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Overexpression of Glycolytic Enzymes and Antioxidant Protein in Differentiated Beta Cells

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ABSTRACT

Due to the importance of proteins in the differentiation and function of cells, the investigation of variation of the protein expression levels can help for the better understanding of the differentiation processes. Cell-therapy provides a promising alternative for the treatment of type 1 diabetes. In our previous studies, we isolated and expanded human skin-derived precursors (hSKPs) and differentiate them into IPCs *in vitro*, through exposure to suitable differentiation factors. Cellular clusters were also able to secrete detectable amounts of insulin and C-peptide in glucose concentration-dependent manner. In the present study, we applied a two-dimensional electrophoresis technique to evaluate variations in protein expression in expanded SKPs and differentiated IPCs. The results showed that the protein pattern of SKPs differs with differentiated IPCs in terms of the number of points and the expression level of protein. The current study demonstrates that IPC differentiation in human SKPs is accompanied by modulation of 13 major proteins with diverse Functions: metabolic, chaperone activity, redox (antioxidant), protein degradation, transcription and signaling cascades. Overexpression of three key enzymes in the glycolytic pathway was observed. Since insulin is a hormone that is involved in glucose metabolism, it is not surprising overexpression of glycolytic enzymes found in differentiated cells. Here we show for the first time the expression of the antioxidant proteins in differentiated cells has increased. Also, augmentation in ubiquitin and cyclophilin A was observed, indicating overexpression of proteins that involved in degradation of old proteins and synthesis and folding of new proteins in the differentiation process.

Keywords: Diabetes, Differentiation, Skin-derived precursors, Insulin-producing cells, Two-dimensional electrophoresis

INTRODUCTION

Today, advances in proteomics technologies can be used to identify maps of protein expression found in different cells. Identification of proteome maps of human stem cells can be used as a useful resource in researches. By attention to the pattern of protein expression in these cells before and after differentiation, we can reveal protein expression changes due to activation or repression of specific pathways during differentiation or proliferation. Many proteins are detected in the proteome of human pancreatic islet cells that refer to functional pathways in the activated differentiation and plasticity processes.

Type I diabetes is an immunologically-mediated disease and results in the destruction of β-cells in pancreatic islets. Current medical care based upon the long-term injection of insulin isn't fully capable of achieving tight management of glucose regulation [1,2]. The use of stem cells for producing functional beta cells might provide an alternative approach [3-7]. One of the best candidates among adult stem cells is Skin-derived precursors cells (SKPs) which would overcome these problems [8,9]. Indeed, skin-derived stem cells represent a highly accessible and potentially autologous source of adult precursors that are capable of generating different functional cell types (such as glial cells, neurons, smooth muscle cells, insulin-producing cells (IPCs) and adipocytes), which makes them a predominant autologous donor source for stem cell therapy [10-15]. Nevertheless, the process for induction of differentiation is not completely understood and may be influenced by different culture conditioning [3].

In the previous study, we reported our experience in culturing human skin-derived precursors (hSKPs), in a suitable IPC differentiation culture medium, resulting in the

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production of insulin. It has been demonstrated the presence of skin-derived precursor cells in the human foreskin that exhibit potential of differentiation to IPCs and that could be considered as a source of IPC ex vivo production [16]. Due to the results, SKPs derived from human foreskin as an easily accessible, autologous source of pancreatic progenitor cells are capable of differentiating into insulin-producing islet-like cell cluster. Therefore, it can be a valuable source of protein pattern studies of pancreatic progenitor cells. We applied proteomic techniques to evaluate variations in protein expression in expanded and differentiated SKPs, influenced by the conditioning process. Proteomic profiling of human pancreatic islet-cells has been reported [17-19] with the identification of 66 different proteins, serving as a reference map of human islet cell populations. These data were however at variance with the reported proteomic data on islet cells of murine and rat origin [19,20]. Proteomic study of pancreatic cancer cells to identify proteins involved in cancer and metastasis has been reported [21]. Detailed proteomic data on mouse and rat models of type II diabetes have been reported in the literature [22,23]. In addition, a report of the proteomic profile of stem cells from pancreatic islets (human islet-mesenchymal stem cells, HI-MSCs) and human bone marrow (bone marrow mesenchymal stem cells, BM-MSCs) were cultured in suitable conditions in order to induce differentiation into Islet-like Cells (ILCs), has been recently described [24]. Nevertheless, detailed data in this field about human SKPs don't have yet to be described, and no proteomic data have been reported on IPCs derived from SKPs.

The comparison of the proteomic assets of SKPs and IPCs may unravel similarities and differences correlated to the differentiation processes, and it might provide new insights into the conditioning protocols required in order to achieve a stable and robust secretion of insulin.

