

Surrounding Cells Affect the Gene Expression Pattern of Human Beta-defensins in Squamous Cell Carcinoma *In Vitro*

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Abstract. *Background/Aim:* Defensins are basic peptides involved in non-immune bio-defense mechanisms in a normal epithelium. Human oral squamous cell carcinoma cells (OSCC) also produce human beta-defensins (HBDs), although their exact function is not clear. This study aimed to analyze the variation in gene expression levels of hBDs in co-cultures of OSCC with murine cells. *Materials and Methods:* Two OSCC cell lines (HSC-3, HSC-4) were co-cultured with mouse embryonic fibroblasts, NIH/3T3 or a mouse chondrogenic cell line derived from teratocarcinoma, ATDC5, for 1.5 days. Expression patterns of the hBD genes were investigated by real-time polymerase chain reaction (RT-PCR). *Results:* hBD1 expression increased when co-cultured with NIH/3T3 but decreased when co-cultured with ATDC5. Expression of hBD2 and hBD4 tended to decrease. OSCC cells formed colonies when co-cultured with NIH/3T3 but were scattered when co-cultured with ATDC5. *Conclusion:* hBDs expression in OSCC is dependent on the type of co-cultured cells and differences in gene expression may be responsible for the morphological differences observed. OSCC may produce HBDs for purposes other than bio-defense by surrounding cells.

Human beta-defensins (HBDs) are basic peptides that play important roles in the non-immune biological defense mechanism in a normal epithelium (1, 2). The response of HBDs occurs faster than the immune response in normal

epidermal tissues (3). Recently, other roles of beta-defensins have been reported. For example, human beta-defensin-1 (HBD1), -2 (HBD2) and -3 (HBD3) have been found in mucocoeles where neither infection nor inflammation was detected (4). There is also a change in the pattern of immunoreactivity for anti-HBD1 and HBD2 antibodies in minor salivary glands in cases of Sjögren's syndrome, an autoimmune disease (5). A change in the pattern of the immunoreactivity of anti-HBDs antibodies has also been observed in the tracheal epithelium of a mouse model of the sick building syndrome induced by toluene exposure (6). These studies indicate that the expression of hBDs does not always correlate with immunological defense.

Using reversed-phase high-performance liquid chromatography (HPLC), Sawaki *et al.* showed that the concentration of HBD2 in OSCC cells is much higher than that in normal oral epithelium (7) and that it may not reflect the essential role of biological defense. These authors also investigated the effect of HBD2 on mitotic activity under a combination culture (co-culture) system. When HSC-4 was co-cultured with normal human epidermal keratinocytes (NHEK), the mitotic index (MI) of NHEK was reduced; however, MI of HSC-4 was not changed. Interestingly, the MI of both HSC-4 and NHEK was not changed in co-culture with the anti-HBD2 antibody (8). These results suggested that HBD2 affected the mitotic activity of NHEK but not that of HSC-4, and it is still not clear why HBD2 does not inhibit mitosis of HSC-4. Using *in situ* hybridization, it was reported that hBD2 mRNA was highly concentrated in an implanted OSCC (9). Interestingly, normal epithelial cells surrounding OSCC aggregates strongly expressed hBD2. It was suggested that HBD2 might lead to death of normal keratinocytes adjacent to OSCC and subsequently assist in the multiplication of tumor cells indirectly.

It has still not been well-defined why OSCC and not normal epithelial cells have an increased expression of hBD genes. There are two hypotheses on the role of HBDs in cancer cell

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proliferation; one is that HBD2 and HBD3 promote proliferation of cancer cells (10) and the other is that HBD1 and HBD2 suppress proliferation of cancer cells (10, 11). Gerashchenko *et al.* reported that high concentrations of HBD4 suppress cancer cell proliferation but low concentrations promote cell-cycle progression (12). HBDs are well-known to have an anti-microbial role, but this suggests that HBDs must also have a role in cancer proliferation, although the mechanism is unknown.

In the present study we analyzed the gene expression of *hBDs* by using real-time polymerase chain reaction (RT-PCR) in OSCC cell lines co-cultured with different cell types derived from mouse. We analyzed the gene expression pattern of *hBD1*, 2 and 4 and attempted to correlate the gene expression pattern of *hBDs* with morphological changes of OSCC cancer cell populations.

