Journal of Cellular Biochemistry 95:769–781 (2005)

Proteomic Analysis of Differential Protein Expression in Response to Epidermal Growth Factor in Neonatal Porcine Pancreatic Cell Monolayers

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Abstract We have proposed that porcine neonatal pancreatic cell clusters (NPCCs) may be a useful alternative source of cells for islet transplantation, and that monolayer cultures might provide an opportunity to manipulate the cells before transplantation. In addition we previously identified 10 genes up-regulated by epidermal growth factor (EGF) in cultured porcine NPCC monolayers. We have now analyzed the intracellular signaling pathways activated by EGF and searched for proteins differentially expressed following EGF treatment of the monolayers, using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). EGF treatment resulted in phosphorylation of both Erk 1/2 and Akt, as well as increased cell proliferation. Five unknown and 13 previously identified proteins were differentially expressed in response to EGF. EGF treatment increased the expression of several structural proteins of epithelial cells, such as cytokeratin 19 and plakoglobin, whereas vimentin, the intermediate filament protein of mesenchymal cells, and non-muscle myosin alkali chain isoform 1, decreased. Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 factor, which promotes epithelial cell proliferation, and hemoglobin alpha I & II also increased, whereas cyclin A1, immunoglobulin heavy chain, apolipoprotein A1, 5,10-ethylenetetrahydrofolated reductase (5,10-MTHFR), angiotensin-converting enzyme 2 (ACE2), co-lipase II precursor, and NAD^+ isocitrate dehydrogenase (NAD^+ IDH) alpha chain proteins decreased. Our results show that EGF stimulates proliferation of pancreatic epithelial cells by simultaneously activating the MAPK and PI-3K pathways. HnRNP A2/B1, hemoglobin, cyclin A1, and ACE2 may play roles in the proliferation of epithelial cells in response to EGF. J. Cell. Biochem. 95: 769–781, 2005.

Key words: neonatal pancreatic cell clusters (NPCCs); epidermal growth factor (EGF); pig; proteomics; two-dimensional gel electrophoresis (2-DE); matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Recently islet transplantation has achieved remarkable results [Shapiro et al., 2000]. However, this form of transplantation is not the ultimate solution as there is a serious shortage of human pancreases. A theoretical alternative would be to engineer β-cell growth and differentiation in vitro prior to transplantation, or to induce β-cell regeneration in vivo. There is much evidence that new islets arise from duct epithelium during the postnatal period and that this pathway of β-cell regeneration mimics or recapitulates the embryonic development of pancreas [Rosenberg and Vinik, 1989; Bonner-Weir et al., 1993; Rosenberg, 1995]. Epidermal growth factor (EGF) is thought to be one of the most important factors, both for embryonic pancreas development and for proliferation of

Grant sponsor: Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea; Grant numbers: 03-PJ10-PG6-GP01-0002, 0405-DB01-0104-0006.

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Received 28 October 2004; Accepted 1 March 2005

DOI 10.1002/jcb.20482

pancreas precursor cells and duct cells and their differentiation into islet cells in vitro [Huotari et al., 1998; Miyagawa et al., 1999; Miettinen et al., 2000; Cras-Meneur et al., 2001].

We have suggested that porcine neonatal pancreatic cell clusters (NPCCs), which mainly consist of duct epithelial cells, could be an attractive alternative source for islet transplantation, because of their potential for growth and their convenience [Yoon et al., 1999]. We showed that porcine NPCCs can be cultured as monolayers and might, therefore, provide an opportunity to manipulate the cells before transplantation, using approaches such as growth factor addition or genetic transduction [Tatarkiewicz et al., 2003]. We also demonstrated by subtractive hybridization that EGF up-regulates the transcripts of five unknown and five known genes [Jeon et al., 2004]. However, our understanding of the molecular mechanisms of pancreas precursor cell proliferation and differentiation remains limited.

Proteome analysis is a powerful tool for analyzing differentially expressed proteins by comparing the two-dimensional gel electrophoresis (2-DE) patterns of proteins under different conditions. To extend our understanding of the role of EGF in pancreas precursor cell proliferation and differentiation, we have analyzed the intracellular signaling pathway involved, and searched for differentially expressed proteins following EGF treatment of monolayer-cultured NPCCs by proteomic techniques.

MATERIALS AND METHODS

Preparation of Porcine NPCC Monolayers

The method used has been described by us previously [Jeon et al., 2004]. Our study was carried out in accordance with the principles of laboratory animal care. Briefly, eight neonatal pigs aged 1 to 3 days were killed and total pancreatectomy was performed. The pancreases were digested mechanically by mincing into 1–2 mm^3 pieces in a 50 ml tube containing M199 medium (Gibco/Invitrogen, Grand Island, NY) and then enzymatically by shaking in a water bath at 37°C for 20 min followed by the addition of collagenase P (2 mg/ml; Boehringer-Mannheim, Indianapolis, IN). After 8 min, and at the end of the digestion, the suspension was handshaken for an additional 1 min. The digested pancreas was filtered through a 500 μm sieve (Sigma-Aldrich, Inc., St Louis, MO). The washing procedure was repeated twice with Hank's balanced salts (HBSS) medium. After the supernatant had been aspirated, 10 ml of Ham's F10 medium was added to the pellet. An additional 5 ml of Ham's F10 medium was added, and the 15 ml of the suspension was transferred into 150 mm × 15 mm bacteriological plates (Becton-Dickinson, Franklin Lakes, NJ). Following the further addition of 20 ml Ham's F10 medium to each plate, they were incubated at 37°C in an atmosphere of 5% CO_2.

Porcine NPCCs that had been cultured overnight were washed three times with HBSS medium. The pellet was incubated in dissociation medium (Sigma-Aldrich, Inc.) containing 0.025% trypsin, 0.001% DNase I, and the cell clusters were broken up by gentle aspiration with a pipette and incubated in a shaking water bath at 37°C for 10 min. The suspension was washed three times more with HBSS medium, and the dispersed cells were plated at a density of 1×10^7 per 150 mm dish, and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified chamber with 5% CO_2 at 37°C. The medium in each plate was replaced with 35 ml of fresh medium on Day 3, and every other day thereafter until Day 7. The cells were treated with EGF for 48 h commencing on Day 7. Each experiment was performed at least three times.

Analysis of Cell Proliferation

Cells were seeded at a density 5×10^4 cells/well in 48-well plates in RPMI 1640 medium containing 10% calf serum. After 48 h, the medium was changed to serum-free medium. The cells were then incubated in medium containing human recombinant EGF (100 μg/L) for 24 and 48 h with 1 μCi/well [^{3}H]-thymidine present during the last 24 h. Thereafter 10% trichloroacetic acid was added, and the cells were maintained on ice for 20 min. They were then washed twice with 80% ethanol and solubilized in 500 μl of cell dissolution solution (containing 0.25 mol/L NaOH and 0.2 % sodium dodecyl sulfate) added to each well, followed after 15 min by 50 μl of 5N HCl. The contents of the wells were transferred to scintillation vials containing 4 ml of scintillation cocktail, and the radioactivity of the samples was measured with a Packard β-counter (Packard Instruments Co., Downers Grove, IL). Each experiment was done in quadruplicate and performed at least three times.

