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MEF2C Protects Bone Marrow B Lymphopoiesis during Stress Hematopoiesis

Author
Wang, Wenyuan

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MEF2C Protects Bone Marrow B Lymphopoiesis during Stress Hematopoiesis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Wenyuan Wang

2016

ABSTRACT OF THE DISSERTATION

MEF2C Protects Bone Marrow B Lymphopoiesis

during Stress Hematopoiesis

by

Wenyuan Wang

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2016

Professor Hanna K.A. Mikkola, Chair

The integrity of the immune system is critical for health as it serves as the guardian to protect an organism from foreign pathogens. Because most mature immune cells have a short lifespan, the lifelong replenishment of the immune system depends on the hematopoietic stem cell (HSC). In order to adapt to various stress situations that occur throughout life, the immune system needs to have mechanisms to protect its integrity and ensure rapid regeneration during such stress situations.

One key protective mechanism that is required to protect the immune system from various stress factors is the DNA repair system. Such mechanisms are of particular importance in the development of B lymphocytes, which are central mediators of humoral immunity. During development, the B cell progenitors in the bone marrow (BM) go through a series of rearrangements of immunoglobulin genes to generate diverse B cell receptors that can recognize

foreign antigens. The nature of this rearrangement process is the generation and repair of DNA double strand breaks (DSBs), and failure of this process causes cell death of BM B lymphoid progenitors, which makes the BM B lymphoid compartment inherently vulnerable.

My thesis research focused on the identification of novel mechanisms that protect B lymphopoiesis, especially upon stress situations. Through my research, I identified transcription factor MEF2C as a guardian of BM B lymphoid progenitors and showed that MEF2C is critical for maintaining the integrity of the immune system through regulation of DNA repair and V(D)J recombination during both homeostasis and stress hematopoiesis.

Hematopoietic deletion of *Mef2c* in mice reduced the survival and cellularity of BM B cell progenitors downstream of common lymphoid progenitor (CLP), while peripheral B cells remained unaltered in homeostatic conditions. Intriguingly, this phenotype was reminiscent of the B lymphoid defects observed during aging. Loss of *Mef2c* severely compromised the recovery of BM and peripheral B cells after sub-lethal irradiation and 5-FU induced BM ablation, while the recovery of T lymphoid and myeloid cells was unaffected. These data imply that MEF2C protects B cell progenitor survival, especially upon proliferative stress. Microarray of *Mef2c* deficient B cell progenitors showed down-regulation of DNA repair genes, including sensors of DSB and effectors in homologous recombination (HR) and non-homologous end joining (NHEJ) repair pathways. Comet assay revealed excessive DNA damage specifically in B cell progenitors in *Mef2c* deficient BM, while CLPs, mature B cells, T cell progenitors or myeloid cells were unaffected. γH2AX staining showed increased DSBs in *Mef2c* deficient pre-B cells. These data show that MEF2C regulates DNA repair specifically in B cell progenitors. NHEJ repair is also required for V(D)J recombination in BM B cell progenitors. Loss of *Mef2c* impaired the proper induction of *Rag* initiators and key NHEJ factors during B cell progenitor

transition, and reduced the recombination efficiency of both heavy and light chains, uncovering a novel function for MEF2C in V(D)J recombination. ChIP-Seq in human B lymphoblasts showed that MEF2C directly binds to genes encoding critical factors of DSB repair and V(D)J machinery. MEF2C binding strongly correlated with the binding of co-activator and enhancer epigenetic marks, suggesting that MEF2C functions by boosting gene activation through these enhancers. These data define MEF2C as a lineage specific regulator of DNA repair machinery that protects B lymphoid progenitor homeostasis.

In the HSC compartment, deletion of *Mef2c* resulted in a reduction of HSCs. Microarray analysis revealed down-regulation of critical HR DSB repair and nucleotide excision repair (NER) factors as well as cell cycle regulators in *Mef2c* deficient HSCs. During stress hematopoiesis induced by irradiation, higher cell death was observed in *Mef2c* deficient HSCs. These preliminary data suggested a potential function of MEF2C also in regulating DNA repair and protecting the integrity of the HSC pool. Future studies will be needed to explore the specific cellular and molecular mechanism that MEF2C utilizes in protecting the HSC compartment.

The dissertation of Wenyuan Wang is approved.

Utpal Banerjee

Gay M. Crooks

Stephen Smale

Hanna K.A. Mikkola, Committee Chair

University of California, Los Angeles

2016

DEDICATION

This dissertation is dedicated to my loving and supportive parents, Jing and Ping, my grandparents Shoukui and Yingzhen, and the rest of my loving family members.

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Biographical Sketch:

WENYUAN WANG

EDUCATION

09/06 – 07/10 B.S. in Life Science, Peking University, China

PUBLICATIONS

Wang W, Org T, Montel-Hagen A, Pioli P, Israely E, Duan D, Malkin D, Flach J, Schiestl R, Mikkola HK. MEF2C Protects Bone Marrow B Lymphoid Progenitors during Stress Hematopoiesis.. (in revision)

Corselli M, Chin CJ, Sahaghian A, **Wang W**, Ge S, Evseenko D, Wang X, Montelatici E, Lazzari L, Crooks GM, Péault B. "Perivascular support of human hematopoietic cells". Blood. 2013 Apr 11; 121(15): 2891-901

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FELLOWSHIPS & HONORS

2015 UCLA Dissertation Year Fellowship

2015 UCLA Molecular Biology Institute Dissertation Year Award

2015 1st Place, UCLA, USC and UCSF Tri-institute Stem Cell Center Retreat Poster Prize

2014 BSCRC CIRM Pre-doctoral Training Grant

2014 UCLA Leadership Certificate in the Business of Science

2013 BSCRC CIRM Pre-doctoral Training Grant

2012 American Society of Hematology 54th Annual Meeting Abstract Achievement Award

2012 The Philip Whitcome Pre-Doctoral Training Grant

2011 The Philip Whitcome Pre-Doctoral Training Grant

INTERNATIONAL CONFERENCE PRESENTATIONS

Wang W*, Montel-Hagen A, Org T, Malkin D,Schiestl R, Mikkola H, "Mef2C Protects B Lymphoid Progenitor during Stress Hematopoiesis". Poster presented at the International Society for Stem Cell Research Annual Meeting, Stockholm, June 2015.

Wang W*, Montel-Hagen A, Malkin D,Schiestl R, Mikkola H, "Mef2C Protects B Lymphoid Progenitor Homeostasis by Enhancing DNA Repair and V(D)J Recombination". Poster presented at the Society for Hematology and Stem Cells 41st Annual Scientific Meeting, Montreal, August 2014.

Wang W*, Montel-Hagen A, Sasidharan R, Mikkola HK. "Mef2C prevents premature aging of the B lymphoid system through the regulation of DNA repair machinery". Talk presented at the University of California, Santa Cruz Stem Cells and Aging Symposium, Santa Cruz, CA, May 2013.

Wang W*, Montel-Hagen A, Sasidharan R, Mikkola HK. "Mef2C maintains B cell homeostasis through the regulation of DNA repair machinery". Talk presented at the American Society of Hematology 54th Annual Meeting, Atlanta, GA, December 2012.

Wang W*, Montel-Hagen A, Sasidharan R, Mikkola HK. "Mef2C maintains B cell homeostasis through the regulation of DNA repair machinery". Poster presented at the Society for Hematology and Stem Cells 41st Annual Scientific Meeting, Amsterdam, August 2012.

WORK & EXTRACURRICULARS

Consulting Intern of Venice Family Clinic Pharmacy, UCLA May 2014 – Oct 2014
Assessed the feasibility for the Venice Family Clinic to open a retail pharmacy, identified opportunities associated with mail order prescription and MTM program, and presented the team findings to the board of directors

Team Leader of Biomedical Venture Competition, UCLA Jul 2013 – Jul 2014
Conducted market and customer assessments of a novel dental desensitizer to evaluate its commercial value. Generated and initiated a market entry strategy and contacted industrial players for potential partnership. Won a $30,000 non-dilutive funding to support the round one clinical trial on human subjects.

President of Advanced Degree Consulting Club, UCLA Jun 2013 – Jun 2014
Led board members to organize case competition, career talk series, mini-MBA groups, consulting firm information sessions and case interview practices.

Teaching Assistant of Stem Cell Biology Class, UCLA Mar 2012 – Jun 2013
Led two weekly sessions to discuss stem cell publications and mentored students on scientific research proposal writing.

Chapter 1:

Introduction

1.1 The Hematopoietic Stem Cell

The immune system is critical for an individual's health, as it protects the organism against attacks from foreign pathogens. As most mature immune cells are short lived, hematopoietic stem cell (HSC) is responsible for the life-long replenishment of all blood cell types (Figure 1.1), and therefore plays a critical role in maintaining the integrity of the immune system.

Like many other stem cell types, HSC is characterized by the capability of self-renewal, which refers to the ability to give rise to an HSC without differentiating into downstream progeny, and multi-potency, which is the ability to differentiate into all blood cell types (Seita and Weissman, 2010). When using mice as a model system, HSCs are identified based on their ability to provide long-term replenishment of all blood cell lineages upon transplantation into lethally irradiated recipients (Domen and Weissman, 1999).

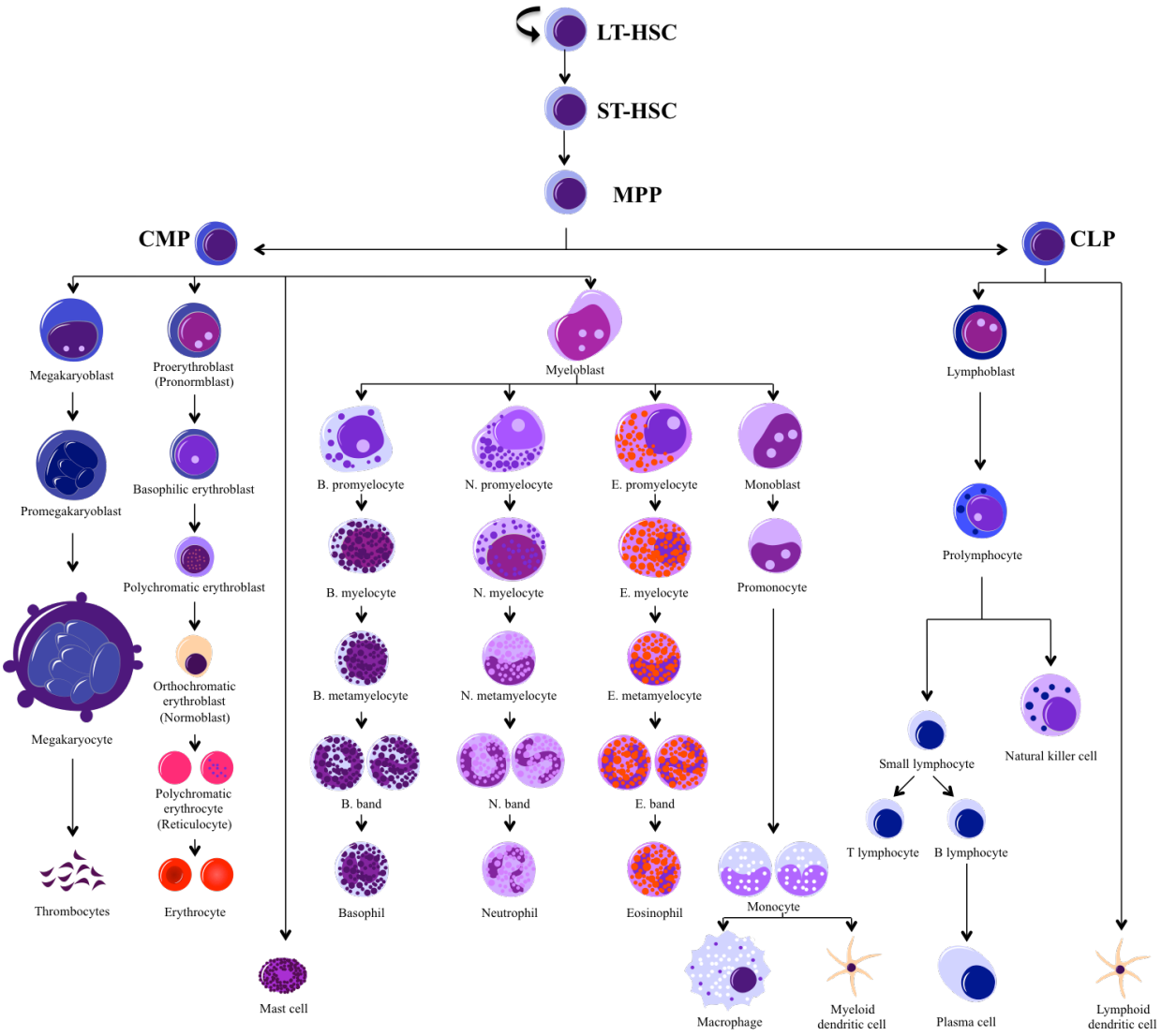

Figure 1.1 The Hematopoietic Hierarchy in Adult

Long-term hematopoietic stem cell (LT-HSC) in the bone marrow gives rise to short-term repopulating hematopoietic stem cell (ST-HSC), which can differentiate into multi-potential progenitor (MPP). MPP will then differentiate into lineage-restricted progenitor with limited potential: common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). These lineage-restricted progenitors will then further develop into different mature blood cell types.

For facilitate HSC identification and isolation, multiple surface markers for HSCs have been discovered. In mice, long-term hematopoietic stem cells (LT-HSCs) are enriched in Lineage$^-$ Sca-1$^+$ cKit$^+$ (LSK) CD150$^+$ CD48$^-$ CD41$^-$ population, while multi-potential progenitors (MPPs) are Lineage$^-$ Sca-1$^+$ cKit$^+$ CD150$^-$ CD48$^-$ (Kiel et al., 2005). These markers allow easier recognition of HSCs by flow cytometry and isolation of HSCs by fluorescence-activated cell sorting (FACS). However, the biological relevance of these markers is still not completely understood and may therefore affect the studies conducted by using them. For example, CD41, which is traditionally thought to only transiently mark fetal HSCs (Ferkowicz et al., 2003), has recently been shown to mark a sub-population of adult HSCs that accumulate with age (Gekas and Graf, 2013). Therefore, the choice of specific combination of different surface markers may affect the results of HSC studies.

In the bone marrow (BM), HSCs locate in a specific microenvironment where signal cross talk between HSCs and the microenvironment, also recognized as HSC niche, maintains the homeostasis of HSCs. The importance of HSC niche has long been realized as previous studies showed that single mutation in the stem cell factor (SCF, also known as Kit ligand) that is expressed by the niche components leads to the dysregulation of HSC self-renewal (Barker, 1997; McCulloch et al., 1965). Despite the importance of the niche for HSC maintenance, the exact cell types within the HSC niche and their distinct functions in maintaining HSC homeostasis are still not completely clear. It has been shown that HSC niche is primarily composed of perivascular cells (see Appendix), which is generated partially by mesenchymal stromal cells (MSCs) and endothelial cells (Morrison and Scadden, 2014). However, this is not the complete picture. For example, it has been shown that the sympathetic nervous cells can regulate the expression of CXCL12 and therefore affect HSC retention in the BM (Katayama et

al., 2006). Even within the perivascular niche, due to the different types of vessels where the niche locates, perivascular cells can provide different functions in HSC maintenance. While sinusoids are the most studied niche due to its abundance and proximity to SCF and CXCL12 expressing cells (Ding and Morrison, 2013), arterioles are also found to play an important role in HSC quiescence maintenance (Kunisaki et al., 2013). Dissecting the distinct functions of different niche components will be critical for understanding HSC self-renewal and differentiation regulation in the future, and therefore contributing to the development of better *in vitro* culture systems to expand or maintain functional HSCs.

1.2 B Lymphoid Development in the Bone Marrow

In presence of foreign pathogens, the immune system induces two types of immune responses to protect the organism: humoral immune response, which is mediated by antibody, and cell-mediated immune response. B lymphocytes are the central mediators of humoral immunity as they are responsible for generating antibodies against foreign antigens.

In order to be able to recognize diverse foreign antigens, B cell progenitors in the BM go through a series of rearrangements of genes that encode the immunoglobulin to generate diverse B cell receptors. As the exons that encode immunoglobulin contains three types of segments: variable (V), diversity (D) and joining (J), and these segments are assembled to generate the functional B cell receptor, the B cell receptor rearrangements are also called the V(D)J recombination (Hesslein and Schatz, 2001; Tonegawa, 1983).

The D to J rearrangement of the immunoglobulin heavy chain starts at the late common lymphoid progenitor (CLP) and lasts till the pro-B cell stage, when the V to DJ rearrangement follows to generate the fully rearranged heavy chain. The heavy chain is then expressed at the protein level and couples with surrogate light chains, lambda-5 (λ5) and VpreB, to form the pre-B cell receptor (pre-BCR) on the surface of late pro-B cell and induces proliferation of these progenitors (Mårtensson and Ceredig, 2000). After the expansion, pro-B cells exist the cell cycle and transit into pre-B cell stage, when the V to J rearrangement of immunoglobulin light chain takes place. (Nemazee, 2006; Schlissel, 2003) Upon the completion of the light chain recombination, fully rearranged B cell receptor will be expressed on the surface of these B cell progenitors, which will then leave the BM for further development in the spleen. (Figure 1.2)

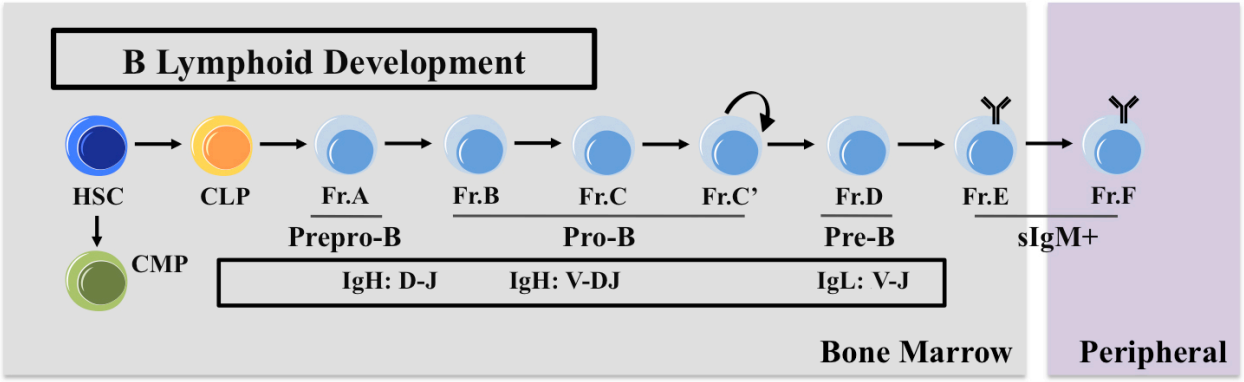

Figure 1.2 B Lymphoid Development in the Bone Marrow

B lymphoid development in the bone marrow requires the rearrangement of both immunoglobulin heavy and light chains. The rearrangement of heavy chain first started at the CLP and prepro-B stage, and then completes at the late pro-B stage to generate pre-BCR. The light chain rearrangement occurs in the pre-B cells to produce fully rearranged BCR.

At the molecular level, V(D)J recombination is initiated by creating DNA double strand breaks (DSBs) mediated by the recombination activating gene (RAG) recombinase activity at the border of two recombining immunoglobulin gene segments (Oettinger et al., 1990; Schatz et al., 1989). The primary components of the RAG protein complex is encoded by two critical genes: *Rag1* and *Rag2*. Specifically, RAG1 is the major DNA-binding component that harbors majority of the recombinase activity, while RAG2 enhances the interaction of RAG1 and DNA segments (Schatz and Ji, 2011). The RAG proteins can recognize the specific recombination signal sequences (RSSs) that flank each V, D and J gene segments. When the RSSs complex pair, a hairpin structure will be formed between a V and a D (or a D and a J) segment, thus enabling the rearrangement of these gene segments (Gellert, 2002; Swanson, 2004). After the rearrangement, the DSBs generated are then repaired by the non-homologous end joining (NHEJ) DSB repair machinery to region the DNA ends (Gu et al., 1997; Nussenzweig et al., 1996). (Figure 1.3) Defective DNA repair during this process results in cell death or in some instances, the creation of genetic lesions such as chromosomal translocations (Mills et al., 2003), making BM B lymphopoiesis an inherently vulnerable process.

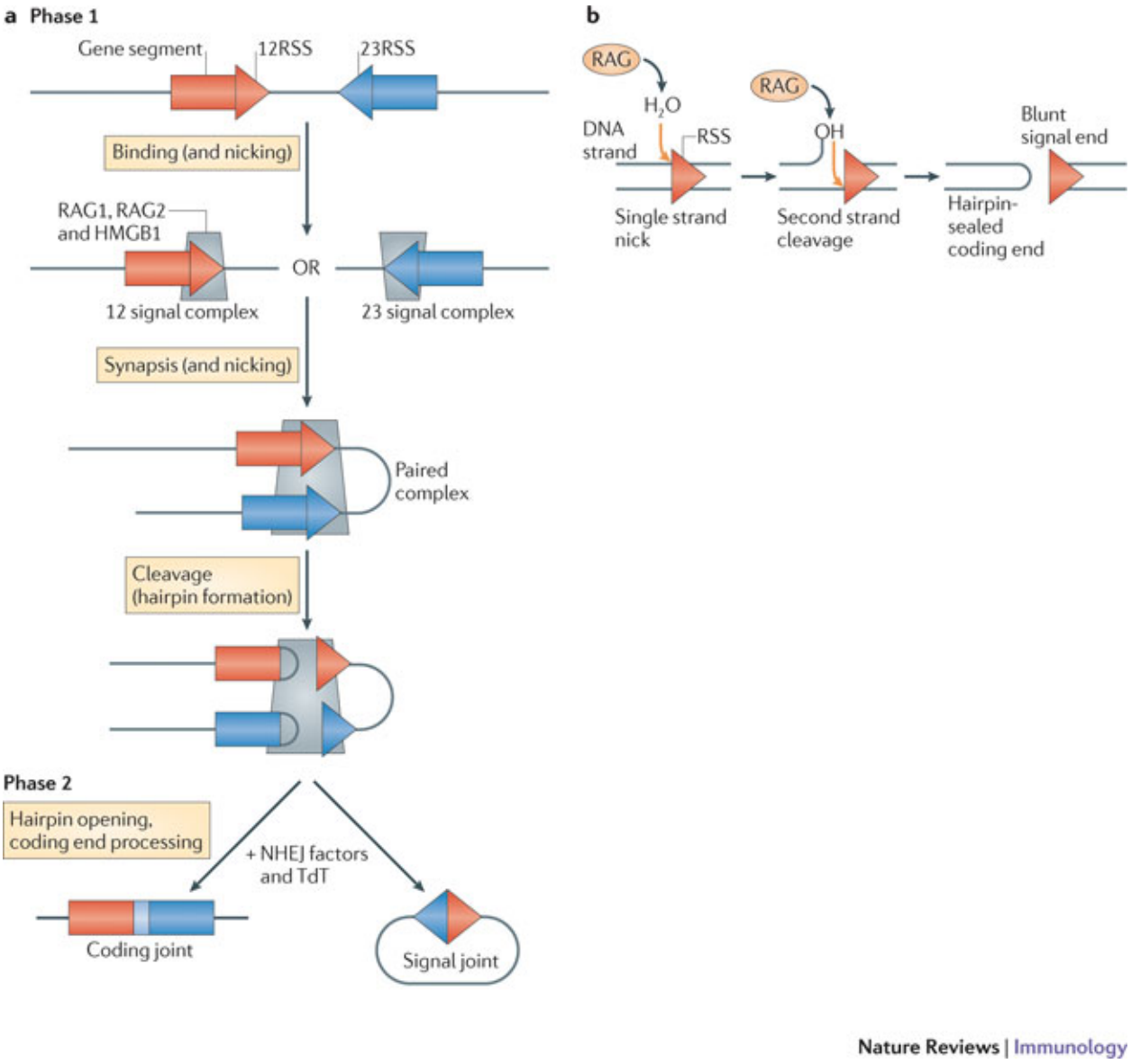

Figure 1.3 The Mechanism of V(D)J Recombination (Schatz and Ji, 2011)

Antigen receptor gene segments are flanked by RSSs. In the first phase, RAG proteins bind to the first RSS, forming a signal complex. Capture of the second RSS results in the formation of the paired complex, within which the RAG proteins introduce double strand breaks between the gene segments and the RSSs. In the second phase, the RAG proteins cooperate with non-homologous end joining (NHEJ) DNA repair factors to rejoin the DNA ends.

1.3 Stress Hematopoiesis

The hematopoietic system is exposed to various stress situations that necessitate rapid proliferation of the stem and progenitor cells to replenish the blood and immune system (Wilson et al., 2008). The regeneration of hematopoietic system under such situations is called stress hematopoiesis, and it can be induced by multiple stress factors, including BM transplantation (Thornley et al., 2001), radiation and chemotherapy (Mauch et al., 1995), heavy bleeding (Cheshier et al., 2007), infection (Baldridge et al., 2010), etc.

As the blood system relies on HSCs for life-long replenishment, significant effort has been devoted to study the effects of different stress factors on the regulation of HSCs. During homeostasis, signals from the bone marrow niche will preserve most HSCs dormant to preserve their long-term self-renewal potential (Wilson et al., 2007). At homeostatic condition, these dormant HSCs divide once every half a year in mice, making them unlikely to significantly contributes to the daily blood replenishment and suggesting that they can be the reserved pool that is responsible for stress induced proliferation (Trumpp et al., 2010). Indeed, it has been shown that hematopoietic stress induced by chemotherapeutic agent 5-fluorouracil (5-FU) can force almost all the dormant HSCs into cell cycle, which then give rise to a large number of downstream progenitors to maintain sufficient blood cell production (Wilson et al., 2008). Similar to chemotherapy, other stress factors that cause massive loss of hematopoietic cells, like irradiation and severe bleeding, have also been shown to induce proliferation of dormant HSCs (Cheshier et al., 2007). Although the specific mechanism that induces dormant HSCs into cycling upon these stress are still not clear, it seems to be transient as the activated HSCs return to dormancy once homeostasis is re-established (Wilson et al., 2008).

In addition to investigating the effects of stress on HSC maintenance, several studies have focused on stress erythropoiesis, which is the rapid development of new erythrocytes in the spleen and liver upon acute anemic stress (Paulson et al., 2011). These efforts led to the identification of multiple unique signals as key regulators of stress erythropoiesis including Hedgehog, bone morphogenetic protein 4 (BMP4), stem cell factor and hypoxia (Perry et al., 2007; Perry et al., 2009).

However, other than stress erythropoiesis, little is known if other hematopoietic lineages rely on distinct regulatory mechanisms to secure proficient progenitor proliferation and differentiation during stress.

1.4 Aging of the Hematopoietic System

As the organism is exposed to various stress factors throughout life, it is not surprising that aging leads to multiple defects within the hematopoietic system (Figure 1.4). These defects contribute to immunosenescence, which refers to the higher susceptibility to anemia, infectious diseases, autoimmunity and leukemia in the aged population (Henry et al., 2011; Weiskopf et al., 2009).

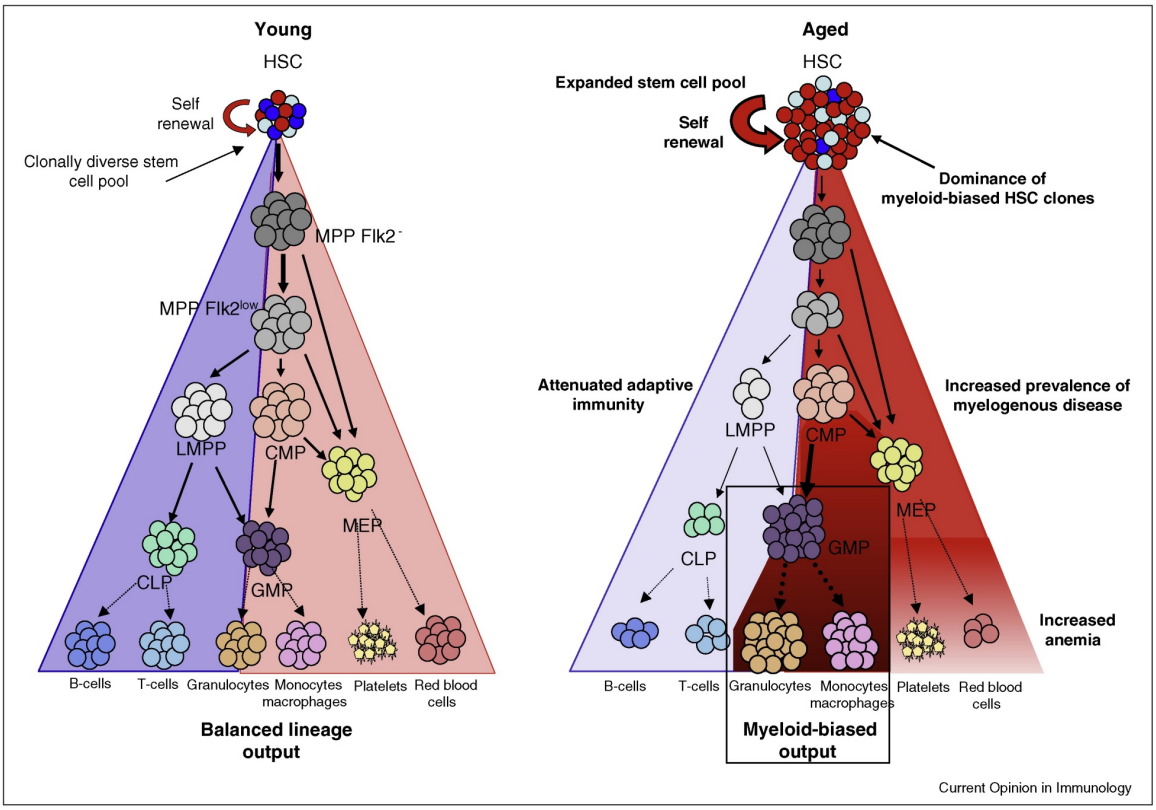

Figure 1.4 Aging of the Hematopoietic System

Aging of the hematopoietic system is accompanied by multiple defects at different levels. During physiological aging, immunophenotypic HSCs accumulate, but exhibit reduced repopulating potential and lineage bias. Combination of the biased lineage potential at the HSC level and defects in the differentiation potential at downstream progenitor level together resulted in a myeloid-biased output in the elderly.

