Targeting RSPO3-LGR4 Signaling for Leukemia Stem Cell Eradication in Acute Myeloid Leukemia

Graphical Abstract

Highlights

- RSPO-LGR4 is essential for stem cell self-renewal in a subset of AML
- LGR4 promotes aberrant self-renewal through cooperation with HOXA9
- RSPO3 acts as a stem cell growth factor to sustain proliferation of AML patient blasts
- Clinical-grade anti-RSPO3 impairs LSC self-renewal in patient-derived xenografts

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In Brief

Salik et al. identify a positive modulator of canonical WNT signaling pathway that drives the self-renewal of leukemia stem cells in a subset of acute myeloid leukemia (AML). An antibody blocking the ligand and receptor interaction of this pathway could be a potential therapeutic to treat aggressive AML.
Targeting RSPO3-LGR4 Signaling for Leukemia Stem Cell Eradication in Acute Myeloid Leukemia

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SUMMARY

Signals driving aberrant self-renewal in the heterogeneous leukemia stem cell (LSC) pool determine aggressiveness of acute myeloid leukemia (AML). We report that a positive modulator of canonical WNT signaling pathway, RSPO-LGR4, upregulates key self-renewal genes and is essential for LSC self-renewal in a subset of AML. RSPO2/3 serve as stem cell growth factors to block differentiation and promote proliferation of primary AML patient blasts. RSPO receptor, LGR4, is epigenetically upregulated and works through cooperation with HOXA9, a poor prognostic predictor. Blocking the RSPO3-LGR4 interaction by clinical-grade anti-RSPO3 antibody (OMP-13IR10/rosmantuzumab) impairs self-renewal and induces differentiation in AML patient-derived xenografts but does not affect normal hematopoietic stem cells, providing a therapeutic opportunity for HOXA9-dependent leukemia.

Significance

A fundamental challenge in the treatment of aggressive AML is relapse caused by the persistence of chemoresistant LSCs. Ablating LSCs by targeting deregulated self-renewal pathways is critical for successful anticancer therapy. Despite the recognized clinical importance, identification of pharmacologically tractable pathways required for self-renewal in a heterogeneous LSC pool remains largely unexplored. Here, we uncover an essential role for a positive modulator of canonical WNT signaling, RSPO-LGR4, in promoting aberrant self-renewal in a subset of AML. RSPO3-LGR4 pathway could be effectively blocked by an anti-RSPO3 antibody (previously used in clinical trials for solid tumors) that abrogates leukemia-initiating capacity of patient-derived LSCs without harming the healthy stem cell compartment, thus providing a therapeutic window to specifically target LSCs.
INTRODUCTION

Self-renewal and differentiation block are two characteristic features of leukemia stem cells (LSCs) contributing to tumor heterogeneity and malignancy in acute myeloid leukemia (AML). Like the normal hematopoietic stem cell (HSC) compartment, LSCs derived from HSCs or committed progenitors are functionally heterogeneous in primary human AML, with varying degrees of self-renewal capacity and developmental potential (Hope et al., 2004). A high relapse rate in AML suggests that current standard intensive chemotherapy does not target quiescent and highly self-renewing LSCs and therefore only provides durable therapy for a minority of patients. Identifying signals critical for driving high self-renewal activity in a heterogeneous LSC pool, which determines disease aggressiveness, is still a largely unexplored research area essential for designing effective cancer therapies.

HOXA9 is a key regulator of normal and malignant stem cells and controls the self-renewal/differentiation switch (Abramovich and Humphries, 2005). High expression of HOXA9 is an indicator of poor survival in a broad range of malignancies and is observed in approximately 50% of AML patient samples with diverse cytogenetic abnormalities, including translocations (Golub et al., 1999). Translocations involving the mixed-lineage leukemia (MLL) gene on chromosome 11q23, a relatively common cytogenetic abnormality in acute leukemia, result in the formation of chimeric MLL fusions (e.g., MLL-AF9) that are associated with poor outcome (Krivtsov and Armstrong, 2007; Muntean and Hess, 2012). In syngeneic mouse models, MLL-AF9 induces leukemic transformation of HSCs or committed progenitors by triggering an aberrant HOX-associated self-renewal gene expression program, including HOXA9 and the HOX cofactor MEIS1 (Krivtsov et al., 2006). HOXA9 and MEIS1 cooperate to block myeloid differentiation pathways before inducing AML in mice (Calvo et al., 2001; Kawagoe et al., 1999; Kroon et al., 1998; Lawrence et al., 1999). We and others have previously demonstrated that WNT/β-catenin signaling is required for self-renewal of LSCs in murine models of AML induced by MLL fusion-transduced bone marrow (BM)/committed progenitors or by coexpression of key MLL fusion targets HOXA9 and MEIS1 in HSCs (Wang et al., 2010; Yeung et al., 2010). However, key molecules involved in driving constitutive activation of β-catenin signaling and their capacity to control LSC functions still remain largely unclear.

WNT/β-catenin signaling is often active in human cancers, including AML (Cleurs and Nusse, 2012; Simon et al., 2005; Ysebaert et al., 2006). Of note, while aberrant activation of β-catenin frequently occurs in human LSCs (Majeti et al., 2009; Yeung et al., 2010) and is associated with poor overall survival in AML patients (Ysebaert et al., 2006), increased expression of WNT proteins has not been observed. Conversely, in normal HSCs, WNT3 treatment activates β-catenin and results in increased proliferation in vitro and enhanced self-renewal in vivo of adult HSCs (Reya et al., 2003; Willert et al., 2003), while WNT3 deletion in fetal liver hematopoiesis leads to decreased HSC numbers and impaired long-term repopulating capacity of HSCs upon serial transplantation in mice (Luis et al., 2009). This suggests that aberrant activation of WNT/β-catenin signaling in AML may require other developmental signaling molecules that function as agonists. The R-spondin ligands are candidates for such a role as they bind to and function through LGR4/LGR5 (leucine-rich-repeat-containing G protein-coupled receptors) and synergize with low levels of WNT ligands to potentiate β-catenin activation in normal adult stem cells from several organs, such as the intestine (Carmon et al., 2011; Lynch and Wang, 2016). While the LGR proteins appear to be physically associated with the WNT receptor complex Frizzled-LRP5/6, the potency of R-spondins in enhancing WNT signals is not affected by the level of WNT receptors, but instead largely depends on the abundance of LGR expression (Carmon et al., 2011; de Lau et al., 2011). Notably, LGR4 is expressed predominantly in fetal liver long-term reconstituting HSCs that have extensive self-renewal capacity, implicating a role for LGR4 in early hematopoietic development (Liu et al., 2014). Among R-spondin ligands (RSPO1-RSPO4), RSPO3 is prominently expressed in hematopoietic organs (Kazanskaya et al., 2008). These observations suggest a possible involvement of RSPO-LGR4 signaling in malignant hematopoiesis.

RESULTS

Inhibition of LGR4 Exerts Origin-Specific Blockade of Aberrant Self-Renewal and AML Progression

Consistent with previous observations, our integrated analysis of five independent microarray datasets (GEO: GSE13159, GSE15434, GSE61804, GSE14468, and The Cancer Genome Atlas [TCGA]) showed increased levels of LGR4 but no noticeable changes of the related family member LGR5 across 481 AML patient samples harboring diverse cytogenetic abnormalities, including 11q23/MLL, compared with normal human HSCs (GEO: GSE42519, p < 0.0291; Figure S1 A) (Bagger et al., 2016). LGR4 plays an essential role during development as genetic ablation of Lgr4 in mice results in embryonic and perinatal lethality (Mazerbourg et al., 2004). To understand the functional involvement of LGR4 in leukemogenesis, we used a lentiviral vector-based short hairpin RNA (shRNA) system to knock down the expression of Lgr4 (Lgr4sh1 and Lgr4sh2) in murine MLL-AF9-HSPC pre-LSCs, which were generated by transducing an HSC-enriched hematopoietic stem/progenitor cell population (HSPCs or LSK: Lin−Sca-1+c-Kit+) with lentivirus encoding the MLL-AF9 fusion oncogene. MLL-AF9 serves as an initiating oncogenic event to promote self-renewal and a myeloid differentiation block in normal HSPCs and MLL fusion oncogenes are critically dependent on β-catenin activity (Krivtsov et al., 2006; Wang et al., 2010; Yeung et al., 2010). Efficient depletion of Lgr4 protein substantially reduced expression of endogenous β-catenin, as well as several key WNT/self-renewal target genes, including c-Fos, Tcf7l2, Ccnd1, and Mef2c, whereas overexpression of a constitutively active form of β-catenin (β-cat*) (Wang et al., 2010) rescued the defect in colony formation caused by Lgr4 depletion (Figures 1A–1D and S1B). This provides evidence supporting WNT/β-catenin targets as essential components downstream of LGR4 signaling.

