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Review

The urokinase receptor and integrins in cancer progression

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Abstract. Enhanced levels of expression of urokinase receptor (uPAR) and certain integrins have been linked to cancer cell progression. This has classically been attributed to matrix degradation via the activation of the urokinase (uPA)/plasmin system and modulation of cell motility and survival through integrin engagement. More recently, uPAR has been shown to play multiple roles independent of protease activity. Specifically, uPAR has been shown to be intimately involved in the regulation of cell adhesion, migration and proliferation in part through interactions with other membrane partners, including integrins. The goal of this review is to summarize recent insights in the function of uPAR/integrin interactions, to provide a framework for understanding the importance of these interactions in the context of cancer, and to highlight its potential as a target for therapeutic intervention.

Keywords. Urokinase, urokinase receptor, uPAR, integrins, cancer.

Introduction

Urokinase receptor (uPAR, CD87), a glycolipid-anchored protein [1], was initially thought to finely regulate the location and extent of plasminogen activation (PA) by focusing urokinase (uPA) on the cell membrane and protecting serine protease activity from PA inhibitors [2, 3]. Enzymes and substrates focusing on the cell surface strongly enhance the proteolytic cascade [4]. uPA is one of the plasminogen activators in mammalian species that convert the extracellular plasminogen into plasmin, which in turn promotes the degradation of all components of the extracellular matrix (ECM) including fibrinogen (Fg), fibronectin (Fn) and vitronectin (Vn) [5–7]. Numerous reports have demonstrated that upregulation of the uPA system is highly correlated with the malignancy of various carcinomas [8–12]. It was widely believed that uPA activity-dependent cell surface plasminogen activation plays the major role in the processes of ECM degradation and basement membrane dissolution that lead to cancer invasiveness and metastasis [13]. Therefore, the attempted cancer therapeutic efforts to target the uPA system have been mainly focused on inhibiting uPA activity or preventing uPA binding to its receptor uPAR, but with limited success [14–17]. The primary inhibitor of plasminogen activators, the type-1 inhibitor (PAI-1), secreted in an active form and stabilized by binding to plasma or ECM Vn, has dramatic effects on cell adhesion, detachment and migration [18], and paradoxically high levels of PAI-1 also correlate with poor prognosis in cancer patients [19–22]. These findings confound the mechanisms by which the uPA system promotes tumor progression.

In the past decade, extensive evidence indicated that uPAR also modulates cell adhesion, migration, and

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proliferation through interactions with other membrane proteins independent of protease activity [8, 23, 24]. In addition to binding uPA, uPAR is also a receptor for Vn, and its binding to Vn is promoted by concurrent uPA association [25, 26]. Binding of uPAR ligands was found to alter cell signaling that leads to the reorganization of the actin cytoskeleton [27, 28]. As a glycosyl-phosphatidylinositol (GPI)-anchored receptor, uPAR lacks transmembrane and intracellular domains and is therefore incapable of transducing intracellular signals alone. It is generally believed that signal transduction originating from uPAR must involve lateral interactions with transmembrane partners. In fact, uPAR-mediated functions and signaling have been suggested to require its interaction with membrane proteins such as integrins [23, 29–34], chemokine receptors [35, 36], and epidermal growth factor receptor (EGFR) [37–41]. It is now becoming clear that uPAR through its interactions with integrins can specifically modify integrin function in matrix binding, adhesion, migration, and signaling.

Integrins are recognized as one of the major families of cell surface adhesion receptors [42, 43]. Via integrins, cells can sense dimensionality and other physical and biochemical properties of the ECM. Direct integrin signaling and indirect integrin modulation of signaling pathways regulate ECM remodeling, which can contribute to tumorigenesis [44]. Integrins can alter cellular behavior through the recruitment and activation of signaling proteins such as non-receptor tyrosine kinases, which bind to and phosphorylate various adaptor proteins [45]. The ability to connect to the actin cytoskeleton is a key part of the adhesive function of integrins. This linkage between integrins and the cytoskeleton involves a large complex of integrin-associated proteins that function in both the assembly and disassembly of the link [46]. Integrin-mediated adhesion controls Rac binding to membranes within cholesterol-enriched membrane domains, which is mediated by caveolae and regulated by caveolin-1 phosphorylated on Tyr 14. This mechanism may account at least in part for the control of multiple pathways by integrins [47, 48].

During tumor development, changes in integrin expression, intracellular control of integrin functions and signals perceived from integrin ligand binding impact upon the ability of tumor cells to interact with their environment and enable metastatic cells to convert from a sessile and stationary phenotype to one that is migratory and invasive [49]. There is increasing evidence that certain integrins associate with receptor tyrosine kinases (RTKs) or other membrane proteins such as uPAR to activate signaling pathways that are necessary for tumor invasion and metastasis [23, 24, 50]. The focus of this review is on the functional and signaling consequences of uPAR/integrin interaction in the context of cancer.

uPAR structure and residues affecting integrin interaction

The cellular receptor for human uPA (uPAR) is anchored to the plasma membrane by a COOH-terminal GPI moiety [51]. It is synthesized as a 313-residue polypeptide containing 28 cysteine residues and organized into three domains with homologous cysteine repeats characteristic of the Ly-6 protein superfamily [52]. The domain I (DI) of uPAR located in N-terminus is believed to be the module for interaction with its ligand uPA [53].

Recently the crystal structure of a soluble form of human uPAR was solved at 2.7 Å resolution with an inhibitory peptide of uPA-uPAR interaction [54]. This structure confirms that uPAR is composed of three consecutive three-finger domains organized in a circular manner, which generates a deep internal cavity and a large external surface. The model reveals that the receptor-binding module of uPA engages the uPAR central cavity, thus leaving the external receptor surface accessible for other protein interactions (Vn and integrins). In the following years, several independent groups solved the uPAR crystal in association with either the amino-terminal fragment (ATF) of uPA [55, 56] or a uPAR antibody [57, 58]. Half of the uPA/uPAR binding interface is located in DI, confirming the previous observations showing the predominant role of this domain in ligand binding [53, 59]. Data from a comprehensive alanine scanning mutagenesis of uPAR (244 mutants) show that the residues for uPA interaction are all located within or at the rim of the central cavity uniquely formed by the assembly of all three domains in uPAR [60]. These results provide structural bases for the conformational changes induced in uPAR and the nature of uPA/uPAR interactions and provide insight into mechanisms by which the cellular responses are induced by uPA binding.

