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Differentially Up-Regulated Genes in Proliferating Porcine Neonatal Pancreas Cells Caused by Epidermal Growth Factor

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Abstract Pancreatic duct cells are considered to be a major source for β-cell regeneration or neogenesis. Although epidermal growth factor (EGF) is a well-known important growth factor for pancreas development, the control of pancreatic duct cell growth and differentiation by EGF is poorly understood. In this study, we focused on identifying the genes that were differentially up-regulated in response to EGF stimulation using monolayer cultured porcine neonatal pancreas cells. Cells were obtained from 1 to 3 day old pigs, dispersed and cultured for 8 days. Monolayer cultured porcine pancreas cells were comprised of duct cells and some endocrine and mesenchymal cells (75.2 ± 15.1, 19.6 ± 4.9, and $9.5 \pm 3.1\%$, respectively). After 16 h in serum free media, cells were treated with 100 μg/L EGF for 24 h. Differentially expressed genes were screened by subtractive hybridization. ^{3}H-thymidine uptake was significantly increased by EGF with time (untreated vs. 24 h treated, untreated vs. 48 h treated: 305.5 ± 3.5 cpm vs. 380.3 ± 17.3 cpm ($P < 0.05$), 309.2 ± 4.51 vs. 929 ± 9.19 cpm, ($P < 0.005$), respectively). Three hundred and fify cDNA clones were obtained by subtractive hybridization and the inserts were confirmed in 161 colonies and then sequenced. Finally, we found increased mRNA expression of five unknown and five known genes, including cytochrome c oxidase subunit I (*COI*), cyclooxygenase-2 (*COX-2*), matrix metalloproteinase-13 (*MMP-13*), Wiskott–Aldrich syndrome protein interacting protein (*WASPIP*), and hyaluronan synthase-2 (*HAS-2*). We confirmed the up-regulation of these genes by Northern blot and semi-quantitative RT-PCR at various time points. The present findings opened new targets for the research on the mechanisms of pancreatic duct cell proliferation by EGF. J. Cell. Biochem. 91: 354–364, 2004.

Key words: pancreas duct cell; subtractive hybridization; epidermal growth factor; pig; matrix metalloproteinase-13

Recent studies on diabetic patients have indicated that tight control of blood glucose is the most important factor in the prevention of chronic diabetic complications [The DCCT Research Group, 1993; Ohkubo et al., 1995; Turner and Holman, 1996]. Because it is difficult to maintain euglycemia for a life-long period, the development of a new way to cure the diabetes mellitus is urgently required. The restoration of insulin secretion with β-cell replacement would be an ideal, particularly in diabetic patients with insulin deficiency. Although successful human islet transplantation has already been established [Shapiro et al., 2000], two main obstacles to successful islet

Abbreviations used: EGF, epidermal growth factor; COI, cytochrome c oxidase subunit I; COX-2, cyclooxygenase-2; MMP-13, matrix metalloproteinase-13; WASPIP, Wiskott–Aldrich syndrome protein interacting protein; HAS-2, hyaluronan synthase-2.

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transplantation, limitation of islet sources, and the immune rejection still remain to be solved. In our previous report, porcine neonatal pancreas cell clusters, which were mainly comprised of duct cells, were expanded and differentiated into β-cells following transplantation [Yoon et al., 1999]. However, our understanding of the molecular mechanisms of pancreas duct cell proliferation and differentiation is so limited.

Betacellulin, a member of the epidermal growth factor (EGF) family, has been detected in primitive duct cells of fetal pancreata, and EGF receptors were also expressed mainly in the islet and duct cells of adult pancreas [Miyagawa et al., 1999]. Furthermore, a recent report showed that EGF was an important factor for pancreas precursor cell proliferation in vitro [Corentin et al., 2001], and that islet cell migration and differentiation were impaired in the mice lacking EGF receptors [Miettinen et al., 2000]. These findings suggest EGF is important in the growth and differentiation of islet cells. Although recent progress has been made concerning the transcription factors implicated in pancreatic development [Edlund, 1998], the control of pancreatic cell growth and differentiation by growth factors is poorly understood.

In this study, we aimed to search differentially up-regulated genes following EGF treatment on the neonatal porcine pancreas cells to understand the role of EGF on duct cell proliferation and differentiation in the pancreas.

MATERIALS AND METHODS

Materials Reagents Used in Culture

To make modified Ham's F10 (Ham's F10), 9.8 g of powdered F10 (GIBCO/Life Technologies, Grand Island, NY) was added to 1 L of distilled water in a Kimax bottle. Additionally the medium included: D-Glucose (702 mg/L), nicotinamide/niacinamide (1,220 mg/L), L-glutamine (146 mg/L), $CaCl_2{\cdot}2H_2O$ (25 mg/L), IBMX (3-isobutyl-1-methylxanthine, 11 mg/L) (Sigma Chemical, St. Louis, MO), and bovine serum albumin (BSA, RIA grade, A-7888, fraction V, Sigma) 5 g/L. To make modified HBSS (HBSS+), 2.6 g/L of HEPES, 10 mg/L of phenol red, and 2.5 g/L of BSA (fraction V, A-3425), M199 (all from Sigma), were added. The dissociation medium, trypsin and DNase were also purchased from Sigma.

