A Nonhematopoietic Erythropoietin Analogue, ARA 290, Inhibits Macrophage Activation and Prevents Damage to Transplanted Islets

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Background. Erythropoietin exerts anti-inflammatory, antiapoptotic, and cytoprotective effects in addition to its hematopoietic action. A nonhematopoietic erythropoietin analogue, ARA 290, has similar properties. The efficacy of pancreatic islet transplantation (PITx) is reduced due to islet damage that occurs during isolation and from the severe inflammatory reactions caused by the transplantation procedure. We investigated whether ARA 290 protects islets and ameliorates inflammatory responses following PITx thus improving engraftment. Methods. The effects of ARA 290 on pancreatic islets of C57BL/6J (H-2b) mice and on murine macrophages were investigated using an in vitro culture model. As a marginal PITx, 185 islets were transplanted into the liver of streptozotocin-induced diabetic mice (H-2b) via the portal vein. Recipients were given ARA 290 (120 μg/kg) intraperitoneally just before and at 0, 6, and 24 hours after PITx. Liver samples were obtained at 12 hours after PITx, and expression levels of proinflammatory cytokines were assessed. Results. ARA 290 protected islets from cytokine-induced damage and apoptosis. Secretion of pro-inflammatory cytokines (IL-6, IL-12, and TNF-α) from macrophages was significantly inhibited by ARA 290. After the marginal PITx, ARA 290 treatment significantly improved the blood glucose levels when compared to those of control animals (P < 0.001). Upregulation of monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, IL-1β, and IL-6 messenger RNA expression within the liver was suppressed by ARA 290 treatment. Conclusions. ARA 290 protected pancreatic islets from cytokine-induced damage and apoptosis and ameliorated the inflammatory response after PITx. ARA 290 appears to be a promising candidate for improvement of PITx.

Pancreatic islet transplantation (PITx) offers a feasible treatment option for type 1 diabetes mellitus patients, especially those with glycemic instability. However, the protocol requires that transplant recipients receive large quantities of viable islets to achieve an insulin-independent state and long-term graft function. This is mainly because most of the islets transplanted into the liver via the portal vein are subsequently destroyed after PITx. 1,2 The islet isolation process itself also triggers a cascade of stressful events in the islets involving apoptosis and the production of proinflammatory molecules that negatively influence islet function and produce detrimental effects after PITx. 3,4 Hence, an efficient strategy for preventing islet damage during the isolation process and islet graft loss after PITx is of great importance for further improvement in clinical outcomes as well as a possible decrease in the islet graft mass needed.

In the context of intraportal PITx, platelet aggregation and the activation of coagulation/complement cascades are immediately induced when transplanted islets come into contact with blood. 5,6 These reactions lead to inflammatory responses driven by mediators such as monocyte chemoattractant protein (MCP)-1, 7 IL-1β, and TNF-α 8 and lead to infiltration of neutrophils and mononuclear cells into the transplanted islets. Macrophages and Kupffer cells, the resident macrophages of the liver, are deeply involved with these reactions. 9,12 Activated macrophages secrete proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ, which can directly injure transplanted islets. 13 Indeed, macrophage depletion has been conceived the experimental design, initiated the projects, interpreted data, and contributed to the discussion. Y.S. performed surgical procedures and conducted in vitro experiments. A.C. provided the ARA 290 to the projects and contributed to planning the experiments. C.G.O. introduced the ARA 290 to the projects and contributed to planning the experiments to the discussion. M.K. conceived the experimental design, participated in the experiments, interpreted the data, and prepared the article. M.W. conceived the experimental design, cared for experimental animals, conducted in vitro experiments, interpreted data, and prepared the article. T.L.

