Bone marrow niche crosses paths with BMPs: a road to protection and persistence in CML

Abstract

Chronic myeloid leukaemia (CML) is a paradigm of precision medicine, being one of the first cancers to be treated with targeted therapy. This has revolutionised CML therapy and patient outcome, with high survival rates. However, this now means an ever-increasing number of patients are living with the disease on life-long tyrosine kinase inhibitor (TKI) therapy, with most patients anticipated to have near normal life expectancy. Unfortunately, in a significant number of patients TKIs are not curative. This low-level disease persistence suggests that despite a molecularly targeted therapeutic approach there are BCR-ABL1 independent mechanisms exploited to sustain the survival of a small cell population of leukaemic stem cells (LSCs). In CML LSCs display many features akin to haemopoietic stem cells, namely; quiescence, self-renewal and the ability to produce mature progeny, this all occurs through intrinsic and extrinsic signals within the specialised microenvironment of the bone marrow (BM) niche. One important avenue of investigation in CML is how the disease highjacks the BM, thereby remodelling this microenvironment to create a niche, which enables LSC persistence and resistance to TKI treatment. In this review we explore how changes in growth factor levels; in particular the bone morphogenetic proteins (BMPs) and pro-inflammatory cytokines impact on cell behaviour, extracellular matrix deposition and bone remodelling in CML. We also discuss the challenges in targeting LSCs and the potential of dual targeting using combination therapies against BMP receptors and BCR-ABL1.

Discovery of Bone Morphogenetic Proteins

Fundamental work carried out in the 1960s on bone regeneration by Dr. Urist lead to the discovery of the bone morphogenetic proteins (BMPs). His work identified the presence of substances naturally occurring in bone facilitating bone repair, which were named based on these bone inductive properties [1,2]. It was only in the 1980s, following advances in molecular biology technologies that BMPs were finally cloned and identified to be members of the transforming growth factor beta (TGF-β) superfamily of cytokines [3,4]. To date more than 20 BMP family members have been discovered, with research indicating they have extensive functions. Studies have shown that BMPs are crucial during embryogenesis especially for bone formation, with other key roles in organogenesis, angiogenesis, and haemopoiesis during developmental processes (reviewed by Wang *et al.* [5]). In adults, they are essential for bone regeneration and help to sustain tissue homeostasis, with deregulation of the BMP signalling pathway linked to a multitude of different cancer types including haematological malignancies [5,6].

Haemopoiesis and the bone marrow microenvironment

Haemopoietic stem cells (HSCs) generate all blood lineages depending on life demands to maintain homeostasis within the body. They reside in a specific microenvironment - the bone marrow (BM) niche. The BM niche itself can be roughly subdivided into two distinct areas, the perivascular [7,8] and the endosteal [9] zones. Within these zones haemopoietic and other non-haemopoietic tissues such as osteolineage cells, sinusoidal endothelium, fibroblasts, Schwann cells, perivascular stromal cells, adipocytes, endothelial cells, mesenchymal stem cells (MSCs) and immune cells reside. The niche architecture is also supported by the

extracellular matrix (ECM), a highly heterogeneous structure containing three classes of insoluble macromolecules: (a) structural proteins such as collagen, laminin and elastin, (b) glycoproteins including fibronectin (FN) and vitronectin (VN) and (c) glycosaminoglycans (GAGs) comprising keratin, hyaluronic acid and chondroitin sulfate, which display hydrophobic properties resulting in buffering and cell adhesion [10],[11]. The niche delivers signals through secreted or bound molecules, and physical cues, such as oxygen tension and shear stress to control cell behaviour [12]. HSCs self-renewal, proliferation, and differentiation into mature blood cells is orchestrated within this local microenvironment through these intrinsic and extrinsic cues. The perivascular zone, which contains sinusoidal endothelial cells and perivascular stromal cells, has been associated with more actively cycling HSCs due to its close proximity to the vasculature resulting in exposure to higher gradients of circulating chemokines, cytokines, growth factors (GF), and oxygen concentrations. The endosteal zone close to the inner surface of the bone cavity has been linked to HSC quiescence and self-renewal [13], due to the more hypoxic environment and interactions with osteoblasts. HSCs specifically interact via N-cadherin and Jagged 1 with the osteoblasts that line the endosteal surface leading to activation of the Notch signalling pathway [14]. In addition, osteoblasts also secrete other important regulators of quiescence and HSC maintenance, such as cytokines, chemokines, WNTs and BMPs [15].

The BM niche is a dynamic environment, with bone remodelling playing an important role in maintaining the integrity of the architecture. The BMP signalling pathway is essential for this process, with several of the BMP ligands inducing osteoblastic differentiation [5]. Several cells within the BM niche produce BMPs these include; stromal cells, osteoblasts, megakaryocytes, platelets, HSCs and major haemopoietic cells [16]. BMP2, BMP4, BMP6 and BMP7 are important orchestrators of this process acting on MSCs, BM stromal cells and osteoblast precursor cells to induce differentiation into osteoblasts [17]. Osteoblasts produce BMP2, BMP3, BMP4 and BMP6 [18,19], whereas HSCs and progenitor cells produce BMP2, BMP4, BMP6 and BMP7 [20],[21]. Once secreted these become incorporated into the ECM and can act in an autocrine or paracrine fashion. Several BMPs have been shown to be important in haemopoiesis, with BMP2, BMP4 and BMP7 involved in HSC self-renewal and maintenance, progenitor cell expansion and haemopoietic differentiation. In the absence of erythropoietin, BMP2 can stimulate HSC to commit and differentiate towards erythropoiesis [22], whereas BMP4 sustains stem cell maintenance and megakaryocytopoiesis by thrombopoeitin (TPO) [23], highlighting their role in haemopoietic commitment and homeostasis.

Stem cell behaviour can also be modulated by microRNAs (miRNA). These short non-coding RNAs play an important role in RNA silencing and post-transcriptional regulation of gene expression, with several miRNAs implemented in negative modulation of the BMP signalling pathway (Table 1 and reviewed by Lowery *et al*. [24]). In the BM niche MSC have the ability to differentiate into adipocytes, osteoblasts, or chondrocytes with these processes induced by specific signalling pathways, including the Wnt and BMP pathways [25]. Silencing of the two key enzymes Dicer or Drosha involved in miRNA processing, in MSCs, indicates that this ability to differentiate is dependent on miRNAs [26]. In particular, miR-196a, miR-29b, miR-2861, miR-3960 and miR-335-5p have been reported to enhance osteogenic differentiation [27–30], whereas miR-143, miR-24, miR-31, miR-30c, miR642a and miR-3p regulate adipogenesis [31–34] Other miRNAs have been shown to negatively regulate differentiation, with miR-26a, miR-133, miR135, miR141 and miR-200a all reported to prevent osteogenic differentiation [35–37]. Epigenetic changes leading to alterations in expression of miRNAs has also been linked to bone remodelling in osteoporosis [38], highlighting the important role miRNAs play in BM homeostasis.

