NEUREXOPHILIN1 SUPPRESSES THE PROLIFERATION OF HEMATOPOIETIC PROGENITOR CELLS

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Acknowledgements

My acceptance to graduate school was made possible by the combined efforts of two families. Due to a negative research experience as an undergraduate, I had resolved not to pursue a career in science. This decision was met with (justified) disbelief by my parents, both of whom have provided me with a deep and abiding love of learning. Growing up, my mother had done her best to impress upon me a love of history (both natural and otherwise), while my father had made a point of demonstrating how awesome chemistry can be – and both enabled all manner of geeky interests, such as robots for as long as I can remember. As I had long expressed a desire to become a scientist, my parents pressured me to apply to graduate school against my wishes. Because actually getting accepted was the last thing I wanted, I made sure to sabotage every application I submitted: some I sent in woefully incomplete, others complete with references from people deeply antipathetic towards me. Not wanting to offend my friend and roommate Jeffery Broxmeyer, I made sure not to send an application to the program headed by his father. While visiting his son, Hal E. Broxmeyer had taken a shine to me and suggested I apply to his program because the floor of our apartment was littered with copies of Science (the subscription given to me by my older brother as a Christmas present). My hopes of escaping without offense did not take Hal's strong-headedness into consideration, whose initial enthusiasm faded into frustration and disappointment (an emotional journey I am confident I allowed Hal to experience several more times over the course of my graduate career). In keeping with his prolific

nature as a scientist, Hal isn't one to leave an unsolved problem unaddressed so under the guise of visiting Jeffery in Indianapolis for a few days over the summer after graduation (Jeff has since assured me that some prompting was involved), I was fortunate enough to tour the Broxmeyer lab and meet many of the fine people I would later work with as well as talk with several faculty members (such as Prof. Bauer and Prof. Yang). With the benefit of hindsight, I can now see this experience as my interview for graduate school. At the end of my trip, I had been offered a place in the incoming graduate class – but still I deferred, asking for more time. It wasn't merely my negative experience in laboratory research which gave me pause, but also a nagging sore throat which interfered with my thought process. Because of my large mouth (inherited from my father), my dentists thought that I would have space for my wisdom teeth and I had never had them removed. They were mistaken and unbeknownst to me, the "sore throat" I was experiencing was a "raging bone infection" which "nearly cost me my jaw" according to the oral surgeon who treated me. In my opiate-addled recovery, I decided that graduate school was a fantastic idea – whether the opiates enabled me to make a decision in keeping with my true desire or drunkenly stumbling along the path of least resistance, I'll allow others to judge.

Similar to the Ph.D. I hope this thesis proves me worthy. Acceptance to graduate school is a beginning, not an end and over my graduate career I have been helped by too many people to list. First and foremost, there is my mentor Hal E. Broxmeyer, of which some has already been written. While there were times he frustrated me (an emotion I am

confident was mirrored), I feel very humbled to have had a mentor who had faith in me when he really shouldn't have and helped me focus the raw creative (and sometimes insane) energy of my brain towards proper science. Likewise, my committee (past and present) was instrumental in my matriculation as a scientist. From rotation, the keen analytic mind of Prof. Pelus has helped me distinguish the publishable avenues of my research from (theoretically interesting) flights of fancy – his approachable demeanor also helped give me the courage to share my problems and concerns with those who outrank me. Likewise, at an early stage Dr. Fu provided friendship and a role model whereby I could see how the frantic energies of creative excess can be tempered into proper science. With his acceptance of a position in Singapore, Prof. Harrington had big shoes to fill and she did so admirably. Her kind and gentle method of mentoring helped enable me to see experimental avenues and applications of my research to which I would have otherwise remained blind. Lastly, Prof. Nakshatri is the sort of man every scientist strives to be. Possessed of a comprehensive knowledge of scientific thought and an insatiable curiosity, I am beyond grateful to have had the benefit of his mind applied to my graduate career. I especially remember a question he asked during my Qualifying Examination about the role of NRXN1α in nicotine addiction citing a paper I was not even aware existed. Thank you, all.

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the system described within this thesis. Jamie, thank you for your support and as a scientist I feel compelled to remind you that for any given experiment a negative result does not carry any normative weight. Gina, thank you for staying by me and supporting me while I was writing, your tolerance is to be commended. Kris Kmitta and Jess Reagan, thank you for not merely being friends but also for being a social nexus for me here in Indianapolis. Lastly, to all the members of Happy Wednesday: thanks for keeping it real and staying in touch.

Abstract

John M Kinzfogl

NEUREXOPHILIN1 SUPRESSES THE PROLIFERATION

OF HEMATOPOIETIC PROGENITOR CELLS

Neurexin I alpha (NRXN1α) and Dystroglycan (DAG1) are membrane receptors which serve as mutual ligands in the neuronal system. Neurexophilins (NXPHs) bind NRXN1α. Both NRXN1α and DAG1 were expressed in primitive populations in human cord blood (huCB) and murine bone marrow (muBM), with high concentrations of NXPHs in huCB plasma. We evaluated effects of these molecules on huCB and muBM hematopoietic progenitor (HPC) and stem (HSC) cells. At both a single and population level *in vitro*, we found that NXPH1 is a potent inhibitor of HPC proliferation acting through NRXN1α, an effect antagonized by DAG1. Injection of recombinant NXPH1 *in vivo* resulted in myelo- and lymphosuppression, with absolute numbers and cycling status of functional and phenotypically defined HPCs dose- and time-dependently decreased, and absolute numbers and cycling status of phenotypically defined longer-term repopulation HSCs increased. Competitive transplants showed an initial decrease in engraftment of NXPH1-treated cells, with an intermediate stage increase in engraftment. The increase in HSCs is at least partially mediated by the mTOR pathway and is thought to be homeostatic in nature. These results demonstrate the presence and function of a regulated signaling axis in hematopoiesis centered on NRXN1α and its modulation by DAG1 and NXPH1.

Hal E. Broxmeyer, Ph.D., Chair

Table of contents

List of tables

List of figures

Abbreviations

Aorta-gonad-mesenpheros	AGM
Acquired Immune Deficiency Syndrome	AIDS
α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate	AMPA
Allophycocyanin	APC
Bcl-2-associated X protein	BAX
B-cell lymphoma	BCL
Burst-forming unit Erythrocyte	BFU-E
Basic helix-loop-helix	bHLH
Bone marrow	BM
Bromo-deoxyuridine	BrdU
Bovine serum albumin	BSA
Cord blood	CB
Cluster differentiation	CD
Colony-forming unit	CFU
CFU-erythroid	CFU-E
CFU-granulocyte	CFU-G
CFU granulocyte/erythrocyte/megakaryocyte/Macrophage	CFU-GEMM
CFU-macrophage	CFU-M
CFU-megakaryocyte	CFU-Mk
CFU-spleen	CFU-S
Common lymphoid progenitor	CLP

Common myeloid progenitor	CMP
Chemokine CXC motif receptor	CXCR
Dalton	Da
Dystroglycan	DAG1
Drosophilia disc large tumor suppressor	DlgA
Dibco phosphor-buffered saline solution	DPBS
Electrochemiluminescence	ECL
Ethylenediaminetetraacetic acid	EDTA
Epidermal growth factor	EGF
Erythropoietin	EPO
E-twenty six	ETS
Embryonic stem cell	ESC
Flourescence-activated cell sorting	FACS
Fetal bovine serum	FBS
Immunoglobulin G Fc Receptor	FcγR
Fluorescein isothiocyanate	FITC
Fms-like tyrosine kinase receptor-3 (ligand)	Flt3L
Proto-oncogene tyrosine protein kinase Fyn	FYN
Granulocyte colony stimulating factor	GM-CSF
γ-aminobutyric acid	GABA
Glucagon-like peptide	GLP
Granulocyte/macrophage colony stimulating factor	GM-CSF

Granulocyte/macrophage progenitor	GMP
Growth factor receptor bound protein	Grb
Gray	Gy
Human	hu
Human immunodeficiency virus	HIV
Hematopoietic progenitor cell	HPC
Hematopoietic stem cell	HSC
Hematopoietic stem/progenitor cell	HSPC
Half maximal inhibitory concentration	IC50
Helix-loop-helix inhibitor of differentiation	Id
Immunoglobulin	Ig
Insulin-like growth factor binding protein	IGFBP
Interleukin	IL
Maximal inhibition	I_{max}
Iscove's modified dulbecco's medium	IMDM
Intermediate repopulating hematopoietic stem cell	ITR-HSC
Low-density fraction	LD
Lineage	Lin
Laminin, Neurexin, Sex-hormone binding domain	LNS
Lin^-, $sca1^+$, $c\text{-}kit^+$	LSK
Alpha-latrotoxin	LT
Long-term repopulating hematopoietic stem cell	LTR-HSC

Murine	mu
Magnetic activated cell sorting	MACS
Membrane associated guanylate kinase	MAGUK
Megakaryocyte/erythroid progenitor	MEP
Multipotent progenitor	MPP
Mesenchymal stem/stromal cell	MSC
Mammalian target of Rapamycin	mTOR
Nicotinic acetylcholine receptor	nAChR
National center for biotechnology information	NCBI
Nuclear factor kappa-light chain enhancer of activated B-cells	NFκB
Nerve growth factor	NGF
N-methyl-D-aspartic acid	NMDA
Non-obese diabetic/severe combined immunodeficiency	NOD/SCID
NOD/Shi-scid/IL-2Rα(null)	NSG
Neurexin	NRXN
Neurexophilin	NXPH
Paired box	PAX
Peripheral blood	PB
Phosph-buffered saline	PBS
Pre-B-cell leukemia homeobox	PBX
PSD95, DlgA, zo-1	PDZ
Phycoerythin	PE

Propidium Iodide	PI
Post-synaptic density protein	PSD
Stem cell factor	SCF
Stromal cell derived factor	SDF
Sodium dodecyl sulfate	SDS
Scr homology containing	SHC
Signaling lymphocytic activation molecule	SLAM
Sarcoma	Scr
SCF, TPO, Flt3L	STF
Short term repopulating hematopoietic stem cell	STR-HSC
Tris-buffered saline and Tween-20	TBST
Thrombopoietin	TPO
Tris(hydroxymethyl)aminomethane	Tris
Zonula occludens	zo

Introduction

Historical overview

In the study of human physiology, blood has always maintained an esteemed position. Hippocrates listed blood as one of the four humors that creates the body. The sanguine humor was associated with a passionate and courageous, if flighty, temperament. The first recognizable medical investigation of blood was performed by Galen (131-201CE), the most influential physician in history. He discovered that blood was carried by the arteries and, combining the philosophy of Hippocrates with observations from dissected animals, hypothesized that the liver was the hematogenic organ, where hematopoiesis begins (Boylan 2007).

These philosophies of blood existed with little challenge until the Scientific Revolution. Blood cells were first observed in 1658 by Jan Swammerdam, though formal study of blood cells did not begin until 1674 with Anton van Leeuwenhoek's work. Despite these early discoveries, it would take over a century before components of blood other than red cells were discovered. As part of his work with the vaginal secretions of prostitutes, Alfred Donné would discover platelets in 1842. White blood cells were simultaneously identified in 1843 by Gabriel Andral in France and William Addison in England. After the discovery of the three main elements of blood, Lionel Beale and others attempted to further characterize them using microscopy. This approach met with little success until

1879 when Paul Ehrlich published his techniques for staining blood films and differential blood counting (Hajdu 2002).

The science of blood transfusions progressed in a similar manner, with a strong early start at around the same time blood was first described, coupled with a combination of scientific and cultural road-blocks that slowed its development. The first recorded successful blood transfusion was performed by Richard Lower on dogs in 1665. After initial experiments, Lower found that stitching the artery of the donor animal to the vein of the recipient was the most favorable combination, so a medium sized dog was bled from the jugular vein until it was close to death. Using a reed, he then connected a neck artery of a large dog to the jugular of the bled animal. He allowed blood to flow into the recipient until the donor died. As if by magic, the smaller dog seemed to come back from the dead and after the jugular vein was sewed close it leapt from the table and rolled in the grass to clean itself from the blood as though nothing had happened (Keynes 2010).

This work would be expanded upon by Jean-Baptiste Denis, who in 1667 performed the first blood transfusion in a human being using calf's blood. The recipient, Antoine Mauroy, was a madman who suffered from violent outbursts and it was thought that the gentle nature of the calf would temper his condition. Antoine Mauroy not only survived the experience but it also seemed to treat his condition: his violent outbursts subsided and the evil which had driven him mad was expelled as black urine. The black

urine is immediately recognizable to modern scientists as a result of hemolysis. The human immune system recognized the calf's blood as foreign and destroyed them, thereby releasing hemoglobin into the bloodstream. However, that his madness was actually treated by the procedure is at first quite surprising though knowledge of the source of Mauroy's madness clarifies this issue somewhat. A regular patron of brothels, it is probable that Mauroy's violent outbursts stemmed from syphilis. *Treponema pallidum* has a poor tolerance for elevated temperatures and the fever caused by the xenotransfusion likely killed the bacteria (Starr 2000).

The publication of Denis' experiment would create a fad of blood transfusion throughout Europe. While the initial success of cross-species transfusions may be surprising to modern readers, the eventual deaths resulting from it are not. In 1670 the French Parliament banned all transfusions involving humans, a move quickly followed in England and Rome. It would be over 100 years before blood transfusion was once again visited by scientists (Ficarra 1942). In 1795 the first human-to-human blood transfusion is thought to have been performed by Philip Syng Physick in Philadelphia, though the first published account was performed by James Blundell in 1818 (Rudmann 2005).

Following the work of Blundell, transfusions during the 19th century relied on surgically connecting the artery of the donor with the vein of the recipient. Because of the high mortality associated with transfusions, this procedure remained relatively rare. The first problem inherent to blood transfusion is blood type – a problem that would persist until

1901 with Karl Landsteiner's characterization of blood types (Society, Hankins et al. 2001). The second problem was the actual method of transfusion. Echoing the experiments of Lower two centuries prior, transfusion in the 19th century required a deft surgeon who could both attach the artery and vein of the donor and recipient as well as detach and close each without killing either the donor or the recipient. This technique was simplified by Dr. Aveling who developed an apparatus consisting of two silver tubes connected by a length of rubber tubing with a squeeze bulb attached (Aveling 1872). No longer the provenance of highly specialized individuals, transfusions could be performed by any surgeon; however, oxygen introduced by the squeeze pump lead to clotting. While various attempts to address this problem existed, a true solution did not present itself until 1915. While it had been known since the 1860s that citrate prevented blood clotting, the standard laboratory concentration of 1% proved lethal to recipients. Performing a series of simple dose-response experiments, Dr. Richard Lewisohn found that a 0.2% concentration was both sufficient to prevent blood clotting, as well as below the threshold of citrate toxicity in recipients (Starr 2000).

The new ease of transfusion would allow for some unusual experiments, such as the use of cadaver blood in the Soviet Union (Yudin 1937), as well as the discovery and identification of plasma. When blood treated with citrate or any other anticoagulant is allowed to sit, it separates into three layers: red cells at the bottom, a thin buffy coat of white cells and platelets, and a layer of clear plasma floating at the top. Plasma was found to be useful in treating patients suffering from traumatic shock, such as suffered

by individuals exposed to gunfire (Tatum, Elliott et al. 1939). Lacking fragile red blood cells, plasma can be stored for longer periods of time than whole blood making it ideal for transatlantic shipping; however, it is nutrient rich and is easily contaminated. Seeking a solution to the contamination issue, Dr. Edwin J. Cohn's laboratory began fractionating plasma into its constituent parts. They characterized the primary protein constituents of plasma as fibrinogen, globulins, and albumin. Albumin was identified as a powerful anti-shock agent, which could be shipped in dried form, thereby solving the problem of contamination (Cohn, Oncley et al. 1944; Cohn, Strong et al. 1946; Cohn, Hughes et al. 1947; Cohn and Wolfson 1948; Cohn 1948).

Like so many other things, the field of hematopoiesis was thrust into modernity through tragedy. After the military deployment of nuclear weapons at the end of the Second World War, Japanese began dying of radiation poisoning. This was unexpected at the time, as it was commonly thought that the primary difference between nuclear and conventional explosives was the explosive force carried per weight of payload. By observing the symptoms of radiation poisoning, medical scientists hypothesized that adult hematopoiesis was a dynamic system supported by primitive cells that could differentiate into different kinds of hematopoietic cells, as well as having the ability to renew themselves. The refinement of this hypothesis gave rise to the modern understanding of hematopoietic stem (HSC) and progenitor (HPC) cells, as well as the hematopoietic hierarchy (Lanza 2004).

Identification of the functional HSC

In keeping with its original discovery, an HSC is defined by its ability to completely restore the hematopoietic system of a lethally irradiated animal. In order for this to occur, HSCs are required to manifest two distinct capacities: hematopoietic multipotency and self-renewal. Hematopoietic multipotency is the ability of HSCs to give rise to all mature blood cell types, while self-renewal is the ability of HSCs to divide with at least one daughter cell being an HSC in order to maintain the hematopoietic stem cell pool throughout the life of the animal. Shortly after the HSC was theorized, it was demonstrated that bone marrow and spleen cells from healthy animals could restore the hematopoietic system in lethally irradiated recipients (Jacobson, Simmons et al. 1951; Lorenz, Uphoff et al. 1951), a process which was further refined over the next decade. The spleens of reconstituted mice developed nodules in proportion with the quantity of donor bone marrow cells injected (Till and McCulloch, 1961). Till and McCulloch hypothesized that these nodules developed from a single cell: the HSC.

Unfortunately, the initial enthusiasm for colony-forming units-spleen (CFU-S) cells was misplaced, and its position in the hematopoietic hierarchy was ultimately clarified. Unlike a true HSC, the CFU-S is only able to restore cells of the myeloid lineage and is additionally unable to reconstitute a lethally irradiated mouse. However, treated mice do live significantly longer than untreated mice and their hematopoietic system is temporarily restored (Till and McCulloch 1964). This suggested that different grades of

HSCs existed. At present, HSCs are divided into three classes. The true, long-term repopulating HSC (LTR-HSC) is capable of fully restoring the hematopoietic system of a lethally irradiated animal through serial transplantation into secondary, tertiary, and so on, recipients (Broxmeyer and Smith 2009). Intermediate-term repopulating HSCs (ITR-HSCs) are able to fully restore hematopoiesis in the irradiated animal for up to 32 weeks, but not through serial transplantation. Finally, short-term repopulating HSCs (STR-HSCs), are capable of fully restoring hematopoiesis in the recipient animal for up to 16 weeks. Beneath the STR-HSCs exists progenitor cells which can only restore specific hematopoietic populations, amongst them the CFU-S (Broxmeyer and Smith 2009). As ITR- and STR-HSCs exhibit very similar functions, many phenotypic definitions of HSCs do not make a distinction between them and simply have LTR- and STR-HSCs with LTR-HSCs defined by their ability to repopulate a mouse for over 24 weeks and successfully undergo secondary transplantation and STR-HSCs defined in contrast (Broxmeyer and Smith 2009). In a primary transplantation assay, the first three months are thought to define the period when STR-HSCs are active and starting with month six defines the period when LTR-HSCs are thought to be active, with ITR-HSCs thought to be active during the interim period (Chen, Astle et al. 1999; Marshak, Gardner et al. 2001; Benveniste, Frelin et al. 2010).

As the CFU-S assay did not describe the HSC, other methods of assessing the quantity and behavior of HSCs were necessary. One early method was limiting dilutions of transplanted cells (Jacobson, Simmons et al. 1951; Lorenz, Uphoff et al. 1951). As HSCs

are defined by their ability to rescue a lethally irradiated animal, different treatments could be compared by minimum number of cells necessary to rescue a lethally irradiated mouse. This technique is still sometimes employed; however it is ethically complicated and fails to account for many of the nuances involved in hematopoietic reconstitution. These issues were addressed by viewing an old technique in a new light. Heterologous transplants had been required to demonstrate that cells from the healthy donor were indeed responsible for reconstituting the hematopoietic system; however this idea was taken to a new level in the 1980s, with the development of the competitive repopulation assay using congenic mice (Harrison 1980). Congenic mice are inbred populations of mice that are genetically identical save for one easily recognized locus, in hematopoiesis this is normally CD45. In a competitive repopulation assay, the lethally irradiated recipient animal (often a CD45.1 BoyJ mouse, though CD45.1/CD45.2 C57Bl/6xBoyJ F1 mice are becoming more common) receives a mixture of cells from a congenic donor animal (often from CD45.2 C57Bl/6 mice) as well as sufficient competitor cells (often from CD45.1 BoyJ mice) to fully restore the hematopoietic system in the recipient. The competitor cells establish a standard whereby the effect of different treatments of donor cells can be compared.

Phenotypic identification of the murine HSC

Having developed a sensitive assay for HSC function, defining the phenotype of HSCs became a possibility. This is an area of on-going study, frustrated not by the paucity of

options but by the large number of possibilities and the seemingly arbitrary nature of cell surface markers. The field of phenotypic markers began in the early 1980s when mature B cells and their precursors were identified using a single antibody (Coffman and Weissman 1981). Building upon this work, lineage (Lin) markers for a variety of mature hematopoietic cells were identified (Muller-Sieburg, Whitlock et al. 1986). However, the absence of lineage markers enriches HSCs but alone is much too heterogeneous a population to be of any real scientific use. The presence of HSCs were further enriched in the Lin negative fraction by the expression of stem cell antigen-1 (Sca1) and Thy1.1 (Spangrude, Heimfeld et al. 1988). The absence of lineage markers and the presence of Sca1 on cells are unambiguous; however, the expression of Thy1.1 was neither positive nor negative but "low", a value difficult to standardize. This problem was resolved by identifying the expression of stem cell factor (SCF) receptor (c-kit) as a replacement for the low expression of Thy1.1 (Ogawa, Matsuzaki et al. 1991; Okada, Nakauchi et al. 1992). At present, there is broad consensus that Lin^-, $Sca1^+$, c-kit^+ (LSK) population is enriched for HSCs. This heterogeneous population can be further resolved by the presence of cluster domain 34 (CD34), with its absence defining a population further enriched for LTR-HSCs and its presence defining a population enriched for STR-HSCs (Osawa, Hanada et al. 1996). As lymphoid populations within the LSK population heavily express CD34, HSCs can be further defined by the absence of interleukin-receptor 7 alpha (IL-7Rα). This framework has the additional advantage of being able to identify several progenitor populations. CLPs are defined as IL-7Rα^+ LSK cells, and the CMP population is defined as Lin^-, IL-7Rα^-, c-kit^+, $Sca1^-$. The CMP population can be further

divided into MEPs and GMPs with the use of FcγRIII/II and CD34 with MEPs being negative for both and GMPs being positive for both (Katsumoto, Aikawa et al. 2006). More stringent phenotypic definitions for each of the three classes of hematopoietic cells exist, most especially the LTR-HSCs which are defined by members of the signaling lymphocyte activation (SLAM) molecule family (Kiel, Yilmaz et al. 2005; Yilmaz, Kiel et al. 2006). SLAM markers CD150, CD48 and CD244 can be used to identify the HSC population, described as $CD150^{+}$, $CD48^{-}$, and $CD244^{-}$. Similar to the LSK-system described earlier, SLAM markers can also be used to identify other populations as well: MMPs are $CD150^{-}$, $CD48^{-}$, $CD244^{+}$, while lineage-restricted progenitor cells are $CD150^{-}$, $CD48^{+}$, $CD244^{+}$ (Van Zant 2006).

Phenotypic identification of the human HSC

As Jacques Monod said, "what is true for *E. coli* is true for the elephants." (Friedmann 2004) and given the conservative nature of evolution it ought to come as no surprise that the human HSC essentially behaves in the same manner as murine HSCs. The differences between the two systems lie in the functional assay for HSCs and the arbitrary and less definitive set of surface markers which serve to phenotypically define HSCs (Weissman and Shizuru 2008).

Phenotypic markers for HSCs are more precisely defined in the murine model. Analyses of human HSCs begin with the $CD34^{+}$ fraction (Civin, Strauss et al. 1984); though

somewhat controversial models do exist where the HSC can be $CD34^-$ (Zanjani, Almeida-Porada et al. 1998). The primary virtue of the single $CD34^+$ marker is the ease of use in clinical settings, where consistent and quick analysis is paramount (Krause, Fackler et al. 1996). This population can be further sub-divided by the expression of CD38 (Terstappen, Huang et al. 1991). The $CD34^+$ $CD38^+$ population is heavily enriched for HPCs while the $CD34^+$ $CD38^-$ population is enriched for HSCs. Though it has not been demonstrated to be superior to CD34, some analyses use CD133 instead (Lu, Baudouin et al. 2007). Similar to the murine system, other markers such as CD59, Thy1, c-kit, and lineage are available to further resolve these heterogeneous populations. For example, a more stringently but still ill-defined HSC in the human system is $CD34^+$, $CD59^+$, $Thy1^+$, $CD38^{lo/-}$, c-$kit^{-/lo}$, Lin^- (Weissman and Shizuru 2008). In keeping with the arbitrary and unpredictable nature of phenotypic definitions, SLAM markers, which have been so effective at defining the murine HSC are insufficient when it comes to the human system (Larochelle, Savona et al. 2011).

As no equivalent transplant assay for HSCs is available for humans, a surrogate system was developed in immunocompromised mice. Either non-obese diabetic severe combined immunodeficient (NOD/SCID) or NSG (NOD/Shi-*scid*/IL-2Rα^{null}) mice are sublethally irradiated to create empty hematopoietic niches available for human cells to engraft (Lapidot, Pflumio et al. 1992; Ito, Kobayashi et al. 2008). Similar to competitive repopulation in the murine model, the murine cells provide a standard whereby the efficacy of different treatments on human cells can be compared.

Hematopoietic progenitors

The benefit of hindsight makes it clear that all hematopoietic cells are ultimately derived from a single pluripotent HSC. Initially, hematopoietic populations were examined in isolation, first through microscopy (Hajdu 2002) and then through clonogenic colony assays (Pluznik and Sachs 1965; Bradley and Metcalf 1966; Pluznik and Sachs 1966). The colony assay in particular was instrumental in clarifying the hematopoietic hierarchy. Through careful study it was found that progenitors able to give rise to either granulocyte (CFU-G) or monocyte/macrophage (CFU-M) colonies are preceded by progenitors that can give rise to both (CFU-GM also known as granulocyte/macrophage progenitor, GMP). Likewise, progenitors able to give rise to proper small erythroid colonies (CFU-E) are preceded by those that generate erythroid bursts (BFU-E). Megakaryocytes are also generated by their own progenitor cell (CFU-Mk). Both BFU-E and CFU-Mk progenitors are preceded by a megakaryocyte/erythroid progenitor (MEP) cell. Both GMPs and MEPs are preceded by colony forming unit granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM), which is synonymous with the common myeloid progenitor cell (CMP). Similar techniques in the lymphoid lineage identified progenitors which specifically gave rise to B and T cells as well as a common lymphoid progenitor (CLP). There exists an even more primitive multipotent progenitor (MPP) which is able to give rise to both CMPs and CLPs, which in turn is preceded by the HSC itself. This structure gives rise to the hematopoietic

hierarchy, sometimes described as the hematopoietic tree, which is diagrammed in Figure 1.

Hematogenesis, hematopoiesis and umbilical cord blood

Adult HSCs reside primarily within the bone marrow, though extramedullary hematopoiesis is possible in the spleen and liver. Intriguingly, hematopoietic differentiation is observed in murine embryonic development between E7.0 and E7.5 whereas condensation of prechodrogenic mesenchymal cells, the first step in bone development, does not begin until E8.5 (Tuan 1998). This means that the eventual site of adult hematopoiesis is distinct from the site where HSCs are initially developed. Several sites and models of embryonic HSC development have been proposed but all are complicated by one of blood's chief virtues: its dispersed nature. Unlike solid tissues restricted to easily identifiable loci, hematopoietic cells are scattered throughout the organism meaning that sites of hematopoietic activity may be distinct from a site of hematopoietic development.

