Diubiquitin (Ubdi) is a susceptibility gene for virus-triggered autoimmune diabetes in rats

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Genetic studies of type 1 diabetes (T1D) have been advanced by comparative analysis of multiple susceptible and resistant rat strains with a permissive class II MHC haplotype, RT1B/Du. LEW.1WR1 (but not resistant LEW.1W or WF) rats are susceptible to T1D induced by a TLR3 agonist polyinosinic-polycytidylic acid followed by infection with parvovirus. We have mapped genetic loci for virus-induced T1D susceptibility, identifying a major susceptibility locus (Iddm37) near the MHC. The Iddm37 homologs on mouse and human chromosomes are also diabetes linked. We report that a major effect gene within Iddm37 is diubiquitin (Ubdi). Gene expression profiling of pancreatic lymph nodes in susceptible and resistant rats during disease induction showed differences in Ubdi transcript abundance. The LEW.1WR1 Ubdi promoter allele leads to higher inducible levels of Ubdi than that of LEW.1W or WF. Using zinc-finger nucleases, we deleted a segment of the LEW.1WR1 Ubdi gene and eliminated its expression. UBDb-deficient rats show substantially reduced diabetes after viral infection. Complementary studies show that there may be another diabetes gene in addition to Ubdi in the Iddm37 interval. These data prove that Ubdi is a diabetes susceptibility gene, providing insight into the interplay of multiple genes and environmental factors in T1D susceptibility.

Keywords: autoimmune diabetes; susceptibility genes; virus-induced; rat model
are in strong linkage disequilibrium, and this is reflected in the ‘B/D’ nomenclature. In addition, a previous genome-wide linkage study was used to identify regions of the rat genome that control T1D due to virus exposure. In this linkage study, (LEW.1WR1 × WF) F2 animals were used to map two major quantitative trait loci, both derived from LEW.1WR1, that promote diabetes susceptibility in rats. In addition to an established susceptibility locus (Iddm14), a new locus (Iddm37) near the MHC on chromosome 4 was found to be a major determinant of T1D disease susceptibility virus-induced T1D. Interestingly, Iddm37 homologs on mouse and human chromosomes are also linked to T1D.

In the present study, we took advantage of congenic strains that are closely related to LEW.1WR1 and informative for the Iddm37 interval. The LEW.1WR1 rat was generated from two LEW MHC congenics, LEW.1W and LEW.1A (Figure 1). On the basis of their genotypes, we predicted that both parental strain rats would be resistant to induction of diabetes by KRV + poly I:C but for different reasons. LEW.1W has the permissive class II MHC (RT1-B/Da) but not the permissive Iddm37, whereas LEW.1A has the permissive Iddm37 but a nonpermissive class II region (RT1-B/Du). LEW.1WR1 rats bear RT1-D/B and (MHC class II) and a recombination distal to the Iddm37 interval and are susceptible.

We confirmed this hypothesis and proceeded to positional gene identification for Iddm37 using the LEW.1WR1 and LEW.1W strains. To do so, we performed gene expression studies of the genes in the interval, and identified two genes for further study. The strain distribution pattern of sequence polymorphisms and expression of each gene, diubiquitin (Ubd), closely resembled the strain distribution pattern of virus-induced diabetes in rats. The Ubd gene was then deleted on the LEW.1WR1 background, which resulted in modestly but significantly reduced diabetes incidence, as would be expected of a single gene in the polygenic context of T1D. To our knowledge, this is the first genetic deletion using zinc-finger nucleases (ZFN) technology of any autoimmune candidate gene in rats.

RESULTS

Susceptibility to KRV-induced T1D in congenic rat strains

We first assessed the susceptibility of three LEW congenic rat strains to virus-induced diabetes. As shown in Figure 2, we documented diabetes susceptibility in LEW.1WR1 rat and diabetes resistance in LEW.1A rats, which bear the nonpermissive RT1-B/Da haplotype. This experiment also confirmed our prediction that LEW.1W congenic rats, bearing a permissive RT1-B/Da but a resistant haplotype for the Iddm37 interval, are T1D resistant in response to viral infection.

