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Development of a pancreas-liver organ-on-chip coculture model

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Abstract

Advances in organ-on-chip technology allowed the recapitulation of two or more organs interaction thanks to dedicated microbioreactors interconnected by microfluidic network. Here, we developed pancreas-liver coculture model in a microfluidic biochip, using rat Langerhans islets and hepatocytes. The behavior and functionality of the model were compared to islets and hepatocytes (with/without insulin) monocultures. We confirmed the insulin, glucagon and C-peptide secretions by islets monoculture and coculture. Furthermore, C-peptide and insulin secretions were higher in coculture after 5 days of culture. The islets coculture presented downregulation of *Pdx1*, *Glut2*, *Gcg*, *App*, *Ins1*, *Neurod*, *Neurog3* and *Gcgr* genes, compared to monocultures. In hepatic compartment, the monocultures without insulin were negative to CK18 staining and displayed a weaker production of albumin when compared to monocultures with insulin. They also presented a moderate protein expression of CYP3A2, GLUT2, INSR and modulation of several hepatic genes. In coculture model, hepatocytes displayed albumin productions similar to those in monoculture with insulin. The hepatocytes cocultures were highly positive to INSR, GLUT2, CK18 and CYP3A2 immunostaining and allowed to recover mRNA levels similar to monoculture with insulin (*Gcgr*, *Insr*, *Hnf4a*, *Igf1* and *Alb*). The result illustrated that islets can produce insulin to supplement the culture medium and recover hepatic functionality. We believe that our model illustrated the potential of organ-on-chip technology to reproduce cross-talk between liver and pancreas.

Keywords: Islets of Langerhans, hepatocytes, coculture, microfluidic biochips, glucose homeostasis

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by deregulation of glucose homeostasis that results from insulin deficiency or systemic insulin resistance (Rogal et al., 2019). DM is one of the most prevalent and costly diseases in the world affecting approximately 463 million people worldwide (1 in 11 adults), according to the latest International Diabetes Federation (IDF) estimation (IDF official web site, 2019). In 2016, World Health Organization (WHO) estimated that 1.6 million deaths were directly caused by diabetes (WHO official web sites, 2020). The complications of diabetes are also associated with multiple medical problems such as blindness, kidney failure, cardiovascular diseases, sexual dysfunction, neuropathy and peripheral vascular disease (Rogal et al., 2019, Jellali et al., 2020, Forbes and Cooper 2013). Type 2 diabetes mellitus (T2DM, called insulin-independent diabetes) is the most common form of diabetes, representing 90% of diabetic patients (415 million people worldwide, Sadu et al., 2019; IDF official web site, 2019). T2DM is caused by glucose target-tissues insensitivity to insulin and impaired insulin secretion (Jun et al., 2019). Currently, there is no therapy curative for T2DM. Most treatments help patients to control the disease and include lifestyle adjustments and drug therapy such as metformin, sulphonylureas, glitazones and GLP-1 (King and Bove 2016; Rogal et al., 2019; Zbinden et al., 2020).

T2DM is a complex disease involving several organs interactions, including pancreas, liver, muscle, adipose tissues, kidney and gut (DeFronzo et al., 2015). One of the key features of DM2 is the insulin resistance state characterized by a drop-down of glucose uptake by the skeletal muscle, fatty tissue and liver (Forbes and Cooper 2013). In pancreas, the state of hyperglycemia and hyperinsulinemia triggers the increase of insulin production and beta cell proliferation as a compensatory effect of insulin resistance state. T2DM occurs when the pancreas fails to adapt to increased blood glucose levels (Bauer et al., 2017). The liver (with muscle) is one of the first organs severely affected by insulin resistance following a chronic excess of dietary energy. The liver responds to chronic systemic hyperglycemia by increasing gluconeogenesis, which is responsible for the increased basal rate of glucose production (Rogal et al., 2019; DeFronzo et al., 2015). T2DM is associated with high prevalence of hepatic comorbidities, such as non-alcoholic fatty

liver disease (NAFLD, Perry et al., 2014). NAFLD is a rapidly growing disease affecting 30 % of general population and around 90% of T2DM patients (Saponaro et al., 2015). Furthermore, it is still controversial whether NAFLD is a consequence or a cause of pancreas disorders. The relevant physio-pathological models involving organ to organ interactions are critical to extract relevant biomarkers and therapeutic solutions. As a result, it is crucial of importance to reproduce both the liver and the pancreas physiologies to control and identify properly the interaction of diabetes development and its systemic relationship with liver.

