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### Authors

Li, Jingyuan  
Chen, Xuqi  
McClusky, Rebecca  
et al.

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# The number of X chromosomes influences protection from cardiac ischaemia/reperfusion injury in mice: one X is better than two

Jingyuan Li<sup>1</sup>, Xuqi Chen<sup>2</sup>, Rebecca McClusky<sup>2</sup>, Maureen Ruiz-Sundstrom<sup>2</sup>, Yuichiro Itoh<sup>2</sup>, Soban Umar<sup>1</sup>, Arthur P. Arnold<sup>2</sup>, and Mansoureh Eghbali<sup>1,3\*</sup>

<sup>1</sup>Department of Anesthesiology, Division of Molecular Medicine, David Geffen School of Medicine at University of California Los Angeles, BH-160CHS, Los Angeles, CA 90095-7115, USA;

<sup>2</sup>Department of Integrative Biology and Physiology, University of California, Los Angeles, Los Angeles, CA, USA; and <sup>3</sup>Cardiovascular Research Laboratories, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, CA, USA

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**Aim** Sex differences in coronary heart disease have been attributed to sex hormones, whereas the potential role of the sex chromosomes has been ignored so far. Here, we investigated the role of the sex chromosomes in causing sex differences in myocardial ischaemia/reperfusion (I/R) injury.

**Methods and results** We used two unique mouse models, the 'four core genotypes' [XX mice with ovaries (XXF) or testes (XXM) and XY mice with ovaries (XYF) or testes (XYM)] and XY\* (gonadal male or female mice with one or two X chromosomes). All mice were gonadectomized (GDX). *In vivo* or isolated Langendorff-perfused hearts were subjected to I/R injury. The *in vivo* infarct size in XY mice was significantly smaller than XX mice regardless of their gonadal type ( $24.5 \pm 4.1\%$  in XYF and  $21.8 \pm 3.3\%$  in XYM vs.  $37.0 \pm 3.2\%$  in XXF and  $35.5 \pm 2.1\%$  in XXM,  $P < 0.01$ ). Consistent with the results *in vivo*, the infarct size was markedly smaller and cardiac functional recovery was significantly better in XY mice compared with XX *ex vivo*. The mitochondrial calcium retention capacity was significantly higher in XY compared with XX mice (nmol/mg protein: XXF =  $126 \pm 9$  and XXM =  $192 \pm 45$  vs. XYF =  $250 \pm 56$  and XYM =  $286 \pm 51$ ,  $P < 0.05$ ). In XY\* mice, mice with 2X chromosomes had larger infarct size (2X females =  $41.4 \pm 8.9\%$  and 2X males =  $46.3 \pm 9.5\%$  vs. 1X females =  $23.7 \pm 3.9\%$  and 1X males =  $26.6 \pm 6.9\%$ ,  $P < 0.05$ ) and lower heart functional recovery, compared with those with 1X chromosome. Several X genes that escape X inactivation (*Eif2s3x*, *Kdm6a*, and *Kdm5c*) showed higher expression in XX than in XY hearts.

**Conclusion** XX mice have higher vulnerability to I/R injury compared with XY mice, which is due to the number of X chromosomes rather than the absence of the Y chromosome.

**Keywords** Sex chromosome • Cardiac ischaemia/reperfusion

## 1. Introduction

It is now well established that the incidence and progression of heart disease is markedly higher in men than in age-matched women before menopause.<sup>1,2</sup> Among all cardiovascular-related diseases, coronary artery disease still remains the leading cause of death in western countries. Sex differences in susceptibility to ischaemia/reperfusion (I/R) injury have also been demonstrated in humans—pre-menopausal women have a lower risk of ischaemic heart disease than age-matched men, whereas after menopause the risk is similar or even higher in women.<sup>3</sup> The lower incidence of coronary artery disease in women

during reproductive age is believed to be related to female sex hormones. In fact, the protective role of oestradiol (E2) has been highlighted in the context of myocardial I/R injury in women as well as in females in experimental models.<sup>4</sup> There is higher incidence of coronary artery disease in young women who had an oophorectomy.<sup>5</sup> Infarct size is larger in ovariectomized (OVX) mice than in gonadally intact mice,<sup>6</sup> and E2 pre-treatment of OVX mice reduces the infarct size.<sup>7</sup> The women's health initiative study, however, failed to support the ability of hormone replacement therapy in postmenopausal women to reduce the risk of ischaemic heart disease.<sup>8,9</sup> These results raise the question whether ovarian hormones alone account for the sex

\* Corresponding author. Tel: +1 310 206 0345. Email: meghbali@ucla.edu; <http://www.anes.ucla.edu/dmm/eghbali/>

differences. Largely ignored thus far is the potential role of the different numbers and types of sex chromosomes in each male vs. female cell. Since XX chromosomes are always confounded with the presence of ovaries, and XY with the presence of a testes, it has not been possible previously to examine the role of sex chromosome complement (XX vs. XY) independent of gonadal hormone effects in susceptibility to I/R injury in experimental models.

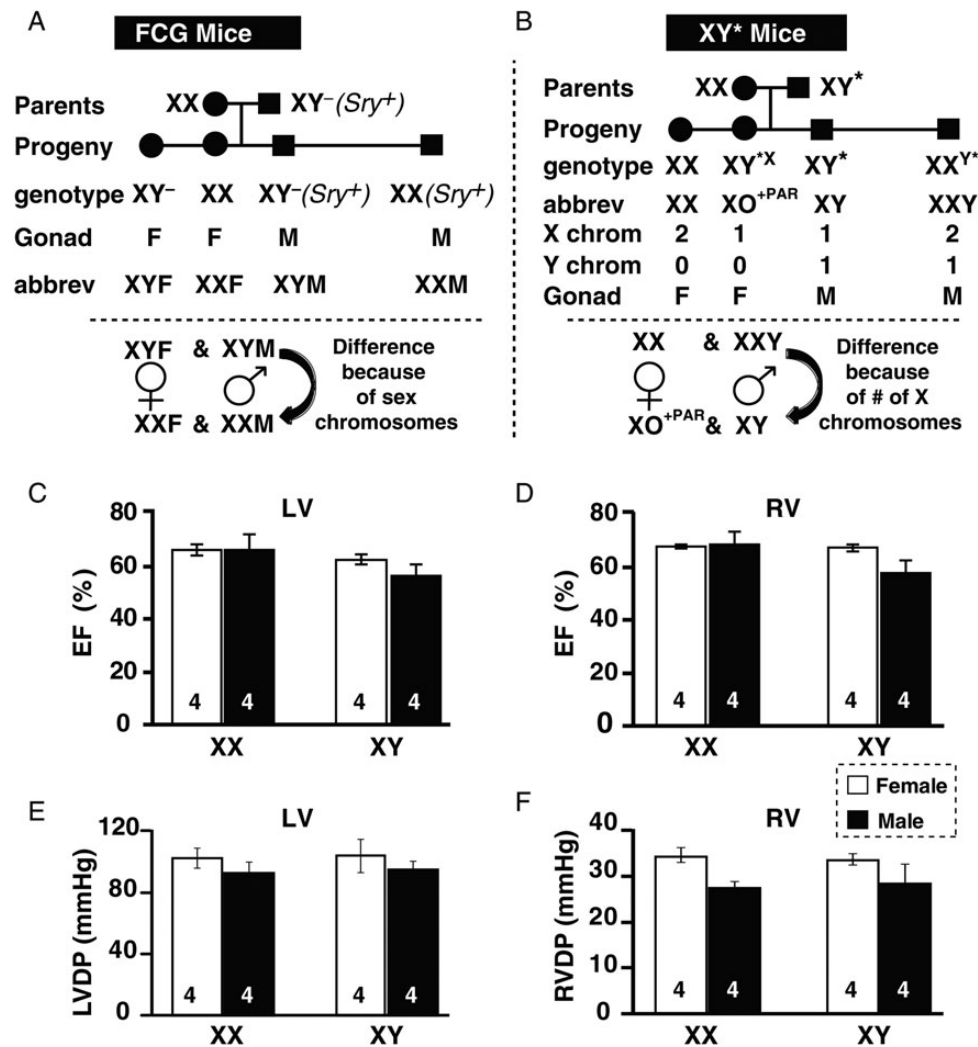
To unravel the role of the sex chromosome on susceptibility to myocardial I/R injury, we used two unique mouse models, the 'four core genotypes' (FCGs) and XY\* (Figure 1A and B). FCG mice were used to compare XX and XY mice that have the same type of gonad, because gonadal type in this model is not controlled by the sex chromosomes. This is accomplished by 'moving' the testis-determining gene *Sry* from the Y chromosome to an autosome, so that XX or XY mice can have either type of gonad. The XY\* model utilizes the Y\* chromosome that has an aberrant pseudoautosomal region (PAR) that recombines variably with the X PAR during meiosis.<sup>10,11</sup> This model produces mice with one X chromosome or two X chromosomes, each in the

