Brief Communication

MOLECULAR METABOLISM

Critical role for adenosine receptor A2a in β-cell proliferation

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ABSTRACT

Objective: Pharmacological activation of adenosine signaling has been shown to increase β-cell proliferation and thereby β-cell regeneration in zebrafish and rodent models of diabetes. However, whether adenosine has an endogenous role in regulating β-cell proliferation is unknown. The objective of this study was to determine whether endogenous adenosine regulates β-cell proliferation—either in the basal state or states of increased demand for insulin—and to delineate the mechanisms involved.

Methods: We analyzed the effect of pharmacological adenosine agonists on β-cell proliferation in *in vitro* cultures of mouse islets and in zebrafish models with β- or δ-cell ablation. In addition, we performed physiological and histological characterization of wild-type mice and mutant mice with pancreas- or β-cell-specific deficiency in *Adora2a* (the gene encoding adenosine receptor A2a). The mutant mice were used for *in vivo* studies on the role of adenosine in the basal state and during pregnancy (a state of increased demand for insulin), as well as for *in vitro* studies of cultured islets.

Results: Pharmacological adenosine signaling in zebrafish had a stronger effect on β-cell proliferation during β-cell regeneration than in the basal state, an effect that was independent of the apoptotic microenvironment of the regeneration model. In mice, deficiency in *Adora2a* impaired glucose control and diminished compensatory β-cell proliferation during pregnancy but did not have any overt phenotype in the basal state. Islets isolated from *Adora2a*-deficient mice had a reduced baseline level of β-cell proliferation *in vitro*, consistent with our finding that UK432097, an A2a-specific agonist, promotes the proliferation of mouse β-cells *in vitro*.

Conclusions: This is the first study linking endogenously produced adenosine to β-cell proliferation. Moreover, we show that adenosine signaling via the A2a receptor has an important role in compensatory β-cell proliferation, a feature that could be harnessed pharmacologically for β-cell expansion and future therapeutic development for diabetes.

Keywords β-cell proliferation; Adenosine; Islet biology; Gestational diabetes

1. INTRODUCTION

Type 1 and type 2 diabetes feature a shortage of functional β-cells [1], resulting from β-cell death, insulin deficiency, or insulin resistance. The current treatments used to counteract this insufficiency include transplantation of pancreas or pancreatic islets or administration of insulin or insulin sensitizers/secretagogues. However, there is a shortage of pancreas transplant donors, and glycemia is difficult to adequately control with pharmacological treatments in severe cases of diabetes. Alternative strategies, preferably curative ones, therefore, are needed. One potentially curative strategy would be to stimulate the remaining β-cells to proliferate. Depletion of β-cells in zebrafish and rodent models of diabetes is followed by significant recovery of the β-cell mass, indicating that the pancreas has the capacity to regenerate [2–4]. Likewise, the human β-cell mass is known to adapt to the high demand for insulin that occurs in states such as pregnancy and obesity [5–7]. Identifying the signals that drive this pancreatic adaptability could potentially enable the development of a curative diabetes treatment based on increasing the number of functional β-cells.

As an adaptive response to the insulin resistance that prevails in pregnancy, which ensures that enough glucose reaches the fetus, the mother's β-cell mass expands [8,9], increasing the production of insulin and ensuring that the mother's blood glucose levels remains under control. Several pregnancy hormones, including prolactin and placental lactogen [10], stimulate β-cell proliferation by inducing the downstream effectors FoxM1, Menin, osteoprotegerin, and serotonin [11–14]. As a result, insulin secretion increases in pregnancy. It is possible that positive feedback loops triggered by this high demand for insulin further potentiate β-cell proliferation. Moreover, because gestational diabetes is associated with an increased risk of developing type 2 diabetes later in life [15,16], the inverse action of the mechanisms underlying the failure of compensatory proliferation of β-cells in gestational diabetes may therefore have potential as novel interventions in the treatment of diabetes.

Bridging developmental biology and drug discovery, we have previously used the zebrafish model to perform *in vivo* screening for drugs, small molecules, and secreted proteins that can induce β-cell regeneration [2]. After screening >10,000 small molecules for

