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Hepatocyte Growth Factor/Scatter Factor (HGF/SF) Is a Regulator of Fibronectin Splicing in MDCK Cells: Comparison between the Effects of HGF/SF and TGF-β1 on Fibronectin Splicing at the EDA Region

Teruhiko Inoue, Kazuki Nabeshima, Yoshiya Shimao, and Masashi Koono[1]
Second Department of Pathology, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan

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EDA-containing fibronectin (EDA + FN) is selectively produced under several physiological and pathological conditions requiring tissue remodeling, where cells actively proliferate and migrate. Only a few growth factors, such as transforming growth factor (TGF)-β1, have been reported to regulate FN splicing at the EDA region. In the present study, we showed for the first time that hepatocyte growth factor/scatter factor (HGF/SF), which is mainly produced by mesenchymal cells and functions as a motogenic and mitogenic factor for epithelial cells, modulates FN splicing at the EDA region in MDCK epithelial cells. HGF/SF treatment increased the ratio of EDA + FN mRNA to mRNA of FN that lacks EDA (EDA − FN) (EDA+/EDA− ratio) more than TGF-β1 treatment did: at a range from 0.02 to 20 ng/ml, HGF/SF increased the ratio in a dose-dependent manner by up to 2.1-fold compared with nontreated control, while TGF-β1 stimulated the EDA+/EDA− ratio by 1.5-fold at the optimum dose of 10 ng/ml. However, TGF-β1 increased total FN mRNA levels by 3-fold at 10 ng/ml, but HGF/SF did not. We previously demonstrated that fibroblasts cultured at low cell density expressed more EDA + FN than those at high cell density. The same effect of cell density was also observed in MDCK cells. Furthermore, at low cell density, HGF/SF stimulated EDA inclusion into FN mRNA more effectively than did TGF-β1, whereas at high cell density, TGF-β1 was more potent than HGF/SF. Simultaneous treatment of cells with HGF/SF and TGF-β1 synergistically stimulated EDA inclusion into FN mRNA. This stimulation of EDA inclusion into FN mRNA by HGF/SF led to increased EDA + FN protein production and secretion by cells, which was demonstrated by immunoblotting. Thus, our studies have shown that HGF/SF is an enhancer of EDA inclusion into FN mRNA as is TGF-β1. However, these two factors were different in their effects at low and high cell densities and also in their effects on total FN mRNA levels.

Key Words: **HGF/SF; TGF-β; fibronectin splicing.**

Fibronectin (FN) is an extracellular matrix (ECM) glycoprotein, which is involved in diverse biological phenomena such as wound healing, development and cancer invasion. FN has many variants which are produced by alternative splicing of its primary transcripts in three regions, termed EDA (EIIIA), EDB(EIIIB), and IIICS (V region); as a consequence theoretically 20 isoforms are generated in human (1, 2). The alternative splicing of the FN pre-mRNA is regulated in a cell-, tissue- and development-specific manner, and is deregulated in malignancies (1–4). EDA-containing FN (EDA + FN) is present in fetal tissue, once decreased in adult, and then reappears in wound healing (5, 6) and a variety of tumor tissues (4, 7–12). Moreover, EDA + FN is approximately twice as potent as FN that lacks EDA (EDA − FN) in the ability to promote cell adhesion and migration (13).

What controls the mechanisms of FN splicing at the EDA region includes extracellular regulatory factors as well as intracellular cis and trans-acting sequences (14). The extracellular regulatory factors include growth factors such as transforming growth factor (TGF)-β1, vitamin D_3 and epidermal growth factor (EGF), which stimulate preferential EDA + FN production in human fibroblasts, osteosarcoma and rat liver epithelial cells, respectively (15–18). Among them TGF-β1 is the most consentaneous factor to modulate FN splicing at the EDA region and the spatial correlation of TGF-β1 and EDA + FN is also shown (19).

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchymal-derived pleiotropic cytokine that shows mitogenic, motogenic and morphogenic activities on various types of cells (20–23). As a motogenic

[1] To whom correspondence and reprint requests should be addressed. Fax: (+81) 985-85-6003.

