

Dominant-negative mutant hepatocyte nuclear factor 1 α induces diabetes in transgenic-cloned pigs

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Abstract Pigs have been recognized as an excellent biomedical model for investigating a variety of human health issues. We developed genetically modified pigs that exhibit the apparent symptoms of diabetes. Transgenic cloned pigs carrying a mutant human hepatocyte nuclear factor 1 α gene, which is known to cause the type 3 form of maturity-onset diabetes of the young, were produced using a combined technology of intracytoplasmic sperm injection-mediated gene transfer and somatic cell nuclear transfer. Although most of the 22 cloned offspring obtained died before weaning, four pigs that lived for 20–196 days were diagnosed as diabetes mellitus with nonfasting blood glucose levels greater than 200 mg/dl. Oral glucose tolerance test on a cloned pig also revealed a significant increase of blood glucose level after glucose loading. Histochemical analysis of pancreas tissue from the cloned

pigs showed small and irregularly formed Langerhans Islets, in which poor insulin secretion was detected.

Keywords Transgenic-cloned pig · Intracytoplasmic sperm injection · Somatic cell nuclear transfer · Diabetic model · HNF-1 α P291fsinsC · Dominant negative

Introduction

Advances in transgenic technologies have enabled the production of animals showing disease progression similar to that seen in humans (Prather et al. 2003). These animals provide models for etiological and pathogenetic research and are also useful tools for the development of new drugs (Larsen and Rolin 2004; Lunney 2007; Prather et al. 2003).

Diabetes mellitus, characterized by high blood glucose levels and various complications such as diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy, is one of the most serious chronic diseases in modern society (Wild et al. 2004). This disease is caused by a physical or functional loss of the β -cell mass, primarily due to an autoimmune process (type 1 etiological process) and/or increased need for insulin due to insulin resistance (type 2 process; Tuomi 2005). Development of diabetes has been attributed to multiple genetic and/or environmental factors (Knip et al. 2005; Taylor et al. 2007). For example, genetic mutation of hepatocyte nuclear

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factor (HNF)-1 α is described as a cause of diabetes (Yamagata et al. 1996; Yamagata 2003). HNF-1 α is a transcription factor expressed in the liver, kidney, small intestine, and spleen, as well as in the pancreas (Miquerol et al. 1994; Nicosia et al. 1990). Mutations in HNF-1 α cause the type 3 form of maturity-onset diabetes of the young (MODY3), which is an early-onset non-insulin-dependent type of diabetes characterized by autosomal dominant inheritance (Yamagata et al. 1996; Yamagata 2003).

Transgenic (tg) mice carrying mutant HNF-1 α have been produced, and those mice show diabetic symptoms such as high blood glucose levels, impaired insulin secretion, and hypoplasia of the islets of Langerhans (Hagenfeldt-Johansson et al. 2001; Watanabe et al. 2007; Yamagata et al. 2002). Small animal models of disease such as in rodents, however, often do not faithfully mimic the relevant human conditions (Lunney 2007). In contrast, diabetic models in pigs, which are well known to display anatomical and physiological similarities to humans, would be useful in research, including the development of new therapies and drugs (Larsen and Rolin 2004; Lunney 2007).

Several porcine models of diabetes induced by streptozotocin administration have been reported (Larsen et al. 2002; Murakami et al. 2007), but there have been no porcine models of diabetes caused by transgenesis to date. The present study produced tg-cloned pigs carrying the human HNF-1 α mutant gene, and demonstrated that these pigs exhibit the pathophysiological characteristics of diabetes.

Materials and methods

Animal care

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC-04-004) and the Animal Code of Bios (ACB-2005; Bios, Kanagawa, Japan).

DNA constructs

An expression vector (Fig. 1) for the mutant human HNF-1 α cDNA (HNF-1 α P291fsinsC) (Yamagata et al. 1996) was constructed as described previously (Watanabe et al. 2007). This DNA vector was constructed based on pBluescript SK(-) (Stratagene, LaJolla, CA, USA) and consists of the enhancer for the immediate-early gene of human cytomegalovirus (CMV-IE, 0.4 kbp) followed by the porcine insulin promoter (0.7 kbp), the human HNF-1 α P291fsinsC cDNA (2.3 kbp), the SV40 poly-adenylation signal (0.1 kbp), and the chicken β -globin insulator (1.2 kbp, Watanabe et al. 2007). The transgene fragment was excised from the plasmid vector by enzymatic digestion, separated by gel electrophoresis, and purified using a GENECLON II Kit (MP Biomedicals, Solon, OH, USA).

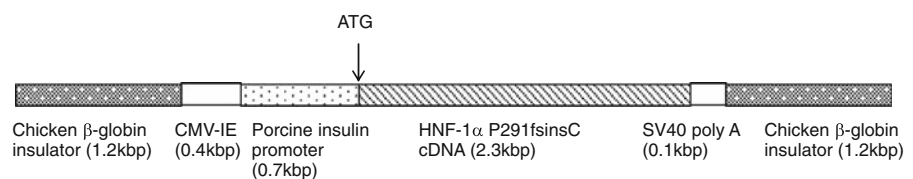
Production of tg-fetuses and somatic cell cloning

Tg-cloned pigs carrying human HNF-1 α P291fsinsC cDNA were produced using a combined method of intracytoplasmic sperm injection (ICSI)-mediated gene transfer and somatic cell nuclear transfer (SCNT) (Kurome et al. 2006). Introduction of the transgene into porcine in vitro-matured (IVM) oocytes by an ICSI-mediated gene transfer method was undertaken as described elsewhere (Kurome et al. 2006, 2007).

