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Epithelial–mesenchymal transition in ovarian cancer

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ABSTRACT

Ovarian cancer is a highly metastatic disease and the leading cause of death from gynecologic malignancy. Hence, understanding of the molecular changes associated with ovarian cancer metastasis could lead to the identification of targets for novel therapeutic interventions.

The conversion of an epithelial cell to a mesenchymal cell plays a key role both in the embryonic development and cancer invasion and metastasis. Cells undergoing epithelial–mesenchymal transition (EMT) lose their epithelial morphology, reorganize their cytoskeleton and acquire a motile phenotype through the up- and down-regulation of several molecules including tight and adherent junctions proteins and mesenchymal markers.

EMT is believed to be governed by signals from the neoplastic microenvironment including a variety of cytokines and growth factors. In ovarian cancer EMT is induced by transforming growth factor- β (TGF- β), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and endothelin-1 (ET-1). Alterations in these cellular pathways candidate them as useful target for ovarian cancer treatment.

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1. Introduction

The ability of cancer cells to undergo invasion and metastasis is regulated through the action of a variety of cellular and signalling proteins. This complex environment includes intracellular and membrane proteins that drive mechanically cell migration and protrusion as well as growth factors produced by stroma and tumor cells. To migrate and to spread within the tissues cells modify their shape, they become polarized and extend protrusions allowing for increased migratory capacity.

The metastatic sequence involves numerous steps, including detachment of cells within a primary tumor, penetration of local stroma, entry of local vascular or lymphatic vessels (intravasation), aggregation with platelets, interaction with and adhesion to distant endothelia, extravasation, recolonization, and expansion [1,2].

It is well established that cancer invasion and metastasis still represent the major causes of the failure of cancer treatment. Furthermore, the identification of molecules and/or signalling pathways involved in these processes is of paramount importance for the development of an appropriate therapy for certain types of cancers like ovarian cancers for which the early-stage detection is still a barrier.

Ovarian cancer is a highly metastatic disease and the leading cause of death from gynecologic malignancy. In 2009 in the United States, it is estimated that ovarian cancer will be diagnosed in 21,550 women with an estimated 14,600 deaths per year (surveillance, epidemiology and end results (SEER) Program of the National Cancer Institute). Despite enormous progress in the understanding of ovarian cancer biology, this disease remains one of the leading cause of cancer death among women in most western countries due to the advanced stage of disease at diagnosis (stages III–IV) when the vast majority of women are diagnosed with disseminated intraperitoneal carcinomatosis.

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Ovarian carcinomas include a large group of neoplasms with a wide range of genetic alterations, morphological characteristics and clinical outcome.

Surface epithelial tumors (carcinomas) account for approximately 60% of all ovarian tumors and approximately 90% of malignant ovarian tumors neoplasms and are thought to arise from the normal ovarian surface epithelium (OSE) or inclusion cysts lined with OSE cells that were exposed to inflammatory stimuli, prolonged gonadotropin stimulation or incessant ovulation [3,4].

OSE covering a nonovulating ovary is a stationary mesothelium that exhibits epithelial and mesenchymal characteristics [5] with the capacity to give rise to inclusion cysts through the loss of mesenchymal characteristics and subsequent acquisition of epithelial characteristics (mesenchymal–epithelial transition, MET) [6].

Additionally, ovarian tumors are also classified in five major subtypes designated as follows: serous, mucinous, endometrioid, clear cell, and transitional cell (or Brenner type). Tumors in each of the categories can be further subdivided into benign, malignant and intermediate (tumor of borderline malignancy, BOT) to reflect or not their capability to invade anatomically distant normal tissues.

2. Epithelial–mesenchymal transition: the role of E-cadherin

Epithelial tumors commonly use collective migration mechanism to infiltrate neighbouring tissue [7], however, several features set apart ovarian cancer spread from other epithelial tumors.

First, due to the lack of an anatomical barrier, ovarian carcinoma can spread directly throughout the peritoneal cavity, mainly by intra-abdominal dissemination and by lymphatic dissemination, enabling in this way the attachment to peritoneum and omentum. Dissemination through the vasculature is rare [8].

