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Chapter 22

Animal Models with a Genetic Alteration of the ACE2/Ang-(1-7)/Mas Axis

Luiza A. Rabelo,[*] Valéria Nunes-Souza,[*] Michael Bader[†]

[*]Max Delbrück Center for Molecular Medicine, Berlin, Germany; Laboratório de Reatividade Cardiovascular (LRC), Setor de Fisiologia e Farmacologia, ICBS, Universidade Federal de Alagoas, Maceió, Al, Brazil; National Institute of Science and Technology in NanoBiopharmaceutics (N-BIOFAR), Brazil, [†]Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany; Charité Medical Faculty, Berlin, Germany; Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; Institute for Biology, University of Lübeck, Germany

INTRODUCTION

The study of hormone systems involved in cardiovascular and metabolic diseases, such as the renin–angiotensin system (RAS), can only be performed in whole organisms due to the complex interplay of different organs, which determines cardiovascular physiology. In contrast to pharmacological interventions in these systems, which often lack specificity, the targeted genetic alteration of the expression of single-hormone system components is the most straightforward method to analyze their functions in cardiovascular and metabolic homeostasis and disorders. Accordingly, the generation of transgenic and knockout (KO) animals was widely used to study the role of RAS components in cardiovascular control and in the pathogenesis of diseases.[1,2] The aim of this chapter is to describe the animal models generated by transgenic technology for the functional analysis of the protective axis of the RAS, consisting of angiotensin-converting enzyme 2 (ACE2), Ang-(1-7), and Mas.

TRANSGENIC ANIMAL TECHNOLOGY: A BRIEF UPDATE

In biomedical research, the use of rats and mice has become a major tool, considering the easiness of breeding, growth, and maintenance and the similarity with human organisms in most cardiovascular and metabolic systems.[3] Currently, the use of transgenic technologies to access animal physiology is routine, but the pioneering work was performed in the 1980s. The first successful genetic modifications of a mouse were achieved by Gordon and colleagues.[4] This group demonstrated that foreign genes can be integrated into the mouse genome by transfer of DNA constructs into the pronuclei of zygotes. By the use of specific promoters, the investigator can direct the expression of the transgene into specific tissues. About 10 years later, the same technology was also established for the rat, interestingly first targeting the RAS component renin.[5]

Since 1986, the elimination of gene expression is also possible by homologous recombination-mediated targeted gene KO in embryonic stem (ES) cells.[6–8] To this purpose, firstly, a targeting vector has to be designed and constructed that contains parts of DNA sequences homologous to the gene of interest and the intended mutation. Following the transfection of this vector into ES cells, a few cells will incorporate it into the endogenous gene via homologous recombination and can be selected by appropriate methods. These successfully targeted ES cells are injected into host blastocysts, and a chimeric animal is obtained. This animal carries the mutation in part of its cells and will eventually pass it on to its offspring, which will be heterozygous or homozygous (KO) for the mutant gene. The KO animals will present the physiological phenotype caused by the absence of the gene product.[6] In general, this method is still the most commonly used for the targeted alteration of the mouse genome. It took more than 20 years for this technology to also became available for the rat by the discovery of a method to establish germ-line-competent ES cells from this species in 2008,[9] and a few KO rat models have been developed since using ES cells.[10,11] However, recently, novel methods for the targeted alteration of genes in the mouse and rat genome have become available, which will probably replace the relatively complicated ES cell method in the near future. They are based on nucleases that are targeted to a certain site in the genome by different methods.[12] Zinc finger (ZFN) and TALE nucleases use protein domains that specifically recognize a DNA sequence of choice, while the CRISPR/Cas9 system uses a guide RNA that binds DNA by specific base pairing. Since only one animal model for the RAS has been described yet produced by ZFN technology, the renin-KO rat,[13] and since the Mas-KO rat has obviously been generated using ZFNs (http://rgd.mcw.edu/rgdweb/report/strain/main.html?id=5131952; access 25.04.2014) but is not yet published, we will not go into more detail on these novel technologies.

