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Selective effect of INGAP-PP upon mouse embryonic stem cell differentiation toward islet cells

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ABSTRACT

We evaluated the effect of islet neogenesis-associated protein pentadecapeptide (INGAP-PP) upon islet β- and non-β cell differentiation from mouse embryonic stem (mES) cells. ES-D3 cell lines were cultured following Lumelsky's protocol with or without INGAP-PP (5 μg/ml) at different stages. Gene expression was quantified using qPCR. mES cells were fixed and immunostained using anti insulin-, somatostatin-, glucagon-, Pdx-1-, Ngn-3-, Nkx-6.1 and PGP9.5 specific antibodies. PCNA was used to measure replication rate. Bcl_2 (immunostaining) and caspase-3 (enzyme activity and gene expression) were determined as apoptosis markers. INGAP-PP increased IAPP, Glut-2, Kir-6.2, SUR-1 and insulin gene expression, and the percentage of insulin-immunostained cells. Conversely, INGAP-PP reduced significantly glucagon and somatostatin gene expression and immunopositivity. While nestin gene expression was not affected, there was a significant reduction in the percentage of PGP9.5-immunostained cells. Pdx-1 gene expression increased by 115% in INGAP-PP treated cells, as well as the percentage of Pdx-1, Ngn-3 and Nkx-6.1 immunopositive cells. Neither caspase-3 (expression and activity) nor Bcl_2 positively immunostained cells were affected by INGAP-PP. Accordingly, INGAP-PP would promote stem cell differentiation into a β-like cell phenotype, simultaneously decreasing its differentiation toward non-β-cell precursors. Therefore, INGAP-PP would be potentially useful to obtain β-cells from stem cells for replacement therapy.

1. Introduction

Type 1 diabetes mellitus (T1DM) is the result of a progressive autoimmune destruction of β-cells [1]. This destruction is of such magnitude that the remaining β-cell mass is unable to maintain normal glucose homeostasis, so that patients are forced to receive continuous insulin treatment to survive [2]. Unfortunately, most patients treated with insulin do not attain an appropriate metabolic control to effectively prevent the development and progression of chronic complications [3]. Consequently, many researchers have actively attempted to replace the lost β-cells by pancreas or islet transplantation. Although significant progress has been made by the development of an islet transplant-protocol that successfully achieves insulin independence in transplanted patients [4], the scarce availability of tissue donors limits its wide application. The report that embryonic stem (ES) cells could differentiate into pancreatic islet-like structures [5] encouraged to use these cells as a potential source of surrogate β-cells for T1DM therapy [6–7]. Differentiation of stem cells toward a specific cell lineage requires the highly regulated as well as tightly and timely controlled transcription of several genes encoding structural and regulatory proteins. Thus, several attempts have been made to optimise the results by modifying the original protocol conditions and using several small bio-organic molecules [8–9].

During the last years our group has been studying one of these molecules, islet neogenesis-associated protein (INGAP), which was first identified in adult hamsters whose pancreas head was wrapped in cellophane [10]. Later, INGAP-immunopositive cells were identified in the pancreas of normal hamsters [11]. These immunoreactive INGAP-cells have also been reported during normal embryonic mouse pancreas commitment, thus providing evidence of its early presence and possible involvement in pancreas development and patterning [12]. Further, transgenic mice with targeted pancreatic expression of INGAP become resistant to diabetes induction by streptozotocin (SZT) injection [13]. Using a model of insulin resistance induced by dietary manipulation, we have consistently shown a simultaneous and significant increase in β-cell mass [11], INGAP-positive cell mass [14], glucose-induced insulin secretion, and the appearance of a possibly early islet precursor cell co-expressing INGAP/Pdx-1 [15]. On the other hand, it has been proved that a pentadecapeptide having the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduces the effect of the intact molecule upon thymidine incorporation into ductal cells and a ductal cell line [16]. Injection of INGAP-PP to either normal or SZT diabetic mice is accompanied by an increase in β-cell mass and signs of islet neogenesis [17]. We have also shown that neonatal and adult

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rat islets cultured with INGAP-PP release more insulin in response to glucose and other secretagogues [18], and increase the expression of genes involved in the process of insulin secretion [19–20].