Age related defects in the immune system originate already from the defective HSCs in the aged. In both murine and human system, physiological aging is associated with increased number of immunophenotypic HSCs (which are defined only by surface marker expression) in the BM. However, compared to young HSCs, these aged HSCs are not functionally equivalent: upon serial transplantation assay and other stress conditions, these aged HSCs exhibit multiple

functional defects such as decreased regenerative potential. (Chambers and Goodell, 2007; Morrison et al., 1996; Rossi et al., 2005) Indeed, this increase in number of HSCs cannot compensate for the decline in functionality, and therefore results in an overall reduced regeneration capacity of the HSC pool in the elderly (Sudo et al., 2000).

Other than the reduced self-renewal capacity, aging in the hematopoietic compartment also leads to biased lineage differentiation at both the HSC and downstream progenitor levels. Specifically, aging is accompanied by a reduction of the adaptive immune system (lymphoid compartment) and an increase of the myeloid compartment (Rossi et al., 2005). At the stem cell level, it has been shown that aged HSCs hold reduced lymphoid and erythroid potential compared to young HSCs, which is at least partially derived from the biased gene expression of lineage deciding factors in the aged HSCs (Rossi et al., 2005).

On top of the lineage bias at the HSC level, downstream lymphoid progenitors also exhibit defects during aging. Reduced B lymphopoiesis and immunoglobulin diversity are major factors underlying immunosenescence (Ademokun et al., 2010; Allman and Miller, 2005; Cancro et al., 2009; Dunn-Walters and Ademokun, 2010; Linton and Dorshkind, 2004; Melamed and Scott, 2012; Signer et al., 2007; Szabo et al., 2003; Szabo et al., 1999). During physiological aging, B cell production in the BM declines (Ademokun et al., 2010; Miller and Allman, 2003; Signer et al., 2007; Stephan et al., 1996; Szabo et al., 1999), although the size of peripheral B cell pool is initially preserved by a compensatory homeostatic maintenance of mature B cells (Kline et al., 1999). Multiple factors have been shown to contribute to reduced BM B lymphopoiesis in the aged, including loss of lymphoid biased HSCs (Cho et al., 2008; Miller and Allman, 2005; Muller-Sieburg et al., 2012) and decreased survival of pre-B cell progenitors (Kirman et al., 1998). (Figure 1.5)

1.5 DNA Repair Pathways

Genomic stability of stem and progenitor cells is critical, as it is required for proper self-renewal and differentiation capacity (Lombard et al., 2005). Throughout life, the immune system is exposed to multiple stress factors that may induce DNA damage, and therefore proper DNA repair mechanism is critical for maintaining the homeostasis of the immune system. In addition, specific DNA repair pathway, the non-homologous end joining (NHEJ) machinery, is also required for normal lymphoid development as described in the 1.2 sections above (Figure 1.5).

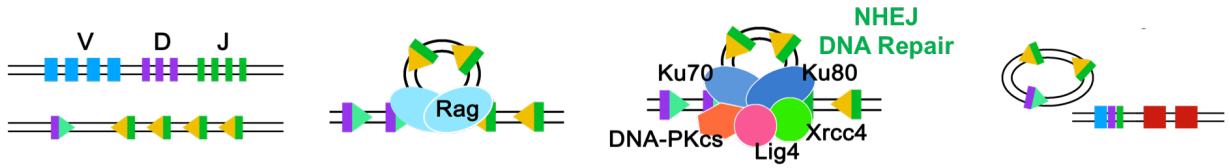

Figure 1.5 NHEJ Repair in V(D)J Rearrangement during B Lymphoid Development

During V(D)J rearrangement in the bone marrow, DNA double strand break (DSB) is generated by the RAG recombinase to allow the rearrangement of the immunoglobulin gene segments. Upon the completion of the rearrangement, the machinery of non-homologous end joining (NHEJ) repair pathway is recruited to the immunoglobulin gene to join the DNA ends.

DNA double strand break (DSB) is one of the most common types of DNA damage that can be induced by endogenous proliferative stress or exogenous insults like irradiation. In the presence of DSB, the sensor MRN complex that's composed of three key players, MRE11A, RAD50 and NBS1, can recognize the DSB through binding, and transduce the signal for downstream DNA damage responses. Two different pathways can be used by the cells to fix the

DNA break: homologous recombination (HR) and NHEJ (Sancar et al., 2004) (Figure 1.6). Eukaryotic cells choose one of these two pathways depending on cell cycle status: HR repair utilizes the sister chromatid as the template for accurate repair, and therefore can only occur in cycling cells; while NHEJ repair has a minimal requirement for homologous template, and therefore can happen in any stage of cell cycle, but is more error prone due to the lack of template (Weinstock et al., 2006).

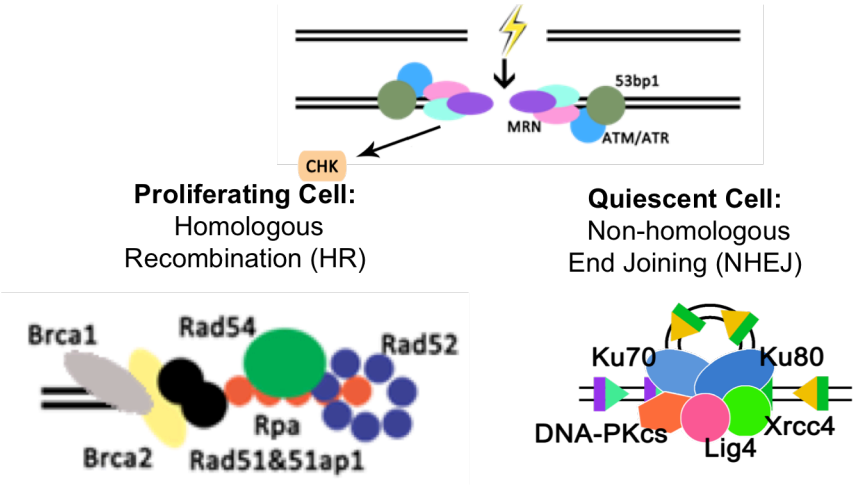

Figure 1.6 HR and NHEJ DNA Double Strand Break Repair Pathways

Eukaryotic cells utilize one of the two pathways for DNA double strand break (DSB) repair. In the presence of DSB, MRN (Mre11a, Rad50 & Nbs1) complex binds to the break end as a sensor, and transduce the signal through ATM/ATR protein to activate downstream repair pathways. In cycling cells, CHEK proteins are then activated to induce cell cycle arrest, and homologous recombination machinery is recruited to join the DNA end based on template DNA.

As majority of the HSCs stay in quiescence during homeostasis, HSCs typically adopt the more error-prone NHEJ repair for DSB repair while downstream progenitors utilize the more

accurate HR repair (Mohrin et al., 2010). However, as the NHEJ repair is more error-prone, it has been suggested that HSCs cannot efficiently protect their genome from mutations resulting from the inaccurate repair, especially during aging process when these mutations can accumulate to induce functional defects. Recent study suggests that some HSCs actually accumulate DNA damage during quiescence to avoid the error-prone NHEJ repair. Instead, these HSCs strategically induce the HR repair when entering into cell cycle (Beerman et al., 2014).

In addition to DNA DSB repair, other types of DNA repair mechanisms are also required to maintain the integrity of the genome: the nucleotide excision repair (NER) is required to excise oligonucleotide fragments surrounding abnormal bases, while the mismatch repair (MRR) machinery recognize single mismatches or misaligned short nucleotide repeats to fix them (Park and Gerson, 2005).

Although DNA repair is an important basic cellular function in most cell types, it is unknown if the cell types such that have unique needs for DNA repair, such as the long-lived hematopoietic stem cells, or the lymphoid progenitors that undergo rearrangement, have any cell type specific regulatory mechanisms that ensure the proper expression of the various components of the DNA machinery.

1.6 MEF2C in Normal Hematopoiesis

Multiple recent studies have identified myocyte enhancer factor 2C (MEF2C) as a key regulator of the B lymphoid system (Figure 1.7). MEF2C is a MADS box transcription factor

that was originally discovered as a regulator of cardiogenesis and myogenesis, as germline deletion of *Mef2c* in mice resulted in embryonic death due to cardiac defects (Lin et al., 1997).

In the adult mice bone marrow, *Mef2c* is highly expressed in common lymphoid progenitors (CLPs) and B cell progenitors, while its expression is barely detectable in T cells, granulocytes or erythrocytes (Stehling-Sun et al., 2009). Deletion of *Mef2c* by B cell specific Cd19-Cre revealed that MEF2C is required for B cell receptor induced proliferation of mature B cells in the spleen (Andrews et al., 2012; Khiem et al., 2008; Wilker et al., 2008). However, as the deletion of *Mef2c* by Cd19-Cre was not complete in BM B cell progenitors, this model could not be used to evaluate the presence of B cell progenitor defect. Deletion of *Mef2c* in the BM using interferon inducible Mx1-Cre and PIPC treatment, followed by transplantation or culture, led to a severe reduction of B lymphoid cells while myeloid cells were increased, proposing a role for MEF2C in myeloid versus lymphoid fate choice(Stehling-Sun et al., 2009). Our lab showed that hematopoietic specific deletion of *Mef2c* using Vav-Cre results in a reduction of BM B cell progenitors, especially pre-B cells, without overtly affecting the cellularity of peripheral B cells during homeostatic conditions (Gekas et al., 2009). A requirement for MEF2C within BM B lymphoid cells was also documented by inducing B cell specific deletion of *Mef2c* by Mb-1-Cre. This led to a reduction of B lymphoid cells in both BM and splenic compartments of neonates (2 weeks of age). Interestingly, while peripheral cellularity of B cells was corrected as the mice reached adulthood, BM B lymphopoiesis still remained compromised (Debnath et al., 2013).

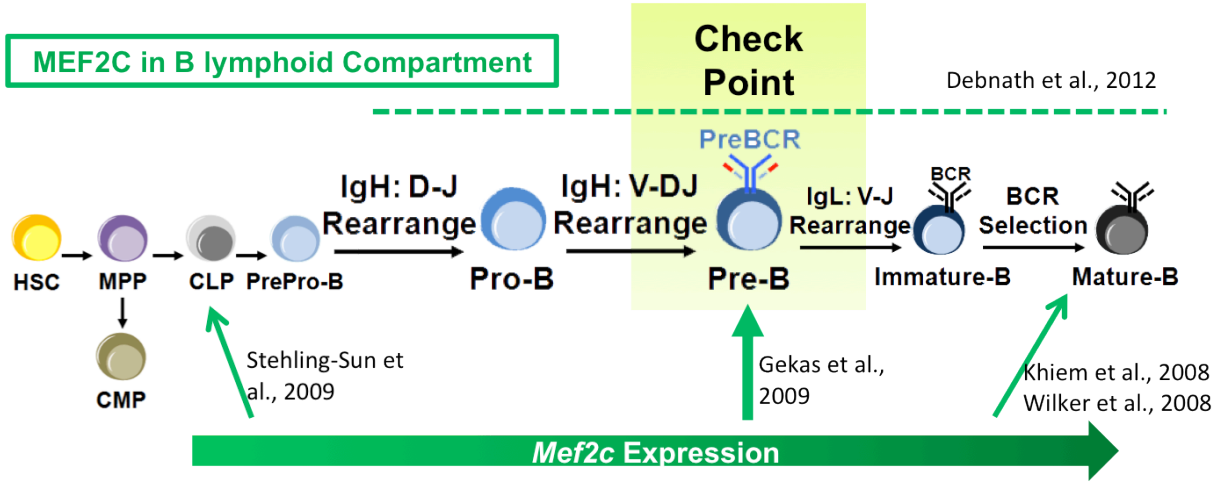

Figure 1.7 Previous Findings of MEF2C in the Hematopoietic System

Previous studies have documented that MEF2C functions at different stages of B-lymphopoiesis.

Although these studies proposed a requirement for MEF2C within BM B lymphoid progenitors, the cellular and molecular mechanisms through which MEF2C protects BM B lymphopoiesis have remained unknown. This question was particularly intriguing as *Mef2c* deficient mice share some features of B lymphoid aging (Gekas et al., 2009), which is characterized as a reduction of BM B cell progenitors while blood and splenic B cell cellularity can initially be maintained through compensatory mechanisms (Kline et al., 1999; Linton and Dorshkind, 2004; Miller and Allman, 2003; Stephan et al., 1996).

Aims of this Dissertation

Define the Underlying Causes for the Reduction of B Lymphoid Progenitors in *Mef2c* Deficient Mice

Previous work in our lab showed that loss of *Mef2c* in the hematopoietic system results in the reduction of bone marrow (BM) B Lymphoid progenitors, causing a phenotype reminiscent of B lymphoid aging. My goal with this thesis is to define the causes for such reduction by first testing if the reduced cellularity correlates with increased cell death of BM B lymphoid progenitors, and by assessing a potential function for MEF2C in regulating DNA repair, V(D)J rearrangement, and other pathways that are critical for the maintenance of B lymphoid progenitors. Gene expression profile is used to identify transcriptional changes in these pathways, and functional assays for each process are conducted to assess the effect of *Mef2c* loss.

Determine if the Requirement for MEF2C to Protect the Bone Marrow B lymphoid Compartment is Heightened upon Stress

As maintaining the integrity of the immune system during various stress situations is critical for an individual's health, I aim to define the requirement for MEF2C in protecting B lymphopoiesis under stress. Stress hematopoiesis is induced in control and *Mef2c* deficient mice by sub-lethal irradiation or chemotherapy treatment, and the recovery of various hematopoietic lineages will be tracked at different time points. Functional analyses for critical cellular process that MEF2C regulates (identified by Aim 1) are conducted in control and *Mef2c* deficient B

lymphoid progenitors during the recovery from stress to examine if MEF2C requirement is increased during stress.

Unveil the Molecular Mechanism that MEF2C Governs in Regulating the Transcription of Key Pathways in Hematopoietic Stem and Progenitor Cells

As MEF2C functions as a transcription factor, we aim to define the downstream target genes that MEF2C regulates to maintain homeostasis of the immune system. We aim to identify which subset of targets is directly regulated by MEF2C by analyzing MEF2C ChIP-sequencing data in B lymphocytes.

Explore the Requirement for MEF2C in Protecting Hematopoietic Stem Cells

As the expression of *Mef2c* is highly enriched also in the hematopoietic stem cell (HSC) compartment, we seek to explore the function of MEF2C in the HSC pool. The frequency of phenotypic and functional HSCs in control and *Mef2c* deficient mice will first be determined to test the requirement for MEF2C in maintaining HSC integrity. Gene expression comparisons will then be conducted to identify potential cellular processes that MEF2C regulates in HSCs.

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Chapter 2:

MEF2C Protects Bone Marrow B Lymphoid Progenitors during Stress Hematopoiesis

MEF2C Protects Bone Marrow B Lymphoid Progenitors during Stress Hematopoiesis

[1, 2, 3] Wenyuan Wang, [1, 2,4] Tonis Org¶, [1, 2] Amélie Montel-Hagen¶, [1, 2] Peter D. Pioli [1, 2] Edo Israely, [1, 2] Dan Duan, [5,6] Daniel Malkin, [7] Johanna Flach, [5,6] Robert H. Schiestl, [1, 2, 3, 6] Hanna K.A Mikkola*

[1]Department of Molecular, Cell and Developmental Biology, University of California Los Angeles, Los Angeles, CA 90095, USA. [2]Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research. [3]Molecular Biology Institute, [4]Institute of Molecular and Cell Biology, University of Tartu, Estonia, [5]Department of Molecular Toxicology, [6]Jonsson Comprehensive Cancer Center, UCLA. [7]The Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, University of California, San Francisco.

¶These authors contributed equally to this work

Contact: Hanna Mikkola, hmikkola@mcdb.ucla.edu, 1-310-825-2565, 615 Charles E. Young Dr S, Los Angeles, CA 90095

Abstract

Lifelong immune cell production requires DNA repair mechanisms to protect genomic integrity of stem/progenitor cells. DNA double strand break (DSB) repair is also critical for V(D)J recombination and B cell receptor generation, which are pre-requisites for B cell progenitor survival. We discovered that MEF2C enhances the expression of DNA repair and V(D)J factors in B cell progenitors, promoting DSB repair, V(D)J recombination and B cell progenitor survival. While *Mef2c* deficient mice maintained relatively intact peripheral B lymphoid cellularity during homeostasis, they exhibited poor B lymphoid recovery after sub-lethal irradiation and 5-fluorouracil injection. MEF2C binding in human B lymphoblasts co-localized with active histone marks at promoters and enhancers of DNA repair and V(D)J factors. Moreover, *Mef2C* deficient mouse pre-B cells showed reduced chromatin accessibility in regulatory regions in these genes. These data imply that MEF2C protects B lymphopoiesis during stress by ensuring proper expression of DNA repair and B cell factors.

Ability to sustain B lymphopoiesis through various stress situations is essential for immune function. B lymphopoiesis occurs in bone marrow (BM) where B lymphoid progenitors undergo V(D)J recombination to generate B cell receptors (BCRs)[1-4]. The success of V(D)J recombination is critical for humoral immunity as diverse BCRs are required to recognize antigens and generate antibodies. V(D)J recombination is initiated by creating DNA double strand breaks (DSBs) by RAG recombinases at the border of recombining gene segments[5,6]. After rearrangement, the DSBs are repaired by non-homologous end joining (NHEJ) machinery[7,8]. Defective DNA repair during this process results in cell death or genetic lesions[9], making B lymphopoiesis inherently vulnerable. To ensure genomic integrity, B lymphoid progenitors adopt a tight regulation of cell survival to exclude cells with abnormal rearrangement[10]. This homeostatic balance is altered during physiological aging[11-13] due to reduced V(D)J recombination efficiency[14,15] and increased B lymphoid progenitor death[16], which contributes to the impaired immune function during aging.

The hematopoietic system encounters various stress factors that necessitate rapid proliferation of stem/progenitor cells to replenish the blood/immune system[17]. The regeneration of hematopoietic system under such situations is called stress hematopoiesis, and it can be induced by BM transplantation[18], radiation and chemotherapy[19], bleeding[20], infection[21], etc. In addition to investigating the effects of stress on HSC maintenance, several studies have focused on stress erythropoiesis and identified multiple unique signals that regulate this process[22]. However, little is known how other hematopoietic lineages secure proficient progenitor proliferation and differentiation during stress.

Recent studies identified myocyte enhancer factor 2C (MEF2C) as a regulator of B lymphoid system. MEF2C is a MADS box transcription factor originally discovered as a

regulator of cardiogenesis and myogenesis[23]. In BM, *Mef2c* is highly expressed in common lymphoid progenitors (CLPs) and B lymphoid cells, while its expression is minimal in T cells, granulocytes or erythrocytes[24]. Deletion of *Mef2c* by B-cell specific Cd19-Cre showed that MEF2C is required for BCR-induced proliferation of mature B cells in the spleen[25-27]; however, as the deletion of *Mef2c* was not complete in BM B cell progenitors, this model could not be used to evaluate the presence of B cell progenitor defect. Deletion of *Mef2c* using Mx1-Cre and PIPC treatment, followed by transplantation or culture, led to a severe reduction of B lymphoid cells while myeloid cells were increased, proposing a role for MEF2C in myeloid/lymphoid fate choice[24]. We showed that hematopoietic specific deletion of *Mef2c* using Vav-Cre results in a reduction of BM B cell progenitors, especially pre-B cells, without overtly affecting peripheral B cell pool during homeostatic conditions[28]. A requirement for MEF2C within BM B lymphoid cells was also documented by using B-cell specific Mb-1-Cre. This led to a reduction of B lymphoid cells in both BM and spleen of neonates (2 weeks of age). While peripheral cellularity of B cells was corrected as the mice reached adulthood, BM B lymphopoiesis still remained compromised[29]. A recent study showed that MEF2C acts in part redundantly with MEF2D, and MEF2C/D are activated by pre-BCR signaling to promote target gene expression[30]. Although these studies proposed a requirement for MEF2C within B lymphoid progenitors, the cellular and molecular mechanisms through which MEF2C protects BM B lymphopoiesis have remained largely unknown. This question was particularly intriguing as *Mef2c* deficient mice share features of B lymphoid aging[28], which is characterized by reduction of BM B cell progenitors while peripheral B cell cellularity can initially be maintained through compensatory mechanisms[11-13].

Here we report that MEF2C protects BM B lymphoid progenitor pool by augmenting the transcription of factors crucial for DNA repair and V(D)J recombination, thereby ensuring efficient BM B lymphopoiesis. Loss of *Mef2c* severely compromised B lymphoid recovery after sub-lethal irradiation or 5-FU (5-fluorouracil) injection, documenting a critical function for MEF2C during regenerative stress. MEF2C binding was observed at genes encoding key DNA repair and V(D)J factors as well as B cell transcription factors in human B lymphoblasts, co-localizing with co-activator p300 and epigenetic marks representing active enhancers and promoters. Moreover, ATAC-sequencing showed that MEF2C enhances chromatin accessibility at regulatory regions of its target genes in mouse pre-B cells. These findings uncover a central role for MEF2C as a transcriptional activator of DNA repair and B cell regulatory machinery in B lymphoid progenitors, and document a unique requirement for MEF2C dependent transcriptional mechanisms to secure efficient BM B cell production during stress hematopoiesis.

Results

MEF2C maintains the cellularity of the BM B lymphoid compartment by securing B lymphoid progenitor survival

To define the processes that MEF2C regulates in B lymphoid cells, *Mef2c*$^{fl/fl}$ was conditionally deleted in mice using Vav-Cre, which deletes loxP-targeted alleles in hematopoietic cells during mid-gestation[28,31]. Unless otherwise stated, middle-aged mice (7-10 months old) were used for the analyses, as our prior study showed that the reduction in *Mef2c* deficient B cell progenitors was most notable at this age[28]. Analysis of larger groups of middle-aged adult mice confirmed a specific reduction of BM B cells in *Mef2c* deficient mice, while B lymphocytes in blood and spleen were not significantly affected (**Supplementary Figure 1**). In addition to the reduction of pre-pro-B and pre-B cells in *Mef2c* deficient mice observed previously[28], analysis of a larger cohort of mice suggested that all BM B lymphoid progenitor stages (pre-pro-B, pro-B and pre-B) were significantly reduced (**Figure 1a**). The reduction of all BM B cell progenitor subsets was also confirmed by immunophenotyping B cell development as defined by Hardy et al.[32] (**Supplementary Figure 2a**). In contrast, the cellularity of mature recirculating B cells (sIgM$^+$ or Fr.F) in BM was not affected (**Figure 1a**). Moreover, Vav-Cre *Mef2c*$^{fl/fl}$ mice showed no significant difference in the percentage of CLPs, defined as Lin$^-$c-Kit^{lo}AA4.1$^+$IL-7Rα^+Flt3$^+$ (**Figure 1b**) or Lin$^-$IL-7R$^+$Sca-1^{lo}c-Kit^{lo} (**Supplementary Figure 2b**). These data suggested that MEF2C has a critical function in maintaining the integrity of BM B lymphoid progenitor compartment.

Analysis of BM B cell progenitors at different ages showed a reduction already in young and middle aged *Mef2c* deficient mice. By 8-10 months of age, *Mef2c* deficient mice exhibited a

comparable decrease in pre-B cell percentages as controls showed by 20-22 months (**Supplementary Figure 3**). These results raised the question if MEF2C controls similar molecular and cellular processes that are negatively affected during B cell aging.

Regulation of cell viability is a critical mechanism by which B lymphoid progenitors maintain quality control[10]. Analysis of annexin V expression revealed increased cell death of all BM B lymphoid progenitor subsets in *Mef2c* deficient mice. This effect was specific to the B cell progenitor compartment as BM $sIgM^+$ mature B lymphocytes, total BM cells and CLPs were unaffected (**Figure 1c,d**). These data suggest that MEF2C plays an important role in protecting BM B lymphoid progenitor survival. *Bcl2l1*(Bcl-x_L*)* is a well-studied pro-survival factor for BM B cell progenitors and has previously been shown to be regulated by MEF2C in mature B cells[26,33]. Q-PCR revealed that MEF2C is required for normal expression of *Bcl2l1* in both pro- and pre-B cells (**Supplementary Figure 2c**), further validating the importance for MEF2C in regulating B cell progenitor survival.

Figure-1

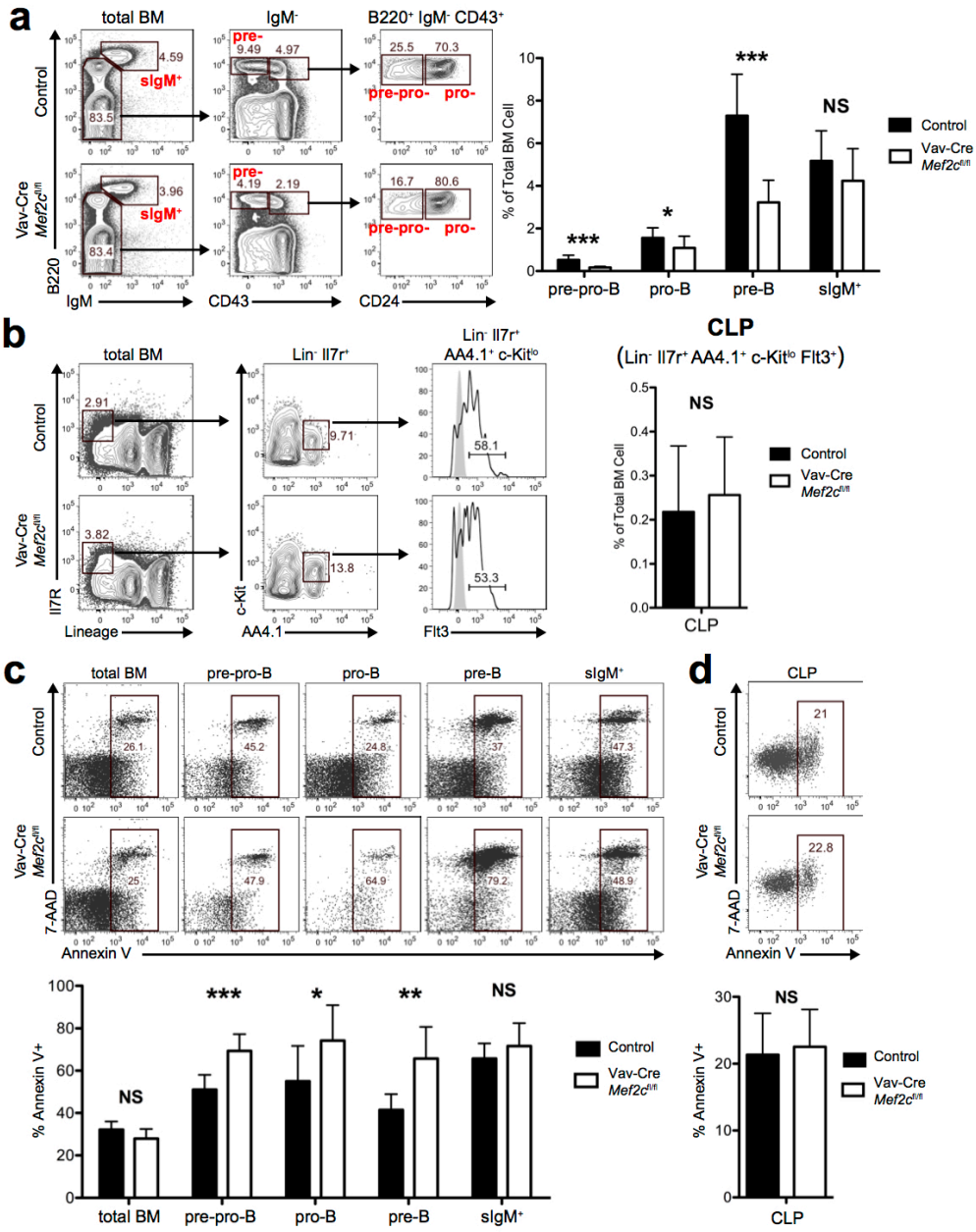

Figure 1. MEF2C maintains the integrity of BM B lymphoid compartment by promoting B cell progenitor survival

(a) Flow cytometric analysis of BM B lymphoid progenitor fractions in Vav-Cre *Mef2c*$^{fl/fl}$ mice as compared to control mice revealed reduction of all B lymphoid progenitors in *Mef2c* deficient mice while the mature B cells in the BM (sIgM^{+}) were not affected (n≥11). **(b)** Flow cytometric

analysis suggested that loss of *Mef2c* does not affect the frequency of BM common lymphoid progenitors (CLP) (n≥7). **(c)** Flow cytometric analysis of annexin V positivity in BM B lymphoid progenitors documented increased cell death in *Mef2c* deficient B lymphoid progenitors while total BM and $sIgM^+$ cells were not affected (n≥7). **(d)** Annexin V positivity was not increased in *Mef2c* deficient CLP (n≥8). All mice were analyzed at 7-10 months of age. Data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

MEF2C is essential for efficient B lymphoid recovery during stress hematopoiesis

Since loss of *Mef2c* compromised the survival and cellularity of BM B lymphoid progenitors during homeostasis, we asked if these defects are exacerbated in stress situations. Sub-lethal irradiation ablates most hematopoietic cells, including B lymphocytes[34], and leads to rapid proliferation and differentiation of stem/progenitor cells. To investigate the function of MEF2C during regenerative stress, the cellularity of different hematopoietic lineages was monitored following 6Gy sub-lethal total body irradiation (**Figure 2a**). 2 days after irradiation, a drastic loss of blood B, T and myeloid cells was observed in both *Mef2c* deficient and control mice (**Figure 2b,c, Supplementary Figure 4a,b**). No significant difference in total white blood cell (WBC) or red blood cell (RBC) counts was observed in *Mef2c* deficient mice compared to controls before or after sub-lethal irradiation. The previously identified reduction in platelets[28] was retained after irradiation (**Supplementary Figure 4c**). However, while control mice regained normal levels of B cells within 6 weeks following irradiation, *Mef2c* deficient mice showed minimal B lymphoid recovery at this stage (**Figure 2b,c**). In contrast, the recovery of T

lymphoid and myeloid lineages in *Mef2c* deficient mice was comparable to that of controls (**Figure 2c, Supplementary Figure 4a,b**). The spleens of *Mef2c* deficient animals also demonstrated impaired B lymphoid recovery, thus excluding the sequestration of B cells in peripheral lymphoid organs as a major contributor to the impaired recovery of circulating B cells (**Figure 2d**).