Advanced new *in vitro* technologies can create ample opportunities for a more modern approach to toxicology and to pharmacology, to replace the traditional “black box” of animal-based and of conventional 2D *in vitro*-based paradigms. The animal models fail to faithfully recapitulate the human condition and lose relevance when extrapolating the results to humans (Ghaemmaghami et al., 2012). *In vitro* cell cultures are mainly performed in 2D cultures using conventional multi-well plates, which do not accurately reflect the physiological *in vivo* microenvironment (Capulli et al., 2014). Consequently, it is essential to improve those basic plates cultures to reproduce, as closely as possible, the characteristics found *in vivo*. This is the reason why many groups are developing tissue-engineering and 3D culture processes in order to provide a more appropriate micro-environment for the tissue maintenance and development.

Among those *in vitro* complex models, organ-on-chip technology seems to be one of the suited methods for reproducing the behavior of an organ or a group of organs, as well as the controlled physiological microenvironment (Bathia and Ingber 2014). In the last years, several teams have proposed organ-on-chip devices that reproduce various behaviors of various organs and tissues including liver, kidney, gut, lung, heart and intestines (Kimura et al., 2018; Polini et al., 2104). While many of organ-on-chip technologies have been developed and advanced for the liver, only few studies were dedicated to pancreas, and even less, to our knowledge, to the liver-pancreas interaction. Among those *in vitro* complex models, organ-on-chip technology has been used to reproduce *in vitro* pancreas-on-chip model (Schulze et al., 2017; Li et al., 2017; Brooks et al., 2016; Lee et al; 2018; Zbinden et al., 2020). However, most of the current microfluidic platforms have mainly been designed for islets quality assessment for subsequent *in vivo* implantation (Rogal et al 2019). In

parallel, few recent works also demonstrated the potential of organ-on-chip technology for complex pancreatic analysis and pancreatic islet–liver cross-talk study (Bauer et al., 2017; Zbinden et al., 2020).

Following those works, we proposed in this paper a new coculture organ on chip microfluidic technology allowing hepatocytes and pancreatic islets long term culture. We presented the hepatic perturbation by removing the insulin from the culture medium. Then, we investigate the restauration of the hepatic function thanks the interaction between the liver and the endocrine pancreas trough the circulating hormones and cofactors in the co-culture configuration.

2. Material and methods

2.1. Device design and fabrication

The microfluidic coculture consists of two different biochips (one for the liver, one for pancreas) serially connected. Each biochip is manufactured with two polydimethylsiloxane (PDMS) layers.

In the pancreas on chip, the micro-structured bottom layer, used for islets trapping, is composed of 600 micro-wells of 400 μm in diameter (depth of 300 μm), and spaced by 50 μm (Fig.1A, Essaouiba et al., submitted). The second PDMS layer, with a reservoir (depth of 100 μm , Fig.1A), is placed on top of the first layer and includes an inlet and outlet for culture medium perfusion. A micro-channels network placed at the inlet and outlet of each layer allows homogenous culture medium distribution in the biochip (Fig.1A).

Concerning the liver on chip, we used a micro-structured bottom layer composed of microchannels and microchambers network in cell culture chamber of 1.2 cm length, 1 cm width and 100 μm height (Fig. 1A). The second PDMS layer, with a reservoir (depth of 100 μm , Fig.1A) is sealed on the top of the first layer. As described above, a micro-channels network is also presents in the inlet and outlet to ensure homogenous culture medium distribution. The design and dimensions of

biochip have been described in our previous work (Baudoin et al., 2011; Jellali et al., 2016).

The PDMS biochips were manufactured by replica molding process. First, photolithography was performed to create the mold masters of the bottom and top layer of the biochips using SU-8 photosensitive resin. Then, PDMS prepolymer (mixture of 10:1 base polymer curing agent; Sylgard 184, Dow Corning) was poured onto SU-8 master and cured 2 h at 75°C. The surfaces of obtained PDMS layers were activated with reactive air plasma (1 min; Harrick Scientific) and brought together immediately to form an irreversible seal.

2.2. Rat Islets and hepatocytes isolation

Pancreatic islets and hepatocytes were isolated from male Wistar rats of 8-9 and 6 weeks old, respectively (CLEA Japan, Inc, Tokyo, Japan). The rats were housed at the University of Tokyo with a 12-h light/dark cycle at 22°C with food and water ad libitum. All animal experimentation procedures were done according the guidelines of the University of Tokyo and the Japanese Ministry of education.

