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Epithelial to mesenchymal transition in the pathogenesis of uterine malignant mixed Müllerian tumours: the role of ubiquitin proteasome system and therapeutic opportunities

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Abstract Malignant mixed Müllerian tumours (malignant mixed mesodermal tumours, MMMT) of the uterus are metaplastic carcinomas with a sarcomatous component and thus they are also called carcinosarcomas. It has now been accepted that the sarcomatous component is derived from epithelial elements that have undergone metaplasia. The process that produces this metaplasia is epithelial to mesenchymal transition (EMT), which has recently been described as a neoplasia-associated programme shared with embryonic development and enabling neoplastic cells to move and metastasise. The ubiquitin proteasome system (UPS) regulates the turnover and functions of hundreds of cellular proteins. It plays important roles in EMT by being involved in the regulation of several pathways participating in the execution of this metastasis-associated programme. In this review the specific role of UPS in EMT of MMMT is discussed and therapeutic opportunities from UPS manipulations are proposed.

Keywords Malignant mixed Müllerian tumours · Malignant mixed mesodermal tumours · Endometrial carcinosarcoma · Ubiquitin proteasome system · Epithelial to mesenchymal transition

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Introduction: malignant mixed Müllerian tumour as a metaplastic carcinoma

Malignant mixed Müllerian tumours (MMMTs), also referred to as endometrial carcinosarcomas or malignant mixed mesodermal tumours, are neoplasms with a debated histogenesis in the past. Currently evidence from various studies points to an epithelial histogenesis with extensive metaplasia that is seen histologically as a sarcomatoid element. Most commonly the epithelial component is serous followed by endometrioid while the most common sarcomatoid component is stromal and less commonly leiomyosarcoma or heterologous elements such as rhabdomyosarcoma, osteo-, chondro- or liposarcoma [1].

The presence of two different elements, epithelial and sarcomatous, had initially prompted various theories for its pathogenesis. The collision theory put forward the presence of two concomitant distinct malignancies while two other non-mutually exclusive theories, the stem cell (also called combination theory) and the metaplastic monoclonal theory (also called conversion theory), argued for a common origin of the two components, either from a pluripotent endometrial stem cell that can create both or from the transformation of part of the epithelial component to morphologically sarcomatous areas. The fact that in some cases the sarcomatous element is heterologous suggests that the metaplastic process is associated with the neoplastic process and is not part of the reactivation of a pluripotent potential in a normal resident stem cell. This may be more consistent with the metaplastic theory rather than the stem cell theory. Nevertheless it remains possible that the initiating lesion takes place in an epithelial stem cell. CD133+ stem cells have been identified in MMMTs [2]. In view of the role of EMT in MMMT (see next section), it is

important to note that the stem cell phenotype is associated with EMT [3].

Stem cell and/or metaplastic origin of MMMT are supported by experimental data arguing for a common initiating source of the two components. Intuitively this is also more probable whereas two neoplasms in the same patient and organ would be unlikely. Epidemiologic data show that MMMT shares risk factors such as obesity and nulliparity with endometrial carcinoma [4], a fact that may imply a common pathogenesis. MMMT natural history with initial metastases through lymph nodes mimics carcinoma. In contrast, uterine sarcomas spread through haematogenous metastases, commonly to the lung [5, 6].

The common origin of the two components is supported by various experimental studies. An *in vitro* evaluation of a MMMT cell line and clones derived from it showed stable expression of epithelial markers as well as vimentin and electron microscopy showed that even spindle shaped cells retained some epithelial characteristics [7]. In a study of epithelial and sarcomatous clones of a MMMT cell line, only the epithelial clone could produce both morphologies in culture while the sarcomatous clone could give rise to only sarcomatous elements [8]. Similarly experiments of this epithelial clone of MMMT cells xeno-transplanted to mice showed that these cells could give rise to tumours with both components *in vivo* [8]. Metaplastic carcinomas of other sites such as the breast are also of monoclonal origin [9, 10].

Epithelial to mesenchymal transition in MMMT

Epithelial to mesenchymal transition (EMT) is a phenomenon that takes place in normal development where cells of epithelial layers lose their attachment to their neighbouring cells, acquire a fibroblast-like morphology and become motile to populate other structures during organogenesis and histogenesis in the foetus. In carcinogenesis, a pathologic EMT allows cancer cells to become invasive at the tissue level and subsequently metastasise [11].

Evidence that MMMT represents a metaplastic carcinoma of the endometrium with extensive EMT is complemented by morphologic observations in a sub-set of endometrial carcinomas mainly of low grade, which, at the periphery, possess glands of a particular morphology called MELF (microcystic, elongated and fragmented) [12, 13]. These glands, as their name denotes, are deformed, elongated and fragmented, and display microcystic elements probably secondary to fragmentation, which creates non-communicating lumens resembling small cysts. Immunohistochemically they partially lose expression of ß-catenin and hormonal receptors for oestrogens and progesterone, while they retain cytokeratin AE1/AE3 and CK7 expression. Their morphology and immunohistochemical profile suggest that MELF represent a form of EMT. It is of interest that some adenocarcinomas of uterine endocervix are characterised by a similar phenomenon, with attenuated glands, sparse clusters of cells or single cells at the periphery showing decreased ß-catenin and E-cadherin staining [14].