The Protective Arm of the Renin–Angiotensin System (RAS). http://dx.doi.org/10.1016/B978-0-12-801364-9.00022-5

TRANSGENIC AND KO RODENT MODELS OF THE ACE2/ANG-(1-7)/MAS AXIS

Our group and others have developed several transgenic and KO rat and mouse models with genetic deletion and/or overexpression of components of the ACE2/Ang-(1-7)/Mas axis. Some of these models produced pleiotropic phenotypes depending on the genetic background of the strain they were generated in.

In the following, we will list these models and summarize the insights into the physiology of the protective RAS axis gained by their analysis (see also Table 1).

ACE2 Models

ACE2 KO Mice

The ACE2 gene is located on the X chromosome, and thus, heterozygous deletion ($ACE2^{-/y}$) already results in the complete absence of the enzyme in male animals. Deletion of ACE2 in mouse leads to several cardiac abnormalities. In an elegant study, Crackower and colleagues provided the first *in vivo* evidence[14] supporting the hypothesis that the loss of ACE2 promotes heart dysfunction.[60] However, later studies on the function of ACE2 in the heart resulted in contradictory observations.[61] Two independent groups showed that the baseline cardiac function and morphology appeared normal in their ACE2-deficient mouse lines.[16,18] Nevertheless, $ACE2^{-/y}$ and even heterozygous female $ACE2^{+/-}$ mice are more susceptible to pressure overload or diabetes-induced cardiac injury.[18–20] Moreover, the lack of ACE2 also exacerbated diabetic and shock-induced kidney injury.[25–27]

There is also still a debate whether the deletion of ACE2 changes basic blood pressure in mice. Most likely, this effect depends strongly on the genetic background of the mice analyzed: 129 mice show hardly any effect, while C57BL/6 or FVB/N $ACE2^{-/y}$ mice are clearly hypertensive[61] (our unpublished results). Nevertheless, C57BL/6 $ACE2^{-/y}$ mice displayed high blood pressure during pregnancy and reduced weight gain and gave birth to smaller pups.[17] Besides an upregulation of oxidative stress in the brain and consequently of the sympathetic nervous system,[41] the cause for the hypertensive effect of ACE2 deletion may be an endothelial dysfunction as evidenced by an impaired acetylcholine-induced aortic vasodilatation.[15]

ACE2 deletion in apolipoprotein E (ApoE) KO mice, a classical model for atherosclerosis, worsened plaque formation and vascular inflammation.[21,22] In low-density lipoprotein receptor KO mice, fed a high-fat diet, ACE2 deletion also aggravated atherosclerosis.[62] Moreover, loss of ACE2 led to increased arterial neointima formation in response to endovascular injury in the femoral artery accompanied by an overexpression of inflammation-related genes.[22]

Several studies have shown an important role of ACE2 in metabolism. C57BL/6 $ACE2^{-/y}$ mice show impaired glucose homeostasis at different ages.[23,24] Also, ACE2 gene deletion aggravated liver fibrosis in models of chronic hepatic injury.[63]

$ACE2^{-/y}$ mice displayed aggravated pathologies in the acute respiratory distress syndrome[28] and in bleomycin-induced lung injury[29] rendering ACE2 an important target for inflammatory lung diseases.

$ACE2^{-/y}$ animals were also instrumental for the surprising finding that the protein is not only an enzyme but also a trafficking molecule in the gut being responsible for the functional expression of the amino acid transporter SLC6A19.[30,31] $ACE2^{-/y}$ mice, therefore, show reduced levels of large amino acids, such as tryptophan, in the circulation, altered gut microbiota, and intestinal inflammation.[32]

Human ACE2 Overexpression in Mouse

Besides being an enzyme and trafficking molecule, ACE2 is also the receptor for the human severe acute respiratory syndrome (SARS) coronavirus. In order to study this function, transgenic mouse models have been generated by several groups that overexpress human ACE2 using the mouse ACE2 promoter,[33] the cytomegalovirus promoter,[36,37] or the cytokeratin 18 promoter specific for the airway and other epithelia.[34,35] As expected, human ACE2 expression led to an increased susceptibility of the transgenic mice to SARS virus infection. In the kidney, ACE2 overexpression protected the mice from shock-induced injury.[26]

