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## Tissue-specific induced DNA methyltransferase 1 (Dnmt1) in endocrine pancreas by RCAS-TVA-based somatic gene transfer system promotes $\beta$ -cell proliferation

Ziqiang Yuan<sup>1,2</sup>, Juliet C. Gardiner<sup>2</sup>, Elaine C. Maggi<sup>2,3</sup>, Asha Adem<sup>2</sup>, George Zhang<sup>4</sup>, Sylvia Lee<sup>2</sup>, Peter Romanienko<sup>2</sup>, Yi-Chieh Nancy Du<sup>4</sup>, Steven K. Libutti<sup>2,5</sup>

<sup>1</sup>Department of Medical Oncology, Robert Wood Johnson Medical School, New Brunswick, NJ, USA

<sup>2</sup>Rutgers Cancer Institute of New Jersey, New Brunswick, NJ, USA

<sup>3</sup>Department of Genetics, Albert Einstein College of Medicine, Bronx, NY, USA

<sup>4</sup>Department of Pathology and Lab Medicine, Weill Cornell Medicine, New York, NY, USA

<sup>5</sup>Department of Surgery, Robert Wood Johnson Medical School, New Brunswick, NJ, USA

### Abstract

We reported that inactivation of menin (the protein product of *MEN1*) increases activity of Dnmt1 and mediates DNA hypermethylation in the development of multiple endocrine neoplasia type 1 (MEN1) syndrome. We have developed a RCAS-TVA-based somatic gene transfer system that enables tissue-specific delivery of Dnmt1 to individual  $\beta$ -cells of the pancreas in a *RIP-TVA* mouse model. In the present study, we mediated Dnmt1 expression in islet  $\beta$ -cells in *RIP-TVA* mice by utilizing the RCAS-TVA system to test if the upregulation of Dnmt1 can promote  $\beta$ -cell proliferation. In vitro, we demonstrated that upregulation of Dnmt1 increased  $\beta$ -cell proliferation. In vivo, our results showed that the levels of serum insulin were increased in the *RIP-TVA* mice with RCASBP-*Dnmt1* infection compared with wild-type control mice with RCASBP-*Dnmt1* infection. Furthermore, we confirmed that mRNA and protein expression of Dnmt1 as well as Dnmt1 enzyme activity were upregulated in the *RIP-TVA* mice with RCASBP-*Dnmt1* infection compared with wild-type control mice with RCASBP-*Dnmt1* infection. Finally, we demonstrated that upregulation of Dnmt1 resulted in hyperplasia through  $\beta$ -cell proliferation. We conclude that the upregulation of Dnmt1 promotes islet  $\beta$ -cell proliferation and targeting Dnmt1 may be a promising therapy for patients suffering from pancreatic neuroendocrine tumors.

### Introduction

MEN1 is a familial cancer syndrome characterized by tissue-specific tumorigenesis via largely unknown mechanisms. Patients with *MEN1* mutations develop tissue-specific

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Steven K. Libutti, Steven.libutti@cinj.rutgers.edu.

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neoplasms in the parathyroid glands, anterior pituitary glands, and the endocrine pancreas [1–6]. Menin, encoded by *MEN1*, is a transcriptional modulator and a tumor suppressor. Menin is involved in the regulation of DNA repair, cell proliferation, and apoptosis [7–12].

Human cancer genomes are characterized by widespread aberrations in DNA methylation patterns [13–16]. Studies have shown that the loss of menin increases DNA hypermethylation at the promoter region of several genes [17, 18]. Using a novel genome-wide methylation analysis method, we recently demonstrated that inactivation of menin results in increased activity of DNA methyltransferase 1 (Dnmt1) by activating retinoblastoma-binding protein 5 (Rbbp5), a menin-associated protein [19]. Despite *Men1* loss in all pancreas cell types, the increased Dnmt1 activity resulted in global DNA hypermethylation and tumorigenesis in endocrine tissues of the pancreas but not in exocrine tissues of the pancreas [19]. Therefore, the characterization of the role of Dnmt1 in endocrine tissues of the pancreas may provide an important insight into the possible role of epigenetic mechanisms in the pathogenesis of MEN1 tumor development.

Tumors develop through multiple stages, implicating the involvement of multiple effectors, but the tools to assess how candidate genes contribute to stepwise tumor progression have been limited due to a lack of a tissue-specific delivery system. We developed a Replication-Competent ASLV long terminal repeat (LTR) with a splice acceptor-tumor virus A (RCAS-TVA) expression system that enables tissue-specific delivery of genes to individual cells of the pancreas in adult mice [20, 21]. Using the tissue-specific rat insulin promoter (RIP) that drives the expression of the receptor for subgroup A avian leucosis virus (TVA), the *RIP-TVA* mice can be infected with avian viruses carrying genes of interest [20, 21]. In this study, we investigate the role of Dnmt1 in promoting  $\beta$ -cell proliferation by tissue-specific Dnmt1 upregulation in endocrine tissues of the pancreas using our established RCAS-TVA somatic gene transfer system.

## Materials and methods

### Cell culture

The N134 cell line was generated from an islet  $\beta$ -cell tumor in a *RIP-Tag; RIP-TVA* mouse [20]. DF1 is a chicken fibroblast cell line used to express RCASBP-*Dnmt1* and RCASBP-*Luciferase*, respectively. The N134 cell line was infected with viruses carrying RCASBP-*Dnmt1* following the established protocol [22]. The mouse DF1 and N134 cell lines were cultured in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin.

### Animal experiments

Generation of RIP-TVA mice has been previously described [20]. All mice were housed in accordance with the institutional guideline. All procedures involving mice were approved by the institutional animal care and use committee of Weill Cornell Medicine. Viral propagation and titer determination were described previously [20, 22]. Two-day-old *RIP-TVA* mice ( $n = 4$ ) and wild-type (WT, in C57BL/6 background,  $n = 3$ ) control mice were injected with RCASBP-*Dnmt1* at  $1 \times 10^8$  IU/ml by intra-peritoneal (IP) injection. All mice were killed at 8 months post-infection.