To define a direct role of LGR4 in LSC regulation and AML development, MLL-AF9-HSPC Lgr4sh pre-LSCs were
Figure 1. Inhibition of LGR4 Exerts Origin-Specific Blockade of Aberrant Self-Renewal and AML Progression in Mice
(A) Schematic outline of the experimental procedure.
(B) Western blots confirming sufficient knockdown of Lgr4 and a resultant reduction of endogenous β-catenin protein in MLL-AF9-HSPC pre-LSCs carrying scrambled control (Scr) versus Lgr4 shRNAs.

(legend continued on next page)
transplanted into sublethally irradiated C57BL/6 (BL6) syngeneic recipient mice. Our results showed that only 2 out of 13 (15%) mice that received Lgr4sh1 pre-LSCs and 4 out of 13 (30%) mice that received Lgr4sh2 pre-LSCs developed AML, while all control mice succumbed to primary AML with a short latency (Figure 1E). These data suggest that inhibition of Lgr4 impairs the initiation and progression of HSPC-derived MLL-AF9 AML in mice. Furthermore, Lgr4 depletion inhibited the ability of pre-LSCs to expand in mouse BM and to infiltrate into the spleen, demonstrated by decreased percentages of GFP+ leukemic cells in these organs as well as lower spleen weights (Figures 1F and S1C). Morphological analysis of BM smears only exhibited abnormal immature blasts in scrambled control (Scr), whereas both leukemic blasts and differentiated myeloid cells (e.g., neutrophils) were observed in Lgr4-deficient leukemic cells indicating Lgr4 depletion-induced differentiation (Figure 1G). LSCs might have been severely compromised by Lgr4 depletion during development since LSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>-c-Kit<sup>hi</sup>CD16/32<sup>hi</sup>CD34<sup>+</sup>) (Krivtsov et al., 2006) flow-sorted from the BM of primary AML lacked the ability to expand in methylcellulose (data not shown). GFP<sup>+</sup> Lgr4-deficient leukemic cells from primary AML could only transiently expand in the mouse BM with a reduced proliferative capacity, as determined by in vivo bromodeoxyuridine (BrDU) cell proliferation assays at 10 days post-transplantation, but eventually failed to induce secondary leukemia (Figures 1H and 1I). In contrast, control leukemic cells could be efficiently expanded in the mouse BM and developed AML with a shorter latency in secondary recipients compared with primary engrafted mice (Figures 1H, 1I, and 1E). Upon sacrifice of Lgr4sh1 and Lgr4sh2 secondary recipients at 122 days after transplantation, GFP<sup>+</sup> leukemic cells were not detectable by flow cytometry in both BM and spleen (data not shown). Thus, inhibition of Lgr4 has potent leukemia-inhibitory effects and prevents the development of HSPC-derived MLL-AF9 AML in vivo.

To determine whether the cell of origin influences the dependence of pre-LSCs on Lgr4 signaling, we generated pre-LSCs originating from more committed progenitors by transducing MLL-AF9-GFP into granulocyte-macrophage progenitors (GMPs: Lin<sup>-</sup>Sca-1<sup>-</sup>-c-Kit<sup>+</sup>CD16/32<sup>hi</sup>CD34<sup>+</sup>) (Krivtsov et al., 2006), which expressed relatively lower levels of Lgr4 than HSPCs (Figure S1D). Unlike Lgr4 depletion in MLL-AF9-HSPC pre-LSCs, which caused a substantial decrease in WNT/self-renewal target genes (Figure 1C), knockdown of Lgr4 in MLL-AF9-GMP pre-LSCs did not significantly alter the expression of c-Fos but had a lesser although still significant effect on expression of Tcf7l2, Ccnd1, and Melc2 as well as endogenous β-catenin (Figures 1J and S1F). Lgr4 depletion in MLL-AF9-GMP pre-LSCs resulted in delayed disease onset and extended survival as well as blast cell differentiation (e.g., neutrophils) and a reduced ability of pre-LSCs to expand in mouse BM; however, it had no influence on spleen weights and pre-LSC infiltration into spleen (Figures 1K–1M and S1F). In addition, unlike the potent anti-proliferative effect of Lgr4 depletion on GFP<sup>+</sup> HSPC-derived leukemic cells in vivo, Lgr4 depletion revealed negligible inhibitory effect on the engraftment of GFP<sup>+</sup> GMP-derived MLL-AF9 leukemic cells in the mouse BM at 10 days post-transplantation (Figure 1N). As a result, Lgr4-deficient GMP-derived leukemic cells were capable of engrafting secondary recipients with a slightly prolonged leukemia latency in mice, indicative of perhaps partially impaired LSC self-renewal (Figure 1O).

Our results demonstrate that dependency on LGR4 signaling may rely largely on the abundance of endogenous LGR4 expression in MLL-AF9 pre-LSCs at the initial stage of LSC development. HSPC-derived AML exhibiting higher LGR4 expression requires LGR4 for LSC functions and leukemia progression; on the other hand, GMP-derived AML has a partial requirement for LGR4. Thus, the cell of origin contributes to tumor heterogeneity in AML by determining functional effects of LGR4 on LSC self-renewal.
Figure 2. LGR4 Cooperates with HOXA9/MEIS1 Producing a Highly Aggressive Short Latency AML

(A) Microarray data analysis of 183 AML patient samples (Cancer Genome Atlas Research Network, 2013) showing a significant positive correlation between expression of LGR4 and HOXA9. p < 0.0001 and r = 0.546.

(B) Western blots revealing higher levels of endogenous Lgr4 and β-catenin proteins in MLL-AF9- HSPC pre-LSCs than in HOXA9/MEIS1 (A9M)-HSPC pre-LSCs.

(C) Western blots confirming overexpression of Lgr4 (Lgr4OE) in A9M-GMP pre-LSCs transduced with MSCV-Lgr4-neo.

(D) Kaplan-Meier survival curves of mice receiving neo versus Lgr4OE A9M-GMP pre-LSCs. 1 x 10^6 pre-LSCs were transplanted into sublethally irradiated BL6 recipient mice for the development of primary AML. n = 12 mice per group. p value was determined by the log rank test.

(E) Kaplan-Meier survival curves of mice receiving neo versus Lgr4OE A9M-HSPC pre-LSCs or MLL-AF9-HSPC pre-LSCs (n = 6 mice). 1 x 10^6 pre-LSCs were transplanted into sublethally irradiated BL6 recipient mice for the development of primary AML. p value was determined by the log rank test.
LGR4 Cooperates with HOXA9/MEIS1 Producing a Highly Aggressive Short-Latency AML

HOXA9 and MEIS1 are considered to be critical targets in MLL-rearranged leukemias. Coexpression of these genes in normal HSPCs induces AML although with a less aggressive phenotype compared with MLL-AF9 in murine models (Dietrich et al., 2014; Krivtsov et al., 2006; Wang et al., 2010). Intriguingly, we observed a positive correlation between expression of LGR4 and HOXA9 in 183 human AML patient samples (Figure 2A; r = 0.546, p < 0.0001) (Cancer Genome Atlas Research Network, 2013). This implies a potential cooperative role for LGR4 in HOXA9-mediated transformation.

