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Apoptosis screening of human chromosome 21 proteins reveals novel cell death regulators

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Abstract The functional analysis of chromosome 21 (Chr21) proteins is of great medical relevance. This refers, in particular, to the trisomy of human Chr21, which results in Down's syndrome, a complex developmental and neurodegenerative disease. In a previous study we analyzed 89 human Chr21 genes for the subcellular localization of their encoded proteins using a transfected-cell array technique. In the present study, the results of the follow-up investigation are presented in which 52 human Chr21 genes were over-expressed in HEK cells using the transfected-cell array platform, and the effect of this protein over-expression on the induction of apoptosis has been analyzed. We found that the over-expression of two Chr21 proteins (claudin-14 and -8) induced cell death independent of the classic caspase-mediated apoptosis. Our results strongly suggest the functional involvement of claudins in the control of the cell cycle and regulation of the cell death induction mechanism.

Keywords Down's syndrome · Transfected-cell array · Apoptosis · Claudins · Microarray

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Introduction

With an incidence of approximately one in 700 live births, Down's syndrome (DS) is the most common genetic cause of mental retardation [1]. DS, or trisomy 21, is caused by an extra, third, copy of all or part of human chromosome 21 and the consequent over-expression of the proteins encoded within it. The phenotype of Down's syndrome includes various organ malformations, stereotypic craniofacial anomalies and brain malformations [2]. The molecular analysis of this syndrome, however, poses a particular challenge because the aneuploid region of Chr21 contains genes of unknown functions. Genomic sequencing and gene expression analyses of human Chr21 [3–7], as well as studies of the transcriptome of DS mouse models [8–12], provide a comprehensive resource for the systematic functional characterization of Chr21 genes. However, the functional characterization at the protein level has been performed most often using protein prediction algorithms. Therefore, the determination of the subcellular protein distribution as well as the effects of protein over-expression on cellular physiology would provide an important insight into the function of human Chr21 genes.

In a previous study, we analyzed 89 human Chr21 genes for the subcellular localization of their encoded proteins using a transfected-cell array technique. The over-expression of several Chr21 genes was found to result in changes in cellular phenotypes, such as the cell morphology and proliferation [13]. To understand the underlying mechanisms of these phenotypes, in the present study we investigated the effects of protein over-expression on induced cell death using the same cell array technique. A novel enhanced apoptosis assay was developed on the cell array platform, allowing the efficient, large-scale screening of apoptosis regulators. This led to the discovery of cell death

induction resulting from the over-expression of two tight-junction proteins, claudin-8 and -14, out of 52 human chromosome 21 proteins. By using multiple assays that utilize different detection principles for dying cells, we were able to reveal a non-classic type of apoptosis induced by claudins-8 and -14.

Materials and methods

Plasmid constructs and transfected-cell array assay

Protein expression constructs for 52 human chromosome 21 genes have been cloned and reverse transfected as described previously [13]. Briefly, each ORF was amplified from cDNA clones and using the Gateway™ cloning technology (Invitrogen), the PCR products were finally cloned into the pDEST™26 vector for protein over-expression with a 6×-Histidine (His_6) tag at the N-terminus. For the reverse transfection on cell arrays, the plasmids were first diluted in a gelatin solution and printed onto standard glass slides that were coated with poly-L-lysine (Sigma). The spotted plasmid-slides were then treated with Effectene transfection reagent (Qiagen) before adding the HEK293T cells on the top for reverse transfection, as described by Ziauddin and Sabatini [14]. The Chr21 proteins were allowed to over-express on cell arrays for 48–72 h before the cells were subjected to the apoptosis detection assay.

Cell culture

Human HEK293T/17 (ATCC, CRL-11268) cells were cultured in the DMEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% (v/v) fetal calf serum (Biochrom, Berlin, Germany), L-glutamine (Sigma) and gentamicin (Gibco Invitrogen, Karlsruhe, Germany) at 37°C in a humidified 5% CO_2 incubator. One day before the transfection, 10 × 10e6 HEK293T cells per 10-cm dish in 10 ml of medium were seeded out. For the cell array, 3.5 × 10e6 HEK293T cells were added on top of each slide for the reverse transfection.

