Nfe2l1-silenced insulinoma cells acquire aggressiveness and chemoresistance

Jingqi Fu1, Hongzhi Zheng2, Qi Cui1, Chengjie Chen1, Simeng Bao1, Jing Sun1, Lu Li1, Bei Yang3, Huihui Wang1, Yongyong Hou1, Yuanyuan Xu1, Yuanhong Xu4, Qiang Zhang5 and Jingbo Pi1

1Program of Environmental Toxicology, School of Public Health, China Medical University, Shenyang, People’s Republic of China
2Department of Geriatric Endocrinology, The First Affiliated Hospital, China Medical University, Shenyang, People’s Republic of China
3Department of Histology and Embryology, School of Basic Medical Sciences, China Medical University, Shenyang, People’s Republic of China
4Department of Pancreatic Surgery, The First Affiliated Hospital, China Medical University, Shenyang, People’s Republic of China
5Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA

Correspondence should be addressed to J Pi: jbpi@cmu.edu.cn or jingbopi@163.com

Abstract

The transcription factor nuclear factor erythroid 2-like 1 (NFE2L1 or NRF1) is involved in various critical cell processes such as maintenance of ubiquitin-proteasome system and regulation of the cellular antioxidant response. We previously determined that pancreatic β-cell-specific Nfe2l1-knockout mice had hyperinsulinemia and that silencing of Nfe2l1 in mouse islets or MIN6 insulinoma β-cells induced elevated basal insulin release and altered glucose metabolism. Hypoglycemia is a major issue with aggressive insulinomas, although a role of NFE2L1 in this pathology is not defined. In the present work, we studied the tumorigenicity of Nfe2l1-deficient insulinoma MIN6 cells (Nfe2l1-KD) and sensitivity to chemotherapy. Nfe2l1-KD cells grew faster and were more aggressive than Scramble cells in vitro. In a mouse allograft transplantation model, insulinomas arising from Nfe2l1-KD cells were more aggressive and chemoresistant. The conclusion was amplified using streptozotocin (STZ) administration in an allograft transplantation model in diabetic Akita background mice. Furthermore, Nfe2l1-KD cells were resistant to damage by the chemotherapeutic drugs STZ and 5-fluorouracil, which was linked to binding of hexokinase 1 with mitochondria, enhanced mitochondrial membrane potential and closed mitochondrial potential transition pore. Overall, both in vitro and in vivo data from Nfe2l1-KD insulinoma cells provided evidence of a previously un-appreciated action of NFE2L1 in suppression of tumorigenesis. Nfe2l1 silencing desensitizes insulinoma cells and derived tumors to chemotherapeutic-induced damage, likely via metabolic reprogramming. These data indicate that NFE2L1 could potentially play an important role in the carcinogenic process and impact chemosensitivity, at least within a subset of pancreatic endocrine tumors.

Introduction

Insulinomas are the most common type of pancreatic endocrine tumors and often cause hypoglycemia due to persistent hyperinsulinemia. Insulinomas are rare and most often (~90%) are small and benign (Ro et al. 2013, Parbhu & Adler 2016). The primary clinical choice to cure a presumed solitary insulinoma is surgical resection (Ro et al. 2013, Parbhu & Adler 2016). However, since these tumors are quite small, preoperative localization is difficult.
and recurrence after resection is high. Furthermore, cases of unresectable malignant insulinoma often present with severe hypoglycemia as a major complication and several strategies are used to alleviate this serious condition (Shao et al. 2016). Orally administered glucose together with chemical inhibition of insulin secretion help reverse the symptoms of hypoglycemia and improve quality of life (Ro et al. 2013). Specifically, diazoxide has been used to inhibit insulin secretion of insulinoma cells (Shao et al. 2016). Beyond this, the chemotherapeutic drug streptozotocin (STZ) is given to directly destroy β-cell-derived insulinoma cells (Schulz et al. 1990, Schnedl et al. 1994), along with general cancer chemotherapeutic drugs, such as 5-fluorouracil (5-Fu) (Kiyozumi et al. 2013, Zheng et al. 2014). STZ is a toxic glucose analog that specifically accumulates in pancreatic β-cells via glucose transporter 2 (GLUT2) (Schnedl et al. 1994, Wang & Gleichmann 1998, Szkudelski 2001). Alkylation of DNA (Szkudelski 2001) and oxidative stress (Perez-Gutierrez et al. 2016) have been implicated as major mechanisms for STZ-induced insulinoma cell apoptosis and necrosis (Schulz et al. 1990). STZ is also frequently used for rodent models of chemically induced type 1 diabetes because of this specific damage to pancreatic β-cells (Szkudelski 2001, Perez-Gutierrez et al. 2016).

