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Brief Report: Elastin Microfibril Interface 1 and Integrin-Linked Protein Kinase Are Novel Markers of Islet Regenerative Function in Human Multipotent Mesenchymal Stromal Cells

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ABSTRACT

Multipotent mesenchymal stromal cell (MSC) transplantation is proposed as a novel therapy for treating diabetes by promoting the regeneration of damaged islets. The clinical promise of such treatments may be hampered by a high degree of donor-related variability in MSC function and a lack of standards for comparing potency. Here, we set out to identify markers of cultured human MSCs directly associated with islet regenerative function. Stromal cultures from nine separate bone marrow donors were demonstrated to have differing capacities to reduce hyperglycemia in the NOD/SCID streptozotocin-induced diabetic model. Regenerative (R) and non-regenerative (NR) MSC cultures were directly compared using isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomics. A total of 1,410 proteins were quantified resulting in the identification of 612 upregulated proteins and 275 downregulated proteins by ± 1.2-fold in R-MSC cultures. Elastin microfibril interface 1 (EMILIN-1), integrin-linked protein kinase (ILK), and hepatoma-derived growth factor (HDGF) were differentially expressed in R-MSCs, and Ingenuity Pathway Analyses revealed each candidate as known regulators of integrin signaling. Western blot validation of EMILIN-1, ILK, and HDGF not only showed significantly higher abundance levels in R-MSCs, as compared with NR-MSCs, but also correlated with passage-induced loss of islet-regenerative potential. Generalized estimating equation modeling was applied to examine the association between each marker and blood glucose reduction. Both EMILIN-1 and ILK were significantly associated with blood glucose lowering function in vivo. Our study is the first to identify EMILIN-1 and ILK as prospective markers of islet regenerative function in human MSCs. STEM CELLS *2016;34:2249–2255*

SIGNIFICANCE STATEMENT

The vast majority of current stem cell based clinical trials are testing the therapeutic capacity of multipotent mesenchymal stem cells for treating a wide range of diseases. One of the critical issues plaguing the progress of these trials is a lack of suitable and reliable methods for measuring the potency of these stem cells. This study uncovers two new potential proteins whose abundance in human bone marrow mesenchymal stem cell cultures provides an accurate indication of their potency for repairing damaged pancreatic islets, as would be found patients suffering from Type I diabetes. We predict these proteins to impact the development of novel stem cell products for treating pancreatic diseases.

INTRODUCTION

Multipotent mesenchymal stromal cells, also called mesenchymal stem cells (MSCs), are strong candidates for regeneration-inducing therapies during diabetes, as these cells promote islet repair and can modulate the immune system, two aspects critical for the treatment of type 1 diabetes [1, 2]. Phase 2 clinical trials using human MSCs for the treatment of type 1 diabetes mellitus are currently underway. Using the well-established streptozotocin (STZ)-treated diabetic NOD/SCID model [3], we have recently shown that transplanted human MSCs can stimulate endogenous islet formation associated

with ductal structures resulting in improvements in islet function and glycemic control [4–6].

MSCs are found at extremely low frequency in the bone marrow and peripheral tissues. Thus, expansion in vitro is necessary prior to clinical use. As a result, current methods for culturing MSCs generate a heterogeneous mixture of stromal cells with variable functional properties. Unfortunately, standards currently used to characterize the purity of MSC cultures do not address this heterogeneity with respect to clinically-relevant secretory functions, such as immune suppression or paracrine islet restoration. This was illustrated in preclinical studies where MSC cultures demonstrated identical properties of surface phenotype and multipotent differentiation capacity, but showed a high degree of donor- and culture duration-dependent variability in their capacity to improve glycemia after transplantation in STZ-treated mice [4, 6]. In addition, both intra- and inter-individual heterogeneity from MSC preparations has been previously reported to affect their functional properties [7–12]. These studies reinforce the importance of identifying functionally relevant potency-associated markers that permit the identification of MSCs with indication-specific therapeutic potential.

We used quantitative proteomics comparing MSC cultures with and without islet-regenerative capacity as a comprehensive system to identify MSC-specific markers associated with islet regeneration. Herein, we establish EMILIN-1 and ILK as markers highly associated with MSC islet-regenerative capacity, suggesting integrin-mediated extracellular matrix signals influence the efficacy of MSC-based cellular therapies in the context of islet regeneration.

