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Introduction: Self-renewal is considered a defining property of stem cells. Self-renewal is essential in embryogenesis and normal tissue repair and homeostasis. However, in cancer, self-renewal pathways, e.g. WNT, NOTCH, Hedgehog and BMP, frequently become de-regulated in stem cells, or more mature progenitor cells acquire self-renewal properties, resulting in abnormal tissue growth and tumorigenesis.

Areas Covered: This review considers the conserved embryonic self-renewal pathways, including WNT, NOTCH, Hedgehog and BMP. The article describes recent advances in our understanding of these pathways in leukaemia and, more specifically, leukaemia stem cells (LSC), how these pathways cross-talk and interact with the LSC microenvironment, and discusses the clinical implications and potential therapeutic strategies, both preclinical and in clinical trial for haematological malignancies.

Expert Opinion: The conserved embryonic self-renewal pathways are frequently de-regulated in cancer stem cells (CSC), including LSCs. There is significant cross-talk between self-renewal pathways, and their downstream targets, and the microenvironment. Effective targeting of these pathways is challenging due to cross-talk, and importantly, because these pathways are important for normal stem cells as well as CSC, adverse effects on normal tissues may mean a therapeutic window cannot be identified. Nonetheless, several agents targeting these pathways are currently in clinical trials in haematological malignancies.

Article Highlights:

- Self-renewal pathways, including WNT, NOTCH, Hedgehog and BMP are frequently de-regulated in haematological malignancies
- There is significant cross-talk between self-renewal pathways and their downstream targets
- Self-renewal pathways remain active in normal tissues and are important for tissue repair, and often targeting these conserved embryonic self-renewal pathways results in toxicity to normal cells in vitro and significant side effects in patients
- Small molecule inhibitors targeting the NOTCH and Hedgehog pathways are being used clinically in some solid tumours (e.g. breast cancer and basal cell carcinoma, respectively) and are in clinical trial in haematological malignancies
- SMO inhibitors, which target the Hedgehog pathway have recently shown some promise in acute myeloid leukaemia and myelofibrosis

Key words: Self-renewal, cancer stem cell, leukaemia stem cell, microenvironment, Hedgehog, Wnt, Notch, bone morphogenic protein
1. Introduction:
Cancer stem cells (CSC) offer the concept that a small population of cells sharing characteristics of differentiation, self-renewal and homeostatic control, allow for the maintenance and dissemination of disease [1, 2]. Within haematological disease, these are referred to as leukaemic stem cells (LSCs). LSCs are mostly quiescent, in G0-phase and out of the cell cycle, and home to the bone marrow microenvironment, in which they are protected from apoptosis and conventional treatments [3-5]. Within a variety of haematological malignancies, including acute myeloid leukaemia (AML) [6], chronic myeloid leukaemia (CML) [5, 7], and multiple myeloma [8], the existence of LSCs has been identified. In vivo and in vitro experimental models have demonstrated that LSCs share many features with haemopoietic stem cells (HSCs) [9], including self-renewal and engraftment potential, but have also offered critical differences in functional properties [10], which allows for a therapeutic index of intervention, that would permit targeting of LSCs, whilst maintaining normal haematopoiesis.

AML LSCs were one of the first CSC populations to be characterised within haematological and solid malignancy [6, 11]. The complexity in morphological, genetic, and epigenetic heterogeneity within AML makes identifying an appropriate therapeutic target challenging, but may allow for LSC characterisation if the AML LSCs have distinct features according to their subtype. However, the immunophenotypic identification of the AML LSC remains a persistent area of debate [6, 12-14]. Historically, LSCs (both in AML and CML) have been characterised within the CD34+CD38- population, where their presence has been shown to be capable of generating leukaemic primary, secondary and tertiary engraftment in non-obese diabetic severe combined immunodeficient mice [6, 11]. However, recent studies have suggested that LSC activity can extend into the CD34+CD38+ population, with data indicating that leukaemia-initiating capacity could be present in a mature CD34- population [15, 16]. This suggests that LSCs can either be derived from HSCs or from progenitor cells that acquire self-renewal properties. Additional markers of AML LSCs have been proposed, including CD123 [17, 18], CD33 [18], CD45RA [19], CD47 [20], CD96 [21], and CD93 [22]. To date, none have been translated into the clinical setting for identification or targeting of the LSC. This is, in part, due to the intra-patient and inter-patient heterogeneity identified within AML LSC populations [16, 23] and has been particularly noted within relapse, where AML LSC frequency and phenotypic diversity has been shown to be much greater compared to the initial diagnostic LSC [24, 25].

