

Connexin32 inhibits gastric carcinogenesis through cell cycle arrest and altered expression of p21^{Cip1} and p27^{Kip1}

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Gap junctions and their structural proteins, connexins (Cxs), have been implicated in carcinogenesis. To explore the involvement of Cx32 in gastric carcinogenesis, immunochemical analysis of Cx32 and proliferation marker Ki67 using tissue-microarrayed human gastric cancer and normal tissues was performed. In addition, after Cx32 overexpression in the human gastric cancer cell line AGS, cell proliferation, cell cycle analyses, and p21^{Cip1} and p27^{Kip1} expression levels were examined by bromodeoxyuridine assay, flow cytometry, real-time RT-PCR, and western blotting. Immunohistochemical study noted a strong inverse correlation between Cx32 and Ki67 expression pattern as well as their location. *In vitro*, overexpression of Cx32 in AGS cells inhibited cell proliferation significantly. G₁ arrest, up-regulation of cell cycle-regulatory proteins p21^{Cip1} and p27^{Kip1} was also found at both mRNA and protein levels. Taken together, Cx32 plays some roles in gastric cancer development by inhibiting gastric cancer cell proliferation through cell cycle arrest and cell cycle regulatory proteins. [BMB Reports 2013; 46(1): 25-30]

INTRODUCTION

Gastric cancer is one of the most important causes of cancer-related death worldwide and remains a major public health concern in eastern Asian countries, including Korea (1, 2). In Korea, gastric cancer has been the most common type of cancer for the last ten years, causing 56.8 deaths per 100,000 individuals annually (2). The development of gastric cancer in response to exposure to carcinogens and/or *Helicobacter pylori* is believed to occur over a long period of time and involve a number of events (3, 4). Disruption of the balance between cell proliferation and apoptosis is an important driving force of gastric cancer develop-

ment (5, 6). Although our understanding of gastric cancer has improved considerably, the precise mechanisms underlying gastric cancer progression remain incompletely understood.

Gap junction channels, which are localized to cell-cell contact sites, are composed of connexins (Cxs) and mediate the intercellular flux of metabolites, nutrients, and second messengers (7-9). This gap junction intercellular communication and Cxs play important roles in organ/tissue homeostasis and cell differentiation (7, 8). Individual Cxs are defined and named based on their molecular weight and differ in both function and expression patterns (7, 9, 10). Cx26 and Cx32 are the main types of stomach Cxs (11, 12), whereas colonic and rectal epithelial cells primarily express Cx26 (13).

Abnormal patterns of Cx expression, such as decreases, loss or abnormal subcellular localizations, have been reported in various tumors (12-15). Recently, we reported that localization of Cx32 expression altered from cell membranes to the cytoplasm or its expression was altogether lost in human gastric cancer in relation to the degree of tumor cell differentiation (16). Moreover, decreased expression of several types of Cxs has been reported in chemically induced mouse lung tumors (17, 18).

Accumulating evidence has clearly demonstrated a role for Cxs in cell proliferation. A comparison of the cellular proliferation with the levels of Cx43 has demonstrated a possible inverse correlation in canine bone tumors (19). Consistent with this, knocking down Cx32 expression was shown to increase cell proliferation in rat hepatoma cell line (20), and Cx43 overexpression was found to significantly decrease proliferation of human lung cancer-derived cell lines (21). It is generally recognized that tumors develop and progress through uncontrolled cell growth due to abnormalities in the cell cycle (22, 23).

In this study, we examined the expression of Cx32 and that of the proliferation marker Ki67 in tissue-microarrayed human gastric cancer tissues and investigated the correlation between their expression patterns. We then examined cell proliferation, cell cycle distribution, and the cell cycle regulator p21^{Cip1} and p27^{Kip1} expression levels after Cx32 overexpression in the human gastric cancer cell line AGS.