Material and Methods

Detailed materials and methods were provided as Supporting Information.

Isolation and Propagation of MSCs from Human Bone Marrow

Human bone marrow was drawn from the iliac crest of normal donors with informed consent from the London Health Sciences Centre, London, Ontario and MSCs were derived as previously described [1, 3, 4, 6, 13]. In-process data for MSC characterization and donor-related factors are provided in Supporting Information Table 1.

Transplantation of MSCs into STZ-treated NOD/SCID Mice

Animal care was in accordance to the Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Western Ontario. Immunodeficient NOD/SCID mice (Jackson Labs) were intraperitoneally-injected (i.p.) with 35 mg/kg streptozotocin (STZ) (Sigma-Aldrich) for 5 consecutive days, as previously described [3–6, 14]. Mice reaching hyperglycemia on day 10 (15–25 mmol/L) were sublethally irradiated (300 cGy) and transplanted by tail vein injection with PBS or MSCs (5×10^5). Systemic blood glucose was monitored weekly for 42 days, as previously described [1, 3–6]. MSCs from individual donors were classified as either "regenerative" (R) or "non-regenerative" (NR) based on their capacity to reduce hyperglycemia in transplanted mice.

Table 1. EMILIN-1 and ILK abundance levels were significantly associated with blood glucose level ("≥ 20 mM" and "< 20 mM") based on the generalized estimating equation modeling

Parameter	Estimate	Standard error	*p*-value
EMILIN-1	159.14	40.83	<.0001
ILK	178.84	40.86	<.0001
HDGF	58.19	37.39	.1196

The results were summarized based on the generalized estimating equation modeling; the independent variables are day and one of the potential markers investigated in our study. The *p*-values of EMILIN-1, ILK, and HDGF are 0.0348, 0.0286, and 0.0579, respectively, in the mixed model.

Sample Preparation for iTRAQ Labeling

iTRAQ was performed using subcellular fractionations (cytosolic, membrane, nuclear) which were generated by QProteome Cell Compartment Kit (QIAGEN) according to the manufacturer's instructions and mass spectrometry analysis was performed with QTof Synapt and LT-FTICR, both coupled to a Waters nanoAcquity UPLC. A total of 1,410 proteins were identified in all conditions, where proteins with ratios either at $>+1.2$ or <-1.2 comparing the islet regenerative versus non-regenerative groups were considered to be differentially expressed, and canonical and non-canonical pathways (p-value $\leq .05$) activated were analyzed using Ingenuity Pathway Analysis (version 23814503).

Quantitative Infrared Western Blotting

Whole protein lysates were prepared for quantitative infrared Western blotting, as described previously [15]. Bands were detected using the ChemiDoc XRS + system for fluorescent secondary antibodies and quantified with Image Lab Software (Bio-Rad Laboratories). The quantification of Western blots was performed using a loading control (i.e. gapdh or alpha-tubulin) according to the method described in Degasperi [16]. Three technical replicates were performed and representative blots are shown.

Statistical Analyses

To examine the association between each identified potential markers and blood glucose level after transplantation, the blood glucose data between day 14 and 42 and the expression-level average values (obtained by Western blot) were examined for EMILIN-1, HDGF, and ILK. Blood glucose was analyzed as binary data (i.e., "≥ 20 mM" and "< 20 mM"), and its association with potential markers was assessed using generalized estimating equation (GEE) modeling. Since a blood glucose level of less than 20 mM is a clinical meaningful and reliable indicator of islet regeneration, the GEE modeling is considered the primary analysis of our study. The GEE modeling allows sample-specific correlation across time (i.e., days after MSC i.v. injection), and an auto-regression correlation structure was chosen for the modeling based on quasi-likelihood information criterion statistics [17, 18]. In the secondary analysis assessing the association, the blood glucose data were analyzed as continuous data or using a mixed model, and an auto-regressive covariance structure was chosen based on interpretability and Akaike's Information Criteria. Statistical models were implemented using SAS EG4.2.