Based on these results, we have currently tested the effect of INGAP-PP upon differentiation of mouse ES (mES) cells toward insulin-producing and other islet cells.

2. Materials and methods

2.1. Reagents

DMEM and DMEM/F-12 tissue culture media, glutamine, non-essential amino acids, bFGF and Pluronic F-68 were obtained from Invitrogen (Karlsruhe, Germany). Fetal calf serum (FCS) embryonic stem cell grade and gentamicin were purchased from PAA (Vienna, Austria); leukemia inhibitory factor (LIF) was obtained from Chemicon (Temecula, CA, USA). Insulin, transferrin, sodium selenite, putrescine and progesterone were from Sigma (St. Louis, MO, USA). All primer pairs, including random hexamer primers, were synthesized by MWG (Munich, Germany). RevertAid™ H⁻M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). Biotherm™ Taq-polymerase as well as the dNTP was from Genecraft (Münster, Germany). SYBR Green I was from Biozym (Hess. Oldendorf, Germany) and the plastic ware for the qPCR reaction was from Abgene (Hamburg, Germany). Ac-DEVD-AMC was obtained from Biosource International (Camarillo, Ca, USA) and AMC from Merck (Darmstadt, Germany). INGAP-PP – a pentadecapeptide with the 104–118 amino acid sequence of INGAP (NH_2-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH) – and a scrambled peptide containing the same INGAP-PP amino acids but in a different sequence (NH_2-Thr-Ser-Asn-Leu-Ile-Gly-Gly-Asp-Pro-His-Gly-Pro-Leu-Ser-His-COOH) were provided by GenScript (Scotch Plains NJ, USA). Quality control of the peptides (amino acid analysis and mass spectrometry) indicated >95% purity and a molecular weight of 1501.63. Unless otherwise mentioned, chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

2.2. Cell lines and culture conditions

The mES cell line ES-D3 [21] was kindly provided by Dr. S. Lenzen (Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany). In order to maintain these cells in an embryonic state, they were cultured on a feeder layer of mouse embryonic fibroblasts in stem cell medium (DMEM) containing 25 mM glucose and supplemented with 15% (v/v) FCS, 2 mM L-glutamine, 100 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 50 μg/ml gentamicin and 1000 U/ml LIF in a humidified atmosphere at 37 °C and 5% CO_2. The cells were transferred for two passages on gelatin-coated tissue culture dishes to remove the feeder layer.

To promote differentiation, cells were cultured following the Lumelsky's protocol [5]. Briefly, one million cells were transferred onto a bacterial culture dish in medium as described above but devoid of LIF. Cells were then grown in suspension for up to 5 days (stage 2). During this time, cells formed embryoid bodies (EBs) which were allowed to settle down on gelatin-coated dishes in serum-free DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, and 100 mM non-essential amino acids for 7 days (stage 3). Thereafter, EBs were dissociated by adding 2 ml trypsin and further incubated for 5–10 min at 37 °C. Fifteen million cells were then cultured for 4 days in the same medium but supplemented with 10 ng/ml bFGF (stage 4). The cells were finally cultivated 7 days in DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 5% FCS, 2 mM L-glutamine, 100 mM non-essential amino acids and 10 mM nicotinamide (stage 5). At every stage the medium was changed daily.

Regarding treated cells, 50 μl of the INGAP-PP stock solution freshly prepared (1 μg/μl in DMEM-F12 medium) was added daily to a 10 ml plate (5 μg/ml final concentration in the plate) from stage 3 to the end of the experiment. In order to discard any possible general unspecific peptide effect upon the cultures, the scrambled pentadecapeptide was used as negative control. This peptide was prepared and added to the control plates in the same way as INGAP-PP.

Six different experiments were done, including in each one three plates for each condition tested.

2.3. qPCR analysis

Total RNA was isolated from mES cells using the Chomczynski protocol [22]. RNA was quantified photometrically and analysed on a denaturing agarose gel. For cDNA synthesis, random hexamers were used to prime the reaction of the RevertAid™ H⁻M-MuLV reverse transcriptase. The QuantiTect SYBR Green™ technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was used. The reactions were performed using the DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). Samples were first denatured at 94 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 62 °C for 30 s, and an extension step at 72 °C for 30 s. Each PCR amplification was performed in triplicate. The optimal parameters for the PCR reactions were empirically defined. The purity of the amplified PCR product was verified by melting curves. Data are expressed as relative gene expression (rge) after normalisation to the beta-actin housekeeping gene, using the *Qgene96* and *LineRegPCR* softwares [23–24]. The sequence of every pair of primers used for q-PCR is listed in Table 1.