### Cloning of RCASBP-*Dnmt1*

RCASBP is a replication competent TVA with a splice acceptor and Bryan-RSV pol gene. RCASBP-*Dnmt1* was generated by Biomatik (Biomatik, Wilmington, DE).

### Viral propagation in DF1 cell line

Five- $\mu$ g of viral RCASBP-*Dnmt1* DNA and RCASBP-*Luciferase* (control) with DMEM (without serum, penicillin, or streptomycin) were diluted, respectively. The diluted RCASBP-*Dnmt1* DNA and RCASBP-control were further transfected to DF1 cells as described [22], with the exception that lipofectamine 2000 (Life Technologies, Grand Island, NY) was used.

### In vitro infection of TVA-expressing cells and In vivo infection of mice

Viral supernatant containing RCASBP-*Dnmt1* or RCASBP-control was collected from confluent DF1 cells and the viral supernatant was passed through a 0.45  $\mu$ m filter to obtain cell-free viruses as previously described [22]. The viral supernatants containing RCASBP-*Dnmt1* or RCASBP-control were used to infect N134 cells, respectively. The infected N134 cells were harvested at 24 h and 48 h post-infection for the in vitro studies. A total of 0.5 million DF1 cells expressing RCASBP-*Dnmt1* were injected into mice for the in vivo studies via IP injection.

### Detection of viral integration in the RCASBP infected cells

Identification of viral integration was used to detect the presence of RCASBP in the infected cells. DNA was extracted from infected cells with RCASBP-*Dnmt1* and RCASBP-control, respectively, using a DNeasy extraction kit (Qiagen, Valencia, CA). The viral integration was detected by PCR as described previously [22]. The primers for viral integration are listed in Supplementary Table 1.

### Laser capture microdissection (LCM)

The pancreatic tissues of mice were harvested and embedded in OCT. The OCT-embedded tissue sections were stained with Arcturus Histogene Frozen Section Staining Kit (Applied Biosystems, Foster City, CA) and the islet tissues were further dissected using an Arcturus XT LCM system per manufacturer's instructions.

### Real-time RT-PCR analysis

Total RNA was extracted from infected cells using the RNeasy extraction kit (Qiagen, Valencia, CA) and from LCM islet tissues of *RIP-TVA* mice and WT control mice using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA). mRNA expression of the *Dnmt1* gene was detected by quantitative RT-PCR. The primers for the *Dnmt1* gene are listed in Supplementary Table 1.

### Western analysis

Total protein was extracted from N134 cells infected with RCASBP-*Dnmt1* or RCASBP-control using a RIPA lysis buffer (Pierce, Rockford, IL). The extracted proteins were quantified and equal amounts of protein were separated by SDS-PAGE for western blotting.

The blots were incubated overnight at 4 °C with primary antibodies: mouse anti-GAPDH (1:1000 dilution) and mouse anti-Dnmt1 (1: 2000 dilution) (Abcam, Cambridge, MA), respectively. The secondary antibody used was: 1:5000 dilution of anti-mouse IgG-HRP (Thermo Fisher Scientific, Rockford, IL). The small differences in loading were corrected by comparison with the loading control, GAPDH.

### **MTS cell proliferation analysis**

To detect  $\beta$ -cell proliferation with a MTS cell proliferation assay kit,  $1 \times 10^3$  cells (N134) were seeded in a 96-well plate and the cells were infected with virus containing RCASBP-*Dnmt1* and RCASBP-control, respectively. After 24 h and 48 h of incubation, cell proliferation was analyzed with a MTS cell proliferation assay kit (Abcam, Cambridge, MA) according to the manufacturer's instruction.

### **Dnmt1 enzymatic activity assay**

Dnmt1 enzyme activity was detected on nuclear complexes extracted from infected cells and mouse pancreatic tissue samples using a Dnmt1 enzyme assay kit (EpiGentek, Farmingdale, NY) per manufacturer's instructions. The enzymatic activity of Dnmt1 was detected with a microplate reader at 450 nm [19].

### **Enzyme-linked immunosorbent assay (ELISA) analysis**

Mouse blood was collected via a retro-orbital bleeding technique following an 18 h fast. Serum levels of insulin were measured by ELISA with the Ultrasensitive Mouse Insulin ELISA Kit (Merckodia, Winston-Salem, NC) as described previously [23].

### **Hematoxylin and Eosin (H&E) staining**

Whole pancreata were frozen and sectioned (5- $\mu$ m) on a rotating microtome (Microm, Walldorf, Germany). H&E staining was performed according to standard procedures. For morphometric analysis, three 100- $\mu$ m sections were digitalized. Sections with maximal tumor diameter in the pancreatic cross-sectional area were determined by computerized pixel counting.

### **Immunofluorescence (IF) staining**

IF staining was used to evaluate the expression levels of Dnmt1 and Ki67 in pancreatic tissues from treated mice. The sections were incubated overnight at 4 °C with primary antibodies: pig anti-insulin antibody (1:500 dilution; DAKO, CA, USA), mouse anti-Dnmt1 (1:200 dilution), and mouse anti-ki67 (1:1000 dilution) (Abcam, Cambridge, MA), respectively. After washing, the slides were incubated with goat anti-mouse Alexa Fluor 488 (Life Technologies, Grand Island, NY) and goat anti-guinea pig Alexa Fluor 647 (Life Technologies, Grand Island, NY) (both 1:200 dilutions) for 45 min in the dark. The slides were assembled in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were obtained with an epifluorescence microscope with an attached camera.

## Statistical analysis

The two-tailed student's *t*-test was performed to assess the significance between experimental groups. GraphPad software (GraphPad Software, Inc, version 5.02) was used for all statistical analyses. Statistically significant *P* values are indicated in the figures.

