

## Review

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# Hypoxia, cytokines and stromal recruitment: parallels between pathophysiology of encapsulating peritoneal sclerosis, endometriosis and peritoneal metastasis

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**Abstract:** Peritoneal response to various kinds of injury involves loss of peritoneal mesothelial cells (PMC), danger signalling, epithelial-mesenchymal transition and mesothelial-mesenchymal transition (MMT). Encapsulating peritoneal sclerosis (EPS), endometriosis (EM) and peritoneal metastasis (PM) are all characterized by hypoxia and formation of a vascularized connective tissue stroma mediated by vascular endothelial growth factor (VEGF). Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is constitutively expressed by the PMC and plays a major role in the maintenance of a transformed, inflammatory micro-environment in PM, but also in EPS and EM. Persistently high levels of TGF- $\beta$ 1 or stimulation by inflammatory cytokines (interleukin-6 (IL-6)) induce peritoneal MMT, adhesion formation and fibrosis. TGF- $\beta$ 1 enhances hypoxia inducible factor-1 $\alpha$  expression, which drives cell growth, extracellular matrix production and cell migration. Disruption of the peritoneal glycocalyx and exposure of the basement membrane release low molecular weight hyaluronan, which initiates a cascade of pro-inflammatory mediators, including peritoneal cytokines (TNF- $\alpha$ , IL-1, IL-6, prostaglandins), growth factors (TGF- $\alpha$ , TGF- $\beta$ , platelet-derived growth factor, VEGF, epidermal growth factor) and the fibrin/coagulation cascade (thrombin, Tissue factor, plasminogen activator inhibitor [PAI]-1/2). Chronic inflammation and cellular transformation are mediated by damage-associated molecular patterns, pattern recognition receptors, AGE-RAGE, extracellular lactate, pro-inflammatory cytokines, reactive oxygen species, increased glycolysis, metabolomic reprogramming and cancer-associated fibroblasts. The pathogenesis of EPS, EM and PM shows similarities to the cellular transformation and stromal recruitment of wound healing.

**Keywords:** CAPD, DAMPs, endometriosis, EPS, HIF, hyaluronan, peritoneal metastasis, TGF- $\beta$ 1, VEGF

## Introduction

Thirty years ago, Harold Dvorak published a seminal paper entitled “Tumors: Wounds that do not heal”. The novelty was based on two findings, namely, the discovery of vascular permeability factor (VPF, subsequently renamed as vascular endothelial growth factor [VEGF]) as a tumour product and the recognition that the chronic vascular hyperpermeability (CVH) induced by VPF/VEGF likely accounted for the fibrin deposited in solid tumours and in early stages of wound healing [1].

Dvorak made the crucial observation that both wounds and tumours are hypoxic. When cells experience shortage of oxygen, they produce a family of transcription factors named Hypoxia-inducible factors (HIF). HIF is instrumental for the survival of cells under hypoxia or physiological stress. HIF induces angiogenesis by stimulating the outsprouting of new vessels from existing vessels, designed to deliver oxygen and nutrients to the growing (or healing) tissue. Overexpression of HIF-1 $\alpha$  was observed in peritoneal dialysis patients [2], in women with endometriosis (EM) [3] and in patients with peritoneal metastasis (PM) [4]. Both HIF-1 $\alpha$  and HIF-1 $\beta$  activate the transcription of the gene encoding VEGF [5]. By binding to its receptor, VEGF stimulates neoangiogenesis. VEGF has multiple interdependent pathways which contribute to chronic inflammation, organ fibrosis and carcinogenesis [6, 7]. These mechanisms are involved in the peritoneal response to injury, particularly epithelial-mesenchymal transition (EMT) and mesothelial-mesenchymal transition (MMT).

Tissue regeneration is regulated by members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, which also regulate cell fate and differentiation in embryonic development. During embryonic development, TGF- $\beta$  can induce EMT. TGF- $\beta$  signalling can either inhibit or foster

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tumour growth [5]. The creation of a vascularized connective tissue stroma in healing wounds and in EM is mediated by VEGF. VEGF was also found to promote PM and ascites formation [7–9].

The recognition of tissue injury and the subsequent inflammatory response is fundamental to the resolution of cellular damage and successful healing. If the initial injury is severe or repetitive, a wound is formed which becomes chronic. This review will highlight the similarities in the pathophysiology of encapsulating peritoneal sclerosis (EPS), EM and PM. The contribution of TGF- $\beta$  superfamily, HIF and VEGF in inflammation, cellular proliferation, stromal recruitment and angiogenesis in these diseases will be reviewed.

## Mechanisms of peritoneal injury

Intact peritoneal mesothelial cells (PMC) express epithelial-like markers and phenotype. They maintain peritoneal homeostasis by controlling fluid and electrolyte transport, initiation and resolution of inflammation, leucocyte migration, fibrinolysis and cellular signalling. The peritoneal mesothelium produces a glycocalyx comprised of surfactants, phospholipids and glycosaminoglycans (GAGs) which provides a slippery protective surface on which the viscera slide. The major component of the glycocalyx is high molecular weight (1,000–2,000 kDa) hyaluronan (HMW-HA), a linear, non-sulphated GAG which covers the mesothelial microvilli [10]. Hyaluronan (HA) is a polymer composed of repeating disaccharides of  $\beta$ -(1, 4) glucuronic acid and  $\beta$ -(1, 3) *N*-acetyl glucosamine [11].

### Disruption of HA is pro-inflammatory

Native HMW-HA has unique viscoelastic, hygrostatic, rheologic, anti-oxidant, anti-inflammatory and anti-angiogenic properties [10]. Disruption of the glycocalyx or damage to the PMC releases low molecular weight hyaluronan (LMW-HA), which initiates a cascade of pro-inflammatory mediators [12]. Such mediators include peritoneal cytokines (TNF- $\alpha$ , interleukin [IL]-1, IL-6, prostaglandins), growth factors (TGF- $\alpha$ , TGF- $\beta$ , platelet-derived growth factor [PDGF], VEGF, epidermal growth factor [EGF]) and the fibrin/coagulation cascade (thrombin, Tissue factor, PAI-1/2) This is part of the damage-associated molecular patterns (DAMPs) response to cellular injury [13]. HA interacts with CD44 (cluster of differentiation cell surface HA binding protein), Hyaluronan Mediated Motility Receptor

(RHAMM) and Toll-like receptor (TLR)-4 on mesothelial and cancer cells, which can promote both EMT and MMT [12, 14, 15]. When activated or injured, PMCs remodel the extracellular matrix (ECM) or invade the basement membrane (BM) by the production of matrix metalloproteases (MMP-2/MMP-9) and degradation of type IV collagen [10]. This may initiate attempted healing of injured peritoneum but, by the same mechanisms, enhance the growth of transcoelomic carcinoma. For example, active CD44 facilitates MMP-9 on the surface of cancer cells, which degrades the ECM and thereby activates latent TGF- $\beta$ 1 and promotes angiogenesis and tumour invasion. TGF- $\beta$ 1 also promotes transformation of fibroblasts to myofibroblasts via CD44v6 signalling [16].

## Mesothelial-mesenchymal transition

MMT is a feature of chronic inflammation of the peritoneum, particularly in EPS. MMT in EPS involves loss of peritoneal mesothelium, impaired fibrinolysis, submesothelial myofibroblast proliferation and fibrous collagen and advanced glycation end products (AGE) deposition in the submesothelial layer. Vascular effects include neoangiogenesis, medial hyalinization and sclerosis of the peritoneal vasculature. The submesothelial myofibroblasts appear to be derived from PMC which have been transformed. MMT is typified by loss of PMC epithelial markers such as intercellular adhesion molecule (ICAM-1), calretinin, CA-125 and E-cadherin (CDH1), with increased expression of mesenchymal markers including N-cadherin, SNAIL, fibronectin, vimentin, fibroblast-specific protein-1, Collagen-1, MMP-2, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) [11, 15, 17]. Acquisition of a mesenchymal phenotype is a non-linear five-dimensional process, controlled by a different transcription factor for each axis involving:

- Increased cellular polarity with fibroblast shape
- BM remodelling
- Loss of cell–cell adhesion
- Apical constriction
- Cell migration [18].

MMT and EMT are induced by AGE-RAGE interaction, HIF, TGF- $\beta$  and repression of E-cadherin. E-cadherin transcriptional repressors include the zinc finger proteins Snail and Slug, zinc finger E-box binding homeobox (ZEB1, ZEB2) and twist basic helix loop helix transcription factor 1 (TWIST1), SIP1, FOXC1, FOXC2. Induction of EMT by these different EMT-Transcriptional factors (EMT-TFs) may drive epithelial and mesothelial cells into different regions in the

five-dimension landscape, leading to variable phenotypical behaviour and epithelial/mesenchymal plasticity [18]. Both the TGF- $\beta$ 1-induced SMAD-dependent and SMAD-independent pathways converge on the activation of Snail, which is a strong inducer of EMT in PMC [19]. Mammalian SMAD is the homologue of *Drosophila* protein MAD (Mothers Against Decapentaplegic) and the *Caenorhabditis elegans* protein SMA (Small body size). The SMAD-independent, non-canonical TGF- $\beta$  pathway also induces long non-coding RNA activated by TGF- $\beta$  (lncRNA-ATB). LncRNA-ATB increases ZEB1 and ZEB2 mRNA and protein levels through competitively inhibiting miR 200s, resulting in EMT [20]. Other cytokines and growth factors involved in MMT/EMT include hepatocyte growth factor (HGF), PDGF and IL-1 $\beta$  [15]. The Wnt/ $\beta$ -catenin signalling pathway which promotes Human epidermal growth factor-2 (HER2) activity, EMT and cell invasion is linked to TGF- $\beta$ 1 by TWIST [21].

