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The Role of Claudin-1 and Claudin-7 in Cervical Tumorigenesis

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Abstract. *Background/Aim: The claudin family of proteins are key constituents of tight junctions and the aberrant expression of these proteins can contribute to de-stabilisation of tight junctions and thus to loss of cell polarity and cohesion. Increased expression of claudin-1 and claudin-7 has been observed in pre-invasive cervical lesions and cervical carcinomas. The present study attempted to assess the effect of claudin-1 and claudin-7 overexpression on the HeLa cervical carcinoma cell line, in terms of cell proliferation/viability, permeability, invasion and migration. Materials and Methods: HeLa cells were stably transfected with expression vectors containing the claudin-1 and claudin-7 genes to produce two separate stable cell lines expressing claudin-1 and claudin-7, respectively. The stable cell lines were examined with regard to their invasion and migration abilities, cell permeability and cell proliferation/viability and compared to non-claudin-1 or -7 transfected HeLa. Results: The present study found that claudin-1 and claudin-7 affected the migratory ability of HeLa cells, reducing their ability to migrate in a gap closure assay compared to non-claudin-transfected HeLa cells. Monolayers of claudin-1 and claudin-7 transfected cells also displayed an increased transepithlial electrical resistance indicating decreased permeability compared to non-claudin-transfected HeLa. The study found that claudin-1 or claudin-7 expression had no effect on the proliferation or viability of HeLa cells. Claudin-1 or -7 expression also did not affect the invasive ability of HeLa cells with both stable cells lines and non-claudin-transfected HeLa cells all showing low invasive ability. Conclusion: The results of the present study indicate that claudin-1 and claudin-7 overexpression alone does not contribute to increased tumorigenesis in cervical carcinoma, instead claudin-1 and -7 expression in HeLa cells contribute to reducing the migratory ability of cells and decrease their permeability.*

Cervical cancer is the second most common cancer in women worldwide, behind only breast cancer (WHO 2013). The disease is associated with a persistent infection of the cervical epithelium by the human papilloma virus (HPV) (1), and like many other epithelial malignancies, a loss of cell polarity and cohesion is thought to play a key role in the development of the disease (2, 3). Tight junctions (TJ) play a key role in maintaining the apical-basolateral polarity and cohesive structure of epithelial cells.

The tight junction is an inter-cellular structure that mediates adhesion between epithelial cells and is required for proper epithelial cell function (4). Tight junctions control paracellular permeability across epithelial cell sheets and also serve as a barrier to intra-membrane diffusion of components between cells apical and basolateral membrane domains (5).

Claudins are tetraspan transmembrane protein components of tight junctions. They determine the barrier properties of this type of cell–cell contact existing between the plasma membranes of two neighbouring cells (6). Claudins can completely tighten the paracellular cleft for solutes, and they can form paracellular ion pores (6). The claudin family consists of at least 24 members, with each showing a specific organ and tissue distribution (4).

Claudin-1 expression is observed in several normal tissue types, where it plays a role in maintaining tight junction integrity and function (7). Studies have found claudin-1 overexpression in certain malignancies, such as oral squamous carcinoma and colon carcinoma, is associated with tumorigenesis (8, 9), whereas in other malignancies, such a lung (10) and gastric (11) claudin-1 expression is associated with tumor-suppressive activity. This suggests a dual function for claudin-1 as both an oncogene and tumor suppressor depending on the tumor type. Similar to claudin-1, claudin-7 can function in both an oncogenic and tumor suppressive manner, depending on the origin of the tumor. In certain malignancies, such as esophageal (12) and breast (13) a reduction of claudin-7 expression has been observed and is associated with tumorigenesis. However in other tumors, such as ovarian malignancies, elevated expression has been observed (14).

Other studies have observed claudin-1 and claudin-7 overexpression in pre-invasive cervical lesions and invasive cervical malignancies (15, 16). The present study attempts to assess the effect of claudin-1 and claudin-7 overexpression in the cervical, claudin-null cell line, HeLa, and to elucidate how

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they may contribute to disease pathogenesis. To achieve this task, HeLa cells were transfected with claudin-1 and claudin-7 vectors and two stable cells lines were generated overexpressing claudin-1 and -7. The invasive, migratory and proliferative ability of these cell lines was determined to assess the effect of claudin overexpression on the cell lines and to elucidate the role of claudin overexpression in cervical neoplasia.