Inspired by the fact that the outside surface of uPAR is available for its partners, multiple groups have attempted to look for integrin binding sites on uPAR. Based on our previous findings and uPAR crystal structure, we have mutated 18 charged groups or serine residues on uPAR that are also located away from the uPA binding site, mostly in DII and DIII. We identified single amino acid mutants of uPAR that fail to associate with either $\alpha3\beta1$ (D262A) or $\alpha5\beta1$ (H249A) but associate normally with uPA [61]. Recently, Reiser's group found that the D262A

mutant uPAR is unable to interact with murine α3β1 integrin [62]. Co-immunoprecipitation of uPAR and α3β1 or α5β1 in both HEK293 cells and tumor cell lines confirms that D262 and H249 sites (Fig. 1, residues colored in orange) in DIII of uPAR are important for interaction with integrin α3β1 and α5β1, respectively [61]. Independently, Ossowski's group found that a 9-mer peptide derived from a sequence in DIII (residues 240–248) binds purified α5β1 integrin [63]. Substituting a single amino acid (S245A) in this peptide, or in full-length soluble uPAR, impairs binding of the purified integrin. Incubation of highly malignant cells with the peptide 240–248, but not the S245A mutant peptide, disrupts the physical association of uPAR and α5β1 integrin. In the uPAR crystal structure Ser-245 (Fig. 1, residue colored in purple) is well separated from the central uPA-binding cavity. The congruent findings from these two groups suggest that residues 240–250 in DIII of uPAR are required for α5β1 binding.

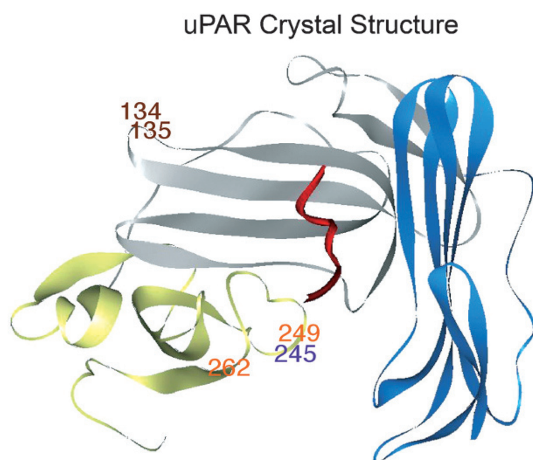

Figure 1. uPAR crystal structure. The crystal structure of uPAR is displayed as a ribbon diagram using the coordinates 1YWH [54]. The individual uPAR domains are colored blue (DI), gray (DII) and yellow (DIII). The uPAR binding peptide is marked red. Putative integrin-binding residues in domains II and III reported by different groups are color-labeled: orange, our group [61]; brown, Blasi's group [64]; purple, Ossowski's group [63]. Residues for integrin binding: α5β1 134, 135, 245, 249; α3β1 262; αVβ3 134, 135.

Blasi's group identified a peptide D2A, derived from a sequence in domain II (DII) ([130]IQEGEEGRPKDDR[142]) of uPAR, that disrupts uPAR-αVβ3 and uPAR-α5β1 co-immunoprecipitation, indicating that this region can bind directly with at least two integrins [64]. Furthermore, E134 and E135 (Fig. 1, residues colored in brown) were found to be the key residues. Indeed, this region lies outside of the uPA binding pocket and is thus accessible to interaction with other molecules. However, their data also suggest that besides the D2A epitope, other sites of interaction between uPAR and integrins may exist and these sites might be located in domain III (DIII). The observations reviewed here imply that the residues in DII of uPAR may be crucial for multiple integrins (α5β1 and αVβ3) and the specific residues in DIII of uPAR may determine the specificity of integrin binding (α3β1 or α5β1). Whether other uPAR-associated integrins bind the same or different sites on uPAR remains to be explored. Additional structural studies may help to reveal the global view of the uPAR/integrin binding interface.

Integrin structure and binding sites for uPAR

Integrins are heterodimers formed by the non-covalent association of α and β subunits, and to date 24 different α-β combinations have been identified at the protein level in mammals [65]. Both subunits are type I transmembrane proteins containing large extracellular domains and mostly short cytoplasmic domains [66, 67]. Initially, the only domain recognized by sequence homology was the α subunit inserted or I domain [68, 69], whose crystal structure was later solved [70–73]. In fact the boundaries and structures of the I-like, β-propeller, and integrin EGF (I-EGF) domains were all predicted before their structures were solved [74–76]. The first integrin heterodimer crystal structure was solved in 2001 on the extracellular portion of integrin αVβ3 at 3.1 Å resolution [77]. In the crystal, the 12 domains of αVβ3 assemble into an ovoid 'head' and two 'tails'. The overall bent conformation for αVβ3 reflects the flexibility that may be linked to integrin regulation. The main intersubunit interface lies within the head, between a seven-bladed β-propeller from αV and an I-like domain from β3. In the following year the same group solved the crystal structure of the extracellular segment of integrin αVβ3 in complex with a cyclic peptide presenting the Arg-Gly-Asp (RGD) sequence [78]. The ligand binds at the major interface between the αV and β3 subunits (headpiece) and makes extensive contacts with both. Ligand binding induces both tertiary and quaternary changes as well as small changes in the orientation of αV relative to β3 [79]. The three-dimensional structure of the ligand-binding headpiece of integrin α5β1 complexed with its ligand fibronectin (Fn) was later determined using molecular electron microscopy (EM) [80]. The Fn fragment binds to the interface between the β-propeller and I-like domains in the integrin headpiece through the RGD-containing module 10. Furthermore, comparisons between crystal, NMR (nuclear magnetic resonances) and EM structures reveal dramatic rearrangements that regulate ligand binding by integrins [67, 81–84].

Besides binding to their ECM ligands and cytoskeletal proteins [85], integrins also interact laterally with other membrane receptors to form distinct membrane complexes that recruit additional signaling molecules via these partners [86–91]. uPAR represents one of the partners for integrin [23, 24] and has been identified to associate with multiple integrin family members, such as β1, β2, β3, β5, and β6 integrins [29–32, 34, 92]. Cell binding or recombinant integrin binding assays identified uPAR as an atypical integrin ligand [33, 93–96].

