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Review

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Signaling pathways influencing tumor microenvironment and their exploitation for targeted drug delivery

Abstract: In the recent years, the "tumor microenvironment" has been receiving growing attention due to its involvement in neoplastic transformation, tumor growth, invasion, and protection of tumor cells from host immune response. All these events are facilitated by chemical signals produced by the tumor as well as the surrounding stromal cells. This review is divided into two main parts in which the first part discusses the receptor tyrosine kinase (RTK)-mediated growth factor signaling, steroid hormone (SH) signaling, ancient signaling pathways, and other molecules that are involved in tumorigenesis and how they interact with each other to create a complex tumor microenvironment. In the second part, we bring together the recent nanocarrier-mediated drug delivery approaches to target the signaling pathways/molecules present in the tumor microenvironment.

Keywords: active targeting; functionalization; nanoparticle-mediated drug delivery; signaling pathways; tumor microenvironment.

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1 Introduction

Cancer can be considered as a developmental disorder because most of the signaling pathways responsible for tumor formation are the ones involved in embryo development. This is evident by the resemblance of aggressive tumor cells with embryonic stem cells by means of their plastic, multipotent nature. Deregulation/dysfunction of developmental pathways in and around tumor cells as well as the absence of many regulatory checkpoints results in aberrant uncontrolled growth of tumor cells. Research over years has contributed substantially to our understanding of the cellular and molecular interactions in the tumor microenvironment that orchestrates tumorigenesis. The constantly evolving tumor microenvironment is rich in growth factors, which elicit a cascade of signaling events through specific cell-surface receptors, leading to rapid proliferation, angiogenesis, resistance to cell death, and endure epithelial-mesenchymal transition (EMT) and metastasis. Our knowledge about the role of tumor microenvironment in cancer has improved significantly, moving from a conceptual framework toward the development of novel strategies to treat cancer. Combining therapies that target not only the tumor cells but also the tumor microenvironment and/or the signaling pathways providing resistance to the cancer cells from responding to chemotherapy, have greater degree of success in cancer treatment [1]. Nanoparticles designed based on the characteristics and specific signaling interaction of the tumor microenvironment is a promising strategy to combat cancer. For instance, nanoparticles sensitive to the acidic pH of the tumor microenvironment provides selectivity to tumor cells over the normal ones, thus enhances specificity and drug delivery efficiency [2, 3]. The first part of this review presents a holistic discussion about the important signaling molecules/pathways such as the receptor tyrosine kinases (RTK), steroid hormones (SH), and the ancient signaling pathways that are altered during cancer and signaling interactions enriching

the tumor microenvironment. Interested readers are also referred to other in-depth reviews on specific topics under most of the sections. The second part consolidates how the signaling molecules discussed in the previous part are exploited to functionalize nanoparticle-mediated therapeutic strategies to treat cancer effectively.

2 Receptor tyrosine kinase (RTK) signaling

Signaling via mutated or constitutively active variant of receptor tyrosine kinase (RTK) function as a potential means for cancer cells to evade host mechanisms and develop tumors. A huge deal of attention has been diverted toward RTK signaling because of their overexpression commonly found in many cancers, their ability to crosstalk between themselves, and importantly, they connect the extracellular cues with intracellular effector pathways. As a result, RTK receptor expression has been extensively used as a prognostic biomarker in many malignancies. There are several RTKs, and only the primary ones upregulated in cancer are reviewed here (Figure 1).

2.1 ErbB family of receptors

The epidermal growth factor receptor (EGFR) is a member of the ErbB family, a subfamily comprised of ErbB1/HER1/EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. The ErbB receptors are prominent cancer drivers, which form active homo- or heterodimers upon ligand binding [4]. ErbB receptors bind to EGF produced by the same cell (autocrine) or other cells (paracrine). After ligand binding, the dimerized receptor's intracellular tyrosine kinase domain will be activated causing phosphorylation of specific tyrosine residues that serve as docking sites for proteins containing Src homology 2 (SH2) domains such as Grb2, Shc1, p85, PLCγ, and JAK1, leading to the activation of several intracellular signaling pathways. These downstream signaling cascades include the Ras/MAPK/extracellular signal-regulated kinase (ERK), PI3K/Akt, JAK/ STAT, and PLCγ/protein kinase-C (PKC) pathways for cell proliferation, survival, and mobility [5, 6]. The intracellular kinase domain of HER3 is thought to be an inactive pseudokinase that lacks several catalytically important residues and so it primarily signals by heterodimerizing with HER2 [7]. However, it was reported to have sufficient kinase activity to trans-autophosphorylate its intracellular region [8]. Recently, HER3 overexpression in various tumors including colorectal, gastric, breast, and ovarian cancers has been associated with worse survival, and its effect on overall survival was significantly higher when HER2 was co-overexpressed [9]. Similarly, ErbB receptors are also expressed at high levels in different cancers, and the levels of gene/protein expression is correlated with the growth, state, and aggressiveness of cancer [10, 11]. For instance, HER2 amplification occurs in 20% of breast cancers [11], and 54% of glioblastoma exhibit EGFR overexpression [12]. Glioblastoma cells often present both the wild-type EGFR gene amplification and the constitutively active variant EGFRvIII, resulting in increased EGFR signaling [12]. However, EGFRvIII expression without EGFR gene amplification is fairly uncommon, suggesting that EGFR gene amplification may precede EGFRvIII mutation [13]. All the aforementioned features make ErbB receptors a potential therapeutic target to treat tumors. A detailed review on targeting ErbB receptors can be found in [14].

2.2 Fibroblast growth factor receptor (FGFR) family

Fibroblast growth factor receptors (FGFR) are transmembrane tyrosine-kinase receptors that coordinate a variety of cellular functions. There are 4 FGFRs (FGFR1-4) and 22 FGF ligands [15]. Binding of FGF ligands to FGFRs activate several downstream signaling pathways, including Ras/MAPK/ERK, PLCγ/PKC, PI3K/Akt, and JAK/STAT. Being a crucial signaling for basic processes such as proliferation, survival, angiogenesis, and migration, deregulated FGF signaling can contribute to the development and progression of tumors [16]. FGFR signaling is altered in many cancers including benign skin tumors [17], prostate [18], bladder, and breast cancers [19–21]. Breast cancer cells have been reported to overexpress FGFR1, 2, 4 and display mutations in FGFR2 and 4 [21]. Moreover, emerging data suggest that in addition to the known functions of FGF signaling in promoting tumor cell proliferation and survival, FGF signaling might also regulate EMT [22], tumor metastasis and lymphangiogenesis in a vascular endothelial growth factor-C (VEGF-C)-dependent mechanism [23]. Overexpression of FGFR1 and its altered splicing mechanisms, leading to increased expression of FGFR1β isoform has been associated with high-grade/stage bladder cancer [24, 25]. Although, activating mutation and overexpression of FGFR3 is a common phenomenon observed in low-grade bladder cancer [19], a switch from its epithelial to mesenchymal isoform with wider ligand affinity is

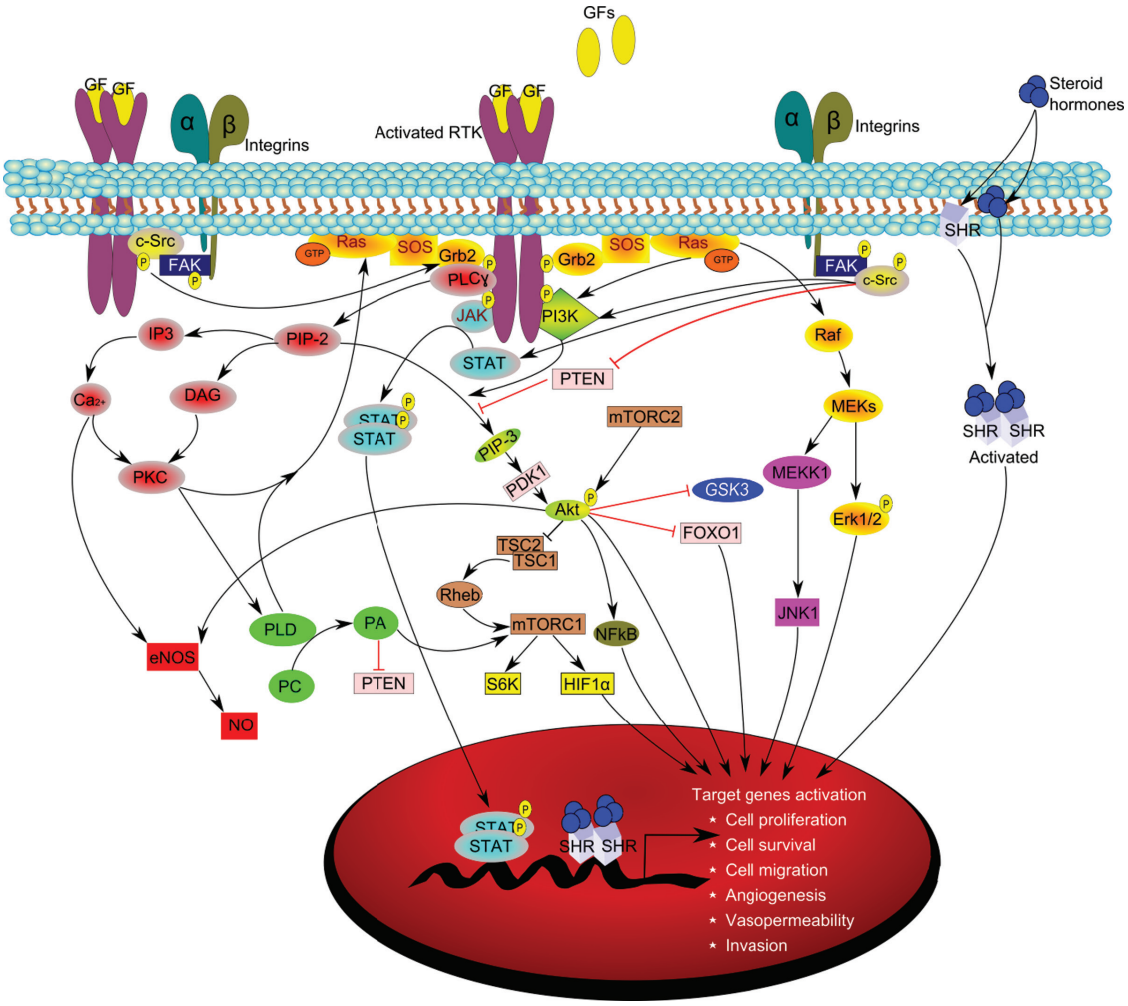
thought to have more deleterious effects [19, 26]. Particularly, FGFR1 has been considered as a potential oncogene in breast cancer because its deregulated signaling contributes to cell proliferation, growth, angiogenesis, EMT, and cell migration in S115 breast cancer [20]. Overall, FGFRs stands as an attractive target for therapeutic intervention in cancer [19, 21, 27].

