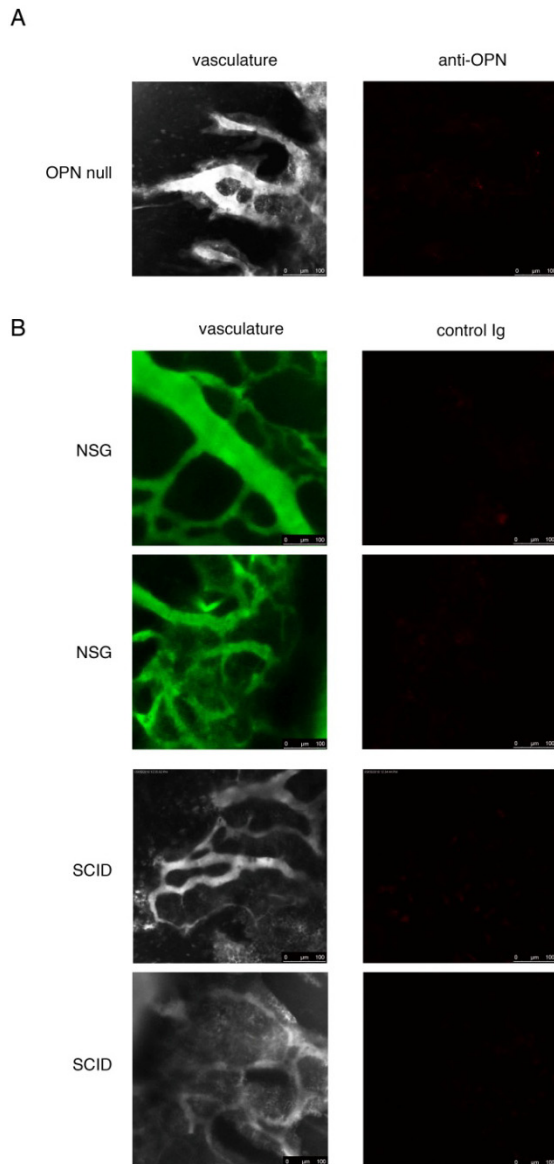


Supplementary Materials

Supplementary Table 1. Primary human ALL BM samples used in for *in vitro* assays and NSG mouse engraftment.

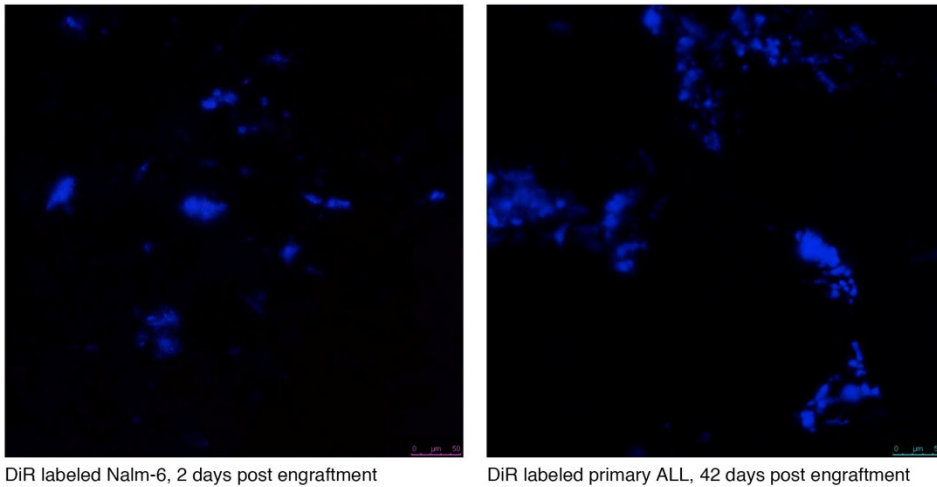
Case Number	Lineage/Maturation	Ph-Status	BM Blasts	Age	Prior Therapy	WBC on Presentation (1,000/ μ l)
1	Pre-B	Negative	93%	3	None	9
2	Pre-B	Negative	90%	31	None	3
3	Pro-B	Negative	96%	71	None	5.9
4	Pre-B	Negative	95%	5	None	7
5	Pre-B	Negative	95%	43	None	22
6	Pre-B	Negative	95%	2	None	23