Table 1: MiRNA involved in regulation of the BMP pathway

BMP2	miR-140 [39]
BMP4	miR-200, miR-145, miR-196a [40–42]
BMP7	miR542-3p, miR-30a/b/c/d [43,44]
BMPR2	miR-17-5p, miR-20a, miR-100, miR-135b, miR-302, miR-125, miR-153, miR-125b, miR-21 [35,45–52]
ACVR1/ALK2	miR-130a, miR-148a [53,54]
SMAD1	miR-30a/b/c/d, miR-26a, miR-155, miR-199*, miR-205 [44,55–62]
SMAD4	miR-26a, miR-205, miR-23b [56–58,62,63]
SMAD5	miR-155, miR-23b, miR-135 [35,59,60,63]

BMP secretion and presentation within the BM niche

BMPs are synthesised inside the cell as precursor proteins, consisting of three distinct domains: the N-terminal signal peptide essential for directing the protein to the secretory pathway; the pro-domain, essential for protein folding and cleavage of the secreted protein and the C-terminal mature domain. The latter contains seven conserved cysteine residues important for forming the cysteine knot structure and dimerization of the mature cleaved peptide along with key sites for N- and O- glycosylation. Bioactivity of BMPs like many GFs is regulated post-translationally, through these modifying events with glycosylation playing an important role in increasing the protein stability, half-life and receptor specificity. Proteolytic cleavage is essential for secretion and the signalling range of the mature dimer. BMP2 and BMP4 share 80% homology, like other BMP family members their pro-domains are cleaved by furin at the consensus –R-X-X-R- site [64], uniquely BMP2 and BMP4 also contain a second site –R-X-R/K-R- at the carboxy-terminal side of this preferred consensus sequence (Figure 1). These two sites have been shown to be important for altering the activity and range of the ligand with simultaneous cleavage at both sites maximising biological activity and increasing the biological range *in vivo* [65,66].

BMPs are active either as homodimers or heterodimers with BMP2 having the potential to form heterodimers with either BMP6 or BMP7, whereas BMP4 forms heterodimers only with BMP7 [67–70]. Both precursors need to be produced simultaneously in the cell, in order for heterodimers to form, as this process occurs internally prior to secretion [71]. Transcriptomic data indicates that HSCs and progenitors express BMP2, BMP4, BMP6 and BMP7 [20],[21].

Heterodimers of BMP2/7 and BMP4/7 have been shown to be 5 to 20 times more potent than their homodimers in osteogenic differentiation assays, and to induce bone regeneration more effectively than homodimers [71,72]. BMP2/6 heterodimers have also been shown to be more potent than their corresponding homodimers at inducing differentiation of human embryonic

stem cells [69]. Specificity of the BMP homodimers and heterodimers is through receptor expression on the target cells with BMPs requiring binding to a type 1 and type 2 receptor to initiate signalling. Type 1 and type 2 receptors differ in their cytoplasmic regions and through the presence/absence of a membrane proximal serine-glycine rich sequence (GS-region) N-terminal to the intrinsic serine/threonine kinase domain, which is only present in the type 1 receptors. The extracellular ligand-binding domains also differ enabling ligand specificity (Reviewed by Yadin *et al.* [73]). BMP2 and BMP4 signal through the type 1 receptors BMPR1A/ALK3 and BMPR1B/ALK6 and the type 2 receptors BMPR2, ActRIIA, ActRIIB. Whereas BMP6 and BMP7 signal through type 1 receptors ACVR1/ALK2, BMPR1A/ALK3 and BMPR1B/ALK6 and type 2 receptors BMPR2, ActRIIA, ActRIIB to activate the canonical BMP signalling cascade. This results in phosphorylation of the R-SMADs namely SMAD 1/5/8 through the serine-threonine kinase activity of the type 1 receptors, pSMAD1/5/8 then complexes with SMAD4, translocate to the nucleus initiating expression of BMP target genes (Figure 1).