2.3 Insulin receptors (IR) and insulin-like growth factor receptors (IGFR) family

The insulin receptors (IR-A and IR-B) and the insulin-like growth factor receptors (IGF1R and IGF2R) are tyrosine kinase membrane-bound receptors that share ~60% sequence homology and regulates glucose homeostasis and growth in response to nutrient availability in cells. IR has two isoforms, IR-A and IR-B, which are predominantly expressed in the fetal and adult tissues, respectively. However, cancer cells preferably overexpress the fetal isoform IR-A, which has the advantages of generating hybrid receptors with IGFIR and to have equal affinity to IGF1/IGF2 like that of IGF1R [28–30]. In fact, the hybrid receptors are reported to possess higher affinity for IGF1 than insulin and function predominantly as an IGF1 receptor [31]. IR-mediated nonmetabolic insulin signaling has been found in human myosarcoma cells [32], colon cancer cells [33], breast, prostate, and colorectal cancers [34–36]. Moreover, IR-associated obesity, -type 2 diabetes mellitus (T2DM) and -hyperinsulinemia are some important risk factors for several malignancies including breast cancer [37]. Upon insulin binding to IR, the activated RTK will phosphorylate insulin receptor substrate proteins (IRS1-4), providing docking sites for effectors/adapter proteins, containing SH2 domains. This triggers a cascade of reactions causing the activation of PI3K/Akt and Ras/MAPK pathways that mediate the metabolic and mitogenic activities of insulin, respectively [38, 39]. The antiapoptotic activity of insulin is reported to involve both the PI3K/Akt and MAPK pathways [40, 41]. Insulin also possesses angiogenic properties in a VEGF-dependent or -independent manner through PI3K/Akt and MAPK pathways [42, 43]. While the ability of insulin to stimulate PI3K is lost in the presence of insulin resistance and hyperinsulinemia, its capacity to activate MAPK pathway is enhanced [39]. Thus, hyperinsulinemia-mediated increased levels of circulating insulin in association with IR-A overexpression in cancer cells may cause abnormal nonmetabolic effects of IR, such as cell survival, proliferation, migration, and angiogenesis, the key events that occur during tumor growth and metastasis [38, 43], making the circulating insulin a risk factor of colorectal, pancreatic, and breast cancers [44, 45].

IGF1R is a potential cellular oncogene through which both IGF1 and IGF2 exert their mitogenic, antiapoptotic, and transforming activities [46]. IGF1R expression is seen as a prerequisite for tumor formation because mouse fibroblasts deprived of IGF1R were unable to be transformed by a number of oncogenes [47, 48]. IGF1R signaling plays critical steps, namely, cell adhesion, migration, invasion, and angiogenesis during the metastatic cascade and is involved in a wide range of cancers including the breast, prostate, pediatric, cervix, and ovarian cancers [37]. Ligand binding to the extracellular subunit of IGF1R causes autophosphorylation and conformational changes of its tyrosine kinase domain, leading to the binding of IRS1-4 and Shc proteins. Phosphorylation of these proteins eventually activates at least two signaling pathways: PI3K/Akt and Ras/Raf/MEK/ERK. The antiapoptotic effect of IGF1R is mainly exerted by the PI3K/Akt pathway activation. Phosphorylated IRS activates PI3K, which helps the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3, the reaction inhibited by phosphatase and tensin homolog (PTEN). PIP3 phosphorylates Akt as well as PKC proteins, both of which regulate the metabolic activities of the cell such as glucose uptake [49, 50]. Importantly, activated Akt interferes with the antiapoptotic and proapoptotic functions of several proteins. Upon phosphorylation by Akt, Bcl-2-associated death promoter (BAD) becomes inactivated and allows the antiapoptotic activity of Bcl-2, promoting cell survival. In addition, phosphorylated-Akt also inhibits the proapoptotic protein caspase-9 and prevents cell death [51]. By activating nuclear factor-κB- (NF-κB), Akt can also regulate the expression of antiapoptotic genes [52]. On the other hand, phosphorylated Shc protein binds to Grb2 that recruits Son of Sevenless (SOS), which in turn activates Ras/Raf/MER/ERK pathway. Activated ERK get translocated to the nucleus and regulates target gene expression, influencing cell proliferation and survival [53].

IGF1 and IGF2 are single-chain polypeptides that share 62% sequence homology and generate multiple transcripts depending on their transcription initiation promoter sites and alternative splicing mechanisms. The availability of free IGF1 to interact with IGF1R is regulated by the levels of the six IGF-binding proteins (IGFBP1-6). Under normal physiological conditions, only 1% of the IGFs circulate freely, while others are bound to the IGFBPs [54]. In addition to IGFBPs, their associated proteases are also important in IGFR signaling because they hydrolyzes IGFBPs, causing the release of bound IGFs,



enabling them to interact with IGF1R. Diet, nutrition, and growth hormones have an influence on IGF1 expression [55]. Similarly, IGF1R expression is also affected by nutrition, growth factors, and SHs [56]. Although other growth factors stimulate IGF1R production, IGF1 functions as its negative regulator [57]. Hyperinsulinemia can also favor the production of IGF1 and increases its bioavailability and IGF1R signaling by modulating IGFBPs [58]. Both IGF1 and IGF2 are overexpressed in an array of cancers such as the colon, prostate, breast, colorectal, thyroid, lung, pancreatic cancers, and several sarcomas [59, 60]. Insulin and IGF1 have the ability to cross-bind to each other's receptor, although with much less affinity than that of their preferred ligand [61]. Unlike IGF1R, IGF2R has no tyrosine kinase activity, and it binds to IGF2 and reduces its bioavailability by sending it for lysosomal degradation [62]. Because of this effect, IGF2R has been considered as a potential tumor-suppressor molecule. In-depth reviews on IR, IGF, and IGF1R in cancer can be found elsewhere [37, 39, 63, 64].

2.4 Platelet-derived growth factor receptors (PDGFR)

There are two types of the platelet-derived growth factor receptors: PDGFRα and PDGFRβ that are activated by five different disulfide-linked dimer ligands: PDGF-AA, -BB, -AB, -CC, and -DD with varying specificity. Although all PDGFs except the PDGF-DD interact with PDGFRα and induce receptor dimer formation, PDGF-AA is the most potent ligand of PDGFRα. PDGF-BB and PDGF-DD interacts with PDGFRβ [65]. Ligand-binding to receptors induces homo- or heteroreceptor dimerization, leading to the activation of their intrinsic tyrosine kinase domain and subsequent recruitment of SH2-domain-containing signaling proteins, which activates the downstream pathways that cause the basic cellular processes like, proliferation, migration, and transformation [66]. Both, PDGFs (-BB and -DD) and the receptors (PDGFRα and PDGFRβ) are overexpressed in the breast [67], prostate [68], kidney [69], lung [70], ovarian [71], glioma [72], melanoma [73], and bone

Figure 1 RTK and SHR signaling.
The activity of growth factors (GF) such as EGF, FGF, IGF, PDGF, and VEGF family members are mediated by the RTK signaling. These receptors are made up of an extracellular region, a single transmembrane spanning region, and a cytoplasmic tyrosine kinase domain. The extracellular domain of the RTK binds to the respective GF ligands that cause receptor dimerization and subsequent autophosphorylation on multiple specific intracellular tyrosine residues, creating binding sites for specific proteins. Autophosphorylated RTKs stimulate small GTP-binding protein, Ras by recruiting SOS and its adapter protein GRB2 to the membrane. This initiates a series of signal transduction cascade. Ras activates PLCγ, which can also be activated by Src in a RTK-dependent or -independent manner through steroid hormone receptors (SHR). Activated PLCγ hydrolyses PIP2 to release the second messengers 1,2-diacylglycerol (DAG) and IP3, in which DAG is the activator of PKC that activates Ras/Raf and thus ERK signaling, leading to the expression of transcription factors related to cell proliferation, migration, and angiogenesis. In addition, PKC also activates PLD that catalyzes the hydrolysis of PC to PA, activator of signaling cascades like mTOR. PA also inhibits PTEN, a tumor suppressor that negatively regulate mTORC1 activity. IP3 activates Ca^{2+} release from the endoplasmic reticulum by binding to its intracellular receptor (IP3R). Thus, accumulated intracellular calcium displaces the inhibitory binding of caveolin to eNOS and induces NO production, which increases angiogenesis and vasopermeability. Another important intracellular pathway activated upon RTK signaling is the PI3K/Akt, which starts with the recruitment of PI3K (p85α/p110α) to the receptor, enabling p110α to phosphorylate PIP2 and PIP3. Binding of PIP3 to Akt, allows Akt phosphorylation and partial activation by PDK1. Thus, partly activated Akt is fully activated by mTORC2. In turn, phosphorylated/fully activated Akt activates mTORC1 either directly or through its inhibitory action on TSC1/TSC2, which inhibits mTOR. mTORC1 regulates S6K and HIF1α, inducing translation of several genes including the ones participating in homeostatic responses to hypoxia. Although Akt signaling can promote cell proliferation, metabolism, migration, and angiogenesis, its important role is to function as an antiapoptotic signal by exerting its effect by phosphorylating a variety of downstream targets including mTOR, NF-κB, eNOS, FOXO1, GSK3, etc. reviewed in [95]. Here, the activities of FOXO1 and GSK3 are suppressed by p-Akt, reliving their inhibitory function on cell proliferation and survival. The activity of Akt is negatively regulated by PTEN, which inhibits phosphorylation of PIP2 to PIP3. Erk/MAPK is an important proliferative pathway, which is activated by Ras/Raf. Phosphorylated Erk dimer can function in the cytosol as well as in the nucleus where it activates many transcription factors related to cell proliferation. GFs may also activate ERK through PLCγ/PKC signals. The JNK pathway is a subgroup of MAP kinases that is phosphorylated/activated by MAP2K isoforms MKK4 and MKK7, which themselves are phosphorylated by MEKK1-4. Phosphorylated JNKs are translocated to the nucleus where it will activate its well-known target, c-Jun and other transcription factors, namely, activating transcription factor 2 (ATF2) and activator protein 1 (AP1). The JNK pathway can either have a pro-oncogenic role by promoting cell proliferation or can behave as a tumor suppressor by its proapoptotic effects or by employing tumor surveillance through the involvement of the immune system in a context-dependent manner (reviewed in [96, 97]). The JAK/STAT pathway also plays significant role in cell growth, survival, and differentiation. Activated RTK dimers allow phosphorylation of JAK proteins, which will activate STATs to form dimers. These dimers then get translocated into the nucleus and activate transcription of specific genes, related to survival and proliferation. Src is a nonreceptor cytoplasmic tyrosine kinase, which gets activated following RTK and/or integrins/FAK stimulation (FAK is a tyrosine kinase, which acts both as a signaling molecule and a scaffold protein). Src could induce activation of different transduction cascades including Ras/MAPK, PI3K/Akt, and STAT pathways [98] and inhibit PTEN [99]. Dysregulated steroid hormone (such as androgen, estrogen, and progesterone) signaling through their respective receptors results in uncontrolled proliferation and survival, leading to tumor initiation and progression. Ligand-induced receptor dimers bind either directly to specific DNA response elements or through other DNA-bound transcription factors to alter the transcription of specific genes. Integration of steroid hormone (SH) and GF signaling occur through Erk/MAPK, Akt/PI3K, PKC, PLC, and STAT pathways (reviewed in [100]).