We first identified a critical non-I-domain binding site for uPAR on the αM subunit (M25; residues 424–440) of αMβ2 integrin by homology with a phage display peptide known to bind uPAR (P25) [93]. In the β-propeller model of α-chain folding, M25 spans an exposed loop on the upper ligand-binding surface of αM. Treatment with M25 or replacing M25 on αMβ2 with the homologous sequence of the αX subunit from another β2 integrin disrupted association of αMβ2 with uPAR. Our group further demonstrated that uPAR can also associate with integrin α3β1 via a surface loop within the β-propeller (W4 BC loop: 242RHRHM246) of the α3 subunit but outside the laminin binding region [97]. A 17-mer peptide derived from the α3 chain-containing uPAR-binding sequence (α325) or alanine mutations within the uPAR-interacting loop (H245A or R244A), prevented uPAR association with α3β1. Sequence homologies of this loop among a couple of β1 integrin-coupled α chains as well as αM suggest that H245 may be the key residue for uPAR interaction with β1 integrins. It is thus conceivable that other β1 integrins bearing homology at this site, such as α6β1, may share a similar uPAR binding property. Inspection of integrin α3β1 and α5β1 models generated based on the atomic coordinates of the αVβ3 crystal structure [77] reveals two loops on the β1 chain (β1P1: 224NLDSPEGGF232; β1P2: 262FHFAGDGKL270) that are very close to the known uPAR binding region on the α chain (Fig. 2) [94]. The β1 peptides or Ser227Ala point mutation on the β1 chain abrogate uPAR/α5β1 complex formation and function. These findings suggest that the uPAR interaction sites on integrins can involve both α and β chains. Integrin structure modeling and integrin-derived functional blocking peptides in combination with site-directed mutagenesis may provide effective tools to study how uPAR interacts with other integrins known to be associated with uPAR.

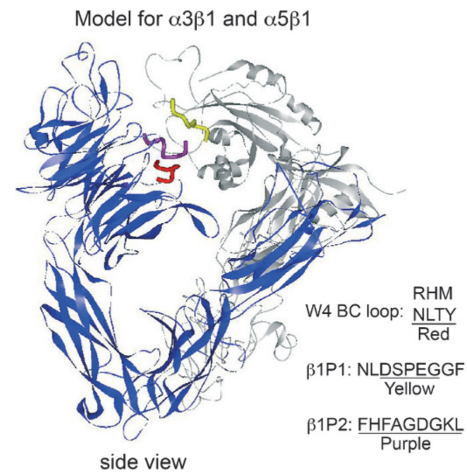

Figure 2. Model structure for α3β1 and α5β1. The energy-minimized models of integrin α3β1 and α5β1 structures were developed based on the atomic coordinates of αVβ3 crystal structure [77]. The α5β1 model is shown here to represent the two. The putative uPAR binding regions are color-marked. One region in the W4 BC loop of the predicted β-propeller structure of the α chain (blue) is marked in red [95, 97]: RHM for α3 and NLTY for α5. The other two regions (β1P1 and β1P2) in β1 chain (gray) are indicated in yellow and purple, respectively [94]. The side view of the model is shown.

uPAR/integrin signaling and function in cancer cells

uPAR and integrins are coordinately expressed by mutually targeting their promoter regions [98–100] and are highly regulated in malignant cancer cells [101–104], and uPAR regulates integrin-mediated cell responses through direct binding to integrins [33, 94, 95]. This has been shown in various cancer cell types, such as lung cancer [61], breast cancer [32, 105, 106], ovarian cancer [104, 107], melanoma [98, 101], head and neck carcinoma [108], and prostate cancer [28, 109]. It is therefore of special interest to review the intracellular signal transduction pathways downstream of uPAR/integrin complexes and the subsequent cellular changes important for cancer progression, including adhesion, migration, differentiation, and proliferation.

Although there are over two dozen different integrins, so far uPAR has only been found to physically and functionally associate with a few β1, β2, and αV integrins. Each integrin has its unique expression, distribution, structure, ligand(s), and functions. Therefore, uPAR binding to each of them by different mechanisms could result in the activation of specific signaling pathways and lead to the regulation of distinct functions. Here the cancer-related functions of the more extensively studied uPAR/integrin complexes are reviewed.

uPAR/α3β1

α3β1 integrin is a laminin receptor with apparently diverse functions [110]. It is involved in binding of the basement membrane and found in adherence junctions. It also plays a role in epithelial development, maintenance, and epithelial-to-mesenchymal transition (EMT) [110]. α3β1 is widely expressed in various cancers, including epithelial carcinoma [110], and integrin-mediated tumor cell invasion of basement membranes is crucial to tumor metastasis [111].

Our group first demonstrated uPAR/α3β1 complex formation in uPAR transfected HEK293 cells and showed that the complex regulates tumor cell adhesion and migration, signal transduction across cell membranes, and cytoskeletal organization. uPAR was found to co-localize with α3β1 in human breast cancer MDA-MB-231 cells, promoting uPA-stimulated cell spreading and focal adhesion kinase (FAK) phosphorylation on Fn or collagen type I (Col) in a heterotrimeric G protein-dependent mechanism of signaling between α3β1 and other β1 integrins [95].

EMT is believed to be a major mechanism by which cancer cells become migratory and invasive [112]. Integrins, Src kinases, and β-catenin are among the interconnected transduction pathways known to be involved in EMT [113]. Repression of E-cadherin by transcriptional regulators such as Snail/Slug or Twist has emerged as one critical step in driving EMT [112, 114]. α3β1 integrin has been shown to regulate E-cadherin-mediated cell-cell adhesion and epithelial cytoskeletal organization [115]. We have found that mouse epithelial cells expressing uPAR and wild-type α3β1 integrin show mesenchymal phenotype in culture, with increased Src activity, upregulation of Slug, and downregulation of E-cadherin and gamma-catenin. Cells expressing H245A or R244A mutant α3β1, unable to interact with uPAR, were unaffected by co-expression of uPAR and retained an epithelial phenotype, suggesting that uPAR association with α3β1 is required for this process. Furthermore, inhibition of Src kinase activity by specific inhibitors or expression of kinase dead dominant negative Src construct restored the epithelial phenotype. These results reveal a uPAR/α3β1- and Src-dependent pathway for EMT and implicate these pathways in the acquisition of a migratory and invasive phenotype in cancer cells [97].

Recent studies have suggested that many G protein-coupled receptors (GPCRs), such as chemokine receptors, are involved in the tumorigenesis and metastasis of multiple human cancers, such as non-small cell lung cancer, breast cancer, prostate cancer, melanoma, gastric cancer and diffused large B cell lymphoma [116]. uPAR cleavage by different proteases in the DI-DII linker region exposes the specific sequence SRSRY (residues 88–92) [117, 118] that binds and activates GPCR FPRL1 (the formyl peptide receptor-like-1), which induces cell migration [36]. By comparing the signaling pathways involved in uPA-stimulated cell migration between cells expressing wild-type (wt) or human cleavage resistant (hcr) mutant uPAR, Blasi's group was able to tease out two different pathways in response to uPA. Wt-uPAR requires both α3β1 integrin and FPRL1 to mediate uPA-induced migration, and wt-uPAR/α3β1 association leads to uPAR cleavage and ERK activation, whereas hcr-uPAR does not activate ERK or engage FPRL1, but rather forms a uPAR/α3β1/EGFR complex that results in the autotyrosine phosphorylation of EGFR [41]. These data suggest that cell migration could be regulated through distinct pathways activated by integrin interaction with different forms of uPAR and the recruitment of specific co-partners. These findings also suggest that a similar outcome can be achieved by different mechanisms.