Endometrial cancer cells extracted from clinical samples exhibit sub-populations with stem cell-like properties that are called side populations and have the ability to efflux the fluorescent dye Hoechst 33342 due to their expression of transporter protein ABCG (also called BCRP1, breast cancer resistance protein 1) [15, 16]. Sub-populations with the same properties derived from an endometrial cancer cell line have the ability to grow more aggressively, are more mobile than the rest of the cells, and form tumours in mice that display both epithelial and sarcomatous components staining for mesenchymal markers vimentin and α-smooth muscle actin and arguing for a potential for dual differentiation in neoplastic epithelial endometrial stem cells [17]. Mesenchymal cells were confirmed to be derived from the xenograft through an EMT and produce their own stroma. Isoforms of the stem cell marker CD44, the hyaluronan receptor, are over-expressed already in endometrioid carcinomas compared with normal endometrium [18].

Thus it appears that EMT is already present in low-grade endometrioid endometrial carcinomas [19] and becomes more prominent in higher grade, where the glands of normal morphology disappear, with MMMT representing the most advanced grade of the spectrum presenting frank areas of sarcomatous morphology.

The ubiquitin proteasome system and EMT

Post-translational modifications of cellular proteins such as phosphorylation, methylation and acetylation play an important role in cellular functions. Ubiquitination is a post-translational protein modification referring to the attachment of the small protein ubiquitin to a lysine residue of a target protein. This modification is more versatile than other post-translational modifications because several ubiquitin molecules can be added to the first, creating chains of ubiquitin on the target proteins that modify the signal [20]. In addition, the ubiquitin molecule possesses seven lysine residues, all of which can serve as attachment sites for this addition of subsequent ubiquitins. Resulting chains have different conformations and lead to various outcomes [21]. A prominent place among these outcomes is occupied by recognition of the ubiquitinated protein by the proteasome and degradation. Recognition by the proteasome is effectuated after a chain of at least four ubiquitins have been attached to the target protein through lysine 48. Sometimes chains of lysine 11-attached ubiquitin may also be recognised by the proteasome [22]. In contrast, ubiquitin attached through lysine 63 plays roles in other functions such as endocytosis, transcription, DNA repair and degradation through the

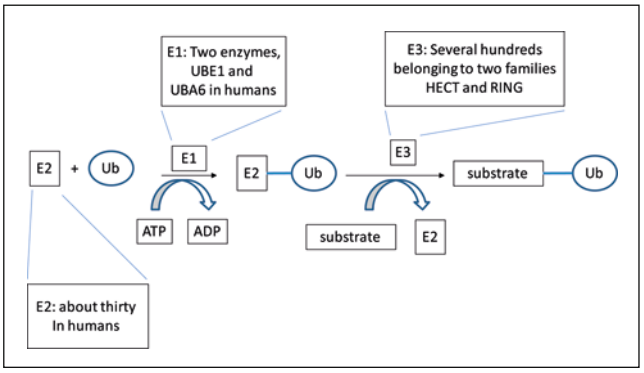

Fig. 1 The ubiquitination cascade. E1 or ubiquitin-activating enzyme binds ubiquitin (Ub) in an ATP-dependent manner and transfers it to E2 or ubiquitin-conjugating enzyme as a thioester. Then E2-linked ubiquitin is transferred to the target protein with the aid of E3 ligase. There are two E1 enzymes, UBE1 and UBA6, in humans. The latter also serves as the ligase for the ubiquitin-like molecule FAT10. There are 30–40 E2s and more than 500 E3s in the human genome. E3s belong to two families, the HECT (Homologous to HPV E6 Carboxyterminal domain) and the RING (Really Interesting New Gene) family. A third family, the U-box containing ligases, is considered a sub-family of RING ligases due to the conformational similarity of the U-box and RING domains

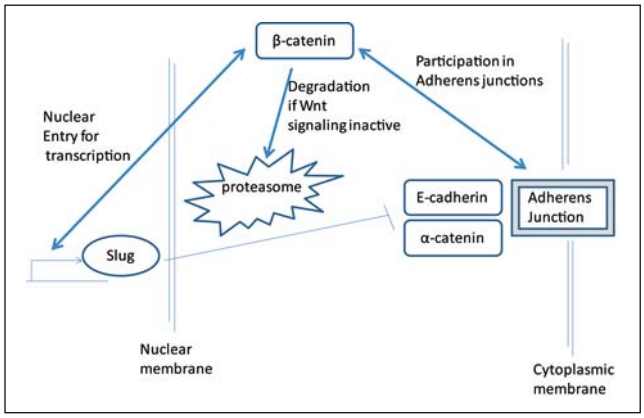

Fig. 2 Cytoplasmic β-catenin constitutes the pool for both nuclear entry to act as a transcription factor and cytoplasmic membrane localisation to act as a component of adherens junctions. If Wnt signalling is inactive, β-catenin is phosphorylated and ubiquitinated to be degraded by the proteasome. Among the target genes of β-catenin-promoted transcription, Slug suppresses E-cadherin transcription and thus promotes junction dissolution and favours β-catenin entry to the nucleus in a positive feedback loop that promotes EMT. Arrows denote activation and inverse ⊤ signs inhibition

lysosome. Ubiquitination is executed in a well described cascade of enzymatic reactions mediated by three types of enzymes (Fig. 1): ubiquitin activating enzymes or E1s (two known enzymes in humans), ubiquitin conjugating enzymes or E2s (about 30 in humans) and ubiquitin ligases or E3s (several hundred in human cells). Specific pairs of E2s and E3s decide what type of ubiquitin attachment will be executed [23]. Ubiquitination, similarly to other post-translational modifications, is reversible and there are five families of de-ubiquitinating enzymes that perform this function [24].

