochondria are considered functionally mature and are indistinguishable from adult mitochondria[158]. From E13.5 to birth, glucose, lactate and fatty acids become the main substrates for mitochondrial oxidative metabolism[158]. By E18.5, over 50% of cardiac ATP is generated from aerobic oxidative metabolism[158] **(Fig. 8)**. After birth, the heart undergoes a shift from *in utero* hypoxia to normoxia. This is accompanied by a well-characterized metabolic shift towards fatty acid oxidation and a surge in mitochondrial biogenesis that mediates an increase in efficiency and capacity for generating ATP[158]. These changes are required to enable the heart to meet the increased workload and associated energy demand in postnatal life.

Although adult cardiac metabolism is relatively stable under normal conditions, the heart undergoes dynamic adaptive as well as mal-adaptive metabolic responses to stress. Several cardiac stressors, such as pressure overload or cardiac ischemia, increase cardiac glycolysis and reduce fatty acid oxidation[159–161]. The initial increase in glycolysis in response to stress leads to increased ATP production, and maintains ionic homeostasis in the heart. Failure to increase glycolysis exacerbates hypertrophy, fibrosis and cardiac dysfunction[162]. Thus, increased glycolysis initially serves as an adaptive response to stress. However, long-term maintenance of this metabolic shift is detrimental. Glycolysis during stress is uncoupled from glucose oxidation and thus leads to the buildup of lactate and protons, which promote cardiac injury. In addition, reduction in mitochondrial oxidative metabolism is a maladaptive response that ultimately results in ATP deficiency and reduced contractility[163]. This switch in myocardial metabolism during disease is the result of transcriptional deregulation in the genes encoding glycolytic and mitochondrial metabolic enzymes[164] as well as insulin resistance that develops at early stages of heart disease[164,165]. Reduced insulin signaling in the diseased heart precedes cardiac dysfunction and is an early marker of disease and a determinant of its progression[164]. Deregulation of insulin signaling can inhibit fatty acid oxidation and glucose oxidation. Insulin is also an important regulator of cardiac substrate selection and metabolism. It is important to note that such changes in cardiac metabolism (i.e. reduced fatty acid oxidation and increased glycolysis) are dependent on disease type and severity, and some stressors such as ANGII induced hypertrophy do not alter cardiac metabolism[161]. Nonetheless, metabolic deregulation is a common hallmark of several cardiac diseases, including pressure overload-induced hypertrophy[161], ischemic insult[166] and high–salt–diet–induced heart failure with preserved ejection fraction[167].

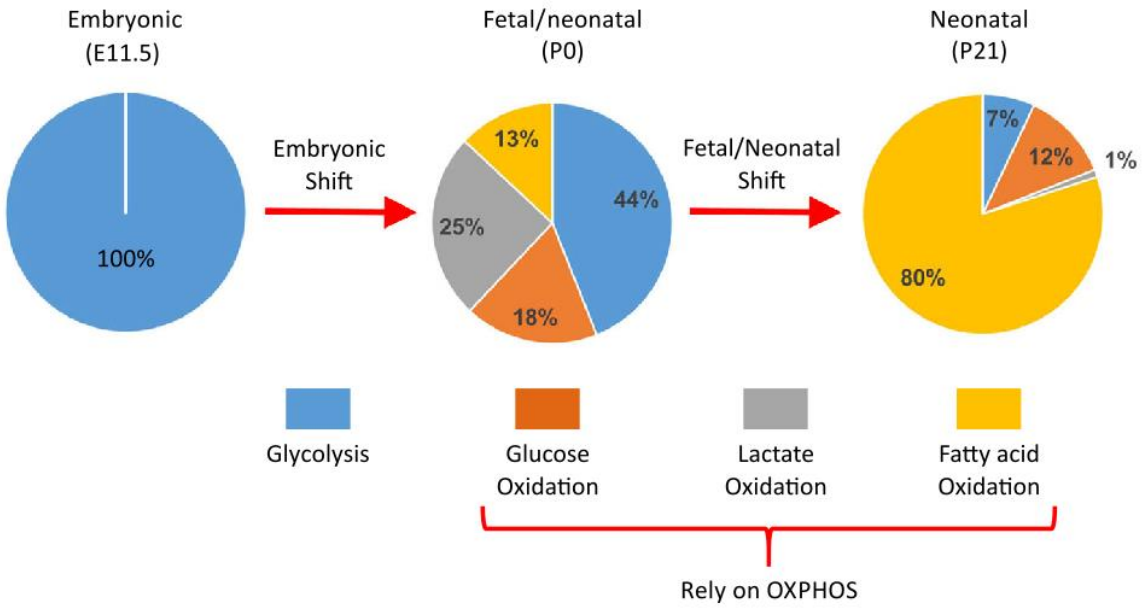



**Figure 8. Cardiac metabolism during fetal and postnatal life.** Percent contributions of glycolysis and oxidative metabolism including glucose oxidation, lactate oxidation, and fatty acid oxidation to cardiac ATP production are shown. E indicates embryonic day; P indicates postnatal day; OXPHOS, oxidative phosphorylation. Figure obtained from [158].

#### 1.7.3.2. Cardiac metabolic pathways are altered in response to maternal obesity

Animal models of maternal obesity and diabetes demonstrate significant changes to fetal cardiac metabolism. Fetal cardiac metabolism is affected by maternal metabolism and nutrient availability. The fetal heart is more dependent on maternal glucose and lactate at mid-late gestation due to higher availability than circulating free fatty acids[157]. This is notable because substrate availability is altered during obesity. Free fatty acids are increased in maternal blood and fetal cord blood of overweight/obese mothers in humans[168]. In animal models, both maternal and fetal plasma glucose are also increased[169–172]. Increased fetal glucose promotes hyperinsulinemia in the fetus that is associated with cardiac hypertrophy[169–172]. Several signaling pathways that regulate metabolism are known to be altered in fetal hearts in response to maternal obesity[25].

Insulin signaling is required for fetal and postnatal cardiac growth, but misregulation of this pathway leads to cardiac hypertrophy and dysfunction[173–175]. Maternal diabetes causes aberrant insulin signaling which is associated with fetal and neonatal cardiac hypertrophy[176–179]. Insulin signaling is also misregulated in the fetal heart in animal models of maternal obesity[170] **(Fig. 9)**. Insulin signaling is initiated by auto-phosphorylation of the insulin receptor beta (IRβ) in response to insulin binding. Activated IRβ leads to phosphorylation of the insulin receptor substrate-1 (IRS1), the key mediator of insulin signaling. The activity of IRS1 is modulated by JNK and p38 MAP kinases, which can phosphorylate IRS1 and impair IRβ-mediated phosphorylation of IRS-1, thereby attenuating sensitivity to insulin. In response to maternal obesity, phosphorylation of IRβ is reduced in the heart at late gestation[170] **(Fig. 9)**, indicating impaired insulin signaling. Consistent with this finding, the activity of JNK, p38 MAPK and phosphorylation of IRS1 are increased in the hearts of fetuses of chronically obese ewes[170] **(Fig. 9)**. Phosphorylation and kinase activity of the IRS1 downstream target Akt, and Akt-mediated phosphorylation of mTOR are also educed in hearts of fetuses of obese ewes[170] **(Fig. 9)**. Akt is a metabolic regulator that promotes glucose uptake[180]. Reduced activity of Akt indicates potential reduction in glucose uptake in the hearts of fetuses of obese ewes during the third trimester. MTOR signaling also regulates glycolytic and mitochondrial metabolism. It is important to note that Akt phosphorylation is not altered during the second trimester [181] and mTOR phosphorylation is mildly but significantly increased in both left and right ventricles during the second trimester of gestation[181] **(Fig. 9)**. This suggests that insulin signaling might be mildly perturbed at mid-gestation in the fetal heart, and more strongly at late gestation, when downregulation of multiple components of the pathway is strikingly evident. Overall, the data highlights that deregulation of insulin signaling may be a sequential and chronologically defined event in fetuses of obese mothers. Combined with the increased availability of fatty acids, insulin signaling mediates increased fatty acid synthesis in the embryo[182] and is associated with increased lipid accumulation in the fetal hearts of obese ewes at late gestation.

The AMPK signaling pathway, which activates fatty acid metabolism, reduces fatty acid synthesis [183,184] and increases glucose uptake [185], is also disrupted in the heart of fetuses of obese pregnant ewes. AMPK activity is decreased while PP2C (the AMPK phosphatase) is increased in the fetal heart in response to maternal obesity **(Fig. 9)**. Reduced fatty acid metabolism is

consistent with the increased cardiac lipid accumulation in fetal heart of obese ewes, at late gestation [156] **(Fig. 9)**. Downregulation of the fatty acid transporter CD36 in the heart[170], suggests that in addition to a potential shift towards reduced fatty acid synthesis, nutrient transport is also altered **(Fig. 9)**. It is still unclear how alteration of these key regulators of glycolytic and oxidative metabolism impact fetal cardiac metabolism. Further studies are required to directly examine the metabolic capacity of fetal hearts in response to maternal obesity. Overall, maternal obesity impacts the activity of several metabolic regulators including AMPK, AKT and insulin signaling, with larger effects at later gestation, which is consistent with the progressive activation of oxidative metabolism during development **(Fig. 8)**.

Maternal obesity also impairs metabolic homeostasis in the adult heart of the offspring. The rate-limiting enzyme for fatty acid synthesis and lipid deposition Stearoyl-CoA Desaturase 1 (SCD1) is increased in the adult hearts of offspring of obese mice, under baseline conditions [186] **(Fig. 9)**. However, lipids are not increased in these hearts. Offspring of obese mothers only present with exacerbated intramyocardial lipid accumulation when challenged with high-fat diets[186] **(Fig. 9)**. This suggests that maternal obesity might poise the heart for lipid accumulation, but other factors such as hyperlipidemia caused by an obesogenic diet, could be necessary to induce significant lipid accumulation in the heart. Phosphorylation of Akt and AMPK, two signaling molecules regulating glycolysis and triglyceride (TG) synthesis, are not decreased by either maternal high fat diet or postnatal high fat diet alone. However, a double insult of high-fat diet feeding of mother and offspring leads to dampened phosphorylation of Akt and AMPK [186], similar to that seen in the fetal heart[170] **(Fig. 9)**. Depressed AMPK signaling in offspring exposed to the pre-and postnatal high fat diet may impede fatty acid oxidation and promote TG synthesis [183–185]. At the molecular level, glucose uptake by CMs in response to maternal high fat diet is not altered in adult mice, but is reduced when the offspring are challenged with an obesogenic diet, suggesting that maternal diet impacts glucose uptake under metabolic stress[186] **(Fig. 9)**. This is consistent with increased insulin resistance commonly occurring in early stages of heart disease. It is unclear if these effects on the cardiovascular system are secondary to the more severe metabolic dysfunction in offspring of obese mothers when challenged with an obesogenic diet. Altogether, these findings indicate that obesity during pregnancy leads to subtle cardiac metabolic abnormalities in the offspring that are exacerbated in response to metabolic stress.

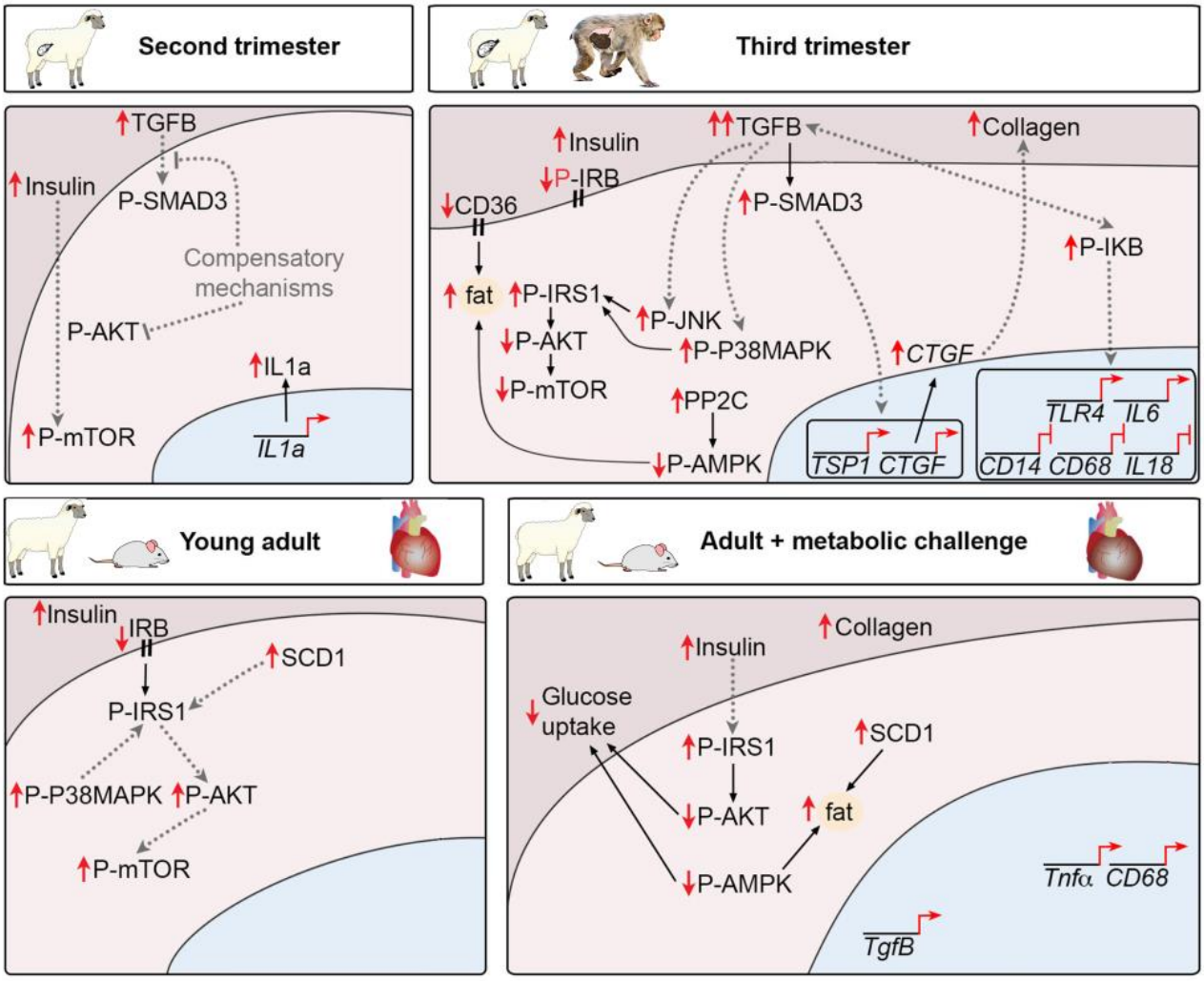



**Figure 9. Obesity during pregnancy perturbs signaling pathways in the heart.**
Inflammation, TGFβ and metabolic signaling pathways are mildly deregulated at mid-gestation and more strongly deregulated at late-gestation. Many of these pathways are persistently misregulated in the adult heart. TGFB signaling is successively activated throughout gestation and is poised for activation in response to metabolic stress in adulthood. Some inflammatory cytokines are also upregulated during gestation, while others are downregulated, but might remain poised for activation in response to metabolic stress in adulthood. Regulators of energy metabolism, including insulin signaling pathway, SCD1, and fatty acid transporter CD36 are misregulated most severely during late gestation, revert in young adulthood and might also remain poised for severe misregulation in response to chronic long-term metabolic stress during adulthood, which is consistent with fat accumulation at late gestation and in challenged adults. The animal models used to obtain these findings are shown on the left side of the labels.

## 1.8. Limitations of previously described animal models of maternal obesity-induced CVD in the offspring

Current animal models of maternal obesity in mice, sheep and primates induce maternal weight gain as well as severe hyperglycemia and hyperinsulinemia in both the mother and the fetus [138,169–172,187,188]. Dissociating the effects of obesity from uncontrolled diabetes is not possible in such models. This is of critical importance because hyperglycemic control has been a common course of clinical intervention for over 70 years[189] and it effectively reduces the risk of perinatal cardiac hypertrophy[190–192], yet the offspring's risk of adult-onset cardiovascular disease in response to obesity persists. In addition, cardiac form and function in fetuses of obese women are compromised even in the absence of gestational diabetes[12,13]. Thus, the effects of obesity in humans are likely dissociated from the effects of hyperglycemic insult.

The effects of maternal hyperglycemia have been thoroughly studied in both human and diabetic animal models. The outcomes in these offspring are distinct from those of obesity insults. Hyperglycemia increases fetal insulin levels resulting in fetal hyperinsulinemia, which has detrimental and well-characterized effects on fetal and postnatal development **(Fig. 10)**. The effects on the cardiovascular system are particularly well established. Since glucose freely crosses the placenta to the fetal circulation, by facilitated diffusion, maternal hyperglycemia leads to fetal hyperglycemia[193,194]. Although maternal insulin does not cross the placenta, the fetal pancreas autonomously secretes insulin in response to hyperglycemia at early gestation (12 weeks in human gestation) resulting in fetal hyperinsulinemia[195–199]. Aberrant insulin signaling is attributed to the onset of transient cardiac hypertrophy in diabetic preganacies[33,197,200]. Both fetal insulin and insulin growth factor (IGF-1) are increased in diabetic hyperglycemic pregnancies[195,201–203]. These ligands bind to their receptors and activate downstream insulin signaling components that directly induce cardiac growth. The expression of genes controlling cell growth and differentiation is regulated by Ras–mitogen-activated protein kinase (MAPK) pathway downstream of insulin. The metabolic actions of insulin are largely mediated by activation of the phosphatidylinositol 3-kinase (PI3K)-AKT-AMPK/mTOR pathway [197]. Importantly, in human pregnancies affected by diabetes, fetal hyperinsulinemia is alleviated immediately after birth when the maternal hyperglycemic intra-uterine environment is no longer present. Thus, although fetal hyperglycemia is associated with neonatal cardiac hypertrophy at

birth, the hypertrophy is transient and dissipates with age as the insulin levels are normalized[33,197].

All animal models of maternal obesity used thus far induce severe and uncontrolled hyperglycemia, and the phenotypes in the offspring recapitulate those of diabetic pregnancy, not obesity. For example, all animal studies on maternal obesity report a complete penetrance of cardiac hypertrophy in young adult offspring of obese mothers[204–207], which is not consistent with the modest increase in CVD susceptibility, or with the late onset of the disease in human offspring of obese mothers (see section 1.2.1.2). In addition, cardiac hypertrophy in the offspring of obese mice is prominent in neonatal mice but progressively decreases until it completely dissipates by 12 weeks of age[206]. This is consistent with transient hypertrophy in neonates born to women affected by hyperglycemia during pregnancy. The activity of the entire insulin signaling pathway including the insulin receptor, IRS, AKT, mTOR, AMPK and MAPK are all reported to be perturbed in fetal hearts of animal models of maternal obesity[170,181] **(Fig. 9)**. The offspring in these animal models also develop exacerbated cardiac dysfunction, hypertrophy and fibrosis when exposed to long-term metabolic stress[139,186,205], which is consistent with increased susceptibility to CVD in offspring of obese[15,16,44] or diabetic preganancies[208]. However, discerning between the effects of obesity and hyperglycemia in these models is not possible.

The possibility of transient hyperglycemia may never be eliminated during obesity, even in the absence of gestational diabetes. Recent evidence demonstrates that maternal glucose levels lower than those used for diagnosis of gestational diabetes can impact fetal growth[209]. Moreover, even acute and moderate hyperglycemia during pregnancy is sufficient to cause cardiac hypertrophy in the unborn offspring in rats[200]. Thus, although chronic hyperglycemia and diabetes are not representative of the human obese condition, subclinical changes in maternal glucose metabolism might mediate some of the effects of maternal obesity. Altered glucose metabolism can never be eliminated in animal models of diet-induced obesity. However, there is a need for improved models of maternal obesity with a more limited glucose imbalance that would better represent the human obese pregnancy and the increased susceptibility for late-onset CVD in the offspring.

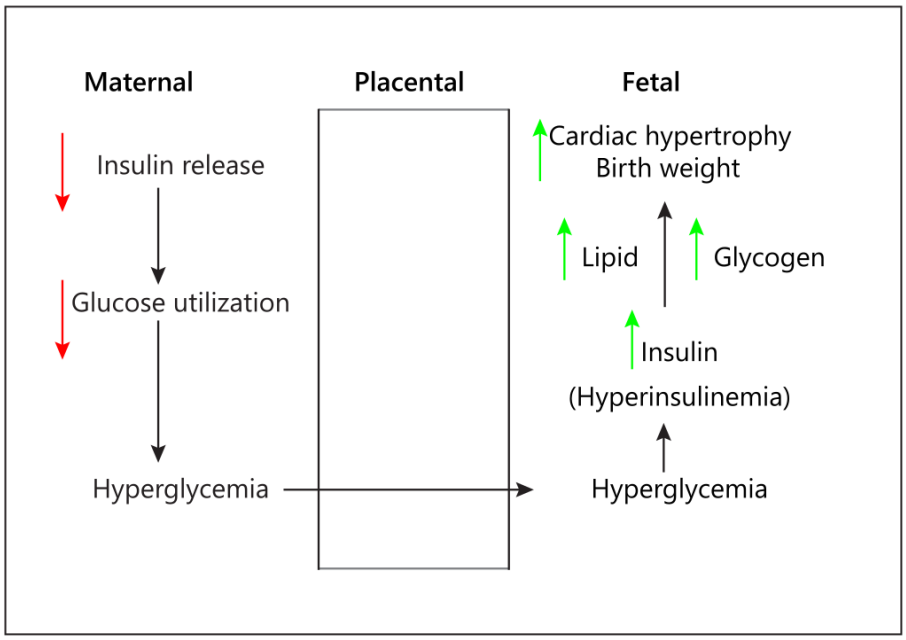



**Figure 10. The effects of maternal hyperglycemia on the fetus.** Increased glucose in maternal blood can cross the placenta barrier leading to fetal hyperglycemia. This leads to an endogenous increase in fetal insulin secretion that mediates lipid and glycogen storage (associated with macrosomia) and cardiac hypertrophy. Figure was obtained and modified from [195].

## *2. Thesis Rationale and Summary*

The developmental origins of adult cardiac disease predisposition in offspring of obese mothers are not fully understood. While multiple functional and molecular pathways are known to be perturbed in fetal hearts of obese mothers at mid-late gestation, the impact of obesity on early stages of cardiac morphogenesis is completely unknown. In addition, how these early cardiac responses to obesity mediate the long-term increase in cardiac disease susceptibility is not known.

Transcriptional misregulation of early cardiac gene networks can determine cardiac disease risk in adult life. Nkx2-5 is a cardiac kernel transcription factor required for establishing gene expression networks in the developing heart and regulating the cardiac stress response in adulthood. Proper function of this transcription factor depends on a homeostatic *in utero* and postnatal environment. I hypothesized that maternal obesity during pregnancy disrupts gene expression in the developing and adult heart, by altering the function of Nkx2-5 thereby increasing susceptibility to adult-onset cardiac disease. Understanding if, and how, obesity alters the gene expression networks in the developing heart and their long-term impact in adulthood will provide insight into the developmental origins of adult-onset cardiac disease. Identifying the specific pathways that respond to maternal obesity during development and how they affect the adult heart is important for understanding how cardiac disease risk is determined.

This lack of knowledge on the impact of obesity on the early developing heart is partially due to inadequate models available. The prevalent and severe hyperglycemia in current obesity models confounds the conclusions regarding the impact of uncontrolled diabetes *vs* obesity. Employing obesity models that have a more moderate metabolic imbalance, better glucose tolerance, and that more closely recapitulate the human effects of maternal obesity phenotypes on the offspring is an imperative first step to reveal the origin of adult-onset heart disease susceptibility. In addition, dissociating between the primary effects of maternal obesity on the heart and the secondary effects of metabolic abnormalities in the offspring is required.

In this thesis, I first established and characterized an obesity model that better recapitulates the effects of maternal obesity in humans on the mother and offspring. I describe a model of maternal obesity with limited glucose imbalance. The offspring of obese mothers in this model

develop metabolic dysfunction similar to that reported in humans born to obese women. Importantly, I found that offspring born to obese mothers develop progressive cardiac dysfunction that is more prominent in older mice but remains subclinical. However, in response to cardiac stress, the offspring of obese mothers mount severe pathological responses. This indicates that a secondary insult during adult life is required for the onset of cardiac disease in offspring of obese mothers.

To identify the embryonic origins of adult-onset heart disease susceptibility, I uncovered the transcriptional response of the developing mouse and human heart to maternal obesity at early and early-mid gestation, respectively. I show that Nkx2-5 and downstream targets regulating cardiac specification and differentiation are significantly misregulated in mouse cardiac progenitors. At later stages of development, once mitochondrial oxidative metabolism is established, I found that pathways controlling lipid metabolism and clearance as well as inflammation are upregulated in human hearts of fetuses of obese donors. To determine if these changes persist in adult hearts and their impact on cardiac stress responses, I profiled the global transcriptional landscape of the adult offspring of obese mothers at several ages, at baseline and under pathological conditions. Adult hearts consistently upregulated pro-fibrotic and pro-inflammatory pathways. In addition, genes controlling metabolic and cardiac conduction-related pathways were repressed. Analysis of gene promoter composition revealed that genes dysregulated in the heart in response to obesity during pregnancy are enriched for Nkx2-5 targets. Accordingly, analysis of Nkx2-5-bound chromatin in fetal and adult hearts revealed that dysregulated genes are enriched for direct targets. Cardiac gene expression changes were not associated with non-cardiac metabolic parameters in the offspring but were correlated with maternal weight and severity of the cardiac defects.

In summary, my work identifies Nkx2-5 as a common upstream regulator of gene expression changes in response to maternal obesity. Few of the Nkx2-5 downstream targets are persistently misregulated throughout development and during adulthood. The majority of misregulated genes are dysregulated in a stage-specific manner, in either fetal or adult hearts. Early cardiac progenitors activate genes regulating differentiation and specification. Thereafter, genes regulating metabolism and ion channels are repressed whereas genes regulating cell adhesion and fibrosis are activated. Hundreds of genes undergoing small changes in expression underlie

subclinical, age-dependent, cardiac dysfunction in offspring of obese mothers. A secondary insult during adulthood exacerbates the transcriptional and functional deregulation in offspring.

This thesis demonstrates the molecular events underlying fetal programming of cardiac disease in response to maternal obesity and provides novel insight with broad implications in the developmental origins of adult-onset cardiac disease. Nkx2-5 and downstream "maternal obesity"-responsive pathways may serve as novel candidates for screening at-risk individuals and potential targets for modulating the risk of CVD. However, the mechanistic basis for deregulation of Nkx2-5 and its targets, the maternal signals that induce this deregulation, and how long-term transcriptional deregulation is established remains unaddressed.

## *3. Materials and methods*

### Mouse genetic backgrounds and housing conditions

All animal procedures were approved by the Animal Care Committee at The Centre for Phenogenomics. Experiments were performed on *Nkx2-5tg* mice[210], unless otherwise indicated. Experiments that specifically investigated the effects of mouse genetic background were performed on C57Bl/6J strains and are clearly indicated. Mice were housed in standard vented cages in temperature- and humidity-controlled rooms with 12-hour light-dark cycles (21–22 °C, 30–60% humidity), and free access to water and food.

### Maternal breeding and feeding strategy

Female mice were fed irradiated diets ad libitum: control diet (10% fat, 1% sucrose kcal; D12450K) or high fat/high sugar diet (33% fat, 30% sucrose by kcal; D13052905). Diets were obtained from Research Diets, Inc. Diet feeding began at 4 weeks of age and was continued throughout their lifetime. Female mice were first mated at 12 weeks of age. The first litters were sacrificed. This was used to identify proven breeders. The second litters were used for all experiments. All male mice used for breeding were fed control diets except during breeding. During gestation and lactation, all female mice were maintained on their respective diets.

### Offspring feeding

Only male offspring were used in the experiments because cardiac function fluctuates with the estrous cycle in female mice[211] which might increase data heterogeneity and confound results. Females were sacrificed at weaning. All male offspring were weaned onto control diets at P21 and continued the same diet till 4 months of age. At 4 months, the entire litter was randomly assigned to one of two groups: control diet or high fat/high sugar diet. Diets were not altered thereafter. Mice were sacrificed at various timepoints 2, 4, and 8 months of age. All mice were sacrificed in a random-fed state, during the light cycle.

### Cross-fostering

Female breeding was timed to ensure that two females (one fed a high fat/high sugar diet and another fed a control diet) delivered pups on the same day. At P0, the pups from the control-fed mother were sacrificed and replaced with the pups from the high fat/high sugar diet-fed mother. The mother under high fat/high sugar diet was sacrificed thereafter.

### Body weight and body composition analysis

Body weight of mothers was measured weekly beginning at 4 weeks of age, the onset of diet feeding. The Offspring weight was measured weekly, beginning at P0. During the first 2 weeks of life, the body weight of both males and females was measured and included in the analysis. After the first two weeks, the sex of the mice could be easily identified and only male mice were weighed and included in the analysis. Body composition of un-anesthetized mice was measured using the body composition analyzer (EchoMRI-100 machine, Echo Medical Systems, Houston, TX, USA). Body weight was measured immediately before the acquisition of body composition parameters. Fat percentage was obtained by dividing the total fat mass by the body weight. Lean percentages were obtained by dividing the total lean mass by the body weight. Body composition analysis was conducted at various time points as specified in the text.

**Glucose tolerance test:**

Glucose tolerance tests were performed on mice after 16 hrs. of fasting, with water ad libitum, by intraperitoneal injection of glucose (1 mg/g of body weight). Blood glucose was measured at 0, 15, 30, 60, and 120 mins post-injection, using a glucometer (Contour NEXT, Bayer HealthCare). Glucose tolerance of mothers and offspring was measured at several time points as specified in the text.

**Adverse myocardial remodeling induction**

Osmotic pumps (ALZET) were filled with isoproterenol (60 mg/kg/day dissolved in PBS) and implanted subdermally in 8-week old mice for 14 days.

**Echocardiography**

Cardiac morphology and function were evaluated in male offspring using the high-frequency ultrasound imaging system Vevo2100 (VisualSonics, Toronto, Canada) with a 30 MHz transducer. All mice were scanned under light isoflurane (1.5%) anesthesia within 20-30 min. The mouse body temperature was carefully monitored using a rectal thermometer and maintained at ~37 degrees using a heated platform and a heat lamp. The left ventricular dimensions, systolic and diastolic functions were measured as previously described[212].

**Blood Pressure and Heart Rate Measurements**

CODA Monitor Noninvasive Blood Pressure System (Kent Scientific Corporation) was used for measuring blood pressure and heart rate on unanesthetized mice. Mice were placed in holders on warming platforms and their temperature was carefully monitored using an infrared

thermometer. All mice were acclimated to the system for 2 days before data acquisition by placing them in the holders for 20 min on the warming platforms with the cuff on their tail. Recordings were collected on days 3, 4 and 5 after acclimation. A total of 20 recording cycles were conducted for each mouse on each day, the last ten recordings were averaged. The average readings of the three days were used for analysis. All acclimations and recordings were conducted between 3-5 pm, to avoid variation known to be induced by circadian rhythms[213]. Appropriate holder and cuff sizes were selected according to the mouse weight, as per manufacturer instructions.

**Mouse embryonic collection**

For mouse embryo studies, the presence of vaginal plugs indicated E0.5. Somites were counted to determine the embryonic stage, with 21-25 somites indicating E9.5. Pregnant female mice were sacrificed at a random fed state during the light cycle. I have carefully described the method used for embryonic dissections in more detail before[214].

**Human fetal heart collection**

All procedures and protocols were approved by the Research Ethics Boards at The Hospital for Sick Children and Mount Sinai Hospital. Samples were obtained from the Research Centre for Women's and Infants' Health BioBank at Mount Sinai Hospital in accordance with Institutional ethics guidelines. All fetal hearts were obtained from donors undergoing elective termination procedures. Terminations due to congenital defects were excluded. Fetal hearts were dissected by a qualified nurse and frozen in RNAlater solution at -80°C. Hearts were later thawed, and I dissected ventricles away from atria and non-cardiac tissue. Ventricles were cut open and rinsed for 10 seconds in PBS to remove blood inside the chambers then immediately placed in Trizol Reagent (Thermo Fisher Scientific).

**Histology**

Freshly dissected hearts in cold PBS were prepared for paraffin embedding as we previously described[215]. Hearts were sectioned at a thickness of 7 μm. Masson's Trichrome staining was performed as per the manufacturer's protocol (Newcomer Supply, Inc, #9176).

**Immunofluorescence**

Dissected hearts were washed in PBS then kept in 30% sucrose/PBS at 4 °C overnight or until the hearts sank. Hearts were embedded in OCT compound and sectioned at 7 μm thickness. For

cell size measurements using Wheat Germ Agglutinin Alexa Fluor™ 594 Conjugate (W11262 Invitrogen), slides were fixed for 10 min at room temperature and washed in PBS three times for 5 minutes each. Slides were incubated with Wheat Germ Agglutinin 10 µg/ml for 30 minutes then washed with PBS 3 times, 10 minutes each. Slides were mounted with VECTASHIELD® mounting medium containing DAPI. Immunostaining was conducted as previously described[215]. Antibodies used were anti-COL5A1 (Santa Cruz Biotechnology, sc-20648, 1:200), anti-GFP (GeneScript, A01694, 1:1000) and anti-ISL1(Developmental Studies Hybridoma Bank, 40.2D6, 1:100).

**FACS**

eGFP positive embryos were identified under a Nikon SMZ1500 microscope. I have detailed the steps for embryonic dissociation and cardiac progenitor isolation before[216]. Briefly, embryos with 20-25 somites were dissociated using TrypLE Express (Gibco) at 37°C for 10 min by pipetting up and down. Digestion was then inhibited by the addition of FBS (for a final concentration of 10% FBS in solution). Cells were centrifuged and resuspended in Red Blood Cell Lysis Solution (130-094-183, Miltenyi Biotec). Lysis was performed at room temperature for 2 min. Cells were then washed and resuspended in 1 % FBS DMEM 1mM EDTA. Cells were sorted into 100% FBS.

Unless otherwise stated, embryos were pooled together before sorting for qPCR or sorted individually for RNA-seq. Approximately 5,000-10,000 eGFP positive cells were obtained from each embryo.

**RNA extraction**

For adult mouse hearts and human fetal hearts, whole ventricles were extracted and frozen in Trizol after dissection. Homogenization was performed using the TissueLyserII (Qiagen) by shaking ventricles within tubes containing stainless steel beads and Trizol Reagent (Thermo Fisher Scientific) at 30kHz for 1 min. Homogenized samples were centrifuged to pellet insoluble tissue. Direct-zol™ RNA MiniPrep kit (Zymo Research) was used to isolate DNA-free RNA. For CPCs, sorted cells were lysed in Trizol Reagent (Thermo Fisher Scientific) and RNA was extracted using the phenol-chloroform extraction method as per the manufacturer's protocol.