To confirm this, we generated HOXA9/MEIS1 pre-LSCs by transforming LSK or GMP cells with HOXA9-GFP and MEIS1-puro followed by transduction with Lgr4. HOXA9/MEIS1-HSPC pre-LSCs expressed relatively lower protein levels of Lgr4 and β-catenin compared with MLL-AF9-HSPC pre-LSCs (Figure 2B). We found that Lgr4 itself was not able to fully transform normal HSPCs (data not shown) or HOXA9/MEIS1(A9M)-transduced GMP (Figures 2C and 2D), but instead could cooperate with HOXA9/MEIS1 in HSPCs to accelerate disease onset and produce leukemia with shortened latencies similar to those in MLL-AF9-HSPC AML (Figure 2E). The enhanced aggressive phenotype in HOXA9-driven AML might be caused by an Lgr4OE-induced increment in proliferation capacity in vivo, as we observed a 5-fold increase in GFP+ leukemic cells observed in the BM at 1-month post-transplantation of pre-LSCs into BL6 recipient mice, as well as by an Lgr4OE-mediated increase in the quiescent (G0) stem-like population accompanied by a decrease in intracellular reactive oxygen species levels in the BM of primary AML (Figures 2F–2H). Western blot analysis confirmed that Lgr4 overexpression in GFP+ HOXA9/MEIS1 leukemic BM cells upregulated endogenous β-catenin, reaching a level similar to that in GFP+ MLL-AF9 leukemic BM cells (Figure 2I). These data are in line with increased basal levels of LGR4 in HSPCs cooperating with HOXA9/MEIS1 to promote leukemia in mice that mirrors the full-blown aggressive MLL-AF9 AML phenotype.

Consistent with our observations in murine models, overexpression of LGR4 substantially increased tumor aggressiveness and reduced survival in NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice xenotransplanted with human MLL-AF9 AML cell line (THP-1) and this was associated with increased levels of non-phosphorylated (active) β-catenin in human CD33+ myeloid cells from the BM of primary recipients (Figures S2A–S2C). Despite abundant expression of LGR4 in both THP-1 cells and the human BCR-ABL-positive chronic myeloid leukemia K562 cell line, depletion of LGR4 decreased tumorigenesis and increased survival of THP-1 xenograft mice, but did not affect survival in K562 xenograft mice (Figures S2D–S2F). The difference in LGR4 function could largely be attributed to endogenous expression of HOXA9, which was substantially higher in THP-1 cells but absent in K562 cells (Figure S2D).

We found that expression levels of HOXA9 affected LGR4 function. Analysis of a published microarray dataset (Faber et al., 2009) supports this notion, showing that depletion of HOXA9 with shRNA caused a significant decrease in expression of LGR4, MEF2C, and MEIS1 in human MLL-AF9 AML cell line (MOLM-14) (Figure 2J). Lgr4 depletion prolonged survival in HOXA9/MEIS1-HSPC mice, albeit to a much lesser extent than that in MLL-AF9-HSPC mice (Figures 1E and 2K). Immunophenotypic analysis revealed reduced Gr-1−/low c-Kithigh LSC-enriched population and induced differentiation (e.g., neutrophils) by Lgr4-depletion in HOXA9/MEIS1 leukemic BM cells (Figures 2L and 2M). We have previously documented that the Gr-1−/low c-Kithigh population is over 100-fold enriched for LSCs as compared with the Gr-1+c-Kithigh population in the heterogeneous LSC pool of HOXA9/MEIS1-HSPC AML (Wang et al., 2010). These results underscore an active and cooperative role of LGR4 in supporting HOXA9-dependent leukemogenesis.

LGR4 Expression Is Upregulated by an Epigenetic Mechanism in Human AML Cell Lines

HOXA9 and MEIS1 are reportedly upregulated by DOT1L-mediated H3K79 methylation in aggressive leukemia driven by oncoproteins, such as MLL-AF9, MLL-AF10, and CALM-AF10 (Bernt et al., 2011; Chen et al., 2013). High levels of H3K79 methylation at the loci of HOXA9 and MEIS1 are associated with high expression levels of these genes. To understand how LGR4 expression is regulated, we analyzed two independent chromatin immunoprecipitation sequencing datasets for occupancy profiles of H3K79me2 and our analysis displayed the enrichment of H3K79me2 on the loci of HOXA9, MEIS1, LGR4, and MEF2C in human MLL-AF9 AML (MOLM-13) cells but not in human promyelocytic leukemia (HL-60) cells, which expressed relatively low levels of LGR4 and HOXA9 (Figures S2D, S3A, and S3B).

Likewise, an analysis of a microarray dataset revealed that DOT1L inhibitor EPZ004777, which decreased H3K79me2 levels (Daignel et al., 2011), substantially reduced expression of HOXA9,
MEIS1, LGR4, and MEF2C in MOLM-13 cells (Figure S3C). Our results confirmed the findings and showed that DOT1L inhibitor SG0946, which reduced H3K7me2, induced suppression of HOXA9, LGR4, and MEF2C expression in MOLM-13 and THP-1 cells, as well as in hCD33+ AML patient-derived xenograft (PDX) cells, but not in HL-60 cells (Figures S3D–S3G). It is thus likely that driver mutations (e.g., MLL-AF9) upregulate LGR4 expression, at least in part, through DOT1L-mediated H3K79 methylation, which is well known for active transcription of target genes in aggressive AML (Bent et al., 2011; Chen et al., 2013).

**Inhibition of LGR4 Blocks RSPO3 Function in Murine Pre-LSCs and Primary AML PDX Model**

LGR4 functions as a receptor of R-spondin ligands (RSPO1–RSPO4), which are potent WNT signal enhancers (Kazanskaya et al., 2004; Kim et al., 2005). Our data showed that RSPO or WNT3 alone was not sufficient to enhance endogenous β-catenin expression in murine pre-LSCs induced by MLL-AF9 or HOXA9/MEIS1 (Figures S4A–S4F). We found that only a combination of WNT3 and RSPO2/RSPO3 exclusively increased endogenous β-catenin expression; conversely, the combination of WNT3 and RSPO1/RSPO4 did not affect β-catenin levels (Figures S4A–S4C). Increasing concentrations of RSPO3 (50–200 ng/mL) or WNT3 (10%–20%, v/v) did not increase β-catenin expression (Figures S4D–S4F). Importantly, inhibition of Lgr4 completely abrogated RSPO3/WNT3-mediated β-catenin activation (Figure S4G), indicating a crucial role for LGR4 in relaying the RSPO signal to exert downstream oncogenic effects.

We next validated the above findings in a clinically relevant AML PDX mouse model using CRISPR/Cas9 technology to knock out LGR4. The AML PDX model was established by xenotransplanting NSG mice with primary human AML cells from a relapsed patient harboring mutations in DNMT3A, RUNX1, KRAS, NRAS, PTPN11, ETVE, and BCOR (Table S1). AML PDX cells expressed high levels of LGR4 and HOXA9 (Figure 3A). LGR4 and non-phospho (active) β-catenin were predominantly expressed in the hCD34+ HSPC population compared with the hCD34+ compartment in AML PDX BM cells (Figures 3B and S4H), implicating a possible role for LGR4 in regulating human leukemia-initiating cells in primary AML. CRISPR/Cas9-mediated knockout of LGR4 not only markedly decreased nuclear active β-catenin and consequently reduced leukemia burden in firefly luciferase (FLuc)-expressing AML PDX mice monitored by in vivo bioluminescence imaging, but also blocked RSPO3/WNT3-induced increase in hCD34+ immature cells ex vivo (Figures 3C–3E, S4I, and S4J). These findings support the clinical relevance of LGR4 oncogenic function and underscore the importance of RSPO-LGR4 signaling in sustaining primary human hCD34+ immature cells.

**RSP02/3 Serve as Stem Cell Growth Factors to Sustain the Differentiation Block in AML Patient Blasts Ex Vivo, whereas Anti-RSPO3 Antibody Reduces Leukemia Burden in NSG Mice Engrafted with Primary AML Patient Specimens**

Primary AML patient blasts depend on external signals, such as growth factors and cytokines from the BM niche for survival and growth (Konopleva and Jordan, 2011); thus, these immature cells are difficult to culture and will differentiate if necessary and sufficient signals are not received. Given our observation that RSPOs as secreted growth factors potentiated WNT/β-catenin activation required for LSC development, we investigated the ability of exogenous RSPO proteins to maintain primary AML blasts in their undifferentiated, immature state. We found that LGR4/HOXA9-coexpressing patient blasts were predominantly dependent on RSPO/WNT3 pathway activation and could be grown ex vivo in the presence of exogenous RSPO2/3 and WNT3 ligands (Figures 4 and S5A–S5D).

