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Modulation of Naïve Mesenchymal Stem/Stromal Cells into Insulin-Producing Cells by Extracellular Vesicles: Optimization of Coculture Conditions

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ABSTRACT

Aim: *This study aimed to optimize the coculture conditions of extracellular vesicles (EVs) derived from surrogate beta cells with naïve mesenchymal stem cells (MSCs).*

Methods: *MSCs were isolated from liposuction aspirates from three healthy donors. These cells were expanded and differentiated into insulin-producing cells (IPCs). EVs were retrieved from the conditioned media of naïve (uneducated) and differentiated (educated) MSCs by ultracentrifugation. The isolated EVs were characterized by transmission electron microscopy, particle size distribution analysis, and flow cytometry. EVs were cocultured with naïve MSCs under different experimental conditions. Cell preparations were evaluated by immunofluorescence, flow cytometry, and RT-PCR.*

Result: *the results revealed that 80 µg of educated EVs cocultured with 1x 10^5 MSCs for 24 h provided the optimal conditions for modulating naïve MSCs into IPCs.*

Conclusion: ***T****he coculture of EVs derived from IPCs with an appropriate concentration and duration could modulate naïve MSCs into IPCs.*

Keywords

Mesenchymal stromal cells, Differentiation, Insulin-producing cells, Extracellular vesicles.

Introduction

Diabetes mellitus (DM) represents a significant global health challenge. The prevalence of DM has increased dramatically, from 108 million cases in 1980 to over 400 million in 2014 [1]. If this trend persists, projections suggest that by 2045, more than 600 million individuals will be affected by DM [2]. The condition manifests in two primary forms: type 1 DM (T1DM), characterized by insufficient insulin production, and type 2 DM (T2DM), marked by the body's inability to use insulin effectively. T1DM develops when the immune system attacks and destroys insulin-producing β-cells in the pancreatic islets.

The primary treatment for T1DM involves the administration of exogenous insulin, which can maintain proper blood sugar levels throughout a patient's life. Nevertheless, this approach has some limitations, including the risk of imprecise insulin delivery leading to poor glycemic control or hypoglycemic episodes. Additionally, some individuals may still develop microvascular complications despite insulin therapy. An alternative to lifelong insulin treatment is the transplantation of either pancreatic islets or a whole pancreas, which offers a potentially ideal solution for T1DM management [3]. Nevertheless, the scarcity of organs from deceased donors and the necessity for immunosuppression remain significant constraints.

T2DM constitutes the bulk of diabetes cases. Initially, this condition can be managed through dietary changes and oral medications. Over time, 27% of diabetic patients become reliant on insulin. Among these individuals, fewer than half achieve the recommended HbA1c levels, as externally administered insulin cannot replicate the precise glycemic control provided by insulin produced in the pancreas [4]. Advancements in regenerative therapies offer the possibility of creating insulin-producing cells (IPCs) from a variety of stem cell sources. Embryonic, neonatal, induced pluripotent, and mesenchymal/stromal cells (MSCs) were used for this purpose [5]. However, cell therapy has several challenges: cell necrosis, immune rejection, and the possibility of teratogenicity [6].

It was demonstrated that culture medium conditioned by MSCs produced therapeutic effects similar to those of the parent cells in rodent models of acute myocardial infarction [7] and lung injury [8]. As a result, the paradigm of MSC-mediated function has shifted from cell engraftment to secretome-based signaling. Eventually, it is now known that MSCs exert their therapeutic effects by releasing various membrane-surrounding vesicles called extracellular vesicles (EVs) into the extracellular milieu [9]. EVs are lipid bilayer vesicles and encompass three main types: exosomes, microvesicles, and apoptotic bodies, depending on their size and biogenesis [10].

