



Targeting Signaling Pathways in Cancer Stem Cells for Therapy of Cancer

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Abstract

Cancer stem cells (CSCs) represent a fraction of cancer cells that has the ability to self-renew and differentiate into a variety of tumor cells. This subset of cancer cells is involved in relapse, metastasis, radiation resistance, and multidrug resistance, all of which contribute to the formation of new tumors. As a result, CSCs are regarded to be the most potential cancer therapeutic target. Several studies indicate dysregulation of developmental and stem cell-specific signaling pathways in cancer stem cells. These signaling pathways can regulate both intrinsic and extrinsic pathways, directly and indirectly, thus regulating the cancer stem cell phenotype. Therefore, gaining a complete understanding of the involvement of the signaling pathways in cancer stem cell functions could lead to development of better cancer treatment strategies. This chapter presents an overview of the major signaling pathway of cancer stem cells and how they modulate various factors involved in the maintenance of cancer stem cells. Moreover, the updates of therapeutics strategies targeting these pathways are also discussed.

Keywords

Signaling pathway · Cancer Stem Cells · Tumor Microenvironment · Immune Evasion · Resistance to Therapy

Introduction

Stem cells are defined as a population of undifferentiated cells with the characteristic ability of self-renewal and differentiation into various cellular progenies. The correlation of cancer to stem cells had long been hypothesized due to its metastatic and re-emergent nature (Ayob and Ramasamy 2018). It was Lapidot et al. that provided the first experimental evidence of CSCs in the year 1994. In acute myeloid leukemia (AML), the tumor-introducing cell was thought to be a cell of the hematopoietic stem cell hierarchy susceptible to leukemic transformation due to various genetic or epigenetic factors. A population of human AML cells was introduced into severe combined immunodeficient (SCID) mice. The proliferation of these cells in the mice model concluded the presence of an enriched population of leukemia-inducing stem cells expressing stem cell surface markers (Lapidot et al. 1994). Various subsequent studies described the heterogeneity of the stem cells present in AML, which led to the possibility of research in the case of various other solid tumors (Lapidot et al. 1994). The CSC populations from the clonogenic core of a cancer is responsible for its excessive growth and progression. These cells, capable of differentiation and self-renewal, give rise to the non-tumorigenic bulk of the tumor.

CSCs have shown a high degree of resistance to conventional cancer treatments such as chemotherapy and radiation and are considered a likely candidate for the initial occurrence and metastasis of cancer. The metastatic nature of CSCs allows solid tumors to migrate and colonize at a different site (Yang et al. 2020). Cancer

stem cells and normal adult stem cells share a number of regulatory pathways that control their normal functions and help in maintaining homeostasis. Mutation or dysregulation of these pathways in CSCs contributes to its remission and resistance to therapy. Various signaling pathways that are dysfunctional in CSCs include Notch, Wnt, Hedgehog, TGF β , AMPK, Autophagy, etc. As yet, the precise mechanism underlying these dysregulated signaling pathways in CSC function has not been elucidated in its entirety. This chapter presents an overview of the major signaling pathway of cancer stem cells and how they modulate various intrinsic and extrinsic factors involved in the maintenance of cancer stem cells. Moreover, the current status of therapeutic strategies targeting these pathways is also presented.

Signaling Pathways in Cancer Stem Cells

Developmental Signaling Pathway

Notch, Hedgehog (Hh), and Wnt are the major developmental signaling pathways that not only play a pivotal role in embryonic development and differentiation but are also involved in the regulation of stem cells. Interestingly, CSCs utilize these signaling pathways, and often the aberrant regulation of these signaling pathways has been observed in cancer stem cells.

The Notch signaling is highly conserved among metazoans and mediates cell fate decisions during development. In mammals, Notch signaling is initiated when canonical Notch ligands (Jag1, Jag2, Dll1, Dll3, and Dll4) stimulate the Notch receptors (Notch1–4) on the adjacent cells (Artavanis-Tsakonas et al. 1999). This interaction leads to the induction of proteolytic cleavage of the Notch receptor resulting in the endocytosis of Notch extracellular domain (NECD) into the ligand-expressing cells and translocation of Notch intracellular domain (NICD) to the nucleus, where it interacts with CSL (CBF-1, Su(H), Lag-1), leading to the transcription of Notch target genes (Gordon et al. 2007) (Fig. 1). Aberrant Notch signaling often leads to developmental defects and is associated with tumorigenesis (Ranganathan et al. 2011). Indeed, numerous investigations on Notch signaling in CSCs have found that it plays a critical role in controlling CSC self-renewal, growth, and metastasis.

