Distinct gene-sex interactions regulate adult rat cardiomyocyte width and length independently

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Boutin-Ganache, I., S. Picard, and C. F. Deschepper. Distinct gene-sex interactions regulate adult rat cardiomyocyte width and length independently. Physiol Genomics 12: 61–67, 2002. First published November 5, 2002; 10.1152/physigeng.00121.2002.——Wistar-Kyoto (WKY) and WKY-derived hyperactive (WKHA) rats are two genetically-related inbred strains of rats that are both normotensive yet exhibit differences in left ventricular mass (LVM). We had shown previously that cardiomyocytes from male WKHA are wider than that of male WKY, and that there was genetic linkage between LVM and a locus on chromosome 5 (RNO5) in the male progeny of a F2 WKHA/WKY cross. We show here that cardiomyocyte width is linked to the same RNO5 locus in male reciprocal congenic rats derived from WKHA and WKY. Contrary to males, we found no genetic linkage between LVM and the RNO5 locus in female rats. However, ventricular hypertrophy in females might be of a different nature, because cardiomyocytes from female WKHA were shorter than their WKY counterparts (with no difference in width). The RNO5 locus contains that of the natriuretic peptide precursor A (Nppa) gene. In male congenic rats, changes in cardiomyocyte width always correlated with reciprocal changes in the LV concentration of atrial natriuretic factor (ANF, i.e., the peptide product of Nppa). Taken together with other functional data, the small size of the RNO5 locus (~63 cR) increased the likelihood that both cardiomyocyte width and LV ANF concentration could be linked to only one gene (possibly Nppa) in male rats. Moreover, our results support the notion that genes and sex interact to regulate cardiomyocyte width from one another.

left ventricular hypertrophy; sex; congenic rat strains; natriuretic peptide precursor A; cardiomyocyte shape

TO IDENTIFY GENETIC FACTORS that regulate cardiac left ventricular mass (LVM) independently of blood pressure, we have previously analyzed the genomes of the male progeny of WKY/WKHA hybrid crosses (10). Both strains are normotensive but exhibit differences in cardiac mass (11). By performing a whole-genome scan of the progeny of the cross, we have found that Rattus norvegicus chromosome 5 (RNO5) harbored in males quantitative trait loci (QTL) linked to left ventricular atrial natriuretic factor (LV ANF) and to LVM with highly significant “log of the odds ratio” (LOD) scores (10). Both QTLs map to the reported position of chromosomal band 5q36 (http://ratmap.gen.gu.se), and their 1-LOD support intervals overlap a region that contains the locus of the natriuretic peptide precursor A (Nppa) gene (i.e., the gene that governs the synthesis of the ANF precursor). However, because of the inherent statistical imprecision of linkage data, it was not possible to determine on the basis of these data alone whether these QTLs corresponded to one identical locus or to 2 contiguous ones.

To test whether the same QTLs can be detected in female rats, we screened the genomic DNA of the female progeny of our WKY/WKHA hybrid cross with markers linked to RNO5.

Moreover, to obtain a physical validation of the statistical linkage data, we generated several congenic rat strains where portions of RNO5 from WKY-derived hyperactive (WKHA) rats were introgressed into the background of Wistar-Kyoto (WKY) rats, and vice versa. To improve the precision of our phenotypic characterization procedures, we also quantitated the width and length of cardiomyocytes isolated from adult hearts. Indeed, we have shown previously that this procedure made it possible to detect interstrain differences that were larger and more reliable than those obtained by just measuring LVM (11).

MATERIAL AND METHODS

Animals. All procedures on animals were approved by the Institut de Recherches Cliniques de Montréal (IRCM) Institutional Animal Care Committee and conducted according to guidelines issued by the Canadian Council on Animal Care. The nomenclature of the strains is in compliance with the recommendations of the International Rat Genetic Nomenclature Committee. The WKHA/Cfd rats originated from a colony maintained at the IRCM, as registered with the Institute of Laboratory Animal Resources. WKY/Cfd rats also originated from a colony maintained at the IRCM and were derived from WKY/Crd parents obtained from Charles River (St. Constant, Quebec, Canada). Adult animals were housed 2–3 per cage and given unlimited access to standard chow and water. All animals were used at 12 wk of age for the purpose of these studies.

