FoxO1-Dependent Induction of Acute Myeloid Leukemia by Osteoblasts in Mice

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Abstract

Osteoblasts, the bone forming cells, affect self-renewal and expansion of hematopoietic stem cells (HSCs), as well as homing of healthy hematopoietic cells and tumor cells into the bone marrow. Constitutive activation of β-catenin in osteoblasts is sufficient to alter the differentiation potential of myeloid and lymphoid progenitors and to initiate the development of acute myeloid leukemia (AML) in mice. We show here that Notch1 is the receptor mediating the leukemogenic properties of osteblast-activated β-catenin in HSCs. Moreover, using cell-specific gene inactivation mouse models, we show that FoxO1 expression in osteoblasts is required for and mediates the
leukemogenic properties of β-catenin. At the molecular level, FoxO1 interacts with β-catenin in osteoblasts to induce expression of the Notch ligand, Jagged-1. Subsequent activation of Notch signaling in long-term repopulating HSC progenitors induces the leukemogenic transformation of HSCs and ultimately leads to the development of AML. These findings identify FoxO1 expressed in osteoblasts as a factor affecting hematopoiesis and provide a molecular mechanism whereby the FoxO1/activated β-catenin interaction results in AML. These observations support the notion that the bone marrow niche is an instigator of leukemia and raise the prospect that FoxO1 oncogenic properties may occur in other tissues.

Introduction

Over the last few years it has become increasingly apparent that stromal cells within the bone marrow microenvironment influence the fate of hematopoietic stem cells (HSC)\(^1\)\(^-\)\(^8\). In particular, osteoblasts, the bone-forming cells, influence hematopoietic stem cell (HSC) fate\(^9\)\(^-\)\(^12\). Osteoblastic cells support and expand HSCs \textit{in vitro} and increase engraftment \textit{in vivo}\(^13\). They can both stimulate\(^11\)\(^,\)\(^12\) and limit HSC expansion\(^14\), promote quiescence\(^3\)\(^,\)\(^15\)\(^,\)\(^16\), initiate HSC mobilization\(^17\)\(^,\)\(^18\), and regulate B lymphopoiesis\(^19\)\(^,\)\(^20\) and myeloproliferation\(^21\). They also integrate sympathetic nervous system signaling and HSC regulation\(^22\). In addition, mesenchymal stem cells (MSCs) of the osteoblast lineage affect HSC function in mouse models of myeloproliferative neoplasms (MPN)\(^23\)\(^,\)\(^24\) resulting in the development of myelodysplasia (MDS) and acute myeloid leukemia (AML)\(^25\). Osteoblastic cells also differentially regulate the progression of chronic versus acute myeloid leukemia\(^26\).

Recent findings have extended the regulatory role of osteoblasts beyond hematopoiesis and into the initiation of hematological disease by showing that genetic alterations in osteoblasts can induce AML in mice and are associated with AML development in humans\(^27\). Indeed, constitutive activation of β-catenin signaling in mouse osteoblasts disrupts hematopoiesis by shifting the differentiation potential of HSC progenitors to the myeloid lineage, compromising B-lymphopoiesis and inducing anemia. As a result, granulocyte/monocyte progenitors accumulate and AML develops which, is characterized by infiltration of blood, bone marrow, spleen and liver with immature myeloid and dysplastic cells. This type of osteoblast-induced AML phenotype can be transferred to healthy mice and is associated with clonal evolution at the cytogenetic level. The pathway transmitting the leukemogenic signal of activated β-catenin in osteoblasts involves induction of Notch signaling in HSC progenitors. These observations are relevant to human disease, because β-catenin nuclear accumulation in osteoblasts and activation of Notch signaling in hematopoietic cells was detected in a population of patients with MDS, AML, and AML that arose from a prior MDS.

The molecular mechanisms underlying the oncogenic function of β-catenin in osteoblasts are incompletely understood. With the goal to improve this knowledge we sought transcription factors that could interact with β-catenin and affect its activity in osteoblasts. Among the many candidates we focused on FoxO1, a member of the FoxO family of Forkhead transcription factors, because it interacts with β-catenin in osteoblasts, as well as in other
cells types, where it acts to oppose β-catenin function\textsuperscript{28–31}. At the same time, FoxO1 is required to maintain the function and immature state of leukemia-initiating cells\textsuperscript{32} raising the possibility that, at least part of the AML-potentiating properties of FoxO1 might be due to its expression in osteoblasts in addition to its expression in hematopoietic cells. These observations led us to hypothesize that, that FoxO1 may also influence the leukemogenic function of constitutive active β-catenin in osteoblasts.

In testing this working hypothesis through genetic and molecular means, we observed that FoxO1 in osteoblasts does interact with β-catenin, to upregulate Notch ligand expression on osteoblasts and to subsequently induce Notch signaling in long term repopulating HSC progenitors. This interaction leads to anemia, myeloid expansion and AML development.

**Materials and Methods**

**Animals**

*FoxO1*\textsuperscript{fl/fl}, \(α1(I)\text{Collagen-Cre}\) [\(α1(I)\text{Col-Cre}\)], and *Catnb*\textsuperscript{+/-lox(ex3)} mice have been reported\textsuperscript{33–36}. Specific deletion of Notch1 and Notch2 in hematopoietic cells was obtained by breeding *Notch1*\textsuperscript{fl/fl} (Purchased from Jax, Stock# 007181) or *Notch2*\textsuperscript{fl/fl} mice\textsuperscript{37} (purchased from the Jackson Laboratory, Stock# 010525) with *Vav-cre* transgenic mice (purchased from the Jackson Laboratory Stock# 008610). The comparative analysis of all histological and flow cytometry measurements was performed at 1 month of age because *Ctnnb1*\textsuperscript{CAosb} and *Ctnnb1*\textsuperscript{CAosb;FoxO1osb−/−} mice die between 4 and 6 weeks of age. Additional details are provided in the supplementary Information. All the protocols and experiments were conducted according to the guidelines of the Institute of Comparative Medicine, Columbia University.