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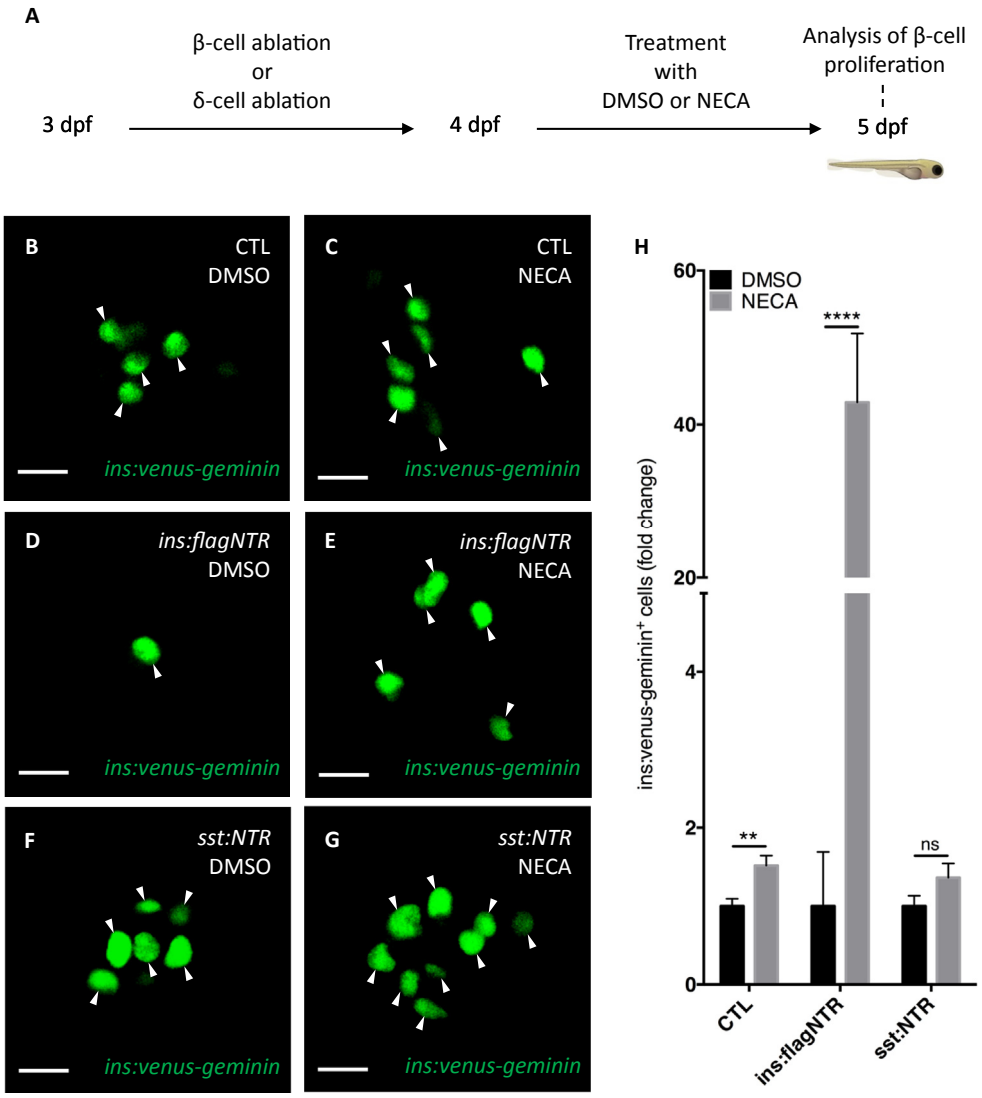

Figure 1: Pharmacological activation of adenosine signaling promotes β-cell proliferation independently of an apoptotic microenvironment. (A) Schema of the analysis of β-cell proliferation in control zebrafish larvae and larvae subjected to β-cell ablation or δ-cell ablation. Ablation was carried out by exposing the nitroreductase (NTR)-expressing transgenics to metronidazole (MTZ) from 3 to 4 days post fertilization (dpf). Islets were analyzed by confocal microscopy at 5 dpf. (B–G) Representative pictures of proliferating β-cells (arrowheads) in islets of *Tg(ins:venus-geminin)* zebrafish treated with DMSO or NECA from 4 to 5 dpf in the presence or absence of β-cell ablation (D–E) or δ-cell ablation (F–G). β-cell ablation was achieved by crossing the *Tg(ins:venus-geminin)* zebrafish with *Tg(ins:flag-NTR)* zebrafish and treating them with MTZ; δ-cell ablation by crossing them with *Tg(sst:NTR)* zebrafish and treating them with MTZ. (H) β-cell proliferation in the absence of cell ablation (CTL), and after β-cell or δ-cell ablation. Each condition was normalized (DMSO = 1), allowing comparison of fold changes. Absolute numbers are shown in Figure S1. $n = 13–32$; scale bars: 10 μm; **$p < 0.01$; ****$p < 0.0001$.

promoters of β-cell regeneration in zebrafish, we found that the most potent 'hits' converged on agonism of the adenosine pathway and thereby promoted β-cell proliferation. These hits included the non-specific adenosine receptor agonist NECA, the adenosine kinase (Adk) inhibitor A-134974, and phosphodiesterase inhibitors. Adk inhibitors increase the levels of endogenous adenosine by preventing the degradation of adenosine, i.e. the phosphorylation of adenosine to AMP. Adk inhibitors were independently found to increase β-cell proliferation in a different screen for β-cell proliferation in rat β-cells [17]. Still unknown is whether endogenously produced adenosine regulates β-cell proliferation—either in the basal state or in states where there is a high demand for insulin.

Here, we show that adenosine signaling through the A2a receptor is required for compensatory β-cell proliferation in mice during pregnancy and is sufficient to promote proliferation of mouse β-cells *in vitro*. Together, our work suggests that adenosine signaling has an important role in generating β-cell homeostasis, a feature that could be therapeutically exploited to increase the number of β-cells in people with diabetes.

2. RESULTS

2.1. Pharmacological activation of adenosine signaling promotes β-cell proliferation independently of an apoptotic microenvironment

Using zebrafish models of β-cell proliferation and regeneration, we previously identified adenosine signaling as a mitogen important in β-cell proliferation during regeneration but not in β-cell proliferation during development [2]. We now sought to determine whether this differential effect is due to the apoptotic microenvironment that results from the ablation of β-cells or to the increased demand for insulin in