Hedgehog signaling (Hh) is another major developmental signaling pathway involved in tissue patterning during embryonic development, Epithelial to Mesenchymal transition (EMT), repairs of normal tissue, etc. (Varjosalo and Taipale 2008). It is majorly composed of Hh ligands, transmembrane patched (PTCH) receptor, smoothed (SMO) protein, transduction molecules, and GLI, a transcription factor. Hedgehog signaling gets initiated when the Hh ligands – Sonic hedgehog (Shh), Desert hedgehog (DHH) or Indian hedgehog (IHH) interacts with the PTCH receptor, which results in the release of an inhibitory effect of PTCH on SMO (located in the cell membrane), leading to the activation and nuclear localization of Gli, where it activates the target genes (Fukushima et al. 2016) (Fig. 1). Dysregulated Hedgehog signaling is often observed in many cancers, and recent reports suggest its crucial role in regulating the properties of CSCs in various cancers such as glioma, multiple myeloma, CML,

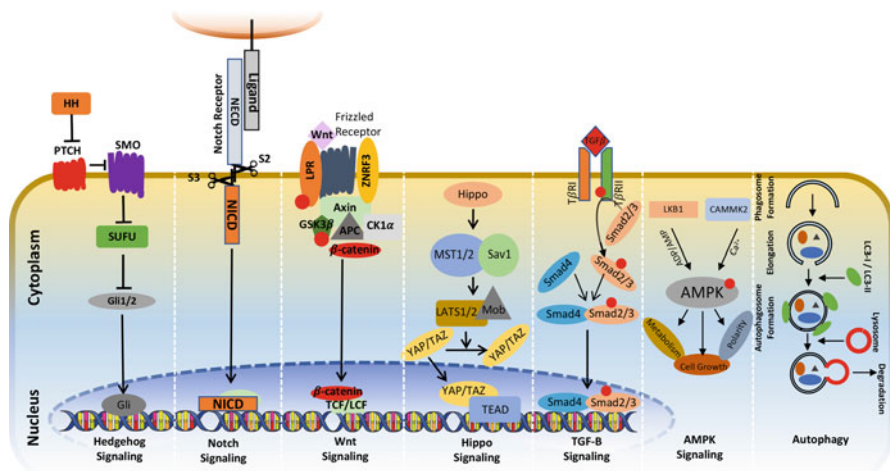


Fig. 1 Simplified view of Notch, Hedgehog, Wnt, TGF- β , HIPPO, AMPK, and Autophagy signaling pathway involved in cancer stem cells. The figure is widely discussed in the text

etc. In glioma and multiple myeloma cells, pharmacological or genetic suppression of SMO has found to decrease CSC proliferation and self-renewal (Zhao et al. 2009).

The Wnt signaling pathway is one more developmental pathway, which is highly complex and evolutionarily conserved among species. It comprises of 19 Wnt ligands and more than 15 receptors (MacDonald et al. 2009). In the absence of Wnt ligands, β -catenin forms a destruction complex with Axin, APC (adenomatous polyposis coli), and GSK-3 β (glycogen synthase kinase-3 β). The phosphorylation of β -catenin by GSK-3 β leads to the recruitment of ubiquitin E3 β -transducin repeat-containing protein (β -TrCP), resulting in the degradation of β -catenin by the proteasome, thus maintaining low cytoplasmic β -catenin level. When Wnt ligands released by cells bind to receptors/co-receptors (Frizzled/LRP5/LRP6) on neighboring cells, canonical Wnt signaling is initiated, causing Dsh to be recruited to the cell membrane. Dsh-induced phosphorylation prevents the formation of the Axin-GSK-3-APC complex, which prevents β -catenin degradation. Free β -catenin translocates to the nucleus, where it interacts with LEF and TCF, causing target genes to be transcribed and expressed (Fig. 1). High Wnt activity has often been associated with the CSCs functionality in several tumors. In colorectal cancer, high Wnt signaling upregulates the c-Myc and cyclin D, which leads to the CSC phenotype. Similarly, significant Wnt activation in CSCs was required for carcinogenesis in squamous cell carcinomas, and deletion of the β -catenin gene resulted in CSC depletion and tumor regression in mice (Malanchi et al. 2008).

