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In vitro Impact of Pro-Senescent Endothelial Microvesicles on Isolated Pancreatic Rat Islets Function

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Introduction

Islet transplantation is a cell therapy proposed in patients with brittle [Type 1 Diabetes \(T1D\)](#), with life threatening issues [1, 2]. Despite improvements in immunosuppression protocols, and improved insulin independence rates, reaching up to 50% at 5 years, a gradual loss of islet function that marks chronic graft dysfunction is reported [3]. Isolated pancreatic islets are injected in the portal vein as a cell suspension, and engraft in liver micro-vessels. Nevertheless, the isolation procedure leads to ischemia and the contact of islets with blood favors an acute inflammatory response termed Instant Blood Mediated Inflammatory Reaction (IBMIR) within the first 24 hours. This particular form of ischemia-reperfusion leads to the immediate loss of 50 % of transplanted islets and damages the endocrine function. It is characterized by local coagulation events and inflammatory responses at the vicinity of the islets that impair the recovery of functional endothelial cells and the islet perfusion [4]. The contribution of such early ischemia-reperfusion to the chronic grafted islets dysfunction remains debated. One of the possible causes would be ischemia-induced senescence of both endocrine as well as hepatic and intra-islet endothelial cells. Indeed, pancreatic islet aging increases the incidence of diabetes due to decreased insulin secretion and increased insulin resistance [5, 6]. Cellular senescence, defined as an irreversible cell cycle arrest [7], is correlated with a progressive decline in proliferative and regenerative capacity reported in pancreatic islets and specifically in insulin secreting cells (β -cells) [6]. Furthermore, aging-related oxidative stress in the vessels and pancreatic tissues [5, 8] combined to the weak antioxidant capacity of the latter contribute to redox-driven alterations of islet morphology and function with the eventual accumulation of senescence markers [9-11]. Vascular aging is known to favor redox-sensitive endothelial senescence pathways [12] that may limit the vaso-protective properties of the endothelium and alter graft perfusion.

Aside from replicative senescence that progressively occurs with ageing, premature or accelerated senescence was associated with ischemia-reperfusion injury in patients with ischemic heart disease and organ transplantation [13-15]. Senescence involves different cell cycle regulators like tumor suppressors and cyclin dependent kinases and leads to cell dysfunction. Another common feature of senescent cells is the release of microvesicles (MVs) acting as pro-senescent inducers [16].

Microvesicles are submicron plasma membrane fragments released into the extracellular space by stimulated cells after plasma membrane remodeling [17]. MVs express bio-active molecules and surface antigens that characterize their cell origin, making them markers of cell damage [18], their release from the cell being directly correlated to cell damage and disease severity [19]. MVs circulating in the blood were shown reliable markers of ischemia insult and of worsen outcome prognosis value in cardiovascular, stem cell, renal [20-22] and islet-transplantations [23, 24]. Indeed, they are a signature of organ ischemia-reperfusion injury [25-28].

Data from earlier studies demonstrate that endothelial MVs are not only biomarkers but also vascular effectors, those released from senescent cells showing thrombogenic potential and promoting endothelial dysfunction [16, 29-31]. We reasoned that ischemia prompted by islet isolation procedures would induce MV-driven endothelial senescence impairing the intra-islet vascular function.

We therefore examined the impact of pro-senescent endothelial MVs on isolated pancreatic islets and whether they may contribute to accelerated senescence and dysfunction. The effects of pro-senescent and young cell-derived endothelial MVs on isolated rat islets were compared.

Material and Methods

Ethics statement

Male Wistar rats (Janvier-Labs, Le Genest-St-Isle, France) were housed in a temperature-controlled (22°C) room and maintained on a standard 12-hours light/dark cycle (lights on at 07:00 am), with free access to food and water. Experiments conformed to the Guide of Care and the Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and were authorized by a local ethic committee. All animal experiments were done in a registered animal yard within the Faculty of Pharmacy (Authorization number F-67-218-26).