Second, metastatic tumor cells undergo morphological and molecular changes during the transition from a benign to a malign phenotype to facilitate the interaction with the peritoneal stroma and mesothelium. The dedifferentiation of epithelial cells toward a mesenchymal state, named epithelial–mesenchymal transition (EMT), has been recognised as a key step during several phases of embryonic development as well as during pathological conditions such as organ fibrosis and cancer metastasis [9].

During EMT, epithelial cells lose their compact organization in colonies, acquire a spindle shaped morphology and transform themselves in a more motile and invasive counterpart acquiring a migratory behaviour. These changes occur together with proteins and genes modifications in epithelial intermediate filaments, whose expression is typically reduced, and in the overexpression of vimentin and matrix metalloproteases (MMPs). In addition, alterations in the expression of cell–cell and cell–matrix adhesion molecules including integrins and E- and N-cadherin seem to have a role during EMT [10–16].

A key feature of EMT is the switch from E-cadherin to N-cadherin, cells undergoing EMT downregulate the expression E-cadherin accompanied by an increased expression

of N-cadherin which promotes the interaction with endothelial and stromal components [11].

E-cadherin is a 120 kDa transmembrane glycoprotein involved in calcium-dependent cell–cell adhesion. It is bound via catenins proteins (α , β and γ) to the actin cytoskeleton. For its role in the maintenance of normal intercellular adhesion, loss of cadherin mediated adhesion has been recognised as a prerequisite for tumor cell invasion and metastasis formation by promoting tumor cells detachment from the primary site and facilitating the arrest of cancer cells at metastatic sites [12]. In the light of this, a reduced expression of E-cadherin was observed in various human malignancies [13,15] and associated with metastasis [16]. Using the tumorigenic cell line RERF-LC-AI, negative for the expression of E-cadherin, it was shown that forced expression of E-cadherin inhibited ovarian metastasis [16].

The mechanism by which loss of E-cadherin expression drives metastasis could be explained in part through the regulation of integrin-mediated contacts with the extracellular matrix (ECM). In ovarian cancer, an *in vivo* experimental study showed that down-regulation of E-cadherin

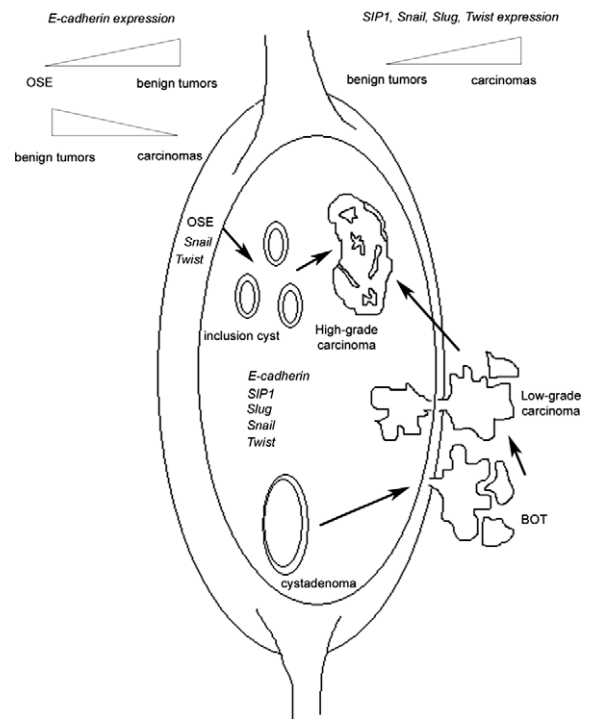


Fig. 1. Schematic illustration of E-cadherin, SIP1, Snail, Slug and Twist during ovarian progression. A model has been proposed by Shih and Kurman in 2004 to explain ovarian cancer progression [83]. In this model, epithelial ovarian tumors have been classified into two broad categories: type I tumors including low-grade serous carcinomas, mucinous, endometrioid, and clear cells carcinomas seem to develop from their precursors, namely borderline ovarian tumors (BOTs), in a stepwise manner; type II including high-grade serous malignancies develop from the OSE or inclusion cysts without a common precursor. OSE cells covering the ovarian surface do not express E-cadherin but are positive for Snail and Twist expression. As depicted, E-cadherin expression changes during ovarian cancer progression showing an inverse correlation compared to SIP1, Snail, Slug and Twist expression.

by siRNA induced an increase in α_5 -integrin mRNA through the EGFR/FAK/ERK–MAPK pathway, associated with the ability of cancer cells to adhere to the mesothelial cells lining the abdominal cavity [17].