The first and most-studied category is exosomes. These exosomes are derived by invading the endosomal membrane to form multivesicular bodies (MVBs), which enclose numerous intraluminal vesicles. MVBs are released as exosomes upon fusion with the plasma membrane and have a size of 50–150 nm. The second major type of vesicles is microvesicles (MVs), which are larger than exosomes and have a size of 100–1000 nm. EVs are released by direct outward budding and fission of the plasma membrane. The third class of EVs are apoptotic bodies formed from cells that undergo programmed cell death and become fragmented. These vesicles are larger, ranging from 500 nm to several microns in size [11]. EVs carry a cargo of proteins, lipids, and different types of RNA that can be transferred from donor cells to recipient cells [12,13]. Pioneering studies by Ratajczak and Valadi demonstrated that functional messenger RNA (mRNA) within the cargo of EVs are transferred to recipient cells and can be translated into proteins [14,15]. This concept was supported by various researchers [16-19]. EVs can also transfer microRNA (miRNAs), proteins, and lipid to target cells [20,21]. A previous study demonstrated that EVs derived from surrogate beta cells can modulate naïve MSCs into IPCs [22]. The purpose of this study was to optimize the coculture conditions of EVs derived from surrogate beta cells and naïve MSCs. The cell/EVs ratio and the duration of the coculture were evaluated.

Materials and Methods

Retrieval, Expansion, and Differentiation of Human Adipose Tissue Mesenchymal Stromal Cells

Ethical approval for this study was obtained from the University of Mansoura Ethical Committee (IRB MS 23.10.2468). Liposuction aspirates were obtained from three healthy, consenting individuals during elective cosmetic procedures. MSCs were retrieved from these aspirates according to our previously published methods [23]. The obtained cells from different donors were pooled and expanded for three passages. The identity of these cells was tested by their morphology, flow cytometry, and their ability to trilineage differentiation. at passage three, the cells were differentiated by our conventional protocol [24].

Harvesting of EVs

EVs were retrieved from the conditioned media of naïve (uneducated) and differentiated (educated) MSCs. The cells were cultured in the same medium supplemented with EVs-depleted FBS for 48 hours. EVs-depleted FBS was carried out by ultracentrifugation of FBS at 100,000×g overnight. Following supernatant collection, the EVs were isolated through sequential centrifugation at 4°C. Initially, the supernatant was centrifuged at 300×g for 10 minutes, 2000×g for 20 minutes, and 10,000×g for 30 minutes to remove large vesicles and cell debris. Subsequently, the supernatant was further processed through ultracentrifugation at 100,000×g for 2 hours. The result was an EVs pellet that was washed with PBS. The EVs preparation was diluted with PBS to obtain EVs harvested from the media of 4×10^7 hMSCs in one milliliter, and the protein concentration was determined by a BCA protein assay kit (EMD Millipore, MA, USA).

Characterization of EVs

EVs were then characterized by transmission electron microscope (Supplementary file: Data S1), particle size distribution analysis by dynamic light scanning (Supplementary file: Data S2), and flow cytometry for specific proteins (Supplementary file: Data S3) according to the International Society for Extracellular Vesicle's recommendations [25]. To demonstrate that these EVs are internalized into MSCs, they were stained with Exoria (Exopharm Ltd, Melbourne, Australia) according to the method described by Terter et al. [26]. (Supplementary file: Data S4).

Coculture of Educated or Uneducated EVs with MSCs

In this study, the optimal coculture conditions for modulation of MSCs by educated EVs were determined in a stepwise fashion. Initially, the perfect quantity (by weight) of EVs relative to the same number of naïve MSCs was explored in 2 experiments. Subsequently, the duration of coculture using the previously identified EVs/MSCs ratio was identified again in 2 experiments. Finally, using the combined results of the aforementioned procedures, cocultured experiments were repeated 7 times to inquire for reproducibility. In all these experiments, the use of uneducated EVs or the culture medium only served as a negative control.

Evaluation of the Cell Preparations

Cells were evaluated by flow cytometry (Supplementary file: Data S5), immunocytochemistry (Supplementary file: Data S6), and relative gene expression by real-time PCR (Supplementary file: Data S7).

Statistical Analysis

Data analysis was analyzed using IBM SPSS statistics 16.0 software (IBM Corp., Armonk, NY, USA). Since the data were nonparametric and unmatched, significant differences between the two groups were analyzed using the Mann-Whitney test. For more than two groups, the Kruskal-Wallis 1-way analysis of variance was used. A p-value of < 0.05 was considered significant.