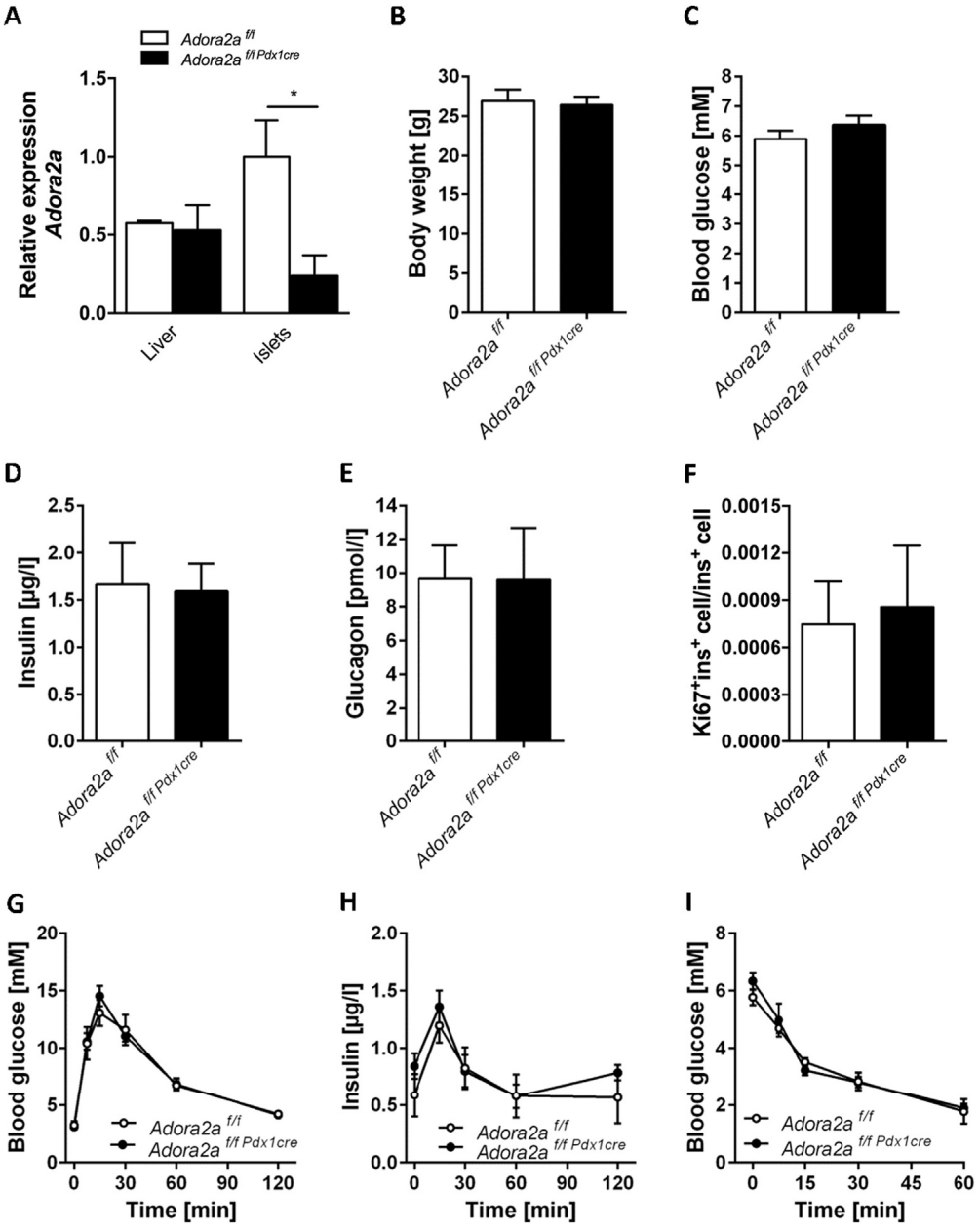

Figure 2: Deletion of *Adora2a* in the pancreas has no effect on glucose regulation and β-cell proliferation in the basal state. (A) Real-time PCR displays a significant reduction of *Adora2a*-expression in islets of *Adora2a*$^{f/f\ Pdx1cre}$ mice compared to controls. The expression of *Adora2a* in liver is not influenced by Pdx1-Cre mediated *Adora2a* deletion. (B–E) There are no differences in body weight (B), blood glucose (C), plasma insulin (D), or plasma glucagon (E), between female *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f}$ mice, at the age of 20 weeks. (F) Sections of pancreata were stained for insulin, glucagon and Ki67. The number of β-cells co-expressing Ki67 and insulin did not vary between female *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f}$ mice. (G–H) Oral glucose tolerance tests did not display any difference in blood glucose (G) or plasma insulin (H) between female *Adora2a*$^{f/f\ Pdx1cre}$ and control mice. (I) Insulin tolerance tests of female *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f}$ mice by intraperitoneal insulin application did not differ between the genotypes. Data represent mean ± SEM. $n = 4–12$; *$p < 0.05$.

the regeneration model. The regeneration model involves ablating the β-cells of zebrafish and observing the regeneration of these cells under different circumstances. Specific ablation of β-cells is achieved by using zebrafish, termed *Tg(ins:flag-NTR)*, that are genetically modified to express the bacterial enzyme nitroreductase (NTR) in β-cells. NTR can convert the prodrug metronidazole (MTZ) to a toxic compound, such that adding MTZ to the water containing the *Tg(ins:flag-NTR)* zebrafish leads to apoptosis of their NTR-expressing β-cells. To efficiently examine β-cell proliferation in zebrafish larvae, we used a reporter line that specifically marks proliferating β-cells, i.e. *Tg(ins:-venus-geminin)*, a system based on FUCCI (fluorescence ubiquitination cell cycle indicator) [18]. As expected, treating these transgenic zebrafish with the adenosine agonist NECA after ablation of their β-cells significantly increased β-cell proliferation, whereas such treatment had only a modest effect on β-cell proliferation in the absence of β-cell ablation (Figure 1A–E). However, when we used the same ablation system to target a different cell type, i.e. somatostatin-producing δ-cells rather than insulin-producing β-cells, we found that