Nuclear factor erythroid 2 like 2 (NFE2L1) is a transcription factor and a member of the cap ‘n’ collar/basic-region leucine zipper (CNC-bZIP) family. NFE2L1 plays critical roles in embryonic development (Chen et al. 2003, Leung et al. 2003), neurogenic ubiquitin-proteasome system maintenance (Kobayashi et al. 2011, Lee et al. 2011), antioxidant response regulation (Xing et al. 2007, Ohtsuji et al. 2008) and hepatic lipid metabolism (Xu et al. 2005, Hirotsu et al. 2012). Previously, we showed that pancreatic β-cell-specific Nfe2l1-knockout mice had severe hyperinsulinemia, and the silencing of Nfe2l1 (Nfe2l1-KD) in MIN6 β-cells and isolated mouse islets caused elevated basal insulin release but reduced glucose-stimulated insulin secretion (GSIS) (Zheng et al. 2015). This impaired glucose metabolism in Nfe2l1-deficient MIN6 insulinoma cells was linked to increased glycolysis and lactate production. The enhancement of glycolysis in MIN6 insulinoma cells was characterized as a ‘Warburg Effect’ (Danhier et al. 2017, Hasanpourghadi et al. 2017). However, a higher oxygen consumption rate (OCR) was also observed in Nfe2l1-deficient MIN6 cells. High bioenergetics profiles (both OCR and extracellular acidification rate (ECAR)) have been previously observed in lymphocyte activation (van der Windt et al. 2013, Caro-Maldonado et al. 2014) and osteoblast differentiation (Guntur et al. 2014).

In contrast, most cancer cells have deregulated bioenergetics, and this appears important to acquisition of the malignant phenotype. For instance, chemical suppression of both OCR and ECAR mitigates malignant behavior (hypermigration) and enhances sensitivity to doxorubicin, a common cancer chemotherapeutic, in breast cancer cells (Vaughan et al. 2014). The impact of the high energetic profile of Nfe2l1-deficient insulinoma cells on their potential tumorigenicity is unknown.

In prior work, we found that hexokinase 1 (HK1) was markedly increased in Nfe2l1-deficient pancreatic β-cells (Zheng et al. 2015). HK1 is the enzyme responsible for the first reaction in glycolysis. HK1 has also recently been reported capable of blocking apoptosis signaling by interaction with the voltage-dependent anion channel (VDAC) of mitochondrial potential transition pore (mPTP) (Schindler & Foley 2013). In the present work, we determined the sensitivity of Nfe2l1-deficient insulinoma cells to chemotherapy-induced apoptosis. We found that the upregulated HK1 was specifically located in mitochondria of the Nfe2l1-KD MIN6 cells, which caused mPTP closure and mitigated STZ-induced apoptosis compared with Scramble control cells. Further in vivo, Nfe2l1-KD-derived insulinomas were insensitive to STZ administration. Thus, Nfe2l1 deficiency in insulinoma cells caused acquired chemoresistance via metabolic changes. In addition, Nfe2l1-KD MIN6 and Scramble control cells were tested in an allograft tumor model using different mouse strains, which include wild-type C57BL/6 mice and mice with type 1 diabetic Akita background. The results showed that the Nfe2l1-deficient insulinoma cells formed tumors that grew more rapidly, acted more aggressively and displayed key metabolic differences that afforded chemotherapeutic resistance. Together, all these data indicate that NFE2L1 potentially plays a role in the tumorigenic process and could potentially impact chemotherapeutic resistance, at least in insulinomas.

Materials and methods

Allograft tumor model

The mice were housed in virus-free facilities on a 12-h light/12-h darkness cycle and were provided diet and distilled water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of China Medical University (Shenyang, China) following all current guidelines for animal care and welfare. Wild-type mice (WT, C57BL/6Nju, J006664) and insulin Akita mutation mice
(Akita, C57BL/6-Ins2<sup>Akita</sup>/Nju, J003548) were purchased from Nanjing BioMedical Research Institute of Nanjing University (NBRI, Nanjing, China). Allograft tumors in WT and Akita mice (at the age of 10–12 weeks) were initiated by subcutaneous inoculation of MIN6 cells (3 × 10<sup>6</sup> cells/mouse), as previously described (Kimura et al. 2002). Cells were suspended in 0.1 mL of phosphate buffered saline (PBS, pH 7.4) and inoculated subcutaneously into the dorsal thoracic region at a single site immediately after cell preparation. In the survival study, WT mice (Fig. 2B) or Akita mice (Fig. 3A and D) received inoculations of either Scramble control or Nfe2l1-KD MIN6 cells subcutaneously in the dorsal thoracic region. Survival was then assessed over 30 days.
during which mice typically died of hypoglycemia. For the tumor mass determination study (Fig. 2D and E), each WT mouse was inoculated subcutaneously with both Scramble control and Nfe2l1-KD MIN6 cells. One cell type was inoculated to the left of the dorsal thoracic midline and the other to the right. This was done to negate any impact individualized circulating factors might have on tumor growth in the mice. As soon as possible after the mouse had died from hypoglycemia, the resulting tumors from both inoculation sites were excised, weighed separately and fixed. The picture of gross tumor size was taken when all the mice in this experiment had died. Tail blood glucose was monitored post cell inoculation using the FreeStyle Blood Glucose Monitoring System (TheraSense, Alameda, CA, USA), which has a determination range of 20–600 mg/dL. The blood glucose of mice that died early was set to ‘0’ for statistical purposes. When ‘High’ was indicated by this glucose meter, this indicated the tested blood glucose was above the highest limit and was set to ‘600 mg/dL’ for statistical purposes. When ‘Low’ was indicated by the glucose meter, the tested blood glucose was below the lowest limit and was set to ‘20 mg/dL’ for statistical purposes.

