The Wnt/β-catenin Pathway Is Required for the Development of Leukemia Stem Cells in AML

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Abstract

A subset of cells called leukemia stem cells (LSCs) possess limitless self-renewal and are responsible for maintenance of leukemia. Selective eradication of LSCs could offer significant therapeutic benefit and there is great interest in identifying the signaling pathways that control their development. Here, LSCs were studied in mouse models of acute myelogenous leukemia (AML) induced either by co-expression of the Hoxa9 and Meis1a oncogenes or the fusion oncoprotein MLL-AF9. We show that the Wnt/β-catenin signaling pathway is required for self-renewal of LSCs derived from either hematopoietic stem cells (HSC) or more differentiated granulocyte macrophage progenitors (GMP). Since the Wnt/β-catenin pathway is normally active in HSCs, but not in GMP, reactivation of β-catenin signaling is required for transformation of progenitor cells by certain oncogenes. β-catenin is not absolutely required for self-renewal of adult HSCs, thus targeting the Wnt/β-catenin pathway may represent a new therapeutic opportunity in AML.

Acute myelogenous leukemia (AML) is the most common acute leukemia in adults and most people with AML are not cured with current therapies. Only a small subset of AML cells are capable of extensive proliferation and self-renewal (1). Such cells are referred to as leukemia stem cells (LSC) because they share properties, including extensive self-renewal potential, with normal stem cells. LSC are being studied as potential therapeutic targets (2, 3), and the signaling pathways that control their development and survival are of particular interest. The Wnt/β-catenin is active in some human leukemias (4–7) and in hematopoietic stem cells (HSC) (8–10). While β-catenin is not required for self-renewal of adult HSC (11, 12), it is unclear if β-catenin is required for LSC development and maintenance in AML. We

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have studied well characterized mouse models to address whether β-catenin is necessary for LSC development from either HSC or more differentiated committed myeloid progenitor cells.

Hox genes have been implicated in the regulation of normal stem cell self-renewal (16, 17), and enforced co-expression of *Hoxa9* and *Meis1a* in murine bone marrow leads to rapid AML development (18). Previous studies demonstrated that leukemogenic fusion proteins, such as those involving the mixed lineage leukemia (MLL) protein e.g. MLL-AF9, can transform non-self renewing granulocyte/macrophage restricted progenitors (GMP) and activate genes encoding homeobox (Hox) proteins such as HoxA9 and Meis1 (13–15). To determine whether a combination of Hoxa9 and Meis1a (HoxA9/M) can transform GMP, we co-transduced sorted $Lin^- c-Kit^+ Sca-1^+$ (KLS) cells, which are enriched for HSC, or $Lin^- c-Kit^+ Sca-1^- FcR\gamma^+ CD34^+$ (GMP) cells with viruses encoding HoxA9 (MSCV-HoxA9-GFP) and Meis1a (MSCV-Meis1a-puro), and used either a bulk approach (15, 18, 26) or a single cell approach (Fig. S1). When single cells were expanded *in vitro* up to 6 weeks, no significant differences were observed between KLS cells transduced with HoxA9/M (KLS-HoxA9/M), GMP transduced with HoxA9/M (GMP-HoxA9/M), or GMP transduced with MLL-AF9 (GMP-MLLAF9) in proliferation, cell cycle profile, apoptosis levels, gross morphology or immunophenotype (Fig. S1). However there was a proliferative defect upon extended culture of GMP-HoxA9/M cells. When cells were injected into recipient mice, 13 of 14 (93%) mice that received GMP-MLLAF9 cells developed AML, 14 of 20 (70%) of mice that received KLS-HoxA9/M cells developed AML, whereas only 1 of 23 mice that received GMP-HoxA9/M cells developed AML (Fig. S2). These differences in leukemogenic potential could not be ascribed to differential expression of *HoxA9* and *Meis1* (Fig. S3)

To characterize the *in vivo* defect in HoxA9/M transduced GMP we assessed for GFP+ cells and their immunophenotypes in recipient mice at multiple timepoints after transplantation. By 18 hours KLS-HoxA9/M and GMP-HoxA9/M cells homed to the bone marrow with similar efficiency (Fig. S2). By eight days both cell types displayed heterogeneity of expression of the stem cell marker, c-Kit ($c-Kit^{high}$ and $c-Kit^{low}$) (Fig. 1a). KLS-HoxA9/M cells sustained the two c-Kit populations through one month until ultimately a lethal leukemia developed (Fig 1a). Conversely, at 1 month post-transplantation, GMP-HoxA9/M only retained the $c-Kit^{low}$ population that was eventually lost from bone marrow (Fig. 1a).

