J. Biochem. 122, 96-100 (1997)

Immortalized Gastric Epithelial Cell Line GSM06 Synthesizes Hyaluronan under the Influence of Simian Virus 40 Large T-Antigen Expression[1]

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Received for publication, February 3, 1997

GSM06 is a cell line established from the stomach of transgenic mouse harboring a temperature-sensitive simian virus 40 (SV40) large T-antigen gene. ^{3}H-labeled macromolecules produced by the cells incubated with [^{3}H]glucosamine were characterized to examine whether or not GSM06 cells synthesize mucin (mucus glycoprotein). The GSM06 cells grew until a confluent monolayer formed at 33°C (the permissive temperature for SV40 large T-antigen expression), and the ^{3}H-labeled macromolecules appeared in both cell extract and medium during culture for at least 1 week. Unexpectedly, almost all ^{3}H-labeled macromolecules, which were excluded from a column of Sepharose CL-4B, were identified as hyaluronan by analyses using Sepharose CL-2B chromatography, cesium trifluoroacetate equilibrium centrifugation, treatment with dithiothreitol, and trypsin, hyaluronidase, and chondroitinase ABC digestion. At a nonpermissive temperature (39°C), GSM06 cells grew only slightly, but produced much more hyaluronan than at 33°C. The results indicate that GSM06 cells produce not mucin, but hyaluronan, and that the expression of large T-antigen may influence hyaluronan synthesis in GSM06 cells.

Key words: gastric epithelial cell, GSM06 cell line, hyaluronan, immortalized cell line, temperature-sensitive SV40 large T-antigen.

Gastric mucus cell produces mucin, a component of mucus which is believed to protect the gastric mucosa from acid, digestive enzyme, and pathogens. In order to examine the biosynthetic pathway of mucin and its control mechanism, a cell culture of the mucus cells is required because gastric mucosa contains many kinds of cells, including acid-producing parietal cells, pepsinogen-producing chief cells, endocrine cells, and so on. Thus, many studies have been performed using primary cultures of gastric epithelial cells (*1-5*). However, primary culture requires the preparation of cells every time. In order to examine the control mechanism of intestinal mucin synthesis, carcinoma-derived cell lines were used (*6, 7*), but only a few gastric cell lines producing mucins are available.

GSM06 is an immortalized gastric cell line established from transgenic mice harboring temperature-sensitive simian virus 40 (SV40) large T-antigen gene (*8*). Based on the morphological and histochemical properties (*8, 9*), the cells were thought to be an epithelial mucus cell line. However, little is known about the synthesis of mucin in this cell line. To clarify this point, GSM06 cells were cultured at 33 or 39°C for several days, then incubated with [^{3}H]glucosamine, and the produced ^{3}H-labeled macromolecules were characterized. In this paper, we show that GSM06 cells produce not mucin, but hyaluronan, and that their synthetic activity may be influenced by the expression of SV40 large T-antigen.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium/Ham's F-12 medium (DME/F-12) was obtained from GIBCO (Grand Island). Fetal bovine serum (FBS) was purchased from SEBAK (Aidenbach). A mixture of insulin, transferrin, ethanolamine, and serinium (ITES) and epidermal growth factor (EGF) were purchased from Wako Pure Chemical (Osaka). Culture flasks (25 cm^2) and 24-well culture dishes, both precoated with type I collagen, were purchased from Iwaki Glass (Tokyo) and Sumitomo Bakelite (Tokyo), respectively. D-[1,6-^{3}H]Glucosamine hydrochloride was obtained from Dupont-New England Nuclear (Boston). *Streptomyces hyalurolyticus* hyaluronidase, *Proteus vulgaris* chondroitinase ABC, hyaluronan, and unsatu-

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CsTFA, cesium trifluoroacetate; EGF, epidermal growth factor; FBS, fetal bovine serum; GuHCl, guanidinium hydrochloride; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SV40, simian virus 40; ⊿Di-HA, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ⊿Di-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ⊿Di-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ⊿Di-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose.

rated disaccharide standards (⊿Di-0S, ⊿Di-4S, and ⊿Di-6S) were purchased from Seikagaku Kogyo (Tokyo). ⊿Di-HA was prepared from hyaluronan by chondroitinase ABC digestion as described below. Trypsin (TPCK-treated) was purchased from Sigma Chemical (St. Louis). Sepharose CL-4B and Sepharose CL-2B were purchased from Pharmacia Biotech (Tokyo).