**STZ administration**

Akita mice were inoculated with Scramble control or Nfe2l1-KD MIN6 cells. Seven days after inoculation, the mice were given 200 mg STZ/Kg of body weight via intraperitoneal injection. STZ was dissolved in 0.025 M sodium citrate buffer (pH 4.5). We collected blood through tail bleeding and immediately analyzed for glucose level using the FreeStyle Blood Glucose Monitoring System.

**Cell culture and reagents**

MIN6 cells were a gracious gift of Dr Marcia Haigis (Harvard University, Boston, MA, USA). Beta-TC-6 insulinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA). MIN6 and beta-TC-6 cells were SV40 large T-antigen-transformed cells, generated using insulinomas from mice overexpressing the SV40 large T-antigen in β-cells from human and rat insulin promoter, respectively (Miyazaki et al. 1990, Poitout et al. 1995). MIN6 and beta-TC-6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, with 15% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5 μL/L β-mercaptoethanol in humidified 5% CO₂.
95% air at 37°C. Scramble and Nfe2I1-KD MIN6 cells were established by MISSION shRNA lentiviral transduction (mouse Nfe2I1 (SHVRSNM_008686) or Scrambled non-target negative control (SHC002V)) as described (Zheng et al. 2015). Briefly, parental MIN6 cells were plated in 6-well plates at 40–60% confluency in complete medium. The following day, hexadimethrine bromide (8μg/mL, Sigma) and viral particles were added to each well. Following a 24-h incubation period, medium containing viral particles was replaced with fresh medium containing 1μg/mL of puromycin. The established stable cell lines were cultured in medium containing puromycin. FBS, PBS and cell culture supplements were purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). β-mercaptoethanol, rotenone, antimycin A, oligomycin, carbonylcyanide chlorophenylhydrazone (CCCP), hexadimethrine bromide and STZ were obtained from Sigma. Cells were counted with a hemocytometer before subculture or after treatments.

**OCR and ECAR measurements**

OCR and ECAR were measured in vitro using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent, Billerica, MA, USA) as described (Fu et al. 2011, Zheng et al. 2015). Scramble and Nfe2I1-KD MIN6 cells were seeded in XF24-well cell culture microplates (Seahorse Bioscience, Agilent) at 5 x 10^4 cells/well in 500μL medium and cultured at 37°C, 5% CO2 overnight. Assays were initiated by replacing the growth medium from each well with 600μL pre-warmed unbuffered DMEM (20mM glucose, Seahorse Bioscience, Agilent). Prior to each measurement, the XF24 analyzer gently mixed the assay media in each well to allow the oxygen tension and pH value to reach equilibrium. Following this mixing, oxygen tension and pH were measured simultaneously in a transient microchamber created by the sensor cartridge for 3mins. Three baseline rates were measured, and the average of these baseline rates was used for the data analysis.

**Cell viability assays**

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Fu et al. 2015), lactate dehydrogenase (LDH) release assay (Fu et al. 2011) and trypan blue exclusion assay (Fu et al. 2013) were used to determine cell viability after various treatments. Scramble and Nfe2I1-KD MIN6 cells were seeded in 96-well plates (5 x 10^4 cells per well) and allowed to adhere for 24h. The medium was then removed and replaced with fresh medium containing the test chemicals for 24h. The treated cells were used for the MTS assay, and the culture medium was used for the LDH release assay. The MTS assay was measured by the Cell Titer Non-Radioactive Cell-Proliferation Assay Kit (Promega). Upon addition of MTS solution to reaction plate and incubation at 37°C for 1h, the absorbance was read at 490nm with the FlexStation 3 Multi-Mode microplate reader ( Molecular Devices, Sunnyvale, CA, USA). Measurements were expressed as a percentage of untreated control cells. The LDH release of cell culture medium after the indicated treatment tests membrane integrity and was used to determine the cytotoxicity of the various test chemicals (Fu et al. 2011). We utilized the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega).

For the trypan blue exclusion assay, Scramble and Nfe2I1-KD MIN6 cells were seeded in 24-well plates (2 x 10^5 cells per well) and exposed to various concentrations of test chemicals for 24h. Medium was then removed and cells were stained with 0.4% trypan blue solution and counted with the hemocytometer (Fu et al. 2013). The percentage of dead cells was used to determine the proportional cell viability.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

RNA isolation and quantitative real-time PCR (qPCR) were performed as described (Fu et al. 2017). Briefly, total RNA was extracted with TRIzol (Life Technologies) and reverse transcribed with PrimeScript RT Reagent with cDNA Eraser (Takara). Relative mRNA abundance was determined by real-time PCR with SYBR Premix Ex Taq (Takara). See Supplementary Table 1 (see section on supplementary data given at the end of this article) for primer sequences. The primers were designed by using Primer Express 4 (Applied Biosystems) and synthesized by Life Technologies. Real-time fluorescence detection was performed using a QuantStudio 6 System.
Antibody Xue Yehuda-Nguyen). Briefly, the cells were stained with calcein-AM for 30 mins to stain viable cells. The decrease in slope of calcein-AM fluorescence post-CoCl₂ treatment was used to indicate the quenching rate of cytosolic calcium. After this, the decrease in the slope of calcein-AM fluorescence quenching curve post ionomycin exposure reflects the release of mitochondrial calcium, and this was used to quantify the opening rate of mPTP.

