

IL-8 Regulates Epithelial-Mesenchymal Transition through pERK1/2 in AGS Cells

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Abstract

Objective and Design: The aim of this project was to evaluate the influence of inflammatory cytokines IL-1 β and IL-8 on gastric epithelial-mesenchymal transition in gastric epithelial cells.

Material: Gastric epithelial adenocarcinoma cell line AGS and recombinant IL-1 β or IL-8.

Treatment: Confluent AGS cells were stimulated with rIL-1 β or rIL-8 for various periods of time.

Methods: The expression of claudin-4, -5, -6, -7, Cdx2, SNAIL, TWIST, α -catenin, β -catenin, and ERK was analyzed by western blot and immunohistochemistry. ELISA was used to evaluate IL-1 and IL-8 concentration in culture supernatants of treated AGS cells.

Results: AGS cells exposed for 1 or 48 h to 500 pg/ml of rIL-1 β secreted dissimilar maximal concentrations of IL-8 (2651 vs 6518 pg/ml, respectively). AGS cells exposed to high rIL-8 concentrations (5800 pg/ml) developed epithelial-mesenchymal transition features such as the hummingbird phenotype and unremitting claudin-4 expression that were associated to increased Cdx2, TWIST, Snail and β -catenin expression, all mediated by the phosphorylation of ERK1/2. EMT features were initially observed in AGS cells treated with 2500 pg/ml of rIL-8; lower rIL-8 concentrations did not induce EMT changes in AGS cells.

Conclusion: Chronic IL-8 mediated inflammation favors epithelial-mesenchymal transition in AGS cells.

Keywords: Claudins; IL-8; Cdx2; Carcinogenesis; ERK

Introduction

The epithelial-mesenchymal transition is a process in which epithelial cells suffer plasticity changes that confer them an invasive mesenchymal phenotype and metastatic behavior [1]. EMT has been associated with hypoxia inducible factor, transcription factors such as Snail, Slug or TWIST, pro-inflammatory cytokines, transforming growth factor β 3, and claudin overexpression or downregulation [2-8].

Chronic inflammation in the stomach due to *H. pylori* infection or other causes involve the up-regulation of pro-inflammatory cytokine IL-1 β [9]. Stomach-specific expression of IL-1 β in mice induces gastric inflammation and gastric carcinogenesis [1]. Gastric adenocarcinoma, the second most common cause of death by cancer, exhibit changes in claudin expression during progression to metastatic adenocarcinoma [10]. Altered expression of claudins 4, 6, 7 and 9 in human gastric cancer cells has been associated with higher cancer mortality, invasiveness and poor survival [8,11-13]. Claudin expression is modulated by several transcription factors, including members of the Cdx gene family [14].

Importantly Cdx2 has been associated with gastric carcinogenesis and modified claudin expression [15-17]. Nevertheless, the precise influence of the pro-inflammatory cytokines on claudin expression in gastric carcinogenesis has not been properly assessed. The aim of our work was to evaluate the effect of IL-1 β on the cellular mechanism involved on claudin expression on the AGS gastric epithelial cell line. Our results showed that IL-1 β induce the secretion of the highly pro-inflammatory cytokine IL-8 by AGS cells and that AGS cells exposed to high IL-8 concentrations activate the ERK1/2 signaling pathway that in turn induces the overexpression of claudin 4 and the epithelial-mesenchymal transcription factors Cdx2, TWIST, SNAIL and β -catenin as well as the transition to the hummingbird phenotype.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), L-glutamine, sodium pyruvate, insulin, Dulbecco's Phosphate-Buffered Saline (PBS), streptomycin-penicillin, bovine albumin and rabbit anti claudin-5 and -7 were from Invitrogen (Life Technologies Corp, USA). Mouse anti claudin-4 (GTX-15104) and -6 (GTX-115183) were from GeneTex (Irvine, CA, USA). PD98059 a selective inhibitor of MAPK/ERK kinase was from Enzo Life Sciences (Farmingdale, NY, USA). Rabbit monoclonal anti-human phosphorylated ERK1/2 (Phospho-p44/42 MAPK [Thr202/Tyr204], Cat No 9101) was from Cell Signaling Techn. (Danvers, MA, USA). Goat, rabbit or mouse IgG HRP- conjugated antibodies were from Zymed-Life Technologies (Grand Island, NY, USA). Alexa 488 conjugated goat anti-mouse IgG or donkey anti-rabbit IgG antibodies were from Abcam (Cambridge, MA, USA). Mouse monoclonal anti Cdx-2 (AM392-5M) was from BioGenex (Fremont, CA, USA). Mouse monoclonal anti TWIST (SC-81417) and anti β -catenin (E-5)(SC-7963) as well as rabbit anti α -catenin (H-297)(SC-7894) and rabbit anti Snail (SC-28199) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tween-20, Triton X-100, phosphatase inhibitor cocktail 1 and 2, trypsin inhibitor, leupeptin, aprotinin and beta-actin antibody were from Sigma-Aldrich (St. Louis, MO, USA). Anti-actin antibody was a kind gift of Dr. José Manuel Hernández, CINVESTAV-México. Protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Goat anti mouse/sheep IgG-FITC conjugated (#515-095-003) was from Jackson Immuno Research Lab. (West Grovw, PA, USA). SuperSignal West Dura Chemiluminescent Substrate was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Recombinant human IL-1 β and IL-8, as well as IL-1 β and IL-8 ELISA MAX Deluxe sets were purchased from Biologend Inc. (San Diego, CA, USA).

Cell culture

1x10⁶ human gastric adenocarcinoma cell line AGS cells (CRL-1739, ATCC, USA) were cultured in sterile P-100 Petri dishes with DMEM supplemented with 5% FBS, 1% streptomycin-penicillin solution, 1mmol/L L-glutamine, insulin and sodium pyruvate solution at 37 °C in a humid environment containing 5% CO₂, until reaching >90% confluence. Afterwards 8x10⁵ cells/well were seeded in 6 well Tissue Culture Plates (Biocompare, San Francisco, CA, USA) for 48 h until they reached 95% confluence, that represent 2x10⁶ cells/well. All the experiments were immediately performed with this confluent AGS cell cultures in their third passage. Western blot analysis was performed using 9x10⁶ confluent AGS cells. Human recombinant IL-1 β or IL-8 was used to stimulate the AGS cultures. The supernatants of these experiments were collected and kept at -70 °C until use. All the experiments were performed with AGS cells grown from 2nd passage frozen vials maintained in liquid nitrogen. Results are expressed as the mean of three different experiments.