**Gene expression analysis**

**Quantitative PCR**

cDNA was prepared using the SuperScript III First-Strand Synthesis Kit (Invitrogen). PCR reactions were prepared with ADVANCED qPCR Mastermix with SUPERGREEN DYE Low Rox, and run on a CFX384 TouchTM Real-Time PCR Detection System (Bio-Rad). All samples were run in triplicate. Data were analyzed using the CFX Manager Software (Bio-Rad).

**Next generation sequencing**

For 8-month old adult hearts, RNA-seq libraries were prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina with the NEBNext® Poly(A) mRNA Magnetic Isolation Module. 1 µg of total RNA was used as starting material. Single-end sequencing (50 bp) was performed on the Illumina HiSeq 2500 platform.

For 2-month old mice subjected to isoproterenol stress tests and their age-matched controls, RNA-seq libraries were prepared using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina with the NEBNext® Poly(A) mRNA Magnetic Isolation Module. 1 µg of total RNA was used as starting material. Single-end sequencing (50 bp) was performed on the Illumina HiSeq 2500 platform.

For cardiac progenitor cells, sequencing libraries were prepared using Ovation® SoLo RNA-Seq Systems (NuGEN) for mouse, with 9 ng of total RNA as starting material.

For human fetal hearts, RNA-seq libraries were prepared using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina with the NEBNext® Poly(A) mRNA Magnetic Isolation Module. 1 µg of total RNA was used as starting material. Single-end sequencing (50 bp) was performed on the Illumina HiSeq 2500 platform.

**RNAseq Analysis**

Raw sequence reads were trimmed using Trimmomatic 0.36[217] and aligned to the reference mouse genome (mm10) or human genome (hg38) using STAR version 2.5.3[218]. Total counts were quantified using FeatureCounts[219] and normalized using DESeq2 version 1.22.21 (with batch correction)[220]. Adjusted *P* values < 0.05 were considered significant. For CPCs, reads were de-duplicated using NuDup (nugentechnologies.github.io/nudup/). All PCA plots were generated using Plotly[221] in R. DE genes were clustered using Cluster 3.0 and visualized with Java Treeview[222]. GO term and KEGG pathway enrichment was analyzed using DAVID v6.8[223,224]. Volcano plots were created using R version 3.5.1. Over-represented transcription factor binding sites were detected by mouse single site analysis with default settings using oPOSSUM v3.0[225].

10,000 bp upstream and downstream of the transcription start site of all the genes that were expressed (normalized counts >50) at the respective timepoints were used as background.

**Gene Set Enrichment Analysis**

Gene set enrichment analysis (GSEA) (http://www.broad.mit.edu/GSEA)[226,227] was performed with 1000 permutations and a weighted enrichment statistic. For GSEA used to identify differentially enriched GO terms and pathways in hearts at 32 weeks of age, all expressed genes (13,906 genes) were ranked by their $-\log_{10}$(adjusted P-value) x ($\log_2$FoldChange) estimated by DESeq2. Gene sets smaller than 5 and larger than 200 were excluded from the analysis. Gene set "Mouse_GO_AllPathways_no_GO_iea_January_01_2020_symbol", compiled by the Bader Lab at http://download.baderlab.org/EM_Genesets was used for GSEA. Cytoscape was used for network visualization[228] with the Enrichment Map[229] and AutoAnnotate[230] plugins as previously described[231]. All labels were edited for simplicity.

For GSEA used for comparison with established models of cardiac stress and human hearts affected by disease, gene sets were generated *de novo* by analysis of publicly available raw RNA-seq data: SRP076218 (isoproterenol-induced stress)[232], SRP055928 (TAC)[233], E-MTAB-727 (exercise-induced hypertrophy) [234], SRP055875 (diabetic cardiomyopathy)[235], SRP093239(Ang-II induced hypertension), SRP186138 (hypertrophic cardiomyopathy)[236], SRP151309(dilated and ischemic cardiomyopathy)[237]. For each model, the differentially expressed genes (DE) were categorized as up- or down-regulated. All significantly DE genes in adult hearts ($P$<0.05) were used as input in a pre-ranked gene list to conduct a more focused analysis given that the gene sets used for input were based only on DE genes. Genes were pre-ranked by their $-\log_{10}$ (adjusted $P$-value) x ($\log_2$FoldChange) estimated by DESeq2. 1000 permutations were conducted using a weighted statistic. The complete sets of genes that were differentially expressed in the different models and human hearts affected by disease were used for the analysis, therefore, a gene set size limit was not set. GSEA was performed using databases of up- and down-regulated genes, separately. We focused on genes with positive enrichment scores when using the upregulated gene set database, and negative enrichment scores when using the downregulated gene set to determine similarity, *i.e*. models with the highest direct correlation with genes DE in my model.

**Gene Network Analysis**

GeneMania[238] was used to build the networks used for the focused analysis of individual pathways. Interactions were visualized on Cytoscape[228]. Gene lists used as input representing the different pathways were manually curated from DE genes by combining KEGG pathways and GO-terms related to the respective pathways.

**MicroRNA target prediction**

Target prediction was performed using TargetScanMouse v7.1[239].

**Phenotypic correlation with gene expression**

Normalized gene expression levels were correlated with phenotypic variables using the Pearson correlation test. Benjamini & Hochberg correction was applied to correct for multiple testing. Samples with missing phenotypic parameters were excluded from the respective correlation. This was repeated using 10,000 size-matched sets of genes randomly selected from the universe of all expressed genes. Bootstrap *P*-value is reported as the number of permutations with the number of significantly correlated genes >= the number observed for DEgenes.

**ChIP-seq analysis**

ChIP-seq data for NKX2-5 in adult hearts was obtained from GSE35151[122], and in cardiac progenitors from GSE77548[84]. Bed files of NKX2-5 peaks were obtained from these studies and NKX2-5 bound genes were determined with Genomic Regions Enrichment of Annotation Tool (GREAT)[240], v4.0.4; mm9 assembly, using default settings. Fisher's exact test was used to determine enrichment. Differentially expressed genes were defined by an adjPval<0.05. Genes whose expression did not significantly change and that were used for the test were defined as all expressed genes with a mean expression greater than that of the lowest DE gene at the respective time point. Bigwigs used to generate the aggregate plots were obtained from GSE35151[122] and GSE77548[84].

**Statistical analysis**

Data are presented as the mean ± S.E.M. unless otherwise indicated. 2 group comparisons were conducted by Student's t-test. For multiple group comparisons or for comparisons of 2 groups over time ANOVA was used. Multiple hypothesis testing correction was performed as indicated in the manuscript. P<0.05 was considered significant. Statistical analysis was conducted either using GraphPad Prism 6 or R.

**Number of mice and litters used for each experiment**

Figure 13 (left)

| Group name | Age (wk) | Number of mice | Number of litters |
|---|---|---|---|
| Cn/Cn | 0-16 | 42 | 14 |
| Cn/Cn | 16-32 | 25 | 8 |
| Cn/Ob | 16-32 | 17 | 6 |
| Ob/Cn | 0-16 | 33 | 9 |
| Ob/Cn | 16-32 | 14 | 4 |
| Ob/Ob | 16-32 | 16 | 5 |
| XOb/Cn | 0-32 | 7 | 1 |

Figure 13 (right)

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 16 | 8 |
| Cn/Ob | 17 | 6 |
| XOb/Cn | 7 | 1 |
| Ob/Cn | 14 | 4 |
| Ob/Ob | 16 | 5 |

Figure 13b-c

| Group name | Age (mo) | Number of mice | Number of litters |
|---|---|---|---|
| Cn/Cn | 2 | 35 | 11 |
| | 4 | 39 | 12 |
| | 6 | 16 | 5 |
| | 8 | 16 | 5 |
| Ob/Cn | 2 | 30 | 8 |
| | 4 | 34 | 9 |
| | 6 | 14 | 4 |
| | 8 | 14 | 4 |
| XOb/Cn | 2 | 7 | 1 |
| | 4 | 7 | 1 |
| | 6 | 7 | 1 |
| | 8 | 7 | 1 |
| Cn/Ob | 6 | 15 | 6 |
| | 8 | 17 | 6 |
| Ob/Ob | 6 | 16 | 5 |
| | 8 | 16 | 5 |

Figure 13d (left)

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 32 | 11 |
| Ob/Cn | 31 | 8 |
| XOb/Cn | 7 | 1 |

Figure 13d (right)

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 16 | 5 |
| Ob/Cn | 14 | 5 |
| XOb/Cn | 7 | 1 |
| Cn/Ob | 16 | 6 |
| Ob/Ob | 16 | 5 |

Figure 14

| Group name | Age (mo) | Number of mice | Number of litters |
|---|---|---|---|
| Cn/Cn | 4 | 8 | 4 |
| | 8 | 8 | 4 |
| Ob/Cn | 4 | 8 | 2 |
| | 8 | 7 | 4 |
| XOb/Cn | 4 | 7 | 1 |
| | 8 | 7 | 1 |

Figure 15a

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 26 | 9 |
| Ob/Cn | 24 | 7 |
| XOb/Cn | 7 | 1 |

Figure 15b

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 15 | 5 |
| Ob/Cn | 11 | 4 |
| XOb/Cn | 7 | 1 |

Figure 16c

| Group name | Age | Number of mice | Number of litters |
|---|---|---|---|
| Cn/Cn | 2 | 9 | 3 |
| Ob/Cn | 2 | 3 | 1 |

| | | | |
|---|---|---|---|
| Cn/Cn | 8 | 24 | 7 |
| Ob/Cn | 8 | 17 | 6 |
| XOb/Cn | 8 | 7 | 1 |

Figure 16e

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 4 | 2 |
| Ob/Cn | 4 | 3 |
| Cn/Cn | 3 | 1 |

Figure 21

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 8 | 5 |
| Ob/Cn | 8 | 4 |

Figure 22

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn | 8 | 5 |
| XOb/Cn | 4 | 1 |

Figure 24d

| Number of mice | Number of litters |
|---|---|
| 15 | 8 |

Figure 26a-b and Figure 27

| Group name | Number of mice | Number of litters |
|---|---|---|
| Cn/Cn – PBS | 6 | 3 |
| Ob/Cn – PBS | 6 | 3 |
| Cn/Cn – ISO | 6 | 4 |
| Ob/Cn – ISO | 6 | 3 |

Figure 29e and Figure 32

| Group name | Number of embryos | Number of litters |
|---|---|---|
| Cn | 8 | 4 |
| Ob | 8 | 4 |

Figure 30

| Group name | Number of embryos | Number of litters |
|---|---|---|
| Cn | 2 | 1 |
| Ob | 2 | 1 |

### *4. Data attribution:*

Many of the result presented in chapters 5 and 7 have been published in:

Ahmed, A., Liang, M., Chi, L., Zhou, YQ., Sled, JG., Wilson, MD., Delgado-Olguín, P. (2020). Maternal obesity persistently alters cardiac progenitor gene expression and programs adult-onset heart disease susceptibility. Mol. Metab. 43: 101116.

Dr. Quetzalcoatl Escalante measured some of the weights presented in **Figure 11b**. Dr. Yu-Qing Zhou performed the echocardiography acquisition used for **Figure 14**. Minggao Liang performed correlation analysis in **Table 4**, generated QQ plots in **Figure 23**, analyzed and generated Figure **24a**, re-analyzed publicly available raw RNAseq datasets and generated PCA plots in **Figure 25a**, generated the genesets which were used to perform GSEA in **Figure 25b**, de-duplicated RNAseq reads of CPCs using NuDup and generated aggregate ChIP-seq signal plots in Figure **31e, h and 38a**. Dr. Lijun Chi acquired images in Figure **28b-d**. All other experiments and analyses were conducted by Abdalla Ahmed.

## 5. *Results 1: Cardiac disease susceptibility is increased in the offspring in a mouse model of maternal obesity*

### 5.1. A moderately obesogenic diet increases weight and fat mass without overt glucose intolerance

Previous analysis of offspring exposed to maternal obesity in mouse, rat and sheep models has been performed in the context of severe diabetic obesity. To analyze the effects of maternal obesity on the offspring's cardiovascular health I aimed to first establish a model of maternal obesity with limited diabetic predisposition to more closely recapitulate the human condition. I tested the effects of feeding a moderately obesogenic (Ob) diet (30% fat and 33% sugar) on female mice and compared it to the effects of a previously used severely obesogenic (SevOb) diet (60% fat).

Female mice fed Ob or SevOb diets gained more weight compared to control (Cn) fed mice, as early as one week after feeding **(Fig. 11a, b)**. The weight gain progressively increased with age **(Fig. 11a, b)**. Notably, mice fed SevOb diet gained significantly more weight in the same period compared to mice fed Ob diet (weight at 10 weeks: 24.7g *vs* 21.7g, p=0.00446). The body fat percentage was increased in females fed Ob or SevOb diets, compared to females fed Cn diet, however, the increase was significantly greater in response to SevOb (36% *vs* 45.2%, p = 0.0459) **(Fig. 11c, d)**. Notably, glucose tolerance was not altered even after 16 weeks of Ob feeding **(Fig. 11e)** but was significantly reduced in response to SevOb after only 6 weeks of feeding **(Fig. 11f)**. Thus, female mice fed the Ob diet recapitulate known obese phenotypes but with less severity compared to SevOb, and they maintain glucose homeostasis. The Ob diet serves as a better model for human maternal obesity and was used for the entirety of the work presented in this thesis.

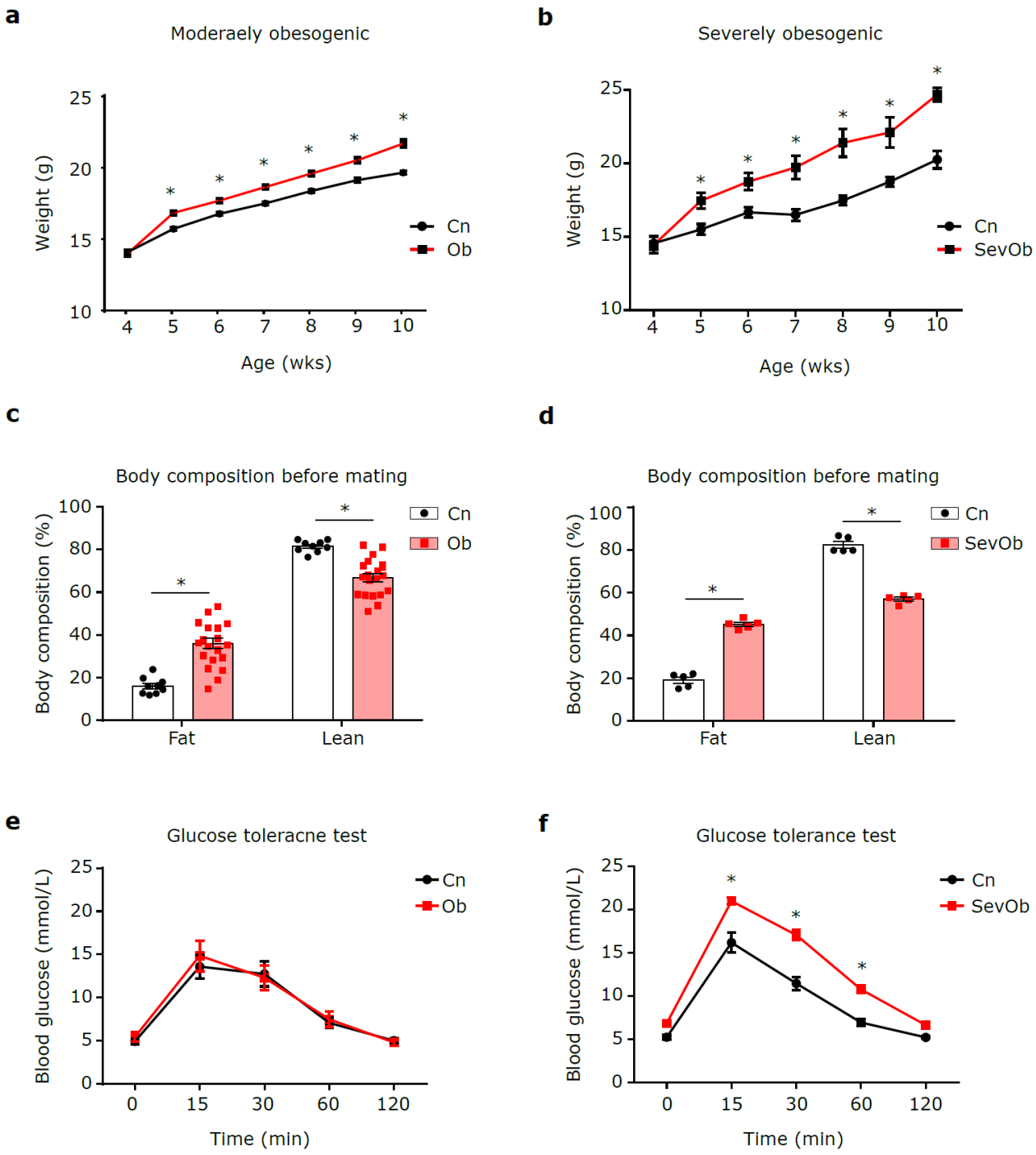



**Figure 11. A moderately obesogenic diet increases female weight and body fat to a lesser extent than a severely obesogenic diet and does not alter glucose tolerance. a)** Weight of female mice fed a control diet (Cn) or a moderately obesogenic diet (Ob). N= 60 mice per group. **b)** Weight of female mice fed Cn or severely obesogenic diet (SevOb). N= 10 mice per group. **c)** Body composition of Cn- and Ob-fed female mice. N=9 and 20 mice per group. **d)** Body

composition of Cn- and SevOb-fed female mice. N=5 mice per group. **e)** Glucose tolerance test on Cn- and Ob-fed mice. No differences are present. N=5 mice per group. **f)** Glucose tolerance test on Cn- and SevOb-fed females showing significantly higher glucose levels in SevOb-fed mice. N=5 mice per group. Bars represent the mean ± SEM. * $P<0.05$.

## 5.2. Maternal obesity augments weight gain, fat deposition and impairs glucose homeostasis in response to metabolic challenge in the offspring

Previous analysis of offspring exposed to maternal obesity has demonstrated increased cardiometabolic risk in humans and in animal models of maternal obesity. However, differences in severity, duration and onset of environmental stress cues has made it difficult to define the specific timing when overnutrition impacts the cardiometabolic fate of the offspring. To identify the critical period and conditions necessary for promoting cardiometabolic disease in the offspring I fed female mice Cn or Ob diet during pre-gestation and gestation **(Fig. 12)**. At birth, the offspring of Ob-fed females were either maintained with their birth mother, or were cross-fostered with a Cn-fed mother **(Fig. 12)**. After weaning, all offspring were fed Cn diets until 4 months of age, after which they were divided into two groups: either maintained on the Cn diet, or fed Ob diet for 4 months **(Fig. 12)**. This resulted in a total of 5 different groups of offspring that differed in the prenatal maternal diet, the postnatal maternal diet and in the diet of the offspring after 4 months of age **(Fig. 12)**.

Although my primary focus is on the cardiac health of the offspring, I first sought to characterize the metabolic phenotype of the offspring to enable better contextualization of potential cardiac phenotypes. Offspring of Ob-fed mothers were born at the same weight as offspring of Cn-fed mothers **(Fig. 13a)**. During lactation, offspring that were maintained with their Ob-fed mother gained more weight by their second week of life compared to offspring of Cn-fed mothers, but cross-fostered offspring (XOb/Cn) did not **(Fig. 13a)**. Consistently, when offspring were weaned and maintained on a Cn diet, only mice whose mothers were fed an Ob diet during both gestation and lactation (Ob/Cn) had increased body weight throughout their adult life **(Fig. 13a)**. When challenged with Ob diet at 4 months of age, offspring of Ob-fed mothers (Ob/Ob) gained significantly more weight compared to offspring of Cn-fed mothers (Cn/Ob) **(Fig. 13a)**. Overall, this indicates that exposure to Ob diet during gestation and lactation

leads to increased weight of the offspring and augments their weight gain in response to the Ob diet.

To determine if weight increase was due to increased fat or lean mass, I measured the body composition of the offspring. Consistent with the increased weight of Ob/Cn, fat and lean mass also increased **(Fig. 13b)**. However, body fat percentage increased, and lean percentage decreased **(Fig. 13c)**, highlighting a shift in the body composition. Cross-fostered mice maintained normal fat and lean mass throughout their lifetime. In response to Ob diet, the offspring of obese mice gained significantly more body fat compared to offspring of Cn-fed mice, but the lean mass was not altered **(Fig. 13b)**. Thus, the increased weight in offspring of obese dams was attributed to a significant increase in body fat throughout their lifetime and an exacerbated response to the Ob diet.

To determine if increased weight and fat mass are associated with altered glucose homeostasis, I used a commonly used screening test to measure the response of the offspring to glucose. Glucose tolerance was not altered in Ob/Cn, or XOb/Cn compared to Cn/Cn at 2, or 6 months of age **(Fig. 13d)**. In response to feeding the offspring with the Ob diet, the glucose tolerance of both Cn/Ob and Ob/Ob decreased compared to the Cn-fed offspring, but Ob/Ob had significantly worse glucose tolerance compared to Cn/Ob **(Fig. 13d)**. This indicates that despite a subtle increase in weight, offspring of obese mothers maintain normal glucose homeostasis when fed Cn diet but develop an exacerbated glucose intolerance when fed Ob diet.

In summary, maternal obesity during gestation alone is not sufficient to promote weight gain increase or alter body composition in the offspring. Ob diet during gestation and lactation predisposes offspring to a modest increase the body weight and fat mass but does not disrupt glucose homeostasis under baseline conditions. In addition, increased body weight, fat mass and impaired glucose homeostasis are exacerbated during metabolic challenge in the offspring of Ob-fed mothers. These results are consistent with those observed in other obesity models[241] suggesting a common mechanism for postnatal programming of weight gain and exacerbated responses to metabolic challenge in offspring born to obese mothers.

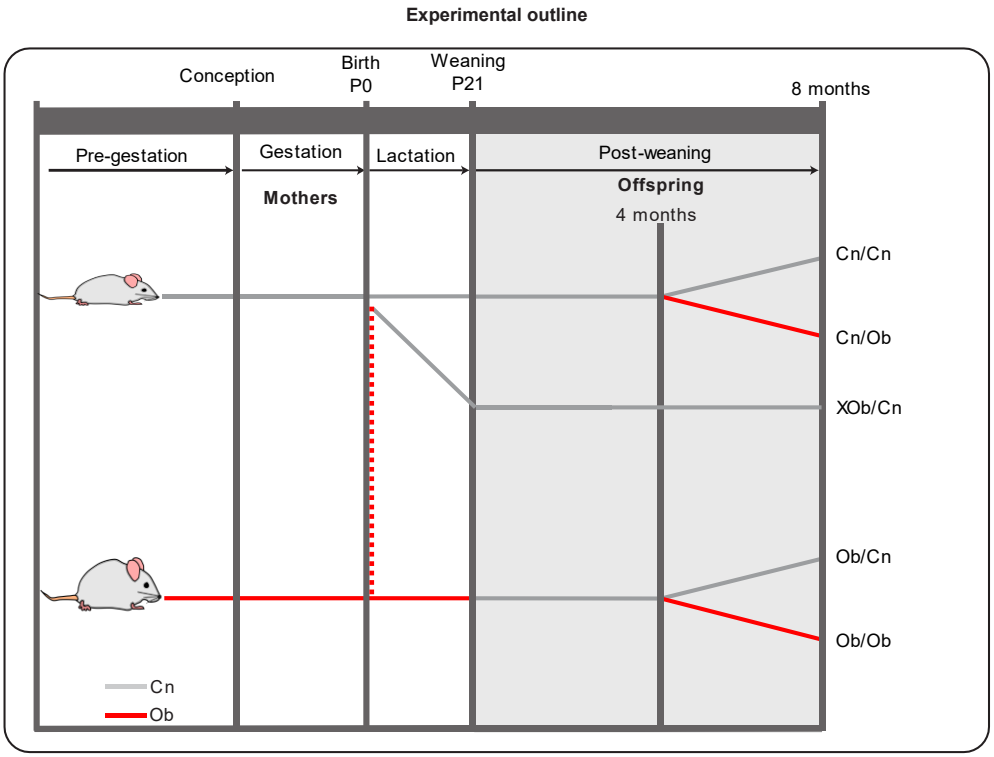



**Figure 12. Feeding scheme of female mice and their offspring.** Female mice were fed a control diet (grey line) or an obesogenic diet (red line) starting at 1 month of age and maintained on their respective diets for 4 months before mating and during pregnancy. All male breeders were exposed to the high fat/high sugar diet only for 1 to 4 days during mating. At birth, one litter from an Ob-fed mouse was cross-fostered with a Cn-fed females which had also delivered offspring on the same day. The offspring of the cross-fostering Cn-fed female was terminated. All other offspring were maintained with their birth mothers until weaning. All offspring were weaned at P21 to the Cn diet. At 4 months of age, offspring were divided into groups, fed either the Ob diet or maintained on the Cn diet. This resulted in a total of 5 groups differing in the prenatal and postnatal maternal diet and in the diet of the offspring after 4 months.

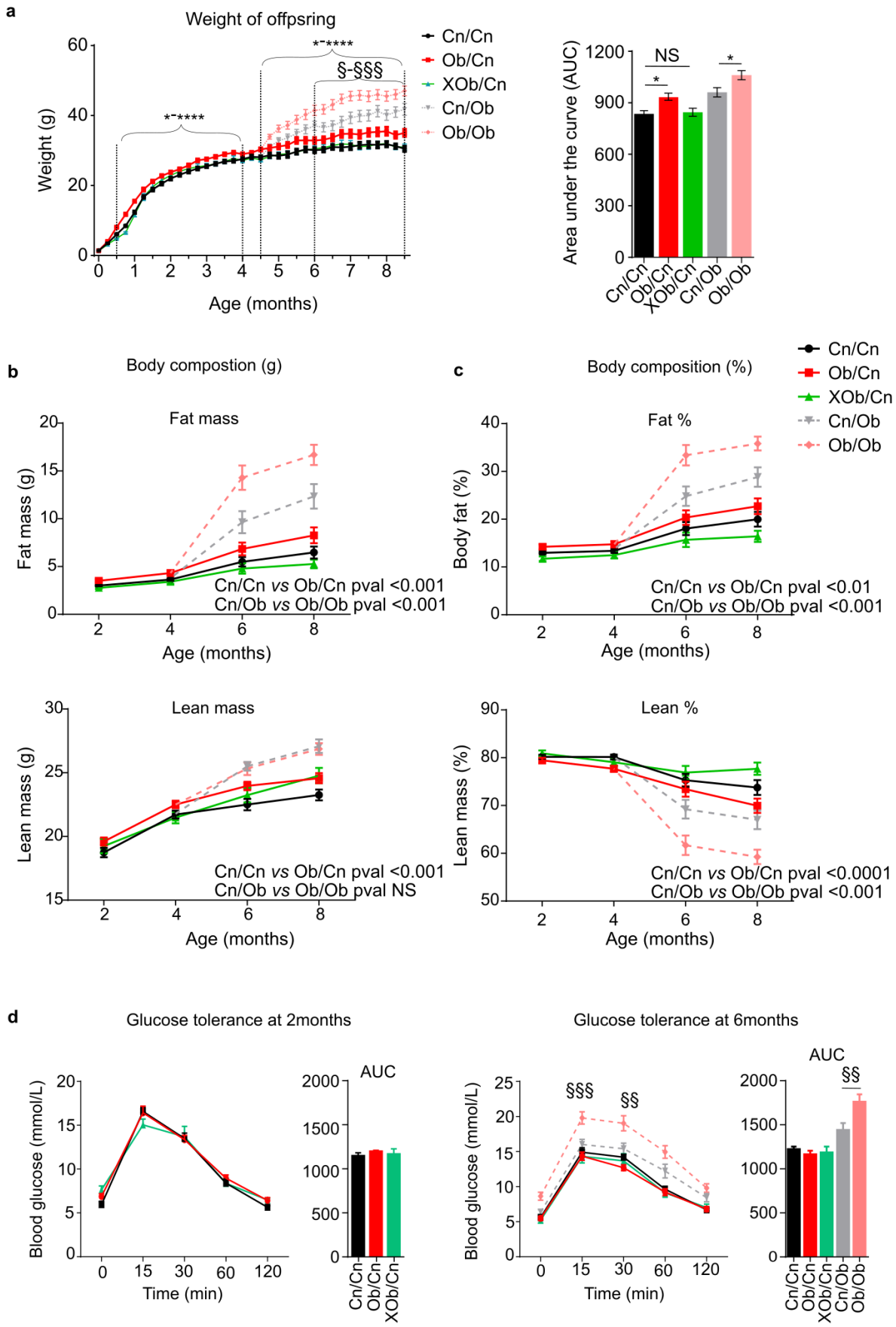

a
Weight of offpsring
Weight (g)
Age (months)
Cn/Cn
Ob/Cn
XOb/Cn
Cn/Ob
Ob/Ob
*-****
§-§§§
Area under the curve (AUC)
NS
b
Body compostion (g)
Fat mass
Fat mass (g)
Cn/Cn vs Ob/Cn pval <0.001
Cn/Ob vs Ob/Ob pval <0.001
Lean mass
Lean mass (g)
Cn/Cn vs Ob/Cn pval <0.001
Cn/Ob vs Ob/Ob pval NS
c
Body composition (%)
Fat %
Body fat (%)
Cn/Cn vs Ob/Cn pval <0.01
Cn/Ob vs Ob/Ob pval <0.001
Lean %
Lean mass (%)
Cn/Cn vs Ob/Cn pval <0.0001
Cn/Ob vs Ob/Ob pval <0.001
d
Glucose tolerance at 2months
Glucose tolerance at 6months
Blood glucose (mmol/L)
Time (min)
AUC
§§§
§§

**Figure 13. Male offspring exposed to prenatal and postanal maternal Ob exhibit mild changes in weight and body composition when fed a control diet but undergo augmented obesogenic and diabetic responses to high-fat diet. A**, Body weight of offspring measured weekly (left). Area under the curve (AUC) of weekly body weight measurements. N=7-16 mice per group. **b)** Body composition in mass and **c)** percentage (%) of offspring at 2, 4, 6 and 8 months of age. N= 7-39 mice per group. **C**, Glucose tolerance tests of offspring at 2 and 6 months of age. N= 7-15 mice per group. Multiplicity adjusted *P* values are indicated above the timepoints. Bars represent the mean ± SEM. Insets show the mean ± SEM of the area under the curve (AUC). * represents $P<0.05$ between Cn/Cn and Ob/Cn. § represents a $P<0.05$ between Cn/Ob and Ob/Ob.

## 5.3. Fetal exposure to maternal obesity leads to decreased diastolic function in young offspring, but long-term dysfunction is dependent on exposure to an an obesogenic environment during lactation

With a better understanding of the metabolic context/outcomes of the offspring, I analyzed the cardiac function in offspring of obese mothers. Of note, the cardiac function of offspring which were challenged with Ob diets (Cn/Ob and Ob/Ob) was also analyzed however, the Ob diet-induced aortic valve plaque formation with variable penetrance in offspring of Ob and Cn-fed mothers (data not shown). The high variability in phenotypes within these two groups precluded further analysis of all offspring that were fed Ob diets.

Offspring of obese mice had normal ejection fraction and fractional shortening at 4 months of age irrespective of the maternal diet during lactation **(Fig. 14a, b)**, indicating maintained systolic function in young adulthood. The isovolumetric relaxation time was significantly increased in offspring of Ob-fed mothers, and cross-fostered offspring **(Fig. 14c, e)**, indicating a reduced diastolic function programmed independently of the post-natal diet. The E/A ratio, another marker of diastolic function, was reduced in Ob/Cn but not in cross-fostered mice **(Fig. 14c, f)**. Thus, maternal obesity does not alter systolic function in young, 4 months old offspring. However, it leads to reduced diastolic function, albeit to a lesser extent in cross-fostered offspring.

Diastolic dysfunction is an early marker of cardiac disease and several studies have demonstrated diastolic dysfunction precedes systolic dysfunction in several models of cardiac stress. To determine if the diastolic dysfunction at 4 months of age progresses with age, I performed follow-up cardiac functional analysis in offspring of obese mothers at 8 months of age. The increase in the isovolumetric relaxation time in Ob/Cn was sustained at 8 months and was coupled with a normalization of the E/A ratio **(Fig. 14d, e, f)**. Pseudonormalization of the E/A ratio is the known progression of reduced E/A ratio and suggests a further reduction in diastolic function. Notably, the ejection fraction decreased by ~10% and fractional shortening decreased by ~5% in offspring of obese mothers **(Fig. 14a, b)**, indicating a reduced systolic function. The cardiac output of the mice was not altered indicating a compensated left ventricular function **(Fig. 14g, h)**. Cross-fostered mice maintained normal systolic function and the increase in IVRT was reverted to normal levels at 8 months of age. Notably, the longer IVRT, a ~10% reduction in ejection fraction, and a ~5% reduction in fractional shortening in OH were mild and likely representative of early, sub-clinical, stages of heart disease.

Overall, my results demonstrate that *in utero* exposure to maternal obesity is sufficient to induce mild diastolic dysfunction in young adults but a second exposure to an obesogenic environment during lactation is required for long-term maintenance and progression of cardiac dysfunction. The progressive decline in cardiac function is mild and is more consistent with subclinical heart disease rather than overt onset of cardiomyopathy.