In ovarian tissue, E-cadherin is regulated at both the mRNA and protein levels in part due to differences in the promoter activation in OSE and ovarian carcinoma cells (Fig. 1) [18]. OSE cells covering the ovarian surface do not express E-cadherin, however, E-cadherin is present in OSE cells covering deep clefts and inclusion cysts and ovarian tumors [19]. *In vitro* experiments have demonstrated that introduction of E-cadherin expression into human immortalized OSE cells induces adherens junctions formation, regulates morphology and migratory activity of OSE cells [18,20].

Down-regulation of E-cadherin was described in a high percentage of BOTs and carcinomas, when compared with benign tumors [21] and associated with high tumor grade, presence of peritoneal seeding and low overall survival rate [22]. Moreover, ascites cells with low E-cadherin expression were more invasive than solid tumor cells [23].

Several transcriptional repressors of E-cadherin have been identified, including the zinc finger factors Snail [24] and Slug [25] and the two-handed zinc factor ZEB-2 (SIP1) [26]. These repressors are regulated by specific pathways (see below) but also under hypoxic conditions [27]. Tumor hypoxia have been found to increase Snail expression, to reduce E-cadherin expression increasing in this way the invasiveness of ovarian cancer cells.

In ovarian neoplasms, the expression of Snail showed a negative correlation with E-cadherin expression and was associated with a poor ovarian carcinoma prognosis (Fig. 1). Immunohistochemical analysis demonstrated that OSE cells were negative for Slug expression and positive for Snail as also confirmed by western blotting and RT-PCR. In addition, the expression of Snail and Slug was increased in benign, borderline and malignant tumors suggesting a role in the development of ovarian carcinoma [28].

3. Pathways leading to EMT

Induction of EMT is driven by a complex interplay between cancer cells and their environment including stroma or extracellular components such as cytokines and growth factors acting in an autocrine or paracrine fashion. Here, we performed a PubMed searching to identify factors promoting EMT in ovarian cancer cells. Five molecules have been described to be involved in this process and here described.

3.1. Transforming growth factor- β (TGF- β)

TGF- β is a secreted cytokines with diverse roles in regulating cellular processes such as proliferation, migration and apoptosis. TGF- β was first identified by Roberts et al. for its ability to induce change in cellular shape and anchorage-independent growth [29]. This family is comprised of different members including activins, inhibins, bone morphogenetic proteins (BMPs), and Mullerian-inhibiting substance (MIS).

TGF- β binds to two different serine/threonine kinase receptors, named type II (T β RII) and type I (ALK1/TSR-1, ALK2/Tsk7L, ALK5/T β RI), which form hetero-tetramers composed of two type II and two type I receptors [30]. Upon TGF- β treatment, the downstream signalling components Smad 2 and Smad 3 are phosphorylated by the TGF- β type I receptor. They form complexes with Smad 4 and subsequently accumulate in the nucleus [31].

In addition to Smad, other pathways exist with a role in the transduction of TGF- β signal. These non-Smad pathways include Rho-like GTPase signalling pathways, PI3 K/Akt pathway and MAPK pathway [32].

Many types of human cancer including basal cell carcinoma, melanoma and colorectal cancer showed an overexpression of TGF- β and a correlation between its expression and clinical findings [33–35]. It was also reported that TGF- β was localized in both ovarian carcinoma cells and stromal tissues and rarely expressed in normal ovarian surface epithelia [36]. Both TGF- β 1 and TGF- β 2 were overexpressed in malignant ovarian carcinomas [37].

TGF- β has a dual role in cancer progression, it promotes cell death during the early phase of tumorigenesis but not during the late phases of carcinogenesis, where it acts as a tumor promoter.