**Microarray**

Total RNA was extracted from primary osteoblasts isolated from mouse calvaria using Trizol reagent (Invitrogen). Microarray analysis was performed using the GeneChip 3′ IVT Express kit and mouse genome 430 2.0 array gene chips (Affymetrix). Detailed protocol is provided in Supplementary Information.

**Hematological measurements and peripheral blood morphology**

Blood was collected by cardiac puncture and cell counts were performed on a FORCYTE Hematology Analyzer (Oxford Science Inc.). Further details are included in Supplementary Information.

**Reporter constructs and luciferase assays**

Mouse *FoxO1*, *FoxO3*, *FoxO4* and β-catenin expression constructs were transfected in HEK293T, OB-6 or primary osteoblasts. Further details about the preparation of reporter constructs and luciferase assays are given in Supplementary Information.

**Antibodies and Flow Cytometry analysis**

Freshly isolated bone marrow cells and spleen cells were resuspended in flow-staining buffer (PBS plus 2% FBS) and primary conjugated antibodies were added. After 30 minutes of
incubation at 4°C, cells were washed twice before flow cytometry analysis. Detailed staining protocol and listing of antibodies are given in Supplementary Information.

**Histological analysis of murine bone, spleen and liver**

Murine long bones, spleen and liver were collected from one month old mice, fixed overnight in 10% neutral formalin solution, embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin (H&E). Murine bones were decalcified prior to paraffin embedding. Immunohistochemistry details are provided in Supplementary Information.

**Bone marrow transplantation**

Ctnnb1<sup>F<sub>Asob</sub></sup> mice, Notch1<sup>f<sub>fl/fl</sub></sup>;vav-cre, Notch2<sup>f<sub>fl/fl</sub></sup>;vav-cre and their WT control littermates were all CD45.2 congenic mice. Therefore, for transplantation experiments, donor derived bone marrow cells were labeled with CellTrace Far Red DDAO-SE fluorescent dye (Invitrogen) according to the manufacturer’s instructions. Further details are given in the Supplementary Information.

**Assessment of chimerism**

Engraftment efficiency in recipients was monitored by donor contribution of cells with red fluorescence in the blood, bone marrow, spleen and thymus of recipients using FACS analysis. Additional details are provided in Supplementary Information.

**Statistical analysis**

All data are represented as mean ± standard deviation. Statistical analyses were performed using a one-way ANOVA followed by Student-Newman-Keuls test and a p value less than 0.05 was considered significant.

**Results**

**FoxO1 promotes β-catenin signaling in osteoblasts**

To determine whether FoxO1 affects β-catenin signaling in osteoblasts, we examined if the two endogenous proteins interact. FoxO1 physically associated with β-catenin in osteoblasts (Figure 1a). Consistent with this observation, expression of the β-catenin transcriptional targets, Axin2, Tcf1, Tcf3 and Lef1 was increased following forced expression of FoxO1 in osteoblasts (Figure 1b). Expression of the same β-catenin target genes was also upregulated in the bone of Ctnnb1<sup>F<sub>Asob</sub></sup> mice (Figure 1c). In contrast, expression of the FoxO1 targets cyclin D1, D2, p27Kip1, Superoxide Dismutase 2 (Sod2) and Gadd45 were not affected by forced expression of β-catenin in osteoblasts (Figure 1d). In vivo, expression of Axin2, Tcf1, Tcf3 and Lef1 was decreased in bones from mice harboring an osteoblast-specific inactivation of FoxO1 (FoxO1<sub>osb</sub>−/−) as compared to wild type (WT) littermates (Figure 1e). In contrast, expression of the FoxO1 targets was not altered in the bone of Ctnnb1<sup>F<sub>Asob</sub></sup> mice expressing the constitutively active β-catenin allele in osteoblasts (Figure 1f). Confirming the purity of the material used to assess gene expression in bone, expression of the blood-specific genes GlycophorinA, CD19 and CD45 was barely detectable in bone (Figures 1g–i). FoxO1 protein levels were not altered in Ctnnb1<sup>F<sub>Asob</sub></sup> mice (Figure 1j). Taken together,
these observations suggest that FoxO1 and β-catenin could form a functional complex in which FoxO1 acts as a coactivator of β-catenin required for β-catenin activity.

**FoxO1 induces anemia and potentiates myeloid expansion in response to activated β-catenin**

To investigate whether FoxO1 synergizes with β-catenin in osteoblasts to deregulate hematopoiesis in Ctnnb1\(^{CAsb}\) mice, we generated mice lacking FoxO1 in osteoblasts by crossing mice harboring a floxed allele of FoxO1 with mice expressing Cre under the control of 2.3 kb of the proximal promoter of the mouse pro-\(\alpha1(I)\)Collagen gene\(^{39}\). Prior to analyzing these mutant mice we verified we had deleted FoxO1 in osteoblasts but not in HSC or other cell types (Figure 2a). The expression of Axin2, Tcf1, Tcf3 and Lef1 was normalized in the compound mutant mice confirming that removing one allele of FoxO1 had normalized β-catenin signaling in osteoblasts (Figures 2b–e).

We reasoned that if the activating mutation of β-catenin in osteoblasts causes leukemia in a FoxO1-dependent manner then removing one FoxO1 allele should normalize hematopoiesis in Ctnnb1\(^{CAsb}\) mice. Consistent with this hypothesis and cell culture results presented in Figure 1, anemia, peripheral monocytosis, neutrophilia and lymphocytopenia, features observed in Ctnnb1\(^{CAsb}\) mice\(^{27}\), were all corrected in Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− mice (Figures 2f–k and supplementary Table 1). Likewise, the decrease in erythroid cells observed in both the bone marrow and spleen of Ctnnb1\(^{CAsb}\) mice were rescued in Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− mice (Figures 2l–o). Total bone marrow cellularity was decreased in Ctnnb1\(^{CAsb}\) mice but was partially rescued in Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− animals (Supplementary Table 1).

