

Interleukin-8 promotes human ovarian cancer cell migration by epithelial–mesenchymal transition induction in vitro

J. Yin · F. Zeng · N. Wu · K. Kang ·
Z. Yang · H. Yang

Received: 15 June 2014 / Accepted: 1 October 2014 / Published online: 6 November 2014
© Federación de Sociedades Españolas de Oncología (FESEO) 2014

Abstract

Purpose It has been well established that high serum levels of interleukin-8 (IL-8) in ovarian cancer result in a poor clinical outcome. Thus, the aim of this study was investigating the role of IL-8 in ovarian cancer development.

Methods Two human ovarian cancer cell lines (SKOV3 and OVCAR3) were cocultured with IL-8 (100 ng/L) for 24 h, then cell migration was determined by transwell assay. Epithelial–mesenchymal transition (EMT)-associated proteins including E-cadherin and β -catenin, and phosphorylation status of β -catenin were investigated by Western blot analysis.

Results After treatment with IL-8 (100 ng/L) for 24 h, transwell assay result showed that the number of migrated ovarian cells increased significantly. Western blot analysis revealed that protein levels of E-cadherin were decreased, while that of β -catenin were elevated both in IL-8 pretreated SKOV3 and OVCAR3 cells. We further found that phosphorylation status of β -catenin were elevated either in cytoplasm or in nucleus of these two ovarian cancer cell lines after treatment with IL-8 for 24 h.

Conclusions Our data suggest that IL-8 induces EMT in ovarian cancer cells and implicates its potential role in enhancing ovarian cancer cell metastasis.

Keywords Interleukin-8 (IL-8) · Ovarian cancer · Metastasis · Epithelial–mesenchymal transition (EMT)

Introduction

Ovarian cancer is the most common cause of gynecologic disease-related death, with a 5-year survival rate about 30 %. About 70 % of patients with ovarian cancer are diagnosed at an advanced stage when the ovarian cancer has been metastasized, because the patients usually showed asymptomatic at early stage. Thus, inhibition of ovarian cancer cell metastasis after diagnosis is helpful for improving prognosis. It is known to all that multiple factors such as growth factors, cytokines and cell signaling proteins in tumor microenvironment play crucial roles in tumor cell metastasis [1]. Therefore, understanding their molecular mechanisms in metastasis processes is vital for ovarian cancer diagnosis and therapy.

Interleukin-8 (IL-8), a pro-inflammatory cytokine, was initially described as a neutrophil and monocyte chemo-attractant and over-expressed in multiple malignancies, leading to a poor clinical outcome [2]. Especially, elevated levels of IL-8 were found in high-invasive tumor cells [3]. Previous reports suggested up-regulation of IL-8 in cancer cells increased tumor cell motility [4–6], contributing to cancer cell migration and metastasis. In preliminary research, we verified that inhibition of endogenous IL-8 expression in ovarian cancer cells attenuated cell migration [7], indicating IL-8 may promote ovarian cancer cell metastasis. However, the role of IL-8 in ovarian cancer cell

Juan Yin and Fanqing Zeng have contributed equally to this work.

J. Yin · F. Zeng · N. Wu · K. Kang · H. Yang (✉)
Department of Gynecology, The Ninth People's Hospital of
Chongqing, 69 JiaLing Road, Chongqing 400700, People's
Republic of China
e-mail: leaftaney@hotmail.com

Z. Yang (✉)
Department of Obstetrics and Gynecology, The Second
Affiliated Hospital of Chongqing Medical University,
Chongqing 400010, People's Republic of China
e-mail: cqyangz@vip.163.com

metastasis has not been verified, and the cellular mechanism of IL-8 in ovarian cancer remains unknown.