In this study, we first performed qPCR analysis on 16 primary AML patient samples and identified 5 patient specimens coexpressing LGR4 and HOXA9 (Figure 4A). Our data showed that 8 out of 16 (50%) samples expressed high levels of LGR4, while 5 out of 8 LGR4-expressing samples revealed relatively high levels of HOXA9 (Figure 4A). We next assessed the impact of endogenous HOXA9 expression on the response of LGR4-expressing patient blasts to RSPO/WNT3 ligands. Seven LGR4-expressing patient specimens (i.e., five with high HOXA9 and two with low HOXA9) were cultured under four different conditions, including medium only, cytokines (a cocktail consisting of stem cell factor, FLT3 ligand, thrombopoietin, interleukin-3 [IL-3], and IL-6), RSPO2/WNT3, or RSPO3/WNT3 (Figure 4B; Tables 1 and S2). AML blasts in all seven LGR4-expressing patient specimens retained the immature phenotype accompanied with increased human CD34+ cells in cytokines, but rapidly differentiated in medium only (Figures S5A–S5G). Unlike cytokines that functioned irrespective of HOXA9 expression, RSPO/WNT3 ligands sustained an immature blast-like phenotype only in LGR4/HOXA9-coexpressing AML patient specimens, such as those harboring 9p deletion or MLL rearrangements (MLL-AF1q, MLL-AF9, or MLL-AF10), but did not maintain the undifferentiated, proliferating state in AML patient specimens with normal karyotype or harboring AML1-ETO that lacked HOXA9, albeit with high levels of LGR4 expression (Figures 4B, 4C, and S5A–S5G; Table S2). These observations not only support a cooperative function of HOXA9 in RSPO-LGR4 pathway activation, but also underline the role of RSPO2/3 as growth factors that increase proliferation and preserve the immature stem cell-like phenotype of primary human AML patient blasts.

Notably, anti-RSPO3 monoclonal antibody (OMP-131R10/romsantuzumab; OncoMed Pharmaceuticals), which has proven to be safe and well tolerated in phase 1 clinical trials in advanced solid tumors, blocked RSPO3/WNT3-induced increase of human CD34+ immature blasts in primary AML patient specimens and PDX cells, phenocopying the inhibitory effect of LGR4 knockout in AML PDX cells (Figures 3D, S5A, S6A, and S6B). The anti-RSPO3-induced phenotype was associated with suppression of endogenous LGR4 and HOXA9 expression (Figure S6C). Altogether, these findings uncover a critical dependency of LGR4/HOXA9-coexpressing primary human AML cells on RSPO/WNT3 ligands for their growth and survival, implicating anti-RSPO3 antibody as a potential therapeutic agent for AML treatment.

We next examined the in vivo therapeutic potential of anti-RSPO3 antibody by treating NSG mice engrafted with primary AML patient specimens (Figure 4D). Among five AML patient specimens coexpressing LGR4 and HOXA9, three harboring 9p deletion, MLL-AF9, or MLL-AF1q were successfully engrafted consistent with an approximate 50% engraftment rate of primary
AML patient samples in NSG mice (Krevvata et al., 2018). In line with our ex vivo observations, all engrafted patient specimens responded to anti-RSPO3 antibody treatment in vivo, resulting in markedly decreased percentages of human CD34+CD45+ LSC-enriched cells as well as reduced spleen weight and size in NSG mice (Figures 4E–4G and S6D–S6F). Likewise, in vivo anti-RSPO3 treatment significantly reduced expression of endogenous LGR4 and HOXA9 (Figure S6E). These findings support the therapeutic value of anti-RSPO3 antibody for targeting primary patient cells in the treatment of AML dependent on HOXA9, an indicator of poor prognosis.

Clinical-Grade Anti-RSPO3 Antibody Targets LSC-Enriched Populations in AML PDXs but Not Normal Human HSPCs in NSG Mice

Consistent with our in vivo observations of primary AML patient samples directly engrafted in NSG mice, the established PDX model of primary AML responded to anti-RSPO3 antibody treatment, which markedly reduced leukemia burden and diminished the percentage of hCD34+ cells in mouse BM (Figures 5A and 5B). The anti-RSPO3-response was primarily attributable to a substantial decrease in nuclear active β-catenin and several key WNT/self-renewal target genes, including c-FOS, TCF7L2, CCND1, MEF2C,
AML patient specimens (n=7)

9p deletion
MLL-AF1q
MLL-AF9
MLL-AF10 (D)
MLL-AF10 (R)
AML1-ETO (P1)
Normal karyotype (P1)

HOXA9 high
(n=5)

HOXA9 low
(n=2)

Figure S5
Table S2

AML patient specimens (n=5)

RSPO/WNT3
Responding
Blast cell retention

In vivo

PDX - 9p deletion

anti-RSPO3

Ex vivo

Medium only

Cytokines

RSPO2/WNT3

RSPO3/WNT3

A

B

C

D

E

F

G

(legend on next page)
and HOX cluster genes HOXA7 and HOXA11 in hCD34+ cells, as well as induced differentiation of hCD33+ myeloid blasts (Figures 5C–5E). Subsequent in vivo serial transplantation showed that hCD33+ myeloid blasts isolated from anti-RSPO3-treated primary mice failed to engraft into secondary NSG recipients (Figure 5F), indicating anti-RSPO3-induced functional impairment in the LSC compartment in the PDX mouse model of primary human AML.

In contrast to the observation in AML PDX mice, anti-RSPO3 treatment (20 mg/kg, intraperitoneally, once per week for 5 weeks) had negligible growth-inhibitory effects in NSG mice engrafted with expanded normal human CD34+ HSPCs (Figures 5G and S7A–S7D), which would be a consequence of intrinsic relative LGR4 independence. Collectively, we conclude that the anti-RSPO3 antibody selectively targets CD34+ LSC-enriched populations in primary human AML and has potential as a therapeutic agent for the treatment of leukemias, which rely on RSPO-LGR4 activation associated with coexpression of LGR4 and HOXA9.

**RSPO-LGR4 Upregulates Key WNT/Self-Renewal Target Genes through the pCREB-CBP Pathway**

We next investigated how RSPO-LGR4 regulates WNT/self-renewal target genes. G protein-coupled receptors often activate downstream signaling pathways through their coupled G protein subunits. We found a strong correlation between expression of LGR4 and GNAS in 1,064 AML patient samples irrespective of their subtypes, by analyzing an integrated gene expression dataset of five independent studies (Figure 6A; r = 0.789, p < 0.0001; GEO: GSE13159, GSE15434, GSE61804, GSE14468, and TOGA) (Bagger et al., 2016). In agreement with this observation, overexpression of G protein z subunit Gaα (encoded by GNAS) not only increased non-phospho (active) β-catenin to the level similar to that induced by RSPO2/WNT3 in human MLL-AF9 AML (THP-1) cells, but also substantially augmented RSPO2/WNT3-induced activation of β-catenin (Figure 6B). Conversely, Gaα knockdown with GNAS shRNAs reduced the level of active β-catenin (Figures 6C and S8A). These results suggest an active involvement of Gaα in RSPO-LGR4 signaling.

Gaα has a known function in cAMP-dependent pathway activation. The cAMP-Glo assay and western blot analyses showed that RSPO2/WNT3 stimulation significantly increased the intracellular cAMP level (Figure S8B), which increased phosphorylation of nuclear transcription factor CREB (cAMP-response element binding protein) at Ser133 in human MLL-AF9 THP-1 cells (Figures 6D). LGR4 depletion resulted in a marked decrease in p-CREB, which is an active transcriptional form (Figure 6E). Knockdown of CREB with shRNAs resulted in reduced levels of p-CREB and decreased expression of both active and total β-catenin (Figures 6F and S8C). The phosphorylation of CREB is associated with poor survival and increased risk of relapse in AML patients (Shankar et al., 2005). Elevated nuclear p-CREB was observed in hCD33+ AML PDX cells; conversely, in vivo anti-RSPO3 treatment inhibited phosphorylation of nuclear CREB in hCD34+ cells isolated from the BM of treated AML PDX mice (Figures 6G and 6H). The phosphorylation event enables CREB to bind to and recruit histone acetyltransferases CBP (CREB-binding protein) and its paralog p300, which increase acetylation of nuclear transcription factors (e.g., β-catenin) to activate downstream target genes (Chrivita et al., 1993; Hoffmeyer et al., 2017).

