Table 1 Genes for rat disease phenotypes identified by positional cloning since 1999

Gene	Phenotype	Susceptible strain	Date	Complementation[b]	Human trait	Supplementary references[c]
Cd36	Insulin resistance, hyperlipidemia	SHR/NCrl	1999, 2001	*In vivo*	Dyslipidemia, insulin resistance, hypertension	S1,S2
Aspa	Spongy leukodystrophy	TRM/Kyo	2000	ND	Canavan disease	S3
Mertk	Retinal dystrophy	RCS/Kyo	2000, 2001	*In vivo*		S4,S5
Atrn	Hypomyelination, mahogany coat color	Zi/Kyo	2001	*In vivo*		S6
Cyp11b1	Blood pressure	SS/Jr	2001	ND	Hypertension	S7,S8
Ncf1	Arthritis	DA/Rhd	2002	*In vivo*	Rheumatoid arthritis	S9,S10
Cblb	Type 1 diabetes	KDP/Tky	2002	*In vivo*		S11
Gimap5	Type 1 diabetes	BB/OK	2002	ND		S12,S13
Pkhd1	Polycystic kidney disease	PKD	2002	ND	Polycystic kidney disease	S14
Rab38	Platelet storage pool disease, renal failure	FH, LE/BluGill, FHH/EurMcwi	2004, 2005	ND		S15,S16
Ciita	MHC expression	DA/Slc	2005	ND	Rheumatoid arthritis, multiple sclerosis, myocardial infarction	S17
Gstm1	Hypertension, oxidant stress	SHRSP/Gla	2005	ND		S18
Anks6	Polycystic kidney disease	PKD/Mhn(cy/+)	2005	ND		S19
Fcgr3	Crescentic glomerulonephritis	WKY/NCrl	2006	ND	Systemic lupus erythematosus, systemic autoimmunity	S20,S21
Tmem67	Polycystic kidneys, brain malformations	WPK	2006	ND	Meckel-Gruber syndrome	S22
Fbxo10, Frmpd1	Mammary cancer	WF/NHsd	2007[a]	ND	Breast cancer	S23
Ephx2	Heart failure	SHHF/Bbb	2007[a]	*In vivo*	Heart failure	S24
Ogn	Left ventricular mass	SHR/NCrl	2007[a]	*In vivo*	Left ventricular mass	S25
Jund	Crescentic glomerulonephritis, macrophage activation	WKY/NCrl	2007[a]	ND	Macrophage activation	S26
Vav1	Neuroinflammation	DA/BII	2007[a]	ND	Multiple sclerosis	S27
Srebf1	Hepatic steatosis	SHR/Olalpcv	2008	*In vivo*		S28

[a]Presented at the Cold Spring Harbor Rat Genomics and Models meeting, December 2007. [b]Where indicated, complementation was by *in vivo* complementation in either rat or mouse. ND, Not done. [c]Supplementary references S1 to S27 are given in the **Supplementary References** online.

nomenclature assignment, map locations, strains or populations used in each study and phenotypes measured—are curated. The mapped traits are visualized in the genome browsers at RGD, Ensembl, UCSC and also in NCBI's map viewer. The comprehensive rat strain catalog maintained at RGD has become the foundation for all knowledge relating to over 1,000 strains, substrains and genetically modified rats. Reflecting the disease-focused nature of much of rat research, RGD has also created disease portals[19] to provide easy access to genes, QTLs, pathways, phenotypes and strains associated with major disease areas such as obesity, cardiovascular, neurological and other diseases.

The community has been working to integrate data from scattered resources using Distributed Annotation Systems (DAS) and BioMart, both of which have proven highly valuable in other model organism and genome databases. Some significant developments centered on data integration are still needed, however. A common reference gene set is needed to ensure consistency across the genome databases. The main gene prediction and gene annotation groups (RGD, NCBI, Ensembl and UCSC) have been working together since mid-2007 to develop a process to tackle this issue. Phenotype data must be captured and described in a consistent manner both within a species and between species. Finally, there is a critical need to expand links between physiological data and genotype data across species, particularly as genome-wide association studies (GWAS) yield huge datasets that provide a rich resource for comparative data mining.

National Rat Genetic Resource Centers. The development of hundreds of inbred and mutant strains and the characterization of a wide range of disease models have necessitated and driven the creation of two major rat resource centers, in the USA and Japan. As a result of recommendations of an international NIH workshop in 1998, the NIH Rat Resource Research Center (RRRC; http://www.nrrrc.missouri.edu/) was established in 2001. A similar approach on a larger scale was undertaken in Japan in 2002 with the National Bio Resource Project for the Rat, (NBRP-Rat; http://www.anim.med.kyoto-u.ac.jp/NBR/). Smaller repositories have been established in Europe at the Hannover Medical School (http://www.mh-hannover.de/2652.html) and by EURATools at the Czech Academy of Sciences (http://www.euratools.eu). The resource centers collect, preserve and supply unique and important rat strains for biomedical research to the local and international research community. In addition, all resource centers carry out research directed at improving repository functions, including better cryopreservation methods and assisted reproductive technologies, and they also develop new diagnostic screening methods for rat pathogens. Researchers at the NBRP-Rat have recently created a tutorial on DVD, in English and Japanese, on cryopreservation of embryos and spermatozoa and their rederivation, including vitrification of rat two-cell embryos and morulas, intracytoplasmic sperm injection (ICSI), cryopreservation of epididymal sperm and assisted reproduction techniques[20].

At present the RRRC contains over 300 transgenic, spontaneous mutant, complex trait models and inbred strains. The NBRP contains over 562 rat strains, and the ERRC and Prague repositories over 140 rat strains. These strain collections include a wide range of healthy inbred strains, as well as mutant, congenic, consomic, recombinant inbred and *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized strains. Because of advances in cryopreservation and the recent development of robust protocols for ICSI and sperm freezing, it has also been possible to preserve ENU mutant lines as sperm and DNA collections. Sperm

from 5,000 ENU mutagenized F344 G1 animals are now integrated into the NBRP repository[21]. These strain repositories are important for the current and future research programs based around rat genetics.

Specialized mapping panels. Recombinant inbred strains have many advantages for QTL mapping, including the ability to increase trait heritability by phenotyping multiple genetically identical rats from each rat strain[22] and the ability to accumulate data from genetically stable strains over years, obviating the need for *de novo* genotyping. Currently, two large recombinant inbred panels are available in the rat: the HXB/BXH set ($n = 31$) derived by reciprocal crosses of SHR/Ola and BN-*Lx* (refs. 3,4) and the LEXF/FXLE set ($n = 34$) by reciprocal crosses of F344/Stm and LE/Stm[23]. Two smaller panels, PXO and LXB, have also been reported[24,25]. The larger LEXF/FXLE and HXB/BXH panels have been proven to produce successful results in genetic mapping, and expression QTL (eQTL) and gene identification studies[4,16,23,26–28].

Rat heterogeneous stock panels allow fine-mapping of QTLs to subcentimorgan intervals, significantly reducing the number of candidate genes found within each QTL. The best characterized rat heterogeneous stock panel was established at the NIH in 1984 and derives from eight genetically distinct and phenotypically diverse inbred strains (ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N and WN/N)[29]. The heterogeneous stock colonies are maintained in a breeding scheme designed to minimize inbreeding and to reduce the size of haplotypes from each strain throughout the genome by recombination. After 50 generations of breeding, the estimated average distance between recombination events approaches a single centimorgan[30], enabling efficient and very high resolution QTL mapping. Heterogeneous stock colonies from this panel are now maintained at the Medical College of Wisconsin (Wisconsin, USA), the Autonomous University of Barcelona (Spain) and the Indiana University School of Medicine (Indiana, USA). Following the landmark study of Flint and colleagues[31], who demonstrated successful fine mapping of 843 QTLs for over 100 phenotypes using heterogeneous stock mice, heterogeneous stock rat studies are now underway within the EURATools consortium and at the Medical College of Wisconsin and a wide variety of behavioral, cardiovascular, metabolic, inflammatory and other traits are being mapped. Many of these traits are preferentially studied in the rat because of accuracy and ease of high-throughput measurements in the rat model and similarity of the traits to human phenotypes.

Consomic rat strains are generated by introgressing an entire chromosome from one inbred strain into the isogenic background of another inbred strain using marker-assisted selection. The PhysGen (http://pga.mcw.edu) Program for Genomic Application at the Medical College of Wisconsin developed two panels of consomic rats using the SS/JrHsdMcwi, the FHH/EurMcwi and the BN/NHsdMcwi strains[5]. Comprehensive characterization (434,845 physiological data points) of these consomic strains, each carrying a chromosome from the sequenced Brown Norway strain, allows for immediate mapping of traits to a particular chromosome without the need for genetic crosses. From the consomic strains, congenic strains can be rapidly bred within 6 months to narrow the region on a specific chromosome to a region that can be targeted for gene identification. Several studies of this nature are now nearing completion. All strains are available from PhysGen.

From rat model to human disease

The infrastructure investment described above and the resulting genome resources have led to many disease gene discoveries in rats, frequently translated to humans, to an extent that could not have been anticipated nine years ago (**Table 1**). In the early part of the past decade, most of the rat traits that were positionally cloned were monogenic, with only a few exceptions of more complex traits, including insulin resistance (*Cd36*)[32], autoimmune diabetes (*Cblb*, *Gimap5*)[33–35] and arthritis (*Ncf1*)[36]. Identification of *Cd36* as an insulin resistance gene was the first example of cloning a complex-trait gene using a combined expression-microarray and linkage approach[32]. In the last 2 years, however, the integrated use of genetic mapping, gene expression and computational analysis has permitted identification of a steady stream of genes underlying rat polygenic traits, including, but not limited to, neuroinflammation[37], glomerulonephritis[38,39], mammary cancer[40], heart failure[41] and left ventricular mass[26]. In all of these studies, associations were also demonstrated to related traits or diseases in humans. The investments in animal models and genomic infrastructure, together with these recent reports, indicate that the discovery rate is accelerating. We predict that continued development of the genome infrastructure will further increase this rate of discovery.

New concepts in genomics are being rapidly applied to rat research, one example being gene CNVs. The observation that the molecular lesions of two of the earliest QTL genes identified in the rat (*Cd36* and *Fcgr3*) were gene CNVs[32,38] has suggested that this type of structural genome variation may be an important evolutionary mechanism mediating development of genetically complex phenotypes in rats and other mammals. A recent genome-wide survey has revealed that at least 1.5% of the rat genome resides in regions that are variable in copy number[42]. Notably, these variable regions share many characteristics with regions that are variable in the human genome. In addition, 80 genes that are implicated in human disease were found to reside in variable copy number regions in both human and rat. Finally, the availability of data from recombinant inbred strains, including genome-wide gene expression data and high-resolution haplotype information, provides a platform for robustly linking copy number status to gene expression changes and phenotypic variability[42]. Construction of a more complete, higher resolution CNV map will provide further insights into the contribution of CNVs to the evolution of, and susceptibility to, complex phenotypes.

Genetic analysis of some rat traits, exemplified by polycystic kidney disease (PKD), has contributed to gene identification in humans and, in the case of PKD, has increased understanding of the pathophysiology to the extent of identifying treatment modalities for clinical trials. In the common infantile form of PKD, autosomal recessive PKD, the gene in the PCK rat model was found by mapping and positional cloning to be orthologous to that responsible for human autosomal recessive PKD, and analysis of the rat model enabled the human disease gene to be identified[43]. Subsequently, the PCK model was shown to have ciliary abnormalities[44], reinforcing the link between primary cilia and PKD. Possible defects in planar cell polarity (mitotic spindle orientation) were also first associated with PKD in this model[45]. The PCK model has also proven effective for screening potential therapeutic agents that have been targeted at the higher cellular amounts of cyclic AMP in PKD by way of the vasopressin V2 receptor (Avpr2) in the collecting duct[46–49] or by use of somatostatin analogs[50]. Clinical trials of a vasopressin receptor antagonist and the somatostatin analog octreotide are in progress.

Transgenesis and gene targeting

Although gene targeting technology remains under development, other technologies for disrupting genes have been adapted from the mouse to generate knockout rat models for functional annotation. Many of these strategies are derived from the mouse mutagenesis programs as recommended (see **Box 1**) and include traditional transgenesis (reviewed in ref. 51), viral vector–mediated DNA transfer[52,53], sperm-mediated DNA transfer[54–57] and RNA interference[58,59]. To date, it has not proved possible

to culture germline-competent rat embryonic stem (ES) cells, although ES-like cells have been isolated and reported to contribute to extraembryonic tissues *in vivo*[60–62]. Nuclear transfer in the rat has also been problematic because cultured oocytes are prone to spontaneous activation, and so far there are few reports of live births after nuclear transfer[63–65]. Meanwhile, chemical mutagenesis using ENU and gene-trap transposon insertional mutagenesis using the Sleeping Beauty transposon system have now generated dozens of mutant rat strains and potential disease models.

Traditional transgenesis by microinjection continues to be used for genetic manipulation of the rat to generate new models of interest. For example, one of the best models for Alzheimer's disease is a triple transgenic rat model that brought together two mutant amyloid precursor protein alleles with human presenilin-1 (ref. 66). Other examples include transgenic models for amyotrophic lateral sclerosis and human tauopathies[67,68].

Chemical mutagenesis. Small- and large-scale random chemical mutagenesis using ENU combined with a gene screening strategy has been used to generate gene knockouts in the rat. For example, two important cancer genes, *Brca1* and *Brca2*, were screened using a simple yeast-based screening assay that identifies premature stop codons introduced by ENU mutagenesis[69], and an *Apc* null line with a phenotype showing close similarity to human colon cancer has been reported[70]. The Cuppen laboratory and the PhysGen program, using an alternative strategy[71] involving high-throughput resequencing of PCR-amplified genomic targets to detect mutations[72], have identified more than 200 ENU-induced mutant strains, including 18 putative knockout mutations. Finally, as reported in this issue and described above, the combination of ENU mutagenesis with a high-throughput screening assay using the Mu-transposition reaction (MuT-POWER) and ICSI for the recovery of the rare heterozygous genotypes from a frozen sperm repository[21] has yielded large numbers of mutant strains and mutations.

