Regulation of CXCL8/IL-8 expression by Zonula Occludens-1

in human breast cancer cells

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Abstract

Accumulating data now suggest that ZO-1, once delocalized from tight junctions, could be implicated in the regulation of tumor promoting genes. Because of their major implication in different steps of tumor progression, we investigated here the influence of ZO-1 on chemokines expression in breast cancer cells. Using GeneArray analysis to compare chemokine mRNA expression in breast tumor cells transfected with a siRNA against ZO-1, we identified CXCL-8/IL-8 as a major potential target of ZO-1 signaling, being strongly downregulated following ZO-1 siRNA transfection. Examining further the relationship between ZO-1 and IL-8, we first demonstrated that CXCL8/IL-8 expression correlates with a relocalization of ZO-1 in several breast cancer cell lines. Moreover, CXCL8/IL-8 is downregulated in invasive BT549 cells transfected with 3 different ZO-1 siRNA and overexpressed in non-invasive BT20 and SKBR3 cells transfected with vectors expressing ZO-1. We also provide evidence for an activation of the CXCL8/IL-8 promoter by ZO-1. Finally, we demonstrate that the regulation of CXCL8/IL-8 by ZO-1 is independent of the β-catenin pathway. Our results thus clearly demonstrate an implication of ZO-1 in CXCL8/IL-8 regulation. Because of the major implications of CXCL8/IL-8 in tumor invasion, such a regulation could play an important role in breast cancer progression.

Introduction

During the metastatic progression of epithelial cancer cells, intercellular adhesion complexes are reorganized and their molecular components are downregulated and/or relocalized. The reorganization of E-cadherin/catenin complexes and more particularly the involvement of the β-catenin pathway in the regulation of tumor-associated genes has been largely documented during epithelial tumor cell invasion (1, 2). Studies are now also accumulating which demonstrate the implication of tight junction (TJ) molecules in tumor progression (3, 4) .

In normal epithelia, TJs are constituted of transmembrane proteins (occludin, claudins, junctional adhesion molecules (JAMs)) linked to the actin network via cytoplasmic molecules including those of the zonula occludens (ZO) family: ZO-1, -2, -3. ZO-1 has been the first described member of the ZOs and is considered as a central scaffolding protein regulating TJ organization. ZO-1 indeed interacts with other TJ molecules through its conserved structural domains: three PDZ (*P*SD95/*D*LG/ZO-1) domains, one src homology 3 (SH3) domain and one guanylate kinase homologous (GK) domain (5-7). For instance, ZO-1 binds transmembrane molecules of the TJ including occludin via its GK domain and several claudins via the first PDZ domain. ZO-1 also interacts with cytoplasmic molecules of the TJ such as ZO-2 or ZO-3 via the second PDZ domain. In addition, ZO-1 binds actin via its carboxyl terminal region and an indirect binding of ZO-1 with the cytoskeleton also occurs through peripherical junctional proteins including cortactin, α-actin, AF6/Afadin or cingulin. Finally, the SH3 domain of ZO-1 binds several signaling proteins such as serine protein kinase (ZAK), the Y-box transcription factor ZONAB (ZO-1-associated nucleic acid-binding protein), the heat shock protein Apg2 and the G proteins Gα12.

In carcinomas, ZO-1 is generally considered as a tumor suppressor. Several studies have thus reported that a reduction of ZO-1 expression correlates with tumor dedifferentiation and

progression in breast and colorectal carcinomas (8). Nevertheless, ZO-1 has also been found overexpressed in different types of cancers including pancreatic cancers, gastric cancers and melanoma (9-11) More particularly, a loss of membrane-associated localization and a cytoplasmic distribution of ZO-1 has been reported in breast carcinomas (8). Also, *in vitro*, migrating epithelial MDCK cells as well as fibroblasts under proliferative and pro-migratory conditions have been reported to display a ZO-1 nuclear localization (12, 13). It has thus been suggested that, as described for β-catenin, ZO-1 can shuttle between the plasma membrane and the cytoplasm or nucleus and exert specific roles in these different subcellular compartments (12, 14, 15). Accordingly, nuclear exporting (NES) and nuclear localization (NLS) signals have been identified on ZO-1 (14). Also, ZO-1 is today considered as a member of the NACos family (nuclear and adhesion complex components) described as adhesion molecules which can also be found in the nucleus (16). However, data reporting nuclear ZO-1 are scarce and a functional role of nuclear ZO-1 has not been clearly established. A relocalization of ZO-1 out of cell-cell contacts and an increased cytoplasmic level of ZO-1 has been implicated in the pro-invasive role of ZO-1 rather than a nuclear shuttling. Supporting this concept, it has been shown that a form of ZO-1 which lacks the GK domain, consequently displaying altered binding to occludin and impaired recruitment to the plasma membrane, accumulates in the cytoplasm and is able to confer tumorigenicity to transfected MDCK cells injected into nude mice (17). Modulating ZO-1 expression by cDNA or siRNA transfections, we and others also demonstrated the tumor invasion promoting activity of ZO-1 in breast and melanoma tumor cell lines respectively (11, 18). Based on these results, the concept has emerged that, in addition to its structural role in TJ organization, ZO-1, once delocalized from TJs, may be involved in intracellular signaling pathways and in gene expression favoring tumor progression.

Chemokines are pro-inflammatory cytokines that can be divided into four classes depending on the number of amino acids separating the first two conserved cysteins: C, CC, CXC and CX_3C chemokines. Chemokines were initially defined as soluble factors regulating directional leukocyte migration during inflammation process but which are now also regarded as potent regulators of cancer development (19, 20). CXCL8/IL-8 has been the first identified chemokine and belongs to the CXC chemokine family. CXCL8/IL-8 activate a large number of intracellular signaling pathways (serine/threonine kinases, tyrosine kinases or Rho-GTPases) through G protein coupled receptors (CXCR1 and CXCR2) and exert many biological effects on different cell types of the tumor microenvironment (21). It has been shown to attract, among others, neutrophils and basophils. CXCL8/IL-8 has also largely been implicated as a positive regulator of angiogenesis, a particular role shared by all other CXC chemokines displaying an ELR motif (22, 23). CXCL8/IL-8 has thus been shown to promote angiogenesis via its interactions with its two receptors CXCR1 and CXCR2 on human umbilical vein endothelial cells (HUVEC) and on human microvascular endothelial cells (HMEC) (24-26). In agreement with its tumor promoting role, CXCL8/IL-8 is found overexpressed in many different types including breast carcinoma (27, 28) CXCL8/IL-8 expression also more precisely correlates with the pathological stage in prostate cancer (29), with a high tumor grade and metastatic potential in breast tumors (30, 31) and with an increased vascularization in gastric carcinoma (32) and breast carcinoma (31).

If the implication of chemokines, and particularly CXCL8/IL-8, in tumor growth and invasion is now well established, the mechanisms contributing to their overexpression by epithelial tumor cells are still poorly understood. In a previous study, we demonstrated that a reorganization of adherens junctions and the activation of the β-catenin pathway regulate chemokine expression (33). In the present study, we have examined the influence of ZO-1 on

chemokines expression in breast cancer cell lines. We thus identified CXCL8/IL-8 as a major potential target of ZO-1 signaling which could be involved in breast tumor progression.

