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# *All-trans* retinoic acid ameliorates glycemic control in diabetic mice via modulating pancreatic islet production of vascular endothelial growth factor-A

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ABSTRACT

Patients with type 1 diabetes mellitus are associated with impairment in vitamin A metabolism. This study evaluated whether treatment with retinoic acid, the biologically active metabolite of vitamin A, can ameliorate diabetes. *All-trans* retinoic acid (*at*RA) was used to treat streptozotocin (STZ)-induced diabetic mice which revealed *at*RA administration ameliorated blood glucose levels of diabetic mice. This hyperglycemic amelioration was accompanied by an increase in the amount of β cells co-expressed Pdx1 and insulin and by restoration of the vascular laminin expression. The *at*RA-induced production of vascular endothelial growth factor-A from the pancreatic islets was possibly the key factor that mediated the restoration of islet vascularity and recovery of β−cell mass. Furthermore, the combination of islet transplantation and *atRA* administration significantly rescued hyperglycemia in diabetic mice. These findings suggest that vitamin A derivatives can potentially be used as a supplementary treatment to improve diabetes management and glycemic control.



## 1. Introduction

Diabetes mellitus (DM), a syndrome of disordered metabolism, is a major public health problem affecting 422 million people worldwide [1]. It is one of the major causes of adult mortality and morbidity. Thus, there is an urgent need to develop strategies to improve the diabetes management and glycemic control. Vitamin A is an essential micronutrient [2]. Deficiency in vitamin An uptake is widely prevalent in developing countries and is possibly one of the major causes of fetal growth restriction, and subsequent risk of insulin resistance and glucose intolerance in adulthood [3]. Recent accumulating evidences suggest that vitamin A is a potent regulator of metabolism and alterations in vitamin A status contribute to the pathogenesis of obesity and diabetes [4]. This has increased the interest in developing pharmacological strategies for controlling the energy balance through natural and synthetic vitamin A derivatives [5].

The majority of type 1 diabetes mellitus (T1DM) is of the immune-mediated type in which the β-cell loss results from the T-cell mediated autoimmune attack [6]. Vitamin A is an essential component for maintaining immune tolerance and T-cell-mediated immunity [7,8]. Therefore, impaired vitamin A metabolism can affect the autoimmune response and development of T1DM [9]. Several clinical studies showed that the individuals with T1D had decreased circulating levels of vitamin A or RBP4 as compared to the non-diabetics [10–12]. Similarly, experimental models of T1DM in rodents have also shown that the development of diabetes is associated with a defect in vitamin A metabolism. For example, serum vitamin A and hepatic levels of retinyl-palmitate (RP) were significantly decreased in streptozotocin (STZ)-induced diabetic

*Abbreviations:* *At*RA, *all-trans* retinoic acid; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; VEGF-A, vascular endothelial growth factor-A; RAR, retinoic acid receptor.