Transposon insertional mutagenesis. Transgenic rats carrying components of the Sleeping Beauty transposon system have been bred to generate transposon-knockout mutations[73,74]. Although the low mutation rate of Sleeping Beauty mutagenesis is less amenable than ENU to forward-genetic screening applications and is generally inapplicable to the site-directed approach, the efficiency of the Sleeping Beauty system is adequate for creating a resource of defined knockout rat strains by random mutagenesis. Combining this technology with visible markers such as fluorescent proteins or β-galactosidase has revealed expression patterns of trapped genes[73] and creates tools for cell lineage tracing and organ systems biology. From all transposon-induced mutations generated so far by PhysGen, more than 30 are being sent to RRRC for storage and distribution.

Although germline-competent ES cells have not been established in the rat system, spermatogonial stem cell lines[75,76] offer an alternative for insertional mutagenesis *in vitro*. By combining transposition-mediated gene insertion, gene-trapping technology, high-throughput culturing and archiving, a library of gene knockouts in rat spermatogonial stem cells could be generated. The use of diverse transposon systems with distinct target site preferences makes full genome coverage with transposon-based insertional mutagenesis a realistic goal.

Phenotyping challenges

Arguably the greatest asset in rat biology is the detailed knowledge generated over many decades by phenotypic analyses of physiological, pathological and pharmacological traits. Most of these studies have been driven by specific hypotheses, and, as a result, rat strains until now have mostly been studied for very specific phenotypes, providing only a narrow glimpse of the (patho)physiology of each strain.

The mouse community has invested in creating standard operating protocols and establishing mouse phenotyping clinics (http://www.eumorphia.org; http://empress.har.mrc.ac.uk; http://www.knockoutmouse.org; http://www.jax.org/phenome), with the aim of permitting comparison of phenotypes across strains and laboratories. The growing number of mutant rat lines generated by ENU mutagenesis, and those anticipated from transposon-mediated mutagenesis, increases the importance of standardized phenotyping in the rat model. So far, the rat community has embarked on a limited number of discrete phenotyping programs, with similar aims to those of the mouse community in optimizing utility and comparability.

The Rat Phenome Project in Japan has recently recharacterized 163 rat strains as models of human diseases and collected strain data for 109 parameters across neurobehavioral, cardiovascular, biochemical, hematological and anatomical phenotypic categories[77]. The PhysGen program has characterized a panel of 44 consomic rat strains for 213 mainly cardiovascular phenotypes measured across the entire consomic panel. Data from both programs are publicly available at http://www.anim.med.kyoto-u.ac.jp/nbr/phenome and at PhysGen. The EURATools consortium has developed a protocol to fine-map over 50 behavioral, metabolic, hemodynamic, hematologic, immunologic, morphometric and expression phenotypes in individual heterogeneous stock rats, and the generation of these phenotypes is now underway.

Development of a more comprehensive international rat phenome project would advance the use of the rat by new investigators and might improve design of existing studies by investigators already using the rat. In developing a phenome project, it will be important to consider the different requirements for screening mutant rat lines compared to phenotyping in mapping studies. The emphasis in screening mutant lines is to identify a single, often extreme abnormality, whereas in mapping panels and inbred strains, extreme values may represent poor-quality data. Another consideration is the challenge attached to obtaining several phenotypes from single animals, as recognized for example in the SHIRPA protocol[78] and in mouse heterogeneous stock mapping studies[31].

The mouse phenome projects offer an example of a coordinated effort in phenotyping. A similar initiative in the rat should benefit from the experiences of the mouse community in refining strategies to expand the efficient characterization of large numbers of inbred and mutant strains and to enhance the use of specialized mapping panels. Furthermore, the use of standardized ontologies (for example, ref. 79) to describe rat phenotypes will facilitate the comparison of disease phenotypes in different model species and humans.

Building the community

The past 10 years have seen the growth of a committed and close-knit rat genetics and genomics community that spans many disease areas and several disciplines. The most important community activities are the establishment of annual international scientific meetings; the development of the genome database, RGD, as described above; and the establishment of a European rat research community around several European Union–funded projects, including the recently funded EURATools consortium.

Following 20 years of international meetings on rat alloantigens and other topics of interest to rat geneticists, the first Cold Spring Harbor Laboratory meeting on Rat Genomics and Models was held in December 1999. Subsequent biennial meetings have been held at Cold Spring Harbor Laboratory since then, with alternating meetings in the intervening years being held in Europe and Asia. Future meetings will be held at Cold Spring Harbor in 2009 and 2011, in Hinxton, UK in 2008 and in Japan in 2010.

In Europe, annual meetings have also been held centered around several consortia funded under the European Union's Sixth Framework Program (http://cordis.europa.eu/era/). Principal investigators from the targeted research projects, STAR (a SNP and haplotype map for the rat; http://www.snp-star.eu) and Med-Rat (New Tools to Generate Transgenic and Knockout Mouse and Rat Models; http://medrat.abc.hu/), and from the larger scale Integrated Project EURATools (European Rat Tools for Functional Genomics; http://www.euratools.eu), represent a critical mass of the European rat research community who have contributed widely to the progress described in this Perspective[16,26,39,41].

Vision for the future

A principal purpose for studying the biology and pathobiology of the rat is to translate this information to humans, particularly in the context of complex traits. Many inbred models have been developed by selectively breeding rats carrying a phenotype of interest, resulting in genetic models that capture natural variation leading to common disease phenotypes including cancer, hypertension, diabetes, arthritis, autoimmunity and alcohol preference.

Because of notable recent advances in human genetics, including identification of scores of susceptibility genes for common human diseases by GWAS, the need for a multispecies platform to integrate and investigate human disease at the level of both genotype and phenotype has become increasingly apparent. For although GWAS have identified many previously unsuspected human disease genes, most of the genes individually have small effects on disease susceptibility. Furthermore, many of the associations are in gene deserts or, alternatively, span several genes. Thus, identifying the exact genes and mechanisms that underlie the GWAS associations often remains a significant barrier to progress.

For genetic studies using animal models, the arrival of human GWAS is a major paradigm shift. For many years, the role of animal systems in genetic studies has been predicated on the increased heritability, flexibility and statistical power of experimental crosses over corresponding studies in humans. Animal studies could therefore map disease genes with greater ease and higher statistical confidence and could then inform the search for disease genes in the complex backdrop of the human genome. The new human GWAS and resequencing programs are now able to sift the genome of the human without the need for animal model data, therefore raising questions about the continuing value of animal genetics studies.

In our view, the case for continuing, and even strengthening, efforts in animal genetics is clear and is based on several fundamental principles:

1. The wealth of literature accumulated over 50–100 years in models such as the mouse and rat offer an unparalleled resource for molecular genetic investigation of mammalian physiology and pathobiology.

2. The genome resources currently and potentially available for animal models have increased the pace of gene discovery such that scores or even hundreds of traits now mapped as QTLs or defined in consomic or minimal congenic regions will be positionally cloned in the next 5 to 10 years.

3. Positionally cloned animal QTL genes offer a unique route to defining underlying mechanisms of gene action in a way that cannot be achieved directly for human disease genes. Animals also offer better systems for in-depth phenotyping and for studying mechanisms of action of human disease genes.

4. Identification of quantitative trait nucleotides that underlie animal QTLs provides insights not only into gene function in health and disease but also into the evolutionary history of animals and animal models and their genomes.

5. Although all genes identified as underlying animal disease phenotypes may not themselves underlie the related human diseases, the rat QTL genes identified so far have shown remarkable relevance to related human disease phenotypes, and either the genes themselves or the pathways in which these genes reside are likely to continue to provide insights into human disease and new approaches to their prevention and treatment.

6. Rat models can and will be built on the basis of clinical disorders and genetic and environmental stimuli, increasing the reliability of these systems for predicting drug responses in humans.

This Perspective has summarized the development of rat genomic resources over the past 10 years and has described how these resources have led to considerable research successes and the establishment of a strong, dynamic and committed research community focused on use of genome tools to advance understanding of rat and human physiology and pathobiology. We believe, however, that the return on the investment of the past 10 years is at an early stage. The acceleration of research progress described in this paper has reached fruition for a handful of QTLs and disease genes, but scores of other rat QTL projects are on the brink of similar discoveries. With continuously advancing genome resources and new mapping strategies, the rate of discovery stands to accelerate further.

This predicted pace of discovery, however, will depend not only on support of the present community resources, but also on use of new technologies to generate more sequence from more strains, use of the emerging strategies for rat mutagenesis and gene targeting, improved use of comparative genomics for data mining, and further development of technologies such as tools for analysis of microRNAs. These approaches will broaden the resources that rat researchers can draw upon to complete QTL gene identification, pursue discovery of gene function, and investigate the evolutionary pressures and pathobiology that make the rat an invaluable animal model for understanding the mechanisms and pathways underlying mammalian health and disease.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to the production of this manuscript.

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1. Jacob, H. Functional genomics and rat models. *Genome Res.* **9**, 1013–1016 (1999).
2. Lindsey, J.R. Historical foundations in the laboratory rat. in *The Laboratory Rat* (eds. Baker, H.J., Lindsey, J.R. & Weisbroth, S.H.) 1–36 (Academic, New York, 1979).
3. Pravenec, M., Klir, P., Kren, V., Zicha, J. & Kunes, J. An analysis of spontaneous hypertension in spontaneously hypertensive rats by means of new recombinant inbred strains. *J. Hypertens.* **7**, 217–221 (1989).
4. Pravenec, M. *et al.* Genetic analysis of "metabolic syndrome" in the spontaneously hypertensive rat. *Physiol. Res.* **53** (suppl. 1), S15–S22 (2004).
5. Cowley, A.W., Jr, Roman, R.J. & Jacob, H.J. Application of chromosomal substitution techniques in gene-function discovery. *J. Physiol. (Lond.)* **554**, 46–55 (2004).
6. Gibbs, R.A. *et al.* Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature* **428**, 493–521 (2004).
7. Dewey, C. *et al.* Accurate identification of novel human genes through simultaneous gene prediction in human, mouse, and rat. *Genome Res.* **14**, 661–664 (2004).
8. Gibbs, R.A. *et al.* Evolutionary and biomedical insights from the rhesus macaque genome. *Science* **316**, 222–234 (2007).
9. Samad, A.H. *et al.* Mapping the genome one molecule at a time—optical mapping. *Nature* **378**, 516–517 (1995).
10. Serikawa, T. *et al.* Rat gene mapping using PCR-analyzed microsatellites. *Genetics* **131**, 701–721 (1992).
11. Jacob, H.J. *et al.* A genetic linkage map of the laboratory rat, *Rattus norvegicus*. *Nat.*

