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Extracellular Matrix Modulates Insulin Production During Differentiation of AR42J Cells: Functional Role of Pax6 Transcription Factor

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ABSTRACT

Extracellular matrix (ECM) modulates differentiation of pancreatic β-cells during development. However, the mechanism by which ECM proteins modulate differentiation is not totally clear. We investigated the effect of ECM proteins on differentiation β-cells in vitro. We investigated the effect of basement membrane ECM on differentiation of AR42J cells and rat ductal cells. First, we examined the effect of reconstituted basement membrane, Matrigel on differentiation of AR42J cells induced by activin and betacellulin. Matrigel augmented insulin production and increased the expression of GLUT2, SUR1, and glucokinase. Among various transcription factors investigated, Matrigel markedly upregulated the expression of Pax6. When Pax6 was overexpressed in cells treated with activin and betacellulin, the expression of insulin was upregulated. Conversely, knockdown of Pax6 significantly reduced the insulin expression in cells cultured on Matrigel. The effects of Matrigel on insulin-production and induction of Pax6 were reproduced partially by laminin-1, a major component of Matrigel, and inhibited by anti-integrin-β1 antibody. Matrigel also enhanced the activation of p38 mitogen-activated kinase induced by activin and betacellulin, which was inhibited by anti-β1 antibody. Finally, the effect of Matrigel on differentiation was reproduced in rat cultured ductal cells, and Matrigel also increased the expression of Pax6. These results indicate that basement membrane ECM augments differentiation of pancreatic progenitor cells to insulin-secreting cells by upregulating the expression of Pax6. J. Cell. Biochem. 112: 318–329, 2011.

KEY WORDS: DIFFERENTIATION; β-CELL; MATRIX; PAX6; ACTIVIN A; BETACELLULIN

Differentiation of pancreatic β-cell is strictly controlled by an integrated network of transcription factors. Homeobox transcription factors are important in regulating differentiation [Jonsson et al., 1994; Harrison et al., 1995; Offield et al., 1996; Ahlgren et al., 1997; Naya et al., 1997; Sosa-Pineda et al., 1997; Sussel et al., 1998; Li et al., 1999; Sander et al., 2000; Olbrot et al., 2002; Artner et al., 2007]. Among them, importance of the paired homeobox family is also demonstrated. Pax4-deficient mice lack differentiated β-and δ-cells, and fail to develop mature islets [Sosa-Pineda et al., 1997]. Severe reduction of all endocrine cell types and disruption of the islet architecture are observed in mice lacking Pax6 [Sander et al., 1997; St-Onge et al., 1997]. These transcription factors do not only regulate development but also act to maintain mature β-cell functions [Murtaugh, 2006]. Determination of the factors and molecular signals that regulate these transcription factors is essential not only for understanding the mechanism of pancreatic development but also for establishing the cell based therapy for diabetes.

The importance of extracellular matrix (ECM) in regulating adhesion, migration, proliferation, differentiation and maintenance of mature cell function is well demonstrated. In the pancreas, roles of the basement membrane and its components for islet development and β-cell functions are extensively investigated. For instance, in embryo, attachment to the basement membrane of fetal aorta promotes induction of pancreatic progenitor cells from undifferentiated endoderm [Lammert et al., 2001; Yoshitomi and Zaret, 2004]. Recent study using knockout mice with islet specific ablation of vascular endothelial cells shows severe reduction of β-cells, due to the loss of cell attachment to the basement membrane [Nikolova et al., 2006]. Also, laminin-1, one of the major components of the

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basement membrane in fetal pancreas, induces differentiation of early pancreatic cells into insulin-positive cells in mice, whereas collagen type IV which is also an ECM component of the fetal pancreas has been shown to inhibit pancreatic development [Jiang et al., 1999].

In addition to the role of ECM, the importance of integrins are suggested. Integrins are heterodimeric glycoproteins composed of 18 α-subunits noncovalently linked to 8 β-subunits and provide a dynamic interaction of environmental cues and intracellular events by binding to their corresponding ECM [Hynes, 2002]. Of various integrins, the β1 integrin is important for islet development [Jiang et al., 2002; Wang et al., 2005; Yashpal et al., 2008]. Indeed, β1 integrin is broadly expressed in pancreatic epithelium, and in mice lacking β1 integrin, islet structure is disrupted [Kren et al., 2007].

In general, signals from ECM or integrin receptors control cell differentiation by altering the expression of transcriptional factors [Riquelme et al., 2001; Suzuki et al., 2003]. Therefore, the role of cell–matrix interaction on the regulatory network of transcription factors that control pancreatic development is postulated. However, information as to this link is limited. Yoshitomi and Zaret [2004] showed that attachment to aortic endothelial cells induces the expression of Ptf1a in dorsal pancreatic endoderm. They also showed that contact with the basement membrane was required for maintenance of Pdx1 expression in dorsal pancreas. This study demonstrates that signals from ECM modulate pancreatic development by induction of transcriptional factors, but the precise role of ECM or their receptor integrins in the regulation of transcriptional factors is unclear.

In the present study, we investigated the effect of the cell–matrix interaction on differentiation of pancreatic β-cells, in particular, its effect on regulation of transcription factors. By using AR42J cells, a model cell system to study the processes of endocrine differentiation [Mashima et al., 1996], we found that reconstituted basement membrane Matrigel promotes endocrine differentiation, at least in part, by inducing Pax6. In addition, we also found that some of these effects are dependent on laminin-1 and transmitted by the β1 integrin signaling.

MATERIALS AND METHODS

MATERIALS

Recombinant human activin A was provided by Dr. Y. Eto (Central Research Laboratory, Ajinomoto, Kawasaki, Japan). Recombinant human betacellulin (BTC) was prepared as described previously [Seno et al., 1996].

CELL CULTURE

AR42J cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) [Mashima et al., 1996]. Cells were incubated with a combination of activin A and BTC to induce differentiation. To evaluate the effect of anti-β1 integrin blocking antibody, cells were resuspended in DMEM containing 10% FBS and preincubated for 15 min with 10 μg/ml anti-β1 blocking antibody (Ha 2/5, BD Biosciences Japan, Tokyo, Japan) or purified hamster IgM before plating.

Rat pancreatic ductal cells were cultured as described previously [Ogata et al., 2004]. More than 95% of the cells were positive for cytokeratin, and cells positive for immunoreactive insulin were not observed before differentiation.

COATING OF DISHES AND SLIDES

Petri dishes (100 mm in diameter) were coated with 25 $\mu g/cm^2$ growth factor-reduced Matrigel, 5 $\mu g/cm^2$ human fibronectin, 10 $\mu g/cm^2$ laminin, 10 $\mu g/cm^2$ collagen type-IV. All extracellular matrices were purchased from BD Biosciences Japan. For morphological and immunohistochemical analyses, glass coverslips were coated with the same concentrations of matrices. In each experiment, 5% bovine serum albumin (BSA) (Sigma–Aldrich Japan, Tokyo, Japan) in phosphate-buffered saline (PBS) was used as a control.

PREPARATION OF RECOMBINANT ADENOVIRUSES

Recombinant adenovirus expressing Pax6 was prepared using a Virapower™ Adenoviral Gateway Expression kit (Invitrogen Japan, Tokyo, Japan). In brief, coding region of mouse Pax6 was digested from plasmid vector encoding mouse Pax6 (pBAT14-mPax6, kindly provided by Dr. Michel German, UCSF, San Francisco, CA) and cloned into an entry vector pENTR3C. To create expression clones and produce recombinant adenovirus, transfer of the mouse Pax6 cassette into the destination vector containing human CMV promoter (pAd/CMV/V5-DEST, Invitrogen Japan) by LR recombination reaction, linearization of plasmid with Pac I and transfection into Adenovirus expressing siRNA for rat Pax6 was constructed using the following oligonucleotide: 5′-CACCGGGTCTGTACCAACGACAATACGAATATTGTCGTTGGTACAGACCC-3′. This oligonucleotide was inserted in the pENTR/U6 entry vector (Invitrogen Japan). Subsequently, U6/shRNAs cassettes were transferred into the destination vector (pAd/BLOCK-iT-DEST, Invitrogen Japan) by LR recombination. Following linearization of plasmid, transfection into 293 cells was performed.

