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ORIGINAL PAPER

Human Connexin30.2/31.3 (GJC3) does not Form Functional Gap Junction Channels but Causes Enhanced ATP Release in HeLa Cells

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Abstract Gap junctional intercellular communication has numerous functions, each of which meets the particular needs of organs, tissues, or groups of cells. Connexins (CXs) are homologous four-transmembrane-domain proteins that are the major components of gap junctions. CX30.2/CX31.3 (*GJC3*) is a relatively new member of the CX protein family. Until now, however, the functional characteristics of CX30.2/CX31.3 have been unclear. To elucidate the properties of CX30.2/CX31.3 channels, their subcellular localization in HeLa cells, their effectiveness in dye transfer, and function on channels were investigated. In the immunofluorescent assay, cells that express CX30.2/CX31.3-GFP exhibited continuous fluorescence along the apposed cell membranes, rather than punctated fluorescence in contacting membranes between two cells. Surprisingly, dyes that can be capable of being permeated by CX26 GJ, according to a scrape loading dye transfer assay in previous studies, are impermeated by CX30.2/CX31.3 GJ, suggesting a difference between the characteristics of CX30.2/CX31.3 GJ and CX26 GJ. Furthermore, a significant amount of ATP was released from the HeLa cells that stably expressed CX30.2/CX31.3, in a medium with low calcium ion concentration, suggesting a hemichannel-based function for CX30.2/CX31.3. Based on these findings, we suggest that CX30.2/CX31.3 shares functional properties with pannexin (hemi) channels rather than gap junction channels of other CXs.

Keywords CX30.2/CX31.3 · GJC3 · Connexin · Gap junction

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Introduction

Gap junction intercellular communication (GJIC) has several functions—each of which is to meet the specific needs of organs, tissues, or groups of cells [1]. Gap junctions (GJs) are clusters of gated intercellular channels that directly connect to the cytoplasma of neighboring cells and thereby allow the passage of small ions, metabolites, secondary messengers, and other small molecules (<1 kDa) among cells [1, 2]. GJ channels are double membrane protein structures that are formed by the head-to-head docking of two half channels to generate hydrophilic pores across the membrane [3]. Each half channel, or connexon, comprises six polytopic transmembrane protein subunits, called connexins (CXs). The CXs within a connexon may be the same (homomeric) or different (heteromeric) and

two CXs that dock together can be identical (homotypic junctions) or different (heterotypic junctions) [4]. Investigations have also shown that CXs can assemble into a functional hexameric connexon in the ER membrane [5]. Subcellular fractionation studies and immunocolocalization analyses suggest that connexins pass through the Golgi apparatus to reach the plasma membrane [6–8].

Human CXs belong to a protein family of more than 21 members, each of which is encoded by a different gene. The numbers assigned to the various CXs refer to their approximate molecular weights [9]. CXs share a common structure of four transmembrane segments, which extend into two extracellular and three cytoplasmic domains [10]. Based on the similarities of nucleotides and amino acids, they can be further classified into subgroups, the *GJA*, *GJB*, *GJC*, *GJD,* and *GJE* gene subgroups [9, 11].

Human CX30.2/CX31.3 (NP_853516) belongs to the *GJC* subgroup that is encoded by the *GJC3* gene (NM_181538). Human CX30.2/CX31.3, orthologs of the mouse Cx29, was first identified by database analysis in 2002 [12]. The gene maps to chromosome 7q22.1, contains two exons with an open reading frame (ORF) of 840 base pairs, and encodes a protein of 279 amino acid residues with a molecular weight of 31.29 kDa. [9, 13]. Northern blot analysis detected the highest expression of Cx29 in the mouse sciatic nerve, with weaker expression in the brain and spinal cord [12]. Cx29 has been demonstrated, using cDNA macroarray hybridization, to be strongly expressed in the mouse cochlea [14]. Animal studies reveal that Cx29 is expressed in the cochlea neurons, spiral limbus, spiral ligament, organ of Corti, stria vascularis, Schwann cells that myelinate the soma, and spiral ganglion neurons in the mouse and rat cochlea [15, 16]. A previous investigation showed that mouse Cx29 did not induce intercellular conductances when expressed alone in mouse neuroblastoma cells [12]. Our earlier study established that mutations of *GJC3* are associated with nonsyndromic hearing loss [17, 18]. Moreover, our data demonstrate that the p.E269D missense mutation of CX30.2/CX31.3 results in the accumulation of the CX30.2/CX31.3 mutant protein in the endoplasmic reticulum, rather than in the cytoplasmic membrane [19].

