Determinants of Embryonic Hematopoietic Stem Cell Emergence and Maturation

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Abstract

A fundamental goal in stem cell biology is to develop therapeutically applicable cells in a dish. The discovery of Yamanaka and others of a platform to transform any somatic cells into pluripotent stem cells makes this an enticing goal for personalized medicine. Hematopoietic stem cell (HSC) is one such highly sought-after cell type useful for treating myriads of refractory hematological malignancies when a donor match is difficult to obtain. Partial match results in complications such as graft-versus-host disease, which is responsible for a significant portion of morbidity and mortality in patients receiving grafts. Advances in understanding the developmental origins of HSCs—precisely the molecular mechanisms promoting their emergence and maturation—may lead to derivation of personalized HSCs, circumventing these issues of immune mismatch.

In mouse, it is widely accepted that HSCs emerge during mid-gestation from the caudal region of the embryo containing the dorsal aorta, which is called the aorta-gonad-mesonephros (AGM). The emergence occurs through an endothelial intermediate via a phenomenon called the endothelial-to-hematopoietic transition (EHT), whereby endothelial cells round up to become HSCs. AGM HSCs subsequently seed the fetal liver and bone marrow near birth. Although many signaling cascades have been implicated in HSC emergence, the likely scenario is a combinatorial effect of biomechanical cues and signaling pathways acting in concert. Prior work from our lab suggests that fluid shear stress, from the initiation of the heartbeat, is responsible for HSC emergence. Here, we determined that the AGM is enriched for expression of targets of Protein Kinase A (PKA)/CREB, a pathway activated by fluid shear stress. By analyzing CREB genomic occupancy from chromatin-immunoprecipitation sequencing (ChIP-seq) data, we identified the Bone Morphogenetic Protein (BMP) pathway as a potential regulator of CREB. By chemical modulation of the PKA/CREB and BMP pathways in isolated AGM VE-cadherin+ cells, which marks hemogenic endothelial cells from mid-gestation embryos, we demonstrate that PKA/CREB regulates hematopoietic engraftment and clonogenicity of hematopoietic progenitors and is dependent on secreted BMP ligands through the type I BMP receptor. Finally, we observed blunting of this signaling axis using Ncx1 knockout (KO) embryos, which lack a heartbeat and intravascular flow. Modulation of the PKA/CREB-BMP signaling axis in Ncx1 KO embryos results in partial rescue of hematopoiesis. Thus, we have identified a novel
inflammatory signaling axis PKA/CREB-BMP acting downstream of shear stress that regulates HSC emergence in the AGM via the EHT.

AGM HSCs are developmentally immature and undergo further maturation as they transit through the fetal liver (FL) to the bone marrow. Compared to FL and adult HSCs, AGM HSCs have reduced repopulation potential in adult bone marrow transplantation—a deficiency shared with pluripotent stem cell-derived HSC. Signaling pathways underlying this deficiency in AGM HSCs are poorly understood. By co-expression gene network analysis we deduced that AGM HSCs are relatively deficient in interferon-alpha/Jak-Stat1-associated gene expression. Here we show that treatment of AGM HSCs with interferon-alpha enhances long-term hematopoietic engraftment and competitiveness without affecting homing or stem cell frequency. We identify AT-rich interactive domain-3a (Arid3a), a factor essential for FL and B lymphopoiesis, as a key transcriptional coregulator of interferon-alpha/Stat1 signaling. Arid3a occupies the genomic loci of Stat1 as well as several interferon-alpha effector genes, acting to upregulate their expression. Accordingly, Arid3aΔ/Δ AGM HSCs had significantly reduced transplant potential, which was rescued by interferon-alpha treatment. Our results identify the inflammatory interferon-alpha/Jak-Stat pathway in the developmental maturation of embryonic HSCs, whose manipulation may lead to increased potency of reprogrammed HSCs for transplantation. The integration of many signaling pathways results in a carefully orchestrated emergence and maturation of HSCs that is yet to be recapitulated in vitro. Both signaling axes are novel inflammatory signals regulating normal HSC development, which have important applications in the derivation of HSCs from pluripotent stem cells.
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RESULTS

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<tr>
<td>AGM</td>
<td>aorta-gonad-mesonephros</td>
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<tr>
<td>ao</td>
<td>dorsal aorta</td>
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<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BM</td>
<td>bone marrow</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>BMT</td>
<td>bone marrow transplantation</td>
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<td>bp</td>
<td>base pair</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>ChIP-Seq</td>
<td>chromatin immunoprecipitation-sequencing</td>
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<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
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<td>CLP</td>
<td>common lymphoid progenitor</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<td>cv</td>
<td>cardinal vein</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ee</td>
<td>embryo equivalent</td>
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<tr>
<td>EHT</td>
<td>endothelial-to-hematopoietic transition</td>
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<tr>
<td>ENCODE</td>
<td>Encyclopedia of DNA Elements</td>
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<tr>
<td>Epo</td>
<td>erythropoietin</td>
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<td>Ery</td>
<td>erythroid</td>
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<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinases</td>
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<tr>
<td>ESC</td>
<td>embryonic stem cells</td>
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<tr>
<td>FACs</td>
<td>fluorescence activated cell sorting</td>
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<td>FDR</td>
<td>false discovery rate</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FL</td>
<td>fetal liver</td>
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<tr>
<td>FLT3L</td>
<td>Flt3 ligand</td>
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<tr>
<td>GEMM</td>
<td>granulocyte-erythrocyte-mono-lymphocyte-megakaryocyte</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GM</td>
<td>granulocyte-macrophage</td>
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<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
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<tr>
<td>GY</td>
<td>gray</td>
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<tr>
<td>H3K4</td>
<td>histone H3 lysine 4</td>
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<td>H3K27</td>
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HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC hematopoietic stem cell
IFNa interferon-alpha
Ifnar1 interferon-alpha receptor 1
IFNg interferon-gamma
Ifngr1 interferon-gamma receptor 1
IL interleukin
IMDM Iscove's Modified Dulbecco's Medium
KO knockout
M myeloid
MAPK mitogen-activated protein kinases
mESC mouse embryonic stem cell
MHC major histocompatibility complex
mRNA messenger ribonucleic acid
nc notochord
nt neural tube
OCT Optimal Cutting Temperature compound
PE R-Phycoerythrin
PGE2 prostaglandin E2
PKA protein kinase A
PI propidium iodide
Pl placenta
PI3K phosphatidylinositol 3-kinase
PSC pluripotent stem cells
PSp para-aortic splanchnopleura
QPCR quantitative PCR
RT-PCR reverse transcription polymerase chain reaction
S133/675 serine-133/675
SCF stem cell factor
SDS sodium dodecyl sulfate
sm somites
TLR toll-like receptor
TNF tumor necrosis factor
TPO thrombopoietin
ur urogenital ridge
VE-cadherin vascular endothelial cadherin
VEGF vascular endothelial growth factor
vm ventral mesenchyme
WT wild-type
WGCNA weighted gene co-expression network analysis
YS yolk sac
Acknowledgements

First and foremost, I would like to thank Dr. George Daley for his support. I enjoyed and appreciated the tremendous amount of academic freedom he has given me in the lab. In times of struggle, he has been the voice of reason that helped to steer me in the right direction. In his laboratory, ‘stumbling in the unknown’ is quite fun and that attests to his leadership and mentorship. My last and only regret is that I do not have time to start more new projects because of my impending graduation in May 2015.

I have come to appreciate the Daley laboratory as an extended family. I would like to thank all members of the Daley laboratory, past and present. I reminisce about the laboratory retreats we have had together as a team. I would like to especially thank M. Willy Lensch, a former member who has been a mentor and a friend. I would also like to thank Beth Kaleta, the lab manager, for putting up with many enumerable requests. I would like to thank Sam Ross, a very talented mouse technician, for helping with many of my mouse experiments.
Statement of Contributions

The work presented here was performed during my Howard Hughes Medical Institute Fellowship (June 2014 – July 2015) between my third and fourth year of medical school under the direct mentorship of Dr. George Daley.

The second chapter is based on a manuscript in press at Journal of Experimental Medicine. I am the first author of this manuscript. P.G.K. and G.Q.D. conceived the study, analyzed the data, and wrote the manuscript. P.G.K. performed all the experiments including the informatics analysis except for the following exceptions. H.N. and A.N. provided the Ncx1 knockout embryos for the experiments in Figure 6 and performed the Ncx1 knockout rescue experiments in Figure 6O. P.P.D. and S.H.O. helped perform the chromatin-immunoprecipitation sequencing on phospho-Creb and phospho-Smad1/5/8. M.J.C. and T.M.S. provided the Sca1-GFP embryos and helped with the analysis in Figure 1D-E. R.G.R., S.S.C., S.J.R. assisted with the transplantations and peripheral bleeds for assessing peripheral chimerism under my supervision. R.G.R. helped with editing the manuscript. K.M.S. and L.I.Z. provided critical reagents and feedback.

The third chapter on Arid3a-Stat1 is based on a manuscript in submission at Journal of Experimental Medicine. I am the first author of this manuscript. P.G.K. and G.Q.D. conceived the study, analyzed the data, and wrote the manuscript. P.G.K. performed all the experiments including the informatics analysis except for the following exceptions. S.J.R. assisted with transplantations and peripheral bleeds under my supervision. C.R. generated Figure 6F. C.R. and J.V.H. helped prepare Arid3a KO embryos for fixation for Figures 6A-D. M.C.C. helped generate viral titres and edit the manuscript. H.O.T. provided critical reagents and constructive feedback.
Chapter 1 - Background

Challenges in bone marrow transplantation

Bone marrow transplantation (BMT) is the only curative therapy for a wide range of hematological malignancies including acute myeloid leukemia and Hodgkin’s disease, red cell disorders such as sickle cell anemia, and other genetic disorders such as severe combined immunodeficiency. Several developments in the 1960s propelled the field of BMT forward. Notably, in 1960, E. Donnall Thomas first pioneered the use of BMT to induce remission in refractory leukemias in two pediatric patients who had identical twin donors (1). In animal studies, Becker, McCulloch and Till identified using spleen colonies the existence of a hematopoietic stem cell (HSC) with clonal potential (2). These advances, among many others, made BMT a feasible option for many patients.

Major hurdles in BMT include immune mismatch and lack of available donors. Allogeneic transplantation—using donor marrow containing non-identical major histocompatibility complex (MHC) from the host—incites an immune reaction in which the donor lymphocytes attack the recipient tissues, damaging organs such as the skin, gastrointestinal tract, liver, kidney and lung. This reaction, called graft-versus-host disease (GVHD), is potentially fatal and leads to severe infectious complications and end-organ damage. Immunosuppressive regimens such as methotrexate can ameliorate GVHD. However, in one recent study, the non-relapse mortality—that is, mortality not attributable to primary malignancy—was high as 60% in severe GVHD within months of BMT (3). Additionally, only a quarter of potential recipients of BMT will have an immune-matched sibling (4), and despite the increase of participants in the bone marrow registry, patients may not be able to wait months to acquire stem cells through the registry. One alternative source of HSCs is cord blood, which remains attractive because less stringent immune matching is required for transplantation (5). However, limitations in banking and quantity often hamper widespread use. Indeed, combining many cords are required to increase engraftment (6). A source of autologous HSCs would alleviate these concerns of immune mismatch and access.

Scientists have looked to embryonic stem cells (ESCs) as a potential source of cells for therapy. The isolation of mouse ESCs by Martin in 1981 (7), and the isolation of human ESCs by Thompson in 1998 (8), are regarded as milestones in stem cell research. ESCs can self-renew or divide without losing their potential, and are pluripotent, having the potential to
give rise to all the cells in the body when differentiated in the right environment. Therefore, HSC derivation from ESCs is a possibility.

The drive in stem cell research has been towards personalized medicine. With a technique called somatic cell nuclear transfer, a differentiated cell from the body could be injected into an unfertilized egg, which made the derivation of personalized ESCs feasible (9). However, this technique, used in the cloning of Dolly the sheep, was fraught with ethical concerns for human therapy. Specifically, the procurement of human eggs and the generation of pluripotent stem cells (PSCs) at the expense of human eggs posed significant challenges. Recently, the advancement by Yamanaka and others to generate PSCs via introduction of four factors—Oct4, Sox2, Klf4 and c-Myc—has alleviated many ethical concerns and provided an abundant source of PSCs for research and personalized medicine (10). Personalized PSCs circumvent issues of immune mismatch and rejection upon transplantation. This has fuelled efforts to derive HSCs from PSCs.

Origins of developmental hematopoiesis

Hematopoietic stem cells (HSC) can self-renew and differentiate into diverse hematopoietic cell types including myeloid lineages such as monocytes, neutrophils, megakaryocytes and erythrocytes and lymphoid lineages such as B, T and natural killer cells (Figure 1). Because a single HSC can reconstitute an organism’s entire blood supply (11), the derivation of these cells from one’s own tissues has been a major goal in the field of developmental hematopoiesis. The derivation of these HSCs from PSCs requires an understanding of the different molecular signals involved in HSC formation during development. We primarily focus here on mouse development as a model of human development.

In early development, the fertilized egg implants in the lining of the uterus and gives rise to a structure called the blastocyst, which contain an inner cell mass capable of giving rise to the embryo proper. The inner cell mass first gives rise to the primitive ectoderm (called the ‘epiblast’), and subsequently forms the mesoderm and endoderm via the formation of the primitive streak. These three germ layers give rise to distinct tissues in the body. In this primitive streak, complex extracellular molecular signals involving Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Wnt ligands and among others determine
the fate of different mesodermal precursors in a concentration-dependent manner (12).
Specifically, the middle third of the primitive streak gives rise to the lateral plate mesoderm,
which later gives rise to the circulatory system. The posterior third of the primitive streak
gives rise to extra-embryonic mesoderm, including the yolk sac. Therefore, the middle to
posterior primitive streak differentiates into the various hematopoietic organs.

The first hematopoietic cells of the erythroid lineage develop in the extra-embryonic
yolk sac at embryonic day 7.5 (E7.5) (13). Because of this restricted lineage potential, the
yolk sac is not thought to be a de novo site of HSC formation in the embryo, and
hematopoietic potential is thought to be ‘primitive’. HSCs can be detected in close proximity
to the dorsal aorta after the onset of circulation at E8.5. At E9, hematopoietic stem cell (HSC)
activity from the para-aortic splanchnopleura (PSp) can be detected when transplanted into
neonatal mice (14). At E10-11, the PSp matures into the aorta-gonad-mesonephros (AGM)
and bone fide HSCs that engraft lethally irradiated adult recipients emerge (15, 16). Some
have suggested de novo generation of HSCs in the placenta (17), but the AGM is widely
regarded as the first site of ‘definitive’ hematopoiesis. These HSCs later colonize additional
organs required for adult hematopoiesis such as the fetal liver and subsequently the bone
marrow before birth (Figure 2).

The association between HSCs and the dorsal aorta is not a mere coincidence.
Lineage tracing studies have demonstrated that labeling endothelial cells in mid-gestation
embryos later marks almost all adult hematopoietic cells (18, 19). Advances in live-imaging
techniques have enabled the visualization of emerging hematopoietic stem and progenitor
cells in zebrafish and mouse from the ventral aspect of the dorsal aorta (20-22) (Figure 3). In
zebrafish, where the anatomy is clearer due to the transparency of the organism, distinct
stages of HSC emergence could be observed: flat endothelial cells round up towards the
lumen and finally dislodge into circulation, losing some of their endothelial characteristics
(21, 22). This process is called the endothelial-to-hematopoietic transition (EHT) and the
cells destined to become HSCs are aptly named hemogenic endothelial cells.

Many signaling pathways regulate the formation of the hemogenic endothelium and
EHT. Interestingly, they can be largely organized as dorsal signals (from the neural tube),
ventral signals (from the gut), or intrinsic signals (within the aortic endothelium) (Figure 3).
Dorsal signals include Sonic Hedgehog molecules emerging as a gradient from the notochord
(23, 24). Sonic Hedgehog is required for angiogenesis (25), and at lower concentrations likely
acts via promoting the secretion of other soluble factors such as Vascular Endothelial Growth Factor (VEGF) (25) or BMPs (23). Hedgehog signals therefore promote hematopoiesis from hemogenic endothelial cells in a non-cell autonomous manner (26).

