Brief Communication

MOLECULAR METABOLISM

Overexpression of ST5, an activator of Ras, has no effect on β-cell proliferation in adult mice

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ABSTRACT

Objective: Both Type I and Type II diabetes mellitus result from insufficient functional β-cell mass. Efforts to increase β-cell proliferation as a means to restore β-cell mass have been met with limited success. Suppression of Tumorigenicity 5 (ST5) activates Ras/Erk signaling in the presence of Epidermal Growth Factor (EGF). In the pancreatic islet, Ras/Erk signaling is required for augmented β-cell proliferation during pregnancy, suggesting that ST5 is an appealing candidate to enhance adult β-cell proliferation. We aimed to test the hypothesis that overexpression of ST5 drives adult β-cell proliferation.

Methods: We utilized a doxycycline-inducible bitransgenic mouse model to activate β-cell-specific expression of human ST5 in adult mice at will. Islet morphology, β-cell proliferation, and β-cell mass in control and ST5-overexpressing (ST5 OE) animals were analyzed by immunofluorescent staining, under basal and two stimulated metabolic states: pregnancy and streptozotocin (STZ)-induced β-cell loss.

Results: Doxycycline treatment resulted in robust ST5 overexpression in islets from 12-16 week-old ST5 OE animals compared to controls, without affecting the islet morphology and identity of the β-cells. Under both basal and metabolically stimulated pregnancy states, β-cell proliferation and mass were comparable in ST5 OE and control animals. Furthermore, there was no detectable difference in β-cell proliferation between ST5 OE and control animals in response to STZ-induced β-cell loss.

Conclusions: We successfully derived an inducible bitransgenic mouse model to overexpress ST5 specifically in β-cells. However, our findings demonstrate that ST5 overexpression by itself has no mitogenic effect on the adult β-cell under basal and metabolically challenged states.

Keywords Diabetes; β-cell proliferation; ST5 (Suppression Of Tumorigenicity 5); Ras/ERK signaling

1. INTRODUCTION

The two main forms of diabetes, Type I and Type II diabetes, can both be attributed to insufficient functional β-cell mass. Identifying methods to promote β-cell proliferation, especially in adults, has been an attractive therapeutic strategy for the treatment of diabetes. β-cells, notoriously reluctant to proliferate in adulthood, are able to re-enter the cell cycle in response to metabolic challenges such as pregnancy [1,2], β-cell damage [3,4], and obesity [5,6], at least in rodent models. Numerous elegant studies have detailed complex pathways governing adaptive β-cell expansion [7–9], providing a basis for further investigations for candidate accelerators of adult β-cell cell cycle.

HNF4α, a gene linked to Maturity Onset Diabetes of the Young (MODY), is one the transcription factors required for adaptive β-cell proliferation during pregnancy in mice [10]. Suppression of Tumorigenicity 5 (ST5), a direct transcriptional target of Hnf4α, plays a role in one of the pathways thought to promote cell proliferation in response to the presence of mitogenic stimuli by mediating the activation of the Ras/ERK signaling cascade [10]. In humans, *ST5* encodes three protein isoforms: p70, p82, and p126 [11–13]. While the short form of human ST5, p70, is associated with reduced tumorigenic phenotype in mammalian cell lines and was the reason for the naming of the gene [12]; the longest form, p126, activates MAPK/ERK in response to Epidermal Growth Factor (EGF) in COS-7 cells [14]. Mechanistically, the C-terminal GEF homology domain of p126 catalyzes the exchange of GDP to GTP in Ras, a small GTPase molecular switch, ultimately triggering the activation of the MAPK/ERK signaling cascade and thus inducing cell cycle entry [14]. Additionally, we have previously demonstrated that attenuated ST5 expression in islets devoid of *Hnf4α* decreases Ras-GTP and phosphorylated ERK (pERK) levels [10].