Figure 1. The centromeric end of rat chromosome 20. We determined informative alleles by determining genotypes of the four congenic strains (Blankenhorn et al.), and this cartoon (not to scale) shows the proximal 25 Mb of chromosome 20 and the approximate borders of intervals retained in each congenic rat strain. LEW.1WR1 is a recombinant descendant of LEW.1A × LEW.1W.

Figure 2. Kaplan–Meier survival plot of virus-induced T1D in LEW RT1-congenic rats. The rat strains show highly significantly different susceptibility to T1D (χ² = 28.99, P < 0.0001). Days PI, days post induction by poly I:C + KRV. For these studies, a total of 18 LEW.1WR1, 12 LEW.1W and 6 LEW.1A rats were induced for T1D when they reached 28–30 days of age.

Microarray

There are 370 known genes and gene elements in the Iddm37 region (Rat genome v4, http://genomeweb.uchc.edu/) and we examined the expression differences for all of them using the Affymetrix RG230 2.0 GeneChip (Affymetrix, Santa Clara, CA, USA). For this experiment, we treated one group of nine LEW.1WR1 rats and two groups of diabetes resistant rats (LEW.1W and WF.Iddm4, nine of each) to induce diabetes. The WF.Iddm4 is a congenic diabetes resistant strain on the WF rat which is susceptible to diabetes induced by poly I:C but not rat virus. Using these animals, we examined global transcript levels in pancreatic lymph nodes across three early time points in disease progression (day 0, after poly I:C; day 3, after KRV; and day 5, after KRV). Comparisons were made between LEW.1WR1 and each of the other rat strains. We accepted a false discovery rate of 0.20, to account for the biological diversity among the three replicates each time.

Nine genes within the Iddm37 interval were differentially regulated in the same direction in comparisons of LEW.1WR1 vs LEW.1W and LEW.1WR1 vs WF.1iddm4, as would be expected for an Iddm37 candidate gene. Confirmatory quantitative RT–PCR was conducted for all nine of these genes (Table 1). We selected for further study, the two most significantly different genes that were concordant and different across all time points: UBD and RT1-N1 (Table 1). RT1-N1 is an MHC class Ib gene (http://rgd.mcw.edu/rgdweb/report/gene/main.html?id=3498). Notably, there was an excess of LEW.1WR1 Ubd transcripts, and an excess of LEW.1W RT1-N1 transcripts on day 0, even before the KRV inoculation.

Iddm37 candidate gene strain distribution patterns (SDP)

to determine which of the two genes was the more likely candidate for Iddm37, it was necessary to characterize their alleles and haplotypes in a number of rat strains (that is, to determine their SDP). To obtain an SDP for the RT1-N1 gene, we first identified polymorphic variants between LEW.1W and LEW.1WR1 by sequencing, which identified one synonymous single nucleotide polymorphisms (at nucleotide 2 785 930 on chromosome 20) between LEW.1W and LEW.1WR1 in exon 2 and a second single nucleotide polymorphisms (nucleotide 2 785 102) in the promoter region that distinguishes LEW.1W and WF from LEW.1WR1. We
Table 1. Fold-change of expression (LEW.1WR1:other rat strain) is shown for nine genes with significant expression in pancreatic lymph nodes on the microarray (chip)

| Day 0 (poly I:C only) | Day 3 | Day 5 |
|-----------------------|-------|-------|
| LEW.1WR1 vs LEW.1W | LEW.1WR1 vs LEW.1W | LEW.1WR1 vs LEW.1W |
| RT1-N1 class Ib | Ubd | Lymphotoxin alpha |
| Ltna | RT1-N1/RT1-N2 | Lta |
| Tnf | Ubd transcript 1 | Tnf |
| Nrm | Valyl-tRNA synthetase | Sialidase 1 |
| Nurim | Leukocyte-specific transcript 1 | Lst1 |

Highlighted in bold type are genes that are significantly upregulated or downregulated in LEW.1WR1. Quantitative RT–PCR was performed on the same triplicate samples from PLN, to determine which of the microarray differences between the rats for expression of these genes is genuine. Only Ubd, RT1-N1, RT1-N1/RT1-N2, and BBDR were characterized by the absence of this additional 35 bp (Table 2).