Regardless of these complexities, the importance of AML LSCs clinically is well established. Most pertinently, within the identification of minimal residual disease (MRD), which resembles that of the diagnostic disease population, allows response to therapy to be followed over time, and offers a prognostic indicator for adverse outcome [26, 27]. It seems reasonable that LSCs reside within this population, having only been minimally impacted by conventional chemotherapy that targets dividing cells, and therefore, it stands that treatments focussing on the elimination of LSCs will reduce MRD and ultimately improve disease outcomes. Furthermore, it has been shown that AML patients with a greater number of LSCs or a more prevalent stem cell phenotype at diagnosis have inferior clinical outcomes compared to those who have fewer LSCs or a less prevalent stem cell phenotype [23, 28].
Although the most historic evidence of LSCs within haematological disease arise within AML, much insight into potential therapeutic strategies can be acquired from CML, where the chronic-phase disease represents a classic example of the stochastic CSC hypothesis model, without the molecular, epigenetic and genetic heterogeneity seen within AML. Furthermore, it is known that as the disease progresses to the acute phase, termed blast crisis (BC), committed progenitors gain self-renewal function; again highlighting the potential for LSC to be derived from a more mature progenitor population [29-31]. Therefore, understanding the LSC in CML may be clinically transferable to other stem cell driven diseases, including AML.

In CML, low-level BCR-ABL positive LSCs, as defined within a CD34+CD38- population, have been identified in the bone marrow of tyrosine kinase inhibitor (TKI)-treated CP-CML patients in deep molecular response (i.e. those that achieve a 4-log or greater reduction of quantitative BCR-ABL expression from standardised baseline over a prolonged period) [32-35]. These cells have been shown to be capable of growth in long-term culture-initiating cell (LTC-IC) assays and have murine engraftment potential, demonstrating their self-renewal capability. The demonstration that CML LSCs persist in the presence of a targeted therapy demonstrates the phenomenon of disease persistence, and highlights that LSCs are BCR-ABL independent, relying on other pathways to sustain their survival [36, 37]. The concept of disease persistence through quiescent LSCs has been further highlighted in recent years by trials exploring the discontinuation of TKIs in CP-CML patients with sustained deep molecular response [38-41]. These trials have demonstrated that discontinuation of therapy can be selectively achieved, with the most recent update of the ‘Stop IMatinib’ (STIM) trial data stating the cumulative incidence of molecular relapse at 60 months was 61% (CI 52-57%), with few cases of late relapse being observed [42]. This suggests that if molecular relapse is to occur, it happens early. It seems reasonable then, that targeting both the LSC population and BCR-ABL will lead to superior curative potential within CML.

A number of challenges remain within both the scientific and clinical communities in the eradication of LSCs, in both AML and CML. Firstly, in the identification of an appropriate specific target, or pan-target, that will enable eradication of the LSCs without a transient response. Secondly, when an appropriate target is identified that is clinically justifiable, the timings of intervention must be deduced, as well as the evaluation of disease persistence. This will remain a huge clinical challenge, especially in view that therapies that are only effective against the stem cell compartment (which represents 1-2% of bulk) may be difficult to evaluate due to the persistence of bulk malignant cells and concordant chemotherapy-based treatment. This review aims to evaluate scientific and clinical approaches for targeting self-renewal pathways in LSCs.

