Claudin-12 is involved in the cell migration during metastasis

Desislava Kolchakova, Dzhemal Moten, Tsvetelina Batsalova and Balik Dzhambazov *

Department of Developmental Biology, Plovdiv University “Paisii Hilendarski”, 4000 Plovdiv, Bulgaria
* Correspondence: balik@uni-plovdiv.bg (B.D.)

Abstract: Claudins are important components of the tight junctions determining the barrier properties, cell polarity and paracellular permeability. Although many functions of claudins in cancer cells are not elucidated, recent studies showed that claudins play an important role in the cell migration and metastasis. Loss of epithelial/endothelial integrity, disruption of tight junctions and increased paracellular leakage are often observed during metastasis. The aim of our study was to investigate the involvement of claudin-12 in the process of cell migration as well as to evaluate the possibility to use this protein as a specific target for regulation of the tumorigenesis. We have performed immunocytochemistry to detect the expression of claudin-12 in different epithelial/endothelial human cell lines and three of them (A549, LS180 and HeLa) were selected for further experiments. By using the Transwell chamber migration assays, we found that anti-claudin-12 antibodies inhibited both migration and proliferation of the claudin-12 expresing cells (A549 and LS180) as well as the migration capacity of Jurkat cells through the monolayers formed from A549 or LS180 cells. In addition, co-culture of Jurkat cells on monolayers from A549 or LS180 cells, in presence of synthetic claudin-12 peptides, representing the extracellular domains of the claudin-12 protein, also reduced the number of migrated Jurkat cells. Two of the tested peptides (p5 and p6) almost completely blocked the migration of Jurkat cells. All migrated Jurkat cells expressed LFA-1 and CD62L, but not CD44. Thus, claudin-12 is a suitable biomarker for tumor progression and metastasis, and an attractive target for antitumor therapy. Anti-claudin-12 antibodies and competitive inhibitory peptides could be useful in the therapeutic approach applied to cancer metastasis in tissues expressing claudin-12.

Keywords: claudin-12, cell migration, antibody treatment, peptide inhibition, metastasis, tight junctions

1. Introduction

Claudins are small (20–34 kDa) tetraspanning transmembrane proteins that are involved in the structure of tight junctions [1,2]. They play a critical role for the regulation of paracellular permeability to ions and small molecules in endothelia or epithelia and the maintenance of cell polarity [3,4]. In addition, claudins are associated with various signaling pathways related to cell proliferation and differentiation [5,6].

The mammalian claudin gene family consists of 27 members, as their expression levels and subcellular localization depends on the tissue and cell type [7,8]. Among claudin proteins, claudin-12 is defined as an unusual member, since it does not possess an intracellular PDZ binding motif [1,9], which mediates the interaction with the cytoskeleton. Claudin-12 is expressed in epithelia and endothelia of gastrointestine, inner ear, brain endothelial cells [1,8,10], as well as in the smooth and striated muscle cells, neurons and astrocytes [11]. It has been shown that *in vitro* expression of claudin-12 is up-regulated by vitamin D, suggesting an essential role of this claudin for the Ca^{2+} absorption between intestinal epithelial cells [12].

Dysregulated expression of claudins has been reported in various cancers, suggesting that they may have an important role in the migration, invasion and metastasis of cancer cells [3,13-17]. More than 90% of cancer-related deaths are due to metastases [18]. The metastatic process requires transendothelial migration of the cancer cells. They have to leave their primary site by intravasation in the lumen of the vasculature, to circulate in the bloodstream, and to extravasate in a secondary site [18,19]. Metastasis is accompanied by disruption of tight junctions, loss of epithelial/endothelial integrity and increased paracellular leakage, providing a space for mobility of the cancer cells [20]. It was reported that overexpression of claudin-12 significantly increased the metastatic properties of the human bronchial epithelial cells BEAS-2B [17].

However, the role of claudins for migration of cancer cells through the tight junctions during metastasis is not fully understood. The aim of this study was to investigate the involvement of claudin-12 in the process of metastasis as well as to evaluate the possibility of using this protein as a specific target for regulation of the tumorigenesis. We hypothesized that cancer cells use claudin-12 to migrate through the tight junctions during metastasis and blocking this protein or competitive binding of cancer cells to peptides derived from the extracellular part of claudin-12 will reduce the metastatic process.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions

The human cell lines A549 (epithelial lung carcinoma cells, ATCC CCL 185, NBIMCC 2404), Caco-2 (epithelial colon adenocarcinoma cells, ECACC 86010202), HT-29 (epithelial colon adenocarcinoma cells, ECACC 91072201), LS180 (Dukes' type B epithelial colon adenocarcinoma, mucin-secreting cells, ECACC 87021202), SK-Hep-1 (endothelial liver adenocarcinoma cells, ATCC HTB 52, NBIMCC 1858), HeLa (epithelial cervix adenocarcinoma cells, ATCC CCL 2, NBIMCC 164) and Jurkat E6.1 (acute lymphoblastic leukaemia T cells, ECACC 88042803) were obtained either from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, United Kingdom) or from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC). All adherent cell lines were cultured in complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and stabilized antibiotic antimycotic solution (all from Merck KGaA, Darmstadt, Germany) at 37ºC, 5% CO_2, 95% atmospheric air in a humidified incubator. The Jurkat cells were maintained at the same conditions using RPMI 1640 medium (Merck KGaA, Darmstadt, Germany) instead DMEM. Prior to the performed assays, the cell lines were expanded in 75 cm^2 culture flasks (TPP, Trasadingen, Switzerland).

2.2. Reagents and Antibodies

All reagents were of analytical quality and obtained from Merck KGaA (Darmstadt, Germany), unless otherwise stated. Six synthetic peptides (p1-peptide, YNIHLNKKFEPVFSFDYA, Cldn-12 (157-174), second extracelluar domain; p2-peptide, NWRKLRLITFNRNEKNLTVY; p3-peptide, TGLWVKCARYDGSSDCLMYD; p4-peptide, TTWYSSVDQLDLRVLQ; p5-peptide, NRNEKNLTVYTGLWVKCARY and p6-peptide, DGSSDCLMYDTTWYSSVDQL, parts of the first extracelluar domain) representing sequences from the extracellular domains of the protein claudin-12 were synthesized from Schafer-N (Copenhagen, Denmark). Monoclonal anti-CLDN12 antibody produced in mouse (clone 2D8, cat. No SAB1403012) and anti-mouse IgG (whole molecule)–FITC antibody produced in goat (cat. No F0257) were purchased from Merck KGaA (Darmstadt, Germany). Monoclonal anti-human CD11a/CD18-APC (LFA-1) antibody (clone m24, cat. No 363410), anti-human CD62L-FITC (L-selectin) antibody (clone DREG-56, cat. No 304804) and anti-human CD44-PE antibody (clone BJ18, cat. No 338808), all produced in mouse, were purchased from BioLegend® (San Diego, CA, USA).