Preparation of Neonatal Porcine Pancreas Cells

The method employed was a modification of that used by Korbutt et al. [1996] and Shapiro et al. [2000]. Neonatal pigs aged 1–3 days were delivered and anesthetized. From a midline incision, a pancreatectomy was performed. This study was carried out in accordance with the principles of laboratory animal care. The pancreas was placed in a 50 ml plastic centrifuge tube containing M199 and placed on ice. The pancreas in 25 ml of M199 containing 10 ml/L of penicillin–streptomycin (Gibco BRL, Grand Island, NY) in a 50 ml plastic centrifuge tube was then minced into 1–2 mm pieces. Three milligram per milliliter of collagenase P (Boehringer-Mannheim, Indianapolis, IN) in M199 was added to the minced pancreas. After hand shaking, the digest was poured with a funnel through a 500 μm sieve (Sigma Chemical). The washing procedure was repeated twice with HBSS. After the supernatant was aspirated, 10 ml of Ham's F10 was added to the pellet. An additional 5 ml of Ham's F10 was added, and then 15 ml of the suspension was transferred into 150×15 mm bacteriological plates (Becton-Dickinson, Franklin Lakes, NJ). Following the further addition of 20 ml Ham's F10 to each plate they were incubated at 37°C in an atmosphere of 5% CO_2.

Dispersion of the Porcine NPCCs

NPCCs cultured overnight were washed three times with dissociation medium, and incubated in a shaking water bath at 37°C for 10 min. After washing, the cell clusters were broken by gentle aspiration with a pipette. The suspension was spun at 700 rpm, and then stopped, to separate individual cells from clumps. After then, supernatant was collected, whereas and the pellet subjected to dissociation for 10 min, as described above. Dispersed cells were plated with a density of 1×10^7 per 150 mm dish, and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Each plate was filled with 35 ml of new media at Day 3, and every other day thereafter until Day 7. EGF was treated for 24 h from Day 7.

Thymidine Uptake

Cells were plated in 6-well plates (5×10^4 cells/well) for 48 h in RPMI1640 containing 10% FCS and 0.5% BSA. After 16 h in serum

starvation, human recombinant EGF (100 μg/L) was added for 24 and 48 h. Then [^{3}H]-methylthymidine (0.5 μCi/ml) and 1 μM unlabeled thymidine were added for the last 24 h. Acid-soluble [^{3}H] thymidine was removed by washing the cells for 30 min with ice-cold 5% trichloroacetic acid. The cells were then washed with 80% ethanol, solubilized in 1 M NaOH, and incubated for 30 min at room temperature. The incorporated radioactivity was determined by the addition of 4 ml of a liquid scintillation cocktail and liquid scintillation counting.

Immunocytochemistry

After dispersion for 8 days, monolayer cultured cells were fixed with 4% paraformaldehyde for 30 min. To determine the phenotype of the culture cells, triple immunofluorescence staining was performed: cytokeratin 7 and pancytokeratin for the duct cells, vimentin for the mesenchymal cells, insulin/cocktail antibodies (anti-glucagon + anti-pancreatic polypeptide + anti-somatostatin antibodies) for the endocrine cells of the pancreas (DACO, Glostrup, Denmark) and DAPI (Sigma Chemical) for the nuclei. Before staining, cells were permeabilized with 0.25% Triton X-100 for 5 min. Normal donkey or goat serum (1:50) was applied for 30 min as blocking antibodies. The dishes were incubated with both primary antibodies overnight at 4°C. After washing with PBS, the dishes were incubated for 1 h at room temperature with the following secondary antibodies: Texas red conjugated affiniPure donkey anti-guinea pig and anti-mouse IgG, FITC conjugated affiniPure donkey anti-rabbit IgG (1:100 dilution, Jackson Immuno-Research Lab., West Grove, PA). After washing, the dishes were mounted with 1 ng/ml DAPI containing anti-fade medium.

Image Acquisition and Quantification of the Cells in the Dishes

For acquisition of digital images at two or three fluorescence emission wavelengths, we used a Bio-Rad confocal microscope. The light source was a multi-photon laser giving excitation wavelengths in the range 368–647 nm, with a select 512×512 pixels scan size. The FITC and Texas red were excited at 488 nm and 568 nm of the multi-photon laser, respectively, and the DAPI was excited at 368 nm. We scanned the FITC, Texas red, and DAPI images separately, with different laser wavelength, and made overlapped images. To quantify the total number of ducts, mesenchymal, and endocrine cells in the dishes, dispersed cells from three pigs were used. Fifteen to twenty images were systematically taken from one 35 mm dish, and stained with insulin/cocktail/DAPI or pancytokeratin/vimentin/DAPI. After counting the numbers of each cell from all the sampled images, we calculated the percentage of the cells in the dishes.