2.4. Cell processing and immunocytochemical studies

Pelleted mES cells were fixed in 4% paraformaldehyde in 0.15 M phosphate buffered saline (PBS), pH 7.3, and embedded in paraffin. At

Table 1
Sequence of pair of primers used for qPCR

Gene		Primer sequence
Insulin	Fw	5′-CCCACCCAGGCTTTTGTCAAACAGC-3′
	Rv	5′-TCCAGCTGGTAGAGGGAGCAGATG-3′
IAPP	Fw	5′-GATTCCCTATTTGGATCCCC-3′
	Rv	5′-CTCTCTGTGGCACTGAACCA-3′
Glucagon	Fw	5′-CAGGGCACATTCACCAGCGACTAC-3′
	Rv	5′-TCAGAGAAGGAGCCATCAGCGTG-3′
Somatostatin	Fw	5′-ATGCTGTCCTGCCGTCTCCA-3′
	Rv	5′-TGCAGCTCCAGCCTCATCTCG-3′
Glut-2	Fw	5′-GAAGACAAGATCACCGGAACCTTGG-3′
	Rv	5′-GGTCATCCAGTGGAACACCCAAAA-3′
Kir-6.2	Fw	5′-TGCTGTCCCGAAAGGGCATTATC-3′
	Rv	5′-TGCAGTTGCCTTTCTTGGACACG-3′
SUR-1	Fw	5′-ACCAAGGTGTCCTCAACAACGGCT-3′
	Rv	5′-TGGAGCCAGGTGCTATGGTGAATG-3′
Pdx-1	Fw	5′-ACCGCGTCCAGCTCCCTTTC-3′
	Rv	5′-CAACATCACTGCCAGCTCCACC-3′
Nkx-6.1	Fw	5′-AGAACCGCAGGACCAAGTGGAGAA-3′
	Rv	5′-TCGTCATCCTCCTCATTCTCCGAAG-3′
Ngn-3	Fw	5′-TAGCAGAACTTCAGAGGGAGC-3′
	Rv	5′-GGGAAAAGGTTGTTGTGTCTC-3′
Nestin	Fw	5′-GAGAGTCGCTTAGAGGTGCA-3′
	Rv	5′-CCACTTCCAGACTAAGGGAC-3′
Caspase-3	Fw	5′-CCTCAGAGAGACATTCATGGGCC-3′
	Rv	5′-GCTGCTCCTTTTGCTATGATCTTCC-3′
Cdc-1	Fw	5′-ACAGCAGGTCTTCGTGCAGA-3′
	Rv	5′-ACTGGATGTTGGCTGCTTCAC-3′
Beta actin	Fw	5′-AGAGGGAAATCGTGCGTGAC-3′
	Rv	5′-CAATAGTGATGACCTGGCCGT-3′

Fw, forward (sense) primer; Rv, reverse (antisense) primer. All amplicons were designed intron-spanning and were in a size ranging from 100–300 bp. Beta actin was used as housekeeping gene.

every condition tested, we performed three different experiments and from each one we obtained three different paraffin blocks. From each one of these blocks we thereafter studied serial sections (5 μm) obtained at three different levels. Each section from a given series was mounted on separate slides to stain adjacent sections for immunocytochemical identification of insulin and non-insulin producing cells. For this purpose, tissue sections were incubated with appropriate dilutions of monoclonal-mouse antibody against human insulin (IgG) Novoclone™ HUI 018 (1:40) and a mixture of rabbit anti-glucagon (1:400, kindly provided by Novo Nordisk, Copenhagen, Denmark) and anti-somatostatin (1:6000, a gift from Dr S. Efendic, Department of Endocrinology, Karolinska Institute, Copenhagen, Denmark) sera. The reaction was completed by the streptavidin–biotin complex method with either peroxidase or alkaline phosphatase, together with carbazole and fast blue, respectively, as chromogens. Controls for serological specificity were made by preincubating a given antiserum with an excess of the corresponding hormone for 24 h at 4 °C.