[74] tumors. Expression of PDGFs and PDGFRs are found even in low-grade gliomas, unlike the EGFR expression found only in high-grade tumors, suggesting an early role for PDGF signaling in gliomas [75]. PDGFR signaling in tumor is primarily associated with angiogenesis and metastasis, like in the case of gliomas and breast cancer [67, 76]. PDGF-B, -C, and -D has been reported to enhance tumor angiogenesis through enhanced VEGF expression [77–79]. Tumor cell-secreted PDGF-B also functions to determine the fate of the mesenchymal stem cells *in vitro* through a transmembrane glycoprotein receptor, neuropilin-1 (NRP-1) signaling [80], and it should be noted that NRP-1 expression is positively correlated with the invasion ability of cancer cells. Recently, it was demonstrated that the knockdown of PDGFRβ in glioblastoma stem cells downregulates the critical angiogenesis regulator VEGF [81]. In this context, VEGF165 has been reported to bind to NRP-1 and trigger the NRP-1/VEGFR2/PI3K/Akt signaling pathway causing tumor angiogenesis, cancer cell invasion, and tumorigenesis [82]. PDGFR also influence the cancer microenvironment by recruiting nearby stromal cells, which facilitate tumor-stromal cell interaction that determines tumor development [83, 84]. The role of PDGFR in cancer has been critically reviewed before [85].

2.5 Vascular endothelial growth factor receptors (VEGFR)

The vascular endothelial growth factor (VEGF) family is crucial for angiogenesis, lymphangiogenesis, and vasculogenesis, and it consists of six members: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). The biological effects of VEGF are mediated

by their interaction with the three protein-tyrosine kinase vascular endothelial growth factor receptors (VEGFR1, VEGFR2, and VEGFR3). The two non-enzymatic receptors, NRP-1 and NRP-2, are proposed to facilitate the binding of various VEGF ligands to their primary receptors [86]. During tumorogenesis, it is vital that the rapidly proliferating tumor grown beyond 1–2 mm^3 receive adequate blood supply through newly generated tumor blood vessels. VEGFs overproduced by tumor cells are essential to drive angiogenesis that enables tumor growth and metastasis [87]. Binding of VEGFs to their appropriate VEGFR induces receptor dimerization that leads to autophosphorylation of the receptor's intrinsic tyrosine residues within the kinase domain-stimulating catalytic activity. This will ultimately activate the intracellular Ras/Raf/MEK, PLCγ, and PI3K/Akt pathways resulting in the survival of immature endothelial cells, growth and migration of vascular endothelial cells, and enhanced capillary vascular permeability through different mechanisms [88]. VEGF signaling through the PI3K/Akt pathway is also known to regulate the expression of metastasis- and fibrosis-related genes belonging to the TGF-β and connective tissue growth factor family [89, 90]. Endothelial isoform of nitric oxide synthase (eNOS), the major source of nitric oxide (NO) can also be stimulated by VEGFR signaling downstream of Akt activation to increase vascular permeability [91, 92]. VEGFs and VEGFRs are overexpressed in various human primary solid tumors including the ovarian, breast, non-small-cell lung carcinomas, colon, and colorectal cancers. Although VEGFR is primarily expressed in tumor vessels and associated with tumor-angiogenesis [93], they are also expressed in tumor cells [93], enabling tumor growth [94].

VEGF-A exerts its activity by binding to VEGFR1 and VEGFR2. VEGFR1 expressed in the endothelial cells primarily functions during development and tumor angiogenesis by binding to VEGF-A, -B, and PIGF [101, 102], and it is overexpressed in tumor cells [103]. Although the expression level of the VEGFR1-specific ligand, PIGF, is increased in many tumors [104], the function of this protein in tumor development is controversial because it has been associated with both tumor suppression [105, 106] as well as enhanced tumor growth [107, 108]. Accordingly, PIGF blockage did not display tumor inhibition in all the tested mouse models for tumor [109]. Although, VEGFR2 has lower affinity for VEGF-A than VEGFR1, VEGFR2 exhibits stronger tyrosine kinase activity in response to its ligands, which makes VEGFR2 the major receptor of VEGF-A [110], and it can function both in an autocrine and paracrine fashion [94]. VEGFR3 expression in the vascular endothelium begins with the purpose of remodeling the primary capillary plexus during embryonic development. But, along development and in adult life, VEGFR3 expression gets restricted to the lymphatic endothelial cells and mainly contributes to lymphangiogenesis [111]. VEGFR3 exerts its signaling by binding to VEGF-C and -D, which are overexpressed in tumors [112]. Signaling through VEGF/VEGFR3 in lymphatic vessels is worth investing because the lymphatic vasculature is a route for tumor metastasis. Recently, Karnezis et al. [113] have shown that the collecting lymphatics serve as an important place for cancer metastasis by linking the signals via the VEGF-D/VEGFR2/VEGFR3 and the prostaglandin pathways. Contrary to its role in tumorigenesis, a soluble form of VEGFR2 (splice variant) was found as an inhibitor of lymphangiogenesis by sequestering VEGF-C and preventing it from activating VEGFR3 [114]. To have a deeper understanding of VEGF signaling in tumor, the readers can refer to Rastogi (2008) [88].

3 Steroid hormones (SH)

Steroid hormones (SH) that are associated with cancer are the ones that can elicit cell proliferation and enable cancer progression. Deregulated estrogen and androgen (also progesterone) signaling is the predominant causative agent of breast, ovarian, testis, and prostate cancers. The role of estrogen and androgen receptors in tumor formation are briefed here (Figure 1).

3.1 Estrogen receptor (ER)

The signaling pathways activated downstream of the estrogen receptor (ER) is critical for the development and growth of breast cancer. Classically, upon binding of the ligand 17β-estradiol (E2) to ER, the dimerized receptor gets translocated into the nucleus. Genomic action of ER is triggered by the binding of the dimerized ERs to the DNA directly in the estrogen response element or indirectly by tethering to other DNA-bound transcription factors, leading to ER target activation. During this process, the E2-ER complex recruits functionally diverse coregulators such as SRC1, AIB1, MTA1, etc. to form multiprotein complexes, which will modulate ER function [115]. In addition, ER can also exert nongenomic signaling through its interaction with cytosolic/membrane-associated signaling proteins [100]. Among the two ER transcription factors (ERα and ERβ), ERα is overexpressed up to 70% in breast tumors compared to normal tissues [100]. Both the genomic and nongenomic actions of ERα play a significant role in breast tumors because of their role in proliferation and metastasis [116, 117]. In fact, bone and lung metastasis of tumor

has been associated with their ERα expression levels [118, 119]. On the other hand, ERβ-mediated signaling in breast tumor cells play a distinct role of antiproliferative [120] and antimigratory function, and its expression level is inversely correlated with invasive breast cancer [121]. EMT is a key process that occurs during the invasion of tumor cells to the surrounding tissues, and ER can influence this process by interacting with the major regulators of EMT, the Snail and Slug [122, 123]. Collectively, deregulated genomic and nongenomic signaling through ERs and their coregulators underlie a majority of human breast cancers, which causes a huge percentage of cancer-related deaths in women.

