

Mouse models of diabetic nephropathy

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Purpose of review

Progress in identification of effective therapies for diabetic nephropathy continues to be limited by the lack of ideal animal models. Here we review the current status of some leading murine models of this disorder.

Recent findings

A consensus statement of the Animals Models of Diabetic Complications Consortium sets forth guidelines and standards for measuring renal function and structural parameters necessary for validating murine models of diabetic nephropathy. Two murine models exploiting endothelial nitric oxide synthase (eNOS) deficiency as a major susceptibility factor for development of diabetic nephropathy are among the very few options for studying features of advanced diabetic nephropathy. Akita and OVE26 mice with mutations that result in Type I diabetes are also useful models of diabetic nephropathy. The recently described BTBR *ob/ob* (leptin deficient) mouse with Type II diabetes demonstrates key features of early podocyte loss and mesangiolysis characteristic of human diabetic nephropathy.

Summary

While there are many murine models of mesangial matrix expansion in the setting of diabetes, few progress to develop advanced diabetic lesions. Mice with eNOS deficiency, OVE26 mice, and the recently described BTBR *ob/ob* mouse currently appear to be the best murine models of advanced disease. A model that allows testing of interventions that modulate podocyte loss and regeneration, such as the BTBR *ob/ob* mouse, may be of particular benefit in developing therapeutics for diabetic nephropathy.

Keywords

animal models, diabetic nephropathy, eNOS, leptin, podocytes

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Introduction

The value of animal models for studies of the pathogenesis and natural history of diabetic nephropathy is beyond question. An ideal animal model would reproduce most or all of the lesions of human diabetic nephropathy as described below and would be susceptible to genetic analysis. For practical reasons such an animal model should be cheap, easy to maintain, and widely available. These last requirements generally preclude consideration of nonhuman primates, dogs, and swine as widely utilizable animal species for model development. For these reasons, we focus on murine systems as the most useful and widely used models, although there are a few examples where investigators have used lower order model systems such as fruit flies and zebrafish to gain mechanistic insights into diabetic type renal injury. In this consideration of animal models of diabetic nephropathy, we begin with this question: what are the features of human diabetic nephropathy that should be present in an animal model?

Structural alterations characteristic of diabetic nephropathy in humans

Diabetic nephropathy is in large part a glomerular disease. Abnormalities of glomerular structure are among the most striking and consistent changes identified in biopsy material from patients with diabetic nephropathy [1]. A well defined sequence of glomerular injury has been identified, based on experimental studies and observations in human patients. In the earliest stages of injury, which correlate at least in part with the hemodynamic changes of hyperfiltration, glomerular (and renal) hypertrophy occurs [1].

Mesangial injury

Increased accumulation of mesangial matrix then develops. This process is generally global – involving all segments of the glomeruli – and diffuse – involving all the glomeruli throughout the cortex. This process of diffuse mesangial sclerosis – or diffuse intercapillary glomerulosclerosis as termed by Kimmelstiel and

Wilson – is the hallmark lesion of diabetic glomerulopathy.

The lesions of diffuse mesangial sclerosis are progressive, culminating in the distinctive nodular accumulations of mesangial matrix, so-called Kimmelstiel–Wilson nodules, generally 15 years or more after the onset of diabetes. Nodular mesangial sclerosis has been estimated to occur in approximately 25% of renal biopsies in patients with advanced diabetic nephropathy, although the precise prevalence of this lesion is not known [1,2].

Related to, and likely central to, the process of nodular mesangial sclerosis are mesangiolysis and capillary microaneurysm formation. Mesangiolysis – the fraying and focal dissolution of the mesangial matrix, and capillary microaneurysms – the ballooning out into the urinary space of glomerular capillaries with frequent accumulations of hyaline in the enlarged capillary lumina – often occur in conjunction with mesangial nodules. Some observers have deduced a sequence of injury of repetitive mesangiolysis, microaneurysm formation, and subsequent capillary collapse that leads to the formation of mesangial nodules [3]. These alterations in mesangial cells and the mesangial matrix have long been considered central to the evolution of diabetic nephropathy.

While mesangial alterations similar to those found in human diabetic nephropathy are the most characteristic and easily recognized abnormalities of diabetic nephropathy, there has been a recent focus on podocyte alterations, since podocytes are reduced in number in both Type I and Type II diabetics with diabetic nephropathy, and this reduction in podocytes may precede and even predict the onset of clinically detectable proteinuria [4]. Other lesions that characterize the pathology of diabetic glomerular injury include diffuse thickening of glomerular basement membranes and loss of charge and permselectivity of the glomerular filtration barrier. The pathophysiologic basis for basement membrane thickening, mesangial expansion, mesangiolysis, microaneurysm formation, and podocyte loss largely remain unknown. An ideal animal model of diabetic nephropathy would recapitulate each of these changes.