Results

Characteristics of MSCs

MSCs adhered to the plastic surface of the culture plates and displayed a spindle-like shape Supplementary file: Data S8). These cells were positive for specific mesenchymal cell markers, including CD73, CD90, and CD105, and negative for hematopoietic stem cell markers, including CD14, CD34, and CD45 (Supplementary file: Data S9). Additionally, their ability to differentiate into adipocytes, chondrocytes, and osteocytes was confirmed (Supplementary file: S10).

Characteristics of isolated EVs

The spherical cup-shaped morphology of MSC-derived EVs was validated using transmission electron microscopy (Supplementary file: S11). The particle size distribution analysis by intensity showed that the largest EVs concentration (88.6%) had an average diameter of 181.9 nm (Supplementary file: S12). Furthermore, the expression of specific associated proteins, including CD9 and

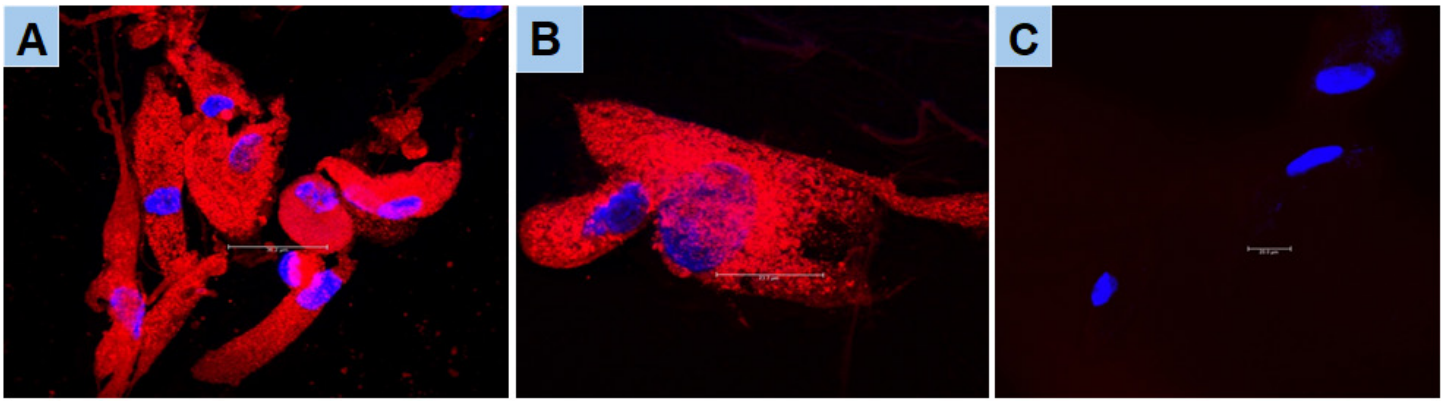


Figure 1: Uptake of EVs by MSCs:

A. Labeled educated EVs (red) are internalized into MSCs.
B. Labeled uneducated EVs (red) are internalized into MSCs.
C. Unlabeled EVs as a negative control.