Ctnnb1\(^{CAsb}\) mice are characterized by a shift in the differentiation of HSCs to the myeloid lineage at the expense of B-lymphopoiesis\(^{27}\). Therefore, we examined whether haploinsufficiency at the FoxO1 locus in osteoblasts would correct deregulation of these two lineages in the bone marrow and spleen. We observed that the increase in the relative percentage of myeloid (CD11b+/Gr1+) cells seen in the Ctnnb1\(^{CAsb}\) bone marrow was normalized in Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− mice (Figures 3a and b). Likewise, the percentage of myeloid cells in the spleen of Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− mice was similar to that of WT animals (Figures 3c and d). The loss of typical splenic architecture, expansion of red pulp and coalescence of white pulp, observed in Ctnnb1\(^{CAsb}\) mice was inhibited in Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− mice (Figure 3e).

Because monocytes and granulocytes originate from the same progenitors, we examined the hematopoietic stem and progenitor cell (HSPC) populations in the bone marrow. The HSPC pool size, defined by the percentage of Lin-Sca+c-Kit+ (LSK) cells, is 2- fold greater than in WT littermates (Figure 3f). FoxO1 haploinsufficiency in osteoblasts of Ctnnb1\(^{CAsb}\);FoxO1\(^{osb}\)+/− mice reversed the defect in LSK cells. Similarly, it prevented the increase in the LSK+/CD150+/CD48− subset of long term repopulating HSC progenitors (LT-HSCs) (Figures 3g and h). In the bone marrow, within the myeloid progenitor population (Lin-Kit+Sca1−), alterations in the granulocyte/monocyte progenitor subset (CD34/FcgRII/III, GMP population), observed in Ctnnb1\(^{CAsb}\) mice were rescued in
Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice (Figures 3i and j). Likewise, Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice showed normal GMP population percentage in the spleen (Figures 3k and l).

Taken together these findings indicate that defects in bone marrow, peripheral and spleen hematopoiesis caused by constitutive active β-catenin signaling in osteoblasts are fully rescued by removing one allele of FoxO1 from osteoblasts.

**Constitutively active β-catenin acts through FoxO1 in osteoblasts to induce AML development**

We next asked whether FoxO1, in addition to mediating deregulation of hematopoiesis in Ctnnb1\textsuperscript{CAsb} mice, is also involved in the development of myeloid malignancy. Histological analysis showed that the cellular alterations observed in Ctnnb1\textsuperscript{CAsb} mice associated with their AML phenotype were rescued in Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} animals. Specifically, monocytic and myeloid cells of the long bones, spleen and liver were similar in Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} and wild type mice (Figures 4a–f). The appearance of blasts and neutrophils with nuclear hypersegmentation in the blood (Figures 4a and b), infiltration of myeloid and monocytic cells, and increased atypical micro-megakaryocytes in the bone marrow (Figure 4c) and spleen (Figure 4d and e), observed in Ctnnb1\textsuperscript{CAsb} animals were all eliminated in Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice. The liver of Ctnnb1\textsuperscript{CAsb} mice is characterized by focal blast infiltration at periportal sites rather than extensive infiltration across the entire tissue. This phenotype is similar to other mouse AML models which present with variable extent of leukemia blast infiltration\textsuperscript{40} such as the AML model of human AML1/MDS1/EVI1 fusion protein\textsuperscript{41}. In the Ctnnb1\textsuperscript{CAsb} model limited infiltration in the liver may be due to the dependency of AML on the abnormal marrow niche. Inactivation of one allele of FoxO1 in osteoblasts abrogated monocyte infiltration or clusters of atypical cells in the liver of Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice (Figure 4f). Myeloperoxidase staining of immature myeloid cells in bone marrow, spleen and liver of Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice, was similar to that of WT animals, establishing that leukemogenesis did not occur in these mice (Figures 4g–i).

The reduction of the lymphoid-biased multipotential LSK+/FLT3+ progenitors in the bone marrow (Figures 5a and b) was fully reversed by FoxO1 haploinsufficiency in osteoblasts of Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice. B-lymphopoiesis was partially reversed in Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice (Figures 5c–f). Lastly, FoxO1 haploinsufficiency in osteoblasts prevented the early lethality phenotype of Ctnnb1\textsuperscript{CAsb} mice since Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice lived and were healthy for at least one year, the entire time that they were observed (Figure 5g). Importantly, reduction of FoxO1 levels in osteoblasts of Ctnnb1\textsuperscript{CAsb}; FoxO1\textsuperscript{osb+/−} mice did not rescue the osteopetrotic phenotype that is observed in Ctnnb1\textsuperscript{CAsb} mice, suggesting that AML development occurs independent of osteopetrosis (Figure 5h). Collectively, these results indicate that the development of AML by constitutive activation of β-catenin in osteoblasts depends on FoxO1. In addition they demonstrate an oncogenic role of FoxO1 in osteoblasts that is triggered by constitutive activation of β-catenin in the same cells and potentiates leukemogenesis.
**A β-catenin/FoxO1 interaction in osteoblasts regulates HSC function through Notch signaling**