Analysis of the area under each glucose tolerance curve was performed with student two-tailed *t*-test (Fig. 1). Analysis

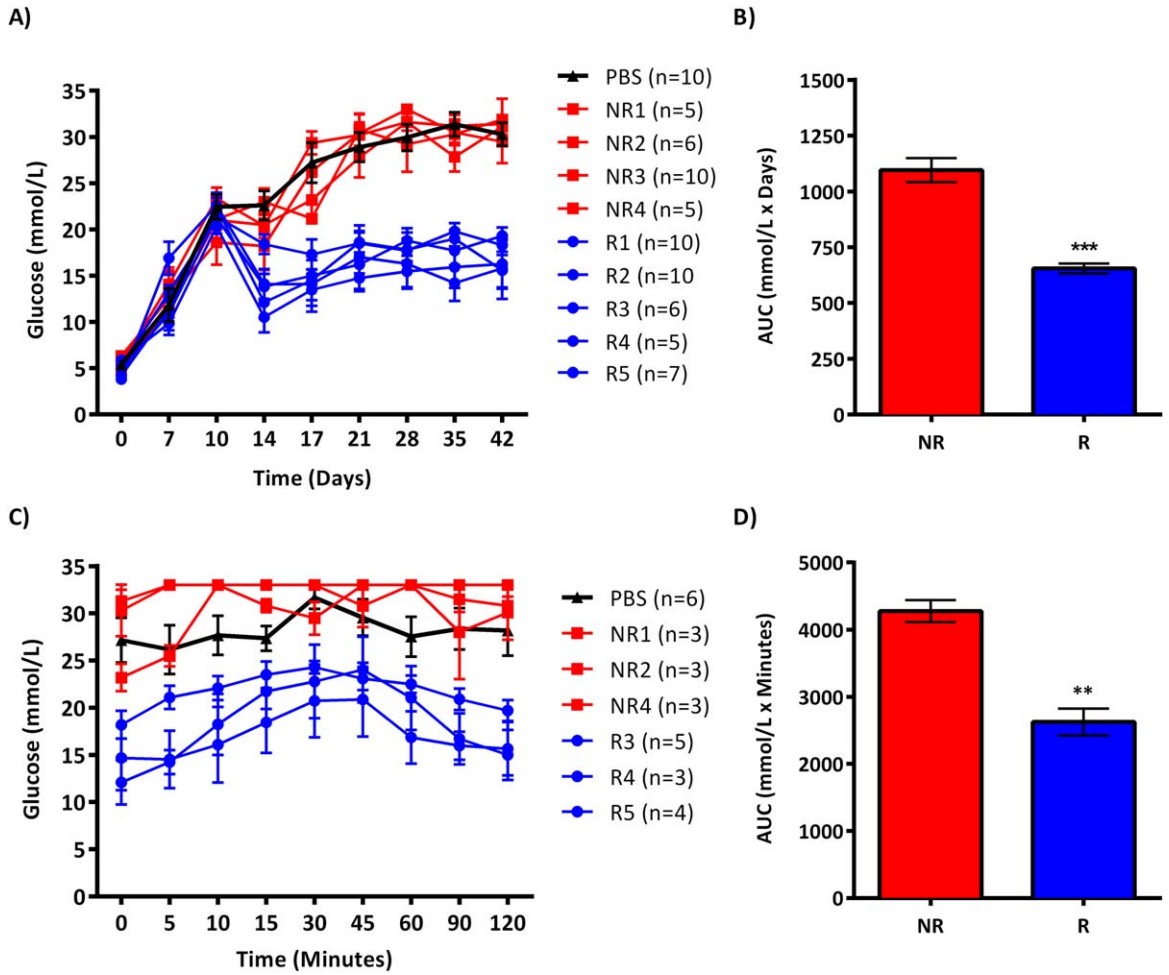

Figure 1. Transplantation of islet regenerative mesenchymal stem cell (MSC) samples induces recovery from hyperglycemia and improved glucose tolerance. **(A)**: MSC cultures derived from nine independent donors ($N = 9$) were transplanted at day 10 into STZ-treated nonobese diabetic/SCID mice and segregated into MSC samples that demonstrated islet-regenerative capacity (R) and those that did not (NR) based on significant ($p < .05$) recovery from hyperglycemia at days 17–42 compared to PBS-injected controls ($n =$ number of mice transplanted/MSC sample). **(B)**: Analysis of the area under each glucose curve was calculated for all mice transplanted with R-MSC and NR-MSC samples. R-MSC samples showed significantly reduced area under the curve. **(C)**: Glucose tolerance tests were performed for 2 hours after intraperitoneal injection of a glucose bolus (2 mg/kg) at day 42 on mice transplanted with 3 R versus 3 NR samples. **(D)**: Analysis of the area under each glucose tolerance curve was calculated for mice transplanted with R-MSCs and NR-MSCs. R-MSC samples showed significantly reduced area under the curve. Analysis of significance was performed by student *t*-test. **, $p < .01$, ***, $p < .001$. Abbreviation: AUC, area under the curve.

