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REVIEW

Porcine models for studying complications and organ crosstalk in diabetes mellitus

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Abstract
The worldwide prevalence of diabetes mellitus and obesity is rapidly increasing not only in adults but also in children and adolescents. Diabetes is associated with macrovascular complications increasing the risk for cardiovascular disease and stroke, as well as microvascular complications leading to diabetic nephropathy, retinopathy and neuropathy. Animal models are essential for studying disease mechanisms and for developing and testing diagnostic procedures and therapeutic strategies. Rodent models are most widely used but have limitations in translational research. Porcine models have the potential to bridge the gap between basic studies and clinical trials in human patients. This article provides an overview of concepts for the development of porcine models for diabetes and obesity research, with a focus on genetically engineered models. Diabetes-associated ocular, cardiovascular and renal alterations observed in diabetic pig models are summarized and their similarities with complications in diabetic patients are discussed. Systematic multi-organ biobanking of porcine models of diabetes and obesity and molecular profiling of representative tissue samples on different levels, e.g., on the transcriptome, proteome, or metabolome level, is proposed as a strategy for discovering tissue-specific pathomechanisms and their molecular key drivers using systems biology tools. This is exemplified by a recent study providing multi-omics insights into functional changes of the liver in a transgenic pig model for insulin-deficient diabetes mellitus. Collectively, these approaches will provide a better understanding of organ crosstalk in diabetes mellitus and eventually reveal new molecular targets for the prevention, early diagnosis and treatment of diabetes mellitus and its associated complications.

Keywords Pig model · Diabetes · Complications · Retinopathy · Cardiomyopathy · Nephropathy · Organ crosstalk · Biobank · Multi-omics analysis · Obesity

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Introduction

Diabetes mellitus (DM) is a complex metabolic disease with a worldwide prevalence of around 425 million people aged 20–79 years in 2017 that is expected to increase by almost 50% to 629 million people in 2045 (International Diabetes Federation 2017). Concomitantly, the number of overweight and obese adults is rising with a total of 604 million adults categorized as obese in 2015 (Afshin et al. 2017). In addition, an increasing number of children and adolescents is affected by DM and/or obesity (108 million obese children in 2015) putting them at an increased risk for the development of chronic complications due to an expanded disease exposure (Afshin et al. 2017; International Diabetes Federation 2017; Lascar et al. 2018). Acute hyperglycemia may lead to life-threatening diabetic ketoacidosis while chronic hyperglycemia is associated with macrovascular complications, increasing the risk for myocardial infarction and stroke and microvascular complications leading to diabetic nephropathy, retinopathy and neuropathy (reviewed in Forbes and Cooper 2013). Although numerous treatment options exist for the different types of DM, the disease is progressive and associated with severe alterations in multiple tissues and organs. For instance, DM is the most prevalent cause of chronic kidney disease, leading to terminal renal failure (reviewed in Alicic et al. 2017). Providing insight into disease mechanisms of diabetic complications is thus among the highest priorities in national and international health research agendas.

To date, DM is classified into four categories (American Diabetes Association 2019): (i) type 1 diabetes, (ii) type 2 diabetes, (iii) gestational diabetes and (iv) other specific types of diabetes. Type 1 diabetes (T1D) has its average onset during childhood or adolescence and accounts for ~5–10% of DM cases. The underlying pathophysiology is a cell-mediated autoimmune destruction of the pancreatic beta cells, leading to an absolute insulin deficiency. The majority of DM cases, i.e., 90–95% fall into category 2. Type 2 diabetes (T2D) mainly affects people > 40 years of age and is associated with various degrees of peripheral insulin resistance accompanied by a progressive decline of beta cell function, leading to a relative rather than an absolute insulin deficiency. Category 3, gestational diabetes (GD), is diagnosed in the second or third trimester of pregnancy and shares similar characteristics with T2D. Both T2D and GD are frequently accompanied by obesity. Category 4 includes monogenic forms of DM affecting, e.g., insulin secretion or insulin action but also DM induced by diseases of the exocrine pancreas, e.g., cystic fibrosis or pancreatitis as well as DM resulting from drug administration, e.g., glucocorticoids (American Diabetes Association 2019).

Since the current DM classification is apparently not sufficient to stratify patients for their risk to convert from a prediabetic to a clinical diabetic state or to develop diabetes complications, attempts have been made to identify subphenotypes of prediabetes (Haring 2016) and of adult-onset diabetes (Ahlqvist et al. 2018). The latter study revealed five different subtypes characterized by defined metabolic parameters as well as risk for diabetes complications in four large study cohorts of newly diagnosed diabetes cases: (i) severe autoimmune diabetes (SAID), (ii) severe insulin-deficient diabetes (SIDD), (iii) severe insulin-resistant diabetes (SIRD), (iv) mild obesity-related diabetes (MOD) and (v) mild age-related diabetes (MARD). This refined classification was established to personalize diabetes treatment with existing therapies and novel treatment approaches.

Currently, available treatment options are not able to stop the progression of the disease or prevent the development of diabetes complications. To refine the current knowledge on disease mechanisms of DM and associated complications and to develop novel diagnostic and treatment strategies, animal models are of inestimable value. The pig is a favorable model organism due to its close similarity to humans in size, as well as in numerous anatomical and physiological aspects (reviewed in Renner et al. 2016b). This is particularly true for the islets of Langerhans, which show major differences in development, structure and function between humans and rodents (reviewed in Liu and Hebrok 2017 and Tritschler et al. 2017). In many aspects, such as islet size distribution, anatomy and vascularization, the proportion of beta cells in the islets and the spatial arrangement and interaction of the endocrine cell types, porcine islets are more similar to human islets than to mouse islets (Dufrane et al. 2005; Hoang et al. 2014). In line with these observations, RNA sequencing studies revealed that porcine and human beta and alpha cells share characteristic molecular and developmental features not observed in the corresponding mouse cells (Kim et al. 2019). Transgenic pigs expressing fluorescent reporter genes in the beta cells (Kemter et al. 2017; Matsunari et al. 2014) will help to improve the resolution of such analyses to the single-cell level.

This article provides an overview of current approaches for the development of porcine models for DM and obesity research, with a focus on genetically engineered models. Currently available models will be discussed, particularly in the context of their relevance for studies of complications and organ crosstalk in DM.

The pig as a model for diabetes and obesity research

Pigs are attractive animal models due to their similarities to humans in anatomy and metabolism. Compared with nonhuman primates, pigs have several important advantages: (i) greater ethical acceptance; (ii) favorable reproduction characteristics, allowing the generation of sufficiently large study groups in a reasonable time frame; (iii) well-established methods for genetic modification including CRISPR/Cas technology; and (iv) similar body dimensions compared to humans, e.g., providing sufficient amounts of blood/tissue material in a lower total number of animals required (3R principle) (reviewed in Renner et al. 2016b). Some of these advantages also hold true for dogs but genetic modification of dogs is far less developed. For diabetes research, a number of specific characteristics of pigs are particularly relevant.

Unlike rodents, pigs share the same circadian rhythm (diurnal) and eating behavior (discontinuously during the day) with humans. Moreover, the diet regimen and gastrointestinal structure of the omnivorous pig and human are similar (Renner et al. 2016b). Pigs have similar skin anatomy and pharmacokinetic characteristics following dermal drug dosing as in humans (Schneider and Wolf 2016). Unlike rodents, energy expenditure via thermogenesis does not depend on brown adipose tissue as pigs do not express uncoupling protein 1 (UCP1) (reviewed in Jastroch and Andersson 2015). Porcine insulin differs from human insulin in only one amino acid and was used for human treatment before it was replaced by recombinant human insulin. Furthermore, islet structure and vascularization, the proportions of endocrine cell types, and beta cell mass are very similar in pigs and humans (reviewed in Bakhti et al. 2019; Hoang et al. 2014; Renner et al. 2016b).

Moreover, the porcine cardiovascular system shows numerous relevant similarities to humans. Heart size, heart-to-body mass ratio, coronary anatomy and hemodynamic (e.g., cardiac output, respiratory rate) and electrophysiological (e.g., heart rate, electrocardiogram characteristics) parameters are more human-like in pigs compared to rodents (reviewed in Clauss et al. 2019). The same is true for characteristics relevant to atherosclerosis, i.e., lipid profile as well as plaque location, morphology and content. In pigs and humans, low-density lipoprotein (LDL) is the major circulating lipoprotein in plasma and both species are susceptible to diet-induced hypercholesterolemia. Lesions are predominantly located in the coronary arteries or terminal aorta and consist of various inflammatory cells, a lipid-rich necrotic core and a fibrous cap (Lee 1986).

Pig eyes share many similarities with human eyes, with a holangiotic retinal vasculature, absence of a tapetum, cone photoreceptors in the outer retina and macular area and similar retinal and scleral thickness (Middleton 2010).

Human and porcine kidneys are similar in size and are multilobular and multipapillary with similar vascularization patterns, whereas rodents have unilobular and unipapillary kidneys (reviewed in Giraud et al. 2011). The total number of nephrons, recognized as a pathogenetically relevant factor for the development of chronic kidney disease, is in the same order of magnitude in pigs ($1.6–4.6 \times 10^6$) and humans ($0.2–2.0 \times 10^6$) but approximately 100 times lower in rodents ($9–18 \times 10^3$ and $13–26 \times 10^3$ in mice and in rats respectively) (Blutke et al. 2016; Rieger et al. 2016; Wang and Garrett 2017).

Generation of porcine models for diabetes research

(Pre)diabetic pig models can be generated (i) surgically, by partial or total pancreatectomy; (ii) chemically, by treatment with selective beta cell toxic substances such as streptozotocin and alloxan; (iii) by dietary intervention, e.g., high-energy, high-fat, high-sugar diet; or (iv) by genetic modification. The choice of model depends on the research question, as no model matches all aspects of this complex metabolic disease. Each approach has advantages and disadvantages as outlined in Fig. 1.

Total pancreatectomy is commonly used as the diabetes model in preclinical islet transplantation research. The excised pancreas is often used for islet isolation, almost completely avoiding warm ischemia and thus resulting in improved islet quality for subsequent islet (auto)transplantation. Concomitant splenectomy can facilitate the complex surgical accessibility of the pig pancreas (Heinke et al. 2016). However, as pancreatectomy is a highly invasive approach, other methods for diabetes induction in the pig are preferred, e.g., for testing of novel compounds.

For chemical induction, streptozotocin (STZ, 2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose), a cytotoxic glucose analogue with alkylating properties that enters the cell via the glucose transporter 2 (GLUT2), is most widely used. Within the cell, the nitrosourea moiety of STZ is cleaved, inducing DNA damage via incorporation of alkyl groups. DNA damage induces activation of poly-ADP-ribose polymerases (PARP) for DNA repair, triggering NAD^+ depletion and production of nitric oxide and free radicals, which finally results in necrotic beta cell death (Lenzen 2008). Different protocols concerning dose and frequency of STZ application, such as single high-dose (e.g., 150 mg/kg BW) or multiple low-dose injections (e.g., 50 mg/kg BW), have been developed (Dufrane et al. 2006; Gerrity et al. 2001; Jensen-Waern et al. 2009). Application of nicotinamide prior to STZ injection has been used to reduce the severity of STZ-mediated beta cell destruction (Larsen et al. 2002; Lee et al. 2012). The low level of GLUT2 expression in porcine beta cells compared to other species like rats and primates (Dufrane et al. 2006), high variation in individual sensitivity to STZ treatment and a substantial degree of beta cell regeneration in young animals make STZ treatment a rather difficult approach to induce DM in the pig. A potential source of inter-individual variation in pigs in the response to STZ is polymorphism in *TCF7L2* (Tu et al. 2018), the most prominent type 2 diabetes susceptibility gene in humans (Grant 2019).

Dietary intervention provides numerous opportunities to modulate metabolism mimicking the Western lifestyle. In the pig, high-energy diets usually enriched with saturated fat of plant and/or animal origin and carbohydrates with a low glycemic index such as sucrose and/or fructose can provoke

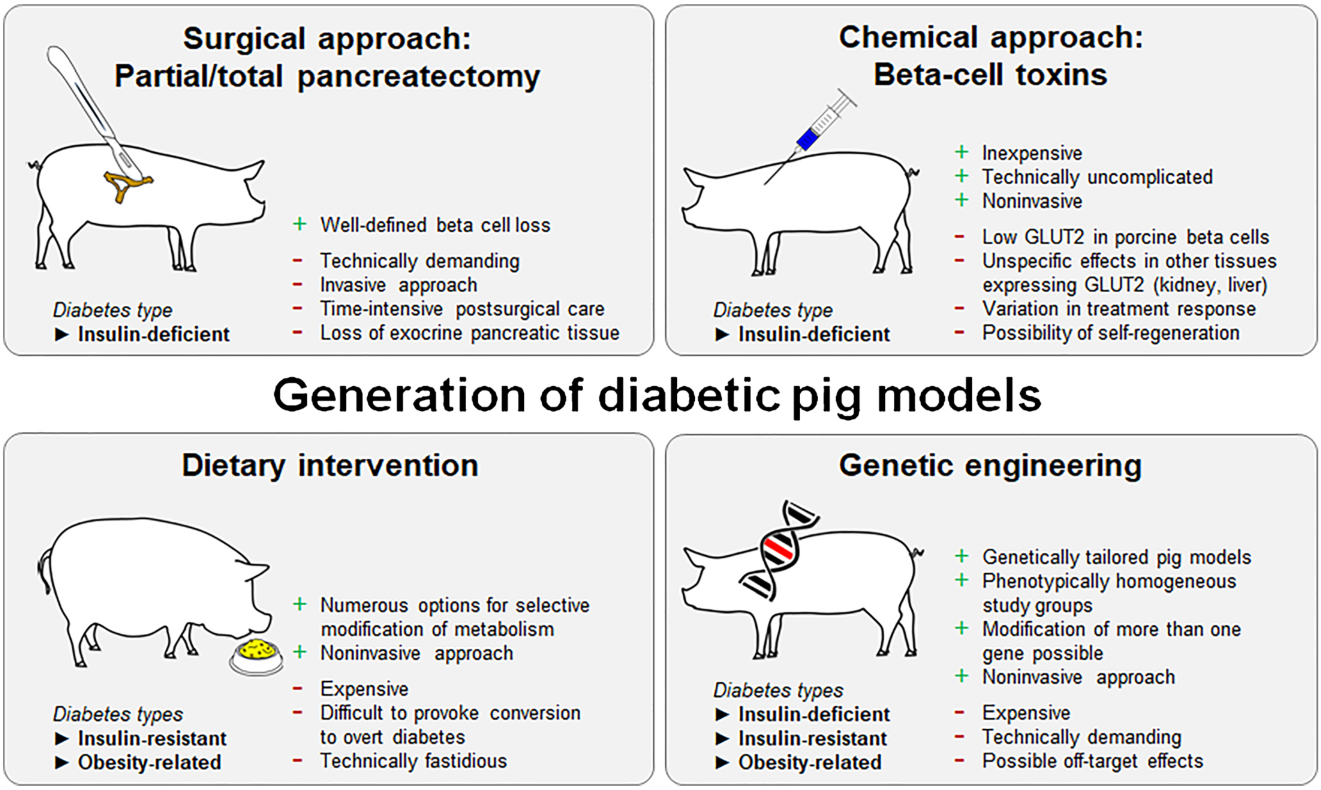

Fig. 1 Advantages and disadvantages of current approaches for the establishment of diabetic pig models and classification to diabetic sub-phenotypes (according to Ahlqvist et al. 2018)

characteristics of the metabolic syndrome, such as generalized obesity, dyslipidemia, reduced glucose tolerance and insulin resistance (reviewed in Renner et al. 2016b). Supplementation of pure cholesterol substantially exacerbates the dyslipidemic condition. However, dietary interventions alone rarely cause substantial hyperglycemia in the pig (Feng et al. 2015; Panasevich et al. 2018). The combination of high-energy diet regimens with STZ treatment to reduce beta cell mass can induce hyperglycemia (Koopmans et al. 2006). However, in addition to the obstacles with STZ treatment as described above, care must be taken that the animals do not become katabolic or ketotic (Koopmans et al. 2011; Ludvigsen et al. 2015a). Furthermore, the genetic background has a major influence on the response of pigs to dietary interventions. Under a high-fat diet, Ossabaw swine developed more features of metabolic syndrome and greater native as well as stent-induced coronary artery disease than Yucatan pigs, which are leaner and more resistant to metabolic syndrome and atherosclerosis (Neeb et al. 2010). After long-term feeding of high-fat and high-carbohydrate diet, combined with limited physical activity, Chinese Bama miniature pigs exhibited a higher incidence of increased fasting blood glucose and insulin levels, a decreased glucose disappearance rate and characteristic pathology especially in liver and skeletal muscle compared with the more resistant Duroc breed (Zhang et al. 2019).

Genetic engineering allows the generation of tailored porcine disease models. The first transgenic pigs were produced more than three decades ago by pronuclear DNA microinjection into fertilized oocytes (Brem et al. 1985; Hammer et al. 1985). This approach is limited by low efficiency and the inability to introduce targeted genetic modifications. Lentiviral vectors are highly efficient in transducing porcine oocytes and zygotes, resulting in a high proportion of transgenic piglets (Hofmann et al. 2003). This approach was used to generate transgenic pigs expressing a dominant-negative receptor for the incretin hormone glucose-dependent insulinotropic polypeptide (GIP) in beta cells, resulting in reduced glucose tolerance and insulin secretion and a progressive reduction of beta cell mass (Renner et al. 2010). Major drawbacks of lentiviral transgenesis are the limited size of the inserts and the potential for multiple independently segregating integration sites in the host genome. The latter problem may also occur when transposons are used for pig transgenesis (Garrels et al. 2011; Jakobsen et al. 2011). A major breakthrough was the establishment of somatic cell nuclear transfer (SCNT) in pigs, which provided for the first time a technological basis for introducing targeted genetic modifications (reviewed in Klymiuk et al. 2016; Kurome et al. 2015). The next important technological step offering new opportunities for genetic modification of pigs was gen(om)e editing (GE) using targeted nucleases. These induce site-specific DNA double-strand breaks, which are repaired by two major mechanisms: non-homologous end-joining (NHEJ) or homology-directed repair (HDR) (reviewed in Doetschman and Georgieva 2017). NHEJ often results in small insertions or deletions (indels), which may lead to a frameshift in the coding sequence, and loss of function of the affected gene. HDR with an exogenous donor template containing homology arms (DNA or single-strand oligodeoxynucleotides) corresponding to the target region can be used to insert specific point mutations or sequences at a desired locus (reviewed in Klymiuk et al. 2016). Three major classes of targeted nucleases have been successfully used for GE in pigs—zinc finger nucleases (ZFNs) (Hauschild et al. 2011; Whyte et al. 2011), transcription activator-like effector nucleases (TALENs) (Carlson et al. 2012) and most recently RNA-guided endonucleases (RGNs) derived from the bacterial clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system (Hai et al. 2014), which is continuously optimized to increase target site availability and target specificity thus avoiding off-target effects (reviewed in Pickar-Oliver and Gersbach 2019).