MORPHOLOGICAL ANALYSIS AND IMMUNOHISTOCHEMISTRY

AR42J cells cultured on BSA or matrix-coated glasses were fixed, treated with 0.1% Triton X-100, and then blocked with Blocking Ace solution (Morinaga, Tokyo, Japan). Primary antibodies used were mouse anti-swain insulin (1:100, Spring Bioscience, Freemont, CA), rabbit anti-rat Pax6 (Covance, Berkeley, CA), and rabbit anti-human glucagon (1:100, Dako, Glostrup, Denmark, CA). Secondary antibodies were as follows: biotinylated anti-mouse IgG and biotinylated anti-rabbit IgG (1:400, Vector Laboratories, Brulingam, CA). The fluorescent signals were detected by avidin-conjugated AlexaFluor488 (Molecular Probes, Inc., Eugene, OR). For double-staining, Alexa Fluor568-conjugated anti-mouse IgG and Alexa568-conjugated anti-rabbit IgG (1:600) were used as secondary antibodies.

RNA ISOLATION AND RT-PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen Japan). Total RNA samples were pretreated with DNase I (Nippon gene, Tokyo, Japan) to remove contamination of genomic DNA. First-strand cDNA was synthesized by the SuperScript™III first strand

synthesis system (Invitrogen Japan). Oligonucleotide-primers and PCR-reaction conditions were as previously mentioned [Zhang et al., 2001], except for rat insulin 1 (5′-TACAATCATAGACCATCAGCAAGC-3′ and 5′-CAGTTGGTAGAGGGAGCAGAT-3′ initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 30 s), rat insulin 2 (5′-AGCCCTAAGTGAACCAGCTACA-3′ and 5′-TGCCAAGGTCTGAAGGTCAC-3′, initial denaturation at 95°C for 5 min followed by 36 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 30 s), neurogenin-3 (5′-TTCGCACAGTTCCTTGCTGC-3′ and 5′-CGCAACACTGGATTAGGTCACTC-3′ initial denaturation at 95°C for 5 min followed by 32 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s), and MafA (5′-GATGAAGTTCGAGGTGAAGA-3′ and 5′-GCTCATCCAGTACAGATCCT-3′ initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s).

REAL-TIME PCR ANALYSIS

One microliter of the first-strand cDNA obtained as described above was used in a 20-μl reaction mixture including 1× SYBR greenER qPCR SuperMix (Invitrogen Japan) and 150 nM primer for rat insulin 2 primer, 200 nM primer for rat Pax6 (5′-CACCGCCCTCACCAACAC-3′ and 5′ GCAGG AGTACGAGGAGGTCTGA-3′), and 200 nM for rat GAPDH (5′-CATGACCACAGTCCATGCCATC-3′; anti-sense, 5′-CACCCTGTTGCTGTAGCCATATTC-3′). The initial cycling conditions involved a hold at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, then 60°C for 60 s. The signal fluorescence magnitude was detected with an ABI PRISM 7500 Sequence Detector System. Data were normalized to GAPDH signals and presented as mean ± SE.

IMMUNOBLOTTING

Cells were suspended in Laemmli buffer and heated to 100°C for 10 min. After centrifugation, the supernatant was collected, and protein concentration was measured by a BCA protein assay kit (Pierce, Rockford, IL). Cell lysate proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membrane (Millipore Japan, Tokyo, Japan). The membranes were washed in Tris buffered-saline containing 0.1% Tween-20 and blocked with 5% nonfat dry milk overnight at 4°C. Immunoblotting was performed with the phospho-p42/44 mitogen-activated protein kinase (MAPK), phopho-p38 MAPK, p42/44 MAPK, and p38 detected by chemiluminescence reagents (LumiGLO, Cell Signaling Technology Japan), and the chemiluminescence signals were detected by an image analyzer system (LAS-3000, Fujifilm, Tokyo, Japan).

ELISA FOR INSULIN

Whole cell extracts were obtained by treatment for 24 h on ice in acid–ethanol. The insulin content was determined using Insulin ELISA kit/Rat Ultra Sensitive using rodent insulin standard (Morinaga Biochemicals, Yokohama, Japan), and rat insulinoma INS-1 was used as a positive control. Insulin concentration was normalized with total cellular protein, measured using the BCA protein assay kit (Pierce).

STATISTICAL ANALYSIS

Values are expressed as mean ± SE. The statistical significance was determined using a two-tailed unpaired Student's *t*-test, and differences were considered to be statistically significant when $P < 0.05$.

RESULTS

EFFECT OF RECONSTITUTED BASEMENT MEMBRANE ON DIFFERENTIATION

Previous studies suggested the importance of the basement for pancreatic development [Jiang et al., 1999; Bonner-Weir et al., 2000; Lammert et al., 2001; Yoshitomi and Zaret, 2004; Nikolova et al., 2006]. We first investigated the role of reconstituted basement membrane, Matrigel, on cell differentiation in AR42J cells. We first characterized cell adhesion and morphology in cells cultured on Matrigel-coated or BSA-coated dish (control dish). As shown in Figure 1A-a, naive AR42J cells were round-shaped on the control dish, and time required for majority of the cells to attach to the dish was approximately 12 h. When cells were cultured on Matrigel-coated dishes, cells adhered quite rapidly, and majority of the cells adhered within 30 min (Fig. 1B). When attached, they rapidly flattened and presented spindle-shaped appearances (Fig. 1A-b) which were comparable to those observed in activin-treated cells as previously reported [Ohnishi et al., 1995]. These relatively flattened and spread-shaped appearances were also observed in cells cultured on Matrigel in the presence of BTC and activin (differentiated cells). In differentiated cells cultured on Matrigel, cell size was increased and relatively longer, and multiple processes compared to those cultured on control dish were observed (Fig. 1A-c,A-d). We next measured the expression of mRNA for pancreatic hormones. As previously described [Ohnishi et al., 1995], we confirmed the absence of pancreatic hormones in naive AR42J cell, whereas induction of insulin-2 and pancreatic polypeptide (PP) in differentiated cells (Fig. 1C). Although these expression patterns were unchanged even in cells cultured on Matrigel, we found a remarkable increase in mRNA for insulin-2 in cells cultured on Matrigel. Quantitative RT-PCR analysis demonstrated an approximately sevenfold higher expression of insulin-2 in cells cultured on Matrigel ($P < 0.05$, Fig. 1D). We next performed immunohistochemistry to examine distribution of insulin signals. As shown in Figure 1E, insulin signals were detected in the tips of cell processes in the majority the cells cultured on control dishes. In contrast, cells cultured on Matrigel showed a broadly cytoplasmic distribution of insulin signals. In addition, the signal intensities of insulin in cells cultured on Matrigel were stronger. On the other hand, insulin signals were not detected in naive cells. Consistent with the results obtained by immunohistochemistry, we found approximately twofold increase in insulin content in differentiated cells cultured on Matrigel ($P < 0.05$, Fig. 1F). We further measured the expression of β-cell-associated molecules including GLUT2, glucokinase, SUR1 and Kir6.2, and neuroendocrine markers including PGP9.5, synaptophysin and tyrosine hydroxylase. As reported previously, the expression of GLUT2, SUR1, and glucokinase (Fig. 1G) was induced by the treatment with activin and BTC. In addition, induction of the expression of mRNA for Kir6.2 and tyrosine