absence or presence of a Y chromosome. To eliminate the role of gonadal hormones in adulthood, all mice were gonadectomized (GDx). Here, we report that the number of X chromosomes also plays an important role in myocardial susceptibility to I/R injury.

## 2. Methods

### 2.1 Animals

All mice were inbred C57BL/6. The FCG model allows comparing XX and XY mice with the same type of gonad (Figure 1A), whereas XY\* mouse model allows comparing mice with one X chromosome (XO<sup>+PAR</sup> or XY\*) with those with two X chromosomes (XX or XXY\*) in the absence or presence of a Y chromosome (Figure 1B).<sup>10,11</sup> The FCG mouse model utilizes the 'Y minus' chromosome (Y<sup>-</sup>) deleted for *Sry*.<sup>12-14</sup> XY<sup>-</sup> mice develop ovaries and are called XYF here. Introducing an *Sry* transgene onto an autosome produces XY<sup>-</sup>(*Sry*+) mice with testes (called XYM here; Figure 1A). The testis-determining autosomal *Sry* segregates independently from the Y chromosome, thus mating XYM with XXF produces the FCG, two with



**Figure 1** GDx FCG mice have similar heart function at baseline. Mouse models. (A) The 'FCG' model produces mice in which gonadal sex is independent of sex chromosome complement (XX vs. XY). (B) The XY\* model is useful for comparing groups that differ in the number of X chromosomes or in the presence/absence of the Y chromosome, to determine which causes XX vs. XY differences. (C and D) LV and RV EF of GDx FCG mice at baseline estimated from M-mode echocardiography. (E and F) LVDP and RVDP of GDx FCG mice at baseline measured directly by a catheter ( $n = 4$  mice/group).

Sry (gonadal males, XYM and XXM), and two lacking Sry (gonadal females, XYF and XXF). In the XY\* model,<sup>10,11</sup> mating XY\* males with XX females produces four types of offspring: (i) XX gonadal females; (ii) XY\*<sup>X</sup> gonadal females that have a single X chromosome plus a small chromosome that is predominantly a PAR—these mice are called XO<sup>+PAR</sup> here (see refs<sup>14,15</sup> for this nomenclature and detailed discussion); (iii) XY\* gonadal males are genetically similar to XY males and called XY here; and (iv) XX<sup>Y\*</sup> gonadal males that are similar genetically to XXY and are called XXY here. Comparison of XX and XXY mice with XO<sup>+PAR</sup> and XY represents a comparison of mice with two vs. one non-PAR regions of the X chromosomes (keeping the number of PARs equal at 2, as in normal XX and XY mice), and comparison of XX and XO<sup>+PAR</sup> mice with XY and XXY represents a comparison of mice without vs. with a Y chromosome, respectively. We used the XY\* model, in combination with FCG mice, to determine whether the sex chromosome effect (difference in XX vs. XY) found in FCG mice is caused by the number of X chromosomes, or by the presence/absence of the Y chromosome. All mice were GDX at Days 72–76, under 1.5 MAC isoflurane anaesthesia, mice were given a subcutaneous injection of carprofen (5 mg/kg), and the gonads were removed. One month later (30–35 days), mice were used for experiments, so all groups had no gonadal hormones and those hormones could not cause XX vs. XY differences at the time of testing. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Animal protocols were approved by the University of California Los Angeles School of Medicine Animal Research Committee.

We used 105 FCG mice and 28 XY\* mice for this study. For *ex vivo* experiments, six FCG mice and five XY\* mice were excluded from the study before even subjecting them to ischaemia due to technical problems. Four FCG mice and three XY\* mice were eliminated from the analysis because of fibrillation during reperfusion. For experiments *in vivo*, five FCG mice were excluded from the study due to technical problems (e.g. bleeding during surgery, fibrillation after ligation). For the gene expression analysis, 15 wild-type (WT) XX female and XY male mice were GDX at Days 70–75 and 2 weeks later were used for the experiment.

## 2.2 Cardiac haemodynamic measurements at baseline *in vivo*

B- and M-mode were performed using a VisualSonics Vevo 2100 equipped with a 30-MHz linear transducer. The left ventricle (LV) and right ventricle (RV) ejection fraction (EF) [(diastolic volume – systolic volume)/diastolic volume] were quantified using M-mode for LV or RV. The pressures were

measured directly by inserting a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, AD Instruments) into the LV or RV right before sacrifice. The left ventricular developed pressure (LVDP) was calculated as  $LVDP = LVSP - LVEDP$ , in which LVSP is LV systolic pressure and LVEDP is LV end-diastolic pressure, and right ventricular developed pressure (RVDP) was calculated as  $RVDP = RVSP - RVEDP$ , in which RVSP is RV systolic pressure and RVEDP is RV end-diastolic pressure.

## 2.3 Left anterior descending coronary artery occlusion and measurement of infarct size

Mice were anaesthetized with ketamine (80 mg/kg i.p.) and xylazine (8 mg/kg i.p.), intubated and ventilated with a ventilator (CWESAR-830/P). The hearts were exposed through a left thoracotomy in the fourth intercostal space. The pericardium was opened, and a 7-0 Prolene suture was tightened around the proximal left anterior descending coronary artery to induce ischaemia. The ligature was removed after 30 min to start the reperfusion, and the mice were allowed to recover from anaesthesia. The mice were anaesthetized again at the end of 24 h reperfusion, and the ligature was tightened to inject 1% Evans Blue dye through the RV. The myocardial ischaemic area at risk (AAR) was identified as the region lacking blue staining. The ventricles of the hearts were sliced transversely into 2 mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 15 min to identify the non-infarcted and infarcted areas. The infarcted area was displayed as the area unstained by TTC. Infarct size was expressed as a percentage of the AAR.