Pdx-1-positive cells were stained with the Pdx-1 antibody (1:1200, kindly provided by Dr C. Wright, Department of Cell Biology, Vanderbilt University, Nashville, TN, USA) and revealed as described above using carbazole as chromogen. Before staining, we treated deparaffinized sections with 250 ml antigen retrieval solution (Vector Laboratories, Burlingame, CA, USA) for 10 min in a 500 W microwave oven [25]. The number of Pdx-1-positive cells was expressed as the percentage of the total stem cells counted.

Ngn-3- and Nkx-6.1-positive cells were identified using the Ngn-3 antibody (1:3000, kindly provided by Dr. M. German, University of California, San Francisco, CA, USA) and Nkx-6.1 (1:1500, kindly provided by Dr. O.D. Madsen, Hagedorn Research Institute, Gentofte, Denmark). Tissue sections were incubated overnight with primary antibodies and 30 min with the secondary biotinylated antibodies. The number of positive cells for each peptide was expressed as the percentage of the total stem cells counted.

Protein gene Product 9.5 (PGP9.5) is a new marker for neurons and cells of diffuse neuroendocrine system. The polyclonal antibody against PGP9.5 stains cell bodies and axons. Positive cells were identified using the PGP9.5 polyclonal rabbit antibody (UltaClone Rossiters Farmhouse Wellow Isle of Wight England) (1:400). Tissue sections were incubated overnight with the primary antibodies and 30 min with the secondary biotinylated antibodies. The area of positive cells for the peptide was expressed as the percentage of the total area measured.

2.5. Cell replication rate: double-immunolabelling studies

Stem cell replication rate was estimated by detecting proliferating cell nuclear antigen (PCNA, 1:4000; Sigma) by a modified avidin–biotin peroxidase method [26]. Thereafter, the replication rate was expressed as the percentage of PCNA-labelled cells among the total stem cells counted.

To identify the replicated cell type, we performed double staining with either PCNA-insulin or PCNA-glucagon + somatostatin antibodies (overnight incubation). We then proceeded to an incubation with the streptavidin–biotin complex (30 min) and peroxidase and alkaline phosphatase, together with carbazole and fast blue, respectively, as chromogens.

2.6. Antiapoptotic index

Antiapoptotic index was measured by incubating tissue sections with the Bcl_2 antibody (1:400, DAKO laboratories) overnight, and a further incubation with the secondary biotinylated antibody for 30 min. Before staining, we treated deparaffinized sections with antigen retrieval solution and microwave oven incubation as described for Pdx-1 (see above). The number of Bcl_2-positive cells was expressed as the percentage of the total stem cells counted.

2.7. Morphometric analysis

Videomicroscopy was used to perform morphometric analysis using a Jenamed 2 Carl Zeiss light microscope and a RGB CCD Sony camera together with the OPTIMAS software (Bioscan Incorporated, Edmons, WA, USA). With this procedure, we measured the volume density (Vvi, defined as the percentage of immunostained area compared with the total area of the section) of cells immunostained with a specific insulin antibody, stained with the combination of glucagon–somatostatin antibodies, and stained with PGP9.5 antibody, as well as the percentage of Pdx-1, of Nkx-6.1, of Bcl_2 and of PCNA immunopositive cells. In every case no less than 10,000 cells were counted.

2.8. Caspase-3 activity

The caspase-3 activity in mES cells was determined by specific cleavage of the fluorescent substrate Ac-DEVD-AMC [27]. 1×10^7 cells were lysed in cell lysis buffer (50 mM Hepes, pH 7.4, 0.1% Chaps, 5 mM DTT, 0.1 mM EDTA) for 5 min at 4 °C and centrifuged for 10 min at 10,000 g. The protein content was subsequently determined with the Bradford assay [28]; 10 μg total protein was added to 80 μl assay buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 1 mM EDTA, 10% glycerol) and to 10 μl of the caspase-3 substrate Ac-DEVD-AMC, providing a final substrate concentration of 0.03 mM. The increase of free fluorescence was quantified fluorimetrically at 360 nm (excitation) and 460 nm (emission) for 3 h at 37 °C. Caspase-3 activity was measured throughout the increase of fluorescence in 60 min. The units were calculated against a standard dilution curve of free AMC (one unit = cleavage of 1 nmol AMC × h^{-1}).