## Results

### RCASBP-TVA system enables delivery of the target gene to cells

In order to assess viral integration in the N134 TVA-expressing cells after RCASBP-*Dnmt1* and RCASBP-control infection, DNA was extracted from infected N134 cells and PCR was performed. Virus integration was identified in N134 cells with both RCASBP-*Dnmt1* and RCASBP-control infection at 24 h and 48 h, respectively (Fig. 1). These data indicate that the RCASBP-TVA system enables delivery of *Dnmt1* to cells.

### Islet $\beta$ -cells with RCASBP-*Dnmt1* infection show increased expression levels and enzyme activity of *Dnmt1*

To test if RCASBP-*Dnmt1* infection can induce *Dnmt1* expression in the N134  $\beta$ -cell tumor cell line, we measured the expression levels and enzyme activity of *Dnmt1* in N134  $\beta$ -cell tumor cells. We confirmed that the mRNA and protein expression levels were significantly increased in the N134 cells with RCASBP-*Dnmt1* infection when compared with the N134 cells with RCASBP-control infection, as measured by real-time PCR and western blot analysis (Fig. 2a, b). Furthermore, we found that *Dnmt1* enzymatic activity was also significantly increased in the N134 cells with RCASBP-*Dnmt1* infection when compared with the N134 cells with RCASBP-*Luciferase* control infection by a functional *Dnmt1* enzymatic activity assay (Fig. 2c). These results indicate that the infection of N134 TVA-expressing cells with RCASBP-*Dnmt1* can successfully deliver *Dnmt1* to individual cells, upregulate *Dnmt1* mRNA and protein expression and enhance *Dnmt1* enzyme activity in vitro.

### Upregulation of *Dnmt1* in N134 $\beta$ -cell tumor cell line with RCASBP-*Dnmt1* infection increased $\beta$ -cell proliferation

Our previous study demonstrated that MEN1 tumors are associated with global DNA methylation and *Dnmt1* overexpression [19]. Using a MTS cell proliferation assay, we demonstrated that upregulation of *Dnmt1* by RCASBP-*Dnmt1* infection promotes cell proliferation of islet cells as compared with RCASBP-control infected cells after 24 h ( $P=0.001$ ) and 48 h ( $P<0.001$ ) post-infection (Fig. 3). These results indicate that overexpression of *Dnmt1* in  $\beta$ -cell can promote  $\beta$ -cells proliferation in vitro.

### Tissue-specific somatic *Dnmt1* delivery in islet $\beta$ -cells increased serum insulin levels in RIP-TVA mice

To determine the effect of *Dnmt1* upregulation in islet  $\beta$ -cells in vivo, we injected DF1 cells expressing RCASBP-*Dnmt1* into newborn RIP-TVA mice or WT control litter-mates (p1 or p2). Eight months later, we used serum insulin as a measurable surrogate for  $\beta$ -cell proliferation. The infected RIP-TVA mice ( $N=4$ ) had significantly higher serum insulin

levels when compared with the infected WT mice ( $N=3$ ), as measured by an insulin ELISA assay ( $P=0.0205$ ) (Fig. 4).

### Tissue-specific somatic Dnmt1 delivery resulted in hyperplasia through $\beta$ -cell proliferation in *RIP-TVA* mice

Using a *RIP-TVA* transgene to express the TVA receptor in  $\beta$ -cells of the pancreas, the candidate gene has been demonstrated to induce the somatic development of neo-plastic  $\beta$ -cell lesions in *RIP-TVA* transgenic mice by infection with the RCASBP vector [20, 21]. To identify the effects of tissue-specific upregulation of Dnmt1 in  $\beta$ -cells of the pancreas, we compared the islet size of the *RIP-TVA* mice to that of the WT control mice when treated with RCASBP-*Dnmt1*. Hyperplasia was observed in the islets of the pancreas and the islet size was significantly increased in the *RIP-TVA* mice compared with the WT control mice ( $2202 \mu\text{m}^2 \pm 708.6$  vs.  $441 \mu\text{m}^2 \pm 44.6$ ;  $P=0.0079$ ) (Fig. 5). Furthermore, we demonstrated that  $\beta$ -cell proliferation was significantly increased in *RIP-TVA* mice infected with RCASBP-*Dnmt1* when compared with the control mice, as measured by Ki67 staining ( $P=0.0071$ ) (Fig. 6). The results indicate that Dnmt1 overexpression in  $\beta$ -cells of the pancreas results in hyperplasia through  $\beta$ -cell proliferation.

### Tissue-specific somatic Dnmt1 delivery in islet $\beta$ -cells increased the expression level and enzyme activity of Dnmt1 in *RIP-TVA* mice

We previously demonstrated the upregulation of Dnmt1 in insulinomas of *Men1* conditional knockout mice [19]. In this present study, we examined the expression of Dnmt1 in pancreatic islet tissues using LCM real-time PCR and IF staining. We demonstrated a significant upregulation of Dnmt1 in the islets of *RIP-TVA* mice infected with RCASBP-*Dnmt1* compared with infected WT control mice ( $P=0.0115$ ) (Fig. 7a, b). Furthermore, we found that Dnmt1 enzymatic activity was significantly increased in the islets of *RIP-TVA* mice infected with RCASBP-*Dnmt1* compared with WT mice infected with RCASBP-*Dnmt1* ( $P=0.0361$ ) (Fig. 7c). These results indicate that the RCAS-TVA expression system enables tissue-specific delivery of the Dnmt1 gene to individual dividing cells of the pancreas in adult mice.