Figure-2

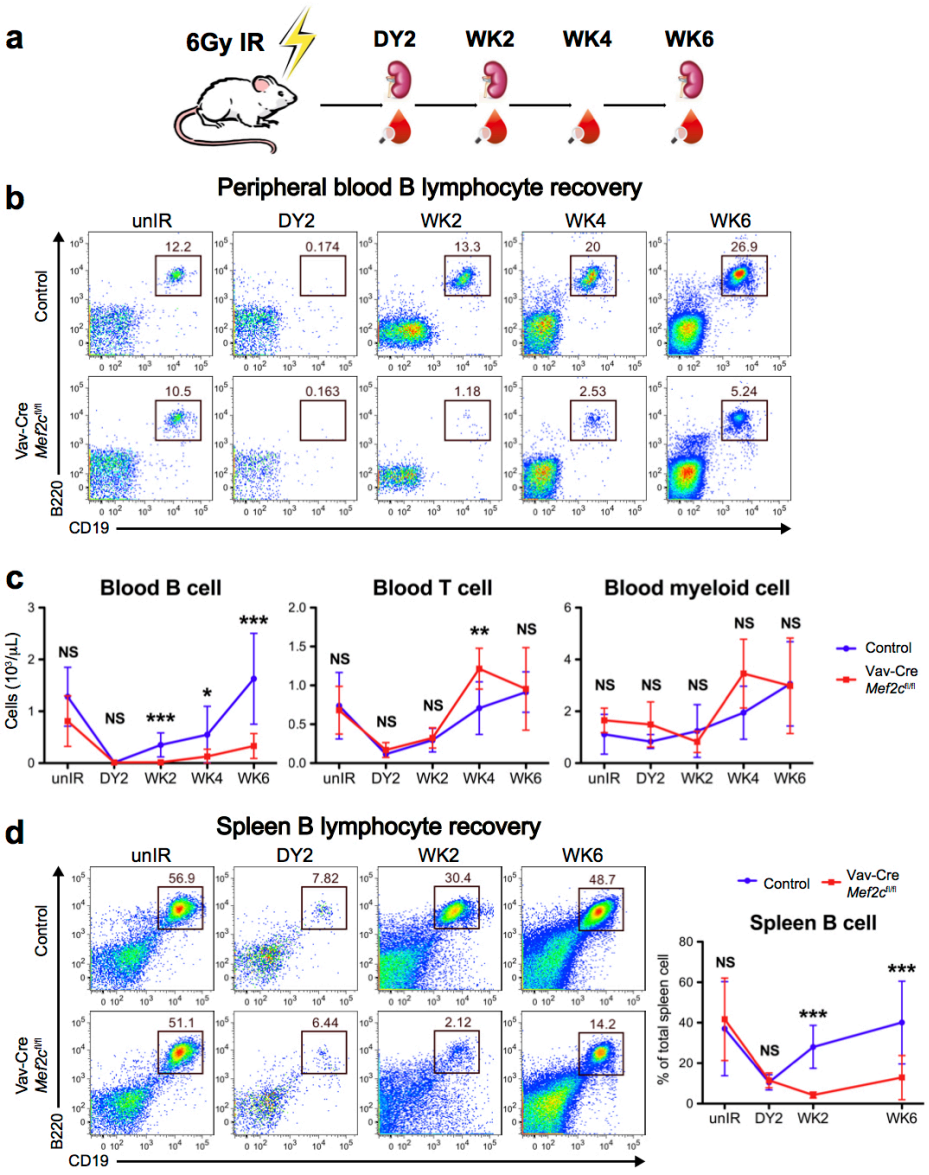

Figure 2. MEF2C is required for efficient peripheral B lymphoid recovery upon sub-lethal irradiation induced stress hematopoiesis

(a) Experimental design of hematopoietic ablation by sub-lethal irradiation: Vav-Cre *Mef2c*$^{fl/fl}$ and control mice received 6Gy of total body irradiation. Peripheral blood was collected for CBC and flow cytometric analysis at day 2 and weeks 2, 4 and 6. Mice were sacrificed and spleens were analyzed by flow cytometry at day 2, and weeks 2 and 6. **(b)** Representative flow cytometric analysis of peripheral blood B cells revealed defective B cell recovery in *Mef2c* deficient mice. **(c)** Quantification of peripheral blood lineage cell count (WBC count from CBC was multiplied with each lineage-specific percentage obtained from flow cytometry) documents that loss of *Mef2c* compromised the recovery of B cells while T and myeloid cells were not affected. **(d)** Flow cytometric analysis of spleen B cells in both Vav-Cre *Mef2c*$^{fl/fl}$ and control mice after irradiation revealed defective B cell recovery in *Mef2c* deficient spleens. All mice were analyzed at 7-10 months of age. Day 2: n=4 mice, data shown are the mean ± SD of two independent experiments; other time points: n≥5, data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

To better understand the reason for the compromised peripheral B lymphoid recovery in *Mef2c* deficient mice during irradiation induced stress, kinetics of BM recovery was assessed (**Figure 3a**). At 2 days after irradiation, the cellularity of BM B lymphoid progenitors was greatly reduced in both *Mef2c* deficient and control mice. Although the recovery of total BM cells was comparable between *Mef2c* deficient mice and controls, the recovery of BM B cells was markedly impaired in the absence of *Mef2c* (**Figure 3b, Supplementary Figure 5a,b**). By 2 weeks after irradiation, the cellularity of all BM B lymphoid progenitors in controls had drastically improved, and by 6 weeks, reached a level comparable to un-irradiated mice (**Figure**

3c). In contrast, there was minimal recovery of all B cell progenitor subsets in *Mef2c* deficient mice at week 2. Additionally, *Mef2c* loss also compromised CLP recovery 2 weeks post irradiation (**Figure 3d**). By week 6, levels of pre-pro-B and pro-B cells in irradiated *Mef2c* deficient mice were similar to those in un-irradiated mice. This was not the case for pre-B cells and $sIgM^+$ B cells as their cell numbers were still severely reduced (**Figure 3c**). These data suggested that the impaired recovery of peripheral B cells in *Mef2c* deficient mice results from inefficient BM B lymphopoiesis that is affected already at the level of CLP, and pinpointed the pre-B cell stage as a major bottleneck delaying B lymphoid recovery in *Mef2c* deficient mice during irradiation-induced stress hematopoiesis.

To investigate whether the poor regeneration of B lymphoid cells in *Mef2c* deficient mice correlated with increased cell death, viability of BM B cell progenitors was assessed. Annexin V staining 2 weeks after irradiation showed increased cell death in *Mef2c* deficient B lymphoid progenitors (**Supplementary Figure 5c**), documenting a critical requirement for MEF2C in protecting BM B lymphoid progenitor survival upon regenerative stress.

To test if MEF2C governs stress hematopoiesis that is caused by other factors than irradiation, we analyzed the BM recovery of control and *Mef2c* deficient mice after 5-FU (5-fluorouracil) injection, which ablates cycling hematopoietic cells and promotes rapid proliferation and differentiation of remaining hematopoietic cells (**Figure 3e**). Similar to the recovery from irradiation, the recovery of BM B cells, but not total BM or BM myeloid cells, was compromised in *Mef2c* deficient mice at week 2 (**Figure 3f**). Analysis of BM progenitor subsets revealed that MEF2C is required for proper B lymphoid progenitor and CLP recovery upon 5-FU induced stress hematopoiesis (**Figure 3g**). These data collectively suggest that

MEF2C is required to protect B lymphoid progenitors during regenerative stress, even in the absence of external DNA damage caused by irradiation.

Figure-3

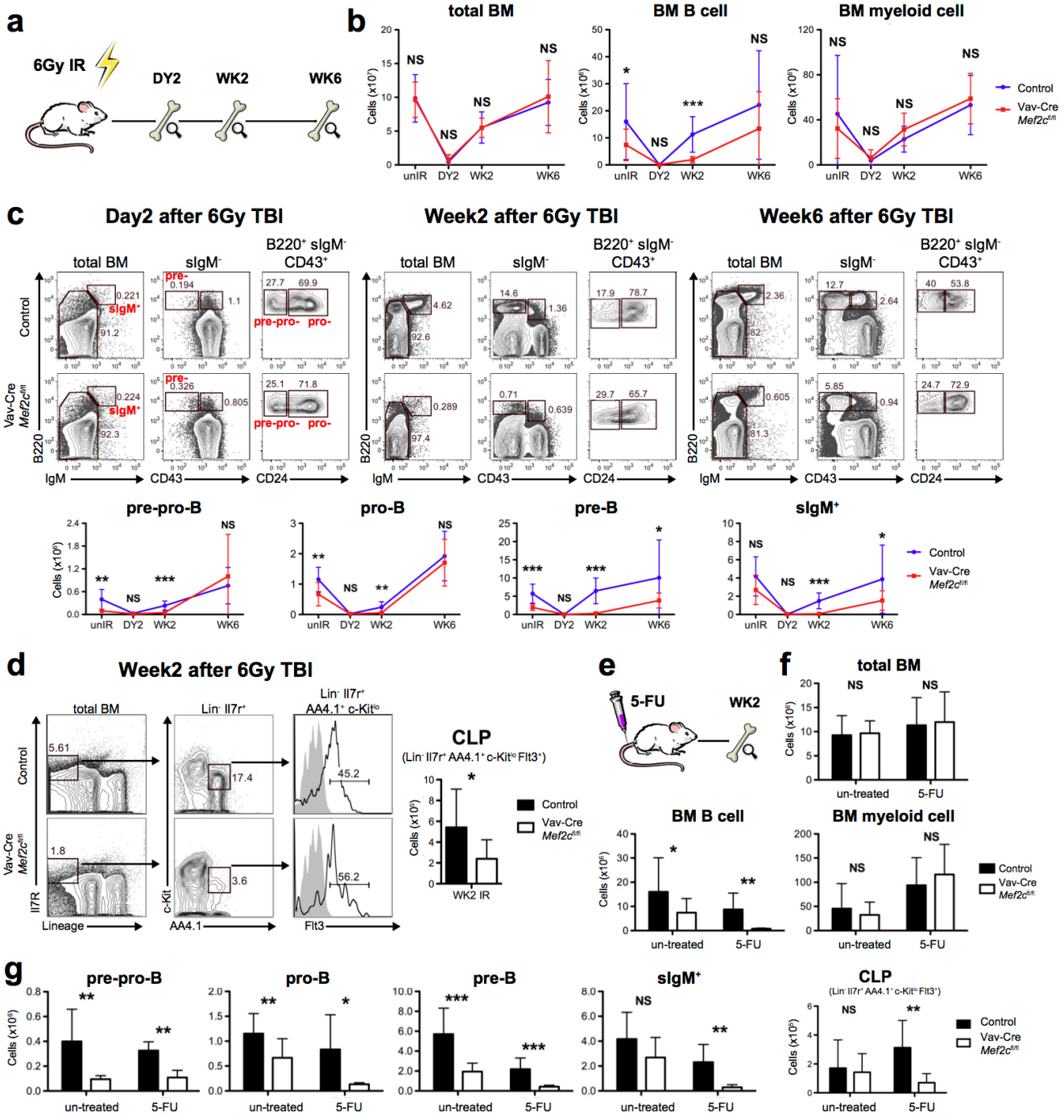

Figure 3. In the absence of *Mef2c*, the BM pre-B cell stage is the major bottleneck for B lymphoid recovery during stress hematopoiesis

(a) Experimental design of BM recovery analysis: Vav-Cre *Mef2c*$^{fl/fl}$ and control mice received 6Gy of total body irradiation. Mice were sacrificed and BM compartments were analyzed by flow cytometry at day 2 and weeks 2 and 6. **(b)** Quantification of total BM, BM B and myeloid cellularity shows comparable total BM and myeloid cell counts in *Mef2c* deficient and control mice while reduced BM B cell counts in *Mef2c* deficient mice during recovery from irradiation. **(c)** Representative flow cytometric analysis and quantification of BM B lymphoid progenitors at 2 days, 2 weeks and 6 weeks post 6Gy irradiation shows that loss of *Mef2c* compromises most drastically the recovery of pre-B cells and downstream sIgM^{+} BM B cells. **(d)** Quantification of CLPs at 2 weeks post 6Gy irradiation shows that *Mef2c* is required for proper CLP recovery upon IR induced stress. **(e)** Experimental design of 5-FU stress analysis: Vav-Cre *Mef2c*$^{fl/fl}$ and control mice received sub-lethal dose of 5-FU and BM was collected at week 2 for flow cytometry. **(f)** Quantification of total BM, BM B and myeloid cellularity shows comparable total BM and myeloid cell counts in *Mef2c* deficient and control mice while reduced BM B cell counts in *Mef2c* deficient mice during recovery from 5-FU. **(g)** Quantification of BM B cell progenitors and CLPs at week 2 post 5-FU injection revealed compromised recovery of all B cell progenitors and CLPs in *Mef2c* deficient mice. All mice were analyzed at 5-10 months of age. Day 2: n=4, data shown are the mean ± SD of two independent experiments; other time points: n≥5, data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

MEF2C regulates DNA double strand break repair in BM B lymphoid progenitors

To determine the molecular basis for the inefficient B lymphopoiesis in *Mef2c* deficient BM, microarray analysis was used to identify the pathways regulated by MEF2C. The analysis was first focused on pre-B cells, as this population is a major bottleneck for B lymphoid recovery from irradiation in *Mef2c* deficient BM.

Lack of *Mef2c* significantly ($|FC| \geq 2.0$, p-value≤ 0.05) reduced the expression of 1,884 genes and increased the expression of 436 genes in pre-B cells (**Figure 4a, Supplementary Table 1**). Gene Ontology (GO) analysis of differentially expressed genes identified cell cycle, DNA repair and transcription processes among the most significantly enriched categories among the genes down-regulated in *Mef2c* deficient pre-B cells (**Figure 4b**), while only few significant GO categories were identified among the genes up-regulated in *Mef2c* deficient cells (**Supplementary Table 1**). Taken together, these data suggest that MEF2C primarily functions as an activator in BM B cell progenitors.

As DNA DSB repair is critical for cell survival and V(D)J recombination, we evaluated the expression of DSB repair genes in *Mef2c* deficient pre-B cells. Analysis of microarray data suggested that loss of *Mef2c* affects both NHEJ and homologous recombination (HR) repair pathways. As such, the transcription of DSB sensors (*Mre11a* & *Rad50*) and effectors of both NHEJ (*Xrcc4*, *Xrcc6* (also known as *Ku70*) and *Lig4*) and HR (*Chek1&2*, *Rad51* and *Rad54l*) were significantly down-regulated in *Mef2c* deficient cells (**Figure 4c**). Reduced expression of selected DSB repair genes (*Mre11a*, *Xrcc4*, *Xrcc6* and *Lig4*) was validated by Q-PCR in pre-B cells (**Figure 4d**). These data raised the question as to whether *Mef2c* deficiency compromises DNA repair in B cell progenitors.

To assess the functional requirement for MEF2C in DNA repair, alkaline comet assay, which detects DNA strand breaks, was performed. Qualitative and quantitative analysis of comet tails revealed a high level of DNA damage in *Mef2c* deficient pre-B cells (**Figure 4e**). To assay DSB repair specifically, immunostaining for γH2AX was conducted. Quantification of γH2AX foci revealed increased DNA DSBs in *Mef2c* deficient pre-B cells (**Figure 4f**), confirming the requirement for MEF2C in proper DSB repair in BM B lymphoid progenitors.

Although pre-B cell stage was a major bottleneck delaying *Mef2c* deficient B lymphoid recovery during stress, the survival of other B cell progenitors was also reduced in *Mef2c* deficient mice both during homeostasis and stress. We therefore asked if MEF2C regulates DNA repair processes in other B cell progenitors. Microarray and Q-PCR analysis of pro-B cells suggested that MEF2C also transcriptionally regulates DNA repair machinery in these cells (**Supplementary Figure 6, Supplementary Table 2**).

To further define the stages of hemato-lymphoid development in which MEF2C functions to enhance DNA repair, alkaline comet assay was performed on multiple hematopoietic subpopulations. Similar to pre-B cells, lack of *Mef2c* led to increased DNA damage in pro-B cells, whereas the upstream CLPs or downstream BM $sIgM^+$ mature B cells were not significantly affected (**Figure 4g**). Moreover, lack of *Mef2c* did not affect the level of DNA damage in thymic T cell progenitors (DN1 & DN2) or BM myeloid cells (**Figure 4g**). These data documented that MEF2C is required for efficient DNA repair specifically in BM B lymphoid progenitors downstream of CLP.

Figure-4

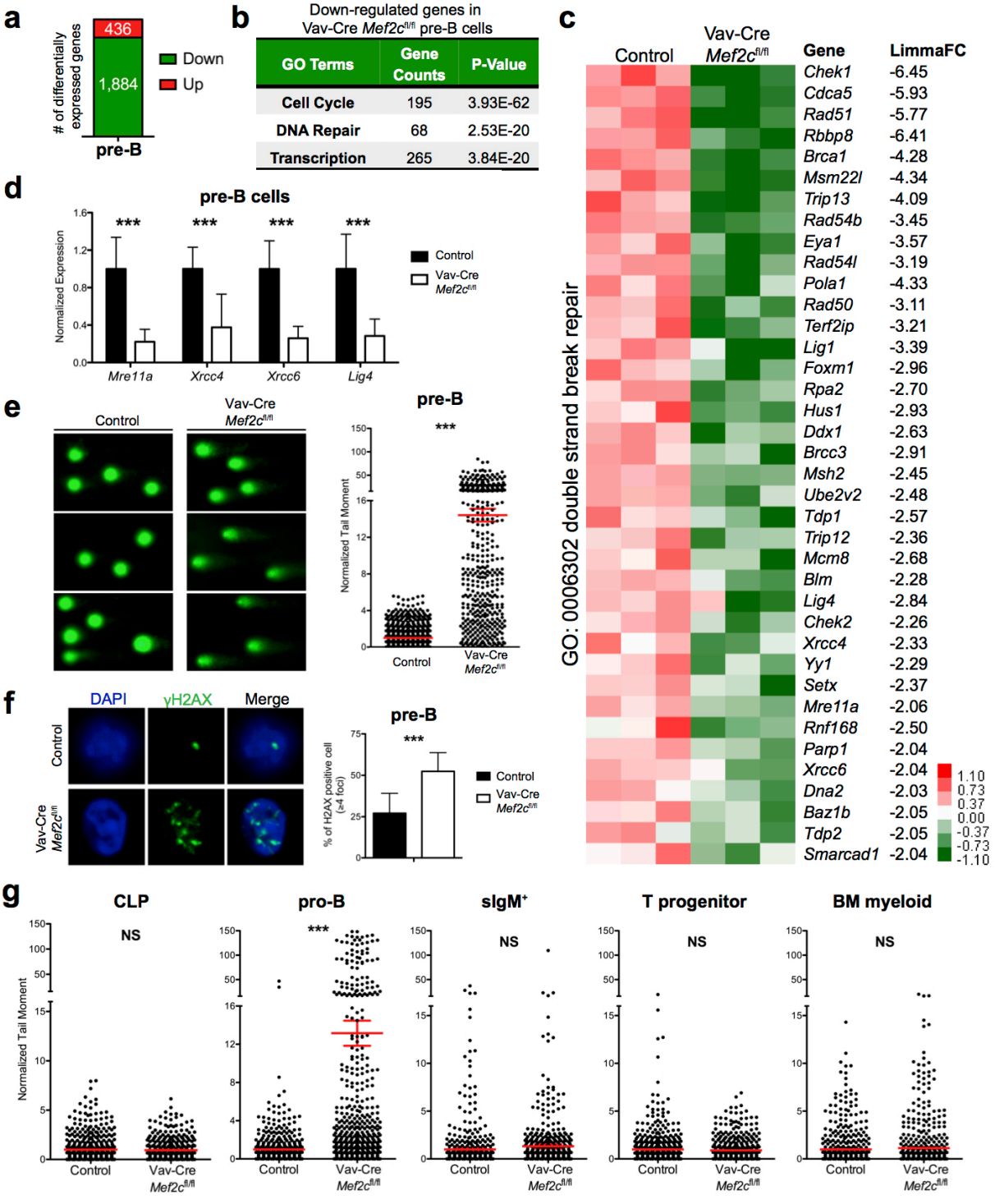
a
# of differentially expressed genes
436
1,884
Down
Up
pre-B
b
Down-regulated genes in Vav-Cre Mef2c^fl/fl pre-B cells
GO Terms | Gene Counts | P-Value
Cell Cycle | 195 | 3.93E-62
DNA Repair | 68 | 2.53E-20
Transcription | 265 | 3.84E-20
c
Control
Vav-Cre Mef2c^fl/fl
GO: 0006302 double strand break repair
Gene | LimmaFC
Chek1 -6.45
Cdca5 -5.93
Rad51 -5.77
Rbbp8 -6.41
Brca1 -4.28
Msm22l -4.34
Trip13 -4.09
Rad54b -3.45
Eya1 -3.57
Rad54l -3.19
Pola1 -4.33
Rad50 -3.11
Terf2ip -3.21
Lig1 -3.39
Foxm1 -2.96
Rpa2 -2.70
Hus1 -2.93
Ddx1 -2.63
Brcc3 -2.91
Msh2 -2.45
Ube2v2 -2.48
Tdp1 -2.57
Trip12 -2.36
Mcm8 -2.68
Blm -2.28
Lig4 -2.84
Chek2 -2.26
Xrcc4 -2.33
Yy1 -2.29
Setx -2.37
Mre11a -2.06
Rnf168 -2.50
Parp1 -2.04
Xrcc6 -2.04
Dna2 -2.03
Baz1b -2.05
Tdp2 -2.05
Smarcad1 -2.04
1.10
0.73
0.37
0.00
-0.37
-0.73
-1.10
d
pre-B cells
Normalized Expression
***
Control
Vav-Cre Mef2c^fl/fl
Mre11a
Xrcc4
Xrcc6
Lig4
e
Control
Vav-Cre Mef2c^fl/fl
pre-B
***
Normalized Tail Moment
f
DAPI
γH2AX
Merge
Control
Vav-Cre Mef2c^fl/fl
pre-B
***
% of H2AX positive cell (≥4 foci)
g
CLP
pro-B
sIgM+
T progenitor
BM myeloid
NS
***
Normalized Tail Moment
Control
Vav-Cre Mef2c^fl/fl

Figure 4. MEF2C regulates DNA double strand break (DSB) repair in BM B cell progenitors

(a) Microarray analysis of pre-B cells from control & Vav-Cre *Mef2c*$^{fl/fl}$ mice (9 months old) revealed 1,884 significantly (|FC|≥2 and p≤0.05) down-regulated and 436 up-regulated genes in the absence of *Mef2c* (n=3). **(b)** Cell cycle, DNA repair and transcription were among the most significantly enriched GO categories of genes down-regulated in *Mef2c* deficient pre-B cells. **(c)** DNA DSB repair genes that are significantly down-regulated in *Mef2c* deficient pre-B cells are shown. **(d)** Q-PCR of key genes encoding DNA repair machinery validated defective expression in *Mef2c* deficient pre-B cells. n≥5 mice, data shown are the mean ± SD of two or more independent experiments. **(e)** Representative figures and quantification of alkaline comets revealed increased DNA damage in *Mef2c* deficient pre-B cells (7-10 months old). n≥6 mice, data shown are the mean ± SEM of three independent experiments. 728 control pre-B cells and 450 *Mef2c* deficient pre-B cells were analyzed. **(f)** Representative IF analysis of γH2AX in pre-B cells (7-10 months old) revealed that MEF2C is required for proper DSB repair in pre-B cells. n≥6 mice, data shown are the mean ± SD of three independent experiments. **(g)** Quantification of alkaline comets revealed increased DNA damage in pro-B cells, but not CLP, sIgM^{+} BM B cells, thymic T progenitors (DN1 and 2) or BM myeloid cells in *Mef2c* deficient mice (7-10 months old). n≥5 mice, data shown are the mean ± SEM of two or more independent experiments. More than 350 cells for each population were analyzed. NS not significant, *** P<0.001.

MEF2C enhances the expression of DNA repair and V(D)J recombination factors while ensuring efficient BCR rearrangement both during normal and stress B lymphopoiesis

In addition to maintaining genomic integrity, DNA repair is also required for B lymphoid differentiation. During V(D)J recombination, DSBs are induced by RAG recombinases[5,6] and repaired by NHEJ machinery[7,8]. Successful BCR rearrangement is critical for B cell progenitor survival, and requires proper expression of recombination factors[35]. A previous study suggested that *Mef2c* loss impairs the induction of *Rag1* in BM multipotent progenitors[24]. Analysis of *Mef2c* deficient pro-B and pre-B cells showed reduced expression of *Rag1*, *Rag2* and genes encoding NHEJ machinery (*Xrcc4&6* and *Lig4*) (**Figure 5a**). These results suggest that MEF2C promotes V(D)J recombination by enabling efficient induction of effectors that execute this process.

To determine if the compromised expression of V(D)J recombination factors in *Mef2c* deficient mice had any downstream effects on immunoglobulin rearrangement, we performed Q-PCR analysis to assess the rearrangement frequency of heavy and light chains. First we assessed recombination of k and l light chains, which occurs at the pre-B cell stage. Genomic DNA was extracted from FACS sorted pre-B cells and then subjected to SYBR green Q-PCR using primers that detect the rearranged k or l alleles but not the un-rearranged versions. The abundance of rearranged k and l chain was determined relative to the constant genomic DNA region of *Actb* and normalized to control mice. Loss of *Mef2c* significantly reduced the rearrangement efficiency of both κ and l light chains (**Figure 5b**).

While light chain rearrangement occurs in pre-B cells, heavy chain rearrangement occurs in pre-pro- and pro-B cells. Since *Mef2c* deficient pro-B cells also showed defective DNA repair and down-regulation of similar biological pathways as in pre-B cells, we tested if MEF2C also

governs heavy chain rearrangement. Q-PCR analysis in *Mef2c* deficient pro-B cells revealed a significant reduction in V to DJ rearrangement frequency of the most frequent IgH family (V_H-J558)[36] in adult mice (**Figure 5c**), while the heavy chain family member V_H7183 demonstrated a non-significant trend towards being reduced in the *Mef2c* knockout. These data suggest that MEF2C is required for efficient heavy and light chain recombination in BM B lymphoid progenitors.

We next quantified the rearranged heavy(μ) and light(κ) chains at protein level. As the ability to successfully execute V(D)J recombination is also a requirement for the rapid regeneration of B lymphoid progenitors during regenerative stress, we performed the analysis both before and 2 weeks after sub-lethal irradiation. Control mice were able to efficiently execute heavy and light chain rearrangement both during homeostasis and regenerative stress (**Figure 5d,e**). In contrast, the protein levels of intracellular μ and κ in *Mef2c* deficient pro- and pre-B cells, respectively, were already reduced at steady state. These deficiencies were more drastic when assessed 2 weeks after irradiation (**Figure 5d,e**). As such, MEF2C is critical for efficient V(D)J recombination in BM B lymphoid progenitors during regenerative stress.

Figure-5

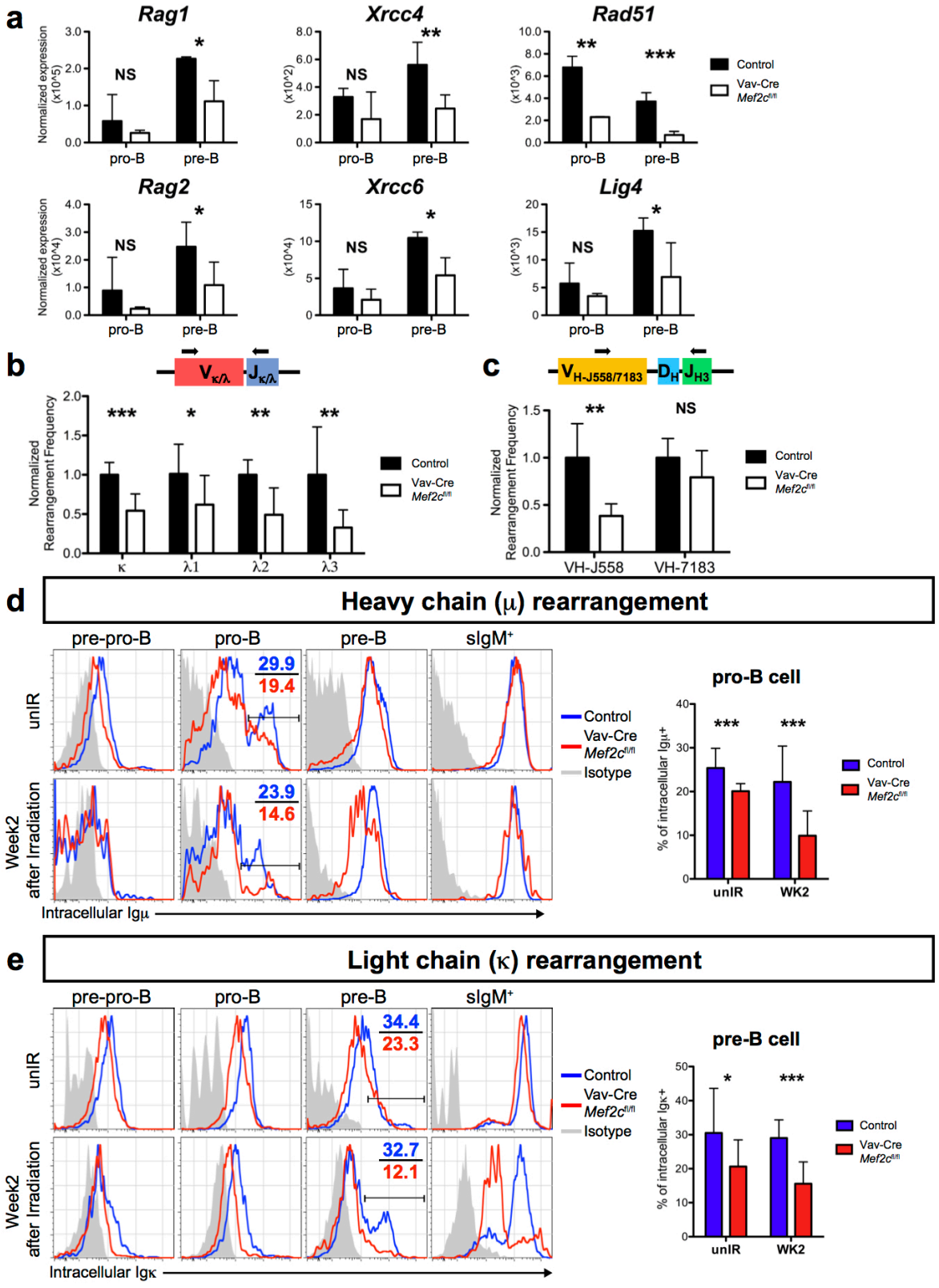
a
Rag1
Xrcc4
Rad51
Rag2
Xrcc6
Lig4
Normalized expression
NS
pro-B
pre-B
Control
Vav-Cre Mef2c fl/fl
b
c
Normalized Rearrangement Frequency
κ
λ1
λ2
λ3
VH-J558
VH-7183
d
Heavy chain (μ) rearrangement
pre-pro-B
pro-B
pre-B
sIgM+
unIR
Week2 after Irradiation
29.9
19.4
23.9
14.6
Intracellular Igμ
Isotype
pro-B cell
% of intracellular Igμ+
WK2
e
Light chain (κ) rearrangement
34.4
23.3
32.7
12.1
Intracellular Igκ
pre-B cell
% of intracellular Igκ+

Figure 5. MEF2C regulates NHEJ repair and V(D)J recombination machinery to promote BCR rearrangement during BM B lymphopoiesis

(a) Analysis of Affymetrix microarray data shows the expression of *Rag1*, *Rag2*, *Xrcc4, Xrcc6*, *Lig4* and *Rad51* in pro-B and pre-B cells in both control and Vav-Cre *Mef2c*$^{fl/fl}$ mice (9 months old). Loss of *Mef2c* compromised the expression of all these factors (n≥2). **(b,c)** Quantitative PCR analysis of κ and l1-3 light chain rearrangement (n≥5 mice) in BM pre-B cells, and V_H-J558 and V_H-7183 to DJ_{H3} heavy chain rearrangement (n≥7) in pro-B cells (7-10 months old) revealed significantly reduced frequencies of κ and l1-3 light chain and V_H-J558 heavy chain recombination in the absence of *Mef2c*. **(d,e)** Representative flow cytometric plots and quantification of intracellular expression of μ and κ in BM B cell progenitors from mice without irradiation and 2 weeks after 6Gy total body irradiation shows compromised rearrangement efficiency already in steady state, and further exaggeration of the defect during stress hematopoiesis (7-10 months old, n≥10). Data shown are the mean ± SD of three or more independent experiments. * P<0.05, ** P<0.01 and *** P<0.001.