## 2.4 Langendorff preparation

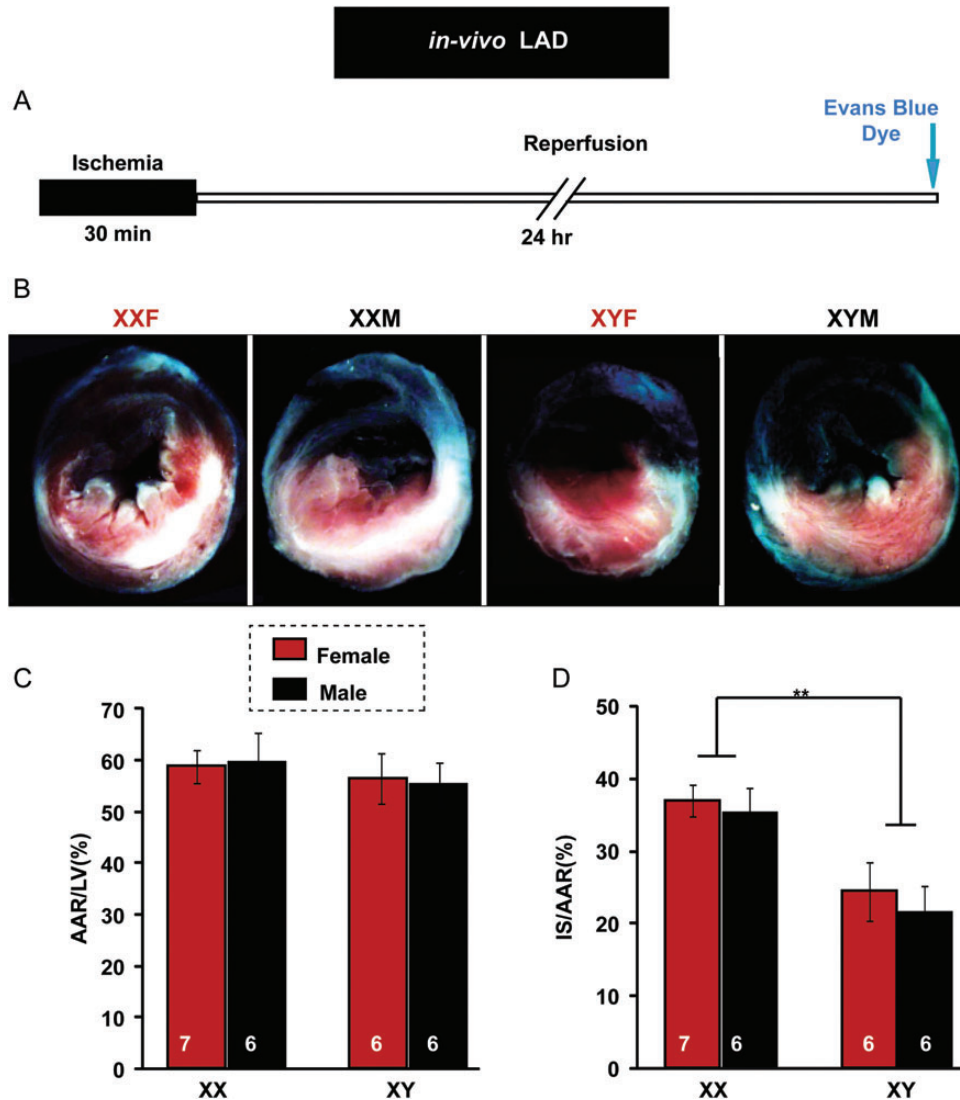
Mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and heparin (200 IU/kg) was injected to prevent blood coagulation. The heart was quickly removed and perfused through the aorta in a Langendorff apparatus with Krebs–Henseleit bicarbonate buffer solution (in mM): glucose 11.1, NaCl 118, KCl 4.7, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, CaCl<sub>2</sub> 2 at pH 7.4 bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C. Once equilibration was achieved, the aorta was clamped for 30 min to induce global normothermic (37°C) ischaemia, followed by 60 min to measure infarct size. For measurement of mitochondrial calcium retention capacity (CRC), the duration of reperfusion was 10 min, when mitochondrial Ca<sup>2+</sup> overload increases strikingly.<sup>16</sup> Thus, we isolated mitochondria at 10 min of reperfusion to measure at an earlier time point the initial mitochondrial dysfunction as we have published recently.<sup>17–19</sup>

**Table 1** Cardiac structure of FCG mice at the baseline

	<b>FXX</b>	<b>MXX</b>	<b>FX Y</b>	<b>MX Y</b>
	<b>Three mice</b>	<b>Four mice</b>	<b>Four mice</b>	<b>Four mice</b>
IVS (mm)				
Diastolic	0.60 ± 0.04	0.70 ± 0.04	0.57 ± 0.04	0.64 ± 0.04
Systolic	1.06 ± 0.14	0.99 ± 0.04	1.02 ± 0.12	0.93 ± 0.02
LV PW (mm)				
Diastolic	0.71 ± 0.04	0.72 ± 0.02	0.64 ± 0.05	0.66 ± 0.04
Systolic	1.12 ± 0.11	1.00 ± 0.09	0.96 ± 0.05	0.98 ± 0.03
LV diameter (mm)				
Diastolic	2.88 ± 0.12	3.15 ± 0.08	3.56 ± 0.20	3.28 ± 0.03
Systolic	1.91 ± 0.05	2.00 ± 0.01	2.32 ± 0.21	2.28 ± 0.07

Values are mean ± SEM. All the parameters were obtained from M-mode images of parasternal short-axis view. There is no significant differences between the groups for any of the parameters.

IVS, intraventricular septum; LV PW, left ventricular posterior wall; LV diameter, left ventricular diameter.



**Figure 2** Larger *in vivo* myocardial infarct size in GDX FCG XX mice compared with XY, irrespective of gonadal type. (A) In the experimental protocol, the LAD is occluded in GDX FCG mice for 30 min followed by 24 h of reperfusion. (B) Representative TTC staining, the white area represents infarcted area, blue shows the non-infarcted area, and red plus white areas show risk area. (C) Percentage of AAR divided by the LV. (D) Infarct size (IS) divided by AAR. \*\* $P < 0.01$ ,  $n = 6-7$ .

## 2.5 Heart functional measurements *ex vivo*

A catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, AD Instruments) directly inserted into the LV to measure LVSP, LVEDP, and heart rate (HR). The LVDP was calculated as  $LVDP = LVSP - LVEDP$  and the rate pressure product (RPP) as  $RPP = HR \times LVDP$ . The maximum rate of rise of LV pressure ( $dP/dt_{max}$ ) and the maximum isovolumetric rate of relaxation ( $-dP/dt_{min}$ ) were directly calculated from the recordings.

## 2.6 Myocardial necrosis *ex vivo*

At the end of reperfusion, the hearts were cut into slices and myocardial necrosis was assessed by measurement of the infarct size using TTC staining.<sup>17-19</sup> The slices were fixed in 4% paraformaldehyde. The area of necrosis was quantified in Photoshop and expressed as the percentage of total ventricular (LV) area.

## 2.7 $Ca^{2+}$ -induced mitochondrial permeability transition

### 2.7.1 Preparation of isolated mitochondria

Mitochondria were isolated as previously described.<sup>17-19</sup> In brief, myocardial sections of *ex vivo* hearts (~0.15–0.22 g) were placed in isolation buffer A (containing in mM: 70 sucrose, 210 mannitol, 1 EDTA, and 50 Tris-HCl, pH 7.4 at 4°C). The tissue were finely minced with scissors and homogenized in the same buffer A (1 mL of buffer/0.1 g of tissue) using Kontes and Potter-Elvehjem tissue grinders. The homogenate was centrifuged at 1300 g for 3 min; the supernatant was filtered through cheesecloth and centrifuged at 10 000 g for 10 min. The mitochondrial pellet was resuspended in isolation buffer B (containing in mM: 70 sucrose, 210 mannitol, 0.1 EDTA, and 50 Tris-HCl, pH 7.4). The mitochondrial protein concentration was measured using the Bradford assay.