2.3. Immunocytochemistry

A549, Caco-2, HT-29, LS180, SK-Hep-1 and HeLa cells were seeded (1.0×10^5 cells/well) on coverslips in 12-well plates (TPP, Trasadingen, Switzerland) and cultured in a humidified incubator at 37ºC, 5% CO_2 in complete DMEM (1 ml/ well). After 48 h incubation period, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and fixed with ice cold acetone:methanol (1:1). Then, cells were stained for 30 min with anti-CLDN12 antibody at room temperature, washed with DPBS and incubated for 15 min with secondary anti-mouse IgG–FITC antibodies in dark. After washing three times with DPBS, the expression of claudin-12 in studied cell lines was observed using a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). For the next experiments, we selected two claudin-12 positive cell lines (A549 and LS180) and one negative (HeLa).

2.4. Cell migration assays

For the cell migration assays of the selected adherent cell lines (A549, LS180 and HeLa), 5.0×10^5 cells/well suspended in 100 μL complete medium (CM) were added as triplicates into the upper chambers of Corning® HTS Transwell® 96 well permeable supports (8.0 μm pore polycarbonate membrane, cat. No CLS3374, Merck KgaA, Darmstadt, Germany) and incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 h. After 24 h of incubation, cell monolayers in the upper chambers were treated with monoclonal anti-CLDN12 antibody (1 μg/mL) for 30 min at 37°C, then washed three times with 150 μL serum-free DMEM and incubated for additional 24 h in 100 μL serum-free DMEM at 37°C in a humidified atmosphere containing 5% CO_2. For last 24 h, in the lower chambers were added 100 μL complete medium. Cells without anti-CLDN12 antibody treatment served as control groups. Migrated into the lower chamber cells were fixed in cold methanol and stained with 0.5% crystal violet for 10 min. All migrated cells were visualized under an inverted light microscope Inverso (Medline Scientific, Chalgrove, Oxon, UK) equipped with a Si-3000 digital camera and software (Medline Scientific, Chalgrove, Oxon, UK). The cells were counted in each transwell of the triplicates and photographed (magnification, x200).

To analyze the migration of Jurkat cells through the tight junctions of the formed monolayers from the selected adherent cells, we used the same transwell system as described above with small modifications. In order to avoid migration of the adherent cells through the transwell membrane, we used Corning® HTS Transwell® 96 well plates with 3.0 μm pore of the polycarbonate membrane (cat. No CLS3385, Merck KgaA, Darmstadt, Germany) instead 8.0

μm. After treatment with monoclonal anti-CLDN12 antibody and washing of the formed into the upper chambers monolayers from A549, LS180 or HeLa cells, Jurkat cells (1.0×10^6 cells/well) suspended in 150 μL FBS-free medium were added and co-cultured for 24 h at 37ºC in a humidified CO_2 incubator. A549, LS180 and HeLa cells without anti-CLDN12 antibody treatment served as control groups. Lower chambers were supplemented again with complete medium containing 10% FBS. Migrated Jurkat cells were photographed, counted and collected for flow cytometric analysis.

2.5. Cell proliferation assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide) assay was used to examine the cell proliferation of studied cell lines (A549, LS180 and HeLa) after treatment with anti-claudin-12 antibodies. Briefly, the cells were seeded (1.0×10^5 cells/well) on 96-well plates (TPP, Trasadingen, Switzerland) and cultured in complete DMEM for 24 h at 37°C, 5% CO_2 and high humidity. Then were added anti-CLDN12 antibody (1 μg/mL) and incubated for a further 24 h. During the last 3 h of the incubation period (48 h), 10 μL MTT solution (5 mg/mL) was added to each well. Subsequently, the MTT containing medium was removed and 100 μL DMSO was added into each well. The cells were incubated for 15 minutes at room temperature on a shaker in order to dissolve the accumulated formazan crystals. Absorbance was measured at 540 nm using Synergy-2 reader (BioTek, Winooski, VT, USA). The results are expressed as a percentage of the untreated control (mean ± SE of triplicates, ** $P<0.01$ vs. the control group).

2.6. Flow cytometric (FACS) analysis

Control Jurkat cells as well as migrated Jurkat cells (after co-culture with the studied adherent cells) were collected and washed with FACS buffer (DPBS containing 5% fetal calf serum and 0.05% NaN_3). Then, the cells were stained with FITC-conjugated anti-human CD62L (L-selectin), APC-conjugated anti-human CD11a/CD18 (LFA-1) and PE-conjugated anti-human CD44 antibodies for 30 min at 4°C in dark. After that, the cells were washed twice with FACS buffer and subjected to flow cytometry using a Cytomics FC500 instrument (Beckman Coulter, USA). The levels of the studied biomarkers were compared between the control and migrated Jurkat cells.

2.7. Cell migration assay with competitive inhibition

To evaluate the role of claudin-12 protein for migration of Jurkat cells through the monolayers from adherent cells (A549, LS180 or HeLa), we performed again the same migration assay described before for migration of Jurkat cells, but without treatment of monolayers with anti-CLDN12 antibody. Here, in the upper chambers of the Transwell® (3.0 μm), to the co-culture from Jurkat and adherent cells were added synthetic peptides (p1, p2, p3, p4, p5, p6) representing parts from the extracellular domains of the protein claudin-12. Each peptide was tested in triplicates in a final concentration of 5 μg/mL. Co-culture transwells without peptides were used as controls.

2.8. Statistical analysis

All experiments were conducted in triplicates and all data are presented as the mean values ± SE. To compare non-parametric data for statistical significance, the Mann-Whitney *U*-test or Kruskal-Wallis test were applied using StatView program (SAS Institute). Values of $p<0.05$ were considered significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). All results were compared to those from the controls.

3. Results

3.1. Expression of claudin-12

In order to select a suitable *in vitro* model for our study, we have tested several human cell lines (Caco-2, LS180, HT-29, A549, HeLa, SK-Hep-1) for expression of claudin-12. Taking in account that this protein is expressing predominantly in the tight junctions of epithelia, we chose two claudin-12 expressing cell lines (Figure 1a-d) with different origin – A549 (alveolar epithelial cells derived from lung tissue) and LS180 (epithelial cells derived from the colon), and one non-expressing claudin-12 cell line (HeLa) as a negative control (Figure 1e-f). In addition to the selected cell lines, Caco-2 and HT-29 cells also expressed claudin-12 although the signal was weaker, but not the cell line SK-Hep-1, which was claudin-12 negative (data not shown). We decided to use the cell line LS180 instead Caco-2 or HT-29 cells because in addition, the LS180 cells produce mucin.