Total RNA Preparation and SMART™ cDNA Synthesis

Total RNA was obtained from cultured duct epithelial cells using Trizol reagent (GIBCO BRL). We generated cDNA from the total RNA samples from the EGF treated and non-treated cells using SMART™ (Switch Mechanism at 5′ end of RNA template) PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). The SMART™ cDNA synthesis technology [Matz et al., 1999] utilizes a combination of two primers in a single reaction. Briefly, 4 μg of total RNA was reversely transcribed in a 20 μl mixture of 200 U of Superscript™ reverse transcriptase (Gibco BRL) using 10 μM of modified oligo (dT) primer (CDS primer): the resulting full-length, single-stranded cDNA contained the complete 5′ end of the mRNA and the sequence complementary to the SMART™ oligonucleotide, which then serves as a long distance PCR priming site to amplify the full-length cDNA. The first strand of cDNA was diluted to a final volume of 40 μl with distilled water. Diluted cDNA (2.5 μl) was used to generate the cDNA by long distance PCR with Advantage™ two DNA polymerase mix (Clontech), using a PCR primer according to the protocol.

SUBTRACTIVE HYBRIDIZATION

cDNA Subtraction

Subtractive hybridization was performed with a PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's protocol using RNA derived from EGF treated (100 μg/L) monolayer cultured pancreas cells, but not from the other samples. Double strand (ds) cDNA was synthesized from mRNA by a PCR-Select™ cDNA subtraction kit (Clontech), and each ds cDNA was digested with *Rsa*I (Clontech) to obtain shorter and blunt-ended molecules, which was then purified. These were divided into two, ligated separately with two

different adaptors as a tester, with cDNA without adaptors used as a driver. Firstly, an excess amount of driver was added to each sample of tester, and hybridized at 68°C for 8 h. For the second hybridization, two primary hybridization samples were mixed together with fresh driver cDNA, and incubated at 68°C overnight. Finally, the secondary PCR amplification was carried out using nested primers to obtain different gene sequences, which were further enriched.

Cloning

After subtractive hybridization, the second PCR products were cloned into the pGEM-T Easy Vector™ (Promega, Madison, WI), and then transformed by heat shock at 42°C for 1 min in 50 μl of TOP 10 *E. coli* competent cells. Colonies were cultured overnight at 37°C on LB agar plates containing ampicillin, X-gal, and IPTG.

Selecting the Different Clones and Sequencing

White colonies were isolated and cultured overnight in 5 ml of ampicillin containing LB medium. Plasmid DNAs were purified with a QIAprep Spin miniprep kit (QIAGEN, Chatsworth, CA) from insert-contained colonies, with the insert of each colony identified by *Eco*RI (Gibco BRL) digestion. Selected clones were sequenced, and their sequences were identified with a BLAST analysis using the GenBank database.

Semi-Quantitative RT-PCR Analysis

Primers were made on the basis of published sequences and sequencing results. The PCR conditions were as follows; after boiling at 94°C for 5 min, amplification was carried out with 30 cycles at 94°C for 30 s, 55 °C for 30 s, and 72°C for 2 min, the mixture was then cycled at 72°C for 10 min to complete the elongation step. The band intensities were normalized with those of the corresponding GAPDH. For confirming our results, every RT-PCR experiment was repeated three times and also analyzed the expression levels of the genes in various time points (before, and 8, 24, 48, and 72 h after EGF treatment). To confirm the similar amount of RNA used for semi-quantitative RT-PCR analysis, a housekeeping gene *GAPDH* was used as a control. The band density, stained with ethidium bromide (EtBr) on 1.5% agarose gel, was measured using a densitrometer VDS analyzer (Pharmacia Biotech AB, Uppsala, Sweden). Then band intensities were normalized with those of the corresponding GAPDH used for a control.

Northern Blot

The mRNA expression of five known genes was studied by Northern blot analysis. Total RNA was isolated from pig pancreas cell after EGF treatment. Twenty micrograms of RNA were electrophoresed on denaturing 1% formaldehyde gels and blotted on nylon membranes (Boehringer Mannheim, Indianapolis, IN). The blots were stained with 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2). Membranes were destained in 0.2×SSC and 1% SDS. The blots were prehybridized for 4 h at 42°C in buffer containing 5×SSC, 1×Denhardt's solution, 100 μg/ml of denatured salmon sperm DNA, 0.1% SDS, and 50% formamide. The probes used for Northern blot analysis were labeled by random primers with [α-^{32}P] dCTP. Hybridization was performed at 42°C overnight in a buffer that contained the same constituents as the prehybridization solution and [α-^{32}P] dCTP-labeled cDNA probes. After hybridization, the blots were washed twice at 68°C in 2×SSC, 0.1% SDS, and 0.1×SSC, 0.1% SDS. The blots were exposed to Kodak X-ray film for 24 h.

Statistical Analysis

Analysis of the data for each gene was performed using Student's *t*-test when two groups were compared. Data were presented as mean± SE and a value of $P < 0.05$ was considered to indicated a statistically significant difference.

