

# Upregulation of Connexin43 Expression in Corneal Fibroblasts by Corneal Epithelial Cells

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**PURPOSE.** The authors have previously shown that the expression of tight-junction proteins in corneal epithelial cells is regulated by corneal fibroblasts in a coculture system. They have now examined the effect of corneal epithelial cells on the expression of the gap-junction protein connexin43 in corneal fibroblasts.

**METHODS.** Human corneal fibroblasts and simian virus 40-transformed human corneal epithelial (HCE) cells were cultured on opposite sides of a collagen vitrigel membrane. Expression of junctional proteins in corneal fibroblasts was examined by reverse transcription-polymerase chain reaction, immunoblot, and immunofluorescence analyses. The effect of a small interfering RNA specific for insulin-like growth factor-1 (IGF-1) mRNA on connexin43 expression was determined by transfection.

**RESULTS.** The amounts of connexin43 mRNA and protein in corneal fibroblasts were increased by the presence of HCE cells. HCE cells had no effect on the expression of the tight-junction proteins ZO-1, occludin, and claudin in corneal fibroblasts. The effect of HCE cells on connexin43 expression was mimicked by exposure of corneal fibroblasts to IGF-1. Furthermore, depletion of IGF-1 in HCE cells by RNA interference largely abolished the effect of these cells on connexin43 expression in corneal fibroblasts. Finally, the upregulation of connexin43 expression in corneal fibroblasts by HCE cells was blocked by inhibitors of signaling by the mitogen-activated protein kinases ERK (PD98059) and p38 MAPK (SB203580).

**CONCLUSIONS.** The presence of corneal epithelial cells upregulated the expression of connexin43 in corneal fibroblasts, suggesting that corneal epithelial cells are important for the maintenance of gap junction-mediated communication among corneal fibroblasts. (*Invest Ophthalmol Vis Sci.* 2009;50:2054–2060) DOI:10.1167/iovs.08-2418

Keratoocytes, or corneal fibroblasts, are the principal cellular component of the corneal stroma, and they actively synthesize and secrete collagen, proteoglycans, and other proteins of the extracellular matrix. These cells thus play an important role in the maintenance of metabolic and physiological homeostasis of the cornea.<sup>1,2</sup> Although they are dispersed

throughout the entire stroma, corneal fibroblasts occupy only 2% to 3% of the total stromal volume.<sup>3</sup> With the use of transmission and scanning electron microscopy, we previously showed that corneal fibroblasts are connected to each other by gap junctions and thereby form a three-dimensional cellular network.<sup>4,5</sup> Gap junctions mediate the intercellular diffusion of ions or molecules smaller than 1 kDa, thereby contributing to the regulation of tissue differentiation and homeostasis.<sup>6</sup> A dye-coupling technique has revealed gap junction-mediated intercellular communication to be operative among corneal fibroblasts in normal and wounded rabbit cornea and in the human cornea *ex vivo*.<sup>7</sup> It is also operative among rabbit corneal fibroblasts or myofibroblasts *in vitro*.<sup>8</sup> Gap junctions are formed by a pair of connexins, each containing six connexin molecules, that are situated in apposing membranes of the connected cells. At least 18 mammalian members of the connexin protein family have been identified by cDNA cloning, and different cell types express different combinations of these connexins.<sup>9</sup> Gap junctions in corneal fibroblasts have been shown to contain connexin43 (Cx43) but not Cx26 or Cx32.<sup>8,10</sup>

In the clinical setting, patients are often encountered in whom loss of the corneal epithelium or delay in the resurfacing of corneal epithelial defects leads to the activation of underlying fibroblasts in the stroma. These clinical observations suggest that epithelial-mesenchymal interaction is important for maintenance of the normal structure and functions of the cornea. We recently established an experimental model in which corneal epithelial cells and fibroblasts are cultured on opposite sides of a collagen (vitrigel) membrane.<sup>11</sup> With this model, we showed that the presence of corneal fibroblasts upregulated the expression of the tight-junction proteins ZO-1, occludin, and claudin in corneal epithelial cells, suggesting that corneal fibroblasts play an important role in the differentiation of corneal epithelial cells.

With regard to the mediators of such functional interactions between epithelial cells and fibroblasts in the cornea, three patterns of expression of the genes for various cytokines, growth factors, and their receptors have been identified in corneal epithelial cells and fibroblasts.<sup>12</sup> The genes for insulin-like growth factor-1 (IGF-1) and its receptor, for example, were found to be expressed in epithelial cells and fibroblasts. IGF-1 has been shown to increase Cx43 expression and gap junction-mediated intercellular communication in astrocytes<sup>13</sup> and vascular smooth muscle cells,<sup>14</sup> suggesting that it might also regulate Cx43 expression in the cornea.

To evaluate further the physiological relevance of interactions between corneal epithelial cells and fibroblasts and to investigate the possible participation of IGF-1 in epithelial-mesenchymal interaction in the cornea, we have now examined the effect of the presence of corneal epithelial cells on Cx43 expression in corneal fibroblasts at both the mRNA and the protein levels in our coculture system. Our results suggest that gap junction-mediated intercellular communication among corneal fibroblasts is promoted by corneal epithelial cells and that this effect might be mediated, at least in part, through IGF-1 released from the epithelial cells.

