

Focal Adhesion Kinase Interacts with the Transcriptional Coactivator FHL2 and Both are Overexpressed in Epithelial Ovarian Cancer

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Abstract. *Abnormal signal transduction arising from integrins and protein tyrosine kinases has been implicated in the initiation and progression of a variety of human cancers. Integrin-mediated signal transduction pathways require regulated cytoplasmic protein-protein interactions. However, little is known about integrin-associated proteins and ovarian cancer. In our study we investigated the association of pp125FAK, a cytoplasmic tyrosine kinase, involved in anchorage-independent growth of tumor cells, and the Four and a Half LIM domain (FHL) protein FHL2, which was recently shown to interact with integrins. Our data demonstrated that pp125FAK and FHL2 form a protein complex in human ovarian carcinoma. Furthermore, we showed that pp125FAK is overexpressed in epithelial ovarian cancer, but virtually absent in normal ovary. Our immunohistochemistry data showed that FHL2 protein expression is also augmented in epithelial ovarian cancer. Taken together, our results demonstrated for the first time FHL2 expression in human ovarian cancer cells, suggesting an important functional role of pp125FAK and FHL2 complex in gynecologic malignancies.*

The transcriptional co-activator FHL2 is a LIM-only member of the LIM protein superfamily. LIM proteins play important roles in a variety of biological processes including cytoskeleton reorganization, cell lineage specification, organ development, and in pathological processes such as tumorigenesis (1,2). LIM proteins are defined by the presence of one or more LIM domains, which mediate protein-protein interactions. Each LIM domain is characterized by two cysteine-composed double zinc finger motifs (3). Based on the presence of additional motifs, LIM proteins are grouped into three classes.

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The Four and a Half LIM domain (FHL) proteins are formed by the five members FHL1, 2, 3, 4 and ACT and belong to the LIM-only protein subclass (4).

FHL2 interacts with transcription factors such as the androgen receptor, CREB/CREM, PLZF, AP-1, as well as various other proteins, e.g. hCDC47 and presenilin 2 (5-10).

FHL2 exhibits a tissue-restricted expression pattern with prominent expression in heart and prostate epithelial cells (5) and its subcellular localization can be both nuclear and non-nuclear. Stimulation of the Rho signalling pathway induces translocation of FHL2 from the cell membrane into the nucleus and subsequent activation of FHL2- and AR- dependent genes (5,11). FHL2 is found in focal adhesion complexes and binds to integrins (12). Integrins present in focal adhesion complexes are known to initiate signal transduction cascades that require protein-protein interactions. The exact role played by FHL2 in such transduction cascades is not well understood yet. Apart from integrins and FHL2, a number of cytoplasmic proteins have been identified in focal adhesion complexes, including the cytoplasmic tyrosine kinase pp125FAK (pp125 Focal Adhesion Kinase) (13). A very early and crucial event in integrin-mediated signal transduction is the phosphorylation of proteins on tyrosine residues and one of the downstream targets is pp125FAK, which binds to the β_1 -integrin subunit (14).

pp125FAK is a nonreceptor tyrosine kinase that is ubiquitously expressed throughout development and widely expressed in adult tissues. pp125FAK takes a key-position in the signal transduction network, since it is activated not only by integrins, but also by growth factor receptors, G-protein-coupled receptors and neuropeptides such as endothelin and bombesin (15). Upon autophosphorylation at tyrosine 397 and subsequent activation, pp125FAK associates with the tyrosine kinase Src by binding to its SH2 (Src homology 2) domain. Phosphorylation of tyrosine 925 of pp125FAK by Src then recruits the Grb2 adaptor protein, which triggers downstream signaling events leading to activation of extracellular signal-regulated kinases *via* Ras (16). Therefore pp125FAK links integrin-generated signals to other signal transduction pathways.

The pp125FAK/Src complex has been shown to phosphorylate downstream substrates such as paxillin and p130cas. In addition, the phosphorylated tyrosine 397 constitutes the major binding site for the SH2 domains of the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and pp125FAK association with PI 3-kinase in cell adhesion triggers activation of PI 3-kinase and its signaling pathways (17). pp125FAK interactions with these and potentially other proteins are believed to mediate pp125FAK's functions in integrin-dependent signal transduction.

