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A role for the transducer of the Hippo pathway, TAZ, in the development of aggressive types of endometrial cancer

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Although TAZ, the final effector of the Hippo pathway that modulates epithelial to mesenchymal transition and stemness, has been implicated in the development of different types of cancer, its role in endometrial cancer has not yet been studied. Thus, we evaluated the expression of TAZ in different types of endometrial cancer by immunohistochemistry. TAZ expression was detected in 76% of undifferentiated endometrial carcinomas, 54% of endometrial carcinosarcomas, 46% of endometrial serous carcinomas, 36% of grade 3 endometrioid carcinomas, and 18% of grade 1-2 endometrioid carcinomas, with statistically significant differences. We analyzed the *WWTR1* gene that encodes TAZ by FISH and MassARRAY spectrometry, ruling out gene amplification and differential promoter methylation as the main mechanisms that modulate TAZ expression in endometrial tumors. However, we did detect a significant association between Scribble hypoexpression and delocalization with TAZ expression. Moreover, we demonstrated that TAZ promoted invasiveness, and it favored cell motility and tumor growth, in endometrial cancer cell lines. In addition, TAZ expression was associated with the transition from an epithelial to mesenchymal phenotype, both *in vitro* and in human tumors. Together, these data reveal a previously unknown role for TAZ and the Hippo pathway in the progression of aggressive subtypes of endometrial cancer.

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In developed countries, endometrial carcinoma is the most common malignant tumor of the female genital tract.[1] On the basis of epidemiological, clinical, pathological, and molecular features, endometrial carcinoma can be classified into at least two main categories:[2] type I estrogen-dependent carcinomas that account for 80–85% of cases and that are typically represented by low-grade (grade 1 and 2) endometrioid carcinomas, and type II endometrial carcinomas that are not estrogen dependent and that are mainly represented by a serous subtype but also by other high-grade histological tumors like clear cell or undifferentiated carcinomas.[2]

In endometrial carcinoma, the epithelial to mesenchymal transition has been associated with high-grade and aggressive features.[3–6] Epithelial to

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mesenchymal transition is a key developmental program by which polarized epithelial cells convert into motile mesenchymal ones, and it is a process that is activated during cancer invasion and metastasis.[7] The epithelial to mesenchymal transition is coordinated by a network of transcription factors that regulate the expression of proteins involved in cell polarity, cell-cell contacts, cytoskeletal structure, and extracellular matrix degradation.[8] The loss of E-cadherin expression is a crucial event in epithelial to mesenchymal transition and indeed, transcription factors associated to epithelial to mesenchymal transition can be grouped according to their ability to directly or indirectly repress E-cadherin.[9] Among the direct repressors, we can find zinc finger proteins like SNAIL1, SNAIL2, ZEB1 (*TCF8*), ZEB2 (*SIP1*), and the bHLH factor E47 (*TCF3*) or KLF8. By contrast, indirect repressors include TWIST bHLH proteins or E22.[8] In addition, the epithelial to mesenchymal transition program is closely linked to the acquisition of stem cell properties in other tumor cells, as defined by the acquisition of the $CD44^{high}/CD24^{low}$ phenotype, *in vitro* self-renewal and *in vivo* tumor initiation capacities.[7,10] The epithelial to mesenchymal transition has also been implicated in the dissemination of primary tumors owing to invasion or intravasation, as well as to the generation of distant tumors by migrating cancer stem cells.[11]

The Hippo pathway has been implicated in epithelial to mesenchymal transition and stemness,[12] and it is a pathway that coordinates cell proliferation, apoptosis, and differentiation associated with the regulation of organ development and regeneration.[13] The pathway involves a conserved kinase cascade, and the final transcriptional co-activators TAZ and YAP have been implicated in tissue homeostasis and the control of organ size through tissue-specific stem cell regulation.[14] Dysregulation of the Hippo pathway is correlated with epithelial to mesenchymal transition and cancer development, mainly driven by TAZ and YAP.[15] These two DNA-binding proteins are normally repressed by Mst2 and Lats2 phosphorylation,[16,17] or they are regulated by the expression and location of the cell polarity protein Scribble.[18] TAZ/YAP and epithelial to mesenchymal transition are thought to maintain a bidirectional relationship, whereby the loss of polarity and cell contacts (key events during the epithelial to mesenchymal transition process) induces the activation of both factors, which in turn participate in the epithelial to mesenchymal transition program.[19] Specifically, the transcriptional co-activator TAZ (*WWTR1*) has been associated with the loss of E-cadherin function in breast cancer.[18] and it also induces the expression of transcription factors associated to epithelial to mesenchymal transition like ZEB1 in retinal pigment epithelial cells.[20] Moreover, it has been shown that TAZ may confer cancer stem cell properties to breast cancer cells,[18] and it is coupled to the loss of polarity in epithelial cells through the membrane delocalization of Scribble during epithelial to mesenchymal transition.[18]

Although TAZ and YAP have been associated to several types of cancer, their role in endometrial carcinoma is still unknown. To gain insight into a possible role of TAZ in endometrial carcinoma, we studied the expression of TAZ and other epithelial to mesenchymal transition regulators in a series of 143 endometrial carcinoma and endometrial carcinosarcoma samples. Enhanced TAZ protein expression was observed in high-grade endometrial tumors and this increase was associated to Scribble delocalization. To further study the role of TAZ in endometrial carcinoma, we overexpressed and silenced this protein in endometrial cancer cell lines, demonstrating that increased TAZ expression is associated with cell motility, invasiveness, and tumor growth. Finally, we found that TAZ expression is associated with molecular features of epithelial to mesenchymal transition in endometrial cell lines and tumors.

Materials and methods

Tumor Samples

In this study, we analyzed 143 formalin-fixed paraffin-embedded samples from 56 grade 1-2 (low grade) and 14 grade 3 (high grade) endometrioid endometrial carcinomas, 26 endometrial serous carcinomas, 21 undifferentiated endometrial carcinomas, and 26 endometrial carcinosarcomas.[3,4,21] We also analyzed 33 frozen samples of 17 low-grade endometrioid endometrial carcinomas and 16 endometrial carcinosarcomas.[21] Histological typing of all the samples was performed according to the World Health Organization (WHO) classification and the tissue samples were obtained from the Departments of Pathology at the Hospital Universitario Virgen del Rocío (Sevilla, Spain), Memorial Sloan-Kettering Cancer Center (New York, USA), and Hospital Universitari Arnau de Vilanova (IRBLLEIDA, Lleida, Spain). Representative areas of these samples were selected to set up six different tissue microarrays,[22] and a summary of the relevant clinicopathological features is provided in Supplementary Table 1. This study was performed following the standard Spanish ethical regulations (Ley de Investigación Orgánica Biomédica, 14 July 2007) and it was approved by the ethic committees of each institution involved.