In addition to the regulation of tumor cell motility and signaling through direct membrane association, uPAR/α3β1 complex has also been found to promote tumor invasion by up-regulating uPA transcription. Expression of uPA and uPAR is highly correlated with tumor invasiveness. Stack's group investigated the functional link between cell adhesion and matrix proteolysis. They found that ligation of α3β1 integrin with an antibody or ligands dramatically co-clusters uPAR on the cell surface and enhances Src/ERK-dependent, EGFR-independent uPA secretion [119, 120]. Furthermore, in squamous cell carcinoma cells, they showed that clustering α3β1 integrin in the presence of a peptide (α325) that disrupts uPAR/α3β1 integrin association or suppressing uPAR expression by RNA interference prevents uPA induction and blocks filopodia formation and matrix invasion. α3 integrin null epithelial cells reconstituted with wild-type α3 integrin, but not H245A mutant α3 integrin unable to bind uPAR, induces uPA expression upon integrin clustering. Together these data demonstrate that ligand-induced clustering of α3β1 integrin promotes uPAR/α3β1 interaction and potentiates cellular signaling, culminating in enhanced uPA expression and uPA-dependent invasive behavior [120]. Whether the expression of uPAR itself or other proteases such as MMPs is regulated by similar mechanism will be interesting future topics.

Moreover, uPAR/α3β1 complex has been found to regulate cell adhesion and signaling on Vn. Cell adhesion and signaling induced by Vn are rather complex. ECM protein Vn binds to multiple receptors, including uPAR and its integrin receptors (such as αVβ3, αVβ5) in cancer cells [25, 121–124], and PAI-1 also competes with both uPAR and integrin for the binding sites on Vn, and blocks Vn adhesion [125,

126]. Data from our group as well as Blasi's group support a model in which the uPAR/α3β1 complex mediates cell adhesion to Vn as this adhesion can be blocked by either α3β1 blocking antibody [95] or uPAR/α3β1 blocking peptide α325 [64]. We believe this adhesion is mediated by direct uPAR binding to Vn and can be regulated by β1 integrin association and uPA engagement. Interestingly, uPAR-dependent and integrin-independent cell binding to Vn was observed in HEK293 cells expressing uPAR with putative integrin-binding sites mutated [127] and in mouse pre-B lymphocytes (BAF3), which do not express integrins known to be involved in Vn adhesion [128]. These results suggest that uPAR can directly mediate Vn adhesion when uPAR/integrin interactions are disrupted or Vn adhesion-related integrin receptors are not expressed. This is not surprising since uPAR direct binding to Vn does not require integrin(s). We believe that Vn engagement by uPAR could occur by distinct mechanisms depending on whether integrin binding partners are present. Since uPAR and uPAR-binding integrins are extensively expressed in many malignant tumors, it is likely that matrix association of tumor cells is largely mediated by uPAR/integrin complexes. Clearly, additional studies on cell adhesion to Vn or Vn-induced signaling could help to sort out whether two or more mechanisms are involved in cancer cell attachment to Vn and to refine our understanding of Vn binding by uPAR and integrins.

uPAR/α5β1

Integrin α5β1 is known as the receptor for fibronectin (Fn). It binds the Arg-Gly-Asp (RGD) motif in the cell-binding domain of Fn and is involved in matrix assembly, cytoskeletal reorganization, signal transduction, regulation of cell adhesion and motility [129], and the induction of proteases such as MMPs [130]. α5β1 has been found to be overexpressed in breast cancer [131], colon carcinoma [132], ovarian cancer [133], head and neck carcinoma [134], and melanoma [135]. In addition, α5 overexpression correlates with poor survival in non-small cell lung cancer patients [136].

Our group has been focused on studying the effect of uPAR on α5β1 integrin-mediated adhesion, migration, Fn matrix assembly, and signaling pathways. Inspired by the finding of Czekay and colleagues that PAI-1 can detach cells from Vn, Fn and type-1 collagen by binding to the uPA present in uPA-uPAR-integrin complexes on the cell surface [126], we further investigated the molecular mechanism by which PAI-1 influences α5β1 integrin function via uPAR [94]. Using recombinant proteins and blocking peptides, we mapped uPAR-binding sequences on the integrin β1 chain near the known α-chain site of uPAR/β1 interaction and revealed these regions as important regulatory sites. The β1 peptides derived from the uPAR-binding region on β1 subunit or a β1 chain Ser227Ala point mutation abrogate the functional effects of uPAR on α5β1. Positioning of the uPAR-binding site near the Fn-binding site of α5β1 not only promotes α5β1 interactions with Fn, but allows PAI-1 to reverse Fn binding and enhance cell migration, suggesting a molecular basis for PAI-1-mediated cell detachment.

Interestingly, we found that direct binding of uPAR to α5β1 does not change overall integrin binding to Fn, but rather switches Fn-binding site from the RGD motif to the adjacent heparin-binding domain (HepII) [94]. Thus, uPAR appears to serve as a molecular switch that regulates two distinct mechanisms of Fn binding by α5β1. Whether a similar switch mechanism of matrix binding applies to other integrins that associate with uPAR is not known and remains to be explored.

The integrin molecule contains a number of flexible joints and connections that permit a broad spectrum of possible conformational states [137]. In cancer cell lines we found that uPAR binding to α5β1 integrin changes its conformation. Suppression of endogenous uPAR expression by small interfering RNA induces the presentation of β1-chain ligand-induced-binding site (LIBS) epitopes on tumor cells, the presence of which represents an extended-active integrin conformation [94]. In fact, uPAR-dependent decrease in β1-chain LIBS epitope has been observed in multiple cancer cell lines, including human fibrosarcoma HT1080, human breast cancer MDA-MB-231, and human ovarian cancer SKOV-3. We believe that in uPAR-expressing tumor cells the lower LIBS antibody binding reflects integrin angulation resulting from uPAR/α5β1 complex formation. And since engagement of Fn by this complex leads to the activation of downstream signaling pathways, we propose a modified 'bent' integrin conformation as an alternative activation state when complexed with uPAR and possibly other cis-acting membrane ligands as judged by enhanced adhesion and Fn matrix assembly. Because LIBS epitopes can be regulated by many stimuli or cellular conditions [138], it is possible that in some cell systems the exposure of LIBS epitopes may be affected predominantly by other factors rather than uPAR association. Nevertheless, these data suggest a uPAR-dependent mechanism permitting fine-tuning of integrin function by both extracellular associations and intracellular signals.