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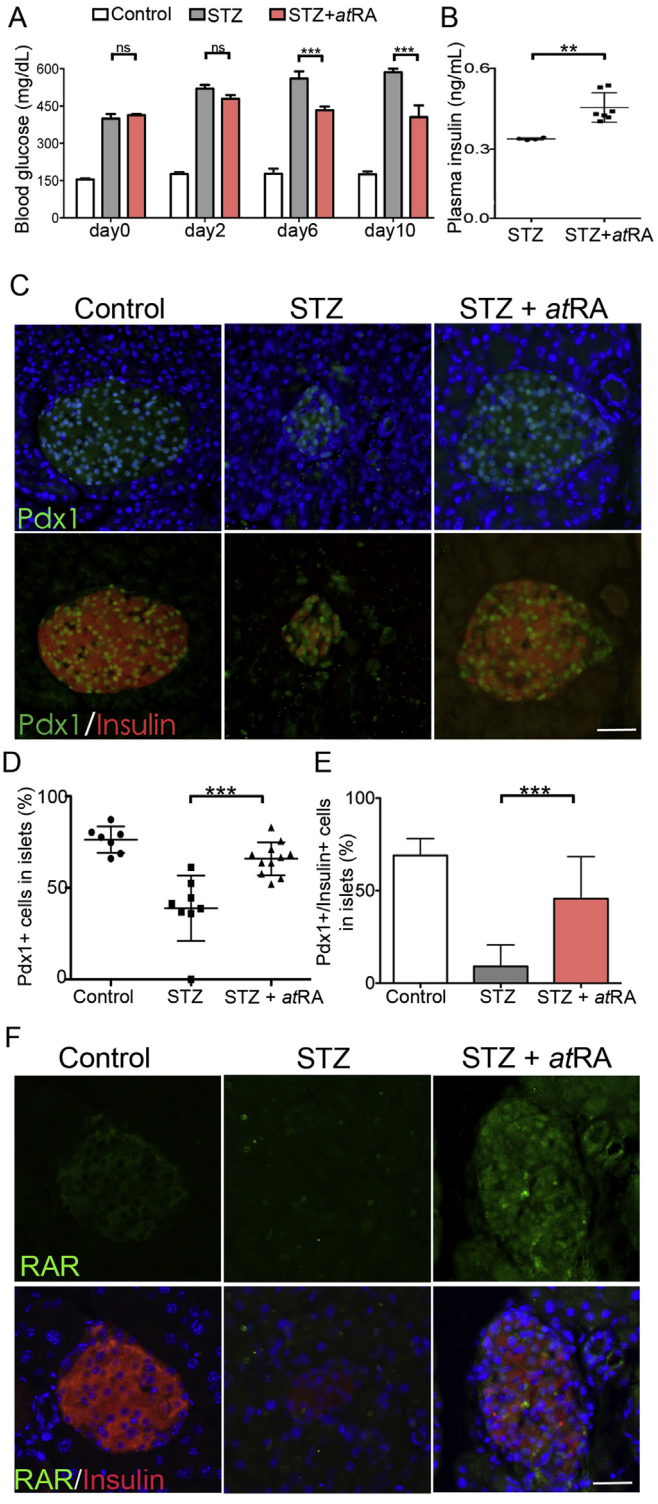


**Fig. 1. *At*RA treatment ameliorated glycemic control in the STZ-induced diabetic mice**. Non-diabetic (control) and STZ-induced diabetic CD-1 mice were treated with or without 1 mg/kg *at*RA every other day. (A) Fasting blood glucose levels of mice in control, STZ, and STZ + *at*RA groups were determined on day 0, 2, 6, and 10. Values are mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.005$ indicate statistically significant differences as compared to the STZ-treated group. (B) Plasma insulin levels of mice in control, STZ, and STZ + atRA groups were determined on day 10. Values are mean ± standard error per group. $^{**}p < 0.01$ indicates that the difference was statistically significant as compared to the STZ-treated mice. (C) The pancreas of control, STZ-treated, and STZ/*at*RA-treated mice was immunostained with anti-Pdx1 (green) and anti-insulin (red) antibodies. Nuclei were counterstained with Hoechst dye (blue). Scale bar, 50 μm. (D) The amount of Pdx1-positive cells and (E) Pdx1/insulin-double positive cells in an islet were calculated. Values are presented as percentage of the total insulin-positive cells in an islet and expressed as mean ± SD. $^{***}p < 0.005$ indicates statistically significant difference as compared to the STZ-treated mice. (F) The pancreas of control, STZ-treated, and STZ/*at*RA-treated mice was immunostained with anti-RAR (green) and anti-insulin (red) antibodies. Nuclei were counterstained with Hoechst dye (blue). Scale bar, 50 μm.

rats and biobreeding (BB) rats [10,13,14]. The findings imply metabolic availability of Vitamin A affects development of diabetes.

Vitamin A-rich foods have the potential of reducing the onset of autoimmune diabetes [15]. For example, it was shown that vitamin A deficiency caused hyperglycemia, loss of β-cell mass, and impairment in glucose-stimulated insulin secretion (GSIS); however, repletion with the biologically active metabolite of vitamin A, *all-trans* retinoic acid (*at*RA), restored GSIS and β-cell mass [16,17]. *At*RA has been shown to possess the capability to enhance insulin secretion and insulin content in the pancreatic β cells [18]. Therefore, the current study aimed to evaluate whether treatment with *at*RA can ameliorate diabetes. We examined if atRA administration can improve glycemic control in STZ-induced diabetic mice and evaluate whether treatment with *at*RA can enhance grafted islet survival and function.

