MATRICELLULAR RECEPTORS AS POTENTIAL TARGETS IN ANTI-CANCER THERAPEUTIC STRATEGIES

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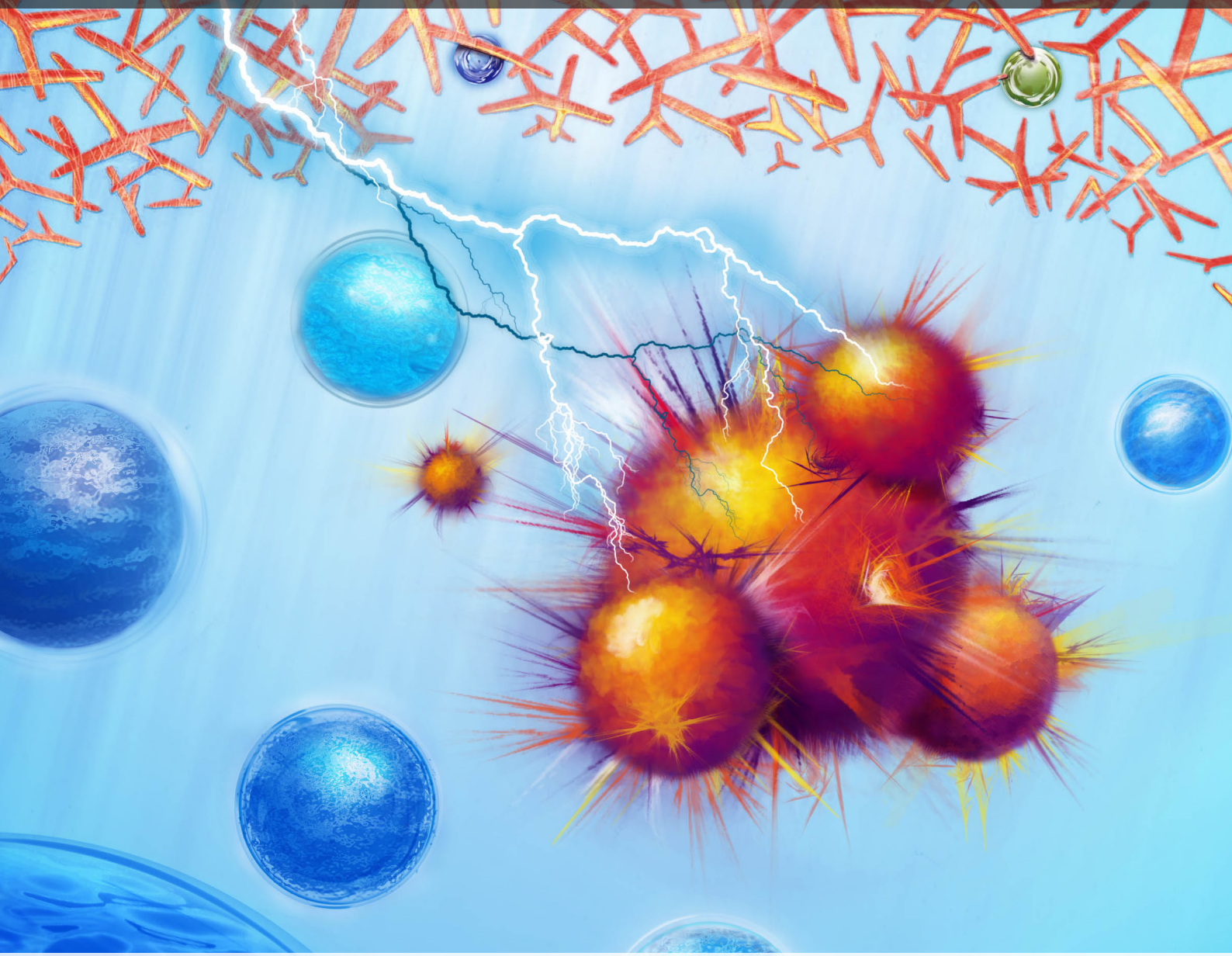

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MATRICELLULAR RECEPTORS AS POTENTIAL TARGETS IN ANTI-CANCER THERAPEUTIC STRATEGIES

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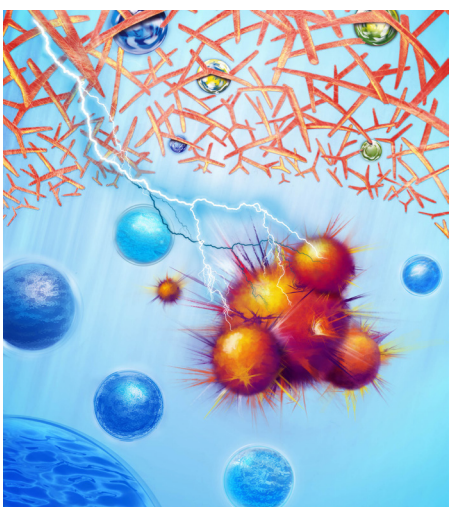

Biological storm: how extracellular matrix targets tumor cells
An original illustration by R. Laurent, Direction de la Communication, Université de Reims Champagne-Ardenne (URCA), Reims, France

The invasive character of a primary cancer is greatly dependent on numerous interactions between tumor cells and their extracellular surroundings. Matricellular receptors are defined as (cell-surface) receptors that bind extracellular matrix (ECM) structural proteins and soluble factors dynamically acting on ECM homeostasis. Matricellular receptors mediate numerous signalings from the extracellular environment to cell nucleus and drive main biological functions that are cell growth, survival and migration. Numerous data from the last decade evidence that matricellular receptors are biosensors that allow to a tumor cell answering to microenvironmental variations, and in this sense they are important contributors to tumor cell malignancy.

Matricellular receptors represent thus valuable targets for the development of original anti-cancer strategies. Original reports, bibliographic reviews or hypotheses are welcome to improve the basic knowledge of matricellular receptor properties, their spatio-temporal regulation, the dynamic formation of complex receptors and the impact of such interactions on the invasive properties of tumor cells. Biological, biophysical and pharmacological, as well as in silico contributions will be appreciated.

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EDITORIAL
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Editorial: Matricellular Receptors As Potential Targets in Anti-Cancer Therapeutic Strategies

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Keywords: discoïdin domain receptor (DDR), elastin receptor, integrin, low-density lipoprotein receptor-related protein (LRP), syndecan, thrombospondin receptor, tyrosine kinase receptor, urokinase receptor

The Editorial on the research topic

Matricellular Receptors as Potential Targets in Anti-Cancer Therapeutic Strategies

Throughout their life, tumor cells proliferate, migrate, overcome obstacles and survive. All these actions require multiple interactions between tumor cells and their extracellular surroundings through specialized cell-surface molecules termed matricellular receptors. We define the matricellular receptors as being receptors that bind extracellular matrix (ECM) structural proteins or soluble factors that dynamically act on ECM homeostasis. Matricellular receptors mediate signalings from the extracellular environment to cell nucleus and drive main biological functions that are cell growth, survival and migration. Numerous data from the last decade provide evidence that matricellular receptors are biosensors that allow to a tumor cell to answer to microenvironmental variations. In this sense they are important contributors to tumor cell malignancy.

Tumor development is associated with an intense remodeling of ECM that generates biologically active fragments, termed matricryptins. Ricard-Blum and Vallet review on matricryptins and their receptor(s) and co-receptor(s), which form a complex network at the surface of tumor and stromal cells. They describe their roles in angiogenesis, tumor growth and metastasis, and their anti-cancer drug potential.

Interaction between cells and the ECM largely involves the well-known cell-surface receptors integrins. Data accumulation during the last 20 years demonstrated that these heterodimeric proteins act as sensors of cell microenvironment by transducing intracellular signals regulating cell fate. They are now considered as critical players in cancer progression. Blandin et al. summarize the current knowledge about integrin involvement in tumor progression and specifically provide informations about $\beta 1$ integrins as therapeutic targets to disrupt hallmarks of cancer.

Beside integrins that bind various ECM macromolecules, the tyrosine kinase receptors Discoidin Domain Receptors (DDR) specifically interact with collagens. Collagens, mainly the fibrillar type I collagen, are major components of the tumor stroma. After summarizing biochemical data on DDR, Rammal et al. pinpoint the roles of DDR1 and DDR2 in the successive phases of a cancer development. Finally, the authors review pharmacological approaches to inhibit DDR1 and DDR2, which might represent valuable targets for anti-cancer therapies.

Elastin is the longest-lived protein in vertebrate and provides elasticity to tissues with high mechanical constraints such as lung or skin. Its degradation during cancer progression not only affects its mechanical properties but also generates elastin-derived peptides (EDP) that are actively involved in the development of cancer. Scandolera et al. describe the role of EDP in tumor

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development and focus their review on the main elastin receptor, an heterotrimer named the Elastin Receptor Complex (ERC), unique by its composition and operating mechanism. They propose anti-ERC therapeutic strategies and describe ERC involvement in cancer-associated processes such as diabetes and thrombosis.

Syndecans are transmembrane proteoglycans expressed at the cell-surface of various cell types. Syndecans are now considered as key regulators of tumorigenesis and cancer progression, especially as being involved in the control of cell proliferation, migration and angiogenesis and in cell-matrix interaction and dynamics. In this regard, the review by Cheng et al. discusses the current state of knowledge of syndecans expression and implication in the field of cancer, with a special and exciting focus on syndecans binding with PDZ domain-containing proteins. The regulation of PDZ binding by phosphorylation of the syndecan cytoplasmic tail is notably debated. Consistently, the experimental data reported by Kashyap et al. focus on syntenin, a scaffold protein containing two PDZ domains and known as an intracellular adaptor for syndecans. To evaluate the potential benefit of anti-syntenin strategies, the authors report the effects of syntenin depletion on various cancer cells from distinct origins. Their results show that syntenin loss of function leads to a significant decrease in tumor cell proliferation, growth and migration in each cancer cell model with a noteworthy defect in the cell-surface expression of active β1-integrin. The authors conclude that syntenin may constitute a molecular target of pharmacological interest in the tumor context.

Growth factors receptors are recognized as critical players in tumor progression by regulating diverse biological activities such as proliferation, migration or survival through their binding on Tyrosine Kinase Receptors (TKR). Among this large family, Erb receptors are often overexpressed, amplified, or mutated in many forms of cancer, making them important therapeutic targets. In their review, Appert-Collin et al. describe the regulation of Erb activity and their role in epithelial-mesenchymal transition. They illustrate the dedicated therapeutic strategies allowing their inhibition with an interesting focus on peptides which mimick transmembrane domains. Beside their role in cancer progression, TKR are involved in the development of various fibrotic diseases. In this regard, these diseases also benefit from advances in the pharmacological strategies developed to fight cancer. Particularly, VEGFR, PDGFR, FGFR, and EGFR kinases appear as potential targets for anti-liver fibrosis therapies. Qu et al. summarize the anti-liver fibrosis effects of multitargeted TK inhibitors and molecular mechanisms. with a specific focus on anti-cancer drugs such as sorafenib and erlotinib.

The urokinase receptor (uPAR) is a key cell-surface receptor generating pericellular proteolysis involved in tissue remodeling processes and triggering intracellular signaling pathways to support various cancer-related events. The mini review by Gonias and Hu gives a synthetic overview of uPAR expression and function in cancer and provides a relevant schematic representation of uPAR-related mechanisms at bothsides of the cell membrane. The authors also address the role of uPAR in the tumor cell resistance to anti-cancer drugs. This part is quite new and attractive and constitutes an exciting area to explore for the future.

While numerous matricellular receptors only exhibit signaling properties, the low-density lipoprotein receptor-related protein-1 (LRP-1) further has endocytic capacities. Van Gool et al. provide an overview of this complex receptor, with a particular focus on the multiple roles played by LRP-1 in cancer progression. Furthermore, the authors present recent (pre)clinical data that suggest applications of LRP-1 as therapeutic tool (in brain cancers, for crossing the blood-brain barrier) and diagnostic/prognostic tool. The amount of LRP-1 at the cell surface is strictly regulated by a proteolytic process termed shedding, which is itself controlled, notably by cell cholesterol level. Dekky et al. present original data that highlight the importance of cell cholesterol distribution in the modulation of LRP-1 shedding. Their results suggest an inverse correlation between intracellular cholesterol concentration and LRP-1 shedding efficiency.

As we have mentioned, these matricellular receptors may constitute relevant targets to fight against malignant diseases. As examples, Jeanne et al. focus on the CD47 and CD36 molecules that function as cell-surface receptors for thrombospondin-1, a large matricellular glycoprotein highly overexpressed within tumor stroma where it promotes an aggressive phenotype. The authors review the various therapeutic options, including antibody-based approaches, therapeutic gene modulation and TSP-1-derived peptides and mimetics. Interestingly, the authors also discuss in detail the more recent and innovative approaches including combination strategies to improve radiotherapy and chemotherapy.

Through (mini) reviews and original reports, this Research Topic highlights matricellular receptors that could represent valuable targets for the development of original anti-cancer strategies. We would like to thank all the authors for their important contribution in these exciting fundamental and applied research fields. We are also grateful to the reviewers and editors for their constructive comments that allowed our issue to reach a high standard quality.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Matricryptins Network with Matricellular Receptors at the Surface of Endothelial and Tumor Cells

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The extracellular matrix (ECM) is a source of bioactive fragments called matricryptins or matrikines resulting from the proteolytic cleavage of extracellular proteins (e.g., collagens, elastin, and laminins) and proteoglycans (e.g., perlecan). Matrix metalloproteinases (MMPs), cathepsins, and bone-morphogenetic protein-1 release fragments, which regulate physiopathological processes including tumor growth, metastasis, and angiogenesis, a pre-requisite for tumor growth. A number of matricryptins, and/or synthetic peptides derived from them, are currently investigated as potential anti-cancer drugs both *in vitro* and in animal models. Modifications aiming at improving their efficiency and their delivery to their target cells are studied. However, their use as drugs is not straightforward. The biological activities of these fragments are mediated by several receptor families. Several matricryptins may bind to the same matricellular receptor, and a single matricryptin may bind to two different receptors belonging or not to the same family such as integrins and growth factor receptors. Furthermore, some matricryptins interact with each other, integrins and growth factor receptors crosstalk and a signaling pathway may be regulated by several matricryptins. This forms an intricate 3D interaction network at the surface of tumor and endothelial cells, which is tightly associated with other cell-surface associated molecules such as heparan sulfate, caveolin, and nucleolin. Deciphering the molecular mechanisms underlying the behavior of this network is required in order to optimize the development of matricryptins as anti-cancer agents.

Keywords: matricryptins, endostatin, matricellular receptors, interaction networks, anticancer drugs

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INTRODUCTION

Matricryptins are biologically active fragments released from extracellular matrix (ECM) proteins and glycosaminoglycans by proteases (Davis et al., 2000). We have extended the definition of matricryptins to the ectodomains of membrane collagens and membrane proteoglycans, which are released in the ECM by sheddases, and to fragments of ECM-associated enzymes such as

Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HA, hyaluronan; MAPK, mitogen-associated protein kinase; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; RHAMM, receptor for hyaluronic acid-mediated motility; TLR, toll-like receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

lysyl oxidase, which initiates the covalent cross-linking of collagens and elastin, and matrix metalloproteinases (MMPs), which contribute to ECM remodeling (Ricard-Blum and Salza, 2014; Ricard-Blum and Vallet, 2015). The molecular functions of matricryptins and the biological processes they regulate have been reviewed with a focus on collagen and proteoglycan matricryptins (Ricard-Blum and Ballut, 2011), on matricryptins regulating tissue repair (Ricard-Blum and Salza, 2014), angiogenesis (Sund et al., 2010; Boosani and Sudhakar, 2011; Gunda and Sudhakar, 2013), cancer (Monboisse et al., 2014), and on the proteases releasing matricryptins (Ricard-Blum and Vallet, 2015).

Synthetic peptides and/or domains derived from matricryptin sequences and recapitulating their biological roles are also able to regulate angiogenesis and/or cancer in various tumor cells and cancer models (Rosca et al., 2011). They include sequences of tumstatin (He et al., 2010; Han et al., 2012; Wang et al., 2015a), laminins (Kikkawa et al., 2013), endostatin (Morbidelli et al., 2003), endorepellin (Willis et al., 2013), and the hemopexin domain of MMP-9 (Ugarte-Berzal et al., 2012, 2014). Neither these peptides nor the ectodomains of membrane collagens and syndecans are described here due to space limitation. We focus on the major matricryptins, which control cancer, metastasis, and angiogenesis, a pre-requisite for tumor growth and a therapeutic target (Folkman, 1971; Welti et al., 2013; Huang et al., 2015), and on their receptors.

REGULATION OF ANGIOGENESIS, TUMOR GROWTH AND METASTASIS BY MATRICRYPTINS

Matricryptins regulate wound healing, fibrosis, inflammation, angiogenesis, and cancer and are involved in infectious and neurodegenerative diseases (Ricard-Blum and Ballut, 2011; Ricard-Blum and Salza, 2014; Ricard-Blum and Vallet, 2015). Most of the matricryptins regulating angiogenesis and tumor growth are derived from collagens IV and XVIII (Monboisse et al., 2014; Walia et al., 2015), elastin (Robinet et al., 2005; Pocza et al., 2008; Heinz et al., 2010), fibronectin (Ambesi et al., 2005), laminins (Tran et al., 2008), osteopontin (Bayless and Davis, 2001; Lund et al., 2009; Yamaguchi et al., 2012), MMPs (Bello et al., 2001; Ezhilarasan et al., 2009), proteoglycans (Goyal et al., 2011), and hyaluronan (Cyphert et al., 2015; **Table 1**). They are released from the ECM by a variety of proteinases (matrixins, adamalysins, tolloids, cathepsins, thrombin, and plasmin; Ricard-Blum and Vallet, 2015; Wells et al., 2015).

Matricryptins regulating angiogenesis and tumor growth target endothelial cells and/or tumor cells (Robinet et al., 2005; Tran et al., 2008; Sund et al., 2010; Boosani and Sudhakar, 2011; Ricard-Blum and Ballut, 2011; Toupance et al., 2012; Kikkawa et al., 2013; Monboisse et al., 2014; Ricard-Blum and Salza, 2014; Monslow et al., 2015; Nikitovic et al., 2015; Ricard-Blum and Vallet, 2015; Walia et al., 2015). Several matricryptins inhibit the proliferation and the migration of endothelial cells, block cell cycle at G1 as shown for anastellin (Ambesi et al., 2005) and endostatin (Hanai et al., 2002) and induce apoptosis. Arresten, derived from the C-terminus of the α1 chain of collagen IV, activates FasL mediated apoptosis for example (Verma et al., 2013). Endostatin and endorepellin, a matricryptin of perlecan, induce autophagy of endothelial cells, the autophagic activity of endorepellin being mediated by a VEGFR2-dependent pathway (Nguyen et al., 2009; Poluzzi et al., 2014). A modified endostatin (Endostar) induces autophagy in hepatoma cells (Wu et al., 2008). Matricryptins normalize tumor vasculature, which improves the delivery of cytotoxic drugs to the tumor and hence the response to anti-cancer treatments (Jain, 2005). Endostatin contributes to the normalization of tumor vasculature in a lung cancer model (Ning et al., 2012), and in esophageal squamous cell carcinoma, where it enhances the effect of radiotherapy and reduces hypoxia (Zhu et al., 2015), possibly by a crosstalk between cancer and endothelial cells mediated by the Hypoxia-Inducible Factor and VEGF expression.

Matricryptins derived from collagens IV and XVIII target tumoral cells. Arresten inhibits migration and invasion of squamous cell carcinoma and induces their death (Aikio et al., 2012). Endostatin inhibits the proliferation of some cancer cells (e.g., the HT29 human colorectal adenocarcinoma cell line) but not of others (e.g., the MDA-MB-231 human mammary adenocarcinoma cell line) (Ricard-Blum et al., 2004). Matricryptins enhance the sensitivity of tumor cells to a cytotoxic drug and even reverse in part their resistance to this drug. A tumstatin peptide increases the sensitivity of non-small cell lung carcinoma cells to cisplatin (Wang et al., 2015c) and Endostar enhances the sensitivity to radiation of nasopharyngeal carcinoma and lung adenocarcinoma xenografts in mice (Wen et al., 2009).

Matricryptins regulate angiogenesis, tumor growth, and metastasis by various molecular mechanisms. The anti-angiogenic activities of tumstatin and endostatin contribute to tumor suppression by p53 *via* the upregulation of the α(II) collagen prolylyl hydroxylase (Folkman, 2006; Teodoro et al., 2006). Endostatin inhibits proliferation and migration of glioblastoma cells by inhibiting T-type Ca^{2+} channels (Zhang et al., 2012), and its ATPase activity contributes to its anti-angiogenic and antitumor properties (Wang et al., 2015b). This matricryptin inhibits hemangioendothelioma by downregulating chemokine (C-X-C motif) ligand 1 *via* the inactivation of NF–κB (Guo et al., 2015).

RECEPTORS AND CO-RECEPTORS OF MATRICRYPTINS

Matricryptins regulating angiogenesis, tumor growth and metastasis bind to several receptors, and co-receptors (**Figure 1**, Faye et al., 2009a) to modulate signaling pathways and fulfill their biological functions (**Table 1**). The other ligands of the receptors (e.g., ECM proteins, proteoglycans, growth factors, and chemokines) are not represented in **Figure 1** for the sake of clarity. Pathways regulated by matricryptins in endothelial or tumor cells *via* unidentified receptors and/or in other cell types are mentioned below but are not listed in **Table 1**.

TABLE 1 | Matricryptins, receptors, and signaling pathways regulated by matricryptins in endothelial and tumor cells.

Receptors	Matricryptins	Signaling pathways	Cells	References
INTEGRINS				
α1β1	Arresten *(α1 chain of collagen IV)*	Inhibition of FAK/c-Raf/MEK1/2/ERK1/2/p38 MAPK pathway; Inhibition of hypoxia-induced expression of HIF 1α and VEGF	ECs	Sudhakar et al., 2005
			HSC-3 human tongue squamous carcinoma cells	Aikio et al., 2012
α2β1	Endorepellin *(C-terminus of perlecan)*	Activation of SHP-1	ECs	Nyström et al., 2009
		Activation of the tyrosine phosphatase SHP-1; Dephosphorylation of VEGFR2; Down-regulation of VEGFA	ECs	Goyal et al., 2011
		Down-regulation of VEGFR2	ECs	Poluzzi et al., 2014
	Procollagen I C-propeptide		HT1080 human fibrosarcoma cells	Weston et al., 1994
α3β1	Tumstatin *(α3 chain of collagen IV)*	Integrin α3β1 is a trans-dominant inhibitor of integrin αv	ECs	Borza et al., 2006
	Canstatin *(α2 chain of collagen IV)*		ECs	Petitclerc et al., 2000
α4β1	N-terminal osteopontin fragment		HL-60 human promyelocytic leukemia cells	Bayless and Davis, 2001
	PEX domain of MMP-9		Human chronic lymphocytic leukemia B cells	Ugarte-Berzal et al., 2012
α4β7	N-terminal osteopontin fragment		RPMI 8866 human lymphoblastoid cell line	Green et al., 2001
α5β1	Endostatin *(α1 chain of collagen XVIII)* K_D = 975 and 451 nM, 2 binding sites, soluble endostatin, immobilized full-length integrin; (Faye et al., 2009b)	Inhibition of FAK/c-Raf/MEK1/2/p38/ERK1 MAPK pathway	ECs	Sudhakar et al., 2003
		Induction of phosphatase-dependent activation of caveolin-associated Src family kinases	ECs	Wickström et al., 2002
		Induction of recruitment of α5β1 integrin into the raft fraction via a heparan sulfate proteoglycan-dependent mechanism. Induction of Src-dependent activation of p190RhoGAP with concomitant decrease in RhoA activity and disassembly of actin stress fibers and focal adhesions	ECs	Wickström et al., 2003
			Hemangioendothelioma-derived cells	Guo et al., 2015
	N-terminal osteopontin fragment		Human colorectal adenocarcinoma (SW480 cells)	Yokosaki et al., 2005
α6β1	Tumstatin *(α3 chain of collagen IV)*		ECs	Maeshima et al., 2000
α9β1	N-terminal osteopontin fragment		Human colorectal adenocarcinoma (SW480 cells)	Yokosaki et al., 2005
αvβ3	Endostatin *(α1 chain of collagen XVIII)* K_D = 1.2 μM and 501 nM, 2 binding sites, soluble endostatin, immobilized full-length integrin; (Faye et al., 2009b)		ECs	Rehn et al., 2001

(Continued)

TABLE 1 | Continued

Receptors	Matricryptins	Signaling pathways	Cells	References
	Canstatin *(α2 chain of collagen IV)*	Induction of two apoptotic pathways through the activation of caspase-8 and caspase-9	ECs	Magnon et al., 2005
		Induction of caspase 9-dependent apoptotic pathway	Human breast adenocarcinoma cells (MDA-MB-231)	Magnon et al., 2005
			ECs	Petitclerc et al., 2000
	Tumstatin *(α3 chain of collagen IV)*	Inhibition of Cap-dependent translation (protein synthesis) mediated by FAK/PI3K/Akt/mTOR/4E-BP1 pathway	ECs	Maeshima et al., 2000; Sudhakar et al., 2003
			ECs	Petitclerc et al., 2000
		Inhibition of the activation of FAK, PI3K, protein kinase B (PKB/Akt), and mTOR It prevents the dissociation of eukaryotic initiation factor 4E protein from 4E-binding protein 1	ECs	Maeshima et al., 2002
		Stimulation of FAK and PI3K phosphorylation	Human metastatic melanoma cell line (HT-144)	Pasco et al., 2000
		Inhibition of the growth of tumors dependent on Akt/mTOR activation (functional PTEN required)	Human glioma cells	Kawaguchi et al., 2006
	Tetrastatin *(α4 chain of collagen IV)* $K_D = 148$ nM (2-state model, soluble tetrastatin, immobilized full-length integrin)		Human melanoma cells (UACC-903)	Brassart-Pasco et al., 2012
	NC1 domain of α6 chain of collagen IV		ECs	Petitclerc et al., 2000
	Procollagen II N-propeptide		Human chondrosarcoma cell line (hCh-1)	Wang et al., 2010
	PEX domain of MMP-2		ECs	Brooks et al., 1998
	N-terminal osteopontin fragment		Human colorectal adenocarcinoma (SW480 cells)	Yokosaki et al., 2005
	VGAPG, VGAP (elastin peptides)		Human melanoma cell lines (WM35 and HT168-M1)	Pocza et al., 2008
αvβ5	Endostatin *(α1 chain of collagen XVIII)*		ECs	Rehn et al., 2001
	Canstatin *(α2 chain of collagen IV)*	Induction of two apoptotic pathways through the activation of caspase-8 and caspase-9	ECs	Magnon et al., 2005
		Induction of caspase 9-dependent apoptotic pathway	Human breast adenocarcinoma cells (MDA-MB-231)	Magnon et al., 2005
			ECs	Petitclerc et al., 2000
	Tumstatin *(α3 chain of collagen IV)*		ECs	Pedchenko et al., 2004
	Procollagen II N-propeptide		Human chondrosarcoma cell line (hCh-1)	Wang et al., 2010
	N-terminal osteopontin fragment		Human colorectal adenocarcinoma (SW480 cells)	Yokosaki et al., 2005
αvβ6	N-terminal osteopontin fragment		Human colorectal adenocarcinoma (SW480 cells)	Yokosaki et al., 2005

(Continued)

TABLE 1 | Continued

Receptors	Matricryptins	Signaling pathways	Cells	References
GROWTH FACTOR RECEPTORS				
VEGFR1	Endostatin *(α1 chain of collagen XVIII)*		ECs	Kim et al., 2002
	Endorepellin *(C-terminus of perlecan)* $K_D = 1$ nM (soluble endorepellin, immobilized ectodomain of VEGFR1)		ECs	Goyal et al., 2011
VEGFR2	Endostatin *(α1 chain of collagen XVIII)*	Inhibition of VEGF-induced tyrosine phosphorylation of VEGFR2 and activation of ERK, p38 MAPK, and p125FAK	ECs	Kim et al., 2002
	Endorepellin *(C-terminus of perlecan)*	Attenuation of VEGFA-evoked activation of VEGFR2 at Tyr^{1175} $K_D = 0.9$ nM (soluble endorepellin, immobilized ectodomain of VEGFR2)	ECs	Goyal et al., 2011
		Attenuation of both the PI3K/PDK1/Akt/mTOR and the PKC/JNK/AP1 pathways	ECs	Goyal et al., 2012
		Induction of the formation of the Peg3-Vps34-Beclin 1 autophagic complexes via inhibition of the PI3K/Akt/mTOR pathway Induction of autophagy through a VEGFR2 dependent but α2β1 integrin-independent pathway	ECs	Poluzzi et al., 2014
EGFR	Laminin-332 EGF-like (domain III) of the γ2 chain	Stimulation of EGFR phosphorylation; Induction of ERK phosphorylation	Human breast adenocarcinoma cells (MDA-MB-231)	Schenk et al., 2003
CHEMOKINE RECEPTORS				
CXCR2	Proline-glycine-proline *(collagen matrikine)*	Activation of Rac1, increase in phosphorylation of ERK, PAK and VE-cadherin	ECs	Hahn et al., 2015
HEPARAN SULFATE PROTEOGLYCANS				
Glypican-1	Endostatin *(α1 chain of collagen XVIII)*		ECs	Karumanchi et al., 2001
Glypican-4	Endostatin *(α1 chain of collagen XVIII)*		ECs	Karumanchi et al., 2001
Syndecan-1	LG45 domain of the α3 chain of laminin-332		HT1080 human fibrosarcoma cells	Carulli et al., 2012
Syndecan-4	LG45 domain of the α3 chain of laminin-332		HT1080 human fibrosarcoma cells	Carulli et al., 2012
ELASTIN RECEPTOR COMPLEX				
Elastin receptor complex	Elastin peptides (xGxxPG sequences)	67 kDa elastin binding protein (an alternatively spliced form of β-galactosidase)	ECs	Robinet et al., 2005
			Human melanoma cell lines (WM35 and HT168-M1)	Pocza et al., 2008
GALECTIN-3 RECEPTOR				
Galectin-3 receptor	VGVAPG and VAPG *(elastin peptides)*		Human melanoma cell lines (WM35 and HT168-M1)	Pocza et al., 2008

(Continued)

TABLE 1 | Continued

Receptors	Matricryptins	Signaling pathways	Cells	References
LACTOSE-INSENSITIVE RECEPTOR				
Lactose-insensitive receptor	VGVAPG *(elastin peptide)*		M27 subline of murine Lewis lung carcinoma	Blood and Zetter, 1993
	AGVPGLGVG and AGVPGFGAG *(elastin peptides)*		Human lung carcinoma cells	Toupance et al., 2012
CD44, RHAMM AND TLR4				
CD44	Hyaluronan oligosaccharides (3–10 disaccharides)	PKC-α phosphorylation of γ-adducin, a membrane cytoskeletal and actin-binding protein, Activation of ERK1/2	ECs	Matou-Nasri et al., 2009
		Stimulation of ERK1/2 signaling Inhibition of CD44 clustering (3–10 disaccharides)	Human breast cancer cells (BT-159, ductal carcinoma)	Yang et al., 2012
	N-terminal osteopontin fragment (Leu^{1}-Gly^{127})	CD44-mediated OPN binding requires β1 integrin	Rat BDX2 fibrosarcoma cells	Katagiri et al., 1999
	C-terminal osteopontin fragment (Leu^{132}-Asn^{278})	CD44-mediated OPN binding requires β1 integrin	Rat BDX2 fibrosarcoma cells	Katagiri et al., 1999
	Osteopontin fragment (5 kDa, residues 167–210)		Human hepatocellular carcinoma cells	Takafuji et al., 2007
	PEX domain of MMP-9		Human chronic lymphocytic leukemia cells	Ugarte-Berzal et al., 2014
LYVE-1	Hyaluronan oligosaccharides (3–10 disaccharides)	Increased tyrosine phosphorylation of protein kinase Cα/βII and ERK1/2	ECs	Wu et al., 2014
TLR4	Hyaluronan oligosaccharides (4, 6, 8-mer HA fragments)		ECs	Taylor et al., 2004
RHAMM	Hyaluronan oligosaccharides (2–10 disaccharides)	Activation of ERK1/2	ECs	Gao et al., 2008
	Hyaluronan oligosaccharides (3–10 disaccharides)	Activation of ERK1/2 Up-regulation of cdk1/Cdc2		Matou-Nasri et al., 2009
CELL SURFACE ASSOCIATED PROTEIN				
Nucleolin	Endostatin *(α1 chain of collagen XVIII)* K_D = 13 nM; (Shi et al., 2007)		Hemangioendothelioma-derived cells	Guo et al., 2015

Receptors identified in other cell types and the associated signaling pathways are mentioned in the text. 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; AP1, activation protein 1; Cdk1/Cdc2, cyclin-dependent kinase-1; CXCR2, CXC chemokine receptor 2; CXCL1, Chemokine (C-X-C motif) ligand 1; EC, endothelial cell; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HIF, hypoxia-inducible factor; JNK, c-Jun N-terminal kinases; LG, laminin G domain-like; LYVE-1, Lymphatic vessel endothelial hyaluronan receptor 1; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; PAK, p21-activated kinase; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PTEN, phosphatase and tensin homolog; RHAMM, receptor for HA-mediated motility; SHP-1, Src homology-2 protein phosphatase-1; TLR4, Toll-like receptor 4; VE-cadherin, vascular endothelial cadherin; VEGFA, vascular endothelial growth factor A; VEGFR, Vascular endothelial growth factor receptor.

Integrins

There are 24 integrins comprised of an α subunit and a β subunit (Barczyk et al., 2010). They lack intrinsic kinase activity and are the major adhesion receptors of the ECM, controlling ECM assembly, cell-matrix interactions, cell migration, and tumor growth (Missan and DiPersio, 2012; Xiong et al., 2013). A number of matricryptins bind to integrins at the surface of tumor and/or endothelial cells (**Table 1**). Matricryptins also interact with purified integrins (e.g., αvβ5 integrin for endostatin; Rehn et al., 2001; Faye et al., 2009b), or on other cell types. The αvβ3 integrin is the main receptor targeted by matricryptins (**Figure 1**).

Anastellin decreases the activation state of α5β1 integrin on endothelial cells (Ambesi and McKeown-Longo, 2014). Arresten interacts with α3β1/αvβ3 and α1β1/α2β1 integrins at the surface of HPV-16-immortalized proximal tubular epithelial cells and mesangial cells respectively, whereas tumstatin binds to immortalized glomerular epithelial cells through α3β1 and α2β1 integrins (Aggeli et al., 2009). The above integrins are also involved in the effects of matricryptins on other

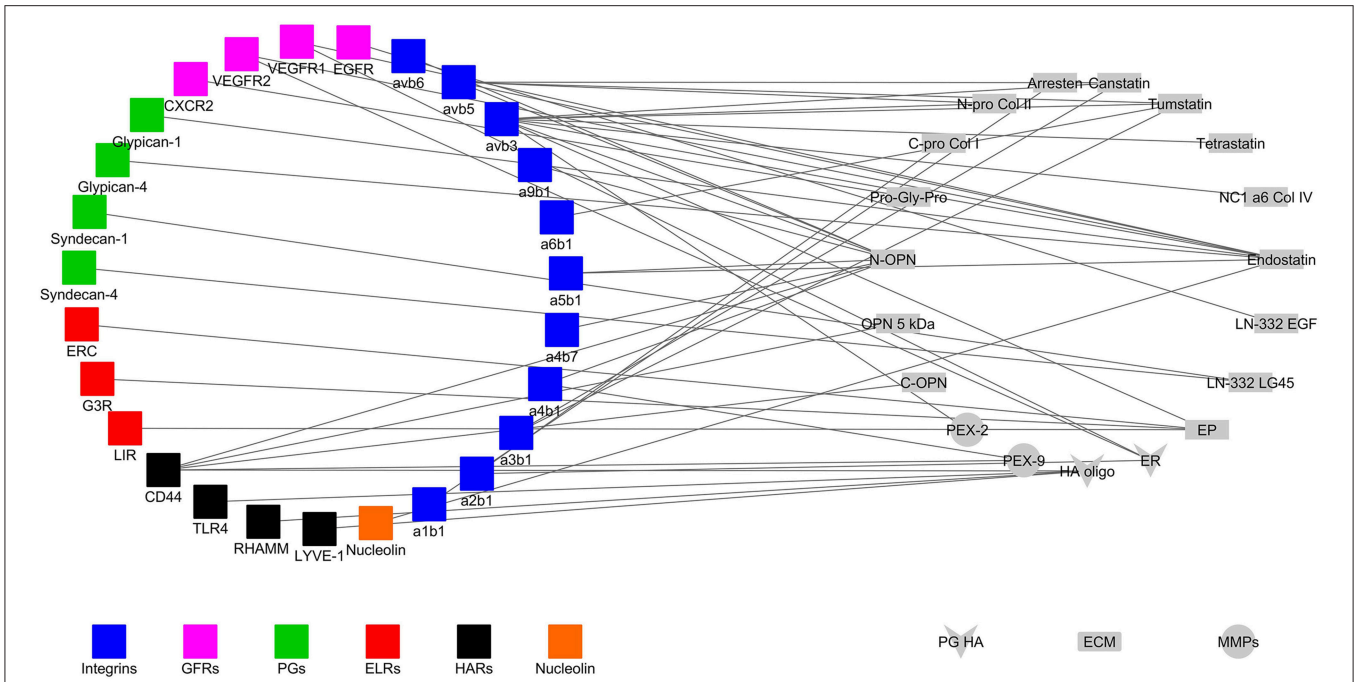

FIGURE 1 | Interaction network of matricryptins (right) and their receptors (left) expressed at the surface of endothelial and cancer cells. ab, alpha and beta integrin subunits; C-Pro Col, C-propeptide of procollagen; CXCR, chemokine CXC receptor; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EP, elastin peptide; ER, endorepellin; ERC, elastin receptor complex; ELR, elastin receptor; ES, endostatin; G3R, galectin-3 receptor; GFR, growth factor receptor; HA oligo, hyaluronan oligosaccharide; HAR, hyaluronan receptor; LN LG45, laminin domain LG45; LIR, lactose insensitive receptor; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; N-Pro Col, N-propeptide of procollagen; MMP, matrix metalloproteinase; NC1, non-collagenous domain; OPN, osteopontin; PEX, hemopexin domain; PG, proteoglycan; RHAMM, receptor for hyaluronic acid-mediated motility; TLR4, toll-like receptor; VEGFR, vascular endothelial growth factor receptor.

cell types. Endostatin, generated by cerebellar Purkinje cells, contributes to the organization of climbing fiber terminals in these neurons by binding and signaling through α3β1 integrin (Su et al., 2012). The adhesion of smooth muscle cells to anastellin is mediated by both β1 integrins and cell surface heparan sulfate proteoglycans, which triggers ERK1/2 activation in these cells (Mercurius and Morla, 2001) and the induction of osteoclast apoptosis by the N-propeptide of procollagen II is mediated by αv or β3 integrin subunits (Hayashi et al., 2011).

Growth Factor and Chemokine Receptors

Growth factor receptors belong to the tyrosine kinase receptor family. They regulate cell proliferation, differentiation, cell cycle, survival and apoptosis and play a role in cancer (McDonell et al., 2015). VEGR receptors 1–3 (Roskoski, 2008; Grünewald et al., 2010; Simons, 2012) and EGF receptor (Lemmon et al., 2014) interact with matricryptins (**Table 1**). Two endostatin peptides bind to VEGFR3 (Han et al., 2012, 2015) and EGF-like repeats of tenascin C interact with EGFR, inducing phosphorylation of the receptor and of ERK MAP kinases in NR6 mouse fibroblasts (Swindle et al., 2001). Endorepellin simultaneously engages VEGFR2 and α2β1 integrin, both expressed by endothelial cells, and regulate angiogenesis and autophagy through a dual receptor antagonism (Goyal et al., 2011; Poluzzi et al., 2014). Anastellin inhibits lysophospholipid (Ambesi and McKeown-Longo, 2009) and VEGF165-dependent signaling in endothelial cells by preventing the formation of the complex containing VEGFR2 and neuropilin-1 at the surface of endothelial cells (Ambesi and McKeown-Longo, 2014). One matricryptin of collagen I interacts with a member of the chemokine receptor family, the CXC chemokine receptor 2 (Stadtmann and Zarbock, 2012; Veenstra and Ransohoff, 2012).

Cell Surface Proteoglycans

Proteoglycans are divided into intracellular, pericellular, extracellular, and cell-surface families (Iozzo and Schaefer, 2015). Syndecans are transmembrane heparan sulfate proteoglycans (Couchman et al., 2015), which play a role in cell adhesion, migration, receptor trafficking, growth factor interactions, angiogenesis (De Rossi and Whiteford, 2014) and cancer (Barbouri et al., 2014). They are enzymatically shed from the cell surface and compete with their membrane forms for ligand binding (Manon-Jensen et al., 2010). They act in synergy with integrins to control cell adhesion and other biological processes (Morgan et al., 2007; Roper et al., 2012; Humphries et al., 2015), and the binding of heparan sulfate chains to integrin α5β1 promotes cell adhesion and spreading (Faye et al., 2009b). Syndecans act as co-receptors of VEGF and control tumor progression in association with integrins (Grünewald et al.,

2010; Soares et al., 2015). Glypicans, membrane-associated proteoglycans with a glycosylphosphatidyl anchor, regulate Wnt, Hedgehog, fibroblast growth factor, and bone morphogenetic protein signaling (Filmus et al., 2008; Iozzo and Schaefer, 2015). One matricryptin, endostatin, binds to glypicans *via* their heparan sulfate chains (Karumanchi et al., 2001).

Elastin Receptors

The Elastin Receptor Complex (ERC) is composed of two membrane associated proteins (the protective protein/cathepsin A and neuraminidase-1) and of the elastin-binding protein, an inactive spliced variant of lysosomal β-galactosidase (Blanchevoye et al., 2013). Elastin peptides activate extracellular signal-regulated kinase 1/2 *via* a Ras-independent mechanism in fibroblasts (Duca et al., 2005), the enzymatic activity of neuraminidase-1 being responsible for signal transduction (Duca et al., 2007). Another, still unidentified, receptor of elastin peptides exists at the surface of macrophages (Maeda et al., 2007). Furthermore, elastin peptides regulate tumor cell migration and invasion through an Hsp90-dependent mechanism (Donet et al., 2014).

CD44, Receptor for HA-Mediated Motility (RHAMM) and Toll-Like Receptors (TLRs)

Hyaluronan, a non-sulfated glycosaminoglycan, has two major receptors, CD44 and RHAMM, which mediate its roles in inflammation and cancer (Toole, 2009; Misra et al., 2015; Nikitovic et al., 2015). The binding to, and activation of, receptors depend on the size of HA, its oligosaccharides stimulating angiogenesis (Gao et al., 2008). CD44, which has several isoforms regulates cell proliferation, adhesion and migration, and is involved in tumorigenesis (Jordan et al., 2015). A sequence in the hemopexin domain of MMP-9 (PEX9) impairs tumor cell adhesion to PEX9/MMP9 through interaction with CD44 (Ugarte-Berzal et al., 2014). RHAMM has splicing variants and is located inside the cell and on the cell surface, where it is anchored *via* a glycosylphosphatidylinositol (Tolg et al., 2014; Misra et al., 2015). Toll-like receptors are pattern recognition receptors involved in innate immunity (Rakoff-Nahoum and Medzhitov, 2009). Low-molecular weight hyaluronan induces the formation of a complex containing CD44, TLR2/TLR4, the actin filament-associated protein AFAP-110, and a myeloid differentiation factor MyD88, which triggers cytoskeleton activation and results in tumor invasion (Bourguignon et al., 2011).

Other Membrane and Cell Surface-Associated Proteins

Matricryptins form complexes with membrane or membrane-associated proteins. Caveolin- participates in the formation of membrane caveolae, which are platforms for signal transduction (Fridolfsson et al., 2014) and forms a complex with α5β1 integrin and endostatin in lipid rafts at the endothelial cell surface (Wickström et al., 2002) (**Table 1**). Nucleolin, a multifunctional protein, localized inside the cell and at the cell surface (Berger et al., 2015), binds to endostatin and triggers its internalization in endothelial cells in association with the urokinase plasminogen activator receptor and the α5β1integrin (Shi et al., 2007; Song et al., 2012).

MATRICRYPTINS AS POTENTIAL DRUGS

Matricryptins are potential anti-cancer drugs, either alone, or in combination with other treatments, but their use in pre-clinical and clinical studies remains challenging. Indeed matricryptins may display opposite activities depending on the context. The anti-tumoral effect of endostatin is enhanced by silencing of the proteoglycan versican, which decreases the inflammatory and immunosuppressive changes triggered by anti-angiogenic therapy (Wang et al., 2015d). However, endostatin may induce the proliferation of carcinoma cells, whereas its effect on cancer invasion is modulated by the tumor microenvironment (Alahuhta et al., 2015). Endorepellin stimulates angiogenesis in a stroke model by increasing VEGF levels, and exerts a neuroprotective effect in this model *via* α5β1 integrin and VEGFR2 (Lee et al., 2011). In addition, endostatin exhibits a biphasic response curve both for its anti-angiogenic and anti-tumoral properties (Celik et al., 2005; Javaherian et al., 2011), which requires to test a large range of concentrations to determine the optimal dose for treatment. Another challenge is that matricryptins may themselves contain cryptic sequences displaying opposite activities as reported for the anti-angiogenic matricryptin endostatin, which contains an embedded pro-angiogenic sequence (Morbidelli et al., 2003). Different matricryptins regulate the same biological process in an opposite way as reported for the regulation of the angiogenic signaling in choroidal endothelial cells by hexastatin and elastokines (Gunda and Sudhakar, 2013), or distinct steps of a biological process as described for anastellin and endostatin (Neskey et al., 2008).

Matricryptins can be modified to extend the half-life, and their efficacy (Xu et al., 2007; Zheng, 2009; Ricard-Blum and Ballut, 2011; Ricard-Blum and Salza, 2014). Most of the examples detailed below concern endostatin, which is extensively studied and has been approved for the treatment of non-small-cell lung cancer in China (Biaoxue et al., 2012) under a recombinant form, Endostar, which contains an extra metal chelating sequence (MGGSHHHHH) at the N-terminus enhancing its solubility and stability (Jiang et al., 2009). PEGylation (Nie et al., 2006; Tong et al., 2010; Tan et al., 2012; Guo et al., 2014), and the fusion of endostatin to low molecular weight heparin or to the Fc region of an IgG enhance its half-life and its anti-angiogenic, or anti-tumoral activities (Lee et al., 2008; Jing et al., 2011; Ning et al., 2012; Tan et al., 2012; Li et al., 2015b).

Tumors may escape from anti-tumoral and anti-angiogenic matricryptins by upregulating factors, which stimulate angiogenesis (Fernando et al., 2008). The combination of matricryptins with inhibitors of pro-angiogenic pathways, chemotherapy, or radiotherapy enhance their therapeutic efficacy. Tumstatin has been fused to another endogenous inhibitor of angiogenesis, vasostatin (Gu et al., 2014) and to tumor necrosis factor α, which has anti-tumoral and anti-angiogenic properties, which results in a more effective fusion protein than tumstatin alone (Luo et al., 2006). Endostatin has

been fused to the proapoptotic domain (BH3) of the BAX protein (Chura-Chambi et al., 2014), to tumor necrosis factor-related apoptosis-inducing ligand (Zheng et al., 2013) and one of its anti-angiogenic sequences to an heptapeptide inhibitor of MMPs (Qiu et al., 2013). Endostatin has also been fused to protein sequences targeting it to tumors and/or tumor vasculature such as humanized antibodies against tyrosine kinase-type receptor HER2 (Shin et al., 2011) or against tumor-associated glycoprotein 72 highly expressed in human tumor tissues (Lee et al., 2015), the RGD integrin-binding sequence (Jing et al., 2011), and a liver-targeting peptide (circumsporozoite protein CSP I-plus (Ma et al., 2014; Bao et al., 2015).

Several approaches improve the delivery of matricryptins to tumors and endothelial cells (Xu et al., 2007; Ricard-Blum and Ballut, 2011) such as conditionally replicating oncolytic adenoviral vector for arresten (Li et al., 2015a), naked plasmid electrotransfer in muscle for tumstatin overexpression (Thevenard et al., 2013), and the nonpathogenic and anaerobic bacterium, *Bifidobacterium longum*, which proliferates in the hypoxic zones within tumors for tumstatin expression (Wei et al., 2015). Endostatin has been delivered in polylactic acid nanoparticles (Hu and Zhang, 2010), in gold nanoshells, which are very efficient on lung cancer cells when associated with near-infrared thermal therapy (Luo et al., 2015) and into microbubbles in combination with ultrasonic radiation in a cancer model (Zhang et al., 2014). Dendrimers mimicking the surface structure of endostatin and loaded with anticancer drug, result in both angiogenesis inhibition by endostatin and death of cancer cells by the anticancer drug (Jain and Jain, 2014).

Clinical trials of endostatin mostly focus on solid tumors in association with cytotoxic drugs (https://clinicaltrials.gov/). These trials include phase I (Lin et al., 2007; Chen et al., 2014), II (Lu et al., 2015), and III trials (Wang et al., 2005). Although endostatin did not improve overall survival, progression-free survival, and objective response rate when combined with etoposide and carboplatin in patients with extensive-stage small-cell lung cancer phase II trial (Lu et al., 2015), it significantly improves the response rate and median time to tumor progression when combined with vinorelbine and cisplatin in advanced non-small cell lung cancer patients compared to chemotherapy alone (Wang et al., 2005). Promising results have been obtained with endostatin associated with paclitaxel and 5-fluorouracile in patients with refractory malignant ascites secondary to ovarian cancer (Zhao et al., 2014).

CONCLUDING REMARKS

Several matricryptins such as the propeptide of lysyl oxidase, which is a tumor suppressor (Min et al., 2007; Ozdener et al., 2015) and the netrin-like domain of procollagen C-proteinase enhancer-1, a new anti-angiogenic matricryptin (Salza et al., 2014), warrant further investigation in angiogenesis, and tumor models to decipher their mechanisms of action at the molecular and cellular levels. Matricryptins are useful for treating fibroproliferative disorders (Yamaguchi et al., 2012; Wan et al., 2013) and *fundus oculi* angiogenesis diseases (Zhang et al., 2015). The finding that a peptide derived from endostatin can be delivered orally *in vivo* and exerts anti-fibrotic activity (Nishimoto et al., 2015) paves the way for the development of new matricryptin drugs with oral bioavailability, which is a preferred administration route for long-term treatment. Matricryptins are also used as biomarkers in serum and in cerebrospinal fluid (Ricard-Blum and Vallet, 2015; Salza et al., 2015) and may serve as imaging agents when labeled with (99m)Tc as shown for endostatin (Leung, 2004) and tumstatin (He et al., 2015) and for tumstatin conjugated to iron oxide nanoparticles (Ho et al., 2012).

AUTHOR CONTRIBUTIONS

SV drafted the Section Receptors and Co-receptors of Matricryptins and **Table 1** and made the figure. SB made bibliographical searches for all the sections and wrote the manuscript.

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β1 Integrins as Therapeutic Targets to Disrupt Hallmarks of Cancer

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Integrins belong to a large family of αβ heterodimeric transmembrane proteins first recognized as adhesion molecules that bind to dedicated elements of the extracellular matrix and also to other surrounding cells. As important sensors of the cell microenvironment, they regulate numerous signaling pathways in response to structural variations of the extracellular matrix. Biochemical and biomechanical cues provided by this matrix and transmitted to cells via integrins are critically modified in tumoral settings. Integrins repertoire are subjected to expression level modifications, in tumor cells, and in surrounding cancer-associated cells, implicated in tumor initiation and progression as well. As critical players in numerous cancer hallmarks, defined by Hanahan and Weinberg (2011), integrins represent pertinent therapeutic targets. We will briefly summarize here our current knowledge about integrin implications in those different hallmarks focusing primarily on β1 integrins.

