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Diabetes models by screen for hyperglycemia in phenotype-driven ENU mouse mutagenesis projects

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Aigner B, Rathkolb B, Herbach N, Hrabé de Angelis MH, Wanke R, Wolf E. Diabetes models by screen for hyperglycemia in phenotype-driven ENU mouse mutagenesis projects. *Am J Physiol Endocrinol Metab* 294: E232–E240, 2008. First published December 4, 2007; doi:10.1152/ajpendo.00592.2007.—More than 150 million people suffer from diabetes mellitus worldwide, and this number is expected to rise substantially within the next decades. Despite its high prevalence, the pathogenesis of diabetes mellitus is not completely understood. Therefore, appropriate experimental models are essential tools to gain more insight into the genetics and pathogenesis of the disease. Here, we describe the current efforts to establish novel diabetes models derived from unbiased, phenotype-driven, large-scale *N*-ethyl-*N*-nitrosourea (ENU) mouse mutagenesis projects started a decade ago using hyperglycemia as a high-throughput screen parameter. Mouse lines were established according to their hyperglycemia phenotype over several generations, thereby revealing a mutation as cause for the aberrant phenotype. Chromosomal assignment of the causative mutation and subsequent candidate gene analysis led to the detection of the mutations that resulted in novel alleles of genes already known to be involved in glucose homeostasis, like glucokinase, insulin 2, and insulin receptor. Additional ENU-induced hyperglycemia lines are under genetic analysis. Improvements in screen for diabetic animals are implemented to detect more subtle phenotypes. Moreover, diet challenge assays are being employed to uncover interactions between genetic and environmental factors in the pathogenesis of diabetes mellitus. The new mouse mutants recovered in phenotype-driven ENU mouse mutagenesis projects complement the available models generated by targeted mutagenesis of candidate genes, all together providing the large resource of models required for a systematic dissection of the pathogenesis of diabetes mellitus.

clinical chemistry; *N*-ethyl-*N*-nitrosourea; glucose; insulin

TYPE 2 DIABETES MELLITUS (T2DM) is characterized by uncontrolled hyperglycemia caused by both insulin resistance and pancreatic β-cell dysfunction. Human T2DM is usually a polyfactorial disease resulting from the interaction of genetic susceptibility and environmental factors. The prevalence of diabetes mellitus has reached an alarming dimension in industrialized countries and is expected to rise substantially within the next decades. Diabetes leads to a reduced quality of life and life expectancy by causing increased risk for blindness, cardiovascular disease, nephropathy, and neuropathy (53). Despite its high prevalence, the pathogenesis of diabetes mellitus is not completely understood. Diabetic mouse mutants are used to identify genes and pathways that regulate glucose homeostasis, contribute to the susceptibility to diabetes, and affect the development and severity of secondary complications. Thus, these models are important to gain insights into all aspects of the pathophysiology of diabetes and also for the development and evaluation of novel prophylactic and therapeutic strategies.

Existing Rodent Diabetes Models

Suitable animal models mirror some of the multiform symptoms of human diabetes. Diabetes models were generated in different genetic backgrounds by dietary induction, toxic β-cell destruction, surgical manipulations, and/or combinations thereof. Specific models appeared by spontaneous mutations that were subsequently analyzed using forward genetics methods. On the other hand, reverse genetics techniques were used to produce genetically modified mice with defined alterations of genes that were suggested to play a role in glucose homeostasis (8, 44, 46). Furthermore, the genome-wide search for causative loci was carried out by examining the genetic polymorphisms that are linked to different plasma glucose phenotypes of the already-existing mouse strains. Mapping of quantitative trait loci (QTL) controlling plasma glucose levels was done in independent and combined crosses of inbred mouse strains (8–10). In addition, genetic polymorphisms of heterogeneous stock mice influencing the plasma glucose homeostasis were examined by high-resolution whole-genome association studies of quantitative traits (51).

Phenotype-driven ENU Mouse Mutagenesis Projects

Random chemical mutagenesis and subsequent screening for clinically relevant phenotypes without a priori assumptions is a powerful approach to derive novel mouse models for biomedical research. The alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) is currently the most powerful mutagen for the production of mutant mice. ENU shows mutagenic action on premeiotic spermatogonial stem cells. This allows the production of a large number of randomly mutant offspring from treated males (20). Compared with other mutagenesis methods, ENU predominantly induces point mutations, thereby leading to allelic series for the functional analysis of genes (47). Mutations induced by ENU are not tagged molecularly as is the case in other random mutagenesis projects e.g., using gene traps. This

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is initially a disadvantage as the causative mutations have to be analyzed by linkage analysis. However, the availability of point mutations is important for the detailed functional analysis of genes. The function of a given gene in glucose homeostasis cannot be described by analyzing only one mutant allele; multiple alleles of the same gene are necessary for this goal. The ENU mouse mutagenesis projects established during the last 10 years serve as platforms for the systematic, genome-wide, large-scale production and analysis of mouse mutants as a model system for inherited human diseases, thereby facilitating the identification and functional characterization of genes that are relevant for the prevention, diagnosis, and therapy of diseases (20).