Based on the above results, we suggest that CX30.2/CX31.3 protein is important to the development of nerve and hearing. However, the functional characteristics of human CX30.2/CX31.3 are unclear. In this investigation, CX30.2/CX31.3 did not produce functional gap junction channels in stably expressing CX30.2/CX31.3 HeLa cells, although it was transported to the plasma membrane. In addition, this investigation study demonstrates hemichannel activity via the release of ATP in these cells at low calcium concentrations. According to these results, a gap junction-dependent function of CX30.2/CX31.3, which may be similar to that proposed for pannexins, is suggested.

Materials and Methods

Wild-type GJC3 Gene-EGFP Expression Constructs

For fusion protein generation, cDNA sequences of the autofluorescent reporter proteins EGFP (p-eGFPN1 vector; Clontech, Palo Alto, CA) were fused in-frame to the C-terminus of wild-type *GJC3* genes. The ORFs of *GJC3* were obtained from the pcDNA3.1 clone [19] after digestion with *Kpn*I and *Sac*II, and then subcloned into the *Kpn*I and *Sac*II restriction sites in vectors p-eGFPN1 (Clontech, Palo Alto, CA, USA). The dideoxy DNA sequencing method, using a DNA Sequencing Kit (Applied Biosystems Corporation, Foster city, CA), an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Corporation, Foster city, CA), and restriction digests were used to confirm the DNA sequence of all constructs.

Expression of CX30.2/CX31.3 Gap Junction Proteins in HeLa Cells

Human epitheloid cervix carcinoma cells (HeLa, ATCC CCL 2; American Type Culture Collection, Rockville, MD, USA) lacking the *GJIC* gene were used throughout this study. Cell lines were maintained in a standard cell culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 units/ml penicillin–streptomycin. Cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator. The vectors p-eGFPN1 containing the DNA fragment encoding the wild-type CX31.3 protein was transfected to HeLa cells using lipofectamine (Invitrogen Corporation, California, USA). In order to obtain HeLa cell colonies that stably expressed CX30.2/CX31.3, 1 mg/ml G418 (Geneticin, Gibco–BRL, Grand Island, NY, USA) was added to the growth medium. The growth medium was renewed at 2–3-d intervals. After 2–3 weeks, single cell colonies were obtained. Under a fluorescence microscope, cells displaying green fluorescence were chosen for further culture. After individual colonies had been chosen, a FACSAria^{TM} cell sorter (BD Biosciences, USA) was used to sort positive cells. The positive stable cell line was used for the subsequent functional analyses.

Immunofluorescence Staining of Post-transfection HeLa Cells

Wild-type CX30.2/CX31.3 protein expression in HeLa cells was analyzed by a direct fluorescent protein fusion method involving fusion of GFP to the C-terminal ends of

the CX30.2/CX31.3 proteins. Briefly, post-transfection HeLa cells grown on coverslips were fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS for 20 min and then rinsed three times in PBS. Then, the coverslips were immersed in 10% normal goat serum and 0.1% Triton X-100 for 15 min. The primary antisera and dilutions were as follows: mouse anti-pan-cadherin antibody at 1:200 (anti-CH19; abcan) for cell membrane. After incubation with primary antiserum at 4°C overnight, the cells were rinsed in PBS three times before adding Alexa Fluor 594 conjugated secondary antibodies (Invitrogen, Carisbad, CA). The nuclei of cells were counterstained with DAPI (2 μg/ml) for 5 min and rinsed with PBS. Mounted slides were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany).

Reverse Transcription-polymerase Chain Reaction (RT–PCR)

Total RNA was isolated from positive stable cell lines using the Total RNA Extraction Miniprep System according to the manufacturer's directions (VIOGENE, Sunnyvale). cDNA was synthesized according to the manufacturer's directions in a reaction volume of 20 μl, containing 2–5 μg RNA, random hexamer primer, and 200 units Improm-II™ Reverse Transcriptase (Promega, San Luis Obispo). With primers specific for the coding region of the *GJC3* gene (forward 5′-ATGTGCGGCAGGT TCCTGAG-3′ and reverse 5′-CATGTTTGGGATCAGCGG-3′), PCR was performed (94°C 30 s, 58°C 35 s, 72°C 1 min) for 35 cycles in a volume of 25 μl containing 1 mM Tris–HCl (pH 9.0), 5 mM KCl, 150 μM $MgCl_2$, 200 μM dNTP, 1 units proTaq DNA polymerase (Promega, San Luis Obispo), 100 ng of cDNA, and 200 μM forward and reverse primers. A fragment of approximately 700 bp was amplified from cDNA of the *GJC3* gene. The PCR products were subjected to electrophoresis in an agarose gel (2 w/v %) stained with ethidium bromide. The signals were detected by an Alpha Image 2200 system (Alpha Image 2200 analysis software).