Keywords: integrins, hallmarks of cancer, proliferation, migrationinvasion, resistance to cell death, angiogenesis, therapeutic target

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INTRODUCTION

In the setting of cancer, six hallmarks enabling a cell to become tumorigenic and ultimately malignant have been defined by Hanahan and Weinberg (2000). As such cancer cells have the abilities to sustain proliferative signaling, to evade growth suppressors, to resist to cell death, to enable replicative immortality, to induce angiogenesis and to activate invasion and metastasis. This list has been extended recently by the authors taking into account the new progresses made in the past decade with the proposal of two new hallmarks comprising the reprogramming of energy metabolism and the evasion of immune destruction (Hanahan and Weinberg, 2011). A recent review emphasized the modulation of these hallmarks by the extracellular matrix (Pickup et al., 2014). Integrins belong to one of the most studied family of matricellular receptors. These heterodimeric αβ cell surface receptors sense the extracellular matrix with high flexibility (Hanein and Horwitz, 2012; Hohenester, 2014) triggering thereby specific answers in both physiological and pathophysiological conditions. In humans, 18 α and 8 β subunits have been characterized enabling about 24 heterodimeric combinations. The Arg-Gly-Asp (RGD) binding integrins belong to the most studied subfamily including αvβ1,αvβ3, αvβ5, αvβ6, αvβ8, α5β1, and αIIbβ3. The RGD motif is available in ECM components such as fibronectin, vitronectin, osteopontin, and fibrinogen. Cell binding to collagen or laminin involves either β1 (α1β1, α2β1, α10β1, α11β1,α6β1…) or β4 (α6β4…) subunits-containing integrins. Integrins signaling operates through the integrin adhesome which appears complex (Winograd-Katz et al., 2014). Interactions of integrins with soluble or membrane-localized elements as well as with cytoplasmic adaptors or catalytic partners

(kinases, phosphatases, proteases . . .) define series of coordinated and spatiotemporal regulated processes. Mechanistic insights into the fine tuning of integrin signaling thereby revealing the high versatility of the cell answer to integrin-driven stimuli were enabled by the development of new technologies (Rossier et al., 2012; Robertson et al., 2015). Expression and activity of integrins and of their adhesome components have been implicated in various diseases including cancer. Integrins are well recognized as valuable tumor therapeutic targets although essentially in preclinical studies (Desgrosellier and Cheresh, 2010; Schaffner et al., 2013). A recent review emphasized their capability to regulate cancer stemness, metastasis, and drug resistance (Seguin et al., 2015). They remarkably impact on the hallmarks of cancer as defined above. In this review, our aim is to update the knowledges with the most recent data in the field focusing particularly on β1 integrins and their roles in the tumor progression. Integrins containing the β1 subunit constitute the largest subgroup and appear overexpressed in several solid tumors compared to control tissues (Paulus et al., 1993; Barkan and Chambers, 2011; Fabricius et al., 2011; Lahlou and Muller, 2011; Schaffner et al., 2013).

INTEGRINS AND A SUSTAINED PROLIFERATIVE SIGNALING

Integrins contribute to the cell cycle progression in physiological and pathological situations (reviewed in Moreno-Layseca and Streuli, 2014). The cross-talk between integrins and growth factor receptors (GFR) is well established especially in sustaining the cell proliferative signaling. Several GFR are concerned of which the epidermal growth factor receptor (EGFR), the hepatocyte growth factor receptor (HGFR/cMet), the platelet-derived growth factor receptor (PDGFR) and the vascular endothelial growth factor receptor (VEGFR). Direct activation of GFR by integrins was first described in normal cells. In endothelial cells, integrins phosphorylate EGFR even in the absence of EGF (Moro et al., 2002). More recently, β1 integrin downregulation decreased the phosphorylation of c-Met and of EGFR in hepatocytes during liver regeneration (Speicher et al., 2014). Keratinocytes stimulation by EGF modulates constituents of focal adhesion complexes including β1/β3 integrins and FAK (Eberwein et al., 2015). The synergistic relationship between integrins and GFR is also highlighted in tumor progression (Ivaska and Heino, 2011). Physical interactions between integrins and GFR have been demonstrated by co-immunoprecipitation experiments (α5 integrin and EGFR; Morozevich et al., 2012) or by FRET analysis on patient tumor slices (β1 integrin and ERBB1; Petras et al., 2013). Interestingly, proliferative cooperation between ECM receptors and GFR may also be achieved through direct ECM-GF interactions (Vlahakis et al., 2005; Oommen et al., 2011; Dong et al., 2014; Han et al., 2014; Zhu and Clark, 2014). The fine molecular tuning of the integrin-GFR interplay implicates other partners either from the ECM or from the cellular compartment. The matricellular protein CCN1 (CYR61/CCN1, cysteine-rich protein 61) inhibits EGFR-dependent hepatocytes proliferation through ROS accumulation induced by α6β1 integrin in liver carcinoma (Chen et al., 2015). Tenascin-C induces a physical association of PDGFR- β and α5β1 integrin resulting in prolonged activation of PDGFR- β and deregulated proliferation of fibroblast cell line (Tanaka et al., 2014). EGFR signaling regulates ILK (Integrin Linked Kinase) to increase gastric cancer cells proliferation (Tseng et al., 2014). In epidermoid carcinoma cells, EGF stimulation modulates α5β1 activation state by phosphorylation of Filamin-A (Vial and McKeown-Longo, 2012). In the same model, α5β1 integrin inhibition reduces EGFR phosphorylation implicated in cell proliferation (Morozevich et al., 2012). Scaffolding proteins such as tensin4 (TNS4) may create a functional complex between cMet and integrin β1 (Muharram et al., 2014). Hepatocellular carcinoma progression has been blocked by nanoparticle-formulated siRNA targeting β1 and αv integrins through reduced activation of MET oncogene (Bogorad et al., 2014). Integrins and GFR thus mainly interact through cross-regulated signaling pathways. In the case of EGFR and α5β1integrin, common way of intracellular trafficking may also potentiate their functions (Caswell et al., 2008). The overexpression of GFR and/or the expression of constitutively active mutants (such as the EGFRvIII mutant; Guo et al., 2015) are hallmarks of different tumor types and boost the proliferation of tumoral cells. Powerful therapeutic strategies may thus include simultaneous integrin/GFR targeting for selected tumors and patients (Eke et al., 2015).

INTEGRINS AND THE EVASION OF GROWTH SUPPRESSORS AND THE RESISTANCE TO CELL DEATH

Evading Growth Suppressor

p53 one of the most prominent tumor suppressor, is mutated in about 50% of cancers (Ciriello et al., 2013). Wild type p53 signaling is nevertheless altered in a large majority of tumors by alternative pathways such as deletions/mutations of endogenous activators or amplifications of inhibitors (Brennan et al., 2013). Integrin signaling may be added to the list of p53 activity regulators. We have shown that α5β1 integrin impairs the p53 activation by chemotherapeutic drugs (Martinkova et al., 2010; Janouskova et al., 2012, 2013; Martin et al., 2012). Similar results obtained by others in breast carcinoma cells showed enhanced expression of p53 upon depletion of α2β1 integrin (Morozevich et al., 2015). In glioblastoma, an overexpression of α5 integrin was recorded in p53 wild type tumors (Janouskova et al., 2012) explaining their resistance to therapies. Conversely, in ovarian tumors with a mutated p53 overexpression of the β4 integrin leads to a metastasis advantage (Lee et al., 2015). In squamous cell carcinoma, cooperation between p53 and αv integrin, impacts on tumor induction and growth (Savar et al., 2015). Integrin signaling pathways have been highlighted in the regulation of p53 activity. Our recent data demonstrated participation of the integrin/AKT/PEA15/caspase8 axis in the inhibition of p53 (Renner et al., 2015). As reported by others, the integrin-downstream kinase FAK, has the capability to inhibit p53 through direct physical interaction in the nucleus or cytoplasm thus linking signaling from the ECM to the nucleus (Golubovskaya

and Cance, 2011; Golubovskaya, 2014). Interestingly, a regulatory loop exists between FAK and p53 (Golubovskaya et al., 2008) similar to the one we described between α5 integrin and p53 (Renner et al., 2015). Finally, abrogation of α5 integrin or FAK signaling concomitantly with activation of p53 leads to tumor cell apoptosis (Gillory et al., 2015; Renner et al., 2015). Reactivation of p53 appears as a pertinent strategy for numerous tumors (Selivanova, 2014) and, as suggested above, blocking either integrins or their downstream signaling pathways may offer new opportunities to synergistically enhance the p53 tumor suppressor effects.

Resisting Cell Death

Maintenance of cell survival through ECM-integrin interactions has been recognized for a long time in development and in tissue homeostasis. Loss in cell adhesion will block the pro-survival integrin-dependent signaling pathways including PI3K/AKT, MEK/ERK, FAK, NFκB, and/or ILK leading to a particular form of apoptosis named anoikis (Griffiths et al., 2011; Vachon, 2011). Resistance to anoikis promotes tumor progression and favors emergence of metastasis (Paoli et al., 2013; Buchheit et al., 2014). The "integrin switch" includes changes in their expression profile and functionality during cell detachment from the ECM thus overcoming anoikis and allowing tumor cell survival and metastasis (Janes and Watt, 2004). New contributors to anoikis resistance through integrin pathway modulations were recently discovered. In melanoma cells, TIMP1, a member of the metalloproteinase inhibitors, was shown to form a complex with CD63 and integrin β1 conferring resistance to anoikis (Toricelli et al., 2013). Depletion of cytoplasmic FER, a non-receptor tyrosine kinase, by increasing the expression of α6β1 integrin decreased anoikis resistance in breast cancer cells (Ivanova et al., 2013). Vacuolar-ATPase inhibitor has been shown to reduce active β1 integrins and to regulate anoikis resistance in several cancer cells (Schempp et al., 2014). Zinc finger transcription factor ZNF304 transcriptionally regulates the β1 integrin and prevents anoikis (Aslan et al., 2015). The miR-26a targeting of α5 integrin promotes anoikis in human hepatocellular carcinoma (Zhang et al., 2015b). Finally, atypical anoikis involving necrosis and autophagy in glioma cells was induced by cilengitide, an αvβ3/β5 integrin inhibitor (Silginer et al., 2014). Very recently, suppression of anoikis was attributed to integrin endosomal signaling (Alanko et al., 2015). These recent examples document the different ways for a tumoral cell to engage for resisting to cell-detachment induced apoptosis by means of modulation of integrin expression and functions.

Resistance to Therapies as a Consequence

As the therapeutic protocols aim to eradicate the tumors and avoid recurrences, the best strategy would be to induce cell death. As supported by their pro-survival capacities, integrins participate to the resistance toward therapies including radio-, chemo- and targeted therapies (Aoudjit and Vuori, 2012; Nistico et al., 2014; Shishido et al., 2014; Eke and Cordes, 2015; Naci et al., 2015).

Research from the group of Cordes largely confirmed that β1 integrins induce radioresistance in head and neck cancers (Eke et al., 2012, 2015; Dickreuter et al., 2015; Steglich et al., 2015) whereas similar results have been reported by others in breast cancer (Nam et al., 2009, 2013; Ahmed et al., 2013). Resistance to radiotherapy has also been linked to αvβ3/β5 integrins (Monferran et al., 2008; Skuli et al., 2009; Ning et al., 2010; Ducassou et al., 2013; Lanvin et al., 2013). β1 integrins also modulate solid tumor responses to chemotherapies (Howe and Addison, 2012; Sorensen et al., 2015). In glioblastoma, we demonstrated the crucial role of α5β1 integrin in the resistance to Temozolomide (Martinkova et al., 2010; Janouskova et al., 2012). IGFBP-2 was involved in this resistance (Holmes et al., 2012; Han et al., 2014). Chemoresistance against doxorubicin by means of α2β1 integrin activation was recently noted in leukemia (Naci et al., 2012). Interestingly, an anchorage-independent form of chemoresistance may exist in leukemia cells implicating only the α integrin subunit and its cytoplasmic tail sequence KXGFFKR (Liu et al., 2013). If confirmed in other tumors, this will constitute a new concept in the field of integrin-dependent chemoresistance.

Integrins are also coopted candidates for innate and acquired resistance provoking tumor recurrence. In melanoma, the mutant BRAF inhibitor, vemurafenib, drives an adhesion signaling network involving α5β1 integrin and implicated in the drug resistance (Fedorenko et al., 2015). BRAF inhibition also activated a β1 integrin/FAK signaling pathway in the fibroblastic tumor stroma promoting tumoral cell survival (Hirata et al., 2015). In breast cancer cells, acquired resistance to tamoxifen is mediated by cancer-associated fibroblast-derived fibronectin which induces β1 integrin-dependent signaling in adjacent tumoral cells (Yuan et al., 2015). Ovarian taxol-resistant tumor populations exhibit an increase in β1 integrin expression and microtubule dynamics (McGrail et al., 2015). One of the most studied resistance mechanism addresses the integrin-GFR crosstalk. The importance of αvβ3 integrin/KRAS axis in the resistance of various solid tumors toward EGFR targeted therapies has been demonstrated (Seguin et al., 2014). β1 integrin is also implicated in resistance to anti-EGFR therapies (Huang et al., 2011; Morello et al., 2011; Eke et al., 2013; Kanda et al., 2013). By contrast, a recent study showed that β1 integrin and EGFR inhibitions are inefficient for radio- and chemo-sensitization of colorectal carcinoma cell *in vitro* (Poschau et al., 2015). Cooperation between β1 integrin and c-Met regulates tyrosine kinase inhibitor resistance in lung cancer (Ju and Zhou, 2013).

In solid tumors, as resistance to therapies can be mediated by GFR and β1 integrin, targeting of β1 integrin simultaneously with GFR inhibitors may be a promising therapeutic approach. In addition, new data stress side-effects of targeted therapies on the tumor-surrounding microenvironment that may affect the integrin signaling pathways to reinforce their resistance mechanisms.

INTEGRINS AND INVASION/METASTASIS

Other key biological process of cancer progression comprises local invasion and metastatic dissemination of tumor cells which present interconnected pathways with resistance to therapies

(Alexander and Friedl, 2012). Cell adhesion to ECM is central to the migration/invasion/metastasis process and implicates largely integrins (Scales and Parsons, 2011; Esposito and Kang, 2014; Naci et al., 2015). It is known for a long time that integrins regulate MMPs (matrix metalloproteinases) facilitating ECM degradation and remodeling. New data extend these findings (Borrirukwanit et al., 2014; Missan et al., 2015; Schlomann et al., 2015). New components are still being discovered contributing to the activity/function of integrins in cancer. Among those, actin-binding proteins or nucleation/assembly factors were recently reported to play crucial roles in the proinvasive activity of integrins. High expression of Profilin-1 (PFN1-a regulator of actin polymerization) was associated to tumor infiltration and lymph node metastasis. In gastric cancer, silencing PFN1 reduced β1 integrin expression and prevented FAK signaling (Cheng et al., 2015). Formin-like 2 (FMNL2—actin nucleation and assembly factor), upregulated in several metastatic cancers, interacts with RhoC to drive α2β1 and α5β1 integrin internalization/trafficking and invasive motility of cancer cells (Wang et al., 2015). Invasive migration of cancer cells into fibronectin-rich 3D ECM was reported to be enhanced following Rab-coupling protein (RCP)-driven endocytic recycling of α5β1 integrin. Invasive cells exhibit dynamic actin spike protrusions that are Arp2/3-independent but requires ROCK-mediated activation of FHOD3 (member of the formin family of protein; Paul et al., 2015). Integrin signaling can be rewired to increase tumor invasiveness during tumor metastasis by a novel mechanism recently described (Leyme et al., 2015). Integrins and G protein-coupled receptor traditionally trigger independent signaling but interestingly it was shown that integrin signaling requires the activation of the trimeric G protein Gαi by GIV or Girdin. In breast cancer cells, GIV colocalize with β1 integrin in invadosomes to recruit Gαi3 to the integrin signaling complex. Expression of GIV in non-invasive cancer cells results in enhanced haptotaxis and invasion. Modulation of expression of integrins is an alternative mechanisms used by cancer cells to control migration, invasion and metastasis. Human telomerase reverse transcriptase (hTERT) expression and telomerase activation are observed in 90% of human malignancies. hTERT plays an important role in cancer invasion by enhancing β1 integrin to promote the invasion of gastric cancer (Hu et al., 2015). The collaboration between integrins and GFR also accelerate tumor cell mobility and invasion. Clinical and functional analyses showed that CD151 and α3β1 integrin were elevated in glioblastoma. Both synergized with EGF/EGFR to accelerate tumor cell motility and invasion (Zhou et al., 2015). β1 integrin/kindlin and EGFR complexes increase breast and lung cancer cell migration (Li et al., 2013; Williams and Coppolino, 2014; Guo et al., 2015). Fibronectin matrix mediates PDGFR-β association with α5β1 integrin in focal adhesions and regulates cell migration (Veevers-Lowe et al., 2011). HGF-mediated c-Met activation induces collective cancer cell invasion through β1 integrin trafficking (Mai et al., 2014). All these data suggest that β1 integrins and GFR share the same signaling pathways to modulate migration of cancer cells. In human colorectal cancer, downregulation of the aryl hydrocarbon receptor nuclear translocator (ARNT or HIF-1β) promoted cancer cell migration and invasion through the activation of the fibronectin/β1 integrin/FAK signaling axis. Chemotherapeutic drugs inhibited ARNT expression and promoted invasion of residual tumor cells (Huang et al., 2015). In head and neck squamous cell carcinoma, disappearance of caveolin-1 expression in primary tumors is predictive of high risk of metastasis and is of bad prognosis. α2β1 and α5β1 integrins, both of which are regulated by caveolin-1, are responsible for the acquisition of motile, invasive, evasive and metastatic traits of tumors (Jung et al., 2015). MiR targeting of integrins represents a new way to endogenously regulate their expression. By targeting directly kindlin-2, miR-200b silenced the kindling-2/β1 integrin/AKT regulatory axis that ultimately suppresses the invasiveness of esophageal squamous cancer cells. miR-25 acts as a tumor suppressor in prostate cancer by direct functional interaction with the 3'UTR regions of proinvasive αv and α6 integrins (Zhang et al., 2015a; Zoni et al., 2015).

The tumor cell dissemination to a particular metastatic niche is dependent on the integrin repertoire expressed at the surface of cancer cells, blood and lymph compartment, vasculature, stromal cells as well as the composition and organization of ECM. For instance, a hepatic microenvironment favors the expression of α2β1 and α5β1 integrins on colorectal cancer cells which prompted colorectal cancer metastases to settle in the liver (Pelillo et al., 2015). αvβ3, α2β1 and α4β1 integrins play a key role in bone metastasis as their ligands are normally expressed by the bone-associated cells (Esposito and Kang, 2014). Knock-out mice for α11β1 integrin, a stromal cell-specific receptor for fibrillar collagen overexpressed by carcinoma-associated fibroblasts (CAF), prevent the metastatic potential of lung adenocarcinoma cells to bone, kidney, or brain (Navab et al., 2015). Lymph node metastasis (LNM) is recognized in clinical medicine as of bad prognosis for HNSCC patient. α2β1, α3β1, α6β1 integrins were identified as specific receptors that mediate the interactions between tumor cells and laminin present in the lymphatic environment (Fennewald et al., 2012; Soares et al., 2015).

INTEGRINS AND NEOANGIOGENESIS

The role of integrins in developmental and pathological angiogenesis has been largely described (Avraamides et al., 2008). As a leader, the αvβ3/β5 integrin was long considered as a primordial player in tumoral neo-angiogenesis and its specific antagonist cilengitide was the first to reach clinical trials as an anti-angiogenic compound (Stupp et al., 2010). Unfortunately, cilengitide failed to improve the overall survival of glioblastoma patients in a multicentric randomized phase III clinical trial (Stupp et al., 2014). The need to understand the fine molecular events supporting integrin biology and functions appears currently as a priority in the field (Atkinson et al., 2014). Recent data indicate that dosage and timing of αvβ3 integrin antagonism are critical to pro- or anti-angiogenesis effects (Robinson et al., 2009; Steri et al., 2014). Hence, proof of principle that low doses of cilengitide, which were shown to promote angiogenesis, may be used in a "vascular promotion therapy," opens a new field in integrin

antagonist usefulness (Wong et al., 2015). Integrin α5β1 was also highlighted as a pro-angiogenic driver with an increased expression in neo-angiogenic tumoral vessels (Schaffner et al., 2013). However, recent data using KO mice models challenged the implication of α5, αv and their matrix ligand fibronectin in the tumor angiogenesis (Murphy et al., 2015). Discrepancies observed between the effects of the gene deletions and those of integrin-matrix adhesion blocking compounds on angiogenesis led to the interesting hypothesis that the latter may induce some anti-angiogenic function in the integrins. A better understanding of the integrin signaling pathways will help to understand their fine tuning in endothelial cells. Recent data explored the molecular regulation of angiogenesis through β1 integrin activation/inhibition and revealed cross-talks between angiopoietin-2, Arf6, VE-cadherin or MAP4K4 and β1 pathway (Hakanpaa et al., 2015; Hongu et al., 2015; Vitorino et al., 2015; Yamamoto et al., 2015).

Integrins also participate to anti-angiogenic therapy resistance. One of the most studied anti-angiogenic therapy is Bevacizumab, a monoclonal antibody against VEGF-A. Addition of Bevacizumab to adjuvant therapies in multiple cancer types improved progression free survival of patients (Ahmadizar et al., 2015). In brain tumors, anti-VEGF therapy led to bevacizumab-resistant recurrent glioblastoma with two different phenotypes, one of which appeared as infiltrative and the other as proliferative (de Groot et al., 2010; DeLay et al., 2012). Interestingly, the former expressed more α5β1 integrin and fibronectine. β1 integrin targeting was shown to disrupt the resistance toward Bevacizumab (Carbonell et al., 2013; Jahangiri et al., 2014).

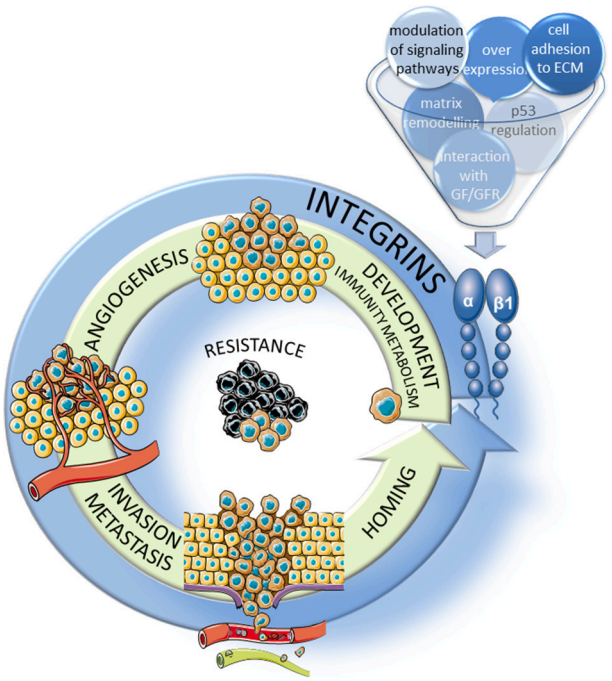

FIGURE 1 | Implication of β1 integrins in the hallmarks of cancer. β1 integrins participate, through several mechanisms, to the major steps of tumor progression including the development of the tumor and of new vessels, migration/invasion into the surrounding stroma and extravasion through neoangiogenic vessels and homing in new tissues to form metastasis. In addition, these integrins participate largely to the resistance to therapies.

INTEGRINS AND REPROGRAMMING OF ENERGY METABOLISM AND THE EVASION OF IMMUNE DESTRUCTION

Unlike normal cells, tumor cells use aerobic glycolysis (the Warburg effect) rather than oxidative phosphorylation (OXPHOS) to generate energy. This reprogramming of glucose metabolism is promoted by Twist through a β1-integrin/FAK/PI3K/AKT/mTOR pathway (Yang et al., 2015). Interestingly, it was shown recently that aerobic glycolysis or OXPHOS deregulation may enhance cancer cell migration and invasion through modulation of β1 integrin pathway (Yang et al., 2014; Nunes et al., 2015). Concerning the immune system, αv integrin upregulation can promote ADCC (antibody-dependent-cell-mediated cytotoxicity) but also link drug resistance with immune evasion (Jinushi et al., 2012; Anikeeva et al., 2014). Local immune response can be abrogated by tenascin C/α5β1 integrin to promote metastasis (Jachetti et al., 2015). A phenomenom named "integrin transregulation" can enhance tumor immunity through an increase in T-cell entry into melanomas (Cantor et al., 2015). Innate immune cells can promote tumor metastasis in dedicated environment. Interestingly, it was proposed that immune cell-derived microparticles may transfer αMβ2 integrin to tumor cells leading to their migration *in vitro* and metastasis *in vivo* (Ma et al., 2013). This recent literature suggests that an exponential growth of data will be available in the future characterizing the roles of integrins in these two new hallmarks of cancer.

CONCLUSIONS

The goal of this review was to give a brief and non-exhaustive overview of the most recent data about the implication of β1 integrins in different hallmarks of cancer (**Figure 1**). Examples given here stress the complexity of the integrin signaling pathways which will largely depend on the tumor context under consideration. Micro environmental cues as well as molecular features of the tumoral cells themselves will determine which integrin(s) may be preferentially targeted. Increasing knowledge on how the integrin expression and functions are modulated is mandatory to propose associated therapies more susceptible to eradicate tumors.

AUTHOR CONTRIBUTIONS

MD planned and edited the manuscript; AB, GR, SM, IL, and ML made the experiments leading to the laboratory publications cited in the review; AB, GR, ML, IL, SM, and MD wrote the manuscript.

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Discoidin Domain Receptors: Potential Actors and Targets in Cancer

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The extracellular matrix critically controls cancer cell behavior by inducing several signaling pathways through cell membrane receptors. Besides conferring structural properties to tissues around the tumor, the extracellular matrix is able to regulate cell proliferation, survival, migration, and invasion. Among these receptors, the integrins family constitutes a major class of receptors that mediate cell interactions with extracellular matrix components. Twenty years ago, a new class of extracellular matrix receptors has been discovered. These tyrosine kinase receptors are the two discoidin domain receptors DDR1 and DDR2. DDR1 was first identified in the *Dictyostelium discoideum* and was shown to mediate cell aggregation. DDR2 shares highly conserved sequences with DDR1. Both receptors are activated upon binding to collagen, one of the most abundant proteins in extracellular matrix. While DDR2 can only be activated by fibrillar collagen, particularly types I and III, DDR1 is mostly activated by type I and IV collagens. In contrast with classical growth factor tyrosine kinase receptors which display a rapid and transient activation, DDR1 and DDR2 are unique in that they exhibit delayed and sustained receptor phosphorylation upon binding to collagen. Recent studies have reported differential expression and mutations of DDR1 and DDR2 in several cancer types and indicate clearly that these receptors have to be taken into account as new players in the different aspects of tumor progression, from non-malignant to highly malignant and invasive stages. This review will discuss the current knowledge on the role of DDR1 and DDR2 in malignant transformation, cell proliferation, epithelial to mesenchymal transition, migratory, and invasive processes, and finally the modulation of the response to chemotherapy. These new insights suggest that DDR1 and DDR2 are new potential targets in cancer therapy.

Keywords: discoidin domain receptors, tyrosine kinase, extracellular matrix, collagen, cell signaling, cancer, targeted therapy

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INTRODUCTION

While advances in treatment have increased the survival rate for many cancers, it is still one of the leading causes of death in the world, particularly in developing countries. Cancer represents a tremendous burden on patients, families and societies. Yet, it is generally accepted that cancer risk actually depends on a combination of genes aspects, environment and lifestyle. After surgery,

radiation therapy (RT) has long been an integral component of cancer care. It is usually employed to locally eradicate tumor cells as well as alter tumor stroma with either curative or palliative intent. Despite many improvements (image guided radiotherapy, intensity modulated radiotherapy, etc.), RT often fails to provide local tumor control, and delivering higher doses of radiation alone is unlikely to solve this problem (Higgins et al., 2015). In addition, neither surgery nor radiotherapy could control the metastatic spread of tumor. Therefore, current efforts have been focusing on understanding the molecular, cellular, and systemic processes driving cancer initiation, progression, heterogeneity, and metastatic spread.

The extracellular matrix (ECM) critically controls cancer cell behavior by inducing several signaling pathways. Besides conferring structural properties to tissues, ECM is able to regulate cell proliferation, survival, migration, and invasion (Lu et al., 2012). As a major part of the tumor ECM, type I collagen exhibits high density and altered architecture in malignant cancer and is causally linked to tumor formation and metastasis (Provenzano et al., 2006, 2008). Until recently, these effects on tumor cells were exclusively attributed to integrins; a major class of receptors that mediate cell interactions with extracellular matrix components. The identification of the Discoidin Domain Receptor (DDR) family as collagen receptors represents a new paradigm in the regulation of collagen-cell interactions and regulation of tumor progression.

DDR1 and DDR2 were initially discovered by homology cloning based on their catalytic kinase domains and were orphan receptors until 1997 when Shrivastava and co-workers, and Vogel and co-workers, reported that different types of collagen are functional ligands for these receptors (Shrivastava et al., 1997; Vogel et al., 1997). Indeed, DDRs belong to the large family of receptor tyrosine kinases based on the presence of a catalytic kinase domain with a distinct extracellular Discoidin (DS) homology domain (Johnson et al., 1993; Alves et al., 1995; Perez et al., 1996). DDR1 was first identified in the *Dictyostelium discoideum* and was shown to mediate cell aggregation (Breuer and Siu, 1981; Springer et al., 1984). DDR2 shares highly conserved sequences with DDR1 (Carafoli et al., 2009). Both receptors are activated upon binding to collagen. DDR1 is activated by various types of collagen including type I, IV, V, VI, and VIII, whereas DDR2 is only activated by fibrillar collagens, in particular collagens type I, III, and type X (Shrivastava et al., 1997; Vogel et al., 1997; Leitinger and Kwan, 2006). In contrast with classical growth factor tyrosine kinase receptors such as the epithelial growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) which display a rapid and transient activation (Dengjel et al., 2007), DDR1 and DDR2 are unique in that they exhibit remarkably delayed and sustained receptor phosphorylation upon binding to collagen (Vogel et al., 1997). Furthermore, many classical tyrosine kinase receptors (RTKs) undergo negative regulation such as receptor/ligand internalization and subsequent degradation or dephosphorylation by phosphatases (Avraham and Yarden, 2011). In the case of DDRs, phosphorylation levels may persist up to 18 h (Vogel et al., 1997).

Both DDRs are expressed early during embryonic development as demonstrated in many *in vivo* studies (Valiathan et al., 2012). Indeed mice lacking DDR1 or DDR2 exhibit major defects in skeletal development (Bargal et al., 2009), reproduction (Matsumura et al., 2009; Kano et al., 2010), inflammation (Olaso et al., 2011), and cardiovascular system (Franco et al., 2010). In addition, they are uniquely positioned to function as sensors for ECM and to regulate a wide range of cell functions such as migration, cell proliferation, cytokine secretion, and ECM homeostasis/remodeling (Valiathan et al., 2012). While activation of DDRs is required for normal development, studies have reported differential expression and mutations of DDR1 and DDR2 in several cancers (Valiathan et al., 2012). In malignant transformation, cell proliferation, epithelial to mesenchymal transition, migration, and invasive processes, the role of DDRs in different aspects of tumor progression will be highlighted. We further discuss recent studies on DDRs as a therapeutic and potential target in cancer but also its role in the modulation of the response to chemotherapy. Hopefully, these useful updates will encourage more research on DDRs in cancer and the possibility to better identify them as promising targets for future therapies.

STRUCTURE, FUNCTION AND REGULATION OF DDRs (FIGURE 1)

Structurally, DDRs are characterized by 4 different domains: an extracellular region composed of an N-terminal DS domain and a DS-like domain which binds to collagen. DDR1 and DDR2 share high degree of sequence identity in DS and DS-like domains with 59 and 51% of similarity, respectively (Carafoli and Hohenester, 2013). The juxtamembrane (JM) domain is composed of extracellular JM regions of about 50 amino acids for DDR1 and 30 for DDR2 followed by large cytosolic JM regions of about 171 amino acids for DDR1, depending on the protein isoform, and 142 for DDR2 (Leitinger, 2011; Carafoli and Hohenester, 2013). Finally, the catalytic tyrosine kinase domain (KD) is composed of 300 amino acids, undergoes phosphorylation and activates downstream signaling. This KD is ended by a short C-terminal peptide of about 8 amino acids for DDR1 and 6 amino acids for DDR2 (Carafoli and Hohenester, 2013). For detailed structural studies, readers are invited to refer to the cited references (Carafoli et al., 2009, 2012; Fu et al., 2013; Li et al., 2015).

Unlike DDR2, five isoforms of DDR1 (DDR1a, b, c, d, and e) have been described as they exhibit differences in the extent of glycosylation (Vogel, 1999), phosphorylation (Vogel et al., 2006; Carafoli and Hohenester, 2013), protein interactions (Matsuyama et al., 2003), expression patterns, and functions (Vogel et al., 2006). DDR1a, b, and c are kinase-active, whereas DDR1d and e are kinase-deficient because of frame shift and truncation (Alves et al., 2001). While the longest isoform (DDR1c) is composed of 919 amino acids, DDR1a and DDR1b, the most abundant isoforms, lack 37 amino acids in the JM domain or 6 amino acids in the KD. DDR1d and DDR1e isoforms are C-terminally truncated receptors. DDR1d lacks exons 11 and

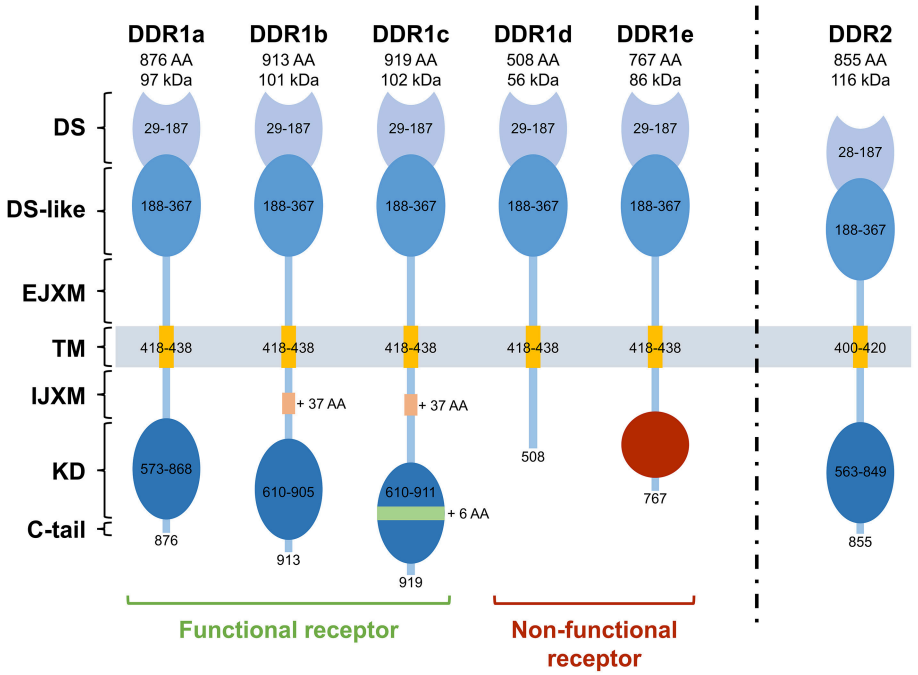

FIGURE 1 | Structure of the different Discoidin Domain Receptors. DDR1a, DDR1b, DDR1c, and DDR2 are enzymatic active receptors, and DDR1d and DDR1e are inactive kinase-deficient receptors. DS, discoidin domain; DS-like, discoidin-like domain; EJXM, extracellular juxtamembrane region; TM, transmembrane segment; IJXM, intracellular juxtamembrane region; KD, kinase domain; AA, Amino Acid.

12 causing a frame-shift mutation that generates a stop codon and premature termination of transcription, whereas DDR1e lacks exons 11 and 12 as well as the first half of exon 10 (Alves et al., 1995, 2001). In 2006, a sixth isoform that lacks a part of the extracellular domain has been described in the postmeiotic germ cells of the rat testis (Mullenbach et al., 2006).

DDRs control important aspects of cell behavior including proliferation, migration, adhesion, and ECM remodeling but are dysregulated in various human diseases. They are both activated by several types of collagen. However, this activation strictly requires collagen to be in its native and triple-helical conformation. Heat-denatured collagen is not recognized by DDRs (Leitinger and Kwan, 2006; Dengjel et al., 2007). Both receptors are commonly activated by various types of collagen (mainly type I) but distinctly activated by type IV for DDR1 and type X for DDR2 (Dengjel et al., 2007; Avraham and Yarden, 2011). Surprisingly, the substitution of five peripheral amino acids in DDR2 with their DDR1 counterparts converts DDR2 into a receptor of type IV collagen (Xu et al., 2011). Another intriguing feature of DDRs is their unusually slow autophosphorylation upon stimulation by the ligand compared with typical RTKs (hours rather than seconds; Dengjel et al., 2007). Furthermore, DDRs dimerization is essential for collagen recognition unlike other RTKs in which ligand binding leads to receptor dimerization (Leitinger, 2003). By contrast to DDR2 which binds to several collagen peptides (Farndale et al., 2008), DDR1 binding is restricted to the GVMGFO motif (Gu et al., 2011). This collagen binding site of DDRs is highly conserved, 11 of the 13 amino acids identified by nuclear magnetic resonance (NMR) by Ichikawa and co-workers are identical between DDR1 and DDR2 (Ichikawa et al., 2007).

Upon collagen binding, specific tyrosines residues present in the activation loop of DDRs tyrosine kinase domain are phosphorylated. Phosphorylation of these tyrosine residues leads to the binding of a number of different Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing proteins (Carafoli and Hohenester, 2013). Moreover, the activated KD of DDRs is believed to autophosphorylate several tyrosines in the JM region, which serve as docking sites for several adaptor proteins such as SH2 domain containing transforming protein 1 (Shc1) (Ikeda et al., 2002), cytoplasmic protein Nck2 (Koo et al., 2006), protein tyrosine phosphatase SHP-2 (Wang et al., 2006), cell division control protein 42 (Cdc42) (Yeh et al., 2009), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Das et al., 2006), extracellular signal-regulated kinase mitogen-activated protein kinase (ERK1/2-MAPK) (Su et al., 2009), activator protein (AP)-1 (Su et al., 2009), and members of the signal transducers and activators of transcription (STAT) family of transcription factors (Wang et al., 2006). Defining DDR signaling pathways has always been a challenging task. Indeed, DDRs bind to multiple collagens and both exhibit unique and common structural and activation properties, but phosphorylate different target receptors (Ongusaha et al., 2003). In addition, DDRs may act in concert with other signaling receptors, including the Wnt5a/Frizzled (Dejmek et al., 2003) and Notch1 (Kim et al., 2011) receptors in the case of DDR1 and the insulin receptor (Iwai et al., 2013a) in the case of DDR2. Finally, DDRs signaling is cell/tissue type-specific and

context-dependent. For example, DDR1 inhibits cell migration in Madin-Darby canine kidney (MDCK) cells (Wang et al., 2006) whereas, in other cellular systems, DDR1 and DDR2 promote cell migration and/or invasion (Yoshida and Teramoto, 2007).

DDRs IMPLICATION IN CANCER (TABLES 1, 2)

DDRs have been linked to tumor progression in several human cancers. In fact, several studies have shown that the expression and activation of DDRs are often dysregulated in such diseases (Valiathan et al., 2012). In addition, somatic mutations of DDRs genes have been found in various cancers (Ford et al., 2007). In the case of DDR2, these mutations are present in 3–4% of patients with lung squamous cell cancer (Hammerman et al., 2011) and have been reported in other cancers at comparable frequencies including lung adenocarcinoma, cervical carcinoma, gastric carcinoma, bladder carcinoma, melanoma, colorectal cancer, head, and neck cancer (Beauchamp et al., 2014). Mutation known as I638F, has been shown to promote resistance to inhibitors of DDR2 kinase function (Hammerman et al., 2011; **Figure 2**). Nevertheless, the picture is still complicated by the fact that DDRs can act also as anti-tumorigenic receptors and their effect is highly dependent on the type of cancer and the nature of the microenvironment. In the following parts, we will try to explore recent data on the role of DDR1 and DDR2 in malignant transformation, cell proliferation, epithelial to mesenchymal transition, migratory, and invasive processes.

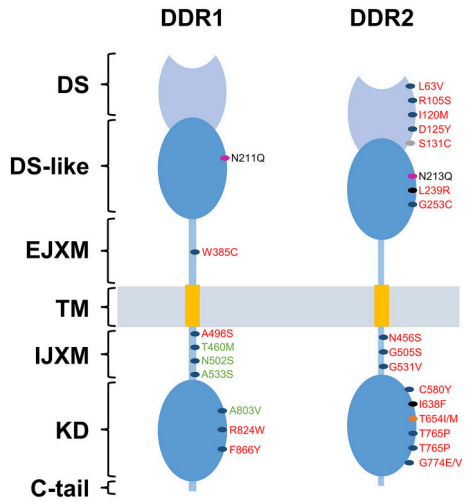

FIGURE 2 | DDRs reported mutations in cancer. Domain distribution of somatic mutations of mutated DDR1 and DDR2 identified in lung cancer (red) and Acute Myeloid Leukemia (green) samples. Blue spots represent mutation with undefined role. Gray spot represent a mutation that promotes tumor cell proliferation and invasion. Pink spots represent a mutation that activates constitutively DDRs. Black spots represent mutations responsible for an increased sensitivity to dasatinib. Orange spot represents the "gatekeeper mutation," responsible for the resistance of tumor cells to dasatinib. DS, discoidin domain; DS-like, discoidin-like domain; EJXM, extracellular juxtamembrane region; TM, transmembrane segment; IJXM, intracellular juxtamembrane region; KD, kinase domain.

CELL PROLIFERATION AND SURVIVAL

The emerging role of DDRs in tumor *cell survival/proliferation* and their crosstalk with oncogenic signaling was previously evaluated through *in vitro* and/or *in vivo* DDRs silencing strategies. Both DDR1 and DDR2 can exhibit pro- (Ongusaha et al., 2003; Das et al., 2006; Yamanaka et al., 2006; Hammerman et al., 2011; Kim et al., 2011, 2014; Cader et al., 2013; Han et al., 2014; Rudra-Ganguly et al., 2014) and anti- (Wall et al., 2005, 2007; Assent et al., 2015) proliferative activities in a cell type and context-dependent manner. Targeting DDR1 with siRNA in glioma (Yamanaka et al., 2006) and pancreatic adenocarcinoma cell lines (Rudra-Ganguly et al., 2014) inhibited tumor cell proliferation *in vitro* and impaired subcutaneous xenograft tumor growth in mice. Upregulation of transforming growth factor beta 1 (TGFβ1), following DDR1 silencing, is thought to induce tumor cell growth arrest (Rudra-Ganguly et al., 2014). Furthermore, inhibition of DDR1 in human colon carcinoma cells (Ongusaha et al., 2003), breast cancer cell lines (Ongusaha et al., 2003; Das et al., 2006) and collagen treated Hodgkin lymphoma cells (Cader et al., 2013) resulted in an increase in cell death in response to induced DNA damage (Ongusaha et al., 2003; Das et al., 2006; Cader et al., 2013). These data suggest that DDR1 expression in tumor cells can confer resistance to chemotherapeutic drugs. This resistance is thought to be due to an activation of NFκB and its downstream effectors, including cyclooxygenase-2 (COX-2) which plays a role in resistance to chemotherapy-induced apoptosis (Cao and Prescott, 2002; Das et al., 2006) or by counteracting p53 mediated apoptosis (Ongusaha et al., 2003). Consistently, studies in human HCT116 colon carcinoma cells showed that DDR1, in response to collagen-induced activation, promotes cell survival in a Notch signaling manner (Kim et al., 2011).

In the case of DDR2, mutations of the receptor were shown to promote cell growth in NIH3T3 mouse embryonic fibroblast cells [(Hammerman et al., 2011) section Supplementary Data, Figure S3-a]. An upregulation of DDR2 followed by an increase in cell proliferation and survival is thought to be induced by an overexpression of COX-2 in U2OS human osteosarcoma cells (Han et al., 2014). In H1299 cells, inhibition of DDR2 activity by overexpressing the juxtamembrane domain containing JM2 suppressed collagen-induced colony formation and cell proliferation. JM2-mediated DDR2 dimerization is likely to be essential for activation of the receptor and cell proliferation. Thus, inhibition of DDR2 function using a JM2-containing peptide may be a useful strategy for the treatment of DDR2-positive cancers (Kim et al., 2014).

In contrast to the above studies, DDR1 and DDR2 can also act as cell growth inhibitors. Indeed, DDR2 has been reported to induce an inhibitory effect on proliferation of human melanoma and fibrosarcoma cells, once cultured on fibrillar collagen, with a growth arrest in the G0/G1 phase of the cell cycle. This process was shown to be induced through $p15^{INK4b}$ cyclin-dependent kinase inhibitor, raising the question whether $p15^{INK4b}$ could be a downstream target of DDR2 signaling (Henriet et al.,

TABLE 1 | Non-exhaustive list of reported DDRs *in vitro* functions in various aspects of cancer progression.

		DDR1 in cancer cells	DDR2 in cancer cells
Positive regulator	Proliferation /survival	- Human glioma: U251, GI-1 and T98G (Yamanaka et al., 2006) - Human pancreatic adenocarcinoma: BXPC3 (Rudra-Ganguly et al., 2014) - Human colon carcinoma: HCT116 (Ongusaha et al., 2003; Kim et al., 2011) - Human osteosarcoma: Saos2 (Ongusaha et al., 2003) - Human breast cancer: MCF-7 (Ongusaha et al., 2003), MDA-MB-435 and T47D (Das et al., 2006) - Human Hodgkin lymphoma: L428 (Cader et al., 2013).	- Human squamous cell lung cancer: H2286 and HCC366 (Hammerman et al., 2011) - Human osteosarcoma: U2OS (Han et al., 2014) - Human squamous cell lung cancer: H1299 (Kim et al., 2014) - Human melanoma: A375 (Badiola et al., 2011) - Human hepatoma: SKHEP (Badiola et al., 2011) - Human colon carcinoma: HT-29 (Badiola et al., 2011).
	EMT	- Human hepatoma: HAK-1A and HAK-1B (Maeyama et al., 2008) - Human non-small cell lung carcinoma: A549 (Walsh et al., 2011) - Human colorectal cancer: LOVE1 and LOVO (Hu et al., 2014) - Human pancreatic adenocarcinoma: BxPC3 (Shintani et al., 2008).	- Human lung carcinoma: A549 (Walsh et al., 2011) - Human breast cancer: MDA-MB-231 (Zhang et al., 2013; Ren et al., 2014), MCF-7 and MDA-MB-468 (Ren et al., 2014).
	Migration	- Human glioma: G140 (Ram et al., 2006) - Human hepatocellular carcinoma: HLE and Huh-7 (Park et al., 2007) - Human non-small cell lung carcinoma: A549 and H358 (Yang et al., 2010) - Human pancreatic cancer: BxPC3 (Rudra-Ganguly et al., 2014) - Human colorectal cancer: LOVE1 and LOVO (Hu et al., 2014) - Human breast cancer: MCF-7 (Huang et al., 2009), MDA-MB-231 (Castro-Sanchez et al., 2010), MDA-MB-468 and T47D (Neuhaus et al., 2011).	- Human melanoma: A375 (Badiola et al., 2011) - Human hepatoma: SKHEP (Badiola et al., 2011) - Human colon carcinoma: HT-29 (Badiola et al., 2011) - Human prostate cancer: PC-3 (Yan et al., 2014) - Human lung carcinoma: A549 (Walsh et al., 2011) - Human nasopharyngeal carcinoma isolated cells (Chua et al., 2008) - Murine melanoma: B16BL6 (Poudel et al., 2015).
	Invasion	- Human glioma: G140 (Ram et al., 2006) - Human hepatocellular carcinoma: HLE and Huh-7 (Park et al., 2007) - Human oral squamous cell carcinoma: A431 (Hidalgo-Carcedo et al., 2011) - Human colorectal cancer: LOVE1 and LOVO (Hu et al., 2014) - Human non-small cell lung carcinoma: A549 (Yang et al., 2010; Miao et al., 2013; Juin et al., 2014) and H358 (Yang et al., 2010) - Human hepatoblastoma: Huh6 (Juin et al., 2014) - Human breast cancer: MDA-MB-231 (Castro-Sanchez et al., 2011; Juin et al., 2014) - Human prostate cancer: PC-3 (Shimada et al., 2008) - Human pituitary adenoma: HP-75 (Yoshida and Teramoto, 2007).	- Human prostate cancer: LNCaP and PC-3 (Yan et al., 2014) - Human squamous cell lung cancer: H1299 (Kim et al., 2014) - Human breast cancer: MDA-MB-231 (Zhang et al., 2013) - Murine melanoma: B16BL6 (Poudel et al., 2015).
Negative regulator	Proliferation /survival	- Human breast cancer: MCF-7 and ZR-75-1 (Maquoi et al., 2012; Assent et al., 2015).	- Human melanoma: A2058 (Wall et al., 2005) and M24met (Henriet et al., 2000; Wall et al., 2005, 2007) - Human fibrosarcoma: HT-1080 (Wall et al., 2005) - Human squamous cell lung cancer: (Iwai et al., 2013b; Miao et al., 2014).
	EMT	- Human breast cancer: Hs578T, MCF-7 and MDA-MB-231 (Koh et al., 2015).	NR
	Migration	- Human breast cancer: MCF-7 (Hansen et al., 2006), MDA-MB-231 (Hansen et al., 2006; Koh et al., 2015) and Hs578T (Koh et al., 2015).	- Murine colon carcinoma: MCA38 (Badiola et al., 2012).
	Invasion	NR	NR

DDR, Discoidin domain receptor; EMT, Epithelial mesenchymal transition; NR, not reported.

TABLE 2 | Insights into DDRs contribution in cancer from *in vivo* studies.

	Cancer cell type	*In-vivo* model	Reported Results	References
DDR1	Human pancreatic adenocarcinoma	BXPC3 mouse tumor xenografts	shRNA-DDR1 silencing reduced the growth of tumor xenografts (∼50% reduction compared to control)	Rudra-Ganguly et al., 2014
	Human colon carcinoma	HCT116 mouse tumor xenografts	shRNA-DDR1 silencing reduced the growth of tumor xenografts (∼30% reduction compared to control)	Kim et al., 2011
	Human breast cancer	Hs587T and MDA-MB-231 cells seeded on upper layer of Chorioallantoic membrane (CAM)	DDR1 overexpression in cells, induced a decreased invasion after 48 h of incubation	Koh et al., 2015
	Human prostate cancer	Androgen independent-LNCaP and LNCaP prostate seeded on CAM	siRNA-DDR1 silencing in cells, induced a decreased invasion after 72 h of incubation	Shimada et al., 2008
DDR2	Human squamous cell lung cancer (SCC)	NCI-H1703, NCI-H2286 or A549 cells athymic nude mouse xenografts	Dasatinib inhibited the proliferation of DDR2-mutated SCC cell lines (NCI-H1703, NCI-H2286 but not A549) in xenograft studies	Hammerman et al., 2011
	Human melanoma	Intrasplenic inoculation of A375R2-70 and A37R2-40 cells in C57BL/6J-Hfn11 nude mice	siRNA-DDR2 silencing in A375R2-70 and -40 reduced experimental liver metastasis development, by 60 and 75%, respectively	Badiola et al., 2011
	Mouse breast cancer	4T1-Luc/GFP cells implantation into the breast tissue of syngeneic Balb/cJ mice	DDR2 depletion led to a reduced metastatic capacity of 4T1-Luc cells	Zhang et al., 2013
	Human breast cancer	MDA-MB-231-luc-D3H2LN cells transplantation into nude mice mammary fat pads	shRNA-DDR2 silencing improved mice lifespan and attenuated cells invasive capacity even 7 weeks after transplantation	Ren et al., 2014
	Human prostate cancer	PC-3 cells intrabone injection in mice metastasis model	DDR2 depletion alleviated PC-3 cells induced osteolytic lesions, signature of bone destruction	Yan et al., 2014
	Murine colon carcinoma	Intrasplenic MCA38 cells injection into DDR2-deficient mice	Increase in cancer cells hepatic colonization efficiency (hepatic occupied volume and number of metastatic foci per area unit)	Badiola et al., 2012

shRNA, Short hairpin ribonucleic acid; siRNA, small interfering ribonucleic acid.

2000; Wall et al., 2005, 2007). SHP-2 has been shown to be a key downstream component of DDR2 signaling. Indeed, Iwaï and co-workers demonstrated that the mutation I638F in the kinase domain of DDR2, leads to an inhibition of SHP-2 phosphorylation and a loss of its cell growth suppression effect, whereas mutations L63V in the discoidin domain and G505S in the intracellular juxtamembrane region don't have any effect on SHP-2 phosphorylation (Iwai et al., 2013b). In addition, the mutation S131C in the DS domain of DDR2 was able to increase squamous cell lung cancer (SCC) proliferation *in vitro* and *in vivo* (Miao et al., 2014; **Figure 2**). Related to DDR1, it has been identified as a key sensor that monitors the cellular microenvironment and triggers apoptosis through the induction of the pro-apoptotic Bcl-2-interacting killer protein (BIK) in luminal breast cancer cells within a collagen three dimensional culture system (Maquoi et al., 2012; Assent et al., 2015).