A total of 164 embryos that developed from sperm-injected oocytes were transferred into two estrus-synchronized recipient gilts after culture for 1 or 2 days in PZM5 (Functional Peptide, Yamagata, Japan). Recipients were autopsied to recover the fetuses at gestational day 36.

Among the ten fetuses obtained, two tg-fetuses (#111-2 and #111-4) were identified by PCR and Southern blot (Fig. 2), and used to collect nuclear donor cells. PCR conditions have been reported previously (Watanabe et al. 2007). Sex of the fetuses was also confirmed by PCR (Kawarasaki et al. 1995). In addition,

Fig. 1 Structure of an expression vector for the mutant human HNF-1 α cDNA



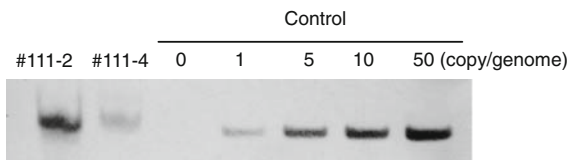


Fig. 2 Southern blotting for the HNF-1 α P291fsinsC gene in nuclear donor cells

the copy number of the transgene was confirmed by Southern blot analysis (Watanabe et al. 2007).

A primary culture of fibroblast cells was established from one of the tg-fetuses by routine cell culture technology and was maintained in Dulbecco's Modified Eagle Medium supplemented with 15% fetal calf serum (FCS). After 3–4 passages, cells were stored frozen until used for nuclear transfer.

SCNT was performed using IVM oocytes as recipient cytoplasts, as described previously (Kurome et al. 2006, 2008). Briefly, oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm using a beveled pipette (diameter 30 μ m) in Tyrode's lactose medium supplemented with 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone in the presence of 0.1 μ g/ml demecolcine, 5 μ g/ml cytochalasin B, and 10% FCS (Yin et al. 2002). Fetal fibroblast cells established from a tg-fetus were used as nuclear donors after cell cycle synchronization by serum starvation for 48 h. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. Membrane fusion of the donor cell and the cytoplasm was induced by applying a single direct current (DC) pulse (200 V/mm, 20 μ s \times 1) and pre and post-pulse alternating current fields of 5 V, 1 MHz for 5 s in a 280 mM mannitol solution (pH 7.2) containing 0.15 mM MgSO₄, 0.01% (w/v) polyvinyl alcohol, and 0.5 mM HEPES. Reconstructed embryos were cultured in NCSU23 (Petters and Wells 1993) for 1–1.5 h, followed by electrical activation. Cloned embryos were surgically transferred into estrus-synchronized recipients after culture for 1 or 2 days in PZM5 medium.

Characterization of tg-cloned pigs

Transcription of the transgene in the organs of tg-cloned pigs was confirmed by RT-PCR (Watanabe et al. 2007). Translation of the transgene was confirmed by western blot analysis as described

previously (Watanabe et al. 2007), except for the use of the polyclonal goat anti-human HNF-1 α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the secondary antibody, alkaline phosphatase-conjugated polyclonal donkey anti-goat IgG (Santa Cruz Biotechnology).

Biochemical analysis of blood components

Venous blood samples were collected in tubes containing heparin to determine the concentrations of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GTP), albumin (ALB), globulin (Ig), total protein (TP), total bilirubin (TB), total cholesterol (T-CHO), triglyceride (TG), phospholipids (PL), creatine kinase (CK), cholinesterase (ChE), blood urea nitrogen (BUN), creatinine (CRE), uric acid (UA), glucose (GLU), calcium (Ca), inorganic phosphate (IP), sodium (Na), potassium (K), and chloride (Cl) using an Auto Chemical Analyzer (JCA-DM8; JEOL, Tokyo, Japan). The concentration of 1,5-anhydroglucitol (1,5-AG) was determined using the enzymatic method (SRL, Tokyo, Japan).

Oral glucose tolerance test (OGTT)

An OGTT was performed on a tg-cloned pig showing high blood glucose level on postpartum day 20. After fasting for 13 h, a glucose solution (3 g glucose/kg body weight) was administered to the pig through an oral catheter. Blood samples were drawn from the cervical vein at 0, 30, 60, 120, 180, and 240 min after glucose loading.

Histochemical analysis and immunohistochemical analysis

Both tg-cloned and control pigs were sacrificed by exsanguination under anesthesia. Dissected organs were fixed in a 10% neutral-buffered formalin solution, embedded in paraffin, sectioned, and finally stained with hematoxylin and eosin (HE).

For immunohistochemistry, sections were incubated with the diluted primary anti-pig insulin polyclonal guinea pig antibody for 30 min at room temperature, followed by incubation with the secondary antibody of "EnVision + Rabbit/HRP" (DakoCytomation,

Glostrup, Denmark) for 30 min, and finally stained with diaminobenzidine (DakoCytomation, Glostrup, Denmark).

Statistics

Values for the biochemical parameters of the blood plasma were expressed as mean \pm SD. Differences were analyzed using a two-tailed unpaired Student's *t* test.

Results

Production of tg-cloned pigs with SCNT

Fibroblast cells established from a male transgenic fetus (#111-2) carried ten copies of the transgene, as confirmed by Southern blot analysis (Fig. 2). These cells were used as nuclear donor cells in SCNT. A total of 927 cloned embryos were produced and transferred into 12 recipient gilts, all of which became pregnant. Although one gilt miscarried on gestational day 36, 11 gilts farrowed a total 22 live and 9 stillborn piglets.

The mean body weight of the tg-cloned piglets was 857 g (range 499–1,310 g). All piglets were confirmed as transgenic by PCR. While 17 of the 22 offspring died within 10 days after birth, five lived 20 days or longer (Fig. 3). Four of these piglets, DI09, DI12, DI17, and DI21, which lived for 30, 22, 196, and 120 days, respectively, were judged as



Fig. 3 A transgenic cloned piglet with diabetes mellitus, 19 days postpartum

diabetic, with nonfasting blood glucose levels greater than 200 mg/dl. DI03 died before measuring the blood sugar level at 20 days after birth.

