Chapter

Self-Renewal Pathways in Acute Myeloid Leukemia Stem Cells

Jonason Yang, Nunki Hassan, Sheng Xiang Franklin Chen, Jayvee Datuin and Jenny Y. Wang

Abstract

Acute myeloid leukemia (AML) is a difficult-to-treat blood cancer. A major challenge in treating patients with AML is relapse, which is caused by the persistence of leukemia stem cells (LSCs). Self-renewal is a defining property of LSCs and its deregulation is crucial for re-initiating a new leukemia after chemotherapy. Emerging therapeutic agents inhibiting aberrant self-renewal pathways, such as anti-RSPO3 monoclonal antibody discovered in our recent study, present significant clinical potential that may extend beyond the scope of leukemogenesis. In this chapter, we provide an overview of normal and malignant hematopoietic stem cells, discuss current treatments and limitations, and review key self-renewal pathways and potential therapeutic opportunities in AML.

Keywords: acute myeloid leukemia, leukemia stem cells, self-renewal, signaling, WNT, β-Catenin, G protein-coupled receptor, GPR84, LGR4, RSPO3, targeted therapy

1. Introduction

AML is a heterogenous clonal disorder characterized by blocked differentiation and increased proliferation of hematopoietic progenitors. The heterogeneity of AML can be attributed to diverse driver mutations that may be present in combination with multiple cells of origin and epigenetic abnormalities [1–3]. The main treatment for AML is chemotherapy, which kills rapidly dividing leukemic blasts but is ineffective against quiescent, self-renewing LSCs leading to relapse and poor clinical outcomes. The discovery of LSCs not only as the origin but also the culprit of therapeutic resistance in AML is a milestone in our understanding of malignancy and thus targeting LSCs is a critical and challenging step in developing anticancer therapy [4]. Recent evidence has shown that aberrant activation of self-renewal pathways, such as WNT/β-catenin and RSPO3-LGR4 pathways, is essential for the initiation and development of LSCs [5, 6], unveiling a potential target for curative therapies in AML.

2. Normal and malignant hematopoietic stem cells

2.1 Hematopoietic stem cells

Normal hematopoiesis is a hierarchically organized process where hematopoietic stem cells (HSCs) can self-renew to produce new copies of themselves via symmetric

division or differentiate into lineage-committed progenitors via asymmetric division [7], which ultimately give rise to all blood lineage cells (**Figure 1**). A tight balance between self-renewal and differentiation is critical for sustaining the functional integrity of hematopoiesis, which prevents HSC exhaustion or hematologic malignancies such as leukemia. HSCs preferentially reside in a hypoxic microenvironment within the bone marrow in which they are maintained in a quiescent state [8]. Quiescence is important to preserve the genetic integrity of HSCs during adult homeostasis as frequent DNA replication may incur oncogenic mutations. Therefore, dysregulation of key HSC properties is essential for leukemia initiation as they enable the accumulation of genetic lesions and promote malignant transformation.

2.2 Leukemia stem cells

The discovery of the first cancer stem cell in AML two decades ago has led to a paradigm shift in our understanding of cancer cell biology and the way cancer can be treated and cured [9]. Cancer stem cells have been subsequently identified in a variety of tumors. Similar to normal HSCs, LSCs are a subpopulation of leukemic cells, which reside at the apex of a malignant hierarchy and possess the ability to self-renew and to differentiate into non-LSC bulk blasts [10]. LSCs with unlimited self-renewal capacity and chemoresistance are responsible for disease initiation and progression and are believed to be the root cause of cancer relapse. It was initially hypothesized that only HSCs could undergo malignant transformation into LSCs due to their inherent ability to self-renew. However, the study of acute promyeloid leukemia (APML), a subset of AML, showed that committed progenitors might be capable of developing into LSCs. The APML-associated fusion gene PML/retinoic acid receptor-α (RARA), resulting from the t(15, 17) balanced reciprocal translocation, was present in CD34-CD38+ cell population but not in CD34+CD38- cell population [11]. As APML only represents a unique subset of AML, further