Genet. **9**, 63–69 (1995).
12. Watanabe, T.K. *et al.* A radiation hybrid map of the rat genome containing 5,255 markers. *Nat. Genet.* **22**, 27–36 (1999).
13. Kwitek, A.E. *et al.* High-density rat radiation hybrid maps containing over 24,000 SSLPs, genes, and ESTs provide a direct link to the rat genome sequence. *Genome Res.* **14**, 750–757 (2004).
14. Wilder, S.P. *et al.* Integration of the rat recombination and EST maps in the rat genomic sequence and comparative mapping analysis with the mouse genome. *Genome Res.* **14**, 758–765 (2004).
15. Zimdahl, H. *et al.* A SNP map of the rat genome generated from cDNA sequences. *Science* **303**, 807 (2004).
16. STAR Consortium. SNP and haplotype mapping for genetic analysis in the rat. *Nat. Genet.* **40**, 560–566 (2008).
17. Hamta, A. *et al.* Chromosome ideograms of the laboratory rat (*Rattus norvegicus*) based on high-resolution banding, and anchoring of the cytogenetic map to the DNA sequence by FISH in sample chromosomes. *Cytogenet. Genome Res.* **115**, 158–168 (2006).
18. Twigger, S.N. *et al.* What everybody should know about the rat genome and its online resources. *Nat. Genet.* **40**, 523–527 (2008).
19. Twigger, S.N., Shimoyama, M., Bromberg, S., Kwitek, A.E. & Jacob, H.J. The Rat Genome Database, update 2007—easing the path from disease to data and back again. *Nucleic Acids Res.* **35**, D658–D662 (2007).
20. Kashiwazaki, N. *et al.* Generation of rat offspring derived from cryopreserved spermatozoa in Japanese National Bioresources. *Reprod. Fertil. Dev.* **19**, 124–125 (2007).
21. Mashimo, T. *et al.* An ENU-induced mutant archive for gene targeting in rats. *Nat. Genet.* **40**, 514–515 (2008).
22. Belknap, J.K. Effect of within-strain sample size on QTL detection and mapping using recombinant inbred mouse strains. *Behav. Genet.* **28**, 29–38 (1998).
23. Voigt, B. *et al.* Evaluation of LEXF/FXLE rat recombinant inbred strains for genetic dissection of complex traits. *Physiol. Genomics* **32**, 335–342 (2008).
24. Kemlink, D., Jerabkova, V., Janku, M., Krenova, D. & Kren, V. PXO set of recombinant inbred strains of the rat: a new strain distribution pattern containing 448 markers. *Folia Biol. (Praha)* **49**, 165–176 (2003).
25. Hedrich, H.J. *Recombinant inbred strains*, 487–494 (Gustav Fisher, Stuttgart, 1990).
26. Petretto, E. *et al.* Integrated genomic approaches implicate osteoglycin (*Ogn*) in the regulation of left ventricular mass. *Nat. Genet.* **40**, 546–552 (2008).
27. Hubner, N. *et al.* Integrated transcriptional profiling and linkage analysis for identification of genes underlying disease. *Nat. Genet.* **37**, 243–253 (2005).
28. Aitman, T.J. *et al.* Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat. Genet.* **16**, 197–201 (1997).
29. Hansen, C. & Spuhler, K. Development of the National Institutes of Health genetically heterogeneous rat stock. *Alcohol. Clin. Exp. Res.* **8**, 477–479 (1984).
30. Mott, R., Talbot, C.J., Turri, M.G., Collins, A.C. & Flint, J. A method for fine mapping quantitative trait loci in outbred animal stocks. *Proc. Natl. Acad. Sci. USA* **97**, 12649–12654 (2000).
31. Valdar, W. *et al.* Genome-wide genetic association of complex traits in heterogeneous stock mice. *Nat. Genet.* **38**, 879–887 (2006).
32. Aitman, T.J. *et al.* Identification of *Cd36* (*Fat*) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat. Genet.* **21**, 76–83 (1999).
33. Yokoi, N. *et al. Cblb* is a major susceptibility gene for rat type 1 diabetes mellitus. *Nat. Genet.* **31**, 391–394 (2002).
34. MacMurray, A.J. *et al.* Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (*Ian*)-related gene. *Genome Res.* **12**, 1029–1039 (2002).
35. Hornum, L., Romer, J. & Markholst, H. The diabetes-prone BB rat carries a frameshift mutation in *Ian4*, a positional candidate of *Iddm1*. *Diabetes* **51**, 1972–1979 (2002).
36. Olofsson, P. *et al.* Positional identification of *Ncf1* as a gene that regulates arthritis severity in rats. *Nat. Genet.* **33**, 25–32 (2003).
37. Swanberg, M. *et al. MHC2TA* is associated with differential MHC molecule expression and susceptibility to rheumatoid arthritis, multiple sclerosis and myocardial infarction. *Nat. Genet.* **37**, 486–494 (2005).
38. Aitman, T.J. *et al.* Copy number polymorphism in *Fcgr3* predisposes to glomerulonephritis in rats and humans. *Nature* **439**, 851–855 (2006).
39. Behmoaras, J. *et al. Jund* is a determinant of macrophage activation and is associated with glomerulonephritis susceptibility. *Nat. Genet.* **40**, 553–559 (2008).
40. Samuelson, D.J. *et al.* Rat *Mcs5a* is a compound quantitative trait locus with orthologous human loci that associate with breast cancer risk. *Proc. Natl. Acad. Sci. USA* **104**, 6299–6304 (2007).
41. Monti, J. *et al.* Soluble epoxide hydrolase is a susceptibility factor for heart failure in a rat model of human disease. *Nat. Genet.* **40**, 560–566 (2008).
42. Guryev, V. *et al.* Distribution and functional impact of DNA copy number variation in the rat. *Nat. Genet.* **40**, 538–545 (2008).
43. Ward, C.J. *et al.* The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat. Genet.* **30**, 259–269 (2002).
44. Masyuk, T.V. *et al.* Defects in cholangiocyte fibrocystin expression and ciliary structure in the PCK rat. *Gastroenterology* **125**, 1303–1310 (2003).
45. Fischer, E. *et al.* Defective planar cell polarity in polycystic kidney disease. *Nat. Genet.* **38**, 21–23 (2006).
46. Gattone, V.H., II, Wang, X., Harris, P.C. & Torres, V.E. Inhibition of renal cystic disease development and progression by a vasopressin V2 receptor antagonist. *Nat. Med.* **9**, 1323–1326 (2003).
47. Wang, X., Gattone, V.H., II, Harris, P.C. & Torres, V.E. Effectiveness of vasopressin V2 receptor antagonists OPC-31260 and OPC-41061. *J. Am. Soc. Nephrol.* **16**, 846–851 (2005).
48. Nagao, S. *et al.* Increased water intake decreases progression of polycystic kidney disease in the PCK rat. *J. Am. Soc. Nephrol.* **17**, 2220–2227 (2006).
49. Wang, X., Wu, Y., Ward, C.J., Harris, P.C. & Torres, V.E. Vasopressin directly regulates cyst growth in polycystic kidney disease. *J. Am. Soc. Nephrol.* **19**, 102–108 (2007).
50. Masyuk, T.V., Masyuk, A.I., Torres, V.E., Harris, P.C. & Larusso, N.F. Octreotide inhibits hepatic cystogenesis in a rodent model of polycystic liver disease by reducing cholangiocyte adenosine 3′,5′-cyclic monophosphate. *Gastroenterology* **132**, 1104–1116 (2007).
51. Tesson, L. *et al.* Transgenic modifications of the rat genome. *Transgenic Res.* **14**, 531–546 (2005).
52. van den Brandt, J., Wang, D., Kwon, S.H., Heinkelein, M. & Reichardt, H.M. Lentivirally generated eGFP-transgenic rats allow efficient cell tracking *in vivo*. *Genesis* **39**, 94–99 (2004).
53. Broser, P., Grinevich, V., Osten, P., Sakmann, B. & Wallace, D.J. Critical period plasticity of axonal arbors of layer 2/3 pyramidal neurons in rat somatosensory cortex: layer-specific reduction of projections into deprived cortical columns. *Cereb. Cortex*, published online 12 November 2007 (doi:10.1093/cercor/bhm189).
54. Dozortsev, D., Wakaiama, T., Ermilov, A. & Yanagimachi, R. Intracytoplasmic sperm injection in the rat. *Zygote* **6**, 143–147 (1998).
55. Hirabayashi, M. *et al.* Factors affecting production of transgenic rats by ICSI-mediated DNA transfer: effects of sonication and freeze-thawing of spermatozoa, rat strains for sperm and oocyte donors, and different constructs of exogenous DNA. *Mol. Reprod. Dev.* **70**, 422–428 (2005).
56. Kato, M. *et al.* Production of transgenic rats by ooplasmic injection of spermatogenic cells exposed to exogenous DNA: a preliminary study. *Mol. Reprod. Dev.* **69**, 153–158 (2004).
57. Hirabayashi, M. *et al.* Offspring derived from intracytoplasmic injection of transgenic rat sperm. *Transgenic Res.* **11**, 221–228 (2002).
58. Young-Pearse, T.L. *et al.* A critical function for β-amyloid precursor protein in neuronal migration revealed by *in utero* RNA interference. *J. Neurosci.* **27**, 14459–14469 (2007).
59. Dann, C.T. New technology for an old favorite: lentiviral transgenesis and RNAi in rats. *Transgenic Res.* **16**, 571–580 (2007).
60. Buehr, M. *et al.* Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol. Reprod.* **68**, 222–229 (2003).
61. Vassilieva, S., Guan, K., Pich, U. & Wobus, A.M. Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. *Exp. Cell Res.* **258**, 361–373 (2000).
62. Demers, S.P., Yoo, J.G., Lian, L., Therrien, J. & Smith, L.C. Rat embryonic stem-like (ES-like) cells can contribute to extraembryonic tissues in vivo. *Cloning Stem Cells* **9**, 512–522 (2007).
63. Popova, E., Bader, M. & Krivokharchenko, A. Full-term development of rat after transfer of nuclei from two-cell stage embryos. *Biol. Reprod.* **75**, 524–530 (2006).
64. Roh, S. *et al.* Birth of rats following nuclear exchange at the 2-cell stage. *Zygote* **11**, 317–321 (2003).
65. Zhou, Q. *et al.* Generation of fertile cloned rats by regulating oocyte activation. *Science* **302**, 1179 (2003).
66. Flood, D.G. *et al.* A transgenic rat model of Alzheimer's disease with extracellular Aβ deposition. *Neurobiol. Aging*, published online 28 November 2007 (doi:10.1016/j.neurobiolaging.2007.10.006).
67. Howland, D.S. *et al.* Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc. Natl. Acad. Sci. USA* **99**, 1604–1609 (2002).
68. Zilka, N. *et al.* Truncated tau from sporadic Alzheimer's disease suffices to drive neurofibrillary degeneration in vivo. *FEBS Lett.* **580**, 3582–3588 (2006).
69. Zan, Y. *et al.* Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. *Nat. Biotechnol.* **21**, 645–651 (2003).
70. Amos-Landgraf, J.M. *et al.* A target-selected Apc-mutant rat kindred enhances the modeling of familial human colon cancer. *Proc. Natl. Acad. Sci. USA* **104**, 4036–4041 (2007).
71. Smits, B.M. *et al.* Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-selected mutagenesis. *Pharmacogenet. Genomics* **16**, 159–169 (2006).
72. Wienholds, E. *et al.* Efficient target-selected mutagenesis in zebrafish. *Genome Res.* **13**, 2700–2707 (2003).
73. Kitada, K. *et al.* Transposon-tagged mutagenesis in the rat. *Nat. Methods* **4**, 131–133 (2007).
74. Lu, B. *et al.* Generation of rat mutants using a coat color-tagged Sleeping Beauty transposon system. *Mamm. Genome* **18**, 338–346 (2007).
75. Hamra, F.K. *et al.* Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. *Proc. Natl. Acad. Sci. USA* **102**, 17430–17435 (2005).
76. Shinohara, T. *et al.* Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination. *Proc. Natl. Acad. Sci. USA* **103**, 13624–13628 (2006).
77. Mashimo, T., Voigt, B., Kuramoto, T. & Serikawa, T. Rat Phenome Project: the untapped potential of existing rat strains. *J. Appl. Physiol.* **98**, 371–379 (2005).
78. Rogers, D.C. *et al.* Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* **8**, 711–713 (1997).
79. Smith, C.L., Goldsmith, C.A. & Eppig, J.T. The Mammalian Phenotype Ontology as a tool for annotating, analyzing and comparing phenotypic information. *Genome Biol.* **6**, R7 (2005).

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Enhanced colitis-associated colon carcinogenesis in a novel *Apc* mutant rat

Kazuto Yoshimi,[1] Takuji Tanaka,[2] Akiko Takizawa,[1] Megumi Kato,[3] Masumi Hirabayashi,[3] Tomoji Mashimo,[1] Tadao Serikawa[1] and Takashi Kuramoto[1,4]

[1]Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto; [2]Department of Oncologic Pathology, Kanazawa Medical University, Uchinada, Ishikawa; [3]National Institute for Physiological Sciences, The Graduate University for Advanced Studies, Okazaki, Japan

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To establish an efficient rat model for colitis-associated colorectal cancer, azoxymethane and dextran sodium sulfate (AOM/DSS)-induced colon carcinogenesis was applied to a novel adenomatous polyposis coli (*Apc*) mutant, the Kyoto Apc Delta (KAD) rat. The KAD rat was derived from ethylnitrosourea mutagenesis and harbors a nonsense mutation in the *Apc* gene (S2523X). The truncated APC of the KAD rat was deduced to lack part of the basic domain, an EB1-binding domain, and a PDZ domain, but retained an intact β-catenin binding region. KAD rats, homozygous for the *Apc* mutation on a genetic background of the F344 rat, showed no spontaneous tumors in the gastrointestinal tract. At 5 weeks of age, male KAD rats were given a single subcutaneous administration of AOM (20 mg/kg, bodyweight). One week later, they were given DSS (2% in drinking water) for 1 week. At week 15, the incidence and multiplicity of colon tumors developed in the KAD rat were remarkably severe compared with those in the F344 rat: 100 versus 50% in incidence and 10.7 ± 3.5 versus 0.8 ± 1.0 in multiplicity. KAD tumors were dominantly distributed in the rectum and distal colon, resembling human colorectal cancer. Accumulation of β-catenin protein and frequent β-catenin mutations were prominent features of KAD colon tumors. To our knowledge, AOM/DSS-induced colon carcinogenesis using the KAD rat is the most efficient to induce colon tumors in the rat, and therefore would be available as an excellent model for human colitis-associated CRC. (*Cancer Sci* 2009; 100: 2022–2027)

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the world. Globally, the CRC mortality was 639 000 in 2004.[1] Chronic inflammation has been identified as a potential risk factor for CRC. Clinical studies have shown that inflammatory bowel disease, such as Crohn's disease[2] and ulcerative colitis,[3] elevates the risk of CRC.

Animal experiments are assumed to simulate or at least provide plausible pathophysiological mechanisms of various diseases, including cancer and chronic inflammatory disorders. For inflammation-related CRC, a two-stage colitis-related mouse colon carcinogenesis model was recently established.[4] In this model, colon carcinogenesis is initiated with carcinogens and then dextran sodium sulfate (DSS), which can induce colonic mucosal inflammation resembling the histopathology of ulcerative colitis, is used as a tumor-promoting agent. Colon carcinogenesis initiated with carcinogens such as azoxymethane (AOM),[4] 1,2-dimethylhydrazine (DMH),[5] and heterocyclic amines[6] is effectively promoted in combination with DSS.

The two-stage colitis-related model has been applied to a rat colon carcinogenesis study. Similar to mice, DSS also promotes DMH-induced[7] and AOM-induced[8]colon carcinogenesis in the F344 rat. These rat models can be utilized to investigate the pathogenesis of colitis-related colon carcinogenesis and to detect carcinogenesis modifiers.[7,8] For more effective colorectal carcinogenesis, however, a novel model, in which much more and larger tumors are induced in a shorter experiment period, is required. It would be preferable to obtain a large volume of tumors, sufficient to be identifiable on macroscopic observation, because this would be an advantage in testing chemotherapeutic efficacy of anticancer drugs as well as chemoprevention ability of anti-inflammatory drugs.

One possible idea to enhance the AOM/DSS model is deficiency of the tumor suppressor adenomatous polyposis coli (*Apc*) gene, which plays a significant role in tumor development in the gut,[9] for example, AOM enhances colorectal carcinogenesis in $Apc^{min/+}$ mice that carry a germline mutation in the *Apc* gene and develop multiple polyps in the intestine.[10] Furthermore, DSS strongly promotes colorectal carcinogenesis in $Apc^{min/+}$ mice.[11] These findings prompted us to examine AOM/DSS-induced colon carcinogenesis in an *Apc* mutant rat. In the rat, an *Apc*-deficient Pirc rat is available.[12] The Pirc rat carries a nonsense mutation in the *Apc* gene and the resultant truncated APC (Δ1137) lacks the β-catenin binding region. The Pirc rat develops multiple tumors with a distribution between the colon and small intestine. The average number of colonic polyps is 8 ± 3 in Pirc rats aged 4–6 months, and most of them are adenomas. *N*-ethyl-*N*-nitrosourea (ENU) treatment enhances colonic polyps, but it takes more than 7 months to obtain them.[12] However, a carcinogenesis test with AOM alone or AOM/DSS has not yet been done in the Pirc rats.

We have recently produced a rat mutant archive consisting of cryopreserved sperm derived from ~5000 ENU-mutagenized male rats and corresponding DNA samples. The mutant archive is estimated to store ~2 million mutations, sufficient to find several mutations in a particular gene of interest.[13]

We present here a novel homozygous *Apc* mutant rat strain, the Kyoto Apc Delta (KAD) rat, from the rat ENU-mutant archive. KAD rats harbor a nonsense mutation in exon 15 that results in premature termination at codon 2523 of the serine residue of APC protein. KAD rats are viable and show no spontaneous tumors in the small intestine or colorectum. Treatment with AOM/DSS revealed an increased susceptibility of KAD rats to colitis-associated colon carcinogenesis in a 15-week experimental period. Also, the development of colorectal tumors can be tracked by endoscopic observation. AOM/DSS-induced colon carcinogenesis in the KAD rats will provide a novel rat model for investigating colitis-related colon carcinogenesis, identifying xenobiotics with modifying effects, and evaluating anticancer drug candidates.