Materials and Methods

Cell cultures. Two cell lines derived from human squamous cell carcinoma of tongue were used in this study: HSC-3 (JCRB0623, Health Science Research Resources Bank (HSRRB), Osaka, Japan) and HSC-4 (JCRB0624, HSRRB, Osaka, Japan). Two cell lines derived from mouse, NIH/3T3 (fibroblastic cell line) and ATDC5 (a chondrogenic cell line derived from teratocarcinoma AT805, kindly provided by Prof. Takigawa, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences) were used as partners of the combination culture (co-culture).

Cells were seeded at a concentration of 1×10^6 cells / ml, in a 3-cm petri dish. Cells were cultured in DMEM/F12 + GlutaMAX (Life Technologies Corporation, Carlsbad, CA, USA) containing 1% antibiotics (penicillin and streptomycin; PAA Laboratories GmbH, Pasching, Austria) and 5% fetal bovine serum (NICHIREI BIOSCIENCE INC., Tokyo, Japan) at 37°C in a humidified chamber with 5% CO₂. After 1.5 days, the cell density reached 95% confluence.

Each cell line was subcultured once every 3 days using 1,500 U/ml Dispase II (Eidia Tokyo, Japan) buffered with Ca²⁺- and Mg²⁺-free Tyrode's solution. Images of cultured cells were taken with a phase contrast microscope (Axiovert 405M, ZEISS, Jena, Germany).

Quantitative real-time RT-PCR. After 1.5 days of co-culture, cells were fixed with RNAlater (Qiagen, GmbH, Hilden, Germany) at 4°C overnight and then RNA was isolated and purified using the RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Valencia, CA, USA). cDNA was synthesized by reverse transcription of each sample with a Ready-To-Go T-primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ, USA).

The sequences of forward and reverse primers for human *hBD1-4* were generated from the sequences of the coding region available through the National Center for Biotechnology Information (NCBI, accession numbers: NM_005218, AF_040153, NM_004942, respectively). Primer sequence of human beta-actin (*ACTB*) was generated from sequences of the coding region available through NCBI (accession no. NM_001101). All primers were designed with the Primer 3 software (<http://primer3.sourceforge.net/>) and are summarized in Table I. These primers were specific for human *hBD* genes. Specificity of PCR was confirmed by sequencing, 1% agarose gel electrophoresis and melting curve analysis.

Real time RT-PCR was performed with a Light Cycler® 1.5 ST300 (Roche Diagnostics, Mannheim, Germany) with SYBR Green detection (SYBR® Premix Ex Taq I, Takara Bio Inc., Shiga, Japan). A cDNA library derived from normal human skin (HD-101, Zyagen, San Diego, CA, USA) was used in this study. PCR products of HBD1-4 were prepared with each primer pair and serial dilution of each product was used as a template to generate a standard curve. *ACTB* was used as a reference gene. We also prepared primer pairs of *hBD3* (NCBI, accession no. NP_001075020.1); however, no PCR products were obtained.

All values are expressed as mean ± standard deviation (SD) (n>25). Data from real-time RT-PCR were evaluated by the Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Immunohistochemistry. Immunoreactivity for anti-HBD1 antibody (diluted 1:500; Peptide Institute, Osaka, Japan) was revealed by 3, 3'-diaminobenzidine (DAB) color reaction using the VECTASTAIN Elite ABC Kit (Vector, Burlingame, CA, USA), according to methods described by Kaneda *et al.* (5). After immunostaining at room temperature, cells were observed by a stereomicroscope (Leica, MZ 125, Wetzlar, Germany) using a bright and a semi-dark condenser without counterstaining.

Results

Comparison of gene expression of *hBDs* in HSC-3:

(A) Expression profile of the *hBD1* transcript. The expression of *hBD1* was significantly increased in HSC-3 co-cultured with NIH/3T3 (mean value: 249% for HSC-3 only, *p*<0.05) (Figure 1A) but significantly decreased in co-culture with ATDC5 (mean: 32% for HSC-3 only value, *p*<0.05) (Figure 1D). There were no significant differences in *hBD1* in HSC-3 when co-cultured with ATDC5.

(B) Expression profile of the *hBD2* transcript. The expression of *hBD2* was significantly decreased in HSC-3 co-cultured with NIH/3T3 (mean value: 38% for HSC-3 only, *p*<0.05) (Figure 1B). There were no significant differences in *hBD2* in HSC-3 when co-cultured with ATDC5 (Figure 1E).