Cytokine detection

IL-1 β and IL-8 concentration was determined by ELISA in supernatants of 1x10⁶ AGS cell cultures in sterile P-100 Petri dishes that had reached a 95% confluence. The ELISA MAX kits were from Biologend's (San Diego, CA, USA) and the detection procedure was performed following the manufacturer's instructions. The plates were read in a BioTek ELx808 (BioTek U.S., Winooski, VT, USA).

Treatment of AGS cells with IL-8 and ERK1/2 inhibitor

Confluent AGS cells were incubated in non-supplemented FBS DMEM for 12 h and then incubated with the ERK 1/2 inhibitor PD98059 (100 μ M) for 1 h before adding different recombinant human IL-8 (100-3800 pg/ml) for selected time periods (1, 2, 4, 6, 8, 12 h). Cells treated for 3 h with human recombinant epithelial growth factor (hEGF; 100ng/mL, Life Technol Corp, USA) were used as pERK1/2 activation control.

Whole cell protein extraction and immunoblot analysis

Cells were scrapped from the plates with lysis buffer (50mM Tris-HCl, 150mM NaCl, 0.1% SDS, 1% sodium deoxycholate, pH 7.6) supplemented with 0.5% of phosphatase inhibitor cocktail 1 and 2, 1mg/ml trypsin inhibitor, 2 μ g/ml leupeptin and 2 μ g/ml aprotinin (Sigma-Aldrich, USA). Cells were sonicated for 1 min before protein concentration was determined. For Western blot analysis 20 μ g of protein was resolved on 12% SDS/PAGE and transferred to nitrocellulose membranes in a Bio-Rad semi-dry blotting system for 1 h at 120 mA. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h, washed twice with TBS and incubated with primary anti- claudin, -Cdx-2, -Snail, -TWIST, - β -catenin or - α -catenin (1:500) antibody diluted in Tween 20/TBS (TTBS) overnight at 4 °C. Membranes were washed with TTBS, and incubated with horseradish peroxidase labelled secondary antibodies diluted 1:500 in TBS for 2 h at room temperature. To evaluate pERK1/2 the primary antibody was used at a 1:500 dilution. PD98059 was used at 100 μ M. After these processes membranes were washed thrice with TTBS and once with TBS before antibody binding was detected by chemiluminiscence. Equal protein loading was confirmed in all the experiments by determining β -actin as loading control. All experiments were run in triplicate.

The differences in claudin or Cdx-2 expression in the cell membrane were determined with images obtained from the chemiluminescence sheets using the MiniBis Pro (DNR Bio-Imaging Systems, Jerusalem, Israel) and analysed using DNR GelQuant software that calculates the intensity of the gel band measuring the amount of pixels/sq.in. The values for the basal and post-treatment results were determined for each band. The results are expressed as mean intensity.

Immunofluorescent microscopy

1×10^6 AGS cells were seeded in 2x2 cm sterile glass covers, placed in 6 well culture plates (one cover per well) and cultured with DMEM supplemented with 5% FBS, 1% streptomycin-penicillin solution, 1mmol/L L-glutamine, insulin and sodium pyruvate solution at 37 °C in a humid environment containing 5% CO₂ until confluence. Cells were collected, washed three times in PBS and incubated with 3,800 pg/ml of human rIL-8 in DMEM for 1, 2, 4, 6, 8 and 12 h periods. Cells were then washed thrice with cold PBS before adding 4% paraformaldehyde for 2 h at 4 °C. Afterwards, cells were washed and permeabilized with 500 ul of 0.1% Triton X-100 in PBS for 10 m at room temperature. After extensive washing, cells were incubated with 2% albumin in PBS for 30 m before extensive washing with PBS/0.05% Tween 20. Cells were then incubated overnight at 4 °C, with anti Cdx2 monoclonal antibody 1:500 in PBS/1% albumin, washed with PBS/0.05% Tween-20, incubated 1 h with the FITC-conjugated secondary antibody (1:500 in PBS/1% albumin), washed with PBS/0.05% Tween-20, and counterstained with 25 µl of Vectashield/DAPI, before being analysed and photographed with a Leica System model TCSSP5 confocal microscope.

Statistical analysis

All experimental results are expressed as the mean \pm standard deviation of the mean. Student's t-test analysis was used to evaluate differences between groups using Sigma Plot v.11 software. Differences were considered to be significant for $p < 0.05$ values.

Results

Effect of IL-1 β on IL-8 secretion by AGS cells

Culture supernatants of AGS cell cultures pre-incubated for 1 h with 500 pg/ml of rIL-1 β showed an initial increase in IL-8 concentration (296 pg/ml) after 30 min that reached its peak after 6 h (2651 pg/ml) (Figure 1). Culture supernatants of AGS cells pre-incubated for 48 h with 500 pg/ml of rIL-1 β showed a very strong basal concentration of IL-8 (6518 pg/ml) that exhibited a slow but continuous time-related decrease as demonstrated by the values determined after 6 h (4813 pg/ml). Control supernatants of non IL-1 β -stimulated AGS cells showed a maximal IL-8 value of 58 pg/ml after 4 h of culture, the remaining values in all time lapses were below 50 pg/ml. The difference in IL-8 concentration in all time intervals and in both experimental settings was statistically significant ($p < 0.01$).

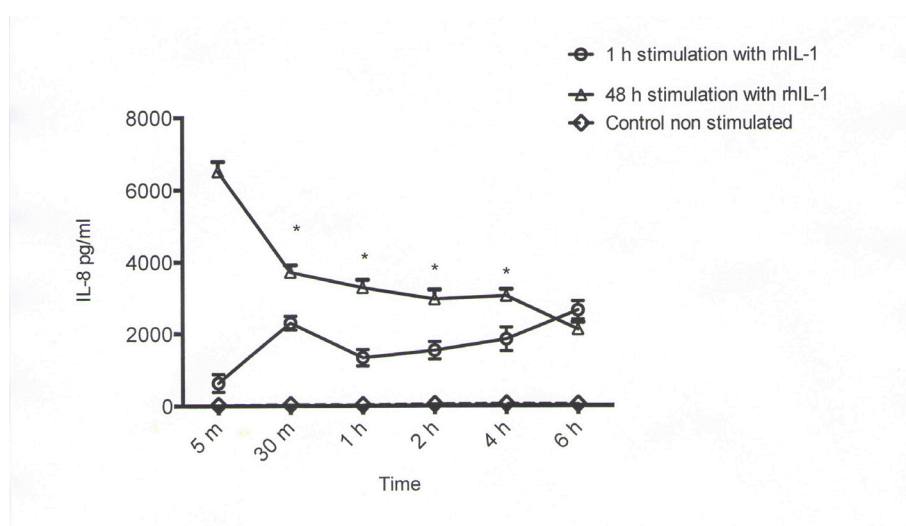


Figure 1: Interleukin-8 secretion by AGS cells pre-incubated with rIL-1 β for 1 and 48 h. Histogram represents the mean value \pm SD of the triplicate experiments at the different experimental times. The difference in IL-8 concentration in all the time lapses and in both experimental settings was statistically significant ($p < 0.01$)