## Discussion

DNA methylation is the most common epigenetic modification in the mammalian genome, and aberrant methylation patterns can be found in human tumors [24–28]. DNA methylation is catalyzed by a family of DNA methyltransferases. Of these, Dnmt1 is a maintenance methylase, making it a key player in the important process of DNA methylation [29–32]. Overexpression of Dnmt1 has been detected in many types of human cancers, including neuroendocrine tumors [33–37]. Recently, we demonstrated that inactivation of menin increases activity of Dnmt1 and mediates global DNA hypermethylation in endocrine tissues of the pancreas and parathyroid glands resulting in MEN1 tumorigenesis [19]. Therefore, further characterization of the role of Dnmt1 upregulation in endocrine tissues of the pancreas appeared an attractive area of investigation. In this study, we tested the role of Dnmt1 in a mouse islet  $\beta$ -cell tumor cell line and islet  $\beta$ -cells of the pancreas in *RIP-TVA* mice by utilizing the RCAS-TVA somatic gene transfer system. The RCAS-TVA somatic



gene transfer system enables tissue-specific delivery of genes to TVA-expressing cells of the pancreas in *RIP-TVA* mice [20, 21]. In the present study, we generated a RCASBP-*Dnmt1* expression vector that expressed Dnmt1 and increased Dnmt1 enzyme activity in the N134  $\beta$ -cell tumor cell line derived from a *RIP-Tag*; *RIP-TVA* mouse and in the islet tissues in *RIP-TVA* mice by RCASBP-*Dnmt1* virus infection. In this well-characterized model, we demonstrated that Dnmt1 can be somatically delivered in a tissue-specific manner under the control of TVA-expressing  $\beta$ -cells of the pancreas. Our results demonstrated that this approach allows for the introduction of somatic genetic changes in a tissue-specific way by generating a vector carrying the gene-of-interest, which is advantageous to the extensive process of generating transgenic mouse models.

We previously showed that increased Dnmt1 activity resulted in global DNA methylation and tumorigenesis in endocrine tissue but not in exocrine tissue of the pancreas. In this current study, we performed phenotypic analysis and demonstrated that the upregulation of Dnmt1 in islet  $\beta$ -cells of the pancreas with RCASBP-*Dnmt1* virus infection can increase serum insulin levels in the *RIP-TVA* mice. The islet hyperplasia was confirmed in *RIP-TVA* mice and  $\beta$ -cell proliferation was also increased in both in vitro and in vivo systems in *RIP-TVA* mice after RCASBP-*Dnmt1* virus infection. Although the low efficiency of in vivo infection has been a limitation of using the RCAS-TVA system, with reports of islet  $\beta$ -cells gradually developing into tumors [20, 21], our present study demonstrated that islet hyperplasia in *RIP-TVA* mice was observed at 8 months post-infection with RCASBP-*Dnmt1*. These results support our observation that Dnmt1 may be an important driver in the initiation of pancreatic neuroendocrine tumors [19] and suggest that further testing of Dnmt1 blockade may be a strategy for treating MEN1 related pancreatic neuroendocrine tumors.

Previously, we demonstrated that the increased activity of DNMT1 mediates global DNA hypermethylation, which results in aberrant activation of the Wnt/ $\beta$ -catenin signaling pathway through inactivation of *Sox* regulatory genes in the development of MEN1 [19]. Several studies have linked decreased expression of the *Sox* family genes with tumorigenesis [38–43]. Therefore, a further investigation into the dysregulated signaling pathways relevant to MEN1 tumorigenesis and resulting from methylation changes by Dnmt1 activity is necessary.

In conclusion, we have assessed the effect of Dnmt1 in pancreatic neuroendocrine tumors using a RCAS-TVA somatic tissue-specific gene transfer mouse model without the need to generate a conditional transgenic mouse. Furthermore, we demonstrated that increasing Dnmt1 activity can promote  $\beta$ -cell proliferation. Our findings suggest that Dnmt1 may be an important driver in the promotion of pancreatic neuroendocrine tumors and therefore could be a potential therapeutic target for their treatment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