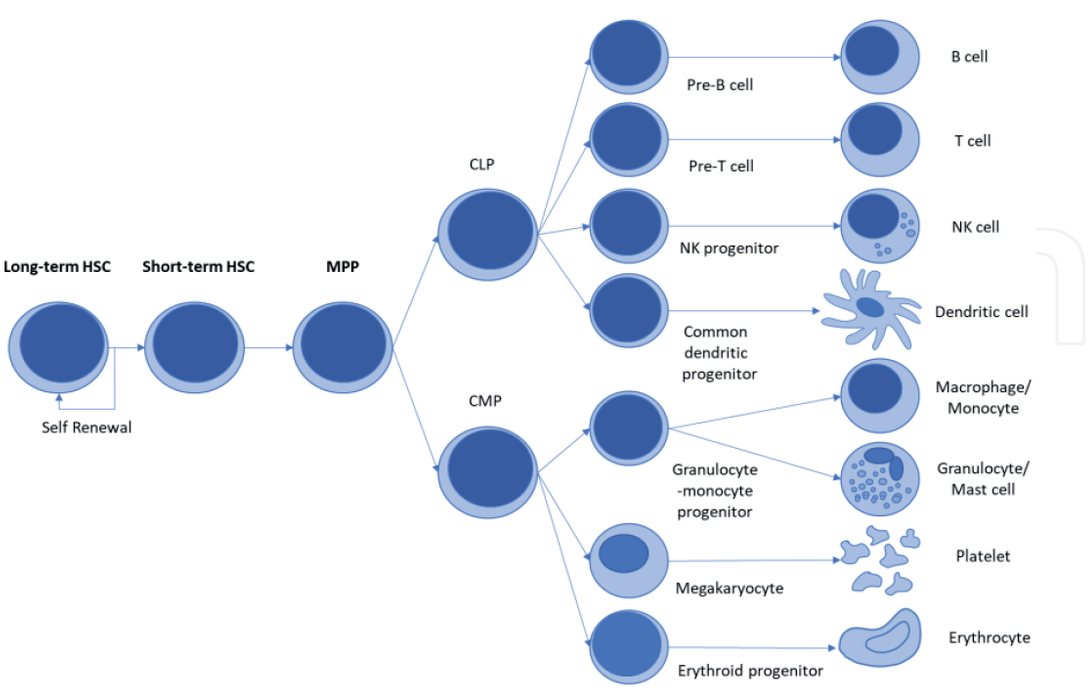

Figure 1.
Hierarchical organization of normal hematopoiesis. HSCs are categorized as long-term HSCs, which are highly self-renewing cells that last a lifespan, and short-term HSCs that self-renew for a limited period and differentiate into multipotent progenitor (MPP). MPP differentiates further into lineage-restricted progenitors including common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) from which all mature blood cells arise. NK, natural killer.

studies looked at mixed-lineage leukemia (MLL) fusion proteins and isolated cell populations of committed progenitors with MLL fusion mutations to examine their leukemogenesis potential. By injecting sublethally irradiated mice with the aforementioned cell population, leukemogenesis was observed and thus the ectopic renewal of genes in committed progenitors associated with self-renewal is a definite possibility [12, 13]. The cancer stem cell model implies that eradication of LSCs is crucial for developing relapse-free therapies and to achieve a long-term remission in AML. However, eradication of LSCs remains a hefty challenge, whose difficulty lies in the therapeutic targeting of key oncogenic pathways driving LSC formation and maintenance without affecting normal adult HSCs and hematopoiesis.