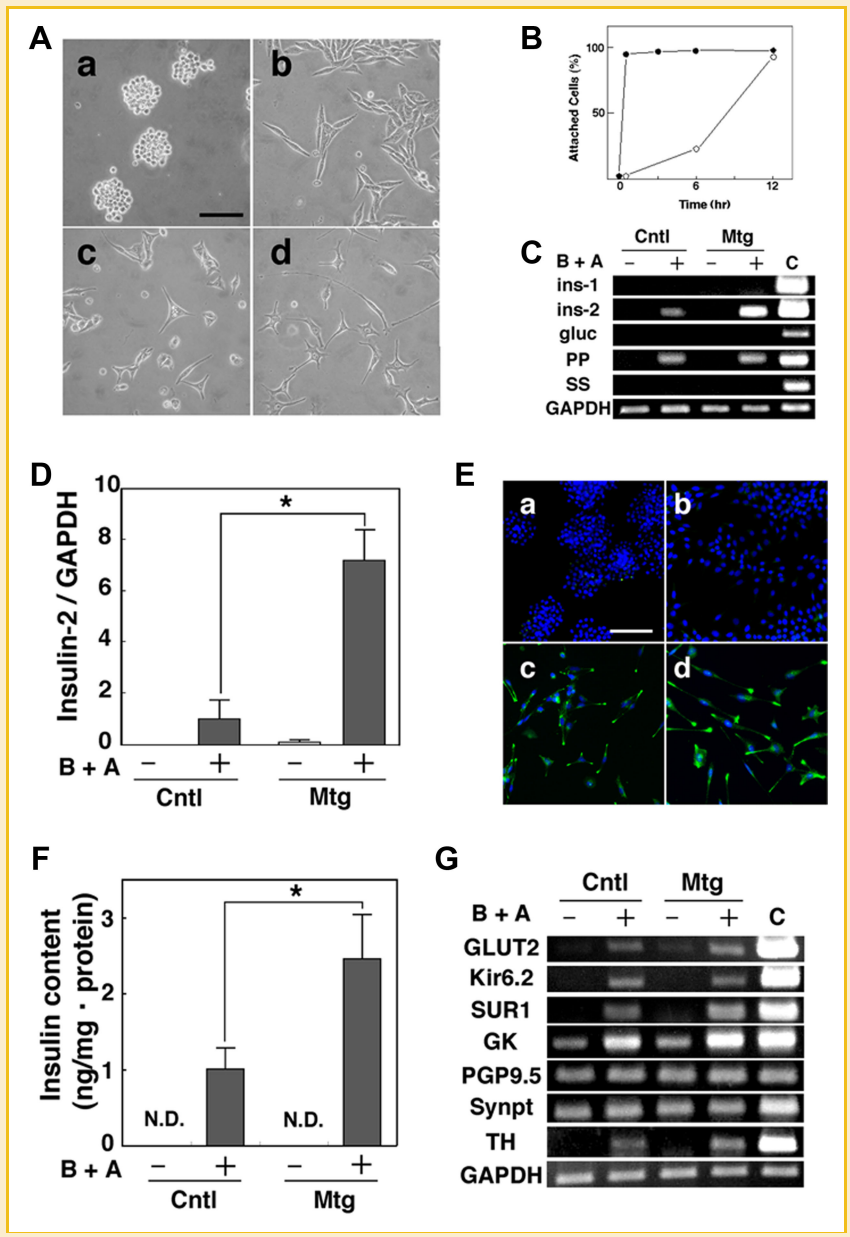

Fig. 1. Effect of reconstituted basement membrane on differentiation of AR42J cells. A: Cell Morphology. AR42J cells (1×10^5 cells/35 mm-dish) were plated on BSA (control matrix) (a,c)-coated and Matrigel (b,d)-coated dishes and incubated for 48 h with (c,d) or without (a,b) 1 nM BTC and 2 nM activin A. Phase contrast micrographs are presented. Bar = 100 μm. B: Time course of effect of matrigel on cell adhesion. Cell were plated on BSA- (○) and matrigel-coated (●) dishes for various periods and the number of attached cells was counted. Values are the mean of three experiments. C: Expression of mRNA for pancreatic hormones. Cells (6×10^5 cells/100 mm-dish) were incubated for 48 h with (+) or without (−) 1 nM BTC and 2 nM activin A (B + A) in control and Matrigel-(Mtg)-coated dishes. The expression of mRNA for insulin-1 (ins-1), insulin-2 (ins 2), glucagon (gluc), pancreatic polypeptide (PP), and somatostatin (SS) was analyzed by RT-PCR. GAPDH serves as a control. mRNA obtained from rat pancreas was used as a positive control (c). Cntl: control, Mtg: Matrigel. D: Quantification of mRNA for insulin-2. Cells were incubated as described in (A), and the expression of mRNA for insulin was quantified by real-time PCR. Results are the mean ± SE for three experiments. $^*P < 0.05$. Cntl: control, Mtg: Matrigel. E: Insulin-staining of differentiated cells. Cells were cultured on control dish (a,c) or Matrigel-coated (b,d) dishes and incubated for 48 h with (c,d) or without (a,b) 2 nM activin A and 1 nM BTC. Staining of insulin by immunofluorescence was obtained. A broad cytoplasmic staining pattern of insulin was observed in differentiated cells cultured on Matrigel (d). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). Bar = 100 μm. Cntl: control, Mtg: Matrigel. F: Changes in the insulin content. Cells were treated as described above, and changes in the insulin content and total cellular protein were measured. The normalized values are the mean ± SE for three independent experiments. ND, not detectable. $^*P < 0.05$. Cntl: control, Mtg: Matrigel. G: Changes in the expression of mRNA for islet-associated Molecules. Cells were cultured on control (Cntl) or Matrigel-coated (Mtg) dishes and incubated for 48 h with (+) or without (−) activin A and BTC. mRNA for GLUT2, Kir6.2, SUR1, and glukokinase (GK) were measured by RT-PCR. C: mRNA obtained from INS-1 cells.

hydroxylase was observed. These expression patterns were also observed in cells cultured on Matrigel, but the expression of mRNA for SUR1, glucokinase, and tyrosine hydroxyrase was increased. The expression of mRNA for PGP9.5 and synaptophysin was detected in cells cultured on control dishes and Matrigel, and there were no significant changes during the differentiation. These results suggest that Matrigel promotes differentiation in AR42J cells.

CHANGES IN THE EXPRESSION OF ISLET CELL ASSOCIATED TRANSCRIPTION FACTORS

To determine whether the effect of Matrigel on β-cell differentiation involves changes in the expression of transcriptional factors, we investigated mRNA for islet-associated transcription factors by RT-PCR. As shown in Figure 2A, we found that the expression of Pdx-1, Nkx2.1, Beta2, cdx2/3, cdx4, and lmx1.2 were detected in cells cultured on control dishes and Matrigel, and there were no significant changes during differentiation. The mRNA for Nkx6.1, Isl-1, HB9 and MafA was undetectable before and after differentiation in cells cultured on control dishes and Matrigel. The mRNA for lmx2, lmx1.1, Pax4, Hox1.11, and neurogenin3 was increased during differentiation in cells cultured on control dishes and Matrigel, and there were no significant differences. The mRNA for Pax6 was up-regulated during differentiation in cells cultured on control dishes and Matrigel. However, the expression level was higher in cells cultured on Matrigel. By quantitative RT-PCR, we found an approximately fivefold higher expression of Pax6 in cells cultured on Matrigel (Fig. 2B).