Little is known about integrin-associated proteins and signaling in human ovarian cancer cells (18). In this study we investigated the expression and interaction of pp125FAK and FHL2 in human ovarian tissue. Our results show, for the first time, that both pp125FAK and FHL2 are overexpressed in human epithelial ovarian cancer. Furthermore, pp125FAK and FHL2 form a protein-protein complex in human ovarian carcinoma.

Materials and Methods

Tissue samples. The tissue samples were obtained at the time of surgery at the Department of Obstetrics and Gynecology, Freiburg University Medical Center, Germany, between April 2000 and October 2002. Specimens from 13 epithelial ovarian carcinomas (stage III and IV) and 6 nonneoplastic tissues from human postmenopausal ovary were obtained. All of the tissue specimens were histologically examined and the pathological diagnoses were confirmed. The ethical standards of the University of Freiburg were followed in all procedures.

RNA analysis. Northern blots (BD Biosciences, Heidelberg, Germany) were hybridized with an FHL2 probe according to the manufacturer's instructions (BD Biosciences). The FHL2 coding region was labeled with StripEZ (Ambion, Austin, USA) and hybridized as recommended. RNA loading was controlled using β -actin or GAPDH as probe (5).

Co-immunoprecipitation assays and Western blot analyses. Total cell extracts from human ovarian carcinoma or OVCAR-3 cells (ATCC HTB 161) were prepared in IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 20% glycerol, 50 mM NaF, 2 mM NaV, 0.2 mM DTT, 0.1 mM Pefabloc). Following centrifugation, pre-cleared supernatants were incubated for 1 h with α -FHL2 antibody (5) or α -pp125FAK antibody (Santa Cruz, Santa Cruz, USA; #sc-558-G) on GammaBind™-Sepharose 4B (Amersham, Freiburg, Germany) in IP buffer. Precipitated protein complexes were then washed 4 times with IP buffer and analyzed on 10% SDS polyacrylamide gels. Western blots were processed as indicated. Secondary antibody and chemoluminescence procedures were performed according to the manufacturer's instructions (Amersham).

GST pull-down assays. Expression vectors for glutathion S-transferase (GST) and FHL2 (GST-FHL2) were described previously (5). pcDNA3.1FAKwt was a gift of C. Hauck. Expression of GST fusion proteins (Amersham) and the coupled *in vitro* transcription-translation reaction (Promega, Mannheim, Germany) were performed according to the manufacturer's instructions. GST pull-down assays were performed as previously

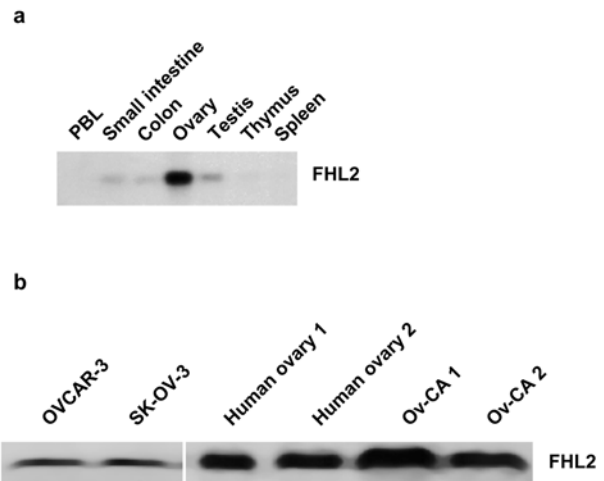


Figure 1. FHL2 is expressed in human ovary. a) Northern blot analysis: FHL2 mRNA expression in a panel of adult human tissues. FHL2 is strongly expressed in normal ovary. PBL: Peripheral blood leucocytes. b) Western blot analyses of cell extracts from two established human ovarian cancer cell lines (OVCAR-3 and SK-OV-3), two normal human postmenopausal ovaries and two ovarian carcinoma samples were performed with a monoclonal α -FHL2 antibody (5). A total of 6 ovarian carcinoma samples and 4 normal ovaries were analyzed with the same result (data not shown).

described (5) except that incubation was performed at 37°C. Ten percent of the *in vitro* translated proteins were loaded as input.