3.2. Anti-claudin-12 antibodies suppress the migration and proliferation of claudin-12 expressing cancer cells

To evaluate whether anti-claudin-12 antibodies can influence the migration of claudin-12 expressing cells (A549 and LS180) were used transwell assays. Results showed a significant reduction in the number of migrated A549 and LS180 cells after treatment with anti-claudin-12 antibodies compared with non-treated cells (Figure 2). The mean number of migrated A549 cells with and without antibody treatment was respectively, 23±4 and 193±17 cells (Figure 2a-c). For the cell line LS180 these values were 6±3 and 283±8 cells (Figure 2d-f). Our data revealed no significant difference in the migration ability between the anti-claudin-12 antibody treated (133±10) and non-treated (165±7) HeLa cells (Figure 2d-f). These analyses suggested that claudin-12 of the tight junctions is involved in the migration of cells expressing this protein.

In addition, to examine the effect of anti-claudin-12 antibodies on the cell viability (respectively cell proliferation), we performed MTT assays using the same strategy of antibody treatment. As shown in Figure 3, antibody treatment resulted in a significant reduction in proliferation of claudin-12 expressing A549 and LS180 cells compared to the controls, and a slight increase in cell proliferation of HeLa cells. After 48 h, the cell viability for A549 cells decreased to 35.47% and for LS180 cells – to 26.42% (Figure 3). MTT assays demonstrated that anti-claudin-12 antibodies suppress the cell proliferation as well.

3.3. Claudin-12 is involved in the migration of Jurkat cells through the tight junctions

To determine the involvement of claudin-12 in the process of metastasis, we next performed similar to the previous transwell assays, but using membranes with 3.0 μm pore size and co-culture of adhesive cells (A549, LS180 or HeLa) and Jurkat cells (Figure 4). The results revealed that Jurkat cells were able to migrate mostly through the tight junctions of the claudin-12 expressing cells (A549 and LS180) that were not pre-treated with anti-claudin-12 antibodies (Figure 4). The mean number of migrated Jurkat cells through A549 and LS180 cells was respectively 310±30 (Figure 4c) and 254±21 (Figure 4f). We found no differences in the co-culture system HeLa-Jurkat, where migrated Jurkat cells were not detected (Figure 4g-i). These results confirmed that claudin-12 is involved in the cell migration during the metastasis.

3.4. Migrating Jurkat cells express lymphocyte function-associated antigen-1 (LFA-1 integrin) and L-selectin (CD62L)

We also performed flow cytometric analysis of the Jurkat cells for expression of LFA-1 and CD62L before co-culture and after migration assay. As can be seen in Figure 5, Jurkat cells constitutively express the integrin LFA-1 (before and after migration), while the expression of L-selectin (CD62L) was induced after co-culture with A549 or LS180 cells. These results

suggest that for migration through the tight junctions Jurkat cells probably use LFA-1 or CD62L, or both molecules. Jurkat cells did not expressed CD44 glycoprotein (data not shown).

3.5. Claudin-12 peptides can block the migration of Jurkat cells through the tight junctions

To examine the potential of claudin-12 peptides derived from the extracellular domains of the claudin-12 protein to reduce the migration ability of Jurkat cells, we repeated the transwell experiments with co-culture systems, but instead anti-claudin-12 antibodies we used synthetic claudin-12 peptides (Figure 6). Our results indicated that two of the used peptides (p5 and p6) completely inhibited the migration of Jurkat cells through the A549 (Figure 6a) and LS180 cells (Figure 6b). Although the other claudin-12 peptides (p1, p2, p3 and p4) slightly reduced the migration of Jurkat cells compared to the control (black bars, without peptides) this inhibition was not significant (Figure 6). Results suggest that Jurkat cells migrate through the tight junctions by binding to claudin-12 and more specifically to the first extracellular domain since the peptides p5 and p6 are parts of this domain. The peptide p1 (red bars) that represented the second extracellular domain did not inhibit the migration of the Jurkat cells.

4. Discussion

Claudin proteins are integral components of the tight junctions maintaining cell polarity, paracellular permeability, cell proliferation, transformation and metastasis [21]. It has been shown that during metastasis, expression of certain claudins could increase or decrease in a tissue specific fashion [17,22-30]. The expression and functions of these proteins are regulated by different mechanisms including disintegration of the cell-cell contacts, cytokines, hormones or other signaling pathways. In our study, we focus on the non-canonical claudin-12 (lacking a PDZ binding domain) and its significance for the cell migration during metastasis. We found that epithelial-derived cancer cell lines from the colon (LS180, Caco-2, HT-29) and from the lung (A549) express claudin-12, while the liver endothelial SK-Hep-1 and cervix epithelial HeLa cancer cells were claudin-12 negative. This is in agreement with results of other studies that showed expression of claudin-12 in the epithelia and endothelia of the gastrointestinal tract [1] or bronchial epithelial cells [17]. Lack of expression of claudin proteins in HeLa cells (claudin-null cell line) was also previously reported [31,32]. This comparative analysis showed that the cell lines (A549, LS180, HeLa) used as models in this study were correctly selected.

Pretreatment of claudin-12 expressing cells (A549, LS180) with anti-claudin-12 antibodies significantly reduced both migration and proliferation of these cells. Even more, such pretreatment blocked the migration of Jurkat cells through the tight junctions of the formed epithelial cell monolayers during the co-culture. These observations are novel and demonstrate the potential capabilities of anti-claudin-12 antibodies to inhibit the metastatic process in claudin-12 expressing tissues. For example, it has recently been reported that claudin-12 is involved in the epithelial-mesenchymal transition and migration of human bronchial epithelial BEAS-2B cells [17]. In addition, these researchers found upregulated expression of claudin-12 in lung squamous cell carcinoma (SqCC) tissues suggesting the *CLDN12* gene as a protooncogene in SqCC [17]. Similarly, Tian *et al*. concluded that cytoplasmic overexpression of claudin-12 promote proliferation and migration ability of osteosarcoma cells [14]. Taking in account the tissue-specific expression of the claudin proteins, Jiang *et al*. [21] noted that they could be used as prognostic and diagnostic biomarkers, e.g. claudin-1 for colon cancers, claudin-3 for ovarian cancers, claudin-10 for hepatocellular carcinomas etc. Thus, claudin-12 may be used as a biomarker for tumor progression and metastasis in the gastrointestine, lung SqCC and osteosarcomas.

On the other hand, claudin proteins were identified as an attractive target for antitumor therapy [21]. Therefore, blocking expression of claudin-12 or anti-claudin-12 antibodies should

have a beneficial effect inhibiting the tumor progression and metastasis in tissues expressing claudin-12. Our results showed that short peptides from the first extracellular domain of the protein claudin-12 are also able to reduce the migration of Jurkat cells through the tight junctions suggesting that competitive inhibition mechanisms could be useful in the therapeutic approach applied to cancer metastasis.