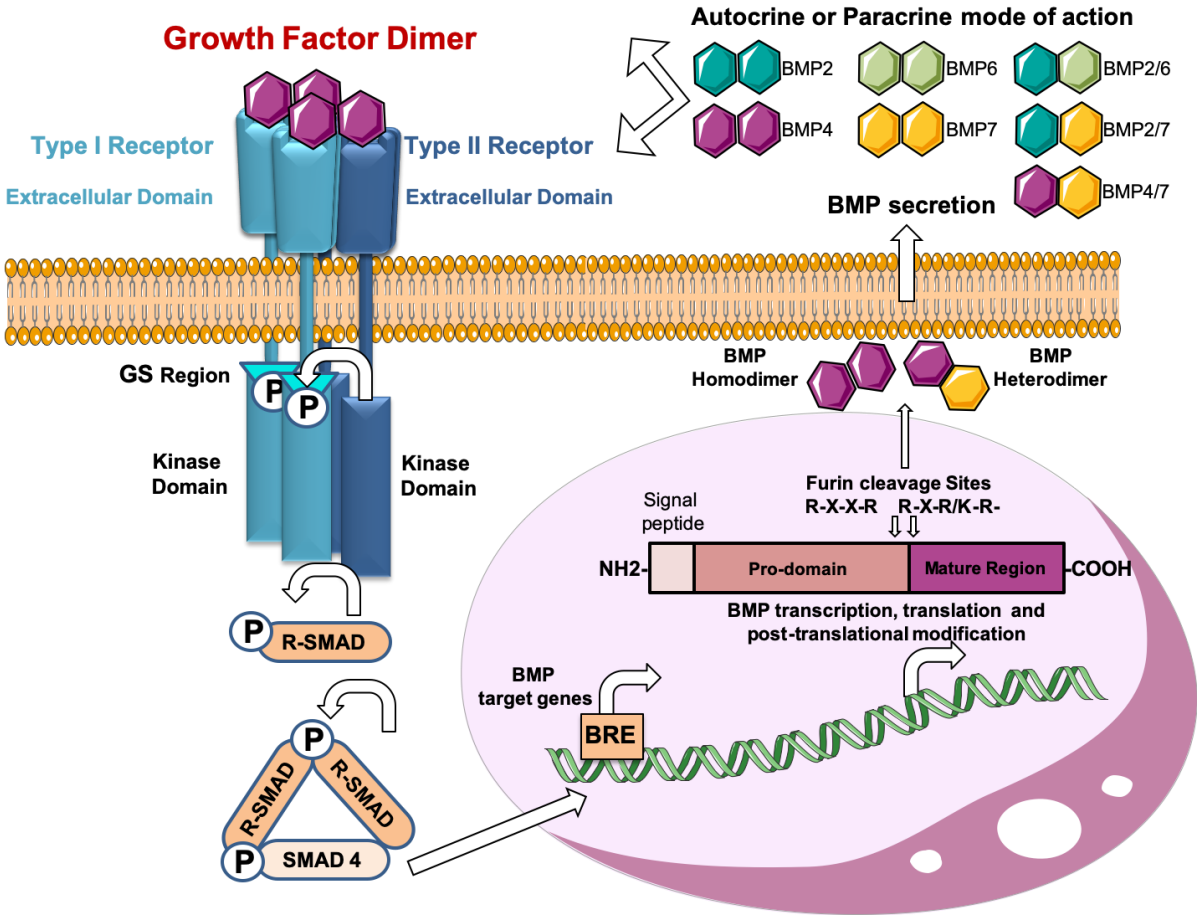

Figure 1: Schematic of BMP2 and BMP4 protein structure and receptor binding.

BMPs are synthesised inside cells as large and inactive precursors, consisting of a N-terminal signal peptide which directs the protein to the secretory pathway, a pro-peptide important for protein folding, and the C-terminal mature peptide. Following post-translational modifications, the precursors dimerize and the pro-domain is proteolytically cleaved by furin at Arg-X-X-Arg to generate the active homodimer or heterodimer. BMP2 and BMP4 also contain a second consensus furin cleavage site Arg-X-Arg/Lys-Arg. On secretion BMPs act in an autocrine or paracrine fashion through binding to their specific Type I and Type II receptors. Ligand binding results in receptor dimerization, phosphorylation and activation of the R-SMADs (SMAD1/5/8) which complex with SMAD4, translocate to the nucleus

and initiate transcription of target genes through binding to BMP responsive elements (BRE) in the promoter region.

Once secreted *in vivo* the soluble forms of BMPs have very short half-lives of only hours, this is even shorter when administered systemically with clearance occurring within minutes. Studies indicate the activity and stability of BMPs are greatly enhanced when bound to ECM proteins, with bound forms of BMPs having half-lives of several months [74–76]. BMPs bind to the glycoprotein family of ECM, in particular FN through the GF-binding region ($FNIII_{12-13}$). This region can sequester several GFs families including; TGF-β, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF). FN when incorporated into the ECM has a fibrillar conformation [77], this fibrillar FN structure enables simultaneous availability of the integrin-binding region ($FNIII_{9-10}$) and BMP presentation enabling cell binding via integrins [10,11,78]. Several integrins engage with FN through $FNIII_{9-10}$, these include αIIbβ3, αVβ3, αVβ6, αVβ1, α4β1, α5β1, and α8β1 [79], with several BM niche cells interacting with FN in this way. Cells, which specifically interact with FN via integrin binding include; fibroblasts, MSCs, BM stromal cells, HSCs and their progenitors [80,81]. The binding of GFs to the ECM in the BM niche enables localised, low dose administration of signals [82–84].

Fitzpatrick *et al.* revealed insight into mechanism of BMP receptor and integrin crosstalk, and mechanotransduction [85]. They showed that when BMP2 and FN are combined this leads to bioactivity, triggering phosphorylation of SMAD1/5/8 and translocation into the nucleus. Furthermore, SMAD signalling was regulated by cell spreading and actin dynamics [85]. Additionally, oscillatory shear stress experiments in endothelial cells revealed an interaction between BMPR1B/ALK6 and $\alpha_v\beta_3$ integrin through the formation of a mechanoreceptor complex [86,87]. Based on tissue stiffness non-haemopoietic cells can change integrin expression levels determining stem cell fate. Positive integrin-BMP cross-talk has been observed in several studies, with increases in expression of integrins in response to BMP signalling [88–91]. Integrin inhibition can also reduce BMP responses and SMAD transcription, without effecting BMP receptor binding or BMPR-integrin localization. Moreover, integrins are also able to repress BMP signalling [92].These findings highlight the importance of integrins regulating cellular responses to BMPs as both react to mechanical properties of the ECM [86]. ECM proteins such as FN may provide a potential new feature for GF presentation and cell interactions in disease modelling. Spatio-temporal controlled expression of highly abundant and deregulated GFs such as the BMPs in the BM microenvironment may allow us to ensure optimal lifespan of molecules, avoid toxic off target effects and can be potentially further incorporated into *in vitro* 3D disease models to predict *in vivo* responses of drug treatments in diseases such as haematological malignancies. In addition, research focussing on the development of materials which regulates the secretory profile on MSCs [82,93,94] will also help move research from 2D to more physiologically relevant 3D approaches.

Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) arises due to a genetic mutation in an HSC in the BM niche and accounts for about 15% of all new diagnosed leukaemia in adults [95]. CML is characterized by the Philadelphia chromosome (Ph^+) arising from the t(9;22)(q34;q11.2) chromosomal translocation that generates the oncogenic fusion protein BCR-ABL1. The molecular consequence is a constitutively active cytoplasmic tyrosine kinase, which affects proliferation and myeloid cell fate, consequently overproducing immature and mature granulocytic cells [96,97]. CML slowly accumulates over time and proceeds in three distinct phases: chronic

phase (CP), accelerated phase (AP) ending in a terminal blast crisis if untreated. There are currently 4 tyrosine kinase inhibitors (TKIs) used as frontline therapy (imatinib, nilotinib, dasatinib, and bosutinib), designed to block the kinase activity of ABL1. However, TKIs are not curative and low-level disease persistence suggests that despite a molecularly targeted therapeutic approach there are BCR-ABL1 independent mechanisms exploited to sustain the survival of a small cell population of leukaemic stem cells (LSCs). LSCs share many features with HSCs and are able to self-renew, as well as produce leukaemic cells. They are able to rebuild and control their microenvironment, creating a more specialised niche for their needs in which they can persist and resist TKI treatment based on stem cell-niche cross talk. Therefore, TKIs fail to eliminate these cells even in patients showing complete cytogenetic remission (CCyR) [98,99]. Single cell transcriptomic analysis discovered sub-populations of therapy-resistant cells that are not apparent in bulk cell-population analysis[100]. These cells are resistant to therapy, meaning when TKI treatment is stopped the disease is likely to return. LSC subpopulation heterogeneity could drive resistance and is dependent on the cues offered by the microenvironment. Clinical trials exploring discontinuation of treatment in patients with major molecular response (MMR, i.e. those that achieve a 4 log or greater reduction of quantitative BCR-ABL1 expression from standardised baseline over a prolonged period) have revealed disease-persistence through quiescent LSCs [101,102]. After treatment withdrawal approximately 30-40 % of patients experienced a molecular relapse defined as the loss of MMR [101,102].