Immunohistochemistry. Stainings were performed using a protocol for antigen retrieval and indirect immunoperoxidase as described (5). The dilutions used were 1:600 for the α -FHL2 polyclonal antibody (11), 1:1200 for the α -pp125FAK (Upstate Biotechnology, Charlottesville, USA; #05-537) mouse monoclonal antibody. Secondary antibody dilution and immunoreactions were performed as recommended by the manufacturer (Vectastain, Alexis, Grunberg, Germany).

Results

FHL2 is expressed in human ovarian tissue. In the present study we were interested in analyzing the FHL2 expression pattern, especially in human ovary. To address this issue we performed Northern blot analysis. As shown in Figure 1a, FHL2 is strongly expressed in the human ovary whereas little FHL2 signal is detected in other tissues. Western blot analyses performed with a monoclonal α -FHL2 antibody (5) show that FHL2 is present in extracts from normal human ovary, human ovarian cancer and in the two ovarian carcinoma cell lines, OVCAR-3 and SK-OV-3 (Figure 1b).

To determine the subcellular localization of FHL2, we performed immunohistochemical analyses of FHL2 in normal human ovarian tissue and ovarian tumor tissue obtained from surgery on postmenopausal women. In normal ovary (Figures 2a-c), FHL2 immunoreactivity is detected in the epithelial and stromal cells. In an epithelial ovarian cancer section