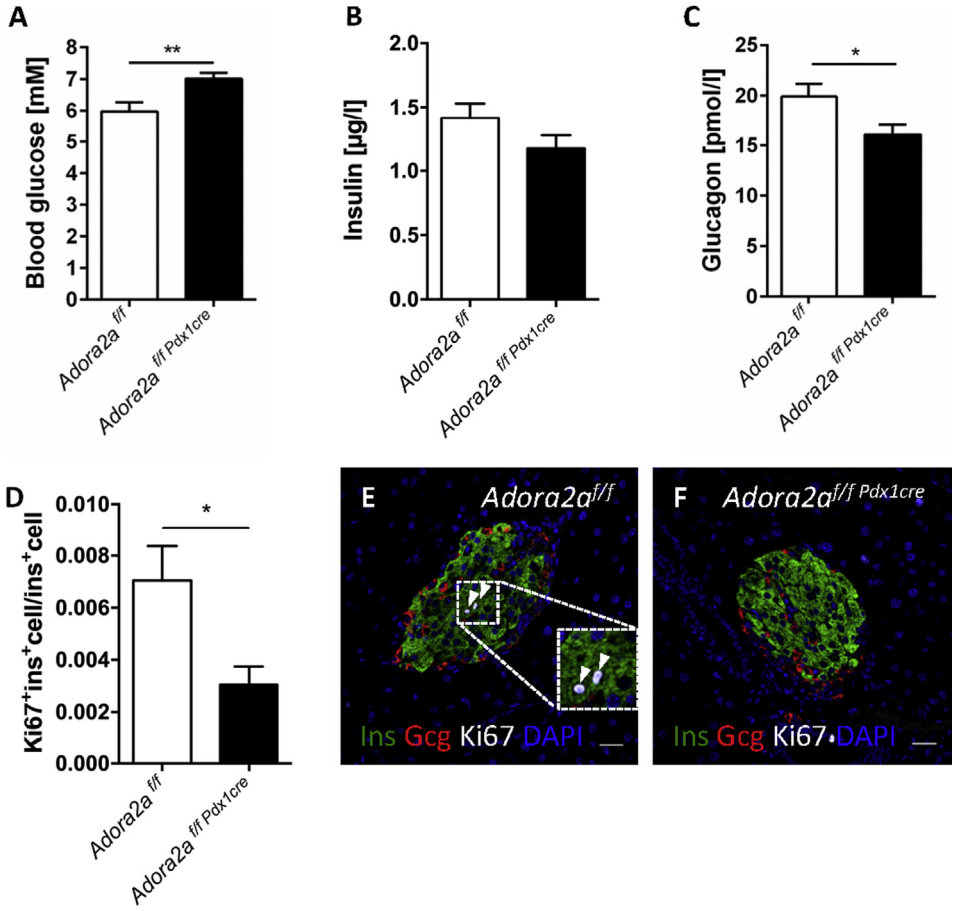

Figure 3: Deletion of *Adora2a* in the pancreas disrupts glucose regulation and β-cell proliferation in pregnant mice. (A–F) Pregnant *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f}$ mice were sacrificed on gestational day 13.5. Pregnant *Adora2a*$^{f/f\ Pdx1cre}$ mice displayed increased blood glucose levels (A), no significant change in plasma insulin (B), and a reduction of plasma glucagon (C), compared to controls. (D) Sections of pancreata were stained for insulin, glucagon, and Ki67. The number of β-cells co-expressing Ki67 and insulin was significantly reduced in *Adora2a*$^{f/f\ Pdx1cre}$ compared to *Adora2a*$^{f/f}$ mice. (E–F) Representative staining of pancreata from pregnant *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f}$ mice visualizing β-cell proliferation (arrowheads). Scale bars: 24 µm. Data represent mean ± SEM. $n = 5–9$; *p < 0.05; **p < 0.01.

NECA did not significantly induce β-cell proliferation in δ-cell-ablated larvae (Figure 1F–G). A direct comparison of the increase in β-cell proliferation (after normalization to control treatment) clearly visualizes the differential effect of NECA under the different ablative conditions (Figure 1H; for absolute numbers see Figure S1). Similar data were obtained when β-cell proliferation was analyzed after 1 or 2 days of regeneration (data not shown). Thus, we did not find any evidence for adenosine signaling being a more potent driver of β-cell proliferation in an apoptotic microenvironment.

2.2. Deletion of Adora2a in the pancreas has no effect on glucose regulation and β-cell proliferation in the basal state

We previously showed that knock-down of *adora2aa*, corresponding to the mammalian adenosine receptor *Adora2a*, abolished the ability of the non-specific adenosine agonist NECA to promote β-cell proliferation in zebrafish [2]. Therefore, we now sought to determine whether the A2a receptor has an endogenous role in regulating glucose levels and β-cell proliferation in mice. To do so, we deleted *Adora2a* in the whole pancreas by crossing a floxed allele of *Adora2a* with Pdx1-Cre (designated *Adora2a*$^{f/f\ Pdx1cre}$). This approach generated mice with a marked deficiency in *Adora2a* expression in islets but normal levels of *Adora2a* expression in the liver (Figure 2A). A comparison between female *Adora2a*$^{f/f\ Pdx1cre}$ mutant and control mice did not show any significant differences in body weight (Figure 2B), blood glucose levels (Figure 2C), plasma insulin levels (Figure 2D), plasma glucagon levels (Figure 2E), β-cell proliferation (Figure 2F), glucose tolerance (Figure 2G–H), or insulin tolerance (Figure 2I), i.e. in the absence of any challenges. Likewise, there was no difference between male *Adora2a*$^{f/f\ Pdx1cre}$ mutants and corresponding controls with regards to body weight, blood glucose levels, plasma insulin levels, plasma glucagon levels, or β-cell proliferation (Figure 2A–G). Together, these findings suggest that adenosine signaling through the A2a receptor in the pancreas does not regulate glycemia or β-cell proliferation in mice in the basal state.

2.3. Deletion of Adora2a in the pancreas disrupts glucose regulation and β-cell proliferation in pregnant mice

To examine whether adenosine signaling has a role in the homeostatic control of glycemia and β-cell proliferation when the demand for insulin is high, we studied pregnant *Adora2a*$^{f/f\ Pdx1cre}$ mutant mice at gestational day 13.5 (G13.5), a time at which the compensatory β-cell proliferation associated with pregnancy is at its peak [11]. We found that pregnant *Adora2a*$^{f/f\ Pdx1cre}$ mutant mice had significantly higher levels of glucose in their blood than pregnant control mice (Figure 3A), despite having comparable levels of insulin (Figure 3B), and significantly lower levels of glucagon (Figure 3C). That the *Adora2a*$^{f/f\ Pdx1cre}$

mice had lower levels of glucagon is consistent with A2a's role in promoting glucagon secretion [23]. Moreover, histological analysis showed that there was a marked reduction in β-cell proliferation in pregnant *Adora2a* mutant mice, as indicated by the number of Ki67-positive β-cells in the pancreas (Figure 3D). The overall histology of the islets was otherwise comparable in pregnant *Adora2a* mutant mice and pregnant controls (Figure 3E–F). Thus, adenosine signaling regulates glucose and hormone levels, as well as compensatory β-cell proliferation during pregnancy, i.e. several intertwined factors that are central for generating homeostasis in pregnancy.

