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Beta-cell regeneration: lineage tracing models

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ABSTRACT

Background

Several immunotherapies have been able to restore normoglycaemia in newly diabetic NOD mice. However, the mechanism behind restoration of the functional beta-cell mass, either through replication, functional recovery or neogenesis of beta cells, remains unclear. Furthermore, previous rodent studies have been conducted in non-autoimmune models: the role of the ongoing islet autoimmunity in this process is unknown. Developing cell lineage tracing models in mice that spontaneous develop an auto-immune form of diabetes could be of value in addressing the regenerative capacity of beta cells in type 1 diabetes.

Methods

We combined the rat insulin promoter with conditional Cre-mediated expression of red fluorescent protein (RFP) in mice of a Non Obese Diabetic (NOD) background. In NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice, absence of Doxycyclin should result in beta cell RFP expression. Alternatively, in NOD.RIPCreER.ROSA-tdRFP mice, beta cells are programmed to express RFP upon tamoxifen treatment. Finally, we combined RFP reporter mice with the rat glucagon promoter for unconditional RFP- expression in alpha cells of NOD.GluCre.ROSA-tdRFP mice.

Results

The individual transgenes did not interfere with diabetes susceptibility. NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice brightly and reliably express RFP in a high percentage (93.0% ± 1.3) of their beta cells in the total absence of Doxycyclin. Presence of Doxycyclin in food and drinking water from birth onwards, adequately suppressed RFP expression (94.3% ± 1.7). NOD.RIPCreER.ROSA-tdRFP beta cells express RFP in an equal high percentage (94.5% ± 1.3) upon tamoxifen treatment. NOD.GluCre.ROSA-tdRFP mice express RFP in a smaller but significant percentage of their alpha cells (58.0% ± 6.0).

Discussion

One of the key goals of future treatments for T1DM is abrogation of auto-immunity combined with restoration of beta cell mass. Our cell lineage tracing models add a novel tool to murine beta-cell regeneration research. These models could be used to address the origin of insulin producing cells after immune-intervention in preclinical studies. Furthermore these models could reveal the role of ongoing autoimmunity on beta-cell regeneration.

BACKGROUND

T1DM is a chronic disease caused by autoimmune mediated destruction of insulin producing beta-cells in the islets of Langerhans in the pancreas. This causes a decrease in beta-cell function and eventually leads to life-long insulin-dependency. Currently there is no cure; treatment consists of insulin administration to mimic beta-cell function. Despite intensive insulin regimes, T1DM still contributes to substantial morbidity and mortality. It is estimated that at diagnose of T1DM, 40-90% of the beta cells have been destroyed. Among the number of issues that need to be addressed in T1DM research, finding a way to put the immune attack to a halt and finding a way to restore beta cell mass/ function are key issues. One way of partially restoring beta cell mass is via replacement with allograft pancreatic tissue, either whole pancreas, or isolated islets of Langerhans. Currently different immune-suppressive strategies are being investigated for prolonged graft survival. However, the worldwide shortage of pancreas/ islet allografts combined with transplantation-related side effects, fuels the exploration of the beta-cell regenerative capacity [1].

There is evidence -both in rodents and humans- that beta cells are able to adapt their cell mass to various physiological and pathological conditions. In NOD mice, beta-cell proliferation initially increases during islet inflammation, even prior to significant changes in beta-cell area or glucose levels [2]. During pregnancy, beta-cell mass in rodents increases 2,5-5 times, resulting from both beta-cell hypertrophy and an increased number of beta-cells suggesting beta-cell replication [3,4]. In humans, older autopsy studies of pregnant women have revealed similar findings, with a significant increase in beta-cell mass (2.4x) with expansion of both islet size and number of beta-cells per islets [5]. However, a more recent study showed beta-cell mass does increase to a lesser extend (1.4x) with an increased number of small new cells, indicative of neogenesis rather than beta-cell replication. [6]. In experimental animal models, with partial or near-total destruction of the endocrine pancreas, regeneration of the beta-cells is seen as to different extends. Nir et al. show beta-cell replication as the main source of new beta-cells after 70-80% chemical beta-cell ablation by diphtheria toxin in a cell lineage tracing model [7]. Xu et al show that endogenous beta-cell progenitors can be activated in the mouse pancreas after partial duct ligation [8]. Near-total chemical ablation by diphtheria toxin in an alternative cell lineage tracing model revealed evidence for alpha-cell dedifferentiation [9]. Differences in outcome may be related to differences in experimental models, particularly concerning the percentage of beta-cell destruction as regenerative stimuli used in different models may be insufficient to trigger a neogenesis pathway. Of note, these studies have been performed on mice on a non-autoimmune background.