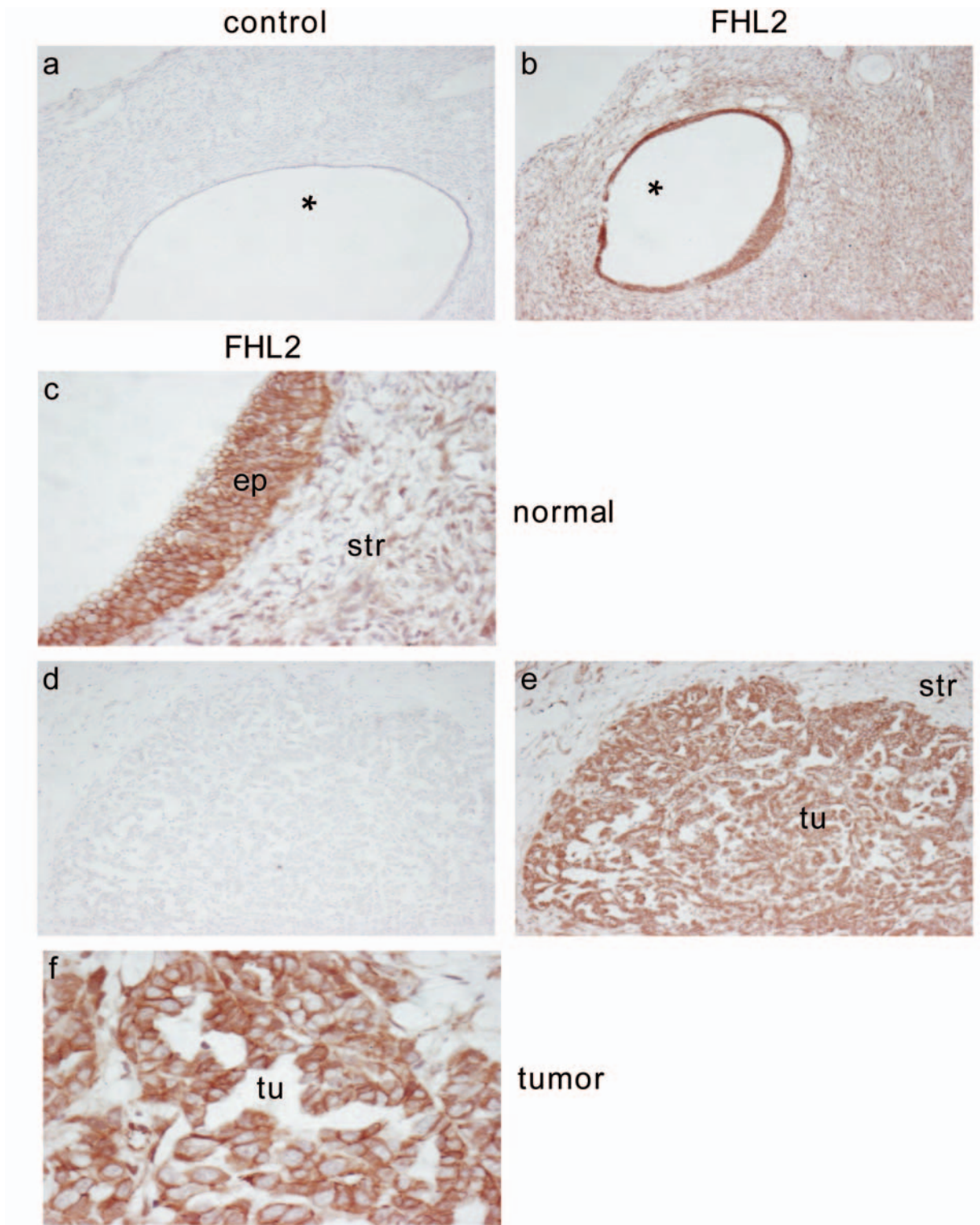


Figure 2. *FHL2* is overexpressed in human epithelial ovarian cancer. Immunohistochemical analyses of *FHL2* in normal human ovary (b,c) and epithelial ovarian cancer (e,f) from postmenopausal women show strong expression in epithelial cells (ep) and weaker expression in stromal cells (str) of normal ovary (b,c). Star: benign cyst. In a serous papillary carcinoma of human ovary (e,f) strong *FHL2* immunoreactivity is detected in the tumor area (tu). The higher magnification (c,f) shows that *FHL2* staining is cytoplasmic with strong immunoreactivity of the cell membrane in epithelial cells. Controls were performed with rabbit IgG instead of the specific antibody and are shown in (a) and (d). A total of 3 normal ovaries and 7 ovarian carcinomas were analyzed with the same result (data not shown).