Immunohistochemistry and Fluorescence *In Situ* Hybridization

Tissue microarray sections were assessed by immunohistochemistry and fluorescence *in situ* hybridization. Immunohistochemistry was performed using the Envision method (Dako, CA, USA) with a heat-induced antigen retrieval step, and using primary antibodies raised against TAZ and Scribble (Supplementary Table 2). In order to assay *WWTR1* (3q25.1) gene amplification, we performed fluorescence *in situ* hybridization as described

previously by Zordan.[23] We used the Spectrum green-labeled Bacterial Artificial Chromosome (RP11-126i10, chr3:150717532–150892549) from the Children Hospital Oakland Research Institute and the Spectrum red-labelled chromosome 3 enumeration probe (Vysis CEP3. Abbott).

DNA Isolation and Sequenom MassARRAY Quantitative DNA Methylation Analysis

DNA was isolated from 33 frozen tissues (17 low-grade endometrioid endometrial carcinomas and 16 endometrial carcinosarcomas). Bisulfite conversion details are shown in Supplementary Methods 1. The purified and treated DNA was amplified in a TProfessional Standard 384 Thermocycler (Biometra, Jena, Germany) using specific PCR primers (Supplementary Table 3). MassARRAY spectrometry of the PCR products was performed according to the manufacturer's protocol and the data were processed with the EpiTyper Analyser software v1.0 (Sequenom, San Diego, CA, USA).

RNA Isolation, mRNA and microRNA Expression Analysis

RNA isolation, total RNA quantification, and reverse transcription of 38 formalin-fixed paraffin-embedded endometrial carcinoma samples (11 low/high-grade endometrioid endometrial carcinomas, 10 undifferentiated endometrial carcinomas, 9 endometrial serous carcinomas, and 8 endometrial carcinosarcomas) and cell lines was performed as described in the Supplementary Methods 2. We used different TaqMan Gene Expression Assays (Applied Biosystems: Supplementary Table 4) for qRT-PCR using the SsoFastProbe Supermix (Bio-Rad) in a CFX96 Real Time PCR Detection System (Bio-Rad). The resulting data were analyzed using the $2^{-(\Delta\Delta Ct)}$ method to estimate gene expression.[24] In addition, the expression of the miR-200 family microRNAs (miR-200a/b/c, miR-141, and miR-429: Supplementary Table 5) was analyzed by qRT-PCR with the TaqMan Universal PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real Time PCR System with the SDS 2.3 software (Applied Biosystems).

Cell Culture and Infection

The function of the *WWTR1* gene was studied in different endometrial cancer cell lines: HEC1A and Ishikawa, derived from a well differentiated endometrioid endometrial carcinoma; AN3CA, derived from a poor differentiated endometrial carcinoma; and SK-UT-1 and SK-UT-1B, derived from an endometrial leiomyosarcoma. To generate stable clones, TAZ overexpression in Ishikawa cells was induced by retroviral infection, and TAZ was silenced by lentiviral infection of AN3CA cells (Supplementary Methods 3).

Western Blotting and Immunofluorescence

Western blots and immunofluorescence were performed as described previously.[25] The antibody details are provided in Supplementary Table 6.[26] Immunofluorescence preparations were examined using an Eclipse 90i microscope system (Nikon) and processed with Image-J analysis software.

Migration and Invasion Assays

The migration and invasion assays were performed as described previously.[25] Briefly, cells were grown to 85% confluence in 60-mm cell culture dishes, and a wound was then made in the culture by scratching the monolayer. After the times indicated, pictures of three selected fields from each plate were taken. Invasion assays were carried out on modified Boyden chambers (8-μm pore filters) coated with 250 μg/ml matrigel, seeding 1×10^5 cells and quantifying the cells on the lower part of the filter 24 h after seeding.

In Vitro Cell Proliferation

Cell proliferation was assayed on 1.5×10^4 cells grown in a 96-well plate according to the Alamar Blue protocol (resazurin: Life Technologies). A Biotek Epoch microplate spectrophotometer was used to determine absorbance at 570 and 600 nm.

In Vivo Tumorigenesis Assay

Ishikawa or AN3CA cells (1×10^6) were injected subcutaneously into both dorsal flanks of 8-week-old female Balb/c immunocompromised mice ($n = 6$ for each condition; Charles River Laboratories). After cell injection, tumor growth was monitored weekly by measuring the two orthogonal external diameters with a calliper. When the tumors reached a size of 0.90–1 cm^3, they were surgically excised and processed for histological analysis. A minimum of four tumors were analyzed for each cell line.

Statistics

Normality tests were used to assess the sample distribution and cross tables were used with a Pearson's chi-squared test to evaluate immunohistochemical differences. Pearson's correlations were evaluated to determine the association between mRNA and miRNA expression, while a Student's *t* test, Fisher's Least Significant Difference test, or one-way ANOVA was used to compare the means of expression between groups. All analyses were performed with SPSS 17.0 for Windows (SPSS) and significance was considered at $P \leq 0.05$.

Results

TAZ Expression Is Characteristic of High-grade Endometrial Carcinomas, Especially Undifferentiated Endometrial Carcinomas

In order to study the potential role of TAZ in endometrial carcinoma, we first analyzed its expression in different subtypes of endometrial carcinoma by immunohistochemistry. We found that TAZ expression was associated more specifically to high-grade endometrial tumors, being detected in 76% (16/21) of undifferentiated endometrial carcinomas, 54% (14/26) of endometrial carcinosarcomas, 46% (12/26) of endometrial serous carcinomas, and 36% (5/14) of high-grade endometrioid endometrial carcinomas. However, only 18% (10/56) of low-grade endometrioid endometrial carcinomas expressed TAZ (Figure 1a and Table 1), and the difference between endometrial serous carcinoma, endometrial carcinosarcoma, or undifferentiated endometrial carcinoma and low-grade endometrioid endometrial carcinomas were significant in pairwise comparisons ($P < 0.05$). Our findings indicate that TAZ is mainly expressed in the aggressive subtypes of endometrial carcinomas.