Animals procedures. On the day of tissue collection, each animal was weighed for determination of whole body weight (BW). The rats were then killed by decapitation, and hearts were collected. Heart ventricles were dissected into right ventricle (without the septal wall) and left ventricle (LV;
including the septal wall). Each part was blotted dry and weighed individually. The apex of each LV was then dissected out, frozen in liquid nitrogen, and kept at -70°C for additional analysis.

**Genetic crosses and genomic analyses.** Total progenies of 345 male and 141 females had previously been obtained by F2 WKY/WKHA hybrid crosses. Genomic DNA was obtained from the spleen of each animal, using the DNeasy tissue kit (Qiagen). To generate RNO5 linkage maps in female rats, the genomic DNA from each member of the progeny was amplified with a total of 14 M13-tailed 32P-labeled microsatellite markers, as described previously (3). These 14 markers were the same as those identified in Fig. 1. Construction of linkage maps and QTL interval mapping were performed with the help of the program Map Manager QTXb15 (Roswell Park Cancer Institute, Buffalo, NY). Significance levels were set as described previously for detection of a QTL for a codominant trait in a rat intercross (21). Linkage maps for the 345 male animals had been performed previously with the help of the MapMAKER/EXP 3.0 software package. For the sake of consistency, the male dataset was reanalyzed for the current study, using the same microsatellite markers as in females as well as the same computer program (Map Manager).

**Generation of congenic strains.** Congenic strains were generated using the marker-assisted breeding method (also known as the "speed congenic" method) (22). For one type of cross, male WKY rats (the receiving strain) and female WKHA rats (the donor strain) were first bred to produce F1 rats (where the Y chromosome originated from WKY). These F1 male rats were then backcrossed to female WKY rats to produce the first backcross generation (N2). N2 male rats were first genotyped for four markers (D5Rat173, D5Wox10, D5Rat204, and D5Rat28) delineating a region of interest on RNO5. Those that were heterozygous WKY/WKHA for each marker were selected and further genotyped with 57 additional markers covering the whole genome. From these analyses, we selected the one N2 male rat that was heterozygous WKY/WKHA for the chromosome 5 region but also contained the largest number of markers that were homozygous for WKY alleles on the remaining part of the genome. That optimal breeder was then backcrossed to 20 female WKY rats to produce the N3 progeny. This sequence of procedures was repeated until generation N5, where the only markers that were detected as heterozygous WKY/WKHA were those delineating three different regions on RNO5. With the help of genotyping with additional markers mapping to the same

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**Fig. 1.** Physical map of RNO5 from each congenic strain. The position of each marker is indicated on the left, with distances indicated either in cM (from the genetic map) or in cR (from the radiation hybrid map maintained at the Rat Genome Database [http://www.rgd.mcw.edu]). Open bars represent regions originating from WKHA, solid bars represent regions originating from WKY, and gray bars represent regions whose origin could not be determined because of the absence of polymorphic markers within the intervals. The marker D5Wox10 (in bold) is comprised within the Nppa gene (whose location is further indicated by arrows).
region, these regions corresponded to the intervals D5Rat173-D5Mgh16, D5Rat45-D5Rat245, and D5Rat28-D5Rat45 (Fig. 1). At that point, ~100% of the genome outside of the introgressed fragment can be considered to originate from the recipient strain (22). The last step involved the crossing of N5 female to N5 male rats, which yielded (after genotypic selection) rats that were homozygous WKHA/WKHA for the region of interest but homozygous WKY/WKY for the rest of the genome. The three corresponding strains were designated WKY.WKHA-(D5Rat173-D5Mgh16), WKY.WKHA-(D5Rat45-D5Rat245), and WKY.WKHA-(D5Rat28-D5Rat45), in compliance with the guidelines issued by the International Committee on Standardized Genetic Nomenclature for Mice and Rats.