Materials and Methods

Immobiline dry strips (pH 3-10 and 18 cm), acrylamide, bisacrylamide, CHAPS and SDS were purchased from Pharmacia. Urea, thiourea, bromophenol blue, DTT, Tris base, ammonium persulfate, glycine, TEMED, silver nitrate and 2-mercaptoethanol, were of analytical grade and purchased from Merck (Germany).

Sample Preparation for Proteomics

SKPs and IPCs cells, control, and differentiated cells, were collected directly from flasks and washed twice in PBS. Cells lysates were obtained using 2DE lysis solution (8 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM Tris) with added cocktail protease inhibitor (Sigma) by gentle lysis methods and shaking for 5 hours at room temperature. Suspensions were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatants were conserved for 2-DE analysis. Protein concentrations were measured using a Bradford Protein Assay method.

Rehydration and In-gel Sample Application

The 18 cm ready-made IPG strips, pH 3-10 NL, were rehydrated with a 350 µl total volume lysis solution (cells lysates) contains 300 µg of protein, 2.5% (v/v) IPG buffer (3-10) and 1% (w/v) DTT. Then IPG strip side down into the lysis buffer and incubated at 37 °C for 15 h in the strip holder. After incubation, the IPG strip was transferred in the groove of immobiline dry strip tray as the acidic end of the strip at the front anode electrode.

First Dimension Separation (Isoelectric Focusing)

Before running isoelectric focusing, 300 ml of IPG cover fluid was poured into the tray to cover the IPG strips. The isoelectric focusing was performed using a Multiphor 2 system (Pharmacia Biotech). The temperature was adjusted at 20 °C by dual temperature circulator (Tamson Zoetermeer-Holl). The IEF voltage was applied using power supply (Pharmacia) according to a pre-set program (Table 1). Focusing was performed at 20 °C with a limit of 10 mA per strip.

Second Dimension Separation (SDS- PAGE)

Once the first dimension separation was terminated, the strip was equilibrated in two steps. The first with 50 mM Tris-HCl, pH 8.8, Urea 6 M, Glycerol 30% (v/v), SDS 3% (w/v) and DTT 1% (w/v) for 30 min, and the second step with the same buffer and the same time but with 5% (w/v) iodoacetamide instead of DTT. For the second dimension separation, 13.5% vertical SDS polyacrylamide gels were used. Gels were stained with silver staining compatible with mass and scanned using Progenesis SameSpots V3.0 (Nonlinear Dynamics) software, some significant spots were

Table 1. IEF Program for IPG Strip (pH 3-10 and 18 cm)

Step	Voltage (V)	Step duration (h)
1	500	1
2	500	2
3	3500	3
4	3500	15

processed for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) analysis.

Silver Staining Compatible with Mass

Silver staining method provides a very sensitive tool for protein visualization with a detection level of 0.3-10 ng/spot. After electrophoresis, gels were fixed inappropriate fixation solution (50% methanol, 12% acetic acid and 0.019% w/v formaldehyde) for 2 h. The gels washed with washing solution (35% ethanol) for 20 min 3 times, Incubated with a sensitizing solution (thiosulphate sodium.5 H_2O 0.062 g in 200 ml deionized water) for 2 min, followed by washes in deionized water for 5 min 3 times. The gels were placed in staining solution (2% silver nitrate, 0.025% w/v formaldehyde) and with gentle shaking for 20 min and rinsed briefly in deionized water. The gel was developed in developing solution (6% w/v sodium carbonate, 0.019% w/v formaldehyde). After detection of protein spots, the gel was placed in the stop solution (50% methanol and 12% acetic acid) for 30 min and finally stored in 1% acetic acid in 4 °C for future analysis. The 2-DE gel images were analyzed with an image analysis software program, Progenesis SameSpots V3.0 (Nonlinear Dynamics) software.

Image Analysis

2-DE image analysis was performed using Progenesis SameSpots V3.0 (Nonlinear Dynamics) software (Bio-Rad, USA) according to the manufacturer's instructions. The normalization of each individual spot was performed according to the total quantity of valid spots in each gel, after subtracting background values. The spot volume was used as the analysis parameter to quantify protein expression.