Figure 1:

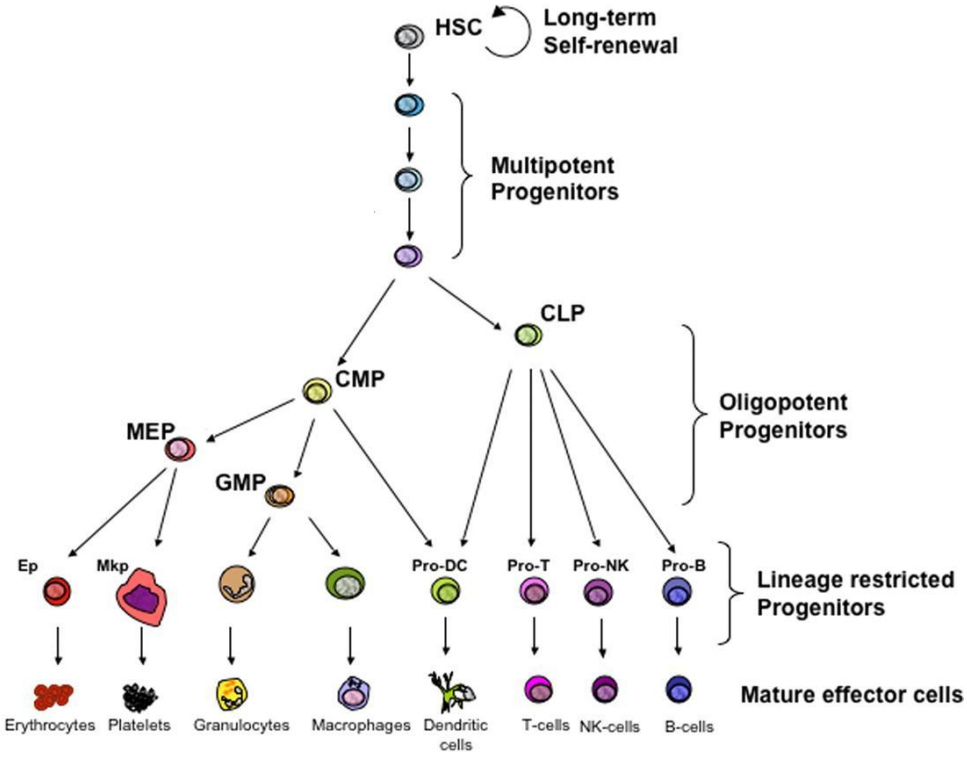

HSC
Long-term
Self-renewal
Multipotent
Progenitors
CLP
CMP
MEP
GMP
Oligopotent
Progenitors
Ep
Mkp
Pro-DC
Pro-T
Pro-NK
Pro-B
Lineage restricted
Progenitors
Mature effector cells
Erythrocytes
Platelets
Granulocytes
Macrophages
Dendritic
cells
T-cells
NK-cells
B-cells

Figure 1: A schematic representation of the hematopoietic hierarchy.

Hematopoietic development is thought to follow a tightly regulated hierarchy beginning with the multipotent hematopoietic stem cell and progressing through a variety of progenitor stages before ultimately developing into a mature hematopoietic cell. While recent advances have suggested the hierarchy may not be as rigid as once thought, the basic model presented here continues to hold. Modified from(Weissman and Shizuru 2008).

The first site of hematopoietic activity to be identified was the yolk sac. In 1907, Wera Dantschakoff was the first to note that endothelial cells in chicken embryos appears to give rise to hematopoietic cells, findings which were quickly repeated and confirmed in a variety of other organisms such as sharks, frogs, turtles, mice, rabbits, pigs, goats, sheep, cows, mongooses and humans (Smith and Glomski 1982). These initial discoveries could not be tested until the development of *in vitro* and *in vivo* assays nearly half a century later. Initial experiments in birds suggested that transplanted yolk sac cells could repopulate the spleen and bone marrow of irradiated avian embryos(Moore and Owen 1965; Moore and Owen 1967). These findings were further refined in mice starting in the 1970s. It was found that the yolk sac contains myeloid progenitors from E7.0 to E9.5, after which CFU-S cells are also present (Moore and Metcalf 1970; Medvinsky, Samoylina et al. 1993). True HSCs are not observed in the yolk sac until E11.5 (Medvinsky, Rybtsov et al. 2011). Despite initially promising data on the yolk sac, true HSCs appear too late for this to be the site of hematogenesis, forcing scientists to look elsewhere.

In 1975 attention turned to a series of experiments performed on birds, where the central area (the future body) of a quail blastoderm was grafted to the peripheral area of a chick yolk embryo (the future yolk sac). In contrast to what others (Moore and Owen 1965; Moore and Owen 1967) had observed, it was found that hematopoietic organs in the chimera where exclusively colonized by cells from the quail (Dieterlen-Lievre 1975). These findings were further clarified in the murine model, where it was

found that the aorta-gonad-mesonephros (AGM) region of the developing embryo body contains HSCs (Godin, Garcia-Porrero et al. 1993). These HSCs are present at E10.5, a full day earlier than in the yolk sac (Gekas, Rhodes et al. 2010). Studies in several species have reaffirmed these studies by demonstrating *de novo* formation of HSCs within the AGM (Gekas, Dieterlen-Lievre et al. 2005; Mikkola, Gekas et al. 2005). However, readily transplantable HSCs in the AGM region are only found in low numbers and during a short developmental time window suggesting that another site is at least partially responsible for HSC development (Kumaravelu, Hook et al. 2002). Intriguingly, blood forming endothelial cells from the yolk sac migrate to the AGM region during development, which may help resolve some of the controversy surrounding both the yolk sac and AGM region and their role in hematogenesis (Oberlin, Tavian et al. 2002). While both the yolk sac and the AGM region contain HSCs and at least the AGM region has been shown to be capable of developing HSCs, the inverted hierarchy of early hematopoietic development in the embryo has led to the hypothesis that there are two waves of hematopoietic development within the embryo (Dzierzak and Speck 2008). The first wave, centered on the yolk sac and AGM region, is capable of giving rise to macrophages, megakaryocytes and primitive erythrocytes (Chasis 2004; Ou, Chae et al. 2011). The second, definitive wave occurs at midgestation and gives rise to the true HSC (Dzierzak and Speck 2008). Having exhausted the yolk sac and the embryo proper, the only remaining site for hematogenesis is the placenta. The placenta is a highly vascularized organ, rich in cytokines and growth factors that promote hematopoietic colony formation (Burgess, Wilson et al. 1977). In 2005, HSCs were identified in the

placenta at E10.5-E11 (Serikov, Hounshell et al. 2009). These HSCs undergo a rapid expansion starting at E12.5-E13.5 and yielding 15-fold more HSCs than found in the AGM region (Serikov, Hounshell et al. 2009), consistent with the two wave hypothesis. Furthermore, the placenta is directly upstream of the liver in fetal circulation, which suggests that the fetal liver may be primarily colonized by placental HSCs. Another intriguing piece of evidence in favor of placental hematogenesis is the unique properties of HSCs derived from umbilical cord blood.

Connecting the placenta to the fetal liver, the umbilical cord was long regarded as medical waste. While HPCs had been identified in umbilical cord blood as early as 1974 (Knudtzon 1974), the unique properties of umbilical cord blood would not be noticed until the late 1980s. At that time, extensive lab work firmly demonstrated that not only are HPCs present in umbilical cord blood, but that umbilical cord blood is heavily enriched in HPCs and that these cells can be sent by overnight mail and cryopreserved (Broxmeyer, Douglas et al. 1989). This led directly to the first cord blood transplantation by Eliane Gluckman at the Hopital St. Louis in Paris, France. In October, 1988, HLA-matched sibling cord blood cells were used to treat a six-year old boy suffering from Fanconi anemia (Gluckman, Broxmeyer et al. 1989). At the time, there were serious concerns that cord blood did not contain sufficient HSCs and HPCs to properly engraft in a patient and that contamination from maternal cells might enhance graft-versus-host disease (Broxmeyer and Smith 2009). Instead, it became clear that umbilical cord blood is a very rich source of clinically relevant HSCs and HPCs and that less, not more, graft-

versus-host disease is observed. The first patient to be treated with umbilical cord blood is still alive today, 23 years after the procedure and has been cured of the hematological manifestations of Fanconi anemia. In addition to a high concentration of HSCs and HPCs and reduced graft-versus-host disease, umbilical cord blood can also be easily stored. The first "proof-of-principle" cord blood bank was established in the Broxmeyer laboratory. Cord blood that has been cryopreserved for as long as 23.5 years has been successfully recovered from this bank with high effectiveness as compared to fresh cord blood (Broxmeyer 2011). Since the first umbilical cord blood transplant, more than 25,000 human cord blood transplants have been performed treating patients with a variety of malignant and non-malignant disorders (Rocha and Broxymeyer 2009). The utility of umbilical cord blood is somewhat limited by the low absolute cell number as well as a long engraftment time for neutrophils and platelets. Because the incidence and severity of graft-versus-host disease is reduced in umbilical cord blood transplantation, there have been attempts to overcome these problems by transplanting cord blood from multiple donors. To date, there has been no rigorous proof that two cord bloods are better than one; indeed, in most cases only one of the two cord blood collections actually engrafts long-term. Additionally, multiple cord blood transplants have been associated with higher levels of graft-versus-host disease, effectively removing one of the advantages this stem cell source presents (Broxmeyer 2011). Instead of increasing the number of cells through multiple donors, another possibility is to increase the activity of stem cells present in the cord blood. Engraftment of HSCs involves at least two separate events: homing of the cells to the

bone marrow and then nurturing them for survival, proliferation, self-renewal and differentiation within the bone marrow microenvironment (Broxmeyer 2011). Increasing the efficacy of stem cell homing could theoretically counteract the small donor size. One promising target to accomplish this feat is stromal cell-derived factor 1 (SDF1/CXCL12) which binds to CXCR4 present on the HSC. Not only does SDF1/CXCL12 act as a chemoattractant, directing the HSC towards the microenvironment, but it also enhances the survival of primitive hematopoietic cells (Shaheen and Broxmeyer 2005). Through chemical inhibition and knock-out mouse studies, it was found that removing CD26/DPPIV activity greatly enhances the effect of both endogenous and exogenous SDF1 on HSC homing (Christopherson, Hangoc et al. 2002; Christopherson, Hangoc et al. 2004). Despite these problems, the field of umbilical cord blood transplantation has already demonstrated great promise for treating disease and holds greater promise still.

The process of hematogenesis remains poorly understood. The early appearance of hematopoietic cells in the yolk sac and AGM region present a case that the initial HSC may be developed there. However, neither site appears to produce enough HSCs to fully populate the developing organism. This has led to a two-wave hypothesis that separates the initial primitive hematopoietic development in the yolk sac and AGM region from the initiation of lifelong hematopoiesis by HSCs created in the placenta (Dzierzak and Speck 2008). Placental HSCs are both numerous and functionally similar to HSCs found in the adult BM. One difference is that placental HSCs found in the umbilical cord appear to be more naïve than adult BM cells, leading to reduced graft-

versus-host disease in recipients of umbilical cord blood transplants. Irrespective of the site and process of hematogenesis, it is known that afterwards HSCs first migrate to the fetal liver and then to the bone marrow where they will remain throughout the life of the organism.

The HSC niche

Efforts to better manipulate cells for enhanced engraftment requires an understanding of HSC/HPC microenvironmental (niche) within the bone marrow. While unable to give rise to HSCs, there exists within the bone marrow a niche which is able to maintain HSC self-renewal. Perhaps because of its supportive and not developmental role, the precise nature of the bone marrow niche was controversial for a very long time. This controversy was no-doubt compounded by the different classes of HSC and the assays used to define them. Early attempts at identifying the niche resulted in two candidate cell types at different locations within the bone marrow. Osteoblasts, located within the endosteum of the bone marrow (Lo Celso, Fleming et al. 2009) and endothelial cells within sinusoidal blood vessels (Kiel, Yilmaz et al. 2005). Both cell types are able to promote hematopoiesis both *in vitro* and *in vivo*, express adhesion molecules consistent with their function as the site of the hematopoietic niche and reside in areas of low oxygen tension (Taichman and Emerson 1994; Li, Johnson et al. 2004). While these sites do play an important role in hematopoiesis, neither alone was able to satisfactorily meet all the criteria of the true hematopoietic niche (Kiel and Morrison 2008). Recent

evidence suggests that the true hematopoietic niche lies in the bone marrow endosteum but rather than interacting with an osteoblast, the HSC interacts with mesenchymal stem/stromal cells (MSC), beta-adrenergic neurons, macrophages, and osteoblasts. Like the niche candidates, the MSC is able to promote hematopoiesis *in vivo* and *in vitro* and furthermore it possesses qualities of both osteoblastic and endothelial cells, resolving the conflict of which cell type better described the niche. Furthermore, the presence of the beta-adrenergic neuron provides a point of contact between the neuronal and hematopoietic systems, a relationship which will be described in greater detail later (Mendez-Ferrer, Michurina et al. 2010; Chow, Lucas et al. 2011). As the discovery of the MSC-niche is a somewhat recent development, information gathered from research pertaining to the older niche-sites will also be examined in this thesis.

Relationship between hematopoietic and neuronal systems

There is an increased understanding of hematopoiesis and its regulation by the neuronal system, information that may be relevant to enhancing engraftment of HSC/HPCs. In addition to the beta-adrenergic neuron at the hematopoietic niche, the hematopoietic and neuronal systems have numerous means of communication with each other. Amongst the best studied of these are adrenergic and γ-aminobutyric acid (GABA)ergic signaling; however cholinergic and glutamatergic signaling have been investigated. The role of adrenergic signaling in hematopoiesis is fairly straightforward. Endogenous

adrenergic signaling molecules, such as epinephrine, norepinephrine as well as synthetic agonists such as methamphetamine stimulate cycling in HS/PCs (Maestroni 1995) and, when injected *in vivo*, stimulate egress from the bone marrow niche (Giudice, Caraglia et al. 2010). While well studied, the role of GABAergic signaling in hematopoiesis remains less clear. Similar to its antagonistic role to adrenergic signaling in the neuronal system, exposure to GABA decreases the cycling status of HPCs (Andang, Hjerling-Leffler et al. 2008) and the presence of GABA inhibits chemotaxis towards SDF1α (Seidel, Niggemann et al. 2007). However, GABA alone is also a chemattractant for HPCs (Zangiacomi, Balon et al. 2009). Intriguingly, the MSC found at the hematopoietic niche have been implicated in GABAergic signaling and can partially compliment the function of GABAergic neurons in brain damaged mice (Bae, Han et al. 2007). The relationship between these elements warrants further study.

Cholinergic signaling has also been shown to play an important role in early hematopoietic development: exposure of embryonic stem cells to nicotine increases differentiation towards the hemagioblast (Serobyan, Jagannathan et al. 2007). In the adult animal, both nicotine and acetylcholine appear to play a proliferative role (Gallicchio, Chen et al. 1983; Koval, Zverkova et al. 2008). Injection of either compound *in vivo* leads to increased leukocyte counts in peripheral blood, bone marrow, and spleen. Furthermore, chronic nicotine exposure results in extramedullary hematopoiesis in the spleen (Chang, Forsberg et al. 2010). In contrast, nicotine decreases the production of cytokines within the niche which support HSC proliferation

such as IL-3, IL-6, G-CSF, GM-CSF, and IGFBP-3 (Koval, Zverkova et al. 2008). Nicotine exposure also decreases CXCR4 expression on hematopoietic cells, which partially explains the decreased engraftment of HSCs in animals exposed to nicotine (Khaldoyanidi, Sikora et al. 2001). Data from knock-out mouse for components of the cholinergic system refines these observations. The absence of β2-containing nicotinic receptors favors early myeloid precursor generation whereas absence of α7-containing nicotinic receptors favors development of mature myeloid and erythroid cells. These findings mean that under physiological conditions, β2-containing nicotinic receptors serve to limit early progenitor generation whereas α7 favors the expansion of immature forms. Interestingly, α4β2 nicotinic receptors are inherent to HPCs and more primitive cells whereas α7-containing receptors are expressed in both immature and mature hematopoietic cells (Koval, Zverkova et al. 2008).

The role of glutamatergic signaling in hematopoiesis is less well understood. At present, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors do not appear to play a role in hematopoiesis based on a search of the NCBI database. However, there are aspects of N-methly-D-aspartic acid (NMDA) receptor function which are related to issues of hematopoietic importance. NMDA receptors have been shown to play a role in megakaryocytopoiesis (Genever, Wilkinson et al. 1999; Hitchcock, Skerry et al. 2003) thought to be independent of thrombopoietin-mediated effects (Zheng, Yang et al. 2008). The NMDA receptor is expressed by osteoclast precursors and activation of the receptor is required for osteoclastogenesis (Merle, Itzstein et al. 2003) – a finding which

may have implications for the osteoblastic niche. Furthermore, NMDA receptors are particularly susceptible to damage from reactive oxygen species (Bracci, Perrone et al. 2001), which given the hypoxic nature of the niche may suggest a role for them. Lastly, there is a positive correlation between NMDA receptor-excitation and CXCR4 expression (Riviere, Subra et al. 1999). Much remains to be discovered in this area, and the early groundwork justifies further study.

In addition to cross-talk between the hematopoietic and neuronal systems, developmental similarities also exist. Of particular interest are the similarities between HSC and neuronal stem cells. The two stem cell populations have a high degree of overlap between both factor and genes involved in their development (Scheffler, Horn et al. 1999). A representative list is provided in Table 1. Because differences in both laboratory techniques available and organ function have led to different emphases in research, knowledge gained in one system can be used as a vehicle for discovery in the other (Quesenberry, Hulspas et al. 1999; Zhang, Rohlmann et al. 2005). One such discovery is the subependymal zone, the proliferative central core of the central nervous system, which was discovered due to its similarity to the bone marrow (Scheffler, Horn et al. 1999). This thesis will employ a similar theoretical approach and examine the role of Neurexin I alpha (NRXN1α), a well-studied neuronal protein, in hematopoiesis.

The Neurexins

The Neurexins are a conserved family of three proteins, each with two isoforms and numerous splice variants for each isoform. Because of this, they are sometimes described as "three genes, 1001 products" (Missler and Sudhof 1998). Neurexin I alpha (NRXN1α), the primary focus of this thesis, was the first member of the family to be discovered (Ushkaryov, Rohou et al. 2008) as the receptor for alpha-latrotoxin (LT), the component of *Latrodectus spp*. venom of mammalian relevance and other members were identified based on sequence homology (Lise and El-Husseini 2006). Neurexins are single transmembrane proteins with a small internal PDZ domain and a large extracellular portion whose structure is determined by the isoform. The larger alpha-isoform ranges in size from 160-220kDa and features six Laminin, Neurexin, Sex-hormone-binding protein domain; also known as a Laminin G domain (LNS) repeats as well as an epidermal growth factor (EGF)-like sequence (Lise and El-Husseini 2006). The shorter beta-isoform ranges from 55-95kDa and lacks the EGF-like sequence and several, though not all, LNS repeats (Sugita, Saito et al. 2001). In both isoforms, splice variants affect the extracellular domain and tend to alter the LNS domains (Ushkaryov, Hata et al. 1994). The receptor is involved in calcium signaling, synaptogenesis, and heterogeneous cell-to-cell adhesion, all mediated by a variety of ligands. Neuroligins generally bind the beta-isoform, while DAG1, the Neurexophilins (NXPHs), and alpha-latrotoxin each bind the second LNS domain, a unique feature of the alpha-isoform (Lise and El-Husseini 2006). After Neuroligin, an intracellular reaction involving

Lin2, a ubiquitously expressed scaffolding protein of the MAGUK family, leads to opening calcium channels on the cell membrane as well as to additional downstream effects mediated by proteins such as Lin7 and Lin10 (Hata, Butz et al. 1996; Craig and Kang 2007). The role of the third family of ligands, the NXPHs, is less well defined; however, data suggests that they inhibit Neurexin-mediated signaling within the neuronal system (Beglopoulos, Montag-Sallaz et al. 2005; Zhang, Rohlmann et al. 2005)

Data from knock-out animals suggests that Neurexins are able to functionally compliment each other (Missler, Zhang et al. 2003), though only NRXN1α appears to be expressed in the hematopoietic system (Ivanova, Dimos et al. 2002). In the neuronal system, the primary role of NRXNs appears to be in directing synapse development (Lise and El-Husseini 2006). The beta-NRXNs and Neuroligands play a mutually supportive, and necessary, instructive role in generating the synapse. After beta-NRXN-Neuroligand binding, the cell expressing the beta-NRXNs will develop into the pre-synaptic cell whereas the cell expressing Neuroligand will develop into the post-synaptic cell. Activation of either beta-NRXN or Neuroligand on neuronal cells is sufficient to cause them to develop into pre- or post-synaptic cells, respectively and absence of NRXN-Neuroligand signaling results in aberrant synapse development (Dean, Scholl et al. 2003).

Table 1:

Growth factors, steroids, cytokines, and their ligands	Epidermal growth factor, fibroblast growth factors, NGF, brain-derived neurotrophic factor, transforming growth factors, cilliary neurotrophic factor, bone-morphogenetic proteins, leukemia inhibitory factor, glial growth factor, platelet-derived growth factor, tumor necrosis factors, insulin-like growth factors, interleukins, colony stimulating factors, KIT, interferon, retinoic acid, T3
Transcription factors, developmental genes, apoptotic and anti-apoptotic genes	bHLH (XATH1), Id2, homeodomain, RU49, PBX1, POU, PAX, nuclear-receptor-type zinc finger, leucine zipper, ETS, rel-NFkB, Ikaros, Notch, Delta, OCT, neurogenin, neuregulin, GLP1, LAG2, Rb, BCL2, BAX, caspases, Fas, Fasl, Musashi, Hu
Extracellular matrix and cell-surface proteins	Tenascin and other glycoprotiens, CD34, proteoglycans, adhesion molecules, polysialylated neural cell-adhesion molecule, N-cadherin, Dystroglycan, Neurexin I alpha, L1 and Thy1
Other	Erythropoietin, thrombopoietin, silencers (glial-cell missing, neuron-restrictive silencer factor), cell-cycle-associated molecules, SHC (SRC-homology-2-domain-containing transforming protein C), neuropoietins, Neurexophilin, and other cell-signaling molecules

Table 1: Representative elements common to both hematopoietic and neuronal development.

A partial list of representative factors and genes common to both the hematopoietic and neuronal systems. Not included are cell cycle-related genes, cyclin-dependent-kinase inhibitors, and many other elements common to progenitors and stem cells of all lineages. Modified from (Scheffler, Horn et al. 1999).

In addition to the importance of NRXN in synapse development, it also appears to play a role in what kind of synapse is developed: glutamatergic and GABAergic synapses are favored by NRXNs (Craig and Kang 2007). Specifically, beta-NRXNs induce clustering of GABAergic post-synaptic scaffolding proteins Gephyrin and Neuroligand 2, whereas Neuroligands 1, 3, and 4 serve as scaffolding proteins for proteins associated with glutamatergic synapses (Kang, Zhang et al. 2008). NRXN1α is also involved in the localization of $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR), which modulates the efficacy of glutamatergic and GABAergic synapses, to the pre-synaptic cleft (Cheng, Amici et al. 2009). Likewise, alpha-NRXNs are able to directly bind the $GABA_A$ receptor, increasing the concentration of GABAergic proteins at the post-synaptic cleft (Zhang, Atasoy et al. 2010).

In addition to their instructive roles in synapse development, alpha-NRXNs play a critical role in modulating the activity of glutamatergic and GABAergic synapses. The post-synaptic N-methyl-D-aspartate (NMDA) receptor, which plays an important role in glutamatergic signaling, requires alpha-NRXNs to function (Kattenstroth, Tantalaki et al. 2004). Similar in function, though opposite in mechanism, alpha-NRXNs are also able to bind $GABA_A$ receptors and silence them (Zhang, Atasoy et al. 2010). Intriguingly, this means that alpha-NRXN causes synapses to differentiate along an inhibitory line but once the synapse is formed alpha-NRXNs serve to support excitatory signaling and muffle inhibitory signaling. Because of this, it is thought that alpha-NRXN serves as a mediator between excitatory and inhibitory neuronal signaling (Graf, Zhang et al. 2004).

In keeping with its important role in synapse development, NRXN has been associated with neurological phenotypes. Schizophrenia has been associated with disruptions of NRXN1 (Kirov, Gumus et al. 2008) as well as Lin2 (Kristiansen, Beneyto et al. 2006), the protein directly downstream of NRXN. NRXN1α also appears to play an important role in autism spectrum disorder, as ultra-rare cohorts of particular missense mutations and splice variants have been associated with the disorder (Kim, Kishikawa et al. 2008; Glessner, Wang et al. 2009); disruption of NRXN1α due to chromosomal breakage has also been linked to autism (Kim, Kishikawa et al. 2008). Interestingly, the chromosomal breakage that disrupted NRXN1α appeared to leave NRXN1β unaffected and while some correlation between specific missense mutations in NRXN1β and autism have been suggested (Feng, Schroer et al. 2006; Kim, Kishikawa et al. 2008); family data suggests that incomplete penetrance occurs (Yan, Noltner et al. 2008). These associations may prove of interest to the investigation of the hematopoietic role of this signaling axis. While the neuronal aspects of these diseases are well documented and involved in diagnosis (Broyd, Demanuele et al. 2009), both diseases also manifest a distinct hematopoietic phenotype. Schizophrenics and autistics posse a pro-inflammatory phenotype coupled with an increase in the absolute number and cycling status of many hematopoietic populations including HPCs (Malacarne and Dallapiccola 1969; Wilke, Arolt et al. 1996; Muller, Riedel et al. 2000; Cohly and Panja 2005).

Because of its many binding partners, a diagram of NRXN1α and its binding partners is provided in Figure 2.

Figure 2:

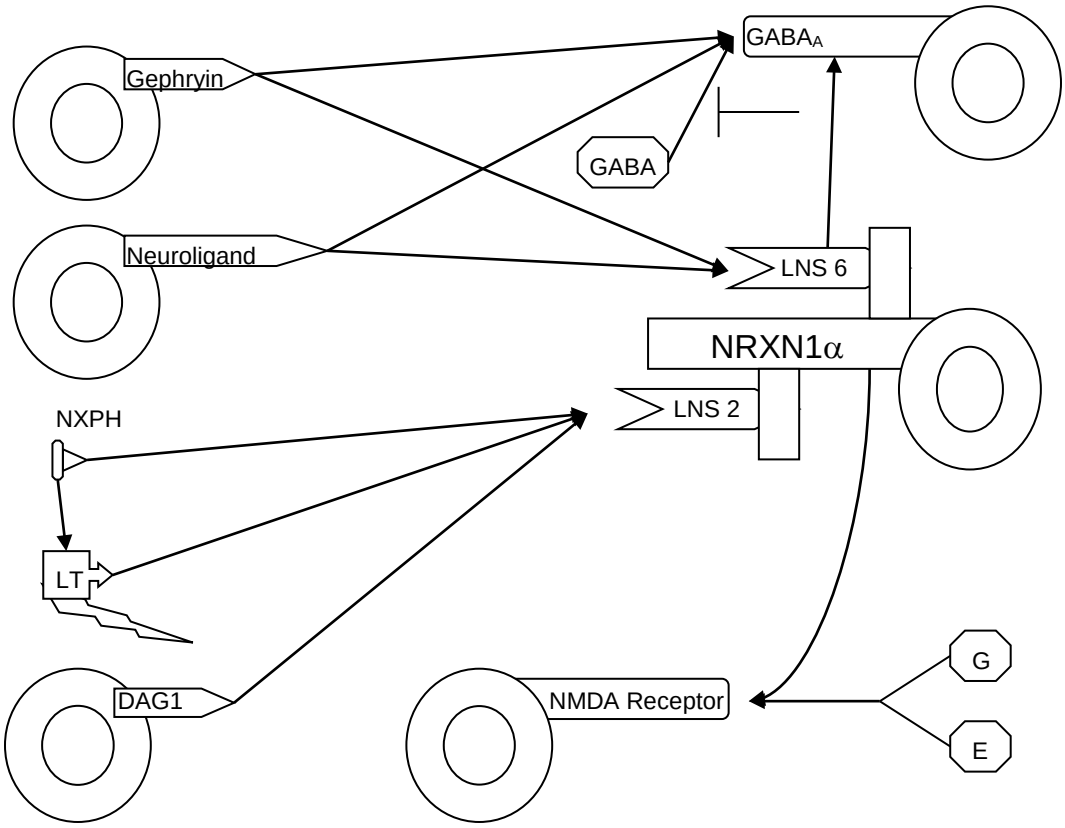

GABA$_A$
Gephryin
GABA
Neuroligand
LNS 6
NRXN1α
LNS 2
NXPH
LT
DAG1
NMDA Receptor
G
E

Figure 2: A schematic representation of the relationship between NRXN1α and its binding partners.

NRXN1α sits at the center of a complex and tightly regulated system. Though it possesses six LNS domains, most ligands bind either the second or the sixth. The sixth LNS domain is primarily involved in GABAergic signaling and binds Gephyrin, Neuroligand 2, and $GABA_A$ receptor. When NRXN1α is bound to $GABA_A$ receptor it is unable to respond to GABA. In addition to binding NRXN1α, Gephyrin and Neuroligand 2 also bind $GABA_A$ receptor and help recruit it to the synapse. The endogenous activity of the second LNS domain is less well understood but it is the principle binding site for LT. In addition to LT, there are two endogenous ligands for the second LNS domain: NXPH and DAG1. NXPH also binds to LT. Lastly, NRXN1α is required for NMDA function though the binding site has not been defined. Drawing not to scale, NRXN1α has been presented as a branching protein for ease of viewing and does not reflect the actual structure.

Dystroglycan

Dystroglycan (DAG1) is a ubiquitously expressed transmembrane glycoprotein encoded by a single gene, *dag1*, whose product is cleaved into a larger 120-190kDa alpha-subunit non-covalently bound to a smaller 43kDa transmembrane beta-subunit. DAG1 is the central component of the dystrophin-associated glycoprotein complex and serves as an interface between the cytoskeleton and extracellular matrix. As such, DAG1 is a receptor for multiple extracellular matrix proteins such as Agrin, Laminin, Perlecan, etc (Williamson, Henry et al. 1997). DAG1 can also bind NRXN1α, which is most commonly observed in the brain where normal components of the extracellular matrix are absent (Sugita, Saito et al. 2001). Within the cell, beta-Dystroglycan function is normally associated with dystrophins and syntrophins (Drysdale and Crosby 2005), though it has also been associated with FYN, C-src tyrosine kinase, Src, NCK1, SHC1, Caveolin-3 (Sotgia, Lee et al. 2000), and Grb2 (Yang, Jung et al. 1995).