Effect of IL-8 on AGS cells claudin expression

To analyze the possible effect of IL-8 on claudin expression, AGS cells were first incubated with 50 pg/ml of rIL-8 for various periods of time. Figure 2 summarizes the results. Claudin-4 expression decreased by 18,7% after 4 h exposure in comparison with the basal value ($p < 0.05$) but there was a slow return to basal levels after 12 h. The expression of claudin-5 and -6 was not modified. Claudin-7 expression showed a transitory increase in the first hour of exposure ($p < 0.05$) but returned to basal values after 2 h.

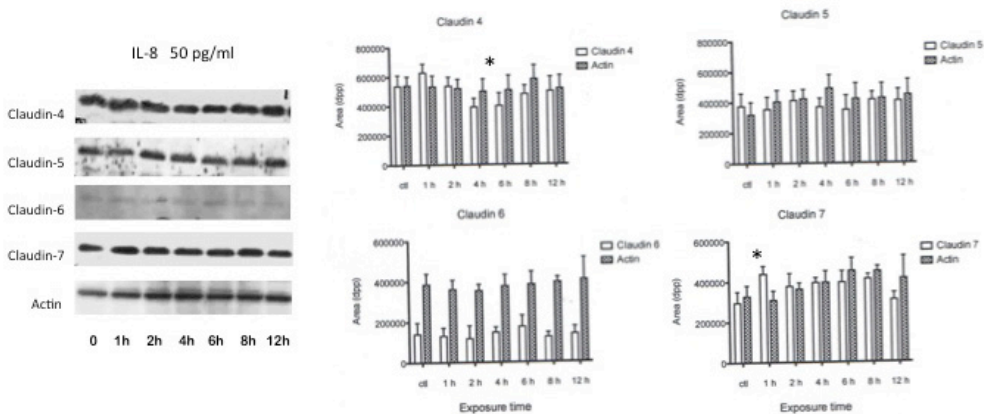


Figure 2: Effect of low dose recombinant IL-8 stimulation on AGS cells claudin expression. Representative image of the Western blot expression of the claudins evaluated in AGS cells. The image’s right hand histograms represent the mean intensity values \pm SD of the densitometry analysis performed in triplicate experiments at the different experimental times. Actin was used as control. * $p < 0.05$

As expected as rIL-8 concentration was increased (from 50-pg/ml to 3800-pg/ml) there was a significant increase ($p < 0.05$) in claudin-4 expression that began to be noticeable from the 200-pg/ml concentrations. The differences in the expression of claudin-5, -6, and -7 were not statistically significant throughout the evaluation period (Figure 3).

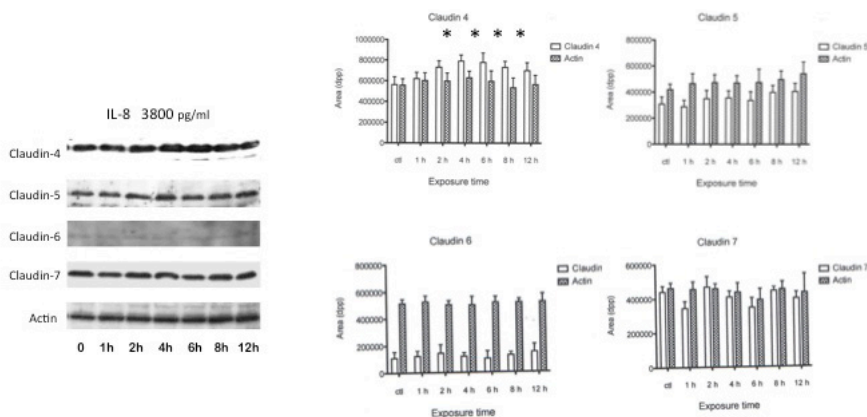


Figure 3: Effect of high dose recombinant IL-8 stimulation on AGS cells claudin expression. Representative image of the Western blot expression of the claudins evaluated in AGS cells. The image’s right hand histograms represent the mean intensity values \pm SD of the densitometry analysis performed in triplicate experiments at the different experimental times. Actin was used as control. * $p < 0.05$

Effect of IL-8 on Cdx2 and claudin 4 expressions

Claudin-4 expression is known to be regulated by the transcription factor Cdx2, therefore we investigated the effect of IL-8 on Cdx2 expression [16]. Our results showed that low IL-8 concentration (50 pg/ml of rIL-8) did not induce an increase in the expression of Cdx2 in AGS cells, whereas high IL-8 concentration (3800 pg/ml) increased Cdx2 protein expression by 120% in contrast to the constitutive level. The increased expression of Cdx2 was nearly abolished in the presence of the MEK inhibitor PD98059 (Figure 4A) at the same concentrations that induced a complete abolishment of ERK1/2 expression (Figure 4B). Confocal microscopy corroborated the differences observed in AGS cells incubated with low and high rIL-8 concentrations (Figure 5A and B, respectively). The inhibition upstream of the ERK1/2 signaling pathway was accompanied by a significant decrease (87%) in Cdx2 expression. Interestingly, AGS cells incubated with 500 pg/ml rIL-1b did not express Cdx2 at any time (Figure 5C).

Activation of Cdx2 enhanced claudin-4 expression through the ERK1/2 signaling pathway. High human rIL-8 concentration induces an increase in claudin 4. Although Western blot analysis demonstrated the persistence of claudin-4 throughout the 12 h period (Figure 6A), confocal microscopy (Figure 6B) showed that claudin-4 expression started to diminish after 8 h. Interestingly, claudin-4 is not over-expressed after co-stimulation of IL-8 and inhibitor; the expression of claudin-4 in the presence of the MEK inhibitor PD98059 decreased, by a mean 70% value, after 12 h.