### Chronic inflammation, TGF- $\beta$ 1 and IL-6

TGF- $\beta$ 1 was first discovered in 1983 when it was shown to stimulate the growth of cultured rat fibroblasts, and is the master cytokine in liver fibrogenesis [22]. In the meantime, the TGF- $\beta$  superfamily is recognized as a fundamental mediator in the development of EPS, organ fibrosis, EM and PM. This superfamily includes at least 40 structurally and functionally related proteins, such as bone morphogenetic proteins (BMPs), activins, inhibins, anti-Mullerian hormone, growth differentiation factors (GDFs), and glial-derived neurotrophic factors [22]. TGF- $\beta$ 1 is regarded as a principle factor in the transformation of PMCs into a mesenchymal phenotype in both *in vivo* and *in vitro* studies. The mammalian TGF- $\beta$  family members (TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3) are induced and activated in a variety of fibrotic diseases [23].

Persistently high levels of TGF- $\beta$ 1 or stimulation by inflammatory cytokines (IL-6) induce peritoneal MMT, adhesion formation and fibrosis [24, 25] (Figure 1). Recently a murine continuous ambulatory peritoneal dialysis (CAPD) model showed exposure to TGF- $\beta$ 1 alone directed naïve CD4+ T cell precursors towards the Treg lineage, whereas the combination of TGF- $\beta$ 1 and IL-6 caused progenitors to adopt a Th17 signature. Peritoneal Treg cells produce IL-10, which is an anti-inflammatory cytokine. TGF- $\beta$ 1 was strongly upregulated in the peritoneal cavity of cd69<sup>-/-</sup> mice exposed to peritoneal dialysis fluid (PDF). The lack of CD69 expression on Treg cells caused Th17 cell expansion, the release of the pro-inflammatory cytokine IL-17 and peritoneal fibrosis. PDF-induced fibrosis was blocked by anti-IL-17 antibodies in cd69<sup>-/-</sup> mice and

enhanced by neutralizing anti-CD69 antibody in wild-type mice [26]. HIF-1 $\alpha$  is also involved in the inflammatory process via the retinoic acid-related orphan receptor- $\gamma$ t to drive Th17 differentiation. Th17 cells undergo clonal expansion and switch to glycolysis to maintain ATP production. This Warburg-like metabolic reprogramming occurs independently of oxygen levels and is controlled by a mammalian target of rapamycin (mTOR)-dependent nutrient-sensing pathway [27]. TNF receptor-associated factor (TRAF6) regulates both canonical TGF- $\beta$ 1/SMAD pathways via cytokines, and SMAD-independent pathways involving TGF- $\beta$ -activated kinase-1 (TAK1)/mitogen-activating protein kinase kinase kinase (MAP3K)/p38. IL-1 $\alpha$  and TNF- $\alpha$  cause Activin A secretion via TAK-1/p38/NF $\kappa$ B signalling. The resulting activin A signals in an autocrine way via ALK/SMAD2,3/AKT to promote myofibroblast formation. Activin A levels were respectively increased by 1,152% and 459% after treatment with IL-1 $\alpha$  and TNF- $\alpha$  [28]. Thus peritoneal sclerosis and MMT proceed as a result of continued inflammatory cytokine stimulation of TGF- $\beta$ 1 release and exaggerated DAMP signalling.

### HA size regulates inflammation and TGF- $\beta$ 1

The molecular size of HA polymers released after cellular injury appears to be a discriminating factor in the initiation and also resolution of tissue inflammation and MMT [10]. Medium size HA fragments (100–250 kDa) promote cell migration and cytokine production. LMW-HA (6–50 kDa) significantly increases TLR-4 expression compared to medium-to-HMW-HA (100–1,800 kDa). Upregulation of the TLR4–Myeloid Differentiation Factor 88 (MYD88) axis results in the down-regulation of BAMB1, a pseudoreceptor which normally antagonises TGF- $\beta$  receptor activation. Very small MW HA fragments (four saccharides) promote chemotaxis. Small HA oligomers (< 6 kDa) upregulate TGF- $\beta$ 1 and TNF- $\alpha$  by 35-fold compared to native HA, and impair cellular reparation after injury, promoting continued inflammation [10]. Myofibroblast differentiation in response to TGF- $\beta$ 1 is dependent on, and facilitated by, HA interactions with CD44 and EGFR. This has downstream effects on extracellular signal-regulated kinase (ERK) phosphorylation and calcium/calmodulin kinase II activation [29].

### TGF- $\beta$ role in chronic inflammation

Chronic inflammatory conditions include retroperitoneal fibrosis, hepatic fibrosis, idiopathic pulmonary fibrosis,

systemic sclerosis (CREST syndrome), Crohn's disease, radiation enteritis, collagenous colitis, EPS and others [19]. Such conditions share a common pathophysiology of pro-inflammatory mediators, TGF- $\beta$  overexpression and myofibroblast generation. TGF- $\beta$  is constitutively expressed by most cells, including fibroblasts, mesothelial and epithelial cells in the peritoneum and gastrointestinal tract [30, 31]. TGF- $\beta$  has a pro-fibrogenic activity and can transform rat mesothelial cells, mouse mesangial cells and human smooth muscle cells into myofibroblasts [32]. These myofibroblasts have the ability to produce collagen and  $\alpha$ -SMA and contract the ECM resulting in wound contraction or tissue fibrosis [19].

Continued platelet activation, deposition of ECM and impaired fibrinolysis also potentiate chronic wounds. Thrombin activation of platelets triggers the release of TGF- $\beta$ 1 from the  $\alpha$ -granules of platelets, and thrombin activity can also release latent TGF- $\beta$ 1 from the ECM [33]. The synthesis of different collagen type I polypeptides is determined by two separate pathways: the TGF- $\beta$ 1 protein activation pathway and the SMAD signalling pathway [34].

## Activation of TGF- $\beta$ 1

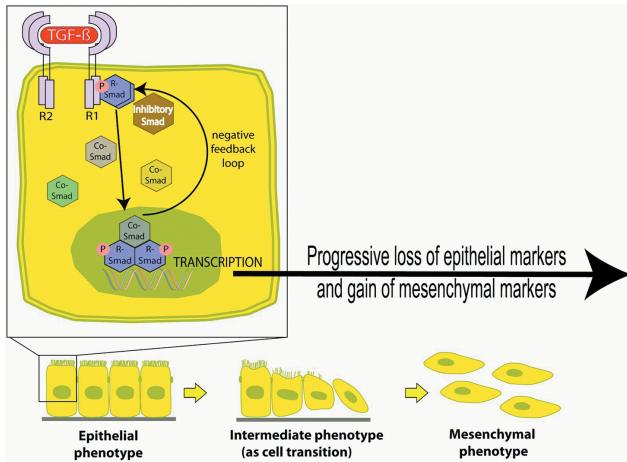
The inactive pro-TGF- $\beta$  complex contains TGF- $\beta$  and the latency-associated peptides (LAP). It is connected to the ECM by the latent-TGF- $\beta$ -binding proteins 1/2 (LTBP 1/2). Thus the ECM is a sequestrant reservoir for latent TGF- $\beta$ 1, which acts as an extracellular sensor and transducer of mechanical forces, oxidative stress or tissue damage [34]. Active extracellular TGF- $\beta$ 1 is released from the pro-TGF- $\beta$  complex under proteolytic activity from plasmin, MMP-2 and MMP-9 or thrombospondin (TSP-1) [30]. Activation can also occur by mild acids (lactate), integrin  $\alpha$ V- $\beta$ 6, reactive oxygen species (ROS), angiotensin II, low-density lipoproteins (LDL), glucose, thromboxane A<sub>2</sub>, calpain, cathepsin D, chymase, elastase, endoglycosidase F, kallikrein, or neuraminidase [31, 35]. The released TGF- $\beta$ 1 then acts on its type I (T $\beta$ R-I) and type II (T $\beta$ R-II) serine/threonine kinase cell surface receptors. TGF- $\beta$ 1 normally inhibits epithelial cell cycling and promotes apoptosis, and as such is regarded as a tumour suppressor in the early stages of carcinoma. TGF- $\beta$ 1 also promotes epithelial to mesenchymal transition, which is a transient stage in the response to injury and acute wound healing. However, dysregulation of TGF- $\beta$ 1 signalling occurs in sustained or severe peritoneal injury or peritoneal malignancy, which contributes to progressive EMT/MMT, angiogenesis and cellular proliferation [31].