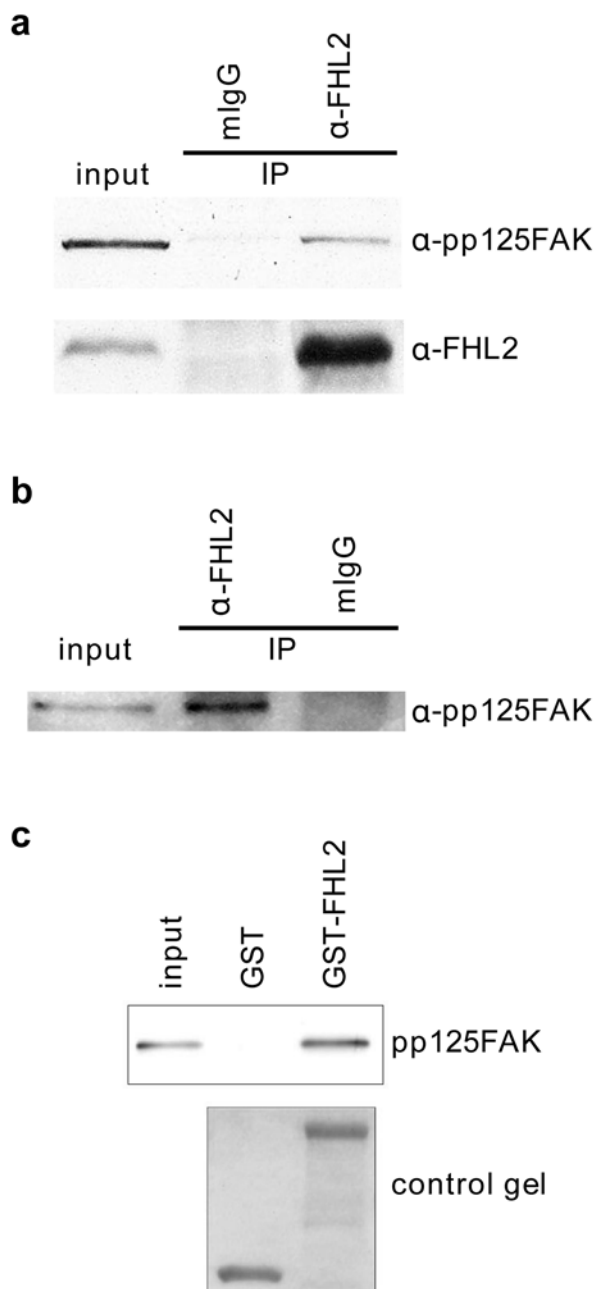


Figure 3. *FHL2* and *pp125FAK* interact *in vivo*. a) Extracts from human ovarian carcinoma were immunoprecipitated with a monoclonal α -FHL2 antibody. Western blots were processed with either monoclonal α -FHL2 or a polyclonal α -pp125FAK antibody. As a control the immunoprecipitation was carried out with mouse IgG (mIgG). b) Extracts of OVCAR-3 cells were immunoprecipitated with a monoclonal α -FHL2 antibody and analyzed in a Western blot with the α -pp125FAK antibody. As a control the immunoprecipitation was performed with mouse IgG. Input was 5% (a) or 10% (b) of the extract used for the immunoprecipitation. c) *pp125FAK* and *FHL2* interact *in vitro*. GST pull-down experiments were performed with [³⁵S]methionine-labeled *pp125FAK* and bacterially expressed GST-*FHL2* fusion protein. GST protein was used as control (upper panel). Same protein amounts of GST and GST-*FHL2* were used as shown in the Coomassie-stained control gel (lower panel).

(Figures 2d-f), FHL2 is specifically expressed in the tumor cells. The surrounding stromal and endothelial cells show clearly less FHL2 immunoreactivity. Again, FHL2 immunoreactivity is cytoplasmic with a strong staining intensity of the cell membrane in the tumor cells resembling a honeycomb-like staining pattern. Immunohistochemical analyses of FHL2 in all epithelial ovarian cancer specimens tested show increased expression compared to normal ovaries. Taken together, our data demonstrate that FHL2 is expressed in human ovarian tissue and that a strong FHL2 immunoreactivity is detected in the tumor areas of human epithelial ovarian cancer sections.

pp125FAK interacts with *FHL2*. Since FHL2 was described to bind integrins, we tested a possible association with other proteins involved in integrin signaling and focal adhesion formation. One of these proteins is the cytoplasmic tyrosine kinase *pp125FAK*.

To test whether *pp125FAK* is associated with FHL2 *in vivo*, we performed immunoprecipitations from cell extracts obtained from human ovarian carcinoma samples. A monoclonal α -FHL2 antibody co-immunoprecipitates endogenous *pp125FAK* (Figure 3a). In control experiments using goat IgG, *pp125FAK* is not detected. To verify further the specificity of the association, the endogenous FHL2/*pp125FAK* complex was co-immunoprecipitated in OVCAR-3 cells. As expected, *pp125FAK* is co-precipitated with the monoclonal α -FHL2 antibody (Figure 3b). To demonstrate that *pp125FAK* and FHL2 interact directly, we performed pull-down experiments. Bacterially expressed FHL2 protein fused to GST was incubated with *in vitro* translated [³⁵S]methionine-labeled *pp125FAK*. As shown in Figure 3c, *pp125FAK* interacts directly with FHL2, but not with the GST control. Taken together, these results demonstrate that FHL2 and *pp125FAK* are associated *in vivo* and *in vitro*. This is the first evidence for a protein-protein interaction between a FHL protein and a cytoplasmic tyrosine kinase.

pp125FAK is overexpressed in human ovarian cancer. To examine the expression level of *pp125FAK* in normal and malignant ovarian tissue and in ovarian carcinoma cell lines, we carried out Western blot analyses with a polyclonal α -*pp125FAK* antibody. *pp125FAK* is strongly expressed in OVCAR-3 and SK-OV-3 cells and in all tested epithelial ovarian cancer samples (Figure 4). In contrast, no *pp125FAK* is detected in normal ovarian tissue.