A second type of cross was reciprocal to the first one. It involved the crossing of male WKHA to female WKY rats to generate F1 rats, followed by repeated backcrossings to WKHA female rats. This procedure generated the WKHA.WKY-(D5Rat45-D5Mgh16) strain (Fig. 1).

ANF radioimmunoassays. Fragments of left ventricular apex (~200 mg) were weighed, powdered under liquid nitrogen, and boiled for 5 min in a volume of 2 ml of 0.2 mol/l acetic acid. The extracts were then centrifuged at 30,000 g for 30 min. Aliquots of 5 μl of supernatant were assayed, using the same procedures and reagents as described previously (16). The content of ANF immunoreactivity in LV tissue was expressed as femtomoles per milligram wet weight.

Isolated cardiomyocytes. Cardiomyocytes were isolated from five male and five female rats from each parental or congenic strain (12 wk of age). The hearts were rapidly removed from anesthetized animals previously injected intraperitoneally with 500 U heparin sulfate, and [Ca2+]tolerant cardiomyocytes were isolated by the Langendorff method (cardiac retrograde aortic perfusion), as described previously (11, 32). Cardiomyocytes were separated from non-cardiomyocytes by sedimentation on a 6% solution of bovine serum albumin, then fixed for 30 min in 0.15 mol/l phosphate buffer and rinsed with 0.15 mol/l phosphate buffer at 4°C. Both solutions have been shown to preserve the volume of fixed cells compared with unfixed ones (15). Fixed cardiomyocytes were allowed to settle in Petri dishes containing 0.15 mol/l phosphate buffer and examined with a Zeiss Axiosvert microscope connected to a video camera that allowed capture of the images as electronic files. With the use of Northern Eclipse version 6.0 software, ~100 cells from each animal were analyzed for determination of cell length (defined as the longest length parallel to the longitudinal axis of the myocyte) and cell surface (calculated on the basis of the manual contour drawn around the myocyte). Cell width was calculated by dividing the value of surface by that of length.

RESULTS

Results of the linkage analyses are shown in Fig. 2. By interval mapping, a QTL linked to LV ANF (QTL/ANFv) was found on RNO5 both for female (LOD = 6.6) and for male rats (LOD = 7.1). Similarly to what had been reported previously, a QTL linked to LV mass (QTL/LVM) was found for males (LOD = 3.7). Both QTLs mapped to the same region of RNO5, since there was partial overlap of their 1-LOD confidence interval. In contrast to our results in males, there was no statistical linkage between LVM and any region of RNO5 in female rats. Of note, the LV mass of female WKHA

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Fig. 2. Quantitative trait loci (QTL) "log of the odds ratio" (LOD) plots of chromosome 5 for ln(LV ANF) and LVM in the female and male F2 progeny from WKY/WKHA rat crosses. Numbers on the x-axis represent distances in cM. Lines have been positioned at the LOD threshold values for statistical linkage of codominant traits in a rat intercross. The hatched boxes represent the 1-LOD support intervals for either QTL/LV ANF or QTL/LVM. Dotted lines indicate the position of the D5Wox10 locus, which coincides with that of the Nppa gene. LVM, left ventricular mass; LV ANF, LV concentration of atrial natriuretic factor.
was about 11% greater than that of female WKY, with that difference being of the same magnitude as that found by us previously between their male counterparts (11).