The proteasome, a barrel-shaped multi-protein structure with a lumen residing both in the cytoplasm and the nucleus, has two distinct parts: a central part called 20S proteasome or core particle (CP), where the enzymatic activities executing proteolysis reside, and a peripheral part covering one or both ends of the CP, called 19S proteasome or regulatory particle (RP). RP sub-units function in ubiquitinated target protein recognition, denaturing, de-ubiquitination and transfer to the CP for degradation. Three enzymatic functions (trypsin-like, chymotrypsin-like and caspase-like) residing in distinct sub-units of CP result in the production of fragments of 4–14 amino acids [25].

The ubiquitin proteasome system (UPS) regulates all processes that are involved in carcinogenesis, among them invasion and metastasis. EMT, the process that endows neoplastic cells with invasive and metastatic potential, is intimately interwoven with other neoplastic processes, being served by several common pathways, several of which are regulated by UPS [26]. In the next section EMT pathways pertaining to MMMT pathogenesis will be discussed.

Common molecular lesions in MMMT: role in EMT induction and regulation by UPS

Epithelial endometrial carcinomas are divided into two general types, called I and II or endometrioid and non-endometrioid respectively [27]. Type II carcinomas are most commonly of papillary serous or clear cell histology and some authors argue that poorly differentiated endometrioid carcinomas should be included with type II because they display similar immunohistochemistry and similar prognosis [28]. Endometrioid or type I endometrial carcinomas commonly display activating mutations of *ß-catenin* and disabled PTEN and less commonly K-ras activation (15–30%), Her2 activation (10–20%) and p53 loss of function (10–20%). Type II or non-endometrioid endometrial cancers are most commonly of serous or clear cell histology and have p53 disabling in 90% of cases. Her2 activation is present in a similar percentage of cases compared with type I carcinomas but PTEN inactivation and K-ras activation or *ß-catenin* mutations are rare. MMMTs present lesions in common with both type I and II endometrial carcinomas, with p53 inactivation and *ß-catenin* pathway activation being the most common [29, 30]. In addition, *C-myc* gene amplification or polysomy of chromosome 8q appears to be a common lesion in uterine MMMTs and ovarian carcinosarcomas [31]. Two other genes frequently amplified in MMMTs are *transforming growth factor ß1 (TGFß1)* and kinase *Akt2* at chromosome 19 [32]. All these lesions must provide, in a sub-set of MMMT cells, the co-operative action that allows epithelial cells to undergo an EMT and give rise to the sarcomatous component.

ß-Catenin activation plays a significant role in EMT regulation by two mechanisms (Fig. 2). As a transcription factor, ß-catenin participates in the induction of transcrip-

tional modulator Slug (Snail2), which is a repressor of E-cadherin. ß-Catenin has another function, as a component of adherens junctions together with E-cadherin and α-catenin. Total cellular ß-catenin amounts are in equilibrium between adherens junctions and nuclear transcription function while cytoplasmic ß-catenin represents the pool that feeds both functions, or alternatively it is phosphorylated and ubiquitinated to be degraded by the proteasome. In MMMT, immunohistochemistry for nuclear ß-catenin has been found to correlate with phosphorylated Akt kinase and Slug, and inversely with expression of E-cadherin [30]. Phosphorylated Akt is activated and phosphorylates, in its turn, multiple substrate proteins, including kinase glucogen synthase kinase 3ß (GSK3ß). The same effect may be obtained by activating mutations or amplification of *Akt2*. Phosphorylated GSK3ß is inhibited and prevented from phosphorylating ß-catenin, which is then stabilised to either enter the nucleus and act as a transcription factor promoting the transcription of Slug or move to the plasma membrane and stabilise adherens junctions through interaction with E-cadherin. In this model Akt can regulate the same pool of ß-catenin that is activated by signals through the canonical Wnt pathway. Of interest in other situations, it appears that inhibition of GSK3ß by Akt or Wnt signalling activates different pools of ß-catenin, an Axin-independent pool in the former case and an Axin-complexed in the latter [33]. Activation of Slug by ß-catenin may provide a feed-forward loop for the establishment of EMT, given that Slug-induced repression of E-cadherin would dissolve adherens junctions and further favour the movement of ß-catenin to the nucleus for transcription activity as long as GSK3ß is inhibited. Thus, Akt activation in MMMT is a strong stimulus for the promotion of EMT. An additional down-stream effector of Akt with a role in EMT is kinase IKK. Activation of IKK by Akt leads to phosphorylation followed by ubiquitination of protein IκB and finally to activation of transcription factor NF-κB. NF-κB is an EMT promoter by induction of Snail and ßHLH (basic helix-loop-helix) transcription factor Twist. Nevertheless, in histochemical evaluation of MMMT specimens, no correlation was found between the NF-κB family factor p65 and phosphorylated Akt or Slug [34], probably reflecting the fact that these pathways are not linear but there is a multiplicity of regulations functioning in conjunction.

p53, a tumour-suppressing transcription factor and one of the most common mutated proteins in cancer, is commonly mutated in MMMT. Its function is activated after DNA damage and other stress signals and leads to cell cycle arrest or apoptosis if damage is too severe to be repaired [35]. Post-transcriptional modifications, availability of co-factors and duration of triggering signal are among the factors that influence the final outcome of p53 activation. Due to these functions that allow the cell to repair reversible DNA damage or promote cell death if damage is irreparable in order to avoid inheritance of mutations in progeny, p53 is named the guardian of the genome. In addition it plays a prime role in counteracting several pathways