FUNCTIONAL ROLE OF PAX6

The above results indicate that Matrigel induces the expression of Pax6 during differentiation. Previous reports showed that Pax6 promoted transcription of insulin [Sander et al., 1997]. We speculated that Pax6 may be involved in the increase in the insulin expression. To assess the significance of Pax6, we first transfected an adenovirus vector encoding Pax6 into cells cultured on control dishes. To effectively identify transfected cells, we measured immunofluorescence using anti-Pax6 antibody. Some Pax6-transfected cells induced morphological changes, which included relatively longer neurite-like processes (Fig. 3A). In addition, these cells presented relatively intense signals of insulin with broadly cytoplasmic localization. Consistent with results obtained by immunohistochemistry, quantitative RT-PCR analysis demonstrated an approximately fivefold increase in the expression of mRNA for insulin-2 in Pax6-transfected cells ($P < 0.05$, Fig. 3B). However, the expression levels of insulin-2 in Pax6-transfected cells were lower than that of untransfected cells cultured on Matrigel ($P < 0.05$). Effect of combination of Matrigel and Ad-Pax6 was nearly identical to that of Matrigel alone. Furthermore, Pax6-transfected cells expressed glucagon and somatostatin by RT-PCR analysis (data not shown). To further investigate the functional role of Pax6, we also

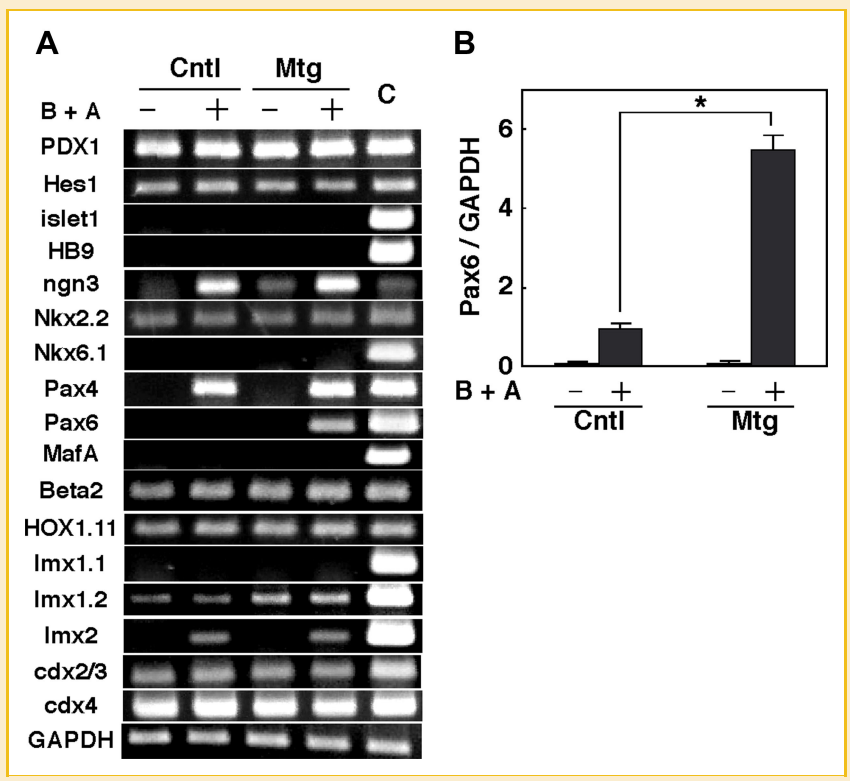

Fig. 2. Effect of matrigel on the expression of mRNA for islet-related transcription factors. A: Changes in mRNA for islet-related transcription factors. Cells were cultured on BSA- (Cntl) or matrigel-coated (Mtg) dishes and incubated for 48 h with (+) or without (−) 1 nM betacellulin and 2 nM activin A (B + A). mRNA from INS-1 cells was used as a positive control (c). B: Quantification of the expression of mRNA for Pax6. Cells were incubated as indicated in (A), and real-time RCR analysis of mRNA for Pax6 was performed. Results are the mean ± SE for four experiments. $^{*}P < 0.05$.