2. Signalling pathways as a target for LSC eradication

As discussed, self-renewal is considered to be the integral property of the LSC, and its deregulation is known to affect the development, maintenance, and persistence of LSCs in both AML and CML. To date, a number of aberrant signalling pathways have been proposed to contribute to the LSC phenotype [43-47]. These pathways, including hedgehog, Notch and Wnt, are known regulators of cell survival, and are often differentially expressed following genetic events. A number of deregulated proteins within these pathways may represent a broadly applicable therapeutic strategy, however, it is well known that these pathways rarely work in isolation, and rely on a web of activity leading to
disease maintenance, persistence, and progression. The complexities in interaction between these pathways are well documented and will be discussed within each heading below (figure 1). To add to this complexity in AML is the genetic variation that is seen, as this can mediate the proliferative and anti-apoptotic signals. Expression of specific oncogenes, such as FLT3, RAS, and MLL, may create new dependencies on specific signalling pathways in LSCs, and activate signalling pathways in isolation or simultaneously [48, 49]. Therefore, developing a therapeutic intervention that is efficacious in all activated pathways remains unachievable. Our best option remains to understand the acquired vulnerability in the mechanisms of the signalling pathways, which may offer a therapeutic window to eradicate the LSCs and plan for clinical translation.

3. Evolutionary conserved self-renewal pathways

Hedgehog signalling

The Hedgehog (Hh) signalling pathway has been shown to be inappropriately activated in a number of malignancies, including AML and CML, where it is intrinsically involved in the maintenance and expansion of the LSC compartment [50-52]. The Hh signalling pathway is an evolutionary conserved signalling pathway that is critical for embryonic development and adult homeostasis [53, 54]. Within haematological malignancy, activation of the pathway has been shown to be linked to primary immotile cilia [55, 56], where the receptor-ligand interaction causes the internalisation of PTCH1 and subsequent activation of Smoothened (SMO). This enables SMO to move into the cilium allowing for the accumulation of the active forms and the activation of key downstream targets [57]. SMO is the critical mediator in the canonical pathway and, therefore, represents a key therapeutic target to prevent the pathway’s activation. SMO can be readily targeted with pharmacological agents, including cyclopamine, and clinical grade agents, such as LDE225 (sonidegib) [58] or PF-04449913 (glasdegib) [57, 59].

However, the pathway’s complexity lies in its interactions and dependency with other key survival pathways. It has been suggested that the survival of CML progenitor cells is maintained by both the auto-activation of Hh and β-catenin [60]. Furthermore, Hh activation modulates NUMB-p53 responses, therefore, Hh suppression will subsequently alter p53 target genes; p53 is referred to as the guardian of the genome, therefore, careful evaluation of modulation of its function needs to be gained [61, 62]. p53 as a modulatory target in leukaemia is an area which is becoming of increasing interest; both in CML [63] and AML [64].

The importance of Hh signalling in CML is well established, with SMO inhibition leading to reduced self-renewal capacity of CML LSCs in both in vitro models with clinical grade SMO antagonists, and in vivo, where Smo-deficient mice have reduced leukaemogenesis in primary and secondary transplantation models [50, 52, 58]. Furthermore, combination of SMO antagonists with TKIs has been demonstrated to lead to a synergistic reduction of chronic phase (CP) CML LSCs in patient samples in vitro and CP and BC CML xenograft transplantation models [58, 65]. Our group has recently demonstrated that LDE225, a small molecule clinically investigated SMO inhibitor, used alone and in combination with the TKI, nilotinib, inhibited the Hh pathway in CD34+ CP-CML cells, reducing the number and self-renewal capacity of CML LSC in vitro [58]. The combination had no effect on normal HSCs and when combined, these agents reduced CD34+ CP-CML cell engraftment in NSG mice.
Furthermore, upon administration to EGFP+/SCLtTA/TRE-BCR-ABL mice, the combination enhanced survival with reduced leukaemia development in secondary transplant recipients.

Importantly, deregulation of Hh may potentially contribute to disease progression, with differential Hh activity increasing as CML progresses to BC [60], and increased gene expression of PTCH1 has been observed in BC samples [46]. Therefore, early targeting of the pathway may be therapeutically viable to reduce disease progression.

Whilst preclinical data appears promising to support the Hh pathway as a therapeutic target in CML, clinical trials utilising SMO antagonists as a therapeutic option have halted in early phases. Within solid tumors, these inhibitors have successfully been translated into clinical practice [66, 67], however, within CML when combined with TKI, toxicity has been a major limitation [68, 69].

In AML, the role of Hh has not been fully elucidated, with limited data available on the implications of Hh deregulation on disease biology, perhaps due to the vast heterogeneity seen within the disease. Activation of Hh, through SMO and GLI expression, has been described within primary AML samples, particularly acute promyelocytic leukaemia (APML) [70, 71]; no SMO mutations have been identified to account for this increase in activity [55]. It is clear, however, that there is a prognostic significance in its expression, with increased GLI1 and GLI2 being associated with reduced overall survival and as a marker of prognosis, respectively [72].