DI17 and DI21 developed quadriplegia at 166 and 113 days, respectively, and subsequently could not move independently. Cause of death was pneumonia for DI09 but was unknown for the remaining three animals.

Transcription and translation of the transferred gene

Analysis was performed to determine if the introduced transgene (HNF-1 α P291fsinsC) was transcribed and translated. Transcription of the transgene was confirmed by RT-PCR in organs including the brain, heart, lung, liver, pancreas, spleen, and kidney. Western blotting using an anti-human HNF-1 α polyclonal antibody detected protein expression of the transgene in the heart, pancreas, and kidney (Fig. 4).

Biochemical analysis of plasma from tg-cloned pigs

As shown in Fig. 5, DI09, one of the five cloned pigs that survived more than 20 days showed a nonfasting blood glucose level of 231 mg/dl at postpartum day

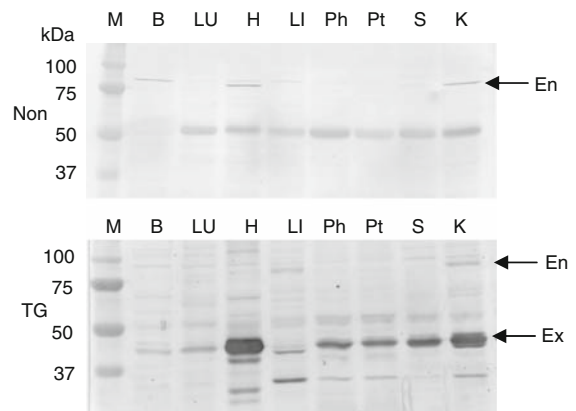


Fig. 4 Western blot analysis of the HNF-1 α P291fsinsC gene product in transgenic (TG) and nontransgenic (Non) piglets. The 315 amino acid HNF1 α P291fsinsC protein (Ex) was detected in the brain (B), lung (LU), heart (H), pancreatic head (Ph), pancreatic tail (Pt), spleen (S), and kidney (K). In the liver (LI) of transgenic piglets, the HNF-1 α P291fsinsC protein was not detected. M: molecular size marker. En: endogenous HNF-1 α

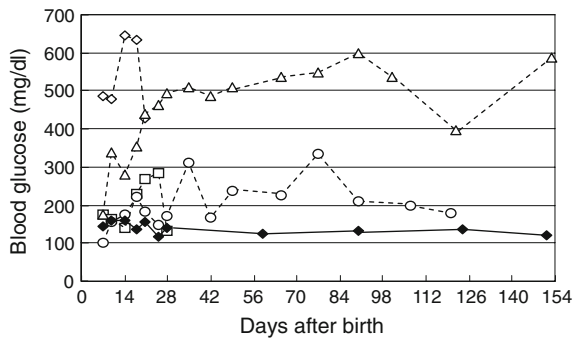


Fig. 5 Nonfasting blood glucose concentrations of the control piglets (—◆—, *n* = 3) and the transgenic cloned piglets, DI09 (—□—), DI12 (—◇—), DI17 (—△—), and DI21 (—○—)

18. This level remained greater than 200 mg/dl until death at day 30 due to pneumonia. DI12 showed a nonfasting blood glucose level of 485 mg/dl at 7 days after birth, and it remained greater than 400 mg/dl until death at 22 days. The blood glucose level of DI17 was 339 mg/dl at 10 days after birth, and it remained about 400 mg/dl until day 164. Nonfasting blood glucose level of DI21 reached 220 mg/dl at 18 days after birth, then it fluctuated below or over 200 mg/dl until day 49. After day 49, it remained greater than 200 mg/dl until day 107.

DI12 underwent OGTT at 20 days after birth. Blood glucose level after fasting for 13 h was 278 mg/dl and increased to 788 mg/dl 2 h after glucose loading (Fig. 6).

The plasma levels of 1,5-AG in tg-cloned pigs and in control wild-type pigs were compared. In contrast to a mean level of $6.8 \pm 1.5 \mu\text{g/ml}$ (range 4.5–11.9 $\mu\text{g/ml}$) in wild-type pigs, the concentration of 1,5-AG was significantly lower in the tg-cloned pigs DI09 and DI12, at 3.3 $\mu\text{g/ml}$ (age 28 days) and 1.1 $\mu\text{g/ml}$ (age 20 days), respectively. In DI17 and DI21, 1,5-AG levels reflected increases in blood glucose and were significantly lower than that in the control group (Fig. 7).

Furthermore, we investigated 22 biochemical parameters in the plasma of tg-animals that lived at least 20 days. Table 1 shows the blood biochemical findings for DI17 and DI21 at 48 and 120 days after birth. Creatinine concentrations for the tg-cloned pigs (0.6–0.8 mg/dl) were comparable to those in control pigs (0.7–0.9 mg/dl), and remained within the normal range until death. This suggests that the kidneys of tg-cloned pigs functioned normally. At the age of

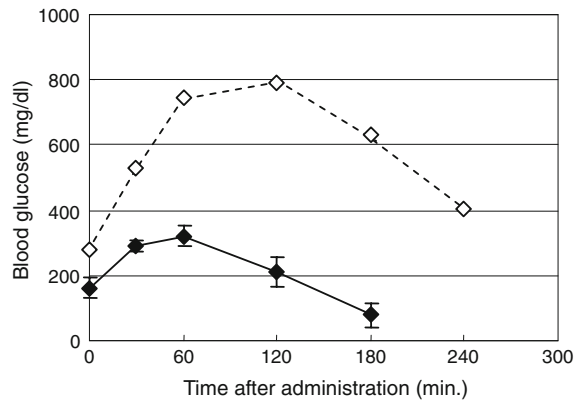


Fig. 6 Oral glucose tolerance test of the control piglets (—◆—, *n* = 3) and the transgenic cloned piglet, DI12 (—◇—) 20 days postpartum