Endothelial primary cell culture

Hearts from 3 months old pigs were collected from the local slaughterhouse (COPVIAL, Holtzheim, France). Endothelial cells (ECs) from the left circumflex coronary arteries were isolated within the next 3 hours [32]. Briefly, the left circumflex coronary artery was excised and cleaned of adhesive conjunctive tissues. ECs were isolated by filling the artery with a 1 mg/ml, type I collagenase solution

(Worthington, Serlabo Technologies, Entraigues sur la Sorgue, France) dissolved in MCDB131 medium (Invitrogen; Carlsbad, CA, USA) with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 µg/ml) and L-glutamine (1mM, all from Lonza, Ozyme, St Quentin en Yvelines, France) for 15 min at 37°C. The ECs were collected after circular massages of the arteries and the remaining cells were flushed with the medium. Collected cells were gently centrifuged at 600 g for 5 min, then the medium was removed and cells were suspended in medium supplemented with 10% fetal calf serum. Extracted cells were seeded in a T25 flask and cultivated at 37°C in a humidified incubator with 5% CO₂. Non-adherent cells were removed from the medium 6-8 hours after seeding. ECs were sequentially passaged from passage P1 (young) to P3 (senescent) cells. The medium was changed every 48 hours. Typically, ECs grow 6 days, between P0 and P1, P1 and P2, P2 and P3 cumulating 18 days of culture before showing a characteristic senescent phenotype (circular shape, largest and better visible nucleus) and Senescence-Associated β-galactosidase (SA-βgal) activity, the common feature to senescent cells [13].

MVs isolation and measurement

MVs were collected from the supernatant of P1 and P3 ECs by differential centrifugation under sterile conditions [33]. Briefly, cells and debris were discarded at 800 g for 10 min and the supernatant further centrifuged at 13000 g, 4°C for 60 min. The final suspension was kept for less than 30 days at 4°C. The MVs measurement was performed by prothrombinase assay after capture onto biotinylated Annexin-5 (Ann-V) [34]. This procedure relies on the high affinity of annexin-5 for phosphatidylserine (PS) on the MVs surface. Samples (100 µl) were added to each well and incubated at 37°C for one hour. After three washings, the insolubilized MVs were measured by prothrombinase assay. In this assay, the degree of exposure of anionic phospholipids (PS) on the surface of MVs was the limiting factor of the conversion of prothrombin into thrombin in the presence of purified human coagulation factors (FII 4.2 µM, FVa 250 pM, FXa 53 pM and CaCl₂ 2 mM). After 10 minutes at 37°C, the generated thrombin is measured using a chromogenic substrate, pNAPEP 0216 (1.52 mM). Absorbance at 405 nm was converted into equivalent phosphatidylserine (PhtdSer eq) by reference to

a standard curve obtained using a suspension of synthetic vesicles of known amounts of PS (33% (w/w) of PS and 67% (w/w) of phosphatidylcholine [35].

Pancreatic islets isolation and treatments

Pancreatic islets were isolated from Wistar male rats (mean weight 250 g) [36, 37]. Rats were anaesthetized by intraperitoneal injection of mixture of Ketamine and Xylazine. A 10 mL of 1 mg/mL Type-XI collagenase (C7657, Sigma-Aldrich, Saint-Quentin Fallavier, France) suspension in Hanks balanced saline solution (HBSS) (Lonza, BE10-527F, Basel, Switzerland) was injected into the common bile duct of the euthanized animals. The pancreas was excised and digestion was stopped by cold HBSS. After centrifugation, the pellet underwent three successive cold washings (4°C) in FBS-supplemented HBSS. Isolation of islets was achieved using a three density gradient Ficoll PM-400 solutions (F4375, Sigma-Aldrich) (densities: 1108, 1096, 1069 g/ml) and centrifugation at 800g for 18 min at 4°C. Islets purity was evaluated after dithizone (D5130, Sigma-Aldrich) labeling by optical microscopy. Islets were re-suspended in RPMI 1640 (Gibco 21875-034, Saint Aubin, France). Handpicked islets showed a typical diameter distribution range (75-250 µm), those above 300 µm were discarded. Collected islets were cultured in non-adherent plates at 37°C in a humidified incubator with 5% CO₂ for 24 hours and thereafter referred to as "freshly isolated islet".

Freshly isolated pancreatic islets were treated for 24 hours by 5 nM PhtdSer EQ P1 or P3 cell-derived MVs (P1, P3-MVs) or hydrogen peroxide 100 µM (H₂O₂) (H1009, Sigma-Aldrich) as a positive control. All data were compared to untreated islets incubated with the identical volume of vehicle. In preliminary experiments, the optimal 5 nM MVs concentration was determined by a concentration-curve response (0-20 nM PhtdSer EQ) established for a 24 hours incubation time.