Scrape Loading Dye Transfer

Positive (CX30.2/CX31.3 confluent expression) stable HeLa cells were grown on coverslips for scrape loading. Lucifer Yellow (0.1%; Sigma, MW: 443 Da, 2− charge), Rhodamin B dextra (1%; Sigama, MW:10000 Da, no charge), or Propidium iodide (0.0067%; Invitrogen; MW: 414 Da, 2+ charge) was washed in PBS, dissolved in PBS, and then added in the dark, and a grid was cut into the coverslips using a $26^{1}_{GX}/_{2}$ needle. Cells were incubated for 5 min, and then washed in PBS that contained 0.9 mM calcium, to terminate GJ communication, and images were captured under a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany). For neurobiotin scrape loading, cells were rinsed in PBS without calcium or magnesium; then, 2% neurobiotin (Invitrogen, MW: 287 Da, 1+ charge; diluted in PBS) or 2% biotin ethylenediamine (Vectashield, MW: 297 Da, no charge; diluted in PBS) was added; the cells were then scraped and incubated for 5 min, washed in PBS, fixed in 4% PFA for 15 min, washed in PBS, blocked in 1% BSA with 0.2% Triton X-100 for 90 min at RT, incubated in streptavidin–Alexa Fluor 594 (Invitrogen, diluted 1:300) at RT for 1 h, and imaged as above.

ATP Release Assay

Positive (CX30.2/CX31.3 confluent expression) stable HeLa cells were cultured to 4×10^5 on six-well plates (Falcon, Becton–Dickinson, Lincoln Park, NJ) and incubated in 300 μl PBS with or without 0.9 mM Ca^{2+} and an additional 2 mM ethyleneglycol-tetraacetic acid (EGTA) for 15 min at 37°C. Following incubation, 10 μl of the supernatant was collected. The ATP concentration was determined using an ATP Determination Kit, following the manufacturer's instructions (Molecular Probes, Invitrogen).

Results

To elucidate the subcellular localization of the CX30.2/CX31.3 protein, lipofectamine was utilized to transfect gap junction-deficient HeLa cells with *GJC3* constructs that were directly "tagged" with GFP at the C-terminal end of the protein. Cells that were stably integrated, the WT CX30.2/CX31.3, was selected. RT–PCR was carried out to evaluate the expression of transgenes in stable cell lines. The stable cell lines expressed a transcript for CX30.2/CX31.3WT, but no CX30.2/CX31.3 was detected in non-transfected HeLa cells (Fig. 1). In the CX30.2/CX31.3WT-GFP stably expressed HeLa cells, fluorescence that was associated with GFP expression was observed along apposed cell membranes. This membrane localization was verified by colocalization with pan-Cadherin (Fig. 2a). The result differs from that obtained in Cx26WT-GFP expressed HeLa cells [20], which yields the typical punctuate pattern of a gap junction channel between neighboring expressed HeLa cells (Fig. 2b). Moreover, HeLa cells that had been transfected with an "empty" expression pGFP plasmid was used as a negative control, and only GFP-tagged protein were found to be uniformly spread throughout the cytoplasm of the HeLa cells (Fig. 2c).

To determine whether CX30.2/CX31.3 forms functional GJ channels, four tracer molecules of different sizes and charges with low molecular weight (<1 KDa) were used to analyze the permeability of CX30.2/CX31.3 in a scrape