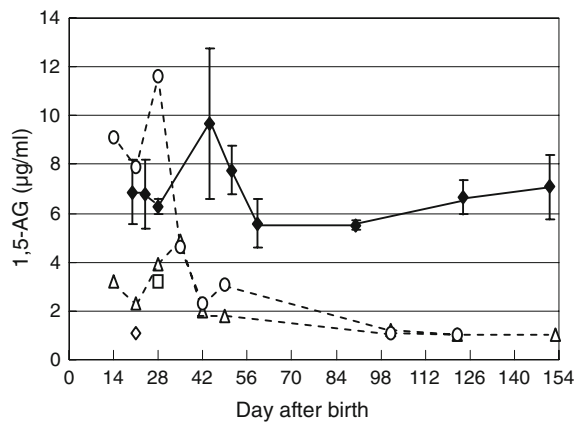


Fig. 7 1,5-AG concentrations of the control piglets (—◆—, *n* = 3) and the transgenic cloned piglets, DI09 (—□—), DI12 (—◇—), DI17 (—△—), and DI21 (—○—)

48 days, AST was within the normal range, but when compared to control pigs, AST was higher at the age of 120 days for the tg-cloned pigs (DI17: 116.3 units/l, DI21: 119.1 units/l vs. control: 20.4 ± 6.8 units/l), suggesting the possibility of hepatic dysfunction. At 120 days values of LDH and CK also showed large difference from controls. Increase of LDH may indicate hepatic dysfunction. High CK value is one of the characteristics of diabetes, though influence of stress caused by blood sampling can not be ruled out.

Histological analysis of tg-cloned pigs

Histological examination of the pancreas, kidneys, and liver was performed in the two pigs with longer

Table 1 Biochemical parameters in the plasma of the transgenic cloned piglets, DI17 and DI21, and control piglets at 48 and 120 days after birth

	48 Days			120 Days		
	DI17	DI21	Control	DI17	DI21	Control
LDH (units/l)	2683.6	2475.7	1922.3 ± 274.6	3122.7	4362.9	1190.4 ± 101.5
AST (units/l)	37.2	61.7	52.9 ± 5.8	116.3	119.1	20.4 ± 6.8
ALT (units/l)	33.3	36.9	34.2 ± 4.9	53.9	71.6	37.4 ± 4.3
ALP (units/l)	1342.3	769.4	1334.0 ± 99.8	408.2	144.5	599.6 ± 217.3
γ-GTP (units/l)	29.7	26.0	40.9 ± 12.8	46.4	30.6	30.5 ± 18.0
ALB (g/dl)	2.3	2.3	3.8 ± 0.2	3.0	3.2	3.9 ± 0.2
Ig (g/dl)	2.5	2.6	1.6 ± 0.2	2.3	2.5	2.2 ± 0.2
TP (g/dl)	4.9	4.9	5.3 ± 0.2	5.3	5.6	6.1 ± 0.0
TB (mg/dl)	0.1	0.1	0.3 ± 0.1	0.3	0.3	0.2 ± 0.0
T-CHO (mg/dl)	92.5	87.4	73.9 ± 13.6	75.4	109.8	89.9 ± 14.3
TG (mg/dl)	26.5	34.1	24.4 ± 11.2	17.6	66.2	24.0 ± 3.6
PL (mg/dl)	107.2	93.5	91.6 ± 25.5	71.6	105.9	86.0 ± 17.4
CK (units/l)	3628.0	3984.6	2212.8 ± 1307.2	3141.1	4297.4	421.7 ± 81.4
ChE (units/l)	241.6	233.0	240.4 ± 26.1	284.9	215.5	197.9 ± 5.6
BUN (mg/dl)	21.3	11.3	9.1 ± 2.0	17.0	21.1	11.9 ± 1.0
CRE (mg/dl)	0.8	0.7	0.7 ± 0.1	0.8	0.6	0.9 ± 0.0
UA (mg/dl)	0.02	0.02	0.04 ± 0.03	0.02	0.02	0.03 ± 0.00
GLU (mg/dl)	508.0	239.1	80.3 ± 14.9	394.8	178.1	134.9 ± 7.5
Ca (mg/dl)	11.1	11.1	11.6 ± 0.4	8.6	9.5	11.1 ± 0.3
IP (mg/dl)	4.4	6.8	9.6 ± 0.5	5.9	6.0	8.4 ± 0.5
Na (mEq/l)	143.2	154.2	140.5 ± 2.7	142.5	146.6	143.1 ± 0.9
K (mEq/l)	5.8	6.3	5.9 ± 0.5	4.6	5.9	5.9 ± 1.5
Cl (mEq/l)	105.7	112.6	101.0 ± 3.2	114.1	109.8	108.3 ± 0.3
1,5-AG (μg/ml)	1.8	3.1	8.8 ± 2.2	1.0	1.0	6.7 ± 0.7

survival with hyperglycemia (DI21 120 days; DI17 196 days) and in DI09 and DI12, which had hyperglycemia but died between 20 and 30 days old. In the tg-cloned pigs, the pancreas contained a mixture of large acinar cells filled with secretory granules and slightly smaller acinar cells (Fig. 8a, b). Immunostaining showed disorganized islets of Langerhans, including numerous degenerative cells (Fig. 8c, d). In DI12, an animal with severe diabetes, the islets of Langerhans were comprised of only insulin-negative cells.