Materials and Methods

Establishment of KAD rat strain. A total of 1735 DNA samples from ENU-mutagenized F344/NSlc rats from the Kyoto

[4]To whom correspondence should be addressed.
E-mail: tkuramot@anim.med.kyoto-u.ac.jp

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University Rat Mutant Archive (KURMA) were screened with seven sets of primers (Table S1). These primers were designed to amplify exons 9, 11, 14, or 15 of the rat *Apc* gene. Approximately 6.27 Mb of genomic DNA was screened. Rats carrying the *Apc* mutation were recovered by intracytoplasmic sperm injection.[13] Male rats carrying the *Apc* mutation were backcrossed five times with female F344/NSlc rats to remove latent mutations induced by ENU.

Western blotting. Proteins were prepared from the brainstems of KAD and control F344/NSlc rats at 5 weeks of age. Western blotting and signal detection were carried out as described.[14] Antibodies against the N terminus of APC (H-290; Santa Cruz Biotechnology, Santa Cruz, CA, USA), the C terminus of APC (C-20; Santa Cruz Biotechnology), and β-actin (AC-40; Sigma-Aldrich Japan, Tokyo, Japan) were used. Secondary antibodies against rabbit IgG (NA934; GE Healthcare Bio-Sciences, Tokyo, Japan) and mouse IgG (NA931; GE Healthcare Bio-Sciences) were used.

Carcinogenesis test. Colon carcinogenic tests were carried out as described.[4] Briefly, male KAD rats ($n = 17$) were divided into three experimental and control groups. Group 1 ($n = 6$) was given a single subcutaneous injection of AOM (20 mg/kg bodyweight) at 5 weeks of age. Starting 1 week after the AOM injection, animals were given 2% DSS in drinking water for 7 days and then no further treatment for 13 weeks. Groups 2 ($n = 5$) and 3 ($n = 3$) were given AOM alone and DSS alone, respectively. Group 4 ($n = 3$) was untreated. Male F344/NSlc rats ($n = 6$) were treated with AOM followed by DSS (group 5) and were controls of group 1. All rats were maintained under the conditions of humidity (50 ± 10%), light (14 : 10 h L : D cycle), and temperature (24 ± 2°C) at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. At 15 weeks after the AOM injection, they were killed by cervical dislocation under anesthesia with isoflurane (Forane; Abbott Japan, Tokyo, Japan). All experimental procedures were approved by the Animal Research Committee of Kyoto University and were carried out according to the Regulation on Animal Experimentation at Kyoto University.

Histopathology and immunohistochemistry. At autopsy, the colorectum of the rats was resected, washed with PBS, and opened longitudinally along the main axis. After careful macroscopic inspection, tumors and the colonic mucosa were dissected and processed for histopathological examination with hematoxylin–eosin staining. Immunohistochemical staining of β-catenin was carried out as described previously.[15]

Endoscopic observation and biopsy. Endoscopic observations were carried out every week after the 8 weeks of the carcinogenesis tests. Anesthesia was administrated through the regulated flow of isoflurane vapor (2%) through a nose cone. The colon was flushed with a tap water enema. The endoscope (BF TYPE 3C40; Olympus, Tokyo, Japan) was inserted into the colon and endoscopic images were acquired. A tumor specimen was biopsied under microscopic observation.

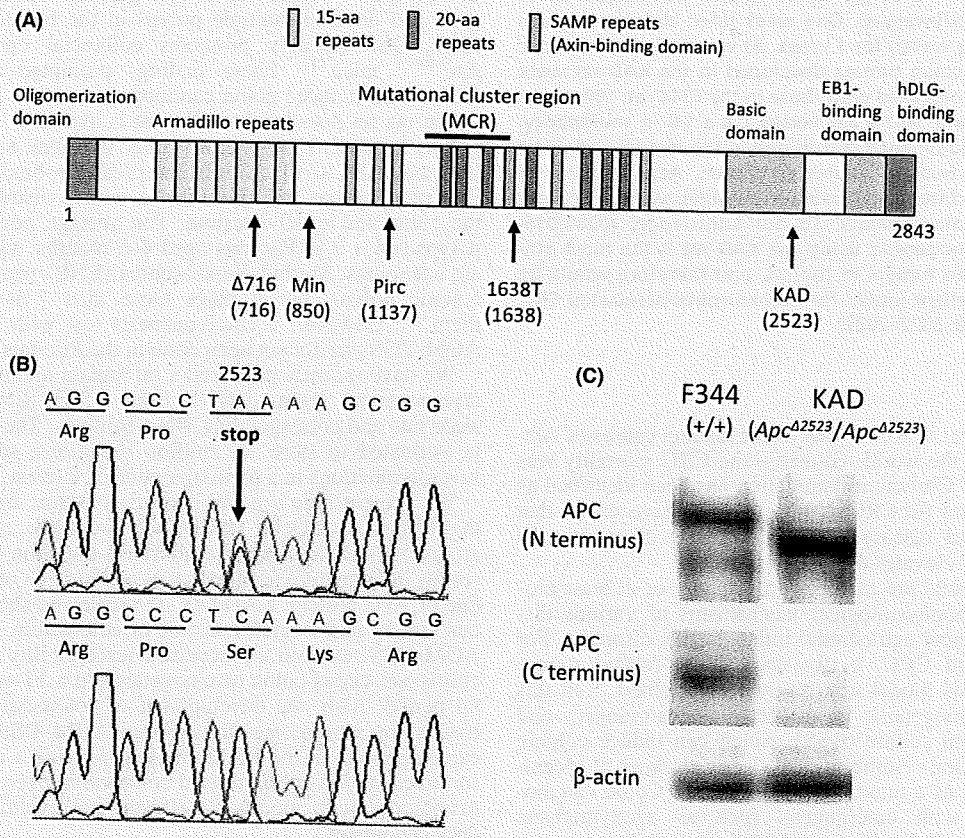

Fig. 1. Establishment of the Kyoto Apc Delta (KAD) rat strain. (A) Schematic diagram that shows multiple domains of full-length adenomatous polyposis coli (APC). Black arrows indicate orthologous locations of truncating mutations in mouse and rat models. The nonsense mutation in the KAD rat is indicated by a red arrow. (B) Sequence trace of a founder rat showing heterozygosity for C-to-A transversion (arrow) at nucleotide 7621 of the *Apc* gene (upper) compared with wild-type littermates (lower). The mutation generated a premature stop codon (TAA) at the 2523 amino acid position of APC. (C) Western blot analysis of APC in KAD and control F344/NSlc rats. Proteins extracted from the brains of F344 (+/+) and KAD ($Apc^{\Delta 2523}/Apc^{\Delta 2523}$) rats were hybridized with anti-N terminus of APC (top), anti-C terminus of APC (middle) antibodies. β-Actin was used as an internal control (bottom). In KAD rats, smaller APC protein was detected with the anti-N terminus APC antibody than F344 rats, and no signal was detected with the anti-C terminus APC antibody.

Mutation detection. Mutations of the β-catenin (*Catnnb1*) or K-ras (*Kras*) genes in tumors were screened by direct sequencing. Genomic DNA was extracted from tissues stored in RNAlater (Applied Biosystems, Inc., Carlsbad, CA, USA). PCR primers were designed to amplify mutational hot spots detected in the AOM-induced colon tumors.[16] The nucleotide sequences of primers were as follows: rCatnnb1-F, GCTGACCTCATGGAGTTGGA and rCatnnb1-R, GCTACTTGCTCTTGCGTGAA; rKras-F, TGAATTCAGAATGCCTTAGAGTTTT and rKras-R, GCACCGATGGTTCCCTATTA. DNA sequencing was carried out as described previously.[17]

Results

Establishment of the KAD rat. A C-to-A point mutation was detected in the DNA archive of KURMA and was predicted to result in premature termination at codon 2523 of the serine residue of the APC protein (Fig. 1A). Rats carrying the mutation were recovered from the corresponding frozen sperm (KURMA sperm archive number: ENU1588) with intracytoplasmic sperm injection.[13] The nonsense mutation (c. 7621C > A, p. Ser2523X) was confirmed in recovered animals, which were F_1 hybrids between recipient F344/NSlc and G_1 donor animals (Fig. 1B); we therefore named this allele $Apc^{\Delta 2523}$. The deduced APC protein was predicted to lack a part of the basic domain, EB1-binding domain, and PDZ domain (Fig. 1A). Because homozygous Min mice and Pirc rats have been reported to be embryonic lethal,[12,18] we crossed $Apc^{\Delta 2523}$ heterozygous mutants to obtain $Apc^{\Delta 2523}$ homozygotes. Rats homozygous for $Apc^{\Delta 2523}$ were viable and survived almost 2 years. We thus designated the $Apc^{\Delta 2523}$ homozygous strain the KAD rat. Western blot analyses indicated a lack of the C terminus of APC protein in the KAD rat (Fig. 1C). Cellular localization of β-catenin protein was not altered in the colon epithelia of the KAD rat, compared with the F344 rat (data not shown).

High susceptibility to colitis-associated colon carcinogenesis in the KAD rat. $Apc^{\Delta 2523}$ homozygous KAD developed no spontaneous tumors in their gastrointestinal tracts even after 20 months of age. We then tried to induce colon tumors in the KAD rats by administrating AOM as a chemical colonic carcinogen and/or DSS as a colitis-inducing agent. The AOM-treated (group 2), DSS-treated (group 3), and non-treated (group 4) KAD rats showed no colon tumors on either macroscopic or microscopic observation. Meanwhile, AOM/DSS-treated KAD rats (group 1) developed multiple colon tumors, of which the incidence, number, and volume could be compared with those of tumors developed in AOM/DSS-treated F344 rats (group 5). Interestingly, the AOM/DSS-treated KAD rats showed a higher incidence of diarrhea than the AOM/DSS-treated F344 rats during a few weeks after the cessation of DSS treatment (Fig. 2).

Macroscopically, all of the AOM/DSS-treated KAD rats showed multiple nodular, polypoid, or caterpillar-like colonic tumors (Fig. 3A), whereas half of the AOM/DSS-treated F344 rats had a few colonic tumors (Fig. 3B). The average number of colorectal tumors in the KAD rat was significantly higher than that of F344 rats (9.5 ± 1.8 vs 1.3 ± 0.8, $P < 0.0001$) (Fig. 4A). The average volume of KAD tumors was not different from that of F344 tumors (33.9 ± 23.0 mm^3 vs 10.3 ± 13.7 mm^3, $P = 0.38$). Colon tumors that developed in the KAD rats that received AOM and DSS were distributed more prominently in the rectum (4.0 ± 1.5) and distal colon (5.2 ± 1.7) than in the middle colon (0.3 ± 0.5) (Fig. 4B). No tumors were observed in the proximal colon, cecum, or small intestine.

Microscopically, tumors induced in AOM/DSS-treated KAD rats were diagnosed as tubular adenoma (Fig. 5A), well or moderately differentiated tubular adenocarcinoma (Fig. 5B), or signet-ring cell carcinoma (Fig. S1). The multiplicity of adenoma of the KAD rat was significantly higher than that of the F344 rat

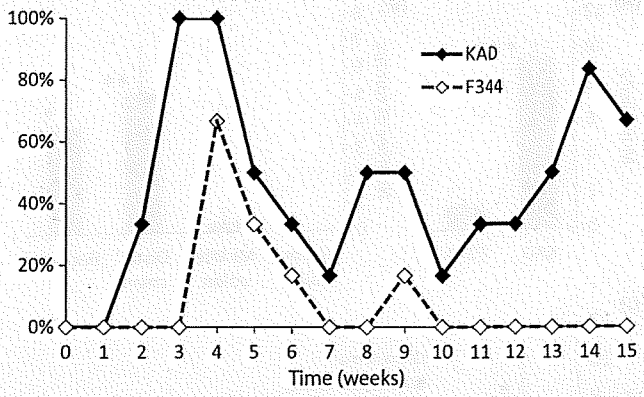

Fig. 2. Incidences of diarrhea observed in azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated Kyoto Apc Delta (KAD) and F344 rats. Percentages of rats showing diarrhea in weekly observations are shown. One-week DSS administration is indicated by a grey box. Note that all KAD rats showed diarrhea in week 3, whereas no F344 rats showed diarrhea.

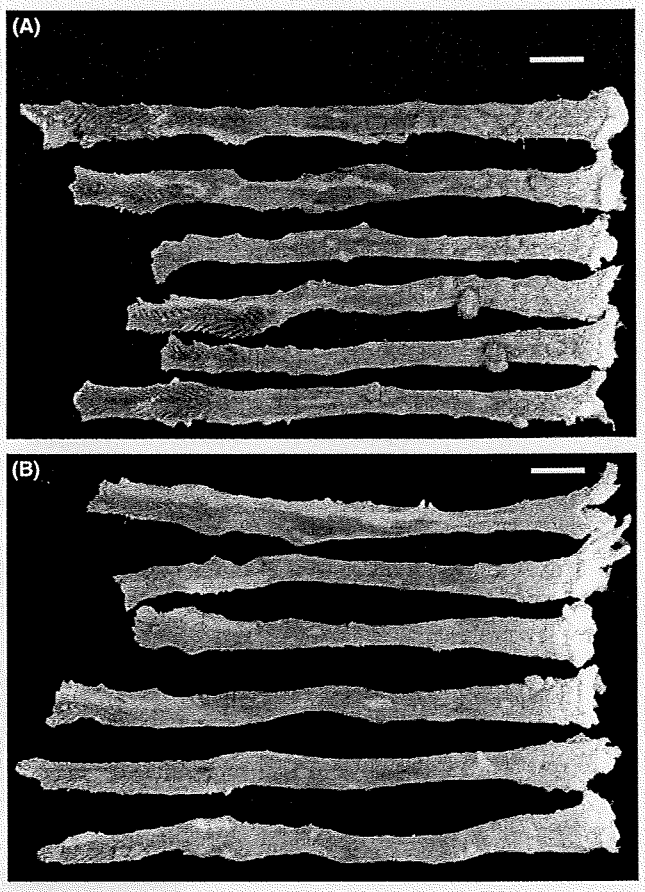

Fig. 3. Macroscopic view of large bowels of azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated (A) Kyoto Apc Delta (KAD) and (B) F344 rats. Scale bars = 2 cm.