We found that the migrating Jurkat cells express both the integrin LFA-1 and L-selectin (CD62L). It is well known that LFA-1 and L-selectin play a major role for the adhesion of circulating leukocytes to the endothelial cells regulating T cell activation and migration through the endothelium [33-35]. Based on our observation that anti-claudin-12 antibodies reduced the number of migrated Jurkat cells, we hypothesized that claudin-12 is a ligand for LFA-1 and/or L-selectin. However, additional studies are needed to prove whether claudin-12 binds to LFA-1 and L-selectin leading to disruption of tight junctions and migration of T cells.

5. Conclusions

We have shown that A549 and LS180 cells express claudin-12 and anti-claudin-12 antibodies inhibit cell migration and proliferation. Furthermore, synthetic peptides representing the first extracellular domain of claudin-12 reduce the migration of Jurkat cells. Migrated Jurkat cells express LFA-1 and L-selectin. Our findings identify the essential role of claudin-12 for migration of cancer cells through the claudin-12 expressing tissues in the process of metastasis.

Author Contributions: Conceptualization, B.D.; methodology, B.D. and T.B.; formal analysis, D.K., D.M., and T.B.; investigation, D.K., D.M., and T.B.; supervision, T.B. and B.D.; writing—original draft preparation, B.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the operational program "Science and education for smart growth" 2014-2020, grant number BG05M2OP001-1.002-0005-C01, Personalized Innovative Medicine Competence Center (PERIMED).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Krause, G.; Winkler, L.; Mueller, S.L.; Haseloff, R.F.; Piontek, J.; Blasig, I.E. Structure and function of claudins. *Biochimica et biophysica acta* **2008**, *1778*, 631-645, doi:10.1016/j.bbamem.2007.10.018.
2. Lal-Nag, M.; Morin, P.J. The claudins. *Genome biology* **2009**, *10*, 235, doi:10.1186/gb-2009-10-8-235.
3. Kwon, M.J. Emerging roles of claudins in human cancer. *International journal of molecular sciences* **2013**, *14*, 18148-18180, doi:10.3390/ijms140918148.
4. Niessen, C.M. Tight Junctions/Adherens Junctions: Basic Structure and Function. *Journal of Investigative Dermatology* **2007**, *127*, 2525-2532, doi:10.1038/sj.jid.5700865.
5. Matter, K.; Balda, M.S. Signalling to and from tight junctions. *Nature Reviews Molecular Cell Biology* **2003**, *4*, 225-237, doi:10.1038/nrm1055.
6. Matter, K.; Aijaz, S.; Tsapara, A.; Balda, M.S. Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. *Current opinion in cell biology* **2005**, *17*, 453-458, doi:10.1016/j.ceb.2005.08.003.
7. Tsukita, S.; Furuse, M.; Itoh, M. Multifunctional strands in tight junctions. *Nature reviews. Molecular cell biology* **2001**, *2*, 285-293, doi:10.1038/35067088.
8. Fujita, H.; Chiba, H.; Yokozaki, H.; Sakai, N.; Sugimoto, K.; Wada, T.; Kojima, T.; Yamashita, T.; Sawada, N. Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **2006**, *54*, 933-944, doi:10.1369/jhc.6A6944.2006.
9. Günzel, D.; Yu, A.S.L. Claudins and the Modulation of Tight Junction Permeability. *Physiological reviews* **2013**, *93*, 525-569, doi:10.1152/physrev.00019.2012.
10. Kitajiri, S.I.; Furuse, M.; Morita, K.; Saishin-Kiuchi, Y.; Kido, H.; Ito, J.; Tsukita, S. Expression patterns of claudins, tight junction adhesion molecules, in the inner ear. *Hearing research* **2004**, *187*, 25-34.
11. Castro Dias, M.; Coisne, C.; Baden, P.; Enzmann, G.; Garrett, L.; Becker, L.; Holter, S.M.; Hrabe de Angelis, M.; Deutsch, U.; Engelhardt, B. Claudin-12 is not required for blood-brain barrier tight junction function. *Fluids and barriers of the CNS* **2019**, *16*, 30, doi:10.1186/s12987-019-0150-9.
12. Fujita, H.; Sugimoto, K.; Inatomi, S.; Maeda, T.; Osanai, M.; Uchiyama, Y.; Yamamoto, Y.; Wada, T.; Kojima, T.; Yokozaki, H., et al. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca2+ absorption between enterocytes. *Molecular biology of the cell* **2008**, *19*, 1912-1921, doi:10.1091/mbc.E07-09-0973.
13. Osanai, M.; Takasawa, A.; Murata, M.; Sawada, N. Claudins in cancer: bench to bedside. *Pflugers Archiv : European journal of physiology* **2017**, *469*, 55-67, doi:10.1007/s00424-016-1877-7.
14. Tian, X.; He, Y.; Han, Z.; Su, H.; Chu, C. The Cytoplasmic Expression Of CLDN12 Predicts An Unfavorable Prognosis And Promotes Proliferation And Migration Of Osteosarcoma. *Cancer management and research* **2019**, *11*, 9339-9351, doi:10.2147/CMAR.S229441.
15. Morin, P.J. Claudin proteins in human cancer: promising new targets for diagnosis and therapy. *Cancer research* **2005**, *65*, 9603-9606, doi:10.1158/0008-5472.CAN-05-2782.
16. Singh, A.B.; Sharma, A.; Dhawan, P. Claudin family of proteins and cancer: an overview. *Journal of oncology* **2010**, *2010*, 541957, doi:10.1155/2010/541957.
17. Sun, L.; Feng, L.; Cui, J. Increased expression of claudin-12 promotes the metastatic phenotype of human bronchial epithelial cells and is associated with poor prognosis in