Islet viability

After 24 hours of MVs incubation, islet viability was assessed by 2 different approaches. Primarily we examined the double red PI / green fluorescein diacetate staining. Using an epi-fluorescence microscope, red necrotic cells and green viable cells were counted within a suspension of 30-labeled islets. The viability of the islets was calculated as the area ratio of green and red fluorescent / total

islet. The experiments were repeated 5 times (i.e. using 5 different islets preparations from 5 different rats).

In the second approach, 30 islets per condition were disassociated to isolated cell suspension and studied by flow cytometry using double PI / Ann-V staining. For this purpose, islets were first centrifuged at 200 g for 10 minutes. The pelleted islets were resuspended in 500 µl of a solution 2x trypsin (340 000 IU/L) and then incubated for 8-10 minutes at 37°C with gentle shaking. After confirmed dissociation by microscopy, the reaction was stopped by addition of 700 µl of supplemented medium followed by a 10 minutes centrifugation at 200 g. The pellet was re-suspended in 500 µl fixation buffer containing 0.5 µg/ml Ann-V-FITC solution and 1 µg/ml of PI. After 10 minutes at 37°C, the cell suspension was analyzed using a flow cytometer set at logarithmic gain. 5000 events were recorded for each sample (FACS calibur, Becton Dickinson, [La Jolla](#), CA, USA). In another set of experiments, the expression of cleaved Caspase-3 was measured by western blot in the protein lysates of 200 islets (see below), using a polyclonal rabbit antibody (1/1000) (Asp175, Cell Signaling Technology, Danvers, MA, USA).

Measurement of islet function by glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) was measured in suspensions, each containing 40 islets in KREBS solution supplemented with 0.5% albumin, 10 mM HEPES and 2.5 mM (0.45 g / L) glucose (basal), or 25 mM (4.5 g/ L) glucose (stimulated). After MVs treatment (see below), islets were transferred for 1 hour into 2.5 mM glucose and centrifuged at 200 g for 10 minutes. The pelleted islets were incubated for another 1 hour in 2.5 mM glucose, centrifuged at 200 g for 10 minutes and their supernatant stored at -80°C until insulin measurement. Islets were finally incubated in 25 mM glucose for 1 hour, centrifuged and their supernatant stored at -80°C. The amount of secreted insulin was measured by ELISA (Millipore cat# EZRMI-13K, Billerica, MA, USA). The glucose stimulation index (SI) was calculated by the ratio of insulin concentration after high glucose concentration stimulation (25 mM) to insulin concentration after low glucose concentration stimulation (2.5 mM). The experiments were repeated 5 times (i.e. using 5 different islets preparations from 5 different rats).

Assessment of MVs integration into islets

MVs collected from P1 ECs (P1-MVs) and P3 ECs (P3-MVs) were labeled by the red fluorescent lipid probe PKH26 (Sigma-Aldrich) and washed twice by centrifugation (13 000 g, 60 min). MVs (5 nM PhtdSer EQ) were incubated with 20 pancreatic islets for 24 hours at 37°C. Accumulation of the probe on target cell surface was observed by fluorescent microscopy and quantified by flow cytometry using a FACS-scan cytometer set at logarithmic gain. 5000 events were recorded for each sample. The experiments were repeated 4 times (i.e. using 4 different islets preparations from 4 different rats).

Assessment of senescence

Senescence was characterized either by conventional microscopy using X-gal, a chromogenic substrate of the Senescence-Associated β -galactosidase (SA- β gal), a common feature of senescent cells, or by analysis of the expression of senescent markers p53, p21 and p16 by western blot.

Pancreatic islets (20 per condition) were washed with PBS and then incubated in the SA- β -gal staining solution (1 mg/ml X-gal, 40 mM citric acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, 2 mM magnesium chloride dissolved in phosphate buffer, pH 6.0) for 8 hours at 37°C in a CO₂-free atmosphere. The percentage of senescent stained islets was determined by microscopy.

For western blot assays, 200 washed islets were analysed per condition. Islet proteins were extracted after 15 min incubation at 4°C with a specific lysis buffer (Tris/HCl 20 mM pH 7.5; Q-Biogene, MP Biomedicals, Illkirch, France) NaCl 150 mM, Na₃VO₄ 1 mM, sodium pyrophosphate 10 mM, NaF 20 mM, okadaic acid 0.01 mM (Sigma-Aldrich) containing a tablet of protease inhibitor (Complete Roche, Basel, Switzerland) and 1% Triton X-100 (Euromedex, 2000-B, Souffelweyersheim, France).