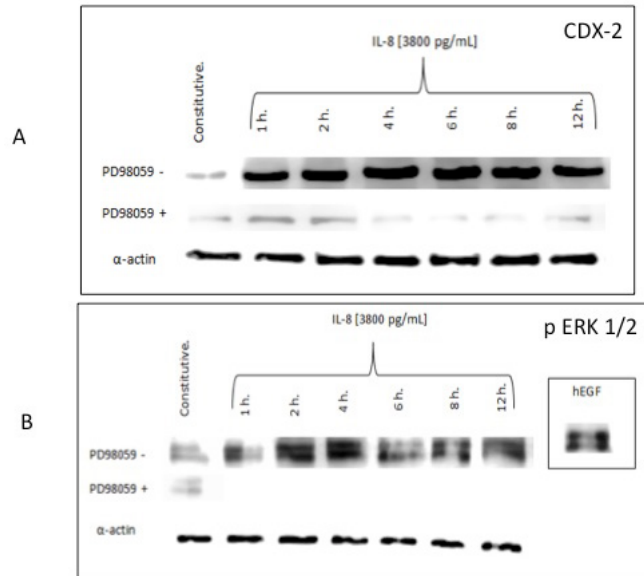


Figure 4: A) Western blot analysis showing the expression of Cdx2 in AGS cells treated with high dose recombinant IL-8 concentration, in the presence or not of the MEK inhibitor PD98095. B) Western blot analysis showing the expression of pERK 1/2 in AGS cells treated with high dose recombinant IL-8 concentration, in the presence or not of the MEK inhibitor PD98095; the image in the right hand side of the figure – positive control- shows that the expression of pERK 1/2 in AGS cells treated with hEGF was not abolished by the presence of the MEK inhibitor. Results represent the mean of three different experiments

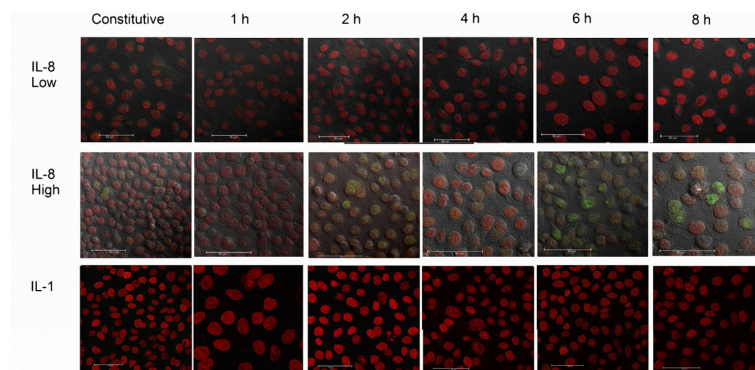


Figure 5: Microphotographs that show the effect of different human recombinant IL-8 and IL-1 β concentrations, on Cdx2 expression, by AGS cells. A) low-dose recombinant IL-8 (50 pg/ml) concentration, B) high-dose recombinant IL-8 (3,800 pg/ml) concentration, and C) recombinant IL-1 β (500 pg/ml) concentration. The images represent the phenomena observed at different time intervals. The red color represents the cell's nuclear staining with PI (propide iodide) whereas the green color represents the fluorescence of the Alexa-488 or FITC-labeled second antibody. The confocal microscopy images were taken with a 60x lens and the white bar represents 50 μ m

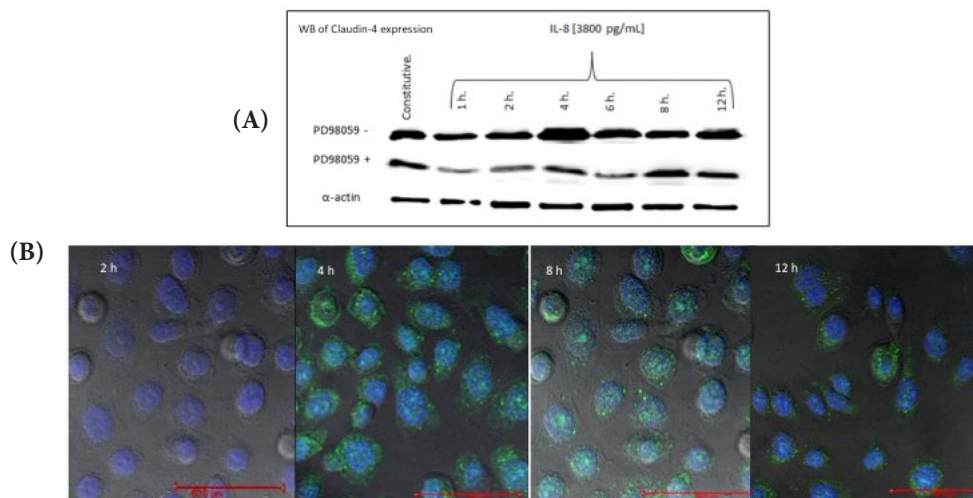


Figure 6: A) Expression of claudin 4 in AGS cells treated with high dose recombinant IL-8 in the presence of the MEK inhibitor PD98095. B) Microphotographs showing the effect on claudin-4 expression, at different time intervals, of AGS cells exposed to high dose recombinant IL-8. The blue color represents the cell's nuclear staining with DAPI (4',6'-diamidino-2-phenylindole,dihydrochloride) and the green color represents the fluorescence of the Alexa-488 or FITC-labeled second antibody. The confocal microscopy images were taken with a 60x lens and the red bar represents 50 μ m

Effect of IL-8 on Epithelial Mesenchymal Transition (EMT) protein expression

High (3800 pg/ml) rIL-8 concentrations induced the overexpression of other important EMT-associated transcription factors (Figure 7). TWIST increased its expression in AGS cells after 1 h and remained elevated for other 4 h. The overexpression of Snail was evident after 1 h and lasted for 8 h, similarly to b-catenin. The expression of Snail was completely abolished by the MEK inhibitor PD98059 whereas that of TWIST and b-catenin decreased by 70% and 86%, respectively. As expected, interleukin 8 had no effect on a-catenin expression whereas PD98059 increased significantly its expression. Confocal microscopy corroborated the increased expression of TWIST, Snail, b-catenin and a-catenin (Figure 8).