lung squamous cell carcinoma. *Experimental and therapeutic medicine* **2019**, *17*, 165-174, doi:10.3892/etm.2018.6964.
18. Fares, J.; Fares, M.Y.; Khachfe, H.H.; Salhab, H.A.; Fares, Y. Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduction and Targeted Therapy* **2020**, *5*, doi:10.1038/s41392-020-0134-x.
19. Massagué, J.; Obenauf, A.C. Metastatic colonization by circulating tumour cells. *Nature* **2016**, *529*, 298-306, doi:10.1038/nature17038.
20. Ouban, A.; Ahmed, A.A. Claudins in human cancer: a review. *Histology and histopathology* **2010**, *25*, 83-90, doi:10.14670/HH-25.83.
21. Jiang, W.G.; Sanders, A.J.; Katoh, M.; Ungefroren, H.; Gieseler, F.; Prince, M.; Thompson, S.K.; Zollo, M.; Spano, D.; Dhawan, P., et al. Tissue invasion and metastasis: Molecular, biological and clinical perspectives. *Seminars in Cancer Biology* **2015**, *35*, S244-S275, doi:10.1016/j.semcancer.2015.03.008.
22. Usami, Y.; Chiba, H.; Nakayama, F.; Ueda, J.; Matsuda, Y.; Sawada, N.; Komori, T.; Ito, A.; Yokozaki, H. Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. *Human pathology* **2006**, *37*, 569-577, doi:10.1016/j.humpath.2005.12.018.
23. Rangel, L.B.; Agarwal, R.; D'Souza, T.; Pizer, E.S.; Alo, P.L.; Lancaster, W.D.; Gregoire, L.; Schwartz, D.R.; Cho, K.R.; Morin, P.J. Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. *Clinical cancer research : an official journal of the American Association for Cancer Research* **2003**, *9*, 2567-2575.
24. Lee, J.W.; Lee, S.J.; Seo, J.; Song, S.Y.; Ahn, G.; Park, C.S.; Lee, J.H.; Kim, B.G.; Bae, D.S. Increased expressions of claudin-1 and claudin-7 during the progression of cervical neoplasia. *Gynecologic oncology* **2005**, *97*, 53-59, doi:10.1016/j.ygyno.2004.11.058.
25. Agarwal, R.; D'Souza, T.; Morin, P.J. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer research* **2005**, *65*, 7378-7385, doi:10.1158/0008-5472.CAN-05-1036.
26. Sun, L.; Feng, L.; Cui, J. Increased expression of claudin-17 promotes a malignant phenotype in hepatocyte via Tyk2/Stat3 signaling and is associated with poor prognosis in patients with hepatocellular carcinoma. *Diagnostic pathology* **2018**, *13*, 72, doi:10.1186/s13000-018-0749-1.
27. Yamada, G.; Murata, M.; Takasawa, A.; Nojima, M.; Mori, Y.; Sawada, N.; Takahashi, H. Increased expressions of claudin 4 and 7 in atypical adenomatous hyperplasia and adenocarcinoma of the lung. *Medical molecular morphology* **2016**, *49*, 163-169, doi:10.1007/s00795-016-0135-6.
28. Che, J.; Yang, Y.; Xiao, J.; Zhao, P.; Yan, B.; Dong, S.; Cao, B. Decreased expression of claudin-3 is associated with a poor prognosis and EMT in completely resected squamous cell lung carcinoma. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **2015**, *36*, 6559-6568, doi:10.1007/s13277-015-3350-1.
29. Miyamoto, K.; Kusumi, T.; Sato, F.; Kawasaki, H.; Shibata, S.; Ohashi, M.; Hakamada, K.; Sasaki, M.; Kijima, H. Decreased expression of claudin-1 is correlated with recurrence status in esophageal squamous cell carcinoma. *Biomed Res* **2008**, *29*, 71-76.
30. Morohashi, S.; Kusumi, T.; Sato, F.; Odagiri, H.; Chiba, H.; Yoshihara, S.; Hakamada, K.; Sasaki, M.; Kijima, H. Decreased expression of claudin-1 correlates with recurrence status in breast cancer. *International journal of molecular medicine* **2007**, *20*, 139-143.

31. Daugherty, B.L.; Ward, C.; Smith, T.; Ritzenthaler, J.D.; Koval, M. Regulation of heterotypic claudin compatibility. *The Journal of biological chemistry* **2007**, *282*, 30005-30013, doi:10.1074/jbc.M703547200.
32. Cunniffe, C.; Brankin, B.; Lambkin, H.; Ryan, F. The role of claudin-1 and claudin-7 in cervical tumorigenesis. *Anticancer research* **2014**, *34*, 2851-2857.
33. Sigal, A.; Bleijs, D.A.; Grabovsky, V.; van Vliet, S.J.; Dwir, O.; Figdor, C.G.; van Kooyk, Y.; Alon, R. The LFA-1 Integrin Supports Rolling Adhesions on ICAM-1 Under Physiological Shear Flow in a Permissive Cellular Environment. *The Journal of Immunology* **2000**, *165*, 442-452, doi:10.4049/jimmunol.165.1.442.
34. Walling, B.L.; Kim, M. LFA-1 in T Cell Migration and Differentiation. *Frontiers in Immunology* **2018**, *9*, doi:10.3389/fimmu.2018.00952.
35. Grailer, J.J.; Kodera, M.; Steeber, D.A. L-selectin: Role in regulating homeostasis and cutaneous inflammation. *Journal of dermatological science* **2009**, *56*, 141-147, doi:10.1016/j.jdermsci.2009.10.001.

Figure legends:

Figure 1. Expression of claudin-12 in A549 (**a,b**), LS180 (**c,d**) and HeLa (**e,f**) cells, cultured for 48 h (**a, c, e** – light microscopy; **b, d, f** – fluorescent microscopy). Fixed cells were stained with purified monoclonal anti-CLDN12 antibody and FITC-conjugated secondary anti-mouse IgG.

Figure 2. Impact of claudin-12 on the migration abilities of cancer cells expressing claudin-12. A549, LS180 and HeLa cells were treated with anti-CLDN12 antibody, and Transwell chamber method was used to evaluate the migration of the treated and non-treated cells. Cells were stained with 0.5% crystal violet (**a, b, d, e, g, h**). Results from the comparative analysis (**c, f, i**) are presented as mean ± standard error of the mean. *** indicates $P<0.001$.

Figure 3. Impact of claudin-12 on the proliferation of cancer cells expressing claudin-12. A549, LS180 and HeLa cells were treated with anti-CLDN12 antibody, and the MTT assay was used to examine the cell proliferation of the treated and non-treated cells. Results are presented as a percent of viable cells compared to the control (non-treated cells) calculated from triplicates. ** indicates $P<0.01$.

Figure 4. Impact of claudin-12 on the migration abilities of Jurkat cells. Monolayers from A549, LS180 and HeLa cells were treated with anti-CLDN12 antibody and co-cultured with Jurkat cells. The Transwell chamber method was used to evaluate the migration of Jurkat cells through the monolayers. Images were taken from the lower chambers where should be the migrated cells (**a, b, d, e, g, h**). Results from the comparative analysis (**c, f, i**) are presented as mean ± standard error of the mean. *** indicates $P<0.001$.

Figure 5. Flow cytometric analysis of migrated Jurkat cells (shaded histograms) and control Jurkat cell (open histograms) for expression of LFA-1 and L-selectin. Cells were stained with anti-human CD11a/CD18 APC-conjugated (for LFA-1) and anti-human CD62L FITC-conjugated (for L-selectin) antibodies. The data are from one representative experiment.

Figure 6. Migration capacity of Jurkat cells after incubation with synthetic claudin-12 peptides. Monolayers from A549, LS180 and HeLa cells were co-cultured for 24 h with Jurkat cells in presence of 5 μg/mL synthetic claudin-12 peptides. Six synthetic claudin-12 peptides (p1-p6) representing the extracellular domains of the claudin-12 protein were tested as competitors of the claudin-12 in the tight junctions. The Transwell chamber method was used to evaluate the migration of Jurkat cells through the monolayers. Results are presented as mean ± standard error of the mean. *** indicates $P<0.001$.