## TGF- $\beta$ 1 and SMAD signalling pathway

TGF- $\beta$ 1 interacts as a ligand with type 1 receptors (T $\beta$ RI and Activin-Like-Kinase 1) and type 2 receptors (T $\beta$ RII). After binding of a TGF- $\beta$  dimer to dimers of type 1 and type 2 receptors, a stable complex is formed and Receptor-regulated SMAD (R-SMAD) is phosphorylated by the kinase of the receptor. SMAD proteins transduce signals from the cell membrane to the nuclear DNA. Some of the SMAD-binding transcription factors in the nucleus are relevant for the function of TGF- $\beta$  superfamily members in cell growth and migration [5]. In endothelial cells, SMAD2/3 signalling inhibits cellular proliferation and migration. In contrast, SMAD1, SMAD5 and SMAD8 signalling promotes these cellular processes.

The type 1 receptor Activin like kinase (ALK5) is the main ALK expressed in epithelial cells and is commonly referred as T $\beta$ RI [36]. TGF- $\beta$ /activin pathways usually signal through SMAD2, SMAD3; and BMP/GDF pathways via SMAD1, SMAD5 and SMAD8. However, TGF- $\beta$ 1 may signal via SMAD1/5 in some cells, including endothelial, fibroblast, immortalized epithelial, adenoma and carcinoma cell lines [37]. Prior to being activated, SMADs are anchored to the cell membrane by SMAD Anchor for Receptor Activation which brings the SMADs close to the TGF receptor kinases [38]. TGF- $\beta$ 1 first binds to T $\beta$ R-II, and then recruitment of T $\beta$ R-I ensues. Both receptors form the T $\beta$ R heteromeric complex in which T $\beta$ R-II phosphorylates and activates T $\beta$ R-I. The active T $\beta$ R-I then phosphorylates SMAD2/3 (R-SMADS). This results in retention of p-SMAD2/3 in the cytoplasm and association with SMAD4 (co-SMAD). The resulting hetero-oligomeric complex is translocated to the nucleus and activates SMAD-dependent transcriptional activity. These include DNA sequence-specific binding sites Activating Transcription Factor-2 and SMAD Binding Element (SBE), which regulate gene expression [39,40–42] (Figure 1). TGF- $\beta$ 1 transcriptional factors include c-jun, p300/CPB, c-myc, cyclin D1, cyclin-dependent kinase 4, cyclin dependent kinase (CDK)-interacting protein 1 (p21cip), cyclin-dependent kinase inhibitor 1B (p27 Kip1), cyclin-dependent kinase 4 inhibitor B/multiple tumour suppressor 2 (p15 INK4B), retinoblastoma protein (Rb), integrins, E-cadherin, collagen and other DNA-binding proteins [41]. Tumour suppressor p53 (TP53) is a cofactor for SMAD-mediated transcription, including the full activation of p21cip. Both p21cip and p15 INK4B, when translated, interfere with cyclin-CDK interactions, which inhibits CDK activation. p15 INK4B also prevents phosphorylation of Rb, resulting in suppression of release of the transcription factor E2F. E2F controls transition from





**Figure 1:** TGF signalling and peritoneal MMT.

G1 to S phase in cell cycling. TGF- $\beta$ 1 causes further cell cycle arrest by transcriptional repression of c-Myc proto-oncogene and upregulation of apoptosis factor Bim [43]. Loss of function, mutations or lack of expression of either T $\beta$ R receptor or their downstream effectors (including TP53, Rb, CDKN1A, or the CDKN2A/B locus) leads to failure of growth arrest, which occurs in advanced epithelial cancers [43, 44].

## Regulation of TGF- $\beta$ 1 signalling

SMAD6 and SMAD7 are inhibitory SMADs which provide negative feedback control of TGF- $\beta$ 1 family signalling activity. SMAD6 preferentially blocks BMP signalling [45]. SMAD7 competes for T $\beta$ R-I binding and inhibits phosphorylation of SMAD2 or SMAD3 [46]. SMAD7 decreases the stability and activity of T $\beta$ R-I by inducing receptor degradation. SMAD7 competes with SMAD4 to associate with R-SMADs and recruits the E3 ubiquitin ligases (including SMAD ubiquitin regulatory factors (SMURF1, SMURF2, PRAJA, WWP1, NEDD4L)), to R-SMADs for polyubiquitination and proteosomal degradation [31, [47]. SMAD7 also blocks the interaction of the hetero-oligomeric SMAD complex with its nuclear targets [45]. Active TGF- $\beta$ 1 not only induces SMAD7 transcription but also promotes the degradation of SMAD7 by activating the SMAD ubiquitination regulatory factor-2 (SMURF2)/arkadia-mediated ubiquitin-proteasome degradation pathway. This involves the formation of a SMURF2/SMAD7 complex, which targets T $\beta$ R-I ubiquitylation-mediated degradation. SMAD and T $\beta$ R-I ubiquitination is reversed by deubiquitination enzymes, which

include ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases and ovarian tumour proteases [48]. Three examples of deubiquitinating enzymes for T $\beta$ R-I are ubiquitin-specific peptidase-4 (USP4), -11 (USP11) and -15 (USP15), all of which antagonize the effect of SMAD7 and strongly induce TGF- $\beta$ 1 signalling [49]. The latent form of TGF- $\beta$ 1 can protect against renal fibrosis and inflammation by upregulating SMAD7 [50]. Expression of SMAD7 by active TGF- $\beta$ 1 is defective in chronic fibrosing conditions including EPS, related to interactions between AP-1, Sp1, SMAD proteins and counterregulation by nuclear factor- $\kappa$ B (NF- $\kappa$ B) [51]. Increased levels of SMURF2 and decreased SMAD7 promote the TGF- $\beta$ 1-induced repression of E-cadherin and upregulation of vimentin, thus promoting EMT/MMT [49]. HSP90 increases TGF- $\beta$ 1 signalling by binding to T $\beta$ R-I and T $\beta$ R-II, which protects these receptors from SMURF2/SMAD7-mediated ubiquitylation [37]. TRAF6-mediated cleavage of T $\beta$ R-I inhibits normal SMAD3 pathways of TGF- $\beta$ 1 signalling. The intracellular retention of the cleaved T $\beta$ R-I fragment and its nuclear translocation promotes angiogenesis and cellular invasion by Snail and Jag1 gene expression [45].

## TGF- $\beta$ 1 and non-SMAD signalling pathways

The SMAD 2/3 independent, non-canonical pathways are directly activated by the TGF superfamily members, including TGF- $\beta$ 1 and Activin [44, 52]. These pathways include NF- $\kappa$ B, Mitogen-activated protein kinases (MAPK) pathway components (ras, raf, ERK1/2, p38 MAPK, JNK), the phosphatidylinositol-3 kinase (PI3K):AKT:mTOR signalling axis, and cadherin junction regulators RhoA and RAC [53]. PI3K binds to and is activated by T $\beta$ R-I, leading to increased ECM production [49]. AKT also enhances TGF-induced SMAD signalling by increasing SMURF2 and USP4, which worsens peritoneal fibrosis in murine models of EPS [49]. PI3K/AKT contributes to HIF-1 $\alpha$  stabilization by provoking expression of heat shock proteins [54]. TAK-1 is a serine/threonine kinase which belongs to the MAP3K family. TAK1 functions in response to TRAF6 and TGF- $\beta$  receptor activation, and promotes downstream activation of JNK/c-Jun [55]. c-Jun transcriptional target genes, including receptor for activated C kinase1 (RACK1) and cyclin D1, lead to promotion of EMT [56]. Another non-canonical pathway is JAK interactions with T $\beta$ R-I to activate STAT3. Phosphorylated STAT3 is required for much of the TGF- $\beta$  target genes in hepatic stellate cells, with SMAD3/STAT3 being crucial in TGF- $\beta$  hepatic fibrosis [57]. c-Src is the mammalian proto-oncogene

homologue of the Rous sarcoma virus transforming protein v-src. The protein product of c-Src is Src, a non-receptor tyrosine kinase required for phosphorylation of T $\beta$ R-II, prior to TGF- $\beta$ -mediated p38 MAPK activation and cell invasion [58, 59]. The integrin-linked kinase (ILK) mediates TGF- $\beta$ -induced EMT through Snail and Slug, which is independent of the SMAD signalling pathway [60]. TGF- $\beta$  signalling via focal adhesion kinase (FAK) is involved in myofibroblast differentiation and remodelling of connective tissue.

TGF- $\beta$ 1 causes fibrosis by three main mechanisms:

- Myofibroblast differentiation via EMT/MMT/endothelial mesenchymal transition (Endo-MT).
- ECM production through canonical (SMAD 2/3-dependent) and non-canonical (non-SMAD dependent) pathways.
- Excessive accumulation of ECM by suppression of MMP and promotion of tissue inhibitor of metalloprotease (TIMP), connective tissue growth factor (CTGF) and PAI [22].

## TGF- $\beta$ , ROS and Redox fibrosis

ROS are formed by mitochondrial metabolism, inflammation, oxidative stress, ischaemia/reperfusion injury or exposure to ionizing radiation, ultraviolet light, chemotherapy or asbestos [34]. ROS activate expression of TGF- $\beta$ 1, and activated TGF- $\beta$ 1 also increases ROS production.