Role of the BMP pathway in sustaining CML LSC in the BM niche

Recent studies indicate both an intrinsic and extrinsic deregulation of the BMP pathway in CML patients, with alterations most prevalent in TKI resistant patients [103–105],[106]. Laperrousaz *et al.* revealed deregulation of BMPR1B/ALK6 and downstream signalling elements, such as *SMAD1*, *SMAD4*, *SMAD5*, *SMAD6*, *ID2* and *RUNX1* in CP CML patients compared to healthy controls [103]. Deregulation of the pathway was also confirmed by Toofan *et al.* who showed that differential expression of BMP pathway genes such as *ACV1C*, *INHBA*, *SMAD7*, *SNAIL1* and *SMURF2* could be linked to treatment response [105]. BMPR1B/ALK6 was identified as the main driver for myeloid progenitor expansion with BCR-ABL1 oncogene being sufficient to increase BMPR1B/ALK6 cell membrane expression in CML [103]. Moreover, a higher abundance of BMP2 and BMP4 in the BM plasma of CP-CML patients at diagnosis was determined compared to normal donors. BMPR1B/ALK6 high cells responded to BMP2 and BMP4 stimulation with an increase in progenitors but the same was not observed in BMPR1B/ALK6 low cells [103]. However, only BMP4 supplementation expanded stem cell numbers, and genotypically all expanded cells were Ph^+ [103]. On a molecular level, BCR-ABL1 overexpression increased BMPR1B/ALK6 surface expression [103], but BCR-ABL1 expression itself was not affected by BMP2 or BMP4 stimulation [104]. Conversely, *TWIST-1* a transcription factor involved in LSC resistance [107], was overexpressed upon BMP exposure. *TWIST-1* overexpression was reported by Wang *et al.*, to support stem cell growth, drug resistance, tumour-initiating properties and to increase clonogenic capacities in acute myeloid leukaemia [108]. *TWIST-1* increased under BMP2 and BMP4 stimulation despite imatinib treatment, indicating BMPR1B/$ALK6^+$ cells may be resistant due to *TWIST-1* mediated upregulation [104]. On the clinical site, 40 % of newly diagnosed patients display higher BMPR1B/ALK6 levels [104]. Upon imatinib treatment BMPR1B/ALK6 expression increases, without expression modification in healthy controls, which could demonstrate a subselection of BMPR1B/$ALK6^+$ immature cells *in vivo* during TKI treatment. Furthermore, BMPR1B/ALK6 high cells survived TKI treatment better than BMPR1B/ALK6 low cells. Higher BMPR1B/ALK6 transcript levels were only observed in BM samples whereas MNC

from peripheral blood displayed normal amounts. These findings indicate that BMP ligands and signalling play an important role in CML pathogenesis and CML disease persistence.

Remodelling of the BM niche in CML

In CML several features of BM remodelling have been reported including increased BM vascularity and fibrosis [45,46,[111]]. Fibrosis is a common complication of myeloproliferative neoplasms (MPNs) and can lead to myelofibrosis, extramedullary haemopoiesis, and ultimately disease progression. In BCR-ABL1 negative MPNs, myelofibrosis is driven by the malignant stem/progenitor clone and an increase in megakaryocytes in the BM leading to a pro-inflammatory phenotype [112]. In CML, patients with high grade fibrosis at diagnosis reportedly have a poorer prognosis with a significant reduction in numbers reaching MMR following TKI treatment [113,114]. Fibrosis is thought to be a secondary event in CML caused in response to the cytokines released by the malignant stem/progenitor cells. It is known that cytokines form a pro-inflammatory environment during CML development, providing an advantage to LSCs by helping mediate TKI resistance [12,115,116]. In CML there is evidence for increased expression of several cytokines including FGF, hepatocyte growth factor (HGF), IL-1α, IL-6, IL-8, PDGF, TGF-β, tumour necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), BMP2 and BMP4 [103,117–124] .

Increased levels of FGF, IL-6 and VEGF have been linked to promoting angiogenesis. IL-6 levels in CML patients were found to directly correlate with BM angiogenesis and to increase during disease progression (reviewed by Valent *et al.* [123]). Moreover, blockage of IL-6 activity can restore treatment sensitivity [120,121]. Furthermore, BCR-ABL1 expression increases VEGF production in CML cells, with high VEGF levels correlating to a decreased survival rate (reviewed by Valent *et al.* [123]). Another common feature of CML is high numbers of megakaryocytes in the BM [125]. In MPNs neutrophils and megakaryocytes have been reported to undergo emperipolesis, whereby neutrophils deliver their enzymes leading to the release of cytokines such as TGF-β, PDGF and FGF by the megakaryocytes. Fibrosis is primarily due to a TGF-β induced production of total collagen, deposition of laminin and adhesive glycoproteins (VN, FN and tenascin) in the BM niche by MSC, megakaryocytes and osteoblasts. BMPs have also been linked to aberrant BM matrix homeostasis in myelofibrosis with BMP1, BMP6, BMP7 and BMPR2 significantly elevated as the disease progresses. BMP molecules are expressed by non-leukaemic cells, such as stromal cells, megakaryocytes, and platelets [23,103] as well as through an autocrine loop by TKI resistant $CD34^+$ leukaemic cells themselves [104]. Long term pressure of TKI treatment increases the secretion of BMP2 by MSCs in CML with MSCs of resistant patients producing higher amounts of BMP4 compared to healthy cells [104,126]. In fibrosis BM stromal cells and megakaryocytes have been shown to be the predominant source of BMP1, which then activates the latent form of TGF-β. Fibroblasts then secrete BMP6 in response to the higher levels of TGF-β in the niche [127]. Expression of PDGF is also associated with BM fibrosis especially in accelerated phase and blast crisis CML [123,128]. This high GF gradient in the BM niche then stimulates fibroblasts to cause fibrosis and endothelial cells to cause neoangiogenesis (Figure 2).