Cell Culture—GSM06 cells were cultured in a collagen-coated plastic culture flask in DME/F12 medium supplemented with 10% FBS, 100 mg/ml streptomycin, 100 units/ml penicillin, 1% ITES, and 10 ng/ml EGF in a humid 5% CO_2 atmosphere as previously described (*8*). The cells were cultured at 37°C for 24 h and then at 33°C until the monolayers became confluent. After confluence, the cells were passaged by 0.25% trypsin-0.53 mM EDTA treatment (*8*). The cells were counted using a hematocytometer after having been harvested by trypsin-EDTA treatment.

Radio-Labeling of Macromolecules—The cells (2.3×10^5) were cultured in a 24-well collagen-coated plastic culture dish at 37°C for 24 h and then at 33 or 39°C for 1-7 days. They were incubated at 37 or 39°C for 24 h with 370 kBq/ml of [^{3}H]glucosamine (1,542.4 GBq/mmol) in DME/F12 medium supplemented with 100 mg/ml streptomycin, 100 units/ml penicillin, 1% ITES, and 10 ng/ml EGF. At the end of the incubation time, the medium was removed, and the cells were rinsed with phosphate-buffered saline, pH 7.4 (PBS, 8 g NaCl, 0.2 g KCl, 0.2 g NaH_2PO_4, and 2.4 g K_2HPO_4 in 1 liter). They were harvested with a rubber policeman and then homogenized by hand in a glass homogenizer in 6 M guanidinium hydrochloride (GuHCl), pH 7.4, containing 2% Triton X-100, 50 mM Tris, 10 mM EDTA, 2 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 mM pepstatin A. The homogenate was stirred for 15 h at 4°C and centrifuged at $8,000\times g$ for 60 min. The supernatants were applied to a column (1×25 cm) of Sepharose CL-4B previously equilibrated with 4 M GuHCl, pH 7.4, containing 0.5% Triton X-100, 50 mM Tris, 10 mM EDTA, 2 mM benzamidine HCl, and 1 mM PMSF. The excluded fractions from the column were further analyzed using a column (1×25 cm) of Sepharose CL-2B equilibrated with the same solution as for the Sepharose CL-4B chromatography. The medium was analyzed using the same column system.

Cesium Trifluoroacetate (CsTFA) Equilibrium Centrifugation—CsTFA equilibrium centrifugation was performed at $152,000\times g$ for 120 h at 10°C with a starting density of 1.42 g/ml as previously described (*10*).

Enzyme Digestion and Chemical Treatment of Macromolecules—^{3}H-labeled macromolecules were obtained as excluded fractions by Sepharose CL-4B chromatography using 50 mM Tris-HCl, pH 7.2, as an eluent and then digested with TPCK-treated trypsin, *Proteus vulgaris* chondroitinase ABC or *Streptomyces hyalurolyticus* hyaluronidase as already described (*11*). The digests were analyzed by Sepharose CL-2B (in the case of trypsin) or Sepharose CL-4B chromatography. The chondroitinase ABC digests were further analyzed by thin layer chromatography. The digests were desalted, then applied to a cellulose-coated plastic sheet (Merck, Darmstadt) and developed with 1-butanol/acetic acid/1 M ammonia (2 : 3 : 1) as described (*12*). The spots of ⊿Di-HA, ⊿Di-0S, ⊿Di-4S, and ⊿Di-6S added as internal markers were detected by UV absorption measurement. The spots were scraped off the cellulose sheet (5 mm width) and the radioactivity was measured.

^{3}H-labeled macromolecules were also treated with 0.1 M dithiothreitol (DTT) at 4°C for 15 h, then with 0.2 M iodoacetamide to alkylate the SH-groups, and applied to a Sepharose CL-2B column.