Materials and Methods

Generation of stable cell lines. Vectors containing either the *claudin-1* or *claudin-7* gene and were acquired from Genecopedia (Rockville, MD, USA). HeLa cells were cultured in 6-well plates to 60-70% confluency before they were transfected with either the claudin-1 or claudin-7 vector using the Lipofectamine 2000 reagent (Life Technologies Grand Island, NY, USA). After incubation for 48 h at 37˚C transfected cells were transferred from 6-well plates to 100-mm plates and incubated for a further 24 h. Geneticin selection was then performed by the addition of 400 μg/ml G418 (Roche, Basel, Switzerland) to the culture medium. After geneticin selection, colonies were picked and screened for the expression of either the claudin-1 or claudin-7 protein using western blotting. HeLa cells identified as stably expressing claudin-1 (C1-HeLa), claudin-7 (C7-HeLa) and HeLa cells without the claudin-1 or claudin-7 vector (Null-HeLa) were then used for further experimental analysis.

Western blotting. Cells were washed twice in PBS before being lysed in RIPA buffer containing 1% protease inhibitors (Merck Millipore, Billerica, MA, USA). Lysates were sonicated for 15 s, to ensure complete lysis and incubated on ice for 15 min and centrifuged at 14,000 rpm at 4˚C for 20 min. The supernatant was removed and an aliquot was stored at –80˚C and the remaining sample used for immunoblotting. Protein concentration was determined using the bradford assay and 30 μg of protein were electrophoresed on a 12% SDS polyacryamide gel. The gel was electrophoresed at 100 V for 3 h before being transferred to a nitrocellulose membrane. The membrane was blocked using 5% skimmed dry milk in TBST for 1 h and then incubated with either rabbit anti claudin-1 (Invitrogen), rabbit anti-claudin-7 (Invitrogen Carlsbad, CA, USA) or mouse anti-beta actin (Sigma Aldrich, St. Louis, MO, USA) at 4˚C overnight. The membrane was washed 3 times with TBST before application of the appropriate anti-mouse or anti-rabbit HRP linked antibodies (Sigma Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Bands were visualized using an enhanced chemilumminescent substrate (Thermo Pierce, Rockford Illinois, USA) as per the manufacturer's protocol.

Invasion assay. Invasion assay was performed using the Cytoselect Cell Invasion Assay, produced by Cell Biolabs (San Diego CA, USA). The invasion chamber plate was allowed to warm to room temperature before the addition of 300 μl of serum-free RPMI to the inner compartment and incubated at room temperature for 1 h. A cell suspension containing cells at a concentration of 1×10^6 per ml was prepared in serum-free RPMI. The serum-free medium from the inner compartment was removed and 300 μl of the cell suspension was added to the inner compartment. 500 μl of RPMI containing 10% foetal bovine serum were added to the outer compartment of the well. The invasion chamber was incubated at 37˚C for 24 h in a 5% CO_2 atmosphere. Following incubation, media were aspirated from the inner compartment and the interior of the compartment was swabbed to remove non-migratory cells. The insert was transferred to a new well containing 400 μl of cell stain solution and incubated for 10 min. The inserts were gently rinsed with water several times before being allowed to air dry. Invasive cells were counted under a light microscope with at least three individual fields per insert observed. Each insert was then transferred to a new well containing 200 μl of extraction solution and incubated on an orbital shaker for 10 min. 100 μl of each sample was transferred to a 96-well microtiter plate and the absorbance measured at 560 nm.

Gap closure assay. Cells were seeded in a 6-well plate and cultured until a fully confluent monolayer had formed. A gap was then created in the monolayer by scraping a p200 pipette tip vertically down the center of the well. The scratch was then repeated horizontally across the well to create a cross shaped gap in the monolayer. Each well was gently rinsed 3 times in PBS to remove loose cells before the addition of complete media. Under a phase-contrast microscope an appropriate area of the gap was identified and an image captured (0-h timepoint). This coordinates of the area was noted for future orientation. The plate was placed in an incubator at 37˚C, 5% CO_2 until the next timepoint. At 24-h and 48-h timepoints the plate was removed and another image was captured at the appropriate coordinates. The experiment was performed a minimum of three separate times.