**Determination of apoptosis**

Cells were seeded in 12-well plates and cultured for 24 h to reach approximately 80% confluence. The cells were treated with STZ at the indicated concentrations for 8 h. Floating cells and attached cells were then harvested for apoptosis analysis. Cells were washed three times with ice-cold PBS and stained using the TACS Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) as described (Zhao et al. 2011). Briefly, the cells were stained with Annexin V-FITC for 15 mins at room temperature in 200 μL binding buffer. Then, 300 μL binding buffer was added, and the cells were stained with propidium iodide (PI) for 30 mins at 4°C. The fluorescence of 10,000 cells was analyzed using the Canto II flow cytometer (Becton-Dickinson). The percentage of apoptotic cells was statistically analyzed using FlowJo 7.6.1 software (FlowJo LLC., Ashland, OR, USA).

**Statistical analysis**

All statistical analyses were performed using Graphpad Prism 4 (GraphPad Software), with a P<0.05 considered as significant. Data were expressed as mean±s.d. For comparisons between two groups, a Student’s t-test was used. For the tumor mass assessments where tumors were developed from the two cell lines (i.e. Scramble or Nfe2l1-KD MIN6 cells) simultaneously at different sites in the same animals, a paired t-test was used (Fig. 2D). For comparisons among multiple groups, two-way ANOVA followed by a Bonferroni multiple comparison tests were used. Kaplan–Meier survival curves were used to analyze the effects of cell genotype, host phenotype and post-inoculation treatments on survival after insulinoma cells inoculation.
Results

mRNA expression of Nfe2l1 in insulinoma cells was comparable to that in pancreatic islets

We previously showed that pancreatic β-cell-specific Nfe2l1-knockout mice have severe hyperinsulinemia (Zheng et al. 2015). In this regard, insulinomas are common pancreatic endocrine tumors and often cause severe hyperinsulinemia. Thus, we sought to compare the abundance of Nfe2l1 in insulinoma cells and normal pancreatic endocrine cells, by determining relative Nfe2l1 mRNA levels in mouse isolated islets and spontaneously developed insulinoma cell lines MIN6 and beta-TC-6 cells (Fig. 1A). The results showed that MIN6 insulinoma cells had Nfe2l1 mRNA levels similar to mouse isolated islets. While beta-TC-6 insulinoma cells showed higher levels than other cell type.

To study any potential role of NFE2L1 in carcinogenic process for insulinomas, we first confirmed Nfe2l1 mRNA and protein levels in the established stable Nfe2l1-deficient MIN6 (Nfe2l1-KD) cells (Fig. 1B and C). There was about a 70% reduction of Nfe2l1 mRNA levels in Nfe2l1-KD MIN6 cells. NFE2L1 protein has a short life and is degraded rapidly by the proteasome system and can be stabilized by proteasome inhibition (Sha & Goldberg 2014). Thus, although a difference in NFE2L1 basal protein levels is not clearly visible between Scramble and Nfe2l1-KD by immunoblotting, treatment with the proteasome inhibitor MG132 dramatically increases protein expression in Scramble cells. In agreement with the observed reduction of Nfe2l1 mRNA, the levels of NFE2L1 protein after MG132 exposure were substantially diminished in Nfe2l1-KD cells (Fig. 1C).

Nfe2l1-KD MIN6 cells showed a high energetic profile and caused severe hypoglycemia and tumor formation after allograft transplantation

In prior work, we found silencing of Nfe2l1 in insulinoma MIN6 cells increased glucose uptake and aerobic glycolysis (Zheng et al. 2015). In this regard, in the current study, it was apparent that Nfe2l1-KD MIN6 cells showed elevated OCR and ECAR under normal culture conditions (Fig. 2A), indicating these Nfe2l1-KD insulinoma cells have a high energetic profile and potentially pointing toward malignant tendencies. To further test this hypothesis, we used an allograft cell inoculation model and inoculated WT mice with Scramble or Nfe2l1-KD MIN6 cells. As shown in Fig. 2B, inoculation of Nfe2l1-KD cells caused markedly diminished survival compared to mice inoculated with Scramble cells. Following the cell inoculation, the circulating blood glucose levels dramatically decreased (Fig. 2C). Thus, it is likely that death after MIN6 cell inoculation was due to severe hypoglycemia. Of note, for statistical purposes, the blood glucose of mice that died early was set to ’0’. Consistent with the survival data, there were more death and ‘0 mg/dl blood glucose’ in Nfe2l1-KD cell inoculation group at day 6, 12, 18, which made the blood glucose levels of mice inoculated with Nfe2l1-KD cells tended to be lower (Fig. 2C). To define the rate of tumor growth with time while negating any individualized circulating effects, we inoculated Scramble and Nfe2l1-KD MIN6 cells at separate sites into a series of the same mice. Upon death, the tumors were excised and weighed. As shown in Fig. 2D and E, we found that tumors derived from inoculated Nfe2l1-KD cells grew much faster than tumors derived from Scramble cells.