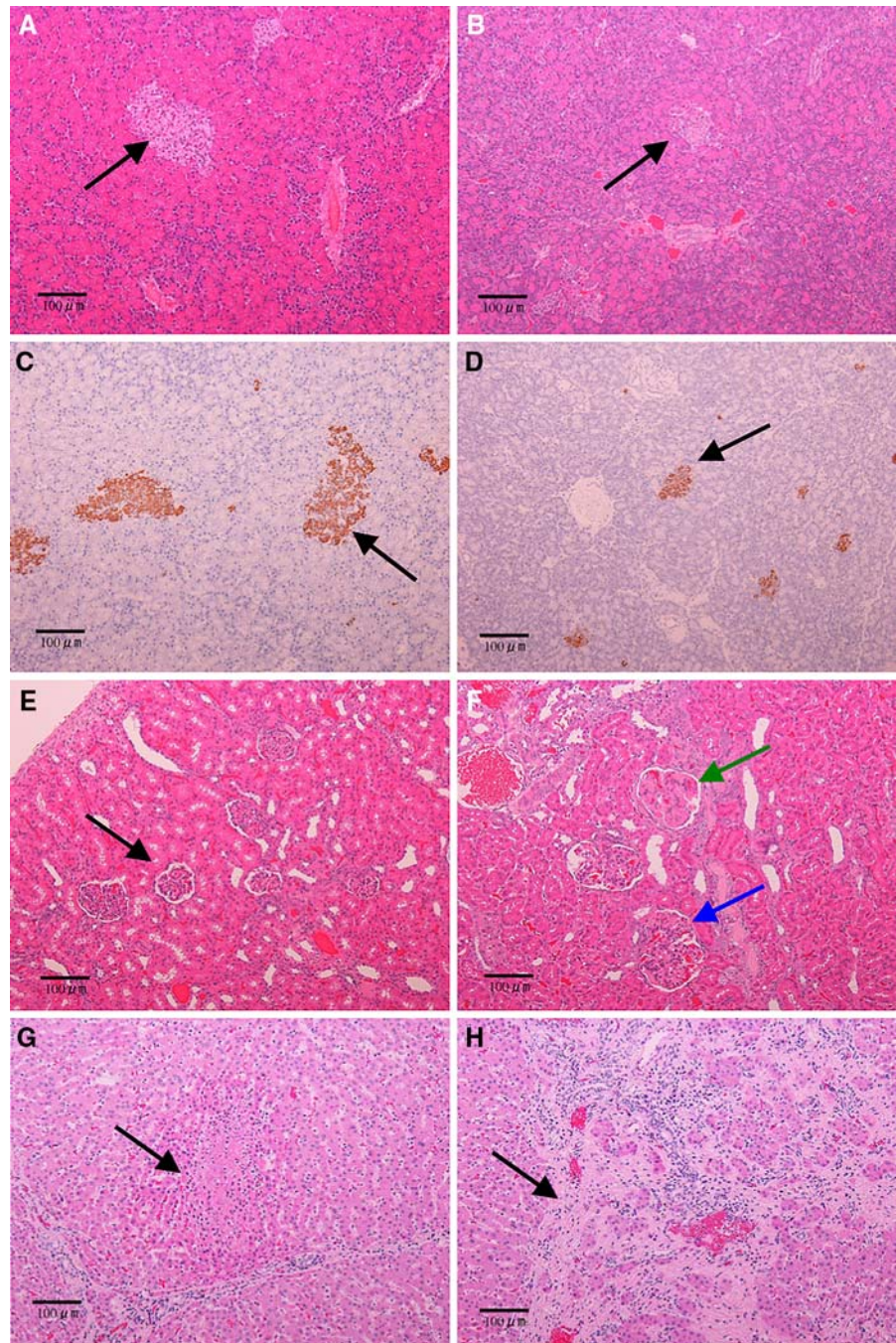
In kidney tissue from DI09 and DI12, HE-stained sections showed relatively immature glomerular structures and vacuolation of proximal tubule epithelial cells. In kidney tissue from DI17 and DI21, some glomerular sclerosis and hypertrophy was evident (Fig. 8e, f).

In DI09 and DI12, liver tissue showed delayed growth with extramedullary hematopoiesis. In DI17, some hepatocyte focal necrosis and fibrosis was observed (Fig. 8 g, h).

Discussion

When developing animal models of human diseases, the extent of genetic modification can have adverse effects on long-term survival and reproductive ability. This not only limits the usefulness of the model animals but also hampers breeding and establishment of strains. In the present study, we first obtained transgenic fetuses using an ICSI-mediated gene transfer method and then produced tg-cloned pigs with established primary culture cells. Even in pig

Fig. 8 Histochemical analysis of the pancreas, kidney and liver of the transgenic cloned piglets. Paraffin-embedded sections of pancreas (**a, b**), kidney (**e, f**) and liver (**g, h**) tissues were stained with HE. Pancreas section was also stained with an antibody against insulin (**c, d**). Langerhans islets (*arrow*) from transgenic cloned piglets (**b, d**) were smaller than those from the controls (**a, c**). The *blue* and *green* arrows in the kidney image of the transgenic cloned piglet (**f**) show representative glomerular hypertrophy and glomerular sclerosis, respectively. A glomerulus of the control piglet is indicated by an arrow in **E**. In the liver of the transgenic cloned piglet focal necrosis (*arrow*: **g**) and fibrosis (*arrow*: **h**) were observed. Scale bar = 100 μ m



disease models with severe phenotypes that limit individual growth and survival, the combined use of ICSI-mediated gene transfer and SCNT can enable the production of individuals from transgenic fetal cells.