2.9. Statistical analysis

Data are expressed as means ± SEM. Unless specifically stated, statistical analyses were performed using ANOVA followed by Dunnett's test for multiple comparisons or *t*-test for correlations using the Prism analysis program (Graphpad, San Diego, USA).

3. Results

3.1. β-cell markers

mES cells treated with INGAP-PP showed a significant increase in the expression of genes (qPCR measurements) considered as β-cell markers, such as Glut-2, Kir-6.2, SUR-1 and IAPP (Table 2). Insulin gene expression was also significantly enhanced in these cells, as well as the Vvi of cells immunostained with a specific insulin antibody (0.24 ± 0.02 vs. 0.12 ± 0.01%; $P<0.05$) (Fig. 1A). No changes were observed in cells treated with the scrambled peptide.

Table 2
Relative gene expression in control and INGAP-PP-treated mES cells

	Control	+INGAP-PP
PDX-1	0.046 ± 0.008	0.099 ± 0.014** (+115)
Nkx-6.1	25.02 ± 1.00	35.12 ± 1.93* (+40)
Glut-2	0.107 ± 0.005	0.2 ± 0.22* (+87)
Kir-6.2	0.082 ± 0.016	0.133 ± 0.009* (+62)
SUR-1	8.628 ± 1.703	26.114 ± 5.736** (+202)
Insulin	0.026 ± 0.003	0.04 ± 0.001* (+53)
IAPP	0.013 ± 0.001	0.028 ± 0.002** (+115)
Somatostatin	4.33 ± 0.65	1.63 ± 0.17** (−62)
Glucagon	0.021 ± 0.003	0.006 ± 0.001** (−71)
Cdc-1	6.3 ± 0.2	4.12 ± 0.4** (−35)

Results are expressed as a relative gene expression against beta-actin × 1000. Values are means ± SEM of five different experiments run in triplicate. Between brackets, percentage of change compared with the control.
* $P<0.01$.
** $P<0.001$.