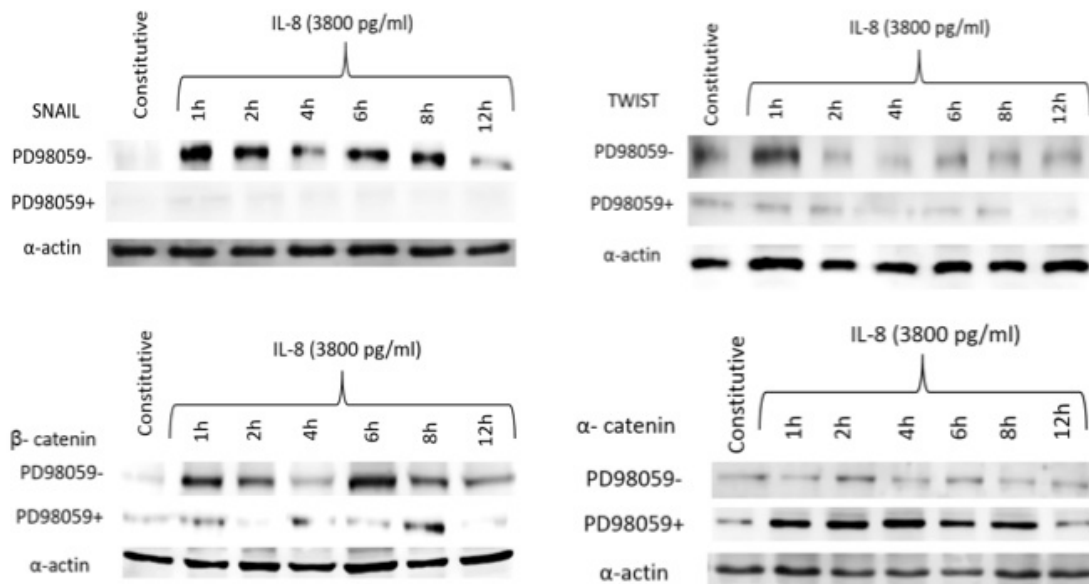


Figure 7: Western blot analysis showing the expression of Snail, TWIST, β - and α -catenin transcription factors in AGS cells treated with high dose recombinant IL-8 concentration, at different time intervals, in the presence or not of the MEK inhibitor PD98095. Results represent the mean of three different experiments

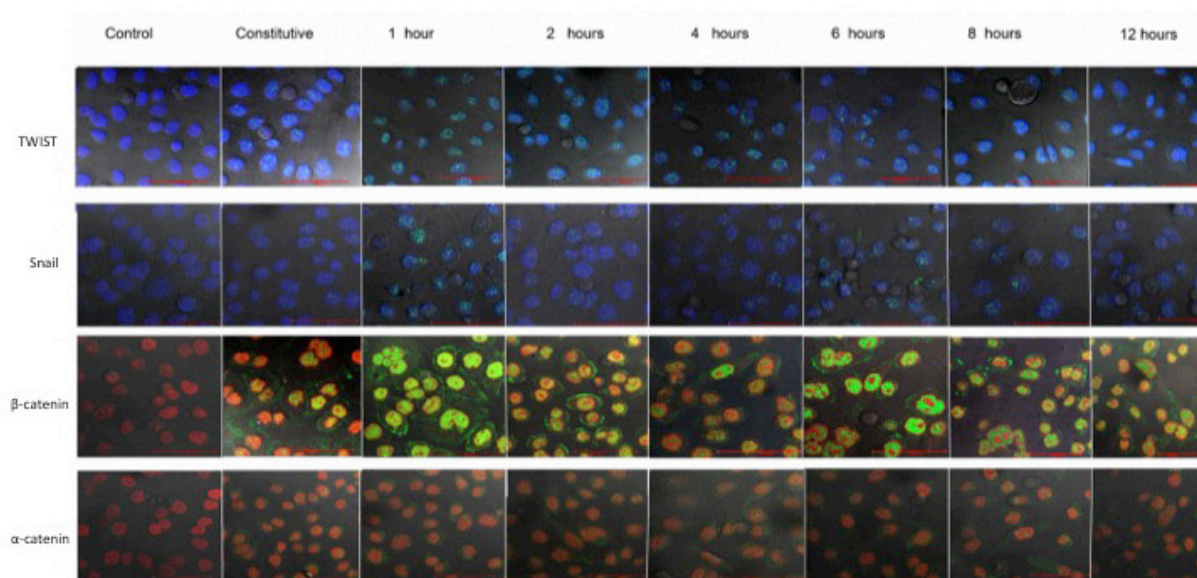


Figure 8: Micrographs demonstrating the expression of TWIST, SNAIL, β -catenin and α -catenin epithelial-mesenchymal transition proteins on AGS cells exposed to high human recombinant IL-8 (3,800 pg/ml) concentrations, at different time intervals. The red color represents the cell's nuclear staining with PI (propide iodide) and the blue color represents the cell's nuclear staining with DAPI (4',6-diamidino-2-phenylindole,dihydrochloride); the green color represents the fluorescence of the Alexa-488 or FITC-labeled second antibody. The confocal microscopy images were taken with a 60x lens and the red bar represents 50 μ m

Effect of IL-8 and ERK1/2 inhibition on AGS cells phenotype

Compatible with the phenotypic modification associated with the EMT process, we observed that an important number of AGS cells exposed to high (3800 pg/ml) rIL-8 concentrations acquired the hummingbird phenotype (Figure 9A-D) after 4 h of continuous exposure to the cytokine. Interestingly, the inhibition of the MAPK/ERK pathway in AGS cells exposed to high rIL-8 concentrations and maintained in culture for 4 h, obliterated the formation of the hummingbird phenotype (Figure 9E-F), although the normal cell phenotype was not retained. These results endorse the importance of this kinase pathway in cellular transformation.