The availability of therapies that decrease insulin requirements and normalize glycated haemoglobin in T1DM patients [10,11,12] and that can fully reverse hyperglycemia in newly diagnosed diabetic NOD mice [13,14] raises the question of how functional beta-cell mass could be restored. For over a century, replication of pre-existing beta-cells and

islet neogenesis, either via transdifferentiation of non-beta cells, or via differentiation of progenitor/ stem cells have been proposed mechanisms of beta-cell regeneration [15,16]. More recently, beta-cell recovery of exhausted cells has been additionally suggested. A prospective phase 1-2 study in recent onset T1DM patients undergoing autologous non-myeloablative hematopoetic stem cell transplantation showed prolonged insulin independency compared to the natural course of the disease. [17]. The mechanism behind the increased beta-cell function allegedly does involve recovery of pre-existing beta-cells but this interpretation may not be sufficient to explain remission lasting more than seven years. A recent study in T2DM patients found double positive endocrine cells (insulin and glucagon/somatostatin) suggesting some degree of beta-cell neogenesis as a compensatory mechanism in newly diagnosed T2DM patients [18]. Although the classical pathophysiology of T2DM clearly differs from T1DM, some overlay in beta-cell inflammation with subsequent apoptosis of beta cell between the two conditions is being found. Spijker et al. demonstrated via cell lineage tracing studies in vitro, conversion of human beta-cells into glucagon producing alpha-cells, emphasizing human endocrine cell plasticity [19].

In the earliest beta-cell regeneration studies, islet cell regeneration was assessed from a morphological point of view. The development of techniques indicating islet cell proliferation, such as 3-H-thymidine incorporation and BrdU labelling, has been a step forward in addressing this issue in preclinical models [15]. However the true origin of new insulin-producing cells remains difficult to prove. The technique of cell lineage is based on the inheritable labelling of individual islet cells [20]. In T1DM research this means that the islet cell of interest, for instance the alpha-, beta- or duct cell can be marked in such a way that this mark will be transferred to its progeny.

Development of the Cre/loxP recombination system has had important implications for lineage tracing studies in mice. Cre recombinase is a small, bacteriophage P1-derived integrase that catalyses defined DNA recombination events between specific target sites, termed loxP (locus of crossover [x] in P1) [21]. The result of Cre-mediated recombination between two loxP sites depends on their specific orientation relative to another: a DNA sequence flanked by two directly repeated loxP elements is cut out as a circular molecule. In contrast, DNA flanked by two oppositely oriented sites will be inverted. By combining Cre/loxP with cell regulatory elements and a reliable and clear reporter, cells can be permanently and heritably marked. By using promoters responsive to the presence or absence of a certain drug, this labelling can be induced (conditional expression).

Cre/loxP systems have been combined with different reporters. Nir et al report approximately 20% beta-cell labeling with Human Placental Alkaline Phosphatase upon Tamoxifen treatment [7]. In a previous publication this group reports 30% labeling of beta cells [22]. Thorel et al report 95% Yellow Fluorescent Protein (YFP) labelling of beta-cells and a 90% labelling efficiency of alpha-cells, using the Rosa-26 YFP reporter [9].

Previous preclinical studies have been conducted in non-auto-immune models: the role

of the ongoing islet autoimmunity on beta-cell regeneration is unknown. Developing reliable cell lineage tracing models in mice that spontaneous develop an auto-immune form of diabetes [23] could be of value in addressing the regenerative capacity of beta-cells in type 1 diabetes, especially when combined with immune intervention studies.

MATERIALS AND METHODS

Mice.

NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP

NOD.RIP-tTA/tet07-Cre mice (from our lab, unpublished data) express a tetracycline-dependent transactivator under the control of a Rat Insulin Promoter. Absence of a Tetracycline (Doxycycline) leads to expression of Cre recombinase in beta-cells (Fig. 1A). This model is also known as 'Tet-off', as the presence of tetracycline turns Cre recombinase expression off.