Genetically engineered pig models for diabetes and obesity research

The rapid progress in genetic engineering — along with the availability of porcine whole - genome sequences (Groenen 2016; Groenen et al. 2012; Warr et al. 2019) — has facilitated the generation of numerous tailored pig models for diabetes and obesity research (Table 1).

Genetically (pre)diabetic pig models

Expression of a dominant-negative glucose-dependent insulinotropic polypeptide receptor

Transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor (GIPR^{dn}) in beta cells mimic the impaired insulinotropic action of the incretin hormone GIP as observed in human type 2 diabetes patients (reviewed in Nauck and Meier 2016). Expression of the GIPR^{dn} that can still bind endogenous GIP but does not induce further signaling leads to reduced glucose tolerance and insulin secretion as well as reduced beta cell proliferation and mass (Renner et al. 2016a; Renner et al. 2010). As GIPR^{dn} transgenic pigs show deterioration of glucose control with increasing age, targeted metabolomics analyses of transgenic animals versus non-transgenic littermates revealed distinct changes of specific amino acids and lipid species that could also be found in humans, thereby representing possible biomarkers for disease progression (Renner et al. 2012). Chronic treatment of GIPR^{dn} transgenic pigs with a GLP1 receptor agonist revealed reduced food intake and body weight gain as well as improved glycemic control and insulin sensitivity (Streckel et al. 2015) but did not have a pro-proliferative effect on beta cells nor on alpha cells or acinus cells, which was reported for cellular and rodent models (reviewed in Renner et al. 2016a).

Expression of mutant insulin transgenes/modifications of the insulin gene

To date, more than 50 different mutations in the human *INS* gene have been described that — depending on their position — lead to permanent neonatal diabetes (PNDM), now termed mutant *INS* gene-induced diabetes of youth (MIDY), maturity-onset diabetes of the young (MODY) or hyper(pro-)insulinemic conditions (Liu et al. 2015; Stoy et al. 2010).

Two different pig models for permanent neonatal diabetes were established—transgenic pigs expressing the mutant insulin C94Y (Renner et al. 2013) or C93S (Renner et al. 2019) (Fig. 2a). Corresponding *INS*/*Ins2* mutations that disrupt the C(B7)-C(A7) interchain disulfide bond (C94Y mutation) or the C(A6)-C(A11) intrachain disulfide bond (C93S mutation) of the insulin molecule also exist in humans and in rodents, i.e., the widely used Akita mouse model (reviewed in Liu et al. 2015) for the C94Y mutation and the Munich *Ins2*^{C95S} mouse model for the C93S mutation (Herbach et al. 2007).

Expression of the mutant *INS*/*Ins2* leads to impaired trafficking of normal proinsulin by formation of high molecular weight complexes with misfolded (pro)insulin, accumulation of misfolded insulin in the endoplasmic reticulum (ER) and ER stress that finally triggers beta cell apoptosis (reviewed in Liu et al. 2015). Accordingly, *INS*^{C94Y} transgenic pigs are characterized by impaired insulin secretion with consequential hypoinsulinemia, increased fasting glucose levels and progressively decreasing beta cell mass as well as reduced growth (Renner et al. 2013). A recent study of the myocardium showed capillary rarefaction and reduced pericyte investment in 5-month-old *INS*^{C94Y} transgenic pigs compared to age-matched WT littermates (Hinkel et al. 2017). Moreover, the retina of *INS*^{C94Y} transgenic pigs (age 24 or 40 months) exhibits several DM-associated morphologic alterations (Kleinwort et al. 2017). Evaluation of the liver of *INS*^{C94Y} transgenic pigs compared to age-matched WT littermates revealed distinct characteristics of an insulin-deficient diabetes on a multi-omics level (Backman et al. 2019).

INS^{C93S} transgenic pigs show lower expression of the mutant insulin compared to *INS*^{C94Y} transgenic pigs (Fig. 2b) and therefore exhibit a milder PNDM phenotype with normal growth (Fig. 2c), mild fasting hyperglycemia and hypoinsulinemia (Fig. 2d, e), reduced glucose tolerance and very subtle beta cell loss (Renner et al. 2019).

Table 1 Genetically modified pig models relevant for diabetes research

Genetic modification	Regulatory sequence	Generation method	Pig background	Phenotypic characteristics	Reference(s)
Transgenic expression of a dominant-negative GIP-receptor (GIPR^{dn})	Rat *Ins2* promoter	Random integration, lentiviral GT	Domestic pig (Landrace-Swabian Hall)	Prediabetic phenotype; reduced glucose tolerance and insulin secretion, reduced beta-cell mass and beta cell proliferation	Renner et al. 2010
Transgenic expression of mutant insulin C94Y	Porcine *INS* promoter	Random integration, SCNT-mediated GT	Domestic pig (Landrace-Swabian Hall)	PNDM; insulin deficiency due to insulin secretion deficit and beta cell apoptosis; cataract, retinopathy, impaired myocardial function and regeneration	Hinkel et al. 2017; Kleinwort et al. 2017; Renner et al. 2013
Transgenic expression of mutant insulin C93S	Porcine *INS* promoter	Random integration + SCNT	Domestic pig (Landrace-Swabian Hall)	Mild PNDM; insulin deficiency due to insulin secretion deficit	Renner et al. 2019
KO of the insulin (*INS*) gene	Porcine *INS* promoter (*INS*)	CRISPR/Cas9 (NHEJ) in cells + SCNT	PWG micropig	Absolute insulin deficiency, severe hyperglycemia, death within 2 days after birth	Cho et al. 2018
Humanization of the porcine insulin sequence	Porcine *INS* promoter (*INS*)	CRISPR/Cas9 (HDR) in cells + SCNT	Bama minipig	Expression of human insulin, no abnormalities in pancreas observed	Yang et al. 2016
Transgenic expression of a dominant-negative mutant hepatic nuclear factor 1α (P291fsinsC)	Cytomegalovirus (CMV) immediate early gene enhancer and porcine *INS* promoter	Random integration + ICSI-mediated GT	Domestic pig (Landrace/Large White x Duroc)	Hyperglycemia, hypoinsulinemia, cataract, renal changes (nodular lesion), retinal changes (cotton-wool spots, hemorrhage, neovascular-membrane like structures)	Umeyama et al. 2017; Umeyama et al. 2009
KO of the neurogenin-3 (*NGN3*) gene	Porcine *NGN3* promoter (*NGN3*)	CRISPR/Cas9 (NHEJ) in cells + SCNT	Not specified	Complete loss of islet alpha and delta cells, severe reduction of beta cells, nonviable due to severe weight loss within the first days of life	Sheets et al. 2018
KO of the pancreatic and duodenal homeobox 1 (*PDX1*) gene	Porcine *PDX1* promoter (*PDX1*)	TALENs (NHEJ) in cells + SCNT	Not specified	Pancreatic agenesis, death within 2 days after birth	Kang et al. 2017
Transgenic expression of hairy and enhancer of split-1 (HES1)	Porcine *PDX1* promoter	Random integration + SCNT	Not specified	Pancreatic agenesis	Matsunari et al. 2013
Transgenic co-expression of • 11-β-Hydroxysteroid dehydrogenase 1 (11β-HSD1) • Human islet amyloid polypeptide (hIAPP) • C/EBP homologous protein (CHOP)	 Porcine apolipoprotein E (*APOE*) promoter Porcine *INS* promoter Porcine *INS* promoter	Random integration + SCNT	Wuzhishan miniature pig	Expression of 11β-HSD1 in liver and of hIAPP and CHOP in pancreatic beta cells; preliminary findings pointed to impaired beta cell development and apoptosis and increased hepatic cholesterol and triglyceride concentrations; further analyses needed to judge the value of the model	Kong et al. 2016
Beta cell specific expression of enhanced green fluorescent protein (eGFP)	Porcine *INS* promoter	Random integration + SCNT	Domestic pig (Landrace-Swabian Hall)	Expression of eGFP in pancreatic beta cells; eGFP expression with no effect on in vitro and in vivo beta cell function	Kemter et al. 2017
Beta cell specific expression of green fluorescent protein (Venus)	Mouse *Pdx1* promoter	Random integration, ICSI-mediated GT, SCNT	Not specified	Expression of Venus in pancreas, functional islets following transplantation under mouse kidney capsule	Matsunari et al. 2014
Transgenic expression of mutant human proprotein convertase subtilisin/kexin type 9 (PCSK9)	Human α1-antitrypsin (*AAT*) promoter + hepatocyte control region (HCR) of the human *APOE* gene	DNA transposition in cells + SCNT	Yucatan minipig	Hypercholesterolemia (VLDL and LDL cholesterol), reduced hepatic LDL cholesterol receptor levels, impaired LDL cholesterol clearance, arteriosclerotic lesions in aorta and iliofemoral arteries under HFHC diet	Al-Mashhadi et al. 2015; Al-Mashhadi et al. 2013

Table 1 (continued)

Genetic modification	Regulatory sequence	Generation method	Pig background	Phenotypic characteristics	Reference(s)
Transgenic expression of mutant chimpanzee PCSK9	Albumin (*ALB*) enhancer + human *AAT* promoter	DNA transposition in cells + SCNT	Ossabaw minipig Landrace domestic pig	Hypercholesterolemia (total and LDL cholesterol), decreased HDL cholesterol, hypertriglyceridemia, more pronounced arteriosclerotic lesions on Ossabaw background; more advanced lesions under HFHC diet	Yuan et al. 2018
KO of the low-density lipoprotein receptor (*LDLR*) gene	Porcine *LDLR* promoter (*LDLR*)	Gene targeting by rAAV-mediated HR in cells + SCNT	Yucatan minipig	Mild (LDLR$^{+/-}$) to severe (LDLR$^{-/-}$) hypercholesterolemia (elevated TC, LDL, VLDL, reduced HDL), arteriosclerotic lesions in abdominal aorta and coronary arteries	Davis et al. 2014
KO of the *LDLR* gene	Porcine *LDLR* promoter (*LDLR*)	Gene targeting (HR) in cells + SCNT	Domestic pig (Landrace × Large White crossbred pigs)	Severe (LDLR$^{-/-}$) hypercholesterolemia (elevated TC, LDL, VLDL, reduced HDL), arteriosclerotic lesions in abdominal aorta and coronary arteries	Li et al. 2016b
Transgenic expression of human apolipoprotein (a) (Apo (a))	*β-Actin* promoter + CMV enhancer	Random integration In cells + SCNT	Clawn miniature pig NIBS miniature pig	Increased plasma level of lipoprotein A	Ozawa et al. 2015; Shimatsu et al. 2016
Transgenic expression of human apolipoprotein C-III (Apo C-III)	Human Apo C-III promoter	Random integration in cells + SCNT	Undefined minipig line	Mild to moderate plasma hypertriglyceridemia, delayed clearance of plasma triglyceride; reduced lipoprotein lipase activity	Wei et al. 2012
Transgenic expression of lipoprotein-associated phospholipase A2 (Lp-PLA2)	Elongation factor 1 alpha (*EF1α*) promoter	Random integration in cells + SCNT	Beijing experimental miniature pig	Elevated plasma triglyceride levels in the postprandial state, elevated expression of inflammatory genes in peripheral blood mononuclear cells	Tang et al. 2015
Transgenic expression of cholesteryl ester transfer protein (CETP)	Human *Apo C-III* promoter	Random integration + SCNT	Not specified	Mild elevation of LDL- and reduction of HDL cholesterol	Chen et al. 2017a
Expression of murine uncoupling protein 1 (UCP1)	*Adiponectin* promoter	CRISPR/Cas9 (targeted KI into porcine *UCP1* locus) in cells + SCNT	Domestic pig	Improved thermoregulation, reduced fat deposition, increased lipolysis	Zheng et al. 2017
KO of the growth hormone receptor (*GHR*) gene	Porcine *GHR* (*GHR*)	CRISPR/Cas9 (NHEJ) in cells + SCNT	Domestic pig (Landrace-Swabian Hall)	Growth retardation, reduced serum insulin-like growth factor 1 (IGF1) levels and IGF-binding protein 3 (IGFBP3) activity, increased IGFBP2 and serum GH concentrations, increased total body fat and decreased muscle to fat ratio	Hinrichs et al. 2018
Transgenic expression of phosphoenolpyruvate carboxykinase-cytosolic form (PEPCK-C)	*α-Skeletal-actin* promoter	Random integration in cells + SCNT	Chinese-Tibetan minipig	Increased ectopic fat storage and fatty acid content in skeletal muscle	Ren et al. 2017

GT gene transfer, *SCNT* somatic cell nuclear transfer, *KO* knockout, *KI* knockin, *HFHC* high-fat high-cholesterol, *NHEJ* non-homologous end-joining, *HDR* homology-directed repair, *HR* homologous recombination, *TC* total cholesterol, *NIBS* Nippon Institute for Biological Sciences

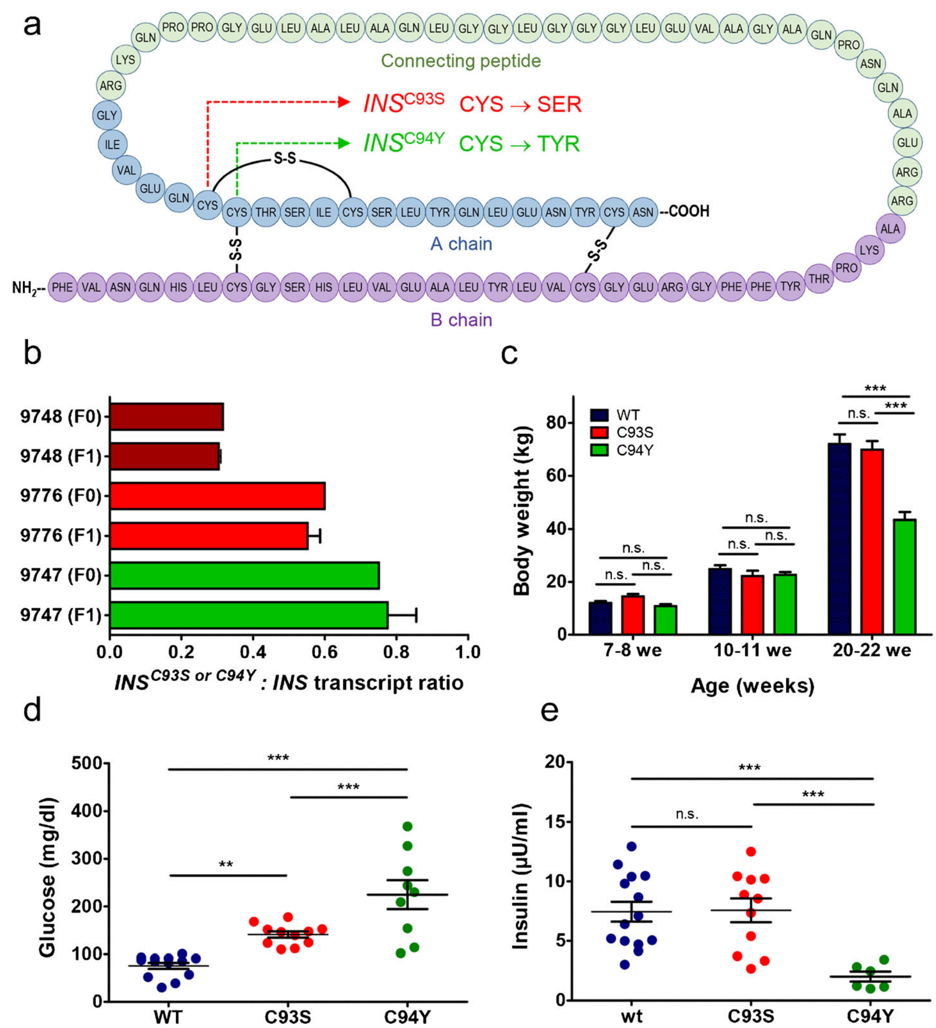

Fig. 2 Phenotypic differences of transgenic pigs expressing the mutant insulin C93S or C94Y. **a** Amino acid sequence of porcine insulin, showing the C93S and C94Y mutations targeting the intrachain and interchain disulfide bonds, respectively. **b** Quantification of *INS*^{C93S} or *INS*^{C94Y} and wild-type *INS* transcripts in pancreatic tissue of *INS*^{C93S} or *INS*^{C94Y} transgenic founders (F0) and F1-offspring by next generation sequencing of RT-PCR amplicons. *INS*^{C94Y} transgenic pigs show at least a 1.4-fold higher expression of the mutant insulin compared to *INS*^{C93S} transgenic pigs. **c** Body weight development of *INS*^{C94Y} and *INS*^{C93S} transgenic pigs compared to non-transgenic controls (WT). **d**, **e** Fasting glucose (**d**) and insulin (**e**) concentrations of *INS*^{C93S} and *INS*^{C94Y} transgenic pigs compared to non-transgenic controls (WT) at 4–5 months of age. Data are means ± SEM; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. not significant

Different lines of *INS* knockout pigs have been generated using the CRISPR/Cas9 system exhibiting absolute insulin deficiency due to bi-allelic nucleotide deletions or insertions (Cho et al. 2018). All founder piglets died shortly after birth so that the model is currently not available for further use.

To prevent possible anti-porcine insulin antibody production (Clark et al. 1982) in the context of islet xenotransplantation, pigs expressing exclusively human insulin have been generated using TALENs or CRISPR/Cas9 technology with single-stranded oligonucleotides (ssODNs) as homology donors for homology-directed repair (Yang et al. 2016). Bi-allelic modification resulting in replacement of alanine by threonine at position B30 was only achieved with CRISPR/Cas9. Pancreases from these “humanized” *INS* pigs did not show any abnormalities (Yang et al. 2016).