of Fig. 3A, 3B was executed by Mann-Whitney two-tailed test and one-way ANOVA followed by Tukey's post hoc test, respectively. A *p*-value $< .05$ was considered statistically significant. The analyses related to Figs. (1 and 3) were performed with GraphPad Prism software version 6.0.

Results

MSCs Derived from Independent Donors Showed Variable Capacity to Reduce Hyperglycemia in STZ-treated NOD/SCID Mice

Using the STZ-treated NOD/SCID model to assess islet regeneration [5, 6], we initially set out to identify islet regenerative (R) and nonregenerative (NR) MSC samples, based on donor-dependent capacity to reduce hyperglycemia after transplantation. MSCs from nine healthy bone marrow (BM) donors were i.v.-injected at passage 4 into NOD/SCID mice at day 10 following STZ-treatment from days 1–5 (Fig. 1A). Blood glucose levels were measured weekly for up to 42 days post STZ-treatment. Compared with PBS-injected controls that remained severely hyperglycemic (>25 mmol/L), injection of MSCs (5×10^5) derived from five independent donors led to significantly improved systemic glycemia from days 14–42 (Fig. 1A, 1B). In contrast, mice injected with MSCs from four other independent donors maintained blood glucose levels similar to PBS injected controls (Fig. 1A, 1B). Glucose tolerance tests were also performed after i.p. injection of a glucose bolus (2 mg/kg) at day 42 on mice transplanted with three NR-MSC versus three R-MSC samples. Mice transplanted with R-MSC demonstrated significantly improved glucose tolerance (Figure 1C, 1D). These data confirm that the ability of transplanted BM-derived MSCs to improve glucose control varied between donors and could be clearly segregated into either islet R or NR sub-groups. Importantly, we have previously established that transplanted human BM-MSCs do not acquire insulin expression in vivo. In

