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DNA damage-induced replication stress mediates cell cycle arrest in postnatal
pancreatic beta cells

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

Tiffany Guan

Committee in Charge:

Professor Maïke Sander, Chair
Professor David Traver, Co-Chair
Professor Kimberly Cooper

2016

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Co-Chair

Chair

University of California, San Diego

2016

DEDICATION

I dedicate this thesis to my family, for their unconditional love, support, and encouragement.

EPIGRAPH

“It’s better to know how to learn than to know.”

Dr. Seuss

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Material included in this master's thesis contains information being prepared for publication in Zeng, Chun; Guan, Tiffany; Sander, Maike. "Replication stress drives proliferative decline in postnatal beta cells".

ABSTRACT OF THE THESIS

DNA damage-induced replication stress mediates cell cycle arrest in postnatal
pancreatic beta cells

by

Tiffany Guan

Master of Science in Biology

University of California, San Diego, 2016

Professor Maïke Sander, Chair

It is well recognized that the regenerative capacity of pancreatic insulin-producing beta cells declines with age, with the most dramatic reduction occurring shortly after birth. However, the mechanism that accounts for this decline remains

poorly understood. We sought to determine the molecular changes that attribute to this decrease in beta cell proliferation. Here, we show that activation of the DNA damage response (DDR) coincides with the decline in beta cell proliferation occurring during early postnatal life. In addition, we discovered a postnatal increase in the expression levels of replication stress-related DNA damage markers such as replication protein A (RPA) and phosphorylated Chk1 (pChk1). Furthermore, inhibition of the downstream effector Wee1 increases beta cell proliferation *in vitro* and *in vivo*. The results of this study provide insight into age-dependent changes in beta cells that lead to the increased incidence of cell cycle arrest. We hope that these findings will not only contribute to uncovering mechanisms underlying the age-dependent decline in beta cell proliferation, but also assist in the identification of factors that could potentially be pharmacologically targeted to improve beta cell function and stimulate their expansion for the management and treatment of diabetes mellitus.

INTRODUCTION

Diabetes mellitus

Diabetes mellitus is an endocrine metabolic disease where the body fails to regulate high blood glucose levels over an extended period of time [1]. There are two variants of diabetes—type I and type II diabetes. Type I diabetes is caused by the autoimmune loss of pancreatic insulin-producing islet cells, known as beta cells [2]. The loss of beta cell mass decreases the ability to maintain blood glucose homeostasis. Although genetics plays a role in the development of type I diabetes, the exact cause remains unknown. Type II diabetes, which is often associated with obesity, sedentary lifestyles, and poor dietary habits, is initiated by the development of insulin resistance [3, 4]. The consistently high blood glucose levels overwhelm the beta cells, which are unable to produce sufficient amounts of the peptide hormone insulin, ultimately leading to persistently high levels of glucose in the bloodstream, a condition known as hyperglycemia. In type II diabetes, peripheral tissues such as fat and muscle also become less responsive to the effects of insulin, a condition known as insulin resistance. This increased demand for insulin production often leads to beta cell malfunction, de-differentiation, and apoptosis [5, 6]. Postmortem studies of elderly type II diabetics have shown these patients to have decreased beta cell mass compared to age-and weight-matched healthy individuals due to increased beta cell apoptosis [7].

Diabetes ultimately results in an increased risk of numerous health issues including heart disease, blindness, stroke, and peripheral neuropathy leading to amputations [6]. The Center for Disease Control (CDC) and the International

Diabetes Foundation estimate that diabetes affects approximately 26 million people in the United States, and over 300 million people worldwide. This increase in morbidity and mortality in such a large population makes the disease an immense public health concern. As a result, there is much motivation to further study diabetes mellitus in order to develop improved management and treatment options.

Diabetes Management Options

There are currently no known cures for diabetes, but there are several management options. Patients with type II diabetes are recommended to adopt healthier diets, exercise regularly, and consume a variety of medications that will either increase insulin sensitivity or decrease blood glucose levels. Type I diabetes patients typically require lifelong glucose monitoring and insulin therapy. An additional management option for type I diabetes patients is transplantation of pancreatic islets of Langerhans into the hepatic portal vein. This method has been shown to quickly increase beta cell mass; transplanted islets begin to produce and secrete insulin, thus establishing normoglycemia and improving glucose level management for several years [8]. However, islet transplantation has many caveats: the supply of human islets from human cadaveric pancreata is severely limited, the procedure is costly, and recipients are to receive lifelong immunosuppression, especially to decrease the autoimmune destruction response in type I diabetic patients [9].

Regeneration of Beta Cells as a Therapy for Diabetes Mellitus

Current therapies for type II diabetes consist of combination of lifestyle changes and oral medications [10]. Although improved diet and exercise regimens along with medications such as metformin and sulfonylureas can improve glycemic control in type II diabetic patients, none of these methods compare to the precision of endogenous beta cells [11]. Given that beta cell mass has been shown to decrease significantly in diabetic patients, with a reduction of up to 60%, an alternative method of approach to improve insulin production is to develop strategies for the regeneration and replication of existing beta cells [7]. New pancreatic beta cells typically develop via the replication of endogenous beta cells rather than stem-cell differentiation [12], so the replication of endogenous beta cells can provide a new source of beta cells to increase beta cell mass, ultimately improving blood glucose control in type II diabetes patients [13].

In addition, type I diabetes patients still retain a small amount of functional beta cells even with the autoimmune loss of beta cells. One study found that 16% of patients still exhibited detectable C-peptide levels, a peptide released during proinsulin processing, illustrating that they still retain a low level of endogenous beta cells [14]. Coupled with methods to prevent autoimmune attack, these patients could potentially benefit from regeneration of beta cells by induced replication as well.

There has been great interest in the field in identifying regulators of beta cell proliferation. Early studies have shown that the cell cycle inhibitor p16^{INK4A} and upstream transcriptional regulators Ezh2 and Bmi1 are age-dependently expressed regulators of beta cell proliferation [15-17]. However, how p16^{INK4A} levels are

regulated in beta cells and whether other cell cycle regulators contribute to this age-dependent decline in beta cell replication remains poorly understood. More recently, studies have identified the nuclear factors of activated T cells (NFAT) as likely mediators of human beta cell proliferation [18]. In addition, recent reports have shown that targeting dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A), which catalyzes the autophosphorylation of serine/threonine and tyrosine residues, and glycogen synthase kinase 3 beta (GSK3B), which mediates high glucose-induced degradation of insulin substrate receptor 1 (IRS-1) can stimulate beta cell proliferation in adult islets [19]. Here, inhibition of DYRK1A and GSK3B causes NFAT nuclear localization, leading to beta cell proliferation and improved glucose tolerance [20, 21].

A study utilizing double knockout mice created by crossing mice deficient in cyclin D2, a regulator of the G1/S cell cycle checkpoint, (D2KO) with either mice deficient in either receptor substrate 1 (IRS1KO) or insulin receptor liver-specific knockout mice (LIRKO) illustrated that both D2KO/IRS1KO and D2KO/LIRKO knockouts exhibited insulin resistance and glucose intolerance [22]. Furthermore, they lacked compensatory beta cell hyperplasia, an increase in the proliferation of beta cells in order to increase beta cell mass and insulin secretion in an attempt to compensate for insulin. Cyclin D2 expression was also shown to coincide with the increased replication rate of endocrine cells and an increase in islet mass [23]. These studies ultimately illustrate that cyclin D2 is essential for promoting proliferation in order to expand beta cell mass.