Mouse models of diabetic nephropathy

There are numerous rodent and rabbit models of spontaneous and acquired (e.g. induction with streptozotocin) diabetes, which have been the subject of recent reviews [5,6,7^{••}]. Most of these models, including NOD mice and mice administered streptozotocin as models of Type I diabetes, and *db/db* mice as the most widely used model of Type II diabetes, develop functional and morphologic renal abnormalities in conjunction with hyperglycemia.

Key points

- Phenotyping of pathologic changes in mouse models of diabetic nephropathy should be comprehensive and include, whenever possible, measures of podocyte number or density, characterization of mesangiolysis and mesangial expansion, and exclusion of concurrent immune complex deposition processes, in addition to extraglomerular alterations in the tubulointerstitium and vasculature.
- There are few murine models of morphologically advanced diabetic nephropathy; currently these include OVE26 mice, diabetic eNOS-deficient mice, and BTBR *ob/ob* mice.
- BTBR *ob/ob* mice with Type II diabetes due to leptin deficiency offer the potential to study reversibility of nephropathy with leptin restoration.

The functional abnormalities include hyperfiltration and proteinuria; morphologic abnormalities include variable degrees of mesangial matrix expansion and glomerular capillary basement membrane thickening. These features replicate the changes of morphologically early diabetic nephropathy, but very few of these models develop the nodular glomerulosclerosis characteristic of advanced human diabetic nephropathy and certainly do not do it quickly or reliably.

The US National Institutes of Health-supported Animal Models of Diabetic Complications Consortium (AMDCC) was created in part to address these deficiencies, and remains engaged in efforts to identify and characterize more comprehensive mouse models of diabetic nephropathy. A consensus publication from this group in 2005 [5], and updated in 2009 [7^{••}], provides explicit standards for phenotyping murine models of diabetic nephropathy and indicates major methodological pitfalls that have made the results from many studies of various models problematic to interpret. One premise of this group is that the standardization of phenotyping methodology will allow better comparisons of results obtained by different groups using different animal models, and ensure that future studies will increasingly use murine models that closely and reliably model human disease. The AMDCC initially proposed the following three criteria for an ideal mouse model: progressive renal insufficiency in the setting of hyperglycemia; albuminuria; and characteristic pathologic changes including basement membrane thickening by electron microscopy, mesangial matrix expansion + nodular mesangial sclerosis, interstitial fibrosis, and arteriolar hyalinosis [5].

These criteria have been updated to include:

- (1) greater than 50% decline in glomerular filtration rate over the lifetime of the animal.

- (2) greater than 10-fold increase in albuminuria compared with controls for that strain at the same age and gender.
- (3) pathologic changes in kidneys, including advanced mesangial matrix expansion $\pm$ nodular sclerosis and mesangiolysis, any degree of arteriolar hyalinosis, glomerular basement membrane thickening by $>50\%$ over baseline, and tubulointerstitial fibrosis [7^{••}].

Additional histologic phenotyping for diabetic nephropathy models with advanced disease should include:

- (1) quantification of mesangial matrix expression, ideally with morphometric analyses, and with mesangiolysis and microaneurysms detected with appropriate tissue stains.
- (2) exclusion of immune complex disease with immunohistochemistry on frozen sections for IgG, IgM, and IgA.
- (3) demonstration of glomerular basement membrane thickening by electron microscopy.
- (4) demonstration of podocyte loss by a reasonable morphometric system [7^{••}].

Demonstration of podocyte loss is critically important in view of the extensive data that podocyte loss is an early and likely essential event in progressive human diabetic nephropathy, yet this is infrequently determined in animal models. Podocyte loss cannot be recognized in routine histologic preparations. Immunohistochemical stains are necessary to identify podocytes and podocyte loss, and the methodology used must account for differences in podocyte density as glomerular volume is altered (e.g. by the hypertrophy characteristic of diabetic nephropathy) [8,9]. These standards also emphasize the importance of going beyond measures of altered mesangial matrix volume alone to characterize the structural abnormalities in models of diabetic nephropathy.