Three congenic strains [WKY.WKHA-(D5Rat173-D5Mgh16), WKY.WKHA-(D5Rat45-D5Rat245), and WKY.WKHA-(D5Rat28-D5Rat45)] were generated by introgression of regions of RNO5 into the recipient WKY strain, whereas one reciprocal congenic strain [WKHA.WKHA-(D5Rat45-D5Mgh16)] was generated by introgression of the region of RNO5 from WKY rats into the recipient WKHA strain (Fig. 1). Table 1 shows the values of LV ANF and of the morphological variables obtained in cardiomycocytes isolated from the hearts of rats from parental or congenic strains. In male rats, the width of cardiomycocytes from WKY is smaller than that of cardiomycocytes from WKHA, whereas there was no difference in length between cells from either strain. Introgression of the D5Rat28-D5Rat45 RNO5 fragment (that laid on RNO5 outside of the QTL as previously defined by statistical linkage) from WKHA into WKY rats resulted in cardiomycocytes whose shape characteristics were not different from that of cells from the receiving WKY strain. The two other WKHA introgressed fragments both contained the QTL previously defined by statistical linkage, but differed in size. Introgression of the larger D5Rat173-D5Mgh16 RNO5 fragment from WKHA into WKY resulted in cardiomycocytes that were statistically larger than that from the receiving strain, but smaller than that from the donor WKHA strain. Introgression of the smaller D5Rat45-D5Rat245 fragment resulted in cardiomycocytes whose width was as large as that of cells from the donor WKHA strain. Reciprocally, introgression of a the D5Rat45-D5Mgh16 fragment from WKY into WKHA resulted in cardiomycocytes whose width was significantly smaller than that of the receiving WKHA strain and also smaller than that of the donor WKY strain. All differences in the width of male cardiomycocytes were accompanied by reciprocal changes in the values of LV ANF. All combined, these data define a RNO5 fragment that extends maximally from D5Rat45 (1,049 cR) and D5Rat245 (1,112 cR) and that is physically associated to both LV ANF and cardiomycocyte width in male rats.

In females, introgression of the D5Rat45-D5Rat245 fragment from WKHA into the WKY background or introgression of the D5Rat45-D5Mgh16 fragment from WKY into the WKHA background correlated well with LV ANF but had no effect on cardiomycocyte width. Unlike what we had observed in male rats, the width of cardiomycocytes from female WKHA was not different from that from female WKY (Table 2). However, the length of cardiomycocytes from female WKHA was statistically shorter than that from female WKY. Intersex comparisons revealed that: 1) the width of cardiomycocytes from female WKY was larger than that of cells from male WKY; 2) the width of female WKHA was narrower that that of cells from male WKHA; but 3) that females from both strains had a higher width/length ratio than corresponding male rats. Altogether, it appeared that the width and length of cardiomycocytes in the various strains varied in a sex- and strain-specific manner.

### DISCUSSION

The data presented herein provide concordant evidence (on the basis of both statistical linkage and physical mapping data) that a region extending maxi-

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**Table 1. Interstrain comparisons**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Strain</th>
<th>Length, µm</th>
<th>Width, µm</th>
<th>W/L</th>
<th>LV ANF, fmol/mg</th>
<th>Animal Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>WKY (receiving strain)</td>
<td>130 ± 2</td>
<td>23.3 ± 0.5</td>
<td>0.184 ± 0.007</td>
<td>643 ± 63</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>WKY.WKHA-(D5Rat28-D5Rat45)</td>
<td>131.7 ± 3.2</td>
<td>22.4 ± 0.4</td>
<td>0.175 ± 0.007</td>
<td>629 ± 69</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>WKY.WKHA-(D5Rat173-D5Mgh16)</td>
<td>127.7 ± 0.7</td>
<td>26.9 ± 0.2</td>
<td>0.218 ± 0.003</td>
<td>217 ± 15</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>WKY.WKHA-(D5Rat45-D5Rat245)</td>
<td>128.6 ± 0.3</td>
<td>29.6 ± 0.4</td>
<td>0.242 ± 0.003</td>
<td>209 ± 30</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>WKHA (receiving strain)</td>
<td>128.8 ± 1.14</td>
<td>28.8 ± 0.5</td>
<td>0.233 ± 0.004</td>
<td>185 ± 15</td>
<td>e</td>
</tr>
<tr>
<td>Male</td>
<td>WKHA (donor strain)</td>
<td>128.8 ± 1.14</td>
<td>28.8 ± 0.5</td>
<td>0.233 ± 0.004</td>
<td>185 ± 15</td>
<td>f</td>
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<tr>
<td></td>
<td>WKHA.WKHA-(D5Rat45-D5Mgh16)</td>
<td>129.3 ± 1.13</td>
<td>20.8 ± 0.5</td>
<td>0.165 ± 0.004</td>
<td>582 ± 54</td>
<td>g</td>
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<tr>
<td>Female</td>
<td>WKY</td>
<td>116 ± 2.8</td>
<td>26.1 ± 0.26</td>
<td>0.234 ± 0.008</td>
<td>604 ± 62</td>
<td>a</td>
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<tr>
<td></td>
<td>WKY.WKHA-(D5Rat45-D5Rat245)</td>
<td>115.8 ± 2.1</td>
<td>26.1 ± 0.5</td>
<td>0.234 ± 0.008</td>
<td>195 ± 30</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>WKHA/WKHA-(D5Rat28-D5Rat45)</td>
<td>107.7 ± 1.1</td>
<td>27.46 ± 0.3</td>
<td>0.264 ± 0.003</td>
<td>638 ± 58</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td>WKHA</td>
<td>107.4 ± 3.6</td>
<td>26.8 ± 0.3</td>
<td>0.261 ± 0.008</td>
<td>268 ± 28</td>
<td>j</td>
</tr>
</tbody>
</table>