(C) Expression profile of *hBD4* transcript. There was no significant difference in *hBD4* expression in HSC-3 co-cultured with NIH/3T3, ATDC5 (Figure 1C, F).

Comparison of gene expression of *hBDs* in HSC-4:

(A) Expression profile of the *hBD1* transcript. The expression of *hBD1* was significantly increased in HSC-4 co-cultured with NIH/3T3 (mean: 517% for HSC-4 only value, *p*<0.05) (Figure 2A) but significantly decreased in co-culture with ATDC5 (mean: 14% of HSC-4 only value, *p*<0.05) (Figure 2D).

(B) Expression profile of the *hBD2* transcript. There were no significant differences in *hBD2* expression in HSC-4 co-cultured with NIH/3T3 and ATDC5 (Figure 2B, E).

(C) Expression profile of the *hBD4* transcript. There were no significant differences in *hBD4* expression in

Table I. Primer pairs of human *hBD1-4* and beta-actin (*ACTB*). Primer pairs of human *hBD1-4* and beta-actin (*ACTB*) used for real time RT-PCR are summarized in Table I. The sequences of primers for the human *BD1-4* were designed from sequences of the coding region available through the National Center for Biotechnology Information mentioned in Materials and Methods.

Name of target cDNA	Accession No.	Primer sequence (5'→3')		Amplicon size (bp)
		forward	reverse	
<i>hBD1</i>	NM_005218	CGC CAT GAG AAC TTC CTA CC	ACA GGT GCC TTG AAT TTT GG	229
<i>hBD2</i>	AF_040153	ATC AGC CAT GAG GGT CTT GT	GAG ACC ACA GGT GCC AAT TT	172
<i>hBD4</i>	NM_004942	CTC AGC TCC TGG TGA AGC TC	GGA TCG CCT ATA CCA CCA AA	116
beta-actin (<i>ACTB</i>)	NM_001101	TCC CTG GAG AAG AGC TAC GA	AGC ACT GTG TTG GCG TAC AG	184

HSC-4 co-cultured with NIH/3T3 (Figure 2C). The expression ratio of *hBD4* was increased significantly in HSC-4 co-cultured with ATDC5 (mean value: 207% of HSC-4 only, $p < 0.05$) (Figure 2F).

Microscopic observation of cultured HSC-3, -4 cells. After co-culturing with NIH/3T3 for 1.5 days, HSC-3 and -4 cells formed small colonies surrounded by NIH/3T3 cells (Figure 3C, G and Figure 4A, C). After co-culturing with ATDC5 for 1.5 days, obvious colonization of HSC-3, -4 cells could not be found (Figure 3D, H). The boundary between HSC-3 or -4 and ATDC5 was not clear. There were no colonies or aggregations of HSC-3 (Figure 3A), HSC-4 (Figure 3E), NIH/3T3 (Figure 3B) and ATDC5 (Figure 3F) cell lines when cultured individually.

Discussion

Beta-defensins are anti-microbial peptides and their expression changes depending on bacterial infection and/or localization of inflammatory cytokines (13, 14). However, change in the pattern of immunoreactivity of HBDs has been reported in several diseases not related to biological defense mechanisms, *e.g.* mucocoeles (4), Sjögren's syndrome (5) and in a model of sick building syndrome (6). Yoshimoto *et al.* reported that an aggregation of OSCC under a mouse back skin expressed *hBD2* by *in situ* hybridization with an *hBD2* pan-probe. However, the mechanism of expression was not clear (9). HBDs are produced generally for biological defense; however, they may have other roles and mechanisms in OSCC. Gerashchenko *et al.* have reported that *in vitro*, *HBD4* enhances or suppresses viability of SCC cell lines depending on its concentration (12). However, the critical level of HBDs that either activate or inhibit cancer growth was not detected.