2.2.1. Islets isolation

Pancreatic islets were isolated following the protocol described by Yonekawa et al. 2006 and Kiba et al. 2013. The rats were anesthetized by isoflurane inhalation solution (Pfizer). After clamping of all irrigation blood vessels, the enzymatic solution (Liberase™ TL, Roche) was injected through the bile duct, previously identified and clamped. After the pancreatectomy, the selective chemical digestion of the organ was done at 37°C for 30 min with the Liberase TL/ ET-K solution (ET-Kyoto solution, Otsuka Pharmaceutical). The digestion was followed by washing and purification steps using a discontinuous OptiPrep® density gradient (Merck/ Sigma-Aldrich). Then, the islets of Langerhans were identified and hand-picked with a Pasteur pipette under a stereomicroscope (Leica S9 D) and transferred to a cold solution made of UW solution (University of Wisconsin, Kneteman et a., 1990) complemented with Miraclid (Mochika pharmacy, Japan) and Heparin (Mochika pharmacy, Japan). After

the islets assessment and counting, the tissue was stored at 4 °C until culture starting.

2.2.2. Hepatocytes isolation

Primary hepatocytes were isolated using the two-step collagenase protocol based in Seglen et al. protocol (Rizki-Safitri et al., 2020; Xiao et al., 2015; Seglen 1976). Briefly, after animal anesthesia by isoflurane inhalation solution (Pfizer), the liver was perfused with buffer solution in order to washout the blood. subsequently, the liver was extracted, deposited in Dulbecco's Modified Eagle Medium (DMEM, Gibco™ – Life Technologies) and the tissue was gently disrupted. The digested tissues were filtered through 100 µm filters (cell strainer 100 µm nylon; Falcon®) and the liver cell suspensions were centrifuged thrice. The resulting pellets were mixed, suspended in Percoll (#P1644, Sigma-Aldrich) and HBSS (#H6648, Sigma-Aldrich) separating solution. Percoll isogradient centrifugation was performed to isolate dead cells and a significant portion of the nonparenchymal cells in a floating top layer that was discarded. Finally, the obtained cells were suspended in seeding medium (William's E medium (Gibco™) supplemented with 10% fetal bovine serum (FBS, Gibco™) and 100 units/mL of penicillin, 100 mg/mL streptomycin (Gibco™). Cell viability was assessed by Trypan blue dye exclusion and hepatocytes culture with a viability of more than 85% were used. The purity obtained is over 98%.

2.3. Biochip culture

The experimental setup used for culture in biochip was composed of a perfusion loop, including the culture medium tank (bubble trap), the peristaltic pump, and one or two biochips. They were interconnected by 0.65 mm interior diameter silicone/Teflon tubing (Fig.S1, supplementary file). The bubble trap contained 2 mL of culture medium and the flow rate was set at 20 µL/min. Before each experiment, the circuit (biochip, tubing and bubble trap) was sterilized by autoclaving and dried in oven.

Four groups of biochips were investigated and compared: (i) hepatocytes biochip monoculture with insulin (hepatocytes monoculture ITS +); (ii) hepatocytes biochip monoculture without insulin (hepatocytes monoculture ITS -); (iii) pancreatic

islets biochip monoculture (islets monoculture) and (iv) hepatocytes/islets biochips coculture without insulin (hepatocytes/islets coculture). The detailed experimental procedure is shown in Fig. 1B.

2.3.1. Pancreatic islets culture in biochip (pancreas on chip)

The biochips were previously filled with culture media in order to remove the air bubbles and moisturize the circuits. The preconditioning process was carried out during one hour at 37° C in the incubator. The pancreatic islets in the preservation solution were washed with cold culture media and gently diluted in the appropriate amount. In order to minimize the islets damage, wide orifice pipette tips with low binding were used during all the handling process. After loading in the biochips, the islets were counted under microscope in order to keep a record of the islets number per biochip (~40 islets/biochip). Then, the circuit was connected to the peristaltic pump and the perfusion started. The entire setup was continuously incubated at 37 °C in a 5% CO₂ supplied incubator. The basal culture medium used for the pancreatic islets culture was a classic RPMI 1640 medium (Gibco™) supplemented with 10% FBS (Gibco™), 100 units/mL of penicillin, 100 mg/mL streptomycin (Gibco™) and GlutaMAX™ (Gibco™) at 10 Mm.

2.3.2. Hepatocytes biochip culture (liver on chip)

After sterilization, biochips were coated with rat tail type 1 collagen (Corning®, 300 µg/mL in phosphate-buffered saline: PBS Gibco™) and incubated at 37°C in an atmosphere supplied with 5% CO₂. After 1h, the collagen solution was washed using the seeding medium and the freshly isolated hepatocytes (5x10⁵ cells/biochip) loaded into the microfluidic device via biochip inlet ports using a micropipette tip. To keep the seeding medium inside the culture chamber, the biochip inlet ports were closed using two syringes (containing 500 µL of seeding medium), and the biochips were placed in an incubator at 37°C and 5% CO₂. After 24 h of static conditions to promote cell adhesion, the seeding medium was replaced by the culture medium and the biochip integrated in the perfusion experimental setup to launch dynamic culture.