MEF2C binds to the enhancers and promoters of genes encoding critical DNA repair and V(D)J factors

To define whether MEF2C can directly bind to the regulatory elements of genes encoding DNA repair and V(D)J recombination factors, we analyzed chromatin immunoprecipitation-sequencing (ChIP-seq) data for MEF2C in human B lymphoblasts (available via Encode[37]). GO analysis of MEF2C bound genes in human B lymphoblasts revealed significant enrichment of transcription regulation, cell cycle regulation and B cell differentiation categories (**Figure 6a**).

Intersection of the human ChIP-seq data with mouse microarray data revealed that a considerable fraction of genes down-regulated in *Mef2c* deficient mouse pre-B cells were associated with MEF2C peaks in human B lymphoblasts. Moreover, this gene set was enriched for genes that function in transcription regulation and DNA repair (**Figure 6a**).

The majority of MEF2C binding sites were distally located (5kb to 500kb) relative to transcription start sites (TSSs) while 8% of MEF2C binding localized to within 5kb of TSSs (**Figure 6b**), indicating that MEF2C can function at both enhancers and promoters. Analysis of human B lymphoblast ChIP-seq data for histone modifications along with the transcriptional co-activator p300 showed that MEF2C binding was strongly correlated with a "pro-transcription" chromatin state. Along these lines, MEF2C binding co-localized with co-activator p300 and histone marks representative of an "activating" transcriptional environment, including H3K4me1, H3K4me3 and H3K27ac. In contrast, no enrichment of repressive histone marks such was H3K9me3 or H3K27me3 was observed around MEF2C binding sites (**Figure 6c**). These data further support the notion that MEF2C primarily acts as a transcriptional activator in B lymphoid cells. Analysis of individual MEF2C candidate target genes revealed that MEF2C directly bound to genes encoding RAG recombinases, DSB sensor components (MRE11A and RAD50), NHEJ effectors (XRCC4 and LIG4) and HR effectors (RAD51AP1). Similar to the global analysis, MEF2C peaks were associated with p300 binding and activation-associated chromatin marks at these genes, suggesting that MEF2C directly promotes their transcription (**Figure 6d**).

In addition to the compromised expression of DNA repair regulators, *Mef2c* deficient pre-B cells also failed to properly induce the transcription of key B cell regulators (**Figure 6e**). These included *Il7r*, which was previously identified as a direct target of MEF2C[24], as well as

Tcf3(*E2a*) and *Ebf1*, master transcriptional regulators of B lymphoid progenitor differentiation and V(D)J recombination[35,38-43]. MEF2C peaks were also identified at genes encoding these regulators in human ChIP-seq data (**Figure 6f**). Interestingly, a recent study suggested that EBF1 may also regulate DSB repair[44]. This raised the question as to whether MEF2C could also regulate B lymphopoiesis cooperatively with TCF3 and/or EBF1. The intersection of MEF2C, TCF3 and EBF1 binding sites in human B lymphoblasts revealed significant co-localization in genes enriched for functions in transcription, cell survival, immune response and cell cycle regulation (**Supplementary Figure 7a**). Furthermore, co-localization of MEF2C binding with EBF1 and/or TCF3 binding was identified at genes encoding effectors of DNA repair and recombination (e.g. RAG1, RAD50, LIG4 and XRCC4). These sites were also associated with p300 binding and high DNase hypersensitivity indicative of these proteins acting as collaborating factors in transcriptional activation of their target genes (**Supplementary Figure 7b**). Together, these data suggest that MEF2C may promote DNA repair and V(D)J recombination both by enabling proper induction of key B cell regulators such as TCF3 and EBF1 and co-operating with these factors to activate target genes that govern these processes.

Figure-6

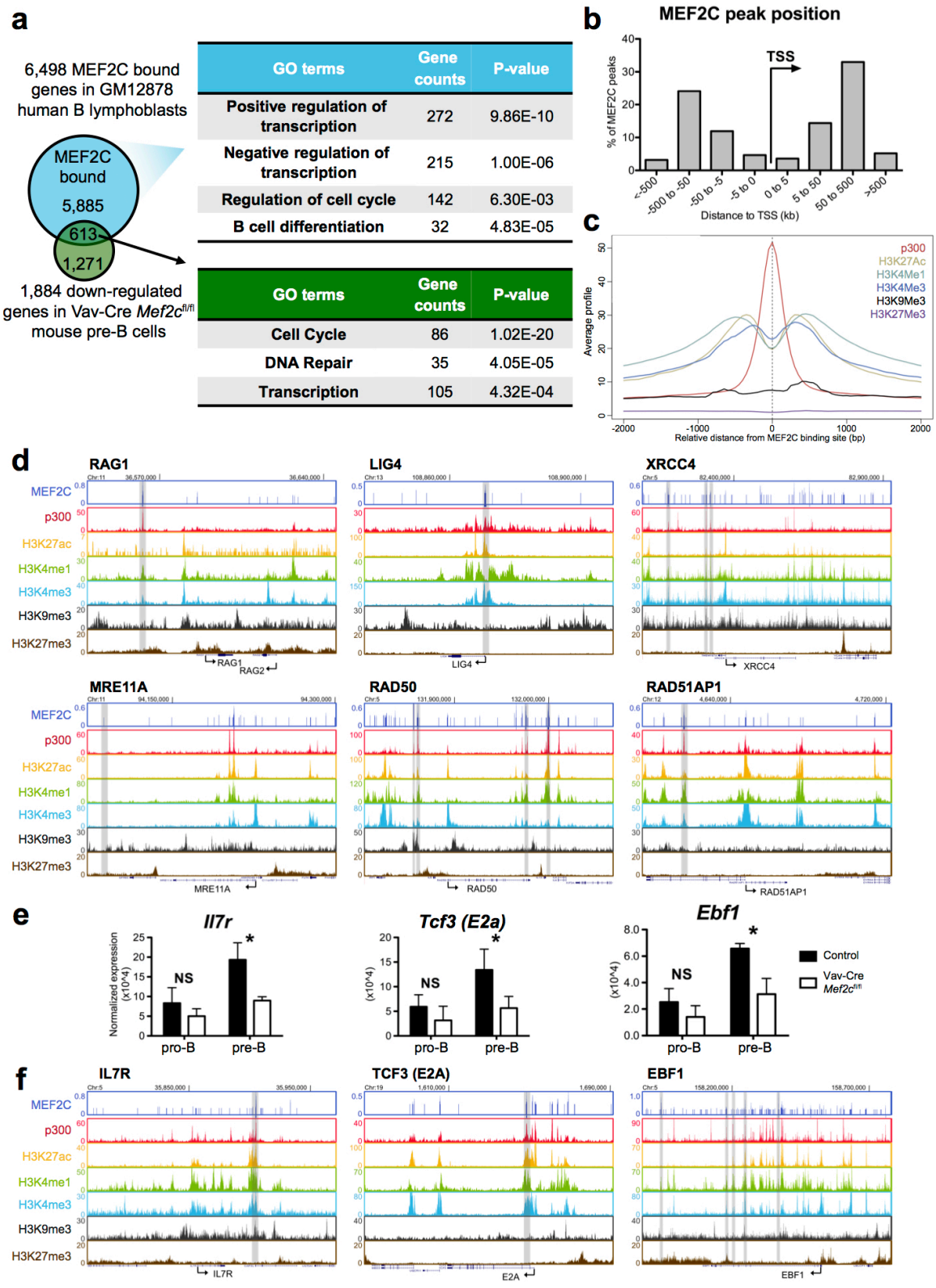
a
6,498 MEF2C bound genes in GM12878 human B lymphoblasts
MEF2C bound
5,885
613
1,271
1,884 down-regulated genes in Vav-Cre Mef2c fl/fl mouse pre-B cells
GO terms | Gene counts | P-value
Positive regulation of transcription | 272 | 9.86E-10
Negative regulation of transcription | 215 | 1.00E-06
Regulation of cell cycle | 142 | 6.30E-03
B cell differentiation | 32 | 4.83E-05
GO terms | Gene counts | P-value
Cell Cycle | 86 | 1.02E-20
DNA Repair | 35 | 4.05E-05
Transcription | 105 | 4.32E-04
b
MEF2C peak position
% of MEF2C peaks
TSS
<-500
-500 to -50
-50 to -5
-5 to 0
0 to 5
5 to 50
50 to 500
>500
Distance to TSS (kb)
c
Average profile
p300
H3K27Ac
H3K4Me1
H3K4Me3
H3K9Me3
H3K27Me3
Relative distance from MEF2C binding site (bp)
d
RAG1
LIG4
XRCC4
MRE11A
RAD50
RAD51AP1
MEF2C
p300
H3K27ac
H3K4me1
H3K4me3
H3K9me3
H3K27me3
e
Il7r
Tcf3 (E2a)
Ebf1
Normalized expression (x10^4)
NS
*
pro-B
pre-B
Control
Vav-Cre Mef2c fl/fl
f
IL7R
TCF3 (E2A)
EBF1

Figure 6. MEF2C binds to active enhancers and promoters of DNA repair and V(D)J recombination genes in human B lymphoid cells

(a) Intersection of MEF2C bound genes in human B lymphoblasts and down-regulated genes in *Mef2c* deficient mouse BM pre-B cells identified DNA repair regulators as candidate direct targets of MEF2C in pre-B cells. **(b)** GREAT analysis showed that MEF2C binding sites are located both around TSS and at distant regulatory elements. **(c)** Distribution of co-activator p300 and epigenetic marks around MEF2C binding sites shows correlation of MEF2C peaks with both active enhancer and promoter marks (H3K4me1, H3K4me3, H3K27ac) but not repressive epigenetic marks (H3K9me3, H3K27me3). **(d)** Genome browser tracks show MEF2C and p300 binding and enrichment of active histone marks at genes encoding for DNA repair and V(D)J recombination factors. MEF2C peaks defined by the ENCODE dataset are highlighted in grey. **(e)** Analysis of Affymetrix data shows that proper expression of *Il7r*, *E2a* (*Tcf3*) and *Ebf1* is dependent on MEF2C (n≥2). **(f)** Genome browser shots show MEF2C and p300 binding and enrichment of active epigenetic marks at genes encoding for IL7R, E2A (TCF3) and EBF1. Regions with MEF2C peaks, defined by the ENCODE dataset, are highlighted in grey. NS not significant, * P<0.05.

MEF2C enhances chromatin accessibility at regulatory regions of its target genes in mouse BM pre-B cells

To explore the molecular mechanism MEF2C utilizes in promoting gene expression, we performed ATAC-sequencing analysis to identify open chromatin regions in control and *Mef2c* deficient BM myeloid and pre-B cells. MAnorm[45] analysis of regions that are differentially (ratio≥4, p-value≤0.01) enriched in control myeloid and pre-B cells revealed that both cell types

possess regulatory regions that are more accessible in that cell type (8671 in pre-B cells, and 4372 in myeloid cells)**(Figure 7a, Supplementary Table 3)**. GO analysis confirmed that regions more accessible in pre-B cells are enriched for B cell differentiation pathways while regions more accessible in myeloid cells are enriched in myeloid differentiation pathways **(Figure 7a)**. These data enable the identification of unique cell types specific regulatory regions and imply that regulation of chromatin accessibility plays a role in lineage differentiation.

To determine if MEF2C is involved in modulating the chromatin accessibility of its target genes in B cell progenitors, we performed MAnorm analysis in control versus *Mef2c* deficient pre-B cells. In agreement with MEF2C being a transcription activator in B cell progenitors, significantly more regions were found to be more accessible in control (5007) than *Mef2c* deficient pre-B cells (455)**(Figure 7b, Supplementary Table 4)**. Importantly, the sites that are less accessible in *Mef2c* deficient pre-B cells include regions associated with MEF2C target genes that are important in B cell differentiation (*Il7r, Tcf3* and *Ebf1*), V(D)J initiation (*Rag1&2*), DSB repair (*Mre11a, Xrcc4, Xrcc6* and *Lig4*) and B cell progenitor survival (*Bcl2l1*). GO analysis of genes associated with regions that are less accessible in the absence of *Mef2c* also revealed B cell differentiation and V(D)J recombination among the top enriched terms **(Figure 7b)**. In comparison, BM myeloid cells had fewer regions that showed differences (ratio≥4, p-value≤0.01) in accessibility upon *Mef2c* loss, as only 346 sites were more accessible in control, and 73 in *Mef2c* deficient myeloid cells. The sites enriched in control myeloid cells were associated with genes encoding phagocytosis and chromatin remodeling, and were distinct of those regulated by MEF2C in pre-B cells. **(Supplementary Figure 8a, Supplementary Table 5)** These data suggest that MEF2C may enhance the expression of DNA repair and V(D)J factors in B lymphoid cells in part through modulating the chromatin accessibility at their regulatory sites.

As the analysis of ENCODE data revealed the possibility of MEF2C cooperation with TCF3 and EBF1, we then conducted motif analysis[46] of regions that are more accessible in control than *Mef2c* deficient pre-B cells. Indeed, E-box (motif for bHLH factors such as TCF3) and EBF were among the top enriched motifs **(Figure 7c)**. Intersection analysis of TCF3 bindings in mouse pro-B cells[47] with regions that are less accessible in *Mef2c* deficient pre-B cells showed significant overlap. Moreover, many of the MEF2C dependent peaks also correlated with cell type specific regulatory regions that are more accessible in pre-B cells compared to myeloid cells **(Figure 7d)**. In contrast, there was minimal overlap with TCF3 binding and MEF2C dependent accessible regions in myeloid cells **(Supplementary Figure 8b)**. These data support the hypothesis that MEF2C co-regulates its target genes required for BM B lymphopoiesis together with B cell specific transcription factors such as TCF3 and EBF1.

Analysis of the ATAC-seq in putative MEF2C direct target genes (based on ENCODE data) that are critical for B cell differentiation (*Il7r*), V(D)J recombination (*Rag1&2*) and DNA repair (*Lig4* and *Mre11a*) identified regions that are more accessible in control than *Mef2c* deficient pre-B cells around all these genes. These regions were identified both around TSSs (promoters) and in distant sites (putative enhancers). Majority of these regions were also uniquely accessible in pre-B cells as compared to myeloid cells, and co-localized with TCF3 bindings in pro-B cells **(Figure 7e)**. Together, these analyses suggest that MEF2C promotes open chromatin conformation in regulatory regions to activate critical B cell regulatory programs during B lymphoid differentiation.

Figure-7

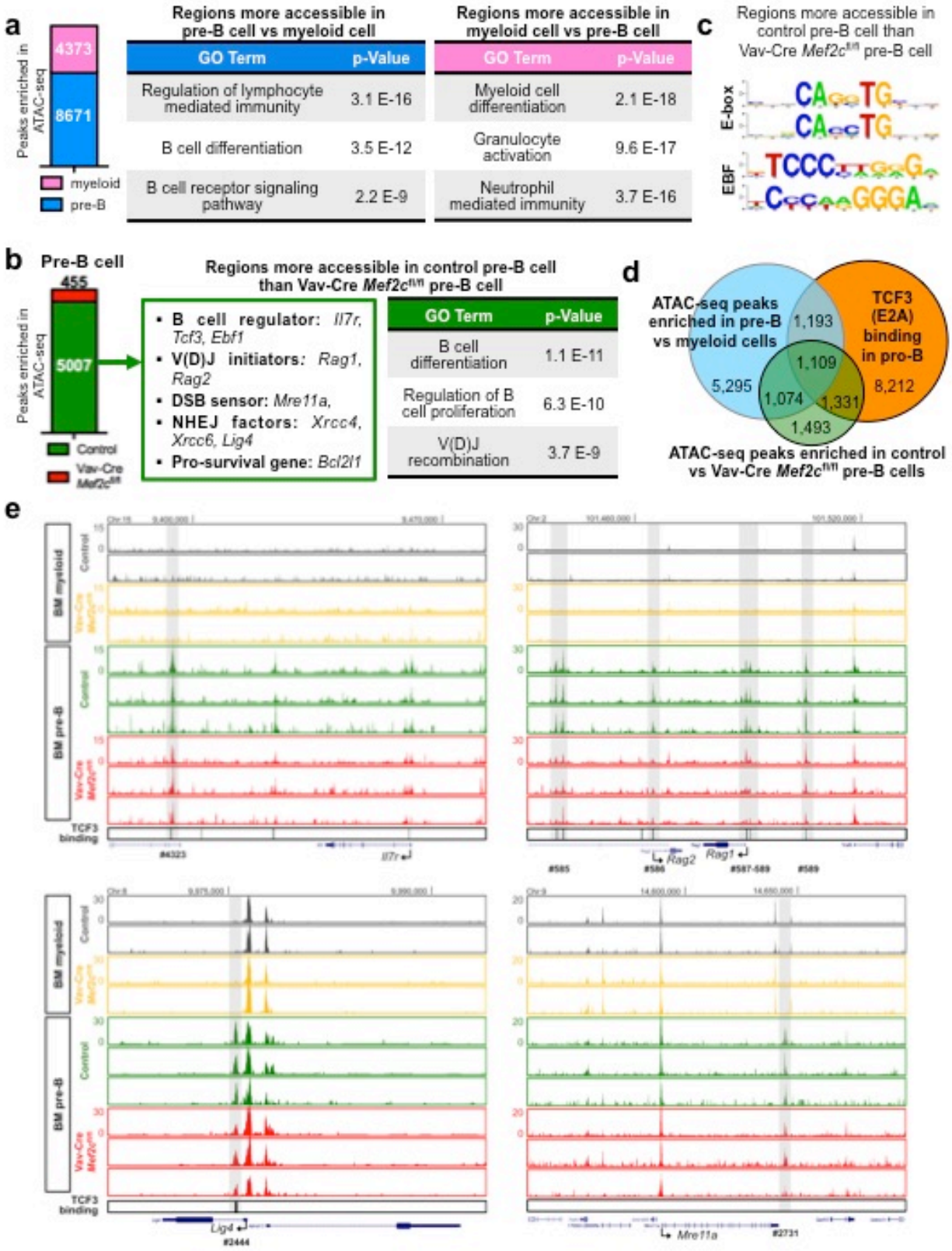
a
Peaks enriched in ATAC-seq
4373
8671
myeloid
pre-B
Regions more accessible in pre-B cell vs myeloid cell
GO Term
p-Value
Regulation of lymphocyte mediated immunity
3.1 E-16
B cell differentiation
3.5 E-12
B cell receptor signaling pathway
2.2 E-9
Regions more accessible in myeloid cell vs pre-B cell
GO Term
p-Value
Myeloid cell differentiation
2.1 E-18
Granulocyte activation
9.6 E-17
Neutrophil mediated immunity
3.7 E-16
c
Regions more accessible in control pre-B cell than Vav-Cre Mef2c fl/fl pre-B cell
E-box
EBF
b
Pre-B cell
455
5007
Peaks enriched in ATAC-seq
Control
Vav-Cre Mef2c fl/fl
Regions more accessible in control pre-B cell than Vav-Cre Mef2c fl/fl pre-B cell
B cell regulator: Il7r, Tcf3, Ebf1
V(D)J initiators: Rag1, Rag2
DSB sensor: Mre11a,
NHEJ factors: Xrcc4, Xrcc6, Lig4
Pro-survival gene: Bcl2l1
GO Term
p-Value
B cell differentiation
1.1 E-11
Regulation of B cell proliferation
6.3 E-10
V(D)J recombination
3.7 E-9
d
ATAC-seq peaks enriched in pre-B vs myeloid cells
TCF3 (E2A) binding in pro-B
1,193
1,109
5,295
8,212
1,074
1,331
1,493
ATAC-seq peaks enriched in control vs Vav-Cre Mef2c fl/fl pre-B cells
e
BM myeloid
BM pre-B
Control
Vav-Cre Mef2c
TCF3 binding
Il7r
Rag2
Rag1
Lig4
Mre11a

Figure 7. MEF2C promotes chromatin accessibility in key regulatory regions at its target genes in mouse pre-B cells

(a) MAnorm analysis of ATAC-sequencing(ATAC-seq) data in control BM myeloid ($Mac1^{+}Gr1^{+}$) and pre-B cells revealed that the accessibility of regulatory sites in lineage specific genes is differentially (ratio≥4, p-Value≤0.01) regulated in these two populations. **(b)** MAnorm analysis of ATAC-seq data in control and *Mef2c* deficient pre-B cells revealed that MEF2C is required to promote chromatin accessibility in regulatory regions of genes required for B cell differentiation. **(c)** The ATAC-seq peaks that are more accessible in control pre-B cells compared to *Mef2c* deficient pre-B cells are enriched for E-box and EBF motifs. **(d)** Venn diagram showing extensive overlap of regions that are more accessible in control pre-B cells vs. *Mef2c* deficient pre-B cells with regions that are more accessible in pre-B cells than myeloid cells, as well as TCF3 bound regions in pro-B cells. **(e)** Loss of *Mef2c* reduced the chromatin accessibility nearby genes that are critical for B cell differentiation and DNA repair. Many of these regulatory sites are also uniquely accessible in pre-B cells as compared to myeloid cells. Regions that are significantly (ratio≥4, p-Value≤0.01) more accessible in control than *Mef2c* deficient pre-B cells are highlighted in grey and labeled with reference numbers co-related to Supplementary Table 4.

Discussion

The ability of a stem/progenitor cell to repair DNA damage is vital to its genomic integrity. In addition to serving housekeeping functions in most lineages, DNA repair, and DSB repair specifically, is critical for normal lymphoid development due to its necessity during V(D)J recombination. It has been unknown, however, if B lymphoid cells possess lineage specific mechanisms that link the activation of DNA repair machinery with B cell differentiation. Moreover, little is known if there are unique requirements for B cell transcriptional machinery during regenerative stress, when efficient DNA repair is required to protect the rapid differentiation and proliferation of stem/progenitor cells. Our work identified MEF2C as a critical transcriptional activator that ensures efficient DNA repair and V(D)J recombination in BM B cell progenitors, which becomes critical for B cell regeneration during stress hematopoiesis.

Our data shows that MEF2C enhances the transcription of genes encoding DSB repair machinery, RAG recombinases and B lymphoid regulators in BM B cell progenitors. Thus, MEF2C enables efficient V(D)J recombination, which is a pre-requisite for B lymphoid progenitor survival (**Figure 8a**). Absence of *Mef2c* reduces the efficiency of both heavy and light chain rearrangement, compromising BM B lymphopoiesis at multiple stages. While *Mef2c* loss led to reduced BM B lymphopoiesis in homeostatic conditions, peripheral B cell numbers were normal most likely due to the homeostatic expansion of mature B cells or eventual saturation of B cell survival niches in secondary lymphoid organs. However, during stress, such as recovery from irradiation or 5-FU treatment, the lack of *Mef2c* becomes a bottleneck for rapid BM B

lymphopoiesis that is required for timely replenishment of peripheral B lymphoid compartment (**Figure 8b**). Although developing T cells also undergo V(D)J recombination, our analysis showed that *Mef2c* loss has essentially no effect on DNA repair in T cell progenitors, nor affected steady state T lymphopoiesis or T cell recovery during stress hematopoiesis. These data thus document a unique requirement of MEF2C dependent transcriptional mechanisms to secure efficient BM B lymphopoiesis during stress. Such mechanisms could be important in various stress conditions such as during the recovery from BM transplantation, radiation or chemotherapy. However, given the high homology between MEF2 family members, it is possible that MEF2D and/or another MEF2 family member expressed in T-cells[48] functions in developing thymocytes in a fashion analogous to MEF2C in B cell progenitors.

Quantification of B cell progenitors showed that the B lymphoid defects in *Mef2c* deficient mice have similar features as observed in aged mice. This includes a profound loss of BM B cell progenitors, especially pre-B cells, without immediate effects on peripheral B cell compartments[11,12]. Although the effects of aging in B lymphoid cells are most notable at pre-B cell stage, defects are already observed at CLP level. Notably, *Mef2c* deficiency also compromised the recovery CLPs during stress. Similar to *Mef2c* deficiency, aging of the B lymphoid compartment is associated with increased B cell progenitor death[16] and defective BCR rearrangement[14]. Such defects have been linked to reduced *Tcf3(E2a)* and *Rag* expression and defective DNA repair in the elderly[49-51], phenomena that we also observed in *Mef2c* deficient mice. It is intriguing that the loss of a single gene, *Mef2c*, leads to similar phenotypes to aging in B cell progenitors. Future studies will be needed to determine at both molecular and cellular levels the degree to which *Mef2c* deficiency models physiological B lymphoid aging.

Figure-8

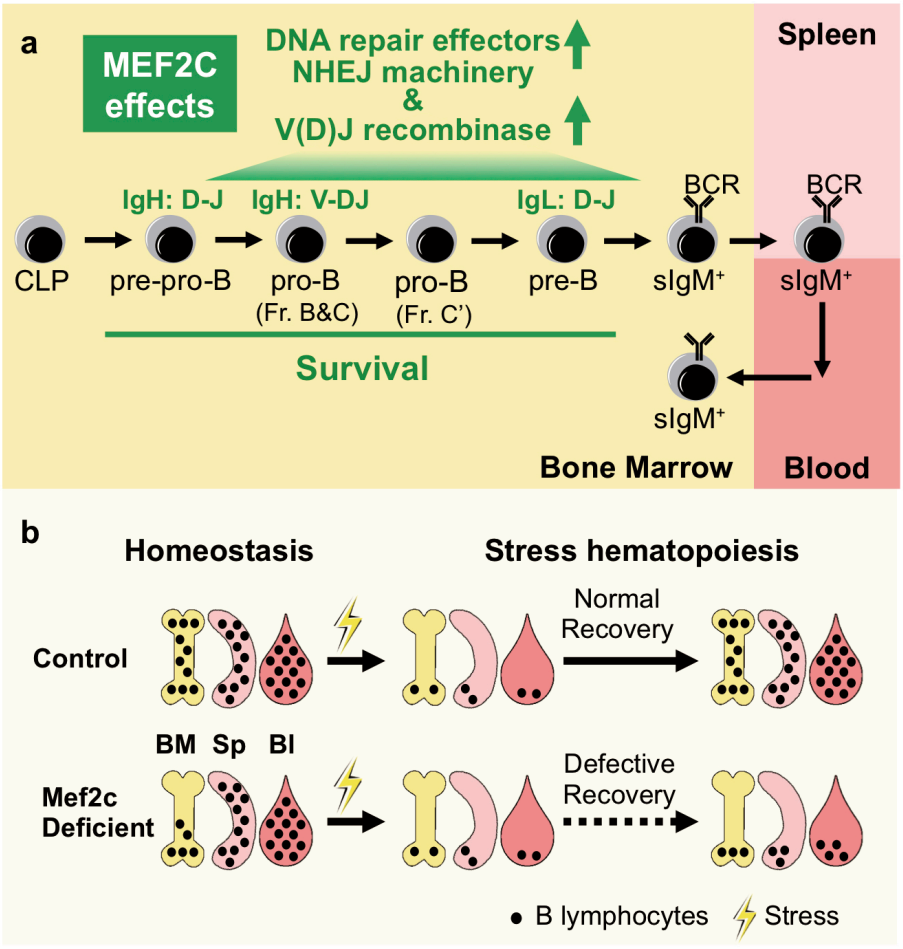

Figure 8. MEF2C enables B lymphoid regeneration during stress hematopoiesis by promoting DNA repair and V(D)J recombination in B lymphoid progenitors

(a) MEF2C ensures efficient BM B lymphopoiesis by enhancing the expression of critical V(D)J recombination initiators and DSB repair machinery thereby promoting the rearrangement of both heavy and light chains, the success of which ensures the survival of BM B cell progenitors. **(b)** While loss of MEF2C compromises BM B lymphopoiesis during homeostatic conditions, this can be compensated for in the periphery to maintain a relatively intact B cell pool in blood and spleen. However, the loss of *Mef2c* severely compromises B lymphoid recovery after irradiation, documenting a critical function for MEF2C in regeneration of the B lymphoid compartment during stress hematopoiesis.