Integrins transmit bidirectional signals across the plasma membrane by coupling extracellular conformational change with intracellular signaling pathways

[139, 140]. Since uPAR association with α5β1 regulates integrin conformation, we reasoned that signaling through the integrin would also be affected. Therefore, we also explored the importance of uPAR/integrin interaction in β1 integrin signaling [61]. Knockdown of endogenous uPAR in tumor cell lines HT1080 and H1299 resulted in markedly reduced Fn and α5β1-dependent ERK activation and metalloproteinase MMP-9 expression. Using site-directed mutagenesis, single amino acid mutations in uPAR DIII were identified to be crucial for either α3β1 (D262A) or α5β1 (H249A) association. Re-expression in uPAR knockdown cells of the H249A mutant, which is deficient in α5β1 binding, failed to reconstitute Fn-mediated signaling, whereas signaling in wild-type and D262A mutant expressing cells was restored. Surprisingly, we have observed that engaging Fn by α5β1 on different sites (RGD versus HepII) activates distinct signaling pathways through α5β1. Tumor cell binding to the RGD site on Fn leads to FAK/Src activation, whereas binding to the HepII site causes Rac 1 activation. Importantly, both RGD- and HepII-initiated signals are required to activate ERK and upregulate MMP-9. Altogether, our data suggest that uPAR/α5β1 interaction is required for maximal response to Fn engagement, which could contribute to tumor cell invasion by induction of MMPs, and that this operates through the Src/Rac/ERK signaling pathway. More studies are needed to determine whether it is a general phenomenon that uPAR association with an integrin alters the integrin-binding site on its ECM ligand and the mechanism through which the integrin signals.

Using human head and neck squamous cell carcinoma cell line HEp3, Ossowski's group undertook a series of studies on uPAR/α5β1 integrin and found that uPAR interaction promotes α5β1-mediated function. First, they found that physical association of uPA/uPAR/α5β1 in HEp3 cells, which express a high level of uPAR, promotes adhesion to Fn and induces basal ERK activity above a threshold to enhance tumor growth *in vivo* on chicken embryo chorioallantoic membranes (CAMs) [108]. Later they discovered that the state of tumorigenicity and dormancy in multiple tumor cell lines could be shifted through the regulation of the balance between ERK and the apoptotic/growth suppressive stress-activated protein kinase 2 (p38MAPK) [141, 142] and that the ERK/p38MAPK activity ratio predicts whether the cells will proliferate or enter a state of dormancy *in vivo* [141]. These results suggest that malignant cancer cells *in vivo* to some extent depend on proliferative signals from surface receptors and the ECM.

The uPAR/α5β1 complex was found to initiate intracellular signaling through FAK and Src leading to ERK activation and tumor growth on chicken embryo CAMs. Downregulation of uPAR or expression of dominant negative FAK-related non-kinase (FRNK) forces human carcinoma cells into dormancy [143]. Furthermore, Ossowski's group demonstrated that EGFR mediates the uPAR/α5β1/Fn-induced growth pathway and that FAK is the link between integrin and EGFR signaling. Disruption of uPAR or EGFR signaling reduces HEp3 proliferation *in vivo* [37]. Collectively, their findings unveil a mechanism whereby uPAR interacts with and activates α5β1 integrin, triggers FAK/Src activation, which also regulates EGFR signaling, and subsequently leads to ERK pathway activation and cancer cell proliferation.

The function and signaling of uPAR/α5β1 complex has been extensively studied in various cancer cells. Depending on the cellular context, uPAR association with α5β1 integrin could yield positive or negative effects on cell behavior. For example, the results from our group and Ossowski's group reviewed above show the positive effects from uPAR/α5β1 association. In the prostate carcinoma cell line LNCaP, positive influence was also observed: uPA binding to uPAR increases uPAR/α5β1 interaction and results in tyrosine phosphorylation of FAK and the crk-associated substrate p130Cas, which enhances cell migration [28]. However, in some cases uPAR/α5β1 association could have a negative impact on cell function. For instance, tetraspanin CD82/KAI1 (a tumor metastasis suppressor) promotes uPAR association with α5β1 integrin and directs the complex to focal adhesions, but turns uPAR into a cryptic form unable to bind uPA, and therefore indirectly inhibits the pericellular plasminogen activation important for cell migration [144]. The results from these studies underscore the importance of uPAR in the regulation of integrin function.

Cell functions can be modulated by physiological or artificial factors that disrupt uPAR/α5β1 interaction. Cleaved high molecular weight kininogen (HKa) physically disrupts the formation of this complex, inhibits *in vivo* neovascularization, and induces apoptosis [145]. In squamous carcinoma cells gangliosides inhibit uPA-dependent cell migration by preventing the association of uPAR with α5β1 integrin or uPAR/α5β1 with EGFR [40]. The uPAR-binding peptide P25 was found to block uPAR/β1 physical association [29, 95] in several cancer cell lines, including human colon carcinoma cell line HCT116 [104], human breast cancer cell line MDA-MB-231 [106], and human head and neck carcinoma HEp-3 [108, 141]. Ossowski's group demonstrated that P25 inhibited uPAR/α5β1 physical association and α5β1-mediated Fn matrix assembly in HEp-3 cells [23]. On the contrary, McKeown-Longo's group observed that P25

binding to uPAR increases Fn matrix assembly in human skin fibroblasts [146] and in the human osteosarcoma cell line MG-63 [147]. Although it is not clear why and how P25 resulted in different effects on Fn matrix assembly in different cell systems, their data suggest that P25 can affect α5β1 integrin function through different mechanisms: direct interfering of uPAR/α5β1 association or indirect effects via uPAR-dependent sequential activation of Src kinase, EGFR, and α5β1 integrin [148]. The factors that determine whether P25 affects α5β1-mediated Fn matrix assembly by direct or indirect mechanisms remain to be defined.

uPAR/αV integrins

αV integrins form a subfamily of five members (αVβ1, αVβ3, αVβ5, αVβ6, and αVβ8) that recognize a group of overlapping ligands which generally contain the canonical RGD recognition sequence [149]. The αV integrins are widely expressed, and their expression is tightly regulated [150]. Apart from αVβ6, all of the αV integrins bind to Vn [151]. Among these Vn receptors, αVβ3 and αVβ5 are believed to contribute to tumor-related angiogenesis [152], and antagonists targeting these integrins have been developed in an attempt to prevent tumor progression [153]. The relationship between the αVβ3 or αVβ5 and uPA/uPAR systems has significant implications for regulation of cell motility events associated with angiogenesis and tumor metastasis.