Next, we sought to investigate the molecular mechanisms through which FoxO1 synergizes with β-catenin to induce AML development. We have previously shown that constitutive activation of β-catenin in osteoblasts induces AML, in part by promoting the expression of Jagged-1 in the same cells. As a result Notch signaling is activated in long term HSC progenitors (LT-LSK cells)\(^\text{27}\). To obtain genetic proof of this mechanism, we inactivated Notch signaling specifically in hematopoietic cells of Ctnnb1\(^{CAosb}\) mice by inactivating either the Notch1 or the Notch2 receptor. Bone marrow cells isolated from C57BL/6J (CD45.2) mice lacking Notch1 or Notch2 in hematopoietic cells (Notch1\(^{fl/fl}\);Vav-Cre and Notch2\(^{fl/fl}\);Vav-Cre mice), or their WT littermates (Notch1\(^{fl/fl}\) and Notch2\(^{fl/fl}\) mice), were labelled with far red florescent dye and transplanted into the liver of 2 day-old, lethally-irradiated CD45.2 Ctnnb1\(^{CAosb}\) mice or WT littermates as previously described. We verified impaired Notch signaling in Notch1\(^{fl/fl}\);Vav-Cre knockout bone marrow cells (Supplementary Figure 1a). Overall chimerism, 2 weeks after transplantation, reached 50%–70% in all examined tissues (Supplementary Figure 1b and c). Our analysis indicated 40%–50% and 67%–80% chimerism within the LSK and LT-LSK populations, the Leukemia Initiating Population from Ctnnb1\(^{CAosb}\) mice, respectively (Supplementary Figure 1d and e). Far red-labeled donor cells were able to differentiate into the myeloid (CD11b+), B-lymphoid (CD19+), and erythroid (Ter119+) lineages (Supplementary Figure 1f–k).

Notch signaling was also assessed in LSK cells following 10 weeks of transplantation. In the transplanted Ctnnb1\(^{CAosb}\) mice Notch signaling was decreased as tested by the expression of Notch transcriptional targets (Figures 6a and b). At this time Notch1 and Notch2 recombination in the marrow and spleen of transplanted Ctnnb1\(^{CAosb}\) mice reached 70%–90% (Figures 6c and d). This degree of recombination along with our observation that host hematopoiesis is ablated in lethally irradiated neonate Ctnnb1\(^{CAosb}\) recipients suggest that the LSK cells used to assess Notch signaling are mainly derived from donor hematopoiesis.

Recipients of Notch2-deficient hematopoietic cells showed heavy infiltration of blood with blasts and dysplastic neutrophils (Figure 6e); their numbers ranged from 18–75% and 20–70%, respectively, within 20 days following transplantation. Transplanted mice maintained low body weight (Figure 6f), and similar to the Ctnnb1\(^{CAosb}\) mice transplanted with wild type bone marrow cells, died within 5 weeks following transplantation (Figure 6g). In contrast, transplantation of Ctnnb1\(^{CAosb}\) neonates with Notch1-deficient hematopoietic cells prevented AML development (Figure 6h), progressively increased body weight (Figure 6i) and rescued lethality as transplanted Ctnnb1\(^{CAosb}\) mice survived for at least 23 weeks (Figure 6j), the entire time they were observed. These results indicate that activation of Notch signaling in HSCs of Ctnnb1\(^{CAosb}\) mice mediates the leukemogenic signal of osteoblasts and that this event is facilitated by Notch1.

In view of this observation, we asked whether β-catenin and FoxO1 act synergistically to activate Jagged-1/Notch signaling and/or whether FoxO1, in response to activated β-catenin induces a distinct pathway that deregulates HSC fate and is also required for AML development.

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To distinguish between these two possibilities we performed an unbiased approach, microarray analysis. To identify potential targets we looked for complementary changes in the expression of secreted molecules in microarrays of Ctnnb1<sup>CAosb</sup> and FoxO1<sub>osb</sub>−/− osteoblasts. The aim of this experiment was to identify FoxO1 targets and to generate compound mutant mice to examine whether β-catenin and FoxO1 synergize in vivo to regulate expression of the identified target. For these genetic epistasis experiments we needed to know the genes that are deregulated in the FoxO1−/− osteoblasts so that we could verify that they were not deregulated in the FoxO1+/− osteoblasts. Microarray analysis of 12,000 genes from Ctnnb1<sup>CAosb</sup> and 34,000 genes from FoxO1<sub>osb</sub>−/− osteoblasts identified only one gene with opposite changes in its expression in both cell types, Jagged-1, a Notch ligand (Supplementary Table 2).

Further experiments confirmed the results of the microarray analysis since, Jagged-1 was upregulated in Ctnnb1<sup>CAosb</sup> and downregulated in FoxO1<sub>osb</sub>−/− compared to WT osteoblasts (Figure 7a). We subsequently examined whether FoxO1 mediates the effects of constitutive activation of β-catenin in osteoblasts to increase Notch signaling in LT-LSK cells, the leukemia initiating cells (LICs) which can transfer the AML of Ctnnb1<sup>CAosb</sup> mice to healthy animals. Similar to the reversal of LSK expansion, removal of one FoxO1 allele from osteoblasts of Ctnnb1<sup>CAosb</sup> mice reversed the increased expression of the Notch targets Hes1, Hes5, Hey1 and Hey2 specifically in the leukemia-initiating LSK+/CD150+/CD48− subpopulation (LT-HSCs) (Figures 7b–e). These results demonstrate that Notch signaling in hematopoietic cells mediates the leukemogenic signal of activated β-catenin in osteoblasts. They also show that FoxO1 is required for activation of the Jagged-1/Notch pathway by β-catenin in osteoblasts and for AML development.