To examine this finding in more detail, we also performed immunohistochemical analyses. In normal ovary *pp125FAK* immunoreactivity is mainly observed in vascular endothelial cells (Figure 5, arrows) as has been described for other tissues (19). However, *pp125FAK* is significantly overexpressed in ovarian tumor cells. In tumor cells the *pp125FAK* staining is predominantly cytoplasmic.

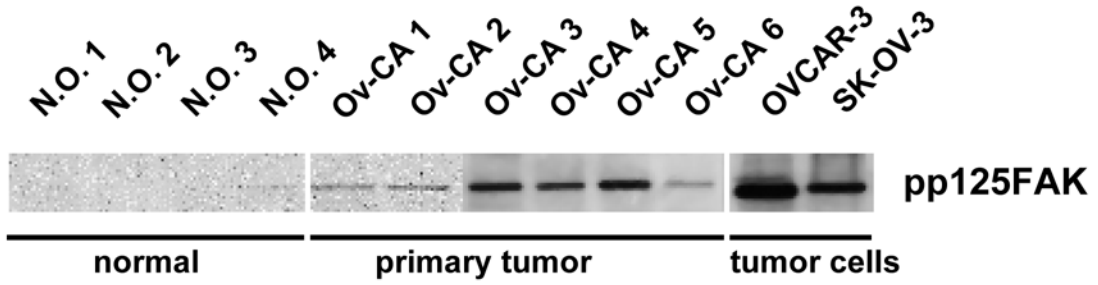


Figure 4. *pp125FAK* is overexpressed in human epithelial ovarian cancer. Extracts of 4 normal ovaries (N.O.), 6 epithelial ovarian cancer specimen (Ov-CA) and the human ovarian cancer cell lines OVCAR-3 and SK-OV-3 were analyzed in Western blots with a polyclonal α -*pp125FAK* antibody.