Ventral to the dorsal aorta, BMP molecules are present in greater quantities. Murine stromal cell lines derived from the ventral mesenchyme of the AGM secrete BMP4 (27). An isoform of Sonic Hedgehog, Indian Hedgehog, is also secreted ventrally (28). Because Indian and Sonic Hedgehog both act through the same receptor and Hedgehog ligands can promote BMP secretion, whether BMP secretion is a result of local Indian Hedgehog signals or notochord-derived Sonic Hedgehog signals has not yet been determined. Dorsal FGF signals seem to restrict this BMP secretion to the ventral region of the aorta, thus limiting BMP signaling to the ventral region (29). BMP likely is responsible for directly inducing hematopoietic transcription factors such as Gata2 and thus activating a hematopoietic program in endothelial cells (30).

Molecular signals intrinsic to the endothelium include Notch, Wnt and retinoic acid signaling. Notch1 is required for HSC formation from endothelial cells (31), and the ligand most likely responsible for Notch1 activation Jagged1 is present in the vicinity of the endothelium (32). This signaling is mediated locally because the ligand and its receptor must be present in cells that are in direct contact. The responding cell cleaves the intracellular domain of the Notch receptor, which translocates to the nucleus and activates many hematopoietic genes including Gata2 (26, 33). Aside from Notch, many other local signals such as Wnt and retinoic acid signaling regulate hematopoietic emergence within the endothelium (34-36). As evidence of intricate crosstalk, Hedgehog signaling molecules can also activate Notch signals (26, 37). Together, these signaling pathways, organized along the dorsal-ventral axis, localizes HSC emergence predominantly to the ventral aspects of the dorsal aorta.

Efforts to derive hematopoietic stem cells in vitro

Directed differentiation is not the only method to derive HSCs. Current efforts to derive hematopoietic stem cells (HSCs) in vitro consist of three distinct approaches: differentiation, teratoma induction, or direct conversion. The goal of the traditional approach of differentiating pluripotent stem cells (PSCs) towards HSCs is integrating lessons from
developmental biology discussed above to recapitulate pro-hematopoietic microenvironments in vitro. Lessons learned from patterning the primitive streak have led to techniques for generating several ‘definitive’ lineages such as the T lymphoid lineage in vitro (38, 39). Alternatively, similar results could be obtained via co-culture of PSCs with OP9 bone marrow stromal cells, which likely mimics the pro-hematopoietic bone marrow microenvironment (40, 41). However, despite many attempts, the successful derivation of HSCs from PSCs via directed differentiation has not been achieved. The application of molecular signals in a combinatorial fashion may be critical.

The second approach to generating HSCs from PSCs involves harnessing the developmental potential of PSCs in vivo via teratoma formation. Teratoma assays can be used to document the pluripotency of PSCs via the injection of PSCs into an immune-compromised recipient mouse. Injected PSCs differentiate in vivo forming all three germ layers and form distinct tissues such as cartilage and secretory epithelium. Although the tissues are formed haphazardly, this approach has been successful at deriving small quantities of HSCs (42, 43). The use of xenotransplantation for the derivation of HSCs as well as the limiting quantities of HSCs obtained will likely make this approach unfeasible for clinical applications.

The last approach to generating HSCs involves using transcription factors to directly convert one cell type into HSCs. This was first demonstrated using the homeobox protein Hoxb4, which conferred long-term lympho-myeloid engraftment potential upon differentiating hematopoietic progenitors derived from PSCs (44, 45). Since then, many transcription factor combinations have been identified that will endow even differentiated B cells with HSC potential (46-49). One major pitfall of this approach is that transgene introduction often requires viral integration, which carries potential oncogenic risks (50). Another significant drawback is that many direct conversions are imperfect, resulting in short-term engraftment or lack of T lymphoid engraftment (47-49). Harnessing the developmental potential of PSCs through differentiation is very challenging. However, derivation of HSCs through these other methods still leads to these unexpected shortcomings such as lack of lymphoid reconstitution that may only be overcome by a deeper understanding of the developmental origins of HSCs. The work presented here will focus strictly on developmental signals promoting HSC emergence and maturation, with an eye towards deriving HSCs in the future via directed differentiation.
Figure 1 – Adapted from a figure by Willy Lensch, a former member of the Daley laboratory.

Figure 1 – Hematopoietic stem cell hierarchy

Hematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs further differentiate into progenitors that form colonies in methylcellulose in the presence of cytokines such as colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) and CFU-granulocyte-monocyte (CFU-GM) lineages.
Figure 2 – Hematopoietic ontogeny

Origins of hematopoiesis depicted on a timeline of mouse embryonic development from embryonic day 3 (E3) until birth. Red indicates regions with detectable HSC activity upon transplantation into lethally irradiated adult recipients.
Figure 3 – Anatomy of the aorta-gonad-mesonephros (AGM) region

Embryonic day 11.5 (E11.5) embryo is depicted without the yolk sac, placenta or vitelline vessels. The AGM is marked with solid lines and highlighted with an arrow. The dotted line corresponds to the cross-sectional anatomy depicted on the right. nt = neural tube, nc = notochord, sm = somites, ao = dorsal aorta, ur = urogenital ridge.
Chapter 2 - Flow-induced PKA/CREB-BMP in HSC emergence

At the time of submission of this dissertation, the work presented in this chapter has been accepted for publication as an Article to Journal of Experimental Medicine.

Title: Flow-induced Protein Kinase A / CREB pathway acts via BMP signaling to promote HSC emergence

Peter Geon Kim¹, Haruko Nakano², Partha P. Das¹, Michael J. Chen¹, R. Grant Rowe¹, Stephanie S. Chou¹, Samantha J. Ross¹, Kathleen M. Sakamoto³, Leonard I. Zon¹, Thorsten M. Schlaeger¹, Stuart H. Orkin¹, Atsushi Nakano², George Q. Daley¹

¹Stem Cell Transplantation Program, Division of Pediatric Hematology/Oncology, Howard Hughes Medical Institute, Boston Children’s Hospital and Dana Farber Cancer Institute; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School; Harvard Stem Cell Institute; Boston, Massachusetts 02115, USA.

²Department of Molecular Cell and Developmental Biology, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, Jonsson Comprehensive Cancer Center, Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90095, USA

³Division of Pediatric Hematology/Oncology, Lucile Packard Children’s Hospital, Stanford University School of Medicine, Stanford, California, USA
Introduction

The development of the murine circulatory system at E8.5 coincides with the development of more definitive hematopoietic compartments including HSCs and the lymphoid lineage. Accordingly, recent studies have linked biomechanical forces such as blood flow-induced shear stress to hematopoietic development (51, 52). In these studies, genetic mutants lacking intravascular circulation were used to demonstrate the reduction in hematopoietic emergence in the AGM, and nitric oxide signaling was implicated in blood flow-dependent AGM hematopoiesis (51-53). Although chimeric analysis demonstrated a cell-autonomous requirement for nitric oxide signaling in zebrafish (52), whether this pathway directly promotes hematopoiesis remains an open question due to the vasodilatory effect of nitric oxide donors and their effects on smooth muscle. Apart from these observations, signaling pathways responsible for flow-dependent hematopoietic induction have not been characterized.

Activation of Protein Kinase A (PKA) and its downstream target CREB by exogenous shear stress has been observed in diverse cell types including chondrocytes and osteocytes (54, 55). The classic mechanism of PKA activation involves the binding of a ligand to a G-protein coupled receptor and activation of adenylyl cyclase, which converts ATP into the second messenger cyclic AMP (cAMP). The binding of cAMP to PKA releases catalytic subunits that phosphorylate CREB in the nucleus. In differentiating mouse embryonic stem cells (mESCs), PKA/CREB has been linked to endothelial and hematopoietic differentiation via binding of CREB to the Etv2 promoter, which upregulates pro-hematopoietic factors such as Gata2 and Scl/Tal1 (56). Moreover, the PKA/CREB signaling pathway has been explored in the context of the Prostaglandin E2 signaling pathway in zebrafish, where it promotes AGM hematopoiesis via activation of the Wnt pathway (34). However, whether this pathway is conserved in the mouse is unclear, especially given conflicting reports on Wnt signaling in AGM hematopoiesis (35, 36). Prostaglandin E2 also directly activates a number of pathways including PI3K-AKT and ERK-MAPK, which makes it difficult to conclude that PKA/CREB is the sole mediator of the pro-hematopoietic effects of this molecule (57). Given the shear-responsiveness of the PKA/CREB pathway and its implication in early embryonic hematopoiesis in other species, we investigated the possible role of shear stress activated PKA/CREB signaling during AGM hematopoiesis in the mouse.
We first verified that this pathway is activated by shear stress in VE-cadherin+ endothelial cells and present in the murine AGM, specifically in the cells lining the dorsal aorta. We then conducted a informatics-based screen using microarray data on CREB overexpression and CREB chromatin immunoprecipitation-sequencing (ChIP-Seq) data using data available at Encyclopedia of DNA Elements (ENCODE) and elsewhere to identify regulators of CREB function in hematopoietic cells (58-63). Using insight gained from informatics, we discover that the Bone Morphogenetic Protein (BMP) signaling pathway acts downstream of PKA/CREB signaling in regulating AGM hematopoiesis. Finally, we show that this is a blood-flow dependent pathway by demonstrating the abrogation of PKA/CREB-BMP signaling axis in Ncx1 null embryos, which lack a heartbeat and intravascular flow. The activation of the PKA/CREB-BMP signaling axis using exogenous chemicals or morphogens can rescue hematopoiesis in Ncx1 null embryos. Our data thus document a blood-flow dependent pathway regulating hematopoietic development.

**Results**

CREB activation in the AGM

To assess the role of the CREB pathway in hematopoiesis, we examined CREB-mediated gene expression programs across various embryonic and adult hematopoietic tissues and found marked enrichment of CREB target genes in the aorta-gonad-mesonephros (AGM) relative to other tissues (Figure 1a) (64). CREB mRNA expression was similar among hematopoietic tissues, suggesting a post-transcriptional mechanism of target gene activation (Figure 1b). Because phospho-CREB at S133 is required for its transcriptional activity (65), we examined the distribution of S133-phosphorylated CREB in the E11.5 AGM, a time point coinciding with HSC emergence from the endothelium (16, 18, 20, 21). Some cells lining the aortic endothelium were S133-phosphorylated (Figure 1c), which raises the possibility of a shear stress-mediated effect. We also examined phospho-CREB in E10.5 embryos and obtained similar results (Figure 1d). Interestingly, most cells that were positive for Sca1-GFP, which marks the emerging HSCs in the endothelium (66, 67), also co-expressed phospho-CREB (Figure 1e). Because other S133-phosphorylated regions also included the ventral mesenchyme, notochord and the neural tube (Figure 1c-d), we examined the relationship between phospho-CREB and shear stress more closely in isolated VE-cadherin+ cells from
differentiated mESCs, which is a more accessible endothelial cell type. Shear stress increased S133-phosphorylation of CREB in a time-dependent manner (Figure 1f). The concomitant phosphorylation of beta-catenin at S675, a unique site for Protein Kinase A (PKA) phosphorylation (68), indicated shear-induced PKA activity (Figure 1f). Therefore, PKA phosphorylation of CREB in the AGM is likely dependent on blood flow.

Genomic binding and interaction of CREB in K562 cells

We next took advantage of published microarray data on CREB overexpression in human leukemic K562 cells as a model of hematopoietic stem/progenitor cells to gain insight into the role of the PKA/CREB pathway (59). To correlate gene transcription with transcription factor binding to the genome, we examined the active promoters of 447 genes that were upregulated by CREB overexpression (p-value cut-off 0.05) for CREB genomic occupancy by analyzing the K562 chromatin immunoprecipitation-sequencing (ChIP-seq) data (Figure 2a) (58). We defined active promoters as having histone H3K4 trimethylation (H3K4me3) and H3K27 acetylation (H3K27ac) but lacking H3K27 trimethylation (H3K27me3) (69, 70). We screened for regulators of the CREB pathway by examining transcription factor co-occupancy (Figure 2b). The top CREB co-occupants within promoters of genes upregulated by CREB overexpression included PLU.1, THAP1, MYC, CEBPD, E2F4 and SMAD1 (Figure 2b). Among these top hits were two direct effectors of BMP signaling. SMAD1 is a direct target of BMP receptors and is responsible for their downstream transcriptional activity (71) and E2F4 is known to be a SMAD cofactor (72). Therefore, we investigated the interaction between CREB and the BMP pathway target SMAD1.

As this analysis was restricted to genes responding to CREB overexpression, we also analyzed global genomic occupancy in active promoters and enhancers. SMAD1 and CREB peaks were enriched near active promoters (Figure 2c). Active enhancers are defined by the presence of H3K4 monomethylation (H3K4me1) and H3K27ac marks in the absence of H3K4me3 (73). CREB bound active enhancers less than promoters in agreement with the preferential promoter distribution of CREB in other cell types (Figure 2d) (74). However, SMAD1 still overlapped these CREB enhancer peaks (Figure 2d). To investigate the biological function of the genes co-bound by both CREB and SMAD1, we analyzed the top 3000 genes with the strongest enrichment for CREB and SMAD1 peaks for PANTHER pathway annotation (75). Genes of co-bound peaks were annotated for hematopoietic cell
activation, angiogenesis, and VEGF signaling pathway (Figure 2e). To strengthen our claim regarding CREB and SMAD1 genomic co-occupancy, we treated K562 cells with forskolin or BMP4 and performed ChIP-sequencing using phospho-CREB or phospho-Smad1/5/8 antibodies and obtained similar results (Figure 2f). Although K562 is a committed hematopoietic cell, the co-bound regions raise the hypothesis that these pathways regulate both hematopoietic and endothelial genes.

We explored this hypothesis further in the context of specific genes. First we examined CREB and SMAD1 occupancy of early BMP response genes of the pathway ID1-3, some of which have known roles in vasculogenesis (76). In the genomic region containing these genes, we found binding of both CREB and SMAD1 in the promoters (marked by H3K4me3) and enhancers (marked by H3K4me1) in highly conserved regions (Figure 2g-i). In addition to the early response genes, we also observed similar co-occupancy within the GATA2 promoter (Figure 2j), a known regulator of AGM hematopoiesis (77); ETV5 (Figure 2k), which is expressed in the AGM HSCs (64); and FOS (Figure 2l), which was shown recently in cooperation with Gata2, Etv6 and Gfi1b to be sufficient to convert fibroblasts to endothelial cells capable of giving rise to hematopoietic cells (78). Taken together, this bioinformatics screen suggests that the PKA/CREB and BMP pathways interact in the AGM, where emerging hematopoietic cells have endothelial characteristics (16).

BMP4 rescues AGM hematopoiesis from PKA/CREB inhibition

We investigated the interaction between the PKA/CREB and BMP pathways during AGM hematopoiesis, as predicted by bioinformatics, by examining the localization of the BMP ligand Bmp4 in the E11.5 AGM. Similar to the distribution of phospho-CREB (Figure 1c-d), Bmp4 was localized in cells lining the dorsal aorta and more strongly in the ventral than the dorsal mesenchyme, as previously reported (Figure 3a) (27). In immediately adjacent sections, the transcriptionally active BMP target protein complexes phospho-Smad1/5 and Smad1/5/8 showed a similar distribution (Figure 3b-c). Upon closer examination of the ventral floor of the dorsal aorta, we observed cells with phospho-CREB in close proximity to Bmp4 (Figure 3d).

To directly modulate the PKA/CREB pathway within embryonic tissues, we dissected the AGM from E11.5 embryos and isolated VE-cadherin+ cells (Figure 3e)—a fraction known to harbor HSCs (16)—and exposed these cells to chemical modulators or extracellular ligands. Using high-content immunofluorescence, we tracked the dynamics of pathway
modulation by quantifying the nuclear localization of proteins at a single cell level using VE-cadherin⁺ cells isolated from differentiating mESCs (Supplemental Figure 1). Exposure of these cells to KT5720, a potent inhibitor of PKA through competitive inhibition of ATP, reduced phospho-CREB in a dose-dependent fashion most strongly at 1 hour post-treatment (Figure 3f). Exposure to 5µM of KT5720 significantly reduced hematopoietic colony-forming unit (CFU) activity of VE-cadherin⁺ cells isolated from the E11.5 AGM (Figure 3g). In support of a PKA/CREB–BMP signaling interaction, BMP4 treatment of 4 ng/ml rescued hematopoietic CFUs when PKA/CREB was inhibited (Figure 3g). We obtained similar results when we repeated the experiment with E10.5 AGM (Figure 3h). However, when we treated VE-cadherin⁺ cells, which includes CD41⁺/CD45⁺ hematopoietic precursors (16, 26), the treatment had no effect, suggesting that the effect is specific to VE-cadherin⁺ cells (Figure 3i). Because KT5720 has nonspecific targets in addition to its effect on PKA, we used another PKA inhibitor, H89, which has no other overlapping nonspecific targets, and found similar effects (0.5-2µM; data not shown) (79). Rescue of hematopoietic CFUs suggests that BMP4 acts downstream of PKA/CREB signaling in ex vivo culture.