MTT assay. CLDN-1, CLDN-7 and untransfected HeLa cells were seeded in 96-well plates (1×10^4 cells per well) and cultured at 37˚C, 5% CO_2. At 24- and 48-h time points the assay was performed in triplicate and in three separate experiments according to the following protocol. A stock MTT solution was prepared at a concentration of 5 mg/ml by dissolving 50 mg MTT (Sigma Aldrich, St. Louis, MO, USA) in 10 ml d.d.H_20. A working solution of MTT reagent was prepared by diluting the stock solution 1:10 in RMPI-1640 media and filter sterilising the solution. At the appropriate time point the media is removed from each well and 100 μl of working MTT solution is added to the wells and the cells were incubated for 3 h. After 3 h, the cells were washed 3 times with sterile PBS and after the last wash all residual liquid was removed from the well. 100 μl of DMSO was then added to each well and the plate was shaken on a rotary shaker for 10 min. The absorbance of each well at 595 nm was then recorded using a spectrophotometer.

Transepithelial electrical resistance. Cells were seeded onto a 1.2-cm diameter transwell insert with a 3.0 μm pore size (Corning Costar) and cultured until a fully confluent monolayer had formed. Transepithelial electrical resistance was then measured using the STX2 electrode and EVOM (World Precision Instruments). Readings for each insert were taken 10 times and the experiment was performed on three separate occasions. Resistance per cm^2 was calculated according to the EVOM manual by subtracting the value of the blank insert from all values then multiplying this value by $\pi d^2/_4$.

Results

Generation of stable cell lines. Following transfection of HeLa cells with expression vectors and genticin selection, two separate cell lines each expressing claudin-1 (C1-HeLa) and claudin-7 (C7-HeLa) proteins were identified using

western blotting (Figure 1). Non-claudin-transfected HeLa cells (Null-HeLa) were confirmed to have no expression of claudin-1 or -7 (Figure 1).

Effect of claudin-1 and claudin-7 transfection on cell invasion. In the present study we attempted to assess the effect of claudin-1 and claudin-7 overexpression on the invasive, migratory, proliferation and cell permeability properties of HeLa cells. C1-HeLa cells showed a very low invasive ability, as evidenced by the low absorbance reading observed upon staining and lysis of invaded cells (Figure 2A). This result correlated with the observation of very few cells on the membrane when observed under a light microscope (Figure 2B). C7-HeLa displayed a similar invasive ability to C1-HeLa cells, with both a low number of invaded cells observed when the membrane was observed under a light microscope (Figure 2C) and low absorbance reading observed when invaded cells stained and lysed (Figure 2A) Null-HeLa, showed slightly higher invasive ability compared to C1-HeLa and C7-HeLa although overall the level of invasion was still very low (Figure 2A).

Effect of claudin-1 and claudin-7 transfection on transepithlial electrical resistance. Claudin-1-transfected cells displayed increased TEER values compared to non-transfected Hela cells, with an average resistance of 27.55 Ω per cm^2 compared to 12.89 Ω per cm^2 for untransfected Hela cells. Claudin-7-transfected cells had an average resistance of 28.26 Ω per cm^2 which is significant increase compared to the resistance of claudin null Hela (Figure 3) and comparable to value observed for claudin-1 transfected cells (Figure 3). Results were analyzed using a one-way anova test and were deemed to be statistically significant ($p<0.05$).

Effect of claudin-1 and claudin-7 transfection on cell proliferation/viability. MTT assay results indicate that claudin-1 and claudin-7 transfection had no effect on the proliferative ability of HeLa cells. Claudin-1 and claudin-7-transfected cells showed a high level of proliferation at the 48-h and 72-h timepoints of the assay, with absorbance values roughly doubling at each timepoint (Figure 4) The assay results from claudin-1 and claudin-7-transfected cells closely matched those of non-claudin expressing HeLa cells (Figure 4) indicating that claudin-1 or -7 did not affect the viability or proliferation of the cells.

