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Transgenerational cardiology: One way to a baby's heart is through the mother

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Abstract

Despite decades of progress, congenital heart disease remains a major cause of mortality and suffering in children and young adults. Prevention would be ideal, but formidable biological and technical hurdles face any intervention that seeks to target the main causes, genetic mutations in the embryo. Other factors, however, significantly modify the total risk in individuals who carry mutations. Investigation of these factors could lead to an alternative approach to prevention. To define the risk modifiers, our group has taken an “experimental epidemiologic” approach via inbred mouse strain crosses. The original intent was to map genes that modify an individual’s risk of heart defects caused by an *Nkx2-5* mutation. During the analysis of >2000 *Nkx2-5*^{+/-} offspring from one cross we serendipitously discovered a maternal-age associated risk, which also exists in humans. Reciprocal ovarian transplants between young and old mothers indicate that the incidence of heart defects correlates with the age of the mother and not the oocyte, which implicates a maternal pathway as the basis of the risk. The quantitative risk varies between strain backgrounds, so maternal genetic polymorphisms determine the activity of a factor or factors in the pathway. Most strikingly, voluntary exercise by the mother mitigates the risk. Therefore, congenital heart disease can in principle be prevented by targeting a maternal pathway even if the embryo carries a causative mutation. Further mechanistic insight is necessary to develop an intervention that could be implemented on a broad scale, but the physiology of maternal-fetal interactions, aging, and exercise are notoriously complex and undefined. This suggests that an unbiased genetic approach would most efficiently lead to the relevant pathway. A genetic foundation would lay the groundwork for human studies and clinical trials.

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1. INTRODUCTION

Congenital heart disease afflicts ~1% of all live births, half of which involve a moderate or severe malformation (Hoffman and Kaplan, 2002). In absolute terms, ~20,000 of the 4 million children born each year in the United States will require medical attention or surgery for their heart defect. Although technical innovations in the latter decades of the 20th century improved survival dramatically, morbidity and mortality remain unacceptably high. For example, hypoplastic left heart syndrome, in which the right ventricle must function as the sole pumping chamber, was lethal before a palliative operation was developed in the 1980's (Norwood et al., 1983). By 2010, transplantation-free survival to one year of age had reached 70% (Ohye et al., 2010). The clinical progress has led to two trends in the current era. First, the rate of improvement in surgical outcomes is decelerating (Erikssen et al., 2015). Technical solutions may be approaching the physiological limits of how well a palliated heart can function. Second, the burden of congenital heart disease is shifting into adulthood. More than 1 million adults in the United States have congenital heart disease, which recently surpassed the number of children (Gilboa et al., 2016). Based on current trends, adults will comprise the majority of hospitalized, congenital heart disease patients in a few years (O'Leary et al., 2013). Prevention could have the greatest impact on these trends. On what basis then can a strategy be developed?

Prevention typically addresses the root causes of a disease. The major causes of congenital heart disease are inherited and *de novo* genetic mutations in the embryo. The first genetic mutations, affecting *NKX2-5* and *TBX5*, were discovered in the 1990's (Basson et al., 1997; Li et al., 1997; Schott et al., 1998). By 2014, mutations of several dozen genes were known (Andersen et al., 2014). Just two years later, the authors of a whole-exome sequencing study of 1213 parent-offspring trios estimated that *de novo* mutations of 392 genes contribute to the pathogenesis of congenital heart disease (Homsy et al., 2015). Etiologic heterogeneity limits the impact that a prevention strategy focused on one gene could have. Chromosomal structural variation or copy number variants (CNV) contribute to ~10% of cases (Lander and Ware, 2014). Deleterious CNV duplications and deletions of the same genomic interval highlight the critical role of gene dosage for normal cardiac development (Thorsson et al., 2015). The narrow range over which gene dosage can safely vary suggests that a method that modulates the activity of a gene would have to be precise.