EPITHELIAL/MESENCHYMAL TRANSITION

The epithelial to mesenchymal transition (EMT) plays crucial role in the differentiation of multiple tissues and organs. EMT also contributes to tissue repair, but it can adversely cause organ fibrosis and promote tumor progression through a variety of mechanisms. EMT is characterized by an increase in cell motility and invasiveness, induction and maintenance of stem cell properties, prevention of apoptosis, senescence, and resistance to therapy (Thiery et al., 2009). Tumor cells that undergo EMT are found to express less epithelial markers such as E-cadherin (Maeyama et al., 2008; Walsh et al., 2011; Zhang et al., 2013; Hu et al., 2014; Ren et al., 2014; Koh et al., 2015) and cytokeratins (Maeyama et al., 2008) but express more mesenchymal markers such as vimentin (Maeyama et al., 2008; Walsh et al., 2011; Hu et al., 2014; Ren et al., 2014; Koh et al., 2015) and N-cadherin (Shintani et al., 2008; Hu et al., 2014), with a possible switch in DDR expression from DDR1 (epithelial) to DDR2 (mesenchymal). These reports have shown that induction of an EMT phenotype results in transcriptional downregulation of DDR1 and that a predominant DDR2 expression reflects a result of EMT process toward more malignant cells (Maeyama et al., 2008; Toy et al., 2015). In addition, the newly expressed DDR2, in several cell lines of human cancer such as liver (HAK-1A and HAK-1B cells) (Maeyama et al., 2008), lung (A549 cells) (Walsh et al., 2011), and breast (MDA-MB-231, MCF-7, SK-BR3, and

TABLE 3 | An update on DDRs inhibitors: compound name, type and reported half maximal inhibitory concentration.

Compound	Half maximal inhibitory concentration (IC_{50}) nM		Inhibitor type
	DDR1	DDR2	
Dasatinib (Day et al., 2008)	0.5 ± 0.2 nM	1.4 ± 0.3 nM	Kinase type I inhibitor
Nilotinib (Day et al., 2008)	43 ± 3 nM	55 ± 9 nM	Kinase type II inhibitor
Imatinib (Day et al., 2008)	337 ± 56 nM	675 ± 127 nM	Kinase type II inhibitor
Ponatinib (Canning et al., 2014)	9 nM	9 nM	Kinase type II inhibitor
Actinomycin D (Siddiqui et al., 2009)	NR	9000 nM	Antibiotic
LCB 03-0110 (Sun et al., 2012)	164 nM	171 nM	Thienopyridine derivative
7rh (Gao et al., 2013)	6.8 nM	101.4 nM	3-(2-(pyrazolo[1,5-a]pyrimidin-6-yl)-ethynyl)benzamides derivative
7rj (Gao et al., 2013)	7 nM	93.6 nM	3-(2-(pyrazolo[1,5-a]pyrimidin-6-yl)-ethynyl)benzamides derivative
2a (Richters et al., 2014)	68 nM	65 nM	Pyrazolo-urea containing compound
4a (Richters et al., 2014)	235 nM	75 nM	Pyrazolo-urea containing compound
4b (Richters et al., 2014)	39 nM	18 nM	Pyrazolo-urea containing compound
DDR1-IN-1 (Kim et al., 2013)	105 nM	413 nM	Kinase type II inhibitor
DDR1-IN-2 (Kim et al., 2013)	47 nM	145 nM	Kinase type II inhibitor

MDA-MB-468 cells) (Zhang et al., 2013; Ren et al., 2014), is phosphorylated upon interaction with type I collagen, suggesting that the induced receptor is physiologically active.

Studies in A549 lung carcinoma cells showed that inhibiting the expression of DDR2 with siRNA is sufficient to alter activity of the NF-κB and the lymphoid enhancer-binding factor 1 (LEF-1) transcription factors and to inhibit EMT and cell migration induced by TGF-β1 (Walsh et al., 2011). While in breast cancer cells, Zhang and co-workers showed that activation of DDR2 regulates SNAIL1 protein stability by stimulating ERK2 activity, in a Src-dependent manner. Activated ERK2 directly phosphorylates SNAIL1, leading to SNAIL1 nuclear accumulation, a decrease in ubiquitination, and an increase in protein half-life. Thus, DDR2 maintains SNAIL1 protein level and its activity in tumor cells, facilitating cell invasion (Zhang et al., 2013). Lately, it has been showed that DDR2 expression and activation in breast cancer cells can be increased by hypoxia, which is well-known to participate in tumor metastatic events (Ren et al., 2014).

While these studies suggest that acquisition of a more mesenchymal-like phenotype is associated with expression of DDR2, other studies suggest that, depending on the cell type, both DDRs can promote EMT. DDR1 has been shown to interact with α2β1 integrin receptors and activate cell signaling pathways, which promote expression of mesenchymal markers (Shintani et al., 2008). Many studies have shown that microRNAs can also regulate the expression of various genes closely associated with invasion and metastasis in colorectal cancer (CRC) pathogenesis. Indeed, overexpressing miR-199a-5p leads to a decrease in the expression of DDR1, matrix metalloproteinase-2 (MMP-2), N-cadherin, and vimentin, and an increase in E-cadherin expression in both LOVE1 and LOVO CRC cell lines. However, down-regulation of miR-199a-5p resulted in the opposite effects (Hu et al., 2014). Zinc finger E-box-binding homeobox 1 (ZEB1) is a transcription factor that is overexpressed downstream of EMT inducers, and plays a critical role in mediating changes in gene expression during EMT, particularly for E-cadherin (Eger et al., 2005). Studies in triple-negative breast cancer cells revealed a novel H-Ras/ZEB1/DDR1 network that contributes to breast cancer progression in Ras-dependent hyperactive signaling. These data showed that oncogenic H-Ras signaling upregulates ZEB1, which in turn suppresses E-cadherin and DDR1, leading to EMT and invasion (Koh et al., 2015).

CELL MIGRATION

As mentioned above, both DDR1 and DDR2 can support EMT and contribute to the adaptation of cells to their new environment by activation, in addition to other receptors, of EMT-induced migration programs. Regulation of cell migration by DDR1 was reported in various cancer cell lines including glioma (Ram et al., 2006), hepatocarcinoma (Park et al., 2007), lung (Yang et al., 2010), pancreas (Rudra-Ganguly et al., 2014), colorectal (Hu et al., 2014), and breast (Hansen et al., 2006; Huang et al., 2009; Castro-Sanchez et al., 2010; Neuhaus et al., 2011) carcinoma. Nevertheless, this regulation is cell type and receptor isoform dependent. Therefore, conflicting reports attributed inhibitory (Hansen et al., 2006; Koh et al., 2015) as well as pro-migratory (Ram et al., 2006; Park et al., 2007; Huang et al., 2009; Castro-Sanchez et al., 2010; Yang et al., 2010; Neuhaus et al., 2011; Hu et al., 2014; Rudra-Ganguly et al., 2014) effects for DDR1 in cell migration. Overexpression of DDR1a (but not DDR1b) in glioma (Ram et al., 2006), hepatocellular carcinoma (Park et al., 2007) and non-small lung cancer cells (Yang et al., 2010) significantly promotes tumor cell motility. Although, the significance of the difference between the migration induced effects of DDR1a and DDR1b is unknown, structural differences and divergent signaling between DDR1a and DDR1b have been suggested (Park et al., 2007). As an essential soluble component of the ECM, TGF-β1 elicits numerous changes in cellular behavior but has a conflicting role in cancer progression. Studies on pancreatic cancer cells showed that the pro-migratory effect of DDR1, in these cells, appears to be in part mediated *via* TGF-β1 downregulation (Rudra-Ganguly et al., 2014). Stimulation of

MDA-MB-231 breast cancer cells with type IV collagen is able to induce cell migration through a DDR1 and CD9-dependent pathway (Castro-Sanchez et al., 2010). In MDA-MB-468 breast cancer cells, DDR1-dependent promotion of cell migration was shown to be induced through a regulation of the migration suppressor tyrosine-protein kinase (SYK) activity (Neuhaus et al., 2011). In MCF-7 cells, full-length DDR1 associated to myosin IIA facilitates the process of cell migration (Huang et al., 2009). DDR1 can also play a negative role in cell migration. Indeed, overexpression of DDR1 in Hs587T breast cancer cells reduced their *in vitro* migratory behavior in type I collagen three dimensional (3D) culture system (Koh et al., 2015). While in MDA-MB-231 breast cancer cells, DDR1 suppresses migration only when co-expressed with its interacting partners, the Dopamine and cAMP-regulated neuronal phosphoprotein-32 (DARPP-32) (Hansen et al., 2006).

DDR2, when activated by type I collagen, was shown to support the migration of human A375 and B16BL6 murine melanoma cells (Badiola et al., 2011; Poudel et al., 2015), SK-HEP hepatoma cells, HT-29 colon carcinoma cells (Badiola et al., 2011), PC-3 prostate cancer cells (Yan et al., 2014), A549 lung carcinoma cells (Walsh et al., 2011), and nasopharyngeal carcinoma cells (Chua et al., 2008). Badiola and co-workers showed, that the c-Jun N-terminal kinases (JNK) pathway is involved in DDR2 inducing cell migration in A375 human melanoma cells (Badiola et al., 2011). While in A549 lung cancer cells, DDR2 inhibition with siRNA was sufficient to inhibit cell migration induced by TGF-β1 (Walsh et al., 2011). Recently, it has been shown that DDR2 promotes migratory phenotype of B16BL6 murine melanoma cells through the regulation of ERK and NF-κB signaling pathways (Poudel et al., 2015). In a single report, DDR2 was shown to be a negative migration regulator. Indeed, culturing MCA38 colon carcinoma cells in presence of conditioned media from untreated DDR2$^{-/-}$ hepatic stellate cells (HSCs) and tumor-activated DDR2$^{-/-}$ HSCs was able to enhance the migration of MCA38 cells, respectively, by 60 and 90% (Badiola et al., 2012).

CELL INVASION

Tumor invasion is a complex process that requires ECM degradation and tissue remodeling. Indeed, this process requires the activation of multiple genes but depends also on the action of key molecules such as ECM-degrading proteases and ECM receptors. Among these receptors, DDR1 has been found to be highly expressed in invasive tumors indicating its critical role as a regulator of cell invasion and subsequent tumor metastasis (Valiathan et al., 2012). Moreover, accumulating evidence using Matrigel or type I collagen as matrix barriers suggests that DDR1 plays a promoting role in invasion of a variety of human cancers including glioma (Ram et al., 2006), hepatocellular (Park et al., 2007), squamous epidermoid (Hidalgo-Carcedo et al., 2011), colorectal (Hu et al., 2014), lung (Yang et al., 2010; Miao et al., 2013; Juin et al., 2014), prostate (Shimada et al., 2008), breast carcinomas (Castro-Sanchez et al., 2011; Juin et al., 2014). This has also been observed for pituitary adenoma (Yoshida and Teramoto, 2007). Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, degrade the basement membrane and ECM, facilitating cell migration, tumor invasion, and metastasis. Among MMPs, MMP-2, and MMP-9 are considered important in the malignant behavior of tumor cells (Shuman Moss et al., 2012). Several reports showed that DDR1 can function as an inducer of MMP-2 (Ram et al., 2006; Park et al., 2007; Yoshida and Teramoto, 2007; Castro-Sanchez et al., 2011; Hu et al., 2014; Juin et al., 2014), or/and MMP-9 (Park et al., 2007; Yoshida and Teramoto, 2007; Shimada et al., 2008; Yang et al., 2010; Castro-Sanchez et al., 2011; Miao et al., 2013) and thus, contribute to the matrix components degradation. Overexpression of the DDR1a but not DDR1b isoform confers an aggressive invasive behavior to glioma cells *in vitro* by increasing their ability to invade Matrigel or type I collagen. DDR1a activation by collagen leads to the conversion of pro-MMP-2 (72 kDa) into its active form (62 kDa) (Ram et al., 2006). Whereas, DDR1a and DDR1b overexpression resulted in an increase of MMP-2 and MMP-9 in hepatocellular carcinoma and non-small lung cancer cell lines, respectively (Park et al., 2007; Yang et al., 2010). Hu and co-workers showed that overexpression of DDR1 induces invasion in colon carcinoma through the up-regulation of MMP-2 (Hu et al., 2014). By contrast, DDR1 in pituitary adenoma cell line induced an increase in both MMP-2 and MMP-9 secretion (Yoshida and Teramoto, 2007). In 2011, Hidalgo-Carcedo and co-workers suggested that the ability of DDR1 to support collective cell invasion of human A431 oral squamous cell carcinoma cells does not require receptor signaling and is independent of its activation by collagen. In these cells, DDR1 through its interaction with the cell polarity regulators Par3 and Par6, induces a decrease in actomyosin contractility and thereby enables collective cancer cell invasion (Hidalgo-Carcedo et al., 2011). Prostate cancer antigen-1 (PCA-1) has been shown to contribute to prostate carcinoma cell invasion through DDR1 (Shimada et al., 2008). In MDA-MB-231 breast cancer cells, type IV collagen induces MMP-2 and MMP-9 secretion and invasion through a DDR1 and Src-dependent pathway (Castro-Sanchez et al., 2011). Moreover, MMP-2 and MMP-9 secretion required protein kinase C (PKC) activity and epidermal growth factor receptor (EGFR) activation (Castro-Sanchez et al., 2011). Frederic Saltel's team proposed that DDR1 could be a sensor used by MDA-MB-231 breast and A549 lung carcinoma cells to interact with fibrillar type I collagen, leading to the organization of linear invadosomes, *via* a Cdc42-Tuba pathway (Juin et al., 2014). Neither DDR1 kinase activity nor Src tyrosine kinase were required for the formation and activity of invadosomes (Juin et al., 2014).

DDR2 has been found to promote invasion in prostate (Yan et al., 2014), non-small cell lung (Kim et al., 2014), breast (Zhang et al., 2013), and metastatic melanoma (Poudel et al., 2015). Zhang and co-workers have identified an intracellular signaling pathway initiated by collagen-mediated DDR2 activation, leading to ERK1/2 activation in a Src-dependent manner and SNAIL1 phosphorylation. This induced SNAIL1 stabilization and promoted MDA-MB-231 cell invasion *in vitro* and *in vivo* (Zhang et al., 2013). Recently, Poudel and co-workers demonstrated that DDR2 was able to modulate MMP-2 and MMP-9 secretion in response to type I collagen and to regulate the invasive phenotype

of murine metastatic melanoma cells through a regulation of ERK1/2 and NF-κB signaling pathways (Poudel et al., 2015).

DDRs INHIBITION AND TARGETED THERAPY IN CANCER (TABLE 3)

The contribution of DDRs in tumor progression clearly indicates that inhibition of these receptors might represent a promising therapeutic strategy. Yet, DDRs inhibitors reported so far are adenosine triphosphate (ATP) competitive inhibitors that bind to either active (type-1 inhibitors) or inactive (type-2 inhibitors) conformations, preventing transfer of the terminal phosphate group of ATP to the protein substrate. Type-1 inhibitors bind in the so-called "open conformation" of DDRs kinase domain, which is characterized by a "DFG-in" configuration of the conserved triad DFG at the beginning of activation loop. In contrast, type-2 inhibitors bind to and stabilize an inactive kinase form that is characterized by "DFG-out" conformation. The "DFG-out" motif opens an additional cavity, a hydrophobic allosteric site that, in addition to the ATP binding pocket, is targeted by type-2 inhibitors (Kothiwale et al., 2015). Using chemical and proteomic approaches, dasatinib, imatinib, nilotinib (Bantscheff et al., 2007; Rix et al., 2007; Day et al., 2008), and ponatinib (Canning et al., 2014) were identified as potent small-molecule inhibitors against DDR1 and DDR2, with IC_{50} values of 0.5, 337, 43, 9 nM and 1.4, 675, 55, 9 nM, respectively (Day et al., 2008; Canning et al., 2014). These four molecules were originally developed to inhibit Bcr-Abl tyrosine kinase in chronic myeloid leukemia. Imatinib, nilotinib, and ponatinib (type-2 inhibitors) are more selective by inhibiting a few tyrosine kinases, whereas dasatinib (type-1 inhibitor) is known to inhibit dozen of tyrosine kinases. In 2011, Hammerman and co-workers have shown that DDR2 is mutated in approximately 4% of lung squamous cell cancer and have reported data to suggest that these mutations induce a gain in DDRs function (Hammerman et al., 2011). The same group has also shown that cell lines harboring these mutations are sensitized to the multitargeted kinase inhibitor dasatinib (Hammerman et al., 2011; Bai et al., 2014). Indeed, dasatinib can efficiently inhibit the proliferation of DDR2- mutated SCC cell lines *in vitro* and *in vivo*, as well as cells ectopically expressing mutant DDR2 (Hammerman et al., 2011). This led to the design of clinical trials testing its efficacy in patients with non-small-cell lung carcinoma (NSCLC) (Haura et al., 2010; Johnson et al., 2010; Pitini et al., 2013; Gold et al., 2014). However, inhibition of DDRs signaling pathways often activates secondary survival mechanisms (Beauchamp et al., 2014). Therefore, combining dasatinib and Src kinase inhibitors, could enhance the efficacy of dasatinib in NSCLC (Khurshid et al., 2012) and could also decrease substantial toxicity associated with dasatinib when administered alone (Brunner et al., 2013; Dy and Adjei, 2013).

Actinomycin D is an antibiotic compound that has been clinically used for a long time as an anticancer drug to treat rhabdomyosarcoma, Ewing's sarcoma, trophoblastic neoplasia, and testicular carcinoma. Yang and co-workers have identified Actinomycin D as an antagonist of the DDR2-collagen interaction. Indeed, this compound selectively inhibited the activation of DDR2 by type I collagen in HEK293 cells. However, its relatively weak inhibitory activity (IC_{50} = 9000 nM) may limit its further application for inhibition of DDR2 (Siddiqui et al., 2009). LCB 03-0110, a thienopyridine derivative, was identified from a chemical library using the kinase domain of DDR2 and has been shown to inhibit collagen-induced activation of DDR1 and DDR2 receptors with IC_{50} values of 164 and 171 nM, respectively. However, this compound is also an effective inhibitor for other tyrosine kinases (Sun et al., 2012). Moreover, Ding and co-workers have reported a series of 3-(2-(pyrazolo[1,5-a]pyrimidin-6-yl)-ethynyl)benzamides which selectively bind and inhibit, with a type II mode, the kinase function of DDR1 and were significantly less potent against many other kinases such as Bcr-Abl. The two most promising compounds in this series 7rh and 7rj inhibited the kinase activity of DDR1, with IC_{50} values of 6.8 and 7.0 nM, respectively. *In vitro* investigations revealed that these compounds potently inhibited the proliferation of cancer cell lines expressing high levels of DDR1, including A549 and NCI-H23 lung carcinoma, MDA-MB-435, MCF-7, and T47D breast carcinoma and HCT116 colon carcinoma cells (Gao et al., 2013). Using a new strategy called "fluorescent labels in kinases" (FLiK), Rauh and co-workers reached to identify a series of pyrazolo-urea containing compounds as new type II inhibitors of DDR2. The inhibitory effects of three compounds (2a, 4a, and 4b) were further validated by an orthogonal activity-based assay. DDR2 was found to be inhibited, by these compounds, with IC_{50} values of 65, 75, and 18 nM, respectively. These molecules were also able to inhibit DDR1 with IC_{50} values of 68, 235, and 39 nM, respectively. Furthermore, compounds 2a and 4b exhibited significant effect against the T654M gatekeeper mutant of DDR2 with IC_{50} values of 2.0 and 1.0 nM, respectively (Richters et al., 2014). Gray and co-workers designed and synthesized a series of type II inhibitors, among which DDR1-IN-1 and DDR1-IN-2 induced a significant inhibitory effect against DDR1 with IC_{50} values of 105 and 47 nM, respectively. These two inhibitors were also able to inhibit DDR1 activation in U2OS cells in the presence of collagen, with EC_{50} values of 86 and 9.0 nM, respectively (Kim et al., 2013). Using fragment based drug design (so-called back-to-front design), Murray and co-workers have recently discovered novel inhibitors of DDR1 and DDR2 that were potent and selective and displayed interesting pharmacokinetic properties. *In vitro* studies showed that DDR2 activity was highly inhibited by these molecules but in contrast to unselective inhibitors such as dasatinib, they were not able to inhibit proliferation of lung SCC cells harboring a mutant DDR2 (Murray et al., 2015). Finally, other ways to inhibit DDRs consist in the use of targeted delivery of miRNAs based therapeutics such as miR-199a-5p (Hu et al., 2014) or monoclonal antibodies including Fab 3E3 (Carafoli et al., 2012), 48B3 (Ram et al., 2006), and H-126 (Castro-Sanchez et al., 2010) that have been shown to bind to the DS-like domain of DDR1.

CONCLUSION

Type I collagen, one of the abundant matrix components and an activator of these receptors, was considered for a long time as a mechanic barrier against cell proliferation and

migration but also as a physical obstacle against chemotherapy by decreasing passive diffusion of anticancer drugs. Herein, the reported studies clearly demonstrate that the interaction between type I collagen and DDRs plays a functional role in the regulation of tumor progression, from cell proliferation/survival to migration/invasion processes. However, effects of DDRs activation on tumor progression are controversial. For cell proliferation, it have clearly been demonstrated that DDR1 and DDR2 act as growth suppressors *via* their activation by type I collagen and specific downstream cell signaling. In the case of DDR1, apoptosis was concurrent with cell proliferation suppression. Moreover, the role of DDR2 in the suppression of cell proliferation has been elegantly demonstrated using receptor mutants. In fact, kinase domain mutants were particularly able to alleviate this suppression by inhibiting the activation of these receptors and their downstream cell signaling. These mutations have been identified as novel drivers contributing to cell proliferation *in vivo* and consequently tumor progression. However, other findings strongly suggested that activation of these receptors resulted also in activation of pro-survival signaling pathways. In the case of cell migration and invasion, several *in vitro* and *in vivo* studies specifically addressed the consequences of DDR1 and DDR2 activation in the initiation of migratory and invasive processes. The majority of these studies tended to attribute a functional role of these receptors in the promotion of cell migration and invasion. Moreover, clinical studies on DDR1 and DDR2 expression and the outcome of several cancer pathologies found a correlation between the expression of these receptors, metastasis, and a reduced survival.

Finally, DDR1 and DDR2 are considered as potential therapeutic targets. Therefore, a considerable effort has been made to design inhibitors against these receptors. For kinase activity inhibitors, several molecules including imatinib and nilotinib were identified as inhibitors of these receptors. However, mutations have been noted in several cancer specimens. In the case of DDR2 mutations in squamous lung cell carcinoma, dasatinib showed particular efficacy. Nevertheless, latest *in vitro* model studies have reported a second site mutation in DDR2 which was able to confer resistance to dasatinib. Therefore, and given the clinical trials of dasatinib and other inhibitors in the future, the establishment of additional models of resistance will be important to design strategies that overcome resistance to these molecules.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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CORRECTION
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Corrigendum: Discoidin Domain Receptors: Potential Actors and Targets in Cancer

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CORRIGENDUM

Following relevant reader's comments and Editor's request, my co-authors and I would like to add some references which have been unintentionally omitted in some sections of the review. We thank the readers and the Editorial Office for pointing this out and helping us to improve the manuscript.

"INTRODUCTION SECTION" (PAGE 2)

After surgery, radiation therapy (RT) has long been an integral component of cancer care. It is usually employed to locally eradicate tumor cells as well as alter tumor stroma with either curative or palliative intent (Hodge et al., 2012; Kwilas et al., 2012).

Therefore, current efforts have been focusing on understanding the molecular, cellular, and systemic processes driving cancer initiation, progression, heterogeneity, and metastatic spread (Ramos and Bentires-Alj, 2015; Semenova et al., 2015).

As a major part of the tumor ECM, type I collagen exhibits high density and altered architecture in malignant cancer and is causally linked to tumor formation and metastasis (Ren et al., 2014).

Until recently, these effects on tumor cells were exclusively attributed to integrins, a major class of receptors that mediate cell interactions with extracellular matrix components. The identification of the Discoidin Domain Receptor (DDR) family as collagen receptors represents a new paradigm in the regulation of collagen-cell interactions and regulation of tumor progression (Marquez and Olaso, 2014).

DDR1 and DDR2 were initially discovered by homology cloning based on their catalytic kinase domains and were orphan receptors until 1997 when Shrivastava and co-workers and Vogel and co-workers, reported that different types of collagen are functional ligands for these receptors (Leitinger, 2014).

In contrast with classical growth factor tyrosine kinase receptors such as the epithelial growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) which display a rapid and transient activation, DDR1 and DDR2 are unique in that they exhibit remarkably delayed and sustained receptor phosphorylation upon binding to collagen (Iwai et al., 2013).

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Furthermore, many classical tyrosine kinase receptors (RTKs) undergo negative regulation such as receptor/ligand internalization and subsequent degradation or dephosphorylation by phosphatases (Fu et al., 2013).

In addition, they are uniquely positioned to function as sensors for ECM and to regulate a wide range of cell functions such as migration, cell proliferation, cytokine secretion, and ECM homeostasis/remodeling (Borza and Pozzi, 2014).

"STRUCTURE, FUNCTION AND REGULATION OF DDRS" SECTION (PAGE 3)

DDRs control important aspects of cell behavior including proliferation, migration, adhesion, and ECM remodeling but are dysregulated in various human diseases. They are both activated by several types of collagen. However, this activation strictly requires collagen to be in its native and triple-helical conformation. Heat-denatured collagen is not recognized by DDRs (Valiathan et al., 2012; Carafoli and Hohenester, 2013).

Surprisingly, the substitution of five peripheral amino acids in DDR2 with their DDR1 counterparts converts DDR2 into a receptor of type IV collagen (Carafoli and Hohenester, 2013).

AUTHOR CONTRIBUTIONS

All authors contributed to the designing, writing and the validation of the last version of the manuscript.

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The Elastin Receptor Complex: A Unique Matricellular Receptor with High Anti-tumoral Potential

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Elastin, one of the longest-lived proteins, confers elasticity to tissues with high mechanical constraints. During aging or pathophysiological conditions such as cancer progression, this insoluble polymer of tropoelastin undergoes an important degradation leading to the release of bioactive elastin-derived peptides (EDPs), named elastokines. EDP exhibit several biological functions able to drive tumor development by regulating cell proliferation, invasion, survival, angiogenesis, and matrix metalloproteinase expression in various tumor and stromal cells. Although, several receptors have been suggested to bind elastokines ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, galectin-3), their main receptor remains the elastin receptor complex (ERC). This heterotrimer comprises a peripheral subunit, named elastin binding protein (EBP), associated to the protective protein/cathepsin A (PPCA). The latter is bound to a membrane-associated protein called Neuraminidase-1 (Neu-1). The pro-tumoral effects of elastokines have been linked to their binding onto EBP. Additionally, Neu-1 sialidase activity is essential for their signal transduction. Consistently, EDP-EBP interaction and Neu-1 activity emerge as original anti-tumoral targets. Interestingly, besides its direct involvement in cancer progression, the ERC also regulates diabetes outcome and thrombosis, an important risk factor for cancer development and a vascular process highly increased in patients suffering from cancer. In this review, we will describe ERC and elastokines involvement in cancer development suggesting that this unique receptor would be a promising therapeutic target. We will also discuss the pharmacological concepts aiming at blocking its pro-tumoral activities. Finally, its emerging role in cancer-associated complications and pathologies such as diabetes and thrombotic events will be also considered.

Keywords: extracellular matrix, elastokines, ERC, neuraminidase-1, therapeutic targets

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CANCER DEVELOPMENT AND EXTRACELLULAR MATRIX

Despite a great progress concerning predictive biomarkers, diagnostic and prognostic strategies, cancer remains the second leading cause of death worldwide after cardiovascular diseases. In 2012, approximately 14 million of new cases and 8.2 million of cancer related deaths have been reported, according to the World Health Organization.

Although, the development of cancer was initially thought to be initiated when a single mutated cell begins to proliferate abnormally leading to the formation of primary tumor (*in situ*), the

polyclonal origin of tumors has now been proposed (Parsons, 2008). Malignant cells then cross the tissue, possibly the basement membrane, and invade the extracellular matrix (ECM). From there, invasive tumor cells can spread throughout the body *via* the lymphatic or circulatory systems creating metastatic tumors.

Extracellular matrix remodeling is crucial for regulating tissue homeostasis but also contributes to disease when it is dysregulated. It is composed of macromolecules such as collagens, elastin, laminins, fibronectin, and proteoglycans. Those components interact with cell receptors, transmitting signals that orientate cell adhesion, migration, proliferation, apoptosis, survival, or differentiation. ECM does not only behave as a simple physical support for tissue integrity and plasticity. It is also a reservoir of growth factors, proteases, and other signaling molecules (Hynes, 2009).

During tumor progression, ECM is modified by proteases secreted by both normal and tumor cells. This degradation generates bioactive fragments called matrikines or matricryptines (Davis et al., 2000; Maquart et al., 2004). Matrikines can modulate cell proliferation, migration, invasion, apoptosis, angiogenesis as well as the production and activation of matrix metalloproteinases (MMPs) and the plasminogen system (Bellon et al., 2004; Maquart et al., 2005). In this review, we will focus on elastin, and especially on pro-tumoral activities of elastin-derived peptide (EDP) through their unique receptor, the elastin receptor complex (ERC).

ELASTIN

Elastic Fibers Components

The elastin synthesis, begins during the fetal period (Uitto et al., 1991) and peaks just before birth. Elastogenesis then decreases rapidly to disappear at puberty (Swee et al., 1995). The half-life of elastin is about 70 years (Petersen et al., 2002) and neo-synthesis is low or inexistent. In addition, the ability to form functional elastic fibers is lost. Elastic fibers are essential components of the ECM and are responsible for elasticity of vertebrate tissues. They are found in abundance in tissues subjected to high mechanical stresses requiring repeated cycles of expansion and back to their original state such as the skin, lungs, tendons, or arteries.

Elastic fibers are complex macromolecular assemblies consisting of a coat of fibrillin-rich microfibrils surrounding a heart of elastin (Kielty et al., 2002). The architecture of mature elastic fibers is extremely complex and tissue-specific, reflecting the particular functions they have in tissues. Elastin is a highly hydrophobic polymer of crosslinked-tropoelastin monomers. Microfibrils are made by glycoproteins such as fibrillin-1, fibrillin-2, microfibril-associated glycoprotein-1 (MAGP-1), emilins, latent transforming growth factor β-binding proteins (LTBPs), microfibrillar-associated proteins (MFAPs), and Fibulins (Mithieux and Weiss, 1995). The tropoelastin sequence is composed of alternating domains of very hydrophobic repeating units (which ensure elasticity) and lysine-rich domains. These lysine residues are essential, since the oxidative deamination of their side chains allows the formation of mature elastin covalent crosslinks, i.e., desmosine and isodesmosine, that confer a great mechanical resistance to the elastomer.

Biosynthesis

Elastin is synthesized and secreted from various cell types such as endothelial cells and fibroblasts (Rodgers and Weiss, 2005).

After a major splicing, mature tropoelastin mRNA is exported out of the nucleus and its translation occurs on the surface of the rough endoplasmic reticulum (RER) forming a polypeptide of about 70 kDa with a N-terminal signal sequence of 26 amino acids which is cleaved when the protein reached the RER lumen (Grosso and Mecham, 1988). After release of the signal peptide, the protein is associated with elastin-binding protein (EBP) to prevent its aggregation and premature degradation (Hinek et al., 1995). The EBP-tropoelastin assembly is then directed to the plasma membrane. EBP is secreted and binding of galactose sugars on its galactolectin site causes the release of tropoelastin, which is then aligned and properly incorporated into the growing elastic fiber (Privitera et al., 1998). After tropoelastin release, EBP is recycled and can accompany another tropoelastin molecule.

ELASTIN DEGRADATION AND ELASTIN PEPTIDES

Elastases cleave insoluble and soluble elastin and include serine-, cysteine-, and metallo-proteinases. The serine proteinases neutrophil elastase (Ela-2), cathepsin G, and proteinase-3 and four members of the cysteine cathepsin family (L, S, K, and V) display elastinolytic activity. Moreover, four MMP are elastases (MMP-2, MMP-7, MMP-9, MMP-12). Some generated EDP harbor a GxxPG consensus motif (where x represents any amino acid) adopting a type VIII β-turn, essential for their bioactivity (Brassart et al., 2001). These bioactive EDP are referred as elastokines and the typical elastokine is the VGVAPG peptide, found in the domain encoded by exon 24 of human tropoelastin. Other bioactive GxxPG motifs, GVYPG, GFGPG and GVLPG, and longer elastokines have been reported (Heinz et al., 2012). For instance, MMP-7, -9, and -12 have been shown to generate the bioactive peptides YTTGKLPYGYGPGG, YGARPGVGVGGIP, and PGFGAVPGA (Heinz et al., 2010).

Elastokines contribute to cancer progression by stimulating several capacities of tumor cells such as an elevated expression and secretion of proteases, strongly potentiating their migration and matrix invasion properties (Brassart et al., 1998; Ntayi et al., 2004; Coquerel et al., 2009; Toupance et al., 2012; Donet et al., 2014; **Table 1**). Interestingly, elastokines present potent chemotactic activity on melanoma cells and their presence at a distant organ might contribute to metastasis (Pocza et al., 2008). EDP have also been reported to induce *in vitro* proliferation of glioblastoma (Hinek et al., 1999), and astrocytoma human cell lines (Jung et al., 1998) as well as murine melanoma cell line (Devy et al., 2010). Our laboratory was the first to demonstrate *in vivo* that EDP enhanced murine melanoma cells growth and invasion (Devy et al., 2010).

Elastokines have also biological effects on normal cells. They stimulate migration and proliferation of monocytes and skin fibroblasts (Senior et al., 1984; Shiratsuchi et al., 2010). They up-regulate MMP expression by fibroblasts inducing a remodeling program in favor of melanoma cell invasion. Elastokines exhibit pro-angiogenic activity through MT1-MMP and NO-mediated increase of endothelial cell migration and tubulogenesis (Robinet et al., 2005; Fahem et al., 2008; Gunda et al., 2013). A wide range of biological effects on immune cells was reported in response to EDP stimulation (Antonicelli et al., 2007). Among them, the chemotactic activity (Nowak et al., 1989; Hance et al., 2002; Houghton et al., 2006; Guo et al., 2011) and elastases production (Hauck et al., 1995; Péterszegi et al., 1997; Varga et al., 1997) amplify elastolysis and increase inflammatory cells recruitment at the tumor site.

RECEPTORS AND SIGNALING

The biological effects of EDP are mediated by their binding to their cell surface receptors. Among them, the ERC is the most prominent but others potential receptors have also been reported, namely galectin-3 (Pocza et al., 2008) and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Rodgers and Weiss, 2004; Lee et al., 2014b).

Galectin-3 is expressed in normal and tumor cells and possesses diverse biological functions associated with inflammatory response such as adhesion, cell differentiation, cell migration, and cytokine production. It also modulates others biological functions linked to tumor development like angiogenesis, tumor progression, proliferation, chemotactic, and cell-matrix interactions (Fortuna-Costa et al., 2014). It was moreover reported that galectin-3 is able to interact with both

TABLE 1 | Cancer-associated biological effects of EDP.

Biological effects	Cell types	EDPs cancer-associated biological effects
Angiogenesis	Endothelial cells	Nackman et al., 1997; Robinet et al., 2005; Daamen et al., 2008; Fahem et al., 2008; Gunda et al., 2013
Apoptosis and cell survival	Fibroblasts	Cantarelli et al., 2009
	Lymphocytes	Péterszegi and Robert, 1998; Péterszegi et al., 1999
Adhesion	Fibroblasts	Hornebeck et al., 1986; Groult et al., 1991; Yamamoto et al., 2002; Rodgers and Weiss, 2004; Bax et al., 2009; Akhtar et al., 2011
	Astrocytoma	Jung et al., 1999
	Carcinoma	Timar et al., 1991; Svitkina and Parsons, 1993
	Melanoma	Timar et al., 1991; Svitkina and Parsons, 1993
Proliferation	Fibroblasts	Ghuysen-Itard et al., 1992; Kamoun et al., 1995; Tyagi et al., 1996; Tajima et al., 1997; Duca et al., 2005; Shiratsuchi et al., 2010
	Lymphocytes	Poggi and Mingari, 1995; Péterszegi et al., 1996
	Melanoma	Devy et al., 2010
	Astrocytoma	Jung et al., 1998
	Glioma	Hinek et al., 1999
	Endothelial cells	Ito et al., 1998; Dutoya et al., 2000
Tumor invasion and proteases release	Fibroblasts	Gminski et al., 1991a,b; Archilla-Marcos and Robert, 1993; Landeau et al., 1994; Brassart et al., 2001; Huet et al., 2001
	Endothelial cells	Robinet et al., 2005; Fahem et al., 2008; Siemianowicz et al., 2010, 2015
	Monocytes	Fülöp et al., 1986; Varga et al., 1997
	Lymphocytes	Péterszegi et al., 1996, 1999
	Melanoma	Ntayi et al., 2004; Pocza et al., 2008; Devy et al., 2010
	Glioma	Coquerel et al., 2009
	3LL-HM carcinoma	Timar et al., 1991
	Lung cancer	Toupance et al., 2012
	HT1080 fibrosarcoma	Brassart et al., 1998; Huet et al., 2002;Donet et al., 2014
Chomotaxis and migration	Keratinocytes	Fujimoto et al., 2000
	Fibroblasts	Senior et al., 1982, 1984; Mecham et al., 1989; Grosso and Scott, 1993b; Duca et al., 2005; Shiratsuchi et al., 2010
	Eodothelial cells	Long et al., 1989; Skeie and Mullins, 2008; Skeie et al., 2012
	Monocytes	Senior et al., 1980, 1984; Bisaccia et al., 1994; Castiglione Morelli et al., 1997; Uemura and Okamoto, 1997; Hance et al., 2002; Houghton et al., 2006
	Macrophages	Kamisato et al., 1997; Guo et al., 2006, 2011
	3LL-HM carcinoma	Timar et al., 1991
	M27 lung cancer	Blood et al., 1988; Blood and Zetter, 1989, 1993; Yusa et al., 1989; Grosso and Scott, 1993a
	Melanoma	Mecham et al., 1989; Pocza et al., 2008
	HT1080 fibrosarcoma	Donet et al., 2014

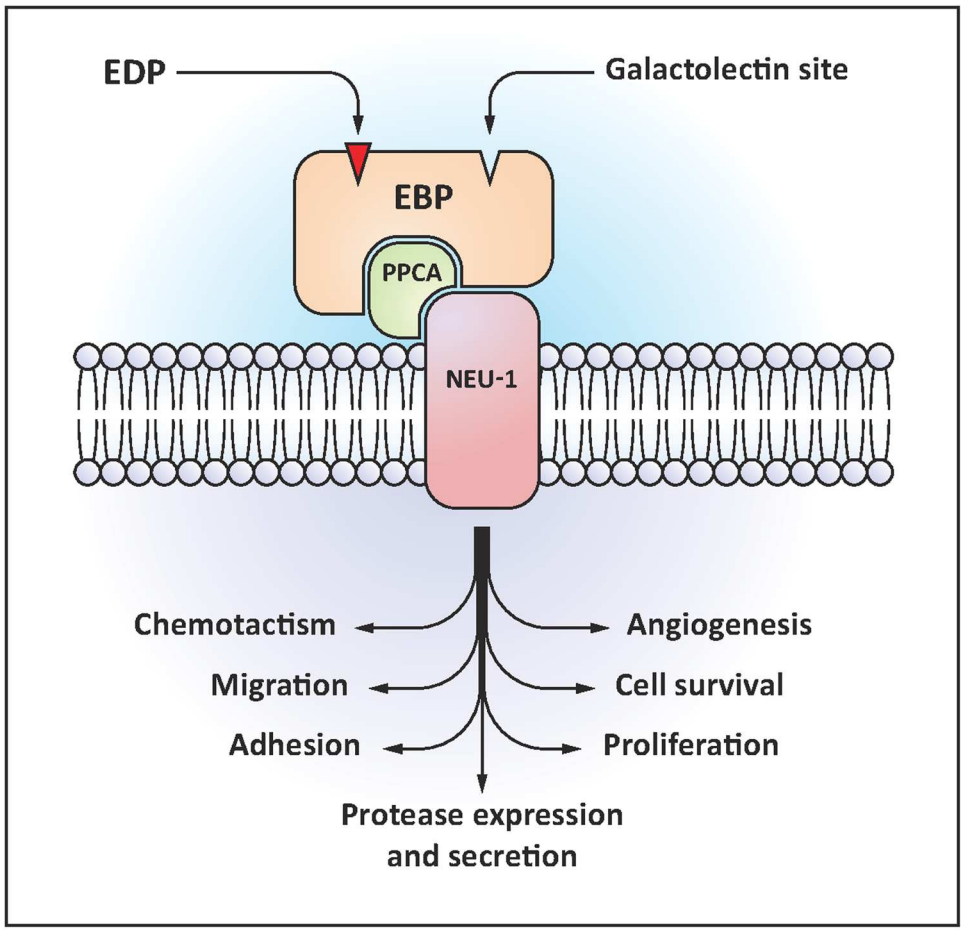

FIGURE 1 | Elastin receptor complex (ERC) structural organization and EDP-induced biological effects.

soluble and insoluble elastin in a lactose-dependent manner (Ochieng et al., 2004). This interaction can modulate tumor development as observed by the ability of some specific EDP, such as VGVAPG and VAPG, to amplify melanoma invasion (Pocza et al., 2008).

Integrin $\alpha_v\beta_3$ regulates diverse biological functions such as cell adhesion, proliferation and migration (Byzova et al., 1998). $\alpha_v\beta_3$ mainly binds ligands through RGD sequence recognition but it can also recognize others ligands that do not harbor this motif. Indeed, integrin $\alpha_v\beta_3$ recognizes with high affinity the RKRK sequence present in the C-terminal domain of tropoelastin (Bax et al., 2009). Moreover, a recent study has shown the ability for $\alpha_v\beta_5$ to bind tropelastin involving the central region of the protein (Lee et al., 2014b).

The ERC is a heterotrimeric receptor binding elastokines presenting the GxxPG consensus sequence (**Figure 1**). This receptor contains a peripheral 67-kDa protein named EBP (accession number P16278-2), and two membrane-associated proteins, the protective protein/cathepsin A (PPCA, 55-kDa, accession number P10619) and neuraminidase-1 (Neu-1, 61-kDa, accession number Q99519; Duca et al., 2007). EBP is an enzymatically inactive spliced variant of lysosomal β-galactosidase (Privitera et al., 1998). EBP possesses two functional binding sites: the elastin site on which EDP binding triggers signaling pathways, and the galactolectin site whom occupancy by galactosugars induces EDP release and dissociation of the complex (Mecham et al., 1991). When EDP bind to EBP, neuraminidase-1 is activated and catalyzes the desialylation of adjacent gangliosides such as GM_3 [*N*-acetylneuraminic-α-(2-3)-galactosyl-β-(1-4)-glucosyl-(1-1′)-ceramide] generating lactosylceramide (LacCer) production (Rusciani et al., 2010; Scandolera et al., 2015). LacCer is a second messenger able to activate intracellular signals. Intracellular signaling pathways modulated by EDP depend on the cell type. Duca et al. (2002) showed that pro-MMP-1 induction mediated by EDP in human dermal fibroblasts involves the activation of MEK1/2/ERK1/2 pathway through a signal dependent on PKA and PI3K. Moreover, complementary works demonstrated that EDP are able to modulate signaling pathways involving modules such as Ras-Raf-1-MEK1/2-ERK1/2, Gi-p110γ-Raf-1-MEK1/2-ERK1/2, cAMP-PKA-B-Raf-MEK1/2-ERK1/2, NO-cGMP-PKG-Raf-1-MEK1/2-ERK1/2 or Gi-p110γ-Akt-caspase9-Bad-Foxo3A. They also induce Ca^{2+} mobilization (Jacob et al., 1987; Faury et al., 1998; Duca et al., 2005; Fahem et al., 2008).

Although EDP are the main ligands of ERC, bioactive xGxxPG motifs are found in numerous matrix protein sequences. For instance, laminin B1 chain harbors a LGTIPG sequence that triggers elastin-like signaling, inducing pro-tumoral activities, and was identified as a ligand of this receptor in melanoma cells. That is why EBP was first called the 67-kD elastin/laminin binding protein (Mecham et al., 1989; Hinek, 1994).

ANTI-ERC THERAPEUTIC STRATEGIES

Limiting or blocking the deleterious effects of EDP/ERC interaction can be achieved either by limiting EDP generation or by acting directly on the ERC and its signaling pathways. As this review is focused on ERC, we will not detail here elastases inhibition strategies.

Targeting EBP

Blocking the binding of EDP on EBP can be achieved either by using the V14 peptide or a galactoside. The V14 peptide (VVGSPSAQDEASPL) is derived from EBP sequence and can bind EDP. As a consequence, excess V14 can trap circulating EDP thereby blocking their effects (Robinet et al., 2007). Alternatively, the use of galactosugars (mostly lactose or chondroitin sulfate) leads to the shedding of EBP from the complex and blocks the corresponding signaling (Blaise et al., 2013). Although V14 and galactosugars helped to better understand EDP biology, their selective delivery at the site of vascular injury is still an issue.

Targeting Neu-1

The catalytic activity of Neu-1 is required for proper EDP signaling. As a consequence, its inhibition blocks EDP-driven signals. The 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (ddNeu5Ac) inhibitor is currently used as a sialidase inhibitor to block EDP effects (Duca et al., 2007). However, this compound also inhibits other sialidases precluding its therapeutic use.

An attempt was made to design and synthesize inhibitors for human neuraminidases (Magesh et al., 2009) but the results were not satisfactory. Indeed, selectivity was not achieved, probably because the structures of human sialidases are not fully described.

Recently, O'Shea et al. (2014) used oseltamivir phosphate to target Neu-1 and disable cancer cell survival in human pancreatic cancer with acquired chemoresistance. This study suggests that Tamiflu could be possibly used to selectively block Neu-1.

Blocking EDP-Mediated Signaling Pathways

In human skin fibroblasts, Neu-1 promotes the local conversion of the GM_3 ganglioside into LacCer following EDP treatment. LacCer can therefore be regarded as the second messenger of the complex (Rusciani et al., 2010). Thus, blocking the signaling pathways triggered by LacCer will suppress EDP effects. In this context, PI3Kg is a promising target as this kinase is central to EDP-related signaling (Duca et al., 2005).

Besides this direct signaling, Neu-1 is also known for its ability to desialylate other membrane residing glycoconjugates, notably receptors. During the last decade, Neu-1 has been shown to modulate insulin receptor signaling (Blaise et al., 2013) and to regulate TLR4 (Amith et al., 2010), Trk A (Jayanth et al., 2010), PDGF-BB and IGF receptors (Hinek et al., 2008), EGF and MUC1 receptors (Lillehoj et al., 2012), and CD31 (Lee et al., 2014a). Consequently, this ERC subunit now emerges not only as a catabolic enzyme but also as a regulator of signaling platforms (Pshezhetsky and Hinek, 2011).

Efforts are now made to understand the intricate network of Neu-1 partners and how they interact each other in order to devise new strategies aiming at selectively impeding these interactions.

ERC INVOLVEMENT IN CANCER-ASSOCIATED PROCESSES

Diabetes

Type 2 diabetes leads to many micro- and macrovascular complications implicating several molecular factors and with significant impact in terms of morbidity and mortality. For example, type 2 diabetes mellitus is associated with an increase in the expression of MMPs, especially MMP-2 and 9, and an increase in the degradation of elastin and, thus, the generation of EDP (Hopps and Caimi, 2012). EDP immunogenic properties favor the formation of anti-elastin antibodies, which concentrations are greatly increased in diabetic patients as compared to non-diabetic subjects (Fulop et al., 1990).

Cancer is a well-known complication of diabetes. Indeed, cancer development is more frequent in diabetic people than in the general population. According to recent studies and meta-analyzes, cancers involving the pancreas (Morrison, 2012), liver (Giovannucci et al., 2010), colon (Larsson et al., 2005), breast (Larsson et al., 2007), urinary tract (Larsson et al., 2006), and the endometrium (Friberg et al., 2007) occur more frequently among patients with type 2 diabetes. In contrast, a recent meta-analysis (Giovannucci et al., 2010) involving a total of nineteen studies, indicates a reduced risk of occurrence of 16% for prostate carcinoma in diabetic patients.

Several mechanisms could be involved in the initiation and/or progression of cancer in diabetes but these mechanisms still remain hypothetical.

Insulin and its associated receptor seem to have a key role, as well as the insulin-like growth factor 1 and its receptor, in the interplay between cancer and diabetes (Cohen and LeRoith, 2012). Furthermore, hyperglycemia could promote tumor progression due to increased intracellular metabolic activity specific to cancer cells and a greater membrane transport of glucose. Interestingly, it has been shown that the activation of pro-tumoral factors such as neutrophil elastase (NE; Moroy et al., 2012) and the accumulation of EDP in blood may represent inducible factors of insulin resistance in mice (Blaise et al., 2013). Indeed, $NE^{-/-}$ mice have increased blood glucose, decreased insulin pathway activity, and increased gluconeogenesis (Talukdar et al., 2012). This insulin resistance might be due to a decrease in the expression of Hsp90 and an increase of the inhibitory protein (IkB) of the transcription

factor NFkB. The pro-inflammatory state present in diabetics could decrease the efficiency of intracellular antioxidants and also participate in carcinogenesis. Some cytokines, such as tumor necrosis factor α (TNF-α), promote tumor growth by activating NF-κB (Szlosarek et al., 2006). Another mechanism related to the pro-inflammatory state, mitochondrial dysfunction, would be present in diabetic patients resulting in decreased energy available for DNA repair. Meanwhile, our laboratory has shown that EDP, which are products of NE activity, induce hyperglycemia and insulin resistance in animals by inhibiting insulin receptor signaling pathways in muscle, liver, and adipose tissue. Although the precise mechanism remains to be elucidated, it appears that this inhibitory effect involves a physical interaction between the insulin receptor and the ERC *via* its Neu-1 subunit (Blaise et al., 2013). Consequently, the ERC could not only exhibit a clear pro-tumoral aspect, but is also involved in the outcome of diabetes influencing cancer development.

Thrombosis

Cancer-associated thrombosis is a major cause of morbidity and mortality in patients with cancer. Thrombotic complications, mostly from venous thromboembolism, are the second cause of death among patients with cancer (Khorana et al., 2007). Several mechanisms have been suggested to contribute to these increased thrombotic complications such as the prothrombotic activity of cancer cells (Mitrugno et al., 2015), the secondary deleterious effects of anti-cancer therapies and the interaction of cancer cells with blood platelets. Indeed, cumulative evidences show that platelets and their activation play important roles in cancer growth and dissemination (Gay and Felding-Habermann, 2011). Therefore, antiplatelet therapy to minimize platelet activation and aggregation, typically reserved for cardiovascular diseases, may have profound implications in cancer treatment (Franco et al., 2015).

In a recent study published by Kawecki et al. (2014), EDP were shown to decrease human platelet aggregation in whole blood and washed platelets. Both EDP and the VGVAPG peptide strongly reduced thrombus formation *in vitro* and *in vivo* in wild-type mice. Moreover, EDP and VGVAPG also prolonged tail bleeding times. The same study also reported that the regulatory role of EDP relies on a dual mechanism that involves effects on platelets, that express a functional ERC able to trigger an increase of platelet sialidase activity, and on the ability of EDP to disrupt plasma von Willebrand factor interaction with collagen. Therefore, it is tempting to speculate that EDP may rather have beneficial effects on cancer-associated thrombosis by reducing platelet aggregation and thrombus formation.

However, if EDP modulate the formation of procoagulant microparticles by malignant cells and tissue factor expression of, the major molecular driver of cancer-associated coagulopathy and thromboembolic disorders (Mitrugno et al., 2015), remains unknown so far. Additional experiments are required to better understand the overall effects of elastin degradation products on cancer-associated thrombosis.

CONCLUSION

It is now admitted that ECM can directly influence cell fate and is involved in the phenotypic modulation of cells during cancer progression. Matrix-derived peptides, originating from tumor microenvironment degradation, are crucial actors involved in the pathology and a potential source of innovative therapy. Thus, among all the matrikines described up to now, bibliographic data show that elastokines and their singular receptor, present important pro-tumoral activities. Consequently, the targeting of the ERC is of particular interest as it is not only directly involved in cancer development where an important elastolysis is observed, but also in cancer-associated processes such as diabetes and thrombosis.

AUTHOR CONTRIBUTIONS

AS, LO, SS, AG, SB, CK, PM, HEB, BR-C, LM, LDe, LDu contributed to the writing of the paper and to its relecture. LO contributed to **Table 1**. AG contributed to **Figure 1**.

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Syndecans as Cell Surface Receptors in Cancer Biology. A Focus on their Interaction with PDZ Domain Proteins

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Syndecans are transmembrane receptors with ectodomains that are modified by glycosaminoglycan chains. The ectodomains can interact with a wide variety of molecules, including growth factors, cytokines, proteinases, adhesion receptors, and extracellular matrix (ECM) components. The four syndecans in mammals are expressed in a development-, cell-type-, and tissue-specific manner and can function either as co-receptors with other cell surface receptors or as independent adhesion receptors that mediate cell signaling. They help regulate cell proliferation and migration, angiogenesis, cell/cell and cell/ECM adhesion, and they may participate in several key tumorigenesis processes. In some cancers, syndecan expression regulates tumor cell proliferation, adhesion, motility, and other functions, and may be a prognostic marker for tumor progression and patient survival. The short cytoplasmic tail is likely to be involved in these events through recruitment of signaling partners. In particular, the conserved carboxyl-terminal EFYA tetrapeptide sequence that is present in all syndecans binds to some PDZ domain-containing proteins that may function as scaffold proteins that recruit signaling and cytoskeletal proteins to the plasma membrane. There is growing interest in understanding these interactions at both the structural and biological levels, and recent findings show their high degree of complexity. Parameters that influence the recruitment of PDZ domain proteins by syndecans, such as binding specificity and affinity, are the focus of active investigations and are important for understanding regulatory mechanisms. Recent studies show that binding may be affected by post-translational events that influence regulatory mechanisms, such as phosphorylation within the syndecan cytoplasmic tail.