2.4. Adenosine signaling increases β-cell proliferation in mouse islets *in vitro*

We next assessed the effect of adenosine signaling through A2a on β-cell proliferation in cultured mouse islets. Interestingly, the baseline level of β-cell proliferation was markedly lower in cultured islets derived from *Adora2a*$^{f/f\ Pdx1cre}$ mutant mice than in those derived from control mice (Figure 4A–C). To distinguish between an islet-autonomous and a β-cell-autonomous role for adenosine signaling in β-cell proliferation, we deleted *Adora2a* specifically in β-cells by crossing the floxed allele of *Adora2a* with mice carrying cre-recombinase under the control of the mouse insulin promoter, thus generating *Adora2a*$^{f/f\ MIPcre}$ mice. Like islets from mice lacking *Adora2a* in the whole pancreas, islets isolated from mice lacking *Adora2a* specifically in β-cells had a marked reduction in basal levels of β-cell proliferation (Figure 4D–F). Together, these data suggest that adenosine signaling through A2a has a direct effect on β-cells, an effect necessary for stimulating β-cell proliferation and maintaining a normal basal level of β-cell proliferation in islets grown *in vitro*.

We next examined whether pharmacological agonism of the A2a receptor is sufficient to induce β-cell proliferation. We compared the effects of the non-specific agonist NECA, which activates all four of the mammalian adenosine receptors, and those of the A2a-specific agonist UK432097 on β-cell proliferation in islets isolated from wild-