The functions of the genes pose a different set of challenges. Causative mutations typically involve transcription factors, epigenetic regulators and cellular signaling. They disrupt developmental pathways in the heart and often other organs (Andersen et al., 2014). Mutations of the same gene can thus cause either an isolated heart defect or a broader syndrome. For example, heterozygous loss-of-function mutations of *NOTCH1* cause isolated heart defects (Garg et al., 2005; Kerstjens-Frederikse et al., 2016) or Adams-Oliver syndrome in rare individuals. Features of the syndrome include scalp cutis aplasia and transverse terminal limb defects in addition to the heart defect (Stittrich et al., 2014). Why two individuals who carry the same mutation can have either an isolated heart defect or multiple anomalies is not understood. The unpredictability of phenotype suggests that the manipulation of a developmental pathway in the hope of a normal heart would be fraught with risk elsewhere in the body.

The benefits might outweigh the risks of an intervention for some individuals, but it is hard to predict who is at risk. Despite the clear genetic basis, most congenital heart defects occur sporadically; family history is not a sufficiently sensitive or specific predictor of risk (Oyen et al., 2009). Furthermore, a defect forms early during embryonic development. Cardiac morphogenesis is complete by the tenth week of gestation when the heart is about 5 mm in diameter (Dhanantwari et al., 2009). Fetal echocardiography can reveal a defect at 17–18 weeks gestation when the heart is 13–15 mm. In early embryonic abnormalities that progress to severe defects are microscopic. The identification of embryos that could benefit from an early intervention is a giant hurdle.

When viewed solely from the perspective of how and when cardiac development goes awry, the prevention of congenital heart disease on a large scale appears unattainable. A narrow focus on the pathogenic mechanisms only seems to magnify the challenges. On the other hand, individuals who carry deleterious genetic mutations or chromosomal abnormalities commonly have normal hearts. For example, Down and DiGeorge syndromes are strongly associated with congenital heart disease, but one half and one quarter of affected individuals, respectively, have a normal heart (Freeman et al., 2008; Ryan et al., 1997). A rational approach to prevention may be suggested by investigating factors that modify risk in the presence of a genetic susceptibility.

2. THE MULTIFACTORIAL BASIS OF A CONGENITAL HEART DEFECT

Decades before the advent of molecular genetics, Nora sought to explain patterns of congenital heart disease in families and twins who appeared to have a heritable, but not simple Mendelian basis. He reasoned that multiple factors beside the cause modify the quantitative risk of a heart defect in an individual. The multifactorial hypothesis, he argued, provided a useful conceptual framework to investigate and hopefully prevent congenital heart disease (Nora, 1968). Nevertheless, scientists in the following decades mainly focused upon the proximate, monogenic causes. Their tools — from linkage analysis to whole-exome sequencing — are better suited to discovering mutations that have large effect. As the nature of the mutated genes became clear (and their potential for targeting in a prevention strategy arguably less so), Nora's hypothesis has attracted renewed attention. To define the modifying factors, investigators have compared groups of affected and normal individuals, both of whom share the same genetic cause for congenital heart disease.

The human studies to date have mainly focused upon polymorphisms that affect risk in Down (Ackerman et al., 2012; Li et al., 2012; Ramachandran et al., 2015; Ramachandran et al., 2015) or DiGeorge (Goldmuntz et al., 2009; Guo et al., 2015; Mlynarski et al., 2015) syndrome. The studies typically include several hundred cases and controls. The sample sizes are large for a study of congenital heart disease but small for human genetic association analysis. Genetic polymorphisms in the VEGF pathway and a couple candidate genes have been implicated as Down syndrome modifiers (Ackerman et al., 2012; Li et al., 2012; Robinson et al., 2003). Common genetic variants do not have a detectable effect, but large, rare CNV deletions may increase risk in Down syndrome (Ramachandran et al., 2015; Ramachandran et al., 2015). In DiGeorge syndrome, rare, deleterious variants of histone-modifier genes and CNVs that duplicate SLC2A3 may increase risk (Guo et al., 2015;

Mlynarski et al., 2015). The genetic modifiers found so far do not suggest a means to reduce risk. There is conflicting evidence for an effect of maternal genetic polymorphisms in the folic acid pathway and DNA methylation in Down syndrome, but whether folic acid supplementation would reduce risk is unclear (Coppede, 2015). Folic acid supplementation probably does not reduce the risk of congenital heart disease in the general population (Leirgul et al., 2015).