In-gel Digestion and MALDI-TOF Analysis

Silver nitrate-stained spots were excised from 2-DE gels; destaining and in-gel enzymatic digestion were performed: Briefly, each spot was destained with 100 ml of 50% v/v acetonitrile in 5 mM ammonium bicarbonate and dried with 100 ml of acetonitrile. Each dried gel piece was rehydrated for 40 min at 4 °C in 10 ml of a digestion buffer containing 5 mM ammonium bicarbonate, and 10 ng ml^{-1} of trypsin. Digestion was allowed to proceed overnight at 37 °C and peptide mixtures were stored at 4°C until assayed. All digests were analyzed by MALDI-TOF (Tof Spec SE, MicroMass) equipped with a delayed extraction unit. Peptide solutions were prepared with equal volumes of saturated a-cyano-4-hydroxycinnamic acid solution in 40% v/v acetonitrile-0.1% v/v trifluoroacetic acid. The MALDI-TOF was calibrated with a mix of PEG (PEG 1000, 2000 and 3000 with the ratio 1:1:2) and mass spectra were acquired in the positive-ion mode. Peak lists were generated with Protein Lynx Data Preparation (Protein Lynx Global Server 2.2.5) using the following parameters: external calibration with lock mass using mass 2465.1989 Da of ACTH, background subtract type adaptive combining all scans, performing deisotoping with a threshold of 1%. The twenty-five most intense masses were used for database searches against the SWISSPROT database (release 2011-01 of 11-Jan-11) using the free search program MASCOT 2.3.02 (http://www.matrixscience.com). The following parameters were used in the searches: taxa Homo sapiens, trypsin digest, and one missed cleavage by trypsin, carbamidomethylation of cysteine as fixed modification, methionine oxidation as variable modification and the maximum error allowed 100 ppm. Only proteins with a Mascot score.55 were taken into consideration.

Statistical Analysis

Data from image analysis were used as values of protein expression in 2-DE experiments as previously described [25]. The two-tailed Student's t-test was used to verify the significance of the variations of expression of proteins in SKPs and IPCs cells, control and differentiated cells. Experiments were performed in triplicate. Statistical significance was set at p values ≤ 0.05. In 2-DE experiments, proteins were classified as differentially expressed if the ratio of the spot intensity between treated cells and control cells was greater than 1.5-fold (protein overexpressed).

RESULTS

Proteins from three independent biological replicas of SKPs and IPCs were extracted and analyzed by 2-DE using a nonlinear gradient of pH 3-10. Resulting gels, stained with silver nitrate, were quantitatively and statistically analyzed by Progenesis SameSpots V3.0 (Nonlinear Dynamics) software as described above (see Material and Methods). Figure 1 shows representative 2-DE gel images, showing the differentially expressed spots identified in each gel. As is considered, two-dimensional electrophoresis patterns obtained by SKPs of human foreskin and differentiated IPCs have a significantly different. Two-dimensional gel image of SKP, as a "reference image" was selected for further analysis. After the alignment of the spots by software and matching them, 907 points in the two study groups were identified by software and visually confirm compliance. After analyzing these points, 100 points, with $P \leq 0.05$ and Fold ≥ 1.5 were identified that were more expressed in SKPs than IPCs and displayed significant differences. Also, 439 points with $P \leq 0.05$ and Fold ≥ 1.5 were identified in the IPCs had more expression than SKPs. Among these spots, we could identify 24 spots who had more concentration than other spots and were induced or reduced by $\geq$2.5-fold following IPCs differentiation. These spots were selected and excised from gels and subjected to in-gel tryptic digestion and identification by MALDI-TOF-TOF.

After analysis by MALDI-TOF-TOF method, 24 spots corresponding to 13 unique proteins were identified and in some case, the different isozyme of proteins was identified in different spots over the gel (*e.g.* CypA or SODM) as shown in Fig. 2. These proteins were identified by peptide mass fingerprinting with the search program Mascot search (Matrix Science). The identity, full and abbreviated name, number of peptides matched, percent coverage, "score," pI and molecular weight of each protein are presented in Table 2. Changes for representative protein features are displayed between two-dimensional gels prepared with protein lysates from undifferentiated and IPCs differentiated cells in Fig. 3.

Overexpression of triose phosphate isomerase, Glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate mutase, three key enzymes in the glycolytic pathway, was observed. Since insulin is a hormone that is involved in glucose metabolism, it is not surprising overexpression of glycolytic enzymes found in differentiated cells. Triose phosphate isomerase has a molecular weight of 26930 Dalton and pI 6.9. Based on the software calculations, its expression in IPCs 2.5 fold was higher than SKPs. Glyceraldehyde 3-phosphate dehydrogenase has a molecular weight of 36202 Dalton and pI 8.26. Its various isoforms form 2 spots on a gel having the same molecular weights and different pIs. Based on software calculations, these spots were increased by 2.5 and 2.9 fold in IPCs, respectively, compared to SKPs. Phosphoglycerate mutase has a molecular weight of 28,900 Dalton and pI 6.67, and its various isoforms are 4 spots on the gel having different pIs. According to the software calculations, these spots were raised 3.8, 2.5, 4.2 and 2.6 fold in IPCs.