Lysate proteins were measured by Lowry protein assay (DC protein assay, Bio-rad, USA), and 25 μ g per condition loaded on a 10% SDS-polyacrylamide gels, run at 100 V for 2h and transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham, Life Sciences, Germany) at 100 V for 2 hours. Aspecific binding was blocked by incubation in TBS buffer (Biorad) containing 5% bovine serum albumin (BSA) (Euromedex, 04-100-812-E, Souffelweyersheim, France), and 0.1% Tween 20 (Sigma-Aldrich) (TBS-T) for 2 hours.

Membranes were then incubated overnight at 4°C in TBS-T containing 5% BSA and polyclonal rabbit antibody raised against p53 (1/1000) (Santa Cruz Biotechnology FL-393 SC-6243, Dallas, Texas, USA), p21 (1/1000) (Abcam ab7960, Cambridge, UK), p16 (1/1000) (Abbiotec, 250804, Escondido, USA). Membranes were washed 3x (TBST) and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and density signals of each band detected using ECL Substrate (170-0561, BIO-RAD, United States). Membranes were incubated with a mouse monoclonal anti- β -tubulin I mouse antibody (Sigma-Aldrich) for normalization purposes. In addition, to allow comparison between different experiments, all data were reported to the untreated control expressed as 100%. The experiments were repeated 2 times for imaging and 5 times for western-blot. For each western-blot experimental condition, 3 rats preparations were pooled before handpicking.

Statistical analysis

All experiments were performed on 4-5 separate occasions, therefore using 4-5 different freshly isolated islet suspensions. Data is expressed as means \pm SEM of the data obtained on these occasions. Statistical analysis was performed [using either analysis of variance \(ANOVA\) one way followed by the Bonferroni post-hoc test \(Figures 1, 2 & 3\) or Student's t test using two-tail Mann-Whitney test \(Figure 4\) using](#) GraphPad Prism version 6.01 (GraphPad Software, San Diego, USA). $p < 0.05$ was considered statistically significant. In case of percentage comparison, values were transformed into their decimal form prior statistical analysis.

Results

MVs integrate into the islet

Fluorescent PKH26-stained MVs were used to [prove](#) the interaction between MVs (5nM PhtdSer eq.) and the pancreatic islets. After 24 hours incubation, PKH26-red labelling was detected in the islets by fluorescent microscopy, suggesting MVs integration (Figure 1A). After islet dissociation, a significant increase in the proportion of fluorescent cells was observed in those islets treated either with P1 or P3-MVs as compared to untreated ones (P1-MVs: $39.5 \pm 0.86\%$, P3-MVs: $42.75 \pm 5.20\%$ vs. untreated islets: $6.91 \pm 1.83\%$, $p < 0.001$) (Figure 1 B).

Pro-senescent endothelial MVs alter islet viability

A trend to reduced pancreatic islets viability was observed after 24 hours treatment with P3-ECs derived MVs (P3-MVs) whereas H₂O₂-induced a significant decrease (P3-MVs: 89.47% ± 1.61; P1-MVs: 93.21% ± 0.81, H₂O₂: 84.09% ± 1.69; vs. untreated: 93.15% ± 0.97, p < 0.05) by double red PI / green fluorescein diacetate staining (Figure 1 C).

After islet dissociation, apoptosis quantification by flow cytometry confirmed the absence of a significant viability loss in MVs-treated islets. P3-MVs led to a slight trend to apoptosis (P3-MVs: 36.66% ± 2.09 vs. 24.80% ± 2.53, p > 0.05 in untreated islets), whereas H₂O₂-induced apoptosis was slightly higher and significant (38.12% ± 4.14 vs. 24.80% ± 2.53 in the untreated islets, p < 0.05), indicating that islets indeed remained sensitive to oxidative stress under our conditions. The degree of islet apoptosis after P1-MVs treatment (27.70% ± 3.04) was similar to that of untreated islets (Figure 2 A). Measurement of cleaved-Caspase 3 performed on a 6 folds amounts of islets as compared to the above labelling (200 vs. 30 islets), confirmed that the degree of caspase-3 activation by pro-senescent inducers (P3-MVs or H₂O₂), remained non-significant as compared to that observed in P1-MVs or untreated islets (Figure 2 B).

Pro-senescent endothelial MVs alter islet function

A significant 41% glucose stimulation index (SI) reduction was observed after treatment by pro-senescent P3-MVs whereas no significant effect could be detected after treatment by P1-MVs (P3-MVs: 1.23 ± 0.06, P1-MVs: 1.76 ± 0.27, vs. untreated: 2.10 ± 0.31, p < 0.05). P3-MVs nearly reached the efficacy of H₂O₂ that led to a 43% SI decrease (H₂O₂: 1.20 ± 0.06 vs. untreated; p < 0.01) (Figure 3).