The primary hepatocytes culture medium was composed of William's E medium (Gibco™) supplemented with 100 units/mL of penicillin / 100 mg/mL streptomycin (Gibco™), GlutaMAX™ (Gibco™) at 10 Mm, 1% non-essential amino acids (Invitrogen), 3% Bovine Serum Albumin (Sigma), 1% Insulin-Transferrin-Selenium ITS-100X (PanBiotech), 0.1 µM Dexamethasone (Wako Pure Chemical Industries), 10 ng/ml mouse Epidermal Growth Factor (Takara Bio), 0.5 mM ascorbic acid 2-phosphate (from magnesium salt n-hydrate; Wako Pure Chemical Industries) and 20 mM HEPES (Gibco™). For the hepatocytes monoculture without insulin (monoculture ITS -), we used the same medium composition excluding ITS.

2.3.3. Hepatocytes/islets cocultures (pancreas/liver on chip)

The liver and pancreas biochips were prepared separately. At first, the hepatocytes were inoculated inside the liver biochip (as in section 2.3.2). After 24h of adhesion, the hepatocytes were cultivated inside the liver biochips for 24h in perfusion (this result to 48h of culture in the liver biochip including 24h for adhesion in static condition and 24h of perfusion). The pancreatic islets biochips were prepared after those 48h. The islets were inoculated in the biochips as described in section 2.3.1. After 1h at rest and the islets sedimentation, the liver perfusions were stopped and one pancreas biochip and one liver biochip were serially connected to each other to create a pancreas – liver coculture model (Fig.1A and Fig.S1, supplementary file). The culture medium for the coculture condition is a mixture 1:1 of pancreatic islets and hepatocytes media, excluding ITS from the last one.

2.4. RTqPCR assays

Total RNAs were extracted and purified from samples using a hybrid protocol that combines Trizol™ Reagent (Life Technologies) and RNeasy Mini Kit (QIAGEN 74104) following the manufacturer's instructions. Concentrations and qualities of extracted RNAs were assessed using a BioSpec-nano (Shimadzu Scientific Instruments). Reverse-transcription into cDNA was performed from 0.5 µg of total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). Real-time quantitative reverse transcription polymerase chain reaction (RTqPCR) was then performed with the THUNDERBIRD SYBR qPCR Mix (TOYOBO) according

to manufacturer's protocol and a StepOnePlus Real-Time PCR system (Applied Biosystems). The primer sequences of genes are shown in Table. S1 and S2. β -Actin was used as the reference gene and fresh isolated hepatocytes and pancreatic islets (days 0) as the reference sample for the normalization of gene expression data.

2.5. Immunostaining

The islets were transferred to an untreated TCPS 24 wells plate. The hepatocytes immunostaining was performed in the biochip. The islets and the hepatocytes were washed with phosphate buffer saline solution (PBS, Gibco) and fixed in paraformaldehyde 4% at 4 °C for 24h. In order to perform the immunostaining in a 3D structure, the islets were permeabilized with 1% Triton X100 in PBS for 3h at 4°C and washed 3 times with PBS for 30 min, while the hepatocytes were permeabilized with 0.1% Triton X100 in PBS for 15 min. Then both, islets and hepatocytes were blocked with a gelatin buffer for 24h at 4°C. Primary antibodies were incubated at 4°C in a BSA/PBS solution for 48h and overnight for pancreatic islets and hepatocytes, respectively. After several washing steps, secondary antibodies were incubated in a BSA/PBS solution at 4°C in the dark (24h for islets and overnight for hepatocytes). Actin filaments were stained with Phalloidin-iFluor 488 Reagent (abcam). Finally, the nuclei were stained with Hoechst 33342 (H342, Dojindo) at 1/800 for 30 min at room temperature in the dark.

All the incubations and washing steps were carried out using a shaker for the islets of Langerhans immunostaining process. For hepatocytes, the biochips were cut with a scalpel removing the PDMS top layer in order to increase the resolution of the image. Observations were made with an Olympus IX-81 confocal laser-scanning microscope. The primary and secondary antibodies used are presented in Table. S3 (supplementary file).

