Male *Men1* heterozygous mice exhibit fasting hyperglycemia in the early stage of MEN1

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited syndrome characterized by multiple tumors in the parathyroid glands, endocrine pancreas and anterior pituitary. Recent clinical studies have revealed a strong association between MEN1 syndrome and the risk of developing diabetes mellitus; however, the underlying mechanisms remain unknown. In this study, heterozygous *Men1* knockout (*Men1*^{+/-}) mice were used as MEN1 models to investigate MEN1-associated glucose metabolic phenotypes and mechanisms. Heterozygous deficiency of *Men1* in 12-month-old male mice induced fasting hyperglycemia, along with increased serum insulin levels. However, male *Men1*^{+/-} mice did not show insulin resistance, as evidenced by Akt activation in hepatic tissues and an insulin tolerance test. Increased glucose levels following pyruvate challenge and expression of key gluconeogenic genes suggested increased hepatic glucose output in the male *Men1*^{+/-} mice. This effect could be partly due to higher basal serum glucagon levels, which resulted from pancreatic islet cell proliferation induced by heterozygous loss of *Men1*. Taken together, our results indicate that fasted male *Men1*^{+/-} mice, in the early stage of development of MEN1, display glucose metabolic disorders. These disorders are caused not by direct induction of insulin resistance, but via increased glucagon secretion and the consequent stimulation of hepatic glucose production.

Key Words

- ▶ *Men1*
- ▶ glucagon
- ▶ gluconeogenesis
- ▶ hepatic glucose output
- ▶ insulin resistance

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited syndrome characterized by multiple occurrences of endocrine tumors affecting the parathyroid glands, endocrine pancreas and anterior pituitary (Thakker *et al.* 2012). Menin, encoded by *MEN1*, is a tumor suppressor, and its loss of function causes MEN1 syndrome (Marx 2005). It is widely expressed in various tissues, including metabolic tissues such as the

pancreas and liver (Guru *et al.* 1999). In their study on preventing gestational diabetes in pregnant mice, Karnik and coworkers reported that menin expression in the islets is physiologically suppressed by prolactin (Karnik *et al.* 2007). Furthermore, acute ablation of *Men1* in β -cells could prevent and/or treat hyperglycemia in diabetic mice (Yang *et al.* 2010a,b). Cheng and coworkers (2011) and Cao and coworkers (2013) demonstrated that hepatocyte-specific

knockout of *Men1* in mice does not affect glucose metabolism during aging and under a high-fat diet (HFD). However, Wuescher and coworkers reported that liver-specific heterozygous deletion of *Men1* leads to glucose intolerance in HFD-fed mice rather than standard diet-fed mice (Wuescher *et al.* 2011, 2012). These studies suggest that menin plays an important role in glucose metabolism.

MEN1 patients exhibit higher prevalence of impaired fasting glucose levels and diabetes mellitus (DM) (Wagner *et al.* 2005, McCallum *et al.* 2006, van Wijk *et al.* 2012). Clinical studies based on homeostasis model assessment (HOMA) have revealed that patients with MEN1 are insulin resistant (Wagner *et al.* 2005, McCallum *et al.* 2006, van Wijk *et al.* 2012). Heterozygous *Men1* knockout (*Men1*^{+/-}) mice develop endocrine tumors similar to those observed in MEN1 patients (Crabtree *et al.* 2001, Bertolino *et al.* 2003, Loffler *et al.* 2007). However, it is currently unknown whether these mice show abnormalities in glucose homeostasis, similar to MEN1 patients. To investigate MEN1-associated glucose metabolic phenotypes and mechanisms and to better understand the clinical manifestations of MEN1, *Men1*^{+/-} mice were used as MEN1 models in this study.

Our data showed that heterozygous deletion of *Men1* resulted in impairment of glucose metabolism in 12-month-old male mice; however, it did not induce insulin resistance. Instead, it promoted glucagon production and increased hepatic glucose output (HGO), which together contributed to increased glucose levels in *Men1*^{+/-} males.