C57Bl/6.ROSA-tdRFP mice are a Red Fluorescent Protein (RFP) reporter strain [24]. A tandem-dimer RFP (tdRFP) was inserted in an anti-sense orientation relative to the ubiquitously expressed ROSA26 locus to diminish leaky reporter expression (Fig. 1B). We backcrossed the targeted allele onto the NOD background for at least 10 generations and then introgressed this allele into NOD.RIP-tTA/tet07-Cre mice to produce NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice. Presence of Cre recombinase under the Rat Insulin Promotor leads to expression of Red Fluorescent Protein in beta-cells.

NOD.RIPCreER.ROSA-tdRFP

NOD.RIPCreER mice (kind gift from D. Melton) express Cre recombinase fused to the Estrogen Receptor under the control of a Rat Insulin Promotor. Cre recombinase is initially kept in the cytosol of beta cells: nuclear translocation only occurs after (Estrogen-Receptor binding) Tamoxifen treatment (Fig. 1C). These mice were crossed with the RFP-reporter mice to produce NOD.RIPCreER.ROSA-tdRFP mice.

NOD.GluCre.ROSA-tdRFP

C57Bl/6.GluCre mice (kind gift from P. Herrera) were backcrossed with NOD mice for at least 10 generations. NOD.GluCre mice express Cre recombinase under the Rat Glucagon Promotor. Their alpha-cells constitutively express Cre recombinase. They were crossed with the RFP-reporter mice described above to produce NOD.GluCre.ROSA-tdRFP mice.

All mice were kept under specific pathogen free conditions. These studies were approved by the Faculty of Medicine's Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

Doxycyclin.

NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice were bred on irradiated Doxycyclin containing food (2.3 g/kg; Bio-Serv) and 1 mg/ml Doxycyclin containing drinking water as negative controls.