In agreement with the inhibitory effect of LGR4 knockout or anti-RSPO3 treatment in AML PDXs (Figures 3C and 5), we observed that treatment with I-CBP112, a selective inhibitor of the CBP/p300 bromodomain, triggered cell differentiation and blocked RSPO3/WNT3-induced increase in hCD34+ immature cells (Figures 6I and S8D). The I-CBP112-induced deficient phenotype was associated with significantly decreased expression of key WNT/self-renewal target genes, including MEIS1, MEF2C, CCND1, and HOX cluster genes HOXA7, HOXA9, and HOXA11, as well as β-catenin transcriptional cofactors c-FOS and TCF7L2 in AML PDX cells (Figure 6J). Collectively, these data suggest that RSPO-LGR4 regulates WNT/self-renewal target genes, at least partially, through the pCREB-CBP pathway in primary AML. We cannot rule out the possibility that LGR4 activates pathways in addition to pCREB-CBP. Thus, blocking the RSPO3-LGR4 interaction by anti-RSPO3 antibody is superior to targeting pathways downstream of LGR4 for the therapeutic purpose.

**DISCUSSION**

AML is a highly heterogeneous and aggressive malignancy initiated with the formation of pre-LSC clones through a range of
Figure 5. Clinical-Grade Anti-RSPO3 Antibody Reduces Leukemia Burden and Compromises Primary LSCs in an AML PDX Mouse Model but Does Not Affect Normal Human HSPCs in NSG Mice

(A) In vivo bioluminescence imaging and total flux (p/s) of AML PDX mice treated with anti-RSPO3 antibody (20 mg/kg, i.p. once per week for 4 weeks). One mouse was excluded from further analysis as the baseline bioluminescent signal was notably higher than the cohort, **p < 0.005. Two-way ANOVA.

(B) Percentages of hCD34+ cells engrafted in the BM of AML PDX mice in response to in vivo anti-RSPO3 treatment. Scatter dot plots are represented as the mean ± SD. Unpaired t test. *p < 0.05.

(C) Representative IF images displaying reduced levels of non-phospho (active) β-catenin in hCD34+ HSPCs isolated from AML PDX mice in response to in vivo anti-RSPO3 treatment. Scale bars, 10 µm.

(D) qPCR showing downregulation of key WNT/self-renewal target genes in hCD34+ cells isolated from AML PDX mice in response to in vivo anti-RSPO3 treatment. Mean ± SD. n = 3 replicates per condition. Unpaired t test. ****p < 0.0001.

(E) Wright-Giemsa staining (x60 magnification) of hCD33+ cells isolated from AML PDX mice showing anti-RSPO3-induced differentiation of immature AML PDX cells. Arrows indicate metamyelocytes and neutrophils.

(F) In vivo bioluminescence imaging and total flux (p/s) of secondary NSG-recipient mice, which were xenotransplanted intravenously with 3 × 10⁴ hCD33+ myeloid cells isolated from primary AML PDX mice treated with control versus anti-RSPO3 antibodies. Note: transplanted secondary recipient mice received no further treatment. ****p < 0.0001. Two-way ANOVA.
Table 1. Clinical Characteristics of AML Patient Samples

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Initiating oncogenic events (e.g., recurrent mutations, gene fusions involving the MLL gene, increased HOXA9/MEIS1), resulting in an aberrant self-renewal program and providing a selective growth advantage over their origin (e.g., HSPCs and GMPs) (Cozio et al., 2003; Krivtsov et al., 2006; Welch et al., 2012). Each pre-LSC may acquire different additional cooperating event(s) (e.g., increased WNT/b-catenin, DOT1L-mediated H3K79 methylation), enabling pre-LSCs to further transform into a heterogeneous LSC pool with enhanced self-renewal activity (Bernt et al., 2011; Krivtsov et al., 2006; Wang et al., 2010; Yeung et al., 2010). Given that persistent LSCs are a major cause of treatment failure and relapse that occurs frequently for certain aggressive AML subtypes, understanding the transformation process and pharmacologically tractable pathways that sustain the LSC pool is of crucial importance for the design of effective cancer therapies.

Previous studies have shown that the cell of origin is an important factor controlling disease phenotype and clinical outcome in AML, where pre-LSCs originated from HSPCs develop a more aggressive subtype of AML than do those originated from GMPs serving as a reservoir of disease in patients with poor survival (Krivtsov et al., 2013; Stavropoulou et al., 2016). Likewise, our results suggest that LSCs derived from HSPCs appear to rely heavily on LGR4 signaling for self-renewal and the establishment of a particularly aggressive phenotype in mice; conversely, LSCs derived from GMPs exhibit only a partial dependency on LGR4 signaling. It is likely that LSCs developed from different origins utilize distinct endogenous signaling mechanisms driving tumorigenesis. We have previously shown that Gaq signaling is required for the maintenance of GMP-derived MLL-AF9 AML, and genetic and pharmacological blockade of Gaq suppresses b-catenin activity leading to impaired leukemogenesis via inhibition of mitochondrial energy metabolism and activation of GADD45α-associated stress response (Lynch et al., 2016). Notably, recent studies demonstrate a direct link between LGR4 and Gaq signaling where LGR4 relays response (Lynch et al., 2016). Thus, GM-P-derived MLL-AF9 AML may require co-activation and cooperation of multiple signaling pathways, including LGR4 and Gaq, to maintain b-catenin activity and self-renewal. In support of this notion, we observe that overexpression of LGR4 in GMP-derived HOXA9/MEIS1 pre-LSCs increases leukemia incidence but does not significantly affect the survival rate in mice; this is different from the potent pro-oncogenic effect of LGR4 in HSPC-derived HOXA9/MEIS1 AML, indicating additional upstream molecules contributing to the activation of b-catenin required for GMP transformation via HOXA9/MEIS1. Our findings underline the importance of identifying key regulatory elements upstream of b-catenin signaling as they have the capacity to manipulate downstream signal events that determine origin-specific tumorigenic effects. The origin-dependent difference in pathway requirements contributes to the heterogeneity of the LSC pool within a tumor.

(G) Percentages of normal hCD45+ cells engrafted in the peripheral blood (PB) and hCD34+ HSPCs in the BM of NSG-recipient mice at 106 days post-transplantation, after in vivo anti-RSPO3 antibody treatment (20 mg/kg, i.p. once per week for 5 weeks). Data are represented as the mean ± SD. n = 3 mice per group. Unpaired t test. NS, not significant (p > 0.05).

See also Figure S7.
Figure 6. RSPO-LGR4 Upregulates Key WNT/Self-Renewal Target Genes through the pCREB-CBP Pathway

(A) Integrated analysis of five independent gene expression datasets (GEO: GSE13159, GSE15434, GSE61804, GSE14468, and TCGA) (Bagger et al., 2016) showing a strong positive correlation between expression of LGR4 and GNAS in 1,064 primary AML patient samples. \( p < 0.0001 \) and \( r = 0.789 \).

(B) Western blots demonstrating overexpression of V5-tagged human Ga\(_s\) protein in human MLL- AF9 (THP-1) cells and resultant changes in non-phospho (active) \( \beta \)-catenin in response to 5 h of culture with 200 ng/mL of RSPO2/WNT3.

(C) Western blots confirming downregulation of active \( \beta \)-catenin caused by Ga\(_s\) knockdown, which was induced by GNAS shRNA in human AML THP-1 cells.

(D) Western blots demonstrating RSPO2/WNT3-induced increase in p-CREB Ser133 relative to total CREB (tCREB) in the nuclear fraction but no change in the cytosol fraction of human THP-1 cells, following 5 h of culture with 200 ng/mL of RSPO2/WNT3. Scatter dot plots represent the band intensity as mean ± SD. \( n=3 \) independent experiments. Unpaired t test. *\( p < 0.05 \); NS, not significant (\( p > 0.05 \)).

(E) Western blots confirming downregulation of p-CREBSer133 induced by LGR4 depletion in human THP-1 cells carrying LGR4 shRNA.

(F) Western blots confirming decreased levels of p-CREBSer133, total \( \beta \)-catenin and active \( \beta \)-catenin in human THP-1 cells carrying CREB shRNA.