Materials and methods

Animal model

All animal protocols were approved by the Animal Ethics Committee of the Xiamen University. *Men1*^{ΔN3-8/+} (C57BL/6) mice heterozygous for the *Men1* locus (*Men1*^{+/-}) were a gift from Francis Collins (National Human Genome Research Institute, Bethesda, MD, USA). They were housed in a pathogen-free environment in a 12h light:12h darkness cycle and given free access to water and standard chow diet. Studies were conducted using the *Men1*^{+/-} mice as a model for MEN1, with age-matched wild-type (*Men1*^{+/+}) mice as controls. Because mice developed multiple endocrine tumors late in life (Crabtree *et al.* 2001, Bertolino *et al.* 2003, Loffler *et al.* 2007), *Men1*^{+/-} mice at the age of 12 months (the early stage of development of MEN1) and 18 months (the middle-advanced stage of development of MEN1) were used in this study. Mice were fasted for 24h, anesthetized with 1.2% avertin (240mg/kg body weight)

and then their blood was collected by cardiac puncture. One hour before administration of the anesthetic, the mice were weighed and injected intraperitoneally with bromodeoxyuridine (BrdU; Sigma-Aldrich) in phosphate-buffered saline (PBS) at a dose of 50mg/kg body weight. The whole pancreas and liver were harvested for histological and molecular biological analyses, respectively. Concentrations of fasting serum hormones, including insulin, glucagon, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), ghrelin, leptin, plasminogen activator inhibitor-1 (PAI-1) and resistin, were estimated with the Bio-Plex Pro Mouse Diabetes Standard 8-Plex Assay (Bio-Rad).

Glucose tolerance test

After an overnight fast, mice were weighed and injected intraperitoneally with a bolus of glucose (2g/kg body weight). Blood glucose levels were determined from tail blood before and, at the indicated time points, after glucose injection with a OneTouch UltraEasy glucometer (LifeScan, Milpitas, CA, USA).

Insulin tolerance test

Mice were fasted for 5 h before receiving an intraperitoneal injection of insulin (0.75 U/kg) in PBS. Blood glucose concentrations were estimated in tail blood before and, at the indicated time points, after insulin injection.

Pyruvate tolerance test

Overnight fasted mice were injected intraperitoneally with sodium pyruvate (2g/kg body weight). Blood glucose values were tested from tail blood before and, at the indicated time points, after sodium pyruvate injection.

Western blotting analysis

After the indicated animal experiments, liver tissues were lysed in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Nantong, China). Protein concentrations were determined with a BCA Protein Assay Kit (Pierce). Tissue lysates (50μg protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difluoride membrane. After blocking for 60min at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, the membranes were incubated overnight at 4°C with anti-pAkt

Table 1 Primers used for qPCR analysis.

Sequence name	Primers	Temperature (°C)	Size (bp)
<i>mG6pc</i> -F	5'-GTGCAGCTGAACGTCTGTCTGT-3'	63	139
<i>mG6pc</i> -R	5'-TCCGGAGGCTGGCATTGT-3'	61	
<i>mPck1</i> -F	5'-GAACTGACAGACTCGCCTATGT-3'	62	165
<i>mPck1</i> -R	5'-GTTGAGGCCCCAGTTGTTG-3'	60	
<i>mGapdh</i> -F	5'-AACAGCAACTCCCACTCTTC-3'	58	111
<i>mGapdh</i> -R	5'-CCTGTTGCTGTAGCCGTATT-3'	58	

(Ser473) antibody (Cell Signaling, #9271), anti-Akt antibody (Cell Signaling, #9272), anti-pIRS-1 (Tyr632) antibody (Santa Cruz, sc-17196), or anti-GAPDH antibody (Epitomics, P04406). The membranes were washed thrice with Tris-buffered saline containing 0.1% Tween 2 and incubated with horseradish peroxidase-conjugated secondary antibodies, and then the protein levels were detected using an enhanced chemiluminescence kit (Millipore).

Quantitative polymerase chain reaction analysis

Total RNA was extracted from frozen liver tissues using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian,

China) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed as described previously (Xu *et al.* 2013) using an ABI StepOne detection system (Applied Biosystems) with the primer sequences, which are listed in Table 1. Mouse *Gapdh* was used as an internal control.