Keywords: syndecan, cancer, PDZ domain, phosphorylation, cytoskeleton, extracellular matrix

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Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; IGF1R, insulin-like growth factor-1 receptor; PDZ, postsynaptic density-95/disc large protein/zonula occludens-1; PDZ-BM, PDZ binding motif; PKC, protein kinase C.

INTRODUCTION

Syndecans are transmembrane proteoglycans that are found on the surface of many types of mammalian cells. The four syndecans in mammals are encoded by four genes, but invertebrates have just one syndecan. Based on chromosomal localization and exon organization studies, all syndecans arise from a single ancestral gene. Syndecans are expressed in a development-, cell-type-, and tissue-specific manner and function either as independent or co-receptors that mediate cell signaling (Bishop et al., 2007; Multhaupt et al., 2009). In these type I transmembrane glycoproteins, the core protein ranges in size from 20 to 45 kDa. Syndecan core proteins include an extracellular domain (ectodomain) that carries either heparan sulfate only or heparan sulfate and chondroitin sulfate a single transmembrane (TM) domain; and a short cytoplasmic domain (**Figure 1A**). The ectodomain can interact with a wide variety of molecules, including growth factors, cytokines, proteinases, adhesion receptors, and ECM components. Syndecan-1 is mainly expressed in mesenchymal and epithelial cells. Syndecan-2 is highly expressed in endothelial and mesenchymal tissues and in liver, neural, and fibroblast cells. Syndecan-3, the longest of the four syndecans, is expressed in neural tissue and developing musculoskeletal system, but is undetectable in epithelial cells. Finally, syndecan-4, which has the shortest core protein, is widely expressed.

Syndecans are implicated in the control of cell–cell, cell–pathogen, and cell–matrix interactions via the recruitment of the actin cytoskeleton, as well as in cellular proliferation, differentiation, and migration. Syndecans can be found in cell protrusions and focal adhesions, where they colocalize with actin (Granés et al., 1999; Berndt et al., 2004). Importantly, they can act as co-receptors of other cell surface receptors like growth factor receptors and integrins (Morgan et al., 2007; Couchman, 2010; Rapraeger, 2013). In this context, syndecans can bind, immobilize, concentrate, and induce conformational changes in growth factors, adhesion molecules, and other signaling molecules via their heparan sulfate chains, thus facilitating their receptor interaction. They can also protect ligands from activation or sequester them away from membrane receptors (Zimmermann and David, 1999; Alexopoulou et al., 2007).

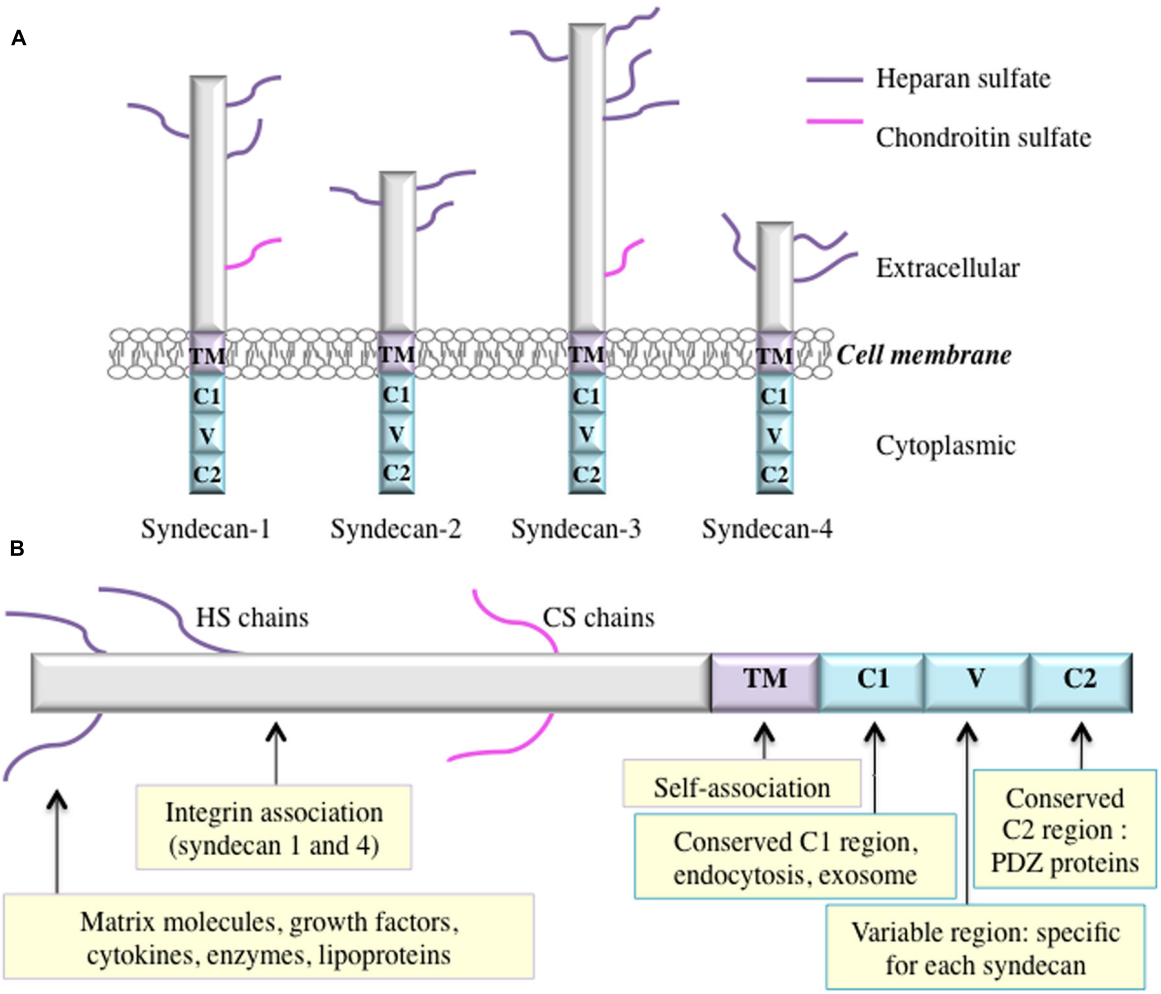
Syndecans undergo regulated physiological shedding of their extracellular domain, a process that may be increased in pathological conditions, thereby allowing them to act as soluble effectors and/or antagonists (Kim et al., 1994; Manon-Jensen et al., 2010). In addition, syntenins/syndecans, in conjunction with the syntenin-binding protein ALIX, are likely to be involved in or to enhance exosome production (Baietti et al., 2012; Friand et al., 2015).

SYNDECANS AND CANCER

Syndecans are involved in cancers, infectious diseases, obesity, wound healing, and angiogenesis. As documented in recent reviews, they are considered key regulators of tumor progression (Barbouri et al., 2014; Couchman et al., 2015; Theocharis et al., 2015). In some cancers, syndecan expression may regulate tumor cell function and serve as a prognostic marker for tumor progression and patient survival. Syndecan-1 expression is dysregulated in a number of cancers, including head and neck, ovarian, breast, and colorectal carcinomas (Teng et al., 2012). Syndecan-1 acts as a tumor suppressor in MDA-MB-231 breast cancer cells (Hassan et al., 2013). Treating these cells with syndecan-1 small interfering RNA not only enhances β1-integrin and focal adhesion kinase activity, leading to increased cellular adhesion and migration, but it also improves cellular resistance to irradiation. A study on pre-invasive breast cancer revealed an inverse correlation between the expression of syndecan-1 and the pro-metastatic microRNA miR-10b, suggesting a potential novel mode of post-transcriptional regulation of syndecan-1 (Hannafon et al., 2011). Studies revealing the negative regulation of syndecan-1 by miR-10b and its pro-invasive consequences in human breast cancer cells, reported syndecan-1 as a new regulatory target of miR-10b (Ibrahim et al., 2012). Other studies revealed that syndecan-1 decreases cell migration in lung epithelium via activation of Rap1, which slows focal adhesion disassembly (Altemeier et al., 2012). Syndecan-1 also plays a role in squamous cell carcinoma collagen-mediated motility and invasion by modulating RhoA and Rac activity, suggesting that decreased syndecan-1 expression during carcinoma progression may enhance tumor cell invasiveness (Ishikawa and Kramer, 2010).

The presence of syndecan-1 is associated with favorable outcomes in both lung cancer and mesothelioma (Kumar-Singh et al., 1998; Anttonen et al., 2001), and the loss of syndecan-1 is a feature of hepatocellular carcinoma with high metastatic potential (Matsumoto et al., 1997). Low syndecan-1 expression correlates with gastric carcinoma invasion and metastasis (Chu et al., 2008). In contrast, studies have reported that high expression levels of syndecan-1 in breast carcinoma are associated with high histological grade, high mitotic count, large tumor size, c-erbB-2 over-expression, and estrogen receptor-negative status. These studies show that high syndecan-1 expression correlates with the most invasive breast carcinomas (Stanley et al., 1999; Barbareschi et al., 2003; Leivonen et al., 2004; Lendorf et al., 2011). Studies using an *in vitro* breast cancer model also suggest that syndecan-1 participates directly in tumor cell spreading and adhesion (Beauvais and Rapraeger, 2003). In prostate cancer, high syndecan-1 expression is a feature of biologically aggressive progression (Zellweger et al., 2003). As stated in recent comprehensive reviews, stromal expression of syndecan-1 may have negative prognostic value, and elevated serum levels of the shed syndecan ectodomain might also be a prognostic indicator (Gharbaran, 2015; Szatmári et al., 2015). Studies have revealed a mechanism by which syndecan-1 and -4 ectodomains, may capture and induce autophosphorylation of the tyrosine kinase receptors HER2 and EGFR respectively, leading to integrin mediated carcinoma cell migration (Wang et al., 2014, 2015).

Nuclear localization of syndecan-1 has been reported, suggesting that it may function as a transcription factor and therefore impact gene regulation affecting cancer pathogenesis



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Syndecan	PDZ protein	References
Syndecan-1	Syntenin	Grootjans et al., 1997; 2000; Sulka et al., 2009
	CASK	Cohen et al., 1998; Maday et al., 2008
	Synectin	Maday et al., 2008
	Synbindin	Maday et al., 2008
	Tiam-1	Shepherd et al., 2010; Liu et al., 2013
Syndecan-2	Syntenin	Grootjans et al., 1997; 2000
	CASK	Cohen et al., 1998; Volta et al., 2010
	Synbindin	Fan et al., 2009
Syndecan-3	Syntenin	Grootjans et al., 1997; 2000
	CASK	Grootjans et al., 2000; Ojeh et al., 2008
	Tiam1	Liu et al., 2013
Syndecan-4	Syntenin	Grootjans et al, 1997; 2000; Kang et al., 2003
	CASK	Bass et al., 2002
	Synectin	Gao et al., 2000

FIGURE 1 | (A) The syndecan family of heparan sulfate proteoglycans. Syndecans consist of a transmembrane core protein with a short cytoplasmic tail and an ectodomain. All syndecan ectodomains are modified with either heparan sulfate only or heparan sulfate and chondroitin sulfate sugar chains. **(B)** A schematic showing the syndecan domains and examples of functions and interacting partners. **(C)** List of known PDZ-containing proteins that interact with syndecans.

(Brockstedt et al., 2002). In addition, heparanase and syndecan-1 may cooperate to drive growth factor signaling and to regulate cell behavior, thus enhancing tumor growth and dissemination (Ramani et al., 2013; Palaiologou et al., 2014; Roucourt et al., 2015). One study found that syndecan-4 inhibited breast carcinoma cell invasion (Beauvais and Rapraeger, 2003), and its expression in human breast carcinoma was described as being associated with good prognosis (Lendorf et al., 2011). In contrast, another study found that syndecan-4 expression correlated significantly with high histological grade and negative estrogen receptor status (Baba et al., 2006) and was therefore a marker of poorer prognosis. Furthermore, a study of pancreatic cancer showed that syndecan-2 was involved in perineural invasion of pancreatic adenocarcinoma cells (De Oliveira et al., 2012). Silencing syndecan-2 expression in these cells significantly reduced motility and invasiveness. Syndecan-2 is upregulated in breast tumors (Lim et al., 2015) and in colon carcinomas (Park et al., 2002; Ryu et al., 2009; Choi et al., 2010). In highly metastatic colorectal cancer cells, syndecan-2 expression is enhanced by fibronectin secreted by stromal cells (Vicente et al., 2013). In colorectal carcinoma, low epithelial expression of syndecan-1 is associated with a higher histological grade, with more advanced clinical stage of the patients, and with potentially more unfavorable prognosis (Lundin et al., 2005; Hashimoto et al., 2008; Mitselou et al., 2012). Results from a recent meta-analysis of colorectal cancer studies demonstrated that loss of syndecan-1 expression in colorectal cancer correlates with histological grade and tumor stage, but not with lymph node or distant metastasis (Wei et al., 2015). The authors also reported that syndecan-1 expression does not have prognostic value in colorectal carcinoma patients. To date, syndecan-3 has not been implicated in cancer. Although the mechanisms are not yet fully understood, these examples highlight the important role of syndecans in tumor progression and suggest that they are relevant and promising therapeutic targets (Ramani et al., 2013; Barbouri et al., 2014; Theocharis et al., 2015). For instance, the anti-tumoral activity of zoledronic acid on breast cancer cells was reported to correlate with a differential modulation of syndecans (Dedes et al., 2012). Synstatin peptides based on HER2 and EGFR interaction motifs on syndecan-1 and -4 respectively can competitively displace receptor tyrosine kinase interaction and disrupt activation of cell motility (Wang et al., 2015). Similar peptides were designed to block IGF1R binding to syndecan-1/αvβ3 integrin complex and inhibit the integrin activity in endothelial and tumor cells (Rapraeger, 2013).

THE TRANSMEMBRANE DOMAIN-INDUCED OLIGOMERIZATION PROPERTIES OF SYNDECANS

Syndecans transmembrane domain is composed of 25 hydrophobic amino acid residues responsible for the molecular interaction that causes homo-oligomerization of syndecan core proteins (Asundi and Carey, 1995; Choi et al., 2005), a step essential for their signaling activation. The conserved GXXXG (where X is any amino acid) motif is involved in this process. Recent studies have revealed the potential of syndecan-2 and -4 to form hetero-oligomers, reducing each syndecan activity (Choi et al., 2015). This hetero-oligomerization capacity may offer insight into an underlying modulating mechanism (Kwon et al., 2015).

The cytoplasmic tail has two conserved regions, C1 and C2, that share common characteristics in all syndecans, plus a central variable region (V) that may regulate cell spreading and actin cytoskeleton assembly (**Figure 1B**; Carey et al., 1996; Chakravarti et al., 2005; Stepp et al., 2015). Each region can support signaling complexes formation (Carey, 1997; Bernfield et al., 1999; Yoneda and Couchman, 2003). The C1 domain is thought to participate in syndecan dimerization (Oh et al., 1997) and in the binding of various intracellular proteins, such as ezrin (Granés et al., 2000). In neuroblastoma, the C1 region of syndecan-3 interacts with a protein complex composed of Src family kinases and the actin-binding proteins cortactin and tubulin (Kinnunen et al., 1998). Likewise, the V region of syndecan-4 interacts with PKCα (protein kinase Cα) as well as with phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P2) (Oh et al., 1997, 1998). The C2 carboxyl-terminal tetrapeptide sequence present in all syndecans consists of the highly conserved tetrapeptide sequence Glu-Phe-Tyr-Ala (EFYA) (Bass and Humphries, 2002; Multhaupt et al., 2009; Rousselle and Letourneur, 2009).

THE INTERACTIONS OF SYNDECANS WITH CYTOSKELETON PDZ DOMAIN PROTEINS

The EFYA sequence binds to PDZ domain-containing proteins, such as syntenin-1 (Grootjans et al., 1997) and CASK (Cohen et al., 1998), which may function as membrane scaffold proteins that recruit signaling and cytoskeletal proteins to the plasma membrane. The EFYA motif thus belongs to the large family of PDZ-binding motifs (PDZ-BMs). Recent work suggests that PDZ interactions are involved in protein trafficking, possibly routing proteoglycans to the cell surface (Wawrzyniak et al., 2012).

There is growing interest in understanding the binding of syndecans to their PDZ domain-containing counterparts (**Figure 1C**). Not only are the interactions involved in cytoskeletal rearrangements in response to the signaling activities, but syndecan-PDZ domain-containing protein complexes may also participate in cell-ECM adhesion and migration. For example, synectin binding to syndecan-4 may modulate in vitro cell migration (Gao et al., 2000; Tkachenko et al., 2006). As well, cell adhesion to fibronectin is regulated by the interaction of syndecan-1 with the PDZ domain of the T-cell lymphoma invasion and metastasis gene 1 protein (Tiam1) (Shepherd et al., 2010). A study of hippocampal neurons revealed that syndecan-2 induces spine formation by recruiting intracellular vesicles toward postsynaptic sites through an interaction with synbindin (Ethell et al., 2000).

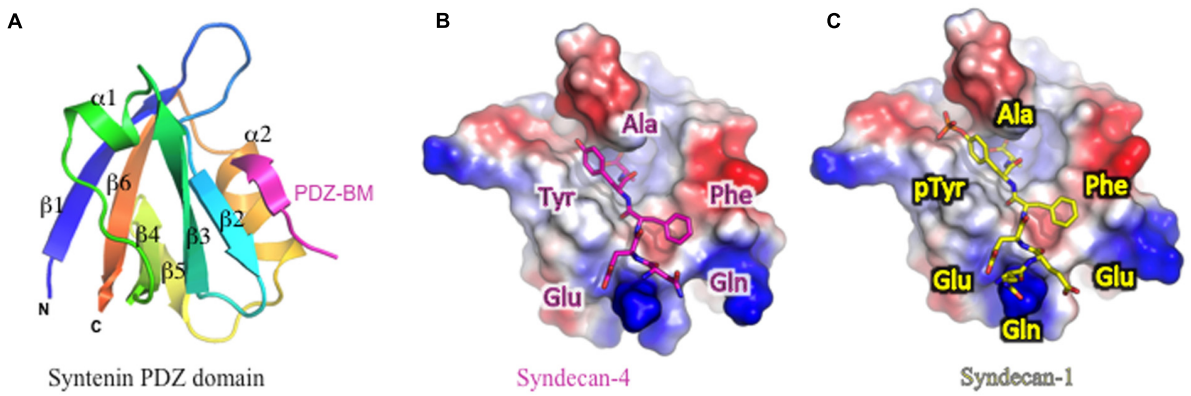
The name PDZ is an acronym derived from the first three proteins in which these domains were identified: PSD-95 (*postsynaptic density PSD-95/SAP90*), DLG (*Drosophila melanogaster tumor suppressor septate junction protein* Disks

large-1), and ZO-1 (*epithelial tight junction protein* Zonula Occludens-1) (Kennedy, 1995; Zimmermann, 2006; Ye and Zhang, 2013). Over 250 non-redundant PDZ domains have been identified in the human proteome (Wang et al., 2010) and are found in proteins involved in diverse cellular functions, such as maintenance of cell polarity, signal transduction in neurons, and cell migration (Harris and Lim, 2001; Sheng and Sala, 2001; Jeleñ et al., 2003).

The number of amino acid residues in a PDZ domain is relatively small (80–100 amino acids) (Hung and Sheng, 2002). Structural analysis of these domains indicates that a canonical PDZ domain consists of five or six β-strands and two or three α-helices (Luck et al., 2012) (**Figure 2A**). In addition, the domain itself folds into a compact globular shape; this maintains the proximity of the N- and C-termini to each other on opposite sides of the PDZ-BM interaction site (Sheng and Sala, 2001; Jeleñ et al., 2003; Lee and Zheng, 2010). The PDZ-BM fits in the groove between the α2-helix and the β2-strand structure such that the α2-helix is anti-parallel to the β2-strand (**Figure 2B**). This interaction site is also known as the carboxylate-binding site because of the highly conserved carboxylate-binding loop at the end of the groove that connects the β1- and β2-strand structures: R/K-X-X-X-G-Φ-G-Φ (where Φ is a hydrophobic residue) (Sheng and Sala, 2001; Hung and Sheng, 2002; Lee and Zheng, 2010).

There are no reports of PDZ domains interacting with syndecans through motifs other than the EFYA sequence. Since the EFYA motif is the only PDZ-BM in syndecans, it seems likely that all four syndecans have similar binding affinity for the same PDZ-containing proteins. For example, all syndecans have similar affinity for the PDZ1-PDZ2 tandem domain of syntenin-1 (Grootjans et al., 2000). However, CASK has a higher affinity for syndecan-2 and syndecan-4 than for syndecan-1 and syndecan-3. The molecular mechanism underlying this difference in affinities is unknown. On the other hand, the PDZ domain of Tiam1 binds to a peptide corresponding to the last eight residues of syndecan-1 and -3, but not to those of syndecan-2 and -4 (Liu et al., 2013).

PDZ domain-containing proteins play essential roles in most aspects of cellular homoeostasis and are implicated in diverse aspects of tumor development and metastasis (Subbaiah et al., 2011). A number of studies have established that MDA-9/syntenin has a pivotal role in cancer development and progression, and suggest that it could be a tumor marker (Philley et al., 2016). Recent data indicate that in addition to its



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	C1	V	C2	Syntenin-1	Tiam1
Syndecan-1	RMKKKDEGSYSL	EEPKQANGGAYQK	PTKQEEFYA	+	+
	RMKKKDEGSYSL	EEPKQANGGAYQK	PTKQEEF$_p$YA	-	+
Syndecan-2	RMRKKDEGSYDL	GERKPSS-AAYQK	APTK-EFYA	+	-
Syndecan-3	RMKKKDEGSYTL	EEPKQAS-VTYQK	PDKQEEFYA	+	+
Syndecan-4	RMKKKDEGSYDL	GKKP-----IYKK	APTN-EFYA	+	-
	RMKKKDEG$_p$SYDL	GKKP-----IYKK	APTN-EFYA	-	-
	RMKKKDEGS$_p$YDL	GKKP-----IYKK	APTN-EFYA	++	-

FIGURE 2 | (A) Structural definition of syndecan binding to the PDZ domain. Ribbon diagram of the syntenin PDZ2 bound to the syndecan-4 peptide TNEFYA (pdb code 1 OBY, Kang et al., 2003), **(B)** Surface charge (blue positive and red negative) representation of the PDZ2 domain with syndecan-4 (magenta) peptide displayed as ball-and-stick. **(C)** Similar representation of PDZ2 with a model of syndecan-1 tyrosine phosphorylated peptide (yellow) showed as ball-and-stick. **(D)** Phosphorylation of Tyr and Ser residues within syndecan cytoplasmic tails and their effects on PDZ protein binding: (–) no interaction; (+) interaction; (++) enhanced interaction. The conserved (C1 and C2) and variable (V) domain organization is from Couchman et al. (2015).

involvement in the migration and growth of tumor cells, syntenin appears to be involved in controlling the plasma membrane localization of active β1-integrin (Kashyap et al., 2015). A recent study showed that co-upregulation of CASK and syndecan-2 in colorectal cancer is associated with an unfavorable prognosis (Wei et al., 2014), suggesting that CASK could be a prognostic factor for colorectal cancer metastasis. Synbindin was shown to contribute to the aggressiveness of gastric cancer by activating the ERK signaling pathway (Kong et al., 2013), while synectin was shown to participate in pancreatic cancer growth (Muders et al., 2006). As a guanine exchange factor for Rac1, tiam1 involvement in cancer biology may be linked to its pivotal function in cytoskeletal dynamics (Vigil et al., 2010). One study reported that syndecan-2 regulates colon carcinoma cell migration through Tiam1-dependent Rac activation (Choi et al., 2010).

REGULATION OF PDZ BINDING BY PHOSPHORYLATION OF THE SYNDECAN CYTOPLASMIC TAIL

The phosphorylation of Ser, Thr, or Tyr residues in the syndecan cytoplasmic tail appears to be a key mechanism that regulates its interactions with PDZ domains (**Figure 2B**). We reported that the formation of membrane protrusions in cells plated on immobilized laminin α3 chain LG45 domain required the dephosphorylation of tyrosine residues in the cytoplasmic tail of syndecan-1 (Sulka et al., 2009; Rousselle and Beck, 2013). Further experiments demonstrated that phosphorylation of the Tyr residue in its EFYA sequence abolished its interaction with syntenin-1 (**Figure 2C**, Sulka et al., 2009). In contrast, phosphorylation of this Tyr residue did not affect the binding of the PDZ domain of Tiam1 (**Figure 2D**, Shepherd et al., 2010; Liu et al., 2013). It is not known whether this holds true for phosphorylated syndecan-3 as well. Based on the examination of other syndecan-binding PDZ domains, the PDZ domains of CASK and synectin are predicted to interact with Tyr-phosphorylated syndecan-1 in a manner similar to that seen in the Tiam1 PDZ-phosphorylated syndecan-1 complex. This mechanism may support syndecan signaling specificity (Liu et al., 2013).

Other regulatory mechanisms involving the phosphorylation of a Ser residue in the carboxyl terminus of PDZ-binding proteins may either disrupts or enhances interactions with PDZ domains (**Figure 2D**, Cohen et al., 1996; Matsuda et al., 1999; Hegedüs et al., 2003). Studies of syndecan-4 revealed that phosphorylation of the Ser residue in the C1 region induces a conformational change in the C2 domain, even though the phosphorylation site is 20 residues away and impedes the PDZ binding ability of syntenin-1 (Horowitz and Simons, 1998; Koo et al., 2006). Furthermore, phosphorylation of the Tyr residue of the syndecan-4 C1 region was shown to enhance syntenin-1 binding and to function as a molecular switch to regulate specific integrin recycling and coordinate focal adhesion dynamics (Morgan et al., 2013).

These findings reinforce the importance of residues upstream of the EFYA motif in the regulation of PDZ domain interactions with syndecans. To date, there are no reports of the phosphorylation of Thr residues in terms of regulation of syndecan binding to PDZ domains. The phosphorylation of Tyr versus Ser residues depends upon which enzymes are involved. For example, Src family kinases and Elk kinases are widely reported to be the enzymes responsible for the phosphorylation of the Tyr residues (Asundi and Carey, 1997; Morgan et al., 2013). In contrast, PKCs are the only enzymes that have been reported to be involved in Ser residue phosphorylation (Prasthofer et al., 1995; Oh et al., 1997; Koo et al., 2006). Moreover, PKC can only recognize the Ser residue in syndecan-2 and syndecan-3, but not those in syndecan-1 and syndecan-4 (Prasthofer et al., 1995). Similarly, endogenously phosphorylated Tyr residues were only found on syndecan-1 and sydecan-4 in B82 fibroblasts, although this cell line also expresses syndecan-2 (Ott and Rapraeger, 1998).

Since phosphorylation is a key mechanism in modulating the interactions of syndecans with cytoplasmic proteins, the process is expected to be tightly regulated and some proportion of syndecans in a cell are expected to be in a phosphorylated state. Indeed, studies have found endogenously phosphorylated syndecans in cultured cells (Asundi and Carey, 1997; Ott and Rapraeger, 1998; Bass and Humphries, 2002; Morgan et al., 2013). These results illustrate the high level of complexity underlying the syndecans "turn on and off" signals.

CONCLUSION

The study of both the structural and biological aspects of the mechanisms underlying PDZ protein binding to syndecans is an exciting field of research. Due to their high level of complexity, the physiological significance of these interactions is not yet fully clarified; however, ongoing and future work will undoubtedly shed light on these important molecular complexes and their roles in cytoplasmic signaling pathways.

AUTHOR CONTRIBUTIONS

PR and BC wrote the manuscript. MM prepared **Figure 1**. LT designed structural models presented in **Figure 2**.

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Syntenin controls migration, growth, proliferation, and cell cycle progression in cancer cells

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The scaffold protein syntenin abounds during fetal life where it is important for developmental movements. In human adulthood, syntenin gain-of-function is increasingly associated with various cancers and poor prognosis. Depending on the cancer model analyzed, syntenin affects various signaling pathways. We previously have shown that syntenin allows syndecan heparan sulfate proteoglycans to escape degradation. This indicates that syntenin has the potential to support sustained signaling of a plethora of growth factors and adhesion molecules. Here, we aim to clarify the impact of syntenin loss-of-function on cancer cell migration, growth, and proliferation, using cells from various cancer types and syntenin shRNA and siRNA silencing approaches. We observed decreased migration, growth, and proliferation of the mouse melanoma cell line B16F10, the human colon cancer cell line HT29 and the human breast cancer cell line MCF7. We further documented that syntenin controls the presence of active β1 integrin at the cell membrane and G1/S cell cycle transition as well as the expression levels of CDK4, Cyclin D2, and Retinoblastoma proteins. These data confirm that syntenin supports the migration and growth of tumor cells, independently of their origin, and further highlight the attractiveness of syntenin as potential therapeutic target.

Keywords: syntenin, PDZ proteins, syndecan, cancer cell migration, cancer cell growth, cancer cell proliferation, cell cycle

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INTRODUCTION

Syntenin is strongly expressed during human fetal life and at relatively low levels in adult tissues (Zimmermann et al., 2001). Loss-of-function studies in Xenopus and zebrafish indicated that syntenin plays an important role in early developmental movements by controlling the non-canonical Wnt-signaling pathway (Luyten et al., 2008; Lambaerts et al., 2012), among others. Loss of syntenin function in mice has comparatively little effects. Indeed, Tamura et al. (2015) recently reported a mild phenotype in intestinal homeostasis.

Syntenin is detectable in adult human tissues, but an increasing number of independent studies indicate that syntenin is overexpressed in various patient tumor samples. Syntenin gain-of-function was first described in metastatic melanoma (Helmke et al., 2004), and more recently in breast cancer (Qian et al., 2013; Yang et al., 2013) and in multiple neuroepithelial tumors (Kegelman et al., 2014) suggesting that syntenin could be a tumor marker. For example, in breast

cancer patients, the correlation between syntenin expression, tumor size, lymph node status, and recurrence appears statistically significant (Yang et al., 2013).

In cellular models, by *in vitro* but also *in vivo* approaches with xenografts, several studies have shown that elevated syntenin expression is particularly relevant for invasion and metastasis (Koo et al., 2002; Boukerche et al., 2005; Das et al., 2013; Liu et al., 2014). Depending on the cellular context, syntenin has been associated with the activation of various signaling pathways, including SRC/p38MAPK/NFkB in human melanoma (Boukerche et al., 2005, 2007, 2008, 2010), in human glioblastoma multiform (GBM) (Kegelman et al., 2014), and in head and neck squamous cell carcinoma angiogenesis (Oyesanya et al., 2014), integrin β1/ERK1/2 in human breast cancer cells (Yang et al., 2013), EGFR/Akt/PI3K in urothelial cell carcinoma (Dasgupta et al., 2013), HIF-1α/IGFBP-2 in human melanoma angiogenesis (Das et al., 2013), and STAT3/PI3K/CTNNB1 in head and neck squamous cell carcinoma angiogenesis (Oyesanya et al., 2014).

Syntenin is a scaffold protein containing two Post synaptic density-95, Disc-large tumor suppressor and Zonula occludens-1 (PDZ) domains that we originally identified as an intracellular adaptor for the syndecan family of heparan sulfate (HS) proteoglycans (Grootjans et al., 1997). HS proteoglycans are highly abundant in adherent cells and their HS chains have numerous ligands, including various morphogens, adhesion molecules, and growth factors, such as Wnts, fibronectin and FGFs, whose deregulated signaling is involved in cancer development and progression (Fuster and Esko, 2005).HS plays an important role in the docking of these factors to cognate signaling receptors and can connect and regulate many signaling systems in a cell-type and cell-context dependent manner. Besides interacting with syndecans, the PDZ domains of syntenin can also directly interact with various membrane proteins and receptors (Beekman and Coffer, 2008), including Frizzled Wnt receptors that can rely on syndecans for their functions (Luyten et al., 2008). In structure–function studies, we demonstrated that syntenin allows syndecans and associated molecules to escape degradation by promoting their recycling to the plasma membrane (Zimmermann et al., 2005) or their secretion as exosomal cargo (Baietti et al., 2012; Ghossoub et al., 2014; Friand et al., 2015; Roucourt et al., 2015). These studies are entirely consistent with the observation that syntenin can boost various signaling pathways when overexpressed in cancer cells. The functional versatility of syndecans also explains that syntenin gain-of-function can support various signaling pathways and that specific effects can be cell-type dependent.

As a starting point to evaluate the potential benefit of anti-syntenin drugs, we here aimed to document and compare the impact of syntenin loss-of-function on the migration, invasion, growth, and proliferation of various model cancer cell lines.

MATERIALS AND METHODS

Cell Culture and Transient Transfections

HT29, MCF7, and B16F10 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HT29 cells were grown in McCoy's medium (Thermofisher Scientific), MCF7 cells in DMEM-F12 medium (Thermofisher Scientific), and B16F10 cells in DMEM medium (Thermofisher Scientific). Media were supplemented with 10% fetal bovine serum (FBS) (Thermofisher Scientific) and cells were incubated at 37°C under 5% CO2. For transient expressions, cells were plated 24 h earlier at a density of 1×10^5 cells per well in six well plates (BD Falcon) with 2 ml medium. 4 μl of Fugene HD reagent (Roche Applied Sciences) were added to 200 μl Opti-MEM solution (Thermofisher Scientific) and 1 μg plasmid DNA. The mixture was incubated for 20 min at room temperature before being added to the cells. For RNAi experiments, cells at a confluence of 50% were transfected with 20 nM RNAi using Lipofectamine RNAiMAX reagent (Life technologies, USA). Cells were analyzed after indicated time.

Expression Vectors and Reagents

RNAis targeting Syntenin and the non-targeting control RNAi (si Ctrl) were purchased from GE healthcare Dharmacon Inc (Human syntenin (5′-GCAAGACCUUCCAGUAUAA-3′), Mouse syntenin smartpool (M-043821-01)). For rescue experiments, syntenin cDNA was cloned in pcDNA3.1/Zeo(+) (Thermofisher Scientific) and mutated by directed mutagenesis on three nucleotides in the sequence targeted by the siRNA (CCTTCCAGT mutated to CCGTCGAGC).

Empty vector, control shRNA and the 29 mer human and mouse shRNA sequences cloned in pGFP-V-RS vector were purchased from Origene [control shRNA GCACTACCAGAGC TAACTCAGATAGTACT (TR30013), Human syntenin shRNA 2 (GCCTAATGGACCACACCATTCCTGAGGTT (TG309594B/G I338370), shRNA 3 (GTGGCTCCTGTAACTGGTAATGATGTT GG (TG309594C/GI338371), Mouse shRNA (TCAGGCTCAAA CTGCTTATTCTGCCAATC (TG512166A/GI574570)].

Statistical Analysis

Statistical Analysis was performed using the standard two-tailed Student *t*-test, and $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered statistically significant. Metamorph, Image J and ColonyDoc-It acquired data were processed with GraphPad Prism software.

Preparation of Cancer Cell Lines Depleted in Syntenin

Empty vector, control shRNA, and syntenin shRNAs expression vectors for mouse and human syntenin were transfected in phoenix packaging cells [ecotropic for Mouse and amphotropic for human cells (Life Technologies)]. Viral supernatants were harvested after 24–48 h intervals and used to infect cells for 48 h. Stable populations were selected for 10 days using optimal concentrations of puromycin as tested firstly on non-transduced cells (minimal concentration for death).

Generation of Syntenin Antibody

For the immunization, two rabbits were injected with two peptides, respectively, NEAEICESMPMVSGA and PSIM-KSLMDHTIPEV, corresponding to sequences found in the

N-Terminal and the C-terminal part of mouse syntenin-1 (Eurogentec). Crude sera from the two rabbits were pooled. Anti-syntenin antibodies were purified using the C-terminal peptide antigen with a carboxy terminal cysteine and a thiopropyl sepharose 6B column (Amersham). The unbound proteins were washed with sodium phosphate buffer. Antibodies were eluted with 0.1 mM glycine pH 2.5. Purified antibodies were immediately brought back to pH 7.5 using a solution of 1.5 mM TRIS-HCl pH 8.5. Antibodies were dialyzed overnight against PBS containing 0.01% sodium azide, 0.1% BSA, pH 7.5 overnight at 4°C and aliquots were stored at −20°C. The purified antibodies were shown to recognize mouse and human syntenin-1 using recombinant proteins. Their reactivity was lost on syntenin-1 CRISPR-Cas9 knock-out human cells and fibroblasts originating from syntenin-1 knock-out mice, attesting for their specificity.

Western Blotting

Cells were plated in 10 cm diameter dishes. After 48 h, cell lysates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare Life sciences). The membranes were blocked with 5% fat free milk and incubated with primary antibodies against Syntenin (homemade, see generation of syntenin antibody paragraph), β Actin (Santa Cruz, sc-69879), Tubulin (Sigma–Aldrich, USA), Cyclin D2 (Santa Cruz, sc-593), CDK4 (Cell signaling #2906), Retinoblastoma (BD Biosciences # 554136), and HRP-conjugated secondary antibodies. The membranes were washed with PBS/0.1% Tween buffer and antibody binding was revealed using enhanced chemiluminesescence (ECL) reagent (Thermo Scientific) according to the recommendations of the manufacturer. Signals were detected on photographic films (GE healthcare).

Wound Healing Assay

Cells were treated with 1 ng/ml mitomycin (Sigma M4287) overnight to inhibit cell division. Treated cells were plated in Ibidi culture-inserts (Ibidi Cat-80206) for 12 h to reach 90–95% of confluence. A wound was created by removing inserts from the dishes. Medium was refreshed to remove dead cells and the cells were observed under an inverted light microscope (Leica SP2) equipped with a camera. Images were taken by MetaMorph software every 10 min for 24 h. Measurements of cell velocity were calculated using the MetaMorph software.

Transwell Migration Assay

Cells were serum starved and treated with 1 ng/ml of mitomycin (Sigma M4287) for 12 h to inhibit cell division. Upper chambers (8 μm pores, Becton Dickinson, USA) cell chambers were placed in 24-well format transwell plates. Starved cells were plated in assay media (200 μl) (20 000 cells/well in the upper chamber). Lower chambers were pre-coated for 2 h with rat tail collagen I (Roche). To initiate migration, media containing 10% serum (600 μl) was placed in the lower chamber as attractant. After 48 h of incubation, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and stained with 0.5% crystal violet for 15 min at room temperature. Inserts were washed with PBS and air dried for 15 min. Cells on the upper side (non-migrating) of the filter were removed by swabbing with cotton wool. Migrating cells were imaged on a Zeiss Axioskop 2 fluorescence microscope using a 10x objective (Zeiss, NA 0.3) and a Spot RT/SE CCD camera (Diagnostics) in mosaic format. Cells were counted using ImageJ software cell counter.

Plate Colony Formation Assay

Cells were counted using Malassez slides and seeded at 1000 cells per 6 cm diameter dish in the usual media for each cell line (see above). The culture media was changed every 5 days. After incubation for 14 days, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and stained with 0.5% crystal violet for 15 min at room temperature. Pictures were taken; visible colonies were counted using an automatic colony counter (UVP, ColonyDoc-It^{TM} Imaging Station).

Soft Agar Colony Formation Assay

Agarose was melted in water and mixed with medium pre-incubated at 37°C at end-concentrations of 0.6 or 0.36%. A lower layer (0.6% agarose) was deposited in each well of a 96 well plates, except for the first and last rows and columns that were filled with PBS to avoid drying. Agarose was allowed to cool for 15 min before addition of the upper layer containing 2000 cells and 0.36% agarose. Plates were incubated at 37°C for 21 days in a 5% CO2 incubator and observed under a microscope at 24 h intervals. At the end of the experiments, cells were tested for their ATP content, an indicator of their metabolic activity, using the Promega kit G7570. In this assay, ATP is used as a co-factor for a luciferase reaction. Signals were analyzed by measuring luminescence with a LUMI starluminometer (BMG Labtech).

Cell Proliferation

1×10^5 cells were plated in 6 cm diameter dishes in six well culture plates. At each time point, a plate from each cell line was trypsinised, cells were collected in 2 ml of media and stained with Trypan blue. Cell counting was performed with Countess® Cell Counting Chamber Slides (Thermo fisher C10228).

Immunofluorescence Staining and Confocal Microscopy

Cells were cultured on glass coverslips, fixed with 4% PFA for 15 min, washed in PBS and then incubated with Integrin β1 antibody (BD 553715) in PBS containing 0.3% BSA and 0.05% saponin. Coverslips were mounted in DABCO/Mowiol and observed with a Zeiss Meta confocal microscope (LSM 510 META, Zeiss, France) with a UV laser and a 40× objective. Confocal images were analyzed and mounted using Photoshop (Adobe, San Jose, CA, USA) software.

Cell Cycle Analysis

MCF7 cells were transfected with non-targeting and Syntenin siRNAs in 10% serum. After 24 h cells were synchronized in G_1 phase by serum starvation for 24 h. After synchronization, cell cycle entry was recovered in fresh media and cells were analyzed at different time points. Therefore, cells were washed with cold PBS and fixed in 70% ethanol at 4°C for 30 min. After fixation

cells were stained with a 20 μg/mL propidium iodide solution (PBS containing 40 μg/mL RNase A) for 30 min at 37°C. Cell cycle distribution in G_1, S, and G_2/M phases was analyzed on the BD LSRFORTESSA (BD Biosciences) flow cytometer. The percentage of cells in each cell cycle phase was analyzed by FACS Diva software.

RESULTS

Generation and Validation of Various Cancer Cell Lines Depleted in Syntenin

To evaluate the impact of syntenin loss-of-function in cancer cells, we selected three different models: mouse melanoma B16F10 (mixture of spindle-shaped and epithelial-like cells, mutated for p53, RAS wild-type, highly metastatic and invasive), Human colorectal adenocarcinoma HT29 (epithelial cells, mutated for p53, RAS wild-type, highly metastatic, and invasive) and Human breast adenocarcinoma MCF7 (epithelial cells, ER-positive, p53, and RAS wild-type, non-metastatic, poorly invasive). B16F10 and HT29 cells, express higher levels of syntenin in comparison to MCF7 cells (**Figure 1A**). To generate cell populations stably depleted for syntenin, we used retroviral-mediated gene transduction of small hairpin RNAs (shRNA). One shRNA molecule (shRNA) and two different shRNA molecules (shRNA2 and 3) were used to efficiently knockdown syntenin expression in mouse and human cells, respectively (**Figure 1B**). After selection, the levels of syntenin were assessed by Western blot. The control shRNA, used as control, did not significantly affect syntenin expression when compared to empty vector transduced cells. shRNA targeting syntenin induced a significant decrease in syntenin expression in B16F10 cells (down by 80% compared to control levels), in HT29 cells (down by 70 and 75% compared to control levels for the two different shRNA, respectively) and in MCF7 cells (down by 60 and 65% compared to control levels for the two different shRNA, respectively) (**Figure 1C**).

Syntenin Loss-of-function Impairs Cell Migration

We then evaluated the effect of syntenin silencing on the migratory potential of the various cell lines. We measured cellular migration in wound healing (**Figure 2A** and Supplementary Figure 1A) and transwell migration assays (**Figure 2B** and Supplementary Figure 1B). To exclude any influence of cell proliferation on cell migration, we performed our experiments in the presence of 1 ng/ml of mitomycin to block cell division (Dow et al., 2007). Control shRNA, used as control, did not affect syntenin migration significantly when compared to empty vector transduced cells. shRNA targeting syntenin induced a significant decrease in cellular migration by wound healing assay in B16F10 cells (down by 44% compared to control levels), in HT29 cells (down by 35 and 37% compared to control levels for the two different shRNA, respectively) and in MCF7 cells (down by 28 and 35% compared to control levels for the two different shRNA, respectively) (**Figure 2A**). shRNA targeting syntenin also induced a significant decrease in cellular migration in transwell migration assays in B16F10 cells (down by 38% compared to control levels), in HT29 cells (down by 28 and 30% compared to control levels for the two different shRNA, respectively) and in MCF7 cells (down by 35 and 36% compared to control levels for the two different shRNA, respectively) (**Figure 2B**). In an attempt to identify mechanisms that could explain perturbed migration, we compared the tubulin cytoskeleton pattern, as well as the membrane distribution of active integrin β1 in control and syntenin-depleted cells in regular 2D cultures. While tubulin cytoskeleton did not display drastic differences (data not shown), the plasma membrane/cell borders staining of the active form of integrin β1 was clearly less marked in syntenin-depleted cells than in controls (shown for B16F10 cells) (**Figure 2C**). Altogether, these results indicate that syntenin expression supports B16F10, HT29 and MCF7 cancer cell migration.

Syntenin Loss-of-function Impairs Anchorage-independent Cell Growth and Cellular Proliferation

The role of syntenin in cell growth was investigated using *in vitro* plate colony formation assays (**Figures 3A,B**) and soft agar colony formation assays (**Figures 3C,D**). The number of colonies was significantly decreased in syntenin-depleted B16F10, HT29, and MCF7 cells compared to control cells, i.e., cells expressing empty vectors. In MCF7 cells, two different shRNA induced a drastic reduction of plate colony formation, decreasing, respectively, by 85 and 88% compared to control. In B16F10 and HT29 cells, the reduction of colony numbers was less drastic but still significant, with a decrease by 30% in B16F10 and by 36 or 38% in HT29 cells depending on the shRNA (**Figures 3A,B**). We also performed soft agar colony formation assays to test cellular abilities to form colonies in three dimensions because this assay is considered as the most stringent test for the detection of anchorage-independent tumor cell growth (Horibata et al., 2015). shRNA targeting syntenin induced a 49% decrease in the growth of B16F10 cells, a 20 or 33% decrease in the growth of HT29 cells and a 44 or 46% decrease in the growth of MCF7 cells (respective values for the two different shRNA) (**Figures 3C,D**). Control shRNA, used as control, did not significantly affect colony formation, neither in plate nor in soft agar assays, when compared to empty vector transduced B16F10 and HT29 cells. Surprisingly, control shRNA significantly affected colony formation of MCF7 cells in both assays. The reason for this specific effect in MCF7 cells is unknown. While *a priori* this observation does not change the conclusion that syntenin depletion affects the growth of MCF7 cells, it indicates that from a quantitative point of view, data should be interpreted with caution.

We also tested the effect of syntenin depletion by transiently downregulating syntenin expression, using small interfering RNA (siRNA). Effective syntenin knockdown by mouse and human syntenin siRNAs over a period of 4 days was validated by Western blot analysis (**Figure 4A**). In these experiments,

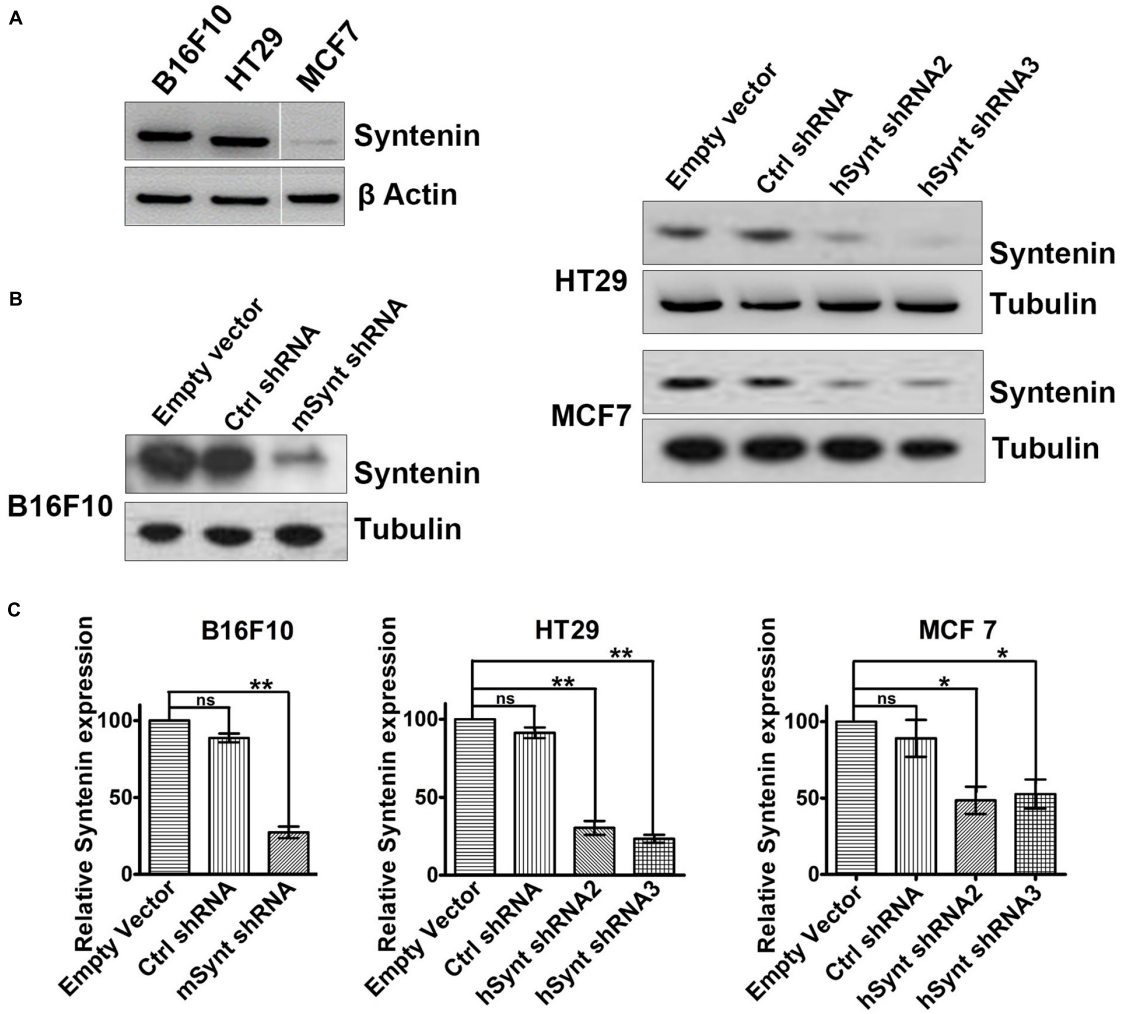

FIGURE 1 | Generation of cells with stable syntenin knockdown. (A) Western blots illustrating Syntenin basal expression in B16F10, HT29, and MCF7 cells, data are from the same blot. Actin was used as a loading control. **(B)** Western blots illustrating Syntenin expression levels after viral transduction with various constructs, as indicated. One small hairpin RNA (mSynt shRNA) and two different shRNA molecules (hSynt shRNA2 and hSynt shRNA3) were used to efficiently knockdown syntenin expression in mouse (B16F10) and human cells (HT29 and MCF7), respectively. Empty vector and ctrl shRNA were used as controls. Tubulin was used as a loading control. **(C)** Bar graphs indicate the average levels of syntenin expression, expressed relative to levels in empty vector transduced cells (taken as 100%). $n = 3$, bars represent mean value ± SD, n.s., non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

syntenin expression was downregulated by 90–95% compared to controls. We tested the proliferation of B16F10, HT29, and MCF7 cells transiently transfected with non-targeting control siRNA (si Ctrl) and syntenin siRNA (si Syntenin) by counting the number of viable cells every day over a period of 5 days (**Figure 4B**). Control B16F10 and HT29 cells (si Ctrl) showed a 30-fold increase in cell number after 5 days, while control MCF7 cells showed a 20-fold increase. Syntenin-depleted B16F10 and HT29 cells (siSyntenin) showed a 15-fold increase in cell number after 5 days, while syntenin-depleted MCF7 cells showed a 10-fold increase. A significant difference between controls and syntenin-depleted cells was observed in all models at day 2 and later times (**Figure 4B**). An effect of syntenin depletion on cell death could be ruled out because we observed in controls and syntenin depleted cells a similar extremely low number of Trypan blue (Supplementary Figure 2) and annexin-V positive cells (Supplementary Figure 3). To further validate syntenin effects on cellular proliferation, we also performed rescue experiments with MCF7 cells. Cells were treated with control (si Ctrl) and syntenin siRNAs (si Syntenin) for 24 h and then transiently transfected with an expression vector for wild-type syntenin mutated for the siRNA targeting sequence (syntenin OE) or the empty vector as a control. Total cell lysates analyzed by Western blot indicated rescue of syntenin expression above the control levels in MCF7 cells at 48h (**Figure 4C**), but these levels were still in the physiological range commonly observed in cell cultures. Rescued MCF7 cells showed a significant improvement of proliferation at day 2 and later, and 30-fold increase in cell number after 5 days of culture (**Figure 4D**). We assume that this increase in proliferation (by a factor 1.5 at day 5 when compared

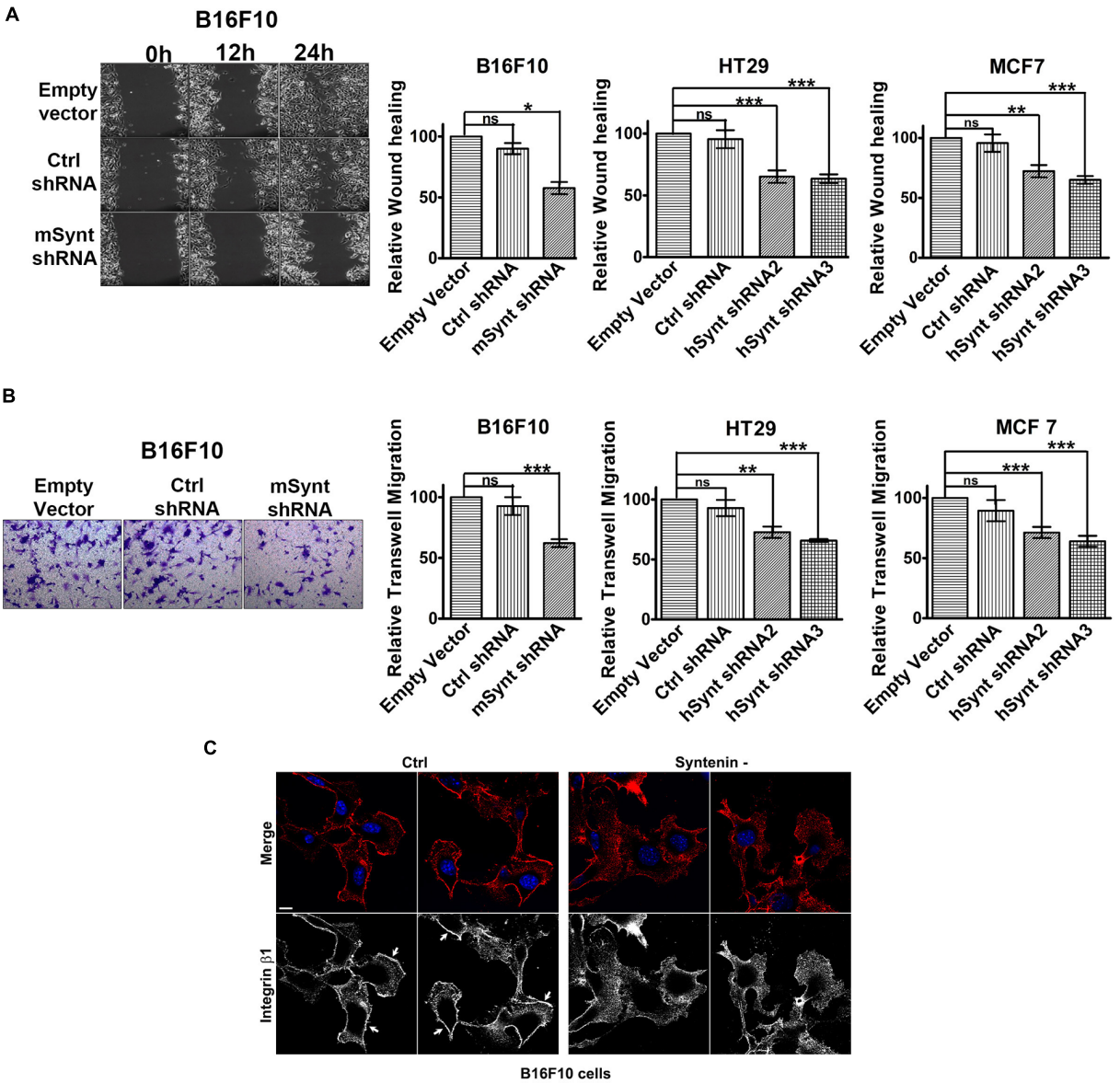

FIGURE 2 | Syntenin loss-of-function reduces the migration of mouse and human cancer cells. (A) Left: Phase-contrast micrographs illustrating migration by wound healing of B16F10 cells monolayers transduced with empty vector, control, or syntenin shRNA. Images were taken at different time points after wounding, as indicated. Right: Bar graphs indicate gap closures relative to closure in empty vector transduced cells (taken as 100%). n = 3, bars represent mean value ± SD, n.s., non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). **(B)** Left: Micrographs illustrating transwell-migration of B16F10 cells transduced with empty vector, control or syntenin shRNA. Right: Bar graphs indicate trans-migration relative to empty vector transduced cells (taken as 100%). Note that syntenin-depleted cells always migrate more slowly than control cells. $n = 3$, bars represent mean value ± SD, n.s., non-significant, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). **(C)** Confocal micrographs illustrating active integrin β1 staining (red in merge, blue corresponds to DAPI staining of the nuclei) in B16F10 cells treated with control (Ctrl) or syntenin siRNA (syntenin -), as indicated. Note the integrin β1 staining at the cell borders in controls (arrows) but not in syntenin-depleted cells. Scale bar, 10 μm.

to control cells-Si Ctrl in **Figure 4B**), might result from the slight gain of syntenin expression in rescued cells. Altogether, the above data indicate that syntenin supports the capacity of single cells to form colonies, anchorage-independent cell growth, and proliferation in B16F10, HT29, and MCF7 cells and that cellular proliferation might be directly correlated to syntenin expression levels.