To determine whether the $c-Kit^{high}$ and $c-Kit^{low}$ populations identified 1 month post injection of KLS-HoxA9/M cells are functionally distinct, we utilized *in vivo* limiting dilution analysis to measure the frequency of leukemia initiating cells (LIC) in these two populations. LIC are defined as cells isolated prior to the development of clinically evident leukemia that can initiate leukemia in subsequent recipient mice. A high frequency of LIC was observed in the $c-Kit^{high}$ population. In contrast, $c-Kit^{low}$ cells were very inefficient at inducing leukemia (Fig. S4). We next assessed for LSC activity in the fully developed HoxA9/M induced leukemias. Limiting dilution analysis demonstrated the $Gr1^{-/(low)}c-Kit^{high}$ population was over 100-fold enriched for LSC as compared to the $Gr1^+c-Kit^{high}$ cells (Fig. S4, S5), and had an immunophenotype similar to MLL-AF9 LSC (L-GMP) (15,19) (Fig. S6). Thus, maintenance of the $c-Kit^{high}$ population is associated with leukemia development, and cellular heterogeneity is found throughout the process of leukemia development. The inability of GMP-HoxA9/M cells to induce leukemia prompted us to search for a self-renewal pathway active in LSC that might be deficient in the GMP-HoxA9/M cells.

We previously demonstrated that $Lin^- Sca-1^- c-Kit^{high} FcR\gamma^+ CD34^+$ cells (termed L-GMP) are enriched for LSC in the MLL-AF9 AML model, and that L-GMP are globally similar to

normal cells at a mid myeloid stage of development but express a subset of genes normally highly expressed in HSC (15). Given the LSC population in HoxA9/M driven leukemia described here has a similar immunophenotype, we reasoned that genes highly expressed in MLL-AF9 L-GMP, HoxA9/M L-GMP, and HSC but not in GMP might pinpoint genes important for leukemic self-renewal. Supervised analysis of gene expression data demonstrated a group of genes that are highly expressed in normal and leukemia stem cells, and expressed at lower levels in normal myeloid progenitors (Fig. 1b). Prostaglandin-endoperoxide synthase 1 (Ptgs1) (also known as Cycloxygenase-1 or Cox-1) and one of the prostaglandin receptors (Ptger1) were upregulated in both GMP-MLLAF9-derived and KLS-HoxA9/M-derived L-GMP, which was confirmed by qPCR (Fig. 1b; Fig. S6). As studies have highlighted a critical connection between prostaglandin synthesis and the Wnt/β-catenin pathway (20–23), we assessed for β-catenin activation. An antibody specific for activated β-catenin demonstrated activity in GMP-MLLAF9-derived and KLS-HoxA9/M-derived L-GMPs but not normal GMP as assessed by immunoblot or immunofluorescence (Fig. 1c; Fig. S7). Also, β-catenin activity was found in GMP-MLLAF9-derived and KLS-HoxA9/M-derived cells grown in vitro but not in GMP-HoxA9/M -derived cells (Fig. S7). Remarkably, activated/nuclear β-catenin is found in c-Kit^{high}, but not c-Kit^{low} cells isolated from mice 1 month after injection with KLS-HoxA9/M cells (Fig. 1d).

Given the activation pattern of β-catenin in GMP-MLLAF9-derived and KLS-HoxA9/M-derived leukemias, and its minimal activation in GMP-HoxA9/M-derived cells, we hypothesized that the inability of HoxA9/M to transform GMP might be due to the inability to sufficiently activate β-catenin. To test this we transduced GMP-HoxA9/M cells with a retrovirus encoding a constitutively active form of β-catenin (βcat*) (Fig. 2a). Transplantation of GMP-HoxA9/M cells transduced with βcat* produced AML similar to KLS-HoxA9/M cells (Fig. 2b). Expression of βcat* in GMP cells did not induce leukemia (Fig. 2b).