Immunofluorescence

The recombinant Chr21 proteins were detected with Penta·His Alexa488 or Alexa555-conjugated mouse antibody (Qiagen). The mouse anti-Bax antibody (clone 3, BD Biosciences) was used to evaluate the expression level of the exogenous Bax protein. After fixation with 3.7% (v/v) paraformaldehyde, the cell arrays were permeabilized with 0.1% (v/v) Triton X-100 or 0.5% (w/v) saponin and subsequently blocked for 1 h at room temperature using 5% (w/v) bovine serum albumin or 5% (v/v) normal serum from the host species of the fluorophor-labeled antibodies. The cell arrays were then incubated with the mouse primary antibodies. For the detection of Bax protein, an incubation with the secondary antibody, Alexa488 or Alexa568 goat anti-mouse IgG antibody (Molecular Probes), was performed. Each cell array slide was incubated with DAPI before the final washing and mounting with Prolong Gold anti-fade reagent (Molecular Probes).

Apoptosis assays

TUNEL

The cell arrays were fixed with 3.7% formaldehyde for 20 min at room temperature. After two PBS washes, the permeabilization was performed with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at room temperature. After washing with PBS, the appropriate volume of freshly prepared TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Roche) was added to the slides and the slides were incubated in a humid atmosphere for 60 min at 37°C in the dark. After three PBS washes, the cell arrays were mounted for fluorescence microscopy or a scanner analysis. Alternatively, AlexaFluor 488-conjugated anti-Fluorescein/Oregon Green goat antibody (Molecular Probes) was used as a secondary antibody to amplify the fluorescence signal while still allowing fluorescein-compatible optics.

Annexin V

The cell arrays were washed once with the PBS containing Ca^{2+} (Gibco, Invitrogen) to remove the culture medium, followed by a wash with the Annexin V binding buffer (BD Pharmingen). The Annexin V working solution was then added to incubate the cells for 20–30 min at room temperature. For Annexin V-biotin (Caltag Laboratories), a 1:40 dilution was found optimal, whereas for Annexin V-FITC (BD Pharmingen), even the original 10× stock solution was found to stain the apoptotic cells weakly. The Annexin V-biotin-labeled cell arrays were rinsed twice in Annexin V binding buffer before being subjected to streptavidin binding. Alexa Flour 594- or 488-conjugated streptavidin (Molecular Probes) was freshly prepared in Annexin V binding buffer to 3–5 μg/ml, and used to incubate the cell arrays for 20 min at room temperature. After the removal of the streptavidin excess by washing the slide with Annexin V binding buffer, the apoptotic cells were stained by Annexin V and examined by fluorescence microscopy. Alternatively, for prolonged storage, the

Annexin V-labeled cell arrays were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature.

Cleaved caspase-3

The labeling of cleaved caspase-3 was performed with the rabbit antibody (Cell Signaling) following the basic immunofluorescence protocol described above. In some cases, the cleaved caspase-3 antibody was also combined with the mouse anti-His or mouse anti-Bax antibody, to reveal the correlations between the activation of caspase-3 and the over-expression of Chr21 and Bax proteins. In this case, the incubation with the primary antibodies was performed overnight at 4°C. The Alexa488-conjugated donkey anti-rabbit IgG and Alexa568-conjugated goat anti-mouse IgG (Molecular Probes) were mixed and used as secondary antibodies for the fluorescence labeling.

Triple apoptosis assay

Three apoptosis assays—Annexin V, cleaved caspase-3 and the TUNEL reaction—were applied together on a single cell array slide to collect the death signals from different apoptosis stages in a single experiment. The cell arrays were always labeled with Annexin V prior to the cell fixation. After the fixation, the cell array slide was stored in Annexin V binding buffer at 4°C until the multiple detection. For the triple apoptosis assay, the cell arrays were first permeabilized with 0.1% Triton X-100 in PBS rather than with 0.1% sodium citrate, which is used in the single TUNEL assay. After blocking in 5% BSA, the arrays were first incubated overnight with rabbit cleaved caspase-3 before proceeding to the TUNEL reaction. The rest of the basic immunofluorescence protocol was then followed, except that the composition of secondary antibodies was modified according to the purpose of each experiment. When it was necessary to combine the signals from caspase-3 and TUNEL, the AlexaFluor488-conjugated goat anti-rabbit and AlexaFluor488-conjugated donkey anti-FITC secondary antibodies were used. Otherwise, the AlexaFluor555-conjugated anti-Rabbit antibody was used rather than the AlexaFluor488-conjugation.