## 2. Material and methods

### 2.1. Reagents

*All-trans* retinoic acid (*at*RA) and streptozotocin (STZ) were purchased from Sigma (St. Louis, MO). VEGF-A was obtained from R&D System Inc. (Minneapolis, MN). *At*RA, was dissolved in DMSO (Sigma) and added to the islet tissue culture at the concentrations indicated in the text.

### 2.2. Animal experiments

All animal experiments were approved by Academia Sinica Institutional Animal Care and Utilization Committee. Mice were housed under specific pathogen free conditions. Male CD-1 mice (6–8 week old) were purchased from BioLASCO Taiwan Co., Ltd. Diabetes was induced by five continuous injections of 40 mg/kg/day STZ. In some experiments, diabetic mice were intraperitoneally administered 1 mg/kg *at*RA every other day.

### 2.3. Immunofluorescence staining

The pancreatic tissues were dissected and fixed in 4% paraformaldehyde overnight at 4 °C. Tissue sections were rehydrated through a graded alcohol series, pretreated with sodium citrate antigen retrieval buffer (10 mM, pH 6.0), permeabilized with 1% (vol/vol) Triton X-100 (in PBS), and incubated in 2% blocking buffer (Roche) containing 0.1% Triton X-100, respectively. The tissue sections were then incubated with primary antibodies overnight at 4 °C followed by incubation with secondary antibodies (supplementary information) for 2 h in the dark at 25 °C. Subsequently, the tissue sections were mounted in Prolong Gold anti-fade reagent (Molecular Probes, Eugene, OR).

### 2.4. Pancreatic islet isolation

The pancreatic islets of 6–8 week old male CD-1 mice were isolated using collagenase P digestion, as previously described [19], and cultured in RPMI 1640 medium containing 11.1 mM glucose, 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% FCS (Invitrogen, Carlsbad, CA). *At*RA (1 μM) and anti-integrinα6

antibody (10 μg/mL, Millipore, Billerica, MA) were added from the second day of culture. All experiments were performed under low-light conditions to avoid retinoid photoisomerization.

### 2.5. Insulin and VEGF-A secretion assays

The blood samples were collected in heparinized tubes by cardiac puncture at the end of the experiment. The blood was centrifuged at 1500 *g* for 10 min at 25 °C, and the obtained plasma was aliquoted and stored immediately at −20 °C until assayed. All samples were thawed at 4 °C at the time of the enzyme-linked immunosorbent assay (ELISA) analysis. Plasma VEGF production was measured using VEGF-A ELISA kit according to the manufacturer's instructions (Raybiotech, Norcross, GA). Measurement of the plasma insulin was carried out with a mouse insulin ELISA kit according to the manufacturer's instructions (Mercodia, Uppsala, Sweden).

### 2.6. Measurement of insulin content

For each independent experiment, 10 islets were plated and exposed to the treatment conditions. Briefly, the islets were lysed in protein lysis buffer and the amount of whole islet protein was measured by BCA protein assay. The insulin content was determined with mouse insulin ELISA kit (Mercodia) and normalized to the total protein content.

### 2.7. Islet transplantation

After STZ treatment, the diabetic mice (blood glucose levels >350 mg/dL) were randomly assigned to three groups and were transplanted with islet mass (200 islets). At the time of transplantation, mice were anaesthetized with Avertin (tribromoethanol). A breach was made in the kidney capsule, and a polyethylene catheter was introduced through the breach along the kidney capsule to generate a subcapsular space. Freshly isolated islets were then injected through the catheter into the subcapsular space and implanted beneath the renal capsule on the dorsal side of the left kidney. After removing the catheter, the opening was cauterized, and the kidney was repositioned, followed by suturing of the muscles and skin [20,21]. At the end of the experiment, the graft-containing kidneys were removed and fixed in 10% (vol/vol) buffered formalin, dehydrated, and embedded in paraffin. Embedded tissues were consecutively sectioned at 5-μm thickness.