To acquire the metastatic ability, tumor cells stimulated by cytokines experience phenotypic changes such as epithelial–mesenchymal transition (EMT). EMT is characterized as reduction of cell–cell adhesion and loss of normal cell polarity in epithelial cells [8]. Loss of E-cadherin and transfer of β -catenin from cytoplasm to nucleus in epithelial cells are characteristic changes of EMT [9, 10]. E-cadherin is a single span transmembrane protein that contains five repeats and cytoplasmic domain. The intracellular part of E-cadherin is linked to β -catenin, forming a cadherin–catenin complex to regulate normal cell shape and dynamics [11, 12]. Recent studies clarified that up-regulated expression of E-cadherin may reduce some tumor metastasis. Moreover, loss of E-cadherin resulted in accumulation of β -catenin in cytoplasm and stimulated WNT-signaling pathway, which is closely correlated with tumor metastasis [13–15]. As a core member of WNT-signaling pathway, β -catenin influences cell–cell adhesion by possible changes of its distribution or the level of self-phosphorylation in other tumors [16, 17].

Previous reports evidenced that some cytokines such as transforming growth factor β (TGF- β) and tumor necrosis factor (TNF- α) played crucial roles in EMT induction [18–20]. Our previous results verified that inhibition of endogenous IL-8 expression in ovarian cancer cells attenuated cell migration [7]. In the present study, we want to know whether IL-8 induces ovarian cancer by EMT induction. We first identify the effect of IL-8 on ovarian cancer metastasis, and then find its possible molecular mechanism of it.

Materials and methods

Chemicals and reagents

RPMI 1640, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine and collagenase were supplied by Gibco-BRL (Rockville, MD, USA). Recombinant human interleukin-8 (8–79) was obtained from SinoBio Biotechnology company (Shanghai, China). Anti-E-cadherin antibody, anti- β -actin antibody and anti-phospho- β -catenin (Ser675) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Lamin B (M-20) antibody were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment

Human ovarian carcinoma cell lines (SKOV3 and OVCAR3) were kindly donated by Ultrasound Institute of

Chongqing Medical University. They were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) in a humidified 5 % CO₂ incubator. When cells reached sub-confluence, they were then pretreated for 24 h with culture medium containing IL-8 (100 ng/mL) that was tested in the experiments.

Transwell assay

The lower chambers were filled with 0.6 mL of RPMI 1640 medium containing IL-8 (100 ng/mL). Cells were re-suspended in RPMI-1640 containing 1 % FBS, added (1×10^6 cells/100 μ L) to the upper chamber with the polycarbonate membrane (8- μ m pore, Corning Life Science). After 24 h incubation, at 37 °C under 5 % CO₂, cells that had not migrated were removed, whereas migrated cells were fixed in 4 % PFA for 10 min at room temperature and stained with hematoxylin. To count the number of migrated cells in lower chambers, an inverted microscope was used (Nikon TEU-2000). Experiments were performed in triplicate with a minimum of 10 grids (400 \times magnification) per filter counted. Data were assayed by two dependent investigators with Image Pro Plus 6.0.

Western blot analysis

Cells were washed twice with ice-cold PBS and scraped in 1 ml of the same buffer. After centrifugation at 10,000 $\times g$, the cell pellet was suspended in ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.2 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), vortexed for 2 min, and then centrifuged at 12,000 $\times g$ for 15 min. The supernatants were transferred to fresh tubes and assayed for total protein content. The nuclear and cytoplasmic cellular extraction was performed by Nuclear and Cytoplasmic Protein Extraction kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China). Protein samples (100 μ g) were electrophoretically fractionated with a discontinuous system consisting of 10 % polyacrylamide resolving gels and 8 % stacking gels, and then transferred to nitrocellulose membranes (Amersham, Buckinghamshire, England) at 100 v and 250 mA (current constant). The membranes were washed, blocked, and then incubated with primary antibodies against E-cadherin, β -actin, β -catenin, phospho- β -catenin (Ser675) (Cell Signaling, Beverly, MA), and Lamin B (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA). The bound horseradish peroxidase-conjugated secondary antibody was detected by an enhanced chemiluminescence procedure. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The results were expressed as mean \pm SEM of at least three independent experiments performed in triplicate. Treatment groups were compared using one-way analysis of variance (ANOVA), and the Newman–Keuls test was used to locate any significant differences identified in the ANOVA. $p < 0.05$ was accepted as significant.

Results

Effect of IL-8 on cell migration

To verify the role of IL-8 in human ovarian tumor cell migration, the transwell assay was employed. We treated ovarian cancer cells with this optimized IL-8 concentration (100 ng/mL) [21] for 24 h. Our results showed a significant increased number of SKOV3 and OVCAR3 cells in the lower chamber of transwell (Fig. 1a, b), when compared with IL-8-free groups (3.1-fold, 2.3-fold, respectively, $p < 0.05$).