Laser scanning and microscopy

To observe the fluorescence signals within each cell cluster (i.e., apoptotic cells and over-expressed proteins), a BioCCD scanning system (Applied Biosystems, Darmstadt, Germany) was used. For the cell morphology analysis and protein subcellular localization, the ImagerZ1 microscope (Zeiss, Jena, Germany) and LSM510 confocal system (Zeiss) were used, together with the Axiovision 4.0 and LSM510 software (Zeiss).

Results

Multiple apoptosis detection on cell arrays

To select a suitable apoptosis detection assay for the cell array, a construct over-expressing the human Bax protein, a key regulatory protein in the mitochondrial apoptotic pathway, was first used as a positive control for the assay optimization. Three apoptosis assays (Annexin V, cleaved caspase-3 antibody, and TUNEL) were found to be compatible with transfected cell arrays adjustments of the respective protocols. As shown in Fig. 1a, different

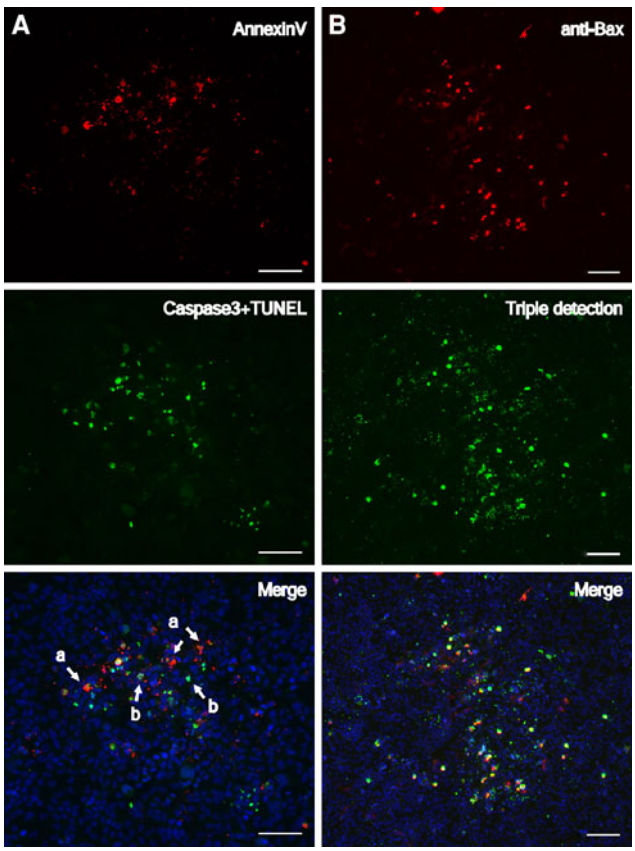

Fig. 1 **a** The different cell death assays (as indicated) target the cells at different stages of apoptosis. Annexin V, anti-activated Caspase-3 antibody and TUNEL were used to label the same apoptotic cell cluster induced by Bax over-expression with different fluorochromes (as indicated). The non-overlapping signals in the merged image (*bottom*) indicate different apoptotic stages of the cells. The cells in *red* (**a**) were stained by Annexin V only, a marker for the early stage of apoptosis; the *green* cells (**b**) were in the middle to late stages of apoptosis, as detected by TUNEL and an antibody against activated caspase-3. **b** The combined apoptosis assays collectively label the asynchronous death signals induced by Bax over-expression. The immunofluorescence labeling using an anti-Bax antibody indicates a cell cluster over-expressing Bax. *Triple detection*: combined Annexin V, activated caspase-3 Ab and TUNEL assays. *Merge*: the combination of anti-Bax staining and the triple death detection. Scale bars, 100 μm