### 2.8. Determination of pancreatic Pdx-1, insulin, CD31 and laminin expression pattern

Pancreatic Pdx-1, insulin, CD31 and laminin expression pattern in the islets derived from each experimental group was determined with double immunofluorescence labeling. Images were captured using a Zeiss or Leica Confocal Microscope and analyzed by Metaphorph software (Universal Imaging, Downingtown, PA).

### 2.9. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test or one-way ANOVA followed by post hoc Bonferroni's multiple comparison test, as appropriate, using GraphPad Prism software. Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. AtRA administration ameliorates the blood glucose level of diabetic mice

Diabetes was induced in CD-1 mice by administering multiple low-doses of STZ. Half of the diabetic mice were intraperitoneally administered 1 mg/kg *at*RA every other day. Non STZ-treated/age matched mice were used as control. The fasting blood glucose level was determined every other day. The average fasting blood glucose levels of control CD-1 mice were below 150 mg/dL (Fig. 1A). After five continuous injections of STZ, the average fasting blood

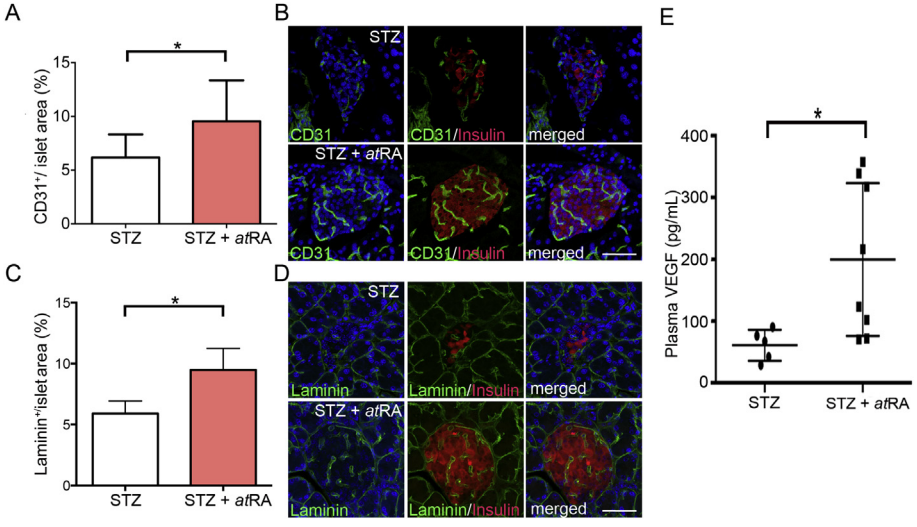


**Fig. 2. *At*RA enhanced islet vascularization in diabetic mice**. STZ-induced diabetic CD-1 mice were treated with or without 1 mg/kg *at*RA every other day. The pancreas of STZ-treated and STZ/*at*RA-treated mice was collected on day 10, immunostained with anti-CD31 (green) and anti-insulin (red) (A–B) or anti-laminin (green) and anti-insulin (red) (C–D) antibodies. Nuclei were counterstained with Hoechst dye (blue). Scale bar, 50 μm. The vascular areas that stained positively for CD31 (A) or laminin (C) were determined by using MetaMorph software. The percentage of CD31-positive area (A) and laminin-positive area in an islet was calculated. Values are presented as percentage of the total laminin-positive area in an islet and expressed as mean ± SD. *$p < 0.05$ indicates statistically significant difference as compared to the STZ-treated mice. (E) Plasma VEGF levels of control, STZ, and STZ + *at*RA mice groups were determined on day 10. Values are mean ± standard error of each experimental group.

