

Title	The function of connexin 43 on the differentiation of rat bone marrow cells in culture.
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Journal	Biomedical Research, 27(6): 289-295
URL	http://hdl.handle.net/10130/32
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The function of connexin 43 on the differentiation of rat bone marrow cells in culture

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(Received 27 September 2006; and accepted 26 October 2006)

ABSTRACT

Connexin (Cx) 43-mediated gap-junctional intercellular communication (GJC) mainly regulates the osteoblastic differentiation, but much of the function of Cx43 on the differentiation of bone marrow cells is unclear. This study is aimed to clarify relationship between the differentiation of rat bone marrow cells and the function of Cx43. Bone marrow cells derived from four-week-old Wistar strain rats were grown in the presence and absence of 18- α -glycyrrhetic acid (AGA, 100 μ M) to inhibit Cx43-mediated GJC. Expression of Cx43 gene and protein, and the level of intracellular cyclic adenosine monophosphate (cAMP) were determined as the assessment of the function in Cx43-mediated GJC, and alkaline phosphatase (ALP) activity and mineralization were measured as the assessment of osteoblastic differentiation. The Cx43 gene expression was first observed at 2 days, but under the condition in which rat bone marrow cells were treated with AGA, there was no significant effect on the Cx43 gene expression. By administrating AGA to rat bone marrow cells, all parameters of maturation but the Cx43 gene expression significantly decreased. The results of this experiment suggest that Cx43-mediated GJC plays a critical role in rat bone marrow cells, progress toward maturation.

Connexin (Cx) comprise a family of gap-junction structural proteins, which are associated as hexamers in plasma membranes of adjacent individual cells to form intercellular channels that tightly assemble (2, 18). Gap junctions are involved in development, differentiation, and homeostasis as intercellular communication channels for small-molecular-weight substances of less than 1 kDa, such as ions, cAMP, and IP3 (13, 16, 17, 35). Cx43 and Cx45 have been detected in cultured osteoblastic cells and calcified tissues, but gap-junctional intercellular communication (GJC) among cultured

osteoblastic cells is best correlated with Cx43 expression (6, 12, 14, 21, 31, 34); Lecanda *et al.* reported that Cx43-mediated GJC can upregulate the expression of osteoblastic differentiation markers, osteocalcin and bone sialoprotein, in cultured osteoblastic cell lines (14). Cx43 has been detected in the developing chick mandible, and it has been noted that Cx43 is expressed before development of the osteogenic phenotype in bone-forming regions (21).

Recent research has shown that age-related impairment of osteogenesis involves a decrease in the proliferation and differentiation capacity of preosteoblasts or mature osteoblasts (1, 8-11, 25) and in the reactivity to growth factors such as transforming growth factor- β (TGF- β) and insulin-like growth factor-I (IGF-I) (22, 24, 26). Haraguchi *et al.* suggested that Cx 43 might be involved in the age-related decrease in the differentiation of bone marrow cells (7). However, few studies have investigated the relationship between age-related changes in bone-

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forming capacity and Cx43 function. Moreover, much of the function of Cx43 on the differentiation of bone marrow cells is unclear. First of all, in this study, we focused on the function of Cx43, and examined that the effect of long-term inhibition of Cx43-mediated GJC on the differentiation of bone marrow cells in culture.

MATERIALS AND METHODS

Animals. Four-week-old male Wistar strain rats (with a body weight of about 100 g each) were used in this study. All animals were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and housed at the Tokyo Dental College at Ichikawa General Hospital, in the research-building animal breeding room. All experiments were performed in accordance with the guidelines for experimental laboratory animals in the animal facility of Tokyo Dental College.

Cell cultures of bone marrow. The bilateral femora of rats were removed and rat bone marrow (RBM) cells were flushed out, pooled, and cultured according to the method of Maniatiopoulos *et al.* at a primary density of one femur per 75-cm² tissue culture flasks (Asahi Techno Glass Corp., Chiba, Japan) (19). RBM cells were grown in the standard culture medium consisted of α -minimal essential medium (α -MEM; Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen Corp.), 10 mM Na- β -glycerophosphate (Sigma Chemical Corp., St. Louis, MO, USA), 10⁻⁸ M dexamethasone (Sigma Chemical Corp.), antibiotics (50 μ g/mL Gentamicin [Sigma Chemical Corp.] and 0.3 μ g/mL Fungizone [Invitrogen Corp.]), and 50 μ g/mL ascorbic acid (Sigma Chemical Corp.) in a humidified, 95% air and 5% CO₂, 37°C incubator. After 6 days of primary culture, RBM cells were trypsinized, counted, and subcultured at a density of approximately 3 \times 10³ cells/cm² for the experiments. The culture medium was changed every 2 days.