Human ACE2 Overexpression in the Mouse Brain

The transgenic mouse overexpressing human ACE2 in the brain using the synapsin promoter has confirmed the important actions of central Ang II in the pathogenesis of cardiovascular diseases and the protective role of Ang-(1-7). The animals are protected from hypertension induced by low peripheral infusions of Ang II[40] and DOCA-salt treatment.[64] Moreover, they show an amelioration of cardiac hypertrophy induced by AngII,[65] of chronic heart failure induced by coronary ligation,[66] and of stroke induced by middle cerebral artery occlusion.[42,67] In most cases, an increase in Ang-(1-7) and a decrease of Ang II in the brain both influencing the levels of local NO and the autonomic nervous system were shown to be instrumental.

TABLE 1 Transgenic Rodent Models of the ACE2/Ang-(1-7)/Mas Axis

Component of the ACE2/Ang-(1–7)/Mas Axis	Promoter	Species	Effect on Expression	Phenotypes/References
ACE2	-	Mouse	Knockout	Cardiac[14] and endothelial dysfunction[15] (our unpublished data) Increased blood pressure (depending on genetic background)[16] Increased blood pressure during pregnancy[17] Increased susceptibility to cardiac damage[18–20] Aggravated atherosclerosis[21,22] Disturbed glucose homeostasis[23,24] Aggravated kidney[25–27] and lung injury[28,29] Amino acid uptake deficiency in the gut[30–32]
ACE2 (human)	ACE2 (mouse)	Mouse	General overexpression	Increased susceptibility to SARS virus infection[33]
ACE2 (human)	Cytokeratin 18	Mouse	Overexpression in epithelia	Increased susceptibility to SARS virus infection[34,35]
ACE2 (human)	CMV	Mouse	General overexpression	Increased susceptibility to SARS virus infection[36,37]
ACE2 (human)	αMHC	Mouse	Overexpression in heart	Increase in ventricular tachycardia and sudden death[38]
ACE2 (human)	Nephrin	Mouse	Overexpression in podocytes	Ameliorated nephropathy induced by diabetes[39]
ACE2 (human)	Synapsin	Mouse	Overexpression in brain	Attenuated neurogenic hypertension[40] Decreased oxidative stress and sympathetic activity[40,41] Protection from brain ischemic injury[42]
ACE2 (human)	SMMHC	Rat (SHRSP)	Overexpression in smooth muscle	Ameliorated hypertension, reduction in oxidative stress[43]
Mas	-	Mouse	Knockout	Increased anxiety (sex-dependent)[44,45] Cardiac[46] and endothelial dysfunction[47,48] Increased vascular and systemic oxidative stress[47,48] Erectile dysfunction[49] Nephropathy[50] Metabolic dysfunction[51]
Mas (rat)	Opsin	Mouse	Overexpression in retina	Degeneration of photoreceptors[52]
PRCP	-	Mouse	Knockout	Decrease in body weight, hypertension, vascular dysfunction[53] (Schadock et al., unpublished results)
Ang-(1–7)	CMV	Rat	Overexpression in testis	Improved cardiac and endothelial function,[54] improved lipid and glycolytic profile[55,56] Protection from heart hypertrophy[57]
Ang-(1–7)	αMHC	Mouse	Overexpression in heart	Protection from heart hypertrophy[58]
Ang-(1–7)	αMHC	Rat	Overexpression in heart	Improved cardiac function and protection from heart hypertrophy[59]

PRCP, prolylcarboxypeptidase; SHRSP, spontaneously hypertensive stroke-prone rat; SMMHC, smooth muscle myosin heavy chain; CMV, cytomegalovirus; αMHC, α-cardiac myosin heavy chain.