3.2 Androgen receptor (AR)

Androgen is a SH that stimulates growth, development, and maintenance of prostate cells by binding to the androgen receptor (AR), which is a member of the steroid-thyroid-retinoid nuclear-receptor superfamily. Prostate cancer is one of the most common forms of cancer in men, and its development and growth mainly depend on androgen in such a way that the ablation of androgen can suppress prostate tumor. However, overtime, they can develop into androgen-independent prostate cancers (AIPC), which is a lethal form that progresses and metastasizes. Although, these are hormone-refractory tumors, they still overexpress AR [124]. Basically, androgens regulate the ratio of proliferating cells over the dying cells by promoting proliferation and inhibiting apoptosis. Testosterone is the main circulating androgen, whose free form is converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase (SRD5A2) in the prostate. DHT is the most active hormonal ligand for AR, and upon its binding, AR homo-dimerizes and bind to the androgen response elements (AREs) in the promoter regions of its target genes. This AR homo-dimer complex will further recruit coregulatory proteins, which can be either coactivators or corepressors depending on which the target genes will be activated or repressed [125]. Most of the AIPCs still express AR but signal in a non-androgen-bound manner [126] through their crosstalk with growth factor (GF) signaling pathways. GFs, such as IGF1, EGF, keratinocyte growth factor (KGF), and FGFs can activate AR in the absence of androgen [127]. For instance, in mice, HER2 is overexpressed in AIPC condition, and it is shown to convert androgen-dependent cell lines into androgen-independent cells upon overexpression [128]. HER2 might mediate this action through the antiapoptotic PI3K/Akt pathway activation [129]. A crosstalk between AR and ERK has also been reported in prostate and molecular apocrine breast cancer, contributing to disease progression [130–132].

4 Ancient signaling pathways in tumor

There are three important highly conserved signaling pathways that are hyperactive in the tumor cells. They are the multifunctional Hedgehog (Hh), Notch, and WNT signaling (Figure 2), which regulate the basic cellular processes such as proliferation, differentiation and survival that underlie most of the critical cell fate decisions.

4.1 Hedgehog (Hh) signaling

Hyperactive Hedgehog (Hh) signaling is an important hallmark of a large number of human cancers, including those of the brain [133], skin [134], lung [135], prostate [136], gastrointestinal track [137], and pancreatic cancer [138]. Hh is a morphogen that can act in a short- and long-range manner. There are three Hh proteins: Sonic Hh, Indian Hh, and Desert Hh, which transduce their signaling through glioma-associated (Gli) family of zinc finger transcription factors (Gli1-3). Gli1 always functions as a strong transcriptional activator; Gli2 and Gli3 have both activator and repressor functions, although Gli2 mostly functions as an activator and Gli3 as a repressor. In the absence of Hh ligand, Gli1 is not transcribed, but Gli2 and Gli3 are expressed; however, they will be subjected to proteolytic cleavage to form the short repressor forms [139]. Different ratios of Gli-activator (Gli-A) to Gli-repressor (Gli-R) have the potential to differentially regulate gene expression during embryo development [140, 141] and tumorigenesis [139]. This combination of Gli proteins is defined as the Gli code, and it is proposed to underlie specific cellular fates [139, 142]. Patched (PTCH1-2) is the major receptor for Hh proteins. Binding of Hh to PTCH, releases PTCH-mediated inhibition on smoothened (SMO), allowing it to transduce Hh signaling intracellularly, causing Gli-A accumulation and nuclear translocation to turn on Hh target gene expression. Hh signaling in vertebrates requires the presence of a nonmotile primary cilium where SMO is accumulated upon Hh signaling activation [143]. Under tumorous conditions, hyperactivation of Hh pathway happens either by mutation of pathway components, namely, PTCH, (receptor and negative regulator); SMO, (signaling mediator); or supressor of fused (SUFU), (prevents nuclear translocation of Gli molecules and also inhibits Gli1-mediated transcriptional activity [144]) or by PTCH [145] or SMO [146] or Hh overexpression [147–149]. Mutation of pathway components results in ligand-independent constitutive pathway activation,

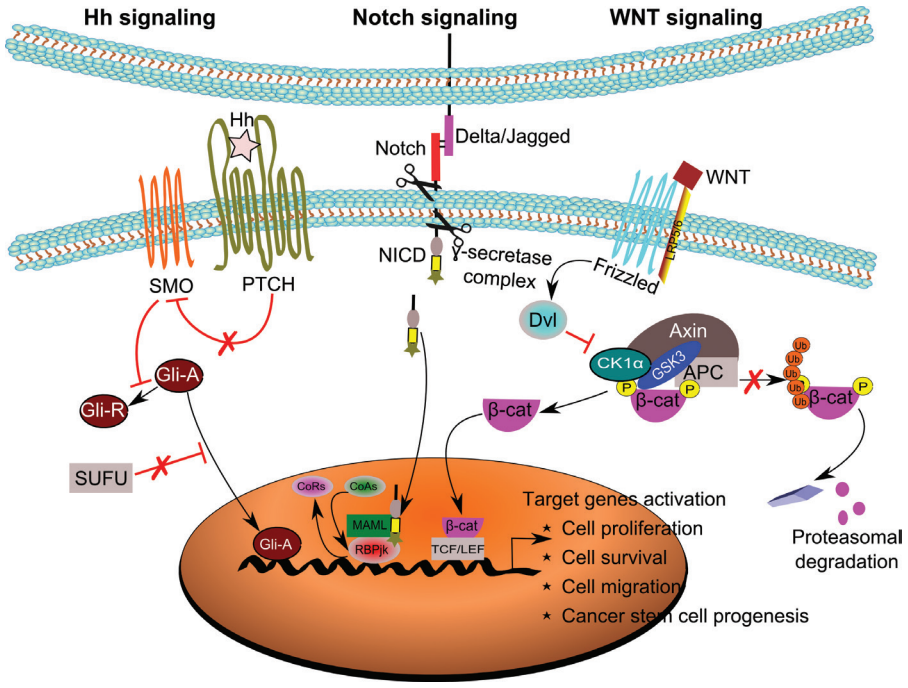


Figure 2 Schematic representation of the ancient signaling pathways, Hh, Notch, and WNT.
Members of the Gli family of transcriptional factors are the effectors of Hh signaling. In the absence of Hh ligand (SHH, DHH, and IHH), the full length Gli proteins (Gli- activator: Gli-A) are proteolytically cleaved into a lower molecular weight transcription repressor forms (Gli- repressor: Gli-R). Binding of Hh to its receptor, PTCH relieves its inhibition on SMO, allowing SMO-mediated accumulation of the full-length Gli-A form and its translocation into the nucleus where it activates Hh target genes. In the absence of Hh ligand, SUFU interacts with Gli proteins, sequestering the Gli-A from in the cytoplasm, preventing their nuclear translocation. Notch is a cell-cell communication pathway in which one cell expresses the plasma transmembrane ligand (Delta/Jagged) and the other expresses the receptor (Notch). Upon ligand binding, a series of proteolytic cleavage events occur, ultimately releasing the NICD into the cytoplasm and subsequent translocation into the nucleus. In the nucleus, NICD binds to RBPjk, a DNA-binding protein along with the transcriptional coactivator MAML1 to recruit transcriptional coactivators (CoAs) in order to initiate transcription of Notch target genes. In the absence of NICD, RBPjk will be in association with corepressors (CoRs) that inhibits Notch target gene transcription. Activation of the WNT signaling cascade begins when the secreted WNT ligands bind to FZD receptor and LRP5/6 coreceptors resulting in downstream stabilization and nuclear translocation of the transcriptional coactivator β-catenin through the activity of Dvl. In the nucleus, prior to WNT signaling, lymphoid-enhancing factor (LEF) and T-cell factor (TCF) are bound to the promoter/enhancer regions of WNT target genes, repressing their expression. Accumulation of β-catenin by WNT signaling leads to binding of β-catenin to TCF/LEF, promoting transcriptional activation of several target genes. In the absence of WNT ligand, β-catenin is associated with a cytoplasmic complex containing CK1α, GSK3, AXIN, and the APC protein. This complex promotes phosphorylation of β-catenin and targets it for ubiquitination and subsequent degradation.

and the latter causes ligand-dependent pathway activation. When the tumor cell overexpresses the ligand, it can promote growth and survival of the neighboring tumor cell by signaling in an autocrine fashion. By this means, the tumor can be controlled by adding pathway inhibitors [135] or can be accelerated by supplementing ligands [137]. Alternatively, Hh-dependent signaling can also occur in a paracrine manner where the ligand produced by the epithelial cells signals to the underlying mesenchymal or stromal cells, which in turn signals back to regulate epithelial cell proliferation and survival, by producing various signaling molecules. Apart from being activated in cancerous cells, hyperactive Gli code is the key factor of human glioma cancer stem cells [133]. Stecca and Ruiz [139] proposed that the naturally repressed form of Gli code is reverted when the tumor suppressors are lost upon mutations/epigenetic changes, resulting in uncontrolled proliferation of the cancer stem cells. Expression of the Hh pathway components has also been detected in human breast cancer stem cells [150], overall pointing to the possibility of therapeutic targeting of the stem cell population that ultimately cause tumor. Detailed reviews on Hh signaling can be found elsewhere [139, 151].

4.2 Notch signaling

Notch is an evolutionarily conserved fundamental signaling pathway that regulates several events during embryo development and tissue homeostasis

in adulthood through its four membrane-bound type I receptors (Notch 1–4) and five transmembrane ligands (Delta1, Delta3, Delta4, Jagged1, and Jagged2). Notch is a short-range signaling, and it requires cell-cell contact with each cell expressing either the receptor or the ligand. Signaling initiation occurs upon ligand-receptor interaction and the proteolytic cleavage of the notch intracellular domain (NICD) by a γ-secretase complex whose key components are presenilin and nicastrin [152]. Thus, liberated NICD gets translocated into the nucleus and binds to the DNA-binding transcriptional mediator protein, C-protein-binding factor 1 (CBF1)/RBPjk [153], trading, therein bound transcriptional corepressors with transcriptional coactivators, allowing transcription of a wide variety of Notch target genes. Mastermind-like transcriptional activator proteins (MAML1-3) are shown to be required for Notch signaling by forming a ternary complex with NICD and RBPjk [154]. The members of mammalian Hairy/Enhancer of Split (*HES*) genes are generally considered as the effectors of Notch signaling [155], but it also has other targets including the cell-cycle regulators, cyclinD1 and p21 [156, 157]. Apart from this canonical RBPjk-dependent Notch signaling, the noncanonical RBPjk-independent Notch signaling also exists, and it can also contribute to tumor formation [158, 159].