After the ENU-mutagenized males were mated to wild-type females, specific pathological states were identified in the offspring by appropriate routine procedures that allow the screen of large numbers of mice for a broad spectrum of parameters. High numbers of screen parameters are usually used to optimize the cost/benefit calculation of ENU mouse mutagenesis projects. Phenotypic screens in major ENU mouse mutagenesis projects often include dysmorphology, clinical chemistry, immunology, allergy, and behavior as well as other screens with a high number of test parameters. However, smaller laboratories have also efficiently set up specialized projects. Screen profiles of clinical chemical blood parameters were established to detect phenotypic variants with defects of various organ systems or changes in metabolic pathways. Breeding of the phenotypically affected mice and screen of the offspring confirmed the transmission of the altered phenotype to subsequent generations, thereby revealing a mutation as cause for the aberrant phenotype. The underlying mutations are then identified by forward genetics strategies and lead to the identification of genes/alleles that may have counterparts relevant for human diseases (Fig. 1). Mutant lines with the causative mutation already identified are successfully used in different areas of biomedical research (19, 20, 37).

Being representative of the basic strategy of other projects, the Munich ENU mouse mutagenesis project was carried out on the inbred C3HeB/FeJ (C3H) genetic background, which is described as a diabetes-resistant strain (10). Ten-week-old male mice were injected intraperitoneally with ENU. The screen for dominant mutations was performed on G1 animals, which were derived by mating of mutagenized G0 males to wild-type C3H females. Inheritance of observed abnormal phenotypes was tested in G2 offspring from the mating of the affected G1 mouse exhibiting the altered phenotype and wild-type C3H mice. G1 males that did not show an abnormal phenotype in the screen for dominant mutations were mated to wild-type females to produce G2 daughters, which were then mated to their sires. The resulting G3 mice were used for the screen for recessive mutations. Inheritance of observed abnormal phenotypes was tested on G4 intercross or G4 $\times$ G3 backcross offspring in the inbred C3H genetic background (36).

ENU mouse mutagenesis projects are done on different inbred genetic backgrounds (BALB/c, C3H, C57BL/6). The chemically mutagenized mice are subsequently mated with mice of the same or of different inbred strains. In both cases, the G1 animals harbor identical genetic backgrounds (inbred vs. hybrid), which is not the case in the animals of the subsequent generations after mating the chemically mutagenized mice with mice of another inbred strain. A defined phenotype may occur in a reproducible manner through subsequent generations in an identical genetic background. A heterogenous genetic background may reveal various interactions of the ENU-induced mutations in different genotypes. Different alleles may cause diabetes in various genetic backgrounds. By using mice with increased diabetes susceptibility like C57BL/6J, which lack the Nnt protein and therefore display glucose intolerance and reduced insulin secretion, sensitized/modifier screens may be carried out to detect additive diabetes susceptibility alleles (12).

Mutation rates in early ENU mouse mutagenesis experiments have been determined by the specific locus test as well as other functional tests, including a limited number of specific genes and loci, which led to results with restricted common validity (Ref. 20 and references therein). Using similar mutagenesis protocols and various inbred strains, studies of different chromosomal regions of ENU-mutagenized mice with different molecular genetic methods [denaturing HPLC, temperature gradient capillary electrophoresis (TGCE), direct sequencing] observed one mutation in 100,000 bp (5) and one mutation in 1.0–2.5 Mbp (3, 11, 33, 39, 43). Thus, molecular genetic analysis may be the most effective approach for the determination of mutation frequencies in ENU mouse mutagenesis projects. After confirmation of the inheritance of the mutant phenotype to the subsequent generations, back-cross of the phenotypic mutant animal to wild-type mice leads to the loss of noncausative mutations. After ten back-cross generations, 20 cM of the originally mutagenized genomic DNA remains, which harbors about 25 mutations, including the causative mutation (45). Considering only a small number of the more than 1,000 mutations per animal as potentially functional by causing phenotypic consequences, at least the largest part of them, except for the causative mutation, is segregated (5, 24, 42).

The establishment of lines showing the mutant phenotype indicates the penetrance of the mutant phenotype and the number of segregating mutations causing the mutant phenotype. The examination of the chromosomal position of the causative mutation is carried out by linkage analysis. Briefly, phenotypic mutants harboring the mutation are bred to wild-type mice of a second inbred strain; the resulting hybrid offspring are analyzed for the abnormal phenotype of the parent, and hybrid mutants are back-crossed to wild-type mice of the second inbred strain (for dominant mutations) or intercrossed (for recessive mutations). The resulting offspring are again analyzed for the abnormal phenotype of the parent, thereby dividing them into phenotypically mutant and nonmutant animals. Genome-wide linkage analysis of the genomic DNA samples using polymorphic markers subsequently reveals the chromosomal position of the mutation. Further linkage analysis on the determined chromosomal site and candidate gene analysis including sequencing techniques are then performed to identify the causative mutation (45). The probability that a confounding mutation is linked to a sequenced mutation in a determined chromosomal region with a length of 5 Mb was calculated to be low ($P < 0.05$). In total, most mutant phenotypes are effectively monogenic (23, 24). Novel genetic backgrounds appear in the G1 and G2 animals of the linkage analysis which might result in variations of the ENU-induced mutant phenotype. This can lead to the loss of the mutant