Human ACE2 Overexpression in the Mouse Heart

Transgenic mice overexpressing human ACE2 in the heart surprisingly showed an increase in ventricular tachycardia and sudden death, which was due to a dysregulation of connexins.[38] The underlying mechanism and the involved peptides could not be elucidated.

Human ACE2 Overexpression in Mouse Podocytes

When human ACE2 was overexpressed in podocytes of mice using the nephrin promoter, the nephropathy induced by diabetes was ameliorated.[39] The authors suggest that this is due to reduced renal Ang II levels leading to a reduced expression of TGF-beta, but they could also not exclude a protective effect of Ang-(1-7).

Human ACE2 Overexpression in the Rat Vascular Smooth Muscle

ACE2 is highly expressed in the endothelium and smooth muscle cells (SMC), and its expression is reduced in the spontaneously hypertensive stroke-prone rat (SHRSP). When human ACE2 was overexpressed in vascular SMC of transgenic SHRSP, endothelial dysfunction and hypertension were ameliorated, which was accompanied by a reduction in oxidative stress linked to a decrease in Ang II and/or an increase in Ang-(1-7).[43]

Mas Models

Mas-KO Mice

In 1998, we generated Mas-deficient ($Mas^{-/-}$) mice on the mixed 129×C57BL/6 background and showed that these animals were healthy in appearance and grew normally and exhibited normal Ang II plasma levels.[44] However, male (but not female[45]) $Mas^{-/-}$ mice displayed increased anxiety on the elevated-plus maze. We also showed not only that long-term potentiation was markedly increased in the hippocampal CA1 region of $Mas^{-/-}$ mice[44] but also that object recognition memory is impaired.[68] Collectively, these data support a role of Mas in behavior.

C57BL/6 $Mas^{-/-}$ mice show a marked cardiac dysfunction both *in vitro*[69] and *in vivo*[46] accompanied by an increase in extracellular matrix proteins, such as collagen I, collagen III, and fibronectin.[46] Furthermore, $Mas^{-/-}$ animals exhibit vascular oxidative stress, endothelial dysfunction, and high blood pressure at least on the FVB/N genetic background.[47,48] Consistently, endothelial function is also impaired in isolated $Mas^{-/-}$ vessels.[70] Consequently, $Mas^{-/-}$ mice exhibit a pronounced decrease in blood flow and a marked increase in resistance in different vascular beds.[71] These functional changes in both regional and systemic hemodynamics in $Mas^{-/-}$ mice suggest that the Ang-(1-7)/Mas axis plays an important role in vascular regulation. A dysregulation of the vascular function in the *corpus cavernosum* is probably also the reason for the erectile dysfunction observed in $Mas^{-/-}$ mice.[49]

Pinheiro et al.[50] showed an imbalance in renal function in $Mas^{-/-}$ mice: reduced urine volume and fractional sodium excretion and increased glomerular filtration rate and proteinuria. Surprisingly, a proinflammatory role for Ang-(1-7) and Mas was reported in a model of unilateral ureteral obstruction in mice[72] in contrast to the anti-inflammatory effects of Ang-(1-7) and Mas in other models of kidney nephropathy.[73] Certainly, additional studies are needed to clarify the role of Ang-(1-7) and Mas in the kidney. The anti-inflammatory actions of Mas were also recently confirmed in a lipopolysaccharide (LPS)-induced endotoxic shock model.[74]

Santos and colleagues[51] have revealed the effects of Mas deficiency on lipid and glucose metabolism. They used $Mas^{-/-}$ mice on the FVB/N background and demonstrated that loss of Mas increases the risk of metabolic complications by causing several features of the metabolic syndrome, such as type 2 diabetes mellitus, hypertension, dyslipidemia, and nonalcoholic fatty liver disease. Mas deletion decreased the responsiveness of adipocytes to insulin accompanied by a decreased expression of PPARγ in adipose tissue.[75] Moreover, Silva et al. recently showed that Mas deletion in $ApoE^{-/-}$ mice leads to dyslipidemia and liver steatosis.[76]