Arsenal of data from developmental and oncogenic studies suggests that Notch signaling can function in a context-dependent manner based on the cell type and stage of differentiation at which it is activated. During development and adult tissue homeostasis, Notch signaling is mandatory to maintain neural, breast, hematopoietic, and intestinal stem cells [160–164]. Apparently, many tumors also possess pluripotent stem cell population, which eventually generates large tumors [165], and Notch signaling actively takes part in controlling the fate of cancer stem cells from several tumors [166]. In fact, emerging pieces of evidence suggest that Notch components are required for the survival of breast and intestinal cancer stem cells [160, 162, 164, 167]. Notch signaling has been associated with a number of hematopoietic and epithelial human tumors including colon, breast, lung, skin, cervical, prostate cancers, leukemia, and neuroblastoma [167–170]. But, the way it works in tumor tissue is complex because in some cancers, it acts like a tumor suppressor and, in others, like an oncogenic factor. For example, Notch2 functions as a tumor suppressor in breast cancer, while other Notch receptors are oncogenic [171]; however, in brain cancer, Notch2 acts as an oncogene, whereas Notch1 has the opposite effect [172].

Notch signaling activation in invasive breast cancer cells is the result of the following one or more events: elevated levels of ligands, receptors, downstream targets, and downregulation of Numb, the inhibitor of Notch signaling [167, 173–175]. These changes lead to cell survival either by reduced apoptosis or increased cell proliferation through Akt/PI3K, ERK/MAPK, and JNK/STAT pathways [176]. Different Notch receptors are upregulated in different cancers: high Notch1 protein expression has been observed in human cervix, colon, lung, pancreas, skin, and brain cancers; Notch2 mRNA and protein are overexpressed in human brain, cervix, colon, pancreas, and skin cancers; Notch3 and Notch4 proteins are overexpressed in human malignant melanoma and human pancreatic cancer; elevated Notch4 mRNA expression has been reported in human breast cancer [176]. Being a regulator of cell fate decision, Notch signaling is known to contribute to resistance against many cancer treatments [167]. The following reviews can be referred for more information on Notch signaling in cancer [167, 176].

4.3 WNT signaling

WNT is another highly conserved pathway that is also frequently deregulated in malignancies. Like Hh and Notch signaling, WNT signaling is also associated with stem cell homeostasis in many tissues, namely, intestine, colon, bone, blood, muscle, hair, and fat [177–179]. This signaling pathway also mediates cell proliferation, migration, differentiation, adhesion, and death [180]. The term WNT is an amalgam of wingless from *Drosophila* (Wg) and its mouse homolog int1. There are 19 WNT proteins in mammals. WNTs are soluble secreted factors that signal through its interaction with cell surface G-protein-coupled receptors, Frizzled (FZD), and the coreceptors LRP5/6. WNTs activate at least three different signaling pathways: the canonical pathway that requires β-catenin activation and WNT/Ca^{2+} and WNT/planar cell polarity (PCP) noncanonical signaling pathways that are independent of β-catenin [181, 182]. In the canonical pathway, WNT-activated FZD will immediately recruit the cytosolic disheveled protein (Dvl1, 2, or 3) and regulate the intracellular concentration of β-catenin by modulating the activity of the β-catenin destruction complex containing axis inhibitor (Axin), adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1) [183]. Mutations in β-catenin and APC are reported in many human cancers [184], and the pathway is deregulated in colorectal and breast cancers [181, 184–186]. Tumor cells either present an upregulation of WNT positive

regulators or downregulation of negative regulators to activate the pathway. For instance, Dvl1 is upregulated [187], and a secreted WNT inhibitor, FZD-related protein 1 (FRP1) is downregulated/deleted in many breast cancers [188]. Sustained expression of FRP1 in human breast cancer cell line dramatically impaired their ability to form tumor xenografts in mammary glands of nude mice [189]. WNT receptor FZD and coreceptors LRP5/6 are also overexpressed in many tumors. FZD1 overexpression in breast cancer cell line is reported to confer multidrug resistance through MRD1 induction [190]. FZD7 expression in colon cancer cell lines has been accounted for canonical WNT pathway activation despite the presence of APC or β-catenin encoding gene (CTNNB1) mutation [191]. Likewise, LRP6 overexpression is defined as the characteristic of a subpopulation of breast cancer, and its silencing significantly reduced WNT signaling, suggesting LRP6 as a potential therapeutic target [192]. Also, LRP5 expression is shown to be required for WNT-dependent mammary tumors [193]. Perturbation of WNT signaling has been shown to inhibit proliferation and impair cell motility of human breast cancer cell lines [185, 186, 189]. Furthermore, during breast cancer metastasis, WNT signals are reported to promote EMT and migration through stabilization of Snail [194], which could be compromised by inhibiting WNT signaling [195].

The noncanonical pathways also play important roles in tumorigenesis [196]. During the metastasis of melanoma, Wnt/Ca^{2+}pathway is involved in EMT through WNT5a [197]. As PCP plays a crucial role in cell adhesion and movement, its dysfunction greatly correlates with tumor metastasis. WNT11- and WNT5a-activated WNT/PCP pathway [198, 199] promote metastasis through Rac, Rho, and JNK in hepatocellular carcinoma, melanoma, gastric, non-small-cell lung, colon, and breast cancers [200, 201]. To add further complexity to the system, WNT5a is reported to function as an oncogene or tumor suppressor in a context-dependent manner, suggesting that PCP might be functioning as a tumor suppressor in the early stages of tumorigenesis and then in more progressed tumors as oncogene [201]. Both the WNT/β-catenin and noncanonical WNT signaling are also implemented in tumor angiogenesis [202]. The following reviews are suggested for deeper understanding of WNT signaling in cancer [183, 184, 201].

5 Other important molecules modulated during cancer

The multifunctional protein Src is an intracellular/membrane-associated tyrosine kinase that regulates cell proliferation, angiogenesis, survival, differentiation, and cell movement by interacting with GF receptors, steroid hormone receptors (SHR), and many other adaptor proteins (Figure 1). Through activation of different transduction cascades including Ras/MAPK/ERK, PI3K/Akt, and STAT pathways, Src is capable of transforming normal cells into malignant ones (reviewed in [203]). Src also mediates adhesion-dependent responses by functioning as an important mediator downstream of integrins [204]. FAK is a tyrosine kinase, which can act as a signaling molecule or as a scaffold protein, enabling the recruitment of Src to integrin. Src-integrin interaction also functions synergistically with RTKs [205]. In agreement, β1 integrin overexpression in non-small-cell lung cancer has been associated with its resistance to gefitinib, which targets the tyrosine kinase activity of EGFR [206]. High c-Src activity is reported in several cancers, such as breast, colon, pancreatic, neural, ovarian, esophageal, gastric, lung, and melanoma [207]. It is often co-expressed with GFs, like in the case of majority of breast cancers (over 70%) where it is co-overexpressed with HER family members [100]. Src can phosphorylate and, thereby, inhibit the tumor-suppressor protein PTEN [99]. Src represents a viable target for antiangiogenesis therapy because it is reported to induce VEGF expression and angiogenesis in pancreatic cancer cells [208]. In addition to augmenting GF signaling, c-Src also mediates signaling through SHR, and it has been proposed to be important for E2-stimulated cellular proliferation through ER [203, 209].

Signal transducer and activator of transcription (STAT1-6) are second messengers of the JAK/STAT signaling pathway in response to the binding of extracellular proteins, including GF, hormones, and cytokines and serves as the integrator of signaling pathways activated by GFs and hormones. Upon tyrosine kinase-mediated phosphorylation, STATs will homo- or heterodimerize and get translocated into the nucleus where it binds to STAT-specific response elements on DNA to regulate transcription of interferon-stimulated genes (ISGs). Dysregulated JAK/STAT signaling leads to tumor formation through increased angiogenesis, enhanced survival, and immunosuppression. Overexpression/activation of STAT3, STAT5a, and STAT5b has been described in many tumors including the lung, prostate, and breast cancers [210–212].

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA), the activator of signaling cascades. There are two PLDs identified in mammals (PLD, PLD2), which are activated downstream of WNT/β-catenin signaling [213, 214]. Polymorphisms or point mutations in PLD2 are found in colon and breast cancers, respectively [215]. PLD-produced

PA, lies in the center of many key cell growth regulator pathways associated with cancer, namely, SOS/Ras [216], Raf/MAPK/ERK, and mammalian target of rapamycin (mTOR) pathways [217, 218].

The highly conserved phosphatidylinositol 3-kinase (PI3K) pathway regulates diverse cellular processes, including metabolism, angiogenesis, growth, survival, proliferation, apoptosis, and cell migration [219]. Akt, the target of PI3K signaling, is activated upon phosphorylation by 3-phosphoinositidedependent kinase (PDK1) or mTORC2 or by other kinases [220]. Several human cancers possess mutations in p110α, the catalytic subunit of PI3K and PTEN at very high frequencies, resulting in increased activity of the PI3K/Akt signaling pathway [221]. Phosphorylated-Akt can augment cancer in several ways: (1) Akt phosphorylates its substrate, FOXO (Forkhead box gene, group O; proapoptotic transcription factor) and enables its retention in the cytosol, causing increased cell proliferation and survival [222]. Inhibition of Akt signal causes FOXO nuclear translocation and subsequent activation of receptor gene expression [223, 224]. (2) Akt can also influence eNOS and potentiate angiogenesis and vascular permeability [88, 225]. (3) Tuberous sclerosis complex 2 (TSC2) is also a substrate of Akt, which along with PTEN and LKB1 are tumor suppressors that negatively regulate mTORC1 activity [221]. (4) Activated Akt inhibits GSK3 by phosphorylation, which might be mediating some of the antiapoptotic effects of Akt [226, 227]. (5) PI3K/Akt pathway can enhance NF-κB-dependent transcription, which regulates cell fate decisions, such as apoptosis and proliferation [228]. (6) PI3K/Akt pathway activation can also confer cell survival signal by suppressing apoptosis, such as the case with anoikis, apoptosis induced by inadequate or inappropriate cell-matrix interactions [229].