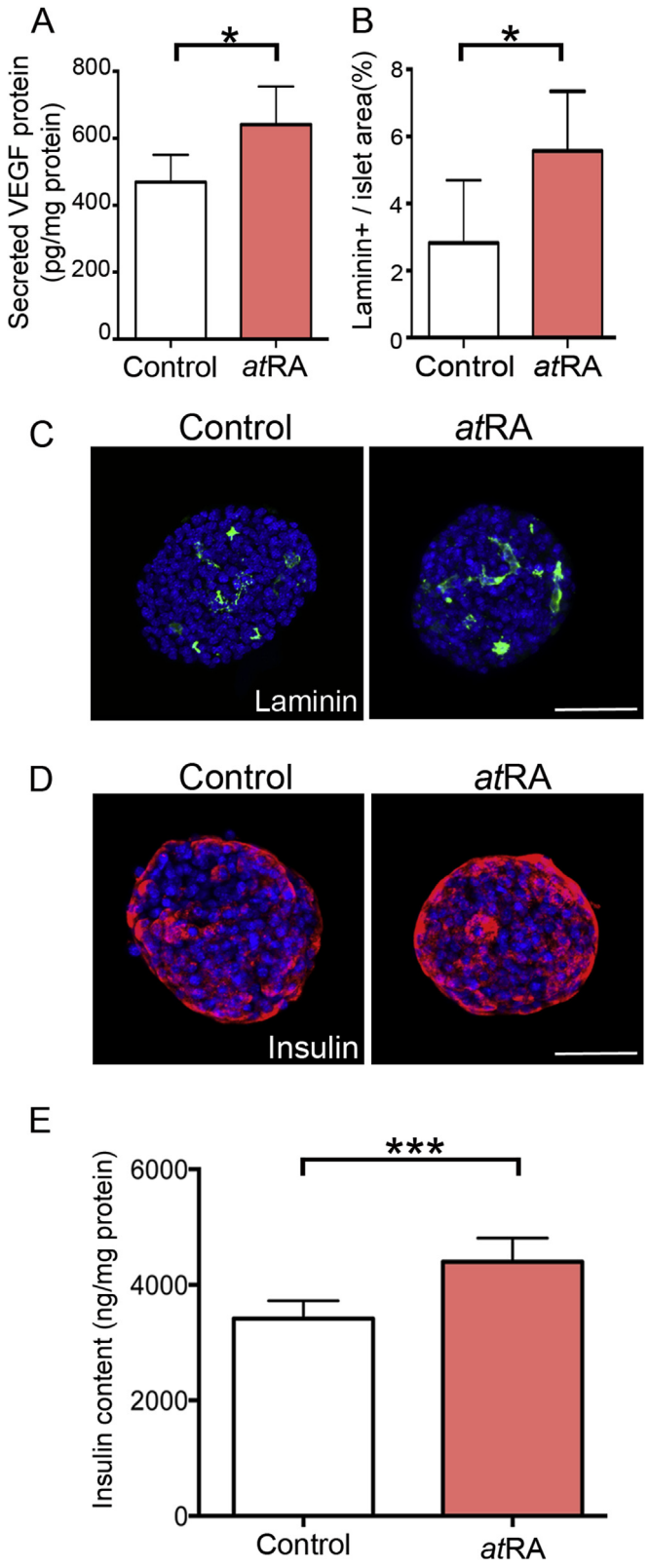


**Fig. 3. *at*RA treatment elevated laminin expression and VEGF-A production in islets**. Islets were isolated from 6 to 8 week old CD-1 mice and cultured in RPMI 1640 medium containing serum. Islets were then treated with or without 1 μM *at*RA for 3 days. (A) Subsequently, the culture medium was collected and the level of VEGF-A in the medium was determined using VEGF165 ELISA kit. Values were normalized to the total cell lysate proteins and presented as pg/mg total protein ±SD. $^{*}p < 0.05$ indicates statistically significant difference as compared to the untreated control islet culture. (B–D) Immunofluorescent staining was performed on the pancreatic islets treated with or without *at*RA for laminin (C, green) or insulin (D, red). Cell nuclei were stained with Hoechst dye (blue). Scale bar, 50 μm (B) Vascular areas that stained positively for laminin in (C) were determined using MetaMorph software. The percentage of laminin-positive area in the islets was calculated. Values are presented as percentage of total laminin-positive area in an islet and expressed as mean ± SD. $^{*}p < 0.05$ indicates statistically significant differences as compared to the untreated control islet culture. (E) The total insulin content of islets treated with or without 1 μM *at*RA for 3 days was determined using insulin ELISA kit. Values were normalized to the total cell lysate protein and presented as ng/mg total protein ±SD. $^{***}p < 0.005$ indicates statistically significant difference as compared to the untreated control islet culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glucose levels of STZ-treated mice were over 350 mg/dL. The STZ-treated mice were then divided into two groups; one group was treated with *at*RA. After 10 days, the *at*RA-treated diabetic mice had significantly lower fasting blood glucose levels than the STZ-treated diabetic mice had (average fasting glucose level >500 mg/dL). In order to validate the effect of atRA treatment, plasma concentrations of insulin were measured. The *at*RA-treated diabetic mice showed higher levels of plasma insulin than the STZ-treated diabetic mice did (Fig. 1B). This explains why the *at*RA treatment can improve glycemic control. Immunofluorescent staining showed that the atRA treatment partially restored insulin and Pdx1 expressions in the injured islet of the STZ-treated mice (Fig. 1C and D). Identification of islet cells co-expressing Pdx1 and insulin indicated that *at*RA treatment could possibly enhance β-cell regeneration in diabetic mice (Fig. 1C and E). Furthermore, the expression of nuclear RARs was determined to evaluate whether *at*RA treatment can activate retinoic acid receptor (RAR) signaling in the islets of diabetic mice. The *at*RA-treated group showed higher levels of nuclear RARs than the control group and STZ-treated group did (Fig. 1F). The results suggested enhancement of β-cell regeneration in islets of diabetic mice by *at*RA treatment is possibly mediated by activated RAR signaling.