The EGF/Ras/ERK axis has long been proposed as a mitogenic pathway in the β-cell. However, transgenic overexpression of EGF or its close family member HB-EGF induces drastic transformation and disorganization of islets rather than substantial β-cell proliferation [15,16]. It remains

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Abbreviations: ST5, Suppression of Tumorigenicity 5; GEF, Guanine Nucleotide Exchange Factor; ST5 OE, ST5-overexpressing; STZ, streptozotocin; MODY, Maturity Onset Diabetes of the Young; EGF, Epidermal Growth Factor; pERK, phosphorylated ERK; PDGF, Platelet-Derived Growth Factor; TRE, Tetracycline Response Element; RIP, Rat Insulin Promoter; rtTA, reverse tetracycline-controlled transactivator

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unclear how increased Ras/ERK activity *in vivo* affects β-cell proliferation in adulthood, in response to normal or high metabolic demand.

In this study, we aimed to test the hypothesis that overexpression of the long isoform of ST5, an activator of the Ras/ERK pathway, is able to promote adult β-cell proliferation. We employed a doxycycline-inducible system to overexpress ST5 in β-cells of adult mice and challenged the animals using two experimental paradigms of high metabolic demand. Our results demonstrate that overexpressing ST5 under both basal metabolic or challenged states is not sufficient to enhance β-cell proliferation.

2. MATERIALS AND METHODS

2.1. Animals

Tre-ST5 transgenic mice were derived by engineering a TRE (Tetracycline Response Element) cassette upstream of the cDNA encoding the longest isoform of human *ST5*, p126. *RIP (Rat Insulin Promoter)-rtTA* mice were purchased from the Jackson laboratory. All mice were maintained on a mixed 129SvEv/C57BL/6 background. Genotyping was performed by PCR analysis using genomic DNA isolated from the tail tips of newborn mice using the following primers:

Gene	Forward Primer	Reverse Primer
RIP-rtTA	5′- GTGAAGTGGGTCCGCGTACAG -3′	5′- GTACTCGTCAATTCCAAGGGCATCG -3′
TRE-hST5	5′- CATCCACGCTGTTTTGACCTC -3′	5′- CTCCAAAGGGTGTGAAATCCA -3′

Adult mice (12–16 wk of age) were used in all experiments unless otherwise noted. To induce ST5 expression, doxycycline (Dox, Sigma #D9891) was administered via the drinking water at 1 g/L and replenished twice a week. For both pregnancy and streptozotocin (STZ) studies, mice were kept on Dox^+ water for two weeks, starting from gestational day 0.5 in females and the first injection day of STZ in males, respectively. For the STZ study, both ST5 OE and control mice were intraperitoneally injected daily for four consecutive days with 85 μg/g body weight of freshly dissolved STZ (Sigma #S0130) in 0.1 M sodium citrate (pH 4.5).

To label proliferating β cells in STZ-treated animals, 1 g/L of BrdU (Sigma #B5002) was administrated via the drinking water for one week prior to sacrifice of the mice. For studies on basal metabolic state and during pregnancy, a shorter BrdU labeling was performed as the follows: animals were treated by intraperitoneal injection of BrdU (30 μg per gram body weight) 16 h prior to sacrifice. All procedures involving mice were conducted in accordance with approved Institutional Animal Care and Use Committee protocols.

2.2. Physiological studies

Blood glucose levels during ad libitum feeding and glucose tolerance tests were measured as previously described [17,18].

2.3. Histological analysis

For all histological studies, pancreata were dissected and fixed in 4% paraformaldehyde overnight at 4 °C before paraffin embedding and sectioning with the maximum pancreatic footprint. Tissues were sectioned to 5 μm thickness. Antigen retrieval was performed in citric acid buffer pH 6.0 in the 2100 Classic Clinical Autoclave (Prestige Medical) for 1 h, followed by 15 min cooling in running water. All slides were blocked with Casblock (Thermo Fisher Scientific #00-8120) for 10 min at room temperature. To quantify β-cell proliferation, dual immunofluorescent staining was performed for insulin and BrdU or Ki67. The primary antibodies [guinea pig anti-insulin (1:1,300, Invitrogen #180067) and rat anti-BrdU (1:500, AbD Serotec #OBT0030G)] or mouse anti-Ki67 (1:500, BD pharmacology #550609) were diluted in Casblock and incubated with the tissue sections overnight at 4 °C. Secondary antibody incubation was performed with species-specific fluorophore-labeled secondary antibodies (1:500, Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (Thermo Fisher Scientific #D1306). β-cell proliferation was calculated by dividing BrdU/insulin or Ki67/insulin double-positive cell number by insulin-positive cell number within islets. At least two sections from each animal were manually counted, and 3–4 animals from each genotype were analyzed. Pdx1 and ST5 antibody staining was performed as described above using the following primary antibodies: goat anti-Pdx1 (1:1,000, Santa Cruz #sc-14664) and rabbit anti-ST5 (1:200, Abcam # ab111556).