3. Cause of chemotherapy failure: leukemia stem cells

Chemotherapy remains the first line treatment for AML, which has been relatively unchanged since its inception more than four decades ago. The chemotherapy drugs often used for AML treatment are a combination of cytarabine and an anthracycline drug such as daunorubicin. Insufficient inhibition of quiescent LSCs may be the culprit behind the failure of chemotherapy for the treatment of AML. Similar to their normal HSC counterpart, most LSCs remain quiescent or in the G0 phase of the cell cycle [14, 15]. This proves challenging as chemotherapy only interferes with DNA replication via DNA polymerase inhibition (e.g. cytarabine) or with DNA restructuring via topoisomerase II inhibition (e.g. daunorubicin) to induce apoptotic cell death in actively replicating cells [16, 17]. It is noted that LSCs can retain chemoresistant adaptations present in HSCs such as expression of P-glycoprotein, an efflux pump that may export chemotherapeutic agents and is associated with multidrug resistance and poor disease outcomes [18]. Consequently, most chemotherapy-induced cell deaths occur within leukemic blasts rather than in LSCs. Thus, the residual disease within patients is often characterized by the subpopulation of LSCs which are often quiescent and resistant to conventional chemotherapy leading to relapse (**Figure 2A**). This highlights the need for stem cell-targeted therapies so that the subpopulation of LSCs can be eliminated allowing for long-term remission (**Figure 2B**).

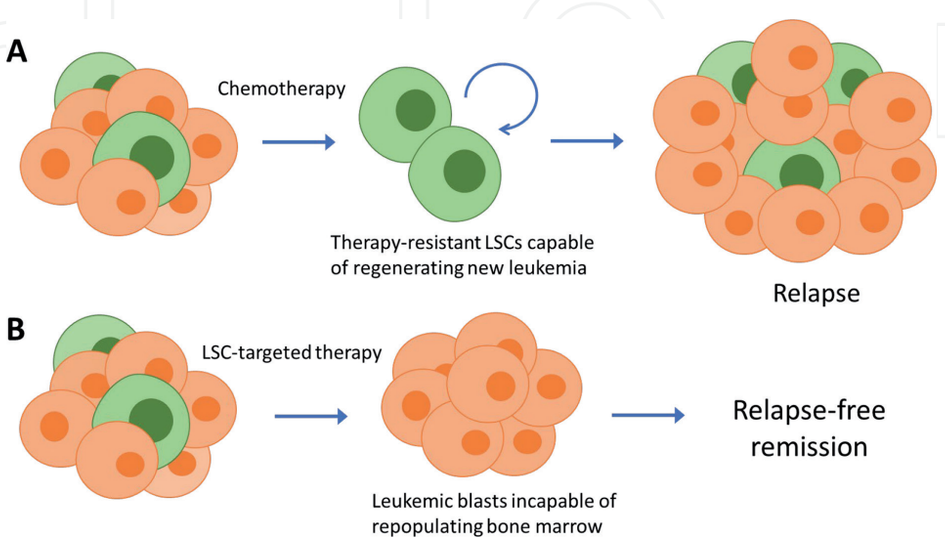

Figure 2.
Conventional chemotherapy versus targeted therapies for AML treatment. (A) Chemotherapy is unable to eradicate LSCs, leading to relapse. (B) Targeted therapy specifically kills LSCs, resulting in relapse-free remission.

4. Self-renewal signaling in leukemia stem cells

Self-renewal is a key property of LSCs and its deregulation is responsible for leukemia initiation and progression, which could be targeted for LSC eradication. Several pathways controlling LSC self-renewal have been identified in AML, such as WNT/β-catenin signaling and G protein-coupled receptors including GPR84 and LGR4 [5, 6, 19, 20].

4.1 WNT/β-catenin signaling pathway

β-catenin was first associated with colon cancer almost thirty years ago via its interaction with adenomatous polyposis coli (APC) [21, 22]. It later became evident that the canonical WNT/β-catenin pathway is dysregulated in various cancers, including AML [23, 24]. We and others have demonstrated that aberrant activation of WNT/β-catenin signaling contributes to the transformation of normal HSCs into LSCs [5, 25]. Our studies show that WNT/β-catenin signaling pathway is required for self-renewal of LSCs derived from either normal HSCs or lineage-committed progenitors in AML [5].

The WNT/β-catenin pathway was initially explored in normal HSCs (**Figure 3**). Constitutive activation of β-catenin results in an increase in the number of HSCs *in vitro* [26]. However, *in vivo* studies challenge the *in vitro* observation, where the conditional deletion of β-catenin in adult HSCs does not cause the self-renewal defect in mice [27].

