Materials and Methods

Experimental Animals and Study Design

This study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The entire experimental protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (IACUC approval No. 14-0187-S1A0).

Eight-week-old Sprague-Dawley rats weighing 260–310 g were randomly allocated to five groups (n = 15 in each group): normal controls (C); those with diabetes treated at 7 weeks (7W), 10 weeks (10W), and 13 weeks (13W) after diabetes induction; and those with untreated diabetes (DM). A single or double intraperitoneal injection of 60 mg kg^{-1} streptozotocin with a citrate phosphate buffer (50 mM sodium citrate, pH 4.5) was used to induce diabetes, and the development of diabetes was confirmed by measuring the blood glucose levels (>300 mg dL^{-1}) at 48 h after the injection. In the treated groups (7W, 10W, and 13W), insulin was injected daily from 7 weeks, 10 weeks, and 13 weeks after diabetes induction, respectively, until 14 weeks. The initial dose was 5–6 units of neutral protamine Hagedorn (NPH), and the dose was adjusted based on the blood glucose levels to maintain proper glycemic control. The blood glucose levels were measured weekly, and the glycated hemoglobin levels were measured at 14 weeks. At 14 weeks after diabetes induction, skin biopsies were performed, with the dorsal skin of rats obtained using a 3-mm punch (length: 6mm; Integra Miltex, York, PA, USA). Pancreatic tissues were also acquired in the same way in order to examine intrinsic insulin production. The flowchart for the overall experimental procedures is demonstrated in Figure 1.

Immunohistochemistry and Masson's Trichrome Staining

A streptavidin-biotin amplification method was performed for immunohistochemistry (IHC). The tissues were incubated with primary antibodies targeting the following molecules: insulin, collagen 1, collagen 3, transforming growth factor-β1 (TGF-β1), insulin-like growth factor 1 receptor (IGF-1R) (Abcam, Cambridge, MA, USA), and vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After rinsing the primary antibodies, an ABC kit (Invitrogen, Carlsbad, CA, USA) containing secondary antibodies was used to detect fluorescence by IHC staining. A Masson's trichrome stain kit Ab150686 (Abcam) was utilized according to the manufacturer's instructions. The intensity was determined by an image analysis program (Leica QWin version 3.5.1; Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

Data from different groups were compared by two-tailed unpaired Student's *t* test or Mann-Whitney test using SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). All data are expressed as mean $\pm$ standard deviation. All experiments were repeated at least thrice with reproducible results. *p* values of <0.05 were considered statistically significant.