It was demonstrated that ovarian carcinoma cell lines (OVCAR-3, HTB-77, 2780, CRL-1572 and CAOV-3) were growth inhibited by TGF- β in a dose related manner [38,39]. Similarly, TGF- β 1 inhibited epidermal growth factor-mediated OVCCR1 cell proliferation by blocking the cell cycle at the G1/S transition but did not affect the growth of IGROV1 cells [40]. By contrast, Yamada et al. showed no inhibition in the cell growth of primary ovarian cancer cell cultures established from solid ovarian carcinomas when treated with TGF- β 1 as assessed by MTT assay [41].

Dunfield et al. showed that TGF- β pathway is functional in primary ovarian cancer cells and cancer cell lines (CaCO3 and SKOV3) but in different manner. In fact, TGF- β 1 treatment inhibited cancer cell growth in primary cells isolated from ascite specimens, probably through the up-regulation of cell cycle regulators such as the cyclin-dependent kinase inhibitor, p15^{Ink4B}, but not in CaCO3 and SKOV3 [42].

Loss of c-Myc repression of p15^{Ink4B} has also a role in the mechanisms of TGF-growth inhibition [43]. TGF- β decreased c-Myc protein and mRNA levels in normal primary cultures, but not in malignant cultures from ovarian carcinomas, allowing in this way the Smad-dependent transcription activation of p15^{Ink4B} which inhibit CDK phosphorylation of the retinoblastoma protein [43]. c-Myc has been shown to regulate p15^{Ink4B} by inhibiting its expression through the transcription factor Miz-1 [44].

In addition, to evade the antiproliferative effects of TGF- β , cancer cells can also modify the expression of genes involved in the regulation of TGF- β pathway as demonstrated by Sunde et al. The authors used data profiling to identify seven genes that were aberrantly expressed in 119 ovarian cancers compared with 10 ovarian surface epithelium samples. Three genes involved in the inhibition of TGF- β signalling (DACH1, EVI1, and BMP7) were up-regulated in cancer specimens and four which enhance its activity (TGFBR1, SMAD4, TFE3 and PCAF) were down-regulated [45].

TGF- β pathway has been targeted through the administration of small-molecule inhibitors of the kinase domains, modification of immune components and soluble-protein or antisense-compound inhibitors [46].

TGF- β is one of the main inducers of EMT in several biological systems as demonstrated both *in vitro* and *in vivo*. Mouse mammary epithelial cells treated with TGF down-regulated the expression of the epithelial markers E-cadherin, ZO-1, desmoplakins I/II and increased the expression of the mesenchymal marker fibronectin [47]. TGF- β has also been described to induce EMT in ovarian adenocarcinoma cells [48], mouse NMuMG breast epithelial tumor cells [49] and human lung adenocarcinoma cell line A549 [50]. In A549 cells, EMT led to increased migratory and invasive abilities [50] and activation of several cytoskeletal proteins including β -actin, cofilin-1, moesin, filamin A and B, heat-shock protein β -1, trangelin-2, calgizarrin and calpactin [51].

We demonstrate that ovarian cancer cell line SKOV3 treated with TGF- β (10 ng/ml, 24 h) increases the expression of cofilin-1 and profilin-1 and modify its cytoskeletal organization as assessed by confocal microscopy analysis (Figs. 2 and 3). After binding to its receptor TGF- β stimulates the re-organization of actin cytoskeleton triggering stress fibers and cellular protrusion formation (Fig. 3).

Studies *in vivo* using transgenic mice with keratinocyte-targeted expression of TGF- β 1 showed that TGF- β expression stimulated the conversion of squamous into spindle cell carcinoma enhancing malignant progression [52].