Numerous issues in human studies, such as small sample size, genetic heterogeneity, and unknown environmental factors, limit the ability to detect rare modifiers of large effect or common modifiers of small effect, let alone establish consistent results between studies. Our group has used inbred mouse strain crosses to circumvent these issues, taking advantage of the well-described variability of mutant cardiac malformation phenotypes between genetic backgrounds (Bruneau et al., 2001; Sakata et al., 2006; Rajagopal et al., 2007). We chose *Nkx2-5*^{+/-} mice as the model. Mutations of *NKX2-5* were first discovered in humans to cause congenital heart defects of diverse types (Benson et al., 1999; McElhinney et al., 2003; Schott et al., 1998). *Nkx2-5*^{+/-} mice develop similar defects (Biben et al., 2000; Tanaka et al., 2002; Winston et al., 2010). Naturally occurring genetic polymorphisms in mice account for their strain-dependent phenotypes. Within an inbred strain all mice are genetically identical. In a cross between two strains, only one or two alleles of a gene exist. Systematic crosses can ensure that the frequency of an allele in the population is exactly one-half or one, so rare alleles are not confounding. In addition, the environment in the mouse colony is held constant. Variation in diet or lifestyle, which frequently clouds the interpretation of human studies, does not exist.

We expected that the enumeration of modifiers would require the analysis of thousands of mice. For a project this large, we had to solve logistical challenges related to physiology and anatomy. First, congenital heart defects are normally compatible with survival *in utero*, but certain defects cause death in the newborn period. In the womb the placenta exchanges oxygen and carbon dioxide between the maternal and fetal circulations. After birth, the lungs perform all gas exchange. In coordination with the change in respiratory physiology, the ductus arteriosus, which permits blood to flow from the pulmonary artery to the aorta, closes by 12 hours after birth. Pulmonary vascular resistance falls to a nadir a day later in the mouse (Nguyen et al., 1997). “Duct-dependent” cardiac lesions require a patent ductus to maintain circulation to either the lungs or body; ductus closure causes affected newborn mice to die within hours of birth. Large “left-to-right” shunt lesions, mainly ventricular septal defects, cause death from pulmonary congestion as pulmonary vascular resistance falls relative to systemic. Mouse mothers normally give birth between midnight and 4 a.m.; litters are occasionally born in the daytime. We inspect all breeding cages for pregnant dams and collect all newborn pups every morning of every day of the year. The daily examination of dams permits precise estimates of delivery dates. We will check a dam later in the day if she fails to deliver on the expected night. Following this routine, newborn *Nkx2-5*^{+/-} pups have been obtained at the expected Mendelian ratio (Winston et al., 2010). Practically no pups are lost to a congenital heart defect; even single-ventricle lesions have been found (Fig. 1).

Phenotyping on a large scale was the second, major challenge. To discover a defect that can be located anywhere in the heart, large studies have taken one of two approaches. One group has performed ultrasound imaging of live fetuses for an ENU mutagenesis screen (Shen et al., 2005). The method uses a combination of two-dimensional imaging of cardiac structures and Doppler imaging of blood flow. Fetal echocardiography is well-suited for the detection of large defects that cause abnormal patterns of blood flow and hence for screening severe mutations (Li et al., 2015; Yu et al., 2004). Common, septal defects are more difficult to detect because of the limited spatial resolution of imaging and the nature of fetal circulatory physiology. Of practical significance, efficient phenotyping in real time requires a highly skilled fetal cardiac ultrasonographer.

The second approach, which we have taken, is the examination of serially sectioned, fixed hearts. Sections can be obtained digitally by magnetic resonance imaging (Pieles et al., 2007) or computed tomography (Degenhardt et al., 2010). We chose instead conventional histology for its simplicity and low cost of data acquisition and storage (i.e., paraffin blocks, hematoxylin-and-eosin staining, and glass slides in boxes). Hearts are completely sectioned at 6 μ m thickness for subsequent evaluation by the team. A student can be taught normal cardiac anatomy quickly. Within one month students can specifically name simple and complex defects. Phenotyping by two or more trained individuals ensures consistent, accurate diagnoses. The team has found examples of common defects, such as secundum atrial septal defects and muscular and membranous ventricular septal defects. They have also found rare, complex defects, including atrioventricular septal defects, tetralogy of Fallot, double-outlet right ventricle, and coronary artery fistulas (Jay et al., 2007; Ng et al., 2009; Winston et al., 2010; Zhang et al., 2007).