apoptotic cells, as a result of the Bax over-expression, were positive to the Annexin V staining, cleaved caspase-3 and TUNEL assays. The staining differences might originate from the biological mechanism underlying the particular assays. Annexin V is known to dominantly bind to the cell plasma membrane in regions where the asymmetry of the membrane structure has been lost. Because cell membranes were more abundant at the early stage of apoptosis, Annexin V binding at this stage was much more intensive compared to the staining of cells with fragmented membranes at later stages of apoptosis. In contrast, most of the TUNEL-sensitive cells were often characterized with collapses in the cytoplasm and nucleus, and most frequently with cell blebbing or fragmentation, features of the very late stage of apoptosis. Activated caspsase-3 was often detected in the cells between the middle and early-late stage, when the cytoplasm shrank and the nucleus lost its integrity, but prior to fragmentation (Fig. 1a). Due to their differential sensitivities, the three assays (Annexin V, cleaved caspase-3 and TUNEL), when used with separate fluorochromes, allowed us to identify different stages of the cell death pathway. On the other hand, all three assays could be used simultaneously (triple death assay) with the same color fluorochrome to collect the asynchronous apoptotic signals ranging from the early to late stage, as shown in Fig. 1b. This was of particular use in this study to investigate the apoptosis-inducing effects of human Chr21 proteins on Hek293T cell arrays, where false-negatives might be present due to the relatively low efficiency of transfection.

The over-expression of two Chr21 proteins (claudin-8 and -14) induced cell death through a non-apoptotic pathway

The triple apoptosis assay was subsequently applied to study Chr21 proteins for possible apoptosis-inducing effect. Cells over-expressing claudin-8 and -14 were found to be extensively labeled, whereas claudin-17 showed negative staining. As shown in Fig. 2, 48 h after the reverse transfection, over 70% of the cells over-expressing claudin-14 were positively stained by the triple assay, whereas none of the cells over-expressing claudin-17 were stained. In the subsequent time-course experiments performed in both Hek293T and HeLa cells at 24, 48 and 72 h after transfection, at least a 70% positive staining was obtained for the claudin-14-transfected cells irrespective of the transfection time and cell lines (data not shown).

To determine whether these results were cell array-dependent, normal transfections of the three claudins were performed in a 6-well plate format and the triple death assay was performed on cells fixed on a glass cover slip. Again, most of the cells over-expressing claudin-14 or -8 were tested positively for cell death, whereas only few

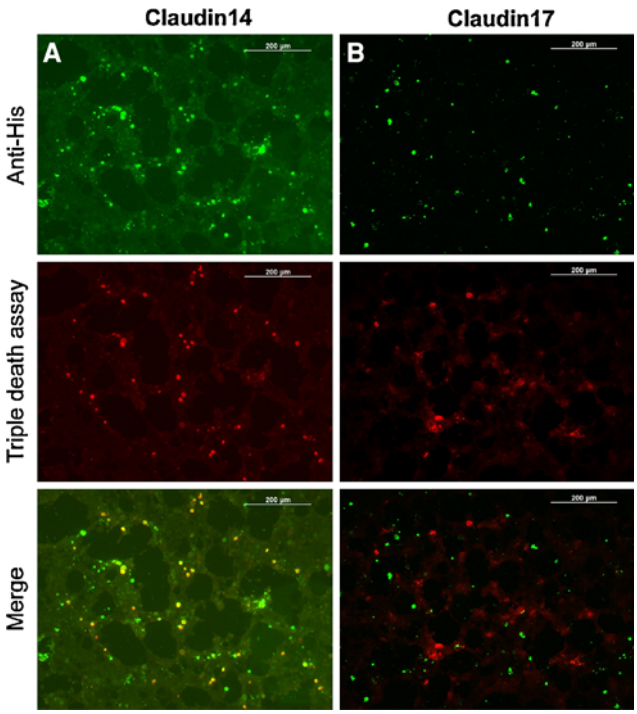

Fig. 2 The over-expression of claudins leads to programmed cell death. The over-expression of claudin-14 (**a**), but not claudin-17 (**b**), resulted in a positive detection by the "triple death assay". *Upper images*: the cell clusters transfected with a plasmid coding for claudin-14 (*upper left*) or claudin-17 (*upper right*). The expressed proteins were labeled with an anti-His antibody that was conjugated to a green fluorophor. *Middle images*: the simultaneous detection of the same cell clusters by Annexin V, the activated Caspase-3 antibody and TUNEL with red fluorescence. *Merged images*: the combination of anti-His staining and death detection. The *yellow cells* in the *left image* indicate cell death as a result of claudin-14 over-expression. Scale bars, 200 μm