(G and H) Representative IF images showing expression of nuclear active p-CREBSer133 in hCD33\(^+\) AML PDX cells (G) and in hCD34\(^+\) cells (H) isolated from Ctr- versus anti-RSPO3-treated AML PDX mice. Scale bars, 10 µm.
The mechanism underlying the enhanced aggressive leukemia phenotype associated with increased LGR4 may involve functional cooperation between LGR4 and HOXA9 in LSCs. High levels of HOXA9 expression is a characteristic feature of AML, including cases with MLL rearrangements, and is associated with poor clinical outcome (Golub et al., 1999). We have previously demonstrated that enforced expression of HOXA9/MEIS1 transforms normal HSPCs where β-catenin is normally active, but is not able to fully transform GMPs that inherently lack β-catenin activity and self-renewal ability (Wang et al., 2010). This implicates an indispensable requirement for β-catenin activation in HOXA9-mediated transformation. Consistent with the role of β-catenin in promoting LSC self-renewal and the role of LGR4 as an essential upstream effector of β-catenin signaling, we observe that LGR4 cooperates with HOXA9/MEIS1 in HSPCs contributing to a highly tumorigenic phenotype characteristic of MLL-AF9 AML. This is in line with a positive correlation between the expression of LGR4 and HOXA9 in AML patient samples and the observation that co-expression of LGR4 and HOXA9 is a necessary prerequisite for RSPO-LGR4 oncogenic activity in primary AML patient specimens. Thus, functional cooperation between LGR4 activation and a HOXA9 gene expression program likely drives in vivo self-renewal and enhanced leukemogenesis.

Here, we report a differential requirement of RSPO-LGR4 signaling between LSCs and normal HSCs. Previous studies have demonstrated that the WNT ligand itself is sufficient to activate canonical WNT/β-catenin signaling in normal HSCs, by forming a ternary complex with the LRP5/6 and Frizzled receptors (Reya et al., 2003; Tamai et al., 2000; Willet et al., 2003). Our findings add support to this notion, showing that in vivo anti-RSPO3 antibody treatment has negligible inhibitory effect on engraftment of normal human CD34+ HSPCs in xenotransplanted mice. In contrast to normal HSCs, we uncover that WNT activation of β-catenin in primary AML critically depends on a second signal provided by an RSPO ligand that drives the self-renewal of LSCs through an LGR4-dependent mechanism. RSPO-LGR4 reportedly forms a supercomplex with the WNT receptors and consequently enhances β-catenin signaling by synergizing with low levels of the WNT ligand (de Lau et al., 2011). The potency of RSPOs in enhancing WNT signals is not affected by WNT receptor levels, but instead largely depends on the abundance of LGR expression (de Lau et al., 2011). We show that pharmacological disruption of the RSPO-LGR4 interaction by clinical-grade anti-RSPO3 antibody decreases leukemia burden in AML PDXs and impairs engraftment of secondary recipients consistent with effective targeting of LSCs. This treatment shows negligible impact on normal human stem cell compartment in NSG mice. While the therapeutic efficacy of anti-RSPO3 antibody will need further preclinical validation in a large number of PDX models with varying mutational profiles, our findings indicate differential dependence of normal and malignant stem cells on RSPO-LGR4 signaling, and suggest that LGR4 levels may provide a biomarker for anti-RSPO3 antibody treatment and that a therapeutic window exists for selective targeting of LSCs.

Overall, our studies have elucidated unique properties of AML LSCs that drive particularly aggressive disease and define a critical role for RSPO-LGR4 signaling in promoting β-catenin activation and AML leukemogenesis. Aberrant activation of RSPO-LGR4 is essential for sustaining self-renewal and a myeloid differentiation block, which contribute to the aggressive leukemia phenotype through cooperation with HOXA9. LGR4 depletion and anti-RSPO3 antibody treatment compromise LSCs and impede AML initiation in both murine models and PDX models, underscoring the therapeutic value of targeting RSPO-LGR4 signaling in AML.

STAR METHODS

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(I) Absolute numbers of viable hCD34+ immature cells from primary AML PDX cells treated with DMSO versus 1 μM I-CBP112 for 24 h of culture with 200 ng/mL RSPO3/WNT3. Scatter dot plots represent the mean ± SD. n = 6 replicates. Unpaired t test. ***p < 0.0001.

(J) qPCR showing reduced levels of key WNT/Self-renewal target genes in primary AML PDX cells in response to treatment with 1 μM I-CBP112 for 24 h of culture with 200 ng/mL RSPO3/WNT3. Mean ± SD. n = 4 replicates per condition. Data are representative of at least three independent repeats. Unpaired t test. *p < 0.05, **p < 0.005, ***p < 0.0005.

See also Figure S8.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ccell.2020.05.014.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

C.M. is an employee of OncoMed Pharmaceuticals and owns stock options in the company. The other authors declare no competing interests.

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REFERENCES


# STAR METHODS

## KEY RESOURCES TABLE

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<td>Mouse IL-3</td>
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<td>Cat# 78042</td>
</tr>
</tbody>
</table>

Critical Commercial Assays

- EasySep™ human CD34 positive selection Kit
  StemCell Technologies | Cat# 18056
- EasySep™ human CD33 positive selection Kit
  StemCell Technologies | Cat# 18287
- APC BrdU Flow Kit
  BD Biosciences | Cat# 552598
- cAMP-Glo assay
  Promega Corporation | Cat# V1501
- RNase Mini Kit
  Qiagen | Cat# 74104

Deposited Data

- H3K79me2 ChIP-seq of human MOLM-13 & HL-60 cells
  Deshpande et al., 2013 | GEO: GSE43063
- H3K79me2 ChIP-seq of human MOLM-13 cells
  Chen et al., 2015 | GEO: GSM1519628
- Microarray of human MOLM-13 cells treated with DMSO versus EPZ004777 (DOT1L inhibitor)
  Daigle et al., 2011 | GEO: GSE29828
- Microarray of human MOLM-14 cells transduced with control versus HOX9 shRNA
  Faber et al., 2009 | GEO: GSE13714
- Microarray of several cohorts of primary AML patients versus normal human HSCs
  Bagger et al., 2016 | GEO: AML patients: GSE13159, GSE15434, GSE61804, GSE14468, and The Cancer Genome Atlas TCGA; Normal human HSCs: GSE42519

Experimental Models: Cell Lines

- Human AML cell line (THP-1)
  ATCC | TIB-202
- Human acute promyelocytic leukemia cell line (HL-60)
  ATCC | CCL-240
- Human CML cell line (K562)
  ATCC | CCL-243
- Human AML cell line (MOLM-13)
  DSMZ | ACC 554
- Human embryonic kidney 293T cell line (HEK293T)
  ATCC | CRL-11268

Experimental Models: Organisms/Strains

- Mouse: C57BL/6
  Australian BioResources Mossvale | N/A
- Mouse: NOD.Cg-Prkdcsid Il2rgrm1Wjl/SzJ (NSG)
  Australian BioResources Mossvale | N/A
- Mouse: Patient-derived xenograft (PDX)
  Vick et al., 2015 | N/A

Oligonucleotides

- Primers, see Table S4
  This study | N/A

Software and Algorithms

- FlowJo Software
  TreeStar | In house license
- ImageJ
  Schneider et al., 2012 | https://imagej.nih.gov/ij/
- GenePattern
  Reich et al., 2006 | http://software.broadinstitute.org/cancer/software/genepattern/
- Integrative Genomics Viewer (IGV)
  Thorvaldsdottir et al., 2013 | https://software.broadinstitute.org/software/igv/
- Living Image Software (v3.0)
  Xenogen | In house license
- GraphPad Prism 7
  GraphPad Software | In house license

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jenny Y. Wang (jwang@ccia.unsw.edu.au).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability
This study did not generate datasets and code. Previously published expression datasets used in this study are available through GEO under GEO: GSE13714, GSE29828, GSE13159, GSE15434, GSE61804, GSE14468 as well as The Cancer Genome Atlas (TCGA). ChIP-seq datasets are available through GSM1519628 and GSE43063.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
All animal studies were conducted in compliance with the Australian Code for the care and use of animals and under UNSW Animal Care and Ethics Committee approved protocols (14/123B, 17/48B and 18/1B). Female C57BL/6 (BL6) and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from Australian BioResources and used for the transplantation experiments between 6 and 8 weeks of age. Mouse models of MLL-AF9-HSPC, MLL-AF9-GMP, HOXA9/MEIS1-HSPC and HOXA9/MEIS1-GMP-driven AML as well as patient-derived and cell line-derived xenografts were established by transplanting leukemic cells into BL6 or NSG mice as previously described (Wang et al., 2010; Dietrich et al., 2014; Gonzales-Aloy et al., 2019; Lynch et al., 2019). Transplanted mice were examined regularly for ill health according to our approved protocols. The PDX model used for in vivo bioluminescence imaging was established by transplanting into NSG mice with firefly luciferase (FLuc)-expressing primary human AML cells from a relapsed patient harboring multiple mutations including DNMT3A, RUNX1, K/NRAS, PTPN11, ETV6 and BCOR (Table S1). Mice were randomly assigned into control or treatment groups.