TAZ Expression Is Associated to Anomalous Scribble Expression but Not to *WWTR1* Gene Amplification or Promoter Methylation

Several mechanisms can control TAZ expression, including *WWTR1* gene amplification, promoter

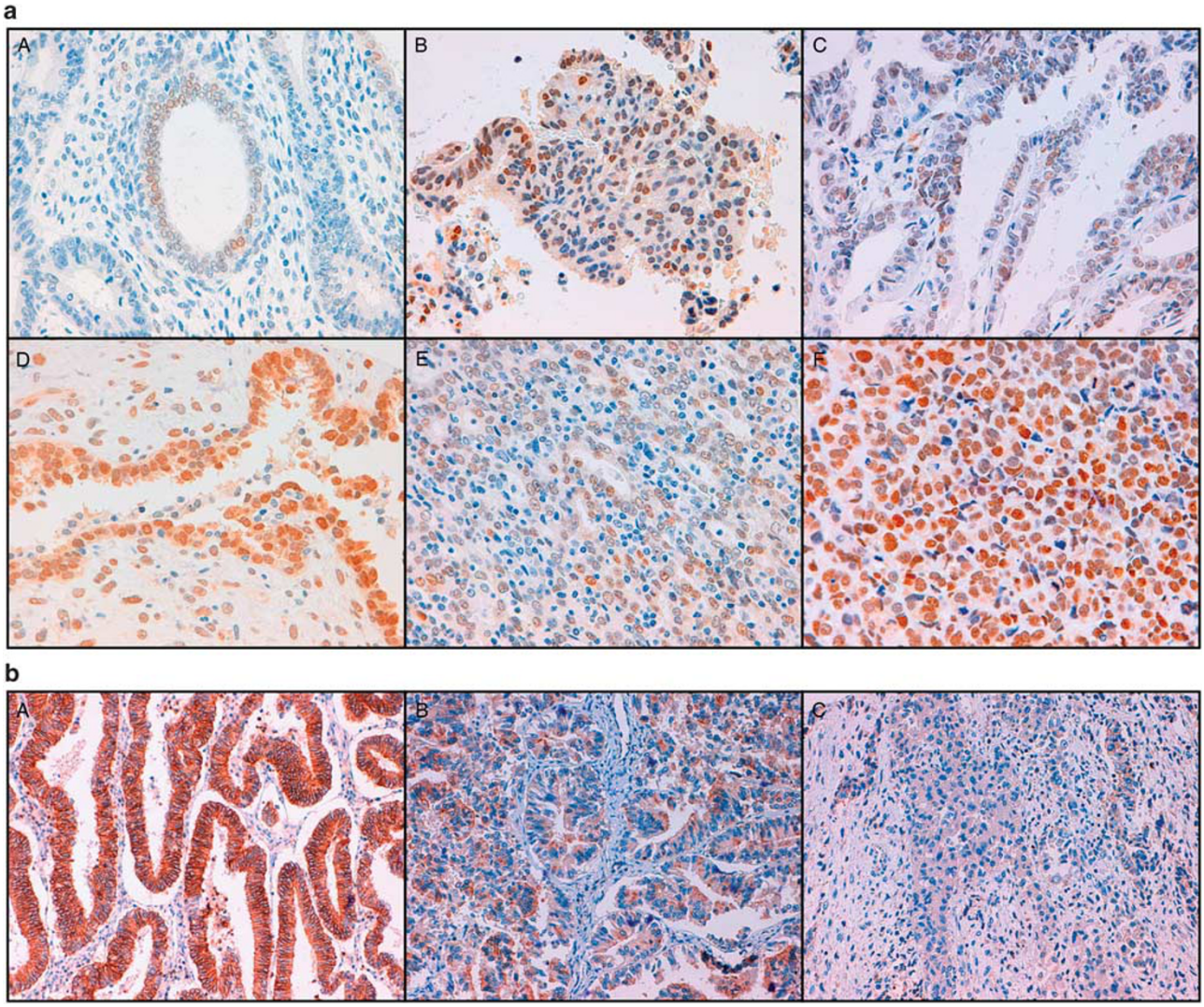

Figure 1 (**a**) Immunohistochemistry of the TAZ protein in different subtypes of endometrial cancer (×20): (A) Low-grade endometrioid endometrial carcinoma; (B) high-grade endometrioid endometrial carcinoma; (C) endometrial serous carcinoma; (D) endometrial carcinosarcoma; (E) and (F) different intensities from positive samples of undifferentiated endometrial carcinoma. (**b**) Immunohistochemistry of the Scribble protein in different subtypes of endometrial cancer (×20). (A) Membrane conserved Scribble expression in a low-grade endometrioid endometrial carcinoma. (B) Redistribution of Scribble in a low-grade endometrioid endometrial carcinoma. (C) Weak Scribble expression and redistribution in an undifferentiated endometrial carcinoma.

Table 1 Frequency of TAZ immunoreactivity in different subtypes of endometrial cancer ($N=143$): low and high-grade endometrioid endometrial carcinomas (LG-EEC and HG-EEC), endometrial serous carcinoma (ESC), endometrial carcinosarcoma (ECS), and undifferentiated endometrial carcinoma (UEC)

Tumor subtype	*TAZ expression*
LG-EEC	18% (10/56)
HG-EEC	36% (5/14)
ESC	46% (12/26)
ECS	54% (14/26)
UEC	76% (16/21)

Samples were considered as positive when TAZ expression was found in more than 5% of the tumor cells. Pairwise comparisons show differences between the low-grade endometrioid endometrial carcinomas and endometrial serous carcinoma, endometrial carcinosarcoma, or undifferentiated endometrial carcinoma group ($P<0.05$) but not between the high-grade groups (high-grade endometrioid endometrial carcinoma, endometrial serous carcinoma, endometrial carcinosarcoma, undifferentiated endometrial carcinoma).

Table 2 Scribble expression and subcellular location in different subtypes of endometrial tumors ($N=106$): low and high-grade endometrioid endometrial carcinoma (LG-EEC and HG-EEC), endometrial serous carcinoma (ESC), endometrial carcinosarcoma (ECS), and undifferentiated endometrial carcinoma (UEC)

Tumor subtype	*Conserved membrane Scribble expression*	*Reduced/delocalized Scribble expression*
LG-EEC	20/28 (71%)	8/28 (29%)
HG-EEC	3/14 (21%)	11/14 (79%)
ESC	7/26 (27%)	19/26 (73%)
ECS	3/22 (14%)	19/22 (86%)
UEC	3/16 (19%)	13/16 (81%)

Pairwise comparisons show differences between low-grade endometrioid endometrial carcinoma and the rest of groups ($P<0.001$) but not between the high-grade groups (high-grade endometrioid endometrial carcinoma, endometrial serous carcinoma, endometrial carcinosarcoma, undifferentiated endometrial carcinoma).

hypermethylation, or inactivation of the Hippo pathway.[18,27] We first analyzed the *WWTR1* locus (3q25.1) in tissue microarray by fluorescence *in situ* hybridization in order to assess whether the *WWTR1* gene was amplified in endometrial carcinoma.[18] We were unable to find *WWTR1* amplification in any of the tumors examined and thus, we ruled out this as a possible influence on TAZ expression (Supplementary Figure 1).