RESULTS

Characterization of ^{3}H-Labeled Macromolecules Synthesized and Secreted by GSM06 Cells Incubated with [^{3}H]-Glucosamine—^{3}H-labeled macromolecules were extracted with 6 M GuHCl containing 2% Triton X-100 from GSM06 cells incubated with [^{3}H]glucosamine and obtained from a Sepharose CL-4B column as the excluded fraction (Fig. 1A). The ^{3}H-labeled macromolecules were also obtained from the culture medium as the excluded fraction by Sepharose CL-4B chromatography (Fig. 1B). The included fractions in the column appear to be small glycoproteins on

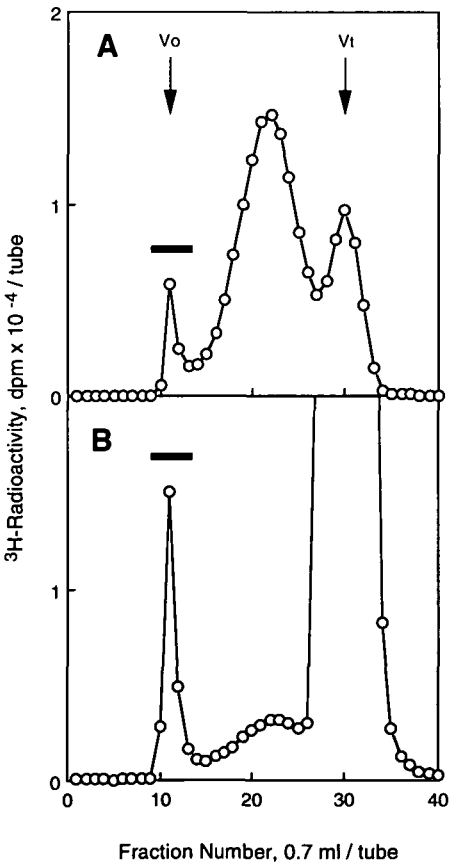

Fig. 1. **Sepharose CL-4B chromatography of the cell extract (A) and the medium (B) obtained from the culture of GSM06 cells in the presence of [^{3}H]glucosamine.** GSM06 cells were cultured for 1 day at 37°C and for 6 days at 33°C, and then cultured for 24 h at 37°C in the presence of [^{3}H]glucosamine. ^{3}H-labeled molecules were extracted from the cells and then applied to a column (1×25 cm) of Sepharose CL-4B previously equilibrated with 4 M GuHCl, pH 7.2, containing 50 mM Tris, 0.5% (v/v) Triton X-100, and protease inhibitors as described in "EXPERIMENTAL PROCEDURES." The culture medium was directly applied to the same column. Each fraction was assayed for ^{3}H-radioactivity. The excluded fractions are indicated by a bar. V_o and V_t, void volume and total volume of the column, respectively.

the basis of their elution positions. CsTFA equilibrium centrifugation has been performed to characterize the ^{3}H-labeled macromolecules. Figure 2 shows that macromolecules obtained from both the cell extract and the medium were recovered at a density of 1.46 g/ml. This result suggests that they may not be mucin because mucin would appear at a density of about 1.43 g/ml under the conditions used (*10*). The possibility that the radio-labeled macromolecule is a nucleic acid or proteoglycan is eliminated because such molecules would have a density higher than 1.46 g/ml under the conditions used.

To determine the size of the ^{3}H-labeled macromolecules excluded from the Sepharose CL-4B column, Sepharose CL-2B chromatography was used. The samples obtained from the medium or the cell extract were excluded from the column (data not shown). The results indicate that the size of this molecule is 10 MDa or more.

The size and density of the ^{3}H-labeled macromolecules indicate that they may be hyaluronan because (i) hyaluronan is known to be extremely large and, therefore, excluded from a Sepharose CL-2B column and (ii) the obtained value of density was consistent with that of hyaluronan. However, it is still possible that the ^{3}H-labeled sample is mucin, because gastric mucin extracted from the rat stomach in the absence of DTT was excluded from a Sepharose CL-2B column (*10*).

To test the above possibilities, the macromolecules were treated with DTT and rechromatographed using the same Sepharose CL-2B column. Mucin was expected to be included in this column after DTT treatment (*13*). However, the ^{3}H-labeled macromolecules obtained from the medium or the cell extract were still excluded from the column after DTT treatment (data not shown). The effect of trypsin digestion was then examined. The macromolecules after trypsin digestion were also excluded from the column (data not shown), although mucin was expected to be included in this column after trypsin digestion (*13*).