To quantify the ratio of $insulin^+$ area to islet area, whole pancreata were removed, laid out flat, and fixed as above. Five-micron longitudinal sections were prepared, with 50-micron intervals between sections. Every sixth section (a total of 3–4 sections per pancreas) was used for hormone immunofluorescent labeling. This sampling method spans over 50% of the pancreatic volume. In addition, care was taken to score equivalent regions of control and mutant specimens. After immunofluorescent staining, 100% of each hormone-labeled section was scanned at 4× magnification. The areas of positive signal and the total pancreatic areas were measured by Image J [19]. β-cell mass was derived from the total pancreas weight multiplied by the percentage of β-cell area relative to the whole pancreatic area.

2.4. RNA extraction and qRT-PCR

We isolated mouse pancreatic islets by intra-ductal collagenase perfusion using standard methods [18]. Total RNA from mouse islets was extracted using RNeasy Mini Kit (Qiagen #74104) and analyzed by qRT-PCR as described previously [20]. qRT-PCR primer sequences are the following:

Species	Gene	Forward Primer	Reverse Primer
Human	ST5	5′- ttaaagccatcgccacaac -3′	5′- catggtcatttcggctctct -3′
Mouse	ST5	5′- cccagactccgtcagtttct -3′	5′- gggcatcattctctgtctgg -3′

2.5. Statistical methods

Statistical analysis between two groups was performed using a two-tailed Student's *t*-test unless noted otherwise. Values were considered significant when $P < 0.05$. Data are shown as mean plus standard error of the mean.

3. RESULTS

3.1. Inducible overexpression of ST5 in the adult β-cell is not sufficient to enhance cell proliferation under basal condition

Because ST5 is a direct transcriptional target of Hnf4α during pregnancy-induced β-cell replication and because ST5 deficiency leads to decreased ERK phosphorylation [10], we hypothesized that overexpression of ST5 could induce cell cycle entry in adult β-cells. To test this notion, we developed a doxycycline-inducible mouse model to