Methylation could inhibit the *WWTR1* promoter, and in this way, it might be associated with TAZ expression levels in endometrial carcinomas. We analyzed a 1.1 kb region of the *WWTR1* gene promoter in 16 endometrial carcinosarcomas and 17 low-grade endometrioid endometrial carcinomas by MassARRAY spectrometry and we were unable to find significant differences in the methylation of this promoter region in the analyzed groups. In addition, methylation status did not appear to be correlated with TAZ expression in these samples (data not shown). Similarly, we failed to detect differences in methylation among the different histotypes when the *YAP1* promoter was analyzed (Supplementary Figure 2).

The expression and subcellular location of Scribble is also an important means to modulate the Hippo pathway[18] and thus, we studied Scribble expression and subcellular distribution by immunohistochemistry to evaluate whether this might influence TAZ expression in endometrial carcinomas. We evaluated 28 low-grade endometrioid endometrial carcinomas, 14 high-grade endometrioid endometrial carcinomas, 26 endometrial serous carcinomas, 16 undifferentiated endometrial carcinomas, and 22 endometrial carcinosarcomas (Figure 1b). Membrane expression of Scribble was conserved in 71% (20/28) of low-grade endometrioid endometrial carcinomas but only in 21% (3/14) of high-grade endometrioid endometrial carcinomas, 27% (7/26) of endometrial serous carcinomas, 19% (3/16) of undifferentiated endometrial carcinomas, and 14% (3/22) of endometrial carcinosarcomas (Table 2). We observed significant differences between low-grade endometrioid endometrial carcinomas and the rest of the endometrial cancer subgroups ($P<0.001$), but no differences were found when we compared high-grade endometrial tumors (high-grade endometrioid endometrial carcinomas, endometrial serous carcinomas, undifferentiated endometrial carcinomas, and endometrial carcinosarcomas). Remarkably, nuclear TAZ was frequently detected in endometrial carcinomas with weak and delocalized Scribble expression, observing a significant correlation between TAZ and Scribble expression ($P<0.05$).

According to these data, TAZ expression is not related to *WWTR1* amplification or methylation status but rather, it appears to be regulated by the expression and subcellular distribution of Scribble in endometrial carcinomas.

TAZ Overexpression in Endometrial Cancer Cells Increases Early Cell Motility, Invasiveness, and Tumor Growth

To further study the role of TAZ in endometrial tumorigenesis, we analyzed the TAZ protein and mRNA levels in a panel of endometrial cancer cell lines (Supplementary Figure 3A and 3B, respectively). Ishikawa cells are derived from a well-differentiated endometrioid endometrial carcinoma and they express TAZ mRNA and protein relatively weakly, in agreement with our findings in human low-grade endometrioid endometrial carcinomas. By contrast, TAZ was expressed strongly in AN3CA cells, which stem from a poorly differentiated endometrial carcinoma, consistent with our observations in undifferentiated endometrial carcinomas. Hence, we selected Ishikawa and AN3CA cells to generate

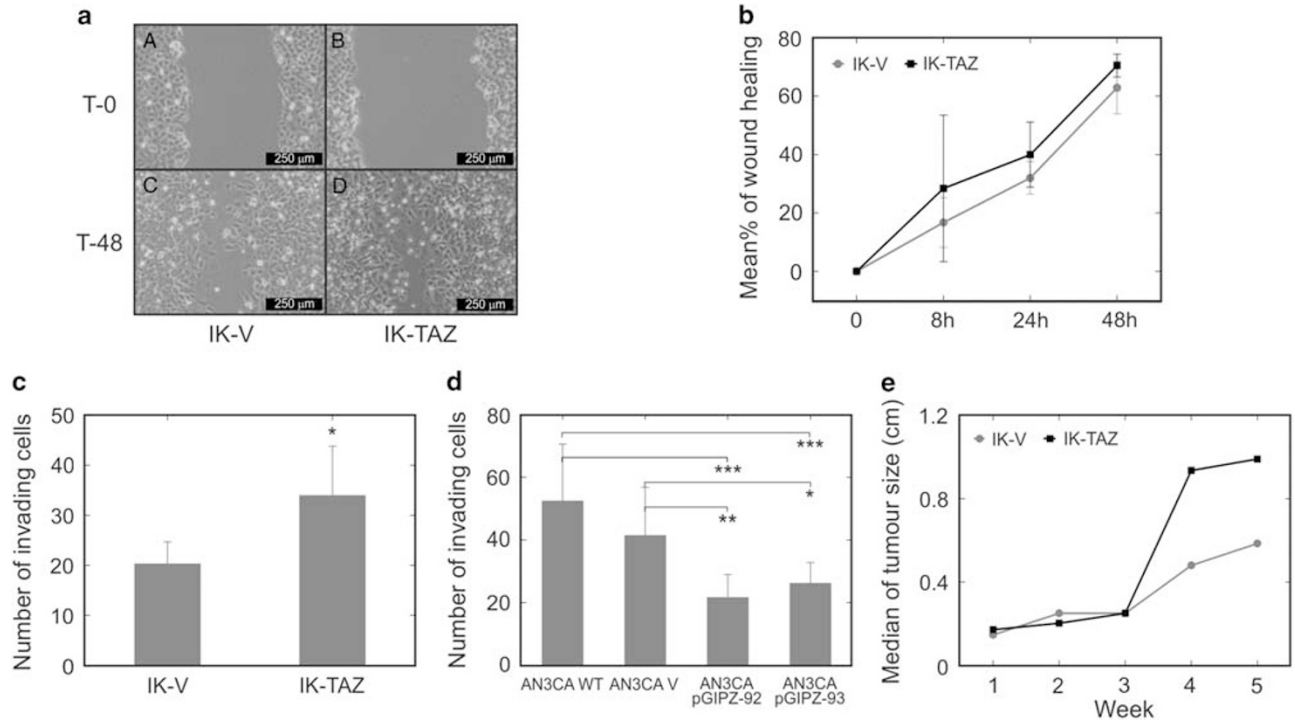

Figure 2 Changes in cell behavior when TAZ was overexpressed. (**a**) Images from a wound-healing assay at T-0 and T-48 h. (**b**) Average wound closing per clone. (**c**) Mean number of invasive Ishikawa-TAZ (IK-TAZ) and Ishikawa-control cells (IK-V). (**d**) Invasive ability of controls AN3CA cells and AN3CA-shTAZ clones: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (**e**) Median of tumor size (cm) over time in the subcutaneous cell injection assay.

stable TAZ-overexpressing and -silenced clones, respectively.