Pro-senescent MVs induce the up-regulation of senescence markers

Conversion of the X-gal substrate into a blue stain indicating the presence of SA-βgal was detected in P3-MVs treated cells (100 % islets staining) but not in P1-MVs treated islets nor in untreated controls, thereby suggesting that only P3-MVs are pro-senescent. In line with these data, P3-MVs significantly up-regulated p53 as compared to P1-MVs or untreated islets (P3-MVs: 1.78 ± 0.15 vs. P1-MVs: 1.10 ± 0.12 or untreated islets: 1, p < 0.01). A similar pattern was observed for p21 (P3-MVs: 2.08 ± 0.38 vs. P1-MVs 0.94 ± 0.11 or untreated islets: 1, p < 0.05). Similarly, the expression of p16, the p21

downstream effector, was solely and significantly increased by P3-MVs (P3-MVs: 1.80 ± 0.28 vs. untreated islets: [1](#), $p < 0.05$). H_2O_2 induced a greater p53 up-regulation than P3-MVs (H_2O_2 : 2.19 ± 0.43 vs. untreated islets: [1](#), $p < 0.05$). By contrast no significant effect of P1-MVs could be detected on the expression level of p53, p21 and p16, suggesting a specific pro-senescent action exerted by P3-MVs (Figure 4).

Discussion

The present study demonstrates that endothelial pro-senescent P3-MVs target freshly isolated islets, induce Senescence-Associated β -galactosidase and prompt the up-regulation of senescence markers while altering islet function assessed by the glucose stimulation index.

Our data confirm and [extend](#) our previous observation of MV-mediated autocrine and paracrine pancreatic crosstalk between β -cells and with endothelial cells in 2D culture models [38].

In the present study, MVs from young and senescent cells similarly integrated into the islet after 24 hours, demonstrating that the MV ability to integrate target cells is not a limiting step in the MV-mediated noxious effects. These data are [in line](#) with our previous observation of an optimal MV integration into isolated β -cells after 24 hours [38] and of their noxious effects on pancreatic cells. Indeed, the ability of β -cell derived MVs to act as early autocrine effectors that reduce insulin secretion within the next 8 hours following cytokine or oxidative stress [33] is in compliance with the present data. Similarly, MVs shed from exocrine pancreatic cells in response to lipopolysaccharide, also altered the β -cell function within 18 hours [39].

The pro-senescent features of endothelial MVs has been reported by our team and others in human coronary and murine aorta endothelial cells [16, 30], the present data constitute a first demonstration of a MV-mediated integration into the multiple lineage structure of the islet without significant alteration of cell viability. Because islet apoptosis remained minimal, the accelerated islet senescence induced by endothelial MV appears a specific process leading to overall islet dysfunction, as previously reported in other 2D MV-mediated cell crosstalk [16, 38].

The β and α cells constitute the majority of the islet cell populations. It is therefore likely that endothelial pro-senescent MVs mainly target mechanisms of early β or α cell response, eventually via

altered paracrine cell crosstalk. Our data further question the eventual impact of MVs on the circadian activity oscillations of α and β -cells within the islets possibly different *in-vivo* and *in-vitro* [40]. Indeed, it was recently proposed that feeding and fasting cycles would drive the temporal insulin and glucagon secretion through differential circadian expression of genes, among which actors of the respiratory chain involved in the redox homeostasis, often disrupted in senescent cells [41].

Our observation of the pro-senescent endothelial MV-driven up-regulation of p53, p21 and p16 is highly suggestive of an accelerated and premature senescence within the isolated islets also confirmed by the presence of SA- β gal activity after 24 hours. Indeed, p53 is a cell cycle regulator involved in controlling intricate pathways of cell death and survival [42]. The activation of p53 and p21 can lead to apoptosis or senescence [43], whereas the down-stream p16 is characteristic of cellular senescence [44]. As a proof of concept, time-dependent p53 inhibition by siRNA silencing was associated with reduced ischemia-reperfusion driven senescence in mice renal tissues [45]. Furthermore, in mice islets, p16 miRNA has been associated with telomeric shortening and aging [46], in accordance with a poorer regenerative potential of older islets that overexpress p16 [47].