Western Blotting

Cells were lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% NP-40, 10 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1 mM PMSF, complete protease inhibitor), and the lysates centrifuged at 15,000 rpm for 5 min at 4°C. Supernatant protein concentrations were measured by the Bradford method with BSA as standard. The proteins (40 μg) were separated by 8% or 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Bioscience, Piscataway, NJ). The membranes were blocked with 5% fat-free dry milk for 1 h in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 0.1% Tween 20 (TBS-T), and then incubated with primary antibody or 1% fat-free dry milk in TBS-T, at 4°C overnight. The antibodies used were: anti-Erk 1/2 polyclonal antibody, anti-phospho-Erk 1/2 polyclonal antibody, anti-phospho-Akt polyclonal antibody, anti-Akt polyclonal antibody, anti-PY 20 polyclonal antibody (all from Cell Signaling Technology, Inc., Beverly, MA), anti-vimentin monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA) and anti-cytokeratin 19 polyclonal antibody (DakoCytomation, Glostrup, Denmark) diluted 1:1000 in 1% BSA in TBS-T, anti-hnRNP A2/B1 (Abcam Ltd, Cambridge, UK), ACE2 and β-actin (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-cyclin A1 (BD-Pharmingen, San Diego, CA) diluted 1:1,000 in 1% BSA The membranes were washed, and then incubated with secondary peroxidase-conjugated anti-rabbit or anti-mouse antibody (from Amersham Bioscience) diluted 1:1000 in 1% fat-free dry milk in TBS-T at room temperature for 1 h. Staining was detected with an enhanced chemiluminescence kit (Amersham Bioscience), and quantified by densitometry with ImageMaster VSD software (Amersham Bioscience).

Two-Dimensional Gel Electrophoresis

Cells were washed three times with sterile phosphate buffered saline (PBS), and homogenized in 400 μl lysis buffer (7M urea, 2M thiourea, 2% CHAPS, 2% Pharmalyte pH 3–10, 100 mM dithiothreitol, complete protease inhibitor). The resulting lysates were sonicated for about 10 s on ice, and DNase I (10 U/L mg proteins, Boehringer-Mannheim) was then added and the lysates incubated at room temperature for 30 min. To remove solid material, the samples were centrifuged at 15,000 rpm for 20 min, and supernatant protein measured by the Bradford method.

Samples (1 mg/ml) were applied to strips that had been rehydrated with sample solution at room temperature for 18 h. After rehydration, isoelectric focusing was performed at 20°C with a current limit of 50 μA/strip as follows: Immobiline DryStrips (18 cm, pH 3–10, Pharmacia, Peapack, NJ) were equilibrated for 20 min by gently shaking in 375 mM Tris-HCl, pH 8.8, containing 6M urea, 20% glycerol, 2% SDS, and 0.01% bromophenol blue with 10 mM tributyl phosphine. Then the second-dimensional gels were overlaid in a solution containing 0.5% agarose, 24.8 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS, and a trace of bromophenol blue. Using vertical SDS gradient slab gels (9%–16%, dimensions 180 mm × 200 mm × 1.5 mm), electrophoresis was performed at a constant current of 10 mA/gel at 20°C. After fixing the proteins in 40% methanol and 5% phosphoric acid for 4 h, the gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA) overnight. Gel images were obtained with a GS-710 image scanner (Bio-Rad Laboratories) and processed with Melanie 5 software (GeneBio, Geneva, Switzerland). Digitized images were compared using a matching method, and differentially expressed spots (more than 1.2-fold increase or decrease in intensity) were identified. Each experiment was performed in quadruplicate and performed at least three times.

MALDI-TOF Mass Spectrometry Analysis

Spots on the gels were excised with end-removed pipette tips to accommodate various spot diameters. The gel slices were destained in microtubes and dehydrated with 50 μl ammonium bicarbonate: acetonitrile (60:40) at room temperature for 10–20 min. After the solvent had been removed, the gels were dehydrated with 50 μl 100% acetonitrile solution, and then dried with a Speedvac at room temperature for 30 min. The dried gels were digested with 140 μl trypsin solution (1 g trypsin containing 50 mM ammonium bicarbonate pH 8.0) at 37°C for 24 h and the tryptic peptides were loaded onto columns [applied with 70% acetonitrile containing POROS R2:oligo R3 (1:1)] using GELoader tips (Eppendorf, Wesseling-Berzdorf, Germany) as described [Gobom et al., 1999].

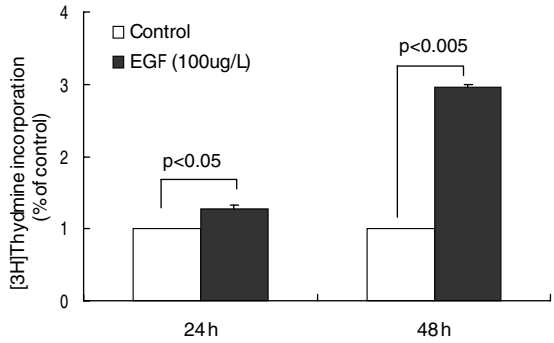


Fig. 1. The effect of EGF on thymidine incorporation by porcine NPCCs. Cells were incubated for 24 and 48 h, and cell proliferation was examined by assaying [^{3}H] thymidine incorporation. Results are expressed as fold increase in [^{3}H]-thymidine incorporation relative to controls. Values are means ± SD. The amount of thymidine incorporation increased gradually with 48 h EGF treatment in porcine NPCCs.

After equilibration with 0.1% TFA, the peptides were eluted in 70% acetonitrile/0.1% TFA solution and dropped onto a MALDI plate (96 × 2; Applied Biosystems, Foster, CA). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with an Applied Biosystems Voyager DE-PRO spectrometer (Applied Biosystems). The instrument was operated at accelerating voltage 20 kV, positive ion reflection mode, voltage grid 74.5%, guide wire voltage 0%, and delay time 75 ns. The spectra were internally calibrated using the trypsin autolysis products (842.51 and 2211.11 Da) and monoisotopic peptide masses were assigned and used for database searches.

Proteins were identified by searching the SWISS-PROT and NCBI databases using MS-Fit (Protein Prospector; http//www.prospector.