Decrease in Beta Cell Proliferation in the Postnatal Time Period

In mice and humans, beta cell replication serves as the prevalent mechanism that ensures rapid expansion of beta cell mass during the postnatal time frame [24]. However, it is well recognized that the regeneration of beta cells becomes increasingly restricted with age. Still, there are certain circumstances when beta cell replication increases once again. For example, pregnancy and acute hyperglycemia recruit beta cells to enter the cell cycle in order to temporarily increase beta cell mass [25, 26]. Regardless, the increasing loss of beta cell proliferative ability with age remains a perpetual issue. However, this compensatory growth of beta cell mass indicates that it may be possible to target and promote beta cell proliferation.

It is well established that the largest regression in the proliferation rate of pancreatic beta cells occurs in the postnatal time immediately after birth, a phenomenon conserved in both mice and humans [27, 28]. Studies of beta cell proliferation have concluded that while approximately 25% of beta cells from postnatal day 5 (P5) mice exhibited proliferation (as established by the incorporation of BrDu, a proliferation marker in their DNA), only 7% of beta cells from postnatal day 16 (P16) mice exhibited proliferation [28]. Eventually, this decline in regenerative capacity in mouse beta cells tapers off, nearing zero in adulthood. Analogously in humans, 3% of fetal beta cells replicate, but by 6 months of age, human beta cells are largely senescent, with only 0.1-0.3% of the cells proliferating intermittently per day [6, 28, 29]. In other words, the aging process somehow restricts the cell cycle entry of pancreatic beta cells.

Although this phenomenon of decreasing beta cell proliferative capability with

age is widely acknowledged, the molecular changes that account for this decline remain undetermined. Deciphering the cellular events and mechanisms that lead to the decrease in beta cell proliferation during aging could help identify strategies for reversing this process and safely increasing beta cell mass. This could ultimately open the door for the development of new methods to manage or cure diabetes mellitus.

DNA Damage Response and Cell Cycle Arrest

More recent studies have suggested a possible role for a DNA damage response in promoted cell cycle arrest in beta cells [30-32]. The DNA damage response is a cellular defense mechanism that protects the cell from adverse mutations during DNA replication and plays a role in the maintenance of genomic integrity. In addition, when a cell is exposed to DNA-damaging agents, the DNA damage response is activated, ultimately causing cell cycle arrest, DNA repair, or apoptosis [33]. In many tissues, the DNA damage response is associated with cell cycle arrest and sometimes even is responsible for mediating the decrease in replicative capacity during aging [34]. Barral and colleagues investigated DNA damage in mouse neurons by measuring levels of histone H2AX (γ H2AX), an early marker for DNA damage [35]. They noticed an increased concentration of γ H2AX signals in the aging adult and senescent cerebral cortex, as well as apoptotic nuclei, demonstrating that DNA damage may be associated with proliferation and apoptosis in neurons.

The fact that the drastic decline in proliferation occurs directly following birth might suggest that environmental changes or signals may be triggering a DNA damage response, which in turn may initiate replicative cessation. For example, using a mouse

model system, Puente and colleagues discovered that cardiomyocyte cell-cycle arrest is induced via the DNA damage response in the postnatal frame [36]. Here, after birth, the conversion from embryonic to postnatal blood circulation results in a switch from anaerobic glycolysis to aerobic oxidative phosphorylation energy production. This conversion contributes to the observed increase in oxidative stress DNA damage and reactive oxygen species (ROS) levels, which in turn decreases replication ability. Previous studies have also illustrated the damaging effects of oscillating glucose levels, causing oxidative stress, likely one of the origins of endothelial damage observed in diabetes [37]. Studies in beta cells also found that nutrient shifts during weaning causes islet-specific microRNA changes, which results in a switch of expression of metabolic enzymes [38]. It is possible that alterations in glucose levels due to changes in diet between the fetal and postnatal phase may also be a possible factor that causes cell cycle arrest. Furthermore, the early decline in beta cell replication occurs at the transition from fetal metabolism, where insulin is supplied by the mother, to postnatal metabolism, where beta cells sense glucose levels and secrete insulin in response to changes in glucose concentration. Consistent with the need for glucose sensing in postnatal but not fetal beta cells, rates of glycolysis are higher in postnatal than in fetal beta cells [39]. Interestingly, increased rates of glycolysis have recently been shown to cause activation of a DDR pathway in beta cells [40, 41]. Given this, it may be that activation of the DNA damage response pathway caused by environmental shifts during the postnatal time period facilitates the cell-cycle arrest of beta cells, which then decreases the proliferative capacity of beta cells.

ATR and ATM DNA Response Pathways

Studies have shown that DNA damage response activation coincides with cell-cycle arrest, and oncogene-induced senescence requires the activation of the ATM and ATR DNA damage pathways, for double-stranded breaks and single-stranded breaks, respectively [42]. This suggests that the DNA damage response plays a role in driving the cells into a senescent state in order to maintain genomic integrity [43].

DNA replication is essential for the process of cell division. However, challenges to the progression of DNA polymerase along the DNA strand can lead to DNA damage in the form of replication stress, ultimately slowing or stalling replication forks and hindering DNA synthesis. Stalled replication forks are a major source of endogenous DNA damage. The DNA damage response signaling pathway is coordinated by the ATM and ATR kinases, which cooperate to control the cells' response to DNA damage, but their functions are distinct and not redundant [44]. When lesions caused by replication stress remain unrepaired, they cause both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) breaks.

While there are instances of replication stress causing dsDNA breaks, it is most frequently caused when DNA helicases continue unwinding the parental DNA double helix, even after the DNA polymerase has halted activity [45]. These regions of ssDNA within the vicinity of the stalled replication fork are recognized and bound by replication protein-A (RPA), which signals the replication stress DNA damage response pathway (Figure 1). Ataxia telangiectasia mutated and Rad3 related (ATR) is activated, which then recruits several other stress-response mediators to form a complex [46, 47]. One of the downstream targets of the ATR pathway is the Ser/Thr

checkpoint-kinase 1 (Chk1), which regulates phosphorylation of cyclin-dependent kinases (CDKs) and influences cell cycle damage checkpoint signaling [48, 49]. In response to genomic integrity, Chk1 phosphorylates and inhibits CDC25 phosphatases while promoting Wee1 activity, ultimately inhibiting CDK1 and blocking cell entry into mitosis [50]. The ATR complex assembled at the stalled replication sites serves to stabilize replication forks in response to replication stress by signaling for proper DNA replication, cell cycle preservation, and DNA damage repair. Continual DNA damage signaling in response to replication stress often leads to cellular senescence, or irreversible cell cycle arrest, even though the cell remains metabolically active [51].

When a cell undergoes the most harmful forms of DNA damage, dsDNA breaks, ATM is rapidly localized to DNA damage location, ATM kinase activity is elevated, and signaling cascades for cell cycle checkpoints and DNA repair are initiated (Figure 1). ATM activation leads to increased phosphorylation of various different substrates, most notably Brca1, Chk2, and p53, ultimately controlling the G1/S and G2/M checkpoints [44, 52]. The ATM complex assembled at the dsDNA break site then acts to mediate DNA repair.

Hypothesis and Aim

It is well recognized that the regeneration of pancreatic insulin-producing beta cells becomes increasingly restricted with age, with the largest decrease in proliferation rate occurring in the postnatal phase. However, the mechanism behind this decline remains poorly understood. A better understanding of the cellular events that lead to beta cell replicative senescence during aging should help identify

strategies for reversing this process and safely increasing beta cell mass.

DNA damage and activation of a cellular response is known to cause cells to stop proliferating. Importantly, recent studies have shown that a DNA damage response can be activated by increased glucose uptake in beta cells. Because there is a shift in glucose uptake after birth, I sought to determine if activation of a DNA damage response causes the decline in beta cell replication early in life. I hypothesize that activation of the DNA damage response in the pancreatic beta cells during the postnatal phase causes cell cycle arrest, resulting in a decline in proliferation capacity. Based on this hypothesis, I focused on the following four aims: (1) to investigate whether a DNA damage response is activated in postnatal beta cells; (2) to determine whether or not the DNA damage response is related to replication stress; (3) to study whether or not inhibition of the replication stress-induced DNA damage pathway will promote beta cell proliferation; and (4) to develop and optimize single-cell gel electrophoresis methods (comet assay) to confirm and evaluate the type of DNA damage that occurs in postnatal beta cells.