Syntenin Loss of Function Impairs G1/S Cell Cycle Transition

To better understand the effect of syntenin loss-of-function on cell growth and proliferation, we tested whether cells were arrested in a particular phase of the cell cycle. Syntenin-depleted (si Syntenin) and control (si Ctrl) MCF7 cells were synchronized

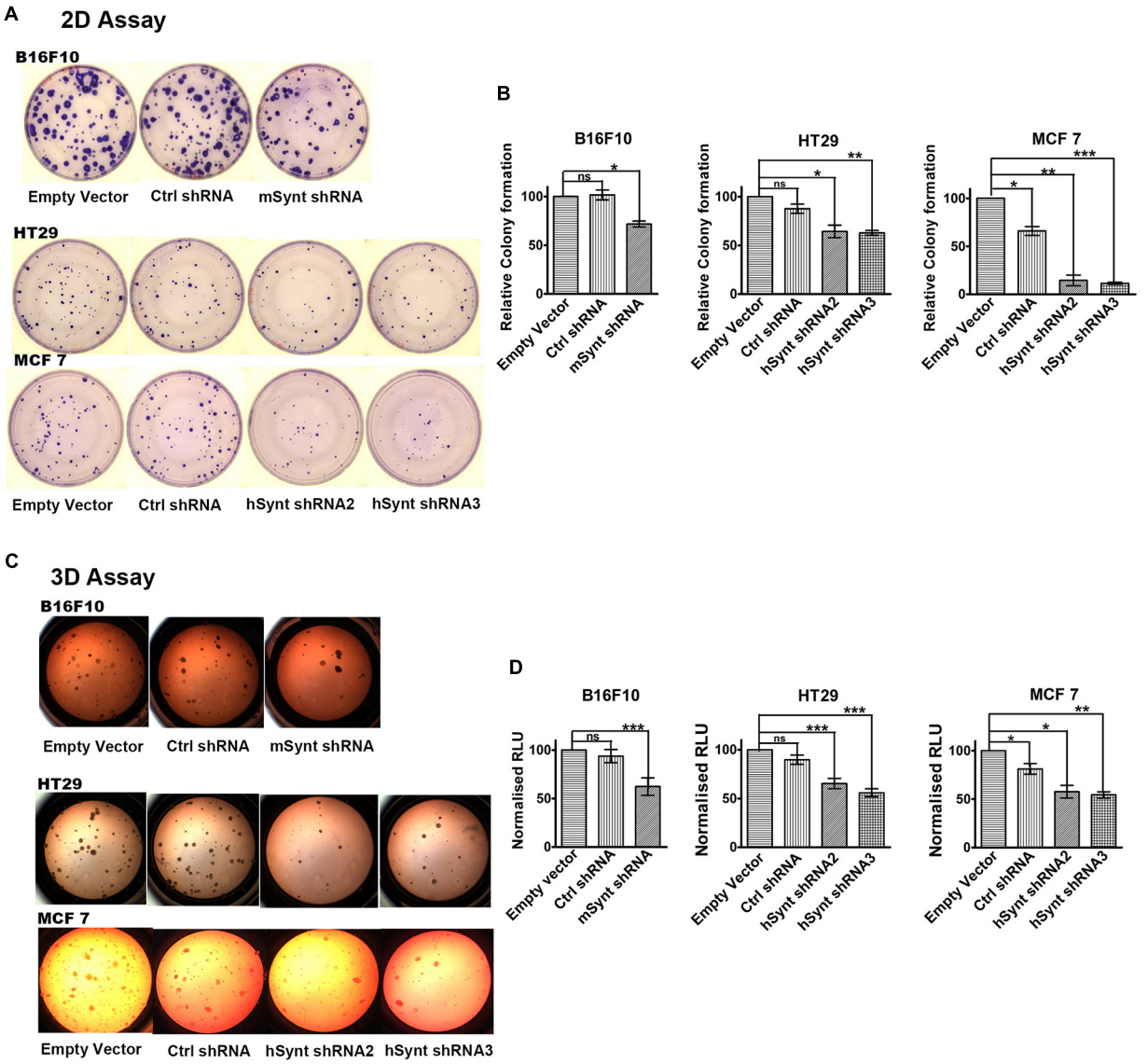

FIGURE 3 | Syntenin loss-of-function leads to reduced growth in different cancer cell models. (A) Representative images of colony formation assays with the different cell models. **(B)** Bar graphs indicate the number of colonies formed relative to colony numbers formed by empty vector-transduced cells (taken as 100%). $n = 3$, bars represent mean value ± SD, n.s., non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). **(C)** Representative images of soft agar colony formation assays with the different cell models. **(D)** Bar graphs indicate luciferase activities expressed in relative light units (RLUs), relative to activities measured in empty vector transduced cells (taken as 100%). Note that syntenin-depleted cells grow more slowly than the control cells. $n = 3$, bars represent mean value ± SD, n.s., non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test).

by serum starvation for 24 h. Cells were then serum-stimulated for 24 h and analyzed for different cell cycle phases by flow cytometry. Syntenin-depleted cells showed a significant increase in G1 phase and a significant decrease in S and G2/M phases when compared to control cells (**Figure 5A**). The effects of serum-stimulation on the different phases of the cell cycle were also analyzed at different time points over a period of 2 days in two independent experiments; see **Figure 5B** for one illustration. At early time points (from 0 to 12 h), control and syntenin-depleted cells did not show drastic differences in G1 phase (on average 73% in controls and 71% in depleted cells) but the percentage of S phase in control cells was twice more important compared to syntenin-depleted cells (on average 5.5% in controls and 2.5% in depleted cells). Starting from 18 h, S phase was increased to reach 19% at 48 h in control cells, while in syntenin-depleted cells, S phase poorly increased over time to reach at maximum 6% after 48h of serum stimulation. Moreover, the ratio of cells in G1, S, and G2/M stayed quite constant all along the experiment in syntenin depletion conditions on the contrary to what we observed in controls (**Figure 5B**). Taken together, these data suggest that syntenin depletion induces a defect in G1/S cell cycle transition. Of prime importance in this process

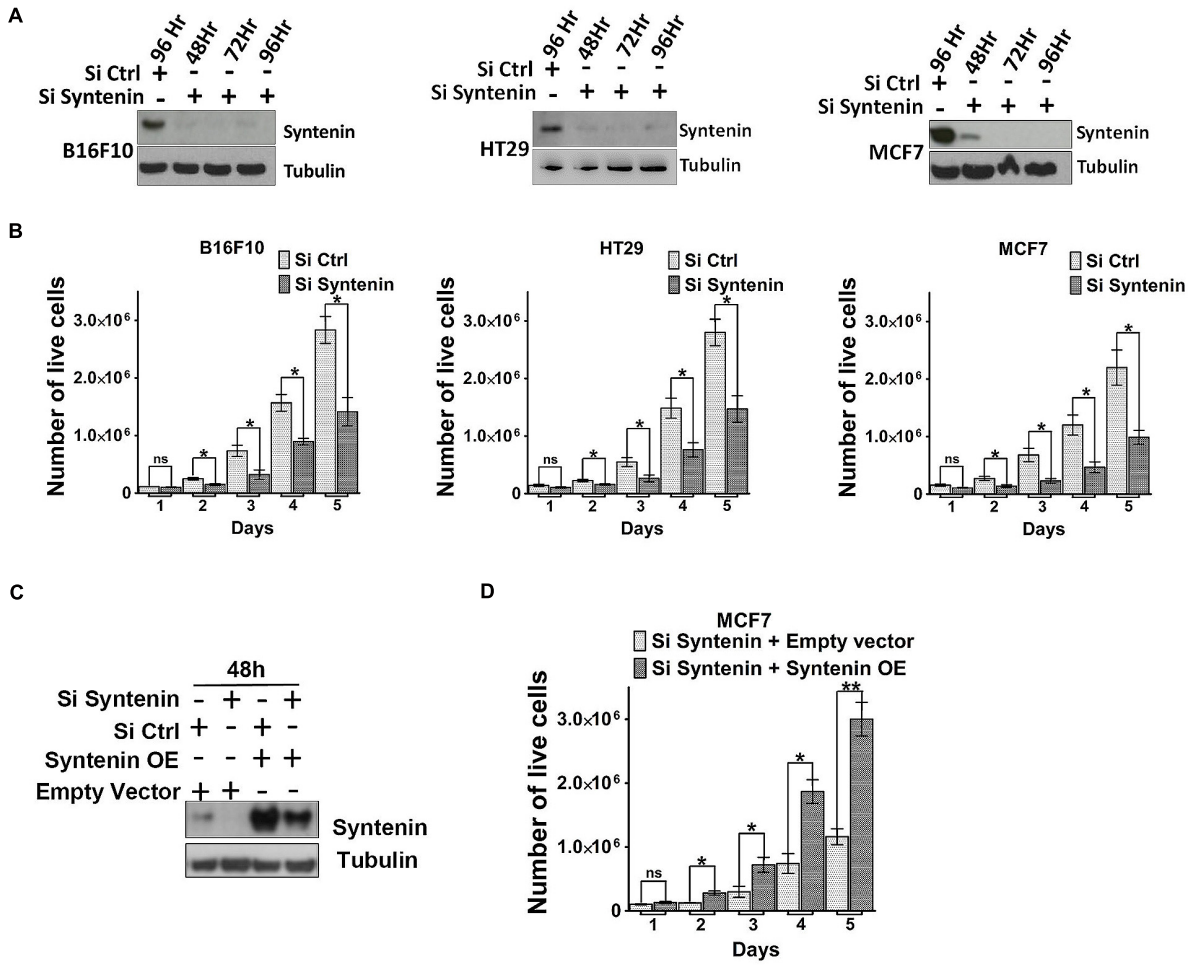

FIGURE 4 | Syntenin loss-of-function leads to reduced proliferation in different cancer cell models. (A) Western blots illustrating syntenin expression levels, at different time point, in B16F10, HT29, and MCF7 cells, after transfection with non-targeting (Si Ctrl) or syntenin (Si Syntenin) siRNAs. Tubulin was used as a loading control. **(B)** Bar graphs indicate the absolute number of living cells measured after different days of culture, as indicated. Note that significant differences were already observed at day 2. $n = 3$, bars represent mean value ± SD, n.s., non-significant, $^{*}P < 0.05$ (Student's *t*-test). **(C)** Western blots illustrating Syntenin expression levels in MCF7 cells 48 h after transfection with different constructs, as indicated. SiRNA Syntenin (Si Syntenin); non-targeting siRNA (Si Ctrl); expression vector for human Syntenin non-tagged and mutated for the siRNA targeting sequence (Syntenin OE); empty expression vector (Empty vector). Tubulin was used as loading control. **(D)** Bar graph indicating the absolute number of living MCF7 cells in Syntenin rescue experiments after different days in culture. $n = 3$, bars represent mean value ± SD, n.s., non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

is cyclin D2, which binds and activates cyclin-dependent kinases 4 (CDK4), thereby phosphorylating Retinoblastoma protein (Rb) and promoting progression from mid to late G1 (Sherr, 1994). We therefore tested for the expression levels of these three cell cycle regulators of G1/S transition by Western blot and observed that they are all significantly downregulated in syntenin-depleted cells compared to control cells (**Figures 5C,D**). Altogether, our data indicate that syntenin might control cell growth by acting on G1/S cell cycle transition.

DISCUSSION

Distinct assays and different cancer cell types (melanoma, colon and breast) from various origins (mouse and human) were used in this study to investigate the effects of syntenin knockdown. All assays showed that syntenin depletion significantly decreases tumor cell migration, growth, and proliferation. Our results with the commonly used B16F10 cells corroborate previously reported studies with other mouse melanoma models showing that syntenin overexpression promotes melanoma invasion, motility and anchorage-independent growth (Boukerche et al., 2005). Additionally, our experiments in human colon HT29 cells, show that depletion of syntenin reduces cellular migration, growth and proliferation. This is consistent with a study from Lee et al. (2011) showing that syntenin gain of function stimulates the migration of HT29 cells, and other colon cancer cells in transwell assays, while syntenin knockdown by siRNAs have the opposite effects (Lee et al., 2011). Although not directly tested in HT29, the same study also illustrated a role for syndecan-2-syntenin

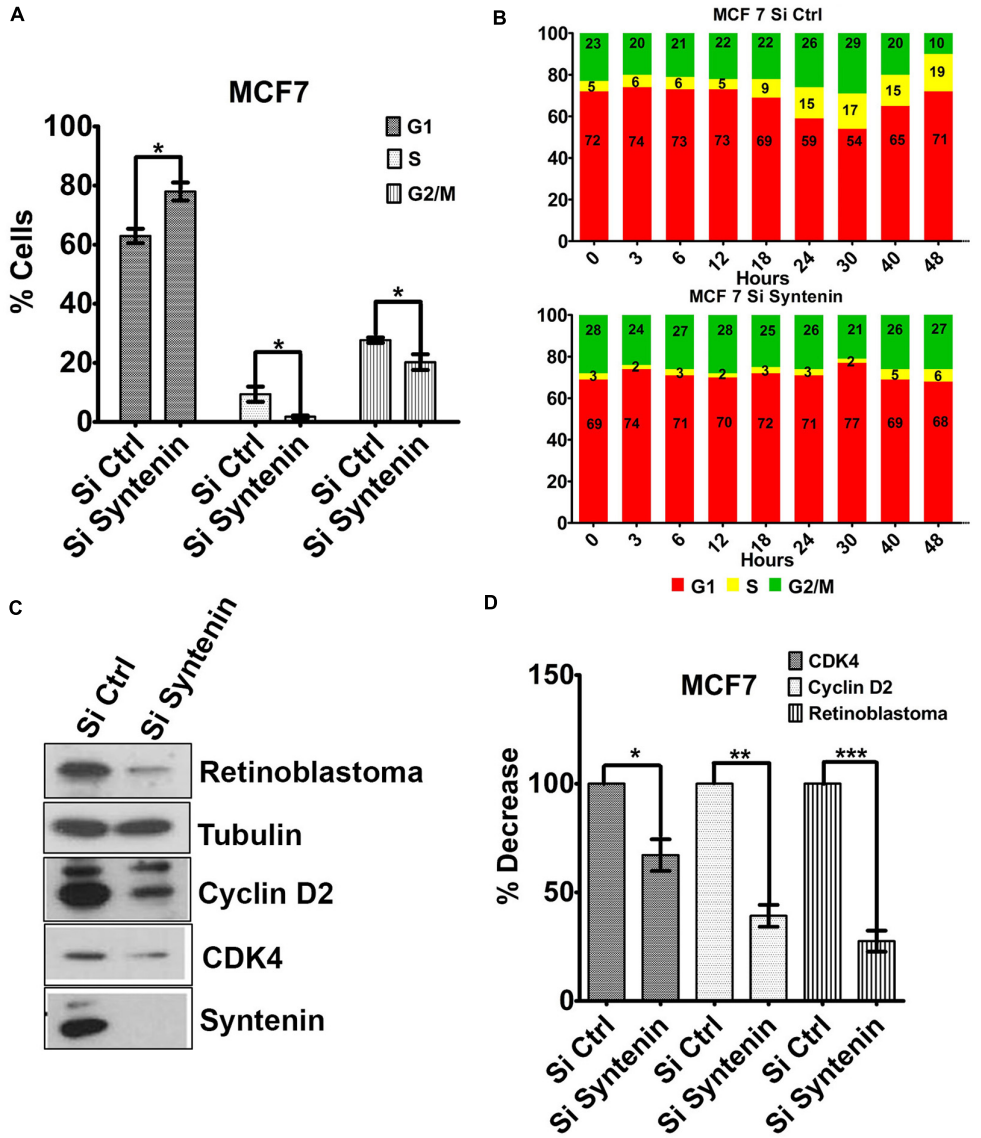

FIGURE 5 | Downregulation of syntenin impairs cell cycle G1/S transition in MCF7 cells. (A) Bar graph representing the percentage of cells in G1, S, and G2/M cell cycle phases after synchronization. Cells were transfected with non-targeting siRNA (Si Ctrl) or Syntenin siRNA (Si Syntenin). $n = 5$, bars represent mean value ± SD, $^*P < 0.05$ (Student's *t*-test). **(B)** Bar graphs illustrate one kinetic experiment indicating the percentage of cells in G1, S, and G2/M cell cycle phases after serum stimulation, at different time points, as indicated, in cells transfected with non-targeting siRNA (top) or Syntenin si RNA (bottom). **(C)** Western blot comparing the expression levels of different cell cycle markers, as indicated, in control cells (Si Ctrl) and Syntenin-depleted (si Syntenin) cells. Tubulin was used as loading control. Note that the expression of Cyclin D2, CDK4, and Rb is downregulated in syntenin-depleted cells. **(D)** Bar graph indicating the expression level of cell cycle markers in Syntenin-depleted cells relative to controls (taken as 100%). $n = 3$, 4, and 5 for Cyclin D2, Rb, and CDK4, respectively, bars represent mean value ± SD, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test).

interaction in the migratory potential of colon cancer cells, an observation directly in favor of our hypothesis that syntenin effects in cancer cells are primarily due to its effects on syndecan metabolism. Our results with the MCF7 breast cancer cells corroborate pioneer data from Koo et al. (2002) and more recent data by Yang et al. (2013). Indeed, Koo et al. (2002) identified syntenin by differential gene expression profile as a metastasis-related gene in breast cancer cells. They showed that gain of syntenin expression in MCF7 cells stimulates their migration and invasion in transwell assays. Moreover they identified syntenin PDZ2 domain, the syndecan high-affinity interacting domain of syntenin (Grootjans et al., 1997, 2000), to be responsible for the migratory stimulating effect. By studying a larger panel of breast cancer cells, Yang et al. (2013) found that syntenin expression levels correlate with the metastatic potential of these cells. Using the MDA-MB-231 breast cancer cell model, they revealed the impact of syntenin gain- and loss-of-function on the migratory and invasive behavior of these cells. Finally, using xenografts, they

showed that syntenin overexpression promotes tumor growth and lung metastasis *in vivo* (Yang et al., 2013).

Consistent with the low migratory rate, the data presented here show that syntenin-depleted cells present a defect in the plasma membrane localization of active β1-integrin. This observation is totally in line with previous data showing that, by allowing syndecan recycling, syntenin might support HS-dependent signaling molecules to be present and active at the plasma membrane (Zimmermann et al., 2005) and that, integrin trafficking is required for cellular adhesion and migration (Humphries et al., 2015). Integrin related or not, we observed a decrease in the percentage of cells in the most proliferative S and G2/M phases and a defect in the cell cycle G1/S transition phase in syntenin-depleted cells. We also documented that the growth regulatory molecules and G1/S key regulators, Cyclin D2 and CDK4 (Sherr, 1994) are downregulated upon syntenin inhibition. Alltogether, these observations are in line with a role for syntenin in tumor progression. Moreover, Cyclin D1 and CDK4 downregulation through syntenin depletion was previously reported in head and neck squamous cell carcinoma (Oyesanya et al., 2014). Cyclins D are synthesized as long as growth factor stimulation persists. Their destruction, in response to growth factors deprivation for example, results in the failure of cells to enter S phase. A potential explanation for the effects of syntenin depletion on Cyclins D and CDK4 would be that syndecan associated growth factor receptor systems such as FGF-2-FGFR1, are directed toward degradation instead of being recycled to the plasma membrane (Zimmermann et al., 2005). In this manner, syntenin-depleted cells would be less able to respond to serum stimulation and thus less able to stimulate Cyclins D synthesis and entry into the S phase. Yet, there is also a possible more direct explanation. Indeed, Cyclin D1 and Cyclin D2 present a class II PDZ-binding motif (PDZBM) at their C-terminal domain (DVDI and DIDL, respectively). A direct interaction between syntenin PDZ domains and Cyclins D PDZBM could also control G1/S transition as syntenin has also been detected in the nucleus (Zimmermann et al., 2001).

In agreement with our results in MCF7 cells, a study from Qian et al. (2013) showed that syntenin promotes MDA-MB-231 breast cancer cells G1/S transition phase (Qian et al., 2013). Interestingly, the authors also propose that syntenin levels in breast cancer cells is inversely correlated to estrogen receptor levels and propose that syntenin maintains the growth of breast cancer cells when estrogen-signaling pathway is not available. Although we similarly observed the negative correlation between syntenin levels and estrogen-receptor status (data not shown), this would not fit our hypothesis that syntenin effects in cancer are supported by its effect on syndecan trafficking. More importantly, our results with estrogen receptor-positive MCF7 cells demonstrate that even low levels of syntenin are sufficient to modulate cancer cell behavior.

CONCLUSION

Exploration of syntenin as a pharmacological target to inhibit cancer progression is probably a valuable objective with potential broad impact.

AUTHOR CONTRIBUTIONS

RK, BR., FL, JF, AC, AR, RG, carried out the molecular, biochemical, and cell biological work; RG, PZ supervised the research and wrote the manuscript; all authors were invited to revise the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2015.00241

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frontiers in Pharmacology

REVIEW
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Role of ErbB Receptors in Cancer Cell Migration and Invasion

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Growth factors mediate their diverse biologic responses (regulation of cellular proliferation, differentiation, migration and survival) by binding to and activating cell-surface receptors with intrinsic protein kinase activity named receptor tyrosine kinases (RTKs). About 60 RTKs have been identified and can be classified into more than 16 different receptor families. Their activity is normally tightly controlled and regulated. Overexpression of RTK proteins or functional alterations caused by mutations in the corresponding genes or abnormal stimulation by autocrine growth factor loops contribute to constitutive RTK signaling, resulting in alterations in the physiological activities of cells. The ErbB receptor family of RTKs comprises four distinct receptors: the EGFR (also known as ErbB1/HER1), ErbB2 (neu, HER2), ErbB3 (HER3) and ErbB4 (HER4). ErbB family members are often overexpressed, amplified, or mutated in many forms of cancer, making them important therapeutic targets. EGFR has been found to be amplified in gliomas and non-small-cell lung carcinoma while ErbB2 amplifications are seen in breast, ovarian, bladder, non-small-cell lung carcinoma, as well as several other tumor types. Several data have shown that ErbB receptor family and its downstream pathway regulate epithelial-mesenchymal transition, migration, and tumor invasion by modulating extracellular matrix (ECM) components. Recent findings indicate that ECM components such as matrikines bind specifically to EGF receptor and promote cell invasion. In this review, we will present an in-depth overview of the structure, mechanisms, cell signaling, and functions of ErbB family receptors in cell adhesion and migration. Furthermore, we will describe in a last part the new strategies developed in anti-cancer therapy to inhibit ErbB family receptor activation.

Keywords: ErbB receptors, cancer, epithelial-mesenchymal transition, migration, cell signaling

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Extracellular matrix (ECM) plays an essential role in the tumor progression. As part of the tumoral cell microenvironment, it contributes in the cell proliferation and migration, promoting tumoral growth and metastasis. Active migration of cancer cells from the primary tumor *via* lymphatic or blood vessel routes is an indispensable prerequisite for metastasis formation. Regulation of cancer cell migration processes is dependent upon many different signaling pathways as well as molecules of various classes and origins such as ECM components. Cytokines and growth factors, which regulate receptor kinases and related receptors with associated kinases play an important role in this regulation (Kedrin et al., 2007). Indeed, the ErbB family of receptor tyrosine kinases (RTK) includes epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and ErbB4 which

are expressed ubiquitously in epithelial, mesenchymal, cardiac, and neuronal cells. They are involved in a variety of cellular processes, including proliferation, survival, angiogenesis, and metastasis in many cancers. This review attempts to give an overview of current knowledge about structure, regulation and cell signaling of ErbB receptors. Finally, we summarize some of the recent developments in understanding the role of EGFR/ErbB signaling in epithelial mesenchymal transition and in cancer cell migration, its contribution to cancer progression, and the possibilities and challenges in targeting EGFR/ErbB signaling in cancer therapy.

STRUCTURE OF ErbB FAMILY RECEPTORS

Epidermal growth factor (EGF) was one of the first growth factors discovered in the early 1960s. It was shown to be a polypeptide able to stimulate growth and differentiation of cells of epidermal and mesodermal origin (Cohen, 1983). Subsequent studies identified the receptor and the receptor's intrinsic kinase activity. EGF was shown to bind with high affinity to a specific receptor located in the cell membrane and stimulate rapid activation of a protein kinase activity. The EGFR was purified and characterized as a ∼170 kDa molecular weight integral membrane glycoprotein, bearing ligand-inducible kinase activity. The EGFR kinase activity was shown to result in phosphorylation of tyrosine residues, the first such demonstration for any receptor. It was also found that ligand binding induces receptor clustering and that antibody cross-linking mimics the effects of EGF, indicating the importance of receptor dimerization/oligomerization in its activation. Cloning of the human receptor was performed in Ullrich et al. (1984). Analysis of the sequence confirmed previous data, confirming the glycoprotein nature of EGFR, and the presence of a tyrosine-specific protein kinase sequence. Molecular cloning of EGFR also revealed a close similarity with the viral v-erbB oncogene, yielding the first indication of a link between growth factor receptors and cancer. Cloning techniques also revealed the existence of three related membranes receptors, which were called ErbB2–4, or Human EGF Receptor (HER) and share the overall primary structure of EGFR. Furthermore, during activation mechanism, the four members of the family can form various heterodimers, potentially yielding a wide array of signaling outcomes. Subsequent work allowed for a more precise delineation of the different domains composing the receptor (**Figure 1A**) (Ceresa and Peterson, 2014; Roskoski, 2014).

Based upon the primary amino acid structure of EGFR, the four ErbB receptors consist of a large extracellular domain, a single hydrophobic transmembrane segment, and an intracellular domain consisting of a juxtamembrane domain, a typical tyrosine protein kinase segment, and a tyrosine-rich carboxyterminal tail. Upon receptor activation, a number of these C-terminal tyrosines are phosphorylated. The extracellular domain itself is made of a tandem repeat of two types of subdomains: domains I and III, which are leucine-rich segments that make up the ligand binding, and cysteine-rich domains II and IV. Domain II participates in homo and heterodimer formation with ErbB family members (see below).

The first crystallographic view of the EGFR kinase domain confirmed its likeness with previous published protein kinase structures, with two lobes defining an ATP-binding cleft. But this provided little insight into how the kinase is activated by receptor dimerization. Analysis of additional crystal structures of the active EGFR kinase domain revealed a characteristic asymmetric dimer (Zhang et al., 2006). In this dimer, the large carboxy lobe of one kinase binds the small amino lobe of the other kinase domain (**Figure 2B**). This is reminiscent of the activation mechanism of the cyclin-dependent kinases (CDKs) and Src family kinases.

NMR structures have also been published for the dimeric transmembrane segments of the ErbB receptors, and their importance in the ligand-induced activation of the EGFR has been confirmed very recently in a series of papers by the Kuriyan group (reviewed in Endres et al., 2014). In this work, an assymetric interaction of the juxtamembrane domains was also described upon ligand-induced rearrangements of the receptor structure, leading to kinase domains interactions and activation.

Regulation of ErbB Activity: Mechanisms of Dimerization and Activation

As previously described (see above), in the general case, growth factors bind RTKs, as ErbB receptors, which induce their dimerization and subsequent activation (Ullrich and Schlessinger, 1990). A multitude of extracellular polypeptide ligands can bind ErbB receptors. Indeed, numerous growth factors have been described as ligands for this receptor family, and these polypeptides are divided into four group. EGF receptors bind to EGF, epigen, transforming growth factor, and amphiregulin. Betacellulin, heparin-binding EGF-like growth factor, and epiregulin bind to EGFR and ErbB4. The third group -which binds to ErbB3 and ErbB4- includes neuregulin-1 and neuregulin-2. The last group of ligands binds to ErbB4 and consists of neuregulin-3 and neuregulin-4 (Roskoski, 2014). ErbB2 has no known ligand. These ligands exist usually in a proform as transmembrane precursors which submit a proteolytic processing to release the soluble, active N-terminal ectodomains (Massague and Pandiella, 1993; Singh and Harris, 2005).

Deeper insights in the structure-function relationship of the ErbB receptors were published in the early 2000s with a series of crystal structures of the EGFR extracellular region with and without bound ligand (**Figure 1B**). The EGFR extracellular region dimerization is mediated entirely by receptor–receptor contacts, by a "dimerization arm" that projects from domain II (Lemmon and Schlessinger, 2010). In fact, two different conformational states have been described for the extracellular region of EGFR, ErbB3, and ErbB4 (**Figure 2**) (Cho and Leahy, 2002; Bouyain et al., 2005; Dawson et al., 2005). The first one is the inactive conformation: in the absence of ligand, the EGFR adopts a monomeric, compact, "tethered" conformation (**Figure 2A**) which presents an intramolecular tether between domains II and IV of the extracellular region (**Figure 2A**). This autoinhibited state prevents interaction between subregions I and III to form

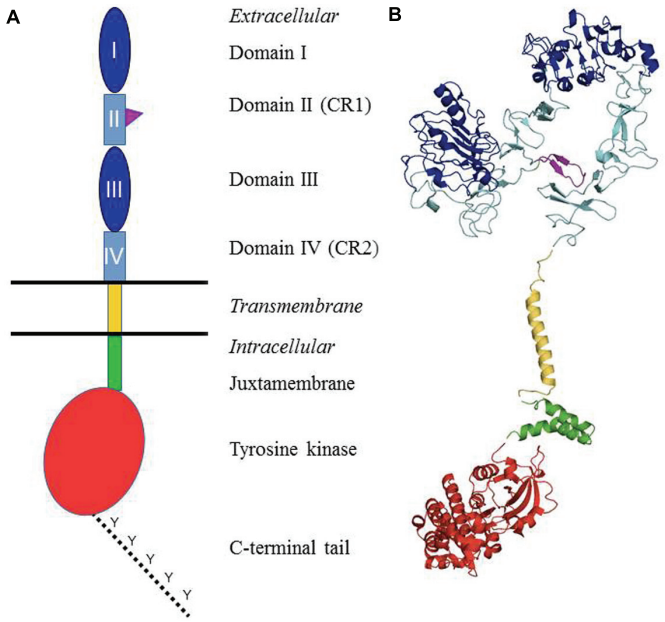

FIGURE 1 | Structural organization of the ErbB/HER receptors. (A) Shows a schematic representation of the different domains. The extracellular part of the receptors is composed of four domains: I and III = ligand-binding domain (dark blue); II and IV = cysteine-rich domains (light blue). The domain II contains the dimerization arm (purple). It is followed by the single transmembrane domain (yellow), a juxtamembrane domain (green), the tyrosine kinase domain (red) and a C-terminal tail which contains the main tyrosines that are phosphorylated upon receptor activation (dotted line). **(B)** Presents the structures of different domains of the human EGFR which have been established through X-ray crystallography or solution RMN. Color coding is identical to **(A)**. Protein Data Bank (PDB) accession codes are as follows: ectodomain (in closed unliganded conformation), 3QWQ, transmembrane region, 2KS1, juxtamembrane domain, 1Z9I; kinase domain, 3W32. No structure is available for the C-terminal tail.

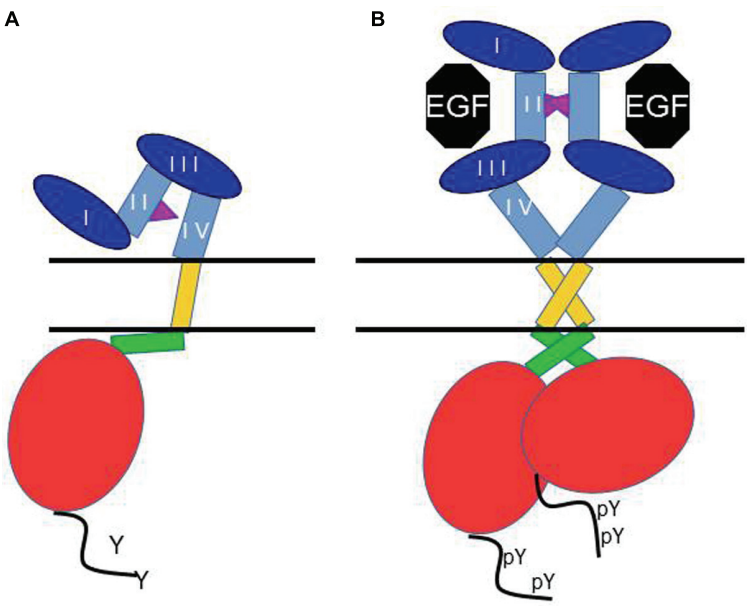

FIGURE 2 | Schematic of the current view of main structural events in the activation of the EGF receptor. In **(A)** the receptor is depicted in its monomeric, unliganded, inactive form. The dimerization arm of the extracellular domain II binds to domain IV, and the juxtamembrane domain interacts with membrane phospholipids. In **(B)** binding of EGF to one monomer to domains I and III induces a conformation change which makes the dimerization arm available for interaction with another extended ligand-bound monomer, and causes dimerization. This conformational change is accompanied by the formation of an anti-parallel interaction between the two juxtamembrane domains, and thus an asymmetric "head to tail" interaction of the two kinase domains, resulting in allosteric activation of the kinase, and C-terminal tail tyrosine phosphorylation.

a ligand-binding pocket which holds the extracellular domain in a closed conformation (Riese et al., 2007; Fuller et al., 2008). In the active conformation, the crystal structures reveal a dimeric, extended conformation where the ligand bridges domains I and III, thereby opening the structure. The subregions I and III rearrangement results in the ligand-binding pocket formation that permits interactions between a single ligand molecule and these domains I and III (**Figure 2B**) (Burgess et al., 2003; Jorissen et al., 2003). The main focal point of movement resides in a "hinge" domain at the junction of domains II and III (Burgess et al., 2003). Thus, in the presence of ligand, there are no more intramolecular interaction between domains II and IV, resulting in exposure of the dimerization arm of domain II. This allows receptor dimerization *via* intermolecular contacts that involve mostly the dimerization arm in subregion II (**Figure 2B**). A small region, C-terminal of the dimerization arm, in domain II as well as part of domain IV are also involved in the dimerization, albeit to a lesser extent (Dawson et al., 2005). ErbB2 differs significantly from this scheme, in that it has no known ligands, but the structure of its extracellular domain shows an extended configuration, seemingly poised for hetero-interactions with other ErbB family members.

Thus, the model for receptor activation which has been proposed is as follows: unliganded EGFR, ErbB3 and ErbB4 receptors exist in an autoinhibited form that undergoes domain rearrangement to an active form after ligand binding. This rearrangement juxtaposes domains I and III breaking the domain II–IV tether and unmasking the domain II to participate in receptor dimerization and activation of signal transduction.

After homo- or heterodimerization, the activation of intrinsic protein kinase activity at the intracellular c-terminus results in the stimulation of the intrinsic catalytic activity of the receptor and phosphorylation of specific tyrosine residues of the receptors (Bennasroune et al., 2004b). These molecular mechanisms associated with RTK activation have been described by biochemical and structural studies, and imply structural modifications (Hubbard, 1999; Hubbard and Till, 2000). The precise molecular mechanism vary somewhat between the different families of RTKs. In many cases (insulin receptor, Eph, PDGF receptor, . . .), it is the autophosphorylation of an activation loop in the kinase domain which is responsible for the transition to the active kinase conformation. This is not the case for ErbB receptors for which the transition to the active form is rather due to the formation of an asymmetric dimer of the kinase domains, in which one kinase allosterically activates the other one. The kinase domains then catalyze the phosphorylation of tyrosine residues (outside the kinase domain in the C-terminal tail) creating docking sites for adaptor proteins or enzymes involved in downstream signal transduction.

Several downstream signaling pathways are activated after specific ErbB receptor activation (by homo- or heterodimerization) resulting notably in actin polymerization and intracellular organization necessary for migration and invasion of epithelial cells (Feigin and Muthuswamy, 2009). When ligands bind to ErbB receptors, they trigger a cascade of biochemical events inducing stimulation of rich signaling pathways. This intracellular signaling involves a variety of molecules known as adaptors and scaffolding proteins (Pawson and Scott, 1997). For example, Grb2 is an important adaptor in the activation of the ras/raf/MAPK pathway. These adaptors often feature several motifs that mediate interactions between intracellular proteins: Phosphotyrosine-binding (PTB) and Src homology 2 (SH2) domains specifically bind to phosphotyrosine, whereas SH3 domain binds to proline-rich sequences of target proteins. Thus, these adaptor molecules permit to recruit specific proteins to establish signaling networks particular to a cascade and a cell location.

Among these signaling cascades, ErbB receptor activation is associated (i) with the phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) pathway which plays a key role in cell survival, (ii) and with the Ras/Raf/MEK/ERK1/2 and the phospholipase C (PLCγ) pathways mediating cell proliferation (Yarden and Pines, 2012). In the following chapter, we will focus on the role of ErbB family receptors in epithelial-mesenchymal transition (EMT), migration, and tumor invasion of cancer cells.

ROLE OF ErbB RECEPTORS IN CANCER AND NEW STRATEGIES DEVELOPED IN ANTI-CANCER THERAPY

ErbB receptors were linked to human cancer pathogenesis by about three decades ago. For example, EGFR and ErbB2 are mutated in many epithelial tumors and clinical studies suggest that they play an important role in cancer development and progression. These receptors have been largely studied, not only to understand the mechanisms underlying their oncogenic potential, but also to exploit them as putative therapeutic targets. In this part, we will focus on the role of ErbB receptors in EMT, migration and tumor invasion. Then, we will summarize the new therapeutic approaches to inhibit ErbB receptor activation in cancer.

Role in Epithelial-mesenchymal Transition, Migration, and Tumor Invasion by Modulating Extracellular Matrix Components

ErbB receptors influence cell proliferation, differentiation, and migration. Not surprisingly, alterations of ErbB familly play a role in the development and progression of several epithelial tumors (Yarden and Sliwkowski, 2001). Cancer cell migration and invasion allow tumor spread into surrounding tissues and circulation which generates metastasis, a significant hallmark of poor prognosis (Friedl and Wolf, 2003). Overexpression of EGF and its receptors has been demonstrated in many breast cancers and was associated with a higher incidence of distant metastases (De Luca et al., 2008; Giltnane et al., 2009).

Two types of cell migration exist: mesenchymal- and amoeboid-type migration (Friedl and Wolf, 2003). Mesenchymal-type cell migration is characterized by protrusion formation such as filopodia and lamellipodia at the leading edge of migrating cells and by adhesions of these protrusions linking the actin cytoskeleton to the extracellular matrix (ECM;

Parsons et al., 2010). Adhesion disassembly at the cell rear and the contraction of actomyosin then allows the cell to achieve cellular movement (Parsons et al., 2010). In carcinoma, the most prevalent form of all human cancers (80–90%), malignant transformation is associated with the loss of differentiated epithelial characteristics and a coinciding increase of less-mature mesenchymal characters during EMT. In cancer, EMT induces tumor progression by affording properties such as invasiveness, the ability to metastasize, resistance to therapy, and possibly the generation of stem-like cancer cells (Mallini et al., 2014).

Members of the ErbB receptor family play prominent roles during carcinogenesis, and most induce EMT when overexpressed both *in vitro* and *in vivo* (Al Moustafa et al., 2012). In line with the hypothesis that EGF family members play a fundamental role in the initial steps of EMT, transformation by HER2/neu resulted in increased CD44high/CD24low immortalized human mammary epithelial cells with many of the stem-like properties of the initial steps of EMT (Morel et al., 2008). In oral squamous cell carcinoma cells, inhibition of EGFR induced a transition from a fibroblastic morphology to a more epithelial phenotype with an accumulation of desmosomal cadherins at cell–cell junctions (Lorch et al., 2004). These studies suggest that EGFR signaling mediates the initial steps of EMT, and that EGFR inhibition may restrain EMT in some cellular contexts. In fact, ligand-independent, constitutively active forms of EGFR can increase motility and invasiveness of tumor cells, and EGFR inhibitors block cancer cell migration *in vitro*. Cellular migration and invasion is inhibited by blocking EGFR and consequently its pathways, by a monoclonal antibody (mAb) or a tyrosine kinase inhibitor (TKI), suggesting a crucial role for EGFR inhibitors in the control of cancer metastasis (Yue et al., 2012; Liu et al., 2014). Furthermore, Jeon et al. (2015) demonstrated that HER2 expression level plays an important role in the induction of fibronectin expression, a major component of ECM, in breast cancer cells that triggers cell adhesion and cell invasion.

Several studies have revealed that HER2 is expressed in circulating tumors cells of early and metastatic breast cancer patients. The consequences of HER2 expression are usually more severe in circulating tumors cells in comparison to the corresponding primary tumors (Kallergi et al., 2008; Fehm et al., 2010). Indeed, circulating tumor cells and metastases of breast cancer present a dynamic *in vivo* pattern of EMT (Bonnomet et al., 2012). $CD44^{+}/CD24^{-}$ subpopulation of tumor cells which overexpressed RAS or HER2 have a phenotype with increased EMT potential (Wang et al., 2012; Bhat-Nakshatri et al., 2013). The $CD44^{+}/CD24^{-/low}$ gene expression signature, identified as a "claudin-low" molecular subtype (Creighton et al., 2009), is characterized by expression of many EMT-associated genes, such as FoxC2, Zeb, and N-cadherin (Morel et al., 2008; Creighton et al., 2009). EMT in breast cancer stem cells could play an important role in the metastatic phenomenon (Korkaya et al., 2012; Wang et al., 2012). Furthermore, several studies have highlighted that HER2 regulates the stem cell population and then contributes to mammary carcinogenesis (Bedard et al., 2009) and that HER2 overexpression in multiple breast cancer cell lines results in an increase of $ALDH1^{+}$ cell fraction, which has a greater capacity to invade and form tumors in immunodeficient mice (Korkaya et al., 2008).

Integrins, focal adhesion kinase (FAK), and Rho GTPases (Rho, Rac, Cdc42) are important regulators in mesenchymal-type migration (Parsons et al., 2010) and may be influenced by EGFR signaling. Indeed, ErbB signaling induces cell adhesion and migration by modulation of e.g., FAK or Rho GTPases (Fichter et al., 2014). For example, Fichter et al. (2014) showed that inhibition of EGFR signaling in esophageal squamous cell carcinomas rearranges the actin cytoskeleton, induces focal adhesions, and limits esophageal cancer cell migration by rapid inhibition of ERK1/2, Akt, STAT3, and RhoA activity. However, as (i) Zhan et al. have shown that only EGFR/ErbB2 heterodimers increased the invasive potential of mammary epithelial cells which is not observed with homodimers (Zhan et al., 2006), and as (ii) Guy et al. (1992) described that EGFR overexpression in mice was not associated with transformation of the entire mammary epithelium, but provoked only focal mammary tumors (sometimes metastatic), these results suggest that additional mechanisms are probably involved in ErbB activation effects on EMT and cell invasion.

Cancer cells secrete EGF-like, growth factors that can play a role directly on endothelial cells (Kuo et al., 2012). The microenvironment can also act on tumor cells. Indeed, EGF-like peptides and angiogenic growth factors that can both act on endothelial cells and activate EGFR in cancer cells are produced by bone marrow stromal cells (Fidler, 2002). EGFR activation in human carcinoma cell lines also increases matrix metalloproteinase-9 (MMP-9) activity, which increases *in vitro* cell invasion by facilitating disintegration of ECM barriers to tumor invasion (Zuo et al., 2011).

During the mesenchymal mode of invasion, the presence of proteases in ECM that can degrade the surrounding ECM (Sahai and Marshall, 2003; Wolf et al., 2003) will cause the liberation of small peptides originating from the fragmentation of ECM proteins. These molecules called matrikines limit EGFR signaling to the perimembrane area of the cytosol, a mode that is preferential for motility (Iyer et al., 2008) and cell survival (Fan et al., 2007; Rodrigues et al., 2013). Tenascin C (TNC) establishes interactions between the epithelium and the mesenchyme during embryonic development, tissue differentiation and wound repair but persistent high levels of TNC are present in various tumor tissues, including brain, bone, prostate, intestine, lung, skin, and breast (Pas et al., 2006). TNC is a hexameric glycoprotein of which each subunit contains: the N-terminal assembly domain, a domain composed of 14.5 EGF-like repeats (EGFL), a domain composed of a varied number of fibronectin type III-like repeats, and a fibrinogen-like sequence on the C terminus (Orend and Chiquet-Ehrismann, 2006). The EGF-like repeats of TNC also have counter-adhesive properties (Spring et al., 1989; Prieto et al., 1992) and have been shown to bind and signal through the EGFR (Swindle et al., 2001; Iyer et al., 2007). Interestingly, the binding of TNC EGFL to EGFR preferentially promotes cell migration by limiting receptor signaling to the perimembrane space (Iyer et al., 2008). Indeed, the binding of TNC EGFL to the receptor does not induce ligand-induced internalization of the receptor (Iyer et al., 2007). Thus, essentially all of the EGFR signaling occurs from

the plasma membrane locale. Based on the results obtained on the signaling, authors propose that plasma membrane-associated signaling of EGFR is preferential for motility. Others matrikines derived from Thrombospondin 1 and Laminin-332 feature EGFL domains that have been shown to bind and activate EGFR (Schenk et al., 2003; Liu et al., 2009).

This section has highlighted the importance of the ErbB family receptors in regulating EMT during cancer. Understanding and defining the initial molecular signals leading to the EMT switch in tumor cells would absolutely participate to the earliest possible clinical detection and therapeutic strategies. While the use of inhibitors delivered individually to ErbB/EGF targets seems reasonable, limited effect suggests that a combinatorial approach could permit substantial improvements in clinical outcome. Enlightening the steps that induce the re-activation of embryonic processes and signaling pathways in cancer, such as those involved in EMT, and best understanding the interactions between cells and their microenvironment, will lastly lead to more rational strategies in our arsenal for targeting cancer.

New Strategies Developed in Anti-cancer Therapy to Inhibit ErbB Family Receptor Activation

Advances in genetic engineering and fundamental research applied to a better understanding of the biology of ErbB signaling in cancer have led to the development of many therapeutic agents including monoclonal antibodies (mAbs), small-molecule TKIs and other agents like peptides, affibodies, nanobodies, etc. (Bennasroune et al., 2004b; Alaoui-Jamali et al., 2015). In this paragraph, we present a partial overview of current development of drugs targeting ErbB receptors. **Table 1** presents several examples of drugs, their targets and their current status in term of clinical trials.

Cetuximab and Panitumumab are mAbs that bind to EGFR, possessing anti-tumor activity. They are frequently used in treatment of metastatic colorectal and head/neck cancer. Cetuximab is a chimeric human: murine immunoglobulin G1 (IgG1) mAb. It binds to EGFR with higher affinity that EGF (Kim et al., 2001) and also binds to the mutant receptor EGFRvIII. The cetuximab promotes EGFR internalization (Sunada et al., 1986). Panitumumab (ABX-EGF), fully human mAb with high EGFR affinity, blocks ligand-binding and induces EGFR internalization (Yang et al., 2001). Activity of Panitumumab has been demonstrated against variety of advanced cancer patients, including renal carcinomas and metastatic colorectal cancer in clinical trials (Douillard et al., 2010). Another antibody against a second member of ErbB receptor family has been developed. Trastuzumab or Herceptin selectively binds to the extracellular domain of HER2 receptors and inhibits downstream signaling pathways. This inhibition results in decreased proliferation of tumor cells. Trastuzumab identifies tumor cells for immune destruction, and then, promotes an antibody-dependent cellular cytotoxicity, causing apoptosis of tumor cells (Molina et al., 2001). Trastuzumab is predominantly used in the treatment of the ErbB2-positive breast cancer subtype where its combination with conventional chemotherapy, had a significant effect on disease free survival of patients with early stage ErbB2+ breast cancer (Hudis, 2007).

Currently, small molecule inhibitors under clinical trials or approved by the US Food and Drug Administration (FDA) are reversible or irreversible inhibitors. They bind to the ATP-binding site in the kinase domain of ErbB receptors and next inhibit their intracellular kinase activity. Most of the existing small molecule TKIs which target RTK are multi-targeted and inhibit a variety of molecules in a non-specific manner. This characteristic has been demonstrated to have several disadvantages. It's why only a few specific and selective TKIs have been approved by authorities for cancer treatment. Several approaches have been developed: TKI that targets a specific member of the ErbB family or TKI that inhibit multiple members of the ErbB family. These last inhibitors bind their targets irreversibly and are currently under evaluation for the treatment of cancer. Erlotinib or gefitinib are the first-generation reversible EGFR-TKIs and are approved first-line therapies for patients with non-small cell lung cancer (NSCLC) presenting activating EGFR mutations. Unfortunately, despite these agents have demonstrated improvement in progression-free survival, patients present resistance to these agents and tumors rapidly regrow (Hirsh, 2015).