To assess the hematopoietic function of PKA/CREB in vivo, we transplanted isolated E11.5 VE-cadherin⁺ cells that were exposed to chemical compounds or ligands into lethally irradiated adult recipients. We monitored donor chimerism based on the percentage of CD45.2⁺ donor cells and assessed lineage contribution based on Gr⁺/Mac1⁺ myeloid cells, CD3⁺ T cells, and B220⁺ B cells (Figure 3j). Exposure to 5µM of KT5720 reduced the percentage of donor chimerism as compared to the DMSO-treated controls (Figure 3k). On the other hand, BMP4 treatment rescued the percentage of donor chimerism from PKA/CREB inhibition (Figure 3k). These results suggest that PKA/CREB is required for in vivo hematopoietic activity and that BMP4 can compensate for the inhibition of PKA/CREB.

Smad signaling is correlated with PKA/CREB activation

We explored the temporal dynamics of PKA/CREB and BMP signaling activation to investigate a link between these pathways. We used high-content immunofluorescence to quantify the levels of phospho-CREB and phospho-Smad1/5/8 on a single cell level over time using VE-cadherin⁺ cells from differentiating mESCs. Activation of the PKA/CREB pathway using forskolin, an adenylyl cyclase activator, increased phospho-CREB in a dose-dependent manner (Figure 4a). When we quantified phospho-Smad1/5/8 over time in forskolin-treated cells, we saw an increase concomitant with an increase in phospho-CREB (Figure 4b). Both
doses of forskolin increased phospho-Smad1/5/8 at two hours post treatment with the higher
dose downtrending towards eight hours and the lower dose uptrending. When compared to
BMP4-treated cells, phospho-Smad1/5/8 in forskolin-treated cells displayed distinct temporal
dynamics. Taken together, phospho-Smad1/5/8 dynamics correlate with that of phospho-
CREB.

Forskolin-treatment promotes AGM hematopoiesis

In the absence of exogenous BMP ligands in culture, the presence of phospho-
Smad1/5/8 in forskolin-treated cells suggests that PKA/CREB activates the BMP pathway to
promote AGM hematopoiesis. We treated VE-cadherin^+ cells from the E11.5 AGM with
forskolin and observed a significant increase in the number and size of multipotent GEMM
colonies (Figure 4c-d) whereas lineage-restricted colonies were less affected. We also
observed a similar trend with BMP4 treatment but the result was not significant (Figure 4c; p
= 0.2). Both forskolin and BMP4 treatments have similar pro-hematopoietic effects on CFUs.

To assess whether PKA/CREB activation collaborates with pathways independent of
BMP to promote hematopoiesis, we combined BMP4 and forskolin treatments. If forskolin
activates hematopoietic pathways other than BMP, then the combination treatment should
increase CFUs above that of forskolin treatment alone. However, 1µM forskolin combined
with BMP4 did not result in additional enhancement (Figure 4c). The higher dose of forskolin
combined with BMP4 had an inhibitory effect on CFUs when compared to the control
(Figure 4c), suggesting dose-sensitivity possibly due to the role of BMP as a concentration-
dependent morphogen.

Activation of adenylate cyclase can result in activation of Epac1/2 in addition to PKA
(80) but Epac1 and Epac2 expression were absent in the AGM (Supplemental Figure 2a-b).
Moreover, forskolin treatment did not result in activation of the Epac target pathway
phospholipase C–epsilon (Supplemental Figure 2c-d), thus excluding its role in AGM
hematopoiesis.

Because CREB activation drives anti-apoptotic signals through induction of bcl-2
expression in B-cells (81), we examined the effect of forskolin or BMP4 treatment on the
viability of AGM VE-cadherin^+ cells. Cells exposed to forskolin or BMP4 did not have
significant changes in the percentage of apoptotic cells compared to the control as assessed
by Annexin V staining (Supplemental Figure 3a). Therefore, it is unlikely that the increase in
hematopoiesis is due to increased cell survival.
BMP4 treatment is known to expand the c-Kit low fraction of AGM cells in culture (82). If forskolin treatment induces the BMP pathway, then forskolin exposure should induce a similar surface phenotype as BMP4 exposure. Accordingly, forskolin treatment resulted in a modest trend towards an increase in c-Kit low fraction that is comparable to BMP4 treatment alone (Supplemental Figure 3b).

HSCs reside in the VE-cadherin\(^+\)CD45\(^+\) fraction of the E11.5 AGM (16). We tested whether the forskolin or BMP4 treatment of AGM VE-cadherin\(^+\) cells increases the absolute number of VE-cadherin\(^-\)CD45\(^+\) cells. However, we did not observe any significant increase in these cells with forskolin treatment (Supplemental Figure 3c-d). This suggests that there is not an appreciable change in the number of phenotypic HSCs but rather a detectable change in HSC functionality.

Pro-hematopoietic effect of forskolin is dependent on BMP type I receptors

Because genomic CREB binding sites overlap with SMAD1 sites (Figure 2b, 2f), we explored whether forskolin activated SMAD1 target genes directly through CREB or whether the effect was dependent on the secretion of a BMP ligand. To test this, we used two different but specific BMP type I receptor ALK2/3/6 inhibitors LDN193189 and dorsomorphin (83, 84). In agreement with previous reports on BMP signaling (23, 27), dorsomorphin at 10µM significantly reduced the hematopoietic CFUs as compared to the control (Figure 4c). LDN193189 at 4µM showed a trend towards reduced hematopoietic activity but was not significant (Figure 4c; p-value = 0.11). When forskolin treatment was combined with LDN193189 or dorsomorphin, CFU activity was significantly diminished as compared to the forskolin treatment alone (Figure 4c). Additionally, CFU activity from the combination treatments was similar to inhibitor treatments alone. A similar result was obtained when we repeated the experiment with E10.5 AGM (Figure 4e). When we treated VE-cadherin\(^+\) cells, which includes CD41\(^+\)/CD45\(^+\) hematopoietic cells, the treatment had no effect, confirming that the effect is specific to VE-cadherin\(^+\) cells (Figure 4f). Together, these data suggest that the pro-hematopoietic effect of forskolin is dependent on BMP type I receptors.

To confirm this with in vivo transplantation experiments, we sorted VE-cadherin\(^+\) cells from the E11.5 AGM and treated with forskolin, BMP4 ligand, or an inhibitor of the BMP type I receptor LDN193189. We then transplanted these treated cells into irradiated recipients and monitored donor chimerism. In agreement with the CFU data, forskolin and BMP4 showed a trend towards increased hematopoietic engraftment and the combination
treatment did not result in an additional increase in chimerism (Figure 4g). Multi-lineage engraftment upon secondary transplantation confirmed the long-term potential of these HSCs (Figure 4h). Moreover, hematopoietic engraftment was modestly decreased with the inhibition of BMP type I receptors (Figure 4g), confirming a ligand-dependent effect on engraftment. Curiously, some LDN193189-treated samples engrafted suggesting that BMP signaling may be required for specification of HSCs but not thereafter (Figure 4g).

Although the hematopoietic effect of forskolin was dependent on the BMP type I receptors, the source of BMP ligands was unknown because serum-free media was used to culture VE-cadherin+ cells. We hypothesized that sorted VE-cadherin+ cells are able to release Bmp4 ligands and signal to other VE-cadherin+ cells via paracrine or autocrine signaling. To confirm this, we sorted VE-cadherin+ cells from the AGM and quantified Bmp4 ligand release via high-content immunofluorescence following PKA/CREB activation with forskolin. Bmp4 secretion per cell was significantly increased with forskolin treatment (Figure 4i). Conversely, inhibition of PKA/CREB signaling via the H89 compound decreased Bmp4 secretion (Figure 4i) as well as the BMP target phospho-Smad1/5/8 (Figure 4j). We found instances of phospho-Smad1/5/8 in VE-cadherin+ cells near cells secreting the Bmp4 ligand as well as phospho-Smad1/5/8 in Bmp4 secreting cells, indicative of both autocrine and paracrine signaling (Figure 4k).

Global transcriptome shift demonstrating BMP pathway activation

To confirm the BMP pathway activation via microarray analysis, we evaluated gene expression changes in forskolin or BMP4-treated VE-cadherin+ cells from the AGM. First we investigated whether forskolin-treated cells upregulated BMP ligands. Indeed, forskolin modestly upregulated Bmp2/4, which are both ligands of the BMP type I receptors (Figure 5a). The expression of the BMP type I receptors Bmpr1a or Bmpr1b were not altered when compared to the control consistent with a ligand-dependent effect (Figure 5a). Forskolin treatment also upregulated genes co-bound by CREB and SMAD1 (Figure 2g-l) but the effect was less prominent than BMP4 treatment alone (Figure 5a).

Because the effect of forskolin on selected BMP targets was weaker than BMP4, we assessed whether we could detect a global upregulation in BMP target genes in the forskolin-treated cells. BMP4 treatment significantly upregulated 147 genes (p-value cut-off 0.01). Forskolin-treated cells also had a significant global upregulation in these BMP target genes.
that are present (Figure 5b; p-value = 0.02), thus confirming that PKA/CREB activation results in global BMP target gene upregulation.

Given the upregulation of the BMP target genes, we expected an overlap in the hematopoietic transcription factors significantly upregulated by forskolin or BMP4 treatment. Surprisingly, there was no overlap of upregulated transcription factors (Figure 5c). We hypothesized that this was due to the short duration of treatment that resulted in upregulation of genes below the significance threshold. To test this further, we examined the genes upregulated by the combination treatment of forskolin and BMP4 and found a stronger activation of factors important for AGM HSC formation, such as \textit{Gata2} (73) and \textit{Gata3} (85) as well as factors important for adult HSC maintenance such as \textit{Pbx1} (86) compared to either treatment alone (Figure 5c).

Finally, we confirmed the upregulation of several BMP target genes (\textit{Id1}, \textit{Id3} and \textit{Gata2}) upon forskolin treatment by quantitative RT-PCR (Figure 5d). Together, these data suggest that forskolin and BMP4 upregulate common BMP targets, including those with known roles in hematopoiesis.

**CREB-BMP in the hemogenic endothelium**

To confirm the activity of CREB-BMP signaling in the hemogenic endothelium, we analyzed published microarray data on the hemogenic endothelium characterized using the Runx1+23 enhancer-GFP transgenic mice (87). At the onset of circulation, both the hemogenic endothelium and nonhemogenic endothelium upregulated selected CREB-BMP targets, whereas hematopoietic progenitors did not (Figure 5e). Global BMP target upregulation was significantly increased only in the hemogenic endothelium as compared to the hematopoietic progenitors but not in the nonhemogenic endothelium (Figure 5f). Together with the immunostaining and CFU data (Figure 1d, 3i, 4f), this suggests that hemogenic endothelial cells respond to CREB-BMP signaling but hematopoietic progenitors do not.

**Shear stress dependence of the CREB-BMP signaling axis**

We hypothesized that the PKA/CREB-BMP signaling axis is a blood-flow dependent pathway because phospho-CREB in VE-cadherin$^+$ cells increased with shear stress \textit{in vitro} (Figure 1f) and because the localization of the factors involved in this signaling axis were expressed close to or within the cells lining the dorsal aorta (Figure 3a). To confirm this, we
examined E9.5 murine embryos lacking a heartbeat and intravascular flow due to a homozygous mutation in the Na\(^{+}/\)Ca\(^{2+}\) exchanger Ncx1 (88). These mice are shown to have shear-stress dependent hematopoietic defects at E9.5 in the para-aortic splanchnopleura (PSp) (51, 89), which later matures into the AGM.

The embryonic lethality of these mice at E9-10 precludes further analysis of definitive hematopoiesis via transplantation (15, 88). Despite this limitation, we examined histologic sections of Ncx1 null embryos to explore the relationship between intravascular flow and the PKA/CREB-BMP signaling axis. In the E9.5 wild type littermates, the two dorsal aortae have fused to a single dorsal aorta in agreement with previous studies (Figure 6a-e) (90). Phospho-CREB was present in the mesenchyme surrounding the dorsal aorta, the cells lining the dorsal aorta and the neural tube (Figure 6b). In agreement with the E11.5 distribution of phospho-CREB and Bmp4 (Figure 3a), phospho-CREB and Bmp4 in the E9.5 embryos were also present at higher levels in the ventral mesenchyme surrounding the dorsal aorta (Figure 6b-c). In the E9.5 Ncx1 null embryos, we observed a delay in the fusion of the dorsal aortae that occurs between E8-9.5 (Figure 6f-j), which is consistent with the growth retardation phenotype previously documented (88). Consistent with the PKA/CREB-BMP axis as a blood flow-responsive pathway, we observed a decrease in phospho-CREB in the mesenchyme surrounding the two dorsal aortae and a stronger decrease in phospho-CREB in the cells lining the dorsal aorta when compared to the neural tube (Figure 6g). Bmp4 was similarly decreased but not absent when compared to the control (Figure 6h-i). Quantification of phospho-CREB\(^{+}\) cells in multiple sections revealed significant decrease with Ncx1 KO embryos (Figure 6k). Similar result was obtained with phospho-Smad1/5/8, a BMP target protein (Figure 6l). Because regions demonstrating incomplete fusion of the dorsal aortae can have different signaling dynamics, we also examined Ncx1 null embryo sections that have a near complete fusion of the dorsal aortae and observed a similar decrease in phospho-CREB and Bmp4 (data not shown).

To determine whether exogenous shear stress can increase PKA/CREB-BMP signaling in the AGM, we sorted VE-cadherin\(^{+}\) cells from the AGM and subjected them to 6 hours of shear stress. Shear stress increased phospho-CREB levels, and this effect was mitigated by 2µM of H89, a PKA inhibitor (Figure 6m). Furthermore, Bmp4 was detected upon application of shear stress but also decreased with H89 treatment (Figure 6m). BMP4 transcript is found in endothelial cells exposed to shear stress, especially oscillatory shear stress (Figure 6n) (91). This may be similar to the retrograde flow experienced before
formation of cardiac outflow tract cushions and valves at E11.5 and E13.5 respectively (92, 93).

Finally, we examined whether PKA/CREB-BMP signaling axis can restore the hematopoietic activity in the absence of shear stress. AGM explants from E9.5 Ncx1 KO embryos have reduced hematopoietic progenitor activity in the PSp due to the lack of blood flow (51). When AGM explants from E9.5 Ncx1 KO embryos were exposed to exogenous BMP4 or forskolin (1µM) for 24 hours, CFU activity was increased, suggesting a partial rescue via modulation of the PKA/CREB-BMP signaling axis (Figure 6o). Taken together, our data suggests that the PKA/CREB-BMP axis is a blood flow dependent signaling axis responsible for hematopoietic development (Figure 7).

**Discussion**

In this study, we have shown that the PKA/CREB pathway is a novel shear-responsive pathway that acts via BMP to promote AGM hematopoiesis. Chemical modulation of the PKA/CREB-BMP signaling axis implicates PKA/CREB signaling as necessary for AGM hematopoiesis, and demonstrates dependence on BMP type I receptors. Surprisingly, VE-cadherin$^+$ cells secrete BMP ligands in response to the activation of PKA/CREB, thus promoting hematopoietic development in neighboring VE-cadherin$^+$ cells in an autocrine-paracrine manner. At the genomic level, the BMP target SMAD1 and CREB co-regulate several factors involved in hematopoietic induction such as Gata2. In Ncx1 null embryos lacking intravascular flow, the PKA/CREB-BMP signaling axis is blunted. Our data establish a mechanism by which mechanical forces are translated into molecular signals for governing HSC emergence.