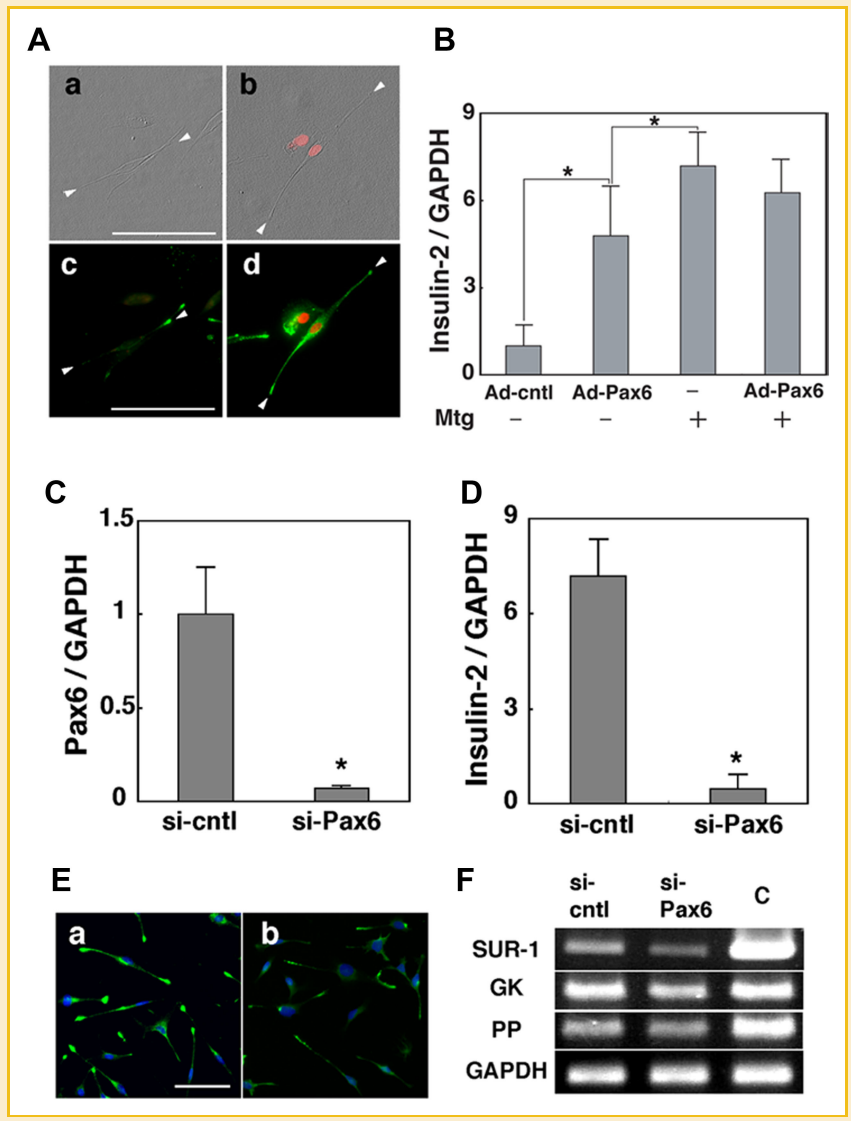

Fig. 3. Effects of overexpression and knockdown of Pax6 on differentiation. A: Morphology of cells overexpressing Pax6. Cells were infected with adenovirus encoding full-length cDNA for mouse Pax6 and incubated for 72 h with 1 nM BTC and 2 nM activin A in BSA-coated dishes. Upper panels (a,b) show morphology of Pax6-transfected cells. Effectively transfected cells were identified by Pax6-specific antibody (red), and the immunostaining of Pax6 was overlaid on Nomarski images (b). Adenovirus encoding lacZ gene was used as control (a). Lower panels (c,d) show overlaid images of Pax6 (red) and insulin (green), which are corresponding images of upper panels. Arrowheads indicated the tip of cell process in a single cell. Bar = 100 μm. B: Effect of overexpression of Pax6 on the expression of mRNA for insulin-2. The expression of mRNA for insulin-2 in Pax6-transfected (Ad-Pax6), LacZ-transfected control (Ad-cntl), non-transfected cells cultured on Matrigel (Mtg) and Pax6-transfected cells cultured on Matrigel was quantified by real-time PCR. Results were normalized to GAPDH. Values are the mean ± SE $^{*}P < 0.05$. C: Knockdown of pax6 mRNA using siRNA specific to rat Pax6. Cells cultured on matrigel and infected with adenovirus encoding specific siRNA for Pax6 (si-Pax6) and control vectors (si-cntl) were treated with 1 nM BTC and 2 nM activin A. The expression levels of endogenous pax6 mRNA were detected after incubation for 72 h by real-time PCR. Results were normalized to GAPDH and values are the mean ± SE $^{*}P < 0.05$. D: Effect of knocking down of Pax6 on the expression of mRNA for insulin-2. Cells were treated as in (C), and real-time RCR analysis was performed to quantify the amount of mRNA for insulin-2. Results were normalized to GAPDH and values are the mean ± SE $^{*}P < 0.05$. E: Effect of siPax on the expression of insulin. Cells transfected with siPax (b) or siLacZ (a) were cultured on Matrigel-coated dishes in the presence of activin A and BTC. Nuclei were stained with DAPI (blue color). Bar = 100 μm. F: Changes in the expression of mRNA for SUR1, glukokinase (GK), and pancreatic polypeptide (PP) in differentiated AR42J cells with or without transfection of si-Pax6.

prepared adenovirus encoding siRNA against Pax6 (Ad-siPax6). Knockdown of Pax6 was significant in Ad-siPax6-transfected cells. The levels of mRNA for Pax6 were reduced to 10% compared to those of the control vector ($P < 0.05$, Fig. 3C). Quantitative RT-PCR analysis showed that silencing of Pax6 significantly reduced the expression of insulin-2 ($P < 0.05$, Fig. 3D). mRNA for insulin-2 in Ad-siPax6-transfected cells was approximately 15% of that of the cells transfected with control vector. Additionally, we found a decrease in insulin immunoreactivities in Ad-siPax6-transfected cells (Fig. 3E). Furthermore, we observed a decrease in mRNA for SUR1 and glucokinase in Ad-siPax6-transfected cells, whereas the expression of mRNA for other hormones including PP, glucagons,

and somatostatin was unchanged (Fig. 3F). Note that we did not observe changes in the expression of other transcription factors (data not shown).

EFFECT OF PURIFIED ECM COMPONENTS ON DIFFERENTIATION

Matrigel contains several basement membrane ECMs including laminin-1, collagen type IV, entactin, fibronectin, and heparin sulfate proteoglycan, and former two are known as major components. To determine which components of the Matrigel regulated differentiation, we examined the effect of laminin-1 and collagen type IV. When the cells were cultured on laminin-1, similar morphological changes as seen in the cells cultured on Matrigel were observed in both undifferentiated (Fig. 4A-c) and differentiated condition (Fig. 4A-g). In addition to morphological changes, we found relatively high levels of mRNA for insulin-2 and Pax6 in differentiated cells cultured on laminin-1 compared to those in cells cultured on plastic dish ($P < 0.05$, Fig. 4B,C) although these effects of laminin-1 were less than those of Matrigel. Furthermore, we also detected relatively high levels and broadly cytoplasmic distributions of insulin immunoreactivities in the cells cultured on laminin-1 (Fig. 4D). On the other hand, obvious morphological changes and upregulation of mRNA for insulin-2 and Pax6 were not observed in the cells cultured on collagen type IV (Fig. 4A-d,A-h,B-D). In addition, we also examined the effect of

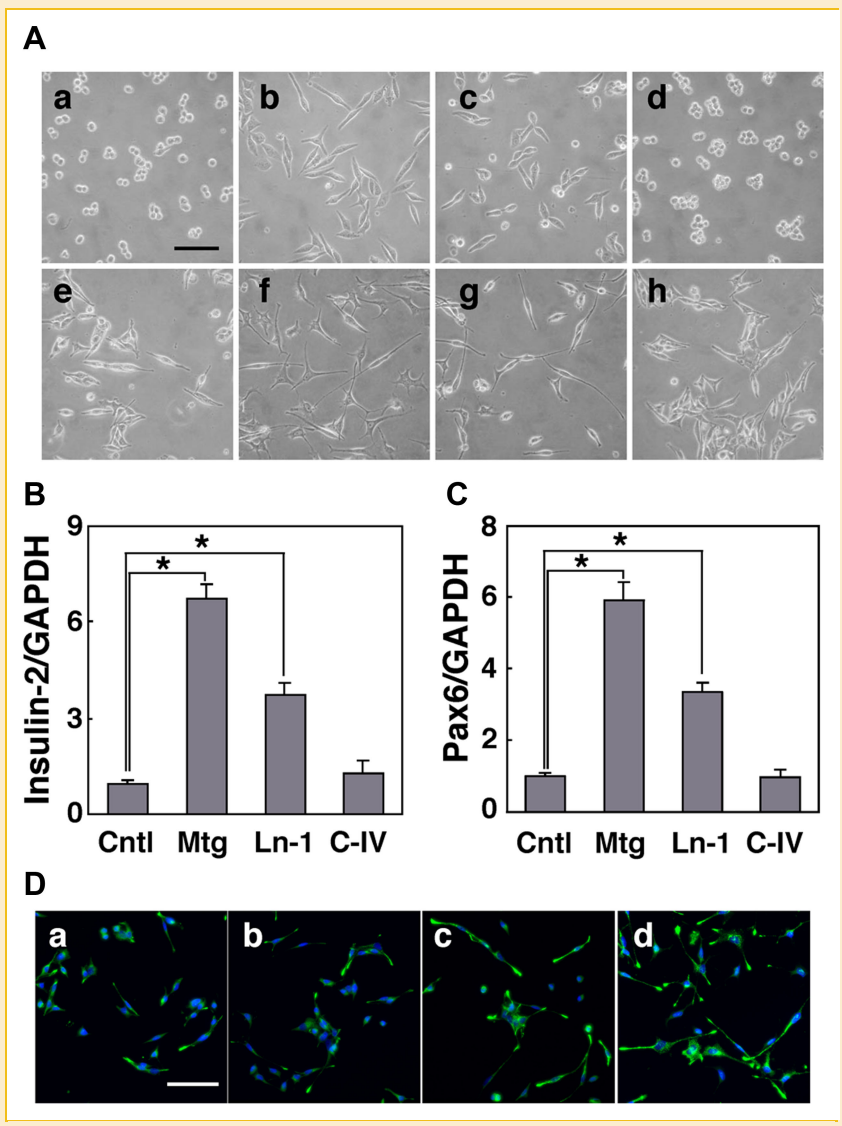

Fig. 4. Effect of purified basement membrane matrix on differentiation. A: Cell morphology. Cells were incubated for 48 h with (e,f,g,h) or without (a,b,c,d) 1 nM BTC and 2 nM activin A on control (a,e), Matrigel-(b,f), laminin-1 (c,g) and collagen type IV (d,h)-coated dishes. Phase-contrast images were presented. Bar = 100 μm. B,C: Quantification of mRNA for insulin-2 and Pax6 in differentiated cells. Cells were incubated for 48 h with 1 nM BTC and 2 nM activin A on control (Cntl), Matrigel (Mtg)-, laminin-1 (Ln-1)- and collagen type IV (C-IV)-coated dishes, and the expression of mRNA for insulin-2 (B) and Pax6 (C) were quantified by real-time PCR. Results are the mean ± SE for three experiments. $^{*}P < 0.05$. D: Insulin-staining of differentiated cells. Cells were cultured as described in (B), and stainings of insulin by immunofluorescence (green) were obtained. A broad cytoplasmic staining pattern of insulin was observed in differentiated cells cultured on Matrigel (d) and laminin-1 (c). a: Control dish. d: Collagen type IV-coated dish. Nuclei were stained with DAPI (blue). Bar = 100 μm.

fibronectin, one of minor component of Matrigel but no obvious changes were observed (data not shown). These data suggest that laminin-1 is the major component of Matrigel responsible for its positive effects on differentiation in AR42J cells although involvement of other signaling molecules in Matrigel is not excluded.