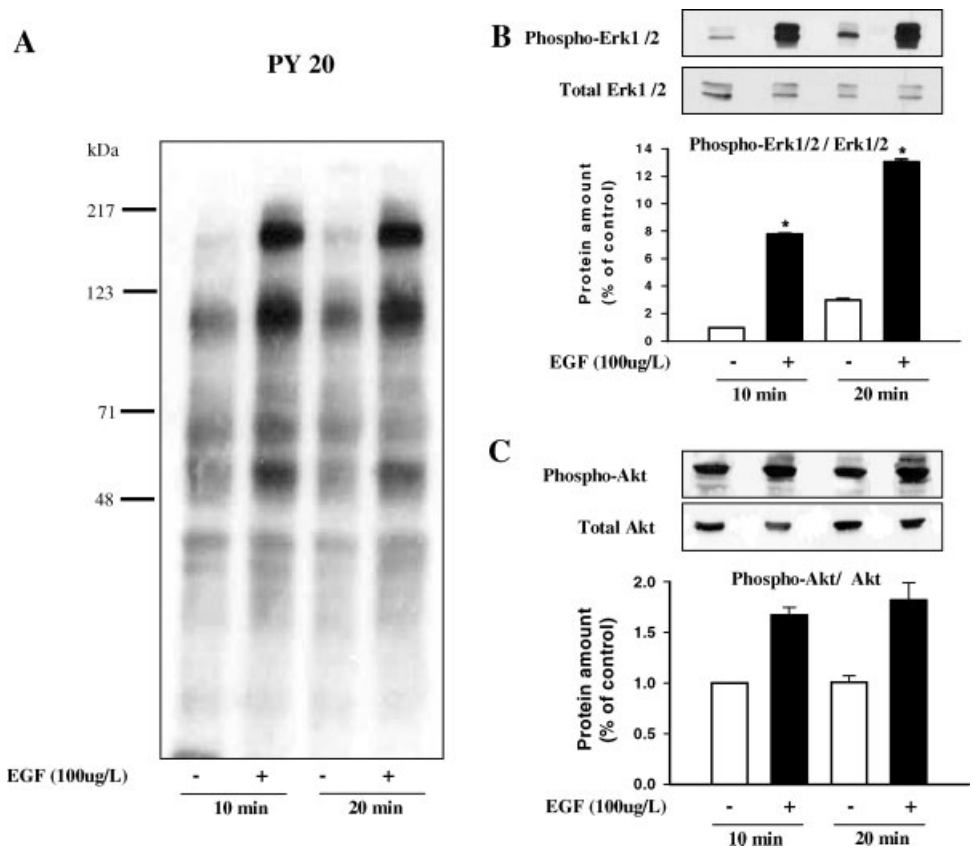


Fig. 2. Intracellular targets of EGF. Cells were grown to 80% confluence, treated with EGF (100 μg/L) for the indicated times, and harvested for Western blotting signaling intermediates. The same amount of total lysate protein was loaded into each SDS–PAGE channel. The membrane was immunoblotted with anti-PY20 antibody (1:2,000), anti-phospho-ERK 1/2 antibody (1:1,000), and anti-phospho-Akt antibody (1:1,000). **A**: The phosphorylation of total tyrosine kinase was increased significantly by EGF. **B**: Erk 1/2 activity also increased within 20 min after 100 μg/L EGF treatment. **C**: Akt activity was also significantly elevated from 10 min after 100 μg/L EGF. Quantitative analysis of gel bands. Each data point represents the mean ± SD of three separate experiments. Student's *t*-test gave $P < 0.05$ between the control and EGF treated groups.

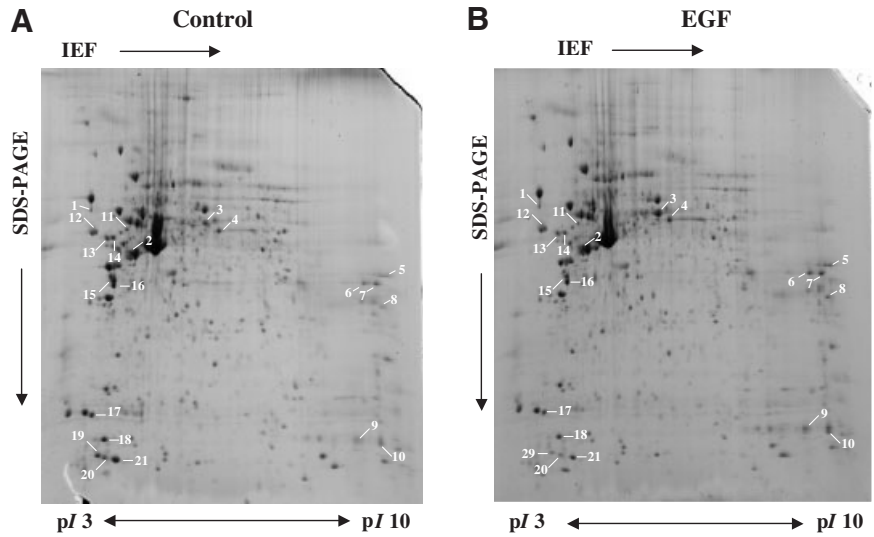


Fig. 3. 2-DE gels of control and EGF-treated cells. Samples of 400 μg protein were applied to pH 3–10 non linear IPG strips (18 cm), and run on 9%–16% gradient SDS–PAGE gels as the second dimension. Gels were visualized with Coomassie brilliant blue G-250 and compared using Melanie 5 software. **A**: 2-DE gel of control porcine NPCCs. **B**: 2-DE gel of porcine NPCCs treated for 48 h with 100 μg/L EGF. Arrows indicate 21 differentially expressed protein spots.

ucsf.edu) and MASCOT (Matrix Science; http// www.matrixscience.com). All searches were analyzed with a 100 ppm mass tolerance.

Statistical Analysis

All experimental results are described as means ± SD. Statistical significance was determined using Student's *t*-test, and $P < 0.05$ was considered significant.

RESULTS

EGF Stimulates Proliferation of Porcine NPCCs

To examine stimulation of porcine NPCC proliferation in response to EGF, the cells were serum-starved for 24 h and incubated for an additional 24 or 48 h with 100 μg/L EGF, and cell growth was examined by [^{3}H]-thymidine incorporation. Thymidine incorporation increased slightly after 24 h of treatment and substantially after 48 h (Fig. 1). The proportion of endocrine, duct, and mesenchymal cells in the dishes was not significantly changed by EGF treatment for 24 h, as previously reported (data not shown).