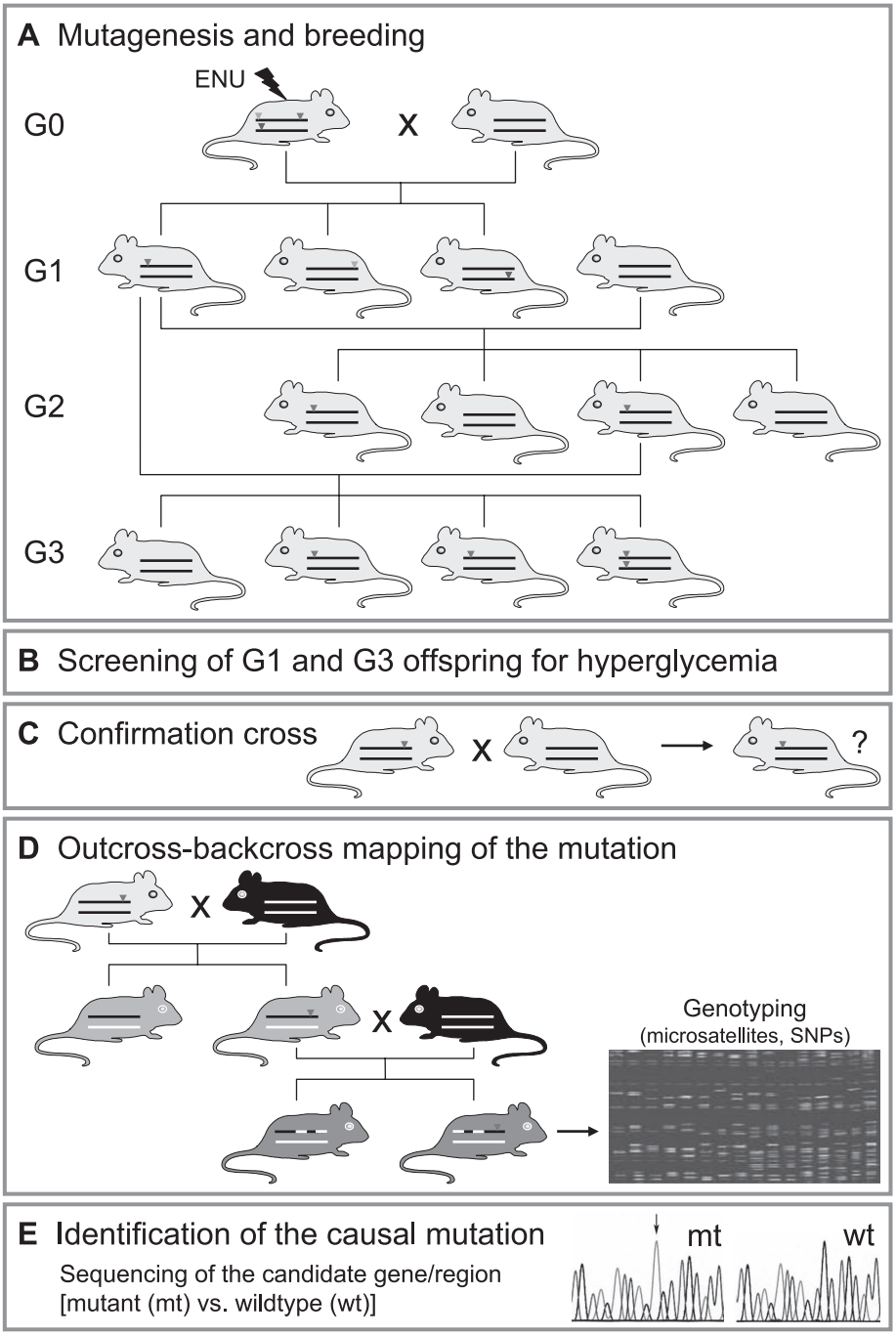

Fig. 1. Generation of novel disease models in phenotype-driven *N*-ethyl-*N*-nitrosourea (ENU) mouse mutagenesis projects. After intraperitoneal treatment of male mice with ENU, defined mating schemes produced the G1 and G3 offspring (*A*), which were phenotypically examined for alterations, e.g., hyperglycemia (*B*). Phenotypical G1 and G3 variants were mated to test the inheritance of the altered phenotype caused by dominant or recessive mutations, respectively (*C*). The final step of establishing new disease models includes identification of the causative mutation by linkage analysis (*D*) and subsequent candidate gene analysis (*E*), as well as in-depth phenotypic analysis.

phenotype; in this case linkage analysis has to be tried with another second inbred strain.

The functional proof that a mutation that lies in the correct genomic interval is responsible for the phenotype may be carried out by demonstrating that the affected protein is absent or inactive, by rescuing the phenotype via introduction of a wild-type copy of the gene, using reverse genetics methods, or by performing complementation tests with another mutant allele (6). The mutants may carry loss-of-function, dominant negative, hypomorphic, or gain-of-function alleles of the affected genes.

Phenotypic Screen for Hyperglycemia

Several ENU mouse mutagenesis projects used plasma glucose as parameter for the high-throughput screen of the mutagenized mice (Table 1). Multiple factors influence the outcome of the projects. Standardization of the phenotype analyses has been greatly improved by establishing specialized mouse phenotyping centers that use defined standard operating procedures (http://www.interphenome.org). As an example, in the Munich ENU mouse mutagenesis project (http://www.gsf.de/ieg/groups/genome/enu.html) (13), clinical chemical anal-

Table 1. *Major public ENU mouse mutagenesis projects using plasma glucose as screening parameter*