AGA and vehicle treatment. RBM cells were treated with 100 μ M 18- α -glycyrrhetic acid (AGA; Sigma Chemical Corp.) in order to prevent the Cx 43-mediated gap-junctional permeability in these cells (5, 27). AGA was prepared as 1-mM stocks in dimethylsulfoxide (DMSO; Wako) and diluted as needed. The final DMSO concentration was always less than 0.1%. On the next day of subculture (day 1), RBM cells were grown in the presence and absence of 100 μ M AGA in the standard culture medium for culture periods ranging from 1 to 27 days. AGA

treatment was also performed every 2 days, and culture medium replacement and AGA treatment of RBM cells did not occur on the same day.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. For RNA isolation, each group of RBM cells (n = 2) was seeded into a 100-mm culture dish (Asahi Techno Glass Corp.). At the time-points indicated in the figures, total RNA was extracted from these cells using the RNeasy[®] Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's specifications. The RNA concentration was determined spectrophotometrically and the RNA was stored at -80°C until use. For analysis of Cx43 gene expression by RT-PCR, 100 ng of total RNA was reverse-transcribed to synthesize complementary DNA (cDNA) and PCR amplification using a SuperScript[™] One-step RT-PCR with Platinum[®] Taq (Invitrogen Corp.). The primer used were rat specific Cx43 (438-bp product: 5'-AAAGGCGTTA AGGATCGCGTG-3' and 5'-GTCATCAGGCC GAGGCCT-3') and glyceraldehyde phosphate dehydrogenase (GAPDH, 832-bp product: 5'-TTGACCT CAACTAVATGG-3' and 5'-TCATACCAGGAAAT GAGC-3') (7). For cDNA synthesis and pre-denaturation, each mixture was incubated at 50°C for 30 min, and heated at 94°C for 2 min. For PCR amplification, they were subjected to 45 cycles at 94°C for 15 sec, 50°C for 30 sec, and 72°C for 1 min. After reaction, PCR-amplified products were analyzed on 1.7% agarose gel containing ethidium bromide.

Immunofluorescence staining. The staining of the RBM cells with Cx43 antibody was done using indirect immunofluorescence. Each group of RBM cells (n = 6) was seeded into a 35-mm culture dish (Asahi Techno Glass Corp.), and at the timepoints indicated in the figures, the localization of Cx43 protein was determined as described previously (7, 32, 33). The mouse anti-Cx43 monoclonal antibody (Chemicon International, Temecula, CA, USA) diluted at 1 : 50 as primary antibody and FITC-conjugated goat anti-mouse IgG (Biosource International, Camarillo, CA, USA) diluted at 1 : 100 as second antibody were used. For a negative control, the specimens were incubated without primary antibody. Cx43-positive spots were observed under a fluorescence microscope (Axioplan 2 Imaging; Carl Zeiss Corp., Göttingen, Germany) with a B excitation spectrum filter. Cx43 immunofluorescence was evaluated by counting from 50 to 100 RBM cells in each area at \times 200 magnification by random sampling, and calculating the percentage of positive

cells as the Cx43 positive ratio.

Measurement of intracellular cyclic adenosine monophosphate (cAMP). Each group of RBM cells ($n = 6$) was seeded into a 48-well microplate (Asahi Techno Glass Corp.). At the timepoints indicated in the figures, the intracellular cAMP concentration in the RBM cells was determined using a cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences Corp., Piscataway, NJ, USA) following the manufacturer's specifications. The intracellular cAMP concentration was determined spectrophotometrically at 450 nm using a microplate reader (Milenia Kinetic Analyzer; Diagnostic Products Corp., Los Angeles, CA, USA) against a standard curve, after the enzymeimmunoassays were completed. To determine the relative amount of intracellular cAMP, the cellular protein concentration in the same supernatant was measured using a Coomassie[®] Plus Protein Assay Reagent Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's specifications. The level of intracellular cAMP was expressed as fmol/ μ g protein.