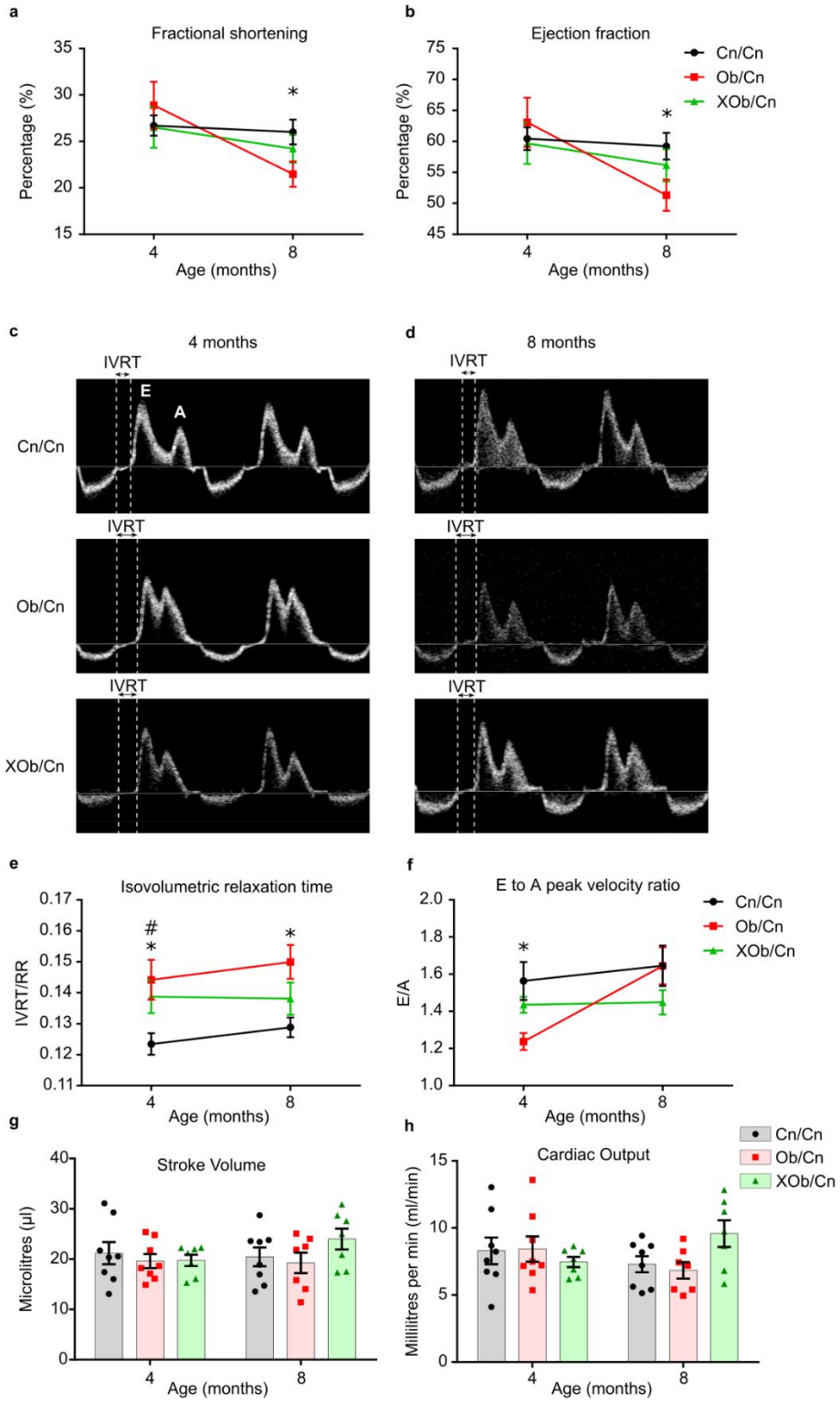



**Figure 14. Exposure to an obesogenic environment during gestation alone reduces early diastolic function in offspring but Ob-feeding during lactation promotes progressive dysfunction. a)** Left ventricular fractional shortening of offspring at 4 and 8 months of age. **b)**

Left ventricular ejection fraction of offspring at 4 and 8 months of age. **c)** Representative pulsed Doppler recording of blood flow across the mitral valve for 2 cardiac cycles in offspring at 4 and **d)** 8 months of age. Isovolumetric relaxation time (IVRT) is indicated between the white lines. Peak velocities of early (E) and late/atrial (A) diastolic filling waves are denoted. **e)** Quantification of the IVRT normalized to the length of one cardiac cycle (RR) **f)** Peak E to A ratios at 4 and 8 months of age. **g)** Left ventricular stroke volume and **h)** cardiac output of offspring at 4 and 8 months of age. N=7-8 mice per group at each timepoint. Bars represent the mean ± SEM. * represents $P<0.05$ between Cn/Cn and Ob/Cn. # represents a $P<0.05$ between Cn/Cn and XOb/Cn.

## 5.4. Maternal obesity does not alter blood pressure but causes bradycardia in the offspring

To determine if cardiac dysfunction in offspring of obese mice is a secondary consequence of hypertension, I measured the blood pressure of unanesthetized offspring of obese mice. Diastolic and systolic blood pressures were not altered at 4 or 8 months of age **(Fig. 15a, b)**.

The heart rate of unanesthetized offspring of obese mice was reduced at 4 months, and a similar trend was maintained at 8 months **(Fig. 15a, b)**. Heart rates of cross-fostered mice tended to decrease at 4 months although no significant changes were detected at 4 or 8 months of age **(Fig. 15a, b)**. This indicates the exposure to maternal obesity does not alter blood pressure in adult offspring. Exposure to maternal obesity during gestation and lactation reduces the heart rate of the offspring.

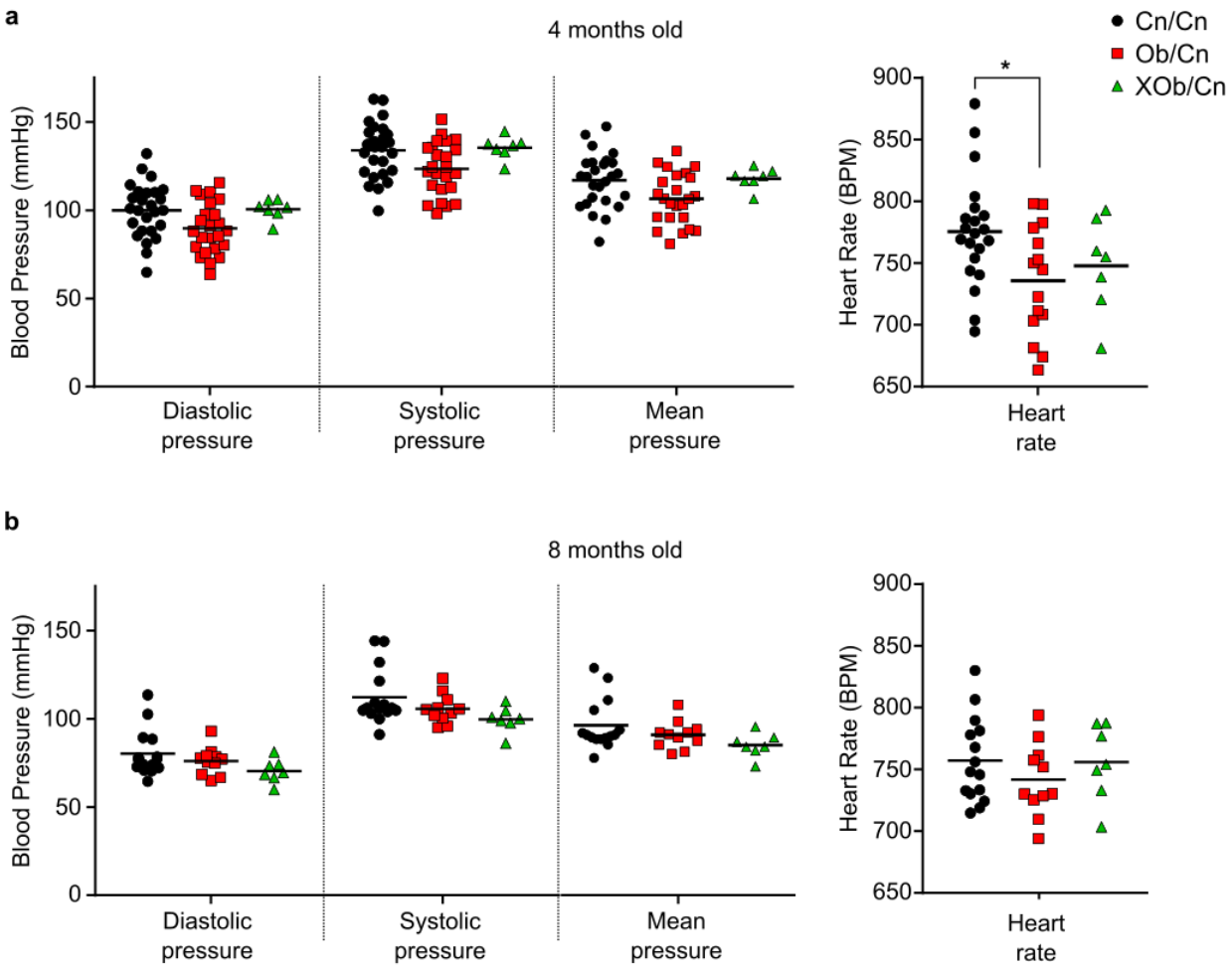



**Figure 15. Maternal Ob-feeding during pregnancy and lactation predisposes offspring to bradycardia but does not alter blood pressure. a)** Blood pressure in males born to mice fed a control diet, or an Ob diet at 4 months of age (Left). Heart rate during blood pressure acquisition (right). **b)** Blood pressure in males born to mice fed a control diet, or an Ob diet at 8 months of age (Left). Heart rate during blood pressure acquisition (right). Bars represent the mean ± SEM. n=7-26 mice per group. * represents $P<0.05$

## 5.5. Maternal obesity causes cardiomyocyte hypertrophy in adult offspring

To determine the morphological and cellular effects of maternal obesity on the heart of offspring of obese dams, I first examined the overall cardiac structure at 8 months. No overt morphological changes, such as septation defects or ventricular hypoplasia, were apparent in the hearts of offspring of obese mice **(Fig. 16a, b)**. At 2 and 8 months of age, the normalized heart weight was mildly increased in mice whose mothers were fed Ob diet during gestation and lactation **(Fig. 16c)**. In contrast, the heart size of 8-month-old cross-fostered mice was not altered **(Fig. 16b)**, consistent with normal cardiovascular function **(Fig. 14)**. To determine if the heart was fibrotic, I assessed the presence of fibrous tissue in offspring hearts at 8 months of age. No overt fibrosis was detected by Mason's trichrome staining in the heart, irrespective of maternal postnatal diet **(Fig. 16b, d)**. To determine if increased heart size was associated with cardiomyocyte hypertrophy, I measured cardiomyocyte cell surface area in the hearts of offspring at 8 months of age. Cardiomyocyte size was increased in Ob/Cn at the apex, but not at the middle or base of the heart **(Fig. 16e)**. Cardiomyocyte size was not altered in cross-fostered mice **(Fig. 16e)**. My results indicate that maternal obesity leads to increased heart size as early as 2 months of age, and persistently at 8 months due to a localized hypertrophic response in the cardiac apex. The localized hypertrophic response coupled with the lack of fibrosis is consistent with subclinical signs of disease.

To determine if cardiac hypertrophy originates during development, I isolated cardiac myocytes from E13.5 ventricles. Cardiac myocytes labeled by staining for the cell surface marker VCAM1were isolated using FACS, which enriches for differentiated cardiac myocytes in the embryonic heart[214,242] **(Fig. 17a)**. Cell size was not altered in cultured E13.5 CMs after 24 or 72hrs, in response to maternal obesity **(Fig. 17b)**. This suggests that cardiomyocytes from the early developing heart of embryos of obese mice are not innately hypertrophied. However, the influence of cell culture conditions on cell size cannot be excluded and future *in vivo* cell size measurements are required to validate this finding.

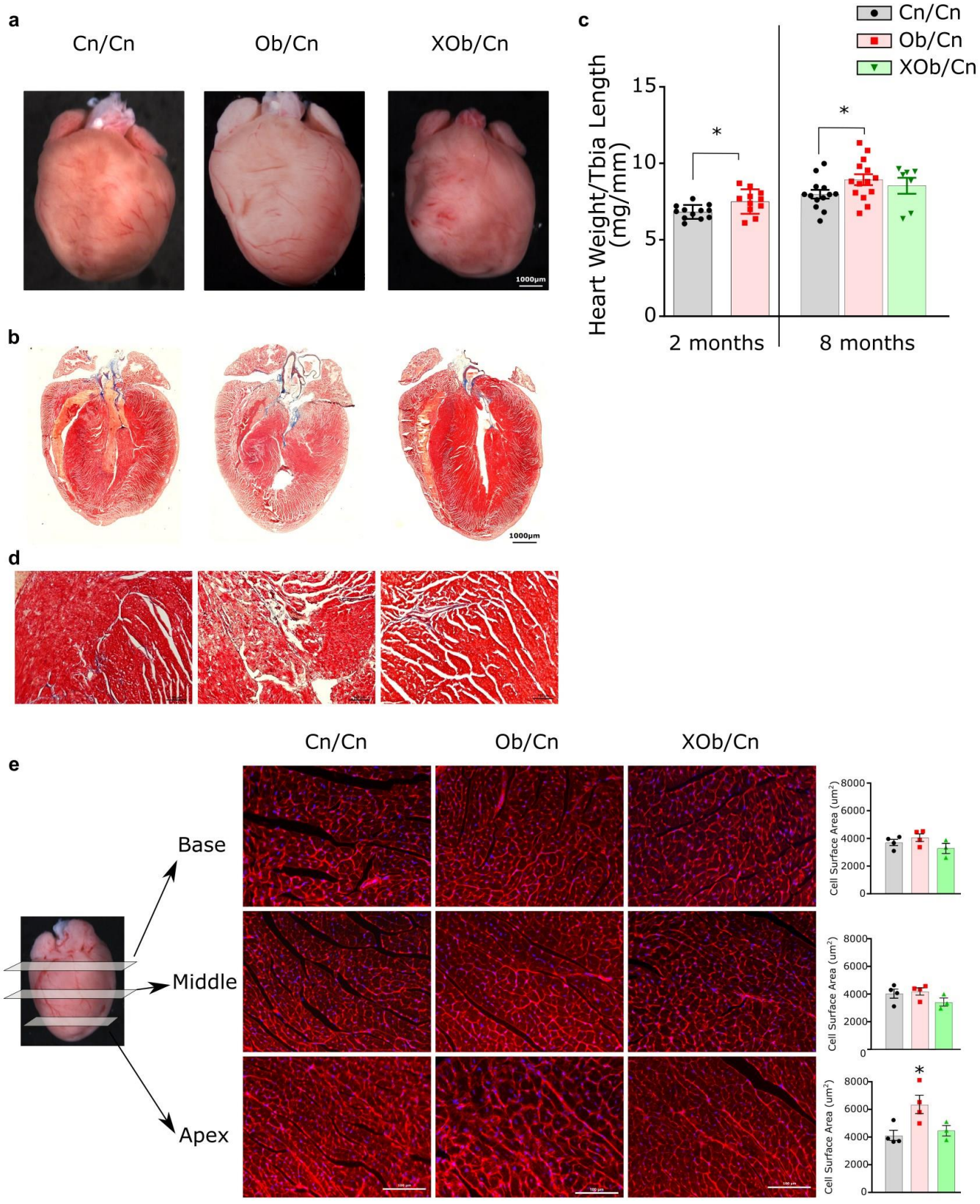

a
Cn/Cn
Ob/Cn
XOb/Cn
1000µm
b
1000µm
c
Cn/Cn
Ob/Cn
XOb/Cn
Heart Weight/Tbia Length (mg/mm)
15
10
5
0
*
*
2 months
8 months
d
e
Cn/Cn
Ob/Cn
XOb/Cn
Base
Middle
Apex
Cell Surface Area (um²)
8000
6000
4000
2000
0
*

**Figure 16. Maternal Ob-feeding during pregnancy and lactation causes early and persistent cardiac hypertrophy in the offspring. a)** Whole mount of the gross cardiac morphology of offspring of Cn-, Ob-, and cross-fostered Ob-fed mice at 8 months of age. **b)** Representative Masson's trichrome staining on sections of hearts of offspring at 8 months of age. **c)** Heart weight normalized to tibia length ratio in offspring and 2 and 8 months of age. **d)** Magnifications of the left ventricular wall shown in b) demonstrating no overt differences in fibrosis. **e)** Left ventricular muscle sections stained with wheat germ agglutinin (WGA) at the apex, middle and base of the heart. Quantification of the cell surface area of cardiomyocytes at the apex, middle and base of the left ventricle is shown on the right. Bars represent the mean ± SEM. * represents $P<0.05$

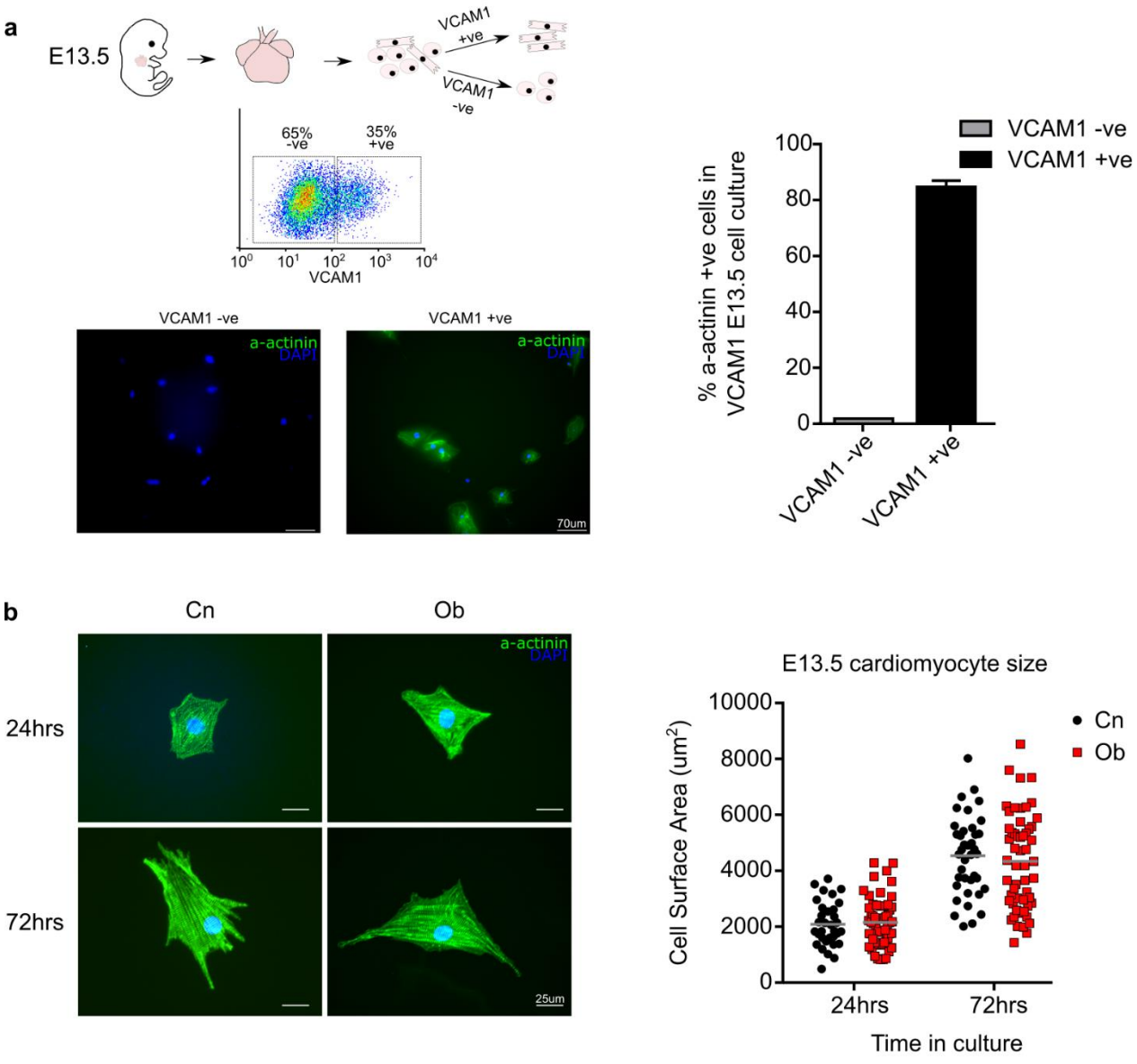



**Figure 17. Maternal Ob-feeding does not induce embryonic cardiomyocyte hypertrophy at E13.5. a)** Schematic representation of cardiomyocyte isolation from E13.5 hearts using FACS against VCAM1 (Top). Representative sorting plots are shown below. Immunofluorescence staining against a-actinin (green) demonstrating the VCAM1 positive cell fraction was enriched in cardiomyocytes and the VCAM1 negative cell fraction was depleted from cardiomyocytes. Quantification of the percentage of a-actinin positive cells in each fraction is shown on the right. **b)** Immunofluorescence staining against a-actinin (green) was used to stain cardiomyocytes and visualize the cell borders. Cells were imaged 24hrs after isolation and 72hrs after isolation to

examine their cell size before and after they have spread. Quantification of the cardiomyocyte cell size obtained from embryos of Cn- or Ob-fed mice is shown on the right.

## 5.6. Offspring of obese mothers develop an exacerbated pathological remodeling in response to pathological stimulus

CVD in humans born to obese women is not fully penetrant. Instead, these individuals are more likely to be hospitalized or die due to cardiovascular events. It is not clear why some offspring develop disease and others do not. My results thus far suggest that a secondary insult in postnatal life, such as exposure to the obesogenic environment during lactation, is required for the development and progression of subclinical cardiac dysfunction. I hypothesized that maternal obesity might poise the offspring for cardiac disease. Therefore, it is possible that further insult during adulthood might be required to trigger cardiomyopathy. To test if the offspring of obese females are poised for cardiac disease, I exposed Ob/Cn and Cn/Cn to a pathological stimulus and examined the functional, morphological and cellular cardiac responses.

At 2 months of age, mice were chronically infused with isoproterenol (ISO) using osmotic pumps, for 14 days **(Fig. 18)**. Isoproterenol is a non-selective beta-adrenergic agonist. Chronic infusion mimics advanced heart failure which is characterized by chronic adrenergic stimulation. In mice, chronic infusion with ISO induces significant hypertrophy and fibrosis after 14 days and heart failure after 21days[243]. At 2 months of age, prior to pump implantation, no differences in systolic or diastolic function were present between offspring of Cn and Ob-fed mothers **(Table 2)**. Similarly, cardiac function was not altered in PBS-treated mice of Ob/Cn compared to Cn/Cn, however, the thickness of the posterior left ventricular wall was increased **(Table 3)**. As expected, 14 days of isoproterenol infusion increased heart rate, cardiac function and ventricular wall thickness in all mice **(Table 3, Fig. 19a)**. Heart rate and systolic function increased by similar levels irrespective of maternal diet **(Table 3)**. Thickening of the ventricular walls was augmented in offspring of obese mothers. In addition, ventricular diameters were reduced in offspring of obese mothers during ISO treatment **(Table 3, Fig. 19a, b, c)**. Similarly, chamber volumes at end-diastole decreased and the volumes at end-systole tended to decrease **(Table 3, Fig. 19a, d, e)**, suggesting exacerbated restrictive hypertrophy. Unlike the offspring of Cn-fed mice, Ob/Cn failed to shorten the IVRT in response to ISO infusion **(Fig. 19f)**. The increase in cardiac output was also diminished **(Fig. 19g)**. Thus, offspring of obese mice are

functionally normal in young adulthood, but in response to pathological insult with ISO they mount a restrictive hypertrophic response and have a reduced diastolic and cardiac output response.

To determine if the pathological changes are associated with cardiac fibrosis, I quantified collagen content and its distribution in the heart. No difference in collagen content was present in the young PBS-treated offspring of Cn or Ob-fed mothers **(Fig. 20a, b)**. The total collagen area in the heart was 2-fold higher in offspring of Ob compared to Cn-fed mothers in response to ISO **(Fig. 20a, b)**. Interstitial and perivascular fibrosis were both increased in response to ISO in control mice **(Fig. 20a, b)**. This indicates that young 2-month-old Ob/Cn are prone to develop augmented fibrotic remodeling in response to stress.

**Table 2. Cardiac morphological and functional parameters detected by echocardiography in offspring of Cn- and Ob-fed mice at 2 months of age.**

| | Mean Cn/Cn | Mean Ob/Cn | P value (Student's t-test) | Benjamini-Hochberg Adjusted P value |
|---|---|---|---|---|
| Ejection fraction | 55.97 | 58.87 | 0.1875 | 0.249988 |
| Fractional shortening | 28.88 | 30.96 | 0.1636 | 0.237978 |
| AW (Systole) | 1.0435 | 1.1510 | 0.0193 | 0.061761 |
| PW (Systole) | 0.9627 | 1.0600 | 0.0043 | 0.069382 |
| AW (Diastole) | 0.7374 | 0.8205 | 0.0274 | 0.073127 |
| PW (Diastole) | 0.7108 | 0.7823 | 0.0094 | 0.050329 |
| ESD | 2.7920 | 2.7518 | 0.6929 | 0.791865 |
| EDD | 3.7871 | 3.7654 | 0.7923 | 0.845158 |
| Stroke volume | 35.0695 | 36.7256 | 0.2811 | 0.345991 |
| Cardiac output | 15.0939 | 16.9312 | 0.0388 | 0.088776 |
| E | 692.228 | 686.912 | 0.8222 | 0.822171 |
| A | 555.262 | 524.834 | 0.1509 | 0.241374 |
| E/A | 1.2526 | 1.3272 | 0.0826 | 0.146787 |
| IVRT/RR | 0.13784 | 0.12887 | 0.019053 | 0.076214 |
| Myocardial Performance Index | 0.70627 | 0.65244 | 0.067788 | 0.135576 |
| HR | 430.18 | 458.02 | 0.005645 | 0.045157* |
| n | 30 | 22 | | |
| Number of litters | 9 | 9 | | |

**Table 3. Cardiac morphological and functional parameters detected by echocardiography in offspring of Cn- and Ob-fed mice at 2.5 months of age (2 weeks after chronic infusion with PBS or ISO).**

| Cardiac parameters | Mean | Mean | Mean | Mean | Adj-Pvalue | | | |
|---|---|---|---|---|---|---|---|---|
| | Cn/Cn - PBS | Ob/Cn - PBS | Cn/Cn - ISO | Ob/Cn - ISO | Cn/Cn - PBS *vs* Ob/Cn - PBS | Cn/Cn - PBS *vs* Cn/Cn - ISO | Ob/Cn - PBS *vs* Ob/Cn - ISO | Cn/Cn - ISO *vs* Ob/Cn - ISO |
| Ejection fraction | 52.49 | 55.34 | 69.71 | 77.79 | 0.9337 | 0.0003* | 0.0003* | 0.1761 |
| Fractional shortening | 26.63 | 28.62 | 39.87 | 46.4 | 0.9592 | 0.0008* | 0.0005* | 0.1979 |
| AW (Systole) | 1.065 | 1.067 | 1.555 | 1.809 | > 0.9999 | < 0.0001 * | < 0.0001* | 0.0101* |
| PW (Systole) | 0.9321 | 0.9592 | 1.478 | 1.731 | 0.9796 | < 0.0001* | < 0.0001* | 0.0005* |
| AW (Diastole) | 0.7609 | 0.773 | 0.9917 | 1.148 | 0.9976 | 0.0001* | < 0.0001* | 0.0136* |
| PW (Diastole) | 0.7054 | 0.8432 | 0.9948 | 1.062 | 0.0229* | < 0.0001* | 0.0002* | 0.2315 |
| ESD | 2.945 | 2.947 | 2.445 | 1.888 | > 0.9999 | 0.0477* | 0.0005* | 0.0274* |
| EDD | 3.86 | 3.883 | 3.924 | 3.534 | 0.999 | 0.9579 | 0.1675 | 0.0244* |
| ESV | 31.95 | 31.17 | 22.17 | 12.93 | 0.9986 | 0.0778 | 0.0052* | 0.1187 |
| EDV | 66.83 | 67.43 | 68.96 | 53.92 | 0.9997 | 0.975 | 0.1915 | 0.0322* |
| Stroke volume | 34.89 | 36.27 | 46.79 | 40.99 | 0.964 | < 0.0001* | 0.3907 | 0.0791 |
| Cardiac output | 15.57 | 16 | 31.72 | 27.87 | 0.995 | < 0.0001* | < 0.0001* | 0.0498* |
| E | 653.3 | 623.8 | 1127 | 1120 | 0.9458 | < 0.0001* | < 0.0001* | 0.9986 |
| IVRT/RR | 0.146 | 0.1371 | 0.1223 | 0.1449 | 0.7451 | 0.0085* | 0.8174 | 0.0158* |
| Myocardial Performance Index | 0.7443 | 0.6345 | 0.5128 | 0.5921 | 0.181 | < 0.0001* | 0.8594 | 0.2145 |
| HR | 445.7 | 440.8 | 678 | 685.1 | 0.9859 | < 0.0001* | < 0.0001* | 0.9283 |
| n | 11 | 6 | 15 | 10 | | | | |
| Number of litters | 7 | 4 | 9 | 7 | | | | |

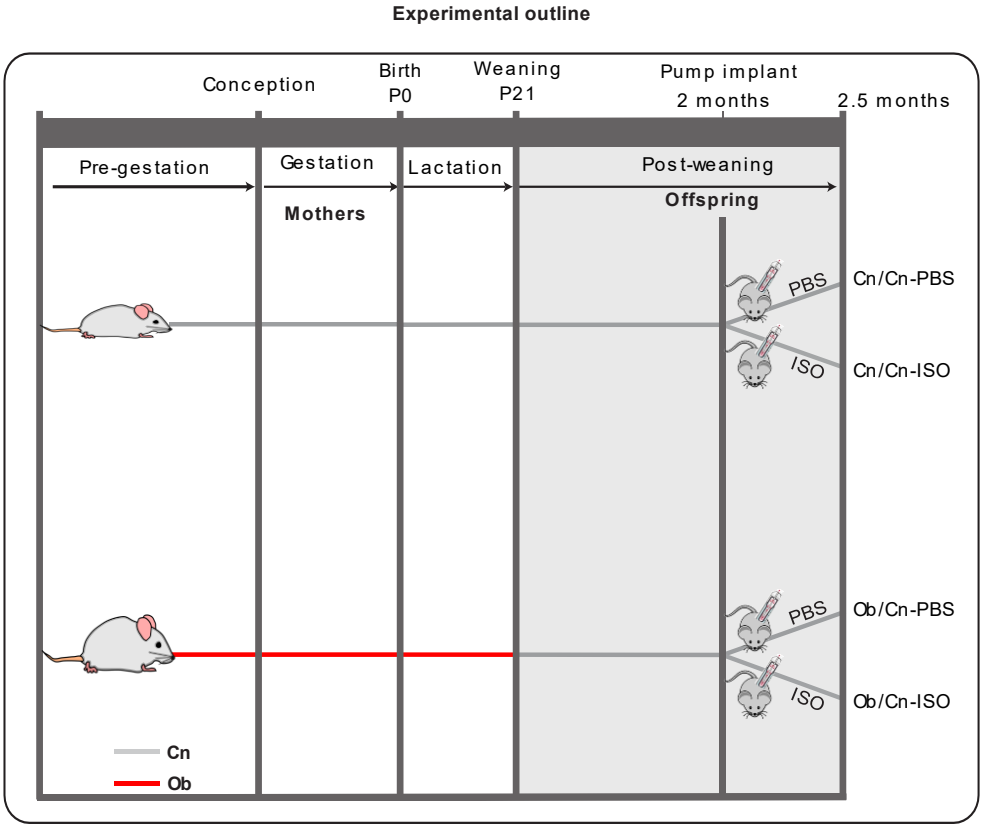



**Figure 18. Experimental scheme to determine the response of young offspring of Ob-fed mice to pathological stress.** Female mice were fed a control diet (grey line) or an obesogenic diet (red line) starting at 1 month of age and maintained on their respective diets for 4 months before mating and during pregnancy. All other offspring were maintained with their birth mothers during lactation and were weaned onto control diets at P21. At 2 months of age, offspring cardiac function was assessed using echocardiography then mice were randomized and implanted with osmotic pumps filled with PBS or ISO. 14 days after pump implantation mice underwent a second follow-up echocardiogram and were terminated thereafter.

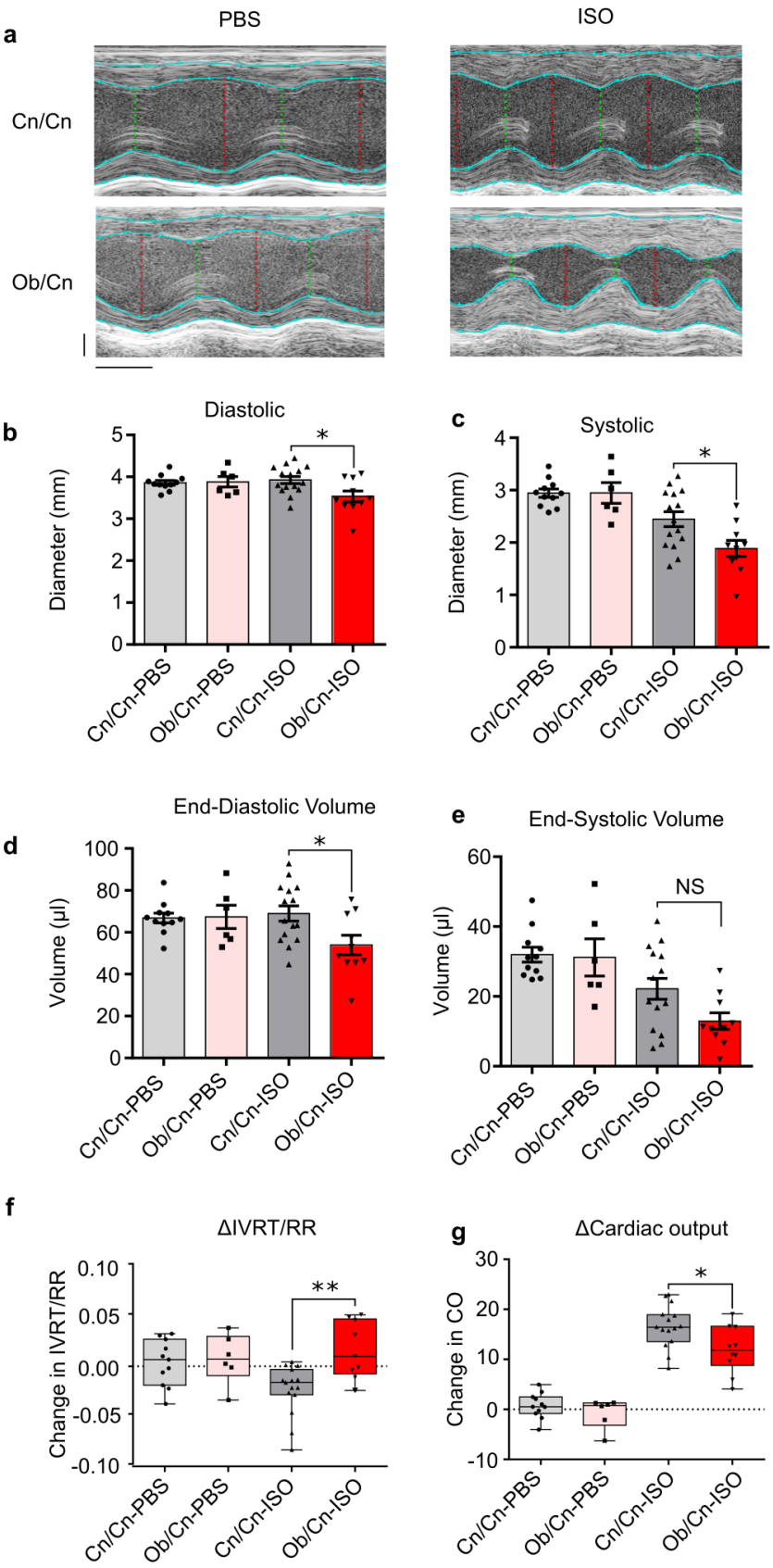

a
PBS
ISO
Cn/Cn
Ob/Cn
b
Diastolic
Diameter (mm)
Cn/Cn-PBS
Ob/Cn-PBS
Cn/Cn-ISO
Ob/Cn-ISO
c
Systolic
Diameter (mm)
d
End-Diastolic Volume
Volume (µl)
e
End-Systolic Volume
Volume (µl)
NS
f
ΔIVRT/RR
Change in IVRT/RR
g
ΔCardiac output
Change in CO

**Figure 19. Male offspring of Ob-fed mice develop exacerbated pathological remodeling in response to isoproterenol infusion. a)** M-mode echocardiogram of the left ventricle of mice born to females fed a control (Cn/Cn) or a high-fat diet (Ob/Cn) that were treated with PBS or isoproterenol (ISO). Horizontal scale bar = 50 msec, vertical scale bar = 1 mm. **b)** End-diastolic diameter, **c)** end-systolic diameter, **d)** end-diastolic volume and **e)** end-systolic volume in mice 2 weeks after pump implant. **f)** Echocardiography was performed on each mouse prior to pump implantation and 2 weeks after implantation. The change in isovolumetric relaxation time normalized to time between heartbeats (IVRT/RR) and **g)** change in cardiac output for each mouse is shown. For **b)** to **g)** N=11, 6, 15 and 10 mice per group, respectively (refer to table 3 for exact numerical values).