To characterize genetic modifiers of the *Nkx2-5*^{+/-} phenotype, we crossed C57BL/6N, *Nkx2-5*^{+/-} males to either FVB/N or A/J wild-type females to produce F1 hybrids. *Nkx2-5*^{+/-} F1 mice were then backcrossed to the parental strains or intercrossed (Fig. 2). Analyses of the various crosses indicate the existence of polymorphisms that either increase or decrease the risk of specific defects; some may modify the risk of more than one defect (Winston et al., 2010). Modifier loci mapped in the C57BL/6N $\times$ FVB/N F2 intercross confirm the presence of alleles and their specificity for particular phenotypes (Winston et al., 2012). Ongoing analyses of F2 and advanced intercrosses aim to fine map loci and to define the complex genetic architecture of congenital heart disease.

Breeding pairs were allowed to mate continuously for the duration of their reproductive lifespan, while their newborn pups were collected for phenotyping. The experimental design led to an unexpected observation in one of our first crosses. Julia Winston noticed a drop in the incidence of heart defects after older mothers were replaced with young females. Although the environment is held constant for the parents, she realized that three environmental factors, maternal age, paternal age and litter size, vary between embryos. Logistic regression analysis on the entire C57BL/6 $\times$ FVB/N F2 intercross confirmed the impression in the *Nkx2-5*^{+/-} offspring. Maternal age had no effect on incidence in the wildtype, which rarely have heart defects. Maternal age thus acts as a modifier rather than a cause of heart defects. Paternal age and litter size did not have a significant or consistent effect. Chromosomal aneuploidy was excluded as the basis of the maternal-age associated

risk. Because the risk accumulates as the mother ages, it can become large relative to the fixed effect of genetic modifiers in the embryo (Winston et al., 2012).

3. THE MATERNAL-AGE ASSOCIATED RISK OF CONGENITAL HEART DISEASE

Epidemiologic studies consistently find a maternal-age associated risk of congenital heart disease even after excluding cases of chromosomal abnormality (Table) (Forrester and Merz, 2004; Hollier et al., 2000; Kidd et al., 1993; Materna-Kiryluk et al., 2009; Miller et al., 2011; Pradat et al., 2003; Reefhuis and Honein, 2004). A similar risk in the *Nkx2-5^{+/-}* mouse model offered the opportunity to investigate the mechanism (Fig. 3). As all of a mother's eggs were produced during her embryonic development, the basis of the maternal-age effect could reside either in the oocyte or mother's soma. To determine which, we performed reciprocal ovarian transplants between young and old mothers. To our surprise, the incidence of heart defects in the resulting *Nkx2-5^{+/-}* offspring matched that expected for a maternal basis. The offspring of young mothers who ovulated from old ovaries had a low incidence, whereas the offspring of old mothers and young ovaries had a high incidence (Schulkey et al., 2015). An oocyte-based mechanism may contribute to the maternal-age effect in humans, but there is no evidence for one in our mouse model.

A maternal factor or pathway related to aging must interact with embryonic cardiac development, leading us to consider other maternal risk factors in humans as the potential basis. Folate or B12 deficiency in older mouse mothers is unlikely. Mouse chow contains a high concentration of folic acid, and there was no difference between young and old mothers in serum homocysteine or B12 levels or red blood cell indices (unpublished data). Maternal hyperglycemia (Priest et al., 2015), pre-pregnancy diabetes (Oyen et al., 2016) and obesity (Cedergren and Kallen, 2003; Gilboa et al., 2010; Madsen et al., 2013) are difficult to disentangle from aging in epidemiologic studies, but easily induced by a high-fat diet in the mouse. The diet had no effect on the incidence of heart defects among the offspring of young mothers, who were markedly hyperglycemic and glucose intolerant. Older mothers developed severe obesity, yet the maternal-age associated risk remained the same (Schulkey et al., 2015). The negative results strongly suggest that the investigation of specific mechanisms one at a time would be risky and inefficient.