Quantification of alkaline phosphatase (ALP) activity. Each group of RBM cells ($n = 6$) was seeded into a 24-well microplate (Asahi Techno Glass Corp.). At the timepoints indicated in the figures, RBM cells were lysed with CellLytic[™]-M (Sigma Chemical Corp.) according to the manufacturer's specifications, and aliquots of the supernatant were used for ALP and cellular protein measurements as described previously (3, 20). ALP activity was determined spectrophotometrically at 405 nm by measuring the conversion to *p*-nitrophenol from *p*-nitrophenylphosphate (*p*NPP; Sigma Chemical Corp.) after combining 20 μ L of supernatant with 180 μ L buffer-substrate solution at 37°C (pH 10.4) in 96-well plates using a microplate reader. The amount of *p*-nitrophenol released was measured every 2 min for a 10-minute incubation period against a standard curve. Values were also normalized to total protein as described in the intracellular cAMP section. ALP activity was expressed as IU/ μ g protein.

Observations of calcified nodules and analysis of mineralization. To observe calcified nodules in the culture dishes, each group of RBM cells ($n = 6$) was seeded into a 35-mm culture dish. At the timepoints indicated in the figures, mineralization was determined as described previously (28, 30). RBM cells were fixed with ice-cold 70% ethanol for 60 min and stained with 1 mL of 40 mM Alizarin Red S

(Sigma Chemical Corp.), pH 4.2, for 10 min. To reduce nonspecific binding of Alizarin Red S, cultures were then washed five times with MilliQ Water and once for 15 min with calcium and magnesium-free phosphate-buffered saline (PBS; Dainippon Pharmaceutical Corp., Osaka, Japan) at room temperature. The mineral-bound Alizarin Red S per unit area of culture dish surface (μ g/cm²) was measured using a spectrophotometer at a wavelength of 562 nm (using a standard curve) after the staining dye was dissolved by a 15-min incubation in 1 mL of 10% cetylpyridinium chloride (Sigma Chemical Corp.) and 10 mM sodium phosphate (Wako, pH 7.0).

Statistical analysis. The data were expressed as the mean \pm SD and were statistically analyzed using Student's *t*-test for each experiment. *p* values less than 0.05 were considered to be significant.

RESULTS

Effect of AGA on GJC function during maturation of RBM cells

To evaluate the function of GJC on the differentiation of RBM cells, we analyzed the expression of Cx43 mRNA and protein, and measured the level of intracellular cAMP in these cells. The results of RT-PCR analysis in RBM cell homogenates are shown in Fig. 1A. Cx43 mRNA expression was first observed in both vehicle and AGA treated cells at 2 days and continually expressed until 13 days of culture. The expression of Cx43 mRNA showed the upward trend as these cells progressed toward a more mature phenotype. Under the condition in which RBM cells were treated with AGA to inhibit Cx43-mediated GJC, there was no significant effect on the Cx43 mRNA expression. Expression of Cx43 protein was examined in RBM cells by immunofluorescence analysis. Cx43-positive spots in both vehicle and AGA treated cells appeared as a punctate immunoreactive pattern, mostly at the cell-to-cell boundaries (Fig. 1B). The percentage of cells with Cx43 positive spots increased as these cells grew toward a more mature phenotype. By administration of AGA to RBM cells, the Cx43 positive ratio significantly decreased at all of the timepoints determined (Fig. 1C). Intracellular cAMP increased until up to 9 days of subculture and decreased thereafter in both vehicle and AGA treated cells. Under the condition in which RBM cells were treated with AGA, intracellular cAMP significantly decreased, compared with the vehicle-treated cells at all timepoints measured (Fig. 2).