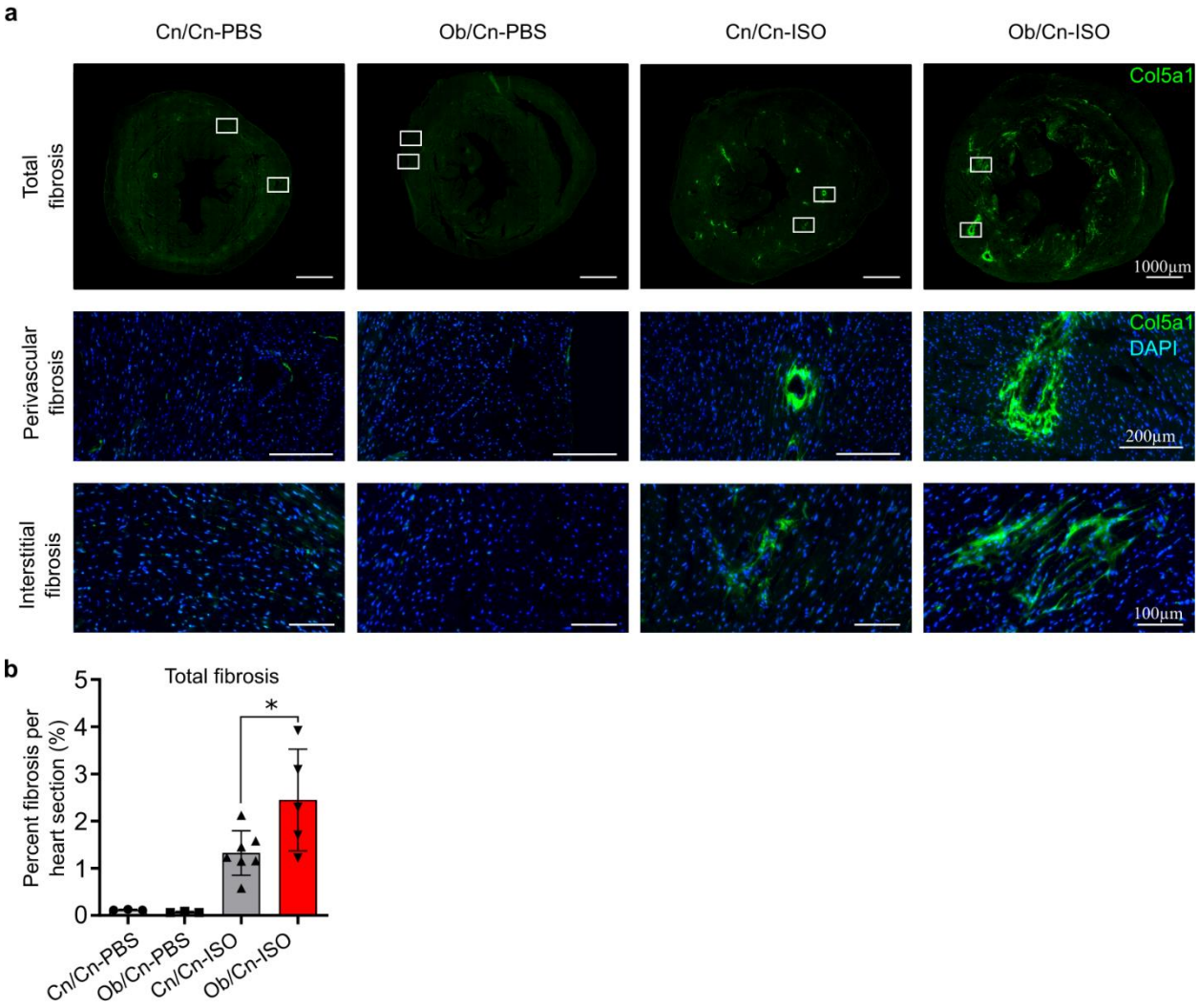



**Figure 20. Male offspring of Ob-fed mice develop augmented cardiac fibrosis in response to isoproterenol infusion. a)** Immunofluorescence against Collagen Type V Alpha 1 (Col5a1) in cross-sections obtained from the middle of the hearts of offspring at 2.5 months of age (top). Lower panels are magnifications of the ventricular wall showing perivascular (middle) and interstitial fibrosis (bottom). **b)** Quantification of total fibrosis area percentage from the entire heart section shown in the top panels of a). N=3, 3, 7 and 5 mice per group, respectively.

## *6. Discussion 1:*

### 6.1. Establishing a mouse model of maternal obesity

Growing evidence from clinical studies points to maternal obesity, independently of diabetes, as an important contributor to cardiovascular disease in the progeny[12–16]. In contrast, all animal models of maternal obesity use aggressive obesogenic diets that induce weight gain concomitantly with diabetes and hyperglycemia[138,169–172,187,188], thus, dissociating between the effects of obesity and diabetes is not possible in current animal models. In my thesis, I demonstrate that long-term feeding of mice with a relatively mild obesogenic diet (30% fat, 33% sucrose) is effective in increasing weight gain while maintaining normal glucose tolerance in females, even after long-term feeding **(Fig. 11)**. Although mice on the mild obesogenic diet gained weight slower than mice that were fed an aggressive obesogenic diet, the mice reached similar weights and body fat percentages as previous obesity models, albeit over a longer period **(Fig. 11)**. Although small increases in glucose levels representing a pre-diabetic state during pregnancy cannot be excluded, normal glucose tolerance test results exclude the effects of chronic maternal hyperglycemia. Thus, I established a model to study the effect of maternal obesity, independently of diabetes, on offspring cardiovascular health.

### 6.2. Programming of metabolic dysfunction in offspring of obese mice is influenced by maternal prenatal and postnatal diet

Offspring of obese women do not develop cardiac disease in isolation of other diseases, but they are also at higher risk for metabolic disorders such as obesity and diabetes[244]. Obesity and diabetes are independently associated with an increased risk of cardiovascular disease[245], however, it is unclear if cardiac disease predisposition is a primary outcome of maternal obesity or a secondary consequence of obesity and diabetes in the offspring. It is also unclear if the risks of obesity and diabetes are programmed during the same developmental time window as CVD.

In my thesis, I characterized the metabolic phenotype of the offspring to help contextualize potential cardiac phenotypes and I also established the developmental time frame at which these metabolic phenotypes are programmed. I demonstrate that when offspring of obese mice are weaned onto control diets, their weight and body fat are mildly increased throughout their lifetime, but their glucose tolerance is normal, as compared to the offspring of lean mothers **(Fig. 13)**. Despite these mild metabolic phenotypes, the offspring were poised for aggravated diabetes and fat accumulation in response to high fat diet feeding **(Fig. 13)**. This is consistent with

increased risk of obesity in human offspring, rather than a predetermined disease trajectory[244]. The mild metabolic phenotypes were established during the perinatal period, not during fetal development **(Fig. 13)**. It has previously been shown that offspring of mice fed severely obesogenic diets during pregnancy and lactation are hyperphagic and develop glucose intolerance[246]. In addition, feeding mice a severely obesogenic diet during lactation alone is known to impair neural innervation in the feeding circuits of the hypothalamus (the paraventricular nucleus) and insulin-secreting cells of the pancreas (the islets)[241]. Reduced innervation is caused by increased glucose and insulin in maternal milk which leads to hyperinsulinemia in the pups during the perinatal period – the period when hypothalamic neural projections are established[241]. Subtle increase in weight and fat deposition in my model is likely due to impaired hypothalamic feeding circuits, however, maintained glucose tolerance in the offspring suggests that reduced pancreatic innervation and glucose intolerance reported in previous models[241,246] might be an artifact of the aggressive obesogenic diets previously used, and a consequence of maternal diabetes. Notably, it has been previously demonstrated that exposure to an obesogenic environment during lactation alone does not cause exacerbated weight gain or diabetes when the offspring are fed obesogenic diets[241]. In my thesis, I demonstrate that a combined fetal and postnatal exposure to an obesogenic environment exacerbates metabolic dysfunction in mice **(Fig. 13)**. This suggests that exposure to maternal obesity during both fetal life and early perinatal life is required for programming the exacerbated metabolic response to obesogenic diets. My study provides a model for dissecting the pre-disposition to aggravated metabolic remodeling in response to maternal obesity. Importantly, when offspring are fed control diets, cardiac phenotypes can be investigated in mice that lack severe metabolic disturbances.

## 6.3. The heart is programmed to develop cardiac dysfunction in response to an obesogenic *in utero* environment

Adult-onset CVD in offspring of obese women is characterized by its late onset and incomplete penetrance[14–16]. Although the offspring of obese women are at higher risk of cardiovascular disease, it is predominantly in older age and most of these individuals do not develop the disease[14–16], which suggests an increased susceptibility to CVD disease rather than a predetermined disease trajectory. Animal models of maternal obesity, confounded by diabetes,

develop completely penetrant cardiac hypertrophy in early life, but the hypertrophy dissipates with age[204–207]. Such findings are consistent with transient neonatal hypertrophy which occurs in offspring of diabetic women[33,197,200]. In my study, maternal obesity in the absence of glucose intolerance did not alter cardiac function in young offspring, but rather caused a mild, progressive reduction in cardiac function in older mice **(Fig. 14)**. At 2 months of age, all offspring of obese mice were phenotypically silent **(Table 2)**. At 4 months, diastolic function was mildly decreased in offspring of obese mice, but systolic function was maintained. At 8 months, both diastolic and systolic function were mildly decreased **(Fig. 14)**. Reduced diastolic function is considered an early predictor of cardiac disease and can precede systolic dyfucntion[39,40]. Mild reduction in systolic function of offspring of obese mice, coupled with early progressive diastolic dysfunction indicates early stages of disease onset. The heart of offspring of obese mice was also hypertrophied at 2 months of age, prior to functional impairment, and maintained the same level of hypertrophy with age **(Fig. 16c)**. A localized apical hypertrophic response with basal sparring was noted **(Fig. 16e)**, similar to that seen in stress-induced cardiomyopathy[243]. It is possible that the increased pressure gradient at the apex of the heart, as compared to the mid and base[247], comparatively predisposes the apex to earlier initiation of hypertrophy, however, this remains to be tested. Overall, my findings demonstrate that maternal obesity leads to mild cardiac hypertrophy in the offspring, which is associated with subclinical diastolic dysfunction in early stages and eventually systolic dysfunction.

The developmental time frame when the risk for cardiac disease is programmed in response to maternal obesity remains unknown. In my thesis, I used a cross-fostering model to establish that exposure to maternal obesity during pregnancy is sufficient to program a reduced diastolic function in the offspring at 4 months of age **(Fig. 14e)**. However, it is not sufficient to promote cardiac arrhythmia (at 4 months of age) **(Fig. 15)**, or long-term maintenance or progression cardiac dysfunction (at 8 months of age) **(Fig. 14)**. Exposure to maternal obesity during lactation was required for the manifestation of cardiac arrhythmia **(Fig. 15)** and the progression of cardiac dysfunction with age **(Fig. 14)**. This contrasts with metabolic disease which is programmed during the perinatal period in mice **(Fig. 13 and** [241]). An interesting question is why the risks for different diseases or phenotypic outcomes are programmed at separate stages in life. Previous work, and the work presented in this thesis, suggests that maternal insults affect the structure or

physiology of organ systems during critical periods of development that coincide with formation or rapid growth of the organ. For example, the incidence of obstructive airway disease is higher in offspring of women that were exposed to famine during mid-gestation, the period when the bronchial tree grows most rapidly[248]. Similarly, obesity and impaired glucose homeostasis are programmed in offspring of mice exposed to an obesogenic environment during lactation, the period when hypothalamic neurocircuits controlling feeding and insulin secretion are formed[241]. The heart undergoes morphogenesis during early gestation, but it also experiences rapid growth and metabolic remodeling during the early postnatal period[157,249]. I found that fetal exposure to maternal obesity is sufficient to program diastolic dysfunction in young mice **(Fig. 14e)**. An obesogenic postnatal environment – maternal obesity during lactation – was required for maintenance and progression of such phenotypes **(Fig. 14)**. This supports the hypothesis that fetal programming of cardiac disease occurs during the critical time windows of cardiac development, fetal and perinatal life.

Although my study does not address the specific fetal stage when disease is programmed in the heart, clinical studies point to early development as a likely candidate[12,250–253]. Indeed, the function of the hearts of fetuses of obese women is impaired as early as the first trimester[12]. In addition, exposure to famine specifically during early gestation, but not mid or late gestation, increases incidence[250,251] and mortality rates for CVD[252,253], and reduces the age of onset of disease[251]. Cumulatively, my results indicate that the fetal heart is programmed to develop cardiac dysfunction in response to an obesogenic *in utero* environment, but the postnatal environment determines if dysfunction is maintained and how it progresses.

## 6.4. Double hit model for manifestation of cardiac disease in response to maternal obesity

My findings support a double hit model wherein obesity during pregnancy sensitizes the offspring to cardiac disease, but a secondary stressor is required to elicit pathological responses during the offspring's postnatal life. In my thesis, I demonstrate that in mice born to obese mothers, further exposure to maternal obesity during lactation promotes arrhythmia (at 4 months) **(Fig. 15)** and long-term maintenance of diastolic dysfunction (at 8 months) **(Fig. 14)**. Aging promotes progressive decline in systolic and diastolic function **(Fig. 14)**. Even in the young (2 months) offspring of obese mice who are phenotypically silent, cardiac insult in the form

isoproterenol-mediated chronic adrenergic stimulation leads to augmented fibrosis **(Fig. 20)** and restrictive hypertrophic remodeling leading to reduced cardiac output, compared to the control isoproterenol-treated mice **(Fig. 19)**. Enhanced relaxation in response to increased inotropy – due to isoproterenol infusion[254] – was not observed, suggestive of diastolic dysfunction. This supports a double hit model wherein a secondary insult is required to elicit a pathological response. Several diseases are known to develop using a two-hit model. For example, hereditary pulmonary arterial (PA) hypertension is caused by a genetic mutation that is phenotypically silent unless an injury is acquired[255]. Similarly, concomitant metabolic and hypertensive stress were recently shown to act in a two-hit model to cause heart failure with preserved ejection fraction (HFpEF)[256]. Prior models of maternal obesity also suggest that a secondary insult might be required. Metabolic challenge in the offspring using a high-fat diet reduced fractional shortening and increased lipid droplets and ROS in the heart, with no changes seen under basal conditions[186]. Although we did not observe the same phenotypic changes in response to ISO or aging, different types of stress might likely elicit specific responses in hearts. Testing the effect of a broader range of cardiac stressors, such as pressure overload or myocardial infarction are necessary to strengthen this conclusion.

Cumulatively, my study addresses how maternal obesity increases the offspring's risk for adult-onset cardiovascular disease. My results suggest that maternal obesity during fetal and perinatal life programs heart disease susceptibility. I demonstrate that young offspring of obese mothers are phenotypically silent under baseline conditions but develop severe and exacerbated pathological remodeling in the heart in response to cardiac stress. This double-hit hypothesis is consistent with the higher risk for cardiovascular disease seen in human offspring of obese mothers[14–16] and explains why some offspring develop the disease and others do not.

## *7. Results 2: Maternal obesity alters the expression of Nkx2-5 target genes in fetal and adult hearts in the offspring*

### 7.1. Maternal obesity activates genes regulating ECM remodeling, inflammation and TGFβ signaling, and represses genes regulating heart rate and metabolic signaling

To uncover the transcriptional pathways underlying heart dysfunction programmed by obesity during pregnancy, I first analyzed the genome-wide mRNA profile of adult ventricles in Ob/Cn and Cn/Cn at 8 months of age by RNAseq. This timepoint represents a subclinical stage of disease progression with mild cardiac dysfunction and hypertrophy and thus could elucidate the transcriptional pathways dysregulated in mildly dysfunctional hearts. The mRNA expression profile of Ob/Cn ventricles clustered separately from Cn/Cn in a principal component analysis (PCA), indicating distinct transcriptional profiles between groups **(Fig. 21b)**. Ob/Cn samples were also separated in the PCA, indicating inter-group heterogeneity in gene expression. Differential expression analysis identified 1,372 significantly dysregulated genes, of which 485 were upregulated and 887 downregulated in Ob/Cn **(Fig. 21c)**. There was variation in the extent of deregulation within the Ob/Cn group however, the mean expression levels of 98% (1,338) of the genes dysregulated in Ob/Cn changed by less than two-fold **(Fig. 21c)**, with an average fold change of 1.32-fold, suggesting that small but broad changes in gene expression underlie subclinical heart dysfunction.

Gene Ontology (GO) analysis revealed that genes upregulated in the adult heart of Ob/Cn are most highly enriched for terms related to ECM remodeling and collagen organization **(Fig. 21d)**. Terms related to inflammatory response, and TGF-β signaling were also significantly enriched **(Fig. 21d)**. The PI3K-Akt signaling pathway **(Fig. 21e)**, which is activated downstream of TGF-β[257] and was previously found to be over-activated in the adult heart in response to obesity during pregnancy[204], was amongst the top 5 upregulated KEGG pathways identified by our analysis.

Consistent with a reduced heart rate in adult Ob/Cn **(Fig. 15)**, regulation of heart rate was amongst the top enriched GO terms in downregulated genes **(Fig. 21d)**. KEGG pathway enrichment identified pathways regulating metabolism, such as insulin signaling and downstream MTOR and AMPK signaling, as the most enriched downregulated pathways **(Fig. 21f)**.

Accordingly, network maps of gene set enrichment analysis (GSEA) showed higher expression of ECM and inflammation-related gene sets, and lower expression of genes in cardiac contraction, insulin, and metabolic pathways, as well as cardiac development-related gene sets **(Fig. 21g)**. qPCR on the ventricles confirmed the upregulation of *Col3a1, Col6a3, Fbn1,* and *MMP14,* which are key regulators of ECM assembly and turnover, genes in the TGF-β pathway *Thbs1* and *Tgif1,* and the pro-inflammatory gene *Egfr*. Downregulation of the ion channel *Scn5a* and the metabolic regulator *AKT1* was also confirmed **(Fig. 21h)**. Cardiac stress markers *S100a4* and *Myh6*[258] were dysregulated in the hearts of offspring of obese mice, however, *Myh7*, *Nppa*, *Nppb*, *Acta1*, and *Gata4* were not **(Fig. 21i)**. This indicates a modest or incomplete activation of cardiac stress pathways. Thus, upregulation of genes controlling ECM, inflammation and TGF-β signaling, and concomitant downregulation of cardiac conduction and metabolic regulators such as insulin signaling defines subclinical heart dysfunction in Ob/Cn.

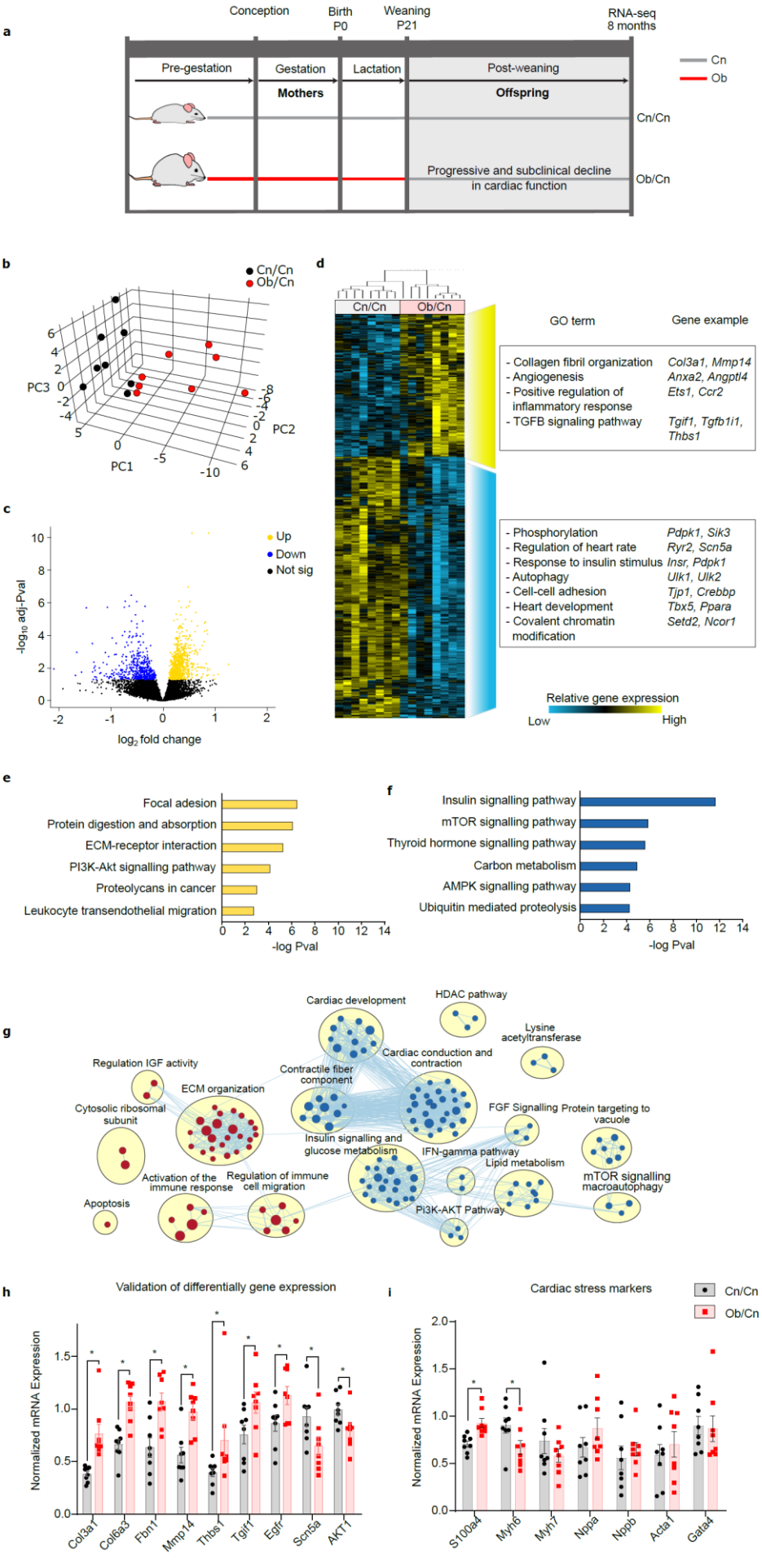

a
Conception
Birth
P0
Weaning
P21
RNA-seq
8 months
Pre-gestation
Gestation
Lactation
Post-weaning
Mothers
Offspring
Cn
Ob
Cn/Cn
Ob/Cn
Progressive and subclinical decline
in cardiac function
b
Cn/Cn
Ob/Cn
PC1
PC2
PC3
c
Up
Down
Not sig
-log10 adj-Pval
log2 fold change
d
Cn/Cn
Ob/Cn
GO term
Gene example
- Collagen fibril organization
- Angiogenesis
- Positive regulation of inflammatory response
- TGFB signaling pathway
Col3a1, Mmp14
Anxa2, Angptl4
Ets1, Ccr2
Tgif1, Tgfb1i1, Thbs1
- Phosphorylation
- Regulation of heart rate
- Response to insulin stimulus
- Autophagy
- Cell-cell adhesion
- Heart development
- Covalent chromatin modification
Pdpk1, Sik3
Ryr2, Scn5a
Insr, Pdpk1
Ulk1, Ulk2
Tjp1, Crebbp
Tbx5, Ppara
Setd2, Ncor1
Relative gene expression
Low
High
e
Focal adesion
Protein digestion and absorption
ECM-receptor interaction
PI3K-Akt signalling pathway
Proteolycans in cancer
Leukocyte transendothelial migration
-log Pval
f
Insulin signalling pathway
mTOR signalling pathway
Thyroid hormone signalling pathway
Carbon metabolism
AMPK signalling pathway
Ubiquitin mediated proteolysis
-log Pval
g
Cardiac development
HDAC pathway
Lysine acetyltransferase
Cardiac conduction and contraction
Contractile fiber component
Regulation IGF activity
ECM organization
Cytosolic ribosomal subunit
FGF Signalling
Protein targeting to vacuole
Insulin signalling and glucose metabolism
IFN-gamma pathway
Lipid metabolism
mTOR signalling macroautophagy
Activation of the immune response
Regulation of immune cell migration
Apoptosis
PI3K-AKT Pathway
h
Validation of differentially gene expression
Normalized mRNA Expression
Col3a1
Col6a3
Fbn1
Mmp14
Thbs1
Tgif1
Egfr
Scn5a
AKT1
i
Cardiac stress markers
Cn/Cn
Ob/Cn
Normalized mRNA Expression
S100a4
Myh6
Myh7
Nppa
Nppb
Acta1
Gata4

**Figure 21. Maternal obesity during pregnancy and lactation is associated with small but broad changes in transcriptional pathways controlling heart disease. a)** Schematic representation of the experimental plan to profile the transcriptome of 8-month-old adult hearts in response to maternal obesity. **b)** Principal component analysis (PCA) plot displaying separation of cardiac transcriptomes by maternal diet. **c)** Volcano plot of all expressed genes. -$\log_{10}$ (adjusted p-value) (y-axis) was plotted against the $\log_2$(fold-change) (x-axis). Significantly downregulated genes are in blue and upregulated in yellow. **d)** Heatmap displaying unsupervised hierarchal clustering of the expression levels of differentially expressed genes between ventricles of Cn/Cn and Ob/Cn. Enriched GO terms and representative genes are shown to the right. **e)** KEGG pathways enriched in genes that were significantly up- and **f)** down-regulated in mice born to obese mothers. **g)** Network clustering of gene sets enriched in upregulated (red) and downregulated (blue) genes. Gene sets were analyzed using GSEA. **h)** qPCR on hearts of Cn/Cn and Ob/Cn at 8 months of age. The genes analyzed are representative of the pathways identified in d). N=8 mice per group. **i)** qPCR on hearts of Cn/Cn and Ob/Cn at 8 months of age. The genes analyzed are known markers of cardiac stress. Bars represent the mean ± SEM. * $P$<0.05.

## 7.2. Limited transcriptional misregulation in cross-fostered offspring of obese mothers despite normalization of cardiac function

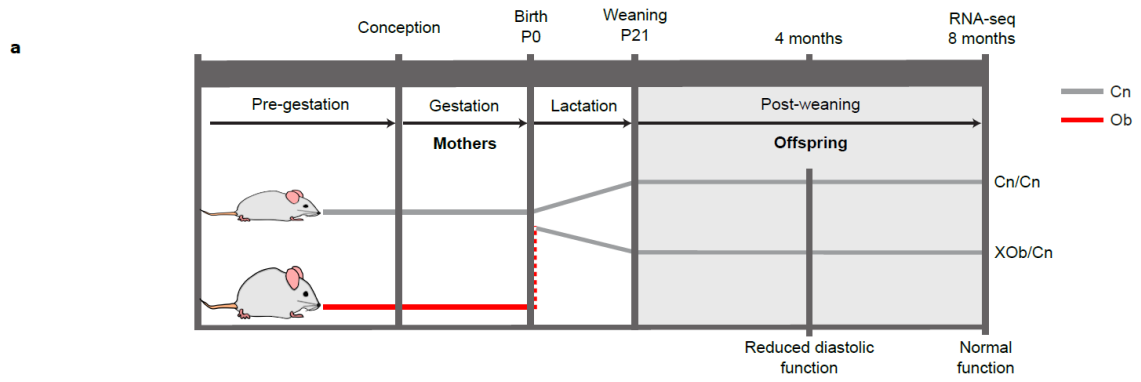
My results suggest that exposure to an Ob diet during fetal development leads to a transient reduction of diastolic function in young (4 months old) cross-fostered mice, but diastolic function is restored at 8 months of age. It is unclear if maternal obesity during gestation leads to long-term transcriptional misregulation, similar to that seen in mice exposed to obesity during gestation and lactation.

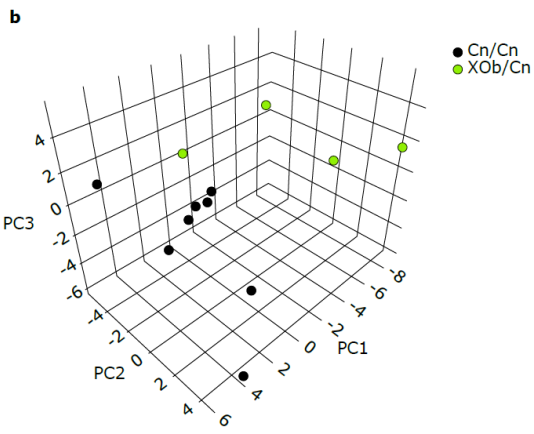
To determine if exposure to an obesogenic environment during lactation is necessary for misregulating gene expressing in 8-month-old hearts, I analyzed the genome-wide mRNA profile of adult ventricles in cross-fostered offspring of obese mothers (XOb/Cn) as compared to offspring of lean mothers (Cn/Cn) **(Fig. 22a)**. Notably, at 8 months, cross-fostered mice had normal cardiac and metabolic function compared to controls. However, the mRNA expression profiles of ventricles of XOb/Cn clustered separately from Cn/Cn in a PCA plot **(Fig. 22b)**, indicating distinct transcriptional profiles between groups. Differential expression analysis

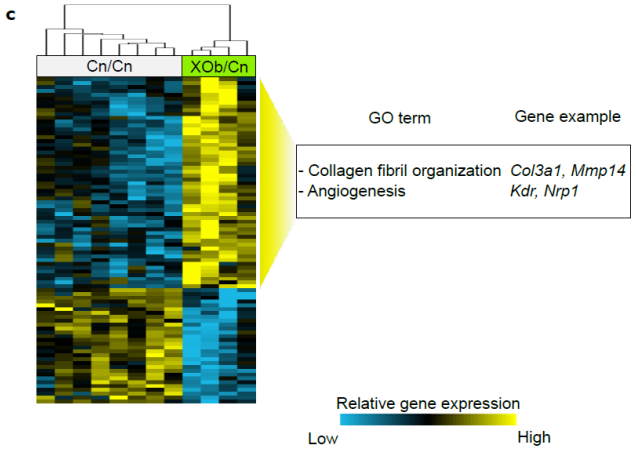
identified 85 significantly dysregulated genes, of which 55 were upregulated and 30 were downregulated **(Fig. 22c, d)**. 88% of genes dysregulated in cross-fostered mice changed by less than two-fold, with an average fold change of 1.65-fold **(Fig. 22d)**, suggesting that *in utero* exposure to obesity is sufficient to induce small changes in gene expression, despite complete restoration of heart and metabolic function.

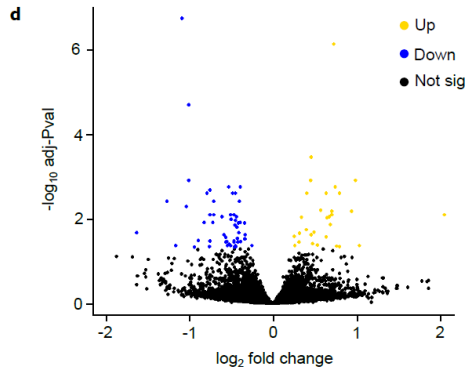
Upregulated genes enriched for ECM remodeling and angiogenesis **(Fig. 22c)**, similar to offspring that were exposed to Ob during lactation **(Fig. 21d)**. KEGG pathway enrichment revealed that genes upregulated in the adult heart of XOb/Cn are most highly enriched for terms related to cell focal adhesion and ECM remodeling, as well as metabolic signaling such as the PI3K-Akt pathway **(Fig. 22e)**. Downregulated genes did not significantly enrich for GO terms or KEGG pathways. However, I noted that the top downregulated genes were regulators of metabolism such as crystallin (*Crym*) and mitogen-activated protein 3 kinase (*Map3k6*)[259].
To determine if the genes misregulated in ventricles of cross-fostered mice represent a subset of all misregulated genes identified in mice exposed to Ob during both gestation and lactation, I cross-referenced misregulated genes in cross-fostered mice and mice born to obese dams that remained with their birth mother until weaning (Ob/Cn). Approximately half of the genes (46%) that were misregulated in cross-fostered mice were also misregulated in Ob/Cn **(Fig. 22f)**. Notably, off the 12 collagen-encoding genes upregulated in Ob/Cn, half of them were also upregulated in XOb/Cn. Off the genes that were uniquely upregulated in XOb/Cn, several were associated with angiogenesis, such as *kdr*, and neural guidance, such as neuropilin 1 and 2, although no specific pathway was significantly enriched.