Analysis of MEF2C ChIP-seq data in human B lymphoblasts identified MEF2C binding at enhancers and/or promoters of critical B cell transcription factors and genes essential for V(D)J recombination and DNA repair, suggesting a direct regulatory function. Our analysis identified two complementary mechanisms of how MEF2C may promote these transcriptional programs: 1) MEF2C facilitates proper induction of B cell transcription factors such as TCF3/E2A and EBF1 and 2) MEF2C cooperates with these factors to enhance the transcription of genes encoding RAGs and DNA repair machinery. ATAC-seq in mouse pre-B cells revealed that MEF2C enhances chromatin accessibility in regulatory regions of its target genes, many of which overlap with TCF3 binding sites in pro-B cells[47]. As the MADS box of MEF2C can recruit p300 to enhance transcription[52], it is plausible that MEF2C recruits p300 or other co-activators to enable efficient gene transcription. However, further examination will be required to mechanistically define how MEF2C ensures open chromatin environment of its target genes. Altogether, our data imply that, MEF2C is not a bona fide lineage-specification factor whose absence would lead to an absolute block in gene activation or lineage differentiation. Rather, MEF2C functions in "volume-control" to enable efficient up-regulation of B cell transcriptional programs, including factors required for V(D)J recombination and DNA repair. .

Mef2c is also known as a cooperating oncogene in leukemia. *Mef2c* became abnormally induced in leukemic GMPs in myeloid leukemia and knockdown of *Mef2c* attenuated the proliferative potential of these cells[53]. In MLL-ENL, loss of *Mef2c* in leukemic cells reduced their homing and invasive capacities[54]. Moreover, MEF2C is emerging as an important oncogene in T-ALL[55-58]. It is still unclear how ectopic activation of *Mef2c* expression promotes leukemia progression, and whether it regulates DNA repair that could lead to survival advantage and/or

therapy resistance. Our finding that MEF2C acts as an amplifier of cell type specific transcriptional programs in B lymphoid cells is consistent with the data that MEF2C is not an oncogene alone, but potentiates the leukemogenic effect of the oncogene it co-operates with[59]. Moreover, as MEF2C functions in normal development of various other tissues (muscle, heart, vasculature and neural progenitors)[23,60-63], it is important to define if MEF2C functions in analogous fashion as in B lymphoid cells to augment cell type specific transcriptional programs in these stem/progenitor cells.

Methods

Mice: Vav-Cre mice were bred with *Mef2c*$^{fl/fl}$ mice to generate Vav-Cre *Mef2c*$^{fl/fl}$ mice. *Mef2c*$^{fl/fl}$, *Mef2c*$^{fl/+}$ or Vav-Cre *Mef2c*$^{fl/+}$ mice of the same age were used as controls. Genotyping analysis was done as previously described[28]. All mice were maintained according to the guidelines of the UCLA Animal Research Committee.

Flow cytometric analysis and isolation of B lymphoid progenitors: Hematopoietic cells were analyzed using antibodies listed in **Supplementary Table 6**. Dead cells were excluded with 7-amino-actinomycin D and cell populations were analyzed using a LSR II or Fortessa flow cytometer. Cell sorting was performed using a FACS Aria cell sorter. Data were analyzed with FlowJo software version 9.2. Fractionation of BM B lymphoid progenitors was based on the surface expression of the following markers: pre-pro-B: $B220^{+}IgM^{-}CD43^{+}CD24^{-}$, pro-B: $B220^{+}IgM^{-}CD43^{+}CD24^{+}$, pre-B: $B220^{+}IgM^{-}CD43^{-}$ and sIgM^{+}: $B220^{+}IgM^{+}$. Hardy fractionation of BM B lymphoid progenitors was based on the surface expression of the following markers: Fr.A: $B220^{+}IgM^{-}CD43^{+}CD24^{-}BP\text{-}1^{-}$, Fr.B: $B220^{+}IgM^{-}CD43^{+}CD24^{+}BP\text{-}1^{-}$, Fr.C: $B220^{+}IgM^{-}CD43^{+}CD24^{-}BP\text{-}1^{+}$, Fr.D: $B220^{+}IgM^{-}CD43^{-}$, Fr.E: $B220^{+}IgM^{+}IgD^{-}$, Fr.F: $B220^{+}IgM^{+}IgD^{+}$.

Irradiation: Sub-lethal (6Gy) total body irradiation was performed with Co-60 pool irradiator.

5-FU administration: 5-FU (Sigma) was dissolved in sterile PBS and mice were intravenously injected with a single dose of 150mg kg^{-1}.

Analysis of peripheral blood counts: Peripheral blood was harvested from the retro-orbital sinus into Vacutainer tubes (BD Biosciences) and sent to UCLA Division of Laboratory Animal Medicine laboratory for complete blood cytometry (CBC) analysis.

Gene expression profiling: Total RNA was isolated from sorted cells using a combination of QIAshredder columns followed by the RNEasy Micro/Mini Kit (QIAGEN). Affymetrix microarray analysis was performed on independent control and *Mef2c* deficient sorted pro-B and pre-B cell samples. The R package Limma provided through the open source project Bioconductor was used to assess differential expression. To calculate absolute mRNA expression levels, the RMA (Robust Multiarray Averaging) method was used to obtain background adjusted, quantile normalized and probe level data summarized values for all probe sets. The Affymetrix Mouse Genome 430 2.0 Array GeneChip platform was used for the analysis. Official gene symbols for probe sets were obtained using the Bioconductor annotation database mouse4302.db. The mas5calls algorithm through the R package of affy was used for calculating PMA detection calls for each array sample. Probes that were absent or only marginally present in more than 4 replicates were excluded from analysis. Differentially expressed genes were uploaded into the DAVID interface to identify significantly over-represented functional GO biological process categories.

Quantiative RT-PCR (Q-PCR): Total RNA was extracted from B cell progenitors (see above). cDNA synthesis was carried out according to the manufacturer's protocol for the Quantitect Reverse transcription kit (QIAGEN), and Q-PCR was performed using a LightCycler 480

(Roche) with LightCycler 480 SYBR Green I Master mix (Roche). Primer sequences are shown in Supplementary Table 7. Samples were normalized to *Actb*.

Immunofluorescence microscopy (γH2AX): IF microscopy of γH2AX was performed as described . In brief, FACS purified cells were pipetted onto poly-lysine coated slides, fixed with 4% PFA for 10 min at room temperature, permeabilized in 0.15% Triton X-100 for 2 min at room temperature and blocked in 10% donkey serum / PBS overnight at 4°C. Slides were then incubated for 1-2 hr at room temperature with anti-phospho-H2AX (Ser 139) (Millipore, 05-636). Slides were washed three times in PBS and incubated for 1 hr at room temperature with AF488-conjugated goat anti-mouse (Life Technologies, A11029) antibody. Slides were then washed three times in PBS and mounted using ProLong® Gold Antifade Reagent with DAPI (Life Technologies, P36935). Microscopy imaging was performed using Zeiss LSM 700 confocal microscope (100x objective) and Nikon ECLIPSE E600 microscope (100x objective). Cells were scored as positive (≥4 foci) or negative (0-3 foci) based on the number of foci observed by eye. All scoring was done blind and more than 50 cells were counted per sample.

Alkaline comet assay: Alkaline comet assay was performed with Enzo comet SCGE assay kits according to the manufacturer's protocols. In brief, FACS purified cells were embedded in low melting point agarose and transferred onto comet slides. Cells were then lysed and treated with freshly made alkaline solution followed by electrophoresis in alkaline electrophoresis solution. Slides were dried for at least 2 days before imaging. Nuclei were stained with SYBR Green I for 20 min. Pictures of individual cells were taken with Nikon ECLIPSE E600 microscope (×40 objective) and analyzed using the CASP software (http://casplab.com/). The tail moments of all

comets analyzed were used to define outliers and non-outliers based on calculated absolute deviation. Cells were defined as outliers when their tail moments absolute deviation was ≥ 3 median absolute deviation.

Quantitative analysis of Ig gene rearrangements: Genomic DNA was extracted from sorted B cell populations (pro-B for heavy chain and pre-B for light chain) with the genomic DNA extraction kit (QIAGEN). Quantitative analysis of V_H-J558 and V_H-7183 heavy chain and κ and l light chain rearrangements were performed by qPCR using published primers[64]. Q-PCR was performed as stated above. Samples were normalized to *Actb*. Rearrangement frequencies were calculated as $2^{\Delta Ct}$ with $\Delta Ct = Ct_{Actb} - Ct_{Ig}$.

ChIP-sequencing (ChIP-seq) analysis: ChIP-seq data from the ENCODE[37] project were used for analysis. Alignment images were generated with the UCSC Genome Browser [65] and identification of peaks was based on the peak calling process of the ENCODE project. Peak intersection was done with Galaxy [66] and peaks were mapped to nearby genes within 200kb of a given TSS using Genomic Regions Enrichment of Annotations Tool (Great)[67]. The following publically available ChIP-seq datasets generated with GM12878 cells from the ENCODE project were used for analyses: MEF2C GSM803420, p300 GSM935562, H3K4me1 GSM733772, H3K4me3 GSM733708, H3K27ac GSM733771, H3K9me3 GSM733664, H3K27me3 GSE50893, E2A GSM1010745, EBF1 GSM803386, DNase HS GSM816665.

ATAC-Sequencing (ATAC-seq) analysis: ATAC-seq was performed in 2-3 biological replicates using 50,000 FACS sorted myeloid cells ($Mac1^+Gr1^+$) and pre-B cells ($B220^+sIgM^-$

CD43$^-$) from the BM of control and Vav-Cre *Mef2c*$^{fl/fl}$ mice, as described before[68]. Briefly, after nuclear extraction, transposition reaction (Nextera DNA Library Prep Kit, Illumina) was carried out at 37^{o}C for 30 minutes. After DNA purification, 12 cycles of PCR amplification was carried out using NEBNext High-Fidelity 2x PCR Master Mix (New England Labs). Purified libraries were subsequently subjected to paired end sequencing using HIseq-2000 (Illumina) to obtain 50 bp long reads. Demultiplexing was carried out using in house Unix shell script followed by mapping to mouse genome (mm9) using Bowtie[69]. Peaks were identified with MACS[70] and MAnorm analysis [45] was used to identify differentially accessible regions between control myeloid and pre-B cells as well as control and *Mef2c* deficient pre-B cells or myeloid cells. Peaks that had at least 4 times more reads in one condition and had p-value equal or less than 0.01 were considered differentially accessible. Centdist analysis[46] was used to identify transcription factor binding motifs enriched within different subsets of differentially accessible regions. GREAT program[5] was used to associate identified regions with genes (using default settings) and to perform GO enrichment analysis.

Statistics: Unless otherwise described, student's unpaired two-tailed t-test was used for statistical analysis and differences with p-values ≤ 0.05 were considered significant.

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Author Contribution

W.W. designed and performed research, analyzed and interpreted data and wrote the manuscript; T.O. performed ATAC-seq analysis for Fig.7 and Supplementary Fig.8, and assisted in genomewide analysis with Fig.6 and Supplementary Fig.7; A.M.H. designed and performed research, analyzed and interpreted data and contributed to Fig.1a&b, Supplementary Fig.1&2

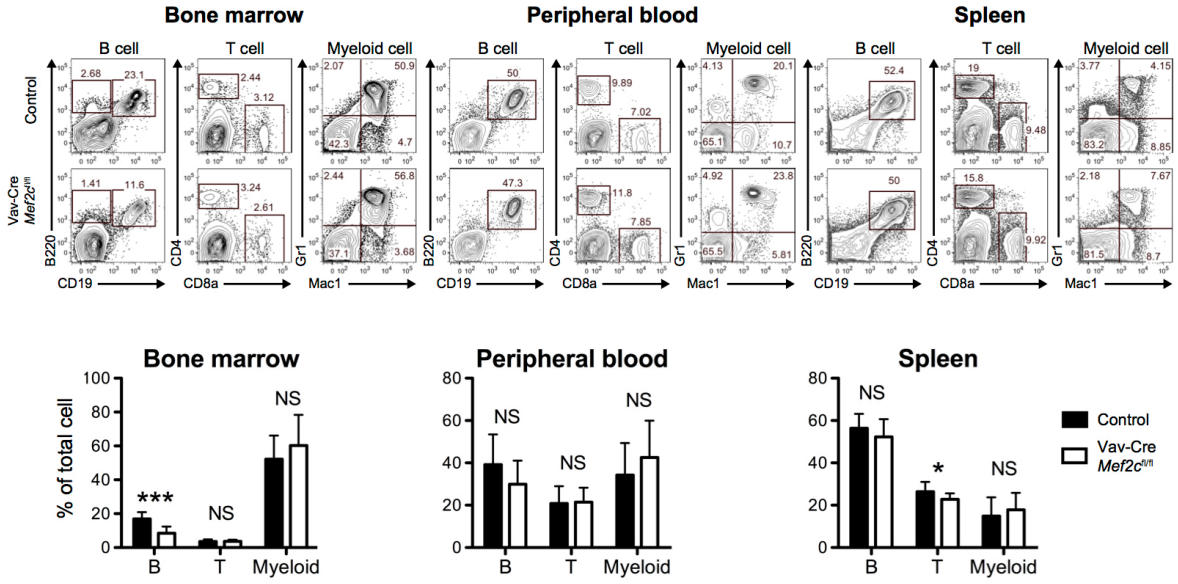
and microarray analysis; P.P and D.D contributed to the genomewide analysis, E.I. contributed to Fig.4e; D.M. and R.H.S. assisted with Fig.4d&f; J.F. helped with Fig.4e; and H.K.A.M. supervised the overall design, conduct and interpretation of experiments, and writing of the manuscript. All authors read and edited the manuscript.

Financial Interest Statement

The authors have declared that no conflict of interest exists.

Supplementary Figures

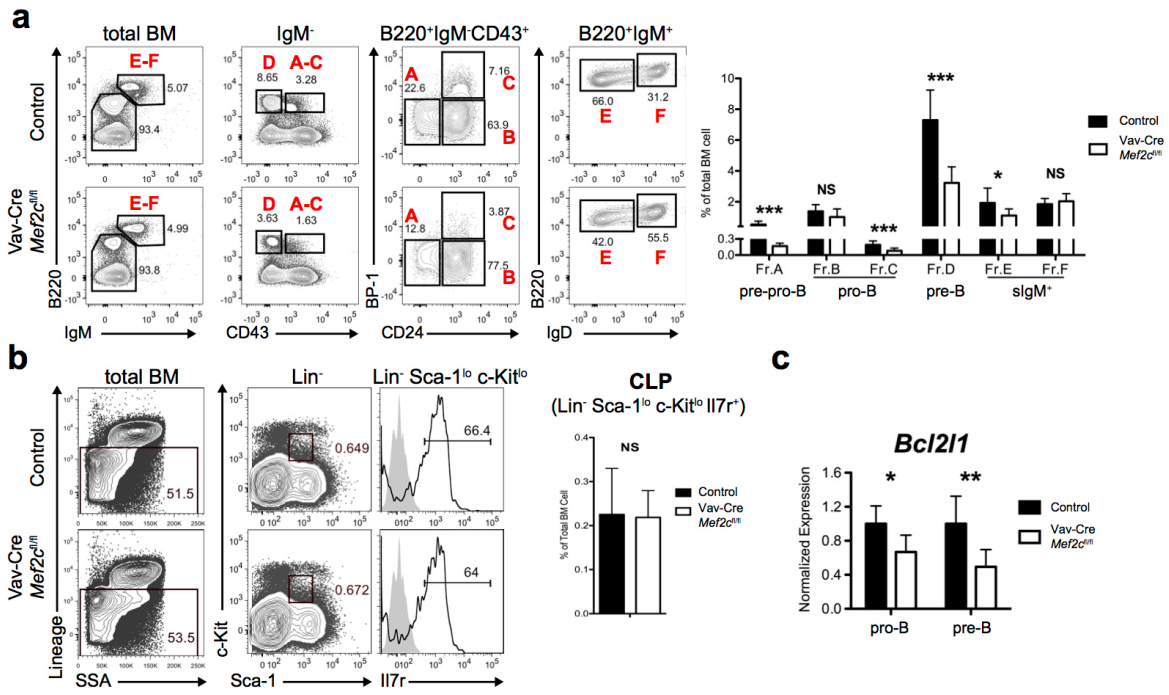
Supplementary Figure-1



Supplementary Figure 1. Loss of *Mef2c* affects the frequency of BM B lymphoid progenitors

Deletion of *Mef2c* in hematopoietic cells in Vav-Cre *Mef2c*$^{fl/fl}$ mice resulted in specific reduction of BM B cells, while the cellularity of blood and spleen B cells was unaffected (n≥7 mice). Representative flow cytometric analysis and quantification of hematopoietic lineages in BM, peripheral blood and spleen are shown. All mice were analyzed at 7-10 months of age. Data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

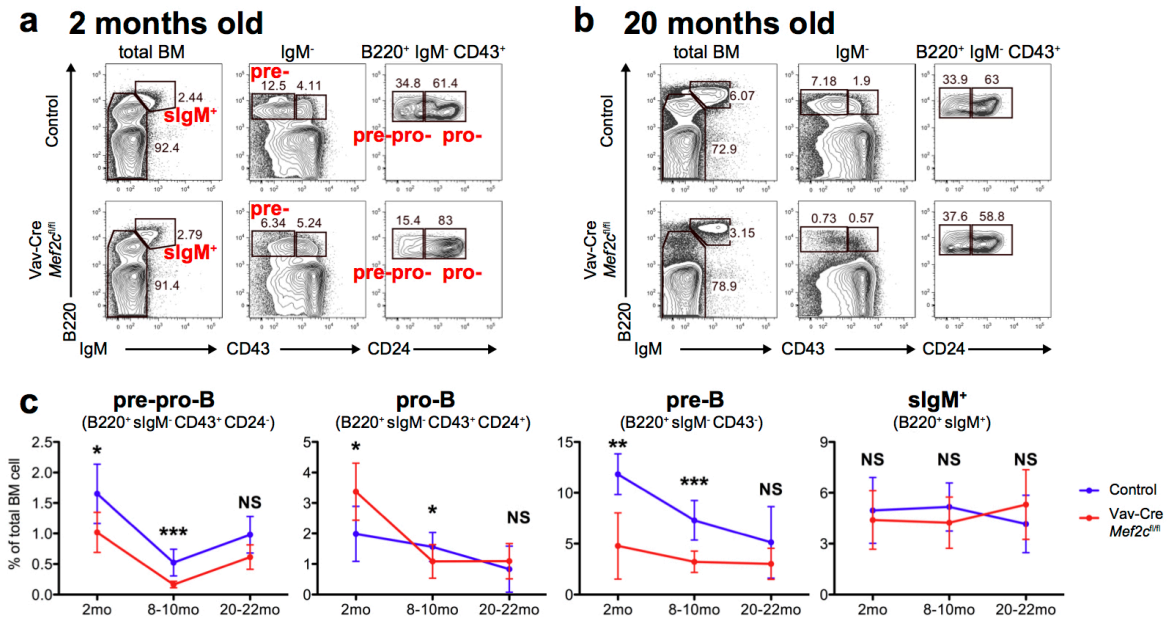
Supplementary Figure-2



Supplementary Figure 2. Phenotyping by alternative surface markers confirms compromised BM B lymphoid progenitor cellularity downstream of the CLP in *Mef2c* deficient mice.

(a) Representative flow cytometric analysis and quantification of BM B lymphoid progenitors using Hardy fractionation confirms reduction of all BM B cell progenitor subsets (n≥11). **(b)** Representative flow cytometric analysis and quantification of BM common lymphoid progenitors, defined as $Lin^{-}IL\text{-}7R^{+}Sca\text{-}1^{lo}c\text{-}Kit^{lo}$ (n≥14), shows no difference in the absence of *Mef2c*. **(c)** Q-PCR analysis of pro-survival gene, *Bcl2l1*, showed that loss of *Mef2c* compromised the expression of *Bcl2l1* in B cell progenitors (n≥5). All mice were analyzed at 7-10 months of age. Data shown are the mean ± SD of two or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

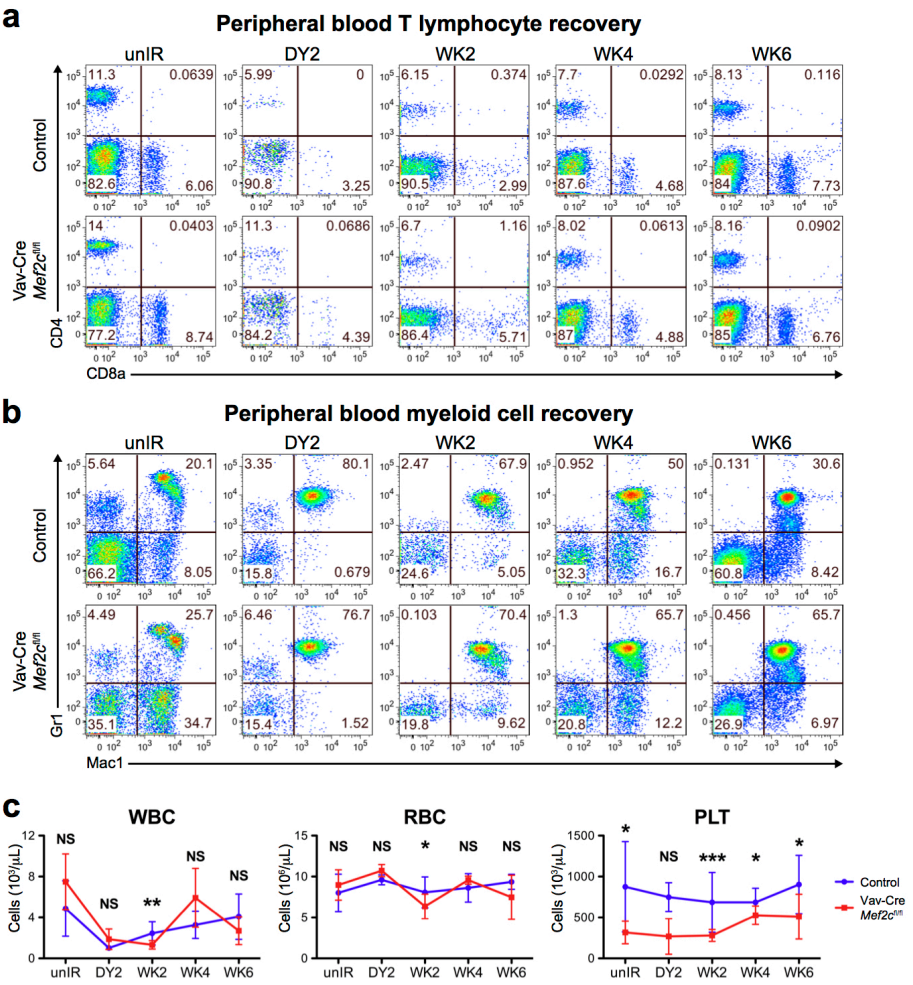
Supplementary Figure-3



Supplementary Figure 3. *Mef2c* deficiency results in premature decline of BM B lymphoid progenitors that resembles B lymphoid aging

(a,b) Representative flow cytometric analysis of BM B lymphoid progenitors from young (2 months) and old (20 months) mice. **(c)** Quantification of BM B lymphoid progenitors in *Mef2c* deficient and control mice of different ages shows that young/middle aged *Mef2c* deficient mice show similar reduction of BM B cell progenitors as observed in control mice during aging (n≥6). Data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

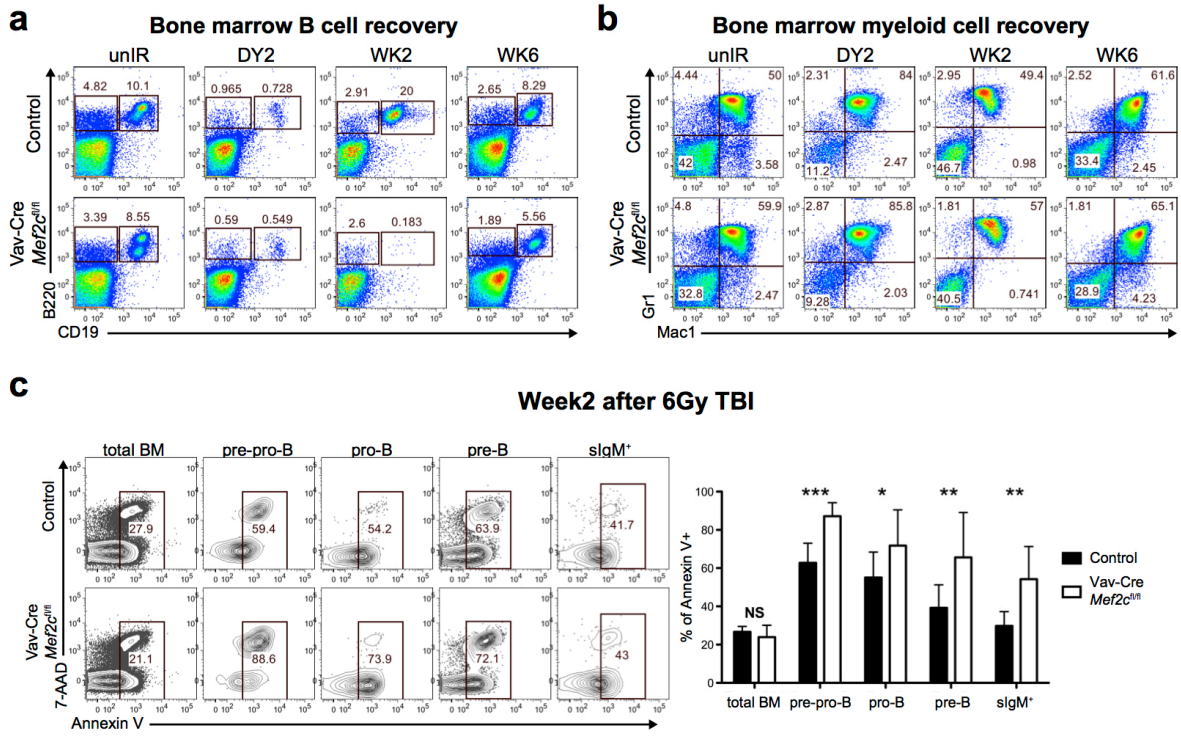
Supplementary Figure-4



Supplementary Figure 4. Recovery of most peripheral blood lineages post irradiation is not affected in the absence of *Mef2c*.

(a,b) Representative flow cytometric analysis of peripheral blood T lymphoid and myeloid cells shows comparable recovery in *Mef2c* deficient mice upon 6Gy sub-lethal irradiation as controls. **(c)** Quantification of peripheral blood total WBC, RBC and platelet (PLT) counts before and after 6Gy total body irradiation shows a significant defect in *Mef2c* deficient mice only in platelets, regardless of the presence of irradiation. Day 2: n=4, data shown are the mean ± SD of two independent experiments; other time points: n≥5, data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

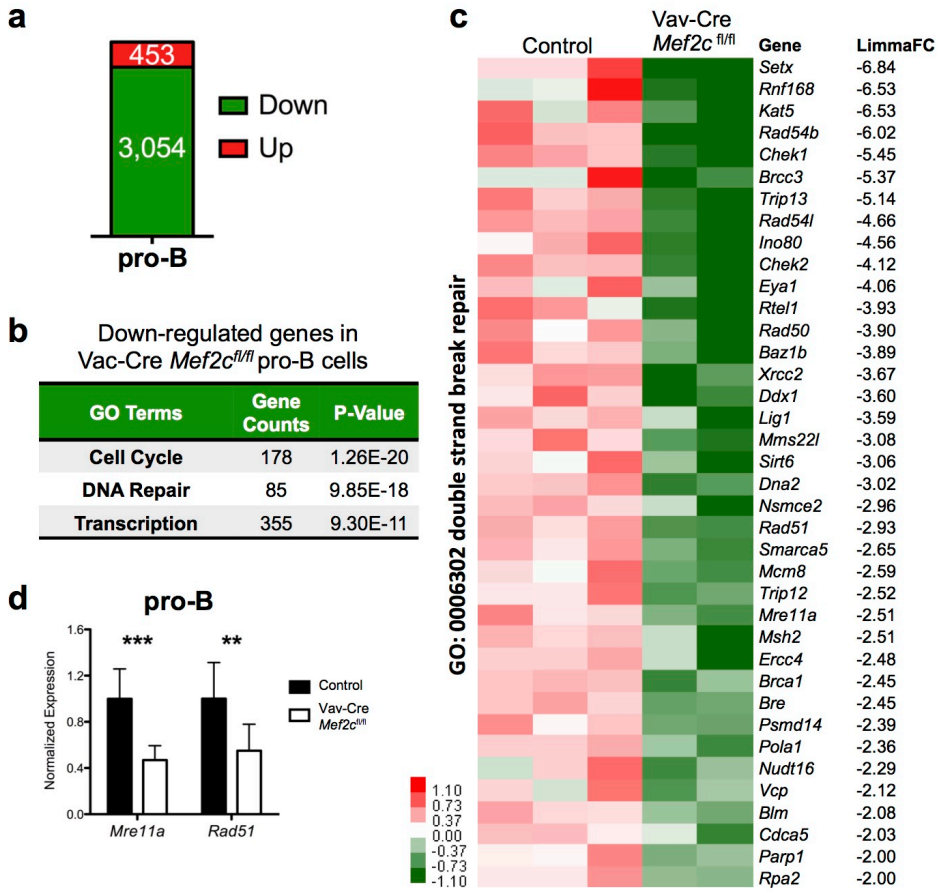
Supplementary Figure-5



Supplementary Figure 5: Loss of *Mef2c* affects the recovery of BM B lymphoid cells but not myeloid cells

(a,b) Representative flow cytometric analysis of BM B lymphoid and myeloid cells shows that loss of *Mef2c* specifically compromises the recovery of BM B lymphoid cells while the recovery myeloid cells is unaffected. **(c)** Representative flow cytometric analysis and quantification of annexin V positivity in BM B lymphoid populations at week 2 after 6Gy irradiation (n≥9) shows compromised cell survival in *Mef2c* deficient B lymphoid populations, but not total BM cells. Data shown are the mean ± SD of three or more independent experiments. NS not significant, * P<0.05, ** P<0.01 and *** P<0.001.