RESULTS

The DNA Damage pathway is activated in the pancreatic beta cell postnatally

Following induction of DNA damage, the specialized histone protein H2A becomes phosphorylated at serine 139 (γH2AX) and rapidly accumulates at the sites of DNA damage lesions forming distinct foci [53-55]. In addition, 53BP1 is phosphorylated at Ser25, Ser29, or other residues to regulate genome stability as a result of DNA damage [56]. H2AX foci formation is followed by recruitment of many other proteins involved in the DNA repair process including the p53 binding protein 53BP1 [54]. To investigate whether a DNA damage response is activated in pancreatic beta cells postnatally, we used immunofluorescence to detect activation of DNA damage markers γH2AX and 53BP1 in postnatal day 1 (P1) and postnatal day 21 (P21) using the transgenic reporter mouse line mIns1-H2B-mCherry (Figure 1). Immunofluorescence of P1 and P21 pancreatic islets showed elevated levels endogenous γH2AX and nuclear relocalization of 53BP1 (Figure 2). These results indicate that the DNA damage response is activated in beta cells during the postnatal period, namely between the ages of P1 and P21.

Postnatal aging is associated with elevated levels of replication stress

Recent studies have revealed that the appearance of γH2AX and 53BP1 is not only associated with DNA damage, but also chromatin organization and cell self-renewal. In addition, they are both activated by the ATM-Chk2 and ATR-Chk1 signaling cascades of the DNA damage response [56, 57]. The ATM-Chk2 signaling cascade is associated with dsDNA breaks. ATM is phosphorylated (pATM) upon

DNA damage activation, and is crucial in the signaling of downstream protein kinases in order to instigate a cell cycle blockade (Figure 1) [58]. The ATR-Chk1 signaling cascade is closely associated with ssDNA breaks. RPA binds to regions of ssDNA near stalled replication forks and activates ATR, which then recruits and activates several other stress-response mediators including Chk1 [46, 47].

To investigate further into the whether DNA damage pathway that is activated in postnatal beta cells is associated with ssDNA or dsDNA breaks, we performed immunofluorescence in P1 and P21 H2B-mCherry mouse pancreata. Here, we stained for the presence of RPA foci and phosphorylation of ATM (pATM), which are markers for ssDNA and DSBs, respectively.

We found no apparent difference in the percentage of pATM positive beta cells between P1 and P21 pancreata (Figure 3). This indicates that the ATM-Chk2 DNA damage pathway associated with dsDNA breaks is not activated. However, more P21 beta cells showed the appearance of RPA foci, which generates a signal for activation of the replication stress response (Figure 3). In addition, we also assessed the phosphorylation of Chk1, a downstream effector of the replication stress response. Consistent with the RPA foci, increased pChk1 was also observed in P21 beta cells compared to P1 beta cells. Together, these results indicate that DNA damage is activated in postnatal beta cells, and the DNA damage is associated with ssDNA breaks, likely replication stress.

Pharmacological inhibition of the replication stress response pathway promotes beta cell proliferation

Based on our preliminary findings showing that ssDNA breaks are found in postnatal beta cells, we hypothesized that replication stress provides a barrier for beta cell proliferation by inducing the DNA damage response. To determine if inhibition of the replication stress response pathway could promote proliferation *in vitro*, we examined whether inhibiting Wee1 activity, which is activated by the DNA damage response and a repressor CDK1-dependent G2-M transition, can increase beta cell proliferation. We isolated and dispersed pancreatic islets from 4-6 week old MIns1-H2B-mCherry mice and cultured them in the presence of the Wee1 inhibitor MK-1775. Pancreatic islets treated with the MK-1775 for 48 hours at low concentrations of 10nM and 100nM exhibited increased levels of proliferation compared to untreated islets (Figure 5). This suggests that inhibition of Wee1 increases beta cell proliferation *in vitro*.

To determine if inhibition of the replication stress response pathway could promote proliferation *in vivo*, MIns1-H2B-mCherry mice were injected daily with MK-1775 from ages P7 to P21. Mice injected with the MK-1775 demonstrated higher levels of beta cell proliferation compared to mice injected with DMSO. Immunostaining of MK-1775 treated P21 mouse pancreata showed increased expression of Ki67-positive beta cells (Figure 6). This suggests that inhibition of Wee1 also increases beta cell proliferation, *in vivo*.

Optimization of the alkaline comet assay in pancreatic beta cells allows for measurement of single-stranded DNA breaks to confirm replication stress.

The alkaline comet assay, a method to measure both ssDNA and dsDNA breaks at a single cell level, was established specifically for pancreatic beta cells. This method of single-cell electrophoresis allows for the direct evaluation and quantification of DNA damage. Here, Min6 pancreatic beta cells were treated with either H₂O₂ or medium. H₂O₂, a reactive oxygen species, causes a variety of DNA lesions, including single and double stranded breaks as well as crosslinking of DNA bases [59]. Cells were harvested, and trypsinized into single cells, and resuspending in LMP agarose (Figure 7A). The Min6 cell-agarose mixture was the coated onto NMP agarose-coated Trevigen CometSlides. Cells were lysed and their DNA denatured using high alkaline conditions. Electrophoresis of this DNA under alkaline (pH >13) conditions allows for DNA without lesions to remain supercoiled within the nucleoid, while broken DNA ends migrate out of the nucleoid forming “comets”. After staining with Sybr Gold, a fluorescent stain that detects nucleic acids, we utilized fluorescence microscopy and visualized larger comets with H₂O₂-treated Min6 cells compared to control cells treated with medium (Figure 7B). Tail % DNA, the intensity of the comet tail relative to the comet head, was used to reflect the number of DNA breaks. Quantification of the comets showed a significant tail % DNA fold change in the treated vs. untreated Min6 cells (Figure 7C). The results indicate that we developed a successful method to quantify DNA damage at the single cell level using the Min6 mouse pancreatic beta cell line. The alkaline comet assay will be useful in confirming that beta cells undergo replication stress by measuring ssDNA

breaks. Furthermore, the presence of ssDNA damage would indicate that replication stress occurs in postnatal beta cells and may contribute to cell cycle arrest with age.

Material included in this master's thesis contains information being prepared for publication in Zeng, Chun; Guan, Tiffany; Sander, Maiké. "Replication stress drives proliferative decline in postnatal beta cells".