Commonly overlooked by investigators and reviewers of studies is that these criteria were established by consensus, and represent goals and not absolute standards. Failure to achieve any one specific standard should not doom a promising mouse model to oblivion, but in aggregate a model of diabetic nephropathy that meets most of these criteria is likely to prove useful as a research tool. As long as this caveat is kept in mind, these standards have begun to gain wide acceptance for being both unambiguous and rigorous [10].

In addition to defining goals for phenotyping mouse models of diabetic nephropathy and reviewing strengths of several models under active development, the AMDCC consensus paper provides a perspective on where the field is moving. One trend followed by both

the consortium and other investigators to overcome the limitations of current models is a strategy of breeding critical diabetogenic mutations into mouse strains found to be susceptible to the development of specific complications such as nephropathy (e.g. the DBA/2 strain) versus strains that are resistant (e.g. C57BL/6). To date a few monogenic mutations [endothelial nitric oxide synthase (eNOS) [11] and bradykinin receptor [12] deficiencies are examples] have been shown to impart significant features of diabetic nephropathy when introduced into susceptible mice, but there have been few such mutations identified to date and none as yet fully model human diabetic nephropathy.

db/db

The *db/db* mouse model of leptin deficiency is currently the most widely used mouse for modeling diabetic nephropathy in settings of Type II diabetes. Leptin deficiency confers susceptibility to obesity, insulin resistance, and Type II diabetes. *db/db* mice have a mutation deletion of the leptin receptor and an underlying genetic background that is susceptible to diabetic complications such as nephropathy [13]. Diabetic nephropathy in these mice is manifest by albuminuria, podocyte loss, and mesangial matrix expansion [5,13,14]. The mesangial expansion develops relatively slowly, often needing a half year or more to fully evolve. In general, these mice do not develop mesangiolysis or nodular mesangial sclerosis, nor do they develop progressive renal insufficiency. Hence, these mice are a good model of the early changes of human diabetic nephropathy, but fail to recapitulate later and morphologically advanced features of diabetic nephropathy.

The importance of strain as a major co-factor for susceptibility to nephropathy is particularly evidenced by the observation that mice of the C57BL/6 KS strain (a strain that has been modified to contain part of the DBA/2 strain genetic endowment) that carry the *db/db* mutation can develop features of diabetic nephropathy, while the identical mutation in mice of C57BL/6 strain, without the contribution of DBA/2, fails to develop significant nephropathy [5].

OVE26 mice

The OVE26 mouse is characterized by transgenic overexpression of calmodulin in pancreatic β cells leading to deficient production of insulin and Type I diabetes [15[•]]. This overexpression of calmodulin, in mice of FVB background, results in significant nephropathy with marked albuminuria far in excess of the standard recommended by the AMDCC. The nephropathy in these mice is characterized by increased mesangial matrix, which can be severe and extends to more global glomerulosclerosis,

diminished podocyte number, and mild degrees of renal fibrosis. The diminished podocyte number, albuminuria, and mesangial matrix increase all are significant strengths for using these mice for studies of diabetic nephropathy. A significant limitation of these mice is the requirement for the mutation to be expressed in the FVB mouse strain to obtain the desired phenotypic features. Albuminuria, mesangial matrix expansion, and fibrosis are all significantly diminished when the transgene is introduced into the C57BL/6 or DBA/2 mouse strains (despite the enhanced susceptibility of DBA/2 mice to develop diabetic nephropathy) [15^{*}]. This particular strain dependence makes it challenging to introduce other genetic mutations from other mouse strains into this model. Although OVE26 mice on an FVB background develop mesangial sclerosing lesions of advanced diabetic nephropathy including nodular mesangial sclerosis, it has been difficult to glean from published studies how this has been quantitated and therefore how uniform and widespread these changes may be. These mice develop reduction in podocyte number and density, but most of this loss appears to develop late in the disease course (as assessed at days 60, 150, and 450) and hence follows, rather than precedes, other manifestations of diabetic nephropathy [16]. Finally, based on the initial promise of the model, a number of laboratories have investigated its use. At least one group other than that of Epstein *et al.* has found that these mice develop features of advanced diabetic nephropathy [17], but difficulties with the viability of these mice also have been reported [7^{**}].