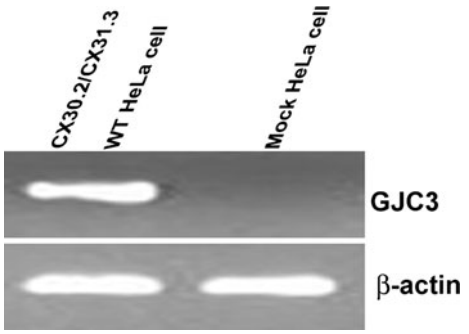


Fig. 1 Expression analysis of *GJC3* mRNA in the transfected HeLa cells by RT–PCR. RT–PCR analysis of total RNA from HeLa cells expressing CX30.2/CX31.3WT-EGFP, and Mock HeLa confirms expression of the corresponding mRNAs in stably transfected HeLa cell lines. *β*-actin served as reference of the loading amount of total RNA for each sample

loading dye transfer assay (Table 1). In addition, tracer molecules, Rhodamin B dextra with a high molecular weight (MW:10 KDa, uncharged) were used as a negative control with no permeability in the GJ channels. The results revealed that all of the tracer molecules with low molecular weight, as well as Rhodamin B dextra did not move away from the scrape line to diffuse to neighboring HeLa cells that stably expressed CX30.2/CX31.3 or mock HeLa cells (Fig. 3). However, HeLa cells that were transfected with CX26 exhibited an efficiency of dye transfer (Fig. 4). These experiments were performed three times with similar results. Our results suggested that permeable function of CX30.2/CX31.3 differs from CX26 in making GJ channels.

When CX30.2/CX31.3 was localized to the plasma membrane, but failed to form morphologic or functional gap junctions, an attempt was made to determine whether CX30.2/CX31.3 induces the formation of functional hemichannels via ATP release. Accordingly, the culture media from confluent CX30.2/CX31.3 monolayers of HeLa cells and mock HeLa cells were replaced with calcium-containing PBS, and samples were collected from the supernatant at indicated times (5, 10, 15 min). As displayed in Fig. 3a, ATP levels within the supernatant did not differ between 5 and 15 min. In contrast, when the culture medium contained

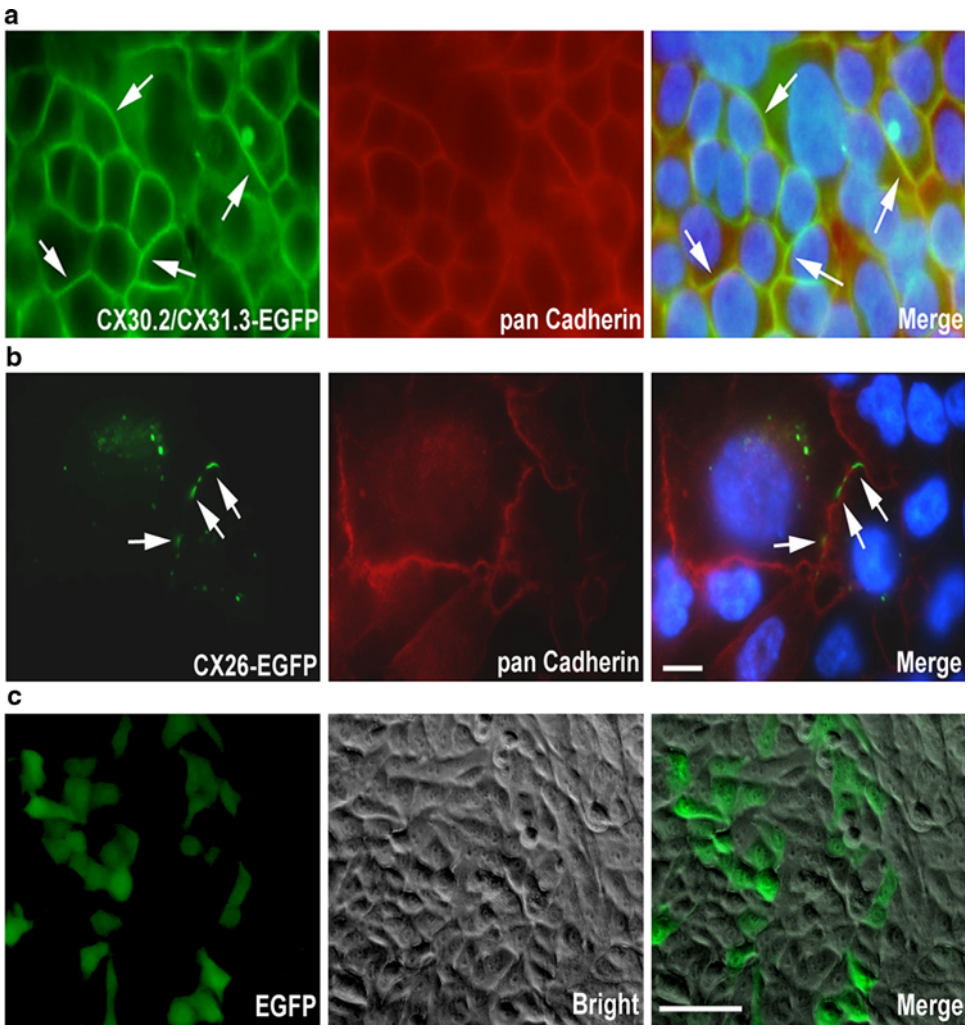
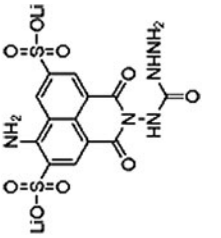
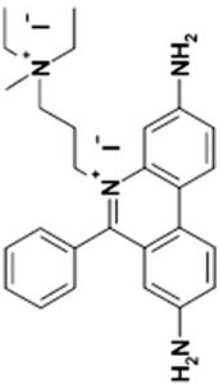
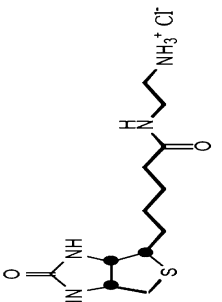
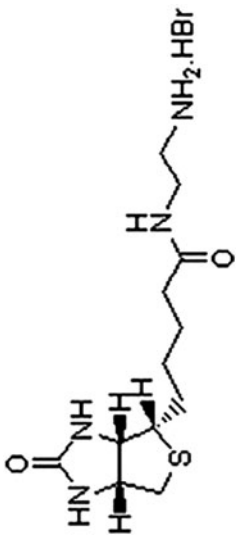