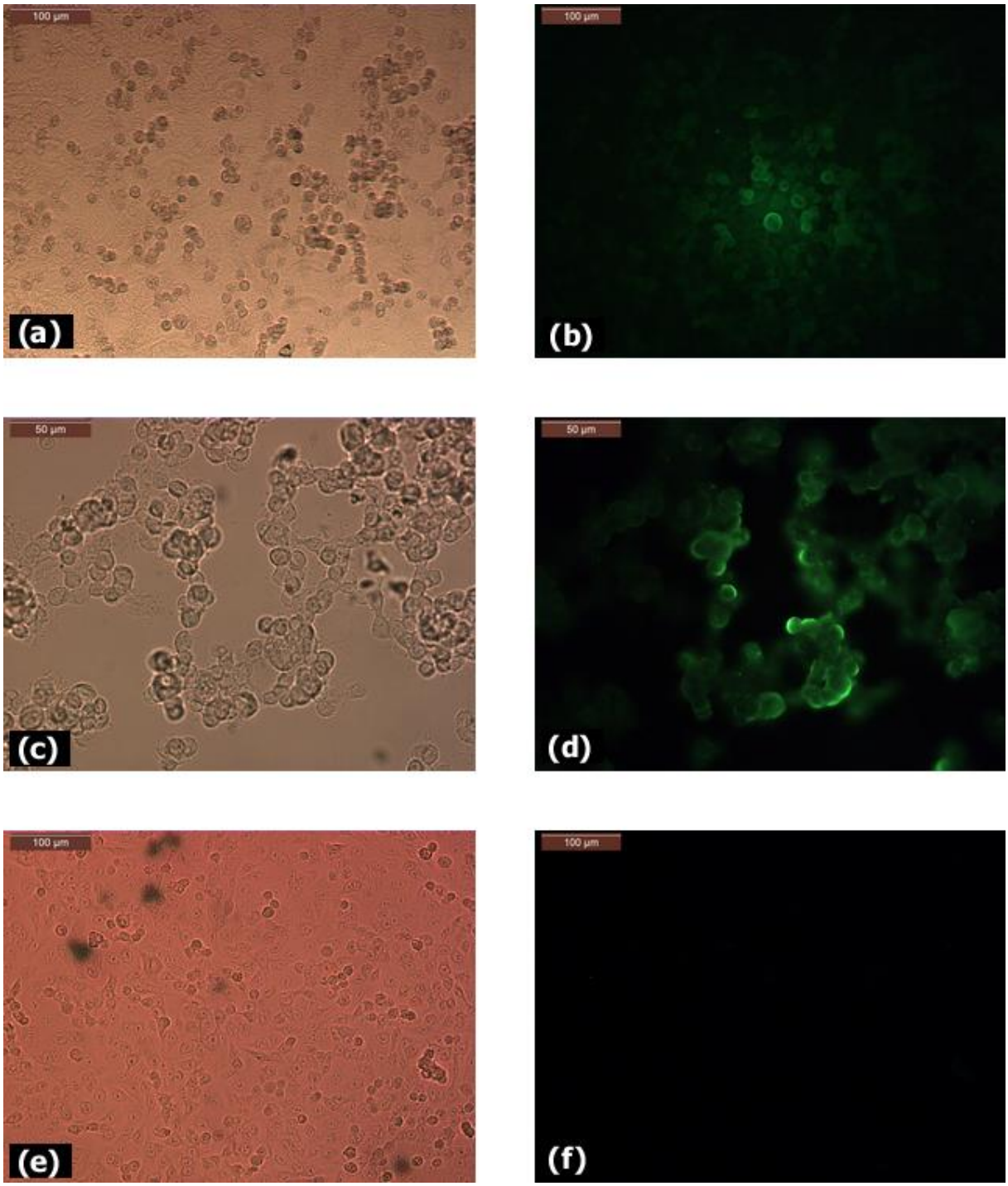

Figure 1. Expression of claudin-12 in A549 (**a,b**), LS180 (**c,d**) and HeLa (**e,f**) cells, cultured for 48 h (**a, c, e** – light microscopy; **b, d, f** – fluorescent microscopy). Fixed cells were stained with purified monoclonal anti-CLDN12 antibody and FITC-conjugated secondary anti-mouse IgG.