Effect of claudin-1 and claudin-7 transfection on cell migration. The effect of claudin-1 and claudin-7 expression on the migratory ability of Hela cells was assessed using a wound healing assay. After creating the gap in the cell monolayer, the size of the gap was measured using the Image J software to ensure each gap were close to equal size. The gap in the C1-HeLa, C7-HeLa and Null-HeLa were 840 μm, 870 μm, 850 μm respectively at the beginning of the experiment (0 h). Images of

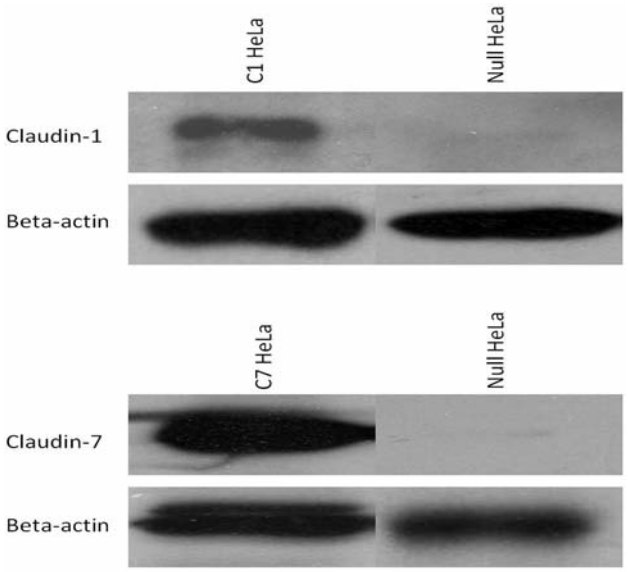

Figure 1. *Western blot of C1-HeLa and C7-HeLa stable cell lines demonstrating expression of claudin-1 and claudin-7 proteins respectively. Non-claudin transfected HeLa (Null-HeLa) displayed no expression of claudin-1 or claudin-7. Beta-actin is used as internal control to verify equal protein loading.*

Null-HeLa show significant migration of cells into the gap after 24 h and complete closure of the gap after 48 h (Figure 5). The size of the gap had reduced after 24 h for C1-HeLa although not to the same extent as the Null-HeLa at 24 h and the gap had not shown complete closure after 48 hours, in contrast to the Null-HeLa cells.

C7-HeLa showed a similar pattern to C1-HeLa with partial closure occurring after 24 h but with gap closure still incomplete after 48 h, unlike the HeLa-null cells which completely infiltrated and filled the gap after 48 h. These results indicate that claudin-1 and claudin-7 transfection may have an effect on the migratory ability of HeLa cells, possibly impeding the motility of cells and their ability to detach from a group of cells and disperse.

Discussion

Loss of cell polarity and adhesion has been well-established as a key feature of tumorigenesis and of facilitating the spread of malignant cells away from the primary tumor to form distant metastasic sites. When tumor metastases form, survival rates and outcomes are significantly poorer than if a tumor remains at its primary site, with over 90% of cancer deaths occurring as a result of tumor metastasis (17). Tight junctions play a key role in the maintaining the cohesive structure of the epithelium and in the regulating the flow of ions and of potentially cancer promoting molecules, such as growth factors through the epithelium. Understanding how tight junctions can become

disrupted and lose their proper function is thus of key importance to gaining insight in the pathogenesis of tumors. As claudins play a major role in maintaining tight junctions, understanding how aberrant expression of these proteins in malignant cells affects the cells invasive, migratory and proliferative ability may offer further insight into the pathogenesis of cervical cancer and offer insight into the identification of new biomarkers and treatments for the disease.

Previous studies have observed increased claudin-1 and claudin-7 in pre-invasive cervical lesions and in cases of cervical carcinomas (15, 16). The present study aimed to investigate the effects of claudin-1 and 7 overexpression on the cervical carcinoma cell line HeLa and elucidate what role claudin-1 and -7 overexpression may play in cervical carcinoma.