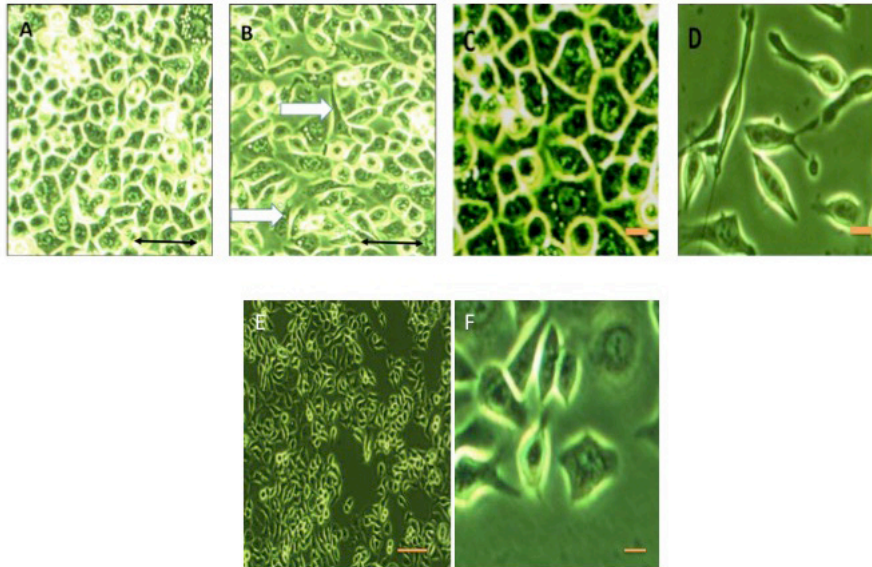


Figure 9: Phenotype modification of AGS cells stimulated with IL-8. A) non-stimulated cells, B) cells stimulated with high dose human recombinant IL-8 (3800 pg/ml) concentrations for 12 h, C) close-up of non-stimulated cells, D) close-up of stimulated cells demonstrating the “hummingbird” phenotype, E) cells stimulated with high dose human recombinant IL-8 in the presence of MEK inhibitor PD98095, F) close-up of cells shown in E where an abnormal cell but not a hummingbird phenotype can be distinguished. Scale bars in A, B and E represent 50 µm. Scale bars in C, D and F represent 10 µm. White arrows in B indicate the characteristic shape change to the “hummingbird” phenotype

Discussion

Epithelial barrier function requires normal expression of tight junction (TJ) proteins, when the TJ structure loses cohesion there can be invasiveness by transformed cells [18]. Claudins are the major membrane TJ proteins and exhibit tissue-specific expression. In order to establish a possible link between inflammatory cytokines and claudin-related cancer development, we evaluated the effect that high concentrations of recombinant IL-1 β and IL-8 could have on claudin expression in AGS cells. IL-1 β induces synovial cells, macrophages and human breast cancer cells to secrete IL-8 [19,20]. The continuous and long-lasting presence of IL-1 β in the gastric adenocarcinoma AGS cells culture induced a substantial secretion of IL-8 by AGS cells. The relevance of this result is highlighted by the fact that in IL-1 β -expressing transgenic mice, gastritis progresses to gastric cancer and that IL-1 β alter TJ barrier in diverse and non-gastric related epithelial cells [21-23]. In the particular case of gastric cancer, where *H. pylori* is recognized as a serious risk factor, several *H. pylori* virulence factors are involved in a complex pro-inflammatory signaling cascade that includes the activation of the ERK/MAPK pathway strongly involved in the regulation of claudin expression and in IL-1 β and -8 production [24].

The results showed that only claudin-4 was overexpressed as a result of the exposure to IL-8 concentrations that resemble those of a chronic inflammatory response. There was a qualitatively different effect of high versus low IL-8 concentrations on claudin-4 expression. The differences we observed in relation to the concentration of IL-8 response by AGS cells are in complete agreement with Mustapha, *et al.* results which describe that freshly obtained gastric epithelial cells versus AGS cells show qualitatively differences in cytokine and chemokine expression [especially CXCL8] [25]. Interestingly, IL-8 amplifies the NF- κ B and MAPKs signaling pathways and it has been clearly associated with cancer development [26,27].

Claudin-4 overexpression is associated with epithelial-mesenchymal transition, a process associated with the Snail transcription factor that contributes to malignancy and regulates interleukin-8 expression [28,29]. Our results demonstrated that high IL-8 concentration also induced the expression of Snail and Twist, potent repressors of E-cadherin expression and other clearly associated epithelial-mesenchymal transition transcription factors such as β -catenin, and that their increase was associated to the ERK/MAPK pathway [3,30]. α -catenin expression traduces the activation of the cancer associated Wnt signaling pathway whereas TWIST is also known to induce rapid dissemination of cytokeratin-positive epithelial cells [31,32]. The transitory increase of expression

of TWIST expression only 1 h of stimulation with IL-8 has been observed in breast cancer cell lines exposed to TGF β [33]. The increase in α -catenin expression when the ERK pathway was inhibited is in accordance with the mechanisms known to favor the ensemble of cell junctions. The qualitative differences that we observed in claudin expression in AGS cells exposed to high IL-8 concentrations might be the consequence of “inflammatory stress” that affects transcriptional activation and protein folding, specially in chronic inflammatory processes [34,35]. Certainly this possibility requires further analysis.

Cell differentiation, especially that of the epithelium, is a highly dynamic system that involves cell generation. p38 MAPKs has emerged as key modulator of cell differentiation processes; this kinase regulates the homeobox transcription factor Cdx2, and in mammals it is inducible by inflammatory cytokines and stress [36-38]. Cdx2 is a gastrointestinal-specific transcription factor highly expressed in multistage tissues of dysplasia and cancer [39]. It has always been assumed that Cdx2 expression was only regulated by the inflammatory cytokine interleukin-6 via an ERK-dependent manner but it has recently emerged that *H. pylori* CagL that regulates IL-1b production via TLR2 also induce Cdx2 expression in the gastric mucosa [40-42]. Our results showed that interleukin-8, another pro-inflammatory cytokine also regulate Cdx2 but, contrary to IL-6 that regulates claudin-2 expression, interleukin-8 regulated claudin-4 expression. The overexpression of claudin-4 is associated to endometrial tumorigenesis, epigenetic de-repression in gastric carcinoma, and ovarian and renal cancer progression [43-46]. Despite the controversy, the role of interleukin 8 in EMT remains the subject of speculation; we believe that our results fit into the current concept of EMT as a process that can be broken in several functional modules, independently regulated and directly affecting cell phenotype [47,48].

Our results also showed that AGS cells exposed to high concentrations of interleukin-1 and -8 diminished claudin-7 expression. Claudin-7 inhibits cell migration through inhibition of the ERK/MAPK pathway so the diminished expression we observed could be associated with enhanced migration ability [49].