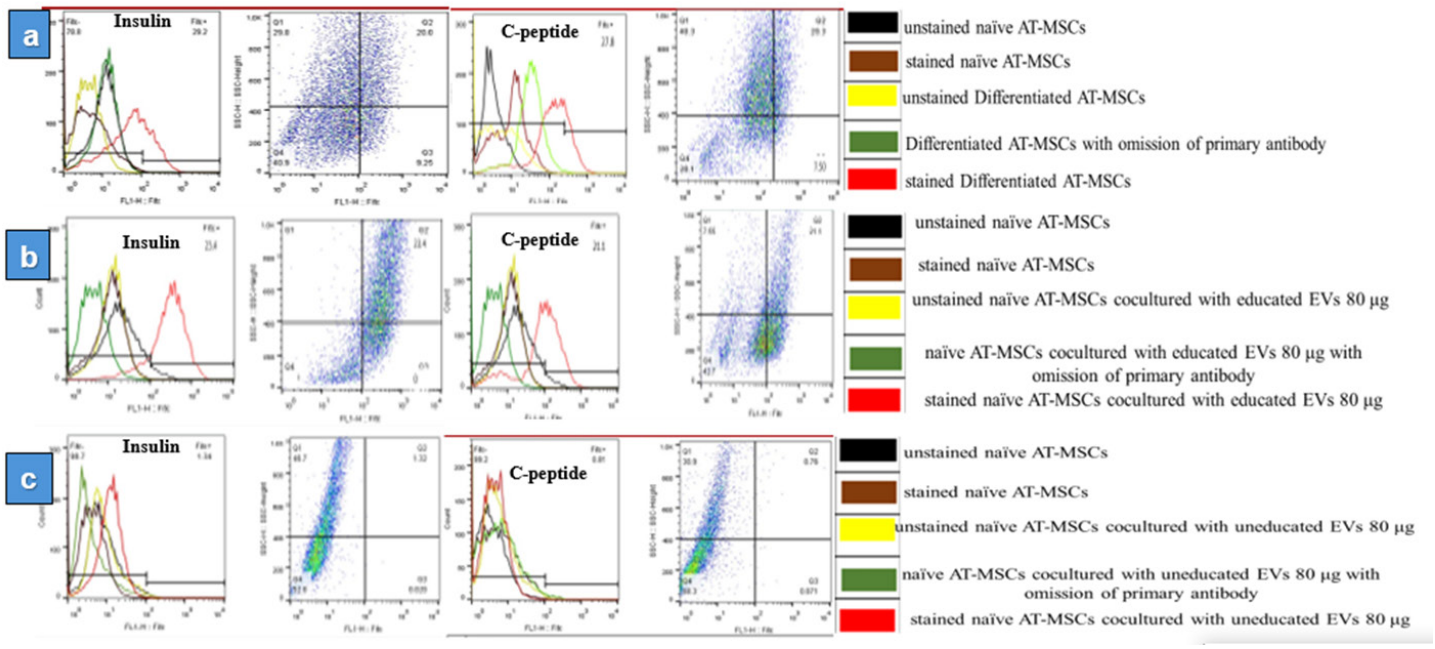


Figure 2: Quantitation of the hormone-positive cells by flow cytometry.

a. MSCs-derived IPCs. The values of insulin and C-peptide positive cells were 21.4% and 20.7% respectively.
b. MSCs were cocultured with 80 µg of educated EVs. The value of insulin positive cells was 18.2% and C-peptide positive cells were 16.2%.
c. MSCs were cocultured with 80 µg of uneducated EVs. The value of insulin positive cells was 0.86% and C-peptide positive cells were 0.50%.

Table 1: Cell preparations by flow cytometry under different concentrations.