Studies suggest that HHIP, a membrane-associated or soluble glycoprotein that functions to bind Hh ligands, can suppress leukaemic cell proliferation [73]; furthermore, within the same study, reduced stromal HHIP expression was shown to contribute towards the development of AML. HHIP can be modulated through standard chemotherapy agents, where it has been demonstrated that 5-azacitadine-mediated amplification of stromal cell HHIP expression led to attenuated leukaemic cell proliferation potential.

The biological significance of pathway modulation has yet to be fully understood, with varying results available. Genetic inactivation of SMO in MLL-AF9-transformed LSCs does not affect AML development in primary recipient mice [74]. Conversely, SMO inhibition with cyclopamine has been shown to reduce proliferation in myelomonocytic cell lines [55]. AML, as a disease, shows great heterogeneity and, therefore, focused evaluation through each of the classified disease entities needs to be undertaken, but it appears that there may be a therapeutic role for Hh inhibitors within a myelomonocytic phenotype. It is more clear that pharmacologic inhibition of Hh signalling appears to enhance AML ‘gold-standard’ therapy by sensitising LSCs to chemotherapy within the bone marrow microenvironment [75], which may lead to clinical advances in the eradication of AML LSCs. Currently, clinical trials are under way to investigate Hedgehog inhibitors in AML, and early phase 1/2 results appear promising [59, 76]. Of note, a subsequent phase 2 clinical trial of LDE225 in AML, NCT01826214, recently closed due to lack of efficacy as a single agent; perhaps highlighting the limited activity of SMO inhibition on bulk disease.

Wnt signalling

The Wnt signalling pathway plays an essential role in the maintenance and differentiation of LSCs and the propagation of malignancies [29, 77]. Its activation has been demonstrated in acute disease, namely AML LSCs and within myeloid BC CML [29, 78]. Furthermore, it has been suggested that a
deletion within β-catenin reduces the ability of mice to develop Bcr-Abl positive leukemias, which is suggestive of a role in the pathogenesis of chronic disease [47]. Therefore, targeting Wnt is a viable option in eradication of the LSC and in the prevention of disease progression and dissemination.

Within CML, loss of β-catenin in a murine model of CML impaired the development of the disease by inhibiting LSC self-renewal, and genetic and pharmacological inhibition of β-catenin activity synergised with TKI to target the loss of CML LSCs [29, 79]. CBP/catenin antagonists have demonstrated efficacy in eliminating the CML and acute lymphoblastic leukaemia (ALL) LSC population in vitro and in vivo [80, 81]. A similar importance is seen within AML, where high expression of Ctnnb1 has been reported to correlate with poor prognosis [82]. Deletion of β-catenin within murine models has been shown to significantly reduce development and transplantation of AML driven by MLL-AF9 or HoxA9 [83, 84]. In turn, within murine models of MLL-rearrangement/AML it has been shown that self-renewal of LSCs is mediated, in part, by Ctnnb1, suggesting that Ctnnb1 may represent a therapeutic target within this subtype. Genetic and pharmacological inhibition of Ctnnb1 leads to decreased leukaemia formation. Interference with prostaglandin signalling has been shown to target the Wnt/β-catenin axis in HSCs [85], and treatment with COX inhibitors, such as indomethacin, has been shown to lead to a decrease of LSCs in secondary recipients [83]. This is mediated through Ctnnb1, although translating this clinically would be a challenge in view of adverse risk of bleeding. Inhibitors of canonical Wnt signalling are currently undergoing phase I clinical trials in AML (NCT01398462) [86].

Again, the interactions with other survival pathways, complicates antagonising Wnt/β-catenin. For example, within CML, TKI exposure has been shown to upregulate CD27 signalling, resulting in activation of Wnt target genes, which include Notch and c-Myc [87, 88]. Wnt-Notch interaction is well documented, particularly within the bone marrow microenvironment, where mutations of Ctnnb1 have been found in osteoblasts resulting in overexpression of Notch ligands and activation of the Notch pathway in HSCs [89].