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shown to prevent systemic increase of proinflammatory mediators and to improve islet graft survival.\(^\text{13}\)

In line with this notion, a number of mechanisms occur during the islet isolation process that lead to undesirable effects on transplanted islets. Isolated islets have been reported to produce and release inflammatory mediators even under standard culture conditions.\(^\text{14,15}\) Beyond the direct toxic effect on \(\beta\) cells,\(^\text{13}\) inflammatory mediators, such as IL-1\(\beta\) and MCP-1, may jeopardize the survival of transplanted islets by enhancing recruitment and activation of macrophages and/or Kupffer cells after PITx. These mechanisms also play a crucial role in the triggering of graft dysfunction and the eventual loss of islets after PITx.\(^\text{27}\)

Erythropoietin (EPO) is well known for its survival and differentiating effects on precursor erythroid cells.\(^\text{16}\) Several studies have shown that EPO also exerts anti-inflammatory, antipoptotic, and cytoprotective effects through its binding to a heterodimeric receptor consisting of EPO receptor (EPOR) and CD131, the β-common subunit (EPOR-βCR).\(^\text{17,18}\) The heteromer has been found in a variety of cells, including myoblasts,\(^\text{19}\) neuronal cells,\(^\text{20}\) kidney cells,\(^\text{21}\) pancreatic islets,\(^\text{22,23}\) and even on monocytes and T cells.\(^\text{24}\) However, clinical use of EPO is the concern of thromboembolic adverse effects because EPO also promotes endothelial activation and platelet reactivity.\(^\text{25}\)

Recently, EPO analogues without hematopoietic function have been developed to overcome this problem. ARA 290, the pyroglutamate helix B surface peptide, is a short peptide of 11 amino acids that has a high specificity for the EPOR-βCR and lacks hematopoietic functions.\(^\text{26-28}\) ARA 290 promotes its effects via activation of the β-common receptor in conjunction with the EPOR, which is locally upregulated following tissue or cell injury.\(^\text{26,29,30}\) It has been shown that ARA 290 improves burn injury and survival following myocardial infarction and that it reduces organ dysfunction in hemorrhagic shock, suppresses development of atherosclerosis, and ameliorates renal ischemia reperfusion injury.\(^\text{31-35}\)

The safety and efficacy of ARA 290 treatment in a humidified atmosphere for 6 hours. The islets and supernatant fluids from each dish were harvested and analyzed.

**Primary Peritoneal Macrophage Preparation, Stimulation, and Culture**

Thioglycollate-elicited primary peritoneal macrophages (PMs) were harvested as described previously.\(^\text{38}\) Briefly, C57BL/6 mice were injected intraperitoneally with 4% Brewer thioglycollate medium (Sigma-Aldrich). At 3 days after the injection, cells were isolated from the mice by flushing the peritoneal cavity with PBS. Cells were seeded on 6-well plates, and adherent PMs were collected and used for subsequent analysis. Peritoneal macrophages were seeded in a 6-well plate at a density of \(5.0 \times 10^5\) cells/mL in Dulbecco modified Eagle medium (Life Technologies) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin and were pretreated with ARA 290 (0, 1.0, 10, 50, and 100 nmol/L) for 40 minutes. After this treatment, PMs were stimulated with a combination of LPS (200 ng/mL) and IFN-γ (5.0 ng/mL) and cultured at 37°C with 5% CO\(_2\) in a humidified atmosphere for 6 hours. Supernatant fluids from each well were collected and used for analysis.

**3-[4, 5-Dimethylthiazol-2-yl]-2, 5-Diphenyltetrazolium Bromide Assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (Sigma-Aldrich) solution was added to pancreatic islets in culture medium at a final concentration of 0.5 mg/mL and incubated for 30 minutes. At the end of the incubation period, the medium was removed, and the converted dye was solubilized with isopropanol. All measurements were performed in triplicate. Absorbance of the converted dye was measured at a wavelength of 550 nm on a Biotek FLx800 Multi-Detection Microplate Reader operated by Gen5 Data Analysis Software.
**Static Glucose-Stimulated Insulin Secretion Tests**

Twenty islets were handpicked from the culture dish and transferred to 24-well Transwell plates (8.0 μm pore size membrane, Corning, Acton, MA) in 1.0 mL of Krebs-Ringer bicarbonate buffer (KRBB) containing 1.67 mM glucose (low-glucose KRBB). The islets were incubated for 60 min with 1.0 mL of low-glucose KRBB followed by incubation with high-glucose KRBB (16.7 mM glucose) for another 60 minutes. The supernatants were collected, and the insulin in the supernatants was quantified using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Mabtech, Nacka, Sweden). A stimulation index was calculated by dividing the total amount of insulin released from the islets cultured in the high-glucose KRBB by the total amount of insulin released from the islets cultured in the low-glucose KRBB. All measurements were performed in duplicate.