The present study successfully produced several tg-cloned pigs with diabetes that exhibited high blood

glucose level. However, most tg-cloned pigs died within 2 weeks after birth. The degree to which pathogenesis induced by the transfer of the mutant HNF1 α gene played a role in this short survival is unknown. In addition, effect of insertional mutation caused by transgene integration on high mortality of

the tg-cloned pigs cannot be ruled out. In clones produced by SCNT, death during the neonatal period due to epigenetic modification is common (Park et al. 2005), so the possible role of a similar modification in early clonal death in our study cannot be excluded. The group of tg-cloned pigs in this study was derived from the same nuclear donor cell, and the marked differences in disease severity and survival among individuals suggest effects of epigenetic modification in somatic cell-cloned pigs.

Diabetes in tg-cloned pigs was confirmed by nonfasting blood glucose levels and an OGTT. Demonstration of persistent, rather than transient, elevation of blood glucose was important. We therefore measured 1,5-AG in plasma which reflects blood glucose concentrations in patients with diabetes within the several previous days, instead of glycosylated hemoglobin (HbA1c). In pigs, hemoglobin contains very little HbA1c due to the limited glucose permeability of erythrocytes (Higgins et al. 1982). Normally, 1,5-AG is filtered and completely reabsorbed in the kidney (Yamanouchi et al. 1989). In diabetes with elevated blood glucose levels, glucose is not completely reabsorbed in the kidney, so serum 1,5-AG is decreased due to competitive inhibition in renal tubular reabsorption (Yamanouchi et al. 1992, 1996). Changes in the concentration of 1,5-AG depend on the duration and degree of glycosuria. Thus, 1,5-AG responds sensitively and rapidly to changes in the concentration of serum glucose, clearly reflecting elevations of glucose within the few previous days (Yamanouchi et al. 1992, 1996). In tg-cloned pigs, the decrease in 1,5-AG, along with increased blood glucose, provides further evidence of chronic hyperglycemia.

HNF1 α mutations have been identified as a cause of MODY3 (Yamagata et al. 1996). In MODY3, hyperglycemia develops due to insufficient insulin secretion by β cells and abnormal islet structure. In addition, the MODY3 phenotype is often associated with impaired renal resorption of glucose, whereas other tissues that also express HNF1 α , including the liver and intestine, do not show clinically significant defects (Iwasaki et al. 1998; Thomas et al. 2002).

Dominant-negative mutant HNF1 α tg-mice have diabetes and abnormal pancreatic islet morphogenesis, but the liver and kidneys are normal (Hagenfeldt-Johansson et al. 2001; Watanabe et al. 2007; Yamagata et al. 2002). These features are similar to

MODY3 in humans (Hagenfeldt-Johansson et al. 2001; Watanabe et al. 2007; Yamagata et al. 2002). In our tg-cloned pigs, as in humans and mice, no renal dysfunction was evident by biochemical analysis. However, the clinical biochemistry values suggested possible hepatic inflammation. The relationship to transgene expression is unknown.

DI09 and DI12, which died on days 30 and 22, respectively, showed immature renal development. While abnormal renal function was not detected based on creatinine data in DI17 and DI21, which exhibited relatively longer survival, histopathological findings confirmed several cases of glomerular hyperplasia. Whether these lesions were attributable to the dominant-negative effects of mutant HNF1 α , secondary changes due to chronic hyperglycemia, or anomalies associated with SCNT is difficult to say with certainty. HNF-1 α knock-out mice exhibit a phenotype that lacks insulin secretion with severe renal dysfunction (Pontoglio et al. 1996, 1998). Although western blotting showed high expression of the mutant protein in the kidneys, polyuria and proteinuria, as confirmed in knockout mice, were not observed. Glomerular degeneration and hypertrophy have been reported as early lesions in diabetic nephropathy (Mahimainathan et al. 2006), so renal lesions in tg-cloned pigs were probably due to chronic hyperglycemia.

RT-PCR confirmed expression of mutant mRNA in the livers of tg-cloned pigs, although western blotting showed no mutant protein. Changes in the liver were thus also likely due to chronic hyperglycemia. Recently, a nonsense-mediated decay (NMD) pathway that degrades mRNA transcripts harboring premature termination codons (PTCs) and prevents expression of mutant proteins has been reported (Frischmeyer and Dietz 1999). The most common mutation, P291fsinsC, is known to show dominant-negative activity when overexpressed in MIN6 mouse insulinoma, C33 human epithelial cervical carcinoma, and INS-1 rat cells in vitro (Vaxillaire et al. 1999; Wang et al. 2000; Yamagata et al. 1998), but is not found in HeLa cells (Thomas et al. 2002). Such diverse effects of P291fsinsC may be ascribed to the NMD pathway (Harries et al. 2004). In the tg-cloned pigs in our study, the absence of significant transgene effects on the liver may be attributable to organ differences in PTC stability, which would influence transgene translation.

In conclusion, the present study suggests the possibility of preparing a porcine diabetes model by applying to pigs the dominant-negative mutant approach verified in mice. As the homology of human and porcine proteins is often high, this strategy of inducing a dominant-negative mutant using a human mutant gene may be used to prepare a wide variety of disease models. In the future, however, it will be necessary to closely analyze interference between porcine endogenous proteins and human mutant proteins originating from transgenes. To develop a practically useful diabetes model, further study will be needed to improve postnatal survival of the tg-cloned pigs.