Nfe2l1-KD cell-derived allograft tumors in Akita mice showed STZ resistance

Pancreatic β-cells in the Akita mouse were shown to be spontaneously damaged by an insulin-2 gene mutation, leading to severe hyperglycemia by the age of 6–10 weeks (Oyadomari et al. 2002). In this study, we found that MIN6 cells inoculation for allograft tumor formation in WT mice markedly reduced blood glucose levels and induced hypoglycemia to the point of death (Fig. 2). To preclude any interference from endogenous pancreatic β-cells, we used Akita mice to mitigate hypoglycemia secondary to MIN6 inoculation in an allograft tumor experiment. As predicted, survival of the MIN6 cells-inoculated Akita mice (Fig. 3A) was significantly longer than WT mice (Fig. 2B). Inoculation with Nfe2l1-KD cells caused earlier death than in mice inoculated with Scramble cells in both WT (Fig. 2B) and in Akita mice (Fig. 3A). Without STZ treatment, the blood glucose levels dropped precipitously with no significant difference between Scramble and Nfe2l1-KD cells-inoculated mice (Fig. 3B). A single dose of STZ on day 7 helped mitigate hypoglycemia induced by MIN6 cells inoculation (Fig. 3C). Comparing with Scramble cells-inoculated mice, Nfe2l1-KD cells-inoculated mice were tolerant to STZ administration determined by blood glucose levels (Fig. 3C). STZ treatment improved survival of mice inoculated with Scramble cells, but failed to impact survival of mice inoculated with Nfe2l1-KD cells (Fig. 3D). This indicates that Nfe2l1-KD cells are resistant to STZ treatment compared with Scramble cells as they form insulinoma in vivo.
Nfe2l1-silenced insulinoma cells grew faster and were more aggressive

To learn the underlying mechanisms, two markers of tumor cell aggressiveness, namely cell proliferation and migration, were studied in MIN6 cells in vitro. As shown in Fig. 4A, Nfe2l1-KD MIN6 cells showed much higher numbers at day 3 compared to Scramble cells. Furthermore, the in vitro ‘wound healing’ assay, indicative of cell migration/invasiveness, also showed clear morphological evidence of cell migration in Nfe2l1-KD MIN6 cells at day 3 (Fig. 4B). Consistent with our previous study (Zheng et al. 2015), expression of glycolysis regulatory genes, including Pkm2 and its inhibitory kinase genes Pdk1, Pdk2 and Pdk3, were all altered in Nfe2l1-KD MIN6 cells. Indeed, silencing of Nfe2l1 also dramatically affected the gene expression of cell migration and tumor progression, including epithelial cell adhesion molecule (Epcam), epithelial splicing regulatory protein 1 (Esrp1), Snail family zinc finger 1 (Snail1), Snail family zinc finger 2 (Snail2) and Zinc finger E-box-binding homeobox 1 (Zeb1) (Fig. 4C), which are associated with cell proliferation and migration phenotypes (Gemmill et al. 2011, Reinke et al. 2012).

Nfe2l1-silenced insulinoma cells were chemoresistant

High bioenergetic profiles, including high OCR and ECAR, are associated with aggressive and chemotherapeutic-resistance cancers (Lin et al. 2012, Vaughan et al. 2014, Pavlova & Thompson 2016). Thus, the susceptibility of Nfe2l1-KD MIN6 cells to acute chemotherapeutic drug-induced cell damage was assessed. As shown in Fig. 5A, Scramble MIN6 cells displayed a typical morphology of cytotoxicity after 8h of STZ treatment, which included cell shrinkage and disruption of cell adhesion. However, Nfe2l1-KD cells similarly treated with STZ showed morphology essentially unaltered from untreated control cells. Consistent with this observation, Nfe2l1-KD cells were also less sensitive to STZ-induced loss of cell viability, as determined by MTS assay (Fig. 5B) and the trypan blue exclusion assay (Fig. 5C). In agreement with the cytotoxicity assays, STZ treatment increased apoptosis in Scramble MIN6 cells in a concentration-dependent fashion (Fig. 6A and B). Importantly, the levels of apoptosis induced in Nfe2l1-KD MIN6 cells by STZ exposure were near baseline (Fig. 6A and B), indicating that Nfe2l1 deficiency instilled resistance to STZ-induced apoptosis. Beyond the pancreatic β-cell-specific chemotherapeutic drug STZ,
Nfe2l1 deficiency did have an impact on resistance to some other general cancer chemotherapeutics. For instance, 5-Fu was less cytotoxic with Nfe2l1 deficiency (Fig. 6C). However, Nfe2l1 deficiency appeared to have no impact on cisplatin-induced cytotoxicity (Fig. 6C).

Many chemotherapeutic drugs have been reported to be mitochondrial toxins (Yang et al. 2016, Guerra et al. 2017). To explore the mechanism of chemoresistance in Nfe2l1-KD MIN6 cells, we tested specific mitochondrial inhibitors. The cell viability of Scramble MIN6 cells was decreased at 24 h by exposure to mitochondrial complex inhibitors, including rotenone, antimycin, oligomycin and mitochondrial uncoupler CCCP in a concentration-dependent fashion (Fig. 7A). These cell viability data by various mitochondrial inhibitor treatments was confirmed by LDH release measurements (Fig. 7B). Notably, compared with Scramble MIN6 cells, Nfe2l1-KD cells showed a significant resistance to the cytotoxicity induced by mitochondrial inhibition (Fig. 7A and B).