We attempted to study the change in the pattern of *hBD* gene expression in colony formation of OSCC using real

time RT-PCR. In this study, we analyzed the gene expression of *hBDs* when HSC-3 or HSC-4 cells were co-cultured with NIH/3T3 or ATDC5. Primers using in the real-time RT-PCR were specific for human *hBD1, 2, 4* genes and co-culture partner of NIH/3T3 or ATDC5 cells are derived from mouse. The morphology of HSC-3 and HSC-4 cell populations was different when the cells were co-cultured with NIH/3T3 or ATDC5 cells. Aggregations of HSC-3, -4 cells were formed in the co-culture with NIH/3T3 (Figure 3C, G) but not with ATDC5 (Figure 3D, H). HSC-3, -4 cells were simply scattered in the co-culture with ATDC5 (Figure 3 D, H). Immunoreactivity for anti-HBD1 antibody was detected intensely in colonies of HSC-4 with NIH/3T3 (Figure 4A) and was detected weakly in the scattered population of HSC-4 with ATDC5 (Figure 4B). These observations were similar to what was found in the report that HSC-4 produces HBDs (9) *in vivo*. The type of cell that was co-cultured with HSC-3 or HSC-4 resulted in different colonizations. Thus, there may be some correlations between morphology of OSCC cell population and gene expression of *hBDs* in co-culture.

The gene expression of *hBD1* was increased in co-cultures of HSC-3, -4 with NIH/3T3, whereas the gene expression of *hBD2* and *hBD4* was reduced or unchanged. The gene expression of *hBDs* was down-regulated when HSC-3 or -4 were co-cultured with ATDC5. The aggregated population of HSC-4 cells expressed an up-regulation of *hBD1* gene; however, the scattered population of HSC-4 cells showed a trend in down-regulation of *hBD1* gene. The characteristics of the two different co-culture partners used in this study are different. NIH/3T3 is a cell line derived from mouse fibroblasts, ATDC5 is derived from mouse teratocarcinoma and has the potential to differentiate into chondrocytes (15, 16). ATDC5 and OSCC cells may have more similarities compared to NIH/3T3. Originally, HBDs were considered as biological defense factors that work prior to the immune