Activation of MAPK and PI-3K Signaling by EGF

Cells were serum-starved for 24 h and incubated for an additional 10 or 20 min with 100 μg/L EGF. We then analyzed tyrosine-phosphorylation of proteins with anti-phosphotyrosine antibody PY 20. Several tyrosine-phosphorylated bands increased in the EGF-treated cells (Fig. 2A). Erk 1/2 activity was also increased from 10 min after 100 μg/L EGF treatment. Erk 1/2 activity persisted over the

TABLE I. Up-Regulated Proteins Induced by EGF

Spot no.	Protein identification	Accession no.	Mass (kDa)	*P*/	Coverage %	Database
1	Unknown					
2	Cytokeratin 19	125,086	43,870	4.9	34	NCBI
3	Plakoglobin [*Sus scrofa*]	18,147,001	81,798	5.8	16	NCBI
4	Unknown					
5	hnRNP A2/B1 isoform B1 [*Homo sapiens*]	14,043,072	37,407	8.9	45	NCBI
6	hnRNP A2/B1 isoform A2 [*Mus musculus*]	32,880,197	32,510	8.7	31	NCBI
7	HnRNP A2/B1 isoform A2 [*Mus musculus*]	32,880,197	32,510	8.7	31	NCBI
8	Unknown					
9	Hemoglobin alpha I & II chain	122,359	14,999	8.9	14	NCBI
10	Hemoglobin alpha I & II chain	122,359	14,999	8.9	20	NCBI

Proteins identified by MALDI-TOF and database searching using MS-Fit and MASCOT.

TABLE II. Down-Regulated Proteins Induced by EGF

Spot no.	Protein identification	Accession no.	Mass (kDa)	*P*/	Coverage %	Database
11	5,10-Ethylenetetrahydrofolate reductase [*Mus musculus*]	3,154,327	74,581	5.1	12	NCBI
12	ACE2	37,182,549	63,782	4.9	13	NCBI
13	Vimentin [*Mus musculus*]	2,078,001	51,533	4.9	57	NCBI
14	Cyclin A1 [*Homo sapiens*]	2,327,153	52,196	4.9	33	NCBI
15	Unknown					
16	Apolipoprotein A1	423,201	30,312	5.4	14	NCBI
17	Smooth muscle and non-muscle myosin alkali chain isoform 1 [*Homo sapiens*]	17,986,258	16,919	4.6	31	NCBI
18	Colipase II precursor	71,956	10,328	5.6	47	NCBI
19	NAD^+ isocitrate dehydrogenase alpha chain	108,335	9,685	4.7	30	NCBI
20	Immunoglobulin heavy chain [*Lama glama*]	4,376,037	13,868	4.9	30	NCBI
21	Unknown					

Proteins identified by MALDI-TOF and database searching using MS-Fit and MASCOT.

incubation period of 20 min ($P < 0.05$, Fig. 2B). EGF had a pronounced effect at 20 min (Fig. 2C).

Proteins Up-Regulated and Down-Regulated by EGF

We performed two-dimensional electrophoresis (2-DE) of proteins extracted from control and 100 μg/L EGF-treated cells to identify proteins affected by EGF treatment. After spot detection, background subtraction and volume normalization, we counted 713 well-resolved spots in the gels from EGF-treated cells. Overall, around 33 protein spots were found consistently up- or down-regulated by over 1.2-fold in triplicate experiments after EGF-treatment for 2 days. We tried to identify round 21 protein spots, encompassing a wide range of molecular weights, pI values, fold changes, and abundance. The location of each spot is labeled with number and an arrow indicating upward or downward regulation by EGF (Fig. 3).

Differentially expressed proteins, nontreated and EGF-treated cells were identified using peptide mass fingerprinting. For all the proteins identified, two search engines (MAS-Fit and MASCOT) gave same proteins hits with high confident scores, and at least two peptides sequenced with good MS/MS spectra. Proteins identified so far are listed in Tables I and II. The up-regulated proteins identified by MAS-Fit and MASCOT were (in decreasing order): cytokeratin 19, plakoglobin, heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, and hemoglobin alpha I & II chain. Three of the 10 up-regulated spots had no homology with known proteins (Fig. 4A,B, Table I).

The down-regulated proteins identified by MAS-Fit and MASCOTT were 5,10-ethylenetetrahydrofolated reductase (5,10-MTHFR), angiotensin-converting enzyme 2 (ACE2), vimentin, cyclin A1, apolipoprotein A1, non-muscle myosin alkali light chain isoform 1, colipase II precursor, NAD^+ isocitrate dehydrogenase (NAD^+ IDH) alpha chain, and immunoglobulin heavy chain. Two additional proteins showed no homology with known proteins (Fig. 5A,B, Table II).

We were able to identify thirteen proteins from the protein database at NCBI; these included skeletal proteins (plakoglobin, cytokeratin 19, vimentin, non-muscle myosin alkali light chain isoform 1), cell cycle-related proteins (hnRNP A2/B1, Cyclin A1), metabolism-related proteins (ACE2, apolipoprotein A1, co-lipase II precursor, 5,10-MTHFR, NAD^+IDH alpha chain), and immunity-related proteins (hemoglobin alpha I & II chain, immunoglobulin heavy chain).

Fig. 4. 2-DE image of proteins up-regulated by 100 μg/L EGF after 48 h. **A**: High magnification views of the regions of the gels framed in Figure 3 are shown. Expression levels were assessed by calculating the ratio of the relative spot volume of a particular protein spot to the total amount of protein on the gel. Arrows indicate 10 protein spots up-regulated in EGF-treated cells. Proteins whose expression increased more than 1.2-fold in EGF-treated cells compared with control were selected for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. **B**: Peptide mass fingerprinting spectra of protein spot nos. 2, 3, 5, 6, 7, 9, and 10 from MALDI-TOF MS. Peptide mass fingerprinting was performed on an Applied Biosystem Voyager DE-PRO spectrometer in reflection mode. The protein were identified as cytokeratin 19, plakoglobin, hnRNP A2/B1, and hemoglobin alpha I and II chain.