Figure 5
Nfe2l1-KD MIN6 β-cells were resistant to the cytotoxicity induced by STZ. (A) Bright field microscopy images (40×) of Scramble and Nfe2l1-KD cells after 8 h treatment with 4 mM STZ. (B and C) MTS cell viability assay after 24 h STZ exposure (B) and trypan blue staining post 8 h STZ exposure (C) showing Nfe2l1-KD confers resistance. n=3. *P<0.05 vs Scramble given the same treatment. #P<0.05 vs vehicle (Veh) of the same cell genotype.

Figure 6
Nfe2l1-KD MIN6 β-cells were resistant to apoptosis induced by STZ. (A) Representative images of flow cytometry-determined apoptosis by Annexin V and PI staining. (B) Quantitative data for apoptosis determination. Cells were treated with various concentrations of STZ for 8 h. (C) Trypan blue staining following a 24 h treatment with 5-Fu or Cisplatin. Data were expressed as trypan blue positive cells (% total cells). n=3. *P<0.05 vs Scramble given the same treatment. #P<0.05 vs vehicle (Veh) of the same cell genotype.
Effects of deficiency of Nfe2l1 on the mitochondrion

STZ exposure results in pancreatic β-cell and insulinoma cell death and apoptosis by induction of oxidative stress and DNA alkylation (Wang & Gleichmann 1998, Szkudelski 2001). While 5-Fu and mitochondrial inhibitors may induce cell damage indirectly, the final apoptotic signal originates within the mitochondrial system (Yang et al. 2016, Guerra et al. 2017). To further study the involvement of Nfe2l1 deficiency in chemotherapeutic resistance and define mitochondrial function in cell-specific chemotherapy-induced cytotoxicity and apoptosis, we performed mitochondrial calcium release assays to measure the opening rate of mPTP. The kinetic slope of mitochondrial calcium decrease after ionomycin incubation was lower in Nfe2l1-KD cells than Scramble MIN6 cells (Fig. 8A), indicating a lower opening rate of mPTP, mitochondrial cytochrome c release blocking and inhibition of mitochondrial depolarization. Consistent with this result, Nfe2l1-KD cells had higher basal mitochondrial membrane potential as determined after probing with TMRM (Yehuda-Shnaidman et al. 2010) (Fig. 8B). We previously showed Hk1 mRNA expression and HK1 protein levels were increased in Nfe2l1-KD cells (Zheng et al. 2015), which blocks mitochondria-derived apoptotic signals by binding to VDAC, a component of mPTP (Schindler & Foley 2013). As shown in Fig. 8C, Western blot analysis showed HK1 in the mitochondrial fraction was highly enriched in Nfe2l1-KD cells. This supports the hypothesis that HK1 may attach to the mitochondria to block mitochondrial toxicant-induced cytotoxicity and apoptosis.

Discussion

NFE2L1 is ubiquitously expressed and has been reported to play critical roles in embryonic development, organ differentiation (Chen et al. 2003, Leung et al. 2003), oxidative stress response (Xing et al. 2007, Ohtsuiji et al. 2008) and hepatic lipid metabolism (Xu et al. 2005, Hirotsu et al. 2012). Our previous findings showed that β cell-specific Nfe2l1-knockout mice (Nfe2l1-(b)KO) have severe hyperinsulinemia and glucose intolerance (Zheng et al. 2015). Furthermore, the silencing of Nfe2l1 in MIN6 β-cells and isolated mouse islets results in elevated basal insulin release but reduced GSIS (Zheng et al. 2015). Specifically, Nfe2l1-KD MIN6 β-cells showed enhanced lactate production and aerobic glycolysis by dramatically upregulating various metabolic enzymes, including HK1.
and lactate dehydrogenase A (Zheng et al. 2015). In the current study, we utilized these same Nfe2l1-KD MIN6 cells and tested the impact of this gene deficiency on tumor forming capacity and formed tumor sensitivity to chemotherapy. The Nfe2l1-deficient MIN6 cells produced more aggressive tumors after allograft inoculation, as they grew much more rapidly and were chemoresistant to various agents compared to tumors produced by Scramble MIN6 cells. In vitro, the Nfe2l1-KD MIN6 cells were also hyperproliferative, showed enhanced migration/ invasiveness and displayed chemotherapeutic resistance.

In this regard, recent clinical evidence indicates that larger insulinas more frequently show malignant characteristics, such as local invasion or metastasis to the liver, than smaller tumors (Ueda et al. 2016). The drug resistance from Nfe2l1-KD deficiency appeared to be due to resistance to chemotherapeutic-induced mitochondrial damage and was due to upregulation of HK1, which was localized in mitochondria and caused mPTP closure. A specific resistance to STZ-induced cell damage was also seen in an allograft Nfe2l1-KD MIN6 cell inoculation model using type 1 diabetic Akita background mice. Thus, NFE2L1 may be key in the carcinogenic process in the pancreas, and its expression appears to impact cancer chemotherapeutic sensitivity, at least for insulinas.