The non-canonical Wnt (i.e. β-catenin independent) signalling pathways are diverse and can be initiated by WNT interaction with Frizzled receptors, or RYK and ROR receptor tyrosine kinases, to regulate small GTPases, as well as calcium flux and kinase cascades [90]. This area is not as well characterised in LSC maintenance as the canonical pathway [91]. Non-canonical signalling has been shown to exert an antagonistic effect on canonical signalling, with Wnt5a promoting GSK3β independent degradation of β-catenin and competing with Wnt3a for binding to the receptor complex [92]. A greater understanding of the non-canonical pathway may decipher an interesting therapeutic approach in β-catenin inhibition.

**Notch signalling**

Notch signalling is involved in a variety of cell-fate decisions that influence the development and function of many organs, including stem cell maintenance, cell proliferation, haemopoiesis and apoptosis [93, 94].

Its role in malignancy has been shown to be cell and tissue-dependent, with the pathway playing both oncogenic and tumour suppressive roles depending on cell and cancer type [95-98]. In haemopoietic malignancies, accumulating evidence demonstrates its importance in growth, differentiation, and apoptosis [96, 97, 99-102], with its role in T-ALL, chronic lymphocytic leukaemia (CLL), and B cell leukaemias and lymphomas well documented [100, 101, 103, 104]. Improved understanding of the
Notch signalling pathway in these malignancies suggests that the Notch pathway may be a prime drug target; however, the therapeutic role of Notch inhibition may be directly dictated by the effects of its inhibition on other cell lineages, including the myeloid lineage [105, 106].

Reports about the role which Notch plays in myeloid disease are conflicting, as Notch activation in myeloid precursors has been shown to promote self-renewal, induce and inhibit differentiation to monocytes, or induce apoptosis [107-110]. Early observations suggested that Notch signalling may play a role in myeloid progression [110-112], with its role best characterised within AML. Importantly, it has been shown that exposing AML cells to plate-bound Notch ligands led to a full range of responses from proliferation to growth arrest that varied with patient sample, suggesting again the difficulty in evaluating signalling pathways due to inter-patient heterogeneity [113]. More recently, observations have supported a tumour suppressive role for Notch signalling in immature LSC compartments of AML disease models [113-117]. Furthermore, in AML cell lines and primary patient blasts, downregulation of Notch1 expression was associated with a decrease in PU.1-mediated differentiation capacity, indicating a pivotal role in maintenance of an immature state [114].

Within CML, the data is limited. In another myeloproliferative disorder, chronic myelomonocytic leukaemia, a tumour suppressor role for the Notch pathway was again described, supporting a loss-of-function hypothesis [116]. Conversely, however, a recent paper has identified an antagonistic role between Notch and TKIs within primitive samples; although the mechanisms have not been fully elicited, this perhaps is representative of cross-talk between signalling pathways [118]. Recent data from our group has suggested the importance of Notch activation within LTC-IC assays, where activation of the CD34+38- population through Jagged1 led to a statistically significant reduction in colonies [119]. It remains to be seen if this pathway has a functional and, indeed, therapeutic role, within CML biology. There are no clinical trials underway evaluating Notch modulation in myeloid disease.

**BMP signalling**

The bone morphogenic proteins (BMPs) belong to the transforming growth factor-beta (TGF-β) superfamily and have been shown to be involved in diverse cellular functions, from apoptosis to self-renewal, in embryonic and adult phenotypes. Dysregulation within the BMP-TGF-β pathway is critical in LSC survival [120-122], particularly mediated by its downstream target genes in the Cdx-Hox axis.