### 2.7.2 Calcium retention capacity

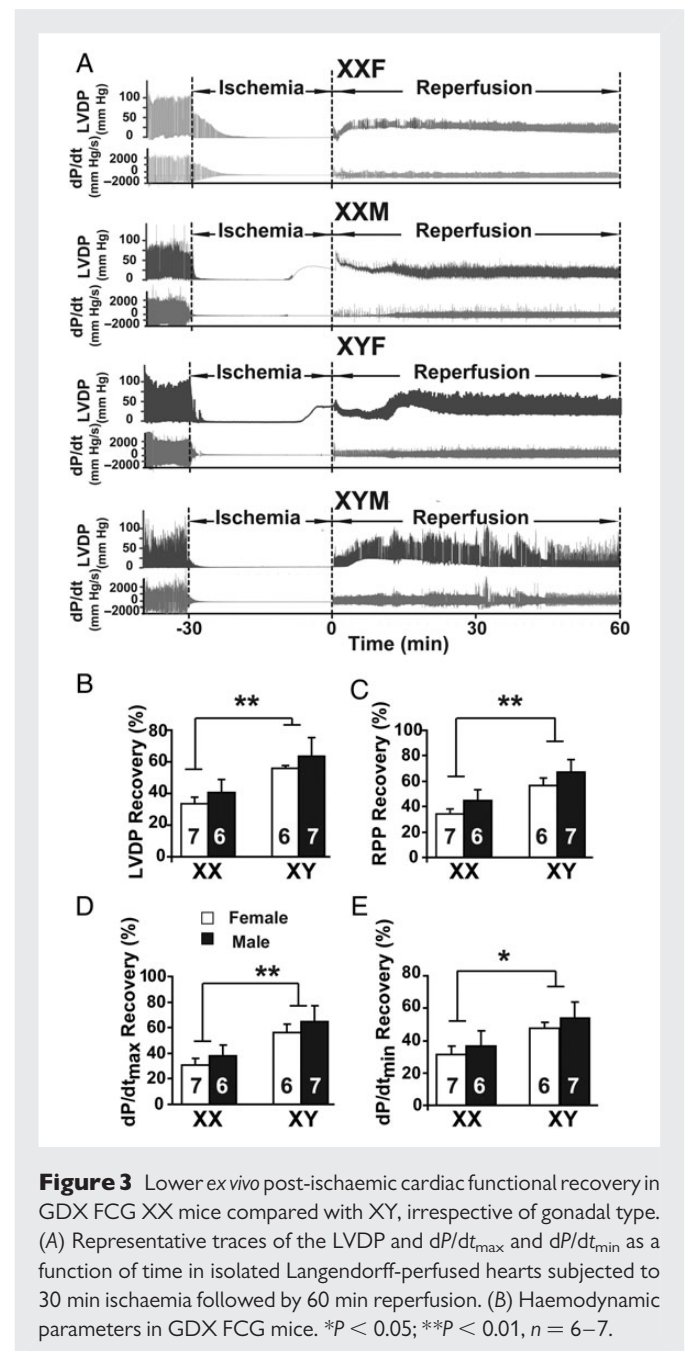
The installation of mitochondrial permeability transition pore (mPTP) opening was assessed following *in vitro*  $\text{Ca}^{2+}$  overload as previously described.<sup>17–19</sup> The free  $\text{Ca}^{2+}$  concentration outside the mitochondria was recorded with 0.5  $\mu\text{M}$  calcium green-5N (Invitrogen, Carlsbad, CA, USA) using excitation and emission wavelengths set at 500 and 530 nm, respectively. Isolated mitochondria (500  $\mu\text{g}$  of protein) were suspended in 2 mL of buffer C (mM, 150 sucrose, 50 KCl, 2  $\text{KH}_2\text{PO}_4$ , 5 succinic acid, and 20 Tris-HCl, pH 7.4). Samples were pre-incubated for 90 s in the spectrofluorometer cell, and  $\text{CaCl}_2$  pulses (20 nmol) were applied every 60 s in the spectrofluorometer. The  $\text{Ca}^{2+}$  pulses induced a peak of extra-mitochondrial  $\text{Ca}^{2+}$  concentration that returned to near-baseline level as  $\text{Ca}^{2+}$  entered the mitochondrial matrix via the uptake by the  $\text{Ca}^{2+}$  uniporter. With increasing calcium loading, the extra-mitochondrial  $\text{Ca}^{2+}$  concentration started accumulating, reflecting a lower capacity for mitochondria  $\text{Ca}^{2+}$  uptake, which was followed by a sustained  $\text{Ca}^{2+}$  increase indicating a massive release of the mitochondria  $\text{Ca}^{2+}$  by the mPTP opening. The CRC was defined as the amount of  $\text{Ca}^{2+}$  required to trigger this massive  $\text{Ca}^{2+}$  release, which was used here as an indicator of the mPTP sensitivity to  $\text{Ca}^{2+}$ . CRC was expressed as nmolar of  $\text{CaCl}_2$  per mg of mitochondrial protein.

### 2.8 Quantitative real-time PCR

WT XX female and XY male mice were GDX and 2 weeks later (3 months old) were used for the experiment. RNA was isolated from whole heart using Trizol (Invitrogen) and treated with RNase-free DNase (Promega, Madison, WI, USA) to eliminate possible genomic DNA contamination. First-strand cDNA synthesis was generated by reverse transcription with SuperScript™ III RNase H-RT (Invitrogen). Quantitative real-time PCR (RT-PCR;  $n = 7$  for males and  $n = 8$  for females) was performed using an ABI 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) with the SensiMixPlusSYBR Green & Fluorescein Master Mix Kit (Quanta, USA). Expression was measured relative to the house-keeping gene *B2M* (beta-2 macroglobulin). Primer sequence for *B2M*: forward: 5'-TGGTGCTTGTCTCACTGACC-3' and reverse: 5'-GTAT GTTCGGCTTCCCATTCC-3'. Five X-linked genes previously reported to escape X inactivation<sup>14,20</sup> were studied here. Primer sequence for *Kdm5c*: forward: 5'-ACCCACCTGGCAAACATTGG-3' and reverse: 5'-ACTGTGCGAAGGGGGATGCTGTG-3'. Primer sequence for *Kdm6a*: forward: 5'-CCAATCCCCGCAGAGCTTACCT-3' and reverse: 5'-TTG CTGGAGCTGTTCCAAGTG-3'. Primer sequence for *Ddx3x*: forward: 5'-GGATCACGGGGTGATTCAAGAGG-3' and reverse: 5'-CTATCT CCACGGCCACCAATGC-3'. Primer sequence for *Eif2s3x*: forward: 5'-TTGTGCCGAGCTGACAGAATGG-3' and reverse: 5'-CGACAGGG AGCCTATGTTGACCA-3'. Primer sequence for *Usp9x*: forward: 5'-GCA TGTACGCGATTTTTCCGAGA-3' and reverse: 5'-CACATAGCTCCA CCAGGCGATG-3'. Quantitative PCR cycling conditions were: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Standard curves for each target gene and control *B2M* were constructed with four serial dilutions.<sup>21</sup>

### 2.9 Statistical analyses

FCG groups were compared using two-way ANOVA [SPSS, version 13.0, (SPSS Inc, Chicago Ill)] with main factors of sex (gonadal male vs. gonadal female, same as Sry present vs. absent) and sex chromosome complement (XX vs. XY). XY\* groups were compared using two-way ANOVA with main factors of number of X chromosomes (1 vs. 2) and the presence or absence of the Y chromosome (present vs. absent). XX vs. XY differences in gene expression were evaluated with a one-way ANOVA. Results were summarized with means  $\pm$  standard errors of the mean (SEM). A  $P$ -value  $< 0.05$  was considered statistically significant.