Materials and Method

Cell lines and culture conditions

All human mammary epithelial cells used were obtained from the American Type Culture Collection (Rockville, MD). Human breast carcinoma cell lines (MCF7, BT20, SKBR3, MDA-MB-231, BT549 and Hs578T) were grown in Dulbecco's Modified Eagle's Medium (Invitrogen, Paisley, UK) supplemented with 10% FCS, 2 mM of L-glutamine and 100 UI/ml of penicillin and streptomycin (Invitrogen). THP-1 monocytic human cells were grown in RPMI medium supplemented with 20% FCS, 2 mM of L-glutamine and 100 UI/ml of penicillin and streptomycin. MCF10A cells were grown in a 1:3 (v/v) mixture of HAM F12 and Dulbecco's modified Eagle's medium (DMEM), supplemented with 20 µg/ml of adenine, 5 µg/ml of insulin, 0.5 µg/ml of hydrocortisone, 2 ng/ml of EGF, 5 µg/ml of transferrin, 1.5 ng/ml of triiodothyronine, 10% FCS and 100 UI/ml of penicillin and streptomycin. MCF10A cells were treated with 5 ng/ml TGF-β (R&D systems Minneapolis, MN) and 10 ng/ml EGF (Sigma, Saint-Louis, MO).

Transmigration assays

THP-1 cells (10^5) were suspended in 200 µl of serum-free RPMI medium supplemented with 0.5% BSA and placed in the upper compartment of a 8 µM transwell (6.5 mm diameter, Costar, N.Y.). The lower compartment was filled with 600 µl of 48h-conditionned serum-free RPMI medium of BT549 cells transfected either with a scrambled control siRNA or an anti-ZO-1 siRNA. After 7 h of incubation at 37°C, THP-1 cells that had migrated in the lower

chamber of the transwell were counted with a cell counter (Cedex, Roche Diagnostics, Mannheim, Germany).

Modified Boyden chamber invasion assay

The *in vitro* invasive properties of cells were assessed using a matrigel-coated modified Boyden chamber assay. 10^5 cells were placed in the upper compartment of the invasion chamber (BD BioCoat Matrigel Invasion Chamber, BD Biosciences, Bedford, MA). The chambers were incubated for 8 hours at 37°C. The filters were then fixed in methanol and stained with hematoxylin. Quantification of the invasion assay was performed by counting the number of cells at the lower surface of the filters (30 fields at 40-fold magnification).

Transfection of small interfering RNA

19-nt specific sequences were selected in the coding sequence of ZO-1 (Genebank accession number: L14837) and of β-catenin (Genebank accession number: NM_003257) to generate 21-nt sense and 21-nt antisense strands of the type (19N)TT (N, any nucleotide). The sense and antisense strands were then annealed to obtain duplexes with identical 3' overhangs. The sequences were submitted to a BLAST search against the human genome to ensure the specificity of the siRNA to the targeted sequence. Corresponding scrambled duplexes which do not recognize any sequence in the human genome were used as control. The 19-nt specific sequences for the three ZO-1 siRNA and for the β-catenin siRNA are as follow: ZO-1 si1 5'-GUUAUACGAGCGAUCUCAU-3', ZO-1 si2 5'-GGAGGAAACAGCUAUAUGG-3', ZO-1 si3 5'-GACGAGAUAAUCCUCAUUU-3', β-catenin si 5'-CTCAACTGAAAGCCGTTTC-3'. For transfection of the siRNA duplexes, $75x10^3$ cells were plated in 6-well plates in 2 ml per well of culture medium. At 24h after plating, the cells were transfected by phosphate calcium precipitation by adding in each well 200 µl of a mixture containing 20 nM of the siRNA

duplexes, 140 mM NaCl, 0.75 mM Na_2HPO_4, 6 mM glucose, 5 mM KCl, 25 mM HEPES and 125 mM $CaCl_2$. Sixteen hours after transfection, the cells were extensively washed with PBS and incubated for 48h in culture medium before they were harvested for Gene Array, RT-PCR analyses and Western blotting. Conditioned media were also collected for ELISA analyses.

Gene Array

The relative mRNA expression of human chemokines and chemokines receptors was analyzed with GEArray Q serie n° HS005.2 (SA Biosciences, Frederick, MD) according to the manufacturer's protocol. Total RNA from BT549 cells transfected with ZO-1 scrambled or ZO-1 siRNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics). An amount of 2 µg RNA was reverse transcribed and double stranded cDNA probes were generated by biotin-16-dUTP incorporation using the AmpoLabeling-LPR kit (SA Biosciences), according to the manufacturer's instructions. The cDNA probes were denatured at 94°C for 2 minutes. The membranes were pre-hybridized in GEAhyb Hybridization Solution (SA Biosciences) supplemented with heat-denatured salmon sperm DNA (Sigma, Saint-Louis, MO) for 4h at 60°C. Labelled cDNA probes were hybridized overnight at 60°C with continuous agitation. Following repetitive washing in salt sodium citrate buffer containing sodium dodecyl sulfate, membranes were incubated with alkaline phosphatase-conjugated streptavidin. Signals were detected using the CDP-Star chemiluminescent substrate (SA Biosciences) and subsequent exposure to X-ray films. After densitometry scanning on a Fluor-S MultiImager (Bio-Rad, Hercules, CA), spots were converted into numerical data using GEArray Expression Analysis Suite software (SA Biosciences) and values were normalized to the signal of the housekeeping gene β-actin.

Plasmids

The human CXCL8/IL-8 promoter constructs have been previously described (34) and were kindly provided by Professor M.A. Buendia (Pasteur Institute, Paris, France). They contain either a 193bp fragment, a 193bp fragment mutated in the β-catenin/TCF binding site or a 173bp fragment lacking the β-catenin/TCF binding site of the 5'upstream regulatory region of the CXCL8/IL-8 gene cloned in the pGL3 firefly luciferase reporter plasmid (Promega, Madison, WI). The TOP-FLASH and FOP-FLASH reporter plasmids, containing three wild-type (TOP-FLASH) or mutated (FOP-FLASH) copies of the β-catenin/TCF binding site upstream of a minimal herpesvirus thymidine kinase promoter driving the firefly luciferase expression, were kindly provided by Professor H.C. Clevers (University Hospital, Utrecht, The Netherlands) (35). The expression vectors encoding wild-type ZO-1 (ZO-1 WT) or the NH2-terminal fragment of ZO-1 comprising the PDZ domains 1-3 (ZO-1 PDZ) have been characterized previously (17).

Transient transfections

Transient transfections were performed with Fugene-6 transfection reagent (Roche Diagnostics) on $1.2x10^5$ BT20 cells and $1.5x10^5$ SKBR3 cells plated in 6-well plates. At 24h after plating, transfection was carried on as recommended by the manufacturer by adding, in each well, a mixture containing 100 µl of serum-free medium, 3 µl of Fugene-6 and 2 µg of the ZO-1 WT or ZO-1 PDZ expression vector. Controls were generated by transfecting the cells with 2 µg the corresponding empty vector pLNCX. The cells were harvested 48h after transfection for RT-PCR or Western blotting analyzes. Conditioned media were also collected for ELISA analyses.

For double transient transfection experiments with ZO-1 expression vectors and β-catenin siRNA, SKBR3 cells were subsequently transfected with the siRNA then with the expression

vectors. Sixteen hours after transfection of the siRNA, the cells were washed twice with PBS, then transiently transfected with the expression vectors for 48h as detailed above.