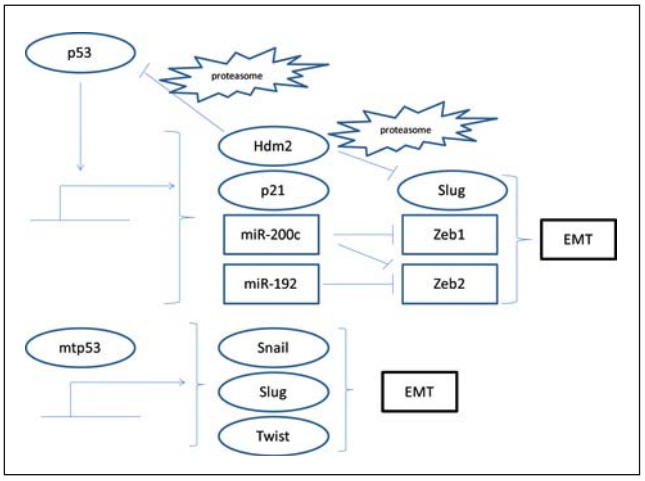

Fig. 3 Wild-type p53 (upper part of the figure) suppresses EMT by inducing transcription of cyclin-dependent kinase inhibitor p21 and micro-RNAs miR-200c and miR-192. It also induces E3 ligase mdm2 (also called Hdm2 in humans), which acts as a negative feedback loop by ubiquitinating p53 for proteasomal degradation, but also as an EMT suppressor by ubiquitinating EMT inducer Slug. In contrast, mutant p53 (lower part of the figure) promotes EMT by favouring transcription of Snail, Slug and Twist. Arrows denote activation and inverse ⊤ signs inhibition

of EMT and thus it can be considered a guardian of metastasis. p53 inactivation, either directly through mutations of its gene or indirectly through lesions interfering with its regulation, not only inhibits apoptosis, promotes the cell cycle and genomic instability, but also promotes invasion and metastasis. Mutant p53 protein often acquires gain of function properties promoting carcinogenesis in general and invasion in particular [36]. EMT is an invasion-promoting process that is inhibited by normal p53 in several ways. p53 directly induces microRNAs of the miR-200 family, miR-200c and miR-141 [37]. As a result, translation of ZEB1 mRNA, which is a target of miR-200c, is suppressed. miR-200c induction concomitantly suppresses polycomb group member BMI1 (B lymphoma mouse Moloney leukaemia virus Insertion region 1) and transcription factor KLF4, both important in the maintenance of stem cell phenotype. Thus p53, through miR-200c, suppresses stemness. This denotes that the two conditions, EMT and stem cell phenotype, may be served by tightly interwoven networks, as is evident also in development [3].

E3 ligase mdm2 (mouse double minute 2, also called Hdm2 in humans) is a transcriptional target of p53. Besides targeting p53 in a negative feedback loop, mdm2 mediates ubiquitination of Slug, being an additional effector of p53-induced EMT suppression (Fig. 3).

p53 down-regulates Slug through promotion of its mdm2-mediated ubiquitination and proteasome degradation, which leads to E-cadherin expression [38]. CDK inhibitor p21, a p53 transcription target, has been found to decrease EMT of breast cancer cells induced by Ras and C-myc [39]. In contrast, cancer-associated mutant p53 promotes Snail, Slug and Twist induction and EMT [40–42].

A main regulation of p53 is executed by the UPS. Tight regulation of its availability and activation is of paramount importance as an importunate activation could lead to untimely cell demise. Under normal non-stress conditions p53 has a short half-life because it is ubiquitinated with the help of E3 ligase mdm2 and degraded by the proteasome. Other E3 ligases, such as Pirh2, ARF-BP1/Mule, COP1 and CHIP, are also implicated in p53 regulation in various conditions [43–46].

p53 immunohistochemical staining, which is usually equivalent with the presence of a mutant form unable to be degraded and as a result having a longer half-life, is present in both epithelial and sarcomatous components of most MMMTs [47–50]. p53 positivity has been reported in the *in situ* epithelial component of MMMTs, suggesting that p53 mutations represent an early molecular lesion at least in a percentage of cases [51].

C-myc is a basic helix-loop-helix leucine zipper family transcription factor with a role in neoplastic transformation and in stem cell maintenance. It heterodimerises with protein Max to bind DNA on specific sequences called E-boxes and trigger recruitment of the transcription machinery [52]. When activated, C-myc promotes both proliferation and apoptosis by up-regulating p53 activator p14ARF. p14ARF activates p53 by binding and inhibiting ligase mdm2. As a result, C-myc is an efficient transformation effector in cells with a disabled p14ARF/mdm2/p53 pathway. In addition, p14ARF inhibits C-myc directly in a negative feedback loop. The same relationship may also exist between ß-catenin and p53 because, at least in some endometrial cell lines, p14ARF is a target of ß-catenin transcription [53] and thus the p14ARF/mdm2/p53 pathway needs to be disabled for efficient transformation in this instance too.

C-myc is regulated by the UPS after ubiquitination with the aid of two E3 ligases. Ubiquitination by E3 ligase Skp2 complex promotes both C-myc transcriptional function and turnover, although ubiquitination is not required for transcription [52]. This is a general theme in transcription where ubiquitination of transcription factors promotes the process by allowing the recruitment of new transcription factor molecules to access the DNA binding site if activating signals persist, in order for transcription to continue. Another ligase, Fbwx7, promotes C-myc degradation independently of promoter binding but dependent on previous phosphorylation by kinases ERK and GSK3 [54, 55]. Phosphorylation of C-myc at a different site by IKK kinases leads to an opposite outcome protecting C-myc from ubiquitination and degradation [56].