Akita mice

Akita mice carry the *Ins2*^{+IC96Y} mutation, a single nucleotide substitution in the *Ins2* gene. This mutation causes abnormal folding of the insulin protein, toxic injury to pancreatic β cells, and diminished capacity to secrete insulin, resulting in Type I diabetes [5,18^{*}]. As originally described in mice of the C57BL/6 strain, diabetic mice with the Akita mutation develop modest levels of albuminuria and modest structural changes of increased mesangial matrix and basement membrane thickening. Depletion of podocytes, due at least in part to increased apoptosis, manifests early in the development of diabetic nephropathy [14]. Gurley *et al.* [18^{*}] have studied the effect of breeding the *Ins2*^{+IC96Y} mutation into strains more susceptible to nephropathy, that is DBA/2 and 129/SvEv. They found that C57BL/6, DBA/2, and 129/SvEv mice with the Akita mutation develop similar degrees of hyperglycemia, but differed in their manifestation of nephropathy. DBA/2 mice had more albuminuria than the others, but only C57BL/6 and 129/SvEv strain mice demonstrated increased mesangial matrix.

These and other previously published studies show that Akita mice have potential to model some features of

diabetic nephropathy, but as is the case with OVE26 mice, these features are heavily dependent on the mouse strain in which the mutation is introduced. To date, none of the published studies of Akita mice, regardless of strain, have demonstrated structural alterations that model advanced human diabetic nephropathy such as mesangiolysis or widespread marked or nodular mesangial sclerosis. A particular caveat to be considered when using these mice is the propensity for Akita mice, for unknown reasons, to develop mesangial deposits of IgA [19]. Since mesangial deposition of IgA is a major cause of mesangial proliferative glomerulopathy in humans, understanding the basis for mesangial matrix increase in Akita mice (diabetes, mesangial immune complexes, or both?) can be problematic and limit the value of studies in which alterations of mesangial volume and matrix accumulation are a key endpoint.

The number of background strains studied with the *Ins2*^{+IC96Y} mutation is small. It can be expected that studies of this mutation in mice of other genetic backgrounds may result in a more robust model of Type I diabetes and nephropathy, and it is possible the confounding issue of IgA deposition may not arise when the mutation is expressed in a different mouse strain.

Podocyte-specific insulin receptor knockout mouse

An exciting recent development has been the report of a mixed mouse strain (with contributions of C57BL/6, 129/Sv, and FVB backgrounds) with podocyte-specific deletion of insulin receptors using both podocin and nephrin promoters to drive cre-recombinase-directed excision of the receptor [20]. These mice develop proteinuria and were reported to develop histologic features of diabetic nephropathy despite maintaining normal insulin levels and normoglycemia. Features of diabetic nephropathy exhibited by these mice included increased podocyte apoptosis at 8 weeks of age, detected at a time when significant proteinuria was becoming established, podocyte foot process effacement, and increased mesangial matrix. However, the published histologic lesions in these mice suggest a nonspecific process of progressive glomerulosclerosis rather than development of classic features of diabetic nephropathy. Features of mesangiolysis and/or nodular glomerulosclerosis were not characterized, nor was podocyte number. Nonetheless, this first publication showed considerable promise for this model as a tool for dissecting important podocyte-specific activities that may mediate some of the structural changes of diabetic nephropathy, and particularly identifies podocyte insulin sensitivity as an important regulatory element in the maintenance of normal glomerular structure and function.

Endothelial nitric oxide synthase deficient mice

There are currently two models of diabetic nephropathy dependent on genetic deletion of endothelial nitric oxide synthase (*eNOS*^{-/-}). eNOS provides the principal means by which nitric oxide is generated in the vasculature; NO is in turn a major regulator of vascular tone. Diminution of NO availability has been considered a major mechanism underlying development of diabetic complications involving the vasculature, including diabetic nephropathy.

A major advance in diabetic nephropathy model development was the introduction of eNOS deficiency into *db/db* mice. *eNOS*^{-/-}/*db/db* mice develop Type II diabetes, obesity, hypertension, albuminuria, marked mesangial expansion, and mesangiolysis [11,21]. These features establish this mouse as one of the very few to develop features of advanced diabetic nephropathy, and hence offering great potential for studying mechanisms underlying progression. However, there have been no subsequent publications on this model following its initial description; we speculate this may be attributable to difficulty in breeding mice with these combined mutations.