In addition, an increase in the expression of proteins involved in protection against oxidative damages in cells such as thiol-specific antioxidant protein, thioredoxin-dependent peroxidase reductase (peroxiredoxin) is observed in differentiated cells. Although manganese superoxide dismutase enzyme is reduced in the differentiated cells, its expression is still impressive. Here we show for the first time the expression of the antioxidant proteins in differentiated cells has increased. Precursor cells also increased the expression of the manganese superoxide dismutase. Thiol-specific antioxidant protein has a molecular weight of 22014 Dalton and pI 6.84. Based on the software calculations, a 2.5-fold increase in IPCs has occurred than SKPs. The molecular weight of thioredoxin-dependent peroxidase reductase (peroxiredoxin) is 28017

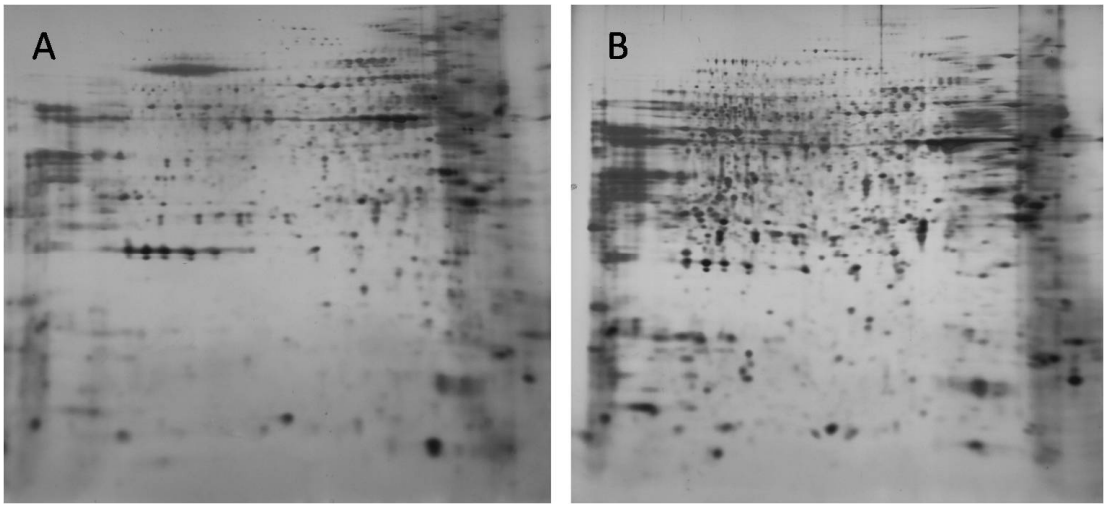

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis. Two-dimensional PAGE was performed with protein lysates prepared from human SKPs cells in the undifferentiated (A) and IPCs differentiated (B) condition 20 days following induction. The gels were stained with silver nitrate compatible with mass.

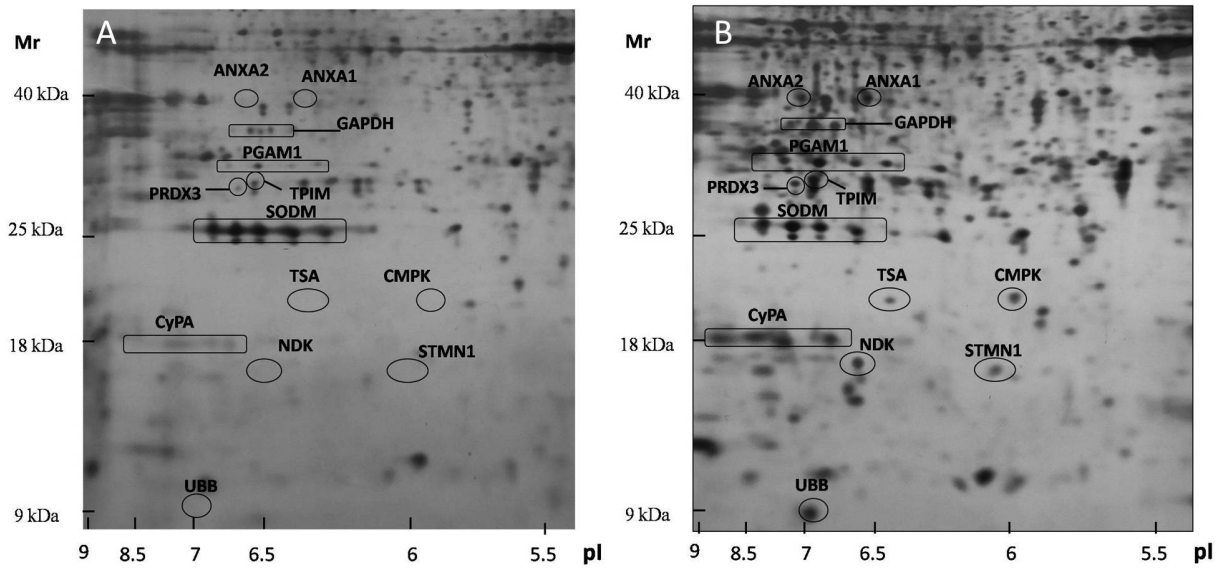

Fig. 2. 2-DE analysis of SKPs and IPCs. Representative image from three independent experiments of silver nitrate stained 2-DE patterns of SKPs (A), IPCs (B). Identified proteins showing differential expression were indicated on both gels. Proteins identified in two groups, Corresponding identifications are reported in Table 2.