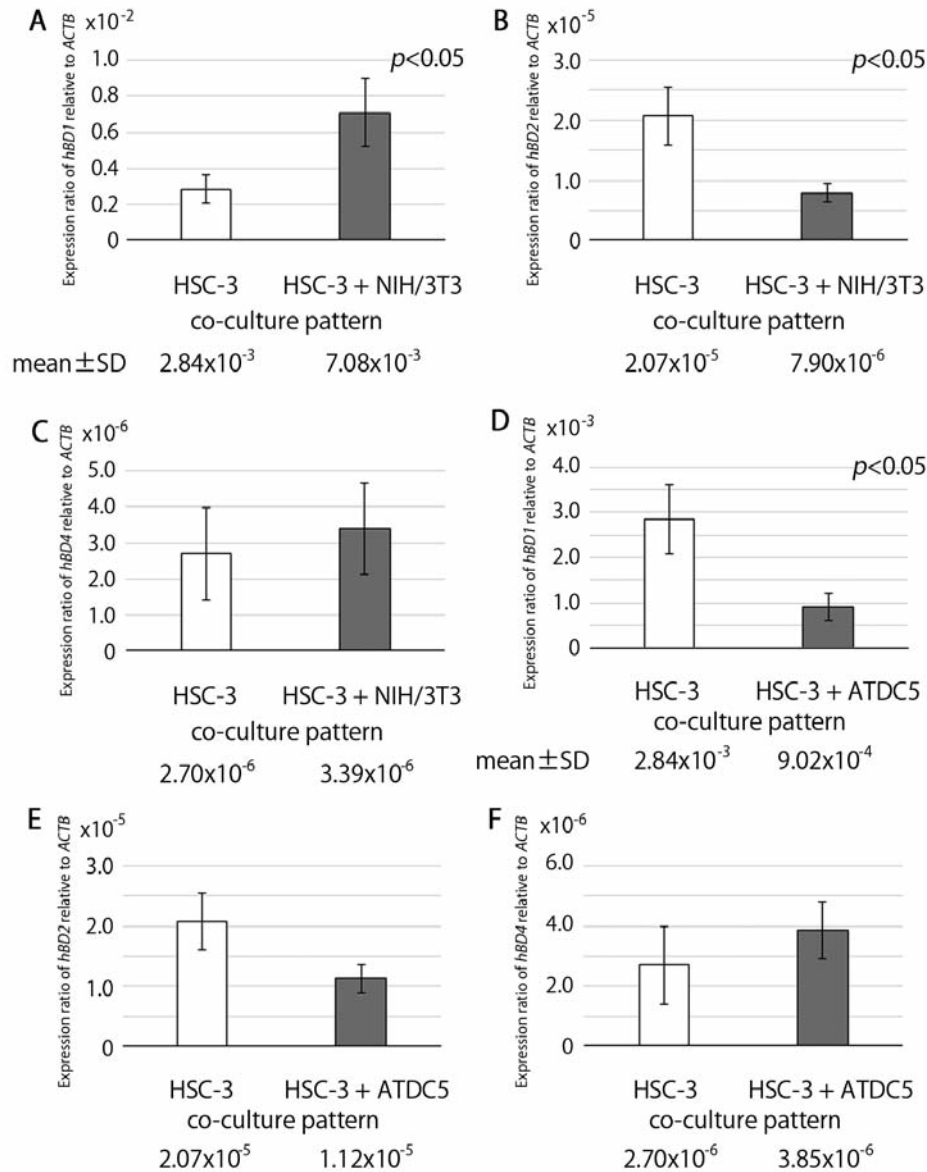


Figure 1. Expression rate of hBD1, 2, 4 genes relative to (ACTB) in HSC-3. (A-C), Expression of hBD1, 2, 4 in HSC-3 in single culture in comparison to co-culture of NIH/3T3; (D-F), expression of hBD1, 2, 4 in HSC-3 in single culture in comparison with co-culture of ATDC5; Abscissa, co-culture patterns and mean \pm SD; Ordinate, shows expression ratio of hBDs relative to (ACTB). The expression of hBD1 was significantly increased ($p < 0.05$) in co-culture with NIH/3T3 but significantly decreased in co-culture with ATDC5. The expression of hBD2 was also significantly decreased in co-culture with NIH/3T3 and ATDC5.

system. However, the original role of HBDs in biological defense might not be applied to co-cultures of OSCC and ATDC5 cells. Additionally, NIH/3T3 can be considered as a part of the subcutaneous tissue *in vitro* (17).

Wenghoefer *et al.* reported that hBD1 expression of OSCC was less than that of healthy gingiva *in vivo* (18). Joly *et al.* compared the expression level of hBD1, 2, 3 of normal gingival cell lines and OSCC cell lines and reported that they

were less in OSCC (19). In this study, we detected changes in the pattern of gene expression of hBD1, 2 and 4 that were produced by human OSCC by real time RT-PCR using co-culture with mouse cell lines. Zhuravel *et al.* suggested that HBD2 suppresses cell growth of malignant epithelial cells *via* arrest of G₁/S transition (11). A study by Winter *et al.* suggests that hBD1 might have a function as a tumor suppressor gene, but that hBD2 and hBD3 might be proto-