An alternate approach to exploit eNOS deficiency has been to use streptozotocin induced Type I diabetes in *eNOS*^{-/-} mice of C57BL/6 background. Although C57BL/6 mice are resistant to development of diabetic nephropathy, Nakagawa *et al.* [22] have shown eNOS deficiency is sufficient to permit development of features of advanced diabetic nephropathy including hypertension, albuminuria, mesangial matrix expansion, mesangiolysis and mesangial nodule formation, and renal insufficiency in these mice. Like *eNOS*^{-/-}/*db/db* mice, this model also offers a means to study mechanisms underlying advanced diabetic nephropathy, but several important caveats need to be considered. Use of high streptozotocin doses in the initial study by Nakagawa also led to high mortality. When a lower dose streptozotocin protocol was used to evaluate the effects of diabetes in *eNOS*^{-/-} C57BL/6 mice, Kanetsuna *et al.* [23] found a milder phenotype with no significant renal insufficiency and only mild mesangial expansion despite the presence of mesangiolysis and albuminuria. *eNOS*^{-/-} mice without diabetes also have a low but baseline background of mesangiolysis and microthrombosis, complicating interpretations of whether the mesangiolysis in diabetic *eNOS*^{-/-} mice models the mesangiolysis of human diabetic nephropathy or is an exacerbation of an alternate injury process. Some support for this scenario comes both from the Kanetsuna study and from a subsequent study of this model by Nakagawa's group that demonstrated the phenotypic features of diabetic glomerulopathy (mesangiolysis and mesangial nodule formation) were the result of hypertension and not diabetes

and could be prevented, despite persistent hyperglycemia, solely by administration of hydralazine to lower blood pressure [24*].

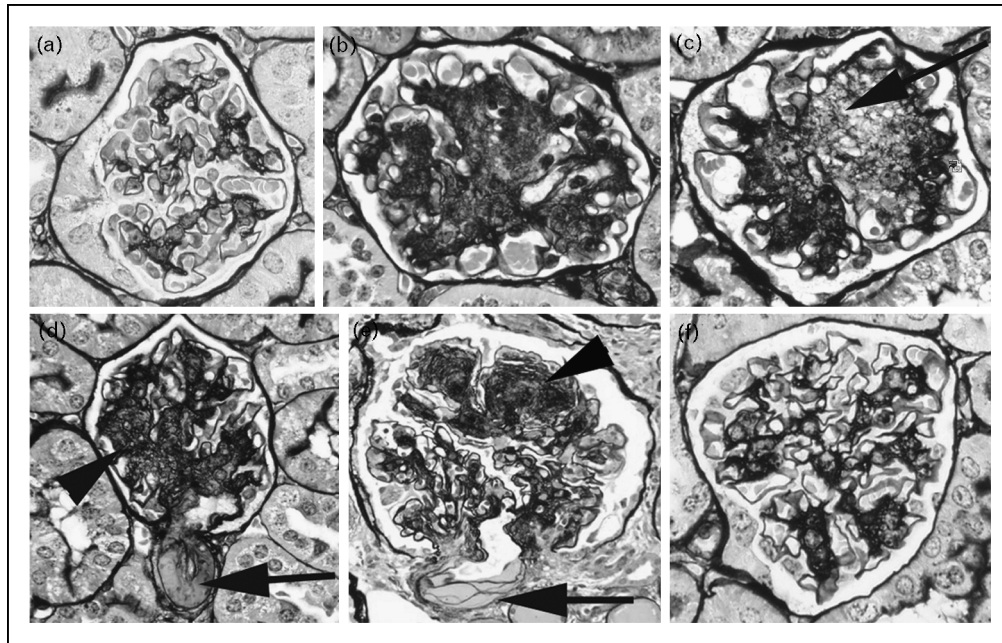
In spite of these limitations, the advanced renal morphologic changes encountered in a diabetic milieu when eNOS is deficient, especially in mice of susceptible genetic background, are rarely encountered in most other diabetic nephropathy models. This makes such mice extremely promising for further model development. Perhaps when this mutation is shifted to an alternate genetic background, or a model is developed that allows regulatable and/or anatomic site-specific deletion of eNOS (e.g. in the glomerular capillaries after an extended period of diabetes), an even more robust model of advanced diabetic nephropathy may emerge.

BTBR *ob/ob* mice

A mouse model of insulin resistance that develops in the BTBR (black and tan *brachyuric*, i.e., short tailed) mouse strain in which the *ob/ob* mutation has been introduced has been created by Clee and Attie [25]. In brief, the characteristics of the BTBR *ob/ob* mice are insulin resistance (measured as early as four weeks of age) with elevated serum insulin levels and pancreatic islet hypertrophy and subsequent sustained hyperglycemia at 350–400 mg/dl. They are largely resistant to significant lowering of blood glucose by insulin administration. The kidney phenotype of these mice includes early loss of podocytes, chronologically coinciding with onset of proteinuria, both of which are detectable by 8 weeks of age [26**]. These changes precede mesangial expansion, identifiable by 10 weeks of age. These features model morphologically early human diabetic nephropathy, and by 18 weeks of age, these mice model progressive, advanced diabetic nephropathy with features of marked proteinuria, more extensive mesangial expansion, mesangiolysis, persistent podocyte loss, basement membrane thickening, and modest but detectable interstitial fibrosis (Fig. 1).