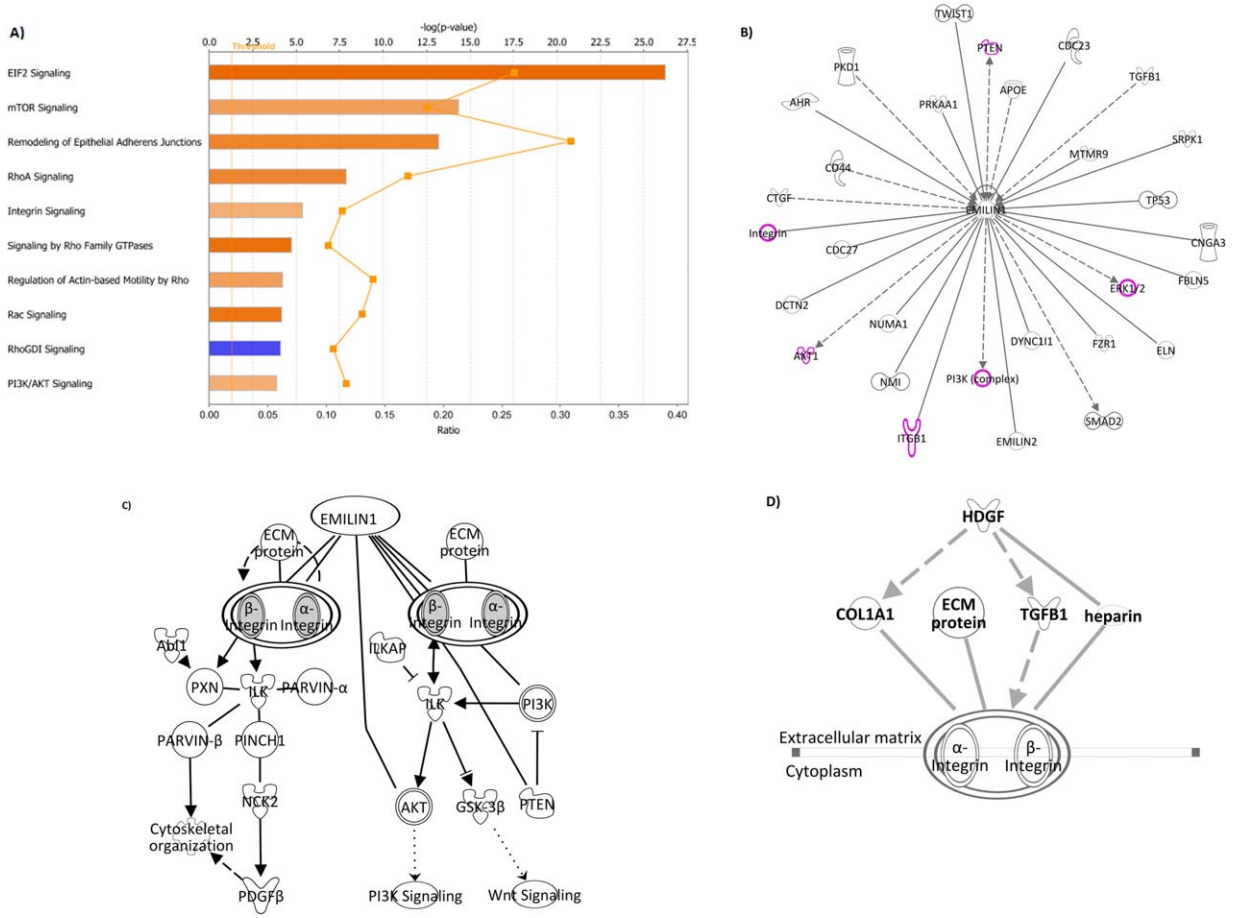

Figure 2. Pathway enrichment analysis showed enriched integrin and EMILIN-1 signaling pathways. **(A)**: Top canonical pathways that were significantly enriched from the differentially regulated protein dataset. The orange straight line represents the threshold for significantly enriched categories, where the *p*-value is .05 (or—log *p*-value of 1.30) found by a modified Fisher's exact test. The top canonical pathways are indicated (cut-off of *p*-value of .001 or −log of *p*-value of 3.0; *z*-score > 1.0). The orange colored bars indicate predicted pathway activation in R-MSC samples and the blue bar indicates pathway inhibition. The overall activation/prediction state of canonical pathways are predicted based on a *z*-score algorithm (i.e., the darker the color of the bars, the higher the *z*-score). The orange points connected by a thin line represent the ratio of proteins in a given category obtained from the differently regulated protein dataset divided by the total number of proteins present in that pathway. **(B)**: EMILIN-1 pathway was found to be a unique noncanonical pathway. EMILIN-1 is represented here with its pathway members. Highlighted in magenta are known targets of the integrin canonical pathway. **(C)**: EMILIN-1 and ILK relationships to the integrin pathway were inquired using the Path Explorer's tool. (**D**): Hepatoma-derived growth factor (HDGF) relationship to the integrin pathway was enquired using the Path Explorer's tool. The shortest paths to integrin are depicted here. Dashed lines represent indirect relationships and nondashed lines represent direct relationships. Data analysis was performed using Ingenuity Pathway Analysis version 23814503. Abbreviations: ECM, extracellular matrix; EMILIN-1, Elastin microfibril interface 1; ILK, integrin-linked protein kinase.

contrast, injected BM MSCs engraft murine tissues and stimulate the formation of small islet clusters in the recipient pancreas via undetermined secretory activities [3–5].

Quantitative Proteomic Analysis of R- and NR-MSC Samples Identified Prospective Markers Associated with Islet Regeneration