Luciferase reporter assays

Transient transfection experiments were performed using Fugene-6 transfection reagent on $5x10^4$ BT20 and SKBR3 cells seeded in a 24-well plate half an hour before the addition of the DNA/Fugene mixture. Each well was incubated with a mixture containing 20 µl of serum-free DMEM, 0.6 µl of Fugene-6, 0.2 µg of the firefly promoter-luciferase reporter constructs (either CXCL8/IL-8 promoter, TOP-FLASH or FOP-FLASH reporter construct), 0.2 µg of the expression vectors (ZO-1 WT, ZO-1 PDZ or β-catenin and TCF4 expression vectors, or the corresponding empty vector) and 0.8 ng of the renilla luciferase expression vector phRG-TK (Promega). At 24 hours after transfection, the cells were lysed in 100 µl of passive lysis buffer and the luciferase activity was determined with a luminometer using the Dual Luciferase Assay System (Promega) on 20 µl of lysate. For each experiment, the firefly luciferase activity was normalized to the activity of the renilla luciferase used as internal control. The results were expressed as fold induction determined by normalizing each firefly luciferase value to the renilla luciferase internal control value and by dividing these normalized values with the mean normalized value of the corresponding reporter construct transfected with the empty expression vector. To assess the β-catenin/TCF/LEF activities, the normalized values obtained with the FOP-FLASH reporter plasmid were subtracted from the normalized values obtained with the TOP-FLASH reporter plasmid. The resulting values were then divided with the mean normalized value of the condition transfected with the empty expression vector. Each experiment was performed at least 3 times in triplicate. Data are expressed as means ± SEM. A one-sample *t*-test was performed and a P value < 0.05 was considered significant.

RT-PCR analysis

Total RNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics). 10 ng of RNA was then reverse transcribed into cDNA and amplified using the GeneAmp Thermostable rTth enzyme (Applied Biosystems, Foster city, CA). The reverse and forward primers for CXCL8/IL-8, ZO-1, MMP-14, vimentin, β-catenin and 28S were designed as follows: CXCL8/IL-8 primers (forward 5'-GAATTCTCAGCCCTCTTCAAAAAC-3' and reverse 5'-GCCAAGGAGTGCTAAAGAACTTAG-3'), ZO-1 primers (forward 5'-GCAGCTAGCCAGTGTACAGTATAC3' and reverse 5'-GCCTCAGAAATCCAGCTTCACGAA-3'), MMP-14 primers (forward 5'-GGATACCCAATGCCCATTGGCCA-3' and reverse 5'-CCATTGGGCATCCAGAAGAGAGC-3'), vimentin primers (forward 5'-GCGTGACGTACGTCAGCAATATGA-3' and reverse-5'-GTTCCAGGGACTCATTGGTTCCTT-3'), β-catenin primers (forward 5'-CGAGGACCAGGTGGTGGTTAATAA-3' and reverse 5'-CAGAATCCACTGGTGAACCAAGCA-3') and 28S (forward 5'-GATTCTGACTTAGAGGCGTTCAGT-3' and reverse 5'-GTTCACCCACTAATAGGGAACGTGA-3'). For quantification, the values obtained for the RNA of interest were normalized to the values obtained for 28S amplification in 3 independent transfection experiments. Results were expressed as fold induction determined by dividing the normalized value of a given stimulated condition with the normalized value of the corresponding control condition. Data are expressed as means ± SEM. A one-sample *t*-test was performed and a P value < 0.05 was considered significant.

Detection of CXCL8/IL-8 by ELISA

CXCL8/IL-8 secretion was measured by ELISA in culture media of the different breast cancer cell lines or of ZO-1 siRNA-transfected BT549 cells. The CXCL8/IL-8 ELISA was performed according to the manufacturer's instructions (DuoSet human IL-8 R&D Systems, Minneapolis, MN). Results are expressed in pg/mg of protein dosed in cell extracts and as means +/- SEM of 3 independent experiments.

In conditioned media of the ZO-1 transfected BT20 and SKBR3 cells, CXCL8/IL-8 secretion was measured using a chemiluminescent ELISA assay according to the manufacturer's instructions (Quantiglo human IL-8, R&D Systems). Results were expressed as fold induction determined by dividing the amount CXCL8/IL-8 detected in ZO-1 siRNA condition with the amount detected in the corresponding scrambled siRNA control. Data are expressed as means ± SEM of 3 independent experiments. A one-sample *t*-test was performed and a P value < 0.05 was considered significant.

Western blotting analysis

Total protein extracts were made in Tris 50 mM pH7.4, NaCl 150 mM, Nonidet P-40 1%, Triton 1%, Deoxycholic acid 1%, SDS 0.1% and Iodoacetamid 5 mM supplemented with a cocktail of proteases inhibitors (Roche Diagnostics). Subcellular fractions were extracted with the Proteoextract subcellular proteome extraction kit according to the manufacturer's instructions (Merck, Darmstadt, Germany). 20 µg of total protein samples or 6 µg of subcellular fractions were separated by electrophoresis on 10% SDS-polyacrylamide gels and then transferred on a PVDF membrane (PerkinElmer, Shelton, CT). Immunoblotting was performed with a monoclonal anti ZO-1 antibody (ZO1-1A12, Zymed Laboratories, San Francisco, CA) followed by an incubation with a horseradish peroxydase-conjugated goat anti-mouse secondary antibody (Dako, Glostrup, Denmark). Signals were visualized using the

Western Lightning Chemiluminescence Reagent kit (Pierce, Rockford, IL). For loading normalization, the blots were then incubated, after extensive washes, with a rabbit anti-actin (Sigma) or a mouse anti-GAPDH antibody, used as a cytosolic marker (Chemicon, Temecula, CA) or with a goat anti-nibrin antibody, used as a nuclear marker (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a horseradish peroxydase-conjugated rabbit anti-goat or goat anti-mouse secondary antibody (Dako). Signals were detected with the Western Lightning Chemiluminescence Reagent kit (Pierce).

Immunofluorescence

Confluent monolayers of cells cultured on glass coverslips were fixed with methanol for 10 minutes at -20°C. The coverslips were then saturated for 10 minutes with 3% bovine serum albumin in PBS. Monolayers were next (after intermediate washes in PBS) incubated for 1h with the monoclonal antibody to ZO-1 and then with a polyclonal goat anti-mouse antibody for 1h. The coverslips were then mounted with Aquapolymount antifading solution (Polysciences, Warrington, PA) onto glass slides and observed under a Zeiss fluorescence microscope.

Results

Screening of chemokine expression by Gene Array

In order to investigate a potential regulation of chemokines by ZO-1, we first used a chemokine Gene Array GEArray Q serie (SA Biosciences) to screen and compare the mRNA expression profile of chemokines in invasive breast tumor BT549 cells transfected with a ZO-1 siRNA and a corresponding scrambled control siRNA. From this screening, CXCL8/IL-8 emerged as a strongly downregulated gene following ZO-1 siRNA transfection (Figure 1a). In order to confirm the results obtained with the GEArray analysis using ZO-1 si1, showing that

ZO-1 regulates IL-8 expression, we transfected BT549 cells with two other ZO-1 siRNA sequences (ZO-1 si2 and ZO-1 si3). As shown in Figure 1b, the 3 siRNA sequences efficiently decreased ZO-1 protein levels. We concomitantly observed that CXCL8/IL-8 mRNA (Figure 1c) and protein (Figure 1d) expression was clearly downregulated in BT549 tumor cells treated with the 3 ZO-1 siRNA. A functional consequence of such chemokine regulation by ZO-1 was shown using a transmigration assay with the monocytic THP-1, previously shown to display a chemotactic response to various chemokine including CXCL8/IL-8 (36). THP-1 cells migrated less towards conditioned medium of ZO-1 siRNA BT549 cells than towards conditioned medium from ZO-1 scrambled control transfectants (Figure 1e).