Fig. 2 Analysis of expression of CX30.2/CX31.3WT in stably transfected HeLa cells based on immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells that express CX30.2/CX31.3-EGFP (**a**) revealed continuous fluorescence along apposed cell membranes. However, HeLa cells that are transfected with CX26WT-EGFP (**b**) exhibit the typical punctuate pattern of the GJ channel between neighboring cells that express CX26. *Arrows* indicate expression of CXs proteins. Fluorescence microscopy of EGFP; **c** HeLa cells exhibit uniform spread expression of these fusion proteins in cytoplasmic HeLa cells. The cells were counterstained with 4′-6-diamidino-2-phenylindole, DAPI, to highlight the nuclei. *Scale bars*: 10 μm

Table 1 Intercellular transfer of fluorescent trace molecular in this study

Trace molecules	Rhodamine B dextran	Lucifer Yellow	Propidium iodide	Neurobiotin	Biotin ethylenediamine
Molecular weight	10,000 Da	443 Da	415 Da	287 Da	297 Da
Charge	No	−2	+2	+1	No
Spectral characteristics	λ_{ex} :570 nm λ_{em} :590 nm	λ_{ex} :428 nm λ_{em} :530 nm	λ_{ex} :562 nm λ_{em} :588 nm	Abs/Em = NA/NA nm	Abs/Em = NA/NA nm
Structure	–				

EGTA, a chelating agent for calcium, the ATP levels within the supernatant rose to approximately 671 nM after 5 min and 804 nM after 15 min in the CX30.2/CX31.3 expression HeLa cells (Fig. 5a). These results indicate that the ATP concentration in the extracellular medium of HeLa cells with stably expressed CX30.2/CX31.3 (804.1 ± 30.4 nM) significantly exceed that in mock HeLa cells (449.5 ± 56.7 nM) at low extracellular calcium concentrations (student *t*-test, $p < 0.001$) (Fig. 5b). Furthermore, the release of ATP by HeLa cells with stably expressed CX30.2/CX31.3 was significantly higher in low extracellular calcium concentrations (804.1 ± 30.4 nM) than under normal calcium conditions (173.3 ± 36.5 nM) (Fig. 5b). This experiment was performed five times with similar results. To determine whether the ATP release was specifically through hemichannels, we performed the same experiments in the presence of 100 or 200 μM 18α-glycyrrhetinic acid (AGA) (Sigma–Aldrich), a hemichannel blocker, diluted in DMSO for 1 h. before ATP assay. The result found that the ATP concentration in the extracellular medium of CX30.2/CX31.3WT HeLa cells with pretreatment AGA is significantly decreased from 813.1 ± 29.1 nM to 382.3 ± 31.5 nM (100 μM AGA) or 182.3 ± 32 nM (200 μM AGA), respectively (Fig. 6).