Values of length, width, and of width/length ratio (W/L) of isolated adult cardiomycocytes and concentrations of ANF in left ventricle (LV) from several strains are means ± SE (n = 5 for cardiomycocytes values; n = 6–10 for LV ANF values). The 3 groups of values represent those that were used for statistical comparisons between strains (ANOVA followed by Fisher’s LSD test). To indicate statistical differences, each type of rat was assigned to an animal code letter (last column). If a variable from a given animal type was statistically different from the corresponding variable from other animal types, then the latter were identified by their respective animal code letters (added as superscripts next to the original variable). In all reported cases, the levels of significance of the differences were P < 0.01. The boxed areas indicate variables whose values are modified (as compared to that in the receiving strain) by introgression of a chromosome fragment from the donor strain.
mally between markers D5Rat45 and D5Rat245 is linked to LV ANF, LVM, and/or cardiomyocyte width in male rats but only to LV ANF in female rats. These markers define a maximal region of 63 cR, as defined by the virtual rat radiation hybrid map (http://www.rgd.mcw.edu). Considering that 1 cR equals ~155 kb (29), this corresponds to a fragment of ~10 Mb. The results from congeneric WKHA WKY-(D5Rat45-D5Mgh16) indicate that region is potentially smaller, but additional polymorphic markers are needed to elucidate the parental origin of the region between D5Rat45 and D5Rat48. These data provide a physical proof of the statistical linkage data obtained previously in male rats.

The shape and volume of isolated cardiomyocytes represent interesting surrogate end points for the phenotypic characterization of cardiac hypertrophy. Indeed, cardiomyocyte volume has been shown to change coordinately with cardiac mass over a large variety of conditions, including pressure and volume overload, hyperthyroidism, and the postmyocardial infarction period (14, 19, 35). Adult cardiomyocytes have a characteristic cylindrical shape (13), whose volume is determined in part by their respective length and width. In physiological conditions, the width/length ratio is tightly regulated within a very narrow range, as it remains unchanged in growing rats with body weights ranging from 75 to 750 g (26). Likewise, the value of this ratio has been shown to be highly conserved in a wide range of mammalian species (6, 12). We have also found that the calculation of the mean width/length ratio of cardiomyocytes provided a more discriminating phenotype with which to compare WKHA and WKY hearts, since the mean values of LV mass in the two strains were separated by only 1.3 SD, whereas the mean values of the width/length ratio were separated by as much as 6.3 SD (9, 11).