Although most epidemiologic studies demonstrate a maternal-age associated risk, some do not (Table) (Baird et al., 1991; Loane et al., 2009; Pradat et al., 2003). Unknown differences between populations could obscure the association. In this regard, genetic polymorphisms between inbred strains plainly determine the quantitative maternal-age associated risk. The risk was first discovered in the C57BL/6N × FVB/N intercross, but undetectable in the C57BL/6N × A/J intercross, and highest in the C57BL/6N strain. The quantitative variation between crosses offers two important insights. First, the alleles of a gene or genes in a maternal pathway must determine the activity of a factor that interacts with cardiac development in the *Nkx2-5^{+/-}* offspring. The zero or low risk in the C57BL/6N × A/J intercross is genetic evidence that modulation of a pathway in the mother can prevent congenital heart disease caused by a mutation in the offspring. Second, the existence of

functional variation permits an unbiased genetic approach to discover the relevant genes and pathways, as discussed below. One need not test specific hypotheses in experiments that take more than a year at the risk of an uninformative, negative result.

Congenital heart disease in the newborn could be interpreted as a sign of aging in the mother. This counterintuitive notion opens up the possibility of targeting age-related pathways in the mother to prevent a birth defect in her offspring. Exercise in particular has well-documented benefits for diverse age-related phenotypes and diseases affecting the cardiovascular system, brain, metabolism and lifespan (Rowe et al., 2014). This raised the intriguing question whether maternal exercise could reduce the risk of congenital heart disease. Running wheels were placed in breeding cages when mothers were either 4-weeks or 8-months old. The mice ran *ad libitum*, but the amount was not quantified. Mice in the wild enjoy running and those in cages with a running wheel will voluntarily run 3–16 kilometers per day (Meijer and Robbers, 2014; Allen et al., 2001).

Exercise, whether begun early or later in life, mitigated the fraction of risk associated with maternal aging. Among the offspring of sedentary, old mothers, defined as >300-days old, the incidence of ventricular septal defects was 20%. With exercise the incidence fell to ~10%, which is equivalent to that observed in the offspring of young, <100-day old mothers. Exercise did not affect the incidence among the offspring of young mothers. Exercise must either decrease the activity of a harmful maternal factor or increase that of a protective one. The benefit of exercise begun relatively late in adulthood indicates that the age-related change in the activity of the unknown factor is not fixed. This finding has practical implications because young women who have not yet considered having children are unlikely to commit to a preventive intervention.

The benefit of exercise is not immediate, however. A detectable effect requires about three months of maternal exercise before birth. To our knowledge, there are no data in humans or animal models regarding the relationship between maternal exercise or fitness and the risk of congenital heart disease. Several groups have examined the effect of voluntary wheel running on mouse offspring metabolic phenotypes, such as insulin sensitivity. Their studies typically demonstrate a positive effect with just one to six weeks of exercise prior to conception and through birth in mouse and rat models (Carter et al., 2013; Carter et al., 2012; Fidalgo et al., 2013; Stanford et al., 2015; Laker et al., 2014; Raipuria et al., 2015). The discrepancy between the duration of exercise required to modify a developmental or metabolic trait is curious. That said, there is growing interest in maternal exercise as a preventive intervention for cardiovascular and metabolic disease in the offspring (Blaize et al., 2015). Insight garnered from studying the effect of maternal exercise on one trait should have bearing upon the others.