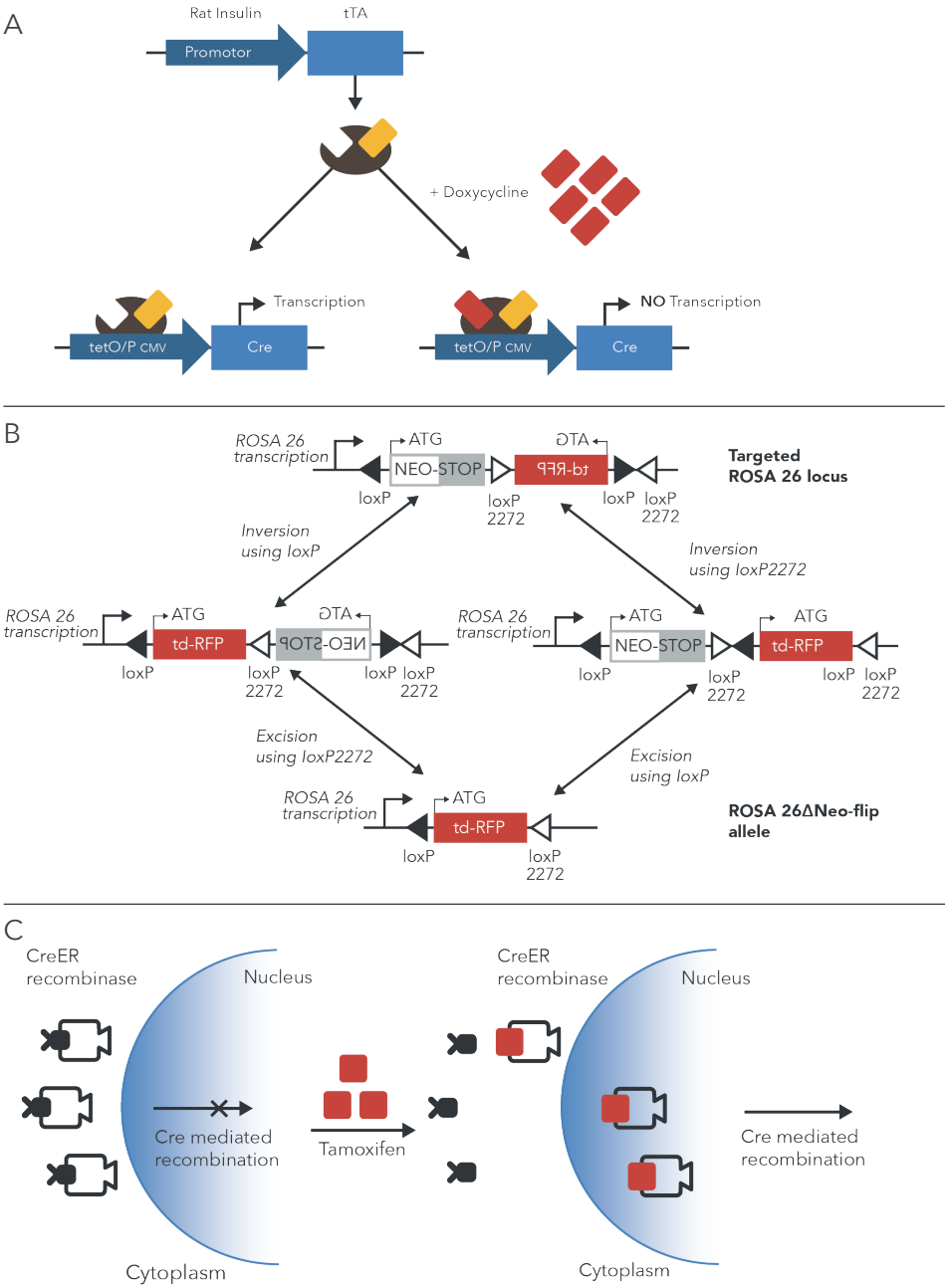

Fig 1 | A. Tetracycline transactivator (tTA) under a Rat Insulin Promoter (RIP). In the absence of Doxycycline, tTA binds to the tetO operator and drives expression of Cre recombinase in beta-cells. **B.** ROSA-tdRFP-reporter: tdRFP has been inversely inserted and is preceded by a stop-codon. Cre recombinase expression leads to removal of the stop-codon and correct orientation and expression of td-RFP [19]. **C.** Cre recombinase fused to the Estrogen Receptor (ER) under a Rat Insulin Promotor. Tamoxifen, which binds to ER, establishes nuclear translocation of Cre recombinase in beta-cells.

Tamoxifen.
Tamoxifen (Sigma) was dissolved in autoclaved corn oil 20 mg/ml containing 10% EtOH 100% at 37 °C using sonication. Tamoxifen was injected intraperitoneal (5 x 4 mg).

Immunopathology.
Pancreases were fixed in 3% paraformaldehyde for two hours before freezing. They were extensively washed in PBS overnight and dehydrated using rising concentrations of sucrose in PBS (5-10-20-30%). The tissue was subsequently frozen directly into Tissue TEC-OCT above ethanol/dry ice, and stored at -80 °C. Cryosections (5 µm) were fixed with 3% paraformaldehyde, stained with guinea pig anti-insulin antibodies and Alexa-488 goat-anti-guinea pig antibodies (Invitrogen) or goat-anti-glucagon antibodies (Santa Cruz Biotechnology) and Alexa 647 donkey-anti-goat antibodies (Invitrogen). The sections were mounted with Prolong gold (Invitrogen) and analyzed with an Olympus FV1000 confocal microscopy system.

Diabetes.
Diabetes was monitored by measuring urine glucose levels twice weekly. Animals were considered diabetic after two consecutive readings greater than or equal to 3+. The average blood glucose levels in mice diagnosed using these criteria are 22.0 ± 3.8 mmol/l, and none of these mice had blood glucose levels below 16 mmol/l.

Statistical Analysis.
Percentage of Cre expression in beta cells was estimated by dividing the amount of insulin+/RFP+ cells by the total amount of insulin+ cells per islet. Percentage of Cre expression in alpha cells was estimated by dividing the amount of glucagon+/ RFP+ cells by the total amount of glucagon+ cells per islet. Percentages are shown ± SEM. We analyzed ~15 islets per mouse and ~6 mice per strain. For NOD.GluCre.ROSA-tdRFP mice we analysed 4 islets per mouse and 4 mice in total.

RESULTS

The individual transgenes did not interfere with diabetes susceptibility.
The individual transgenes of both the conditional beta-cell tracing models and the unconditional alpha-cell tracing model, did not interfere with diabetes susceptibility. Diabetes incidence curves were comparable to wildtype NOD mice (Fig. 2).

NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP mice specifically express RFP in their beta cells.
Transgenic NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice reliably and brightly express RFP in 93.0% ± 1.3 of their beta-cells in the total absence of Doxycyclin, as shown by simultaneous insulin staining (Fig. 3A+B). Conversely, presence of Doxycyclin in food and drinking water of the mother from early pregnancy onwards, adequately suppressed RFP expression in litters (94.3% ± 1.7) (Fig. 3A+B).

In transgenic NOD.RIPCreER.ROSA-tdRFP mice we tested different Tamoxifen administration routes. Administration via gavage (20-20-10 mg) caused toxicity symptoms and was therefore discontinued. Tamoxifen (5 x 4 mg) administered intraperitoneal caused significant higher RFP expression than equal doses injected subcutaneously (results not shown). RFP expression in beta cells of NOD.RIPCreER.ROSA-tdRFP mice, as tested by simultaneous insulin staining, was comparable to our NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP model with 94.5% ± 1.3 of the beta-cells expressing RFP upon Tamoxifen treatment (Fig. 3C).

Initially we experienced difficulties confirming RFP expression. Comparing different tissue handling regimes, we discovered that the intracellular RFP protein is only maintained when pancreases are fixed in 3% paraformaldehyde prior to freezing. Fresh-freezing methods turned out to interfere with preservation of RFP expression.

No RFP expression was found in pancreatic non-beta cells of either NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP or NOD.RIPCreER.ROSA-tdRFP mice, as tested by simultaneous glucagon staining for alpha-cells (Fig. 3B). In addition, no RFP staining was found in non-endocrine tissue, as tested by confocal analysis of splenic and thymic tissue (data not shown).

NOD.RIPCreER.ROSA-tdRFP mice specifically express RFP in their alpha cells.
NOD.GluCre.ROSA-tdRFP mice unconditional express RFP in a smaller but significant percentage of their alpha-cells (58.0% ± 6.0 %) (Fig. 3D) as shown by simultaneous glucagon staining.

No RFP expression was found in pancreatic non-alpha cells of NOD.GluCre.ROSA-tdRFP mice, as tested by simultaneous insulin staining for beta-cells (Fig. 3D). In addition, no RFP staining was found in non-endocrine tissue, as tested by confocal analysis of splenic and thymic tissue (data not shown).

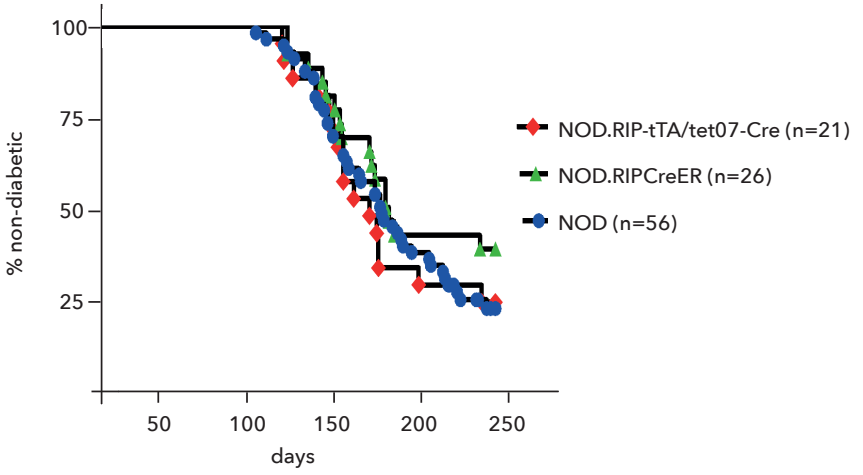

Fig 2 | T1DM incidence NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP mice are comparable to wildtype NOD mice.

