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Annexin A1 Is a Key Modulator of Mesenchymal Stromal Cell–Mediated Improvements in Islet Function

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We have previously demonstrated that coculture of islets with mesenchymal stromal cells (MSCs) enhanced islet insulin secretory capacity in vitro, correlating with improved graft function in vivo. To identify factors that contribute to MSC-mediated improvements in islet function, we have used an unbiased quantitative RT-PCR screening approach to identify MSC-derived peptide ligands of G-protein–coupled receptors that are expressed by islets cells. We demonstrated high expression of annexin A1 (ANXA1) mRNA by MSCs and confirmed expression at the protein level in lysates and MSC-conditioned media by Western blot analysis and ELISA. Preculturing islets with exogenous ANXA1 enhanced glucose-stimulated insulin secretion (GSIS), thereby mimicking the beneficial influence of MSC preculture in vitro. Small interfering RNA–mediated knockdown of ANXA1 in MSCs reduced their capacity to potentiate GSIS. MSCs derived from ANXA1$^{-/-}$ mice had no functional capacity to enhance GSIS, in contrast to wild-type controls. Preculturing islets with ANXA1 had modest effects on their capacity to regulate blood glucose in streptozotocin-induced diabetic mice, indicating that additional MSC-derived factors are required to fully mimic the beneficial effects of MSC preculture in vivo. These findings demonstrate the feasibility of harnessing the MSC secretome as a defined, noncellular strategy to improve the efficiency of clinical islet transplantation protocols.

There is a growing body of evidence that mesenchymal stromal cells (MSCs) can enhance the functional survival of islet grafts after transplantation, offering a potential therapeutic method for improving the outcomes of islet transplantation as a therapy for type 1 diabetes. A number of MSC-derived trophic factors have been shown to influence the graft niche by modifying the responses of host immune, endothelial, or progenitor cells to reduce inflammatory or immune responses (1,2) and to improve graft revascularization (3–5). However, we (6–8) and others (4,9–11) have demonstrated that MSCs also have direct effects on donor islet cells to improve their survival and secretory function. Thus, we previously used direct contact coculture of islets with MSCs derived from kidney (6) or adipose tissue (7) to enhance glucose-stimulated insulin secretion (GSIS) in vitro and demonstrated that this results in superior in vivo function for islet-alone grafts at the experimental renal subcapsular (6) and clinically preferred intraportal transplantation site (7).

Previous studies suggest that the beneficial effect of MSCs on islet function is at least partly mediated by soluble bioactive molecules (12), so we have now applied a nonbiased screening approach to identify novel MSC-derived secretory products that may influence islet function. We have based the current screen on our recent demonstration that islets express 293 different G-protein–coupled receptors (GPCRs) that are known to be activated by more than 250 identified ligands (13). In this study, we have used a quantitative (q)RT-PCR approach to focus on the 131 peptides or proteins that have been identified previously as ligands for islet GPCRs to identify MSC-derived molecules that may contribute to MSC-mediated improvements in islet function. This strategy has identified therapeutic factors that may be used in a noncellular approach to direct defined modifications to islet transplantation protocols, ensuring reproducible improvements in islet function and thus improved islet transplantation outcomes.

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RESEARCH DESIGN AND METHODS

Identification of MSC-Derived Islet GPCR Ligands

Total RNA was extracted from cultured adherent mouse MSC monolayers using RNeasy mini kits and RNase-free DNase sets (Qiagen, Manchester, U.K.) and reverse transcribed into cDNAs using an Applied Biosystems high-capacity reverse transcription kit (Life Technologies, Ltd., Paisley, U.K.). Pooled biological replicates of mouse adipose- or kidney-derived MSC cDNAs were screened by qRT-PCR for a total of 131 potential candidate ligands of known islet GPCRs (13) to create a mRNA expression profile of MSC-encoded ligands specific to islet GPCRs. Melt curve analysis was performed after amplification, and qPCR reactions showing positive melt curves were further analyzed by agarose gel electrophoresis to confirm that the qPCR product was the appropriate size. qRT-PCR of MSC biological replicate cDNAs was performed using QuantiTect primers (Qiagen). Relative expression of mRNAs was determined after normalization against GAPDH as an internal reference and calculated by the $2^{-\Delta\Delta Ct}$ method. To allow discrimination between different levels of expression, each ligand mRNA was classified by its Ct value relative to GAPDH-Ct 18 as high (Ct $<$26), medium (Ct 26–30), or low (Ct $>$30) levels of expression.

Islet Isolation and Culture

Mouse islets were isolated by collagenase digestion (1 mg/mL; type XI; Sigma-Aldrich, Poole, U.K.), followed by density gradient separation (Histopaque-1077; Sigma-Aldrich). After washing with RPMI-1640, islets were picked into groups of 100 for culture alone, with MSCs, or with recombinant annexin A1 (ANXA1) (R&D Systems, Abingdon, U.K.) for 3 days, unless otherwise specified. For MSC preculture, we used a direct-contact monolayer configuration, as previously described (6,7). Media was changed after 2 days for all culture groups and ANXA1 replenished in the ANXA1 preculture group.

Measurement of ANXA1 Expression and Release

To confirm the expression of ANXA1 protein in MSC lysates and to determine whether ANXA1 is released into the MSC culture media, MSCs were seeded into Nunclon 35-mm petri dishes to mimic the experimental setup used for our direct contact islet-MSC preculture configuration (6,7). After 72 h, MSCs were trypsinized and resuspended in ice-cold PBS supplemented with cOmplete ULTRA Mini protease inhibitors (Roche Diagnostics, Burgess Hill, U.K.) and then sonicated. The MSC-conditioned media (CM) from each petri dish was also collected and concentrated ×12 using 10,000 nominal molecular weight limit Amicon Ultra 0.5-mL centrifugal filters (Merck Millipore, Middlesex, U.K.). Control samples were the MSC culture media alone, which was also concentrated ×12. ANXA1 was measured in MSC-lysates and -CM using an ELISA kit (USCN Life Sciences Inc., Wuhan, China).