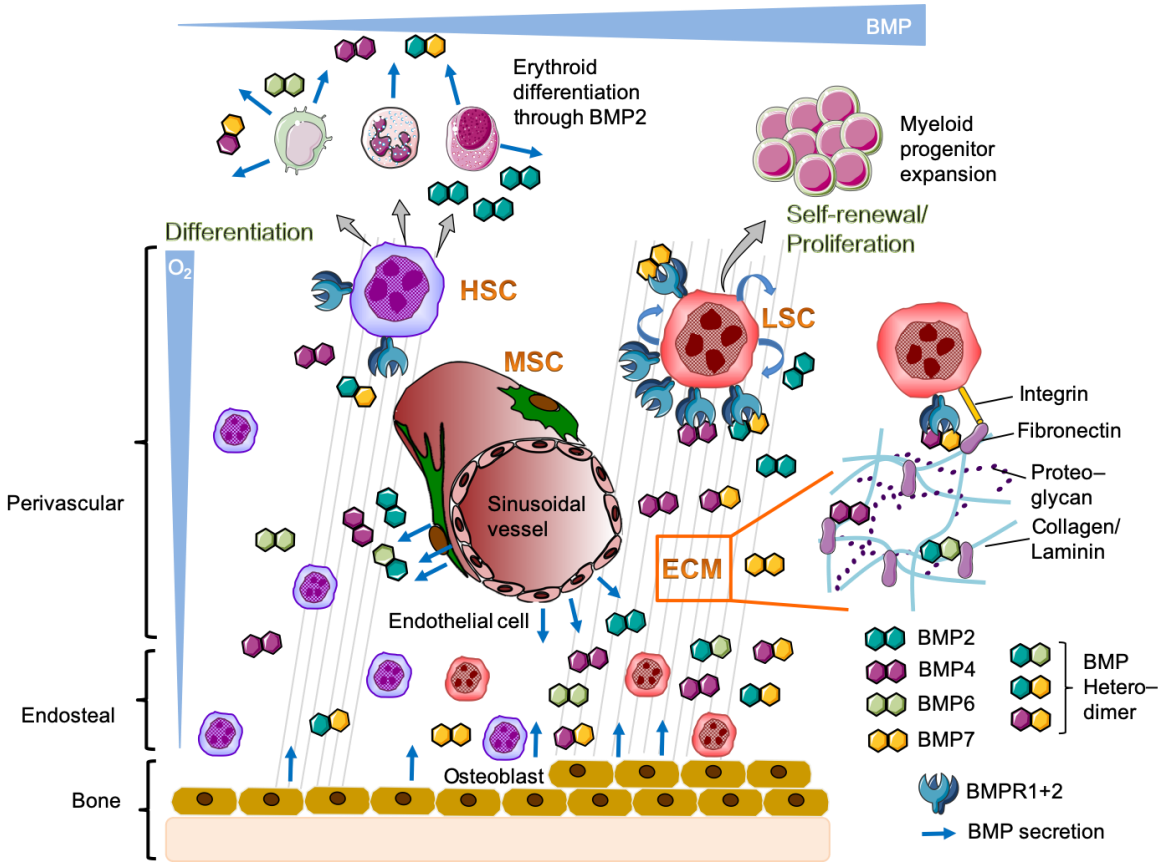

Figure 2: Bone marrow niche remodelling in CML.

HSCs interact with and are regulated by an intricate and vibrant multicellular BM niche. They have close anatomical and functional relationships with cells in the BM. The players in the BM niche include GFs such as the morphogenetic proteins, chemokines and cytokines, the ECM, adhesion factors, and the sympathetic nervous system. BMP ligands are highly abundant in the CML BM microenvironment and can be produced by osteoblasts, MSC, endothelial cells, major haemopoietic cells, LSC and progenitor cells by an autocrine loop. This coupled with the increased levels of pro-inflammatory cytokines released by LSC and progenitor cells can lead to bone remodelling, fibrosis and neoangiogenesis in CML. The release of BMPs and pro-inflammatory cytokines also contributes to LSC's resistance to TKIs. Interactions between LSCs and osteoblasts favours quiescence, the higher numbers of osteoblasts generated by bone remodelling in response to these changes in GF levels in CML is therefore likely to favour LSC self-renewal and survival by supporting a quiescent stage.

Another process linked to myelofibrosis is bone remodelling with increased numbers of osteoblasts resulting in osteosclerosis and neoangiogenesis as the disease progresses. High levels of IL-1α have been linked to inhibiting osteoclastogenesis [129], this is through IL-1α stimulating production of osteoprotegerin by stromal and endothelial cells. High osteoprotegerin can lead to unbalanced osteoblast proliferation. In CML BM, monocytes and endothelial cells reportedly express high levels of IL-1α, with higher than normal levels of the cytokine detected in BM aspirates [122]. These observations coupled with the increase levels of BMP2 and BMP4 reported in CML suggest that osteoblastogenesis is favoured over osteoclastogenesis. Higher osteoblast numbers would favour LSC self-renewal and survival in CML. Furthermore, several of these cytokines confer growth advantage to LSCs including TGF-β [130] BMP2 and BMP4 [103,104,131–133]. CML LSCs demonstrate high levels of IL-1R expression with IL-1α potentiating TKI resistance whereas inhibiting the pathway is able to

restore TKI sensitivity [122]. Moreover, BMP4 increases haemopoietic progenitor adhesion to the stroma, leading to controlled stem cell behaviour [23,91,126] . BMPR1B/ALK6 signalling pathway also allows long-term HSC adhesion to spindle-shaped N-cadherin CD45.2 osteoblastic (SNO) cells by the molecules N-cadherin and beta-catenin and regulates niche size[134] [135].

GF secretion and signalling in the BM niche can alter in response to intrinsic and extrinsic factors to meet the haemopoietic demands of the body. Through tight regulation this process sustains homeostasis over time. If the dynamics of this environment alters due to a haematological malignancy, then this balance will be perturbed resulting in remodelling of the niche due to alterations in GF levels impacting on cell behaviour. Data suggests that the long latency in CML development and the alteration in the GF levels in the BM niche results in an environment whereby the altered secretome leads to changes in the BM matrix, cell numbers and overall dynamics of the BM niche.