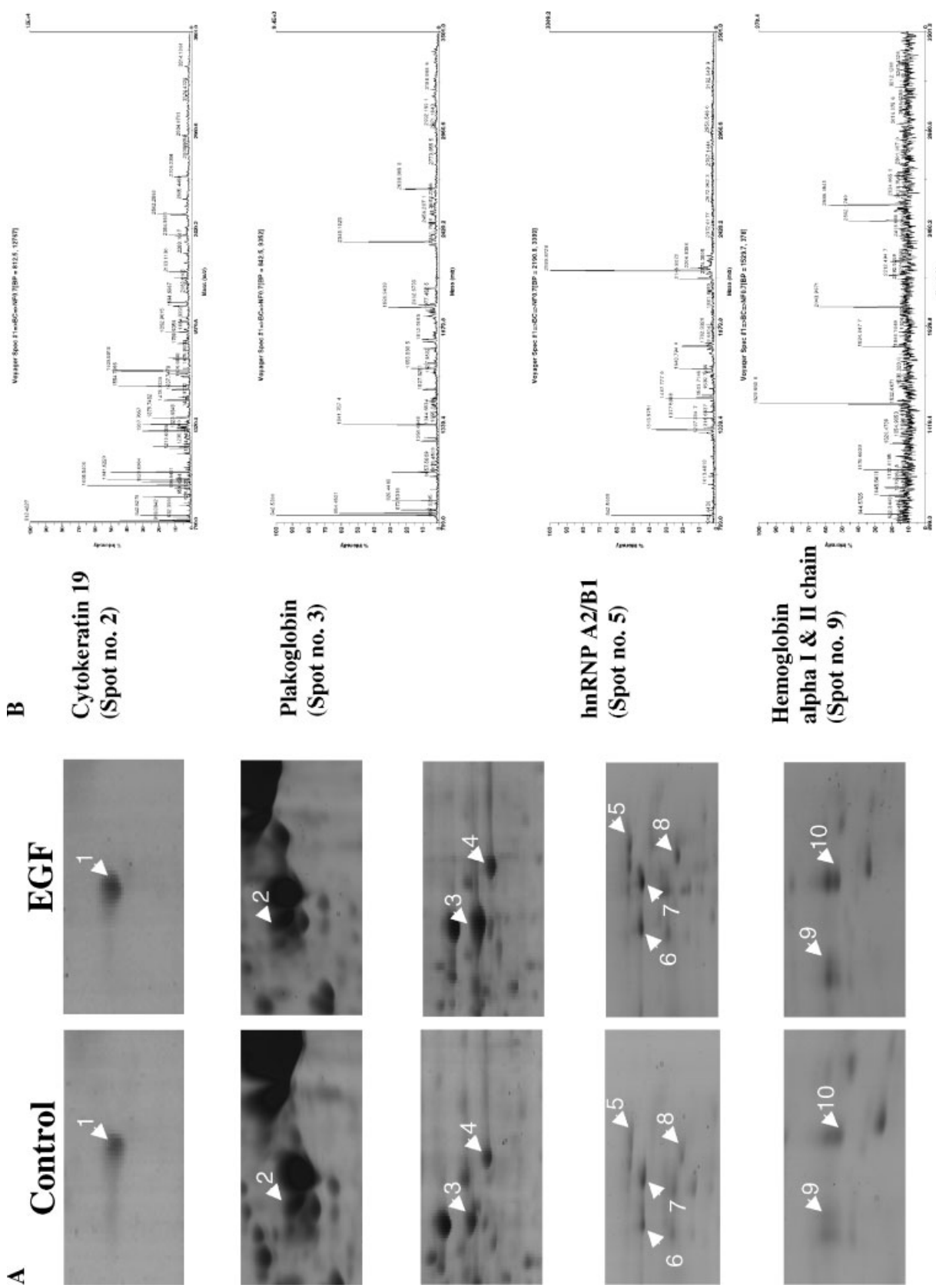
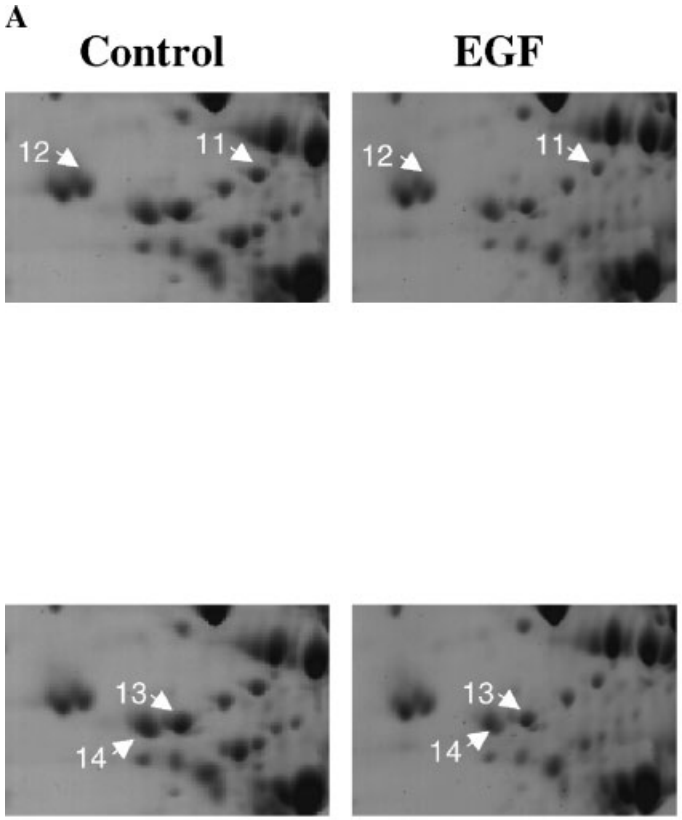

A
Control
EGF
1
2
3
4
5
6
7
8
9
10
B
Cytokeratin 19
(Spot no. 2)
Plakoglobin
(Spot no. 3)
hnRNP A2/B1
(Spot no. 5)
Hemoglobin
alpha I & II chain
(Spot no. 9)

Fig. 4.

A


Control
EGF
12
11
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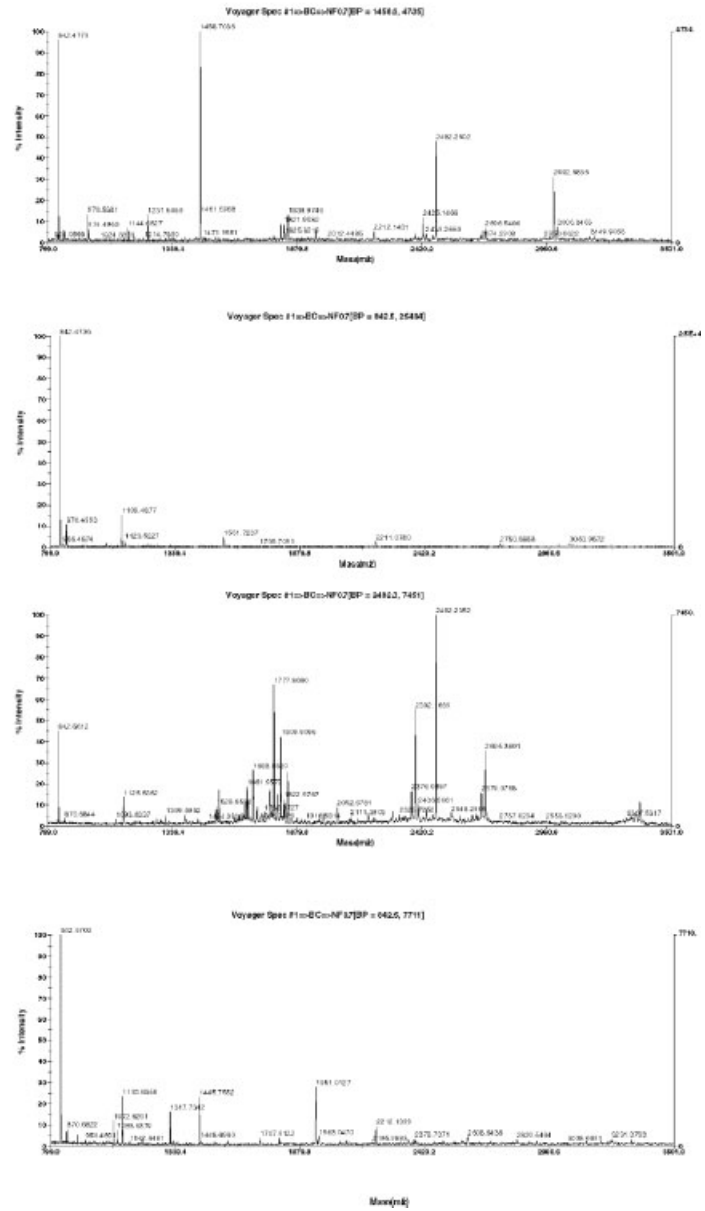
B