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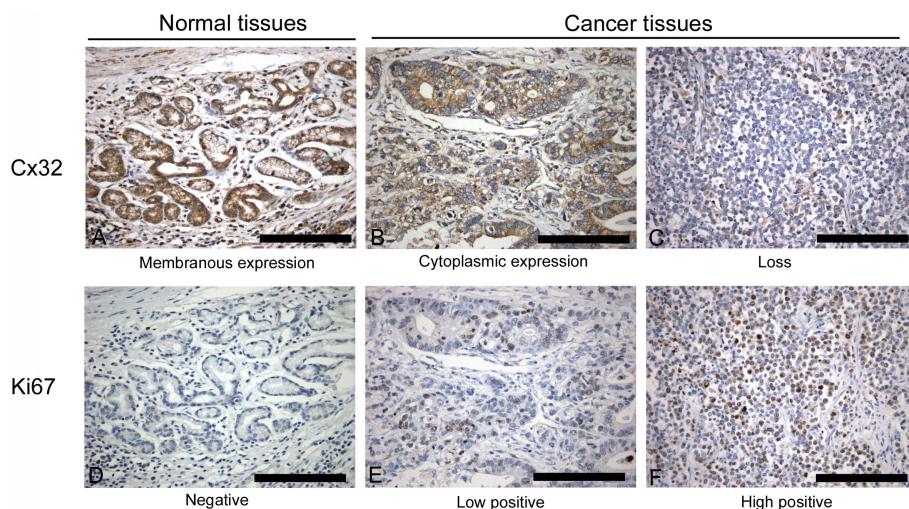


Fig. 1. Immunohistochemical staining for Cx32 and Ki67 in normal gastric tissues (A and D) and gastric cancer tissues (B, C, E, and F). (A) normal mucosa showed intercellular expression; (B) cancer cells in moderately differentiated adenocarcinoma showed intracytoplasmic expression; (C) cancer cells in poorly differentiated adenocarcinoma showed negative staining; (D) normal gastric mucosa showed negative staining for Ki67; (E) cancer cells in moderately differentiated adenocarcinoma showed nuclear expression; (F) cancer cells in signet ring cell carcinoma showed nuclear expression; All bar = 50 μ m.

RESULTS

Cx32 expression in human gastric cancer and normal tissue

We recently investigated Cx32 expression in human normal as well as gastric cancer tissues (16). As previously found, normal gastric mucosa predominantly showed intercellular Cx32 expression (Fig. 1A), whereas cytoplasmic expression (Fig. 1B) and loss of expression (Fig. 1C) were often noted in cancer tissues. The expression of Cx32 at intercellular junctions gradually decreased, whereas cytoplasmic expression or loss of expression increased in proportion to the degree of neoplastic cell differentiation.

The relationship between Cx32 and Ki67 expression in human gastric cancer and normal tissue

Nuclear Ki67 expression was evident in both normal (Fig. 1D) and cancer tissues (Fig. 1E, F). Our results showed that $10.15 \pm 7.57\%$ of cells in normal tissues were Ki67-positive compared with $18.99 \pm 17.41\%$ in gastric cancer tissue. Thus, the percentage of Ki67-positive cells was significantly increased in gastric cancer, a difference that was significant ($P < 0.01$).

An examination of Ki67-positivity in relation to the pattern of Cx32 expression in normal gastric tissues and carcinoma tissue showed an inverse correlation between Cx32 and Ki67 expression (Fig. 2). This correlation held for normal tissue (Spearman $\rho = -0.269$; $P = 0.034$) and cancer tissue (Spearman $\rho = -0.430$; $P < 0.01$) analyzed separately. Specifically, the frequency of Ki67-positive cells was increased as Cx32 localization shifted from a membranous to cytoplasmic pattern, and was further increased with loss of Cx32 expression.

Cell proliferation and cell cycle distribution following overexpression of Cx32 in the AGS gastric cancer cell line

Because a negative correlation was found between cell proliferation and Cx32 expression, we performed *in vitro* experi-

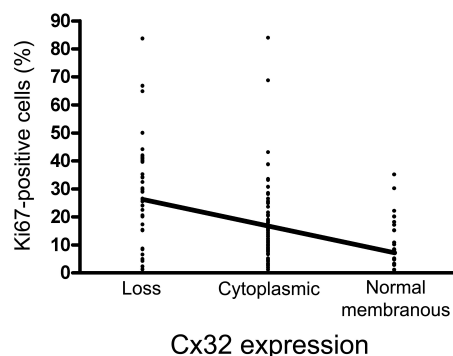


Fig. 2. The relationship between Ki67 and Cx32 expression in normal gastric tissues and cancer tissues. There is a correlation between Ki67 positivity and Cx32 expression (Spearman $r = -0.421$; $P < 0.01$).