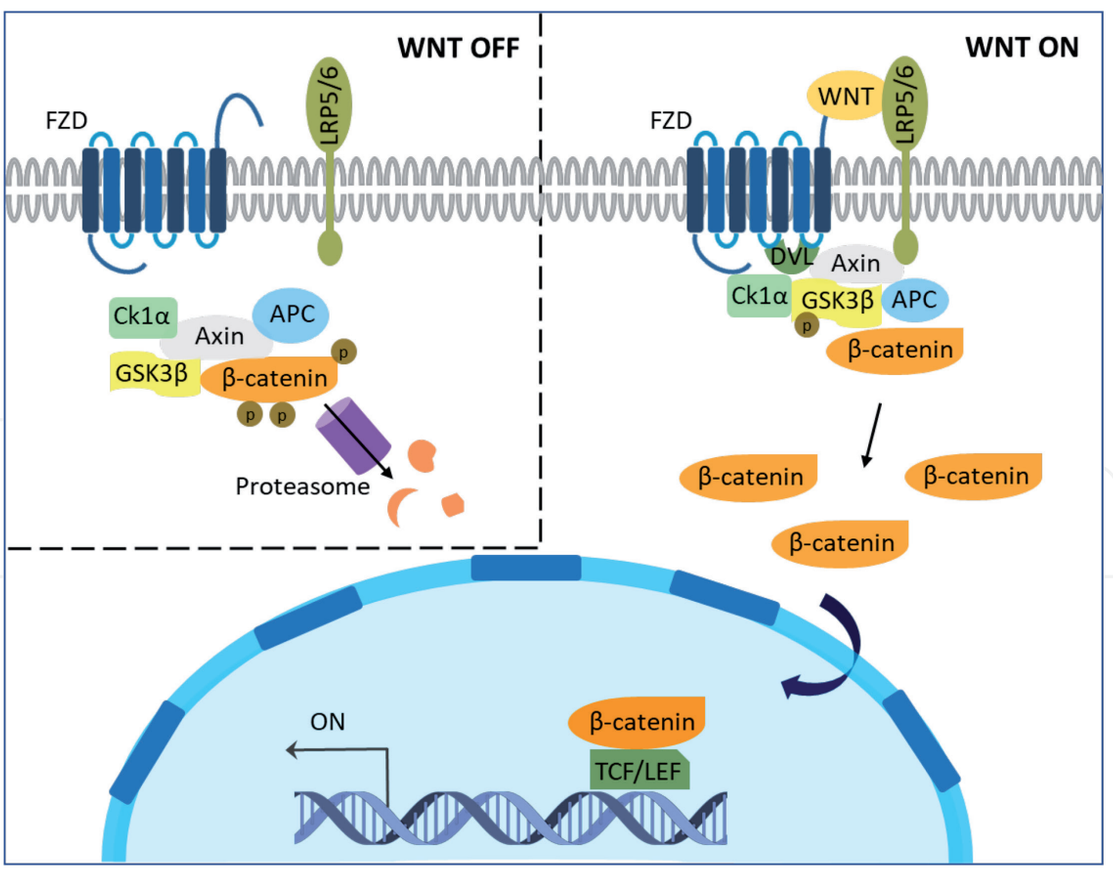

Figure 3.
Schematic diagram of the canonical WNT/β-catenin signaling pathway. In the OFF state, defined by the absence of a WNT ligand, β-catenin is repressed via a destruction complex composed of APC, scaffold protein Axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) that phosphorylates β-catenin leading to its proteasomal degradation [28–30]. In the ON state, a WNT ligand binds to the receptor complex consisting of lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and frizzled receptors (FZD), which recruits the destruction complex, leading to inhibition of β-catenin phosphorylation and therefore ensuring nuclear translocation and the subsequent accumulation of active β-catenin [31].

On the other hand, several lines of evidence including our own have demonstrated the importance of WNT/β-catenin pathway in regulation of LSC self-renewal in AML [5, 25]. Our studies have shown that deletion of β-catenin impairs LSC function and blocks AML progression in mice [5]. β-catenin can promote leukemogenesis through cooperation with homeobox (HOX) oncoproteins in HSCs [5]. Conversely, lack of endogenous β-catenin in lineage-committed progenitors limits the ability of HOX genes to efficiently transform the progenitor cells [5]. Despite the recognized importance, treatments directly targeting WNT/β-catenin pathway have been elusive, mainly due to our limited understanding of precise mechanisms of the pathway and lack of key druggable molecules involved in driving constitutive activation of the pathway.