Interaction between Wnt and BMP signalling regulate the Cdx family of homeobox transcription factors – the master regulators of Hox gene expression [123]. Cdx2 is aberrantly expressed in AML and promotes leukaemia propagation through deregulation of Hox genes [124], with its overexpression demonstrated in 90% of AML patients and overexpression *in vivo* leading to increased engraftment in NSG murine models. Aberrant expression of HOX genes has been linked to both AML and CML [124, 125], with overexpression of HoxB3 [126], HoxB8 [127], or HoxA10 [126] leading to the generation of acute leukaemia in murine models, as well as being associated with expansion of the HSC compartment *in vitro* and *in vivo* models [128, 129]. Although this represents an interesting target of both Wnt and BMP, no translational evidence is available for an antagonistic effect in LSC regression.
Genomic studies within primary CD34+ CML samples suggest that components of the pathway, including target genes, are downregulated [31, 130, 131]. This raises the possibility that the pathway can be activated through extrinsic mechanisms, and emphasises the role of the bone marrow microenvironment in the protection of LSCs against TKI-mediated apoptosis. It has been shown that type 1 receptors are present on LSCs in primary CML samples, with an associated downregulation of BMP ligands [120, 131]. CML aspirate and trephine bone marrow samples had significantly higher levels of BMP2 and BMP4 compared to normal donors. This suggests that there is the ability to upregulate the BMP pathway and that it is via extrinsic mechanisms within the diseased bone marrow microenvironment/niche. Lapenrouzaz et al [120] demonstrated that expression of BMP2 and BMP4 varied depending on niche cell type, with BMP2 and BMP4 being more highly expressed in polymorphonuclear cells and endothelial sinusoid cells, respectively. In response to increased levels of soluble BMP2 and BMP4, they showed that CML LSCs maintained their primitive phenotypes and enhanced long-term colony formation potential, indicating that the BMP pathway can suppress differentiation and potentiate LSC survival.

**The Bone Marrow Microenvironment**

Because LSCs home to the bone marrow microenvironment, it seems pertinent that the bone marrow is considered within cell-to-cell interactions and activation of aberrant self-renewal signalling. The interactions between LSCs and the bone marrow remain an important area of research and may determine the best strategy for eradication of the LSC. In many myeloid leukaemias’ there is enhanced osteoblastic proliferation and a marked increase in LSCs and progenitor expansion [132]. LSCs rely on the bone marrow niche for their survival and modulate it to enhance survival, and a number of key interactions with self-renewal pathways contribute to the chemo-resistance that is seen. Deregulation of BMP has been shown in murine models where Bmpr1a/Alk3 conditional knockout mice have impaired BMP signaling, which leads to increased niche size and thereby enhanced numbers of HSCs [133]. Furthermore, a number of ligand-receptor mediated pathways regulate CML LSC, and in turn alter signaling pathways responsible for their maintenance, including MPL which regulates JAK/STAT signaling [134]. MPL has been shown in high levels to lead to reduced TKI sensitivity in CML, although, in turn, a higher sensitivity to JAK inhibitors. Expansion of the osteoblast layer of the CML bone marrow microenvironment can contribute to creating a hostile environment for HSC, mediated through alterations in TGFβ, NOTCH and pro-inflammatory signalling [132, 135]. However, there are difficulties in utilising the bone marrow microenvironment therapeutically – how should the pathways within the marrow be targeted? Would peripheral injections suffice to a large enough concentration of blockade without leading to adverse effects?

4. Conclusion

Aberrant signalling proteins have been extensively identified and evaluated within LSC biology. These represent a smaller number of signalling pathways. Preclinical data suggests that targeting these unifying pathways may offer an attractive, and more broadly applicable, therapeutic strategy to eradicate the LSC compared to chemotherapy alone. However, these signalling pathway interactions make them inherently complex, and modulating one may down- or up-regulate another, with biologically significant consequences. Furthermore, there is limited understanding of the biology of the LSC following treatment, whether that be with systemic or targeted chemotherapy, with most preclinical studies utilising treatment-naïve patient samples, or murine models. Further work is
needed to understand these intricacies. With increased scientific understanding, the next question will relate to translating these targets from bench to bedside, and with this many questions will unfold.

5. Expert Opinion:
The above suggests that as LSCs retain dependency on self-renewal pathways, they could be selectively targeted if the complexities in their interactions are fully understood. Disappointingly, despite strong *in vitro* and *in vivo* preclinical data, drugs against these targets have yet to be implemented in the clinical setting as a standard of care. This is, in part, due to trial design, as well as toxicities that are generated, particularly in areas with high cell turnover, such as the gastrointestinal tract.

The clinical need for alternative and more effective therapies in AML and CML are different. AML represents a disease where there has been little progress in therapeutic strategies, with improvement in survival likely secondary to better supportive measurements rather than improvements in standard chemotherapeutics. The need for targeted and individualised treatments is a necessity in the disease for improved outcome and eventual cure. However, the targeting of LSCs is likely only to be effective in a minority of patients and will vary, dependent on the sub-class of disease. The difficulty remains in 1) identification of LSC in bulk samples with no established immunophenotype, and 2) understanding the differences in signalling pathways within an epigenetic, genetic, and morphologically heterogeneous disease.