Based on above results, we suggest that CX30.2/CX31.3 can form calcium-dependent hemichannels and release ATP, in the HeLa cells with stably expressed CX30.2/CX31.3.

Discussion

The function of GJ channels and hemichannels that were formed by CX30.2/CX31.3 was tested in stably transfected HeLa cells. Like other CXs, CX30.2/CX31.3 comprises four transmembrane domains that are connected by one cytoplasmic and two extracellular loops, with cytoplasmic C- and N-terminal ends [21]. Most CXs proteins can form typical plaques in the plasma membrane of neighboring cells. Gap junction plaques are clusters of cell–cell channels, which can be packed at densities of over 10,000/μm^2 and the fraction of the cell surface that is covered by the channels is generally considerably relatively low in most cells [11]. Moreover, previously obtained data have demonstrated that plaque formation is a cooperative self-assembly process [22, 23]. However, this investigation instead identified detectable signals (continuous fluorescence) along apposed cell membranes in the HeLa cells that stably expressed CX30.2/Cx31.3-EGFP. The result is similar to that obtained for pannexins in NRK cells [24] and mouse Cx23 in HeLa cells [25]. Accordingly, we suggest that the function of CX30.2/CX31.3 may differ from that of other CXs, as it can form plaques in the plasma membrane, as does CX26, CX30, and CX43.

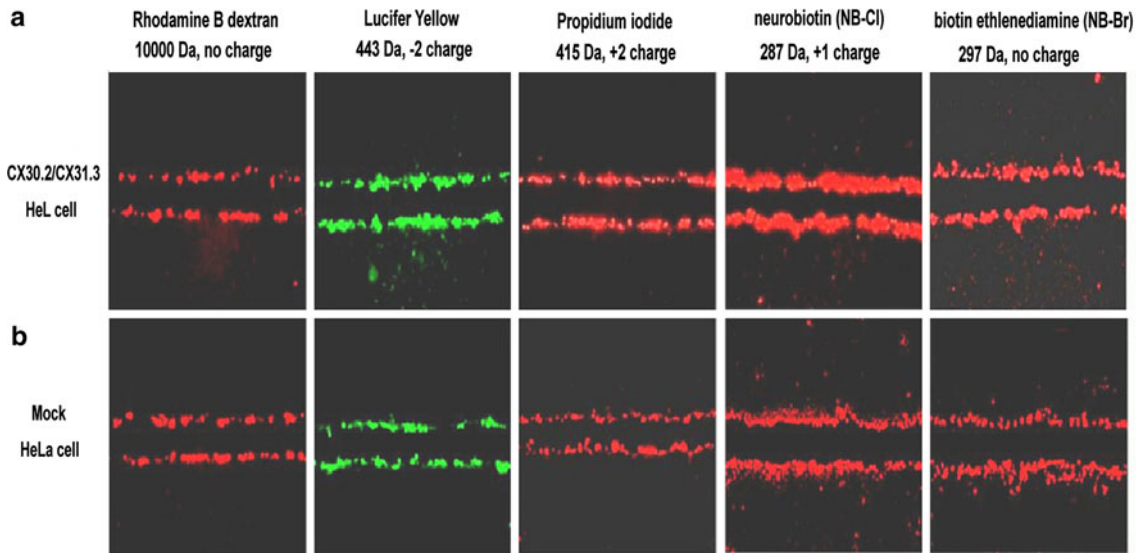


Fig. 3 Dye transfer after scrape loading HeLa cells that stably express CX30.2/CX31.3. Digital fluorescence images of HeLa cells that stably express CX30.2/CX31.3 (**a**) and mock HeLa cell (**b**). Cells were incubated in Lucifer Yellow (LY; 443 Da, −2 charge), propidium iodide (PI; 415 Da, +2 charge), neurobiotin (NB-Cl; 287 Da, +1 charge) or biotin ethylenediamine (NB-Br; 297 Da, uncharge). Rhodamine-dextran (10,000 Da) was the negative control. The wounded cells took up the dye in all cases, but no dye was transferred from wounded parental cells to neighboring cells