A wound-healing assay was used to characterize the *in vitro* migration and motility of the transfected cells. An increase in the migration of Ishikawa-TAZ cells was observed over 8 h, while Ishikawa-TAZ cells closed the wound area by an average of 28.4% in this time, and the control cells closed just 16.7% of the wound. However, after 48 h, the differences between these two cell types were smaller and both clones had completely closed the wound after nearly 72 h (Figure 2a and b). Although the differences were not statistically significant, these data indicated that TAZ may enhance the migratory capacity of Ishikawa cells. We did not find any significant differences in the migration of AN3CA clones in which TAZ was silenced with respect to their controls (data not shown).

When we studied the invasion capacity of the different clones, TAZ overexpression on Ishikawa cells appeared to be correlated with an increase in invasiveness ($P < 0.05$, Figure 2c). By contrast, in the pGIPZ-92 and pGIPZ-93 AN3CA silenced clones, we detected fewer invading cells than in the wild-type control cells and those that received the empty pGIPZ ($P < 0.05$: Figure 2d). These data suggested that TAZ expression enhances the invasive capacity of endometrial cancer cells. Importantly, these differences in cell migration and cell invasion were not related to any alteration in cell proliferation (Supplementary Figure 4A and 4B).

To obtain further information on the biological function of TAZ in endometrial carcinoma, we studied tumorigenesis *in vivo*. The Ishikawa and AN3CA control cells gave rise to tumors at most injections sites (67%; 12/18) and while Ishikawa-TAZ cells showed a similar tumor growth rate as control cells at the outset, this had increased significantly 3 weeks after injection (Figure 2e). No histological changes were evident in the isolated lesions derived from Ishikawa-TAZ and control cells, the size of the tumor represented the only significant difference. These results suggest a new role for TAZ as a tumor growth enhancer once a primary tumor has been established. By contrast, the size of the tumors produced by AN3CA-shTAZ clones did not show differences with respect to that of the controls (data not shown).

TAZ Expression Is Associated with Epithelial to Mesenchymal Transition Features in Endometrial Cancer Cell Lines and Human Tumors

As the acquisition of a motile and tumorigenic phenotype with low proliferative activity is a feature of cells showing epithelial to mesenchymal transition, and TAZ expression promotes epithelial to mesenchymal transition in other tumors,[16,18,27–30]

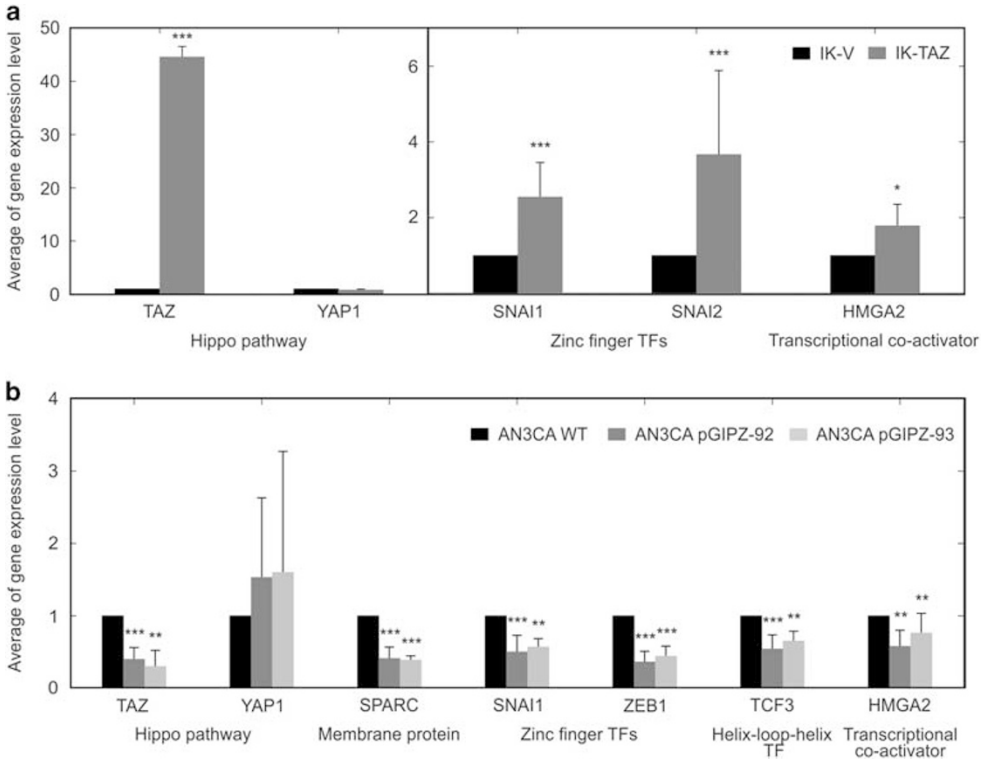

Figure 3 Changes in the expression of epithelial to mesenchymal transition-related genes upon TAZ overexpression and silencing. (**a**) Changes in gene expression upon TAZ overexpression in Ishikawa cells. T-test with dCT values: $*P<0.05$; $**P<0.01$; $***P<0.001$. (**b**) Changes in gene expression upon TAZ silencing in AN3CA cells with two different shRNAs (pGIPZ-92 and pGIPZ-93). Least Significant Difference-test with dCT values: $*P<0.05$; $**P<0.01$; $***P<0.001$.

we assessed whether TAZ expression was associated with modifications of epithelial to mesenchymal transition features in endometrial cancer cells. The expression of a set of previously described epithelial to mesenchymal transition-related genes[8] was evaluated in both types of endometrial cancer clones and we found that some epithelial to mesenchymal transition genes were expressed more strongly in Ishikawa-TAZ cells, such as *SNAI1* ($P<0.001$), *SNAI2* ($P<0.001$), and *HMGA2* ($P<0.05$: Figure 3a). By contrast, when TAZ expression was silenced in AN3CA cells by either of the shRNAs, we observed a decrease in the expression of the membrane factor *SPARC* ($P<0.001$), *SNAI1* ($P<0.01$), *ZEB1* ($P<0.001$), *TCF3* ($P<0.01$), and *HMGA2* ($P<0.01$: Figure 3b). These changes may reflect a role for TAZ in the induction of the epithelial to mesenchymal transition program[19] in endometrial cancer cell lines.