The relationship between biomechanical forces and hematopoietic development has only recently begun to be elucidated (51-53). The striking correlation between the onset of a heartbeat at E8.5 in the mouse, and the emergence of HSCs with the ability to engraft various types of recipients—such as neonatal recipients at E9 (14, 94), and adult recipients at E10-11 (15, 16)—suggests that biomechanical forces and hematopoietic development are intricately linked. Past studies have focused on the nitric oxide pathway as an important mediator of shear-stress (51-53). Blastula transplantation experiments in zebrafish have demonstrated a cell autonomous requirement for nitric oxide signaling during hematopoietic development (52), but further work is required to decipher whether nitric oxide has a direct inductive effect.
on hematopoietic precursors or whether this is a secondary effect due to vasodilation. Shear-dependent programs in zebrafish and mouse may also differ because blood flow is not required for the initiation of runx1+ definitive hematopoiesis in zebrafish (53), but is required for hematopoietic colony formation from the E9 mouse PSp, an early precursor to the AGM (51). Here we suggest that the PKA/CREB-BMP signaling axis is a novel shear-dependent pathway that has a direct inductive effect on VE-cadherin+ precursors. This induction of a hematopoietic program in VE-cadherin+ cells involves a phenomenon called the endothelial-to-hematopoietic transition because our effects are specific to the VE-cadherin+ but not the VE-cadherin- fraction, which contain mature hematopoietic cells. Indeed, most of the emerging hemogenic endothelial cells as defined by Sca1-GFP are also phospho-CREB+. Interestingly, activation of PKA results in upregulation of Foxc2, which has been recently identified to play a role in the hemogenic endothelium (95). The endothelial-to-hematopoietic transition has garnered recent interest because lineage tracing and live-imaging studies have shown that HSCs emerge from the VE-cadherin+ vascular endothelium in the AGM (18, 20, 21). Efforts to understand this phenomenon in human have culminated in various protocols for ESC differentiation (38).

Numerous studies on BMP signaling and AGM hematopoietic development have hinted at its role in the endothelial-to-hematopoietic transition. Bmp4 was originally identified in the murine stromal lines derived from the urogenital ridges of the AGM and have been found to increase the in vivo repopulating ability of AGM HSCs when co-cultured with stromal lines for 3 days (27). In their model, Bmp4 is a ventral signal arising from the mesenchyme that promotes hematopoietic development. Zebrafish data largely fine-tunes this theory by suggesting that the BMP ligands from the ventral mesenchyme and the pronephros region polarize the dorsal aorta opposed with Hedgehog ligands from the notochord (23). In our study, BMP ligands are found also ventrally but we propose a new source of BMP ligands in the AGM that has been previously under-appreciated. We show that BMP ligands are generated autonomously within VE-cadherin+ cells upon PKA/CREB stimulation via shear stress (Figure 4i, 5a). Combined with other signals from the mesenchyme, BMP ligands activate a hematopoietic transcriptional program in neighboring VE-cadherin+ cells that have been exposed to shear stress, thus promoting HSC emergence. Emerging hemogenic endothelial cells have the signature of global BMP target gene upregulation. Evidence suggests that CREB can directly bind to the promoter of genes encoding BMP ligands in other cell types (96). However, the question of direct binding of CREB to this promoter
remains to be answered in the rare AGM cells that give rise to HSCs. Although we did not deplete the rare CD41 or CD45 positive hematopoietic fractions, the observed hematopoietic output post 8 hour exposure to chemicals and ligands is suggestive of cell fate transitions rather than hematopoietic cell division. To confirm this, we calculated the mean doubling time of AGM hematopoietic cells using published data (97). Since E11.5 AGM-derived cells undergo expansion from \( \sim 3640 \) to \( \sim 32130 \) cells in a 96-hour period, we estimated that the doubling time is approximately 30 hours, which is much greater than our exposure time.

The ligand and mechanosensors responsible for the activation of PKA is still in question. Many different signaling modules can activate PKA including prostaglandins (e.g. PGE2) through the prostanoid receptor (34), inflammatory cytokines through interferon signaling (98), calcium flux, purinergic and \( \beta \)-adrenergic signaling. Due to recent evidence suggesting that PGE2 is synthesized in response to shear stress (54, 55), and the proposed role of PGE2 in embryonic hematopoiesis (34), PGE2 is a candidate upstream of PKA for the exploration of shear stress-mediated signaling. Indeed, PGE2 can upregulate \( BMP4 \) in human \( CD34^+ \) cord blood cells (99). However, as many of these pathways can activate PKA, further studies will be required to dissect the relative contributions of individual pathways that signal through PKA/CREB in distinct cell types.

Biomechanical forces likely have a multifactorial effect on emerging HSC precursors that cannot be replicated by the modulation of a single signaling pathway. Although the PKA/CREB-BMP signaling axis is necessary for HSC emergence, the modest and incomplete rescue of Ncx1 KO embryos upon exogenous administration of BMP ligands or PKA agonists suggest that other pathways such as Wnt or Notch pathways are also at play.

This current study shows using sorted VE-cadherin\(^+\) cells from the mouse AGM as well as \( Ncx1 \) null embryos that the PKA/CREB-BMP signaling axis is a blood-flow dependent pathway that has hematopoietic inductive effects on VE-cadherin\(^+\) cells. Further studies will help explain the concentration-dependent effects of the PKA/CREB and BMP pathways and address why only rare cells are endowed with the required gene expression programs to become HSCs. Study of the signaling pathways linking biomechanical stimuli to intracellular events will help elucidate the pathways required to promote HSC formation from ESCs for cellular therapy.
Figure 1 adapted from Kim et al.
Figure 1 – Phosphorylated CREB is present in the AGM and increased by shear stress.

A) Gene set enrichment analysis for CREB target genes using the two-sample Kolmogorov–Smirnov test comparing each hematopoietic tissue against an embryonic stem cell-derived embryoid-body (EB). BM = bone marrow; FL = fetal liver; YS = yolk sac; EPOCH = HoxB4 induced. Data is from McKinney-Freeman et al. 2012 (GSE37000).

B) Expression of CREB across various hematopoietic tissues.

C) Immunofluorescence section of E11.5 AGM showing the localization of phospho-CREB (pCREB) in the cells lining the endothelium and ventral mesenchyme. ao = dorsal aorta; nc = notochord; nt = neural tube, vm = ventral mesenchyme. Scale bar = 100um.

D) Immunofluorescence section of E10.5 AGM showing the localization of pCREB and Sca1-GFP. ao = dorsal aorta; nc = notochord; nt = neural tube, vm = ventral mesenchyme. Scale bar = 100um.

E) Quantification of pCREB+ in Sca1+ population and Sca1+ in pCREB+ population in cells lining the dorsal aorta. n = 5

F) Immunoblot blot of VE-cadherin+ cells from differentiated mESCs that were exposed to shear stress for 6 hours.
Figure 2 adapted from Kim et al.
Figure 2 – Genomic interaction of CREB and SMAD1 in K562 cells.

A) Experimental schema showing the strategy for analysis of CREB-overexpression and genomic occupancy.

B) Top two quartiles of top CREB co-occupants in promoters of genes responding to CREB overexpression. Rows indicate genes upregulated by CREB-overexpression and columns indicate binding of TFs.

C) Global CREB and SMAD1 promoter co-occupancy at active promoters.

D) SMAD1 co-occupancy at CREB-bound enhancers.

E) Annotation of top 3000 scoring CREB and SMAD1 co-bound genes using PANTHER PATHWAY annotation.

F) Validation of CREB and SMAD1 genomic occupancy with phospho-CREB and phospho-Smad1/5/8 ChIP-seq.

Genomic view of CREB and SMAD1 binding in the G) ID1, H) ID2, I) ID3, J) GATA2, K) ETV5, L) FOS gene region. Phastcons track shows species conservation between 46 species.
Figure 3 adapted from Kim et al.
Figure 3 – BMP4 rescues hematopoiesis from PKA/CREB inhibition.

A) Immunofluorescence section of the E11.5 AGM showing the co-localization of phospho-CREB and Bmp4. ao = dorsal aorta; nc = notochord; nt = neural tube. Scale bar = 100um.

B) Immunofluorescence of adjacent section to A) showing the localization of phospho-Smad1/5/8.

C) Immunofluorescence of adjacent section to A) showing the localization of phospho-Smad1/5.

D) Zoomed view of the ventral mesenchyme indicated by an arrow in A).

E) Experimental schema for working with AGM-derived VE-cadherin+ cells.

F) Normalized high-content immunofluorescence of the phospho-CREB dynamics upon KT5720 treatment over 8 hours. Scale bar = 100um. n = 2.

G) Colony-forming unit assay (CFU) of sorted VE-cadherin+ cells from E11.5 per embryo equivalent (e.e) treated with various compounds for 8 hours. n = 3 – 5.

H) CFU assay of sorted VE-cadherin+ cells from E10.5 per e.e treated with various compounds for 8 hours. n = 3.

I) CFU assay of sorted VE-cadherin- cells from E10.5 per e.e treated with various compounds for 8 hours. n = 3.

J) Example of peripheral blood analysis for donor chimerism for myeloid and lymphoid cell contribution using flow cytometry.

K) Donor chimerism levels at 12-weeks post-transplantation. 2.5 e.e. were used per recipient. Averages are shown in red. One-sided Wilcoxon rank-sum test was performed on log-transformed values.
Figure 4 adapted from Kim et al.
Figure 4 – Pro-hematopoietic effect of forskolin is dependent on BMP type I receptors.

A) Normalized high-content immunofluorescence of the phospho-CREB dynamics upon forskolin treatment over 8 hours. Scale bar = 100um. n = 2.

B) Normalized high-content immunofluorescence of the phospho-Smad1/5/8 dynamics upon forskolin or BMP4 treatment over 8 hours. n = 2.

C) CFU assay of sorted VE-cadherin+ cells per embryo equivalent (e.e) treated with various compounds for 8 hours before assay. n = 3 – 9.

D) Representative multipotent CFUs from C) at 12 days post-plating.

E) CFU assay of sorted VE-cadherin+ cells from E10.5 per e.e treated with various compounds for 8 hours. n = 3.

F) CFU assay of sorted VE-cadherin cells from E10.5 per e.e treated with various compounds for 8 hours. n = 3.

G) Donor chimerism levels at 12-weeks post-transplantation. Averages are shown in red. One-sided Wilcoxon rank-sum test was performed on log-transformed values.

H) Donor chimerism levels at 12-16 weeks post-secondary transplantation from top four engrafted recipients from Figure 3I, 4E. Averages are shown in red.

I) Violin plots showing the quantification of BMP4 via immunofluorescence upon forskolin or H89 treatment in VE-cadherin+ cells isolated from the E11.5 AGM. Significance was assessed using the two-sided Wilcoxon-rank sum test.

J) Violin plots showing the quantification of phospho-Smad1/5/8 via immunofluorescence upon forskolin or H89 treatment of VE-cadherin+ cells isolated from the E11.5 AGM. Significance was assessed via the two-sided Wilcoxon-rank sum test.

K) Immunofluorescence for Bmp4 and phospho-Smad1/5/8 in VE-cadherin+ cells isolated from the E11.5 AGM and treated with forskolin or H89. Red arrowhead = phospho-Smad1/5/8 near a Bmp4 secreting cell. White arrowhead = Phospho-Smad1/5/8 in the same cell secreting Bmp4. Scale bar = 50um.
Figure 5 adapted from Kim et al.
Figure 5 – Transcriptome analysis of PKA/CREB-BMP signaling in VE-cadherin+ cells.

A) Upregulation of BMP ligands and target genes upon forskolin treatment. n = 2.
B) Violin plot of BMP4-responsive target genes that are detectable via Presence/Absence calls upon forskolin treatment. Statistical significance via the two-sided Wilcoxon-rank test. n = 2.
C) Transcription factors significantly upregulated by each treatment (F = forskolin; B = BMP4). Numbers indicate genes significantly upregulated genes. n = 2.
D) Quantitative RT-PCR on selected BMP target genes Id1-3 and Gata2 upon forskolin treatment. n = 4.
E) Selected CREB and BMP target genes analyzed in the non-hemogenic endothelium, hemogenic endothelium and hematopoietic progenitors (GSE52075) (87). n = 3.
F) Violin plot of BMP4 target genes similar to B) in the non-hemogenic endothelium, hemogenic endothelium and hematopoietic progenitors (GSE52075) (87). Statistical significance via the two-sided Wilcoxon-rank test. n = 3.
Figure 6 adapted from Kim et al.
Figure 6 – Shear stress-dependent regulation of CREB-BMP axis.

A-E) Frozen sections of the E9.5 wild-type para-aortic splanchnopleura (PSp) stained with phospho-CREB and Bmp4. D-E) shows merged image of A-C. E) is a zoomed in view of the square in D. ao = dorsal aorta; nt = neural tube; vm = ventral mesenchyme. Scale bar = 100μm. White outline contains cells facing the lumen of the dorsal aorta.

F-J) Frozen sections of the E9.5 Ncx1 null para-aortic splanchnopleura (PSp) with two dorsal aortae stained with phospho-CREB, Bmp4. I-J) shows merged image of F-H. J) is a zoomed in view of the square in I. White outline contains cells facing the lumen of the dorsal aorta.

K) Quantification of phospho-CREB excluding cells in the neural tube in WT and Ncx1 KO embryos. Quantification based on three independent embryos.

L) Quantification of phospho-Smad1/5/8 excluding cells in the neural tube in WT and Ncx1 KO embryos. Quantification based on three independent embryos.

M) Immunoblot showing the effect of shear stress and/or PKA inhibitor H89 on phospho-CREB, Bmp4 on VE-cadherin+ cells sorted from the AGM.

N) Differential effect of laminar shear stress (LS) and oscillatory shear stress (OS) on the expression of BMP4 in endothelial cells (GSE20739) (91). n = 3.

O) Rescue of defective hematopoiesis in E9.5 Ncx1 PSp with forskolin or BMP4 treatment. n = 4.
Figure 7 – A model of hematopoietic induction via the PKA/CREB-BMP axis.

Shear stress induction via blood flow activates PKA/CREB, which promotes BMP ligand secretion via an unknown mechanism. The secreted BMP ligand binds to the type I BMP receptor and results in phospho-Smad1/5/8 transduction via autocrine or paracrine mechanism. Along with CREB, Smad cofactors promote hematopoietic induction in the VE-cadherin$^+$ AGM. The grey regions marked with question marks are hypothesized based on the literature and data presented in the current study.
Supplemental Figure 1 adapted from Kim et al.
Supplemental Figure 1 – Schema for processing high-content immunofluorescence data using CellProfiler.

A) Example of a DAPI signal.
B) Detection of nuclear outlines via processing of DAPI-stained nuclei.
C) Example of a secondary antibody stain.
D) Detection of a secondary antibody signal using the nuclear outlines as seeds for exploration of borders.
E) Histogram of fluorescence intensities per cell.
Supplemental Figure 2 adapted from Kim et al.
Supplemental Figure 2 – Forskolin induction does not result in Epac1 activation.

A) *Epac1* mRNA is below the detection threshold as defined by Presence/Absence calls in embryonic hematopoietic tissues but not in more adult tissues such as the fetal liver or bone marrow. Expression data is from McKinney-Freeman et al. 2012 (GSE37000).

B) *Epac2* mRNA is below the detection threshold as defined by the Presence/Absence calls in all hematopoietic tissues. Expression data is from McKinney-Freeman et al. 2012 (GSE37000).

C) Examination of the Epac target pathway Phospholipase C-epsilon reveals lack of gene set enrichment by the two-sample Kolmogorov–Smirnov test.

D) Heatmap of genes in the Phospholipase C-epsilon pathway confirming B).
Supplemental Figure 3 adapted from Kim et al.
Supplemental Figure 3 – Effects of forskolin and BMP4 treatments on VE-cadherin\(^+\) cells.

A) Quantification of cell viability using propidium iodide and Annexin V by flow cytometry after forskolin or BMP4 treatment. \(n = 2 - 3\).

B) Quantification of c-Kit and VE-cadherin positive cells after forskolin or BMP4 treatment by flow cytometry.

C) Strategy for quantification of CD45\(^+\)VE-cadherin\(^+\) cells from cultured E11.5 AGM VE-cadherin\(^-\) cells.