We also used sequencing to characterize the Ubd haplotypes. In our previous report, we presented two haplotypes for the coding region of Ubd.25 We now report an additional Ubd haplotype for the BBDR rat and several other strains that have been classified for T1D susceptibility26 (Supplementary Figure 1). Sequencing the promoters and 5'-UTR of Ubd from LEW.1W, LEW, WF and LEW.1WR1 haplotypes revealed two major allelic variants among these strains. There is a polymorphic microsatellite region at –775 relative to the transcriptional start site, and in all the resistant strains, an insertion of 59 nucleotides just upstream of the 5'-UTR. This 59-nt insertion is a short interspersed element (B2-SINE) as described by the UCSC database (rat genome v4, genome.ucsc.edu).

The polymorphisms of Ubd—including the SINE—were compiled to make an SDP of Ubd haplotypes for comparison with the SDP for RT1-N1 sequence polymorphisms and the SDP of diabetes susceptibility (Table 2). The results show that RT1-N1 is a less probable candidate gene than Ubd; because BBDR rats do not have the same RT1-N1 allele as LEW.1WR1, yet both are susceptible to virus-induced T1D. The Ubd SINE, on the other hand, has an identical SDP for T1D in these rat strains. All the RT1-N1 rat strains that are diabetes resistant have low Ubd expression, and Ubd is highly expressed (and cytokine inducible25) in both KRV–T1D-susceptible rat strains, whereas RT1-N1 expression is discordant. In addition, no functionally relevant polymorphisms were found in the RT1-N1 sequence between LEW.1W and LEW.1WR1, strengthening the notion that Ubd is the gene that more likely underlies the Iddm37 susceptibility locus.

Ubd promoter polymorphisms control allele-specific Ubd expression

The gene expression data showing reduced Ubd gene expression in LEW.1W and WF.Iddm4 rats were not surprising, as we have previously demonstrated that expression of UBD in draining lymph nodes of poly I:C+ KRV inoculated animals is fourfold higher in LEW.1WR1 rats than in WF rats; this difference is even more dramatic in splenocytes.25 In that study, we proved that this was not due to a deficiency of required inflammatory cytokines, which were expressed at similar levels in both rats, indicating that rats of both strains had comparable cytokine responses to poly I:C and to the KRV infection.

It was important to link the phenotype (low Ubd mRNA expression) to the allelic polymorphism seen in LEW.1W. We analyzed the SINE insertion and the region surrounding it using a program to highlight transcription factor binding sites (Transfac, http://www.gene-regulation.com/index2.html); the result suggested that insertion of the SINE could add new transcription factor binding sites and/or distance native Ubd promoter elements from the transcriptional start site. The contribution of the microsatellite polymorphism in the upstream region of the Ubd promoter, however, was unknown. To distinguish which of the two polymorphic promoter elements is responsible for the UBD expression difference, luciferase reporter vectors were constructed (Supplementary figure 2) comprising each variant region in the promoter from either the LEW.1WR1 or the LEW.1W rat, and measured luciferase expression. When tested in vitro in Huh7 cells, the level of luciferase in cells transfected with the SINE-containing promoter from LEW.1W was significantly lower compared with the level of luciferase driven by the Ubd promoter from LEW.1WR1 (Figure 3). The region containing the microsatellite polymorphism...
founder rats. One founder (LEW.1WR1-UbdemUmass or UBD-KO) had heterozygosity at the deletion in LEW.1WR1 rats. Nucleotide sequencing of tail DNA for the top candidate for Iddm37, These results suggested but did not prove that expression of UBD-KO rats

were crossed to produce heterozygous and homozygous UBD-KO acceptor site (Supplementary Figure 3). Progeny from this founder a 65-base pair deletion of intron 1/exon 2 that eliminates a splice

g SINE inhibits cytokine induction of promoter is highly cytokine-inducible, and the presence of the promoter, in open circles are LEW.1W results. Mean ± s.d. are shown per condition per strain, performed once (LEW.1A strain) or twice (for LEW.1W and LEW.1WR1 strains).

did not drive expression of the reporter (not shown). The transfected cells were also grown in the presence of TNF-α, IFN-γ or IFN-β, all of which have been demonstrated to induce Ubd transcription in vitro. The results show that the LEW.1WR1 Ubd promoter is highly cytokine-inducible, and the presence of the SINE inhibits cytokine induction of Ubd in LEW.1W (Figure 3).