The enhanced aerobic glycolysis in Nfe2l1-KD MIN6 insulina cells is similar with the metabolic preference of the ‘Warburg Effect’, a characteristic of most of malignant tumors (Danhi et al. 2017, Hasanpourghadi et al. 2017). The Warburg effect is thought to be a consequence of dysfunctional mitochondria in cancer cells and adaption to low oxygen environments (Vander Heiden et al. 2009, Levine & Puzio-Kuter 2010). We have shown that expression of glycolysis-related enzymes is dramatically enhanced in Nfe2l1-deficient MIN6 cells, including HK1 and LDHA (Zheng et al. 2015) and Pkm2 and its inhibitory kinase Pdks (current study). These glycolysis-related enzymes are all associated with tumor migration and progression in various ways and considered tumor markers (Azoitei et al. 2016, Kudryavtseva et al. 2016, Wang et al. 2017). Surprisingly, higher oxygen consumption and enhanced mitochondrial metabolism rate were also observed in Nfe2l1-deficient MIN6 cells, indicating a high bioenergetic profile. This metabolism status switch is also seen in immune cell activation (van der Windt et al. 2013, Caro-Maldonado et al. 2014), osteoblasts differentiation (Guntur et al. 2014) and insulin secretion (Zheng et al. 2015), where cellular metabolism is reprogrammed and high ATP production is required. In this regard, the blood glucose levels in WT or Akita mice were reduced by MIN6 cell inoculation regardless of the cell genotype (Nfe2l1-KD or Scramble). All the inoculated MIN6 cells grew into tumors causing severe hypoglycemia often to the point of death (Kimura et al. 2002). However, the more highly energetic Nfe2l1-KD MIN6 cells when inoculated in WT mice produced tumors that grew more rapidly and aggressively, as reflected by tumor size and survival (Fig. 2). The findings are confirmed by the survival data in Akita mice (Fig. 3). This enhanced tumorigenicity is consistent with prior work showing enhanced carcinogen...
potential in Nfe2l1-deficient liver cells (Xu et al. 2005, Ren et al. 2016) and indicates NFE2L1 status may be key factor in tumor formation in some cells.

Surgical resection is the primary choice to cure solitary insulinomas in the clinic (Ro et al. 2013, Parbhoo & Adler 2016). In patients with unresectable malignant insulinomas, chemotherapeutic drugs, such as STZ (Schulz et al. 1990, Shao et al. 2016), are employed to reverse the manifestations of chronic hypoglycemia associated with insulinomas. In our allograft tumor model using diabetic Akita mouse, STZ administration did reverse the hypoglycemia and enhanced the survival of mice inoculated with Scramble insulinoma cells, but had no impact on the survival of mice inoculated with Nfe2l1-KD cells. The resistance to STZ treatment in mice inoculated with Nfe2l1-KD cells is consistent with in vitro data. DNA alkylation and oxidative stress are implicated as major mechanisms for STZ-induced pancreatic β-cell destruction. Mitochondrial-derived apoptosis and/or necrosis are consequences of STZ-induced DNA alkylation and oxidative stress (Szkudelski 2001, Wu & Yan 2015). Other chemotherapeutic drugs can be used, such as 5-Fu that directly damages tumor cells or mitochondrial inhibitors that indirectly kill cells from mitochondrial-derived apoptosis and/or necrosis (Lopez & Tait 2015, Yang et al. 2016, Guerra et al. 2017). In the current study, Nfe2l1-KD insulinoma cells were also resistant to these general chemotherapeutic drugs and mitochondrial inhibitors. The mechanisms involved appear to be elevated mitochondrial function and aerobic glycolysis in the Nfe2l1-KD insulinoma cells (Fig. 9).

Both higher OCR and glycolysis rate in Nfe2l1-KD MIN6 cells were detected in the present work. In agreement with findings, we found a higher mitochondrial membrane potential, but no increase in mitochondria mass (Zheng et al. 2015). Enhanced membrane potential is clearly correlated with higher OCR and ECAR, but also cause inhibition of ion leaking through the mitochondrial pores, especially mPTP (Lopez & Tait 2015). Glucokinase (GCK) is the glucose sensor of pancreatic β-cells, which

![Potential mechanisms for aggressive tumor formation and chemoresistance in Nfe2l1-deficient insulinoma cells. In normal (Scramble) insulinoma cells, glucokinase (GCK) primarily phosphorylates glucose to glucose-6-phosphate (G-6-P). G-6-P metabolizes to pyruvate in cytoplasm, and then pyruvate is transported to mitochondria for further oxidation. In Nfe2l1-deficient insulinoma cells, the switch of low-affinity GCK to high-affinity HK1 occurs. The upregulation of HK1 in Nfe2l1-KD MIN6 cells accelerates glucose metabolism and reprograms high energetic (OCR and ECAR) insulinoma cells, which become aggressive. mPTP is a voltage-dependent channel complex in mitochondria. HK1 is specifically upregulated and attached to mitochondria in Nfe2l1-deficient insulinoma cells to block chemotherapeutic drugs-derived, cytochrome c release and apoptotic signaling, which is related to chemotherapy resistance. A full color version of this figure is available at http://dx.doi.org/10.1530/ERC-17-0458.](http://erc.endocrinology-journals.org)
phosphorylates glucose to glucose-6-phosphate (G-6-P). G-6-P metabolizes to pyruvate in cytoplasm, and then pyruvate is transported to mitochondria for further oxidation (Wilson 1995). Previously, we identified the switch of GCK to HK1 as the reason for dysfunction of Nfe2l1-KD and KO β-cells (Zheng et al. 2015). HK1 is also a known anti-apoptotic protein, which specifically binds with VDAC (Linden et al. 1982, Wilson 1995, Pedersen et al. 2002, Schindler & Foley 2013) of mPTP to impact release of cytochrome c and ion permeability, stimulating a closed state for mPTP and thus blocking mitochondrial induced apoptosis (Azoulay-Zohar et al. 2004, Schindler & Foley 2013). In accord with our prior work (Zheng et al. 2015), in the current study, HK1 specifically upregulated and attached to mitochondria in Nfe2l1-KD insulinoma cells. This supports the notion that HK1 is a major factor toward mitigation of mitochondrial induced apoptosis (Fig. 9). mPTP is a voltage-dependent channel complex, which contains the VDAC in the outer membrane, ANT in the inner membrane and cyclophilin D on the matrix side (McCommis & Baines 2012, Hurst et al. 2017). Opening of mPTP can lead to mitochondrial swelling and cell death via apoptosis or necrosis. The closure of mPTP, which decreases mitochondrial membrane permeability, causes mitochondria to become further polarized and resistant to mitochondrial induced apoptosis or cytotoxicity (Hurst et al. 2017).