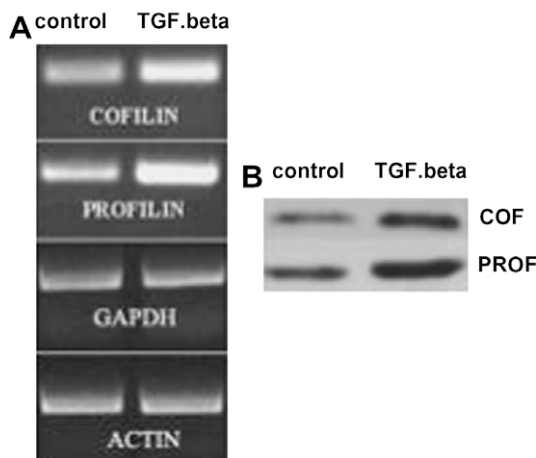


Fig. 2. (A) Expression of cofilin-1 and profilin-1 mRNA in SKOV3. After 24 h of TGF- β stimulation (as previously described), total RNA was extracted from treated and control cells using the SV Total RNA Isolation Kit (Promega). Total RNA was reverse transcribed using oligo (dT) primers and amplified using specific primers. GAPDH and actin were used as loading controls. The expression of cofilin-1 and profilin is higher in SKOV3 treated with TGF- β than in control cells. (B) Western blotting analysis of cofilin-1 and profilin-1 in control and TGF- β treated SKOV3. SKOV3 cells were treated with TGF- β as described above. Fifty micrograms of total proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were then incubated with antibodies specific for cofilin-1 (Sigma) and profilin-1 (Sigma) overnight at 4 °C. These results were representative of two independent experiments.

3.2. Epidermal growth factor (EGF)

EGF is known to induce EMT following binding to its receptor, EGFR. EGFR is expressed in 70% of malignant ovarian tumors and correlated to serous histology, poor prognosis and chemoresistance [53,54]. Given its importance in both ovarian cancer growth and progression many inhibitors and monoclonal antibodies were developed over the past several years. Several selective EGFR tyrosine kinase inhibitors have been shown to offer clinical benefit including gefitinib, erlotinib, lapatinib as well as humanized monoclonal antibodies, such as cetuximab [55,56].

EGF-induced EMT in OSE and cancer cells and have a role during ovarian cancer pathogenesis and metastasis (Fig. 4).

Inclusion cysts derived from OSE after MET represent possible sites of a metaplastic and neoplastic transformation, EMT of OSE might reduce the chances of OSE-derived malignant transformation.

Ahmed et al. shown that a fibroblast-like-morphology can be induced in OSE cells after EGF stimulation suggesting that EGF stimulation may prevent the formation of epithelial inclusion cysts.

They also demonstrated that EMT transition was associated through inhibition of keratin expression and increased mobility as assessed by wounding assay. Furthermore, EGF enhanced the expression of pro-MMP-2/-9 and induced the activation of ERK and integrin-linked kinase (ILK) pathways [57].

EGF-induced EMT has also been implicated in cancer metastasis. EMT-like transitions have been shown to increase the motility of SKOV3 and to downregulate the expression of neutrophil gelatinase-associated lipocalin (NGAL) [58]. EGF also enhanced the expression of α 2, α 6, β 1 integrin subunits, leukaemia inhibitory factor (LIF) and IL-6. Moreover, EGF-induced the activation through phosphorylation of JAK/STAT3 pathway in both OVCA 433 and SKOV3 cells. JAK/STAT3 activation was required for EGF-induced motility of cancer cells but its inhibition did not revert the fibroblast-like morphology of cancer cells [59,60].

3.3. Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is a stromal-derived factor that activates various signal transduction pathways including MAPK, PI3 K and AKT.

In vivo, HGF is found at high levels in malignant ovarian cysts and ascitic fluid from women with ovarian carcinomas [61] and its tyrosine kinase receptor, cMET, is often overexpressed in ovarian carcinomas [62].

Activation of p70 S6 kinase (p70^{S6K}) by MEK-ERK1/2 and PI3 K-AKT is essential for HGF-induced epithelial cell proliferation and can be obtained [63].

In ovarian cancer cells, HGF signalling increased cell scattering through the phosphorylation of p70^{S6K} by PI3 K/AKT. In contrast, both ERK1/2 and PKC were not required for HGF to phosphorylate and activate p70^{S6K} [64].