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## METHODS

### Antibodies and Reagents

Rabbit polyclonal antibodies to ZO-1, occludin, or claudin were obtained from Zymed (Carlsbad, CA), and those to Cx43 were from Chemicon (Billerica, MA). Goat polyclonal antibodies to IGF-1 were obtained from R&D Systems (Minneapolis, MN), and mouse monoclonal antibodies to  $\alpha$ -tubulin were from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated secondary antibodies were from Promega (Madison, WI), and AlexaFluor 488-conjugated secondary antibodies were from Molecular Probes (Carlsbad, CA). Recombinant human forms of epidermal growth factor (EGF) and fibroblast growth factor (FGF) were obtained from R&D Systems, and recombinant human IGF-1 was from Clontech (Mountain View, CA). A small interfering RNA (siRNA) specific for human IGF-1 mRNA and transfection reagent (siPORT *NeoFX*) were obtained from Ambion (Austin, TX). PD98059 and SB203580 were from Calbiochem (San Diego, CA).

### Culture of Human Corneal Fibroblasts

Human corneal fibroblasts were prepared from the tissue remaining after corneal transplantation surgery and were cultured as described previously.<sup>15</sup> In brief, human corneas were obtained from Mid-America Transplant Service (St. Louis, MO), Northwest Lions Eye Bank (Seattle, WA), or the Eye Bank of Wisconsin (Madison, WI). Donors were white males and females ranging in age from 4 to 65 years. The cells prepared from each cornea were maintained separately in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). They were passaged after they had achieved approximately 90% confluence. Cells in the third to seventh passages were used for the experiments described in the present study. The purity of the cell cultures was assessed on the basis of the distinctive morphology of human corneal fibroblasts and their reactivity with antibodies to vimentin in immunofluorescence analysis. All cells were positive for vimentin and negative for cytokeratin, suggesting the absence of contamination by epithelial cells. Human tissue was used in strict accordance with the Declaration of Helsinki.

### HCE Cell Culture

Simian virus 40-transformed human corneal epithelial (HCE) cells, originally established and characterized by Araki-Sasaki et al.,<sup>16</sup> were obtained from RIKEN Biosource Center (Tsukuba, Japan). The cells were maintained in supplemented hormonal epithelial medium (SHEM), which comprises DMEM-F12 (50:50, vol/vol) supplemented with 15% heat-inactivated FBS, bovine insulin (5  $\mu$ g/mL), cholera toxin (0.1  $\mu$ g/mL), recombinant human EGF (10 ng/mL), and gentamicin (40  $\mu$ g/mL).

### Coculture of HCE Cells and Corneal Fibroblasts on a Collagen Membrane

Corneal fibroblasts ( $1 \times 10^5$ ) were seeded on a collagen vitrigel membrane (Asahi Technoglass, Tokyo, Japan) in DMEM supplemented with 10% heat-inactivated FBS. After 24 hours, the membrane was turned upside down in another dish, and HCE cells ( $2 \times 10^5$ ) were seeded on the empty side of the membrane, also in DMEM supplemented with 10% heat-inactivated FBS. After 6 to 8 hours, the membrane was again turned upside down in another dish containing DMEM supplemented with 0.5% heat-inactivated FBS. Cells were then cultured at 37°C in a humidified incubator containing 5% CO<sub>2</sub> and 95% air. As a control, corneal fibroblasts were seeded on a vitrigel membrane without HCE cells.

### RT-PCR Analysis

Total RNA was isolated from corneal fibroblasts on a collagen vitrigel membrane with the use of a purification kit (RNeasy; Qiagen, Valencia, CA), and portions (0.5  $\mu$ g) of the RNA were subjected to reverse transcription (RT) and polymerase chain reaction (PCR) analysis with

an RT-PCR kit (One-Step; Invitrogen, Carlsbad, CA) based on the *Taq* system (Platinum; Invitrogen). The PCR protocol was designed to maintain amplification in the exponential phase. Sequences of the PCR primers (sense and antisense, respectively) were 5'-GGCGTTAAG-GATCGGGTTAA-3' and 5'-CGACGACCTGTACTTAGTGTGCGT-3' for Cx43, 5'-TGCCATTACACGGTCTCTG-3' and 5'-GGTTCTGCCTCAT-CATTCCTC-3' for ZO-1, 5'-AGTGTGATAATAGTGAGTGCTATCC-3' and 5'-TGTACATCTGTCATCTTCTC-3' for occludin, 5'-TTCTCG-CCTTCCTGGGATG-3' and 5'-CTTGAACGATTCTATTGCCATACC-3' for claudin, and 5'-ACCACAGTCCACGCCATCAC-3' and 5'-TCCAC-CACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (G3PDH, internal control). RT and PCR incubations were performed with a PCR system (GeneAmp PCR System 2400-R; Perkin-Elmer, Foster, CA). RT was performed at 50°C for 30 minutes, and PCR was performed for 25 cycles, with each cycle consisting of incubations at 94°C for 2 minutes, 58°C for 30 seconds, and 72°C for 1 minute. The reaction mixture was then cooled to 4°C. Amplification products were fractionated by electrophoresis on a 1.5% agarose gel and were stained with ethidium bromide. For RT and real-time PCR analysis, total RNA was subjected to RT with a kit (Promega), and the resultant cDNA was subjected to real-time PCR analysis by rapid cycling in glass capillaries with the use of a thermocycler (Light-Cycler; Roche Molecular Biochemicals, Indianapolis, IN).