TGF β Pathway

The transforming growth factor-beta (TGF β) signaling pathway is crucial in controlling various cellular behavior and driving developmental processes associated

with organism's growth and embryogenesis. These include cell proliferation, differentiation, apoptosis, homeostasis, and other cellular functions (Batlle and Massague 2019). The TGF β s are a superfamily of more than 30 multifunctional cytokines having conserved cysteine residues. They can be segregated into two subfamilies based on structural similarities and function: the Bone Morphogenetic Protein (BMP) subfamily and the TGF- β subfamily. The TGF β receptors (T β R I and T β R II) are serine-threonine kinases and their activation by TGF β ligands can either trigger a canonical SMAD-dependent signaling cascade or activate other cellular signaling pathways to induce a cellular response (Bellomo et al. 2016). A combination of Smad and non-Smad-mediated processes dictate the eventual consequence of TGF β activation (Fig. 1).

TGF β is known to have a dichotomous role in cancer where the context dictates the function of TGF β as a tumor suppressor or tumor promoter. TGF β signaling pathway is known to have tumor-suppressive activity due to its ability to regulate processes such as cell death and cell cycle arrest, and genes of this pathway are known to get mutated and inactivated in specific types of cancer (Pickup et al. 2013). On the other hand, TGF β is present at high levels in tumor environment and is known to facilitate tumor progression through its ability to inhibit immune cell development, promote cell dedifferentiation, and encourage vascular growth (Padua and Massague 2009). TGF β pathway aids the process of metastasis through its effects on the tumor microenvironment and the cells associated with the microenvironment such as cancer stem cells, tumor-associated fibroblasts, and immune cells (Bellomo et al. 2016).

Hippo Pathway

The Salvador–Warts–Hippo pathway was first identified in isolates from organ tissue cultures of *Drosophila melanogaster* showing characteristic abnormal size (Mo et al. 2014). Although it plays a central role in organ growth development and their homeostasis and the regulation of cell proliferation, differentiation, migration, and apoptosis (Mo et al. 2014), the upstream mechanism that leads to its activation is not completely understood. The mammalian Hippo pathway comprises of kinases mammalian Ste2-like kinases (MST1/2) and large tumor suppressor kinase 1/2 (LATS1/2) (Mo et al. 2014). Activation of the Hippo pathway mediates phosphorylation and inactivation of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), by the kinase LATS1/2. The phosphorylated YAP/TAZ protein is targeted for degradation by ubiquitin-proteasome-dependent pathway. Unphosphorylated and active YAP/TAZ would translocate to the nucleus, associate with transcription factors of the TEAD family, and activate genes involved in cell differentiation and proliferation (Mo et al. 2014).

Dysregulation of this pathway has been identified in a wide variety of human cancers. Mutations in genes coding for the central protein can lead to overexpression of YAP/TAZ-regulated proteins leading to rapid cell proliferation, excessive cell growth, and inhibition of apoptosis. Methylation events on genes coding for MST1/2 and LATS1/2 have been reported in cases of soft tissue sarcoma and breast cancer,

respectively (Pan 2010). Overexpression of the YAP/TAZ-induced genes is a common occurrence in cancer cell lines (Hao et al. 2014). YAP and TEAD are overexpressed in CSCs of medulloblastomas (Fernandez et al. 2009), and TAZ is known to be overexpressed and confer stem cell traits in CSCs of breast cancers (Cordenonsi et al. 2011).

AMPK Pathway

AMP-activated protein kinases (AMPK) are vital regulators of energy metabolism present in essentially all eukaryotes and appear to have evolved early during eukaryotic evolution (Lin and Hardie 2018). In mammals, AMPKs exist as heterotrimeric complexes consisting of catalytic ($\alpha 1$ and $\alpha 2$) and regulatory ($\beta 1$ and $\beta 2$) ($\gamma 1$, $\gamma 2$, and $\gamma 3$) subunits (Ross et al. 2016b). AMPKs are activated by elevated cellular ratios of AMP and ADP over ATP which occur when ATP production is compromised and ATP turnover is enhanced (Lin and Hardie 2018). An increase in AMP levels results in phosphorylation of the α subunit, at the Thr172 position, by the kinase LKB1, a well-known tumor suppressor (Ross et al. 2016a). This suggests the role of AMPK in cancer. AMPK can be activated via a noncanonical mechanism through Ca^{2+} /calmodulin-dependent kinase, CaMKK2. Upon activation, AMPK promotes ATP production by activating catabolic ATP-generating pathways and curbs cell growth and proliferation by inhibiting biosynthetic pathways. AMPK is also known to activate glycolysis, characteristic of cancer cells, by phosphorylation of the glycolytic enzyme phosphofructokinase isozymes (Marsin et al. 2000) (Fig. 1).