(7.7 ± 2.9 vs 0.3 ± 0.5, $P < 0.005$) (Fig. 4C). The multiplicity of adenocarcinoma of the KAD rat was also significantly higher than that of the F344 rat (3.0 ± 0.9 vs 0.5 ± 0.8, $P < 0.001$) (Fig. 4C). Twenty-two of 64 colon tumors induced in the KAD rats invaded the submucosa, muscularis propria, or serosa

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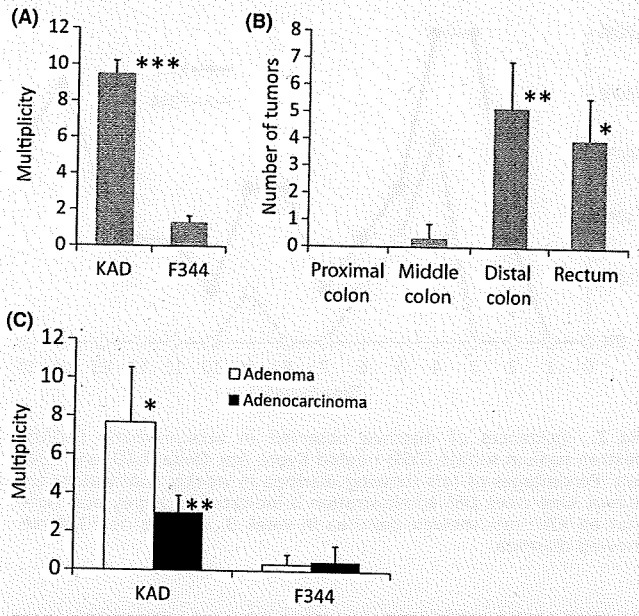

Fig. 4. Increased induction of colon tumors in azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated Kyoto Apc Delta (KAD) rats. (A) Multiplicity of tumors observed macroscopically (mean ± SD) at week 15. ***$P < 0.0001$. (B) Distribution of colon tumors in AOM/DSS-treated KAD rats (mean ± SD) at week 15. **Distal colon versus middle colon, $P < 0.001$; *rectum versus middle colon, $P < 0.005$. (C) Multiplicities of adenoma and adenocarcinoma developed in KAD rats were significantly higher than in F344 rats at week 15. *$P < 0.005$, **$P < 0.001$.

(Fig. 5C), whereas none of the five colon tumors in F344 rats invaded the submucosa or deeper. Four signet-ring cell carcinomas were observed in the KAD rats. Apart from colonic tumors, colonic dysplasia was observed in all of the rats in groups 1 and 5. The average number of dysplasias in the KAD rats (11.2 ± 8.0) was greater than that of the F344 rats (3.7 ± 4.1), but the difference was insignificant ($P = 0.069$). No dysplastic lesions developed in groups 2–4.

Altered cellular localization of β-catenin protein is frequently observed in AOM- or AOM/DSS-induced colorectal tumors.[4,16] Strong β-catenin expression was seen in the cytoplasm and/or nucleus of adenoma and adenocarcinoma cells (Fig. 5D), which indicated the activation of Wnt signaling in these cells.

Endoscopic observation and biopsy of colon tumors. Endoscopic examination was done in the anesthetized KAD rats to determine whether the development of colorectal tumors can be observed without necropsy. We could observe colorectal lesions displaying differences from normal mucosa, including polypoid lesions, as early as the eighth week after AOM administration (Fig. S2A) and could monitor the development of both the number and volume of them during the carcinogenesis test (Fig. S2B). At week 8, the average number of lesions detected by endoscopy was 3.3 ± 1.2. The number of lesions gradually increased with the experimental period and reached 17.3 ± 4.5 at week 14, which was much higher than that of macroscopic observations. Such a discrepancy might be caused by the disappearance of inflammatory polyps at week 14. The biopsy of a tumor specimen under endoscopic observation was successful and the specimen was diagnosed histopathologically (Fig. S2C).

Highly frequent mutations of the β-catenin gene but no mutation of the K-ras gene in colon tumors. Mutation of the β-catenin gene in its glycogen synthase kinase (GSK) 3β phosphorylation consensus motif and K-ras mutation at codon 12 are features of AOM-induced rat colon tumors.[15,16] Direct sequencing of PCR products revealed 29 missense mutations in 29 of 39 (74.4%) colon tumors induced by AOM/DSS in KAD rats (Table 1). The mutation spectrum detected in the present study was quite similar to that detected in the AOM-induced rat colon tumors,[16] which indicated that a common molecular pathway to initiate colon carcinogenesis was shared in AOM- and AOM/DSS-treated colons. Meanwhile, no K-ras mutation at codon 12 was detected in the 39 colon tumors.

Discussion

A two-stage colitis-related colon carcinogenesis model provides a powerful tool for the induction of colon tumors in rats.[7,8] In the current study, to establish a more efficient colon carcinogenesis model, we produced a novel *Apc*-mutant KAD rat. The

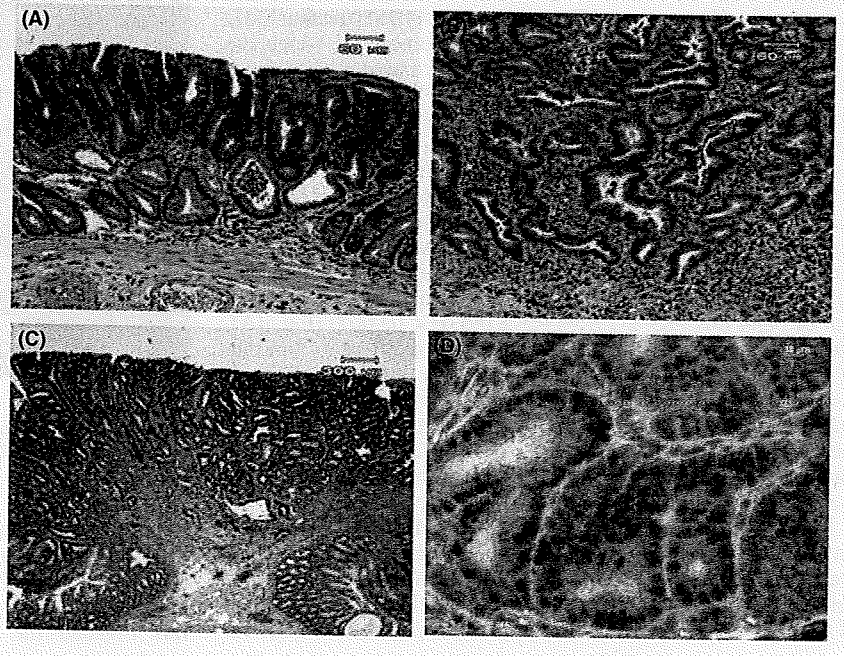

Fig. 5. Histopathology of colonic tumors developed in azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated Kyoto Apc Delta (KAD) rats. (A) Tubular adenoma, (B) well-differentiated adenocarcinoma, and (C) moderately differentiated adenocarcinoma invading the submucosa. Hematoxylin–eosin stain. (D) β-Catenin immunohistochemistry in colonic adenocarcinoma.

Table 1. Mutations in the GSK3β phosphorylation consensus motif of the *Catnnb* gene in colon tumors

Mutated codon	Base change	Amino acid substitution†	No. mutations detected
32	GAT → AAT	Asp → Asn	8
33	TCT → TTT	Ser → Phe	4
34	GGA → GAA	Gly → Glu	8
37	TCT → TTT	Ser → Phe	2
41	ACC → ATC	Thr → Ile	4
44	CCT → CTT	Pro → Leu	1
45	TCC → TTC	Ser → Phe	2
Total			29

†Serine residues in codons 33, 37, and 45 and the threonine residue in codon 41 are GSK3β phosphorylation sites.

KAD rat harbored a nonsense mutation resulting in the truncated APC protein (Δ2523), in which the β-catenin-binding region was retained. The KAD rat was viable and showed the normal distribution of β-catenin in colon epithelium and no spontaneous colon tumors. These findings suggested that Wnt signal might not be activated in the non-treated colon epithelium of KAD rats. In humans, a subset of attenuated familial adnomatous polyposis harbors C-terminal-truncated APC mutations such as Δ2644 and Δ2663.[19,20] The Δ2644 APC protein failed to activate Wnt signaling,[21] and these patients are rarely related to the occurrence of colonic polyposis, but are responsible for the development of extracolonic lesions, including desmoids, gastric fundic grand hyperplastic polyposis, and osteomas. Although we have not yet observed such extracolonic lesions in the KAD rat, further examinations will allow us to establish the KAD rat as a model for attenuated familial adnomatous polyposis.

The AOM/DSS-treated KAD rat showed colon tumors in 100% incidence, much higher in multiplicity (~10-fold) and more advanced in malignancy than the control F344 rat. These tumors can be obtained in only a short period of 15 weeks. We therefore concluded that AOM/DSS colon carcinogenesis was extensively enhanced in the KAD rat. This carcinogenesis model has also several advantages over the Pirc rat model. The epithelial malignancy of our model is more significant than the Pirc model: our model could induce adenocarcinomas (multiplicity is 3.0 ± 0.9), whereas most tumors developed in the colon of the Pirc rat were adenomas. This suggests that we could obtain multiple colon tumors in a shorter period (<15 experimental weeks). Next, we can evaluate the effects of potential carcinogens on colon carcinogenesis more strictly, because KAD are free from spontaneous tumors. Additionally, we can prepare tumor-bearing animals in accordance with our needs, which is a major concern in practical studies. It has been thought that an ideal colon carcinogenesis model would involve not only the efficient induction of tumors but also similar tumor characteristics and good availability for clinical application. The colon tumors developed in the KAD rat showed a predominant distribution in the rectum and distal colon and the accumulation of β-catenin protein, similar to human CRC. Furthermore, the tumors induced were large enough to be observed by endoscopy and biopsied tumor specimens were successfully diagnosed. These findings indicate that the KAD colorectal carcinogenesis model has the potential to mimic clinical operations for human CRC. Our results described here strongly suggest that AOM/DSS-induced colon carcinogenesis with the KAD rat model is ideal and provides an excellent tool to investigate basic and clinical studies on colitis-related CRC. For example, this model enables efficient evaluation of the effects of novel anticancer drugs on tumor regression as well as the effects of anti-inflammatory agents on tumor development. Combination with recently developed fluorescence probes that image viable cancer cells[22] would provide clearer images of tumors and further insights into the pathogenesis of CRC.

In contrast with the AOM/DSS-treated KAD rats, neither AOM-treated nor DSS-treated KAD rats developed colon tumors. It is well known that no colon tumors occurred in the AOM-treated or DSS-treated F344 rats within as short as 15 weeks by the carcinogenesis test.[7,23,24] All AOM/DSS-treated KAD rats developed colon tumors. They also had significant diarrhea for a few weeks after cessation of the DSS exposure. These findings indicate that the KAD rat is susceptible to inflammation provoked by a colitis-inducing agent, DSS, and suggest that severe inflammation of the colon epithelia might be involved in the enhancement of colon carcinogenesis in the KAD rat.

DSS-induced colitis occurs mainly in the distal colon,[25] which is consistent with the predominant distribution of tumors to the distal colon and rectum in the AOM/DSS-treated KAD rat. Additionally, no K-ras mutation was found in the tumors of AOM/DSS-treated KAD rats. K-ras mutation plays a role as a promoter through enhancing COX-2 and iNOS expression in the presence of inflammatory stimuli.[16] However, it is likely that, in our model, DSS enabling the induction of severe inflammation might replace the K-ras mutation. In fact, no mutations of K-ras and a high incidence of substitutions of *Apc* and *p53* genes were found in the colonic tumors induced by a colonic carcinogen, DMH, and a colitis-inducing compound, trinitrobenzene sulfonic acid.[26] These findings may support our idea that inflammation provoked by DSS plays an important role in colon carcinogenesis in the KAD rat, and the C terminus of APC, which is lacking in the KAD rat, might be involved in the effect of DSS on tumor development.

The C terminus of APC, which is lacking in the KAD rat, comprises a 321-amino acid polypeptides and contains a part of the basic domain, EB1-binding domain, and PDZ domain,[27] by which APC interacts with a variety of cytoskeletal proteins, such as microtubules, the microtubule plus end binding protein (EB1), and the mammalian homolog of Discs large.[28–30] With these domains, APC contributes directly and/or indirectly to cell migration, adhesion, chromosome segregation, spindle assembly, and apoptosis in the epithelium of the gut.[31,32] In the DSS colitis model, microbiota alteration, epithelial cell toxicity, increased intestinal permeability, and macrophage activation have been proposed as potential pathogenesis mechanisms of colitis.[33,34] Although so far there is no direct evidence linking these colitis pathogeneses to the functions of APC domains, it is expected that cell migration or adhesion occurring in response to DSS treatment might be disturbed in KAD by the lack of the C-terminal domains. Alternatively, the responses of epithelial cells to cytokines released from macrophages induced by DSS might be altered. Further pathophysiological analysis of the KAD rat colon epithelium would provide insights into the association of the C terminus of APC with colitis. Importantly, other rodent *Apc* mutant models, such as Min mice and Pirc rats, completely lose all protein interaction sites located in the C-terminal half of the protein. Thus, it is very difficult to determine whether the susceptibility to DSS-induced colitis would result from the effects of the C-terminal or central regions of APC.

In summary, we established an enhanced rat AOM/DSS-induced colitis-related colon carcinogenesis model using a novel *Apc* mutant KAD rat. This colon carcinogenesis model system, to our knowledge, is the most effective in the experimental induction of colon tumors and therefore will contribute greatly to promote experimental studies on the pathogenesis, prevention, and treatment of CRC. The KAD rat also provides insights into the involvement of the C terminus of APC in the development of colitis-related CRC.