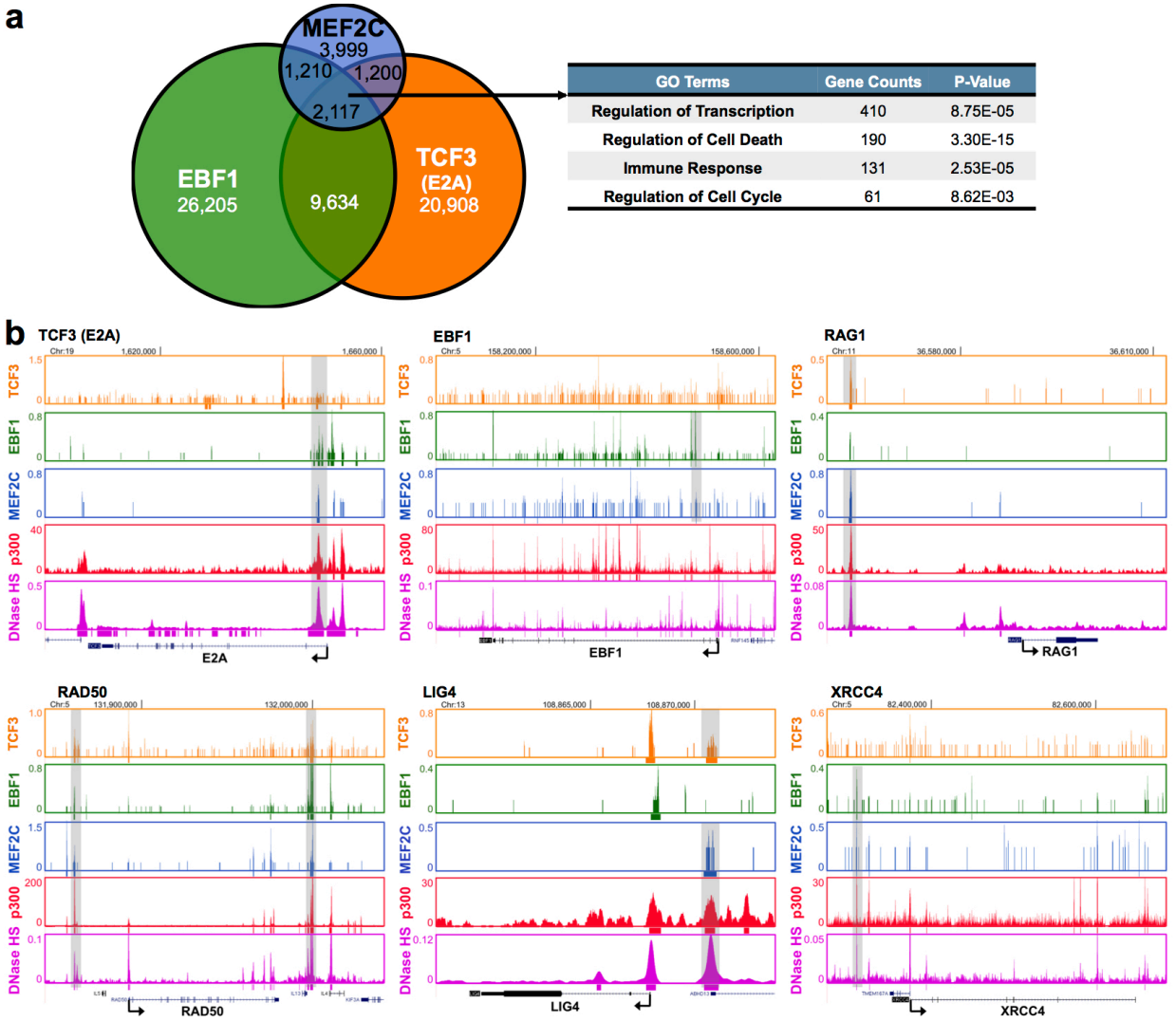
Supplementary Figure-6



Supplementary Figure 6: MEF2C regulates DNA repair pathways also in BM pro-B cells

(a) Microarray analysis of BM pro-B cells from control and Vav-Cre Mef2c$^{fl/fl}$ mice (9 months old) identified 3,054 significantly (|FC|≥2 and p≤0.05) down-regulated and 453 up-regulated genes in the absence of *Mef2c* (n≥2). **(b)** Cell cycle, DNA repair and transcription were among the most significant GO categories down-regulated also in *Mef2c* deficient pro-B cells. **(c)** DNA double strand break repair factors that are significantly down-regulated in *Mef2c* deficient pro-B cells are shown. **(d)** Q-PCR of key genes encoding DNA repair machinery validated defective expression in *Mef2c* deficient pro-B cells. n≥5 mice, data shown are the mean ± SD of two or more independent experiments.

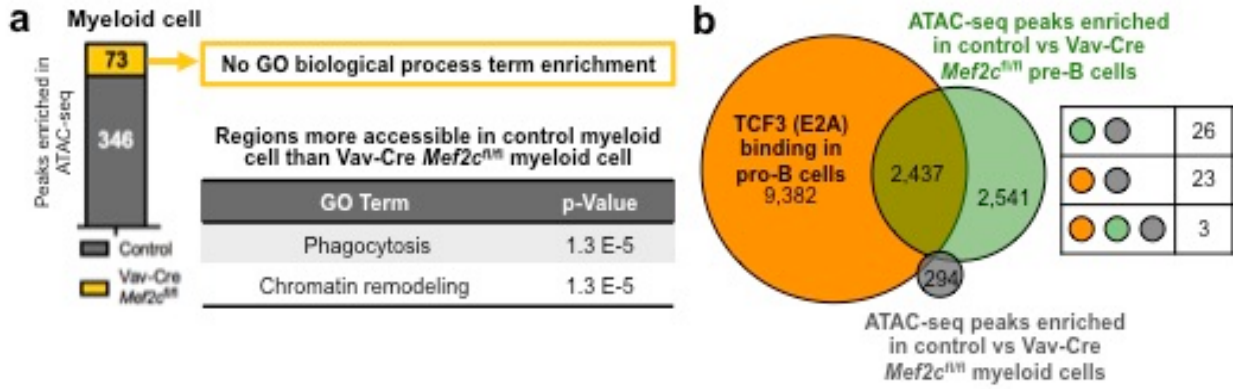
Supplementary Figure-7



Supplementary Figure 7. MEF2C co-localizes with E2A and EBF1 binding at critical DNA repair and V(D)J recombination factors in human B lymphoblasts

(a) Intersection of MEF2C, E2A and EBF1 bound genes in human B lymphoblasts revealed significant overlap. **(b)** E2A, EBF1, MEF2C, p300 binding sites and DNase hypersensitive sites (DNase HS) at genes encoding for key B cell regulators, DNA repair and V(D)J recombination factors are shown. MEF2C peaks that are co-localized with E2A or EBF1 peaks, defined by ENCODE database, are highlighted in grey.

Supplementary Figure-8



Supplementary Figure 8. MEF2C promotes chromatin accessibility in small subset of regulatory regions in myeloid cells that are distinct from MEF2C regulated sites in B lymphoid cells

(a) MAnorm analysis of ATAC-sequencing (ATAC-seq) data in control myeloid and *Mef2c* deficient myeloid ($Mac1^{+}Gr1^{+}$) cells revealed that *Mef2c* loss only significantly (ratio≥4, p-Value≤0.01) affected accessibility of a small number of regulatory sites in BM myeloid cells. **(b)** Venn diagram showing minimal overlaps between regions that are more accessible in control myeloid cells vs. *Mef2c* deficient myeloid cells, with regions that are more accessible in control pre-B vs. *Mef2c* deficient pre-B cells, or TCF3 bound regions in pro-B cells.

Chapter 3:

MEF2C Regulates Hematopoietic Stem Cells in an Age-Dependent Fashion

MEF2C Regulates Hematopoietic Stem Cells in an Age-Dependent Fashion

Introduction

The immune system is critical for health as it protects the organism against attacks from foreign pathogens. Because majority of the mature immune cell types have relatively short lifespan, the hematopoietic stem cell (HSC) is responsible for the life-long replenishment of the immune system.

HSC is characterized by its capability of self-renewal and multi-potency (Seita and Weissman, 2010). In the murine model, long-term HSCs (LT-HSCs) can be identified by the surface expression of Lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) CD150$^+$ CD48$^-$ CD41$^-$, while multi-potential progenitors (MPPs) are thought to be Lineage$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^-$ (Kiel et al., 2005) (Figure 3.2A). Although these markers allow enrichment of HSCs, they do not always represent functionality. The gold standard for assessing functional HSCs in mice is still testing the capability to provide long-term replenishment of all blood cell lineages upon transplantation into lethally irradiated recipients (Domen and Weissman, 1999).

In order to serve as the life-long supply of all blood cell lineages, HSCs need to maintain dormant status to preserve self-renewal capacity and prevent exhaustion (Kiel and Morrison, 2008; Wilson and Trumpp, 2006). The HSC niche, which refers to the specific microenvironment in the BM where HSCs locate, is critical for maintaining the balance between HSC dormancy and self-renewal (Morrison and Spradling, 2008). It has been shown that HSC niche is primarily composed of perivascular cells (see Appendix). At the molecular level, it has

been shown that the HSC niche depends on a several conserved developmental pathways to regulate HSC quiescence, including TGF-β (Dickson et al., 1995), Wnts (Luis et al., 2009), Notch (see Appendix) and Hedgehog (Hh) (Cridland et al., 2009). Other than these signaling pathways, a wide range of environmental factors are also critical for modulating HSC quiescence, including CXCL12/SDF-1 (Nagasawa et al., 1996), stem cell factor (SCF) (Barker, 1997; McCulloch et al., 1965), angiopoietin-1 (Ang-1) (Arai et al., 2004), and thrombopoietin (Qian et al., 2007).

Besides the cell-extrinsic factors in the niche, multiple cell-intrinsic mechanisms are critical for regulating HSC quiescence. The G0 to G1 cell cycle entry of quiescent HSCs is primarily regulated by the activity of cyclin D-Cdk4/6 complex (Pietras et al., 2011). The cyclin D family has three members: *Ccnd1*, *Ccnd2*, and *Ccnd3*, which are all expressed in HSCs (Passegué et al., 2005). Deletion of a single D-cyclin, or one of the two Cdks, has minimal effects on the hematopoietic system, indicating the functional redundancy of these factors (Fantl et al., 1995; Malumbres et al., 2004). However, deletion of all three D-cyclins or both Cdks in mice resulted in embryonic death due to hematopoietic failure caused by HSC exhaustion (Kozar et al., 2004; Malumbres et al., 2004). Multiple upstream signaling pathways have been shown to modulate the activity of Cyclin D-Cdk4/6 complex, including PI3K/Akt/mTOR, Ink CKI, PTEN and TSC1/2. However, as all these experiments are conducted in models of embryonic death, it is not yet clear whether the Cyclin D-Cdk4/6 complex performs a similar function in adult HSCs.

The hematopoietic system is exposed to various stress factors that require rapid proliferation of HSCs to replenish the blood and immune system (Wilson et al., 2008). The regeneration of the hematopoietic system under such situations is called stress hematopoiesis, and it can be induced by multiple stress factors, including BM transplantation (Thornley et al.,

2001), radiation and chemotherapy (Mauch et al., 1995), heavy bleeding (Cheshier et al., 2007), infection (Baldridge et al., 2010), etc.

Various efforts have been devoted to study the effects of different stress factors in regulating HSCs. It has been shown that hematopoietic stress induced by chemotherapeutic agent 5-fluorouracil (5-FU) can force almost all the dormant HSCs into cell cycle to give rise to a large number of downstream progenitors to sustain blood cell production (Wilson et al., 2008). Similar to chemotherapy, other stress factors that cause massive loss of hematopoietic cells, such as irradiation, severe bleeding and viral infection, have also been shown to induce proliferation of dormant HSCs (Cheshier et al., 2007; Essers et al., 2009). Once the mature blood cells are generated, a negative feedback signal is sent back to the BM, and the injury-activated HSCs can then travel back to endosteum niche and return to dormancy (Wilson et al., 2008).

The injury induced HSC proliferation, as well as other internal and external stress factors, can induce DNA damage in HSCs. As the genomic stability of stem and progenitor cells is required for proper self-renewal and differentiation capacity (Lombard et al., 2005), HSCs require properly functioning DNA repair machinery.

Although it has long been known that stress responses and DNA repair are critical for maintaining HSC homeostasis and preventing age-dependent defects, little is known if the HSCs possess specific regulatory mechanism to enhance these pathways.

Our preliminary studies have identified Myocyte Enhancer Factor 2C (MEF2C) as a candidate regulator that protects the HSC pool. In the hematopoietic system, MEF2C was shown to function at multiple stages of normal hematopoiesis (Andrews et al., 2012; Debnath et al., 2013; Gekas et al., 2009; Khiem et al., 2008; Stehling-Sun et al., 2009; Wilker et al., 2008). However, none of the previous study explored the potential involvement of MEF2C in protecting

the HSC homeostasis. As our recent study revealed unique functions of MEF2C in regulating DNA repair process to protect the B lymphoid compartment (Chapter 2), we therefore asked if MEF2C also functions in protecting the HSC compartment, possibly through DNA repair regulation.

Results

MEF2C Regulates Bone Marrow Hematopoietic Stem Cell Pool in an Age-Dependent Fashion

To define the cellular and molecular processes that MEF2C regulates in adult HSCs, *Mef2c* was conditionally deleted in mice by breeding the *Mef2c*$^{flox/flox}$ with the Vav-Cre transgenic mice. Vav-Cre *Mef2c*$^{flox/flox}$ mice undergo efficient deletion of loxP-targeted alleles in the hematopoietic cells during mid-gestation of mouse development (Gekas et al., 2009; Stadtfeld and Graf, 2005). We first focused our study on the middle aged adult mice (7-11 months old) as our previous study in the B lymphoid compartment showed that the *Mef2c* deficiency was most notable at this age (Gekas et al., 2009, Chapter 2).

To examine the frequency of phenotypic HSCs in control and Vav-Cre *Mef2c*$^{flox/flox}$ mice, we analyzed the surface expression of HSC markers (Figure 3.1A) by flow cytometry. Although loss of *Mef2c* did not significantly affect the total hematopoietic stem and progenitor pool (defined by Lineage^{-} Sca-1^{+} c-Kit^{+}, also called LSK) in middle aged mice, further analysis of the long-term stem cells (LT-HSC) using the additional SLAM family surface markers, CD150, CD41 and CD48 (Kiel et al., 2005), revealed significant reduction of LT-HSCs (LSK CD150^{+} CD41^{-} CD48^{-}) in Vav-Cre *Mef2c*$^{flox/flox}$ mice comparing to controls (Figure 3.1B). These data suggested that MEF2C is required for the integrity of the adult LT-HSC pool, but dispensable for the MPP stage during homeostatic conditions.

To examine the requirement for MEF2C in lineage restricted progenitors, we then performed flow cytometry analysis of common lymphoid progenitors (CLPs), common myeloid

progenitors (CMPs), megakaryocyte / erythroid progenitors (MEPs) and granulocyte / monocyte progenitors (GMPs) in control and *Mef2c* deficient mice based on the surface expressions of previously identified markers (Table 3.1). Indeed, loss of *Mef2c* did not significantly affect the frequency of these lineage-restricted progenitors either (Figure 3.1C), suggesting that MEF2C is not critical in regulating the lineage potential of the HSC pool in middle-aged adults

Table 3.1 Surface Markers Used for Flow Cytometric Analysis of Lineage-restricted Progenitors in Mice Bone Marrow Cells

Progenitor Population	Surface Marker Combination
Common Lymphoid Progenitor (CLP)	Lin^{-} IL-7R^{+} Sca-1^{lo} c-Kit^{lo}
Common Myeloid Progenitor (CMP)	Lin^{-} Sca-1^{-} c-Kit^{+} CD34^{+} FcγRIII/II^{low}
Megakaryocyte / Erythroid Progenitor (MEP)	Lin^{-} Sca-1^{-} c-Kit^{+} CD34^{-} FcγRIII/II^{-}
Granulocyte / Monocyte Progenitor (GMP)	Lin^{-} Sca-1^{-} c-Kit^{+} CD34^{+} FcγRIII/II^{+}

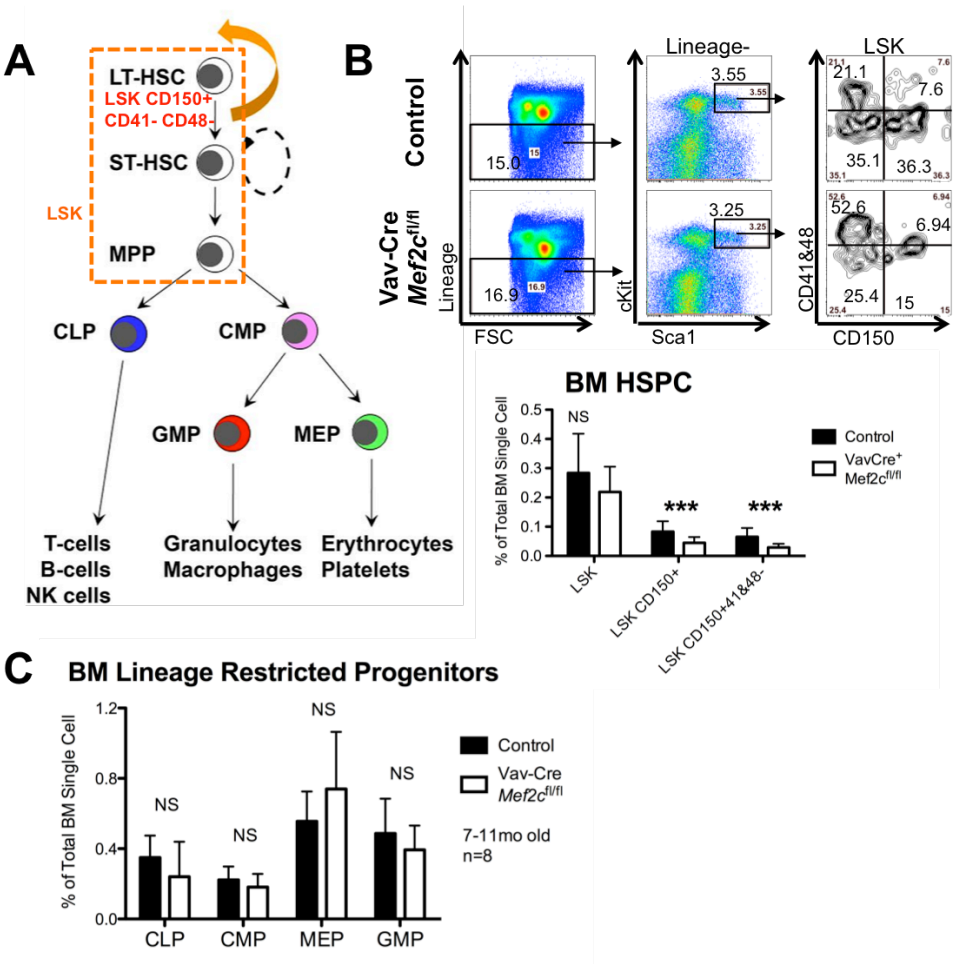


Figure 3.1 Loss of *Mef2c* Resulted in Reduction of Long-Term Hematopoietic Stem Cells in the Middle Aged Adults

(A) In mice, long-term hematopoietic stem cells (LT-HSCs) can be identified by surface expression of Lineage$^-$ c-Kit$^+$ Sca-1$^+$ (LSK) CD150+ CD41- CD48-, while the pool of multi-potential progenitors together with HSCs in the bone marrow (BM) can be identified by staining for LSK. **(B)** Representative flow cytometric analysis and quantification of hematopoietic stem and progenitor cells in the BM of middle aged control and *Mef2c* deficient mice (n≥16). **(C)** Quantification of the flow cytometric analysis of lineage-restricted progenitors: common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), megakaryocyte / erythroid progenitors (MEPs) and granulocyte / monocyte progenitors (GMPs) in the BM of middle aged control and *Mef2c* deficient mice (n≥8). NS: not significant. ***: p-value ≤ 0.001.

The Requirement for MEF2C in Protecting HSC Pool Becomes Higher during Physiological Aging

One important aspect of HSC maintenance is to protect the age-associated HSC defects. As our previous study showed that *Mef2c* loss causes a B lymphoid phenotype that resembles premature aging, we asked if MEF2C is also required to protect the HSC compartment during physiological aging.

To study the importance of MEF2C in physiological aging of HSCs, we analyzed the frequency of MPP (LSK), and LT-HSC (LSK $CD150^+$ and LSK $CD150^+$ $CD41^-$ $CD48^-$) population in both control and Vav-Cre $Mef2c^{flox/flox}$ mice at different ages. Similar to the phenotype observed in middle-aged mice, loss of *Mef2c* did not significantly affect the frequency of MPP pool in young (1-2 months old) or aged (17-20 months old) mice either (Figure 3.2), suggesting that MEF2C is dispensable for maintaining the MPP pool throughout adulthood.

Unlike in middle-aged adults, loss of *Mef2c* in young mice (1-2 months old) only significantly reduced the frequency of most refined LT-HSCs (LSK $CD150^+$ $CD41^-$ $CD48^-$) but not LSK $CD150^+$ population. Consistent with previous studies, we observed an increase of phenotypic LT-HSCs in aged (17-20 months old) control mice compared to the middle-aged or young control mice. However, loss of *Mef2c* significantly compromised this increase of phenotypic LT-HSCs in aged mice, resulted in an age-dependent reduction of LT-HSCs in *Mef2c* deficient mice compared to control mice. (Figure 3.2) These data together suggested a heightened requirement for MEF2C in maintaining LT-HSC pool during physiological aging.

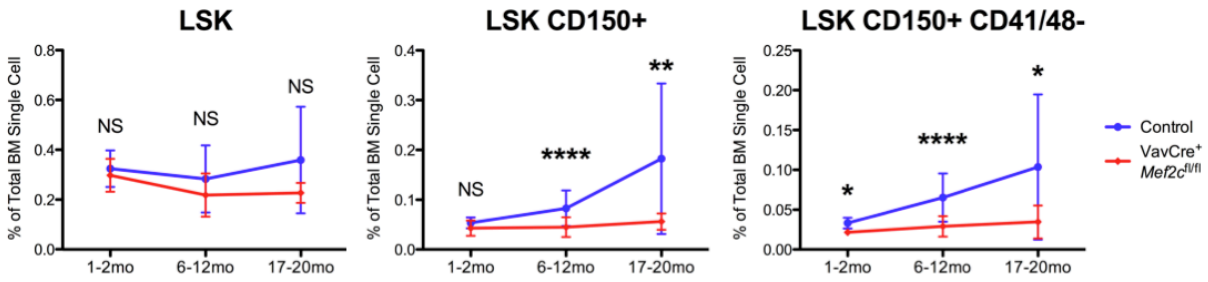


Figure 3.2 Loss of *Mef2c* Compromised the Age-Dependent Increase of Phenotypic Hematopoietic Stem Cells in Mouse Bone Marrow

Quantification of flow cytometric analysis of hematopoietic stem cell and multi-potential progenitors in the bone marrow of control and Vav-Cre *Mef2c*$^{flox/flox}$ mice at different ages of life (n≥6 at each age group). NS: not significant. *: p-value ≤ 0.05. **: p-value ≤ 0.01. ***: p-value ≤ 0.001. ****: p-value ≤ 0.0001.

Loss of *Mef2c* Compromised the Long-Term Repopulating Capability of Bone Marrow

Although surface markers are important in identification of HSCs, they do not always represent functionality, and the gold standard for testing functional HSCs in mice is the capability to provide long-term replenishment of all blood cell lineages upon transplantation into lethally irradiated recipients (Domen and Weissman, 1999). As loss of *Mef2c* resulted in the reduction of phenotypic LT-HSCs, we analyzed the requirement for MEF2C in maintaining the BM repopulating capability by competitive transplantation (Figure 3.4A). Analysis of the peripheral blood engraftment of middle-aged *Mef2c* deficient and control mice revealed that loss of *Mef2c* did not affect the early blood engraftment at week 4, while it significantly reduced the long-term blood engraftment at both week 11 and 15 (Figure 3.3B). These data confirmed the functional requirement for MEF2C in maintaining the integrity of LT-HSC pool, and once again showed that the requirement for MEF2C in MPP function is minimal.

As all the peripheral blood lineages were derived from BM stem and progenitors, we therefore analyzed the BM engraftment at 15 weeks after transplantation. Consistent with the peripheral blood engraftment analysis, loss of *Mef2c* significantly compromised the total BM engraftment from middle-aged donors (Figure 3.3C). Furthermore, analysis of the donor lineage engraftment in the BM revealed that loss of *Mef2c* significantly reduced the engraftment of both B and T lineages. Although the myeloid engraftment was not significant, it also showed the same trend. (Figure 3.3D) These results once again confirmed the requirement for MEF2C in maintaining the repopulating capability of HSC pool. However, future studies of the phenotypic engraftment of MPP and LT-HSCs at multiple time points after transplantation are required to deeply analyze the functional requirement for MEF2C in HSC homing, engrafting, and repopulating activities.

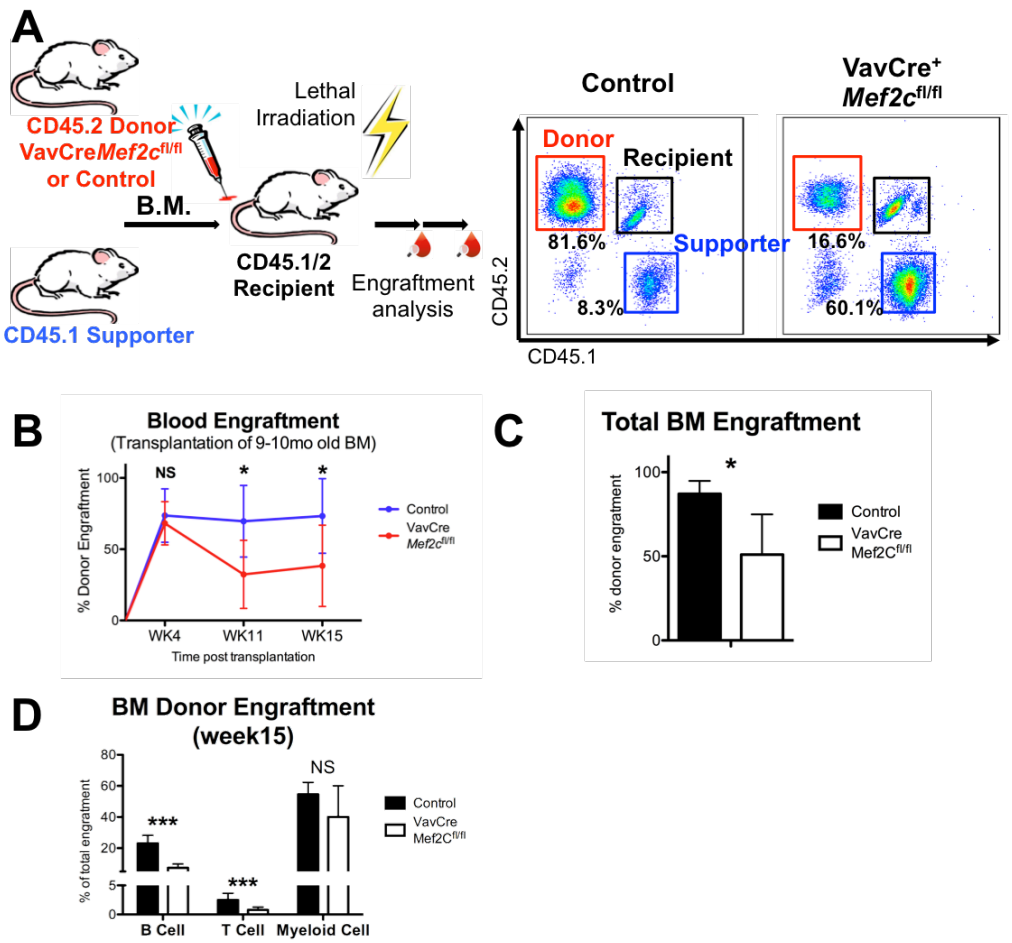


Figure 3.3 Transplantation of Total Bone Marrow from Middle-Aged *Mef2c* Deficient Mice Results in Decreased Engraftment

(A) Experimental design of competitive transplantation analysis of control and *Mef2c* deficient total bone marrow cells. Specifically, CD45.2 donor BM and CD45.1 supporter BM was mixed together and transplanted into lethally irradiated CD45.1/2 recipient mice. Peripheral blood is collected at multiple time points post transplantation and CD45.1 and CD45.2 are stained to differentiate the blood cells that are originated from donor BM. **(B)** Quantification of peripheral blood engraftment of donors that are middle-aged. **(C)** Quantification of total BM engraftment of middle-aged donors at week15 after transplantation. **(D)** Quantification to donor lineage engraftment at week 15 after transplantation. (n≥5) NS: not significant. *: p-value ≤ 0.05. **: p-value ≤ 0.01. ***: p-value ≤ 0.001.

MEF2C Regulates the Transcription of DNA Repair Genes in Long Term Hematopoietic Stem Cells

As loss of *Mef2c* resulted in the reduction of both phenotypic and functional LT-HSCs in middle-aged mice, we determined the cellular pathways that MEF2C regulates in the LT-HSCs. We performed Affymetrix microarray analysis in BM LT-HSCs (LSK $CD150^{+}$ $CD41^{-}$ $CD48^{-}$) from middle-aged (9 months old) control and Vav-Cre $Mef2c^{flox/flox}$ mice.

Lack of *Mef2c* in LT-HSCs significantly ($|LimmaFC| \geq 2$ and Limma p-value ≤ 0.05) reduced the transcription of 1055 genes and increased the expression of 851 genes (Figure 3.4A). Gene Ontology (GO) analysis of differentially expressed genes identified transcription regulation, cell cycle, apoptosis regulation, stress response and DNA repair categories among the most significantly enriched categories down-regulated in *Mef2c* deficient LT-HSCs (Figure 3.4B). As all these processes are important in maintaining the integrity and homeostasis of the HSC pool, these results suggest a possible function for MEF2C in regulating these processes LT-HSCs.

As DNA repair is a critical process that is required for maintaining genomic integrity (Lombard et al., 2005) and is also thought to be one of the key underlying causes for age-dependent deficiencies in LT-HSCs (Sperka et al., 2012), we further zoomed into the gene expression assessment of this GO category (Figure 3.4C). Interestingly, loss of *Mef2c* resulted in the down-regulation of multiple key DNA repair effectors in both nucleotide excision repair (NER) pathway (*Rad23*, *Ercc6*, *Ercc8*, *Gtf2h* and *Pold*, Figure 3.6D) and homologous recombination (HR) double strand break repair pathway (*53bp1*, *Rad51*, *Rad51ap1*, *Rad54*, and *Brca1*, Figure 3.4E). These analysis nominated MEF2C as a DNA repair regulator in LT-HSCs.