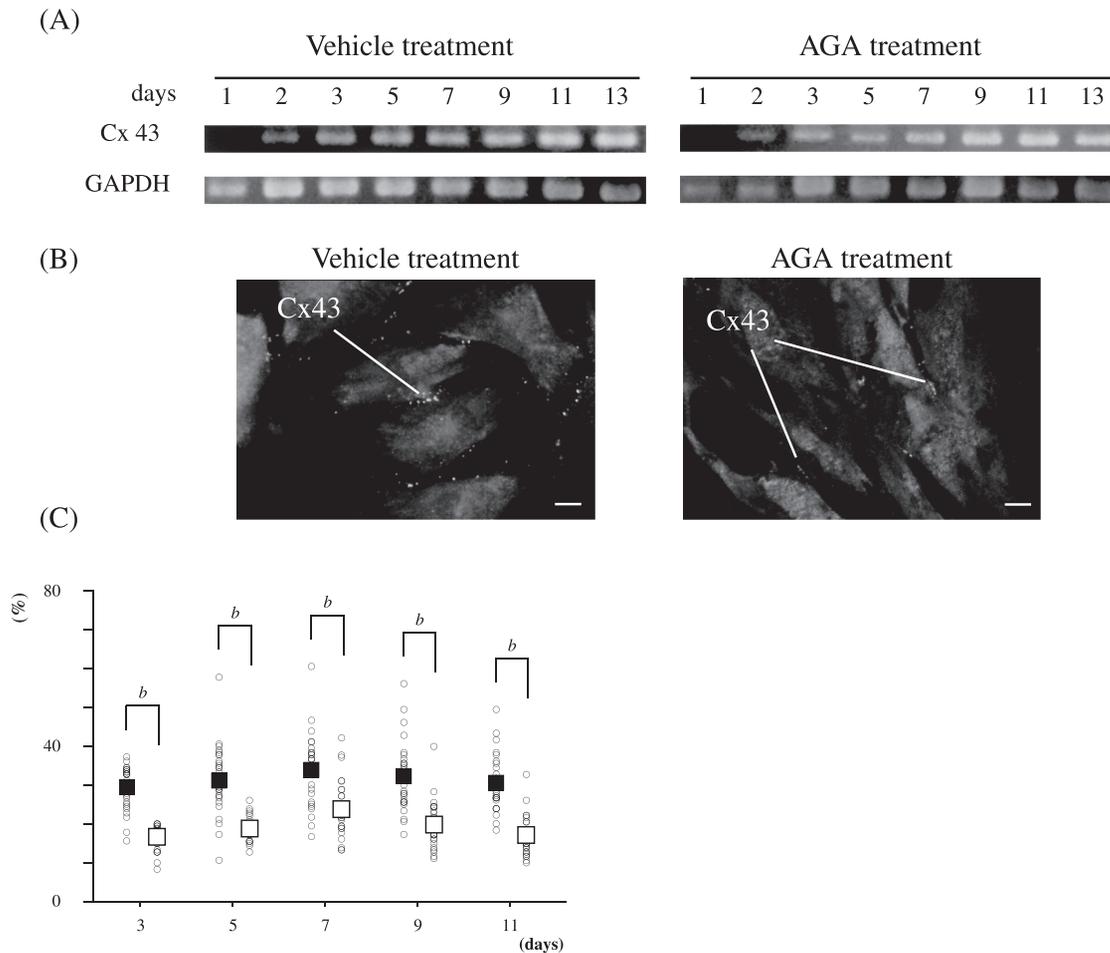


Fig. 1 Expression of Cx43 on RBM cells which were treated with AGA or vehicle. (A) RT-PCR analysis of Cx43 mRNA at the indicated times. (B) Immunofluorescence staining of Cx43 at 5-day of culture. Cx43 positive spots were recognized at cell-to-cell contact. Bar = 10 μ m. (C) Time-dependent changes of Cx43 positive ratio. The calculation of Cx43 positive ratio was shown as described in the Materials and Methods section. Value represents the mean \pm SD ($n = 6$); ^b, $p < 0.01$ from the vehicle treatment (Student's *t*-test). Black-square and white-square show vehicle treated cells and AGA treated cells, respectively.