In addition to its role in muscle and brain tissue, DAG1 also plays a role in the hematopoietic system. Blocking DAG1 in the thymus prevents T-cells from undergoing positive selection (Zhang, Wang et al. 2006). DAG1 is also the second most highly expressed protein on the surface of cells thought to form the osteoblastic niche for hematopoietic stem cells (Hines, Nielsen et al. 2008). Additionally, DAG1 knock-out mice fail to develop a mesoderm, the embryonic tissue from which hematopoietic cells arise (Williamson, Henry et al. 1997). Lastly, beta-Dystroglycan is a target for cleavage

by MMP-9 (Michaluk, Kolodziej et al. 2007) a protease involved in HSC homing during transplant (Lapidot, Dar et al. 2005).

The Neurexophilins

Neurexophilins were first discovered in the hunt for the receptor for LT (Davletov, Krasnoperov et al. 1995; Geppert, Khvotchev et al. 1998): in addition to NRXN1α, a small 29kDa protein bound LT on the column. Further study revealed that this protein, NXPH1, also binds NRXN1α (Missler, Hammer et al. 1998). Through comparison of genomic DNA sequences, four homologous proteins were found within mammals, though members of the family exist throughout the animal kingdom. Of these, NXPH1, 2, and 3 were found to found to bind NRXN1α and can compliment each other in knock-out animals (Missler and Sudhof 1998). NXPH4, on the other hand, is not normally included within the family as it has different binding partners and function: the name remains as a historical artifact (Beglopoulos, Montag-Sallaz et al. 2005; Lise and El-Husseini 2006).

Aside from binding NRXN1α, the function of NXPH family members remains unclear. Double knock-out mice are viable and do not appear to have any clear abnormalities aside from an increased startle response (Beglopoulos, Montag-Sallaz et al. 2005). In mice, NXPH1 is located primarily in the spleen, NXPH2 is found in the kidney and NXPH2 and NXPH3 are secreted by a small subset of inhibitory neurons in the brain (Petrenko,

Ullrich et al. 1996; Clarris, McKeown et al. 2002; Craig and Kang 2007). Furthermore, microarray data has implicated high NXPH expression in macrophages during immune system exhaustion (Rodriguez, Chang et al. 2004) and NXPH expression in many cancers correlates with a negative prognosis (Warnat, Oberthuer et al. 2007; Faria, Miguens et al. 2008; Knight, Skol et al. 2009; Song, Ramus et al. 2009). Both NXPH and DAG1 bind to the second LNS domain on the alpha-NRXNs, suggesting a possible antagonism between the two ligands (Beglopoulos, Montag-Sallaz et al. 2005).

Hypothesis

Given its inhibitory role in a neuronal context as well as its presence in immunosuppressive conditions, we hypothesized that NXPH plays an inhibitory role in hematopoiesis. This inhibition could be either a direct function of NXPH-NRXN1α binding, or by indirectly antagonizing DAG1-NRXN1α interaction. Using recombinant rat NXPH1 and a well-established DAG1 blocking antibody, we investigated a role for the DAG1-NRXN1α axis in human and murine hematopoiesis. In mice *in vivo*, our results suggest that NXPH1 plays a myelo- and lymphosuppressive role. *In vitro*, NXPH1-mediated inhibition of HPC proliferation is antagonized by endogenous DAG1.

Materials and methods

Antibodies and reagents

For flow cytometry, anti-mouse (mu) antibodies against c-Kit, Sca-1, FcγR III/II, IL-7Rα, lineage cocktail (CD3, CD14, CD16, CD19, CD20, and CD56), and isotype control antibodies were from BD Biosciences (San Diego, CA). Anti-mu CD34 antibody was from eBioscience (San Diego, CA) and anti-mu sca-1 and lineage cocktail were purchased from BioLegend (San Diego, CA). Anti-human (hu) CD34, and CD38 were from Biolegend and Miltenyi Biotec (Auburn, CA) and an anti-hu lineage cocktail was from BD Biosciences. In human tissues, DAG1 was analyzed using IIH6 antibody (Santa Cruz; Santa Cruz, CA) and a PE-conjugated anti-mouse IgM (Santa Cruz); NRXN1α was analyzed using N-16 antibody (Santa Cruz) and APC-conjugated anti-goat IgG antibodies (R&D Biosciences; Minneapolis, MN). In murine tissue, DAG1 was analyzed using abcam ab43120 (San Francisco, CA) and an APC-conjugated anti-rabbit IgG (Thermo Scientific, Barrington, IL) for flow cytometry and an HRP-conjugated anti-rabbit IgG antibody (Thermo scientific) for immunohistochemistry; NRXN1α was analyzed using N-16 antibody (Santa Cruz) and APC-conjugatred anti-goat IgG antibodies (R&D Biosciences) for flow cytometry and Synaptic Systems (Goettingen) antibody 175 002 and Thermo Scientific HRP-conjugated anti-rabbit antibody for immunohistochemistry. Prior to sorting, the $CD34^+$ population of huCB and the lineage negative population of mu bone marrow (BM) were enriched via MACS (Miltenyi Biotec, Auburn, CA). Antibodies for Western blots were: anti-

NRXN1α mouse monoclonal antibody (BD Pharmigen; San Diego, CA), anti-NRXN1α goat polyclonal antibody, anti-NXPH1 goat polyclonal antibody (Santa Cruz Biosystems, Santa Cruz, CA), and anti-beta-actin mu monoclonal antibody (Sigma, St. Louis, MO). Recombinant rat NXPH1 and NRXNα were from R&D Systems (Minneapolis, MN). All murine recombinant cytokines including stem cell factor (SCF), granulocyte macrophage-colony stimulating factor (GM-CSF), and interleukin-3 (IL-3), as well as human recombinant interleukin-8 (IL-8) were from R&D systems. Human recombinant cytokines including SCF, GM-CSF, IL-3, thrombopoietin (TPO), and fms-like tyrosine kinase receptor-3 ligand (Flt3L) were from either BioVision (Mountain View, CA) or R&D Systems. Recombinant human erythropoietin (EPO) was from Amgen (Thousand Oaks, CA). Fetal Bovine Serum (FBS) was from Hyclone (Waltham, MA). Growth media including IMDM and RPMI-1640 were from Gibco (Invitrogen, Carlsbad, CA) and McCoy's 5A was from Sigma. Dulbecco's Phosphate Buffered Saline (DPBS) was from Gibco, and only used during *in vivo* injections; otherwise Phosphate Buffered Saline (PBS) from Lonza (Walkersville, MD) was used. Pokeweed mitogen mouse spleen cell conditioned medium (PWMSCM) was produced (Cooper and Broxmeyer 1991). Propidium iodide and bromo-deoxyuridine (BrdU) were purchased from Sigma.

Mice

C57Bl/6 mice were purchased from Jackson laboratories (Bar Harbor, ME), and housed in a conventional animal facility. BoyJ mice and C57/BoyJ F1 mice were bred at Indiana

University Laboratory Animal Resource Center. Studies were approved by the Indiana University Animal Care and Use Committee. In some cases, mice were intravenously injected with either sterile pyrogen-free DPBS or NXPH1, in the presence or absence of BrdU. Some mice were intraperitonously injected with rapamycin (Sigma) in addition to the other compounds listed here. All materials were purchased certified endotoxin free.

Human CB and peripheral blood (PB)

Cord blood and PB were obtained with institutional review board approval. Low-density (LD) mononuclear CB cells and plasma were isolated by density gradient centrifugation over Ficoll-Plaque Plus (Amersham Pharmacia Biotech). $CD34^+$ cells were enriched via MACs. After enrichment, cells were sorted by FACS to a final purity of >98% $CD34^+$. Red Blood Cell (RBC) lysis buffer was used in all cases to remove erythroid cells.

Expansion of $CD34^+$ cord blood cells

Enriched $CD34^+$ cells were expanded as previously described (Broxmeyer, Hangoc et al. 1992) with some minor modifications. Cells were cultured in RPMI-1640 in 1% FBS in the presence of 50ng/ml rhu SCF, 100ng/ml rhu TPO, and 100ng/ml rhu Flt3L. Cells were then washed and used directly in colony assays or labeled with anti-human antibodies and examined via FACS.

Flow cytometry analysis of surface markers, sorting, and plating

Freshly harvested murine bone marrow cells were washed and resuspended in PBS + 2mM EDTA (ethylenediaminetetraacetic acid) + 0.5% bovine serum albumin (BSA), incubated for 10 minutes at room temperature with Fc-block (Miltenyi Biotech) and then incubated for one hour at 4^{o}C with the recommended volume of anti-murine antibodies: lineage cocktail conjugated to APC, c-kit conjugated to PE/Cy5, sca1 conjugated to PE, and CD34 conjugated to FITC. After incubation, cells were washed and resuspended in PBS and kept at 4^{o}C until sorting by FACSAria (BD Biosciences Immunocytometry Systems, San Jose, CA). Both freshly harvested and cytokine expanded human $CD34^{+}$ cells were stained with anti-human antibodies: CD34 conjugated to either FITC or PE. Sorted cells were normally collected into a solution of PBS + 2mM EDTA + 0.5% BSA, washed, and then resuspended at an appropriate working concentration in IMDM. In the case of single cell plating, single $CD34^{+}$ cells were directly sorted into one well of a 96-well microtiter plate containing 100µl methylcellulose culture medium. Cultures contained IMDM, 1% methylcellulose, 30% FBS, as well as several different cytokines, each alone or in combination. At least 216 wells were used per experimental point per experiment.

Phenotypic Analysis

The following stem and progenitor populations were assessed: long-term repopulating HSCs (LTR-HSC; lineage$^-$,sca-1$^+$,c-kit$^+$, IL-7Rα^-, CD34$^-$), short-term repopulating HSCs (STR-HSC; lineage$^-$, sca-1$^+$, c-kit$^+$, IL-7Rα^-, CD34$^+$) common lymphoid progenitor (CLP; lineage$^-$, sca1$^+$, c-kit$^+$, IL-7Rα^+), megakaryocyte/erythroid progenitor (MEP; lineage$^-$, sca1$^-$, c-kit$^+$, CD34$^-$, FcγR$^{-/lo}$), common myeloid progenitor (CMP; lineage$^-$, sca1$^-$, c-kit$^+$, CD34$^+$, FcγR$^{-/lo}$), and granulocyte/macrophage progenitor (GMP; lineage$^-$, sca1$^-$, c-kit$^+$, CD34$^+$, FcγR$^{+/hi}$). Bone marrow from mice was collected and stained with antibodies to surface markers as previously described (Katsumoto, Aikawa et al. 2006). Data was collected from the samples using a LSR II (BD) instrument and BD FACSDiva software (BD,San Diego, CA). Data was analyzed using WinList software (Verity Software House, Topsham, ME).

HPC colony assay

Methylcellulose and agar HPC assays were performed as described previously (Broxmeyer, Orschell et al. 2005). Mouse BM cells were plated at 5x10^4 cells/ml, while murine spleen cells were plated at 5x10^5 cells/ml. Cultured in triplicate, the murine cells were incubated in a humidified atmosphere, 5% CO_2 and lowered (5%) O_2 at 37^{o}C for 7 days. Low-density huCB cells were plated between 1-5x10^4 cells/ml while CD34$^+$ enriched cells were plated between 1-5x10^2 cells/ml. Human cells were cultured under the same conditions as the murine cells, but with human cytokines, and colonies

counted after 14 days incubation. After specified time in culture, cells were scored for colonies formed from colony forming unit-granulocyte-macrophage (CFU-GM). In the presence of Epo, burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte, erythroid, macrophage, megakaryocte (CFU-GEMM), and total colony forming units (CFU-Total) were counted in addition to CFU-GM. Absolute numbers of murine HPCs per femur were calculated based on the total nucleated cellularity per femur and the number of HPC colonies in that number of nucleated cells. High specific activity tritiated thymidine kill assay was used to estimate the cycling status of HPC (= percent of HPC in S-phase) (Broxmeyer, Orschell et al. 2005). To address the issue of DAG1 presence on hu and mu hematopoietic cells, cells were treated for one hour using the well described (Zhang, Wang et al. 2006) IIH6 blocking antibody and then plated. Survival during delayed growth factor addition was assayed by plating hu and mu cells as normal in IMDM, methylcellulose, glutamine, and FBS absent any growth factors. After 0 (control), 24, or 48 hours a standard concentration of growth factors and growth factor combinations was added. Colonies were scored as normal.

Migration assay

Chemotaxis assays were performed as previously described (Basu, Ray et al. 2007) with both mu total BM (final concentration: $5x10^4$ cells/ml) and hu $CD34^+$ cells (final concentration: $5x10^4$). Mu total BM cells were plated in semisolid culture, whereas human $CD34^+$ cells were first counted via flow cytometry.

Western blotting and ELISA

For protein expression, both LD muBM and huCB cells were cultured in RPMI + 10% FBS and incubated overnight in a humidified atmosphere, 5% CO_2 and 5% O_2 at 37^{o}C with growth factors shown. Cells were washed with ice-cold PBS and lysed. After lysis, the solution was exposed to centrifugation at 14000g for 30 minutes at 4^{o}C. Protein content of lysates was quantified using bicinchonic acid protein assay reagent (Pierce, Rockford, IL) and samples were adjusted to equal protein concentration. Total cell lysates were resolved in 4-20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels (Invitrogen) and transferred to Hybond membrane (Millipore, Bedford, MA). Filters were blocked using 5% BSA in Tris (tris(hydroxymethyl)aminomethane)-buffered saline/Tween-20 (TBST) for 1 hour and incubated overnight with antibodies. Membranes were washed with TBST and incubated with secondary antibodies conjugated to horseradish peroxidase, and antibody binding was detected by enhanced chemiluminescence (ECL) reaction (GE). Enzyme-linked immunosorbent assay (ELISA; reagents from BD Pharmingen or R&D Systems) was performed as previously described (Mathur, Chang et al. 2006) to measure concentrations of NXPH in muBM, both hu and mu PB plasma, as well as huCB plasma. Polyclonal antibody raised against full-length recombinant rat NXPH1 (R&D Systems) was used to ensure a high degree of cross-species reactivity, likely with little resolution between NXPH1 and other NXPH-family members.

Cell cycle analysis

In addition to the high specific activity tritiated thymidine kill assay, cycling status of phenotyped HSCs and HPCs was assayed via PI and BrdU uptake (Gratzner, Leif et al. 1975). C57Bl/6 mice were injected *i.v.* with either carrier (DPBS) plus 1mg BrdU or 5μg NXPH1 plus 1mg BrdU. BrdU (0.8mg/ml) was also added to the drinking water at the time of injection. Twenty-four hours post-injection, BM cells were harvested, washed, and stained for sorting via FACS. After sorting, cells were washed and resuspended in a small volume of ice-cold FBS. Cells were then gently vortexed while ice-cold fixative (95% EtOH and 5% acetic acid) was added to the solution. Cells were allowed to fix for two hours at 4^{o}C under fluorescent lights. Fixed cells were washed and resuspended in PBS and RNase. After RNase digest, DNA was denatured by resuspension in 2N HCL plus Triton X-100. After 30 minutes, cells were pelleted and washed with 0.1M $Na_2B_4O_7$ to neutralize any remaining acid. Fixed cells were washed three times and resuspended in PBS + 1% tween + 2% BSA and probed with FITC conjugated anti-BrdU antibodies and stained with PI. Cells were then analyzed via flow cytometry.

PB analysis

At either twenty-four or forty-eight hours after *i.v.* injection of NXPH1, PB was collected by cardiac puncture. Blood was analyzed using a Hemavet (Drew Scientific Inc, Oxford, CT).

Competitive repopulation assay

This was performed as described (Campbell, Basu et al. 2009). Briefly, $5x10^5$ unsorted BM cells from C57/Bl6 mice (CD45.2) treated twenty-four hours earlier with either 5μg NXPH1 or carrier (DPBS) were mixed with $5x10^5$ BoyJ (CD45.1) BM cells and injected by tail vein into C56/Bl6:BoyJ F1 double-positive recipients (CD45.1:CD45.2) which had been total body irradiated with 9.5 Gy. After seven months, BM was analyzed by flow cytometry using fluorescent conjugated anti-CD45.1 and CD45.2 antibodies and lineage specific markers (BD Biosciences). After seven months, 5 secondary recipients per treatment group were transfused with pooled BM from at least 4 members of the corresponding primary transplant group. Four months after the secondary transplant, BM was harvested and analyzed for percent chimerism was analyzed

Immunohistochemistry

Mouse femurs were dissected and demineralized in a solution of 10% EDTA and 4% phosphate-buffered formalin and then infiltrated with paraffin and sectioned. Detection of DAG1 and NRXN1α expression on paraffin-embedded tibiae from 8-16-week-old mice was performed as previously described (Rhee, Allen et al. 2011). Briefly, sections were deparaffinized, treated with 3% H_2O_2 to inhibit endogenous peroxidase

activity, blocked with goat serum, and then incubated with either a 1:500 dilution of rabbit polyclonal anti-mouse α-DAG1 antibody or rabbit polyclonal anti-mouse NRXN1α antibody. Sections were then incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Color was developed with a diaminobenzidine substrate chromogen system and counterstained with methyl green dye. Non-immune IgGs were used as negative controls.

Statistical analysis

Student unpaired 2-tailed *t* test was used for statistical analysis, except for the HPC colony assay where single $CD34^+$ cells were plated per well, in which case Fischer's exact test was employed. In experiments involving multiple variables, analysis of variance (ANOVA) was employed between the groups to control for false positives. The level of significance is indicated by *P* value. Non-linear regression analysis was used to generate trend lines. The goodness of fit is indicated by R^2 value.

Results

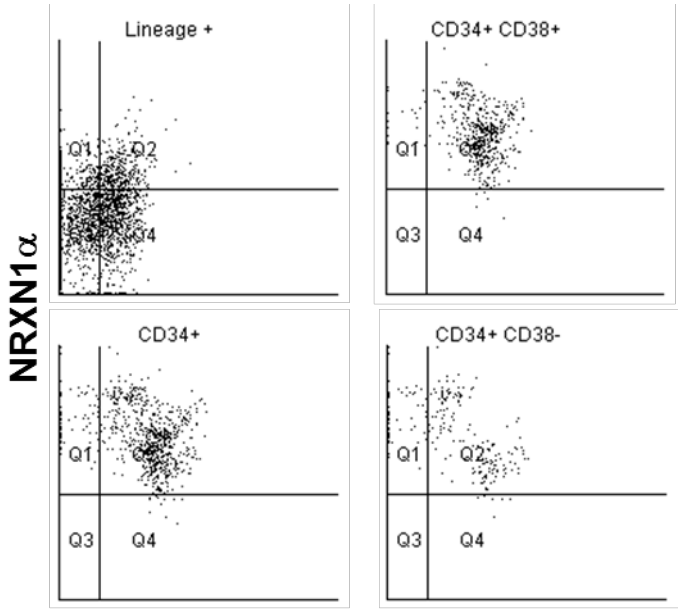
Expression of DAG1, NRXN1α and NXPH in huCB

Previous studies in both the human (hu) and murine (mu) systems suggest that DAG1 (Williamson, Henry et al. 1997; Hines, Nielsen et al. 2008) and NRXN1α (Ivanova, Dimos et al. 2002) may play a role in hematopoietic development. Furthermore, DAG1 has already been shown to be present on a high percentage of $CD34^+$ cells (Steidl, Bork et al. 2004). We found DAG1 and NRXN1α present on nearly all $CD34^+$ cells. Intriguingly, the more primitive $CD34^+$ $CD38^-$ population can be further subdivided into two distinct populations based on DAG1 and NRXN1α expression (Figure 3); however these populations do not appear to differ in their proliferation *in vitro*, nor in their expression of molecules thought to be important to stem cell function such as c-kit, Thy1, CD150, CXCL12 and E-cadherin (used as a control). Percentage of cells expressing DAG1 and NRXN1α decreased as cells differentiated: only a third of lineage positive cells expressed either protein. We could not identify a significant population expressing NXPH via extracellular flow cytometry; however ELISA reveals a high concentration (461.7±59.7ng/ml) of NXPH in huCB plasma as compared to FBS (8.1±2.1ng/ml) and adult huPB plasma (5.8±0.8ng/ml).

Recombinant NXPH1 inhibits colony formation *in vitro* and is antagonized by endogenous DAG1.

Having established the presence of NRXN1α in huCB HPCs, recombinant NXPH1 was used to assay its function. When low-density huCB cells are plated at a concentration of $1x10^4$cells/ml, NXPH1 (200ng/ml) selectively inhibits proliferation stimulated by GM-CSF+SCF (Figure 4) but not SCF, GM-CSF, IL-3, IL-3+SCF and GM-SCF+IL-3+SCF+Epo. Since DAG1 and NRXN1α are involved in calcium signaling, and calcium ionophores are known survival agents, we hypothesized that DAG1-NRXN1α interaction elicited a pro-survival effect from HPCs, an interaction which was antagonized by recombinant NXPH1. To test for this, huCB cells were pre-incubated with a DAG1 blocking antibody. Consistent with this hypothesis, cells stimulated with GM-CSF+SCF proliferated less when pre-incubated with the DAG1 blocking antibody than with the isotype control (Figure 5A). Unexpectedly, when the blocked cells were exposed to recombinant NXPH1, they were inhibited under all growth conditions save SCF alone (Figures 5A and 5B).

Figure 3:



	Percent Cells Expressing:		
Cell Type	**DAG1**	**NRXN1α**	**DAG1+ NRXN1α**
Lineage+	28.5±6.1	33.8±12.0	21.3±7.1
CD34+	95.4±5.1	95.3±4.4	90.9±8.1
CD34+ CD38+	95.2±4.8	93.6±5.2	90.5±6.8
CD34+ CD38-	93.7±8.7	85.8±16.1	81.8±17.5

Figure 3: NRXN1α and DAG1 are expressed on primitive and more differentiated hematopoietic cells in huCB.

Top: Representative FACS plot of the expression of NRXN1α and DAG1 in huCB Lin^+, $CD34^+$, $CD34^+$ $CD38^+$, and $CD34^+$ $CD38^-$ populations.

Bottom: Chart displaying percentages of huCB populations expressing NRXN1α and DAG1 (combined data from at least three independent experiments; mean±SD).

Figure 4:

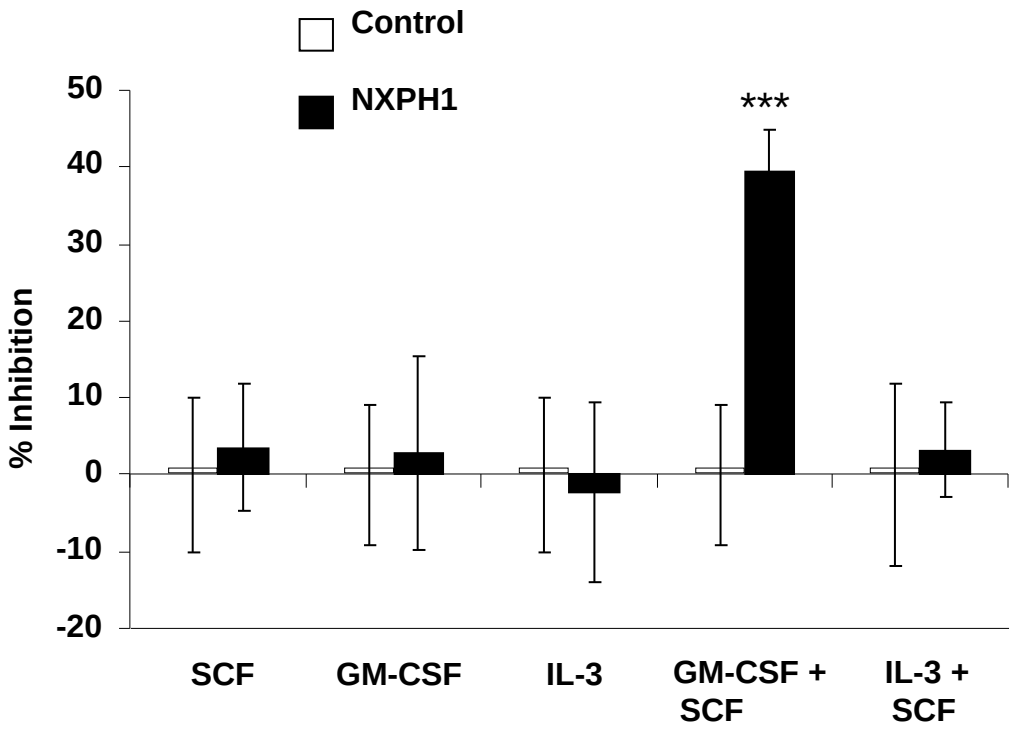
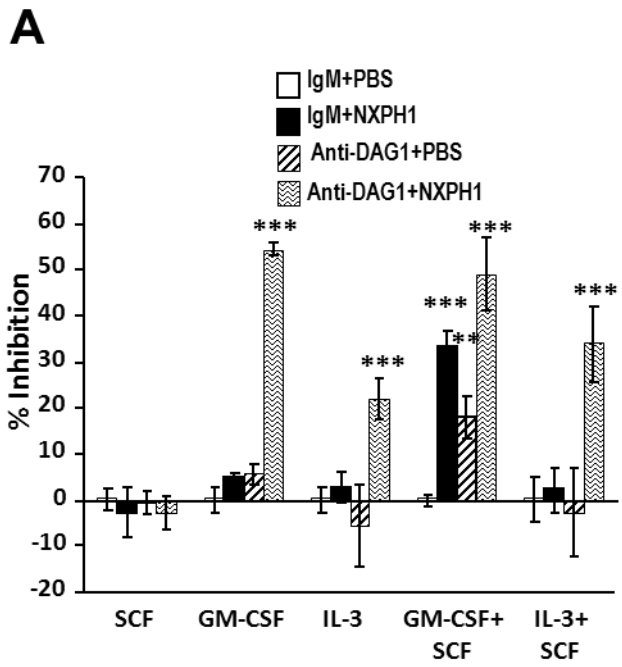

Control
NXPH1
***
50
40
30
20
10
0
-10
-20
% Inhibition
SCF
GM-CSF
IL-3
GM-CSF + SCF
IL-3 + SCF

Figure 4: Recombinant NXPH1 selectively inhibits proliferation stimulated by GM-CSF+SCF in huCB cells *in vitro*.

Percent recombinant NXPH1-mediated inhibition of CFU-GM colony formation in primary huCB cells plated as a population under a variety of factor-stimulated growth conditions (3 combined independent experiments, each done in triplicate; percent inhibition is calculated from the control defined as zero percent inhibition; mean±SD).

Figure 5:


A
IgM+PBS
IgM+NXPH1
Anti-DAG1+PBS
Anti-DAG1+NXPH1
% Inhibition
70
60
50
40
30
20
10
0
-10
-20
***
***
***
**
***
***
SCF
GM-CSF
IL-3
GM-CSF+ SCF
IL-3+ SCF

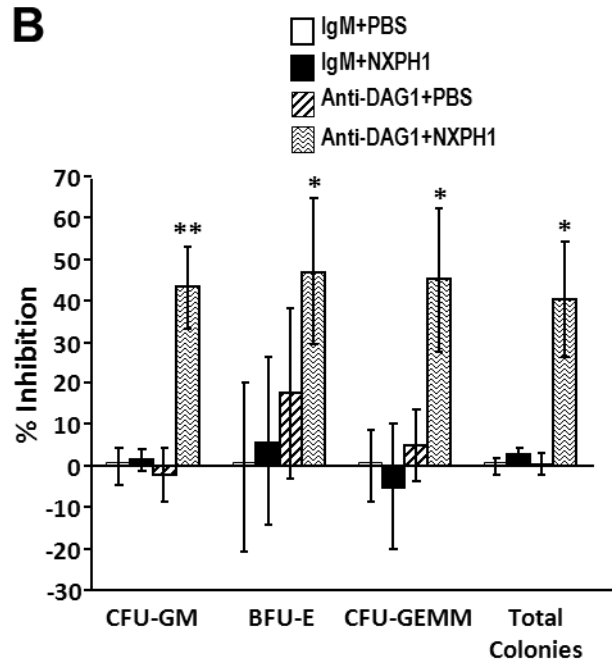

B
IgM+PBS
IgM+NXPH1
Anti-DAG1+PBS
Anti-DAG1+NXPH1
% Inhibition
70
60
50
40
30
20
10
0
-10
-20
-30
**
*
*
*
CFU-GM
BFU-E
CFU-GEMM
Total Colonies

Figure 5: DAG1 antagonizes NXPH1-mediated inhibition *in vitro*.

A: Percent recombinant NXPH1-mediated inhibition of CFU-GM, BFU-E, and CFU-GEMM colonies produced by huCB-derived HPCs stimulated by GM-CSF, IL-3, SCF, and Epo, in the presence and absence of anti-DAG1 blocking antibody when plated in population (combined data from three independent experiments performed in triplicate; percent inhibition is calculated from the control defined as zero percent inhibition; mean±SD).

B: Percent recombinant NXPH1-mediated inhibition of CFU-GM colonies produced by huCB-derived HPCs stimulated by specific growth factors, alone or in combination, in the presence and absence of anti-DAG1 blocking antibody when plated in population (combined data from three independent experiments performed in triplicate; percent inhibition is calculated from the control defined as zero percent inhibition; mean±SD).