A change in AGS cells morphology corroborated TJ modifications induced by IL-8. As it has already been shown, we also observed the hummingbird phenotype in AGS exposed to high IL-8 concentrations [50]. Our results also showed that the induction of the IL-8-induced hummingbird phenotype of the AGS cells was partially abolished when the MAPK/ERK kinase mediated pathway was inhibited with a specific inhibitor. The activation of the ERK pathway in epithelial cells induce phenotypic changes characterized by loss of apical-basal polarity and cell scattering that requires remodeling of actin cytoskeleton [51,52]. Similarly it has been reported that *H.pylori* pathogenicity island CagA induces the production of IL-8 and disrupts scaffolding proteins and cell polarity, compromising cell architecture and giving rise to the so-called “scattering/hummingbird” phenotype associated to epithelial-mesenchymal transition [1,50,53]. Our results strongly suggest that actin reorganization and loss of epithelial characteristics associated to invasive pseudopodia resembling those seen in epithelial-to-mesenchymal transition is mediated by IL-8 via the MAPK/ERK pathway [54,55]. ERK1/2 activation induces the expression of genes encoding cell cycle regulatory proteins associated to cell proliferation [56].

Conclusion

Our results demonstrate that a AGS cells exposed to high concentrations of the proinflammatory cytokine IL-8 activates the expression of the Cdx2 transcription factor via the ERK1/2 signaling pathway, and consequently claudin-4, SNAIL, TWIST and β -catenin expressions, all of which play a significant role in the initiation of the epithelial-mesenchyme neoplasm transition. Our results underline the notion that carcinogenesis is the consequence of the uninterrupted micro environmental presence of inflammatory cytokines.

Acknowledgement

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References

1. Bessede E, Staedel C, Acuna Amador LA, Nguyen PH, Chambonnier L, et al. (2014) Helicobacter pylori generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. *Oncogene* 33: 4123-31.
2. Yang MH, Wu KJ (2008) TWIST activation by hypoxia inducible factor-1 (HIF-1): implications in metastasis and development. *Cell Cycle* 7: 2090-6.
3. Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7: 415-28.
4. Xue H, Lin B, Ni P, Xu H, Huang G (2010) Interleukin-1B and interleukin-1 RN polymorphisms and gastric carcinoma risk: a meta-analysis. *J Gastroenterol Hepatol* 25: 1604-17.
5. Eftang LL, Esbensen Y, Tannaes TM, Bukholm IR, Bukholm G (2012) Interleukin-8 is the single most up-regulated gene in whole genome profiling of *H. pylori* exposed gastric epithelial cells. *BMC Microbiol* 12: 9.

6. Medici D, Hay ED, Olsen BR (2008) Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell* 19: 4875-87.
7. Kyuno D, Yamaguchi H, Ito T, Kono T, Kimura Y, et al. (2014) Targeting tight junctions during epithelial to mesenchymal transition in human pancreatic cancer. *World J Gastroenterol* 20: 10813-24.
8. Resnick MB, Gavilanez M, Newton E, Konkin T, Bhattacharya B, et al. (2005) Claudin expression in gastric adenocarcinomas: a tissue microarray study with prognostic correlation. *Hum Pathol* 36: 886-92.
9. McNamara D, El-Omar E (2008) *Helicobacter pylori* infection and the pathogenesis of gastric cancer: a paradigm for host-bacterial interactions. *Dig Liver Dis* 40: 504-9.
10. Nagini S (2012) Carcinoma of the stomach: A review of epidemiology, pathogenesis, molecular genetics and chemoprevention. *World J Gastrointest Oncol* 4: 156-69.
11. Soini Y, Tommola S, Helin H, Martikainen P (2006) Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse subtype. *Virchows Arch* 448: 52-8.
12. Zavala-Zendejas VE, Torres-Martinez AC, Salas-Morales B, Fortoul TI, Montano LF, et al. (2011) Claudin-6, 7, or 9 overexpression in the human gastric adenocarcinoma cell line AGS increases its invasiveness, migration, and proliferation rate. *Cancer Invest* 29: 1-11.
13. Rendon-Huerta E, Teresa F, Teresa GM, Xochitl GS, Georgina AF, et al. (2010) Distribution and expression pattern of claudins 6, 7, and 9 in diffuse- and intestinal-type gastric adenocarcinomas. *J Gastrointest Cancer* 41: 52-9.
14. Duprey P, Chowdhury K, Dressler GR, Balling R, Simon D, et al. (1988) A mouse gene homologous to the *Drosophila* gene *caudal* is expressed in epithelial cells from the embryonic intestine. *Genes Dev* 2: 1647-54.
15. Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, et al. (2003) Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. *J Pathol* 199: 36-40.
16. Satake S, Semba S, Matsuda Y, Usami Y, Chiba H, et al. (2008) Cdx2 transcription factor regulates claudin-3 and claudin-4 expression during intestinal differentiation of gastric carcinoma. *Pathol Int* 58: 156-63.
17. Irvani O, Tay BW, Chua PJ, Yip GW, Bay BH (2013) Claudins and gastric carcinogenesis. *Exp Biol Med* 238: 344-9.
18. Martin TA, Mason MD, Jiang WG (2011) Tight junctions in cancer metastasis. *Front Biosci* 16: 898-936.
19. Choi HM, Oh da H, Bang JS, Yang HI, Yoo MC, et al. (2010) Differential effect of IL-1beta and TNF alpha on the production of IL-6, IL-8 and PGE2 in fibroblast-like synoviocytes and THP-1 macrophages. *Rheumatol Int* 30: 1025-33.
20. Naldini A, Filippi I, Miglietta D, Moschetta M, Giavazzi R, et al. (2010) Interleukin-1beta regulates the migratory potential of MDAMB231 breast cancer cells through the hypoxia-inducible factor-1alpha. *Eur J Cancer* 46: 3400-8.
21. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, et al. (2008) Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14: 408-19.
22. Rozlomiy VL, Markov AG (2010) Effect of interleukin-1beta on the expression of tight junction proteins in the culture of HaCaT keratinocytes. *Bull Exp Biol Med* 149: 280-3.
23. Duffy HS, John GR, Lee SC, Brosnan CF, Spray DC (2000) Reciprocal regulation of the junctional proteins claudin-1 and connexin43 by interleukin-1beta in primary human fetal astrocytes. *J Neurosci* 20: RC114.
24. Backert S, Naumann M (2010) What a disorder: proinflammatory signaling pathways induced by *Helicobacter pylori*. *Trends Microbiol* 18: 479-86.
25. Mustapha P, Paris I, Garcia M, Tran CT, Cremniter J, et al. (2014) Chemokines and antimicrobial peptides have a cag-dependent early response to *Helicobacter pylori* infection in primary human gastric epithelial cells. *Infect Immun* 82: 2881-9.
26. Sakitani K, Hirata Y, Hayakawa Y, Serizawa T, Nakata W, et al. (2012) Role of interleukin-32 in *Helicobacter pylori*-induced gastric inflammation. *Infect Immun* 80: 3795-803.
27. Laiakis EC, Baulch JE, Morgan WF (2008) Interleukin 8 exhibits a pro-mitogenic and pro-survival role in radiation induced genomically unstable cells. *Mutat Res* 640: 74-81.
28. Ikenouchi J, Matsuda M, Furuse M, Tsukita S (2003) Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci* 116: 1959-67.
29. Hwang WL, Yang MH, Tsai ML, Lan HY, Su SH, et al. (2011) SNAIL regulates interleukin-8 expression, stem cell-like activity, and tumorigenicity of human colorectal carcinoma cells. *Gastroenterology* 141: 279-91, 91 e1-5.
30. Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, et al. (2011) Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. *Cancer Res* 71: 245-54.
31. Pereira L, Mariadason JM, Hannan RD, Dhillon AS (2015) Implications of epithelial-mesenchymal plasticity for heterogeneity in colorectal cancer. *Front Oncol* 5: 13.
32. Shamir ER, Pappalardo E, Jorgens DM, Coutinho K, Tsai WT, et al. (2014) Twist1-induced dissemination preserves epithelial identity and requires E-cadherin. *J Cell Biol* 204: 839-56.
33. Bendinelli P, Maroni P, Matteucci E, Desiderio MA (2015) HGF and TGFbeta1 differently influenced Wwox regulatory function on Twist program for mesenchymal-epithelial transition in bone metastatic versus parental breast carcinoma cells. *Mol Cancer* 14: 112.
34. Chen Y, Zhao L, Li Q, Wheeler DC, Varghese Z, et al. (2014) Inflammatory stress reduces the effectiveness of statins in the kidney by disrupting HMGCoA reductase feedback regulation. *Nephrol Dial Transplant* 29: 1864-78.
35. Cao SS (2015) Endoplasmic reticulum stress and unfolded protein response in inflammatory bowel disease. *Inflamm Bowel Dis* 21: 636-44.
36. Houde M, Laprise P, Jean D, Blais M, Asselin C, et al. (2001) Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. *J Biol Chem* 276: 21885-94.
37. Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911-2.
38. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* 103: 239-52.
39. Song X, Chen HX, Wang XY, Deng XY, Xi YX, et al. (2013) H. pylori-encoded CagA disrupts tight junctions and induces invasiveness of AGS gastric carcinoma cells via Cdx2-dependent targeting of Claudin-2. *Cell Immunol* 286: 22-30.