ments to examine further the direct relationship between Cx32 expression and cell proliferation. The value of bromodeoxyuridine (BrdU) absorbance decreased approximately 30-40% in the AGS cells overexpressing Cx32 compared to that in wild-type AGS cells or AGS cells transfected with a control vector ($P < 0.05$). There was no significant difference in the value between wild-type AGS cells and AGS cells transfected with a control vector ($P > 0.05$) (Fig. 3A). These results suggested that Cx32 overexpression inhibits cell proliferation in AGS cells.

Having demonstrated that Cx32 overexpression negatively regulated cell proliferation, we next quantified the cell cycle distribution (Fig. 3B). The percentage of G₁-phase cells was significantly greater in AGS cells transfected with Cx32 vector than in wild-type AGS cells or AGS cells transfected with control vector ($P < 0.05$). In addition, the percentage of S-phase cells was significantly less in AGS cells overexpressing Cx32 than in wild-type AGS cells or AGS cells transfected with control vector

($P < 0.05$). No differences were evident for the percentage of both G₁- and S-phase cells between wild-type AGS cells and AGS cells transfected with a control vector ($P > 0.05$). These results suggested that the Cx32-dependent inhibition of cell proliferation is related to G₁ arrest.

p21^{Cip1} and p27^{Kip1} expression following overexpression of Cx32 in the AGS gastric cancer cell line

Because Cx32 overexpression affected the cell cycle distribution, we sought to determine whether the expression of cell cycle regulators differed among three groups, focusing on changes in p21^{Cip1} and p27^{Kip1} (Fig. 4). Stable cell lines were screened for

the amount of Cx32 protein by immunoblotting. Western blot analyses revealed that Cx32 expression in AGS cells transfected with Cx32 vector was approximately 2- to 3-fold greater compared to wild-type AGS cells or AGS cells transfected with control vector. The expression of p21^{Cip1} at the mRNA level increased 2- to 2.5-fold and the expression of p21^{Cip1} protein was approximately 30-50% greater in AGS cells overexpressing Cx32 compared to that of wild-type AGS cells and AGS cells transfected with control vector ($P < 0.05$). p27^{Kip1} expression at the mRNA level increased 60-90% and the content of p27^{Kip1} protein was also approximately 2- to 3-fold greater in AGS cells overexpressing Cx32 than the control cell lines ($P < 0.05$). The