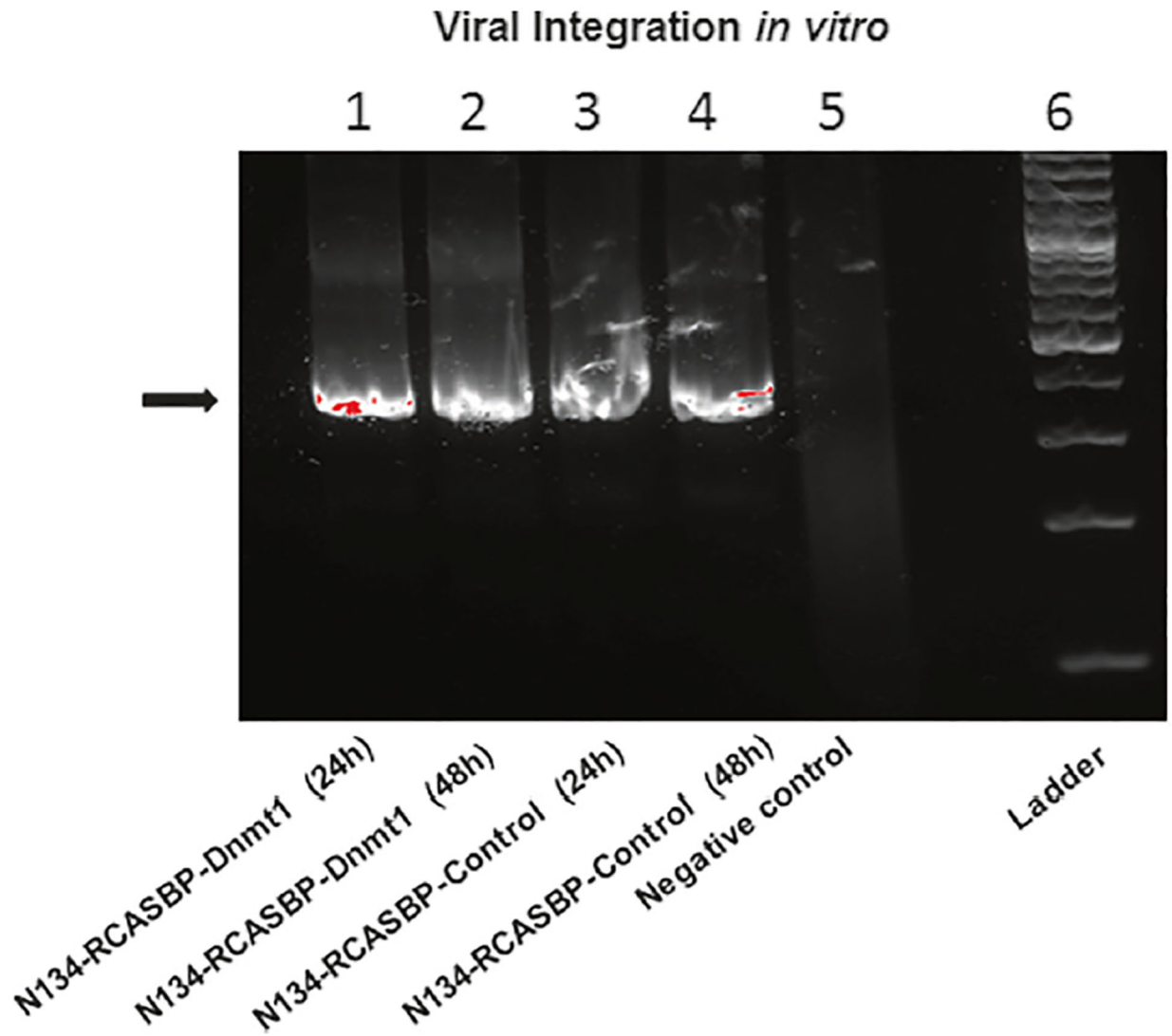
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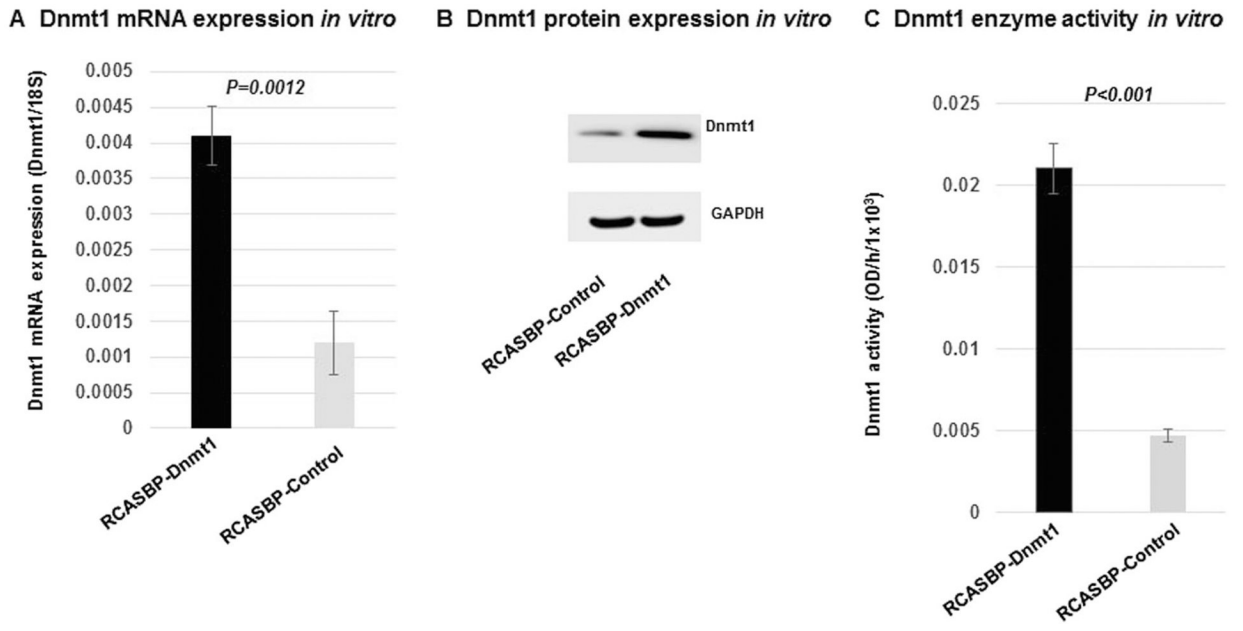