Studies on AMPK in cancer have provided supporting roles of AMPK as a tumor suppressor as well as a tumor promoter. When AMPK was knocked out in B and T cell lymphoma mouse models prior to tumor initiation, it resulted in accelerated tumor formation, confirming AMPK's role as a tumor suppressor. In T cell leukemia mouse model, knocking out of AMPK after the initiation of the disease resulted in less severe and amelioration of the disease, whereas knocking out AMPK prior to development of the disease led to early onset and severe disease (Kishton et al. 2016). Studies have shown high levels of AMPK activities in colorectal cancer (CRC) stem cells, and AMPK inhibition leads to cell death in CRC stem cells.

Autophagy

Autophagy is a highly conserved process whose main function is to maintain cellular homeostasis during stress conditions. It is associated with cell survival by degrading unnecessary cellular components and supplying recycled metabolites for cell growth. Cellular stresses, including amino acid deprivation, ER stress, and other signals, are known to promote autophagy by activating the AMPK signaling pathway and inhibiting the mTOR pathway (Egan et al. 2011). However, dysregulation of autophagy is implicated in several diseases, including neurodegenerative diseases and various cancers. In cancer, autophagy plays a paradoxical role having both tumor-suppressive and tumor-promoting functions in a context-specific manner.

During initial stages, autophagy plays a protective role against cancer development mainly by preventing DNA damage and maintaining genome integrity (Lorin et al. 2013). However, as the tumor develops, autophagy can be triggered as an adaptive response and contributes to the survival of cancer cells by meeting their increased demand for building blocks of macromolecules (Lorin et al. 2013) (Fig. 1).

Recently, the dependence of CSCs on autophagy has also been demonstrated in various cancers, including breast cancer, pancreatic cancer, and colorectal cancers (Garcia-Prat et al. 2016). In fact, autophagy is upregulated in CSCs, where it helps to maintain pluripotency and regulate their migration and metastasis. In breast cancer, autophagy-related gene ATG4 regulates breast CSC populations by promoting their self-renewal *in vitro* and tumor growth *in vivo*. Furthermore, autophagy can be induced in non-cancer cells of the tumor microenvironment to promote the growth of tumor cells. Autophagy also helps tumor cells to avoid immune detection and contributes to resistance to chemotherapy drugs. Therefore, autophagy seems as a promising target for cancer therapy. Although the dual roles of autophagy as a tumor suppressor and tumor promoter present a major challenge in targeting autophagy, recent studies suggest that by sensitizing CSCs to therapy through inhibition/activation of autophagy can be encouraging (Lim et al. 2021).

Properties of CSCs Regulated by Signaling Pathways

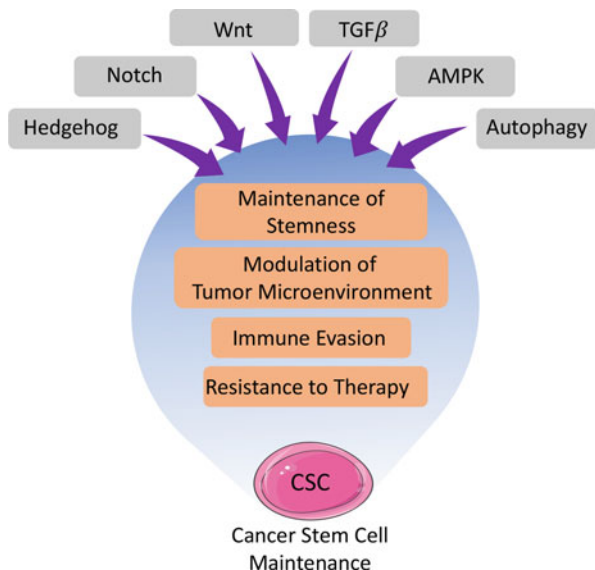
Stem Cell Maintenance

Cancer stem cells have the ability to self-renew as well as differentiate. This ability of CSCs is important in cancer initiation, development, and resistance to numerous anticancer therapies. Although the mechanism underlying this phenomenon is not fully understood, there are numerous evidences suggesting the involvement of the developmental signaling pathway and other signaling pathways in the maintenance of the CSCs (Fig. 2).

