

Regulation of Connexin31 Gene Expression upon Retinoic Acid Treatment in Rat Choriocarcinoma Cells

R. GRÜMMER,¹ P. HELLMANN, O. TRAUB,* M. J. SOARES,† M. E. EL-SABBAN,‡ AND E. WINTERHAGER

*Institute of Anatomy, University Hospital, 45122 Essen, Germany; *Institute of Genetics, University of Bonn, 53117 Bonn, Germany;*

†Department of Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7400; and

‡Division of Nephrology, Winthrop University Hospital, New York, New York 11577

The controlled invasiveness of the trophoblast is based on the balance between invasive properties at implantation and the differentiation program of the developing placenta. During placental development in rats a switch of connexin gene expression has been observed in parallel to the switch from the invasive to the differentiated phenotype of trophoblast cells. To investigate the role of connexin expression for trophoblast invasion, proliferation, and differentiation, we studied one rat trophoblast (HRP-1) and one rat choriocarcinoma cell line (Rcho-1). The choriocarcinoma cells were characterized by expression of cx31 and a lack of E-cadherin, corresponding to the invasive trophoblast *in vivo*, whereas HRP-1 cells expressed cx43, normally found in the spongiotrophoblast and in late giant cells, and E-cadherin. Upon retinoic acid treatment, Rcho-1 cells irreversibly lost cx31 expression, accompanied by a loss of functional coupling. No changes in regard to connexin expression and cell-cell communication could be observed in HRP-1 cells. In addition, treatment of Rcho-1 cells with retinoic acid for 7 days upregulated expression of cx43 transcript, but no protein could be found. Proliferation was clearly reduced and the mean volume of cells doubled from Day 4 to Day 7 of retinoic acid treatment in Rcho-1 cells, while both parameters were not affected in HRP-1 cells. Both cell lines showed a similar invasion rate using a Matrigel invasion assay, and invasion was equally suppressed upon retinoic acid treatment. Thus the different connexin expression appears more likely to play a role in regulating proliferation and differentiation along the multilineage pathway than invasiveness of rat trophoblast cells. © 1996 Academic Press, Inc.

INTRODUCTION

The appropriate development of the placenta plays a critical role in the maintenance and progression of pregnancy [2]. During the initial phase of implantation

the trophoblast cells penetrate the endometrial epithelium and invade deep into the maternal stroma to reach the maternal blood supply. In regard to invasive properties, trophoblast cells resemble tumor cells. Trophoblast invasion, however, is temporally and spatially regulated. During early pregnancy stromal cells of the rat endometrium differentiate into the decidua as a response to the invading trophoblast. This compact tissue seems to control the invasive properties of the early trophoblast [3]. Termination of invasion, however, seems to be regulated by the endogenous differentiation program of the trophoblast cells [4]. They differentiate continuously throughout embryogenesis along a multilineage pathway, yielding several different cell types including cells of the labyrinth, spongiotrophoblast cells, and trophoblast giant cells [5]. In general, differentiation processes can be coordinated, at least in part, by direct cell-to-cell communication via gap junctions. By allowing transfer of small diffusible molecules like cAMP and IP₃, and ions up to 1 kDa like [Ca²⁺], into neighboring cells [6, 7], gap junctions are considered to play important roles in cell differentiation, proliferation, and tissue organization [8] as well as during development [9–11]. Gap junction channels consist of two hemichannels, each of them composed of six protein subunits, the connexins (cx), of which 13 members of a multigene family in the murine genome are now known [12, 13]. The expression of each connexin depends on both cell type and time of development [14]. The diversity of connexins results in different channel properties [15], but it is still not understood how this diversity is related to their function. Targeted mutagenesis of the cx43 gene led to heart defects in the mouse embryo that were incompatible with survival of the newborns [16]. Recent publications could demonstrate that the connexin pattern switches during trophoblast differentiation in a spatial and temporal pattern during pregnancy in the rat [1, 17]. Cx31 expression characterizes the invasive trophoblast of the ectoplacental cone whereas start of differentiation of the labyrinthine trophoblast is indicated by expression of abundant cx26 [1, 18] and of the spongiotropho-

¹ To whom correspondence and reprint requests should be addressed. Fax: 49 201 7235916.

blast by cx43 expression [1]. Concomitantly with this, the adhesion molecule E-cadherin is downregulated during trophoblast invasion and is reexpressed during labyrinthine formation. This confirms the findings of other groups that loss of E-cadherin is correlated with a gain of invasiveness of tumor cells [19–22]. Thus a lack of this cell adhesion molecule and expression of cx31 are correlated with the invasive and highly proliferative cells of the ectoplacental cone, and the expression of E-cadherin, cx43, and cx26 is correlated with more differentiated trophoblast subpopulations. This may point to an important role of these molecules in regulating the differentiation process of trophoblast lineages. To have a better approach to investigate factors leading to different trophoblast subpopulations, we used two established rat trophoblast cell lines, HRP-1 and Rcho-1. The HRP-1 cell line was derived from the labyrinthine region of normal midgestation chorioallantoic rat placenta [23, 24] and expresses trophoblast-specific markers [5]; the Rcho-1 cell line was derived from a transplantable rat choriocarcinoma [25] which was originally generated by Teshima *et al.* [26]. The latter can be manipulated to differentiate along the trophoblast giant cell pathway and has been shown to express trophoblast-specific members of the rat placental prolactin family in a temporal pattern which is similar to that seen during placental development *in vivo* [25, 27]. Here we describe cx31 expression in Rcho-1 choriocarcinoma cells which is suppressed upon retinoic acid (RA) treatment, whereas expression of cx43 in HRP-1 cells is not regulated. The two cell lines demonstrate different cell physiology in response to RA treatment, which could be related to their different cell programs.