*$p<0.05$, **$p<0.005$, ***$p<0.0005$.

As endogenous DAG1 appear to be a complicating factor, we sorted individual huCB $CD34^+$ cells to single wells to determine if the suppressive effect was at level of progenitor cells. Recombinant NXPH1 inhibited proliferation of CFU-GM, BFU-E and CFU-GEMM progenitors stimulated by GM-SCF+IL-3+SCF+Epo (Figure 6A). Because recombinant NXPH1 may have been interacting with a previously unrecognized partner, cells were also cultured in the presence of recombinant NRXN1α. Recombinant NRXN1α did not appear to affect proliferation; however it did decrease the inhibitory effect of recombinant NXPH1 suggesting that recombinant NXPH1 does signal through NRXN1α and exogenous NRXN1α competes with endogenous NRXN1α for binding. It remains unknown whether NRXN1α is internalized under these conditions; however, we feel it is unlikely. If recombinant NRXN1α were being internalized, we would expect to see an increase in the inhibitory effect of recombinant NXPH1 in the samples where both recombinant NRXN1α and NXPH1 are present and this is not observed. Furthermore, NRXN1α is a very large protein (165-175kDa), making internalization difficult for the cell. To ensure DAG1 had no effect on individual $CD34^+$ cells, $CD34^+$ cells were treated with DAG1 blocking antibody (Figure 6B). Consistent with our hypothesized role for DAG1, no difference was observed was observed between the treated and untreated cells at a single cell level. The effect of recombinant NXPH1 on individually plated $CD34^+$ cells cultured in the presence of SCF, GM-CSF, IL-3, GM-CSF+SCF and IL-3+SCF was also assessed. At a single cell level, recombinant NXPH1 suppresses colony forming ability under all conditions save SCF alone (Figure 7), which is consistent with the huCB cells plated in a population after treatment with anti-DAG1

Figure 6:

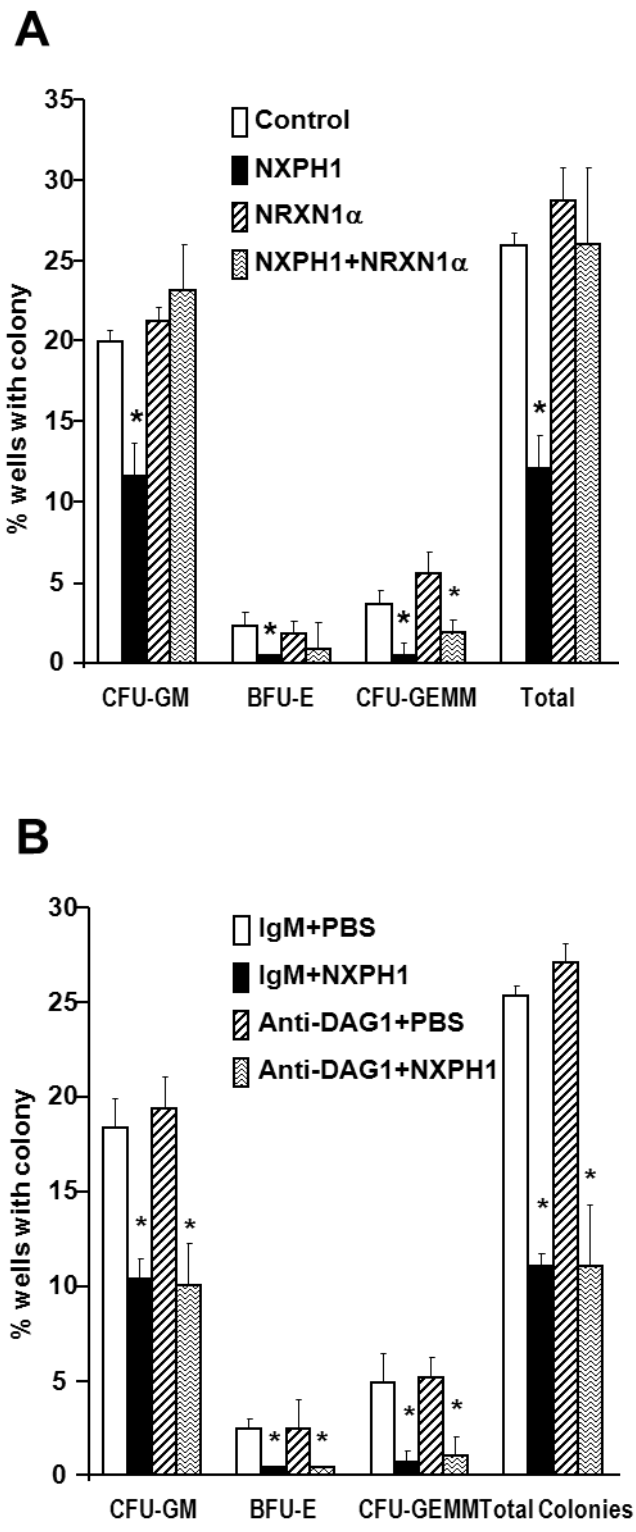

A
Control
NXPH1
NRXN1α
NXPH1+NRXN1α
% wells with colony
35
30
25
20
15
10
5
0
CFU-GM
BFU-E
CFU-GEMM
Total
B
IgM+PBS
IgM+NXPH1
Anti-DAG1+PBS
Anti-DAG1+NXPH1
% wells with colony
30
25
20
15
10
5
0
CFU-GM
BFU-E
CFU-GEMM
Total Colonies

Figure 6: Recombinant NXPH1 directly inhibits colony forming ability of primary huCB HPCs.

A: Percent of wells containing individually plated huCB $CD34^+$ cells producing CFU-GM, BFU-E, and CFU-GEMM colonies in the presence of recombinant NXPH1, and/or NRXN1α (252 wells were evaluated for each point; mean±SD).

B: Percent of wells containing individually plated huCB $CD34^+$ cells producing CFU-GM, BFU-E and CFU-GEMM colonies in the presence of recombinant NXPH1 and/or anti-DAG1 blocking antibody (252 wells were evaluated for each point; mean±SD).

*$p<0.05$

Figure 7:

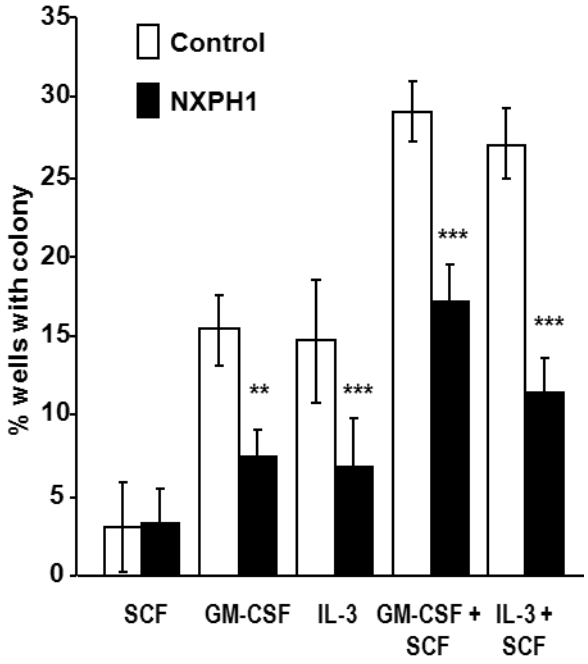

Control
NXPH1
% wells with colony
35
30
25
20
15
10
5
0
**
***
***
***
SCF
GM-CSF
IL-3
GM-CSF + SCF
IL-3 + SCF

Figure 7: Inhibition of huCB HPC colony formation by recombinant NXPH1 is not growth factor dependent.

Percent of wells containing individually plated huCB $CD34^+$ cells grown under a variety of conditions in the presence and absence of recombinant NXPH1 producing CFU-GM colonies (over 500 wells were evaluated for each point; mean±SD).

**p<0.005, ***p<0.0005.

blocking antibody (Figure 5). Furthermore, colonies that developed in the presence of recombinant NXPH1 were smaller than those in the controls (Figure 8).

Though single cell assays suggested that the effect of NXPH1 on HPCs was a direct one, the difference between single cell and population culture warranted further investigation. If the inhibitory effect of NXPH1 on HPCs stimulated by GM-CSF+SCF in a population of cells is fundamentally different from other growth factor and growth factor combinations, pre-treatment might provide some insight on this matter. However, pulse pre-treatment of huCB cells with NXPH1 did not affect HPC proliferation (Figure 9A). We also considered the possibility of an indirect effect. To account for possible secondary effects, conditioned medium from LDCB cells exposed overnight to NXPH1 was generated. Low-density huCB cells were cultured in 20% conditioned medium alone, with SCF, GM-CSF, or GM-CSF+SCF. Conditioned medium from cells cultured in the presence of recombinant NXPH1 was not inhibitory to growth stimulated by conditioned medium alone, SCF, GM-CSF, or GM-CSF+SCF (Figure 9B).

Consistent with our previous results, recombinant NXPH1 directly inhibits HPC proliferation and endogenous DAG1 is the agent that blocks its effects *in vitro*. Together, these results suggest that recombinant NXPH1 binds NRXN1α and directly inhibits huCB HPC proliferation. This inhibitory effect is blocked by the presence of

Figure 8:

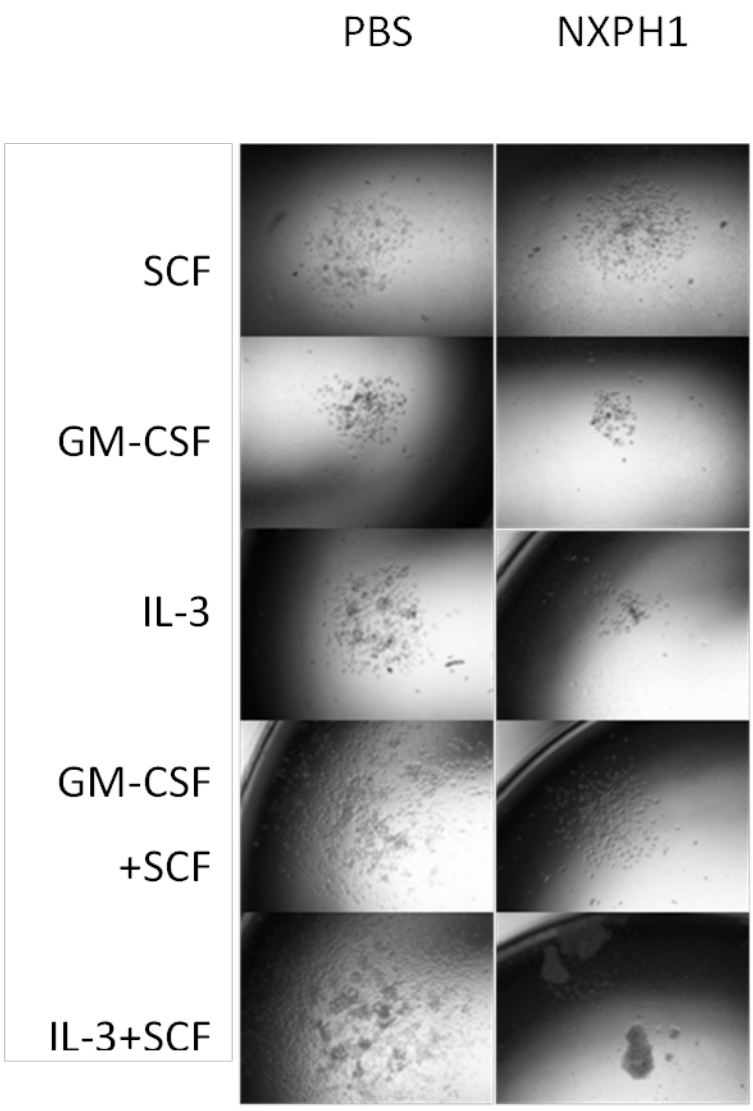


Figure 8: Recombinant NXPH1 decreases CFU-GM colony size.

Size of CFU-GM colonies produced by individually plated huCB $CD34^+$ cells grown under a variety of conditions in the presence and absence of recombinant NXPH1 (representative colonies were randomly selected for display).

Figure 9:

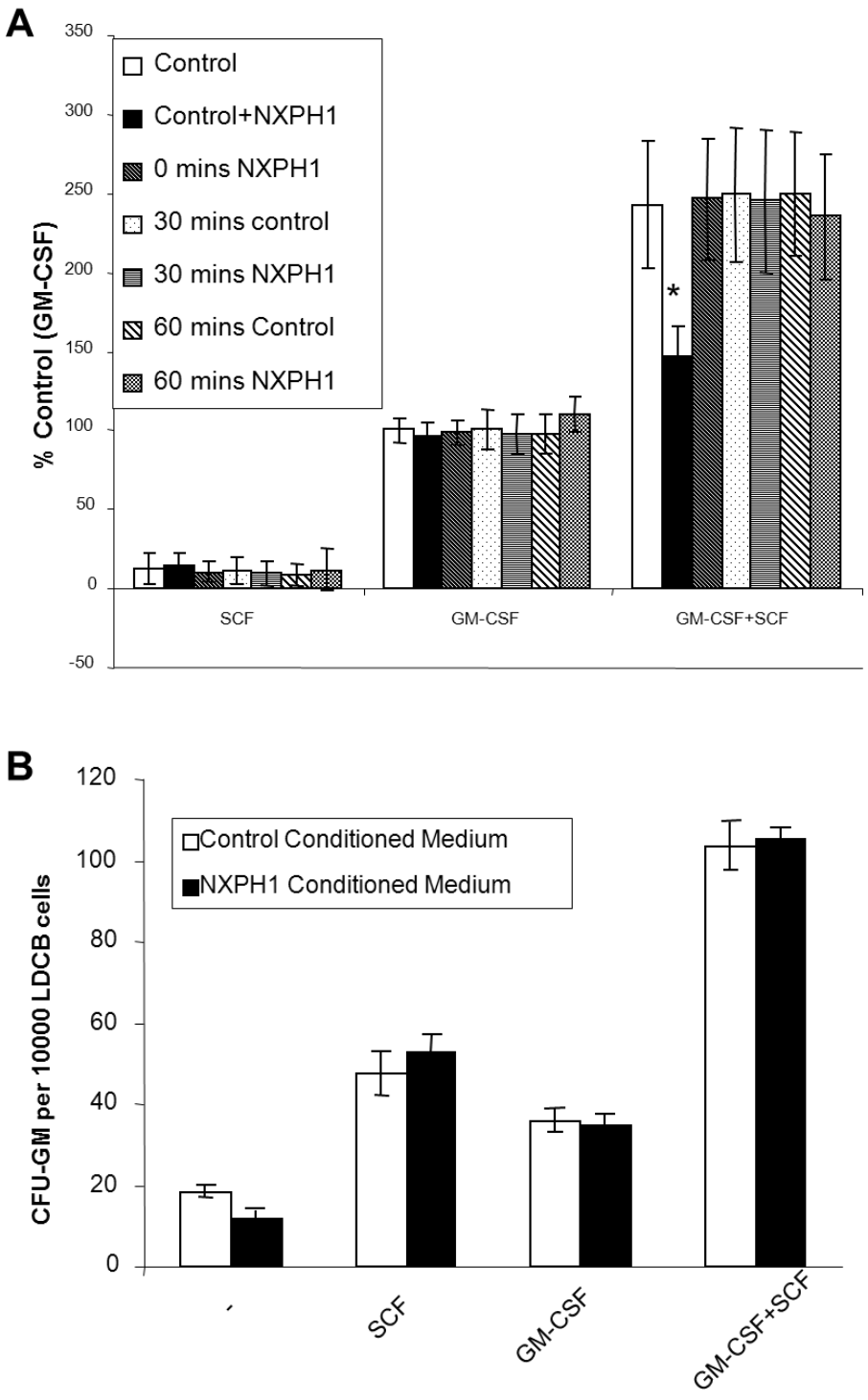

A
% Control (GM-CSF)
350
300
250
200
150
100
50
0
-50
Control
Control+NXPH1
0 mins NXPH1
30 mins control
30 mins NXPH1
60 mins Control
60 mins NXPH1
*
SCF
GM-CSF
GM-CSF+SCF
B
CFU-GM per 10000 LDCB cells
120
100
80
60
40
20
0
Control Conditioned Medium
NXPH1 Conditioned Medium
-
SCF
GM-CSF
GM-CSF+SCF

Figure 9: Recombinant NXPH1 directly inhibits proliferation of HPCs stimulated by GM-CSF+SCF.

A: The effect of recombinant NXPH1 pulse pre-treatment on huCB proliferation expressed as percent control (GM-CSF). Control cells were plated with either DPBS carrier or 500ng/ml NXPH1. Experimental cells were pre-treated with either DPBS carrier or 500ng/ml NXPH1, washed and then plated. (At least three independent experiments performed in triplicate were used for calculating data points; mean±SD).

B: The effect of conditioned medium from NXPH1 treated cells expressed as CFU-GM colony forming ability. $1x10^6$ LDCB cells/ml were cultured in RPMI-1640 + 10% FBS medium in the presence or absence of 500ng/ml NXPH1. Conditioned medium was collected after 24 hours and sterile filtered. 20% conditioned medium was used in conjunction with nothing (-), SCF, GM-CSF, or GM-SCF+SCF.

*$p<0.05$

DAG1 under all conditions save GM-CSF+SCF, (events evaluated later in the murine system).

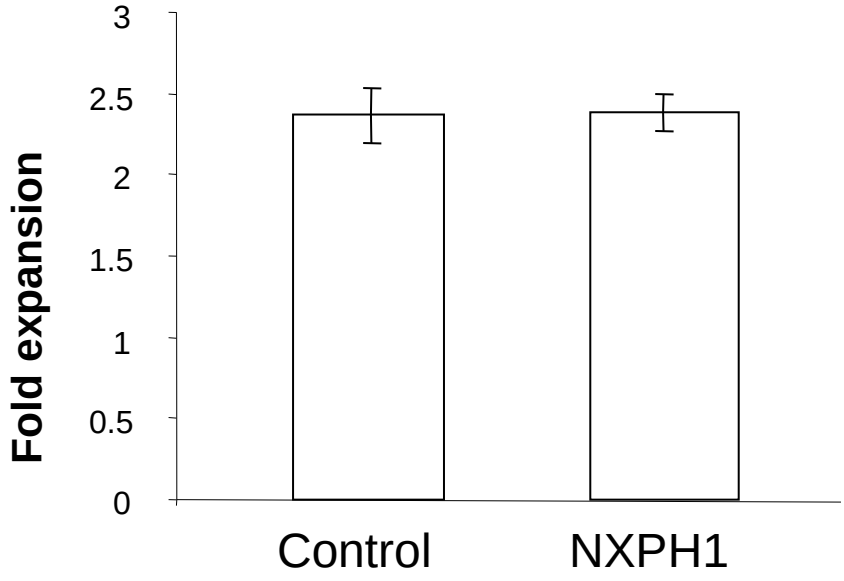
Because NXPH is present in huCB plasma, we hypothesized that it might have an effect on *ex vivo* culture of HPCs. To test this, huCB $CD34^+$ were isolated via MACS and cultured as a population of $1x10^4$cells/ml for three days in the presence of SCF (50ng/ml), TPO (100ng/ml), and Flt3L (100ng/ml) plus or minus NXPH1 (200ng/ml). After the initial culture, single $CD34^+$ cells from the two conditions were sorted via FACS and, as per above, plated as a single cell per well in the presence or absence of 200ng/ml NXPH1 under maximally stimulatory conditions (Epo, SCF, IL-3, and GM-CSF). The presence of NXPH1 did not have an effect on the expansion of HPCs in terms of fold expansion and colony forming ability and the cells produced were responsive to inhibition by recombinant NXPH1 (Figure 10). This argues against the involvement of NXPH in the self-renewal of HSCs derived from huCB.

Alpha-latrotoxin has a pro-survival effect on HPCs which is blocked by NXPH1

In addition to NXPH and DAG1, the second LNS domain of NRXN1α also binds LT. In the neuronal system, LT binds NRXN1α and inserts itself into the cellular membrane forming a calcium ionophore, which is responsible for the effects of *latrodectus spp.* venom on

mammals. Because calcium ionophores have been previously shown to have a pro-survival effect on HPCs, we tested the effect of LT on HPC survival under delayed growth factor addition. Similar to other calcium ionophores, LT did not affect HPC proliferation under normal conditions but did act as a pro-survival factor in the context of delayed growth factor addition *in vitro* (Figure 11A). In addition to binding the same LNS domain as NXPH, LT also binds NXPH. These interactions lead us to question whether recombinant NXPH1 would affect the observed effect of LT *in vitro*. We observed that the pro-survival effect of LT is blocked by the addition of recombinant NXPH1 (Figure 11B). Because these studies were conducted in a population of cells, we were curious whether endogenous DAG1 played a role. Rather than examine the effect of LT on individually plated $CD34^+$ cells, GM-CSF+SCF was used as a proxy culture condition where the effect of endogenous DAG1 is not observed. Alpha-latrotoxin does not affect recombinant NXPH1-mediated inhibition in cells treated with GM-CSF+SCF (Figure 12A) nor do growth factor starved cells behave differently when exposed to GM-CSF+SCF (Figure 12B). Intriguingly, LT does not appear to increase survival of cells treated with IL-3 or IL-3+SCF after delayed growth factor addition.

Figure 10:


Fold expansion
3
2.5
2
1.5
1
0.5
0
Control
NXPH1

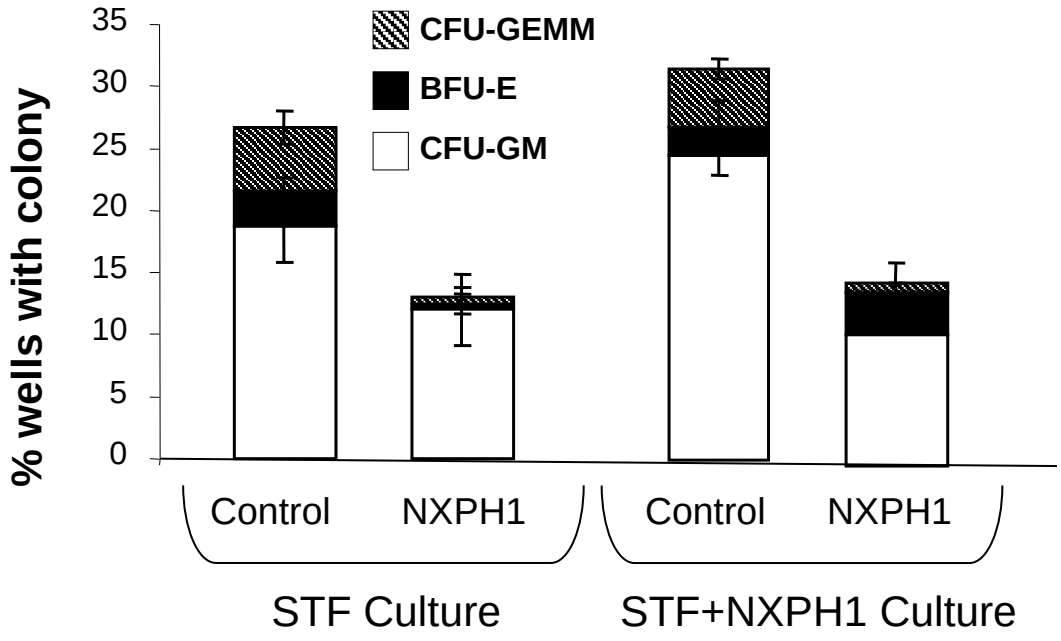

% wells with colony
35
30
25
20
15
10
5
0
CFU-GEMM
BFU-E
CFU-GM
Control
NXPH1
Control
NXPH1
STF Culture
STF+NXPH1 Culture

Figure 10: Recombinant NXPH1 does not affect *ex vivo* expansion of huCB $CD34^+$ cells.

MACS separated huCB $CD34^+$ cells were expanded *ex vivo* in 50ng/ml SCF, 100ng/ml TPO, and 100ng/ml Flt3L (STF) in the presence or absence of 200ng/ml NXPH1 for three days. Cells were then stained for $CD34^+$ and analyzed.

Top: Fold expansion of $CD34^+$ cells after three days of expansion in the presence and absence of 200ng/ml recombinant NXPH1. (3 combined independent experiments; mean±SD).

Bottom: Percent of wells containing individually plated expanded huCB $CD34^+$ cells producing CFU-GM, BFU-E, and CFU-GEMM colonies in the presence of recombinant NXPH1 (252 wells were evaluated for each point; mean±SD).

Figure 11:

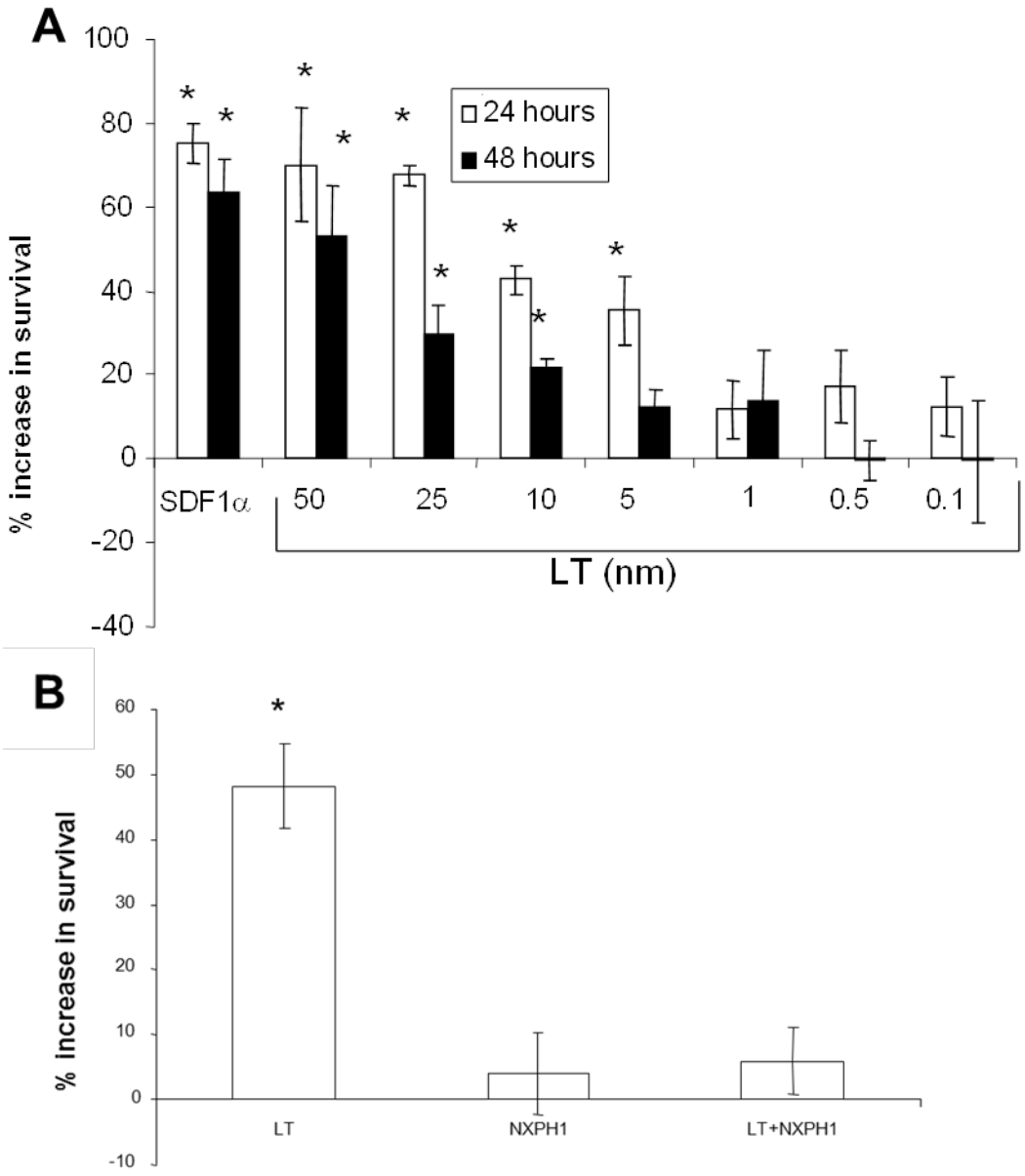

A
100
80
60
40
20
0
-20
-40
% increase in survival
24 hours
48 hours
SDF1α
50
25
10
5
1
0.5
0.1
LT (nm)
B
60
50
40
30
20
10
0
-10
% increase in survival
LT
NXPH1
LT+NXPH1

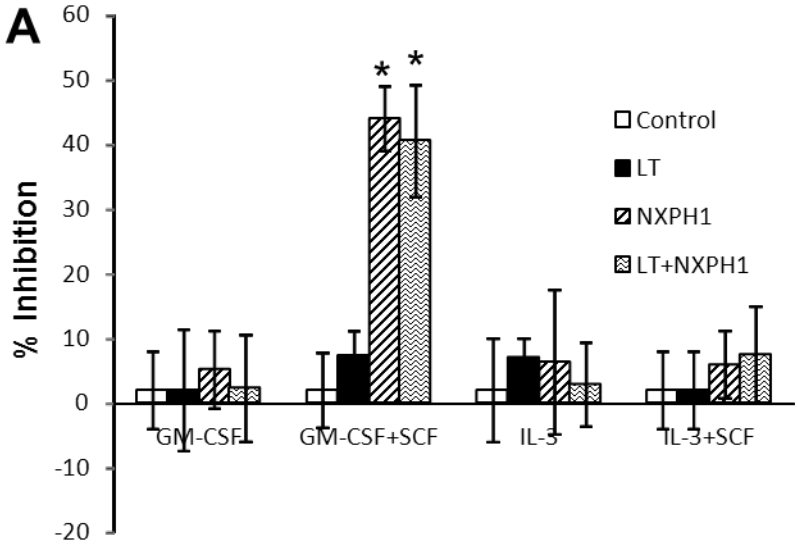
Figure 11: Alpha-latrotoxin has a pro-survival effect on growth-factor starved HPCs.