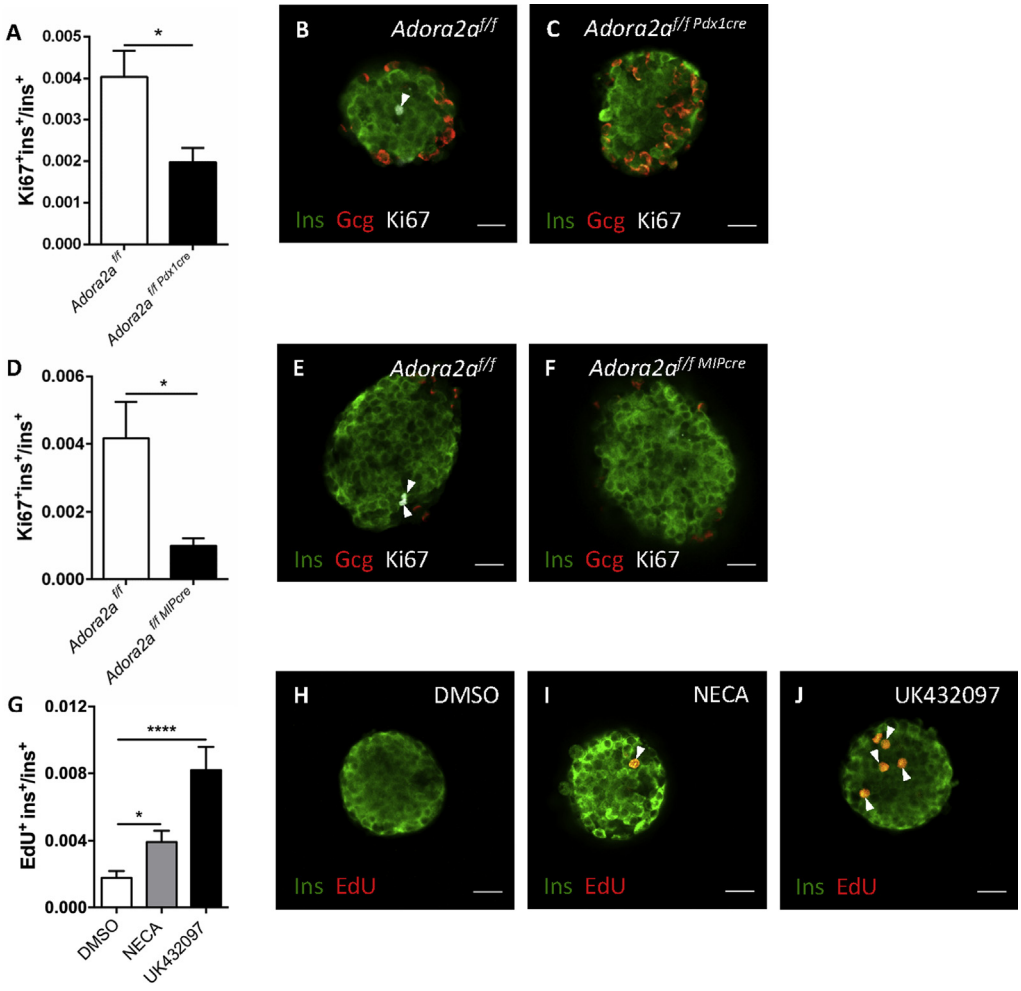

Figure 4: Adenosine signaling increases β-cell proliferation in mouse islets *in vitro*. (A) Isolated islets of female *Adora2a*$^{f/f\ Pdx1cre}$ mice display a reduction in the number of proliferating β-cells co-expressing Ki67 and insulin, compared to control islets. (B–C) Representative stainings of islets from *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f}$ mice. Scale bars: 24 μm. (D–F) The β-cell-autonomous effect of *Adora2a* was determined by β-cell-specific mutagenesis, using MIP-CreER (referred to as *Adora2a*$^{f/f\ MIPcre}$). Isolated islets of *Adora2a*$^{f/f\ MIPcre}$ mice display a reduction in the number of proliferating β-cells co-expressing Ki67 and insulin, compared to control islets. (E–F) Representative stainings of islets from *Adora2a*$^{f/f\ MIPcre}$ and *Adora2a*$^{f/f}$ mice visualizing β-cell proliferation (arrowheads). Scale bars: 24 μm. (G–J) Isolated islets from wild-type mice were incubated for 72 h with DMSO, NECA (10 μM), or UK432097 (1 μM) in the presence of EdU, to determine the proliferative capacity of the different adenosine agonists. (G) Quantification of β-cell proliferation was performed following staining for EdU, insulin and nuclei (DAPI). Representative stainings of islets incubated with DMSO (H), NECA (I), or UK432097 (J), visualizing β-cell proliferation (arrowheads). Scale bars: 24 μm. Data represent mean ± SEM. *$p < 0.05$; ****$p < 0.0001$.

type mice. UK432097 potently increased β-cell proliferation, whereas NECA did not increase β-cell proliferation to the same extent (Figure 4G–J). These results suggest that an increase in β-cell proliferation is better achieved by stimulating specifically the A2a receptor, rather than all adenosine receptors.

3. DISCUSSION

In this study, we uncover a critical role for adenosine signaling in regulating β-cell homeostasis. By studying β-cell regeneration in zebrafish, compensatory β-cell proliferation in pregnant mice, and β-cell proliferation in cultured mouse islets, we show that both exogenous adenosine receptor agonists and endogenous adenosine signal via the A2a receptor to regulate β-cell homeostasis. These findings pave the way for the development of A2a-activating drugs that can increase β-cell proliferation.

While others found that ATP regulates homeostasis of β-cell proliferation [19], we show that adenosine signaling is important for both β-cell regeneration [2] and compensatory β-cell proliferation (this study). ATP is known to regulate the β-cell mass through effects on β-cell polarization; glucose catabolism increases intracellular ATP levels, which leads to closure of K-ATP channels and depolarization of β-cells, and hence to an increase in β-cell proliferation [19]. It is possible that ATP and adenosine work in concert in regulating homeostasis of the β-cell mass. Indeed, because ATP degrades to form adenosine, adenosine may in fact mediate part of the effect attributed to ATP, because ATP is co-secreted with insulin and degrades to form adenosine.

We show that adenosine has only a minor role in regulating β-cell proliferation under basal conditions but a major one in regulating the β-cell proliferation when there is great demand for insulin secretion (which is coupled to ATP secretion and hence adenosine production), such as during pregnancy in mice or β-cell depletion in zebrafish. Our finding that basal β-cell proliferation is dependent on A2a-adenosine signaling in cultured islets (which have no insulin resistance or β-cell depletion) but not *in vivo* (also in the absence of insulin resistance or β-cell depletion) suggests that there are additional signals *in vivo* that can promote basal β-cell proliferation and compensate for the absence of A2a-adenosine signaling. However, this can be different when there is high demand for insulin. For example, in insulin-resistant states, adenosine could possibly synergize with other factors regulating β-cell proliferation. In such a scenario, insulin resistance induces the secretion of both insulin (and hence ATP) from the β-cells and growth factors, e.g. SerpinB1, from the liver [20]. ATP then degrades to adenosine, which synergizes with the secreted growth factors in activating a network of downstream mediators promoting β-cell proliferation. Signals that regulate homeostasis of the β-cell mass in insulin-resistant states could potentially be exploited therapeutically for the regeneration of the β-cell mass in people with diabetes.

Mammals have four adenosine receptors, A1, A2a, A2b, and A3. A1 and A3 couple primarily to Gαi and thus decrease intra-cellular levels of cAMP, whereas A2a and A2b couple primarily to Gαs and thus increase levels of cAMP. Zebrafish, however, have twelve adenosine receptors as a result of genomic duplications. Through *in vitro* studies with an A2a-specific agonist and *in vivo* studies with mice lacking A2a in their pancreas or β-cells, we found that it is the A2a receptor that mediates β-cell proliferation in response to adenosine signaling. Furthermore, we show that A2a-specific agonists have a stronger effect on mouse β-cell proliferation than NECA, perhaps because NECA also activates the A1 and A3 receptors, which, in turn, activate Gαi and thereby counteract the downstream effects of A2a signaling (which is mediated by Gαs). This explanation is also consistent with findings showing that adenosine signaling is affected by phosphodiesterases (which degrade cAMP) and how adenosine promotes the basal level of β-cell proliferation *in vitro* [2,21,22]. Moreover, NECA was recently shown to increase human β-cell proliferation [24]; it would be interesting to examine whether A2a-specific agonists are more potent than non-specific adenosine agonists in increasing β-cell proliferation in human islets grown *in vitro* or transplanted to immunodeficient mice. Knowing which receptor mediates adenosine's effects on β-cell regeneration and proliferation in mammalian models is critical for potential future therapeutic development.

Adk inhibitors increase the levels of endogenous adenosine by preventing the degradation of adenosine, i.e. the phosphorylation of adenosine to AMP. Recent findings suggest that the adenosine pathway might also be important for glucose control in humans. Intriguingly, 9 of 11 individuals with homozygous null mutations in *ADK* have recurrent hypoglycemia [25], some already at birth—a finding that may reflect an increase in β-cell proliferation and β-cell mass in these individuals. ADK serves to degrade adenosine; indeed, several of these individuals with homozygous null mutations in *ADK* have abnormally high levels of adenosine in their urine, most likely reflecting a similar increase in levels of adenosine in their blood. Moreover, a similar link was reported in a child with hypoglycemia: an association between an activating mutation in glucokinase (which increases ATP and hence adenosine levels) and the presence of hypoglycemia, unusually large islets and β-cell proliferation [26]. Together with our findings, these observations suggest that adenosine is central to β-cell homeostasis and glucose control, as it is in zebrafish and mice.