Activation of TGF- $\beta$ 1 by ROS appears to be direct, as release of recombinant latent TGF- $\beta$ 1 can occur in the absence of cellular machinery by ionizing radiation or by metal ion-catalysed ascorbate reactions. Activation of TGF- $\beta$  by ROS only occurs with TGF- $\beta$ 1 and not TGF- $\beta$ 2 or TGF- $\beta$ 3. This is related to ROS oxidation of the LAP-beta1 protein at methionine<sup>253</sup> [35]. The selective activation of TGF- $\beta$ 1 suggests specific DAMPs signalling for different latent TGF- $\beta$  family members. Increased ROS production by activated TGF- $\beta$ 1 is mainly due to induction of NADPH oxidases (NOX). TGF- $\beta$ 1-induced NOX 4 expression is associated with idiopathic pulmonary fibrosis and hepatic fibrosis. TGF- $\beta$ 1 upregulates NOX4 expression and the NOX4-dependent generation of hydrogen peroxide, which is required for TGF- $\beta$ 1-induced myofibroblast differentiation, ECM production and contractility [35]. Furthermore, TGF- $\beta$ 1 decreases antioxidant enzyme activity including Superoxide Dismutase 1 (SOD1), SOD2, Glutathione (GSH) peroxidase, GSH and Catalase (CTL). These are particularly involved in the suppression of Fenton reaction generation of hydroxyl radical, hydrogen

peroxide and superoxide [35]. The persistent activation of LTGF- $\beta$ 1 by oxidative stress and TGF- $\beta$ 1-mediated net persistence of ROS may represent a ROS/TGF- $\beta$ 1 positive feedback loop, leading to amplification of cellular damage, carcinogenesis and fibrosis [34]. Such disturbance in ROS generation and scavenging is referred to as “redox-fibrosis” [35].

## ROS, TGF- $\beta$ and platelets

Platelets contain 40–100 times the levels of TGF- $\beta$ 1 than other, non-neoplastic human cells. When released, 99% of the platelet-derived TGF- $\beta$ 1 is LTGF- $\beta$ 1 complexed with LAP. This is activated in a similar way to ECM TGF- $\beta$ 1 by exposure to integrins, MMP, plasmin or ROS. ROS and glucose degradation product (GDP)-related damage to the peritoneum is characterized by increased levels of 8-iso-prostaglandin F $_{2\alpha}$  (8-iso-PGF $_{2\alpha}$ ) [61–64]. 8-iso-PGF $_{2\alpha}$  is produced from arachidonic acid via non-enzymatic lipid peroxidation, catalysed by oxygen-free radical action on cell membranes and LDL. It is independent of cyclo-oxygenases (COX) and thus unaffected by COX inhibitors such as aspirin and indobufen. High glucose levels and protein glycation enhance LDL oxidation by metal ions, and these reactions also produce AGEs [62]. 8-iso-PGF $_{2\alpha}$  causes irreversible platelet aggregation in the presence of collagen, ADP, arachidonic acid, and PGH $_2$ /TXA $_2$  analogues that fail to aggregate platelets when acting alone [62]. Activated platelets release TGF- $\beta$ 1, VEGF, PDGF, fibroblast growth factor, EGF, HGF, and insulin-like growth factor (IGF) from their cytoplasmic  $\alpha$ -granules. This leads to increased migration of endothelial cells, neoangiogenesis, vascular permeability, fibrosis and MMT and EMT in the peritoneum [65]. Other agents that activate platelets include glycoprotein Ib alpha, sialyl Lewis x/sialyl Lewis a, Necl-5, integrins, TSP-1, high mobility group protein B1 (HMGB1) and podoplanin/Aggrus [65].

## Hypoxia induces HIF-1 $\alpha$ generation

In most human tissues under physiological conditions, the concentration of molecular oxygen lies between 10 and 30  $\mu$ M. Under these normoxic conditions, a member of the HIF- $\alpha$  prolyl hydroxylase family (PHD) hydroxylates one or both of the critical proline residues (pro<sup>402</sup> or pro<sup>564</sup>) in HIF-1 $\alpha$  chains [5]. HIF is a protein dimer, in which one of three different  $\alpha$  chains combines with one or two  $\beta$  chains.

Among HIFs, HIF-1 $\alpha$  and HIF-2 $\alpha$  are the best characterized. Hydroxylation of specific HIF- $\alpha$  proline residues increases the affinity of HIF- $\alpha$  peptide for the von Hippel-Lindau protein (pVHL)-elonginB-elonginC (VBC) complex by at least three orders of magnitude [66, 67]. Polyubiquitinated HIF- $\alpha$  is degraded by the 26S proteasome. Thus, under normoxic conditions, there are usually minimal HIF- $\alpha$  cellular levels due to rapid metabolism of HIF-1 $\alpha$  and HIF-2 $\alpha$  by ubiquitin-proteasomal pathways [68].

## Hypoxic conditions

Because tumours often have a hypoxic central “core” with inadequate diffusion of nutrients and a vascularized periphery, HIF generation is enhanced, which drives growth and proliferation of the cancer cells and the transformed stroma [69]. Under hypoxic conditions, the  $\alpha$  chain is no longer hydroxylated, HIF-1 $\alpha$  is stabilized and HIF-1 $\alpha$  cellular level increases. When stabilized, HIF- $\alpha$  is transported to the cell nucleus where it combines with HIF- $\beta$  to form the heterodimeric HIF. HIF activates the hypoxia response element (HRE) via transcriptional coactivator complexes, including p300/CBP and steroid receptor coactivator (SRC). The HRE has over 100 validated downstream transcription targets [70]. (Table 1) (Figure 2)

## HIF target genes affect glucose metabolism

The metazoan HIF pathway has evolved to sense cellular hypoxia, but also detect failure of oxidative phosphorylation and ATP production, the presence of mitochondrial oxidative stress, heat shock or molecular damage, loss of redox potential or iron deficiency. The HRE provides adaptive capability via transcriptional response pathways, including switching from mitochondrial respiration to anaerobic glycolysis as an alternative energy source. ATP yield of oxidative phosphorylation is almost 20 times higher with oxidative than with anaerobic glycolysis. HIF regulates the transcription of almost all the glycolytic enzymes and membrane glucose transporters (GLUTs). The upregulation of glycolytic enzymes by HIF and EMT-TF is also directly involved in EMT [71].

The “glycolytic switch” is mediated by HIF and Snail. Snail inhibits mitochondrial respiration by repression of cytochrome C oxidase via wnt/Snail signalling. Snail directly targets Fructose-1,6-bisphosphatase (FBP1), the rate limiting enzyme in gluconeogenesis. FBP1 repression also occurs via Zeb1. Loss of FBP1 decreases

**Table 1:** Target genes of HIF.

Function	Transcription target	Reference
Angiogenesis	VEGFA, VEGFR-1, PAI-1 production	[5, 81]
Erythropoiesis	EPO	[68]
Glucose metabolism	Hexokinase II (HK2), Phosphofructokinase-1, glucose 6 phosphate dehydrogenase (G6PD), Aldolase A (ALDOA), $\alpha$ -enolase (ENO1), phosphoglycerate kinase 1 (PGK1), pyruvate kinase M2(PKM2), pyruvate dehydrogenase kinase 1 (PDK1), Lactose dehydrogenase (LDHA) and SLC2A1	[71]
Cell growth	EGF, IGF-1, Nip3, Cyclin D/Cdk4	
Iron absorption and transport	Transferrin, ceruloplasmin, hemoxygenase-1, divalent metal transporter-1, duodenal cytochrome b, ferroportin	[68]
Drug resistance	Multidrug resistance transporter P-glycoprotein	[70]
Extracellular matrix production	Procollagen prolyl hydroxylase $\alpha$ 1, CTGF	[35]
Hormonal regulation	Leptin	[74]
Cell migration	c-Met, CXCR4, carbonic anhydrase IX (CAIX)	[27, 35]
Cell fate	RAGE expression	[27, 66, 68]
Epithelial-mesenchymal transition	Snail, Twist and Zeb1	[71]
Recognition of PAMPs and DAMPs	TLR2/4	[27]
Hypoxia-induced autophagy	HIF-1 $\alpha$ -STAT3-Src axis	[58]
Immune response	Programmed death-ligand 1 (PDL-1), Th17 differentiation	[27]
Lactate shuttling	Monocarboxylate transporter 4 (MCT 4)	[71]
Cytokine production	Interleukin 1 $\beta$ (IL-1 $\beta$ )	[27]

mitochondrial oxygen consumption and increases glucose uptake, glycolysis and lactate generation. Twist has been shown to upregulate LDHA, PKM2, HK2 and G6-PD in Twist-overexpressing cancer cells [71]. Loss of Rb or E2F-1 function leads to increased PDK4 expression and decreased glucose oxidation [72].