**Measurement of Caspase 3/7 Activity**

Apoptosis was assayed using the Caspase-Glo 3/7 Assay kit (Promega Corp., Madison, WI). In total, 20 islets in 30 μL of RPMI-1640 medium were transferred to a 96-well plate. The same volume of 30 μL of Caspase-Glo 3/7 reagent was added and incubated at room temperature for 1 hour. The luciferase activity was measured with a luminometer (Biotek FLx800 Multi-Detection Microplate Reader operated by Gen5 Data Analysis Software). To correct for the number of cells in each sample, the amount of double-stranded DNA was measured by dyeing the sample with the Quant-iT PicoGreen double-stranded DNA Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. All measurements were performed in triplicate. Fluorescence activity was measured with a fluorometer (Biotek FLx800 Multi-Detection Microplate Reader operated by Gen5 Data Analysis Software).

**Diabetes Mellitus Induction**

B6 islet-recipient mice were rendered diabetic by intraperitoneal administration of 180 mg/kg streptozotocin (STZ) (Sigma-Aldrich) 5 to 7 days before PITx. Blood samples were collected via the tail vein, and the blood glucose levels were monitored with the Accu-Check blood glucose monitor (Medicarrier). Diabetes was considered to be established when the blood glucose level of 2 consecutive measurements exceeded 25 mmol/L.

**PITx and Treatment Protocol**

The isolated pancreatic islets were cultured overnight and washed with RPMI-1640 medium containing 10% FBS, counted, and transplanted into the recipient liver via the portal vein as previously described. The recipient animals were transplanted with 185 pancreatic islets treated with ARA 290 or PBS intraperitoneally, were obtained at 12 hours after PITx, snap-frozen in liquid nitrogen, and stored at −80°C until use. Tissue samples were subjected to total RNA extraction using TRIzol reagent (Ambion Life Technologies, Waltham, MA) using the manufacturer’s protocol, and the isolated RNA was dissolved in 50 μL RNase-free water. Complementary DNA synthesis from 1 μg RNA was performed using Applied Biosystem’s high-capacity complementary DNA reverse transcription kit. Quantification of messenger (m)RNA was performed using TaqMan real time PCR on an Applied Biosystems 7500 Fast Real-Time PCR System. All samples were analyzed in triplicate. Relative mRNA expression was calculated from the Ct-values against the housekeeping gene peptidyl-prolyl cis-trans isomerase A using the comparative delta-Ct method. The following primers targeting specific mRNAs were used: Mm00434228_m1 (mouse IL-6), Mm00444124_m1 (mouse MCP-1), Mm00446190_m1 (mouse IL-6), and Mm00443111_m1 (mouse macrophage inflammatory protein [MIP]-1β).

**Statistical Analysis**

Quantitative results are presented as mean values ± SD. Statistical analysis of nonparametrically distributed variables was carried out using the Friedman test or Mann Whitney U test. Differences in the normoglycemic rate between groups were evaluated by log-rank test using a Kaplan-Meier method. A P value less than 0.05 was considered statistically significant, and all calculations were performed using GraphPad Prism software version 6 (GraphPad Software Inc., San Diego, CA).

**RESULTS**

**ARA 290 Protects Isolated Pancreatic Islets from Cytokine-Induced Damage**

The pancreatic islet isolation procedure exerts significant stress on islets by releasing proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ, which result in the loss of β-cell function and the induction of apoptosis. We initially investigated the tissue protective effects of ARA 290 in an in vitro islet-culture model. Isolated...
FIGURE 1. ARA 290 protects isolated pancreatic islets from cytokine-induced damage and apoptosis. The isolated pancreatic islets were cocultured together with IL-1β, TNF-α, and IFN-γ in the presence or absence of ARA 290 for 6 hours. A, In an MTT assay, relative O.D. values compared to those of islets cultured with medium alone are shown (mean ± SD). The addition of ARA 290 maintained islet viability during culturing together with pro-inflammatory cytokines. B, Islets function was evaluated after 6 hours of coculturing. The insulin secretion from the cocultured islets was well preserved when ARA 290 was present. C, To evaluate the antiapoptotic effects of ARA 290 on pancreatic islets, caspase 3/7 activity was also assessed. The addition of ARA 290 significantly suppressed the caspase 3/7 activity at 6 hours after coculturing with the cytokines compared to the vehicle-treated control group (*P < 0.05, n = 4 in all groups; Mann-Whitney U test, respectively). O.D. indicates optical density.