Lapatinib (Tykerb/Tyverb®), developed in Xia et al. (2002), is an orally active reversible dual TKI of EGFR/HER2. It binds covalently to the Cys 773 of EGFR and Cys 805 of HER2 (Howe and Brown, 2011). Studies *in vitro* and *in vivo* using xenografted mice with cell lines over-expressing EGFR and HER2 have shown that lapatinib inhibits tyrosine phosphorylation in catalytic domain of EGFR and HER2 and prevents ERK1/2 and AKT activation which induces apoptosis of tumor cells (Xia et al., 2002). In 2007, Lapatinib was approved by the FDA for patients with breast cancer as second-line treatment. Lapatinib, in combination with an aromatase inhibitor, was also used as first-line therapy for treatment of postmenopausal women with estrogen/HER2 receptor-positive breast cancer. Another inhibitor, Dacomitinib (PF-00299804, PF299), is currently under development. Dacomitinib is a selective, quinazalone-based irreversible pan-HER inhibitor of EGFR/ErbB1, ErbB2/HER2, and ErbB4/HER4–TKI and is in phase III of clinical development for the treatment of NSCLC. Furthermore, other small molecule inhibitors targeted against ErbB receptors are currently in clinical trials (Hirsh, 2015).

Recently, anticancer strategies that involve smaller antibody fragments such as Fragment antigen-binding domain (Fabs), single-chain variable fragment (ScFvs) and nanobodies are in development (Holliger and Hudson, 2005). Nanobodies consist of single-domain antigen-binding fragments derived from the camelids heavy-chain only antibodies (Muyldermans, 2001). Nanobodies have several advantages: they are significantly smaller in size (15 kDa) than scFv (28 kDa) or Fab (55 kDa), and then potentially providing higher tissue dispersion and superior tissue penetration than their counterparts (Holliger and Hudson, 2005); they are also significantly more stable

TABLE 1 | Some examples of drugs targeting ErbB receptor family.

Drug	Company	Receptor	Description	Status	Indication
Cetuximab (Erbitux)	ImClone Systems	EGFR	mAb directed against EGFR	First approval by FDA in 2004	Colorectal, head, neck and pancreas cancers
Panitumumab (Vectibix)	Amgen	EGFR	mAb directed against EGFR	First approval by FDA in 2006	Metastatic colorectal cancer
Erlotinib (Tarceva)	Roche/Genentech/OSI	EGFR	Inhibitor of EGFR signaling	First approval by FDA in 2004	Non-small cell lung cancer, pancreatic cancer
Gefitinib (Iressa)	AstraZeneca	EGFR	Inhibitor of EGFR signaling	First approval by FDA in 2003	Non-small cell lung cancer, esophageal cancer
Lapatinib (Tykerb/Tyverb)	GlaxoSmithKline	EGFR/ HER2	Inhibitor of EGFR/HER2 signaling	First approval by FDA in 2007	Metastatic breast cancer
Dacomitinib	Pfizer	EGFR/ HER2/HER4	Pan-inhibitor of ErbB receptors signaling	Phase III	Non-small cell lung cancer
Trastuzumab (Herceptin)	Genentech	HER2	mAb directed against HER2	First approval by FDA in 1998	HER2-positive breast cancer HER2-overexpressing metastatic gastric or gastroesophageal junction adenocarcinoma
Pertuzumab (Perjeta)	Genentech	HER2	mAb directed against HER2	First approval by FDA in 2012	Breast cancer
Margetuximab (MGAH22)	MacroGenics	HER2	mAb directed against HER2	Phase I	Breast, gastroesophageal and other HER2-positive tumors
Patritumab (U3-1287)	Daiichi Sankyo Pharmaceutical Development and Amgen	HER3	mAb directed against HER3	Phase I–II	Non-small cell lung cancer

mAb, monoclonal antibody.

than V_H (Heavy chain) domains (Stijlemans et al., 2004). Nanobodies specific for EGFR have recently been developed. They inhibit the binding of EGF to the receptor by different mechanisms either by blocking ligand binding to EGFR in a manner similar to cetuximab or by binding an epitope near the EGFR domain II/III junction and then by preventing receptor conformational changes required for high-affinity ligand binding and dimerization (Schmitz et al., 2013). Nanobodies binding to EGFR thereby inhibits EGFR signaling (Roovers et al., 2007, 2011). Several studies have shown that several EGFR-specific nanobodies have the potential to reproduce the clinical efficacy of mAbs such as cetuximab. Moreover, these molecules are more stable and less costly to produce than mAbs. In addition, potent multivalent nanobodies can be produced and can bind a number of targets (Jahnichen et al., 2010; Roovers et al., 2011), allowing to design multivalent agents that combine several modes of EGFR or other cancer target inhibition.

Affibody molecules are derived from the B-domain in the Ig-binding region of *Staphylococcus aureus* protein A (Nygren, 2008). Affibodies are highly soluble, chemically and thermally stable and rapidly removed from the circulation. The single protein chain of affibodies facilitates direct fusion with various proteins such as toxins and fluorophores, radioactive labels, or chemical groups for immobilization. The first affibody molecule, developed *in vivo,* was directed against HER2. This molecule binds to HER2 receptor on an epitope different from that of trastuzumab and with an affinity constant of 50 nM (Friedman et al., 2007). Since co-expression of HER2 and EGFR has been reported to be related with a poor prognosis in several types of cancer, a bispecific affibody directed against these receptors was generated (Friedman et al., 2009). In-depth binding studies have shown that this bispecific affibody can interact at the same time with both target receptors. Other affibody molecules which present different affinities for EGFR and HER2 were also developed to study their selectivity and their cooperativity between the two binding sites. Studies have shown that an affitoxin composed of a HER2-specific affibody linked to a truncated version of *Pseudomonas* exotoxin A was able to bind to HER2 with nanomolar affinity. This affitoxin eliminated HER2-positive cells with IC_{50} values three orders of magnitude lower than the corresponding HER2-negative cells, and induce a rapid shrinkage of BT-474 breast cancer xenograft tumors. This study demonstrates that HER2-affitoxin is an encouraging new therapeutic approach for HER2-overexpressing cancers that are non-responsive to currently available therapies (Zielinski et al., 2011).

Furthermore, new strategies has been developed this last decade to disturb ErbB receptor family dimerization and activation by targeting the transmembrane domain of these receptors: indeed, short synthetic peptides which mimick TM domains are able to inhibit specifically kinase activity and cell signaling induced by EGF and ErbB2 receptors in cancer cells (Bennasroune et al., 2004a). More recently, it has been shown that transmembrane domain targeting peptide antagonizing ErbB2/Neu exhibit anticancer properties by inhibiting breast tumor growth and metastasis in genetically engineered mouse model of breast cancer (Arpel et al., 2014).

CONCLUSION

Even if the involvement of ErbB receptor family by overexpression or activating mutations in oncogenesis is

well described since 25 years, numerous processes concerning the role of these proteins in dysregulation of cell proliferation and migration are not widely understood. Moreover, the cancer progression is accompanied by an extensive remodeling of ECM components. According to the current status of knowledge, several proteins of the ECM as decorin or matrikines may be used both as diagnostic markers and as targets in cancer therapy. Indeed, studying the role of ECM components and their interactions with ErbB receptors in cellular processes of growth, invasion and metastasis should permit the development of new inhibitor classes.

AUTHOR CONTRIBUTIONS

AB: conception and drafting of the review; AA-C: conception and drafting of the review; PH: drafting of the review and final approval; GC: Final approval of the version to be published.

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MINI REVIEW
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New Insight into the Anti-liver Fibrosis Effect of Multitargeted Tyrosine Kinase Inhibitors: From Molecular Target to Clinical Trials

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Tyrosine kinases (TKs) is a family of tyrosine protein kinases with important functions in the regulation of a broad variety of physiological cell processes. Overactivity of TK disturbs cellular homeostasis and has been linked to the development of certain diseases, including various fibrotic diseases. In regard to liver fibrosis, several TKs, such as vascular endothelial growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor, and epidermal growth factor receptor kinases, have been identified as central mediators in collagen production and potential targets for anti-liver fibrosis therapies. Given the essential role of TKs during liver fibrogenesis, multitargeted inhibitors of aberrant TK activity, including sorafenib, erlotinib, imatinib, sunitinib, nilotinib, brivanib and vatalanib, have been shown to have potential for treating liver fibrosis. Beneficial effects are observed by researchers of this field using these multitargeted TK inhibitors in preclinical animal models and in patients with liver fibrosis. The present review will briefly summarize the anti-liver fibrosis effects of multitargeted TK inhibitors and molecular mechanisms.

Keywords: tyrosine kinase inhibitors, liver fibrosis, clinical trials, preclinical study, molecular mechanisms

INTRODUCTION

Liver fibrosis is a chronic medical condition in which the normal liver architecture is replaced by fibrous tissue, scar and regenerative nodules leading to loss of liver function due to various etiologies including infection, drug, cholestasis, metabolic disorder, or immune attack (Hernandez-Gea and Friedman, 2011). Liver fibrosis affects 100s of millions of patients worldwide, which ultimately resulting in cirrhosis, hepatocellular carcinoma (HCC), or even death. Although liver fibrosis is generally recognized being potentially reversible and a number of therapies have been investigated in animal models, those diverse anti-fibrotic therapies are not seemingly effective from bench to bedside. Till date, treatment of liver fibrosis depends upon the stage of the disease, and liver transplantation is the only curative therapy for end stage of liver cirrhosis (Adam and Hoti, 2009). A thorough understanding of the underlying mechanism is critical for developing effective therapeutic approach for cirrhotic patients.

TYROSINE KINASES INVOLVED IN LIVER FIBROGENESIS

Grateful thanks to the decades of relevant experiments and researches, a numerous molecules and signaling pathways involved in the liver fibrogenesis were unveiled and corresponding therapeutics were taken root (Friedman, 2008). Among them, a family of proteins called tyrosine kinases (TKs) are found to be involved in this process. TKs can be divided into two subgroups, receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). RTKs include vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and epidermal growth factor receptor (EGFR) kinases. Meanwhile, nRTKs include c-Abl and Src kinases. Both RTKs and nRTKs are found to be essential for cellular signal transduction networks (Xu and Huang, 2010).

The RTKs are membrane receptors that activate intracellular signaling pathways upon ligand binding to their extracellular domains. These receptors are single-transmembrane proteins comprising an extracellular ligand-binding domain and a linked cytoplasmically oriented, catalytic domain (Almendro et al., 2010). The activation process of RTKs is triggered by the dimerisation of two RTK monomers as well as autophosphorylation of the intracellular phosphatase domain to increase the catalytic activity, which consequently generates a biochemical message and activates intracellular signaling pathways.

In contrast to RTKs, nRTKs (Src, c-Abl, and RhoA) lack extracellular and transmembrane domains, and only include a catalytic domain and a regulatory domain (Arora and Scholar, 2005; Zander and Hallek, 2011). nRTKs modulate signaling pathways after activation in the cytoplasm use different regulatory mechanisms. Additionally, it is also found that nRTKs can be activated by RTKs (**Figure 1**). The interaction between RTKs and nRTKs therefore contribute together to modulate cellular differentiation and proliferation.

Tyrosine Kinases as Modulators of Hepatic Stellate Cells Activation

It's widely accepted that a hallmark of liver fibrogenesis is the transdifferentiation of resting hepatic stellate cells (HSCs) into a myofibroblastic cell type. It was found that many TKs were expressed in activated HSCs. Moreover, the expression of several TKs, especially PDGFR (Heldin, 2014), VEGFR (Yoshiji et al., 2003), and EGFR (Fuchs et al., 2014), were significantly increased during the course of liver fibrosis development. Because of the critical roles in key signal transduction, TKs therefore harbor a mitogenic potential, which when activated, result in the transformation of resting HSCs to active HSCs. As shown in **Figure 1**, multiple downstream signaling pathways, such as MEK/ERK and PI3K/Akt pathways, are found to be activated by TKs during HSC activation. Many TK targeting agents exhibit significant inhibitory effects on chemotaxis, activation and collagen synthesis pathways in HSCs.

Tyrosine Kinases as Modulators of Intrahepatic Angiogenesis

Alterations in the hepatic vasculature are also defined as a crucial component during liver fibrogenesis. Established evidence clearly indicates that microvascular abnormalities promotes portal hypertension and liver fibrosis progression (Medina et al., 2004; Thabut and Shah, 2010). In parallel with capillarization of hepatic sinusoids, intrahepatic angiogenesis giving rise to shunts between pre- and post- sinusoidal vessels would lead to increased portal vascular resistance and decreased effective hepatocyte perfusion (Yoshiji et al., 2003). To date, many TKs have been identified joining in angiogenesis during liver fibrosis progression. Among these, VEGFRs are the most potent in the angiogenesis process (**Figure 1**). VEGFR expression significantly increased during the course of liver fibrosis development in experimental studies (Yan et al., 2015). Anti-VEGFR treatment using either antibodies (Yoshiji et al., 2003) or agents (Yang et al., 2014) significantly attenuates liver fibrosis progression. Additionally, PDGFRs were also considered as proangiogenic molecules involved in portal hypertension and might be potential targets for anti-fibrotic therapy (Rosmorduc, 2010).

ANTI-FIBROTIC ACTIVITY OF MULTITARGETED TYROSINE KINASE INHIBITORS

Over the past decade, numerous small molecule inhibitors targeting TKs have been developed (**Table 1**). Initially, these synthesized drugs were developed for anti-tumor therapy. In recent years, the application of multi-targeted TK inhibitors has also dramatically changed the conventional treatment modes for many other non-malignant diseases, especially for fibrotic diseases (Beyer and Distler, 2013; Heldin, 2014). Given the central role of TKs in liver fibrosis, blockade of the TKs appears to be a promising anti-fibrotic treatment approach. Currently, significant benefits of multitargeted TK inhibitors in liver fibrosis have been observed in preclinical experiments on animal models (Rossler et al., 2008; Grimminger et al., 2010). In the following part, we will summarize recent findings of anti-liver fibrosis effects of TK inhibitors (**Table 2**).

TK Inhibitors in Clinical Trials as Anti-liver Fibrosis Agents

Sorafenib

As one of the most intensively investigated multitargeted TK inhibitors, Sorafenib mainly targets Raf/ERK, VEGFR, and PDGFR-β pathways. Mechanistic investigation demonstrated that sorafenib exhibited potential anti-cancer activities by inhibiting cellular proliferation, suppressing angiogenesis and inducing apoptosis in various tumor types (Plastaras et al., 2007). Clinical trails further revealed that sorafenib can be used alone as the first treatment for advanced HCC. Interestingly, during the course of anti-HCC treatment, clinicians observed positive side effects of sorafenib on liver cirrhosis (Mejias et al., 2009). The anti-fibrotic effect of sorafenib is clearly

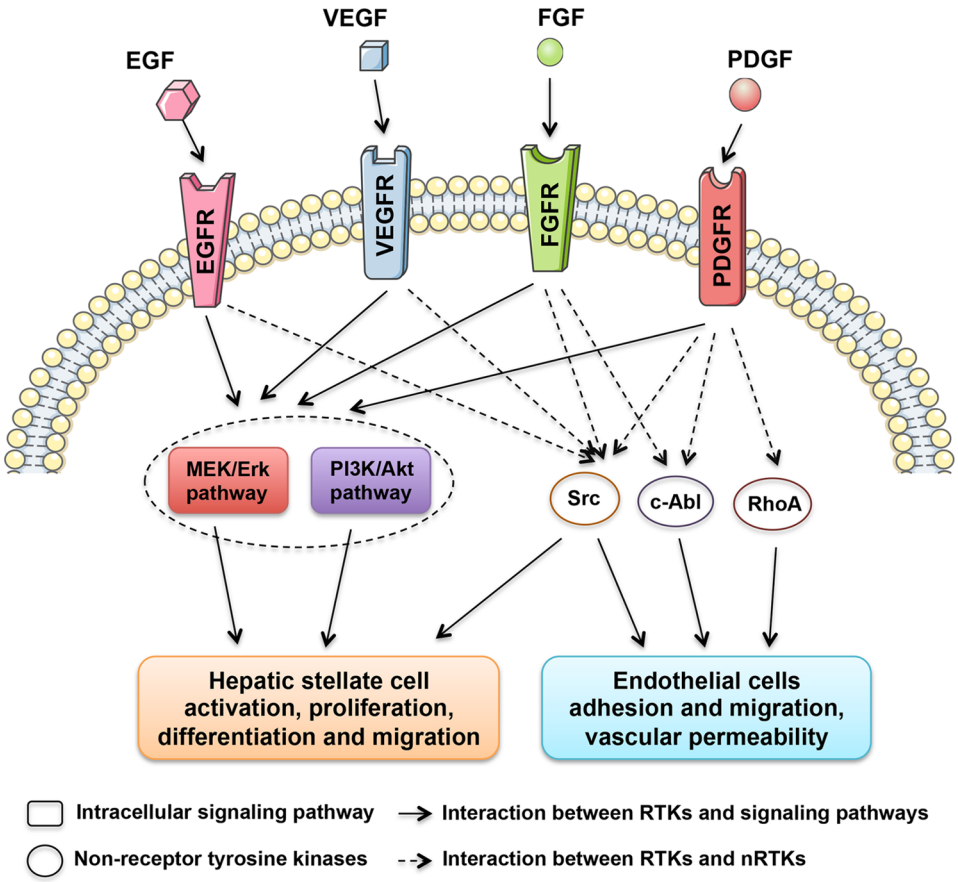

FIGURE 1 | Tyrosine kinases with central roles in liver fibrosis. The regulatory network is composed of receptor tyrosine kinases, including EGFR, VEGFR, FGFR, and PDGFR kinases, and non-receptor tyrosine kinases, such as Src, c-Abl, and RhoA kinases, which stimulate the activation of HSCs and intrahepatic angiogenesis.

TABLE 1 | IC50 values for TK inhibitors inhibition *in vitro*.

TK inhibitors	IC50 values for TK inhibitors inhibition *in vitro* (nM)									
	VEGFR-1	VEGFR-2	VEGFR-3	PDGFR-α	PDGFR-β	FGFR-1	EGFR	c-Kit	Fit-3	Bcr/Abl
Sorafenib	NR	15	20	NR	57	580	>10000	68	58	NR
Erlotinib	NR	NR	NR	NR	NR	NR	2	NR	NR	NR
Imatinib	>10000	>10000	>10000	100	100	NR	NR	100	>10000	600
Sunitinib	NR	80	NR	NR	2	>1000	>1000	NR	NR	NR
Nilotinib	NR	NR	NR	NR	NR	NR	NR	NR	NR	<30
Brivanib	380	25	NR	NR	>1000	148	>1000	NR	NR	NR
Vatalanib	77	37	660	NR	580	NR	NR	730	NR	NR

TK inhibitors, tyrosine kinase inhibitors; NR, not reported. Data taken from http://www.selleckchem.com/.

demonstrated by numerous experiment studies. In nearly all animal models of liver fibrosis, such as carbon tetrachloride (CCl_4), bile duct ligation (BDL), dimethylnitrosamine (DMN), diethylnitrosamine (DEN), or thioacetamide (TAA) induced models, sorafenib exhibits anti-liver fibrosis effects (Hennenberg et al., 2009; Wang et al., 2010; Thabut et al., 2011; Hong et al., 2013; Westra et al., 2014a; Liu et al., 2015; Stefano et al., 2015; **Table 2**).

Hepatic stellate cells are recognized as the main matrix-producing cells and being responsible for excessive deposition of extracellular matrix components during liver fibrogenesis. Mechanistic investigations revealed that sorafenib inhibited PDGF-BB-induced cellular proliferation in a dose-dependent manner in HSCs (Wang et al., 2010). The anti-proliferation of sorafenib on HSCs are found to be mediated by downregulating expression of cyclins and cyclin dependent kinases (CDKs) and

TABLE 2 | Summary of anti-liver fibrosis effects of TK inhibitors in preclinical studies.

TK inhibitors	Effects on fibrotic processes		Animal models of liver fibrosis used for evaluation								
	HSC activation	Angiogenesis	CCl_4	BDL	TAA	DEN	DMN	NASH	PCLS	Pig serum	Parasite
Sorafenib	√	√	√	√	√	√	√	√	√		
Erlotinib	√		√	√		√					
Imatinib	√		√	√	√				√	√	√
Sunitinib	√	√	√						√		
Nilotinib	√		√	√	√						
Brivanib	√	√	√	√	√			√			
Vatalanib	√		√								

CCl_4, carbon tetrachloride; BDL, bile duct ligation; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; TAA, thioacetamide; NASH, non-alcoholic steatohepatitis model; PCLS, precision-cut liver slices from fibrotic livers; Parasite, Schistosoma mansoni.

TABLE 3 | Tyrosine kinase inhibitors in clinical trials as anti-liver fibrosis agents.

TK inhibitors	ClinicalTrials.gov identifier	Recruited parcitipants	Intervention	Study phase	Status
Sorafenib	NCT01714609	Liver cirrhosis participants with portal hypertension	Sorafenib 400mg p.o. twice daily	Phase II	Completed
Erlotinib	NCT02273362	Liver cirrhosis participants following HCC resection	Erlotinib p.o. for 7 days	Phase I	Recruiting

inhibiting the phosphorylation of ERK and Akt (Plastaras et al., 2007; Tomizawa et al., 2010). Recently, increasing evidence have shown that enhanced intrahepatic angiogenesis is associated with faster fibrosis progression and thus has been identified as a crucial contributor to the fibrogenesis. Thabut et al. (2011) also reported that sorafenib was capable of inhibiting the Kruppel-like factor (KLF6)-Angiopoietin-1 (Ang1)-fibronectin molecular triad, thereby suppressing intrahepatic angiogenesis and attenuating liver fibrosis (Thabut et al., 2011).

In preclinical experiments, sorafenib is also found to attenuate the complications of liver cirrhosis. Portal hypertension is a life-threatening complication of liver disease defined by a portal venous pressure gradient exceeding 5 mm (Lee and Kim, 2007). Preclinical studies showed that sorafenib treatment resulted in a reduction in portal pressure and angiogenesis in BDL rats without affecting systemic blood pressure (Tugues et al., 2007; Mejias et al., 2009; Rosmorduc, 2010). Hennenberg et al. (2009) found that the effect of sorafenib on intrahepatic angiogenesis and portal hypertension is mediated by Rho kinase activity (Hennenberg et al., 2009). Additionally, it is also be observed that sorafenib may influence hepatopulmonary (Chang et al., 2013; Yang et al., 2014) and hepatic encephalopathy syndrome (Hsu et al., 2012) in cirrhotic rats.

In early clinical trials of sorafenib as anti-HCC agent, it was observed that patients with liver cirrhosis who received sorafenib therapy had a decrease in portal venous flow of at least 36% (Coriat et al., 2011). Similarly, in a small clinical study, Pinter et al. (2012) also reported the protective effect of a 2-weeks sorafenib treatment on portal hypertension in HCC patients with liver cirrhosis (Pinter et al., 2012). Additionally, Theysohn et al. (2012) found that sorafenib reduced hepatopulmonary shunt in patients with liver cirrhosis, which might greatly improve the prognosis of these patients (Theysohn et al., 2012). Recently, a multi-center, placebo-controlled randomized clinical trial of the effect of sorafenib on portal pressure in patients with cirrhosis was carried out (NCT01714609, **Table 3**). Researchers recruited patients with cirrhosis who have high portal vein pressure and treated them using sorafenib (400 mg twice daily) or placebo. Results from this clinical trial might supply evidence for clinicians to use sorafenib as anti-liver fibrosis agent.

Erlotinib

Erlotinib was the second EGFR TK inhibitor approved by the FDA for non-small cell lung cancer (NSCLC). Fuchs et al. (2014) observed that erlotinib, used at doses equivalent to or less than those used in humans, significantly reduced fibrogenesis in three different animal models of progressive cirrhosis: DEN or BDL induced rat model and CCl_4 induced mouse model. They also found that erlotinib reduced the number of activated HSCs by depressing EGFR phosphorylation in HSCs. An undergoing clinical trial (NCT02273362, **Table 3**) is conducted to evaluate the effects of erlotinib in fibrogenesis inhibition and HCC prevention.

Other TK Inhibitors Exhibited Potential Anti-liver Fibrosis Activity in Preclinical Experiments

Imatinib

Imatinib (also known as STI571), is a potent, competitive 2-phenylamonioyrimideine class inhibitor of three TKs, PDGFR, Bcr-Abl, and c-Kit. It is initially developed for the treatment of chronic myeloid leucemia (CML) and gastrointestinal stroma tumors (by targeting c-Kit). Given its inhibitory capacity on PDGFR which plays an critical role in the activation of fibroblasts, imatinib therefore is considered as a potential therapeutic candidate for the treatment of fibrotic diseases. Akhmetshina et al. (2009) found that imatinib did not only

prevent but also reverse established fibrosis in systemic sclerosis models. Apart from SSc, the anti-fibrotic effects of imatinib were consequently observed in pulmonary, renal and liver fibrosis (Daniels et al., 2004; Abdollahi et al., 2005; Wang et al., 2005; Yoshiji et al., 2005). In many animal models of liver fibrosis, such as CCl_4, BDL, TAA, or Schistosoma mansoni induced liver fibrosis, imatinib exhibits anti-liver fibrosis effects (Yoshiji et al., 2005; Neef et al., 2006; El-Agamy et al., 2011; Shaker et al., 2011a; Kuo et al., 2012; Shiha et al., 2014). In a pig serum-induced liver fibrosis model, Yoshiji et al. (2005) found that imatinib attenuated liver fibrosis via suppressing HSCs activation. In addition, imatinib exhibits increased anti-liver fibrosis activities when used in combination with an angiotensin-converting enzyme inhibitor (ACE-I), perindopril, which suppresses TGF-β1 expression (Yoshiji et al., 2006). Westra et al. (2014a,b) conducted an *in vitro* model using prolonged culture of precision-cut liver slices to screen antifibrotic drugs. It was also found that Imatinib could significantly decrease the expression of fibrosis markers, such as α-SMA, Pcol1A1, and Hsp47 (Westra et al., 2014b).

It should be noted that, different from sorafenib, imatinib seems to only reduce early liver fibrogenesis but does not prevent progression in the long term. In a study reported by Neef et al. (2006), it was found that prophylactic imatinib markedly reduced fibrosis in the first 3 weeks after BDL. Early imatinib treatment induced a 50% decrease of MMP-2 activity and TIMP-1 expression in HSCs, but left numbers of activated HSCs unchanged (Neef et al., 2006). Moreover, when imatinib was used in advanced fibrosis models, it neither reduced numbers of activated HSCs nor inhibit extracellular matrix production.

Sunitinib

Sunitinib is an oral indolin-2-one structural analog, which inhibits multiple RTKs such as VEGFR1/2/3, PDGFR-α/β, FGFR, and c-Kit (Faivre et al., 2007). Clinical trials revealed that sunitinib had potent anti-tumor and anti-angiogenesis effects in multiple cancer types. In liver fibrosis models, sunitinib has been shown to decrease inflammatory infiltration and expression of fibrosis markers in fibrotic livers (Tugues et al., 2007; Westra et al., 2014a). A *in vitro* study conducted by Majumder et al. (2013) revealed that sunitinib inhibited collagen synthesis in HSCs by 47%, attenuated HSC contraction by 65%, and reduced cell migration by 28%. In addition, they also found that sunitinib suppressed angiogenic capacity of endothelial cells (ECs). Similarly, it was also observed that sunitinib could decrease the number of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) positive staining hepatic vessels, and consequently reduced portal vein pressure in cirrhotic rats (Tugues et al., 2007).

Nilotinib

Nilotinib, a selective BCR-ABL TK inhibitor, is shown to be 30-fold more potent than imatinib in preclinical *in vitro* studies. Shaker et al. (2011a,b,c, 2013) found that nilotinib had a promising anti-fibrotic activity in experimental models of liver fibrosis by inhibiting activation of HSCs (Shiha et al., 2014). Liu et al. (2011) also reported that nilotinib significantly inhibited PDGF and TGF-β-simulated activation of ERK and Akt and consequently reduced collagen deposition and α-SMA expression in CCl_4 and BDL-induced fibrotic models.

Brivanib

Brivanib is an orally available dual inhibitor of VEGF and FGF signaling. Nakamura et al. (2014) evaluated the anti-liver fibrosis effects of brivanib on three experimental fibrotic mouse models, including BDL, CCl_4, and chronic TAA induced mouse models of fibrosis. Lin et al. (2014) further found that brivanib markedly suppressed intrahepatic angiogenesis and portal hypertension in cirrhotic rats. Similarly, Yang et al. (2014) also observed brivanib improved hepatic blood flow and inhibited ascites formation in NASH-cirrhotic rats.

Vatalanib

Vatalanib (also known as PTK787/ZK22258) is found to mainly target VEGFR-1 and VEGFR-2, and it also inhibits the activity of PDGFR-β, Flt-4, c-kit, and c-fms with less potency. In liver fibrosis models, Liu et al. (2009a,b) reported that vatalanib attenuated stellate cell activation and liver fibrosis progression by inhibiting VEGF signaling as well as targeting of the PDGF and TGF-β-signaling pathways.

HEPATOTOXICITY OF TK INHIBITORS: AN IMPORTANT ISSUE LIMITED THEIR CLINICAL USE

Most of TK inhibitors are metabolized in liver by hepatic cytochrome P450 enzyme system (Druker, 2003; Lathia et al., 2006; Peer et al., 2012), implying a potential hepatotoxicity when they are administrated in patients. Iacovelli et al. (2014) conducted a meta-analysis base on 3691 patients who received TK inhibitors treatment and found hepatotoxicity occurred in 23–40% of patients treated with TK inhibitors. It is been found that hepatotoxicity usually occurred within the first 2 months after TK inhibitors treatment (Shah et al., 2013). Fatality from TK inhibitor-induced hepatotoxicity is less common compared to hepatotoxic drugs in other classes, but may lead to unfavorable events including liver cirrhosis and even liver failure (Cross et al., 2006; Schramm et al., 2008; Tonyali et al., 2010; Shah et al., 2013). In the following aspect, we summarized the hepatotoxicity of TK inhibitors that observed in clinical cases.

Sorafenib is reported to exhibit a high degree of inter-individual variability in pharmacokinetics and clinical efficacy. The magnitude of variability on sorafenib exposure (area under the plasma concentration-time curve, AUC) ranged from 5 to 83%, and the peak plasma concentrations varied from 33 to 88% at oral doses of 200 or 400 mg administrated twice daily. The median time to peak plasma concentration varied from 2 to 9.5 h (Awada et al., 2005; Clark et al., 2005; Moore et al., 2005; Strumberg et al., 2007). Hepatotoxicity was reported during the therapy periods in some clinical cases. Llanos et al. (2009) reported a case of sorafenib-induced severe hepatotoxicity in a 73-years-old man with Child-Pugh A hepatitis-C virus-related cirrhosis and multinodular HCC. Schramm et al. (2008) also reported a case of sorafenib-induced liver failure. In addition,

acute liver failure caused by imatinib and sunitinib have also been observed (Cross et al., 2006; Tonyali et al., 2010; Shah et al., 2013).

Due to the activity of TKs plays an essential role in many physiological processes and its inhibition by TK inhibitors may lead to side effects as discussed above. Therefore, targeting liver fibrosis via specific delivery of TK inhibitors to HSCs might reduce side effects. Gonzalo et al. (2007) conducted a HSC-selective carrier mannose-6-phosphate modified human serum albumin (M6PHSA) to combine with a TK inhibitor which exhibited potent anti-fibrotic effects. Their findings supply a promising approach to attenuate liver fibrogenesis using TK inhibitors.

PERSPECTIVE

Many intracellular signaling pathways are activated inappropriate during fibrogenesis, in which the activation of TKs is recognized the initial trigger for HSC activation and intrahepatic angiogenesis. The treatment for liver fibrosis, in the past, tends to focus on only one target. As a result, poor benefits obtained despite non-corresponding efforts. Nowadays, accumulating preclinical experiments of multitargeted TK inhibitors made it possible to analyze and look forward to whether TK inhibitors have beneficial effects on not only malignant tumors but also fibrotic disease. Clinical trials of two TK inbhitors (sorafenib and erlotinib) have been carried out and encouraging results have already obtained. Based on the advantages of multitargeted TK inhibitors, targeted therapy might become major approaches for treating liver fibrosis in future.

It also should point out that the usefulness of TK inhibitors for long term treatment of liver fibrosis depends on the severity of the side effects. Although the most common adverse effects of TK inhibitors including rash, gastrointestinal symptoms, fatigue, edema, and neurological symptoms are generally mild and tolerable for liver fibrosis patients, liver function impairment and even acute liver failure have been observed in some clinical cases. Specific delivery of TK inhibitors to selective cells, such as HSCs, might be promising approach to attenuate liver fibrosis in future. Besides, the high price of TK inhibitors might also limit their application on liver fibrosis.

Given together, TK inhibitors are efficient not only on malignant tumors, but also on some non-malignant diseases, especially liver fibrosis. In the near future, clinical application of TK inhibitors on liver fibrosis will turn out to be not merely an efficient but also safety treatment.

AUTHOR CONTRIBUTIONS

All authors fulfill the authorship requirements and have approved the final version of the anuscript. KQ, ZH, and CL developed the paper design and revised the manuscript; SL and HC collected samples and performed literature search; ZY and HZ participated in research work and analyzed data; KQ, ZH, and TL wrote the first draft of the manuscript to which all authors made significant subsequent contributions.

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Urokinase receptor and resistance to targeted anticancer agents

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The urokinase receptor (uPAR) is a GPI-anchored membrane protein, which regulates protease activity at the cell surface and, in collaboration with a system of co-receptors, triggers cell-signaling and regulates gene expression within the cell. In normal tissues, uPAR gene expression is limited; however, in cancer, uPAR is frequently over-expressed and the gene may be amplified. Hypoxia, which often develops in tumors, further increases uPAR expression by cancer cells. uPAR-initiated cell-signaling promotes cancer cell migration, invasion, metastasis, epithelial-mesenchymal transition, stem cell-like properties, survival, and release from states of dormancy. Newly emerging data suggest that the pro-survival cell-signaling activity of uPAR may allow cancer cells to "escape" from the cytotoxic effects of targeted anticancer drugs. Herein, we review the molecular properties of uPAR that are responsible for its activity in cancer cells and its ability to counteract the activity of anticancer drugs.

Keywords: uPAR, plasmin, fibrinolysis, epithelial-mesenchymal transition, cancer stem cell, metastasis, cellular senescence

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Introduction

Adhesion receptors that mediate interactions between adjacent cells or with extracellular matrix (ECM) and at the same time, initiate cell-signaling include the integrins and members of the cadherin superfamily (Giancotti and Ruoslahti, 1999; Angst et al., 2001). The urokinase receptor (uPAR) is functionally similar to adhesion receptors in that it binds to the provisional ECM protein, vitronectin (Wei et al., 1994), and robustly activates cell-signaling (Blasi and Carmeliet, 2002). The second ligand for uPAR is the fibrinolysis protease, urokinase-type plasminogen activator (uPA), which like vitronectin, activates cell-signaling (Busso et al., 1994; Koshelnick et al., 1997). The signaling response elicited by uPA requires the amino-terminal region of uPA and not the uPA active site (Nguyen et al., 1998). Although the cell-signaling responses elicited by binding of either vitronectin or uPA to uPAR may be distinct, when uPAR is expressed at high levels, a composite response is observed, in which signaling factors controlled downstream of uPA and vitronectin are activated collectively (Eastman et al., 2012). From the structural standpoint, understanding the signaling activity of uPAR has been a fascinating challenge given that this 55-kDa, three-domain receptor is coupled to the cell surface only by a glycosylphosphatidylinositol anchor (Roldan et al., 1990; Ploug et al., 1991).

We now understand that the cell-signaling activity of uPAR controls many aspects of cell physiology that are pivotal in cancer progression. Clinical trial data support the hypothesis that uPAR is associated with cancer progression. In pancreatic cancer, the gene encoding uPAR may

Abbreviations: ECM, extracellular matrix; EGFR, EGF receptor; EGFRvIII, EGF receptor variant III; EMT, epithelial-mesenchymal transition; ERα, estrogen receptor-α; GBM, glioblastoma; MMP, matrix metalloprotease; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; uPA, urokinase-type plasminogen activator; uPAR, urokinase receptor.

be amplified and this event substantially deteriorates prognosis (Hildenbrand et al., 2009). In astrocytic brain tumors, uPAR expression correlates with tumor grade (Yamamoto et al., 1994). Recent results suggest that uPAR may play an important role promoting cancer cell survival during cancer chemotherapy. We hypothesize that: *Developing new uPAR-targeting therapeutics may be advantageous to improve the efficacy of currently available anticancer agents*. Support for this hypothesis is found in numerous basic and translational studies that have explored molecular aspects of uPAR function. Unfortunately, efforts to develop uPAR-targeting drugs are still in a formative stage.

Regulation of Cell Surface Plasminogen Activation

Early studies demonstrated that uPA-binding to uPAR increases the catalytic efficiency (k_{cat}/K_M) of plasminogen activation (Ellis and Danø, 1991). Plasminogen activation is further stimulated by the simultaneous binding of plasminogen to the cell surface, which is mediated by any of a number of membrane-associated proteins (Miles and Parmer, 2013). Anchoring of the single-chain or zymogen form of uPA to uPAR also accelerates its conversion to the enzymatically active, two-chain variant, in a reaction most frequently catalyzed by membrane-associated plasmin (Ellis, 1996). This positive feedback loop may generate large amounts of active plasmin at the cell surface.

Although best understood as the principal protease responsible for lysis of fibrin clots, plasmin has diverse glycoprotein substrates and thus, has been implicated in diverse activities including ECM remodeling, angiogenesis, cell migration, and cancer invasion (Mignatti and Rifkin, 1993). Key plasmin substrates, in addition to fibrin and ECM proteins, include latent transforming growth factor-β (Lyons et al., 1988) and pro-forms of matrix metalloproteases (MMPs; Murphy et al., 1999). Many plasmin activities may be facilitated by binding to cellular plasminogen receptors (Miles and Parmer, 2013).

Diverse cancer cells express high levels of uPAR (Mazar, 2008; Smith and Marshall, 2010) and also express plasminogen receptors that function in plasmin generation (Gonias et al., 2001). As cancers enlarge, they frequently outgrow their blood supply, causing hypoxia in the tumor core, which is a known inducer of uPAR expression (Graham et al., 1999; Lester et al., 2007). The effects of hypoxia on uPAR expression are mediated by hypoxia-inducible factor-1, which binds to the hypoxia-responsive element in the uPAR promoter (Krishnamachary et al., 2003). Although this is probably a compensatory response, meant to promote cell survival, once uPAR expression is induced, all the activities of uPAR described herein may be activated, including the ability of uPAR to promote plasminogen activation and potentiate tissue remodeling.

uPAR Signaling Requires a Multiprotein Receptor Complex

To activate cell-signaling in response to uPA or vitronectin, uPAR utilizes a system of co-receptors, which are dynamically assembled to generate qualitatively differing responses (Jo et al., 2005). Formyl peptide receptor-1 (FPR1) is a G protein-coupled receptor and essential co-receptor for cell-signaling downstream of membrane-anchored uPAR (Resnati et al., 2002). FPR1 also mediates cell-signaling in response to soluble forms of uPAR (Resnati et al., 2002). In cells that lack FPR1, FPR2 may substitute to mediate uPAR signaling (de Paulis et al., 2004). Diverse integrins also have been implicated in uPAR signaling, including $\alpha_3\beta_1$, $\alpha_5\beta_1$; $\alpha_v\beta_1$, $\alpha_v\beta_5$, and β2 integrins such as Mac1 (Yebra et al., 1996; May et al., 1998; Carriero et al., 1999; Nguyen et al., 1999; Wei et al., 2001, 2005). Mechanistically, uPAR physically associates with integrins to regulate integrin activity in cell adhesion, cell migration, and in assembly of cell-signaling complexes. Src family kinases and focal adhesion kinase are instrumental upstream signaling factors for uPAR and probably associate with uPAR indirectly through integrins (Koshelnick et al., 1997; Nguyen et al., 2000).

gp130 associates with uPAR in some cell types and may control activation of the JAK1-STAT1 pathway (Koshelnick et al., 1997). Receptor tyrosine kinases (RTKs) also may be important in uPAR signaling. In vascular smooth muscle cells, platelet-derived growth factor receptor-β has been implicated (Kiyan et al., 2005). In many other cell types, the EGF receptor (EGFR) plays an important role (Liu et al., 2002; Jo et al., 2003, 2005). In cancer cells, the EGFR functions with uPAR to activate ERK1/2, which promotes tumor cell survival and release from states of dormancy (Ma et al., 2001; Liu et al., 2002). The EGFR and uPAR also cooperate to activate the mitogenic transcription factor, STAT5b (Jo et al., 2005). Other cell-signaling factors, which have received considerable attention as key downstream targets of uPAR signaling, include PI3K and Rho GTPases such as Rac1 (Blasi and Carmeliet, 2002; Smith and Marshall, 2010). There is considerable overlap between the pathways controlled by RTKs and uPAR. This redundancy may partially explain the ability of uPAR to substitute for RTKs and promote cell survival in tumors treated with RTK-targeting drugs.

uPAR Expression and Function in Cancer

Targeting uPAR in cancer is intriguing given that in normal quiescent human tissues, uPAR expression is limited (Mazar, 2008; Smith and Marshall, 2010). Increased uPAR expression may be observed in activated non-neoplastic cells, including endothelium, smooth muscle cells, and immune system cells, especially in processes such as tissue injury or inflammation. By contrast, uPAR is highly expressed by diverse cancer cells and by non-malignant cells that infiltrate cancers (Mazar, 2008; Smith and Marshall, 2010). When expressed in malignancy, uPAR typically worsens the prognosis irrespective of whether the cell of origin is the tumor cell or the stromal cell.

In addition to pancreatic cancer, *uPAR* gene amplification is observed in breast cancer. In breast cancers that are HER2-positive, *uPAR* and *HER2* tend to be amplified in the same cells (Uhr, 2008). In colorectal cancer, uPAR expression by non-malignant stromal cells is correlated with a negative prognosis (Boonstra et al., 2014). Obviously, different mechanisms are

operational when cancer progression is accelerated by tumor cell uPAR versus stromal uPAR. One possible pathway by which stromal cell uPAR may promote cancer progression is by releasing soluble uPAR, which is biologically active and may regulate cancer cell physiology (Gilder et al., 2015).

Early models attributed the ability of uPAR to promote cancer progression to its control of extracellular proteolysis at the cancer cell surface (Blasi and Carmeliet, 2002; Dano et al., 2005). However, we and others have shown that uPAR promotes cell migration by activating Rac1 and ERK1/2 (Nguyen et al., 1998; Kjoller and Hall, 2001; Ma et al., 2002). In a mouse xenograft model system, uPAR promoted metastasis exclusively by controlling cell-signaling factors such as Rac1 (Jo et al., 2009b). This result does not discount the importance of uPAR in extracellular proteolysis but instead, proves the importance of uPAR-dependent cell-signaling in cancer progression in an *in vivo* model system.

The ability of uPAR to promote cell survival is particularly relevant to its activity in cancer treatment. In cell culture model systems, uPA-binding to uPAR inhibits apoptosis by maintaining an increased level of phosphorylated ERK1/2 (Ma et al., 2001). Similarly, uPAR-initiated cell-signaling prevents anoikis *in vitro* by transcriptional activation of the anti-apoptotic BCL-2 family member, BCL-xL (Alfano et al., 2006). uPAR signaling also regulates BIM, which is a second BCL-2 family member that promotes apoptosis (Wykosky et al., 2015). Glioblastoma (GBM) cells may be driven into apoptosis by suppressing uPAR signaling, which elevates BIM.

Cellular senescence and tumor cell dormancy are important concepts in cancer therapy (Campisi and d'Adda di Fagagna, 2007; Gewirtz, 2009). In response to radiation or chemotherapy, tumors cells may enter senescence as opposed to undergoing apoptosis. Replicative arrest is characteristic of cellular senescence; however, so is sustained survival. From the standpoint of cancer treatment, senescence within a sub-population of tumor cells implies a diminished capacity for tumor growth but also, a decreased opportunity for cancer eradication. There is still debate regarding whether cellular senescence is fully irreversible in cancer. Ossowski and colleagues showed that uPAR controls entry of cancer cells into states of dormancy and release from dormancy (Yu et al., 1997; Aguirre Ghiso et al., 1999) and thereby demonstrated the capacity of uPAR to regulate checkpoints in the life cycle of a cancer cell.

uPAR-initiated cell signaling promotes epithelial-mesenchymal transition (EMT) and this process appears to be reversible (Lester et al., 2007; Jo et al., 2009a). Hypoxia facilitates EMT by increasing uPAR expression (Lester et al., 2007). uPAR may play a central role in the mechanism by which gene products, such as the transcription factor, Forkhead Box M1, promote EMT (Huang et al., 2014). uPAR-activated cell-signaling also induces stem cell-like properties in cancer cells (Jo et al., 2010). Finally, uPAR controls gene expression in cancer cells, promoting expression of factors such as interleukin-4 and transforming growth factor-β, which condition immune system cells so that the tumor microenvironment is more conducive for tumor growth (Hu et al., 2014a). The activities of cancer cell uPAR are summarized in **Figure 1**.

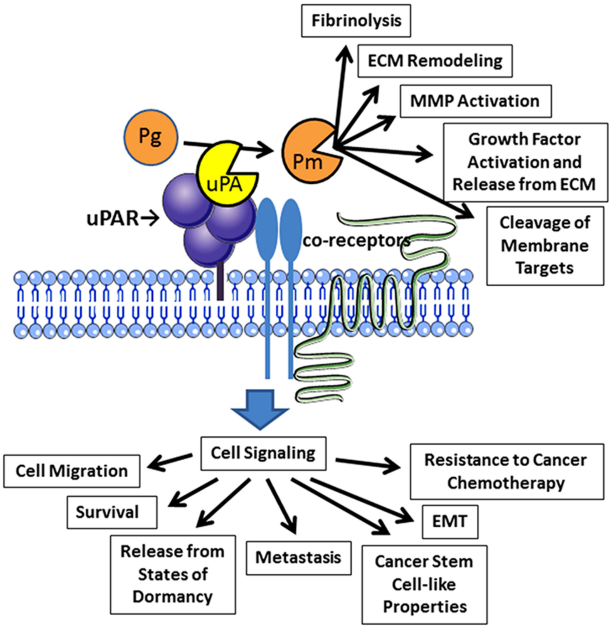

FIGURE 1 | Activities of uPAR in cancer cells. Binding of uPA to uPAR promotes activation of plasminogen (Pg) to form plasmin (Pm). Plasmin then expresses diverse activities near the cell surface. uPAR also collaborates with a system of co-receptors to activate cell-signaling. Reported outcomes of uPAR-initiated cell-signaling in cancer cells are shown.

uPAR and Chemoresistance in Cancer

In studies with cell culture model systems, Alfano et al. (2006) showed that silencing uPA increases the extent of apoptosis observed when cells are treated with cisplatin or UV irradiation. In small cell lung cancer in patients, uPAR expression is associated with resistance to diverse traditional chemotherapeutic agents (Gutova et al., 2007). In squamous cell carcinoma of the head and neck and in malignant mesothelioma, uPAR confers resistance to cisplatin (Cortes-Dericks et al., 2010; Huang et al., 2013). Because uPAR appears to confer some degree of resistance to almost all forms of traditional cancer therapy, each round of treatment may select for tumor cells that have the highest uPAR expression levels. As a result, uPAR-positivity may become increasingly problematic in patients that require multiple rounds of cancer treatment with different modalities.

In breast cancers in which tumor cells express estrogen receptor, anti-estrogen therapeutics such as tamoxifen have served as effective "targeted" anticancer agents (Massarweh and Schiff, 2006). Because as many as 70% of all breast cancers are estrogen receptor-positive, understanding why some malignancies acquire resistance of anti-estrogen drugs is of considerable importance. Activation of cell-signaling pathways downstream of the EGFR, HER2, and Insulin-like Growth Factor Receptor-1 has received attention (Massarweh and Schiff, 2006). In a series of 691 breast cancer patients treated with tamoxifen, progression-free survival correlated inversely with expression of uPAR and uPA (Meijer-van Gelder et al., 2004). To study this phenomenon, we examined estrogen-dependent breast cancer

cell lines (Eastman et al., 2012). In the presence of estrogen, estrogen receptor-α (ERα) functioned as a major receptor responsible for sustaining ERK1/2 activation. When estrogen was withdrawn, ERK1/2 phosphorylation decreased. To model how uPAR may regulate this process, we over-expressed uPAR in our ERα-expressing breast cancer cells. When estrogen was present, uPAR did not regulate ERK1/2 phosphorylation; however, in the absence of estrogen, uPAR provided a rescue pathway, sustaining ERK1/2 activation and promoting cell survival (Eastman et al., 2012). Similar results were obtained when we utilized a xenograft model system in mice. MCF-7 breast cancer cells typically require estrogen supplementation to establish xenografts in SCID mice; however, when MCF-7 cells were transfected to over-express uPAR, the estrogen requirement was attenuated (Eastman et al., 2012). Although more work is clearly required, these early studies support a model in which changes in uPAR expression in breast cancer cells may release tumors from control by estrogen receptor-targeting therapeutics in patients.

uPAR and EGFR in Glioblastoma

Glioblastoma is a highly aggressive astrocytic tumor of the brain in which the gene encoding the EGFR is frequently amplified, driving tumorigenicity (Heimberger et al., 2005). In the context of *EGFR* gene amplification, *EGFR* mutations are common including a truncation mutation that generates a form of the receptor called EGF receptor variant III (EGFRvIII). This EGFR variant does not bind EGF but demonstrates constitutive enzymatic activity in the absence of growth factor (Nishikawa et al., 1994; Heimberger et al., 2005). Given the robust effects of *EGFR* gene amplification and EGFRvIII on GBM progression, it would be reasonable to assume that EGFR-targeting therapeutics would be effective in treating GBM; however, although temporary responses may be observed, tumors typically escape from control (Voelzke et al., 2008).

To understand why EGFR-targeting therapeutics do not demonstrate greater efficacy in GBM, together with our colleagues, we evaluated three models of acquired resistance to EGFR-targeting drugs. Mukasa et al. (2010) developed the first model, applying a genetic approach. EGFRvIII was expressed in U373MG GBM cells under the control of a doxycycline-repressible promoter. Tumors were developed in mice. Once the tumors were established, EGFRvIII expression was neutralized *in vivo* forcing the tumors into a state of dormancy. Many of these tumors emerged from dormancy, re-establishing growth. Wykosky et al. (2015) developed two additional model systems in which GBM cells were treated with the EGFR-targeting tyrosine kinase inhibitors (TKIs), erlotinib, and gefitinib, either in three dimensional cell culture or in xenografts *in vivo*. TKI resistance developed and was readily documented in cell viability and proliferation assays.

In all three model systems, neutralization of EGFRvIII activity induced expression of uPA, activating uPAR-dependent cell-signaling (Hu et al., 2011; Wykosky et al., 2015). uPAR assumed a major role sustaining ERK1/2 activation (Hu et al., 2011; Wykosky et al., 2015). As a result, apoptosis was prevented and the GBM cells survived. BIM was a major target for ERK1/2, downstream of uPAR in GBM cells. Silencing uPA in TKI-resistant GBM cells increased BIM levels and promoted apoptosis. Inhibiting MEK or treating cells with a BH-3 mimetic, which counteracts the activity of anti-apoptotic Bcl-2 family members, restored sensitivity to TKIs in GBM cells. These results suggest that the uPA-uPAR signaling system may provide a major escape pathway for GBM cells when tumors are treated with EGFR-targeting therapeutics. Interestingly, when EGFRvIII was neutralized in GBM cells, GBM cell migration was potentiated (Hu et al., 2014b). The compensatory response of the GBM cells, which involved activation of uPAR signaling to promote cell survival, also promoted cell migration, which is a well described consequence of uPAR-activated cell-signaling. These results suggest that changes in uPAR expression in cancer cells, induced or selected for by anticancer therapies, may unintendedly increase the capacity of the cancer cells to invade or metastasize.

Concluding Comments

Urokinase receptor regulates activities that are relevant to cancer progression on both sides of the plasma membrane. At the cell surface, uPAR stimulates tissue remodeling. Cell-signaling pathways, activated downstream of uPAR, stimulate many activities implicated in cancer progression. As a response to conventional or targeted anticancer agents, uPAR signaling may be activated. Alternatively, anticancer drugs may select for cancer cells in which uPA or uPAR are most highly expressed. Because cell-signaling pathways that support cell survival also may promote cell migration, activation of uPAR-dependent cell-signaling in treated cancer cells may not only prevent cancer eradication but also promote cancer progression.