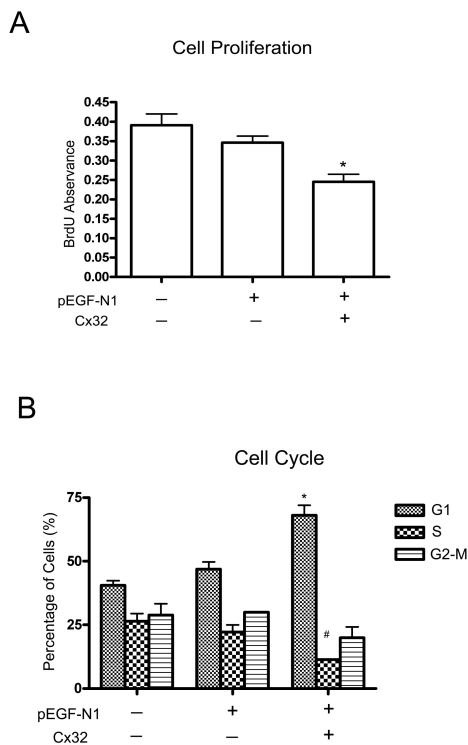


Fig. 3. Cell proliferation and cell cycle distribution analyses showed that Cx32 overexpression inhibited cell proliferation through G₁ arrest in AGS cells. (A) Bromodeoxyuridine assay. The bars represent the means \pm standard deviations of difference of absorbance. Cell proliferation was decreased in AGS cells overexpressing Cx32 compared to the control groups. * $P < 0.05$; relative to control groups. (B) Cell cycle distribution. The bars represent the means \pm standard deviations of the percentage of cell-cycle stage. The percentage of G₁-phase cells was significantly greater in AGS cells transfected with Cx32 vector ($68.06 \pm 3.93\%$) than in wild-type AGS cells ($40.54 \pm 1.80\%$) or AGS cells transfected with control vector ($46.91 \pm 2.78\%$) ($P < 0.05$). In addition, the percentage of S-phase cells was significantly less in AGS cells overexpressing Cx32 ($11.32 \pm 0.24\%$) than in wild-type AGS cells ($26.44 \pm 3.04\%$) or AGS cells transfected with control vector ($22.13 \pm 2.86\%$) ($P < 0.05$). * $P < 0.05$; Vs relative to the control groups in G₁-phase. # $P < 0.05$; relative to control groups in the S-phase.

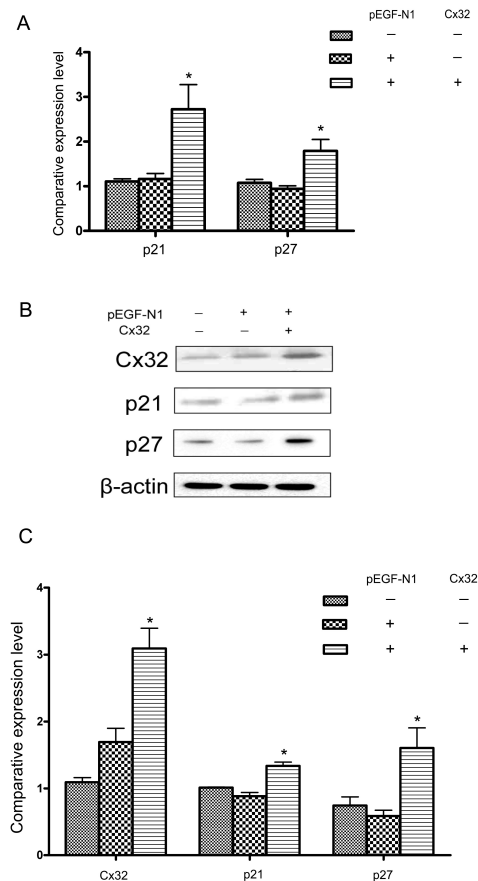


Fig. 4. Real-time RT-PCR and Western blotting analyses showed that Cx32 overexpression increased the expression of p21^{Cip1} and p27^{Kip1} at mRNA and protein levels in AGS cells. (A) The bars represent the means \pm standard deviations of ratio of p21^{Cip1} and p27^{Kip1}/ β -actin in the real-time RT-PCR data. Expression of p21^{Cip1} and p27^{Kip1} was significantly higher than control groups; (B) Cx32, p21^{Cip1}, and p27^{Kip1} protein levels were analyzed by Western blotting using β -actin as the loading control; (C) The density graph indicates the ratios of Cx32, p21^{Cip1} and p27^{Kip1}/ β -actin in the Western blot data. Expression of Cx32, p21^{Cip1}, and p27^{Kip1} was significantly higher than control groups. * $P < 0.05$ relative to the control groups.

levels of all proteins in wild-type AGS cells and AGS cells transfected with control vector were not significantly different ($P > 0.05$). Our results thus showed that Cx32 overexpression in AGS cells induced an increase in p21^{Cip1} and p27^{Kip1} expressions.

DISCUSSION

Similar to other previous studies, we recently found that Cx32 expression is often shifted from an intercellular to an intracytoplasmic location, or is even lost, in human gastric cancer (16). Although altered expression of Cxs has been reported in various malignant tumors, including gastric cancer, the exact role of Cx32 in gastric carcinogenesis has not yet been clearly defined yet. We investigated whether altered Cx32 expression might impact gastric cancer development, placing special emphasis on disruption of normal cell proliferation.

The involvement of Cxs in the regulation of tumor cell proliferation has been suggested by a number of recent studies. A study to knock down Cx32 expression in rat hepatoma cells has shown that the magnitude of cell proliferation is inversely proportional to the level of Cx32 expression (20). Furthermore, the incidence of hepatic and pulmonary neoplasms was found to be higher in Cx32-deficient and Cx43-deficient mice, respectively, than in their wild types littermates (24, 25). An immunohistochemical analysis of *H. pylori*-associated mouse gastric tumors showed an inverse relationship between Cx32 and Ki67 expression (16). Our *in vitro* Cx32 overexpression study is in full agreement with these studies.