MATERIALS AND METHODS

Cell culture. HRP-1 cells were derived from the normal midgestation chorioallantoic placenta of the outbred Holtzman rat [23, 24]; the Rcho-1 cell line was derived from a transplantable rat choriocarcinoma [25], originally generated by Teshima *et al.* [26]. The cells were routinely cultured as monolayers through serial passaging in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 50 μ M β -mercaptoethanol (Merck), 1 mM sodium pyruvate (Sigma), 100 units/ml of penicillin, and 100 μ g/ml of streptomycin (Boehringer) at 37°C in 95% air, 5% CO₂ in 25-cm² plastic cell culture flasks (Falcon). Cells were shown to be free of mycoplasma contamination. Culture medium was changed at 2-day intervals. When the cultures became confluent they were passaged by exposure to 0.05% trypsin/0.02% EDTA in phosphate-buffered saline (PBS; Biochrom).

To test the influence of RA, cells were incubated in medium containing all-*trans*-retinoic acid (Sigma, Germany), in the range from 10⁻⁵ to 10⁻⁹ M. Stock solutions of RA were prepared in ethanol and stored at -80°C.

Growth rates and volume of trophoblast cell lines were determined in three experiments by using an electronic Coulter counter (CASY 1, Schärfe System, Reutlingen, Germany). For these studies culture medium was changed at 24-h intervals.

Immunohistochemistry. For immunohistochemistry, cells were plated on glass coverslips. After 3 days, cultures were fixed in acetone: methanol (1:1) for 5 min and rinsed in PBS containing 0.5% bovine serum albumin (BSA) for 30 min to reduce nonspecific staining. Indirect immunocytochemistry was performed using affinity-purified rabbit antibodies directed to the C-terminus of rat cx31 at a dilution of 1:10 [28], polyclonal rabbit antibodies directed to the C-terminus of rat cx43 at a dilution of 1:25 [29], and polyclonal rabbit antibodies to E-cadherin at a dilution of 1:100 [30]. Controls were performed using rabbit preimmune serum instead of the primary antibody. Cells were incubated with an appropriate secondary antibody conjugated to fluorescein isothiocyanate after rinsing in PBS mounted using glycerol containing phenylene diamine (Sigma). Cells were examined with a Axiophot microscope (Zeiss) equipped for epifluorescence.

Cell coupling. Coupling of gap junctions was demonstrated by microinjection of 4% Lucifer yellow CH (LY; Sigma) in 150 mM LiCl for 60 s in cells growing in petri dishes. The solution of LY was injected by iontophoresis into cells through glass microelectrodes with 0.5- μ m tips with a negative current of about 20 nA. Three minutes after microinjection, the spread of the dye fluorescence to neighboring cells was scored under a Zeiss photomicroscope equipped with phase-contrast and epifluorescence illumination.

To measure changes in intracellular calcium ion levels, a continuous image subtraction method with Indo-1-AM (Sigma) was used. Cells were incubated in culture medium containing 5 mM Indo-1-AM for 2 h at 37°C. After loading, cells were washed with PBS, incubated in fresh medium, and set on the stage of a laser scanning confocal microscope (Nikon RCM8000). Images of Indo-1-AM fluorescence excited at 351 nm were detected by a videocamera connected to an image intensifier, observed through an image processor, and stored on a computer hard disk. With the aid of the processor, a reference image at one moment was stored in memory, and the difference between the live image and the reference image was continuously enhanced and expressed. Although this continuous image subtraction method cannot measure the absolute concentration of intracellular calcium ions, it is useful for real-time imaging of intra- and intercellular calcium mobilization. To observe intercellular calcium mobilization, a weak mechanical stimulation of a single cell was performed by briefly deforming the cell membrane with a fine round-tip glass capillary needle with a heat-blunted microelectrode. Changes in calcium ion levels leading to a change in fluorescence in injected and adjacent cells were measured by the continuous subtraction of images of Indo-1-AM excited at 351 nm. [Ca²⁺] imaging was performed in cooperation with the laboratory of D. C. Spray, Albert Einstein College of Medicine, New York.

Northern hybridizations. Total RNA was isolated from cells by the guanidinium isothiocyanate/cesium chloride method [31], and hybridization was performed as described previously [32, 33]. Briefly, 5 μ g RNA was separated by electrophoresis through denaturing agarose gels and blotted onto nylon membranes (Hybond-M, Amersham-Bucher GmbH). Connexin-specific cDNA probes were random-prime labeled with [α -³²P]dCTP and hybridized with the RNA blots overnight at 42°C in a solution containing 55% deionized formamide, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μ g/ml salmon sperm DNA. The following cDNAs were used: a 1.1-kb cDNA from the coding region of rat cx26 (clone 26-1, [34]), a 1.4-kb cDNA from the coding region of rat cx43 (clone G2B, [35]), a 1.5-kb cDNA corresponding to the coding region of the rat cx32 gene [36], a 1.5-kb cDNA corresponding to part of the region of mouse cx37 (codon number 24-333, [37]), a 1.6-kb cDNA corresponding to the mouse cx31 gene [38], a 0.6-kb cDNA fragment from the rat cx40 gene [39], and a 2.8-kb cDNA from the mouse E-cadherin gene (kindly provided by R. Kemler, Freiburg, Germany). Blots were washed at 60°C in 1 \times SSC, 0.1% SDS for 1 h, in 0.5 \times SSC, 0.1% SDS for 30 min and in 0.1 \times SSC, 0.1% SDS for 30 min. Exposure to Kodak XAR-5 films took

place at -80°C with intensifying screens. After exposure, each blot was again hybridized with a human β -actin-specific probe [40].