Expression of a dominant-negative mutant hepatocyte nuclear factor 1-alpha

In humans, mutations of the HNF1 homeobox A alias hepatocyte nuclear factor 1-alpha (*HNF1A*) gene are inherited in an autosomal-dominant fashion and are known to cause maturity-onset diabetes of the young type 3 (MODY3). MODY3 is characterized by an early-onset (< 25 years of age) hyperglycemia, impaired insulin secretion with little or no effect on insulin action, absence of obesity and no need for insulin treatment. Expression of mutant human *HNF1A*^{P291fsinsC} under control of cytomegalovirus immediate-early gene enhancer and porcine insulin promoter sequences in transgenic pigs resulted in early-onset hyperglycemia (2 weeks of age) due to reduced insulin secretion (Umeyama et al. 2009). In addition, kidney and ocular lesions (see below) were observed in the *HNF1A*$^{\mathrm{P291fsinsC}}$ transgenic pigs (Hara et al. 2014; Umeyama et al. 2017).

Disruption of endocrine cell development in the pancreas

Pancreas development in mammals is guided by a cascade of tightly regulated transcription factors (reviewed in Liu and Hebrok 2017; Tritschler et al. 2017).

The basic helix-loop-helix transcription factor neurogenin-3 (NGN3) is important for the development of the endocrine cells of the islets of Langerhans. Its expression is tightly regulated and occurs in two successive waves during pancreas development in mice, that is, during the primary and secondary transitions of endocrine cell development (Rukstalis and Habener 2009) but in only one wave in humans (Jennings et al. 2013). Studies of pancreas development in the pig revealed similar transcription factor activation and beta cell transcriptomes to humans (Kim et al. 2019). However, more detailed studies evaluating the exact timing of transcriptional control during pancreas development as a whole are needed in pigs and humans. NGN3 is temporally involved in the determination of differentiation into the four distinct endocrine cell lineages of the islets. Either disrupted or forced expression of *Ngn3* early in mouse pancreas development abrogates the formation of islets (Rukstalis and Habener 2009). Sheets et al. (2018) inactivated the *NGN3* gene in pigs using CRISPR/Cas technology. At mid-gestation (day 60), NGN3 deficient pig fetuses exhibited a loss of expression of the putative NGN3-downstream target genes *NEUROD1* and *PAX4* and lacked the islet cell hormones insulin, glucagon, somatostatin and pancreatic polypeptide. NGN3 deficient piglets were born alive but had to be euthanized within the first 2 days of life due to extreme weight loss. In their pancreas, alpha cells and delta cells were completely missing and the number of beta cells was highly reduced, underlining the important role of NGN3 in porcine endocrine pancreas development.

Besides NGN3, pancreatic and duodenal homeobox 1 (PDX1) is another transcription factor essential for the regulation of endocrine pancreas development, beta cell differentiation and maintenance of mature beta cell function (reviewed in Liu and Hebrok 2017; Tritschler et al. 2017). PDX1 deficiency leads to pancreatic agenesis in mice (Jonsson et al. 1994) and humans (Stoffers et al. 1997). Similarly, PDX1-deficient pigs generated by Kang et al. (2017) using TALEN-driven gene editing exhibited pancreatic agenesis, whereas their gastrointestinal system and all other internal organs appeared normal. These piglets were born alive but died within the first 2 days.

A similar apancreatic phenotype was observed in transgenic pigs expressing a *PDX1*-hairy and enhancer of split 1 (*HES1*) transgene (Matsunari et al. 2013). *HES1*, a target gene of the Notch signaling pathway, acts as a transcriptional repressor of genes such as *NGN3* (Sumazaki et al. 2004).

While pancreatogenesis disabled pig strains are — due to their early postnatal lethality—of limited value for diabetes research, they may be valuable as hosts for the generation of human pancreas tissue by embryo or organ complementation with human stem cells (discussed in Suchy and Nakauchi 2018).

Multitransgenic approach to model T2D in pigs

Kong et al. (2016) aimed to generate a multitransgenic T2D model on a Wuzhishan minipig background by (i) liver-specific overexpression of 11-beta-hydroxysteroid dehydrogenase 1 to induce hepatic insulin resistance; (ii) beta cell specific expression of human islet amyloid polypeptide (hIAPP) to facilitate amyloid deposition that does not occur with porcine IAPP; and (iii) beta cell specific expression of C/EBP homologous protein (CHOP), a key regulator of endoplasmic reticulum stress-induced apoptosis to induce beta cell damage/apoptosis. Evaluation of piglets (one stillborn and one 8-day-old live piglet) revealed expression of all three transgenes. Preliminary findings suggested impaired beta cell development and apoptosis and increased hepatic cholesterol and triglyceride concentrations in the transgenic piglet. More detailed analyses in a larger number of animals are pending.

Pigs expressing reporter genes for studies of beta cell development and function

For studies of porcine beta cell maturation in vitro and in vivo, transgenic pigs expressing enhanced green fluorescent protein (eGFP) under control of the porcine *INS* gene promoter were generated (Kemter et al. 2017). Expression of eGFP in the beta cells did not have any negative effects on insulin secretion as examined by static glucose-stimulated insulin secretion (GSIS) assays of isolated islets. Furthermore, after intramuscular transplantation *INS*-eGFP transgenic islets were equally potent as wild-type islets to restore normoglycemia in STZ-induced diabetic mice. Longitudinal in vivo imaging of neonatal *INS*-eGFP transgenic islets over 16 weeks after transplantation into the anterior chamber of the mouse eye revealed an increase in granulation, eGFP-positive fraction and islet vessel fraction, demonstrating the potential of this system for monitoring beta cell and islet maturation in vivo (Kemter et al. 2017). Moreover, eGFP expressing beta cells can be recovered by fluorescence-activated cell sorting and processed for omics analyses like single-cell RNA sequencing. Systematic analyses of beta cells derived from different pre- and postnatal stages may provide new molecular insights into porcine beta cell development and eventually reveal new markers and strategies to improve the maturation of neonatal porcine islets and to assess the quality of islet products for xenotransplantation (discussed in Kemter et al. 2018; Kemter and Wolf 2018).

Matsunari et al. (2014) generated transgenic pigs expressing the GFP variant Venus under the control of the murine *Pdx1* promoter. *Pdx1*-Venus transgenic pigs developed normally and had blood glucose and insulin levels within the normal ranges, indicating that the reporter transgene did not impair beta cell function. While in the fetal pancreas the expression of the *Pdx1*-Venus transgene was observed in acinar cells, it was restricted to the beta cells in postnatal specimens. Like *INS*-eGFP transgenic pigs (Kemter et al. 2017), *Pdx1*-Venus transgenic pigs are an interesting model for studies of pancreas development and regeneration and for islet (xeno)transplantation.

Pig models with dyslipidemia

Dyslipidemia, characterized by high plasma triglyceride levels, low high-density lipoprotein (HDL)-cholesterol and elevated low-density lipoprotein (LDL)-cholesterol concentrations, results from increased fatty acid fluxes as a consequence of insulin resistance and/or insulin deficiency. Dyslipidemia is a hallmark of the metabolic syndrome and a major risk factor for the development of atherosclerosis and cardiovascular disease in diabetic patients (reviewed in Mooradian 2009). Several genetically modified pig models display dyslipidemia.

Expression of the gain-of-function mutant proprotein convertase subtilisin/kexin type 9

Al-Mashhadi et al. (2013) generated transgenic Yucatan minipigs overexpressing the human gain-of-function mutation D374Y of the human proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene under control of the human α1-antitrypsin (*SERPINA1*) promoter and a hepatocyte control region (HCR) from the human apolipoprotein E (*APOE*) gene. PCSK9 plays a crucial role in the regulation of LDL receptor (LDLR) recycling. The PCSK9 complex binds LDLR, leading to its internalization, lysosomal degradation and consequently reduced clearance of circulating LDL cholesterol. Patients with loss-of-function mutations of *PCSK9* reveal very low plasma concentrations of LDL cholesterol, associated with a markedly reduced risk of atherosclerotic cardiovascular disease, while gain-of-function mutations of *PCSK9* are a cause of autosomal-dominant hypercholesterolemia (reviewed in Shapiro et al. 2018). On a standard diet, $PCSK9^{D374Y}$ transgenic pigs showed—depending on the level of $PCSK9^{D374Y}$ expression—elevated plasma concentrations of very low-density lipoprotein (VLDL)- and LDL cholesterol that increased further when the animals were fed a high-fat-high-cholesterol (HFHC) diet. After 46 weeks on the HFHC diet, $PCSK9^{D374Y}$ transgenic pigs showed accelerated progressive atherosclerotic lesions in the aorta and iliofemoral arteries compared to wild-type animals. Interestingly, diabetes induction by STZ did not aggravate the atherosclerotic burden or severity of the lesions (Al-Mashhadi et al. 2015). Ossabaw pigs expressing chimpanzee $PCSK9^{D374Y}$ in the liver showed more pronounced atherosclerotic lesions compared to landrace pigs with the same transgene (Yuan et al. 2018), demonstrating the additive effect of the genetic modification with a susceptible genetic background. Moreover, $PCSK9^{D374Y}$ transgenic Ossabaw pigs receiving a standard diet had up to 2-fold higher plasma lipids than wild-type Ossabaw pigs on a HFHC diet, associated with accelerated development of atherosclerosis. HFHC diet augmented the development of dyslipidemia and atherosclerosis in $PCSK9^{D374Y}$ transgenic Ossabaw pigs compared to standard diet.

Knockout of the low-density lipoprotein receptor gene

Loss-of-function mutations of the *LDLR* gene in humans (reviewed in Burnett and Hooper 2008) result in familial hypercholesterolemia and an increased risk for atherosclerosis and cardiovascular disease (Susan-Resiga et al. 2017). A spontaneous *LDLR* mutation (R84C), which affects the binding affinity of the receptor and leads to hypercholesterolemia and atherosclerotic lesions, has also been described in large domestic pigs (Hasler-Rapacz et al. 1998; Rapacz et al. 1986) but the phenotypic variability and the large size of adult animals limit its value as an animal model. Davis et al. (2014) generated Yucatan minipigs with a mono- or bi-allelic knockout of the *LDLR* gene using recombinant adeno-associated virus (rAAV) assisted gene targeting and SCNT. On a standard diet, $LDLR^{+/-}$ pigs revealed mild and $LDLR^{-/-}$ pigs severe hypercholesterolemia with increased levels of LDL- and VLDL cholesterol but decreased levels of HDL cholesterol already at birth prior to first colostrum uptake. While plasma concentrations of total cholesterol, LDL- and HDL cholesterol remained stable, the increase in VLDL cholesterol concentrations in $LDLR^{-/-}$ pigs continued from 6-fold at birth to 50-fold at 6 months compared with age-matched controls. Atherosclerotic lesions were observed in the coronary arteries and were more pronounced in the abdominal aorta of $LDLR^{-/-}$ pigs at 7 months of age. After feeding a HFHC diet for 90 and 180 days, elevations of plasma total LDL- and VLDL cholesterol in $LDLR^{+/-}$ and specifically in $LDLR^{-/-}$ pigs were more pronounced compared to standard diet feeding. Additionally, $LDLR^{-/-}$ pigs showed extensive atherosclerotic lesions in the abdominal aorta following 90 days HFHC feeding and coronary arteries with complicated human-like plaques were observed following 180 days HFHC feeding. $LDLR^{-/-}$ pigs on a domestic pig background generated by conventional gene targeting (deletion of exon 4) and SCNT (Li et al. 2016b) showed a similar phenotype but developed more complex atherosclerotic lesions at an earlier age compared with $LDLR^{-/-}$ Yucatan pigs. Statin treatment of $LDLR^{-/-}$ domestic pigs fed a HFHC diet resulted in reduced atherosclerotic plaque burden and severity, although plasma cholesterol concentrations were not reduced (Li et al. 2016b). In contrast, statin treatment of $LDLR^{+/-}$ Yucatan minipigs, which received a high-fat diet, was capable to reduce total and HDL cholesterol but not LDL cholesterol concentrations (Amuzie et al. 2016).

Overexpression of apolipoproteins

Apolipoproteins play crucial roles in lipoprotein metabolism by (i) serving as structural components, (ii) acting as ligands for lipoprotein receptors, (iii) guiding the formation of lipoproteins and (iv) serving as activators or inhibitors of enzymes involved in the metabolism of lipoproteins (reviewed in Feingold and Grunfeld 2018).

Apolipoprotein (a) [Apo (a)] is synthesized in the liver and is only found in humans and nonhuman primates. Apo (a) is linked to apolipoprotein B-100 (Apo B-100) via a disulfide bond and is part of lipoprotein (a), an LDL-like particle produced in the liver (Kronenberg and Utermann 2013). Increased plasma concentrations of lipoprotein (a) are linked to an increased risk for coronary heart disease (Saleheen et al. 2017). Transgenic pigs (two different minipig strains) expressing human Apo (a) under the control of an ubiquitously active promoter (Ozawa et al. 2015; Shimatsu et al. 2016) revealed increased plasma concentrations of lipoprotein (a) while total cholesterol, triglyceride, LDL and HDL concentrations were unaltered compared to non-transgenic controls. The effects of human Apo (a) expression on the susceptibility to atherosclerosis in these pigs has not yet been evaluated.

Apolipoprotein C-III (Apo C-III) is an inhibitor of lipoprotein lipase (LPL) and inhibits the interaction of triglyceride-rich lipoproteins with their receptors. Loss-of-function mutations in *APOC3* lead to decreases in serum triglyceride levels and to a reduced risk of cardiovascular disease even in LPL deficient patients (reviewed in Feingold and Grunfeld 2018), whereas increased Apo C-III production was found in patients with hypertriglyceridemia (Cohn et al. 2004). Wei et al. (2012) generated a transgenic minipig model overexpressing human Apo C-III in liver and intestine. The plasma triglyceride levels in these pigs were up to 3-fold elevated in the fasting and postprandial state as well as during an oral fat-load test with no effect on total cholesterol and HDL levels, thus representing the most frequently observed mild to moderate hypertriglyceridemia in humans.

Overexpression of lipoprotein-associated phospholipase A2

Lipoprotein-associated phospholipase A2 (Lp-PLA$_2$) is produced by inflammatory cells, associates with circulating LDL and hydrolyzes oxidized phospholipids in LDL, thus generating bioactive pro-inflammatory lipid mediators that promote atherogenesis (reviewed in Zalewski and Macphee 2005). Transgenic pigs overexpressing Lp-PLA$_2$ under the control of the elongation factor 1 alpha (EF1α) promoter revealed elevated triglyceride levels in the postprandial state but no change in total cholesterol and cholesterol subfractions in the fasting or fed state. Interestingly, in peripheral blood mononuclear cells (PBMCs), the expression of pro-inflammatory genes such as interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor-α (TNF-α) was increased in Lp-PLA$_2$ transgenic vs. control pigs (Tang et al. 2015).

Expression of human cholesteryl ester transfer protein

Cholesteryl ester transfer protein (CETP) promotes the bidirectional transfer of cholesteryl esters and triglyceride between all plasma lipoprotein particles (Barter et al. 1982). Attempts have been made to reduce cardiovascular risk by inhibiting CETP and thereby increase HDL cholesterol (Barter and Rye 2012). Like rodents, pigs lack CETP prohibiting evaluation of CETP inhibition in these species (Barter et al. 1982). Chen et al. (2017a) thus generated transgenic pigs expressing human CETP, which revealed a mild elevation of LDL cholesterol and reduced HDL cholesterol levels.

Pig models with altered adipose tissue homeostasis

In addition to genetic attempts to generate dyslipidemic pig models, several pig models with altered adipose tissue homeostasis have been generated.

Expression of uncoupling protein 1

Uncoupling protein 1 (UCP1) expression is a hallmark of brown adipose tissue (BAT) and is localized in the inner mitochondrial membrane. It uncouples ATP synthesis from proton transit across the inner mitochondrial membrane and is therefore responsible for thermogenesis not requiring muscle activity, i.e., non-shivering thermogenesis. Activation of BAT in humans is associated with improved metabolic parameters such as insulin sensitivity and has therefore been discussed as a potential treatment for obesity and diabetes (reviewed in Betz and Enerback 2018). In contrast to humans, pigs lack UCP1 (Hou et al. 2017; Lin et al. 2017; McGaugh and Schwartz 2017) and functional BAT. Zheng et al. (2017) restored UCP1 expression in pigs by inserting an expression cassette with murine *Ucp1* under the control of an adiponectin promoter into the mutated porcine *UCP1* locus, using CRISPR/Cas technology. The resulting *UCP1* knockin (KI) pigs showed improved thermogenesis and thermoregulation during a 4-h cold exposure, consistent with increased mitochondrial respiratory capacity in differentiated adipocytes. Physical activity, body weight gain, daily energy expenditure and food conversion ratio were not altered. However, the proportion of adipose tissue was significantly reduced, associated with signs of increased lipolysis (increased free fatty acid and decreased triglyceride concentrations in plasma, increased protein levels of phosphohormone-sensitive lipase and adipose triglyceride lipase in adipose tissue (Zheng et al. 2017). Thus, *UCP1* KI pigs are an interesting model for further studies of UCP1 effects on metabolic control in the context of diabetes and obesity.

Growth hormone receptor deficiency

Laron syndrome is a rare autosomal recessive disorder caused by loss-of-function mutations in the growth hormone receptor gene (*GHR*). The most prominent characteristics of affected individuals are short stature and obesity. Nevertheless, Laron syndrome patients reveal a reduced incidence of type 2 diabetes mellitus and of malignancies (Guevara-Aguirre et al. 2011), making them an interesting cohort for diabetes and cancer research.

