LETTER

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The maternal-age-associated risk of congenital heart disease is modifiable

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Maternal age is a risk factor for congenital heart disease even in the absence of any chromosomal abnormality in the newborn[1–7]. Whether the basis of this risk resides with the mother or oocyte is unknown. The impact of maternal age on congenital heart disease can be modelled in mouse pups that harbour a mutation of the cardiac transcription factor gene *Nkx2-5* (ref. 8). Here, reciprocal ovarian transplants between young and old mothers establish a maternal basis for the age-associated risk in mice. A high-fat diet does not accelerate the effect of maternal ageing, so hyperglycaemia and obesity do not simply explain the mechanism. The age-associated risk varies with the mother's strain background, making it a quantitative genetic trait. Most remarkably, voluntary exercise, whether begun by mothers at a young age or later in life, can mitigate the risk when they are older. Thus, even when the offspring carry a causal mutation, an intervention aimed at the mother can meaningfully reduce their risk of congenital heart disease.

Congenital heart disease remains a leading cause of childhood morbidity and mortality despite dramatic clinical advances. Discoveries regarding the pathogenic mechanisms have likewise abounded, but translating knowledge about the causes of the disease in the embryo to improve outcomes will not be simple. The ideal would be to develop a broadly implemented prevention strategy, just as folic acid is prescribed to expectant mothers to prevent neural tube defects[9]. Therefore, we have focused upon genetic or environmental modifiers of heart defects caused by *Nkx2-5* haploinsufficiency in the mouse embryo[8,10]. Mutations of the cardiac transcription factor NKX2-5 that cause congenital heart disease were first discovered in humans[11,12]. The identification of modifiers, which affect risk but do not cause disease *per se*, can point the way towards therapies that do not necessarily target the main cause[13] but are effective nonetheless.

A substantial fraction of the ventricular septal defects (VSDs) seen among *Nkx2-5*$^{+/-}$ mice can be attributed to the effect of modifiers[8]. These modifiers include genetic polymorphisms that quantitatively affect penetrance[8,10]. In the C57BL/6N × FVB/N hybrid strain background, maternal age also affects the risk of VSD in *Nkx2-5*$^{+/-}$ but not wild-type pups; Extended Data Fig. 1 depicts the breeding scheme. The risk is independent of genetic polymorphisms in the offspring and not due to chromosomal aneuploidy[8]. These laboratory and epidemiological observations motivated investigation of the maternal age effect in the mouse model.

The basis of the maternal age effect could reside in either the mother or oocyte. To determine which, we performed reciprocal ovarian transplants between young and old mothers that were first generation (F1) hybrids of the inbred strains C57BL/6N and FVB/N (Fig. 1). F1 parents were bred to produce F2 offspring. The incidence of VSD was significantly greater among the *Nkx2-5*$^{+/-}$ offspring of older mothers bearing young ovaries. Next, we calculated the number of VSDs that would be expected for either a maternal or oocyte basis by looking at the effect of maternal age that was quantified in the 2,262 *Nkx2-5*$^{+/-}$ offspring of C57BL/6N × FVB/N F1 mothers who ovulated from their native ovaries. The observed numbers in each transplanted group were consistent with a maternal basis of the age effect (Fig. 1). We focused on VSDs, the most common defect in *Nkx2-5*$^{+/-}$ mice, because their frequency provides sufficient power for the statistical analyses we carried out[8,10]. Atrial septal defects (ASDs), the second most common defect, showed a pattern consistent with a maternal basis, but their numbers were insufficient to draw statistical conclusions (Extended Data Fig. 2). The results point to a maternal pathway that either produces a factor or mediates a process that interacts with cardiac development in the mutant embryo. A harmful factor would rise with age, whereas a protective one would fall.

Factors related to diabetes or obesity are plausible suspects; both conditions are associated with ageing and human congenital heart disease[14].