Small Interfering RNA–Mediated Knockdown of ANXA1 Expression in MSCs

SMARTpool ON-TARGETplus small interfering (si)RNA (50 nmol/L; Thermo Fisher Scientific Biosciences, GmbH) directed against mouse ANXA1 was transfected into MSCs for 24 h in the presence of DharmaFECT 1 transfection reagent (Thermo Fisher Scientific Biosciences, GmbH), using a siRNA-to-DharmaFECT ratio of 2:1. Control MSCs were transfected with ON-TARGETplus Non-targeting Control siRNA. Transfected MSCs were cultured in antibiotic-free DMEM supplemented with 10% (vol/vol) FCS for the initial 24 h after transfection. siRNAs were removed after 24 h, and MSCs were cultured alone or direct contact islet-MSC cocultures were set up. MSC lysates were collected at 24, 48, and 72 h after transfection for assessment of ANXA1 mRNA. ANXA1 protein expression was assessed by Western blotting using a rabbit anti-ANXA1 monoclonal antibody (1:1,000 dilution; Cell Signaling Technology, Hertfordshire, U.K.), with equal loading confirmed by immunoprobing with a rabbit monoclonal anti-GAPDH antibody (1:5,000 dilution; Cell Signaling Technology). MSCs were disrupted in RIPA lysis buffer (Sigma-Aldrich), total protein was measured using a bicinchoninic acid assay, and 20 μg protein was loaded onto 12% SDS gels (Life Technologies Ltd.).

Islet Secretory Function In Vitro

Insulin secretion in vitro was assessed in static incubations of isolated islets. Islets were preincubated for 2 h in RPMI containing 36 mg/dL glucose. Groups of three islets were transferred into 1.5-mL Eppendorf tubes and incubated at 37°C in a bicarbonate-buffered physiological salt solution, containing 2 mmol/L $CaCl_2$ and 0.5 mg/mL BSA (14) and either 36 or 360 mg/dL glucose. Samples of the incubation medium were taken after 1 h and stored at −20°C until assayed for insulin content using in-house radioimmunoassay (15,16).

Islet Apoptosis In Vitro

To assess caspase 3/7 activity, luciferase activity dependent on caspase 3/7–mediated generation of luciferase substrate was measured (Promega, Southampton, U.K.). Islets were precultured alone or with 5 nmol/L ANXA1 for 72 h. For the final 20 h of the culture period, half of the islets in each culture dish were exposed to mixed cytokines (50 units/mL interleukin-1β, 1,000 units/mL interferon-γ, and 1,000 units/mL tumor necrosis factor-α; PeproTech, London, U.K.) and half of the islets served as controls without cytokines. Islets were picked into groups of five islets per well, and Caspase-Glo 3/7 reagent (Promega) was added. Light emission was detected after 1 h using a Turner BioSystems Veritas luminometer (Promega).

Experimental Animals

Male CD1 mice (Charles River Laboratories, Margate, U.K.), aged 8–12 weeks, were used as islet donors for all in vitro investigations. Male C57BL/6 mice (Harlan, Huntingdon, U.K.), aged 8–12 weeks, were used as donors and recipients

for syngeneic islet transplantation experiments. Mice were made diabetic by intraperitoneal streptozotocin injection (180 mg/kg; Sigma-Aldrich), and those with a nonfasting blood glucose concentration of ≥360 mg/dL were used as recipients. Renal subcapsular islet transplantations were done, as previously described (6,7). Before commencing the study, we performed sample size calculations using data from our previous studies in this minimal mass model of islet transplantation (6,7), which predicted that a power of 0.8 would be achieved using 10 animals in each treatment group. All animal procedures were approved by our institution's ethics committee and performed under license, in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986.

ANXA1$^{-/-}$ knockout mice were generated on a C57BL/6 background, as previously described (17), and bred at Charles River Laboratories. Age- and sex-matched male C57BL/6 wild-type (WT) mice were used as controls for MSC isolation and expansion. MSCs were isolated, expanded, and characterized as previously described (6,7,18).

Graft Function In Vivo

The body weight and blood glucose concentrations of recipient mice were monitored every 3 to 4 days. Reversal of hyperglycemia was defined as nonfasting blood glucose concentrations of ≤200 mg/dL for at least two consecutive readings, without reverting to hyperglycemia on any subsequent day. All mice with blood glucose of <200 mg/dL were given an intraperitoneal glucose tolerance test (IPGTT) 1 month after transplantation. The graft-bearing kidney was removed for analysis of hormone content.

Statistical Analysis

Statistical analysis used the Student *t* test or ANOVA, as appropriate. Two-way repeated-measurement ANOVA was used with the Bonferroni post hoc test to analyze repeated measurements in the same animal at different time points. A Kaplan-Meier survival curve was used to identify differences in the time to cure between groups. A *P* value of <0.05 was considered significant. All data are expressed as means ± SEM.

RESULTS

Identification of MSC-Derived Islet GPCR Ligands

MSC-derived mRNAs encoding peptide ligands for islet GPCRs (13) were identified using a panel of 131 potential ligands to screen adipose- and kidney-derived MSC populations that we have previously demonstrated to enhance islet function in vitro (6–8) and to improve the outcomes of islet transplantation in vivo (6–8,18). Adipose MSCs expressed 36 mRNAs for islet GPCR ligands (Fig. 1*A*), of which, 7 were expressed at high levels (Ct <26), 18 had medium expression levels (Ct 26–30), and 11 were expressed at low levels (Ct >30). Kidney MSCs expressed 26 mRNAs for the ligands (Fig. 1*B*), of which, 3 showed high levels of expression, 14 showed medium expression levels, and 9 were expressed at low levels. The full data set for both MSC populations is summarized in Supplementary Tables 1 and 2 for adipose and kidney MSCs, respectively. ANXA1, stromal cell–derived factor-1 (SDF-1)/chemokine (C-X-C motif) ligand (CXCL) 12, and collagen 3A1 (COL3A1) were all expressed at high levels in both MSC populations, identifying them as potential candidate molecules for the MSC-dependent improvements in islet function we have previously reported for both MSC populations (6,7).