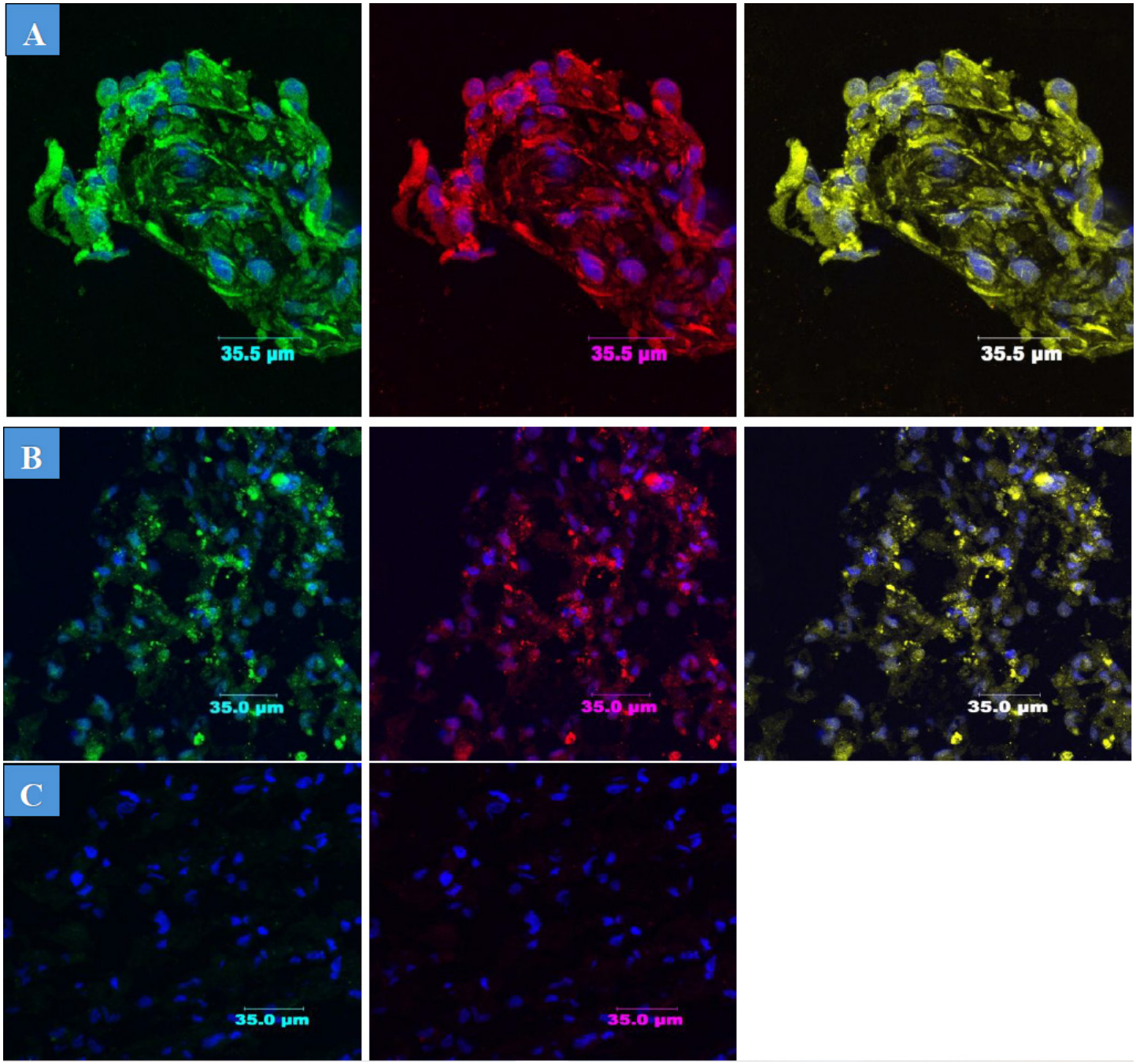
	Doner cells				EVs/ml/total cells for 24 hours	Naïve MSCs cocultured with educated EVs	
	Naïve MSCs		Differentiated cells				
	Insulin	C-peptide	Insulin	C-peptide		Insulin	C-peptide
Exp 1	0.83	0.76	19.4	18.2	10µg/ml/1x10^5	7.6	5.5
					20µg/ml/1x10^5	1.09	0.73
					40µg/ml/1x10^5	8.56	6.79
					80µg/ml/1x10^5	19.1	17.2
					120µg/ml/1x10^5	13.2	11.7
					160µg/ml/1x10^5	16	15
					200µg/ml/1x10^5	11.6	9.32
Exp 2	0.95	0.85	23.8	21.4	10µg/ml/1x10^5	7.82	4.28
					20µg/ml/1x10^5	9.74	6.35
					40µg/ml/1x10^5	15.6	10.7
					80µg/ml/1x10^5	18.7	16.2
					120µg/ml/1x10^5	14.6	11.5
					160µg/ml/1x10^5	16.4	14.8
					200µg/ml/1x10^5	13.6	14.4

Table 2: Cell preparation by flow cytometry under different duration using 80µg educated EVs.

Doner cells				Duration	Naïve MSCs cocultured with educated EVs 80µg/ml/1x10^5	
Naïve MSCs		Differentiated cells				
Insulin	C-peptide	Insulin	C-peptide		Insulin	C-peptide
0.73	0.56	21.4	14.2	24 hours	11.2	8.91
				72 hours	16.3	14.4
				Every 3 days	13	11.2
4.7	3.4	29.6	20.4	24 hours	19.4	18.2
				72 hours	19.4	16.2
				Every 3 days	15.7	13.8

Table 3: Proportion of hormone positive cells by flow cytometry.