In this context, our data underline the eventual impact of pro-senescent endothelial MVs shed into the islet and their eventual pro-oxidative action [38, 48]. Because redox-sensitive pro-senescent and thrombogenic MVs promote endothelial inflammation [16, 30, 41, 49], and favor the expression of tissue factor, it is tempting to speculate that they might contribute to the initial intra-islet ischemia-reperfusion injury of the grafted islets. However, our model only describes ischemia-driven responses and no conclusion can be drawn on the contribution of reperfusion.

The pharmacological prevention of the generation of noxious MVs from isolated islets appears crucial to limit ischemia-induced endothelial senescence. Furthermore, in the particular form of ischemia-reperfusion prompted by islet transplantation, a similar pro-senescent MV-driven vascular inflammatory response of the hepatic vessels is likely to occur where transplanted islets engraft, favoring coagulation events and negative outcome [33, 50]. Interestingly, β -cell cytoprotection was achieved *in-vitro* by switching the noxious characteristics of endothelial MVs to protective ones, following drug endothelial targeting as previously demonstrated using Activated Protein C-treated endothelial cells (APC, Xigris[®]) [38]. Indeed, the feasibility of a pharmacological treatment to

prevent the release of pro-senescent MVs from organs has been demonstrate in middle aged and old rats [51].

Conclusions

In conclusion, our study demonstrate a deleterious paracrine effect of the pro-senescent endothelial P3-MVs that accelerate islet senescence and dysfunction. These data highlight a noxious MVs mediated-crosstalk between senescent endothelial and pancreatic islet cells. Pro-senescent endothelial MVs appear early pathogenic markers of isolated islet dysfunction and a therapeutic target in the prevention of islet ischemia-driven accelerated senescence and dysfunction.

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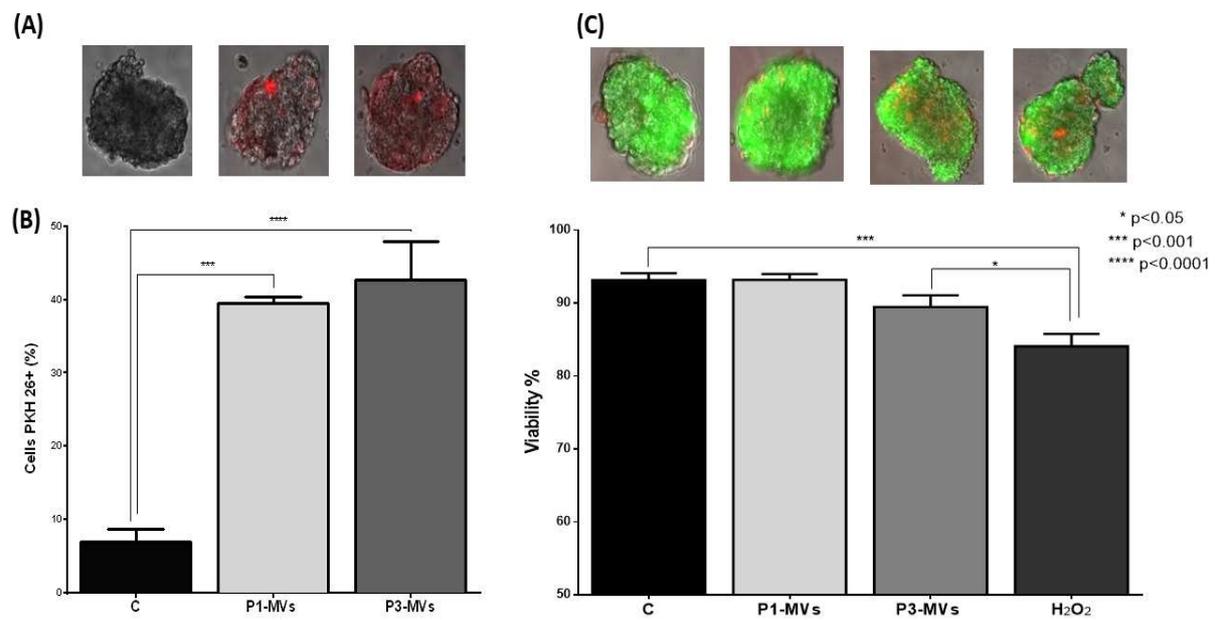
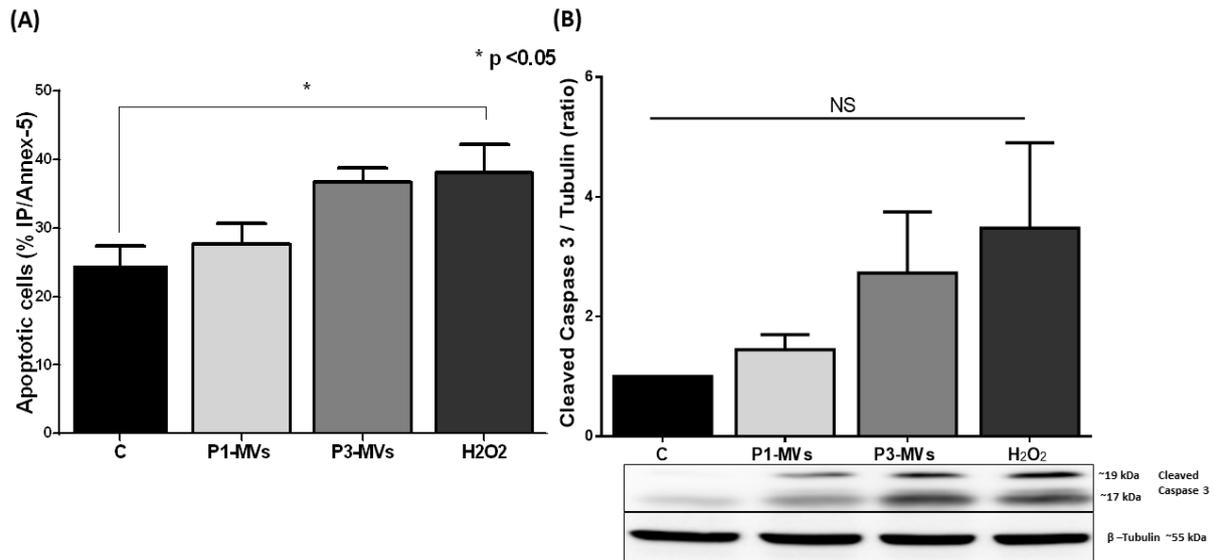