UBD-KO rats

These results suggested but did not prove that expression of Ubd, the top candidate for Iddm37, is a modifier of diabetes susceptibility. To test this hypothesis, we targeted Ubd for genetic deletion in LEW.1WR1 rats. Nucleotide sequencing of tail DNA for heterozygosity at the Ubd locus identified a number of candidate founder rats. One founder (LEW.1WR1-Ubdmmumass or UBD-KO) had a 65-base pair deletion of intron 1/exon 2 that eliminates a splice acceptor site (Supplementary Figure 3). Progeny from this founder were crossed to produce heterozygous and homozygous UBD-KO rats for comparison with the wild-type (WT) rats on the LEW.1WR1 background. Rats bearing a homozygous deletion of Ubd were viable and expressed no Ubd transcripts in their spleens (data not shown).

Absence of UBD expression reduces susceptibility to virus-induced T1D in LEW.1WR1 rats

We first compared diabetes susceptibility of LEW.1WR1 rats with that of LEW.1W rats with either heterozygous or homozygous deletion of Ubd. As shown in Figure 4, overall diabetes frequency in WT LEW.1WR1 rats was highest of the three strains, LEW.1WR1-UBD-KO heterozygotes were less susceptible, and the homozygous LEW.1WR1-UBD-KO rats were the least susceptible. The diabetes-free survival proportions are significantly increased in the heterozygotes (13%) and homozygotes (26%) (χ² test for trend, P = 0.016, Figure 4b). Latency to onset in rats that became diabetic was similar in the three groups, and for this reason, the Kaplan–Meier plot (Figure 4a) of the three groups showed a trend that did not reach statistical significance (P < 0.2).

Genetic complementation enhances susceptibility of resistant LEW.1W rats to virus-induced T1D

One advantage of using the knockout strategy is that the resulting genetically deficient animals can be bred to a strain bearing a putative defective allele to determine if the deleted allele is identical to the defective one. We hypothesize that LEW.1W carries a defective allele of the Ubd gene because we have shown that it cannot be upregulated normally by cytokines and thus is likely to be hypofunctional in the setting of diabetes. To test this hypothesis, we bred LEW.1W rats to heterozygous LEW.1WR1-UBD-KO rats. If Iddm37 is Ubd, then the Ubd-KO-bearing chromosome from the heterozygous parent will not be able to 'complement' the defective LEW.1W Iddm37 allele, and the hybrid (LEW.1W/LEW.1WR1-UBD-KO) rat will remain resistant to diabetes. If Iddm37 is not Ubd, the 'authentic' Iddm37 gene on the UBD-KO chromosome (derived from LEW.1WR1) will bear a susceptible complementing allele for Iddm37, and the hybrid will be as susceptible as the normal F1-hybrid between LEW.1W and LEW1.WR1. The other WT chromosome from the LEW.1WR1-UBD-KO heterozygous parent serves as an internal control for the experiment, providing a confirming comparison for the known T1D susceptibility of Iddm37 heterozygotes.

The results of the complementation experiment were revealing. The UBD-KO showed a significant effect on the LEW.1W background (Kaplan–Meier statistic, P = 0.013; Figure 5a). The hybrid rat bearing a deletion of Ubd on one chromosome and a defective allele from LEW.1W on the other (LEW.1W/LEW.1WR1-UBD-KO) shows greatly increased diabetes-free survival in rats (60%) compared with their WT control, LEW.1W/LEW.1WR1 (25%).