We next analyzed the expression of several proteins linked to the epithelial to mesenchymal transition process in the clones generated. Ishikawa-TAZ cells contained less E-cadherin and more N-cadherin than their control cells (Figure 4a), consistent with the acquisition of mesenchymal features. Conversely, TAZ silencing in AN3CA cells reduced their vimentin expression and increased claudin-1 expression (Figure 4b), indicating a shift at the molecular level to a more epithelial phenotype, although no such morphological changes were observed. Because not only the expression level but also the location of some proteins may change upon the onset of epithelial to mesenchymal transition, we analyzed the subcellular distribution of TAZ and Scribble in Ishikawa-TAZ and control cells by immunofluorescence, as well as the distribution of the epithelial to mesenchymal transition-related proteins vimentin and β-catenin. TAZ overexpression not only enhanced vimentin expression but it also decreased the expression of Scribble and β-catenin, driving their translocation from the membrane to the cytoplasm (Figure 4c).

To assess the relationship between TAZ expression and epithelial to mesenchymal transition features in human endometrial carcinomas, we compared the expression of epithelial to mesenchymal transition genes between low-grade endometrioid endometrial carcinoma (representing the prototype of endometrial carcinoma with a more epithelial phenotype and the subtype in which TAZ expression is least often detected) and endometrial carcinosarcoma (an endometrial carcinoma in which a complete epithelial to mesenchymal transition has been undergone[3] and one of the subtypes that most frequently expresses TAZ). Some genes related to the epithelial to mesenchymal transition were expressed distinctly in these two types of tumor, such as *CDH1*, *SNAI1*, *SNAI2, HMGA2, ZEB1,* and *ZEB2* ($P<0.05$: Figure 5). Moreover, when we compared the expression of the *WWTR1* gene and

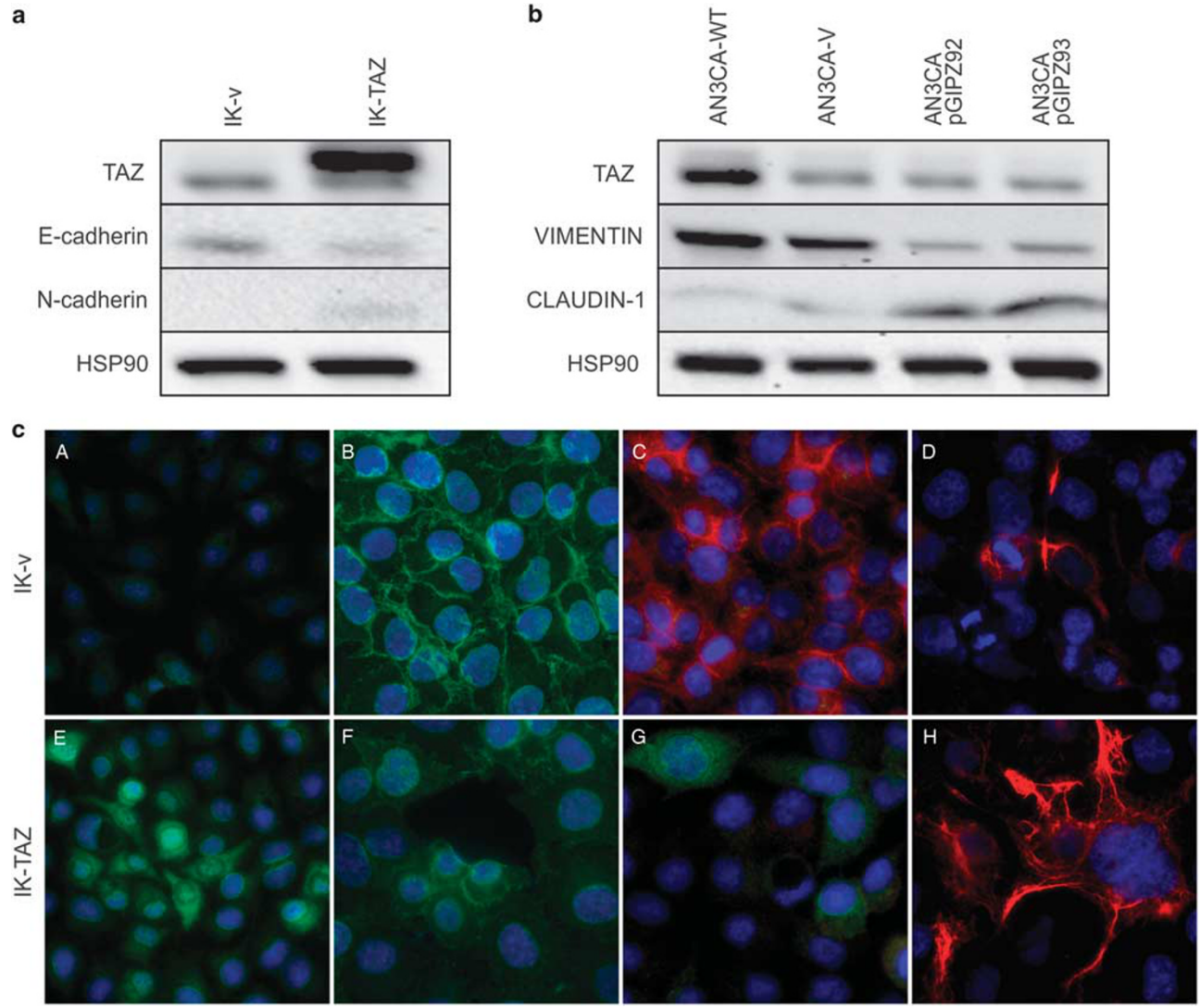

Figure 4 Changes in protein expression and localization. (**a**) Protein expression in Ishikawa clones assessed in western blots. (**b**) Protein expression in AN3CA wild type and silenced clones assessed in western blots. (**c**) Immunofluorescence of Ishikawa clones for different proteins: (A) and (E) TAZ (green); (B) and (F) Scribble (green); (C) and (G) B-catenin (red) and TAZ (green); (D) and (H) Vimentin (red).