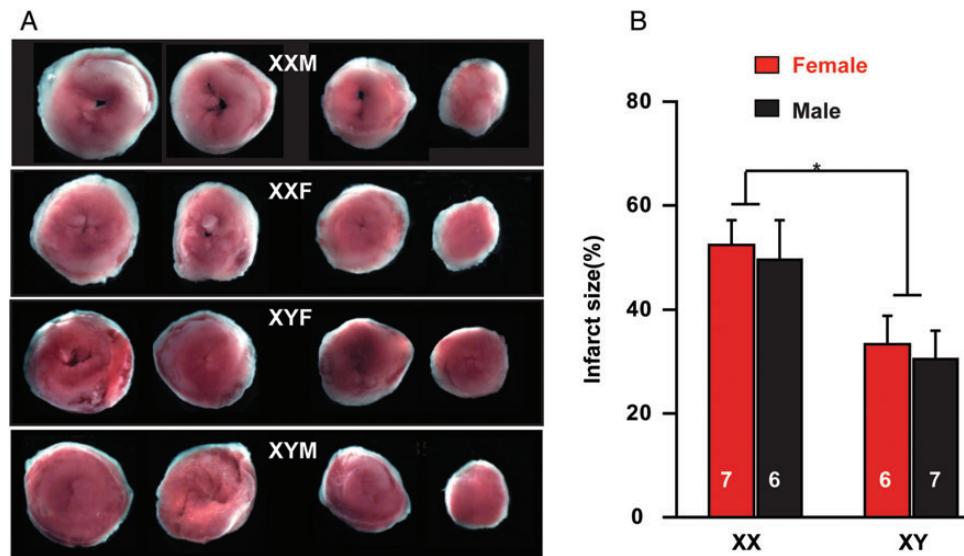


**Figure 3** Lower *ex vivo* post-ischaemic cardiac functional recovery in GDX FCG XX mice compared with XY, irrespective of gonadal type. (A) Representative traces of the LVDP and  $dP/dt_{\max}$  and  $dP/dt_{\min}$  as a function of time in isolated Langendorff-perfused hearts subjected to 30 min ischaemia followed by 60 min reperfusion. (B) Haemodynamic parameters in GDX FCG mice. \* $P < 0.05$ ; \*\* $P < 0.01$ ,  $n = 6-7$ .

## 3. Results

### 3.1 Similar heart function and structure of GDX FCG mice at baseline

The heart structure and function of GDX FCG mice at baseline was compared among four groups. *Table 1* summarizes that cardiac structure was similar between the four groups. There were no significant differences between LV EF and RV EF in XX mice compared with XY mice (both gonadal males and females; *Figure 1C and D*). Direct measurements of developed pressure in LV and RV also revealed no significant differences in these parameters between four groups of FCG mice (*Figure 1E and F*). These results confirm that heart structure and function of GDX FCG mice at baseline is comparable among the four groups.



**Figure 4** Larger *ex vivo* myocardial infarct size in GDX FCG XX mice compared with XY, irrespective of gonadal type. (A) Four slices of the same heart in each group after TTC staining. The white area represents the infarct zone and the red shows the viable area. (B) The area of necrosis as the percentage of total ventricular area. \* $P < 0.05$ ,  $n = 6-7$ .

### 3.2 Myocardial infarct size is larger in GDX FCG XX than in XY mice *in vivo*, irrespective of gonadal type

We first examined the susceptibility of GDX FCG mice to I/R injury in the *in vivo* model (Figure 2A). Representative cross sections of GDX FCG hearts are shown in Figure 2B. Although all the four groups of mice were subjected to a comparable degree of ischaemic risk (AAR/LV:  $56.4 \pm 4.3\%$  in XYF and  $55.4 \pm 4.9\%$  in XYM vs.  $58.8 \pm 5.4\%$  in XXF and  $59.9 \pm 3.3\%$  in XXM; Figure 2C), the infarct size was significantly smaller in XY mice compared with XX mice, irrespective of gonadal type (infarct size:  $24.5 \pm 4.1\%$  in XYF and  $21.8 \pm 3.3\%$  in XYM vs.  $37.0 \pm 3.2\%$  in XXF and  $35.5 \pm 2.1\%$  in XXM,  $P < 0.01$ ; Figure 2D).

### 3.3 Lower haemodynamic cardiac functional recovery after ischaemia in XX mice compared with XY mice, irrespective of gonadal sex

Next, we used isolated Langendorff-perfused hearts to examine the effect of sex chromosome on post-ischaemic heart function and myocardial infarct size *ex vivo*. Typical examples of LVDP and  $dP/dt$  are shown in Figure 3A. Although the LVDP and  $dP/dt$  were comparable in all four groups before ischaemia, the functional recovery after ischaemia was very poor in XX mice compared with XY mice, regardless of their gonadal sex. The LVDP recovery at the end of 60 min reperfusion was significantly lower in XX mice (XXF =  $31.1 \pm 5.5\%$  and XXM =  $40.4 \pm 8.3\%$ ,  $P < 0.01$ ) compared with XY mice (XYF =  $55.9 \pm 1.9\%$  and XYM =  $63.4 \pm 11.9\%$ ; Figure 3B). The RPP was also significantly lower in XX mice ( $31.2 \pm 5.8\%$  in XXF and  $44.7 \pm 8.8\%$  in XXM,  $P < 0.01$ ) than in XY mice ( $56.6 \pm 5.8\%$  in XYF and  $67.2 \pm 10.1\%$  in XYM) at the end of reperfusion (Figure 3C).