β-cell regeneration, as well as compensatory increases in β-cell number, can occur through different mechanisms—e.g. proliferation [27], neogenesis [28], and reprogramming [4]. In this regard, our previous unbiased screens have identified enhancers of β-cell regeneration through different mechanisms; e.g. adenosine signaling promotes β-cell regeneration via increasing β-cell proliferation [2], whereas IGFBP1 promotes β-cell regeneration via increasing α-to-β-cell transdifferentiation [29]. Together, this line of research highlights the importance of performing unbiased screens for identification of potent endogenous pathways and mechanisms that can increase the number of β-cells, as well as translating initial phenotypic findings in discovery models to functional outcomes in mice and ultimately humans.

4. MATERIALS AND METHODS

4.1. Zebrafish experiments

All studies involving zebrafish were performed in accordance with local guidelines and regulations and approved by Stockholms djurförsöksetiska nämnd. The following previously published transgenic zebrafish lines were used: *Tg(ins:Flag-NTR)*^{s950} [2], *Tg(sst2:NTR,cryaa:Cerulean)*^{KI102} [29] and *Tg(ins:Venus-zGeminin; cryaa:Venus)*^{KI107} [29]. We ablated β- or δ-cells in *Tg(ins:Flag-NTR)* or *Tg(sst:NTR)* zebrafish larvae by incubating the larvae in E3 supplemented with 10 mM metronidazole (MTZ; Sigma–Aldrich), 1% DMSO (VWR), and 0.2 mM 1-phenyl-2-thiourea (PTU; Acros Organics) from 3 to 4 days post fertilization (dpf).

4.2. Fluorescent imaging of zebrafish larvae

Fluorescent imaging was performed according to standard procedures and analyzed with a Leica SP8 confocal microscope. The whole endocrine portion of the pancreas was scanned in all larvae analyzed. Confocal stacks were analyzed with ImageJ software. β-Cell proliferation was assessed by counting the number of β-cells marked by *Tg(ins:venus-geminin)*.

4.3. Mouse experiments

All studies involving mice were performed in accordance with local guidelines and regulations and approved by Stockholms djurförsöksetiska nämnd. To delete *Adora2a* in the pancreas (thus generating mutant mice termed *Adora2a*$^{f/f\ Pdx1cre}$), we crossed *Adora2a*^{tmDyj} mice (Jackson Laboratory) [30] with mice carrying cre-recombinase under the control of the mouse *Pdx1* promoter (Tg(Pdx1-cre)89.1Dam/Mmucd) mice [31]. To delete *Adora2a* specifically in the β-cells of mice (thus generating *Adora2a*$^{f/f\ MIPcre}$ mice), we crossed *Adora2a*^{tmDyj} mice with mice carrying cre-recombinase under the control of the mouse insulin promoter (B6.Cg-Tg(Ins1-cre/ERT)1Lphi/J) [32]. *Adora2a*$^{f/f}$ from respective breeds was used as control mice, i.e. mice that do not carry cre-recombinase but in which both *Adora2a* alleles are floxed. Ideally, one should also examine mice carrying only the Cre lines as independent controls. However, we find that deletion of *Adora2a* has the same effect on basal β-cell proliferation in cultured islets from two different Cre lines, suggesting that the *Adora2a*-mutant phenotype we see is not an artefact arising from the integration of Cre.

Genotyping of mice was done by PCR using the following primers: *Adora2a*, forward primer 5′-GGGCAAGATGGGAGTCATT-3′, reverse primer 5′-ATTCTGCATCTCCCGAAACC-3′; and cre forward primer 5′-GCG GTC TGG CAG TAA AAA CTA TC-3′, reverse primer 5′-GTG AAA CAG CAT TGC TGT CAC TT-3′. To activate the cre-recombinase in *Adora2a*$^{f/f\ MIPcre}$ mice, we gave them an oral gavage of 100 μl of 20 mg/ml tamoxifen (T5648, Sigma—Aldrich) once per day for 5 days; control *Adora2a*$^{f/f}$ mice were treated in the same way. The tamoxifen was dissolved in 10% v/v ethanol and 90% v/v corn oil. Recombination efficiency was assessed by crossing *Tg(Ins1-cre/ERT)1Lphi/J)* with a transgenic reporter mouse *Tg(Rosa-CAG-LSL-tdTomato-WPRE)* that expresses Tomato after recombination [33]): recombination occurred in 78% of β-cells (as analyzed in 3 independent mice). For analysis of the effects of pregnancy on the pancreas, pregnant mice were euthanized on day 13.5 of pregnancy. Mice were housed in standardized cages with *ad libitum* access to standard diet and water, and with a 12-hour day—night cycle. FreeStyle lite (Abbott) was used for monitoring blood-glucose values.

4.4. Glucose and insulin tolerance tests

To analyze glucose tolerance, we fasted mice overnight and then gave them 2 g glucose/kg bodyweight (49163, BioUltra glucose solution, Sigma—Aldrich) via oral gavage. Blood parameters were measured directly before the administration of glucose, as well as at 7.5, 15, 30, 60, and 120 min after the administration of glucose. To study insulin tolerance, we gave mice 1 IU insulin/kg body weight (Actrapid penfill insulin, Novo Nordisk) by intraperitoneal injection and measured blood-glucose levels before and 7.5, 15, 30 and 60 min after the insulin injection.

4.5. Measurement of plasma parameters

Blood samples were collected in EDTA-coated tubes (microvette, 16.444.100, Sarstedt) centrifuged for 10 min at 12,000 rpm, and the plasma supernatants were then collected for analysis. Enzyme-linked immunosorbent assays (ELISA) were used for determining insulin (#90080, Ultra-Sensitive Mouse ELISA Kit, Crystal Chem) and glucagon levels (10128101, Mercodia).