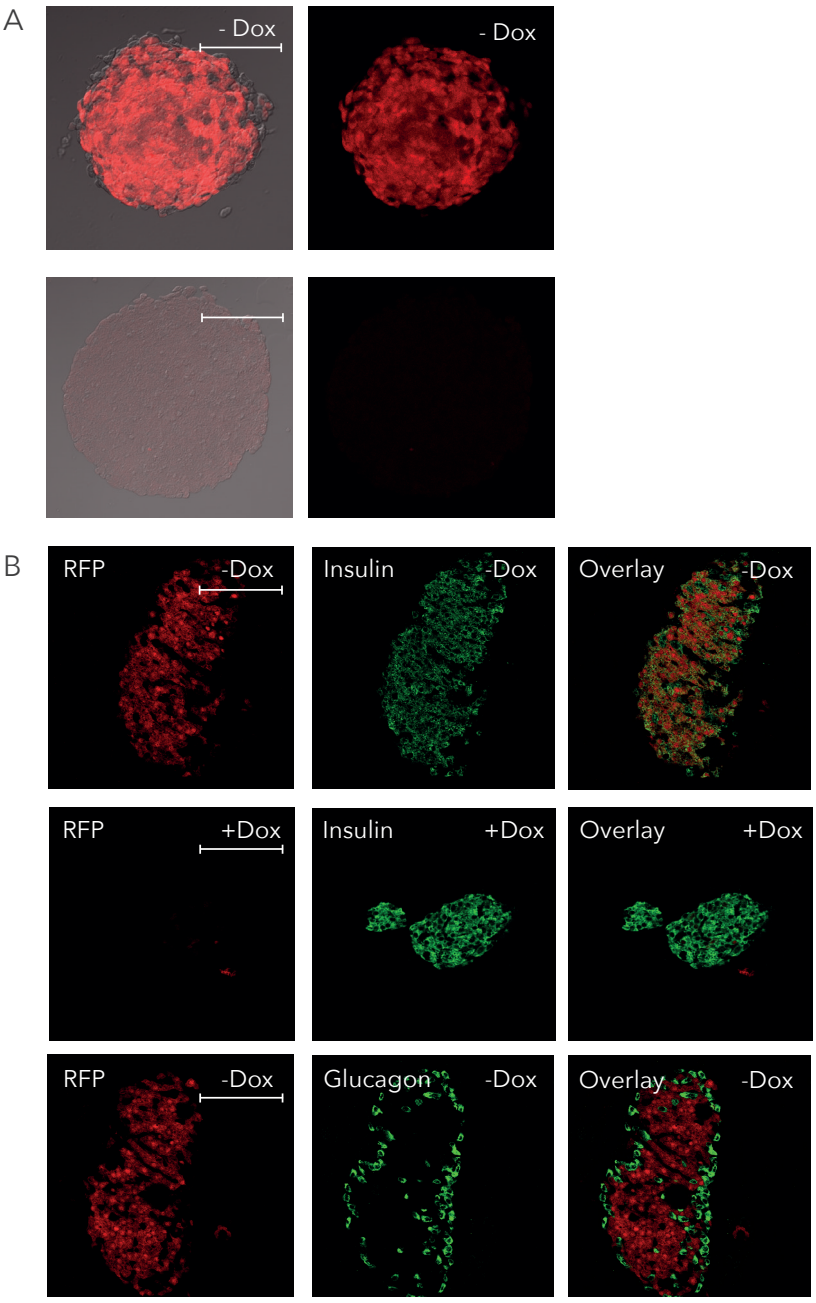

Fig 3 | A. Upper row: RFP expression in isolated islets of NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice in the absence of Doxycyclin (left: brightfield + Cy3, right: Cy3). Lower row: isolated islets of NOD control **B.** RFP expression, insulin (upper two rows, in green)/ glucagon (lower row, in green) staining and overlay in islet cryosections of NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice in the absence and presence of Doxycyclin.