A: Percent increase in survival of huCB HPCs plated with either 200ng/ml SDF1α or varying concentrations of alpha-latrotoxin (LT) under delayed growth-factor addition for 24 or 48 hours (3 combined independent experiments each performed in triplicate; mean±SD).

B: Percent increase in survival of huCB cells plated with either 50nm LT, 200ng NXPH1, 50nm LT and 200ng NXHP1 or PBS control. After a 24 hour growth factor starvation, GM-CSF, IL-3, Epo, and SCF were added to the experimental and control plates (3 combined independent experiments each performed in triplicate; mean±SD).

*$p<0.05$

Figure 12:


A
% Inhibition
60
50
40
30
20
10
0
-10
-20
*
*
Control
LT
NXPH1
LT+NXPH1
GM-CSF
GM-CSF+SCF
IL-3
IL-3+SCF

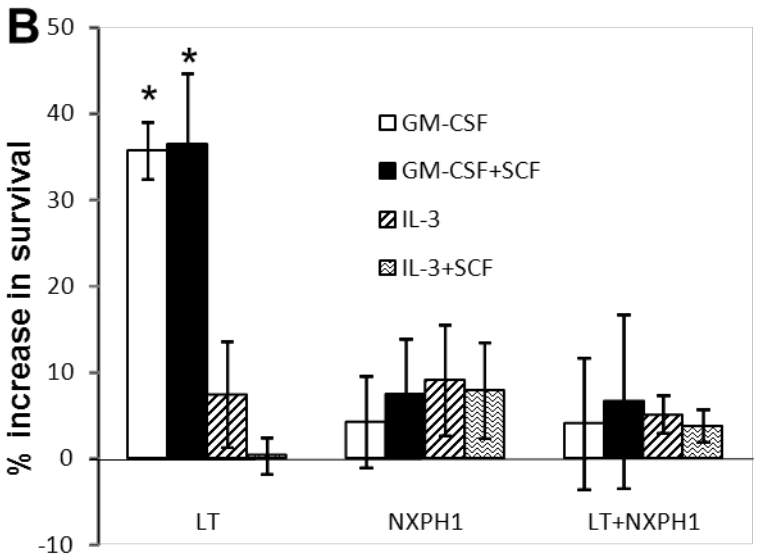

B
% increase in survival
50
40
30
20
10
0
-10
*
*
GM-CSF
GM-CSF+SCF
IL-3
IL-3+SCF
LT
NXPH1
LT+NXPH1

Figure 12: Recombinant NXPH1 blocks the pro-survival effect of LT

A: Percent inhibition of huCB cells plated with 50nm LT and/or 200ng NXPH1 and various growth factors (2 combined independent experiments each performed in triplicate; mean±SD).

B: Percent increase in survival of huCB cells plated with 50nm LT and/or 200ng NXPH1. After a 24 hour growth factor starvation, the growth factors shown were added to the experimental and control plates (2 combined independent experiments each performed in triplicate; mean±SD).

*$p<0.05$

Expression of DAG1, NRXN1α and NXPH1 in the murine hematopoietic system

We observed interesting similarities and differences between the huCB and the murine hematopoietic system. The pattern of NRXN1α expression in adult muBM mirrors that found in huCB with a higher percentage of primitive cells expressing NRXN1α while a high percentage of many populations express DAG1, particularly lymphoid progenitors (Table 2). Because NRXN1α expression correlates with a primitive hematopoietic phenotype and DAG1 has been implicated in the hematopoietic niche (Hines, Nielsen et al. 2008), we examined the expression pattern of NRXN1α and DAG1 in the bone marrow using immunohistochemistry. DAG1 is clearly expressed on active osteoblasts within the bone marrow (Figure 13). Cells expressing high levels of NRXN1α in the bone marrow appear in close proximity with cells expressing DAG1, including stromal cells near the osteoblasts. NXPH is not found at concentrations above background in muBM cells and mu peripheral blood plasma; however previous studies have implicated NXPH in the spleen. Since NXPH is secreted, intracellular staining was used to examine which populations within the spleen express NXPH. A small percent of splenocytes (5.2±0.3%) appear to express NXPH, with the majority of the expression confined to the lineage positive fraction (7.8±1.0% positive for NXPH). A small percentage of CMPs in the spleen (3.4±0.7%) also contained NXPH.

Table 2:

	Percent Cells Expressing:	
	NRXN1α	DAG1
LTR-HSC	41.7±3.9	35.9±12.6
STR-HSC	36.7±5.5	61.7±11.8
MEP	21.3±3.4	40.0±9.6
GMP	10.5±1.4	53.3±11.1
CLP	33.6±10.5	95.6±2.1
Lineage +	0.8±0.1	19.4±7.7

Table 2: Expression of NRXN1α and DAG1 in murine bone marrow.

The expression of NRXN1α and DAG1 on stem and progenitor populations within the muBM. Populations are defined as: long-term repopulating HSCs (LTR-HSC; lineage$^-$, sca-1$^+$, c-kit$^+$, IL-7Rα^-, CD34$^-$), short-term repopulating HSCs (STR-HSC; lineage$^-$, sca-1$^+$, c-kit$^+$, IL-7Rα^-, CD34$^+$) common lymphoid progenitor (CLP; lineage$^-$, sca1$^+$, c-kit$^+$, IL-7Rα^+), megakaryocyte/erythroid progenitor (MEP; lineage$^-$, sca1$^-$, c-kit$^+$, CD34$^-$, FcγR$^{-/lo}$), common myeloid progenitor (CMP; lineage$^-$ sca1$^-$, c-kit$^+$, CD34$^+$, FcγR$^{-/lo}$), and granulocyte/macrophage progenitor (GMP; lineage$^-$, sca1$^-$, c-kit$^+$, CD34$^+$, FcγR$^{+/hi}$). Data was collected from samples using an LSR II (BD) instrument and BD FACSDiva software and analyzed using WinList software.

Figure 13:

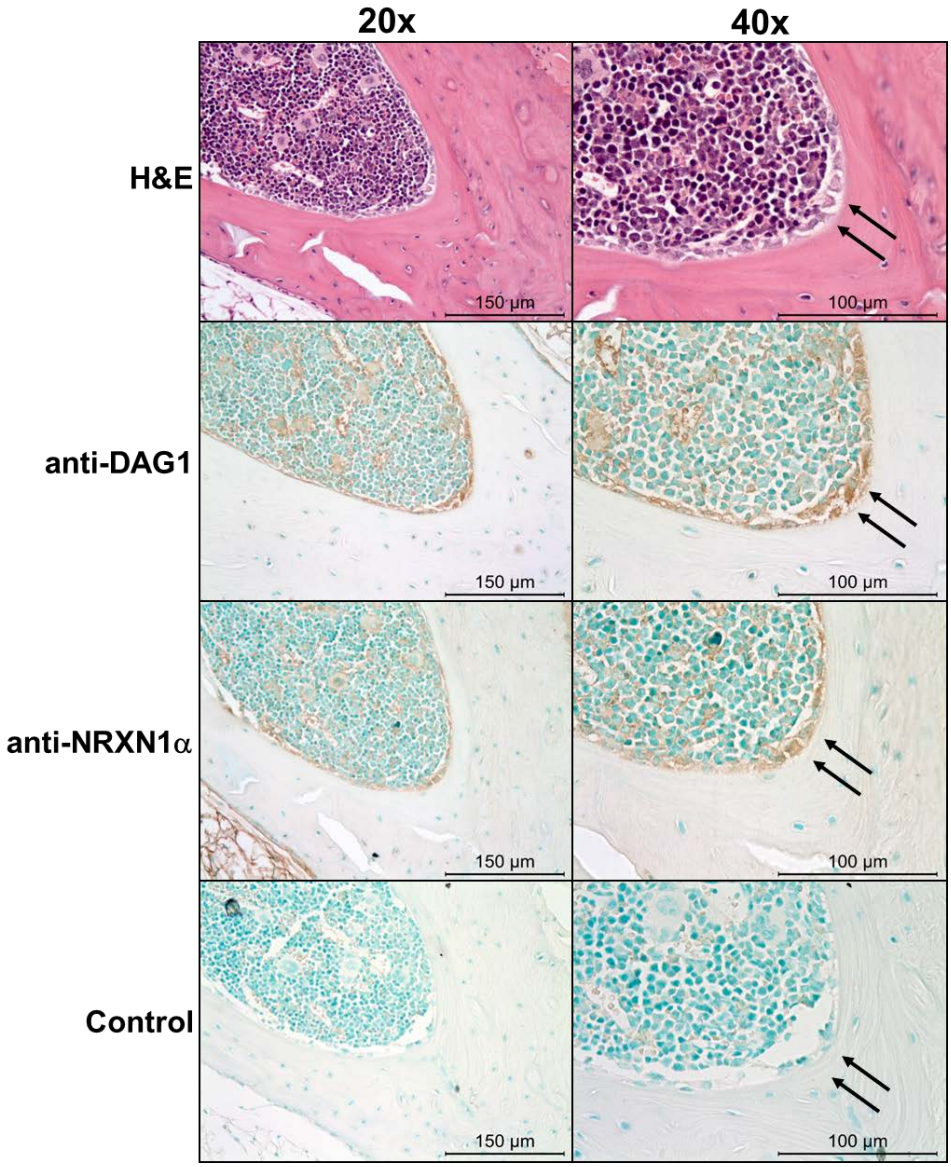


Figure 13: DAG1 and NRXN1α co-localize near osteoblasts in muBM.

Low (20x) and higher (40x) power microphotographs of serial sections mounted using Permount mounting medium and stained with hematoxylin and eosin (H&E) or anti-α-DAG1, anti-NRXN1α, rabbit IgG isotype antibodies. DAG1 and NRXN1α co-localized to active osteoblasts (marked with an arrow). Immunoreactivity was also observed along the periphery of megakaryocytes and erythrocytes for both DAG1 and NRXN1α. Note the lack of immunoreactivity in osteocytes and stromal cells. Micrographs were taken on a Leica BM3000 microscope with PLAN objective x20 (NA 0.4) or a PLAN objective x40 (NA 0.65) at room temperature. Images were captured using Leica Application Suite v3.6.0 from Leico Microsystems.

GM-CSF maintains NRXN1α expression in muBM cells

In vitro exposure of muBM cells plated together with recombinant NXPH1 (Figure 14) mirrors results found in huCB cells. However, NRXN1α is expressed at a high enough level in muBM to be detected by Western analysis, which provided us with a powerful tool to investigate the effect of recombinant NXPH1 on cells stimulated by GM-CSF+SCF. Freshly harvested muBM cells were cultured under a variety of growth conditions for 24 hours and the expression of NRXN1α was examined (Figure 15). NRXN1α is apparent in freshly isolated cells but when cultured with SCF, IL-3 and IL-3+SCF, expression level decreases significantly. However, expression of NRXN1α is maintained in cells cultured with either GM-CSF or GM-CSF+SCF. As DAG1 is less expressed on mature cells (Figure 3, Figure 13, Table 2), we considered that the ratio of NRXN1α to DAG1 may be altered in favor of available NRXN1α. If true, cells plated in a population would require a higher concentration of recombinant NXPH1 in order for equivalent levels of inhibition to be observed. Human CB and muBM cells respond similarly to recombinant NXPH1 in population with maximal inhibition observed at 500ng/ml. However, individually plated human $CD34^+$ huCB cells reach their maximal inhibition at 200ng/ml. An examination of the dose-response curves (Figure 16) reveals that the affinity of NXPH1 to NRXN1α on individually plated $CD34^+$ cells (IC_{50}=68.8±5.9ng/ml) is the same as for NXPH1 to NRXN1α on huCB and muBM cells plated in a population (IC_{50}=68.7±1.8ng/ml and 71.0±4.8ng/ml, respectively).

Figure 14:

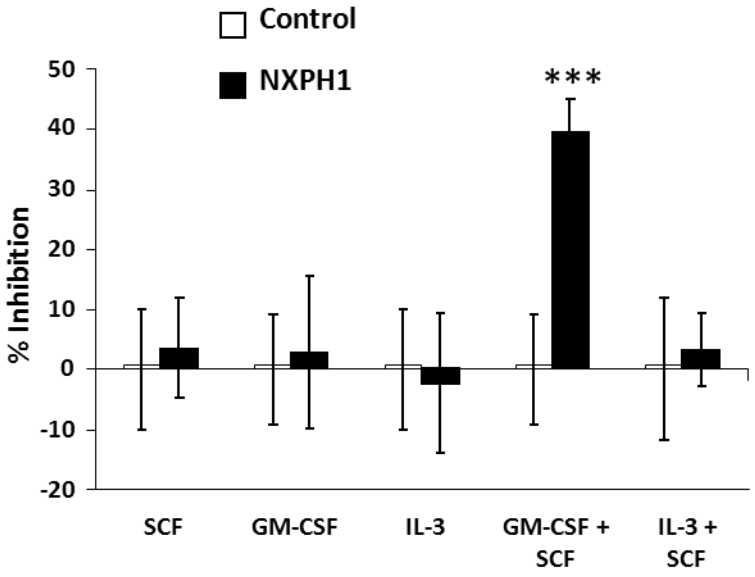

Control
NXPH1
***
% Inhibition
50
40
30
20
10
0
-10
-20
SCF
GM-CSF
IL-3
GM-CSF + SCF
IL-3 + SCF

Figure 14: Recombinant NXPH1 selectively inhibits proliferation stimulated by GM-CSF+SCF in muBM cells *in vitro*.

Percent recombinant NXPH1-mediated inhibition of CFU-GM colony formation in primary muBM cells plated as a population under a variety of factor-stimulated growth conditions (3 combined independent experiments, each done in triplicate; mean±SD)
***p<0.0005

Figure 15:

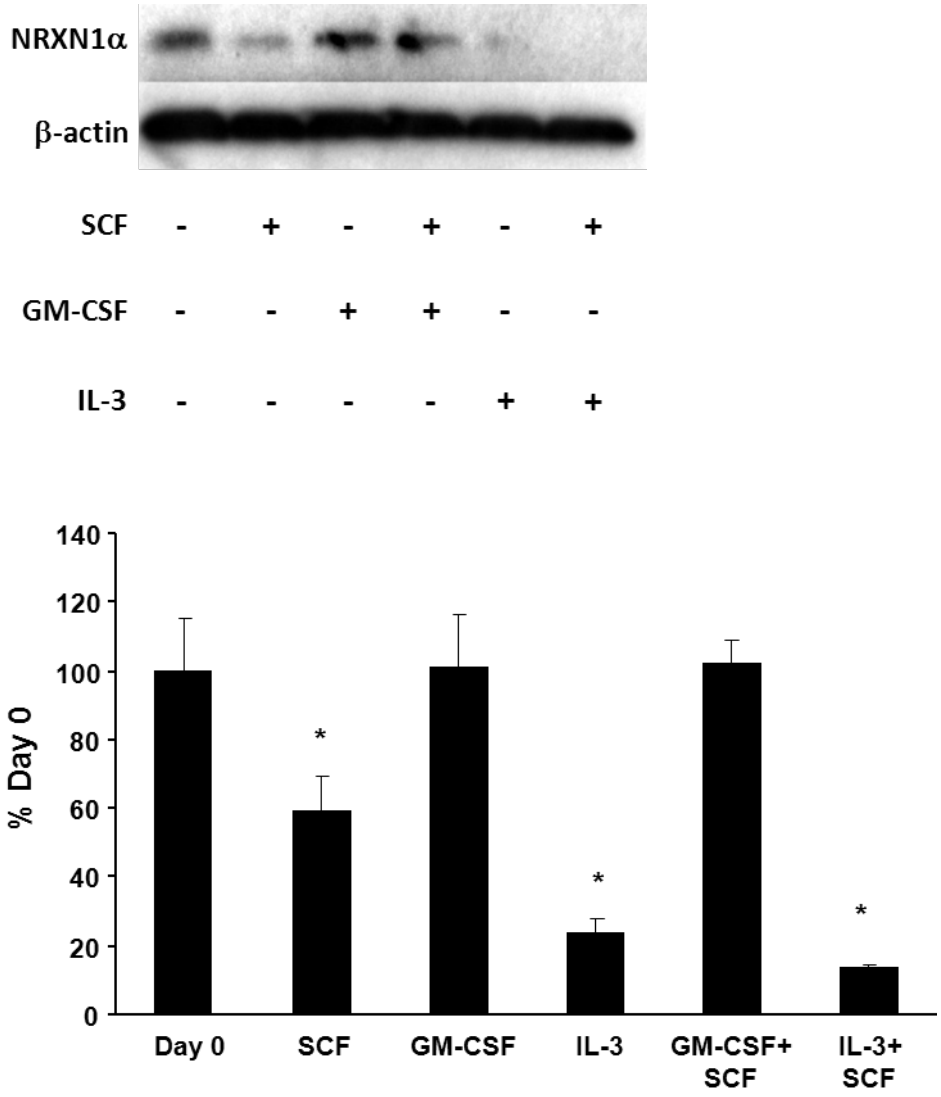

NRXN1α
β-actin
SCF - + - + - +
GM-CSF - - + + - -
IL-3 - - - - + +
% Day 0
140
120
100
80
60
40
20
0
*
*
*
Day 0
SCF
GM-CSF
IL-3
GM-CSF+ SCF
IL-3+ SCF

Figure 15: Expression of NRXN1α is maintained by GM-CSF and downregulated by IL-3.

Top: Representative Western blot of lysate from primary muBM cells cultured for 24 hours under a variety of conditions. The first lane represents lysate from freshly harvested muBM. β-actin is shown as a loading control.

Bottom: Densitometric analysis of NRXN1α expression in Western blots. Values were normalized to β-actin and represented as a percent of the Day 0 control (4 combined independent experiments; mean±SD).

*$p<0.05$

Figure 16:

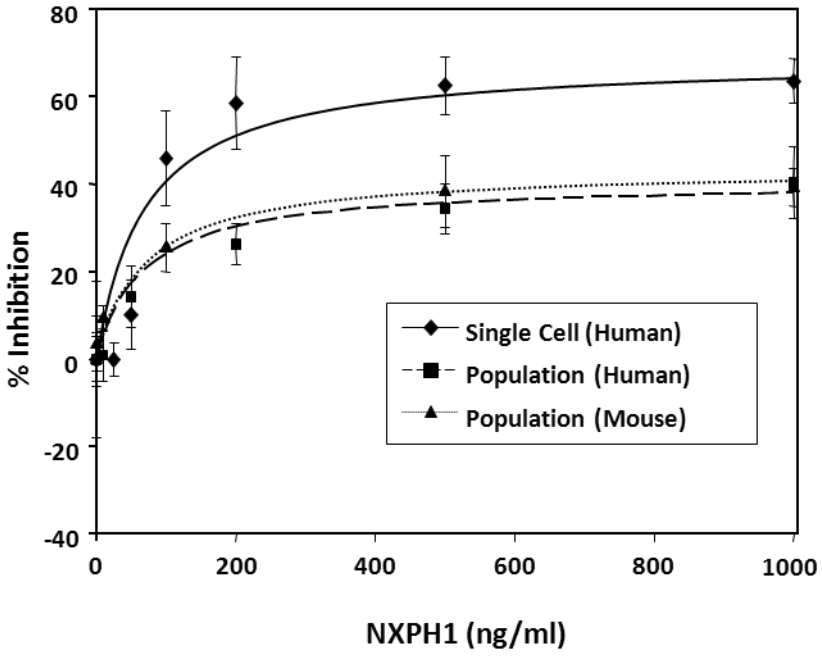

80
60
40
20
0
-20
-40
% Inhibition
0
200
400
600
800
1000
NXPH1 (ng/ml)
Single Cell (Human)
Population (Human)
Population (Mouse)

Figure 16: DAG1 is an allosteric inhibitor of NXPH1 function.

Calculated dose-response curves modeling the NXPH1-mediated inhibition of CFU-GM colony formation in the presence of GM-CSF+SCF. HPCs were derived from either individually plated huCB $CD34^+$ (R^2=0.95), huCB plated in population ($1x10^4$cells/ml) (R^2=0.99), or muBM plated in population ($5x10^4$cells/ml) (R^2=0.99) (288 wells were evaluated for each data point in experiments involving a single plated cell; at least three independent experiments performed in triplicate were used for calculating data points involving cells plated in a population; mean±SD).

However, the efficacy of NXPH1 on individually plated $CD34^+$ (I_{max}=68.7±2.7 % inhibition) is much greater than when huCB and muBM cells are plated in a population (I_{max}=40.7±3.3 and 43.7±2.5 % inhibition, respectively). From this we conclude that DAG1 decreases the efficacy of NXPH1 but not its affinity, suggesting that DAG1 is a non-competitive agonist. Non-competitive antagonism coupled with the knowledge that DAG1 and NXPH1 bind different areas within the second LNS domain of NRXN1α (Lise and El-Husseini 2006) strongly suggests that an allosteric interaction is at play. While the inhibitory effect of blocking DAG1 on GM-CSF+SCF (Figures 5 and 14) complicates these findings and demands that other factors are present, we feel these findings help explain the particular effect of recombinant NXPH1 on cells treated with GM-CSF+SCF. Additionally, the near-absolute downregulation of NRXN1α in growth conditions containing IL-3 helps explain the inability of LT to promote survival in cells treated with IL-3 or IL-3+SCF.

Recombinant NXPH1 exhibits suppressive effects *in vivo*

To determine the effect of recombinant NXPH1 on the hematopoietic system *in vivo*, normal C57Bl/6 mice were injected *i.v.* with endotoxin-free recombinant NXPH1. Twenty-four to 48 hours after injection of recombinant NXPH1, the total number of circulating leukocytes, as well as neutrophils, lymphocytes, monocytes, basophils and platelets were significantly decreased (Figure 17).

Figure 17:

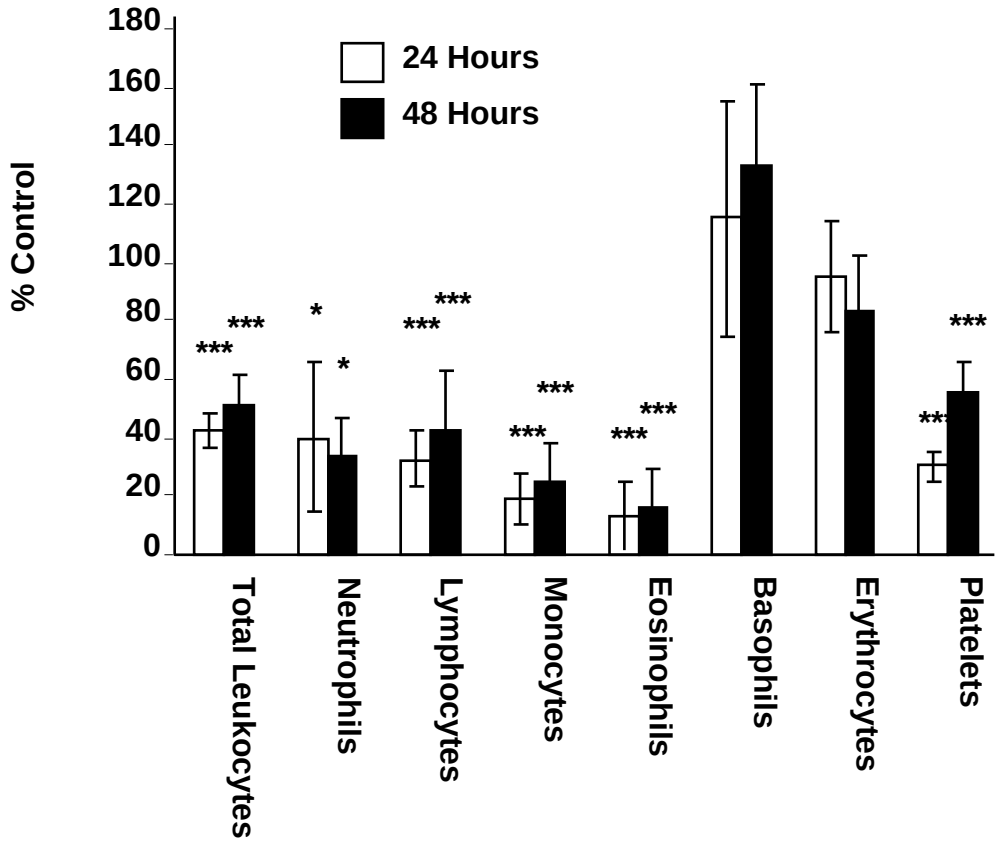

% Control
180
160
140
120
100
80
60
40
20
0
24 Hours
48 Hours
Total Leukocytes
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils
Erythrocytes
Platelets

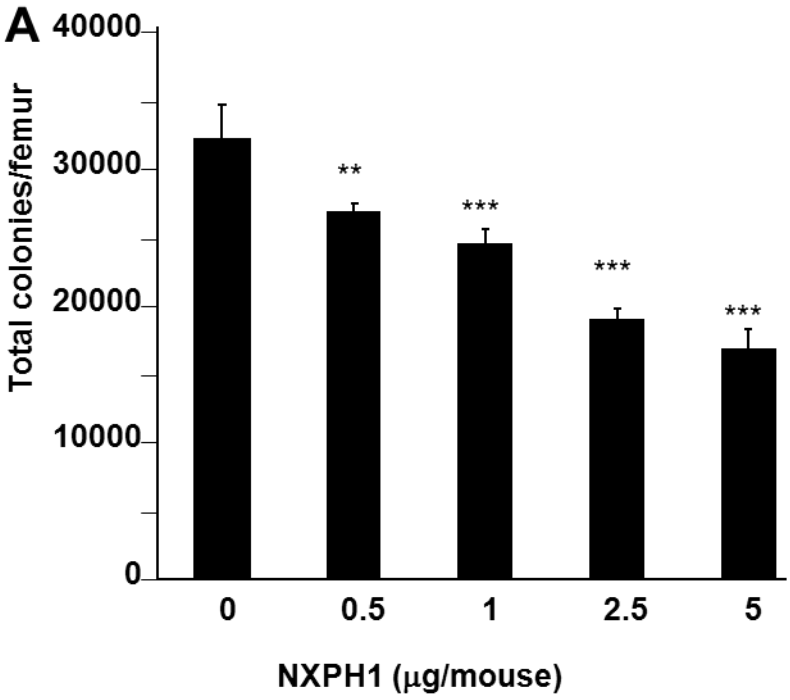
Figure 17: *In vivo* injection of NXPH1 decreases the absolute number of many mature hematopoietic populations in mouse peripheral blood.

Change in peripheral blood populations after *i.v.* injection of recombinant NXPH1 expressed as a percentage of control (at least three independent experiments performed in triplicate; mean±SD).

*p<0.05, **p<0.005, ***p<0.0005.

Absolute numbers and cycling status of HPCs in the BM were decreased in a dose- and time-dependent manner (Figure 18 and 19, respectively). At a maximally inhibitory dose (2.5-5μg NXPH1/mouse), absolute numbers of HPCs were inhibited by 50.8% at 24 hours, effects mirrored by decreases at 24 hours in cycling HPCs (measured by high specificity activated tritiated thymidine kill assay). After 48 hours, absolute numbers of HPCs were increased but still significantly inhibited: HPC numbers completely returned to normal levels 72 hours after injection of NXPH1, a trend mirrored in the cycling status of BM HPCs. Consistent with functional data, phenotypically defined BM HPCs were also suppressed by recombinant NXPH1 (Figure 20) at levels similar to those observed in functional assays. In contrast to the BM, the spleen was less responsive to NXPH1 exposure. At a maximally inhibitory dose (5μg NXPH1/mouse) absolute numbers of splenic HPCs were unaffected for the first two days and increased slightly after the third day (Figure 21A). The cycling status of splenic HPCs was decreased 24 hours after injection but returned to baseline after 48 hours, and was slightly increased after 72 hours (Figure 21B).