Targeting the BMP pathway

When considering CML therapy we need to take into account GF signalling in the BM niche as this is tightly connected with kinase phosphorylation and LSC cell survival, thereby giving us a potential platform for drug targeting. Data from our group indicates that combinatorial treatment of a BMP receptor inhibitor and a TKI is a promising avenue for the future treatment of CML. Our results showed that dual targeting of BMP pathway signalling and BCR-ABL1 leads to altered cell cycle gene transcription, irreversible cell cycle arrest, along with increased apoptosis compared to single treatments. More importantly, inhibition resulted in fewer cell divisions, reduced numbers of $CD34^+$ cells and colony formation from CML patient samples compared with healthy controls [105] . These promising results suggest that this approach warrants further investigations. There are several avenues to explore to target the BMP pathway in the future (Figure 3). At present, there is powerful toolbox of small molecule inhibitors or neutralizing antibodies inhibiting a handful of ligands (BMP2, BMP4, BMP6, BMP7, and BMP10), and receptors (ACVRLI /ALK1, ACVR2A, and ACVR2B), as well as the ability to use recombinant proteins of the antagonists (noggin and gremlin) [24,136,137]. Firstly, BMR1B/ALK6 receptor binding sites can be directly blocked by inhibitors such as dorsomorphin[138] or LDN-193189 [24]. Secondly, secreted BMP molecules by leukaemic or non-leukaemic cells can be inactivated by trapping them using small antagonistic molecules, such as Noggin, Chordin or Gremlin to prevent binding with their cognate receptors [24,139], or BMP ligand expression can be silenced by RNA interference [137]. Newly designed analogues are constantly being developed and screened to make more specific compounds, such as K02288 [140] or LDN-212854 [141]. They are both similar to LDN-193189, however they display higher selectivity against ACVR1/ALK2 and are very effective at low concentrations. Furthermore, K02288 specifically inhibits BMP-induced SMAD pathway without effecting TGF-β signalling. Chemical alterations of already known compounds provide a powerful tool to investigate BMP signalling in more depth and is the basis for pre-clinical drug development [140,142]. Another route is the delivery of recombinant BMP antagonists via gene transfer or exogenous products to increase extracellular antagonist concentrations [137] [24]. Alternatively, BMP-BMPR interaction can be mimicked by using decoy receptors, which comprise only the receptor binding domain of individual BMP receptors and thus can enhance particular binding affinity and decreases signalling [24]. However, BMPs exist both in solution and bound to ECM, which presents it to surrounding cells and can also sequester BMPs when needed. Sequestration of BMPs by ECM can prevent BMP receptor binding due to conformational changes [143].

These two modes of presentation can distinctly regulate cell behaviour and their role in the BM microenvironment, and need to be taken into account when inhibiting BMP signalling [85].

Deregulation of BMP signalling in CML upon TKI pressure needs to be further investigated in the future with the aim to identify key downstream target genes and to understand the crosstalk between BMP receptors, integrins and the ECM within the BM niche. Preliminary experiments have shown that combinatorial treatment of TKIs along with BMP receptor antagonists are a promising therapeutic approach to target the deregulated intrinsic and extrinsic BMP pathway in CML [105].
However, many current BMP receptor inhibitors display off target effects and often inhibit more than one type of receptor. Therefore, new and more specific antagonists or derivatives of already known compounds need to be developed. Companies such as M4K Pharma have been connecting scientists with different expertise across the globe in an open science policy to fast and efficiently develop new ALK inhibitors with the hope to effectively treat diffuse intrinsic pontine glioma (DIPG) and other BMP driven diseases. Future studies need to investigate not only combination therapy of TKIs and BMP antagonist, but also the physical cues and presentation of GF by the ECM in the BM microenvironment, as these are used by the LSCs to circumvent treatment effects and promote LSC self-renewal and persistence. In order for promising compounds to be developed clinically robust pre-clinical testing is required *in vivo*. Several pre-clinical mouse studies investigating the deregulation of BMP signalling in other diseases have proven the safety and efficiency of BMP pathway inhibitors. Dorsomorphin and LDN-193189 increased cancer survival and decreased cell viability in a dose-dependent manner in a mouse intraperitoneal xenograft model of epithelial ovarian cancer. In a mouse model of fibrodysplasia ossificans progressive (FOP), LDN-1893180 dramatically reduced heterotopic ossification [6,144]. Furthermore, spontaneous mouse models of metastatic breast cancer displayed a reduction in primary tumours burden using DMH1, an effective inhibitor of ACVRLI/ALK1, ACVR1/ALK2 and BMPR1A/ALK3 [6,145]. Mouse model studies in AML using subcutaneously implantable materials with human MSCs are able to create a humanized microenvironment with niche properties useful for haemopoietic studies. Adoption of similar platforms would be useful to study BMP pathway inhibitors as well as the expression of specific BMPs by MSCs [146] [147]. Such approaches could be employed to investigate targeting of the BMP pathway in CML in the future.

Table 2: BMP pathway inhibitors

Natural Antagonists	Target/mode of action	Reference(s)
Chordin	Inhibition of BMP and BMP-receptor interaction; interacts with BMP2, BMP4, BMP5, BMP6, BMP7 but not INHBA	[24,139,148]
Noggin	Blocks binding of BMPs to their receptor; binds to BMP2, BMP4, BMP5, BMP6, BMP7, BMP13, and BMP14	[139,149–151]
Gremlin	Inhibits predominantly BMP2 and BMP4; less potent to BMP7	[139,151–154]
Follistatin	Blocks activin receptor interaction and forms an inactive BMP and BMP receptor complex; binds BMP2, BMP4, BMP6 and BMP7 (but BMP2,4,6 with low affinity); does not inhibit BMP4 binding to its receptor, instead it forms a	[149,155–158]