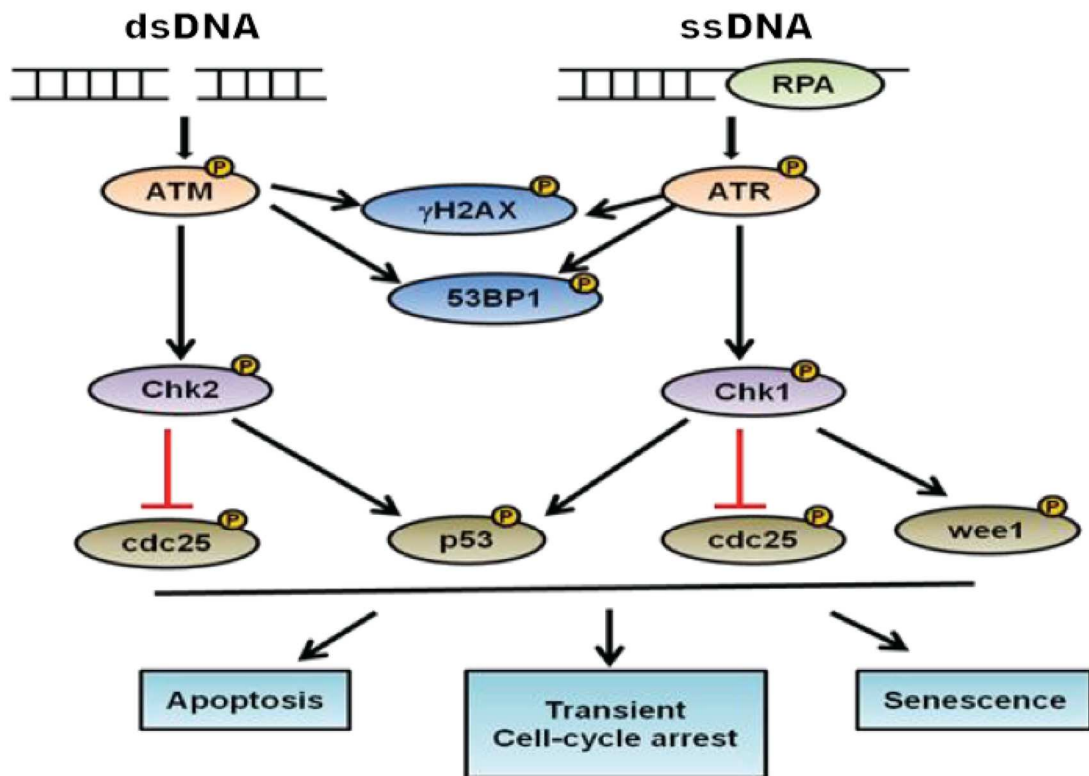


Figure 1. The DNA damage response pathway. The DDR pathway is coordinated primarily by two distinct kinase signaling cascades, the ATM-Chk2 and ATR-Chk1 pathways, which are activated by double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) breaks, respectively. When activated, these signaling pathways phosphorylate and activate the downstream effectors wee1, p53, or inhibit cell cycle division cycle 25 phosphatase (CDC25), resulting in apoptosis, transient cell-cycle arrest, or senescence. The ATR-Chk1 pathway plays a key role in the response to DNA replication stress, the slowing or stalling of replication fork progression, arising from replication barriers, such as repetitive sequences, DNA lesions, and misincorporation of ribonucleotides. Replication stress often results in the generation of Replication protein A (RPA) bound ssDNA at the site of stalled replication forks, which is a sensor for the ATR-Chk1 pathway.

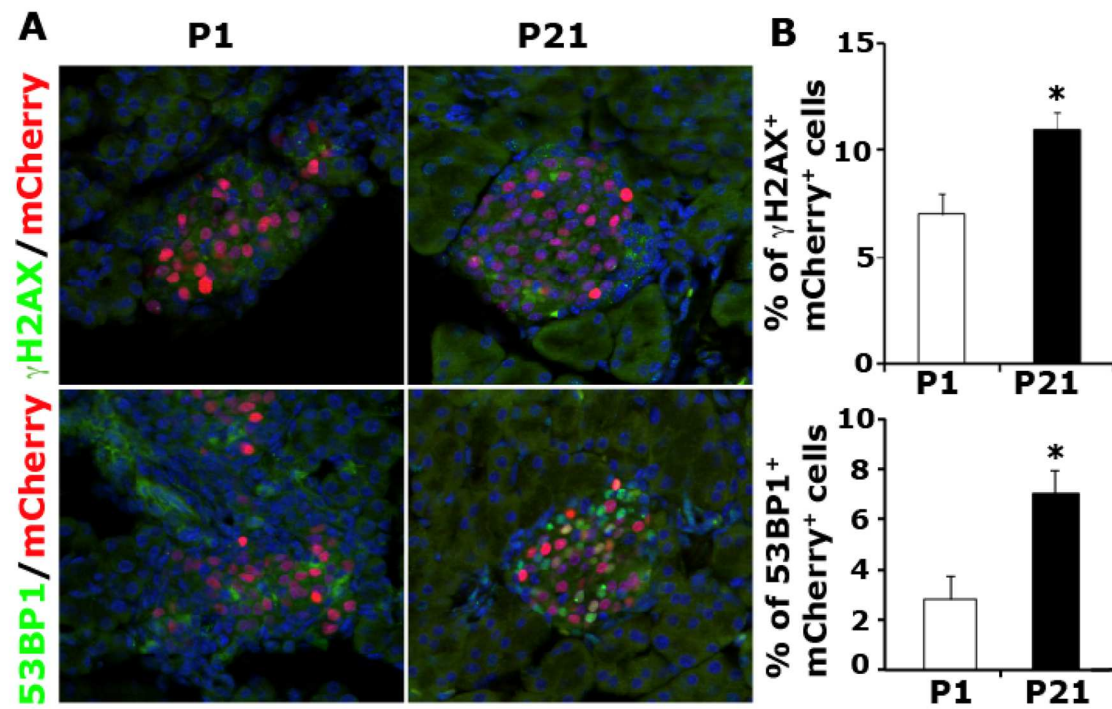


Figure 2. DNA Damage Response is activated in postnatal pancreatic beta cells. (A) Immunostaining of γ H2AX and 53BP1 in P1 and P21 pancreata from mIns1-H2B-mCherry mice. (B) Quantification of mCherry⁺ γ H2AX and 53BP1 foci. n>4, *p<0.05.