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factor, HGF/SF induces scattering (dispersion of coherent clustered cells into single cells) of various epithelial cells and tumor cells *in vitro* (20, 23). HGF/SF also plays an important role as a morphogen during organogenesis (23).

In this study, we for the first time showed that HGF/SF was a FN splicing regulator at the EDA region in MDCK epithelial cells: HGF/SF stimulated inclusion of the EDA sequence into FN mRNA. Differences between the effects of HGF/SF and TGF-β1 were also shown.

MATERIAL AND METHODS

Reagent. Human recombinant (r-) TGF-β1 and human r-HGF/SF were purchased from Boehringer-Mannheim Biochemica (Indianapolis, IN) and TOYOBO (Osaka, Japan), respectively.

Cell culture. MDCK (Madin–Darby canine kidney) cells were obtained from the RIKEN cell bank (Tsukuba, Japan) and maintained as monolayer culture in growth medium, Dulbecco's Modified Eagle Medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), streptomycin and penicillin G, pH 7.35. The culture was passed with 0.125% trypsin and 0.5 mM EDTA at a split ratio of 1:10 and incubated in a fully humidified atmosphere of 5% CO_2–95% air.

Reverse-transcription/polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured MDCK cells using Trizol (Gibco) and reverse-transcribed using random hexamer primers. The enzymatic reaction was performed in 20 μl reaction mixture containing 1 μg total RNA, 75 mM KCl, 50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 4 mM total dNTP (1 mM each dNTP), 200 pmol of hexamers, 200 U of M-MLV reverse transcriptase (Gibco), 1 unit/μl of ribonuclease inhibitor (Takara, Tokyo, Japan), and 1 mM dithiothreitol, at 37°C for 1 h and heated at 95°C for 10 min.

PCR-based analysis of alternating splicing of FN pre-mRNA at the EDA region was performed according to Magnuson *et al.* (18) using ^{32}P-end labeled antisense primers with some modifications. RT product (2 μl) was diluted with PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% (W/V) gelatin) to a final volume 10 μl, which contained 2 pmol of primer pairs and 0.25 unit of Ampli *Taq* DNA polymerase (Takara). PCR was run in a thermocycler (Perkin–Elmer Cetus, Norwalk, CT) using cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min.

For semi-quantitative RT-PCR, we used less than 1.0 μg of total RNA for each RT reaction (24). In addition, since the amounts of PCR products for FN linearly increased between 18 and 26 cycles and little difference in the ratios of the EDA+ PCR products to EDA− PCR products was noted within these cycles, all PCRs were carried out at 20 cycles. Their triplicate products were electrophoresed on 5% polyacrylamide gel, and the product bands were subjected to analysis using FUJIX BAS2000 BioImage Analyzer (Fuji Photo Film, Tokyo, Japan). For internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by using the same RT products and analyzed in the same manner. The relative abundance of total FN mRNA was expressed as the following: (the intensity of the EDA+ band plus the intensity of the EDA− band/the intensity of the corresponding GAPDH band). Results were expressed as the mean ± SE. Statistical significance was determined using an unpaired Student's *t* test, and significance was set at $P < 0.01$.

A primer pair, HA1 (antisense, 5′-AGAGCATAGACACTCACTTCATATTT-3′) and HA2 (sense, 5′-AAACAGAAATGACTATTGAAGGCTTG-3′), was used to amplify the EDA region of FN (18). HA1 and HA2 are present in exon III_{12} and III_{11} of the FN gene, respectively. Amplification of the correct sequence was confirmed by the size of PCR products and also by nested PCR using internal primer pairs. The sequences of forward and reverse primers for GAPDH were 5′-GTGAAGGTCGGAGTCAACG-3′ and 5′-GGTGAAGACGCCAGTGGACTC-3′, respectively. This PCR product of GAPDH was 300 bp.

HGF/SF or TGF-β1 treatment of MDCK cells. MDCK cells were cultured in 24-well plates (Falcon, Lincoln Park, NJ) at various densities from 0.25 to 2.0 $\times$ 10^5 cells per well containing 0.5 ml growth medium. The cells were allowed to attach for 12 h at 37°C in a humid atmosphere of 5% CO_2–95% air, then the media were replaced with fresh growth medium containing various concentrations of HGF/SF or TGF-β1. The cells were cultured for 24 h and subjected to RNA extraction and RT-PCR. Each experimental condition was done in triplicate wells.