D) Absolute quantification of CD45\(^-\)VE-cadherin\(^+\) per embryo equivalent (e.e.) after forskolin and BMP4 treatment. \(n = 2\).
Supplemental Tables

Supplemental Table 1 – Sequence Read Archive (SRA) reference IDs for ChIP-sequencing.

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Trompouki et al. Cell 2011 (61)
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<td>Primerbank (Wang et al. NAR 2012): 26327367a1 (100)</td>
</tr>
</tbody>
</table>
**Experimental Procedures**

**Cell Culture and Differentiation**

mESCs were cultured on irradiated CF1s with Leukemia Inhibitory Factor (LIF) as described (26). Embryoid body differentiation was performed as described (26). Briefly, mESCs were aggregated via hanging drop method, then collected on day 2-2.25, and then collected on day 6 of differentiation and dissociated using DNase I, Collagenase IV and Hyaluronidase for further analysis. For shear stress applications, oscillatory shear stress of approximately 10 dynes/cm²—corresponding to an order of magnitude of previous calculations (51)—was applied to a 10 cm tissue culture dish via 150 rpm of orbital shaking. Maximum shear stress ($\tau_{\text{max}}$) was approximated via $\tau_{\text{max}} = a(\rho \eta (2\pi f)^3)^{1/2}$ where $a$ is the orbital radius, $\rho$ is the fluid density, $\eta$ is the fluid viscosity, and $f$ is the frequency of orbit (101). Variations in shear stress were decreased via peripheral cell attachment (102).

**Mouse Embryo Culture**

E9.5, E10.5 or 11.5 staged mouse embryos were obtained using timed pregnancies of C57BL/6 females unless specified. The AGM region was isolated under a dissecting microscope and dissociated to single cells using DNase I, Collagenase IV and Hyaluronidase for 20 minutes. The cells were then passed through a 40 µm filter and washed with IMDM. Cells were first stained with 1:10 dilution of VE-cadherin (11D4.1) then labeled with anti-rat IgG2a-PE (RG7/1.30) for sorting on FACSARia or anti-PE / anti-Rat MicroBeads (Miltenyi) for magnetic sorting. For magnetic sorting, each staining step was performed for 15 minutes in 2% bovine serum albumin (BSA) / IMDM (vol/vol). All antibodies were obtained from BD Biosciences unless indicated otherwise. Cells obtained via sorting were cultured in serum-free media containing 2% BSA / IMDM (vol/vol) supplemented with Insulin-Transferrin-Selenium (ITS-G; Life Technologies), 25 µg/ml ascorbic acid, 450 µM monothioglycerol, 2 mM penicillin/streptomycin/glutamate and of 20 ng/ml VEGF (R&D), 50 ng/ml IL3 (R&D), 50 ng/ml IL6 (R&D), 50 ng/ml SCF (R&D), 50 ng/ml FLT3L (R&D), 50 ng/ml TPO (R&D), 50 ng/ml IL6R (SBH Sciences). The sorted cells were reaggregated in the presence of chemicals or ligands for 8 hours in a 96-well V-bottom plate. The cells were
then dissociated with enzyme-free dissociation buffer, washed with IMDM before further analysis.

Colony Forming unit (CFU) Assays

Cells were plated into 1.5-2 ml of methylcellulose media containing interleukin-3 (IL-3), IL-6, erythropoietin (Epo) and SCF (M3434; StemCell Technologies) as described (26). CFUs [definitive erythroid (Ery), myeloid (M), granulocyte-macrophage (GM), multilineage granulocyte-erythrocyte-monocyte-megakaryocyte (GEMM)] were scored based on morphology 10 days post-plating.

Transplantation and Peripheral Blood Analysis

B6.SJL-Ptprca mice were used at 6-10 weeks of age. Mice were irradiated with a split dose of 9GY prior to transplantation. Each recipient received 1 embryo equivalent of sorted cells via tail vein injection along with 2 x 10^4 bone marrow or 2 x 10^5 splenic helper cells per experiment group from B6.SJL-Ptprca mice unless indicated otherwise. For secondary transplantations, CD45.2^+ cells were isolated from recipient bone marrow and 3 x 10^5 cells were transplanted per recipient with 2 x 10^5 splenic helper cells. Peripheral blood was collected retro-orbitally at the indicated time point post-transplantion. Red blood cells were removed with 1% dextran sulfate/0.5% EDTA/PBS (wt/vol) and treated with RBC lysis buffer (Sigma). Leukocytes were then stained in 2% serum/PBS (vol/vol) for CD45.1-FITC (A20; BD), CD45.2-PE-Cy7 (104; Biolegend), Mac1-Alexa Fluor 700 (M1/70; Biolegend), Gr1-PE (A20; BD), CD19-APC-Cy7 (6D5; Biolegend), CD3-APC (145-2C11; eBiosciences), Ter119-PE-Cy5 (TER-119; eBiosciences), B220-Pacific Blue (RA3-6B2; BD) and propidium iodide (PI). Engraftment was determined to be percentage of PI and Ter119 negative CD45.2 cells within the CD45 positive population.

Western Blotting

Cells were lysed in RIPA buffer with protease and phosphatase inhibitors (Pierce). Proteins were separated via polyacrylamide gel and transferred to an activated PVDF Membrane. Membrane was blocked for 1 hour using 5% milk or 5% BSA in PBST or Odyssey Blocking
Buffer (LI-COR) and probed with primary antibodies overnight at 4°C. After washing with PBST, HRP-conjugated secondary antibodies were used for 1 hour at room temperature (GE Healthcare). Protein level was detected using SuperSignal West Pico and Femto Luminol reagents (Thermo Scientific). The following antibodies were used: phospho-CREB (Cell Signaling #9198), CREB (Cell Signaling #9197), phospho-S675-β-Catenin (Cell Signaling #4175), active β-Catenin (Cell Signaling #4270), β-Catenin (BD #610154), Bmp4 (Millipore #MAB1049).

High-content Immunofluorescence Microscopy

Cells were cultured in 96-well optical-bottom plates (Costar #3904). Cells were fixed with 2% paraformaldehyde for 15 minutes and permeabilized with methanol at -20°C for 10 minutes. Cells were blocked with Odyssey Blocking Buffer (LI-COR) for 1 hour at room temperature. Cells were stained in this blocking buffer. Primary antibody was incubated overnight and secondary antibody was incubated at room temperature for 1 hour. Cells were imaged with ImageXpress Micro. Nine image fields were collected for each well and processed with image segmentation routine derived from CellProfiler (103), with Otsu global thresholding for nuclear identification. Single cell fluorescence intensity was calculated as mean fluorescence intensity per nucleus or per cell.

Histological Analysis

For paraffin sections, E10.5 or E11.5 mouse embryos were fixed in 4% paraformaldehyde on ice for 1 hour before embedding in paraffin and sectioning. Paraffin embedded sections were dewaxed with xylene and rehydrated with decreasing percentages of ethanol. Antigen retrieval was performed using 10mM sodium citrate buffer pH 6 using a pressure cooker at 95°C for 30 minutes.

For frozen sections, E9.5 mouse embryos were fixed in 4% paraformaldehyde on ice for 2-3 hours, followed by equilibration in 30% sucrose in PBS solution overnight. The tissues were placed in 1:1 30% sucrose/OCT (Tissue-Tek, Electron Microscopy Sciences) solution for 1 hour, 100% OCT for 1 hour, then embedded in 100% OCT compound. The blocks were
immediately frozen on dry ice with isopropanol and stored at -80°C. The sections were cut at 20 µm with a Leica CM3050 S cryostat.

Cells were permeabilized with Triton X-100 (0.01%) and blocked for 1 hour with 10mM glycine in host serum corresponding to the secondary antibody before staining. Immunostaining was performed with the following primary antibodies overnight: phospho-CREB (Cell Signaling #9198), CREB (Cell Signaling #9197), Bmp4 (Millipore #MAB1049), phospho-Smad1/5 (Cell Signaling #9516), phospho-Smad1/5/8 (Cell Signaling #9511), GFP (Abcam #ab6673). For secondary antibody staining, Alexa 488-, Alexa568- or Alex 647-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies were used. DAPI stained cells were mounted with VECTASHIELD mounting medium (Vector Labs). All images were obtained with Nikon Eclipse 90i microscope with a 20x or 10x objective with CoolSNAP HQ2 (Photometrics) using NIS-Elements. Images were processed using ImageJ.

Gene Expression Analysis

RNA from isolated VE-cadherin+ cells was isolated with the RNeasy Micro Kit (Qiagen). RNA was hybridized to Affymetrix Mouse Genome 430 2.0 Array. Microarray data were analyzed using R/Bioconductor. Raw microarray signal intensities were RMA-summarized, quantile-normalized, and filtered for duplicate genes using nsFilter to 20757 genes before analysis (104). Microarray data from this study has been deposited to GEO database under accession number GSE62517. For quantitative RTPCR confirmation, RNA from isolated VE-cadherin+ cells was isolated with the RNeasy Micro Kit (Qiagen). cDNAs were prepared with SuperScript III reverse transcriptase (Invitrogen). Quantitative RTPCR was performed with Brilliant SYBR Green QPCR Mix (Strategene) on the MX3000/6P machine using primer sequences listed in Supplemental Table 2.

ChIP Sequencing and Library Generation

K562 cells were crosslinked with 1% paraformaldehyde for 15 min at 20°C. Reaction was quenched with final concentration of 0.125M glycine. Cells were washed with PBS, pelleted, and resuspended in SDS-ChIP buffer (20 mM Tris-HCl pH 8.0, 150mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) with protease inhibitor. Chromatin was sheared to ~200-500 bp with 30s x 5, 40s x 12 cycles of sonication. Sonicated chromatin was pre-cleared with
Protein-G agarose beads (Roche) and incubated with 10 µg antibody overnight at 4°C. Following antibodies were used: p-CREB (scbt: sc7978X), p-Smad1/5/8 (scbt: sc12353X). G agarose beads were added to the ChIP reactions and incubated for 2 hours at 4°C. Beads were washed twice with 1 ml of low salt wash buffer (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with 1 ml of high salt wash buffer (50mM HEPES pH 7.5, 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with 1 ml of LiCl wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP-40, 250 mM LiCl), and twice with 1 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0). The chromatin was eluted twice in 150ul of SDS elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) at 72°C. Eluted chromatin was reverse-crosslinked at 65°C overnight. An equal volume of TE was added the next day (300µl). ChIP DNA was treated with 1µl of RNaseA (10mg/ml) for 1 hour at 37°C, and with 3µl of proteinase K (20mg/ml) for 3 hours at 37°C, and purified using phenol-chloroform extraction, followed by QIAquick PCR purification spin Columns (Qiagen). Input ChIP samples were held after the pre-clear step and processed similarly from reverse cross-linking step to the end. Purified ChIP DNA was measured in Qubit (Invitrogen). 2-10ng of purified ChIP DNA was used to prepare sequencing libraries (NEB next generation ChIP sequencing Kit). Libraries were checked on Bio-analyzer for quality control. ChIP sequencing was performed using Illumina TM Hiseq 2000.

ChIP-Seq Analysis

Raw sequence read archive (SRA) files were obtained and data sets were aligned using Bowtie (version 1.0.0) (105) to build version hg19 of the human genome using the parameters -k 2 -m 1 -n 2 -best. SRA file IDs are compiled in Supplemental Table 1. Duplicates were removed and reads were extended by 200 bp before visualization using IGVtools (106). For peak-calling, we used MACS (Model based analysis of ChIP-Seq) version 1.4.1 (107) algorithm to identify statistically significant regions of ChIP-Seq enrichment using the threshold p-value 1e-8. To display binding at promoters, we calculated reads per million mapped reads per bp in 50 bp bins ± 3 kb around the transcription start site of transcribed genes. Analysis was performed on the HMS Orchestra High Computing Cluster.
Statistical Analysis

n represents the number of biological replicates. Two-tailed unpaired Student’s t-tests were used unless indicated. Error bars show standard error. Statistical significance is indicated by \* \( P = 0.05 \), \** \( P = 0.01 \), \*** \( P = 0.001 \).
Chapter 3 - Arid3a-interferon/Stat1 signaling promotes AGM HSC maturation

At the time of submission of this dissertation, the work presented in this chapter has been submitted for publication as an Article to Journal of Experimental Medicine.

Title: Arid3a-interferon/Stat1 signaling promotes AGM HSC maturation

Peter Geon Kim, Samantha J. Ross, Catherine Rhee, June V. Harriss, Hayley O. Tucker, George Q. Daley

1Stem Cell Transplantation Program, Division of Pediatric Hematology/Oncology, Howard Hughes Medical Institute, Boston Children’s Hospital and Dana Farber Cancer Institute; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School; Harvard Stem Cell Institute; Boston, MA 02115, USA.

2Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712, USA
Introduction

In the developing mouse embryo, the first hematopoietic stem cells (HSCs), as defined by their ability to repopulate lethally irradiated adult recipients, are detected in the aorta-gonad-mesonephros (AGM) region at embryonic day 11.5 (E11.5) (15, 108). These HSCs later migrate to the fetal liver (FL) before reaching the bone marrow (BM) to sustain adult hematopoiesis. HSCs at various embryonic stages differ molecularly and functionally, reflecting a developmental maturation program. When their expression profiles are analyzed by a Bayesian classifier, AGM HSCs resemble macrophages and are enriched for gene-ontology terms such as “cell communication”, and “positive regulation of angiogenesis”, reflecting their migratory proclivity and endothelial origin (64). Such a molecular signature captures a known phenomenon termed the endothelial-to-hematopoietic transition, in which AGM-derived HSCs emerge through an endothelial intermediate (18-22). In contrast, FL HSCs resemble BM HSCs and their gene expression signature clusters away from AGM HSCs (64). When competing against BM helper cells, AGM HSCs demonstrate reduced repopulation potential in irradiated adult recipients compared to FL and BM HSCs (14). In contrast, transplantation of AGM HSCs is more permissive in neonatal than adult recipients (14). Hence, AGM HSCs are functionally less ‘mature’ than FL and BM HSCs in their capacity to engraft in the adult bone marrow niche.

One of several known regulators of HSC development is Sox17, a transcription factor that is required for the maintenance of embryonic and neonatal HSCs but not adult HSCs (109). However, the signaling pathways that promote the developmental maturation of AGM HSCs remain poorly characterized. Understanding the differences between AGM and adult HSCs is an important and challenging goal. Initial studies using HSCs derived from pluripotent stem cells via ectopic induction of the homeobox gene Hoxb4 demonstrated long-term primary and secondary transplant capability; however, skewed myeloid lineage potential and aberrant surface antigen phenotypes suggested their developmental immaturity (44, 110). Molecular profiling has implicated defective Notch signaling underlying this difference (64), but further experiments are needed to fully interrogate these findings. Additional attempts to faithfully recapture HSCs via transgene induction from pluripotent cell-derived sources revealed similar defects in lymphoid reconstitution (47, 48). Curiously, HSCs reprogrammed from committed adult blood cells seemed unaffected by these limitations (46). The correlation between the cell-of-origin of these induced-HSCs and the nature of identified
developmental defects highlights our imperfect understanding of the molecular programs governing the difference between AGM HSCs and adult HSCs.

Recent studies have suggested that inflammatory pathways mediated by interferon-gamma (IFNg) and tumor necrosis factor (TNF) signaling are important for HSC emergence in the AGM (111-113). Here we focus on the role of interferon-alpha (IFNa) and its effects on the functional maturation of AGM HSCs.

Results

Jak-Stat deficiency in AGM HSCs predicted by weighted gene co-expression network analysis

To identify co-regulated sets of genes (called modules) during hematopoietic development, we performed a weighted gene co-expression network analysis (WGCNA) of our previously published microarray dataset for HSCs at different developmental stages (64, 114). In contrast to McKinney-Freeman et al. in which 66 modules were reported, here we merged similar modules in an effort to identify a single module that was deficient in AGM HSCs but gradually increased in FL and adult BM HSCs (Figure 1a-b). This analysis yields greater statistical power to detect trends by gene ontology analysis or KEGG pathway analysis (115, 116). Through gene ontology analysis, we identified enrichment for terms such as “immune system process,” “leukocyte activation,” and “lymphocyte activation” (Figure 1c). KEGG pathway analysis identified the two highest enriched terms as “Jak-Stat signaling pathway” and “cytokine-cytokine receptor interaction” (Figure 1d). Thus, we determined that a co-expressed gene set is linked to the Jak-Stat signaling pathway accounting for key differences between AGM and FL/adult BM HSCs.