	Doner cells				Recipient cells				Naïve MSCs in HG media only	
					80µg/ml/1x10^5 EVs/ml/total cells					
	Naïve MSCs		Differentiated cells		Naïve MSCs cocultured with educated EVs		Naïve MSCs cocultured with uneducated EVs			
	Insulin	C-peptide	Insulin	C-peptide	Insulin	C-peptide	Insulin	C-peptide	Insulin	C-peptide
Exp 1	0.83	0.76	19.4	18.2	16.9	14	0.76	0.45		
Exp 2	0.83	0.76	19.4	18.2	18.2	16.3	0.89	0.43		
Exp 3	0.94	0.77	28.3	21.4	15.4	14.5			1.38	1.07
Exp 4	0.4	0.375	21.4	20.7	15.9	13.4	0.84	0.50	1.01	0.78
Exp 5	0.95	0.85	23.8	21.4	18.7	16.2	0.96	0.52	0.96	0.66
Exp 6	0.75	0.65	20.3	17.3	19.1	17.2	0.76	0.50	0.45	0.13
Exp 7	0.47	0.5	29.2	27.8	23.4	21.1	1.34	0.8	0.99	0.48
Lower	0.4	0.38	19.39	17.3	15.4	13.4	0.76	0.43	0.42	0.13
upper	0.95	0.85	29.2	27.8	23.4	21.1	1.34	0.8	1.38	1.07
median	0.83	0.76	21.4	20.7	18.2	16.2	0.86	0.5	0.99	0.66



Figures 3: Immunocytochemistry for insulin and C-peptide.

A. Differentiated IPCs. The cells were positive for insulin (green) and C-peptide (red). A merged image of insulin and C-peptide (yellow), indicates that insulin and C-peptide are expressed within the same cells.

B. MSCs cocultured with educated EVs. The cells were positive for insulin (green) and C-peptide (red). A merged image of insulin and C-peptide (yellow), indicates that insulin and C-peptide are expressed within the same cells.

C. MSCs cocultured with uneducated EVs. The cells were negative for insulin and C-peptide. Only blue DAPI stained nuclei were visible.