Immunoblot analysis. After MDCK cells were treated with HGF/SF or/and TGF-β1 as above, their conditioned media (CM) were collected and supplemented with the proteinase inhibitor cocktail, Complete (Boehringer-Mannheim, Germany). After centrifugation (110*g*, 5 min and 4000*g*, 20 min), supernatants were concentrated up to 8-fold using Centricon 10 (Amicon, Beverly, MA). Aliquots adjusted by the cell number (5×10^4 cells) were loaded per lane onto 6.5% SDS–polyacrylamide gel under reducing condition, followed by electrophoretical transfer to Immobilon membrane (Millipore, Bedford, MA). After the non-specific sites were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.6) containing 0.05% Tween 20 (TBS-T) at 37°C for 3 h, the membrane was incubated with mouse monoclonal antibody to human EDA + FN (Harlan Sera-lab, Cawley Down, England) overnight at 4°C. The membrane was washed three times with TBS-T and incubated for 1 h with peroxidase-conjugated anti-mouse IgG. The color was developed with chemiluminescence reagents (Dupon NEN, Boston, MA) according to the manufacture's instructions. The bands on the film were subjected to image analysis (Adobe Photoshop).

RESULTS

Morphology of MDCK cells treated with HGF/SF or TGF-β1. First we observed *in vitro* morphological changes induced by HGF/SF or TGF-β1 under phase-contrast microscopy. MDCK cells without any treatment formed coherent clusters (Fig. 1a). HGF/SF is well known to induce scattering in MDCK cells (20, 25). Twenty-four-hour treatment with 20 ng/ml HGF/SF induced complete scattering of MDCK cells, with cell-cell contacts lost in a large part (Fig. 1b). On the other hand, TGF-β1 treatment induced more spread and flattened appearance compared with non-treated cells as described (26). However, TGF-β1-treated cells maintained cell–cell contact with one another (Fig. 1c). Thus, MDCK cells showed normal morphological responses to HGF/SF and TGF-β1 in our assays.

Effects of TGF-β1 and HGF/SF on FN splicing at the EDA region and total FN mRNA levels. To examine the effects of HGF/SF and TGF-β1 on FN splicing at the EDA region and total FN mRNA levels in MDCK cells, we performed semiquantitative RT-PCR using ^{32}P-end labeled antisense primers. Two PCR products of 604 and 334 bp correspond to EDA + FN and EDA − FN mRNA, respectively (Fig. 2a). The intensities of these bands were quantified, and changes of alternative splicing was analyzed by means of EDA + FN mRNA/EDA − FN mRNA ratio (EDA+/EDA− ratio)