Active transport of glucose into cells occurs via the glucose transporter (SLC2A1/GLUT), which is dependent

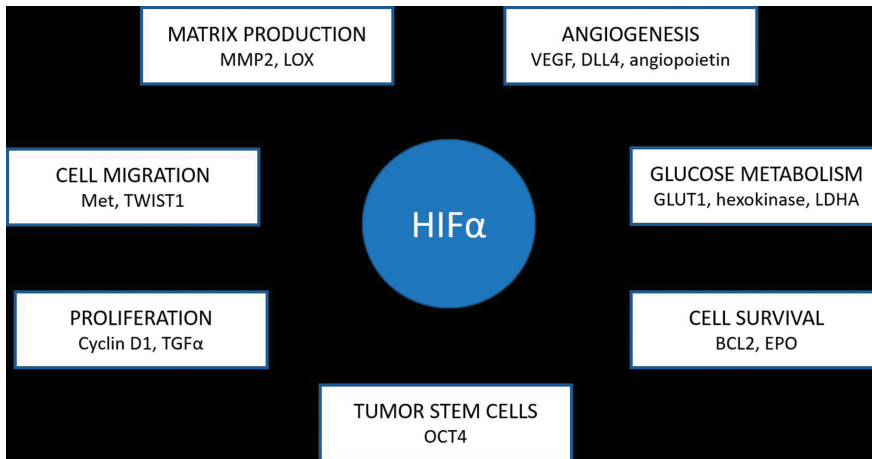


Figure 2: Target genes of HIF.

on ATP and thiamine for energy. Hexokinase II and Phosphofructokinase drive flux of glucose down the hexokinase pathway. G6-PD is important for ribose and NADPH production via the Pentose Phosphate Pathway, which is utilized by rapidly proliferating cells to increase their biomass synthesis [73]. Aldolase A (ALDOA) catalyses the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Pyruvate kinase (PK) converts phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP [71] in the final rate limiting step of glycolysis [74]. Lactate dehydrogenase A (LDHA) converts pyruvate to lactate, and pyruvate dehydrogenase kinase 1 (PDHK1) inhibits the conversion of pyruvate to acetyl co-enzyme A by PDH. Acetyl Co-A is required for normal functioning of the Krebs' cycle in mitochondria. PDHK1 causes build-up of the Krebs' cycle intermediates citrate, fumarate or succinate, leading to further normoxic stabilization of HIF-1 $\alpha$  [27]. Increased glucose transport and glycolytic enzymes, together with inhibition of mitochondrial oxidative phosphorylation, contribute to lactate production and the Warburg effect [75, 76]. Oncogenic growth signalling pathways such as PI-3K, Ras and Wnt are also involved in normoxic stabilization of HIF-1 $\alpha$  and the metabolic reprogramming of cancer cells [73]. Leptin is another target of HRE and also promotes cancer cell EMT, activates the PI3K/AKT signalling pathway and upregulates PKM2 expression [74].

### Role of glycolytic enzymes in EMT

Apart from their role in rapid generation of ATP and lactate, the glycolytic enzymes have also been found to promote EMT and carcinogenesis. These include ALDOA,

ENO1, PKM2, LDHA and PDK1. ALDOA upregulates N-cadherin and vimentin and downregulates E-cadherin [71]. Over expression of ENO1 is associated with tumour progression and poor prognosis in pancreatic cancer, cholangiocarcinoma, neuroendocrine tumours, neuroblastoma, prostate cancer, thyroid carcinoma, hepatocellular carcinoma (HCC) and breast cancer. ENO1 has been shown to promote cell proliferation, cycle progression, cancer migration and invasion [77]. ENO1 expression is upregulated by HIF under hypoxia. It is associated with increased plasmin and Ki67 activity and inversely correlated with p53 expression in pancreatic cancer tissues. This was reflected in larger tumour size, lymph node involvement and a poorer prognosis (HR = 2.469; 95% CI: 1.348–4.522;  $p = 0.003$ ) in pancreatic cancer patients with high pancreatic ENO1 levels. High expression of ENO1 was found on IHC in 47% of pancreatic cancer tissue specimens [78].

PKM2 is not normally found in adult human cells, but is overexpressed in HCC and oesophageal cancer [71]. PKM2 translocates to the nucleus under the effect of ERK and EGF, and acts as a protein kinase via interactions with Src,  $\beta$  catenin and C-myc. Activated  $\beta$  catenin increases cyclin D1 and the expression of SNAIL and vimentin, and decreases expression of E-cadherin. EMT is also mediated by PKM2 interacting with TGF- $\beta$ -induced factor homeobox 2, which recruits histone deacetylase 3 to the promoter of E-cadherin and represses its expression [71]. PKM2 phosphorylates STAT3 using PEP as the phosphate donor, a process independent of Jak2 and Src pathways. p-STAT3 activates downstream EMT genes involved in the migration and motility of many cancer types. STAT3 is usually activated by inflammatory cytokines such as IL-6, and phosphorylation by PKM2



provides a plausible pathway for constitutive STAT3 activation in cancers [79]. C-Myc promotes the expression of heterogeneous nuclear ribonucleoproteins which favours alternative splicing of PK to generate PKM2 isoform in tumour cells. PKM2 has low activity in the production of pyruvate from PEP, as compared to the high activity of native PK. Thus PKM2 directs glucose metabolism towards lactate production rather than mitochondrial oxidative phosphorylation [80].

PKM2 is a direct target gene of HRE, and HIF-1 controls the expression of PKM2. PKM2 is hydroxylated at Pro403 and Pro408 by PHD3, which enhances the interaction of PKM2 with HIF-1 $\alpha$ . PKM2 also enhances HIF-1 $\alpha$  occupancy and histone acetyltransferase p300 recruitment, which may prevent negative regulation by FIH-1. PKM2 promotes the HIF activated HRE targets in glycolysis including genes that mediate increased glucose uptake (GLUT1), increased lactate production (LDHA), decreased oxidative metabolism (PDK1), and also the HRE target genes for increased angiogenesis (VEGF) [81]. The activation of HIF-1 $\alpha$  by PKM2 and downstream transcription of PKM2 by the HRE represents a positive feedback loop that cancer cells utilize to enhance glycolysis, lactate production, cellular proliferation, angiogenesis, EMT and the Warburg effect. (Figure 2)

## TGF- $\beta$ 1 and HIF-1 $\alpha$ are interdependent

HIF-1 $\alpha$  expression is increased 2.2-fold by TGF- $\beta$ 1 alone under normoxic conditions. Under hypoxic conditions, TGF- $\beta$ 1 enhances hypoxia-stimulated HIF-1 $\alpha$  expression 1.4-fold above that seen with hypoxia alone [82]. TGF- $\beta$ 1 decreases the expression of HIF PHD2 via a SMAD2/3-dependent mechanism, resulting in HIF-1 $\alpha$  stabilization and up-regulation of collagen I and PAI expression in renal epithelial cells [83]. Both SMAD3 and HIF-1 $\alpha$  are interdependent contributors to TGF- $\beta$ 1-stimulated collagen expression under cellular normoxia. Inhibiting HIF-1 $\alpha$  decreases TGF- $\beta$ 1-induced SBE-Luc activation, collagen I mRNA expression and promoter activity. Disruption of SMAD3 signalling decreases normoxic, TGF- $\beta$ -stimulated, HIF-1 $\alpha$ -mediated induction of HRE activity [82]. HIF-1 $\alpha$  protein stability is also regulated through an O<sub>2</sub>-independent pathway involving the interaction of HSP90 and RACK1 [84].

EMT of renal tubular epithelial cells into myofibroblasts and tubulointerstitial fibrosis is related to the downstream targets of HRE, stromal cell-derived factor-1 also named

CXCL12 and its receptor CXCR4 [35]. Fibrosis is correlated with expression of HRE target genes for ECM formation including PAI-1, TIMP-1 and CTGF [35]. Late TGF- $\beta$ 1-induced deposition of ECM contributes to hypoxia in the peritoneal submesothelial fibroproliferative zone. This induces a secondary hypoxic response of HIF-1 $\alpha$  expression and further angiogenesis (Figure 2). The mTOR inhibitor rapamycin blocks these late hypoxia/VEGF-induced angiogenic effects, but does not influence the direct TGF- $\beta$ -mediated fibrosis and peritoneal VEGF angiogenesis in EPS [85].

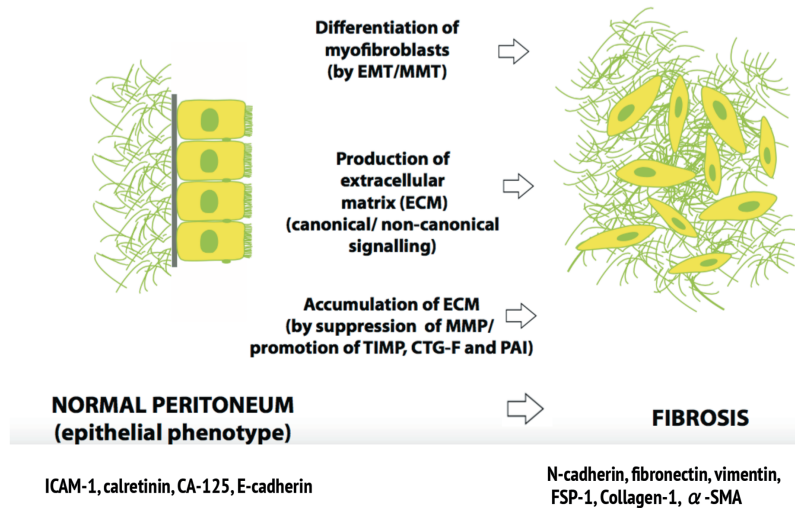
## Pathophysiology of EPS

In this section, we will focus on the effect of non-physiologic conditions on the peritoneum, including peritoneal dialysis and intraperitoneal chemotherapy (IPC) solutions. EPS is a chronic fibrosing condition of the peritoneum with sclerosis, calcification, thickening or encapsulation of the small intestine [86]. EPS was first described by Owtschinnikow in 1907 as “peritonitis chronica fibrosa incapsulata” [87]. EPS is classified as primary or secondary. Secondary EPS is most commonly caused by CAPD. It has also been described after major abdominal surgery, generalized peritonitis, peritoneal malignancy, IPC, EM, peritoneal lavage with sclerosing antiseptics such as chlorhexidine gluconate,  $\beta$ -blocker treatment (practolol/propranolol), Familial Mediterranean Fever, protein S deficiency, abdominal tuberculosis, autoimmune diseases or sarcoidosis [87–89]. Simple peritoneal sclerosis tends to affect the parietal peritoneum without intestinal encapsulation, and is common in CAPD [90]. It is unclear whether EPS is related solely to progression of simple sclerosis [91]. However, it was shown in a mouse model that MMT and peritoneal fibrosis progress in direct proportion to the duration of CAPD [17] (Figure 3).