Dalton and pI 7.67. Based on the software calculations, this spot was 2.7 fold higher in IPC than SKP. Manganese superoxide dismutase has a molecular weight of 24866 Dalton and pI 8.35. Its various isoforms form 4 spots on gel having different pIs. According to the software calculations, these spots have been increased by 1.6, 1.7, 1.9, 3.4 and 1.9 in SKP compared to IPC, respectively.

Cyclophilin (CyPA) is a peptidyl-prolyl *cis/trans*

Table 2. Proteins of Human SKPs and Differentiated IPCs 2-D Gels, Which were Identified by Peptide Mass Fingerprinting Using MALDI-TOF MS

Protein full name	Ab. Name	MW (Da)	pI	Sequence coverage (%)	Score	No. of unique peptides
Cyclophilin A (Peptidyle-prolyl cis-trans isomerase)	CyPA	18217	7.68	17	128	2
Annexin A1	ANXA1	39031	6.57	21	673	6
Stathmin	STMN1	17292	5.76	8	90	1
Phosphoglycerate mutase 1	PGAM1	28900	6.67	30	546	6
Triose phosphate isomerase	TPIM	26930	6.90	5	90	1
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	36202	8.26	8	168	2
Thiol-specific antioxidant protein	TSA	22014	6.84	14	213	2
Nucleoside-diphosphate kinase A1	NDK	17364	6.92	14	153	2
Annexin A2	ANXA2	38808	7.57	5	104	1
Manganese superoxide dismutase	SODM	24866	8.35	15	223	2
Ubiquitin B (isoform CRA-b)	UBB	8720	6.8	14	147	2
UMP-CMP kinase (cytidin mono phosphate kinase)	CMPK	22493	6.02	6	67	1
Thioreduxin-dependent peroxidase reductase (Peroxireduxin)	PRDX3	28017	7.67	4	90	1

isomerase was also augmented after differentiation. The enzyme that catalyzing the cis-trans isomerization of proline residues (in peptide bonds amino terminal) in proteins. Four spots on a 2-DE gel were identified with same molecular weight and different pIs. These 4 spots were identified as the cyclophilin-A enzyme. Its molecular weight is 18217 daltons and its pI is 7.68. Other isoforms of the enzyme have a mean pI of 7.4 and 9.1. These four spots with the same molecular weight and different pI are isoforms of this protein. According to the software calculations, these spots were raised 2.8 to 3.4 fold in IPC, respectively.

Altered expression of ubiquitin, stathmin, annexin A1 and annexin A2, UMP-CMP kinase, nucleoside-diphosphate kinase A1 has been identified in this experiment.

Ubiquitin is a small protein that exists in all eukaryotic cells. The ubiquitin-proteasome system is responsible for the degradation of most intracellular proteins and therefore plays an essential regulatory role in critical cellular processes including cell cycle progression, proliferation, differentiation, angiogenesis, and apoptosis. The molecular weight is 8720 Dalton and pI 6.79. Based on software calculations, this spot was 3.1 fold higher in IPC than SKP.

Stathmin is a ubiquitous soluble protein whose phosphorylation is associated with the intracellular