FIGURE 2. ARA 290 inhibits cytokine-induced macrophage activation. A-C, Thioglycolate-elicited mouse primary macrophages were pretreated with ARA 290 for 40 minutes and then incubated with LPS (200 ng/mL) and IFN-γ (5.0 ng/mL) for 6 hours. The supernatants from each dish were then collected and protein levels of IL-6, IL-12, and TNF-α were evaluated by ELISA (mean ± SD). ARA 290 significantly suppressed the production of IL-6, IL-12, and TNF-α compared to the vehicle-treated control group in a dose-dependent manner (*P < 0.05 vs vehicle-treated control group; Friedman test, n = 4 in each concentration).
mouse pancreatic islets were cultured together with proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) in the presence or absence of ARA 290, and the islets were harvested after 6 hours of culture. The viability and function of the islets were assessed by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay and static glucose-stimulated insulin secretion tests. The addition of ARA 290 maintained islet viability during the exposure to proinflammatory cytokines (Figure 1A). Furthermore, the glucose-stimulated insulin secretion from the cultured islets was well preserved when ARA 290 was added (Figure 1B).

**ARA 290 Protects Isolated Islets from Cytokine-induced Apoptosis**

High-dose EPO has antiapoptotic effects on pancreatic islets that are exposed to proinflammatory cytokines. To evaluate the antiapoptotic effects of ARA 290 on pancreatic islets, activity of the apoptotic enzyme caspase 3/7 in the cultured islets was evaluated. The caspase 3/7 activity increased in the islets cocultured with IL-1β, TNF-α, and IFN-γ. In contrast, as shown in Figure 1C, the addition of ARA 290 significantly suppressed caspase 3/7 activity, indicating that ARA 290 protects pancreatic islets from cytokine-induced apoptosis.

**ARA 290 Inhibits Cytokine-Induced Macrophage Activation**

Activated macrophages play a key role in the early graft damage after PITx, and macrophage depletion has been shown to prevent systemic increases of proinflammatory mediators and to improve islet graft survival. To investigate the effects of ARA 290-mediated inhibition against macrophages, we used an in vitro coculture model including mouse primary PMs together with LPS (200 ng/mL) and IFN-γ (5.0 ng/mL). The pretreatment with ARA 290 significantly suppressed the protein levels of IL-6, IL-12, and TNF-α in the culture supernatants when examined at 6 hours after culture compared to the vehicle-treated control group (Figures 2A-C).

**FIGURE 3.** Treatment with ARA 290 ameliorated blood glucose levels in a marginal mouse PITx model. After 185 pancreatic islets from a B6 mouse were transplanted into the liver of STZ-induced diabetic B6 mice, non-fasting blood glucose levels were monitored (A–C). Based on these blood glucose data, the area under the curve of nonfasting blood glucose levels (D) and normoglycemic rate (E) in each group are shown. Recipients were given either 100 μL PBS as vehicle treatment (n = 7 [A] and black circles in panel E) or ARA 290 (120 μg/kg) intraperitoneally just before and at 0, 6, and 24 hours after PITx (n = 7 [B] and white circles in panel E). As an ARA 290 treatment control, ARA 290 was administered intraperitoneally just before and at 0, 6, and 24 hours after the sham operation without PITx (n = 6, [C] and crosses in panel E). In this marginal PITx model, vehicle-treated control recipient mice rarely accepted the islet grafts and showed high blood glucose levels during the observation period. In sharp contrast, treatment with ARA 290 ameliorated the blood glucose levels soon after the PITx (D; **P < 0.001; Mann-Whitney U test, values are depicted as lower quartile, median, and upper quartile (boxes) with minimum and maximum ranges), and maintained the normoglycemic state during the observation period (E; **P < 0.001 vs vehicle-treated control group; Kaplan-Meier log-rank test).
We examined whether ARA 290 treatment can ameliorate islet graft loss during PITx by using a mouse syngeneic marginal PITx model. One hundred eighty-five islets from C57BL/6 (H-2<sup>b</sup>) mice were transplanted into the liver of STZ-induced diabetic mice (H-2<sup>b</sup>) via the portal vein. Recipients were given ARA 290 (120 μg/kg) intraperitoneally just before and at 0, 6, and 24 hours after PITx, and nonfasting blood glucose levels were monitored. Control recipient mice that were administered vehicle (PBS) rarely accepted the islet grafts and showed high blood glucose levels; normoglycemic state could not be induced in any animals after PITx (normoglycemic rate; 0%, Figures 3A, D, and E). In contrast, ARA 290 treatment just before and shortly after PITx significantly ameliorated the blood glucose levels and rendered normoglycemic state in 6 of 7 recipient animals (normoglycemic rate, 85.7%; Figures 3B, D, and E). ARA 290 treatment without PITx did not improve glucose metabolic control of diabetic mice (Figures 3C, D, and E). No apparent adverse effects were noted in any of the animals during the study.