Figure 18:


A
40000
30000
20000
10000
0
Total colonies/femur
**
***
***
***
0
0.5
1
2.5
5
NXPH1 (μg/mouse)

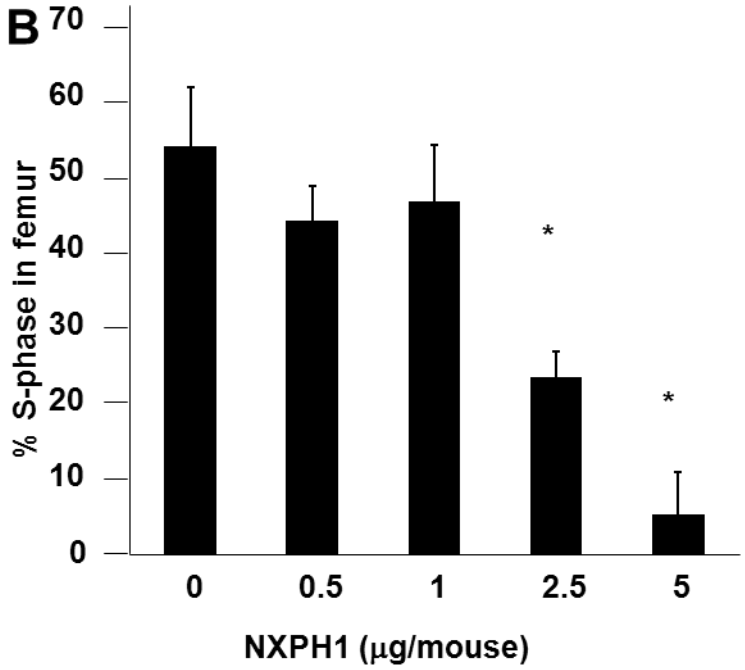

B
70
60
50
40
30
20
10
0
% S-phase in femur
*
*
0
0.5
1
2.5
5
NXPH1 (μg/mouse)

Figure 18: Recombinant NXPH1 decreases the absolute number and cycling status of muBM HPCs in a dose-dependent manner.

A: Absolute colony number of muBM-derived HPCs 24-hours after exposure to varying doses of recombinant NXPH1 (combined data of three independent experiments each performed in triplicate; mean±SD).

B: Cycling status of muBM-derived HPCs 24-hours after exposure to varying doses of recombinant NXPH1 (combined data of three independent experiments each performed in triplicate; mean±SD).

*$p<0.05$, **$p<0.005$, ***$p<0.0005$.

Figure 19:

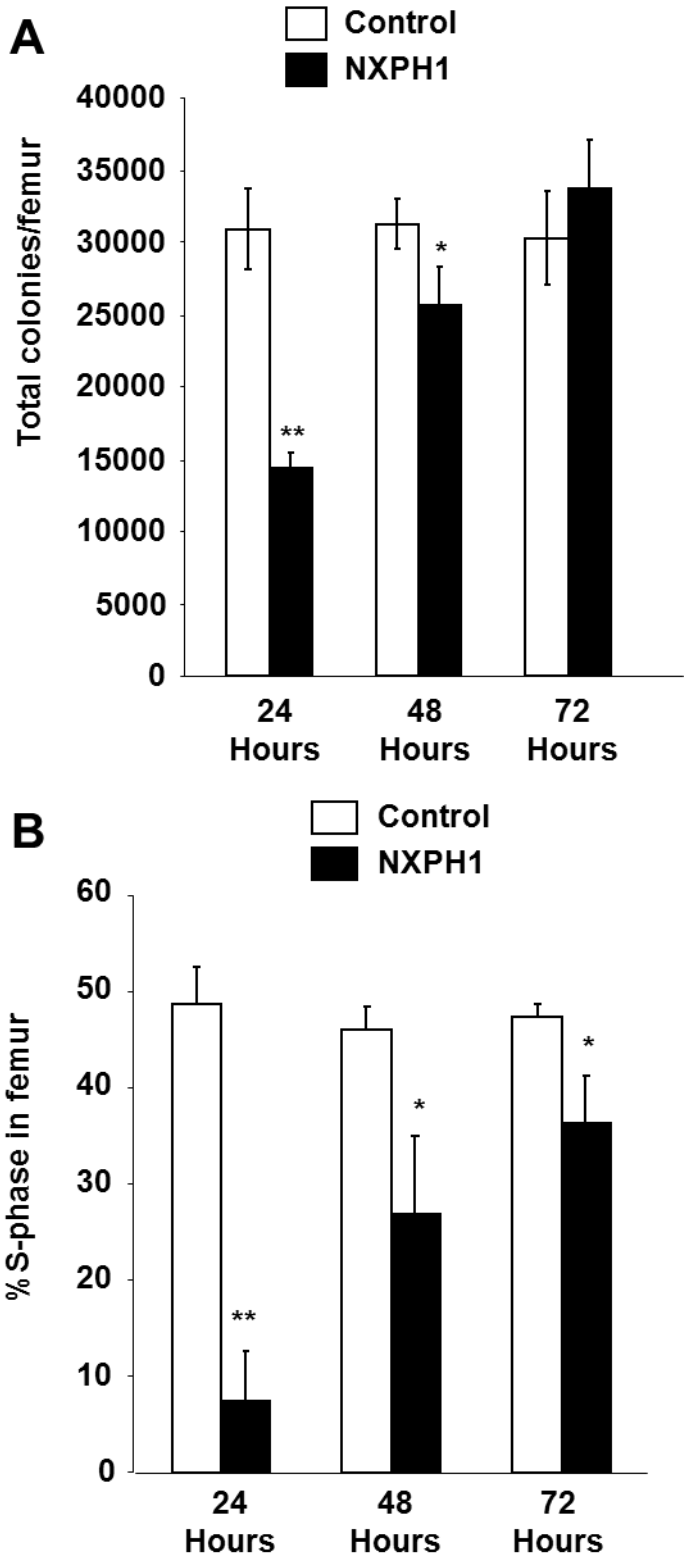

A
Control
NXPH1
Total colonies/femur
40000
35000
30000
25000
20000
15000
10000
5000
0
**
*
24 Hours
48 Hours
72 Hours
B
Control
NXPH1
% S-phase in femur
60
50
40
30
20
10
0
**
*
*
24 Hours
48 Hours
72 Hours

Figure 19: Recombinant NXPH1 decreases the absolute number and cycling status of muBM HPCs in a time-dependent manner.

A: Absolute colony number of muBM-derived HPCs 24, 48, and 72 hours after exposure to DPBS carrier or 5μg/mouse NXPH1 (combined data of three independent experiments each performed in triplicate; mean±SD).

B: Cycling status of muBM-derived HPCs 24, 48, and 72 hours after exposure to DPBS carrier or 5μg/mouse NXPH1 (combined data of three independent experiments each performed in triplicate; mean±SD).

*$p<0.05$, **$p<0.005$

Figure 20:

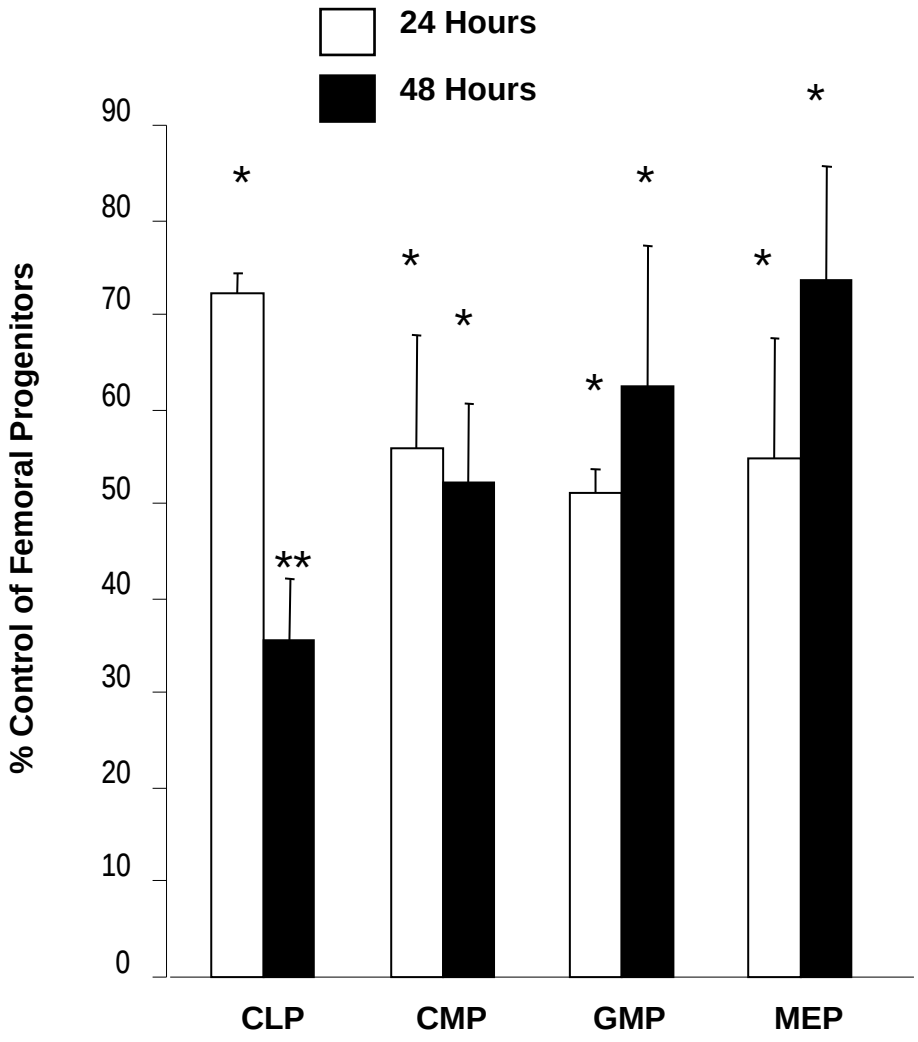

24 Hours
48 Hours
% Control of Femoral Progenitors
90
80
70
60
50
40
30
20
10
0
*
**
*
*
*
*
*
*
CLP
CMP
GMP
MEP

Figure 20: Recombinant NXPH1 decreases phenotypically-defined progenitor cells in a time dependent manner.

Change in phenotypically defined progenitor populations 24 hours after intravenous exposure to DPBS carrier or 5μg/mouse NXPH1 expressed as a percent control (combined data of three independent experiments each performed in triplicate; mean±SD).

*$p<0.05$, **$p<0.005$.

Figure 21:

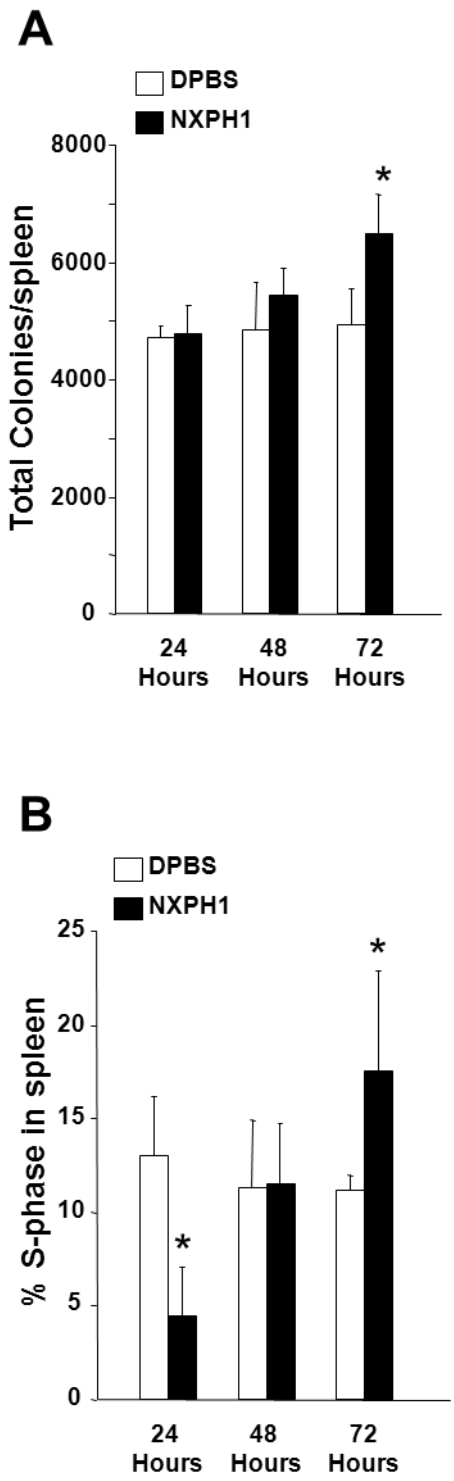

A
DPBS
NXPH1
Total Colonies/spleen
8000
6000
4000
2000
0
*
24 Hours
48 Hours
72 Hours
B
DPBS
NXPH1
% S-phase in spleen
25
20
15
10
5
0
*
*
24 Hours
48 Hours
72 Hours

Figure 21: Recombinant NXPH1 has fewer effects on splenic HPCs *in vivo*.

A: Absolute colony number of spleen-derived HPCs 24, 48, and 72 hours after exposure to DPBS carrier or 5μg/mouse NXPH1 (combined data of three independent experiments each performed in triplicate; mean±SD).

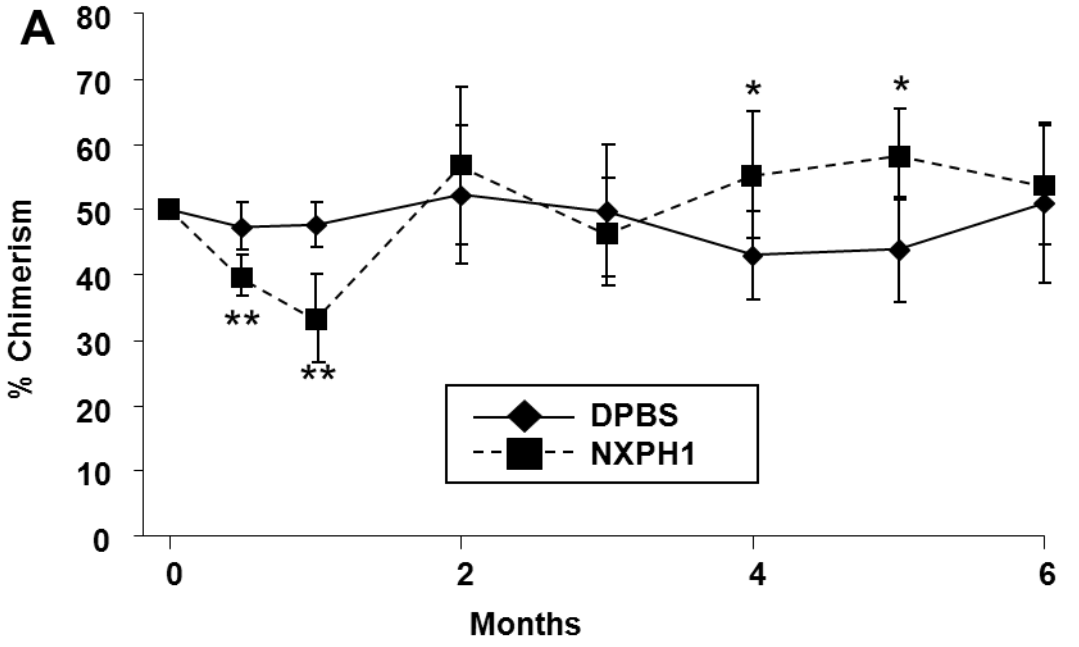
B: Cycling status of spleen-derived HPCs 24, 48, and 72 hours after exposure to DPBS carrier or 5μg/mouse NXPH1 (combined data of three independent experiments each performed in triplicate; mean±SD).

*$p<0.05$

Primitive HSCs are bound to DAG1 *in vivo*

To determine effects of NXPH on functional HSCs, C57Bl/6 mice ($CD45.2^+$) were intravenously injected with either 5μg recombinant NXPH1 or DPBS carrier. Bone marrow was collected from these treated mice after 24 hours, and these cells combined at equal cell numbers with untreated competitor BM cells from BoyJ mice ($CD45.1^+$). Lethally irradiated C57/BoyJ F1 mice had their hematopoietic system reconstituted using these cells. Donor cells from NXPH1-treated mice manifested significantly decreased short-term repopulation potential, balanced by a transient increased in intermediate repopulation potential (Figure 22). After seven months, no effect on long term competitive repopulation potential was observed during the primary transplant (Figure 23A). To further characterize the effect of NXPH1 on the LTR-HSC compartment, $2x10^6$ total BM cells from the primary recipients were transfused into a second set of lethally irradiated C57/BoyJ F1 mice. After four months, the secondary recipients were sacrificed and the percent chimerism in the total BM as well as specific lineage populations was examined (Figure 23B). Consistent with the data from month seven of the primary transplant, no difference was found between the experimental condition and control. This suggested that NXPH1 does not have an effect on the long-term repopulating potential of primitive HSCs.

Figure 22:


A
% Chimerism
Months
DPBS
NXPH1
0
10
20
30
40
50
60
70
80
0
2
4
6
**
**
*
*

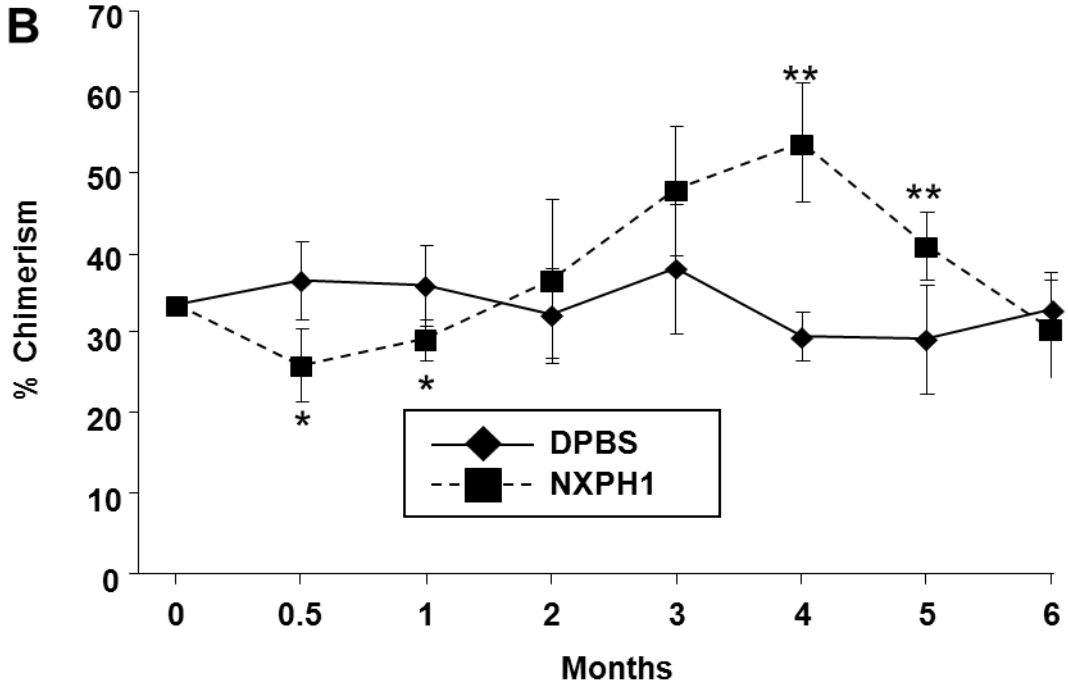

B
% Chimerism
Months
DPBS
NXPH1
0
10
20
30
40
50
60
70
0
0.5
1
2
3
4
5
6
*
*
**
**

Figure 22: Pre-treatment of donor animals with recombinant NXPH1 has different effects on STR- ITR- and LTR-HSCs.

Peripheral blood chimerism of donor cells derived BM from pool of 3 mice per treatment group combined with BoyJ competitor cells 24 hours after treatment of mice with DPBS carrier or 5μg/mouse recombinant NXPH1 transplanted into 5 treatment groups. (mean±SD).

A: 1:1 competitor:donor cell ratio

B: 0.5:1 competitor:donor cell ratio

*$p<0.05$, **$p<0.005$.

Figure 23:

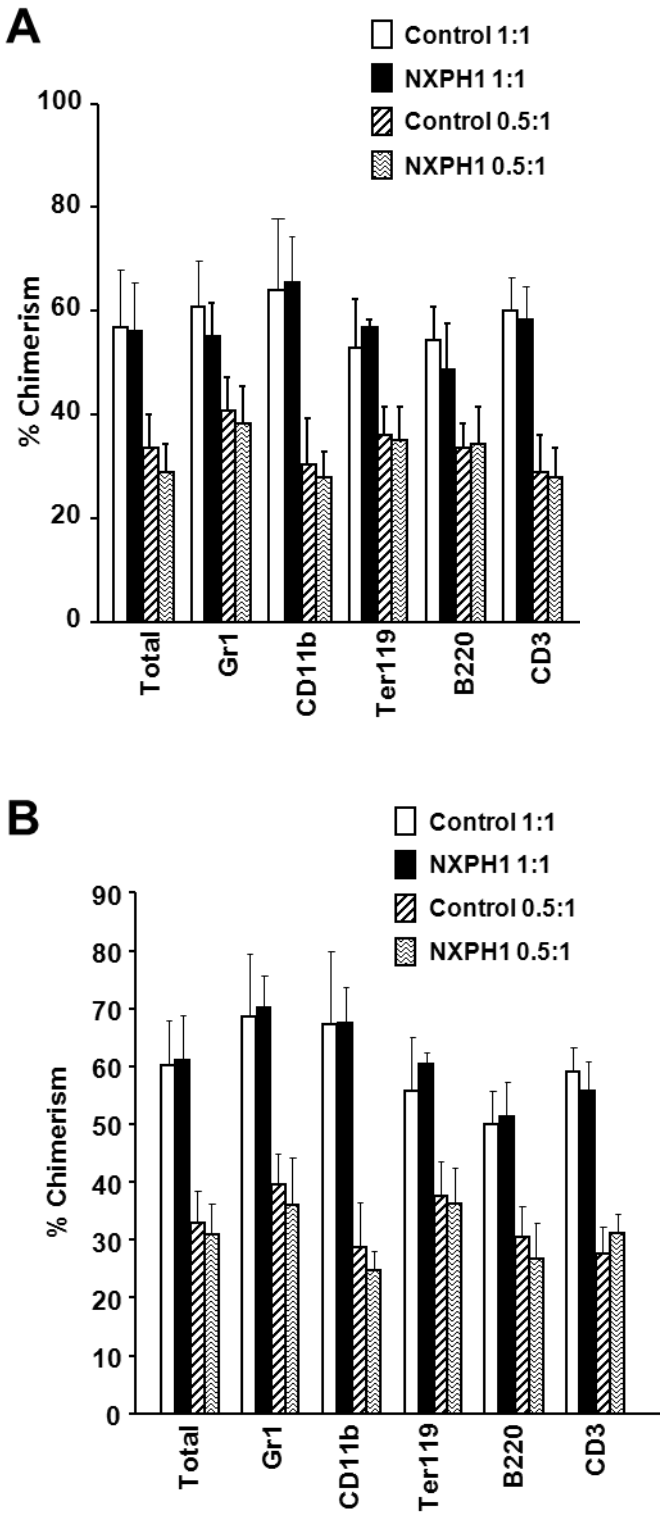

A
Control 1:1
NXPH1 1:1
Control 0.5:1
NXPH1 0.5:1
% Chimerism
100
80
60
40
20
0
Total
Gr1
CD11b
Ter119
B220
CD3
B
Control 1:1
NXPH1 1:1
Control 0.5:1
NXPH1 0.5:1
% Chimerism
90
80
70
60
50
40
30
20
10
0
Total
Gr1
CD11b
Ter119
B220
CD3

Figure 23: Pre-treatment of donor mice with recombinant NXPH1 does not affect long-term engraftment.

A: Percent chimerism of donor cells in total BM, as well as several lineage restricted populations in primary recipients after seven months. In the competitive repopulation assay, pooled BM from 3 C57Bl/6 mice per treatment group was combined either 1:1 or 0.5:1 with BoyJ competitor cells 24 hours after treatment of mice with DPBS carrier or 5μg/mouse recombinant NXPH1 and transplanted into 5 mice per treatment group (mean±SD).

B: Percent chimerism in total BM, as well as several lineage restricted populations in secondary recipients derived from either 1:1 or 0.5:1 primary competitive repopulation assay. After seven months, 5 secondary recipients per treatment group were transfused with pooled BM from at least 4 members of the corresponding primary transplant group. Four months after the secondary transplant, BM was harvested and analyzed for percent chimerism was analyzed (mean±SD).

We had demonstrated using single cell culture and DAG1 blocking antibodies that DAG1 counteracts the suppressive effects of NXPH1 on HPCs *in vitro*. As absolute numbers of femoral HPCs are suppressed by *in vivo* administration of NXPH1 while the long-term repopulating potential of primitive HSCs was unaffected, we hypothesized that only primitive HSCs are bound to DAG1 *in vivo*. To test this, muBM cells harvested from control and NXPH1-treated mice were treated with DAG1 blocking antibody prior to *in vitro* culture in presence of NXPH1. If HPCs were influenced by DAG1 *in vivo*, HPCs removed from mice exposed to NXPH1 *in vivo* ought to be further inhibited by anti-DAG1+NXPH1 *in vitro*. Conversely, if HPCs are not influenced by DAG1 *in vivo*, *in vitro* exposure to anti-DAG1+NXPH1 ought not be inhibitory, due possibly to either maximal inhibition or receptor down-regulation. We found no further inhibition of HPCs after *in vitro* exposure to anti-DAG1+NXPH1, supporting the hypothesis that HPCs are not likely influenced by DAG1 *in vivo* (Figure 24).

To determine which populations were affected by NXPH1 *in vivo*, phenotypically-defined BM HSCs/HPCs were examined 24 and 48 hours after mice were injected *i.v.* with recombinant NXPH1. Consistent with HPC data, STR-HSCs were suppressed at both time-points. Cells categorized in the longer-term population of HSCs experienced significant increase in numbers after 24 hours, but had returned to base-line after 48 hours (Figure 25).

Figure 24:

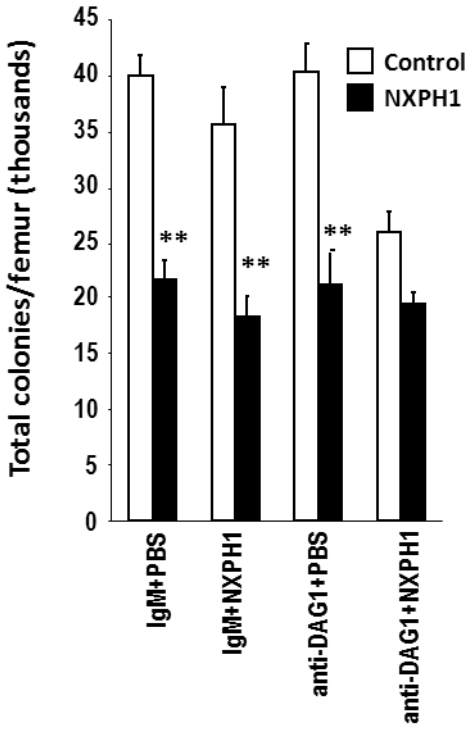

Total colonies/femur (thousands)
45
40
35
30
25
20
15
10
5
0
Control
NXPH1
**
**
**
IgM+PBS
IgM+NXPH1
anti-DAG1+PBS
anti-DAG1+NXPH1

Figure 24: Murine BM HPCs are not exposed to DAG1 *in vivo*.

Effect of *in vitro* exposure to recombinant NXPH1 on muBM total CFUs derived from mice treated with recombinant NXPH1 *in vivo.* Mice were *i.v.* injected with either DPBS carrier or 5µg recombinant NXPH1. Twenty-four hours after *in vivo* exposure, BM was harvested and treated with either mouse IgM or anti-DAG1 blocking antibody *in vitro* and then plated in SCF, GM-CSF, PWMSCM and Epo ± 200ng/ml recombinant NXPH1 (combined data of three animals each analyzed in triplicate; mean±SD).

**$p<0.005$

Figure 25:

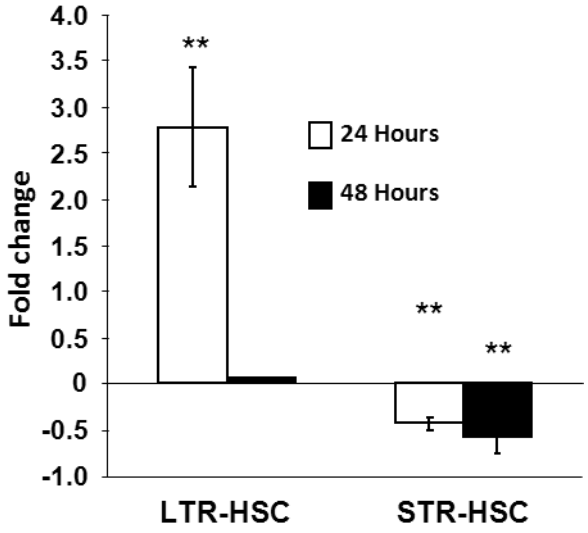

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
-1.0
Fold change
**
**
**
24 Hours
48 Hours
LTR-HSC
STR-HSC

Figure 25: Absolute numbers of primitive HSCs are transiently increased in mice exposed to recombinant NXPH1 *in vivo*.

Fold change *in vivo* of phenotypically-defined HSCs 24 and 48 hours after injection of 5μg/mouse recombinant NXPH1 (combined data from two independent experiments performed in triplicate; mean±SD).