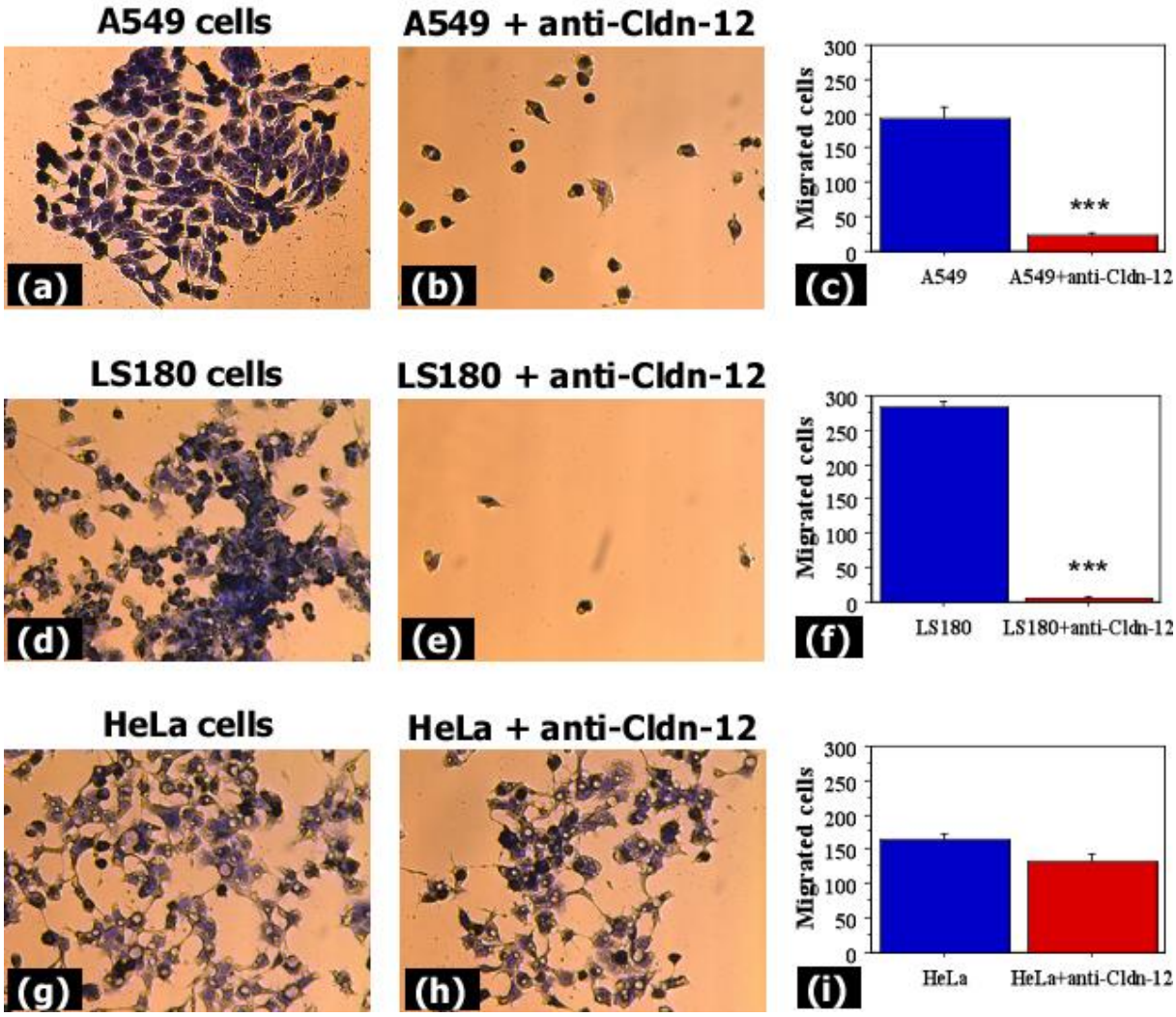

Figure 2. Impact of claudin-12 on the migration abilities of cancer cells expressing claudin-12. A549, LS180 and HeLa cells were treated with anti-CLDN12 antibody, and Transwell chamber method was used to evaluate the migration of the treated and non-treated cells. Cells were stained with 0.5% crystal violet (**a, b, d, e, g, h**). Results from the comparative analysis (**c, f, i**) are presented as mean ± standard error of the mean. *** indicates $P<0.001$.

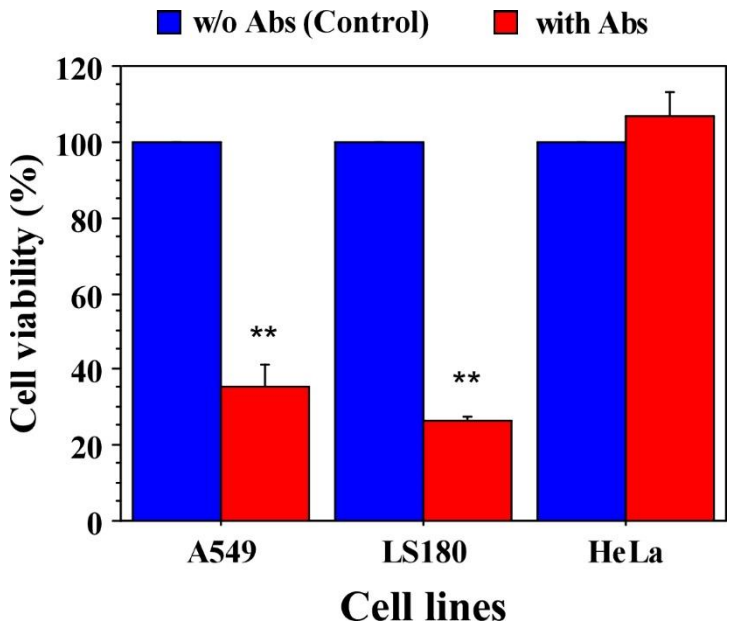

Figure 3. Impact of claudin-12 on the proliferation of cancer cells expressing claudin-12. A549, LS180 and HeLa cells were treated with anti-CLDN12 antibody, and the MTT assay was used to examine the cell proliferation of the treated and non-treated cells. Results are presented as a percent of viable cells compared to the control (non-treated cells) calculated from triplicates. ** indicates $P<0.01$.