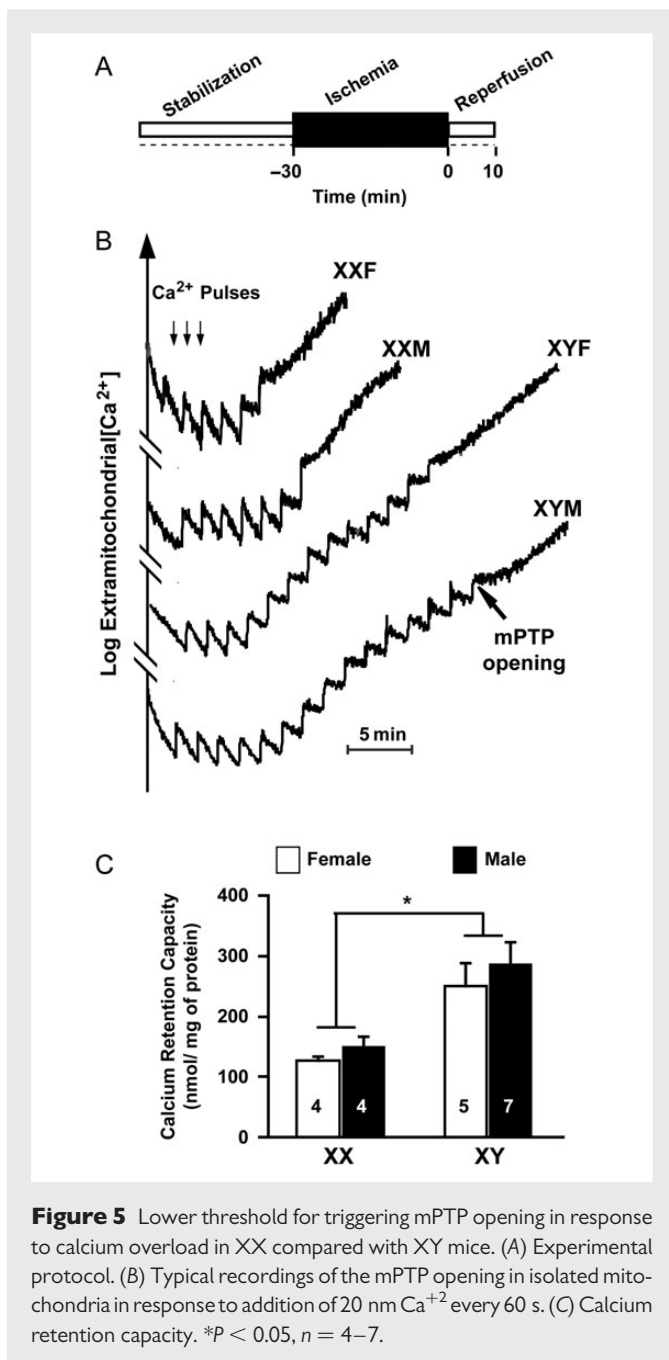
The XY mice also showed a much higher LV  $dP/dt_{\max}$  and LV  $dP/dt_{\min}$  compared with XX mice ( $dP/dt_{\max} = 56.3 \pm 6.6\%$  in XYF and  $64.5 \pm 12.7\%$  in XYM vs.  $30.1 \pm 5.7\%$  in XXF and  $37.6 \pm 9.0\%$  in XXM,  $P < 0.01$ ;  $dP/dt_{\min} = 47.4 \pm 3.7\%$  in XYF and  $53.9 \pm 10.1\%$  in XYM vs.  $30.5 \pm 5.5\%$  in XXF and  $36.6 \pm 9.6\%$  in XXM,  $P < 0.05$ ; Figure 3D and E). There were no significant differences in the HRs among the four groups before ischaemia ( $220 \pm 22$  bpm in XXF;  $234 \pm 29$  bpm in XXM;  $229 \pm 23$  bpm in XYF; and  $230 \pm 15$  bpm in XYM) and at the end of the reperfusion ( $214 \pm 20$  bpm in XXF;  $257 \pm 32$  bpm in XXM;  $226 \pm 20$  bpm in XYF; and  $256 \pm 17$  bpm in XYM).

### 3.4 Larger myocardial infarct size in GDX FCG XX than XY mice, irrespective of gonadal type

Consistent with our *in vivo* data, the infarct size was significantly larger in XX than XY GDX mice *ex vivo* ( $52.4 \pm 4.7\%$  in XXF and  $49.4 \pm 7.6\%$  in XXM vs.  $33.2 \pm 5.6\%$  in XYF and  $30.5 \pm 5.5\%$  in XYM,  $P < 0.05$ ; Figure 4).

### 3.5 Lower threshold for triggering mPTP opening in response to calcium overload in XX compared with XY

Since the mPTP plays a key role in setting the level of myocardial injury, we compared the threshold for triggering cardiac mPTP opening in response to  $Ca^{2+}$  overload in mitochondria isolated from the hearts of GDX FCG mice subjected to I/R injury (Figure 5A). A typical example of the time course of  $Ca^{2+}$  concentration in the mitochondrial external medium in four groups of FCG mice is shown in Figure 5B. The number of Ca pulses to trigger mPTP opening was significantly higher in XY mice compared with XX mice (13 pulses in XYF and 15 pulses in XYM vs. 6 pulses in XXF and XXM). The CRC was about two-fold higher in XY than XX mice (nmol/mg protein:  $250 \pm 56$  in XYF and  $286 \pm 51$  in XYM vs.  $126 \pm 9$  in XXF and  $192 \pm 45$  in XXM,  $P < 0.05$ ; Figure 5C).



**Figure 5** Lower threshold for triggering mPTP opening in response to calcium overload in XX compared with XY mice. (A) Experimental protocol. (B) Typical recordings of the mPTP opening in isolated mitochondria in response to addition of 20 nM  $\text{Ca}^{2+}$  every 60 s. (C) Calcium retention capacity. \* $P < 0.05$ ,  $n = 4-7$ .

### 3.6 The higher susceptibility of XX mice to I/R is due to the number of X chromosomes rather than the absence of the Y chromosome

To explore whether the higher susceptibility of XX mice to I/R injury is due to the number of X chromosomes and/or the absence of the Y chromosome, we used the XY\* model to compare susceptibility to I/R injury in mice having one X chromosome (XY,  $\text{XO}^{+\text{PAR}}$ ) with mice having two X chromosomes (XX, XXY). The functional recovery of mice with two copies of the X chromosome was significantly lower than mice with a single copy, irrespective of gonadal sex. The LVDP recovery at the end of 60 min reperfusion was significantly lower in mice with two X chromosomes ( $30.3 \pm 9.8\%$  in XX gonadal females and  $27.5 \pm 12.4\%$  in XXY gonadal males) compared with mice with

one X chromosome ( $70.5 \pm 19.5\%$  in  $\text{XO}^{+\text{PAR}}$  females and  $48.2 \pm 14.0\%$  in XY males,  $P < 0.05$ ; Figure 6A). The RPP recovery was markedly lower in two X chromosomes compared with one X chromosome ( $34.0 \pm 10.3\%$  in XX gonadal females and  $28.0 \pm 12.1\%$  in XXY gonadal males vs.  $60.3 \pm 13.5\%$  in  $\text{XO}^{+\text{PAR}}$  females and  $60.5 \pm 13.8\%$  in XY males,  $P < 0.05$ ; Figure 6B).  $dP/dt_{\text{max}}$  and  $dP/dt_{\text{min}}$  were also significantly lower in mice with two copies of X compared with mice with one copy of X ( $dP/dt_{\text{max}}$ :  $24.8 \pm 9.6\%$  in XX gonadal females and  $21.8 \pm 11.5\%$  in XXY gonadal males vs.  $56.3 \pm 17.5\%$  in  $\text{XO}^{+\text{PAR}}$  females and  $45.7 \pm 15.9\%$  in XY males,  $P < 0.05$ ;  $dP/dt_{\text{min}}$ :  $25.3 \pm 7.8\%$  in XX gonadal females and  $30.6 \pm 11.8\%$  in XXY gonadal males vs.  $64.4 \pm 18.4\%$  in  $\text{XO}^{+\text{PAR}}$  females and  $46.9 \pm 14.0\%$  in XY males,  $P < 0.05$ ; Figure 6C and D). There were no significant differences in the HRs among the groups before ischaemia ( $180 \pm 16$  bpm in XXF;  $178 \pm 18$  bpm in XXM;  $179 \pm 24$  bpm in XYF; and  $193 \pm 19$  bpm in XYM) and at the end of reperfusion ( $190 \pm 19$  bpm in XXF;  $195 \pm 16$  bpm in XXM;  $167 \pm 24$  bpm in XYF; and  $241 \pm 26$  bpm in XYM).