MSCs have been reported previously to express SDF-1/CXCL12 and COL3A1; however, to our knowledge, it has not been previously reported that MSCs express significant levels of ANXA1. Our subsequent investigations thus focused on the potential role of MSC-derived ANXA1 in regulating islet function.

ANXA1 Content and Release by MSCs

To assess ANXA1 expression levels, we used ELISA to quantify ANXA1 immunoreactivity in lysates prepared from adipose MSCs and in samples of MSC-CM. MSC extracts contained 522 ± 7 pg ANXA1 per 200,000 cells (n = 7), and analysis of MSC-CM showed that ~30% of the ANXA1 protein expressed by MSCs was released into the medium over a 72-h incubation period (165 ± 12 pg/well, n = 7). Control samples of MSC culture media maintained for 72 h without MSCs contained negligible amounts of ANXA1 immunoreactivity (<0.1% of MSC-CM). These observations demonstrate that MSCs synthesize and release ANXA1, consistent with it acting as a mediator through which MSCs influence islet function.

In Vitro Function of Islets Precultured With Exogenous ANXA1

To determine whether exogenous ANXA1 alone was sufficient to mimic the effects of MSCs to potentiate GSIS, we measured GSIS from islets that had been precultured for 72 h with nmol/L concentrations of recombinant ANXA1 that were subsequently challenged with glucose in the absence of exogenous ANXA1, as shown in Fig. 2. ANXA1 preculture had no effect on basal (36 mg/dL glucose) insulin secretion but significantly potentiated GSIS (360 mg/dL) in a concentration-dependent manner with maximal effects at 5 nmol/L (Fig. 2*A*). This effect was reproducible between different islet isolations, and Fig. 2*B* shows the mean results from three separate experiments in which ANXA1 preculture (5 nmol/L) significantly enhanced GSIS. The stimulatory effects on insulin secretion of preculture with recombinant ANXA1 was similar to that observed when islets were precultured with monolayer MSCs in a direct-contact configuration (6,7), as shown in Fig. 2*C*, consistent with ANXA1 being causal for the MSC-mediated effects on insulin secretion.

Preculture of islets with ANXA1 conferred protection against cytokine-induced apoptosis, consistent with the reported anti-inflammatory effects of ANXA1 (19). Figure 2*D* shows that preculture for 72 h in the presence of 5 nmol/L ANXA1 significantly reduced caspase 3/7 activity induced by 20-h exposure to a cocktail of inflammatory cytokines but had no effect on basal levels of caspase