The expression of essential genes involved in CSC self-renewal and survival can be directly regulated by major developmental pathways including Notch, Hh, and Wnt signaling pathways. In this context, it was shown that Notch can directly regulate some of the stem cell markers such as CD44, Nestin, etc. in T-ALL and glioma (Shih and Holland 2006). Similarly, HH induces the expression of several stemness-related genes, such as Oct4, Bmi1, Sox2, etc., thereby maintaining stemness signature in various cancers. Wnt signaling has also been shown to directly regulate several stemness genes directly, such as Lgr5 (Fan et al. 2010).

In addition to these signaling pathways, several other pathways have been shown to maintain the self-renewal capacities of CSCs. TGF β has been demonstrated to play a critical role in the self-renewal/stemness of CSCs in a variety of malignancies via distinct mechanisms. For example, TGF β promotes self-renewal capacity in cancer stem cells in breast cancer and glioblastoma through an ILEI/LIFR signaling axis. In liver CSCs, upregulation of TGF β 1 induces the expression of CD133 (stemness

Fig. 2 Various signaling pathways modulate intrinsic and extrinsic factors that are involved in the maintenance of cancer stem cells (CSC). The figure is widely discussed in the text



marker), together with SMAD4. In a variety of human malignancies, stimulation of Hippo pathway results in the induction of CSC characteristics (Song et al. 2014).

Furthermore, SOX2 activation of YAP is critical for the maintenance of CSCs in osteosarcoma and glioblastoma. In turn, TAZ, a Hippo effector, promotes cancer stemness in head neck squamous cell carcinoma via transcriptional activation of SOX2. Together, there is ample amount of evidence that indicates the vital role of YAP/TAZ in the maintenance of CSCs and cancer progression. However, the upstream regulatory mechanisms of the YAP/TAZ and Hippo pathways in CSCs are unknown. AMPK is one of the key regulators of metabolic stress that plays a crucial role in energy and nutrient homeostasis. The involvement of AMPK in CSC metabolism has been extensively characterized in different malignancies. For example, in prostate CSCs, AMPK was shown to be necessary for maintaining glucose balance and thereby the stemness phenotype. Autophagy has recently been identified as a key trait that CSCs use to preserve their self-renewal capacity and survival. Autophagy inhibition has a deleterious impact on the expression of staminal markers and the ability of CSCs to self-renew. Several studies on the molecular mechanism suggested that autophagy acts through different signaling pathways to maintain CSCs, such as EGFR/Stat3 and TGFβ/Smad signaling pathway, FOXO3, FOXA2, and DNA damage pathway (Nazio et al. 2019).

Tumor Microenvironment

The tumor mass consists of a heterogeneous population of cancer cells and other cellular and noncellular components known as the tumor microenvironment (TME).

TME encompasses endothelial cells (ECs), immune cells, cancer-associated fibroblasts (CAFs), extracellular matrix (ECM), and various cytokines and chemokines. There is continuous signaling cross talk between cancer cells and the surrounding TME, which impacts tumor growth and development. The endothelial cells provide nutritional support for tumor growth and development and also protect tumor cell from the immune system (Hanahan and Weinberg 2011), whereas the immune cells, such as macrophages, can suppress antitumor responses. In addition, fibroblasts allow the migration of cancer cells for metastasis and promote angiogenesis.

In recent years, several studies have shown that the CSCs are strongly associated with their microenvironment or “niche” which plays an important role in the regulation of the self-renewal and differentiation properties of CSCs. In colorectal cancer, fibroblasts in the niche secrete HGF (hepatocyte growth factor) that maintain the “stemness” of CSC by activating the Wnt pathway. Furthermore, TME influences other processes such as metastasis, invasion, angiogenesis, extracellular matrix remodeling, immune evasion, and drug resistance through the production of various growth factors, chemokines, and cytokines. The relationship between the CSC and TME seems to be bidirectional, that is, the CSCs can modify their microenvironment, and in turn, the cellular fate of cancer cells can be altered by the TME. For example, in glioblastoma, CSCs secrete VEGF that promotes angiogenesis, and the endothelial cells induce Notch signaling in CSCs by secreting nitric oxide (Charles et al. 2010).

Hypoxia is one of the characteristic features of TME. The rapid proliferation of cancer cells results in an imbalance between increased demand and decreased oxygen supply leading to the reduced oxygen level in tumors. The hypoxic environment is responsible for genetic instability and the upregulation of various genes including hypoxia-inducible factor 1 alpha (HIF1- α) associated with angiogenesis and metastasis. Hypoxia also triggers CSC plasticity, that is, switching non-CSCs to CSCs and vice versa, which contributes to tumor heterogeneity and drug resistance (Das et al. 2020). Since TME plays an important role in every aspect of CSCs progression, targeting TME presents a promising strategy for treatment of cancer. The development of small-molecule inhibitors that target the specific components of TME is an emerging area for cancer therapy (Zhong et al. 2020) (Fig. 2).