Based on this in vivo platform, MSCs from R-MSCs ($n = 4$) and NR-MSCs ($n = 2$) samples were expanded to generate sufficient cell numbers to allow quantitative proteomic comparison between sub-groups. We applied iTRAQ-based quantitative proteomics following upstream protein fractionation of cytosolic, nuclear and membrane subcellular fractions with subsequent purification of peptides based on isoelectric point using off-gel fractionation. A total of 1,410 proteins were identified and quantified by 1D LC-MS-MS, where 612 proteins were observed to be upregulated and 275 downregulated in R-MSCs by ± 1.2 fold. To further filter the data using a non-biased approach, we analyzed differently regulated proteins using Ingenuity Pathway Analysis (IPA) to search for canonical and non-canonical signaling pathways significantly modulated in R-MSCs (Fig. 2A). These analyses revealed that nine canonical pathways were significantly activated in R-MSCs, including EIF2 and mTOR signaling, remodeling of epithelial junctions, RhoA signaling and integrin signaling (Fig. 2A, orange bars, $p < .00001$), and that 1 pathway was significantly repressed in R-MSCs, namely the RhoGDI signaling pathway (Fig. 2A, blue bar, $p < .0001$). Of these 10 enriched pathways, 8 (mTOR, RhoA, Integrin, Rho family GTPases, Actin-based motility by Rho, Rac, RhoGDI and PI3K/AKT) are known to be engaged following ECM-integrin activation. Interestingly, both the Target of Rapamycin (mTOR) and Ras homology, member A (RhoA) pathways can be activated by integrin engagement via the activity of integrin-linked kinase (ILK) and focal adhesion kinase (FAK) [19], respectively. Based

on these results highlighting the importance of ECM-integrin engagement from R-MSCs, candidate proteins involved in the integrin signaling pathway were further scrutinized using IPA to identify proteins for further functional validation as potential markers of R-MSCs. Several canonical intracellular integrin pathway proteins were found to be differentially regulated in R-MSCs, as highlighted in Supporting Information Fig. 1. More notably, examination of differentially regulated proteins participating in non-canonical integrin pathways uncovered the extracellular matrix protein EMILIN-1 (Fig. 2B). As depicted in Fig. 2C, EMILIN-1 interacts directly with integrin alpha and beta subunits, as well as with other players involved in the integrin-ILK pathway, including PTEN, PI3K, and AKT, but do not directly interact with ILK. A second extracellular matrix protein upregulated in R-MSCs, heparin-binding HDGF, was also shown to have an indirect relationship with the integrin signaling pathway through TGFB1, COL1A1 or heparin, putting further accent on the potential importance of integrin pathways (Fig. 2D). Based on these bioinformatics results along with the previously reported importance of the ECM-integrin signaling complex in islet regeneration [4], EMILIN-1, HDGF, and ILK were selected for further validation as predictive markers of islet regenerative capacity.

Validation by Western Blot Showed that EMILIN-1, ILK, and HDGF were Prospective Markers Associated with Islet-regenerative Potential

To validate the quantitative iTRAQ LC-MS/MS results and to directly assess the importance of three prospective markers associated with islet regeneration, EMILIN-1, ILK, and HDGF total abundance levels were examined by Western blotting using independently derived whole cell lysates from 4 R- and 4 NR-MSC cultures. All three prospective markers showed significantly increased abundance levels in R-MSCs when compared with NR-MSCs (Fig. 3A). Our previous work has also demonstrated that prolonged expansion of R-MSCs resulted in loss of MSC potency for the capacity to reduce hyperglycemia after transplantation [4]. These studies reported that islet regenerating capacity was consistently maintained for up to four passages and then progressively decreased with cell expansion until being lost completely by passage eight. As a measure of prospective biological validation for the protein markers discovered, we used quantitative Western blotting to measure the abundance levels of EMILIN-1, HDGF, and ILK in islet-regenerative MSC cultures subjected to prolonged culture. This experiment was performed using an R-MSC sample (R2) that was shown to progressively lose glucose lowering function with extended passage in a previous publication [5]. All three of the prospective markers showed a progressive and significant decrease in relative abundance levels with increased passage number (Fig. 3B). By passage 8, when glucose lowering function was completely lost, abundance levels of EMILIN-1, HDGF, and ILK had reduced by 1.80-fold, 1.52-fold, and 1.41-fold, respectively. These results indicated that decreased abundance of EMILIN-1, HDGF, and ILK in R-MSCs correlated with passage-induced loss of islet regenerating function after transplantation in vivo.

To examine the association between each potential markers to the reduction of blood glucose level after MSC transplantation, the abundance level values obtained for each marker (Fig. 3A) and the blood glucose data between day 14 and 42 (Fig. 1A) were used in the primary analysis using a generalized estimating equation (GEE) modeling (Table 1). A statistically significant association between EMILIN-1 and ILK abundance and reduced blood glucose concentration after transplantation was revealed (blood glucose analyzed as binary data, p-value: EMILIN-1, $< .0001$; ILK, $< .0001$) (Table 1). Furthermore this correlation was also upheld when blood glucose concentration was analyzed as continuous data (p-value: EMILIN-1 $< .0348$; ILK $< .0286$). While these analyses also suggested that HDGF may also serve as a prospective marker, its association with blood glucose concentration did not quite reach statistical significance using GEE (p-value: blood glucose as binary data, 0.1196; as continuous data, 0.0579) (Table 1).