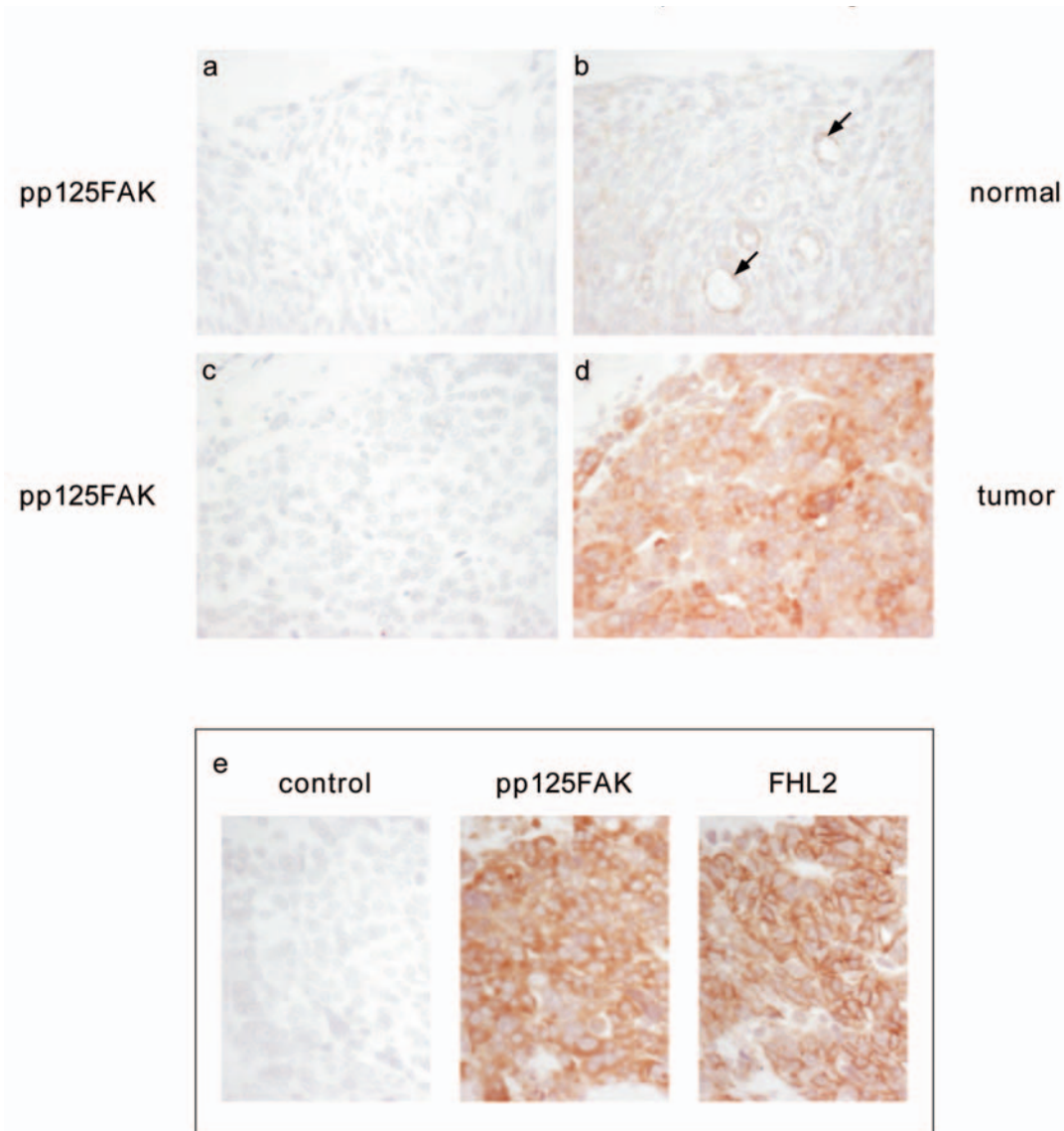


Figure 5. Immunohistochemical analysis of *pp125FAK* overexpression in epithelial ovarian cancer cells. Immunohistochemical staining of *pp125FAK* in normal ovary (b) and epithelial ovarian cancer (d) from postmenopausal women. Controls using mouse IgG instead of specific antibody are shown in a) and c). In normal ovarian tissue *pp125FAK* immunostaining is confined to vascular endothelial cells (b, arrows). Epithelial ovarian cancer cells display strong cytoplasmic *pp125FAK* immunostaining (d). Three normal ovaries and 5 ovarian carcinomas were studied. A typical section of the analyzed cases is shown. e) Consecutive sections of the same epithelial ovarian cancer demonstrate *pp125FAK* and FHL2 immunostaining in the tumor area.

In addition, FHL2 is also strongly expressed in ovarian tumor cells (Figure 2 and 5e). Taken together, these data demonstrate overexpression of pp125FAK and FHL2 in human ovarian cancer.

Discussion

Tumorigenesis is a multistep process of genetic alterations driving the transformation of normal cells into malignancy (20). Abnormal signal transduction has been implicated in the initiation and progression of a variety of human cancers, including gynecologic malignancies (21). Several classes of proteins involved in signal transduction, such as integrins and pp125FAK, are altered in cells possessing invasive or metastatic capabilities (20,22).

Recent studies suggest that FHL2, a LIM-only protein with a restricted expression pattern, is implicated in integrin-mediated signal transduction, focal adhesion formation and regulation of transcription (5,6,8,12). We were interested in investigating interactions between FHL2 and other proteins involved in integrin signaling and focal adhesion formation and to analyze FHL2 expression in human ovarian tissue. Elucidating such protein-protein interactions in normal and cancer cells might lead to a better understanding of signal transduction and cancer.

Northern blot analyses demonstrate that FHL2 mRNA is strongly expressed in human ovarian tissue. We barely detected FHL2 mRNA in the other tissues tested. This is in accordance with the restricted expression pattern of FHL2 (23) and suggests an important functional role of FHL2 in human ovarian tissue.