2.6. Insulin, glucagon, C-peptide and albumin measurements

The hormones and albumin release in the culture medium samples from the different culture conditions were assessed using ELISA assays, following the manufacturer's protocol. The following kits were used: insulin (Mercodia rat Insulin

ELISA kit, 10-1250-01), glucagon (Glucagon DuoSet ELISA kit, DY1249 and DuoSet ELISA Ancillary Reagent Kit 2, DY008), C-peptide (Mercodia rat C-Peptide ELISA kit, 10-1172-01) and albumin (Rat Albumin ELISA quantification set from Bethyl, Cat. No. E110-125 combined with the Enzyme Substrate, TMB, Cat. No. E102). Results were obtained with an iMark microplate reader (Bio-Rad, Osaka, Japan) set to a wavelength of 450nm.

2.7. Glucose and lactate measurement

Glucose and lactate were measured using an YSI 2950 Biochemistry Analyzer. For that purpose, 160 μ L of culture medium were inserted into the analyzer. Measurements were based on a direct reading of L-lactate (L-lactic acid) and glucose in the culture medium by the YSI enzyme sensors, as the enzymes L-lactate oxidase, and glucose oxidase are respectively immobilized in the lactate and glucose sensors.

2.8. Statistics

All experiments were repeated at least three times. The data are presented as the mean \pm standard deviations (SD) of 9 biochips (3 biochips from 3 different experiments, $n=3 \times 3$). The Kruskal Wallis test was performed for the statistical analysis using GraphPad software (Prism 6). Data with P -values < 0.05 were identified as statistically significant and highlighted in the figures.

3. Results

3.1 Cellular morphologies analysis

The hepatocytes were successfully attached in the collagen-coated biochip after few hours of cultures. They were kept at static condition in incubator. After 24h, the hepatocytes created a confluent tissue and displayed typical cuboidal hepatocyte phenotypes (Fig. S2, supplementary file).

The hepatocytes morphology at the end of the experiments (24h in static condition and 6 days of perfusion) are presented in Fig. 1. The hepatocytes monoculture with insulin (hepatocytes monoculture ITS +) displayed typical hepatic monolayer cultures (cells with typical cuboid shape). The cell monolayer was

maintained over 7 days of culture, including 24h at rest and 6 days in perfusion (Fig. 2A). Without insulin (hepatocytes monoculture ITS -), the hepatocytes cultures were heterogeneous from one biochip to another. A few hepatic cultures in biochips without insulin presented similar morphologies when compared to the hepatic culture with insulin (Fig. 2B). In most of the biochips, the hepatocytes formed some aggregated structures or degraded tissue (Fig. 2C). Finally, in the hepatocytes/islets biochips cocultures, the typical hepatic phenotypes (similar to hepatocytes monoculture ITS +) were recovered as shown in Fig. 2D.

Concerning islets cultures, we did not detect a modification of the morphologies of the pancreatic islets, when we compared the islets monocultures and the islets/hepatocytes cocultures. The islets were trapped in the microwells of the pancreas biochips, and displayed the typical spheroid shapes of islets of Langerhans (Fig. 2E and F). Furthermore, we counted the number of islets at the end of experiments (5 days of perfusion). The result showed that the number of islets collected at the end of the perfusion remained similar to the number of islets seeded .

3.2 Immunostaining analysis

Immunofluorescence staining was performed at the end of the experiment (day 5 and 7 for islets and hepatocytes, respectively). For hepatocytes, we selected to stain several hepatic markers: CYP3A2 (one of the most abundant cytochrome P450 in the liver), CK18 (differentiation marker), INSR (insulin receptor) and GLUT2 (glucose transporter). We also stained two important markers in the pancreatic islets: insulin and glucagon that are markers of β -cells and α -cells, respectively.

The immunostainings of the hepatic cells at day 7 are presented in the Fig. 3. The immunostainings have demonstrated that the hepatocytes monocultures with insulin and the hepatocytes/islets cocultures led to positive cell population expressing CYP3A2 and CK18. Those results illustrated the maintenance of the differentiation of the hepatocytes in both type of cultures. On contrary, the CK18 was not expressed and the CYP3A2 was moderately expressed in hepatocytes monocultures without insulin (Fig. 3A). In parallel, the hepatocytes monocultures with insulin and the

hepatocytes/islets cocultures showed an intense positive cell population to INSR and to GLUT2, whereas the expressions of those markers were weaker in hepatocytes without insulin (Fig. 3B).

In the case of pancreatic islets, the level of insulin and glucagon expression inside the islets after extraction (day 0) appeared variable from one rat to another, probably due to the rat fed state at the moment of the extractions (Fig.S3, supplementary file). Then, in pancreatic islets monocultures and islets of pancreas/liver cocultures, we found an expression of both glucagon and insulin hormones at the end of the experiments (Fig. 4). Furthermore, the levels of insulin appeared over expressed in cocultures when compared to the islets monoculture levels. The glucagon was similarly expressed, in both monoculture and coculture of islets ,as far as we did not detect any difference between the immunostaining images.