Discussion

Our study demonstrates that EMILIN-1 and ILK are novel markers of islet regenerative capacity in cultured MSC samples. HDGF likely represents a potential secretory marker as well; however, due to limited sample size, a statistically significant association between HDGF and blood glucose concentration was not achieved. Although the identification of the specific role of these markers in islet regeneration warrants further mechanistic study, the work presented here highlights the variability that exists among stromal cultures and the importance of finding reliable markers related to therapeutic effectiveness after transplantation. To date, few proteins have been identified that show varied abundance levels in stromal cultures correlating directly with in vivo regenerative function. Selection of candidate markers for further validation was based on our bioinformatics results and previous findings highlighting the importance of ECM-integrin and angiogenesis in islet regeneration [4]. Indeed, EMILIN-1, ILK, and HDGF proteins selected for the validation work are all known interactors of the integrin pathway, as demonstrated by pathway enrichment analysis, and also represent known effectors of angiogenesis [20–23], a process that has been postulated to play a crucial role in MSC mediated tissue repair [4–6]. In addition, EMILIN-1 and ILK are important regulatory factors in cell proliferation, survival, matrix interaction and adhesion via integrins [24–26]. By being an upstream activator of the integrin pathway, EMILIN-1 could influence the activity of ILK.

EMILIN-1 is an extracellular matrix (ECM) glycoprotein previously shown to regulate elastogenesis [27], lymphatic valve formation and maintenance [24] and cell proliferation via the interaction with integrins $\alpha4\beta1$ and $\alpha9\beta1$ [28]. HDGF is a known heparin-binding protein shown to induce the chemotaxis and growth of smooth muscle cells [29, 30]. ILK is a 59-kDa ankyrin-repeat containing serine/threonine protein pseudo-kinase that interacts with the cytoplasmic domain of $\beta1$ and $\beta3$ integrin receptors and is therefore a critical component of the integrin-ECM signaling complex [31]. In line with these findings, Bell et al. had previously reported the up-regulation of genes translating to known secreted proteins from R-MSCs involved in regulating the ECM, including several matrix metalloproteases (MMP1 and MMP13) and targets implicated in angiogenesis (HGF, FGF16 and ANGPTL4), as well as transcripts encoding integrins (integrin $\beta8$ and $\alpha8$) [4].

Summary

Based on the known functions of the markers that we identified, we postulate that increased abundance of EMILIN-1 and