### Table 2. Strain distribution patterns (SDP) of genotypes, KRV-induced T1D, and gene expression in related rats

| Strain | LEW.1WR1 | BBDR | LEW.1W | WF | LEW.1AR1 | LEW.1A | LEWIS |
|--------|---------|------|--------|----|----------|--------|-------|
| RT1 Haplotypes (A - B/D) | U-U | U-U | U-U | U-U | A-U | A-A* | L-L* |
| KRV-T1D | Susceptible | Susceptible | Resistant | Resistant | Resistant | Resistant | Resistant |
| UBD mRNA expression | Absent | Absent | Present | Present | Present | Present | Present |
| Ubd exon haplotype | A | BB | L | L | A | L | A |
| RT1-N1 haplotype | A | U | U | U | A | A |
| RT1-N1 expression | Low | High | High | High | Low | nd |

Abbreviation: Nd, not determined. Rats in bold font are susceptible to T1D induced by KRV + Poly I.C. RT1-N1 haplotypes were determined by comparison of alleles at two SNPs and one microsatellite region. LEW.1WR1, LEW.1A and LEW are designated RT1-N1*. Rat strains carrying the RT1-N1 haplotype (WF, LEW.1W and BBDR) carry RT1-N1**. Ubd haplotypes were declared on the basis of the presence or absence of a SINE element and two non-synonymous SNPs in exon 2. The Ubd - haplotype has no SINE, and G and A at SNP1 and SNP2, respectively; Ubd - haplotype is SINE absent, A; A; and Ubd - haplotype bears the SINE element, and has A and C at SNPs 1 and 2. Gene expression was ascertained by qRT–PCR on spleen mRNA samples. aRT1 haplotypes that are nonpermissive for T1D induction. Other strains (LEW.1WR1, LEW.1W and LEW.1A) were tested in the present report or in previous publications. 24 b unpublished data.

Figure 3. The Ubd allele from LEW.1WR1 but not LEW.1W promotes both high constitutive as well as induced UBD expression. In closed circles are results from reporter plasmids containing the LEW.1W promoter, in open circles are LEW.1WR1 results. Mean ± s.d. are shown per condition per strain, performed once (LEW.1A strain) or twice (for LEW.1W and LEW.1WR1 strains).

Figure 4. A) Kaplan–Meier plot (Figure 4a) of the three groups showed a trend that did not reach statistical significance (P < 0.2).
compound heterozygote (LEW.1W/LEW.1WR1UBD-KO) was not as resistant as the LEW.1W/LEW.1W homozygote (100% diabetes-free survival), meaning that the UBD-KO partially complemented the defective allele in LEW.1W. The fact that the LEW.1W defect was partially but not fully complemented by the LEW.1WR1 chromosome carrying the Ubd knockout allele means that Ubd and an additional unidentified gene in the Iddm37 interval both contribute to diabetes. As in the LEW.1WR1 crosses, there is a highly significant difference in incidence in LEW.1W crosses (Figure 5b, \(\chi^2\)-test for trend, \(P=0.0023\)). For these studies, 6 LEW.1W, 11 heterozygous LEW.1WR1Ubd-KO /LEW.1W and 12 heterozygous LEW.1WR1/LEW.1W littermate rats were studied as litters reached 28–30 days of age.

**DISCUSSION**

Diabetes-susceptible rat strains have very high fidelity to the clinical pathology seen in human autoimmune diabetes. This is especially true for testing the gene–environment interactions that induce the disease. Such interactions are difficult to discern in the NOD mouse model of T1D because all environmental perturbations including viral infections that have been studied uniformly prevent the disease. The recently developed technologies that allow targeting of genes for deletion in the rat have made this species exceptionally useful for the dissection of the genetic requirements for diabetes susceptibility. In this report, we have taken the diabetes quantitative trait loci Iddm37 from a map position on the proximal arm of chromosome 20 that encompasses about 4 Mb to a lead candidate gene, Ubd. Deletion of the Ubd gene substantially and significantly reduced the incidence, but not the latency, of T1D in UBD-KO rats as compared with their respective WT controls in two large cohorts of rats. This is to our knowledge, the first successful genetic knockout of a rat autoimmunity gene.