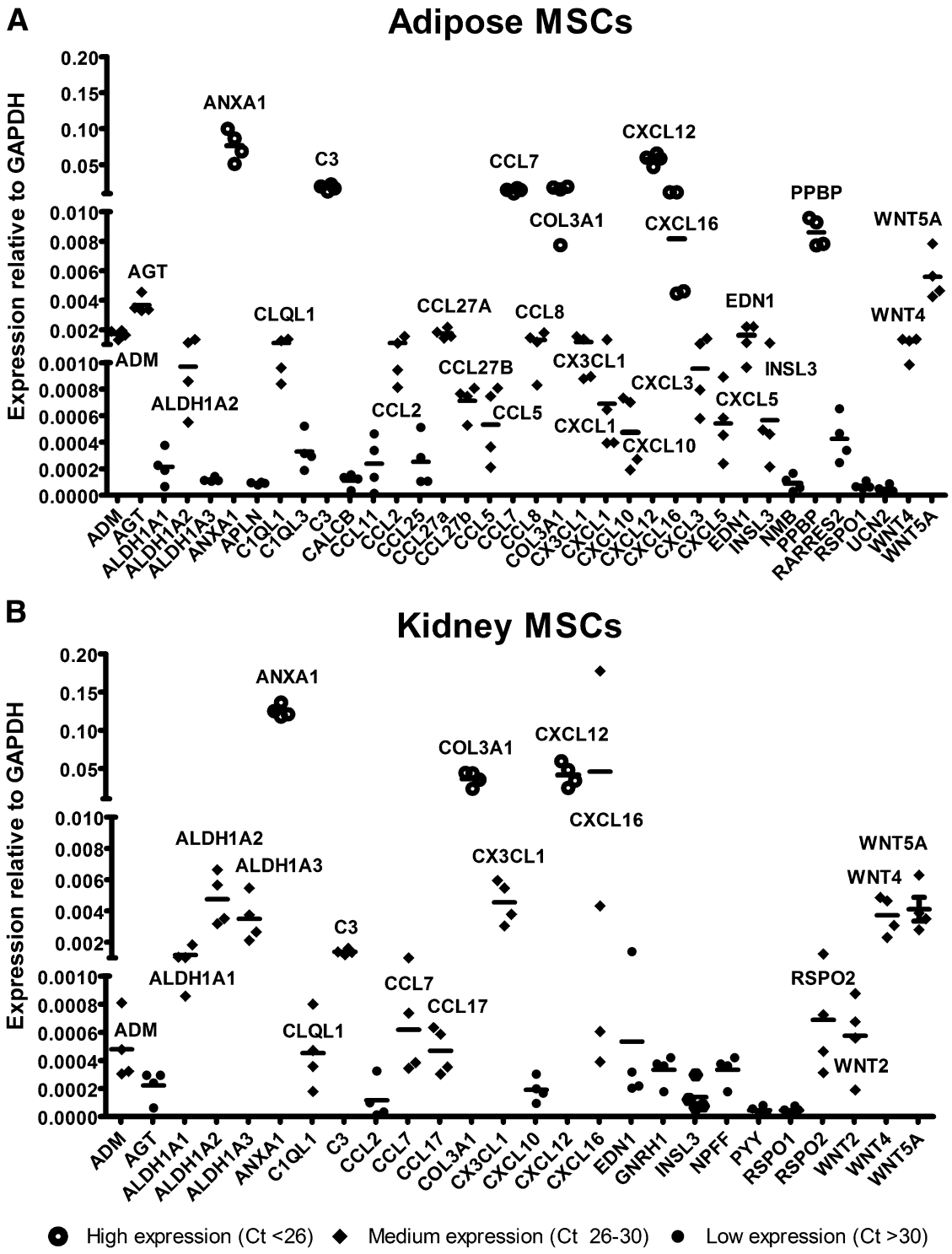

Figure 1—The expression by MSCs of mRNAs for peptide ligands of known islet GPCRs. MSCs derived from mouse adipose (*A*) and kidney (*B*) tissue were cultured as adherent monolayers before harvesting lysates for screening and quantification by qRT-PCR. Data are displayed as the mean expression from four biological replicates, where individual points represent each biological replicate and the horizontal line represents mean value. C3, complement component 3; CCL, chemokine (C-C motif) ligand; PPBP, proplatelet basic protein. The full data set ranked according to Ct value is summarized in Supplementary Tables 1 and 2 for adipose and kidney MSCs, respectively.

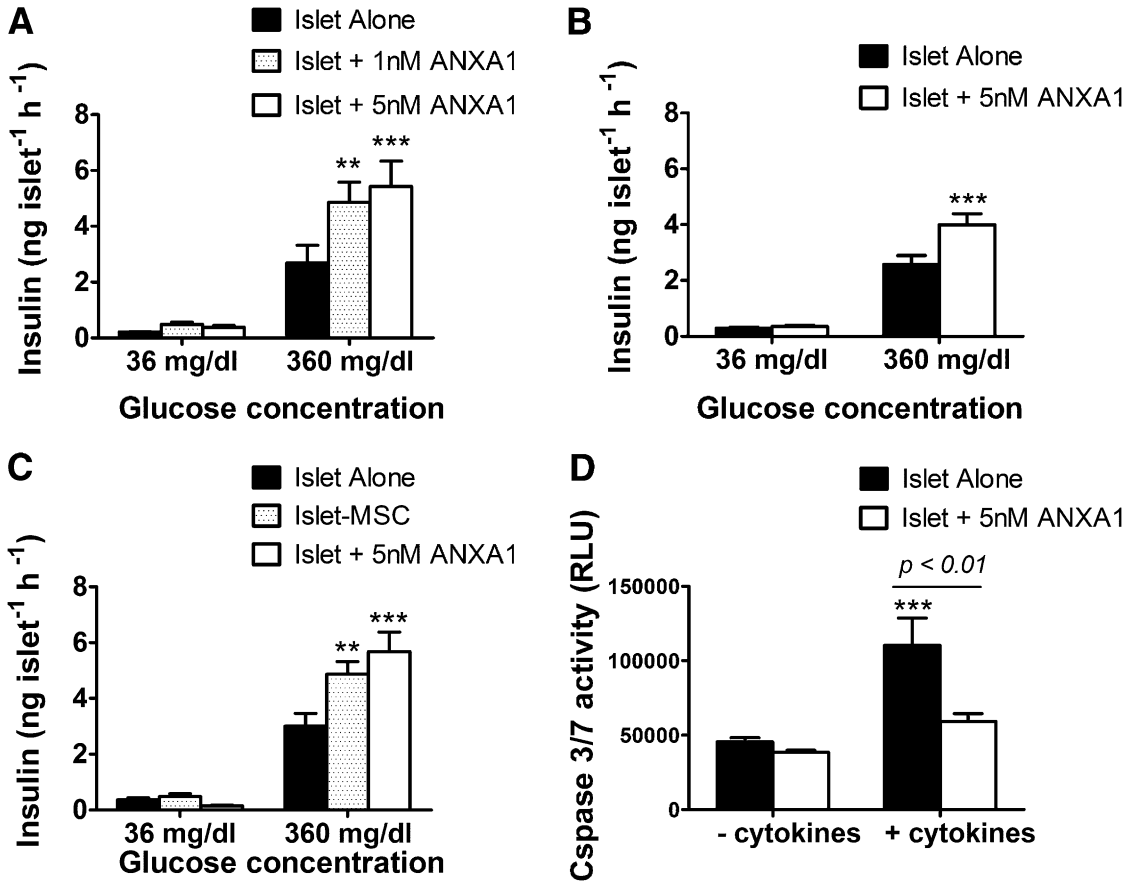

Figure 2—Preculturing islets with exogenous ANXA1 potentiates GSIS and protects from cytokine-induced apoptosis. *A*: Insulin release at 36 mg/dL and 360 mg/dL glucose of 30 replicates of 3 islets per Eppendorf tube, precultured alone, with 1 nmol/L ANXA1, or with 5 nmol/L ANXA1. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. islets cultured alone at the same glucose concentration. *B*: Insulin release at 36 mg/dL and 360 mg/dL glucose of 20–30 replicates of 3 islets per Eppendorf tube, precultured alone or with 5 nmol/L ANXA1. Data presented are representative of three independent experiments. $^{***}P < 0.001$ vs. islets cultured alone at the same glucose concentration. *C*: Insulin release at 36 mg/dL and 360 mg/dL glucose of 20 replicates of 3 islets per Eppendorf tube, precultured alone, with MSCs using a direct-contact preculture configuration, or with 5 nmol/L ANXA1. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. islets cultured alone at the same glucose concentration. *D*: Protection of islets from cytokine-induced apoptosis. Data are representative of three independent experiments in which 6–12 replicates of 5 islets per well in each culture group were assayed. $^{***}P < 0.001$ vs. islets cultured alone in the presence of cytokines for the final 20 h of the 3-day culture period. The *P* values (*A*–*D*) were calculated using two-way ANOVA with the Bonferroni post hoc test.

activity in the absence of cytokines. Islet insulin content was higher in ANXA1 precultured islets (72 h) compared with control islets cultured in the absence of ANXA1 (52.8 ± 5.3 vs. 35.8 ± 3.7 ng/islet, $n = 8$; $P < 0.05$).

siRNA-Mediated Knockdown of ANXA1 Expression in MSCs Reduces Their Functional Capacity to Potentiate GSIS In Vitro

To determine the importance of MSC-derived ANXA1 for MSC-mediated potentiation of islet insulin secretory function in vitro, we used siRNA-mediated knockdown of ANXA1 expression in adipose MSCs. Transient (24-h) exposure to siRNA directed against ANXA1 caused a significant reduction in the expression of ANXA1 mRNA by 24 h compared with MSCs transfected with control nontargeting siRNA (Fig. 3*A*). The knockdown of ANXA1 mRNA was maintained after the removal of the siRNA from the culture media at both 48 h (Fig. 3*B*) and 72 h after transfection (Fig. 3*D*). Immunoblot analysis confirmed that ANXA1 protein was also greatly reduced at 72 h after transfection (Fig. 3*C*).