### 3.2. AtRA enhances islet vascularization in diabetic mice

Previous work demonstrated pancreatic islet production of vascular endothelial growth factor-A (VEGF-A) regulates islet vascularity and, consequently, affect the amount of insulin delivered into the circulation [20]. We therefore hypothesized that *at*RA could improve hyperglycemia through enhance islet vascularization. To assess the islet vascularization, expression of CD31 antigen and vascular laminin in the islets of STZ-treated mice were determined using immunofluorescent staining. STZ treatment not only caused islet damage, but also significantly reduced the levels of CD31 antigen and vascular laminin (Fig. 2). In contrast, treatment with *at*RA restored the levels of CD31 antigen and vascular laminin in STZ-induced diabetic mice (Fig. 2A–D). Quantitative analysis revealed that *at*RA treatment significantly increased the percentage of CD31 and laminin-expressing areas in the islets of the diabetic mice (Fig. 2A and C). Since *at*RA treatment restored the islet vascularity, its effect on the plasma levels of VEGF-A was evaluated. As shown in Fig. 2E, treatment with *at*RA increased the levels of circulating VEGF-A.

### 3.3. AtRA elevates vascular endothelial growth factor production in islets

To validate whether *at*RA can regulate the pancreatic islet production of VEGF-A, islets were isolated from 6 to 8 week old mice, and cultured in medium with or without addition of 1 μM *at*RA for 3 days. Subsequently, the medium was collected and the level of VEGF-A in the medium was determined. As shown in Fig. 3A, the

amount of VEGF-A released into the culture medium was significantly increased (by 36%) in the *at*RA-treated group (Fig. 3A). This increase was accompanied by an enhanced laminin production (Fig. 3B and C) and insulin levels (Fig. 3D and E). Next, we validated whether the *at*RA-induced upregulation of laminin is the key factor that increases insulin content in the islets. For this purpose, laminin-integrin interactions were blocked by addition of neutralizing anti-integrin$\alpha$6 antibodies to the islet cultures. This significantly inhibited the *at*RA-induced upregulation of laminin expression and insulin content (Supplementary Fig. 1A–D). The average insulin content in the control islets was 3418 ± 306.6 ng/mg protein after 3-day culture. The insulin content in the *at*RA-treated islets was 4402 ± 405 ng/mg protein (29% increase as compared to the control islets) (Fig. 3E). In contrast, addition of the neutralizing antibodies against integrin $\alpha$6 downregulated the insulin content to 3639 ± 123.8 ng/mg protein (Supplementary Fig. 1D).