As proliferative stress during irradiation induced hematopoietic ablation can induce ectopic DNA damage in injury activated LT-HSCs, we therefore aimed to identify the functional requirement for MEF2C in protecting LT-HSCs during stress hematopoiesis. Sub-lethal total body irradiation (4Gy) was given to both control and Vav-Cre *Mef2c*$^{flox/flox}$ mice. At week 6 after irradiation, BM was isolated from both irradiated and un-irradiated mice and Annexin V expression analysis for apoptosis was conducted for HSPCs. In mice that were not irradiated, loss of *Mef2c* did not significantly affect the cell survival of either MPPs or LT-HSCs. However, in irradiated mice, *Mef2c* loss resulted in an increased early apoptosis (AnnexinV+ 7AAD-) only in the LT-HSC (LSK CD150^{+}) compartment, but not in the MPP (LSK) pool or total BM cells. (Figure 3.4F)

These data together suggested that MEF2C is important for homeostasis maintenance and cell survival for LT-HSCs upon stress hematopoiesis, possibly through modulating DNA repair and cell cycle pathways. Combining the age-dependent defects observed in *Mef2c* deficient mice, it is plausible that the compromised HSC pool observed in *Mef2c* deficient mice during aging results from accumulation of stress-induced DNA damage. However, future studies are still needed to validate this hypothesis.

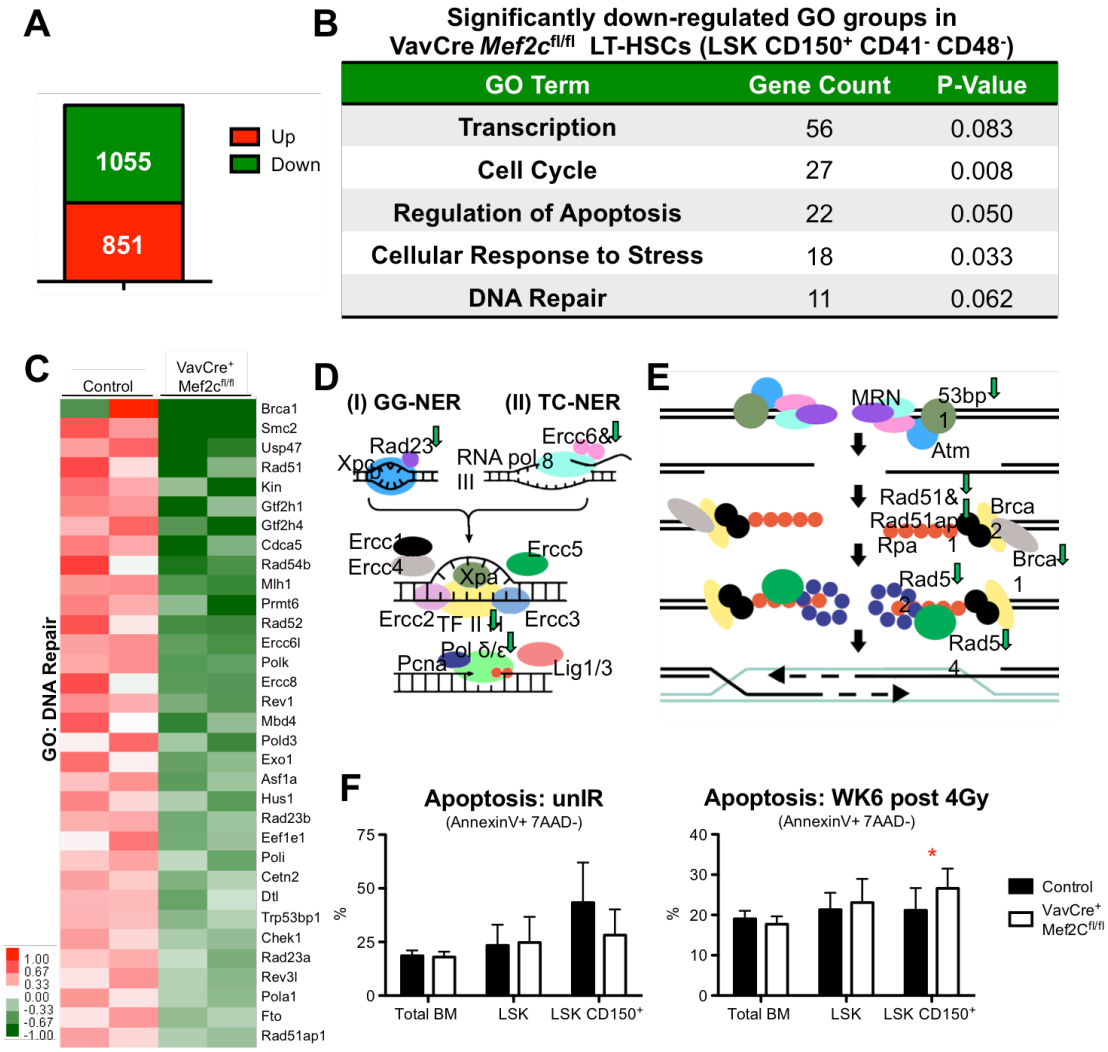


Figure 3.4 Loss of *Mef2c* Results in Down-Regulation of Genes Required for Hematopoietic Stem Cell Maintenance

(A) Quantification of total significantly (|LimmaFC| ≥ 2, p-value ≤ 0.05) up- and down- regulated genes in *Mef2c* deficient LT-HSCs comparing to control. **(B)** Top GO categories that are down-regulated in VavCre *Mef2c*$^{fl/fl}$ LT-HSCs (LSK CD150+ CD48- CD41-). **(C)** Gene expression of significantly changed DNA repair machinery in control and *Mef2c* deficient LT-HSCs. **(D,E)** Model of nucleotide excision repair (NER) and homologous recombination (HR) double strand break repair pathways. **(F)** Quantification of apoptosis of total BM and HSCs in mice that are not irradiated or 6 weeks after 4Gy total body irradiation. *: p-value ≤ 0.05.

Discussion

The capability of hematopoietic stem cells (HSC) to maintain genomic integrity is critical as the entire immune system relies on long-term HSC (LT-HSC) for life long replenishment. Previous studies have identified that the quiescent LT-HSCs adopt error-prone non-homologous end joining (NHEJ) pathway for DNA double strand break (DSB) repair (Mohrin et al., 2010). Multiple hypotheses have been made about how DNA damage accumulation can contribute to HSC aging, which results in reduced repopulating potential and biased lineage potential. Recent studies showed that some subset of LT-HSCs accumulate DNA damage during quiescence, and induce homologous recombination (HR) pathway upon entering cell cycle (Beerman et al., 2014). Although the choice of different DNA repair pathways in HSC has been studied in depth, little is known if HSCs employs cell type specific transcriptional mechanisms in regulating DNA repair and other stress response pathways. More studies need to be conducted to identify key regulators that protect the LT-HSC pool, especially during proliferative stress induced by irradiation, chemotherapy or other stress factors.

Here we identified MEF2C as a candidate transcriptional activator that regulates DNA repair in LT-HSCs to protect the HSC compartment through life. Specifically, data presented here reveals that MEF2C is required for the proper expression of essential components of both HR and NER pathways. Loss of *Mef2c* resulted in the reduction of both phenotypic and functional LT-HSCs while minimally affecting the MPP pool. *Mef2c* deficiency was exaggerated during physiological aging, possibly due to the accumulation of DNA damage through time. Upon irradiation induced stress hematopoiesis, *Mef2c* deficient LT-HSCs showed increased

apoptosis, suggesting that MEF2C function is required to protect the LT-HSC pool during proliferative stress.

However, the data shown in this thesis are preliminary, and future studies are necessary to confirm the different aspects of MEF2C function in LT-HSCs. As the gene expression analysis revealed the potential regulatory requirement of MEF2C in DNA repair, cell cycle and stress response pathways, functional analysis of these processes in control and *Mef2c* deficient LT-HSCs and MPPs are important to confirm these requirements. Specifically, BrdU or Ki67 flow cytometry analysis should be conducted to explore if *Mef2c* loss indeed affects cell cycle regulation in LT-HSCs at both homeostasis and during stress hematopoiesis. In addition, DNA damage analysis by comet assay and γH2AX immune-fluorescent staining should be conducted in future to validate the requirement for MEF2C in DNA repair in LT-HSCs. Gene expression and functional tests for cell cycle and DNA repair should also be performed in *Mef2c* deficient LT-HSCs during physiological aging to test how the requirement for MEF2C becomes enhanced during aging.

As MEF2C functions as a transcription factor, ChIP-seq analysis of MEF2C in wild-type LT-HSCs should be conducted to confirm if MEF2C directly regulates the expression of key DNA repair factors. Analysis of published MEF2C ChIP-seq in human B lymphoblast revealed that MEF2C can directly bind to the regulatory region of key HR and NER factors in that cell type (Figure 3.5), suggesting the possibility of direct binding also in HSCs. In addition, ChIP-seq analysis of active histone marks and co-activator p300 in both control and *Mef2c* deficient LT-HSCs will help reveal the molecular mechanism that MEF2C employs in regulating gene expression in LT-HSCs (also see previous discussion for chapter 2).

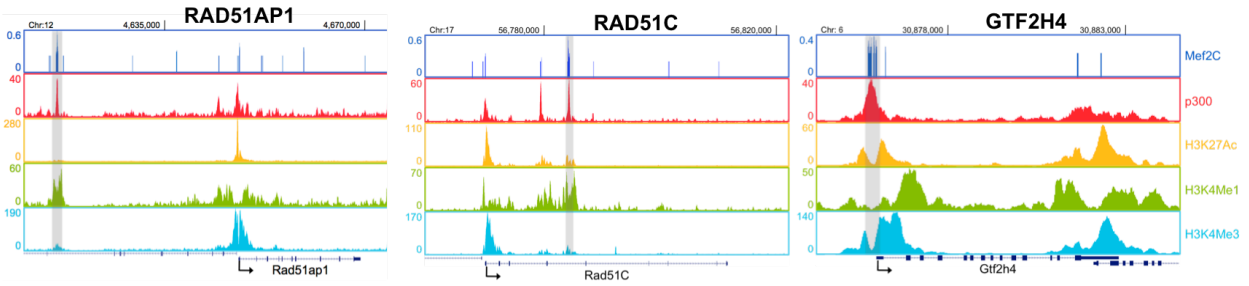


Figure 3.5 MEF2C Directly Binds to Nucleotide Excision Repair and Homologous Recombination Double Strand Break Repair Factors in Human B Cells.

MEF2C binding site at the enhancer / promoter region of key nucleotide excision repair (NER) and homologous recombination (HR) double strand break repair effectors with in the human GM12878 cell line. (Data obtained from the ENCODE project)

In addition to emerging as a key regulator of normal immune cell development, *Mef2c* is also known as a cooperating oncogene in multiple types of leukemia (Canté-Barrett et al., 2013). *Mef2c* became abnormally induced in leukemic GMPs In MLL-AF9 driven myeloid leukemia, *Mef2c* has been shown to be ectopically induced. Importantly, knockdown of *Mef2c* attenuated the proliferative potential of these cells (Krivtsov et al., 2006). In another model of myeloid leukemia (MLL-ENL), the loss of *Mef2c* in leukemic cells reduced their homing and invasive capacities (Schwieger et al., 2009). Moreover, MEF2C is emerging as an important oncogene in T-ALL (Homminga et al., 2011; Nagel et al., 2008; Nagel et al., 2011; Zuurbier et al., 2014). Not surprisingly, MEF2C promotes the survival of T-ALL cells via the down-regulation of Nur77, a critical mediator of apoptosis during thymopoiesis. Unlike in the examples presented above, MEF2C is normally expressed in pre-B cells. Based on our data, a logical question is whether MEF2C contributes to the development and/or progression of pre-B ALL. Given that many

chemotherapeutics (e.g. cisplatin, doxorubicin) exploit DNA damage to induce cellular apoptosis, it is provocative to envisage pre-B ALL as possibly having constitutive expression of an inherent drug resistance pathway (i.e. DNA repair). As such, any chemotherapeutics utilizing DNA damage as a mechanism of action would be predicted to be poorly efficacious(Helleday et al., 2008). Analysis of pre-B leukemic models in which MEF2C expression is manipulated will be key in addressing this possibility.

Moreover, it will be important to define whether the ability of MEF2C to enhance DNA repair is restricted to the hemato-lymphoid system, or is also utilized in stem/progenitor cells in other tissues, such as muscle, heart, vasculature, neural progenitors and neural crest, where MEF2C is required for their normal development (Agarwal et al., 2011; Black and Olson, 1998; Lin et al., 1997; Okamoto et al., 2000; Verzi et al., 2007). In neural progenitors, MEF2C is also required for progenitor cell survival as inhibition of MEF2C led to increased apoptosis in differentiating neural progenitors (Okamoto et al., 2000). Thus, understanding the mechanisms of how MEF2C augments transcription of its target genes during the development and differentiation of stem/progenitor cells in various tissues may have broader implications in regenerative medicine.

Method

Mice: Vav-Cre mice were bred with *Mef2c*$^{fl/fl}$ mice to generate Vav-Cre *Mef2c*$^{fl/fl}$ mice. *Mef2c*$^{fl/fl}$, *Mef2c*$^{fl/+}$ or Vav-Cre *Mef2c*$^{fl/+}$ mice of the same age were used as controls. Genotyping analysis was done as previously described (Gekas et al., 2009). All mice were maintained according to the guidelines of the UCLA Animal Research Committee.

Flow cytometric analysis and isolation of hematopoietic progenitors: Hematopoietic cells were analyzed using different antibodies. Dead cells were excluded with 7-amino-actinomycin D and cell populations were analyzed using a LSR II or Fortessa flow cytometer. Cell sorting was performed using a FACS Aria cell sorter. Data were analyzed with FlowJo software version 9.2.

Irradiation: Sub-lethal (4Gy) or lethal (9.5Gy) total body irradiation was performed with Co-60 pool irradiator.

Competitive transplantation: CD45.2 control or Vav-Cre *Mef2c*$^{flox/flox}$ mice were used as donors, while CD45.1 wild type mice were used as supporters. Total BM cells were isolated from both donor and supporter, and then mixed together to be retro-orbitally injected into lethally irradiated CD45.1/2 recipient mice. At different time points after transplantation, peripheral blood was collected from recipient mice, and flow cytometric analysis of CD45.1 and CD45.2 are used to identify cells that are originated from donor BM (CD45.1$^+$ CD45.2$^-$), supporter (CD45.1$^-$ CD45.2$^+$) and recipient (CD45.1$^+$ CD45.2$^+$). Donor engraftment is then calculated by the following formula:

$$Donor\ engraftment = \frac{\%\ of\ Donor\ Cells}{(\%\ of\ Donor\ Cells + \%\ of\ Supporter\ Cells)} \times 100\%$$

Gene expression profiling: Total RNA was isolated from sorted cells using a combination of QIAshredder columns followed by the RNEasy Micro/Mini Kit (QIAGEN). Affymetrix microarray analysis was performed on independent control and *Mef2c* deficient sorted HSC samples. The R package Limma provided through the open source project Bioconductor was used to assess differential expression. To calculate absolute mRNA expression levels, the RMA (Robust Multiarray Averaging) method was used to obtain background adjusted, quantile normalized and probe level data summarized values for all probe sets. The Affymetrix Mouse Genome 430 2.0 Array GeneChip platform was used for the analysis. Official gene symbols for probe sets were obtained using the Bioconductor annotation database mouse4302.db. The mas5calls algorithm through the R package of affy was used for calculating PMA detection calls for each array sample. Differentially expressed genes were uploaded into the DAVID interface to identify significantly over-represented functional GO biological process categories.

Statistics: Student's unpaired two-tailed t-test was used for statistical analysis and differences with P values of 0.05 or less were considered significant.

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Chapter 4:

Summary and Discussion

Summary and Discussion

Chapter 2: MEF2C Protects B Lymphoid Progenitors during Stress Hematopoiesis

The capability of stem and progenitor cells to repair DNA damage is critical for maintaining genomic integrity. DNA repair is especially critical for B lymphoid progenitors, as they require NHEJ repair machinery to perform B cell receptor rearrangement. In addition, immune system is challenged by various factors that induce regenerative stress, which is associated with higher risk for DNA damage. It is therefore important to understand if B lymphoid progenitors utilize any lineage specific mechanism to regulate the DNA repair system and maintains normal B lymphopoiesis at both steady state and stress situations.

In the current study, we have identified MEF2C as a key regulator of DNA repair machinery and V(D)J recombination in BM B lymphoid progenitors. MEF2C enhances the transcription of genes that encode for RAG recombinase and DSB repair machinery in B cell progenitors to protect the integrity of DNA repair and V(D)J recombination processes. At steady state, *Mef2c* loss resulted in defective DNA repair and immunoglobulin rearrangements, which together contributes to increased cell death. The defects in BM can be compensated by unknown mechanism to maintain an intact peripheral B cell pool. However, MEF2C requirement is heightened during the recovery from stress, and loss of *Mef2c* resulted in compromised B lymphoid recovery in both BM and peripheral organs (spleen and blood). These data together suggested that MEF2C is an important guardian of B lymphopoiesis especially during stress hematopoiesis.

T and B lymphopoiesis share many common steps. Interestingly, although MEF2C is critical for protecting B lymphoid compartment, it is barely expressed in T lymphoid

compartment (Stehling-Sun et al., 2009), and loss of *Mef2c* does not significantly affect the integrity of T lymphoid compartment at either steady state or stress recovery phase. It is therefore intriguing if T lymphoid compartment uses a different factor that performs similar functions as MEF2C does in B lymphoid cells.

In addition to regulating the hematopoietic system, MEF2C is also critical for the normal development of cardiac and neural systems. As DNA repair is generally required for stem and progenitor cells to maintain genomic integrity, it is therefore interesting to explore if MEF2C also regulates DNA repair machinery in any other lineages.

As the phenotype of *Mef2c* deficient mice resembles pre-mature B lymphoid aging, it is important to further explore in detail if the cellular and molecular pathways regulated by MEF2C are the underlying cause for age-associated defects in the B lymphoid compartment. Further analysis of the key MEF2C target pathways that are compromised during physiological aging may reveal new targets in treating immunosenescence.

Finally, as MEF2C is a transcription factor, it is important to explore the exact molecular mechanism that MEF2C utilizes in regulating the DNA repair and V(D)J recombination factors. Previous studies in other systems revealed that the MADS box of MEF2C is capable of binding and recruiting p300 to directly enhance gene expression (Canté-Barrett et al., 2013; Ma et al., 2005). It is plausible that MEF2C recruits p300 to establish H3K27Ac at key target genes to enhance their transcription efficiencies (Figure 4.1). Alternatively, as the MEF2C binding in human B lymphoblast is also associated with H3K4Me3, MEF2C may recruit methyltransferases such as MLL to establish the promoter histone landscape and activate gene transcription (Figure 4.1). Future studies of co-IP in control and *Mef2c* deficient cells will be required to test if MEF2C recruits p300 or MLL, while ChIP-seq analysis of different histone marks in control and

Mef2c deficient cells will further reveal the requirement for MEF2C in modulating the histone landscape.

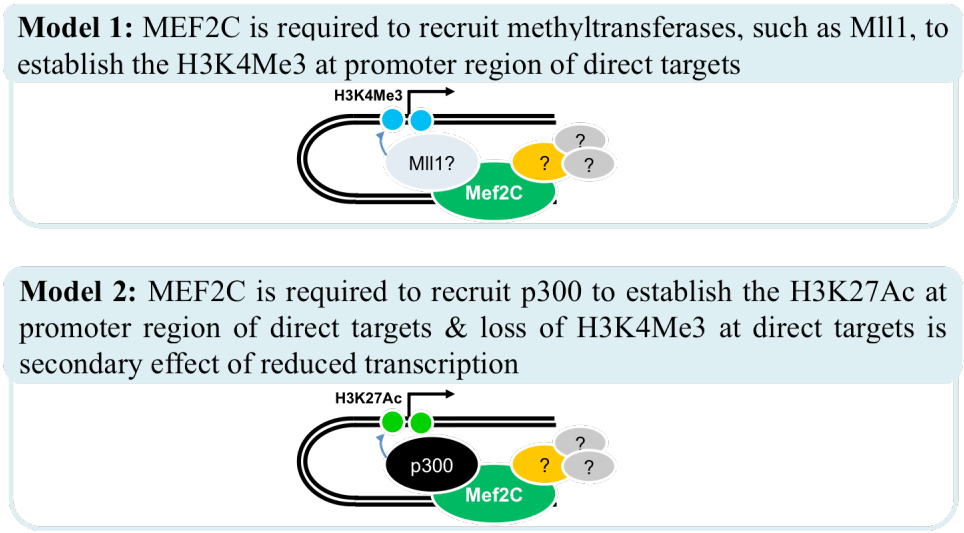

Figure 4.1 Possible Models of the Molecular Mechanism of MEF2C in Regulating Direct Target Gene Expression

Two possible models are proposed to explain the molecular mechanism of MEF2C: (1) MEF2C is required to recruit methyltransferases, such as Mll1, to establish the H3K4Me3 at promoter region of direct targets; (2) MEF2C is required to recruit p300 to establish the H3K27Ac at promoter region of direct targets.

Chapter 3: MEF2C Regulates Hematopoietic Stem Cells in an Age-Dependent Fashion

Our data in Chapter 2 revealed the importance of MEF2C in protecting B lymphoid progenitors through DNA repair regulation. As DNA repair is also critical for HSC to maintain

its homeostasis, we therefore explored the potential function of MEF2C in the HSC compartment. Our preliminary analysis suggested that MEF2C is critical in maintaining the integrity of LT-HSC pool, but dispensable for the downstream MPP compartment. Loss of *Mef2c* compromised the age-dependent induction of phenotypic HSCs during aging. Gene expression analysis nominated DNA repair pathways, specifically HR and NER, as potential targets of MEF2C in LT-HSCs. Future functional studies of these DNA repair processes will be needed to test if MEF2C truly regulates these cellular processes in LT-HSCs. Moreover, analysis of cell cycle, oxidative stress and other process in *Mef2c* deficient LT-HSCs will help identify the potential mechanism that MEF2C uses in protecting the LT-HSC pool.

In addition to emerging as a key regulator of normal immune system development, multiple studies have revealed critical functions for MEF2C in inducing leukemia (Canté-Barrett et al., 2013; Homminga et al., 2011; Krivtsov et al., 2006; Nagel et al., 2008; Nagel et al., 2011; Schwieger et al., 2009). It is therefore important to explore if any of the normal functions of MEF2C is hijacked by the leukemic cells during malignant transformation.

It is unknown how MEF2C regulates the diverse functions in different cell types. Our data suggests that rather that functioning as a lineage specification factor per se, MEF2C may function as a "volume controller", that assists lineage specific factors to enhance the transcription of key genes required for critical processes in the specific cell type. Our studies in the B lymphoid compartment suggest that MEF2C co-binds with EBF1 and TCF3, key B cell factors, to regulate important B lymphoid genes. It is therefore interesting to explore in future studies if MEF2C also co-binds with other HSC factors to regulate HSC homeostasis.

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Appendix:

Perivascular Support of Human

Hematopoietic Stem/Progenitor Cells

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Perivascular support of human hematopoietic stem/progenitor cells

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Regular Article

HEMATOPOIESIS AND STEM CELLS

Perivascular support of human hematopoietic stem/progenitor cells

Mirko Corselli,[1,2] Chee Jia Chin,[3] Chintan Parekh,[4] Arineh Sahaghian,[3] Wenyuan Wang,[5] Shundi Ge,[3] Denis Evseenko,[1,2,6] Xiaoyan Wang,[7] Elisa Montelatici,[8] Lorenza Lazzari,[8] Gay M. Crooks,[2,3,7] and Bruno Péault[1,2,9]

[1]University of California at Los Angeles (UCLA) and Orthopaedic Hospital Department of Orthopaedic Surgery and the Orthopaedic Hospital Research Center, [2]Broad Stem Cell Research Center, and [3]Department of Pathology and Laboratory Medicine, UCLA, Los Angeles, CA; [4]Division of Pediatric Hematology/Oncology, Children's Hospital Los Angeles, Los Angeles, CA; [5]Molecular Biology Institute, [6]Jonsson Comprehensive Cancer Center, and [7]Department of Biostatistics, UCLA, Los Angeles, CA; [8]Cell Factory, Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; and [9]Center for Cardiovascular Science and Center for Regenerative Medicine, University of Edinburgh, Edinburgh, United Kingdom

Key Points

- Perivascular cells maintain HSPCs ex vivo.

Hematopoietic stem and progenitor cells (HSPCs) emerge and develop adjacent to blood vessel walls in the yolk sac, aorta-gonad-mesonephros region, embryonic liver, and fetal bone marrow. In adult mouse bone marrow, perivascular cells shape a "niche" for HSPCs. Mesenchymal stem/stromal cells (MSCs), which support hematopoiesis in culture, are themselves derived in part from perivascular cells. In order to define their direct role in hematopoiesis, we tested the ability of purified human CD146$^+$ perivascular cells, as compared with unfractionated MSCs and CD146$^-$ cells, to sustain human HSPCs in coculture. CD146$^+$ perivascular cells support the long-term persistence, through cell-to-cell contact and at least partly via Notch activation, of human myelolymphoid HSPCs able to engraft primary and secondary immunodeficient mice. Conversely, unfractionated MSCs and CD146$^-$ cells induce differentiation and compromise ex vivo maintenance of HSPCs. Moreover, CD146$^+$ perivascular cells express, natively and in culture, molecular markers of the vascular hematopoietic niche. Unexpectedly, this dramatic, previously undocumented ability to support hematopoietic stem cells is present in CD146$^+$ perivascular cells extracted from the nonhematopoietic adipose tissue. (*Blood.* 2013;121(15):2891-2901)

Introduction

Blood and vasculature are indispensable to embryonic development, and are thus the first differentiated tissues produced in life. Incipient human hematopoiesis adapts to the rudimentary anatomy of the embryo and proceeds first in the yolk sac, then transiently in the placenta and liver before being stabilized in fetal bone marrow (FBM). Definitive hematopoietic stem and progenitor cells (HSPCs) first emerge in the aorta-gonad-mesonephros region of the embryo.[1] Therefore, several organs of distinct germline origins, structures, and eventual roles converge functionally to produce blood cells during development. What remains however remarkably constant through pre- and postnatal life is the physical association of incipient hematopoietic cells with blood vessels. In the yolk sac, erythroid cells emerge within intravascular blood islands.[2] It is now also well accepted that, from fish to humans, specialized blood-forming endothelial cells present in the dorsal aorta and possibly other organs supply the embryo with hematopoietic cells,[3-7] an ontogenic transition that has been modeled in human embryonic stem cells.[8] In addition to this direct developmental affiliation between embryonic endothelial cells and HSPCs, there is evidence that vascular cells nurture blood cells in pre- and postnatal life. The cellular and molecular mechanisms involved in this support can be analyzed in cocultures of stromal and hematopoietic cells.[9-11] For instance, cultured endothelial cells use angiocrine factors to regulate HSPC differentiation or self-renewal.[12-14] Mesenchymal stem/stromal cells (MSCs), the multilineage mesodermal progenitors spontaneously selected in long-term cultures of unfractionated cells from bone marrow and other tissues,[15-18] can also, to some extent, sustain hematopoiesis in vitro.[19-24] However, the relevance of this support to physiologic blood cell production in vivo has been unknown because MSCs have long eluded prospective identification.[25] Similarities between MSCs and pericytes, which ensheath capillaries and microvessels in all organs, have been described.[26-28] In an experimental approach combining stringent cell purification by flow cytometry and differentiation in culture and in vivo, we have demonstrated that human CD146$^+$ perivascular cells represent ubiquitous ancestors of MSCs.[29]

Although hematopoietic stem cells (HSCs) were originally detected in the endosteal regions of the bone marrow,[30] recent findings have suggested the existence of a distinct, perivascular niche for HSPCs.[31-34] Perivascular reticular cells expressing CXCL12 were found to play a role in murine HSC maintenance.[35] In a seminal study by Méndez-Ferrer et al,[36] the function and identity of perivascular niche cells were further defined. The authors showed the existence in murine bone marrow of perivascular nestin$^+$ MSCs associated with HSCs. Ablation of nestin$^+$ MSCs led to a significant reduction in the number and homing ability of HSCs. The direct role for perivascular cells in hematopoiesis regulation was confirmed in a recent study by Ding et al.[37] Selective shutoff of c-kit ligand expression in leptin receptor

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(Lep-R) positive cells surrounding murine bone marrow blood vessels significantly reduced the frequency of long-term reconstituting HSCs.[37]

In the present study, we demonstrate that CD146$^+$ perivascular cells express in vivo nestin, CXCL12, and Lep-R in human FBM as well as in adult adipose tissue. We also report for the first time that human CD146$^+$ perivascular cells are a subset of MSCs able to directly support the ex vivo maintenance of human HSPCs. We further demonstrate that cultured CD146$^+$ perivascular support HSPCs through cell-to-cell contact and activation of Notch signaling. Conversely, conventional unfractionated MSCs or the CD146$^-$ subset of MSCs favor differentiation at the expense of stemness. CD146$^+$ perivascular cells can therefore be considered as the bona fide human equivalents of the hematopoietic perivascular niche components recently described in the mouse.

Methods

Isolation of human primary stromal cells

Human stromal cells were derived from human lipoaspirate specimens (n = 4) and FBM (n = 2) as previously described.[17,29] Lipoaspirates were obtained as discarded specimens without identifiable information, therefore no institutional review board approval was required. Fetal bones (16-18 weeks of pregnancy) were obtained from Novogenix. One hundred milliliters of lipoaspirate were incubated at 37°C for 30 minutes with digestion solution composed by RPMI 1640 (Cellgro), 3.5% bovine serum albumin (Sigma), and 1 mg/mL collagenase type II (Sigma). Adipocytes were discarded after centrifugation while the pellet was resuspended and incubated in red blood cell lysis (eBioscience) to obtain the stromal vascular fraction (SVF). Fetal bones were split open to flush the bone marrow cavity. The bones were placed in digestion solution for 30 minutes at 37°C. Mononuclear cells (MNCs) were isolated using Ficoll-Paque (GE Healthcare). Hematopoietic cells were excluded by magnetic immunodepletion of CD45$^+$ cells as per manufacturer's instructions (Miltenyi Biotec). An aliquot of SVF or CD45-depleted MNCs was plated in tissue-culture–treated flask for the expansion of conventional MSCs.[17] Another aliquot of SVF or CD45-depleted MNCs was processed for fluorescence-activated cell sorting (FACS). Cells were incubated with the following antibodies: CD45–allophycocyanin (APC)–cy7 (BD Biosciences), CD34-APC (BD Biosciences), and CD146–fluorescein isothiocyanate (FITC; AbD Serotec). The viability dye 4,6 diamidino-2-phenylindole (DAPI; Sigma) was added before sorting, on a FACSAria III (BD Biosciences), DAPI$^-$CD45$^-$CD34$^-$CD146$^+$ perivascular cells, or DAPI$^-$CD45$^-$CD34$^+$CD146$^-$ cells, as previously described.[29,38] In some experiments, CD146$^-$ cells were purified from cultured MSCs.