Cx expression per se can reduce the proliferation of cancer cells, an effect that is independent of localization to the plasma membrane and formation of gap-junction plaques by transfected Cxs (26). Other studies reinforce this interpretation, showing that some Cx mutants that are incapable of plasma membrane insertion are nonetheless capable of down-regulating the cell proliferation (27, 28). In canine mammary tumors, malignant tumors showed increased cytoplasmic staining for both Cx26 and Cx43, whereas hyperplastic and benign neoplastic glands showed only membranous expression. Moreover, the expression and distribution of Cx26 and Cx43 were inversely correlated to cell proliferation in malignant tumors (14). Consistent with these observations, we found a relationship between Cx32 localization and cell proliferation in our immunohistochemical study. Thus, Cxs clearly play some role in proliferative aspects of gastric cancer development but further studies are needed to decipher how actually or what pathways are involved. Depending upon such mechanisms, Cxs might be good target for cancer therapy.

Our investigation of the Cx32 effect on the cell cycle showed that Cx32 inhibits cell cycle progression. Similarly, overexpression of Cx43 has been shown to suppress the proliferation of human osteosarcoma U2OS cells through the inhibition of cell cycle transition from the G₁- to the S-phase (29). In addition, the forced expression of Cx43 and Cx32 was reported to decrease the growth of neoplastic mouse lung and rat liver epithelial cells *in vitro* in association with a reduction in the exit of

cells in the G₁-phase (30). These data suggest that Cx32 regulates cell proliferation, at least in part, through G₁-phase arrest. We then investigated the expression levels of p21^{Cip1} and p27^{Kip1} following Cx43 in AGS cells. We found that the degrees of p21^{Cip1} and p27^{Kip1} expression were significantly increased both at the mRNA and protein levels. In rat glioma cells, tolbutamide was shown to increase Cx43 protein synthesis, an effect that was accompanied by the up-regulation of p21^{Cip1} and p27^{Kip1} (31). As shown in Zhang *et al.*, increased synthesis as well as post-transcriptional reduced degradation of p27^{Kip1} was evident in human osteosarcoma cell line U2OS (29). Cx43 overexpression can inhibit cell proliferation in association with a decrease in the stability of S-phase kinase-associated protein 2, which is involved in cell-cycle regulation. This study raises another intriguing possibility that Cxs might have other roles, such as the direct transcriptional regulation of various genes that might include p21^{Cip1} and p27^{Kip1} (32). The regulation mechanism of Cxs in p21^{Cip1} and p27^{Kip1} expression might vary according to tumor type and Cx isoform. Hence, additional studies are needed for better understanding of p21^{Cip1} and p27^{Kip1} expression mechanisms by Cxs in gastric cancer.

In conclusion, our immunohistochemical analysis of patient-matched normal and cancerous gastric tissues demonstrated an inverse relationship between Cx32 expression and proliferation, and our *in vitro* study of the effects of Cx32 overexpression showed that Cx32 inhibited the proliferation of gastric cancer cells through cell cycle arrest and up-regulation of p21^{Cip1} and p27^{Kip1}.

MATERIALS AND METHODS

Immunohistochemical staining for Cx32 and Ki67

Tissue-microarrayed slides containing a total of 105 gastric adenocarcinoma and 62 normal gastric tissues, purchased from SuperBioChips Laboratories (Seoul, Korea) and ISU ABXIS Co., Ltd. (Seoul, Korea) were used for immunohistochemistry.

The degrees and pattern of Cx32 and Ki67 expressions were studied immunohistochemically in replicate sections of tissue-microarrayed slides, using mouse anti-rat Cx32 (Chemicon International Inc., Temecula, CA, USA) and rabbit anti-human Ki67 (Dakocytomation, Glostrup, Denmark) antibodies. Immunoreactive proteins were detected using a Bond Polymer Refine Detection kit and BOND-MAX automated immunostainer (Leica Microsystems, New York City, NY, USA). Briefly, the sections were subject to antigen retrieval and endogenous peroxidase activity was quenched with hydrogen peroxide. The slides were blocked using the blocking serum provided in the staining kit. Thereafter, slides were incubated with primary anti-Cx32 (1 : 200) and anti-Ki67 (1 : 100) followed by incubation with post primary blocker and polymer as described by the manufacturers'. 3,3'-diaminobenzidine was used as the chromogen.