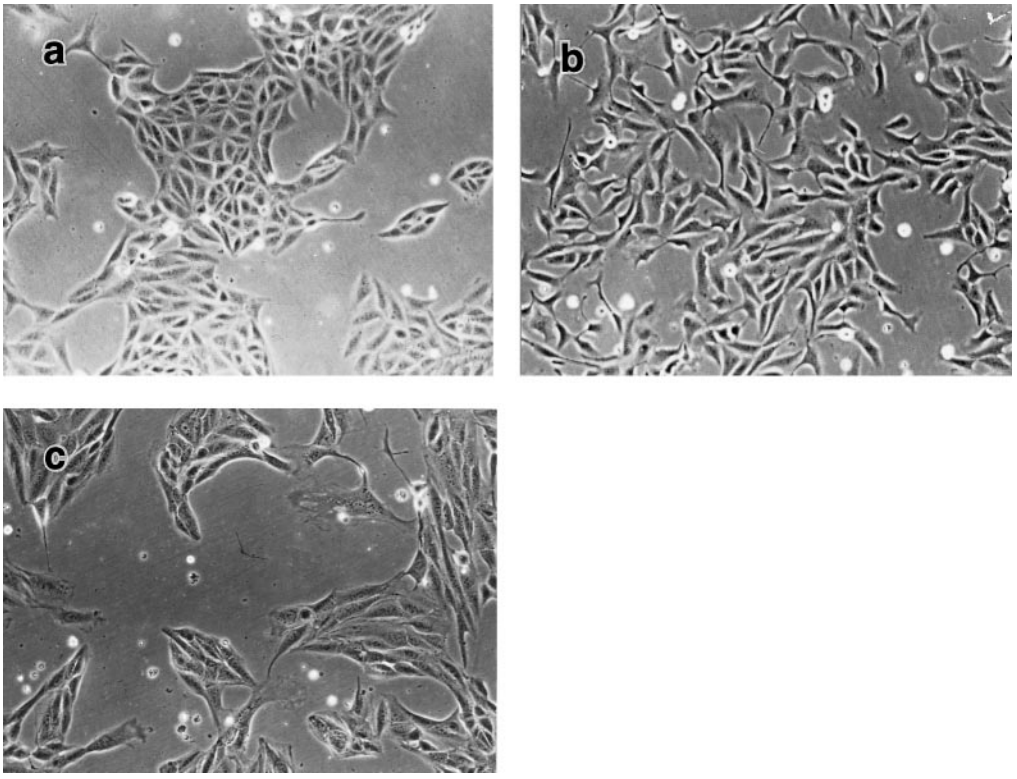

FIG. 1. Morphological changes of MDCK cells induced by treatment with HGF/SF and TGF-β1. MDCK cells were cultured in the absence (a) or the presence of 20 ng/ml HGF/SF (b) or 10 ng/ml TGF-β1 (c) for 24 h and then photographed (20×) under phase-contrast microscopy.

according to Cramer *et al.* (27). The ratio means a frequency of inclusion of the EDA sequence per one exclusion of the domain. Both HGF/SF and TGF-β1 affected FN splicing to express more EDA + FN (Figs. 2b and 2c). At concentrations from 0.02 to 20 ng/ml, HGF/SF increased EDA+/EDA− ratio in a dose dependent manner, and peak response (4.00 ± 0.04, $n = 3$) was obtained at 20 ng/ml. EDA inclusion into FN mRNA was increased by 2.1-fold at this peak concentration compared with non-treated control (Fig. 2b). TGF-β1 also stimulated the EDA-inclusion, with low peak response (2.58 ± 0.06, $n = 3$) at 10 ng/ml. EDA+/EDA− ratio was increased by 1.5-fold at this peak concentration compared with non-treated control (Fig. 2c). Furthermore, TGF-β1 increased total FN mRNA levels in a dose-dependent manner at concentrations from 0.01 to 10 ng/ml (Fig. 2e). At 10 ng/ml, the total FN mRNA level was increased by 3.3-fold compared to non-treated control. On the other hand, HGF/SF did not alter total FN mRNA levels significantly (Fig. 2d).

Effects of cell density on FN splicing at the EDA region. Recently we have reported that EDA inclusion occurs more frequently in fibroblasts cultured at low cell density than those at high cell density (28). Here we studied whether cell density also affects FN splicing in MDCK cells. EDA+/EDA− ratio increased from 1.98 ± 0.04 ($n = 3$) to 3.21 ± 0.03 ($n = 3$), as cell density decreased from 2.0 to 0.5 × 10^5 cells/well (Figs. 3a and 3b). At low cell density (0.5 × 10^5 cells/well), MDCK cells formed small-sized nests or single cells, with cell-cell contact frequently disrupted, whereas at high cell density (2.0 × 10^5 cells/well), they formed a confluent sheet. Total FN mRNA levels were not affected by cell density (Fig. 3c). These findings in MDCK cells were almost the same as those observed in fibroblasts (28).