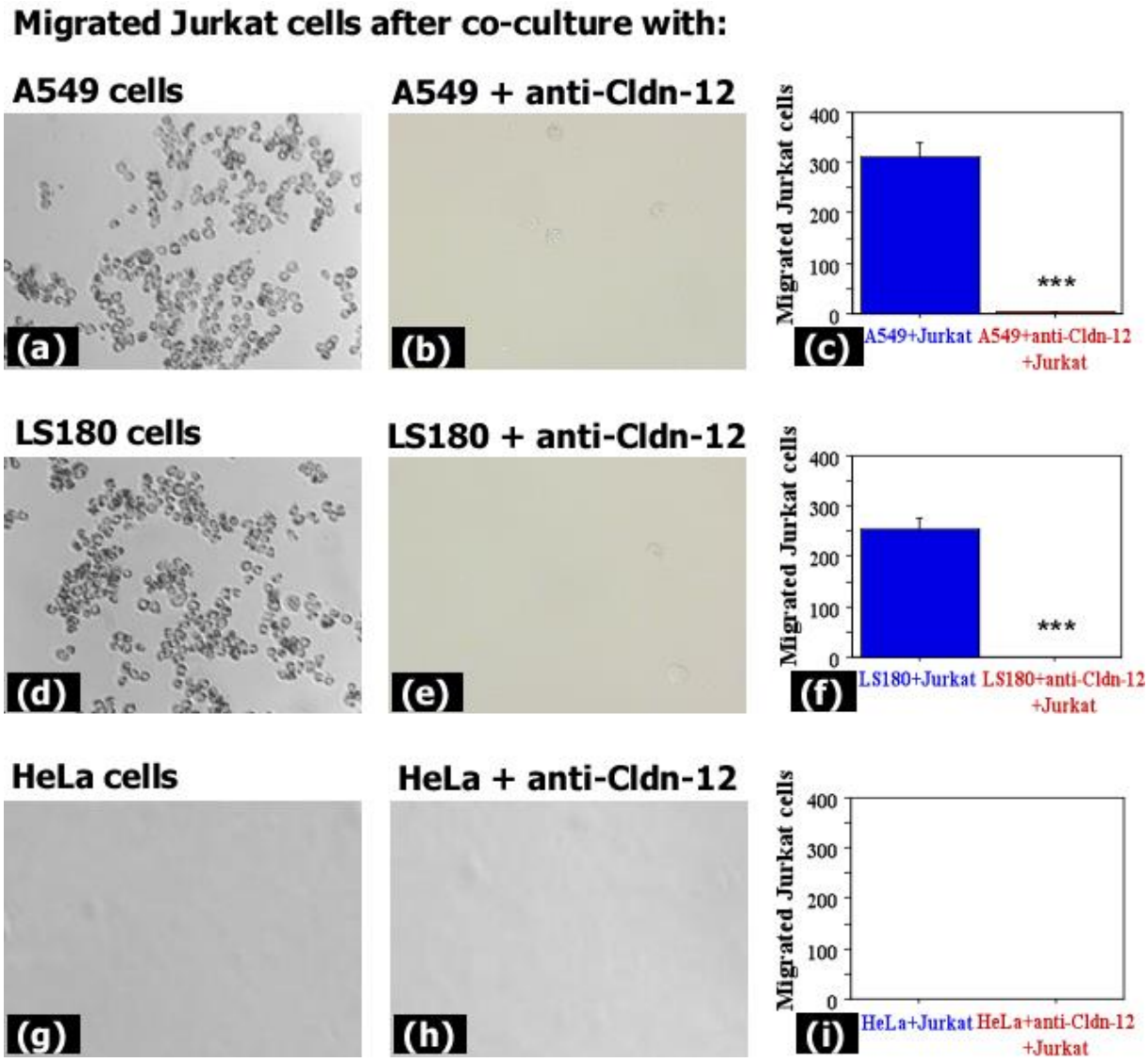

Figure 4. Impact of claudin-12 on the migration abilities of Jurkat cells. Monolayers from A549, LS180 and HeLa cells were treated with anti-CLDN12 antibody and co-cultured with Jurkat cells. The Transwell chamber method was used to evaluate the migration of Jurkat cells through the monolayers. Images were taken from the lower chambers where should be the migrated cells (**a, b, d, e, g, h**). Results from the comparative analysis (**c, f, i**) are presented as mean ± standard error of the mean. *** indicates $P<0.001$.

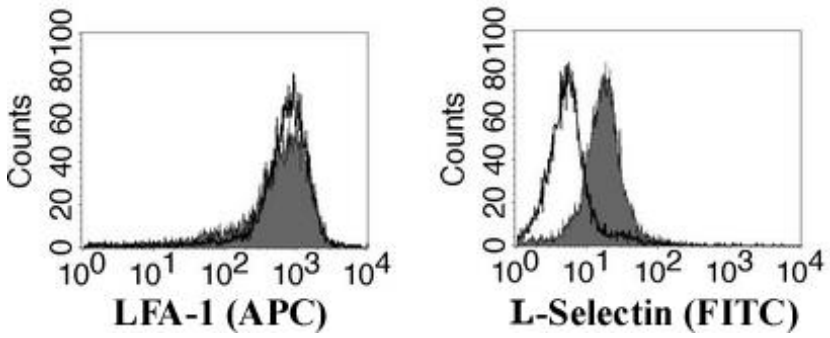

Figure 5. Flow cytometric analysis of migrated Jurkat cells (shaded histograms) and control Jurkat cell (open histograms) for expression of LFA-1 and L-selectin. Cells were stained with anti-human CD11a/CD18 APC-conjugated (for LFA-1) and anti-human CD62L FITC-conjugated (for L-selectin) antibodies. The data are from one representative experiment.

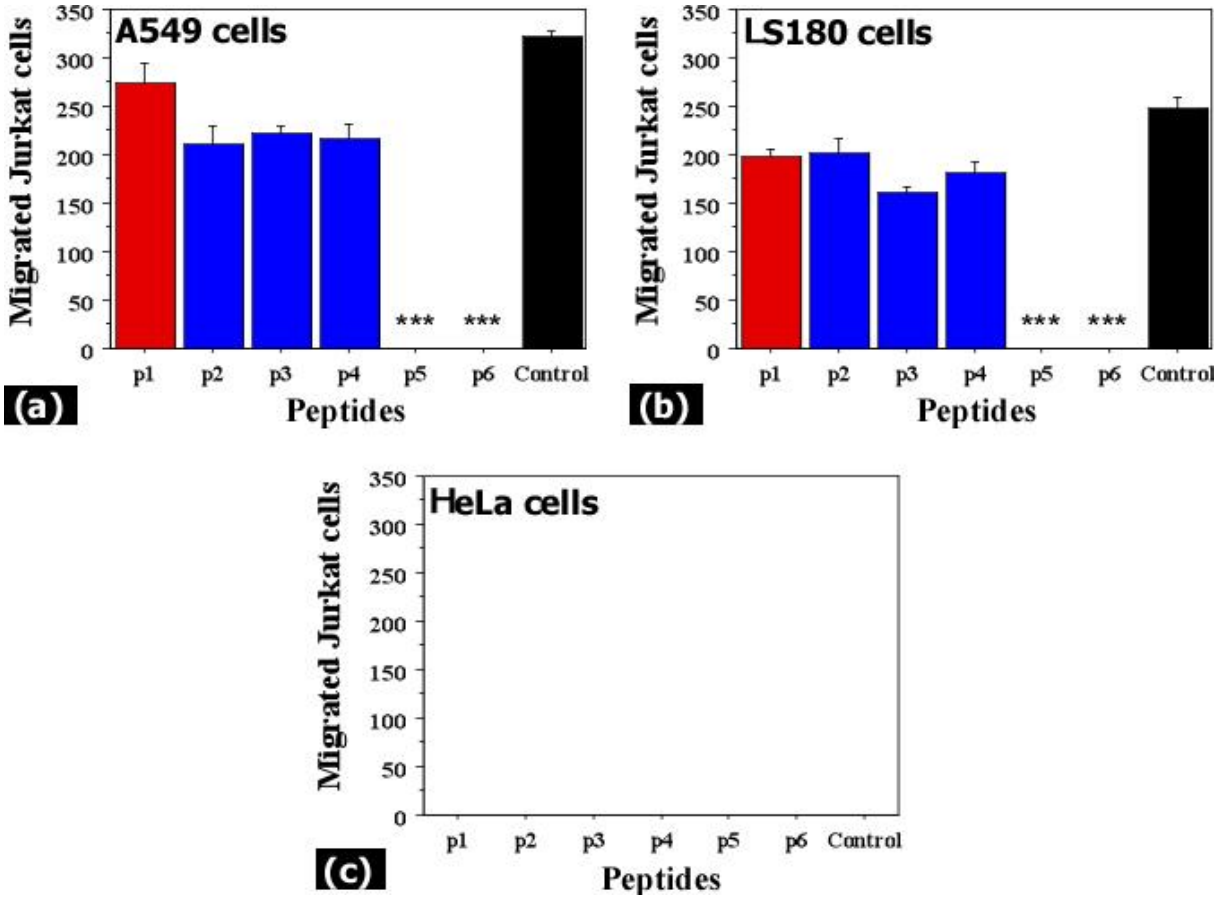

Figure 6. Migration capacity of Jurkat cells after incubation with synthetic claudin-12 peptides. Monolayers from A549, LS180 and HeLa cells were co-cultured for 24 h with Jurkat cells in presence of 5 μg/mL synthetic claudin-12 peptides. Six synthetic claudin-12 peptides (p1-p6) representing the extracellular domains of the claudin-12 protein were tested as competitors of the claudin-12 in the tight junctions. The Transwell chamber method was used to evaluate the migration of Jurkat cells through the monolayers. Results are presented as mean ± standard error of the mean. *** indicates $P<0.001$.