C-myc over-expression promotes EMT in cancer cells *in vitro* and *in vivo* [57]. C-myc over-expressing cells display Snail up-regulation due to both increased transcription and decreased ubiquitination and proteasome degradation, E-cadherin down-regulation and a fibroblast-like configuration [58]. C-myc further destabilises intercellular adhesions through induction of micro-RNA miR-9, which suppresses translation of both E-cadherin and α-catenin [59]. In addition, it suppresses expression of endometrial differentiation, promoting F box transcription factor FOXO1 [60]. Dissolution of adherens junctions promotes ß-catenin transcription function as ß-catenin is shifted to the nucleus if the GSK3ß/axin/APC destruction complex is inhibited. As C-myc is a ß-catenin target gene, a feed-forward EMT-promoting loop is completed [61]. In a series of MMMTs and ovarian carcinosarcomas C-myc amplification by FISH was detected in the majority of cases [31].

Oestrogen and TGFß crosstalk in MMMTs

Low-grade endometrioid endometrial carcinomas express the α sub-type of oestrogen receptor (ERα) and are oestrogen responsive. High oestrogen exposure is a risk factor for these carcinomas. ERß sub-type is expressed normally in uterus together with ERα during development where it antagonises ERα effects, but its expression decreases in normal adult endometrium [62]. This minor role of ERß in endometrial physiology is supported by studies of knockout mice [63]. As grade increases and in serous carcinomas and MMMTs, ERα expression decreases and expression of receptor ERß increases [64–66]. Activation of this sub-type is not efficient in stimulating proliferation in the endometrium [67] and thus high-grade carcinomas and MMMTs become oestrogen-independent and unresponsive. Alternatively spliced forms of ERß incapable of binding the ligand oestradiol are expressed in endometrial carcinomas of all grades and in carcinomas of higher grade, where ERα is not present, and may contribute to oestrogen refractoriness [68]. In low-grade ER-positive endometrial cancer cell line, Ishikawa, oestrogen stimulation has been found to promote EMT [69]. ERα expression in low-grade endometrioid endometrial carcinoma may promote EMT independently of its genomic/transcription factor action by inhibiting TGFß signalling [70]. This action is mediated by facilitation of degradation by the proteasome of Smad2 and Smad3, which are receptor-type intracellular transducers of TGFß signalling cascade [70]. Ubiquitination is executed with the aid of E3 ligases Smurf1 and Smurf2. Reciprocally Smad4, another transducer in the TGFß cascade, in complex with Smad3 may inhibit ERß transcription in breast cancer cells [71]. In contrast, in high-grade endometrial cancer cells HEC-1-A TGFß signalling is important for survival and migration [72]. A dominant negative TGFß receptor II inhibited growth and EMT of these cells. Given the well known dependence of TGFß signalling on the stage of malignancy, with an anti-neoplastic effect predominating in early carcinogenesis and a pro-carcinogenic effect in more advanced stages where other pathways such as K-ras are concomitantly activated [73], it is conceivable that in Ishikawa cells representing a low-grade, early carcinogenesis step, TGFß signalling is anti-carcinogenic and thus its inhibition by ER promotes instead of inhibits EMT. In contrast, in most advanced grades, as represented by the HEC-1-A cell line, TGFß may promote EMT by interact-

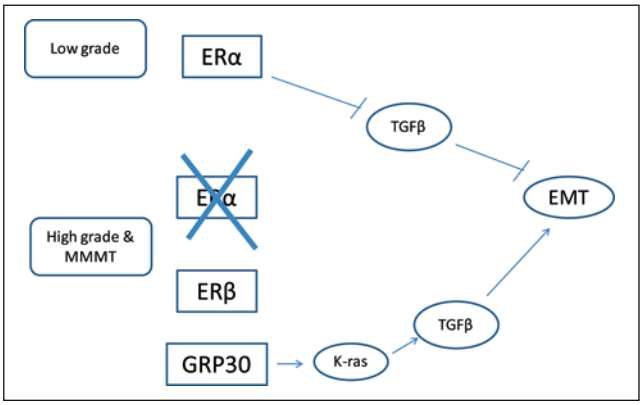

Fig. 4 In low-grade endometrial carcinomas (upper part of the figure) ERα is expressed and promotes EMT by inhibiting TGFβ, which on this occasion is an EMT inhibitor. In higher-grade endometrioid carcinomas, carcinomas of other histologies and MMMTs, ERα is not expressed, replaced by ERβ (including alternatively spliced forms) and GPR30 (lower part of the figure). GPR30 activates K-ras, which transforms TGFβ to an EMT promoting signaling. Arrows denote activation and inverse ⊤ signs inhibition

ing with Snail and suppressing transcription of E-cadherin and other junctional components such as Occludin and Claudin-3 [74]. In higher-grade histologies and MMMTs where ERα is down-regulated, TGFß is left unopposed and at the same time it becomes pro-carcinogenic and EMT-promoting (Fig. 4).

An additional layer of complexity is conferred because non-genomic actions of oestrogens in MMMTs can be mediated by a membrane-associated ER, G-coupled protein receptor GPR30 (also known as GPER, G-coupled protein oestrogen receptor), which is expressed in this malignancy in parallel with ERß and increases as stage advances [75]. GPR30 over-expression detected by immunohistochemistry has been proposed as an adverse prognostic factor in endometrial carcinomas [76]. GPR30 is a seven transmembrane domain receptor and signals through activation of MAPK and PI3K pathways [77] and downstream mediators and transcription factors [78]. Its physiologic ligand is oestradiol and it can also be activated by tamoxifen and fulvestrant but has a much lower affinity for estrone and estriol and does not bind progesterone, glucocorticosteroids or testosterone. In a breast cancer cell line expressing also ER, GPR30 activation by oestradiol resulted in TGFß signalling inhibition [79], but in another cell line lacking ERα and ERß, GPR30 activation by oestradiol promoted proliferation and invasion [80]. In clinical breast cancer samples, GPR30 expression was associated with tumour resistance to tamoxifen [81]. In addition, in endometrial cancer, GPR30 activation promoted proliferation and invasion of both ER-positive and -negative cells [82]. A role for tamoxifen in promoting MMMT development in some breast cancer patients has been reported [83] and may be mediated by GPR30.

