FONDAZIONE DIABETE RICERCA

TRAVEL FELLOWSHIP REPORT

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Report

My fellowship in the Kulkarni lab at Joslin Diabetes Center has been a positive and amazing experience.

The opportunity to complement my own studies in a lab with experts in investigation of human islet function, has given me the experience to improve my scientific skills.

The weekly meetings with the lab members, and the shared meetings with other Joslin investigators, represented constructive experiences which stimulated new ideas and curiosity in different fields of diabetes research, and have improved my ability to judge and criticism of research.

The expertise of the Kulkarni lab, in investigating human islet function and pancreas immunohistochemistry, gave me the chance to complement my own previous work in humans and to learn techniques that will be essential for the future plans in my project and in my future scientific research.

My aim in this fellowship period was to compare in vivo results of my study with several in vitro beta cells features, trying to better define mechanisms that eventually regulate the beta cells adaptation to impaired metabolic state.

I compared islets from non-diabetic patients with failed islets from newly diabetic patients in order to detect molecular changes and regulators involved in the development of diabetes. We believe that the analysis of gene expression and proteomics of human islet samples from patients with different metabolic status (and variable numbers of trans-differentiated cells) might provide a suitable tool to investigate pathways and mechanisms regulating cell fate changes within the islet.

To pursue my goal, I learned these techniques:

Light and electron microscopy

Standard procedures were performed for the preparation of samples to be used in light microscopy evaluations. The immunohistochemistry of analysing pancreas morphology and histological features is fundamental. For immunohistochemistry studies, I used tissue resections fixed in 10% buffered formalin and embedded in paraffin.

Cocktail staining for insulin, glucagon and somatostatin

We imaged all islets with 200x magnification (20 x objective). By analysing these pictures, I measured the positive per insulin, glucagon and somatostatin in every single islet, using a dedicated software. To determine the pancreatic fractional beta cell area, as well as alpha and delta cell area, the entire pancreatic section was imaged at 40x magnification (x objective). The ratio of the beta cell area, exocrine area, as well as, alpha cell and delta cell areas was digitally quantified using a software package.

In order to identify the resource of beta cells in response to alterated metabolic state, we want to determine the contribution of duct cells to the total islet cell mass and show the presence of transdifferentianting α cells(double positive cells for insulin and glucagon) in imparied glucose metabolic state.

Laser capture microdissection (LCM)

I also learned the laser capture microdissection technique to obtain B-cell preparations from frozen samples. The frozen pancreatic tissue were sectioned at 8µm in a cryostat. Immediately prior to LCM, the sections were dehydrated in 70% ethanol for 30 seconds, 100% ethanol twice for 1 min, and xylene for 4 minutes. The sections were then completely air-dried and LCM were performed using an ArcturusXTMicrodissection System (Molecular Devices, Sunnyvale, CA). LCM will be performed by melting thermoplastic films mounted on transparent LCM caps (Molecular Devices) on selected populations of cells. To obtain adequate spot size, the laser beam were set to the following parameters: 35 mW the power, 4.5 msec the pulse duration, and 7.5 µm the spot size. The thermoplastic film containing the microdissected cells were incubated with 10 µl of a guanidine thiocyanate and polyethylene glycol octylphenol ether based buffer for 30 minutes at 42°C and total RNA were extracted using PicoPure RNA Isolation Kit (Molecular Devices). Total RNA quantity will be evaluated by absorbance readings at 260 nm and 280 nm using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and the quality will be assessed by running the samples on Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).

RNA extraction

RNA quantity will be evaluated by absorbance readings at 260 nm and 280 nm using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and the quality will be assessed by running the samples on Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Total RNA will be extracted using Rneasy Micro Kit 50 (Qiagen) after using a Qiazol lysis reagent (Qiagen). Genomic DNA contamination will be removed by treating the samples with DNase I (RNase Free DNase Set, Qiagen). RNA with RIN>6 will be used for downstream experiments.

I validated the RNA extraction protocol in a pilot study in which I compared the efficiency of different RNA extraction kit on LCM human islets preparations. In order to do that I collected about 100 islets from frozen pancreas samples of both no diabetic and diabetic subjects, underwent to partial pancreatectomy, using Laser capture microdissection technique and I used picked islets from one diabetic and one healthy donors as positive controls. Total amount of RNA from each microdissected sample was evaluated with Nanodrop by absorbance at 260 and 280 nm, subsequently 1 µl of RNA was analyzed by the Agilent 2100 Bioanalyzer to assess the RNA quality.

In particular we compare three different protocols on LCM specimens of one NGT and one glucose intolerant subject compare to handpicked islets respectively from a no diabetic donor and a diabetic donor. In all the protocols we used Qiazol as a lysis cells buffer. In the first we didn't use any columns to minimize the loss of material but even if the samples had a consistent concentration, assessed with Nanodrop, the Bioanalyzer showed a relative low RIN both in no diabetic (Fig1;1/4) and diabetic subjects (Fig2;1/4), probably due to ethanol contamination and not adequate washing of the pellets. In the second protocol we add to the first process a step of RNA cleaning using the Minielute Cleanup kit by Qiagen which allow to

purify and concentrate at least 100 µg of total RNA (≥ 200 nucleotides) in an elution volume of 30-100 µl, reporting an increase in RNA quality both in no diabetic and diabetic subjects (Fig.1;2/6 and Fig.2;2/6). While a better performance is reported following the third protocol (Fig.1;3/6 and Fig.2;3/6), through the availment of RNA Micro Kit (Qiagen) containing a poly-A RNA for use as carrier RNA; in part because when added to lysates from very small samples, the carrier RNA may in some cases improve the recovery of total RNA and in part probably because the columns provided in this kit show a greater binding capacity of the MiniKit columns.