### Immunoblot Analysis

Corneal fibroblasts on a collagen vitrigel membrane were washed twice with phosphate-buffered saline (PBS) and lysed in 200  $\mu$ L of a solution containing 150 mM NaCl, 2% SDS, 5 mM EDTA, and 20 mM Tris-HCl (pH 7.5). Cell lysates were fractionated by SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane and exposed consecutively to primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immune complexes were detected with enhanced chemiluminescence reagents (GE Healthcare UK, Little Chalfont, UK). Band intensities in the linear range were quantitated by densitometric scanning of film with the use of image analyzer software (Multi Gauge V3 [2]; Fuji Film, Tokyo, Japan).

### Immunofluorescence Analysis

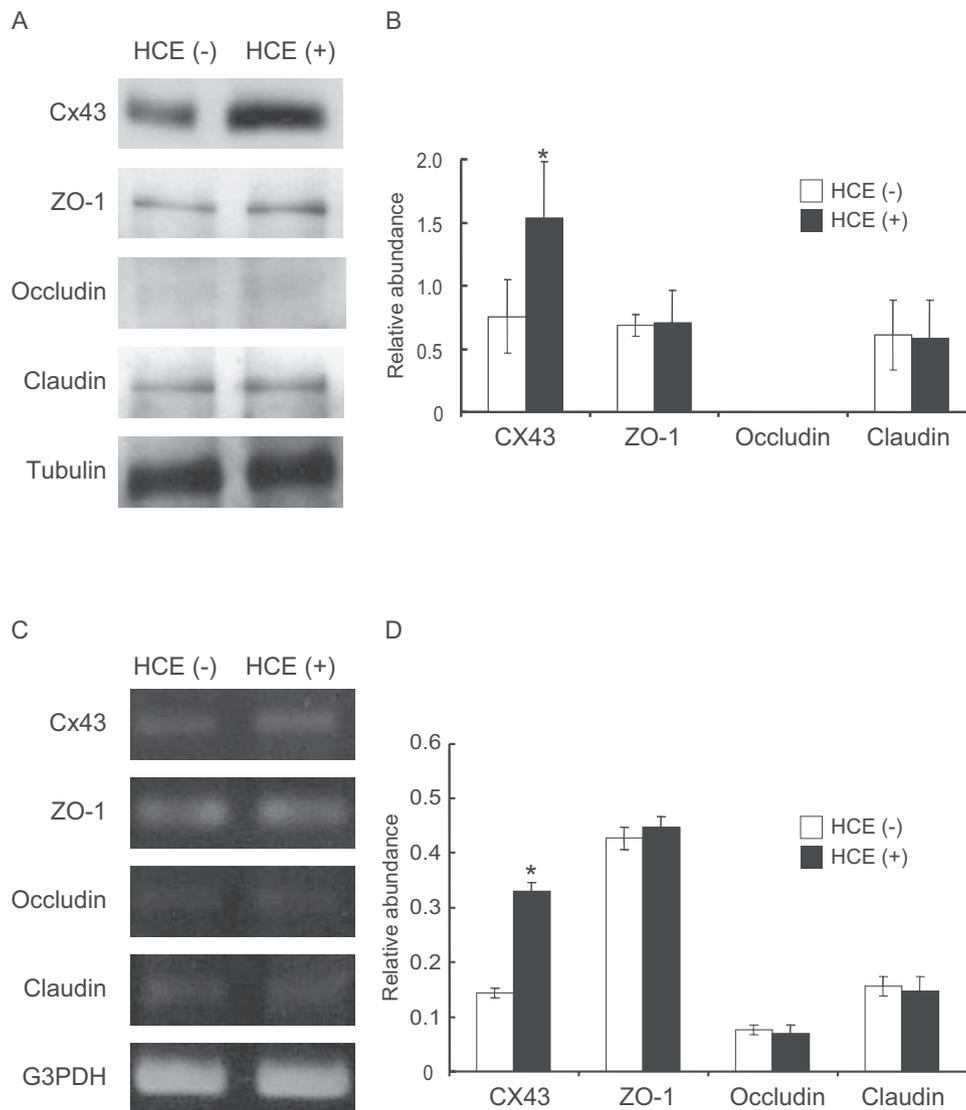
Corneal fibroblasts cultured on a collagen vitrigel membrane were fixed for 15 minutes at room temperature with 3.7% formalin, washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS (PBS-), permeabilized for 5 minutes with 0.1% Triton X-100 in PBS(-), and incubated for 1 hour at room temperature with 1% bovine serum albumin (BSA) in PBS(-). They were then incubated for 1 hour with antibodies to Cx43 at a dilution of 1:100 in PBS(-) containing 1% BSA, washed with PBS(-), and incubated for 1 hour with AlexaFluor 488-conjugated secondary antibodies (1:1000 dilution) in PBS(-) containing 1% BSA. The cells were then examined with a laser confocal microscope (LSM5; Carl Zeiss, Jena, Germany).

### Transfection of siRNA

HCE cells ( $2 \times 10^5$ ) were seeded in 30-mm dishes and cultured for 24 hours to 50% to 60% confluence. siRNA specific for human IGF-1 mRNA (1  $\mu$ M) was mixed with 5  $\mu$ L transfection reagent (siPORT *NeoFX*; Ambion) and diluted with 200  $\mu$ L medium (Opti-MEM; Invitrogen-Gibco, Carlsbad, CA). After incubation for 10 minutes at room temperature, the mixture was added to the culture dishes, and the cells were cultured for an additional 24, 48, or 72 hours. Given that the depletion of IGF-1 was most pronounced after transfection for 48 hours, the siRNA-treated HCE cells were cocultured with corneal fibroblasts after this time.