Effect of AGA-mediated inhibition of GJC on the differentiation of RBM cells

The increase in ALP activity and mineralization was used as a differentiation index of RBM cells. The ALP activity in both vehicle and AGA treated RBM cells increased for up to 11 days of subculture and decreased thereafter. By administrating AGA to RBM cells, the ALP activity significantly decreased at 5 to 13 days (Fig. 3). Calcified nodules stained by Alizarin Red S were first observed at 17 days of subculture in both vehicle and AGA treated cells. To quantify the amount of mineralization in both types of cells, mineral-bound Alizarin Red S was measured. The mineralization increased most notably multiplied from the 19th day of subculture downward to the end of the culture period. Under the

condition in which RBM cells were treated with AGA, the mineralization significantly decreased at all of the timepoints determined (Fig. 4).

DISCUSSION

Osteoblasts are derived from undifferentiated mesenchymal stem cells, and differentiate through osteogenic mesenchymal cells and preosteoblasts into osteoblasts. Bone marrow also contains undifferentiated mesenchymal cells and preosteoblasts. In this study, RBM cells were cultured according to the method of Maniatopoulos *et al.*, and we speculate that RBM cells may differentiate into osteoblasts through two step differentiation mechanism; (1) administration of dexamethasone lead RBM cells to

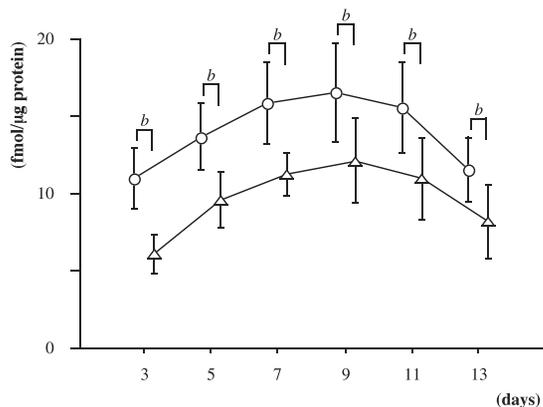


Fig. 2 Time-dependent changes of intracellular cAMP in the presence and absence of AGA. Value represents the mean \pm SD ($n = 6$). Notice the significant difference in cAMP levels between the vehicle and AGA treatment cells (^b; $p < 0.01$, Student's t -test). Vehicle treated cells show as circle, and AGA treated cells show as triangle.

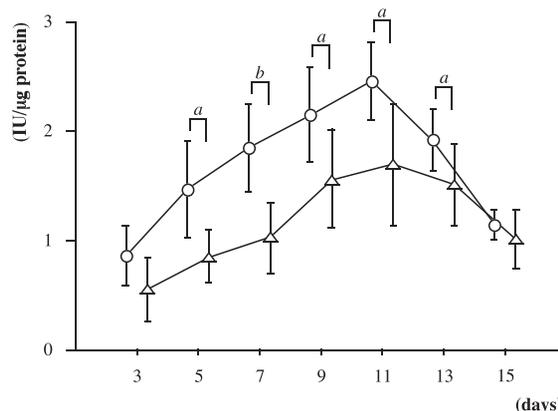


Fig. 3 Time-dependent changes of ALP activity in the presence and absence of AGA. Value represents the mean \pm SD ($n = 6$). ALP activity of AGA treated cells was significantly lower than that of vehicle treated cells at 5 to 13 days (^a; $p < 0.05$, ^b; $p < 0.01$, Student's t -test). Vehicle treated cells show as circle, and AGA treated cells show as triangle.

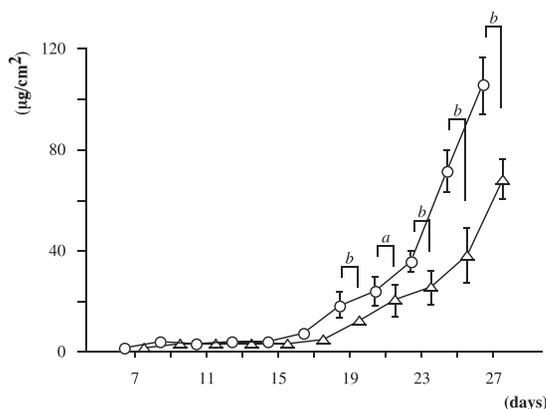


Fig. 4 Time-dependent changes of mineral-bound Alizarin Red S. Value represents the mean \pm SD ($n = 6$). When RBM cells were exposed to AGA, the mineralization significantly decreased at each time tested (^a; $p < 0.05$, ^b; $p < 0.01$, Student's t -test). Vehicle treated cells show as circle, and AGA treated cells show as triangle.

differentiate into preosteoblasts by signaling on a receptor and (2) preosteoblasts proliferate and adhere together, cAMP communicate through gap junctions into adjacent cells, and then preosteoblasts differentiate into osteoblasts. Under the condition in present experiments, the level of intracellular cAMP and ALP activity peaked from the 9th and 11th day, respectively, suggesting that the generality of RBM cells may differentiate into osteoblasts until 11 days.