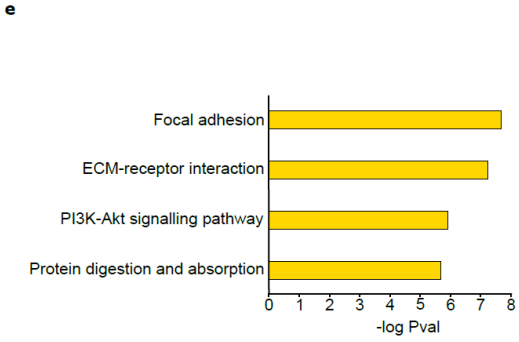
In summary, *in utero* exposure to obesity in the absence of lactation by an obese mother leads to the activation of genes regulating ECM organization, similar to Ob/Cn, but with fewer genes undergoing transcriptional misregulation. Thus, lactation by an obese mother is not required for maternal obesity-induced long-term gene dysregulation in the heart of the offspring.


a
Conception
Birth
P0
Weaning
P21
4 months
RNA-seq
8 months
Pre-gestation
Gestation
Lactation
Post-weaning
Mothers
Offspring
Cn
Ob
Cn/Cn
XOb/Cn
Reduced diastolic function
Normal function


b
Cn/Cn
XOb/Cn
PC3
PC2
PC1


c
Cn/Cn
XOb/Cn
GO term
Gene example
- Collagen fibril organization
Col3a1, Mmp14
- Angiogenesis
Kdr, Nrp1
Relative gene expression
Low
High


d
Up
Down
Not sig
-log10 adj-Pval
log2 fold change


e
Focal adhesion
ECM-receptor interaction
PI3K-Akt signalling pathway
Protein digestion and absorption
-log Pval

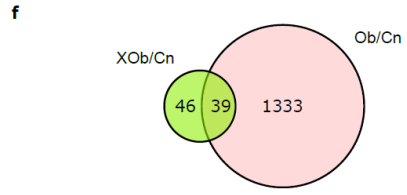

f
XOb/Cn
Ob/Cn
46
39
1333

**Figure 22. Maternal obesity during pregnancy is sufficient to induce transcriptional deregulation in the adult heart, which partially overlaps with Ob/Cn. a)** Schematic representation of the experimental plan to profile the transcriptome of 8-month-old adult hearts of cross-fostered offspring of obese mothers. **b)** Principal component analysis (PCA) plot displaying separation of cardiac transcriptomes by *in utero* exposure to maternal obesity. **c)** Heatmap displaying unsupervised hierarchal clustering of the expression levels of differentially expressed genes between ventricles of Cn/Cn and Ob/Cn. Enriched GO terms and representative genes are shown to the right. **d)** Volcano plot of all expressed genes. $-\log_{10}$ (adjusted p-value) (y-axis) was plotted against the $\log_2$(fold-change) (x-axis). Significantly downregulated genes are in blue and upregulated in yellow. **e)** KEGG pathways enriched in genes that were significantly up-regulated in cross-fostered mice. **f)** Venn diagram of genes dysregulated in cross-fostered offspring (XOb/Cn) and offspring exposed to obesity during pregnancy and lactation.

## 7.3. The magnitude of maternal weight gain is directly correlated with the extent of gene deregulation and diastolic function in the offspring

Given the variation in the magnitude of gene expression fold change in the heart of offspring of obese mothers **(Fig. 21b, d, h),** I sought to identify phenotypic factors that might be associated with this heterogeneity in gene expression responses. I hypothesized that the extent of gene deregulation could be associated with the severity of maternal adiposity and might also be secondary to the extent of cardiovascular or metabolic dysfunction in the offspring.

To identify phenotypic traits associated with the extent of gene deregulation, I correlated the mRNA levels of differentially expressed genes with all phenotypic parameters collected on mothers and offspring. A total of 55 phenotypic parameters were assessed of which only 10 significantly correlated with gene expression changes in the ventricles **(Fig. 23; Fig. 24a; Table 4)**. Maternal weight at the time of conception correlated with the largest number of differentially expressed genes. The mRNA levels of 1135 of the 1372 differentially expressed genes (83%) significantly correlated with maternal weight as a continuous variable (range 21.1-29g) **(Fig. 24a; Table 4)**. Accordingly, the ventricles of offspring born to the heaviest mothers clustered distinctly from ventricles of offspring born to lean mothers **(Fig. 24b)**. The heart weight and IVRT/RR were also correlated with gene expression of 226 and 116 genes, respectively **(Fig.**

**24a; Table 4)**. Notably, 45 phenotypic parameters, including the percentage of fat and lean mass in Ob/Cn did not correlate with gene expression changes, excluding such phenotypes as potential drivers of gene dysregulation in the heart **(Fig. 23; Table 4)**.

Closer examination of differentially expressed genes in the most enriched pathways revealed that a higher maternal weight correlated with pre-dominant upregulation of ECM components and downregulation of insulin and heart rate regulators **(Fig. 24c)**. A higher heart weight in Ob/Cn correlated with lower expression of genes in the insulin signaling pathway, *e.g. PPARα,* whose deficiency promotes cardiac hypertrophy[260], and less with dysregulated ECM components **(Fig. 24c)**. A longer IVRT correlated with upregulation of ECM components such as *Col1a2* and *Col6a2* **(Fig. 24c)**, which is associated with increased stiffening of the ventricular wall[261,262]. Furthermore, maternal weight positively correlated with IVRT **(Fig. 24d)** suggesting that the extent of maternal obesity is associated with the magnitude of cardiac diastolic dysfunction in offspring.

In summary, a higher maternal weight is the strongest predictor of dysregulation of genes controlling pathological remodeling in preclinically dysfunctional hearts in Ob/Cn.

**Table 4. Top 12 phenotypes that correlate with the differential gene expression in 8-month old adult hearts.**

| Trait | # of Significantly Correlated Genes | Permutation *P*-value |
|---|---|---|
| Maternal Weight at Conception | 1135 | 0.0001 |
| IVRT/RR | 226 | 0.0001 |
| Heart Weight | 116 | 0.0001 |
| Mean Blood Pressure at 4 months | 66 | 0.0001 |
| Systolic Blood Pressure at 4 months | 58 | 0.0001 |
| AUC GTT at 4 months | 54 | 0.0001 |
| Diastolic Blood Pressure at 4 months | 33 | 0.0001 |
| Fat Mass at 2 months | 30 | 0.0001 |
| Final Peak Weight at 8 months | 29 | 0.0001 |
| AUC Body Weight | 10 | 0.0001 |
| Fat mass at 4 months | 1 | 0.1115 |
| Lean % at 4 months | 1 | 0.1957 |


Body weight
Maternal Weight at Conception
Offspring Weight at 8 months
Offspring AUC Body Weight
GTT
AUC GTT at 4 months
AUC GTT at 6 months
AUC GTT at 8 months
Cardiac function at 8 months
IVRT/RR
Ejection Fraction
IVCT/RR
Fractional Shortening
DT/RR
Cardiac Output
ET/RR
Stroke Volume
IVRT/DT
E (mm/s)
IVCT/ET
A (mm/s)
Myocardial Performance Index
E/A
Heart size and morphology at 8 months
Heart Weight
AW Thickness (Diastolic)
PW Thickness (Diastolic)
AW Thickness (Systolic)
PW Thickness (Systolic)
End-diastolic Diameter
End-systolic Diameter
Aortic Area
Aortic Diameter
Aortic VTI
Blood pressure
Mean Blood Pressure at 4 months
Systolic Blood Pressure at 4 months
Diastolic Blood Pressure at 4 months
Mean Blood Pressure at 8 months
Systolic Blood Pressure at 8 months
Diastolic Blood Pressure at 8 months
Heart Rate at 8 months
Body composition
Fat Mass at 2 months
Lean Mass at 2 months
Fat Mass at 4 months
Lean Mass at 4 months
Fat Mass at 6 months
Lean Mass at 6 months
Fat Mass at 8 months
Lean Mass at 8 months
Fat % at 2 months
Lean % at 2 months
Fat % at 4 months
Lean % at 4 months
Fat % at 6 months
Lean % at 6 months
Fat % at 8 months
Lean % at 8 months
Q-Q plot
Observed P-val distribution
Expected P-val distribution

**Figure 23. Quantile-Quantile plot for the correlation between phenotype severity and gene deregulation in the hearts.** The observed p-value (after adjusting for multiple comparisons) was plotted on the y-axis against the null hypothesis (x-axis). The red diagonal line denotes the expected pattern under the null hypothesis. Significant deviations from the null hypothesis are highlighted in green.

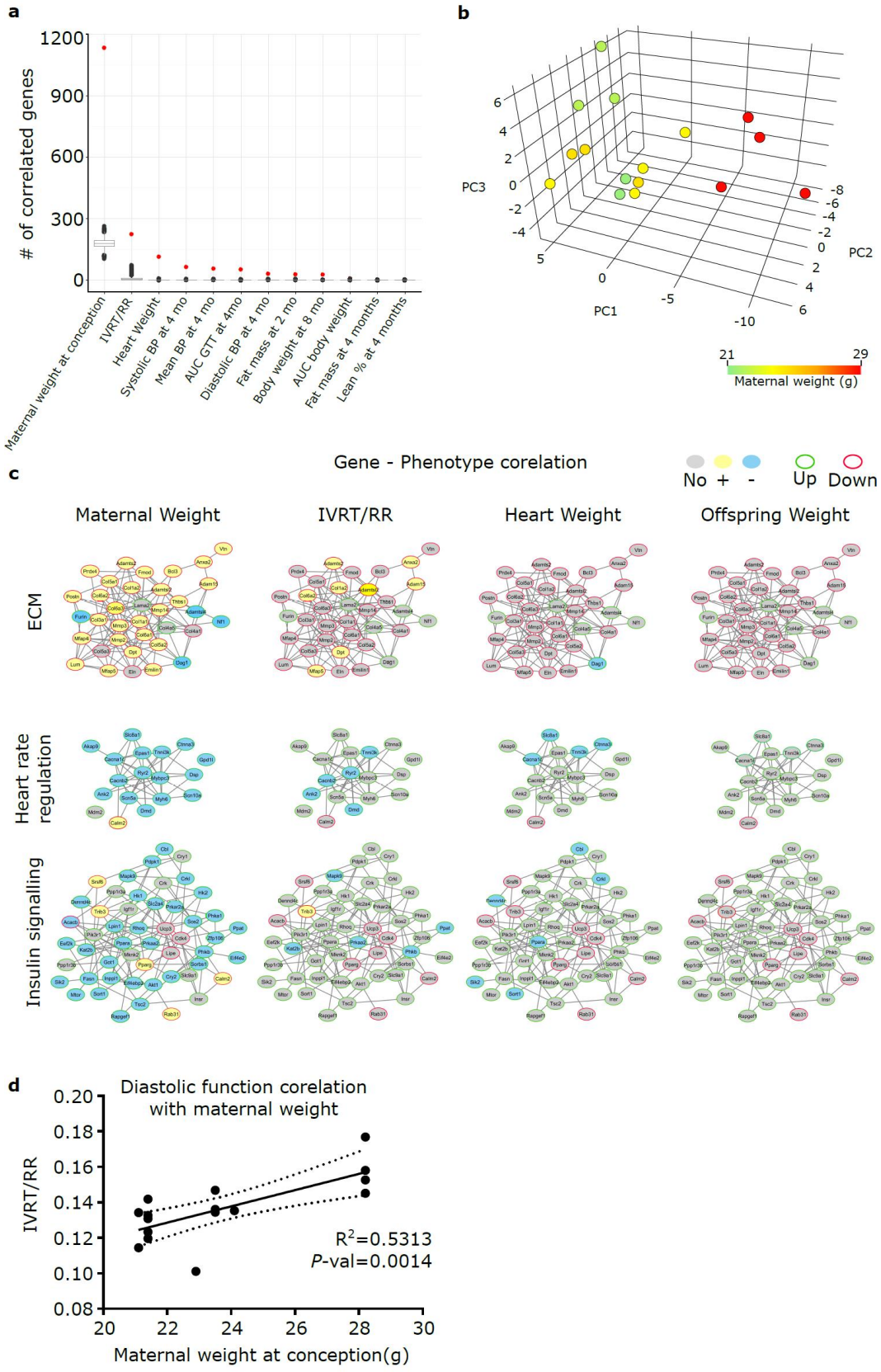

a
# of correlated genes
1200
900
600
300
0
Maternal weight at conception
IVRT/RR
Heart Weight
Systolic BP at 4 mo
Mean BP at 4 mo
AUC GTT at 4mo
Diastolic BP at 4 mo
Fat mass at 2 mo
Body weight at 8 mo
AUC body weight
Fat mass at 4 months
Lean % at 4 months
b
PC3
PC1
PC2
21
29
Maternal weight (g)
c
Gene - Phenotype corelation
No + -
Up Down
Maternal Weight
IVRT/RR
Heart Weight
Offspring Weight
ECM
Heart rate regulation
Insulin signalling
d
Diastolic function corelation with maternal weight
IVRT/RR
0.20
0.18
0.16
0.14
0.12
0.10
0.08
20
22
24
26
28
30
Maternal weight at conception(g)
R2=0.5313
P-val=0.0014

**Figure 24. Maternal weight at conception correlates with the magnitude of gene dysregulation and diastolic function in hearts of adult offspring. a)** Number of genes that significantly correlated with various maternal and offspring phenotypic parameters. Red dots represent the observed number of differentially expressed genes correlated with each phenotype (Bootstrap *P*-value <0.05). Box plots depict 10,000 permutations of randomly selected genes. **b)** Principal component analysis of transcriptomes color-coded by their maternal weight at conception. **c)** Gene networks displaying differentially expressed genes in the top three enriched processes: ECM, heart rate regulation and insulin signaling. Genes that correlated with maternal weight, heart weight, isovolumetric relaxation time and offspring weight are highlighted. up = genes upregulated, down = genes downregulated in Ob/Cn compared to Cn/Cn. Positive (+) and negative (-) correlations refer to a significant Pearson correlation coefficient (adjP<0.05) between gene expression levels and the respective phenotype, after Benjamini & Hochberg correction for multiple testing. no = genes that did not significantly correlate with the respective phenotype. **d)** Pearson correlation between maternal weight at conception and IVRT/RR.

## 7.4. Gene expression profiles of adult hearts of offspring of obese mice recapitulate the expression profile of diabetic and hypertrophic cardiomyopathies

Given the sub-clinical phenotypes identified in Ob/Cn at 8 months, the specific identity of long-term disease outcome was not clear. Subclinical reduction in diastolic followed by progressive systolic dysfunction and cardiac hypertrophy are common phenotypic manifestations of several cardiomyopathies but are not sufficient to classify the identity of the disease. To more precisely define the disease associated with heart dysfunction in offspring born to obese mice, I compared the expression profile of the 1,338 differentially expressed genes in Ob/Cn against publicly available expression profiles of several mouse models of cardiac stress and human hearts affected by disease. I analyzed mouse models of angiotensin-II (AngII) -induced hypertension, diabetic cardiomyopathy[235], chronic isoproterenol (ISO)-induced adrenergic stimulation[232], transverse aortic constriction (TAC) [233], and physiological hypertrophy[234]. Human hearts with dilated cardiomyopathy (DCM)[237], hypertrophic cardiomyopathy (HCM)[236], and ischemic cardiomyopathy (ICM)[237] were also analyzed. The transcriptional profile of hearts of

Ob/Cn at 8 months of age was most similar to the hearts of mice with diabetic cardiomyopathy and isoproterenol-induced adrenergic stimulation, followed by hypertrophic cardiomyopathy, as determined by PCA **(Fig. 25a)**. One caveat of PCA is that it is susceptible to clustering bias due to similarities in sequencing platforms, library construction chemistries and analysis methodology employed by different studies[263]. Although differences in analysis parameters were eliminated by re-analyzing published raw data using the same parameters used for my RNA-seq studies, it does not address the remaining caveats. To overcome clustering bias, I employed GSEA to first identify up- and down-regulated genes within each model. I then used these unique gene sets to calculate the level of enrichment for genes misregulated in response to maternal obesity in my model. Consistent with the PCA results, GSEA revealed that gene sets related to diabetic, hypertrophic and ISO-induced cardiomyopathy are enriched in genes dysregulated in Ob/Cn **(Fig. 25b)**. Notably, gene dysregulation is not secondary to hypertension or diabetes because blood pressure and glucose tolerance were not significantly different between Cn/Cn and Ob/Cn **(Fig. 15, 13d)**. Thus, genes controlling the cardiac response to diabetic, hypertrophic and adrenergic stress are mildly dysregulated in preclinically dysfunctional hearts in Ob/Cn, which is consistent with a state of heart disease susceptibility.

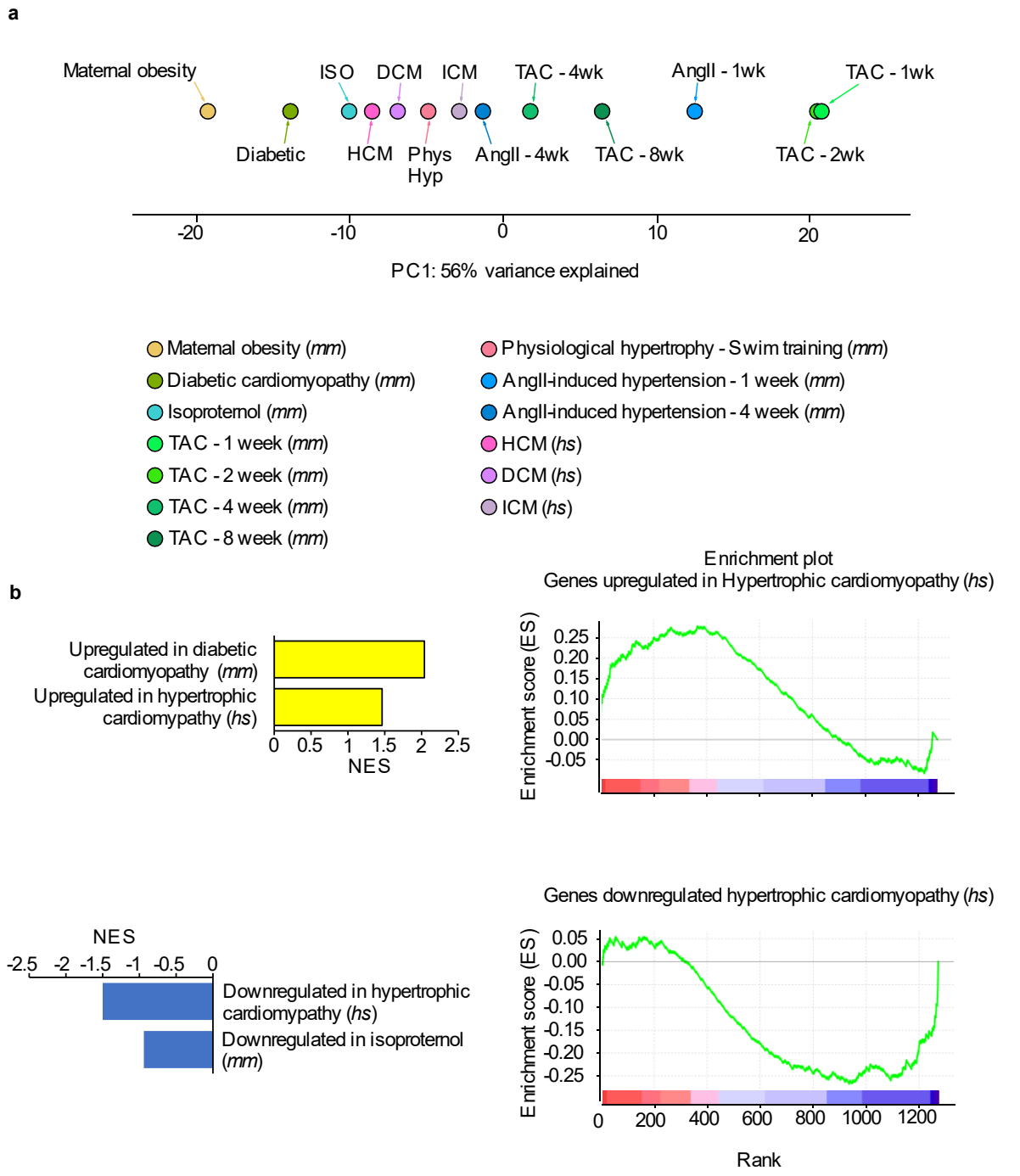



**Figure 25. Transcriptomic profiles of hearts from offspring of obese mice bear similarities with models of hypertrophic, diabetic, and ISO-induced cardiomyopathy. a)** PCA plot of RNA-seq datasets from mouse and human hearts affected by different diseases. Self represents our model of maternal obesity-induced heart disease predisposition. **b)** Gene set enrichment

analysis of hearts affected by different diseases compared against genes up and downregulated in our model. Positively and negatively enriched gene sets are on the left, enrichment plots of hypertrophic cardiomyopathy, one of the top enriched terms, are on the right.

## 7.5. Dynamic changes in gene expression during cardiac insult in response to maternal diet

To uncover the processes mediating an exacerbated response to stress in Ob/Cn, I compared the transcriptome of hearts of 2-month-old offspring of Cn- and Ob-fed mothers in response to PBS or ISO treatment **(Fig. 18)**. PCA separated the samples by treatment group (PBS *vs* ISO) on principal component 1 (PC1), and maternal diet on principal component 2 **(Fig. 26a)**. Among the top 50 genes that explain the largest proportion of principal component 2, regulators of inflammation and ECM components were overexpressed in offspring of Ob-fed mothers **(Fig. 26b)**. qPCR analysis confirmed that genes controlling inflammation (*e.g. Nr4a1, Igtp*) and ECM components (*e.g. Col5a3, Fbn1*) were upregulated in response to maternal diet **(Fig. 26c)**. *Nr4a1* was upregulated in response to maternal obesity irrespective of the treatment group. *Col5a3 and Fbn1* were upregulated in offspring of obese dams exclusively in the ISO-treated mice (but also in untreated 8-month-old offspring), whereas *Igtp* was upregulated in offspring of obese dams only in PBS-treated mice. This indicates that some of the effects of maternal obesity on gene expression are only detectable during stress, and others at baseline, but the overall pathways regulating ECM and inflammation are commonly upregulated.

To further elucidate the dynamic responses at baseline and during ISO-induced cardiomyopathy, I performed unsupervised hierarchal clustering of the genes that were differentially expressed between Cn/Cn and Ob/Cn treated with PBS or ISO **(Fig. 27a)**. Consistent with transcriptional changes in untreated 8 month-old hearts in Ob/Cn **(Fig. 21)**, genes upregulated in Ob/Cn (clusters 1-4) enriched for GO terms related to ECM and heart development; while downregulated genes (clusters 5-8) enriched for GO terms related to metabolism and BMP/TGFβ inhibition **(Fig. 27a)**. Subsets of these genes responded differently to ISO. For example, genes regulating immune cell migration and cell apoptosis were upregulated in PBS-treated Ob/Cn and remained upregulated (cluster 2) to a similar extent under stress. Such genes are normally upregulated during cardiac stress as evidenced by upregulation in

Cn/Cn-ISO, suggesting that these specific disease pathways are activated prior to cardiac stress in Ob/Cn. Genes regulating cardiac development and lipid metabolism were upregulated in Ob/Cn at baseline but became downregulated under stress conditions, reaching similar levels to controls (cluster 4). Such genes might represent stress-specific responses that might be excessively deregulated in response to alternative stressors. Genes regulating SMAD inhibition such as *Smad6* and *Pprm1*, which are known to inhibit TGFβ signaling, were downregulated in Ob/Cn under stress-free and stressed conditions (cluster 5), suggesting reduced inhibition of TGFβ signaling in Ob/Cn.

Notably, genes encoding ECM components and its regulators were very modestly upregulated under baseline conditions but were excessively upregulated in response to ISO (cluster 1), consistent with findings of aged 8-month old hearts **(Fig. 21d, e, g, h)**. Moreover, genes encoding mitochondrial and metabolism-related functions were strongly upregulated in Cn/Cn in response to stress but failed to undergo similar upregulation in Ob/Cn (cluster 8) **(Fig. 27a)**. Such genes were already repressed under baseline conditions (cluster 8) **(Fig. 27a)**. This suggests that failure to activate glycolytic and mitochondrial genes during stress is preceded by early repression. Thus, some of the transcriptional responses to maternal obesity are only apparent during stress in the young adult heart (e.g. ECM upregulation) and others are significantly altered even under stress-free conditions (*e.g.* downregulation of metabolic regulators). These results suggest that obesity during pregnancy sensitizes the heart to overexpression of ECM components and attenuated activation of metabolic regulators to program dysregulated stress responses in the heart.

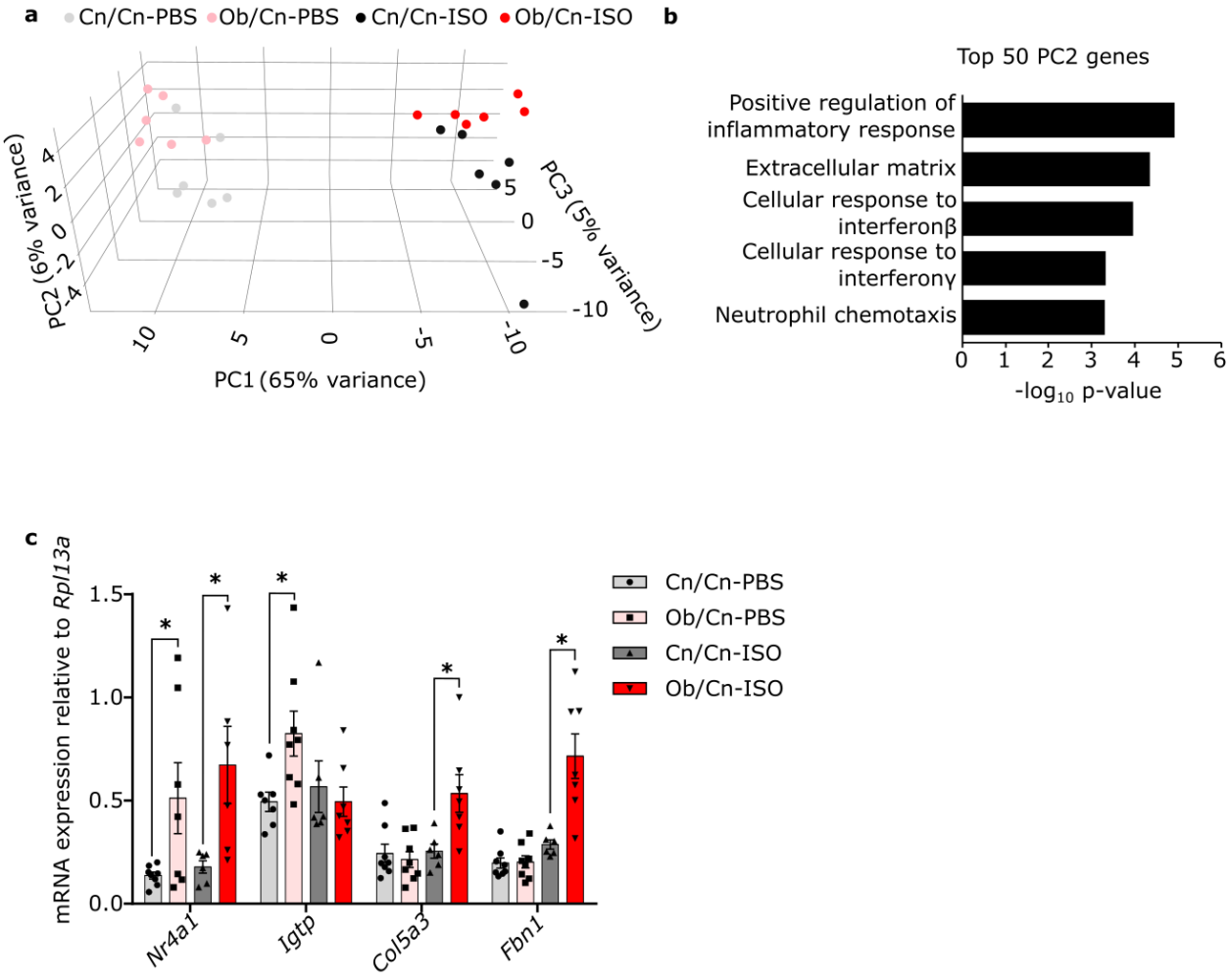



**Figure 26. Dynamic changes in gene expression regulated by maternal diet during the cardiac response to stress. a)** Principal component (PC) analysis separated cardiac transcriptomes by treatment on PC1 and maternal diet on PC2. **b)** GO-term enrichment for the top 50 genes determining PC2. **c)** qPCR of representative genes on PC2 in a). Bars represent the mean ± SEM. N=6-8 mice per group.

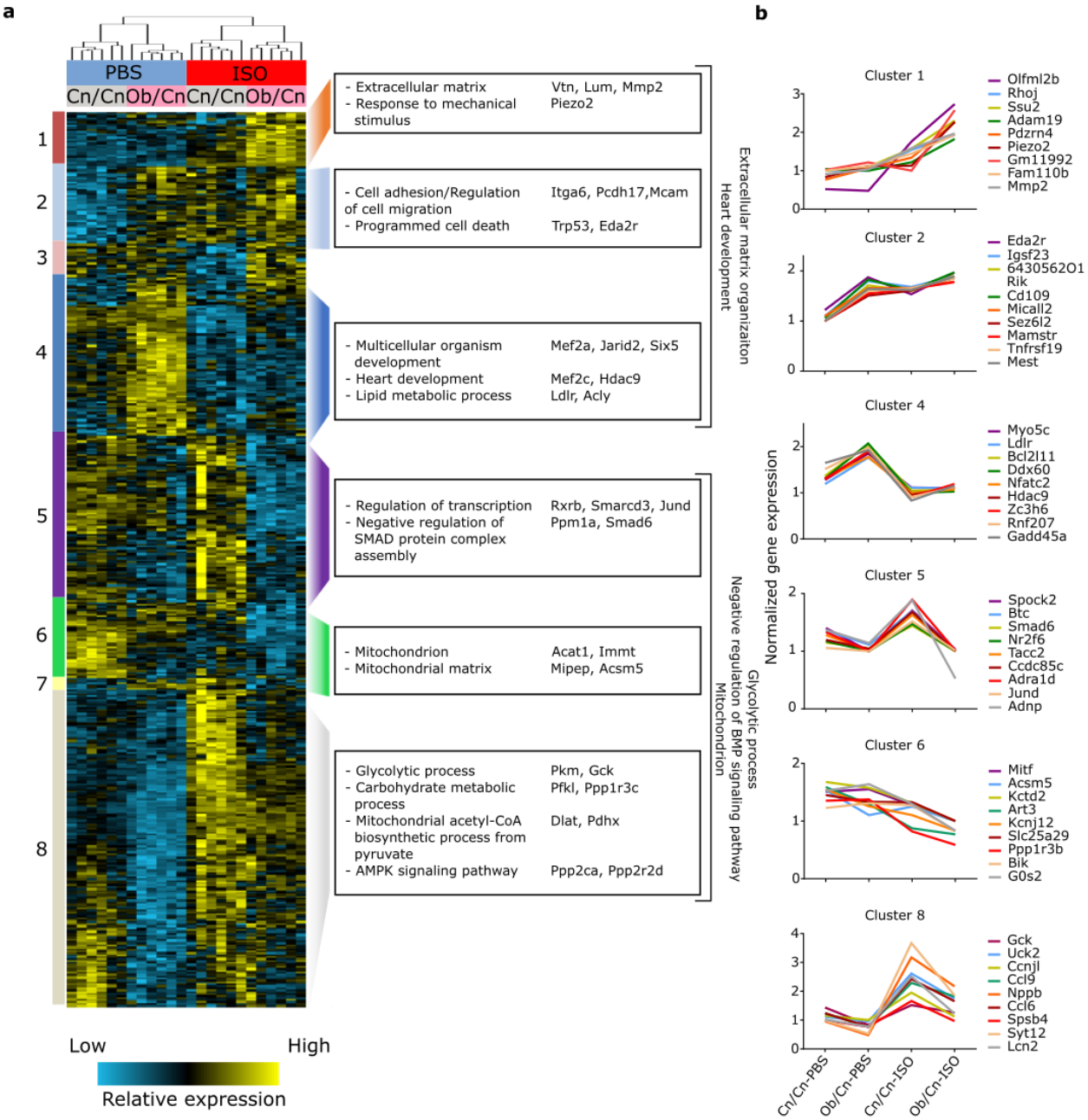



**Figure 27. Maternal obesity reduces metabolic gene expression and augments extracellular matrix genes in response to stress. a)** Unsupervised hierarchal clustering of genes that were differentially expressed between ventricles of Cn/Cn and Ob/Cn treated with PBS or ISO at 2.5 months of age. Enriched GO terms and representative genes are shown to the right. **b)** Expression profiles (magnitude normalized count values) of genes within clusters 1, 2, 4, 5, 6 and 8.

## 7.6. Maternal obesity activates genes regulating cardiac development and represses epigenetic regulators in cardiac progenitor cells

To examine some of the earliest cardiac responses to maternal obesity, I isolated cardiac progenitor cells (CPCs) from embryos of obese and lean mice, at E9.5. At this stage, heterogenous populations of cardiac progenitors of the first and second heart fields are differentiating into cardiomyocytes, endothelial cells, and smooth muscle cells. CPCs derived from the second heart field are delayed in differentiation compared to CPCs of the first heart field. Thus, CPCs from E9.5 represent a mixed population of differentiating progenitors as well as differentiated cardiac cells. I used *Nkx2-5tg* transgenic reporter mice, which express a BAC encoding emerald GFP (eGFP) at the native human *NKX2-5* open reading frame, flanked by the 60kb upstream sequence, and 130 kb downstream sequence from the *NKX2-5* open reading frame **(Fig. 28a)**. This preserves all regulatory sequences up and downstream of the gene body. The eGFP is preceded by a 2A ribosomal skip site **(Fig. 28a)** which allows the eGFP to be released from the preceding peptide sequence during translation[210], thus preserving post-transcriptional regulatory sequences in the 5' untranslated region. This allows the reporter to better recapitulate endogenous gene expression.

To confirm that the BAC *NKX2-5* reporter line (also referred to as *Nkx2-5tg*) efficiently and specifically labels mouse cardiac progenitors, I examined the expression of eGFP in E9.5 embryos. eGFP labeled the left and right ventricles and co-localized with Isl1 in the branchial arches **(Fig. 28b-d)**. Isl1 marks the second heart field, and the branchial arches are the site where CPCs of the second heart field migrate from. In addition, eGFP-labeled cells from dissociated whole embryos increased expression of endogenous *Nkx2-5* and *Isl1* as well as *eGFP*. This data confirms that *Nkx2-5tg* efficiently and specifically labels a broad population of CPCs from the first and second heart fields that are present in the looping heart tube and the branchial arches.

To elucidate the developmental origins of adult cardiac disease susceptibility in offspring of obese mothers, I isolated CPCs from embryos of mice fed Cn or Ob diets at E9.5, and I analyzed their mRNA expression profile by RNAseq. CPCs were isolated from individual embryos of dams weighing between 23 and 26g **(Fig. 29a)**. Prior to sequencing, samples were selected based on similarity in their expression of the cell death marker *TP53*, and red blood cell marker *Hbb-bs*. Both of these markers can vary due to technical variability in embryo

dissociation, red blood cell lysis and cell sorting equipment used for the embryos. All samples chosen for sorting had no difference in cell death marker gene expression or red blood cell contamination **(Fig. 29b)**. To further validate the proper enrichment CPCs, I examined the mRNA expression levels of known cardiac and non-cardiac lineage markers, using the RNAseq data. The most highly expressed genes in eGFP positive cells encode cardiac sarcomeric proteins such as *Ttn*, *Myh7* and *Myh6*, whereas ectoderm markers: *Nkx2-9, Otx2, Tubb3, Sox1* and endoderm markers: *Hesx1, Sox17, FoxA2 and Cdx2* were lowly expressed **(Fig. 29c)**. The top 100 most highly expressed genes in the eGFP positive cells enriched for functions related to cardiac morphogenesis and cardiac function **(Fig. 29d)**. Overall, the transcriptional profile of the eGFP positive cell population is consistent with a cardiac progenitor gene signature, thus validating the enrichment of CPCs by eGFP expression.