Fig.1 Evaluation of RNA purity with Bioanalyzer obtained by the 3 different protocols; 1,2,3 LCM samples of NGT subject, 4,5,6,picked islets of no diabetic donor.

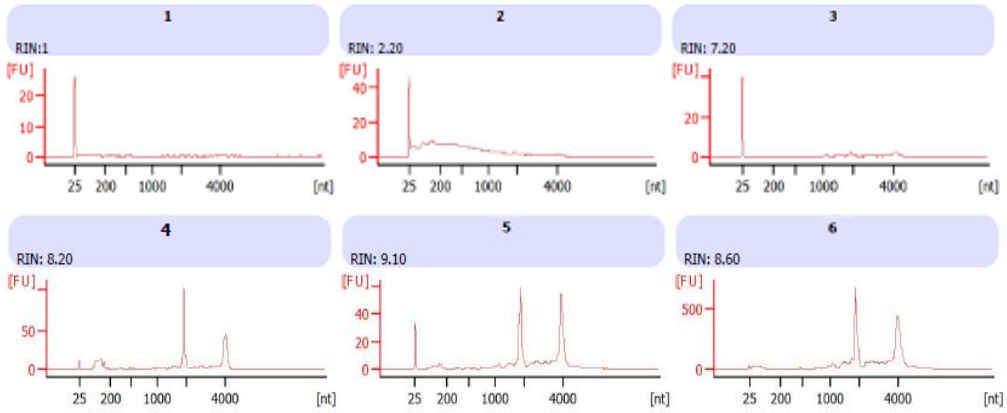
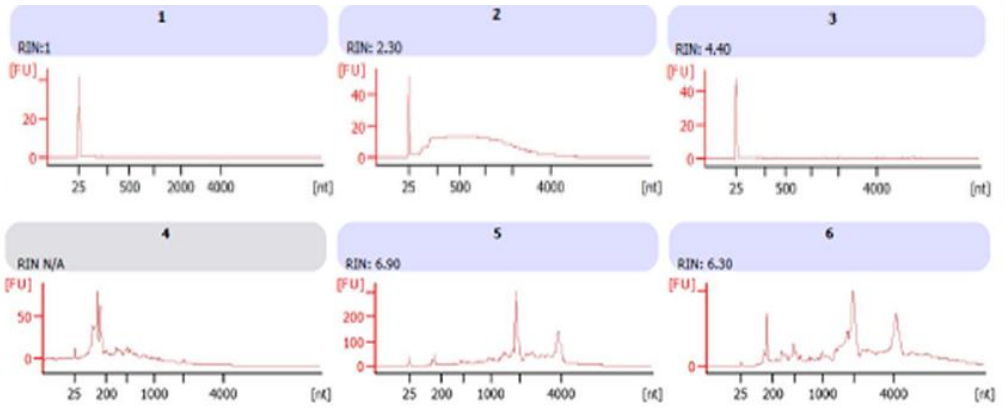


Fig.2 Evaluation of RNA purity with Bioanalyzer obtained by the 3 different protocols; 1,2,3 LCM samples of T2D subject, 4,5,6,picked islets of diabetic donor.



Real-time PCR

PCR measurements of selected genes identified by bioinformatic and computational analysis will be determined. cDNA templates will be synthesized from 200 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). TaqMan Universal PCR Master Mix (Applied Biosystems) will be used to perform real-time PCR in the presence of 0.3 ng cDNA, 1 µM primers and 0.25 µM probe in a total volume of 20 µl. Primers and probes will be obtained from Assay on Demand (Applied Biosystems) or will designed using Primer Express software (Applied Biosystems). The experiments will be performed on an Applied Biosystems 7300 Real Time PCR System.

Proteomics analysis

Protein digestion. Islet samples will be homogenized and digested using a 2,2,2-trifluoroethanol (TFE)-based protocol (Wang H, et al. J Proteome Res 2005).Briefly, islets will be dissolved in 30 ul of 50% TFE / 50% 25 mM NH_4HCO_3 by 3 min sonication in 5510 Branson ultrasonic water bath (Branson Ultrasonics, Danbury, CT) with ice cold water bath. Protein concentration will be determined by BCA assay. About 40 µg islet proteins from each pancreas samples will be denatured in 50% TFE for 105 min at 60 °C, reduced by 2 mM DTT for 60 min at 37 °C, diluted by 5 fold with 50mM NH_4HCO_3, and digested by 0.8 µg trypsin (1:50 w/w trypsin-to-protein ratio) for 3 hours at 37ºC. The digestion will stopped by 0.1% TFA. All peptide samples will be dried down in speed vacum to remove TFE, and resuspended in 25 mM NH_4HCO_3 for LC-MS/MS analysis. Subsequently LC-MS/MS analyses will be performed on a custom-built automated LC system coupled on-line to an LTQ-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) via a nanoelectrospray ionization interface.