cells, undergoing spontaneous apoptosis, were found in the case of the claudin-17 transfection (data not shown). The findings from both the cell array and normal transfection experiments strongly suggest that the over-expression of claudin-8 and -14 but not claudin-17 leads to extensive cell death. Interestingly, in the previous protein subcellular localization study, claudin-8 and -14 had been found predominantly in the ER instead of the plasma membrane, where tight-junction proteins (including claudin-17) localize [13]. Hence, the ER retention of claudin-8 and -14 likely correlates with the cell death induced by their over-expression.

Separate use of the assays revealed a non-apoptotic form of cell death

Despite the cell shrinkage and nuclear DNA degradation, the additional typical apoptosis features, such as cell blebbing, were not observed in the dying cells following the claudin-8 or -14 over-expression. We next asked

whether the cell death induced by the two claudins was a classic type of apoptosis. Annexin V, activated caspase-3, and TUNEL assays were separately applied to determine the origin of the detection signals and thus to elucidate the type and possible mechanism of the induced cell death. In the cells transfected by claudin-8 or -14, Annexin V rather than the other two assays was found to largely contribute to the positive labeling within the "triple death assay". Under a higher magnification, Annexin V was found to bind specifically to the outer leaflets of the cell membrane that wrapped the condensed ER where claudin-14 localized (Fig. 3a). Moreover, the nuclear DNA of the dying cells appeared to degrade as the DAPI staining greatly decreased. For the cells at the late death stage (the cytoplasm extremely condensed), the nuclear DNA was barely stained by DAPI (Fig. 3b). TUNEL-positive staining, however, was only observed in few cells over-expressing the claudins (Fig. 4), suggesting the DNA fragmentation is not compulsory for the claudin-induced cell death. Surprisingly, caspase-3, an essential processor for classic apoptosis, was found not to be actively cleaved in the dying cells induced by the two claudins (Fig. 5). Moreover, the typical apoptotic phenotypes, such as cell blebbing and the formation of apoptotic bodies, were not found in the case of the claudin-induced cell death. These results were confirmed by normal transfections subjected to separate detection assays. Thus, it might be hypothesized that the cell death process resulting from claudin-8 and -14