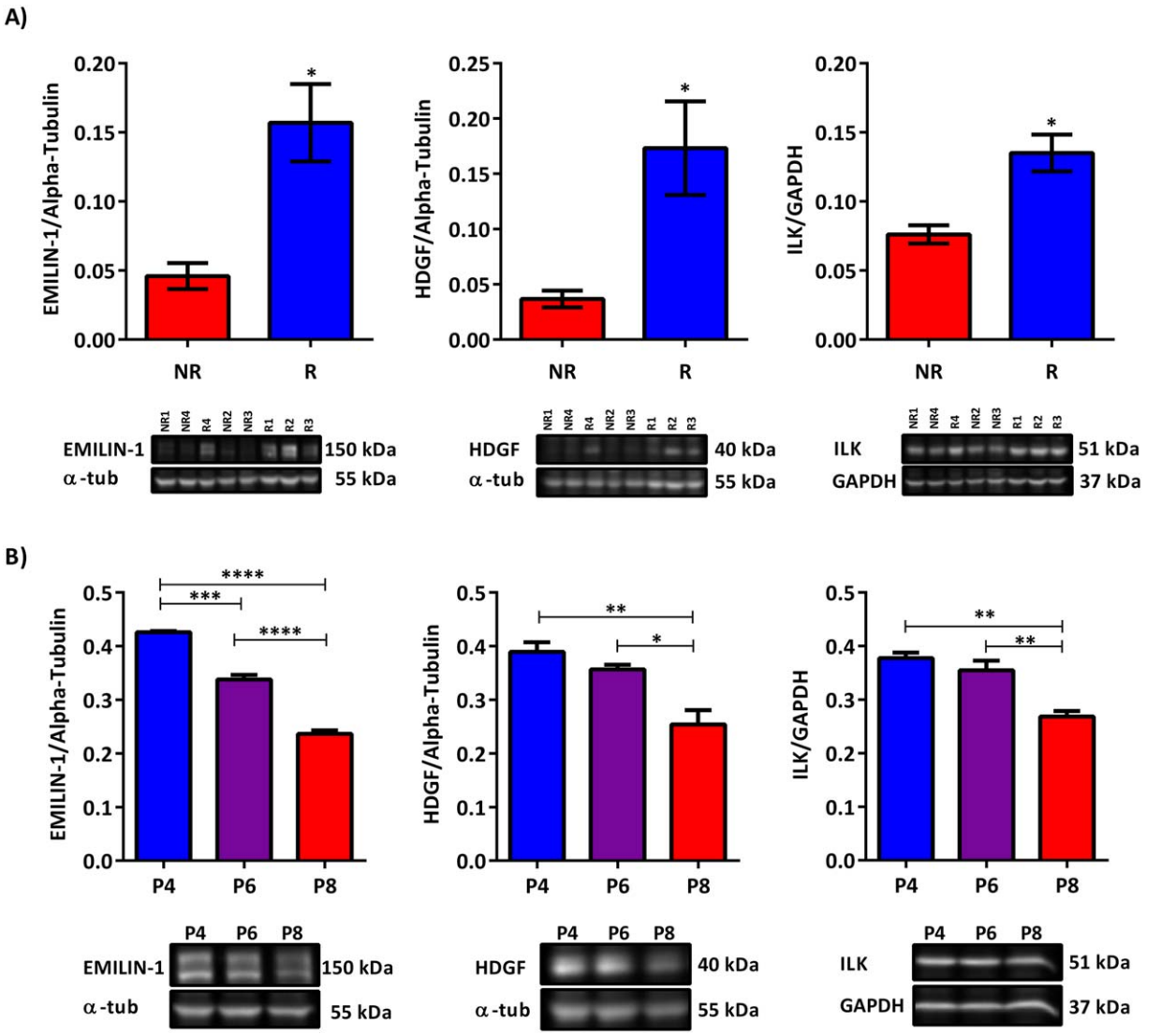

Figure 3. EMILIN-1, ILK, and HDGF are MSC prospective markers associated with islet-regenerative potential. **(A)**: Western blot analyses of putative islet regenerative MSC markers expressed in islet regenerative ($N = 4$) versus nonregenerative ($N = 4$) MSC samples. Data were obtained from three independent experiments and are expressed as mean ± S.E.M. Mann-Whitney two-tailed test was performed. *, $p < .05$. **(B)**: We have previously shown that islet regenerative capacity is decreased with prolonged MSC expansion in culture. MSC from islet regenerative sample (R2) was expanded for up to eight passages and Western blots were performed at increasing passage (P4, P6, and P8). Abundance levels for each putative marker were quantified by relative band intensity densitometry. Data were obtained from three independent experiments and are expressed as mean ± S.E.M. One-way ANOVA with Tukey's multiple comparison post-test was performed. *, $p < 0.05$,**, $p < .01$, ***, $p < .001$. GAPDH: glyceraldehyde 3-phosphate dehydrogenase, NR: non-regenerative, P: passage, R: regenerative, α-tub: Alpha-Tubulin. Abbreviations: EMILIN-1, Elastin microfibril interface 1; HDGF, hepatoma-derived growth factor; ILK, integrin-linked protein kinase; MSC, mesenchymal stromal cell.

HDGF in transplanted R-MSCs acts to increase MSC survival after transplantation through integrin engagement and may modify the ECM in the regenerating pancreas to promote islet recovery/regeneration after beta cell injury in vivo. The data presented here indicates for the first time that increased abundance levels of EMILIN-1 and ILK may provide a useful means for the standardization of human stromal cultures and a basis for the development of in vitro potency assays for MSC-based secretory products to stimulate islet regeneration.

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Author Contributions

L.J.R.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript. J.R.L was funded by a NSERC Visiting Fellowship in Canadian Government Laboratories; C.M.C.: Conception and design, collection and/or assembly of data,

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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