Mas Overexpression in Retina

When rat Mas is overexpressed in the retina of transgenic mice using the opsin promoter, degeneration of photoreceptors is the consequence, which is probably induced by proliferative signaling pathways activated in these cells due to the constitutively active Mas protein.[77]

Prolylcarboxypeptidase KO Mice

Prolylcarboxypeptidase (PRCP, EC 3.4.16.2) is another enzyme that can generate Ang-(1-7) from Ang II, but it is also not specific for angiotensin peptides. PRCP-KO mice show elevated levels of α-melanocyte-stimulating hormone (α-MSH),

an anorexigenic neuropeptide, in the hypothalamus. The phenotype of these animals is characterized by a decrease in body weight and body length under normal diet, accompanied by decreased white adipose tissue.[53] The lean phenotype is also observed after high-fat diet-induced obesity (Schadock et al., unpublished results).

Moreover, PRCP-KO mice display hypertension and vascular dysfunction probably due to an increase in reactive oxygen species and uncoupled eNOS.[52] In addition, PRCP also regulates angiogenesis and vascular repair.[78] How much of these cardiovascular actions are due to alterations in Ang II or Ang-(1-7) levels remains to be elucidated.

Ang-(1-7) Models

Based on an elegant system to generate RAS peptides by release from an artificial protein, which is processed by furin during secretion,[58,79] several transgenic animal models with altered Ang-(1-7) levels were generated.

Transgenic Rats Overexpressing Ang-(1-7)

The transgenic rat TGR(A1-7)3292 expresses the Ang-(1-7)-producing protein mainly in the testis.[80] In this model, Ang-(1-7) levels are chronically elevated in plasma and testis ~2.5-fold and ~4.5-fold, respectively. Surprisingly, this chronic increase in Ang-(1-7) did not alter basal blood pressure levels measured by telemetry. However, TGR(A1-7)3292 rats displayed a marked reduction in isoproterenol-induced heart hypertrophy and an improvement of postischemic systolic function.[54] Botelho-Santos and colleagues[57] showed changes in systemic and regional hemodynamic parameters in these rats, resulting in an increase of vascular conductance in several tissues and a decreased total peripheral resistance. Furthermore, the transgenic rats showed a significant increase in stroke volume and cardiac index[57] and a reduction in basal urinary flow, leading to increased urinary osmolality and osmolal clearance.[80] Furthermore, chronic elevation of circulating Ang-(1-7) levels considerably improved the lipid and glycolytic profile and lowered the fat mass accompanied by a decrease in triglycerides and cholesterol in plasma and an improved glucose tolerance and insulin sensitivity and reduced gluconeogenesis in the liver.[55,56]

Transgenic Mice and Rats Overexpressing Ang-(1-7) in the Heart

Transgenic mice carrying the Ang-(1-7)-releasing construct under the control of the alpha-cardiac myosin heavy-chain promoter exhibit about an eightfold increase of the peptide in the heart and show a normal basic cardiac function but are protected from hypertensive cardiac hypertrophy induced by Ang II infusion[58] but not from myocardial infarction.[81] Transgenic rats generated with the same construct showed a slightly improved resting cardiac function and were also protected from hypertrophy in this case induced by isoproterenol.[59]

CONCLUSIONS AND OUTLOOK

Transgenic and KO rodent models were pivotal for our understanding of the protective functions of the novel RAS axis, ACE2/Ang-(1-7)/Mas. Transgenic overexpression of the components of this axis in general led to ameliorated cardiac and vascular damage in disease states and to an improved metabolic profile. KO models for ACE2 and Mas, however, show aggravated cardiovascular pathologies and a metabolic-syndrome-like state. In particular, the local production of Ang-(1-7) in the vascular wall, in the heart, and in the brain was found to be of high physiological relevance by the use of transgenic animals overexpressing ACE2 or Ang-(1-7) in these tissues. Inducible and cell-type-specific KO models for Mas and ACE2 will be helpful in the future to deepen our understanding of the ACE2/Ang-(1-7)/Mas axis.

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