CXCL8/IL-8 is expressed by invasive breast cancer cells

To further explore a potential relationship between ZO-1 and CXCL8/IL-8 expression, we examined their expression in a set of breast tumor cell lines displaying various degrees of invasiveness. The invasive phenotype of these breast cancer cells, assessed by a modified Boyden chamber assay, has been extensively characterized previously (37) and confirmed in the present study (Figure 2a). Examining CXCL8/IL-8 mRNA expression by RT-PCR analysis, we observed that invasive cells (MDA-MB-231, BT549, Hs578T) expressed high levels of CXCL8/IL-8 compared to non-invasive cell lines (MCF7, BT20, SKBR3) (Figure 2b). CXCL8/IL-8 mRNA in invasive cell lines was also correlated to the expression of other pro-invasive markers such as vimentin or matrix metalloproteinase (MMP-14) previously shown to be target genes of ZO-1 signaling (Figure 2b) (17, 18). This higher expression of CXCL8/IL-8 in invasive breast cancer cell lines was confirmed at the protein level by ELISA analysis (Figure 2c). Looking at ZO-1 expression by Western blotting, we found it was expressed in all the cell lines (Figure 2d) as multiple bands, likely corresponding to different

isoforms or degradation products. By immunofluorescence analysis, however, we observed that ZO-1 subcellular localization differs from one cell line to another. In contrast to invasive cell lines, non-invasive cell lines rapidly form clusters of cells. In these non-invasive cell lines, ZO-1 labeling thus varies with cell density and was mainly found at the cell membrane in intercellular contacts of cell clusters. In contrast, in invasive breast tumor cell lines expressing CXCL8/IL-8, which do not form clusters, ZO-1 staining was mostly cytoplasmic independently of cell confluency. This is illustrated for BT549 (representative of invasive cells) and BT20 cells (representative of non-invasive cells) in Figure 2e.

In order to correlate further CXCL8/IL-8 expression and ZO-1 relocalization, we used the MCF10A cells which were previously shown to undergo alterations of tight junctions following long term TGF-β treatment (38). Here, using a combined treatment of EGF and TGF-β, we observed an induction of CXCL8/IL-8 both at the protein and RNA level which coincided with a diminution of the typical honeycomb membrane staining of ZO-1 (Figure 3). In these different cell systems, a cytoplasmic localization and/or a diminution of membrane-associated localization of ZO-1 is thus associated with enhanced CXCL8/IL-8 expression, supporting our observations that ZO-1 siRNA transfection in invasive BT549 downregulates CXCL8/IL-8.

CXCL8/IL-8 expression is regulated by ZO-1

In order to complement our results obtained with ZO-1 siRNA, showing a downregulation of CXCL8/IL-8 by ZO-1 siRNA, we inversely transiently transfected non-invasive BT20 and SKBR3 breast cancer cells with expression vectors encoding the full length ZO-1 (ZO-1 WT), the NH_2-terminal fragment of ZO-1 comprising the PDZ domains 1-3 (ZO-1 PDZ) that is unable to bind occludin and thus presents a diminished ability to localize at the TJ complexes, or with the empty vector as control. These transfections enhanced ZO-1 expression both at the

protein level as shown by immunofluorescence (Figure 4a) and at the RNA level by RT-PCR (Figure 4b). Using RT-PCR analysis, we observed a significant induction of CXCL8/IL-8 mRNA after transfection of either ZO-1 full length or the PDZ construct in both cell lines (Figure 4b). Indeed, we observed a 2.10 (± 0.21) and 2.18 (± 0.22) fold induction of CXCL8/IL-8 mRNA expression respectively after ZO-1 WT and ZO-1 PDZ transfection of BT20 cells compared to the empty vector. Similarly, the transfection of SKBR3 cells with the ZO-1 WT and ZO-1 PDZ plasmids lead to a significant induction of CXCL8/IL-8 expression (1.96±0.26 and 2.23±0.07 fold respectively). A significant accumulation of CXCL8/IL-8 protein was accordingly detected by ELISA in the conditioned media of BT20 and SKBR3 particularly after ZO-1 PDZ transfection (Figure 4c). Since ZO-1 could have a pro-migratory/invasive signaling role once delocalized from the cell membrane complexes, we examined the subcellular localization of ZO-1 in cells transiently transfected with ZO-1 WT or ZO-1 PDZ expression vectors. As shown by Western blotting analyses on subcellular protein exctracts, an increase of the cytoplasmic pool but also of the nuclear pool of the ZO-1 WT or the truncated ZO-1 PDZ protein was observed in the BT20 and the SKBR3 cells following the transfection of the corresponding expression vectors (Figure 5).

CXCL8/IL-8 promoter is activated by ZO-1

Previous studies have shown that transfection of ZO-1 to enhance its cytoplasmic level and activate the β-catenin pathway (17, 18). Moreover, Levy et al. have demonstrated that CXCL8/IL-8 is regulated by the β-catenin pathway through a specific TCF/LEF binding site (34). We thus further investigated if the β-catenin pathway is implicated in the regulation of CXCL8/IL-8 by ZO-1. We used a luciferase reporter plasmid containing a 193bp fragment of the CXCL8/IL-8 promoter as well as the corresponding construct in which the β-catenin/TCF binding site has been mutated in order to inhibit the binding of β-catenin/TCF complexes (193

pro mut). A truncated form of 173bp which lacks the β-catenin/TCF binding site was also used. These promoter constructs have been previously described (34). Co-transfection experiments were performed in BT20 and SKBR3 cells using the 193bp, the mutated 193bp or the 173bp human CXCL8/IL-8 promoter reporter plasmids and expression vectors encoding ZO-1 WT, ZO-1 PDZ or the empty vector (pLNCX) as control. In BT20 and SKBR3 cells, co-transfection of the 193bp CXCL8/IL-8 promoter construct with ZO-1 WT and ZO-1 PDZ led to the induction of the promoter activity by 3.9±0.4 and 5.5±0.6 fold and 2.5±0.6 and 4±0.8 fold, respectively. The 193bp mutated and the 173bp reporter constructs were also significantly activated following the transfection of the ZO-1 WT or the PDZ expression vectors (Figure 6). This suggests that the β-catenin pathway is not implicated in the regulation of CXCL8/IL-8 by ZO-1.

CXCL8/IL-8 regulation by ZO-1 does not implicate the β-catenin pathway

In order to confirm this lack of implication of the β-catenin pathway in CXCL8/IL-8 regulation by ZO-1, we co-transfected BT20 and SKBR3 cells with the TOP-FLASH or FOP-FLASH reporter plasmid and the ZO-1 WT or ZO-1 PDZ expression vectors. As shown in Figure 7a, the activity of the TOP-FLASH/FOP-FLASH reporter system was accordingly not enhanced following the transfection of the ZO-1 WT or PDZ expression vectors.