INHIBITORY EFFECT OF ANTI *B*-1 INTEGRIN ANTIBODY ON DIFFERENTIATION

To investigate the molecules that integrate the signals from ECM, we focused on the role of β1-integrin since it is a common integrin receptor isoform corresponding to laminins and is believed to be important for islet cell development [Jiang et al., 2002; Wang et al., 2005; Kren et al., 2007; Yashpal et al., 2008]. As shown in Figure 5A, we found that the expression of β1-integrin was detected in naive cells. Interestingly, the expression of β1-integrin was markedly increased in differentiated cells. The increases in β1-integrin expression in differentiated cells were observed in those cultured on Matrigel, and the expression levels between these cells were not different (data not shown). We then analyzed the effect of anti-β1-integrin antibody on the morphology of the cells cultured on Matrigel. In non-differentiated condition, anti-β1 integrin antibody inhibited morphological changes induced by Matrigel, and cells remained round-shaped (Fig. 5B-b). Cells treated with control antibody were not affected (Fig. 5B-a). In a differentiated condition, cells incubated with anti-β1-integrin antibody were less spread out with shorter processes (Fig. 5B-e,B-f). In contrast, cells cultured on control dish were not affected by anti-β1-integrin antibody in either non-differentiated and differentiated conditions (Fig. 5B-c,B-d,B-g,B-h). These inhibitory effects of anti-β1 integrin antibody were also observed in the cells cultured on laminin-1 (data not shown). By quantitative RT-PCR analysis, we also found a significant reduction of the expression of mRNA for insulin-2 and Pax6 in differentiated cells cultured on Matrigel and laminin-1 with anti-β1-integrin antibody (Fig. 5C,D). Conversely, no remarkable changes in insulin and Pax6 were observed in differentiated cells incubated with anti-β1-integrin antibody when the cells were cultured on control dish. Furthermore, we also confirmed decrease in the insulin signal in differentiated cells cultured on Matrigel treated with anti-β1-integrin (Fig. 5E-a,

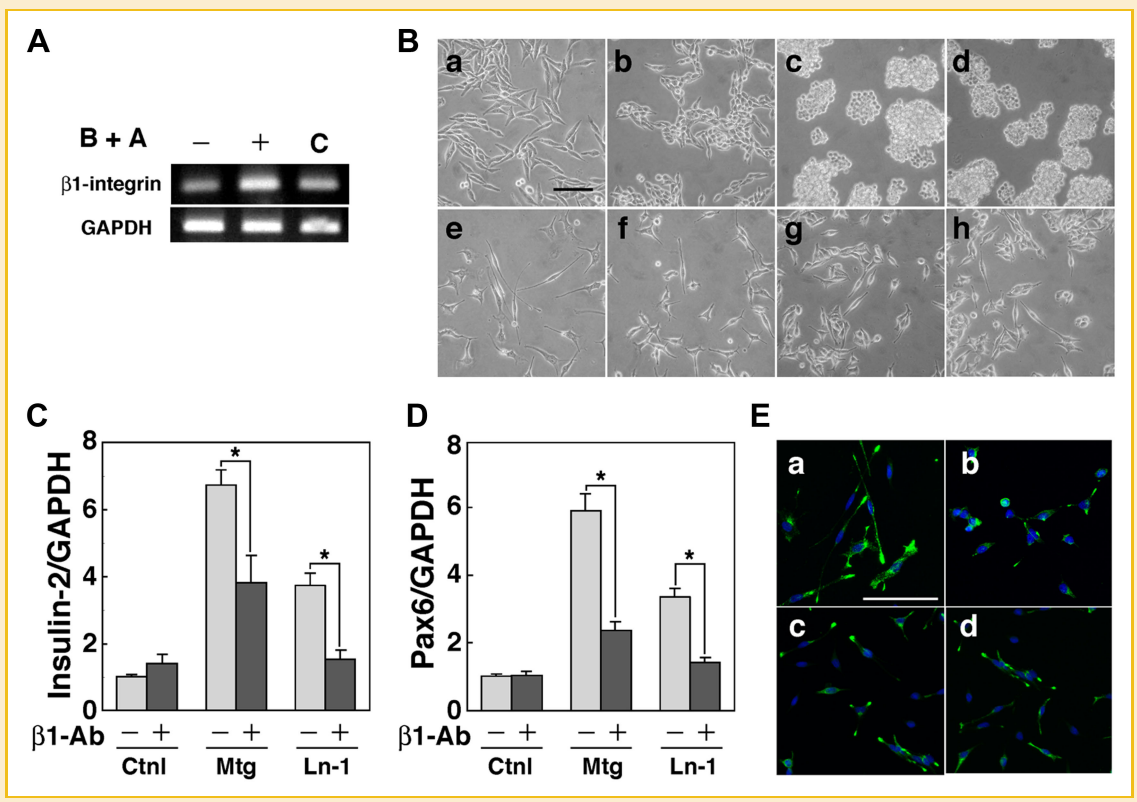

Fig. 5. Effect of anti-β1 integrin antibody on morphological appearance and endocrine differentiation on matrigel and laminin-1. A: Changes in the Expression of mRNA for β1 Integrin. Cells were incubated for 48 h with (+) or without (−) activin A and betacellulin (B + A) on BSA-coated dishes. mRNA for β1 integrin was measured by RT-PCR and mRNA from INS-1 was used as a control (C). B: Effect of Anti-β1 integrin antibody on morphology. Cells cultured on Matrigel (a,b,e,f) or BSA-coated (c,d,g,h) dishes were treated with anti-β1 integrin antibody (b,f,d,h) or control IgM (a,c,e,g). Phase contrast images were obtained 12 h after treatment with (e–h) or without (a–d) activin A and BTC. Bar = 100 μm. C,D: Quantification of mRNA for insulin-2 and Pax6. Cells cultured on Matrigel- (Mtg) and laminin-1 (Ln-1)-coated dishes were incubated for 48 h with activin A and BTC in the presence (+) or absence (−) of anti-β1 integrin antibody (β1-Ab). mRNA for insulin-2 (C) or Pax6 (D) was quantified by real-time PCR. Results were normalized to GAPDH and values are the mean ± SE of three independent experiments. $^{*}P < 0.05$. E: Immunofluorescence staining of insulin in differentiated cells. Cells were cultured on Matrigel- (a,b) or BSA-coated (c,d) (control) dishes in the presence of anti-β1 integrin antibody (b,d) or control IgM (a,c). They were then incubated with activin A and BTC for 48 h. Immunoreactive insulin is shown in green. Nuclei were stained with DAPI (blue). Bar = 100 μm.

E-b), whereas no remarkable change in insulin signal was observed in those cultured on plastic dish (Fig. 5E-c,E-d). The reduction of insulin signals were also observed in anti-β1 integrin antibody treated cells cultured on laminin-1 (data not shown). These results suggest that β1-integrins are involved in the stimulatory effect of Matrigel and laminin-1 on differentiation.