Recent studies from multiple groups suggest that uPA/uPAR/αVβ3 complex is important for tumor cell function and signaling. Blasi's group reported that uPAR activates αVβ3-dependent signaling pathways such as the Janus kinase (Jak)/Stat pathway and regulates Vn-induced cell migration by lateral interaction with αVβ3 [64]. The uPAR/αVβ3 complex can be regulated by physiological factors. For example, calcitonin (CT) selectively increases uPAR/αVβ3 association in highly metastatic prostate cancer PC-3M cells and enhances disaggregation and migration of PC-3M spheroids on Vn [109]. Cleaved HKa disrupts the uPAR/αVβ3 complex and induces apoptosis of cells grown on Vn [145]. Czekay et al. demonstrated that PAI-1 deactivates and disengages uPA/uPAR/αVβ3 complex in HT1080 cells and triggers cell detachment from Vn [126]. The finding that PAI-1 also detaches cells from Fn and type-1 collagen suggests that this pathway may represent a general mechanism and may explain the relevance of high PAI-1 levels to tumor metastasis.

Other than direct uPAR interaction with αVβ3, uPA also binds to αVβ3 with low affinity. Takada's group recently demonstrated that uPA binds to αVβ3 integrin via its kringle domain and induces cell migration. Their results suggest that uPA binding to αVβ3 integrin through the kringle domain plays an important role in both plasminogen activation and uPA-induced intracellular signaling [154]. The uPA kringle domain binds to β1 (α4β1 and α9β1) [154] and β2 [155] integrins as well. It is not clear whether the low affinity interaction between uPA kringle domain and αVβ3 integrin coordinates with high affinity interaction between uPA growth factor domain (GFD, residues 1–49) and uPAR.

Interestingly, Stoppelli's group recently discovered that uPA can also interact with αVβ5 integrin directly, but via a different uPA domain. They demonstrated that uPA binds to αVβ5 via its connecting peptide domain (Cpp, residues 132–158) and binds with uPAR through its GFD domain simultaneously, promoting cytoskeletal rearrangements and directional cell migration [156]. Together these data indicate that the formation of uPAR/uPA/αV integrin ternary complex initiates and potentiates uPAR-dependent signaling and migration. Whether the uPA-connecting peptide domain also contributes to uPAR/uPA/αVβ3 complex formation or whether the uPA kringle domain is involved in the formation of uPAR/uPA/αVβ5 complex remains to be addressed.

uPA/uPAR and αVβ5 integrin are known to be physically and functionally linked. uPA, uPAR, and αVβ5 integrin were first found to co-localize in focal contacts in the human keratinocyte cell line HaCaT [157]. In human pancreatic carcinoma FG cell line, growth factor TGF-α or phorbol ester PMA-mediated induction of uPA/uPAR promotes cell migration on Vn mediated by integrin αVβ5, which is blocked by either soluble uPAR, an antibody that disrupts uPA binding to uPAR, or a monoclonal antibody to αVβ5 [158]. The functional effects of this association were studied using human fibrosarcoma HT1080 and human breast carcinoma MCF-7 cell lines, both of which exhibit uPA-dependent physical association between uPAR and αVβ5 [32, 105]. Gonias's group demonstrated that uPA promotes cellular migration via a uPAR/αVβ5-dependent signaling cascade in which Ras, MAP kinase kinase (MEK), ERK, and myosin light chain kinase (MLCK) serve as essential downstream effectors [105]. Stoppelli's group further confirmed the physical association of uPAR with αVβ5 integrin in these tumor cells by immunoprecipitation and demonstrated the importance of the complex for uPA-induced cytoskeletal rearrangements and tumor cell migration [32]. The importance of uPA in uPAR/αVβ5 function is further supported by the observation that a variant uPA with Ser138/303/Glu di-substitution was found unable to activate Src family kinase, induce uPAR/αVβ5 association, or stimulate MCF-7 cell migration, suggesting that

phosphorylation of uPA on Ser138/303 could regulate uPAR-dependent signaling [159].

In many malignant cancer cells both uPAR and integrins are upregulated. In human melanoma cells, αVβ3 integrin was coordinately expressed with uPAR. Ligation of αVβ3 integrin results in the activation of PKCβ, an increase in uPAR and PAI-1 transcription, cell surface-associated plasmin levels, and cell invasion [98]. In ovarian cancer cells, however, Vn induces downregulation of uPA and uPAR [100], and overexpression of αVβ3 integrin also downregulates uPAR expression [99]. αVβ3 integrin signaling is thought to regulate uPA and uPAR transcription in cancer cells through Rel protein-binding sites in uPA promoter region and Ets-binding sites in uPAR promoter region [99, 100]. Conversely, uPA binding to uPAR modulates integrin expression in tumor cells [124]. By analyzing the expression levels of uPAR and αVβ5 integrin in 35 human breast carcinomas and 5 benign breast lesions, Stoppelli's group demonstrated a positive correlation between the expression levels of these two proteins. They also reported that uPA binding to uPAR upregulates αV and β5 chains in a PKC-dependent manner in MDA-MB-231 and MCF-7 breast carcinoma cell lines. The Ser-Arg-Ser-Arg-Tyr (SRSRY) chemotactic uPAR sequence was found to increase uPAR/αVβ5 physical association and stimulate PKC activity and ERK phosphorylation supported by crosstalk between N-formyl-Met-Leu-Phe receptor (FPR) and αVβ5 [160]. uPA engagement with uPAR may activate signals targeting the cis-acting elements on the αV promoter region such as Sp1 and Ets [161], thereby increasing integrin promoter activity. Whether the generation of signals leading to the regulation of uPAR or integrin gene transcription requires physical interactions between uPAR and integrin is unknown. Nevertheless, the mutual regulation of adhesion and proteolysis receptors suggests a functional relationship between them and is an important topic in cancer research.