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frontiers in Pharmacology

MINI REVIEW
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The Matricellular Receptor LRP1 Forms an Interface for Signaling and Endocytosis in Modulation of the Extracellular Tumor Environment

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The membrane protein low-density lipoprotein receptor related-protein 1 (LRP1) has been attributed a role in cancer. However, its presumably often indirect involvement is far from understood. LRP1 has both endocytic and signaling activities. As a matricellular receptor it is involved in regulation, mostly by clearing, of various extracellular matrix degrading enzymes including matrix metalloproteinases, serine proteases, protease inhibitor complexes, and the endoglycosidase heparanase. Furthermore, by binding extracellular ligands including growth factors and subsequent intracellular interaction with scaffolding and adaptor proteins it is involved in regulation of various signaling cascades. LRP1 expression levels are often downregulated in cancer and some studies consider low LRP1 levels a poor prognostic factor. On the contrary, upregulation in brain cancers has been noted and clinical trials explore the use of LRP1 as cargo receptor to deliver cytotoxic agents. This mini-review focuses on LRP1's role in tumor growth and metastasis especially by modulation of the extracellular tumor environment. In relation to this role its diagnostic, prognostic and therapeutic potential will be discussed.

Keywords: LRP1, cancer, extracellular matrix, metastasis, signaling, endocytosis

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INTRODUCTION

The matricellular receptor low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) is a multifunctional receptor implicated in both endocytosis and signaling pathways (Lillis et al., 2008). Numerous ligands, both structurally and functionally diverse, bind to LRP1 and the endocytosis of many of these ligands is coupled to activation of signal pathways. Together with its broad expression pattern, the multifunctionality of this receptor accounts for its involvement in various physiological and pathological processes including extracellular matrix modulation, transport across the blood–brain barrier (BBB), coagulation, inflammation, Alzheimer's disease, atherosclerosis, etc. The role of LRP1 in many of these processes is discussed in detail in recent reviews (Kanekiyo and Bu, 2014; Strickland et al., 2014). Following upon a short general description of the structure and the function of LRP1, the present mini-review, however, focuses on the often indirect role of LRP1 in tumor growth and metastasis by modulation of the extracellular tumor environment.

GENERAL ROLE OF LRP1 IN ENDOCYTOSIS AND CELL SIGNALING

Lipoprotein receptor related-protein 1, a type I transmembrane receptor, is a member of the LDL-receptor gene family (Lillis et al., 2008). The LRP1 precursor is cleaved by furin in the *trans*-Golgi to generate a 515 kDa N-terminal α-subunit and an 85 kDa C-terminal β-subunit. In the mature two-chain structure, the entirely extracellular α-subunit, containing the ligand binding domains, is non-covalently linked to the transmembrane-containing β-subunit. After maturation, arrival at the cell surface and ligand binding it undergoes highly efficient constitutive endocytosis via clathrin-coated pits and recycling. The dominant signals for endocytosis are YxxL and dileucine motifs in the cytoplasmic or intracellular domain of the β-subunit (Li et al., 2000), whereas two NPxY motifs, of which the latter overlaps with the YxxL motif, are secondary endocytosis signals and binding sites for adaptor proteins involved in signaling (Trommsdorff et al., 1998; Li et al., 2000; Loukinova et al., 2002). Analyses of knock-in mice and derived MEFs carrying inactivating mutations of the proximal NPxY and the distal NPxYxxL motifs revealed that, besides for endocytosis and signaling, these motifs are also relevant for slow recycling of LRP1 from the perinuclear compartment to the plasma membrane and even for early steps in LRP1 biosynthesis, preventing premature proteasomal degradation of precursor LRP1 (Roebroek et al., 2006; Gordts et al., 2009, 2012; Reekmans et al., 2010).

Lipoprotein receptor related-protein 1 ligands include proteases, protease inhibitor complexes, extracellular matrix proteins, growth factors, toxins, and viral proteins (Lillis et al., 2008). Via clearing of proteases, like (matrix-)metalloproteinases and other secreted proteins, like coagulation FVIII, LRP1 contributes to the homeostasis of many secreted proteins and the integrity of the extracellular matrix (**Figure 1A**). LRP1 regulates, however, also the abundance of many other proteins, including receptors present at the plasma membrane. For example, the urokinase-type plasminogen activator (uPA)-plasminogen activator inhibitor-1 (PAI-1) complex is a bivalent ligand, which triggers urokinase receptor (uPAR) internalization and regulates the uPAR signaling by bridging extracellularly uPAR and LRP1 (Gonias et al., 2011). Fe65 and PSD-95 are intracellular adaptor proteins (**Figure 1B**) that interconnect LRP1 to β-amyloid precursor protein (β-APP; Pietrzik et al., 2004) and *N*-methyl-D-aspartate (NMDA) receptor (May et al., 2004; Martin et al., 2008) respectively, stimulating APP endocytosis and amyloid (Aβ) generation (Pietrzik et al., 2004), and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling (Martin et al., 2008).

LRP1 AND CANCER: A LONG BUT DIFFICULT MARRIAGE

Lipoprotein receptor related-protein 1 has already been attributed a role in cancer shortly after its discovery in 1988 (Herz et al., 1988). Initially, several groups reported decreased LRP1 expression (**Figure 1C**) levels in various cancer cell lines and tissues, thus assigning a tumor suppressive role to this receptor (Kancha et al., 1994; de Vries et al., 1996; Gilardoni et al., 2003). These findings provided a rationale for earlier studies in which decreased binding and uptake of α_2-macroglobulin (α_2M), an LRP1 ligand, were observed in multiple cancer cell lines (Van Leuven et al., 1979; Saksela et al., 1981, 1984; Jensen et al., 1989). It should be noted, however, that under normoxia cell culture conditions cancer cell lines *in vitro* might show a reduction in LRP1 expression compared to hypoxic conditions (Montel et al., 2007). As in many tumors *in vivo* hypoxic conditions exist, this observed decrease in LRP1 expression should be interpreted with caution. Nonetheless, more recent work supports a reduction in LRP1 expression in cancer. Amos et al. (2007) compared LRP1 expression between low-grade astrocytoma and high-grade astrocytoma (glioblastoma). They correlated a decrease in LRP1 expression with more advanced tumor grade and enhanced uPA-dependent cell invasion. Previously however, Yamamoto et al. (1997) and Baum et al. (1998) have described opposite results: LRP1 expression was predominantly detected in glioblastoma and to a lesser extent in lower grade astrocytomas. *In vitro*, LRP1 expression appears to vary substantially among different glioblastoma cell lines (Maletinska et al., 2000). In hepatocellular carcinoma, colorectal carcinoma and lung adenocarcinoma, reduced LRP1 expression levels were linked to a poor prognosis and more advanced tumor stages (Obermeyer et al., 2007; Meng et al., 2011; Huang et al., 2012). Recently, it was shown that LRP1 acts in response to ApoE as an endogenous suppressor of the metastatic phenotype in melanoma (Pencheva et al., 2012). However, contrasting evidence exists suggesting a role for LRP1 in supporting thyroid and breast cancer cell invasion and metastasis (Chazaud et al., 2002; Montel et al., 2007; Dedieu et al., 2008; Fayard et al., 2009). Moreover, increased LRP1 expression was found to be predictive of more aggressive tumor behavior and associated with higher histological grade in endometrial carcinomas (Catasus et al., 2011).

Post-translational regulation of LRP1 by proteolytic cleavage (also named shedding) is a critical mechanism in regulating cell-surface LRP1 expression, especially in tumor context (**Figure 1D**). Since the first identification of the extracellular part of LRP1 (LRP1-ECD) solubilized in human plasma (Quinn et al., 1997), proteolytic enzymes from different classes have been identified as LRP1 sheddases (Etique et al., 2013). These include metalloproteinases such as MT1-MMP and ADAM-10 and -12, the serine proteinase tPA and BACE-1. Shedding of LRP1-ECD allows the release from the plasma membrane by γ-secretase of the intra-cytoplasmic domain of LRP1 (LRP1-ICD), which could act as signaling mediator (May et al., 2002). Accumulation of extracellular proteolytic activities associated to the tumor microenvironment could explain at least in part why cell-surface LRP1 is generally found decreased in advanced tumors. However, the significance of LRP1 shedding is not really understood in the field of malignant diseases.

Only a few *LRP1* polymorphisms or mutations were identified in cancer specimens. Benes et al. (2003) associated the C766T polymorphism with an increased risk to develop breast cancer in Caucasian women. Although this change into a thymine nucleotide does not result in an amino acid substitution, this silent mutation has previously also been linked to Alzheimer's

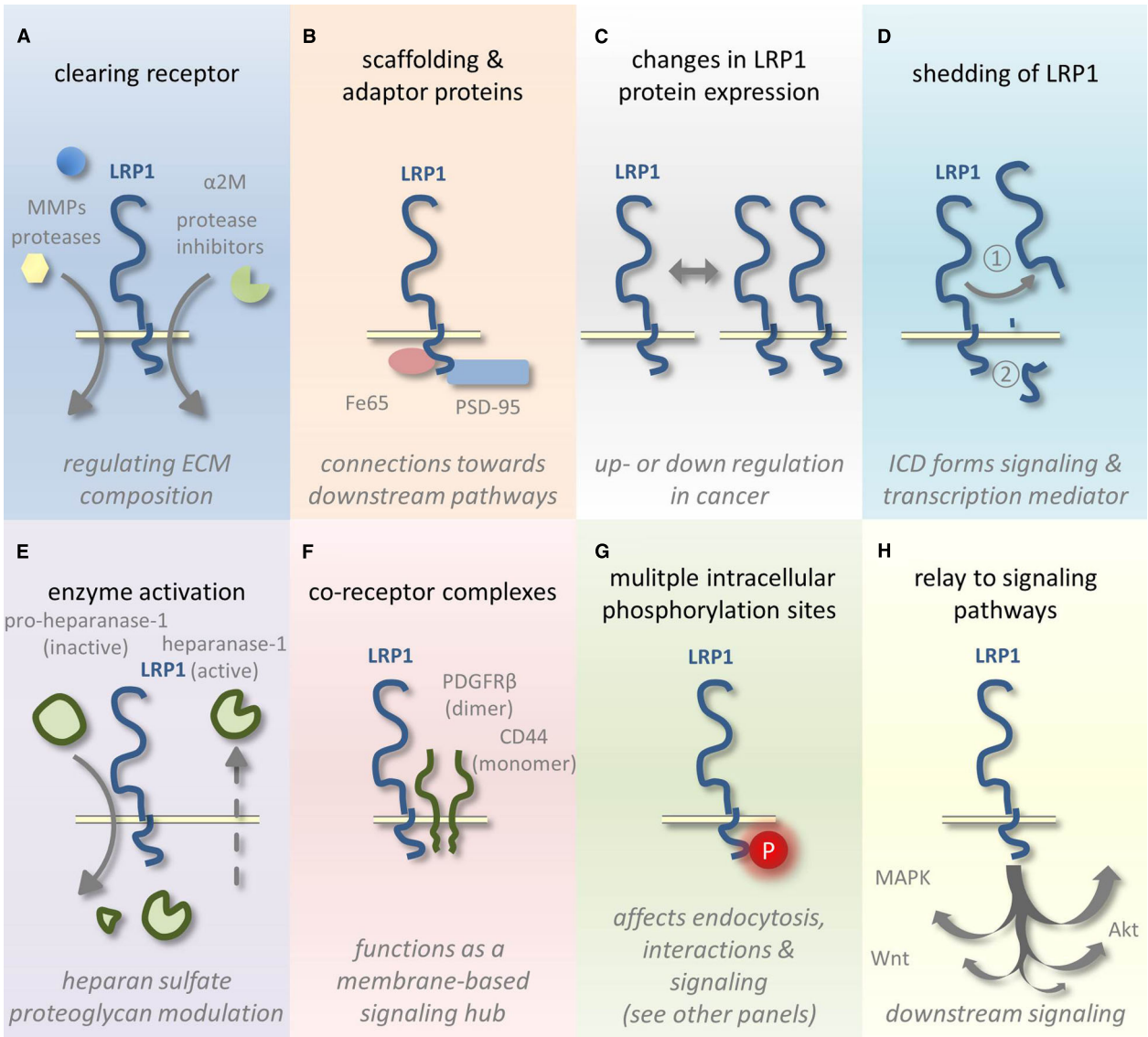

FIGURE 1 | Schematic representation of LRP1-mediated tumor growth and metastasis fine tuning. (A) LRP1 clears various cancer-related ligands from the ECM by endocytosis. **(B)** The LRP1 ICD also interacts with several adaptor and scaffolding proteins. **(C)** LRP1 expression levels vary among different tumor types and tumor stages and **(D)** the receptor can undergo shedding and subsequent release of the ICD. **(E)** Heparanase-1 activation is affected by LRP1-mediated uptake of its inactive precursor. **(F)** The formation of co-receptor complexes with LRP1 influences signaling and **(G)** also the phosphorylation of the LRP1 ICD influences signaling and regulates endocytosis. **(H)** LRP1-mediated signaling affects several well-known pathways linked to cancer.

(Kolsch et al., 2003) and coronary artery disease (Pocathikorn et al., 2003) but also conflicting data were published (Benes et al., 2001; Pritchard et al., 2005). Recently, a LRP1-SNRNP25 fusion gene was identified in two osteosarcomas (Yang et al., 2014). Only the first eight exons including the promoter region of LRP1 are implicated in the fusion gene. Although the relevance of LRP1 expression to osteosarcoma is currently unknown, *in vitro*, however, LRP1-SNRNP25 promotes invasion and migration. LRP1-SNRNP25 expression was increased in both tumors via the LRP1 promoter activity of the fusion gene compared to the wild-type SNRNP25 expression in other osteosarcomas specimen.

A MULTITUDE OF CANCER-MODIFYING PATHWAYS

Remodeling of the ECM is essential for both tumor growth and metastasis. As a matricellular receptor, LRP1 is involved in the regulation of several ECM modifying pathways.

(Matrix)-metalloproteinases (MMPs) are key enzymes in physiological but also in cancer-related modulation of ECM and basement membrane components. Their proteolytic function mostly results in inactivation or degradation of many of their different substrates. MMPs are, however, also found involved in signaling functions in a non-proteolytic manner

(Kessenbrock et al., 2010, 2015; Yamamoto et al., 2015). LRP1 mediates endocytosis of MMP-2, -9, -13, ADAMTS-4 and ADAMTS-5 and clears these proteases from the ECM (Emonard et al., 2005; Yamamoto et al., 2014, 2015). Endocytosis by LRP1 can depend on complex formation: (pro)MMP-2:TSP-2 (thrombospondin-2), proMMP-2:TIMP-2 (tissue inhibitor of metalloproteinases 2), and proMMP-9:TIMP-1 complexes are all ligands to LRP1 and cleared by this receptor (Emonard et al., 2005; Yamamoto et al., 2015). Furthermore, other MMPs are being regulated by LRP1, although indirectly, via the clearance of TIMP-1, -2, and -3 by LRP1 whether bound to an MMP (Emonard et al., 2005; Yamamoto et al., 2015) or alone (TIMP-1 and -3; Scilabra et al., 2013; Thevenard et al., 2014). These TIMPs also display signaling functions via the ERK and Wnt pathways (Liu et al., 2003; Egea et al., 2012). Also the broad spectrum protease inhibitor α_2M binds to LRP1 followed by subsequent internalization (Andersen et al., 2000). Not only metalloproteinases are a target of this glycoprotein but also serine-, carboxyl-, and thiol proteinases are blocked from interacting with their respective substrates (Rehman et al., 2013). Besides its activity as a protease inhibitor, α_2M was recently shown to stimulate angiogenesis via activation of stem cells through FGF-2 and nitric oxide via LRP1-mediated signaling (Sauer et al., 2013).

Heparanase-1 is another matrix modifying enzyme that is endocytosed by LRP1, both for its activation and clearance (**Figure 1E**). This enzyme cleaves heparan sulfate proteoglycans (HSPG), one of the core components of the ECM (Ilan et al., 2006). HSPGs not only play a role in the integrity of the ECM but also act as a storage depot for growth factors, chemokines, cytokines and enzymes. Heparanase-1 is synthesized as an inactive precursor. Activation requires proteolytic cleavage that is partly dependent on LRP1-mediated pro-heparanase-1 internalization (**Figure 1E**; Vreys et al., 2005). Also mature heparanase-1 can be endocytosed by LRP1 targeting it for degradation or recycling (Vreys and David, 2007).

uPA-uPAR signaling is another migration- and invasion-related pathway regulated by LRP1 that can promote cell invasion and migration (Webb et al., 2000; Amos et al., 2007; Gonias et al., 2011). uPA and tPA proteinase activity are implicated in the plasminogen activator system and as such mediate plasmin-dependent degradation of ECM proteins (Gonias et al., 2011). Interaction of uPA with PAI-1 on uPAR stimulates uPAR-LRP1 complex formation and subsequent endocytosis (Czekay et al., 2001). This affects uPAR presence at the plasma membrane with consequences for ECM degradation via the plasminogen activation system and uPAR-integrin interaction, both important for cell migration. Also for angiogenesis the uPA-plasmin system is highly relevant (Raghu et al., 2010). Furthermore, LRP1 was shown to promote maturation of the integrin β1 precursor thereby increasing the level of integrin β1 at the cell surface (Salicioni et al., 2004). LRP1 also binds to $\alpha_M\beta_2$ thereby altering integrin function. In macrophages, LRP1 is important for $\alpha_M\beta_2$ internalization thereby possibly influencing macrophage-mediated inflammation (Ranganathan et al., 2011).

Migration of malignant cells is further affected by LRP1-CD44 complexes in the cell membrane (**Figure 1F**). LRP1 was recently shown to control the adhesion in tumor cells via interaction with, and internalization of CD44, a transmembrane glycoprotein (Perrot et al., 2012). CD44 mediates cell adhesion to the ECM, migration and is probably involved in tumor and metastasis initiation. Like LRP1, CD44 acts as an interface for signal transduction at the cell surface as recently reviewed (Orian-Rousseau, 2015). A lowering in LRP1 expression as observed in certain cancers (see supra) could thus result in CD44 accumulation at the cell surface and enforced cancer cell attachment.

Besides this, probably far from complete, overview of LRP1-related ECM modifying processes, LRP1 also forms co-receptor complexes (**Figure 1F**) at the cell surface with receptors involved in cancer-related pathways. A good example is the association between LRP1 and the platelet-derived growth factor receptor-β (PDGFR-β). LRP1 not only mediates PDGF internalization and degradation, in two accompanying papers, PDGF-BB was shown to mediate the phosphorylation (**Figure 1G**) of the Tyr_{63} in the distal NPxY motif of LRP1 located in caveolae (Boucher et al., 2002; Loukinova et al., 2002). This process is dependent on PDGFR activation and on the kinase activity of the c-Src family of proto-oncogenic tyrosine kinases. This relationship links LRP1 to Ras, c-Myc, MAPK, and Akt/PI3K signaling, well known pathways implicated in oncogenesis (**Figure 1H**). Later, LRP1 was shown to directly associate with PDGFR-β to form a signal transduction complex (Newton et al., 2005; Muratoglu et al., 2010). As such PDGF signaling is influenced by LRP1 and *vice versa*. Recently, the group of May demonstrated that LRP1's ICD also modulates the crosstalk between PDGF-BB and sphingosine-1 which is important for modulation of PDGF-BB induced cell migration and blood vessel maturation (Nakajima et al., 2014). The possible relevance for tumor angiogenesis is yet to be determined. LRP1 also affects angiogenesis among other things via is regulatory role in VEGF signaling. The complex of the angiogenic inhibitor thrombospondin-1 and VEGF is internalized via LRP1 (Greenaway et al., 2007).

THE RELEVANCE OF LRP1 FOR THE INTERACTION BETWEEN MALIGNANT CELLS AND THE TUMOR (MICRO)ENVIRONMENT

Both LRP1 expressed in malignant cells themselves and LRP1 expressed in non-tumorous cells present in the tumor (micro)environment are relevant for modulation of the above described cancer-modifying pathways. These pathways are involved in processes like growth and survival of tumor cells, angiogenesis, extravasation of tumor cells, invasion and metastasis. The relative expression of LRP1, its ligands and co-receptors, irrespective whether expressed by the tumor cells themselves or other cells in the tumor (micro)environment determine the modifying role of LRP1 in these different, but linked processes, which may in fact result in opposing effects on cancer progression.

Montel et al. (2007) silenced LRP1 in tumor cells only and host LRP1 was left untouched. They observed the failure of metastatic

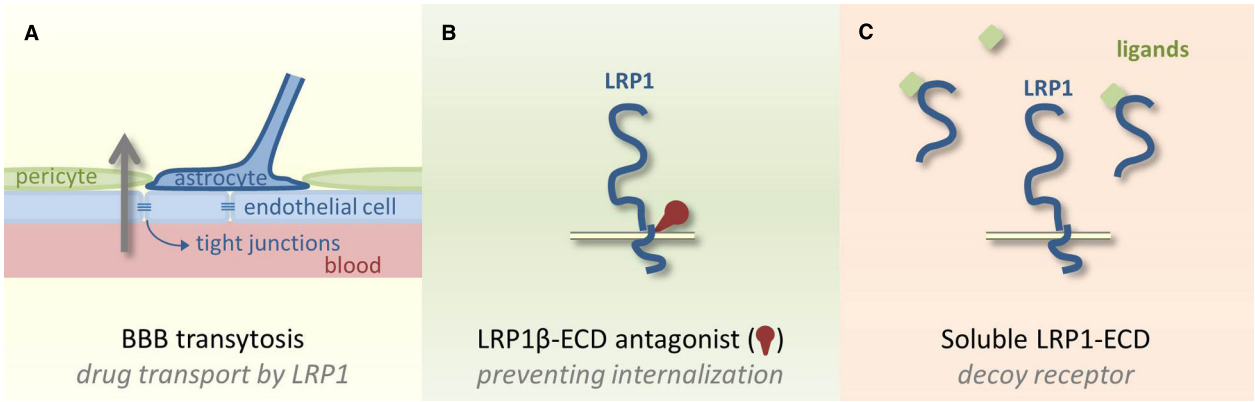

FIGURE 2 | Potential clinical applications of LRP1. (A) Using LRP1 as a cargo receptor to sluice chemotherapeutic agents through the tightly controlled blood–brain barrier via transcytosis is undergoing clinical testing. **(B)** LRP1 antagonists to the ECD could prevent LRP1 internalization and catabolization. **(C)** The ECD of LRP1 could also be used as a soluble decoy receptor to capture LRP1 ligands and avoid ligand-receptor interaction.

foci to grow in the lungs from xenografts of CL16 cells in SCID mice thus illustrating the relevance of LRP1 expression in tumor cells themselves.

Also the importance of LRP1 expression in non-tumor cells in the tumor environment has been demonstrated. In the breast tumor microenvironment, it was reported that the pro-cath-D protease, highly secreted by tumor cells, may trigger mammary fibroblast outgrowth in a paracrine LRP1-dependent manner (Beaujouin et al., 2010). The molecular mechanism engaged appears atypical as pro-cath-D interacts with the extracellular part of LRP1 β-subunit mediating the inhibition of LRP1-regulated intramembrane proteolysis in mammary fibroblasts (Derocq et al., 2012; Laurent-Matha et al., 2012). Recently, Staudt et al. (2013) demonstrated that the recruitment of LRP1-deficient monocytes into subcutaneous and orthotopic pancreatic tumors were significantly increased. The secretion of chemokines by LRP1-deficient macrophages is enhanced (especially CCL3), resulting in an increased number of tumor-associated macrophages (TAM) in the tumor site. The authors provided evidence that the LRP1-deficient TAM collectively contribute to an increased VEGF amount into the tumor microenvironment, leading to increased tumor angiogenesis.

The aforementioned role of LRP1 in heparanase activation and uptake implicates a potential regulatory role for LRP1 in exosomes biogenesis. As reviewed elsewhere (De Toro et al., 2015), exosomes are nanovesicles secreted by various cell types, including cancer cells, that serve in cell–cell communication. They can be isolated from body fluids and are regarded potential biomarkers for diagnosis and prognosis. As recently shown, syndecan heparan sulfate (HS) proteoglycans and heparanase are involved in exosome production (Baietti et al., 2012; Roucourt et al., 2015). Trimming of HS chains on syndecan molecules by heparanase appears to affect the formation of multimeric complexes of syndecans, co-receptors and the intracellular adaptor protein syntenin triggering the generation of intraluminal vesicles in multivesicular bodies (MVBs), eventually resulting in the release of exosomes. Heparanase apparently does not only regulate secretion of tumor-cell derived exosomes, but also its composition and function (Thompson et al., 2013). As such, LRP1-mediated control on active heparanase availability could effect exosome production and function.

DRUG DELIVERY ACROSS THE BLOOD–BRAIN BARRIER

Current studies on therapeutic strategies involving LRP1 focus on using it as a cargo receptor to treat brain metastases. The aforementioned expression of LRP1 in glioblastoma and other brain cancers (Yamamoto et al., 1997; Baum et al., 1998) or metastasis combined with LRP1's expression at the BBB (Pflanzner et al., 2011) is crucial to this strategy. The capability of LRP1 to mediate transcytosis of a broad range of ligands through the BBB (**Figure 2A**) could be the long-awaited sluice for chemotherapeutic agents into the brain as BBB penetration is currently the Achilles' heel in brain cancer therapies (Jovčevska et al., 2013). Uptake of paclitaxel through the BBB followed by endocytosis into tumor cells was shown to be increased after conjugating the taxane paclitaxel to a 19 amino acid sequence named angiopep-2 (Bertrand et al., 2011). This peptide was derived from the Kunitz domain, a known ligand of LRP1. A phase I clinical study showed that this conjugate (GRN1005) is well tolerated (Kurzrock et al., 2012; Drappatz et al., 2013). Therapeutic concentrations could be reached in the tumor and three patients where prior taxane therapy was unsuccessful showed partial response with GRN1005. After an initial phase II study, additional phase II studies are currently ongoing for patients with brain metastases from breast cancer and high grade glioma. Also other constructs are evaluated preclinically including an anti-HER2 antibody conjugated to angiopep-2 to treat brain metastasis from HER2 positive breast cancers (Regina et al., 2015). As demonstrated recently *in vitro* and in animal studies, angiopep-2 could also aid active transport of polymersomes through the BBB via LRP1 mediated trancytosis suitable for antibody delivery to the brain (Tian et al., 2015).

Apart from angiopep-2, also peptides containing a serine-arginine-leucine (SRL) sequence bind LRP1 and were recently

shown to aid PAMAM nanoparticle transport across the BBB (Zarebkohan et al., 2015). These LRP1 targeted particles could become a valuable tool for non-invasive gene targeting to the brain.

Although highly challenging, developing strategies aiming at LRP1 targeting should be relevant in certain tumor microenvironments. We might consider new LRP1 antagonists targeting the extracellular part of the LRP1 β-subunit to avoid LRP1 itself being internalized and catabolized (**Figure 2B**). Another alternative could be to use the soluble LRP1-ECD as a decoy receptor to interfere with endocytic and signaling activities of cell-surface LRP1 (**Figure 2C**). The proof of concept exists for TIMP-3. Bound to LRP1-ECD, TIMP-3 becomes resistant to endocytosis and degradation and retains its inhibitory activity against metalloproteinases (Scilabra et al., 2013). LRP1 ligand-binding domains II and IV are probably the most critical regions that could serve as molecular and structural models for designing new therapeutic tools.

POTENTIAL OF LRP1 IN DIAGNOSIS AND PROGNOSIS

As discussed previously, in some cancer types, LRP1 expression was correlated with invasiveness, tumor stage, and even clinical outcome. However, although it has been suggested that LRP1 could be a potential biomarker (Meng et al., 2011), so far, there seems to be lots of variability and discussion. As mentioned before, LRP1 expression in cell cultures is also debatable as the *in vitro* conditions could affect LRP1 expression. Recent work on data from tumor samples identified LRP1 as a hub in a biomarker network for multi-cancer clinical outcome prediction (Martinez-Ledesma et al., 2015). This further illustrates the involvement and possible prognostic value of LRP1 in various cancers. Future large scale studies on patient samples could provide more insights and demonstrate the true relevance of LRP1 in diagnosis and prognosis of cancer.

CONCLUSION

Via a diverse array of interactions LRP1 modulates various pathways involved in cancer. Especially its role in modifying the ECM could be crucial for tumor growth and metastasis. However, considering the sometimes contradicting studies LRP1 cannot be considered a master switch as some prototype oncogenes or tumor suppressor genes are. Rather, it acts as an interface to fine-tune various cancer-related pathways. Its effects appear to be dependent on both the tumor type and the tumor environment. This complicates LRP1 research and calls for good model systems that integrate the diverse set of LRP1 activities. These should answer the question whether LRP1 could be a valuable target for diagnosis, prognosis and therapeutics in cancer as well as other diseases.

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Cellular Cholesterol Distribution Influences Proteolytic Release of the LRP-1 Ectodomain

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Low-density lipoprotein receptor-related protein-1 (LRP-1) is a multifunctional matricellular receptor composed of a large ligand-binding subunit (515-kDa α-chain) associated with a short trans-membrane subunit (85-kDa β-chain). LRP-1, which exhibits both endocytosis and cell signaling properties, plays a key role in tumor invasion by regulating the activity of proteinases such as matrix metalloproteinases (MMPs). LRP-1 is shed at the cell surface by proteinases such as membrane-type 1 MMP (MT1-MMP) and a disintegrin and metalloproteinase-12 (ADAM-12). Here, we show by using biophysical, biochemical, and cellular imaging approaches that efficient extraction of cell cholesterol and increased LRP-1 shedding occur in MDA-MB-231 breast cancer cells but not in MDA-MB-435 cells. Our data show that cholesterol is differently distributed in both cell lines; predominantly intracellularly for MDA-MB-231 cells and at the plasma membrane for MDA-MB-435 cells. This study highlights the relationship between the rate and cellular distribution of cholesterol and its impact on LRP-1 shedding modulation. Altogether, our data strongly suggest that the increase of LRP-1 shedding upon cholesterol depletion induces a higher accessibility of the sheddase substrate, i.e., LRP-1, at the cell surface rather than an increase of expression of the enzyme.

Keywords: LRP-1, low-density lipoprotein receptor-related protein-1, ectodomain, cholesterol, shedding, Raman microspectroscopy

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INTRODUCTION

The low-density lipoprotein receptor-related protein-1 (LRP-1) is a large heterodimeric receptor composed of an heavy extracellular chain, the 515-kDa α-chain, non-covalently associated with a light transmembrane chain, the 85-kDa β-chain (Emonard et al., 2014). The extracellular α-chain exhibits four cystein-rich complement-type repeats which bind more than 40 ligands, including proteinases and proteinase:inhibitor complexes (Etique et al., 2013). Motifs of the intracellular part of the β-chain activate endocytosis and signaling pathways (Lillis et al., 2005), which drive numerous biological functions and play a key role in the development of many pathological disorders (Lillis et al., 2008; Van Gool et al., 2015). LRP-1 invalidation in mice is lethal at early stage of embryogenesis (Herz et al., 1992). We previously demonstrated that LRP-1 promotes invasion of malignant cells by modulating focal complex composition (Dedieu et al., 2008).

Low-density lipoprotein receptor-related protein-1 is broadly expressed in multiple cell types such as mesenchymal and epithelial cells (Emonard et al., 2014). Its expression is regulated by hormones and growth factors that induce different responses depending on cell types. Cell surface LRP-1 is cleaved by shedding to generate soluble LRP-1 ectodomain composed of the entire extracellular α-chain linked to the extracellular part of the β-chain which was first discovered in plasma (Quinn et al., 1997). The first LRP-1 sheddase was characterized in human choriocarcinoma BeWo cells as a metalloproteinase (Quinn et al., 1999). More recently our group identified a disintegrin and metalloproteinase-12 (ADAM-12) and membrane-type 1 matrix metalloproteinase (MT1-MMP; Selvais et al., 2009, 2011). Several proteolytic enzymes belonging to other proteinases families have also been identified (for a review, Emonard et al., 2014).

Shedding is a closely regulated process that controls most of types I and II transmembrane proteins levels at cell surface (Hartmann et al., 2013). Cellular cholesterol depletion stimulates shedding of the interleukin-6 receptor (Matthews et al., 2003) and CD30 antigen (von Tresckow et al., 2004). By comparing two cell lines exhibiting different levels of cholesterol (conventional human fibrosarcoma HT1080 cells and an epithelioid variant with a twofold higher cell cholesterol content), we previously showed that low cell cholesterol level promotes LRP-1 shedding (Selvais et al., 2011).

Cholesterol is widely expressed at cell surface of mammalian cells but can also be located in the cytosolic compartment where it could play a role in transmembrane protein trafficking (Mukherjee et al., 1998). In the present study, we evaluated the efficiency of LRP-1 shedding process in cell lines expressing either cholesterol at plasma membrane or in cytosolic compartment (Nieva et al., 2012). We demonstrated by using different imaging approaches, that efficient extraction of cholesterol and increased LRP-1 shedding occur predominantly in cells exhibiting cholesterol at cell surface.

MATERIALS AND METHODS

Reagents and Antibodies

Dulbecco's modified Eagle medium (DMEM) and other cell culture reagents were purchased from Thermo Fisher Scientific (Illkirch, France). Fetal calf serum (FCS) was from Dutscher (Brumath, France). Filipin, methyl-β-cyclodextrin (MβCD) and other chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Phosphate-buffered saline (PBS)-B (131 mM NaCl, 5.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) was from bioMérieux (Craponne, France). Anti-LRP-1 α-chain (mouse, clone 8G1) was from Calbiochem (Merck Biosciences, distributed by VWR International, Strasbourg, France). Goat polyclonal antibodies directed against β-actin were from Abcam (Paris, France). Horseradish peroxidase (HRP)-conjugated anti-mouse antibodies were from Cell Signaling Technology (distributed by Ozyme, Montigny-Le-Bretonneux, France) and HRP-anti-goat antibodies from Sigma-Aldrich.

Cell Culture

Human breast cancer cell lines MDA-MB-231 and MDA-MB-435 were obtained from the American Type Culture Collection. MDA-MB-231 and MDA-MB-435 cells were cultured in DMEM containing 1 and 4.5 g/l glucose, respectively. Culture media were supplemented with 10% FCS, 100 units/ml penicillin and 10 mg/ml streptomycin. For cell imaging, FCS was depleted in lipoproteins following a procedure adapted from the Havel et al. (1955). Cells were grown at 37°C in a humid atmosphere (5% CO_2 and 95% air). As cellular cholesterol content depends, at least in part, on cellular confluency state (Takahashi et al., 2007), all experiments were carried out at similar cell densities.

MβCD Treatment and Cholesterol Assay

The water-soluble MβCD forms soluble inclusion complexes with cholesterol, enhancing thus its solubility in aqueous solution (Pitha et al., 1988) and is classically used to extract cholesterol from cultured cells. In the present study, cells were treated with MβCD (0–20 mM) in FCS-free medium for 30 min at 37°C. Cells were then harvested in reaction buffer (0.1 M potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM cholic acid, and 0.1% Triton X-100) and sonicated. Cholesterol content was quantified using the Amplex Red cholesterol assay kit (Invitrogen distributed by Thermo Fisher Scientific), as recommended by the manufacturer. Reactions proceeded for 20 min at 37°C. Alternatively, after treatment with MβCD cells were washed with PBS and further incubated in FCS-free medium for 24 h. Twenty four-hour conditioned media were concentrated and shedding of LRP-1 was analyzed by western blotting.

Raman Microspectroscopy Analysis

Cells (3×10^4) were seeded in 6-well plates containing CaF_2 substrates (Crystan, Ltd., Dorset, UK), and 48 h later cells were fixed with 4% cold paraformaldehyde (PFA) for 30 min at room temperature. After fixing, cells were washed three times with PBS and water, before drying to be analyzed with Raman spectroscopy.

Raman spectra were acquired from each sample using a LabRAM Raman spectrometer (Jobin Yvon, Horiba, Lille France). The setup contained a laser diode at 660 nm supplying an excitation beam of 25 mW at the sample. The laser beam was focused onto the sample using a Leica HCX PL FluoTar x100 objective (NA = 0.75). The same objective collected the light scattered from the sample. An edge filter permitted to reject the laser reflection and the Rayleigh scattering. A grating of 1200 g/mm ensured the dispersion of the Raman Stokes signal with a spectral resolution of 4 cm^{-1}. The intensity of the Raman vibrations was measured using a deep depletion charge-coupled device (CCD) detector. The spectra were collected on a total spectral range from 400 to 4000 cm^{-1}, with an acquisition time of 40 s per spectrum. For each cell, a number of five spectra were collected at the level of the cytoplasm. Raman data were then baseline-corrected using linear segments, slightly smoothed (three points averaging) and normalized on the basis of the total integrated intensity. The five spectra recorded on each cell were averaged. Up to this point, the acquisition and processing

of the data were performed using Labspec 5 software (Horiba Jobin Yvon, Lille France). Then, Raman data were submitted to statistical multivariate processing corresponding to principal component analysis (PCA). PCA operated via a home-made interface using Matlab Toolbox (MathWorks®). Mean-centered data were used for PCA.

Cholesterol Staining and Cell Imaging

Cells (5×10^4) were seeded onto gelatin-coated glass slides and cultured in media containing lipoproteins-depleted FCS for 24 h at 37°C. Then, cells were treated or not with MβCD and fixed in 3% paraformaldehyde for 60 min at room temperature. After three washes in PBS, cells were incubated in glycine (1.5 mg/ml in PBS-B) for 10 min and then stained with filipin (0.05 mg/ml in PBS-B) for 2 h at room temperature. Filipin-stained cell preparations were analyzed using a Zeiss LSM 710 confocal laser scanning microscope with the 63x oil-immersion objective and Zen operating system (Zeiss, Heidelberg, Germany). All acquisitions were performed with UPlan x 63, 1.4 numerical aperture objective by exciting filipin with a chameleon infrared laser tuned at 740 nm. Emitted fluorescence was detected through the appropriate filter and twenty images were captured with a 0.25-μm z-step. DIC images were acquired simultaneously with the reflected light images using a TPMT module. Images were treated with Amira™ software (v6.0.1, FEI visualization Sciences Group, Merignac, France) and projection through each z-stack was merged with DIC images.

RNA Isolation, RT-PCR, and Real-Time-PCR

Total RNAs were isolated and purified with Extract-All kit (Eurobio Laboratories, Courtaboeuf, France). Reverse transcription (RT) and real-time PCR were performed with Verso SYBR 2-Step QRT Rox kit (AB-4113/A) and Absolute QPCR SYBR Green Rox (AB-1162/B), respectively (Thermo Fisher Scientific). Quantitative PCR was carried out on a Chromo4 Real-Time Detector (Bio-Rad Laboratories, Marne-la-Vallée, France). Data were normalized to ribosomal proteins L32 (RPL32) and S18 (RS18) or to β-actin. Primers for LRP-1 (Dedieu et al., 2008) and β-actin (Langlois et al., 2010) were previously described. Primers were synthesized as follows: for MT1-MMP, AACCAAGTGATGGATGGATACC and CTCCTTAATGTGCT TGGGGTAG; for transmembrane form of ADAM-12, ADAM-12L, GGGCTGTAGCTGTCAAATGG and CTGACTTCCGGCA GGTTAAA; for RPL32, CATTGGTTATGGAAGCAACAAA and TTCTTGGAGGAAACATTGTGAG; for RS18, GCAGAAT CCACGCCAGTACAA and GCCAGTGGTCTTGGTGTGCT (Eurogentec France, Angers, France). Results shown are representative of three independent experiments.

Sample Preparation and Western Blot Analysis

Cells treated or not with MβCD were then washed with PBS and further incubated with FCS-free medium for 24 h. Twenty four-hour-conditioned media were cleared by centrifugation (1,000 *g* at 4°C for 10 min) and concentrated 50-fold with Vivaspin centrifugal concentrators (Sartorius Stedim Biotech, distributed by Dutscher) following manufacturer's recommendations. Cells were scraped in ice-cold lysis buffer (10 mM CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonylfluroide supplemented with proteinase inhibitor cocktail from Sigma-Aldrich). After sonication, the remaining pellet was discarded by centrifugation (10,000 *g* at 4°C for 10 min).

Western blotting was performed as previously described (Selvais et al., 2011) using 5% polyacrylamide gel for LRP-1 515 kDa α-chain and 10% polyacrylamide gel for β-actin. Primary antibodies were used at 1/4000 for LRP-1 α-chain and 1/1000 for β-actin. Samples were normalized with respect to cell protein amount, which was determined using BC assay protein quantitation kit (Thermo Scientific, distributed by Interchim, Montluçon, France). Each lane was loaded with cell lysates equivalent of 40 μg protein, or corresponding amounts of conditioned medium. Immunoreactive bands were revealed using the ECL chemiluminescence kit (Amersham Biosciences, distributed by Dutscher), acquired using the Odyssey® Fc Dual-Mode LI-CORE Imaging System (Biosciences Biotechnology, distributed by Eurobio Laboratories) and quantified using ImageJ software. β-actin antibodies were used for normalization. Immunoblots presented are representative of at least three independent experiments.

Statistical Analysis

Data were analyzed using unpaired two-tailed Student's *t*-test. Differences were considered significant for $P < 0.05$. Values are reported as mean ± SD.

RESULTS

We previously reported in the human fibrosarcoma HT1080 cell line the correlation between cell cholesterol amount and efficiency of LRP-1 shedding (Selvais et al., 2011). In the present study we investigated the possible role of cholesterol distribution by using two different breast cancer cell lines MDA-MB-231 and MDA-MB-435 cells, one expressing cholesterol in the cytosol and the other at the plasma membrane (Nieva et al., 2012).

MDA-MB-231 and MDA-MB-435 Breast Cancer Cells Exhibit Different Lipid Phenotypes

We first explored the lipid phenotype of MDA-MB-231 and MDA-MB-435 cells by Raman microspectroscopy. Spectra collected on the cytoplasmic compartment of the two cell lines were processed by PCA, an exploratory unsupervised method of multivariate data processing. PCA is commonly used to explore the intra- and inter-group variabilities based on the Raman signals of the cells (Poplineau et al., 2011). A distinction between the two cell types is clearly visible on the score plot constructed on the two first components (**Figure 1A**). The distinction relies mainly on the first principal component (PC1) that exhibits signals assigned to lipid vibrations (**Figure 1B**). Indeed, the spectral zones grayed on the display of the first component,

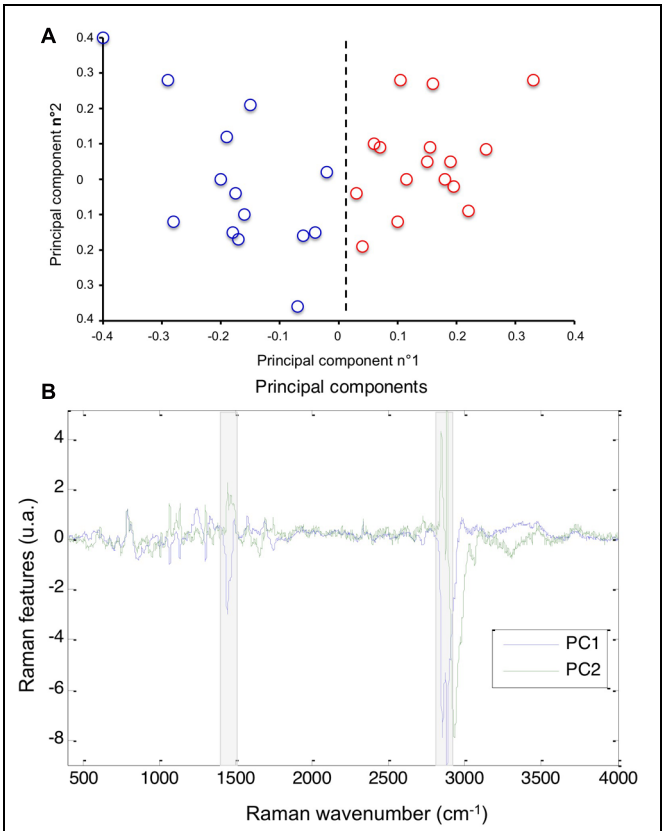

FIGURE 1 | Cytoplasmic lipid profiling of MDA-MB-231 and MDA-MB-435 cells by Raman analysis. (A) Score plot resulting of the PCA processing of Raman spectra collected on MDA-MB-231 (red circles) and MDA-MB-435 (blue circles) cells. The scores were projected on the two first principal components. **(B)** Display of the two first principal components. The Raman features comprising the main variability of the spectral data, were highlighted by the grayed underlining.

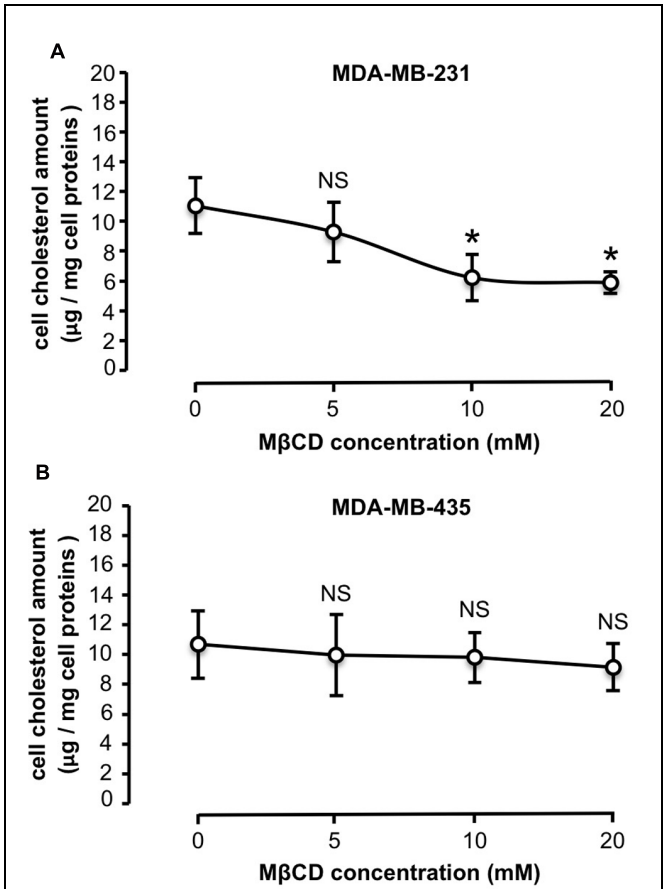

FIGURE 2 | Differential efficiency of MβCD for depleting cholesterol. MDA-MB-231 **(A)** and MDA-MB-435 **(B)** breast cancer cells were treated as described under Section "Materials and Methods" with increasing concentrations of MβCD and cellular cholesterol content was then measured. Values expressed as µg cholesterol/mg cell protein are mean ± SD ($n = 6$ for each cell line). NS, not significant, $^*P < 0.05$; Student's *t*-test.

centered at 1450 and 2850 cm^{-1}, are assigned to bending and stretching vibrations of lipid CH_2 and CH_3 groups respectively. This analysis reflects that the lipid contribution of the cytoplasm as probed by Raman spectroscopy allows distinguishing MDA-MB-231 and MDA-MB-435 cells. The same Raman features are also recovered on the second principal component (PC2), reflecting their involvement in the intra-group variability of these cellular samples.

Efficiency of Cholesterol Depletion Depends on its Cellular Distribution

We next investigated using biochemical and cellular imaging analyses whether the biophysical analysis findings showing different cytoplasmic lipids-based discrimination between MDA-MB-231 and MDA-MB-435 cells was confirmed for cholesterol.

Similar cholesterol content was quantified in untreated MDA-MB-231 and -435 cells, with 11.1 ± 2.9 µg cholesterol/mg cell protein and 10.3 ± 2.9 µg cholesterol/mg cell protein, respectively. MDA-MB-231 and -435 cells were then treated with increasing concentrations of MβCD (5, 10, and 20 mM) to extract cholesterol and the effect on cell cholesterol depletion was measured (**Figure 2**). Five millimolar MβCD had no effect on cell cholesterol amount in the two cell lines. In MDA-MB-231 cells a depletion peak of cell cholesterol was observed at 10 mM of MβCD (**Figure 2A**). In contrast, in MDA-MB-435 cells cell cholesterol amount did not vary upon MβCD treatment (**Figure 2B**). Filipin-labeled cells revealed that cholesterol was predominantly distributed in cytosol for MDA-MB-231 cells (**Figure 3A**, white arrow) and in plasma membrane in MDA-MB-435 cells (**Figure 3C**, red arrow). Interestingly, efficient extraction of cholesterol upon MβCD treatment was observed in MDA-MB-231 cells (**Figure 3B**) but not in MDA-MB-435 cells (**Figure 3D**).

Decrease of Cell Cholesterol Content Potentiates Shedding of LRP-1 without Affecting the Expression of LRP-1, MT1-MMP, and ADAM-12

We then investigated if modulation of cell cholesterol amount by MβCD treatment had an impact on LRP-1 shedding process in

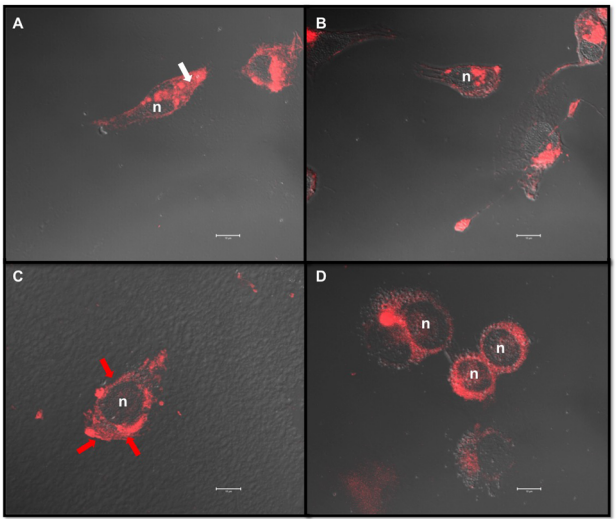

FIGURE 3 | MβCD exhibits different effects on cholesterol depletion in MDA-MB-231 and MDA-MB-435 breast cancer cell lines. MDA-MB-231 **(A,B)** and MDA-MB-435 **(C,D)** cells were treated as described under Section "Materials and Methods" with vehicle alone **(A,C)** or 10 mM MβCD **(B,D)**. Cells were fixed in 3% PFA and treated with filipin (50 μg/mL) for 2 h at room temperature. Projection of each z-stack acquired through confocal microscopy images was merged with DIC images. Filipin labels free cholesterol present in the membranes (red arrow) and in the cytosol (white arrow). Scale bar: 10 μm, n, nucleus.

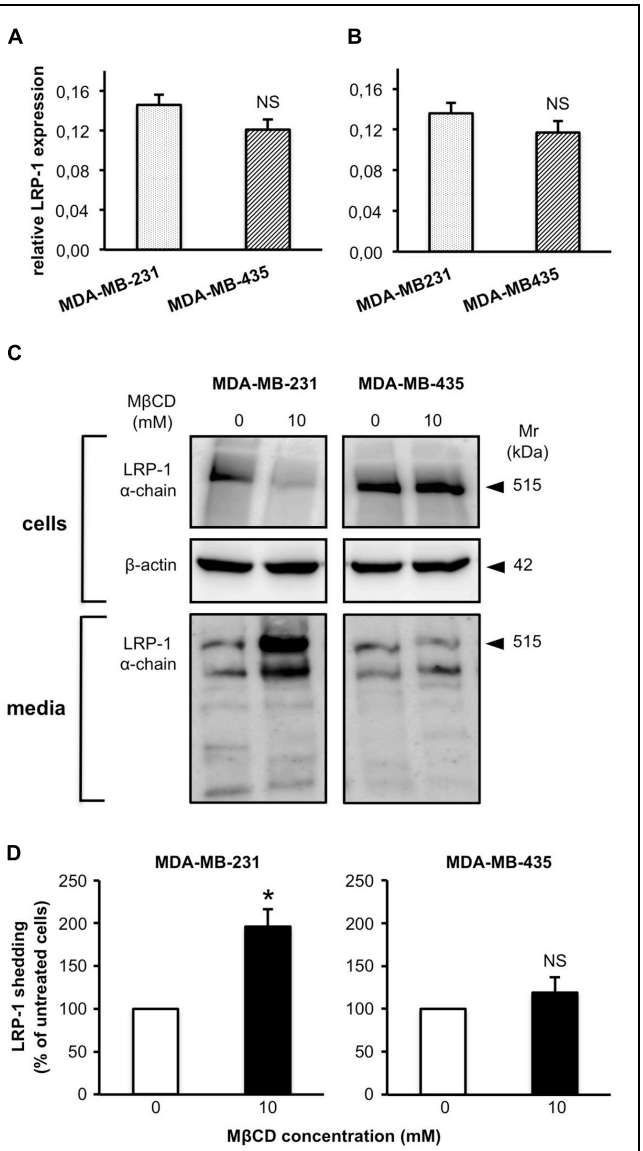

FIGURE 4 | Depletion of cellular cholesterol by MβCD increases shedding of the LRP-1 ectodomain. MDA-MB-231 and MDA-MB-435 cells were treated as described under Section "Materials and Methods" with vehicle alone or 10 mM MβCD. Basal level expression of LRP-1 was measured by quantitative real-time PCR. Data were normalized to ribosomal proteins RPL32 **(A)** and RS18 **(B)**. Western blotting of LRP-1 α-chain and β-actin was performed from cell lysates, and blotting of LRP-1 α-chain in corresponding amounts of 24-h conditioned medium. Data are from a representative experiment **(C)**. LRP-1 ectodomain shedding was quantified on Western blots of LRP-1 α-chain released in the concentrated conditioned medium **(D)**. NS, not significant, $*P < 0.05$; Student's *t*-test.