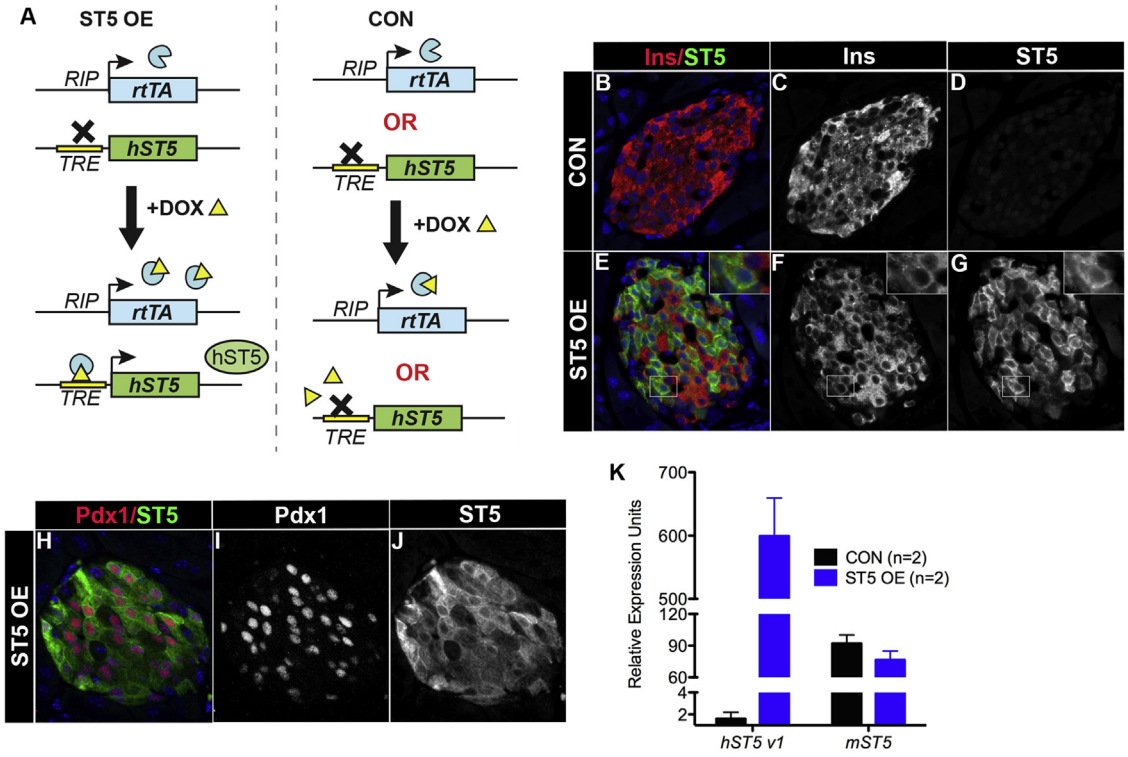

Figure 1: Human ST5 expression in *RIP-rtTA*;*TRE-hST5* double transgenic mice. A. Schematic of the doxycycline-inducible ST5 overexpression (ST5 OE) mouse line. Both ST5 OE and single transgenic control (CON) animals were kept on doxycycline for 2 weeks prior to analyses. **B-G.** Immunofluorescence staining for insulin (red) and ST5 (green) in islets of 12–16 week-old control (B–D) and ST5 OE (E–G) animals. Expression of ST5 was limited to insulin$^+$ cells. ST5 overexpressing and ST5 negative cells have comparable insulin expression levels (insets in E–G). **H–J.** Pdx1 expression (red) in cells overexpressing ST5 (green). **K.** Quantitative real-time PCR analysis of human (hST5v1) and endogenous mouse *ST5* (mST5) mRNA levels in control and ST5-overexpressing islets at 12-weeks of age.