HGF can also lead to EMT through activation of p70^{S6K}. CaOV-3 and SKOV3 cells transfected with constitutively active p70^{S6K} assumed a fibroblast-like appearance by

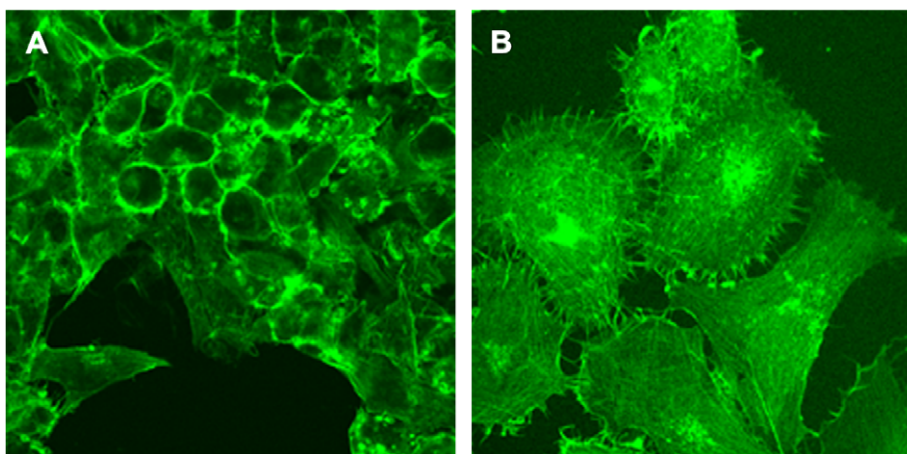


Fig. 3. The human epithelial ovarian cancer cell line SKOV3 was grown in RPMI-1640 medium with glutamine, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were treated with TGF-β (10 ng/ml⁻¹) for 24 h in the absence of serum (B). Control cells were left untreated (A). For confocal microscopy analysis, cells were fixed for 5 min with 3.7% formaldehyde in phosphate-buffered saline (PBS) solution, permeabilized with a 0.1% solution of Triton X-100 in PBS followed by a 30 min incubation at room temperature with phalloidin-TRITC (Sigma). (A) Control cells have a typical epithelial-like morphology in culture flask/Petri with the tendency to form dense colonies. The filamentous actin cytoskeleton shows circumferential organization. (B) Protrusive structures and actin stress fibers are clearly visible only in treated cells.

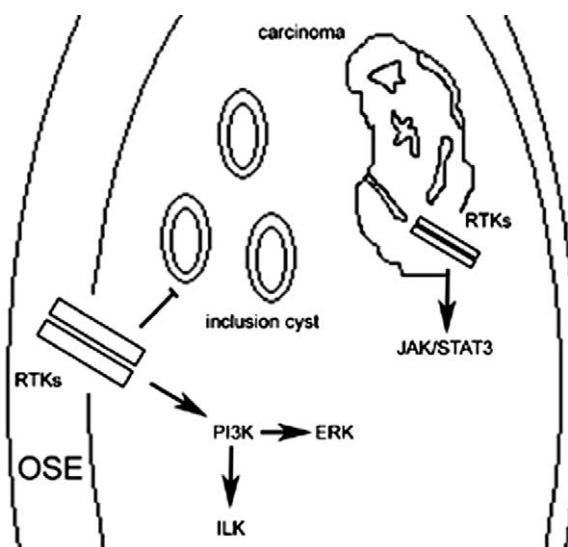


Fig. 4. A simplified overview of signalling network regulating EGF-induced EMT. In OSE cells, activation of the EGF receptor tyrosine kinases (RTKs) by EGF results in activation of the phosphatidylinositol 3-kinase (PI3 K), which activates ILK and ERK pathways. EGF treated OSE cells display a molecular signature characteristic of EMT and are less likely to undergo a conversion in inclusion cysts. JAK/STAT3 pathway is required to induce EMT in ovarian cancer cells. Ovarian cancer cells that undergo EMT lose the expression of E-cadherin and NGAL and show an increased motility.

increasing the expression of Snail and regulating its nuclear localization. The regulation of Snail expression by p70^{S6K} was confirmed also *in vivo*, where ovarian tumor region showing strong expression of activated p70^{S6K} showed Snail expression too [65].