3.3 RTqPCR analysis

At the end of the experiments, we analyzed the expression levels of selected hepatic genes in the different culture conditions (monoculture ITS+, monoculture ITS- and coculture). The comparison of the mRNA levels between the hepatocytes cultivated in monocultures without insulin and with insulin is presented in Fig. 5A (ratio of mRNA levels in monoculture ITS- versus monoculture ITS+). We found that the mRNA level of *Alb* was downregulated in monocultures without insulin (fold change, FC of 0.55). On contrary, the expression levels of *Hnf4a* (FC 40), *Insr* (FC 4.5), *Igf1bp1* (FC 2.5) and *Pck1* (FC 10) were significantly increased. A large variability in the levels of *Gcgr*, *Glut2* and *Cyp3a2* in monocultures without insulin was measured which led to no statistical difference between the two culture conditions on those genes.

The comparison was then performed for the gene expression in hepatocytes cultivated in cocultures with pancreatic islets and the hepatocytes monoculture with insulin. As showed in Fig. 5A, the coculture contributed to recover similar mRNA levels regarding the *Gcgr*, *Insr*, *Hnf4a*, *Igf1bp1* and *Alb* when compared to monoculture of hepatocytes done with insulin. However, the mRNA level of *Cyp3a2*, *Cyp1a2* appeared lower when compared to hepatocyte monoculture with insulin (FC

of 0.1 and 0.35 respectively). Conversely the mRNA level of *Pck1* remained high in coculture when compared to hepatocyte with insulin monoculture and at similar levels when compared to hepatocyte monocultures without insulin.

Finally, we investigated the effect of coculture with hepatocytes on expression levels of pancreatic islet-specific genes. The comparison of the mRNA levels is shown in Fig. 5B (ratio of mRNA levels in coculture versus islets monoculture). We found that the coculture with the hepatocytes contributed to downregulate the levels of *Gcgr*, *Neurod*, *Neurog3*, *Glut2*, *Ins1*, *Gcg*, *App* and *Pdx1* (FC between 0.1 and 0.36). In contrast, expression of *Reg3a*, *Ins1* and *Glp1r* were 5 to 15.5 times higher in the coculture compared to the islets monoculture. However, a large variability was founded for *Ins1* and *Glp1r* in both conditions of culture.

3.4. Functional assays revealed higher insulin and C-peptide secretions in the islets in cocultures with hepatocytes when compared to islets monocultures

The basal functionality of the pancreatic islets in both culture modes (islets monoculture and coculture with hepatocytes) was investigated by the measurement of the levels of insulin, C-peptide and glucagon secretions. At the end of each experiment, the islets were counted to normalize the data. The daily secretions of the three hormones at day 3 and day 5 (end of the experiment) are presented in Fig. 6.

We found that the insulin concentration in the culture medium decreased between day 3 and day 5, in both culture conditions (monoculture and coculture). However, the cocultures with hepatocytes contributed to maintain relative high level of production when compared to monoculture (Fig. 6A). Especially at day 5, we found production of 45 ng/islet/day and 170 ng/day/islet in monoculture and coculture respectively. Similarly, we measured a higher production of C-peptide in coculture when compared to monoculture (Fig. 6B). The secretion was about 1.5 (20 pmol/islet/day) and 5 (29 pmol/islet/day) times higher in the islets coculture with hepatocytes at day 3 and 5, respectively. The levels of glucagon remained constant in the coculture during the 5 days of experiment, close to 800 pg/islets/day, but with a large dispersion at day 3 (Fig 6C). Conversely, the level of glucagon in islets monoculture dropped from 840 to 512 pg/islet/day between day 3 and day 5 of culture.

The hepatocytes functionality was assessed by the measurement of the levels of albumin in the three culture modes (monoculture ITS+, monoculture ITS- and coculture). The hepatocytes in monoculture with insulin and in coculture with pancreatic islets produced similar quantities of albumin (constant over time, from day 3 to day 7, the end of the experiment). Conversely, without insulin in the culture medium, the hepatocytes monoculture presented a drop of the albumin production between day 3 and day 7, as shown in Fig. 7A. This led to reach a production of albumin closed to $750 \pm 100 \mu\text{g}/10^6$ cells in hepatocytes/islets biochip coculture and in liver biochip monoculture with insulin, whereas the values drop closed to $440 \pm 230 \mu\text{g}/10^6$ cells in liver biochip monoculture without insulin.