We next tested the effect of indomethacin (Indo), a reversible COX inhibitor which blocks activation of the Wnt pathway by suppressing β-catenin expression (22). Exposure of LIC-enriched c-Kit^{high} cells to indomethacin reduced β-catenin levels in vitro (Fig. 3a), and mice treated with indo showed a dramatic reduction in the c-Kit^{high} population after 7 days of treatment (Fig. 3b). We performed a limiting dilution assay using flow-sorted GFP+ cells from BM of control and Indo-treated mice which demonstrated a 100-fold decrease in LIC after indo treatment (Fig. 3c). Furthermore, Indo treatment of a fully developed MLL-AF9 leukemia partially suppressed β-catenin (Fig. S8) and reduced LSC frequency (Fig 3d). Thus, indomethacin treatment can suppress the LIC compartment critical for development of AML and also influences established LSC suggesting that AML is dependent on β-catenin signaling. However, the therapeutic effect appears greatest in situations of minimal disease (Fig. 3c) as compared to treatment of more extensive disease (Fig. 3d).

Next, we examined the ability of HoxA9/M to induce leukemia from β-catenin-deficient KLS cells using BM from βcat$^{loxP/loxP}$ mice (24). We transduced βcat$^{loxP/loxP}$ KLS cells with HoxA9/M, sorted single cells (KLS-HoxA9/M βcat$^{loxP/loxP}$), and subsequently transduced the cells with a retroviral vector encoding either Cre recombinase, or an empty vector. Cells transduced with Cre recombinase (KLS-HoxA9/M βcat $^{-/-}$) were devoid of wild-type β-catenin (Fig. S9). These cells were injected into recipient mice and we monitored expansion of KLS-HoxA9/M βcat$^{loxP/loxP}$, KLS-HoxA9/M βcat $^{-/-}$ and control cells in bone marrow. KLS-HoxA9/M βcat$^{loxP/loxP}$ cells were able to expand efficiently in murine BM (Fig. 4a) and ultimately produced leukemia. However, loss of β-catenin led to impaired expansion, and KLS-HoxA9/M βcat $^{-/-}$ cells ultimately lost their ability to expand in bone marrow (Fig. 4a). Importantly βcat* rescued the proliferative defect of KLS-HoxA9/M βcat $^{-/-}$ cells (Fig. S9). Additionally, βcat^{WT} KLS-HoxA9/M cells, βcat^{WT} KLS-HoxA9/

M cells expressing cre recombinase, and KLS-HoxA9/M βcat$^{loxP/loxP}$ cells induced AML in all recipients with similar latencies, whereas KLS-HoxA9/M βcat $^{-/-}$ cells were dramatically impaired in their ability to initiate AML (Fig. 4b). Three mice injected with KLS-HoxA9/M βcat $^{-/-}$ cells did ultimately develop AML, however in the 2 cases that were captured prior to the mouse succumbing to the disease, there was incomplete excision of β-catenin (Fig. S9). Finally, a similar experiment demonstrated a dramatic reduction in the ability of MLL-AF9 to induce leukemia from GMP (Fig. 4c).

These data demonstrate that β-catenin is required for either *Hox*-gene mediated transformation of HSC or MLL-AF9 mediated transformation of committed progenitor cells. Furthermore, lack of β-catenin activation in normal GMP limits the ability of *Hox* genes to efficiently transform committed progenitor cells. This is in contrast to MLL-AF9 that activates sufficient β-catenin to transform GMP. This supports the concept that activation or maintenance of HSC-associated developmental pathways in downstream myeloid cells is an important component of AML development. Given that β-catenin activity is important for HSC during fetal development, but not for adult HSC survival/proliferation (9, 11, 12), targeting the β-catenin pathway may be an attractive therapeutic avenue in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References and Notes