Figure 3*E*–*G* shows that siRNA-induced knockdown of ANXA1 significantly impaired the ability of MSCs to enhance GSIS. Thus, as expected, coculture of islets with control MSCs transfected with nontargeting siRNA enhanced GSIS secretion after 48 h and 72 h (Fig. 3*F* and *G*, respectively), without affecting basal (36 mg/dL glucose) secretion. In contrast, islets precultured with MSCs in which ANXA1 expression was reduced by siRNA treatment showed significantly reduced GSIS at all three assessments compared with islets precultured with control ANXA1-expressing MSCs (Fig. 3*E*–*G*). These observations demonstrate that reducing ANXA1 expression in MSCs reduces their functional capacity to potentiate islet insulin

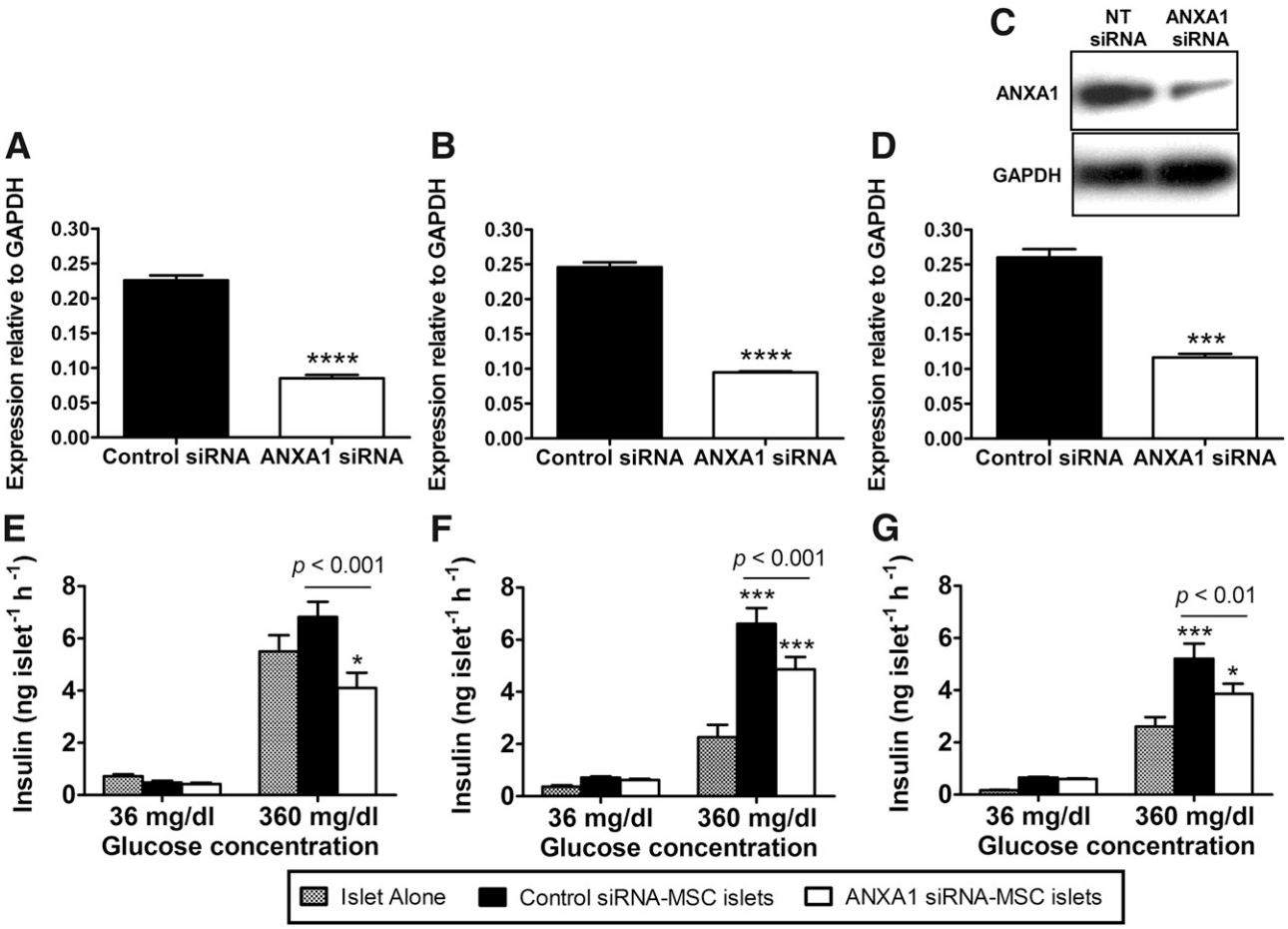

Figure 3—siRNA-mediated knockdown of ANXA1 expression in adipose-derived MSCs reduces their functional capacity to potentiate GSIS in vitro. Expression of ANXA1 in adipose-derived MSCs at 1 day (*A*) and 2 days (*B*) after transfection with control nontargeting or ANXA1 siRNA (n = 3). ****P < 0.0001 (calculated by Student t test) vs. MSCs transfected with control nontargeting siRNA. *C*: ANXA1 immunoreactivities (38 kDa) were detected by immunoblots in adipose MSCs transfected with nontargeting (NT) or ANXA1 siRNA at 3 days after transfection. ANXA1 was normalized against GAPDH (35.8 kDa). *D*: Expression of ANXA1 in adipose-derived MSCs at 3 days after transfection, as in *A* and *B* (n = 3). ***P < 0.001 vs. MSCs transfected with control nontargeting siRNA. Insulin release at 36 mg/dL and 360 mg/dL glucose of 30 replicates of 3 islets per Eppendorf tube, precultured alone, with control nontargeting siRNA transfected MSCs, or with ANXA1 siRNA transfected MSCs for 1 day (*E*), 2 days (*F*), or 3 days (*G*). ***P < 0.001 and *P < 0.05 vs. islets precultured alone at the same glucose concentration. The P values were calculated using two-way ANOVA with the Bonferroni post hoc test.