Invasion assays. For invasion assays transwell chambers with 6.5-mm-diameter polycarbonate filters of 8- μm pore size (Costar) were used which consisted of a separate filter containing a top well and a corresponding lower well. Filters were prepared according to Repesh [41]. Each filter was coated with 100 μl of a 1:20 dilution of cold matrigel (Collaborative Research) in cold RPMI 1640 medium, dried overnight at room temperature, and reconstituted the following day by incubating each well with 100 μl RPMI 1640 at 37°C for 1 h. After removing the medium 4×10^4 cells were added to triplicate wells (top well) in a volume of 200 μl and the lower well was filled with 700 μl of culture medium. After 2 days of culture, the number of cells traversing the filter was quantified by assessing the number of cells attached to the lower side of the filter by visual counting of the whole filter under the light microscope. For this purpose filters were fixed with methanol for 5 min and stained with hematoxylin-

eosin. The medium in the lower well was proved not to contain cells. The invasion rate was determined as the number of invaded cells per 1000 cells. Cells were treated with RA for 3 days before they were used in invasion assays. Invasion assays were performed over 2 days without addition of RA. Culture medium was changed at 24-h intervals.

RESULTS

Connexin and E-Cadherin Expression in Rcho-1 and HRP-1 Cells

When grown as monolayer on tissue culture plastic, HRP-1 cells were of similar size and showed the typical cobblestone appearance of epithelial cells (Fig. 1A). No

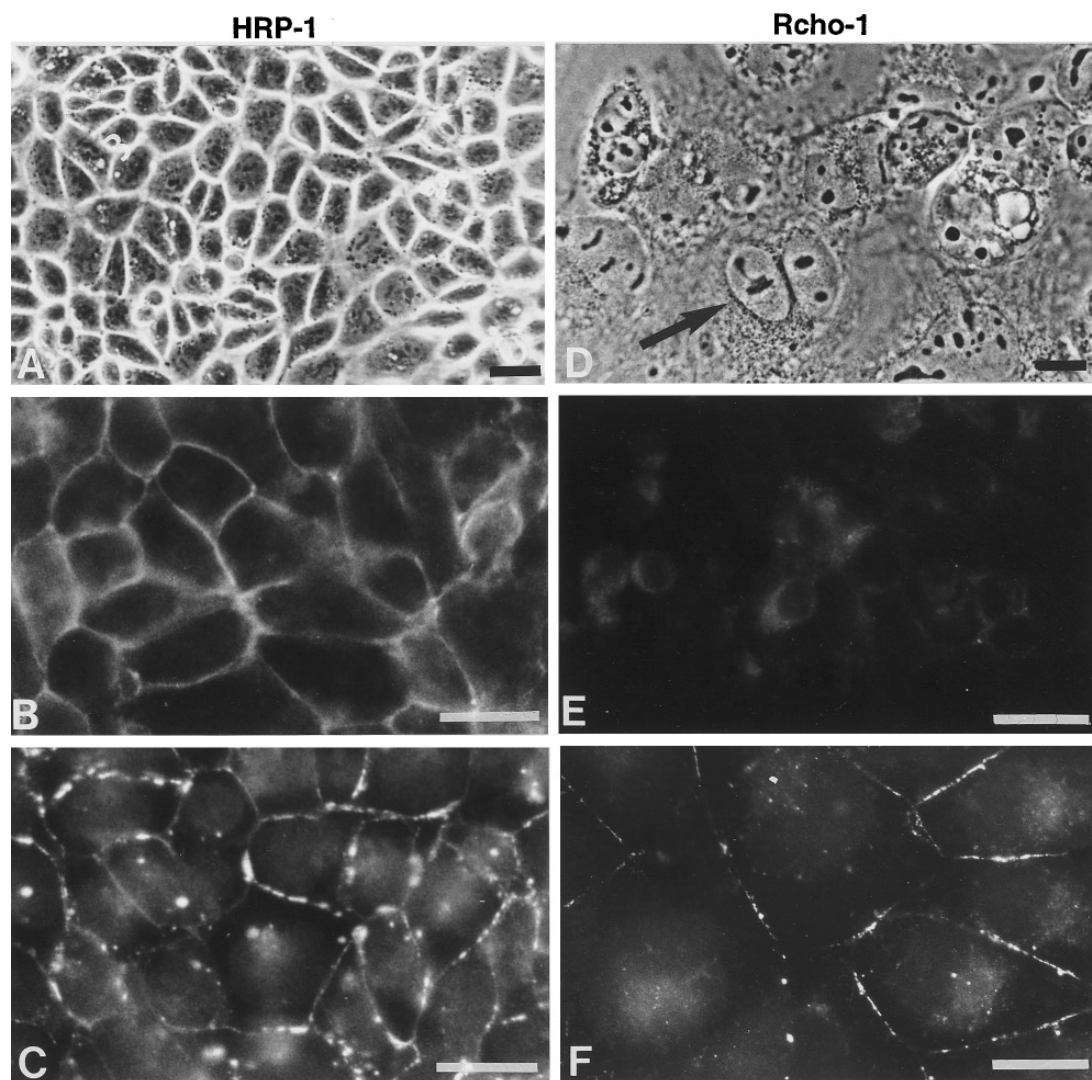


FIG. 1. Immunohistochemical staining for E-cadherin and connexins. HRP-1 cells (A, phase contrast) reveal a staining of cell membranes for E-cadherin (B) and a typical punctate reaction for cx43 (C). Rcho-1 cells (D, phase contrast; arrow indicates giant cell) show no staining for E-cadherin (E) and a positive reaction for cx31 (F). Bars, 20 μm .