Next, the excluded fraction from the Sepharose CL-4B column was treated with *S. hyalurolyticus* hyaluronidase and the digests was rechromatographed on the same column. The ^{3}H-labeled macromolecules obtained from the medium were completely digested and eluted around the column volume (Fig. 3). Similar results were obtained from the cell extract (data not shown). When chondroitinase ABC was used instead of hyaluronidase, a similar degradation was observed (data not shown). The results strongly suggest that the macromolecules produced by GSM06 cells are hyaluronan, because *S. hyalurolyticus* hyaluronidase digests only hyaluronan and chondroitinase ABC also digests hyaluronan under the conditions used. Further evidence was obtained from the analysis of the products of chondroitinase ABC digestion by thin layer chromatography. The products co-migrated with authentic ΔDi-HA on the thin layer plate. Thus, it was concluded from all the results that the ^{3}H-labeled macromolecule was not mucin, but hyaluronan.

Effects of Temperature on Cell Growth and Hyaluronan Synthesis—GSM06 cells are an immortalized cell line harboring the SV40 large T-antigen gene (*8*). The gene product is temperature-sensitive, that is, it acts at 33 or

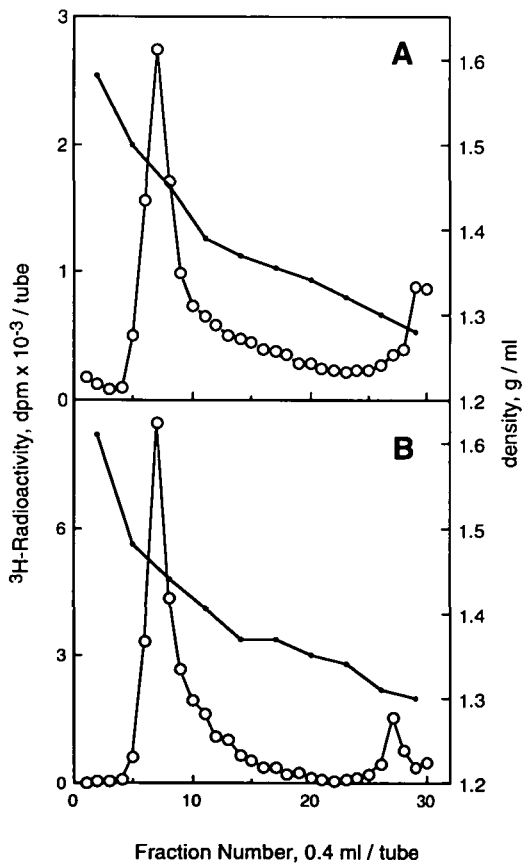

Fig. 2. **CsTFA equilibrium centrifugation of ^{3}H-labeled macromolecules.** ^{3}H-labeled macromolecules were obtained as the excluded fraction from a Sepharose CL-4B column and then centrifuged. Centrifugation was performed at 152,000 × *g* for 120 h at 10°C with a starting density of 1.42 g/ml as already described (*10*). Each fraction was assayed for density (closed circles) and radioactivity (open circles). A, cell extract; B, medium.

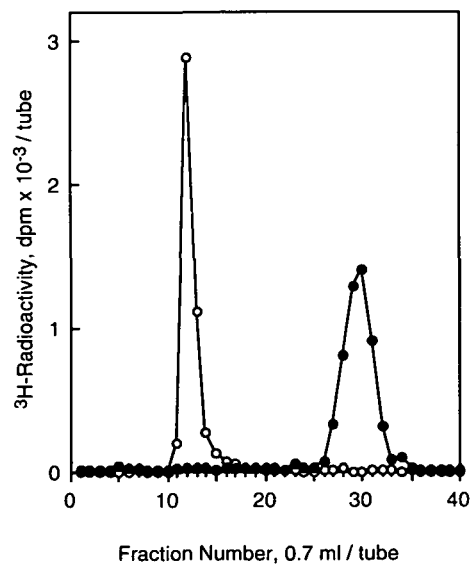

Fig. 3. **Effect of *Streptomyces hyalurolyticus* hyaluronidase digestion.** ^{3}H-labeled macromolecules obtained from the medium as the excluded fraction from a Sepharose CL-4B column were rechromatographed on the same column before (open circles) and after (closed circles) enzyme digestion.