Effect of IL-8 on E-cadherin protein expression

Loss of E-cadherin is one characteristic of EMT, leading to tumor metastasis; therefore, we investigated the influence of IL-8 on E-cadherin in ovarian cancer cells. After treated

with IL-8 for 24 h, the protein level of E-cadherin reduced by fourfold in SKOV3 cells (Fig. 2a) and sixfold in OVCAR3 cells (Fig. 2b), when compared with control groups.

Effect of IL-8 on β -catenin protein expression

Because E-cadherin combined with β -catenin forms a cadherin/catenin complex to keep a physical cell–cell contact, we tested the protein expression of β -catenin in IL-8 co-cultured ovarian cancer cells. As shown in Fig. 3, compared with control groups, protein levels of β -catenin were increased both in SKOV3 (Fig. 3a) and OVCAR3 (Fig. 3b) cells (3.5-fold, 1.5-fold, respectively, $p < 0.05$).

Effect of IL-8 on phosphorylation status of β -catenin

It has been well established that WNT-signaling pathway plays an important role in tumor metastasis by possible change of β -catenin phosphorylation [14–16]. We investigated the effect of IL-8 on β -catenin phosphorylation in ovarian cancer cells. As shown in Fig. 4, compared with control groups, phosphorylated β -catenin protein level increased both in cytoplasm and nucleus (twofold, 2.5-fold, respectively, $p < 0.05$), when SKOV3 cells were treated with IL-8 (100 ng/mL) pretreated for 24 h (Fig. 4a). IL-8 dramatically induced β -catenin phosphorylated in both cytoplasm and in nucleus of OVCAR3 cells (Fig. 4b) (2.4-fold, 2.9-fold, respectively, $p < 0.05$).

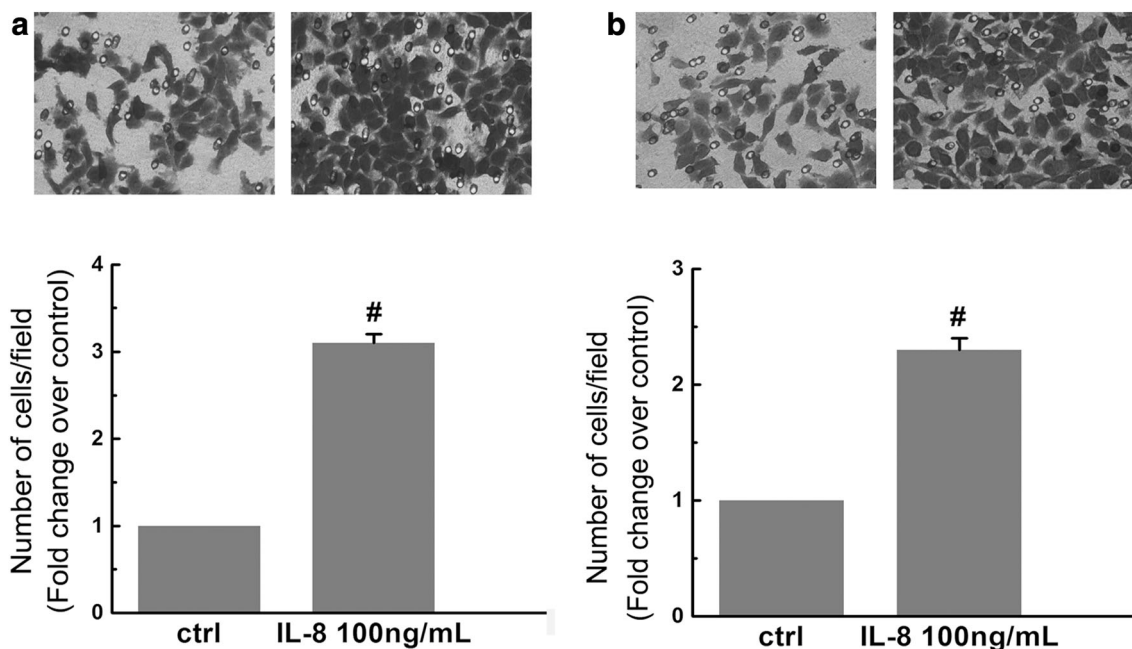


Fig. 1 Effect of IL-8 on ovarian cancer cell migration. SKOV3 (a) and OVCAR3 (b) cells were pretreated with IL-8 (100 ng/ml) for 24 h. Cell migration was determined by transwell assay. Compared

with IL-8-free groups, the number of SKOV3 and OVCAR3 cells in the lower chamber of transwell increased 3.1-fold and 2.3-fold ($p < 0.05$)