GHR-deficient (*GHR* KO) pigs were generated as a large animal model for the human Laron syndrome (Hinrichs et al. 2018), revealing markedly reduced serum insulin-like growth factor 1 (IGF1) levels and reduced IGF-binding protein 3 (IGFBP3) activity but increased IGFBP2 levels. Serum GH concentrations were significantly elevated compared with control pigs. *GHR* KO pigs had a normal birth weight. Growth retardation became significant at the age of 5 weeks. At the age of 6 months, the body weight of *GHR* KO pigs was reduced by 60% compared with controls. In agreement with observations in patients with Laron syndrome (Laron et al. 2006) and *Ghr* KO mice (Berryman et al. 2010), *GHR* KO pigs displayed an increase in total body fat and a decrease in the muscle to fat ratio. The physiological adipocyte lipid turnover may be disturbed at different levels. First, the lipolytic action of GH via an increase in adipose tissue hormone-sensitive lipase (HSL) activity (reviewed in List et al. 2011; Vijayakumar et al. 2010) is lost in the absence of GHR. Second, the synthesis of storage lipids in adipocytes is likely to increase in *GHR* KO pigs. This phenomenon requires hydrolysis by LPL of the triglyceride component of circulating chylomicrons and VLDL into free fatty acids and 2-monoacylglycerol, which can be taken up by adipocytes. Adipose tissue LPL activity is increased by insulin but inhibited by GH (reviewed in Wang and Eckel 2009). In addition to the role of GH in adipose tissue homeostasis, *GHR* KO pigs are an interesting model for studying the causes of juvenile hypoglycemia in patients with Laron syndrome (Hinrichs et al. 2018).

Ectopic expression of the cytosolic form of phosphoenolpyruvate carboxykinase

In an attempt to increase the intramuscular fat content for high-quality pork production, Ren et al. (2017) generated Chinese-Tibetan minipigs overexpressing the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C) under the control of a porcine alpha-skeletal-actin gene promoter. Indeed, PEPCK-C^{mus} transgenic pigs revealed increased ectopic fat storage and fatty acid content in the skeletal muscle. As ectopic fat storage, i.e., fat storage in non-classical fat stores, has negative metabolic effects and plays a role in the pathogenesis of type 2 diabetes and cardiovascular diseases, PEPCK-C^{mus} transgenic pigs may be an interesting model for diabetes research but a detailed metabolic characterization is pending (Ren et al. 2017).

Diabetes-associated alterations in porcine models

Ocular complications

Diabetes mellitus can lead to pathologies in many structures of the eye, with both a systemic chronic metabolic disease and a microangiopathic character (Kiziltoprak et al. 2019). Cataract is one of the major causes of visual impairment in diabetic patients (Drinkwater et al. 2019). Diabetic cataracts develop because the enzyme aldose reductase catalyzes the reduction of glucose into sorbitol, which accumulates intracellularly and leads to osmotic changes resulting in hydropic lens fibers that degenerate and form sugar cataracts. In addition, osmotic stress in the lens induces apoptosis of lens epithelial cells leading to the development of cataracts (reviewed in Pollreisz and Schmidt-Erfurth 2010).

INS^{C94Y} transgenic pigs develop cataracts within the first week of age (Renner et al. 2013), rapidly progressing to mature cataracts with completely opaque lenses (Fig. 3a, b) (Kleinwort et al. 2017). In *HNF1A*^{P291fsinsC} transgenic pigs, cataracts were observed from 2 months of age (Umeyama et al. 2017). Histopathologic examination of lenses from 4.5-month-old pigs revealed vacuolization of the lens fibers in the equatorial region of the lens and swelling of the lens fibers in the posterior pole of the lens. In addition, nucleated cells were found at the posterior pole of some lenses (Umeyama et al. 2017). STZ-induced diabetic or galactose-fed rats are currently the most studied animal models for cataracts and rapidly develop cataracts (Chen et al. 2017b). The pig will probably be a good model to test novel therapeutics to prevent diabetic cataracts or to test novel treatment strategies, because the anatomy and visual ability of their eyes is very similar to humans.

Retinal alterations reported in *HNF1A*$^{\mathrm{P291fsinsC}}$ transgenic pigs from an age of 4 months included retinal hemorrhage and cotton-wool spots, which are early signs of diabetic retinopathy (Umeyama et al. 2017). Because of the early development of cataracts, the onset of retinal changes in *INS*$^{\mathrm{C94Y}}$ transgenic pigs is unclear but after 2 years of hyperglycemia, there were profound changes in the retina (Kleinwort et al. 2017). These changes included several severe features of diabetic retinopathy in humans that to our knowledge are not found in this combination in other animal models of DM (Olivares et al. 2017). There was a substantial, selective thickening of the nerve fiber layer (Fig. 3c–h). These findings are different from observations in a porcine diabetic-hypercholesterolemic (DMHC) pig model, i.e., STZ-induced diabetic pigs that were fed a hyperlipidemic diet, where a thinning of retinal layers was reported (Acharya et al. 2017). Both findings were recently described in humans (Marques et al. 2019). In the same retinopathy severity group, retinal thinning (neurodegeneration) or retinal thickening (edema) were detectable with similar prevalence (Marques et al. 2019). Therefore, both porcine models are useful to study different courses of disease and probably to aid stratification of different disease pathways in the future.

Additionally, retinal Müller glial cells change their phenotype in *INS*$^{\mathrm{C94Y}}$ transgenic pigs, especially in the endfeet (Fig. 3g–j). An increase of glial fibrillary acidic protein (GFAP) expression, a feature already described in patients with diabetic retinopathy (Mizutani et al. 1998), was also detected in the DMHC pig model (Acharya et al. 2017). Vascular complications of DM are the most serious manifestation of the disease in humans (Garside et al. 2019; Rask-Madsen and King 2013). Pigs were shown to have a retinal vasculature that closely resembles that of man (Bloodworth et al. 1965). *INS*$^{\mathrm{C94Y}}$ transgenic pigs show typical intraretinal microvascular abnormalities (IRMAs) of diabetic retinopathy patients (Kleinwort et al. 2017; Rask-Madsen and King 2013), which is different from an alloxan-induced diabetic Yucatan miniature pig model (Hainsworth et al. 2002). In the latter model, significant thickening of retinal capillary basement membranes but no microaneurysms were detected (Hainsworth et al. 2002). In *INS*$^{\mathrm{C94Y}}$ transgenic pigs, retinal vessels changed in their diameters, in the composition of their walls and in their courses (Kleinwort et al. 2017) (Fig. 3k, l). Studies with isolated retinal arterioles of STZ-induced diabetic pigs provided evidence for mechanisms that were impaired after 2 weeks of hyperglycemia, like endothelium-dependent nitric oxide-mediated dilation of the arterioles (Hein et al. 2012). Furthermore, enhanced vasoconstriction in retinal venules was observed under hyperglycemic conditions in STZ-induced diabetic pigs, which could be prevented by blockade of the reverse-mode sodium-calcium exchanger in vitro (Chen et al. 2019).

Galactose-fed dogs were shown to develop diabetes-like retinal vessel changes associated with both the early and moderately advanced stages of retinopathy in man. However, severe retinopathy takes 3–5 years to develop in this model, accompanied by occasional intra-retinal neovascularization (Robinson et al. 2012). Moreover, because of high-maintenance requirements and ethical concerns, dogs are less frequently used as models (Robinson et al. 2012).

A further severe complication of DM is proliferative retinopathy, which progresses from wider retinal venular calibers and subsequent lower fractal dimensions (Broe 2015; Broe et al. 2014). Signs of proliferative retinopathy also developed in *INS*$^{\mathrm{C94Y}}$ transgenic pigs (Kleinwort et al. 2017).

Interestingly, the retinas of 2-year-old *INS*$^{\mathrm{C94Y}}$ transgenic pigs had changes in their cones (Fig. 3m, n), associated with reduced expression of cone arrestin that regulates signaling and trafficking of proteins that contribute to high acuity color vision (Kleinwort et al. 2017). Impaired color vision is a common observation in diabetic retinopathy patients, starting in early, uncomplicated stages and dramatically increasing with macular edema, where two-third of the patients have color discrimination abnormalities (Chous et al. 2016).

Because the duration of diabetes is the strongest predictor for development and progression of retinopathy, the longevity of the pig model is favorable for studying disease mechanisms and testing novel therapeutic interventions (Fong et al. 2004). Interestingly, severe insulin-deficient diabetes (SIDD), the human sub-phenotype modeled by *INS*$^{\mathrm{C94Y}}$ transgenic pigs, is associated with the highest risk for retinopathy (Ahlqvist et al. 2018).

Cardiovascular complications

Diabetes mellitus is associated with an increased risk for the development of cardiovascular diseases. Patients with diabetes have a 2- to 5-fold higher risk of developing atherosclerosis and other vasculopathies (Filardi et al. 2019). These pro-atherogenic effects of diabetes are predominantly mediated through induction of endothelial dysfunction and inflammation that ultimately lead to myocardial infarction, arrhythmias and heart failure.

Our understanding of cardiovascular alterations in the context of DM is based on clinical trials and epidemiological studies in patients, as well as on experimental data obtained in animal models. The advantage of animal models with a well-defined onset of diabetes is that progressive changes in the cardiovascular system during disease development can be assessed. Here, we will describe the current knowledge about cardiovascular disease development in diabetic pig models.

Diabetes mellitus and myocardial ischemia/heart failure

Based on the current European Society of Cardiology (ESC) guidelines, heart failure is divided into three categories based on ejection fraction: (i) heart failure with reduced ejection fraction (HFrEF); (ii) heart failure with preserved ejection

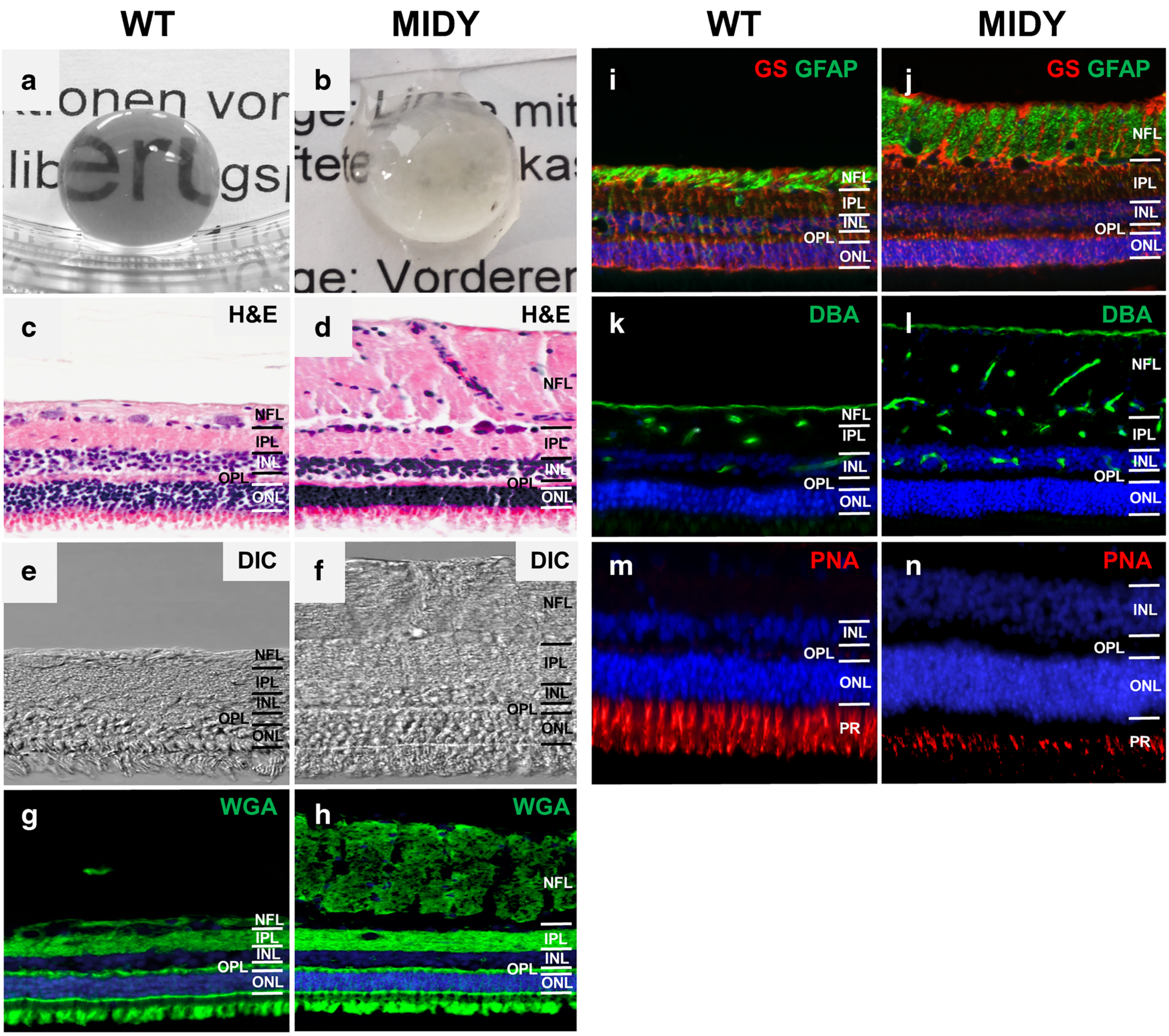

Fig. 3 Ocular changes in long-term diabetic INS^{C94Y} transgenic (MIDY) pigs. **a**, **b** Normal lens (**a**), lens of INS^{C94Y} transgenic pig (**b**). **c**, **d** H&E staining of retinas of a control pig (**c**) and an INS^{C94Y} transgenic pig (**d**). **e**, **f** Differential interference contrast images (DIC) of retinas of a control pig (**e**) and an INS^{C94Y} transgenic pig (**f**); **g**, **h** WGA-lectin (green) indicates thickening of nerve fibre cell layer (NFL) in an INS^{C94Y} transgenic pig (**h**) compared to a control pig (**g**). **i**, **j** Glutamine synthase (GS, red) and glial fibrillary acidic protein (GFAP, green) in a control pig (**i**) and an INS^{C94Y} transgenic pig (**j**). **k**, **l** DBA-lectin (green) detects tortuosity of vessels in MIDY pigs (**l**) but not in control pigs (**k**). **m**, **n** PNA-lectin (red) staining demonstrates different protein expression in cone sheets of INS^{C94Y} transgenic pigs (**n**) compared to non-transgenic controls (**m**)

fraction (HFpEF); and (iii) an intermediate category, heart failure with mid-range ejection fraction (Ponikowski et al. 2016). Diabetes is associated with a higher incidence, prevalence, morbidity and mortality in all categories of heart failure (Lehrke and Marx 2017). The higher incidence of HFrEF is due to the increased susceptibility to the development of atherosclerotic plaques and resultant myocardial ischemia (Beckman et al. 2002; Marso et al. 2010). However, the observation that heart failure in diabetic patients can develop without overt coronary atherosclerosis, which has led to the term “diabetic cardiomyopathy,” is less well understood (Kenny and Abel 2019; Lehrke and Marx 2017). It is increasingly recognized that this diabetic cardiomyopathy may involve a variety of adverse effects on coronary microvascular endothelium, cardiomyocytes, fibroblasts and inflammatory cells that contribute to changes in the left ventricular structure with hypertrophy and interstitial fibrosis (Fig. 4). Following a subclinical phase with asymptomatic left ventricular diastolic dysfunction, diastolic dysfunction may evolve into HFpEF and eventually systolic dysfunction may also ensue (Filardi et al. 2019; Jia et al. 2018).

Diabetes mellitus and arrhythmias

Epidemiological studies demonstrated that diabetes mellitus is one of the major risk factors for the onset and/or persistence of arrhythmias (Benjamin et al. 1994; Kannel et al. 1998). Although there is a growing body of evidence that both atrial and ventricular arrhythmias (Cardoso et al. 2003) can be triggered by diabetes, the majority of studies have focused on the most common arrhythmia—atrial fibrillation (AF). It has been shown that patients with prediabetes have a 20% increased risk (Aune et al. 2018) and patients with diabetes have a 28–40% increased risk to develop AF compared to non-diabetic patients (Aune et al. 2018; Huxley et al. 2011). Interestingly, although the meta-analysis by Aune et al. (2018) suggested a dose-dependent relationship between the risk for AF and blood glucose levels, intensified strategies to lower blood glucose have been shown not to affect the prevalence of AF (Fatemi et al. 2014). Studies in rats revealed that fluctuations in blood glucose levels rather than absolute levels may mediate the onset of AF (Saito et al. 2014). Furthermore, several studies in diabetic patients suggested that hypoglycemia can contribute to arrhythmogenesis (Ko et al. 2018; Odeh et al. 1990) whereas insulin resistance does not play a role as shown by the Framingham Heart Study (Fontes et al. 2012).

Population-based studies demonstrated a clear link between diabetes and arrhythmias and arrhythmogenesis has been intensively studied over the last decades but specific diabetes-related cellular and molecular mechanisms leading to arrhythmias remain incompletely understood. The major hallmarks of arrhythmogenesis are so-called remodeling processes including electrical, contractile, structural and autonomic remodeling (Clauss et al. 2015) and diabetes mellitus has been demonstrated to induce several of these processes both directly and indirectly (see below).