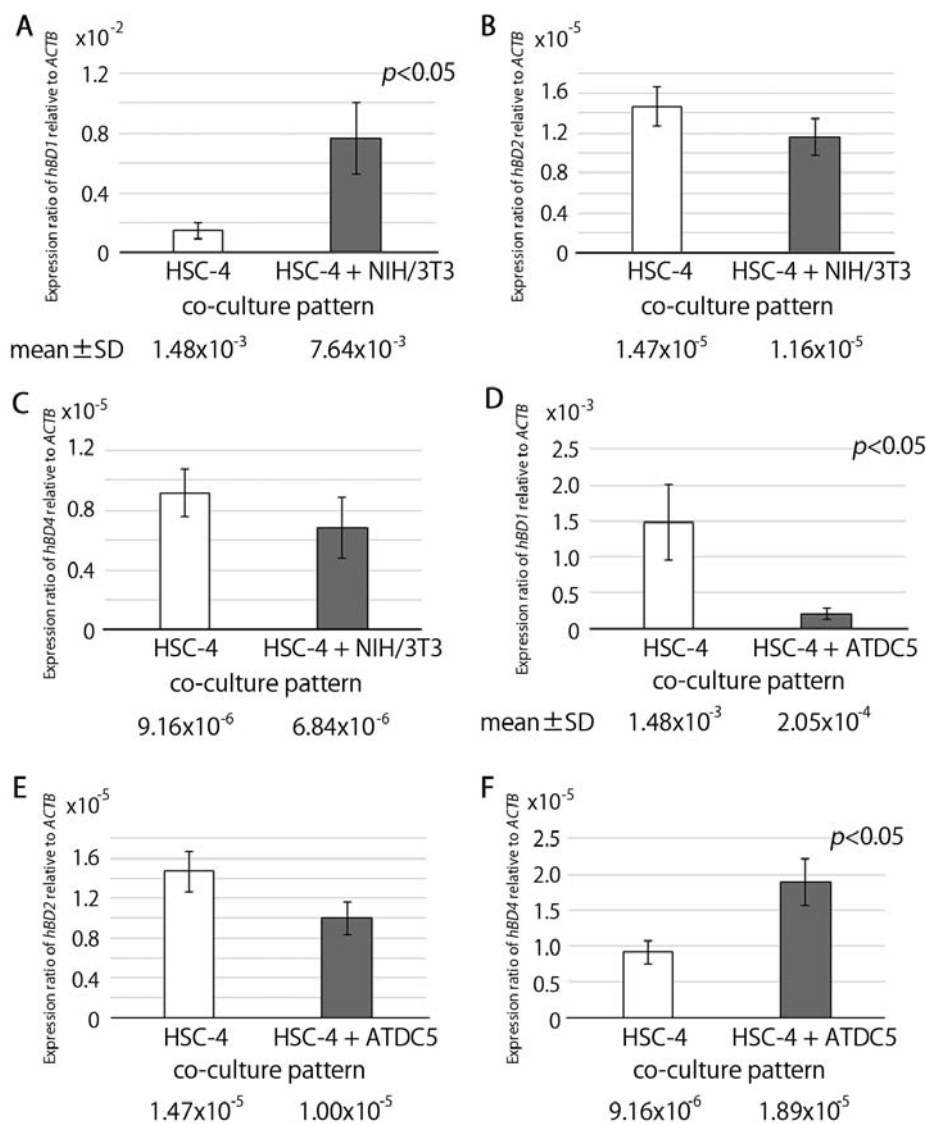


Figure 2. Expression of hBD1, 2, 4 relative to (ACTB) in HSC-4. (A-C), expression of hBD1, 2, 4 in single cultures of HSC-4 in comparison with co-culture with NIH/3T3; (D-F), expression hBD1, 2, 4 single cultures of HSC-4 in comparison with co-culture with ATDC5; Abscissa, co-culture patterns and mean \pm SD; Ordinate, show expression ratio of hBDs relative to (ACTB). The expression of hBD1 in co-cultures with NIH/3T3 was significantly increased but the expression of hBD1 significantly decreased in co-culture with ATDC5. The expression of hBD4 was significantly increased in co-culture with ATDC5. There were no significant differences in hBD2 expression in co-culture with NIH/3T3 and ATDC5 or hBD4 expression in co-cultures with NIH/3T3.

oncogenes in OSCC (10). It has also been proposed by Abiko *et al.* that HBDs produced by cancer are related to susceptibility to bacterial infection (20).

The results of the present study show that HBD1 affects the aggregation (colony) formation of OSCC when co-cultured with NIH/3T3. It is necessary to block *hBD* gene expression in co-culture systems, for instance using siRNA or anti HBD antibodies, to clarify the relationship between the gene expression of *hBDs* and the aggregation formation of OSCC.

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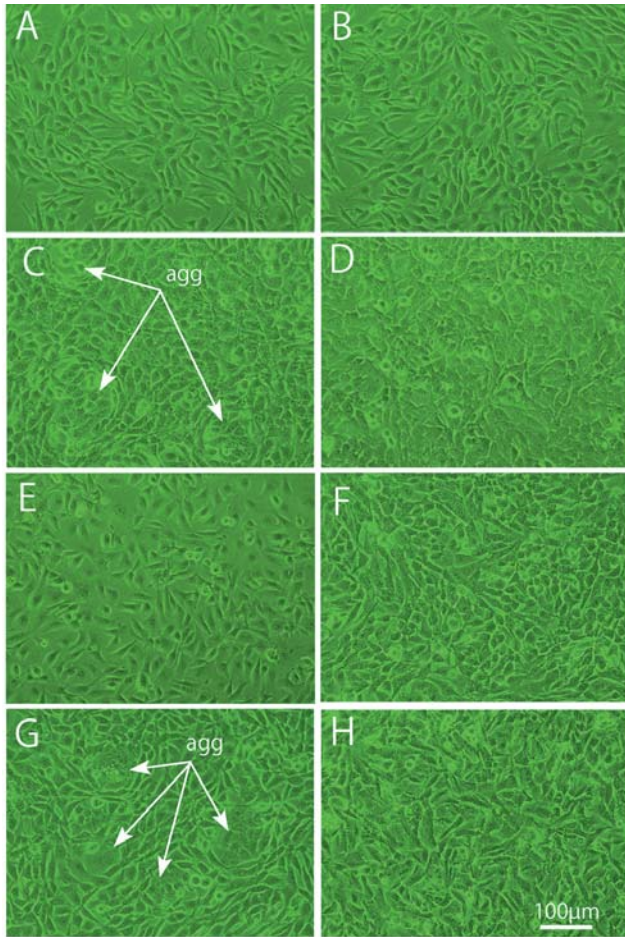