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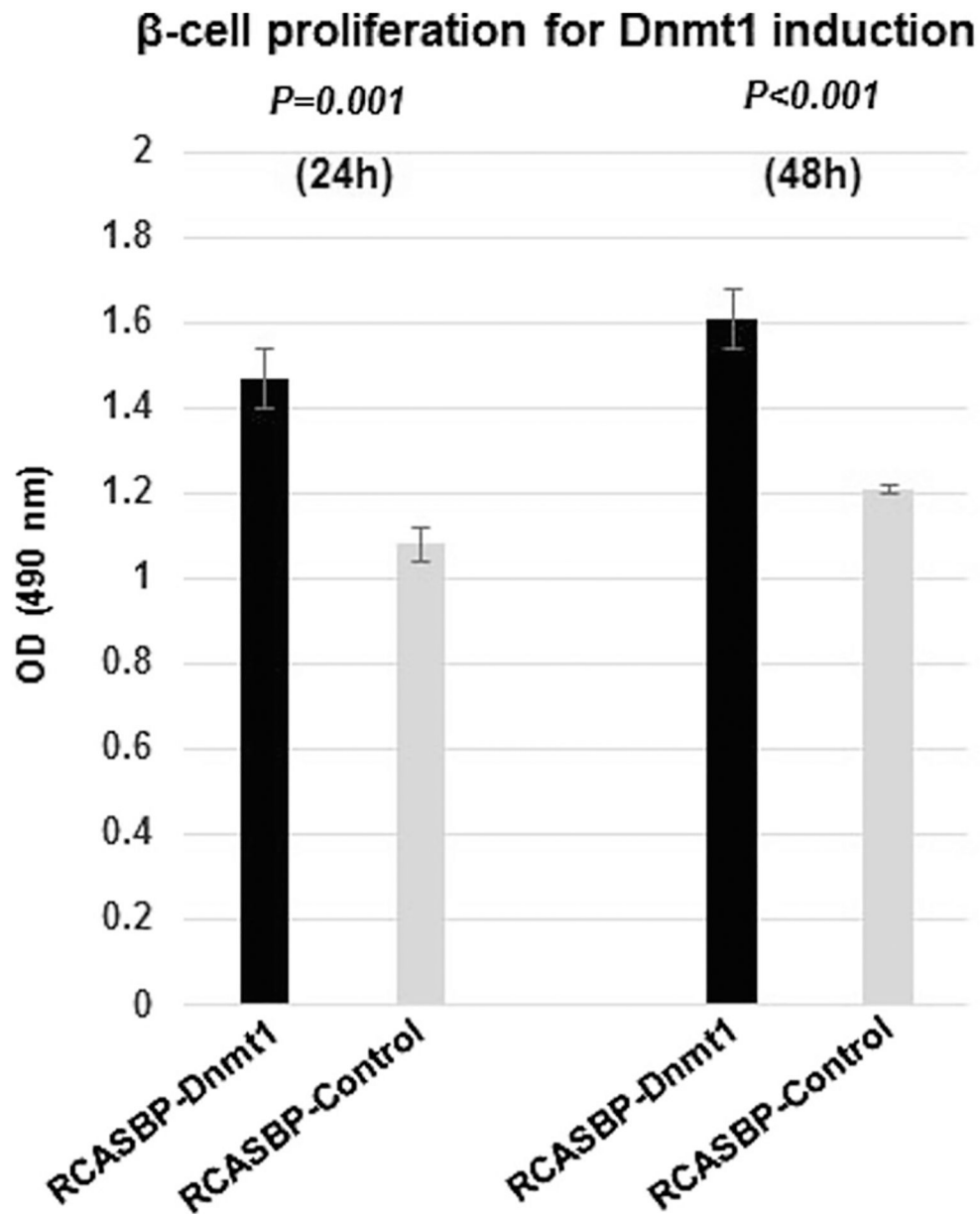
**Fig. 1.**