Of note, while aberrant activation of β-catenin frequently occurs in human LSCs [25, 32] and is associated with poor patient outcomes in AML [33], increased expression of WNT proteins has not been observed. This suggests that aberrant activation of WNT/β-catenin signaling in AML may require other developmental signaling molecules that function as agonists.

4.2 G protein-coupled receptors serving as modulators of WNT/β-catenin signaling

G protein-coupled receptors (GPCRs) are a large superfamily of cell surface signaling proteins that bind extracellular ligands to transduce signals into cells via heterotrimeric G proteins [20]. GPCRs can act as modulators of WNT/β-catenin signaling and have a critical role in embryonic development and stem cell maintenance [20, 34, 35]. Dysregulation of GPCRs have detrimental consequences including malignant transformation and have emerged as crucial players in promoting tumor growth and metastasis [6, 19, 20].

The mechanism of action of GPCRs when stimulated by an appropriate ligand (or agonist) is understood in conjunction with its interaction with the G protein heterotrimer, which contains α, β and γ subunits. Ligands binding to GPCRs stimulate conformational changes within the receptor causing the Gα subunit to exchange bound guanosine diphosphate (GDP) for guanosine-5′-triphosphate (GTP). This step is key to the activation of the G protein as it causes the dissociation between the α subunit and the βγ subunit [36]. Each of the G protein subunits is responsible for several downstream effects. The Gα subunits have been classified into four families: $G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ each with a distinct function. Typically, $G\alpha_s$ and $G\alpha_i$ moderate cyclic AMP (cAMP) levels by stimulating or inhibiting adenylyl cyclase respectively [37]. $G\alpha_{13}$ typically activates the Rho family of GTPases and $G\alpha_q$ stimulates phospholipase Cβ (PLCβ), leading to activation of intracellular Ca^{2+} [20]. The βγ dimer subunits activate downstream signaling partners such as Src, phospholipase C, adenylyl cyclase, phosphodiesterases and ion channels [36]. Aberrant activation of GPCRs has a profound effect on many cellular processes and may ultimately lead to malignant progression.

Although therapeutic targeting of GPCR signaling pathways in non-cancer disease is not a new phenomenon, our limited understanding of the role played by these receptors in tumorigenesis has hindered the development of therapeutic approaches for cancer treatment.

4.2.1 G protein-coupled receptor 84

G protein-coupled receptor 84 (GPR84) is often described as a pro-inflammatory receptor. We have documented a crucial role for GPR84 in sustaining LSC self-renewal through positive regulation of β-catenin signaling in established AML [19]. Overexpression of GPR84 augments activation of β-catenin and its transcriptional co-factors TCF7L2 and c-FOS, and positively modulates a subset of genes associated

with WNT activation and *in vivo* dissemination as well as oncogenic potential of AML cells. On the other hand, GPR84 depletion impairs LSC function and inhibits the development of an aggressive and drug-resistant subtype of AML. The GPR84-deficient phenotype is dependent on β-catenin status as the restoration of β-catenin activation is capable of rescuing the functional defeat [19]. In addition, levels of GPR84 expression are significantly upregulated in human and mouse AML LSCs compared with normal HSCs, thus providing a therapeutic window to selectively target LSCs while sparing normal HSCs [19, 38]. These observations demonstrate a strong rationale for inhibiting GPCR/β-catenin signaling as a novel therapeutic strategy to target drug-resistant malignant stem cells in cancer.

GPR84 is also implicated in pro-inflammatory and fibrotic processes due to its action in macrophages, fibroblasts/myofibroblasts, and epithelial cells [39, 40]. Whilst the precise mechanism of action is unclear, GPR84 antagonist GLPG1205 has been developed to treat idiopathic pulmonary fibrosis patients in a phase II clinical trial (NCT03725852). However, the efficacy of GLPG1205 in AML is yet to be investigated.