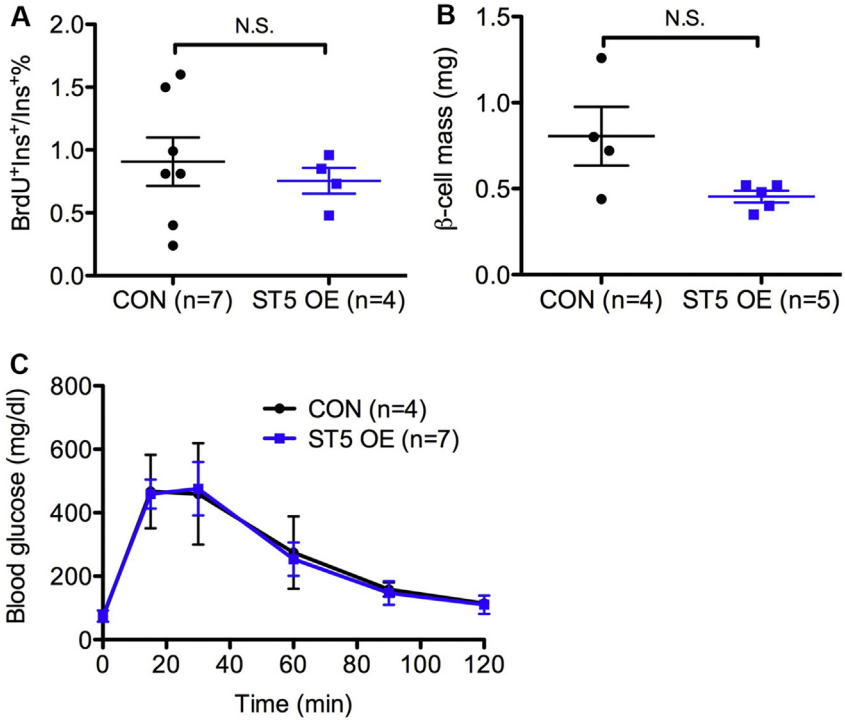

Figure 2: ST5 overexpression does not promote β-cell proliferation under basal metabolic conditions. A. Percentage of proliferating β-cells in 12–16 week-old control and ST5 OE mice. Proliferating β-cells were calculated by quantifying the number of BrdU$^+$/insulin$^+$ double positive cells over the total number of insulin$^+$ cells. **B.** β-cell mass in control and ST5 OE mice. β-cell mass was derived from the total pancreas weight multiplied by the percentage of β-cell area (as detected by insulin positive staining) relative to the whole pancreatic area. **C.** Glucose clearance traces of a glucose tolerance test performed on 12–16 week-old control and ST5 OE males.

overexpress the longest isoform of human ST5, which is known to activate Ras/ERK signaling in the presence of EGF [14], in β-cells. This model is comprised of two transgenic lines: TRE-ST5 and RIP-rtTA. Doxycycline induces ST5 expression in double transgenic animals specifically in the β-cells. We kept 12–16 week-old double (ST5 OE) and single transgenic animals (control) on doxycycline (1 g/L) in drinking water for two weeks (Figure 1A). The majority of β-cells in islets from ST5 OE mice displayed robust ST5 expression, whereas β-cells from control animals had non-detectable ST5 immunoreactivity, as revealed by dual immuno-labeling of insulin and ST5 (Figure 1B–G). In ST5^{+} β-cells, we observed strong membrane and cytoplasmic signals for ST5, consistent with its previously reported role in Ras activation [14] and the notion that activated Ras is anchored to the cytoplasmic face of the plasma membrane [21]. Gene expression analysis of RNA from isolated mouse islets further confirmed the induction of human version of *ST5*, without affecting the endogenous murine *ST5* levels (Figure 1K).

Upon successful induction of ST5 expression, we sought to determine whether the β-cell is able to tolerate high levels of ectopic protein expression. Immuno-fluorescent staining showed strong insulin (Figure 1E-G insets) and Pdx1 protein expression in both ST5^{+} and ST5^{-} β-cells (Figure 1H-J). Therefore, ST5 overexpression does not appear to adversely affect β-cell identity.

Having established a reliable model for inducible ST5 expression in adult β-cells, we next sought to test the sufficiency of ST5 for driving adult β-cell proliferation. We labeled β-cells actively synthesizing DNA during S-phase by injection of BrdU 48 h prior to sacrifice of animals. ST5 OE mice showed a comparable percentage of BrdU^{+}Insulin^{+}/Insulin^{+} cells (Figure 2A) and β-cell mass (Figure 2B) as control mice. Furthermore, ST5 OE and control mice behaved similarly in glucose tolerance tests (Figure 2C). These results demonstrate that ST5 overexpression alone does not provide increased β-cell proliferation under basal metabolic states.

3.2. ST5 overexpression does not augment β-cell proliferation during pregnancy

It is well documented that both intrinsic and extrinsic signals are required to initiate and complete the cell cycle in β-cells. Given that EGF is required for ST5 induced activation of Ras, we speculated that a metabolically demanding state might provide the necessary extracellular stimuli to unlock the mitogenic potential of ST5. EGF signaling is necessary for β-cell mass expansion during pregnancy by activating the Ras/ERK pathway and others in response to nutrient excess [22]. To determine the mitogenic potential of ST5, we employed the pregnancy model during which β-cell proliferation increases four-fold to meet the augmented metabolic load. Quantification of β-cell proliferation at gestational day 14.5 (GD14.5), the peak of β-cell proliferation in pregnant mice [1], showed a similar ratio of Ki67^{+}Ins^{+}/Ins^{+} cell numbers between control and ST5 OE animals (Figure 3A–C). In addition, β-cell mass was indistinguishable between the two groups despite robust ST5 overexpression (Figure 3D).