Immunohistochemistry

Four-micrometer-thick sections of formalin-fixed, paraffin-embedded mouse pancreatic tissues were cut and placed consecutively onto silanized glass slides, and were routinely dewaxed and rehydrated. After rinsing with PBS, the sections were immersed in 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase activity. For DNA denaturation, sections were placed in 2M HCl for 30 min. Antigens were retrieved in 0.1% trypsin solution for 20 min at 37°C. Nonspecific binding was prevented by incubation in 5% normal goat serum for 15 min in a humidified chamber. The sections were

Figure 1

Male *Men1*^{+/-} mice exhibit fasting hyperglycemia. (A) Blood glucose levels after 24 h of fasting in 12-month-old and 18-month-old mice ($n=20$ and 6 , respectively). (B) Serum insulin levels 24 h after fasting in 12-month-old *Men1*^{+/-} and *Men1*^{+/+} mice ($n=10$). (C) Glucose tolerance test in 12-month-old vs 18-month-old male mice ($n=6$). Data are presented as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$.

Figure 2

Insulin resistance is not induced in male *Men1*^{+/-} mice. (A) Body weights of 12-month-old *Men1*^{+/+} and *Men1*^{+/-} mice ($n=20$). (B) The activity (phosphorylated serine 473 on Akt) and total levels of Akt and the tyrosine phosphorylation of IRS-1 in the liver were evaluated after 24 h of fasting ($n=10$). (C) An insulin tolerance test was performed ($n=6$), in 12-month-old *Men1*^{+/+} vs *Men1*^{+/-} male mice. Data are presented as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

then incubated with anti-BrdU antibody (Sigma, B2531) overnight at 4°C. After washing with PBS, antibody binding was detected with horseradish peroxidase-conjugated secondary antibody (Fuzhou Maixin Biotechnology Co, Ltd, Fuzhou, China) at 37°C. The sections were visualized with diaminobenzidine solution, and then lightly counterstained with hematoxylin. As DNA denaturation is not required for anti-glucagon antibody (Abcam, ab10988), the antigens were retrieved in 10mM citrate buffer (pH 6.0) for 15 min in a pressure cooker.

Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). For data at single time point, statistical differences were evaluated by unpaired Student's *t*-test or one-way analysis of variance (ANOVA), as appropriate. Datasets with multiple time points (e.g., GTT, ITT and PTT) were analyzed by repeated-measures ANOVA. A *P* value of <0.05 was considered significant.

Results

Male Men1^{+/-} mice exhibit fasting hyperglycemia

Because the association between MEN1 and DM risk was primarily established by measuring fasting plasma glucose levels, we first evaluated the fasting glucose and insulin levels in mice at 12 months of age. As shown in Fig. 1A and B, relative to wild-type controls, female Men1^{+/-} mice showed normal glucose regulation, whereas both fasting

blood glucose and serum insulin levels were significantly increased in male Men1^{+/-} mice. Nevertheless, heterozygous loss of Men1 in male mice did not induce significant impairment in the ability to clear a glucose load during GTT (Fig. 1C). To confirm impaired glucose metabolism in the middle-advanced MEN1 mouse model, we estimated blood glucose levels in the mice at 18 months of age. Surprisingly, fasting blood glucose level in the Men1^{+/-} male mice was similar to that in the Men1^{+/+} controls (Fig. 1A). A small but significant improvement in glucose tolerance was observed in Men1^{+/-} male mice during GTT (Fig. 1C).

Insulin resistance is not induced in male Men1^{+/-} mice

The increased fasting blood glucose and serum insulin levels observed in humans with MEN1 is classically interpreted to be a consequence of insulin resistance. Obesity is known to cause insulin resistance and alterations in glucose metabolism (Kahn *et al.* 2006). In this study, no significant difference in body weight was observed between male Men1^{+/-} mice and the Men1^{+/+} control group, and female Men1^{+/-} mice weighed even less than their controls (Fig. 2A). These findings suggest that the effect of heterozygous lack of Men1 on metabolism is not mediated by obesity.