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References

1 WHO. *WHO Media Center, Cancer*, Fact sheet No. 297. Geneva: WHO, 2009.
2 Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G7–17.
3 Rutter M, Saunders B, Wilkinson K *et al.* Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; **126**: 451–9.
4 Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* 2003; **94**: 965–73.
5 Kohno H, Suzuki R, Sugie S, Tanaka T. β-*Catenin* mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. *Cancer Sci* 2005; **96**: 69–76.
6 Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, Wakabayashi K. Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and dextran sodium sulfate in male ICR mice possess β-*catenin* gene mutations and increases immunoreactivity for β-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. *Carcinogenesis* 2005; **26**: 229–38.
7 Onose J, Imai T, Hasumura M, Ueda M, Hirose M. Rapid induction of colorectal tumors in rats initiated with 1,2-dimethylhydrazine followed by dextran sodium sulfate treatment. *Cancer Lett* 2003; **198**: 145–52.
8 Tanaka T, Yasui Y, Tanaka M, Tanaka T, Oyama T, Rahman KMW. Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats. *Chem Biol Interact* 2009; **177**: 128–36.
9 Kinzler KW, Nilbert MC, Su LK *et al.* Identification of FAP locus genes from chromosome 5q21. *Science* 1991; **253**: 661–5.
10 Suzui M, Okuno M, Tanaka T, Nakagama H, Moriwaki H. Enhanced colon carcinogenesis induced by azoxymethane in min mice occurs via a mechanism independent of beta-catenin mutation. *Cancer Lett* 2002; **183**: 31–41.
11 Tanaka T, Kohno H, Suzuki R *et al.* Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc$^{Min/+}$ mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. *Int J Cancer* 2006; **118**: 25–34.
12 Amos-Landgraf JM, Kwong LN, Kendziorski CM *et al.* A target-selected *Apc*-mutant rat kindred enhances the modeling of familial human colon cancer. *Proc Natl Acad Sci USA* 2007; **104**: 4036–41.
13 Mashimo T, Yanagihara K, Tokuda S *et al.* An ENU-induced mutant archive for gene targeting in rats. *Nat Genet* 2008; **40**: 514–15.
14 Imai Y, Inoue H, Kataoka A *et al.* Pael receptor is involved in dopamine metabolism in the nigrostriatal system. *Neurosci Res* 2007; **59**: 413–25.
15 Takahashi M, Fukuda K, Sugimura T, Wakabayashi K. β-catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Res* 1998; **58**: 42–6.
16 Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Sci* 2004; **95**: 475–80.
17 Gohma H, Kuramoto T, Kuwamura M *et al.* WTC deafness Kyoto (*dfk*): a rat model for extensive investigations of *Kcnq1* functions. *Physiol Genomics* 2006; **24**: 198–206.
18 Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990; **247**: 322–4.
19 Couture J, Mitri A, Lagace R *et al.* A germline mutation at the extreme 3′ end of the APC gene results in a severe desmoid phenotype and is associated with overexpression of beta-catenin in the desmoid tumor. *Clin Genet* 2000; **57**: 205–12.
20 Matsubara N, Isozaki H, Tanaka N. The farthest 3′ distal end *APC* mutation identified in attenuated adenomatous polyposis coli with extracolonic manifestations. *Dis Colon Rectum* 2000; **43**: 720–1.
21 Morin PJ, Sparks AB, Korinek V *et al.* Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* 1997; **275**: 1787–90.
22 Urano Y, Asanuma D, Hama Y *et al.* Selective molecular imaging of viable cancer cells with pH-activatable fluorescence probes. *Nat Med* 2009; **15**: 104–9.
23 Ghirardi M, Nascimbeni R, Villanacci V, Fontana MG, Di Betta E, Salerni B. Azoxymethane-induced aberrant crypt foci and colorectal tumors in F344 rats: sequential analysis of growth. *Eur Surg Res* 1999; **31**: 272–80.
24 Paulsen JE, Loberg EM, Olstorn HB, Knutsen H, Steffensen IL, Alexander J. Flat dysplastic aberrant crypt foci are related to tumorigenesis in the colon of azoxymethane-treated rat. *Cancer Res* 2005; **65**: 121–9.
25 Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990; **98**: 694–702.
26 Santiago C, Pagan B, Isidro AA, Appleyard CB. Prolonged chronic inflammation progresses to dysplasia in a novel rat model of colitis-associated colon cancer. *Cancer Res* 2007; **67**: 10 766–73.
27 Aoki K, Taketo MM. Adenomatous polyposis coli (*APC*): a multi-functional tumor suppressor gene. *J Cell Sci* 2007; **120**: 3327–35.
28 Munemitsu S, Souza B, Muller O, Albert I, Rubinfeld B, Polakis P. The APC gene product associates with microtubules *in vivo* and promotes their assembly *in vitro*. *Cancer Res* 1994; **54**: 3676–81.
29 Su LK, Burrell M, Hill DE *et al.* APC binds to the novel protein EB1. *Cancer Res* 1995; **55**: 2972–7.
30 Matsumine A, Ogai A, Senda T *et al.* Binding of APC to the human homolog of the *Drosophila* discs large tumor suppressor protein. *Science* 1996; **272**: 1020–3.
31 McCartney BM, Nathke IS. Cell regulation by the Apc protein: Apc as master regulator of epithelia. *Curr Opin Cell Biol* 2008; **20**: 186–93.
32 Hanson CA, Miller JR. Non-traditional roles for the adenomatous polyposis coli (APC) tumor suppressor protein. *Gene* 2005; **361**: 1–12.
33 Boismenu R, Chen Y. Insights from mouse models of colitis. *J Leukoc Biol* 2000; **67**: 267–78.
34 Elson CO, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG, Weaver CT. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* 2005; **206**: 260–76.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Signet-ring cell carcinoma observed in the colon of AOM/DSS-treated KAD rats. Four signet-ring cell carcinomas were observed in AOM/DSS-treated KAD rats (group 1).

Fig. S2. Endoscopic observation of KAD colon tumors and biopsy. (A) Endoscopic image of a colon tumor in a KAD rat at week 11. Bleeding from this tumor was found (arrow). (B) Development of colorectal lesions in KAD rats treated with AOM and DSS (group 1). The average numbers of lesions observed by endoscopy were plotted. (C) Microscopic view of a specimen biopsied under endoscopic observation. The specimen was diagnosed as well-differentiated adenocarcinoma. Bar: 60 μm.

Video S1. Biopsy of a colorectal tumor induced by AOM/DSS two-stage colitis-related carcinogenesis in the KAD rat.

Table S1. Primers used in screening for *Apc* mutation in KURMA ENU-mutagenized DNA archives.

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A set of highly informative rat simple sequence length polymorphism (SSLP) markers and genetically defined rat strains

Tomoji Mashimo, Birger Voigt, Toshiko Tsurumi, Kuniko Naoi, Satoshi Nakanishi, Ken-ichi Yamasaki, Takashi Kuramoto and Tadao Serikawa*

Address: Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto 606–8501, Japan

Email: Tomoji Mashimo - tmashimo@anim.med.kyoto-u.ac.jp; Birger Voigt - birger@anim.med.kyoto-u.ac.jp; Toshiko Tsurumi - ttsurumi@anim.med.kyoto-u.ac.jp; Kuniko Naoi - knaoi@anim.med.kyoto-u.ac.jp; Satoshi Nakanishi - satoshi@anim.med.kyoto-u.ac.jp; Ken-ichi Yamasaki - yamaken@anim.med.kyoto-u.ac.jp; Takashi Kuramoto - tkuramot@anim.med.kyoto-u.ac.jp; Tadao Serikawa* - serikawa@anim.med.kyoto-u.ac.jp

* Corresponding author

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Abstract

Background: The National Bio Resource Project for the Rat in Japan (NBRP-Rat) is focusing on collecting, preserving and distributing various rat strains, including spontaneous mutant, transgenic, congenic, and recombinant inbred (RI) strains. To evaluate their value as models of human diseases, we are characterizing them using 109 phenotypic parameters, such as clinical measurements, internal anatomy, metabolic parameters, and behavioral tests, as part of the Rat Phenome Project. Here, we report on a set of 357 simple sequence length polymorphism (SSLP) markers and 122 rat strains, which were genotyped by the marker set.

Results: The SSLP markers were selected according to their distribution patterns throughout the whole rat genome with an average spacing of 7.59 Mb. The average number of informative markers between all possible pairs of strains was 259 (72.5% of 357 markers), showing their high degree of polymorphism. From the genetic profile of these rat inbred strains, we constructed a rat family tree to clarify their genetic background.

Conclusion: These highly informative SSLP markers as well as genetically and phenotypically defined rat strains are useful for designing experiments for quantitative trait loci (QTL) analysis and to choose strategies for developing new genetic resources. The data and resources are freely available at the NBRP-Rat web site [1].

Background

Over the last several years, rat genomic data has grown dramatically, including the genomic sequence [2], single nucleotide polymorphisms (SNPs) [3], and microarray gene-expression profiles [4]. Several rat genetic markers, such as simple sequence length polymorphisms (SSLPs), expressed sequence tags (ESTs) and sequence-tagged sites (STSs), have also been developed and integrated into a high-density rat radiation hybrid map [5], resulting in the comparative mapping of different species, such as between humans and mice. Such infrastructure development of rat genomic resources provides important infor-

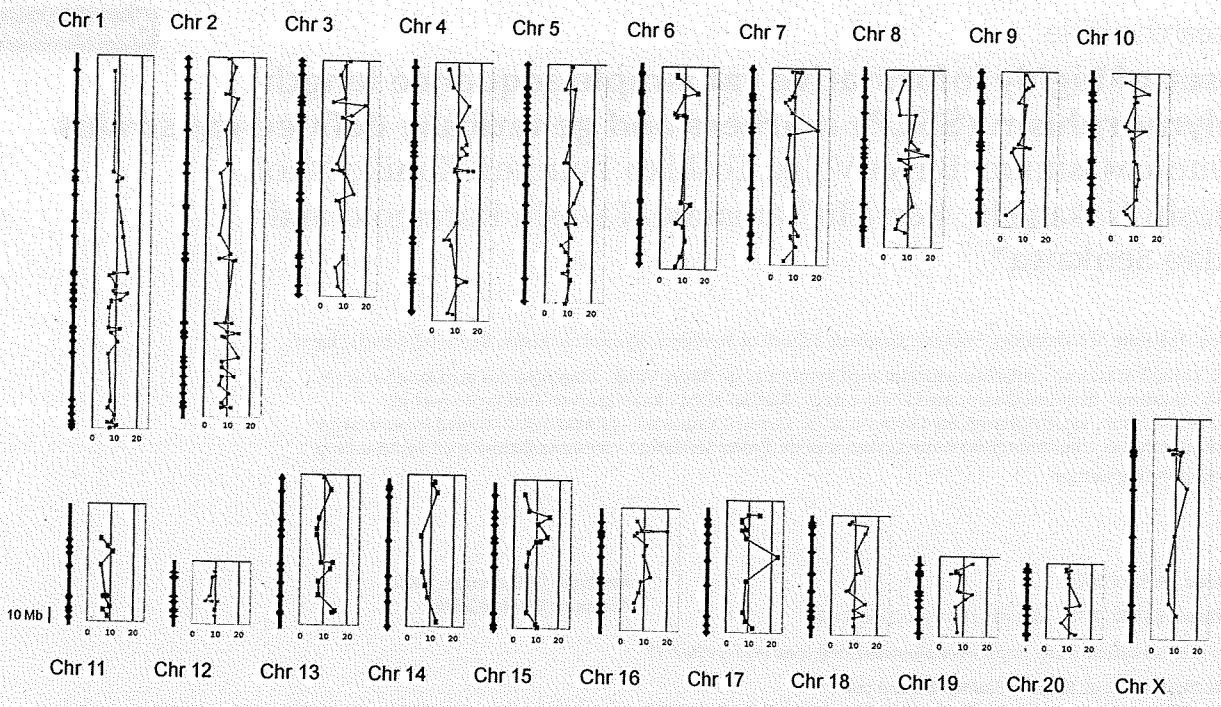

Figure 1

Figure 1

A physical map of 357 rat SSLP markers. The blue lines represent the chromosomal physical map derived from the Rat Genome Browser [15][16] and the blue dots represent the physical position of SSLP markers along the chromosome. The right side graphs of the chromosome indicate the number of alleles among genotyped 122 strains as red dots.

mation for improving the functional annotation of genomic sequences underlying rat QTL and identifying candidate genes for human complex diseases. Although more than 1000 quantitative trait loci have been mapped thus far onto rat chromosomal regions [6], only a few of the mapped QTLs have been identified at the molecular level, such as those for type-I [7] and type-II [8] diabetes, arthritis [9], or fatty acid metabolism [10]. There is a need for functional infrastructure improvement not only for genomic resources and experimental tools, but also for animal resources themselves.

The National Bio Resource Project for the Rat (NBRP-Rat) was started in July, 2002 [11]. The major aim of this project is to improve the experimental environment of rat research by collecting existing rat strains, cryopreserving their sperm and embryos, and distributing them to interested researchers. Until now, more than 300 rat strains have been deposited into the NBRP-Rat. They are all indexed in a publicly accessible database [12]. The biological resource at NBRP-Rat provides the opportunity to supply any deposited rat strain on request to interested scientists around the world, allowing researchers to accelerate their research pace, and to conserve animals and money. Furthermore, to enhance the value of the collected strains and to supply well-characterized rats to the research community, we are promoting the Rat Phenome Project [13], which describes many deposited rat strains with wide-ranging phenotypic measurements comprising 109 parameters: functional observational battery (neurobehavior), behavioral studies, blood pressure, biochemical blood tests, and hematological, urological, and anatomical parameters. These data can be systematically viewed by 'strain ranking' for each parameter, which allows researchers to easily and simultaneously compare phenotypic values of multiple strains and to identify new rat models for specific experiments.