4. AN UNBIASED GENETIC APPROACH TO THE MATERNAL EFFECTS ON OFFSPRING PHENOTYPE

Many plausible hypotheses can explain how maternal age affects the risk of congenital heart disease in offspring who carry a causative mutation. They can be divided into one of two categories. The first hypothesizes the existence of a molecule(s) from the mother that crosses

the placenta and affects cardiac development. Glucose and phenylalanine are prototypical examples in which supraphysiologic maternal levels can cause cardiac malformation (Hachisuga et al., 2015; Levy et al., 2001). Interestingly, the mothers of babies who have tetralogy of Fallot have a mildly elevated level of glucose at midgestation (Priest et al., 2015). A subclinical, quantitative alteration in the concentration of a maternal molecule may not cause a heart defect but could increase risk in offspring who have a genetic predisposition. Glucose alone does not mediate the maternal-age associated risk in the *Nkx2-5^{+/-}* mouse, but other metabolites may alone or in combination with glucose. The concentrations of a growing number of metabolites have been reported to vary with age, exercise and fitness (Tomas-Loba et al., 2013; Chaleckis et al., 2016; Cheng et al., 2015; Lewis et al., 2010; Lustgarten et al., 2013; Muhsen Ali et al., 2016). Age and exercise should have opposing effects on the concentration of a metabolite involved in the maternal-age associated risk. The effect of exercise suggests skeletal muscle, adipose tissue or liver as potential sources of the molecule. The second general category of hypotheses encompasses any that do not invoke maternal molecules acting on the embryo. For example, aging could somehow disturb placental function, which would in turn increase the risk of cardiac malformation. Abnormalities of placental size and architecture have been reported in association with complex congenital heart defects, although the causal relationships are unknown (Jones et al., 2015; Andescavage et al., 2015).

Whatever the mechanism is, the fact that maternal genetic polymorphisms affect the gestational milieu for cardiac development offers a foothold into the unknown pathway. Besides genes involved in diabetes and phenylketonuria, only a few maternal genes are suspected to affect cardiac development. They have been suggested mainly by association studies in humans and have in general not been replicated or experimentally validated (Li et al., 2014; Chowdhury et al., 2012; Mitchell et al., 2015). It is unknown whether any of them could mediate the maternal-age associated risk of congenital heart disease. Clearly, maternal-fetal interactions, aging and exercise involve myriad pathways. At least one that is common to all three processes mediates the maternal-age associated risk, but the investigation of any one of them would probably be fruitless, as the high-fat diet experiment described above attests (Schulkey et al., 2015). An unbiased genetic approach to discover the relevant genes and pathway would probably be more efficient than focusing upon a specific hypothesis or mechanism.

An ongoing effort in our labs thus seeks to map the genes in an advanced intercross of the Large and Small inbred strains. In the 1940's, Goodale and MacArthur selectively bred the largest or smallest mice in a litter to assess the genetic constraints on body size (Goodale, 1941; MacArthur, 1944). The inbreeding experiment produced the Large (LG) and Small (SM) inbred mouse strains. At age 60 days LG and SM weigh 37.4 and 13.7 g, respectively, a ~3-fold difference (Chai, 1956; Chai, 1956). In the 1990's, Cheverud and colleagues began to characterize the genetic architecture of body size in an F2 intercross of LG and SM (LGxSM), first by mapping and quantifying the effects of QTLs in the F2 individuals (Cheverud et al., 1996; Kramer et al., 1998; Vaughn et al., 1999). There is abundant empirical evidence for the role of maternal genetic factors acting in the womb that affect body size and growth (Ernst et al., 2000; Rhees et al., 1999; Cowley et al., 1989; Brumby, 1960; Atchley et al., 1991; Walton and Hammond, 1938), but no phenotypic variation in an

F2 intercross can be attributed to maternal genetic effects because all the mothers are genetically identical F1 hybrids of the two parental strains. Maternal-effect QTLs for growth and body size have been detected in advanced intercross generations (Wolf and Cheverud, 2012; Wolf et al., 2002; Wolf et al., 2011). Their discoveries illustrate how QTLs that affect the maternal-age associated risk of congenital heart disease can be found.

In an advanced intercross, randomly selected pairs of mice from the F2 and subsequent generations are mated to produce a population of genetically unique individuals. In the F3+ generations, breeding between siblings or cousins is avoided so as to maximize recombination frequency (Fig. 4). With each generation, t , the resolution of mapping increases by a factor of $t/2$ over short genomic distances in an advanced intercross relative to the F2 (Darvasi and Soller, 1995). An advanced intercross takes considerable effort to produce but offers many advantages for the analysis of complex genetic traits (Gonzales and Palmer, 2014).