EFFECT OF BASEMENT MEMBRANE INVOLVES p38 MAP KINASE

Our previous studies demonstrated the involvement of p42/44MAPK and p38MAPK but not PI-3kinase and JNK in endocrine differentiation of AR42J cells [Furukawa et al., 1999; Kitamura et al., 2007]. Integrin exerts its cellular responses by regulating these intracellular signaling molecules [Mainiero et al., 2000; Bhowmic et al., 2001]. We therefore studied the effects of Matrigel on the phosphorylation of p38 and p42/44 MAP kinases during differentiation. As shown in Figure 6A, a relatively high amount of phosphorylated p38 was observed in cells cultured on Matrigel. In contrast, no remarkable differences in the amount of phosphorylated p42/44 MAP kinase were observed. Similar patterns of phosphorylations of p38 and p42/44 were also observed in the cells cultured on laminin-1 (data not shown). Additionally, when the cells were incubated with anti-β1 antibody, the increase in the amount of phosphorylated p38 MAP kinase induced by Matrigel was reduced (Fig. 6B). Also, addition of SB203580 attenuated Matrigel-induced activation of p38 MAP kinase (Fig. 6C) and completely blocked the expression of insulin.

EFFECT OF ECMS ON DIFFERENTIATION OF PANCREATIC DUCTAL CELLS

Finally, we addressed whether basement membrane affects differentiation of pancreatic ductal cells to insulin-producing cells in vitro. Ductal cells were cultured on Matrigel and treated with activin and BTC. Cells were incubated for 7 days and insulin content was measured. As depicted in Figure 7A, the expression of mRNA for insulin in cells cultured in Matrigel-coated dishes was markedly higher than those cultured on control dish. Similarly, the insulin content was increased in cells cultured on Matrigel-coated dishes (Fig. 7B). As shown in Figure 7C, the expression of Pax6 was upregulated in cells cultured on Matrigel-coated dishes. The increase in the insulin content was inhibited by anti-β1-integrin antibody (data not shown).

DISCUSSION

We showed that appropriate culture system promoted β-cell differentiation in both pancreatic progenitor-like cell lines and

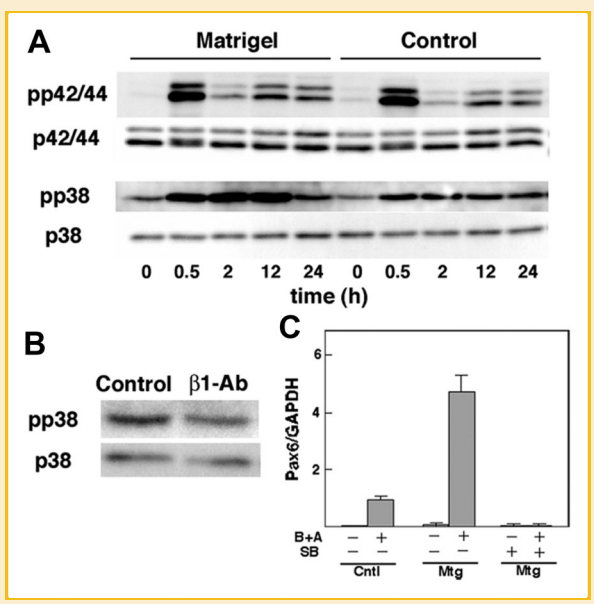

Fig. 6. Effect of matrigel on the activation of mitogen-activated kinases. A: Analysis of phosphorylation of p42/44 MAPK and p38 MAPK during differentiation. Cells cultured on matrigel or BSA (Control) were incubated for indicated time with a combination of activin A and BTC, and phosphorylation of p42/44 and p38 MAP kinases was analyzed by immnoblotting. B: Effect of anti-β1 integrin antibody on phosphorylation of p38 MAPK. Cells were preincubated with or without anti-β1 integrin antibody (β1 Ab) for 15 min and cultured on Matrigel in the presence of BTC and activin A. The cells were harvested 2 h after the treatment with BTC and activin A and Western blot analysis for p38 was performed. Typical images among the three independent analyses are shown. C: Effect of SB203580 on the expression of Pax6. Cells cultured on BSA-coated (Cntl) or Matrigel-coated (Mtrg) dishes were stimulated by activin A and BTC in the presence and absence of 10 μM SB203580 and the expression of Pax6 was measured by quantitative PCR.

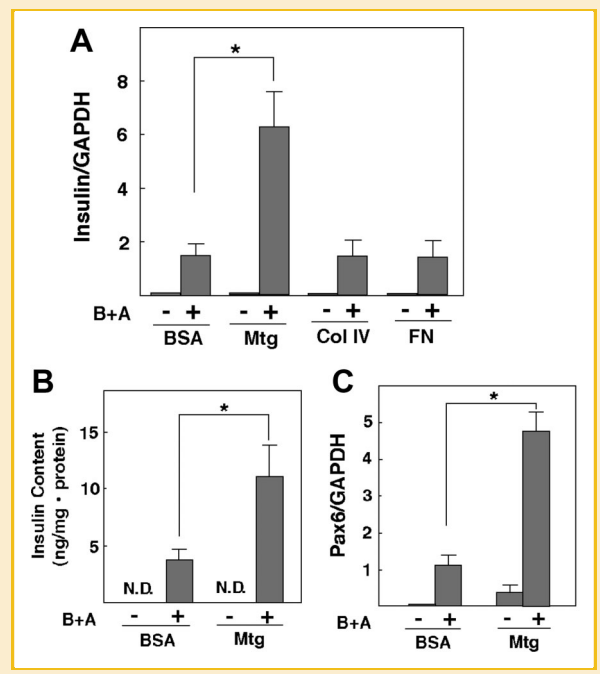

Fig. 7. Effect of ECM on differentiation of ductal cells. A: Expression of mRNA for insulin. Ductal cells were incubated for 7 days with 1 nM BTC and 2 nM activin A (B + A) in control-, Matrigel- (Mtg), collagen type IV- (col IV) and fibronectin-coated (FN) dishes. The expression of insulin was analyzed by real-time PCR. Results are shown as the mean ± SD for four experiments; $^{*}P < 0.05$. B: Changes in the insulin content. Ductal cells were incubated as indicated above and the insulin content was measured. Results are the mean ± SD for four experiments; $^{*}P < 0.05$. C: Changes in the expression of Pax6. Ductal cells were incubated with activin A and BTC for 48 h and the expression of Pax6 was measured by real-time PCR. Results are shown as the mean ± SD for four experiments; $^{*}P < 0.05$.

cultured duct epithelial cells. We found that Matrigel exerted the effect on β-cell differentiation through induction of Pax6, and the effect was reproduced at least partly by an individual ECM component, laminin-1. Additionally, using the blocking antibody we demonstrated that these favorable effects of basement membrane on β-cell differentiation were exerted through the interaction with β-1 integrin. Our data suggest that the basement membrane plays a crucial role in the regulation of β-cell differentiation and appropriate expression of transcriptional factors.