The mice with two X chromosomes also had larger infarct size compared with those with one X ( $41.4 \pm 8.9\%$  in XX gonadal females and  $46.3 \pm 9.5\%$  in XXY gonadal males vs.  $23.7 \pm 3.9\%$  in  $\text{XO}^{+\text{PAR}}$  females and  $26.6 \pm 6.9\%$  in XY males,  $P < 0.05$ ; Figure 6E and F).

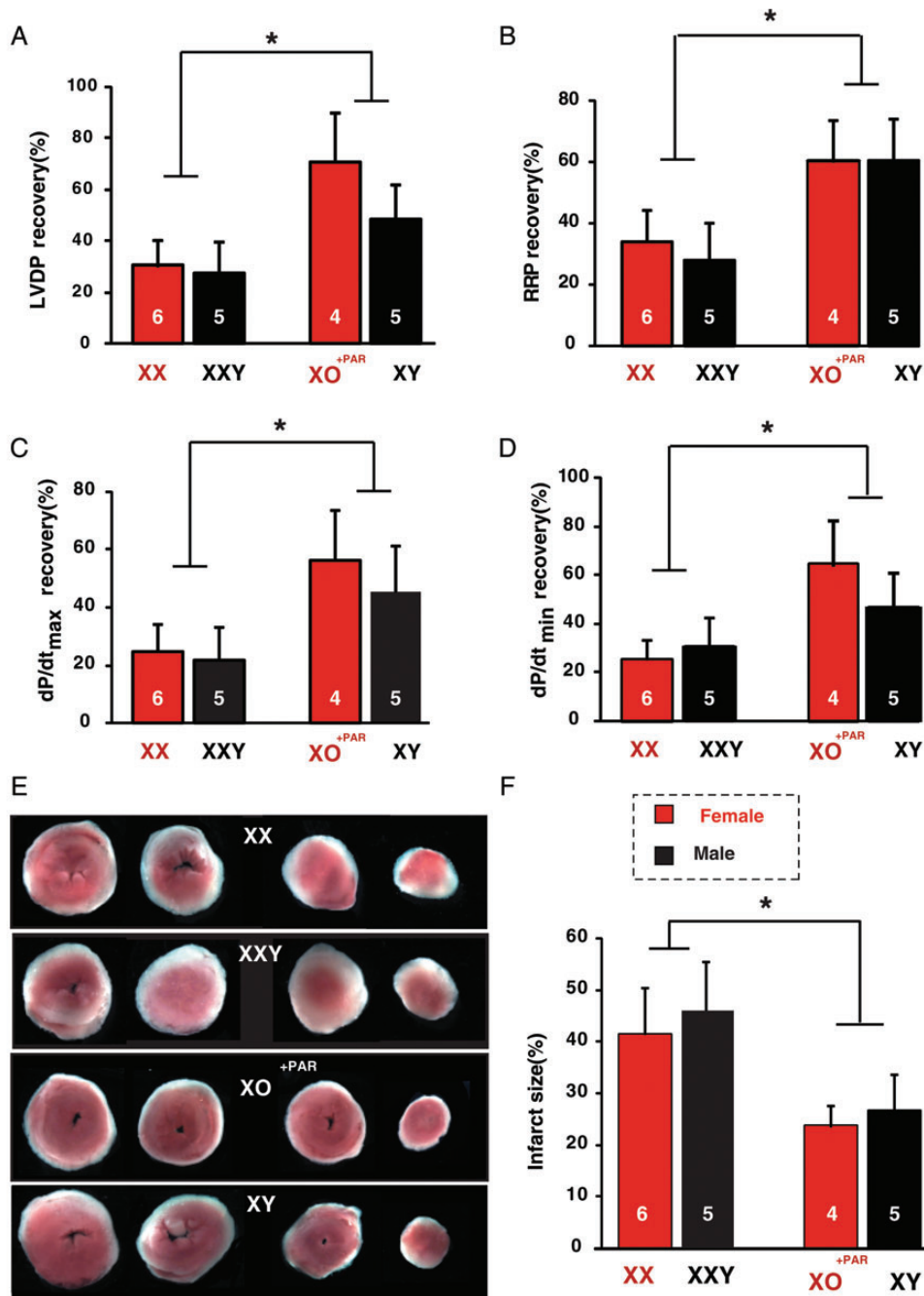
### 3.7 The expression of four X genes escaping inactivation was higher in XX than XY

One hypothesis is that genes that escape X inactivation cause the effects of X chromosome dose found here. Several such X 'escapees' are routinely found to be expressed higher in mice with two X chromosomes than those with one X chromosome, in a variety of tissues.<sup>14,20,22,23</sup> We examined the expression levels in XX and XY hearts of five X escapees. We found higher expression of four of these genes in the heart tissue from XX relative to XY mice: *Eif2s3x*, *Kdm6a*, *Kdm5c*, and *Usp9x* ( $P < 0.05$ ; Figure 7). *Ddx3x* showed higher expression in XX than XY, but the difference was not statistically significant. These results point to at least four of the five genes as likely candidates for causing the X chromosome effects reported here.

## 4. Discussion

Using the two novel mouse models, FCG and XY\*, we uncovered a surprisingly large effect of sex chromosome complement on myocardial I/R injury. Although GDX FCG mice have similar heart function at baseline, XX mice have much larger myocardial infarct size, both *in vivo* and *ex vivo*, compared with XY mice, irrespective of their gonadal sex. The higher susceptibility of XX mice to I/R injury was associated with lower mitochondrial CRC for triggering mPTP opening. Our data also demonstrate that the higher susceptibility of XX mice to I/R injury, compared with XY, is due to the number of X chromosomes rather than the absence of a Y chromosome. We identify four genes escaping inactivation that are constitutively expressed higher in the hearts of mice with two X chromosomes compared with those with one X chromosome, which are therefore candidate genes causing the X chromosome effect. The use of two different mouse models provided independent replication of the sex chromosome effect, indicating that it is not an artefact of either model. These data represent the first discovery that the number and type of sex chromosomes dramatically affects susceptibility to I/R insult. Thus, a genetic difference that differentiates every female from male cell appears to have an important influence in the heart.



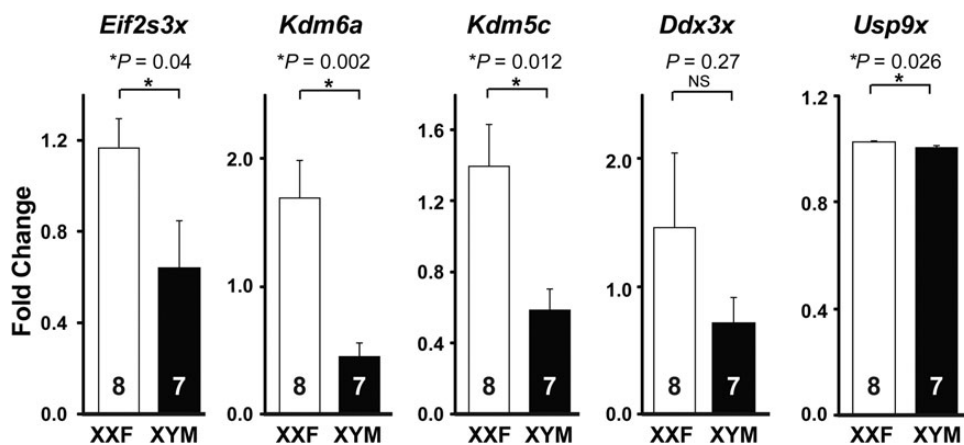


**Figure 6** Lower heart functional recovery and larger infarct size of mice with 2X chromosomes compared with mice with 1X chromosome. (A) LVDP, (B) RPP, (C)  $dP/dt_{max}$ , and (D)  $dP/dt_{min}$  in XY\* mice. \* $P < 0.05$ ,  $n = 4-6$ . (E) Four slices of the same heart in each XY\* mice after TTC staining. The white area represents the infarct zone and the red shows the viable area. (F) The area of necrosis as the percentage of total ventricular area. \* $P < 0.05$ ,  $n = 4-6$ .