Ubiquitination and the UPS have essential and complex roles in transcription, as also discussed in the section on c-myc, and nuclear receptors and ER in particular are no exception. The complexity of the regulation is outlined by the fact that proteasome inhibition enhances some but suppresses the expression of other ER target genes [84]. Proteasome function is required for ERα transcriptional activity by promoting mono-ubiquitination of histone H2B and facilitating transcription elongation in breast cancer cells [85]. Interestingly, knocking-down of the E3 ligase involved in histone H2B ubiquitination, RNF40, led to oestrogen-independent cell proliferation and activation of the PI3K/Akt and MAPK pathways, providing an additional link between ER and K-ras-initiated signalling. Deubiquitination also plays a role in ER transcription. The deubiquitinating enzyme of the OTU (ovarian tumour) family OTUB1 is part of the ER transcription complex and acts in this deubiquitination of the receptor, which results in modulating transcription of target genes and the stability of ER itself [86]. In addition, ubiquitination is involved in endocytosis of G-protein coupled receptors that may lead either to lysosomal degradation or re-cycling to the surface [87] and this has been shown specifically for GPR30 [88].

TGFß signalling is also regulated by the UPS in multiple levels [89]. Several E3 ligases such as Smurf1 and 2 and NEDD4 of the HECT domain family, and ßTrCP and Fbwx1 of the RING family, among others, regulate the stability of proteins participating in TGFß signalling [90–92].

In conclusion, and despite unresolved issues in an evolving field, oestrogen signalling, which may promote proliferation and EMT in lower-grade endometrial carcinoma through inhibition of TGFß anti-neoplastic signalling, becomes down-regulated in higher-grade carcinomas and MMMTs where ERα is not expressed and ERß is probably less effective in counteracting TGFß signalling. In these cases TGFß signalling is transformed to a pro-invasion programme due to concomitant MAPK and Akt activation by GPR30 and other receptors, and promotes EMT (Fig. 4).

EMT inducing transcription factors ZEB, Snail and Twist in MMMT

EMT is promoted by a set of core transcription factors such as ZEB (Zinc finger E-box Binding homeodomain), Snail, Slug and Twist that result in down-regulation of key components of intercellular adhesions, which is a prerequisite for the acquisition of motility.

The zinc finger homeodomain factor ZEB1 is an EMT inducer. It has been reported to be expressed in the normal adult endometrium and its expression is oestrogen-dependent [93]. ZEB1 is over-expressed in the stroma of low-grade endometrial carcinomas [94] but its gene is deleted in the low-grade epithelial cancer component [93]. In grade 3 endometrioid endometrial carcinomas, papillary serous endometrial carcinomas and MMMTs, ZEB1 is often expressed in the epithelial neoplastic cells and becomes oestrogen-independent [93]. Moreover, forced expression

of ZEB1 in endometrial cancer cell lines representative of low-grade carcinomas promotes their migratory potential in an *in vitro* wound healing assay and reduces the expression of E-cadherin [95]. Conversely, partial silencing of ZEB1 by shRNA in a high-grade endometrial cancer cell line reduced its *in vitro* migratory potential although no increase in E-cadherin expression was noticed [95].

High expression of ßHLH transcription factor Twist has been associated with decreased E-cadherin expression and deeper myometrial invasion and with decreased survival in endometrioid endometrial carcinomas [96]. An endometrial cell line undergoing irradiation showed Twist up-regulation and EMT morphologic changes in parallel with an increased migratory potential [97]. Knock-down of Twist with siRNA reversed the increased irradiation-induced migration. Another endometrial cell line did not show prominent EMT changes under the same experimental conditions. These findings support the role of an additional EMT inducer, Twist, in the aggressiveness of endometrial carcinoma. Although no histologic features of MMMT were observed in the cases with high Twist expression *in vivo*, the *in vitro* study suggests that, in some cases, mesenchymal morphologic changes are present.

The homeobox gene HoxA10 is a specific differentiation gene for the endometrium and is induced in the secretory phase of the endometrial cycle by progesterone. HoxA10 is down-regulated as the grade of endometrioid endometrial cancer increases, being most suppressed in grade 3 endometrioid as well as in papillary serous cancers [98]. HoxA10 is a suppressor of Snail and thus its down-regulation through promoter methylation in high-grade endometrial cancers leads to E-cadherin down-regulation, providing a link between increasing grade and EMT/invasion [98]. An association of Snail with higher grade and reduced E-cadherin expression was observed in metastatic lesions of endometrial carcinomas [99]. The related transcription factor Slug (also called Snail2) is up-regulated by ß-catenin signalling and suppressed E-cadherin in MMMT, as already discussed [30].