Figure 3. Phase-contrast microscopic images of cultured cells. Phase-contrast microscopic images of cultured cells. A, single cultured HSC-3; B, single cultured NIH/3T3; C, co-cultured HSC-3 with NIH/3T3; and D, co-cultured HSC-3 with ATDC5; E, single cultured HSC-4; F, single cultured ATDC5; G, co-cultured HSC-4 with NIH/3T3; and H, co-cultured HSC-4 with ATDC5. Arrows with "agg" in C or G indicate HSC-3 or -4 cells that were aggregated like colonies in the co-culture with NIH/3T3. Aggregates of HSC-3 or -4 cells were surrounded by NIH/3T3. However, there were no aggregations of HSC-3 (D) or -4 (H) cells in the co-culture with ATDC5. Magnifications of A to H are the same; scale bar 100 µm.

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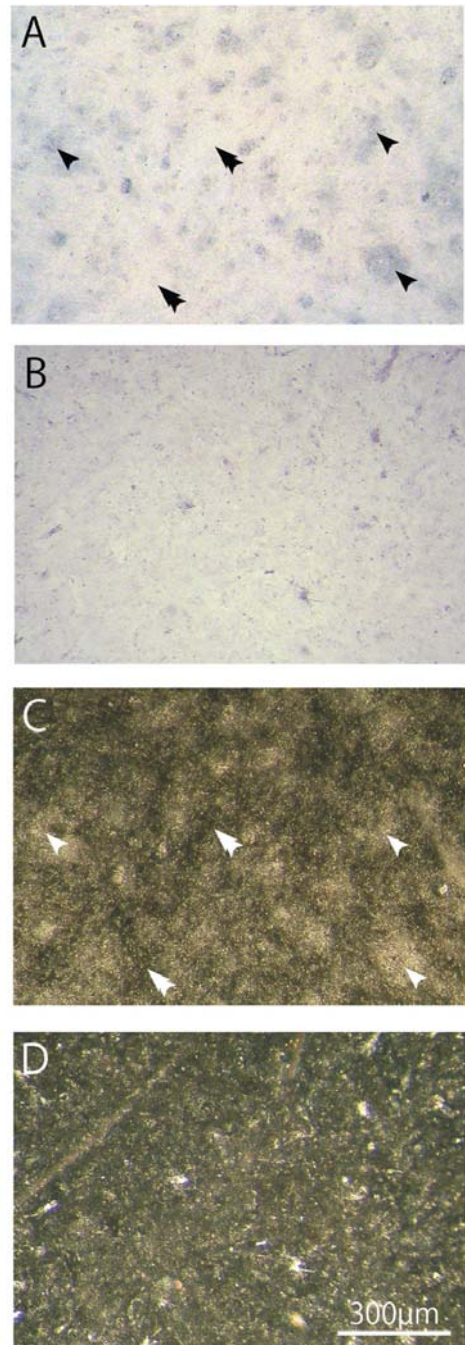


Figure 4. Immunoreactivity for anti-HBD-1 antibody in HSC-4 cells. Microscopic observations of immunoreactivity for anti-HBD-1 antibody. Dark brown-colored positive signals for anti-HBD-1 antibody were found in A and B; C and D, same images of A and B using a semi-dark field condenser; A and C, cultured HSC-4 with NIH/3T3; B and D, co-cultured HSC-4 with ATDC5 for 1.5 d. The double arrowheads indicate NIH-3T3, arrows indicate ATDC5. Dark brown cells indicate positive signals for anti-HBD-1 antibody. No immunoreactivity for HBD-1 was found in NIH/3T3 or ATDC5 cells. Cell aggregates of HSC-4 (arrowheads in A, C) that appeared as colonies were observed in co-culture with NIH/3T3. No aggregates of HSC-4 cells were observed in co-culture with ATDC5 (B, D). Magnifications of A to D are the same; scale bar 300 µm.

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