Immunohistochemical analyses demonstrate that FHL2 protein is present in both normal ovary and in epithelial ovarian cancer. In normal ovary FHL2 expression is confined to the cytoplasm and the cell membrane in epithelial cells, but also detected in stromal cells. Importantly, FHL2 is overexpressed in epithelial ovarian cancer. Again, FHL2 immunoreactivity is cytoplasmic with a strong and circular staining of the cell membrane. However, protein blots of ovarian cancer samples were not enriched for epithelium and thus we cannot conclude that FHL2 is necessarily up-regulated exclusively in the epithelial fraction.

To our knowledge this is the first report showing FHL2 expression in human ovarian cancer. Müller *et al.* showed that translocation of FHL2 to the nucleus occurs in human prostate tissue during progression towards malignancy (11). Interestingly, we barely detected any nuclear FHL2 staining either in normal ovaries or in epithelial ovarian cancer. In summary, our data demonstrate the expression of FHL2 in adult human ovary, in ovarian carcinoma cell lines (OVCAR-3, SK-OV-3) and strong expression in epithelial ovarian cancer.

Next, we investigated a possible interaction between FHL2 and the cytoplasmic tyrosine kinase pp125FAK.

FHL2 and pp125FAK are localized to focal adhesion complexes (1,12,24) and both proteins associate with the cytoplasmic tails of integrins (12,14). In our studies we demonstrated that FHL2 associates with pp125FAK *in vivo* in human ovarian carcinoma as well as *in vitro* as shown in pull-down experiments, suggesting a functional role of the FHL2/pp125FAK complex in signal transduction.

Both, FHL2 and pp125FAK are components of multiprotein complexes making them suitable for the integration of signaling events. FHL2 can be recruited to cell adhesion complexes where it is clustered together with integrins and vinculin at the ends of actin stress fibers (1,12). Several proteins from the LIM family, such as paxillin or zyxin, are adaptors involved in scaffolding of focal adhesion complexes or of the cytoskeleton (25-27). Paxillin and its homologue Hic-5 are known to interact with pp125FAK (27). Other LIM-only proteins such as PINCH and CRP1 are also recruited to integrin signaling complexes through interactions with integrin-linked kinase (28) or *via* zyxin and α -actinin (25), respectively. Hence, FHL2 may fulfil a similar function. Since FHL2 itself seems not to possess kinase activity, the activation of pp125FAK by tyrosine phosphorylation or binding of pp125FAK to downstream signaling molecules (16,17) could be necessary for the FHL2/pp125FAK complex to regulate further signaling pathways. The question as to whether phosphorylation influences the FHL2/pp125FAK complex remains to be analyzed.

In Western blot analyses we showed that pp125FAK is expressed in both ovarian carcinoma cell lines and ovarian cancer samples. Barely any pp125FAK protein was found in normal ovarian tissue, which is in accordance with Judson *et al.* (18). In normal ovary pp125FAK expression was confined to vascular endothelial cells with no pp125FAK expression in the ovarian stromal cells from postmenopausal women. However, the pp125FAK expression pattern in ovaries from premenopausal women remains to be analyzed. Our immunohistochemical analyses confirmed overexpression of pp125FAK in epithelial ovarian cancer with predominantly cytoplasmic immunoreactivity. It has been shown that pp125FAK is involved in cell cycle regulation (29), apoptosis (30), tumorigenesis and metastasis of various human tumors, such as breast cancer or cervical cancer (31-33). Elevated tyrosine phosphorylation of pp125FAK was described for lung cancer and correlated with nodal involvement and disease-free survival (32). Our data favor the hypothesis that this FHL2/pp125FAK complex is a specific property of tumor cell signaling.

In summary, the overexpression of pp125FAK and FHL2 in epithelial ovarian cancer, together with the association of both proteins *in vivo*, indicate a functional role of FHL2/pp125FAK in gynecologic malignancies. We suggest that cytoplasmic tyrosine kinases, such as pp125FAK and associated proteins such as FHL2 may serve as targets of future therapeutics.

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