Multiple cytokines, including interleukins (IL), tumor necrosis factor (TNF) and IFNs, can activate the Jak-Stat signaling pathway and many of these pathways have known roles in inflammation. Recent reports indicate that the TNF and IFNg signaling pathways are required for the emergence of AGM HSCs (111-113), suggesting that inflammatory signaling plays a role during embryonic hematopoiesis. Therefore, we performed gene set enrichment analyses (GSEA) to analyze gene sets differentially expressed in AGM HSCs compared to FL and adult BM HSCs in response to ILs, TNF, and IFNs (Figure 1e) (117). Additionally, because numerous Stat proteins exist, we examined Stat1, 3 and 5A responsive gene sets (Figure 1e). We discovered that AGM HSCs are enriched for the activation of the IL3 and
IL-6 signaling pathways as well as their downstream target Stat3 as compared to FL and adult HSCs (Figure 1e, f). This finding is consistent with published reports implicating IL3 and IL6 in AGM HSC emergence (118, 119). In accordance with a recent study (112), the TNF-responsive genes and the downstream NF-κB pathway were both enriched in AGM HSCs as compared to FL and adult BM HSCs (Figure 1e, g). Interestingly, the IFN pathway—including the downstream Stat1-responsive genes—were overall relatively deficient in the AGM HSCs compared to the level of enrichment seen with the IL3 and IL6 signaling pathways (Figure 1e, h). Collectively, the informatics analyses predicted that the Jak-Stat pathway mediated by IFN/Stat1 signaling is relatively deficient in AGM HSCs.

Deficiency in IFN signaling in the AGM

To validate this prediction, we examined cross sections of the AGM via immunofluorescence at E11.5, a time-point when HSCs are first detectable when transplanted into adult recipients (15). We found that both IFNa and IFNg stained weakly near the dorsal aorta in E11.5 sections (Figure 2a-b). E13.5 FL sections revealed increased expression of IFNs, especially IFNa (Figure 2c-d) while IFNg displayed a more punctate expression pattern (Figure 2d). We confirmed these observations by quantitative RT-PCR using whole tissue samples (Figure 2e). To examine phospho-Stat1 expression in individual phenotypic HSCs, we sorted using the same surface antigen profiles used to obtain gene expression profiles of HSCs at different developmental stages (64). In agreement with the informatics analysis (Figure 1), AGM HSCs were deficient in phospho-Stat1 as compared to FL and BM HSCs as determined by relative mean fluorescence intensity (Figure 2f).

To determine which cells in the E11.5 AGM are responsive to IFN signaling, we performed flow cytometry for Ifnar1 and Ifngr1, receptors for IFNa and IFNg, respectively. We observed that both receptors were expressed in the majority of hematopoietic CD45+ cells (Figure 2g). To determine which Stat proteins become phosphorylated in response to IFNa treatment, we incubated AGM cells with IFNa (0.5 ng/ml) for 90 minutes in the presence of serum. Upon IFNa treatment, hematopoietic CD45+ cells including VE-cadherin+CD45+ HSCs responded robustly through phosphorylation of Stat1 (Figure 2i). CD41- cells, but not CD41+ cells, also responded significantly to IFNa treatment, suggesting that CD41+ primitive embryonic hematopoietic progenitors are do not respond to IFNa signaling (Figure 2i) (120). IFNa treatment also uniquely phosphorylated Stat1 as opposed to Stat3 or Stat5, indicating that IFNa signaling is Stat1-mediated (Figure 2i-k). Moreover, the AGM had a larger
percentage of positive phospho-Stat3 cells at baseline than phospho-Stat1 (Figure 2j), consistent with the informatics analysis suggesting active signaling via IL3, IL6 and Stat3 in the AGM (Figure 1e-g). Taken together, our data indicate that AGM HSCs are deficient in Jak-Stat1 signaling but can respond to IFNa signaling.

IFNa enhances AGM HSC transplantation

Previous work on IFNg signaling in the mouse AGM demonstrated that IFNg receptor deficiency reduces HSC transplantation frequency (111). This leaves open a potential contributory role of IFNa, which is expressed proximal to the dorsal aorta (Figure 2a-b) and at higher levels in E13.5 FL (Figure 2e). We investigated its effect at E11.5, a stage at which HSCs that can engraft irradiated adults first arise in the embryo. As Stat1 signaling is lacking in the AGM HSCs compared to the FL HSCs (Figure 2f), we hypothesized that IFNa signaling might promote the engraftment of AGM HSCs.

In adult HSCs, acute IFNa treatment in vivo drives dormant HSCs to enter active cell cycle progression, whereas chronic IFNa treatment prompts HSC exhaustion (121). Because IFNa exhibits exquisite dose-sensitivity (122), we titrated the IFNa-dose according to its ability to phosphorylate Stat1 in adult splenocytes, and determined that 0.5ng/ml of IFNa resulted in an optimal level of Stat1 phosphorylation (Figure 3a). Pulse treatment of IFNa at this optimized dose was capable of stimulating modest hematopoietic colony-forming unit (CFU) activity (Figure 3b).

Next, we transplanted AGM HSCs from two embryo equivalents (2 e.e) exposed for 90 minutes to two different doses of IFNa (0.05ng/ml and 0.5ng/ml) into irradiated adult recipient mice. We monitored transplant outcomes for donor engraftment as the percentage of CD45.2+CD45.1− within the total CD45+ population and recorded B (B220+), T (CD3+), and myeloid (Gr1+ or Mac1+) contributions (Figure 3c). IFNa treatment (0.5ng/ml) promoted long-term engraftment at 21 weeks post-transplant as compared to control and sub-threshold IFNa dose (0.05ng/ml), although the difference was modest at this non-limiting dose of 2 e.e. (Figure 3d). When we examined the BM, we observed primarily donor-derived cells following IFNa treatment (0.5ng/ml), in contrast to controls or the sub-optimal dose of 0.05ng/ml (Figure 3e). In contrast to reports suggesting lineage skewing in HSCs exposed to IFN (123), we detected no significant lineage skewing at 21 weeks post-transplant (Figure 3f). We also performed secondary transplantations using 2 x 10^6 BM cells derived from the primary transplants along with 3 x 10^5 recipient BM cells, which revealed more dramatic
differences. At 12 weeks post-secondary transplantation, recipients treated with the optimized dose of IFNa had significantly higher donor chimerism than those treated with the suboptimal or control dose (Figure 3g). Collectively, our results show that IFNa enhances long-term transplantation of AGM HSCs in irradiated adult recipients.

Increased competitiveness of IFNa-treated AGM HSCs

The above results prompted us to revisit whether IFNa treatment had an effect on stem cell frequency, homing, or competitive engraftment potential. Thus, we performed a limiting dilution assay with IFNa-treated and untreated AGMs but observed no significant differences in the stem cell frequency (Figure 4a). We repeated the limiting dilution assay with \textit{Ifnar1}−/− AGMs, which lack the interferon-alpha receptor (124), but did not observe any differences in stem cell frequency (Figure 4a).

We next investigated whether IFNa treatment, as with a number of other inflammatory molecules such as prostaglandin E2, could promote homing of AGM HSCs to the BM (125). We transplanted unsorted GFP+ AGMs (3 e.e.) from Ubiquitin-GFP transgenic mice, which allowed us to recover GFP+CD45-Lineage− hematopoietic cells from the BM 18 hours post-transplantation—a time at which the majority of stem and progenitor cells have not undergone cell division (126-128). We observed no significant differences in hematopoietic cell homing to the BM after IFNa treatment of AGM HSCs (Figure 4b).

We then performed competitive transplants with AGMs derived from wild type or Ubiquitin-GFP transgenic mice on the C57Bl/6 background using IFNa versus control treated AGMs (Figure 4c). AGM HSCs treated with 0.5ng/ml IFNa displayed a competitive advantage over untreated, which was significant at 14 weeks post-transplant.

In our previous study, we noted a relative engraftment deficiency when AGMs were transplanted with 3 x 10^5 adult BM cells (14). We repeated these AGM transplantations in the presence of 3 x 10^5 adult BM competitor cells and observed that the competitive engraftment potential of AGM-HSCs was enhanced by IFNa treatment (Figure 4d). Conversely, transplantation of \textit{Ifnar1}−/− AGMs in the presence of these adult BM competitor cells resulted in a dramatic reduction in engraftment (Figure 4d).

A possible explanation for the increased competitiveness of IFNa-treated HSCs in irradiated adults may be that MHC Class I antigens, which are normally expressed at low levels in midgestation embryonic tissues, are upregulated and thereby prevent natural killer-mediated destruction (129, 130). Moreover, it has been documented that pro-inflammatory
cytokines such as IFNγ can induce MHC class I molecules in neurons and lymphoid cells (131, 132). However, immediately before transplantation or at 6-weeks post-, we observed no significant changes in expression of MHCb, which includes H-2Kb and H-2Db (data not shown). Thus, differences in MHC Class I expression do not account for the differences in IFNα-induced competitiveness.

The higher expression of IFNα in the fetal liver suggests that functional maturation of AGM HSCs occur in the FL via exposure to IFNα (Figure 2e). To test this hypothesis, we sorted 5000 Sca1+ cells from the wild-type and Ifnar1−/− E14 FLs and transplanted them into irradiated recipients. Accordingly, hematopoietic engraftment from the Ifnar1−/− FL was significantly lower than that of the wild-type FL (Figure 4e).

Arid3a transcription factor is expressed in the AGM

Reasoning that defects in the maturation of AGM HSCs would manifest prominently in the fetal liver, we considered several candidate transcription factors implicated in the FL HSC biology and focused on Arid3a as a potential regulator of IFN/Stat1 signaling due to the presence of numerous predicted Stat binding sites within the Arid3a locus which might provide a potential feedback mechanism (Figure 5a) (133). Furthermore, Arid3a undergoes post-translational modification by Sumo1 (134) and directly interacts with PIAS1, which not only catalyzes the sumoylation reaction (134, 135), but also acts as a negative regulator of Stat1-mediated gene expression (136). Finally, Arid3a regulates several parameters of B cell function that are also regulated by IFNs, including immunoglobulin production (137, 138).

Examination of Arid3a protein levels in E11.5 embryonic tissues indicated that, in addition to the FL, Arid3a is expressed primarily in its ~102kDa sumoylated form in additional sites of embryonic hematopoiesis, including the AGM, yolk sac, and placenta (Figure 5b). To exclude the possibility that our detection of Arid3a expression in the AGM results from contamination of surrounding tissues, we dissected the adjacent ventral, dorsal, and rostral areas and immune-blotted for Arid3a (Figure 5c). Arid3a was detected only in the ventral FL-containing tissue as previously established (139), eliminating contamination artifacts and confirming its bona fide residence within the AGM. Immunofluorescence staining of E11.5 AGM cross-sections demonstrated that Arid3a is highly expressed in cells surrounding the dorsal aorta, which are likely to be hematopoietic (Figure 5d-e) (140, 141). We detected no signals in the AGM sections from Arid3a KO embryos, thus excluding nonspecific staining (Figure 5f). To determine whether Arid3a is expressed in endothelial or
hematopoietic cells, we stained the AGM with CD45 and VE-cadherin and sorted by flow cytometry into the four quadrants (Figure 5g). Post-sort staining for Arid3a indicated that Arid3a+ cells fractionated almost exclusively within the CD45+ population (Figure 5j), which includes the CD45+VE-cadherin+ HSC population (Figure 5i), and rarely within the double negative or single CD45+VE-cadherin+ endothelial population (Figure 5j, k). Moreover, Arid3a+ expression within the rare CD45+VE-cadherin+ endothelial population was heterogeneous, non-nuclear, and weak (Figure 5k). Together these data suggest a function for Arid3a within the same AGM HSC CD45+ hematopoietic cells that undergo IFNa/Stat1 signaling.

IFNa rescues the hematopoietic defect in Arid3a KO embryos

Arid3a KO mice die between E11.5 and E13.5 as a result of defects in erythroid lineage differentiation (139). Arid3a KO mice have FL HSC defects (139), but whether they also are impaired in AGM hematopoiesis is unknown. We first determined whether hematopoietic progenitors are present in the Arid3a KO AGM by immunostaining for Runx1 (Figure 6a, b). Runx1+ cells were significantly reduced in the dorsal aorta as compared to wild-type AGMs in both E10.5 and E11.5 embryos (Figure 6c, d). To determine whether AGM HSCs are impaired by Arid3a deficiency we compared their transplantation efficiency with those of wildtype and Arid3a+/- AGM controls. As shown in Figure 6e, Arid3a+/- donor chimerism performed at 1e.e input was severely compromised at weeks 6, 10, and 14 post-transplantation. This indicates that the FL hematopoietic defect previously observed in Arid3a KO mice originates within the AGM—the first site of definitive hematopoiesis.

To determine if IFNa treatment can rescue the above hematopoietic defects, we treated wild-type and Arid3a KO AGMs with IFNa and quantified the number of CFUs. Indeed, IFNa rescued the CFU output from Arid3a KO embryos (Figure 6f). To determine whether IFNa can rescue HSC function, we split Arid3a KO AGMs into 0.5 e.e. each and treated one arm with IFNa prior to transplantation. In agreement with the CFU data, we observed a rescue of donor chimerism in mice transplanted with IFNa-treated Arid3a KO AGMs as early as 6 weeks post-transplantation (Figure 6g). We observed one instance of low chimerism from INFa-treated Arid3a KO embryos at 12 weeks post-transplantation that disappeared at 16 weeks; this might correspond to the rare (<2%) Arid3a KO embryos that circumvent embryonic lethality and survive to adulthood (139). Taken together, IFNa signaling can rescue the hematopoietic defect in Arid3a KO embryos.
Arid3a interacts with Stat1 to co-activation transcription of IFN effector genes

Next we investigated whether the IFNa rescue of the hematopoietic defect in Arid3a deficient AGM is directly linked to IFNa-mediated gene regulation. Consistent with this hypothesis, transcript levels of IFNs (Ifng, Ifna), IFN receptors (Ifngr1, Ifnar1) and select IFN target genes (Irf1, Stat1) were significantly reduced in Arid3a KOs (Figure 7a). We also confirmed by immunofluorescence staining that the phosphorylation of Stat1, a consequence of IFNa activation, is deficient in Arid3a KO AGMs (Figure 7b).

To interrogate whether IFNa/Stat1 signaling is downstream of Arid3a or a parallel pathway that converges on IFNa effector genes, we analyzed published chromatin-immunoprecipitation sequencing (ChIP-seq) data from the human hematopoietic line K562 (58). Assessment of ARID3A recruitment to select IFN/Stat1-related loci revealed no binding to any of the IFN cytokine genes, including IFNA4 (Figure 7c and data not shown), suggesting that the defect in IFN transcription observed in the embryo is an indirect process (Figure 7a). However, ARID3A occupied the genomic loci of STAT1, IFNAR1, and IRF1 at positions marked by euchromatic histone modifications associated with active chromatin (e.g., H3K4m3 and H3K4m1) (Figure 7d-f) (142). Furthermore, STAT1-binding sites bore a striking overlap in IFNa-induced ARID3A binding sites but not with an unrelated protein TR4 (Figure 7g), strongly implicating ARID3A as a direct transcriptional activator of these IFN effector genes. Finally, we employed endpoint ChIP-PCR to both confirm the ChIP-seq data and to quantitatively delineate positions of enrichment at both putative enhancers and promoters (Figure 7h-i). Taken together, our data indicate that ARID3A plays no direct role in IFN secretion, but instead is recruited to IFN effector genes to activate their transcription.

Discussion

Little is known about the signaling pathways that promote the developmental maturation of AGM HSCs. Previously, we observed a deficiency in the capacity for AGM HSCs to engraft irradiated adults relative to neonates, suggesting developmental immaturity (14). Our study shows that AGM HSCs are relatively deficient in IFN-mediated Jak-Stat1 signaling in contrast to FL and adult HSCs, and that treatment of AGM HSCs with IFNa improves long-term hematopoietic transplantation and competitiveness of AGM HSCs into irradiated adults without significantly affecting homing or stem cell frequency. We further
identify a novel genetic interaction between Arid3a and IFNa/Stat signaling, and show that Arid3a KO mice have defective AGM hematopoiesis that can be rescued by IFNa treatment. Arid3a regulates this inflammatory pathway during normal embryogenesis via the modulation of IFN effector genes. These data explain how the embryo enlists an inflammatory gene regulatory network to promote the developmental maturation of nascent HSCs.