secretory responses in vitro, suggesting that ANXA1 is a mediator of this effect.

ANXA1 Knockout MSCs Lack the Functional Capacity to Potentiate GSIS In Vitro

To validate our observations using siRNA to knockdown ANXA1 expression in MSCs, we also used MSCs derived from ANXA1 knockout ($ANXA1^{-/-}$) mice. Adipose and kidney MSCs were isolated from $ANXA1^{-/-}$ mice, as previously described (6,7,18), and compared with those isolated from control WT mice. The morphology of MSCs derived from $ANXA1^{-/-}$ mice was similar to that of WT MSCs, and islets maintained a similar morphology during preculture on WT and $ANXA1^{-/-}$ MSC populations (Supplementary Fig. 1). RT-PCR amplification using primers to detect mRNA encoding ANXA1 produced a product of the appropriate size (170 bp) from WT MSC cDNA. In contrast, no product was detected in $ANXA1^{-/-}$ MSC cDNA (Supplementary Fig. 2), confirming the expected phenotypes for the MSC populations.

Adipose and kidney MSCs derived from $ANXA1^{-/-}$ mice lacked the functional capacity to enhance islet GSIS, as shown in Fig. 4. As expected, preculture for 72 h with MSCs isolated from WT mice significantly enhanced subsequent GSIS, without affecting basal secretion (Fig. 4). In contrast, a similar preculture with $ANXA1^{-/-}$ MSCs had no significant effect on GSIS or on basal secretion. These findings are in accordance with our observations using siRNA-mediated knockdown of ANXA1 expression in MSCs, suggesting that ANXA1 is required for the MSC-mediated enhancement of insulin secretion in vitro.

We also isolated islets from $ANXA1^{-/-}$ mice and WT C57BL/6 controls to determine whether ANXA1 deficiency

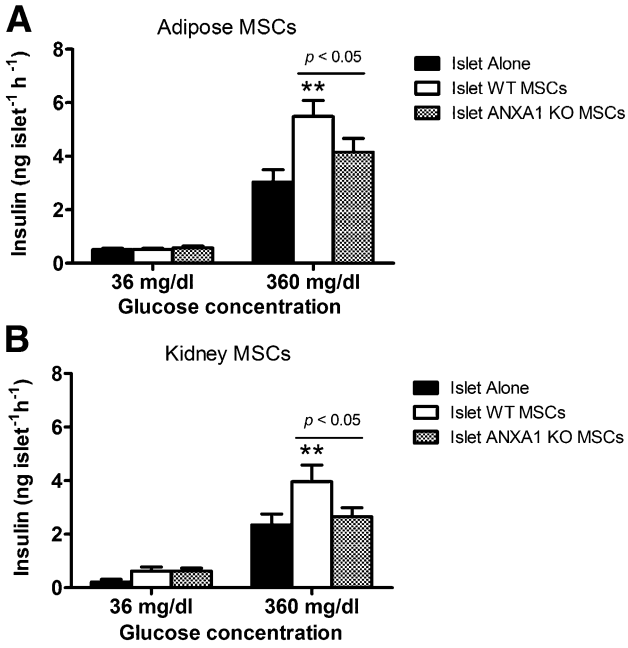

Figure 4—ANXA1$^{-/-}$ MSCs have no functional capacity to potentiate GSIS in vitro. *A* and *B*: Insulin release at 36 mg/dL and 360 mg/dL glucose of 30 replicates of 3 islets per Eppendorf tube, precultured alone, with WT MSCs, or with ANXA1$^{-/-}$ MSCs for 3 days. Data presented are representative of three independent experiments. $^{**}P < 0.01$ (calculated by repeated-measures ANOVA with Bonferroni post hoc test) vs. islets precultured alone at the same glucose concentration.

alters their functional phenotype in vitro. There were no differences in basal or GSIS between ANXA1$^{-/-}$ and WT islets (WT GSIS: 384 ± 67% basal; ANXA1$^{-/-}$ GSIS: 312 ± 48% basal, $n = 30$; $P > 0.4$). The insulin content of freshly isolated islets was also comparable between WT and ANXA1$^{-/-}$ knockout mice (WT 66.7 ± 7.3 ng/islet; ANXA1$^{-/-}$ 62.8 ± 4.7 ng/islet, $n = 8$; $P > 0.6$).

In Vivo Function of ANXA1 Precultured Islets

Preculturing islets with ANXA1 (72 h at 5 nmol/L) at a concentration that enhanced GSIS in vitro had modest effects on their ability to regulate blood glucose in vivo, as shown in Fig. 5. The average blood glucose levels of mice transplanted with ANXA1 precultured islets were consistently lower than those of mice transplanted with islets precultured in the absence of ANXA1 at all post-transplantation time points (3–28 days, Fig. 5*A*), but this effect did not reach statistical significance.