Primary AML Patient Specimens
AML patient samples were obtained from the Sydney Children’s Tumour Bank Network and were consented for research purpose. Ethical approval was obtained from the Sydney Children’s Hospitals Network Human Research Ethics Committee. Frozen BM samples from AML patients at the time of diagnosis, relapse or remission were used under institutional review board-approved protocols. Clinical characteristics of AML patient samples with 9p deletion, MLL-AF1q, MLL-AF9, MLL-AF10 (diagnosis and relapse), AML1-ETO and normal karyotype were summarized in Table 1. Normal adult human CD34+ BM cells were obtained from Lonza (Mt Waverley, VIC, Australia).

Cell Lines
All cell lines were obtained from the sources indicated in the Key Resources Table. MOLM-13 (source: male), THP-1 (source: male), HL-60 (source: female) and K562 (source: female) cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO2. HEK293T cells were used for virus production and were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were tested regularly for mycoplasma. THP-1 and MOLM-13 were authenticated using short tandem repeat profiling and other cell lines were used within 10 passages without further authentication.

METHOD DETAILS

Ex Vivo Culture of Primary AML Patient Cells
Primary AML patient specimens or AML PDX cells were thawed in RPMI-1640 medium (Thermo Fisher Scientific, North Ryde, NSW, Australia) and cultured in various media conditions, such as (1) medium only (RPMI-1640 supplemented with 1% FBS); (2) cytokines (StemSpan™ CD34+ Expansion Supplement [x10] consisting of hSCF, hFLT3-L, hTPO, hIL-3 and hIL-6; StemCell Technologies, 02691); (3) 200 ng/mL RSPO2/WNT3 in medium; (4) 200 ng/mL RSPO3/WNT3 in medium; or (5) 200 ng/mL RSPO3/WNT3 with...
150 μg/mL anti-RSPO3 antibody in medium for 24-96 h. The percentage of hCD34+ cells in primary AML patient blasts was determined by FACS analysis using Alexa Fluor 647 anti-human CD34 (343508) from BioLegend (Balcatta, WA, Australia).

Flow-Sorting and FACS Analysis
Cells were stained with antibodies at 4°C for 30 min before being subjected to flow sorting using BD Influx™ cell sorter or BD FACSAria™ III sorter (BD Biosciences, North Ryde, NSW, Australia) and to FACS analysis using BD LSRFortessa™ cell analyzer or BD FACSCanto™ II (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

FACS Analysis of Human LGR4 Expression
hCD34+ and hCD34- PDX cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and then permeabilized in 0.1% Triton X for 15 min. Cells were washed and subsequently stained with 2 μg/mL LGR4 primary antibody (Abcam, Cambridge, UK) and human CD34 positive selection Kit (StemCell Technologies, 18287), as per manufacturers’ protocols. The purity (>98%) of the isolated population was confirmed using FACS analysis. Murine HSC-enriched LSK cells were flow- sorted as Lin- (CD3, CD4, CD8a, CD33) positive selection Kit (StemCell Technologies, 18287), as per manufacturers’ protocols. The purity (>98%) of the resulting isolated population was confirmed using FACS analysis. Murine HSC-enriched LSK cells were flow- sorted as Lin- (CD3, CD4, CD8a, CD19, B220, Gr-1, Ter119, IL-7R)c1-Kit+c-Kit+ (BioLegend) and myeloid progenitor GMP cells were isolated as GMPs: Lin-c1-Kit+CD16/32-CD34+ (BioLegend) from mouse BM as previously described (Akashi et al., 2000; Krivtsov et al., 2006).

Anti-RSPO3 Monoclonal Antibody
Anti-RSPO3 monoclonal antibody (OMP-131R10/rosmantuzumab; OncoMed Pharmaceuticals, CA, USA) was generated by immunizing mice with purified recombinant human RSPO3 followed by hybridoma generation and characterization (Chartier et al., 2016). The anti-RSPO3 antibody has been well tolerated as a single agent in a phase 1 clinical trial in advanced solid tumors and dose-limiting toxicities were not observed (Bendell et al., 2016).

In Vivo Treatments and Analysis of Tumor Burden
For the established PDX model, genetic characterization of the AML PDX was performed using a targeted, multiplexed amplicon re-sequencing approach (Haloplex, Agilent, Boeblingen, Germany), as described previously (Metzeler et al., 2016; Vick et al., 2015). Non-invasive bioluminescence imaging was used to monitor and assess in vivo engraftment of human AML cells. PDX mice were injected with D-luciferin (150 mg/kg of body weight, Xenogen, Alameda, CA) via intraperitoneal (i.p) injection. Images were subsequently taken and signal output was measured 10 min after injecting the substrate. Signal intensity quantification and analysis were performed using the Living Image software version 3.0 (Xenogen). The bioluminescent signal was recorded as radiation photons/sec/cm²/steradian (p/sec/cm²/sr), represented in pseudocolor to indicate the signal intensities. Mice were excluded from analysis if baseline bioluminescent signal was notably higher than the cohort or if mice had to be euthanized due to causes other than leukemia. NSG mice with baseline bioluminescent signals were randomly assigned into experimental groups. Anti-RSPO3 antibody was then administered at 20 mg/kg once weekly via i.p. injection. Imaging was thereafter performed once weekly. At the end of each experiment, BM cells were assessed by FACS analysis for leukemic cell engraftment and by confocal immunofluorescence and qPCR analysis for target expression.

For anti-RSPO3 treatment of newly-established PDX models, NSG mice were transplanted with primary AML patient samples harboring 9p deletion (7x10⁶ cells per mouse), MLL-AF9 (1.6x10⁶ cells per mouse) or MLL-AF1q (5.7x10⁶ cells per mouse) followed directly by in vivo treatment with control versus anti-RSPO3 antibodies (20 mg/kg, i.p. every 4 days). Primary AML patient specimens harboring diagnosis or relapse MLL-AF10 (7.1x10⁵ or 1.7x10⁶ cells per mouse) did not engraft in NSG mice. Indicated numbers of transplanted patient cells were determined by their availability in our patient tumor bank and equal numbers of patient cells were transplanted into recipient mice. Mice were randomized into control versus treatment groups for each patient specimen. Researchers were not blinded to the treatment groups. BM cells were harvested for FACS analysis post-transplantation at time-points as indicated in the figure legends.

Leukemic Cell Stimulation with RSPO/WNT3 Ligands
Wnt3 or control medium (Reya et al., 2003) was generated by sterile filtering the cultured medium of L-Wnt3 (CRL-2647) or L cells (CRL-2648) as described by the ATCC protocol. Recombinant mouse Rspo1 (3474-RS), Rspo2 (6946-RS), Rspo3 (4120-RS), and recombinant human WNT3 (RDS5036WN010) were purchased from In Vitro Technologies. Recombinant human RSPO2 (120-43-20) and RSPO3 (120-44-20) were obtained from Lonza (Mount Waverley, VIC, Australia). For Western blot analysis of murine cells, 5 x 10⁵ pre-LSCCs were seeded in 24-well plates with IMDM media supplemented with Wnt3 (or control) medium and/or Rspo with indicated concentrations as well as mIL3, and incubated at 37°C for 24 h. For Western blot and
immunofluorescence analysis of human AML cell lines, 3-5 x 10^5 leukemic cells were plated in a 6-well plate with 2 mL RPMI-1640 medium per well in the presence of 200 ng/mL of human WNT3 and/or RSPO2, as well as 1 μM l- CBP112 (Sigma-Aldrich; 1134), and incubated at 37°C for the indicated times.