Insulin resistance affects multiple tissues, including the liver and muscle (Bajaj & DeFronzo 2003). Overproduction of glucose by the liver, despite the presence of hyperinsulinemia, is primarily responsible for fasting hyperglycemia (Roden & Bernroider 2003). Therefore, we examined insulin signaling in the liver by using the phosphorylation of Akt on serine 473 as a marker of insulin action. Relative to wild-type controls, the phosphorylation of serine 473 on Akt in the liver of Men1^{+/-} males is found to be significantly increased (Fig. 2B). No change in total Akt level was observed. Thus, the activity of Akt, a downstream target of insulin, is not inhibited in Men1^{+/-} males. Consistent with this, we observed a significant increase in the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), which is another crucial target of insulin signaling (Fig. 2B). These data demonstrate that the heterozygous absence of Men1 does not cause direct impairment of insulin signaling in mice. To further investigate the effect of heterozygous ablation of Men1 on insulin sensitivity, we performed ITT in male mice. Insulin sensitivity was significantly enhanced in male Men1^{+/-} mice (Fig. 2C). These data suggest that the increased glucose and insulin levels observed in the male Men1^{+/-} mice are not caused by the underlying insulin resistance.

Figure 3

Male Men1^{+/-} mice display increased hepatic glucose output. (A) Pyruvate tolerance test was performed (*n* = 6), and (B and C) the expression levels of gluconeogenic genes (*mG6pc* and *mPck1*) in the liver were analyzed after 24 h of fasting (*n* = 10), in 12-month-old Men1^{+/+} vs Men1^{+/-} male mice. Data are presented as the mean ± S.E.M. **P* < 0.05, ***P* < 0.01.

Male Men1^{+/-} mice display increased HGO

The heterozygous deficiency of *Men1* does not induce insulin resistance, but causes an increase in glucose and insulin levels. An obvious reason for this increase is that heterozygous knockout of *Men1* in males promotes HGO, which is primarily derived from gluconeogenesis under fasting conditions (Wahren & Ekberg 2007). Pyruvate is a major substrate for gluconeogenesis; therefore, to determine if the male *Men1*^{+/-} mice exhibited increased glucose production, we performed PTT. As expected,

Men1^{+/-} mice exhibited higher blood glucose levels upon pyruvate administration, suggesting increased hepatic glucose production (Fig. 3A). The gluconeogenesis pathway is catalyzed by several key enzymes, including phosphoenolpyruvate carboxykinase (PCK1) and glucose-6-phosphatase (G6PC), which are the first and last rate-limiting enzymes of the process, respectively (Zhang *et al.* 2014). Consistent with the results of the PTT, hepatic mRNA levels of *G6pc* were significantly higher in fasted *Men1*^{+/-} mice than those in fasted *Men1*^{+/+} mice (Fig. 3B). Although *Pck1* mRNA was also slightly

Figure 4

Fasting hyperglycemia is associated with islet proliferation and increased fasting serum glucagon levels in male *Men1*^{+/-} mice. (A) Histological analysis was performed in pancreatic tissues by hematoxylin–eosin (H&E) staining (*n*=20). (B) Islet area was measured using the Image-Pro Plus software (*n*=20) (Media Cybernetics, Silver Spring, MD, USA). (C) Proliferation of islet cells was assessed by immunohistochemical staining with BrdU antibody (*n*=20). (D) Serum glucagon concentrations were examined (*n*=10). (E) Glucagon immunohistochemical staining was analyzed in pancreatic tissues (*n*=20), 24 h after fasting in 12-month-old *Men1*^{+/+} and *Men1*^{+/-} mice. Data are presented as the mean ± S.E.M. **P*<0.05, ***P*<0.01.

Figure 5

Other serum hormones (A–F) were evaluated after 24 h of fasting in 12-month-old *Men1*^{+/+} and *Men1*^{+/-} mice (*n* = 10). Data are presented as the mean $\pm$ S.E.M. **P* < 0.05, ***P* < 0.01.

upregulated, no significant difference between the two genotypes was observed (Fig. 3C). These data suggest that heterozygous loss of *Men1* promotes gluconeogenesis and basal glucose production in the liver.