I compared the gene expression profiles of CPCs from E9.5 embryos of mice fed Cn or Ob diets. I identified only 9 genes that were differentially expressed in response to Ob diet **(Fig. 29e)**. Protogenin (*Prtg*) and *miR5125* were the only annotated genes, both of which were upregulated. The remainder 7 genes were all predicted genes that are validated or tested. *Prtg* is an immunoglobulin that regulates mesodermal cell migration by augmenting cell adhesiveness[264], neural crest migration, and cell survival[265]. miR5125 was first identified to be expressed during B-cell differentiation however its functions are unknown. To determine if miR5125 is potentially mediating gene repression in CPCs and adult hearts, I performed target prediction for miR5125 and cross-referenced predicted targets with differentially expressed genes identified in CPCs and adult hearts. The analysis identified *Usp34*, the second-ranking predicted target **(Fig. 29f)**, to be downregulated in adult hearts, however, none of the predicted targets were dysregulated in CPCs, and it is unclear whether this miRNA is persistently misregulated in adulthood. Overall, this suggests that a maternal Ob diet *per se* does not lead to altered global gene expression in differentiating CPCs.

However, BMI in humans proportionally correlates with the risk of cardiovascular disease and mortality in the offspring[14–16]. This is consistent with a higher maternal weight correlating with a greater number of dysregulated genes in the hearts of Ob/Cn **(Fig. 24)**. Accordingly, a focused comparison of CPCs from embryos of the leanest Cn-fed dams against the heaviest Ob-fed dams revealed a significantly broader effect on gene expression. A total of

823 genes were dysregulated, of which 586 were upregulated and 237 downregulated **(Fig. 30a)**. Upregulated genes were most highly enriched for ontologies related to transcriptional regulation and heart development. TGF-β signaling was also amongst highly enriched terms **(Fig. 30a)**. KEGG pathways related to hypertrophic, dilated and arrhythmogenic cardiomyopathy were amongst the most significantly enriched terms **(Fig. 30b, c)**. In contrast, downregulated genes were enriched for epigenetic processes including nucleosome assembly and DNA methylation **(Fig. 30a)** suggesting potential mechanisms for gene dysregulation.

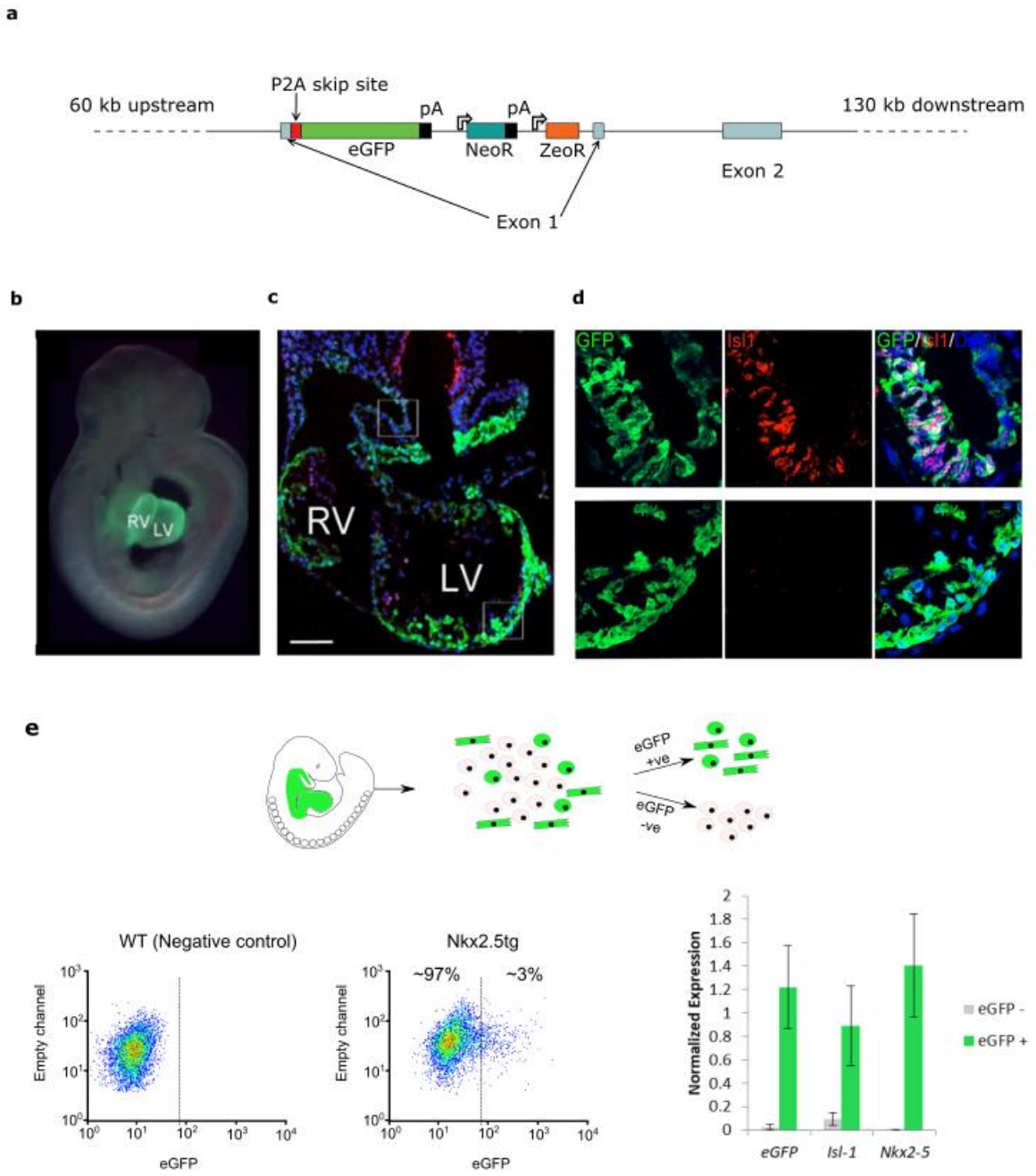
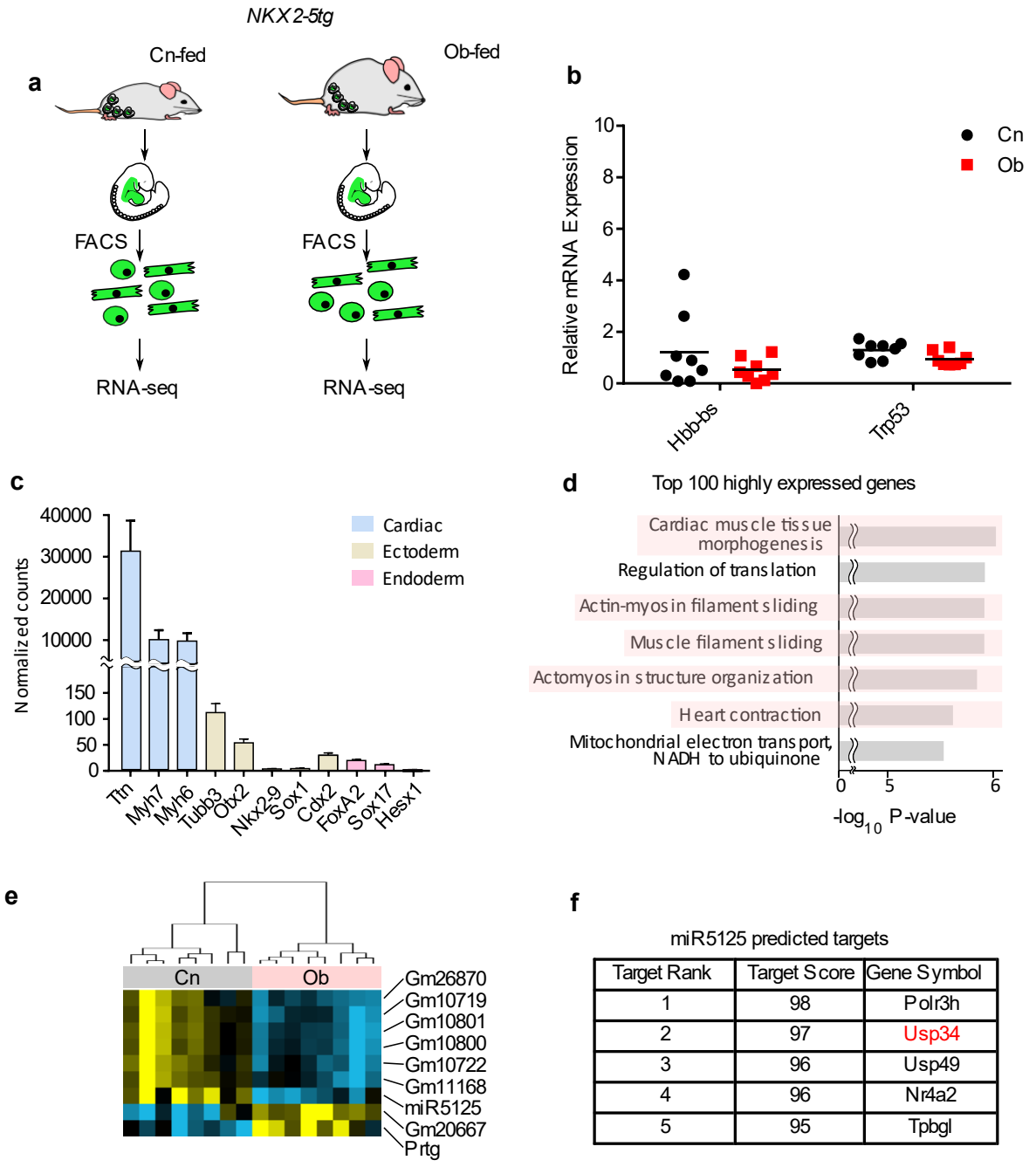



**Figure 28.** ***Nkx2-5-tg*** **labels first and second heart field derivatives with eGFP. a)** Schematic representation of the NKX2-5 Emerald GFP BAC reporter. The fluorescent tag is composed of a 2A sequence preceding the eGFP. The tag is placed within the first exon of the NKX2-5 BAC, which contains the entire human NKX2-5 gene, 60kb upstream and 130kb downstream sequence. Separate bacterial and eukaryotic selection markers are also present. **b)** Whole-mount imaging of *Nkx2-5-tg* embryos at E9.5 showing eGFP fluorescence in the looping heart tube and

pharyngeal mesoderm. **c)** Transverse sections were stained for GFP and Isl1 immunofluorescence. Nuclei were counterstained with DAPI. **d)** Insets of top and bottom boxes shown in c) demonstrating eGFP in first and second heart field derivatives **e)** Schematic representation of cardiac progenitor isolation from E9.5 embryos using FACS (Top). Representative sorting plots showing an eGFP negative (-) embryo on the left, and an eGFP positive (+) embryo on the right. qPCR of *eGFP* and the markers of differentiating CPCs *Isl1* and *Nkx2-5* on sorted eGFP- and eGFP+ cells are shown (bottom right). Error bars represent the mean ± SD of three replicates.




| Target Rank | Target Score | Gene Symbol |
|---|---|---|
| 1 | 98 | Polr3h |
| 2 | 97 | Usp34 |
| 3 | 96 | Usp49 |
| 4 | 96 | Nr4a2 |
| 5 | 95 | Tpbgl |

**Figure 29. Transcriptomic analysis of cardiac progenitor cells from Ob-fed and Cn-fed mice. a)** Schematic representation of the experimental plan to profile the transcriptome of cardiac progenitor cells in response to maternal obesity. **b)** qPCR analysis on cardiac progenitor cells to examine blood cell marker, *Hbb-bs*, and cell death marker, *Trp53*, confirms no changes in blood cell contamination or cell death between cells from Cn- or Ob-fed mice. **c)** Normalized

counts obtained from RNAseq analysis focusing on cardiac, ectoderm, and endoderm markers. **d)** GO term enrichment on the top 100 most highly expressed genes in CPCs. Cardiac muscle terms are highlighted in red. **e)** Heatmap of the mRNA levels of 9 genes differentially expressed in CPCs of embryos from Ob-fed mice. **f)** Predicted targets of the differentially expressed microRNA, *miR5125*. Genes dysregulated in adult hearts are highlighted in red. Bars represent the mean ± SEM.

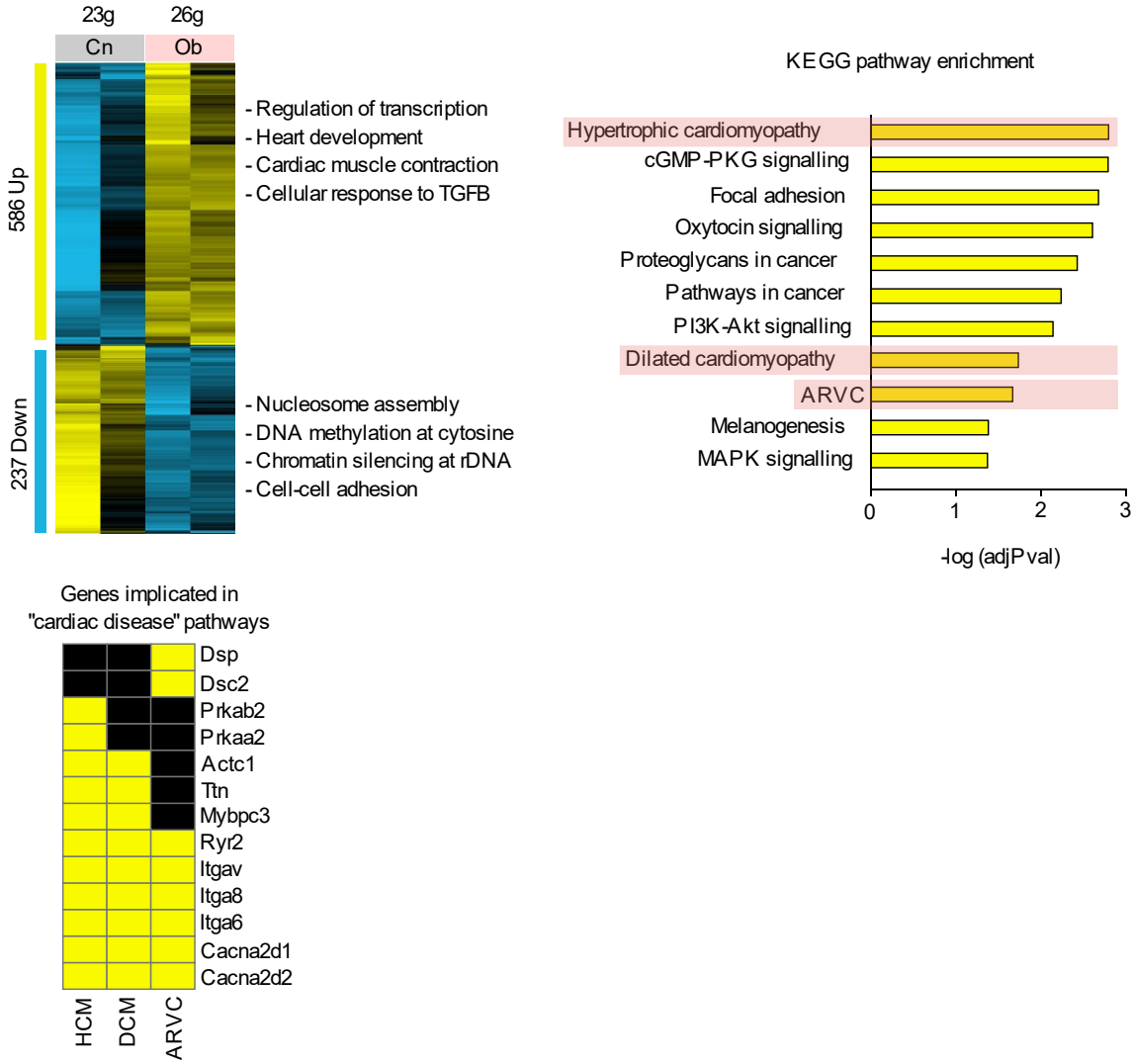



**Figure 30. Maternal obesity alters heart development gene networks and activates cardiac disease pathways in cardiac progenitor cells. a)** Heatmap of genes dysregulated in CPCs of embryos of the heaviest *vs* the leanest mice. **b)** KEGG pathway enrichment on the dysregulated genes. **c)** The specific identity of genes implicated in disease pathways is shown. Yellow indicates a gene is implicated in the respective disease. Black indicates the gene is not implicated. HCM=hypertrophic cardiomyopathy. DCM=dilated cardiomyopathy. ARVC=arrhythmogenic right ventricular cardiomyopathy.

## 7.7. Nkx2-5 targets genes that are misregulated in response to maternal obesity and its expression inversely correlated with maternal weight

Maintaining core transcription networks in CPCs and their descendants is required for cardiogenesis and stable long-term postnatal heart function[84,123]. We sought to identify genes and pathways that are perturbed in CPCs and persistently into adulthood in response to maternal obesity. The majority of misregulated genes were dysregulated in a stage-specific manner **(Fig. 31a)**. However, 112 genes were commonly dysregulated in embryonic CPCs and adult hearts from Ob/Cn **(Fig. 31a)**. Commonly misregulated genes were enriched for pathways directly related to heart disease including hypertrophic cardiomyopathy, ECM-receptor interactions, and metabolism **(Fig. 31b)**. These results suggest that maternal obesity affects cardiac gene expression starting early in development.

To uncover transcription factors controlling heart disease risk, I searched for common transcription factor binding motifs in the regulatory regions of genes dysregulated in the CPCs as well as adult hearts. The binding motif of Nkx2-5 was the most enriched in the 10 kb region upstream and downstream of the transcription start sites, in both CPCs and adult hearts **(Fig. 31c, f)**. To determine if genes dysregulated in CPCs and adult hearts of offspring of obese mice are directly bound by Nkx2-5, I analyzed published Nkx2-5 ChIP-seq datasets from differentiating CPCs[84] and adult mouse hearts[122]. I found that genes differentially expressed in response to maternal obesity in the hearts of obese mothers are enriched for direct Nkx2-5 targets **(Fig. 31d, g)**. Nkx2-5 targeted both upregulated and downregulated genes in CPCs and adult hearts **(Fig. 31e, h)**. However, Nkx2-5 preferentially targeted upregulated genes in CPCs and downregulated genes in adult hearts **(Fig. 31e, h)**, which is consistent with functions of Nkx2-5 as both an activator and repressor of gene expression[84,122,266].

To determine if the misexpression of Nkx2-5 targets is associated with direct changes in Nkx2-5 levels, I quantified gene expression levels of *Nkx2-5* in CPCs. *Nkx2-5* was downregulated in CPCs of embryos from Ob-fed females **(Fig. 31i)**. Furthermore, *Nkx2-5* transcript levels negatively correlated with maternal weight at conception **(Fig. 31j)**. Thus, disruption of Nkx2-5-controlled transcriptional pathways could sit high in the hierarchy of events embryonically programming heart disease susceptibility in response to maternal obesity.

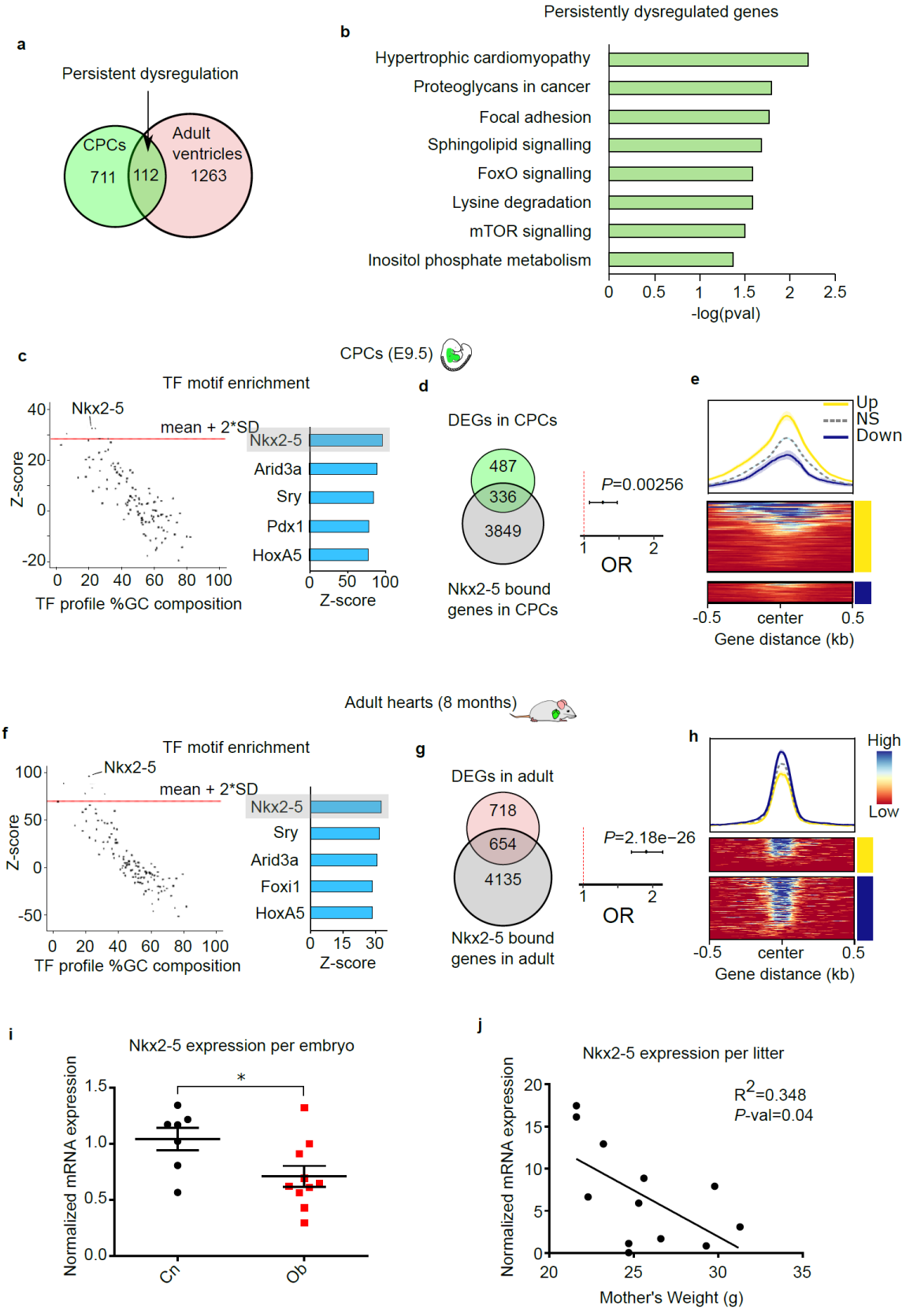

a
Persistent dysregulation
CPCs
711
112
Adult ventricles
1263
b
Persistently dysregulated genes
Hypertrophic cardiomyopathy
Proteoglycans in cancer
Focal adhesion
Sphingolipid signalling
FoxO signalling
Lysine degradation
mTOR signalling
Inositol phosphate metabolism
0 0.5 1 1.5 2 2.5
-log(pval)
c
CPCs (E9.5)
TF motif enrichment
Nkx2-5
mean + 2*SD
Z-score
TF profile %GC composition
Nkx2-5
Arid3a
Sry
Pdx1
HoxA5
Z-score
d
DEGs in CPCs
487
336
3849
Nkx2-5 bound genes in CPCs
P=0.00256
OR
e
Up
NS
Down
-0.5 center 0.5
Gene distance (kb)
Adult hearts (8 months)
f
TF motif enrichment
Nkx2-5
mean + 2*SD
Z-score
TF profile %GC composition
Nkx2-5
Sry
Arid3a
Foxi1
HoxA5
Z-score
g
DEGs in adult
718
654
4135
Nkx2-5 bound genes in adult
P=2.18e−26
OR
h
High
Low
-0.5 center 0.5
Gene distance (kb)
i
Nkx2-5 expression per embryo
Normalized mRNA expression
*
Cn
Ob
j
Nkx2-5 expression per litter
R2=0.348
P-val=0.04
Normalized mRNA expression
Mother's Weight (g)

**Figure 31. Maternal obesity induces dysregulation of Nkx2-5 target genes in cardiac progenitor cells and persistently in the adult heart. a)** Venn diagram of genes dysregulated in CPCs and adult (8-month-old) hearts. **b)** KEGG pathway enrichment on the 112 persistently misregulated genes. **c)** Motif enrichment analysis on 10kb upstream and downstream of the TSS of all genes dysregulated in CPCs, conducted using oPOSSUM v3.0[225]. **d)** Number of differentially expressed genes that are directly bound by Nkx2-5 in CPCs (left). Odds ratio and *P*-value for differentially expressed genes bound by Nkx2-5 compared to that expected by chance, obtained using Fisher's exact test (right). Bars show 95% confidence intervals. **e)** Nkx2-5 ChIP-seq peaks proximal to differentially expressed genes in CPCs. Aggregate plots (top) show the mean ± SEM of the signal of each category. Heatmaps (bottom) show the Nkx2-5 signal centered on the TSS of genes lacking a promoter-proximal peak. **f)** Motif enrichment analysis on 10kb upstream and downstream of the TSS of all genes dysregulated in adult hearts. **g)** Number of differentially expressed genes that are directly bound by Nkx2-5 in adult hearts (left). Odds ratio and *P*-value for differentially expressed genes bound by Nkx2-5 compared to that expected by chance, obtained using Fisher's exact test (right). Bars show 95% confidence intervals. **h)** Nkx2-5 ChIP-seq peaks proximal to differentially expressed genes in adult hearts. Aggregate plots (top) show the mean ± SEM of the signal of each category. Heatmaps (bottom) show the Nkx2-5 signal centered on the TSS of genes lacking a promoter-proximal peak. **i)** qPCR of *Nkx2-5* in CPCs of embryos of Cn- and Ob-fed mice. Each dot represents CPCs from an individual embryo. Bars represent the mean ± SEM. N=7 and 10, respectively. Student t-test was used to determine *P*-value. **j)** Pearson correlation test of *Nkx2-5* mRNA expression in CPCs *vs* maternal weight at conception. Each dot represents pooled CPCs from embryos of one litter. N=5 and 7, respectively. UP=upregulated. Down=downregulated. NS=not significantly changed.

## 7.8. Acute increases in maternal blood glucose are associated with increased gene deregulation in cardiac progenitor cells

Given the variation in the magnitude of gene expression fold changes within CPCs of embryos of Ob-fed mice **(Fig. 29e),** I sought to identify potential maternal phenotypic factors that may underlie this heterogeneity in gene expression responses. I hypothesized that the extent of gene deregulation could be associated with the severity of maternal metabolic dysfunction,

similar to that observed in adult hearts **(Fig. 24)**. Several metabolic parameters are altered by obesity. As a first step towards elucidating the identity of maternal factors associated with transcriptional misregulation in the offspring, I correlated the mRNA levels of differentially expressed genes with the maternal weight at conception and maternal blood glucose levels at the time of embryo collection (E9.5). It is important to note that, unlike the mothers of the offspring that were used for transcriptomic profiling of adult (8-month-old) hearts, the CPCs were obtained from mothers which gained much less weight during Ob diet feeding (CPCs maternal weight range: 23-26 grams, adult heart maternal weight range: 21-29 grams). This small increase in maternal weight at the time of conception did not correlate significantly with gene expression changes in CPCs **(Fig. 32a)**. In contrast, maternal blood glucose levels correlated strongly with gene expression variability **(Fig. 32b)**. Of the 14,609 genes that are expressed in CPCs, 1,149 genes (8%) correlated with maternal blood glucose levels. Notably, of the 823 differentially expressed genes in CPCs in response to maternal obesity, the mRNA levels of 438 genes (53%) correlated with maternal blood glucose levels **(Fig. 32c)**. This indicates that an Ob diet leads to altered expression of these predicted "glucose-responsive genes".

Notably, the glucose tolerance of Ob-fed mice was not altered, thus altered expression of "glucose-responsive genes" was not expected. I hypothesized that, during early stages of obesity, when mice have little weight gain and glucose tolerance is normal, it is possible that acute increases in maternal blood glucose during pregnancy may be an indicator of fetal transcriptional misregulation in early stages of obesity. I examined the blood glucose levels of pregnant mice (at E9.5) fed Ob or Cn diets. Unfasted blood glucose levels of Ob-fed females, at E9.5, was increased **(Fig. 32d)**, indicating acute but moderate hyperglycemia in Ob-, compared to Cn-, fed females at E9.5. Thus, maternal Ob diet leads to moderate increases in blood glucose levels, that are associated with transcriptional dysregulation in CPCs.

To better characterize these glucose-responsive genes in the context of obesity, I examined the function of the 438 glucose-responsive genes that were dysregulated in CPCs in response to maternal obesity. Glucose responsive genes enriched for regulators of heart development, muscle differentiation and contraction as well as vasculogenesis **(Fig. 32e)**. These genes also enriched for cardiac disease pathways, including hypertrophic cardiomyopathy **(Fig. 32)**, consistent with the predisposition to develop cardiac hypertrophy in adulthood.

Differentially expressed genes which did not correlate with maternal blood glucose levels enriched for genes that regulate epigenetic repression, including DNA methylation, and histone K27 tri-methylation **(Fig. 32f)**. These genes were largely repressed in response to maternal obesity. My results indicate that acute increases in glucose levels even in the absence of overt glucose intolerance, are strongly associated with activation of genes regulating cardiac development and disease in CPCs of Ob-fed mice, however other factors are likely involved in mediating gene repression.

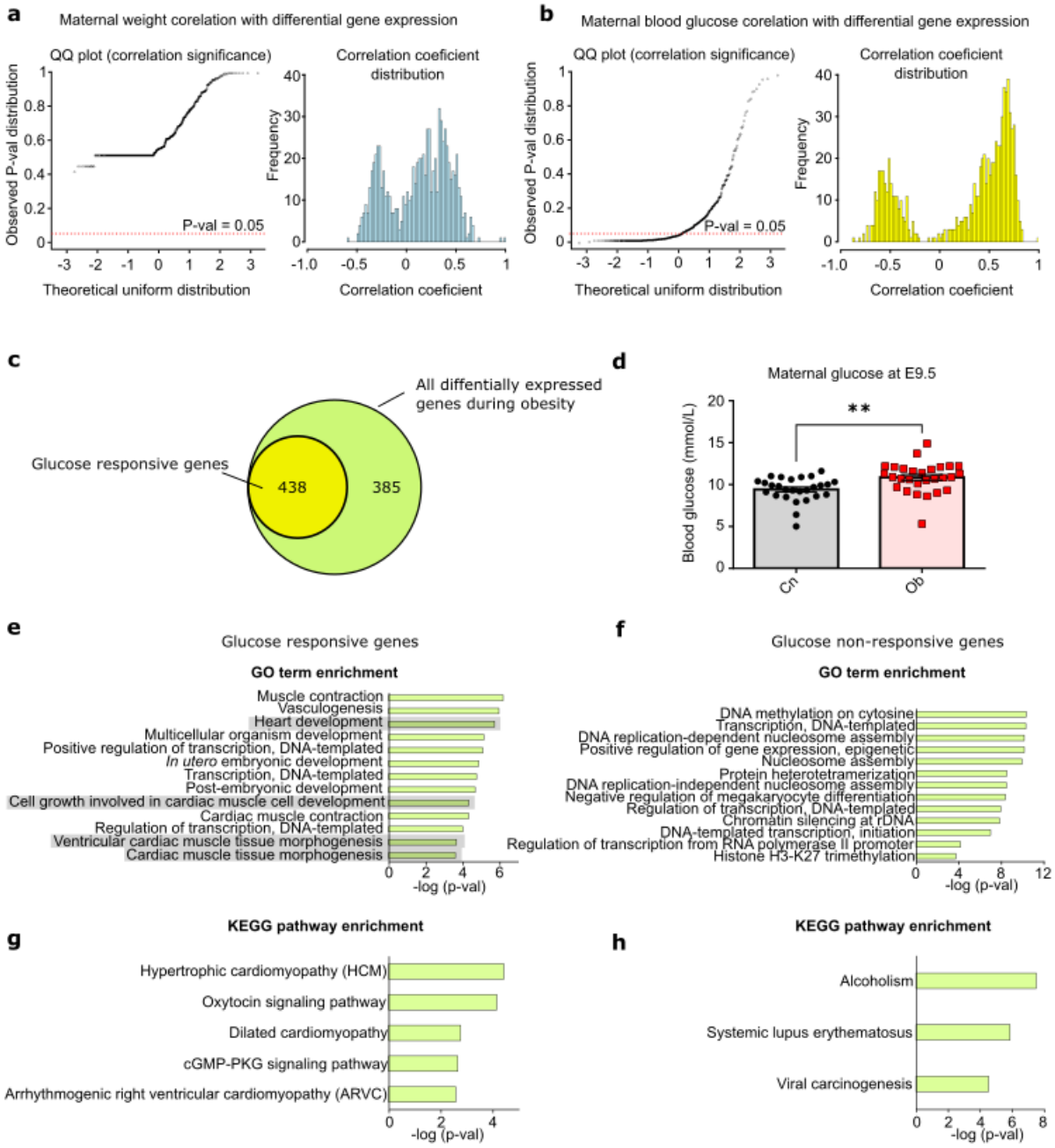


**Figure 32. Increased maternal blood glucose at E9.5 is associated with dysregulation of genes regulating cardiac development and disease. a)** QQ plot (left) for the correlation between maternal weight at conception and the extent of gene deregulation in the hearts. The observed p-value (after adjusting for multiple comparison) was plotted on the y-axis against the null hypothesis (x-axis). Each dot represents a single gene. The red horizontal line denotes a p-value of 0.05. Frequency distribution (right) of the correlation coefficients between maternal weight at conception and gene expression. **b)** QQ plot (left) for the correlation between maternal unfasted blood glucose at E9.5 and the extent of gene deregulation in the hearts. The observed p-value (after adjusting for multiple comparison) was plotted on the y-axis against the null hypothesis (x-axis). Each dot represents a single gene. The red horizontal line denotes a p-value of 0.05. Frequency distribution (right) of the correlation coefficients between maternal blood glucose and gene expression. **c)** Venn diagram of genes dysregulated in CPCs (shown in Fig. 30) and genes that significantly correlated with maternal blood glucose levels. **d)** Maternal blood glucose levels at E9.5, immediately prior to embryo collection. Readings were obtained in an unfasted state. N=27 and 28 mice respectively. **e)** GO term enrichment on glucose-responsive genes. Terms related to cardiac development are highlighted in grey. **f)** GO term enrichment on glucose non-responsive genes. **g)** KEGG pathway enrichment on glucose-responsive and **h)** non-responsive genes. Bars represent the mean ± SEM. ** represents $P<0.01$.