**Transplanted Islet Graft Function After Marginal Syngeneic PITx**

The IPGTT was performed 14 days after PITx to evaluate the function of transplanted islet grafts. In the ARA 290-treated islet recipients, blood glucose levels during the IPGTT were lower than those of the vehicle-treated control islet recipients at every time point. Treatment with ARA 290 significantly improved the function of islet grafts (Figure 4).

**ARA 290 Inhibits Intrahepatic Inflammatory Responses After PITx**

To assess inflammatory responses at the transplant site after PITx, liver samples were obtained from islet-recipient mice 12 hours after PITx. The mRNA expression levels of proinflammatory cytokines were evaluated by using the whole liver. Upregulation of TNF-α, MCP-1, MIP-1β, IL-1β, and IL-6 mRNA expression levels within the liver were suppressed by ARA 290 treatment compared with the mRNA levels in the control group (P < 0.05 vs control; Mann-Whitney U test, n = 4; values are depicted as lower quartile, median, and upper quartile (boxes) with minimum and maximum ranges).
animals at 12 hours after PITx, and expression levels of proinflammatory cytokines were assessed by reverse transcription polymerase chain reaction. Upregulation of TNF-α, MCP-1, MIP-1β, IL-1β, and IL-6 mRNA expression levels within the liver after PITx were suppressed by ARA 290 treatment (IL-6, MCP-1, and MIP-1β, $P < 0.05$; TNF-α, $P = 0.0857$; and IL-1β, $P = 0.0571$) compared with mRNA levels in the control group (Figures 5A-E).

**DISCUSSION**

ARA 290 is a newly developed peptide modelled from the 3-dimensional structure of the binding site of EPO on the EPOR-βcR complex, and ARA 290 induces anti-inflammatory, antiapoptotic, and tissue-protective effects through interaction with the innate repair receptor without any hematopoietic or prothrombotic effects. Mechanically, EPOR-βcR is associated with activation of the PI3K-Akt signalling pathway, which subsequently suppresses the NF-κB-driven gene transcription of pro-inflammatory mediators and also leads to the phosphorylation of endothelial nitric oxide (NO) synthase. In parallel with PI3K-Akt signalling, the canonical JAK2-STAT5 pathway is also activated leading to the transcription of STAT5-dependent genes. Several studies have indicated that EPO signalling may have significant biological effects on β-cells by preventing apoptosis and promoting proliferation and angiogenesis within the pancreatic islets. Here, we have demonstrated that treatment with ARA 290 at just before and for a short time after PITx improves glucose metabolism in the recipients when using a mouse marginal PITx model in which only 185 islets were transplanted into the liver via the portal vein. This effect was associated with significantly reduced mRNA expression levels of proinflammatory cytokines, which are known to induce direct islet cell damage, in the islet-recipient’s liver. In addition to the downregulation of proinflammatory cytokines, we also found that ARA 290 maintained the viability and function of isolated pancreatic islets during culture in the presence of pro-inflammatory cytokines. The addition of ARA 290 significantly suppressed caspase 3/7 activity at 6 hours after coculturing with the cytokines compared with the vehicle-treated control group.