### 3.4. *AtRA improves islet graft vascularization and ameliorates hyperglycemia in diabetic mice*

To further analyze the beneficial effect of *at*RA treatment on glycemic control, STZ-induced diabetic mice were transplanted with 200 islets. Half of the islet-transplanted diabetic mice were treated with *at*RA every other day (STZ w/islet + atRA group). Fasting blood glucose levels of each experimental group were measured on day 0, 4, and 10 post islet transplantation. The blood glucose levels of diabetic mice that received islet transplantation and *at*RA treatment were restored to the normal range (123.67 mg/dL) on day 4 (Fig. 4A). In contrast, the diabetic animals that received only islet transplantation still suffered from moderate hyperglycemia (353.5–328.25 mg/dL) (Fig. 4A). To examine whether *at*RA improves functional performance of islet grafts, the plasma levels of insulin were determined after glucose injection at 10 days post-islet transplantation. In response to the glucose challenge, significantly higher levels of plasma insulin (3.17 folds) were detected in diabetic mice that received islet transplantation and *at*RA treatment than in the corn oil-treated diabetic mice (2.09 vs. 0.66 μg/L; $p < 0.05$) (Fig. 4B). The plasma levels of VEGF-A were determined to validate whether atRA treatment also enhances the VEGF-A production in the islet-transplanted diabetic mice. The plasma VEGF-A levels in the diabetic mice that received islet transplantation and *at*RA treatment were higher than in the STZ-only group diabetic mice (147.43 vs. 24.32 pg./mL) (Fig. 4C). Furthermore, atRA treatment improved the grafted islet functionality and vascularity as shown by the enhanced expression of C-peptide and laminin (Fig 4D and E).

## 4. Discussion

This study showed that the *at*RA-induced upregulation of VEGF-A produced by the islets mediated the restoration of islet vascularity and recovery of β–cell mass. Combination of islet transplantation and *atRA* administration significantly rescued hyperglycemia in diabetic mice. These findings provide evidence to support the idea that vitamin A derivatives can be used as a