Modulation of effects of TGF-β1 and HGF/SF on FN splicing by cell density. Next we studied how cell density affects the effect of HGF/SF and TGF-β1 on FN splicing. At low cell density (0.5 × 10^5 cells/well), HGF/SF (20 ng/ml) and TGF-β1 (10 ng/ml) increased EDA+/EDA− ratio by (1.83 ± 0.06)-fold and (1.59 ± 0.04)-fold compared to non-treated control, respectively (Figs. 4a and 4b). On the other hand, at high cell density (2.0 × 10^5 cells/well), HGF/SF and TGF-β1 increased the ratio by (1.46 ± 0.02)-fold and (1.91 ± 0.04)-fold, respectively (Figs. 4a and 4c). Thus, HGF/SF was more effective at low cell density, while TGF-β1 was more potent at high cell density.

Synergistic effect of HGF/SF and TGF-β1 on FN splicing at the EDA region. Simultaneous treatment with HGF/SF and TGF-β1 has been shown to stimulate cancer cell invasion and migration additively or syner-

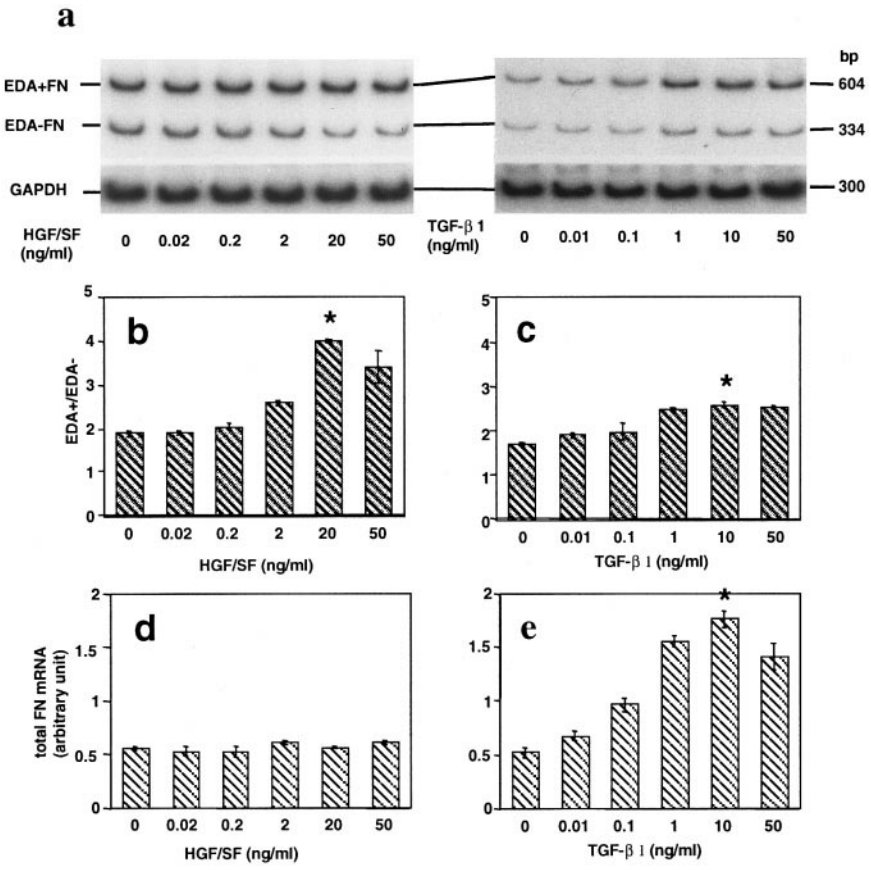

FIG. 2. (a) RT-PCR analysis of alternative splicing at the EDA region of the FN gene. Total RNA was prepared from MDCK cells treated with various concentrations of HGF/SF (0, 0.02, 0.2, 2, 20, and 50 ng/ml) or TGF-β1 (0, 0.01, 0.1, 1, 10, and 50 ng/ml) for 24 h. Two PCR products of 604 and 334 bp correspond to EDA + and EDA − FN, respectively. Products of GAPDH (300 bp) are shown as internal control. (b, c) Results of semiquantitative analysis of (a). Values represent EDA + FN mRNA/EDA − FN mRNA ratio (EDA+/EDA− ratio) (mean ± SE, $n = 3$) induced by HGF/SF (b) or TGF-β1 (c). (d, e) The relative abundance of total FN mRNA in MDCK cells treated with HGF/SF (d) or TGF-β1 (e). The values represent (the intensity of the EDA+ band plus the intensity of the EDA− band/the intensity of the corresponding GAPDH band) (mean ± SE, $n = 3$). *Statistically significant compared with control, $P < 0.01$.