37°C (permissive temperature), but not at 39°C (nonpermissive temperature). The cells grow at the permissive temperature, but not at the nonpermissive temperature (*8, 9*). On the basis of this fact, the hyaluronan synthesis after culture at 33 or 39°C for several days was examined. The cell growth curve (Fig. 4A) shows that the cell grew at 33°C and formed a confluent monolayer after 4 days as previously mentioned. After having been cultured for various times, the cells were incubated for 24 h with [^{3}H]glucosamine. ^{3}H-labeled hyaluronan was obtained from both the cells and medium by Sepharose CL-4B chromatography, and the radioactivity was measured. The hyaluronan synthetic activity (sum of the cell extract and the medium) at this temperature increased time-dependently. However, the synthetic activity per cell did not significantly change during 8 days of culture (Fig. 4B).

In contrast, the cells grew only slightly at 39°C during 8 days of culture. However, the cells synthesized and secreted ^{3}H-labeled macromolecules which were identified as hyaluronan by the hyaluronidase digestion study (data not shown). Despite the nearly constant cell number, hyaluronan synthetic activity increased during the first 3 days, and at Day 3, about 3 times more hyaluronan was synthesized at 39°C compared to the culture at 33°C (Fig. 4C). At Day 5, a nearly equal amount of hyaluronan to Day 3 was synthesized, but the amount was decreased at Day 8.

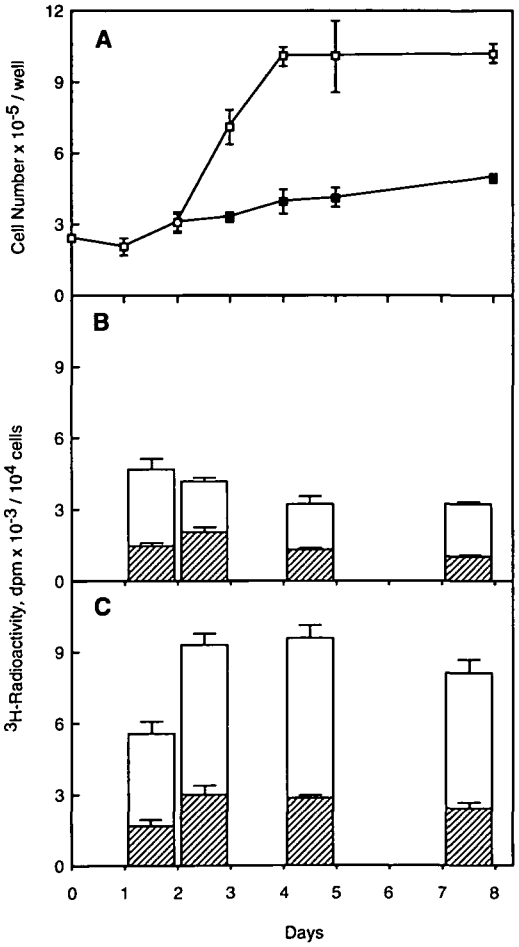

Fig. 4. **Changes in cell number and hyaluronan synthetic activity of GSM06 cells during culture at 33 or 39°C.** GSM06 cells (about 2×10^5/well) were cultured for 1 day at 37°C and then for various days at 33°C (B and open squares in A) or 39°C (C and closed squares in A). One to seven days after the start of culture, cells were cultured for 24 h in the presence of [^{3}H]glucosamine at 37 or 39°C. At the end of culture, the cell was harvested, and the ^{3}H-labeled macromolecules were obtained as the excluded fraction from a column of Sepharose CL-4B. The ^{3}H-labeled macromolecules were also obtained from the culture medium. Open columns in B and C indicate the macromolecules obtained from the medium while hatched columns indicate those from cell extract. Data are the mean ± SD ($n=4$).