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References

- Frischmeyer PA, Dietz HC (1999) Nonsense-mediated mRNA decay in health and disease. *Hum Mol Genet* 8:1893–1900. doi:10.1093/hmg/8.10.1893
- Hagenfeldt-Johansson KA, Herrera PL, Wang H, Gjinovci A, Ishihara H, Wollheim CB (2001) β -Cell-targeted expression of a dominant-negative hepatocyte nuclear factor-1 α induces a maturity-onset diabetes of the young (MODY) 3-like phenotype in transgenic mice. *Endocrinology* 142:5311–5320. doi:10.1210/en.142.12.5311
- Harries LW, Hattersley AT, Ellard S (2004) Messenger RNA transcripts of the hepatocyte nuclear factor-1 α gene containing premature termination codons are subject to nonsense-mediated decay. *Diabetes* 53:500–504. doi:10.2337/diabetes.53.2.500
- Higgins PJ, Garlick RL, Bunn HF (1982) Glycosylated hemoglobin in human and animal red cells. Role of glucose permeability. *Diabetes* 31:743–748. doi:10.2337/diabetes.31.9.743
- Iwasaki N, Ogata M, Tomonaga O, Kuroki H, Kasahara T, Yano N, Iwamoto Y (1998) Liver and kidney function in Japanese patients with maturity-onset diabetes of the young. *Diabetes Care* 21:2144–2148. doi:10.2337/diacare.21.12.2144
- Kawarasaki T, Kohsaka T, Sone M, Yoshida M, Bamba K (1995) Detection of Y-bearing porcine spermatozoa by in situ hybridization using digoxigenin-labeled, porcine male-specific DNA probe produced by polymerase chain reaction. *Mol Reprod Dev* 40:455–459. doi:10.1002/mrd.1080400409
- Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Ålerblom HK (2005) Environmental triggers and determinants of type 1 diabetes. *Diabetes* 54:S125–S136. doi:10.2337/diabetes.54.suppl_2.S125
- Kurome M, Ueda H, Tomii R, Naruse K, Nagashima H (2006) Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. *Transgenic Res* 15:229–240. doi:10.1007/s11248-006-0004-5
- Kurome M, Saito H, Tomii R, Ueno S, Hiruma K, Nagashima H (2007) Effects of sperm pretreatment of on efficiency of ICSI-mediated gene transfer in pigs. *J Reprod Dev* 53:1217–1226. doi:10.1262/jrd.19069
- Kurome M, Hisatomi H, Matsumoto S, Tomii R, Ueno S, Hiruma K, Saito H, Nakamura K, Okumura K, Matsumoto M, Kaji Y, Endo F, Nagashima H (2008) Production efficiency and telomere length of the cloned pigs following serial somatic cell nuclear transfer. *J Reprod Dev* 54:254–258. doi:10.1262/jrd.20038
- Larsen MO, Rolin B (2004) Use of the Göttingen minipig as a model of diabetes, with special focus on type 1 diabetes research. *ILAR J* 45:303–313
- Larsen MO, Wilken M, Gotfredsen CF, Carr RD, Svendsen O, Rolin B (2002) Mild streptozotocin diabetes in the Göttingen minipig. A novel model of moderate insulin deficiency and diabetes. *Am J Physiol Endocrinol Metab* 282:E1342–E1351
- Lunney JK (2007) Advances in swine biomedical model genomics. *Int J Biol Sci* 3:179–184
- Mahimainathan L, Das F, Venkatesan B, Choudhury GG (2006) Mesangial cell hypertrophy by high glucose is mediated by downregulation of the tumor suppressor PTEN. *Diabetes* 55:2115–2125. doi:10.2337/db05-1326
- Miquerol L, Lopez S, Cathalie N, Tulliez M, Raymondjean M, Kahn A (1994) Expression of the L-type pyruvate kinase gene and the hepatocyte nuclear factor 4 transcription factor in exocrine and endocrine pancreas. *J Biol Chem* 269:8944–8951
- Murakami H, Matsumoto M, Inoue H, Kaji Y (2007) Digestive enzyme activities of pancreas and intestinal digesta in streptozotocin-induced diabetic piglets. *Anim Sci J* 78:55–60. doi:10.1111/j.1740-0929.2006.00404.x
- Nicosia A, Monaci P, Tomei L, De Francesco R, Nuzzo M, Stunnenberg H, Cortese R (1990) A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA binding structure of LFB1. *Cell* 61:1225–1236. doi:10.1016/0092-8674(90)90687-A
- Park M, Cho S, Lee S, Choi Y, Park J, Kwon D, Son W, Paik S, Kim T, Han Y, Kim J (2005) A rare and often unrecognized cerebromeningitis and hemodynamic disorder: a major cause of sudden death in somatic cell cloned piglets. *Proteomics* 5:1928–1939. doi:10.1002/pmic.200401079
- Petters RM, Wells KD (1993) Culture of pig embryos. *J Reprod Fertil Suppl* 48:61–73
- Pontoglio M, Barra J, Hadchouel M, Doyen A, Kress C, Bach JP, Babinet C, Yaniv M (1996) Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal fanconi syndrome. *Cell* 84:575–585. doi:10.1016/S0092-8674(00)81033-8