5,10-
MTHFR
(Spot no. 11)
ACE2
(Spot no. 12)
Vimentin
(Spot no. 13)
Cyclin A1
(Spot no. 14)

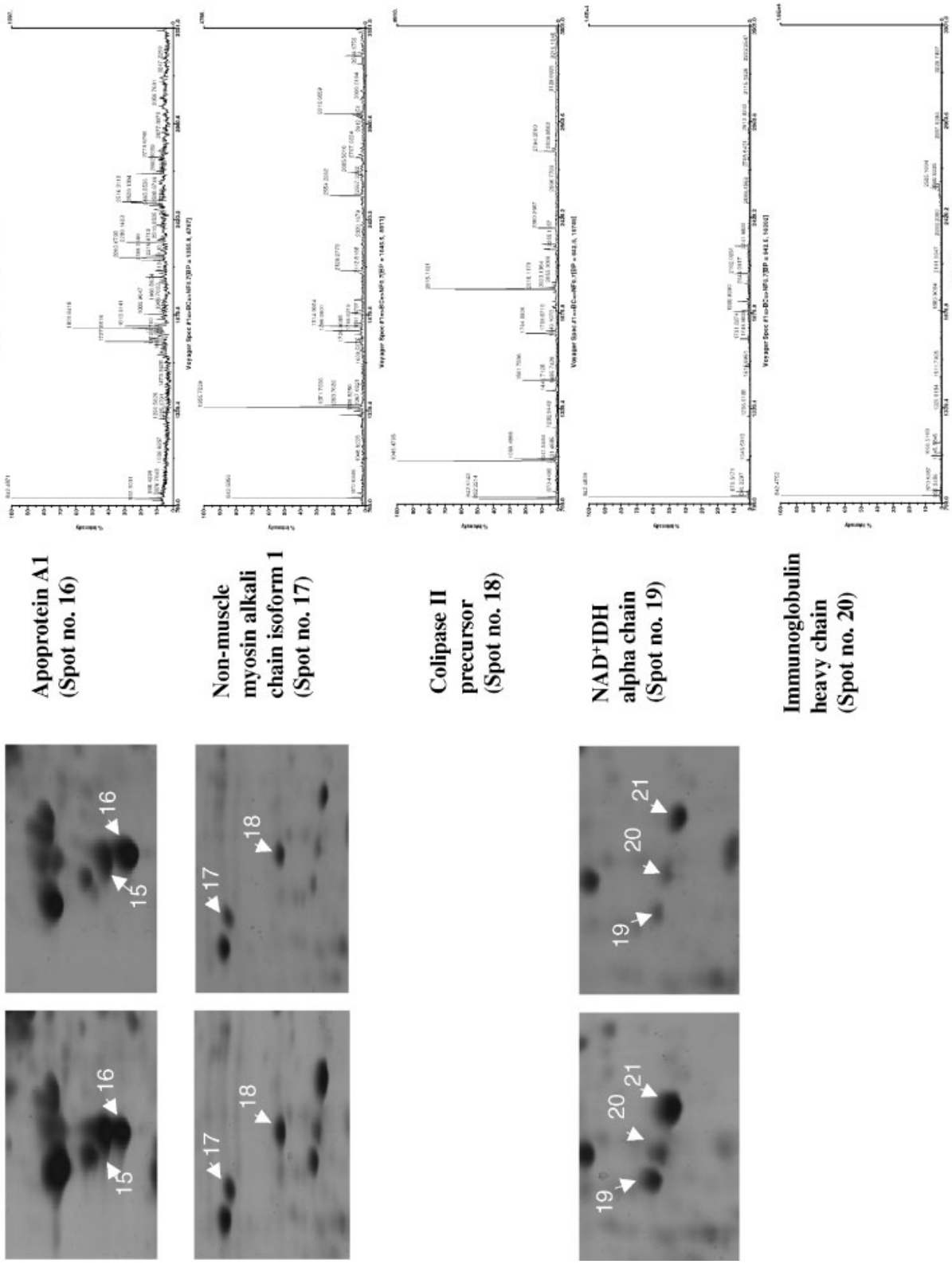

Apoprotein A1
(Spot no. 16)
Non-muscle
myosin alkali
chain isoform 1
(Spot no. 17)
Colipase II
precursor
(Spot no. 18)
NAD+IDH
alpha chain
(Spot no. 19)
Immunoglobulin
heavy chain
(Spot no. 20)
15
16
17
18
19
20
21

Fig. 5.

Western Blotting Analysis

To check the accuracy of our 2-DE system, we also separated proteins by 8% or 10% SDS–PAGE and performed Western blotting for vimentin as a marker of mesenchymal cells, and cytokeratin 19 as a marker of epithelial cells (Fig. 6). Cytokeratin 19 increased in response to EGF ($P < 0.05$); whereas vimentin decreased ($P < 0.05$). Similarly HnRNP A2/B1 increased while ACE2 and cyclin A1 decreased ($P < 0.05$). These effects are in good agreement with expression data obtained by 2-DE and Western blotting.

DISCUSSION

We have presented evidence that EGF treatment induces epithelial cell proliferation via the MAPK and PI-3K pathways, and have observed 21 differentially regulated proteins in monolayer cultured porcine NPCCs. We also identified 13 proteins of the proteins from the NCBI database, including skeletal proteins, cell cycle-related proteins, metabolism-related proteins, and immune-related proteins (see Fig. 7).

During development or regeneration of the pancreas, many kinds of growth factors are observed [Bockman et al., 1978; Miyagawa et al., 1999; Miettinen et al., 2000; Cras-Meneur et al., 2001]. EGF in particular has an important role in the proliferation of pancreatic precursor cells, islet cell differentiation, and mesenchymal–epithelial cell interactions [Miettinen et al., 2000]. The receptor for EGF is a transmembrane glycoprotein. After binding ligand, the receptor has been shown in several systems to initiate a kinase cascade that activates members of the Ras/Raf/Erk MAPK family as well as the PI-3K/Akt pathway [Zatechka and Lou, 2002]. The same is clearly true of porcine NPCCs: we observed that treatment of monolayer cultured NPCCs with EGF led to phosphorylation and subsequent activation of both Erk and Akt.