gistically (29, 30). Thus, we examined the effect of simultaneous treatment with HGF/SF and TGF-β1 on FN splicing at the EDA region in MDCK cells cultured at low cell density. Treatment with HGF/SF alone and TGF-β1 alone increased EDA+/EDA− ratio by 1.6 ± 0.08 ($n = 4$) and 0.97 ± 0.02 ($n = 4$), respectively, while simultaneous treatment with HGF/SF and TGF-β1 increased the ratio by 3.1 ± 0.13 ($n = 4$) compared with non-treated control (Figs. 5a and 5b), indicating the synergistic stimulation of EDA inclusion into FN mRNA in MDCK cells by the simultaneous treatment.

Stimulation of FN protein secretion by HGF/SF. Finally, we examined whether stimulation of EDA inclusion into FN mRNA by HGF/SF leads to increased EDA + FN protein production and secretion by MDCK cells. EDA + FN protein produced by MDCK cells was predominantly secreted into CM, and 24 h treatment with HGF/SF (20 ng/ml) or TGF-β1 (10 ng/ml) increased the secreted EDA + FN protein level by 55 ± 10 or 150 ± 43% ($n = 2$), respectively, compared with nontreated control (Fig. 6). Moreover, simultaneous treatment with HGF/SF and TGF-β1 synergistically increased the secreted protein level by 260 ± 23% ($n = 2$). DMEM with 10% FCS, which was used in this assay, did not contain detectable EDA + FN (data not shown).

DISCUSSION

We presented here for the first time that HGF/SF regulates FN splicing at the EDA region in MDCK epithelial cells so that FN mRNA includes more EDA sequences, without affecting total FN mRNA levels. TGF-β1, which is the most consentaneous molecule to regulate FN splicing, also increased EDA inclusion into FN mRNA in MDCK cells, but with a prominent increase in total FN mRNA levels. In other words, TGF-β1 is a qualitative and quantitative regulator of FN expression, while HGF/SF causes only qualitative changes of FN. Its biological significance is not deter-

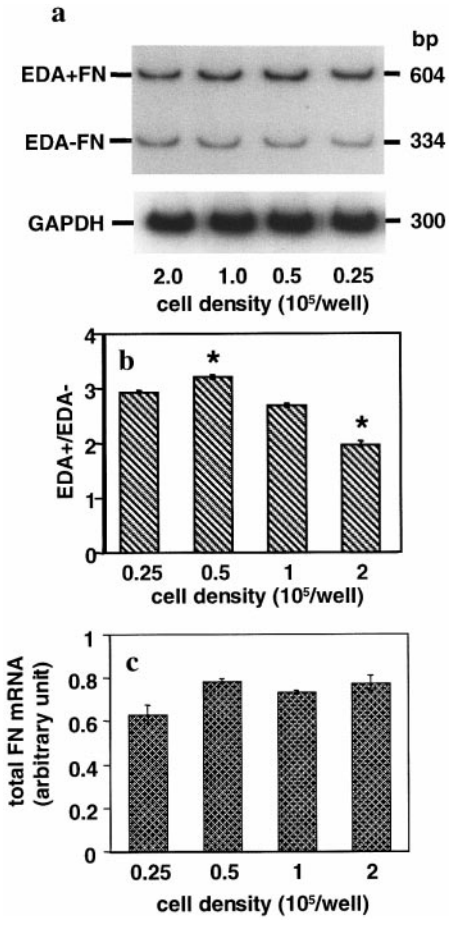

FIG. 3. (a) RT-PCR analysis of FN splicing at the EDA region in MDCK cells plated at various cell densities (2.0, 1.0, 0.5, and 0.25 $\times$ 10^5 cells/well) and cultured for 24 h. (b, c) Results of semiquantitative analysis of (a).Values represent EDA+/EDA− ratio (mean ± SE, n = 3) (b) and total FN mRNA levels (mean ± SE, n = 3) (c). Total FN mRNA levels are expressed as described in Fig. 2. *Statistically significant compared with control, $P < 0.01$.