Finally, we measured the glucose and lactate levels during the culture in the different conditions (Fig 7B). The data displayed a large dispersion, but the tendency illustrated higher glucose consumption in coculture compared to liver monoculture conditions with and without insulin. Furthermore, this consumption (in hepatocytes/islets coculture) seems to be constant between day 3 and day 7. Concerning lactate production, we could not distinguish a significant difference between the different culture conditions, with a lactate production around 0.6-0.8 mmol/L.

Discussion

We have developed a model of coculture of rat hepatocytes with rat Langerhans islets using an organ-on-chip technology. The liver biochip results from our previously developed liver on chip technology that was already successfully applied for rat and human primary hepatocytes (Baudoin et al., 2014; Jellali et al., 2016). We coupled this liver on chip technology with a recent update of our technology to pancreas on chip model (Essaouiba et al., submitted). The interconnection of both organ-on-chip technologies led to produce a new model of interaction between the organs that are the liver and the pancreas. The coculture of two cell/tissue types required an adapted coculture medium able to maintain their characteristics. Preliminary tests were carried out to define the culture medium adapted to islets/hepatocytes coculture: i) hepatocytes monoculture in William's E

medium (standard medium for rat hepatocytes culture) and in a mixture 1:1 of William's E/RPMI 1640; ii) islets monoculture in RPMI 1640 medium (standard medium for islets culture) and in a mixture 1:1 of William's E/RPMI 1640. The performed analyzes (viability, morphology, gene expression of selected markers, albumin and insulin secretion) showed that islets and hepatocytes monocultures in a mixture of William's E and RPMI 1640 maintained their characteristics (when compared to islets and hepatocytes cultivated in their standard medium). Consequently, a mixture 1:1 of William's E/RPMI 1640 media was chosen for the islets/hepatocytes coculture experiments.

In this study, the hepatocytes monoculture without insulin led to a downregulation of the *Alb* mRNA and to a weaker albumin production in culture medium. The decline of albumin production and *Alb* mRNA level in lack of insulin was already reported in the literature of *in vitro* data in rat (Kimball et al., 1995; Flaim et al., 1985) and *in vivo* in mice (Chen et al., 2016). In our study, the lack of insulin also led to a weaker CYP3A2 expression and to the lack of CK18 expression at the protein level (the *Cyp3a2* was also downregulated at mRNA level). Both markers are hepatic differentiation markers. It is reported that rat hepatocytes presented a reduction of about 30% of the mRNA level of CYP3A in the absence of insulin (Woodkrooft et al., 1999). Although there is no literature result about the effect of insulin on CK18 immunostaining, various reports mentioned the crosstalk between CK18 and insulin resistance such as in diabetic patients (Civera et al., 2010) or patients with non-alcoholic steatohepatitis (Kitade et al., 2009), in which CK18 fragments are elevated in plasma. HNF4 transcription factor is involved in the mechanism of liver differentiation *via* the HNF1, HNF4, PXR and CYP450 axis (Chiang, 2009) but it is also involved in the glucose homeostasis (Stoffel et al., 1997), in liver pancreas interactions and into diabetes (Lau et al., 2018). HNF4 is targeted and repressed by insulin in hepatocytes (Hirota et al., 2003; Xie et al., 2009) with was consistent with our result as far as HNF4A was upregulated without insulin.

The hepatocytes monoculture without insulin also presented a modulation of the *Igfbp1* (Insulin-like growth factor binding proteins, up regulation of mRNA level), of the *Insr* (lower expression of the protein in the immunostaining and upregulation of the mRNA level), of *Pck1* (mRNA upregulation). Interestingly our results are consistent with the literature reporting that the insulin inhibits *Igfbp1* in liver (Powell et

al. 1991). *Pck1* catalyzes the first step in gluconeogenesis. In silencing *Pck1* mice, the insulin signaling was improved in liver (Gomez et al, 2008). Conversely insulin is also reported to reduce the mRNA level of *Pck1* (Zhang et al., 2011). Once more those data appeared consistent with our biochip findings. Finally, the overall hepatic biochip culture without insulin illustrated a consistent behavior of our hepatic model when compared to literature.