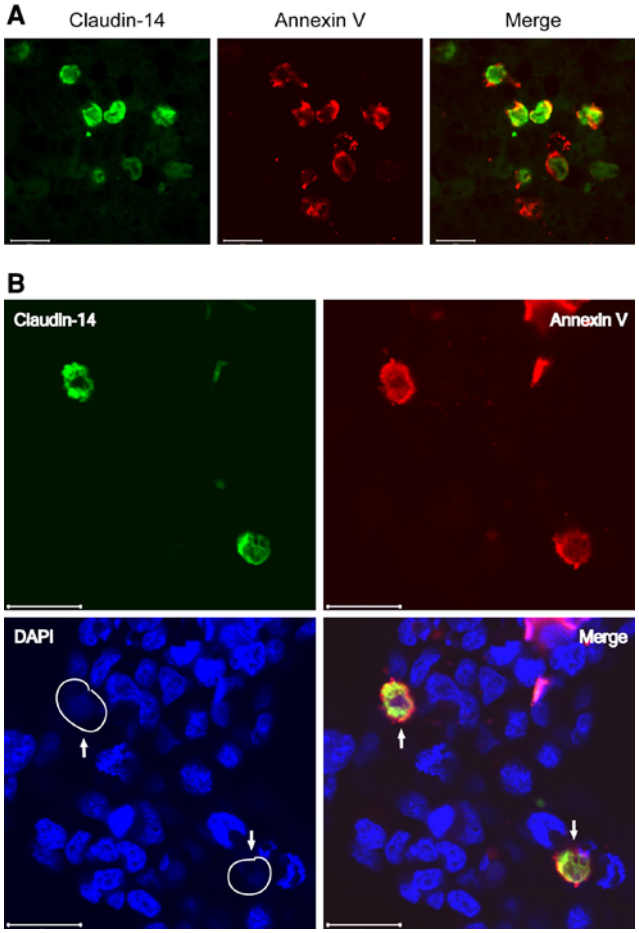

Fig. 3 The cell death induced by claudin-14 over-expression is positive to Annexin V. Annexin V was found to bind specifically to the outer leaflets of the cell membrane where the membrane asymmetry was lost due to claudin-14 over-expression (**a**, **b**). In the cells over-expressing claudin-14, the nuclear DNA degraded and was barely stained by DAPI (*arrow*). *Green fluorescence*: the over-expressed claudin-14 labeled with an Alexa488-conjugated antibody against the His6 epitope fused to claudin-14. *Red fluorescence*: biotin-conjugated Annexin V bound to Alexa594-conjugated streptavidin. *Merge*: the combination of claudin-14, Annexin V, and DAPI staining. Scale bars, 20 μm

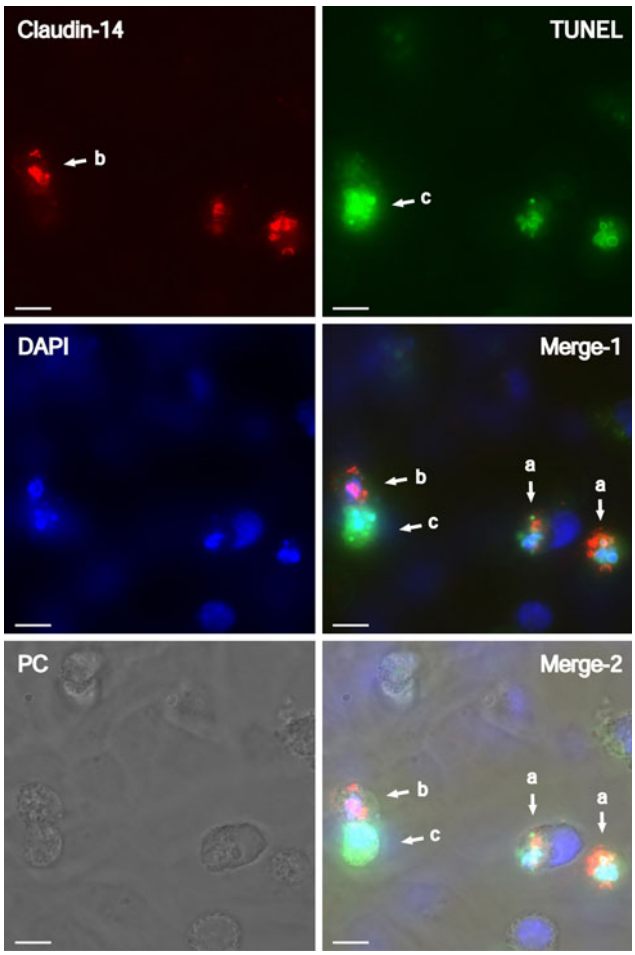

Fig. 4 The cell death induced by claudin-14 over-expression is only partially positive to TUNEL. The DNA fragmentation detected by TUNEL reaction was observed in some (a) but not all of the cells over-expressing the claudins (b). (c) A spontaneous apoptotic cell positive to the TUNEL reaction. *Red fluorescence*: the over-expressed claudin-14 labeled with Alexa555-conjugated antibody against the His6 epitope fused to claudin-14. *Green fluorescence*: the FITC-conjugated TUNEL reaction. *PC*: Phase contrast. *Merge-1*: the combination of claudin-14, TUNEL, and DAPI staining. *Merge-2*: the combination of merge-1 and phase contrast. Scale bars, 20 μm