ENU Project	URL
Baylor College of Medicine, Houston, TX	http://www.mouse-genome.bcm.tmc.edu
GSF, Neuherberg, Germany	http://www.gsf.de/ieg
Jackson Laboratory, Bar Harbor, ME	http://pga.jax.org
John Curtin School of Medical Research, Canberra, Australia	http://jcsmr.anu.edu.au; http://www.apf.edu.au
MRC, Harwell, UK	http://www.mgu.har.mrc.ac.uk
RIKEN GSC, Yokohama, Japan	http://www.gsc.riken.go.jp/Mouse
University of Toronto, Toronto, Canada	http://www.cmhd.ca

ENU, *N*-ethyl-*N*-nitrosourea

ysis, including plasma substrates, proteins, electrolytes and enzymes, was carried out with blood samples from 3-mo-old G1 and G3 C3H mice fasted overnight that were obtained by puncture of the retroorbital sinus under short-term general anesthesia. Plasma from Li-heparin-treated blood was analyzed using an Olympus AU400 autoanalyzer (Olympus, Hamburg, Germany) and the adapted reagents (Olympus). Plasma glucose concentrations were examined using the enzymatic hexokinase assay with the reagent OSR6121 (Olympus) in the linear measurement range of 10–800 mg/dl (0.6–45 mmol/l) (40, 41).

Prerequisite to the detection of hyperglycemia in mutagenized mice is the determination of the physiological values in the genetic background used. Several values for plasma glucose are available for some of the genetic backgrounds used in ENU mouse mutagenesis projects (http://www.interphenome.org). The published values are generally useful for the choice of the appropriate mouse strain according to the aims of the proposed project. Compared with C3H mice, C57BL/6J animals show lower physiological plasma glucose values, which may vary depending on environmental and/or experimental factors (http://www.jax.org/phenome) (26). The great number of critical variables that cannot be completely standardized between different laboratories limits the use of the published data sets of clinical chemical values for specific projects. Therefore, the physiological range of plasma glucose, and subsequently the cut-off level to determine hyperglycemia, has to be measured in each project. In the Munich ENU mouse mutagenesis project, mean plasma glucose levels measured over several time periods in a large number of male and female C3H controls 3 mo post partum after overnight fasting of the animals corresponded to 128 and 123 mg/dl, respectively. The 95% range of the values was similar to the data range including two standard deviations above and below the mean, which indicated the Gaussian distribution of the data. Compared with published blood glucose values from experiments carried out in a short time period and/or using small numbers of animals (http://jax.org/phenome) (25), the range of the values varied broadly, leading to high standard deviations. This may have been due to the increased influence of environmental and experimental changes in long-term studies. In our screen, the upper value of the 90% range (60–208 mg/dl in males and 48–201 mg/dl in females) instead of the 95% range was used to define hyperglycemia (30). This should prevent the overlooking of more subtle phenotypes in the diabetes-resistant C3H strain. Thus, male and female mice exhibiting plasma glucose concentrations higher than 200 mg/dl in two measurements within a 3-wk interval were assumed to be hyperglycemic (Table 2). Other ENU mouse mutagenesis projects used similar cut-off levels to determine hyperglycemia in the C3H × BALB/c (17) and C57BL/6J × DBA/2J (21) genetic background without fasting of the mice.

In the Munich ENU mouse mutagenesis project, screening of more than 15,000 G1 offspring and 500 different G3 pedigrees of ENU-treated G0 mice for dominant and recessive mutations, respectively, resulted in the occurrence of variants showing hyperglycemia in two measurements. To date, the analysis has confirmed the inheritance of plasma glucose levels above 200 mg/dl to the subsequent generations in four (33%) of 12 G1 variants mated, which produced offspring in the screen for dominant mutations, and in none of nine G3 variants mated that produced offspring in the screen for recessive mutations. A fifth hyperglycemia line (GLS008) derived from the screen for dominant mutations is currently being examined to further confirm the inheritance of the phenotype (not shown). The establishment of hyperglycemia lines harboring a recessive mutation was successful in other ENU mutagenesis projects (see below). A high ratio in the failure of the transmission of increased plasma substrate levels to the offspring was also found for other phenotypic deviations in our clinical chemistry screen (2, 36). Compared with the variants that did not transmit the hyperglycemia phenotype to the offspring (mean 225 and 226 mg/dl plasma glucose in the G1 and G3 variants, respectively), the four variants that produced the hyperglycemia lines showed higher plasma glucose levels (mean 319 mg/dl). In the hyperglycemia screens of other ENU mutagenesis projects using different genetic backgrounds, the hyperglycemia phenotype of the G1 or G3 founder animals of successfully established lines was also reported with values below 300 mg/dl (21, 50) (see below).

Comparison of the efficiency of the production of hyperglycemia lines between the ENU mouse mutagenesis projects might lead to an optimized protocol for the generation of ENU-induced hyperglycemia lines but this is practically prevented by several pitfalls. Interaction of unrecognized laboratory-specific factors in mouse genotype, husbandry and experimental procedure may lead to significant deviations in the results of highly standardized experiments (26). Major in vivo and in vitro interfering factors include age, body weight, activity, health status, nutrition, social interference, interference with humans, personnel, time point and site of blood collection, as well as sample preparation and analysis. Furthermore, use of stringent cut-off levels according to a determined wild-type physiologic data range may improve the establishment of mutant lines from the recognized variants but prevent the identification of additional variant animals.

Table 2. *Physiological range of plasma glucose (mg/dl) in 3-month-old C3H wild-type mice of the Munich ENU project*

Sex	*n*	Mean	SD	Mean ± 2SD	95% Range	90% Range
Male	192	128	47	34–222	56–229	60–208
Female	233	123	47	29–217	36–218	48–201

Cut-off level for males and females: 200 mg/dl.