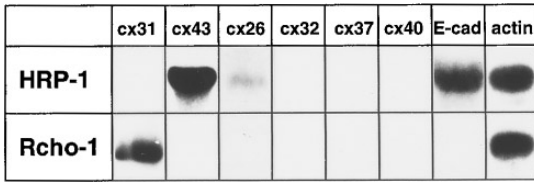


FIG. 2. Northern blot of HRP-1 and Rcho-1 mRNA probed for different connexins and E-cadherin. HRP-1 cells show a strong expression of cx43 and E-cadherin and a weak expression of cx26. Rcho-1 cells only express cx31. The filter was reprobed with a human β -actin-specific probe to assess sample loading.

giant cells could be observed in these cultures. In contrast, Rcho-1 cells were composed of a mixture of morphologies (Fig. 1D) including small cells growing in clusters and giant cells possessing very large nuclei as already described by Faria and Soares [25]. Investigation of the cell adhesion molecule E-cadherin showed a clear expression of the E-cadherin protein at the lateral cell membranes of HRP-1 cells (Fig. 1B), while Rcho-1 cells lacked any E-cadherin expression (Fig. 1E). The results found by immunohistochemistry could be confirmed for both cell lines at the transcript level (Fig. 2).

Screening for different connexin transcripts revealed that cx32, cx37, and cx40 failed to be detected in both cell lines (Fig. 2). There was a faint band of cx26 mRNA detectable in HRP-1 cells (Fig. 2) but the corresponding protein was missing using immunohistochemistry. HRP-1 cells, however, demonstrated large amounts of cx43 transcripts, and the antigen was located at the lateral cell borders (Fig. 1C). Thus these cells expressed the type of connexin normally found in the spongiotrophoblast of the differentiated placenta. Apart from size and shape Rcho-1 choriocarcinoma cells are characterized by the expression of cx31 as revealed by immunohistochemistry (Fig. 1F) and Northern blot (Fig. 2), and thereby resemble trophoblast cells of the invasive ectoplacental cone.

Influence of Retinoic Acid Treatment

Connexin expression. RA treatment of Rcho-1 cells resulted in a downregulation of cx31 with increasing concentration. A drastic decrease in amount of cx31 transcript was found after 3 days of RA treatment at a concentration from 10^{-7} to 10^{-5} M RA, whereas only a slight effect could be seen at a concentration between 10^{-8} and 10^{-9} M (Fig. 3). Thus a concentration of 10^{-6} M was chosen for all following experiments. The time course of RA effects on cx31 expression gave evidence that reduction of transcripts started from 24 h onward with a clear decrease after 72 h (Fig. 4a). Immunohistochemistry revealed that gap junction protein was absent after 3 days of treatment with RA (compare Fig.

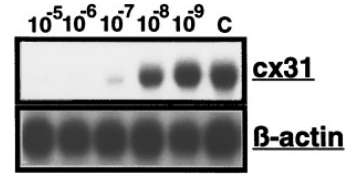


FIG. 3. Northern blot of Rcho-1 mRNA probed for cx31. RNA was prepared from cells treated with RA at a concentration of 10^{-5} to 10^{-9} M for 3 days. Cx31 expression was clearly suppressed from a concentration of 10^{-7} M onward.

1F with Fig. 5B). To determine whether this effect of RA treatment is reversible, Rcho-1 cells were grown in normal medium after treatment with 10^{-6} M RA for 3 days. Interestingly the suppressive effect of RA on cx31 expression was not recovered even within 8 days of culturing (Fig. 6). From 7 days of RA-treatment onward, cx43, but not cx26, was induced in Rcho-1 cells as shown in Northern blot analysis (Fig. 7). The corresponding protein, however, could not be detected in up to 14 days of RA treatment. HRP-1 cells were treated using the same experimental design. No effect of RA on cx43 expression could be observed in these cells even after 5 days of treatment, neither at the mRNA level (Fig. 4b) nor at the protein level (Fig. 5A).