Symptoms and signs of EPS include bloody ascites, anorexia, nausea, diarrhoea and abdominal pain. Early EPS can be associated with inflammatory features of elevated CRP, fever, malaise, anaemia and hypoalbuminaemia. Progression of intestinal encapsulation leads to constipation, abdominal mass, weight loss, severe malnutrition and intermittent subacute bowel obstruction. Diagnosis is based on clinical presentation, radiological imaging, peritoneal biopsy and analysis of peritoneal dialysate fluid [92].

## EPS in CAPD – the genesis of a wound

The frequency of EPS in CAPD is directly related to the duration of peritoneal dialysis. A 1998 Australian



**Figure 3:** Progression of peritoneal fibrosis mediated by TGF.

multicentre, retrospective, observational cohort study of 7,374 patients reported the EPS risk with time was 1.9%, 6.4%, 10.8% and 19.4% after 2, 5, 6 and 8 years of CAPD [93]. The subsequent 2010 Binational Australasian Registry of 7,618 patients found the EPS risk with time had fallen to 0.3%, 0.8% and 3.9% after 3, 5 and 8 years of CAPD [92]. This may be related to changes in PDF biocompatibility and dialysis practices in this time period. However, the EPS risk varies greatly between different countries, ranging from an incident rate/1,000 patient years of 1.8 in Australia and New Zealand (1995–2007) to 13.6 in Scotland (2000–2007). The mortality of EPS is substantial (25–56%), and in some case series up to 60% of patients may die within 4 months of diagnosis [87, 94].

The use of acidic, hyperosmolar, hyperglycaemic, bio-incompatible dialysis fluid generates the initial damage to the peritoneum in CAPD. This is mediated in part by uraemia, lactate, GDP and AGEs [95, 96]. AGEs are formed by the Maillard reaction, a process of irreversible non-enzymatic glycation and oxidation of proteins, nucleic acids, and lipids [97, 98]. Further damage to the peritoneum promotes the development of EPS. This “second-hit” injury includes recurrent infectious peritonitis (*Pseudomonas*, *S. aureus* or fungal spp.), endotoxins (LPS) or recurrent haemoperitoneum in CAPD [17]. Similarly, when the peritoneum is repeatedly or severely injured in other disease processes such as PM, IPC, EM or chemical peritonitis, EPS may also result. EPS is typified by progressive loss and MMT of PMC, inflammatory cell infiltrate with persistent secretion of cytokines, growth factors, and chemokines, and a complex feedback network of chronic peritoneal inflammation.

The signalling pathways involved in EPS include TGF- $\beta$ 1 (and TGF- $\beta$ 1 receptors), integrin-linked kinases (ILK), TRAF6, IL-6, NF- $\kappa$ B, AGE-RAGE, HRE, Src, tyrosine kinase receptors, protein kinase C (AKT), glycogen synthase 3, Wnt/ $\beta$ -catenin, platelet activation and VEGF [37].

### Indicators of EPS: peritoneal cytokeratin markers and cytokine levels

Indicators of EPS in CAPD include failure of PD ultrafiltration [99], declining small solute clearance, decreased glucose reabsorption (D2/D0), higher dialysate-to-plasma urea ratio (D/Purea) [87, 100, 101], low levels of CA-125 and high levels of cytokines IL-1 $\beta$ , IL-6, IL-8, HGF, PDGF, TGF- $\beta$  and VEGF in the dialysate [91, 102, 103]. Abrahams et al. [104] reported increased gene expression of CCN2 (35-fold), TGF- $\beta$ 1(24-fold) and VEGF (77-fold) in the peritoneal membrane of EPS patients compared to CAPD patients without EPS. Sampimon et al. [105] analysed the time course of low CA-125 and elevated IL-6 preceding the diagnosis of EPS. In CAPD patients with ultrafiltration failure, a dialysate appearance rate of CA-125 less than 33 U/min and IL-6 greater than 350 pg/min had a sensitivity of 70% and a specificity of 89% for the diagnosis of EPS. The disappearance of CA-125 from CAP dialysate is related to the mesenchymal transformation of PMC (MMT) and loss of epithelial cytokeratin characteristics [95]. Levels of IL-6 in dialysate efflux reflect the continuing peritoneal inflammatory response and predict the loss of individual peritoneal membrane solute transport function [103].

## Role of TGF- $\beta$ in EPS

Bio-incompatible dialysis fluid, reactive carbonyl species/GDPs (e.g. formaldehyde, glyoxal, methylglyoxal, 3-deoxyglucosone) and formation of AGEs stimulate production of TGF- $\beta$ 1 [106, 107]. Both hyperglycaemic PDF and cellular damage/necrosis cause release of the nuclear protein HMGB1 also named amphoterin. HMGB1 normally resides in the nucleus and regulates DNA replication, transcription, recombination and repair. When released into the extracellular space, HMGB1 also acts as a DAMPs signalling ligand. This is dependent on HMGB1 binding to pattern recognition receptors (PRR), including TLR-2, TLR-4 and RAGE. There is cross ligand binding of different PRRs by endogenous “alarmin” molecules including AGEs, HMGB1, macrophage-1 antigen (MAC-1) and calcium binding protein (S-100), as well as exogenous LPS from gram negative bacteria. Microbial-derived ligands such as LPS are called pathogen-associated molecular patterns (PAMPs). Both DAMPs and PAMPs can synergize in the synthesis and secretion of pro-inflammatory cytokines such as IL-1 $\beta$  in CAPD peritonitis [108]. Activation of RAGE and TLR-4 strongly upregulates MAPK/NF- $\kappa$ B and protein kinase C (AKT) signalling pathways, and CTGF and TGF- $\beta$  release during peritoneal inflammation [98].

NF- $\kappa$ B is a transcription factor which is kept inert by its cytoplasmic inhibitor protein, I- $\kappa$ B. Degradation of I $\kappa$ B and the subsequent nuclear translocation of NF- $\kappa$ B results from cell/PRR/RAGE stimulation by a variety of agents, examples of which include HMGB1, AGEs, DAMPS, PAMPs, oxidized LDL, ROS, inflammatory cytokines, mitogens, angiotensin II, HSP60, BMP2/4, erythropoietin receptor and HA [109, 110]. HMGB1 stimulates MCs to secrete monocyte chemoattractant protein 1 (MCP-1) and IL-8, which causes recruitment of leukocytes to the peritoneum. In addition to MCP-1 and IL-8, endothelial cells also secrete TNF- $\alpha$  in response to HMGB1 [111]. Release of TGF- $\beta$ 1 promotes peritoneal angiogenesis via MMT of PMC, myofibroblast differentiation and VEGF release, and also by the expansion of hypoxic submesothelial ECM/tissue expressing HIF-1 $\alpha$ . Normal peritoneum does not contain myofibroblasts [19]. Lineage tracing shows myofibroblasts can be derived from mesothelial cells (MMT), epithelial cells (EMT), resident pericytes (fibroblast to myofibroblast transition), endo-MT, Th17 lymphocytes or CD34+ bone marrow-derived cells. *In situ* MMT may also contribute to submesothelial myofibroblast differentiation via cellular and ECM signalling [112].

Blocking the cell surface receptor for AGE (RAGE) with monoclonal antibodies (anti-RAGE Ab) reduced TGF- $\beta$ 1-related peritoneal MMT, interstitial fibrosis and vascular sclerosis in a murine uraemia model. Peritoneal AGE accumulation in uraemia results from reactive carbonyl compounds derived from either carbohydrates or lipids. Heat sterilization of glucose containing dialysis fluid may contribute to GDPs and AGE formation. GDPs have strong oxidant properties and can be directly cytotoxic to mesothelial cells or cause AGE accumulation [113].