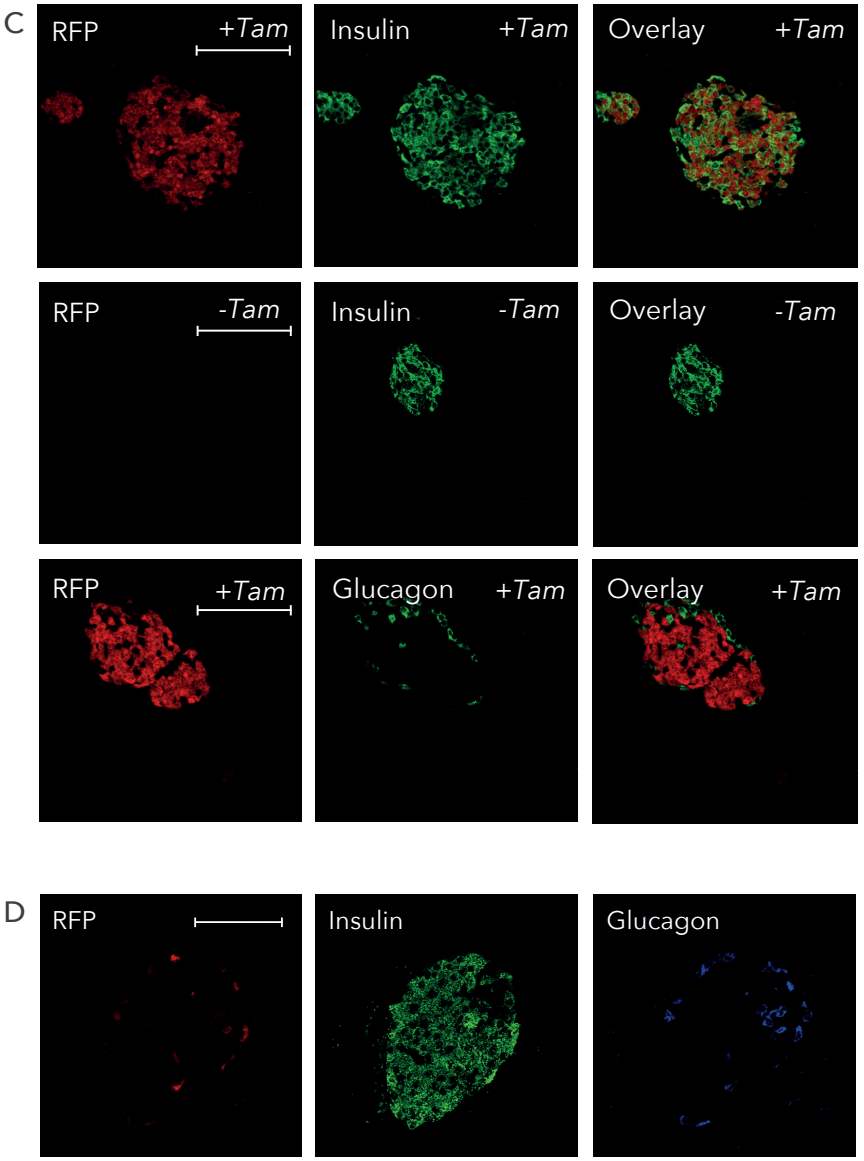

Fig 3 | C. RFP expression, insulin (upper two rows, in green)/ glucagon (lower row, in green) staining and overlay in islet cryosections of Tamoxifen treated NOD.RIPCreER.ROSA-tdRFP mice and untreated controls. **D**. RFP expression, insulin (green) and glucagon (blue) staining in islet cryosections of NOD. GluCre.ROSA-tdRFP mice.

DISCUSSION

One of the key goals of future treatments to cure T1DM is abrogation of auto-immunity combined with restoration of beta-cell mass. The availability of therapies that can restore beta-cell function/ mass initiated the search for the origin of these new insulin producing cells. In addition, the worldwide shortage of pancreas/ islet allografts combined with transplantation-related side effects [25], fuels the exploration of the beta-cell regenerative capacity.

Reliable cell lineage tracing models might help to distinguish between replication, functional recovery or neogenesis of beta-cells as predominant mechanism behind restoration of the functional beta-cell mass. Bright, inheritable labelling of pre-existing beta-cells (or pre-existing alpha-cells) in mice that spontaneously develop an auto-immune form of diabetes and are successfully treated with immune therapy, could help to unravel regeneration mechanisms, especially when combined with Bromodeoxyuridine (BrdU) labelling. This thymidine analogue can be incorporated in the newly-synthesized DNA of replicating cells. Upon successful immune-intervention in either NOD.RIP-tTA/ tet07-Cre.ROSA-tdRFP or Tamoxifen-treated NOD.RIPCreER.ROSA-tdRFP mice, finding RFP+/ insulin+/ BrdU+ cells would suggest replicating pre-existing beta cells as the predominant insulin producing source. RFP+/ insulin+/ BrdU- cells on the contrary would imply recovery of pre-existing beta cells whereas RFP-/ insulin+ cells would point in the direction of a non-beta cell source. In addition, finding RFP+/ insulin+ cells in NOD. GluCre.ROSA-tdRFP mice after successful immune intervention would suggest alpha-cells as the predominant source of new beta-cells, whereas RFP-/insulin+ cells would make this unlikely. Our current cell lineage tracing models only enable us to trace beta- and alpha-cells, if non-beta, non-alpha-cells are suggested as predominant source of new beta-cells, additional studies would be required. Due to neurotoxicity, continuous BrdU administration for longer periods of time is not considered safe. Pulse BrdU administration is a possible solution, with the downside of partial cell replication labelling.