Fasting hyperglycemia is associated with islet proliferation and increased fasting serum glucagon levels in male *Men1*^{+/-} mice

Glucagon regulates HGO in the fasted state by increasing gluconeogenesis and glycogenolysis in the liver (Hancock *et al.* 2010, Ramnanan *et al.* 2011). *Men1* is crucial for the development (Fontaniere *et al.* 2008) and proliferation of pancreatic islets (Karnik *et al.* 2005, Schnepf *et al.* 2006), and therefore plays an important role in regulating basal insulin and glucagon secretion in mice. Under the experimental conditions of this study, we observed hyperplasia of pancreatic islets and development of islet tumors in *Men1*^{+/-} mice (Fig. 4A). Moreover, compared with *Men1*^{+/+} controls, male *Men1*^{+/-} mice displayed a marked increase in pancreatic islet area and cell proliferation (Fig. 4B and C). Higher fasting serum glucagon levels and glucagon immunohistochemical staining were observed in male *Men1*^{+/-} mice, indicating that the increased glucagon level contributed significantly to the elevated HGO and blood glucose levels in the male *Men1*^{+/-} mice under fasting conditions (Fig. 4D and E).

Effects of other serum hormones involved in lipid and glucose metabolism

To enhance our understanding of metabolic phenotypes in *Men1*^{+/-} mice, we also examined the levels of other serum hormones involved in lipid and glucose metabolism

(Fig. 5). Female *Men1*^{+/-} mice had higher serum leptin and lower serum ghrelin levels than the *Men1*^{+/+} controls (Fig. 5C and D). No significant differences were found in the levels of other hormones between the two genotypes.

Discussion

Glucose homeostasis involves the maintenance of a delicate balance between its production and utilization. Failure of glucose homeostasis leads to diseased states, including hyperglycemia and DM. Type 1 diabetes arises from autoimmune-induced β -cell damage (Bluestone *et al.* 2010), whereas type 2 diabetes develops mainly because of insulin resistance (Kahn *et al.* 2014). Eventually, insulin production is insufficient for maintaining normal blood glucose levels, leading to hyperglycemia and secondary complications, including cardiovascular disease, kidney dysfunction and blindness (Bluestone *et al.* 2010, Kahn *et al.* 2014). In the current animal studies, we observed that, compared with 12-month-old male *Men1*^{+/+} mice, male *Men1*^{+/-} mice had impaired glucose metabolism, characterized by high fasting blood glucose and serum insulin levels. These findings are consistent with previous clinical reports (Wagner *et al.* 2005, McCallum *et al.* 2006, van Wijk *et al.* 2012). Clinical studies based on HOMA have indicated that insulin resistance leads to glucose metabolic disorder in patients with MEN1 (Wagner *et al.* 2005, McCallum *et al.* 2006, van Wijk *et al.* 2012). However, we provide evidence that the effects of heterozygous loss of *Men1* on glucose metabolism are not mediated by inducing insulin resistance. In the ITT, male *Men1*^{+/-} mice displayed significantly enhanced glucose clearance rates. The activation of Akt in the liver also suggested that the *Men1*^{+/-} males were not insulin resistant.

The increase in blood glucose levels in the absence of insulin resistance suggests that heterozygous knockout of *Men1* in male mice promotes HGO. Excess production of glucose from the liver is achieved by either increasing gluconeogenesis or by stimulating glycogenolysis (Roden & Bernroider 2003). Under physiological conditions, during prolonged fasting, hepatic gluconeogenesis plays a crucial role in maintaining normal plasma glucose levels (Wahren & Ekberg 2007). Compared with the *Men1*^{+/+} controls, male *Men1*^{+/-} mice displayed increased glucose levels after pyruvate administration. Consistent with the results of the PTT, hepatic mRNA level of the gluconeogenic gene, *G6pc*, was significantly higher in fasted male *Men1*^{+/-} mice than in their control male mice, providing further evidence that heterozygous knockout of *Men1* promotes gluconeogenesis and hepatic glucose production, leading to an elevation in fasting plasma glucose concentration.