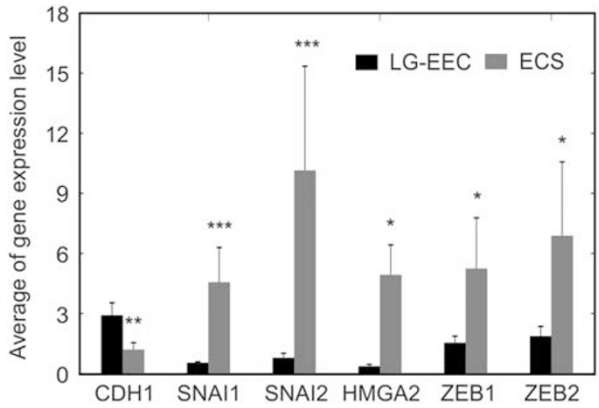

Figure 5 Epithelial to mesenchymal transition gene expression. mRNA analysis of 15 low-grade endometrioid endometrial carcinoma (LG-EEC) and 20 endometrial carcinosarcomas (ECS). T-test with dCT values: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

that of the epithelial to mesenchymal transition-related genes, we observed a negative correlation between *WWTR1* and *CDH1* ($P < 0.05$), and a positive relationship between *WWTR1* and *SNAI1* ($P < 0.01$) or *HMGA2* ($P < 0.05$: Supplementary Figure 5). This pattern was consistent with the changes observed in Ishikawa-TAZ cells, and these results show that TAZ expression was associated with the expression of epithelial to mesenchymal transition-related factors and with the acquisition of epithelial to mesenchymal transition features in endometrial tumors.

We further studied how TAZ contributes to the regulation of epithelial to mesenchymal transition by analyzing the miR-200 family of microRNAs, the deregulation of which is involved in epithelial to mesenchymal transition.[31,32] We found that all miR-200 microRNAs were significantly downregulated in endometrial carcinosarcomas when compared

with low-grade endometrioid endometrial carcinomas ($P < 0.01$: Supplementary Figure 6), consistent with the mesenchymal phenotype of these tumors. When we compared microRNA expression with that of epithelial to mesenchymal transition-related genes, we found a positive relationship between the expression of all the members of miR-200 family and *CDH1* ($P < 0.001$), and a negative relationship with *HMGA2* ($P < 0.05$), *SNAI1* ($P < 0.05$), *SNAI2* ($P < 0.05$, except for miR-200c), *TWIST1* ($P < 0.05$, except for miR-200a), *ZEB1* ($P < 0.05$ except for miR-200b/c), and *ZEB2* ($P < 0.05$ for miR-200a and -141: data not shown). Interestingly, an analysis of the available gene array data from NCI60 cell lines suggested that members of the miR-200 family may target the *WWTR1* 3′-UTR region.[33] Accordingly, we found that *WWTR1* expression was negatively correlated with that of all miR-200 microRNAs (Supplementary Figure 7): miR-141 ($P < 0.001$), miR-200a ($P < 0.01$), miR-200b ($P < 0.05$), miR-200c ($P < 0.01$), and miR-429 ($P < 0.05$).

Discussion

TAZ is a transcriptional co-activator of the Hippo pathway that is involved in epithelial to mesenchymal transition and stemness in different tumors. For instance, it has been demonstrated that TAZ is implicated in promoting migration and invasion in breast cancer cell models,[15] and it has also been shown that TAZ knockdown provokes defects in the maintenance of pluripotency in embryonic stem cells.[34] Hence, it would appear that TAZ is a potent activator of cell transformation and tumorigenesis.[35,36] On the basis of this evidence, and given the importance of epithelial to mesenchymal transition and stemness in the carcinogenesis of certain subtypes of endometrial tumors,[3,4] we analyzed TAZ expression in a series of 143 endometrial carcinoma samples. We found that TAZ expression was associated with high-grade endometrial tumors, mainly represented by undifferentiated endometrial carcinomas and endometrial carcinosarcomas.

Different mechanisms have been proposed to modulate TAZ expression in human cancers. Amplification of the *WWTR1* locus (3q25.1) was detected in approximately 8% of breast cancers,[37] and this was related with increased TAZ/YAP activity.[18] Provisional data from The Cancer Genome Atlas (http://www.cbioportal.org) reported a 5% amplification of *WWTR1* in endometrial carcinosarcomas, however, we did not find any amplification of *WWTR1* in the undifferentiated endometrial carcinoma or endometrial carcinosarcoma tissue in our series. This could reflect the low amplification frequency and the relative low number of cases analyzed.

TAZ has been closely associated with a mesenchymal signature in glioblastomas,[27] and weak TAZ expression was correlated with CpG island hypermethylation in the *WWTR1* promoter of low-grade gliomas.[27] However, we did not find significant differences in the methylation status of *WWTR1* and its paralogue *YAP1* in endometrial carcinosarcoma and endometrioid endometrial carcinoma samples, suggesting that this modification is unlikely to control the expression of TAZ. Together, these data rule out the possibility that TAZ expression in endometrial carcinoma is regulated by gene amplification or promoter methylation as the most common or unique regulatory mechanisms.

Activation of the Hippo pathway has been proposed as one of the main mechanisms that modulates TAZ stability and its subcellular location.[18,38] This pathway may be affected by mechanical stimuli such as cell contact or cytoskeletal dynamics.[39] Indeed, when the cell polarity protein Scribble is located at the membrane, Hippo signalling is activated and TAZ expression repressed. However, if cell polarity is lost during tumor progression or as a result of the induction of epithelial to mesenchymal transition, Scribble becomes delocalized and consequently, enhancing TAZ expression.[18] Scribble may regulate TAZ at the posttranscriptional level, because silencing Scribble in breast cancer cells increases the TAZ protein in cells without affecting its mRNA levels or their epithelial morphology.[18] Our *in vivo* results confirm this cross-talk in endometrial carcinoma, showing that Scribble delocalization is associated with TAZ nuclear expression in high-grade endometrial tumors. Reduced and delocalized Scribble expression in endometrial carcinoma has been associated with poor prognostic factors, such as advanced stages, histopathological differentiation, and lymph node metastasis,[40] in accordance with our data. Moreover, we also observed a reciprocal regulation *in vitro* because, in Ishikawa-TAZ cells, Scribble was translocated from the membrane to the cytoplasm. This redistribution might reflect an alteration in cell polarity as a consequence of the acquisition of mesenchymal properties upon TAZ overexpression.