Effects of diabetes mellitus: endothelial dysfunction

The endothelium can secrete a number of factors including nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor, endothelin and vascular endothelial growth factor (VEGF). Thereby, the endothelium plays a key role in vascular health, in that it regulates vascular tone and vascular growth as well as coagulation and immune responses. In addition, many of these factors exert a paracrine effect on tissue beyond the vasculature (Roberts and Porter 2013). In addition to its role in glucose homeostasis, insulin regulates vascular function. In large epicardial coronary arteries from healthy pigs, insulin-induced vasodilation was endothelium-independent and mediated through activation of K^+-channels in vascular smooth muscle, while in coronary arterioles, both endothelium-derived NO and to a lesser extent tetraethylammonium (TEA)-sensitive K^+-channels mediate the vasodilator response to insulin (Hasdai et al. 1999; Hasdai et al. 1998). Studies in a variety of species have shown that insulin regulates endothelial function by activation of the PI3K-AKT pathway resulting in activation of endothelial nitric oxide synthase (eNOS), while the MAPK-ERK pathway, resulting in endothelin-1 production, is suppressed (reviewed in Roberts and Porter 2013). In diabetes, however, the PI3K-AKT pathway is suppressed resulting in increased activation of the MAPK-ERK pathway and vascular dysfunction. This vascular dysfunction is further exacerbated by an increase in oxidative stress as well as by PKC-β activation in hypercholesterolemia, which induces upregulation of the vascular

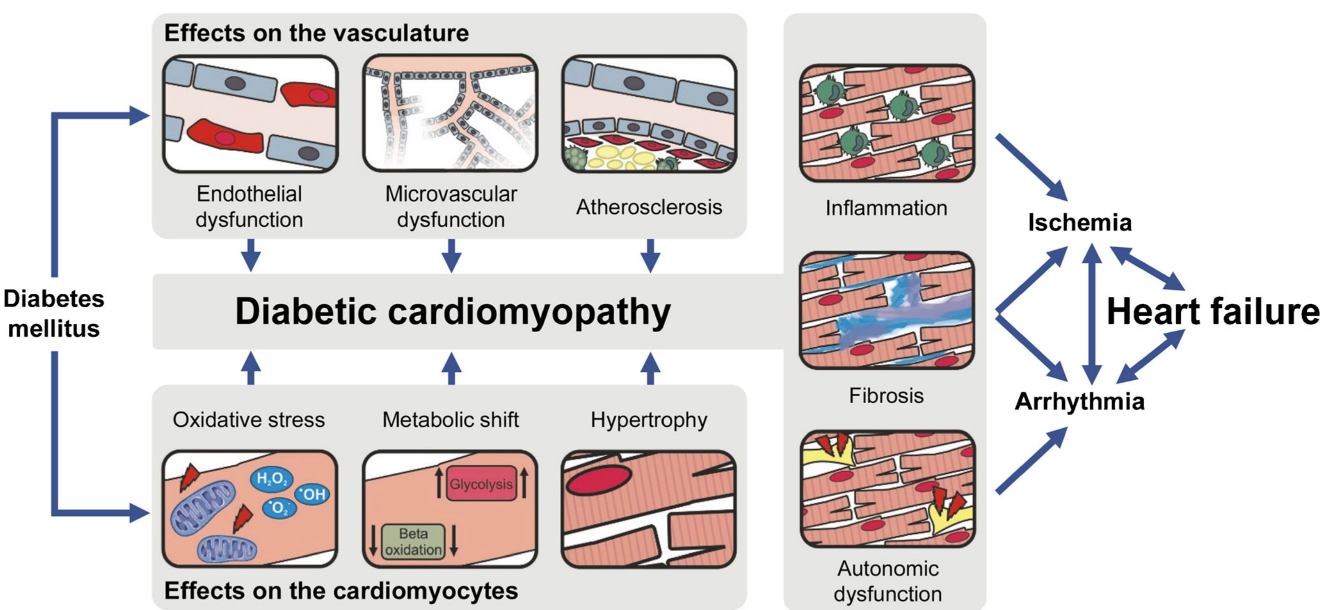

Fig. 4 Pathophysiology of diabetic cardiomyopathy. Cellular and molecular mechanisms induced by diabetes mellitus leading to diabetic cardiomyopathy with subsequent myocardial ischemia, arrhythmias and heart failure

adhesion molecules VCAM and ICAM (Roberts and Porter 2013). The ensuing endothelial dysfunction is a hallmark of diabetes.

Effects of diabetes mellitus: atherosclerosis (coronary/peripheral)

Pigs spontaneously develop atherosclerosis with age (Skold et al. 1966). When pigs are older than 18 months, plaques appear in the abdominal aorta and the iliac arteries but the incidence is initially low. It takes about 4 years for a large proportion of the abdominal aorta and the iliac arteries to be covered with plaques (Skold et al. 1966). Although the predilection sites and the plaque composition in pigs are similar to humans (Gerrity et al. 2001) and pigs are therefore a good animal model to study mechanisms of atherosclerosis, the slow development of the condition in normal commercial pigs is a disadvantage.

Although 4 weeks after induction of diabetes in Yucatan minipigs by STZ injection, PKC-β2 was already upregulated in the heart and the aorta (Guo et al. 2003), it was not investigated whether this upregulation was associated with endothelial dysfunction. Diabetes failed to induce significant atherosclerosis in the coronary arteries by 24 weeks (Hamamdzic et al. 2010; Hamamdzic and Wilensky 2013) and up to 48 weeks no lesions were seen in the abdominal aorta (Gerrity et al. 2001). However, these studies also showed that the combination of diabetes and hypercholesterolemia has a synergistic effect on plaque formation (Gerrity et al. 2001; Hamamdzic et al. 2010) and that plaque burden and severity of plaque phenotype in the coronary vasculature increase over time in both Yorkshire × Landrace hybrids (van Ditzhuijzen et al. 2016) and Göttingen minipigs (Ludvigsen et al. 2015a). Similarly, diabetes exacerbated coronary atherosclerosis and induced a more severe plaque phenotype with calcified lesions in Ossabaw swine with metabolic syndrome (Badin et al. 2018). Moreover, more advanced coronary artery disease was associated with hypo-activation of the AKT pathway, endothelial cell-activation, increased presence of vasa vasorum and local activation of the pro-inflammatory transcription factor NF-κB (Hamamdzic et al. 2010). However, diabetes did not aggravate circulating markers of inflammation (CRP, TNF-α, PAI-1) and oxidative stress (oxLDL) as compared to hypercholesterolemia alone (Ludvigsen et al. 2015a; van den Heuvel et al. 2012).

Furthermore, neither (i) the vasoactive response of isolated coronary conduit arteries, (ii) the endothelium-dependent vasodilator bradykinin, (iii) the endothelium-independent vasodilator S-nitroso-*N*-acetylpenicillamine, nor (iv) the vasoconstrictor response to endothelin-1 were altered when diabetes was superimposed onto hypercholesterolemia (van den Heuvel et al. 2012; van Ditzhuijzen et al. 2016).

Effects of diabetes mellitus: coronary microvascular dysfunction

The microvasculature appears to be more sensitive to the detrimental effects of diabetes and hyperglycemia than the conduit coronary arteries. Using a flow wire to measure coronary blood flow, a reduced baseline coronary blood flow and a reduced maximal coronary blood flow were observed in the absence of diameter changes in the conduit coronary arteries of Yucatan minipigs after 20 weeks of diabetes and hypercholesterolemia (Mokelke et al. 2005). A recent study showed that after just 8 weeks of diabetes, myocardial perfusion at rest and during adenosine stress were impaired as measured with cardiac magnetic resonance (CMR) perfusion scans in diabetic swine (Park et al. 2019). This reduced maximal myocardial perfusion was accompanied by a reduced capillary density (Boodhwani et al. 2007b; Hinkel et al. 2017; Park et al. 2019) and diameter (Park et al. 2019), decreased pericyte coverage (Hinkel et al. 2017) and increased capillary tortuosity as measured histologically (Park et al. 2019).

Isolated vessel measurements showed alterations in coronary microvascular function. Fifteen weeks of diabetes alone impaired the vasodilator response to the NO donor sodium nitroprusside, which was due to a reduction in soluble guanylate cyclase and enhanced phosphorylation of myosin light chain (Clements et al. 2009). Furthermore, diabetes aggravated microvascular dysfunction in pigs with hypercholesterolemia. In that model, 10 weeks of diabetes and hypercholesterolemia but not hypercholesterolemia alone, resulted in an impaired vasodilator response to bradykinin due to loss of NO and an impaired vasoconstrictor response to endothelin-1 in coronary small arteries, whereas no change in response was observed in conduit coronary arteries of the same animals (van den Heuvel et al. 2012). Conversely, prolonged exposure (15 months) to diabetes and hypercholesterolemia potentiated the vasoconstrictor response to endothelin-1, which was associated with an increased expression of the endothelin receptors ET_A as well as ET_B in the coronary microvasculature. Furthermore, coronary small arteries from these animals showed a preserved endothelium-dependent vasodilator response to bradykinin, with an increased contribution of NO and a reduced contribution of endothelium-derived hyperpolarizing factor, which was consistent with the observation that expression of small conductance potassium channels was decreased (Sorop et al. 2016). This reduction in small conductance potassium channels may also underlie the decreased spontaneous potassium current observed in diabetic, hypercholesterolemic pigs (Mokelke et al. 2005).

The changes in microvascular structure and function in diabetes also impair the angiogenic response to chronic myocardial ischemia. Capillary density was reduced in the ischemic territory distal to slowly progressive stenosis both 4

(Hinkel et al. 2017) and 7 weeks (Boodhwani et al. 2007b) after ameroid placement, which was consistent with an increased expression of the anti-angiogenic mediators angiostatin and endostatin, a reduced expression of the pro-angiogenic signal molecules VEGF and Tie-2 as well as a decrease in eNOS phosphorylation, which suggests an impaired NO production (Boodhwani et al. 2007b). Coronary blood flow to the perfusion territory of the stenosed circumflex coronary artery was reduced by diabetes, both at rest and during stress (pacing). This aberrant response was accompanied by decreased contractility and relaxation as well as by impaired endothelium-dependent vasodilation (ADP, substance P and VEGF) of the coronary arterioles. Altogether, these data imply a perturbed angiogenic balance in diabetes. A subsequent study by the same group showed that the negative effects of diabetes can be improved by treatment with insulin (Boodhwani et al. 2007a), suggesting that hyperglycemia plays an important role in these processes.

Effects of diabetes mellitus: altered metabolism/substrate shift and mitochondrial production of reactive oxygen species

The healthy heart is metabolically flexible in that ATP is predominantly derived from free fatty acid oxidation but glucose oxidation also contributes significantly depending on the circumstances. Furthermore, lactate, ketone bodies and branched-chain amino acids could be oxidized to produce ATP (Jia et al. 2018; Lehrke and Marx 2017; Sowton et al. 2019). In heart failure, however, a shift in substrate utilization from free fatty acids to glucose oxidation and glycolysis upon the presence of insulin resistance can be observed (Lehrke and Marx 2017; Sowton et al. 2019). In diabetic cardiomyopathy, metabolic disturbances precede deterioration of cardiac function. Hyperglycemia, insulin resistance and hypertriglyceridemia reduce substrate flexibility by impairing the ability of the heart to use glucose (Jia et al. 2018; Sowton et al. 2019). This is associated with increased leakage of reactive oxygen species (ROS) from the mitochondrial transport chain and increased oxidative stress (Jia et al. 2018).

It was already observed in the 1990s that diabetes was accompanied by alterations in myocardial metabolism. Twelve weeks of STZ-induced diabetes resulted in a decreased cardiac uptake of glucose and lactate in Yucatan minipigs (Hall et al. 1996). The decreased glucose and lactate uptake was associated with decreased expression of the glucose transporters GLUT1 and GLUT4. Moreover, the decreased lactate uptake in the diabetic animals correlated linearly with pyruvate dehydrogenase activity both under basal conditions and during dobutamine stress. Furthermore, diabetic animals exhibited a large increase in tracer-measured lactate output during dobutamine stress, suggesting that diabetic pigs exhibit accelerated glycolysis and impaired pyruvate oxidation during increased cardiac work (Hall et al. 1996). Similar observations were made during ischemia (Stanley et al. 1997), indicating that substrate utilization flexibility is indeed reduced in the diabetic porcine heart.

Effects of diabetes mellitus: altered cardiac geometry/structural remodeling/fibrosis

Diabetic cardiomyopathy is primarily associated with increased stiffness of the myocardium. Hyperglycemia-induced metabolic alterations, oxidative stress with subsequent loss of NO and inflammation not only impact the vasculature but also the cardiomyocytes as well as the fibroblasts and immune cells within the myocardium.

Several processes have been demonstrated to contribute to these hyperglycemia-induced myocardial changes, including alterations in the mitochondrial transport chain and activation of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (NOX) activation, both leading to oxidative stress. Furthermore, hyperglycemia increases formation of advanced glycation end products (AGE) through non-enzymatic reactions between reducing sugars and free amino groups on proteins and lipids, which can subsequently activate the receptor of AGE (RAGE) on cardiomyocytes and induce a pro-inflammatory response with increased connective tissue production and fibrosis. In addition, both activation of the innate immune system, leading to recruitment of inflammatory cells, and activation of the renin-angiotensin-aldosterone system (RAAS), with upregulation of the pro-inflammatory angiotensin receptor type 1 (AT1 receptor) and downregulation of the anti-inflammatory AT2 receptor, contribute to a pro-inflammatory response in the myocardium (Jia et al. 2018; Lehrke and Marx 2017). Altogether, oxidative stress and inflammation induce changes in calcium handling by myocytes, altered expression and phosphorylation of titin isoforms as well as an increase in perivascular and interstitial fibrosis (Jia et al. 2018; Lehrke and Marx 2017). Indeed, after 12 weeks of STZ-induced diabetes in Yucatan minipigs, collagen crosslinking was significantly elevated in the subendocardium (Martinez et al. 2003). A more extensive study on matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) in Chinese Guizhou minipigs 6 months after induction of diabetes with STZ showed increased levels of TIMP1 and TIMP4 that contributed to reduced MMP2 and MMP9 activity, while MMP2 and MMP9 protein expression was unaltered in the diabetic myocardium. These alterations in MMP and TIMP expression were accompanied by increased mRNA expression of plasminogen activator inhibitor-1 (PAI1), indicating that the pro-inflammatory transforming growth factor beta (TGFB) pathway was activated (Lu et al. 2008). These data are similar to a recent study in a diabetic rat model, also showing downregulation of MMP2, activation of the TGFB pathway and increased interstitial fibrosis (Wang et al. 2019) and are consistent with reduced MMP2 activity in the myocardium of diabetic humans (Sodha et al. 2009).

Furthermore, B-type natriuretic peptide (BNP) was increased in diabetic Chinese Guizhou minipigs, which was accompanied by a hypertrophic response of particularly the interventricular septum and surprisingly by an increase in the echocardiographically measured E/A ratio, suggesting that diastolic dysfunction was not present in these animals (Lu et al. 2008).

Interstitial fibrosis, reduced MMP2 expression, myocyte hypertrophy and a shift in titin isoform expression were also observed in a porcine model of diabetes combined with hypercholesterolemia and chronic kidney disease and correlated with oxidative stress and inflammation. These myocardial changes were accompanied by increased stiffness of the myocardium, impaired relaxation and hence left ventricular diastolic dysfunction (Sorop et al. 2018). However, the contributions of the individual risk factors (diabetes, hypercholesterolemia, chronic kidney disease) were not determined in this study.

Effects of diabetes mellitus: electrical alterations and autonomic dysfunction

Electrical remodeling is due to alterations in ion channels or calcium homeostasis leading to enhanced automaticity and ectopic electrical impulses or altered conduction velocity. In this regard, prolongation of the action potential duration (APD) and the QT interval has been observed in diabetic patients (Ewing and Neilson 1990; Ramirez et al. 2011; Sivieri et al. 1993) potentially mediated by reduced potassium currents (Xu et al. 1996). Further electrical remodeling in diabetes includes changes in gap junction biology (Mitasikova et al. 2009) or altered calcium homeostasis (Jain et al. 2014; Zhong et al. 2017) as demonstrated in various animal models.

The diabetes-related mechanisms of arrhythmogenesis have been mainly studied in rodents or rabbits (Grisanti 2018), with only a few studies performed in pigs to date. Mesangeau et al. (2000) induced diabetes in pigs by injection of STZ and showed a significantly increased heart rate after 6 months, indicating autonomic remodeling. This effect was confirmed by Mokelke et al. (2003) who induced diabetes by injection of alloxan into Yucatan minipigs. In the latter study, patch-clamp experiments revealed that potassium current densities are increased in diabetes, an effect that could be prevented by exercise.

Autonomic remodeling as indicated by a reduced heart rate variability also seems to be an important contributor, since patients with diabetes show a prolongation and dispersion of the P wave, which suggests altered atrial electrophysiology predisposing to arrhythmias (Bissinger et al. 2011; Singh et al. 2000). Sympathoadrenal activation by hypoglycemia has also been associated with lethal arrhythmias, further confirming the role of autonomic remodeling in arrhythmogenesis (Reno et al. 2013).

Effects of antidiabetic drugs in the context of myocardial ischemia

Besides studies in diabetic pigs, several groups used non-diabetic pigs to evaluate potential pro- or anti-arrhythmic effects of antidiabetic drugs in the context of myocardial ischemia-reperfusion. Chinda and colleagues treated non-diabetic farm pigs prior to myocardial infarction (LAD occlusion for 90 min followed by 120 min of reperfusion) with a single dose of the DPP-4 inhibitor vildagliptin. Vildagliptin treatment resulted in attenuation of the ischemia-induced shortening of the refractory period, a reduced number of premature capture beats and an increased ventricular fibrillation threshold (Chinda et al. 2013). Metformin treatment for 2–3 weeks prior to myocardial ischemia also resulted in significantly reduced incidence of ventricular fibrillation (12% vs. 50%), an effect that was driven by the preservation of the ATP content within the myocardium resulting in attenuation of ischemia-induced APD shortening and APD heterogeneity across the myocardium (Lu et al. 2017). Besides these benefits, i.e., anti-arrhythmic effects of these two drugs, other antidiabetics seem to be proarrhythmic: 60 min of rosiglitazone infusion prior to myocardial infarction increased the incidence of ventricular fibrillation (58% vs. 10%) although the infarct size related to the area at risk was significantly decreased (Palee et al. 2011). Treatment with the peroxisome proliferator-activated receptor (PPAR)-γ activator troglitazone 1 hour before myocardial infarction caused a shortening of the QTc interval and an increased incidence of ventricular fibrillation (Xu et al. 2003).

Similar to these studies, Diemar et al. (2015) evaluated the influence of blood glucose levels on ischemia-related arrhythmogenesis. In non-diabetic pigs, blood glucose levels were clamped at either a hypoglycemic, normoglycemic, or hyperglycemic level prior to induction of a myocardial infarction. No difference could be observed between groups regarding infarct size, incidence of ventricular fibrillation or mortality, suggesting that acute short-term alterations of blood glucose levels might not have an effect on electrophysiology in vivo.

Future perspectives

Taken together, there is a growing body of evidence demonstrating that diabetes mellitus is a major driver for cardiovascular diseases (summarized in Fig. 4). Despite recent advances in understanding the underlying mechanisms, several questions remain that cannot be answered by epidemiologic studies or clinical trials involving patients with diabetes but rather require suitable animal models mimicking the human diabetic phenotype. Although some fundamental mechanisms can be studied in rodents or rabbits, specific cardiovascular investigations such as hemodynamic assessment or electrophysiology studies are ideally studied in large animal models. In the past, dogs have predominantly been used in cardiovascular research but several disadvantages including differences in lipid profile (Kaabia et al. 2018), coronary anatomy and presence of an extensive collateral circulation as compared to humans (Kato et al. 1987), the lack of genetically modified strains as well as increased resistance to the use of dogs as experimental animals, have limited their use. In this regard, pigs seem to be advantageous since their cardiac and coronary anatomy, hemodynamics and cardiac electrophysiology are similar to humans (Clauss et al. 2019), their use is more socially acceptable in western societies and genetically modified pig models are available.