Fig. 2 Effect of IL-8 on protein level of E-cadherin. SKOV3 (a) and OVCAR3 (b) cells were pretreated with IL-8 (100 ng/mL) for 24 h. After the treatment, protein level of E-cadherin was determined by Western blot analysis. Compared with control groups, the protein level of E-cadherin reduced by fourfold in SKOV3 cells (a) and sixfold in OVCAR3 cells (b) ($p < 0.05$)

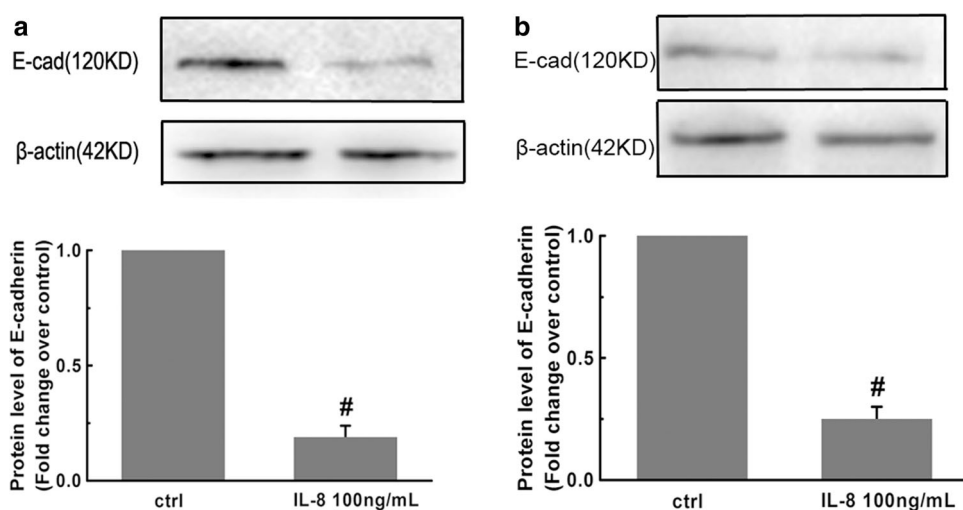
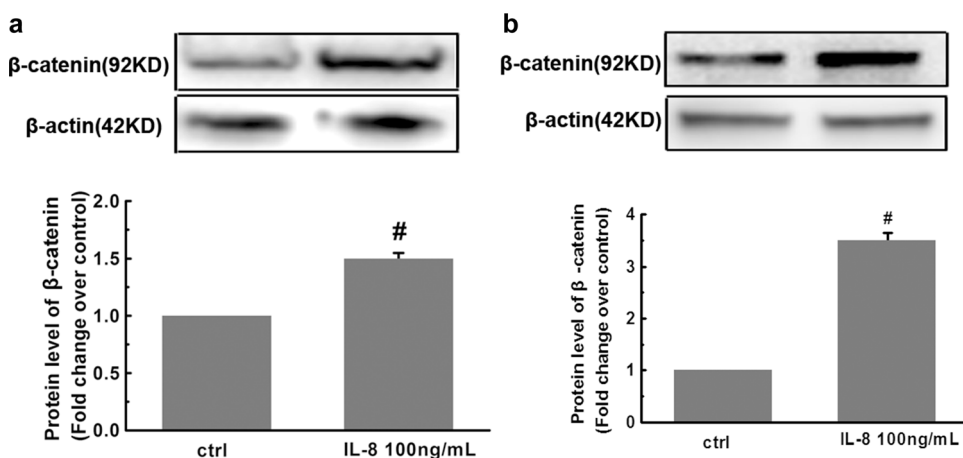