Dye and $[Ca^{2+}]$ transfer. To prove whether cell-cell communication is reduced after downregulation of cx31 upon RA treatment, functional analysis was performed by microinjection of LY as well as by $[Ca^{2+}]$ imaging. After injection into HRP-1 cells, LY spread out from the injection site to the neighboring cells (Figs. 8A and 8B). The number of coupled cells was almost not affected by RA treatment for 3 days (Figs. 8C and 8D; Table 1). Compared to HRP-1 cells, LY transfer into neighboring cells was clearly lower in Rcho-1 cells (Figs. 8E and 8F, Table 1). Cells treated with RA for 3 days lacked any transfer of LY (Figs. 8G and 8H; Table 1). It has been shown [42, 43] that different gap junc-

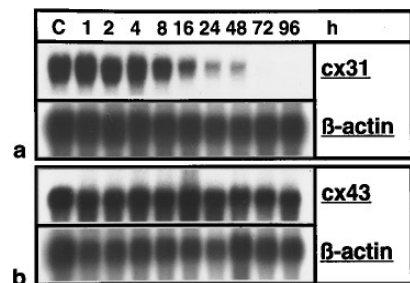


FIG. 4. Northern blot of trophoblast cells treated with RA (10^{-6} M) for up to 96 h. (a) Rcho-1 mRNA probed for cx31. Cx31 expression declines from 24 h onward and is clearly suppressed from 72 h onward. (b) HRP-1 mRNA probed for cx43. No influence of RA on cx43 expression can be observed.

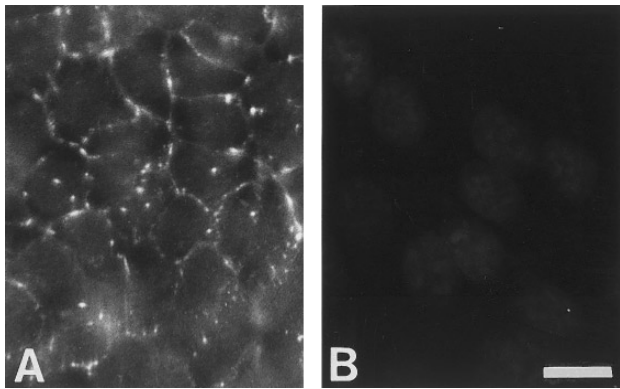


FIG. 5. Immunohistochemistry reveals that cx43 protein can be detected at the lateral membranes in HRP-1 cells (A) while cx31 antigen is not stained in Rcho-1 cells (B) after 3 days of RA treatment. Bar, 20 μ m.

tional connexins show different selectivity for positively or negatively loaded molecules. Therefore we performed $[Ca^{2+}]$ imaging, which, in comparison to LY injection, is a more sensitive method to investigate coupling of Rcho-1 cells. With this method we could demonstrate that untreated Rcho-1 cells were well coupled as $[Ca^{2+}]$ was transferred to adjacent cells within 10–30 s (Figs. 9A–9C). In cells treated with RA for 3 days, no $[Ca^{2+}]$ transfer to neighboring cells could be observed even within 100 s (Figs. 9D–9F). Thus treatment of Rcho-1 cells with RA for 3 days resulted in a disruption of intercellular communication.

E-cadherin. An induction of the cell adhesion molecule E-cadherin could not be induced by RA in the Rcho-1 choriocarcinoma cell line even after 10 days of treatment (Fig. 7). Expression of E-cadherin was not affected in HRP-1 cells treated in the same way (data not shown).

Proliferation and invasion of trophoblast cells. Seeding cells at the same densities and monitoring cell growth for up to 11 days revealed that Rcho-1 cells showed a lower proliferation rate than HRP-1 cells (Fig. 10A). While HRP-1 cell proliferation was not significantly affected by RA treatment, Rcho-1 cell growth

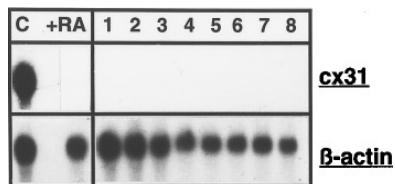


FIG. 6. Northern blot of Rcho-1 cells treated with RA for 3 days (+RA) and of cells transferred to RA-free medium for 1 to 8 days after treatment with RA for 3 days (lanes 1–8), probed for cx31. Cx31 is not reexpressed during this time period.

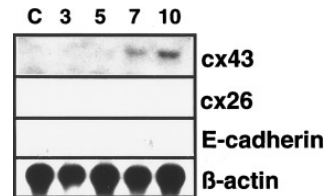


FIG. 7. Northern blot of Rcho-1 cells treated with RA for 10 days. Transcripts for cx43 can be seen from Day 7 of treatment onward. Cx26 expression and E-cadherin expression are not induced by RA treatment.

was strongly retarded from Day 6 of treatment onward (Fig. 10A). With regard to the size of trophoblast cells, RA treatment had no effect on the mean volume of HRP-1 cells, while the volume of Rcho-1 cells doubled from Day 4 to Day 7 of RA exposure (Fig. 10B). To investigate the invasive capacity of both cell lines a reconstituted basement membrane matrix was used for the invasion assay. Figure 11 represents the invasion profile of the different cell lines with and without RA treatment. These data include the amount of cells that completely traversed the matrigel-coated barrier after 2 days of incubation. Without RA treatment both cell lines demonstrated a similar invasion rate. After treatment with RA for 3 days the invasion rate of both cell lines was drastically reduced compared to untreated controls (Fig. 11).