Detection of viral integration in RCASBP infected cells. Virus production was measured by PCR and the PCR products were run in 1% agarose. Viral integration was present in the infected N134 cells with both RCASBP-*Dnmt1* (lane 1 and 2) and RCASBP-control (lane 3 and 4) at 24 h and 48 h, respectively

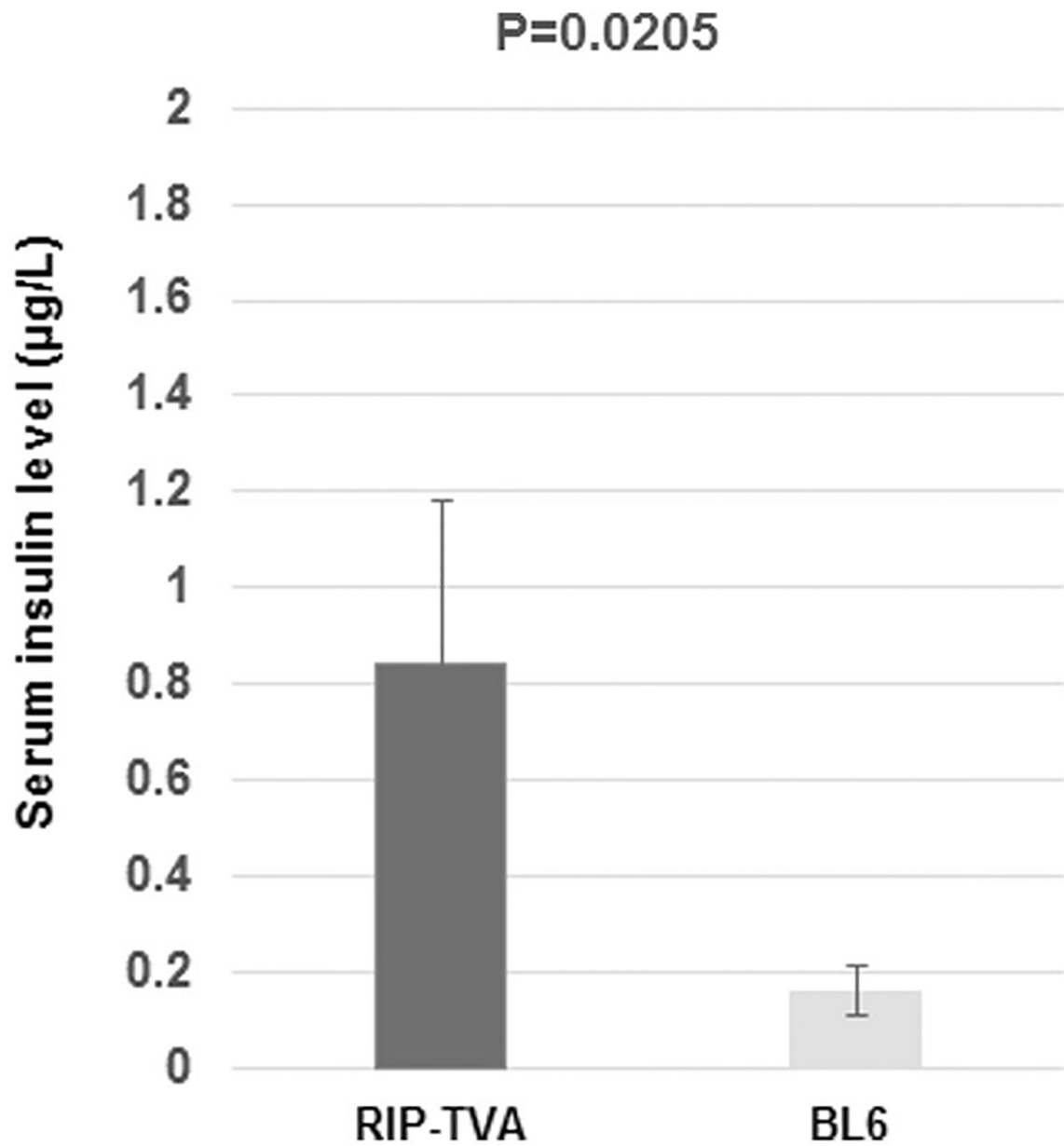


**Fig. 2.**

Upregulation of the expression and enzyme activity of Dnmt1 in the N134  $\beta$ -cell tumor cell line with RCASBP-*Dnmt1* infection. There was a significant increase in the expression levels of Dnmt1 RNA (a) ( $P=0.0012$ ) and protein (b) as well as Dnmt1 enzyme activity (c) ( $P<0.001$ ) in the RCASBP-*Dnmt1* infected N134 cells when compared with those infected with RCASBP-control

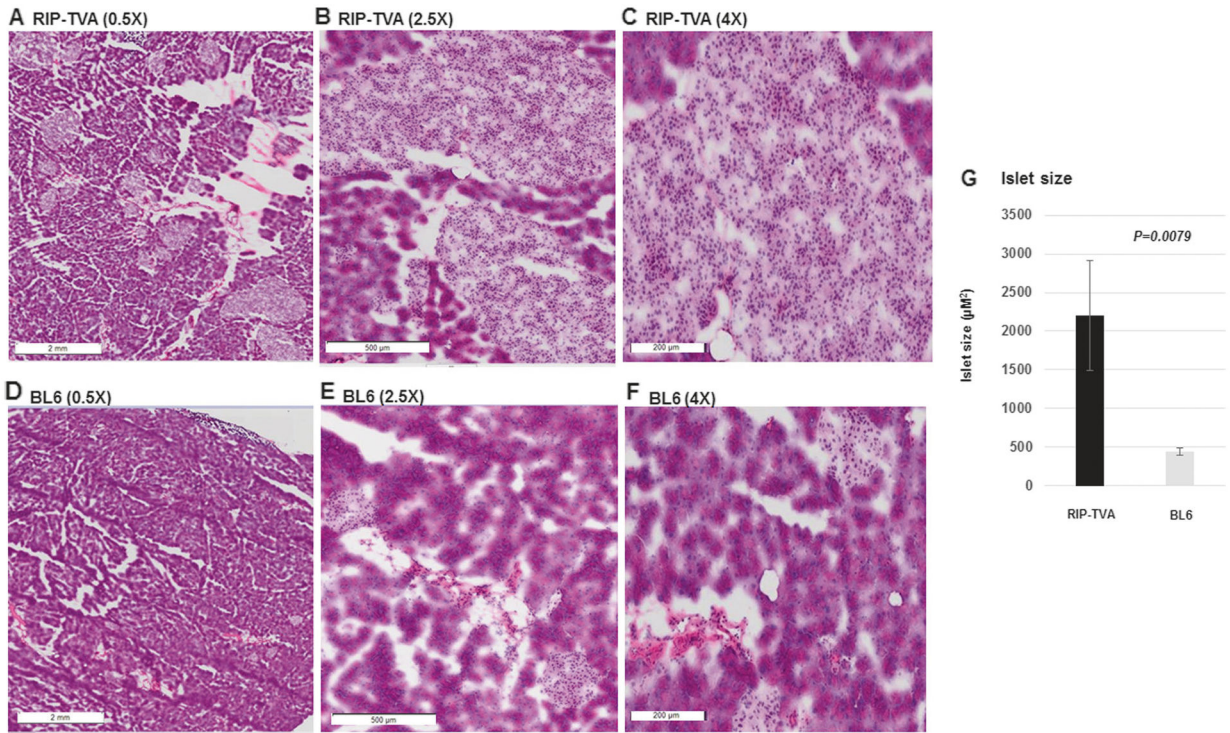


**Fig. 3.** Increased β-cell proliferation after infection with RCASBP-*Dnmt1* by a MTS assay. In the N134 β-cell tumor cell line, cell proliferation was measured after infection with RCASBP-*Dnmt1* and RCASBP-control for 24 h and 48 h. The MTS assays showed that the cell proliferation was significantly increased in the N134 cells with RCASBP-*Dnmt1* infection at 24 h ( $P=0.001$ ) and 48 h ( $P<0.001$ ) as compared with the N134 cells with RCASBP-control infection



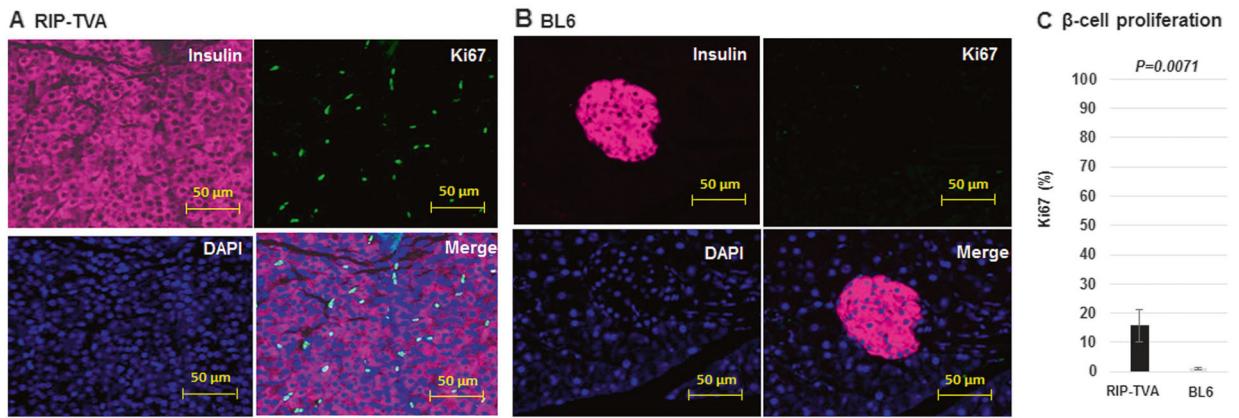
**Fig. 4.** Serum insulin levels were increased in *RIP-TVA* mice with *RCASBP-Dnmt1* infection. Mouse blood was collected following an 18 h fast and serum levels of insulin were measured by ELISA. There was a significant increase in serum insulin in the *RIP-TVA* mice ( $N=4$ ) compared to WT mice ( $N=3$ ) infected with *RCASBP-Dnmt1* ( $P=0.0205$ )