The tumor suppressor *Rassf1a* encodes for a negative effector of Ras, disrupting pro-proliferative Ras/ERK signaling and preventing G1/S phase transition in normal epithelial tissues, leading to cell cycle arrest [23,24]. Because Rassf1a has been shown to prevent endocrine cell expansion in knock-in mice overexpressing an activating mutation of Kras [25], we next examined whether Rassf1a is induced in our pregnancy model, which may partially explain why ST5 OE does not increase β-cell proliferation. Indeed, we found nuclear Rassf1a in a population of β-cells in pregnant GD14.5 females (Figure 3G-H)that is absent in non-pregnant females (Figure 3E-F), suggesting that Rassf1a induction

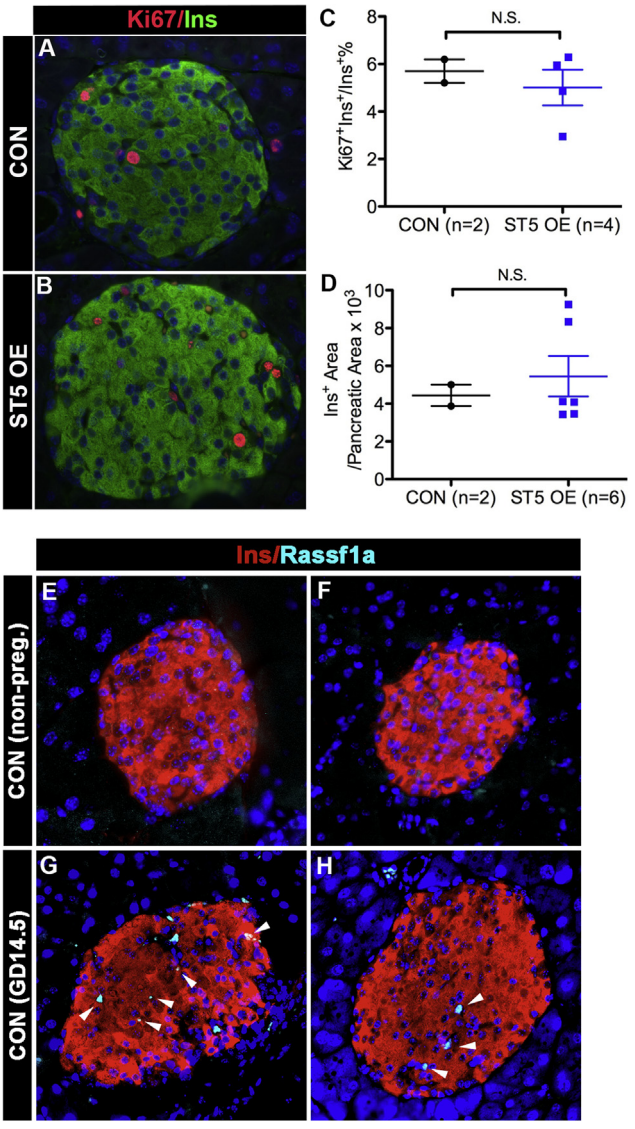

Figure 3: Increased mitogenic stimuli during pregnancy do not induce increased β-cell proliferation in ST5 OE mice. A-B. Immunofluorescence staining for Ki67 (red) and insulin (green) from gestational day (GD) 14.5 control and ST5 OE females. **C.** β-cell proliferation in GD 14.5 control and ST5 OE females. **D.** β-cell mass in GD 14.5 control and ST5 OE females. **E–H.** Immunofluorescence staining for Rassf1a (cyan) and insulin (red) from non-pregnant (E-F) and GD14.5 (G-H) control females. β-cells with nuclear Rassf1a are indicated with white arrows.

during pregnancy may have prevented the activation of the Ras/ERK pathway by ST5 despite the presence of EGF. We thus conclude that ST5 is insufficient to enhance β-cell proliferation during pregnancy.