This study found that claudin-1 or claudin-7 expression had no significant effects on the invasive ability of HeLa cells (Figure 2). Studies have identified that claudin-1 overexpression is associated with increased invasiveness in oral carcinoma cells through up-regulation of several matrix metalloproteinases (8, 18). Claudin-1 expressing colon carcinoma cells that underwent siRNA-mediated knockdown of claudin-1 displayed significantly decreased anchorage-independent growth and invasion with a significant decrease in MMP-9 activity (9). Conversely, claudin-1 overexpression suppresses metastasis and invasion in lung cancer cells (10). The role of claudins in up-regulating MMPs likely has a key influence on the invasive ability of malignant cells as the role of MMPs in digesting the extracellular matrix and promoting invasion has been well-established. Studies have previously reported that HeLa cells lack the alpha beta 3 integrin receptor (19) which through its interaction with membrane type-1 metalloproteinase-2 (MT1-MMP), and tissue inhibitor of metalloproteinase-2 (TIMP-2) aids the activation of MMP-2 (20). A lack of MMP-2 activity in HeLa cells may explain why they show low invasive ability and why the claudin-1 and -7 overexpression in HeLa cells had no significant effect on the invasive ability of Hela cells (Figure 1). These results suggest that any possible pro-invasive effect of claudin-1 and -7 in malignant cervical cells may be reliant on concurrent expression of MMPs.

The association between claudin-1 and claudin-7 expression and cell migration is also highly dependent on tumor type, with claudin-1 expression being associated with increased cell migration in breast cancer cells (21) and, conversely, associated with an inhibition of cell migration in lung cancer cells (10). Claudin-7 expression is associated with an increased migratory ability in ovarian carcinoma cells (14) but is associated with reduced migratory ability in lung carcinoma cells (22). The present study shows that claudin-1 and claudin-7 overexpression reduces the migratory ability of HeLa cells. These results suggest there may be establishment of homotypic claudin interaction between neighboring cells leading to increased adhesion, or that claudin-1 and -7 transfection had a downstream effect on another motility related pathway. The exact mechanisms by which claudins contribute to cell migration and motility are still not fully-understood. The process is likely similar to the process by which claudins can promote and inhibit cell invasion. Promotion of migration may occur through up-regulation of MMPs and subsequent degredation of the extra-cellular matrix (23). Another mechanism through which claudins may influence cell motility and migration is through the N-WASP and ROCK pathway. Loss of claudin-5 in the breast cancer cell line MDA-MB-231 inhibited cell motility through involvement in signaling pathway of N-WASP and ROCK (24).

Aside from contributing to disruption in cellular cohesion, tight junction breakdown is thought to contribute to tumorigenesis through disorganised paracellular permeability allowing for an unregulated flow of various potentially oncogenic molecules, such as growth factors and cytokines, through the epithelium (25). The present study found that claudin-1- and claudin-7-transfected cells showed increased TEER readings suggesting decreased permeability. Claudin-1 and claudin-7 are reported to increase TEER when transfected into cultured epithelial cells (26) although claudin-1 transfected colon carcinoma cells showed reduced TEER readings (27).

The role of claudins in tumorigensis is still not fully-understood, part of which relates to the fact that claudins can function as a tumor suppressors and as oncogenes depending on the tumor type. Immunohistochemistry-based studies of pre-invasive cervical lesions and cases of cervical carcinoma found an increase in expression of claudin-1 and claudin-7 compared to normal cervical epithelium (15, 16), which would suggest that in the case of cervical malignancies claudin-1 and claudin-7 function in an oncogenic manner. The findings of this study, that claudin-1 and claudin-7 expression reduces HeLa cell migration, increases TEER and has no effect on cell invasion or proliferation, suggest that claudin-1 and claudin-7 expression alone is not responsible for tumorigenesis in cervical malignancies, but likely plays a role as part of a larger process of tight junction disruption and cellular transformation. The process of tight junction dysregulation is a complex process likely involving the aberrant expression of several tight junction proteins. Claudin-1, -2, -4, -7 have been previously reported to be overexpressed in cervical pre-invasive lesions and cases of carcinoma (15). Other non-tight junction related proteins that are involved in cellular adhesion, such as E-cadherin and beta-catenin, are also reported to be aberrantly expressed in cervical malignancies (28). The nature of any possible interactions between these proteins and how they contribute to tumorigensis in cervical malignancies is yet to be fully-understood, although experiments in other cell models may offer some insights. In colon carcinoma cell lines claudin-7 forms a complex with epithelial cell adhesion molecule (EpCAM), CD44v6 and CO-O29, that confers a higher degree of apoptosis resistance than