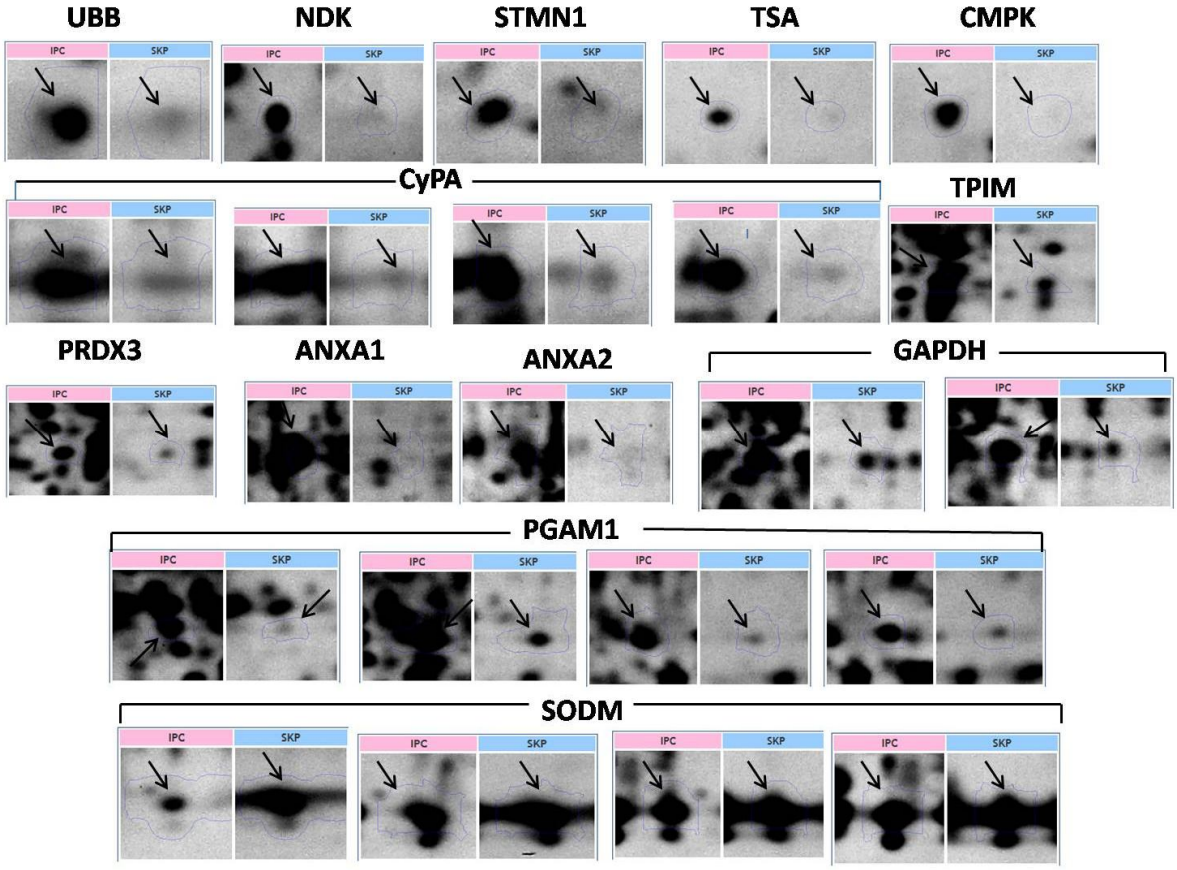

Fig. 3. Protein features from two-dimensional gels prepared with protein lysates from undifferentiated and differentiated human SKPs. Changes for representative protein features are displayed between two-dimensional gels prepared with protein lysates from undifferentiated and IPCs differentiated cells. The arrows indicate the location of the protein features.

mechanisms involved in the regulations of cell differentiation and functions by extracellular effectors. The protein has a molecular weight of 17292 Dalton and a pI of 5.76. Based on software calculations, this spot was 2.7 folds higher in IPCs than SKPs.

Annexins are a family of calcium- and phospholipid-binding proteins found in nearly all eukaryotes. They are structurally highly conserved and have been implicated in a wide range of cellular activities. The annexin A1 protein has a molecular weight of 39031 Dalton and pI 6.57, and Annexin A2 has a molecular weight of 38808 Dalton and pI 7.57. According to the software calculations, these spots were increased by 5.1 and 3.1 fold in IPCs compared to SKPs, respectively.

UMP-CMP kinase: this enzyme catalyzes the conversion of cytidine-monophosphate or uridine-monophosphate to cytidine-diphosphate or uridine-diphosphate by ATP and plays a critical role in providing nucleotides precursors for DNA and RNA synthesis. UMP/CMP kinase plays a critical role in the pathway that supplies nucleotide precursors for DNA and RNA synthesis. The molecular weight is 22493 Dalton and pI is 6.02. Based on software calculations, this stain has increased by 4.1 fold in IPC relative to SKP. Therefore, this increase is significant.

Nucleoside-diphosphate kinase A1: The overall effect of NDKs is to transfer a phosphate group from a nucleoside triphosphate to a nucleoside diphosphate. The protein has a

molecular weight of 17364 Dalton and pI 6.92. Based on software calculations, this 3.6-fold increase in IPC was higher than that of SKPs.

DISCUSSION

In our previous study, we revealed that hSKPs have the potential to be differentiated into functional IPCs in the presence of multiple factors in a four-stage procedure [16]. The differentiated cells were capable of expressing insulin after induction. We tested the functionality of the in vitro-generated IPCs from human foreskin by measuring insulin and C-peptide secretion in response to glucose concentration changes. They were also able to secrete detectable amounts of insulin and C-peptide in glucose concentration-dependent manner [16]. Cells undergoing pancreatic beta cell differentiation exhibit well characterized morphologic changes that are reflected in the cell proteome. We applied the 2-DE electrophoresis technique to evaluate variations in protein expression in expanded and differentiated SKPs, influenced by the conditioning processes.