Table 3. *Mutant lines showing hyperglycemia derived from the Munich ENU mouse mutagenesis project using inbred C3H mice*

	Offspring		Increased Plasma Glucose,‡ mg/dl			
Line	Mut.*	Tested,† *n*	%Penetrance	*n*	Range	Mean
GLS001	D	216	58	63	200–319	245
GLS004	D	147	66	45	200–619	335
GLS006	D	18	100	14	200–701	363
GLS007	D	34	88	15	232–383	286

*D, dominant mutation. †Offspring after mating heterozygous mutants to wild-type mice except for line GLS006, where heterozygous mutants were mated. ‡Plasma glucose cut-off level, 200 mg/dl. 100% phenotypic penetrance is defined in the case of the appearance of 50 and 75% offspring exhibiting hyperglycemia after mating phenotypic mutants to wild-type mice and breeding heterozygous mutant mice, respectively. *n*, Absolute numbers of observed phenotypic mutants.

In the four lines harboring a dominant mutation (GLS001, GLS004, GLS006, GLS007), mutant offspring showing pathological plasma glucose levels were produced by mating heterozygous mutants to wild-type animals except for line GLS006, which was previously suggested to harbor a recessive mutation (Table 3). The phenotypic mutants of the four lines showed mean plasma glucose levels between 245 and 363 mg/dl. The phenotypic penetrance of the hyperglycemia in the lines was analyzed by defining 100% phenotypic penetrance in the case of the appearance of 50 and 75% offspring exhibiting the phenotypic deviation after mating phenotypic mutants to wild-type mice and breeding heterozygous mutant mice, respectively. A suitable phenotypic penetrance of the pathological glucose levels above 50% was observed in all four lines, which facilitates the effective subsequent phenotypic and molecular genetic analyses. Reanalysis of animals after a few weeks increased the phenotypic penetrance of pathological glucose levels, thereby indicating that the penetrance was generally underestimated in the analysis after 12 wk of age. Further in-depth analysis of the line GLS004 revealed complete penetrance of the diabetic phenotype (16) (see below), which indicated the inherent impossibility of carrying out complete standardization in long-term, large-scale phenotyping projects. However, incomplete phenotypic penetrance was also seen in established diabetic lines of other ENU mutagenesis projects (17). The lines GLS001 and GLS004 had already been bred for more than ten generations starting from the ENU-mutagenized G0 founder animal without losing the abnormal phenotype, whereas the lines GLS006 and GLS007 were recently established. In all four lines, the numbers of animals exhibiting plasma glucose levels above the cut-off level were significantly (χ^2-test, $P < 0.001$) higher than the expected 5% of the C3H mouse population (Table 3).

Established ENU-Induced Hyperglycemia Models

Several hyperglycemia lines derived from phenotype-driven ENU mouse mutagenesis projects with the causative mutation already identified have been published (Table 4). All lines harbor a dominant mutation. Most of them are glucokinase (*Gck*) mutants. *Gck* phosphorylates glucose in the first step of glycolysis and plays a critical role in the glucose-responsive insulin secretion of pancreatic β-cells by functioning as a "glucose sensor". In humans, almost 200 heterozygous mutations in the *GCK* gene have been reported to cause maturity-onset diabetes of the young type 2 (MODY2), characterized by mild hyperglycemia. Homozygous inactivating *GCK* mutations result in permanent neonatal diabetes mellitus (PNDM). In addition, heterozygous activating *GCK* mutations causing hypoglycemia have been observed (14, 31). In mice, targeted global *Gck* knockout mutations resulted in mild hyperglycemia in the heterozygous mutants and extreme hyperglycemia and embryonic to postnatal lethality during the first week after birth in the homozygous mutants (4, 15).

The hyperglycemic line GENA348 was established on the C3H × BALB/c genetic background using a G1 founder with plasma glucose values of 240 and 220 mg/dl between 8–12 wk of age. The causative missense mutation in exon 9 of the glucokinase gene (Gck^{I366F}) was the first glucokinase missense mutation reported in mice. It resulted in a semi-dominant glucose intolerance phenotype where 67 and 14%

Table 4. *Published hyperglycemia lines harboring a dominant mutation derived from phenotype-driven ENU mouse mutagenesis projects*

Gene	Chr.	Allele/Name	Reference
Glucokinase	11	Gck^{I366F}/Gena348	(17, 50)
Glucokinase	11	Gck^{V182M}/Rgsc392	(21)
Glucokinase	11	Gck^{T206M}/Rgsc735	(21)
Glucokinase	11	Gck^{C220Y}/Rgsc341	(21)
Glucokinase	11	Gck^{M224R}/Rgsc272	(21)
Glucokinase	11	Gck^{T228A}/Rgsc236	(21)
Glucokinase	11	Gck^{F419L}/Rgsc552	(21)
Glucokinase	11	$Gck^{Y273Stop}$/Rgsc475	(21)
Glucokinase	11	$Gck^{R345Stop}$/Rgsc702	(21)
Glucokinase	11	$Gck^{IVS1A+1G\rightarrow T}$/Rgsc210	(21)
Glucokinase	11	$Gck^{IVS3-3C\rightarrow A}$/Rgsc149	(21)
Glucokinase	11	$Gck^{IVS8+2T\rightarrow C}$/Rgsc553	(21)
Glucokinase	11	Gck^{S127P}/M100960	http://www.gsc.riken.go.jp/Mouse
Insulin 2	7	$Ins2^{C95S}$	(16)
Insulin receptor	8	$Insr^{L1105R}$/M101777	http://www.gsc.riken.go.jp/Mouse
Insulin receptor	8	$Insr^{N1154S}$/M100294	http://www.gsc.riken.go.jp/Mouse
Insulin receptor	8	$Insr^{E1196G}$/M100824	http://www.gsc.riken.go.jp/Mouse
Single-minded homolog 1	10	Splice site mut./M100646	http://www.gsc.riken.go.jp/Mouse