## Progression of EPS

Podoplanin-positive myofibroblasts derived from PMC MMT in a methylglyoxal-induced murine peritoneal injury model may reflect the progression of fibrosis in human CAPD [114]. In patients with EPS, peritoneal podoplanin is expressed by activated calretinin-positive mesothelial cells, lymphatic endothelial cells, and  $\alpha$ -SMA-positive myofibroblasts. The level of podoplanin expression in peritoneal biopsies can be useful in distinguishing simple peritoneal fibrosis from EPS [90]. Peritoneal fibrosis often accelerates after renal transplantation, as calcineurin (CN) inhibitors such as Tacrolimus and Cyclosporin upregulate TGF- $\beta$ 1 [91]. Use of m-TOR inhibitors such as Everolimus instead of CN inhibitors after renal transplantation may reduce the risk of EPS [95]. Histological changes in hyalinizing vasculopathy and EPS, and the use of diagnostic peritoneal biopsy have been recently reviewed in this journal [89].

## Pathophysiology of PM

TGF- $\beta$ 1 is a potent inducer of apoptosis and suppressor of proliferation and immortalization in normal epithelial cells [36]. However, TGF- $\beta$ 1 can lose this cytostatic effect and promote EMT, cancer-associated fibroblasts (CAFs) and continued production of ECM. This is referred to as the TGF- $\beta$  paradox, which is a feature of the tumour micro-environment and is related to multiple factors [37, 39, 53]. (Table 2, Figure 4)

Upregulation of TGF- $\beta$ 1 signalling and HIF expression occurs in colon, esophageal, gastric, hepatocellular and pancreatic carcinoma. This is associated with tumour progression, metastasis, angiogenesis and a poor prognosis [128–130]. For example, elevated TGF- $\beta$ 1 signalling and increased p-SMAD2/3 staining was found in 16% of

Table 2: TGF- $\beta$ 1 paradox.

Action	Mechanism	Ref.
Loss of TGF tumour suppression	TP53/Rb mutations leading to aberrant TGF- $\beta$ 1 signalling	[43]
Loss of TGF tumour suppression	TGF mutations (40–50% for SMAD4, 3–10% for TGFBR1), overexpression of TGF $\beta$ 1,2,3 and Src in pancreatic ductal cancer	[115]
HIF promotion	TGF- $\beta$ normoxic stabilization of HIF/production of SNAIL	[82]
VEGF	Association of HIF-1 $\alpha$ and SMAD3 cooperatively activating the VEGF promoter.	[116]
Src activation	Activation of Src by 17 $\beta$ -estradiol and enhanced TGF- $\beta$ 1/SMAD signalling and cyclin D	[117]
Glycolysis	HIF-induced glycolytic switch.	[68, 70]
Lactate	Lactate production from the Warburg effect and shuttling of lactate via MCT4 and CAFs.	[75, 118]
EMT	Glycolytic enzyme induced EMT.	[71]
Stromal recruitment	Recruitment of tumour-associated macrophages (TAM), tumour associated neutrophils (TANS), Th17 and CAFs by TGF- $\beta$ 1 in the TME.	[119, 120, 136]
USP4	Stabilization of vimentin by USP4 in gastric cancer and associated down-regulation of miR-320a. Overexpression of USP4 is found in colorectal, ovarian and lung adenocarcinomas.	[121]
HSP90	Stabilization of T $\beta$ R-I and T $\beta$ R-II receptors by HSP90.	[37]
RAGE	Persistent RAGE activation by its ligands and feed forward expression of RAGE via RAGE-NF- $\kappa$ B signalling.	[97, 122]
TRAF	TRAF6 activation of SMAD/non-SMAD signalling and Redox fibrosis.	[61]
ILK	ILK activity-dependent formation of the ILK/Rictor complex, which is required for TGF- $\beta$ 1-mediated EMT. This occurs in epithelial cancers but not in normal epithelial cells.	[60]
IL-17	Immune evasion due to TGF- $\beta$ 1 secreted from regulatory T cells (Tregs) up-regulating IL-17rb through downstream SMAD2/3/4 signalling.	[123]
Activin	TGF- $\beta$ -induced activin pathway activation selectively enhancing cancer cell migration and not TGF- $\beta$ -induced growth suppression. Both tumoural TGF- $\beta$ 1 and activin expression in human colorectal cancer = shorter survival (median OS 11.5 months vs. 67 months, $p \leq 0.05$ )	[44, 52]
CAFs	T $\beta$ R-I down-regulation in cancer cells and continued TGF- $\beta$ 1 response in CAFs. Excessive TGF- $\beta$ 1 in the TME due to CAF-TGF- $\beta$ 1 feed forward autocrine effect, and tumour cell-platelet aggregates.	[136]
Trogocytosis	Trogocytosis and release of platelet-derived TGF- $\beta$ 1. Activated platelets coat tumour cells with a cell-fibrin-platelet aggregate shield, protecting them from shear forces and host immune attack by natural killer T lymphocytes. Transference of MHC class I proteins and glucocorticoid-induced tumour necrosis factor receptor-related (GITR) ligand from platelets to the tumour cells ( <i>trogocytosis</i> ) also assists in evasion from host immunosurveillance. Trogocytosis disrupts the recognition of tumour cell missing self, resulting in impaired cytotoxicity and IFN- $\gamma$ production by NK cells.	[124–126]
Platelet-derived TGF- $\beta$ 1	Platelet-derived TGF- $\beta$ 1, released upon tumour cell-platelet interaction, also inhibits NK-cell mediated immunosurveillance through down-regulation of the activating NK receptor NKG2D. Formation of ovarian cancer spheres, chemoattraction, cancer cell migration, EMT and stem cell markers is increased by platelets. This enhances both haematogenous and transcoelomic metastasis.	[124, 127]

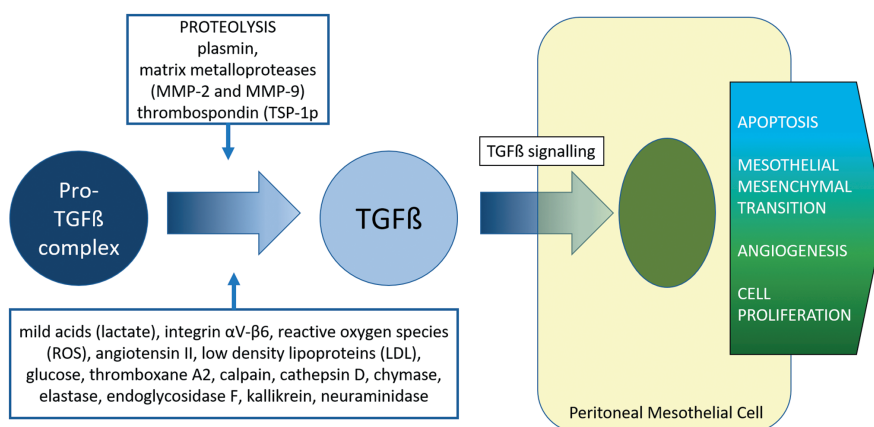


Figure 4: The TGF paradox.



metastatic colorectal cancers. This was associated with the worst outcome, no response to chemotherapy (cisplatin, oxaliplatin or 5-FU), and a mesenchymal phenotype [131]. Increased lncRNA-ATB production via autocrine TGF- $\beta$ /ZEB/miR-200 signalling was also shown to be an independent risk factor for invasion, lymph node metastasis, distant metastasis, higher tumour stage and poorer disease free survival, recurrence free survival and overall survival in gastric cancer, CRC and HCC. High levels of lncRNA-ATB were also associated with trastuzumab resistance in breast cancer patients [132–134].

The initial adhesion of carcinoma cells to the mesothelium is mediated by “cross talk” involving ligand-receptor pairs, including  $\alpha$ 5 $\beta$ 1 integrins–fibronectin,  $\alpha$ V $\beta$ 3 integrins–vitronectin (VN), and CD44–hyaluronic acid. Disruption of the mesothelial glycocalyx and HA oligomer release by ROS, physical trauma, peritoneal desiccation, hypoxia or reperfusion injury enhances carcinoma cell adhesion, inflammatory cytokine release, and peritoneal MMT [12, 15]. The non-aggressive ovarian carcinoma cell line (FOC3) phenotype could be transformed to that of metastasizing ovarian cancer (MFOC3) by exposure to IL-1 $\beta$ . Endogenous IL-1 $\beta$  production by MFOC3 cells stimulated mesothelial cell surface  $\beta$ 1 integrin expression and enhanced cancer cell adhesion via a paracrine IL-1 $\beta$ / $\beta$ 1 integrin axis [135].

Inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  act synergistically with TGF- $\beta$ 1 in upregulating VEGF and IL-6 production in MCs. Normal murine peritoneum which has been activated by TGF- $\beta$ 1 is more receptive to subsequent intraperitoneal ovarian cancer cell inoculation and tumour growth. Even the calretinin positive staining peritoneum distant to malignant implants in metastatic ovarian cancer patients showed nuclear expression of pSMAD3. This indicated that the TGF- $\beta$ 1 pathway had already been widely activated in the peritoneal cavity [136]. Autocrine/paracrine pathways of tumour cell IL-1 $\beta$ / $\beta$ 1 integrin, TGF- $\beta$ 1/TGF- $\beta$ 1R and VEGF/VEGR ligand binding also promote peritoneal inflammation, cancer cell adhesion and MMT. PMC are transformed into CAFs [137] which promote cell migration, ECM invasion, MMP-2 and -9 release, cleavage of ECM proteins, release of ECM latent VEGF and TGF- $\beta$ 1 and further carcinoma cell invasion. Ovarian and gastric cancer xenografts grow more rapidly when co-injected with omental MCs than with cancer cells alone [138, 139]. Such recruitment and transformation of PMCs into CAFs by cancer cells results in a neovascularized stroma, which is the advancing front of PM and ascites formation [137].