microRNAs constitute physiologic important post-transcriptional regulators of protein expression and play a role in carcinogenesis. In MMMT a specific microRNA signature has been revealed in the mesenchymal component compared with the epithelial part of the tumour [100]. Prominent in this signature is the miR-200 family of miRNAs, which is down-regulated in the mesenchymal component. The miR-200 family is composed of five members, miR-200a, miR-200b, miR-200c, miR-141 and miR-429, which are post-transcriptional repressors of ZEBs and, as a result, their down-regulation allows ZEBs to suppress E-cadherin transcription. Indeed, E-cadherin expression was completely lost and p120 cadherin decreased in the mesenchymal component of MMMTs, while mesenchymal markers vimentin, SPARC (secreted protein acidic and rich in cysteine) and fascin were up-regulated. Also up-regulated were EMT inducers TGFß1 and TGFß2. TGFß1 induces EMT in endometrial cell lines and up-regulates Slug, adhesion molecule L1CAM and vimentin [101]. L1CAM is associated in histochemical evaluation of endometrial carcinomas with ER and progesterone receptor (PR) negativity and E-cadherin negativity and is sometimes observed in the invasive front of tumours. Other relevant EMT targets of miR-200c which are suppressed in aggressive endometrial cancer cell lines include fibronectin, moesin, an actin cytoskeleton connector to the plasma membrane, and the receptor tyrosine kinase TrkB [102]. In addition, when compared with endometrioid endometrial cancers, the epithelial component of MMMTs displays higher expression of several mesenchymal markers and lower expression of E-cadherin [100], arguing for the presence of a predisposition or early stages of EMT already in this component. Another miRNA, miR-194, which did not sort out in the above signature, was found to down-regulate the polycomb group protein BMI-1 and to reduce EMT and invasion potential *in vitro* in endometrial cell lines [103]. BMI-1 is a promoter of invasion and metastasis from various primaries and also a target of miR-200c, as previously discussed.

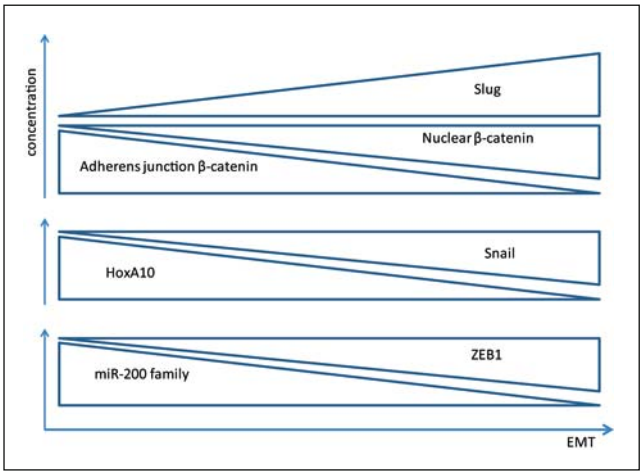

Fig. 5 Schematic representation of key EMT transcription factor concentrations in relationship to important regulators. When β-catenin shifts from membrane localisation as an adherens junction component to the nucleus, it promotes transcription of Slug. Suppression of HoxA10 due to promoter methylation de-represses transcription of Snail. Down-regulation of miR-200 family members promotes translation of ZEB transcription factors. Co-ordinate increase in the concentrations of these transcription factors triggers EMT. The UPS is involved in the regulation of these factors at multiple points and may tip the balance in either direction, as discussed in the text

Collectively these data paint a picture in which transcription factors of the core EMT circuitry are up-regulated in MMMT while differentiation promoting factor HoxA10 and miRNAs normally counter-acting them are down-regulated with increasing grade as the balance shifts towards the EMT/metastatic phenotype (Fig. 5). The UPS modulates the stability of all core EMT transcription factors sometimes in a co-ordinated manner. This is the example of core EMT transcription factors Snail, Slug, Twist and Zeb2, which are all regulated by ubiquitination with the aid of a Skp ligase complex having the F-box protein Ppa

(Partner of paired or FBXL14 in humans) as the substrate recognising sub-unit [104–106].

Intercellular adhesions and cell-matrix adhesions

Intercellular adhesions are important defining elements of epitheliums and need to be dissolved during EMT in order for cells to move. Adherens junctions are made of E-cadherin molecules that span the cytoplasmic membrane and have both extracellular and intracellular domains [107, 108]. With their extracellular domains, E-cadherins make homotypic contacts with E-cadherin molecules of neighbouring cells. The intracellular domain associates together with α-catenin and ß-catenin to the actin cytoskeleton. Other proteins such as p120 catenin, EPLIN (epithelial protein lost in neoplasm), ZO1 (Zonula Occludens 1), Afadin, Vinculin, Paxillin and α-actinin may participate in initiating and strengthening the interactions of catenins with the actin cytoskeleton. Adherens junctions are dynamic and E-cadherin molecules are continuously incorporated in cell membranes and removed by clathrin-mediated endocytosis. Endocytosis of E-cadherin is triggered after phosphorylation by c-src kinase, which is followed by ubiquitination with the aid of c-cbl family E3 ligase Hakai [109]. p120 catenin prevents E-cadherin endocytosis by masking Hakai interaction sites in the juxta-membrane area of its molecule. Hakai over-expression in epithelial cells results in Paxillin down-regulation and decreased cell-matrix adhesion [110].

E-cadherin is a central target of EMT-inducing transcription regulators and is down-regulated by ZEB and Snail transcription factors as well as ß-catenin in high-grade uterine carcinomas and MMMTs [111, 112]. In addition ß-catenin, as mentioned, participates in a feed-forward loop by becoming available to act as a transcription factor when adherens junctions are resolved.