GJ channels are critical intercellular pathways through which ions or small molecules that are usually smaller than 1,000 Da are passed, regulating several physiological and developmental processes [26]. Previously, trace molecules of different sizes and charges and with low molecular weights have been adopted to examine the permeability properties of GJ channels, including CX26, CX30, CX31, and Cx43, by microinjection or scrape loading dye transfer assay [27–31]. The physiological properties of the various CX proteins have not been fully elucidated but they differ in the size and charge characteristics of their associated channels, and in their regulatory properties [30]. For example, HeLa cells that expressed CX26 were permeable to dyes up to 622 Da, but HeLa cells that expressed CX30 were permeable to dyes up to 457 Da [28]. A scrape loading dye transfer study indicated that the dyes with low molecular weight, including Lucifer Yellow (MW: 443, 2− charge), Propidium iodide (MW: 414, 2+ charge), neurobiotin (MW: 287, 1+ charge), and biotin ethylenediamine (MW: 297, uncharge), failed to diffuse away from the scrape line to neighboring cells in the HeLa cells that stably expressed CX30.2/Cx31.3-EGFP. The data demonstrate the lack of permeability of GJ channels formed by CX30.2/CX31.3 in stably transfected HeLa cells. These results are consistent with a previous study, in which lack permeability to Lucifer Yellow and neurobiotin in HeLa cells that express Cx31.3 is observed [32].

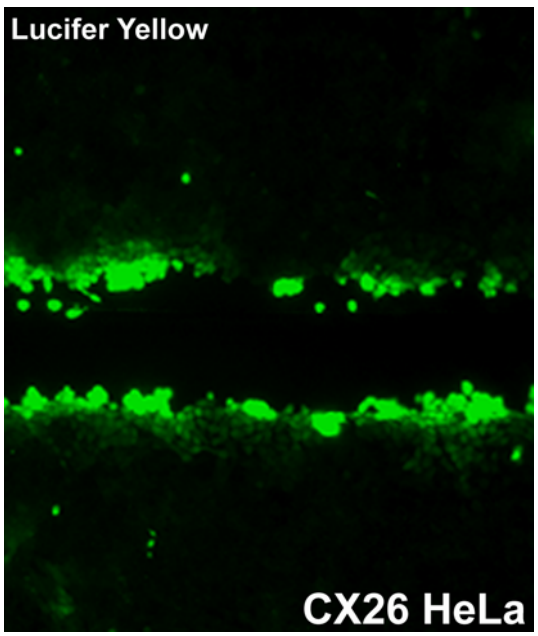


Fig. 4 Dye transfer after scrape loading HeLa cells that stably express CX30.2/CX31.3. Cells were incubated in Lucifer Yellow (LY; 443 Da, −2 charge). The wounded cells took up the dye in all cases and dye was transferred from wounded parental cells to neighboring cells

Many CXs and pannexins, such as CX30, CX45, CX46, CX50, and PxI, exhibit functional hemichannels in Xenopus oocytes or mammalian cell lines, and are thought to be critical to paracrine signaling. [33–35].