Renal complications

Up to 20–40% of patients with DM develop diabetic nephropathy (DN), representing one of the most important microvascular complications of DM with high morbidity and mortality rates. Worldwide, DN has become the single most common condition found in patients with chronic kidney disease (CKD) regularly progressing to end-stage renal disease (ESRD) with the need for dialysis or transplantation (Alicic et al. 2017; Gheith et al. 2016). As for most entities of chronic kidney diseases, a slow and persistent progressive loss of intact kidney nephrons and kidney function that leads to terminal renal failure is characteristic of DN.

Usually, DN first manifests about 10–15 years after diagnosis of DM. Clinically, DN presents as a syndrome composed of albuminuria and progressive decrease in renal function, i.e., declining glomerular filtration rate (Meier and Haller 2004).

Histomorphologically, DN is commonly marked by the development of glomerulosclerosis, tubular atrophy and interstitial fibrosis that ultimately affects all structures of the kidney (Kopel et al. 2019; Lee et al. 2019; Matoba et al. 2019). The first morphological changes detectable in kidney biopsies affect the renal glomeruli. DM-associated glomerular alterations include podocyte hypertrophy and thickening of the glomerular basement membrane (GBM), followed by glomerular enlargement (i.e., glomerular hypertrophy) with mesangial expansion and effacement of podocyte foot processes (Herbach et al. 2009; Johnstone and Holzman 2006; Lee et al. 2019). Detection of subtle morphological alterations, such as GBM-thickening and podocyte foot process effacement, usually requires electron microscopic analysis. For an unbiased quantitative characterization of such pathogenetically relevant morphological key parameters, appropriate quantitative stereological sampling and analysis methods have to be applied (Albl et al. 2016; Blutke et al. 2016; Blutke and Wanke 2018; Nyengaard 1999). Advanced glomerular alterations are characterized by ongoing glomerular hypertrophy, mesangial sclerosis and hypercellularity, collapse of glomerular capillary lumina, capillary hyalinosis and advanced podocyte damage and loss, resulting in synechial attachments of the glomerular tuft to the capsule of Bowman (Shankland 2006; Wanke et al. 2001). These alterations often lead to the formation of the characteristic nodular glomerular lesion pattern in DN, originally described by Kimmelstiel and Wilson in 1936 (Kimmelstiel and Wilson 1936). Glomerular alterations are accompanied by albuminuria, misfiltration of urine/albumin into the renal interstitium and tubulo-interstitial lesions including tubular atrophy, interstitial fibrosis and inflammation.

The complex etiopathogenesis of DN is still not completely understood. Several genetic factors appear to be predisposing components for DN (Wolf 2004), such as mutations, ethnicity, or the congenital individual number of nephrons in the kidney (Gross et al. 2005; Hoy et al. 2005). Different pathomechanisms, such as intraglomerular hypertension and hyperfiltration (Brenner 1983), glomerular hypertrophy (Fogo and Ichikawa 1989) with subsequent podocyte damage (Wanke et al. 2001; Wiggins 2007) and disturbance of the glomerular permselectivity (Remuzzi and Bertani 1990) collectively contribute to the progression of glomerulosclerosis and tubulo-interstitial damage in DN (Wolf 2004). On the molecular level, numerous different factors are recognized to be involved in the development and progression of DN. These include metabolic abnormalities, such as hyperglycemia, dyslipidemia and accumulation of AGE in the kidney, hemodynamic changes, activation of the renin-angiotensin system, oxidative stress and complex inflammatory alterations (Kopel et al. 2019; Lee et al. 2019; Matoba et al. 2019; Wolf 2004; Yacoub and Campbell 2015).

In translational diabetes research, a plethora of experimental animal models of DM has been developed in recent decades, including "classical" rodent as well as large animal models (Alpers and Hudkins 2011; Betz and Conway 2016; Kleinert et al. 2018; Kong et al. 2013; Renner et al. 2016b). However, no single experimental animal DM model reproducibly develops the full spectrum of morphological, functional, clinical and molecular alterations seen in advanced human DN (Alpers and Hudkins 2011; Betz and Conway 2016; Kong et al. 2013). Indeed, there are few established mouse models that develop relevant glomerulosclerotic kidney lesions solely on the basis of DM (Alpers and Hudkins 2011; Betz and Conway 2016; Herbach et al. 2009; Hudkins et al. 2010; Inagi et al. 2006; Kong et al. 2013; Zhao et al. 2006). Additionally, the genetic background of mice often significantly affects the manifestation of diabetes-associated renal alterations (Alpers and Hudkins 2011; Betz and Conway 2016; Gurley et al. 2010; Kong et al. 2013; Popper 2014).

Compared to rodents, the porcine kidney is more similar to the human kidney and therefore, pigs could be considered as promising large animal models for DN mechanistic studies (reviewed in Giraud et al. 2011; Renner et al. 2016b). In addition to comparable absolute size, human and porcine kidneys share several embryonic, anatomical and physiological features that are not adequately reflected by rodent models (see above, reviewed in Giraud et al. 2011). However, similar to rodent DM models, advanced glomerulosclerotic kidney alterations comparable to human DN are not observed in diabetic pig models. Nevertheless, several studies have described DM-associated renal lesions in diabetic pig models, including genetically modified, as well as chemically (STZ)- and diet-induced DM models.

DM-associated kidney lesions in genetically modified pig models

In 2013, we reported the generation of transgenic pigs (Landrace × Swabian Hall hybrids) expressing a mutant porcine insulin gene (INS^{C94Y}) (Renner et al. 2013; Wolf et al. 2014; Wolf 2004) (see above). At 4.5 months of age, INS^{C94Y} transgenic pigs displayed a robust diabetic phenotype with significant hyperglycemia, reduced fasting insulin levels and a massively decreased beta cell mass, as compared to controls. The body weight of INS^{C94Y} transgenic pigs was significantly (41%) lower than in non-transgenic control pigs and the weights of most organs were proportionately reduced. An exception was kidney weight, which was reduced by only 15%, leading to a significant increase of the relative kidney weight of INS^{C94Y} transgenic pigs, indicating renal hypertrophy. However, this was not accompanied by detectable DM-associated histopathological alterations of the kidney. Relevant key morphological parameters of renal glomeruli were examined, using *state-of-the-art* quantitative stereological analyses of perfusion-fixed kidney tissue samples on the level of light and electron microscopy (Albl et al. 2016; Renner et al. 2013). However, the mean glomerular volume and the GBM thickness in 4.5-month-old INS^{C94Y} transgenic pigs were not increased compared to control pigs. Although the relative mean glomerular volume (related to body weight) in INS^{C94Y} transgenic pigs was significantly higher than in control animals, this finding might well be explained by the lower body weight of the diabetic pigs (Renner et al. 2013).

Older INS^{C94Y} transgenic pigs (2–4 years of age) that were examined in subsequent studies (Blutke et al. 2017; Haesner 2018) and single necropsy cases (unpublished data) did not display significantly increased absolute or relative kidney weight, mean glomerular volume, mean podocyte volume, or glomerular basement membrane thickness and showed no evidence of manifest DM-associated morphological kidney alterations. Interestingly, development of DM-associated morphological renal lesions in the homologous mouse model, the “Akita-mouse,” is also largely restricted to early glomerular alterations, such as increased GBM thickness and mesangial matrix expansion, whereas the typical structural lesions seen in advanced human DN (i.e., glomerulosclerosis) do not develop, regardless of the mouse strain/genetic background (Gurley et al. 2010; Kitada et al. 2016).

A higher discrepancy of the renal phenotypes of diabetic mouse and homologous pig models was found in murine and porcine models expressing a dominant-negative receptor for the glucose-dependent insulinotropic polypeptide ($GIPR^{dn}$) in pancreatic beta cells (*see above*) (Herbach et al. 2009; Renner et al. 2010). Whereas distinct genetic strains of diabetic $GIPR^{dn}$ transgenic mice reproducibly develop advanced glomerulosclerotic kidney lesions closely resembling aspects of human DN (podocyte hypertrophy, GBM thickening and glomerular hypertrophy progressing to glomerular sclerosis and tubulo-interstitial lesions) and associated clinical alterations (increased serum creatinine and urea levels, progressive albuminuria) (Herbach et al. 2009; Popper 2014), $GIPR^{dn}$ transgenic pigs bred on a Landrace × Swabian Hall hybrid background show only a mild prediabetic phenotype (Renner et al. 2010; Wolf et al. 2014) and do not develop diabetes-associated kidney damage (own unpublished findings).

The finding of nodular glomerular lesions in kidneys of transgenic pigs expressing a dominant-negative hepatocyte nuclear factor 1-alpha (Hara et al. 2014; Umeyama et al. 2017; Umeyama et al. 2009), a model for maturity-onset diabetes of the young type-3 (MODY3), has attracted much attention in the scientific community of DN researchers. $HNF1A^{\mathrm{P291fsinsC}}$ transgenic pigs carry the most frequent causative mutation found in human MODY3 patients (Yamagata 2003; Yamagata et al. 1996). The transcription factor HNF1A is critically involved in pancreatic islet development and is, among other tissues, expressed in the liver, the pancreas and proximal kidney tubules (but not in renal glomeruli) (Hara et al. 2014; Pontoglio 2000). $HNF1A^{\mathrm{P291fsinsC}}$ transgenic pigs (Landrace/Large White × Duroc genetic background) display a severe diabetic phenotype due to hypoplasia of pancreatic beta cells and also develop ocular lesions (retinopathy, cataract). At 4–5 months of age, the transgenic pigs present a characteristic pattern of morphologic lesions in the kidney, primarily affecting glomeruli located in the deep renal cortex. These glomerular alterations were characterized by significantly increased glomerular tuft section profiles and formation of diffuse glomerular nodules containing mesangial matrix collagens, AGE, necarboxymethyllysine and TGFB. However, other characteristic features of human DN, such as proteinuria, GBM thickening, exudative glomerular lesions, segmental glomerulosclerosis, synechia formation, tubular atrophy, interstitial fibrosis and vascular hyalinosis, did not develop. The pathogenesis of the glomerular alterations in $HNF1A^{\mathrm{P291fsinsC}}$ transgenic pigs is not completely understood. As the expression of the mutant $\mathrm{HNF1A}^{\mathrm{P291fsinsC}}$ in this model is not restricted to pancreatic beta cells (Umeyama et al. 2009), the observed renal changes may be a consequence of local expression of $\mathrm{HNF1A}^{\mathrm{P291fsinsC}}$ in the kidney rather than caused by the diabetic condition of the animals. In this context, the observed glomerular lesion pattern that only affects glomeruli in the deep cortex might probably be related to the different vascular anatomy and peculiarities in the circulation regulation of juxtamedullary glomeruli, making them more vulnerable to insults than glomeruli located in the superficial renal cortex (Denton 2000; Evans et al. 2004). In their initial study, Umeyama et al. (2009) examined *HNF1A*$^{\mathrm{P291fsinsC}}$ transgenic pigs generated by SCNT. The renal lesions might, therefore, as well be attributable to anomalies associated with SCNT (reviewed in Kurome et al. (2013)). However, the renal phenotype of *HNF1A*$^{\mathrm{P291fsinsC}}$ transgenic pigs remained stable also in a second study, where transgenic pigs generated by in vitro fertilization or intrafallopian insemination were examined (Umeyama et al. 2017). Finally, chronic hyperglycemia alone has not been sufficient to induce formation of comparable glomerular alterations in any other diabetic pig model, so far.

Kidney lesions in pig models with diet-induced DM

Based on their analyses in a diet-induced pig model of type II DM, Liu et al. (2007) reported development of moderate glomerular lesions resembling early states of human DN in male Chinese Bama minipigs fed a high-fat/high-sucrose/high-cholesterol diet for 2–5 months. These pigs developed significant hyperglycemia, hyperinsulinemia, elevated serum levels of triglycerides and cholesterol, insulin resistance and significantly increased urinary albumin/creatinine ratios. Histopathological renal lesions were assessed qualitatively and semi-quantitatively and comprised glomerular enlargement, hypercellularity and mesangial expansion, graded as minimal to moderate "glomerulosclerosis" (Liu et al. 2007).

Similar findings were reported by Feng et al. (2015), who examined another diet-induced DM model of Chinese Bama minipigs. At 1 year of age and after 8 months on a high-sugar and high-fat diet, the animals had developed significantly increased fasting blood glucose and insulin levels, as well as significantly elevated levels of serum triglycerides and cholesterol and histological analysis indicated development of glomerular hypertrophy (Feng et al. 2015).

Kidney lesions in pig models with chemically induced DM

Maile et al. (2014) analyzed renal alterations in an STZ-induced DM model of male Yorkshire pigs fed a high-fat diet for 4 weeks. Renal morphology was examined in samples of perfusion-fixed kidney tissue harvested from pigs of approx. 6 months of age. Compared to control animals, diabetic pigs displayed significantly increased GBM thickness and podocyte foot width (indicating foot process effacement), as determined by electron microscopy. Using a model-based quantitative stereological approach, the authors also identified a significant increase of the glomerular mesangial matrix volumes by ~ 20%. These early-stage DM-associated glomerular alterations could be reversed by administration of F(ab)2 antibody fragments specifically blocking the activation of αVbeta3 integrin in endothelial glomerular cells (Maile et al. 2014).

Khairoun et al. (2015) induced development of microvascular damage in a STZ-induced porcine DM model (Landrace × Yorkshire, T-line) by administration of an atherogenic diet for 11 months. While the urinary albumin/creatinine ratios remained unaltered, these pigs developed mild glomerular lesions at 14 months of age, comprising mesangial proliferation and matrix expansion. Additionally, the GBM thickness was found to be increased in some animals (Khairoun et al. 2015).

Future perspectives

The availability of a porcine DM model that develops the full spectrum of clinical and morphological alterations typically seen in human DN (progressive glomerulosclerosis) would undoubtedly be of tremendous value in translational DM research. However, in most of the current porcine models of DM, the development of glomerular kidney lesions is largely restricted to morphological alterations that occur in very early disease stages of DN (GBM thickening, glomerular mesangial expansion), or are not specific for DN (i.e., commonly seen in diverse entities of chronic human kidney disease).

But why is it that DN-typical advanced glomerulosclerotic and tubulo-interstitial lesions are not observed in diabetic pigs? And can the reproducible development of such lesion patterns be triggered experimentally in diabetic pigs? If we want to take full benefit of the potential of porcine animal models in translational DM research, intensive efforts are necessary to address these important questions in future studies.

The genetic background of the commonly used pig strains/lines probably has a similarly large influence on the susceptibility for development of DM-associated kidney alterations as it is in mice. It is likely that several of today's domesticated pig lines might have developed an inherent/natural resistance to the development of DM (e.g., to obesity-related insulin resistance) and its sequelae over hundreds of generations of breeding, during which pigs have been deliberately selected for their ability to efficiently utilize food energy for accumulation of body mass (for later human consumption), while being exposed to a "diabetogenic" pigsty-environment of relative physical inactivity and energy abundance (Gerstein and Waltman 2006).

Highlighting the importance of the genetic background of porcine models in DM and obesity research, "Ossabaw pigs" represent a prime example of the evolution of a genetic susceptibility to metabolic consequences of a diabetogenic environment in pigs (Gerstein and Waltman 2006; Whitfield 2003). These pigs are the feral progeny of a colony of domesticated pigs released by Spanish seafarers on the remote Ossabaw Island (GA, USA), approximately 500 years ago. Being exposed to a harsh environment that demands high physical activity while only providing a seasonal and discontinuous food supply, these pigs (re-)adopted a "thrifty genotype." Despite their domesticated ancestors, Ossabaw pigs now exhibit an (evolutionarily acquired) genetic propensity to obesity and hyperglycemia when fed a high-calorie diet in a low-activity environment. Accordingly, Ossabaw pigs have been used as a porcine model for diet-induced obesity that also develops features of the metabolic syndrome (see above), including visceral obesity, insulin resistance, impaired glucose tolerance, dyslipidemia and hypertension (Dyson et al. 2006), as well as related cardiovascular (Bender et al. 2009), steatohepatitic (Panasevich et al. 2018), atherosclerotic (Neeb et al. 2010), renal glomerular (Li et al. 2011) and retinal (Lim et al. 2018) alterations. Another example demonstrating the potential importance of the genetic background of porcine DM models is the Bama miniature pig, a highly inbred laboratory miniature pig line based on the primitive Bama Xiang pig breed. After 30 years of selective inbreeding, Bama miniature pigs display a high degree of genetic homozygosity (Zhang et al. 2019). They are considered to be quite susceptible to development of high-sucrose, high-fat diet-induced insulin resistance and have thus frequently been used as animal models in DM research (Chen et al. 2009; Liu et al. 2007; Liu et al. 2017; Ruan et al. 2016). Recently, Zhang et al. (2019) generated a chromosome-level reference genome sequence of Bama miniature pigs. In comparison to genome sequences of Duroc pigs, representing a common commercial pig breed, the authors identified several variations in genetic loci potentially associated with the decreased "diabetic resistance" of Bama miniature pigs (Zhang et al. 2019).

In the future, more detailed studies are needed to define the genetic basis underlying the variable resistance or susceptibility of different pig lines to development of DM and diabetic complications when being exposed to a "diabetogenic" environment.

Another possible reason for the lack of studies observing true DN alterations in porcine DM models might be the long period of time necessary for establishment of DN in diabetic pigs, by far exceeding the time frames usually scheduled for scientific experiments. However, long-term observations in diabetic pig models with necessary permanent clinical monitoring and insulin substitution are expensive, work-intensive and might also raise animal welfare concerns. Here, generation of pig models carrying multiple genetic modifications, or treatment of established pig models of DM with challenge treatments, e.g., with special diets and/or chemical compounds, or surgical intervention, might accelerate the development of renal lesions secondary to DM.

Finally, it is most important to validate the comparability of results in different studies reporting on renal alterations in porcine DM models. Therefore, standardized tissue sampling, processing and analysis methods should be applied, particularly for quantification of subtle morphological changes by unbiased quantitative stereological analyses (Albl et al. 2016; Blutke and Wanke 2018; Nyengaard 1999).