To map maternal-effect QTLs, phenotypic variation in the offspring is related to genetic variation in the mothers. This contrasts with direct genetic effects, in which a phenotype is mapped to genes in the same individual. Early studies of F3 LGxSM offspring showed that maternal-effect QTLs, i.e., genetic variation in the F2 mothers, accounted for 31.5% of the variance in growth between the first and second week of life. Direct-effect QTLs in the offspring accounted for just 11.8% (Wolf et al., 2002). Maternal effects on growth could be related to either pre- or postnatal effects, such as milk production for the latter. The two effects were experimentally dissected by cross-fostering, in which half of the newborn pups in each litter were nursed by a different mother. Pre- and postnatal effect QTLs could thus be mapped in the biological and foster mothers. Both types of QTLs contribute significantly to the variance in body weight into adulthood. Interestingly, prenatal QTLs have a stronger, more persistent effect on weight than postnatal QTLs (Wolf et al., 2011).

Major maternal effects on offspring phenotype are recognized across many species and on adult cardiovascular and metabolic disease in humans, but their genetic basis is largely unexplored (Mousseau and Fox, 1998; Barker, 1990). The analysis of complex traits requires large sample sizes, so the additional complexity of mapping phenotypes in one individual to genotypes in a different individual can make maternal effects seem even more daunting. The quantitative genetic analysis of maternal effects is tractable, however, using inbred mouse strains, experimental crosses and controlling for the direct effects of an offspring's own genome on its phenotype. This work has provided a foothold into maternal genetic contributions to offspring traits. In studies from multiple groups, the analyses of the offspring from a few hundred mothers have defined maternal QTLs and genetic effects not only for body size and growth but also for postnatal survival, glycemic indices, lipid levels, and obesity (Peripato et al., 2002; Jarvis et al., 2005; Liljander et al., 2006; Hadsell et al., 2012). After two decades of propagating the LGxSM advanced intercross, the mapping resolution is ~600 kb in the F56 mothers being used to map QTLs for the maternal-age associated risk of congenital heart disease, an interval usually containing just a handful of genes. Preliminary results indicate that the incidence of heart defects increases among the offspring of 300 mothers as the mothers age. Work in progress will evaluate the variation in the quantitative age-associated risk between mothers and the association with genotypes at

loci. A short list of candidate genes would direct attention to specific pathways and maternal phenotypes, such as the serum concentration of a metabolite.

CONCLUSION

Over the past few decades, an intense focus on the embryo or child who has congenital heart disease has enabled incredible scientific insights and improved clinical outcomes. To build upon this progress, we advocate a wider perspective, one that considers the mother, with the goal of developing a means to prevention. Genetic mutations in the embryo are the major cause of heart defects, but it is not at all clear how they can be addressed on a meaningful scale. On the other hand, maternal factors that contribute to the total risk in genetically predisposed offspring could be attractive targets for a prevention strategy. A modifiable pathway in mothers indeed exists, as illustrated by the salutary effect of genetic polymorphisms and exercise in mitigating the maternal-age associated risk of congenital heart disease in *Nkx2-5^{+/-}* mice. Mapping of maternal genetic polymorphisms that mediate the risk is probably the most efficient means to define the pathway. Although the existence of the pathway was discovered in the context of maternal aging in one mutant model, it seems more likely than not to have relevance to human congenital heart disease more broadly. The maternal-age associated risk is observed in humans who have diverse causes of congenital heart disease, and pathways in aging and exercise overlap with those affected in obesity and diabetes, which are also maternal risk factors. Hence, characterization of the maternal genes and their effect on the environment of the developing embryo in a mouse model should begin to lay the groundwork for observational human studies, such as genetic risk stratification of mothers, and hopefully clinical trials.

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- Risk factors for congenital heart disease may suggest a means of prevention.
- Maternal age is a risk factor in humans and in a mouse model.
- The mechanistic basis of the risk resides in the mother and not the oocyte.
- Maternal exercise and genetic polymorphisms mitigate the age-associated risk.
- An unbiased genetic approach should most efficiently define the maternal pathways.