**Transcriptional control of Jagged-1 by β-catenin/FoxO1 signaling in osteoblasts**

The regulation of Jagged-1 expression in osteoblasts by canonical Wnt signaling and FoxO1 suggested that it might be a direct target of TCF-1 and/or FoxO1 in osteoblasts. A close inspection of the DNA sequence of the Jagged-1 promoter region revealed the presence of multiple potential TCF-1 (C/TCTTTG) and FoxO1 (TGTTTT) elements located up to nucleotide −4075 (TCF-1: −4075, −3072, −2626, −2578, −2343, −1993, −1957, −1566, −1232, −1221, −782, FoxO1: −3875, −3861, −3270, −2805, −2442, −2048, −1847, −1835, −1430, −1294). Chromatin immunoprecipitation assays in osteoblasts has shown recruitment of β-catenin to the proximal 2kb promoter region of Jagged-1 (27). An antibody against β-catenin could efficiently immunoprecipitate the −2038/−1691 and −1431/−1085 region of the Jagged-1 promoter spanning elements −1993/−1988, −1957/−1952, −1232/−1227 and −1221/−1216 while weaker binding was observed using primers amplifying the distal −4317/−4015 and −3089/−2776 region of Jagged-1 promoter (Figure 7f). No binding was detectable using primers to amplify the −2675/−2306 or the most proximal −864/−610 region of the promoter. Similarly, using an antibody against FoxO1, recruitment was shown to the −2038/−1691 and −1431/−1085 region of the Jagged-1 promoter while binding to the −4317/−4015 and −3089/−2776 region was not significant. Upon FoxO1 deficiency binding of β-catenin to the −2038/−1691 and −1431/−1085 region of the Jagged-1 promoter was markedly reduced. As a control, FoxO1 antibody did not bind to FoxO1-deficient osteoblasts.
In order to confirm the existence of β-catenin/FoxO1 complex in the same DNA region, rechIP experiments were performed. In these assays, chromatin was first precipitated with antibodies against β-catenin, then was eluted from the beads and subjected to a second immunopurification with antibodies against FoxO1 and vice versa. As shown in Figure 7f, specific signals were observed with both combinations of sequential immunoprecipitations using primers amplifying the −2038/−1691 and −1431/−1085 region of the Jagged-1 promoter. There is no significant rechIP signal even if β-catenin alone is present at the −4317/−4015 region of the promoter showing the simultaneous presence of the two proteins at the elements located within the proximal 2kb region of the Jagged-1 promoter (Figure 7f). The biological importance of these sites was examined by transient transfection assays in FoxO1-deficient or wild type primary osteoblasts using a reporter construct containing the 4.1 Kb of the Jagged-1 promoter fused to the luciferase gene (Jag1-4.1-luc). Overexpression of β-catenin/TCF-1 alone in FoxO1-deficient osteoblasts could not transactivate the Jagged-1 promoter; while re-introduction of FoxO1 induced a 5-fold increase (Figure 7g). Thus, β-catenin is able to form a complex with FoxO1 that enhances β-catenin binding and co-operatively contribute to Jagged-1 upregulation.

Taken together, these observations suggest that β-catenin activation in osteoblasts in conjunction with FoxO1 induces expression of the Notch ligand, Jagged-1, which in turn triggers downstream activation of Notch signaling in adjacent HSCs.

**FoxO3 and FoxO4 compensate FoxO1 homozygous deficiency in Ctnnb1<sup>CAosb</sup>;FoxO1<sup>−/−</sup> osteoblasts and lead to AML development**

In addition to FoxO1, osteoblasts express FoxO3 and FoxO4 and all 3 transcription factors are involved in the regulation of bone mass. Based on this expression pattern and the fact that the three isoforms have redundant functions when expressed in hematopoietic cells, we examined whether FoxO3 and FoxO4 can compensate for the leukemogenic function of FoxO1 in its absence. For this purpose we generated mice expressing constitutive active β-catenin but lacking both FoxO1 alleles in osteoblasts (Ctnnb1<sup>CAosb</sup>;FoxO1<sub>osb</sub>−/− mice). Ctnnb1<sup>CAosb</sup>;FoxO1<sub>osb</sub>−/− mice showed deregulated hematopoiesis and presence of blasts and dysplastic cells in the blood, marrow, spleen and liver, indicating that they had developed AML (Supplementary Figures 2a–h). Ctnnb1<sup>CAosb</sup>;FoxO1<sub>osb</sub>−/− mice have a compound bone phenotype: they are osteopetrotic due to activation of β-catenin but also have low osteoblast numbers due to FoxO1 deficiency in osteoblasts (Supplementary Figures 3a–c). Bone volume was similar to that of Ctnnb1<sup>CAosb</sup> mice, probably because the decrease in osteoclast numbers is pronounced enough to compensate for the decrease in osteoblast numbers.

To understand the mechanism of AML development we measured Jagged-1 expression in their osteoblasts and found that it was increased in the osteoblasts of Ctnnb1<sup>CAosb</sup>;FoxO1<sub>osb</sub>−/− mice, to the same level as in osteoblasts of Ctnnb1<sup>CAosb</sup> mice (Supplementary Figure 4a). Dose response assays comparing the ability of FoxO1, FoxO3 and FoxO4 to activate a Jagged-1 reporter construct show that FoxO1 is a more potent activator than FoxO3 and FoxO4 with 3.5- to 10-fold activity at concentrations of 0.01 – 0.5 μM. (Supplementary Figure 4b). Moreover, combination of FoxO3 and FoxO4 at equal
doses of 0.06 or 0.2 μM increased Jagged-1 promoter activity to similar levels as 0.2 μM of FoxO1. Taken together, these results indicate that decreased levels of FoxO1 in Ctnnb1<sup>Caosb</sup>;FoxO1<sup>osb</sup>+/- osteoblasts prevent FoxO3 and FoxO4 from binding to the Jagged-1 promoter and upregulate Jagged-1 expression and therefore prevent AML development. Homozygous deletion of FoxO1 in Ctnnb1<sup>Caosb</sup>;FoxO1<sup>osb</sup>-/- osteoblasts allows FoxO3 and FoxO4 to compensate in upregulating Jagged-1 expression by β-catenin and the AML phenotype to develop.