### Statistical Analysis

Data are presented as mean  $\pm$  SE from three independent experiments and were analyzed by Student's *t*-test. *P* < 0.05 was considered statistically significant.



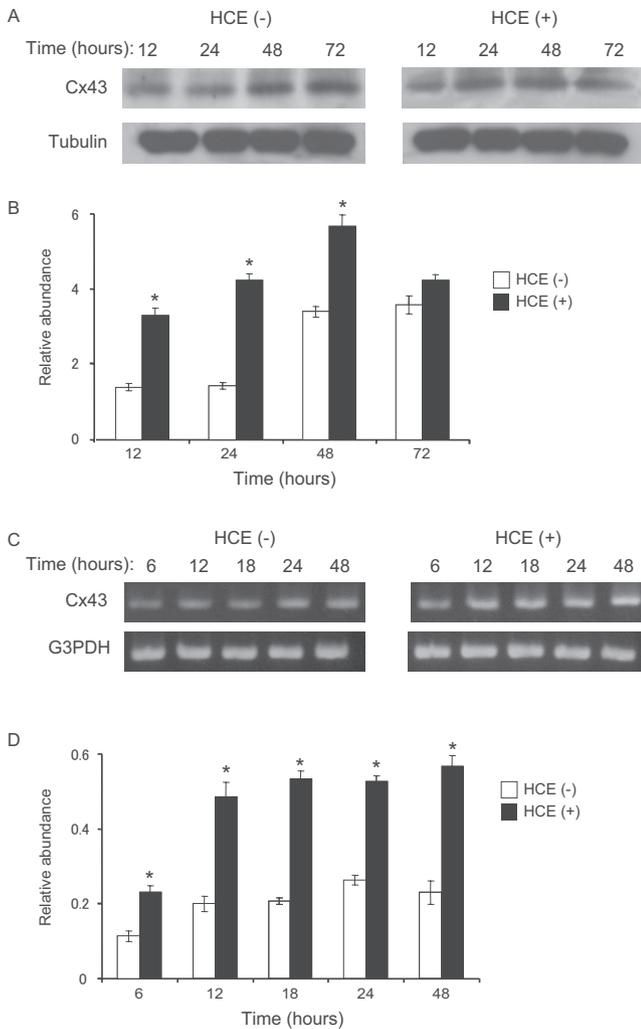
**FIGURE 1.** Expression of junctional proteins in corneal fibroblasts cultured with or without HCE cells. (A) Lysates of corneal fibroblasts cultured for 24 hours on a collagen vitrigel membrane in the absence or presence of HCE cells were subjected to immunoblot analysis with antibodies to Cx43, ZO-1, occludin, claudin, or  $\alpha$ -tubulin (loading control). (B) The abundance of each junctional protein in experiments similar to that shown in (A) was quantified by densitometric scanning of immunoblots and normalized by the corresponding amount of  $\alpha$ -tubulin. (C) Total RNA prepared from corneal fibroblasts cultured for 12 hours on a collagen vitrigel membrane in the absence or presence of HCE cells was subjected to RT-PCR analysis of Cx43, ZO-1, occludin, claudin, and G3PDH mRNA. (D) The abundance of mRNA for junctional proteins in experiments similar to that in (C) was quantified by RT and real-time PCR analysis and normalized by the corresponding amount of G3PDH mRNA. Data in (B) and (D) are mean  $\pm$  SE from three separate experiments. \* $P < 0.05$  versus the corresponding value for corneal fibroblasts incubated in the absence of HCE cells.

## RESULTS

### Effects of HCE Cells on the Expression of Junctional Proteins in Corneal Fibroblasts

We investigated the effect of the presence of HCE cells on the expression of the gap-junction protein Cx43 in corneal fibroblasts. Immunoblot analysis revealed that the amount of Cx43 in corneal fibroblasts cultured for 24 hours in the presence of HCE cells was approximately twice that in those cultured without HCE cells (Figs. 1A, B). RT-PCR and real-time PCR analyses revealed that the abundance of Cx43 mRNA in corneal fibroblasts cultured for 12 hours in the presence of HCE cells was similarly increased compared with that in corneal fibro-

blasts cultured alone (Figs. 1C, D). The presence of HCE cells had no effect on the expression of the tight-junction proteins ZO-1, occludin, and claudin in corneal fibroblasts at the mRNA or protein level (Fig. 1). The effect of HCE cells on Cx43 expression in corneal fibroblasts was time dependent, with maximal amounts of Cx43 protein and mRNA apparent after 24 hours (Figs. 2A, B) or 12 to 48 hours (Figs. 2C, D) of coculture, respectively. Changing the number of HCE cells from  $2 \times 10^5$  to  $1 \times 10^5$ ,  $5 \times 10^5$ , or  $1 \times 10^6$  did not substantially influence the stimulatory effect of these cells on the expression of Cx43 in corneal fibroblasts at the protein (Figs. 3A, B) or mRNA (Figs. 3C, D) level, indicating that the effect was maximal under the standard plating condition.