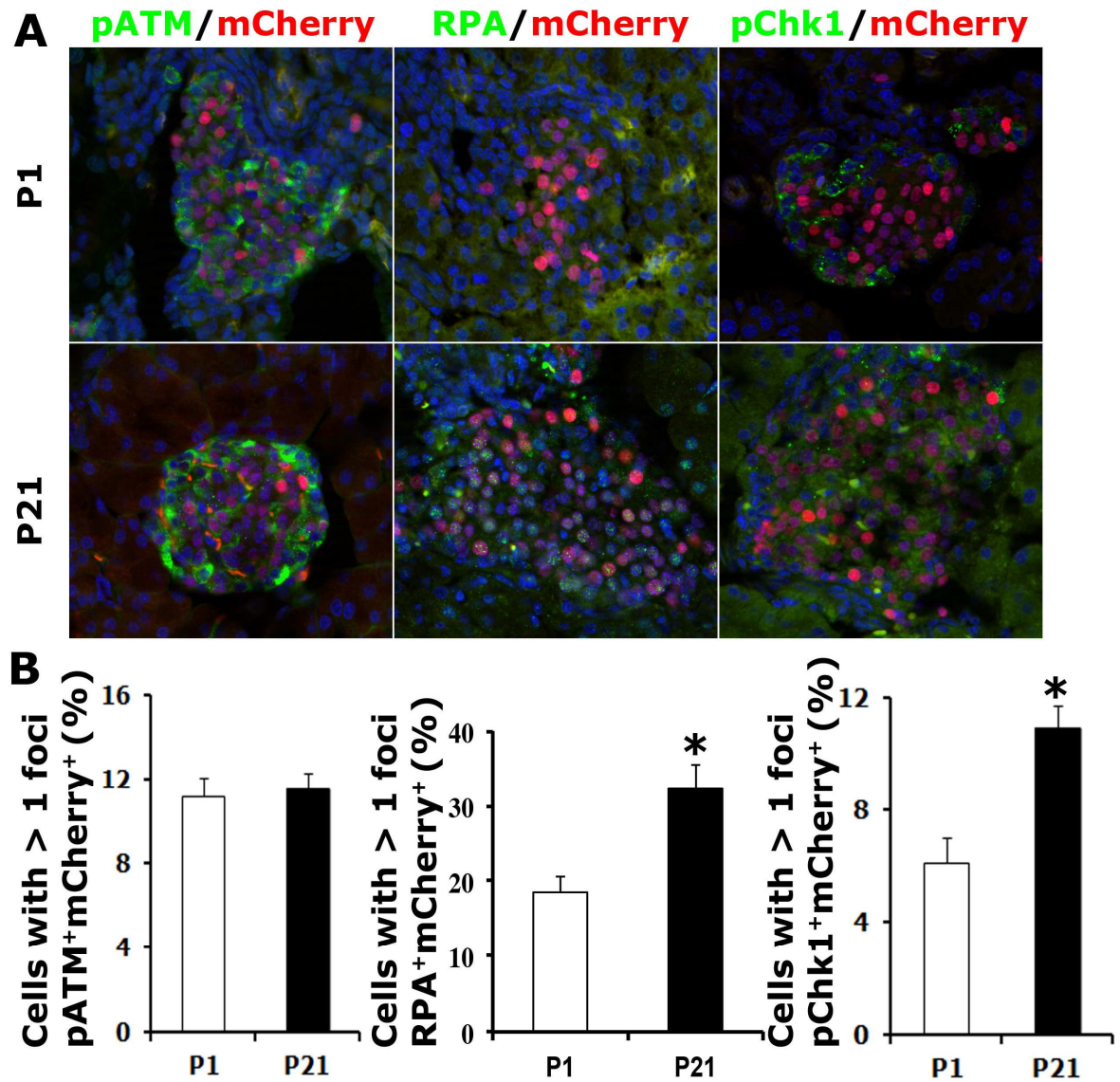


Figure 3. Replication stress markers are elevated in postnatal pancreatic beta cells. (A) Immunostaining of pATM, RPA, and pChk1 in P1 and P21 pancreata from mIns1-H2B-mCherry mice. (B) Quantification of pATM, RPA, and pChk1 foci. $n > 3$, $*p < 0.05$.

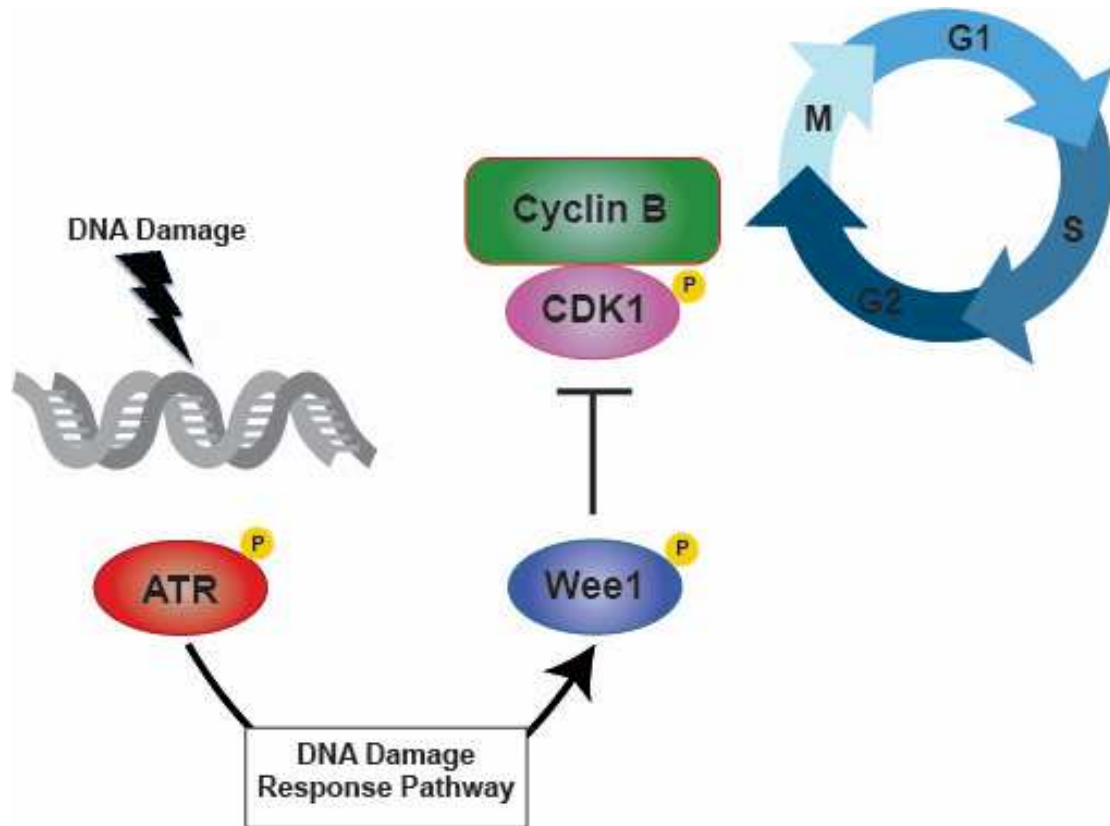


Figure 4. Inhibition of Wee1 of the ATR-Chk1 DNA damage response pathway causes cell-cycle arrest. DNA damage initiates the ATM-Chk1 kinase-signaling cascade of the DNA damage response and activates Wee1 kinase. Wee1 phosphorylation inhibits cyclin-dependent kinases (CDKs), causing cell-cycle arrest.

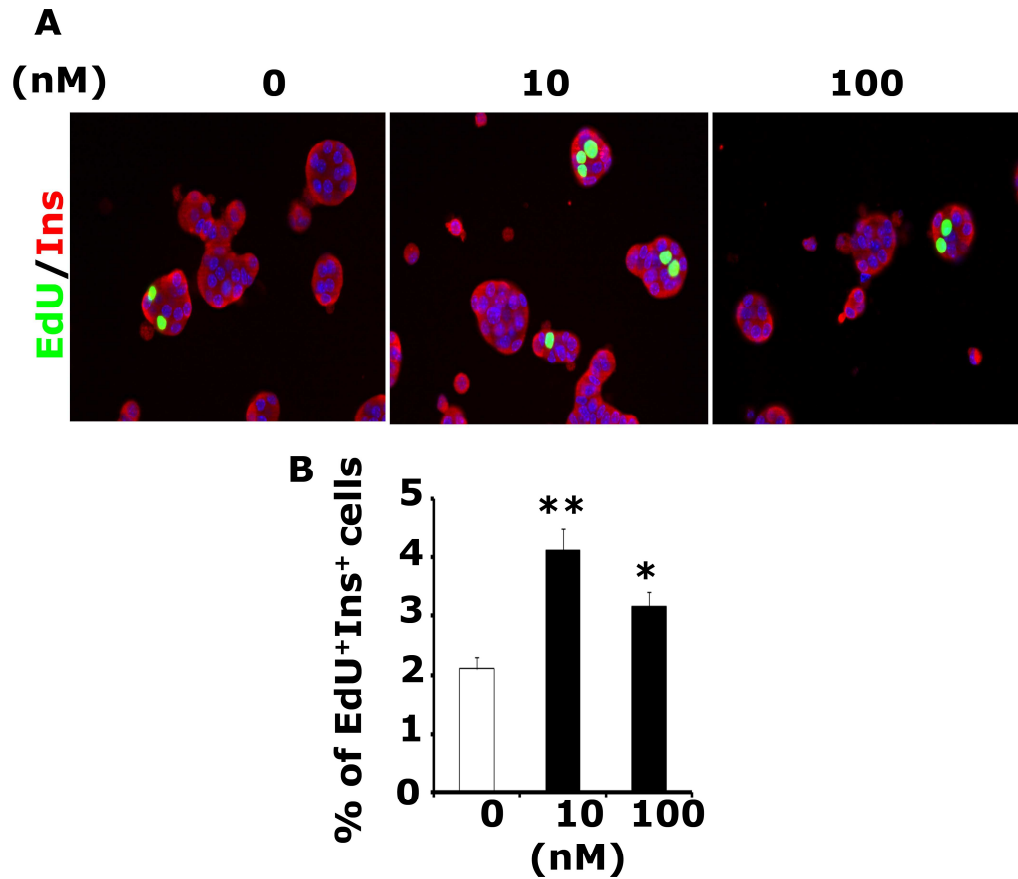


Figure 5. MK-1775 treatment of pancreatic islets *in vitro* promotes beta cell proliferation. (A) Immunostaining of EdU and Ins in isolated P21 pancreatic islets. (B) Quantification of EdU⁺ Ins⁺ cells showed increase proliferation in MK-1775-treated islets. *p<0.05, **p<0.01