DISCUSSION

In this study we could demonstrate that the two rat trophoblastic cell lines (HRP-1 and Rcho-1) express different connexins which could be previously shown to be markers of different developmental stages of the rat placenta [1]. The choriocarcinoma cell line referred to as Rcho-1 expresses cx31 and lacks the adhesion molecule E-cadherin, characteristic features of the early trophoblast forming the ectoplacental cone. Thus these cells are likely to represent the invasive trophoblast population of the early placenta. Due to their expression of cx43, cx26, and E-cadherin, HRP-1 cells resemble more closely the cells originating from the labyrinth, which express E-cadherin and cx26, or the spongiotrophoblast, characterized by cx43 expression. In contrast to HRP-1 cells, Rcho-1 cells are able to differentiate along the trophoblast giant cell pathway [25, 27], which represents one possible endpoint of the trophectoderm multilineage pathway [44, 45]. At least part of the progression along the trophoblast differentiation pathway is under modulation by factors such as RA [46–50]. We examined the changes in connexin expression that accompany RA treatment of trophoblast cells and their influence on proliferation and invasion. RA

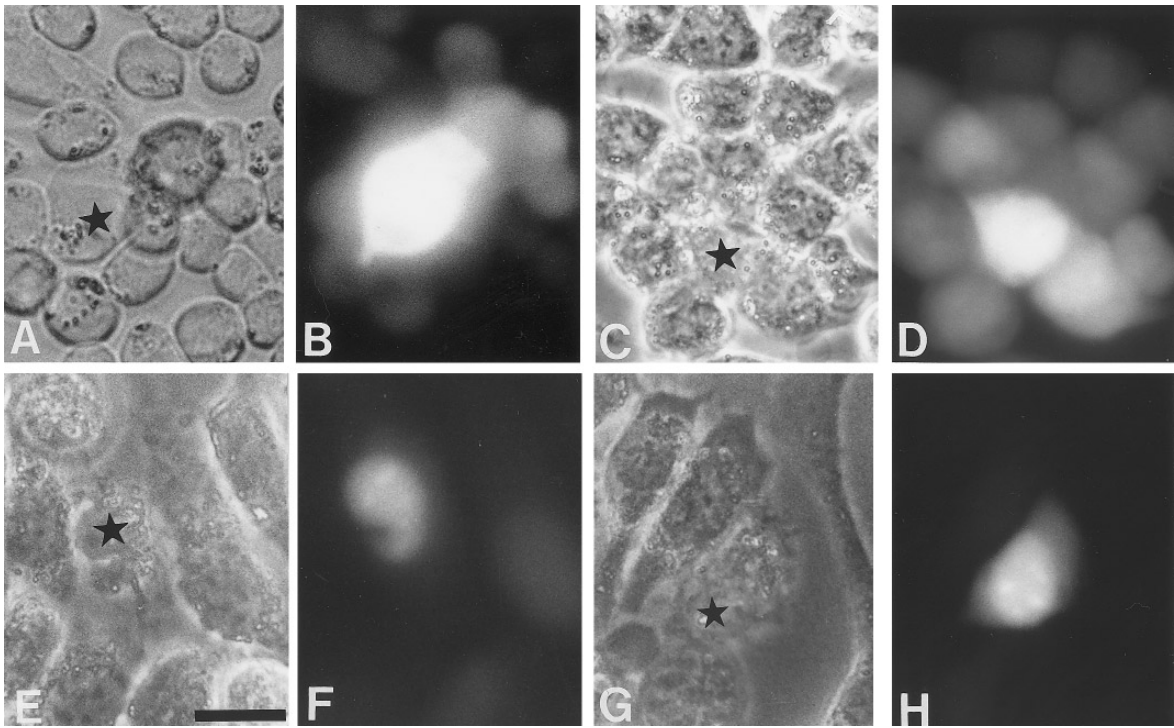


FIG. 8. Dye-coupling with LY. Untreated HRP-1 (A, B) as well as Rcho-1 cells (E, F) show dye spread to neighboring cells. After RA treatment for 3 days dye coupling is unchanged in HRP-1 cells (C, D), while no cell-to-cell transfer of dye can be observed in Rcho-1 cells (G, H). Stars represent injected cells. Bar, 20 μm .

treatment led to a downregulation of cx31 in rat choriocarcinoma cells whereas expression of cx43 was not affected in HRP-1 trophoblast cells. We predict that there is a significant change in the activity of trophoblast-specific regulators of cx31 expression. In parallel with the changes of cx31 expression during RA treatment, the intercellular transfer of second messengers like $[\text{Ca}^{2+}]$ and that of LY are significantly reduced. Thus, the switch in gap junction protein expression in trophoblast cells is accompanied by selective changes in cell-cell communication that

may play an important role in the coordinate control of the differentiation process. These results are in contrast to previous findings of Brissette *et al.* [51], who demonstrated a downregulation of cx26 and cx43 in combination with an upregulation of cx31 with differentiation in mouse keratinocytes in primary culture. Similar contradictory results have been found for the influence of RA on gap junctional communication, both inhibiting [52, 53, 54] and stimulating [52, 55] dye spreading in different cell cultures. This points to a tissue-specific regulation of cx31.

The different regulation of cx31 and cx43 may point to functional differences of these connexins in the cells investigated. In HRP-1 cells we found neither effects of RA on connexin expression nor on proliferation; in Rcho-1 choriocarcinoma cells, in contrast, cx31 expression as well as proliferation were strongly reduced. It is known that retinoids can both inhibit and stimulate growth [56, 57] and may be instrumental in the control of growth [6]. Most investigations, however, showed that cell growth decreases as junctional permeability increases [52]. Here we could show a decrease in cx31 expression combined with an inhibition of proliferation and a change in cell volume. This correlates with the *in vivo* observation that the proliferative trophoblast cell population of the ectoplacental cone expresses cx31, which is lost during differentiation to spongiotro-

TABLE 1
Different Effect of Retinoic Acid on Gap Junctional Permeability in HRP-1 and Rcho-1 Cells

Cells	Number of fluorescent neighboring cells ^a	
	-RA	+RA
HRP-1	18.4 \pm 4.1	17.0 \pm 8.1
Rcho-1	5.2 \pm 0.9	0.0 \pm 0.0

^a Number of fluorescent neighboring cells was measured 3 min after injection of LY into a single cell ($n = 10$; three sets of experiments). Cells were cultivated either without (-RA) or with 10^{-6} M retinoic acid (+RA) for 3 days.