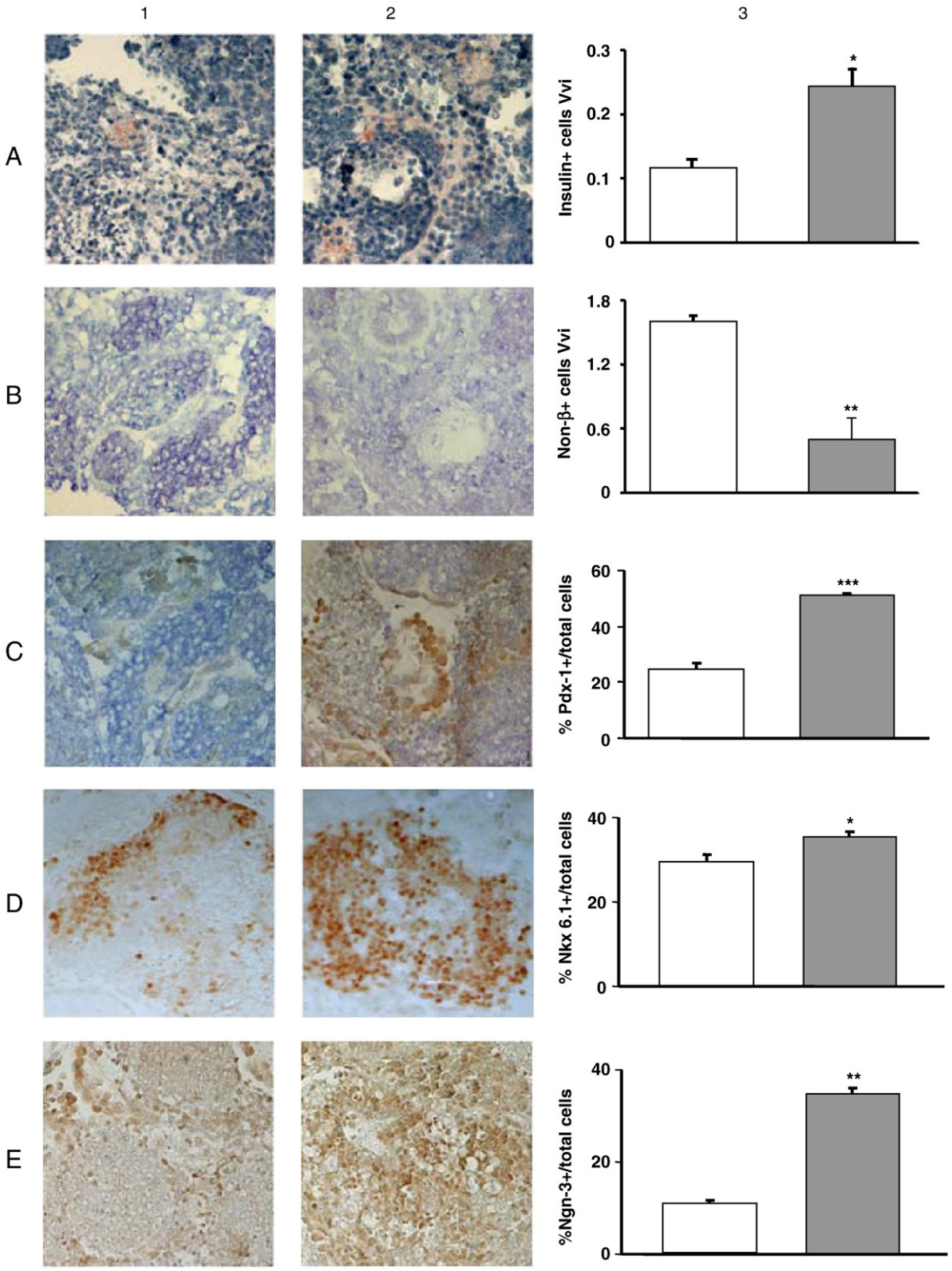

Fig. 1. In each graph, panel 1 represents control mES cells, panel 2 mES cells treated with INGAP-PP, and panel 3 the quantitative immunocytochemical measurement. A. Tissue sections stained with insulin antibody (red cells). Vvi of insulin-positive cells is significantly larger in INGAP-PP-treated cells (panel 3) ×40. B. Non-β-cell (cells stained with the glucagon–somatostatin antibodies) Vvi is significantly smaller in INGAP-PP-treated cells (panel 3). No differences between groups were observed in the size of these cells ×40. C. Tissue sections stained with Pdx-1 antibody (red nuclei cells) and glucagon–somatostatin antibody mixture (blue cells). Pdx-1 increased markedly in INGAP-PP treated mES cells (panel 3) ×40. The number of Pdx-1 positive cells was expressed as the percentage of the total stem cells counted (no fewer than 10,000 each). D. Tissue sections stained with Nkx-6.1 antibody. INGAP-PP induced a significant increase in the number of Nkx-6.1 positive cells ×40. The number of Nkx-6.1 positive cells was expressed as the percentage of the total stem cells counted (no fewer than 10,000 each). E. Tissue sections stained with Ngn-3 antibody. INGAP-PP induced a significant 3-fold increase in the number of Ngn-3 positive cells ×40. The number of Ngn-3 positive cells was expressed as the percentage of the total stem cells counted (no fewer than 10,000 each). Each bar represents mean ± SEM of the measurements performed at three different levels of three blocks corresponding to three different experiments. Control (empty bars) and INGAP-PP treated cells (black bars). *$P<0.05$; **$P<0.01$; ***$P<0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Islet non β-cell markers

INGAP-PP, but not the scrambled peptide, decreased significantly the expression of glucagon and somatostatin genes (Table 2). When these cells were fixed and stained with a mixture of glucagon–somatostatin antibodies, we found a significant decrease in the Vvi of non-β cells (cells immunostained with the combined glucagon–somatostatin antibodies) as compared to their corresponding controls (0.5±0.2 vs. 1.6±0.05%, respectively; $P<0.01$) (Fig. 1B). No differences in cell size were observed between groups.