- Pontoglio M, Sreenan S, Roe M, Pugh W, Ostrega D, Doyen A, Pick AJ, Baldwin A, Velho G, Froguel P, Levisetti M, Bonner-Weir S, Bell GI, Yaniv M, Polonsky KS (1998) Defective insulin secretion in hepatocyte nuclear factor 1 α -deficient mice. *J Clin Invest* 101:2215–2222. doi:[10.1172/JCI2548](https://doi.org/10.1172/JCI2548)
- Prather RS, Hawley RJ, Carter DB, Lai L, Greenstein JL (2003) Transgenic swine for biomedicine and agriculture. *Theriogenology* 59:115–123. doi:[10.1016/S0093-691X\(02\)01263-3](https://doi.org/10.1016/S0093-691X(02)01263-3)
- Taylor KD, Norris JM, Rotter JI (2007) Genome-wide association. Which do you want first: the good news, the bad news, or the good news? *Diabetes* 56:2844–2848. doi:[10.2337/db07-1324](https://doi.org/10.2337/db07-1324)
- Thomas H, Lemm I, Lausen J, Kind L, Roosen S, Ellard S, Hattersley AT, Ryffel GU (2002) Evidence for haploinsufficiency of the human HNF1 α gene revealed by functional characterization of MODY3-associated mutations. *Biol Chem* 383:1691–1700. doi:[10.1515/BC.2002.190](https://doi.org/10.1515/BC.2002.190)
- Tuomi T (2005) Type 1 and type 2 diabetes. What do they have in common? *Diabetes* 54:S40–S45. doi:[10.2337/diabetes.54.suppl_2.S40](https://doi.org/10.2337/diabetes.54.suppl_2.S40)
- Vaxillaire M, Abderrahmani A, Boutin P, Bailleul B, Froguel P, Yaniv M, Pontoglio M (1999) Anatomy of a homeoprotein revealed by the analysis of human MODY3 mutations. *J Biol Chem* 274:35639–35646. doi:[10.1074/jbc.274.50.35639](https://doi.org/10.1074/jbc.274.50.35639)
- Wang H, Antinozzi PA, Hagenfeldt KA, Maechler P, Wollheim CB (2000) Molecular targets of a human HNF1 α mutation responsible for pancreatic β -cell dysfunction. *EMBO J* 19:4257–4264. doi:[10.1093/emboj/19.16.4257](https://doi.org/10.1093/emboj/19.16.4257)
- Watanabe M, Umeyama K, Kawano H, Izuno N, Nagashima H, Miki K (2007) The production of a diabetic mouse using constructs encoding porcine insulin promoter-driven mutant human hepatocyte nuclear factor-1 α . *J Reprod Dev* 53:189–200. doi:[10.1262/jrd.18068](https://doi.org/10.1262/jrd.18068)
- Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes. *Diabetes Care* 27:1047–1053. doi:[10.2337/diacare.27.5.1047](https://doi.org/10.2337/diacare.27.5.1047)
- Yamagata K (2003) Regulation of pancreatic β -cell function by the HNF transcription network: lesson from maturity-onset diabetes of the young (MODY). *Endocr J* 50:491–499. doi:[10.1507/endocrj.50.491](https://doi.org/10.1507/endocrj.50.491)
- Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H, Vaxillaire M, Southam L, Cox RD, Lathrop GM, Boriraj V, Chen X, Cox NJ, Oda Y, Yano H, Le Beau MM, Yamada S, Nishigori H, Takeda J, Fajans SS, Hattersley AT, Iwasaki N, Hansen T, Pedersen O, Polonsky KS, Turner RC, Velho G, Chèvre J, Froguel P, Bell GI (1996) Mutations in the hepatocyte nuclear factor-1 α gene in maturity-onset diabetes of the young (MODY3). *Nature* 384:455–458. doi:[10.1038/384455a0](https://doi.org/10.1038/384455a0)
- Yamagata K, Yang Q, Yamamoto K, Iwahashi H, Miyagawa J, Okita K, Yoshiuchi I, Miyazaki J, Noguchi T, Nakajima H, Namba M, Hanafusa T, Matsuzawa Y (1998) Mutation P291fsinsC in the transcription factor hepatocyte nuclear factor-1 α is dominant negative. *Diabetes* 47:1231–1235. doi:[10.2337/diabetes.47.8.1231](https://doi.org/10.2337/diabetes.47.8.1231)
- Yamagata K, Nammo T, Moriwaki M, Ihara A, Iizuka K, Yang Q, Satoh T, Li M, Uenaka R, Okita K, Iwahashi H, Zhu Q, Cao Y, Imagawa A, Tochino Y, Hanafusa T, Miyagawa J, Matsuzawa Y (2002) Overexpression of dominant-negative mutant hepatocyte nuclear factor-1 α in pancreatic β -cells cause abnormal islet architecture with decreased expression of E-cadherin, reduced β -cell proliferation, and diabetes. *Diabetes* 51:114–123. doi:[10.2337/diabetes.51.1.114](https://doi.org/10.2337/diabetes.51.1.114)
- Yamanouchi T, Minoda S, Yabuuchi M, Akanuma Y, Akanuma H, Miyashita H, Akaoka I (1989) Plasma 1, 5-anhydro-d-glucitol as new clinical marker of glycemic control in NIDDM patients. *Diabetes* 38:723–729. doi:[10.2337/diabetes.38.6.723](https://doi.org/10.2337/diabetes.38.6.723)
- Yamanouchi T, Moromizato H, Shinohara T, Minoda S, Miyashita H, Akaoka I (1992) Estimation of plasma glucose fluctuation with a combination test of hemoglobin A1c and 1, 5 anhydroglucitol. *Metabolism* 41:862–867. doi:[10.1016/0026-0495\(92\)90168-A](https://doi.org/10.1016/0026-0495(92)90168-A)
- Yamanouchi T, Ogata N, Tagaya T, Kawasaki T, Sekino N, Funato H, Akaoka L, Miyashita H (1996) Clinical usefulness of plasma 1,5-anhydroglucitol in monitoring glycemic control. *Lancet* 347:1514–1518. doi:[10.1016/S0140-6736\(96\)90672-8](https://doi.org/10.1016/S0140-6736(96)90672-8)
- Yin XJ, Tani T, Yonemura I, Kawakami M, Miyamoto K, Hasegawa R, Kato Y, Tsunoda Y (2002) Production of cloning pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. *Biol Reprod* 67:442–446. doi:[10.1095/biolreprod67.2.442](https://doi.org/10.1095/biolreprod67.2.442)