Supplementary Figure 1



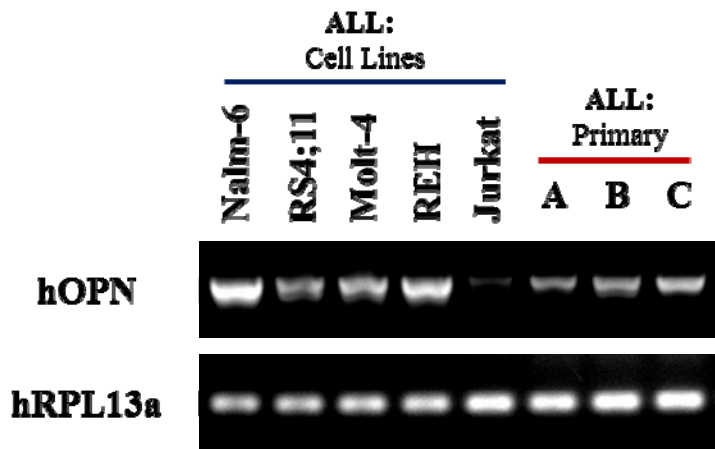
The OPN Ab used for intravital imaging is specific to OPN. (A) An OPN null mouse was injected with AF647-labeled OPN imaging antibody to demonstrate no stromal reactivity in the absence of OPN expression. **(B)** NSG and SCID mice were injected with AF647-labeled isotype control antibody to demonstrate that the observed OPN staining pattern was specific to OPN detection.

Supplementary Figure 2



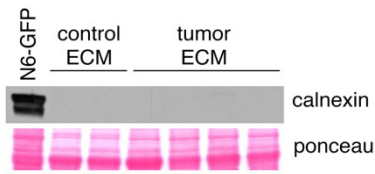
Dye-retaining primary ALL cells are bright and highly dormant. NSG mice were engrafted with DiR-labeled Nalm-6 or primary ALL cells. The Nalm-6 engrafted mouse was imaged 2 days post engraftment, while the primary ALL engrafted mouse was imaged 40 days later. Primary ALL blasts had equivalent fluorescence intensity at D42 as newly-engrafted Nalm-6, demonstrating they had not undergone multiple cell divisions and were highly dormant.

Supplementary Figure 3



OPN mRNA transcripts are detected in ALL cell lines and primary human ALL. RT-PCR analysis shows detectable levels of human osteopontin (hOPN) mRNA transcripts in select ALL cell lines and in BM of NSG mice engrafted with primary human pre-B ALL cells.

Supplementary Figure 4



Bone marrow ECM preparations are void of human cytosolic content. Bone marrow ECM preparations from control and tumor engrafted mice were analyzed via Western blot for the presence of human cytosolic protein. Nalm-6 whole cell lysate was used as a positive control and membranes were probed with a human calnexin Ab.

Supplementary Methods

Flow cytometry analysis. For *in vitro* cell surface receptor staining, 1×10^6 cells were stained with antibody for 1 hour and analyzed on a BD LSRII cytometer. For *in vivo* analysis, femurs and iliac crests from tumor engrafted mice were ground in RPMI containing 10% FBS, and 4×10^6 cells were stained as above. To stain for the Ki67 antigen, 4×10^6 cells were first stained for CD10 and then fixed in 1% paraformaldehyde for 1 hr at 4°C, permeabilized in 75% ethanol overnight at -20°C, and incubated with anti-human-Ki67. Live cell gating was accomplished using Live/Dead Fixable viability stain (Invitrogen).