RESULTS

Twenty Four Hours Thymidine Uptake

Thymidine uptake was increased with 100 μg/L EGF in a time dependent manner (Fig. 1). Before EGF treatment, the thymidine uptakes of 24 and 48 h were 305.5 ± 3.5 and 309.2 ± 4.51 cpm, respectively. After 24 and 48 h of treatment with 100 μg/L EGF, thymidine uptakes were increased significantly up to 380.3 ± 17.3 and 929 ± 9.19 cpm ($P < 0.05$, $P < 0.005$).

Characterization and Quantification of the Cells in the Dishes

A day after the start of culturing, single or small cell clusters started to attach on the

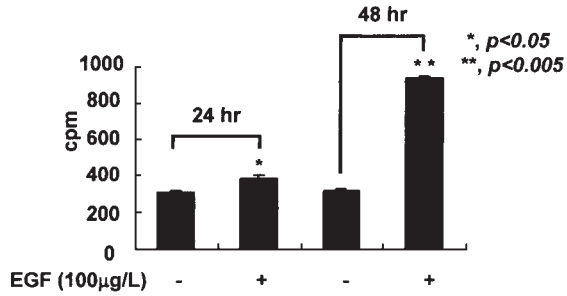


Fig. 1. The changes in thymidine uptake during EGF treatment, in NPCCs, in relation to time. The amount of thymidine uptake was gradually increased with 48 h EGF treatment in NPCCs. Each value represents the mean ± SE (*$P < 0.05$ and **$P < 0.005$ compared with untreated EGF, respectively).

dishes, and proliferated rapidly. After 3 days, we observed several cellular phenotypes in the culture dishes, which were categorized into three different groups: various shapes of epithelial cells, scattered or grouped small round cells, and comma shaped cells. The small, almost round cells were stained with pancreas hormones (Fig. 2A) and the comma shaped cells stained only with vimentin, as a marker of mesenchymal cells. Because various epithelial cells were intensively stained with cytokeratin 7, a specific marker of the duct cells in pig, confirmed as duct cells (Fig. 2B). Just before EGF treatment, the percentage of duct, endocrine and mesenchymal cells in the dishes were 75.2 ± 15.1, 19.6 ± 4.9, and 9.5 ± 3.1%, respectively. The composition of cells in the dishes after 24 h EGF treatment was not significantly changed (data was not shown).

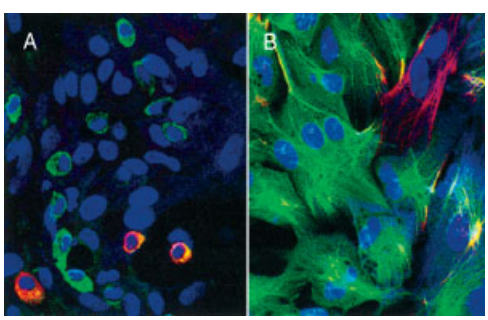


Fig. 2. Triple immunofluorescence staining of monolayer the cultured NPCCs. **Panel A**: Insulin (red)/cocktail (green) antibodies (mixture of anti-glucagon, anti-pancreatic polypeptide, and anti-somatostatin antibodies) and DAPI (blue) for the nuclei. All the scattered small round cells, stained with hormones, and all the insulin positive cells, costained with insulin and cocktail antibodies, were suggestive of active proliferation and differentiation of endocrine cells. **Panel B**: Pancytokeratin (Green)/vimentin (red) and DAPI (blue) for the nuclei. Most of the cells were strongly stained with the pancytokeratin antibody, whereas a few cells were stained with the vimentin only.

Subtractive Hybridization Analysis

To compare the difference between the genes expressed in the porcine neonatal pancreas cells due to EGF treatment and non-treatment, we performed subtractive hybridization. The cDNA with ds cDNA from the EGF treated neonatal porcine pancreas cells were used as testers, and the non-treated neonatal porcine pancreas cells as drivers. After transformation with subtractive PCR products, we had a total of 350 white colonies. Of these, 161 potentially different clones were selected and cultured. Plasmid DNAs were purified from these colonies, and inserts of each colony were identified by *Eco*RI digestion. When we digested with *Eco*RI, the sizes of the inserts were quite variable. These sequences were searched for homology using the GenBank. Five known genes; cytochrome C oxidase subunit I (*COI*), cyclooxygenase-2 (*COX-2*), matrix metalloproteinase-13 (*MMP-13*), Wiskott–Aldrich Syndrome protein integrating protein (*WASPIP*), and hyaluronan synthase-2 (*HAS-2*) were repetitively detected among the 161 randomly selected clones. We also found five sequences without significant homology to genes within the GenBank database (Table I). The COI and COX-2 cDNA fragments found had 95–99% homology with the pig gene from the GenBank database.

In the cases of the MMP-13, WASPIP, and HAS-2, no known gene sequences exist for pig species. Therefore, we compared those genes with *Homo sapiens* genes in the GenBank database. From this the genes were detected with 94–97% homology with *Homo sapiens* genes. We carried out RT-PCR with primers generated for these genes.