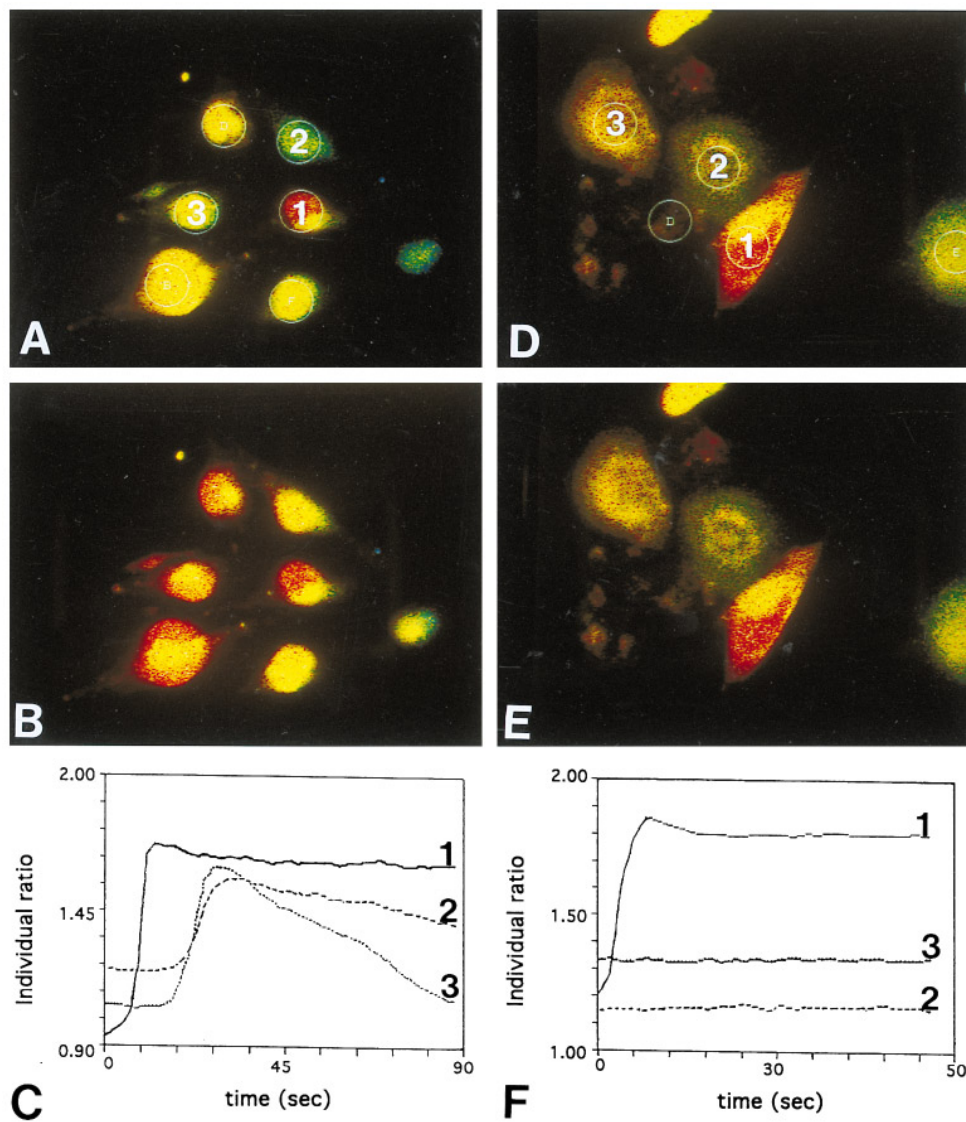


FIG. 9. $[Ca^{2+}]$ imaging of Rcho-1 cells. Red color demonstrates high, yellow/green color demonstrates low $[Ca^{2+}]$. In untreated cells $[Ca^{2+}]$ was transferred from the stimulated cell to neighboring cells within 30 s (A–C). After RA treatment for 3 days, no transfer of $[Ca^{2+}]$ could be observed within 100 s (D–F). Curves (C, F) demonstrate the transfer of $[Ca^{2+}]$ from the stimulated cell (1) to neighboring cells (2, 3).

phoblast or labyrinthine cells and to trophoblast giant cells [1]. In general, cx31 is expressed in only a few adult tissues [38, 58] and is mainly found in skin coexpressed with other connexins [38, 59]. In contrast to cx26 and cx43, cx31 is characterized by its inability to form compatible channels with half channels composed of a different connexin [60]. Thus cells expressing cx31 may escape the control of the surrounding tissues, e.g., control of proliferation. This could especially hold true for regulation of trophoblast invasion as there are hints that the establishment of heterologous cell–cell communication may play a role in regulating invasion processes [61, 62].