1. Bonnet D, Dick JE. Nat Med. Jul.1997 3:730. [PubMed: 9212098]

2. Dick JE. Blood. Dec 15.2008 112:4793. [PubMed: 19064739]

3. Park CY, Tseng D, Weissman IL. Mol Ther. Feb.2009 17:219. [PubMed: 19066601]

4. Jamieson CH, et al. N Engl J Med. Aug 12.2004 351:657. [PubMed: 15306667]

5. Majeti R, et al. Proc Natl Acad Sci U S A. Mar 3.2009 106:3396. [PubMed: 19218430]

6. Muller-Tidow C, et al. Mol Cell Biol. Apr.2004 24:2890. [PubMed: 15024077]

7. Ysebaert L, et al. Leukemia. Jul.2006 20:1211. [PubMed: 16688229]

8. Reya T, et al. Nature. May 22.2003 423:409. [PubMed: 12717450]

9. Zhao C, et al. Cancer Cell. Dec.2007 12:528. [PubMed: 18068630]

10. Malhotra S, Kincade PW. Cell Stem Cell. Jan 9.2009 4:27. [PubMed: 19128790]

11. Jeannet G, et al. Blood. Jan 1.2008 111:142. [PubMed: 17906078]

12. Cobas M, et al. J Exp Med. Jan 19.2004 199:221. [PubMed: 14718516]

13. Cozzio A, et al. Genes Dev. Dec 15.2003 17:3029. [PubMed: 14701873]

14. Huntly BJ, et al. Cancer Cell. Dec.2004 6:587. [PubMed: 15607963]

15. Krivtsov AV, et al. Nature. Aug 17.2006 442:818. [PubMed: 16862118]

16. Sauvageau G, et al. Genes Dev. Jul 15.1995 9:1753. [PubMed: 7622039]

17. Abramovich C, Pineault N, Ohta H, Humphries RK. Ann N Y Acad Sci. Jun.2005 1044:109. [PubMed: 15958703]

18. Kroon E, et al. Embo J. Jul 1.1998 17:3714. [PubMed: 9649441]

19. Somervaille TC, Cleary ML. Cancer Cell. Oct.2006 10:257. [PubMed: 17045204]

20. Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS. Science. Dec 2.2005 310:1504. [PubMed: 16293724]

21. North TE, et al. Nature. Jun 21.2007 447:1007. [PubMed: 17581586]

22. Goessling W, et al. Cell. Mar 20.2009 136:1136. [PubMed: 19303855]

23. Eisinger AL, Prescott SM, Jones DA, Stafforini DM. Prostaglandins Other Lipid Mediat. Jan.2007 82:147. [PubMed: 17164142]

24. Brault V, et al. Development. Apr.2001 128:1253. [PubMed: 11262227]

25. Orido T, et al. J Pharmacol Sci. Nov.2008 108:389. [PubMed: 18987430]

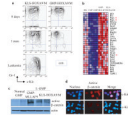26. Please refer to Supplemental Data and Methods for details.

Figure 1. β-catenin is activated in developing leukemia cells and L-GMP derived from KLS-HoxA9/M and GMP-MLLAF9

a, Immunophenotypic analyses of GFP+ cells from bone marrow of mice transplanted with pre-leukemia KLS-HoxA9/M or GMP-HoxA9/M cells at 8 days, 1 month or 4 months (leukemia) post-transplantation.

b, The heat map displays the top 30 probe sets for genes that show increased expression in the HSC population (KLS cells) and L-GMP using Comparative Marker Selection and permutation testing (200 probe sets passed a cutoff of P < 0.002). Note expression of Ptgs-1 (Cox-1) and Ptger1 (prostaglandin E receptor 1).

c, Immunoblot analysis for active (dephosphorylated) β-catenin in normal GMP or L-GMP derived from GMP-MLLAF9 or KLS-HoxA9/M mediated leukemias.

d, Immunofluorescence assessment for active β-catenin in c-Kit[high] or c-Kit[low] populations isolated from mice 1 month after injection of pre-leukemia KLS-HoxA9/M cells. (active β-catenin: red, Hoechst: blue; active β-catenin/Hoechst: merge).
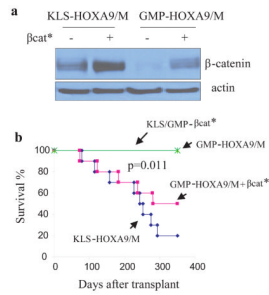
Figure 2. Constitutively active β-catenin cooperates with HoxA9/M to induce AML from GMP cells

a, Constitutively active βcat* was transduced into pre-leukemia KLS-HoxA9/M or GMP-HoxA9/M cells and protein levels were assessed by immunoblot analysis.