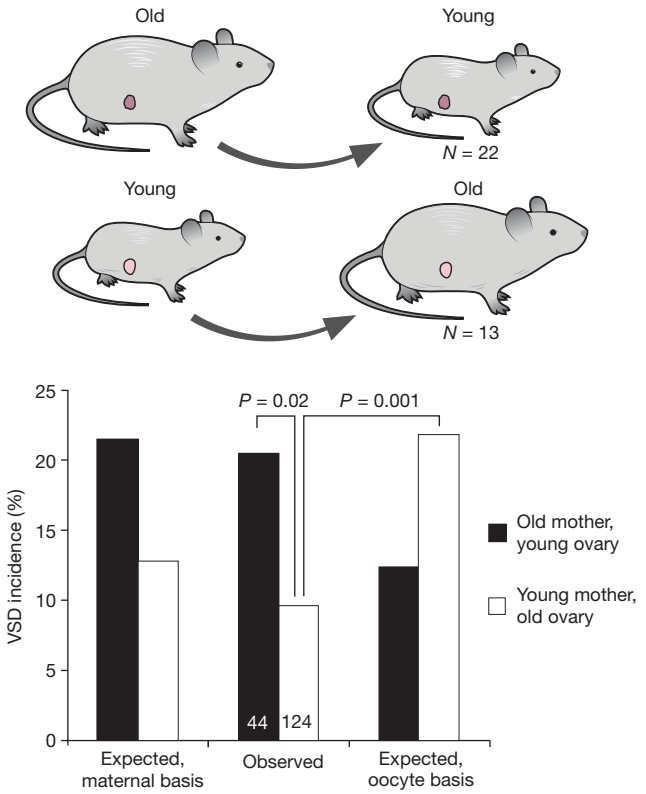

Figure 1 | Reciprocal ovarian transplants between young and old mothers localize the basis of the maternal-age-associated risk to the mother. The incidence of VSD for the offspring of old mothers with young ovaries is significantly greater than that of young mothers with old ovaries. The observed incidence in the offspring of recipient mothers matches that expected for a maternal but not an oocyte basis of the age effect. The observed and expected incidences were compared in a chi-squared goodness-of-fit test. The number of recipient mothers and the number of pups in each age group are shown.

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To assess their contribution to the maternal-age-associated risk, we placed young females on a high-fat diet 4 weeks before mating. The mothers remained on the diet thereafter. Young mothers on a high-fat diet had impaired glucose tolerance and fasting hyperglycaemia, yet their offspring had an incidence of VSD equal to those of mothers on a normal, chow diet (Fig. 2a, b, d). In addition, old mothers developed marked adiposity. Still, the incidence of VSD in their offspring was not significantly increased (Fig. 2c, d, and Extended Data Fig. 3). Despite large effects on glucose homeostasis and obesity, a high-fat diet did not significantly increase the maternal-age-associated risk of congenital heart defects (Fig. 2e and Extended Data Fig. 4). A logistic regression analysis that considered an interaction between diet and age also failed to detect an effect. Any potential effect of the high-fat diet on the maternal-age-associated risk is small relative to the effects on glycaemic indices or body composition.

Most epidemiological studies identify maternal age as a risk factor for congenital heart disease, but the risk is not always detected, perhaps because of genetic variation among populations[6,15,16]. We thus compared the maternal-age-associated risk in three mouse strain backgrounds: the inbred C57BL/6N strain, and the F1 hybrids of C57BL/6N × A/J and C57BL/6N × FVB/N mice (Extended Data Fig. 1). The inbred C57BL/6N strain bears the greatest risk. That risk is significantly greater than in the C57BL/6N × A/J hybrid, which shows no significant risk. The maternal-age-associated risk in the C57BL/6N × FVB/N hybrid is significant and intermediate to the other two backgrounds (Fig. 3). The maternal-age-associated risk is a quantitative genetic trait. For example, the net effect of A/J polymorphisms is to reduce the risk associated with ageing, whereas C57BL/6N polymorphisms generally increase risk. Genetic polymorphisms may affect the activity of the maternal factor hypothesized to interact with embryonic cardiac development.

A/J and C57BL/6 mice are known for their phenotypic differences in a number of complex metabolic traits, including the effects of diet[17,18]. We thus wondered whether exercise, which has beneficial effects on metabolism, could decrease the risk of congenital heart disease, just as A/J polymorphisms decrease the risk of adverse metabolic phenotypes. Running wheels were placed in the cages of C57BL/6N × FVB/N F1 mice at the onset of breeding, when the females were 4 weeks old. The mice were allowed to run *ad libitum* during their entire reproductive