Adherens junction components P-cadherin, E-cadherin, p120 and ß-catenin have been investigated as prognostic markers in a histochemical study of all endometrial carcinomas diagnosed in a Norwegian region between 1981 and 1990 [113]. Decreased expression of E-cadherin and p120 was more commonly seen in higher-grade and clear cell carcinomas while mesenchymal P-cadherin high expression was more commonly seen in these types [113]. High E-cadherin and low P-cadherin expression were associated with a better prognosis in endometrial carcinomas.

EMT is promoted by up-regulation of matrix metalloproteinase MMP-3 (Stromelysin 1) in endometrial cancer, which results in E-cadherin degradation and is associated with vascular invasion and more aggressive tumours [114]. MMP-7 (matrilysin), another matrix metalloproteinase, is expressed in the epithelial component of 70% of cases of MMMTs examined, while expression is lost in corresponding sarcomatous components [115]. Thus, it appears that, after intercellular adhesion dissolution, motion of epithelial cells acquiring EMT phenotype and properties is accompanied by changes in the profile of expressed matrix-modifying enzymes with up-regulation of some MMP-3 and down-regulation of others. MMP-3 and MMP-7 production is stimulated by MAPK cascade activation and may, as a result, be modulated by the UPS given that several proteins taking part in this cascade are regulated by the UPS [116, 117].

Therapeutic opportunities

MMMT can be considered a high-grade epithelial endometrial cancer with extensive metaplasia due to lesions activating EMT pathways. UPS plays a key role in regulating these pathways as discussed in the previous sections. This patho-physiological insight offers opportunities to reverse or counteract EMT by modulating UPS function. Given that EMT is served in many instances by overlapping pathways with other carcinogenesis-enabling properties, reversal of EMT through UPS modifying interventions may have more global anti-neoplastic effects.

Inhibition of the proteasome is already used in cancer therapy. Bortezomib, a specific inhibitor of the chymotrypsin-like proteasome activity, constitutes a well established treatment for multiple myeloma and sub-types of non-Hodgkin lymphoma [118]. Newer inhibitors of the enzymatic cleavage activity of proteasome such as carfilzomib and NPI-0052 are under development [119, 120]. Inhibition of the deubiquitinising activity of 19S RP is another way of inhibiting the proteasome and such inhibitors are in earlier study [121]. Proteasome inhibition, despite the specificity of the enzymatic molecular reaction that is involved, has broad effects in cellular homeostasis and finally produces non-specific cytotoxicity due to perturbations of hundreds of proteins regulated by the proteasome. Nevertheless malignancies with specific molecular lesions could be particularly sensitive to this broad inhibition due to dependence of the lesions on proteasome function or to triggering of different down-stream pathways. For example, cells deficient in p130Cas, an adaptor protein of cellular adhesions, are resistant to apoptosis after bortezomib treatment and trigger autophagy, while cells expressing this protein are sensitive to proteasome inhibition [122]. Thus p130Cas expression may serve as a marker of bortezomib sensitivity. On the other hand, given that p130Cas-deficient cells are concomitantly resistant to doxorubicin, it remains possible that this resistance is generalised and related to adhesion destabilisation and EMT and may not represent a marker of resistance to particular treatments. Indeed EMT is associated with drug resistance related to the aforementioned intertwining of its pathways, with pathways involved in apoptosis prevention and self-sufficiency of survival signals in neoplastic cells [123]. Nevertheless a sub-set of cancer patients could remain sensitive to particular strategies such as proteasome inhibition even in the presence of EMT. In the case of MMMT, a particular patient could be

sensitive to proteasome inhibition if it possesses a mutant C-myc with gain of function but still able to be degraded by the proteasome and concomitantly a wild-type p53 that is functionally inhibited due to instability from increased degradation, e.g., secondary to mdm2 amplification or stabilisation. In this example EMT related to C-myc hyperactivity and p53 disabling could be reversed by proteasome inhibition, which would inhibit C-myc transcription function, producing c-myc turnover stalling on DNA and p53 activity promotion through reversal of its instability.

An alternative to proteasome inhibition strategy is to interfere with other points of the UPS that would offer greater specificity. An example of such an alternative strategy is inhibition of NEDDylation, which refers to the ligation of a target protein with the ubiquitin-like small protein NEDD8 (Neural precursor cells-Expressed Developmentally Down-regulated 8). This is a post-translational modification that often takes plays in the Cullin component of the SCF type RING ligases and helps the E2 enzyme binding to the ligase complex in the ubiquitination cascade. MLN4924, a small molecule inhibitor of the NEDD8 activating enzyme (NAE, the E1 for NEDD8) is in early clinical development [124]. Nevertheless, the SCF ligase family is extensive and many proteins involved in EMT of MMMT (ß-catenin, C-myc, NF-κB, Snail) are regulated by ligases of this family. In addition, NEDDylation has other targets, such as p53 and the core apoptosis effectors caspases [125, 126]. Thus NEDDylation inhibition could interfere with multiple points in EMT progression and the net result of the inhibition, and whether there are sub-sets of MMMT patients that could benefit from it remains to be investigated.

Inhibiting E3 ligases could offer greater specificity. For example, inhibition of mdm2 could be a strategy to explore [127, 128], although in MMMT it would be expected to be effective only in the sub-set with wild-type p53. In patients with mutant p53 these inhibitors may not only be inactive but could have deleterious effects due to inhibition of degradation of Slug. This fact underlines the importance of correctly delineating the sub-set of patients that could benefit from a certain drug.

Defining sub-sets of neoplasms that, due to particular pathogenic lesions, are sensitive to particular drugs is a major challenge in oncology but one that would provide significant advancement in personalised treatment of cancer patients.

Conflict of interest The authors declare that they have no conflict of interest relating to the publication of this manuscript.

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