Figure 1. MVs integration and islet viability

MVs integration into islets was assessed after 24 hours treatment by 5 nM PhtdSer EQ of P1-MVs or P3-MVs previously labeled with the red PKH26 lipid probe. (A) Representative images by fluorescent microscopy (magnification 20 x). (B) Quantification of the labeled PKH26 probed pancreatic islet cells after trypsin dissociation by flow cytometry. (C) Islet viability was assessed by double



propidium iodide (PI) / Fluorescein diacetate double labeling. Viability was calculated as the percentage of green and red fluorescent / total islet area (magnification 20 x), quantification was performed using Image J software, results expressed as mean \pm SEM. Experiments performed on 5 separate occasions, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. Apoptosis in isolated pancreatic islets

Islet apoptosis was measured after trypsin dissociation into single cell suspension following a 24 hours treatment by 5 nM PhtdSer EQ of P1-MVs, P3-MVs or 100 μ M H₂O₂. Cell suspension was analyzed by flow cytometry after PI / FITC-Ann-V double labeling (A) or by western blot after cell lysis (B). Results are expressed as means \pm SEM. Experiments performed on 5 (A) or 4 separate occasions (B), * p<0.05.

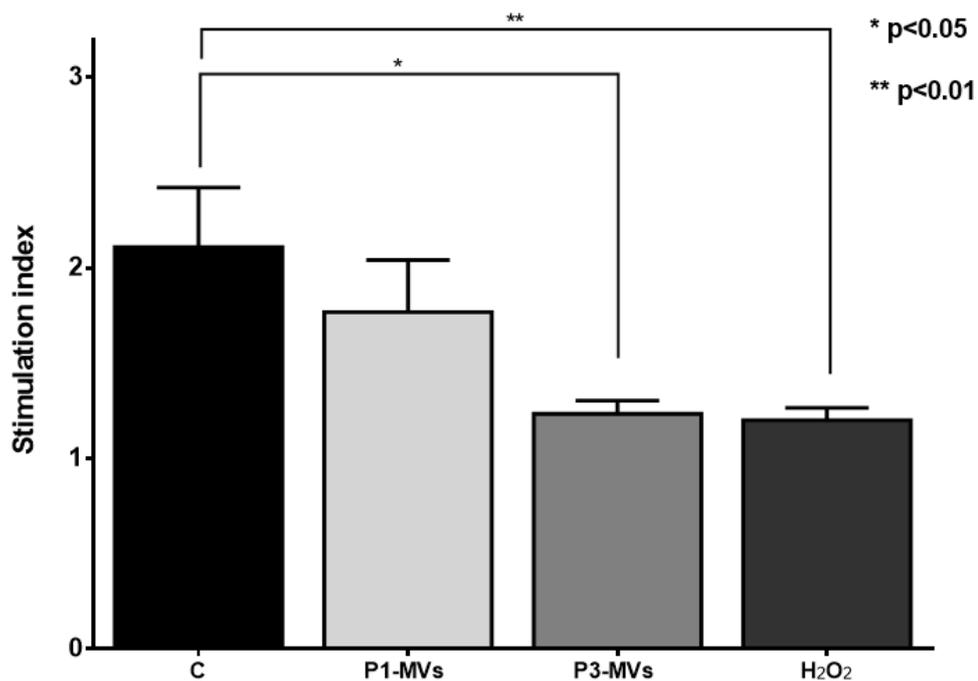