Viral Vector Production, Cell Transduction and Bone Marrow Transplantation
MSCV-MLLAF9-GFP, MSCV-HOXA9-GFP, MSCV-MEIS1-puro and MSCV-β-catenin (β-cat*)-neo have been previously described (Krivtsov et al., 2006; Wang et al., 2010). MSCV-Lgr4-neo and LeGO-IT2-LGR4 vectors were generated by GenScript (NJ, USA). LeGO-IT2 was a gift from Boris Fehe (Addgene plasmid, 27343) (Weber et al., 2008). GNAS-pLX307 was a gift from William Hahn and Sefi Rosenbluh (Addgene plasmid, 98339) (Rosenbluh et al., 2016). Stable knockdown/knockout were achieved by using mouse Lgr4 shRNA sequences (Lgr4sh1: MSH040504-3-LVRU6MP; Lgr4sh2: MSH040504-4-LVRU6MP; scrambled control: CSHCTR001-LVRU6MP; GeneCopoeia, Rockville, MD, USA), human LGR4 shRNA sequences (LGR4sh1: TRCN0000273532; LGR4sh2: TRCN0000285015; scrambled control: SHC216; Sigma-Aldrich, Castle Hill, NSW, Australia) and lentiviral CRISPR/Cas9 plasmids (human LGR4 gRNA: HS0000422096; non-targeting control: CRISPR12, Sigma-Aldrich). Viruses were produced by transfecting HEK293T with standard packaging vectors using lipofectamine 2000 (Life Technologies, Mulgrave, VIC, Australia) for murine AML cells and using lipofectamine 3000 (Life Technologies) for human leukemic cells. Viral supernatants were harvested 48-72 h following transfection, filtered through a 0.45-μm membrane and then concentrated by centrifugation. 1-5 x 10^5 cells were incubated with concentrated virus supplemented with 8 μg/mL polybrene (Sigma-Aldrich). Four-hour transduction was performed for human PDX cells, overnight transduction for human leukemic cell lines and two rounds of transduction for murine leukemic cells. For BM transplantation, pre-LSCs or GFP+ leukemic cells flow-sorted from BM of AML mice were transplanted into sublethally irradiated (6 Gy) syngeneic BL6 recipient mice through intravenous (i.v.) injection.

Colony Formation and Serial Replating Assay
LSCs (Lin-ScA-1-C-Ki67(+)/CD16/32(+)/CD34+), also known as L-GMP or GMP-like LSCs (Krivtsov et al., 2006), which were flow-sorted from the BM of AML mice, or pre-LSCs were seeded at a density of 1 x 10^3 cells per 35 mm dish in methylcellulose supplemented with mL-3. Colonies were counted after 5 days of incubation at 37°C. For the serial replating assay, colonies were harvested and 1 x 10^3 cells were subsequently replated in fresh methylcellulose. Three rounds of serial replating were performed for each experiment.

In Vivo Bromodeoxyuridine (BrdU) Cell Proliferation Assay
GFP+ origin-specific leukemic BM cells from primary AML were transplanted into sublethally irradiated BL6 recipient mice for in vivo BrdU assays at 10 days post-transplantation. In vivo BrdU labeling was done by intraperitoneal (i.p.) injection of allophycocyanin (APC)-conjugated BrdU (1 mg/100 μl in PBS per mouse; BD Biosciences, 552598) for 2 h prior to bone marrow harvest following the manufacturer’s protocol.

Cell Cycle Analysis
Cells were fixed in ice-cold 70% ethanol for 2 h at -20°C, stained with Alexa Fluor 647 mouse anti- Ki67 (BD Biosciences; 558615) and 7-amino-actinomycin D (7AAD, BD Biosciences) staining solution for 30 min at room temperature and analyzed by FACS analysis.

ROS Production
ROS levels were detected by labeling 5 x 10^5 LSCs for 30 min at 37°C with 2.5 μM MitoSOX™ Red (Life Technologies) in PBS. Labeled cells were washed with PBS and analyzed by FACS.

The cAMP-Glo Assay
The assay is based on the principle that Ga(s)-coupled receptors can promote direct activation of adenylyl cyclase leading to increased levels of cAMP, which in turn binds to and activates the exogenously added PKA causing changes in ATP concentration and light production. The cAMP-Glo Assay (Promega Corporation, Alexandria, NSW, Australia; V1501) was performed according to the manufacturer’s instructions. The luciferase activity was measured with a luminometer (Victor3 plate reader; PerkinElmer, Waltham, MA, USA).

Western Blot Analysis
Western blots were performed according to standard laboratory protocols, using antibodies directed against LGR4 (Abcam; ab75501), phospho-Ser133-CREB (p-CREBSer133, Merck; 06-519), CREB (Sapphire Bioscience, Redfern, NSW, Australia; 3360R), β-catenin (BD Biosciences; 610154), active β-catenin (non-phospho-Ser33/Ser37/Thr41; Genesearch, Arundel, QLD, Australia; 8814), V5-Tag (Genesearch; 13202), H3K79me2 (Abcam; ab3594), total H3 (Abcam; ab1791), GAPDH (Abcam; ab8245) or ACTIN (Sigma-Aldrich; A2066). Blots were visualized and quantified using the ChemiDoc MP Imaging System (Bio-Rad) and the ImageJ software (Schneider et al., 2012). Relative protein band intensity was normalized against loading control (ACTIN, GAPDH or H3) and compared to relative control.
Confocal Immunofluorescence
4 x 10^4 cells were cytopun onto glass slides, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and then washed three times for 5 min in PBS with 0.1% Tween-20 (PBS-T). Non-specific antibody binding was blocked with blocking media (10% goat serum and 2% BSA in PBS-T) for 1 h. Slides were stained overnight at 4°C with a primary antibody (i.e., active β-catenin, p-CREBser133; CBP, MetaGene, Brisbane, QLD, Australia, sc-7300) at a dilution of 1:100, washed three times for 5 min in PBS-T, and then stained for 1 h with Alexa Fluor 568- or Alexa Fluor 647-conjugated secondary antibody (Abcam; ab175472 and ab150079), followed by staining with DAPI (1:4000, Thermo Fisher Scientific) for 30 min. Coverslips were mounted with ProLong Gold antifade mountant (Thermo Fisher Scientific) and edges were sealed with nail polish to prevent desiccation. Immunofluorescence images were taken with a TCS SP5 MP-STED confocal microscope (Leica Microsystems, Mannheim, Germany) and processed using NIH ImageJ software.

Quantitative Real-Time PCR (qPCR)
Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen, Chadstone Centre, VIC, Australia). cDNA was synthesized from total RNA using oligo (dT) and M-MLV Reverse Transcriptase (Life Technologies). qPCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) or SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Gladeville, NSW, Australia). Target gene expression values were normalized against the house-keeping gene GAPDH.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics
Statistical significance was determined by two-tailed unpaired Student’s t-test to compare two groups of samples, one-way ANOVA and Dunnett test for multiple comparisons, and two-way ANOVA and Bonferroni correction for comparisons among groups with different time points. Kaplan-Meier plot and log-rank (Mantel-Cox) test were used to assess survival curves. Correlation analysis was performed by calculating the Pearson correlation coefficient. Throughout the manuscript, the following notation was used to indicate statistical significance: * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001. Statistical parameters including the exact values of n and precision measure (mean ± SD) and statistical significance are reported in the figures and figure legends. Statistical analysis and graph construction were performed using GraphPad Prism v7.04 (GraphPad Software, La Jolla, CA, USA).

Pearson Correlation Analysis of Microarray Data for AML Patients
Affymetrix U133 Plus2.0 (HG133P2) array data has previously been published for patients in the USA cohort (Cancer Genome Atlas Research Network, 2013) and is available via the National Cancer Institute’s Genomic Data Commons Portal (https://portal.gdc.cancer.gov). Matched clinical annotations and expression data was available for 183 AML patient samples including 47 classified into the adverse molecular risk group and 40 assigned into the European Leukemia Net adverse risk group (Dohner et al., 2010; Beck et al., 2018). The raw expression files of all 183 patient samples were processed using Partek Genomics Suite software v6.6 (Partek Inc., St. Louis, MO, USA) and normalized expression levels were generated after pre-processed including background subtractions, quantile normalization and log2 transformation. The ComBat algorithm (Johnson et al., 2007) was used to remove experimental variation associated with different array batches. The Pearson correlation coefficient was calculated using GraphPad Prism between the expression level of the probesets representing LGR4 and HOXA9. The significance level of this correlation was tested using a t-distribution, to assess whether the correlation differed significantly from zero (threshold of p < 0.05).

Genome-wide Profiling Analysis
Microarray data, including GSE29828 (Daigle et al., 2011) and GSE13714 (Faber et al., 2009), were analyzed using the GenePattern software (Reich et al., 2006) as previously described (Wang et al., 2010). ChIP-seq data, including GSM1519628 and GSE43063 (Chen et al., 2015; Deshpande et al., 2013), were visualized with the Integrative Genomics Viewer (IGV) software (Thorvaldsdottir et al., 2013).