DISCUSSION

GSM06 cells have been thought to be gastric mucus cells because (i) the cell line was established from the stomach, (ii) morphological observation indicated that the GSM06 cells are epithelial cells, and (iii) GSM06 cells were stained by periodate-Schiff (PAS) staining (*8, 9*). Therefore, it was expected that the GSM06 cells would synthesize mucin. The incubation of GSM06 with [^{3}H]glucosamine showed that GSM06 does synthesize and secrete ^{3}H-labeled macromolecules. However, the macromolecule was identified as hyaluronan, not mucin, based on the fact that: (i) the size of the ^{3}H-labeled macromolecules was nearly 10 MDa or more, (ii) the density of the ^{3}H-labeled macromolecules was about 1.46 g/ml in CsTFA equilibrium centrifugation, (iii) the macromolecules were resistant to DTT treatment and to trypsin digestion, (iv) the macromolecules were completely digested with *S. hyalurolyticus* hyaluronidase and (v) digestion of the macromolecules with *P. vulgaris* chondroitinase ABC under the appropriate conditions produced ⊿Di-HA.

Because the macromolecules produced by GSM06 cells were not mucin, these cells may not be mucus cells. If this is the case, it may be difficult to explain why GSM06 cells produce PAS-positive materials (*8, 9*). However, the fact that GSM06 cells fixed with Carnoy solution (ethanol/chloroform/acetic acid, 5 : 3 : 1 by volume), which fixes the mucus gel layer more efficiently than other fixatives (*14*), were fairly well stained with PAS (Goso and Hotta, unpublished observation) may resolve the problem. PAS-positive materials are probably hydrophobic compounds, possibly glycolipids. Dohi *et al.* indicated that GSM06 cells produce mucin-like glycoproteins, which could be detected by wheat germ agglutinin on SDS-PAGE in combination with Western blotting (*15*). However, they also indicated that the amount and size of the molecules were different from those of mucin produced by mouse gastric mucosa. Their observations are consistent with the findings in this paper that GSM06 cells did not produce ^{3}H-labeled mucin in a size and amount corresponding to those of gastric mucin.

Hyaluronan is found in the epithelia although it has been mainly regarded as an extracellular component of soft connective tissues. Among epithelia, stratified squamous epithelium, such as epidermis (*16, 17*) and esophagus (*18*), have a high content of hyaluronan. Rodent stomach, which is relatively rich in hyaluronan (*19*), consists of fundus, antrum and forestomach, and forestomach is composed of squamous epithelia. If GSM06 cells differentiated to forestomach squamous epithelial cells, the cells might synthesize hyaluronan. This is possible, because GSM06 cells showed a squamous epithelial-like morphology when they were cultured on a collagen membrane (Goso and Hotta,

unpublished observation). In this study, GSM06 cells cultured on collagen-coated dishes seemed to express some properties of squamous epithelial cells, *i.e.*, hyaluronan production without morphological change. It is unknown at this time whether GSM06 cells have the potential to differentiate to mucus cells producing large mucins under appropriate conditions. Further studies are needed to clarify this point.

GSM06 cells synthesized a greater amount of hyaluronan at 39°C than at 33°C. The results imply that the expression of SV40 large T-antigen may control hyaluronan synthesis, because the antigen was expressed only at a permissive temperature (*8*). The mechanism is unknown, but GSM06 cells may synthesize hyaluronan when cell proliferation is prohibited and they begin to differentiate. Wang *et al.* showed that the parenchymal cells in well or moderately differentiated squamous cell carcinomas expressed hyaluronan, whereas poorly differentiated forms were devoid of it (*18*). Thus, they suggested that hyaluronan could be an indicator of squamous differentiation. However, this seems inconsistent with the observations that hyaluronan production increases in proliferating cells and that hyaluronan may play a role in mitosis (*20*). The discrepancy may be explained by the differences in cell type, *i.e.*, fibroblasts and epithelial cells. Hyaluronan may have many roles in different tissues. Further examination of GSM06 cells may lead to clarification of the function of hyaluronan in epithelial differentiation.

The authors are grateful to Tsukiko Sugaya for her technical assistance and to Mieko Hamano for her helpful advice on cell culture.

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