of the enzyme activity remained in the heterozygous mutants and homozygous mutants, respectively. There was no significant effect of the mutation on body weight in either sex of heterozygous mutants and homozygous mutants. Homozygous mutants are viable. In summary, impaired glucose phosphorylating activity resulted primarily in impaired β-cell glucose sensing and glucose-stimulated insulin release and, consequently, impaired glucose tolerance and diabetes (50).

In another ENU mutagenesis project using C57BL/6J × DBA/2J mice, 20 dominant hereditary diabetic mutants were obtained from 43 hyperglycemic G1 variants that had been subjected to the inheritance tests. Eleven independent *Gck* mutant lines were observed, including six missense mutations, two nonsense mutations, and three splice site mutations. One additional *Gck* mutant harboring a missense mutation (M100960: Gck^{S127P}) has been published online (http://www.gsc.riken.go.jp/Mouse). The cut-off glucose level determining hyperglycemia was 200 mg/dl. Ad libitum-fed serum glucose levels of the 11-wk-old G1 mutant founder mice were between 199 and 369 mg/dl. Four (Gck^{V182M}, Gck^{T206M}, Gck^{T228A}, $Gck^{IVS1A+1G\rightarrow T}$) of the 11 ENU mutations have also been found in MODY2 patients and another mutation ($Gck^{IVS8+2T\rightarrow C}$) in PNDM patients. *Gck* mRNA and protein expression were further analyzed in six mutant lines (Gck^{V182M}, Gck^{C220Y}, Gck^{M224R}, $Gck^{Y273Stop}$, $Gck^{R345Stop}$, $Gck^{IVS1A+1G\rightarrow T}$). The heterozygous mutants of all lines examined showed mild hyperglycemia. Homozygous mutants were analyzed in the three lines Gck^{V182M}, $Gck^{R345Stop}$, and $Gck^{IVS1A+1G\rightarrow T}$. They suffered from severe hyperglycemia soon after birth. Depending on the line, the homozygous mutants died within the first week after birth or survived at least 5 wk without insulin treatment. In total, the six mutant lines, like the human patients, suffered from impaired glucose-responsive insulin secretion, which causes hyperglycemia. The high frequency of 12 *Gck* mutations in 20 dominant hereditary diabetic mutants may be due to the phenotype monogenically associated with *Gck* mutations, the high penetrance of *Gck* gene defects, or a greater sensitivity of glucokinase activity to amino acid changes, or it may be due to the possibility that haploinsufficiency leads to the phenotypes caused by *Gck* mutations (21).

We recently identified a novel insulin 2 (*Ins2*) allele in the hyperglycemic line GLS004 derived from the Munich ENU mouse mutagenesis screen. In humans, insulin gene mutations were found as rare disorders (48, 49). In mice, homozygous knockout animals lacking either the *Ins1* or the *Ins2* gene had normal plasma insulin levels and normal values in the glucose tolerance test (27). The Akita mouse serves as an *Ins2* mutant model on the C57BL/6N genetic background, which dominantly develops early onset diabetes mellitus without insulitis or obesity. The animals exhibit a spontaneous G→A transition at nt 1907 in exon 3 of *Ins2*, leading to the amino acid exchange C96Y, the disruption of the A7-B7 interchain disulfide bond, and the appearance of a severe defect in insulin secretion and hypoinsulinemia in heterozygous mutants. Males show a more severe phenotype. The accumulation of mutant insulin in β-cells is thought to be responsible for the onset of diabetes mellitus, rather than the initial lack of active insulin (52). Homozygous Akita mice showed increased postnatal lethality (22).

Our ENU-induced diabetic line GLS004 on the C3H genetic background was named Munich $Ins2^{C95S}$ and exhibits a T→A transversion in the *Ins2* gene at nt 1903 in exon 3, which leads to the amino acid exchange C95S and loss of the A6-A11 intrachain disulfide bond. From 1 mo of age onward, blood glucose levels of heterozygous Munich $Ins2^{C95S}$ mutant mice were significantly increased compared with controls. The fasted and postprandial serum insulin levels of the heterozygous mutants were indistinguishable from those of wild-type littermates. However, serum insulin levels after glucose challenge, pancreatic insulin content, and homeostasis model assessment (HOMA) β-cell indexes of heterozygous mutants were significantly lower than those of wild-type littermates. Initial blood glucose decrease during insulin tolerance tests was lower and HOMA insulin resistance indexes were significantly higher in male mutants, indicating the development of insulin resistance. The total islet volume, the volume density of β-cells in the islets, and the total β-cell volume of heterozygous male mutants was significantly reduced compared with wild-type mice (Fig. 2). Electron microscopy of the β-cells of male mutants showed virtually no secretory insulin granules, the endoplasmic reticulum was severely enlarged, and mitochondria appeared swollen. Thus, Munich $Ins2^{C95S}$ mutant mice may serve as a model to study the pathophysiological alterations of β-cells during the development of diabetes mellitus (16).