Our recent studies have been aimed at exploring the differences between AGM HSCs and FL or BM HSCs. Not surprisingly, nascent AGM HSCs exhibit molecular signatures reminiscent of their endothelial origin and need further maturation steps to reach the functional maturity of FL or adult HSCs (14, 64). To achieve such a goal, the embryo must compartmentalize sites of HSC formation from maturation. Relative to the FL and BM, the AGM is enriched in IL3 and IL6 signaling pathways, which in concert with other pathways such as Notch signaling, specialize in hematopoietic emergence (64, 118). This is likely because the AGM is enriched for shear-stress mediated prostaglandin E2 and PKA/CREB signaling (Diaz et al. in press; Kim et al, in press), which can activate IL6 (34, 143, 144). Through exposure to IFNa, AGM HSCs acquire expanded potential for engraftment in adults, akin to FL or adult HSCs. This may explain the increased long-term repopulating activity observed when co-culturing AGM HSCs with nonhematopoietic cells from E14.5 liver in the presence of oncostatin M, which matures fetal hepatocytes and may reflect some aspects of the FL microenvironment (125, 145). Indeed, the regulation of IFNs under normal developmental conditions is complex. The AGM and the FL must keep up with the increasingly hypoxic environment and the metabolic demands of a rapidly developing embryo by maintaining blood flow and erythroid output (146). One hypothesis is that this highly hypoxic environment is pro-inflammatory, and that hypoxia and the stabilization of hypoxia inducible factor may contribute to IFNa production in hematopoietic tissues (147, 148).

Previous work in zebrafish and mice suggest that IFNg is required for HSC emergence in midgestation embryos (111, 113). However, distinct roles for type I and type II IFNs have been previously described (149, 150), and it is possible that distinct IFNs are utilized in different contexts for hematopoietic development. Our data showing that IFNa is more highly expressed in the FL than IFNg may support the hypothesis that IFNa has a role in AGM HSC maturation whereas IFNg has a role in HSC emergence. Indeed, we find that IFNa does not have significant effects on stem cell frequency in the AGM by limiting dilution analysis. One possible explanation is that IFNg signaling results in the atypical
activation of Stat3 (113), which has known functions in HSC emergence (118, 119). In our experiments involving IFNa, we observed Stat1 activation but did not observe any significant activation of Stat3 in the AGM.

What is the nature of the interaction between Arid3a and IFN signaling? In contrast to the IFNa receptor KO mice, which survive to adulthood although with immune compromise (124), the hematopoietic phenotype of Arid3a KO is likely more severe because Arid3a regulates a more complex expression network encompassing IFNa and IFNg, their receptors, and downstream effector proteins. This implies that Arid3a has a broader role outside the context of IFNa signaling. Indeed, in B lymphocytes Arid3a is known to bind to specific ATC-rich matrix-associated regions (MARs) within the immunoglobulin heavy chair enhancer (Eμ) and a subset of variable region (VH)-associated promoters (137, 151-153). MARs compartmentalize specific loops of chromatin (Cockerill and Garrard, 1986) and in this case, juxtapose VH promoters with Eμ to mediate high level transcription of the locus during development (154-156). As with other members of the AT-rich interactive domain (ARID) family (157), Arid3a has also been shown to mediate chromatin remodeling (137, 151, 152).

In agreement with this hypothesis, most Arid3a-expressing and IFNa-responsive cells are CD45+, suggesting that the interaction between Arid3a and IFN/Stat1 occurs within mostly hematopoietic cells. Although we documented the downregulation of Ifna and Ifng in Arid3a KO embryos, the regulation of IFNa and IFNg transcription is unlikely to be directly mediated by Arid3a. This conclusion is based on our observation that in human hematopoietic K562 cells, ARID3A failed to occupancy the genomic loci of IFNa cytokines. In contrast, ARID3A genomic occupancy overlapped with euchromatin histone marks within the STAT1 locus, as well as within the loci of a number of IFN effector genes, including IFNAR1, and IRF1. This indicates that the interaction between Arid3a and IFN signaling is nonlinear, but instead, a more complex interaction involving IFNa induction of direct ARID3A transactivation of IFN effectors. Thus, it was unexpected that a pulse dose of 90-minute IFNa treatment was sufficient to rescue the hematopoietic defect of Arid3a KO. Perhaps similar to the role played by Arid3a in regulating the accessibility of Eμ (152), Arid3a may function in the IFNa/Stat1 pathway by catalyzing the chromatin accessibility of IFN effector genes. Thus, only by increasing the accessibility and, consequentially, the activation of Stat1 can IFNa overcome the hematopoietic defect in Arid3a KO embryos.
Defining additional functions for Arid3a in CD45$^+$ biology and dissecting precise molecular relationships between Arid3a and IFN/Stat1 signaling will be subjects of future studies.

In summary, we identified a novel genetic interaction between Arid3a and IFN/Stat1 signaling essential to the developmental maturation of AGM HSCs. Understanding and appreciating differences between embryonic and adult HSCs may lead to novel ways to increase the potency of HSCs for transplantation, especially when using HSCs from developmentally immature cell sources like pluripotent stem cells.
Figure 1.
Figure 1 – Screen for signaling pathways corresponding to developmental HSC maturation.

A) Weighted gene co-expression analysis. The horizontal bar represents all genes in the sample. Identified co-expressed genes (modules) are assigned colors. Red indicates up-regulation and blue indicates down-regulation in each sample. Module of interest is indicated by the arrow. YS = yolk sac; FL = fetal liver; BM = bone marrow.

B) Expression levels of genes in this module during embryonic development.

C) Top 5 gene ontology biological process terms identified from genes in this module.

D) Top 5 KEGG Pathway terms identified from genes in this module. Red highlights Jak-Stat pathways.

E) Gene set enrichment analysis of samples compared against the AGM HSCs. Red indicates that the gene set is more highly enriched in the AGM compared to tissues indicated. Blue indicates that the gene set is less enriched in the AGM compared to tissues indicated.

F) Gene set enrichment analysis plot for the IL6 pathway comparing AGM HSCs to E14.5 FL HSCs.

G) Gene set enrichment analysis plot for the TNF pathway comparing AGM HSCs to E14.5 FL HSCs.

H) Gene set enrichment analysis plot for the IFN pathway comparing AGM HSCs to BM HSCs.
Figure 2.
Figure 2 – AGM is relatively deficient in Jak-Stat1 signaling but responds to IFN signaling.

A) Immunostaining of E11.5 AGM for IFNa. ao = dorsal aorta. Scale bar = 100µm.
B) Immunostaining of E11.5 AGM for IFNg. ao = dorsal aorta. Scale bar = 100µm.
C) Immunostaining of E13.5 fetal liver (FL) for IFNa. Scale bar = 100µm.
   Autofluorescence was detected via the FITC channel and subtracted from the original image with ImageJ.
D) Immunostaining of E13.5 FL for IFNg. Scale bar = 100µm. Autofluorescence was detected via the FITC channel and subtracted from the original image with ImageJ.
E) Quantitative RT-PCR for IFNa and IFNg transcripts in the E11.5 AGM and E11.5 and E13.5 FL. n = 4.
F) Relative mean fluorescence intensity (MFI) for phospho-Stat1 in E11.5 AGM, E13.5 FL and BM HSCs. Total MFI was divided by MFI of IgG control to obtain normalization. n = 2-3
G) Flow cytometry for IFN receptors in the CD45+ AGM. n = 3.
H) Example of phospho-Stat1 staining in E11.5 AGM cells treated with IFNa.
I) Phospho-Stat1 response in the different cell compartments in the E11.5 AGM. n = 3.
J) Phospho-Stat3 response in the different cell compartments in the E11.5 AGM. n = 3.
K) Phospho-Stat5 response in the different cell compartments in the E11.5 AGM. n = 3.
Figure 3.
Figure 3 – IFNa treatment promotes long-term engraftment.

A) Immunoblot for phospho-Stat1 in response to dose-titrations of IFNa in adult splenocytes.

B) Effect of various doses of IFNa on colony-forming unit activity from the E11.5 AGM. 
   \( n = 4 \).

C) Example of donor chimerism analysis for multi-lineage engraftment.

D) Effect of IFNa on long-term hematopoietic engraftment of E11.5 AGM HSCs in the peripheral blood. 2 e.e. were transplanted with \( 2 \times 10^5 \) helper splenocytes. Two-way ANOVA was performed. \( n = 4-6 \).

E) Effect of IFNa on long-term hematopoietic engraftment of AGM HSCs in the BM. Wilcoxon rank-sum test was performed comparing 0.5ng/ml IFNa treatment with others.

F) Quantification of lineage contributions of B, T and myeloid cells at 21-weeks post-transplantation. \( n = 4-6 \).

G) Secondary transplantation of \( 2 \times 10^6 \) BM cells from IFNa-treated and control AGMs in Figures D-E with \( 3 \times 10^5 \) competitor BM cells. Two-way ANOVA was performed. \( n = 4-6 \).
Figure 4.
Figure 4 – Enhanced competitive transplantation from interferon-treated AGM.

A) Limiting dilution assay with IFNa treated AGM vs untreated AGM. n = 10. Total number of mice transplanted = 69.

B) Homing assay for detection of CD45$^+$GFP$^+$Lin$^+$ and CD45$^+$GFP$^+$Lin$^-$ in the BM 18-hours post transplantation. n = 5.

C) Fraction of donor chimerism attributable to control or IFNa-treated AGM during transplantation. n = 8.

D) Competitive transplant with IFNa treated, untreated or Ifnar1$^{-/-}$ AGM against 3 x 10$^5$ competitor BM cells. Two-way ANOVA was performed. n = 3-6.

E) Competitive transplant with 5000 Sca1$^+$ cells from E14.0 FL from wild-type or Ifnar1$^{-/-}$ against 2 x 10$^5$ competitor BM cells. Two-way ANOVA was performed. n = 9.
Figure 5.
Figure 5 – Expression of Arid3a in the mid-gestation embryo.

A) Screen for regulators of IFN reveals Stat1 binding sites in the Arid3a locus.
B) Immunoblot of Arid3a in the aorta-gonad-mesonephros (AGM), yolk sac (YS), and placenta (PLA).
C) Immunoblot of Arid3a in tissues surrounding the AGM such as the ventral gut, neural tube, or rostral region of the embryo.
D) Immunofluorescence for Arid3a in E11.5 AGM section counterstained with DAPI. Scale bar = 100µm.
E) Zoomed in image of the ventral aspect of the dorsal aorta in D.
F) Immunofluorescence showing negative staining of Arid3a in the Arid3a KO AGM.
G) Sorting schema of the VE-cadherin⁺/CD45⁺ compartments. Scale bar = 100µm.
H) Immunofluorescence for Arid3a after cytospin and percentage of Arid3a⁺ cells in the VE-cadherin⁻/CD45⁻ fraction. Scale bar = 100µm.
I) Immunofluorescence for Arid3a after cytospin and percentage of Arid3a⁺ cells in the VE-cadherin⁻/CD45⁺ fraction. Inset images are zoomed in images of the cells highlighted by the red arrows. Scale bar = 100µm.
J) Immunofluorescence for Arid3a after cytospin and percentage of Arid3a⁺ cells in the VE-cadherin⁻/CD45⁻ fraction. Scale bar = 100µm.
K) Immunofluorescence for Arid3a after cytospin and percentage of Arid3a⁺ cells in the VE-cadherin⁻/CD45⁺ fraction. Scale bar = 100µm.
Figure 6.
Figure 6 – Hematopoietic defect in Arid3a KO embryos rescued by IFNa.

A) Immunofluorescence for Runx1 in the WT E10.5 AGM. Scale bar = 100µm.

B) Immunofluorescence for Runx1 in the Arid3a KO E10.5 AGM.

C) Quantification of nuclear Runx1 in the E10.5 AGM of Arid3a +/- and -/- sections. n = 13 – 19.

D) Quantification of nuclear Runx1 in the E11.5 AGM of Arid3a +/+, +/- and -/- sections. n = 6 – 9.

E) Donor chimerism of Arid3a +/+, +/- and -/- E11.5-12.5 AGMs transplanted at 1 embryo equivalent (1 e.e.) analysed at 6, 10 and 14 weeks post-transplantation. 5 x 10^5 splenic helper cells were used.

F) CFU assays from E11.5 Arid3a WT and KO AGMs. n = 3.

G) Donor chimerism of E11.5 Arid3a +/-, +/- and -/- AGMs transplanted at 0.5 embryo equivalents (0.5 e.e.) and analyzed at 6, 12 and 16 weeks post-transplantation. 2 x 10^5 splenic helper cells were used. Two-way ANOVA was performed.
Figure 7.
Figure 7 – Genomic interaction between ARID3A and STAT1

A) Quantitative RT-PCR of Arid3a and IFN-related genes in Arid3a +/+ , +/- and +/- E11.5 AGM. n = 3-8.

B) Immunostaining of E11.5 Arid3a WT and KO AGMs for phospho-Stat1. ao = dorsal aorta. cv = cardinal vein. nc = notochord. ur = urogenital ridge. Scale bar = 100µm.

C) Chromatin-immunoprecipitation sequencing (ChIP-seq) tracks ARID3A and STAT1 at the genomic loci of IFNA4 in K562 cells.

D) ChIP-seq tracks ARID3A and STAT1 at the genomic loci of STAT1 in K562 cells.

E) ChIP-seq tracks ARID3A and STAT1 at the genomic loci of IFNAR1 in K562 cells.

F) ChIP-seq tracks ARID3A and STAT1 at the genomic loci of IRF1 in K562 cells.

G) ChIP-seq data showing overlapping binding sites between ARID3A and STAT1 but not the hormone receptor TR4.

H) ARID3A immunoprecipitation in K562 cells.

I) Confirmation of ChIP-seq via quantitative ChIP-PCR normalized by input control. Correct product sizes are between 150-200bp as shown in the 2% agarose gel below after the quantitative PCR reaction. n = 4.
### Supplemental Table 1 – Primers for Quantitative RT-PCR

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**ChIP-PCR primers**

IFNA4-0kb_F  ATAGGAGGGCCTTGATACCTC
IFNA4-0kb_R  TCTCATGGAGGACAGAGATG
IFNA4-2kb_F  GGAAGGAAAGCTGTAGGATTAT
IFNA4-2kb_R  TAACTAGCCTGTTGCTAATG
IFNA4-5kb_F  GGCAAGAGCTGTGAGAAA
IFNA4-5kb_R  GTGTTATGTGGGTTCCCTAATC
IFNAR1-0kb_F  TCACAATGCACCTGGATCTATTTA
IFNAR1-0kb_R  ATGGTCCCTTGGAGAGAA
IFNAR1-10kb_F  CTCATCCTCCTCCTTCCAATAC
IFNAR1-10kb_R  TGTCCTGGCCTCATCTTCT
IFNAR1-5kb_F  AGAGGTCAGGGTTGAAA
IFNAR1-5kb_R  CTAATTGGTGGAAGACACTC
STAT1-0kb_F  AGCAGAGCCCCAAAGAAA
STAT1-0kb_R  GGAACGTGGGGAAGATATAATG
STAT1-5kb_F  TCCTCAGCATGACCTTTATTG
STAT1-5kb_R  GATCTCCTGACCTCGTGAT
STAT1-9kb_F  CACCCCTCAGATCCCTCAAAC
STAT1-9kb_R  GGAGCTAAGGAATGACTGAAAG
IRF1-0kb_F  CTCTACAACAGCCTGATTCC
IRF1-0kb_R  ACGTCTTGCCCTCGACTAA
IRF1-5kb_F  GGACTTTCTTTCTCCAGTTAG
IRF1-5kb_R  TTCCAGGTCGAAAGTAGTAG
IRF1-2kb_F  GGGAAGGACAAGGCTATTT
IRF1-2kb_R   GACACTCTGGAATGGAAAGG

Genotyping

Arid3a KO-F   GGA GTC TGC AGG TGC TTG AA
Arid3a KO-R   GAT CAG CAG CCT CTG TTC CA
Arid3a WT-F   TGA GTT CCC AAG GTC TGT GTG TTC
Arid3a WT-R   GGA TCT CGT ACC GTA AAT GGC AGT
**Experimental Procedures**

**Mouse embryo culture**

E11.5 or E10.5 staged mouse embryos were obtained using timed pregnancies of C57BL/6 females, unless indicated otherwise. The AGM region was isolated under a dissecting microscope and dissociated to single cells using DNase I, Collagenase IV and Hyaluronidase for 15 minutes at 37°C and then washed with IMDM. Dissociated cells were cultured with IFNa (PBL interferon source #12100-1 or Sigma #I8782) for 1.5 hours in 10% fetal calf serum (FCS)/IMDM (vol/vol) in a 96-well V-bottom plate. The cells were then dissociated with enzyme-free dissociation buffer (Gibco), and washed with IMDM before further analysis.