Semi-Quantitative RT-PCR

Total 10 differential expressed genes were detected by the subtractive hybridization were confirmed in the original EGF treated, and non-treated, neonatal porcine pancreas cells by semi-quantitative RT-PCR. They were *COI*, *COX-2*, *MMP-13*, *WASPIP*, *HAS-2*, and five unidentified genes. The newly found five unknown genes showed a different level of expression between EGF-treated and non-treated cell at 24 h (Fig. 3A). Also, semi-quantitative RT-PCR analysis indicated that known genes were more expressed in EGF-treated NPCCs (Fig. 3B). For confirming our results, semi-quantitative

TABLE I. List of EGF-Specific Genes of NPCCs With Homology Sequences in the GenBank

Clone	Size of insert cDNA fragments (bp)	Identified homology genes	Location
1	203	*COI*	5,630–5,833
2	161	*COX-2*	744–905
3	298	*MMP-13*	806–508
4	853	*WASPIP*	421–1,274
5	233	*HAS-2*	468–701
6	216	Unknown	—
7	236	Unknown	—
8	263	Unknown	—
9	318	Unknown	—
10	302	Unknown	—

RT-PCR was performed with the mRNA samples extracted at the various time points after EGF treatment. Although there were no significant differences of expression levels for the genes of unknown 1, 3, and *COI* genes at 24 h treatment of EGF, we could observe a tendency of increased mRNA expression of those genes (Figs. 4A and 5A). The mRNA expressions of three genes (unknown 1, 3, and *COI*) were significantly increased in 8 h after EGF treatment. These findings suggested that those three genes seemed to be induced early by EGF. Other genes (unknown 2, 4, 5, *COX-2*, *MMP-13*, *WASPIP*, and *HAS-2*) were more expressed in EGF-treated pancreas cell than non-treated one at various time points (Figs. 4A and 5A). The ratios were obtained through dividing band densities of each gene by those of GAPDH and shown as graphic image (Figs. 4B and 5B).

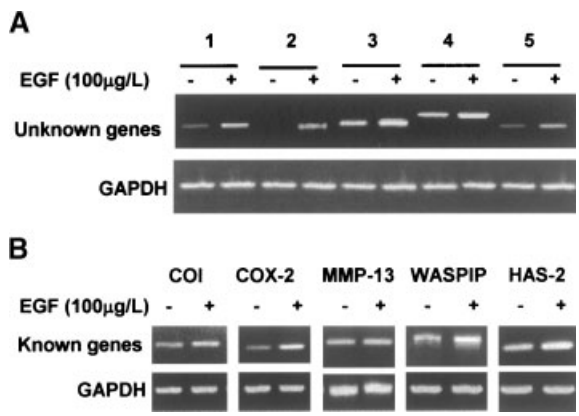


Fig. 3. Semi-quantitative RT-PCR analysis. **Panel A**: Five unknown genes expressed at different levels by RT-PCR. They did not match, following BLAST sequence analysis in the GenBank database. **Panel B**: The homology genes that were differentially expressed in the EGF-treated NPCCs using RT-PCR. The following five gene fragments were detected; *COI* (cytochrome C oxidase subunit I), *COX-2* (cyclooxygenase-2), *MMP-13* (matrix metalloproteinase-13), *WASPIP* (Wiskott–Aldrich Syndrome protein integrating protein), and *HAS-2* (hyaluronan synthase-2).