Furthermore, we have also analyzed CXCL8/IL-8 regulation by ZO-1 following β-catenin RNAi inhibition in SKBR3 cells. We thus analyzed CXCL8/IL-8 expression in SKBR-3 cells successively transfected with a siRNA against β-catenin (or a control siRNA) then with the expression vector for ZO-1 cDNA (or the vector control). As shown in figure 7b, the transfection of the β-catenin siRNA did not modify CXCL8/IL-8 induction by ZO-1. Confirming our luciferase assays, these data also suggest that β-catenin is not involved in CXCL8/IL-8 regulation by ZO-1

Discussion

In the present study, we identified CXCL8/IL-8 as a potential target of ZO-1 regulation. We first demonstrated that CXCL8/IL-8 is highly expressed only in invasive breast cancer cells or in EGF /TGF-β-treated MCF10A cells, in which ZO-1 does not localize at cell-cell contacts but rather displays a cytoplasmic staining. We further documented a downregulation of CXCL8/IL-8 in invasive breast tumor cells transfected with ZO-1 siRNA. Inversely, transfection of ZO-1 expression vectors in non-invasive cells induced CXCL8/IL-8 expression and activated its promoter.

Although this is the first report correlating ZO-1 localization and CXCL8/IL-8 expression, independent studies have associated CXCL8/IL-8 expression with high invasive properties. De Larco et al. have demonstrated that CXCL8/IL-8 is produced at high levels in MDA-MB-231 and MDA-MB-435 compared to MCF7 and T47D cells (27) and is associated with their high metastatic potential. Freund et al. have also reported that CXCL8/IL-8 mRNA expression is 80-fold higher in MDA-MB-231 than in MCF7 cells (39). Furthermore, our data are in agreement with studies reporting CXCL8/IL-8 expression in a variety of human cancer cell lines including renal carcinoma cells (40), melanoma cells (26, 41, 42), breast cancer cells (43), prostate cancer cells (44), colon carcinoma cells (45) and bladder cancer cells (46). Also, these studies associated CXCL8/IL-8 expression and the metastatic potential of the cancer cells. Moreover, CXCL8/IL-8 has been associated with a poor prognosis of breast carcinoma (28, 31, 43), gastric carcinoma (47), melanoma (48) and nasopharyngeal carcinoma (49).

On the other hand, our data are in agreement with the literature showing a relocalization of ZO-1 associated with high migratory/invasive potential of epithelial cells (8, 12, 50).

However, beyond establishing a correlation between CXCL8/IL-8 expression and the cytoplasmic localization of ZO-1, we have functionally involved ZO-1 in the regulation of CXCL8/IL-8 expression. Indeed, we have shown that CXCL8/IL-8 expression is downregulated in invasive BT549 cells transfected with 3 different ZO-1 siRNA and upregulated in BT20 and SKBR3 cells transfected with ZO-1 cDNA. Only a few studies have documented gene regulation by ZO-1. Balda et al. described ZO-1 as a regulator of ErbB-2 expression and cell proliferation in breast cancer cells (51). The induction of an epithelial-to-mesenchymal transition associated with a decrease of epithelial gene expression and an increase in the expression of several mesenchymal genes has also been reported in MDCK cells transfected with ZO-1 expression vectors and correlated to increased invasiveness (17). We recently reported an induction of MMP-14 (membrane type 1-matrix metalloproteinase) expression by ZO-1 in breast cancer cells (18). Taken together, these results therefore suggest that, once delocalized from the junctional membrane adhesion complexes, ZO-1 can contribute to signaling events leading to the activation of the transcription of tumor promoting genes such as CXCL8/IL-8.

The molecular mechanisms involved in such gene regulation by ZO-1 remain however unclear. ZO-1 can interact with several molecular partners, which could directly or indirectly contribute to its ability to regulate gene expression. For example, the regulation of ErbB-2 by ZO-1 involves a direct interaction between ZO-1 and the Y-box transcription factor ZONAB at the cell membrane, inducing a membrane sequestration of this factor which is a repressor of the ErbB-2 promoter (51). Recently, a sequestration of ubinuclein (ubn-1) by ZO-1 has also been described which could impair the nuclear activity of this NACos, also known to interact with the transcription factor AP-1 (52, 53). In these studies however, ZO-1 was rather shown to control gene expression by sequestering nuclear factors at the cell membrane. In contrast, the data which have established a pro-invasive signaling role of ZO-1 strongly implicate the

relocalization of ZO-1 out of cell-cell contacts and an increased cytoplasmic level but not particularly a nuclear shuttling. Thus, an activation of the β-catenin/TCF/LEF transcription pathway has been documented in MDCK cells and in breast tumor cells following transfection of ZO-1 expression vectors which led to enhanced cytoplasmic levels of ZO-1 (17, 18). We and others have reported that chemokines could also be the targets of the β-catenin/TCF pathway. This has been shown for CXCL8/IL-8 by Levy et al. in human hepatoma cells (34), by Masckauchán et al. in human endothelial cells (54) and by our group for MCP-1/CCL2 in breast tumor cell lines (33). In the light of these studies, we have also examined a potential implication of the β-catenin pathway in the regulation of CXCL8/IL-8 by ZO-1. However, our present data suggest that the β-catenin pathway is not implicated in the regulation of CXCL8/IL-8 by ZO-1. A β-catenin-independent regulatory mechanism is thus rather likely to be involved in CXCL8/IL-8 regulation by ZO-1. Along these lines, the human CXCL8/IL-8 promoter can be activated by a complex cooperation of other transcription factors like NFkB, AP-1, PEA3 and c/EBP in human breast cancer cells (39, 55, 56) and a potential direct or indirect interaction between ZO-1 and these transcriptional pathways in different cell compartments (both in the cytoplasm and in the nucleus) cannot be excluded and is under investigation.

In conclusion, we have identified CXCL8/IL-8 as a potential target of ZO-1 signaling in breast tumor cells. Considering the important implication of CXCL8/IL-8 and the reorganization of tight junctions during tumorigenesis, such a regulation of CXCL8/IL-8 by ZO-1 might play an important role in breast cancer progression.

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Legends to figures

Figure 1: CXCL8/IL-8 is downregulated in BT549 cells transfected with ZO-1 siRNA

(a) Analysis by GEArray of the expression of chemokines and chemokines receptors in BT549 cells transfected with a siRNA against ZO-1 (ZO-1 si1) compared to a corresponding scrambled siRNA (ZO-1 Scr si1). The circle represents the CXCL8/IL-8 expression.

(b) Western blot analysis of ZO-1 expression in BT549 cells transfected with 3 siRNA against ZO-1 (ZO-1 si1, 2, 3) or corresponding control siRNA (ZO-1 scr si1, 2, 3). β-actin is shown as a control.

(c) Representative RT-PCR analysis of CXCL8/IL-8 and 28S expression in BT549 cells transfected with the 3 siRNA against ZO-1 (ZO-1 si1, 2, 3) or the control siRNA (ZO-1 scr si1, 2, 3). Quantification of the RT-PCR analyses of CXCL8/IL-8 expression normalized to the 28S levels is shown for 3 independent siRNA transfection experiments. Data are presented as fold induction relative to the control condition *(*P<0.05, **P<0.01, ***P<0.001).*

(d) ELISA analysis of CXCL8/IL-8 present in conditioned media obtained from 3 independent experiments of BT549 cells transfected with ZO-1 siRNA or the corresponding scrambled control siRNA. Data are presented as fold induction relative to the control condition (***P*<0.01).

(e) Analysis of the migration of the monocytic THP-1 cell towards conditioned medium of ZO-1 siRNA BT549 cells or conditioned medium of ZO-1 scrambled control transfectants . Data are presented as fold induction relative to the control condition in 3 independent experiments (***P*<0.01).

Figure 2: CXCL8/IL-8 is expressed in invasive breast cancer cells displaying a cytoplasmic staining of ZO-1

(a) Analysis of the invasive properties of different mammary cell lines in a modified Boyden chamber invasion assay. Results are expressed as the mean of three different experiments ± SEM.