***In vivo* OPN neutralization studies.** Male SCID mice between 6-8 weeks of age were injected via tail vein with 1 mg/kg anti-mouse OPN and 2 mg/kg anti-human OPN or 3 mg/kg control goat Ig (Day 0). 24 hours post antibody injection, mice were engrafted with 10×10^6 Nalm-6-GFP (Day 1). Mice received subsequent antibody injections on Day 7 and Day 14 and were processed as described above for flow cytometry analysis on Day 21.

***In vivo* confocal and multiphoton microscopy.** Mice were anesthetized and a small incision was made in the scalp. Nalm-6 or primary ALL cell homing and engraftment in BM of the skull was then analyzed using a Leica SP5 confocal and multiphoton microscope with a 10x or 20x/0.40 NA objective lens. The system utilizes a femto-second Titanium:sapphire laser (Chameleon) for multiphoton or single photon excitation and multiple cs lasers (including an Argon laser, a HeNe laser and 561 nm and 633 nm diode lasers) for single-photon excitation. The images were captured using Leica LAS-AF software using line and frame averaging. DiR was excited with a Ti:sapphire laser at 740 nm and detected using an avalanche photo diode.

***In vivo* OPN neutralization and Ara-C MRD studies.** Mice were engrafted with 10×10^6 DiR labeled Nalm-6-GFP on Day 0. On Day 10, mice were injected via tail vein with 1 mg/kg anti-mouse OPN and 2 mg/kg anti-human OPN or 3 mg/kg control goat Ig. 4 hours post antibody injection, mice were treated with Ara-C via IP injection. Mice received Ara-C injections on Days 10-14, were untreated on Days 15-23, received Ara-C injections on Days 24-28, were untreated on Days 29-35, and were imaged as described above on Day 35. DiR positive cells were manually counted.

Statistical Analysis. All statistical analyses were performed using Graphpad Prism software. All p values were determined using a two-tailed student's T-test and data is presented as mean +/- SEM. T-tests were performed as paired analyses for the data presented in Figure 4.

RNA extraction and PCR analysis. For cell lines, 10×10^6 cells in logarithmic growth phase were lysed in Trizol (Invitrogen) and RNA was extracted following manufacturer's protocol. cDNA was generated using a Super Script III cDNA Synthesis kit (Invitrogen), and RT-PCR analysis was performed using the OPN primers FWD: caaacgccgaccaaggaaaactcact and REV: gtagcatcagggtactggatgacagg. For murine and human OPN analysis from femurs, femurs were dissected, immediately flash frozen in liquid nitrogen, and ground using a mortar and pestle. Ground femurs were then homogenized in Trizol (Invitrogen) and RNA was extracted and cDNA generated as above. For human OPN analysis, RT-PCR was performed using the above-describe hOPN primer set as well as the following endogenous controls: human-specific mRPL13a and pan-specific β -actin. For murine OPN quantitative real-time PCR analysis, we

utilized Taqman Gene Expression Master Mix (Applied Biosystems) and the following Taqman primer probe sets: mOPN: Mm00436767_m1 and mB2M: Mm00437762_m1. Reactions were run on a Roche Lightcycler 480, and fold change was determined using the $\Delta\Delta C_t$ method with mB2M as the endogenous control.

***In vitro* adhesion assay.** Recombinant human OPN was cleaved using the Thrombin Cleavage Capture Kit (Novagen) following manufacturer's protocol. 96-well plates were coated overnight at 4°C with recombinant bovine fibronectin (Sigma), full length recombinant human OPN, or thrombin-cleaved recombinant human OPN (RnD) at a concentration of 20 $\mu\text{g}/\text{mL}$. Wells were blocked for non-specific binding with 3% BSA in PBS. Nalm-6-GFP or primary ALL cells were resuspended at a concentration of $1 \times 10^6/\text{mL}$ in adhesion buffer containing 1M Tris, 1M CaCl_2 , 5M NaCl_2 , 1 μM PMA and 2mM MnCl_2 . Wells were pre-incubated at with 20 $\mu\text{g}/\text{mL}$ OPN neutralizing antibody or control goat Ig. Cells were plated at 100,000/well and allowed to adhere for 2 hours. Bright field images were taken of multiple fields and adhesion was determined by counting the number of cells that had adhered and spread versus those that remained round and in suspension. For VLA-4 neutralization, cells were pre-incubated in 10 $\mu\text{g}/\text{mL}$ anti-VLA-4 antibody for 30 minutes at 37°C prior to plating.