3.3. Transcription factors

mES cells treated with INGAP-PP, but not with the scrambled peptide, showed an enhanced expression of Pdx-1 (115%) and Nkx-6.1 (40%) genes compared with their corresponding controls (Table 2). They also evinced a significant increase in the protein level of Pdx-1 (immunocytochemistry; Fig. 1C; $P<0.001$) and Nkx-6.1 (Fig. 1D; $P<0.05$). On the other hand, while INGAP-PP induced a significant 3-fold increase in Ngn-3 immunostaining (35.2±1.9 vs. 10.3±0.1%; $P<0.01$) (Fig. 1E), no changes were observed using qPCR.

3.4. Neural markers

While mES cells treated with INGAP-PP showed no changes in nestin gene expression, we measured a significant decrease in the Vvi of PGP9.5 immunostained cells as compared with their corresponding controls (0.37±0.01 vs. 0.22±0.01%; $P<0.05$) (Fig. 2).

3.5. Apoptosis and replication markers

No significant differences were found in caspase-3 gene expression and activity between control and INGAP-PP treated cells. Similarly, no significant changes were measured in Bcl_2 immunopositive cells between control and treated mES cells (22.5±1.3 vs. 20±1.8% respectively).

On the other hand, INGAP-PP induced a significant decrease in both Cdc-1 gene expression (Table 2) and PCNA positive-immunostaining (control vs. INGAP-PP, 31.2±4.4 vs. 16.5±1.3%; $P<0.05$).

3.6. Cell structure

After differentiation, we observed a heterogeneous picture concerning differentiation and structural preservation. More than 30% of the differentiated mES cells showed signs of apoptosis, such as nuclear changes or necrosis. These features were similar in cells treated or not with INGAP-PP. Further, no differences were observed in the percentage of cells undergoing apoptosis or necrosis between groups.

4. Discussion

Our study provides the first experimental evidence that the addition of INGAP-PP to cultured mES cells induces significant changes in gene expression (qPCR) and protein levels (immunocytomorphometric analysis) of these cells. Since such changes were not observed after addition of the scrambled pentadecapeptide, they can be attributed to INGAP-PP rather than to a general unspecific peptide effect.

The above mentioned changes were observed when the peptide was added from the early – but not later – steps of the differentiation protocol; thus, mES cells were only sensitive to INGAP-PP stimulus at the time when there is an increase in the number of nestin-positive cells (stage 3 of Lumelsky's protocol). Consequently, under our experimental conditions, cells pre-differentiated or already committed to a set of phenotypes would be no longer sensitive to INGAP-PP stimulus.

The main depicted effect of INGAP-PP was a simultaneous and significant decrease in both gene expression and protein levels of islet non-β cell markers (glucagon and somatostatin), together with a significant increase in β-cell marker genes such as IAPP, Glut-2, Kir-6.2, SUR-1, and insulin, together with a significant increase in the percentage of insulin-immunostained cells. Therefore, INGAP-PP would shift the differentiation process toward cells portraying characteristics of a mature insulin-producing cell, namely, the glucose sensing machinery and insulin gene expression. A similar enhancement of Kir-6.2 and SUR-1 gene expression was measured in newborn rat islets cultured with INGAP-PP for a week [19,20].

INGAP-PP also increased the expression of transcription factor genes present in β-cell precursors such as Pdx-1, Nkx-6.1 and Ngn-3. While we were unable to measure Ngn-3 mRNA, we detected its protein by immunostaining. Since false immunopositivity was carefully discarded, we might assume that the negative results obtained with qPCR were due to the different time course of both the transcription and the translation processes. It should be noted that Ngn-3 is the earliest islet cell-specific transcription factor during embryonic pancreas development, and that its expression is also markedly increased in injured pancreases from adult animals [29]. Related to the latter condition, Dor et al. posed a question on whether INGAP could enhance Ngn-3 expression [30]; our current data provide the evidence that this is truly the case, at least at the embryonic stage.

While INGAP-PP did not produce any measurable effect upon the apoptotic rate (qPCR, immunocytochemistry and enzymatic assays), it did induce a significant reduction in cell replication rate; these data suggest that under INGAP-PP stimulus, mES cells switch to a more differentiated phenotype reducing their capacity of fast cell replication, without affecting the rate of cell death.