40. Suzuki T, Yoshinaga N, Tanabe S (2011) Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J Biol Chem* 286: 31263-71.
41. Kim DJ, Park JH, Franchi L, Backert S, Nunez G (2013) The Cag pathogenicity island and interaction between TLR2/NOD2 and NLRP3 regulate IL-1beta production in *Helicobacter pylori* infected dendritic cells. *Eur J Immunol* 43: 2650-8.
42. Bornschein J, Malfertheiner P (2014) *Helicobacter pylori* and gastric cancer. *Dig Dis* 32: 249-64.
43. Pan XY, Li X, Che YC, Li HY, Li X, et al. (2013) Overexpression of claudin-4 may be involved in endometrial tumorigenesis. *Oncol Lett* 5: 1422-6.
44. Kwon MJ, Kim SH, Jeong HM, Jung HS, Kim SS, et al. (2011) Claudin-4 overexpression is associated with epigenetic derepression in gastric carcinoma. *Lab Invest* 91: 1652-67.
45. Li J, Chigurupati S, Agarwal R, Mughal MR, Mattson MP, et al. (2009) Possible angiogenic roles for claudin-4 in ovarian cancer. *Cancer Biol Ther* 8: 1806-14.
46. Lechpammer M, Resnick MB, Sabo E, Yakirevich E, Greaves WO, et al. (2008) The diagnostic and prognostic utility of claudin expression in renal cell neoplasms. *Mod Pathol* 21: 1320-9.
47. Palena C, Hamilton DH, Fernando RI (2012) Influence of IL-8 on the epithelial-mesenchymal transition and the tumor microenvironment. *Future Oncol* 8: 713-22.
48. Savagner P (2015) Epithelial-mesenchymal transitions: from cell plasticity to concept elasticity. *Curr Top Dev Biol* 112: 273-300.
49. Lu Z, Ding L, Hong H, Hoggard J, Lu Q, et al. (2011) Claudin-7 inhibits human lung cancer cell migration and invasion through ERK/MAPK signaling pathway. *Exp Cell Res* 317: 1935-46.
50. Su B, Ceponis PJ, Sherman PM (2003) Cytoskeletal rearrangements in gastric epithelial cells in response to *Helicobacter pylori* infection. *J Med Microbiol* 52: 861-7.
51. Caro-Gonzalez HY, Nejsum LN, Siemers KA, Shaler TA, Nelson WJ, et al. (2012) Mitogen-activated protein kinase (MAPK/ERK) regulates adenomatous polyposis coli during growth-factor-induced cell extension. *J Cell Sci* 125: 1247-58.
52. Caslavsky J, Klimova Z, Vomastek T (2013) ERK and RSK regulate distinct steps of a cellular program that induces transition from multicellular epithelium to single cell phenotype. *Cell Signal* 25: 2743-51.
53. Lin SN, Ayada K, Zhao Y, Yokota K, Takenaka R, et al. (2005) *Helicobacter pylori* heat-shock protein 60 induces production of the pro-inflammatory cytokine IL8 in monocytic cells. *J Med Microbiol* 54: 225-33.
54. Suzuki M, Mimuro H, Suzuki T, Park M, Yamamoto T, et al. (2005) Interaction of CagA with Crk plays an important role in *Helicobacter pylori*-induced loss of gastric epithelial cell adhesion. *J Exp Med* 202: 1235-47.
55. Bagnoli F, Buti L, Tompkins L, Covacci A, Amieva MR (2005) *Helicobacter pylori* CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proc Natl Acad Sci USA* 102: 16339-44.
56. Grill C, Gheyas F, Dayananth P, Jin W, Ding W, et al. (2004) Analysis of the ERK1,2 transcriptome in mammary epithelial cells. *Biochem J* 381: 635-44.

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