***In vivo* Nalm-6 cell homing studies.** SCID mice were injected via tail vein with an antibody cocktail containing 1 mg/kg anti-mouse OPN and 2 mg/kg anti-human OPN or 3 mg/kg control Ig. 24 hours post antibody injection, mice were engrafted with 10×10^6 DiR-labeled Nalm-6-GFP. Another 24 hours post cell engraftment, the calvarial BM of mice was imaged as described

above. Cell homing was quantified by determining the total DiR signal area in merged images of the calvarial marrow using Fiji image analysis software.

In vivo immunofluorescence imaging. Antibodies were conjugated to AF647 (Invitrogen) according to the manufacturer's protocol and purified from unbound reagent on Dye Removal columns (Pierce). Labeled anti-OPN or isotype control antibodies were injected intravenously into mice at doses of 1 mg/kg. *In vivo* confocal microscopy was then performed approximately 24 hours post-injection as described above. The AF647 probes were excited with a 633 nm diode laser and detected through a trichromic diode filter. To determine co-localization of dormant cells with stromal OPN, mice were engrafted with 10×10^6 DiR labeled Nalm-6-GFP or 1.75×10^6 primary ALL cells. 41 days post engraftment, mice were injected first with 1 mg/kg unlabeled IgG_{2A} isotype to block non-specific binding and then with AF647 labeled anti-OPN antibodies 2 hours later. Mice were imaged on post-engraftment day 42, approximately 20 hours after injection with anti-OPN antibodies.

ECM preparation, ELISA and Western blotting. For ECM protein preparation, BM was aspirated from control or tumor-engrafted femurs using chilled PBS. Following a 5-minute incubation on ice, cellular content was removed via a series of centrifugation steps at 4°C (3 spins at 1000xg for 5 minutes, followed by 1 spin at 2000xg for 10 minutes). The resultant supernatant was concentrated using Amicon Ultra spin concentrators (Millipore) with a 10,000 Da MW cutoff. For Western analysis, 50 µg of lysate was loaded onto precast Criterion SDS-PAGE gels (BioRad) and transferred to nitrocellulose membrane following standard procedure. Human calnexin detection was performed using a human-specific calnexin antibody (clone 2433,

Cell Signaling) and enhanced chemiluminescence following manufacturer's protocol. Human OPN levels in Nalm-6 conditioned media as well as the ECM preps were obtained using the ImmunoSet Osteopontin ELISA Kit (Assay Designs) following manufacturer's protocol.

Immunohistochemistry. Human BM core biopsies (University of Chicago IRB #11-0179) and mouse femurs were fixed in B5, decalcified and embedded in paraffin. Immunohistochemistry on mouse femurs was performed using anti-OPN antibody at a 1:250 dilution according to standard protocol. Immunohistochemistry on human biopsies was performed using anti-human OPN antibody at 1 1:250 dilution according to standard protocol.

Primary Patient Samples. Cryopreserved primary ALL samples were obtained from the University of Chicago Cancer Cytogenetics Laboratory under IRB protocol #11-0179. Cells were thawed immediately prior to use, stained using Invitrogen's Live/Dead Viability Assay and viable blasts collected by FACS for use in *in vitro* OPN adhesion assays and *in vivo* engraftment studies in NSG mice.

Animal use. 6 to 8 week old male SCID mice were xenografted with 10×10^6 Nalm-6-GFP via tail vein. All animal experiments were performed following guidelines approved by the Institutional Animal Care and Use Committee at the University of Chicago – protocol #71675.