We previously noted 10 genes up-regulated by EGF [Jeon et al., 2004]; we used NPCCs in the experiment of proteomics using same way to prepare for subtractive hybridization, however, comparison of the results of subtractive hybridization and the proteomic analysis revealed some difference between effects at the mRNA and the protein level. We could summarize the differences between subtractive hybridization and proteomics with some possibilities. First, this may be due to the sensitivity of each method to detect and the characteristics of the target molecules such as abundance, molecular weight and so on. Second, proteomics can pick up only actively translated proteins, so its difference could be explained with the difference of post-translational modifications such as methylation, glycosylation, or phosphorylations in contrast to RNA expressions. Third, the time differences would exist between subtractive hybridization and proteomics method as described above. So, we suggest that proteomic and genomic measurement done on the same system provide more comprehensive information.

EGF stimulated the synthesis of epithelial cell specific cytoskeletal proteins such as plakoglobin and keratin, whereas vimentin, the intermediate filament protein of mesenchymal cells, and non-muscle myosin alkali light chain isoform 1, were down-regulated. Remodeling of the extracellular matrix (ECM) is essential in many processes involving cell migration, cell–cell interaction, proliferation, and differentiation. MMPs can either degrade or modify basement membrane proteins, and this might directly initiate epithelial–mesenchymal transformation (EMT) by influencing cell–cell and/or cell–matrix interactions [Blavier et al., 2001]. Expression of MMP-13 is limited to situations in which rapid and effective remodeling of collagenous ECM takes place. In addition to increased MMP-13 mRNA levels [Jeon et al., 2004], it is believed that increases in cytokeratin 19 and plakoglobin are essential for maintaining epithelial integrity. Thus these products that respond to EGF are thought to contribute to the proliferation of pancreatic duct epithelial cells.

EGF also increased the cell-cycle protein, HnRNP A2/B1, which plays a role in events such as splicing and transport of mRNA in normal cells [Kamma et al., 1991]. EGF was known to

Fig. 5. (*Overleaf*) 2-DE image of proteins down-regulated response to 100 μg/L EGF after 48 h. **A**: High magnification views of the regions of framed in Figure 3 are shown. Arrows indicate 11 protein spots down-regulated in EGF-treated cells. **B**: Peptide mass fingerprinting spectra of protein spot nos. 11, 12, 13, 14, 16, 17, 18, 19, and 20 from MALDI-TOF MS. The protein were identified as 5,10-MTHFR, ACE2, vimentin, cyclin A1, apoprotein A1, non-muscle myosin alkali chain isoform, colipase II precursor, NAD^+IDH alpha chain, and immunoglobulin heavy chain.

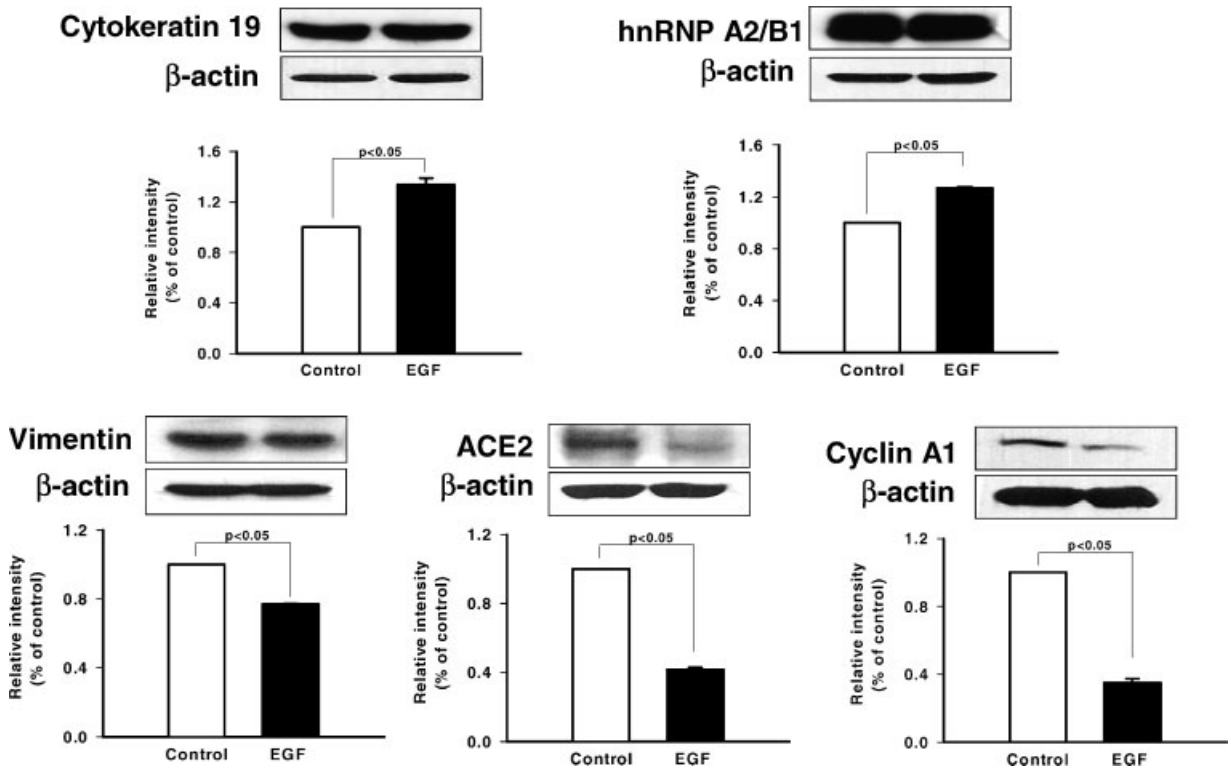
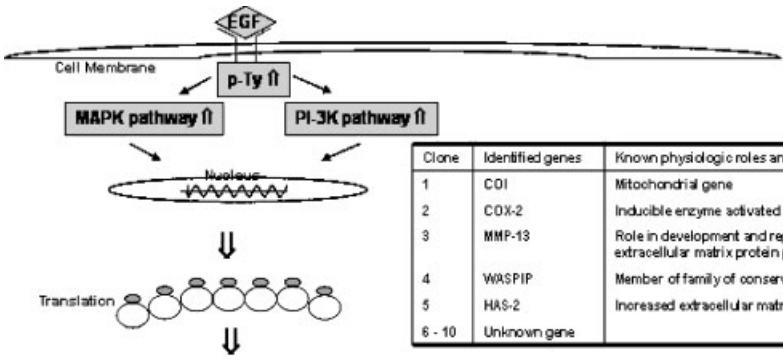


Fig. 6. Evaluation of the expression of hnRNP A2/B1, cytokeratin 19, vimentin, ACE2, and cyclin A1 inresponse to EGF for 48 h. The membrane was immunoblotted with anti-hnRNP A2/B1, anti-cytokeratin 19, anti-vimentin, anti-ACE2, anti-cyclin A1, and β-actin. Other detains as in the legend to Figure 4. Cytokeratin 19 and hnRNP A1/B2 protein expression were elevated following treatment with 100 μg/L EGF, however, vimentin, ACE2, and cyclin A1 were significantly decreased ($P < 0.05$).

induce hemoglobin synthesis in the murine hematopoietic cell line SKT6, suggesting that the presence of activated inflammatory cells contributes to stimulating the proliferation of the tumor cells at the same time as increasing cyclooxygenase-2 (COX-2) expression. COX-2 expression is also up-regulated by EGF in a pancreatic carcinoma cell line as well as in porcine duct cells [Molina et al., 1999; Jeon et al., 2004], we observed here that expression of one of the cell cycle proteins, cyclin A1, was down-regulated by EGF. According to our results, hnRNP A2/B1 protein, cyclin A1, hemoglobin alpha I & II chain, and COX-2 may contribute to the effect of EGF on duct epithelial cell proliferation [Cok et al., 2004].