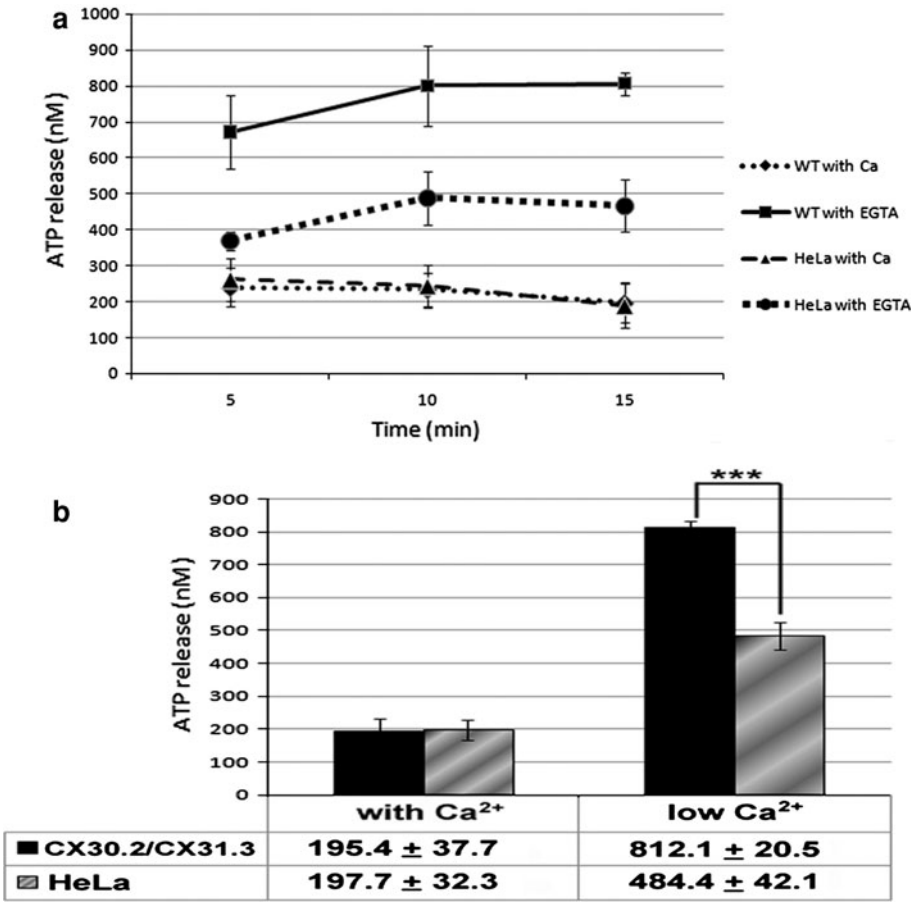


Fig. 5 Analysis of activity of CX30.2/CX31.3 hemichannels based on ATP release. **a** To examine extracellular release of ATP from HeLa monolayers of confluent CX30.2/CX31.3 (WT), cells were washed and the culture medium was replaced with calcium or EGTA-containing PBS. The ATP content of their supernatant was sampled at indicated times and quantified using a luminometric ATP detection assay. **b** HeLa cells that stably expressed CX30.2/CX31.3 released a significantly larger amount of ATP into the extracellular medium at low calcium ion concentration than did HeLa mock cells. Data represent average ± SD ($n = 5$), ***$p<0.001$

Connexin hemichannels, P2x7 receptors (P2x7Rs), pannexin channels, anion channels, vesicles, and transporters are putative conduits for released ATP [36].

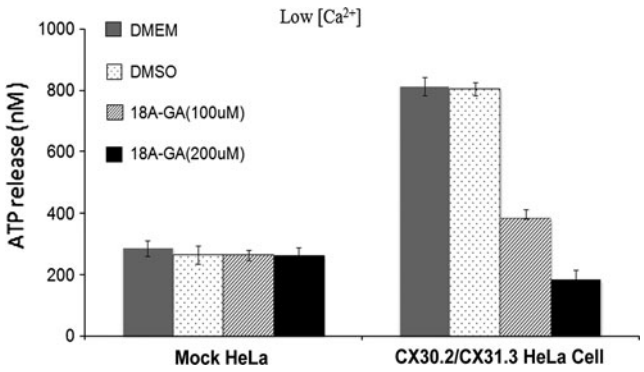


Fig. 6 The effect of AGA on extracellular ATP levels. HeLa cells that stably expressed CX30.2/CX31.3 were cultured in the presence of 100, 200 μM AGA medium and DMSO, respectively. After 1 h, cells were washed and the culture medium was replaced with calcium or EGTA-containing PBS. A culture medium was sampled for ATP content at determined time points (15 min). Our results indicated that ATP concentration in the extracellular medium of CX30.2/CX31.3 HeLa cell with pretreatment AGA is significantly decreased than CX30.2/CX31.3 HeLa cell with no-treatment AGA. In addition, ATP concentration are no noticeable altered in the CX30.2/CX31.3 HeLa cell by only DMSO pretreatment. Data represent average ± SD ($n = 5$)

Calcium has a key role in cellular communication, in which it has been identified as a key cytoplasmic factor in controlling junctional communication, with higher intracellular calcium levels associated with weaker communication in the GJ channels [37]. The data in this investigation demonstrate that HeLa cells express CX30.2/CX31.3 released ATP at a significantly higher level than mock HeLa cells at low calcium ion concentrations. This result agrees with the results of an earlier study, in which connexin hemichannels were activated at low calcium concentrations [38]. This investigation is the first to demonstrate the release of ATP by the hemichannels of the HeLa cells that stably expressed CX30.2/Cx31.3-EGFP.

In summary, the results herein reveal that the function of CX30.2/CX31.3 is dissimilar to that of other CXs that form GJ plaques or functional GJs, and is hemichannel-based. Knowledge of the function of CX30.2/CX31.3 will provide important insights into the mechanisms of human disease that involve mutations in the *GJC3* gene.

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