After 28 days, 7 of 10 mice transplanted with ANXA1 precultured islets had cured, compared with only 3 of 10 mice transplanted with islets precultured alone (Fig. 5*B*). This effect of ANXA1 preculture on graft curative capacity did not achieve statistical significance, although there was a strong trend toward an ANXA1-dependent effect ($P = 0.07$). The glucose tolerance of cured mice was similar in recipients of ANXA1 precultured and control grafts at 28 days after transplantation (Fig. 5*C*), in accordance with our previous observation using MSC-pretreated islets (6). The insulin content of grafts retrieved after 28 days was variable, ranging from 0.4 to 9.2 and from 0.7 to 10.4 μg/graft for recipients of control islets and islets precultured with 5 nmol/L ANXA1, respectively. The mean graft insulin content was ~30% higher in grafts consisting of ANXA1 precultured islets, although this was not statistically significant (Fig. 5*D*).

DISCUSSION

MSCs possess a number of properties that make them excellent candidates for improving the functional survival of transplanted tissues. They act as mobile multidrug dispensers, migrating to the site of tissue damage to release immunomodulatory and anti-inflammatory molecules that influence cells of the innate and acquired immune systems (20–22). MSCs also secrete a wide range of factors that induce tissue repair and angiogenesis (23); release microvesicles containing microRNAs, lipids, and proteins (24); and act as a stromal cell support system by laying down extracellular matrix (ECM), which may provide a physical niche for transplanted cells (25). Recent studies, including our own (6–8), have established that MSCs have the capacity to enhance islet function in vitro (4,9–11) and have demonstrated that cotransplantation of islets and MSCs into diabetic animals improves islet functional survival and glycemic control (3,5,8,18,26–29).

However, dissecting out the mechanisms underlying these in vivo effects is difficult because of the pleiotropic functionality of MSCs. We recently reported that preculture of islets with MSCs in a direct-contact coculture system confers beneficial effects on islet function that persist in islet-alone grafts (6,7), demonstrating that MSCs have direct influences on donor islet cells independent of their effects on the host tissues within the graft niche. These observations present a way of enabling the beneficial properties of MSCs to be incorporated into clinical islet transplantation protocols. Thus, although coengrafting islets and MSCs is technically feasible at the experimental kidney site, this obvious therapeutic strategy of cotransplanting islets and MSCs is confounded by the choice of the hepatic portal vein as the preferred clinical implantation site. Coinfused islets and MSCs disperse throughout the portal vasculature and are therefore unlikely to engraft together, with islets lodging in the hepatic microcirculation, whereas MSCs will most likely pass through the liver and engraft in the lung microcirculation (30). Because codelivering effective numbers of MSCs with the islet graft is not practical, an alternative strategy is to determine how MSCs influence islet function and devise mechanisms for delivering similar effects in an MSC-free system.

Previous studies suggest that the beneficial effects of MSCs on islet function are at least partly mediated by soluble bioactive molecules (12), so we have used an unbiased qRT-PCR screening approach to identify MSC-derived peptide ligands for GPCRs expressed by islets

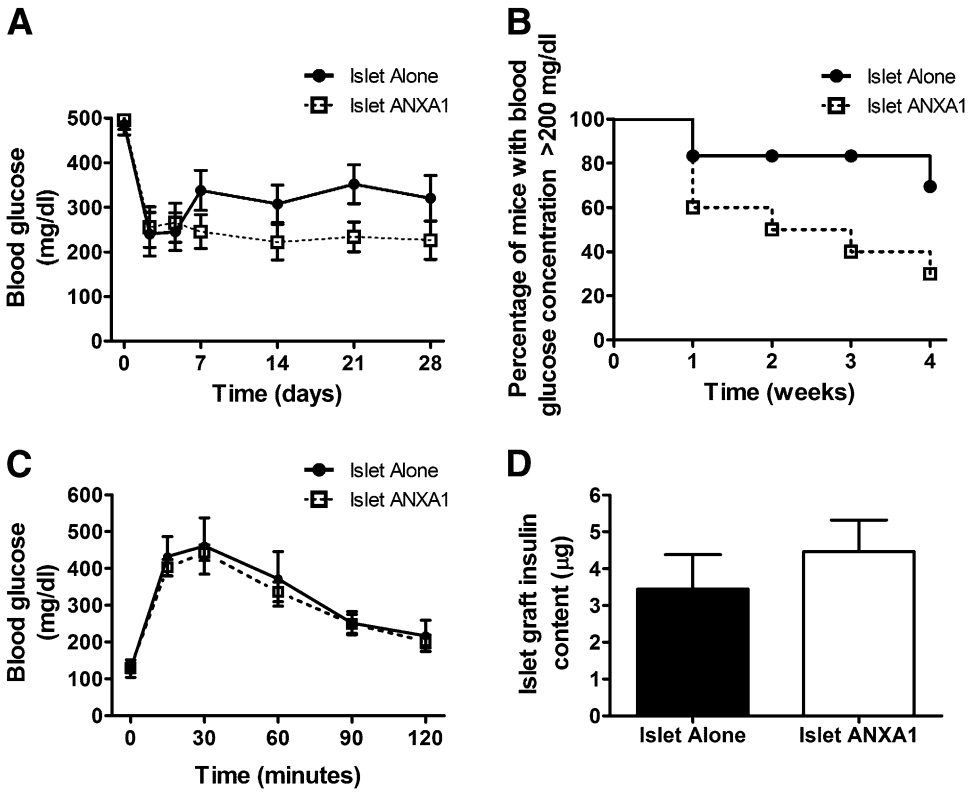

Figure 5—In vivo graft function of ANXA1 precultured islets. *A*: Blood glucose concentrations of mice transplanted with 150 islets precultured alone or with 5 nmol/L ANXA1 for 3 days (n = 10). $P > 0.05$ (calculated by repeated-measures ANOVA with Bonferroni post hoc test). *B*: Graft curative capacity. Percentage of mice remaining diabetic (blood glucose concentration >200 mg/dL) after transplantation as above (*A*) (n = 10 for both transplant groups). $P = 0.07$ (Kaplan-Meier). *C*: IPGTTs in all cured mice at 1 month after transplantation (n = 3–7). $P > 0.05$ (calculated by repeated-measures ANOVA with Bonferroni post hoc test). *D*: Islet graft insulin content at 1 month after transplantation (n = 10). $P > 0.05$ by Student t test.