Altogether, the data reviewed in this section suggest a mechanism in which uPA is intimately involved in the formation of complexes between uPAR and aV integrins. In addition, these studies demonstrate that formation of the uPAR/uPA/αV integrin ternary complex may be a key step in the regulation of tumor cell motility and signaling.

uPAR/β2 integrins

Unlike the other integrins reviewed here, β2 integrins are leukocyte-specific membrane receptors that are crucial for host defense, such as promoting neutrophil recruitment into inflamed tissue and pathogen phagocytosis [162]. uPAR expression has been linked to several hematological malignancies, such as acute leukemia and multiple myeloma [163, 164]. Both uPAR and β2 integrins can be upregulated by cytokine or growth factor stimulation. For example, myelomonocytic U937 cells express integrin αMβ2 (Mac-1, CD11b/CD18) and uPAR upon stimulation with transforming growth factor beta 1 and 1, 25-$(OH)_2$ vitamin D3.

uPAR directly binds αMβ2 integrin, and the binding site is mapped to a non-I-domain-binding site on αM chain (M25; residues 424–440). Disrupting this interaction was found to inhibit leukocyte adhesion to Fn, Vn, and cytokine-stimulated endothelial cells [93]. The lectin domain near residues 943–1047 within the C-terminus of the αM subunit was also suggested for uPAR interaction and regulation of αMβ2-mediated neutrophil adhesion [165].

Leukocyte attachment to Vn and Fg and transendothelial migration were shown to require both αMβ2 and uPAR [30, 166, 167]. uPAR/αMβ2 association enhances cell adhesion to Fg possibly through activation of FAK and MAPK pathways [168]. Whether association of uPAR and β2 integrins rather than uPAR expression level relates to myeloid and monocytic malignancies and how uPAR/β2 complexes affect their signaling and behavior will be important topics for investigations in the future.

In addition to the uPAR/αMβ2 complex, which has been more extensively studied, uPAR association with other β2 integrins has also been explored. The formation of uPAR complexes with lymphocyte function-associated antigen-1 αLβ2 (LFA-1, CD11a/CD18) was demonstrated on monocytes by co-immunoprecipitation and receptor co-capping [169]. αLβ2 expression mediates transmembrane signaling and promotes the induction of T cell membrane uPAR by stabilizing uPAR mRNA [170].

Both uPAR and β2 integrin expression on myeloid cells are induced upon differentiation stimulation [30], and tumor-associated macrophages of invasive breast carcinomas show elevated uPAR levels [171, 172]. Whether uPAR/β2 complexes play a role in immature myeloid cell function and potential cancer-induced immune suppression has not been explored. How uPAR/β2 complexes affect the function of tumor-associated macrophages is also unknown. Understanding how tumors affect myeloid cell precursor differentiation and inhibit T cell responses could help to develop new approaches for cancer therapy.

uPA in uPAR/integrin interactions and its signaling in cancer cells

uPA is translated into a 55-kDa single-chain proenzyme and converted into the two-chain active form by a single cleavage at Lys158-Ile159. The N-terminal A chain (residues 1–158) is comprised of the epi-

dermal growth factor (EGF)-like domain (residues 4–43, GFD) and the triple-disulphide-containing structure called the kringle domain (residues 47–135). The C-terminal B chain contains the serine-protease domain and is unable to bind uPAR [173]. Recent structure analyses confirm that uPA mainly binds to uPAR domain I (DI) via its GFD or amino-terminal fragment (ATF) (residues 1–135) [53–55, 57]. Some secondary binding sites are located in DII and DIII of uPAR [59, 174, 175]. Recent X-ray studies of the structure of uPAR in complex with either its ligand or inhibitory peptide demonstrate the flexibility of the domain organization of the receptor, suggesting that unbound uPAR has a different conformation from its ligand-bound form [176]. By its unique structural assembly, uPAR can orchestrate the fine interplay with the partners that are required to guide uPA-focalized proteolysis on the cell surface and control cell adhesion and migration.

uPA, its inhibitor PAI-1, and uPAR are tumor-associated proteolytic factors [10]. The enzyme activity is required for several events that contribute to tumor motility such as plasminogen activation, cell detachment, and uPAR cleavage. Receptor-bound uPA can efficiently initiate the plasmin protease cascade and degrade ECM, an important step for tumor cells to migrate. PAI-1 binding to active uPA modulates its enzymatic activity and triggers the internalization of the uPA-receptor-inhibitor complex [177]. More importantly, PAI-1 binding to uPA induces cell detachment, which may be an important contributing factor to cancer metastasis [94, 126]. PAI-1-induced cell detachment was found to require matrix-engaged uPA-uPAR-integrin complexes. Active uPA cleaves uPAR at the DI-DII linker region, and the soluble form of D2D3 produced by uPA induces chemotaxis by binding to G-protein-coupled receptor FPRL1 [36, 178, 179]. Blasi's group has demonstrated that uPA-induced uPAR cleavage requires uPAR/α3β1 integrin/EGFR association and ERK activation [41].

In addition to these proteolytic properties, uPA also exerts several other important biological effects in a non-proteolytic fashion [8]. The binding of uPA to uPAR stimulates cellular signaling and promotes cell migration in various cancer cells [26, 28, 40, 105, 158]. Takada's group demonstrated in Chinese hamster ovary (CHO) cells that uPA binding to uPAR subsequently promotes uPA/uPAR binding to α5β1 integrin and regulates cell adhesion, intracellular signaling, and cell migration [180]. uPA can also enhance uPAR/integrin interaction and function by forming multicontact trimolecular complexes. For example, simultaneous binding of uPA with both uPAR and αMβ2 regulates cell adhesion and migration and enhances uPA-mediated plasminogen activation [155]. uPAR-uPA-αVβ5 or uPAR-uPA-αVβ3 ternary complexes have also been shown to initiate and potentiate intracellular signaling and migration [156]. These results suggest that independent of its catalytic activity uPA can enhance uPAR/integrin association by changing uPAR conformation or forming trimolecular complexes with uPAR and integrins, thereby promoting cancer cell adhesion and migration.

Moreover, binding of uPA to cell surface uPAR can lead to the activation of various intracellular signaling molecules, such as tyrosine-and serine-protein kinases. For instance, uPA binding to uPAR was found to activate multiple kinases including ERK, and promote tumor cell invasion and migration [108, 142, 181–184]. uPA has also been found to increase cell survival by activating the ERK pathway [185]. For example, Gonias' group demonstrated that uPA binding to uPAR, activated the basal level of ERK and inhibited apoptosis in MDA-MB-231 breast cancer cells [186]. Stoppelli's group reported that uPA/uPAR promotes cell survival by activating Bcl-xL transcription through the MEK/ERK- and phosphatidylinositol 3-kinase (PI-3K)/Akt-dependent pathways [187]. More importantly, several reports suggest that uPA-induced ERK phosphorylation in cancer cells require uPAR/β1 integrin complexes. uPA/uPAR/α5β1 complex in human carcinoma HEp3 cells was found to stimulate ERK activation and tumor cell proliferation [108]. Inhibition of uPAR and/or β6 integrin in human ovarian cancer cells resulted in the inhibition of uPA-mediated ERK phosphorylation and subsequent cell proliferation [34]. Disruption of uPAR/β1 integrin complex in human colon carcinoma HCT116 cells inhibited uPA-mediated ERK activation and cell migration/invasion [107]. uPAR/α3β1 complex resulted in ERK activation and FPRL1-dependent cell migration [41]. These data suggest a signaling paradigm in which uPA signaling through uPAR occurs through signaling complexes that include both uPAR and integrins.