Deficiency of Nfe2l1 can lead to genetic instability (Oh et al. 2012), further suggesting that NFE2L1 normally suppresses emergence of cancer. Formation of tumors is a multistep process, including initiation, promotion and progression (Barcellos-Hoff et al. 2013), and the exact role of NFE2L1 in some cells the process is not fully clear. Prior reports demonstrated that hepatocyte-specific deletion of Nfe2l1 in mice (Nfe2l1(L)-KO) causes spontaneous hepatic apoptosis, inflammation and steatohepatitis at young age and liver tumors by 12-months of age (Xu et al. 2005, Ohtsui et al. 2008). In this study, Nfe2l1-KD insulinoma cells-derived tumors showed faster, more aggressive growth, were more lethal and resistant to specific and general chemotherapeutics than tumors derived from control cells. The MIN6 cells were originally generated from a mouse insulinoma by overexpressing the SV40 large T-antigen, which promotes malignant transformation in insulin-expressing cells (Miyazaki et al. 1990). Both Nfe2l1-KD insulinoma cells and the Scramble insulinoma cells form allograft tumors after inoculation. In contrast, Nfe2l1(b)-KO mice do not show evidence of insulinoma formation (no change in islet size, no overt tumors, etc.) at least by 17 weeks (Zheng et al. 2015). This may well be too short a time span (Zheng et al. 2015) for tumors to form in vivo. This lack of tumor formation from the pancreatic β-cells of Nfe2l1(b)-KO mice in vivo and yet rapid Nfe2l1-KD MIN6 cells tumorigenicity after allograft inoculation may well be because the MIN6 cells have already been transformed and immortalized, critical early steps in the process of tumor formation. Presumably, in vivo pancreatic β-cells Nfe2l1 deficiency alone may be insufficient to initiate transformation toward tumorigenesis, at least over just 17 weeks. Clearly, further experiments should be performed in Nfe2l1(b)-KO mice with longer time periods, and perhaps using pancreatic tumor initiators or promoters. These experiments should help sort out whether insulinoma occurs in Nfe2l1(b)-KO mice and at what stage or stages of the carcinogenic process NFE2L1 may function as a tumor suppressor.

In summary, although NFE2L1 normally regulates glucose metabolism in pancreatic β-cells, we find when cells are made Nfe2l1 deficient, it impacts insulinoma cell allograft tumor growth and aggressiveness. Nfe2l1-deficient insulinoma cells show a marked chemotherapeutic resistance through the HK1-mPTP-related metabolic pathway. The fact that Nfe2l1 deficiency in inoculated cells resulted in a more rapid tumor growth and aggressiveness potentially indicates an impact on tumor progression as recent evidence indicates that larger insulinomas are more frequently malignant than smaller ones (Ueda et al. 2016). This study provides additional evidence that NFE2L1 is involved in chemotherapeutic resistance and indicates NFE2L1 modulation could be an important therapeutic target in the future to help prevent or cure certain cancers. However, the precise mechanisms by which NFE2L1 regulates metabolic reprogramming in precancerous cells will need further investigation to fully define.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0458.

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.

Funding
This work was supported by National Natural Science Foundation of China 81402635 (J F), 81573106 (J P), 81602825 (B Y) and 81572360 (Y X), China Medical University Training Program for National Natural Science Fund for Excellent Young Scholars (YQ20170001, J F), the Startup Funding of
China Medical University (J P), Liaoning Pandeng Scholar Program (J P) and Program for Liaoning Innovative Research Team in University (No. LT2015028).

Author contribution statement
J F, H Z, Q C, C C and S B performed the research and analyzed the data. J F and J S generated the stable knockdown cells. J F, C C, J S and L L performed the mouse allograft experiments. B Y, H W, Y H, Y X and Y Y revised the manuscript. J F, H W, Y H, Y X and J P planned and supervised the experiments. J F, Q Z and J P wrote the manuscript.

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Received in final form 20 November 2017
Accepted 4 December 2017
Accepted Preprint published online 4 December 2017