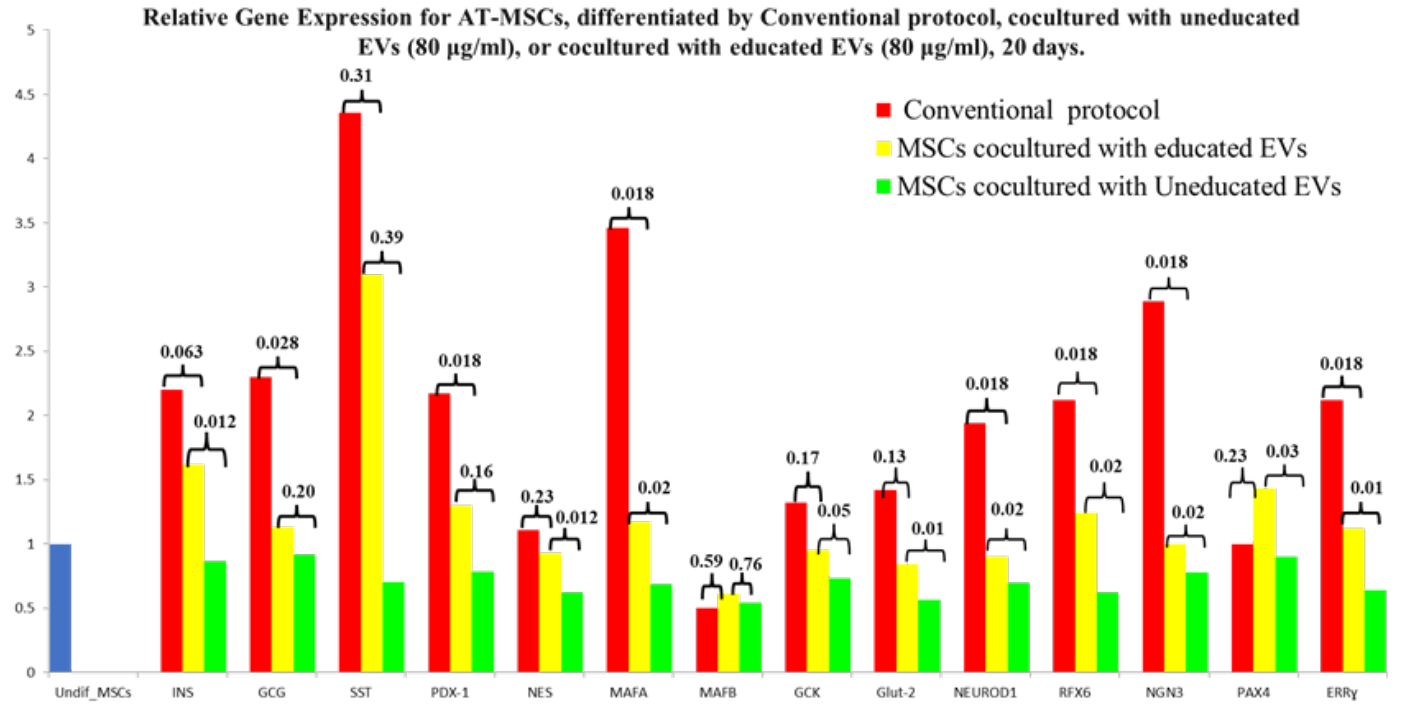


Figure 4: Relative Gene Expression for AT-MSCs, differentiated by Conventional protocol, cocultured with uneducated EVs (80 µg/ml), or cocultured with educated EVs (80 µg/ml), 20 days.

CD63 was confirmed by flow cytometry (Supplementary file: S13). The internalization of the Exoria-labeled EVs into MSCs was confirmed (Figure 1).

Evaluation of Cell Preparations

Quantification of the Hormone-Positive Cells by Flow Cytometry

Experiments for the determination of the optimal EVs/MSCs ratio (10, 20, 40, 80, 120, 160, or 200 µg of educated EVs) revealed that coculture of 80 µg educated EVs with 1× 10^5 MSCs provided the maximal modulation (Table 1). Thereafter, this dose was tested for different durations: 24h, 72, or every 3 days for coculture. The results showed no difference in modulation relative to the duration of coculture (Table 2).

Flow cytometry analysis of differentiation of the parent MSCs showed that the median value of insulin-and C-peptide-positive cells was 21.4% and 20.7% respectively (Figure 2a). The median value for insulin-and C-peptide-positive cells following the optimized MSC/EVs was 18.2% and 16.2%, respectively (Figure 2b). In contrast, the median value following cocultured with uneducated EVs for insulin-and C-peptide-positive cells was 0.86% and 0.5%, respectively (Figure 2c). Additionally, MSCs cultured in high glucose media showed a neglectable percentage of insulin-and C-peptide-positive cells (Table 3).

Immunocytochemistry for Insulin and C-Peptide

Insulin and C-peptide were co-expressed among differentiated MSCs (Figure 3a) as well as, MSCs cocultured with educated EVs also co-expressed insulin and C-peptide (Figure 3b). In contrast, MSCs cocultured with uneducated EVs did not co-express insulin or C-peptide (Figure 3c).

RT-PCR

All relevant pancreatic endocrine genes were expressed among differentiated cells. Naïve MSCs cocultured with 80 µg/ml of educated EVs also expressed all the relevant pancreatic endocrine genes but less than that in differentiated cells. In contrast, naïve MSCs cocultured with 80 µg/ml of uneducated EVs expressed negligible values of insulin and all relevant pancreatic genes (Figure 4) (raw data: Supplementary file: Data S14).