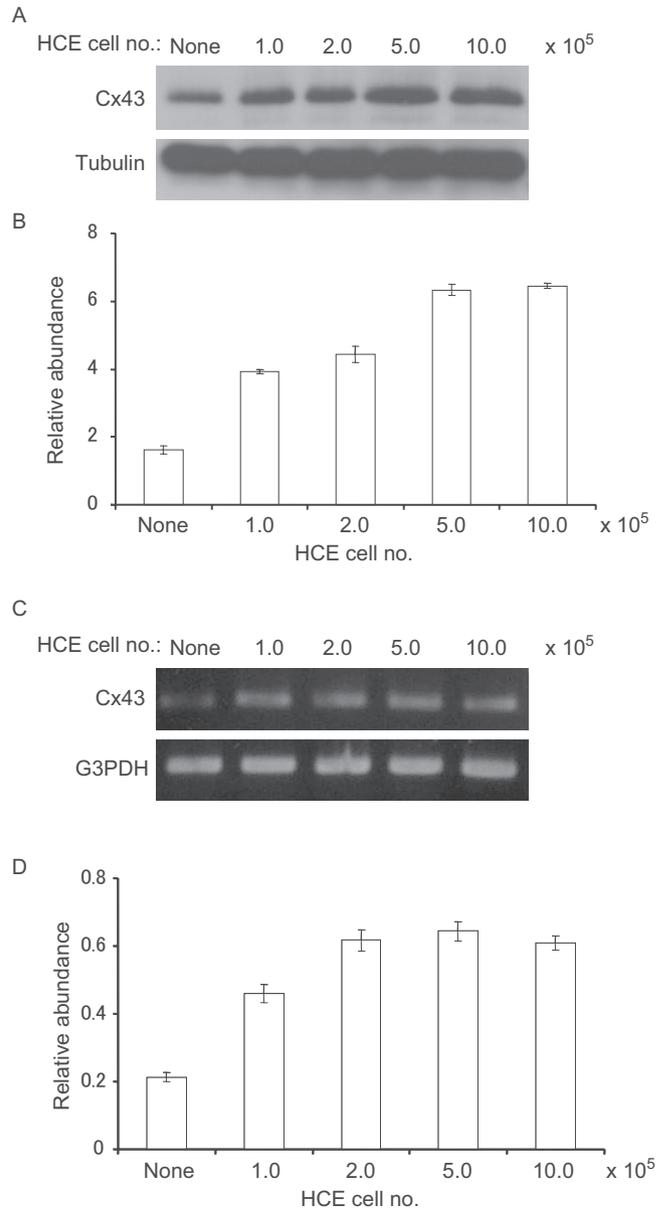
**FIGURE 2.** Time course of Cx43 expression in corneal fibroblasts cultured with or without HCE cells. (A) Lysates of corneal fibroblasts cultured for 12 to 72 hours on a collagen vitrigel membrane in the absence or presence of HCE cells were subjected to immunoblot analysis with antibodies to Cx43 or to  $\alpha$ -tubulin. (B) The abundance of Cx43 in experiments similar to that shown in (A) was quantified by densitometric scanning of immunoblots and normalized by the corresponding amount of  $\alpha$ -tubulin. (C) Total RNA prepared from corneal fibroblasts cultured for the indicated times on a collagen vitrigel membrane in the absence or presence of HCE cells was subjected to RT-PCR analysis of Cx43 and G3PDH mRNA. (D) The abundance of Cx43 mRNA in experiments similar to that in (C) was quantified by RT and real-time PCR analysis and normalized by the corresponding amount of G3PDH mRNA. Data in (B) and (D) are mean  $\pm$  SE from three separate experiments. \* $P < 0.05$  versus the corresponding value for corneal fibroblasts incubated in the absence of HCE cells.

### Effects of Growth Factors on the Expression of Cx43 in Corneal Fibroblasts

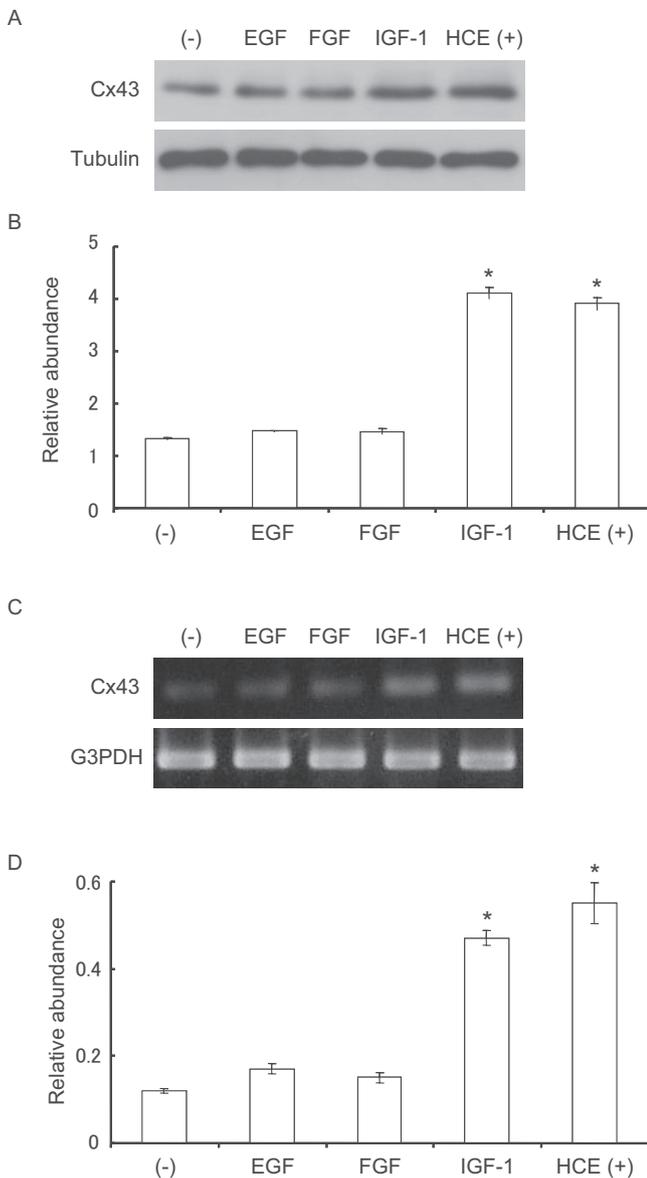
To identify the factor derived from HCE cells that was responsible for the upregulation of Cx43 expression in corneal fibroblasts, we first examined the effects of several growth factors on corneal fibroblasts cultured alone. IGF-1 (10 ng/mL) increased the amounts of Cx43 protein (Figs. 4A, B) and mRNA (Figs. 4C, D) in corneal fibroblasts to an extent similar to that observed with HCE cells. In contrast, EGF (10 ng/mL) and FGF (10 ng/mL) had no effect on Cx43 expression in corneal fibroblasts at the protein or mRNA level (Fig. 4).