## 7.9. Maternal BMI correlates with the expression of genes controlling mitochondrial oxidative metabolism, ECM-cell interaction, and cell adhesion in the human fetal heart

To determine if maternal obesity alters the fetal cardiac transcriptome in humans and to identify BMI-responsive genes, I performed RNAseq on ventricles of 53 fetuses from underweight, healthy, overweight and obese pregnant donors. The women ranged in BMIs from 17 to 50 kg/m$^2$ and fetal gestational age ranged from 8 to 19 weeks. These time points are the earliest timepoints when collection of human fetal hearts was possible, but they largely represent post-septation stages **(Table 1)**. PCA separated samples by gestational age on PC1 and sex on PC2, which explained 42% and 19% of variation, respectively. This indicates that age and sex explain the largest degree of variation amongst the fetal hearts **(Fig. 33a)**. Similarly, heatmap

clustering by Euclidean distance resulted in 4 main groups which were largely defined by gestational age **(Fig. 33b)**. Group 1 comprised hearts from 8 to 9.9 weeks of gestation. Groups 2, 3 and 4 spanned more broadly from 10 to 12.9, 13 to 15.9, and 16 to 18.5 weeks, respectively **(Fig. 33b)**.

To identify genes whose expression is responsive to BMI, I incorporated age and sex in the differential expression model, and I tested for genes whose expression is responsive to BMI as a continuous variable or obesity as a categorical variable. Expression of only 3 genes significantly correlated with BMI as a continuous variable: *GALNT14*, a protein glycosyltransferase, and two pseudogenes *PRSS46P* and *MTATP8P1* **(Fig. 34a)**. Similarly, the expression of 3 genes was significantly associated with obesity as a categorical variable: *MTRNR2L12*, *MTRNR2L1*, and *MTCO3P12* **(Fig. 34b)**, all of which are encoded in the mitochondrial genome. The altered expression of these six genes represents a conserved response to maternal obesity across all ages and sexes examined. These results suggest that obesity *in utero* does not broadly alter gene expression in the human fetal heart independently of sex and age.

Given the limited overlap of differentially expressed genes across timepoints i.e. CPCs, and 8 months old hearts **(Fig. 31a)**, it is apparent that transcriptional cardiac response to maternal obesity varies across different developmental and postnatal time points. I hypothesized that human fetal hearts might also mount stage-specific responses to maternal obesity. I stratified the fetal transcriptomes by gestational age. Two groups, one from 8 to 9.4 weeks (14 samples) and another from 15.3 to 16.6 weeks (9 samples) allowed comparison of very similar transcriptomes, within each group, over a broad BMI range **(Fig. 33a, b)**.

In the youngest age group, 8 to 9.4 weeks, 203 genes correlated with BMI as a continuous variable (referred to as early responsive), of which 128 increased and 76 decreased per unit increase in BMI **(Fig. 35a)**. Positively correlated genes enriched for functions that promote increased lipid metabolism and mitochondrial function such as electron transport chain and mitochondrial translation **(Fig. 35b)**. Both nuclear and mitochondrially encoded regulators of oxidative phosphorylation increased. Such genes include aldehyde dehydrogenase (*ALDH2*), which is a major enzyme in the oxidative metabolism of aldehydes, and NADH dehydrogenase 3

(*MT-ND3*), which promotes electron transport in the mitochondria. Genes that negatively correlated with BMI enriched for terms related to cell adhesion and muscle development **(Fig. 35c)**. For example, the expression of filamins A and C, which anchor membrane proteins to actin filaments and maintain muscle integrity [267] decreased as BMI increased.

In the older age group, 15.3 to16.6 weeks, 108 genes correlated with maternal BMI (referred to as late responsive), of which 83 increased and 25 decreased for every increase in BMI **(Fig. 35d)**. Similar to hearts at 8 to 9.4 weeks of gestation, positively correlated genes enriched for functions that promote oxidative metabolism and mitochondrial function, and negatively correlated genes enriched for regulators of cell-ECM and synapse regulatory pathways **(Fig. 35e-f)**. These terms were more significantly enriched in hearts at later compared to earlier gestation **(Fig. 35b, e)**. These results suggest that in association with a higher maternal BMI, pathways controlling mitochondrial oxidative metabolism are persistently overactivated and pathways regulating cell adhesion are persistently repressed in the fetal heart from early to mid-gestation.

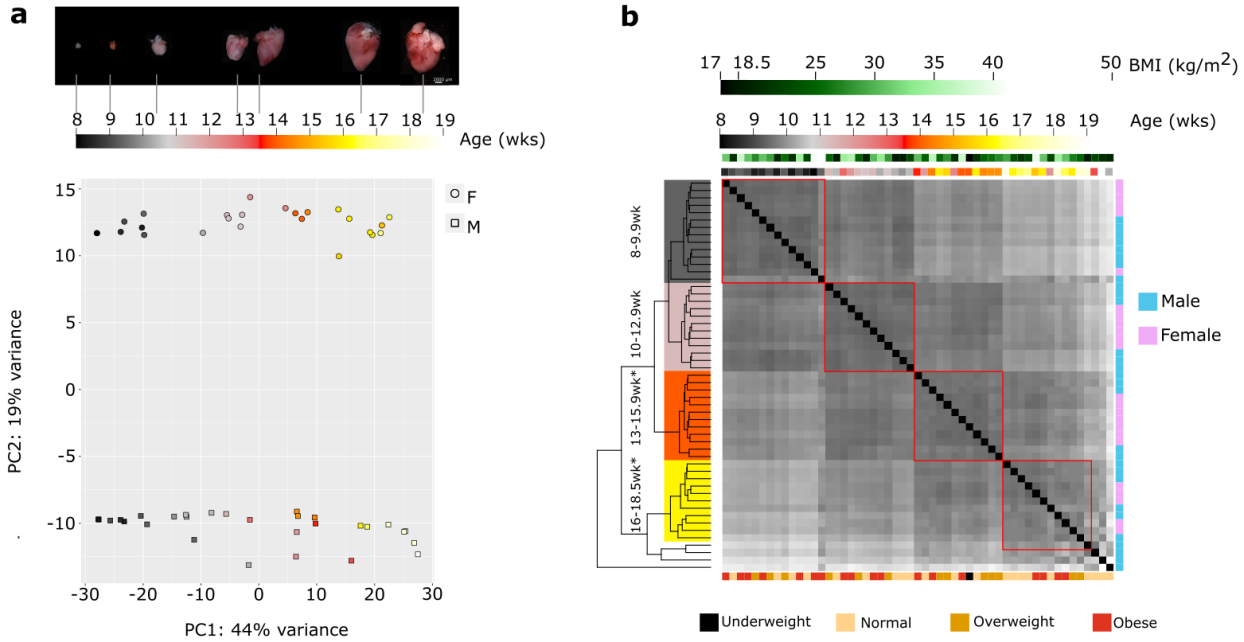



**Figure 33. Gestational age and sex of the fetus determine the largest inter-sample variation in the transcriptional profile of the human fetal heart. a)** PCA plot separated cardiac transcriptomes by gestational age on PC1 and sex on PC2. Representative pictures of the human hearts are shown (top) **b)** Unsupervised hierarchal clustering identified 4 main groups largely

defined by gestational age. * indicates that 2 samples within this category did not match the respective age.

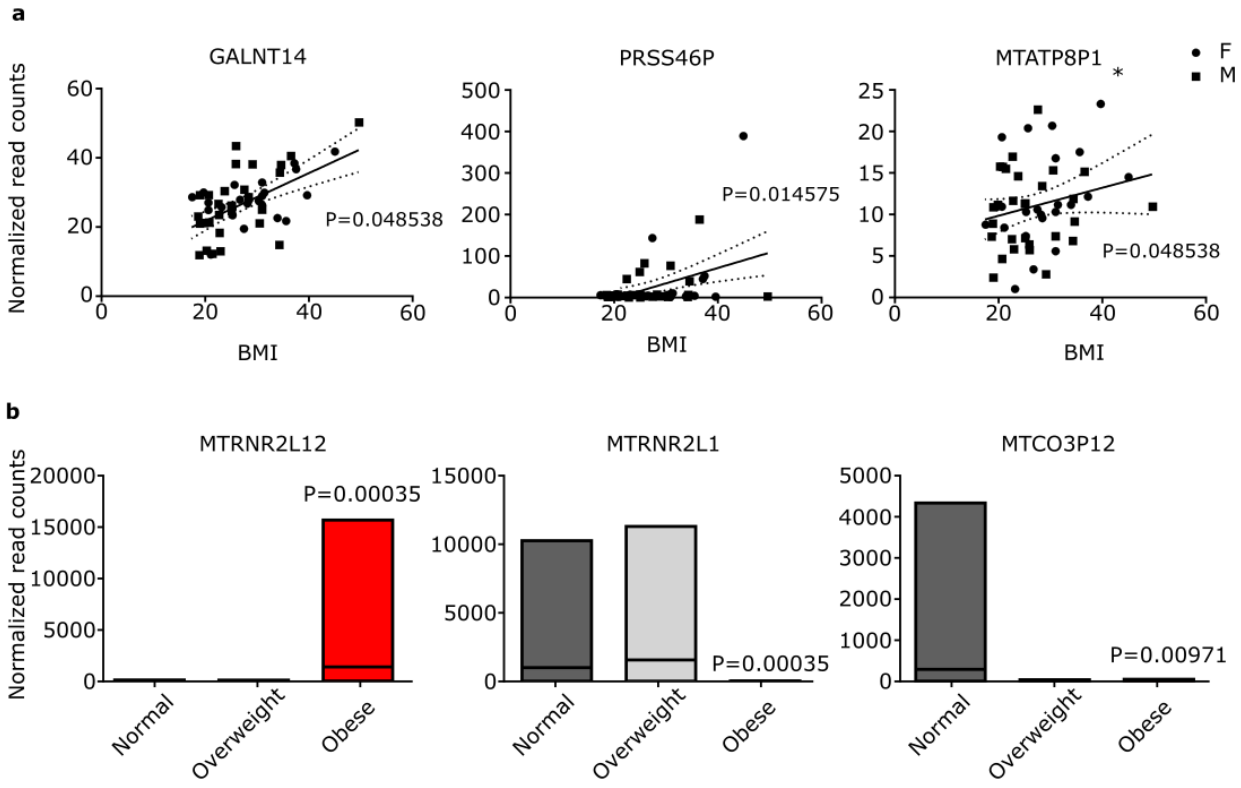



**Figure 34. Obesity *in utero* alters the expression of six genes independently of sex and age in the human fetal heart. a)** Expression of genes that were correlated with BMI irrespective of age and sex. **b)** Expression of genes that were differentially expressed in the hearts of fetuses of obese *vs*. healthy donors as categorical variables.

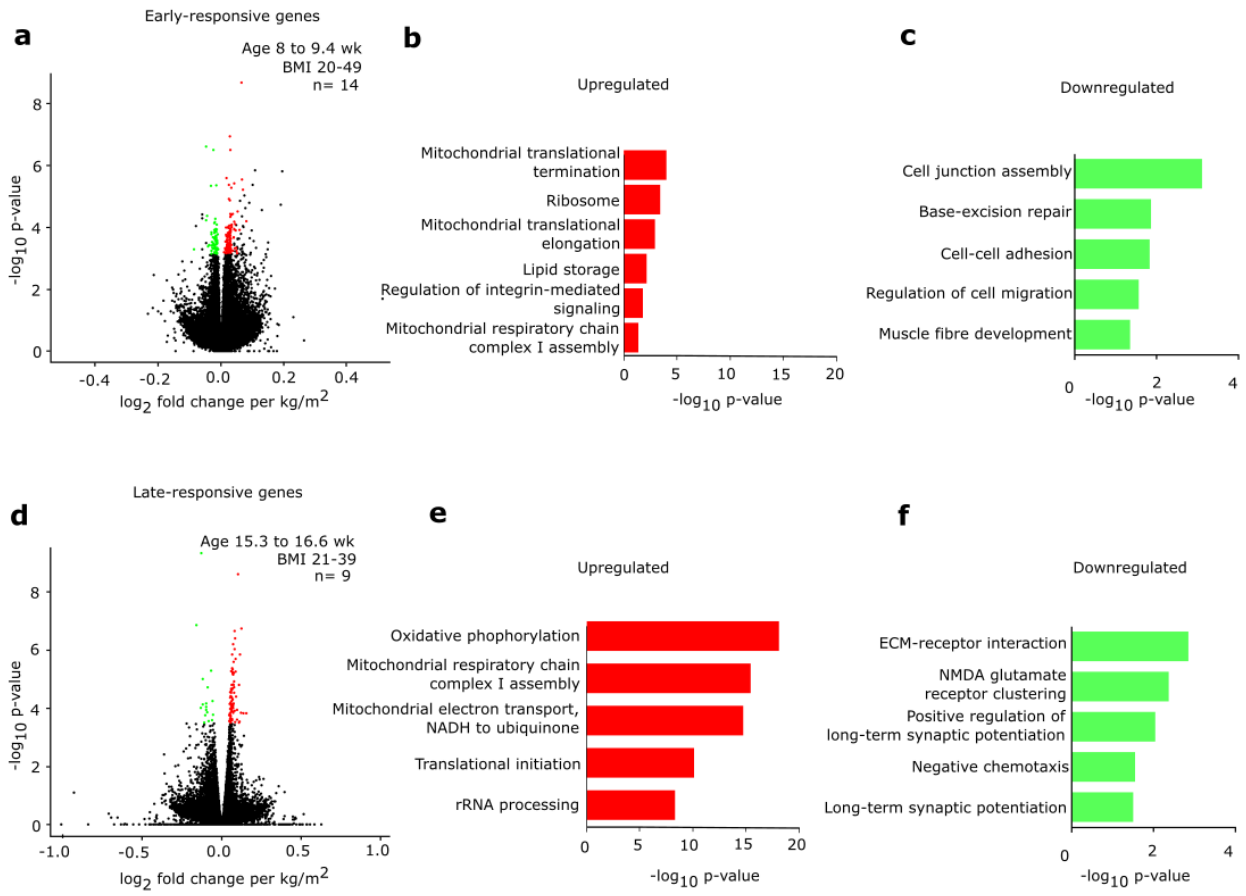



**Figure 35. Maternal BMI correlates with altered expression of genes controlling mitochondrial oxidative metabolism, and cell adhesion in the human fetal heart during early and mid-gestation. a)** Volcano plot of all expressed genes in 8-9.4 weeks-old hearts. Genes that are significantly downregulated per unit increase in BMI are in green, and upregulated genes are in red. **b)** GO term enrichment in genes that were upregulated (red) or **c)** down-regulated (green) in fetal hearts at early gestation, in response to increased maternal BMI. **d)** Volcano plot of all expressed genes in 15.3-16.6 weeks old hearts. Genes that are significantly downregulated per unit increase in BMI are in green, and upregulated genes are in red. **e)** GO term enrichment in genes that were upregulated (red) or **f)** down-regulated (green) in fetal hearts at mid-gestation, in response to increased maternal BMI.

## 7.10. BMI-responsive genes in the human fetal heart are developmentally regulated

Although the same pathways were deregulated during early and mid-gestation, it is unclear if the same genes regulating these pathways are persistently misregulated. To determine if the same genes are misregulated from early to mid-gestation, I first focused on the 202 early-responsive genes and examined their differential expression profiles in hearts obtained at mid-gestation. I found that 91% of early-responsive genes which positively correlated with BMI at 8 to 9.4 weeks maintained the same trend two months later, at 15.3 to 16.6 weeks **(Fig. 36a)**. Similarly, 81% of early-responsive genes which negatively correlated with BMI at 8 to 9.4 weeks tended to decrease as BMI increased at mid-gestation **(Fig. 36a)**. I then examined the differential expression profiles of the 108 late-responsive genes in fetal hearts at early gestation. 94% of all late-responsive genes had the same trends for gene deregulation already at early gestation **(Fig. 36b)**. Despite the maintenance of these trends and the overlap in enriched pathways **(Fig. 35b, c, e, and f)**, only 6 genes (*MT-ND3, MZT2B, MICOS13, SLC8A1-AS1, ANKRD36C, SYCE1L*) reached significance at both early (8 to 9.4 weeks) and mid (15.3 to 16.6 weeks) gestation. This indicates, despite sharing the same misregulated pathways across gestation, different gene sets regulating these common pathways are misregulated, in large magnitudes, at different developmental ages. However, small magnitude changes in these defined gene sets were detected throughout development **(Fig. 36a, b)**. This may suggest that, in response to an increase in maternal BMI, the developing heart maintains a transcriptional trajectory and a memory, in the form of small magnitude changes in gene expression, months before or after larger magnitude changes are present.

Consistently, I found that the expression of early- and late-responsive genes is developmentally regulated. The expression level of genes that positively correlated with BMI was low at early stages of development and increased at later development **(Fig. 36c)**. In contrast, the expression of negatively correlated genes was higher at early gestation but continuously decreased as gestation progressed **(Fig. 36c)**. This suggests that obesity-responsive genes are temporally regulated during heart development. Maternal obesity represses genes that are more highly expressed during early cardiac development and activates genes that are more highly expressed during later stages of cardiac development.

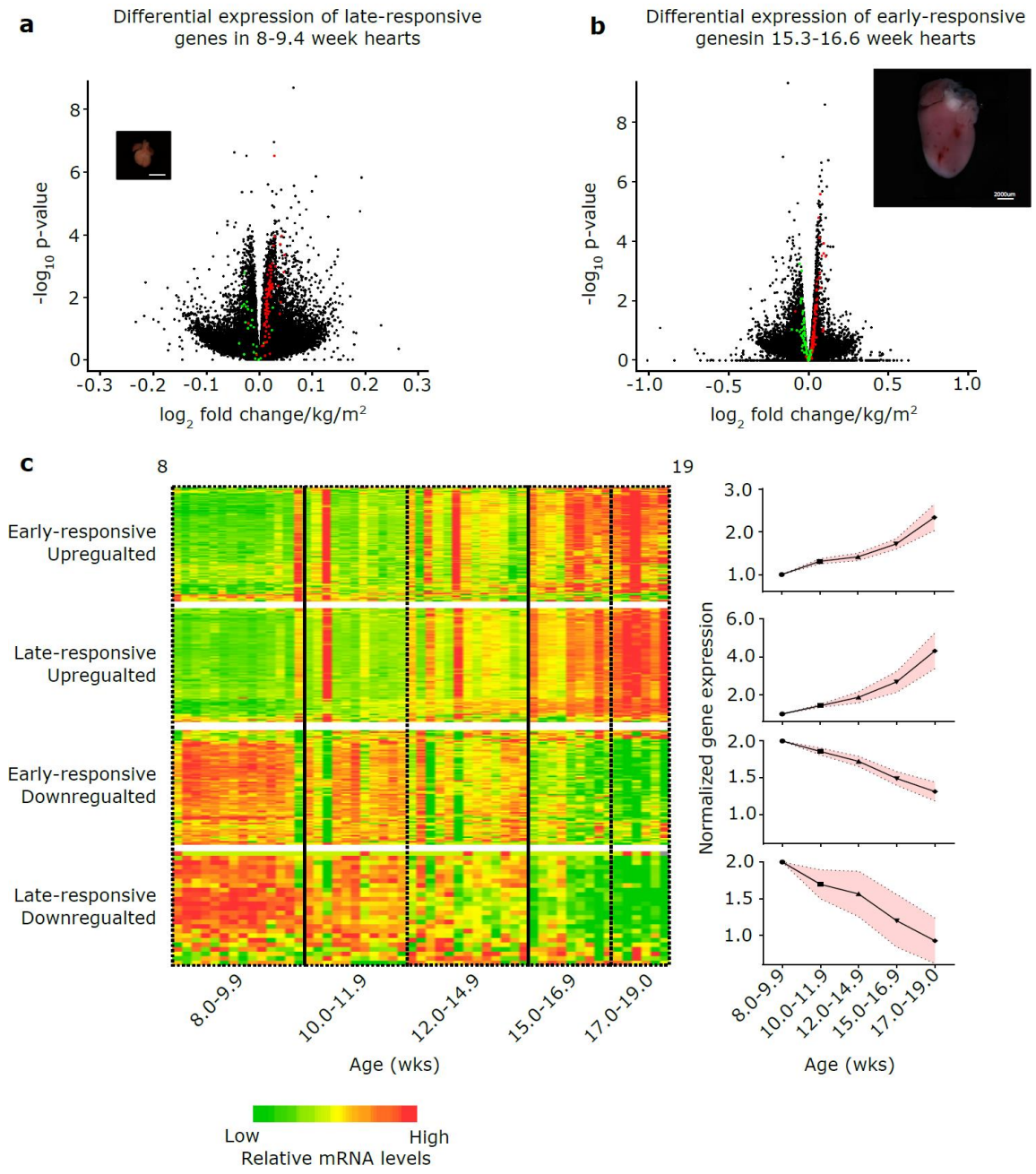



**Figure 36. Developmentally regulated genes are abnormally expressed in the human fetal heart in response to exposure to obesity *in utero*. a)** The identity of all 108 late-responsive genes are highlighted in a volcano plot demonstrating the gene expression fold changes per increase in BMI in 8-9.4wk old hearts. The 83 upregulated late-responsive genes are in red. The 25 downregulated late-responsive genes are in green. **b)** The identity of all 204 early-responsive genes are highlighted in a volcano plot demonstrating the gene expression fold changes per increase in BMI in 15.3-16.6wk old hearts. The 128 upregulated early-responsive genes are in

red. The 76 downregulated early-responsive genes are in green. **c)** Heatmap displaying gene expression level of early- and late-responsive genes at different developmental stages. Normalized gene expression is shown on the right panel.

## 7.11. Genes controlling metabolism, ECM-cell interaction, and cardiac development are dysregulated in human fetal hearts in response to morbid maternal obesity

Gene expression in mouse CPCs and adult hearts was most drastically dysregulated in the offspring of the heaviest dams **(Fig. 24, 30)**. To determine whether morbid obesity is associated with more extreme gene expression changes in the human fetal heart, I compared the hearts of fetuses of morbidly obese (BMI >40) donors against age-matched (8 to 9.4 weeks) healthy (BMI 20.7-22.6) donors. I identified much broader changes in gene expression in response to morbid obesity. After accounting for age and sex, 600 genes were dysregulated in hearts of fetuses of morbidly obese donors. Fatty acid-binding protein 2 (*FABP2*), which directs fatty acid transport to the fetus and previously shown to be dysregulated in livers of mice born to obese dams[268,269], was the most significantly misregulated gene **(Fig. 37a)**. Accordingly, GSEA revealed regulators of mitochondrial fatty acid oxidation as highly upregulated in the hearts of fetuses of obese donors **(Fig. 37b)**. Genes associated with ribosomal biogenesis, and ECM remodeling such as Ribosomal Protein S24 (*RPS24*) and Elastin (*ELN*), respectively, were also significantly upregulated **(Fig. 37b)**, consistent with their upregulation in 8 months old mouse hearts. In contrast, genes regulating cardiac development, as well as cell adhesion were downregulated **(Fig. 37b)**. Notably, these enriched pathways are largely the same pathways identified when correlating BMI as a continuous variable at early and late gestation **(Fig. 35)**. However, comparing samples from opposite ends of the BMI spectrum enabled the detection of many more genes which may have lower fold changes in less obese donors. Thus, hearts of fetuses of morbidly obese donors at 8 to 9.4 weeks of gestation over-activate gene networks regulating mitochondrial metabolism and translation, and concomitantly downregulate networks controlling cell adhesion and heart development.

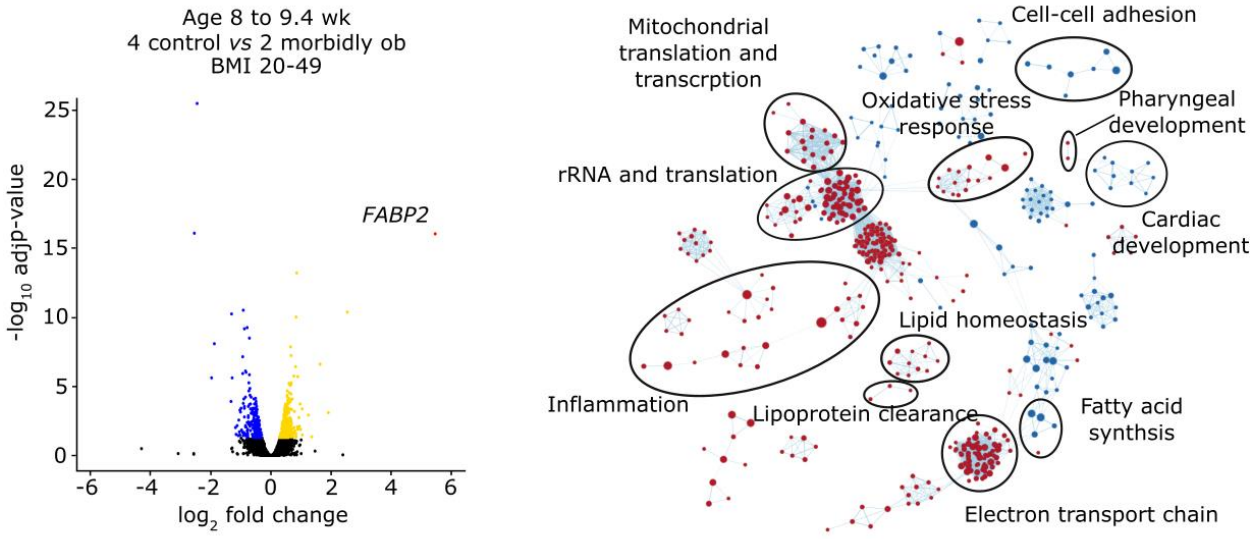



**Figure 37. Morbid maternal obesity leads to broad activation of metabolic and inflammatory gene expression and repression of genes regulating cardiac development and cell adhesion. a)** Volcano plot of all expressed genes in 8-9.4 weeks-old hearts from morbidly obese *vs.* healthy donors. Upregulated genes are in yellow and downregulated in blue. **b)** Network clustering showing gene sets (from GSEA) enriched in upregulated (red) and downregulated (blue) genes.

## 7.12. Genes responsive to maternal BMI in human fetal hearts are enriched for NKX2-5 targets

To determine if NKX2-5 targets genes that are dysregulated in the human fetal heart in response to obesity *in utero*, I cross-referenced dysregulated genes against published NKX2-5 ChIP-seq datasets of differentiated human CMs[266]. Genes downregulated in the fetal hearts of morbidly obese donors were enriched for direct NKX2-5 targets **(Fig. 38a)**. Downregulated NKX2-5 targets include *FLNC*, involved in cell-ECM interactions[267], and cardiomyopathy-associated 1 (*XIRP1*)[270,271], both of which were also downregulated in adult mouse hearts born to obese dams. In contrast, NKX2-5 targets were relatively depleted within upregulated genes, compared to genes that were not differentially expressed **(Fig. 38a)**. Nonetheless, ~20% of upregulated genes were still bound by NKX2-5 **(Fig. 38b)**. For example, several genes regulating lipid uptake and oxidative metabolism including *FABP2* and *ALDH2* were significantly upregulated in fetal hearts of obese donors and were also bound by NKX2-5. Other canonical Nkx2-5 targets such as BMP10[101] were also significantly upregulated in fetal hearts at mid-gestation, in response to increased BMI and are bound by NKX2-5. This suggests that, although NKX2-5 predominantly targets genes that are repressed in response to exposure to obesity *in utero*, it may mediate upregulation of some genes as well. These results are consistent with the enrichment for Nkx2-5 targets in downregulated genes of adult mice born to obese dams **(Fig. 31h)** but contrast with CPCs **(Fig. 31e)**, indicating that the function of Nkx2-5 may be different in CPCs compared to later stages of development. Cumulatively, I found that NKX2-5 preferentially targets genes that are repressed in the human fetal heart in response to exposure to obesity *in utero*. Disruption of NKX2-5-controlled transcriptional pathways sits high in the hierarchy of events embryonically programming heart disease susceptibility in response to maternal obesity.

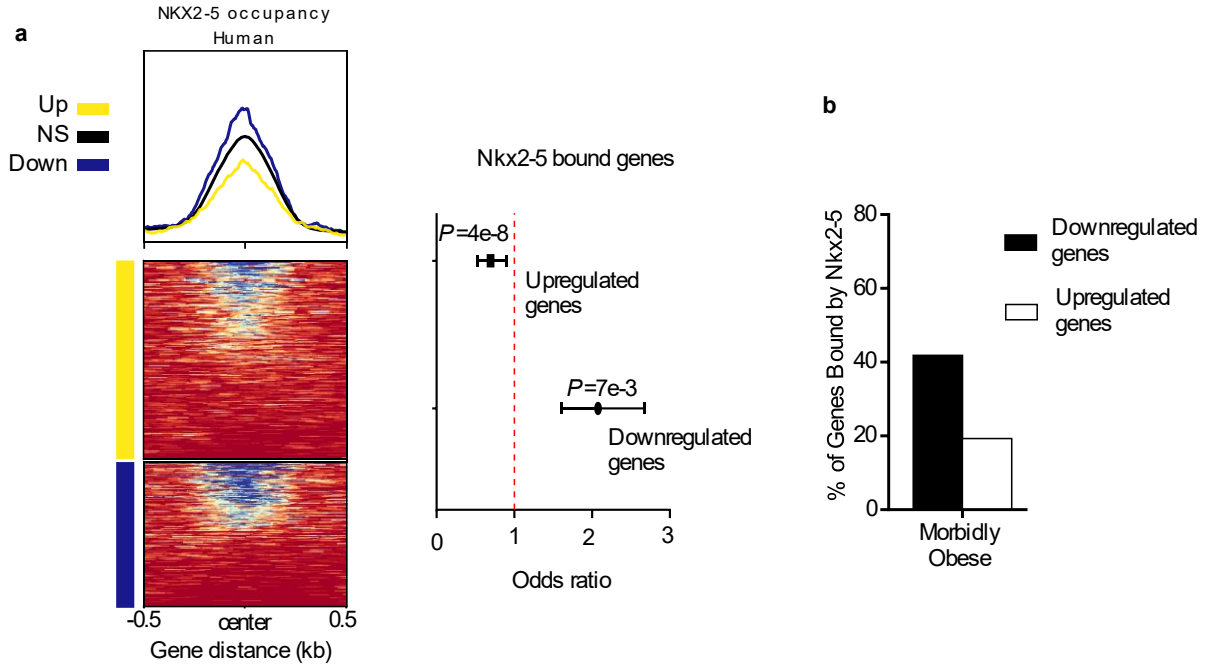



**Figure 38. NKX2-5 targets genes that are abnormally expressed in the human fetal heart in response to exposure to obesity *in utero*. a)** Nkx2-5 ChIP-seq peaks proximal to differentially expressed genes in human hearts. Aggregate plots (top) show the mean signal of each category. Heatmaps (bottom) show the Nkx2-5 signal centered on the TSS of genes lacking a promoter-proximal peak. Odds ratio and *P*-value for differentially expressed genes bound by Nkx2-5 compared to that expected by chance, obtained using Fisher's exact test (right). Bars show 95% confidence intervals. **b)** Percentage of differentially expressed genes bound by NKX2-5.

## *8. Discussion 2:*

### 8.1. Long-term function of Nkx2-5 is altered in the heart in response to maternal obesity

Long-term dysregulation of cardiac transcription factors can program adult-onset heart disease[123]. In my thesis, I demonstrate that *Nkx2-5* expression is reduced CPCs from embryos of obese mice **(Fig. 31i, j)**. Consistently, transcriptomic analysis of CPCs (at E9.5) from embryos of obese mice, human fetal hearts of obese donors (early- and mid-gestation), and in adult hearts (8 months) of offspring of obese mice revealed dysregulation of Nkx2-5 target genes across all stages **(Fig. 31, 38)**. CPCs of embryos of obese mice activated genes regulating cardiac development such as *Hand1*, *Pitx2*, *Vcam1* and other kernel TFs including *Gata4* and *Mef2c* which are known to be repressed by Nkx2-5 **(Fig. 30)**. Conversely, adult hearts repressed genes regulating metabolism and ion channels, such as *PPARα* and *Scn5a*, which are known to be activated by Nkx2-5 **(Fig. 21, 24)**. This is consistent with the known function of Nkx2-5 as a repressor of genes regulating cardiac specification and differentiation during early cardiac development[96,266] and an activator of genes regulating metabolism and ion channels in adulthood[104,105]. *Nkx2-5* knockout mice activate genes regulating cardiac progenitor specification between E7.5-E9.5[96]. In contrast, genes regulating metabolism and ion channels are repressed in the hearts of adult mice bearing a hypomorphic *Nkx2-5* mutation[104,105]. Nkx2-5's dual role as an activator or repressor of gene expression could explain our finding that Nkx2-5 preferentially targets genes that are downregulated in CPCs and upregulated in human fetal hearts and mouse adult hearts in response to obesity during pregnancy **(Fig. 31e, h and Fig. 38)**.

In summary, abnormal expression of *Nkx2-5* and its target pathways defines a state of heart disease susceptibility and a transcriptional signature that could be useful for the early identification of individuals at a higher risk of heart disease. Of note, while many of the misregulated genes were direct targets of Nkx2-5, hundreds of misregulated genes are not. For example, genes regulating ECM remodeling which were activated in the adult hearts **(Fig. 21)** are not known targets of Nkx2-5. Although Nkx2-5 can regulate the expression of other TFs[97,99,119] and thus can indirectly mediate changes in gene expression, other signaling pathways are likely involved in mediating gene expression changes independently of Nkx2-5.

## 8.2. Functions of dysregulated genes are consistent with cardiac phenotypes of the offspring

The link between the genes misregulated in response to maternal obesity and the phenotypes of the offspring fit well in the context of known functions of those genes. Cumulatively, across all embryonic and adult time points examined in my thesis, genes regulating ECM remodeling, TGF-β, inflammatory, and metabolic pathways are dysregulated in the heart in response to maternal obesity **(Fig. 21, 27, 30, 35)**. Genes regulating ECM deposition were upregulated in the adult heart of offspring (at 8 months) **(Fig. 21)**, and in young hearts exposed to stress (at 2 months) **(Fig. 27)**. Upregulation of genes encoding ECM was modest in "unstressed" Ob/Cn at 2 and 8 months of age but was augmented in response to ISO **(Fig. 27)**. Increased ECM deposition is known to increase cardiac stiffness, which can compromise ventricular compliance and reduce diastolic function[261,262]. Indeed, the expression of genes encoding ECM regulators was proportional to the reduction in IVRT at 8 months of age **(Fig. 24)**. The augmented increase in expression of genes encoding ECM regulators was also associated with excessive fibrosis and impaired diastolic response to ISO in young offspring of obese mice **(Fig. 19, 20)**. Thus, it is possible that increased ECM deposition could mediate the reduced diastolic function in our model.