**p<0.005

To further investigate this, normal C57Bl/6 mice underwent the same injection procedure, but with addition of BrdU. BrdU was co-injected with recombinant NXPH1 and placed in drinking water after mouse exposure to NXPH1. Twenty-four hours after exposure of mice to NXPH1 or control media, BM cells from treated and untreated mice were sorted into: KSL $CD34^-$ (c-kit^+, $sca1^+$, $lineage^-$ $CD34^{lo/-}$) and KSL $CD34^+$ cells. Mirroring functional and phenotypic data, KSL $CD34^+$ cells experienced a 38% decrease in percentage of cells in S-phase, while KSL $CD34^-$ cells from NXPH1-treated mice had a 3.7 fold increase in cycling cells (Figure 26A). In both conditions, the cells entering S-phase appeared to come from cells in G_1/G_0 as a corresponding decrease in total percentage was observed in these populations suggesting cell-cycle arrest. Because cell cycle arrest is often associated with apoptosis, we examined extracellular Annexin V expression on the phenotypically defined populations in treated and untreated mice; however no increase in apoptosis was observed in the treated mice (data not shown).

There are two possible interpretations for increases in numbers and cycling status of phenotyped LTR-HSCs: either NXPH1 directly induces cycling, or cycling is perhaps induced to re-assert homeostasis challenged by inhibitory effects of NXPH1 on HPCs. In the hematopoietic system, the mTOR pathway is involved in stress responses (Campbell, Basu et al. 2009) consistent with the second interpretation, so cycling status of both the LTR- and STR-HSCs was examined in mice treated in the same manner as above but with *i.p.* injection of Rapamycin or ethanol-carrier immediately prior to NXPH1 *i.v.* injection. Exposure of mice to Rapamycin inhibited increases in cycling of KSL $CD34^-$ cells while

Figure 26:

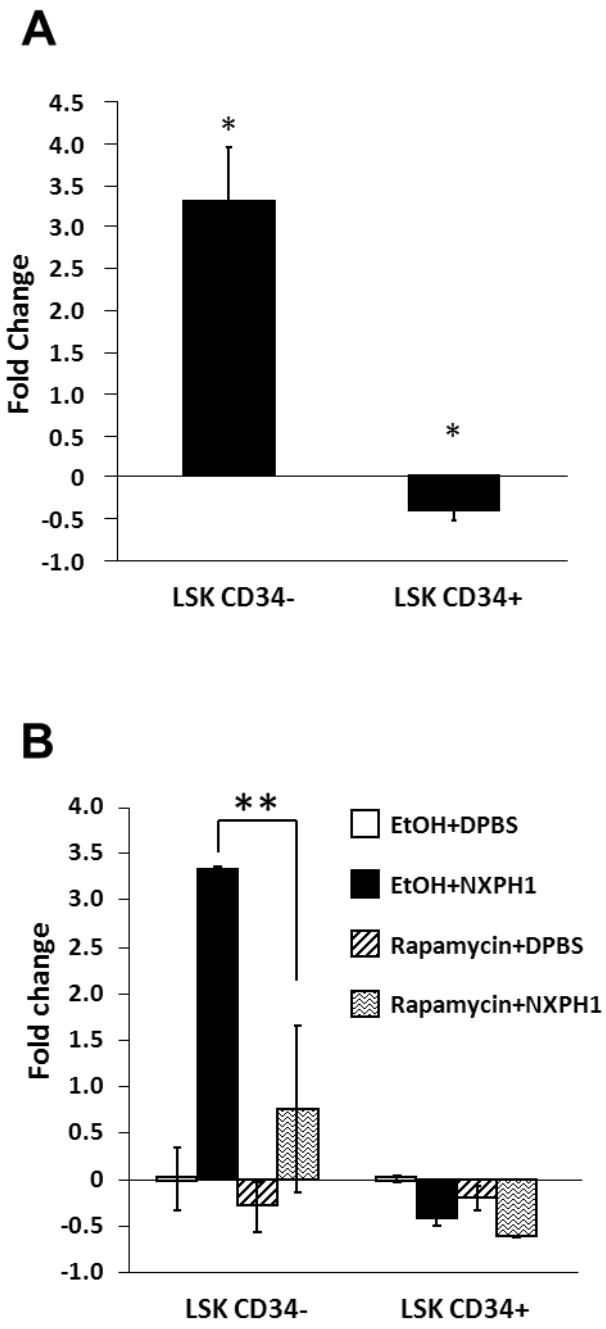

A
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
-1.0
Fold Change
*
*
LSK CD34-
LSK CD34+
B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
-1.0
Fold change
**
EtOH+DPBS
EtOH+NXPH1
Rapamycin+DPBS
Rapamycin+NXPH1
LSK CD34-
LSK CD34+

Figure 26: The increase in primitive HSC in response to recombinant NXPH1 is homeostatic in nature.

A: Fold change in cycling status of populations heavily enriched for long (LSK $CD34^-$) or short (LSK $CD34^+$) term engrafting HSCs 24 hours after injection of 5μg/mouse recombinant NXPH1(combined data from two independent experiments performed in triplicate; mean±SD).

B: Fold change in phenotypically defined HSCs 24 hours after 5μg/mouse *i.v.* recombinant NXPH1 exposure and 5mg/mouse *i.p.* rapamycin injection (combined data from two independent experiments performed in triplicate; mean±SD).

*$p<0.05$, **$p<0.005$

leaving KSL $CD34^+$ cells unaffected (Figure 26B). Because our results show that mTOR is at least partially involved in stimulation of LTR-HSCs in response to NXPH1 exposure *in vivo*, but is not involved in NXPH1-mediated inhibition of STR-HSCs, and because induction of the mTOR pathway in LTR-HSCs has been associated with a homeostatic stress response (Campbell, Basu et al. 2009), we believe that LTR-HSCs are not directly affected by NXPH1 and the observed increase in phenotypically defined HSCs is due to a homeostatic response mediated at least partially through the mTOR pathway.

Results summary

A signaling axis centered on the second LNS domain of NRXN1α is present and functional in the hematopoietic system. High expression levels of NRXN1α and DAG1 are found in primitive hematopoietic populations but not mature hematopoietic populations. Therefore, expression of NRXN1α and DAG1 correlates with primitive hematopoietic populations in both huCB and muBM (Figure 3, Table 2) and NXPH is found in high concentrations in huCB plasma but not the plasma of adult humans or mice. Having demonstrated their presence, the next step was to define their function *in vitro* and *in vivo*.

In vitro, recombinant NXPH1 was shown to have a directly inhibitory effect on HPC proliferation (Figures 4-8, 14); however NXPH1 activity was allosterically inhibited by

endogenous DAG1 (Figure 16) thus, with one exception, NXPH1 inhibitory activity was observed on either individually plated cells (Figures 6-8) or cells exposed to anti-DAG1 blocking antibodies (Figure 5). The lone exception was the ability of NXPH1 to inhibit the proliferation of HPCs stimulated by GM-CSF+SCF which was observed under all conditions tested (Figures 4, 5, 7, 9, 12, 14). Because GM-CSF maintains NRXN1α expression (Figure 15) and DAG1 expression decreases as cells differentiate (Figure 3), we hypothesize that the high degree of proliferation stimulated by GM-CSF+SCF alters the ratio of NRXN1α:DAG1 in such a manner as to allow NXPH1 to interact with some, though not all, HPCs and thereby elicit a significant degree of observed inhibition (Figure 16). The *in vitro* interaction between endogenous NRXN1α and DAG1 with recombinant NXPH1 is diagrammed in Figure 27A.

Mirroring the *in vitro* results, *in vivo* exposure to recombinant NXPH1 has a suppressive effect on murine hematopoiesis. NXPH1 decreased the absolute numbers of mature hematopoietic cells in the peripheral blood (Figure 17) as well as both the absolute numbers and cycling status of muBM HPCs (Figures 18-20) in a time- and dose-dependent manner. Intriguingly, the absolute number and cycling status of HSCs was increased after NXPH1 injection (Figures 25 and 26A). Because endogenous DAG1 is able to antagonize NXPH1-mediated inhibition *in vitro* (Figure 5), we hypothesized that HSCs but not HPCs were bound to DAG1 *in vivo* and the observed increases were a homeostatic response. The involvement of the mTOR pathway in the behavior of HSCs but not HPCs (Figure 26B) supports the hypothesis that response of HSCs to NXPH1

injection is homeostatic in nature. Likewise, the inability of HPCs from mice injected with NXPH1 to be further inhibited by NXPH1 *in vitro* suggests that HPCs are not bound to DAG1 *in vivo* (Figures 23 and 24). Immunohistochemistry further supports the hypothesis that HSCs are bound to DAG1 *in vivo* (Figure 13) where NRXN1α-expressing stromal cells are found in the vicinity of DAG1-expressing osteoblasts. The *in vivo* interaction between endogenous NRXN1α and DAG1 with recombinant NXPH1 is diagrammed in Figure 27B.

The characterization of a heretofore unrecognized signaling axis in the hematopoietic system has a variety of implications which should be considered.

Figure 27:

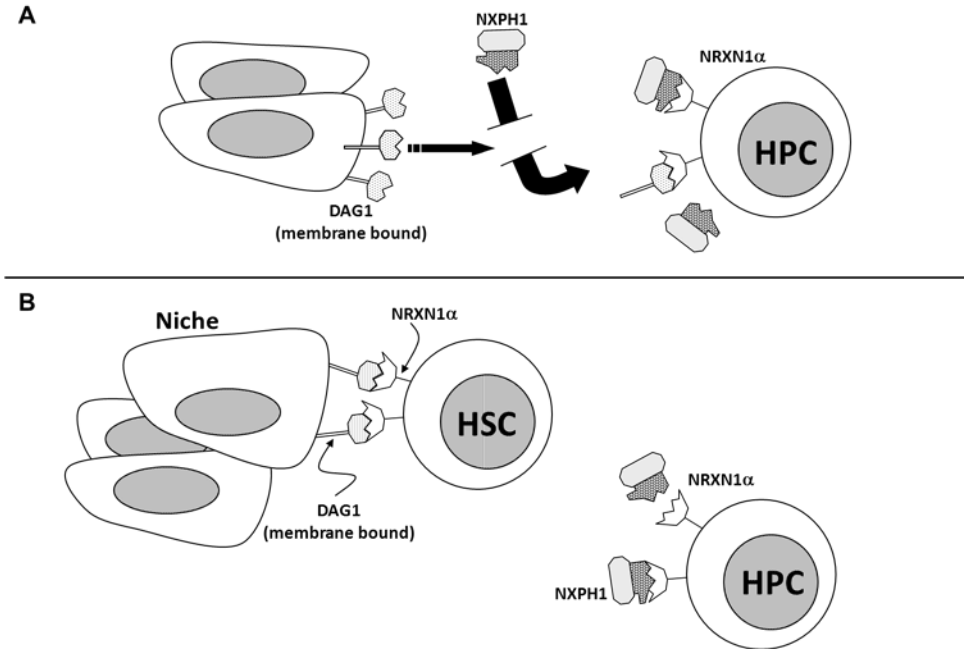

A
NXPH1
NRXN1α
HPC
DAG1
(membrane bound)
B
Niche
NRXN1α
HSC
DAG1
(membrane bound)
NRXN1α
NXPH1
HPC

Figure 27: Diagrammatic role of NRXN1α, DAG1, and NXPH1 in the hematopoietic system.

A: NRXN1α, DAG1 and recombinant NXPH1 as they behave in the hematopoietic model *in vitro*.

B: Hypothetical interaction of NRXN1α, DAG1 and recombinant NXPH1 *in vivo*.

Discussion

A signaling axis centered on the second LNS domain of NRXN1α is present and functional in the hematopoietic system. Expression of NRXN1α correlates with primitive hematopoietic populations, while DAG1 is heavily expressed on active osteoblasts in the bone marrow and is also present on many hematopoietic cells. The third member of the axis, the NXPH family, is found in huCB plasma and within lineage positive cells in the mouse spleen. We found that recombinant NXPH1 acts as a potent inhibitor of HPC proliferation *in vivo* and *in vitro*; however this inhibition is allosterically inhibited by the presence of DAG1. We suggest that NXPH directly acts to suppress hematopoiesis, and a role of DAG1 is to occupy NRXN1α, thereby preventing excess inhibition.

Physiological role of the NRXN1α axis in hematopoiesis

The role of this axis in healthy animals remains unclear; however, human disease models involving disrupted members of this signaling axis and a hematopoietic phenotype may provide insight. While the precise cause of schizophrenia in humans remains unknown one, of four conditions usually applies: disruption of NRXN1 (Kirov et al., 2008), disruption of Lin2 (Kristiansen, Beneyto et al. 2006)– the protein directly downstream of NRXN1, over-expression of IL-3 (Chen, Wang et al. 2007; Xiu, Chen et al.

2008), or an overactive IL-3R (Chen, Wang et al. 2007; Lencz, Morgan et al. 2007; Sun, Wang et al. 2008; Sun, Wei et al. 2009). We have shown that IL-3 downregulates NRXN1α expression *in vitro*, so it seems reasonable to suggest that under conditions pertaining to schizophrenia, NRXN1α is either absent or non-functional. Schizophrenics have a higher number of leukocytes, especially monocytes and neutrophils as compared to normal individuals (Wilke, Arolt et al. 1996). While the lymphocyte levels remain normal, a higher percentage of them are in S-phase (Malacarne and Dallapiccola 1969) and they have an abnormal ratio between T-helper types 1 and 2 (Wilke, Arolt et al. 1996; Monji, Kato et al. 2009). This hematopoietic phenotype resembles the hematopoietic phenotype of autistics, so the relevance of both autism and schizophrenia to the research presented in this thesis will be presented together after the hematopoietic phenotype of autism has been described..

NRXN1α also appears to play an important role in autism spectrum disorder, as ultra-rare cohorts of particular missense mutations and splice variants have been associated with the disease (Kim, Kishikawa et al. 2008; Glessner, Wang et al. 2009); disruption of NRXN1α due to chromosomal breakage has also been linked to autism (Kim, Kishikawa et al. 2008). Interestingly, the chromosomal breakage that disrupted NRXN1α appeared to leave NRXN1β unaffected and while some correlation between specific missense mutations in NRXN1β and autism have been suggested (Feng, Schroer et al. 2006; Kim, Kishikawa et al. 2008); however family data suggests that incomplete penetrance occurs

(Yan, Noltner et al. 2008). Similar to schizophrenia, autism is associated with high monocyte counts; additionally, autistics have an abnormal CD4:CD8 T-cell ratio (Sweeten, Posey et al. 2003).

The hematopoietic phenotypes of schizophrenics and autistic neatly mirrors that of mice intravenously injected with NXPH1. Schizophrenics and autistics exhibit increased numbers and cycling status of many hematopoietic populations; whereas mice intravenously injected with NXPH1 have both a decreased total leukocyte number and a higher percentage of non-cycling progenitors. The absence of the inhibitory effect of NXPH1 in schizophrenics and autistics due to the absence of its receptor could explain the myeloid phenotype. The explanatory power of this model in the lymphoid compartment is weaker, as one would expect a higher number of lymphoid cells in patients with these diseases based off the mouse data (Coffman and Weissman 1981; Muller, Riedel et al. 2000; Monji, Kato et al. 2009; Stefansson, Ophoff et al. 2009; Enstrom, Onore et al. 2010). While the expected phenotype is not observed, both diseases are strongly pro-inflammatory. The hematopoietic exuberance of these diseases coupled with the inhibitory effect of recombinant NXPH1 injected *in vivo* argues in favor of the hypothesis that the primary role of this axis is to suppress hematopoiesis.

The question is whether the observed inhibition is due to presence of recombinant NXPH1 or to absence of DAG1 on cells expressing NRXN1α. Previous work in the neuronal system (Sugita, Saito et al. 2001) as well as the similar behavior between recombinant NXPH1 and DAG1-blocking antibodies on cells exposed to GM-CSF+SCF favor the former interpretation. However, there are problems with this model. Were DAG1 required for survival, one would expect to observe a decrease in the colony forming ability of individually plated progenitors and progenitors exposed to anti-DAG1 blocking antibody. With the exception of the aforementioned GM-CSF+SCF condition, a decrease is not observed. Additionally, DAG1 is widely expressed in a variety of tissues *in vivo*, yet *in vivo* administration of NXPH exhibits an inhibitory effect consistent with *in vitro* data arguing that HPCs are not bound to DAG1 *in vivo*. Lastly, recombinant NXPH1 cannot bind NRXN1α in the presence of DAG1 due to non-competitive antagonism making it extremely unlikely that NXPH could disrupt the DAG1-NRXN1α bond. Taken together with the hematopoietic phenotypes of schizophrenia and autism, this data argues that the NRXN1α axis is suppressive in nature and that NXPH binding inhibits proliferation.

A most interesting member of this signaling axis may be the NXPH-family, particularly NXPH1. When injected *in vivo* recombinant NXPH1 acts in a myelo- and lymphosuppressive manner, decreasing absolute numbers and cycling status of muHPCs, as well as circulating populations of more mature hematopoietic cells. In

contrast, absolute numbers and cycling status of phenotypically defined HSCs was increased after recombinant NXPH1 administration. The rapamycin sensitive nature of these increases suggests that HSCs are not directly induced by NXPH1, and the increase may be a homeostatic response to replenish the affected populations below in the hematopoietic hierarchy. Since many populations downstream in the hematopoietic hierarchy are affected by recombinant NXPH1 administration, unaffected upstream populations are called upon to maintain homeostasis and transiently leave their quiescent state. The absolute numbers of phenotypically defined HSCs are increased after 24 hours and have returned to baseline after 48 hours. The absolute numbers of functionally defined HPCs are greatly reduced after 24 hours, slightly depressed after 48 hours, and fully recovered after 72 hours. In light of the hematopoietic hierarchy where a small number of primitive cells differentiate into a large number of mature cells, the transient nature of this increase coupled with a restoration of normal BM HPC populations shortly thereafter argues in favor of a homeostatic response. Transplant engraftment studies in which competitive repopulation ability of BM cells taken from NXPH1-treated mice is only transiently effected suggest that the true LTR-HSC compartment may be unaffected by the increase observed within the phenotypically defined population. Another interpretation of the data consistent would be that an as-of-yet unrecognized population of HS/PCs transiently adopts a set of immunohistological markers similar to LTR-HSCs as they respond to a hematopoietic challenge. Either way, these results suggest that LTR-HSCs may exist in a privileged state, unaffected by NXPH1. Since NRXN1α is present at the cell surface of

phenotypically-defined LTR-HSCs, this privileged state may reflect two scenarios: either NXPH1 binds NRXN1α and the message is not conveyed due to downstream elements or NXPH1 cannot bind NRXN1α. The *in vitro* antagonism of DAG1 on NXPH1-mediated inhibition may shed light on which of these interpretations better model the system. With the exception of GM-CSF+SCF, the presence of endogenous DAG1 is sufficient to prevent NXPH1-mediated inhibition *in vitro*. Furthermore, the efficacy of NXPH1 *in vivo* (I_{max}=58.2±4.5 % inhibition) is significantly greater than with GM-CSF+SCF *in vitro* (I_{max}=43.7±2.5 % inhibition). The activity of HPCs exposed to NXPH1 *in vivo* most closely resemble HPCs exposed to NXPH1 in the absence of DAG1 *in vitro*. In contrast, phenotypically-defined HSCs are not inhibited by *in vivo* administration of NXPH1; instead they proliferate in what may be a homeostatic response. While indirect elements are almost certain, we feel that it is reasonable to hypothesize that primitive HSCs may be bound to DAG1 in the niche, whereas less primitive HSCs and HPCs tend not to be bound to DAG1 *in vivo*. This hypothesis is supported by the high expression level of DAG1 on active osteoblasts coupled with the close proximity of NRXN1α expressing hematopoietic stromal cells in the BM to DAG1 expressing osteoblasts. From this we propose a model (diagrammed in Figure 27), suggesting that the role of DAG1 in hematopoiesis is to “protect” primitive hematopoietic cells from exposure to members of the NXPH-family.

The next question is to what end this protection and inhibition serve. Under normal conditions the only hematopoietic tissues where NXPH-family members were identified are the spleen and umbilical CB. Both of these tissues have a relatively quiescent hematopoietic compartment and their HPCs exhibit a low level of cycling. This phenotype is consistent with the observed effects of recombinant NXPH1. These data suggest that NXPH plays a role in maintaining the quiescent nature of HPCs in these tissues. If NXPH is involved in suppressing the proliferation of hematopoietic cells in the spleen, it would be necessary for this effect to be reversible during extramedullary hematopoiesis. An interesting candidate for this role is IL-3. A pro-inflammatory cytokine, IL-3 is expressed during periods of hematopoietic stress such as would precede extramedullary hematopoiesis in the spleen. Additionally, splenic cells induced to proliferate by pokeweed mitogen produce high levels of IL-3. As IL-3 downregulates NRXN1α, HPCs would be unable to respond to NXPH and thus proliferate.

Another potential explanation would be survival-dependent selection of primitive hematopoietic cells. It is intuitively appealing to think that in the initial stages of life HSCs undergo a strong selective process thereby protecting the organism against variety of potential hematopoietic disorders. If life-long, true, HSCs do originate in the placenta, the umbilical cord might be the location of this selection. Likewise, splenic HSCs remain quiescent until called upon in extreme situations. While the specifics of the challenge that awakens splenic HS/PCs are varied, the desperate nature of

extramedullary hematopoiesis may demand a similar selective process. This thesis provides no conclusive data in favor of this hypothesis; however, DAG1 has been shown to play a role in the positive selection of T-cells (Zhang, Wang et al. 2006), so any interpretation dismissing the potentially selective role of the NRXN1α axis in hematopoiesis is premature.

Of particular interest is the relationship between NXPH and huCB. Unlike myelosuppressive chemokines (Lu, Xiao et al. 1993), recombinant NXPH1 is able to suppress the colony forming ability of HPCs from freshly isolated huCB. Additionally, we found high concentrations of NXPH-family members in huCB plasma, but not in adult human and murine peripheral blood. Taken together, these findings may be of some meaning in the context of huCB transplantation. While offering many advantages as compared to other sources of HSCs, reconstitution time is longer in patients who have received huCB transplants. It is possible, but not yet determined that members of the NXPH-family may play a role in the engraftment time of huCB cells in a clinical context.

Which cells produce NXPH in the spleen and umbilical CB remains unknown. Microarray data from other laboratories present macrophages as a potential candidate (Rodriguez, Chang et al. 2004). This does not contradict our finding significant intracellular expression of NXPH in the lineage positive fraction of splenocytes as well as splenic CMPs. If true, macrophages as a source of NXPH may be of relevance to the

hematopoietic niche (Chow, Lucas et al. 2011), a possibility whose relevance will be further detailed in future sections.

Thus, the role of the NRXN1α axis in hematopoiesis appears to be suppressive. The inhibitory effect of recombinant NXPH1 on HPC proliferation both *in vivo* and *in vitro* coupled with the high proliferation and pro-inflammatory phenotype of diseases where NRXN1α is absent argues that the primary actor in this axis is the NXPH-family. The other ligand, DAG1, does not have an effect of its own; rather it serves as an endogenous antagonist of NXPH-mediated inhibition. The suppressive effect of NXPH serves to maintain the quiescent state of HPCs in tissues such as cord blood and the spleen. In the spleen, NXPH keeps HPCs in reserve until such a time when they are needed when they are activated by IL-3.

The role of the NRXN1α axis in the hematopoietic niche

In contrast to umbilical cord blood and the spleen, members of the NXPH family were not identified in the bone marrow compartment. However, the expression of DAG1 on active osteoblasts coupled with the potential co-localization of NRXN1α-expressing cells to that site may have interesting implications for the hematopoietic niche. While active osteoblasts do not mark the true hematopoietic niche, important information on the

true niche can be garnered from the osteoblastic niche (Mendez-Ferrer, Michurina et al. 2010; Chow, Lucas et al. 2011). In this case, the localization of phenotypically primitive hematopoietic cells to osteoblasts suggests that DAG1-NRXN1α interaction may play a role in niche function.

In the neuronal system, NRXN1 serves as a mediator between GABAergic and adrenergic signaling and is involved in the formation of the GABAergic synapse. The implications of NRXN1α as a mediator between GABAergic and adrenergic signaling are great when the nature of the true hematopoietic niche is considered. In the true bone marrow niche, HSC activity hinges on an MSC and a beta-adrenergic neuron, with an osteoblast and a macrophage serving as intermediaries. As noted previously, MSCs bear a great deal of similarity to post-synaptic GABAergic neurons and are able to compliment them in brain damaged mice. In the hematopoietic system, GABA functions as a chemoattractant and cell cycle inhibitor for primitive hematopoietic cells, likewise it inhibits the activity of known mobilizing agents such as SDF1α. These properties describe aspects of what one would expect to find in the hematopoietic niche: an attractant for homing, a cell cycle inhibitor to keep HSCs in a quiescent state, and an inhibitor of mobilization to keep cells within the niche. However, in order for HSCs to function in a dynamic system HSCs must also be able to leave the niche and proliferate, both of which are induced by adrenergic signaling. This creates the opportunity for a multifaceted system. In this system, NRXN1α present on the HSC is able to mediate between the GABAergic MSC and beta-

adrenergic neuronal signaling, thereby providing a bridge between properties necessary for HSC maintenance and maintenance of a functional hematopoietic system. This network is further refined by DAG1 expressed on the osteoblast, which serves a scaffolding function maintaining the HSC in place so that it can mediate between excitatory and inhibitory signaling. Lastly, the NXPH-secreting macrophage is able to keep the system in check by inducing a quiescent state in precocious HSCs. While highly speculative, this model is presented as a vehicle for future discovery.

For example, the instructive role of NRXN1 in GABAergic synapse development may be of relevance to the niche. Because the bone marrow niche is not the site of hematogenesis, during development HSCs must migrate to the bone marrow niche where a hematopoietic synapse is formed between an HSC, an MSC, an osteoblast, as well as a beta-adrenergic neuron and a macrophage. In the neuronal system, activation of NRXN1 is sufficient for GABAergic synapse development. The cell expressing NRXN1 will differentiate into the pre-synaptic cell while the other will differentiate into the post-synaptic cell. The resemblance of MSCs to post-synaptic GABAergic neurons creates the exciting possibility that NRXN1 on HSCs plays an important role in the creation of the hematopoietic synapse. If true, NRXN1 present on freshly generated HSCs could induce MSCs to produce part of the niche environment. This would also help explain the similarities between niche MSCs and GABAergic neurons as their

development is instructed by the same signaling axis. Furthermore, DAG1 expressed on the osteoblast may serve a scaffolding function, helping recruit the necessary factors.

Our results present the possibility that the NRXN1α axis may be important in the hematopoietic niche. Expression of NRXN1α on hematopoietic cells correlates with a primitive phenotype. In the bone marrow stroma, cells expressing NRXN1α are in close proximity to active osteoblasts expressing DAG1. While we recognize that osteoblasts are an imperfect model for the true hematopoietic niche, these results present the possibility that DAG1-NRXN1α interaction is involved in the hematopoietic niche. If true, DAG1 expressing osteoblasts may serve as scaffolds enabling the ability of NRXN1α, found on HSCs, to mediate between adrenergic and GABAergic signaling found in the niche. This may provide insight into the relationship between the HSC, the MSC, the beta-adrenergic neuron and the osteoblasts present in the niche. Additionally, the instructive role NRXN1 plays in GABAergic development may have implications on the development of the hematopoietic niche, especially as it applies to the MSC.

NRXN1α axis and disease

In addition to the conditions of schizophrenia and autism, the NRXN1α-axis may be of use in treating cancer. High levels of NXPH expression in myeloid leukemia (Knight, Skol

et al. 2009), neuroblastomas (Warnat, Oberthuer et al. 2007), papillary glioneuronal tumors (Faria, Miguens et al. 2008), as well as ovarian and breast cancer (Song, Ramus et al. 2009) correlates with a poor survival outcome. In fact, NXPH expression has been suggested as a prognostic factor in judging the severity of cancers (Kawasaki, OHIRA et al. 2005). The suppressive effect of recombinant NXPH1 on hematopoiesis may explain this correlation.

Our findings show that recombinant NXPH1 is a potent inhibitor of hematopoiesis. Neurexophilin secreted by cancers would inhibit HPCs and mature hematopoietic cells involved in the immune response. Because of this, we would expect cancers expressing high levels of NXPH to have enhanced immune system suppression and therefore evasion. However, in order for this explanation to work the cancer must not be affected by secreted NXPH. Cancers share many similarities to primitive stem cells, including HSCs and our results suggest that HSCs are not affected by NXPH-mediated inhibition. This presents the possibility that cancers are similarly not affected by NXPH.

Protection of cancer cells from NXPH-mediated inhibition could occur in two ways: either protection via DAG1 similar to HSCs or via downregulation of NRXN1α or other proteins downstream of the NXPH-NRXN1α interaction. The role of DAG1 in cancer argues against the former explanation. In nearly all human cancers analyzed, expression of DAG1 is reduced or absent (Cross, Lippitt et al. 2008). As DAG1 is not available to

occupy NRXN1α, it is likely that the advantages of NXPH-expression selects for cancer cells that do not express NRXN1α.

The loss DAG1 in cancer presents another way in which proteins involved in this thesis may be of clinical relevance. In addition to noted loss of DAG1 expression in cancer cells, restoring DAG1 expression in prostate carcinoma cell lines appears to reduce growth and invasiveness of the cancer (Sgambato, Camerini et al. 2007; Sgambato, De Paola et al. 2007). Because the role of DAG1 in cancer appears to involve a change in the relationship between the cancerous cell and the ECM, NRXN1α is likely not involved as DAG1 preferentially binds normal components of the ECM such as Argin and Perlecan. While beyond the scope of this thesis, further connections between DAG1 and cancer are of clinical interest.