These mice offer a unique opportunity to study mechanisms that may lead to new therapies aimed at regression of diabetic nephropathy, and not just its stabilization. Reversibility of morphologically advanced diabetic nephropathy has not been previously demonstrable in other animal models or in human disease, except in the case of longstanding (10 years) pancreatic transplantation in humans. One particular advantage of BTBR *ob/ob* mice as a model of diabetic nephropathy is the relatively rapid time course for development of advanced disease, which reduces the time needed to conduct interventional studies that may ameliorate advanced diabetic nephropathy after it is already established. The mutation of leptin deficiency (versus deficiency of the leptin receptor in

Figure 1 Comparison of mesangial expansion in BTBR *ob/ob* mice and BTBR wild type mice



BTBR *ob/ob* mice show distinct mesangial expansion at 14 weeks (b) compared to BTBR wild type (a). These mesangial lesions are progressive as shown at 21 weeks (c and d) and result in part from mesangiolysis, with lucency and dissolution of the mesangial matrix (c, arrow). The glomerulus in (d) shows diffuse and focally nodular mesangial sclerosis (arrowhead) and hyalinosis of an arteriole (d, arrow). Compare to human diabetic nephropathy in (e) with characteristic hyalinosis of arteriole (arrow) and nodular sclerosis (arrowhead), and to the limited mesangial change in leptin receptor deficient *db/db* mice (f), currently the most widely used murine model of diabetic nephropathy in Type II diabetes, at 22 weeks. (a–f) Silver methenamine stain. Modified from Hudkins *et al.* [26**].

db/db mice) allows for studies of mechanisms underlying potential reversibility of diabetic nephropathy by utilizing leptin replacement as a strategy. Demonstration that this potential can be realized in these mice has been recently reported (in abstract form) by Pichaiwong *et al.* [27], who demonstrated full reversal of the lesions of advanced diabetic nephropathy by continuous infusion of leptin via osmotic minipump. This may be a model for other models of diabetic nephropathy, by setting a goal for new models that they should be suitable for studies of interventions that ameliorate or even reverse established and advanced diabetic nephropathy, as commonly encountered in patients, and not just prevent or ameliorate diabetic nephropathy in its earliest stages.

The BTBR *ob/ob* mouse, as other models, has significant limitations. Like other mice with leptin deficiency, these mice are infertile and breeding mice in sufficient numbers for interventional studies is labor intensive and expensive. The BTBR strain is not commonly studied, and so introduction of genetic mutations into these mice to test specific pathophysiologic concepts requires extensive and time-consuming backcrossing strategies. However, given the unique possibilities to study reversibility of diabetic nephropathy, and the limitations of alternative

models, these mice are likely to be a worthwhile research tool for the foreseeable future.

Conclusion

There are multiple murine models, modified by strain and superimposed genetic mutations, of diabetic nephropathy. We have considered selected currently used animal models. Some mutations that have been important for recent pathophysiologic studies of mouse models of diabetic nephropathy, but not considered here, include genetic deletions of decorin [28], bradykinin receptors [12], the receptor for advanced glycation end products (RAGE) [17], and osteopontin [29]. Mouse models of diabetes that reliably recapitulate early morphological changes of human diabetic nephropathy, principally mesangial matrix expansion and in some cases, podocyte loss, include *db/db* and Akita mice. There are few models that recapitulate features of morphologically early and late diabetic nephropathy; of these, mice with deficiency of eNOS, OVE26 FVB mice, and BTBR *ob/ob* mice appear to be the most robust. The 2009 review of the AMDCC suggested that *eNOS*^{−/−}/*db/db* mice may be the best current mouse model for recapitulating the functional and structural changes of morphologically advanced human diabetic nephropathy [7**]. The

recently described BTBR *ob/ob* mouse model rivals the *eNOS^{-/-}/db/db* mouse in this regard. The relative rapidity in which lesions develop in this model make it well suited for interventional studies. Despite these advances, all of the mouse models available to date possess important limitations. Designing better models of diabetic nephropathy that will allow identification of underlying mechanisms, or testing of therapeutic interventions, must continue.

Acknowledgement

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 324–325).

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