### Discussion

In this study we identified a novel function of FoxO1 in osteoblasts as a leukemia potentiator and deciphered the molecular bases of this function. Specifically, we show that FoxO1 synergizes with activated β-catenin in osteoblasts to initiate a program of gene expression that disrupts hematopoiesis by altering HSC lineage determination, ultimately leading to AML. This entire signaling cascade, which mediates the crosstalk between osteoblasts and HSCs, is initiated by activation of canonical Wnt signaling in osteoblasts. In turn, stabilized β-catenin acts through FoxO1 to promote expression of Notch ligands in osteoblasts, leading to activation of Notch signaling in HSCs. These findings demonstrate that osteoblasts are able to produce signals that affect the fate of HSCs and simultaneously induce an oncogenic process. They also show that FoxO1, a known tumor suppressor, assumes the opposite role in osteoblasts, where it acts as a tumor inducer capable of triggering AML in response to activated β-catenin signaling.

Similar to our finding that FoxO1 is pro-leukemogenic when expressed in osteoblasts, and in spite of the fact that FoxOs are primarily known as tumor suppressors, indications of a tumorigenic role for them have been previously reported in chronic and acute myeloid leukemia. High expression of FOXO3 has been associated with adverse prognosis in AMLs exhibiting normal cytogenetics. Furthermore, genetic ablation of FoxO3 reduced disease burden in a mouse model of chronic myeloid leukemia. Lastly, activation of both FoxO1 and FoxO3 has been implicated in human AML as means of inducing myeloid block. Therefore, whether it acts through osteoblasts or through leukemia blasts FoxO1 is an inducer or potent modulator of myeloid leukemia.

Our observations, together with current evidence indicate that FoxO1 has different functions in activating β-catenin signaling in osteoblasts depending on the stage of osteoblast differentiation, and these distinct functions may differentially regulate normal and malignant hematopoiesis. Indeed, FoxO1 inhibits β-catenin signaling by tethering it away from its transcriptional complex in osteoblast progenitors which are implicated in normal hematopoiesis by affecting HSC lineage determination survival and proliferation, HSC expansion and quiescence, erythroid lineage and B lymphopoiesis. In contrast, we show that FoxO1 synergizes with β-catenin in osteoblasts to induce malignant hematopoiesis and the development of AML. These observations suggest that cell differentiation along the osteoblast lineage is a complex process and that manipulating FoxO1/β-catenin signaling in the osteoblast or its progenitor can have different repercussions on hematopoiesis.
An “osteoblastic niche” has been proposed as a specialized microenvironment which provides a quiescent state for the maintenance of hematopoietic cell stemness. Changes in it may precede and promote the initiation of genetic events by creating a pre-malignant state characterized by disruption of quiescence-inducing signals or increases in proliferating signaling. Our study directly implicates osteoblasts in the development of leukemogenesis. Similarly, deletion of Dicer1 in Osterix-expressing osteoblast progenitors has been shown to induce MDS with pancytopenia and emergence of secondary leukemia. These two studies address two different pathways: one that involves microRNA regulation of gene expression, and one that affects expression of canonical Wnt signaling targets. Currently it is not known whether the two pathways intersect, since specific microRNAs expressed in osteoblasts and affecting hematopoiesis have not been identified. This important question needs to be addressed in the future.

The fact that Ctnnb1CAosb mice have an osteopetrotic phenotype raised the possibility that part of the hematopoietic dysfunction may be due to narrowing of the bone marrow cavity. However, our observations demonstrate that leukemogenesis occurs independent of osteopetrosis in Ctnnb1CAosb mice. Inactivation of one FoxO1 allele as well as pharmacological inhibition of Notch signaling rescued dysfunctional hematopoiesis and leukemia in Ctnnb1CAosb mice without affecting osteopetrosis. In support of these observations, HSC progenitors are maintained in osteopetrotic mice and osteoclasts do not affect HSC maintenance or mobilization. Additionally, in contrast to the pancytopenic phenotype observed in Ctnnb1CAosb mice, narrowing of the bone marrow cavity in osteopetrosis mouse models does not affect peripheral blood parameters. Thus, compromised HSCs function, leukemogenesis and mortality observed in Ctnnb1CAosb mice occur independent of osteopetrosis.

The mechanisms through which osteoblasts affect hematopoiesis are now being elucidated. As they emerge, they suggest a variety of signals that can affect different aspects of hematopoiesis. A functional interaction between osteoblasts and HSCs that involve engagement of Notch1/Jag1 signaling promotes HSC proliferation. One involving non-canonical Wnt signaling maintains quiescent long-term HSCs, whereas, inactivation of Wnt signaling in osteoblasts disrupts stem cell quiescence, leading to a loss of self-renewal potential through a Shh-mediated pathway. Recently, disruption of HIF signaling in osteoprogenitors was shown to directly modulate erythropoiesis. These studies underscore the importance of understanding the entire spectrum of functions of osteoblasts in regulating HSC activity and indicate that identifying the mechanisms through which such functions occur can have implications in hematological diseases.

Targeting the hematopoietic niche is currently a new strategy for eradicating persistent and drug-resistant leukemia stem cells. Our observations show that a new niche-specific interaction between activated β-catenin and FoxO1 which takes place in osteoblasts is sufficient to initiate a complex phenotype of disordered hematopoiesis that is reminiscent of AML in humans. In view of these data, we propose that targeting of FoxO signaling may inhibit osteoblast-induced AML or render the osteoblastic niche hostile to CML or AML cells in other models.