The success of a murine cell lineage tracing model in T1DM research, depends on several aspects. First, none of the introduced transgenes should interfere with the T1DM incidence of the NOD mice. We have shown that Cre transgenes do not influence T1DM incidence. Second, Cre-mediated recombination should be cell type specific. Our immunohistochemistry studies indicate that RFP expression in our models is indeed limited to the cells that are driven by either an insulin or a glucagon promotor. And third, the efficiency of Cre-induced expression should be high. Compared to Cre-induced expression reported in the literature, both our NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP models, and to a lesser extend our NOD.GluCre.ROSA-tdRFP mice efficiently express RFP in their respective beta- and alpha-cells. With higher expression rates, less statistical assumptions have to be made on the origin or fate of the targeted cells. This expression efficiency is directly linked to the brightness and reliability of the tdRFP reporter that is being used.

We successfully generated cell lineage tracing models with bright, inheritable labelling of pre-existing beta- or alpha-cells in mice of a NOD background. These mice spontaneous develop autoimmune diabetes and could therefore be used as a tool in T1DM research. Furthermore these models could be used to assess the role of the ongoing islet autoimmunity on beta cell regeneration. We predict that ongoing auto-immunity prevents any form of regeneration emphasizing the need for successful immune strategies.

Although there is evidence in both rodents and humans that beta-cells are able to adapt to various conditions, prudence in translating results from mouse models is required. After partial or near-total destruction of the mouse pancreas in a non-auto-immune environment, regeneration of beta-cells is seen as to different extends [7,8,9]. Interestingly, no evidence for beta-cell proliferation or regeneration was found in a study performed on human pancreatic tissue collected from 13 patients who underwent partial (50%) pancreactomy [26]. Differences in outcome may be related to differences in the percentage of beta cell destruction, which might be insufficient to trigger a neogenesis pathway. In addition, chronic pancreatic inflammation was the underlying cause in the majority of the patients, which might influence beta-cell regeneration capacity. Another explanation however could be that regeneration capacity and regeneration pathways might differ between mice and men, emphasizing precaution in translation of results. There are a number of reasons why animal studies currently are being conducted to address the questions raised above. First of all, there is limited accessibility of human pancreases during the course of the disease. Second, we can only measure beta-cell functional responses and not beta-cell mass in patients. Third, we do not have reliable biomarkers to assess the auto-immune process in humans [27]. And fourth, as opposed to mice, we have not been able to cure diabetes yet in humans and therefore do not know what happens to beta cells when the immune attack is terminated. Awareness of model limitations and prudence in translating preclinical studies are in order. However cell lineage tracing studies might give some clues and guidance as to what regenerative pathways could be pursued.

Remaining islet cells have been histologically demonstrated in individuals suffering from T1DM, even for as long as 50 years. This not only emphasizes the heterogeneous course of T1DM but could also be seen as an argument in favour of beta-cell recovery possibilities [28,29].

Identifying possible sources of insulin producing cells after future successful immune intervention could have significant clinical implications. If pre-existing beta-cells are the only source of restored beta-cell mass, the residual beta-cell mass at the time of intervention is expected to predict the outcome of any immune intervention. On the contrary, if other cells serve as beta-cell precursors, outcome should be independent residual beta cell mass [21]. Currently, different strategies are being undertaken to create insulin producing beta-cells from stem cells (either embryonic stem cells or induced pluripotent stem cells) and from endocrine progenitors [30]. In addition to

this, identifying regenerative pathways could eventually result in developing strategies capable of enhancing effectiveness of promising immune therapies.

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Conflicts of interest: none

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