The mPTP is a large non-selective conductance pore located in the inner membrane of mitochondria. The mPTP remains closed during ischaemia, but opens during the reperfusion period.<sup>24</sup> The opening of the mPTP during reperfusion has been implicated in cell death.<sup>25,26</sup> Our data demonstrate that the higher susceptibility of XX hearts to I/R injury is associated with higher mPTP sensitivity to  $Ca^{2+}$ , as the mitochondrial calcium uptake required for the opening of the mPTP was significantly lower in XX hearts compared with XY. The homeostasis of cardiomyocytes in regulating calcium overload is

decreased in XX hearts, resulting in a lower threshold for triggering the opening of the mPTP.

Investigating the sex-specific or sex-biased factors that protect the heart from disease could uncover unexplored protective mechanisms that might be targets for novel therapies. Historically, most animal studies have focused primarily on the important role of gonadal hormones, especially oestrogens, on myocardial I/R injury.<sup>4</sup> Oestrogens exert their cardioprotective action against I/R injury in OVX mice<sup>7</sup> and also reduce the infarct size in male mice.<sup>27</sup> The lower incidence of



**Figure 7** Higher cardiac expression levels of *Eif2s3x*, *Kdm6a*, *Kdm5c*, and *Usp9x* in XXF compared with XY male. Fold-change expression levels of five X genes thought to escape inactivation in the heart of GDX XX female and XY male. \* $P < 0.05$ ,  $n = 7-8$ .

myocardial I/R injury in pre-menopausal women compared with men has also been attributed to oestrogens.<sup>3</sup> The fact that young women who had an oophorectomy had higher incidence of coronary artery disease<sup>5</sup> further supports the protective role of female gonadal hormones. The women's health initiative study, however, failed to support the ability of hormone replacement therapy in postmenopausal women in reducing the risk of ischaemic heart disease.<sup>8,9</sup> Regarding the role of testosterone on post-ischaemic heart function or infarct size, however, the reports are conflicting. Testosterone therapy of GDX female or male rats has been shown either to have no deleterious effects on myocardial function or infarct size after ischaemia,<sup>28</sup> or to protect the male rats from myocardial injury.<sup>29</sup> These contradictory results on the role of sex hormones raise the question whether sex hormones alone account for the sex differences. Unfortunately, it was not possible previously to explore whether the number or the type of sex chromosomes also play a role since the XX sex chromosome complement was always confounded with ovaries, and the XY complement with testes. Using mouse models that break that confound, we have discovered that sex chromosomes play an important role in susceptibility to myocardial I/R injury in GDX mice. The results suggest that the number and type of sex chromosomes dramatically affects susceptibility to I/R insult, revealing a protective mechanism that is different in the two sexes.

Gonadal hormones have previously been thought (based on classic sexual differentiation theory) to cause all sex differences in non-gonadal phenotypes.<sup>30,31</sup> Sex steroid hormones such as oestrogens and androgens are also produced in non-gonadal tissues such as adipose tissue. However, E2 levels in ovariectomized mice are ~1 pg/mL, well below the physiological levels in intact mice, suggesting that the gonads are the main source of oestrogens.<sup>32</sup> Our experiments were performed 4 weeks after gonadectomy, a time when there is no XX vs. XY difference in body weight and adiposity of the four genotypes of FCG mice (ref.<sup>14</sup> and unpublished). Thus, the larger infarct size in XX mice compared with XY mice 4 weeks after gonadectomy in our study is not due to differences in body weight or adiposity. Whether sex chromosome complement causes differences in hormonal secretions from non-gonadal tissues in GDX mice is unknown, and thus non-gonadal hormonal mechanisms are among the possible downstream mediators of the sex chromosome effect.

Our results are found in the absence of gonadal hormones in adult mice. The results suggest that similar XX vs. XY differences may occur in humans, especially under hypogonadal conditions such as after menopause or in aging men. Nevertheless, the chromosomal difference is present throughout the lifetime of individuals and could bias cell function at numerous life phases and conditions of health and disease. The greater susceptibility to I/R injury of XX hearts, relative to XY, goes in the opposite direction from the sex difference in susceptibility in young adult humans, where females are protected relative to males. That observation underscores the idea that there are multiple sex-biasing factors, biological (hormonal and genetic) and social, that might counteract each other,<sup>12</sup> and that understanding the effect of each is required to harness the information for better treatment of cardiovascular disease. In particular, future studies are required to understand how the effects of gonadal hormones synergize with, or antagonize, the sex chromosome effects found here.

Several types of genetic factors could account for the differences in cardiac functional recovery after I/R injury in mice with two vs. one X chromosomes.<sup>30,33</sup> These include the presence of a paternal imprint on X genes in XX but not in XY cells, and an indirect effect of the inactive X chromosome, only in XX cells, on the epigenetic status and expression of autosomal genes.<sup>34</sup> The most likely difference, however, may be that XX cells show constitutively higher expression of a small number of genes that escape X inactivation, in contrast to the large majority of X genes that are inactivated and thus expressed at similar levels in XX and XY cells.<sup>14,20,22</sup> In the present study, in hearts of XX relative to XY GDX mice, we found significantly higher expression of four of the genes that escape inactivation: *Eif2s3x*, *Kdm6a*, *Kdm5c*, and *Usp9x* ( $P < 0.05$ ; Figure 7). *Ddx3x* showed higher expression in XX than XY, although the difference was not statistically significant. These results point to at least four genes as possible candidates for causing the sex chromosome effects reported here. To corroborate these findings, we also analysed expression of the same genes in hearts from gonadally intact male and female mice, using appropriate multiway ANOVAs, as reported in online microarray profiling studies (GEO databases: GSE18224, GSE25700, and GSE23294). In agreement with our findings, expression of *Eif2s3x*, *Kdm6a*, and *Kdm5c* were significantly higher in XX than XY in at least two of the three studies. Expression of *Usp9x* was not significantly higher in XX than XY, a

finding that corresponds to our finding of very similar levels of expression in XX vs. XY, even though the small difference was statistically significant in our study. In microarray analyses, *Ddx3x* was significantly higher in females than in males, reinforcing the possibility that the higher expression in our study might become statistically significant if more samples were measured. Differences among the studies are attributable to different experimental conditions, different methods for measuring transcript levels (microarray vs. quantitative RT-PCR), and the difference in gonadal status. The X escapees include *Eif2s3x*, a translation initiation factor, and two histone demethylases, *Kdm5c* and *Kdm6a*, that likely affect transcription of numerous autosomal genes. *Kdm6a* is required for proper embryonic development of the heart.<sup>35</sup> *Usp9x*, a ubiquitin-specific protease, is implicated cell death pathways and negatively regulates mTOR,<sup>36</sup> which is reported to protect from I/R injury in heart and other tissues.<sup>37</sup> These four genes are potentially powerful regulators and thus represent attractive candidates for genes causing the sex chromosome effects reported here.

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