	non-functional BMP-BMP receptor complex; can form an inactive complex with BMP15	
Cerberus	Direct binding to Nodal, Wnt, and BMP4 through distinct domains	[151,155,159,160]
Usag1	Binds and inhibits BMP2, BMP4, BMP6 and BMP7, also Wnt antagonist	[151,155,161]
Sclerostin (SOST)	Direct binding to BMP5, BMP6 and BMP7 with high affinity and to BMP2 and BMP4 with a lower affinity; only inhibits BMP6 and BMP7 activities; prevents binding of BMPs to their receptor	[155,159,162,163]
Coco	Binds and inhibits BMP4	[155]
PRDC/GREM2	Efficiently inhibits BMP2 and BMP4, and weakly inhibits BMP6 and BMP7; however, heparin interferes with binding of PRDC to BMP-2	[151,155,164,165]
DAN/NBL1	Directly binds and blocks BMP2, BMP4 and BMP7	[151,155,166]
Twisted Gastrulation (TWSG1)	Promotes and inhibits BMPs; binds BMP2, BMP4, and BMP7, but only inhibits BMP7; forms a ternary complex with BMPs and chordin to enhance the inhibitory activity of chordin on BMP	[151,155,167,168]
Crossveinless 2/BMPER	Binds BMP2, BMP4, BMP6, BMP7, BMP9; masking BMP2- receptor type I and type II binding interfaces in BMP2; inhibit BMP2- and BMP4-dependent osteoblast differentiation	[155] [169] [169]
Brorin/Vwc2 and Brorin-like	Inhibit BMP2 and BMP6	[139,170,171]
Tsukushi	BMP antagonist that acts in cooperation with Chordin; binds BMP4	[139,172,173]
BMPR inhibitors		
Dorsomorphin	Inhibition of ACVR1/ALK2, BMPR1A/ALK3, and BMPR1B/ALK6 – BMP type I receptors; significant off target effects (VEGF-2/KDR and PDGFR-β); pre-clinical study in epithelial ovarian cancer; dorsomorphin is still in preclinical development stage and no clinical trial is ongoing currently	[6,24,174–176]
LDN-193189 (LDN)	Targets ACVRLI/ALK1, ACVR1/ALK2, BMPR1A/ALK3 and BMPR1B/ALK6; more potent (ca 100fold) and metabolic stable than dorsomorphin; less off-target effect	[6,24,140,176,177]

	(ca 10 fold to PDGFR-β); only weakly inhibits ACVR1B/ALK4, TGFBR1/ALK5, and ACVR1C/ALK7; off target effects such as BMPR2 and TGFBR1/ALK5; Ongoing research further develops LDN-193189 in preparation for clinical testing in patients with FOP and autoimmune disease	
LDN-212854	Preferred selectivity for ACVR1/ALK2 in preference to ACVRLI /ALK1 and BMPR1A/ALK3 compared to LDN-193189; reduced off target effects compared to LDN; ongoing research in preparation for clinical testing in patients with FOP	[6,24,141,178]
LDN-214117	Higher selectivity for ACVR1/ALK2 than LDN-193189 or K02288 and lower cytotoxic profile; no clinical trials	[6,24,179]
1LWY	Enhanced selectivity for ACVR1/ALK2, reduced off-target effects compared to dorsomorphin and LDN, also selective for BMPR1A/ALK3 and BMPR1B/ALK6; no clinical trials	[24,180]
DMH1	Dorsomorphin analogue; highest selectivity for BMP pathway; very selective for ACVR1/ALK2; also selective for BMPR1A/ALK3, ACVRLI/ALK1, and BMPR1B/ALK6; no activity against TGFBR1/ALK5; no clinical trials	[6,24,181]
DMH2	Dorsomorphin analogue; pan type-inhibitor selective for BMPR1B/ALK6, BMPR1A/ALK3 and ACVR1/ALK2; off target effects such as ACVR1B/ALK4, TGFBR1/ALK5 and BMPR2; no clinical trials	[6,24,181] [182]
DMH3	Dorsomorphin analogue; reduced off targets effect compared to dorsomorphin and LDN; no clinical trials	[6,24,181]
ML347	Enhanced selectivity for ACVRLI/ALK1, ACVR1/ALK2; can discriminate between type 1 BMP receptors: >300-fold selectivity for ACVR1/ALK2 compared to BMPR1A/ALK3; reduced off target effect compared to dorsomorphin and LDN; no clinical trials	[6,178,183]
VU5350	Pan-type 1 BMP receptor inhibitor, targeting BMPR1A/ALK3, ACVR1/ALK2 and BMPR1B/ALK6, off target effects such as BMPR2; no clinical trials	[24,180]
K02288	Inhibits BMP-stimulated phosphorylation of SMAD1/5/8 without affecting TGF-β signalling; no clinical trials	[6,140]
PF-03671148	TGFBR1/ALK5 inhibitor, also shown to be selective against ACVRLI /ALK1; no clinical trials	[142,184]

PF-03446962	Inhibitor of ACVRLI /ALK1; In several clinical trial: patients with urothelial cancer, advanced malignant pleural mesothelioma and hepatocellular carcinoma	[185–187]
Compound 19	TGFBR1/ALK5 inhibitor; no clinical trials	[142,188,189]
Quinazolinone 1	Inhibition of ACVR1/ALK2 kinase domain; no clinical trials	[142] [190]
Neutralizing antibodies and extracellular domains (ECD)		Reviewed in [24]
Anti-BMP2 Ab	Blockage of BMP2	[191,192]
Anti-BMP4 Ab	Blockage of BMP4	[192–194]
Anti-BMP6 Ab	Blockage of BMP6	[195–197]
Anti-BMP7 Ab	Blockage of BMP7	[198,199]
Anti-BMP10 Ab	Blockage of BMP10	[200]
Anti-ALK1 Ab	Competes highly efficiently with the binding of the ACVRLI /ALK1 ligand BMP9 and TGF-β to ACVRLI/ALK1	[201]
BMPR1a-ECD	Inhibition of action of GDF9 and BMP15	[202]
Hemojuvelin-ECD	Inhibits BMP6	[195,203,204]
Dragon-ECD	Potent inhibitor of BMP2 or BMP4 but a less potent inhibitor of BMP6	[196]
ALK1-ECD	Binds with high affinity to BMP9 and BMP10; currently in clinical trial as cancer therapy	[205–209]
ALK3-ECD	prevent receptor-mediated signalling by sequestering BMP ligands; bind BMP2/4 specifically and with high affinity and prevent downstream signalling	[210–213]
ACVR2A-ECD	Inhibits BMP10 and BMP11	[214]
ACVR2B-ECD	Sequesters activins	[214,215]