Systemic analyses of organ crosstalk in porcine models of diabetes and obesity

Since complications in different tissues occur frequently together, there must be a common mechanistic basis, presumably related to disturbed energy metabolism and a reduced defense against toxic products generated during energy flux using glucose and/or lipids. This is expected to result in altered cell-cell and organ-organ crosstalk (discussed in Kim 2016) and finally in an irreversible cascade leading to organ failure. A few recent studies supported this hypothesis but the analyses were limited to specific tissues, such as pancreatic islets (El Ouaamari et al. 2013; Gerst et al. 2017; Shirakawa et al. 2017), adipose tissue (reviewed in Romacho et al. 2014) or skeletal muscle (reviewed in Roden 2015; Whitham and Febbraio 2016), or to specific molecule classes such as advanced glycation end products (Yamagishi and Matsui 2016; Yamagishi et al. 2015). A study analyzing multiple tissues of diabetic mouse models performed only RNA expression analyses (Samdani et al. 2015) and thus has limited potential to shed light into organ crosstalk.

Molecular profiling techniques, e.g., at the transcriptome, proteome and metabolome levels, facilitate the investigation of intermediate molecular ("omics") phenotypes in disease-related cells, tissues and organs (reviewed in Meng et al. 2013). While a single layer of "omics" can only provide limited insights into the biological mechanisms of a disease, multi-omics approaches that integrate data obtained from different omics levels were proposed to understand their interrelation and combined influence on the disease processes (reviewed in Sun and Hu 2016). Systems biology approaches such as integrative analyses of multi-omics data sets aim to provide novel mechanistic insights and to identify therapeutic targets and biomarkers (Caie and Harrison 2016; Hasin et al. 2017; Sun and Hu 2016; Yan et al. 2017).

Central gene expression data repositories such as NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) or EMBL-EBI ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) are important sources for capturing transcriptome alterations in diabetic patients (e.g., Kodama et al. 2012), but are mostly limited to one or a few tissues per study (e.g., blood cells and adipose tissue in Emilsson et al. 2008). The Human Diabetes Proteome Project (HDPP) was launched with an initial focus on islets of Langerhans, insulin-producing cell lines and blood samples from diabetes-related patient cohorts (Topf et al. 2013). Moreover, targeted and non-targeted metabolomics approaches are available for diabetes research and have been used for analyzing human samples and samples from model organisms (reviewed in Sas et al. 2015).

Although cross-tissue networks with a limited spectrum of tissues have been constructed in several studies, integration of multi-omics data with expanded tissue coverage would markedly benefit disease-related network analyses on an organism-wide scale (Meng et al. 2013). This is particularly true for metabolic diseases such as diabetes and obesity where multiple tissues/organs may be causally involved in and/or affected by disease-relevant tissue crosstalk (reviewed in Stern et al. (2016)).

For ethical reasons, the spectrum of tissues available from diabetic patients is limited. In addition, confounding factors such as age, comorbidities and variance introduced by tissue sampling and storage procedures may complicate the analysis and interpretation of omics data from human samples. Samples from diabetic rodent models are less variable but the amount of tissue available for multi-omics analyses and translation of results to humans is limited.

The Munich MIDY pig biobank — a unique resource for studying organ crosstalk in insulin - deficient diabetes mellitus

As a resource for studying systemic consequences of chronic insulin insufficiency and hyperglycemia, we established a comprehensive biobank, the Munich MIDY Pig Biobank (Blutke et al. 2017) (Fig. 5) of long-term diabetic *INS*^{C94Y} transgenic pigs (Renner et al. 2013), a model of mutant *INS* gene-induced diabetes of youth (MIDY) and of wild-type (WT) littermates (highlighted in *Nature* News in Focus; Abbott 2015). Female MIDY pigs were maintained with suboptimal insulin treatment for 2 years, together with female WT littermates. While insulin treatment restored basal insulin in MIDY pigs to the level of WT, fasting plasma levels of glucose and fructosamine, a valid parameter for the evaluation of medium-term glucose control over 2–3 weeks (reviewed in Renner et al. 2016b), were highly elevated. C-peptide levels decreased with age and were undetectable at 2 years, in line with an 82% reduced total beta cell volume compared to WT. Plasma glucagon and beta hydroxybutyrate levels of MIDY pigs were chronically elevated, reflecting hallmarks of poorly controlled diabetes in humans (Blutke et al. 2017) (Fig. 6).

At age 2 years, all pigs were euthanized, necropsied and a comprehensive biobank including ~ 1900 samples of different body fluids (blood, serum, plasma, urine, cerebrospinal fluid, and synovial fluid) as well as ~ 17,000 samples from ~ 50 different tissues and organs were established (Blutke et al. 2017). To ensure generation of representative, high-quality tissue samples, suitable for a broad range of analyses and standardized sampling procedures (Albl et al. 2016) were used (Fig. 5). Samples designated for molecular profiling analyses were collected and snap-frozen within 20 min of death.

The broad spectrum of well-defined biosamples in the Munich MIDY Pig Biobank provides a unique resource for systematic studies of organ crosstalk in insulin-deficient diabetes mellitus in a multi-organ, multi-omics dimension.

A similar biobank has been recently established from morbidly obese Göttingen minipigs fed a high-energy, high-fat diet that were chronically insulin-resistant and hyperinsulinemic (Renner et al. 2018).

Multi-omics insights into physiological derangements of the liver in insulin-deficient diabetes mellitus

In view of the central role of the liver in metabolism and its biochemical crosstalk with multiple organs and tissues (http://www.nature.com/nm/e-poster/Liver-Organ-Normal.html), we started the analysis of the Munich MIDY Pig Biobank by multi-omics profiling of liver samples from MIDY and wild-type (WT) pigs (Backman et al. 2019).

RNA sequencing with reduced representation of ribosomal RNA revealed 533 transcripts that were differentially abundant between MIDY and WT pigs, 320 with increased and 213 with decreased abundance in the MIDY samples. A gene set enrichment analysis (GSEA) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database identified gene sets related to amino acid metabolism, gluconeogenesis/glycolysis, glucagon signaling, retinol metabolism, peroxisome proliferator-activated receptor (PPAR) signaling and peroxisome enriched in the MIDY samples, whereas gene sets associated with immune function and extracellular matrix interactions were enriched in the WT samples (Backman et al. 2019).

Quantitative LC-MS/MS-based proteomics of the same samples identified a total of 2,535 proteins, 60 with significantly higher and 84 with significantly lower abundance in MIDY vs. WT samples. Proteins more abundant in MIDY samples have functions in amino acid metabolism, gluconeogenesis/glycolysis and tricarboxylic acid (TCA) cycle, while those less abundant are involved in pathogen defense response, response to cellular stress, or in cell signaling and genetic information processing (Backman et al. 2019).

A targeted metabolomics analysis of the liver samples detected increased concentrations of lysine, methionine and of the branched-chain amino acids leucine, isoleucine and valine in the MIDY samples, while the concentration of serine was reduced. Arginine was not detected in the MIDY samples,

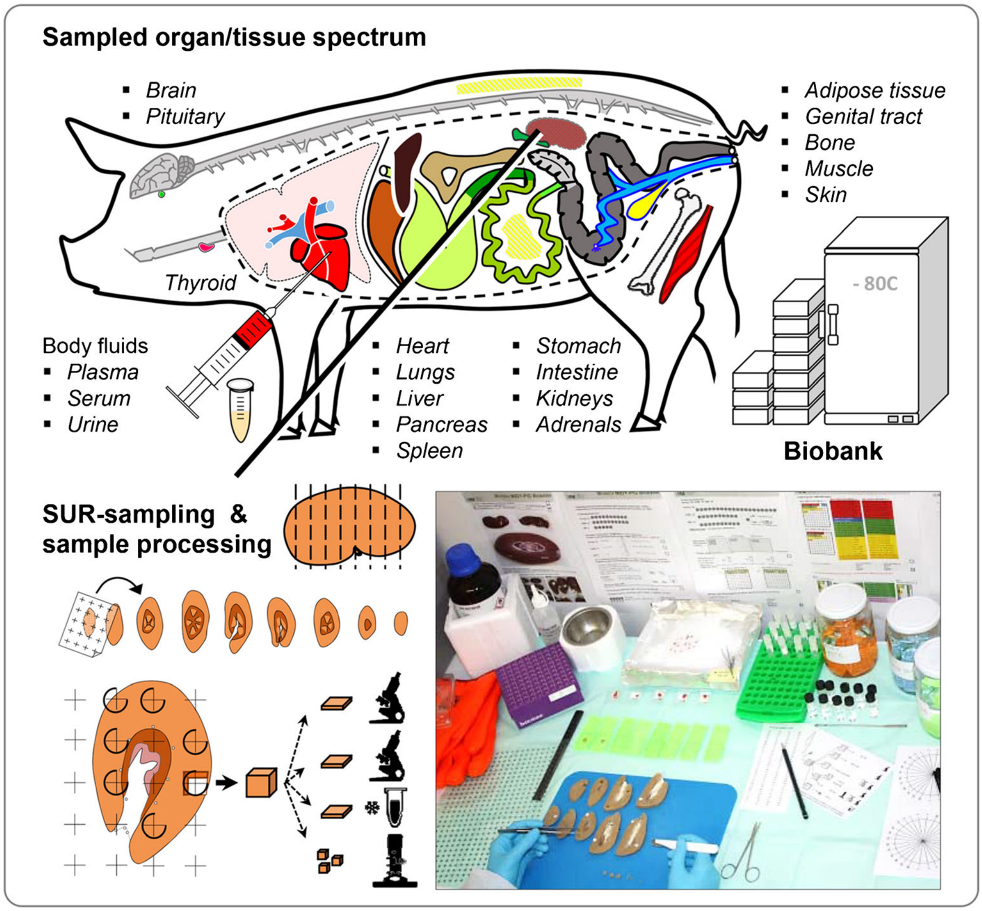

Fig. 5 Biobank design for genetically modified porcine translational models. A broad spectrum of organs and tissues is sampled. Representative tissue specimens are generated, applying organ-adapted systematic uniform random (SUR) sampling designs. Sub-samples of the sampled tissue locations are differentially processed for various downstream analysis types, such as cryo-histology, immunohistochemistry, paraffin-histology, in situ hybridization, electron microscopy and molecular analyses (i.e., DNA-, RNA-, protein-, metabolite- and lipid analyses) and stored under appropriate conditions until analysis. Detailed descriptions of applicable sampling designs are provided in Albl et al. (2016) and Blutke et al. (2017)

although it was detected at low concentrations in the majority of the WT samples. The concentrations of long-chain acylcarnitines (C16, C18) and the ratio of (C16 + C18) to free carnitine (C0) were significantly increased, while the levels of short-chain acylcarnitines (C2, C3, C4, C5) and the ratios of acetylcarnitine (C2) to C0 and of short-chain acylcarnitines (C2 + C3) to C0 were significantly decreased in MIDY samples. Total sphingomyelin (SM) and hydroxy-sphingomyelin (SM-OH) levels as well as the ratio of SM to phosphatidylcholines (PC) were significantly decreased in MIDY samples. A shotgun lipidomics analysis confirmed the metabolomics results in that PC were unchanged, while SM were reduced in MIDY samples. Concentrations of cholesterol (Chol) and phosphatidylserine (PS) were slightly reduced and lysophosphatidylserine (LPS) and phosphatidic acid (PA) more markedly reduced. In contrast, diacylglyceride (DAG) and triacylglyceride (TAG) levels were increased in MIDY samples (Backman et al. 2019).

An integrated analysis of changes on the different omics layers (Backman et al. 2019) provided new insights into functional alterations of the liver in insulin-deficient DM (Fig. 6) and identified candidate molecules driving these changes.

Retinol dehydrogenase 16 — a potential key driver of increased gluconeogenesis

The protein with the highest abundance increase in MIDY samples was retinol dehydrogenase 16 (RDH16; log2 fold change, 4.7) (Backman et al. 2019). Retinol dehydrogenases catalyze the first reaction of the two-step activation of vitamin A (retinol) into all-trans-retinoic acid (atRA), which stimulates—among many other genes—the expression of *PCK1* encoding phosphoenolpyruvate carboxykinase, the rate-limiting enzyme of gluconeogenesis (reviewed in Obrochta et al. 2015). Indeed, the concentrations of PCK1 and of several other enzymes involved in gluconeogenesis were significantly increased in the MIDY samples (Fig. 7). In human hepatoma cells, insulin decreased the expression of the retinol dehydrogenase genes *RDH10* and *RDH16* via activation of the PI3K/AKT pathway, leading to phosphorylation and degradation of forkhead box O1 (FOXO1), an essential transcription factor for the expression of *RDH* genes (Obrochta et al. 2015). The marked abundance increase of RDH16 in MIDY samples supports the role of insulin as a negative regulator of RDH16 expression in vivo (Fig. 7). Significantly increased

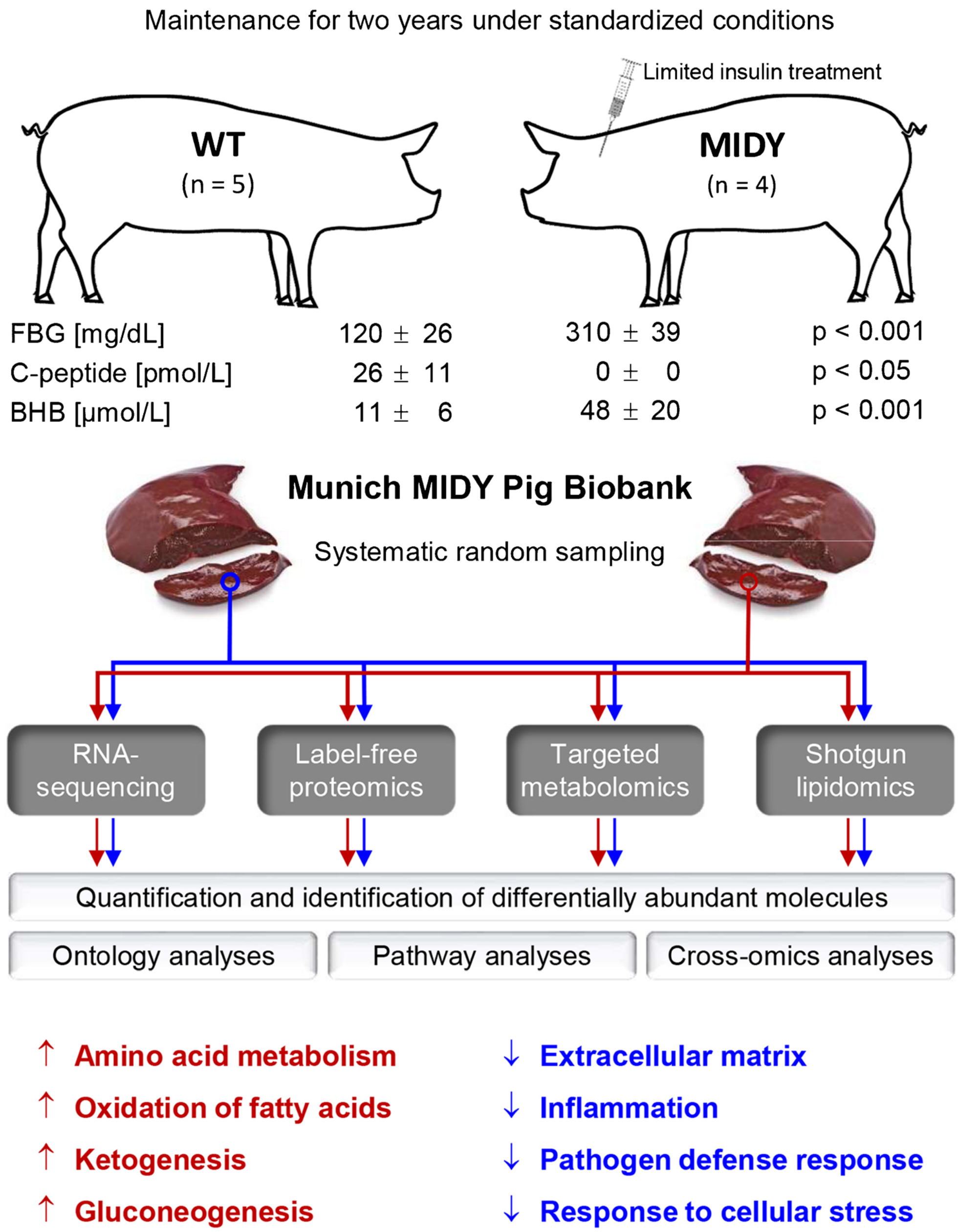

Fig. 6 Multi-omics analyses of liver tissue from long-term diabetic pigs and non-diabetic controls. *INS*^{C94Y} transgenic (MIDY) pigs and non-transgenic controls were maintained under standardized conditions for a period of 2 years. During that period, MIDY pigs were provided with limited insulin treatment. After necropsy, liver tissue was processed by multi-omics analyses (transcriptomics, proteomics, metabolomics, lipidomics) revealing four major upregulated (amino acid metabolism, oxidation of fatty acids, ketogenesis, gluconeogenesis) and downregulated (extracellular matrix, inflammation, pathogen defense response, response to cellular stress) pathways in MIDY pigs compared to controls (Backman et al. 2019)

concentrations of retinal and retinoic acid in MIDY samples demonstrate the biological relevance of increased RDH16 levels, since the abundance of *RDH10* mRNA was not altered in MIDY samples (Backman et al. 2019).

Increased amino acid catabolism and ketogenesis

Stimulated gluconeogenesis was associated with increased expression of enzymes involved in the degradation of specific amino acids. Amino acids need to be deaminated before their carbon skeletons can be used as substrates for gluconeogenesis or ketogenesis. The final acceptor of the α-amino group is α-ketoglutarate, resulting in glutamate that undergoes oxidative deamination. The released ammonia is detoxified via the urea cycle. Consistently, the mRNA levels for several urea cycle enzymes, i.e., carbamoyl-phosphate synthase 1 (CPS1), ornithine carbamoyltransferase (OTC), argininosuccinate synthase 1 (ASS1) and arginase 1 (ARG1), were significantly increased in MIDY samples and considerably increased protein concentrations of OTC and ARG1 were revealed (Backman et al. 2019). The latter may explain why arginine was not detected in MIDY liver samples.