Our study was aimed to clarify modification of hSKPs towards pancreatic beta cell differentiation via culture media induction of differentiation. Modification of the protein asset during these medium-induced modifications may be of interest in order to correlate the regulatory mechanisms acting in the differentiation process. Moreover, it may possibly give an insight into the medium induced stem cells modifications that would be useful for a better control of cell manipulations through safe and conservative approaches.

The current study demonstrates that IPC differentiation in human SKPs is accompanied by modulation of 13 major protein with diverse Functions: metabolic, chaperone activity, redox (antioxidant), protein degradation and signaling cascades (Table I).

Overexpression of Triose phosphate isomerase, Glyceraldehyde 3-phosphate dehydrogenase and Phosphoglycerate mutase, three key enzymes in the glycolytic pathway, was observed. Since insulin is a hormone that is involved in glucose metabolism, it is not surprising overexpression of glycolytic enzymes found in differentiated cells. Proteomics studies on beta cells have significantly shown overexpression of two types of proteins compared to other tissues: proteins responsible for the biosynthesis and folding of other proteins and enzymes involved in glycolysis pathway [19]. Glycolytic enzymes in beta cells, on average, 50 percent more than other enzymes are expressed. Published proteome profiles of mouse pancreatic islets, exposed for 24 h to high glucose (16.7 mM) versus basal (5.6 mM), the major adaptations in high glucose-exposed beta cells were increased expression of the glycolytic enzyme.

In mammals, the beta cells of the pancreatic islets of Langerhans sense changes in the nutritional state of the organism and respond by modulating synthesis and secretion of insulin, the signal for energy storage. Glucose and its metabolite glyceraldehyde 3-phosphate are the most potent nutritional secretagogues of insulin. Because glucose analogs that cannot be metabolized by beta cells do not stimulate insulin secretion, whereas glyceraldehyde and other energy sources do stimulate insulin secretion [26,27], it has been concluded that some final common end product(s) of catabolism may be required to stimulate secretion.

In liver cancer, breast cancer [25], prostate cancer [28] and lung cancer [29,30] overexpression of Phosphoglycerate mutase, as a new metabolic enzyme involved in malignancy was found and blocking its expression shows antiproliferative effects and decreased the growth of cancer cells. The metabolism of cancer cells differs significantly from that of normal cells [31]. Cancer cells are able to maintain high rates of aerobic glycolysis even under the high-oxygen (20%) conditions of normal tissue culture. This property, known as the "Warburg effect", has been recognized for over 70 years [32]. In this context, maintaining a high level of glycolysis is indispensable for the survival and growth of cancer cells [33,34].

In general, an increase in the expression of proteins involved in protection against oxidative damages in cells such as thiol-specific antioxidant protein, thioredoxin-dependent peroxidase reductase is observed in differentiated cells. Although superoxide dismutase enzyme is reduced in the differentiated cells, its expression is still impressive. Here we show for the first time the expression of the antioxidant proteins in differentiated cells has increased.

Precursor cells also expressed a high level of the superoxide dismutase. SODM (mitochondrial superoxide dismutase) is an essential component of the cellular defense mechanism against oxidative stress (ROS), which contributes to the damage to beta cells that plays a role in the pathogenesis of type 2 diabetes [35]. Total protection of stem cells and their differentiated derivatives is mediated by increased expression of these proteins due to a systematic protection against free radical.

Overexpression of Cyclophilin A (CyPA) was also observed in IPC. Takahashi *et al.* (1989) have shown that cyclophilin A is Peptidyl-prolyl *cis-trans* isomerase, an enzyme that accelerates the *cis*-trans isomerization of prolyl-peptide bonds [36]. CyPA plays key roles in several different aspects of cellular physiology including the immune response, transcription, mitochondrial function, cell death, and chemotaxis. Cyclophilin A is expressed highly in several cancer types and correlates with the poor outcome of the patients. The protein can promote cancer cell proliferation, influences cell migration and tumor cell invasion in various cancer cell types, and can counteract also cell death by apoptosis. Li M *et al.* (2006) have reported that Cyclophilin A is overexpressed in human pancreatic cancer cells and stimulates cell proliferation through its receptor [37]. Yang *et al.* (2003) have reported overexpression of cyclophilin A in non-small cell lung cancer [38]. Zheng *et al.* (2008) have shown that Cyclophilin A is necessary for the activation of Janus kinase JAK2 induced by prolactin and the progression of human breast cancer [39]. Of course, stem cells have unlimited cell proliferation, similar to cancer cells. So it's no surprise that many of the proteins that overexpressed in these stem cells also have a high expression in a variety of cancers. Therefore, it can be linked to the unlimited cell proliferation process.