Many studies have reported that Cx43-mediated GJC regulates the osteoblastic function and differentiation in culture (12, 14, 16, 21, 31, 34). However, much of the function of Cx43 on the differentiation

of bone marrow cells is unknown. In this experiment, we examined the expression of Cx43 gene and protein by RT-PCR and immunofluorescence analysis and the level of intracellular cAMP by enzymeimmunoassay as evaluations of Cx43-mediated GJC function in RBM cells. The increase in the expression of Cx43 gene was followed by an increase in the Cx43 protein expression and subsequent increment of intracellular cAMP was before or in parallel with an increase in the ALP activity.

To evaluate the function of Cx43, we cultured RBM cells in a similar way, except for the addition of 18 α -glycyrrhetic acid (AGA, 100 μ M), a specific inhibitor of Cx43-mediated GJC (5, 27). When RBM cells were exposed to AGA, there was no significant effect on the Cx43 gene expression, but the Cx43 protein expression that mainly existed in osteoblasts significantly decreased. cAMP is one of the second messengers in the intracellular communication system and, in addition to calcium ions (29), plays a critical role in osteoblastic differentiation (12, 14, 21, 27, 28, 31, 34). These second messengers travel between adjacent cells directly through the gap junctions. In this experiments, we measured the level of intracellular cAMP as evaluations of Cx43-mediated GJC function. The AGA-induced blockage of GJC significantly reduced the intercellular cAMP levels in the RBM cells. Similarly, the administration of AGA inhibited the Cx43-mediated GJC, reducing the ALP activity and mineralization. These experimental results are supported by the report of Li *et al.* that the blockage of Cx43-mediated GJC

inhibited the expression of differentiation markers such as ALP, osteopontin, and osteocalcin in mature osteoblasts (15), and suggested that administration of AGA suppressed for preosteoblasts to differentiate into osteoblasts under guidance by dexamethasone. Owen *et al.* reported that preosteoblasts include a population of committed cells capable of osteoblastic differentiation under certain conditions and a population of cells in a less differentiated stage which is induced by external stimuli (23). In this experiment, under the conditions of continuous treatment with AGA from the first day after passage, mineralization in RBM cells significantly decreased. Schiller *et al.* reported that the use of AGA in the late stage of culture (days 20–25) during the culture period of 31 days failed to inhibit the mineralization of osteoblastic cell line (27). These observations together suggest that the early stage of culture is crucial in determining the direction of the differentiation of RBM cells.

In this experiment, blockage of Cx43-mediated GJC could not completely inhibit the differentiation of RBM cells, presumably because AGA is a reversible inhibitor, and generally, the signals brought to cells is captured by cell surface receptors and transported into cells through the second messengers. The binding of ligands to the receptors activates adenylyl cyclase through activated G protein, and increases intracellular cAMP. cAMP-dependent protein kinase (PKA), activated by cAMP, moves into the nucleus, and phosphorylates cAMP-responsive element-binding protein (CREB) to activate cAMP-responsive element (CRE), ultimately leading to gene expression. As Dulin *et al.* reported the presence of the I κ B-PKA pathway which activates PKA without involving G-protein mediation (4), the present study might suggest the presence of a pathway for RBM cell differentiation other than the Cx43-mediated GJC.

In conclusion, from the results of this experiment, we speculate that during differentiation of the RBM cells toward a more mature phenotype, the transduction of signals from the outside, including culture conditions, is involved in information exchange mainly through Cx43.

Acknowledgments

We are grateful to Dr. Satoru Kaneko, Department of Obstetrics and Gynecology, Ichikawa General Hospital, Tokyo Dental College, for valuable technical advice and helpful suggestion with respect to the manuscript.

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