cells (13). Comparing mRNA expression profiles in populations of MSCs with proven beneficial effects on islet function allowed us to identify highly expressed molecules common to the different populations as potential candidates for influencing islet function, with the three most highly expressed mRNA species being ANXA1, SDF-1/CXCL12, and COL3A1. CXCL12 is an immunomodulatory molecule that has been widely investigated because of the functional importance of CXCL12/CXC receptor type 4 interactions for MSC homing to injured tissues. CXCL12 has an established role in protecting islets from cytokine-induced apoptosis (31,32), and it has recently been reported that codelivery of CXCL12 enhanced islet graft survival and function in a mouse model of diabetes (33). However, this effect was attributed to CXCL12 modulating cells of the host immune system rather than influencing islet cell function. MSCs actively produce ECM in vitro (33), and several reports have demonstrated the functional importance of ensuring a rapid reestablishment of islet-ECM interactions for isolated and transplanted islets (34–36). We previously demonstrated that cotransplanting MSCs with islets in the renal subcapsular site leads to extensive deposition of ECM, which may enhance function by maintaining correct islet architecture (18). However, there is no evidence that MSC-derived COL3A1 acts as a soluble, receptor-mediated regulator of insulin secretion, so we focused our investigation on the potential role of ANXA1 as an MSC-derived regulator of islet function.

ANXA1, also known as lipocortin 1, is a 37 kDa member of the superfamily of Ca^{2+} and phospholipid-binding proteins, with a widespread tissue localization (37). The high expression levels of ANXA1 in cells of the hematopoietic lineage are consistent with its well-documented anti-inflammatory properties (38) and explain the antiapoptotic effects observed in the current study when islets were precultured with ANXA1 before exposure of inflammatory cytokines. However, our results also demonstrate that ANXA1 directly influences islet β-cell secretory function and that those effects persist in vitro after ANXA1 is removed from the culture medium. Acute effects of ANXA1 on insulin secretion have been reported previously, but these were only seen in the presence of ANXA1 (39–41).

Exogenous ANXA1 may influence islet cells through the formyl peptide receptor (FPR) family of GPCRs. However, annexins can also exert GPCR-independent effects (38) by binding to phosphatidylserine residues on

cell membranes (42). ANXA1 is endogenously expressed in islets, where it is localized to insulin secretory granules, and has been suggested to influence GSIS in an autocrine or paracrine manner (43,44). Our measurements of insulin secretion from ANXA1$^{-/-}$ islets suggest a redundant role for endogenous ANXA1 in the acute control of GSIS. Our studies consistently suggest that ANXA1 is a key mediator of the beneficial effects of MSCs on islet function induced by coculture of islets and MSCs. Although ANXA1 does not contain a secretory signal sequence, it can be exported from cells via noncanonical pathways to have localized effects, and we have demonstrated that MSCs synthesize ANXA1 and release it into the culture medium. In our MSC/islet coculture studies, the MSCs grow as an adherent monolayer, and islets are cocultured in direct contact on top of the monolayer MSCs (6,7). This configuration is likely to expose the islets to localized high concentrations of secreted ANXA1 at the interface between MSCs and cocultured islets. It is also well established that MSC-secreted products can bind to ECM or cell surfaces, producing localized high concentrations of biologically active molecules (45,46). A similar mechanism could enable MSCs to present biologically effective concentrations of ANXA1 to adjacent islet cells in coculture or cotransplant configurations. Reductions in ANXA1 production by MSCs reduced the effects of MSC preculture on insulin secretion, and the extent of this effect correlated with the degree of suppression of ANXA1 production. Thus, the partial reduction in ANXA1 expression achieved using siRNA knockdown was associated with a reduced effect of the MSCs to enhance insulin secretion, whereas the genetic deletion of ANXA1 completely abolished the effects on insulin secretion of islet/MSC coculture. Together, these observations are consistent with MSC-derived ANXA1 being a key mediator of the effects of MSCs to enhance GSIS in vitro.

Preculture of islets with ANXA1 before transplantation had only modest effects on their ability to regulate blood glucose in streptozotocin-induced hyperglycemic mice. We have previously used this experimental model and this number of experimental animals to demonstrate statistically significant improvements in glycemic control and curative capacity of islet grafts after preculture with MSCs, whether implanted in the renal subcapsular (6) or intraportal (7) site, and our sample size calculations based on data from these previous studies predicted a power of 0.8 using 10 animals in each treatment group. The current observations therefore demonstrate that ANXA1 preculture alone is not sufficient to fully mimic the beneficial effects of islet/MSC coculture on transplantation outcomes and suggest that MSCs influence islet function through mechanisms in addition to ANXA1 production. This is not surprising given the pleiotropic effects of MSCs and the numerous secreted products identified in our screening assay, which was limited to putative GPCR peptide ligands and did not address molecules that may influence islet function through other classes of receptor. However, ANXA1 preculture alone was sufficient to mimic the effects of islet/MSC coculture on insulin secretory responses in vitro, suggesting that the additional beneficial effects conferred by MSCs on islet function in vivo are most likely to be primarily via effects on islet cell survival in a hostile inflammatory, hyperglycemic microenvironment. Further studies will be directed toward identifying the molecules involved in these effects and using a combinatorial approach to establish a defined "cocktail" of MSC-derived secretory products to support islet functional survival after transplantation.

In summary, we have used measurements of islet GPCR ligand mRNA expression levels in MSCs to identify ANXA1 as one biologically active molecule through which MSCs influence islet function and have demonstrated that maintaining islets in culture in the presence of exogenous ANXA1 alone is sufficient to mimic some of the beneficial effects of MSCs. The identification of ANXA1 and similar molecules as MSC-derived secretory products offers the potential for devising a defined and cell-free approach to improving islet transplantation protocols while avoiding the problems inherent in variability between MSC populations (47) and the clinical, safety, and regulatory disadvantages of incorporating another cell type (MSCs) into current islet transplantation protocols.

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