b, Survival curves of mice receiving KLS or GMP cells transduced with active βcat* (controls), GMP-HoxA9/M, GMP-HoxA9/M cells transduced with active βcat*, or KLS-HoxA9/M. 5×10^5 cells were transplanted into sublethally irradiated recipients (n=10 in each group). P determined using the log-rank test
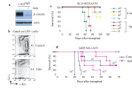
Figure 3. Pharmacological inhibition of β-catenin impairs LSC function
a, Immunoblot analysis of β-catenin levels in c-Kit^{high} cells sorted from bone marrow at 1 month post-injection of mice with KLS-HoxA9/M cells which were subsequently grown in methylcellulose with and without exposure to indomethacin for 3 weeks (25,26).
b, Immunophenotypic analysis of bone marrow GFP+ cells after injection of KLS-HoxA9/M cells and treatment with either vehicle or indomethacin (repeated with 2 independent clones) for 7 days. Treatment began at day 3 post-injection and mice were sacrificed at day 10 post-transplantation (22,26).
c,d Survival curves of mice (5 mice for each group) injected with the indicated number (10 – 10^4) of GFP+ marrow cells sorted from control or Indomethacin (Indo)-treated mice that had received pre-leukemia KLS-HoxA9/M cells **(c)** or leukemic GMP-MLLAF9 cells **(d)** (repeated with 2 independent clones).
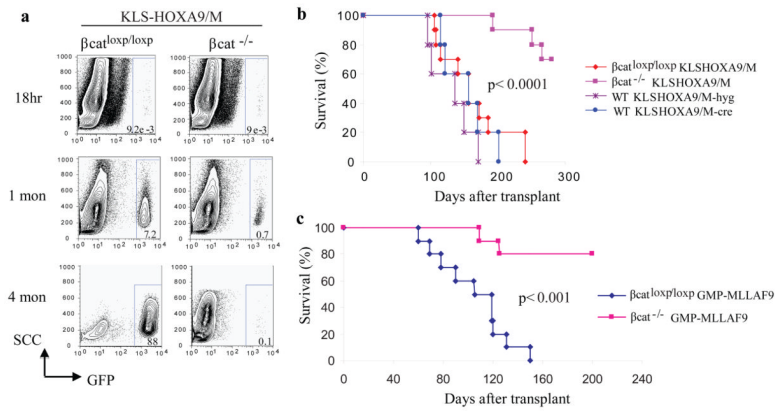
Figure 4. Deletion of β-catenin impairs the development of KLS-HoxA9/M and GMP-MLLAF9 induced AML

a, GFP + cells in bone marrow of mice injected with floxed β-catenin (βcat$^{loxp/loxp}$) or β-catenin-deficient (βcat$^{-/-}$) KLS-HoxA9/M cells at 18 hr, 1 month, 4 months post-transplantation. HoxA9/M-βcat$^{loxP/loxP}$ KLS cells were transduced with a retroviral vector encoding Cre recombinase to generate βcat $^{-/-}$ KLS-HoxA9/M cells or an empty vector to generate control βcat$^{loxp/loxp}$ KLS-HoxA9/M cells. After selection, 1×10^6 infected cells were transplanted into sublethally irradiated recipients (n=5 in each group). Mice were sacrificed at the indicated time points to assess for GFP+ cells.

b, Survival curves of mice transplanted with βcat$^{-/-}$ KLS-HoxA9/M cells, βcat$^{loxp/loxp}$ KLS-HoxA9/M cells, and empty vector or Cre-infected WT KLS-HoxA9/M (KLS cells were sorted from wild-type C57BL/6 mice and transformed with HoxA9/M to assess the effect of Cre on KLS-HoxA9/M leukemia development; n=10 for each group).

c, Survival curves of mice transplanted with βcat$^{-/-}$ GMP-MLLAF9 cells and βcat$^{loxp/loxp}$ GMP-MLLAF9 cells (n=10 for each group). P determined using the log-rank test