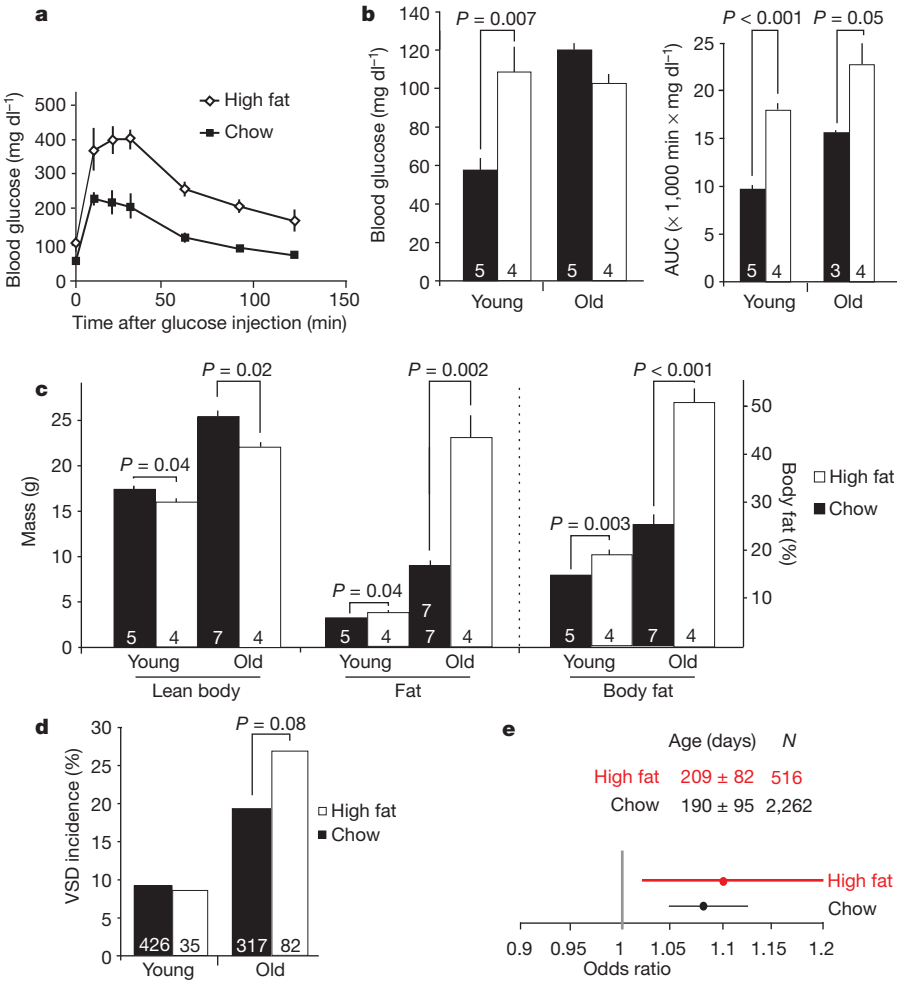

Figure 2 | A high-fat diet does not accelerate the onset of the maternal age effect. **a**, A high-fat diet causes a higher peak glucose and impaired clearance in glucose tolerance tests. Data are shown here from young mothers. **b**, A high-fat diet induces hyperglycaemia in young mothers and impaired glucose tolerance in young and old mothers, as quantified by the area under the curve (AUC) in glucose tolerance tests. **c**, Lean body mass decreases with a high-fat diet, while fat mass and percentage increases. Marked adiposity develops in old mothers on a high-fat diet. **d**, The incidences of VSD in the offspring of young mothers are the same whether on a high-fat or normal diet. The incidence for the offspring of old mothers on a high-fat diet is not significantly increased. Young and old mothers are defined as <100 and >300 days old, respectively. Glucose tolerance tests and magnetic resonance imaging (MRI) quantification of body composition were performed on a subset of the 19 high-fat- or 156 chow-diet-fed mothers who produced the offspring for analysis. **e**, The maternal-age-associated risk is unaffected by a high-fat diet. Odds ratios are presented with the 95% confidence interval; the relative widths of the confidence intervals are related to the sample sizes. Glucose and body composition data are given as mean ± standard error of the mean (s.e.m.); values were compared in two-sided *t*-tests. The age of the mothers (mean ± standard deviation (s.d.)) and the number of offspring in each cohort are shown.

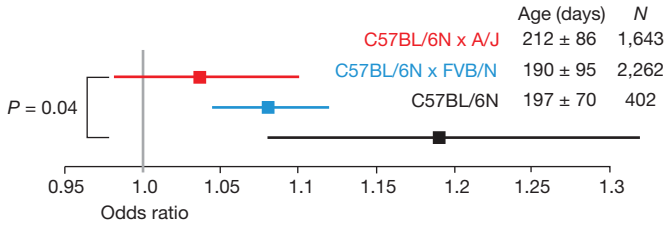

Figure 3 | The maternal-age-associated risk of VSD is a quantitative genetic trait. Risk is significantly greater in the inbred C57BL/6N strain than in the C57BL/6N × A/J F1 hybrid. The C57BL/6N × FVB/N F1 hybrid bears intermediate risk. Odds ratios are presented with the 95% confidence interval; the relative widths of the confidence intervals are related to the sample sizes. The age of mothers (mean ± s.d.) and the number of offspring in each cohort are shown. Odds ratios were compared in a logistic regression analysis that included genetic background as a covariate in addition to maternal age.

lifespan[19]. Early onset exercise did not lower the incidence of VSD in the *Nkx2-5*$^{+/-}$ offspring of young mothers but did in those of old mothers (Fig. 4a). When all the offspring of mothers from young to old age were included in a logistic regression analysis, the maternal-age-associated risk was insignificant in the setting of early onset exercise (Fig. 4b).

Early onset exercise clearly reduces risk for *Nkx2-5*$^{+/-}$ offspring but may be difficult to implement in clinical practice. We therefore assessed the effect of exercise beginning in late adulthood. Running wheels were placed in cages when the mothers were 8 months old. The subsequent incidence of VSD was significantly reduced in the *Nkx2-5*$^{+/-}$ offspring of mothers older than 300 days. Late onset exercise reduced the incidence nearly as much as early onset exercise (Fig. 4a). Consistent with the multifactorial basis of a congenital heart defect, exercise mitigates the fraction of risk associated with maternal ageing but does not eliminate risk entirely, as shown by the equivalent incidences of VSD in the offspring of exercised and sedentary young mothers.