### Role of IGF-1 in the Upregulation of Cx43 Expression in Corneal Fibroblasts by HCE Cells

To examine further the possible role of IGF-1 in the upregulation of Cx43 expression in corneal fibroblasts by HCE cells, we determined the effect of its depletion in HCE cells by RNA interference (RNAi). The time course of IGF-1 depletion in HCE



**FIGURE 3.** Expression of Cx43 in corneal fibroblasts cultured with HCE cells at various densities. (A) Lysates of corneal fibroblasts cultured for 24 hours on a collagen vitrigel membrane in the presence of the indicated numbers of HCE cells were subjected to immunoblot analysis with antibodies to Cx43 and to  $\alpha$ -tubulin. (B) The abundance of Cx43 in experiments similar to that shown in (A) was quantified by densitometric scanning of immunoblots and normalized by the corresponding amount of  $\alpha$ -tubulin. (C) Total RNA prepared from corneal fibroblasts cultured for 12 hours on a collagen vitrigel membrane in the presence of the indicated numbers of HCE cells was subjected to RT-PCR analysis of Cx43 and G3PDH mRNA. (D) The abundance of Cx43 mRNA in experiments similar to that in (C) was quantified by RT and real-time PCR analysis and was normalized by the corresponding amount of G3PDH mRNA. Data in (B) and (D) are mean  $\pm$  SE from three separate experiments.



**FIGURE 4.** Effects of growth factors on Cx43 expression in corneal fibroblasts. **(A)** Corneal fibroblasts cultured on a collagen vitrigel membrane were deprived of serum for 24 hours and incubated in the absence or presence of the indicated growth factors (10 ng/mL) for 24 hours. For comparison, corneal fibroblasts were cocultured with HCE cells for 24 hours. Lysates of the corneal fibroblasts were then subjected to immunoblot analysis with antibodies to Cx43 and to  $\alpha$ -tubulin. **(B)** The abundance of Cx43 in experiments similar to that shown in **(A)** was quantified by densitometric scanning of immunoblots and normalized by the corresponding amount of  $\alpha$ -tubulin. **(C)** Corneal fibroblasts cultured on a collagen vitrigel membrane were deprived of serum for 24 hours and incubated in the absence or presence of the indicated growth factors (10 ng/mL) for 12 hours. For comparison, corneal fibroblasts were cocultured with HCE cells for 12 hours. Total RNA was then isolated from the corneal fibroblasts and subjected to RT-PCR analysis of Cx43 and G3PDH mRNA. **(D)** The abundance of Cx43 mRNA in experiments similar to that in **(C)** was quantified by RT and real-time PCR analysis and was normalized by the corresponding amount of G3PDH mRNA. Data in **(B)** and **(D)** are mean  $\pm$  SE from three separate experiments. \* $P < 0.05$  compared with the corresponding value for corneal fibroblasts incubated in the absence of growth factor or HCE cells.

cells revealed that the effect of the IGF-1 siRNA was most pronounced after transfection for 48 hours (Fig. 5A). RT and real-time PCR analysis revealed that the amount of IGF-1 mRNA

was reduced by 60% to 70% after the transfection of HCE cells with the IGF-1 siRNA for 24 hours (Fig. 5B). The morphology of the siRNA-treated HCE cells did not differ from that of control HCE cells (data not shown). Immunoblot analysis revealed that transfection of HCE cells with the IGF-1 siRNA for 48 hours before coculture with corneal fibroblasts largely abolished the stimulatory effect of the HCE cells on the expression of Cx43 in corneal fibroblasts (Fig. 5C). Immunofluorescence analysis confirmed that HCE cells upregulated the expression of Cx43 in corneal fibroblasts and that this effect was prevented by RNAi-mediated depletion of IGF-1 in HCE cells (Fig. 5D). These results thus indicated that IGF-1 derived from HCE cells mediates the stimulatory effect of these cells on Cx43 expression in corneal fibroblasts.