The inhibitory effect of recombinant NXPH1 on hematopoiesis helps to explain the correlation between high NXPH expression in cancers and a negative prognosis. By secreting members of the NXPH family cancers are able to suppress the immune system and better evade destruction. However, in order for this to work cancers must be immune to the effect of NXPH. Because DAG1 is downregulated in most cancers, it is unlikely that cancers protect themselves from NXPH-mediated inhibition in the same manner as HSCs, rather it seems more likely that NRXN1α or some other factor downstream of NRXN1α is downregulated.

Members of the NXPH family may also be of interest to HIV research since immune system exhaustion is an important difference between HIV infection in humans and SIV infection in apes. One of the critical differences between disease progression of SIV and HIV is immune system exhaustion which occurs in the human but not the simian system; immune system exhaustion is associated with the onset of AIDS (Pandrea, Sodora et al. 2008). This is interesting because during immune system exhaustion in mice, the transcription level of NXPH2 is upregulated in macrophages (Rodriguez, Chang et al. 2004). Given the suppressive effect of NXPH1 on hematopoiesis, it is reasonable to suggest that NXPH family members play a role in immune system exhaustion. If true, inhibiting NXPH may serve to delay the onset of AIDS.

Application of results in the neuronal context

In a neuronal context, DAG1-NRXN1α interaction is mutually reinforcing in development of the neuronal synapse (Sugita, Saito et al. 2001). The hematopoietic system stands in contrast to this, where stimulation of DAG1 by recombinant NRXN1α has no effect on HPC proliferation. However, consistent with its hypothesized antagonistic role in neuropoiesis, NXPH1 acts in an inhibitory manner. *In vitro*, NXPH1 specifically inhibits proliferation of HPCs stimulated by GM-CSF+SCF when cells are plated in population.

However, when progenitor cells are either plated as a single cell in isolation, or in a population where DAG1 has been rendered inaccessible through anti-DAG1 blocking antibodies, NXPH1 is inhibitory to HPC stimulated by a number of different cytokines, alone or in combination. In addition to identifying a role for NRXN1α signaling in hematopoiesis, our results also shed light on the binding of both DAG1 and NXPH1 to NRXN1α. While both DAG1 and NXPH1 bind the second LNS domain of NRXN1α (Lise and El-Husseini 2006), they bind different sites within this region. As the affinity of DAG1 varies greatly between different splice variants of NRXN1α (Sheckler, Henry et al. 2006), the allosteric antagonism of NXPH1 by DAG1 could be modulated without affecting NXPH1-binding to NRXN1α. These may shed further light on this system in neuronal system function. Though DAG1-NRXN1α interaction is implicated in brain development and is thought to play a positive role (Michele, Barresi et al. 2002; Satz, Ostendorf et al. 2010), the precise function of DAG1 remains unclear (Missler, Zhang et al. 2003; Graf, Zhang et al. 2004). Similar to the hematopoietic system, NXPH1 has been associated with an inhibitory role in the neuronal system (Beglopoulos, Montag-Sallaz et al. 2005; Zhang, Rohlmann et al. 2005). It is possible that the function of DAG1 as an allosteric inhibitor may resolve some of the controversy surrounding DAG1 function in the brain. If true, these data would further re-affirm the complementary nature of the neuropoietic and hematopoietic models.

Future directions

The largely descriptive narrative presented within this thesis presents three obvious questions for future research: what is going on within the cell during NXPH-mediated inhibition, how can this information be used to treat human disease and how does this axis relate to the hematopoietic niche? In the following section, I will attempt to outline an approach to study each of these questions.

Mechanism of NRXN1α function in hematopoiesis

The work presented in this thesis offers a descriptive analysis of the relationship between NRXN1α, DAG1, and NXPH1: the mechanism of action for NXPH-mediated inhibition remains unknown. As NRXN1α is most commonly associated with voltage-gated signaling in the brain, any analysis of NRXN1α function should begin there. NRXN1α forms a complex with both N- and P/Q-type Ca^{++} channels (Zhang, Rohlmann et al. 2005) as well as Na^{+}/K^{+} ATPase (Laprise, Lau et al. 2009). While Ca^{++} signaling has been associated with survival in hematopoiesis (Yang, Tan et al. 2006), Na^{+} and K^{+} signaling is more promising. Lithium, an inhibitor of Na^{+}/K^{+} ATPase activity has been shown to specifically stimulate granulopoiesis in response to GM-CSF *in vitro* (Gallicchio, Chen et al. 1983; Gallicchio 1986). As these studies were performed on cells plated in

population, the specific effect of Lithium on cells stimulated by GM-CSF is similar to the specific effect of NXPH1 on cells stimulated by GM-CSF+SCF. Because of this, we hypothesize that NXPH1 binds to NRXN1α and activates the Na^+/K^+ ATPase, thereby inhibiting HPC proliferation. If true, studies with lithium as well as other Na^+/K^+ ATPase inhibitors such as diazepam and propranolol should be able to prevent NXPH1-mediated inhibition of HPCs both *in vivo* and *in vitro* (Eroglu, Keyer-Uysal et al. 1984).

If Na^+/K^+ ATPase-inhibitors are unable to prevent NXPH1-mediated inhibition, another possible avenue of study involves GABAergic and cholinergic intermediaries. The effect of GABA on hematopoiesis superficially resembles the effect of recombinant NXPH1 on hematopoiesis: GABA decreases the cycling status and proliferative potential of HPCs (Andang, Hjerling-Leffler et al. 2008). The failure of conditioned medium from cells treated with NXPH1 to inhibit HPC proliferation coupled with the effect of NXPH1 on single $CD34^+$ huCB cells argues against this hypothesis. However, conditioned medium is an imprecise tool and firm conclusions, where possible, require a rigor beyond that demonstrated in this thesis. Likewise, studies involving delayed NXPH1 addition to cells treated with GM-CSF+SCF in population *in vitro* (data not shown) which showed some inhibition, coupled with the high stability of NXPH1 may allow for secondary effects despite the single cell data. Indeed, an indirect response may explain the ability of recombinant NXPH1 to inhibit huCB HPCs: by the time the indirect inhibitory effects of recombinant NXPH1 are able to influence huCB HPC proliferation the HPCs have already

been induced into cycle by the growth factors present. Because GABA acts as a chemoattractant (Zangiacomi, Balon et al. 2009) and inhibits chemoattracton to SDF1α (Seidel, Niggemann et al. 2007), migration studies should reveal whether GABA plays any role. On its own, recombinant NXPH1 does not appear to be chemattractive nor does it appear to inhibit chemotaxis towards SDF1α (data not shown). While these studies need to be repeated they argue against the role of GABAergic signaling in NXPH function.

Cholinergic signaling is another possibility, though it is not as promising as those previously discussed. Neither nicotine nor acetylcholine have been shown to have an effect on HPCs in methylcellulose culture (Koval, Zverkova et al. 2008) which argues against the involvement of cholinergic signaling in NXPH1 function; however, these studies were conducted on cells plated in a population so DAG1 may obscure the effect. Elements in favor of this hypothesis involve $\alpha4\beta2$ nicotinic receptor and LT. NRXN1 is involved in the localization of the $\alpha4\beta2$ nicotinic receptor in the brain (Cheng, Amici et al. 2009), and in the hematopoietic system receptor presence inhibits HPC proliferation (Koval, Zverkova et al. 2008). Additionally, the neuromuscular response to LT is cholinergic (Lelyanova, Thomson et al. 2009). If recombinant NXPH1 were to modulate cholinergic function it could explain its inhibition of LT mediated survival, though the role of LT as a calcium ionophore is a far more likely mechanism (Ushkaryov, Rohou et al. 2008). The first step to test this hypothesis would be to identify the nature of

NXPH1-mediated inhibition of LT induced survival similar to how the relationship between DAG1 and NXPH1 was established. Since both NXPH1 and LT bind the second LNS domain of NRXN1α and NXPH1 associates with LT, competitive inhibition is likely. Especially considering the independence of LT action and its antagonism by NXPH1 from DAG1, it may well be that NXPH1 and LT form a complex which cannot bind the hematopoietic NRXN1α splice variant. If true, pre-incubation of LT and NXPH1 together in addition to normal dose-response assays is required. In order to determine whether NXPH1 acts through cholinergic signaling, the first step is to perform an ELISA for cholinergic signaling molecules in response to cells treated with anti-DAG1 and recombinant NXPH1. If present, *in vivo* injection of nicotine as well as α2 and β7 knock-out mice would be useful tools in determining whether these cholinergic signals play a role in NXPH1-mediated inhibition.

Lastly, there are other possibilities presented by Lin2, the protein directly downstream of NRXN1α (Hata, Butz et al. 1996; Craig and Kang 2007). As a member of the MAGUK-family, Lin2 has a large number of potential binding partners and intersects with many different signaling pathways (Anderson 1996). Any list of proteins interacting with Lin2 would be incomplete, so it is more expedient to provide several protein domains with which Lin2 has been shown to interact: Mint1-interacting CaM kinase, Veli-interacting, PDZ, SH3, 4.1-band protein binding, and guanilate kinase (Maximov, Sudhof et al. 1999). In the hematopoietic system, over-expression of Lin2 has been shown to inhibit cell

growth by blocking cell-cyle progression via Id1 (Qi, Su et al. 2005), which itself has been shown to be involved in both lymphopoiesis (Kersten, Dosen et al. 2006) and granulopoiesis (Buitenhuis, van Deutekom et al. 2005). Initial investigation of Lin2 ought focus on Id1, using both knock-out and over-expression systems. If Id1 is not involved in recombinant NXPH1-action, high throughput analysis is recommended for investigating this system due to the number of possibilities present. Methods include transposon mutagenesis of immortalized hematopoietic cell lines expressing NRXN1α. Ideally, the cell line used would express NRXN1α but not DAG1; if no such line exists one could be created by knocking-out DAG1. This would ensure that DAG1 antagonism of recombinant NXPH1 does not interfere with the interpretation of the results.

The role of DAG1 in NXPH-mediated inhibition also needs to be further clarified. Our narrative presents DAG1 as merely an allosteric antagonist; however the evidence for our hypothesis is somewhat circumstantial and descriptive. The use of DAG1 blocking antibodies *in vivo* could theoretically be used to support our hypothesis; however DAG1 plays an important role in muscle function, including cardiac muscle and as such *in vivo* experimentation cannot be recommended. While DAG1 -/- mice are not viable due to absence of mesoderm formation and haploid mice suffer from complications due to muscular dystrophies, the role of DAG1 in hematopoiesis could be further clarified using embryonic stem cells in a system similar to the one described in work previously published from the Broxmeyer laboratory (Ou, Chae et al. 2011). Additionally, lineage

specific knock-out mice could be developed using a Cre-lox system to further investigate the role of DAG1 in hematopoiesis.

In addition to the inhibition of HPCs by recombinant NXPH1, the mechanism whereby LT promotes HPC survival during growth factor starvation also remains unknown. In order to proceed, first the pro-survival effect of LT must be shown to be anti-apoptotic in nature. A simple annexin V/PI analysis would of growth factor starved cells would suffice to demonstrate this. Provided that a decrease in apoptosis is observed, it is likely that calcium influx is responsible. Calcium influx is likely responsible because in the neuronal system LT inserts itself into the cellular membrane and forms a calcium ionophore and in the hematopoietic system calcium ionophores have been shown to have a pro-survival effect. Calcium flux in hematopoietic cells has been previously examined using pluronic acid (Basu, Ray et al. 2007) and a similar approach could be applied here. However, that particular assay primarily provides information about the intracellular release of calcium from the endoplasmic reticulum. While there is some intracellular calcium release in neuronal cells after LT binding, the response is primarily from extracellular calcium influx. Because of these facts, a more ideal mechanism would be to use patch-clamp analysis on individually sorted NRXN1α expressing HPCs. This technique is how the mechanism of LT was demonstrated in the neuronal system and with minimal modifications could theoretically be adapted to hematopoietic cells. However, I am unaware of patch-clamp analysis ever having been attempted on

hematopoietic cells so a great deal of optimization may be required before this approach yields substantial results. Patch-clamping is further complicated by the heterogeneous nature of phenotypically defined hematopoietic cells so even after optimization the results may be difficult to interpret. Provided that either pluronic acid and/or patch-clamping are able to demonstrate that the pro-survival effect of LT on HPCs is due to an increase in calcium flux, further investigation would reveal whether AKT, CREB, PLC or all three provide the mechanism whereby growth-factor starvation induced apoptosis is averted.

However, there is reason to question whether LT works in the same manner in the hematopoietic system as in the neuronal system. The maximal stimulatory dose of LT is 100 times higher on HPCs than neuronal cells. Furthermore, a 5nm concentration of LT, the lowest concentration at which an effect is observed on HPCs, membrane integrity of neuronal cells is completely compromised. Because NRXN1α has many splice variants and splice variants have been shown to affect NRXN1α-ligand interaction, it is probable that this difference stems from splice variants in the hematopoietic and neuronal system. This hypothesis could be confirmed by mRNA analysis. Should this hypothesis prove true, microarray analysis would be required to identify potential signaling pathways which may be involved. Depending on which pathways are identified, different approaches involving a combination of knock-out mice and various pathway inhibitors could be employed to further understand the mechanism of action. Another

possibility is DAG1. Since all experiments involving LT were performed in a population of cells, the presence of DAG1 on other cells in the culture may compete with LT for NRXN1α binding.

Another potential area of future research is the interaction between LT and Concanavalin A. In the neuronal system, Concanavalin A binds NRXN1α and, while not interfering with LT-NRXN1α binding it does prevent LT insertion into the cellular membrane. The ability of Concanavalin A to inhibit the insertion of LT into the cellular membrane is of particular interest if the pro-survival effect of LT is not due to its activity as a calcium ionophore. If the pro-survival effect of LT were still observed in the presence of Concanavalin A, the signal would have to be mediated by NRXN1α. The interaction between Concanavalin A and NRXN1α has other potentially interesting implications for hematopoiesis as Concanavalin A is a mitogen whose mechanism of action has not yet been characterized. Including Concanavalin A in future *in vitro* experiments, similar to those already performed involving LT and recombinant NXPH1, could clarify these questions.

Similar to other areas of overlap between the hematopoietic and neuronal system, the differences between them are as illuminating as the similarities. The difference in effective LT dosage between the hematopoietic and neuronal systems suggests that different splice variants of NRXN1α are expressed. The existence of NRXN1α splice

variants also presents the possibility that LT cannot bind hematopoietic NRXN1α and the pro-survival effect seen is mediated by a different, less sensitive LT receptor, such as CIRL. Further investigation of this system using reagents such as Concanavalin A is warranted.

The intracellular mechanisms whereby the observed effects of the NRXN1α-axis are mediated remain unknown. Similar to the initial justification for investigating NRXN1α, the function of NRXN1α, DAG1, NXPH and LT in the neuronal system provides a framework whereby future research can operate. Several possibilities present themselves for the mechanism of NXPH1-mediated inhibition including voltage-gated signaling as well as several different neuronal signaling molecules. Further clarification is also called for in the case of DAG1. While our data suggests that DAG1 functions merely as an allosteric inhibitor of NXPH-mediated inhibition, other interpretations remain possible. As DAG1 knock-out (-/-) mice are not viable, the role of DAG1 in hematopoiesis may best be clarified using embryonic stem cells. The mechanism for the toxin LT is slightly more straight-forward due to the pro-survival effect of calcium ionophores on HPCs; however the existence of other mechanisms is considered and Concanavalin A is presented as a candidate for studying these alternatives. Should the NRXN1α-axis described here prove important in the hematopoietic niche or human disease, the mechanisms whereby its members operate will be vital in developing our understanding.

Clinical applications of the NRXN1α axis.

The involvement of the NRXN1α axis in hematopoiesis may prove to be of clinical relevance. Both schizophrenia and autism involve a disruption of the normal NRXN1α-axis and this disruption may help explain and treat their hematopoietic phenotypes. While these genetic diseases are somewhat rare, cancer and AIDS also potentially intersect with the NRXN1α-axis through NXPH. Of further importance to cancer and potentially other hematopoietic diseases, the high concentration of NXPH in umbilical CB may have implications for CB transplantation.

The existence of viable human populations deficient in NRXN1α-signaling capacity coupled with a recognizable phenotype presents many opportunities for research. While murine knock-outs do exist and should be studied, primary human data is of greater clinical relevance. Additionally, the comparison of human and murine knock-out systems serves to more fully elucidate the role of the system in hematopoiesis. As such, any discussion of experiments involving knock-out models *in vitro* should be thought to involve both human and murine systems. Additionally, most of the studies describing the hematopoietic phenotypes of schizophrenics and autistics are old and descriptive. Re-visiting these systems with modern techniques could reveal a great deal about the role of NRXN1 in hematopoiesis. Furthermore, drugs for the treatment of schizophrenia and autism could be rationally designed to target factors downstream of NRXN1α

potentially providing a better means of care than those presently in use. Of particular interest would be the repopulating potential of mobilized SRCs from schizophrenics and autistics, as it would clarify many questions the data gathered here raises. The proliferative phenotype of schizophrenics and autistics suggests that their cells would exhibit a higher degree of contribution per HPC. Similar to other proliferative systems, this may result in exhaustion of the stem cell compartment (Campbell, Basu et al. 2009); however, NRXN1α does not appear to be involved in HSC proliferation so exhaustion may not occur. More importantly, the hypothetical role of the NRXN1α-axis in the hematopoietic niche (described later) could be observed through sequential serial-transplantation and homing experiments.

As has been previously noted, the expression level of NXPH correlates with a poor prognosis in cancer. We hypothesize that NXPH secreted by cancers would inhibit HPCs and mature hematopoietic cells involve in immune response, which could play a role in immune system suppression and evasion by cancer. Before testing of this hypothesis could even begin, primary cancer samples and immortalized cell lines should be examined for NXPH expression. If NXPH family members are expressed in some primary cancer samples and cell lines, several candidate cell lines should be identified. Ideally, these cell lines would be paired based on the type of cancer they represent with one either expressing or expressing high levels of NXPH and the other either expressing low levels or no NXPH. In each pair, NXPH expression would be silenced in the high

expressing sample and overexpressed in the non-expressing member. The different cancer cell lines would then be injected into different groups of mice and the survival of mice over time would be examined. Ideally, mortality would correlate to NXPH levels expressed by the cell lines. If true, experiments involving Freund's adjuvant and NXPH1 as cancer therapy would be investigated. Knock-out mice are also a possibility; however they likely possess a pro-inflammatory phenotype that could complicate experimental interpretation.

The expression of NXPH also increases during immune system exhaustion. This is interesting because one of the critical differences between disease progression of SIV and HIV is immune system exhaustion which occurs in the human but not the simian system; immune system exhaustion is associated with the onset of AIDS (Pandrea, Sodora et al. 2008). Given the suppressive effect of NXPH1 on hematopoiesis, it is reasonable to suggest that NXPH family members play a role in immune system exhaustion. If true, inhibiting NXPH may serve to delay the onset of AIDS. Inducing immune system exhaustion into NXPH knock-out mice would shed light on this issue. Alternatively, anti-NXPH1 antibodies could be used to delay the onset of immune system exhaustion in wild-type mice inflected with *Leishmania*. If immune system exhaustion were delayed, a similar approach for treating HIV patients could be explored.

Of particular clinical interest is the relationship between NXPH and huCB. Unlike myelosuppressive chemokines (Lu, Xiao et al. 1993), recombinant NXPH1 is able to suppress the colony forming ability of HPCs from freshly isolated huCB. Additionally, we found high concentrations of NXPH-family members in huCB plasma, but not in adult human and murine peripheral blood. Taken together, these findings may be of some meaning in the context of huCB transplantation. While offering many advantages as compared to other sources of HSCs, reconstitution time is longer in patients who have received huCB transplants. It is possible, but not yet determined that members of the NXPH-family may play a role in the engraftment time of huCB cells in a clinical context. To test this hypothesis, huCB cells will be resuspended in sterile filtered huCB plasma, FBS, and FBS augmented with varying concentrations of NXPH1 and different concentrations will be injected into sublethally irradiated NOD/SCID mice. To control for the long reconstitution time being an intrinsic property of huCB HSCs, the experiment will be performed in tandem with muBM cells. Limiting dilutions of C57Bl/6 competitor cells will be injected and the effect of NXPH on repopulation will be examined.

The function of NXPH in huCB remains mysterious. Given the suppressive effects of NXPH on HPC proliferation, it is possible that NXPH is present to inhibit the development of more mature cells in huCB, thereby maintaining an environment more conducive to HSC maintenance. Indeed, this hypothesis is supported by the fact that

the population of hematopoietic cells in huCB is considered relatively naïve. Because the huCB environment is conducive to HS/PC maintenance, huCB is able to be stored at room temperature for long periods of time with minimal impact on the colony-forming potential of HPCs (Broxmeyer, Douglas et al. 1989). If NXPH contributes to the environment which supports this maintenance, exposing huCB samples to anti-NXPH1 antibodies would lead to faster HPC degradation. Another, more powerful, study would be to examine the potential of HS/PCs derived from the placentae of NXPH knock-out mice, provided pilot studies demonstrated that NXPH is present in murine placentae. Double knock-outs for NXPH1 and 3 exist, though evidence suggests that it is NXPH2 that is expressed in the murine hematopoietic system so a triple knock-out would need to be developed. If NXPH is involved in HS/PC maintenance we would expect a decrease in the absolute number and quality of HS/PCs in the knock-out. A third study would be to expand huCB $CD34^+$ cells in the presence of NXPH1 in FBS as well as in huCB plasma with its NXPH depleted via A/G agarose and examine the SRCs in NOD/SCID mice. While NXPH1 does not affect the *in vitro* performance of expanded $CD34^+$ cells, if NXPH helps to maintain stemness *in vitro* assays would be unable to detect it.

While much work remains to be done, the work presented here creates a foundation for future clinical work. The NRXN1α-axis is involved in schizophrenia, autism, cancer and HIV. Additionally, the presence of NXPH in huCB may be of relevance to huCB

transplantation as well as HSC maintenance. Further study of the proteins characterized here may yield new treatments for disease as well as new transplantation techniques.

The NRXN1α-axis and the hematopoietic niche

An interesting finding was DAG1 expression in the bone marrow. While DAG1 appears to be expressed on many cells in the bone marrow, histology reveals that it is most heavily expressed in osteoblasts. The co-localization of DAG1 and NRXN1α in the BM observed together with the physiological response of hematopoietic cells to recombinant NXPH1 exposure leads us to hypothesize that primitive HSCs are bound to DAG1 in the niche. The presence of NRXN1α on the HSC in the niche has interesting implications for both niche function and development.

We hypothesize that NRXN1α expressed on the HSC is able to mediate between GABAergic signaling from the MSC and adrenergic signaling from the beta-adrenergic neuron in the niche. A variety of different avenues exist for studying this system to determine if it is true. The most direct route would be to thaw the NRXN1α -/- as well as NRXN1 -/- mouse embryos and examine their hematopoietic system, especially in response to GABAergic and adrenergic compounds. While only NRXN1 expression has been observed at the RNA level in hematopoietic cells, due to complementation it may

be prudent to examine triple knock-outs eliminating the entire NRXN family, though developmental problems complicate this study. Another avenue is analysis of $CD34^+$ $CD38^-$ huCB populations. FACS analysis of NRXN1α and DAG1 expression of $CD34^+$ $CD38^-$ huCB cells suggests that two separate populations of cells exist. Both populations express NRXN1α and DAG1; however the brightness of the two proteins is different. In one population, NRXN1α is very bright whereas DAG1 is dim; whereas in the other population DAG1 is bright and NRXN1α is dim. While these populations express similar levels of other stem cell markers and do not differ in their *ex vivo* proliferation, *in vivo* analysis is required to truly assay stem cell function. Based on our results in the mouse system *in vivo*, one might expect the NRXN1α dim population to describe the more primitive population; however such thinking may be premature due to the role of NRXN1α in synaptogenesis. In the neuronal system, NRXN1α serves an instructive role in GABAergic development. Taken together with the fact that the adult hematopoietic niche is not the site of development for HSCs and the resemblance of MSCs to post-synaptic GABAergic neurons, it is possible that the adult hematopoietic niche is created through a process similar to GABAergic synaptic development. If true, that one would expect the population with the higher expression level of NRXN1α to contain either more or better SRCs. Because huCB transplant involves relatively little GVHD, it would be possible to test this hypothesis by a sex-mismatched competitive repopulation assay. If this hypothesis were to demonstrate some promise, the next step would be to shift the system over to a model more conducive to developmental studies, such as zebrafish.

Conclusion

Similarities between mammalian tissues represent a powerful tool for directing experimental discovery (Terskikh, Easterday et al. 2001). Here we have demonstrated that three previously identified elements of the neuronal system, DAG1, NRXN1α and NXPH1, and perhaps other members of the NXPH-family, are present and functional within the hematopoietic system and form a tightly regulated axis. DAG1 appears to bind NRXN1α and protect HPCs from NXPH1 binding and subsequent inhibition. *In vivo* this protection is observed on phenotypically defined LTR-HSCs, suggesting that the DAG1-NRXN1α axis may play a significant role in the hematopoietic niche. In addition to their scientific value, these results have a variety of potential clinical implications. The high concentrations of NXPH present in huCB may at least partially be involved in the time to engraftment for CB transplants, while high NXPH expression, which correlates with a negative in cancer, may be involved in immune system evasion by cancer. Further studies in this area are of future experimental and clinical interest.

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Zhang, J., Y. Wang, et al. (2006). "Agrin is involved in lymphocytes activation that is mediated by alpha-dystroglycan." FASEB J **20**(1): 50-58.

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Zheng, C., R. Yang, et al. (2008). "TPO-independent megakaryocytopoiesis." Crit Rev Oncol Hematol **65**(3): 212-222.

Curriculum Vitae

John M Kinzfogl

Education:

Ph.D. Microbiology and Immunology, Indiana University. November 2011
B.S. Bacteriology, University of Wisconsin, Madison. Spring 2005

Research Experience:

Laboratory of **Hal E. Broxmeyer**
Graduate Research Fellow, Indiana University, 2005-2011

- Engaged in independent research on the role of neuronal proteins in hematopoiesis
- Discovered a novel hematopoietic signaling axis
- Resolved conflicting results into a coherent model

Laboratory of **Nancy Keller**
Undergraduate Researcher, University of Wisconsin, 2003-2004

- Investigated lipid metabolism and secondary metabolite biosynthesis in *Aspergillus nidulans*

Laboratory of **Julius Adler**
Undergraduate Researcher, University of Wisconsin, 2001-2002

- Investigated decision making and chemotaxis in *Drosophila melanogaster*

Publications:

Kaplan, M. H., Glosson, N. L., Stritesky, G. L., Yeh, N., **Kinzfogl, J**., Rohrabaugh, S. L., Goswami, R., Pham, D., Levy, D. E., Brutkiewicz, R. R. (2011). "STAT3-dependent IL-21 production from T helper cells regulates hematopoietic progenitor cell homeostasis." Blood 117(23): 6198.

Kinzfogl, J., Hangoc, G., Broxmeyer, H. E. (2011). "Neurexophilin 1 suppresses the proliferation of hematopoietic progenitor cells." Blood 118(3): 565-575.

Broxmeyer, H. E., Kappes, F., Mor-Vaknin, N., Legendre, M., **Kinzfogl, J.**, Cooper, S., Hangoc, G., & Markovitz, D. M. (2011). "DEK Regulates Hematopoietic Stem Engraftment and Progenitor Cell Proliferation." Stem Cells Dev.

Grants, Fellowships, and Awards:

NIH T32 DK007519 "Regulation of Hematopoietic Cell Production"
P.I. Hal E. Broxmeyer

- Participating researcher from July, 2009 to August 2011

NIH HL007910 "Basic Science Studies on Gene Therapy of Blood Diseases"
P.I. Hal E. Broxmeyer

- Participating researcher from July, 2006 to June, 2009

ASH Travel Award

- Received $500 from the American Society of Hematology to help defray annual meeting travel expenses in 2010

Holstrom Environmental Research Scholarship

Mentor: Nancy Keller

- Awarded $4,000 in 2004 by UW alumnus Carleton Holstrom for environmental research

Congress-Bundestag Youth Exchange

- Sponsored by the United States Congress and German Bundestag to cover all expenses for attending the 2000-2001 academic year in a German High School (Gymnasium)

Presentations:

Annual American Society of Hematology Meeting, Orlando, FL (2010)

- Poster: "The Neurexin I alpha/Neurexophilin axis as an anti-proliferative factor in human cord blood and murine bone marrow hematopoeisis."

Annual Midwest Blood Club Symposium, Indianapolis, IN (2010)

- Oral: "The Neurexin I alpha/Neurexophilin axis as a myelosuppressive factor in human cord blood and murine bone marrow hematopoiesis."

Annual American Society of Hematology Meeting, Atlanta, GA (2009)

- Poster: "The Neurexin I alpha/Neurexophilin 1 axis as a myelosuppressive factor in human cord blood and murine bone marrow hematopoiesis."

Teaching Experience:

Nursing Microbiology Laboratory, Teaching Assistant, Indiana University School of Medicine. Indianapolis, IN (Fall 2008).

- Instructed 1st and 2nd year nursing students
- Discussed and clarified lecture material to students
- Organized independent review sessions for interested students