The increased HGO observed in this study was unexpected, because the male *Men1*^{+/-} mice displayed increased glucose and insulin levels, which normally act to suppress HGO (Edgerton *et al.* 2006, Ramnanan *et al.* 2010). As Akt phosphorylation was activated in the livers of male *Men1*^{+/-} mice, insulin resistance may not be the reason for the increased HGO. Therefore, the obvious reason for the increased HGO is the increase in glucagon levels in male *Men1*^{+/-} mice, despite the high levels of glucose and insulin. In 12-month-old male *Men1*^{+/-} mice, increased fasting glucagon levels were observed as a result of pancreatic islet cell proliferation induced by heterozygous ablation of *Men1*. In mice, the majority of cells within the islets are insulin-secreting β -cells (approximately 65–75%), whereas glucagon-secreting α -cells represent less than 20% of the individual islet area (Brissova *et al.* 2005). Therefore, dilatation of α -cells via proliferation would be expected to have a greater physiological impact on glucose homeostasis than dilatation of an equivalent or even greater number of β -cells. This would explain the increase in fasting serum glucagon concentration and, consequently, impaired fasting blood glucose, despite the elevated fasting serum insulin levels.

Male *Men1*^{+/-} mice exhibited fasting hyperglycemia at the age of 12 months, but demonstrated improved glucose tolerance at the age of 18 months. Previous clinical studies on patients with MEN1 have revealed that most early islet lesions tested positive for glucagon, whereas the advanced hormone-secreting islet tumors detected in the same patients were mainly insulinomas (Anlauf *et al.* 2006, Perren *et al.* 2007). These results

indicate that glucagon-positive early lesions may be replaced or overtaken by insulinomas and mixed islet tumors during tumorigenesis. This hypothesis is supported by the recent reports that *Men1* ablation in α -cells leads to the formation of insulinomas (Lu *et al.* 2010, Shen *et al.* 2010), which might provide a possible explanation for the dynamic change in glucose metabolism in *Men1*^{+/-} males.

Although no difference in body weight was observed between the genotypes in male mice, female *Men1*^{+/-} mice weighed significantly less than their controls and exhibited higher serum leptin and lower serum ghrelin levels. The reduction in body weight may have been caused by either a decrease in food intake or an increase in energy expenditure. Because leptin and ghrelin can act centrally to decrease and increase feeding, respectively, the higher leptin and lower ghrelin levels in female *Men1*^{+/-} mice may reflect their lower food intake (Klok *et al.* 2007, Nogueiras *et al.* 2008).

In this study, all mice were under fasting condition. Whether higher blood glucose levels emerge in female *Men1*^{+/-} mice challenged with feeding and whether refeeding alters the metabolic phenotype in male *Men1*^{+/-} mice remain to be elucidated. Moreover, we noticed differences in glucose metabolic phenotypes and body weight between male and female mice. Currently, the reason(s) underlying the observed sexually dimorphic metabolic phenotypes remain unclear. Furthermore, studies on gonadectomized or ovariectomized mice need to be conducted to eliminate the influence of sex hormones.

In summary, we elucidated the glucose metabolic phenotype of mice exhibiting the early stage of development of MEN1. In male *Men1*^{+/-} mice, pancreatic islet proliferation induced by heterozygous knockout of *Men1* increased fasting basal serum glucagon concentration. It subsequently promoted hepatic gluconeogenesis, resulting in impaired fasting blood glucose levels, despite an increase in insulin concentration and insulin sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

G-H J and M-Q X designed the experiments and revised the manuscript; Z-X-Z G, LZ, W-T X, S-Q W, X-R B, Y-L G and H-J Z performed the experiments and acquired the data; Q-Z H, Y C and Z-E W contributed to the separation of the desired tissues; Z-X-Z G, M-Q X and G-H J analyzed the data; and Z-X-Z G wrote the manuscript.

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