To estimate the duration of exercise sufficient for an effect, the observed incidence of VSD was compared to the expected incidence in a range of intervals binned according to the number of days a mother had exercised by a pup's birth date. Given a 20-day gestational period, 3 months of exercise before conception exerts a detectable effect on incidence (Fig. 4c). Variables not examined here, such as the mother's age at the onset of exercise and the intensity of exercise, could affect the magnitude of the effect. Nevertheless, a modest duration of exercise appears sufficient to reduce the risk of congenital heart disease.

The benefit of exercise for the offspring is not associated with overt or large changes in the mother. Neither early nor late onset exercise significantly affected glucose metabolism, lean or fat body mass or weight in old mothers (Fig. 4d, e and Extended Data Fig. 3). Furthermore, body weight was not independently associated with risk under any of the experimental conditions, that is, sedentary control, high-fat diet, early or late onset exercise. In humans, good evidence indicates that maternal diabetes and obesity are risk factors for congenital heart disease[14]. The fraction of children of mothers who have diabetes or obesity who actually have a heart defect is still small, however, so the true risk factor may be present in just a subset of women. Exercise may ameliorate a risk factor associated with ageing, dysglycaemia or obesity without a noticeable effect on glucose metabolism or body composition in the mouse mother.

The present results reveal a maternal pathway that could potentially be targeted to prevent congenital heart disease in offspring who carry a deleterious mutation. Whether this pathway affects the development of defects other than VSD or that are not caused by *Nkx2-5* mutation remains unanswered, although consistent evidence supports a maternal-age-associated risk for ASD in the mouse (Extended Data Figs 2 and 4) and for other defects in humans. Very large studies are necessary to prove or exclude confidently a maternal-age-associated risk for less common defects. Experimental dissection of the pathway may be a more efficient means forward. The results could indicate whether distinct

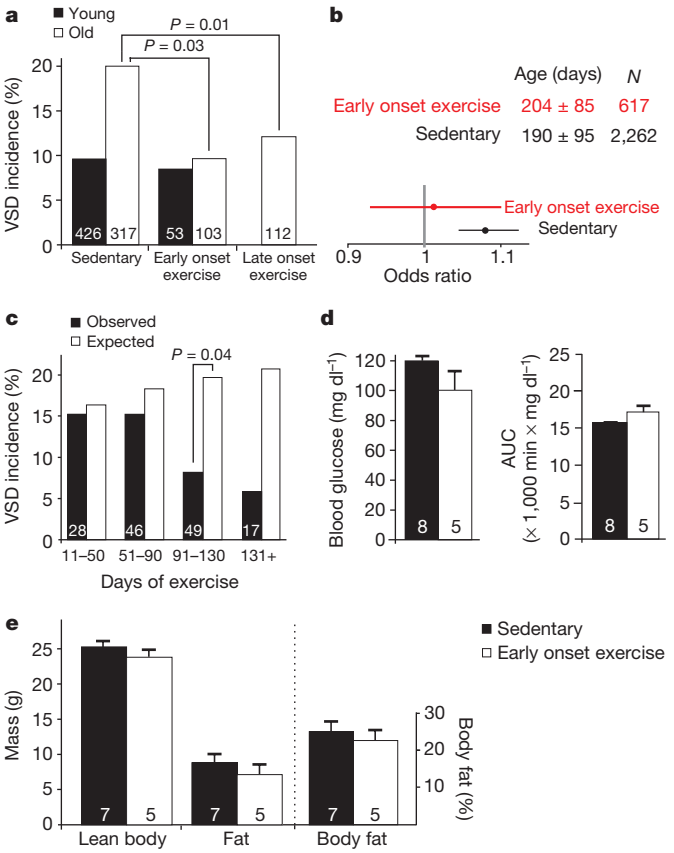

Figure 4 | Exercise can mitigate the risk associated with maternal ageing. **a**–**e**, Running wheels were placed in breeding cages when mothers were 4 weeks or 8 months old (early or late onset exercise groups). **a**, Both early and late-onset exercise decrease the incidence of VSD for the offspring of old compared to those of young mothers. The number of mouse pups in each group is shown. **b**, Early onset exercise makes the risk associated with ageing insignificant. Odds ratios are presented with the 95% confidence interval; the relative widths of the confidence intervals are related to the sample sizes. The age of the mothers (mean ± s.d.) and the number of offspring in each cohort are shown. **c**, The incidence of VSD is shown binned according to the number of days a mother in the late onset group had exercised by the pup's birthdate. The expected incidences are calculated for age-matched, sedentary control mothers. Three months of exercise before conception results in a detectable reduction in the incidence of VSD. **d**, Early onset exercise does not alter fasting glucose levels or glucose tolerance in old mothers. **e**, Lean and fat body mass and body fat percentage are also not appreciably affected. Glucose tolerance tests and MRI quantification of body composition were performed on a subset of the mothers in the early onset exercise and sedentary groups. The data are reported as mean ± s.e.m. The incidences of defects were compared by chi-squared tests.