Immune Evasion

The immune system of our body can recognize and eliminate tumor cells. However, most of the tumors induce immune tolerance through several mechanisms and can thus escape immune surveillance. The immune cells such as T cells, dendritic cells (DCs), macrophages, and natural killer (NK) cells are infiltrated into tumors and are correlated with tumor development, treatment outcome, and recurrence. The tumor’s fate is determined by genetic factors and by the functional properties of the immune cells infiltrating into the tumor (Muller et al. 2020). Several studies have suggested that the crosstalk between CSCs and the immune system is bidirectional. CSCs can modulate various immune cells’ composition and properties and shape the TME into

an immunosuppressive and pro-tumorigenic state. In turn, infiltrating immune cells release growth factors and cytokines that promote self-renewal, tumorigenicity, and metastasis of CSCs (Muller et al. 2020). Thus, inhibition of immune signaling can be an effective way to block the self-renewal property of CSCs and improve therapeutic outcomes.

The various mechanisms employed by CSCs to exhibit immunosuppressive effects are ineffective antigen processing and presentation by downregulation of MHC molecules, modulation of NK-cell activity, inhibition of cytolytic molecules, secretion of immunomodulatory factors, such as TGF β , IL-6, and CCL20 (Tsuchiya and Shiota 2021). CSCs can drive tumor-associated macrophages (TAMs) toward M2 polarization to maintain immunosuppressive milieu at the tumor site by activating the signal transducer and activator of transcription 3 (STAT3) and nuclear factor- κ B (NF- κ B) pathways and various cytokines. Regulatory T cells (Tregs), a subtype of CD4⁺ T cells that inhibit effector T cells and other immune cells, show a positive association with CSCs to promote immunosuppressive environment. Furthermore, CSCs upregulate programmed death-ligand 1 (PD-L1), an immune checkpoint protein, which interacts with PD-1 on immune cells and suppresses their effector functions. In colorectal CSC, altered PD-L1 expression promotes CSC stemness and expansion by activating HMGA1-dependent signaling pathways or PI3K/Akt pathway. Immune checkpoint inhibitors against the checkpoint molecules (PD-1, CTLA-4) to derepress the immune response have emerged as a promising tool in cancer therapeutics (Ferguson et al. 2021) (Fig. 2).

Resistance to Therapy

Resistance to therapy is one of the greatest ongoing challenges to cancer treatment. There are two types of resistance: intrinsic resistance, which is an inherent ability to resist a spectrum of drugs, and acquired resistance, which develops in response to treatment. Several studies have shown that CSCs are the primary cause of therapy failure due to their intrinsic resistance to chemotherapy and radiotherapy (Steinbichler et al. 2018). The therapeutic resistance of CSCs is due to its many features such as evasion of death, low ROS level, tumor microenvironment, quiescence, efficient drug efflux and detoxification ability, EMT, upregulated CSCs survival and stemness signaling pathways, increased autophagy, and enhanced DNA damage response and repair mechanisms. CSCs share resemblance with stem cells and employ similar mechanisms to evade death by senescence and apoptosis. CSCs prevent apoptosis by increased levels of anti-apoptotic proteins such as c-FLIP, Bcl-2, and Bcl-x via the stemness signaling pathways Notch and Hedgehog. Due to its quiescent state, the CSCs have a longer time to repair DNA damage induced by therapy. CSCs have also shown to have enhanced DNA repair activity (Carruthers et al. 2018). CSCs express high numbers of ATP-binding cassette (ABC) transporters on their cell's surface that facilitate to pump out toxic chemicals from the cells.

CSCs exhibit increased autophagy which shows improved survival and therapy resistance. Inhibition of autophagy in breast cancer cell lines resulted in tamoxifen-

resistant cells to tamoxifen-induced killing (Samaddar et al. 2008). Circulating tumor cells in metastatic patients co-expressed EMT and CSC markers. There is evidence of the relationship between EMT phenotype and gefitinib resistance through the Notch-1 signaling pathway. CSCs exist within a hypoxic tumor micro-environment that activates aldehyde dehydrogenase (ALDH) activity, an enzyme that is involved in the oxidation of intracellular aldehydes as well as detoxifying reactive oxygen species (ROS) and alkylating agents such as paclitaxel (Fig. 2).