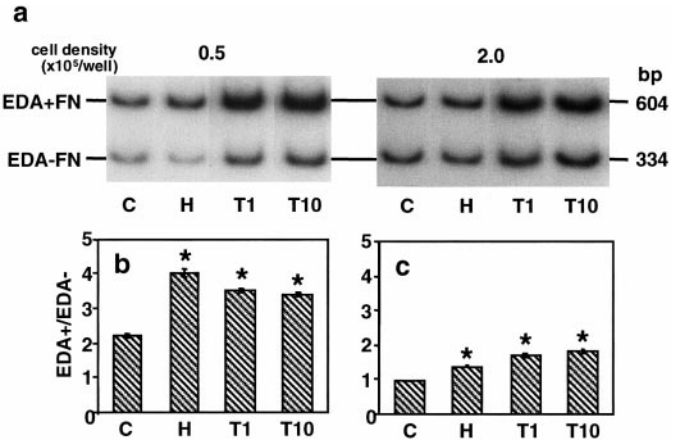

FIG. 4. (a) RT-PCR analysis of FN splicing at the EDA region in MDCK cells treated with HGF/SF (20 ng/ml) (H) or TGF-β1 [1 (T1) or 10 (T10) ng/ml]. Cells were plated at low (0.5 $\times$ 10^5 cells/well) (0.5) or high (2.0 $\times$ 10^5 cells/well) (2.0) cell density. (b, c) Semiquantitative analysis of (a). Values represent EDA+/EDA− ratio (mean ± SE) for triplicate experiments, which were done in cells plated at low cell density (b) or at high cell density (c). C, nontreated control; *statistically significant compared with control, $P < 0.01$.

mined yet, but we speculate that HGF/SF could induce effective cell migration and proliferation via upregulation of EDA + FN, since it is reported that inclusion of the EDA sequence up-regulates integrin-binding affinity of FN and thereby enhances cell migration and proliferation (13, 31). Recently we have shown that HGF/SF induces not only scattering, but also cohort migration, which is cell locomotion as *en masse*, in human colorectal adenocarcinoma cells (32). For this cohort-type migration, FN, especially EDA + FN, produced by carcinoma cells themselves was essential (32, 33). Motogenic activity of HGF/SF might be associated with FN production and its alternative splicing.

Splicing and transcription of the FN gene is coordinately regulated (14, 27). Nuclear speckles, where transcription takes place, concentrate not only RNA polymerase II and poly $(A)^+$ RNA but also molecules involved in RNA splicing (14). Moreover, changes in promoter structures strongly affect splice site selection in the FN gene (27). These lines of evidence indicate that transcription and splicing are coordinately regulated. In our study, however, TGF-β1 stimulated both splicing (EDA inclusion) and transcription of the FN gene, but HGF/SF only splicing. This suggests that there is a way to regulate splicing of the FN gene selectively without affecting its transcription levels.

Effect of HGF/SF on FN gene expression levels appears to be dependent on cell types. In this study, total FN mRNA levels were not altered by HGF/SF treatment in MDCK cells. Constitutive expression of HGF/SF in renal epithelial cells stimulated expressions of its specific receptor gene c-met and also FN gene, which was totally blocked by incubation with a neutralizing anti-HGF/SF but not an anti-TGF-β antibody (34). On the other hand, in human and mouse tumor cell lines, HGF/SF-Met signaling resulted in a great decrease in FN expression at both mRNA and protein levels (35).

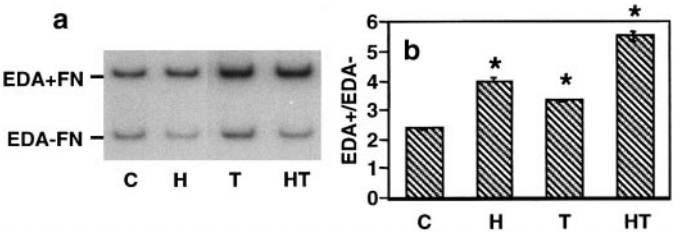

FIG. 5. (a) RT-PCR analysis of FN splicing at the EDA region in MDCK cells treated with HGF/SF (20 ng/ml) alone (H), TGF-β1 (10 ng/ml) alone (T) or both of them simultaneously (HT). Values represent EDA+/EDA− ratio (mean ± SE) for triplicate experiments. C, nontreated control; *statistically significant compared with control, $P < 0.01$.