Northern Blot Analysis

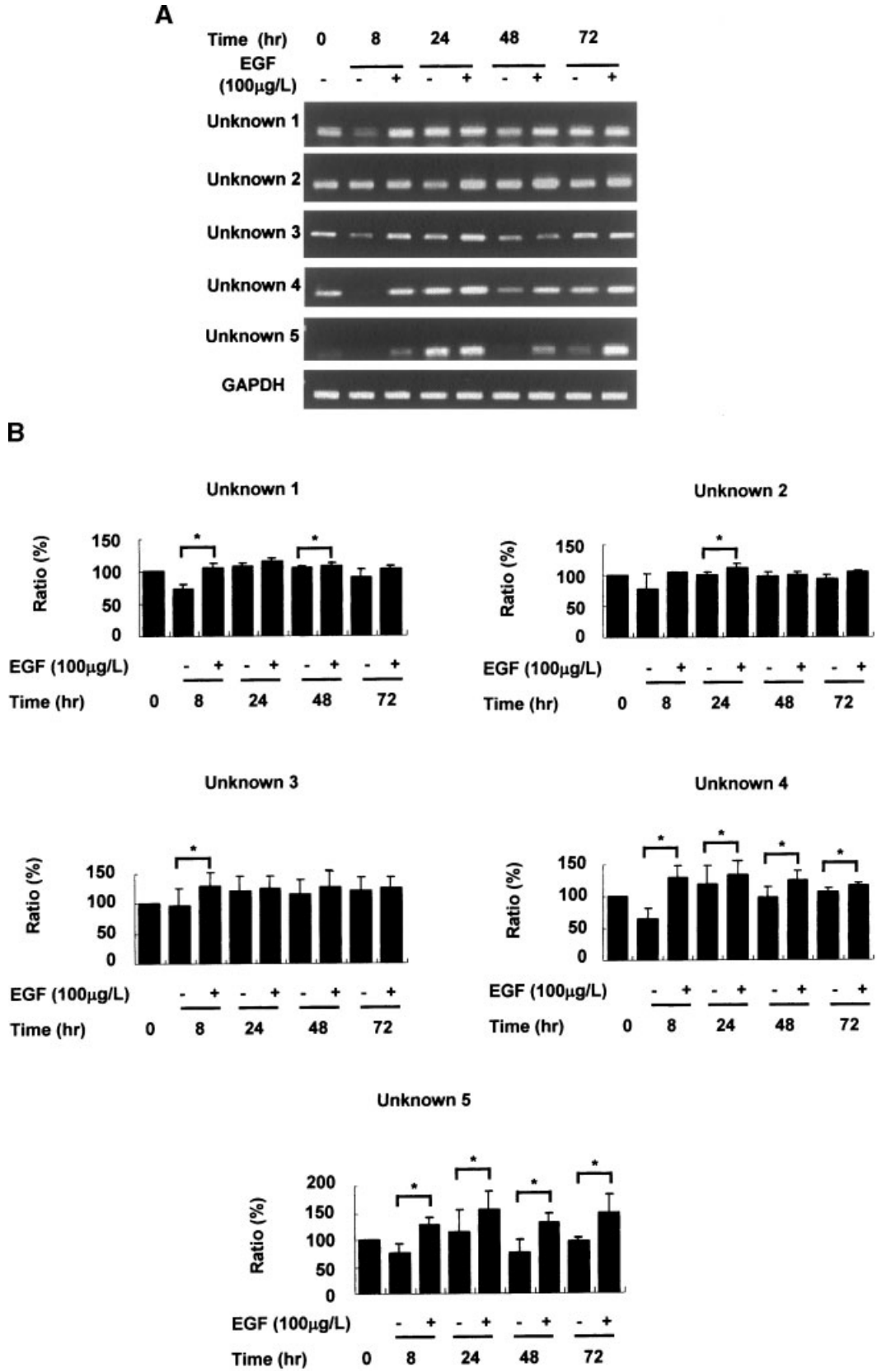
We confirmed again the up-regulation of the five known genes by Northern blot. They were *COI*, *COX-2*, *MMP-13*, *WASPIP*, and *HAS-2* genes (Fig. 6).

We could confirm the significantly increased levels of four known genes (*COX-2*, *MMP-13*, *WASPIP*, and *HAS-2*) in EGF-treated pancreas cell. As a result of semi-quantitative RT-PCR result, COI expression level hadn't showed the difference at 24 h after EGF treatment by Northern blot too.

DISCUSSION

In the early stages of development, all pancreas epithelial tissues are originated from clumps of duct cells [Larsson, 1998]. Although it has been assumed that the principal means of increasing both endocrine and exocrine tissues of the pancreas after birth are replicating of preexisting differentiated cells, neogenesis of pancreatic islets also occurs normally during the regeneration of the pancreas in some animal models and human pancreas [Edstrom and Falkmer, 1967; Rosenberg et al., 1983; Willemer et al., 1987; Brockenbrough et al., 1988; Rosenberg and Vinik, 1989; Bonner-Weir

Fig. 4. (*Overleaf*) The time course of mRNA expression level in monolayer cultured pig pancreas cells. **Panel A**: mRNA expression level according to the time course in five unknown genes by RT-PCR. **Panel B**: Semi-quantitative expression analysis of five unknown genes in monolayer cultured pig pancreas cells with (+) and without (−) EGF treatments. The ratios were determined by dividing the band density of each gene by that of GAPDH. Values are mean ± SE (n = 3). $*P < 0.05$ represents a statistically significant difference.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EGF (100μg/L) - - + - + - + - +
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Fig. 4.