4.6. Isolating and *in vitro* culturing mouse islets

Wild-type C57Bl/6J mice (11—15 weeks old), *Adora2a*$^{f/f}$, *Adora2a*$^{f/f\ Pdx1cre}$ and *Adora2a*$^{f/f\ MIPcre}$ mice fed a standard diet were euthanized by cervical dislocation. Collagenase P (Roche), at 0.3 mg/ml in Hank's buffered salt solution, was injected into the common bile duct such that it was distributed to the whole pancreas. The collagenase-expanded pancreas was dissected out and digested in a collagenase P-containing solution for 10 min in a 37 °C water bath. After several washes, the islets were handpicked and incubated in RPMI 1640 media (Gibco) supplemented with 10% FBS (Gibco), 1% penicillin-streptomycin (Gibco), and 1% L-glutamine (Gibco) for 22 h at 37 °C in 5% CO_2. The islets were then incubated in RPMI 1640 containing either DMSO (VWR) alone as vehicle control or different concentrations of 5′-N-ethylcarboxamidoadenosine (NECA; 1691, Tocris bioscience) or UK432097 (1193, Axon Medchem BV) for 72 h. For analysis of the proliferation rate of mutant β-cells, 5-ethynyl-2′-deoxyuridine (EdU, C10640, Life Technologies) was added to the incubation media during the one day in culture.

4.7. Immunofluorescence staining of mouse pancreata and cultured mouse islets

Freshly isolated mouse pancreata were fixed in 4% formaldehyde, dehydrated, embedded in paraffin, and sectioned. Sections from three different levels of the pancreas were used for staining for insulin, glucagon, Ki67 and nuclei (DAPI), as described for mouse islets grown *in vitro*. Quantification of islet cells was performed with ImageJ. All nuclei (DAPI$^+$), insulin-positive (ins$^+$), glucagon-positive (gcg$^+$), and Ki67$^+$ cells, as well as double-positive cells expressing insulin and Ki67 were counted manually. All islets with more than four ins$^+$ cells were analyzed.

Mouse islets cultured *in vitro* were fixed in 4% formaldehyde for 20 min, washed twice in PBS supplemented with 2.5% bovine serum albumin and 10 mM NaN_3, and then permeabilized in a 0.3% Triton-X 100-PBS solution for 3 h. The islets were next incubated in blocking solution (5% BSA, 0.15% Triton-X100 in PBS) for one hour. Primary antibodies—guinea pig anti-insulin at a concentration of 1:600 (A0564, DAKO), mouse anti-glucagon at 1:200 (G2654, Sigma—Aldrich), and rabbit anti-Ki67 at 1:250 (ab15580, Abcam)—were added to the antibody diluent solution (1% BSA, 0.2% Triton-X100 in PBS) and the incubation continued overnight at 4 °C. Islets were then washed and incubated with Alexa Fluor® secondary antibodies (Life Technologies) and DAPI (D1306, Life Technologies) overnight. Islets treated with EdU were additionally subjected to a 1-hour incubation period with the EdU Click-iT development solution (Invitrogen). The stained islets were mounted in Vectashield® (Vector Laboratories Inc.) and imaged with a Leica SP8 or Zeiss LSM 800 confocal microscope. To calculate β-cell proliferation rate in mouse islets grown *in vitro*, we divided the number of ins$^+$Ki67$^+$ or ins$^+$EdU$^+$ cells in a single islet by the number of ins$^+$ cells in the same islet. In each treatment group, islets from 4 to 7 different mice were used.

4.8. RNA isolation, cDNA synthesis and real-time PCR

RNA was extracted from snap frozen liver by using PureLink® RNA Mini Kit (12183018A, Ambion, Carlsbad, CA, US), and from islets by using the RNAqueous®-Micro Total RNA Isolation Kit (1931, Ambion), according to the manufacturers' instructions. For transcribing RNA to cDNA, a master mix of SuperScript® IV Reverse Transcriptase (18090050, Thermo Fisher), Random Hexamers (N8080127, Thermo Fisher), RNaseOUT™ Recombinant Ribonuclease Inhibitor (10777-019, Thermo Fisher), and dNTPs (R0191, Thermo Fisher) was prepared according to manufacturers' instructions. For quantitative analysis of *Adora2a* expression, a master mix of 12.5 ng cDNA, 10 μM primer and iTaq™ Universal SYBR® Green Supermix (172 5124, BioRad) was prepared and analyzed with ViiA™ real-time PCR (Applied Biosystems).

Table 1 — Oligonucleotides used for *real-time PCR*.

Primer	5′- → -3′
Adora2a forward	TTCCACTCCGGTACAATGGC
Adora2a reverse	CGATGGCGAATGACAGCAC
B2m forward	CTGACCGGCCTGTATGCTAT
B2m reverse	TATGTTCGGCTTCCCATTCT
Tbp forward	CTGGAATTGTACCGCAGCTT
Tbp reverse	ATGATGACTGCAGCAAATCG
Actb forward	TGTGATGGTGGGAATGGGTCAG
Actb reverse	TTTGATGTCACGCACGATTTCC

TATA-box binding protein (Tbp; used in Figure 2A), Beta-2 microglobulin (B2m) and beta-actin (Actb) were processed as endogenous control genes. Data analysis was done as described previously [34] (Table 1).

4.9. Statistical analysis

Results are presented as mean values ± SEM. p Values < 0.05 were considered statistically significant. Statistical analyses were carried out by *t*-tests, ANOVA or Mann—Whitney *U* test as justified.

AUTHOR CONTRIBUTIONS

N.S. performed the studies of mutant mice, isolated mouse islets, and analyzed data. K.C.L. performed zebrafish experiments. J.C., C.M., L.T. and D.T. performed mouse experiments. O.A. conceived the study, analyzed data and wrote the manuscript, with help from all authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.09.006.

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