Immunohistochemistry scoring and analysis

For immunohistochemical analyses, a total of approximately

1,000 tumor or normal cells from each microarrayed spot were evaluated. Cx32 expression patterns were classified into three categories: normal membranous expression, cytoplasmic expression or loss (16). A spot with less than 10% Cx32 was positivity regarded as negative (loss). The Ki67-labeling index (%) was determined by dividing the number of positive cells by the total number of cells, and multiplying by 100.

Cell culture and Cx32 transfection

The AGS human gastric cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), and antibiotic-antimycotic (Invitrogen Biotechnology, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO₂.

A cDNA insert containing the entire coding region of human Cx32 (NM_000166) (33) was subcloned into the *Xho*I – *Hind*III site of the expression vector pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA, USA). The sequence of the resulting Cx32 expression plasmid (pEGFP-N1-Cx32) was confirmed by DNA sequencing. AGS cells were transfected with pEGFP-N1-Cx32 or pEGFP-N1 (control vector) using Metafectene Pro transfection reagent according to the manufacturer's instructions (Biontex Laboratories, Martinsried, Germany). After selection for 14 days with 1 mg/ml of G-418 (Sigma-Aldrich Co., St. Louis, MD, USA), a single-cell clone was established and screened for Cx32 expression by immunoblotting.

Cell proliferation and cell cycle analysis

Cell proliferation was measured using BrdU-based cell proliferation assay (Millipore, Temecula, CA, USA) according to the manufacturer's instructions.

For cell cycle analysis, AGS cells wild-type, AGS cells transfected with control vector, and AGS cells transfected with Cx32 vector were trypsinized, and then fixed and incubating at –20°C. After washing, the cells were incubated in phosphate-buffered saline containing RNase for 30 min at 37°C. Then, a solution of propidium iodide was added to the cell suspension, and the cells were analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA, USA).

Western blotting

For preparation of total protein lysates, AGS cells of three groups were lysed (1 M Tris-HCl, 5 M NaCl, 0.5 M EDTA, and NP-40) and microcentrifuged. The supernatants were separated by electrophoresis on polyacrylamide gels, transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), and probed with antibodies against Cx32, p21^{Cip1} (Santa Cruz biotechnology Inc., Santa Cruz, CA, USA), or p27^{Kip1} (Oncogene, Cambridges, MA, USA). The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (GE Healthcare UK Limited, Buckinghamshire, UK) or horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA). β -actin (Cell Signaling Technology Inc., Danvers, MA,

USA) was used as internal control.

RNA isolation and quantitative real-time RT-PCR

The total RNA from transfected cells was extracted by RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. About 1 μ g of total RNA from each sample was subjected to cDNA synthesis using QuantiTeck Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The cDNA was analyzed by real-time PCR using Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and the following primer pairs: p21^{Cip1} forward 5'-TCCAGCGACCTTCCTCATCCAC-3', reverse 5'-TCCA TAGCCTCTACTGCCACCATC-3'; p27^{Kip1} forward 5'-CGCTCG CCAGTCCATT-3', reverse 5'-ACAAAACCGAACAACAAAG-3'; β -actin forward 5'-CCCACTGTGCCCATCTACG-3'; reverse 5'-AGGATCTTCATGAGGTAGTCAGTCAG-3'. Targets were amplified and mRNA was quantified using a Rotor-Gene Q and the manufacturer's software (Qiagen, Hilden, Germany). The amount of mRNA was calculated using β -actin as the endogenous control.

Statistical analysis

Statistical analyses were performed using SPSS Statistics (version 18.0; SPSS Inc., Chicago, IL, USA). Proliferation indexes (Ki67 labeling indexes) were expressed as means \pm standard deviations; data were compared using a two-tailed Student's *t*-test. The relationship between Ki67 and Cx32 expression was analyzed using Chi-square and Spearman's rho correlation tests. In tests on AGS cells, the data were expressed as means \pm standard deviations of at least three independent experiments ($n = 3$); data were compared using an unpaired two-tailed Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

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