(b) RT-PCR analysis of CXCL8/IL-8, MMP-14 and vimentin expression in different human breast cancer cell lines (MCF7, BT20, SKBR3, MDA-MB-231, BT549, Hs578T). RT-PCR of the 28S mRNA is shown as a control. Each experiment was performed at least 3 times and one representative experiment is shown.

(c) ELISA analysis of CXCL8/IL-8 in 48h conditioned media obtained from the different breast cancer cell lines. Each experiment was performed at least 3 times.

(d) Western blot analysis of ZO-1 expression in these cell lines. β-actin is shown as a control.

(e) Immunofluorescence analysis of ZO-1 expression in non-invasive BT20 cells and in invasive BT549 cells. Confluent monolayers were analyzed. Nuclei are stained with DAPI. Bar=25µm.

Figure 3: CXCL8/IL-8 is overexpressed in MCF10A cells treated with EGF and TGF-β displaying a perturbed membrane ZO-1 staining

(a) Immunofluorescence analysis of ZO-1 in MCF10A exposed to EGF/TGF-β. Bar = 50µm.

(b) RT-PCR analysis of CXCL8/IL-8 expression in MCF10A exposed to EGF/TGF-β. Quantification of the RT-PCR analyses of CXCL8/IL-8 expression normalized to the 28S levels is shown for 3 independent experiments. Data are presented as fold induction relative to the control condition (*P<0.05).

(c) ELISA analysis of CXCL8/IL-8 levels in conditioned media obtained from 3 independent experiments of MCF10A exposed to EGF/TGF-β. Data are presented as fold induction relative to the control condition (**P<0.01).

Figure 4: ZO-1 upregulates CXCL8/IL-8 in BT20 and SKBR3 cells

(a) Immunofluorescence analysis of ZO-1 in BT20 and SKBR3 cells transfected with the ZO-1 expression vector (ZO-1 WT), the ZO-1 PDZ expression vector (ZO-1 PDZ) or the corresponding control vector (pLNCX). Bar=10μm.

(b) Representative RT-PCR analysis of CXCL8/IL-8, ZO-1 and 28S expression in BT20 and SKBR3 cells 48 hrs after transfection with the ZO-1 expression vector (ZO-1 WT), the ZO-1 PDZ expression vector (ZO-1 PDZ) or the corresponding control vector (pLNCX). Quantification of the RT-PCR analyses of CXCL8/IL-8 expression normalized to the 28S levels is shown for 3 independent transfection experiments. Data are presented as fold induction relative to the control condition (*P<0.05, **P<0.01, ***P<0.001).

(c) ELISA analysis of CXCL8/IL-8 levels in conditioned media obtained from 3 independent experiments of BT20 and SKBR3 cells 48 hrs after transfection with ZO-1 WT, ZO-1 PDZ or the control vector (pLNCX). Data are presented as fold induction relative to the control condition (*P<0.05, **P<0.01).

Figure 5: Transfection of ZO-1 expression vectors enhances the cytoplasmic and nuclear pool of ZO-1 proteins

Western blot analysis of ZO-1 expression in subcellular fractions of BT20 and SKBR3 cells transfected with ZO-1 WT (lane 2), ZO-1 PDZ (lane 3) or the control vector (lane 1). GAPDH and nibrin were used as cytoplasmic and nuclear markers, respectively.

Figure 6: ZO-1 and ZO-1 PDZ activate the CXCL8/IL-8 promoter

Luciferase activity of the 193bp promoter (193 pro), the 173bp promoter (173 pro) or the 193bp promoter mutated in the β-catenin/TCF binding site (193 pro mut) co-transfected with the empty expression vector (pLNCX), the ZO-1 expression vector (ZO-1 WT) or the ZO-1 PDZ expression vector (ZO-1 PDZ) into BT20 and SKBR3 cells. Results are expressed as fold induction relative to the co-transfection of the reporter plasmid with the empty expression vector pLNCX (**P*<0.05, ***P*<0.01, ****P*<0.001).

Figure 7: ZO-1 and ZO-1 PDZ do not activate the β-catenin pathway

(a) The reporter plasmids TOP-FLASH and FOP-FLASH were co-transfected with the ZO-1 expression vector (ZO-1 WT), the ZO-1 PDZ expression vector (ZO-1 PDZ) or the empty vector (pLNCX) as a control into BT20 and SKBR3 cells. The normalized FOP values were subtracted from the normalized TOP values. Co-transfection of the β-catenin and TCF-4 expression vectors was performed as a positive control. Data are expressed as fold induction relative to the values obtained in cells transfected with the empty control vector.

(b) RT-PCR analyses of ZO-1, β-catenin and CXCL8/IL-8 in SKBR3 cells transfected with a β-catenin siRNA (β-cat si) or a scrambled control (β-cat Scr si) then with the expression vector for ZO-1 WT (ZO-1 WT) or the control vector (pLNCX). Quantifications of the RT-PCR analyses of CXCL8/IL-8 and ZO-1 expression normalized to the 28S levels are shown for 3 independent experiments. Data are presented as fold induction relative to the control condition (**p*<0.05).