Clinical Targeting of Signaling Pathways in CSC

Encouraged by the results from preclinical studies indicating that therapeutic resistance could be overcome by targeting different cancer stem cell signaling pathways, many small molecule inhibitors of these pathways have been investigated in clinical trials. These clinical studies include molecules designed for the specific pathway or repurposed drugs that are investigated either as mono-therapeutics or in combination with classical anticancer drugs. An overview of the drugs targeting various signaling pathways related to cancer stem cells evaluated for their clinical suitability and efficacy are summarized in Table 1.

Several drugs designed to target different regulators of Notch signaling has received wide attention. All notch inhibitors investigated so far as mono-therapeutics have been found to be well tolerated and safe with acceptable toxicity (Yang et al. 2020). Due to their ability to inhibit pan-Notch activity, gamma-secretase inhibitors entered clinical trials very early ahead of other molecules. Although they are low-cost compounds and can be orally administered with encouraging bio-distribution, the gastrointestinal tract toxicity has hampered its progress in advancing to Phase III trials and approval for clinical use (Andersson and Lendahl 2014). Antibody targeting notch 1, 2, and 3 as well as the ligand DLL4 have been investigated either as standalone chemotherapeutic agents or as part of a combined regimen comprising chemotherapeutic agents for the treatment of many aggressive and disseminated malignancies (Takebe et al. 2014). Phase I clinical trials evaluating safety and feasibility of combining notch inhibitors with different standard of care chemotherapeutics have not reported any undesirable toxicities, except cardiotoxicity when combined with carboplatin. However, the lack of encouraging clinical benefits has precluded the further progress of clinical studies with these drugs and combinations (Strosberg et al. 2012). A lack of correlation between induced activation of notch1 and response to combinations of chemotherapeutic agents observed in T-cell acute lymphoblastic leukemia (Van Vlierberghe and Ferrando 2012) also suggests the need for a critical evaluation on the role of Notch signaling status and clinical response to therapy. Therefore, designing or identifying (as a repurposing strategy) novel drugs with encouraging efficacy and acceptable toxicity needs to be developed in future that convincingly establishes the clinical benefit of targeting notch signaling in the clinical management of certain human malignancies.

Table 1 Overview of the clinical trials with drugs targeting developmental signaling pathways

Pathway	Neoplasms included	Targets	Mono therapy	Combined therapies	Trial stage	Outcome
Notch	Solid tumors; leukemia	Notch ligand DLL3, DLL4; γ secretase; Pan notch signaling	+	+	I–II	Well tolerated with acceptable toxicity and moderate efficacy
Wnt	Solid tumors; leukemia	Porcupine; β -catenin/CBP; frizzled receptor; Fzd8-Fc fusion protein	+	+	I–II	Tolerated with minimal toxicity, but poor efficacy
Hedgehog	Solid tumors; leukemia	Smoothened	+	+	I–II	Tolerated, but not with encouraging clinical benefit
TGF β	Solid tumors (primary, metastatic, and recurrent)	Ligands (TGF β 1/2/3) and receptors (T β RI/RII)	+	+	I–II	Tolerated with minimal toxicity and modest efficacy
AMPK	Solid tumors; leukemia	PI3K and Bcl-2	+	+	I–II	Moderate toxicity with limited efficacy
Autophagy	Solid tumors; leukemia	Lysosome, autophagy flux (early and late phases)	+	+	I–II	Cardio and retinal toxicity. Efficacy not available
Hippo	Solid tumors; leukemia	YAP and TAZ	+	+	I–II	Toxicity as muscle loss. Efficacy not known

Porcupine inhibitors and agents targeting β -catenine/CBP and frizzled receptors are among the widely investigated drugs that target Wnt signaling. Clinical trials examining the safety, tolerance, and toxicity of these drugs in the management of chronic myeloid leukemia and malignancies in different anatomical locations have been encouraging with good patient compliance and acceptable toxicity. However, systematic Phase III clinical trials to evaluate the efficacy of these drugs are yet to be completed or initiated. Vismodegib, sonidegib, and glasdegib investigated so far for their clinical feasibility target the smooth head receptors (Smo inhibitors) in the

hedgehog signaling pathway. Unfortunately, resistance acquired by tumors against Smo inhibitors and undesirable toxicities associated with these drugs have severely compromised their evaluation for efficacy (Du et al. 2019). Further, limited benefit has been found in the clinical trials that investigated the combinations of these inhibitors with standard of care anticancer chemotherapeutic drugs in the treatment of malignant glioma (Xie et al. 2019). Preliminary findings with arsenic trioxide that inhibits Gli transcription factor and hedgehog signaling in the treatment of certain solid tumors and hematological malignancies have been encouraging (Xie et al. 2019).