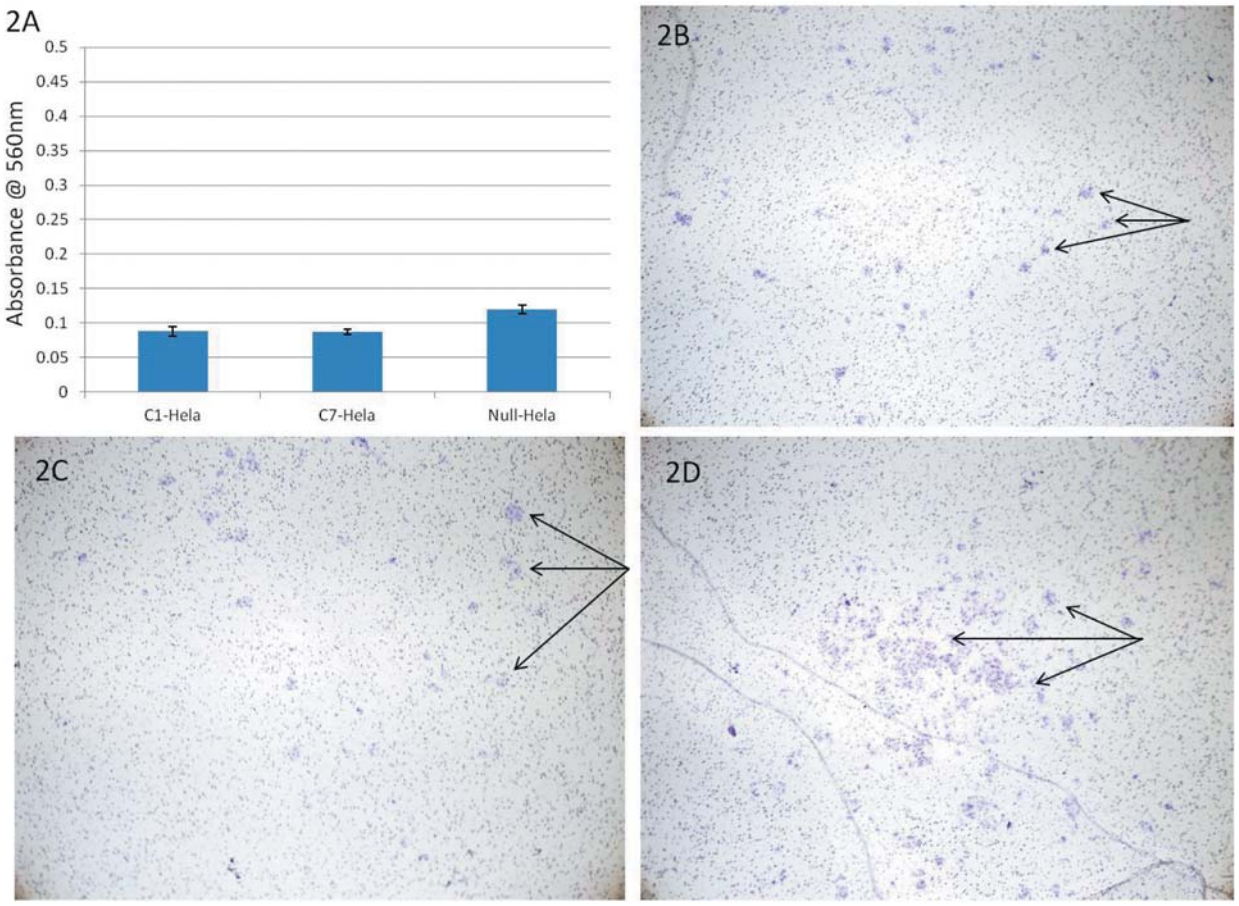

Figure 2. *Graph showing quantification of invaded claudin-1, claudin-7-transfected and untransfected Hela cells at 560 nm following staining and extraction. Error bars show±standard deviation (2A). Brightfield image of invaded claudin-1 transfected (2B), claudin-7-transfected (2C), and untransfected HeLa (2D) (original magnification, ×40) (arrows indicate invasive cells).*