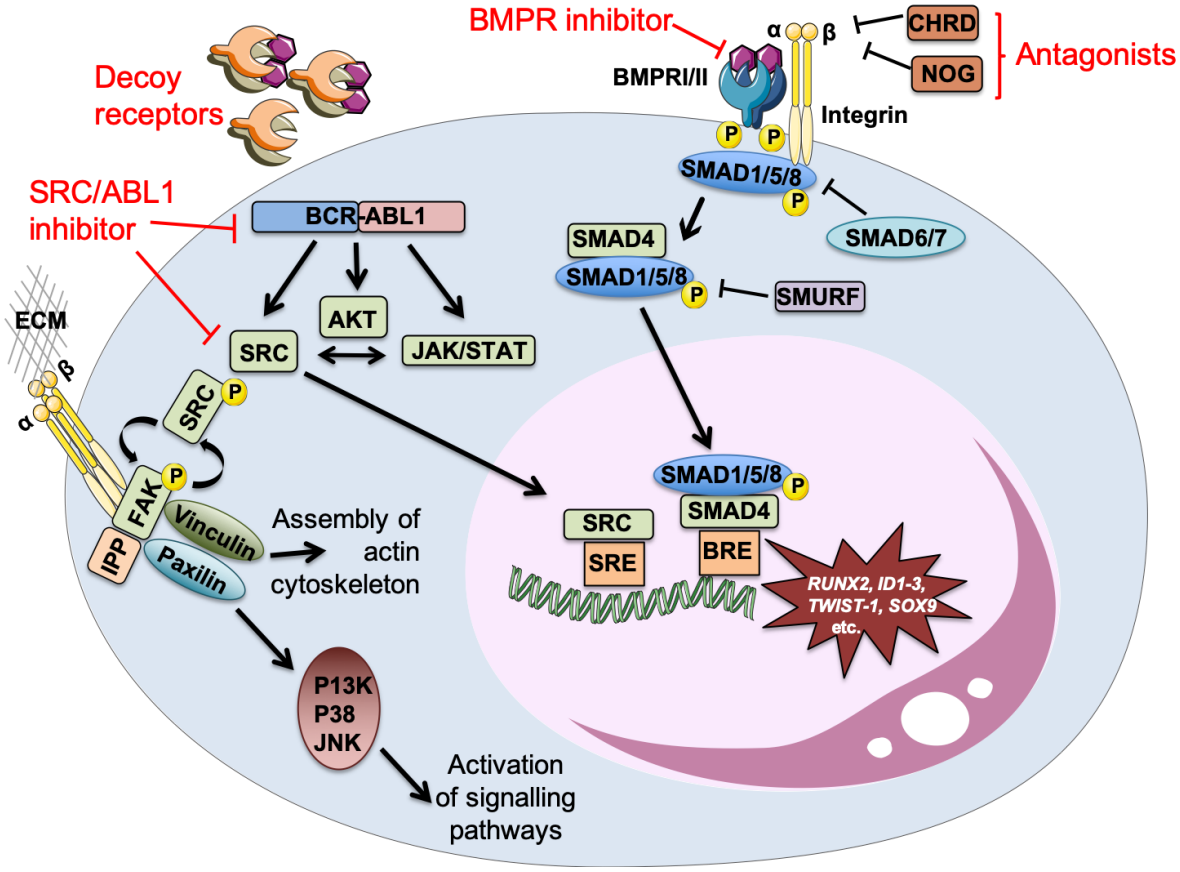

Figure 3: Current approaches to inhibit BMP/SMAD and BCR-ABL1 signalling.

There are several mechanisms that can be employed to prevent BMP pathway signalling and upregulation of early response genes, such as *RUNX2*, *ID1-3* or *TWIST-1* through BCR-ABL1 dependent and independent mechanism. The early response gene expression of *ID1-3* can be enhanced through BCR-ABL1 mediated STAT and SRC signalling with the genes having a SRC-responsive element (SRE) upstream of the translational start site. The same set of genes can be further activated through BMP/SMAD signalling that regulates gene expression through a BMP responsive element (BRE) in its promoter. Furthermore, BMPs as well as integrins react to mechanical properties of the ECM and together can form a mechanoreceptor complex (BMPR1B/ALK6 and αvβ3). The expression of integrins can increase in response to BMP signalling and integrin inhibition can reduce BMP responses and SMAD transcription. Inhibition or promotion of BMP signalling via integrins highlights the importance of mechanotransduction and thus activation of survival pathways. BMP molecules can be trapped by antagonists such as Noggin (NOG) or Chordin (CHRD) to prevent BMPR binding or BMPR inhibitors can block the binding site at the receptor itself. Furthermore, decoy receptors with enhanced binding affinity are able to mimic BMP-BMPR interaction and decrease signalling. This combined with TKIs against ABL1 and SRC to target BCR-ABL1 signalling is a promising avenue to explore in CML.

- **Importance of the field:** CML patient outcome has improved significantly since the introduction of TKIs, however resistance mechanism and permanent reliance of patients on treatment highlights the need to explore new therapeutic pathways. Intrinsic and extrinsic deregulation of the BMP pathway has been shown to be most prevalent in TKI resistant patients. Understanding GF signalling in the BM niche, particularly BMP

pathway deregulation and how this cross talks with BCR-ABL1 to mediate downstream effects on genes involved in self-renewal, proliferation and differentiation can open potential avenues for exploring new treatment strategies to improve patient outcomes in the future.

- **Summary of current thinking**: Higher abundance of BMP2 and BMP4 in CP-CML patients at diagnoses, persistence of BMPR1B/ALK6 high cells following TKI treatment and differential expression of BMP pathway related genes in CML indicates an important role for BMP pathway signalling in the pathogenesis of the disease. A variety of cells in the BM including stromal cells, megakaryocytes, platelets, osteoblasts and HSCs are able to produce BMP ligands, which in haematological malignancy can be perturbed and result in remodelling of the niche. Adverse amounts of BMPs impact on LSC behaviour such as self-renewal, proliferation and dormancy. Furthermore, the altered secretome can lead to changes in ECM composition, the balance in cell numbers and ultimately the dynamics within the niche. Bone remodelling itself plays an essential role in maintaining the niche's integrity and architecture with overexpression of BMP ligands such as BMP2, BMP4, BMP6 and BMP7 inducing osteoblastic differentiation, which could be important for sustaining LSCs persistence following TKI treatment.

- **Future directions:** When considering new treatments, it is necessary to take into account BM niche remodelling, including changes in GF levels and signalling combined with the ECM composition. One avenue to explore is the important cross-talk between BMP/SMAD and BCR-ABL1 signalling in CML (Figure 3). SRC is a key downstream kinase activated via BCR-ABL1 signalling. BMP signalling can cooperate with SRC to activate downstream targets especially the ID family of early transcription factors. ID genes support cell cycle progression, self-renewal and early differentiation of stem cells and contain both SRC responsive element (SRE) and BMP responsive element (BRE) upstream of the promoter region. Targeting both of these mechanisms using a dual ABL1/SRC TKI combined with inhibiting key ALKs (ACVR1/ALK2, BMPR1A/ALK3 and BMPR1B/ALK6) warrant further investigation in CML.

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