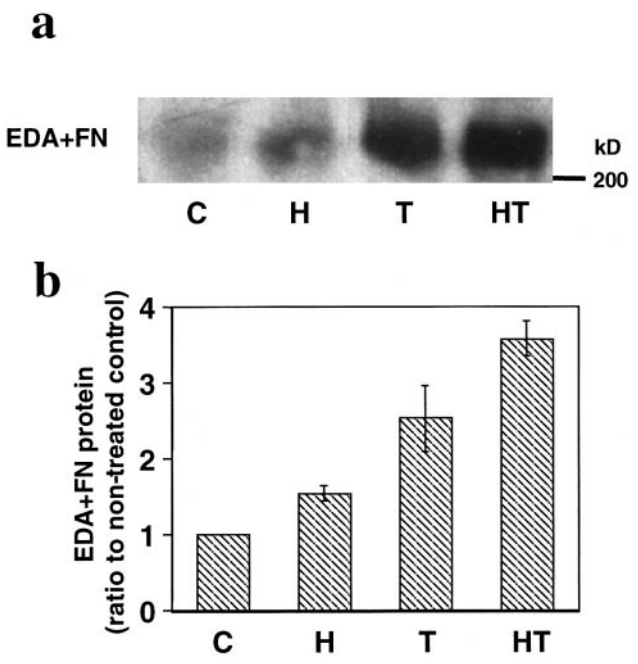

FIG. 6. (a) Immunoblot analysis of EDA + FN protein secretion in MDCK cells. CM was collected from MDCK cells treated for 24 h with HGF/SF (20 ng/ml) alone (H), TGF-β1 (10 ng/ml) alone (T) or both of them simultaneously (HT), concentrated, adjusted by the cell number (5×10^4) and then loaded to each lane. Immunoblotting was done with anti-EDA + FN monoclonal antibody. C, nontreated control; (b) Semiquantitative analysis of (a). Values represent the relative abundances of EDA + FN protein compared to nontreated control (mean ± SE) for duplicate experiments.

Cell density influences FN protein production (36) and also FN splicing at the EDA region in fibroblasts (28). In this study, like in fibroblasts, FN splicing at the EDA region was also influenced by cell density in MDCK epithelial cells. At low cell density, both fibroblasts and MDCK cells stimulate EDA inclusion more effectively compared with at high cell density. Cells are sparse and intercellular communication is frequently lost at low cell density. Furthermore, both TGF-β1 and HGF/SF inhibit intercellular communication mediated by gap junction (37–39). Thus, we speculate that loss of cellular communication could stimulate EDA inclusion in both fibroblasts and epithelial cells.

Cell density also affects the effect of regulator cytokines that control FN splicing at the EDA region in fibroblasts (28). This was the same in MDCK epithelial cells in this study. HGF/SF increased EDA inclusion more effectively at low cell density compared with at high cell density. At low cell density, HGF/SF can induce cell scattering more effectively, causing loss of cell-cell contact. Effect of HGF/SF on FN splicing might be associated with cell scattering.

In the present study, simultaneous treatment of TGF-β1 and HGF/SF synergistically stimulated EDA inclusion into FN mRNA and secretion of EDA + FN protein in MDCK cells. TGF-β1 is secreted by both epithelial (24) and mesenchymal cells (30) and stimulates both types of cells, while HGF/SF is produced predominantly by mesenchymal cells and stimulates epithelial and endothelial cells (20, 23). Since both TGF-β1 and HGF/SF work as a morphogen during development and tissue remodeling, which require abundant deposition of EDA + FN (3, 5, 23, 40), their synergistic effect on EDA inclusion might play an important role there.

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