Angiotensin-converting enzyme 2 (ACE2) is a novel mammalian metallocarboxypeptidase [Turner et al., 2002]. ACE2 is a homologue of angiotensin-converting enzyme, it also increases levels of angiotensin II [Donoghue et al., 2000; Huang et al., 2003], a well-known inducer of TGF-β expression and mesenchymal cell proliferation. The induction of ACE2 by EGF might suppress mesenchymal cell proliferation and result in a decrease of mesenchymal cell structural proteins such as vimentin and non-muscle myosin alkali light chain isoform 1. We could confirm a reduction in the level of vimentin in EGF-treated porcine NPCCs. Angiotensin II and EGF synergistically induce COX-2 expression in the IEC-18 cell line, a model of intestinal epithelial cells; however, the signaling pathways initiated by Angiotensin II and EGF differ [Slice et al., 2004]. The slight difference between ACE2 and COX-2 expression in porcine NPCCs may be associated with the fact that EGF maintains the viability or differentiation of epithelial cells as well as mesenchymal cells.

Co-lipase II precursor and apolipoprotein A1, which is related to co-lipase, were reduced by EGF. Pancreatic co-lipase and its precursor, pro-co-lipase, facilitate interfacial lipid hydrolysis catalyzed by pancreatic lipase, by acting as cofactors of the enzyme [Momsen et al., 1995; D'Agostino and Lowe, 2004]. Thus, the decreased expression of co-lipase and



Clone	Identified genes	Known physiologic roles and effects
1	COI	Mitochondrial gene
2	COX-2	Inducible enzyme activated by EGF at inflammation site
3	MMP-13	Role in development and regeneration of pancreas, extracellular matrix protein production
4	WASPIP	Member of family of conserved cytoskeletal regulators
5	HAS-2	Increased extracellular matrix protein production
6 - 10	Unknown gene	

Functional Group	Name of protein (Spot number)	2-D gel	Function in porcine neonatal pancreatic cell
Cytoskeleton	Cytokeratin (2)	up	Proliferating epithelial cell
	Plakoglobin (3)	up	Structural and functional organization of epithelial cell
	Vimentin (13)	Down	Supressing mesenchymal cell
	Smooth muscle and non-muscle myosin alkali chain isoform 1 (17)	Down	Regulating the structure and function of cytoskeleton
RNA processing	hnRNP A2/B1 (5,6,7)	Up	RNA processing and transport, related with tumor formation
Cell cycle	Cyclin A1 (14)	Down	Anti-apoptosis, increasing epithelial cell proliferation
Metabolism	Apolipoprotein A1 (16)	Down	Removing cholesterol from cell and increasing cell proliferation
	Colipase II precursor (18)	Down	Cofactor of apolipoprotein A1
	ACE2 (12)	Down	Decreasing fibroblast cell proliferation
	NAD*-isocitrate dehydrogenase (19)	Down	
	5,10-Methylenetetrahydrofolate reductase (11)	Down	
Inflammation	Hemoglobin alpha I & II chain (9,10)	Up	Activation of inflammatory action of EGF
	Immunoglobulin heavy chain (20)	Down	

Fig. 7. Schematic diagram of the relationship between the proliferation of mesenchymal and epithelial cells in response to EGF. The major proteins identified in this study are shown. The potential effects of EGF on the proliferation of mesenchymal cells or epithelial cells in pancreatic ducts are summarized. Our previous results with gene expressions using suppression subtractive hybridization are included, and expressed relative to total protein. COI, cytochrome C oxidase subunit I; COX-2, cyclooxygenase-2; MMP-13, matrix metalloproteinase-13; WASPIP, Wiskott-Aldrich syndrome protein integrating protein; HAS-2, Hyaluronan synthase-2; p-Ty, phophorylated tyrosine kinase.

apolipoprotein A1 in pancreas cells suggests that EGF may suppress the trans-differentiation of pancreatic precursor cells into exocrine cells.

In contrast to the increase in cytochrome c oxidase, expression of the NAD^{+}-IDH alpha chain, which is found in the mitochondrial fraction, was suppressed by EGF. This discrepancy needs to be clarified. Levels of 5,10-MTHFR and the immunoglobulin heavy chain were also reduced by EGF for unknown reasons.

Although the changes in mRNA and protein levels relate to EGF stimulation, there were few remarkable differences between duct cells and endocrine cells in our monolayer cultures of porcine NPCCs. We showed that hnRNPA2/B1 had similar physichochemical parameters (37 kDa, pI 8.9; 33 kDa, pI 8.7; 33 kDa, pI 8.7) and the differential expression and distribution in 2-DE gel image. According to hnRNP A2/B1, the apparent changes in CK19, myosin, and vimentin expression in the 2-DE analysis may be due to shifts induced by post-translational modifications rather than by changes in transcriptional. Structural proteins like CK and actin can be considered to integral proteins as a transcription initiation factor [Turck et al., 2004]. These nuclear proteins by differential display resulted in proliferative or differentiated cells response to EGF. This suggests that EGF stimulated the proliferation of both duct cells and endocrine cells during the period of culture.

We obtained the NPCCs cells from eight pigs, and the fold-increase of protein response to EGF was somewhat diverse. A small population of NPCCs responded dramatically to EGF upto 1.5-fold (hnRNP: 1.5-fold; vimentin: 1.9-fold), however, larger population of cells showed a modest increase about 1.2 fold in total protein expression. Though a relative increase or decrease of >1.2 fold in intensity was considered to be significant, we thought that certain cell population within the NPCC may respond differentially to EGF stimulation.

In summary, EGF stimulated porcine neonatal pancreatic epithelial cell proliferation by activating the MAPK and PI-3K pathways, and

resulted in altered expression of some proteins related to cell proliferation. Our findings are the first report of EGF effects at the protein level in cultured NPCCs. Further study of these proteins may provide clues for clinical applications.

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