We show that TAZ overexpression enhances the ability of cells to migrate, as well as produce a significant increase in their invasive capacity without affecting proliferation, suggesting that TAZ fulfills a role in the development of the epithelial to mesenchymal transition *in vitro*. Our *in vivo* tumorigenesis assay in mice also suggests that TAZ induces tumor growth once the tumor has been established. Indeed, the gene expression analysis suggests that TAZ overexpression promotes the expression of the epithelial to mesenchymal transition inducers *SNAI1*, *SNAI2*, and *HMGA2*, whereas the silencing of TAZ decreases that of *SPARC*, *SNAI1*, *ZEB1*, *TCF3,* and *HMGA2*. Accordingly, TAZ would appear to directly or indirectly regulate the transcription of these epithelial to mesenchymal transition and epithelial dedifferentiation markers. These *in vitro* results were confirmed *in vivo*, because *WWTR1* expression was correlated with that of *CDH1*, *SNAI1*, and *HMGA2* in the endometrial tumors analyzed. We also show that

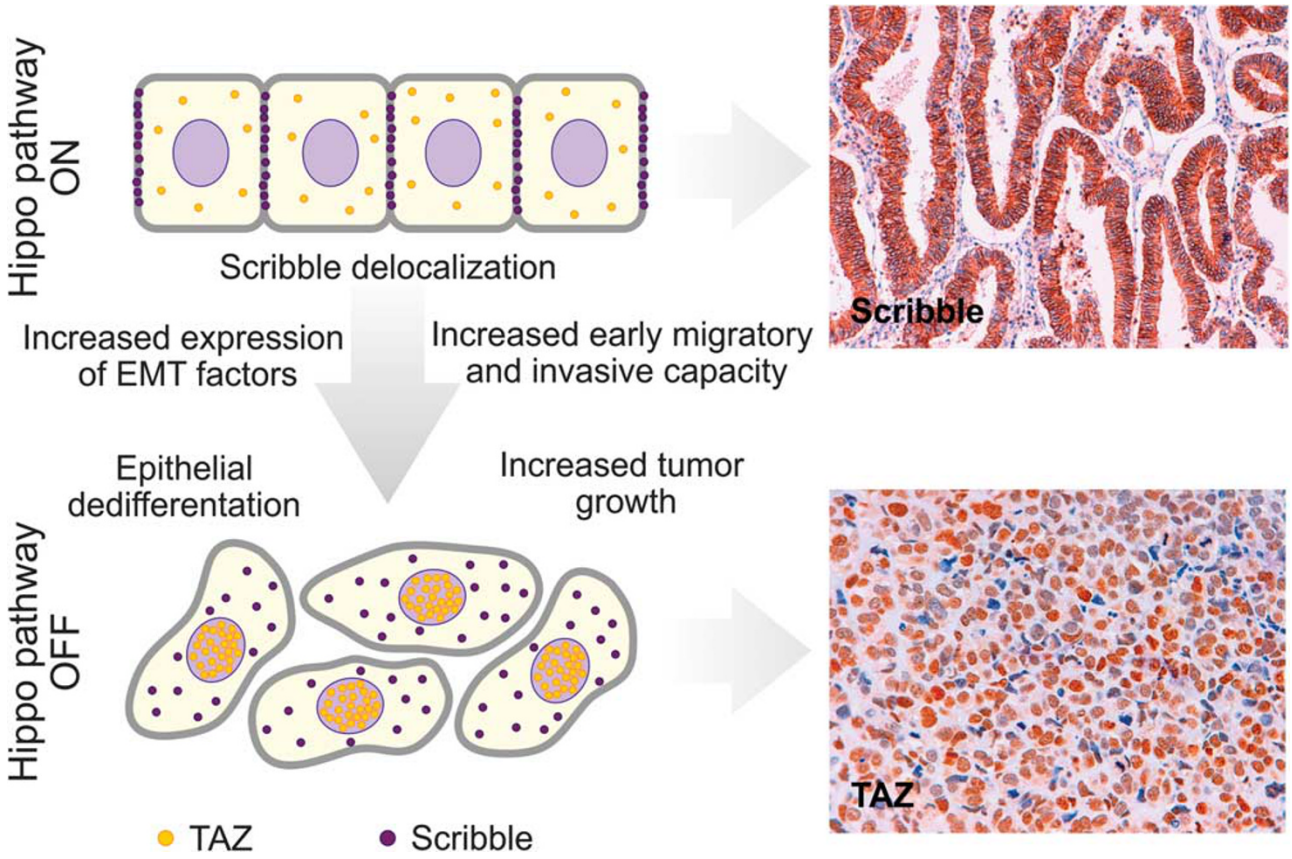

Figure 6 Scheme of the role of the Hippo pathway on the behavior of endometrial cancer cells (based on the mechanism proposed by Cordenonsi *et al*.[18] *Cell*, 2011), showing Scribble expression on an endometrioid endometrial carcinoma and TAZ expression on an undifferentiated endometrial carcinoma.

TAZ overexpression is associated with a loss of E-cadherin while augmenting the N-cadherin protein in the endometrial cancer cell lines, and that TAZ silencing decreases vimentin expression while increasing that of claudin-1. The loss of E-cadherin could be associated with the loss of Scribble activity,[41] consistent with the effects on cell polarity and the triggering of epithelial to mesenchymal transition provoked by TAZ overexpression. In addition to this loss of epithelial features, we detected a negative correlation between the expression of miR-200s and *WWTR1*, showing that miR-200s expression is dampened during the transition from endometrioid endometrial carcinoma to endometrial carcinosarcoma, while *WWTR1* expression increases. This relationship is reinforced by the significant correlation found between miR-200s expression and the expression of *HMGA2, SNAI, SNAI2, TWIST1, ZEB1,* and *ZEB2* mRNA in the tumors analyzed, some of which are directly correlated with *WWTR1* expression.

In summary, TAZ expression is associated with the most aggressive forms of endometrial carcinoma, such as undifferentiated endometrial carcinoma, endometrial carcinosarcoma, or endometrial serous carcinoma. In these tumors, TAZ expression is at least partially modulated by the expression and subcellular location of Scribble. TAZ overexpression favors the expression of epithelial to mesenchymal transition-related factors, and it induces an increase in the early migratory and invasive capacity of cells. Moreover, TAZ also favors tumor growth *in vivo* (Figure 6). These findings reveal a previously unknown role for the Hippo pathway in the development and progression of different subtypes of endometrial carcinoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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