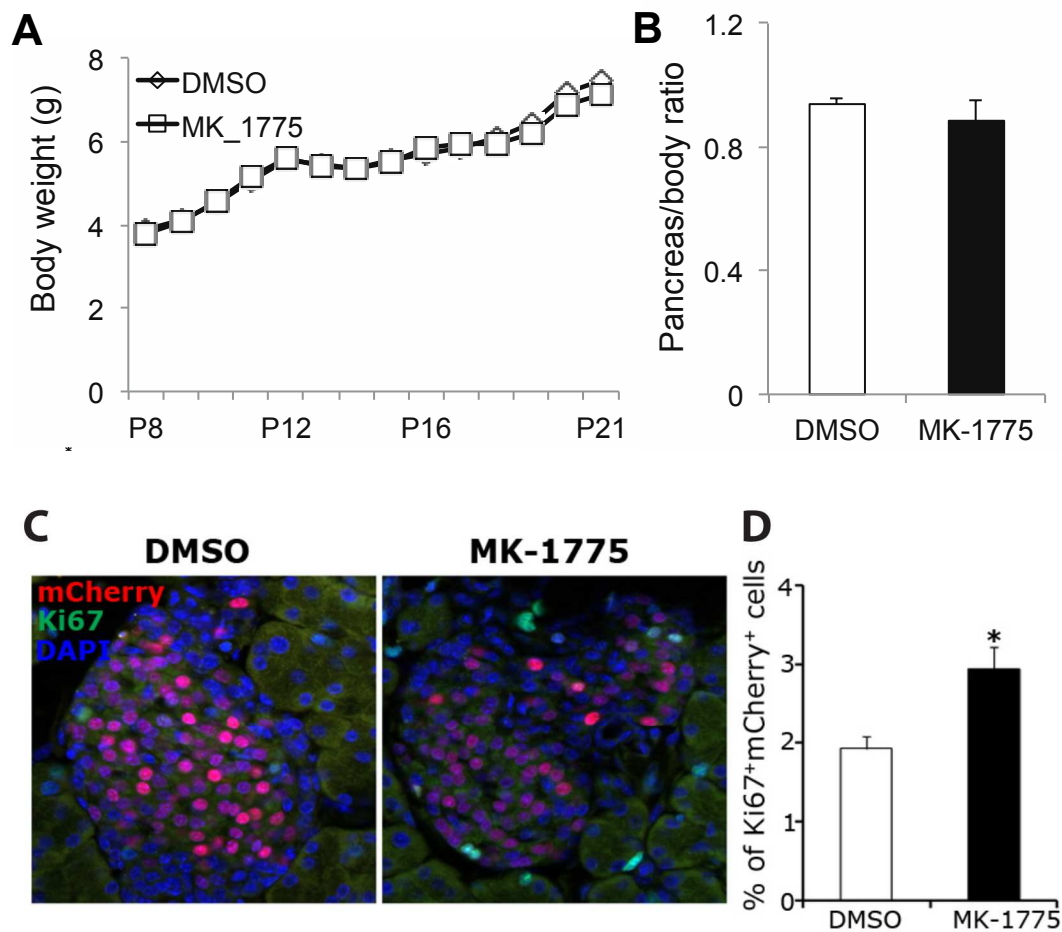


Figure 6. MK-1775 treatment in vivo promotes beta cell proliferation. (A) Body weights of DMSO and MK-1775 treated MIns1-H2B-H2B-mCherry mice from ages P8 to P21. (B) Pancreas/body ratio of DMSO and MK-1775 treated mice at time of pancreas tissue harvest. (C) Immunostaining of Ki67 in P21 mIns1-H2B-mCherry mouse pancreata after injection of MK-1775 *in vivo* daily from P7 to P21. (D) Quantification of Ki67⁺ mCherry⁺ cells. *p<0.05

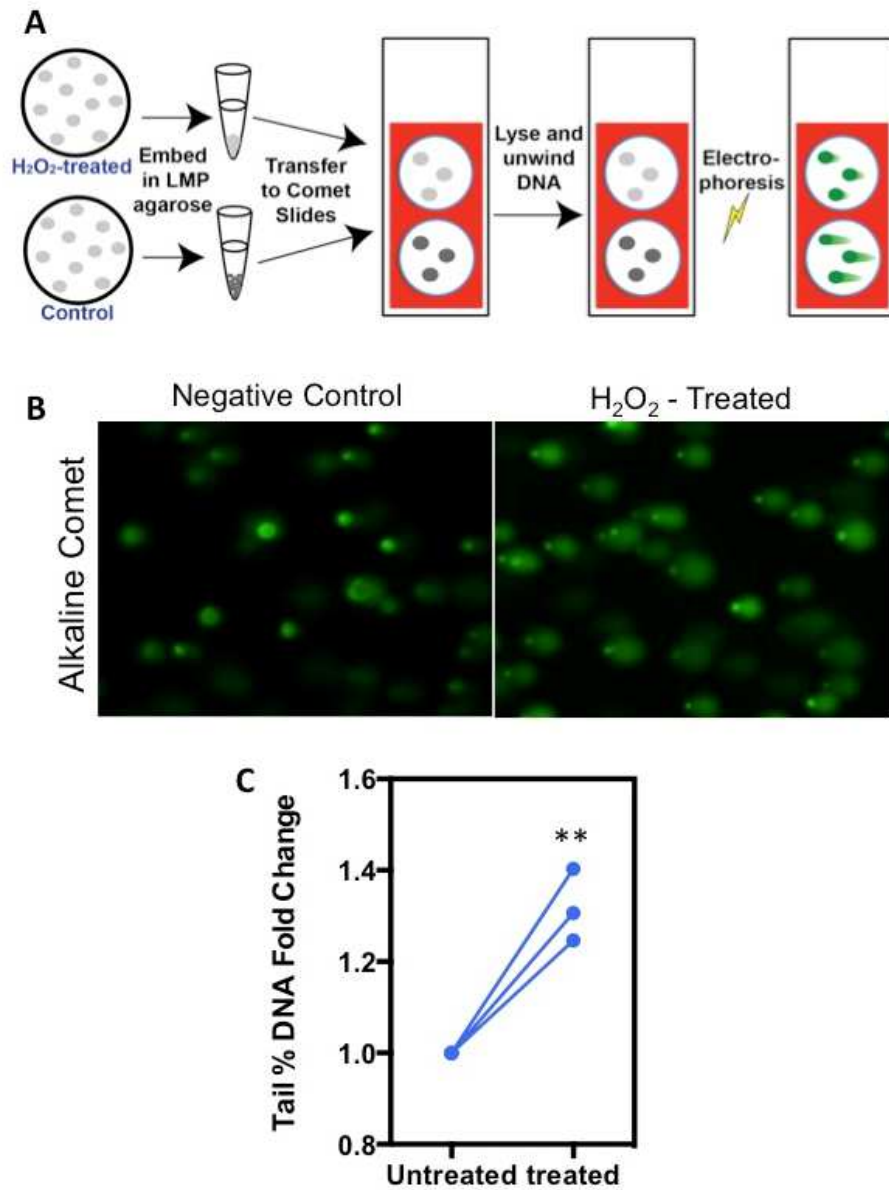


Figure 7. Development of alkaline comet assay for pancreatic beta cells. The alkaline comet assay was developed for pancreatic beta cells using the Min6 cell line to confirm ssDNA via replication stress. (A) Min6 cells were treated with H₂O₂ to induce DNA strand breaks before being suspended in LMP agarose and transferred onto NMP agarose-coated Trevigen CometSlides. Cells were lysed and DNA was unwound prior to electrophoresis in order to compare relative ssDNA damage levels. (B) Alkaline comets treated under control versus H₂O₂ conditions visualized using Sybr Green. (C) Tail % DNA fold change of control versus H₂O₂-treated Min6 cells. n=3, **p<0.01.