The pancreatic islets in our cocultures were able to produce insulin to counter balance the suppression of ITS. However, the level of insulin detected (about 3000 $\mu\text{g/L}$) where lower than the insulin level used in “conventional” hepatocyte culture model (in the present experiment, the hepatocytes monoculture with insulin were performed at 10mg/L of insulin via ITS supplementation in the medium). That could explain why, at the mRNA level of the hepatic marker in hepatocyte coculture such as *Alb*, *Cyp3a2*, remained lower and *Pck1* remained higher than the level in hepatocytes monoculture with insulin. Nevertheless, at the protein level the expression of *Cyp3a2*, CK18 and the production of albumin were restored in coculture. The mRNA levels of insulin related genes such as *Igf1* and *Insr* were also restored, including *Hnf4a*. In addition, the protein expression of *Insr* and *Glut2* appeared similar in the immunostaining in both hepatocyte monoculture with insulin and in islets/hepatocytes cocultures. Those data illustrate the functional cross talk between the pancreas and the liver. It also demonstrated the partial restoration of the expected effect of insulin on hepatocytes. Nevertheless, additional experiments, by tuning the number of isles, to increase the production of insulin would be required to confirm the full recovery of *Pck1* and *Cyp3a2* mRNA levels.

High glucose level normally leads to high production of insulin. In the present culture conditions, the glucose level in medium was of 2,5 mM and did not decrease strikingly between two time points of culture medium change (*nb* similar glucose concentration was used in all culture modes). This led to a continuous stimulation for insulin production in the pancreatic islets monoculture and during the islets/hepatocytes coculture (and thus contribute to restore the hepatic functions in the coculture by insulin secretion stimulated by glucose). Regarding the effect of hepatocyte coculture on the pancreatic islets, we found that the coculture downregulated *Gcgr*, *Glut2*, *Ins1*, *Neurod*, *Neurog3*, *App*, *Gcg*, *Pdx1* and upregulated

Glp1r at the mRNA level. *Pdx1*, *Neurod* and *Neurog3* are important markers of the differentiation of the islets (Gu et al., 2010; Mc Grath et al., 2015). *Neurod* is an important gene (an insulin trans activator) required to maintain functional maturity in pancreatic beta cells, including insulin production through *INS1* (Gu et al., 2010). Consistently, we found both downregulation of *Ins1* and *Neurod* in coculture biochips (which is consistent with *Ins1* silencing in *Neurod* KO mice, Gu et al., 2010). *Neurod* KO-mice expressed *Ins2* gene and are thus able to produce insulin in glucose tolerance test, which also appeared consistent with our finding in which *Ins2* was over expressed in pancreas biochip coculture (Gu et al., 2010). *Pdx1* has pivotal important gene in β cells. *Pdx1* is a homeobox-containing transcription factor that plays a key role in pancreatic development and adult β -cell function (Gao et al., .2014). Depletion of *Pdx1* lead to hyperglycemia in mice, to a cell reprogramming in mice islets, glucagon overexpression in Min6 β cells (Gao et al., .2014). Furthermore, *Pdx1* deficient β cells led to the reduction of the transcript level of *Pdx1*, *Ins1* and *Glut2* and the maintenance of glucagon one (Gao et al., 2014). This result was partially consistent with our dataset in which we found a concomitant downregulation of *Pdx1*, *Ins1* and *Glut2* genes in coculture.

Glucagon is produced during hypoglycemia to stimulate hepatic glucose output. In our study, the glucose concentration remained high in the culture medium, leading to detect a high insulin/glucagon ratio which is consistent with the downregulation of the levels of *Gcg* (glucagon) and *Gcgr* (glucagon receptor) mRNA. However, we also measured high level of *Glp1r* mRNA. It is reported that paracrine glucagon stimulates insulin secretion through both *Gcgr* and *Glp1r*. More particularly the activity of glucagon and GLP-1 receptors were reported to be essential for β cell secretory responses *via* paracrine intra-islet glucagon actions to maintain appropriate insulin secretion (Svendsen et al., 2018), which is consistent with our findings in coculture. Although we described those behaviors in the pancreatic tissue as a result of the hepatic coculture, we did not clearly identify the underlying mechanisms and the endocrine liver signaling driving such cross talk. As a result, additional analysis would be required to complete our investigation including metabolome and proteome analysis.

Conclusion

In this study, we proposed a new liver/pancreas interaction model in biochips to investigate the cross talks between both organs. The characteristic functions of hepatocytes/islets coculture model were evaluated comparing with those of islets or hepatocytes (with and without insulin) monocultures. The hepatocyte monoculture without insulin led to a modulation of the glucose homeostasis targets and of the hepatic differentiation markers. Conversely, the coculture with pancreatic cells producing insulin contributed to recover the hepatic function illustrating the benefice of the two organs model. For pancreatic functions, the presence of the hepatocytes in coculture model contributed to modify the islet response via the increase of the insulin production and the modification of mRNA levels of the gene involved in the insulin/glucagon homeostasis. The pancreas-liver organ on chip model presented here was able to recapitulate several physiological responses, and demonstrated the potential of our approach to reproduce and investigate complex *in vivo* patterns using alternative *in vitro* methods.

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