3.3. ST5 overexpression does not affect β-cell proliferation in response to STZ-induced β-cell damage

Next, we tested whether ST5 mediates β-cell proliferation during regeneration following STZ-induced β-cell damage. We adopted a low-dose STZ injection protocol [3], which triggers overt diabetes (Ad lib glucose $>$ 200 mg/dl) one week after the first STZ injection (Figure 4A-B), indicative of severe β-cell damage. ST5 OE and control mice were given BrdU in the drinking water for a full week following STZ injection in order to label all cell cycle entry events during the recovery phase under prevailing hyperglycemia. While increased β-cell workload

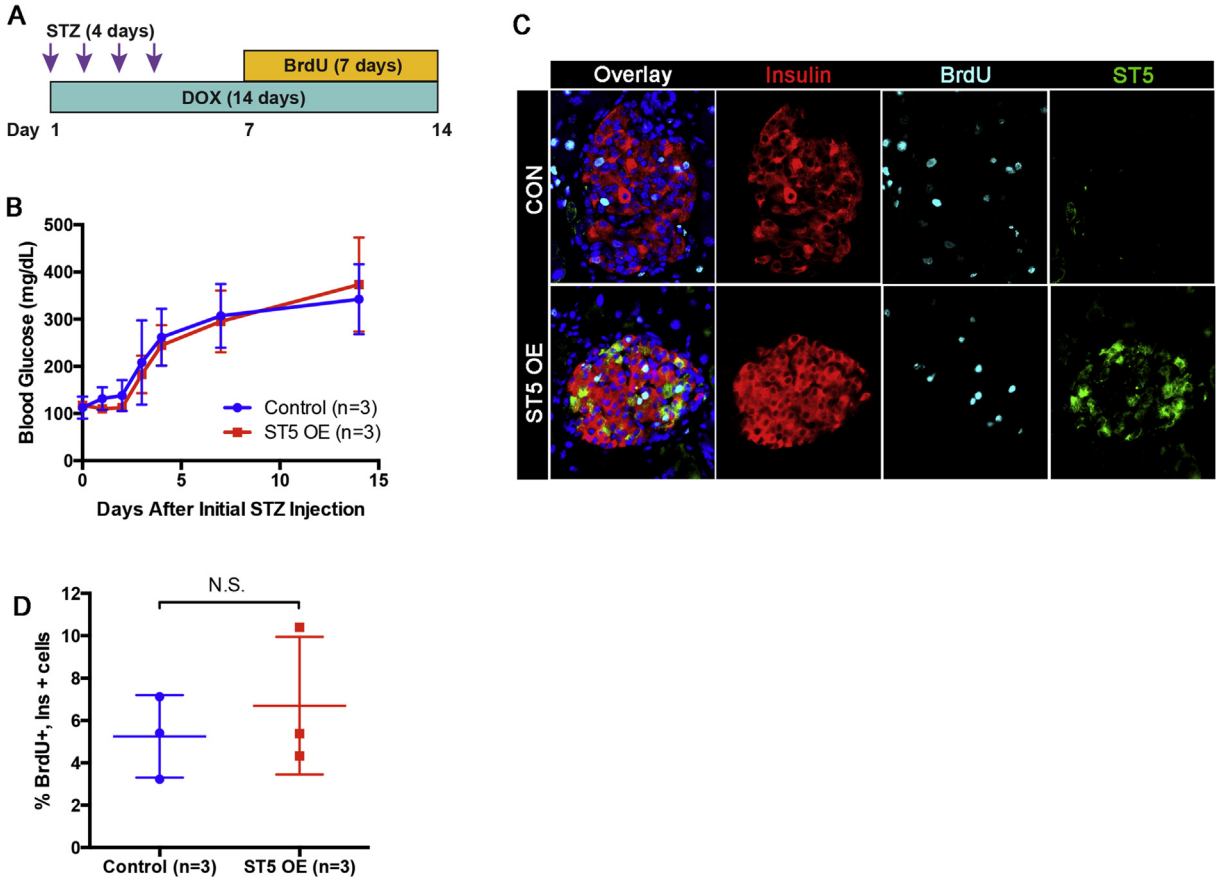

Figure 4: ST5 overexpression does not induce β-cell proliferation in response to STZ-induced β-cell damage. A. Schematic of experimental timeline. 12–16 week-old control and ST5 OE mice were maintained on doxycycline throughout the course of the experiment. The mice were injected with STZ the first 4 days of the study. During the second week, BrdU was added to the Dox^{+} drinking water. **B.** Glucose levels of control and ST5 OE mice during and after STZ induction. By the fifth day, the mice were hyperglycemic (Ad lib glucose > 200 mg/dl), indicating significant β-cell loss. **C.** Representative insulin (red), BrdU (cyan), and ST5 (green) immunofluorescence staining in a control and ST5 OE islet. **D.** β-cell proliferation in control and ST5 OE males.

stimulated β-cell replication as expected, quantification of the percentage of BrdU^{+}Insulin^{+}/total Insulin^{+} cells revealed that there was no difference in β-cell proliferation between ST5 OE and control mice (Figure 4C–D), suggesting that ST5 may not play an important role in β-cell regeneration under severe injury.

4. DISCUSSION

In this study, we examined whether the long isoform of ST5, a Ras activator, is sufficient to drive β-cell proliferation in adult mice during both quiescent and metabolically stressed states. Taking advantage of a doxycycline-inducible mouse model, we were able to temporally overexpress the mitogenic isoform of human ST5 in adult β-cells.

Under basal conditions, ST5 overexpression does not promote proliferation of adult β-cells. Despite a robust upregulation of *ST5* mRNA and protein levels, ST5 overexpression failed to stimulate the Ras/ERK pathway in the islet (data not shown). This is likely due to the lack of extracellular stimuli such as EGF, which are required for Ras activation by ST5. Therefore, it is not surprising that ST5 did not lead to increased β-cell proliferation under basal metabolic conditions.