In the ENU mutagenesis project producing 20 dominant diabetic mutants including the 12 *Gck* mutant lines (21) (see above), additional mutant lines were described online using C57BL/6J × DBA/2J mice without in-depth phenotypic description. Three lines harbored mutations in the insulin receptor (*Insr*) gene ($Insr^{L1105R}$, $Insr^{N1154S}$, $Insr^{E1196G}$), and one line showing obesity had a mutation in the single-minded homolog 1 (*Sim1*) gene (exon 9 splicing donor site mutation) (http://www.gsc.riken.go.jp/Mouse). Targeted knockout mice exist for both genes. Heterozygous *Insr* knockout mice showed normal glucose tolerance, whereas homozygous knockout mice died within the first week of age (1). Heterozygous *Sim1* knockout mice revealed hyperphagia, obesity, and hyperinsulinemia, whereas homozygous knockout mice showed perinatal lethality (34, 35).

In total, the dominant ENU-induced mutations identified to date caused novel alleles of genes already known to be involved in glucose homeostasis. All but one of the glucokinase mutations are derived from a single project. It is assumed that the high number of glucokinase mutations is caused by the specific interaction of factors of the genetic background, environment and experiment in this project and therefore will not appear in other projects. However, the predominant appearance of mutants of another single gene may occur within a given project with constant in vivo and in vitro factors. The predominant appearance of mutations in a single gene may be controlled by modifying various parameters of the project. After the establishment of mutant lines, the ad hoc prescreen of the lines for mutations in this gene may be done.

Additional ENU-Induced Diabetic Lines

In addition to the hyperglycemia lines with the causative mutation already identified, ENU-induced hyperglycemia lines

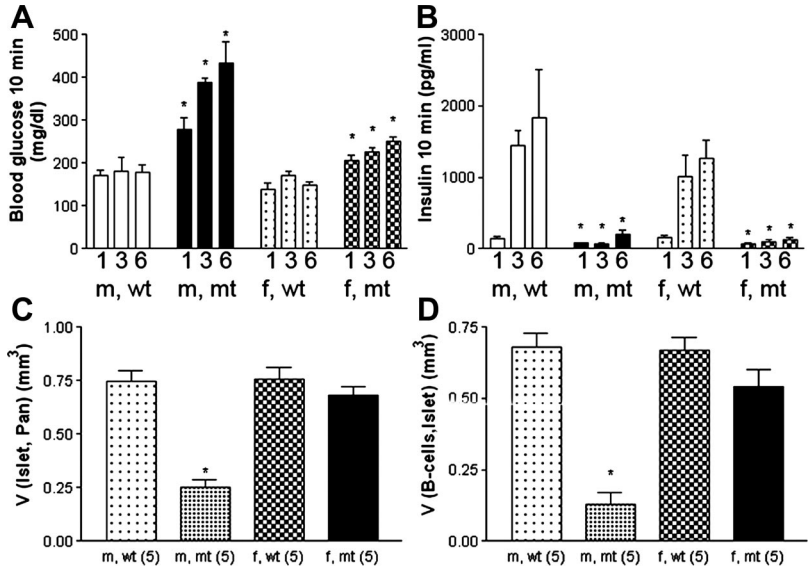

Fig. 2. Heterozygous mutant Munich *Ins2*^{C95S} mice as an example for the establishment of novel diabetes models in ENU mouse mutagenesis projects. Plasma glucose (*A*) and insulin (*B*) levels 10 min after glucose challenge at 1, 3, and 6 mo of age in mutant (mt) mice vs. wild-type controls (wt). Quantitative stereological analysis of the pancreas at 6 mo of age is shown by total islet volume [$V_{(Islet,\ Pan)}$; *C*] and the calculated total β-cell volume [$V_{(\beta\text{-cells},\ Islet)}$; *D*]; m, male; f, female; *n*, no. of animals examined. Data represent means and SE, $*P < 0.05$ (16).

from screens for dominant and recessive mutations with the causative mutation still unknown are published online (Table 5). Causative mutations in the coding region of suitable candidate genes that lie in the linked chromosomal region of mouse lines derived from phenotype-driven ENU mutagenesis projects are inherently identified earlier compared with mutations in genes or noncoding functional sequences previously unknown to influence glucose homeostasis. This is obviously more time consuming. The potential use of high numbers of SNPs as polymorphic markers (http://www.ncbi.nlm.nih.gov/SNP) improves the linkage analysis. Expression analysis of genes in the linked chromosomal region by microarray techniques may assist the selection of suitable candidate genes. The current progress of the sequencing techniques may be used in future analyses by sequencing extensive parts of ENU-mutagenized mouse genomes after breeding them for a low number of generations to wild-type mice.