defects should be analysed separately, and help to focus the aims of human studies. Well-known pathways may not be involved, given that deranged glucose homeostasis and obesity do not exacerbate the maternal age effect. Interestingly, maternal genetic polymorphisms in other metabolic pathways have recently been associated with the risk of conotruncal heart defects in humans[20]. A maternal genetic basis for the age-associated risk in *Nkx2-5*$^{+/-}$ pups likewise supports the concept of gene × gene interactions between the mother and embryo. The present results do not exclude the possibility of other complex molecular, genetic and epigenetic mechanisms, but mapping the genes that affect risk could provide insight into the maternal pathway. In addition, the effect of exercise provides a functional clue. Like ageing, exercise has complex effects on physiology and metabolism. Exercise and aerobic fitness affect the concentration of dozens of metabolites[21–26]. Any of them could be the hypothesized age-related factor. The pertinent factor

or pathway in the mouse, however, must fit a profile delimited by ageing and genetic background.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Forrester, M. B. & Merz, R. D. Descriptive epidemiology of selected congenital heart defects, Hawaii, 1986–1999. *Paediatr. Perinat. Epidemiol.* **18,** 415–424 (2004).
2. Hollier, L. M., Leveno, K. J., Kelly, M. A., McIntire, D. D. & Cunningham, F. G. Maternal age and malformations in singleton births. *Obstet. Gynecol.* **96,** 701–706 (2000).
3. Kidd, S. A., Lancaster, P. A. & McCredie, R. M. The incidence of congenital heart defects in the first year of life. *J. Paediatr. Child Health* **29,** 344–349 (1993).
4. Materna-Kiryluk, A. *et al.* Parental age as a risk factor for isolated congenital malformations in a Polish population. *Paediatr. Perinat. Epidemiol.* **23,** 29–40 (2009).
5. Miller, A., Riehle-Colarusso, T., Siffel, C., Frias, J. L. & Correa, A. Maternal age and prevalence of isolated congenital heart defects in an urban area of the United States. *Am. J. Med. Genet. A* **155,** 2137–2145 (2011).
6. Pradat, P., Francannet, C., Harris, J. A. & Robert, E. The epidemiology of cardiovascular defects, part I: a study based on data from three large registries of congenital malformations. *Pediatr. Cardiol.* **24,** 195–221 (2003).
7. Reefhuis, J. & Honein, M. A. Maternal age and non-chromosomal birth defects, Atlanta—1968–2000: teenager or thirty-something, who is at risk? *Birth Defects Res. A Clin. Mol. Teratol.* **70,** 572–579 (2004).
8. Winston, J. B. *et al.* Complex trait analysis of ventricular septal defects caused by *Nkx2-5* mutation. *Circ Cardiovasc Genet* **5,** 293–300 (2012).
9. MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* **338,** 131–137 (1991).
10. Winston, J. B. *et al.* Heterogeneity of genetic modifiers ensures normal cardiac development. *Circulation* **121,** 1313–1321 (2010).
11. Schott, J. J. *et al.* Congenital heart disease caused by mutations in the transcription factor *NKX2-5*. *Science* **281,** 108–111 (1998).
12. Benson, D. W. *et al.* Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. *J. Clin. Invest.* **104,** 1567–1573 (1999).
13. Nadeau, J. H. Modifier genes in mice and humans. *Nature Rev. Genet.* **2,** 165–174 (2001).
14. Jenkins, K. J. *et al.* Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. *Circulation* **115,** 2995–3014 (2007).
15. Baird, P. A., Sadovnick, A. D. & Yee, I. M. Maternal age and birth defects: a population study. *Lancet* **337,** 527–530 (1991).
16. Loane, M., Dolk, H. & Morris, J. K. Maternal age-specific risk of non-chromosomal anomalies. *BJOG* **116,** 1111–1119 (2009).
17. Burrage, L. C. *et al.* Genetic resistance to diet-induced obesity in chromosome substitution strains of mice. *Mamm. Genome* **21,** 115–129 (2010).
18. Singer, J. B. *et al.* Genetic dissection of complex traits with chromosome substitution strains of mice. *Science* **304,** 445–448 (2004).
19. Allen, D. L. *et al.* Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. *J. Appl. Physiol.* **90,** 1900–1908 (2001).
20. Li, M. *et al.* Detecting maternal–fetal genotype interactions associated with conotruncal heart defects: a haplotype-based analysis with penalized logistic regression. *Genet. Epidemiol.* **38,** 198–208 (2014).
21. Bye, A. *et al.* Serum levels of choline-containing compounds are associated with aerobic fitness level: the HUNT-study. *PLoS ONE* **7,** e42330 (2012).
22. Chorell, E., Svensson, M. B., Moritz, T. & Antti, H. Physical fitness level is reflected by alterations in the human plasma metabolome. *Mol. Biosyst.* **8,** 1187–1196 (2012).
23. Krug, S. *et al.* The dynamic range of the human metabolome revealed by challenges. *FASEB J.* **26,** 2607–2619 (2012).
24. Lewis, G. D. *et al.* Metabolic signatures of exercise in human plasma. *Sci. Transl. Med.* **2,** 33ra37 (2010).
25. Lustgarten, M. S. *et al.* Identification of serum analytes and metabolites associated with aerobic capacity. *Eur. J. Appl. Physiol.* **113,** 1311–1320 (2013).
26. Mukherjee, K. *et al.* Whole blood transcriptomics and urinary metabolomics to define adaptive biochemical pathways of high-intensity exercise in 50–60 year old masters athletes. *PLoS ONE* **9,** e92031 (2014).

Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.Y.J. (jay_p@kids.wustl.edu).

METHODS

Mouse strains. The C57BL/6N and FVB/N inbred strains were purchased from Charles River, and the A/J strain from the Jackson Laboratory. *Nkx2-5*$^{+/-}$ mice were maintained in the C57BL/6N background; *Nkx2-5*$^{+/-}$ males were crossed to FVB/N or A/J females to produce first generation (F1) hybrids[10,27]. *Nkx2-5*$^{+/-}$ F1 mice were then crossed to produce the F2 offspring (Extended Data Fig. 1). No randomization was necessary among genetically identical mice. The animal studies committee at Washington University School of Medicine approved the experiments.

Preparation and phenotyping of newborn mouse hearts. Newborn mouse pups were collected within hours of birth. Their hearts were fixed in formalin, and the pups were genotyped for the *Nkx2-5* knockout allele. *Nkx2-5*$^{+/-}$ hearts were embedded in paraffin, serially sectioned at 6 μm thickness and stained with haematoxylin and eosin. The sections were examined for heart defects as previously described[10]. Phenotyping was performed by individuals blinded to the age of a pup's mother or, in the transplantation experiment, blinded to the age of the ovary as well. Every heart that was phenotyped is included in statistical analyses.

Ovarian transplantation. Mice were anaesthetized with a cocktail (0.5–0.7 ml kg^{-1}) of 3:3:1 ketamine (100 mg ml^{-1}), xylazine (20 mg ml^{-1}) and acepromazine (10 mg ml^{-1}) via intraperitoneal injection. A small incision was made in the dorsolateral flank to access the ovarian bursa. The ovary was removed from the donor and then transplanted into the recipient. The native, contralateral ovary of the recipient was left in place, while the oviduct was ligated. Transplants were performed between C57BL/6N × FVB/N F1 females that were either old (range 244–377 days old, mean 317 days old) or young (range 30–81 days old, mean 48 days old). The recipient females were allowed to recover for 3 weeks before breeding commenced. Pregnant females were noted to carry their fetuses in the uterine horn on the same side as the transplanted ovary.

High-fat diet. Weanling *Nkx2-5*$^{+/-}$ C57BL/6N × FVB/N F1 females were placed on a high-fat diet for 4 weeks before the onset of breeding. The mice remained on the diet while breeding. Calories in the diet derived from 59% fat, 25% carbohydrate and 15% protein (AIN-76A w/58% Fat Energy/Sucrose/Red, catalogue no. 1810835, Test Diet). Calories in the normal, control diet derived from 13% fat, 62% carbohydrate and 25% protein (Pico Rodent Diet 20, catalogue no. 0007688, Lab Diet).

Exercise. Running wheels were placed in breeding cages when the females were either 4 weeks old (early onset group) or 8 months old (late onset group). The mice ran *ad libitum* for the remainder of their reproductive lives.

Intraperitoneal glucose tolerance testing. Mice were fasted overnight (14 h) on paper bedding before glucose challenge (2 g kg^{-1} intraperitoneal). Blood was obtained from the tail vein for glucometry. Samples were measured with a Bayer Contour TS glucometer before and at 10, 20, 30, 60, 90 and 120 min after injection.

Lean and fat body mass quantification. Lean and fat body mass was measured in live mice by quantitative MRI on an EchoMRI 3-in-1 instrument (Echo Medical Systems). Fat body mass measurements were calibrated against canola oil standards.

Statistics. The maternal-age-associated risk of a defect within an experimental condition was calculated by logistic regression analysis. The phenotype of a pup, for example, VSD present or normal, was the dependent variable, and the mother's age in months on the pup's birthdate was the independent variable. The data were fit to the inverse of the logistic function or logit using the Generalized Linear Model in R (http://www.r-project.org/). Fitting yielded an estimate of the maternal age coefficient, from which the odds ratio is calculated. Odds ratios are reported with the 95% confidence interval.