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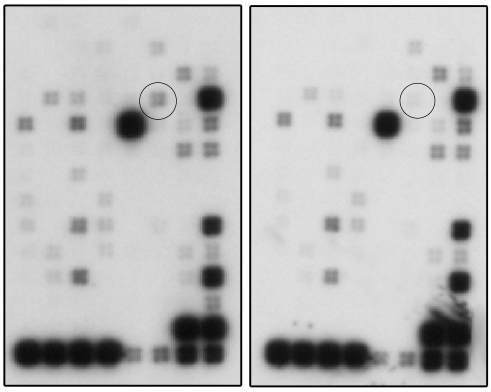
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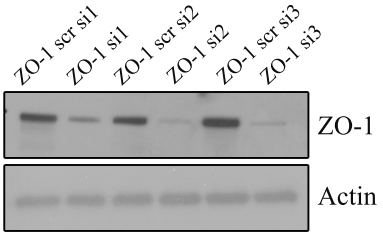
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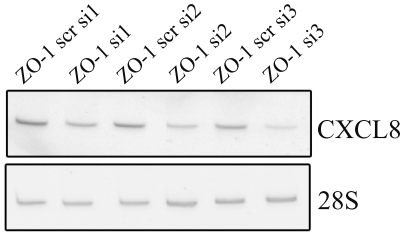
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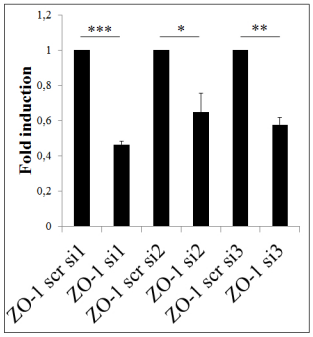
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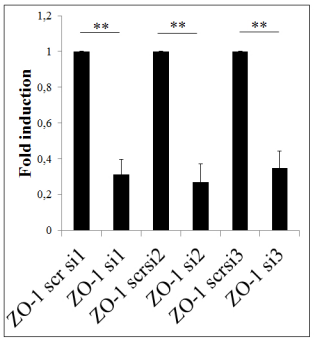
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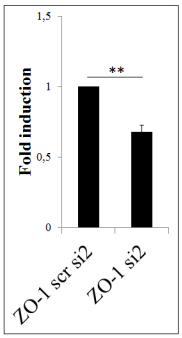
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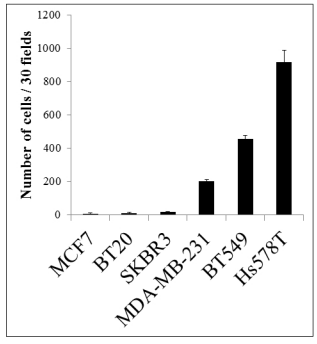
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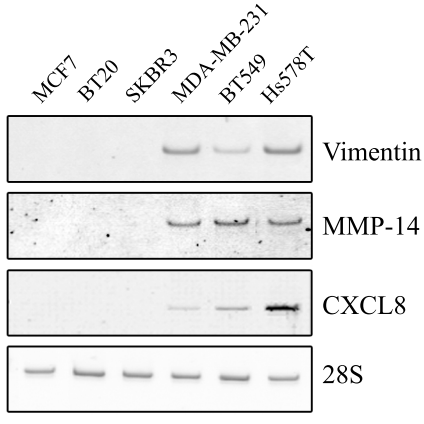
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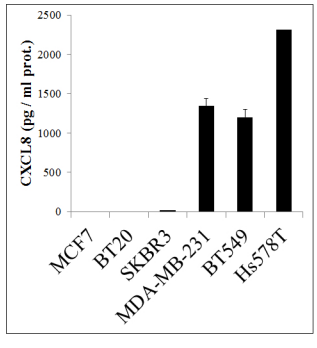
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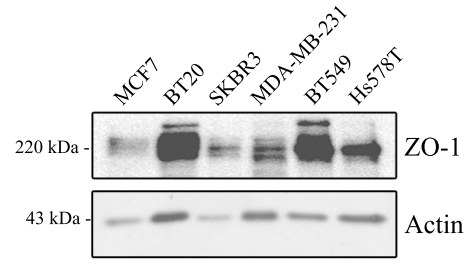
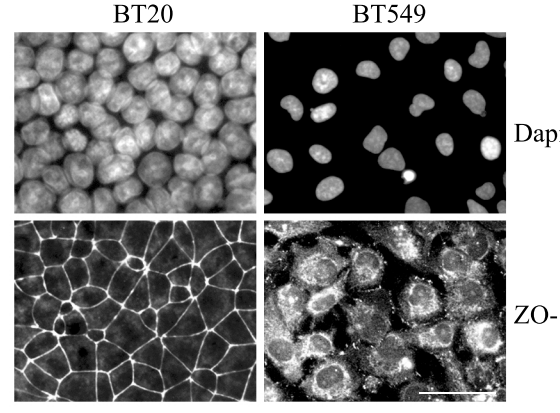
Hs578T