Under certain pathophysiological conditions, the width and/or length of cardiomyocytes may change in a disproportionate fashion. For instance, the width (and/or cross-sectional area) of cardiomyocytes increases disproportionately during pressure overload, whereas their length increases disproportionately in the course of dilated cardiac failure (12). There are also previous examples where the shape of cardiomyocytes was shown to vary in a strain- and sex-specific manner. Indeed, the cross-sectional area/length ratio has been found to differ between outbred Sprague-Dawley rats from two different colonies (5). Likewise, the length of cardiomyocytes from Sprague-Dawley rats from one specific outbred colony has been found to be shorter than that of their male counterparts (1). Each of these shape-related changes may be associated with specific functional consequences. Indeed, increases in the width of cardiomyocytes correspond to the addition of sarcomeres in parallel, which may lead to increased contractility because of the increased density of contractile units per unit of muscle tissue (2). Conversely, it has been shown that increased cell length correlates closely with ventricular dilation and/or impaired mechanical performance in hearts during progression toward failure (20, 30).

Contrary to males, cardiomyocytes from female WKHA and WKY differed in length and not in width. However, the relative width of female cardiomyocytes (when normalized to length) was larger than in males. Of note, the progression from hypertrophy to failure is more rapid in males than in females in spontaneously hypertensive heart failure (SHHF) rats, which correlates well with the shorter length of female cardiomyocytes (31). Furthermore, the contractility of cardiac papillary muscles from Wistar rats has been shown to be greater in females than in males (7), which may correlate with the greater relative width of female cardiomyocytes. These sex-specific differences in shape and/or function may be due in part to differences in sex hormones, since gonadectomy has been shown to alter the contractile performance of hearts from both males and females (25).

All changes in cardiomyocyte width in males correlated well with inverse changes in LV concentration of ANF (whose Nppa gene is contained within the introgressed fragment) in the same strains. Interestingly, there is evidence that ANF may be functionally linked to LVM since: 1) mice lacking the genes for either Nppa or Npr1 [coding for natriuretic peptide receptor A (NPRA), a cGMP-coupled ANF receptor] show an increase in cardiac mass that is exaggerated in the context of the very modest increase in blood pressure seen in the same animals (8, 18, 24); 2) in vitro, ANF and/or analogs of cGMP attenuate the hypertrophic effects of norepinephrine on cultured neonatal rat cardiomyocytes (4, 27), whereas an ANF antagonist enhances these effects (17); and 3) transgenic mice overexpressing constitutive guanylate cyclase in a heart-specific manner are protected against catecholamine-induced hypertrophy (34). Altogether, it is therefore possible

![Table 2. Intra- and Intergender comparisons of cardiomyocytes from parental strains](image-url)
that changes in the width of cardiomyocyte from male rats may be due to changes in LV ANF and/or of its second messenger cGMP. Although a 63-cR locus should be expected to contain a few hundreds of genes, the fact that Nppa (which physically maps to the locus) can also be functionally linked to LVM makes it a strong candidate for linkage to differences in LVM.

In females, the RNO5 locus was linked to LV ANF, but not to LVM. However, ventricular hypertrophy in females might be of a different nature, because cardiomyocytes from female WKHA were shorter than their WKY counterparts (with no difference in width). Accordingly, the RNO5 locus was linked to LV ANF concentration in congenic females but not to cardiomyocyte width (which was not different in the female parental strains to start with). One possible explanation for the lack of effect of the RNO5 locus on the width of female cardiomyocytes is that ANF could activate in males a mechanism that is already activated in females, and hence will have no additional effects. Consistent with this interpretation, it has been shown that the production of nitric oxide synthase (and thus presumably of cGMP) is increased in the heart of female rats. However, ventricular hypertrophy in females might be of a different nature, because cardiomyocytes from both strains. This might be consistent with the hypothesis that different genes and/or molecular pathways regulate the length and the width of cardiomyocytes differentially, along with different functional consequences (12). Given the possible functional and separate consequences of each dimension change, these effects may be pertinent to the reduced incidence of cardiovascular disease in premenopausal women (28).

In summary, our data show that a region on the 5q36 band of RNO5 that contains the Nppa gene and whose length is ~63 cR is linked to both cardiomyocyte width and to LV ANF in male rats. Taken together with other functional data, this increases the probability that alterations of a single gene within that locus (possibly Nppa) are linked to both cardiovascular phenotypes in male rats. Our data also illustrate how genes and sex may interact to regulate cardiomyocyte width and length independently from one another.

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