In both the pregnancy and STZ-induced diabetes models, during which the metabolic demand for insulin and β-cell function is heightened, ST5 overexpression does not increase the rate of proliferation in β-cells. We were able to detect substantially stronger ERK activity in islets from pregnant ST5 OE mice compared to those from pregnant controls (data not shown), indicating that ST5 is sufficient to stimulate Ras/ERK pathway under metabolic stressful conditions. However, enhanced Ras/ERK signaling does not translate into an increased β-cell proliferation rate.

Several recent studies have demonstrated that negative regulators of the Ras/ERK signaling cascade are simultaneously activated with activation of Ras and are capable of overriding the pro-proliferative effect of Ras/ERK signaling. Menin, a tumor suppressor in endocrine organs and inhibitor of Ras and ERK activation, has been shown to inhibit β-cell proliferation and expansion in *db/db*, STZ-induced, and gestational diabetes mouse models [26–28]. It is possible that the augmented activation of Ras by ST5 overexpression could not counteract Menin-mediated suppression of the Ras/ERK pathway in the context of STZ-induced diabetes.

Rassf1a also inhibits Ras/ERK signaling and cell cycle progression and is silenced in many cancers [24,29,30]. A recent study demonstrated that the constitutively active form of Kras paradoxically reduced β-cell proliferation in embryonic and neonatal mice despite the activation of Ras/ERK signaling [25]. This is explained by the significant increase in Rassf1a activation, which counteracted the pro-proliferative downstream output of pERK. The same study also showed that the addition of Exendin-4, a potent β-cell mitogen that acts on the GLP-1 incretin pathway, did not induce β-cell proliferation unless Rassf1a activity was

disrupted. This study is consistent with our finding that Rassf1a was activated in the pregnancy model, which may explain why overexpression of ST5 and increased EGF/Ras/ERK signaling did not increase β-cell proliferation beyond its normal increase in GD14.5 females.
Our study illustrates the challenges of identifying activators of β-cell replication under quiescent and stressed *in vivo* conditions. Further investigation is needed to dissect how β-cells integrate pro-proliferative and anti-proliferative signals in response to heightened metabolic demand and cell loss in order to identify effective strategies to increase β-cell mass for the treatment of diabetes.

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CONFLICT OF INTEREST

None declared.

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