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**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FoxO1 interacts with β-catenin in osteoblasts

(a) Coimmunoprecipitation (IP) of β-Catenin and FoxO1 in cell lysates from osteoblast cells followed by immunoblot analysis. Blots were representatives of n=3. (b–f) Real-Time PCR analysis of (b) Axin-2, Tcf-1, Tcf-3 and Lef-1 expression in osteoblasts transfected with increasing concentrations of FoxO1 construct. EV denotes Empty vector. Data are representative of 3 independent experiments. * p < 0.05 versus EV-transfected cells. (c) Real-Time PCR analysis of Axin-2, Tcf1, Tcf-3 and Lef-1 in the bones of wild type and Ctnnb1\textsuperscript{CAsb} mice (Identical data are shown in Figure 2b–e). (d) Real-Time PCR analysis of Cyclin D1, Cyclin D2, p27kip1, Sod2 and Gadd45 expression in osteoblasts transfected with increasing concentrations of β-catenin. EV denotes Empty vector. Data are representative of three independent experiments. * p < 0.05 versus EV-transfected cells. (e) Quantitative Real-Time PCR analysis of Axin-2, Tcf1, Tcf-3 and Lef-1 in the bones of wild type and FoxO1\textsubscript{osb}\textsuperscript{−/−} mice. Total RNA was isolated from flushed long bones. n=4. * p < 0.05 versus wild type. (f) Quantitative Real-Time PCR analysis of Cyclin D1, Cyclin D2, p27kip1, Sod2 and Gadd45 gene expression in bones of wild type and Ctnnb1\textsuperscript{CAsb} mice. Total RNA was isolated from flushed long bones. (g–i) Real-Time PCR analysis of (g) glycophorin A (h) CD45 (i) CD19 in the flushed long bones and bone marrow. n=4. * p < 0.05 versus WT. (j) Immunoblot analysis showing FoxO1 protein levels in bones lysates of wild type and Ctnnb1\textsuperscript{CAsb} mice. Blot is representative of n=3. Results are mean ± SD. All mice were 1 month of age.
Figure 2. FoxO1 haploinsufficiency in osteoblasts rescues anemia and peripheral blood leukocytosis and monocytosis of Ctnnb1<sup>CAosb</sup> mice

(a) FoxO1 recombination in the femur, spleen, liver and indicated hematopoietic populations from wild-type and FoxO1<sub>osb</sub>−/− mice. Bone marrow cells were flushed from the femur prior to DNA extraction. (b–e) Real-Time PCR analysis of (b) Axin2 (c) Tcf1 (d) Tcf3 and (e) Lef1 gene expression in wild type, Ctnnb<sup>fCAosb</sup>, Ctnnb<sup>fCAosb,FoxO1<sub>osb</sub></sup>+/− and FoxO1<sub>osb</sub>+/− mice. (f–j) Total count of (f) WBCs, (g) RBCs, (h) Monocytes (i) Lymphocytes and (j) Neutrophils in the blood. (k) Relative percentage of white blood cell populations in the peripheral blood of wild type and Ctnnb<sup>fCAosb</sup> mice. (l) Flow cytometry analysis showing erythroid cells and (m) Percentage of Ter119+ cells in the bone marrow. (n) Representative images of Flow cytometry analysis showing erythroid cells and (o) Percentage of Ter119+ population in the spleen. In (b–e) N- 4 mice per group. In (f–o) N=6 mice per group. * p < 0.05 versus WT. and # p < 0.05 versus Ctnnb<sup>fCAosb,FoxO1<sub>osb</sub></sup>+/−. Results are mean ± SD. All mice were 1 month of age.
Figure 3. FoxO1 haploinsufficiency rescues myeloid lineage expansion in Ctnnb1CAosb mice
(a) Representative flow cytometry image and (b) Flow cytometry analysis showing percentage of CD11b+/Gr1+ cells in the bone marrow. (c) Representative FACS image and (d) Percentage of CD11b+/Gr1+ population in the bone marrow. (e) Normal splenic architecture in Ctnnb1CAosb;FoxO1osb+/− mice. White arrows indicate coalescence of white pulp and black arrows indicate expansion of red pulp. Scale bars, 500μm. (f) Percentage of LSK cells in the bone marrow (g) Representative FACS image of LSK+ subpopulations/CD150/CD48 population in the bone marrow and (h) Flow cytometry analysis showing percentage of LT-HSCs, ST-HSCs and MPPs in the bone marrow. (i and j) Flow cytometry analysis showing (i) Representative FACS images and (j) Percentage of myeloid progenitor populations in the bone marrow. (k) Representative images of flow cytometry analysis showing myeloid progenitor populations and (l) Percentage of myeloid progenitor populations in the spleen. N=6 mice per group. *p < 0.05 versus WT and # p < 0.05 versus Ctnnb1CAosb;FoxO1osb+/−. MNC: mononuclear cells. Results are mean ± SD. All mice were 1 month of age.
Figure 4. FoxO1 haploinsufficiency in osteoblasts prevents AML in Ctnnb1<sup>CAosb</sup> mice
(a and b) Lack of (a) blasts (white arrows) and (b) hypersegmented neutrophils in the blood of Ctnnb1<sup>CAosb;FoxO1<sub>osb</sub>+/−</sup> mice. Scale bars, 10 μm. (c) Normal megakaryocytes in bone marrow and (d) Normal spleen histology in Ctnnb1<sup>CAosb;FoxO1<sub>osb</sub>+/−</sup> mice. Solid arrows in Ctnnb1<sup>CAosb</sup> spleen indicate atypical megakaryocytes. Dotted arrow in Ctnnb1<sup>CAosb;FoxO1<sub>osb</sub>+/−</sup> spleen indicates normal megakaryocyte. (e) Normal megakaryocytes in the spleen of Ctnnb1<sup>CAosb;FoxO1<sub>osb</sub>+/−</sup> mice. (f) Normal liver histology in Ctnnb1<sup>CAosb;FoxO1<sub>osb</sub>+/−</sup> mice. Arrow indicates a cluster of immature cells in the liver of Ctnnb1<sup>CAosb</sup> mice. (g and h) MPO staining of (g) bone marrow and (h) spleen showing invasion of myeloid cells and in (i) Liver showing focal aggregation of immature myeloid cells in Ctnnb1<sup>CAosb</sup> but not in Ctnnb1<sup>CAosb;FoxO1<sub>osb</sub>+/−</sup> littermates. In figures c–i, scale bars, 20 μm. All mice were 1 month of age.
Figure 5. FoxO1 haploinsufficiency in osteoblasts attenuates altered B-lymphopoiesis and prevents lethality in Ctnnb1<sup>CAosb</sup> mice