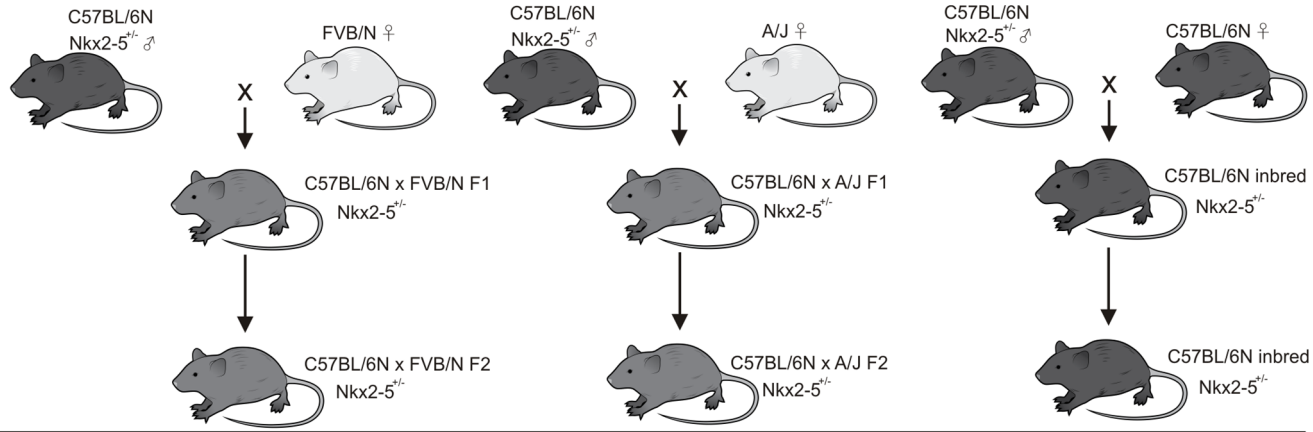
For the reciprocal ovarian transplant and late onset exercise experiments, the expected incidence of a defect was calculated from the estimates of the coefficients in the logit for the C57BL/6N × FVB/N sedentary control group and either the age of the mother or ovary for every pup. The probability of a defect, as given by the logistic function, was calculated for each observed pup. The sum of probabilities across all the pups was the expected number for the experimental group, given the hypothesized or null model. The expected and observed numbers of defects were compared in a chi-squared goodness-of-fit test. To assess the effect of other variables, such as genetic background, diet or the interaction of either with age, the variables were included in the logistic function as covariates in addition to maternal age. The significance of a covariate was determined using the Generalized Linear Model in R.

Two-sided *t*-tests were performed for blood glucose and body composition measurements.

Experimental sample sizes in the ovarian transplantation, high-fat diet, and exercise experiments and analysis of the C57BL/6N strain were planned based on the incidences of VSD in the offspring of young and old C57BL/6N × FVB/N F1 mothers. Every heart collected for an experimental condition was included in the statistical analysis unless poor histology precluded a diagnosis.

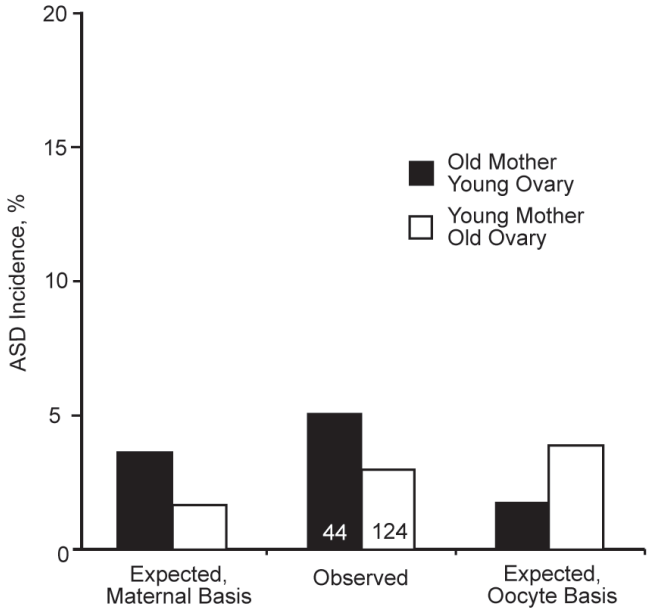
Results are reported as mean ± s.e.m. except for maternal age, which is reported as mean ± s.d. Statistical significance was defined as $P < 0.05$.

27. Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. & Izumo, S. The cardiac homeobox gene *Csx/Nkx2.5* lies genetically upstream of multiple genes essential for heart development. *Development* **126,** 1269–1280 (1999).