Phosphorylation of TSC2 by Akt relieves its negative regulatory effect on mTOR, making it as a primary effector of Akt signaling [230]. mTORC1 activation causes phosphorylation of its effector ribosomal protein S6 kinase 1 (S6K1), which further phosphorylates the ribosomal protein S6 that allows translation of mRNAs encoding different proteins [231]. mTORC1 also regulates VEGF by phosphorylating hypoxia-inducible factor α (HIF-1α) leading to its accumulation in tumor cells [232]. HIF-1α is predominantly responsible for the adaptation of solid tumors to hypoxia by mediating angiogenesis and anaerobic metabolism [233].

Tissue factor (TF)/ protease-activated receptor (PAR)-mediated signaling shapes the tumor microenvironment by inducing several cytokines, chemokines, and GFs in addition to their involvement in tumor cell migration [234, 235].

There are also other factors that influence tumor development and progression. Homologous recombination (HR) is a fundamental cellular process, which upon dysfunction could cause genomic instability leading to malignancies. Mutations in HR regulators, BRCA1 and BRCA2, are also reported to cause hereditary breast and ovarian cancers [236]. Similarly, mutation in another HR regulator, RAD51C is also associated with breast and ovarian cancer [237], implying a crucial role for HR and its regulatory genes in cancer prevention.

6 The tumor microenvironment

The tumor microenvironment could be defined as the supportive environment existing around the tumor that facilitates growth, survival, and invasion of tumor cells by providing appropriate signaling molecules, chemokines, soluble factors, and extracellular matrix. These cues come from the surrounding stromal cells, which include endothelial cells, necessary for tumor angiogenesis; fibroblasts that produce chemokines and involved in extracellular matrix remodeling; and inflammatory cells. Owing to the dynamics in stromal cells, metabolic alterations, and modulations in the extracellular matrix, the tumor microenvironment is under constant evolution. The network between the tumor and the nearby stromal cells are very crucial to establish tumors. Moreover, the tumor microenvironment is also known to regulate the behavior of cancer stem cells [1, 238]. The tumor microenvironment is influenced by crosstalks between the aforementioned signaling pathways.

Target genes of SHH/Gli signaling can also directly or indirectly lead to the synthesis of signaling molecules, some of which may enrich the tumor microenvironment facilitating tumor growth and progression [239–241]. Hh signaling can be modulated by GFs like EGF [242]. In epidermal cells, EGFR-mediated Raf/MEK/ERK intracellular pathways cooperate with Gli1/2 proteins to regulate the Notch ligand, *jagged2* transcription, linking GF, Hh, and Notch signaling [243]. Schreck et al. [244] showed that the effector of Notch signaling, Hes1, can directly bind to Gli1 promoter and repress its transcription causing low Hh activity in glioblastomas and suggested that targeting both pathways simultaneously may be more effective in the elimination of glioblastoma cells. Jagged1 downregulation was also accounted for reduced *gli2* expression in ovarian cancer cells in a Notch-independent fashion. Interestingly, this relationship between Jagged1 and Gli2 worked both ways as knockdown of Gli2 diminished *jagged1*

expression level [245]. Furthermore, *jagged1* expression has been considered as a potential link between Notch and WNT signaling pathways in ovarian [246] and colorectal [247] cancers. Gli's can also be modulated by other signaling pathways: TGF-β/SMAD3 pathway in association with WNT/β-catenin signaling can directly transcribe Gli2, which upregulates Hh target genes including *gli1* expression in an Hh-independent manner [242, 248]. Notch, being a cell-cell communication signaling, can occur between tumor cells and stromal cells [249, 250], promoting angiogenesis [251]. For instance, the Notch ligand, *jagged1*, is expressed both in the stromal (endothelial cells) and the tumor compartments of ovarian cancer and serves as a putative target for therapies. Selectively targeting Jagged1 in the tumor stroma significantly reduced microvessel density, and its combined inhibition in stromal as well as ovarian tumor cells greatly reduced the overall tumor size [245], suggesting the role of Jagged1 in angiogenesis and cell proliferation. WNT signaling from the stromal cells also has its role to play in tumor progression (colorectal cancer: [252]), differentiation, and migration of the tumor cells [253]. Recently, Notch2 was identified as the target of WNT/β-catenin signaling in colorectal cancer cells [254]. But another study in colorectal cancer uncovered an unexpected suppressive role of Notch1 on WNT/β-catenin target genes [255]. Owing to such strong interactions between the ancient Notch, WNT, and Hh signaling pathways, recent studies suggest that inhibiting these pathways in combination with traditional chemotherapies may provide enhanced chemosensitivity [183, 256, 257].

GF constitute an important mode of communication between the tumor epithelium and stromal components [258]. PDGF, released by the tumor cells, signals through the stromal cell-expressed receptors, and in turn, they receive growth inductive signals from the stromal cell-secreted IGF1 [38]. The stromal cell-derived chemokine SDF1 and its receptor CXCR4-mediated signaling play influential role in the metastasis of ERα-positive invasive breast cancers [259, 260]. In addition, GF can also contribute to the aberrant growth of tumor stem cells as it has been recently illustrated for glioblastoma-derived stem-like cells [238]. The influence of various factors on tumor microenvironment has been reviewed in the following articles: SHH: [240], Notch: [251, 261], WNT: [262].

The tumor microenvironment being a birthplace for the activation of various signaling pathways provides the perfect environment for dormant metastases to flourish. Metastasis is a deadly process in malignancies that contributes to the majority of cancer-related deaths. In order to metastasize, the tumor cell should separate itself from the primary tumor, navigate the stromal tumor microenvironment through vasculature and/or lymphatic channels, and invade to a new location to establish the micrometastasis at a distant site [263]. EMT is a crucial step in this process, which is a result of convergent activation of several transcription factors (Snail, Slug, Twist, ZEB1/2, and SMADs) by multiple signaling pathways, namely, TGF-β, WNT, Notch, and Hh [264]. Often, the increasingly complex tumor microenvironment also accounts for therapeutic resistance. The stromal tissue-derived CXCR4 signaling is sufficient to drive metastasis of ERα-positive breast cancers and foster endocrine therapy resistant via increased MAPK signaling [260]. Mostly, drug resistance is associated with MAPK, PI3K/Akt, and PKCγ pathway activation. Owing to these reasons, combination of therapies targeting different factors are beneficial than targeting a single tumor inducer. This is the case in the treatment of breast cancer, where both ER and HER2 are targeted. These discussions suggest that it is necessary to carefully analyze the tumor microenvironment to provide proper treatment.

7 Functionalization of nanoparticles for cancer therapy

Nanoparticles are submicron-size carrier systems composed of natural or synthetic polymers with the size range of 10–1000 nm in which the drug may be dissolved, entrapped, encapsulated, or attached. Nanoparticle-mediated early diagnostic methods and targeted therapies serve as a potential tool to fight cancer because of their ability to achieve site-specific action of the drug at therapeutically optimal rate and dose while reducing the unwanted toxic side effects [265]. In order to achieve these qualities, the nanoparticles are designed considering several parameters as discussed below.

7.1 Challenges in nanodrug delivery and strategies to overcome them

The physiology of every human organ is designed to perform their respective functions at optimum level and to prevent the invasion of toxins, antigens, and pathogens. These protective functions are executed by physical and biochemical barriers, which are also responsible for hampering drug delivery to the targeted site. The physical barriers include the cell membranes, tight junctions between adjacent epithelial cells, extracellular matrix, mucus layer,

etc., while the biochemical barrier comprises of the efflux pumps, catabolic enzymes that leads to drug metabolism/detoxification, drug sequestering to acidic compartments, and drug deactivation mechanisms [266]. As a result, only a small percentage of drugs will finally reach the targeted cells. This limited delivery is not only true for the conventional cancer drugs but also for gene therapy, which stands as an attractive therapeutic approach for cancer. In gene therapy, functional DNA molecules or small interfering RNA (siRNA) are effectively delivered into malfunctioning cells to replace the missing/mutated gene or to induce posttranscriptional gene silencing, respectively [267]. Intravenously injected DNA-containing nanoparticles must be able to successfully circulate in the bloodstream by avoiding serum proteins that may bind to the particles and increase their size, paving the way for them to be eliminated by Kupffer cells present in the reticuloendothelial system. Subsequently, the circulating nanoparticles should extravasate into the tumor tissue and contact the cell surface by crossing the physical/extracellular barriers (cell membranes, tight junctions, and extracellular matrix). Once internalized by the cell, the DNA within the nanoparticle must escape the biochemical and intracellular barriers (lysosomal degradation, endocytic vesicles, degradation by cytosolic nucleases) and find its way into the nucleus and target the transcription active regions.

Another prominent barrier is the blood-brain barrier (BBB), which continues to be a challenge in the treatment of brain cancer [268]. The brain, being the central organ of the human body, have capillaries that have evolved as a natural defense mechanism by restricting the movement of molecules between blood and brain. Successful passage of molecules across the BBB is constrained by tight junctions between capillary endothelial cells, efflux transport proteins expressed in the luminal (blood) side of the BBB, and degrading enzymes present in the cytoplasm of endothelial cells. However, small molecules with appropriate lipophilicity, molecular weight, and charge can pass through the BBB. This action is facilitated by transporters expressed at the luminal and basolateral (brain) side of the endothelial cells, specific receptors expressed on the luminal side of the endothelial cells, and by passive diffusion. Among the several strategies applied to bypass BBB, employing nanoparticles functionalized based on the native receptors or transporters localized in the luminal (blood) side of the endothelial cells have been promising to date, in brain cancer therapies [268–270]. The intravenously injected nanoparticles are mostly transported across the BBB by endocytosis, which will then undergo transcytosis. Usually, polyethylene glycol added (PEGylated), surfactant coated (PS 80), targeting molecule attached biodegradable and nonbiodegradable nanoparticles have been used in *in vitro* and *in vivo* brain-targeting studies [270]. Biologically active polymer core/shell nanoparticles self-assembled from TAT-PEG-b-cholesterol (TAT-PEG-b-Chol) were synthesized and successfully used to deliver ciprofloxacin antibiotic across the BBB [271]. Polyethylene glycol conjugated (PEGylated) gold nanoparticles functionalized with EGF was used to selectively deliver therapeutic drug and phthalocyanine 4 (Pc 4) to brain glioma tumors for photodynamic therapy (PDT) [272]. Recent development in the field of drug delivery to the central nervous system has been thoroughly discussed in the following reviews [268, 270]. Thus, to increase the therapeutic efficacy of nanoparticles, they must be targeted to the required site through appropriate approaches.