To elucidate the mechanisms underlying the ARA 290-mediated effects after PITx, we focused on macrophages. It has been well demonstrated that macrophage activation is one of the key triggers to induce inflammatory or innate immune reactions that lead to early islet grafts loss after PITx. We have shown that ARA 290 inhibited cytokine secretion by macrophages after stimulation by LPS and IFN-γ; addition of ARA 290 at a dose range of 50 to 100 nmol/mL significantly inhibited IL-6, IL-12, and TNF-α secretion from mouse macrophages that had been collected from the peritoneal cavity after Brewer’s thioglycolate injection. This inhibitory effect was in line with the findings shown of Nairz et al who showed that EPO inhibits the induction of pro-inflammatory genes including TNF-α and inducible NO synthase in activated mouse macrophages, which is mechanistically attributable to blockade of NF-κB p65 activation.

A previous study has shown that RAW 264.7 cells, a murine macrophage-like cell line, express EPOR and behave similarly to primary macrophages. However, there is also controversy concerning the effects of EPO on macrophages. In terms of the efficacy on macrophages, ARA 290 did not significantly inhibit proinflammatory cytokines secretion by LPS-activated RAW 264.7 cells even with high concentrations of ARA 290 (data not shown). Moreover, the treatment with ARA 290 did not ameliorate the normoglycemic rate when syngeneic rat islets were transplanted under the kidney capsule, which is low invasive PITx model. We have also examined the effect of ARA 290 by using rat islets isolated from the pancreas that were cold-stored for 18 hours. ARA 290 was added in the University of Wisconsin solution and/or in the digestion solution for islet isolation, however, no improvement was shown in the yield of islets, islet viability, or islet function (manuscript submitted). ARA 290 exerts its effects via activation of the β-common receptor in conjunction with the EPOR; however, these receptors are upregulated only following tissue or cell injury and the EPOR and EPOR-βcR are not expressed on the surface of cells under steady-state conditions. Taken together, these findings indicate that ARA 290 binds to the EPOR-βcR complex that is upregulated by exposure to proinflammatory cytokines, and thus ARA 290 protects islets from the damage caused by the cytokines released by activated macrophages and/or intraportal PITx itself.

After PITx, macrophages, neutrophils, dendritic cells, and T-cells are also activated, and these activated cells produce proinflammatory cytokines, such as IL-1β, TNF-α, IL-6, IL-8, IL-12, and MCP, which amplify the immune response thereafter. As the understanding of innate and adaptive immunology progresses, the importance of preventing inflammatory reactions shortly after PITx is increasingly being recognized, not only for avoiding early islet grafts loss but also for reducing the subsequent adaptive immune response that leads to allograft rejection. In addition, it has been demonstrated that EPO downregulates proinflammatory immune effector pathways in response to LPS stimulation, chemotherapeutic damage, and infection. Taken together with the pivotal role of macrophages in inflammation and our findings in this study, further studies of ARA 290 on allogeneic transplant settings, or on the efficacy of ARA 290 in combination with conventional immunosuppressants would be crucial for clinical application of ARA 290, such studies are currently under progress.

The EPO also promotes endothelial activation and platelet reactivity, therefore thromboembolic complications after ARA 290 treatment is a concern. In the present study, no histopathological evidence of thromboembolism was observed in any animal during ARA 290 treatment, after drug cessation, or at the time of sacrifice (data not shown). In addition, clinical studies of ARA 290 in patients with sarcoidosis or diabetes mellitus have shown promising efficacy of ARA 290 in ameliorating neuropathic pain without any safety issues, including thromboembolic complications.

In summary, we found that inhibition of initial inflammatory activities by a novel EPO analogue, ARA 290, allowed transplanted islets to be engrafted and improved glucose metabolic control even after a marginal PITx. ARA 290 may facilitate development of a treatment strategy that
enables successful PITx using a single donor for multiple diabetic recipients. ARA 290 appears to be a promising agent for enhanced outcome after PITx.\textsuperscript{24,33}

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REFERENCES