Several studies suggested the importance of the basement membrane in β-cell development [Lammert et al., 2001; Yoshitomi and Zaret, 2004]. In addition, the effectiveness of Matrigel in adult pancreatic duct cells and fetal pancreatic cells is reported [Jiang et al., 1999; Bonner-Weir et al., 2000]. These approaches using pancreatic explant may reflect physiological conditions, but it is unfavorable to study the molecular signalings due to heterogeneous cell population. In the present study, we used a simple model cell line reproducing β-cell differentiation. In agreement with previous studies, we demonstrated that reconstitute basement membrane, Matrigel was an effective substrate for differentiation in AR42J cells. Matrigel induced morphological changes and enhancement of the insulin expression during differentiation. Interestingly, we found the morphological changes comparable to those observed in activin-treated cells even in Matrigel-exposed cells without differentiation factors. Although Matrigel-exposed cells did not convert to endocrine cells expressing insulin or PP, these cells showed decrease in amylase expression in parallel with the increase in cytokeratin-20, a marker of pancreatic ductal cells (data not shown). Thus, Matrigel by itself may act on the initial step of differentiation although signals of differentiation factors are still required to convert to endocrine cells. This notion is supported by the concept that pancreatic progenitor cells in vivo are derived from ductal epithelium [Bonner-Weir and Weir, 2005]. In addition to enhancement of the insulin expression, Matrigel-exposed cells express more mature functions. This was evident by the following findings: upregulation of the expression of β-cell markers including SUR1, glucokinase and Pax6 [Sander et al., 1997; St-Onge et al., 1997; Ashery-Padan et al., 2001]; and intense and broadly cytoplasmic accumulation of insulin immunoreactivities. These results strongly suggest that the basement membrane component promotes differentiation. Our findings obtained in AR42J cells are advantageous in several respects compared to those of the previous data obtained in vivo and in vitro. In particular, since AR42J is a clonal cell line, they provide a good experimental system to study the regulation of differentiation and to identity the genes involved [Mashima et al., 1999; Kren et al., 2007]. Indeed, using this model system, we demonstrated the contribution of Pax6 (see below). Note that AR42J is slightly different from progenitors observed in vivo. For example, differentiated AR42J cells express low amount of insulin and lack some transcription factors including islet-1, Nkx6.1 and Maf A. Nonetheless, because we observed similar responsiveness to the basement membrane in ductal cells, AR42J is a useful model for investigating cellular signaling during β-cell differentiation.

Using this cell model, we addressed the intracellular signaling molecules involved in the basement membrane-induced enhancement of differentiation, in particular, the contribution of transcription factors. Of various transcription factors investigated, upregulation of Pax6 was demonstrated. This was observed only when cells were treated with differentiation factors and Matrigel, suggesting that cooperative signalings from extracellular matrix and growth factors are required for its expression. Interestingly, severe reduction of Pax6 expression in Xenopus embryonic endoderm is observed when the embryo is dissociated from the basement membrane of dorsal aorta [Lammert et al., 2001]. These findings strongly indicate that signals from the basement membrane are required for appropriate expression of transcription factor. We demonstrated the role of Pax6 in two ways: by a gain of function study and by a knockdown study. The gain of function study of Pax6 demonstrated up-regulation of insulin both in mRNA and protein levels. Overexpression of Pax6 also induced morphological changes comparable to these observed in Matrigel-exposed cells. However, Pax6-overexpressing cells also produced other hormones including glucagon and somatostatin. These findings indicate that induction of Pax6 reproduces differentiation-inducing activity of Matrigel, but the effect was not completely the same. The induction of glucagon and somatostatin in Pax6-overexpressing cells is not surprising since Pax6 also regulates transcription of these hormones as well as insulin [Sander et al., 1997; Cissell et al., 2003]. Unphysiologically excessive amounts of Pax6 may affect transcriptional regulation of these hormones. Consistent with this notion, we found a marked reduction of Pax4 and Nkx2.2 in Pax6-overexpressing cells (data not shown). Additionally, transgenic mice overexpressing Pax6 in islets exhibit glucagon and insulin double-positive cells and disruption of normal islet structures in the pancreas [Yamaoka et al., 2000]. In contrast to the results of the gain of function experiment, knockdown of Pax6 clearly attenuated upregulation of insulin and other β-cell markers. These effects were due to specific reduction of Pax6 since the expressions of other transcriptional factors were not changed. Taken together, Pax6 plays a key role in Matrigel-mediated differentiation in this model system. Pax6 is known to have a pleiotropic role in the development of the endocrine pancreas, and it is also thought to play an important role in α-, β-, δ-, and PP-cells [Sander et al., 1997; St-Onge et al., 1997]. With regard to the β-cell functions, Pax6 directly and indirectly regulates the expression of mature β-cell markers such as insulin, GLUT2, glucokinase, and IAPP [Sander et al., 1997; Ashery-Padan et al., 2001; Cissell et al., 2003]. However, Pax6 knockout mice do not show complete loss of β-cells [Sander et al., 1997; Murtaugh, 2006]. It is therefore considered to regulate maturation of β-cells rather than determination of the cell fate. The results of the present study are consistent with those aspects of Pax6. Indeed, AR42J cells could differentiate into insulin-producing cells independent of the presence of Pax6 [Zhang et al., 2001]. Collectively, the functions of Pax6 in basement membrane-mediated differentiation may be mainly due to promotion of maturation rather than determination of the cell fate. It is unclear which signals induce the expression of Pax6 and how Pax6 promotes β-cell differentiation.

We have also found that integrin-activated signal transduction pathways are responsible for the regulation of differentiation induced the by the basement membrane. Specific antibody against

β1-intengrin abrogated upregulation of insulin and Pax6 induced by Matrigel and laminin-1. Particularly, the effect of laminin-1 is almost completely abolished by the treatment with blocking antibody. Note that anti-β1 integrin blocking antibody did not affect differentiation in cells cultured on control dish. This indicates that differentiation of AR42J cells itself occurred independently of β1-integrin signaling. Alternatively, it also possible that AR 42J cells do not produce effective ECMs binding to β1 integrin enough to promote differentiation. Consistent with this hypothesis, we confirmed only low levels of laminin expression in AR42J cells (data not shown). These lines of evidence support the importance of exogenous supplementation of basement membrane for appropriate β-cell differentiation in vitro. Our data agree with the previous study showing that reduction of insulin expression in neonatal islet cells in which β1-integrin interaction is abrogated by gene silencing and the treatment with the blocking antibody [Yashpal et al., 2008]. Our results also extend by showing the role of β1 integrin in regulating the expression of transcription factors.

We also demonstrated the involvement of p38 during differentiation. We found relatively high amounts of phosphorylated p38 in cells cultured on Matrigel and they were abrogated by blocking antibody against β1-integrin (Fig. 6A,B). This indicates that p38 is in downstream of the β1-integrin signaling. These crosstalks of growth factor and β1-integrin signaling in modulating stress kinases are demonstrated by several studies [Mainiero et al., 2000; Bhowmic et al., 2001]. Given that overexpression of transforming growth factor-β-activated kinase 1, an upstream regulator of p38, promotes endocrine differentiation [Ogihara et al., 2003], we postulate that p38 activation is involved in basement membrane-induced differentiation. Interestingly, activation of p38 enhances transcriptional activities of Pax6 through the phosphorylation of its transactivation domain [Mikkola et al., 1999]. Also, β1-integrin-dependent induction of Pax6 during lens development is demonstrated in mice with lens-specific ablation of β1-integrin [Simirskii et al., 2007]. These molecules may modify the function of Pax6, which contributes to promotion of β-cell differentiation induced by the basement membrane. Further studies are needed to clarify the downstream signals of integrin receptor that control regulatory network of the transcriptional factors.

Our findings provide a new insight into the understanding of the role of ECMs in β-cell differentiation. Several lines of evidence indicate that signals from ECM contribute to improved β-cell functions including insulin secretion, glucose responsiveness, and survival [Wang et al., 2005; Parnaud et al., 2006; Pinkse et al., 2006]. However, most of these studies focus on the contribution of ECM to the maintenance of cell functions in isolated β-cells. Our data demonstrate an additional role of the basement membrane as a modulator of β-cell differentiation, and Pax6 is involved in this action. These findings may be helpful to establish cell replacement therapy for diabetes using precursor cells. For instance, when the pancreatic precursor cells are used for the source of β-cell transplantation, the use of basement membrane may promote differentiation to β-cells. Our approach using natural agents is safer than virus-based methods which are well-used to obtain insulin-producing cells. Positive roles of ECMs in β-cell differentiation have been reported in ES cells [Schroeder et al., 2006].

In summary, basement membrane promoted differentiation of AR42J and ductal cells into insulin-producing cells. This effect may be exerted through upregulation of Pax6. These findings provide a new insight into our understanding of the mechanisms for ECM-mediated β-cell differentiation.

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