7.2 Passive and active targeting of nanoparticles

In order to create nanoparticles that exclusively target tumor cells, two basic strategies are employed: passive and active targeting methods [273]. In passive targeting, the pathophysiologic features of cancer tissue are exploited for the accumulation of nanoparticles in tumor sites. One such important parameter is the newly formed leaky blood vessels that supply nutrients and oxygen to tumors exceeding 2 mm^3 in size [274]. In addition, tumor cells also present higher compound retention time than healthy cells, which allow the retention of nanoparticles in tumor cells for a prolonged period of time [275]. Together, these parameters provide an enhanced permeability and retention (EPR) effect, the major determinant of passive targeting. This way of delivering nanoparticles is reported to be an apt strategy for gene therapy. Considering the endosomal/nuclease degradation and the negative charge of DNA molecules, it is a challenging task to deliver the DNA or RNA to the target cells, make them to cross cell membrane and enter the nucleus. Although, virus-mediated DNA delivery is widely used to achieve high expression rates, they have the limitations of being toxic, immunogenic, and expensive. Alternatively, biodegradable, functionalized polymeric nanoparticles are utilized in therapy to meet this requirement. Owing to safety, sustained release capacity, and the ability to rapidly escape the endolysosomal pathway, poly-(lactic-co-glycolic acid) (PLGA) nanoparticles have been suggested as a good gene delivery system [276]. Accordingly, pigment epithelial-derived factor (PEDF) gene-loaded PLGA nanoparticles have been demonstrated to be an

innovative therapy for colon carcinoma by inducing apoptosis, decreasing microvessel density, and inhibiting angiogenesis [277]. Modifying the surface of the gene carriers with hydrophilic, flexible, non-ionic polymers like PEG and conjugating targeting moieties are efficient strategies to improve circulation time and site-specific delivery, respectively. However, PEGylation can invariably compromise the specificity of nanoparticles [278]. So, it is vital to balance between the specificity of nanoparticles and their delivery efficiency to achieve optimal results. Passively endocytosed logic gate nanoparticles, developed with a dual pH-responsive random copolymer (poly-β-aminoester ketal-2), has been validated as a novel gene delivery system by Morachis et al. [279]. These nanoparticles possess the ability to remain hydrophobic at physiological pH (pH 7.4) but undergo a switch from hydrophobic to hydrophilic at low endosomal pH, triggering their rapid fragmentation followed by concomitant release of the encapsulated DNA. However, inadequate EPR effect due to variations in the permeability of tumor blood vessels is a limiting step in achieving optimal nano drug delivery through passive method. Active targeting method could be applied to overcome this limitation.

In active targeting method, the nanoparticles are attached to specific moieties, namely, antibodies, peptides, or other small molecules to increase their specificity to the target site. Surface-functionalized nanoparticles developed by impregnating tumor-specific ligands or novel tumor biomarkers on the surface of the nanoparticles significantly improve their targeting efficiency. Enhanced *in vitro* cellular toxicity, achieved by docetaxel-encapsulated PLGA-b-PEG nanoparticle surface functionalized with the A 10 2′-fluoropyrimidine RNA aptamer that recognizes the prostate-specific membrane antigen (PSMA)'s extracellular domain is a good example for the utilization of biomarkers as targeting moiety [280]. However, it should be noted that most of the biomarkers highly expressed in tumor cells are also expressed in healthy cells at comparatively lower levels. For this reason, it is crucial to choose receptors that are overexpressed between 10^4 and 10^5 copies/cell in the tumor cells than in the normal cells [281]. Nowadays, monoclonal antibody-conjugated nanoparticle-mediated delivery of antineoplastic agents has achieved extraordinary potential in cancer therapy [282]. For instance, docetaxel containing PEGylated chitosan nanocapsules conjugated to a monoclonal antibody against the transmembrane tumor-suppressor protein TMEFF-2 presented a delayed and prolonged action on non-small-cell lung carcinoma mouse xenografts compared to the free drug [283]. Although circumventing multiple drug resistance (MDR) is an advantage of using active over the passive targeting strategy [284], multifunctional nanoparticles can have nonspecific interactions with healthy cells, triggering immunogenicity and subsequent nanoparticle clearance [278]. However, many new formulations are developed to match the requirement of the disease and the body, with diagnostic and therapeutic applications. Recently, a new formulation combining the properties of liposomes and nanoporous particles called "protocells" were developed to treat human hepatocellular carcinoma [285]. These protocells exhibited 10,000-fold greater affinity toward carcinoma cells than the healthy hepatocytes, endothelial, or immune cells and displayed ameliorated capacity, stability, specificity, and controlled release of multicomponent cargos at high concentrations within the cytosol of cancer cells [285].

Most of the ligand-receptor interactions mentioned in the first section of this review functions as a putative route for interaction and internalization of drug-loaded nanoparticles by endocytosis (Figure 3). Here, we brief some examples on the utilization of these signaling pathways/molecules for nanoformulation-mediated targeted drug delivery, and many other formulations are provided in Table 1. A chimeric protein, GFP-FRATtide-conjugated silica nanoparticles were designed to target WNT signaling pathway. FRATtide is an inhibitor of GSK3, and its delivery to the human embryonic kidney cells and rat neural stem cells greatly affected WNT signaling cascade by increasing β-catenin levels and transcription of WNT target genes, such as *c-Myc* [307]. Functionalized nanoparticles have also been employed to target tumor angiogenesis as a means to reduce tumor growth. Ruthenium-modified selenium nanoparticles (Ru-SeNPs) have been shown as potential antiangiogenic agents in human umbilical vascular endothelial cells through inhibition of FGFR1 and its downstream ERK and Akt pathways [299]. Even SHRs can be targeted by functionalized nanoparticles. ER-α located on the cell membrane [315] was targeted by thiol-PEGylated tamoxifen derivative plasmonic gold nanoparticles, which exhibited 2.7-fold enhanced drug potency compared to the free drug in ER-positive breast cancer cells [302]. RTK also facilitates effective nano drug delivery. Rapamycin-loaded PLGA nanoparticle surface conjugated with EGFR-antibodies presented superior antiproliferative activity over unconjugated nanoparticles and native rapamycin, due to higher cellular uptake on malignant breast cancer cells overexpressing EGFRs [228]. Paclitaxel, actively targeted to EGFR-overexpressing cancer cells by utilizing chimeric anti-EGFR monoclonal antibody cetuximab surface-conjugated O-carboxymethyl chitosan nanoparticles was reported to enhance cell death [294]. c-Src can effectively activate EGFR, and it

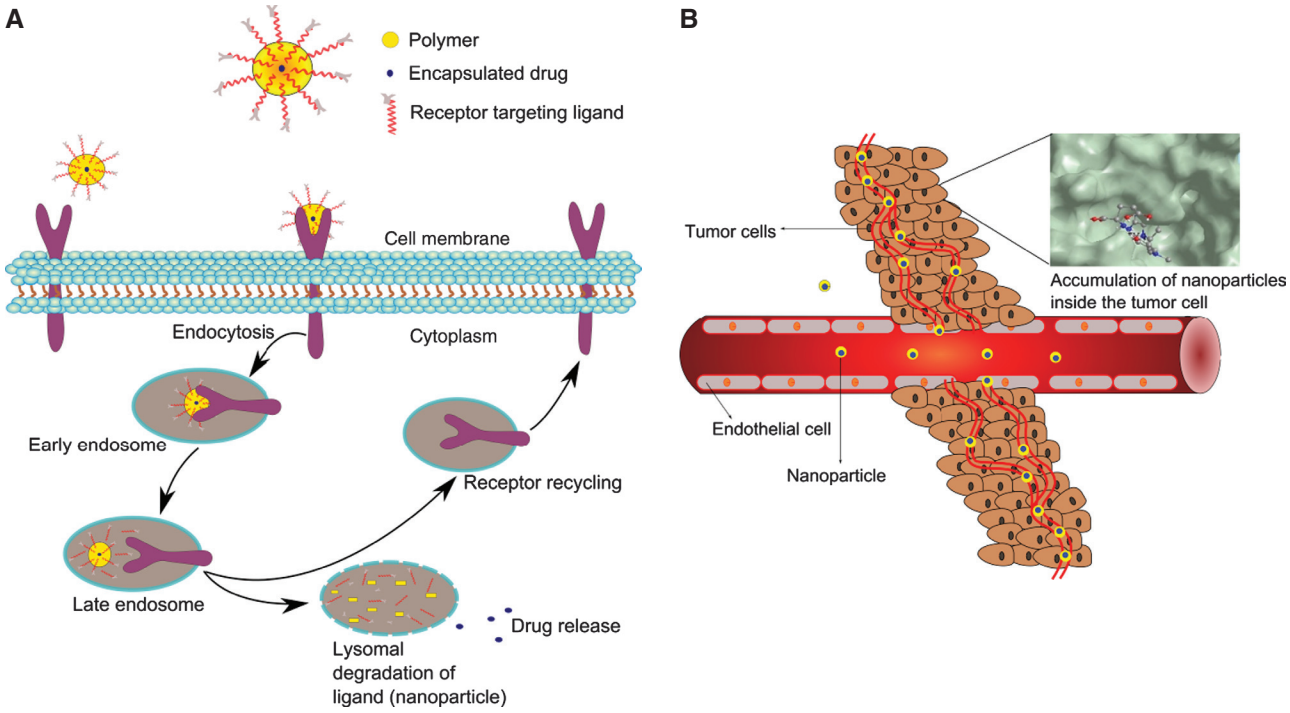


Figure 3 Schematic representation of active (A) and passive (B) targeting. (A) In the active targeting method, the nanoparticles conjugated with specific moieties such as antibodies, peptides, or other small molecules targeting various cell surface receptors are internalized into the cell through receptor-mediated endocytosis. The acidic nature of the endosomes will destabilize its membrane resulting in the release of different components of the internalized nanoparticle as well as the entrapped drug into the cytosol of the cell. (B) Formation of blood vessels is crucial to supply nutrients and oxygen to solid tumors. Thus, newly formed blood vessels possess several gaps in between the endothelial cells making a leaky vasculature. In the passive targeting strategy, the nanoparticles take advantage of these leaky blood vessels to reach the tumor. Moreover, the absence of a well-defined lymphatic system in tumor tissue also improves the compound retention time. These properties of the tumor cells together make the ERP effect that facilitate the accumulation of nanoparticles and therein-entrapped drug at higher concentration in the tumor site.

was recently targeted by c-Src antisense oligonucleotide complexed with PAMAM denderimes. This formulation reduced c-Src and EGFR-dependent target gene expression in human colon cancer cells [316].