DISCUSSION

Replication Stress-Induced DNA Damage Response in Pancreatic Islets

It is well recognized that the regeneration of pancreatic insulin-producing beta cells becomes increasingly restricted with age, with the most drastic change occurring shortly after birth [27, 28]. However, the molecular mechanism that causes this decrease in proliferation remains undetermined. It has also been shown that activation of the DNA damage response often leads to cell cycle arrest [33]. Our studies show that the decrease in beta cell proliferation coincides with activation of multiple DNA damage response markers, namely γ H2AX and 53BP1, leading us to believe that the decrease in proliferation rate is caused by activation of a DNA damage response pathway (Figure 2).

To further investigate the activation of a DNA damage response, we studied the ATM-Chk2 DNA and ATR-Chk1 DNA damage pathways associated with dsDNA and ssDNA breaks, respectively. We found that neonatal beta cells are associated with ssDNA lesions, but not dsDNA lesions. Activation of the ATR-Chk1 DNA damage pathway indicates that replication stress may be a driver for the decline of beta cell growth postnatally.

Inhibition of the Replication Stress Response Pathway Promotes Beta Cell Proliferation

We found that treatment with the Wee1 inhibitor *in vitro* and *in vivo* initiated proliferation of beta cells, as shown by EdU and Ki67 staining, respectively. By inhibiting of the replication stress response pathway, we were able to confirm the role

of replication stress in decreasing beta cell proliferation capacity during the in young postnatal mouse beta cells.

Various cancer studies have shown that inhibition of Wee1 can actually lead to greater tumor growth inhibition, ultimately decreasing proliferation and enhancing anti-tumor efficacy [60, 61]. However, other studies have shown that inhibition of Wee1 increases CDK2 activity and accelerates the cell cycle, thereby overall increasing proliferation [62, 63]. One study using malignant melanoma cells showed that a downregulation of miR0195 causing a decrease in Wee1 expression may help the cells to overcome cell cycle arrest during stress conditions. This may allow for increased cell growth in local tissue environment. Although we used a different method to inhibit Wee1 expression, our studies also show that Wee1 inhibition leads to an increase in proliferative rate in beta cells. Taken together, these studies show that it may be possible to manipulate Wee1 expression beta cells undergoing DNA stress in order to promote proliferation.

Pharmacological targeting to promote beta cell proliferation

By finding the mechanism behind the decrease in beta cell proliferation in the postnatal phase, we can potentially discover specific targets to activate or inhibit in order to reverse this occurrence. Furthermore, we found that replication stress-related DNA damage markers RPA and pChk1 are activated in beta cells postnatally, and inhibition of the downstream effector Wee1 increases beta cell proliferation *in vitro* and *in vivo*. Thus, targeting factors that mediate replication stress responses in beta cells may be a useful strategy to effectively foster regeneration. Since our studies

show that Wee1 inhibition caused an increase in the proliferation in beta cells, this makes it a potential therapeutic candidate for pharmacological targeting for diabetes.

Alkaline Comet Assay

Single-cell electrophoresis was used to directly measure and quantify DNA damage. We have been successful in establishing an alkaline comet assay in pancreatic beta cells using the pancreatic beta cell Min6 cancer line. Quantification of tail % DNA in H₂O₂-treated Min6 cells exhibited higher levels of DNA damage compared to untreated samples, which shows that the developed protocol is effective in measuring relative DNA damage levels.

The alkaline comet assay is a more direct method compared to immunofluorescence studies in order to determine and confirm ssDNA and dsDNA damage lesions. This is due to the fact that many of the markers used to confirm activation of the DNA damage pathway are also involved in various other pathways. For example, γ H2AX is sometimes involved in chromatin remodeling as well as self-renewal in mouse embryonic stem cells, even in the absence of DNA damage [64]. 53BP is also involved in cellular transcription regulation independent of DNA damage [65]. By confirming and understanding the type of DNA damage occurring in beta cells, we can determine which pathways are involved. The presence of ssDNA damage would confirm that replication stress occurs in postnatal beta cells.

Future Directions

There are several experiments to perform in order to follow up on the results

obtained through the studies so far. The alkaline comet assay will be essential to confirming if single strand DNA breaks occur in beta cells. In analyzing the data from the alkaline comet assay in DNA damage induced Min6 cells, we noticed that the fold change for of the tail % DNA increased at a consistent ratio. However, there were variations in the exact tail % DNA values between trials. Previous studies have shown that the alkaline comet assay is influenced by variations in different parameters, including temperature during alkaline treatment and electrophoresis, DNA unwinding time, agarose density, and lysis time [66-69]. For example, recommended DNA unwinding time can range from one hour to overnight. To move forward with this portion of the project, we hope to continue to standardize more of the conditions of the alkaline comet assay to decrease variations in results.

In addition, we plan on utilizing the alkaline comet assay to detect DNA damage in isolated neonatal mouse pancreatic islets. However, one caveat to this experiment is that in order to carry out the tests, we need P1 and P21 age-matched cohorts of mice; it is challenging to schedule the breeding of mice so that they provide P1 and P21 mice at the exact same time. In addition, it is difficult to obtain a large number of P1 islets, as each neonatal mouse provides very few pancreatic islets. One method to overcome these two caveats is to order mice of the specific ages for the purposes of the single-cell electrophoresis experiments instead of waiting for the birthing schedules to align. Finally, an additional caveat is that the isolated islets will not contain a pure beta cell population as they will also contain alpha, delta and PP cells [70]. We can utilize fluorescence-activated cell sorting (FACS) with mIns1-H2B-mCherry to circumvent this obstacle.

In order to continue studying whether or not Wee1 is a viable target to promote beta cell proliferation, we plan to test the effects of MK-1775 in adult mice *in vivo* under diabetic conditions. If Wee1 increases beta cell proliferation, I would expect to see an increase in beta cell mass and an increase in insulin production. Accordingly, in measuring the blood glucose levels of the diabetic mice throughout MK-1775 treatment, I would anticipate to see decreased blood glucose levels and reversal of other diabetes-related symptoms.

In addition, there are many different known sources that cause replication stress, including chemotherapeutics, oncogene activation, nucleotide deficiency, and oxidative stress. A future goal is to look further into the source of replication stress in postnatal pancreatic beta cells that is activating the ATR pathway and causing cell cycle arrest [71]. A study by Puente and colleagues showed that reactive oxygen species (ROS) are the source of decreased proliferation in cardiomyocytes [36]. Following suit, one step will be to investigate whether or not oxidation stress is the factor that activates the replication stress response by 8-oxoguanine (8-oxoG), an oxidative stress marker [72]. We would also like to test whether or not providing various nucleotides to isolated pancreatic islets will promote entry through the cell cycle.

In sum, the studies described thus far along with aforementioned future studies will help elucidate mechanisms underlying age-dependent decline in beta cell replication, as well as identify factors that could potentially be targeted to improve beta cell function and stimulate their expansion.