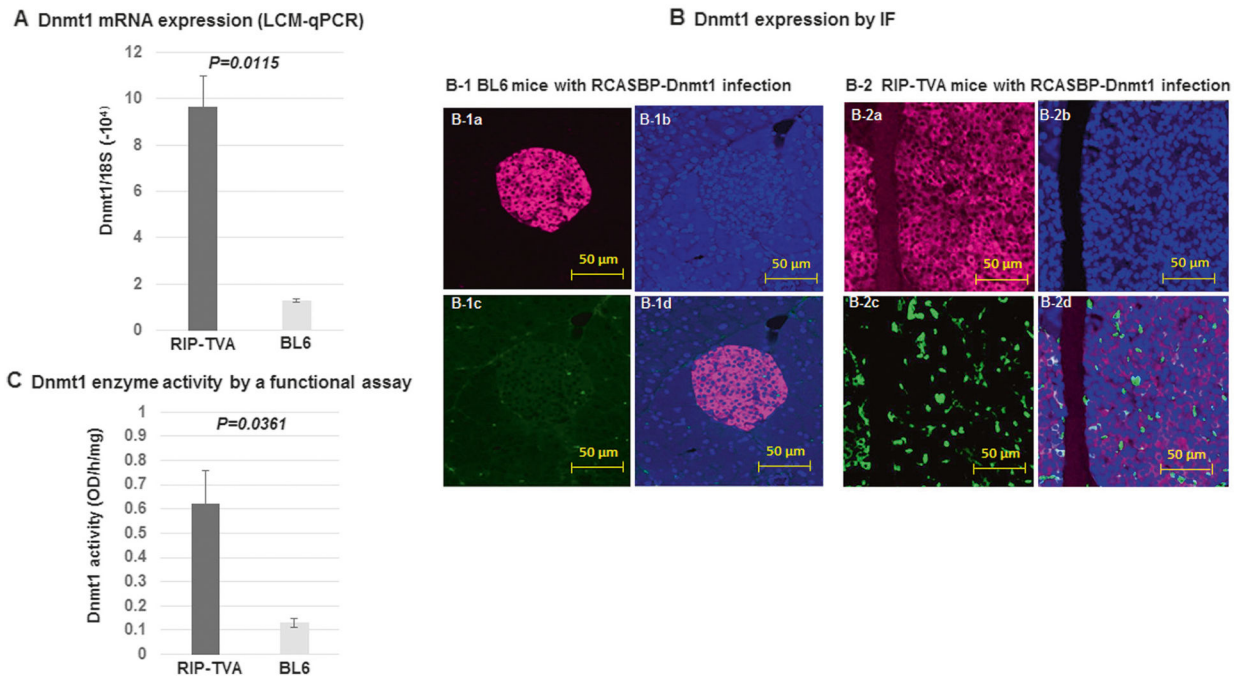


**Fig. 5.**

The islet size was significantly increased in the *RIP-TVA* mice with *RCASBP-Dnmt1* injection. H&E staining showed that the *RIP-TVA* mice infected with *RCASBP-Dnmt1* had a significant increase in islet size at magnification  $0.5 \times$  (**a**), magnification  $2.5 \times$  (**b**), and magnification  $4.0 \times$  (**c**) compared with the WT mice infected with *RCASBP-Dnmt1* (**d**, **e**, and **f**). Quantification was achieved by measuring islet size (**g**) ( $P = 0.0079$ )



**Fig. 6.** The islet  $\beta$ -cell proliferation is significantly increased in the *RIP-TVA* mice with RCASBP-*Dnmt1*. The islet  $\beta$ -cell proliferation was significantly increased by Ki67 analysis in the *RIP-TVA* mice with RCASBP-*Dnmt1* injection (a) compared with the WT mice with RCASBP-*Dnmt1* injection (b). Immunostaining of insulin (pink), Ki67 (green), and nuclei counterstained with DAPI (blue). Quantification was achieved by counting the fluorescent proliferating cells (c) ( $P = 0.0071$ ) (magnification: 20 $\times$ )



**Fig. 7.** Increased expression and enzyme activity of Dnmt1 in the endocrine tissue of the pancreas from *RIP-TVA* mice with *RCASBP-Dnmt1* infection. There was a significant increase of Dnmt1 mRNA ( $P=0.0115$ ) (a), Dnmt1 protein (b), and Dnmt1 enzyme activity ( $P=0.0361$ ) (c) in the endocrine tissue of the pancreas from *RIP-TVA* mice infected with *RCASBP-Dnmt1* compared with the WT control mice infected with *RCASBP-Dnmt1*, as measured by LCM-PCR, IF staining, and Dnmt1 enzyme activity functional assay. Immunostaining of insulin (pink), Dnmt1 (green), and nuclei counterstained with DAPI (blue) (magnification: 20 $\times$ )