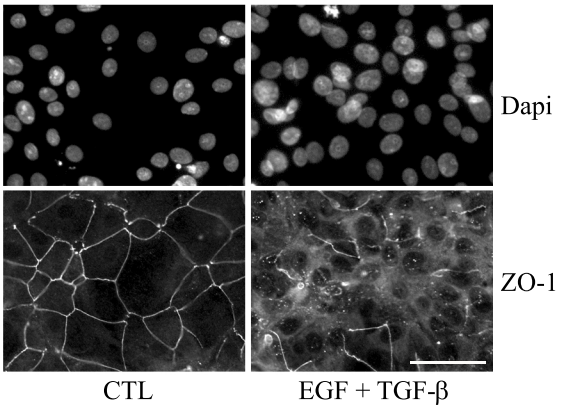
d


MCF7
BT20
SKBR3
MDA-MB-231
BT549
Hs578T
220 kDa
ZO-1
43 kDa
Actin

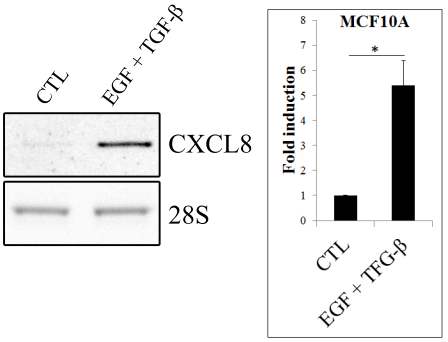
e


BT20
BT549
Dapi
ZO-1

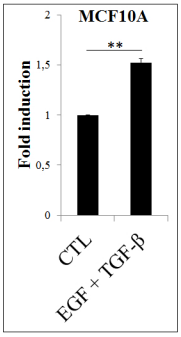
a

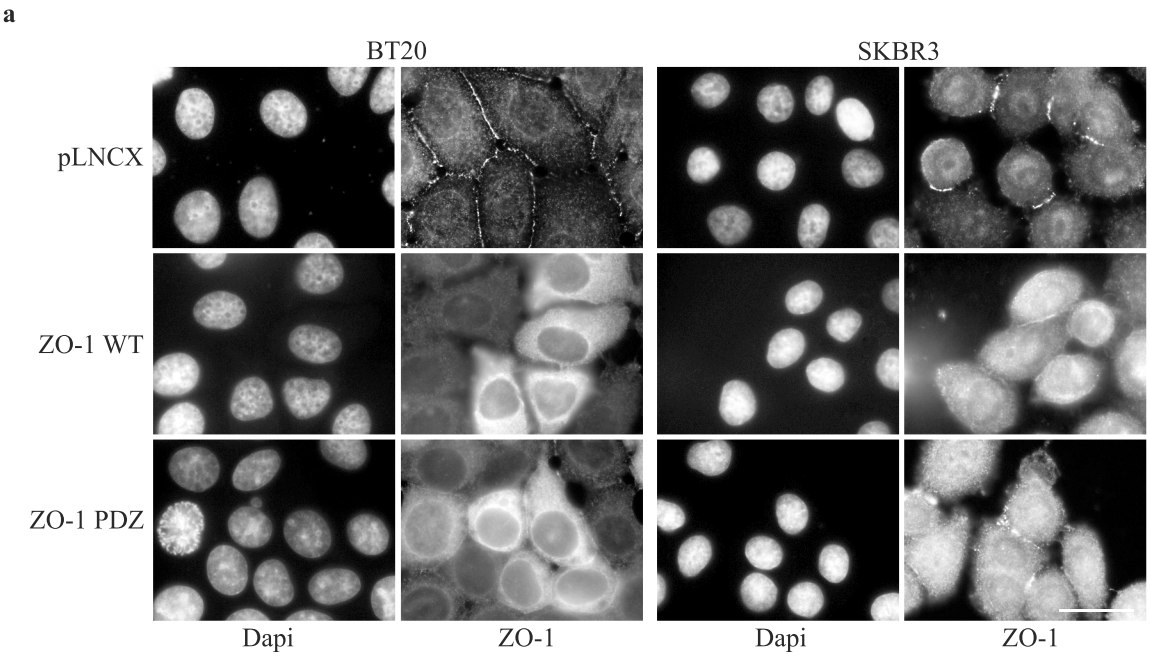

Dapi
ZO-1
CTL
EGF + TGF-β

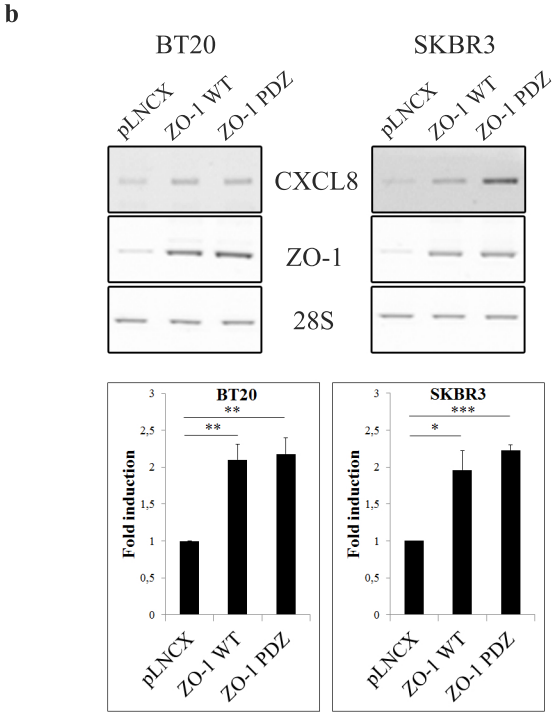
b

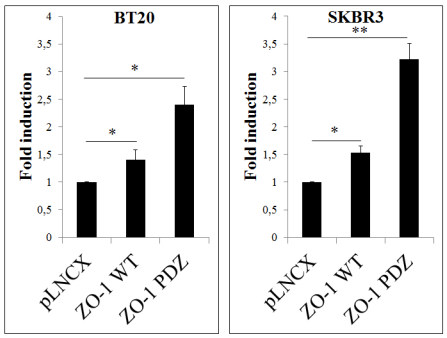

CTL
EGF + TGF-β
CXCL8
28S
MCF10A
Fold induction
*
CTL
EGF + TFG-β

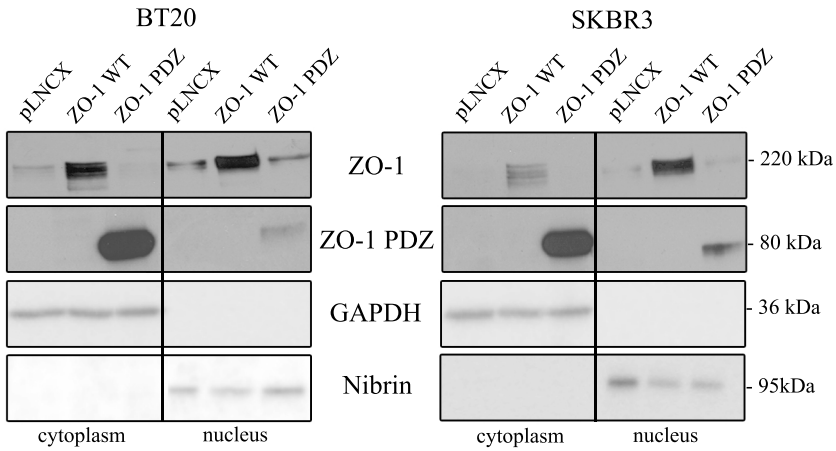
c

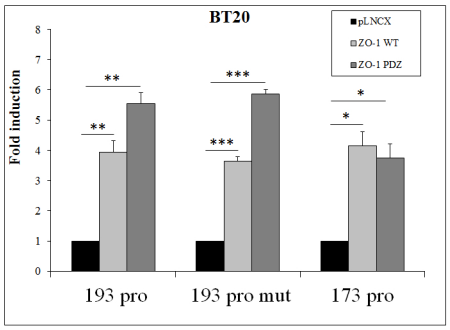

MCF10A
Fold induction
**
CTL
EGF + TGF-β

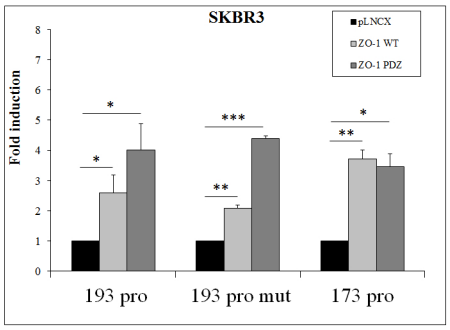

a
BT20
SKBR3
pLNCX
ZO-1 WT
ZO-1 PDZ
Dapi
ZO-1
Dapi
ZO-1


b
BT20
SKBR3
pLNCX
ZO-1 WT
ZO-1 PDZ
CXCL8
ZO-1
28S
BT20
SKBR3
Fold induction
pLNCX
ZO-1 WT
ZO-1 PDZ

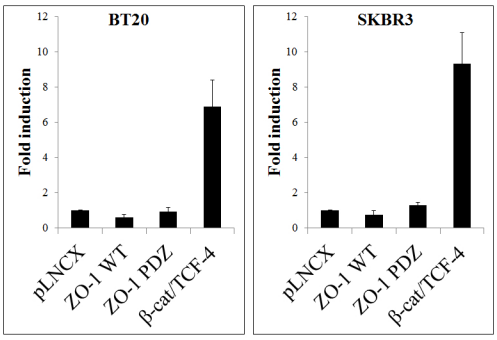

c
BT20
SKBR3
Fold induction
pLNCX
ZO-1 WT
ZO-1 PDZ


BT20
SKBR3
pLNCX
ZO-1 WT
ZO-1 PDZ
ZO-1
ZO-1 PDZ
GAPDH
Nibrin
220 kDa
80 kDa
36 kDa
95kDa
cytoplasm
nucleus

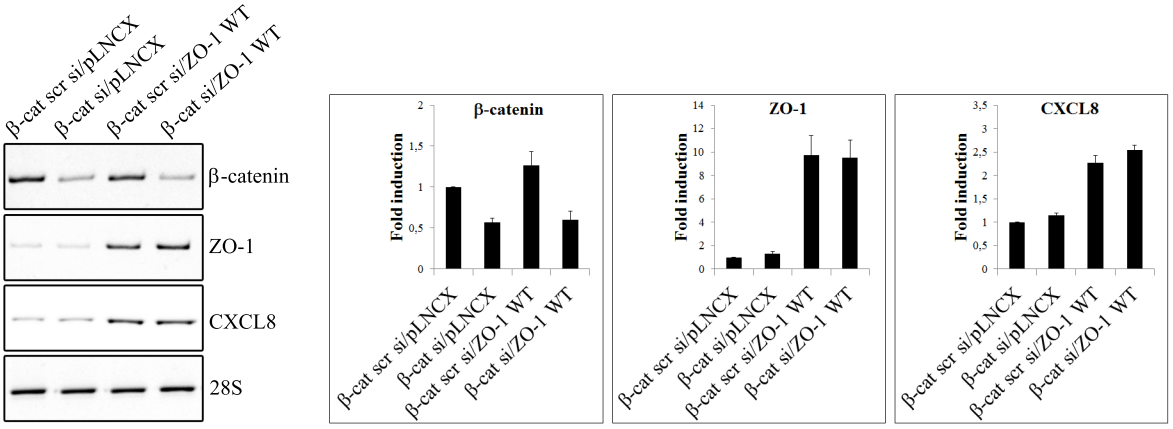

BT20
pLNCX
ZO-1 WT
ZO-1 PDZ
Fold induction
0
1
2
3
4
5
6
7
8
**
**
***
***
*
*
193 pro
193 pro mut
173 pro


SKBR3
pLNCX
ZO-1 WT
ZO-1 PDZ
Fold induction
0
1
2
3
4
5
6
7
8
*
*
***
**
*
**
193 pro
193 pro mut
173 pro

a


BT20
SKBR3
Fold induction
pLNCX
ZO-1 WT
ZO-1 PDZ
β-cat/TCF-4

b


β-cat scr si/pLNCX
β-cat si/pLNCX
β-cat scr si/ZO-1 WT
β-cat si/ZO-1 WT
β-catenin
ZO-1
CXCL8
28S
Fold induction