4.2.2 Leucine-rich repeat-containing G protein-coupled receptor 4

Leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) is another GPCR that we have recently reported to act as a positive modulator of WNT/β-catenin signaling in AML initiation and progression [6]. The cell of origin determines the dependence of LSCs on LGR4 signaling. LSCs derived from HSCs depend primarily on LGR4 signaling for self-renewal and the establishment of an aggressive phenotype in mice; conversely, LSCs derived from lineage-committed progenitors only reveal a partial dependency on LGR4 signaling [6]. LSCs derived from different cellular origins may utilize distinct endogenous signaling mechanisms driving tumorigenesis, where LGR4 in HSC-derived LSCs relays signals via coupling to $G\alpha_s$ to upregulate WNT/self-renewal target genes but LGR4 in progenitor-derived LSCs signals through $G\alpha_q$ to potentiate WNT/β-catenin activation [6, 41]. The origin-dependent difference in pathway requirements contributes to the heterogeneity of an LSC pool within a tumor.

The mechanism underlying the enhanced aggressive leukemia phenotype associated with elevated LGR4 may involve functional cooperation between LGR4 and HOXA9 in LSCs [6]. High level of HOXA9 expression is a characteristic feature of AML, including cases with MLL rearrangements, and is associated with poor patient outcome [42]. Enforced expression of HOXA9/MEIS1 transforms normal HSC-enriched cells where β-catenin is often active, but cannot fully transform committed progenitors that inherently lack β-catenin activity and self-renewal ability [5]. This indicates an indispensable requirement for β-catenin activation in HOXA9-mediated transformation. Consistent with the role of LGR4 as an essential upstream effector of β-catenin signaling, we uncover that LGR4 cooperates with HOXA9/MEIS1 in HSC-enriched cells contributing to a highly tumorigenic phenotype characteristic of MLL-rearranged AML in mice. Thus, functional cooperation between LGR4 activation and a HOXA9 gene expression program drives LSC self-renewal and leukemogenesis in AML [6].

4.2.3 Therapeutic targeting of LGR4-RSPO3 signaling

The LGR family members (e.g. LGR4) have been identified as receptors for R-spondin (RSPO) proteins [43]. RSPOs function as WNT agonists [44] that synergize with low levels of WNT to potentiate β-catenin activation [45, 46]. An important feature of RSPOs is their coexpression with and dependence on WNT ligands during mouse development where RSPO expression is reduced by the double knockout of WNT1 and WNT3 [47], and the RSPO activity is inhibited by depletion of WNT3