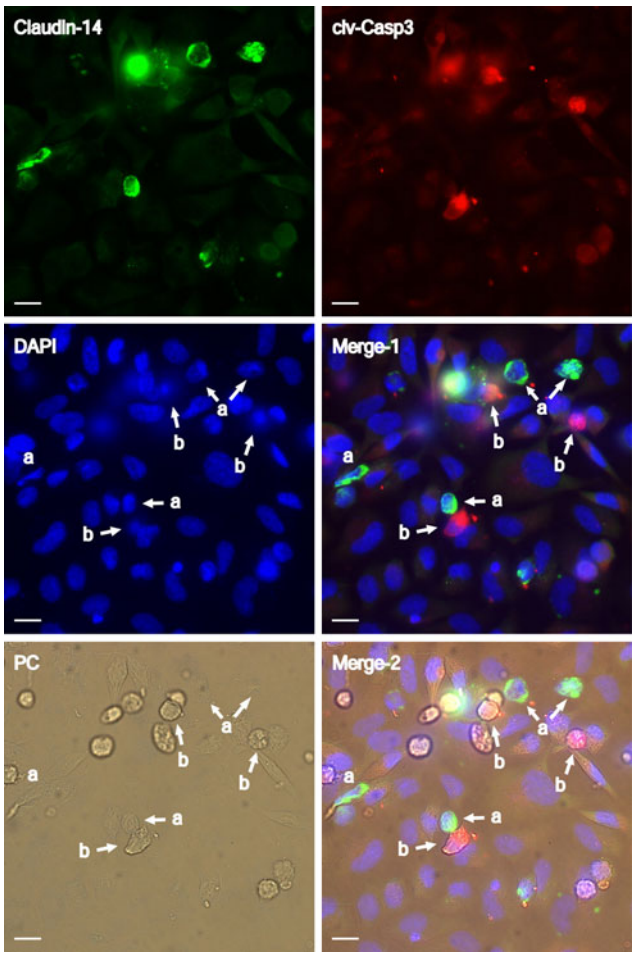

Fig. 5 The cell death induced by claudin-14 over-expression is caspase-3-independent. Cleaved caspase-3, an essential processor for classic apoptosis, was not found in the dying cells induced by the claudin over-expression (a), but was present in the spontaneously apoptotic cells (b). *Green fluorescence*: the over-expressed claudin-14 labeled with the Alexa488-conjugated antibody against the His6 epitope fused to claudin-14. *Red fluorescence*: the spontaneously apoptotic cells labeled by the antibody against cleaved Caspase-3 (clv-Casp3). *PC*: Phase contrast. *Merge-1*: the combination of claudin-14, cleaved caspase-3, and DAPI staining. *Merge-2*: the combination of merge-1 and phase contrast. Scale bars, 20 μm

over-expression occurs via an alternative pathway that is independent of the classic caspase-mediated apoptosis.

Discussion

The claudin multigene family comprises tetraspan membrane proteins which are important structural and functional elements of tight junctions. Claudins have important roles in regulating paracellular permeability and maintaining cell polarity in epithelial and endothelial cells [15]. In mammals, there are 24 members of the claudin family, showing a complex pattern of tissue-specific expression [16]. Claudins extracellular loops from neighboring cells interact with each other to maintain integrity of the cellular sheet. They also regulate paracellular transport between the luminal and basolateral compartments. The claudins interact with a number of proteins and are involved in signal transduction to and from the tight junction [17]. Loss of human claudin-14 is known to cause autosomal recessive deafness [18].

The claudin-related non-classic cell death pathway was previously described during epidermal development in the early embryogenesis of vertebrates. Claudin-1 was found to localize in the periderm and the subperiderm of the chicken embryo, and its function in tight junctions was involved in the formation of the embryonic diffusion barrier, which is lost by desquamation at the end of the embryonic period. Interestingly, a special form of programmed cell death was found to account for the desquamation of the embryonic diffusion barrier. The cell death occurring at the periderm and the subperiderm was partially positive to TUNEL, whereas the activity of caspase-3, -6, and -7 was found to be absent [19]. This type of cell death was very similar to the one induced by claudin-8 and -14 over-expression in this study.

Taken together, these results suggest a previously undescribed functional relationship between the claudins and the control of the cell cycle and regulation of the cell death induction mechanism.

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