Fig. 3 Effect of IL-8 on protein level of β -catenin. SKOV3 (a) and OVCAR3 (b) cells were pretreated with IL-8 (100 ng/mL) for 24 h. Compared with control groups, protein levels of β -catenin were increased both in SKOV3 (a) and OVCAR3 (b) cells (3.5-fold, 1.5-fold, respectively, $p < 0.05$)



Discussion

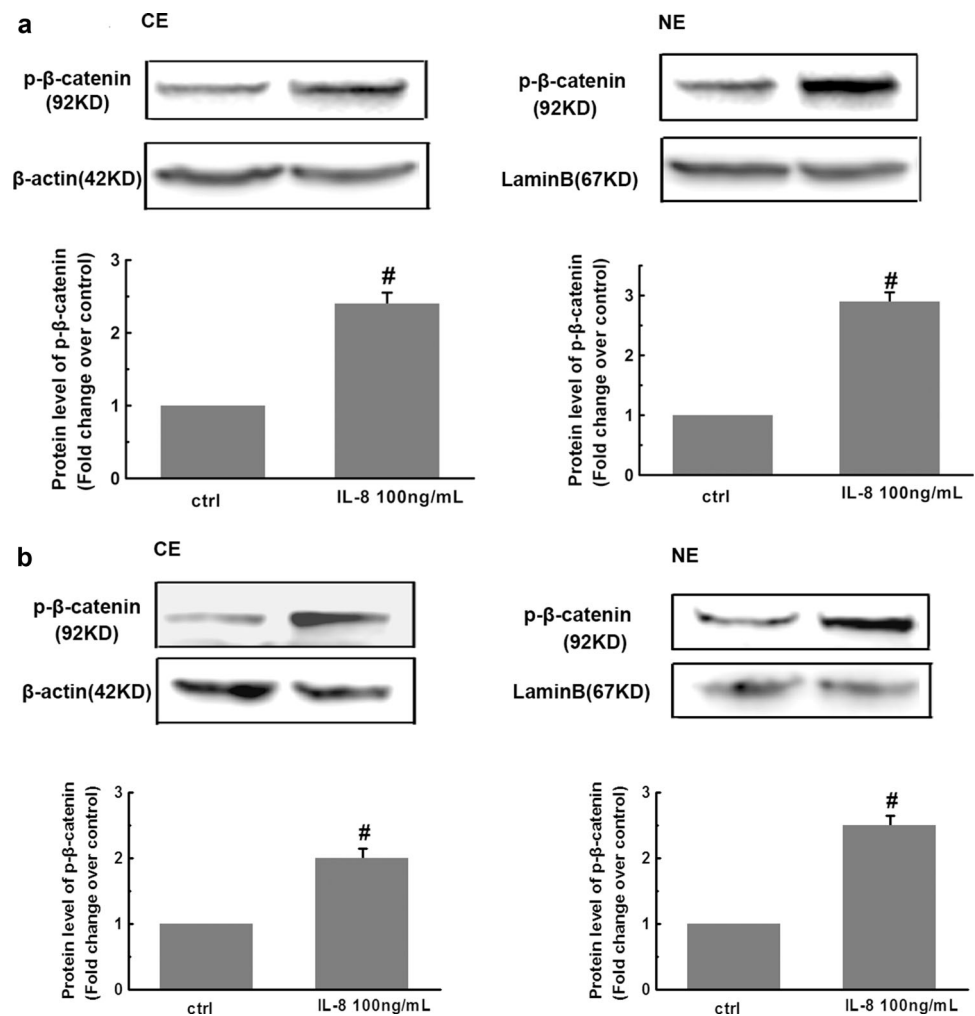
Metastasis is the leading cause of high mortality for patients with ovarian cancer. In the previous study, we have verified that inhibition of endogenous IL-8 expression attenuated cell migration in ovarian cancer [7], indicating IL-8 may promote ovarian cancer cells metastasis. In the present study, we try to further identify the effect of IL-8 on invasive ability in ovarian cancer cells and its possible molecular mechanism.