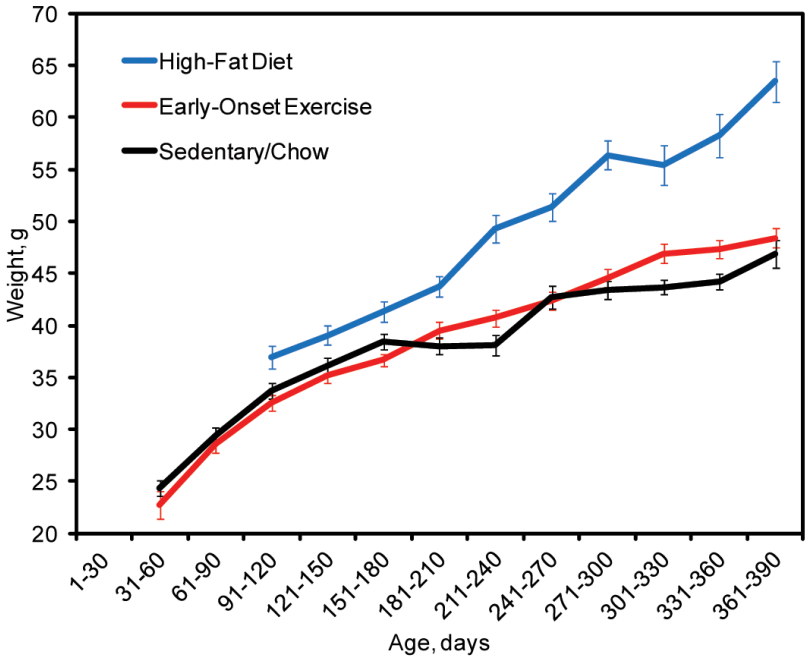


Experimental Condition	Mothers, N		
	C57BL/6N x FVB/N F1	C57BL/6N x A/J F1	C57BL/6N inbred
Sedentary/Chow	156	66	31
High-Fat Diet	19		
Early-Onset Exercise	15		
Late-Onset Exercise	12		

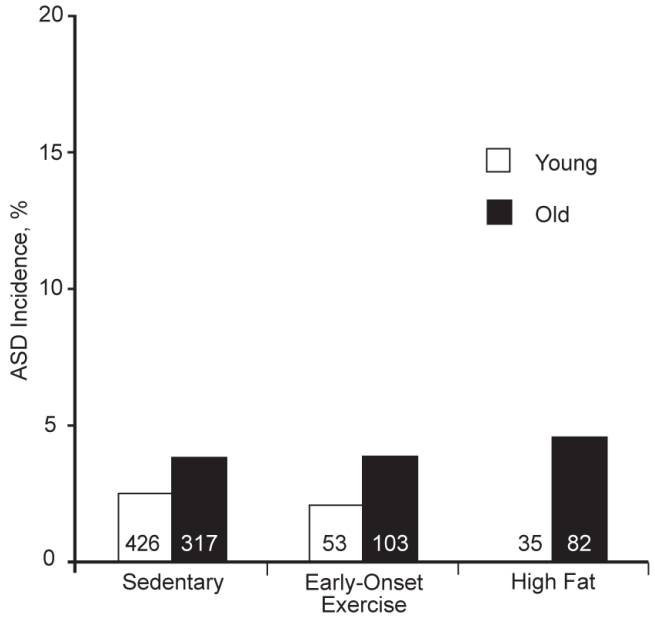
Extended Data Figure 1 | Breeding scheme and experimental conditions. *Nkx2-5*$^{+/-}$ offspring from several maternal genetic backgrounds and experimental conditions were phenotyped. *Nkx2-5*$^{+/-}$ C57BL/6N males were crossed to FVB/N or A/J females to produce F1 hybrids. The cross to a C57BL/6N female maintains the inbred strain. *Nkx2-5*$^{+/-}$ F1 hybrids were intercrossed to produce the F2 progeny. The hearts of newborn *Nkx2-5*$^{+/-}$ F2 pups were phenotyped to calculate the incidence of a defect and the effect of maternal age. C57BL/6N × FVB/N F1 hybrid mothers were bred in either sedentary/chow, high-fat diet, early or late onset exercise conditions. C57BL/6N × A/J F1 hybrid and C57BL/6N inbred mothers were studied only in the sedentary/chow condition. The number of mothers in each cross and experimental condition that were used to produce pups in this study are shown.



Extended Data Figure 2 | Incidences of ASD in the reciprocal ovarian transplant experiment. The relative incidences of ASD in the reciprocal ovarian transplant experiment are consistent with a maternal basis of the age-associated effect. The differences that were significant in the VSD data, however, are not significant here because of the lower incidence of ASD, as depicted by the *y*-axis drawn on a scale comparable to that for VSD. The total number of pups in each group is shown.



Extended Data Figure 3 | Growth charts for C57BL/6N × FVB/N F1 mothers under sedentary, early onset exercise and high-fat diet conditions. C57BL/6N × FVB/N F1 mothers on a high-fat diet develop marked obesity as they age. They weigh substantially more than mothers in the sedentary or early onset exercise groups. Mothers in the latter two groups weigh the same.



Extended Data Figure 4 | Incidences of ASD in the offspring of C57BL/6N × FVB/N mothers. Maternal age may affect the risk of ASD, but the lower incidence of ASD and other defects that are less common than VSD preclude firm statistical conclusions. For example, the incidences of ASD are shown for the *Nkx2-5*$^{+/-}$ offspring of young and old C57BL/6N × FVB/N mothers in the sedentary, early onset exercise, and high-fat diet conditions. The *y*-axis is drawn on a scale comparable to that for VSD incidence. ASD incidences are higher, but not significantly, among the offspring of old mothers compared to young mothers. The incidences are not significantly different between experimental conditions. The total number of pups in each group is shown.