7.3 Nanoparticles in photodynamic therapy (PDT)

In addition to the aforementioned applications, nanoparticles can also function as photosensitizer carriers in photodynamic therapy (PDT). PDT is an established cancer therapy particularly for superficial tumors, where the previously administered photosensitizer, accumulated in the tumor site, will be excited by nonthermal light (635–760 nm) irradiation. Thus, excited photosensitizer along with molecular oxygen, generates singlet oxygen ($^1O_2$), which mediates PDT-induced cell death. Thus, the efficiency of PDT is determined by the successful formation of $^1O_2$ (2). For this purpose, both biodegradable and nonbiodegradable nanoparticles are useful. When biodegradable nanoparticles are used, the photosensitizer released by the particle will be excited to produce $^1O_2$. But, when the nonbiodegradable photosensitizer is used, the photosensitizer will remain inside the particle, which allows efficient $^1O_2$ diffusion [317]. Owing to its several advantages over other polymers used in PDT [276], Gomes et al. used PLGA-loaded bacteriochlorophyll-a (BChl-a) photosensitizer and obtained almost complete phagocytosis in just 2 h of incubation with macrophage cells [318, 319]. PLGA has also been utilized to load hydrophobic photosensitizer molecule zinc phthalocyanine (ZnPc) and illustrated to exhibit tumor regression in tumor-bearing mice, compared to free ZnPc [320]. However, polylactic acid (PLA) nanoparticles performed better than PLGA particles when the hydrophobic natural photosensitizing compound (Hypericin, Hy) from *Hypericum perforatum* was applied in ovarian cancer cells [321]. The nondegradable nanoparticles used in PDT are mostly ceramic-based (example: organically modified silica or organically modified silicate – ORMOSIL [322]) or metallic-based (example: gold [323]) or made from polyacrylamide polymers [324]. Moreover, nonbiodegradable

Table 1 Comprehension of nanoparticle-based approaches to target the aforementioned signaling pathways/molecules.

Nanoparticles	Functional molecule	Reason for functionalization	Target	References
Glutaraldehyde cross-linked albumin nanoparticles	Single variable domain of a EGFR antibody (Ega1)	EGFR-positive 14C squamous head and neck cancer cells	EGFR	[286]
Gold nanospheres and nanorods	IgG antibody	Oral cancer	EGFR, HERI, ErbB1	[287, 288]
PLGA-PEG-PCL nanoparticles	EGFR peptide	MDR in breast and ovarian cancer	EGFR	[289]
Poly (ethylene glycol)-poly(ε-caprolactone) block copolymer micelles	GE11 peptide	Active targeting of EGFR-overexpressing cancer cells	EGFR	[290]
Lipid-based nanoparticles	Nickel	Epidermoid carcinoma cells A431	EGFR	[291]
Rapamycin-loaded PLGA nanoparticles	EGFR antibody	Breast cancer	EGFR	[292]
Catanionic solid lipid nanoparticles	EGFR antibody	Human brain malignant glioblastomas cells (U87MG)	EGFR	[293]
PTXL loaded O-carboxymethyl chitosan nanoparticles	Cetuximab monoclonal antibody	Lung cancer	EGFR	[294]
pH-sensitive immunoliposomes	EGFR antibody	Lung cancer	EGFR	[295]
PEG-PCL-cetuximab-immunomicelles	Anti-EGFR monoclonal antibody.	EGFR-overexpressing tumor cells	EGFR	[296]
Gold nanoparticles	Highly stable FGF1 variant	FGFR-overexpressing cancers	FGFRs	[297]
Cisplatin-loaded gelatin nanoparticles	Heparin	Breast cancer	FGFR2	[298]
Selinum nanoparticles	Ruthinum (11) polypyridyl	Liver cancer	FGFR1, ErK, Akt	[299]
Gold nanoparticle	VEGF antibody	Kill B-chronic lymphocytic leukemia cells	VEGF pathway	[300]
Doxirubicin-loaded liposomes	Estrogen	Breast and uterus cancers	ER	[301]
Plasmonic gold nanoparticles	Thiol-PEGylated tamoxifen derivative	Breast cancer	ER	[302]
Lipid nanoparticles	AR- si RNA	Prostate cancer	AR	[303]
PLGA conjugated with PEG nanoparticles	HPI-1 (Gli1antagonist)	Medulloblastomas, hepatocellular carcinoma	Hh signaling	[304, 305]
PLGA nanoparticles	DCAMKL-1-specific siRNA	Colon cancer	Notch signaling	[306]
Silica nanoparticles	FRATtide peptide	HEK 293 cells	WNT signaling	[307]
LY294002-encapsulated PLGA nanoparticle	–	Inhibition of PI3K-mediated angiogenesis in melonama cells	PI3K pathway	[308]
Liposome-polycation-hyaluronic acid (LPH) nanoparticle	GC4 single-chain variable fragment (scFv) tumor-targeting human monoclonal antibody	c-Myc, MDM2, and VEGF-siRNA and miRNA to lung metastasis murine model	MAPK signaling	[309]
PEG-coated core-cross-linked polymeric micelles	EphB4-binding peptide TNYL-FSPNGPIARAW and labeled with Cy7 and indium 111	Fluorescence imaging of EphB4 in prostate cancer cells	EphB4	[310]
Magnetic iron oxide nanoparticle	PTEN gene expression plasmid	PTEN gene delivery for to reverse cisplatin resistance in lung cancer	PTEN gene delivery	[311]
Polyamidoamin polymers, PAMAM nanoparticals	Antisense oligo c-Src	Knocking-down c-Src in colon carcinoma cell line	c-Src pathway	[239]
Large unilamellar vesicle (LUV) nanoparticle	PEGylated particle containing the Jak3 tyrosine kinase inhibitor, WHI-P131	Leukemias with constitutive Jak3-STAT3/STAT5 activation	Jak3/STAT pathway	[312]
PEGylated chitosan (CS) nanocapsules	Monoclonal antibody anti-TMEFF-2	To treat non-small-cell lung carcinoma	TMEFF-2	[283]
Liposomes	Mitochondrial-targeting molecule-Dequalinium polyethylene glycol-distearo ylphosphatidylethanolamine conjugate	To treat non-small-cell lung carcinoma	To enhance cytotoxic effect in mitochondria	[313]
Star-shaped PLGA-vitamin E TPGS copolymer nanoparticles	Cholic acid	To treat cervical cancer	For better biocompatibility,	[314]

nanoparticles have the potential to perform multiple functions in combination with PDT [317, 325]. A polyacrylamide multifunctional platform with a contrast enhancer for magnetic resonance imaging (MRI), photosensitizer (Photofrin1) for PDT, PEG surface coating and targeting moiety (the integrin-targeting RGD peptide) was synthesized, and each functionalization aspect was demonstrated to be successful by Kopelman et al. [326]. Hybrid gold-iron oxide nanoparticles [327] and lanthanide-doped upconversion nanoparticles [328] are among the many other new formulations that possess diagnostic and PDT tools.

8 Conclusions

Signaling interactions enriching the tumor microenvironment and altered signaling molecules in tumor cells provide potential strategies for targeted nano drug delivery. Over the past years, substantial effort has been made toward the development and advancement of multifunctional nanoparticles for cancer diagnosis and therapeutic purposes. As it has been discussed above, functionalization of nanocarriers by modifying their surfaces with various targeting moieties, namely, antibodies, peptides, and other small molecules, has significantly improved their targeting as well as delivery efficiency. Antibody-conjugated nanoparticles seem as a straightforward way to achieve receptor-mediated endocytosis of the particle at the disease site. In addition to being a route for entry, it is also possible to activate an array of intracellular pathways leading to cell death or proliferation or angiogenesis, etc., when the receptors are targeted. Furthermore, there are many upcoming multifunctional nanoformulations such as magnetic nanoparticles, which through their real-time monitoring ability look promising for clinical use in the area of disease diagnosis and drug delivery to cancer cells [329]. Emerging data suggest that it is possible to simultaneously target two important pathways to improve the treatment efficiency. A bispecific antibody, anti-PDGFR-B/VEGF-A, capable of attenuating angiogenesis through two distinct pathways was reported by Mabry et al. [330], and it is yet to be applied for nano delivery method, which could further improve the efficacy. As cancer is a disease of dysregulated signaling pathways, there are much more to explore at the level of basic research, and also, there is a huge possibility to adapt the known knowledge for the nano applications. Although, biomarkers expressed on tumor cells could be used to design personalized nanoparticles to treat cancer, it is important to optimize the particles to have balanced targeting and delivery competence because overloading the carriers with targeting moieties can trigger immunogenicity and subsequent clearance. However, knowledge accumulated from years of research has enabled many nanoparticle-based drugs to be approved or to be tested in the clinic [273]. It is time to acknowledge that the nanoparticle approach is a wiser way to fight cancer in a robust and personalized manner with minimal side effects.

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