In parallel with the Rat Phenome Project, we are genotyping deposited rat strains. The two major objectives of this study were (1) to choose highly polymorphic SSLP markers that would facilitate genome-wide scans in as many as possible crosses between inbred strains of rat, and (2) using these markers to genetically evaluate rat strains

Table 1: Polymorphic rate between 25 commonly used inbred rat strains

	ACI	BB	BN	BUF	DA	DON	F344	FH	GK	IS	KYN	LE	LEC	LEW	NER	OM	PVG	RCS	SHR	TM	W	WAG	WBN	WKY	WTC	**Total**
ACI/Nkyo	-	76	83	65	31	84	65	75	79	88	85	75	78	69	70	76	71	73	84	75	83	70	71	81	80	**74**
BB/WorTky		-	80	66	78	83	73	68	79	87	84	77	76	60	68	74	69	67	77	72	76	60	71	77	82	**74**
BN/SsNHsd			-	80	81	92	83	80	86	91	90	85	85	78	82	86	85	82	91	83	82	78	81	87	89	**83**
BUF/NacJcl				-	64	86	61	71	76	89	85	75	76	64	67	73	66	65	80	66	79	62	69	77	80	**72**
DA/Slc					-	83	64	75	77	89	85	73	74	67	70	74	68	66	83	74	81	64	74	78	76	**77**
DON						-	84	87	62	73	73	90	90	85	84	88	85	87	57	81	78	84	86	53	55	**76**
F344/Stm							-	72	80	89	86	73	73	66	64	69	62	64	81	72	80	61	67	77	78	**74**
FH/HamSlc								-	79	86	85	67	69	64	77	78	76	73	84	69	81	67	74	82	80	**76**
GK/Slc									-	79	75	83	83	76	76	82	79	79	67	76	66	73	77	60	58	**78**
IS/Kyo										-	80	90	90	88	90	89	87	89	73	87	83	87	87	75	75	**84**
KYN/Hok											-	86	87	86	83	87	84	85	76	86	65	81	85	75	74	**80**
LE/Stm												-	50	73	76	72	77	73	86	68	81	72	75	83	85	**77**
LEC/Hok													-	74	79	75	78	77	86	69	85	73	76	84	83	**76**
LEW/SsNSlc														-	65	73	69	67	83	71	73	59	69	75	79	**76**
NER															-	71	63	65	81	73	74	68	67	78	82	**76**
OM/NSlc																-	69	64	82	75	78	67	73	82	84	**75**
PVG/Seac																	-	48	83	75	77	63	67	81	83	**73**
RCS/kyo																		-	80	73	77	53	70	78	81	**78**
SHR/Izm																			-	79	74	83	82	50	52	**74**
TM/Kyo																				-	78	69	74	76	77	**77**
W/Kyo																					-	76	82	69	70	**70**
WAG/RijKyo																						-	62	77	80	**75**
WBN/KobSlc																							-	81	83	**76**
WKY/Izm																								-	30	**75**
WTC																									-	**75**

deposited into NBRP-Rat. These highly informative SSLP markers in combination with phenotypically defined rat strains are powerful tools for researchers to design various experiments, such as for assessing QTL phenotypes.

Results and discussion

Selection of rat SSLP markers

A panel of 384 SSLP markers was first selected from publicly available data in the Rat Genome Database [14] with two objectives: (1) to obtain the maximum polymorphisms among the listed rat inbred strains and (2) to cover the rat genome except for chromosome Y. Out of 384 markers, 18 failed to be or were poorly amplified by PCR, 7 were amplified as double or multiple bands, and 2 turned out to be designed for the same microsatellite region as other markers. A total of 357 markers were finally used for subsequent genomic screening of rat inbred strains (Fig. 1 and Supplementary Table 1), of which 323 were identified by their physical position in two genome sequence databases, the Ensembl Genome Browser [15] and UCSC Genome Browser [16]. All 34 unidentified markers of the rat genome sequence have been mapped onto a previously reported linkage map (SHRSP × BN cross) [17] or on the radiation hybrid map (RH map version 3.4) [17] published by RGD [14]. The average distance of the marker was 7.59 Mb with the largest gap of 63.0 Mb around the center region of chromosome 6, which roughly corresponds to 28 cM. Detailed information on the SSLP marker set used is available on our website [18].

Genomic profiles and chart tools

We genotyped 122 rat strains with 357 SSLP markers, including 65 inbred strains, 22 substrains, 29 recombinant inbred (RI) strains, and 6 wild rats. The allele size data for each strain are available as genetic profiles on our SSLP database [18]. On the genomic profiles pages, the genotyped strains can be sorted according to their allele size for each marker to easily compare their genetic differences. Researchers can also select any pairwise combination or multiple strains to a maximum of 5 strains to retrieve informative markers for each possible cross.

In addition to the genomic profiles, we have introduced a pedigree-like charting tool that displays genetic differences among the genotyped rat strains (Fig. 2). By selecting one rat strain, its genetic background is instantly compared against all of the strains genotyped at NBRP-Rat. This allows for the selection of a crossing partner strain for QTL analysis or for the easy construction of congenic strains. For instance, BN strains would be good candidates to be crossed with SHR/Izm for genetic analysis, owing to their large genetic diversity (Fig. 2).

Single nucleotide polymorphisms (SNPs) have been assumed to be the best source of genetic variations, accounting for quantitative phenotypic differences between individual strains, and thus became the most promising genetic markers for QTL mapping on dense physical maps [19]. Recently, more than 11,000 rat SSLP markers have been constructed and are publicly available [20]. However, SSLP markers have proved and continue to be a useful genetic tool, particularly in initial mapping studies, owing to their great variety in product size, their easier detection and equal distribution on the genome. In our project, we selected 357 SSLP markers which are equally distributed through the whole rat genome for genetic screening of various rat strains (Fig. 1). The average number of marker alleles among the 122 rat inbred strains tested here was 9.78 ± 2.87, indicating high polymorphisms of the markers. This allows researchers to select informative SSLP markers throughout the rat genome to evaluate their genetic background.

A set of genetically defined rat strains

The 122 rat strains tested here comprise 7381 potential crossing pairs. The polymorphic rate between all possible pairs is shown in supplementary Table 2. Among all crossing pairs, 19.3% have more than 300 informative markers and 87.9% have at least 200 informative markers out of our set of 357 markers (supplementary Fig. 1). The greatest number of informative markers is 341 (96%), derived from the pair between the German wild rat G3 and SHRSR/Ta. The average number of informative markers for a potential cross was 259 ± 21 SD (72.5% of 357 markers), indicating a higher polymorphic rate of our SSLP marker set than that for previously reported data [21,22].

Table 1 indicates the polymorphic rate among 25 commonly used rat inbred strains. All pairs between the strains have at least 107 polymorphic markers (more than 30%). The average number of markers for crosses between 25 commonly used laboratory strains was 271 ± 11 SD (76.0%), which corresponds to an average marker density of 10.0 Mb or 5.6 cM. The highest rate of polymorphisms was 92% between BN and DON. BN, whose genome was closely sequenced [2], has often been used as a crossing partner for linkage and QTL analysis because of its genetic diversity from other inbred rat strains. In contrast to this general assumption, our data suggest it could be promising for certain genetic studies to use the IS strain because of its higher average rate of polymorphisms and various interesting phenotypes (hypotonic, low cholesterol, etc.), compared to commonly used rat strains such as BN. Given that the IS strain was established by crossing a Japanese wild male and a Wistar female as a model for vertebral malformation [23], this strain is a useful but almost unused genetic resource.

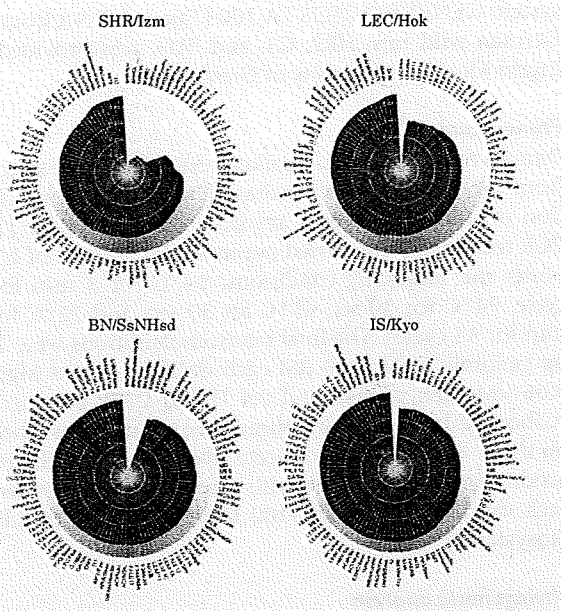

Figure 2

Figure 2
A pedigree-like charting tool on the SSLP database. A pedigree-like charting tool that displays SSLP differences among the genotyped rat strains on the SSLP database [18]. Researchers can select a rat strain to instantly compare its genetic background against all rat strains typed at NBRP-Rat.

Phenotype data are also available for the above genetically defined rat strains on our phenome database [24]. A listing, known as 'strain ranking' (Fig. 3), allows the sorting of more than 100 rat strains according to their phenotypic values. For instance, Figures 3A and 3B indicate that commonly used inbred strains have a wide variability of values for phenotypic parameters, such as body weight and blood pressure, respectively. The charting tool can also provide a column chart or a scatter plot for two selected parameters to indicate the correlation between selected rat strains (Figs 3C and 3D). These phenotype and genotype data allow for the easy selection of appropriate rat strains for various research fields [13].

A family tree of inbred rat strains

Information about the relationships between inbred rat strains is useful for determining the extent of polymorphisms between strains when designing crosses and for evaluating the genetic background of strains when assessing phenotypes. Using the genotype profile derived by our 357 SSLP marker set, we constructed a phylogenetic tree for 93 rat strains (Fig. 4). This tree includes all genotyped strains except for recombinant inbred (RI) ones. Although German and Japanese wild rat strains have not yet been deposited in NBRP-Rat, we used their DNA for phylogenetic analysis to extend the genetic range of our approach and to classify the genetic basis of commonly used laboratory strains. Maximum parsimony analysis was implemented through a heuristic search in PAUP 4.0b10 [25].

As shown in Fig. 3, the phylogenetic relationships of rat strains in the family tree were well resolved and appeared to be mostly consistent with their known histories [26] and previously reported rat family trees [21,22]. As expected, Japanese and German wild rats as well as BN strains, which originated from a brown mutant in a stock of wild rats maintained by DH King and P Aptekman in Philadelphia [26], were distant from each other and from other strains of commonly used laboratory rats. It is assumed that a large proportion of laboratory rat strains originated from the Wistar strain, which were brought to the Wistar Institute by Donaldson in 1906. However, the origin of this strain is still unknown [27]. In the family tree, all Wistar strains including those bred in Japan as well as in other countries, are apparently very different from the group of wild rat strains and BN strains. Further genetic analysis of wild rats derived from other countries, such as China or India, or fancy rats might clarify the origin of the laboratory rat, especially the Wistar-derived strains. In mice, the majority of laboratory strains not derived from wild species are assumed to have originated from a relatively small group of ancestral subsets [28]. In the rat family tree, the laboratory rat strain KZ-*Lepr*^{fa}, which originated from Zucker-fatty rats, belongs to a subgroup of wild rats. Furthermore, IS and KYN strains are historically known to be established by crossing Wistar rats with Japanese wild rats [23]; [29], and this is supported by their longer branch length in our topological tree and relatively higher polymorphic rate from other laboratory strains (Table 1). These observations mean that such inbred strains of rat can provide alternative genetic variations when assessing QTL phenotypes.

Conclusion

The most unique and important point of this study is the availability of many inbred rat strains, which have been genetically defined with a set of equally distributed SSLP markers present throughout the rat genome. Rat strains have also been systematically characterized regarding phenotypic measurements comprising 109 parameters in the Rat Phenome Project [13]. These genetically and phenotypically characterized rat strains can be freely acquired from NBRP-Rat with a contracting material transfer agreement (MTA). Researchers can easily search for their strains of interest according to their phenotypic and genetic requirements by comparing more than 100 inbred strains using visual charting tools in our Rat Phenome and SSLP database. A catalogue of these rat strains provides the opportunity to identify the most suitable parental strains for setting up an appropriate cross to identify QTLs. In

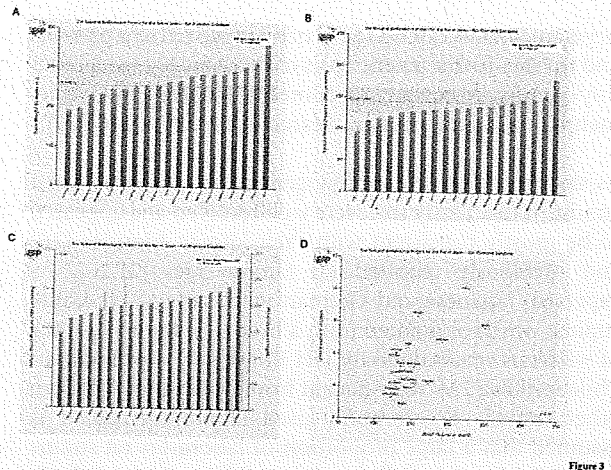

Figure 3
A graphical charting tool on the Phenome database. A graphical charting tool that displays phenotypic values for the deposited rat strains at NBRP-Rat on the Phenome database [13][24]. Various rat strains can be sorted according to their values for a selected parameter, such as body weight (A) and blood pressure (B). Column chart (C) or a scatter plot (D) are also two available options in case two parameters are selected to compare their correlation across all examined rat strains.

addition, this unique catalogue shows the general range of phenotype and genotype data for many inbred strains.

Methods

Animals

All deposited rats at the NBRP-Rat are maintained in specific pathogen-free (SPF) areas or are cryopreserved. Detailed information concerning deposited strains, the depositor, origin, generations, references, deposition status, usage restrictions etc., can be obtained from our website [1]. MITB, MITC, and MITE are Japanese wild-rat strains (partially inbred) of Mitake B, C, and E [30], while HR2, G3, and RU1 are individual males of German wild rats [31]. Although these wild-rat strains and some commercial strains have not yet been deposited in NBRP-Rat, we used their DNA as controls and for genetic enrichment for our phylogenetic analysis. For all markers that appeared to be bi-allelic, in particular for the wild German rats, we always considered only the shorter allele for phylogenetic calculations.

The genomic DNA of the deposited rat strains at NBRP-Rat was extracted from the tail. The tip of the tail was digested with lysis buffer (100 mM Tris- HCl, 12.5 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.8 mg/ml proteinase K) at 50°C overnight. Genomic DNA was extracted using the automatic DNA purification system GENEXTRACTOR TA-100 (Takara). DNA from the German wild rats, HR2, G3, and RU1, was provided by Ingrid Klöting (University of Greifswald).