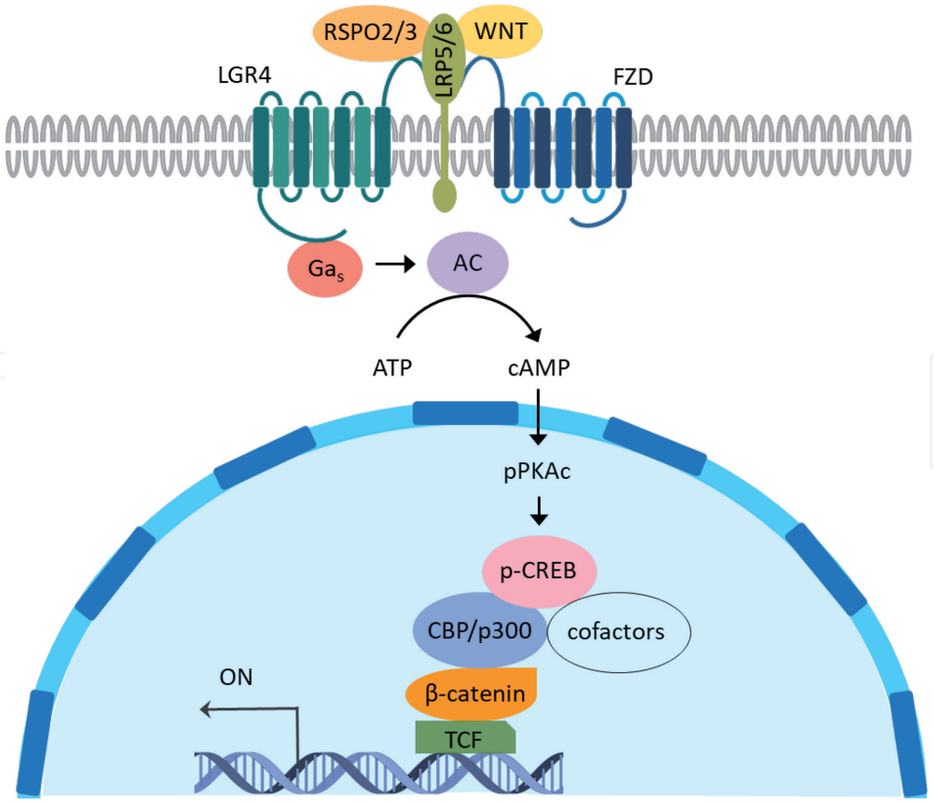

Figure 4.
Schematic diagram describing RSPO2/RSPO3-mediated augmentation of the canonical WNT/β-catenin signaling through an LGR4-dependent mechanism in HOXA9-dependent AML [6].

[46, 48] or WNT antagonists (i.e. DKK1) [49]. Although all four RSPO proteins (RSPO1–4) augment WNT/β-catenin signaling, RSPO2 and RSPO3 appear more effective than RSPO1, and RSPO4 is relatively inactive [50]. RSPO3 is prominently expressed in hematopoietic organs [51]. Our study has demonstrated that RSPO2 and RSPO3 can serve as stem cell growth factors to block differentiation and promote proliferation of primary AML patient blasts, and are capable of potentiating β-catenin activation in AML LSCs via an LGR4-dependent mechanism (**Figure 4**) [6].

The link between RSPO-LGR4 and WNT/β-catenin pathway has opened a new opportunity to specifically target key self-renewal signaling for LSC eradication. We have discovered that RSPO3-LGR4 pathway can be effectively inhibited by a clinical-grade anti-RSPO3 monoclonal antibody (rosmantuzumab), which disrupts the RSPO3-LGR4 interaction and abrogates leukemia-initiating capacity of patient-derived LSCs without affecting the healthy stem cell compartment [6]. Rosmantuzumab has proven to be safe and well tolerated in Phase I clinical trials conducted on patients with advanced solid tumors [52]. While the therapeutic efficacy of anti-RSPO3 antibody will need further preclinical validation in a large number of patient-derived xenograft models with different mutational profiles, these findings indicate differential dependence of normal and malignant stem cells on RSPO-LGR4 signaling and underline a therapeutic opportunity for selective targeting of AML LSCs.

5. Limitations of current studies

As our understanding of cancer continues to grow, we increasingly realize it to be a myriad of heterogenous diseases that defy simple classifications and AML is no exception. As a result, current studies tend to focus on specific tumor markings or specific genes present within tumors. Even within the subgroup of LSCs, there

exists multiple unique pathways that may work together to achieve a tumorigenic effect. In this regard, the notion of having a single drug to treat AML is archaic. The development of novel therapeutic agents is therefore necessary in order to treat an array of aberrant pathways. Thus, future research must not only focus on the effectiveness of treatment, but also how they relate to specific patient factors.

6. Conclusion

The discovery of LSCs has resulted in a paradigm shift in our understanding of leukemia biology and the way we treat AML. LSCs play a significant part in the origin, drug resistance and relapse of leukemia. In recent years, researchers have shed light on this elusive subpopulation of cells by uncovering the cellular pathways that drive the action of LSCs. Pathways such as Wnt/β-catenin and GPCR signaling are not only vital in our understanding of LSC biology but are also critical in providing avenues for the development of effective therapeutics. Currently there are some promising agents in clinical and pre-clinical trials. In the future these agents may constitute combination therapies personalized to a patient's genetic profile. As our knowledge of the precise mechanisms behind LSCs improves, the future of research lies in developing novel targeted therapies that obsolete the single use of chemotherapy in AML.

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Conflict of interest

The authors declare no conflict of interest.

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