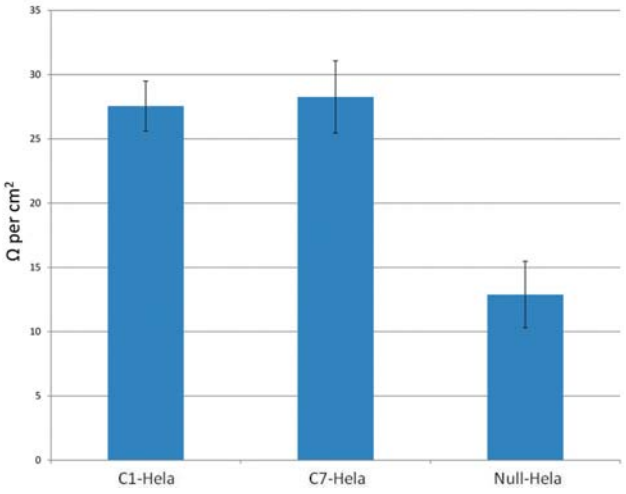

Figure 3. *Graphs show average resistance per cm² in ohms for cultured monolayers of Claudin-1 transfected, Claudin-7 transfected and untransfected Hela cells. Error bars indicate±standard deviation.*

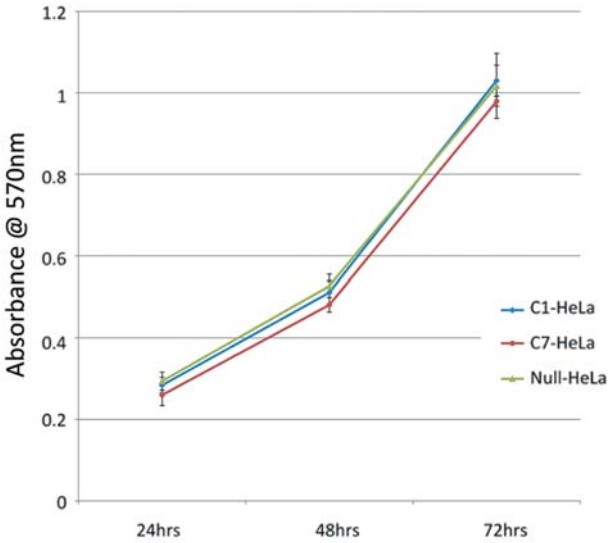

Figure 4. *Graph showing proliferation of C1-Hela, C7-Hela and Hela-Null cell lines using MTT assay at 24 , 48 and 72 h time points. Error bars are ±SD.*