Stimulated ketogenesis in MIDY pigs was evident by significantly increased plasma concentrations of beta hydroxybutyrate (Blutke et al. 2017). The multi-omics analysis of liver samples showed a marked upregulation of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), which catalyzes the first reaction of ketogenesis (reviewed in Newman and Verdin 2014). Insulin physiologically inhibits *HMGCS2* expression via the PI3K-AKT pathway, leading to phosphorylation and nuclear export of the forkhead box transcription factor FOXA2 (Wolfrum et al. 2003) and thus impaired transcription of *HMGCS2* (reviewed in Newman and Verdin 2014). This inhibitory cascade is lifted in MIDY pigs owing to their insulin deficiency. Interestingly, enzymes catalyzing the subsequent steps in ketogenesis, i.e., 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) and 3-hydroxybutyrate dehydrogenase 1 (BDH1) were not increased in abundance in MIDY vs. WT liver samples, supporting the notion that upregulation of HMGCS2 was sufficient for

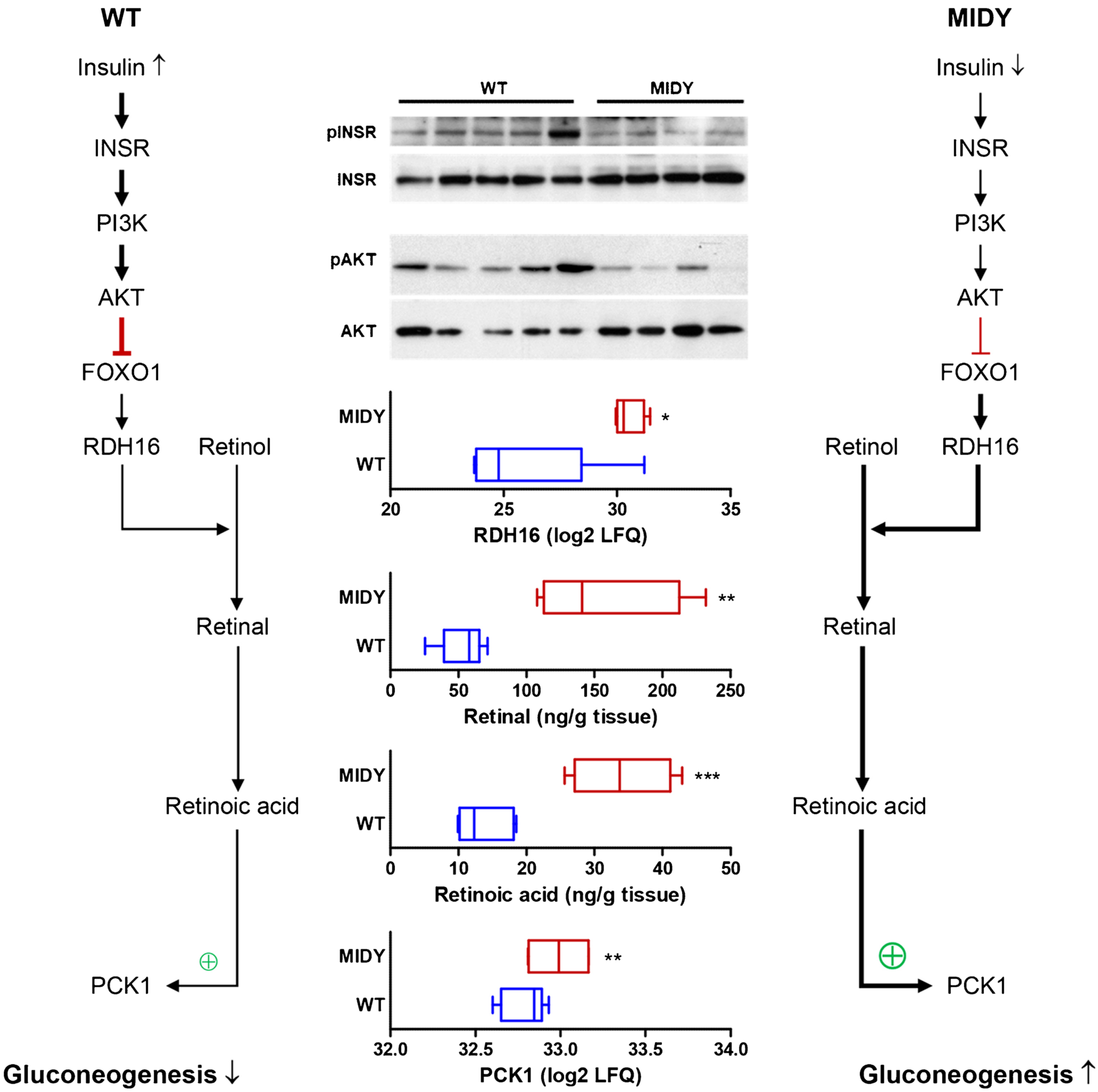

Fig. 7 Increased expression and activity of retinoldehydrogenase 16 (RDH16) as a potential key driver of stimulated gluconeogenesis in insulin-deficient DM. In the wild-type (WT) pig liver, insulin—via the PI3K-AKT pathway—leads to phosphorylation and degradation of FOXO1, a transcription factor stimulating the expression of *RDH16*. RDH16 catalyzes the first reaction of the two-step activation of vitamin A (retinol) into all-trans-retinoic acid (atRA), which stimulates—among many other genes—the expression of *PCK1* encoding the rate-limiting enzyme of gluconeogenesis. In the insulin-deficient MIDY pig liver, the inhibitory effect on *RDH16* expression is absent, leading to markedly increased levels of RDH16 and of retinal and atRA, associated with increased PCK1 levels. INSR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; FOXO1, forkhead box protein O1; PCK1, phosphoenolpyruvate carboxykinase. Data are from Backman et al. (2019)

stimulated ketogenesis in the liver of MIDY pigs. In hepatic ketogenesis, fatty acids are metabolized to acetyl-CoA via mitochondrial or peroxisomal beta-oxidation (reviewed in Newman and Verdin 2014). An increased ratio of long-chain acylcarnitines to free carnitine [(C16 + C18)/C0] in MIDY liver samples indicated an increased activity of carnitine palmitoyltransferase 1A (CPT1A), which catalyzes the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to L-carnitine, thereby facilitating the transfer of long-chain fatty acids from the cytosol into mitochondria for subsequent beta-oxidation (reviewed in Longo et al. 2016). An increased concentration of dicarboxylated acylcarnitines in MIDY samples suggested a higher level of omega-oxidation of fatty acids compared to WT samples (reviewed in Longo et al. 2016).

Increased antioxidative activity

Several antioxidative enzymes, such as glutathione S-transferase mu 2 (GSTM2) and glutathione peroxidase 1 (GPX1), were increased in abundance in the MIDY vs. WT samples (Backman et al. 2019), potentially in response to increased oxidative stress that is caused by hyperglycemia in the liver and many other tissues (reviewed in Mohamed et al. 2016). The abundance of isocitrate dehydrogenase 1 (IDH1), which has antioxidant properties by producing NADPH for the regeneration of glutathione (Itsumi et al. 2015), was also increased in MIDY samples (Backman et al. 2019).

Increased hepatic triacylglycerides (TAGs)

The increased content of TAGs in liver samples from MIDY pigs may be related to their elevated plasma concentrations of non-esterified fatty acids. Another contributing factor may be the altered expression of several apolipoproteins, in particular increased expression of APOA5, which is related to TAG transport and facilitates cytosolic storage of TAG in hepatocytes (Shu et al. 2010). Nevertheless, there was no histological evidence for fatty liver disease in MIDY pigs, although up to 40% of adult patients with type 1 diabetes were reported to have nonalcoholic fatty liver disease (NAFLD) (reviewed in Targher et al. 2018). This discrepancy is most likely due to the natural resistance of pigs against fatty liver disease, even in morbid obesity (Renner et al. 2018).

Reduced inflammatory and immune activity

Interestingly, several pathways related to immune functions were found to be less active in MIDY vs. WT liver samples (Backman et al. 2019). For instance, the transcript abundance for C-reactive protein (CRP), an indicator of inflammation (Cui et al. 2014; Lu et al. 2016), was significantly decreased. Furthermore, the concentration of high mobility group protein B1 (HMGB1), an early inflammatory mediator and a well-established damage-associated molecular pattern (DAMP) that activates Toll-like receptor 4 (TLR4), was decreased in MIDY samples. A number of proteins known to be upregulated upon TLR4 stimulation (Abbas et al. 2005), such as proteasome activator complex subunit 2 (PSME2), GMP reductase 1 (GMPR), protein transport protein Sec61 subunit beta (SEC61B) and 2′-5′-oligoadenylate synthetase 2 (OAS2), were found at lower abundance in MIDY vs. WT liver samples. The same was true for several other proteins known to be involved in or regulated by TLR signaling, including Rac family small GTPase 1 (RAC1), protein phosphatase 2 scaffold subunit A alpha (PPP2R1A), ubiquitin-conjugating enzyme E2 D2 (UBE2D2), S100 calcium-binding protein A1 (S100A1), legumain (LGMN) and mitogen-activated protein kinase 3 (MAPK3). A possible explanation is the reduced activation of AKT in the liver of MIDY pigs, which may restrain TLR-mediated PI3K-AKT-mTOR pathway signaling and consequently influence innate immune homeostasis (Backman et al. 2019). In addition, the expression of several major histocompatibility complex class 1 (SLA-1, SLA-2 and SLA-3) and class 2 (SLA-DQA1, SLA-DQB1, and SLA-DRA) genes was reduced in the MIDY liver samples. Collectively, these findings indicated that inflammatory and immune-related functions of the liver were downregulated in insulin-deficient DM. One pathway of activating liver-associated immune functions is the gut-liver axis, in which trace amounts of microbial products reach the liver via the portal circulation and are scavenged by hepatocytes and Kupffer cells (resident hepatic macrophages) (reviewed in Szabo 2015). Future studies including analyses of the gut microbiome need to clarify if changes in the gut-liver axis contributed to the reduced activation of inflammatory and immune functions in the MIDY liver samples.

In conclusion, the first multi-omics analysis of liver in insulin-deficient DM (Backman et al. 2019) identified key drivers of known functional consequences of insulin deficiency and revealed previously unknown consequences especially for inflammatory and immune functions of the liver. The multi-omics data set generated in this study provides a valuable resource for comparative studies with other experimental or clinical data sets.

Future perspectives

Although diabetes-associated alterations have been described in several organs and tissues of pig models, the full spectrum of complications as observed in humans is still not fully studied in pigs. Hyperglycemia only as, e.g., in MIDY pigs or STZ-induced diabetic pigs may not be sufficient or the time of exposure may not have been long enough to induce severe diabetic complications. To overcome this limitation, additional hypertension could be induced by drugs such as deoxycorticosterone acetate (Han et al. 2017) or by surgical interventions such as constriction of the aorta by extravascular banding, implantable adjustable occluders in the suprarenal aorta or renal arteries, or intravascular devices in the renal arteries (reviewed in Lerman et al. 2019). Recently, a novel spontaneously hypertensive Guizhou minipig model with elevated plasma catecholamine concentrations and increased levels of angiotensin-converting enzyme and angiotensin II type 1 receptors in the rostral ventrolateral medulla (RVLM) was described (Li et al. 2016a). Another frequent co-morbidity of DM is dyslipidemia that can be induced by special diets (e.g., Ludvigsen et al. 2015b; Renner et al. (2018) or genetic modifications (Al-Mashhadi et al. 2013; Chen et al. 2017a; Davis et al. 2014; Fang et al. 2018; Huang et al. 2017; Li et al. 2016b; Yuan et al. 2018). Figure 8 shows a study design outlining how the individual and additive effects of DM, hypertension and dyslipidemia could be dissected in a multi-organ, multi-omics approach.

A recent study suggested that the human-specific loss of CMP-*N*-acetylneuraminic acid hydroxylase (CMAH), which synthesizes sialic acid *N*-glycolylneuraminic acid (Neu5Gc), may be a risk factor for developing atherosclerosis in association with DM, dyslipidemia and/or hypertension (Kawanishi et al. 2019). A genetically engineered mouse model of atherosclerosis (*Ldlr*$^{-/-}$) showed more pronounced lesions when the *Cmah* gene was additionally inactivated (*Ldlr*$^{-/-}$/*Cmah*$^{-/-}$). CMAH deficient pigs have been generated as donors of cells, tissues and organs for xenotransplantation to prevent rejection induced by human natural antibodies against Neu5Gc (reviewed in Kemter et al. 2018). However, to our knowledge, *CMAH* knockout pigs have not been exposed to high-fat diet or DM to see whether they are more susceptible to vascular complications than pigs with intact *CMAH* alleles. This would provide additional important data to clarify the hypothesis that the evolutionary loss of CMAH in humans contributes to atherosclerosis (Kawanishi et al. 2019).

While our multi-omics study of liver tissues from MIDY and WT pigs (Backman et al. 2019) provided a comprehensive overview of molecular changes induced by chronic insulin deficiency and hyperglycemia, identification of the cell types involved in these changes was not addressed. Single-cell transcriptome profiling may help to overcome this limitation. Recently, a human liver cell atlas was established by single-cell RNA sequencing of about 10,000 cells from normal liver tissue from nine human donors, revealing previously unknown subtypes of endothelial cells, Kupffer cells and hepatocytes (Aizarani et al. 2019). Importantly, the analysis pipeline worked with fresh and cryopreserved liver tissue, offering the possibility to perform single-cell transcriptome profiling of organ samples in existing biobanks, such as the Munich MIDY Pig Biobank. In addition, single-cell transcriptomics pipelines have been established for murine (Park et al. 2018) and human kidney tissue (Young et al. 2018),

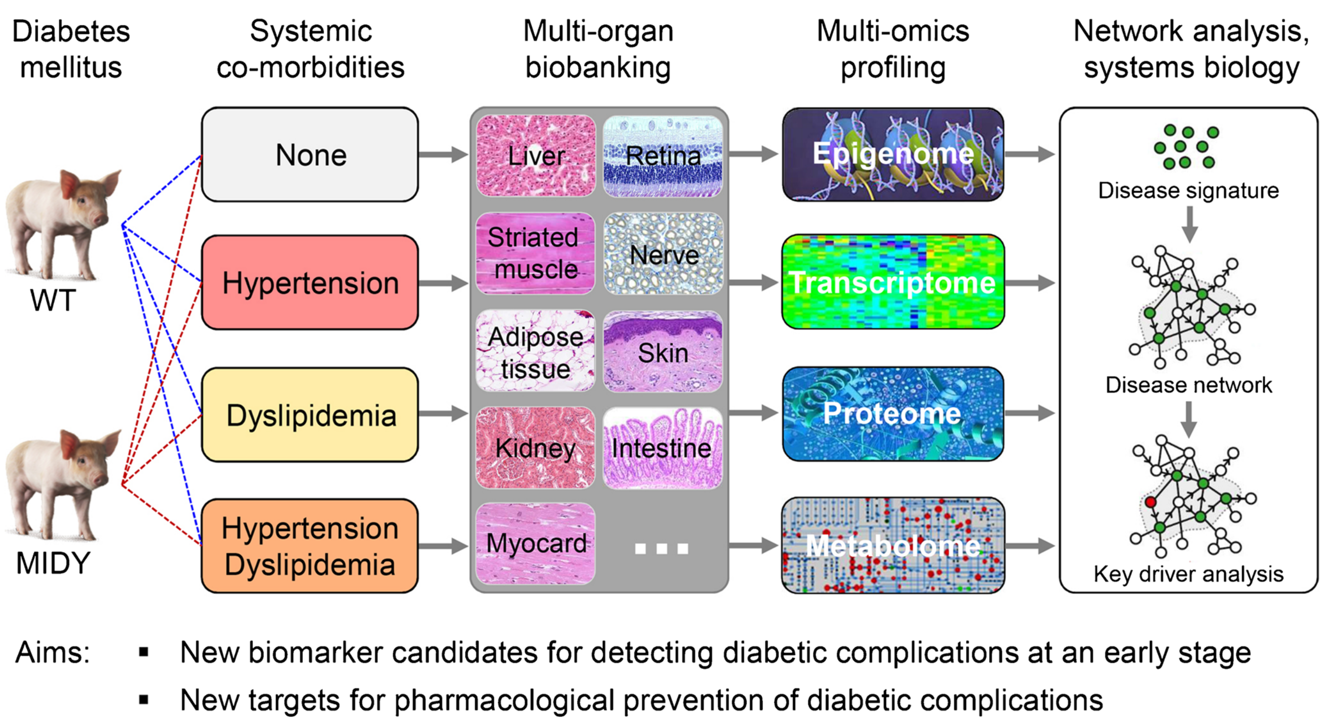

Fig. 8 Study design to dissect individual and additive effects of DM, hypertension and dyslipidemia in a multi-organ, multi-omics approach. A Biobank from MIDY pigs with and without induced comorbidities such as dyslipidemia and/or hypertension and non-transgenic littermates is established and selected tissues processed by a multi-omics approach to identify disease mechanisms and key players that can be potential biomarkers or targets for novel compounds

covering another important target organ for lesions induced by DM.

Pigs are not only interesting models for diabetes and obesity research but may also serve as a source of cells, tissues and organs for patients who suffer from terminal organ failure. The number of donated human organs and tissues falls far short of demand, a situation that threatens the life of many potential recipients. Alternative techniques to allotransplantation such as xenotransplantation are therefore urgently needed. The pig is the preferred donor species for a number of reasons, including size, anatomical and physiological similarities with humans and efficient techniques for genetic engineering/gene editing. More than 40 different genetic modifications have been introduced into pigs to prevent immune rejection of xenografts, to overcome physiological incompatibilities and to reduce the risk of transmitting zoonotic pathogens (reviewed in Wolf et al. 2019). Technical advances in the generation of genetically multi-modified pigs and new developments in the field of immunosuppression helped to significantly improve pig-to-nonhuman primate xenotransplantation in many areas. This has been demonstrated for cell and tissue xenografts (e.g., pancreatic islets; reviewed in Kemter et al. 2018; Kemter and Wolf 2018), and also for vascularized organs, especially the kidney (survival > 1 year; reviewed in Cooper et al. 2018) and the heart (survival for up to 945 days in a non-life-supporting model; Mohiuddin et al. 2016 and more than 180 days in a life-supporting model; Langin et al. 2018). Xenotransplantation of porcine tissues and organs can thus be considered as a realistic therapeutic option in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed.

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