Primers and PCR

Primers for the 384 SSLP markers tested here were selected from the Rat Genome Database [14]. Detailed information on the primer set is available on our website [18]. PCR reactions were carried out in a total volume of 25 μl under the following conditions: 94°C for 3 min for 1 cycle, 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min for 35 cycles. The final reaction conditions were 100 ng genomic DNA, 200 μM each dNTP, 1.0 mM $MgCl_2$, 0.66 μM each primer, and 0.4 U Taq DNA polymerase (GibcoBRL). The forward primer of each pair was labeled on the 5' end with a fluorescent dye. The size of the PCR products was determined with internal size standards in each capillary on an ABI3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis

A phylogenetic tree of rat strains was obtained through maximum parsimony analysis implemented in PAUP 4.0b10 [25]. First, the allele size of the 357 SSLP markers was transformed to discrete characters in such a way that any size allele differed by one step. A heuristic search

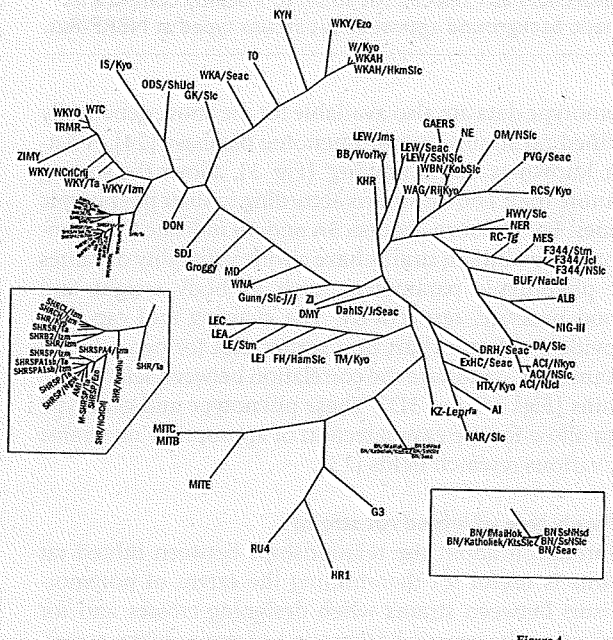

Figure 4
A family tree of 93 inbred rat strains. A phylogenetic tree was developed through a heuristic search for maximum parsimony implemented in PAUP 4.0b10 [25]. TreeView [32] was used to display the radial tree.

method was used in PAUP to search for optimal trees because the genotype data set for the 122 rat strains was too large for single-step computer analysis. Unordered characters were defined such that any state was capable of transforming directly to any other state with equal cost (Fitch parsimony). Two basic strategies are combined in the heuristic search strategy: a stepwise addition to obtain an initial tree, and branch swapping for rearrangement to find shorter trees. Under the analysis, 100 random addition-sequence replications were done, followed by a tree bisection-reconnection (TBR) branch swapping algorithm with the COLAPSE option on to collapse any zero-length branches and with the STEEPEST DESCENT option off. Tree stability was estimated by bootstrap analysis of 100 replicates, sampling characters with equal probability. TreeView [32] was used to display the radial tree.

Authors' contributions

TM selected SSLP markers, performed phylogenetic analysis and wrote the manuscript. BV constructed the website, developed a pedigree-like charting tool, and assisted with the computational analysis of the results. TT contributed to the initial analysis of genotyping data. KN contributed to collecting animals. SN prepared genomic DNA and performed PCR reactions. KY contributed to breeding animals. TK participated in protocol development and results' interpretation. TS conceived, designed, and coordinated the study, and revised the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

supplementary table 1Information on 384 SSLP markers selected in this study.

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[http://www.biomedcentral.com/content/supplementary/1471-2156-7-19-S1.xls]

Additional File 2

supplementary table 2 Polymorphic rate between all possible pairs of 122 rat strains.

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[http://www.biomedcentral.com/content/supplementary/1471-2156-7-19-S2.xls]

Additional File 3

supplementary Figure 1The number of informative markers per cross. Pairwise combinations of 122 inbred rat strains were analyzed to determine the number of markers that were polymorphic for each cross. The number of informative crosses was plotted against the number of informative markers for each pair of strains. The tail on the left side of the histogram displays crosses between closely related substrains, including a cluster of ACI, BN, F344, and SHR strains. The mean number of informative markers per cross for all pairs of strains analyzed was 259 ± 21 SD (72.5% of 357 markers).

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[http://www.biomedcentral.com/content/supplementary/1471-2156-7-19-S3.pdf]

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References

1. **National Bio Resource Project for the Rat in Japan** [http://www.anim.med.kyoto-u.ac.jp/NBR]
2. Rat Genome Sequencing Project Consortium: **Genome sequence of the Brown Norway rat yields insights into mammalian evolution.** *Nature* 2004, **428**:493-521.
3. Zimdahl H, Nyakatura G, Brandt P, Schulz H, Hummel O, Fartmann B, Brett D, Droege M, Monti J, Lee YA, *et al.*: **A SNP map of the rat genome generated from cDNA sequences.** *Science* 2004, **303**:807.
4. Walker JR, Su AI, Self DW, Hogenesch JB, Lapp H, Maier R, Hoyer D, Bilbe G: **Applications of a rat multiple tissue gene expression data set.** *Genome Res* 2004, **14**:742-749.
5. Kwitek AE, Gullings-Handley J, Yu J, Carlos DC, Orlebeke K, Nie J, Eckert J, Lemke A, Andrae JW, Bromberg S, *et al.*: **High-density rat radiation hybrid maps containing over 24,000 SSLPs, genes, and ESTs provide a direct link to the rat genome sequence.** *Genome Res* 2004, **14**:750-757.
6. Twigger S, Lu J, Shimoyama M, Chen D, Pasko D, Long H, Ginster J, Chen CF, Nigam R, Kwitek A, *et al.*: **Rat Genome Database (RGD): mapping disease onto the genome.** *Nucleic Acids Res* 2002, **30**:125-128.
7. Yokoi N, Komeda K, Wang HY, Yano H, Kitada K, Saitoh Y, Seino Y, Yasuda K, Serikawa T, Seino S: **Cblb is a major susceptibility gene for rat type 1 diabetes mellitus.** *Nat Genet* 2002, **31**:391-394.
8. Watanabe TK, Suzuki M, Yamasaki Y, Okuno S, Hishigaki H, Ono T, Oga K, Mizoguchi-Miyakita A, Tsuji A, Kanemoto N, *et al.*: **Mutated G-protein-coupled receptor GPR10 is responsible for the hyperphagia/dyslipidaemia/obesity locus of Dmo1 in the OLETF rat.** *Clin Exp Pharmacol Physiol* 2005, **32**:355-366.
9. Olofsson P, Holmberg J, Tordsson J, Lu S, Akerstrom B, Holmdahl R: **Positional identification of Ncf1 as a gene that regulates arthritis severity in rats.** *Nat Genet* 2003, **33**:25-32.
10. Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, *et al.*: **Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats.** *Nat Genet* 1999, **21**:76-83.
11. Serikawa T: **Colourful history of Japan's rat resources.** *Nature* 2004, **429**:15.
12. **NBRP-Rat Strain database** 2004 [http://www.anim.med.kyoto-u.ac.jp/NBR/strainsx/Strains_list.aspx].
13. Mashimo T, Voigt B, Kuramoto T, Serikawa T: **Rat Phenome Project: the untapped potential of existing rat strains.** *J Appl Physiol* 2005, **98**:371-379.
14. **Rat Genome Database** 2005 [http://rgd.mcw.edu].
15. **Ensembl Genome Browser** [http://www.ensembl.org]
16. **UCSC Genome Browser** [http://genome.ucsc.edu]
17. Steen RG, Kwitek-Black AE, Glenn C, Gullings-Handley J, Van Etten W, Atkinson OS, Appel D, Twigger S, Muir M, Mull T, *et al.*: **A high-density integrated genetic linkage and radiation hybrid map of the laboratory rat.** *Genome Res* 1999, **9**:AP1-8. insert.
18. **NBRP-Rat Genome database** [http://www.anim.med.kyoto-u.ac.jp/NBR/Genotyping.htm]
19. Guryev V, Berezikov E, Malik R, Plasterk RH, Cuppen E: **Single nucleotide polymorphisms associated with rat expressed sequences.** *Genome Res* 2004, **14**:1438-1443.

20. Wilder SP, Bihoreau MT, Argoud K, Watanabe TK, Lathrop M, Gauguier D: **Integration of the rat recombination and EST maps in the rat genomic sequence and comparative mapping analysis with the mouse genome.** *Genome Res* 2004, **14**:758-765.
21. Canzian F: **Phylogenetics of the laboratory rat Rattus norvegicus.** *Genome Res* 1997, **7**:262-267.
22. Thomas MA, Chen CF, Jensen-Seaman MI, Tonellato PJ, Twigger SN, Canzian F: **Phylogenetics of the laboratory rat Rattus norvegicus.** *Mamm Genome* 2003, **14**:61-64.
23. Ishibashi M: **History and summary of Ishibashi rats (ISR).** *Jikken Dobutsu* 1979, **28**:599-600.
24. **NBRP-Rat Phenome database** [http://www.anim.med.kyoto-u.ac.jp/NBR/Phenotyping.htm]
25. Swofford DL: *PAUP: phylogenetic analysis using parsimony, version 3.1* Illinois: Champaign; 1991.
26. Festing MFW: *Inbred strains* New York: Academic press, Inc.; 1979.
27. Lindsey JR: *Historical Foundations* New York: Academic press, Inc.; 1979.
28. Beck JA, Lloyd S, Hafezparast M, Lennon-Pierce M, Eppig JT, Festing MF, Fisher EM: **Genealogies of mouse inbred strains.** *Nat Genet* 2000, **24**:23-25.
29. Shoji R, Harata M: **Abnormal urogenital organs occurring spontaneously in inbred ACI and Kyoto-notched rats.** *Lab Anim* 1977, **11**:247-249.
30. Kondo Y, Ohno K, Oda S, Serikawa T: **Genetic profiles of three inbred strains derived from wild rats (Rattus norvegicus) trapped in Japan.** *Exp Anim* 1996, **45**:405-409.
31. Voigt B, Kovacs P, Vogt L, Kloting I: **How heterozygous are wild rats (Rattus norvegicus)?** *Transplant Proc* 1997, **29**:1772-1773.
32. Page RD: **TreeView: an application to display phylogenetic trees on personal computers.** *Comput Appl Biosci* 1996, **12**:357-358.

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ORIGINAL ARTICLE

Lack of Bcl11b tumor suppressor results in vulnerability to DNA replication stress and damages

K Kamimura[1], Y Mishima[1,2], M Obata[1,2], T Endo[1], Y Aoyagi[1] and R Kominami[1,2]

[1]*Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan and* [2]*Center for Transdisciplinary Research, Niigata University, Niigata, Japan*

Bcl11b/Rit1 **is involved in T-cell development and undergoes chromosomal rearrangements in human T-cell leukemias. Thymocytes of** ***Bcl11b*** $^{-/-}$ **newborn mice exhibit apoptosis at a certain developmental stage when thymocytes re-enter into the cell-cycle. Here, we show that Bcl11b-knockdown T-cell lines, when exposed to growth stimuli, exhibited apoptosis at the S phase with concomitant decreases in a cell-cycle inhibitor, p27 and an antiapoptotic protein, Bcl-xL, owing to transcriptional repression. This repression was a likely consequence of the impairment of Sirt1, a nicotinamide adenine dinucleotide-dependent deacetylase associating with Bcl11b. Activation of the apoptotic process cleaved the mediator protein, Claspin, and inhibited phosphorylation of cell-cycle checkpoint kinase 1 (Chk1) that plays a central role in sensing and responding to incomplete replication.** ***Bcl11b*** $^{-/-}$ **thymocytes also failed to phosphorylate Chk1 when UV irradiated. These results implicate Bcl11b in the remedy for DNA replication stress and maintenance of genomic integrity.**

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Keywords: DNA replication stress; Chk1; p27; apoptosis; Bcl11b tumor suppressor

Introduction

Eucaryotic cells maintain genomic integrity by monitoring DNA for damage or incomplete replication. In the event of aberrant DNA structures being detected, cells activate regulatory pathways to delay cell-cycle progression and to allow the damage to be repaired or replication to be completed (Abraham, 2001). Cells can also activate pathways leading to apoptosis and the removal of a damage cell from a tissue. The balance between the two pathways determines the survival of individual cells and the maintenance of genomic stability. Mammalian cells have two protein kinases of the phosphoinositide 3-kinase-related kinase family, ataxia telangiectasia mutated (ATM) kinase and ATM-Rad3-related kinase (ATR), which play central roles in sensing and responding to chromosomal insults (Abraham, 2001). Downstream of ATM and ATR kinases are the cell-cycle checkpoint kinases (Chk)1 and Chk2, activation of which requires the mediator family of proteins such as Claspin and BRCA1.

DNA replication stress occurring in S phase triggers activation of ATR that phosphorylates Chk1 at Ser317 and Ser345 and upregulates its kinase activity (Abraham, 2001; Chen and Sanchez, 2004). Activated Chk1 inhibits the protein phosphatase Cdc25A and Cdc25C by phosphorylation, halting ongoing DNA replication and initiating DNA repair. Cdc25A and Cdc25C control inhibitory phosphorylation sites on cyclin-dependent kinases (cdks), critical regulators of cell-cycle transitions (Walworth *et al.*, 1993; Sanchez *et al.*, 1997). As Chk1 has a critical role in maintaining genomic stability, Chk1 inactivation in somatic cells results in accumulation of structural chromosomal aberrations, eventually leading to tumorigenesis (Kastan and Bartek, 2004; Lam *et al.*, 2004). Consistently, study of *Chk1* knockout mice shows that *Chk1* is a haploinsufficient tumor suppressor gene (Lam *et al.*, 2004).

$p27^{Kip1}$, a cdk inhibitor, is a rate-determining component of cell-cycle exit in a number of cell types and plays a key role in coordinating the activity of cyclin E-cdk2 with accumulation of cyclin D-cdk4 (Nakayama *et al.*, 2004). The amount of p27 increases in quiescent cells, whereas mitogens and extracellular matrix adhesion signals can promote p27 degradation, allowing quiescent cells to re-enter the cell-cycle. Consistent with a role for p27 in cell proliferation, *p27* knockout mice develop hyperplasia in multiple organs but are relatively free from malignancy with the exception of pituitary hyperplasia (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). Thus, p27 might act as a weak tumor suppressor of a narrowly defined group of cells, largely the decision of cells to exit the cell-cycle (Fero *et al.*, 1998; Di Cristofano *et al.*, 2001).

Bcl11b/Rit1/Ctip2 is a tumor suppressor gene isolated by positional cloning using γ-ray-induced mouse thymic lymphomas (Shinbo *et al.*, 1999; Wakabayashi *et al.*, 2003a; Sakata *et al.*, 2004). Recurrent chromosomal rearrangements at *BCL11B* locus are also found in

Correspondence: Dr R Kominami, Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences and Center for Transdisciplinary Research, Niigata University, Asahimachi 1-757, Niigata 951-8510, Japan.
E-mail: rykomina@med.niigata-u.ac.jp
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