It has been reported that Paclitaxel-induced phosphorylation of p70^{S6K} at Thr⁴²¹ and Ser⁴²⁴ and suppressed its activity in a dose and time dependent manner. Both phosphorylation and inactivation induced by paclitaxel were

found to be regulated by different pathways including mTOR, PKC, JNK and Ca⁺⁺ signalling pathways [66].

3.4. Endothelin-1 (ET-1)

Endothelin-1 (ET-1) is vasoconstrictor peptide isolated from the first time from the culture media of porcine endothelial cells [67]. ET-1 acts by binding to two G-protein-coupled transmembrane receptors, ET_A and ET_B, and it has been implicated in several physiological and pathological conditions, including cancer [68].

ET-1 mRNA was detected in 90% of primary and 100% of metastatic ovarian carcinomas and its mRNA expression was significantly higher in tumors than in normal ovarian tissues [69]. Elevated levels of ET-1 was also present in the ascitic fluids of ovarian cancer patients and correlated with elevated ascitic concentrations of vascular endothelial growth factor (VEGF) [70]. Furthermore, ET-1 induced cyclooxygenase-2 and related prostaglandin E2 release upon the activation of ET_A receptor. Selective inhibition of ET_A by atrasentan significantly reduced the levels of cyclooxygenase-2 protein expression [71].

Rosanò et al. reported that ET_A has a crucial role in determining the ET-1-induced EMT in ovarian carcinoma cells. In human ovarian carcinoma cell line HEY, ET-1 induced the up-regulation of Snail and the nuclear translocation of β-catenin through the inhibition of glycogen synthase kinase-3β (GSK-3β). In addition, ET_A expression was higher in grade 3 and 4 cancers than early-grade ovarian cancers correlated with the down-regulation of E-cadherin and with the enhanced expression of N-cadherin [72].

3.5. Bone morphogenetic protein 4 (BMP4)

Bone morphogenetic protein 4 (BMP4) belongs to the TGF family of secreted ligands. BMP4 mRNA was expressed in a variety of ovary cell types including OSE covering the

ovulated follicle, when OSE mesenchymal features become more prominent compared to epithelial features, as well as ovarian cancer cells [73,74].

BMP4-stimulated OSE and ovarian cancer cell lines increased the expression of distinct target genes, including members of the *ID* (inhibitor of differentiation/DNA binding) gene family, *ID1* and *ID3*, with a more pronounced response in OvCa compared with OSE cells (B). *ID* protein expression have been implicated in ovarian cancer and associated with a poor clinical outcome [75].

BMP4 induced EMT in primary OvCa cells through distinct mechanisms including the re-organization of actin fibers, up-regulation of Snail and Slug and down-regulation of E-cadherin. Treated cells showed also an increased expression of several integrin receptors, extracellular matrix (ECM) proteins, focal adhesion proteins (FAPs) genes and an activation of the Rho GTPases (Rho, Rac1, Cdc42). BMP4 treatment enhanced cell mobility and invasion in OvCa cells but not in normal OSE [76].

4. Conclusions

The role of EMT in cancer invasion and metastasis is strongly supported by several cellular models. Although the role of EMT *in vivo* is still debated among pathologists [77,78], specific inhibition of these signalling pathways proved their clinical efficacy.

Recent studies link EMT with the induction of stem cell markers with new implication in the treatment in ovarian cancer and cancer in general [79–81]. Experimental findings reported that immortalized human mammary epithelial cells (HMLEC) induced to undergo EMT acquired a CD44^{high}/CD24^{low} expression pattern, two cell-surface markers associated with neoplastic mammary stem cells, and an increase ability to form mammospheres [79]. The acquisition of stem cell properties seems to be driven by EMT following the activation of the Ras–MAPK pathway [81].

In ovarian cancer cells, under condition of stress, up-regulation of the transcriptional repressors Snail and Slug were associated to radio- and chemoresistance through the inactivation of components of the p53 pathway and derepression of specific stem cell genes [82]. The authors proposed a probable model by which EMT provides a mechanism of escape to a new, less adverse niche, evading apoptosis and ensuring cell survival in conditions of stress.

Conflicts of interest

None declared.

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