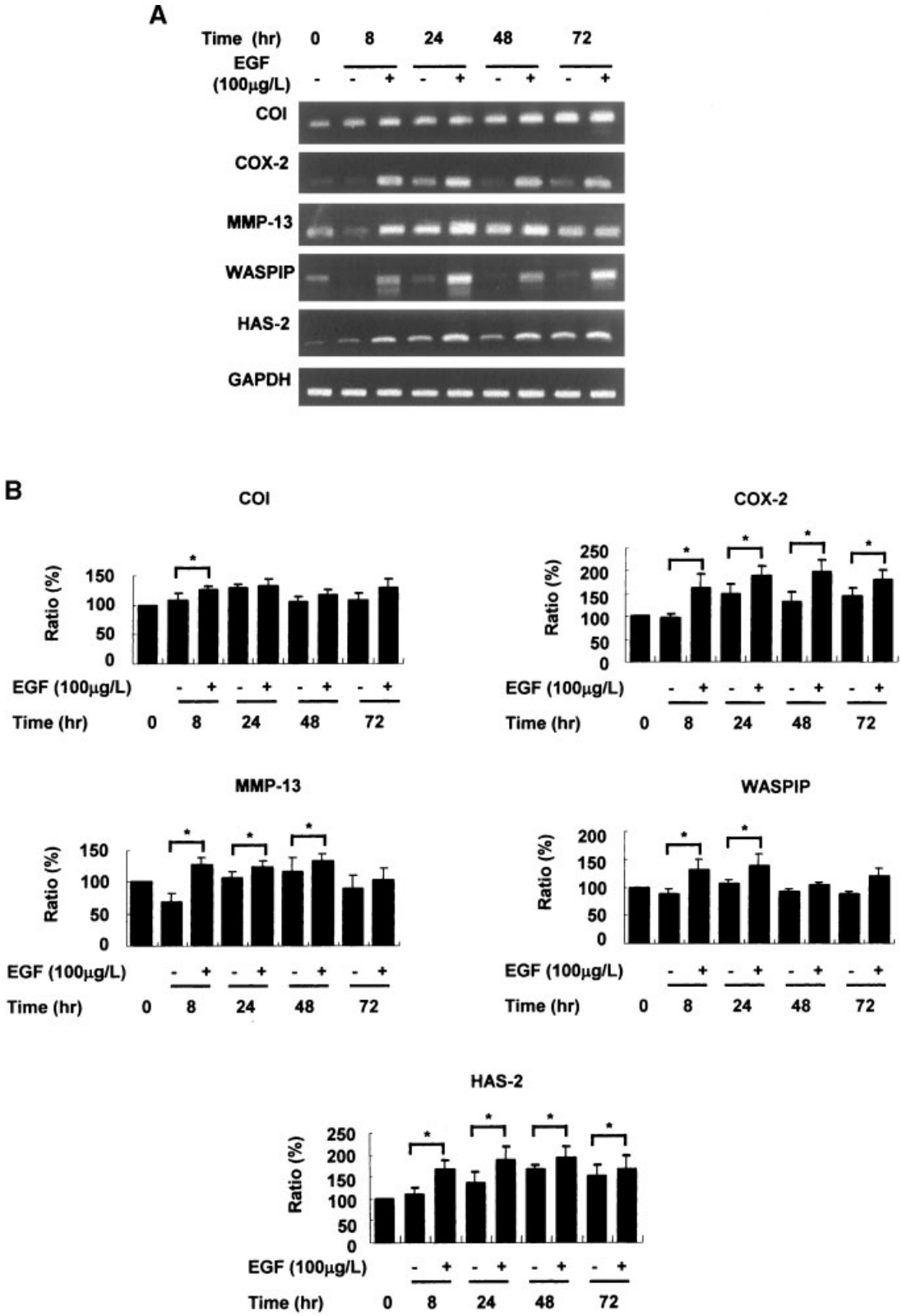

Fig. 5. The time course of mRNA expression level in five known genes. **Panel A**: Quantitative analysis of mRNA expression by RT-PCR. **Panel B**: Semi-quantitative analysis of mRNA five genes (*COI*, *COX-2*, *MMP-13*, *WASPIP*, and *HAS-2* genes) expression after 8, 24, 48, and 72 h treatment with (+) and without (−) EGF treatment in monolayer cultured pig pancreas cells. The ratios were determined by dividing the band density of each gene by that of GAPDH. Values are mean ± SE (n = 3). $^*P < 0.05$ represents a statistically significant difference.

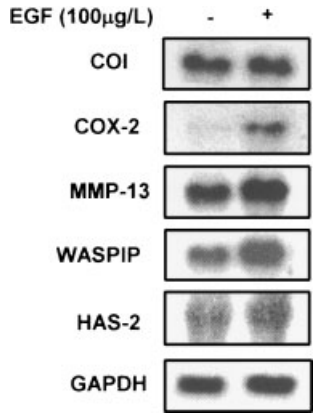

Fig. 6. Northern blot analysis to detect expression of five known genes mRNA. Twenty micrograms of total RNA isolated from monolayer cultured pig pancreas cells. Blots were probed for COI, COX-2, MMP-13, WASPIP, and HAS-2 each other.

et al., 1993; Wang et al., 1995; Rosenberg, 1998]. These results suggest that our understanding on the ontogeny of β-cells has been limited, and pancreatic duct cells in adults could proliferate and differentiate into β-cells under specific stimuli. During the development or regeneration of the pancreas, many kinds of growth factors are related, including EGF [Bockman et al., 1978; Miyagawa et al., 1999; Miettinen et al., 2000; Corentin et al., 2001].