The significant increased numbers of SKOV3 and OVCAR3 cells in the lower chamber of transwell chambers in IL-8-treated group indicated that IL-8 promotes the invasive ability of ovarian cancer cells, which might be responsible for ovarian cancer metastasis. Previous studies showed that IL-8 promoted EMT in hepatocellular carcinoma and contributed to worse prognosis in patients [22]. Because the change of E-cadherin is the marker phenomenon in EMT, we investigated the changes of E-cadherin with IL-8 treatment. Our results showed that protein level

of E-cadherin was down-regulated by IL-8. We elucidated that IL-8 might induce EMT in the ovarian cancer metastasis. Because deletion of E-cadherin in other tumor cells damages cell adherens junctions and makes number of tumor cells detach from original foci to migrate and invade the surrounding tissues [23], the low level of E-cadherin regulated by IL-8 in ovarian cancer cells might be one of the possible mechanisms for ovarian cancer cell metastasis.

Since change of β -catenin is an important factor for EMT, we detected the change of β -catenin. Our result showed that both total protein level of β -catenin and phosphorylated β -catenin was elevated in ovarian cancer cells. E-cadherin defect may allow β -catenin to accumulate in the cytoplasm, which might lead to nucleus translocation, where it combines with T cell factor (Tcf)/Lef transcription factors to trigger transcription of metastasis-associated genes [24]. Thus, increased β -catenin level might account for the ovarian cancer metastasis induced by IL-8. Interestingly, some data conferred that the promoter sequence of IL-8 contains a unique consensus Tcf/Lef site,

Fig. 4 Effect of IL-8 on phosphorylation level of β -catenin in cells. CE means cytoplasmic extracts, NE means nuclear extracts. SKOV3 (a) and OVCAR3 (b) cells were exposed to IL-8 (100 ng/mL) for 24 h. After the treatment, cytoplasmic and nuclear fractions were analyzed for detection of phosphorylated β -catenin by Western blot analysis. Compared with control groups, phosphorylated β -catenin protein increased both in cytoplasm and nucleus (twofold, 2.5-fold, respectively, $p < 0.05$ (a) in SKOV3 cells and OVCAR3 cells (2.4-fold, 2.9-fold, respectively, $p < 0.05$) (b)



which is binding site for β -catenin to induce IL-8 in hepatoma cells, resulting in enhancing cell migration [25]. Thus, IL-8/ β -catenin pathway might be regulated by a positive feedback in tumor cell metastasis. In addition, IL-8 not only elevated β -catenin phosphorylation level of cytoplasm, but also of nuclear phosphorylation of ovarian cancer cell in our work. Status of β -catenin determines its fate and affects its function in holding the cytoskeletal networks [26]. Accumulated phosphorylated β -catenin could be transferred to the nucleus and trigger TCF/Lef transcription factors to involve in the IL-8/ β -catenin to participate in EMT. Hence, changes in β -catenin induced by IL-8 play a role in EMT, which might contribute ovarian cancer metastasis.

In summary, our data indicated that defect of E-cadherin/ β -catenin complex induced by IL-8 may play a critical role in EMT, impacting cell migration and contributing to ovarian cancer progression. As a secretory protein, the level of IL-8 might be a biomarker for ovarian cancer surveillance.

Acknowledgments Supported by the National Natural Science Foundation of China (Grant No. 81300467).

Conflict of interest There is no conflict of interest.

References

- Kim SW, Hayashi M, Lo JF, Fearn C, Xiang R, Lazennec G, et al. Tidl negatively regulates the migratory potential of cancer cells by inhibiting the production of interleukin-8. *Cancer Res.* 2005;65:8784–91.
- Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev.* 2001;12:375–91.
- Luca M, Huang S, Gershenwald JE, Singh RK, Reich R, Bar-Eli M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increase tumor growth and metastasis. *Am J Pathol.* 1997;151:1105–13.
- De Larco JE, Wuertz BR, Rosner KA, Erickson SA, Gamache DE, Manivel JC, et al. A potential role for interleukin-8 in the metastatic phenotype of breast carcinoma cells. *Am J Pathol.* 2001;158:639–46.
- Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, et al. CD44+/CD24– breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res.* 2006;8:R59.
- Singh RK, Gutman M, Radinsky R, Bucana CD, Fidler IJ. Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. *Cancer Res.* 1994;54:3242–7.
- Yin J, Yu C, Yang Z, He JL, Chen WJ, Liu HZ, et al. Tetramethylpyrazine inhibits migration of SKOV3 human ovarian carcinoma cells and decreases the