Figure 1.

A wide variety of congenital heart defects have been observed in newborn *Nkx2-5*^{+/-} mouse pups. They range from mild to severe and are anatomically identical to those found in humans. An example of tricuspid atresia is shown here. All four cardiac chambers are present, but the right ventricle is smaller than the left. The (A) pulmonic, (B) aortic and (C) mitral valves are present. The absence of the tricuspid valve between the right atrium and ventricle is indicated by the asterisk (*, C). There is also a ventricle septal defect (VSD). RA, LA, RV, LV, right and left atrium and ventricle.

Figure 2.

Inbred strain crosses reveal the effects of genetic modifiers and maternal age on the risk of congenital heart defects in *Nkx2-5*^{+/-} mice. *Nkx2-5*^{+/-} in the C57BL/6N background have a variety of congenital heart defects, but the F1 hybrid offspring of a cross to either FVB/N or A/J have a much lower incidence. Heart defects are observed again in the *Nkx2-5*^{+/-} F2 offspring of F1 backcrosses to the parental strains or intercross (F1 × F1). Each inbred strain carries a combination of susceptibility or protective alleles at multiple loci that affect the risk of specific anatomic types of defects. Recombination, as symbolized by the shading of each chromosome, permits mapping of the loci. The presence of heart defects in a particular strain or cross is indicated by the hole in the associated heart. Normal hearts indicate a very low or undetectable incidence of heart defects. The effect of maternal age was quantified in the F2 intercrosses of C57BL/6N × FVB/N and C57BL/6N × A/J and the C57BL/6N inbred strain. Reciprocal ovarian transplant, high-fat diet, and exercise experiments were performed in the C57BL/6N × FVB/N F2 intercross.

Figure 3.

The maternal-age associated risk of congenital heart disease is modifiable. Maternal-age affects the risk of congenital heart disease in the *Nkx2-5*^{+/-} mouse (Control: C57BL/6N × FVB/N F2 intercross, normal diet, sedentary cage condition). Reciprocal ovarian transplant experiments between young and old mouse mothers indicate that the mechanism resides in the mother. A high-fat diet does not alter the risk, which suggests that the mechanism is not related to hyperglycemia or obesity. Maternal genetic polymorphisms can either increase or decrease the risk. Voluntary exercise by mouse mothers, whether started early or later in life, mitigates the risk, thus proving that it is possible to prevent congenital heart disease even if the intervention does not target the causative genetic mutation.

Figure 4.

Development of the LG $\times$ SM advanced intercross. Large and Small inbred mice were crossed to produce F1 hybrids, which were then intercrossed to produce the F2 generation. Mice from the F2 to Fn generations are randomly mated. Brother-sister and cousin mating are avoided. Recombination at each generation divides the chromosomes into smaller intervals. Maternal-age associated risk QTLs are being mapped in mothers from the F56 generation. The resolution of mapping in the F56 is ~600 kb.

TABLE

Epidemiologic studies consistently document a maternal-age associated risk of congenital heart disease, but a few have not.

Locale	Population size	Risk	Reference
Hawaii	283 K	* Yes	Forrester and Merz, 2004
Dallas, TX	102 K	* Yes	Hollier et al, 2000
Australia	343 K	* Yes	Kidd et al, 1993
Poland	902 K	* Yes	Materna-Kiryluk et al, 2009
Atlanta, GA	1.3 M	* Yes	Miller et al, 2011
California	2.22 M	* Yes	Pradat et al, 2003
Atlanta, GA	1.1 M	* Yes	Reefhuis and Honein, 2004
Ontario, Canada	1.87 M	Yes	Agha et al, 2011
Costa Rica	77 K	Yes	Benavides-Lara et al, 2011
Guangdong, China	1.0 M	Yes	Wu et al, 2014
British Columbia, Canada	577 K	* No	Baird et al, 1991
Europe	1.7 M	* No	Loane et al, 2009
Sweden	1.27 M	* No	Pradat et al, 2003

*, These studies excluded cases that were associated with a chromosomal abnormality or syndrome.