The current results are in agreement with those obtained in previous studies related to the effect of INGAP-PP upon islet cells and functions, namely: a) addition of INGAP-PP to normal islets isolated from newborn

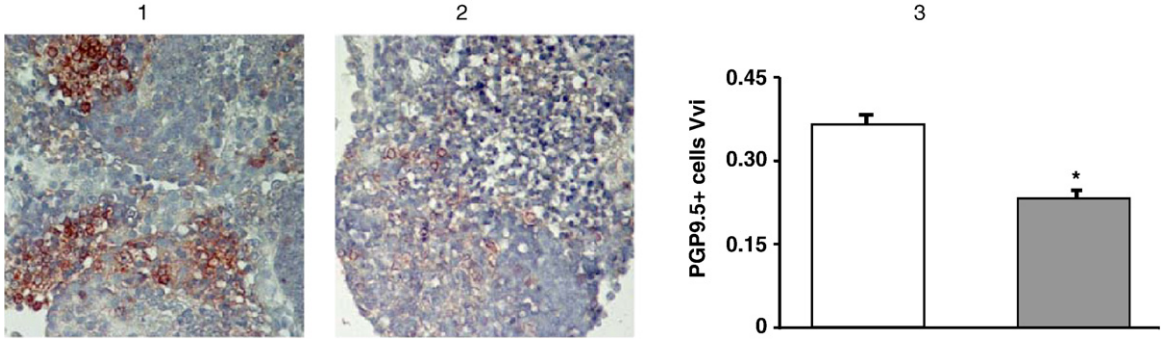

Fig. 2. Panel 1, control mES cells. Panel 2, mES cells treated with INGAP-PP. Panel 3, quantitative immunocytochemical measurement. Tissue sections stained with Protein gene Product 9.5 (PGP9.5) antibody (red cells). There is a significant decrease in the Vvi of PGP9.5 positive cells in INGAP-PP-treated cells (panel 3) ×40. Each bar (mean±SEM) represents the Vvi of PGP9.5 positive cells with respect to the total section area. Empty bars, control; black bars, INGAP-PP treated cells. *$P<0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and adult rats enhanced the release of insulin in response to glucose and other secretagogues [18], as well as the expression of genes involved in the process of insulin secretion [19,20]; b) the intraperitoneal injection of this peptide to either normal or STZ-induced diabetic mice increased significantly β-cell mass, with concomitant signs of increased neogenesis [17], and c) hamsters with insulin resistance induced by dietary manipulation showed a simultaneous and significant increase in the mass of β- and INGAP-positive cells [14]. Interestingly, this increase correlates with the appearance of precursor cells co-expressing INGAP-Pdx-1 [15]. Altogether, the current and our previous reported results suggest that INGAP could play a role in the differentiation of ES cells as well as of adult stem cells toward a β-cell like phenotype.

We must admit, however, that the transcriptional profile of some of the markers induced by INGAP-PP can also be expressed by neural-cell types [31–33]. However, the significant decrease in the percentage of PGP9.5 immunostained cells as well as the absence of significant changes in nestin gene expression in treated cells, argues against a driving effect of INGAP-PP towards neural differentiation.

The effect upon endocrine cell-precursors, together with the marked reduction in non-β cell expression and neural cell markers, suggests that INGAP-PP would exert an opposite on and off effect upon β- and non-β-cell precursors, respectively.

Evidence reported in the literature demonstrates that stem cell differentiation toward insulin producing cells requires the appearance of key factors at a given concentration, at a determined time, and in a precise chronological sequence [34]. This complex net of signals might include those arising from neighbour cells that are not committed to form β-cells, but can influence progenitor cell differentiation into β-cells [35–38]. Based on the evidence mentioned above, we could suggest that INGAP-PP can be one of these modulatory signals that promote, at an early stage of development, stem cell differentiation into a β-like cell phenotype, simultaneously decreasing its differentiation toward non-β-cell precursors. Although knowledge of the underlying mechanism involved in triggering such effects deserves further studies, the current data open new perspectives on the potential usefulness of INGAP-PP to obtain β-cell differentiation from stem cells for replacement therapy.

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