In addition, uPA binding was found to activate the Jak/Stat signal transduction pathway by activating different Stats dependent on cell types, such as Stat1 in vascular cells [188, 189], Stat3 in prostate cancer cells or lung epithelial cells [190, 191], and Stat5b in CHO cells [192]. The PI-3K and Akt/GSK-3β-signaling pathways were also reported to be activated in response to uPA binding to smooth muscle cells [193]. Whether these pathways activated by uPA binding to its receptor depend on uPAR/integrin association remain to be defined.

In cancer cells uPA expression is highly regulated, and this event seems to require uPAR/integrin association.

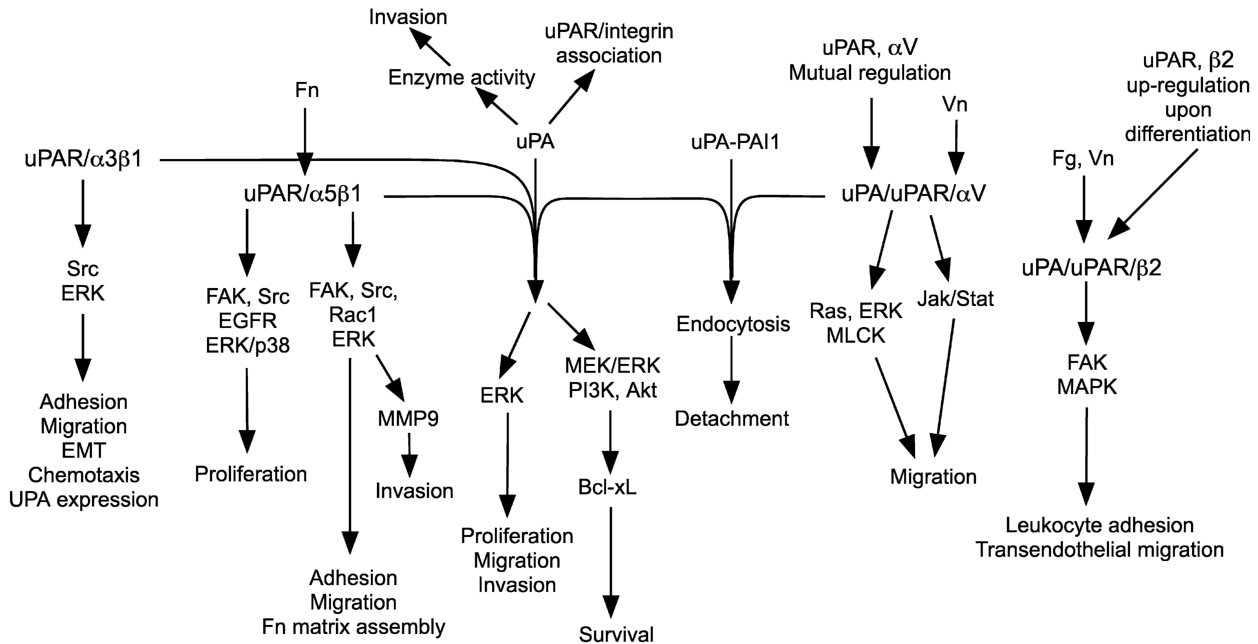

Figure 3. Diagram of signaling and functional consequences from uPAR/integrin interactions. This simplified flow diagram illustrates the well-studied signaling pathways downstream of representative uPAR/integrin complexes and the regulated cell functions reviewed. The contribution of uPA and PAI-1 as well as the mutual regulation of uPAR and integrin expression are also summarized.

In the case of squamous carcinoma cells uPAR/α3β1 was found to contribute to the regulation of uPA transcription [120]. Although uPAR-independent uPA activity [194] and signaling [191] were reported, the large body of experimental evidence suggests that to efficiently mediate function and signaling uPA may need to bind to its receptor uPAR or uPAR/integrin complex.

Thus, uPA plays a pivotal role in promoting tumor invasion and metastasis. It may act in several aspects: 1) increase cell invasion through its enzyme activity; 2) regulate cell function by enhancing uPAR interaction with its partners, such as integrins; and 3) trigger signal transduction, which modulates cell motility, growth, and survival.

Conclusion

In the previous chapters we have summarized the current studies on uPAR interaction with various integrins and their functional effects on cancer progression (Fig. 3). It is becoming clear that the uPAR/integrin complexes can contribute to multiple cancer-related events, including adhesion, migration, proliferation, chemotaxis, and possibly tumor-induced immune suppression from immature myeloid cells. The expression of proteases, such as uPA and MMP, requires uPAR/integrin association, and uPA binding to uPAR further augments the association and their cellular functions. Moreover, recently solved crystal structures of uPAR and integrin have opened new avenues of inquiry as to how exactly these surface receptors may interact. With the congruent findings from independent groups regarding integrin binding sites on uPAR, a coherent model of how uPAR/integrin interactions mediate multiple functional and signaling modalities relevant to cancer is becoming clearer.

Although the complex process of tumor progression is not yet fully understood, it is known to depend in part on the interplay of adhesion molecules and their regulatory partners. There is heterogeneity of cell types within a tumor, and expression levels of uPAR and specific integrins can vary in cancer cells, stromal cells, and infiltrating immune cells. Therefore within this context, uPAR interaction with multiple integrins could be involved on the same cell and perhaps intercellularly within the same cell type or between different cell types. The uPAR/integrin effects can be fine tuned by other partners such as receptor tyrosine kinases and chemokine receptors. Thus, in future studies the extent of uPAR/integrin function with regard to cancer progression *in vivo* should be more thoroughly investigated. In some cases, the unengaged integrins may act independently and uPA may affect integrins or elicit protease activity independent of uPAR. Nevertheless, with respect to cancer therapies, the results of most of the studies reviewed in this article suggest that the more effective anti-cancer

strategies should be developed to target multiple uPAR/integrin complexes customized to specific tumor types and possibly in combination with reagents targeting other relevant pathways.

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