Within CML, there has been an overall improvement in survival of approximately ten-fold since the introduction of targeted therapy against *BCR-ABL* [136]. This has led to the majority of patients achieving close to normal life expectancy, when treated with TKIs. The need for eradication of the LSC is necessary to enable complete eradication of CML and subsequent cure, allowing patients to stop therapy, and to alleviate a financial burden within healthcare systems. Like AML, a biomarker of the LSC needs to be identified and sensitive enough to identify CML LSCs at low level following treatment. A number of potential biomarkers have been described, including CD26 [137], and IL1-RAP [138].

Our group has described the role of CD93 as a potential biomarker of the quiescent LSC population within CML [139]. Although identification of the CML LSC appears to be within reach, little is known about the biology of the LSC in those patients that are in deep molecular response, with much of our understanding and the previous data within a drug-naïve population. Clinically, the therapeutic need will be in those that are on TKI therapy.

The biological understanding of aberrant self-renewal pathways within the LSC and potential targets is well underway, however the preparation for translation into the clinical setting needs to be considered. Firstly, as stated above, within each disease the immunophenotypic characterisation of the LSC needs to be established. When this is verified and supported internationally, there are considerations that need to be addressed to translate targeted inhibition or activation clinically.

Firstly, decisions regarding evaluation of response to self-renewal modulation. Within an *in vivo* and *in vitro* setting, these responses are often generated by utilising immature populations of sorted cells or genetically manipulating murine models. Within a clinical setting, the response of bulk disease cannot be used as a surrogate for the clinical effect on the LSC population as there are differential sensitivities between the bulk and LSC populations, nor can evaluation of survival be used as a surrogate in treatment evaluation. Clearly, established markers of the LSC (e.g. murine engraftment,
LTC-IC assessment, replating efficiency) may be used as a surrogate for response, or an MRD evaluation could be used to generate some understanding of the proportion of LSC at diagnosis and then following treatment.

Secondly, comparing new targeted approaches to standardised chemotherapy regimens needs to be carefully executed. The use of gemtuzumab ozogamicin in AML has directed understanding of the evaluation of a targeted response compared to standard treatments [140, 141], with a realisation that often a targeted therapeutic approach within bulk samples will require a longer treatment period to achieve a clinical response. Can overall survival be used as an endpoint if standard chemotherapy eradicates bulk disease, whereas targeted approaches are acting as an adjunctive therapy that requires longer duration to see an effect?

Thirdly, treatment timings of intervention will be essential in the understanding of disease biology. The treatments could be started simultaneously to allow for the likely longer duration needed for the targeted therapy to achieve a response. But this would cause difficulties in an appropriate primary clinical end-point and evaluation of the LSC response to therapy. Alternatively, the treatments could be sequenced to allow for identification of those patients, particularly with CML, where targeted therapy is not needed, and in AML, in those that have MRD positivity despite chemotherapy. This would, however, open the potential for clonal evolution of the disease, that may render it more difficult to treat and skew results of an LSC-targeted approach. With a concurrent approach, it is likely that drug toxicities may be a clinical issue, rendering the approach undeliverable as a standard-of-care.

Therefore, not only is an in-depth understanding of aberrant signalling pathway biology within LSCs required for generation of appropriate and justifiable therapeutic targets in the eradication of LSCs, careful consideration in the isolation and identification of LSCs, endpoint response, and timing of therapy is needed to enable translation of the therapeutic targets into a clinical setting.
Figure Legends

Figure 1. Complex interactions between self-renewal pathways. The interconnectivity between self-renewal pathways is well documented within the LSC. It is known that proteins within each of the pathways, namely hedgehog, Wnt, and Notch, can both antagonise and agonise the other pathways by cross talk leading to both up- and downregulation of downstream targets.

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Canonical Wnt signalling

- DVL
- GSK3
- B-catenin

Notch signalling

- JAG1
- JAG2

Downstream target genes

Hh signalling

- SMO
- GLI1

HES1