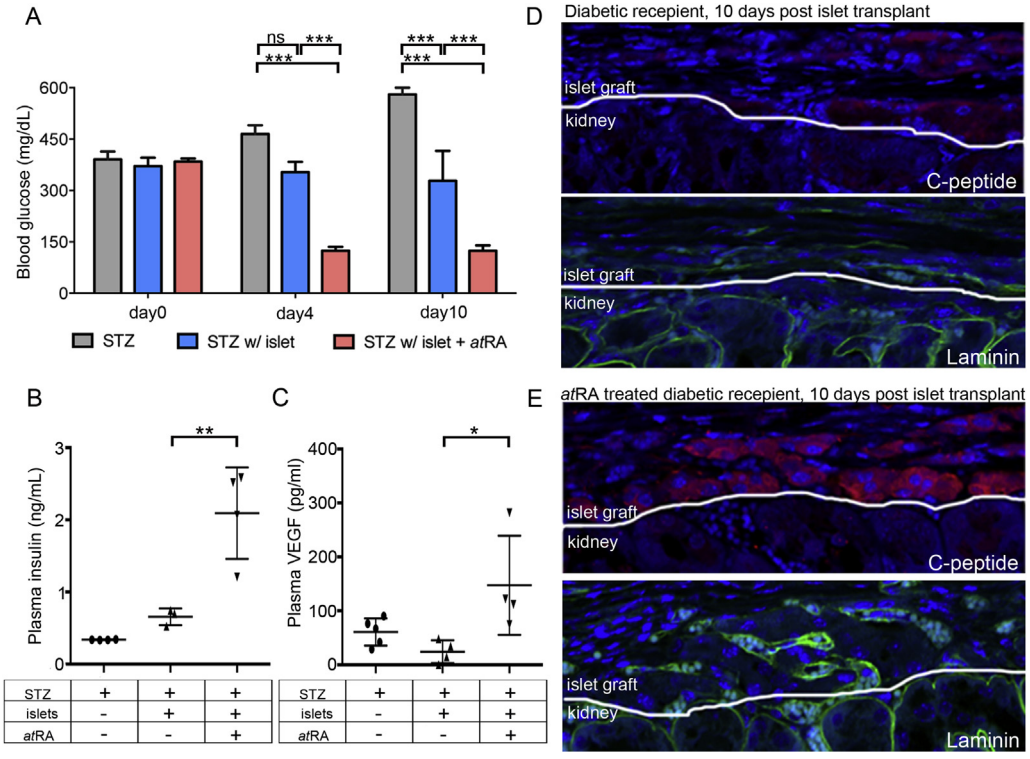


**Fig. 4. *At*RA improved islet graft vascularization and ameliorated hyperglycemia in diabetic mice**. STZ-induced diabetic mice were randomly divided into three groups. In two experimental groups (STZ w/islet, STZ w/islet + atRA), each STZ-induced diabetic mouse was transplanted with 200 islets. Half of the islet-transplanted diabetic mice were also administered with 1 mg/kg *at*RA every other day (STZ w/islet + atRA group). (A) Fasting blood glucose levels of each experimental group (STZ, STZ w/islet, STZ w/islet + atRA) were measured on day 0, 4, and 10 post islet transplantation. Values are mean ± SEM. ***$p < 0.005$ indicates statistically significant difference between the compared groups. (B–C) The plasma insulin and VEGF-A levels of all experimental groups (STZ, STZ w/islet, STZ w/islet + atRA) were determined on day 10. Values are mean ± standard error per group. *$p < 0.05$ and **$p < 0.01$ indicate statistically significant differences as compared to the *at*RA-treated groups. (D, E) The kidney islet grafts of corn-oil treated (D) or *at*RA-treated (E) diabetic recipient mice were collected 10 days post-transplantation and immunostained with anti-C-peptide (red) and anti-Laminin (green) antibodies. Nuclei were counterstained with Hoechst dye (blue).

supplementary treatment to improve diabetes management and glycemic control.

Several previous studies showed that both islet development and maintenance of islet survival after transplantation require signals produced by the endothelial microenvironment [22,23]. In fact, proper vessel formation is a necessary step for generating functional islets [24,25]. Among matrix components and vascular factors produced by the endothelial microenvironment, laminin can stimulate integrin α6β1 to promote β cell differentiation and upregulate the insulin gene expression [26]. In the current study, expression of vascular laminin increased after *at*RA treatment in the STZ-induced diabetic mice (Supplementary Fig. 1A–B) suggesting that *at*RA treatment activates laminin-integrin interaction. Addition of neutralizing antibodies against intergrinα6 inhibited the *at*RA-triggered increase in the insulin content of the islet cultures (Supplementary Fig. 1C–D). These results indicate that *at*RA-mediated enhancement in the laminin expression has a potential role in promoting β-cell differentiation and upregulating the insulin gene expression in the residual β-cells of diabetic mice.

Previous studies using bovine aortic endothelial cells or human umbilical vein endothelial cells demonstrated that *at*RA and RAR agonists significantly enhanced neovascularization [27–29]. This suggests that retinoids can regulate angiogenesis. In this study, *at*RA treatment upregulated the plasma levels of VEGF-A in the STZ-induced diabetic mice. Moreover, using isolated islets, we validated that *at*RA can upregulate the VEGF production from islets. Pancreatic islets are highly vascularized and are known to express VEGF-A [22,26]. Furthermore, the development of microvasculature occurs concomitantly with islet neogenesis [29]. VEGF-A is a major regulator of the islet vascularization during pancreatic development. Inhibition of VEGF-A expression not only suppresses the pancreatic β cell proliferation and insulin expression, but also impairs the glucose-stimulated insulin secretion [24]. The islet transplantation is a suitable therapeutic option in cases of failure of pancreatic β cells to secret insulin, as in diabetes. For successful transplantation, islets must receive enough nutritional and physical support from the host through the formation of new blood vessels around and within the graft to restore islet function. However, isolated islets lose their natural vasculature and specialized extracellular matrix [30]. Furthermore, the density of newly formed vessels after transplantation is much lower than that in the native islets [31]. Therefore, factors modulating the islet vascularity may regulate the survival and function of the grafted islets. In the current study, combined islet transplantation and *at*RA treatment showed improvement in glycemic control. Importantly, *at*RA treatment improved the functionality and vascularity of the grafted islets. Taken together, the findings of this study imply that vitamin A derivatives can be used as a supplementary treatment to improve diabetes management and glycemic control.

## Conflict of interest

All co-authors have seen and agreed with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other journal.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.06.151.

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