Discussion

Earlier findings indicated that EVs released from donor cells can modify the function of recipient cells [14,15]. A previous study showed evidence that EVs derived from IPCs can modulate naïve MSCs into surrogate beta cells [22]. Due to a lack of previous guidelines, we have tried to optimize the coculture conditions to obtain maximal effect in this study. Two variables were evaluated, mainly EVs/MSCs ratio and duration of coculture. Our results indicated that 80 µg of educated EVs cocultured with 1x 10^5 MSCs for 24 h provided the optimal modulation of naïve MSCs into IPCs. Evidence of modulation of naïve MSCs into IPCs under these experimental conditions was evaluated by immunofluorescence, flow cytometry, and RT-PCR. By immunofluorescence, the modulated cells were stained positive for insulin and c-peptide. The

coexpression of these two proteins within the same cell confirms the intrinsic synthesis of insulin. By flow cytometry, the proportion of insulin-positive cells in 7 experiments ranged between 15.4% and 23.4%, with a median of 18.2%. At the same time the proportion of c-peptide-positive cells ranged between 13.4% and 21.1%, with a median of 16.2%. All the relevant pancreatic endocrine genes were expressed by the modulated, though with lower values than those cells differentiated by our conventional protocol.

The mode of function of EVs in the recipient cells is controversial. Initially, transfer of RNAs was suggested [14,15,19]. Subsequently, the role of micro RNAs was proposed [27,28]. Collino et al. reported that EVs released from human MSCs contain more abundant miRNAs than in the cell of origin, suggesting they provide specific function [29]. EVs-mediated transfer of miRNAs regulates protein translation and modulates the expression of gene products in recipient cells [30]. The transfer of genetic information by RNAs and/or miRNAs was denied by Toh and associates [31]. They suggested that proteins in MSC-derived EVs are present at sufficient functional levels to elicit a relevant biological response. Alternatively, EVs receptor-ligand interaction on the surface of the recipient cell can induce downstream events that result in changes in cell function [32].

Guo et al. cocultured exosomes derived from a mouse insulinoma cell line (MIN6) with human-induced pluripotent stem cells (iPSCs) for 21 days. After seven days of coculture, the proportion of insulin-positive cells was higher in the exosome-treated group compared to the control group, as determined by flow cytometry. The treated iPSCs also showed increased expression of relevant pancreatic endocrine genes. Additionally, the expression of miR-706, miR-709, miR-466-c-5p, and miR-423-5p were upregulated in the exosome-induced iPSCs. There was a decrease in β cell-specific genes among the siAgo2-treated exosomes. The study concluded that exosomes induce iPSC differentiation *in vitro* and that this process is mediated by exosomal miRNAs [33].

A study by Bai et al. focused on beta cell generation from iPSCs using EVs secreted by beta cells. i-Beta cells were produced *in vitro* using a four-stage protocol previously outlined by Pagliuca et al. [34]. In the final phase of the experiment, the differentiated iPSCs were cocultured with EVs derived from human β cells. These EVs were replenished continuously every 3 days over 15 days. The researchers observed that this coculture approach facilitated the differentiation of iPSCs into i-β cells. *In vitro* studies revealed that these i-β cells displayed functional characteristics resembling pancreatic β cells. Notably, there was a significant increase in insulin production, which was reversed upon inhibiting microRNAs miR-212 and miR-132. These microRNAs enhanced β cell differentiation by stabilizing NGN3 expression [35].

Our promising results have to be translated into an in *vivo* setting. An ongoing experiment in our laboratory. EVs derived from IPCs are administrated to STZ-diabetic nude mice. The dose, frequency of administration, and therapeutic benefit will be studied. If this experiment will provide evidence that systematic administration of educated EVs can achieve euglycemia in a diabetic animal model, clinical translation can be justified. The use of EVs provides a distinct advantage over stem cell therapy for the treatment of diabetes. EVs can be administered intravenously, and treatment can be repeated. EVs are known to be hypo-immunogenic and are not subjected to rejection. EVs are also immunomodulatory; this function can be useful in immune-dependent T1DM. Since EVs have no nuclei there is no possibility for tumorigenesis. EVs can be frozen, stored, and used as off-the-shelf bases.

In conclusion educated and uneducated EVs uptake by MSCs confirmed that EVs can internalize into MSCs and can modulate them. Coculture 80 µg of educated EVs with 1×10^5 MSCs once for 24 h and then culture in high glucose media for a total 20 days yielded the optimal modulation.

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