The balance between protein synthesis and degradation is one of the important homeostatic factors in eukaryotic cells. Protein degradation is essential for the cells to remove excessive proteins (such as enzymes and transcription factors that are no longer needed) or exogenous proteins transported into the cells. Increasing in Ubiquitin expression occurs in IPCs. Ubiquitin is a small protein that exists in all eukaryotic cells. It performs its myriad functions through conjugation to a large range of target proteins. A variety of different modifications can occur. The ubiquitin-proteasome system is responsible for the degradation of most intracellular proteins and therefore plays an essential regulatory role in critical cellular processes including cell cycle progression, proliferation, differentiation, angiogenesis, and apoptosis. Besides involving in normal cellular functions and homeostasis, the alteration of proteasomal activity contributes to the pathological states of several clinical disorders including inflammation, neurodegeneration, and cancer. It has been reported that human cancer cells possess an elevated level of proteasome activity and are more sensitive to proteasome inhibitors than normal cells, indicating that the inhibition of the ubiquitin-proteasome system could be used as a novel approach for cancer therapy [40,41].

Stathmin is a ubiquitous soluble protein whose phosphorylation is associated with the intracellular mechanisms involved in the regulations of cell differentiation and functions by extracellular effectors [42]. The proliferation, differentiation, and functions of cells are regulated by a large number of converging extracellular signals, many of which acts through specific membrane-bound receptors by generating intracellular second messengers. These second messengers activate a cascade of intracellular reactions, among which protein phosphorylation-dephosphorylation reactions play a major role in all biological systems. Stathmin is a ubiquitous soluble protein (Mr = 19,000) whose phosphorylation could be related, in several biological systems (anterior pituitary, muscle, neurons, PC12 cells), to the intracellular mechanisms involved in the regulation by extracellular effectors of the differentiation and the functions of the cells considered. Of several identified downstream effector targets, activation of p21ras and an associated cascade leading to activation of mitogen-activated protein kinase (MAPK) 1 family members play an essential role in processes mediating neuronal differentiation. In good agreement with this model, the expression of stathmin is also highly regulated during early and late embryonic and postnatal development, as well as in relation to cell proliferation [43] and differentiation [44,45]. Numerous data suggest that stathmin dysfunction might be associated with tumorigenesis. Stathmin expression and phosphorylation are probably linked to the control of cell

differentiation [44,45] and proliferation [43]. The state of stathmin phosphorylation changes markedly during the cell cycle, and cell division also appears to require multisite phosphorylation of this protein. Overexpression of the protein has been regularly observed in acute leukemia [46-48], lymphomas [47,48] and various carcinomas [48] and human malignant breast tumors [49].

Annexins are a family of calcium- and phospholipid-binding proteins found in nearly all eukaryotes. They are structurally highly conserved and have been implicated in a wide range of cellular activities. Altered expression of Annexin A2 has been identified in a wide variety of cancers. Annexin A2 is overexpressed in many cancers and correlates with increased plasmin activity on the tumor cell surface, which mediates the degradation of extracellular matrix and promotes neoangiogenesis to facilitate tumor growth [21,50].

UMP-CMP kinase: this enzyme catalyzes the conversion of cytidine-monophosphate or uridine-monophosphate to cytidine-diphosphate or uridine-diphosphate by ATP and plays a critical role in providing nucleotides precursors for DNA and RNA synthesis. UMP/CMP kinase plays a critical role in the pathway that supplies nucleotide precursors for DNA and RNA synthesis.

Nucleoside-diphosphate kinase A1: The overall effect of NDKs is to transfer a phosphate group from a nucleoside triphosphate to a nucleoside diphosphate.

Overexpression of glycolytic enzymes, three antioxidant proteins, and Cyclophilin A together with the rest of the proteins like Stathmin, annexin A, Cytidine monophosphate kinase, nucleotide diphosphate kinase and ubiquitin which involved in cell signaling pathways and their presence is seen in cell proliferation and differentiation processes, represents their roles in the IPC differentiation process through signaling pathways. In addition, increased expression in cancers of the pancreas, breast, liver and have been seen. This can be somewhat disturbing, Suggests probability tumorigenesis of these cells. Therefore, prior to transplantation of these cells, it is essential to examine this aspect.

Conditioning by means of a differentiation medium has several limitations. Nevertheless, a more detailed description of protein modifications induced only by culture media conditioning may constitute the basis for a rational and safe manipulation of stem cells derived from different sources. The search for small molecules that interfere with signaling pathways may open new useful opportunities for clinical usage in several human disease conditions.

The conditioning approach, so far, is effective in inducing insulin production by SKPs, and further improvements may be forecast in this direction. This phenomenon may underscore the actual limits of a culture conditioning approach that may stimulate some degree of differentiation, but not change the direction of this process. However rational manipulation of culture conditions and the quest for possible inducing molecules might open new perspectives for the use of SKPs from easily accessible sources for clinical usage in human pathology.

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