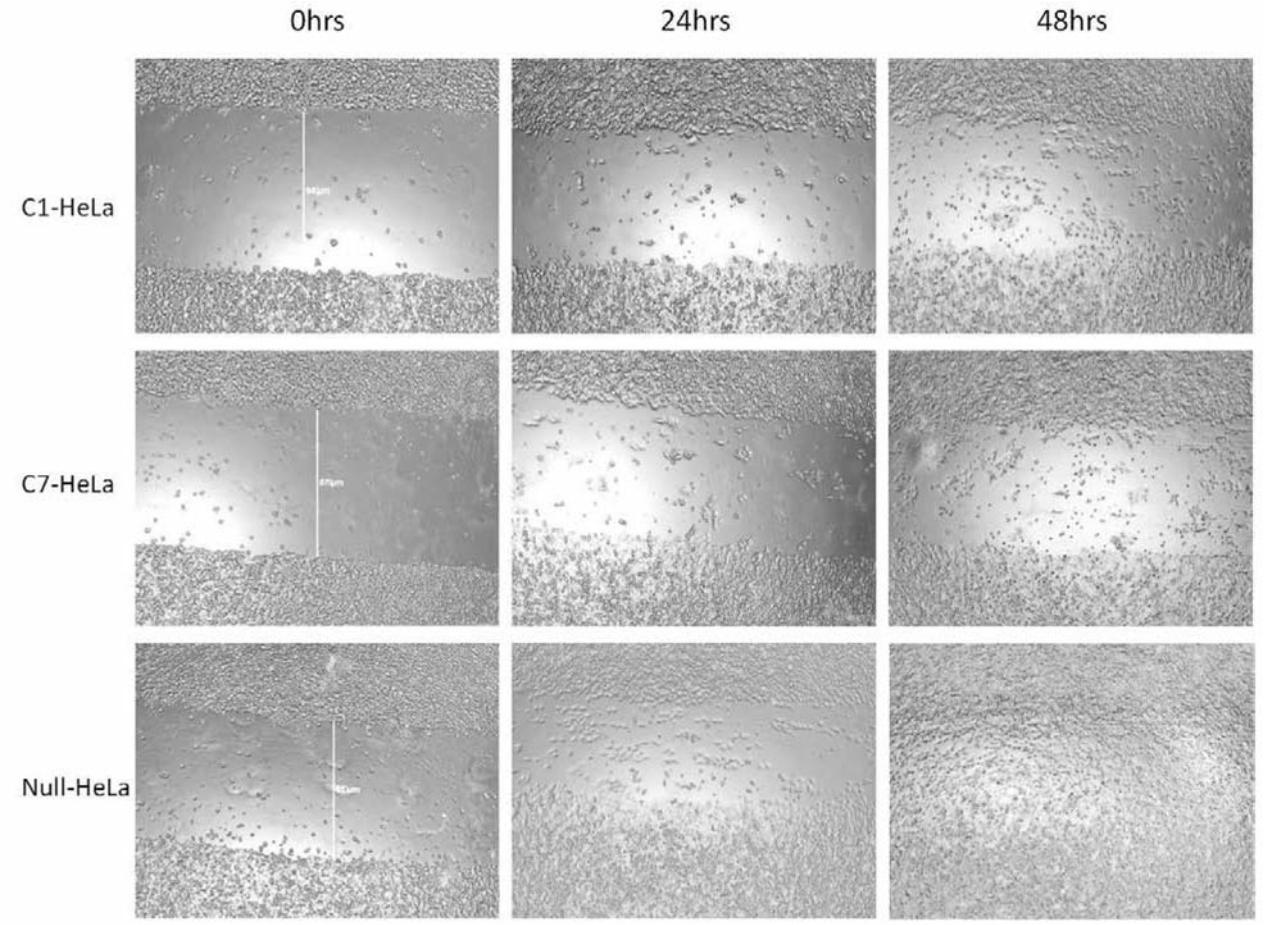

Figure 5. *Gap closure assay showing migration of cells over 48 h time period. Initial width of gap was measured at 0-h timepoint as being 840 μm, 870 μm and 850 μm for C1-HeLa, C7-HeLa and Null-HeLa respectively. Null-HeLa showed partial migration of cells into the gap after 24-h and complete gap closure at 48-h timepoint. C1-HeLa showed partial gap closure at 24 h and 48 h but incomplete gap closure over 48 h period. C7-HeLa showed a similar pattern to C1-HeLa with partial but incomplete gap closure after 48 h time period.*

lines devoid of any one of the four molecules (29). A recent study also found that claudin-7 associates with claudin-1 and facilitates incorporation of claudin-1 into EpCAM-containing complexes, and that TJ formed readily after EpCAM knockdown; the acquisition of trans-epithelial electroresistance was enhanced, and TJ showed increased resistance to disruption by calcium chelation (30).

This study demonstrated that although claudin-1 and -7 expression has been observed to be increased in cases of cervical neoplasia and carcinoma, claudin-1 and claudin-7 overexpression in HeLa cells acts in a tumor suppressive way by reducing cell migration and increasing transepithlial resistance. Further studies should focus in the investigation of the co-expression of different claudins and of claudin co-expression with other tight junction and adhesion proteins to assess what effect these interactions, if any, have on cell migration, invasion, proliferation and permeability.

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