MDA-MB-231 and -435 cells (**Figure 4**), as previously reported for the human fibrosarcoma HT1080 cells (Selvais et al., 2011). In the absence of MβCD treatment, LRP-1 expression is similar in MDA-MB-231 and -435 cells (**Figures 4A,B**). LRP-1 levels are also comparable in CHAPS extracts from MDA-MB-231 and -435 cells as well as in their respective conditioned media (**Figure 4C**). By using MβCD at 10 mM, a concentration that efficiently depleted MDA-MB-231 cells in cholesterol (**Figure 2A**), we observed a large decrease of LRP-1 in the CHAPS extracts of MDA-MB-231 cells that was accompanied by a twofold increase of soluble LRP-1 in conditioned media (**Figures 4C,D**). In contrast MβCD treatment, which did not modify cholesterol amount in MDA-MB-435 cells (**Figure 2B**), had no effect on LRP-1 shedding in these cells (**Figures 4C,D**).

To exclude that differences of LRP-1 shedding levels that we observed between the two breast cancer cell lines could be attributed to modulations of LRP-1, MT1-MMP and/or ADAM-12, its main sheddases (Selvais et al., 2011), we tested the effect of MβCD treatment on the expression of these three molecules. Neither LRP-1 mRNA (**Figure 5A**) nor MT1-MMP mRNA (**Figure 5B**) and ADAM-12 (**Figure 5C**) levels were affected by 10 mM MβCD treatment.

DISCUSSION

In the present study we investigated the relationship between cholesterol cell distribution and LRP-1 shedding efficiency. For this purpose, we used MDA-MB-231 and MDA-MB-435 cells, two cancer cell lines recently described for exhibiting different patterns of cholesterol localization, respectively in the cytoplasm and in the plasma membrane (Nieva et al., 2012). Treatment by MβCD decreased amount of cholesterol that was mainly localized in cytoplasm and stimulated removal of cell-surface LRP-1. In contrast, such a treatment had no effect on cholesterol levels predominantly distributed at the plasma membrane and on the

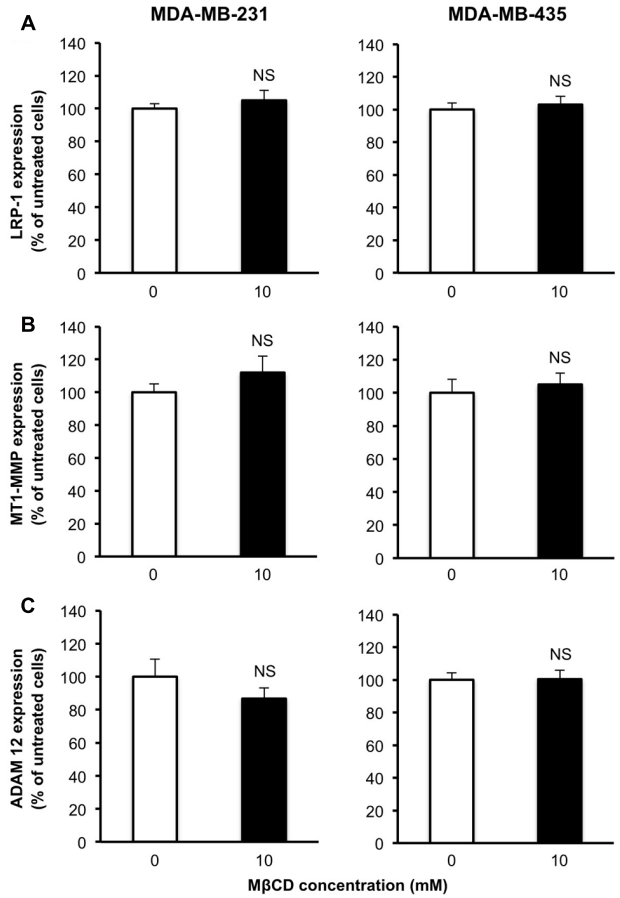

FIGURE 5 | Depletion of cellular cholesterol by MβCD has no effect on the expression of LRP-1 or its sheddases MT1-MMP and ADAM-12. Quantitative real-time PCR of LRP-1 **(A)**, MT1-MMP **(B)** and ADAM-12 **(C)** mRNA levels. After normalization to β-actin mRNA amounts (Langlois et al., 2010), data were presented as a percentage of untreated cells. Values are mean ± SD ($n = 3$ for each cell line). NS, not significant; Student's *t*-test.

release of the LRP-1 ectodomain. These discrepancies are not related to modified expression of LRP-1 and/or of its sheddases, MT1-MMP and ADAM-12. Altogether, these data suggested that cell distribution of cholesterol affects the shedding of LRP-1 from the cell surface.

The lipid profiling of MDA-MB-231 and MDA-MB-435 breast cancer cells was first investigated using Raman microspectroscopy. Raman microspectroscopy was performed on single living cells. The non-destructive and label free spectral analysis permitted to highlight the lipid contribution of the cytoplasmic compartment as a distinctive biochemical characteristic between the two cell types. The discriminant potential of this biophotonic approach was shown by a standard PCA. This unsupervised processing revealed also a marked intra-group variability as visible on the score plot of **Figure 1**. The origin of this variability could be investigated by carrying out spectral imaging at the cellular level (Abramczyk et al., 2015). Innovative devices, based on stimulate Raman scattering, have been recently proven to map the cellular lipid distribution in video-rate imaging (Ramachandran et al., 2015). Raman microspectroscopy allowed us to partially discriminate the two cell lines on the basis of their cytoplasmic spectral signature of lipids, including cholesterol as previously described (Nieva et al., 2012). Recent data obtained by fluorescence microscopy study after filipin staining indicated that cholesterol was mainly concentrated in cytoplasm of MDA-MB-231 cells while it was mostly distributed in plasma membrane of MDA-MB-435 cells (Nieva et al., 2012). In the present study, the observation of filipin-stained MDA-MB-231 and MDA-MB-435 cells by confocal microscope confirmed such findings.

The efficient depletion of cytoplasmic cholesterol in MDA-MB-231 cells after treatment with MβCD indicates that such a compound can pass through plasma membrane for extracting cholesterol from membranes of cytosolic vesicles, as previously reported for removal of lysosomal cholesterol in skin fibroblasts (Swaroop et al., 2012). Unability of MβCD to extract cholesterol mainly distributed in plasma membrane of MDA-MB-435 cells (**Figures 2** and **3**) is rather surprising. MβCD treatment has indeed been often associated with lipid raft disintegration (Zimina et al., 2005). The lipid rafts result of the interaction between cholesterol with sphingolipids in the outer exoplasmic leaflet of the lipid bilayer of cellular membranes. Cholesterol also interacts with phospholipids in the inner cytoplasmic leaflet of the lipid bilayer. Lipid rafts are considered to be present as a liquid-ordered phase while phospholipid-rich domains are in a disordered state (Simons and Ehehalt, 2002). Giant plasma-membrane vesicles represent a valuable physiological tool to investigate lipid phase separation (Baumgart et al., 2007). Using this model, Levental et al. (2009) demonstrated a cholesterol dependence of phase separation in complex membranes at physiological conditions. Moreover, using the same experimental model Sanchez et al. (2011) demonstrated that MβCD preferentially removed cholesterol from a liquid disordered phase. A computational microscopy study recently confirmed that cholesterol was preferentially extracted from the disordered regions compared to liquid-ordered domains of lipid model membranes (López et al., 2013). Altogether, these data suggest that MβCD-resistant cholesterol in membranes of the MDA-MB-435 cells reflects their richness in lipid rafts. This will be evaluated in a future experiment by atomic force microscopy, as recently proposed (Cremona et al., 2015).

The increase of transmembrane receptor shedding was often related to decrease of cell cholesterol amount, possibly by disintegration of lipid rafts and dynamic interactions of the sheddase and its target (Matthews et al., 2003; von Tresckow et al., 2004; Zimina et al., 2005). We showed similar correlation of increase of LRP-1 shedding by MT1-MMP and cell cholesterol decrease upon MβCD treatment in HT1080 cells (Selvais et al., 2011). Interestingly, our present study highlights a relationship between cholesterol cell distribution and LRP-1 shedding efficiency. Fluorescence imaging in living CHO cells clearly evidenced that intracellular cholesterol is mainly distributed in the endocytic recycling compartment and the *trans*-Golgi network (Mukherjee et al., 1998). However, the

multiplicity of cholesterol transport systems makes difficult the establishment of specific trafficking route (Chang et al., 2006). Previous studies demonstrate that cholesterol intracellular trafficking and distribution, rather than total cholesterol levels, are regulatory factors in the β-amyloid precursor protein processing (Marzolo and Bu, 2009). Malnar et al. (2012) proposed that cholesterol regulates the β-amyloid precursor protein processing by modulating APP expression at the cell surface. To our knowledge, no relationship between intracellular cholesterol distribution and LRP-1 localization has been proposed so far.

CONCLUSION

Our data suggest that intracellular cholesterol depletion may increase intracellular trafficking to cell surface of newly synthesized LRP-1 and/or recycled LRP-1 after endocytosis process. Consequently, enhancement of LRP-1 shedding upon cholesterol depletion should reflect a higher disponibility of the sheddase substrate, i.e., LRP-1, at the cell surface. However, the question whether intracellular cholesterol depletion has an impact on LRP-1 localization remains to be elucidated.

AUTHOR CONTRIBUTIONS

BD, AW, HS, MF, J-FA contributed to the acquisition and analysis of data for the work; SD, OP, and JD contributed to the conception, design of the work and to the analysis and interpretation of data for the work; HE contributed to the conception, design of the work and to the analysis and interpretation of data for the work and written the manuscript.

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frontiers in Pharmacology

REVIEW
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Original insights on thrombospondin-1-related antireceptor strategies in cancer

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Thrombospondin-1 (TSP-1) is a large matricellular glycoprotein known to be overexpressed within tumor stroma in several cancer types. While mainly considered as an endogenous angiogenesis inhibitor, TSP-1 exhibits multifaceted functionalities in a tumor context depending both on TSP-1 concentration as well as differential receptor expression by cancer cells and on tumor-associated stromal cells. Besides, the complex modular structure of TSP-1 along with the wide variety of its soluble ligands and membrane receptors considerably increases the complexity of therapeutically targeting interactions involving TSP-1 ligation of cell-surface receptors. Despite the pleiotropic nature of TSP-1, many different antireceptor strategies have been developed giving promising results in preclinical models. However, transition to clinical trials often led to nuanced outcomes mainly due to frequent severe adverse effects. In this review, we will first expose the intricate and even sometimes opposite effects of TSP-1-related signaling on tumor progression by paying particular attention to modulation of angiogenesis and tumor immunity. Then, we will provide an overview of current developments and prospects by focusing particularly on the cell-surface molecules CD47 and CD36 that function as TSP-1 receptors; including antibody-based approaches, therapeutic gene modulation and the use of peptidomimetics. Finally, we will discuss original approaches specifically targeting TSP-1 domains, as well as innovative combination strategies with a view to producing an overall anticancer response.

Keywords: TSP-1, CD47, CD46, cancer, angiogenesis, innovative therapeutic strategies

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STATE OF THE ART

In view of the relatively short-lived benefits observed in targeted therapies that aim at facing advanced primary cancers, the current main therapeutic challenge is to identify original molecular targets in order to limit tumor burden without allowing resistance acquisition (van Beijnum et al., 2015). Indeed, the advent of systems biology over recent years underlined the limits of therapeutic agents designed to block a single pathway and/or growth factor, inevitably leading to the activation of compensatory mechanisms which allow tumor escape and restore disease progression (Wilson et al., 2015). To face the complexity and massive redundancy of signaling pathways and regulatory

Abbreviations: 3TSR, three thrombospondin-1 type 1 repeats; ECM, extracellular matrix; EOC, epithelial ovarian cancer; FGF-2, fibroblast growth factor-2; MET, metronomic; MTD, maximum tolerated dose; PD, pharmacodynamic; PK, pharmacokinetic; RBCs, red blood cells; SIRPα, signal regulatory protein alpha; TCR, T cell receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TSP-1, thrombospondin-1; VEGFR2, vascular endothelial growth factor receptor 2.

processes underlying tumor progression, growing attention is accorded to matricellular proteins and their cell receptors as they function as multiple integrators of tumor progression signals at the tumor/microenvironment interface (Murphy-Ullrich and Sage, 2014). According to their definition first given by Paul Bornstein 25 years ago, matricellular proteins regulate a wide range of both malignant and stromal cell functions through interactions with cell-surface receptors or by acting in a coordinated manner with other ECM components or soluble molecules (Sage and Bornstein, 1991). TSPs may be regarded as the archetypes of the matricellular protein group, with TSP-1 first identified from human platelets in 1978 (Lawler et al., 1978).

Thrombospondin-1 is considered a main actor within a tumor microenvironment, while it exerts intricate and sometimes opposite effects on tumor progression. Elevated circulating levels of TSP-1 were early observed in patients presenting breast, lung, gastrointestinal or even gynecological malignancies (Tuszynski et al., 1992; Nathan et al., 1994). In patients receiving myelosuppressive anti-cancer chemotherapy, TSP-1 blood concentrations assessments strongly correspond with platelet counts (Starlinger et al., 2010). Of note, such correlation is also observed in non-malignant processes where platelet activation is high, such as sickle cell disease (Novelli et al., 2012). Nevertheless, others have noted that elevation of circulating TSP-1 in a cancer setting could be even noticed in absence of plasma contamination by platelet activation (Byrne et al., 2007), thus suggesting that TSP-1 plasma levels might originate from sources other than platelets. Therefore, additional work is needed to determine the exact origins of high TSP-1 plasma concentrations, particularly as many non-platelet sources are known to produce TSP-1 such as endothelial cells, cancer cells, or even circulating immune cells (Dawes et al., 1988). Among the range of possibilities, tumor-originating TSP-1 may provide a plausible explanation to elevated levels detected in patients. Indeed, increased TSP-1 mRNA and/or protein levels were observed within the stromal compartment of breast and gastric carcinoma (Clezardin et al., 1993; Brown et al., 1999; Lin et al., 2012). On the contrary, carcinoma cells express undetectable to low levels of TSP-1 in these studies, and loss of TSP-1 expression by cancer cells is described as an important feature of the "angiogenic switch" in a wide range of solid tumors (Naumov et al., 2006). Indeed, TSP-1 expression is typically down-regulated by oncogenes whereas it is promoted by tumor suppressor genes such as p53 (Dameron et al., 1994). Recently, oncogenic Ras was confirmed as being likely to induce phosphorylation of Myc, thus leading to TSP-1 repression and acquisition of an angiogenic phenotype (Watnick et al., 2015). While TSP-1 expression is lost during malignant progression in a wide variety of major cancer types, a few exceptions need nevertheless to be considered. By way of example, TSP-1 is over-expressed by invasive and metastatic melanoma cells, in which it actively contributes to an epithelial-to-mesenchymal transition program (Jayachandran et al., 2014; Borsotti et al., 2015).

Like other matricellular proteins, TSP-1 is a multi-modular and multifunctional protein able to bind a wide variety of ligands, thus considerably increasing the complexity of its translational potential. As a consequence, it seems obvious that strategies blindly targeting a specific function such as gross matricellular protein silencing, or the use of blocking antibodies, may induce severe adverse effects. Indeed the beneficial properties of the multifunctional protein may also be lost under such treatment. Here, we will focus on TSP-1 and two of its receptors viz. CD47 and CD36, to review pre-clinical and clinical outcomes that could be achieved with current developments. Then, we will discuss future directions to target these cell-surface receptors, using small molecules and peptides able to interfere with TSP-1/CD47 as well as TSP-1/CD36 signaling axis in order to reach an overall anticancer response.

TSP-1: A MAIN ACTOR WITHIN TUMOR MICROENVIRONMENT

Thrombospondin-1 has long been considered to play a role in tumor progression; several studies carried out 20 years ago found it to be overexpressed within tumor stroma and in high circulating levels in several cancers (Qian and Tuszynski, 1996; Bertin et al., 1997; Brown et al., 1999). TSP-1 was also reported to contribute to metastatic spread by promoting tumor cell emboli formation (Incardona et al., 1995). In recent years, an increasing number of studies have tended to present TSP-1 as a poor prognosis and recurrence marker in many cancer types including glioma (Perez-Janices et al., 2015), melanoma (Borsotti et al., 2015) as well as ovarian and pancreatic carcinomas (Lyu et al., 2013; Nie et al., 2014; Pinessi et al., 2015). Among TSP-1 ligands, CD36 and CD47 cell-surface receptors act as key integrators of multiple signals regulating tumor growth and dissemination both positively and negatively. Indeed, TSP-1/CD36/CD47 trimolecular signaling platform dynamics as well as interactions involving co-receptors and soluble ligands exert pleiotropic activities on cancer progression, by directly modulating cancer cells behavior or by acting on tumor microenvironment stromal cells (Kazerounian et al., 2008; Sick et al., 2012).

Modulation of Angiogenesis by TSP-1

Thrombospondin-1 is widely known as an endogenous inhibitor of angiogenesis by negatively regulating NO-mediated signaling in endothelial cells, vSMC and platelets (Isenberg et al., 2008a, 2009b). TSP-1 inhibition of NO/cGMP-related pathways and subsequent antiangiogenic activities are mediated by its interaction with two cell-surface receptors: CD47 and CD36 (Isenberg et al., 2009c; Zhang et al., 2009). CD47 is a ubiquitous 50 kDa receptor consisting of a single N-terminal IgV extracellular domain, five membrane-spanning segments and a short C-terminal cytoplasmic tail (Sick et al., 2012). Although it is now commonly referred to by its immunological marker name, CD47 receptor was first identified through its association with $\alpha_V\beta_3$ integrin, therefore justifying its former name IAP (integrin associated protein; Brown et al., 1990). At the same time, the ovarian tumor antigen OA3 was characterized (Campbell et al., 1992) and subsequently shown to be the

same protein as CD47 (Mawby et al., 1994). CD47 is widely considered as a marker of "self," and is therefore highly expressed by circulating hematopoietic stem cells, erythrocytes and many malignant cells (Oldenborg et al., 2000; Jaiswal et al., 2009). Notably, CD47 was described as a marker of tumor-initiating cells in leukemia as well as in bladder and liver cancer (Chan et al., 2009; Willingham et al., 2012a; Lee et al., 2014). In cancer, CD47 acts as a "don't eat me" signal by engaging its macrophage phagocytic counter-receptor SIRPα (signal regulatory protein alpha; Vernon-Wilson et al., 2000; Chao et al., 2012). Thus, CD47 binding to SIRPα present on immune cells causes a dephosphorylation cascade avoiding synaptic myosin accumulation and thereby preventing engulfment (Tsai and Discher, 2008). However, broad evidence now sustains that CD47 signaling functions go well beyond this simple antiphagocytic passive role, with CD47 acting as a sensor for cell–cell and cell–microenvironment signals. Indeed SIRPα can interact with CD47 receptors in *cis* or in *trans*, and CD47/SIRPα signaling should not be considered as unidirectional in so far as SIRPα binding can in turn affect intracellular signaling through CD47 (Latour et al., 2001), which has further been called "reverse" signaling (Sarfati et al., 2008). While this provides an exciting area for future research, numerous studies of CD47 signaling functions that have been published so far focused on CD47 activation by TSP-1. Indeed, TSP-1 available within the ECM is a key regulator of CD47 signaling. CD47 ligation by TSP-1 C-terminal domain dissociates its constitutive association with VEGFR2 and allows inhibition of both early eNOS-activating signals and NO-independent VEGFR2 signaling, thus leading to subsequent antiangiogenic responses (Kaur et al., 2010; Soto-Pantoja et al., 2015). Remarkably, TSP-1:CD47 interaction also redundantly inhibits NO signaling at the level of such downstream effectors as soluble guanylate cyclases (sGC; Isenberg et al., 2006) and cGMP-dependent protein kinases (cGK; Isenberg et al., 2008a,c). Of note, co-immunoprecipitations experiments were recently conducted identifying for the first time TSP-1 as a new ligand for SIRPα, which may result in stimulation of SIRPα phosphorylation and downstream signaling in non-phagocytic cells (Yao et al., 2014). While this process is likely to involve the joint contribution of CD47, it raises the exciting likelihood of a CD47-independent SIRPα signaling under TSP-1 ligation. As cell-free binding assays indicated that TSP-1:SIRPα interaction does not imply the C-terminal domain of TSP-1, further studies considering recombinant fragments as well as molecular docking experiments would be of particular interest to better characterize this newly identified interaction. To further increase the complexity of TSP-1/CD47/SIRPα signaling axis, it has to be noted that both CD47 and SIRPα ectodomains could be target of sheddases and thus provide additional ligands for TSP-1, CD47, and SIRPα (Ohnishi et al., 2004; Maile et al., 2008; Toth et al., 2013).

In addition to CD47-induced effects, a central region of TSP-1 called 3TSR (three TSP-1 type 1 repeats) binds itself to the CD36 membrane receptor, also leading to angiogenesis inhibition. CD36, first identified from platelets as glycoprotein IV (GpIV; Clemetson et al., 1977), is a class B scavenger receptor (Calvo et al., 1995) also acting as fatty acid translocase (Pohl et al., 2005). It is mostly expressed by microvascular endothelial cells and vSMC (Dawson et al., 1997; Silverstein and Febbraio, 2009) in which TSP-1 ligation promotes CD36 association with β1 integrin and VEGFR2 dimer in a tripartite complex, resulting in decreased VEGFR2 phosphorylation under VEGF stimulation (Zhang et al., 2009). Besides, TSP-1 binding to CD36 inhibits NO-related signaling at the level of eNOS by preventing myristate uptake and also its downstream effects. Indeed, both TSP-1 and a peptide derived from the 3TSR as well as a CD36 "agonist" mAb are able to modulate the fatty acid translocase activity of CD36 by preventing myristate uptake in vascular cells (Isenberg et al., 2007). As CD36 is expected to be the main cell membrane protein involved in fatty acid uptake (Koonen et al., 2005), one should bear in mind that targeting this receptor may affect lipoprotein and glucose metabolism, and therefore lead to cardiovascular complications. In humans, CD36 deficiency is associated with phenotypic expression of the "metabolic syndrome," i.e., hypercholesterolemia, hyperglycemia, insulin resistance, and higher blood pressure (Hirano et al., 2003). Besides, TSP-1 and TSP-1-derived agents that inhibit myristate uptake through CD36 activation are also likely to affect non angiogenesis-related signaling pathways as post-translational myristoylation regulates many protein and cell functions (Martin et al., 2011). While TSP-1 is a high affinity ligand for CD47, binding to CD36 requires higher concentrations that overcome physiological levels. Apart from this 100-fold difference in binding affinities, results from null cells and animals also indicate that while TSP-1 ligation of either CD36 or CD47 is sufficient to inhibit NO-stimulated vascular responses, only CD47 is necessary for such TSP-1 activity at physiological concentrations (Isenberg et al., 2006). However, considering that TSP-1 protein levels in tumor and surrounding tissue are found to be elevated in several cancers (for review, see Kazerounian et al., 2008), one can assume that CD36-activating concentrations are reached within a tumor microenvironment.

TSP-1 Direct Impact on Cancer Cell Behavior and Tumor Immunity

Far from being restricted to angiogenesis modulation, the effects of TSP-1 on tumor progression are multifaceted and sometimes even opposite depending on the molecular and cellular composition of the microenvironment. Indeed, its ability to interact with multiple ligands enables TSP-1 to regulate a wide range of processes such as tumor cell adhesion (Li et al., 2006), proliferation (Sick et al., 2011), survival or apoptosis (Manna and Frazier, 2004; Saumet et al., 2005; Rath et al., 2006a,b), tumor invasion and metastatic dissemination (Jayachandran et al., 2014; Borsotti et al., 2015), inflammation, immune response (Grimbert et al., 2006) and response to treatment (Lih et al., 2006; Bi et al., 2014). Such pleiotropic effects may be governed by TSP-1 concentration as well as by its origin, whether it originates from tumor cells or the stroma compartment (Pinessi et al., 2015). Reverse responses may also be observed depending on the cancer type. For instance, CD47 ligation by TSP-1 induces killing of breast cancer cells (Manna and Frazier, 2004) while it was reported to inhibit apoptosis

and promote drug resistance in thyroid carcinoma cells (Rath et al., 2006a,b). Besides, TSP-1 can also trigger cancer cell death by interacting with the CD36 receptor as recombinant 3TSR fragments of TSP-1 were shown to inhibit proliferation and to induce apoptosis of murine epithelial ovarian cancer cells (EOC; Russell et al., 2015). Therefore, TSP-1 effects on malignant cells are dependent on receptor expression profiles that are likely to vary between different malignant subpopulations or even depending on their differentiation degrees (Zheng et al., 2015).

Apart from TSP-1-related direct modulation of cancer cell behavior through interactions with membrane receptors, the TSP-1/CD47/SIRPα axis is also strongly implicated in controlling tumor immunity, with both positive and negative roles. A widely held opinion is that tumor cells express high CD47 levels to inhibit phagocytosis by signaling through SIRPα found on macrophages and dendritic cells (DCs; Zhao et al., 2011; Chao et al., 2012; Willingham et al., 2012a). Accordingly, restoration of CD47 expression in CD47-deficient leukemia cells increases xenograft aggressiveness (Jaiswal et al., 2009). To date, CD47/SIRPα is the only known negative regulator of phagocytosis at the immunological synapse and it is known to play an important physiological role in maintaining hematopoietic cells and platelets homeostasis (Olsson et al., 2005; Sick et al., 2012). In addition, CD47:SIRPα interaction may also indirectly promote tumor dissemination through binding of tumor cells to macrophages that reside at the level of potential extravasation sites within the vascular wall (Chao et al., 2011b). Several structural and mutagenesis studies highlighted that according to their respective spatial configuration within CD47 extracellular domain, the TSP-1 and SIRPα interaction sites may not be redundant (Floquet et al., 2008; Hatherley et al., 2008; Jeanne et al., 2015; Soto-Pantoja et al., 2015). However, direct binding assays provided contradictory evidence as both TSP-1 and a function-blocking CD47 antibody inhibit CD47:SIRPα interaction (Isenberg et al., 2009a). Furthermore, recent studies underlined that TSP-1 may also interact with SIRPα (Yao et al., 2014), thus accentuating the impression that the above-mentioned studies asserting an essential contribution of the CD47:SIRPα "don't eat me" signal remains incomplete, especially as none of this work was done controlling the absence or presence of TSP-1.

Aside from the previously exposed modulation of innate immunity by the CD47/SIRPα antiphagocytic axis, TSP-1 interaction with CD47 existing on immune cells mostly inactivates antitumor adaptive immunosurveillance. Indeed TSP-1 was shown to directly inhibit TCR-mediated T cell activation (Li et al., 2001) by engaging CD47 (Li et al., 2002). Secreted TSP-1 that binds CD47 on T cells inhibits both the NO/cGMP pathway (Ramanathan et al., 2011) and H_2S signaling (Kaur et al., 2015), therefore concomitantly resulting in an homeostatic inhibitory role of TSP-1:CD47 interaction on T-cell activation. On the other hand, there are cross-talks between the above described mechanisms and VEGF signaling in T cells. Thus, CD47 ligation by TSP-1 inhibits VEGFR2 phosphorylation hence limiting VEGF-induced inhibition of T cell proliferation and TCR signaling (Kaur et al., 2014). Otherwise, TSP-1 binding to CD47 also inhibits differentiation of naïve T cells into Th1 (Bouguermouh et al., 2008), whereas Tregs formation is induced by promoting Foxp3 transcription factor expression (Grimbert et al., 2006; Baumgartner et al., 2008). In cancer, CD47 blockade was shown to enhance antitumor immunity by stimulating CD8+ cytotoxic T cells (Soto-Pantoja et al., 2014). In combination with ionizing radiotherapy that enhances T cell antitumor immunity (Demaria and Formenti, 2012), CD47 blockade in effector T cells is therefore sufficient to inhibit tumor growth, thus offsetting the widely spread opinion that CD47 blockade anticancer effects are attributed to phagocytosis of cancer cells by macrophages. CD47 signaling also regulates natural killer (NK) and DC functions that orchestrate adaptative immunity, leading to tolerogenic signals toward tumor under TSP-1 ligation (Kim et al., 2008; Weng et al., 2014).

Considering these contradictory data, one should realize that TSP-1 roles in cancer progression and metastatic dissemination are complicated and intricate, often leading to paradoxical signals. Indeed, for the purpose of designing new therapeutics, one should bear in mind that several ECM soluble factors and/or cell-surface receptors could bind simultaneously and act as competitors, or even allosterically influence each other's binding and signaling. Therefore, it seems obvious that TSP-1 and/or its receptors massive blockade or silencing may lead to inevitable adverse effects, closely related to the pleiotropic nature of matricellular proteins and their ligands. Despite these considerations, a few strategies have shown promising results in animal cancer models and some of them already moved to clinical trials.

THERAPEUTIC TARGETING OF TSP-1-RELATED SIGNALING

Therapeutic strategies targeting TSP-1 signaling and its CD47/CD36 membrane receptors have already been extensively reviewed over the last 5 years (Belotti et al., 2011; Henkin and Volpert, 2011; Sick et al., 2012; Soto-Pantoja et al., 2013b). Thereby, this review is not meant as a comprehensive overview, but rather as a snapshot of current pre-clinical to clinical developments. The range of new therapeutic methods support the sharply expanding interest in targeting TSP-1-related signaling with a view to regulating its function during cancer progression. After describing the most advanced strategies (summarized in **Table 1**) as well as their benefits and limitations, we will discuss more original and sophisticated approaches which aim at modulating TSP-1/CD47/CD36 signalization either directly or indirectly in order to provide an overall anticancer response. Then we will consider future directions and treatment optimizations with the objective of improving further clinical outcomes.

Antibody Blockade and Gene Therapeutics

The use of monoclonal antibodies (mAbs) is an obvious way to therapeutical target cell-surface receptors. Considerable efforts

TABLE 1 | Therapeutic strategies targeting TSP-1-related signaling.

Compound	Origin/sequence	Target	Stage	Reference
CD47-blocking mAb		CD47	Phase 1	clinicaltrials.gov (four trial studies ongoing)
CD47 antisense morpholino		CD47	Pre-clinic	Maxhimer et al., 2009
Peptides				
4N1/4N1-K	TSP-1 (K-RFYVVMWK-K)	CD47	Pre-clinic	Kalas et al., 2013
7N3	TSP-1 (FIRVVMYEGKK)	CD47	*In vitro*	Maxhimer et al., 2009
PKHB1	i.e., 4N1K, with D counterparts for N- and C-terminal lysines	CD47	Pre-clinic	Martinez-Torres et al., 2015
TAX2	CD47 (CEVSQLLKGDAC)	TSP-1	Pre-clinic	Jeanne et al., 2015
Psap-derived peptide	Prosaposin (DWLPK)	TSP-1 upregulation in $Gr1^+$ cells	Pre-clinic	Catena et al., 2013
TSP-1 recombinant fragment	**TSP-1**			
3TSR	Type 1 repeats	CD36	Pre-clinic	Zhang et al., 2005
3TSR/TRAIL fusion protein	Type 1 repeats, TRAIL	CD36 and TRAIL receptor	Pre-clinic	Choi et al., 2015
Peptidomimetics				
ABT-526 (Abbott)	TSP-1 type 1 repeats (GVITRIR)	CD36	Pre-clinic	Rusk et al., 2006
ABT-510 (Abbott)	TSP-1 type 1 repeats (GVITRIR)	CD36	Phase 2	Baker et al., 2008
ABT-898 (Abbott)	TSP-1 type 1 repeats (GVITRIR)	CD36	Pre-clinic	Campbell et al., 2011
CVX-022 (Pfizer)	TSP-1 mimetic + scaffold Ab	CD36	Pre-clinic	Coronella et al., 2009
CVX-045 (Pfizer)	TSP-1 mimetic + scaffold Ab	CD36	Phase 1	Molckovsky and Siu, 2008
Non-peptide small molecule				
sm27	TSP-1 type 3 repeats	FGF-2	Pre-clinic	Taraboletti et al., 2010
Others				
Trabectedin (ET-743, Yondelis)	Marine natural product	TSP-1 upregulation by tumor cells	Approved	Monk et al., 2012; Dossi et al., 2015
Velcro-CD47 (N3612)	CD47 extracellular domain	SIRPα	*In vitro*	Ho et al., 2015

have focused on developing CD47-targeting mAbs to block the CD47/SIRPα antiphagocytic pathway established between tumor cells and immune cells. Such CD47-blocking mAbs were shown to be effective by allowing the decrease of tumor burden in several preclinical cancer models including acute myeloid leukemia (Majeti et al., 2009), lymphoma (Chao et al., 2010) and osteosarcoma (Xu et al., 2015). While the decrease in tumor growth is mainly attributed to enhanced tumor cell clearance by macrophages under CD47:SIRPα disruption (Willingham et al., 2012a), other studies have noted that alternative mechanisms may explain the antitumor activities of CD47-blocking antibodies. Particularly, the use of intact IgG (such as B6H12 anti-CD47 Ab) in the previously mentioned *in vivo* experiments may also induce Fc-mediated cytotoxicity (Zhao et al., 2011). Of note, one of the CD47-blocking antibody that reduced tumor growth (clone miap410; Willingham et al., 2012b) raised doubts as to its ability to block CD47:SIRPα interaction (Han et al., 2000; Willingham et al., 2012b). Altogether, these data suggest that increased macrophage phagocytosis is not sufficient to explain antitumor activities of CD47-targeting mAbs and that other actors are involved (Soto-Pantoja et al., 2012a; Zhao et al., 2012). Particularly, *in vitro* and *in vivo* studies have shown that macrophages are able to prime an effective CD8+ T cell response following anti-CD47 treatment-mediated phagocytosis of cancer cells, by concomitantly inducing a reduction in regulatory T cell population (Tseng et al., 2013). To date, at least four first-in-man phase 1 clinical trials considering anti-CD47 humanized mAbs are underway, according to clinicaltrials.gov website (identifiers NCT02216409, NCT02447354, NCT02488811, and NCT02367196). Given the ubiquitous expression of CD47, systemically administered anti-CD47 mAbs will inevitably come across a huge number of CD47 copies on red blood cells (RBCs). To avoid phagocytic-induced excessive reduction in erythrocytes count, it has been suggested to use a priming-dose of anti-CD47 that would result in "aged" RBCs removal and subsequent erythropoiesis stimulation (McCracken et al., 2015). Such suggestion is obviously questionable, as many other clearance mechanisms are known to be preponderant in triggering removal of senescent RBCs (Lutz and Bogdanova, 2013). One should note that experiments considering CD47 targeting agents in mice did not induce any significant anemia, which also runs counter to a major role for antiphagocytic

"don't eat me" signal disruption in these studies. Besides, CD47 plays fundamental physiological roles by limiting NO signaling in RBCs, platelets, and endothelium (Soto-Pantoja et al., 2015). Indeed, CD47 antibody targeting may affect NO pathway modulation and subsequent angiogenesis regulation, since a commonly used CD47-blocking antibody (clone B6H12) was previously shown to concomitantly disrupt both TSP-1:CD47 and CD47:SIRPα interactions (Isenberg et al., 2009a). As pre-clinical data suggests that high circulating TSP-1 levels produced by tumor stroma may indirectly increase tumor perfusion while decreasing peritumoral and systemic blood flow, CD47-targeting mAbs are therefore likely to counteract these effects through regional stimulation of NO signaling (Isenberg et al., 2008b, 2009b). On the other hand, anti-CD47 antibodies may also interfere with CD36-mediated modulation of NO signaling, as CD47 is required for CD36 activation under TSP-1 ligation (Isenberg et al., 2006). According to this, systemic administrations of anti-CD47 mAbs for cancer treatment would probably lead to severe adverse events such as hypertension and thrombosis. Therefore, we are not fully persuaded by the use of CD47 antibodies as an alternative to current anticancer drugs, while their local use is much more promising for instance in ischemia prevention (Lin et al., 2014).

As RBCs have prolonged circulating lifetimes without any membrane protein turnover (Mohandas and Gallagher, 2008), other groups have suggested that acute genetic modulation of CD47 expression may represent a surrogate to some of the antibody-based strategies side-effects. Indeed CD47 antisense morpholino potently reduced tumor burden in patient-derived hepatocellular carcinoma xenografts (Lee et al., 2014). This study highlighted that the use of morpholino against CD47 mRNA may be of particular interest in combination with conventional chemotherapy as it potentialized the effects of doxorubicin. In the context of syngeneic melanoma allografts, morpholino suppression of CD47 expression induced only a modest decrease of tumor growth (Maxhimer et al., 2009). There again, more beneficial effects were reached when combining morpholino treatment with radiotherapy in the same allograft model. It should be noted that similar inhibition of tumor growth is observed when irradiating TSP-1 null mice, thus suggesting that anticancer targeting of TSP-1:CD47 interaction would be of a greater relevance than disrupting CD47:SIRPα (Isenberg et al., 2008c; Soto-Pantoja et al., 2013b). Accordingly, TSP-1 silencing in DCs by shRNA interference exhibited antitumor effects in a bladder cancer syngeneic model, by increasing tumor-infiltrating CD4+ and CD8+ T cells (Weng et al., 2014). There again, TSP-1 wide-spread silencing may be a double-edged sword for cancer therapy as TSP-1 exerts opposite effects in endothelial cells and DCs. Therefore, the use of TSP-1 recombinant fragments or small antagonistic molecules may be of a better interest.

TSP-1-derived Peptides, Recombinant Fragments, and Mimetics

Several synthetic peptides derived from the C-terminal domain of TSP-1 were early identified as containing a critical VVM motif proposed to be essential for CD47 binding (Gao and Frazier, 1994). Among them, the widely used 4N1 (^{1016}RFYVVMWK^{1024}) and 7N3 (^{1102}FIRVVMYEGKK^{1112}) are able to reproduce some of TSP-1-mediated biological effects in *in vitro* models (Rath et al., 2006a; Maxhimer et al., 2009). However, 4N1 should no longer be considered as a CD47-specific targeting agent as several studies pointed out 15 years ago its ability to induce cellular responses in a CD47-independent fashion (Tulasne et al., 2001; Barazi et al., 2002). In addition, there is a dearth of convincing *in vivo* data concerning these peptides, probably due to moderate affinity to CD47 and poor pharmacokinetic properties, thus requiring high dose treatments. Indeed repeated administrations of an extended version of the 4N1 peptide named 4N1K (K-^{1016}RFYVVMWK^{1024}-K) induce only modest changes in tumor growth (Kalas et al., 2013), while 4N1K exhibits low stability in plasma. Consideration of 4N1K as a CD47 agonist is all the more controversial as its VVM motif is actually buried within a hydrophobic β-strand arrangement of C-terminal TSP-1, therefore, avoiding accessibility to CD47 without significant conformational changes (Kvansakul et al., 2004). However, normal mode analysis and energy minimizations helped to identify large amplitude motions of TSP-1 signature domain, leading to opening of the hydrophobic cleft and allowing solvent exposure of the 4N1 sequence (Floquet et al., 2008). Some studies highlighted differences in 4N1K-induced responses between CD47+/+ and CD47−/− cells or considering CD47 blocking mAbs (Fujimoto et al., 2003; McDonald et al., 2004), which remains unexplained in view of the above-mentioned studies demonstrating 4N1 non-specificity. Despite these considerations, others have suggested that some of the *in vitro* effects of 4N1K are likely to be due to its hyper-adhesive nature rather than its interaction with CD47, particularly as CD47-deficient cells are able to bind immobilized 4N1K (Leclair and Lim, 2014). Interestingly, a recently identified serum-stable analog of 4N1K named PKHB1, in which natural L-amino acids are replaced by their D counterparts, was demonstrated to induce a twofold reduction in human chronic lymphocytic leukemia xenografts growth (Martinez-Torres et al., 2015). Nevertheless, caution should be observed about such TSP-1-derived CD47 agonists as they might also induce adverse inhibitory effects on host DCs immunity (Weng et al., 2014).

Among TSP-1 multiple domains, the main antiangiogenic sequences are thought to reside within the type 1 repeats involved in CD36 binding (Belotti et al., 2011). Indeed recombinant 3TSR fragments potently inhibit tumor growth in both syngeneic melanoma and orthotopic human pancreatic carcinoma models (Miao et al., 2001; Zhang et al., 2005). Subsequently, TSP-1-derived peptidomimetics were developed and even reached phase 2 clinical trials. ABT-526 (Abbott Laboratories) was the first to be described as a modified peptide based on the GVITRIR sequence of the second TSP-1 type 1 repeats (Haviv et al., 2005), and yield impressive disease regression without any significant adverse effects in tumor-bearing dogs (Rusk et al., 2006). Thereafter a more soluble enantiomer with better PK/PD profile, named ABT-510, entered clinical trials. After showing

relevant PK properties in phase 1 trials (Hoekstra et al., 2005; Gietema et al., 2006), ABT-510 failed to give clear evidence of efficacy in phase 2 and led to severe adverse events such as thrombosis and pulmonary embolism (Ebbinghaus et al., 2007; Baker et al., 2008). ABT-510 is consequently no longer tested in clinical development, however, a second-generation mimetic named ABT-898 has recently emerged with improved therapeutic activity in dogs with soft tissue sarcoma (Sahora et al., 2012). While ABT-898 treatment efficiently allowed the regression of established ovarian tumors in mice (Campbell et al., 2011), it has not entered human development so far. Conjointly, CVX-22 and CVX-045 (Pfizer) were developed fusing TSP-1-derived peptidomimetics with a proprietary scaffold antibody (Levin et al., 2007; Coronella et al., 2009). While CVX-045 showed efficacy in tumor xenografts by reducing MVD and increasing necrotic cores (Li et al., 2011), only limited benefits were observed during phase 1 clinical trials in association with severe adverse events (Molckovsky and Siu, 2008), which probably explains why such "peptibodies" have been discontinued from Pfizer pipeline in 2014 (Rader, 2014).

Original Strategies and Current Developments

Aside from peptidomimetics based on sequences from the type 1 repeats, sm27 is a non-peptide small molecule mimicking the FGF-2 binding site located in the type 3 repeats of TSP-1 (Taraboletti et al., 2010) that exhibits *in vitro* and *ex vivo* antiangiogenic properties (Colombo et al., 2010). Since 2010, several computational studies have been conducted aiming to optimize sm27:FGF-2 binding dynamics (Pagano et al., 2012; Meli et al., 2014), and newly designed derivatives will presumably be evaluated *in vivo* in future experiments. More recently, we characterized a cyclic peptide derived from CD47, named TAX2, that directly binds TSP-1 to antagonize TSP-1:CD47 interaction (Jeanne et al., 2015). TAX2 administration led to a decrease in viable tumor volume in melanoma allograft and potently inhibited pancreatic carcinoma xenograft growth, together with a disruption of tumor-associated vascular network. *In vitro* studies using CD36 blocking mAbs indicated that the unpredicted antiangiogenic properties of TAX2 are likely to be mediated by CD36 activation. According to the TAX2 proposed mechanism of action, such peptide may induce a TSP-1 binding switch from CD47 to CD36. Appropriately, TAX2 antitumor effects are consistent with those observed using TSP-1 recombinant fragments targeting CD36 in similar experimental models (Miao et al., 2001; Zhang et al., 2005). The use of recombinant 3TSR as a CD36-activating treatment recently showed promising results in preclinical models of glioblastoma and ovarian carcinoma (Choi et al., 2015; Russell et al., 2015), therefore it seems relevant to assess the therapeutic potential of TAX2 in the context of such pathologies. In addition, ABT-898 was shown to be especially potent in the female reproductive tract (Campbell et al., 2011), and CD47 was early considered as an ovarian tumor marker (Campbell et al., 1992). Through its original mechanism of action which supposes concomitant disruption of TSP-1:CD47 interaction and enhancement of CD36 activation by TSP-1, TAX2 may inhibit tumor progression while limiting many of the undesired side effects of broadly inhibiting important physiological functions of CD47. Indeed, as TAX2 was designed to target TSP-1 specifically at the CD47 binding site, both TSP-1 and CD47 are presumed to remain free to interact with their respective alternative ligands. Nevertheless, some putative side-effects of using TAX2 as an anti-cancer agent still need to be explored, particularly as TSP-1 interaction with CD47 and/or CD36 is also known to modulate platelet aggregation (Isenberg et al., 2008d). While ABT-510 lack of efficacy in clinical trials is likely due to its inability to mimic the activity of full-length TSP-1 (Ebbinghaus et al., 2007; Markovic et al., 2007), we are convinced that original strategies, viz. the use of TAX2 or the identification of new inhibitors that would target pathway leading to TSP-1 repression, may provide realistic treatment alternatives by finely controlling full-length protein signaling. Interestingly, TAX2 was shown to inhibit endothelial cell cGMP production under NO stimulation. According to our assumptions, TAX2 may target the NO/cGMP pathway downstream from eNOS through stimulation of TSP-1:CD36 interaction. Hence, unlike bevacizumab or other VEGF-targeting blockbuster drugs, TAX2 may also inhibit downstream signals resulting from angiogenic signals other than VEGF such as NO production by stromal cells (Roberts et al., 2007). Currently, further work is being done to improve TAX2 translational potential.

Other molecules were also shown to cause an overall anticancer response by involving the action of TSP-1. Indeed trabectedin (ET-743, Yondelis), a marine natural product approved as a second-line treatment of recurrent ovarian cancer (Monk et al., 2012), exhibits antiangiogenic activities by upregulating tumor cell expression of TSP-1 (Dossi et al., 2015). Besides, a five-amino acid peptide derived from prosaposin (DWLPK) was recently shown to inhibit lung metastatic colonization through upregulation of TSP-1 in $Gr1^+$ myeloid cells (Catena et al., 2013). Therefore, direct stimulation of TSP-1 or even strategies that indirectly increase bioavailable TSP-1 within the pulmonary microenvironment could therefore represent a relevant translational antimetastatic approach. However, attenuation of NO and activated CD47 corroborate with pulmonary hypertension (Xu et al., 2004; Bauer et al., 2012) while TSP-1 is a characteristic component of coronary atherosclerotic plaques (Riessen et al., 1998). Therefore, inducing TSP-1 may lead to cardiovascular complications, especially in the lung (Rogers et al., 2014). In addition, caution should be exercised in generalizing the benefits in other host organs, as they might be dependent on the cytokine environment. Particularly, Lee and collaborators reported that ADAMTS1-mediated processing of TSP1 into antiangiogenic fragments occurs differently for liver and lung metastases (Lee et al., 2010).

Besides the above-described TSP-1-related therapeutic strategies, much has also been done to propose alternative methods to target CD47/SIRPα signaling. While current

approaches have principally targeted the ubiquitously expressed CD47, thus inevitably leading to off-target effects, a novel engineering development has recently emerged aiming to target SIRPα specifically. The so-called "Velcro-CD47" (N3612) consists of a high affinity variant of the human CD47 extracellular domain extended at the N-terminus with a short three amino-acid peptide in order to increase binding affinity to SIRPα (Ho et al., 2015). Velcro-CD47 already proved its ability to enhance macrophage phagocytosis of tumor cells *in vitro* and to target the monocyte subpopulation specifically, and its putative anticancer efficacy will be further evaluated in pre-clinical models.

Future Directions

In order to reach an optimal control of tumor progression, future directions will aim to associate innovative approaches targeting TSP-1/CD47 and TSP-1/CD36 signaling with existing anticancer treatments. Indeed morpholino suppression of CD47 expression was shown to markedly increase radiation-induced delay in tumor growth considering two syngeneic models of melanoma and squamous cell carcinoma (Maxhimer et al., 2009; Ridnour et al., 2015). While it sensitizes the tumor to ionizing radiation, CD47 deficiency concomitantly confers radioprotection to normal tissues through activation of autophagy (Soto-Pantoja et al., 2012b). This may be of particular interest in the field of blood cancer treatment with the aim of minimizing the adverse effects of total body irradiation, especially as morpholino-induced CD47 gene silencing was demonstrated to preserve circulating peripheral blood cells and to protect gastrointestinal tissue from ionizing radiation (Soto-Pantoja et al., 2013a). Therefore, future studies will determine the appropriate strategies targeting CD47 with the purpose of radiomitigation, with the potential of being translated into clinical practice.

CD36-activating 3TSR treatment efficacy was also evaluated on top of conventional chemotherapy. While intermittent bursts of MTD chemotherapy are currently considered in the treatment of ovarian cancer, combination with 3TSR may facilitate the uptake of drugs delivered at low-dose MET scheduling in order to reach higher tumor regression rates in patients with advanced EOC (Russell et al., 2015). Indeed combination of 3TSR with carboplatin and paclitaxel MET chemotherapy considerably promotes survival in a syngeneic murine model of EOC. Interestingly, 3TSR is more effective than ABT-598 in this model, thus supporting the concept that the full function of the type 1 repeats cannot be mimicked by a single short peptide (Campbell et al., 2011). Another promising strategy consists of combining the antiangiogenic property of 3TSR with the pro-apoptotic TRAIL in order to target both tumor and tumor-associated vessels (Ren et al., 2009). Such 3TSR/TRAIL fusion protein was recently demonstrated to improve survival of mice bearing intracranial human glioblastoma xenografts, therefore, suggesting a potent translational potential of 3TSR/TRAIL therapies into clinics (Choi et al., 2015).

Finally, TSP-1 peptidomimetics may be considered not only for their direct therapeutic use, but also to enhance the therapeutic delivery of cytotoxic drugs. Notably, a D-reverse peptide derived from the native KRFKQDGGWSHWSPWSSC motif within the TSR of TSP-1 was first demonstrated to inhibit breast tumor growth in a mouse xenograft model (Guo et al., 1997). More recently, an aspartimide analog based on the same TSP-1 sequence was shown to potentiate the activity of doxorubicin in colon carcinoma xenografts. Indeed, such a peptide is able to support the adhesion of doxorubicin-containing liposomes to both tumor cells and endothelial cells, thus leading to increased antiproliferative and antiangiogenic activities (Rivera-Fillat et al., 2010).

CONCLUSION

To date, mAbs targeting CD47 are the best advance toward clinical development and much interest is accorded to massive anti-CD47 blocking strategies, even within non-scientific skilled communities. Accordingly, a growing number of almost sensational reports excessively praise the therapeutic potential of CD47-targeting anticancer immunotherapies on social networks, video sharing platforms or popular-science writings (Williams, 2012; Foley, 2013), probably with a promotional and fund raising purpose. Noteworthy work of Weissman and collaborators in immunodeficient mice has proved preclinical efficacy of anti-CD47 mAbs in a wide range of xenograft models including leukemia (Chao et al., 2011a), lymphoma (Chao et al., 2011b), multiple myeloma (Kim et al., 2012), and several solid tumors (Edris et al., 2012; Willingham et al., 2012a). However, we are deeply convinced that genetic ablation or antibody blockade of CD47 may not represent a fully satisfying anticancer therapeutic alternative due to adverse effects and/or concomitant attenuation of beneficial functions, and that a more nuanced picture could be exposed to cancer patients. Indeed, massive extinction of any protein/receptor/signaling pathway might lead to adverse effects and resistance, while more accurate strategies are needed to regain the baseline. In particular, a global vision of the numerous molecular and cellular actors involved should be adopted when considering matricellular proteins and their receptors in anticancer drug development. Anti-CD47 mAbs could offer clear benefits in the treatment of cardiovascular diseases, however their use as anticancer drugs is likely to encounter the same limitations as bevacizumab, i.e., hypertension, thromboembolism, and tumor recurrence (Gil-Gil et al., 2013). While genetic modulation of CD47 expression could represent an alternative to antibody-based strategies, further clinical development of previously described morpholino-based approaches may require repeated administrations of high doses, due to morpholino oligonucleotides poor cell and tissue uptake as well as their rapid renal clearance (Moulton and Moulton, 2010). Besides, siRNA and miRNA-based strategies may provide viable alternative to morpholino-based CD47 silencing (Wang et al., 2015).

In our opinion, future research should focus on small molecules that allow a finer and more accurate regulation, thus leading to adequate responses and limited adverse effects. Among the variety of innovative approaches, peptides represent a

fast-growing class of new therapeutics (Diao and Meibohm, 2013) and many structural modification strategies have been developed recently to improve their performance as drugs (Di, 2015). The combination of such cutting-edge strategies with conventional anticancer agents will help optimize dosing schedules, whose influence on resistance acquisition is often under evaluated, particularly among anti-VEGF approaches (Clarke and Hurwitz, 2013). Looking ahead, original and selective TSP-1-related antireceptor strategies could improve long-term benefits by overcoming many undesired effects. The next challenges will concern the translation of these small molecules into the clinic, as well as the identification of optimal combinatorial strategies with standard chemotherapy and radiotherapy.

AUTHOR CONTRIBUTION

CS and LM contributed to write the manuscript; AJ and SD wrote the manuscript; SD supervised the work.

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