Genes encoding regulators of TGF-β signaling were misregulated in CPCs and adult hearts of offspring of obese mice which might mediate increased ECM deposition and other pathological changes in the offspring. Genes regulating TGF-β signaling were activated in CPCs **(Fig. 30)** and adult (8 months) hearts of offspring of obese mice **(Fig. 21)**. In addition, inhibitors of TGF-β were downregulated in adult (2 months) hearts of offspring of obese mice, in response to ISO **(Fig. 27)**. Over activating the TGF-β pathway promotes pathologic remodeling with increased fibrosis, inflammation, and hypertrophy in infarcted and pressure overloaded hearts[272,273]. This could underlie the increased fibrosis and restrictive hypertrophic response to ISO. Notably, during development, TGF-β signalling is mediated by different lignads and downstream effectors that induce cardiac morphogenesis and differentiation compared to the adult mediators of TGF-β signalling which promote pathological remodeling **(section 1.7.1.1.)**. The dynamic roles of TGF-β as a regulator of cardiac development during embryogenesis or a mediator of pathological remodeling in the adult heart could explain the fetal-specific activation of genes regulating

cardiac development and the adult-specific activation of genes regulating ECM remodeling in the heart.

Genes regulating inflammation were also found to be increased in fetal and adult hearts of offspring of obese females. Notably, these genes were not activated in CPCs, but were activated during later development in human fetal hearts **(Fig. 37)** and adult mouse hearts in response to maternal obesity **(Fig. 21, 26)**. Inflammatory cytokines, such as *Il6*, *Tlr4,* and *TNFα* were not expressed in the CPCs at E9.5 (data not shown). In addition, it is known that cardiac macrophages do not infiltrate the heart until E11.5[274]. This could explain why genes encoding pro-inflammatory signaling proteins were not upregulated in CPCs but were upregulated in all subsequent stages: human fetal heart at early- and mid-gestation, as well as adult hearts at 2 and 8 months of age. Several inflammatory cytokines are known to be upregulated in fetal hearts of sheep models of over-nutrition, at mid and late gestation[156]. My results suggest that not only is activation of inflammatory genes a conserved response to maternal obesity across species, but it originates during the first trimester in human fetuses of obese women. It is unclear if increased inflammation is due to an increase in the recruitment of immune cells to the heart or whether the cardiomyocytes increase the expression of pro-inflammatory genes. Future experiments are required to elucidate the changes in cell composition/recruitment in the heart and the cell type-specific changes in gene expression in response to obesity.

The only pathways to be altered across all stages examined were metabolic signaling pathways. Genes in the PI3K-AKT were upregulated in mouse CPCs **(Fig. 30)** and adult hearts **(Fig. 21)**. This pathway starts with the binding of insulin or insulin growth factors to insulin receptors which in turn activates PI3K and AKT[159,275]. Increased AKT activation promotes increased glucose oxidation and reduces fatty acid oxidation[159,275]. CPCs at E9.5 do not have a functional ETC and cannot oxidize fatty acids in their immature mitochondria[158], thus, an increase in AKT activity would likely result in increased glucose uptake and energy production, with little impact on mitochondria. At later developmental stages, once the cardiac mitochondria have matured, genes in the PI3K-AKT signaling pathway were not upregulated in the human fetal heart, however, genes regulating the ETC, mitochondrial translation and transcription and lipid clearance were activated, whereas genes regulating fatty acid synthesis were repressed **(Fig. 35, 37)**. The contrast between the increase in genes regulating fatty acid oxidation and the

decrease in genes regulating fatty acid synthesis suggests that fatty acids might originate from an extra-cardiac source, possibly from the obese donor. Indeed, the most highly upregulated gene in fetuses of morbidly obese women was Fatty Acid Binding Protein 2 (*FABP2)* **(Fig. 37)**, which facilitates cellular uptake and intracellular transport of fatty acids[268,269].

Notably, despite the consistent upregulation of genes within the ETC and oxidative metabolism pathways, the genes which are activated in the human fetal heart between 8 to 9.4 weeks of gestation were largely distinct from those activated between 15.3 to 16.6 weeks of gestation **(Fig. 36a, b)**. The expression of genes regulating cardiac metabolism during development is highly dynamic, and the genes undergo significant changes in expression during development[276]. Indeed, I demonstrate that the genes which are differentially expressed at early gestation get downregulated as gestation progresses, whereas contrastingly, the genes which are differentially expressed at mid-gestation are activated as gestation progresses **(Fig. 36c)**. It is unclear what the functional impact of this increased expression in genes regulating mitochondrial fatty acid oxidation in the fetal heart. The activation of mitochondrial oxidative metabolism in the fetal heart is largely unexplored.

Interestingly, increased expression of genes regulating mitochondrial metabolism is not persistent in adult hearts. The young adult heart (2 months) downregulated genes that localize to the mitochondria and genes which regulate glucose metabolism **(Fig. 27)**. Such genes are normally upregulated during cardiac disease and are part of the adaptive cardiac response to stress[159–162]. However, in response to maternal obesity, they were not upregulated even after cardiac stress **(Fig. 27)**. Thus, maternal obesity might prevent adaptive metabolic remodeling during stress in the offspring's hearts. In older mice, under stress-free conditions, the heart also downregulated genes regulating glucose metabolism, particularly genes in the insulin signaling pathway **(Fig. 21, 24)**. For example, *Akt1* and downstream *PPARα*, were repressed **(Fig. 21, 24)**. Similarly, *Akt* targets within the AMPK and MTOR signaling pathways were also repressed **(Fig. 21)**. Inhibition of PPARa and insulin signaling can impair fatty acid oxidation and lead to hypertrophy and ultimately heart failure[159]. This suggests reduced fatty acid oxidation and reduced insulin signaling, which in turn might reduce glucose metabolism in the heart. Overall, my results indicate that misregulation of genes regulating cardiac metabolic homeostasis is a persistent outcome in response to maternal obesity.

Finally, cardiac ion channels were aberrantly repressed in adult hearts at 8 months of age **(Fig. 21)**. Several ion channels, such as *Ryr2* and *Scn5a*, were exclusively downregulated in the 8-month-old adult heart in Ob/Cn **(Fig. 21, 24)**. Reduced expression of ion channels has been implicated in the development of arrhythmia during heart disease[277], which is consistent with our finding of bradycardia in Ob/Cn **(Fig. 15)**. Bradycardia was previously reported in a mouse model of maternal obesity and progressed to tachycardia with age [246]. This phenotype was hypothesized to be due to a defective response of baroreceptors in the heart [246]. However, our findings suggest that cardiac arrhythmia might be a primary phenotype resulting from abnormal ion channel expression. The impact of bradycardia on the health of the offspring is not clear. Previous studies have demonstrated that reduced heart rates are associated with increased risk for CVD and mortality in humans[278–282]. Thus, reduced expression of ion channels might underlie cardiac arrhythmia and possibly increased cardiac susceptibility in the offspring of obese mice.

An important question is how these different gene expression changes which regulate various pathways are linked to cardiac disease susceptibility in the adult offspring. In my thesis, I demonstrate that the transcriptional signature of the adult hearts of offspring of obese mice is most similar to diabetic cardiomyopathy, cardiac hypertrophy and ISO-induced stress **(Fig. 25)**. Indeed, as observed in Ob/Cn hearts, expression of ECM components and insulin signaling regulators is perturbed in the diabetic mouse model[283]. Diabetic cardiomyopathy has a long latent phase during which stress marker genes and ECM components are expressed normally[283]. During the latent phase the heart is mildly hypertrophied and subclinical diastolic dysfunction is present[283]. As the disease progresses, systolic function is compromised[283], similar to that seen in our model **(Fig. 14)**. Similar to diabetic cardiomyopathy, genes controlling ECM remodeling and inflammation are also upregulated in models of hypertrophic cardiomyopathy and ISO-induced adrenergic stimulation[232,284–286]. Consistently, the offspring of obese mice were more susceptible to ISO-induced stress even in young adulthood **(Fig. 19, 20)**. Although it is not known how the heart of offspring of obese mothers would respond to diabetes or hypertrophic cardiomyopathy, my results suggest that it likely has an exacerbated negative impact on the heart. Whether the offspring are most susceptible to these forms of pathological stimuli, and whether the offspring are sensitized to alternative stressors requires further investigation. Overall, genes in pathways controlling the response of the heart to stress and pathological remodeling are mildly

dysregulated in preclinically dysfunctional hearts in Ob/Cn, revealing a state of heart disease susceptibility.

Collectively, the functions of misregulated genes and pathways identified in my study align with phenotypic presentations of the offspring. This may explain some of the underlying etiology of the disease predisposition in response to maternal obesity. Small increases in the expression of ECM components could cause stiffening of the ventricular wall leading to subclinical diastolic dysfunction. Increased activation of genes regulating TGF-β and inflammatory signaling could underlie mild hypertrophy and systolic dysfunction. Excessive upregulation of these pathways coupled with reduced expression of genes regulating glucose and lipid metabolism, and reduced metabolic remodeling in response to stress could mediate excessive pathological remodeling during stress. A limitation to the results is the use of whole ventricles which do not take into account changes in the cell composition of the heart that might arise due to obesity. Future experiments are required to delineate the cell-specific changes in gene expression and heterogeneities in the cell composition of the heart in response to obesity. Importantly, although the causative role of these pathways in CVD has been validated in previous studies, their role in CVD predisposition needs to be experimentally proven in the context of maternal obesity.

## 8.3. Long-term changes in the chromatin landscape of cardiac progenitor cells might poise genes for deregulation in the adult heart

An unexpected finding from my results is that many genes found to be misregulated in the adult heart were not misregulated during embryogenesis. Indeed, despite the overlap in pathways, there was minimal overlap in the identity of genes misregulated across all stages analyzed, and no single gene was significantly misregulated across all embryonic and adult stages. One possible explanation for this is that epigenetic alterations in the fetal CPCs might alter chromatin accessibility which does not elicit an immediate transcriptional response but might poise genes for deregulation later on. Long-term maintenance of this altered chromatin landscape might enable poised genes to be deregulated in response to the appropriate stimuli. This hypothesis is supported by the downregulation of nucleosome assembly and DNA methylation processes in CPCs **(Fig. 30)**. In addition, it is known that gene repression mediated by trimethylation of lysine 27 and global DNA methylation are reduced in the heart in offspring born to obese dams[287]. These processes stabilize gene expression and might be involved in

poising genes for dysregulation in the adult heart in our model. Nkx2-5 might also be mediating epigenetic changes through its interactions with pioneer factor Gata4 and other chromatin modifiers[80,118,288–291]. Nkx2-5 levels and function are altered in response to dietary and metabolic changes such as hyperglycemia **(section 1.5.4)**. Downregulation of *Nkx2-5* observed in CPCs can lead to failure to regulate the chromatin landscape at downstream targets of Nkx factors in differentiating CPCs. This can cause gene misregulation throughout postnatal life leading to adult-onset heart disease **(section 1.5.3)**.

Nutritional and dietary control of the chromatin landscape is a fundamental mechanism strongly interconnected with gene expression regulation. Methylation of histones and DNA is dependent on the abundance of the universal methyl donor *S*-adenosylmethionine (SAM), which is synthesized from methionine and ATP. SAM donates it methyl group and is converted to *S*-adenosylhomocysteine (SAH), which conversely inhibits methyltransferases. Conversion of SAH back to methionine requires folate and cobalamin (i.e. vitamin B12)[292–294]. Accordingly, consumption of food that is rich in methyl-donating nutrients (SAM, folic acid, and vitamin B) increases DNA methylation and mediate repression of gene expression[295].

Mitochondrial oxidative metabolism is another key determinant of the activity of chromatin-modifying enzymes. Several metabolic intermediates of the TCA cycle are cofactors for histone-modifying enzymes. Fatty acid β-oxidation and metabolism of ketone bodies generate acetyl-CoA, from which histone acetyl transferases transfer an acetyl group to histone tails[292–294]. Class I and IIa histone deacetylases are inhibited by β-hydroxybutyrate (βHB) and butyrate, which are also produced by fatty acid metabolsim[292–294]. Class III deacetylases, including sirtuin 1 (Sirt1) and Sirt2, require $NAD^+$, the oxidized form of NADH (NADH is an electron donor in the ETC), for their activity[292–294]. High $NAD^+$ levels activate sirtuins whereas nicotinamide (NAM), a precursor of $NAD^+$, mediates their inhibtion[292–294]. The Jumonji C (Jmjc) family of histone demethylases and the ten-eleven translocation (Tet) methylcytosine hydroxylases require α-ketoglutarate (α-KG), another metabolic intermediate of the TCA cycle, for their function[292–294]. High levels of glucose oxidation increase α-KG levels, leading to demethylation of DNA and several modified histone tails, including H3K27me3, H3K9me3, and H4K20me3[296,297]. Other TCA intermediates such as succinate, fumarate and βHG inhibit TET and JMJC enzymes[292–294].

Glucose is also used as a substrate for epigenetic modifications. It is used to generate *O*-GlcNAc which is derived from the hexosamine biosynthetic pathway that integrates glucose, amino acid, fatty acid, and nucleotide metabolism. *O*-GlcNAcylation is a non-canonical glycosylation that involves the attachment of single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) moieties to serine and threonine residues of cytoplasmic, nuclear and mitochondrial proteins[292–294]. Subunits of Polycomb repressive complex 1 (PRC1) and PRC2, which are required for maintenance of repression of genes in the CPCs and the adult heart[123], are regulated by *O*-GlcNAcylation that targets PRCs to specific sets of genes[298]. This highlights only a few examples of dietary and metabolic control of chromatin and demonstrates that fluctuations in glucose and fatty acid oxidation can influence DNA and histone methylation status.

Thus, changes in expression of genes regulating glucose and oxidative metabolism in fetal hearts in response to maternal obesity, as well as altered metabolic substrate availability, might alter the chromatin landscape in CPCs and their derivatives. Extensive metabolomic and chromatin landscape analysis will be required to identify the changes in chromatin architecture and determine the exact chromatin-modifying enzymes mediating such changes.

## 8.4. What causes the fetal heart to alter its transcriptome?

An important question remains regarding how the fetus can sense the maternal diet and the identity of the maternal signaling molecules that are transmitted to the fetus to mediate misregulation of gene expression. I have determined that adult cardiac response to feeding the mothers an obesogenic diet is not an "all or nothing" response, but that it is a proportional response to the absolute maternal weight at conception. 83% of the genes that are misregulated in adult (8 months) hearts of offspring of obese mice were misregulated proportionally to the maternal weight **(Fig. 24a)**. In addition, IVRT was positively correlated with maternal weight **(Fig. 24d)** indicating a direct correlation between maternal weight and the extent of diastolic dysfunction in the offspring. Consistently, the risk for adult-onset CVD in human offspring of obese women is also proportional to the maternal BMI[14–16]. Fetal hearts of human donors misregulated genes proportionally with the donor's BMI, as early as the first trimester **(Fig. 35)**, suggesting that the developing fetal heart can sense and respond to maternal BMI. This indicates that it is not maternal diet *per se* that determines gene misregulation and disease risk in offspring,

but the maternal response to the diet. The small number of genes that were affected in CPCs or human fetal hearts when considering maternal obesity as a categorical variable **(Fig. 29, 34)** illustrates the challenge of using group comparisons with assumptions of homogeneity as a tool to decipher maternal obesity-induced risk. Considering phenotype as a continuous variable would facilitate searching for predictors of disease risk programmed by maternal obesity. A key question is whether a threshold body weight or BMI beyond which genes and pathways affecting heart function are dysregulated in the offspring can be defined. Larger studies analyzing broader maternal weight and BMI distributions will be necessary to address this issue.

## 8.5. Moderate but acute hyperglycemia could alter the fetal cardiac transcriptome

Because obesity impacts various organ systems it is difficult to pinpoint the specific physiological changes that program CVD disease risk in the offspring. Increased BMI is associated with changes in several physiological changes including impaired glucose handling, dyslipidemia, hypertension, and others[299]. Systemic characterization of maternal phenotypes and their impact on the offspring will be important in narrowing down the physiological factors that program CVD. However, the identity of such factors, which require the ability to cross the placental fetal barrier to mediate such changes, remains unknown. Glucose, as well as fatty acids, can cross the placental barrier and mediate inflammation and perturbation of insulin signaling[300]. Although glucose tolerance prior to pregnancy was normal in our mice model of obesity **(Fig. 11e)**, small increases in glucose representing a pre-diabetic state during pregnancy cannot be excluded.

In my thesis, I examined the acute blood glucose levels of mice at E9.5, at the time of embryo collection. I found a significant association between maternal blood glucose levels and the mRNA levels of "obese-responsive" genes in CPCs **(Fig. 32)**. The genes that correlated with maternal blood glucose enriched for functions related to cardiac development and cardiac disease **(Fig. 32)**. Several cardiac developmental genes are known to be regulated by glucose levels in the fetal environment. Hyperglycemia impairs heart tube formation by repressing early cardiac kernel TFs including *Nkx2-5* and *Gata4*, leading to impaired migration CPCs from the cardiogenic mesoderm the heart tube, in chick embryos[301]. Hyperglycemia also represses genes regulating cardiac morphogenesis such as *Pitx2* and *Nodal* at E8.5-9, leading to impaired left-

right axis formation at in a mouse model of diabebtes[302]. *Nkx2-5* is downregulated at E13.5-18.5 in response to gestational diabetes and its downstream targets, such *BMP10* are increased[303]. Although my mouse model maintained normal glucose homeostasis during glucose tolerance tests which are conducted in a fasted state **(Fig. 11e)**, the unfasted blood glucose levels of the mothers at E9.5 was significantly higher than the control fed mothers **(Fig. 32d)** suggesting moderate and acute increases in un-fasted blood glucose levels.

It has recently been shown that moderate or acute increases in blood glucose levels are sufficient to increase cardiomyocyte hypertrophy in fetal hearts of rats[200]. In addition, blood glucose levels below those used for diagnosis of gestational diabetes can impact fetal growth[209]. My results suggest that acute increases in blood glucose levels might be the underlying cause of the effects of maternal obesity on the offspring. However, an important limitation is the lack of comprehensive analysis of maternal glucose and insulin serum levels as well as their tolerance tests during pregnancy. Complete metabolic profiling of pregnant women is required to determine the glucose homeostasis as well as other physiological parameters that might be associated with offspring risk of CVD. Linking phenotypes to specific molecular signatures in obese mothers and their offspring would facilitate predicting such responses and identifying individuals at a higher risk of cardiovascular disease.

## *9. Future Directions:*

### 9.1. Analyze the genome-wide distribution of Nkx2-5 in the hearts of offspring of obese mice and determine its requirement for programming adult-onset disease

While Nkx2-5 and its downstream targets are misregulated in response to maternal obesity, experimental analysis of Nkx2-5 binding to the genome and its role in disease remains to be addressed. Direct analysis of Nkx2-5 binding to the genome in CPCs and adult hearts of offspring of obese and lean mothers using chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) will elucidate how maternal obesity alters Nkx2-5 target specificity in the heart of the offspring. Validated antibodies for Nkx2-5 have previously been used in adult hearts[122] as well ES-derived CPCs[84]. For E9.5 embryos, I can isolate 5,000 CPCs from each embryo. Due to the limited cell number, whole litters were pooled together to obtain higher cell yields. I have already collected over 100,000 CPCs from embryos of obese and lean mice. Protocols used in our lab require a minimum of 100,000 cells for ChIP-seq, and as such, short-term completion of this experiment is feasible. I have already collected hearts, at 8 months of age, from offspring of obese mice, and collection of hearts from offspring of lean mice is planned in the coming months. Cross-referencing RNA-seq and ChIP-seq datasets will elucidate if, and how, the "obese-responsive" genes in the hearts of offspring are controlled by Nkx2-5.

The requirement for Nkx2-5 to mediate cardiac susceptibility to disease in the offspring also remains to be addressed. To test if reduced Nkx2-5 function in CPCs is sufficient to program disease, the cardiac function of heterozygous *Nkx2-5* knockout mice should be examined. Heterozygotes for *Nkx2-5* are viable and have a normal cardiac function in young adulthood[304]. The work presented in this thesis suggests that reduced function of Nkx2-5 may program cardiac disease susceptibility. Challenging the Nkx2-5 heterozygotes with pathological stimuli, such as ISO, and analyzing their response will help determine if these hearts have an increased susceptibility to cardiac disease. I expect these mice to develop an exacerbated restrictive hypertrophy and cardiac dysfunction in response to ISO, similar to that seen in our model of maternal obesity **(Fig. 19, 20)**. This analysis could establish if dysregulation of Nkx2-5 is sufficient for disease programming

## 9.2. Assess the functional consequences of gene misregulation, and perform rescue experiments to determine causality

Genes regulating the expression in the TGFβ signaling pathway were activated in CPCs and adult hearts of offspring of obese mice. TGFβ overactivation is sufficient to promote hypertrophy and fibrosis in the heart[129,131,272]. I propose that activation of genes controlling the TGF-β signaling pathway lead to increased susceptibility to cardiac disease. Preventing long-term overactivation of TGFB in embryos and/or postnatally in offspring of obese dams could therefore prevent adult-onset cardiac hypertrophy and dysfunction.

To test this hypothesis, activation of TGF-β signaling first needs to be validated by determining the localization and phosphorylation levels of SMAD proteins – the effectors of the TGFβ pathway. Inhibition of TGF-β will be required to determine if reduced TGF-β signaling is sufficient to rescue the subclinical dysfunction as well as exacerbated pathological remodeling in response to stress. I have attempted to perform this rescue experiment by pharmacological inhibition of TGFβ using Losartan potassium – previously shown to inhibit TGF-β in a mouse model of Marfan syndrome[305]. Beginning at 6 days of gestation, pregnant lean or obese mice received Losartan (0.6g/liter) in their drinking water and treatment continued throughout gestation. The aim was to examine cardiac hypertrophy and function in 4 and 8-month-old offspring. This dosage of losartan, based on a previous study, was effective in reducing p-SMAD2 levels in the embryo **(Fig. 39)**, indicating effective inhibition of TGFβ, however, all embryos died during gestation and subsequently, all pregnant females also died. The dose was titrated to 0.3g/liter. This dosage did not affect the viability of the adult mice and did not result in gross anomalies – mice did not appear lethargic and their weight gain throughout the losartan treatment was not altered. However, this dosage also resulted in the death of all embryos. Further optimization of this treatment regimen is required to assess the viability of using TGFβ inhibitors to reduce disease risk in the offspring of obese mothers.

Another important finding is the altered expression in genes controlling metabolism throughout life, in response to maternal obesity. Future experiments focusing on profiling the metabolome of the hearts as well as measuring the functional effects of such changes on cardiomyocyte metabolism is required. Functional characterization of the cardiomyocytes can be conducted using XFe96 Extracellular Flux Analyzer from Seahorse Bioscience, which can

measure oxidative metabolism and glycolysis in real-time in isolated cardiomyocytes from fetal and adult hearts[306,307]. I have successfully isolated fetal **(Fig. 17)** and adult cardiomyocytes (data not shown) which enables the use of this system. Future experiments should use this platform to measure the functional outcome of metabolic gene dysregulation on fetal and adult cardiomyocytes. Non-targeted metabolomic profiling would also be required to elucidate if this altered expression of genes regulating various metabolic pathways can lead to changes in cardiac metabolites. Overall, complete metabolic profiling of the hearts of the offspring of obese mice is required to determine the effects of the deregulated metabolic gene expression.

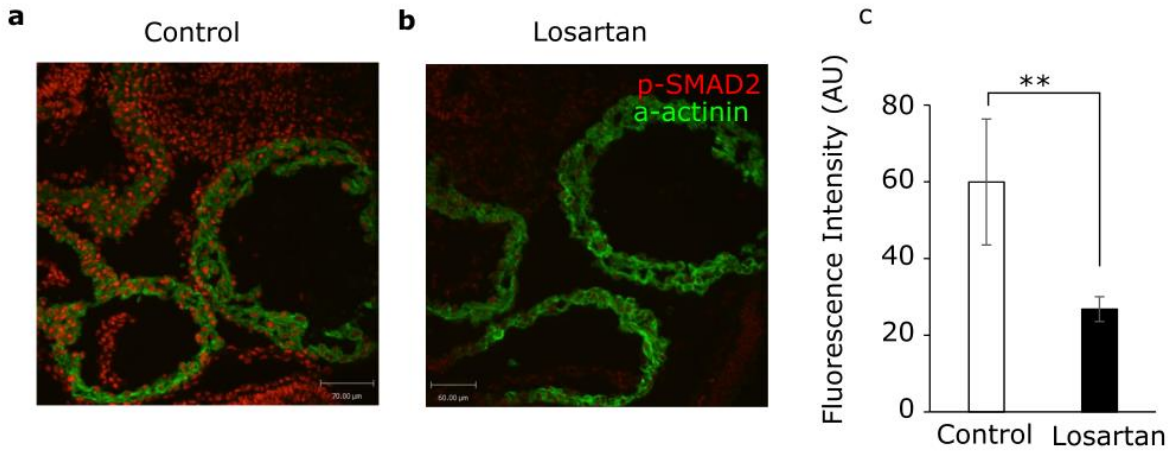



**Figure 39. Losartan inhibits TGFβ signaling.** Immunofluorescence staining for phosphorylated Smad2 (p-Smad2) in red, and a-actinin (cardiomyocyte marker) in green, on E9.5 embryos of mice treated or untreated with losartan in their during water (0.6g/liter). Quantification of p-SMAD2 fluorescence intensity confirmed reduced phosphorylation in response to losartan. Bars represent the mean ± SD. ** indicates p-val <0.01.

## 9.3. Determine if acute increases in maternal glucose levels can recapitulate the transcriptional profile of cardiac progenitor cells of embryos from obese mice

A key question remains to be addressed regarding the identity of the maternal factor or signaling molecule that can communicate the magnitude of maternal BMI to the fetus and induce the transcriptional misregulation reported here. My results suggest glucose as a potential candidate. I propose that acute but moderate increases in glucose levels could mediate fetal

programming of disease. It will be important to prove that maternal glucose levels can cross the placental barrier to reach the embryo and that such increases can mediate transcriptional changes reported in this thesis. Little is known about the nutrient exchange in the early mouse embryo, however, it is known that primitive fetal placental circulation begins at E8.5 (via the umbilical veins) and the embryo also acquires nutrients from the yolk (via the vitelline veins)[308,309]. To determine if maternal glucose can reach the embryo, I measured the glucose levels in the yolk of E9.5 embryos 15 minutes after injecting pregnant female mice (in the peritoneum) with glucose and compared to non-injected mice. The glucose levels increased in the yolk of all embryos in injected mice **(Fig. 40)**. This indicates that maternal glucose levels can alter glucose levels in the embryo. Due to the limited amount of blood in the embryo, I could not directly measure the blood glucose levels of the E9.5 embryos. Future experiments should directly investigate whether increased glucose levels during pregnancy – by direct glucose injection – could alter the levels of Nkx2-5 and its downstream targets, similar to that seen in my model.

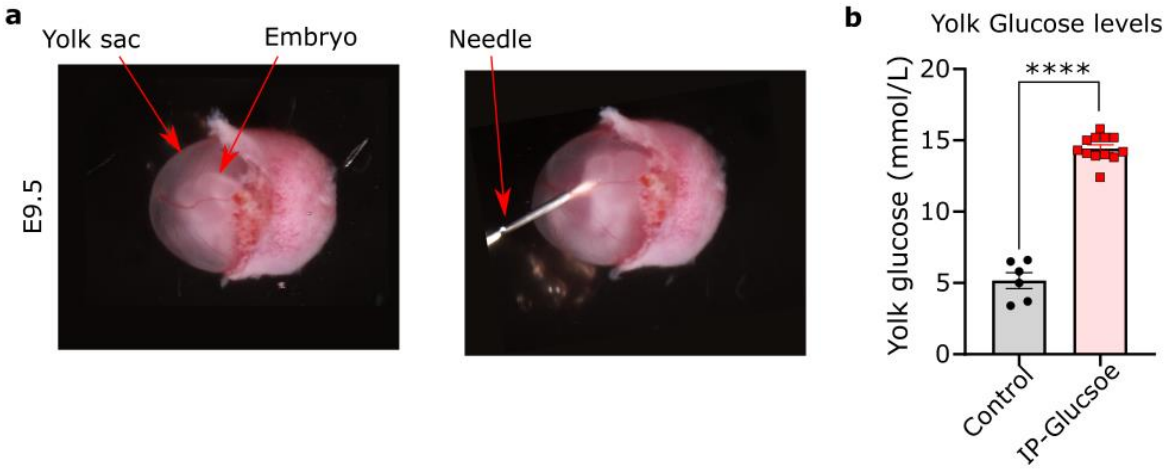



**Figure 40. Uptake of glucose from maternal blood by the yolk at E9.5. a)** Embryos were dissected from the uterus at E9.5. The uterine wall was removed to expose the yolk sac and the embryo within it. A needle was used to puncture the yolk sac and withdraw the yolk. **b)** Glucose levels of the yolk 20 minutes after IP-injection of the mother with glucose, compared to non-injected mothers. Bars represent the mean ± SEM. **** represents $P<0.0001$

## 9.4. Determine the effects of maternal obesity on embryonic cell lineages

Maternal obesity affects several organ systems in the offspring. In my thesis, I found that maternal obesity impacted both the cardiac and metabolic function in the offspring. Previous studies have demonstrated that several other organ systems are affected in the adult offspring, including the pancreas, bone, brain, liver, and lung[310–312]. My study highlights the embryonic origins of adult-onset cardiac disease in response to maternal obesity. However, it is unclear if other organ systems are also impacted *in utero* by maternal obesity. In addition, the use of whole hearts and bulk RNA sequencing was an important limitation for the identification of cell type-specific effects. My lab is currently investigating the embryonic origins of disease risk programming in response to maternal obesity by conducting single-cell RNA sequencing (scRNA-seq) on embryos of obese mice. This will help elucidate some of the earliest effects of maternal obesity on the primordium of various organ systems and embryonic cell lineages.

### *10. Limitations and alternative interpretations*

Obesity is a clinically-centric term which has specific connotations in disease. In humans, it is a chronic health condition with multiorgan involvement, including the pancreas, liver, kidneys, brain, heart, reproductive organs, muscles, and joints[313]. There is no mouse model of obesity that completely recapitulates the full spectrum of disease outcomes or their time span[313]. The mouse model of maternal obesity used in this thesis represents a more acute form of obesity and is defined by the increase in weight and fat mass relative to controls. Although these criteria *i.e*. increased weight and fat mass, are the standard approaches for assessing obesity in mouse, it is important to note their limitation. Increased weight and fat mass do not always infer a disease propensity[314,315] and there is no set cutoff beyond which increasing weight is known to be detrimental. Secondary effects of obesity on other organs, and its systemic effects, such as inflammation, which are potential contributors to cardiac disease programming, were not characterized in this thesis. This limitation in offering a complete model of maternal obesity is common in the field of obesity research. Mice from different genetic backgrounds[316], fed different control or obesogenic diets[313,317], and even maintained in different animal housing facilties[318] have different weights, microbiota and exhibit high variations in their response to obesogenic diets due to both genetic and environmental factors. Thus, weight gain compared to controls is commonly used to define obesity in mouse models despite its limitation as an indicator of a disease state. The interpretations of my results pertain to the definition of obesity as an increase in weight and fat mass, relative to controls. This offers a limited insight to the full spectrum of potential consequences that maternal obesity may have on the cardiac transcriptome and function in the offspring.

In addition, the response of individual mice to the same obesogenic diet is varied. Although many mice gain weight and fat mass when fed obesogenic diets, others maintain a similar body weight trajectory to Cn-fed mice, the latter are referred to as diet-resistant (DR)[319,320]. In my thesis, I noted that several females fed an Ob-diet had a similar body fat and lean mass compared to controls, indicating they are likely diet-resistant **(Fig. 11c)**. Such mice were not excluded and thus could induce a source of variation/heterogeneity within the obese group. This is evidenced by the reduced changes in gene expression observed in embryonic and adult hearts of offspring from females with mild weight gain **(Fig 24, 29e)**. Excluding DR mice may result in a more

homogenous experimental group and uncover more changes in gene expression and phenotypes. However, analysis of the full cohort of Ob-fed mice revealed that it is not the diet consumption *per se* which programs cardiac disease in the offspring, but that maternal weight gain and disruption in glucose homeostasis are more closely associated with such risk. Nonetheless, it is possible that including DR mice in our analysis may have masked some of the effects of obesity on the offspring.

Lastly, in this thesis, I could not separate the cell type-specific effects of maternal obesity from global changes within the entire heart. Recent evidence using scRNA-seq technology has demonstrated heterogeneity in transcriptional responses between cell types and within subpopulations of the same cell type during heart disease[321]. The use of bulk RNA-seq poses a limitation in identifying heterogeneity in gene expression. It is likely that maternal obesity, like all other cardiovascular stressors that have been investigated to date at the single-cell level[321], also exerts cell type-specific effects. The small magnitude of transcriptional changes identified in our study in the adult heart **(Fig. 21)** might not be representative of the magnitude of changes in all cardiac cell types. This is supported by the local hypertrophic response in the apex of the heart, which indicates spatial heterogeneity in cardiac phenotypes. Spatially-restricted phenotypic outcomes in the heart may originate from early heterogeneity in gene expression within clonal cardiac progenitor that are poised for transcriptional deregulation. Indeed, the transcriptional profile of cardiac cells in the developing mouse embryo reflects their anatomical patterning throughout development[322]. However, whether maternal obesity disrupts the transcriptome of spatially restricted populations and the clonal origins of transcriptional deregulation in the adult hearts could not be assessed in this thesis.

Despite these limitations, the work presented in this thesis represents the first evidence of transcriptional deregulation in response to maternal obesity during early heart development both in mouse and human hearts. In addition, my results indicate that obesity persistently alters the transcriptome in adulthood and increases disease susceptibility.

## *11. Conclusion:*

David Barker argued in one of his last speeches (given at MRC centenary celebrations, Southampton, 2013): “The next generation does not have to suffer from heart disease or osteoporosis. These are not mandated by the human genome. They barely existed a hundred years ago. They are unnecessary diseases. We could prevent them had we the will to do so.” Although Dr. Barker was referring to the effects of maternal undernutrition on the offspring, maternal obesity is a growing health concern that is predicted to increase the risk of cardiac diseases in future generations. Through my work, I have made significant progress towards elucidating how maternal obesity during pregnancy increases the risk of cardiac disease in the offspring. Using a mouse obesity model, in the absence of diabetes, I have found that maternal obesity alters the transcriptome in the heart throughout life – in CPCs and adult hearts – leading to subclinical dysfunction under baseline conditions and excessive pathological remodeling in response to stress. Nkx2-5 and its downstream targets are misregulated in CPCs of embryos of obese mice. *Nkx2-5* targets are also misregulated in human fetal hearts of obese donors, and persists to adulthood in the mouse. Furthermore, I have identified the developmental period when cardiac disease risk is programmed. Exposure to obesity during fetal life is sufficient to program subclinical diastolic dysfunction and long-term transcriptional misregulation in adulthood, however, exposure during lactation is necessary for the development of cardiac arrhythmia and systolic dysfunction. My findings have bridged a gap in knowledge regarding the transcriptional responses to maternal obesity, its impact on the heart, and the developmental time frame when cardiac disease risk is programmed. The next generation does not have to suffer from heart disease. We could prevent it had we the will or tools to do so. Future research should focus on modulating the expression and the activity of genes and pathways identified in this thesis to help reduce the risk of cardiac disease.

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