In identifying this gene, we have uncovered a new pathway relevant to autoimmune disease susceptibility. UBD is much more highly expressed in the LEW.1WR1 rat than in the resistant strains. The SINEs in the UBD promoter in resistant strains likely plays a role as a transcriptional repressor of UBD. It has been shown that SINEs can influence the expression of nearby genes in other contexts. In this study, the repressive mechanism in the Ubd promoter is unknown. The link between higher UBD expression and higher disease incidence suggests that UBD could be required for the recognition or processing of viral or self-antigens, or for activating T cells that recognize them. Although UBD, a diubiquitin with ubiquitin-like properties, is likely expressed in pancreas, antigen-presenting cells, B cells and T cells in rats, humans and mice, its
functional importance in these tissues has not yet been defined. The human homolog of UbD is called FAT10, for HLA-F-adjacent transcript number 10. It is known that dendritic cell maturation induces FAT10 in human cells.57,44 In addition, CD40L-CD40 ligation, TLR agonists such as LPS or poly I:C and some cytokines cause upregulation of FAT10 in dendritic cells.47 FAT10:protein conjugates undergo rapid proteasome-dependent degradation in dendritic cells,59 supporting a potential role for UBD in antigen presentation. FAT10/UBD is also associated with resistance to apoptosis in lymphocytes.54,55

It is of interest that our complementation data suggest further that there is a second gene in addition to UbD in the iddm37 interval.50,51 When we analyze the entire set of rat UbD-KO crosses tested for T1D susceptibility, there is a highly significant trend associated with both the alleles of UbD or the absence of UbD and with a second, as yet unidentified gene (‘gene 2’) (Table 3, $\chi^2$-test for trend, $P < 0.0001$). If UbD were the sole gene underlying the effect of iddm37, the complementation experiment would have shown complete resistance in the LEW.1WR1-KO/LEW.1W hybrid rat, instead of the observed 60% resistance (line 4 of Table 3). This second gene is being sought in our congenic animals. These data indicate additivity of multiple genes, each of relatively small effect size, underlying diabetes. This finding will in future studies allow us to analyze in detail T1D susceptibility as a function of the number of susceptible alleles as we have done for iddm37 (Table 3). This combinatorial analysis of genetic elements in the rat is similar to the cumulative genetic risk analysis for human T1D developed by Winkler et al, who were able to predict diabetes risk in the BABY DIAB cohort based on non-HLA-risk allele scores.3 The rat models we now have will allow us to generate this kind of risk for individual genotypes and apply them to different pathways leading to T1D-viral infection, Treg deficiency and other stressors. This should eventually allow us to enrich the repertoire of candidate interventions for halting the progress to T1D in children at risk.

As an example, the identification of UbD as a diabetes susceptibility gene should reanimate studies of the role of FAT10/UbD in children at risk for T1D by virtue of HLA haplotype and family history. The Eisenbarth group has reported linkage to the region containing UbD in their study of Type 1 Diabetes Genetics Consortium (T1DGC) cases and controls.50,51 This human research, coupled with our analyses of the relative necessity of UbD for diabetes induced by virus in rats, leads us to propose that UbD is a good candidate gene for T1D, but quite possibly only in the setting of an environmental perturbant. Our studies clearly demonstrate the interaction of genetic requirements of the disease and environmental factors, and comparable analyses may be necessary to achieve a more complete understanding of the origins of T1D in human populations.

| Table 3. Two gene model of iddm37 |
|-----------------------------------|
| UbD | Gene 2 | Strain | iddm37$^a$ copies | %T1D |
|------|--------|--------|------------------|------|
| S/S  | S/S    | LEW.1WR1 | 45 | 100 |
| KO/S | S/S    | LEW.1W1/LEW.1W1-KO | 35 | 87 |
| KO/  | S/S    | LEW.1W1-KO/LEW.1W1 | 25 | 75 |
| KO   | S/R    | LEW.1W1-KO/LEW.1W | 25 | 40 |
| R/R  | S/R    | LEW.1W1/LEW.1W | 15 | 0 |

Incidence of KRV + poly I:C induced diabetes rats of all iddm37 classes in this study. $\chi^2$-test for trend: $P < 0.0001$.