(a and b) Flow cytometry analysis showing (a) Representative images of LSK+/FLT3+ population and (b) Percentage of LSK+/FLT3+ cells in the bone marrow. (c) Flow cytometry analysis showing representative images for B-cell populations in the bone marrow. (d) Proportion of B-cell populations in the bone marrow. (e) Flow cytometry analysis showing representative image for B-cell populations in the spleen. (f) Proportion of B-cell populations in the spleen. N=6 mice per group. * p < 0.05 versus WT and # p < 0.05 versus Ctnnb1<sup>CAosb</sup>;FoxO1<sup>osb</sup>+/−. (g) Kaplan-Meier survival curve showing life span in indicated groups. Wild type (n=10), Ctnnb1<sup>CAosb</sup> (n=8), Ctnnb1<sup>CAosb</sup>;FoxO1<sup>osb</sup>+/− (n=10) and FoxO1<sup>osb</sup>+/− (n=10). (h) H&E staining of paraffin embedded long bone sections. Scale bars, 250 μm. Results are mean ± SD. MNC: mononuclear cells. In a–f and h, mice were 1 month of age.
Figure 6. Notch1 inactivation in hematopoietic cells inhibits AML development in Ctnnb1<sup>CAosb</sup> mice

Bone marrow cells were obtained from mice with a deletion of Notch1 or Notch2 in the hematopoietic population, (Notch<sup>1fl/fl</sup>;Vav-Cre and Notch<sup>2fl/fl</sup>;Vav-Cre mice), or control littermates, (Notch<sup>1fl/fl</sup> and Notch<sup>2fl/fl</sup> mice). From each respective donor mouse, 1×10<sup>5</sup> bone marrow cells were transplanted into the liver of each lethally-irradiated (600 rads, split dose) neonate Ctnnb1<sup>CAosb</sup> or wild type recipient. (a and b) Quantitative Real-Time PCR analysis of Notch target genes Hes 1, Hes 5, Hey 1 and Hey 2 in the LSK population of wild type mice transplanted with (a) Wild type and Notch2-deficient bone marrow cells and (b) Wild type mice transplanted with bone marrow cells from wild type and Notch1-deficient bone marrow cells, 10 weeks following transplantation. (c and d) PCR analysis of (c) Notch2 and (d) Notch1 allele excision in transplanted recipients. (e) Blasts in the blood of Ctnnb1<sup>CAosb</sup> mice transplanted with Notch2 deficient bone marrow cells. Scale bars, 10 μm. (f) Body weight of mice transplanted with Notch2-deficient or Notch2 wild type control.
(Notch2^{fl/fl}) bone marrow cells. (g) Survival curve in mice transplanted with Notch2 deficient bone marrow cells. (h) Lack of blasts in the blood of Ctnnb1^{CAosb} mice transplanted with Notch1 deficient bone marrow cells. Scale bars, 10 μm. (i) Body weight of mice transplanted with Notch1-deficient or Notch1 wild type control (Notch1^{fl/fl}) bone marrow cells. (j) Survival curve in mice transplanted with Notch1 deficient bone marrow cells. In A–B, n=3 mice per group. In g and j, n= 6 mice per group. In f, n=8 mice in WT-WT, Notch2 - WT transplanted groups at all indicated time points. n= 8 mice in WT-Ctnnb1^{CAosb} transplanted group at week 1, 2, 3 and n=3 mice at week 4. n =8 mice in Notch2- Ctnnb1^{CAosb} transplanted group at week 1, 2, 3 and n=4 mice at week 4. *p < 0.05 versus Notch1- Ctnnb1^{CAosb} transplanted group. In i, n=6 mice per group in WT-WT, Notch1-WT and Notch1- Ctnnb1^{CAosb} transplanted groups at all indicated time points. n=6 mice in WT- Ctnnb1^{CAosb} transplanted group at week 1, 2, 3 and n=3 mice at week 4. *p < 0.05 versus Notch 1- Ctnnb1^{CAosb} transplanted group. In A and B *p < 0.05 versus WT-WT transplanted mice. Data are pooled from two independent experiments. Results are mean ± SD. Solid arrows indicate blasts and dotted arrows indicated hypersegmented neutrophils.
Figure 7. β-catenin/FoxO1 interaction in osteoblasts regulates HSC function through Notch signaling

(a) Expression levels of Jagged-1 in WT, Ctnnb1CAosb and FoxO1osb−/− bone. (b–e) Expression of (b) Hes1 and (c) Hes5 (d) Hey1 and (e) Hey2 in LSK+ subpopulations. In (a–e) n=4 mice per group and * p < 0.05 versus WT. (f) Re-Chip analysis of β-catenin and FoxO1. Complexes immunoprecipitated with the indicated first antibodies were eluted from the protein G-sepharose beads and after dilution were immunoprecipitated with the indicated second antibodies. Left panel: FoxO1-deficient osteoblasts were subjected to a single chromatin immunoprecipitation in the absence (no ab) or the presence of the anti-β-catenin antibody. Non immunoprecipitated chromatin was included (input). Promoter occupancy was assessed by PCR amplification using primers corresponding to the (−2039/−1691), (−1431/−1085), or the (−4317/−4015) region of Jagged1 promoter. Right panel: Lack of FoxO1 antibody binding to extracts from FoxO1-deficient osteoblasts. (g) Luciferase activity in primary osteoblasts isolated from WT and FoxO1osb−/− mice co-transfected with β-catenin, Lef1, FoxO1 and Jagged1-Luc (−4112/+130) reporter constructs. Results show
fold induction over empty vector. *p < 0.05 versus respective Jagged1-Luc. n=3 replicates. Results are mean ± SD. All mice were 1 month of age.