The cessation of Rcho-1 cell proliferation and the loss of cell coupling may be due to RA-induced differentiation to giant cells. Rcho-1 cells are known to yield giant cells [25], and the strong increase in volume of RA-treated cells points to an increase in number of giant cells. In parallel to the loss of cx31 expression, those cells start expressing cx43-mRNA. This refers to the *in vivo* situation, where rat trophoblast giant cells change their expression pattern from cx31 to cx43 [1]. In RA-treated Rcho-1 cells we could not demonstrate cx43 protein. Whether there is a defect in posttranslational processing of cx43 or the amount of cx43 protein in those cells was too low to be de-

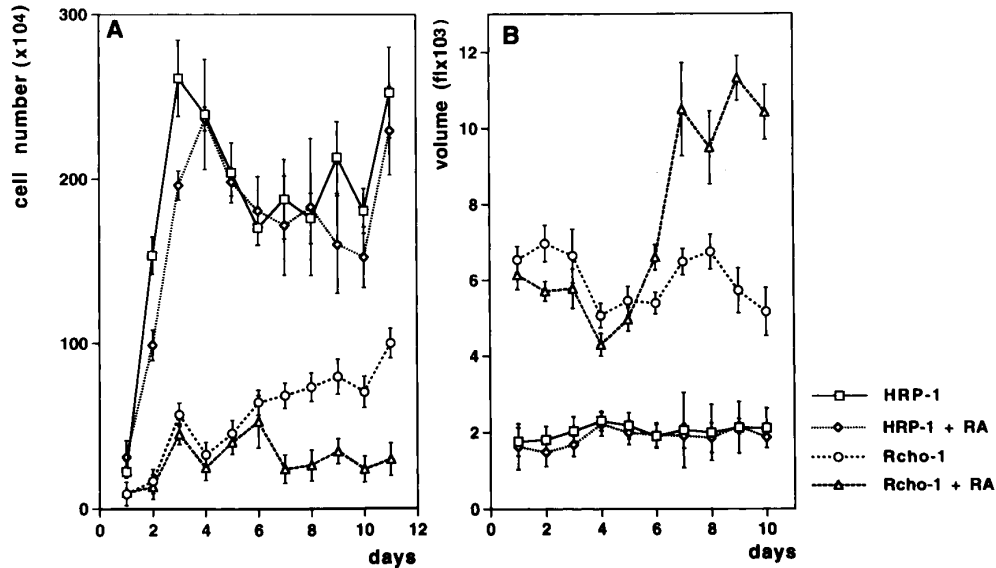


FIG. 10. (A) Proliferation rate of untreated cells and cells treated with RA for 3 days (+RA). Retinoic acid treatment had no influence on proliferation of HRP-1 cells, while proliferation clearly decreased in Rcho-1 cells from Day 6 of treatment onward. (B) Mean volume of untreated cells and cells treated with RA for 3 days (+RA). Retinoic acid treatment had no influence on the mean volume of HRP-1 cells, while volume increased in Rcho-1 cells from Day 6 of treatment onward.

tected by immunohistochemical methods has yet to be investigated.

Quantitative measurement of invasion in a matrigel invasion assay showed that there was little difference in invasion properties as well as in RA-induced inhibition of invasion between the two cell lines. Matrigel contains several components found in extracellular matrices and basement membranes [63]. Obviously, both cell lines were capable of penetrating this barrier to a similar extent, and despite the differences in connexin

expression, the invasion rate of both cell lines was clearly decreased after RA treatment. Thus connexin expression does not appear to correlate with invasive properties but may be involved in proliferation and differentiation of trophoblast cells. The role of cx31 in proliferation and differentiation processes has to await further investigations. It is not known how the effects of RA in Rcho-1 cells are mediated. Whether a specific interaction of RA with cellular receptors [64] is responsible for the observed effect on gap junction expression remains to be clarified. It can be suggested that there is transcriptional regulation of cx31 expression, as RA-induced suppression is irreversible during the observed time period of 8 days. Further mapping of the promoter will help to clarify the regulatory pathways involved in controlling gene expression. Such information on the transcriptional control of genes activated during the early stages of trophoblast differentiation will, it is hoped, provide access to regulatory pathways controlling trophoblast differentiation, and RA may act as one morphogene in this placental development.

The rat trophoblast cell lines HRP-1 and Rcho-1, which show similar connexin expression as trophoblast cells *in vivo*, seem to be a useful model for investigating the regulation of connexin gene expression as well as other genes responsible for placental development.

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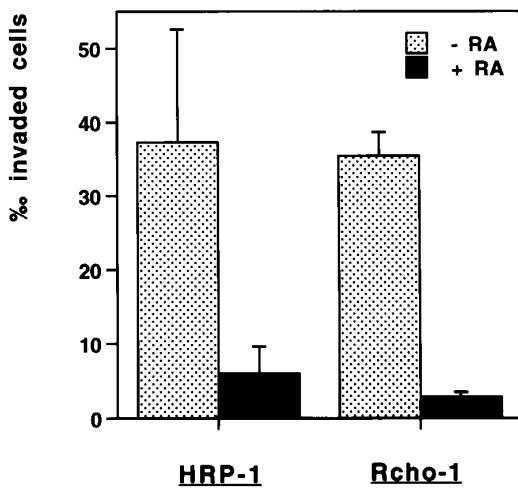


FIG. 11. Matrigel invasion assay of untreated cells and cells treated with RA for 3 days. Invasion rate of both cell lines, HRP-1 and Rcho-1, was strongly reduced after RA treatment.

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