**Colony forming unit (CFU) assays**

Cells were plated into 1.5-2 ml of methylcellulose media containing interleukin-3 (IL-3), IL-6, erythropoietin (Epo) and SCF (M3434; StemCell Technologies) as described (26). CFUs [definitive erythroid (Ery), myeloid (M), granulocyte-macrophage (GM), granulocyte-erythrocyte-monocyte-megakaryocyte multilineage (GEMM)] were scored based on morphology 8-12 days post-plating.

**Flow cytometry and peripheral blood analysis**

Dissociated AGM cells were stained with VE-cadherin (11D4.1; BD) with anti-rat IgG2a-PE (RG7/1.30; BD) secondary antibody, CD45.2-PE-Cy7 (104; Biolegend), Ifnar1 (MAR1-5A3; Biolegend), and Ifngr1 (2E2; Biolegend). Peripheral leukocytes were stained with CD45.1-FITC (A20; BD), CD45.2-PE-Cy7 (104; Biolegend), CD11b-Alex Fluor 700 (M1/70; Biolegend), Gr1-PE (A20; BD), CD19-APC-Cy7 (6D5; Biolegend), CD3-APC (145-2C11; eBiosciences), Ter119-PE-Cy5 (TER-119; eBiosciences), B220-Pacific Blue (RA3-6B2; BD) and propidium iodide (PI). For GFP chimeric analysis, CD45.1-FITC (A20; BD) was omitted and B220-Pacific Blue (RA3-6B2; BD) was substituted with CD45.1-Pacific Blue (A20; BD). For MHC Class I molecule detection, H-2Kb-FITC (AF6-88.5.5.3; eBioscience), H-2Kk-eFluor450 (AF3-12.1.3; eBioscience), H-2Kk-eFluor450 (AF3-12.1.3; eBioscience), H-2Db-APC (28-14-8; eBioscience), and H-2Kd/H-2Dd-PE (34-1-2S; eBioscience) antibodies
were used. Cells were stained in 2% FCS/PBS (vol/vol) on ice for 40 minutes. Engraftment percentage was determined to be percentage of PI and Ter119 negative CD45.2 CD45.1 cells within the CD45 positive population. Limiting dilution analyses were performed using the Extreme Limiting Dilution Analysis software (158).

Intracellular flow cytometry

Isolated cells were fixed with 2% paraformaldehyde for 10 minutes at room temperature and permeabilized with 75% ethanol for 8 minutes at 4°C. Cells were quickly rehydrated with 3x volume of PBS, and then collected via centrifugation before staining overnight at 4°C in 5% FCS/PBS (vol/vol). Cells from the FL and BM were stained first with c-Kit-PE-Cy5 (1:10; 2B8; Biolegend) for 15 minutes at 4°C, washed and stained with Anti-Rat IgG MicroBeads (1:10) for 15 minutes at 4°C, and enriched using magnetic columns before fixation to reduce background staining. Cells from the AGM were stained with CD34-FITC (RAM34; eBioscience), CD41-PE (MWReg30; BD), Biotin-conjugated VE-cadherin (BV13; Biolegend) with Streptavidin PE-Cy5 (BD), c-Kit-APC (2B8; Biolegend), CD45.2-PE-Cy7 (104; Biolegend), and DAPI for live-dead discrimination. Cells from the FL or BM were stained with CD48-PE (HM48-1; BD), c-Kit-PE-Cy5 (2B8; Biolegend), CD150-AF647 (TC15-12F12.2; Biolegend), Sca1-PE-Cy7 (D7; Biolegend), and a lineage cocktail consisting of CD11b- Pacific Blue (M1/70; Biolegend), Gr1-Pacific Blue (A20; BD), CD19-Pacific Blue (6D5; Biolegend), CD3- Pacific Blue (145-2C11; eBiosciences), Ter119-Pacific Blue (TER-119; ebiosciences), B220-Pacific Blue (RA3-6B2; BD) and DAPI for live-dead discrimination. Intracellular antibodies include phospho-Stat1 (1:10; Cell Signaling; #9167), phospho-Stat3 (1:10; Cell Signaling; #9145), and phospho-Stat5 (1:10; Cell Signaling; #4322) with Pacific Orange goat anti-rabbit antibody (Invitrogen).

Transplantation and peripheral blood analysis

B6.SJL-Ptprca Pepticb/Boy mice were used at 6-10 weeks of age. Mice were irradiated with a split dose of 10GY separated by 2.5 hours prior to transplantation. Each recipient was transplanted via tail vein injection along with 2 x 10^5 splenic helper cells per experiment group from B6.SJL-Ptprca Pepticb/Boy mice unless indicated otherwise. Peripheral blood was collected retro-orbitally at the indicated time points post-transplantation. Red blood cells
were removed with 1% dextran sulfate/0.5% EDTA/PBS (wt/wt/vol) and lysed with RBC lysis buffer (Sigma) before analysis.

Western blotting

Cells were lysed in RIPA buffer with protease and phosphatase inhibitors (Pierce). Proteins were separated via polyacrylamide gel and transferred to an activated PVDF Membrane. The membrane was blocked for 1 hour at 25°C using 5% milk or 5% BSA in PBS-Tween (0.1% (v/v) Tween 20) and probed with primary antibodies overnight at 4°C. After washing with PBST, HRP-conjugated secondary antibodies were used for 1 hour at 25°C (GE Healthcare). Protein levels were detected using SuperSignal West Pico and Femto Luminol reagents (Thermo Scientific). The following antibodies were used at recommended dilutions: phospho-STAT1 (Cell Signaling #9167), STAT1 (Cell Signaling #9175 or #9172), actin (Cell Signaling #4967), tubulin (Cell Signaling #2128). Polyclonal rabbit Arid3a was generated as described (137).

Histological Analysis

For paraffin sections, E10.5 or E11.5 mouse embryos were fixed in 2% paraformaldehyde on ice for 1 hour before being embedded in paraffin and sectioned. Paraffin embedded sections were dewaxed with xylene and rehydrated with decreasing percentages of ethanol/PBS (vol/vol). Antigen retrieval was performed using 10mM sodium citrate buffer (pH 6) using a pressure cooker at 95°C for 30 minutes. Cells were blocked for 1 hour with 0.01% Triton X-100/0.01M glycine/10% secondary antibody host serum in PBS. Immunostaining was performed with the following primary antibodies overnight: IFNa (1:100, R&D #32100-1), IFNg (1:100, Abcam #ab133566 or BD #554409), Runx1 (1:100; #2813-1 Epitomics), Arid3a (1:100), and phospho-Stat1 (1:10; Cell Signaling; #9167). For phospho-Stat1 staining, sections were treated with Avidin/Biotin Blocking Kit (Vector Labs), then processed with Vectastain Elite ABC Kit (PK-6100), and lastly stained with Alex 647-conjugated goat anti-rabbit. For others secondary antibody stainings, Alexa 488-, Alexa568- or Alex 647-conjugated donkey or goat anti-rabbit, anti-mouse or anti-goat secondary antibodies were used. Cells were mounted with antifade mounting medium (Vectashield) after DAPI staining.
Microarray analysis

Data was obtained from GSE37000 (64). Microarray data were analyzed using R/Bioconductor. Raw microarray signal intensities were RMA-summarized, quantile-normalized, and filtered for duplicate genes using the nsFilter function, ComBat batch corrected before analysis (104, 159, 160). We used Weighted Gene Co-Expression Network Analysis (WGCNA) to find sets of positively correlated genes (modules) using beta = 10 to reach a scale-free model fit of $R^2 = 0.886$ (114). We used the blockwiseConsensusModules function with minModulesSize = 30 and mergeCutHeight = 0.25, minKMEtoStay = 0, maxBlockSize = 21000, networkType = “signed” instead of the default parameters. Gene set enrichment analysis was performed using deposited gene sets from the Broad Institute (117).

Quantitative RT-PCR

AGM tissues were dissected in the presence of RNAlater (Life Technologies). RNA was isolated with the RNeasy Micro Kit (Qiagen). cDNAs were prepared with SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with Brilliant SYBR Green QPCR Mix (Strategene) on the MX3000/6P machine using primer sequences listed in Supplemental Table 1.

ChIP-PCR

K562 cells were grown in 10% fetal calf serum/RPMI. K562 cells were crosslinked with 1% paraformaldehyde for 15 min at 20°C. Reaction was quenched with final concentration of 0.125M glycine. Cells were washed with PBS, pelleted, and resuspended in SDS-ChIP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) with protease inhibitor. Chromatin was sheared to ~500 bp with 30s x 5, 40s x 8 cycles of sonication. Sonicated chromatin was pre-cleared with Protein-A agarose beads (Roche) and incubated with the primary antibody overnight at 4C. Protein A agarose beads were added to the ChIP reactions and incubated for 2 hours at 4C. Beads were washed twice with 1 ml of low salt wash buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with 1 ml of high salt wash buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with 1 ml
of LiCl wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP-40, 250 mM LiCl), and twice with 1 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0). The chromatin was eluted in 300ul of SDS elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) at 72ºC. Eluted chromatin was reverse-crosslinked at 65ºC overnight. An equal volume of TE was added the next day (300µl). ChIP DNA was treated with 1µl of RNaseA (10mg/ml) for 1 hour at 37ºC, and with 3µl of proteinase K (20mg/ml) for 3 hours at 37ºC, and purified using phenol-chloroform extraction, and then purified via MicroChIP DiaPure columns (Diagenode). Input ChIP samples were held after the pre-clear step and processed similarly from reverse cross-linking step to the end. Purified ChIP DNA was measured in Qubit (Invitrogen). Quantitative PCR was performed with Brilliant SYBR Green QPCR Mix (Strategene) on the MX3000/6P machine using primer sequences listed in Supplemental Table 1.

ChIP-Seq Analysis

Raw sequence read archive (SRA) files were obtained and data sets were aligned using Bowtie (version 1.0.0) (105) to build version hg19 of the human genome using the parameters -k 2 -m 1 -n 2 -best. Duplicates were removed and reads were extended by 200 bp before visualization using IGVtools (106). For peak-calling, we used MACS (Model based analysis of ChIP-Seq) version 1.4.1 (107) algorithm to identify statistically significant regions of ChIP-Seq enrichment using the threshold p-value 1e-8. For Stat1 peak-calling, a lower threshold of 1e-5 was used. To display ARID3A binding at STAT1 loci, we calculated reads per million mapped reads per bp in 50 bp bins ± 3 kb around the transcription start site of transcribed genes. Analysis was performed on the HMS Orchestra High Computing Cluster.

Statistical analysis

n represents the number of biological replicates. Two-tailed unpaired Student’s t-tests were used, unless indicated. Error bars show standard error. Statistical significance is indicated by * P = 0.05, ** P = 0.01, *** P = 0.001.
Chapter 4 - Conclusions and future directions

In this body of work, we have demonstrated that inflammatory signals involving biomechanical shear-stress (i.e. PKA/CREB-BMP pathway) and interferon signaling (i.e. Arid3a-interferon/Stat1 signaling) are involved in HSC emergence and maturation respectively. Despite the discovery of many signaling pathways and genes involved in the emergence of HSCs, the derivation of \textit{bona fide} HSCs via directed differentiation remains elusive. Here, we speculate on the future directions of this field of developmental hematoipoiesis and focus on a few emerging themes.

First, recent data on the derivation of ‘definitive’ hematopoietic lineages highlights the importance of early patterning (38). Kennedy et al. showed that the early modulation of the Activin/Nodal signaling in differentiating PSCs can have profound consequences in T cell development. The likely reason for this finding is that different regions of the primitive streak have different potentials for differentiation. Hence, differentiation potential is therefore locked early in development. Many studies, including our body of work, focus on a specific stage of embryonic hematopoiesis such as E11.5 AGM. Signaling pathways elucidated by means of mechanical dissection of embryos derived from timed-pregnancies take for granted the normal development leading up to the embryonic stage of interest. However, differentiation of PSCs in a culture dish is a disorganized process lacking: 1) the axial information inherent in the embryo; 2) selective apoptotic cues to regulate organ size; 3) the circulatory system for optimal nutrient delivery; and finally 4) biomechanical signals such as shear stress and stretch from the cardiac cycle. Therefore, the signaling pathways described here must be activated in the right cell type to achieve the desired effect. In this light, future work should not assume that the right type of mesoderm (e.g. lateral plate mesoderm) has been formed without proper identification via genetic tools. One such tool would be a fluorescent protein knocked into that gene locus, thus serving as a reporter for the activation of that gene locus. The cell lines bearing these fluorescent reporters would aid in identifying the appropriately differentiated cells, which can be enriched by cell sorting. As such, studying the determinants of the lateral plate mesoderm and its precursors should be just as important as studying signaling pathways involved in HSC emergence and maturation.

However, genetic engineering of human PSCs has been hampered by difficulties in gene editing, which makes the establishment of mesodermal or hematopoietic fluorescent reporter cell lines difficult. Homologous recombination rate in human PSCs, in contrast to
mouse PSCs, is significantly lower, which lower the efficiencies of gene editing techniques that rely on homologous recombination (161). Moreover, despite the use of small molecules such as the Rho-associated kinase (ROCK) inhibitor, Y-27632, human PSCs are much more difficult to culture at a single cell level than mouse PSCs (162). Single cell isolation is critical to isolate rare cells that have undergone gene editing events. These differences between human and mouse PSCs are attributable to different states of pluripotency that exist between mouse and human PSCs. In other words, human PSCs depend on FGF signaling and resemble a more differentiated epiblast form in the early embryo whereas mouse PSCs resemble a less differentiated state called the inner cell mass (163). Recent derivation of human PSCs sharing the characteristics of mouse PSCs may increase gene engineering efficiencies (164, 165). Future derivation of these reporter lines will aid in the identification of the steps involved in the emergence of HSCs and other cell types.

Lastly, the integration of many different signaling pathways and biomechanical pathways is a difficult process. When signaling pathways are discovered, future work is aimed at combining several known pro-hematopoietic signals to achieve the pro-hematopoietic output. Because signaling pathways often target the same downstream targets (e.g. FGFs and BMPs both target SMAD proteins) in a concentration-dependent manner when two signals are inputted, one must rediscover the optimal concentrations that are permissive for enhanced hematopoiesis. The costs and labor associated with redrawing these ‘phase diagrams’ often hinder this line of research. However, results are likely to be fruitful. If HSCs can be obtained albeit at low frequencies via haphazard differentiation inherent to teratoma formation (42, 43), achieving HSC derivation via directed differentiation is equally hopeful. Recent work on the derivation of insulin-secreting beta cells follows this similar line of reasoning of combinatorial signaling pathway modulation in its initial screens (166). In HSC derivation, only one functional HSC is required to provide multi-lineage engraftment and thus chemical screening using concentration gradients of several different pro-hematopoietic signals at the same time is appealing. Our study on shear-stress hints that traditional molecular signals can also be combined with biomechanical signals to promote HSC emergence.

Despite the challenges in deriving HSCs for therapeutic purposes, the field holds many promises. Recent derivations of ‘definitive’ hematopoietic lineages from human PSCs and the derivation of engraftable HSCs from differentiated B-cells are some examples of significant advances in the field (38, 46). In our continued work, ongoing efforts at chemical
and biomechanical induction of PSCs have led to tantalizing results of short-term unilineage hematopoietic engraftment in irradiated recipients that require further characterization, refinement, and optimization.
References


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10.1152/ajpheart.00829.2010. PubMed PMID: 21378144; PubMed Central PMCID: PMC3094086.