- expression of interleukin-8 via the ERK1/2, p38 and AP-1 signaling pathways. *Oncol Rep.* 2011;26:671–9.
8. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial–mesenchymal transition during tumor progression. *Curr Opin Cell Biol.* 2005;17:548–58.
 9. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial–mesenchymal transitions. *Nat Rev Mol Cell Biol.* 2006;7:131–42.
 10. Jeanes A, Gottardi CJ, Yap AS. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene.* 2008;27:6920–9.
 11. Eger A, Stockinger A, Park J, Langkopf E, Mikula M, Gotzmann J, et al. beta-Catenin and TGFbeta signalling cooperate to maintain a mesenchymal phenotype after FosER-induced epithelial to mesenchymal transition. *Oncogene.* 2004;23:2672–80.
 12. Nelson WJ. Regulation of cell–cell adhesion by the cadherin–catenin complex. *Biochem Soc Trans.* 2008;36:149–55.
 13. Gumbiner BM. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol.* 2006;6:622–34.
 14. Rakha EA, Abd E, Rehim D, Pinder SE, Lewis SA, Ellis IO. E-cadherin expression in invasive non-lobular carcinoma of the breast and its prognostic significance. *Histopathology.* 2005;46:685–93.
 15. Baranwal S, Alahari SK. Molecular mechanisms controlling E-cadherin expression in breast cancer. *Biochem Biophys Res Commun.* 2009;384:6–11.
 16. Cai J, Guan H, Fang L, Yang Y, Zhu X, Yuan J, et al. MicroRNA-374a activates Wnt/ β -catenin signaling to promote breast cancer metastasis. *J Clin Invest.* 2013;. doi:10.1172/JCI65871.
 17. Zappulli V, De Cecco S, Trez D, Caliarì D, Aresu L, Castagnaro M. Immunohistochemical expression of E-cadherin and β -catenin in feline mammary tumours. *J Comp Pathol.* 2012;147:161–70.
 18. Cianfrocca R, Tocci P, Spinella F, Di Castro V, Bagnato A, Rosanò L. The endothelin A receptor and epidermal growth factor receptor signaling converge on β -catenin to promote ovarian cancer metastasis. *Life Sci.* 2012;91:13–4.
 19. Mikami Y, Yamauchi Y, Horie M, Kase M, Jo T, Takizawa H, et al. Tumor necrosis factor superfamily member LIGHT induces epithelial–mesenchymal transition in A549 human alveolar epithelial cells. *Biochem Biophys Res Commun.* 2012;428:451–7.
 20. Sullivan NJ, Sasser AK, Axel AE, Vesuna F, Raman V, Ramirez N, et al. Interleukin-6 induces an epithelial–mesenchymal transition phenotype in human breast cancer cells. *Oncogene.* 2009;28:2940–7.
 21. So J, Navari J, Wang FQ, Fishman DA. Lysophosphatidic acid enhances epithelial ovarian carcinoma invasion through the increased expression of interleukin-8. *Gynecol Oncol.* 2004;95:314–22.
 22. Yu J, Ren X, Chen Y, Liu P, Wei X, Li H, et al. Dysfunctional activation of neurotensin/IL-8 pathway in hepatocellular carcinoma is associated with increased inflammatory response in microenvironment, more epithelial mesenchymal transition in cancer and worse prognosis in patients. *PLoS One.* 2013;8:e56069.
 23. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. *Immunol Today.* 1992;13:265–70.
 24. Cowin P, Rowlands TM, Hatsell SJ. Cadherins and catenins in breast cancer. *Curr Opin Cell Biol.* 2005;17:499–508.
 25. Lévy L, Neuveut C, Renard CA, Charneau P, Branchereau S, Gauthier F, et al. Transcriptional activation of interleukin-8 by beta-catenin-Tcf4. *J Biol Chem.* 2002;277:42386–93.
 26. Park MH, Kim DJ, You ST, Lee CS, Kim HK, Park SM, et al. Phosphorylation of β -catenin at serine 663 regulates its transcriptional activity. *Biochem Biophys Res Commun.* 2012;419:543–9.