In this study, we used the porcine neonatal pancreas cells harvested from 1 to 3 day-old pigs, and over 70% of them were comprised duct cells, while endocrine cells and fibroblasts also existed. Because of the mixed population of cells, the message from our results might not be clear enough to interpret. In our previous study, however, porcine neonatal pancreas cells were remarkably expanded and differentiated into the β-cells following transplantation [Yoon et al., 1999]. Furthermore, both the epithelial-mesenchymal interaction [Larsson, 1998], and the effect of EGF, on the surrounding mesenchymal cells, might be important for proliferation and differentiation of the duct cells. From this point of view, neonatal porcine pancreas cells seem to be a good ex vivo model for investigation of the development and regeneration of pancreata.

To gain an insight into the molecular mechanisms of duct cell proliferation induced by EGF, possibly responsible genes for duct cell proliferation should be identified. Because remarkable change in cellular composition was reported to be observed from the eighth day of culture [Tatarkiewicz et al., 2003], we treated EGF for a relatively short time to minimize the change in cellular composition, which has a crucial influence on the pattern of gene expression for subtractions PCR.

Recently, cDNA chip analysis has become the most powerful and popular method for identifying differentially expressed genes, but unfortunately it is not commercially available for pigs yet. We applied the suppression subtractive hybridization method, which is a sensitive method for identifying differentially expressed genes. After EGF treatment, we found 10 differentially up-regulated genes in monolayer culture porcine neonatal pancreas cells. Five showed no homology with known genes from the GenBank, whereas another five had homology with genes, such as *COI*, *COX-2*, *MMP-13*, *WASPIP*, and *HAS-2*.

Mutations of the mitochondrial genes, including cytochrome c oxidase, are associated with a wide spectrum of clinical phenotypes, including diabetes mellitus in humans [Rotig et al., 1993]. Silva et al. [2000] generated a mouse model for mitochondrial diabetes, and found a reduced insulin release in response to glucose stimulation and β-cell mass in older mutants. This suggested that mitochondrial genes play an important role, not only in ATP generation related with insulin secretion, but also in β-cell regeneration.

COX-2 is homologous to COX-1, and an inducible enzyme responsible for the production of prostaglandin at sites of inflammation. It is activated by a variety of growth factors, including EGF [Hamasaki et al., 1993] and tumor promoters, and has been implicated in cancer progression [Eberhart et al., 1994; Ristamaki et al., 1997; Hida et al., 1998; Kokawa et al., 2001; Merati et al., 2001]. And it has been reported that COX-2 was expressed in adenomas of intraductal papillary mucinous tumors, as well as in carcinomas. According to our data, *COX-2* gene expression was increased in monolayers cultured porcine pancreas cells by EGF treatment and EGF treatment increased cell proliferation. These results suggest COX-2 might be one of the mediator of EGF growth promoting effects on the cultured pancreas cells.

MMP-13 (Collagenase-3) is specifically expressed by various tumor cells, and it apparently plays an important role in tumor cell invasion and metastasis [Balbin et al., 1999; Yamaoka and Itakura, 1999; Ala-aho et al.,

2002; Monvoisin et al., 2002]. However, recent reports have shown that MMP-13 also played a role in wound healing, endothelial growth, and fibroblast overgrowth [Stumpf et al., 2002; Wernicke et al., 2002; Zaragoza et al., 2002]. Although MMP-13 expression in the pancreas has been observed in only adenocarcinoma [Ala-aho et al., 2002], we suggested that MMP-13 could be induced by EGF and play an important role during the development or regeneration of the pancreas. WASPIP, which is involved in Wiskott–Aldrich syndrome, a hematological disorder, is a member of a family of conserved cytoskeletal regulators [Takenawa and Miki, 2001; Caron, 2002]. During the culture, we observed rapid changes in the expression of intermediate filament proteins in the pancreas duct cells. In large and flat duct cells (Fig. 2B), showing rapid proliferation, cytokeratin expression was remarkably increased, and vimentin, a marker of mesenchymal cells, was also expressed in many of the duct cells that were shown as a yellow color. This suggests that WASPIP, induced by EGF, could play a role in up-regulation, and in changing the cell specific expression pattern of the cytoskeletal protein during duct cell proliferation. Hyaluronan (hyalunoric acid), a ubiquitous extracellular matrix component, is considered to have a dynamic role in many biological processes such as modulating the cell migration and differentiation, which regulates extracellular matrix organization and metabolism, metastasis, wound healing and inflammation [Weigel et al., 1997; Johnsson et al., 2000; Theocharis et al., 2000]. Therefore, the increased *HAS-2* gene expression by EGF might promote hyaluronan synthesis, and the increased extracellular matrix protein could enhance the proliferation and migration of the duct cells. According to our data, the enhanced cytochrome c oxidase activity, caused by EGF might be related to the EGF-induced proliferation of the porcine neonatal pancreas cells. We found 10 up-regulated genes in monolayer cultured NPCCs following treatment with EGF. The *MMP-13* and *HAS-2* genes were thought to be related to the formation of an extracellular matrix, the *WASPIP* gene with cytoskeleton expression, and the *COX-2* and *COI* genes with duct cell proliferation.

In conclusion, our findings have opened new targets for research on the mechanisms of duct cell proliferation and differentiation caused by EGF. It needs studies about the detailed function of five known genes, and identification of the unknown gene fragments.

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