Interestingly, the VHL gene is known a tumour-suppressor gene. Inactivating germline mutations of the

gene predisposes affected individuals to the development of several hereditary tumours. Familial VHL syndrome is related to loss of VHL protein, failure of ubiquitylation of HIF- $\alpha$  and promotion of vascular, neuroendocrine and renal cell tumours. This has similarities to the normoxic stabilization of HIF- $\alpha$  by TGF- $\beta$ 1 which enables downstream VEGF release in PM [140].

The Warburg effect may, in part, explain the cancer cachexia syndrome seen in patients with advanced peritoneal metastatic disease. Weight loss can be exacerbated by peritoneal carbonyl stress or continued IL-1 release, causing anorexia and food avoidance. Cancer cachexia and age-related sarcopenia may also be related to TGF- $\beta$ 1 stimulation of myostatin, activin A and activin B, which results in profound muscle wasting [28, 141].

## Common pathophysiological features in EPS, EM and PM

For the clinician, diseases such as EPS, EM and PM may appear unrelated pathological entities. The unprepared reader may not suspect that their underlying pathophysiology is indeed quite similar. The pretext for understanding the pathophysiology of these diseases is to realize that damage signalling and wound healing mechanisms are involved in the peritoneal response to various kinds of injury, including EMT and MMT.

EPS is a chronic fibrosing condition of the peritoneum with sclerosis, calcification, thickening or encapsulation of the small intestine [86]. EPS is most commonly due to repeated injury of the peritoneum caused by CAPD. On histology, EPS is typified by progressive loss and MMT of PMC, inflammatory cell infiltrate with persistent secretion of cytokines, stromal growth factors, and chemokines, and a complex feedback network of chronic peritoneal inflammation [17].

EM, a chronic estrogen-dependent gynecological disease, occurs in 10–15% of reproductive-age women. During the proliferative phase of the menstrual cycle, endometrial cells proliferate under the influence of 17 $\beta$ -estradiol, which activates telomerase activity [142, 143]. In EM, this stem cell-like self-renewal ability is enhanced, together with estrogen effects on VEGF release. 17 $\beta$ -Estradiol production is increased due to aromatase activity in EM [142], which drives EMT via HGF, and TNF- $\alpha$  and IL-6 release from peritoneal macrophages [144]. Markers of EMT including overexpression of SLUG, SNAIL, TWIST1 and repression of E-cadherin (CDH1) are found in ectopic compared to eutopic endometrium. E-cadherin-

negative, N-cadherin-positive endometriotic epithelial cells show invasive growth *in vitro*. Increased expression of MYC, Cyclin D1 and Ki67 genes in EM reflects higher levels of proliferation and loss of cell cycle regulation [144, 145]. EM may share common features with type 1 ovarian cancer, including replicative advantage, evasion of apoptosis, genetic changes and tumour suppressor gene mutations [143]. Type 1 ovarian carcinoma (OC) includes EM-associated carcinoma (Clear cell ovarian cancer (CCC)) and endometrioid ovarian tumour, and has different genomic profiles to the more common Type 2 or high grade serous ovarian cancer (HGSC) [146].

Loss of heterozygosity (LOH) or somatic loss of function mutations in tumour suppressor genes including phosphatase and tensin homolog (PTEN), TP53, p16Ink4, human MutL homolog 1 (hMLH1) and B cell lymphoma-2 (BCL-2) have been reported in both EM and type 1 OC. Other genes that may be affected by LOH include the estrogen receptor, progesterone receptor, Apolipoprotein A2, and SOD genes [142, 147]. Endometrioid ovarian cancer has frequent mutations in PTEN, catenin  $\beta$ 1 (CTNNB1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$  (PIK3CA) and AT-rich interaction domain 1A (ARID1A). PIK3CA and ARID1A are also commonly mutated in CCC together with hepatocyte nuclear factor-1 $\beta$  (HNF-1 $\beta$ ) overexpression [146]. PTEN is an important suppressor of the PI3K pathway, and loss of PTEN was found in 0% of normal endometrium, 20.6% of EM cysts, 0–5% of CCC and 14–31% of endometrioid ovarian tumours [148]. Loss of PTEN and K-ras mutations appear to act synergistically in the progression from EM to type 1 ovarian cancer [143, 149]. Methylation of human androgen receptor gene (HUMARA) and phosphoglycerate kinase 1 (PGK-1) genes on the X chromosome are used as markers for monoclonal EM associated ovarian cancers arising from EM. This supports the concept of the EM-type 1 OC sequence [142, 150].

The presence of ectopic menstrual blood and high levels of free iron ions in endometriotic cysts leads to the production of ROS through the Fenton reaction. ROS and IL-1 are also released from activated tissue-associated macrophages. This may contribute to release of PGF2 $\alpha$  into the peritoneal micro-environment, which promotes pelvic pain and oncogene and inflammatory cytokine activation [142]. NF- $\kappa$ B and AP molecular pathway activation by ROS are mediated by convergence of the molecular signals received from activation of receptor activator of NF- $\kappa$ B and TGF- $\beta$ 1 receptors [142]. Down-regulation of miR-33b in EM compared to normal uterine endometrium was found to enhance endometrial proliferation and

VEGF and MMP-9 release [151]. Thus EM lesions grow by EMT, BM invasion, TGF- $\beta$ 1-induced transformation and stromal recruitment, in a similar way to PM [151].

Peritoneal metastases are a deadly mode of transcoelomic tumour spread in gastrointestinal and gynecological cancer. In order to survive, PM induce the vascularized connective tissue stroma that they require. The formation of a vascularized connective tissue stroma is mediated by VEGF. VEGF increases vascular permeability of endothelial cells with a potency 50,000 times that of histamine, allowing egress of plasma and platelets into the extravascular space and exposure to the ECM. VEGF release in PM thus initiates a chain of events which resemble that of wounds, including CVH, fluid extravasation, platelet activation, fibrin deposition, chemotaxis and inflammatory cytokines [7].

TGF- $\beta$ 1 induces a Warburg effect by switching cellular energy production from mitochondrial phosphorylation to cytosolic glycolysis in EM and peritoneal malignancies, resulting in excess lactic acid production and stabilization of HIF- $\alpha$  [27]. The lactate appears to be derived from transformed PMC adjacent to endometriotic lesions, metastatic carcinoma or tumour-associated macrophages (TAM). Lactate is pumped out of cells into the extracellular space by monocarboxylate 4, which contributes to extracellular acidosis in the coelomic micro-environment [46, 71]. TGF- $\beta$ 1 is released from the latency-associated peptide (LAP) and LTBP when exposed to factors such as ROS, plasmin or acid [22]. Increased lactate can activate latent TGF- $\beta$ 1, leading to a positive feedback loop of increasing TGF- $\beta$ 1 and lactate. This causes further mesenchymal transformation of PMC, promoting EM cell invasion or progression of peritoneal metastases [75]. Extracellular acidosis aids lamellipodia formation, genome instability, cysteine protease, MMP and glycosidase breakdown of the ECM, tumour invasion, angiogenesis, drug resistance, and immune evasion [71]. Thus the transformed PMC create an acidic, pseudohypoxic micro-environment which is conducive to continued inflammation, fibrosis and tumorigenesis [71, 152].

## Conclusions

The peritoneal response to various kinds of injury involves loss of PMC, activation of PRRs, EMT and MMT. EPS, EM and PM are all characterized by hypoxia and formation of a vascularized connective tissue stroma mediated by VEGF. TGF- $\beta$ 1 is constitutively expressed by the PMC and plays a major role in the maintenance of a transformed, inflammatory coelomic

micro-environment in PM, but also in EPS and EM. Persistently high levels of TGF- $\beta$ 1 or stimulation by inflammatory cytokines (IL-6) induce peritoneal MMT, adhesion formation and fibrosis. TGF- $\beta$ 1 enhances hypoxia-stimulated HIF-1 $\alpha$  expression, which drives cell growth, ECM production and cell migration. Disruption of the peritoneal glycocalyx and exposure of the BM release LMW-HA and ECM components, initiating a cascade of pro-inflammatory mediators, including peritoneal cytokines (TNF- $\alpha$ , IL-1, IL-6, prostaglandins), growth factors (TGF- $\alpha$ , TGF- $\beta$ , PDGF, VEGF, EGF) and the fibrin/coagulation cascade (thrombin, Tissue factor, PAI-1/2). Chronic inflammation and cellular transformation are mediated by DAMPs, PRR, AGE-RAGE, extracellular lactate, pro-inflammatory cytokines, ROS, increased glycolysis, TGF- $\beta$ 1 release, metabolomic reprogramming, TAM and CAFs. The pathogenesis and chronic progression of EPS, EM and PM have common pathways, related to dysregulated mechanisms of wound healing, just as Harold Dvorak originally postulated in 1986.

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