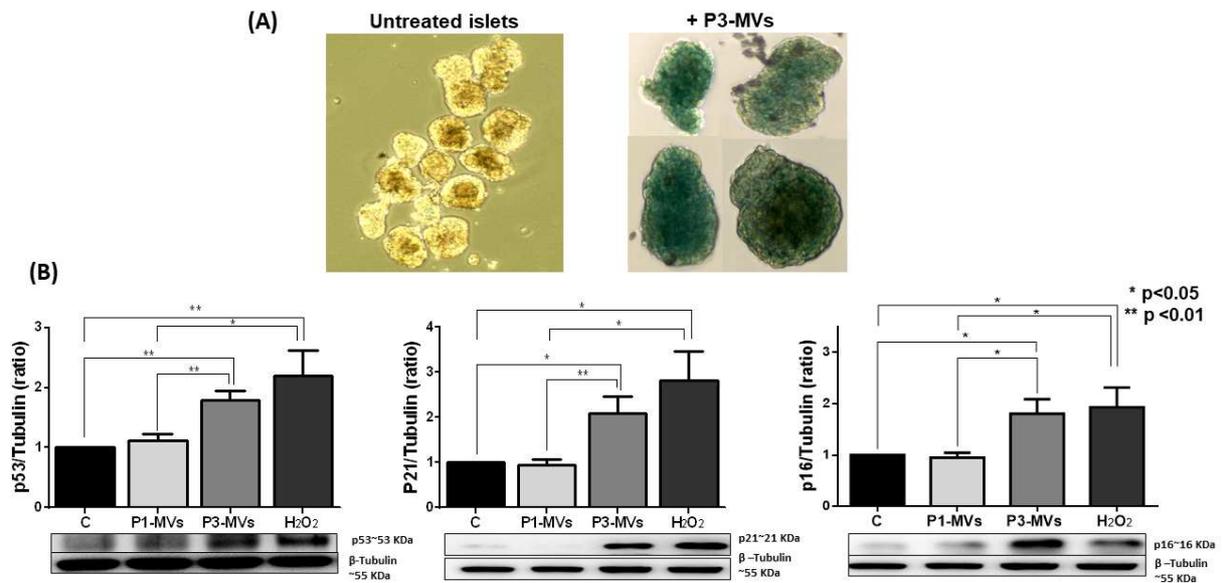


Figure 3. Islet function assay

Islet function was evaluated by the stimulation index (insulin amount secreted after incubation in KREBS solution supplemented with 25 mM glucose / amount of insulin secreted after incubation in KREBS solution supplemented with a 2.5 mM glucose) after 24 hours treatment by 5 nM PhtdSer EQ



of P1-MVs, P3-MVs or 100 μ M H₂O₂. Results are expressed as mean \pm SEM. Experiments performed on 5 separate occasions, * p<0.05, ** p<0.01.

Figure 4. Detection of senescence in MVs-treated pancreatic islets

Detection of senescence in isolated islets after 24 hours MVs incubation by conventional microscopy using X-gal as a chromogenic substrate of the Senescence-Associated β -galactosidase (SA- β gal) (A), or by analysis of the expression of senescent markers p53, p21 and p16 by Western blot (lower panel representative blot, upper panel: cumulative data) (B). Islets were treated with 5 nM PtdSer EQ P1-MVs, P3-MVs or 100 μ M H₂O₂. Quantification was expressed as a ratio of protein of interest/housekeeping protein chemiluminescence (Tubulin) and analyzed using Image J software. Results are expressed as means \pm SEM. Experiments performed on 2 separate occasions (A) or 5 separate occasions (B), * $p < 0.05$, ** $p < 0.01$.