### Effects of MAPK Inhibition on Upregulation of Cx43 Expression in Corneal Fibroblasts by HCE Cells

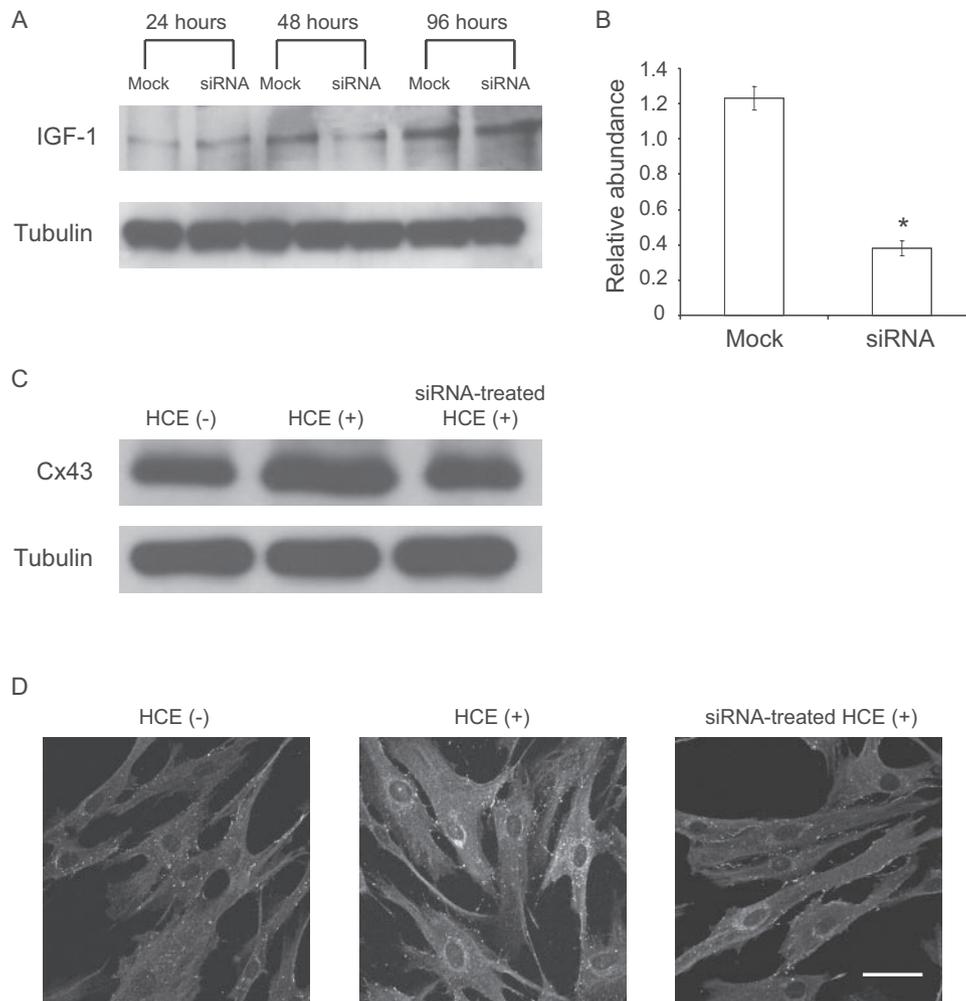
Finally, we investigated the signaling pathways responsible for the upregulation of Cx43 expression in corneal fibroblasts by HCE cells. Corneal fibroblasts were cultured for 12 hours on a collagen vitrigel membrane in the absence or presence of HCE cells and were then exposed to 10  $\mu$ M PD98059 or 10  $\mu$ M SB203580 for 12 hours before the preparation of cell lysates. The HCE cell-induced increase in the amount of Cx43 in corneal fibroblasts was blocked by PD98059 and SB203580 (Fig. 6), which are inhibitors of signaling by the mitogen-activated protein kinases (MAPKs) ERK (extracellular signal-regulated kinase) and p38 MAPK, respectively. These results thus implicate ERK and p38 MAPK in the upregulation of Cx43 expression in corneal fibroblasts by HCE cells. Neither PD98059 nor SB203580 affected the abundance of Cx43 in corneal fibroblasts cultured without HCE cells or that of ZO-1, occludin, or claudin in corneal fibroblasts cultured with or without HCE cells (Fig. 6).

### DISCUSSION

With the use of a coculture system based on a collagen vitrigel membrane, we have shown that the presence of HCE cells upregulated the expression of the gap-junction protein Cx43 in corneal fibroblasts. Depletion of IGF-1 by RNAi in HCE cells largely abolished the stimulatory effect of these cells on Cx43 expression in corneal fibroblasts, suggesting that IGF-1 released from HCE cells is responsible, at least in part, for this effect. We further showed that MAPK signaling pathways participate in this interaction between HCE cells and corneal fibroblasts.