MATERIAL AND METHODS

Harvesting of Mouse Pancreas Tissue

Mouse pancreas tissue was harvested and fixed with 4% paraformaldehyde (PFA) at 4°C. Postnatal day 1 (P1) tissues were fixed for 4 hours and postnatal day 21 (P21) tissues were fixed overnight. Pancreas tissue was washed with 1xPBS, immersed in 30% sucrose overnight at 4°C, and embedded in optimal cutting temperature compound (OCT) (VWR).

Cryosection of Mouse Pancreas Tissue

Mouse pancreas tissue embedded in OCT was cryosectioned at a thickness of 10µm using a Leica M3050 S research cryostat and stored at -80°C until use.

Immunofluorescence Detection of γ H2AX, 53BP1, pChk1, and RPA

For immunofluorescence analysis of γ H2AX, 53BP1, pChk1, and RPA, tissue sections were fixed in a 1:1 methanol: acetone solution at -20°C for 20 min and steamed with an IHC steamer in citrate buffer for antigen retrieval. Tissue sections were then permeabilized with 0.15% Triton-X 100, blocked with 5% Normal Donkey Serum (NDS) in PBST, incubated with primary antibodies overnight at 4°C, and incubated with secondary antibodies for 1 h at room temperature. DAPI at 1:3,000 was used to stain cellular nuclei, and fluorescence was preserved using Vectashield mounting medium (Vector).

Immunofluorescence Detection of pATM

For immunofluorescence analysis of pATM in mouse tissue, we utilized the Vector M.O.M. immunodetection kit (Vector). Mouse tissue sections were incubated at 37°C for 1 h in citrate buffer, permeabilized with 0.15% Triton-X-100 for 1 h, blocked with M.O.M. Mouse Ig Blocking Reagent, and incubated with M.O.M. Diluent before incubation with primary antibodies overnight at 4°C. Slides were then treated with M.O.M. Biotinylated anti-Mouse IgG Reagent and incubated with streptavidin-conjugated fluorophores and other secondary antibodies for 1 h at room temperature. DAPI at 1:3,000 was used to stain cellular nuclei, and fluorescence was preserved using Vectashield mounting medium.

MK-1775 treatment *in vitro*

mIns1-H2B-mCherry mouse pancreata were harvested at age P21. Isolation of islets from 4-6 week old mice is described previously [73]. In brief, pancreata were perfused through the common bile duct with Literase TL (Roche), and islets were purified by centrifugation using a Histopaque gradient (Sigma). Islets were isolated and picked under a microscope, and dissociated with 0.05% trypsin (Sigma) for 5 min in a 37°C water bath. Dispersed islets were plated onto poly-L-Lysine coated 12mm coverslips in 24 well plates and allowed to recover overnight. MK-1775, the indicated Wee1 inhibitor, was added into the medium. One day later, MK-1775, was added once more into the medium, this time along with 10 μ M EdU. On the third day, the medium was discarded, and the islets were washed with PBS twice. Islets were fixed

with 4% PFA for 30 min at room temperature and subsequently washed 3 times with PBS. Isolated islets were then stained with insulin and EdU.

Immunofluorescence detection of EdU

Islets were blocked and permeabilized with 5% NDS and 0.15% Triton-X-100 for 1 h at room temperature and washed twice with 3% BSA in PBS. Islets were incubated with the Click-iT reaction cocktail (Life Technologies) composed of 1 x Click-iT reaction buffer, CuSO₄, Alexa Fluor azide, and reaction buffer additive for 30 minutes at room temperature, protected from light. The reaction cocktail was discarded and islets were washed with 3% BSA in PBS. DAPI at 1:3,000 was used to stain cellular nuclei, and fluorescence was preserved using Vectashield mounting medium.

MK-1775 injection *in vivo*

mIns1-H2B-mCherry mice were injected with 2.5 mg/kg Wee1 inhibitor MK-1775 subcutaneously daily from day 6 to day 12, followed by intraperitoneal injection until day 21. Body weight was monitored daily. Mouse pancreata were harvested, weighed, and fixed with 4% paraformaldehyde (PFA) at 4°C overnight. Tissue was washed 3x with 1 x PBS and immersed in 30% sucrose overnight at 4°C. Pancreas tissue was then embedded in optimal cutting temperature compound (OCT) and stained with Ki67 to detect proliferation rates in beta cells.

Immunofluorescence detection of Ki67

Tissue sections were treated with citrate buffer for antigen retrieval and incubated for 1 h at 37°C. Samples were washed with 1 x PBS, permeabilized with 0.15% Triton-X-100, blocked with 5% NDS, incubated with the Ki67 primary antibody for 1 h at 4°C overnight, and incubated with donkey anti-rabbit Alexa Fluor 488 secondary antibody for 1 h at room temperature.

Alkaline Comet Assay using Min6 Pancreatic Beta Cell Line

Min6 cells were washed a minimum of 2 times with 1 x PBS and trypsinized to minimize cell clusters. The cells were prepared at a density of 1×10^5 cells/mL and combined with 1% low melting point agarose at 37°C at a ratio of no more than 1:10. 50µL of cell suspension was evenly coated onto Trevigen CometSlides, allowed to solidify at 4°C in the dark for 30 min, and gently immersed into pre-chilled lysis buffer (Trevigen) overnight at 4°C. Trevigen CometSlides were immersed in freshly prepared alkaline unwinding solution for 1 h at room temperature. CometSlides were placed in an electrophoresis chamber filled with 1x alkaline comet assay electrophoresis buffer and a voltage of 10 volts was applied for 30 min at 4°C. Samples were washed with dH₂O, immersed in 70% EtOH, dried at 37°C, and stained with Sybr Gold at 1:10,000.

Tail % DNA, the intensity of the comet tail relative to the comet head, was used to quantify the relative number of DNA breaks. Tail % DNA was measured using OpenComet, an open-source software tool and plugin for ImageJ.

Immunofluorescence Microscopy

Images were captured using a Zeiss Axio Observer Z1 microscope with an ApoTome module and processed using Zeiss Zen 2 (blue edition).

Data Analysis

Each set of data presented in the figures is representative of at least three replicate experiments. All averages, SEMs, and significance p values (Student's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) were calculated and graphed with Microsoft Excel. All images were processed and figures were assembled using Adobe Photoshop CS5.1, Adobe Illustrator software CS5.1, and Microsoft PowerPoint.

Table 1. Buffers and Solutions

Name	Components
Alkaline Unwinding Solution	3 M NaOH 1 mM EDTA *pH >13
Alkaline Comet Assay Electrophoresis Buffer	3 M NaOH 1 mM EDTA, pH 8
Antigen Retrieval Citrate Buffer	9.5 mM citric acid 42 mM sodium citrate
Antigen Retrieval EDTA Buffer	1mM EDTA 0.05% Tween-20
Islet Isolation Buffer	10% FBS HBSS 2.8mM glucose
Islet Media	500 mL RPMI 1640 – no glucose 8mM D-Glucose 10% FBS 2mM L-glutamine 100µg/mL PS 1mM Sodium Pyruvate 10mM HEPES 0.25µg/mL Amphoterecin

Table 2. List of primary antibodies used in immunofluorescence staining

Primary Antibodies				
Antigen	Host	Dilution	Source	Catalogue #
γ H2AX	Rabbit	1:100	Biologend	613404
53BP1	Rabbit	1:1000	Novus Biologicals	NB100-304
pChk1	Rabbit	1:200	Cell Signaling	2348S
RPA	Rabbit	1:200	Fisher	PIPA521976
pATM	Mouse	1:400	Santa Cruz	SC-47739
Insulin	Guinea Pig	1:1000	Dako	A056401

Table 3. List of secondary antibodies used in immunofluorescence staining

Secondary Antibodies			
Antigen	Conjugation	Dilution	Source
Guinea Pig	Cy3	1:2000	Jackson ImmunoResearch
Rabbit	A488	1:1000	Jackson ImmunoResearch

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