Table 5. *Published hyperglycemia lines with the causative mutation unknown derived from phenotype-driven ENU mouse mutagenesis projects*

Name/Allele	Inheritance	Reference
Candy	NS	http://strains.emmanet.org
Candy2	NS	http://strains.emmanet.org
Candy3	NS	http://strains.emmanet.org
Candy4	NS	http://strains.emmanet.org
Candy5	NS	http://strains.emmanet.org
GENA263	Dominant	(17)
GENA394	NS	http://strains.emmanet.org
GENA396	NS	http://strains.emmanet.org
GENA397	NS	http://strains.emmanet.org
Glu^{m05Jus}	Recessive	http://www.mouse-genome.bcm.tmc.edu
HLB62	Recessive	http://pga.jax.org
HLB289	NS	http://www.informatics.jax.org
HLB290, Chr. 11	Recessive	http://pga.jax.org
HLB407	Heritable?	http://pga.jax.org
HLB437	Heritable?	http://pga.jax.org
IGT4	Dominant	http://www.mgu.har.mrc.ac.uk
IGT6	Dominant	http://www.mgu.har.mrc.ac.uk
IGT10	Dominant	http://www.mgu.har.mrc.ac.uk
IGT15	Dominant	http://www.mgu.har.mrc.ac.uk
M100025	Dominant	http://www.gsc.riken.go.jp/Mouse
M100160	Dominant	http://www.gsc.riken.go.jp/Mouse
Oedsml, Chr. 2	NS	http://www.mgu.har.mrc.ac.uk
PEDM/14	Recessive	http://www.mgu.har.mrc.ac.uk
PEDM/35	NS	http://strains.emmanet.org
TM/4	Dominant	http://www.mgu.har.mrc.ac.uk
type 2 diabetes	Recessive	(18)
type 2 diabetes	Recessive	(18)

ns, not specified.

Furthermore, a dominant sensitized/modifier ENU mutagenesis screen was established to identify genes and mutations that interact with insulin resistance by using an insulin-resistant genetic background. Various established lines (IGT4, IGT6, IGT10, IGT15) are currently under characterization (http://www.mgu.har.mrc.ac.uk/research/diabetes/sens.html).

Obesity-associated insulin resistance is a major risk factor for T2DM. Therefore, "secondary" hyperglycemia may be observed in ENU-induced mouse mutants that were selected and bred using obesity as a parameter in dysmorphology screens. Search for obese phenotypes in published chemically induced (ENU) mutants (as of august 24, 2007: 1,392 alleles, 1,164 genes/markers) of the "phenotypes and alleles" database in the Mouse Genome Informatics website (http://www.informatics.jax.org/searches/allele_form.shtml) revealed a number of lines showing obesity, e.g., in mice harboring novel alleles for the alstrom syndrome 1 homolog [Alms1^{bbb} (http://www.apf.edu.au), Alms1^{L2131X} (28)], leptin receptor [Lepr$^{db\text{-}1R}$ (http://tnmouse.org), Lepr^{m1Btlr} (http://www.mmrrc.org)], melanocortin 4 receptor [Mc4r^{Glu3} = Glu^{m03Jus} (http://www.mouse-genome.bcm.tmc.edu), Mc4r^{m1Btlr} (http://www.mmrrc.org) (32)], and proprotein convertase subtilisin/kexin type 1 (Pcsk1^{N222D}) (29). In addition, lines showing obesity with the mutation not yet linked and/or discovered are listed: Bgby, Mity, Pkcp (Center for Functional Genomics, Northwestern University); Blb2 (http://www.mouse-genome.bcm.tmc.edu); Hlb44, Hlb52, Hlb80, Hlb81, Hlb93, Hlb124, Hlb125, Hlb131, Hlb132, Hlb147, Hlb163, Hlb181, Hlb197, Hlb199, Hlb230, Hlb349A (http://pga.jax.org); Nmf15 (http://www.jax.org/nmf).

Improvement of the detection of diabetic variants in the screening procedure of the G1 and G3 offspring may be achieved by using more sophisticated screening parameters as indicators of insulin resistance, including elevated fasting insulin levels, in-

creased fasting glucose, and impaired glucose tolerance. This may be done using the HOMA, meal tolerance test (MTT), intraperitoneal or oral glucose tolerance test (IPGTT, OGTT), and intraperitoneal insulin sensitivity test (IPIST) (7). For example, detection of insulin resistance was used as a high-throughput method to establish mutant lines in an ENU mutagenesis project (http://www.mgu.har.mrc.ac.uk/research/diabetes/sens.html).

The screening procedure may also be improved by analyzing the animals more frequently and/or at a higher age. Screening mice at an increased age might lead to a higher level of phenotypic penetrance of the mutation as well as to additional deviations in the plasma glucose due to alternative ENU-induced mutations. Diet challenge tests may reveal interactions between genetic and environmental factors in the development of hyperglycemia. Change of environmental factors is used or intended to be used in ongoing ENU mouse mutagenesis projects, and the outcome may be published in the future. Strain-specific susceptibility to diabetes and/or strain-specific variations in the plasma glucose concentration (38) (http://www.interphenome.org) have been found. Therefore, generation and subsequent analysis of hybrids and/or congenic strains may give rise to variations in the appearance of diabetes.

Summary

Using hyperglycemia as a high-throughput screening parameter, novel dominant alleles of genes already known to be involved in glucose homeostasis, like glucokinase (*Gck*), insulin 2 (*Ins2*), and insulin receptor (*Insr*), were identified in phenotype-driven, large-scale ENU mouse mutagenesis projects started a decade ago. This led to allelic series for the functional analysis of the respective genes. Additional ENU-induced hyperglycemia lines harboring dominant and recessive mutations are currently under genetic and phenotypic analysis. Due to the triggering of random mutations by ENU, new genes and/or alleles not yet known to be involved in glucose homeostasis may be discovered with these mouse mutants. Ongoing ENU mutagenesis projects also use more sophisticated screen methods and breeding strategies, which will reveal additional ENU-derived diabetic mouse models for biomedical research.

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