Extensive preclinical studies showing the potential of targeting various components of TGF β signaling for improving the efficacy of cancer therapy have prompted the evaluation of clinical utility of drugs targeting TGF β signaling. Phase I/II clinical trials have been initiated using TGF β targeting drugs either alone or in combination with other drugs, including immunotherapeutic agents in many primary, metastatic, and recurrent tumors (Ciardiello et al. 2020). These drugs and combinations target different forms of TGF β ligands (TGF β 1/2/3) and receptors (T β RI/RII). The bifunctional antibody, Bintrafusap, that simultaneously targets T β RII and PD-L1 has also been extensively investigated in many malignant tumors (Ciardiello et al. 2020). A cancer vaccine, comprising radiation sterilized non-small cell lung carcinoma cells (NSCLC) transfected with antisense TGF β gene, is also well-tolerated without adverse effects in patients with NSCLC tumors and shows good response.

Chloroquine and hydroxychloroquine originally approved for the treatment of malaria have been investigated for their usefulness in the treatment of breast cancer and leukemia while pantoprazole in combination with doxorubicin has been evaluated in prostate cancer. Both these groups of drugs have been recently established to target autophagy. Although results of their efficacy on the tumors are still awaited, Phase I/II clinical trials have shown cardio and retinal toxicity with the chloroquine compounds, while dose-limiting toxicity in the form of fatigue, neutropenia, and leukopenia were noted with pantoprazole (Al-Bari 2015). Verteporfin, a photosensitizer approved for photodynamic therapy that inhibits the early events in autophagy, has also been evaluated either alone or in combination with chemotherapy (gemcitabine) or radiotherapy for its clinical efficacy in Phase I/II trials in pancreatic ductal carcinoma (Huggett et al. 2014). Absence of any adverse effects when administered before or after chemo or radiotherapy is encouraging and has prompted further efficacy trials. Currently, a Phase I/II trial is ongoing with Visudyne (a liposomal preparation of verteporfin) for the treatment of primary and recurrent EGFR mutated glioblastoma ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04590664) Identifier: NCT04590664).

Many of the tumors related as well as systemic role of AMPK in many cancers established in preclinical studies have prompted the initiation of clinical trials examining the impact of combining AMPK inhibitors (like metformin, phenformin, and salicylates) with radiotherapy and chemotherapy to improve the efficacy, besides examining them as monotherapeutic agents (Steinberg and Carling 2019). Results of these clinical trials indicate a limited efficacy of these AMPK inhibitors and suggest more extensive and long-term studies to establish their long-term utility in the management of cancer. Among the many potential targets in the hippo signaling

pathway, the repurposed drug Atorvastatin that targets TAZ has been investigated for its usefulness in the therapy of breast cancer. Drug-related toxicity in the form of muscle tissue loss has been reported, while results on its efficacy are awaited (Prado et al. 2011).

Conclusion

Cancer stem cells are a subset of cells in the tumor micro-environment that have the potential to initiate, facilitate progression, and metastasis which purportedly confer resistance against therapy, besides contributing to the relapse. Widely used standard of care chemo and radiotherapy efficiently kill only the active proliferating tumor cells, while the quiescent and resistant CSCs escape therapy, thereby leading to relapse or metastasis. CSCs are a heterogeneous cell population; however, they have certain common characteristics such as quiescence, resistance, evade death, pro-survival ability, and stemness. A complex web of signaling pathways are responsible for the distinctive characteristics of the CSCs. CSCs also exist in special niches in the tumor that promote survival and are not that well understood. Understanding these signaling pathways and tumor microenvironment and their role on CSCs is important for developing novel therapies against CSCs. Few inhibitors that target signaling pathways in CSCs have shown promising results in *in vitro* studies and preclinical cancer models; however, only some have reached Phase 1 and 2 clinical trials. This could be because *in vitro* and preclinical models do not mimic the biological complexity of the tumor and its microenvironment as seen in the clinical scenario. These signaling pathways are also required for the several cellular processes in normal tissues, and targeting these pathways could lead to undesirable toxicity. Therefore, there is a need to understand CSCs and tumor microenvironment in more depth to develop selective inhibitors specific to the CSCs and the tumor microenvironment. Efforts are required to develop synergistic drug combinations of CSCs specific inhibitors along with the conventional therapies to prevent the development of resistance and facilitate the use of lower dosages to improve the therapeutic index.

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