Materials and Methods

Rats

Inbred LEW.1WR1, LEW.1W and LEW.1A rats were obtained from BRM, (Worcester, MA, USA). As depicted in Figure 1, LEW.1WR1 (RT1-A<sup>1</sup>, B<sup>D</sup>, C<sup>B</sup>) is an MHC-recombinant congenic strain derived from LEW.1W (RT1<sup>A</sup>) and LEW.1A (RT1<sup>B</sup>) rats.53 RT1-A and RT1-C are Class I and Class I1b MHC genes in the rat, respectively. All of these share the diabetes susceptibility allele of iddm4 (Tcrb-V1531A)54 (iddm4). Congenic WF.iddm4 rats (RT1<sup>F</sup>) were developed and maintained at the University of Massachusetts Medical School; they express the diabetes-susceptible allele of iddm4 but are resistant to KRV + poly I:C-induced diabetes.54 Rats of both sexes were used in roughly equal numbers and were approximately 4-weeks-old at the time of experimentation. Animals were housed in viral antibody-free conditions, confirmed monthly to be serologically free of rat pathogens54 and maintained in accordance with institutional (University of Massachusetts School of Medicine Institutional Animal Care and Use Committee) and national guidelines.55

Diabetes induction

Rats were injected with poly I:C (1 μg g<sup>-1</sup> body weight) three times (on days -3, -2 and -1) and then inoculated with 10<sup>5</sup> plaque forming units of KRV. This dose of poly I:C is not itself diabetogenic, but increases the penetrance of virus-induced diabetes in susceptible rats from ~40% to up to 100%. Rats were monitored for glycosuria three times weekly; diabetes in glycosuric rats was diagnosed on the basis of blood glucose concentration > 250 mgdl<sup>-1</sup> on consecutive days using a hand held glucose meter (One Touch Ultra, LifeScan Inc, Milpitas, CA, USA). Animals were killed when diabetes was diagnosed or on day 40 after viral infection. Liver, pancreatic draining lymph nodes, spleens and pancreata were harvested for further analysis.

Genotyping

Genomic DNA was isolated from tail and liver samples using GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St Louis, MO, USA) and analyzed as in our previous publications.27,28,50

Sequencing

Genes of interest were amplified from genomic DNA of both diabetes-susceptible and resistant rats using hi-fidelity taq polymerase. The PCR products were purified and sequenced by Genewiz (South Plainfield, NJ, USA). The sequences were analyzed by 4peaks software (http://nucleobiotics.com/index.php/4peaks) and aligned by CLUSTALW (http://www.genome.jp/tools/clustalw/).

Quantitative real-time PCR (qRT-PCR)

Rats were treated to induce diabetes as above. Pancreas, pancreatic lymph nodes and spleen were harvested for RNA isolation (Ultraspex, Biotecx and RNeasy RNA kit (Qiagen, Valencia, CA, USA)) on day 0, 3 and 5. CDNA was prepared from total RNA using the ABI High capacity CDNA RT Kit (Life Technologies, Grand Island, NY, USA). PCR was carried out using Applied Biosystems SYBR Green PCR mix and the Perkin Elmer/Applied Biosystems Division 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA), using the same samples for which global gene expression analyses were done (below).

GeneChip analyses

Total RNA was isolated from pancreatic lymph nodes of poly I:C + KRV-treated susceptible and resistant rats treated to induce diabetes as above on day 0, 3 and 5. RNA was DNAse-treated and assessed for quality. For comparison of global gene expression differences, RNA (~100 nanograms) was amplified/labelled (Affymetrix two-cycle cDNA synthesis kit), (Affymetrix) and then hybridized to the Affymetrix RG230 2.0 array, which interrogates > 30,000 transcripts and variants, in accordance to the manufacturers’ protocol. After hybridization, arrays were washed and stained with Affymetrix fluidic protocol and scanned with a 7G Affymetrix GeneChip Scanner. Image data were analyzed with Affymetrix Expression Console 1.1.2 software and normalized with Robust Multichip Analysis (www.bioconductor.org) to determine signal log ratios. The statistical significance of differentially induced transcription was assessed false discovery rates using Partek Genomics Suite 6.5 (Partek, St Louis, MO, USA) and use of a nonparametric rank product test.27
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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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