The upregulation of Cx43 expression in corneal fibroblasts by HCE cells was not accompanied by a similar effect on the expression of the tight-junction proteins ZO-1, occludin, and claudin, suggesting that the presence of HCE cells specifically affects the formation of gap junctions in corneal fibroblasts. The interaction of the intact corneal epithelium with underlying corneal fibroblasts might thus play an important role in homeostasis of the corneal stroma. The effect of HCE cells on Cx43 expression in corneal fibroblasts was apparent at the protein and mRNA levels and was time dependent. We previously showed that the proinflammatory cytokine tumor necrosis factor  $\alpha$  downregulated the expression of Cx43 and dye coupling in corneal fibroblasts cultured as monolayers.<sup>17</sup>

The collagen vitrigel system for cell culture is based on a membrane produced from type I collagen.<sup>18</sup> We applied this system to the coculture of HCE cells and corneal fibroblasts, with the two cell types cultured on opposite sides of the membrane. Given that epithelial cells and fibroblasts do not



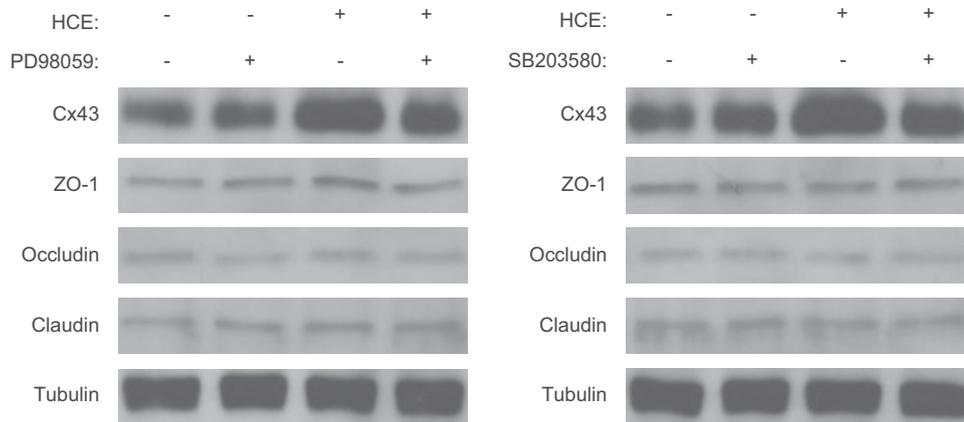
**FIGURE 5.** Inhibition of the stimulatory effect of HCE cells on Cx43 expression in corneal fibroblasts by RNAi-mediated depletion of IGF-1. **(A)** HCE cells were transfected with siRNA specific for IGF-1 mRNA or were subjected to mock transfection for the indicated times. Cell lysates were then subjected to immunoblot analysis with antibodies to IGF-1 and to  $\alpha$ -tubulin. **(B)** Total RNA isolated from HCE cells transfected as in **(A)** for 24 hours was subjected to RT and real-time PCR analysis for quantification of IGF-1 mRNA. Data were normalized by the corresponding amount of G3PDH mRNA and are mean  $\pm$  SE from three separate experiments. **(C)** Corneal fibroblasts were cultured for 24 hours on a collagen vitrigel membrane in the absence or presence of normal HCE cells or of HCE cells that had been transfected with IGF-1 siRNA for 48 hours. Lysates of the corneal fibroblasts were then subjected to immunoblot analysis with antibodies to Cx43 and to  $\alpha$ -tubulin. **(D)** Corneal fibroblasts cultured as in **(C)** were subjected to immunofluorescence analysis with antibodies to Cx43. Scale bar, 50  $\mu$ m.

come into direct contact with each other in this system, it is likely that a factor (or factors) released by the epithelial cells is responsible for the upregulation of Cx43 in the fibroblasts. We found that IGF-1, but not EGF or FGF, mimicked the effect of HCE cells on the expression of Cx43 in corneal fibroblasts. IGF-1 has previously been shown to upregulate the expression of Cx43 in astrocytes and vascular smooth muscle cells.<sup>13,14</sup> Furthermore, we found that RNAi-mediated depletion of IGF-1 in HCE cells largely prevented the stimulatory effect of these cells on Cx43 expression in corneal fibroblasts. These results thus implicate IGF-1 released by HCE cells as a mediator of the upregulation by these cells of Cx43 expression in corneal fibroblasts. IGF-1 has been shown to induce the recruitment of corneal fibroblasts to the site of corneal wounds *in vivo*<sup>19</sup> and the proliferation of these cells.<sup>20</sup>

We have shown previously that IGF-1 promotes corneal epithelial migration *in vitro* and corneal epithelial wound closure *in vivo* in the presence of the neurotransmitter

substance P.<sup>21,22</sup> We have also shown that IGF-1 at high concentrations is sufficient to promote corneal epithelial migration<sup>23,24</sup> and that this growth factor protects HCE cells from apoptosis.<sup>25</sup> These previous and our present observations thus suggest that IGF-1 may play an important role in corneal wound healing through its effects on corneal epithelial cells and corneal fibroblasts.

We found that the inhibition of signaling by ERK or p38 MAPK in our coculture system blocked the upregulation of Cx43 in corneal fibroblasts by HCE cells. These MAPKs have previously been shown to mediate the stimulatory effect of IGF-1 on Cx43 expression in vascular smooth muscle cells.<sup>14</sup> Our results thus suggest that the activation of ERK and p38 MAPK signaling pathways, likely in corneal fibroblasts, is required for the upregulation of the gap-junction protein Cx43 in corneal fibroblasts by HCE cells. The functional consequences of the stimulatory effect of HCE cells on Cx43 expression in corneal fibroblasts remain to be determined.



**FIGURE 6.** Effects of PD98059 and SB203580 on the HCE cell-induced upregulation of Cx43 expression in corneal fibroblasts. Lysates of corneal fibroblasts cultured on a collagen vitrigel membrane in the absence or presence of HCE cells for 12 hours before exposure to 10  $\mu$ M PD98059 or 10  $\mu$ M SB203580 for 12 hours, as indicated, were subjected to immunoblot analysis with antibodies to Cx43, ZO-1, occludin, claudin, or  $\alpha$ -tubulin.

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