For the animal studies, an animal care and use committee protocol (ARC no. 2008-175-11) was approved for the injection of human cells into immunodeficient mice and for the analysis of engraftment of transplanted cells.

Isolation of human CD34$^+$ cells from CB

Umbilical cord blood (CB) was collected from normal deliveries without individually identifiable information, therefore no institutional review board approval was required. MNCs were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Enrichment of CD34$^+$ cells was then performed using the magnetic-activated cell sorting system (Miltenyi Biotec) as per the manufacturer's instructions.

Immunophenotype analysis of stromal cells

Cultured MSCs, CD146$^+$ cells, and CD146$^-$ cells (between passages 3 and 10) were analyzed on an LSR II flow cytometer (Becton Dickinson). Cells were stained with monoclonal antibodies: CD146-FITC (AbD Serotec), CD31-APC (Biolegend), CD44–phycoerythrin (PE), CD73-PEcy7, CD105-PE, CD90-APC, and CD45-FITC (all from BD Biosciences). Unstained samples were used as negative controls. Data were analyzed using FlowJo software (Tree Star).

Mesodermal lineage differentiation assays

The ability of cells to differentiate into mesodermal lineages was tested in osteogenic or adipogenic differentiation medium (Hyclone). After 3 weeks of culture in differentiation conditions, cells were stained with Alizarin red or Oil red O (Sigma) for the detection of mineral deposits or lipids as previously described.[29]

Quantitative RT-PCR

Five hundred thousand cultured cells were processed for RNA extraction using a Qiagen micro kit. An Omniscript reverse transcriptase (RT) kit was used to make complementary DNA, which was subjected to quantitative polymerase chain reaction (qPCR) using Sybr green probe–based gene expression analysis (Applied Biosystems) for 2 housekeeping genes, TBP and GAPDH, and the target genes CD146, nestin, α-SMA, and NG2. A 7500 real-time PCR system was used (ABI). Data were analyzed using the comparative C(T) method.

Western blotting

Cells were lysed in denaturing cell extraction buffer (Invitrogen) containing protease inhibitor tablets (Roche). Proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed using the XCell II western blot system (Invitrogen). Rat anti-human Jagged-1 (Abcam, 1:50) and monoclonal mouse anti-β actin (Sigma, 1:5000) antibodies were used. Donkey anti-rat horseradish peroxidase (HRP) and donkey anti-mouse HRP (Jackson Immunoresearch Inc; 1:5000) were used as secondary antibodies. The blots were developed using ECL Plus Western Blotting Substrate (Pierce).

Coculture of stromal cells and CB CD34$^+$ cells

Cultured stromal cells (between passages 3 and 8) were irradiated (20 Gy) and plated on 96 multi-well plates at 1.5×10^4 cells per well. Twenty-four hours later, CB CD34$^+$ cells ($5\text{-}7 \times 10^4$ per well) were plated on top of the stromal layer. Stroma-free cultures were performed seeding CB CD34$^+$ cells on recombinant retronectin (RN; Lonza) coated wells. Cocultures were performed in RPMI 1640, 5% fetal bovine serum, 1× penicillin/streptavidin. No supplemental cytokines were ever added. Cells were harvested after 1, 2, 4, and 6 weeks. Cocultures in the absence of cell-to-cell contact were performed in 96 multi-well transwell plates (Corning). For the inhibition of Notch, 10μM DAPT (N-[N-(3, 5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester; Sigma) or 10 μg/mL of anti-human Notch 1 neutralizing antibody (Biolegend) were added to each well every 48 hours. An equal volume of dimethylsulfoxide (DMSO; Sigma) or an equal concentration of mouse unrelated IgG (Biolegend) was added to wells as negative controls for DAPT and anti-Notch1 antibody, respectively.

Flow cytometric analysis of cultured CB CD34$^+$ cells

After 1, 2, 4, and 6 weeks of coculture, cells were harvested and stained with the following antibodies: CD45-APC-cy7, CD34-PE-cy7, CD14-APC, CD10-APC, CD33-PE, CD19-FITC (all from BD Biosciences). Dead cells were identified with propidium iodide (PI; BD Biosciences).

Colony forming unit assay

After 1, 2, 4 and 6 weeks of coculture cells were harvested and 2.5×10^3 cells were plated in methylcellulose (Methocult; Stem Cell Technologies). Colonies, here reported as the sum of the progeny of colony forming unit (CFU) granulo-macrophage, burst-forming unit erythroid, and CFU mixed, were scored after 14 days.

In vivo repopulation assay

CB CD34$^+$ cells were cocultured with MSCs or CD146$^+$ cells for 2 weeks in RPMI 1640, 5% fetal bovine serum, 1× penicillin/streptavidin. An equal number of CD45$^+$ cells (10^5) obtained from the cocultures was intratibially injected in sublethally irradiated (250 cGy), 6- to 8-week-old NSG mice (The Jackson Laboratory). Mice were sacrificed 6 weeks posttransplantation. Engraftment of human hematopoietic cells was evaluated by FACS analysis after staining with anti-human specific monoclonal antibodies: CD45-APC-cy7, HLA (A/B/C)–PE, CD34-PE-cy-7, CD19-FITC, CD14-APC, CD15-APC, CD33-APC (all from BD Biosciences). For secondary transplantation, bone marrows from 2 engrafted mice were pooled and intratibially injected into a secondary host (n = 4). Engraftment was evaluated 4 weeks posttransplantation.

Immunocytochemistry and immunohistochemistry

For immunofluorescence analysis, human adipose tissue-frozen sections, cells cultured in chamber slides (Millipore) or cytospun on microscope slides, were fixed with cold methanol/acetone (1:1) for 5 minutes at room temperature prior to incubation with blocking solution (phosphate-buffered saline 5% donkey serum) for 1 hour at room temperature. Overnight incubation at 4°C was performed with unconjugated primary antibodies: mouse anti-human CD146 (BD Biosciences), mouse anti-human CD45 (eBioscience), rat anti-human Jagged-1, rabbit anti-human N1ICD, mouse anti-human nestin, rabbit anti-human CXCL12, rabbit anti-human Lep-R, rabbit anti-human CD146 (all from Abcam). Tissue sections or cells were incubated for 2 hours at room temperature with FITC-conjugated mouse anti-human von Willebrand factor (VWF; US Biological). Tissue sections or cells were incubated for 1 hour at room temperature with the following conjugated antibodies: donkey anti-rabbit–Alexa 488, donkey anti-rabbit– Alexa 647, donkey anti-rat–Alexa 594 or donkey anti-mouse–Alexa 594 (all from Jackson Immunoresearch Inc). For immunohistochemistry on human FBM, fetal bones (16-18 weeks of pregnancy) were fixed in 4% paraformaldehyde (Sigma-Aldrich). Fixed tissues were embedded in paraffin and sections were stained with the same antibodies against nestin, CXCL12, Lep-R, and CD146. Secondary HRP-conjugated IMPRESS anti-rabbit and anti-mouse antibodies and 3, 3′-diaminobenzidine (Vector Laboratories) were used for revelation. As negative controls, tissue sections or cells were incubated only with secondary antibodies. Images were acquired on an Axiovision microscope (Carl Zeiss; software version 4.8) equipped with ApoTome.2 modules for Axio Imager.2 and Axio Observer, with 10×, 20×, 40×, and 63× (1.4 NA) objectives.

Statistical analysis

Mean and SDs were used to summarize continuous variables. Bivariate cross-sectional comparisons of continuous variables were performed using paired *t* tests. Continuous outcomes such as total numbers of CD45$^+$ and CD34$^+$ cells, frequency of CD34$^+$Lin$^-$ cells and CFUs were collected over time. The experimental design involved 2 within-experiment factors, MSCs and pericytes, and time (week 1, 2, 4, 6), which corresponded to a strip-plot design. A mixed-model approach was used. Within the mixed-model framework, we performed hypothesis testing for the comparison of MSCs and pericytes at different time points. Pearson's correlation (r) was reported to assess the linear correlation between CD34$^+$lin$^-$ cells and CFUs. For the qPCR data, ΔCT values were calculated for each marker. A randomized block design model was fitted on ΔCT values. Donors were treated as random effects while stromal cells groups were treated as fixed effects. For all statistical investigations, tests for significance were 2-tailed. To account for type I error inflation due to multiple comparisons, *P* values were adjusted by Bonferroni correction. The Fisher exact test was performed to compare engraftment and not engraftment ability. Statistically significant threshold of *P* value was set at .05. Statistical analyses were carried out using SAS version 9.2 (SAS Institute).

Results

Human CD146$^+$ perivascular cells express nestin, CXCL12, and Lep-R in hematopoietic and nonhematopoietic tissues

Recent studies have described murine perivascular cells as key players for the maintenance of HSPCs. Perivascular niche cells, displaying MSC features, have been identified based on the expression of CXCL12,[34] nestin,[36] and Lep-R.[37] We have previously demonstrated that pericytes, surrounding microvessels, and capillaries, can be detected in multiple human tissues on expression of CD146.[29] Consistent with our previous findings, immunohistochemistry performed on human FBM revealed the presence of CD146-expressing perivascular cells (Figure 1A). Nestin, CXCL12, and Lep-R, markers of the perivascular niche previously described in murine studies, were also expressed in human perivascular cells in FBM (Figure 1B-D). We further investigated the expression of the same stromal cell markers in human adult adipose tissue, considered as an abundant source of MSCs and recently suggested to also be a reservoir of HSCs.[39] Nestin, CXCL12, and Lep-R were all expressed in cells immediately adjacent to VWF-positive endothelial cells (Figure 1E-G). Multicolor immunofluorescence showed that CD146$^+$ pericytes, surrounding microvessels and capillaries, coexpress nestin, CXCL12, and Lep-R (Figure 1H-S). Thus, human CD146$^+$ perivascular cells express in situ markers previously identified in murine studies to mark the perivascular hematopoietic niche.

Purified and ex vivo–expanded CD146$^+$ perivascular cells maintain expression of markers of the perivascular niche

We then analyzed the expression of the perivascular niche markers in purified and ex vivo–expanded CD146$^+$ perivascular cells as compared with unfractionated MSCs and CD146$^-$ cells. MSCs were conventionally derived from the adipose tissue SVF by plastic adherence, while CD146$^+$ perivascular cells and CD146$^-$ cells were purified by FACS as previously described (Figure 2A).[29,38] CD146$^+$ perivascular cells demonstrated expression of cell-surface markers typical of unfractionated cultured MSCs, such as CD44, CD105, CD73, and CD90 and did not express the hematopoietic and endothelial cell markers CD45 and CD31 (supplemental Figure 1a-b, available on the *Blood* website). Also similar to unfractionated MSCs, cultured CD146$^+$ cells were able to differentiate into osteoblasts and adipocytes in culture (supplemental Figure 1c-f). CD146$^+$ perivascular cells retained uniform CD146 expression in culture, as did a small fraction of MSCs, while CD146$^-$ cells remained negative for CD146 expression in culture (Figure 2B). Quantitative RT-PCR analysis of established cultures confirmed that CD146$^+$ cells expressed higher levels of the perivascular cell markers CD146, α-SMA, NG2, and nestin than did either unfractionated MSCs or CD146$^-$ cells derived from fat (Figure 3A) or FBM (Figure 3B). Furthermore, immunocytochemistry demonstrated that cultured CD146$^+$ perivascular cells isolated from fat or FBM express higher levels of nestin and CXCL12 than CD146$^-$ cells do. No significant difference in the expression of Lep-R was observed between cultured CD146$^+$ and CD146$^-$ cells (Figure 3C-N).

CD146$^+$ perivascular cells support HSPCs ex vivo

The ability of distinct stromal cells to support HSPCs ex vivo was assessed by coculturing CB-derived CD34$^+$ cells (CB CD34$^+$) in direct contact with either CD146$^+$ perivascular cells, unfractionated MSCs, or CD146$^-$ cells all obtained from both lipoaspirate

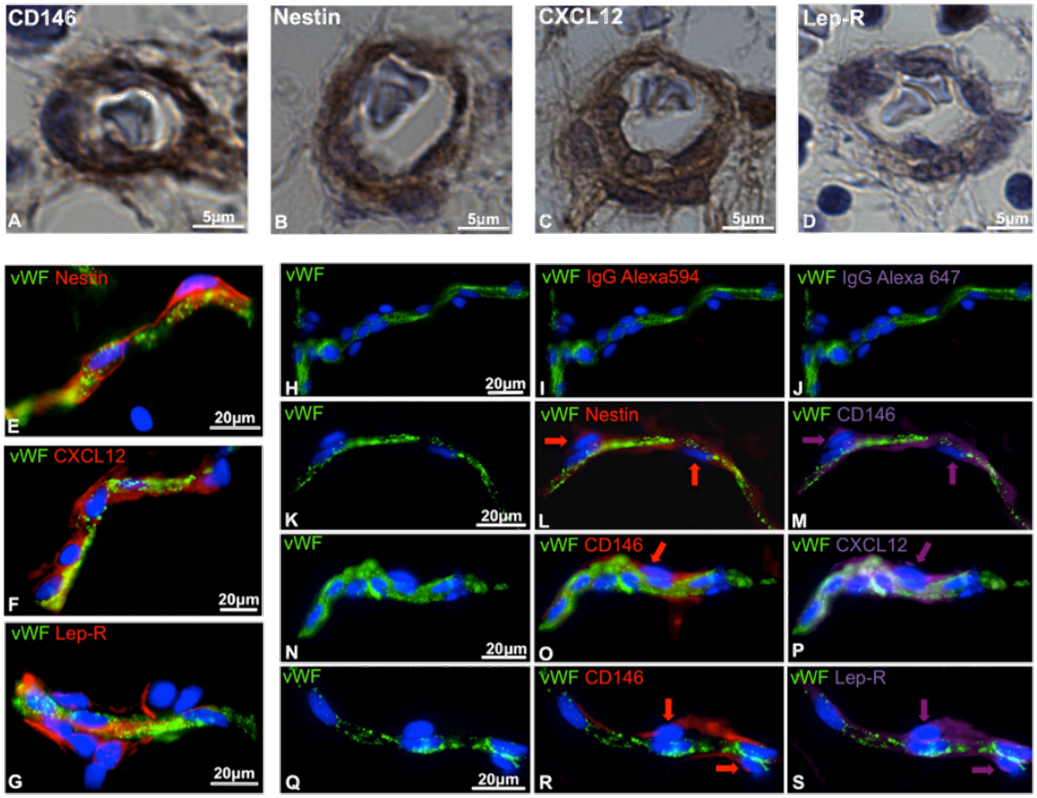

Figure 1. In situ expression of hematopoietic niche markers by human perivascular cells. (A-D) Immunohistochemistry performed on paraffin-embedded sections of 17-week-old human FBM. Pericytes surrounding microvessels express (A) CD146, (B) nestin, (C) CXCL12, and (D) leptin receptor (Lep-R) (original magnification, ×63). (E-S) Immunohistochemistry performed on human adipose tissue cryosections. (E-G) VWF-positive endothelial cells (green) are surrounded by perivascular cells expressing (E) nestin, (F) CXCL12, and (G) Lep-R. (H-S) Triple-staining immunohistochemistry performed on human adipose tissue cryosections shows coexpression of CD146 with (K-M) nestin, (N-P) CXCL12, and (Q-S) LepR. (H-J) Single staining with anti-VWF antibody followed by incubation with conjugated IgG controls revealed the lack of autofluorescence (original magnification, ×40). IgG, immunoglobulin G.

specimens and FBM. These cultures were performed in basal medium with a low concentration of serum (5%) and in the absence of any supplemental cytokines, so that the specific effect of each stromal cell subset could be assessed with minimal influence of exogenous factors. In the absence of any stromal cells or cytokines, hematopoietic cells cultured on RN died within the first 2 weeks, whereas $CD45^+$ cells survived for up to 6 weeks in the presence of either MSCs or $CD146^+$ perivascular cells (Figure 4A). The total number of $CD45^+$ cells recovered from $CD146^+$ cell cocultures remained significantly higher at any time of culture when compared with MSC cocultures (Figure 4A). A similar pattern was observed for the total number of $CD34^+$ cells (Figure 4B). CD34 expression identifies human hematopoietic cells without discriminating between HSCs and lineage-committed progenitors. The most immature progenitors present in cocultures were further defined as $CD34^+Lin^-$ cells based on expression of CD34 and lack of the early myeloid cell marker CD33 and lymphoid cell markers CD10 and CD19. $CD146^+$ cell cocultures contained a significantly higher frequency and number of $CD34^+Lin^-$ cells at all time points (Figure 4C-D). Consistent with these findings, culture in the presence of MSCs resulted in accelerated differentiation of CB $CD34^+$ cells into $CD14^+$ myeloid cells and $CD10/CD19^+$ lymphoid cells, relative to coculture with $CD146^+$ cells (Figure 4E-F). The increased frequency of myeloid and lymphoid cells was counterbalanced by the lower numbers of $CD45^+$ cells in MSC cocultures, hence no significant difference in the absolute numbers of myeloid or lymphoid cells was observed (Figure 4E-F). Furthermore, the number of clonogenic cells detected after 1, 2, 4, and 6 weeks was significantly higher when CB $CD34^+$ cells were cocultured with $CD146^+$ perivascular cells compared with MSCs (Figure 5A).

$CD146^+$ perivascular cells isolated from either FBM or adipose tissue sustained significantly more $CD34^+Lin^-$ cells and CFUs from CB $CD34^+$ cells than $CD146^-$ stromal cells did (supplemental Figure 2a-d), thus confirming that within the heterogeneous MSC population, the ability to support HSPCs is confined to the subset of $CD146^+$ perivascular cells, regardless of the tissue of origin.

$CD146^+$ perivascular cells maintain human HSPCs with repopulating ability and self-renewal potential

We next investigated whether coculture with MSCs or $CD146^+$ perivascular cells retains functional HSPCs. Sublethally irradiated

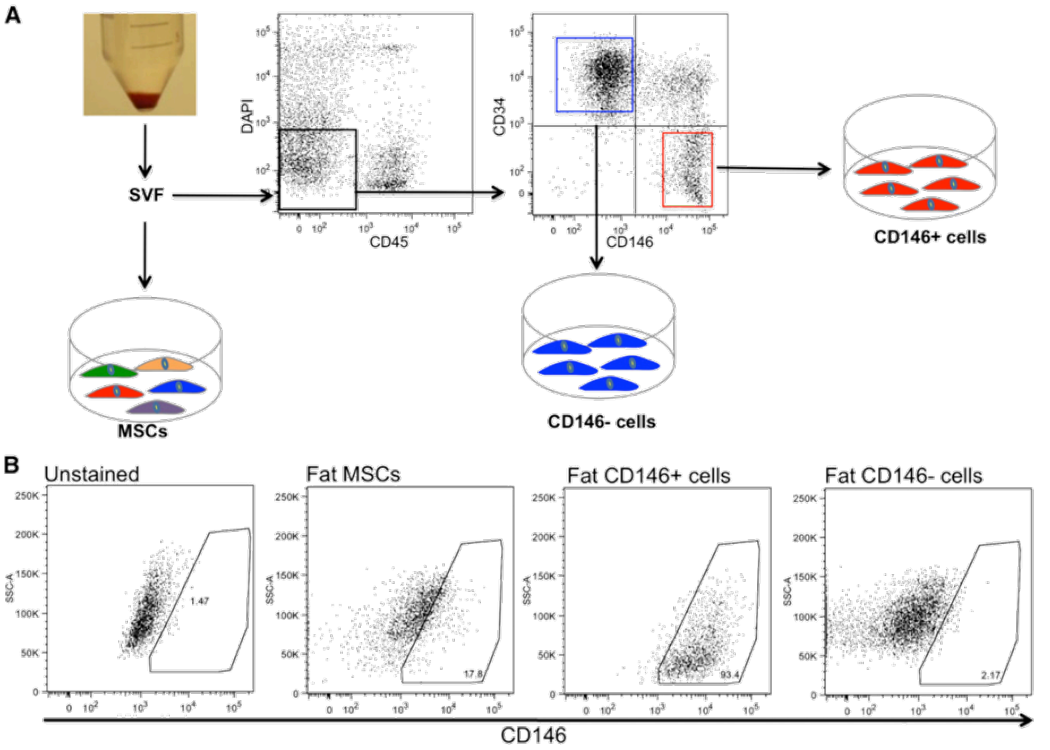

Figure 2. Isolation and culture of MSCs and stromal subsets from lipoaspirate. (A) SVF was obtained from human lipoaspirate specimens (n = 4 donors). An aliquot of SVF was directly seeded in tissue-culture plates for the isolation of conventional MSCs by plastic adherence. Another aliquot of SVF was processed for FACS sorting of DAPI$^-$CD45$^-$CD34$^-$CD146$^+$ perivascular cells and DAPI$^-$CD45$^-$CD34$^+$CD146$^-$ cells. (B) FACS analysis of cultured fat-derived MSCs, CD146$^+$ perivascular cells, and CD146$^-$ cells. After 9 passages in culture, MSCs retain a low percentage of CD146$^+$ cells, while purified CD146$^+$ perivascular cells and CD146$^-$ cells retain a stable phenotype homogeneously positive and negative for CD146, respectively.

NOD/SCID/IL-2 receptor γ-chain null (NSG) mice were injected with hematopoietic cells cocultured with CD146$^+$ perivascular cells or MSCs for 2 weeks in low-serum concentration without added cytokines. Strikingly, all mice transplanted with hematopoietic cells cocultured with perivascular cells exhibited human hematopoietic cell engraftment 6 weeks posttransplantation, whereas no engraftment was observed in any of the mice transplanted with hematopoietic cells cocultured with MSCs (n = 11 mice per group, n = 3 individual experiments; Fisher exact test, $P < .0001$) (Figure 5B-C). Human CD34$^+$ progenitors, CD19$^+$ lymphoid cells and CD14$^+$ myeloid cells were detected in the chimeric mice (Figure 5D). Human CD45$^+$HLA$^+$ cells were not only detected in the medullary site of injection, but also in the contralateral tibia, thus suggesting that HSPCs cocultured with CD146$^+$ perivascular cells maintained the ability to migrate and home to distant sites after initial engraftment (Figure 5E). To assess the self-renewal potential of HSPCs cultured in the presence of CD146$^+$ perivascular cells, bone marrow from chimeric mice was transplanted into secondary NSG mouse hosts. Lymphoid and myeloid engraftment of human cells was still detectable in secondary hosts (Figure 5F-I), demonstrating that the CD146$^+$ cell fraction of MSCs is uniquely able to sustain human HSPCs with multilineage repopulating capacity and self-renewal ability.

Contact with CD146$^+$ cells is required for HSPC maintenance

In addition to the phenotypic and functional differences described above (Figures 2 and 3), a different morphology and spatial distribution was observed between hematopoietic cells cocultured with MSCs or CD146$^+$ perivascular cells. When CD146$^+$ cells were used as a feeder layer, hematopoietic cells appeared small, rounded, and clustered (supplemental Figure 3a). In the presence of MSCs, hematopoietic cells were larger, less uniform in size, and scattered throughout the cultures, consistent with more vigorous hematopoietic differentiation (supplemental Figure 3c). Immunocytochemical analysis confirmed the presence of clusters of CD34$^+$ cells in contact with underlying CD146$^+$ cells in perivascular cell cocultures but not in MSC cocultures (supplemental Figure 3b,d). Based on these observations, we next investigated the role of cell-to-cell contact on HSPC maintenance. When direct contact between CD146$^+$ cells and CB CD34$^+$ cells was prevented in a transwell culture system, the total number of CD45$^+$ cells was dramatically reduced after 1 week of coculture (supplemental Figure 3e). In these noncontact conditions, hematopoietic cells were barely detectable after 2 weeks and the limited number of cells recovered did not allow us to perform further immunophenotypic or functional analyses.

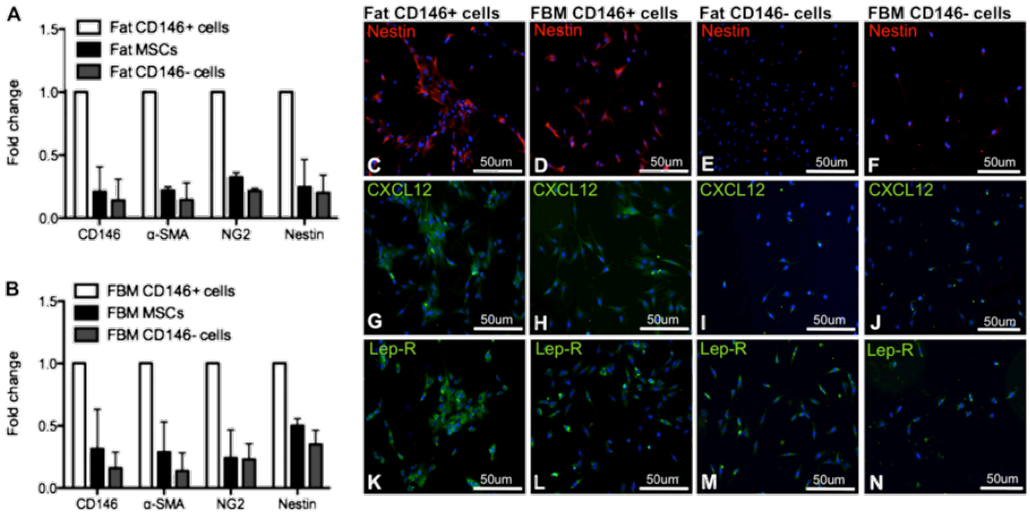

Figure 3. Cultured CD146$^+$ perivascular cells express markers of hematopoietic perivascular niche cells. (A-B) Ex vivo–expanded CD146$^+$ perivascular cells purified from fat and FBM similarly express higher levels of mRNA of perivascular cell markers when compared with MSCs and CD146$^-$ cells (n = 2 donors for each tissue). (C-N) Fat and FBM-derived CD146$^+$ perivascular cells similarly and almost exclusively express (C-F) nestin and (G-J) CXCL12 in culture compared with CD146$^-$ cells. (K-N) No difference in Lep-R expression was observed between CD146$^+$ and CD146$^-$ cells from either fat and FBM (original magnification, ×20). mRNA, messenger RNA.

CD146$^+$ perivascular cells express Notch ligands and activate Notch in hematopoietic cells

Transwell culture experiments suggested that CD146$^+$ perivascular cells sustain hematopoietic cells through cell-to-cell contact rather than by secretion of soluble factors. As Notch signaling is one of the key pathways through which the microenvironment affects growth and differentiation of HSPCs during development,[40] we investigated whether CD146$^+$ perivascular cells sustain HSPCs through the activation of Notch. Immunocytochemistry revealed that all cultured CD146$^+$ perivascular cells express high levels of the Notch ligand Jagged-1. In contrast, only rare MSCs expressed Jagged-1, the majority of which also expressed CD146 (Figure 6A). Western blot analysis detected Jagged-1 expression at high levels in CD146$^+$ perivascular cells compared with unfractionated MSCs (Figure 6B). Expression of other Notch ligands (Jagged-2, DLL-1, and DLL-4) was also detected by qPCR in MSCs, albeit at a lower level compared with CD146$^+$ perivascular cells (Figure 6C).

We used an antibody recognizing an epitope exclusively exposed after Notch 1 receptor cleavage (NICD) to measure the frequency of hematopoietic cells activating Notch in the presence of CD146$^+$ cells or MSCs (supplemental Figure 4a-b). As expected, Notch activation was not observed when direct contact between CD146$^+$ cells and hematopoietic cells was inhibited in transwell cocultures (supplemental Figure 4c-d). MSCs, which express all 4 Notch ligands tested, were able to activate Notch1 in ~50% of hematopoietic cells and progenitors (Figure 6D-E). The percentage of NICD$^+$CD45$^+$ cells was significantly higher in CD146$^+$ cell cocultures than in cocultures with total MSCs or CD146$^-$ cells, regardless of the tissue of origin (FBM or fat) (Figure 6D). Furthermore, Notch activation was significantly stronger in CD34$^+$ progenitors cocultured with CD146$^+$ cells compared with those cocultured with MSCs or CD146$^-$ cells (Figure 6E).

Notch inhibition in CD146$^+$ cell/HSPC cocultures reduces progenitor cell numbers and stimulates B-cell differentiation

To further assess the functional role of Notch activation in HSPCs, CB CD34$^+$ cells, and CD146$^+$ perivascular cells were cocultured in the presence of the γ-secretase inhibitor DAPT. Notch inhibition resulted in significantly reduced total number of CD45$^+$ cells, CD34$^+$Lin$^-$ cells, and CFUs compared with control cocultures performed in the presence of the DMSO solvent alone (Figure 7A-C). A significantly higher frequency of PI$^+$ dead cells was measured after Notch inhibition (Figure 7D). Of note, the frequency of PI$^+$ cells was not increased when CD146$^+$ perivascular cells or CB CD34$^+$ cells were treated separately with DAPT in the absence of supplemental cytokines, thus excluding nonspecific cytotoxicity from DAPT (supplemental Figure 5a). Notch inhibition also resulted in a dramatic increase in B-cell differentiation (Figure 7E). A comparable decrease in output of total CD34$^+$Lin$^-$ cells and increase in B-lymphoid cells (supplemental Figure 5b-d) was also observed when cocultures were performed in the presence of an antibody to specifically block the Notch-1 receptor. However, the effect was less pronounced when compared with DAPT treatment. To explain this difference, we determined the levels of Notch inhibition following DAPT or anti-Notch-1 blocking antibody treatment. Although Notch activation was totally abrogated by DAPT, low-level activation was still detected in a few cells after antibody treatment, confirming that the latter treatment is less efficient than chemical inactivation of Notch (supplemental Figure 4e-g).

Altogether, these results show that CD146$^+$ perivascular cells are a subset of MSCs able to support HSPCs and regulate lineage

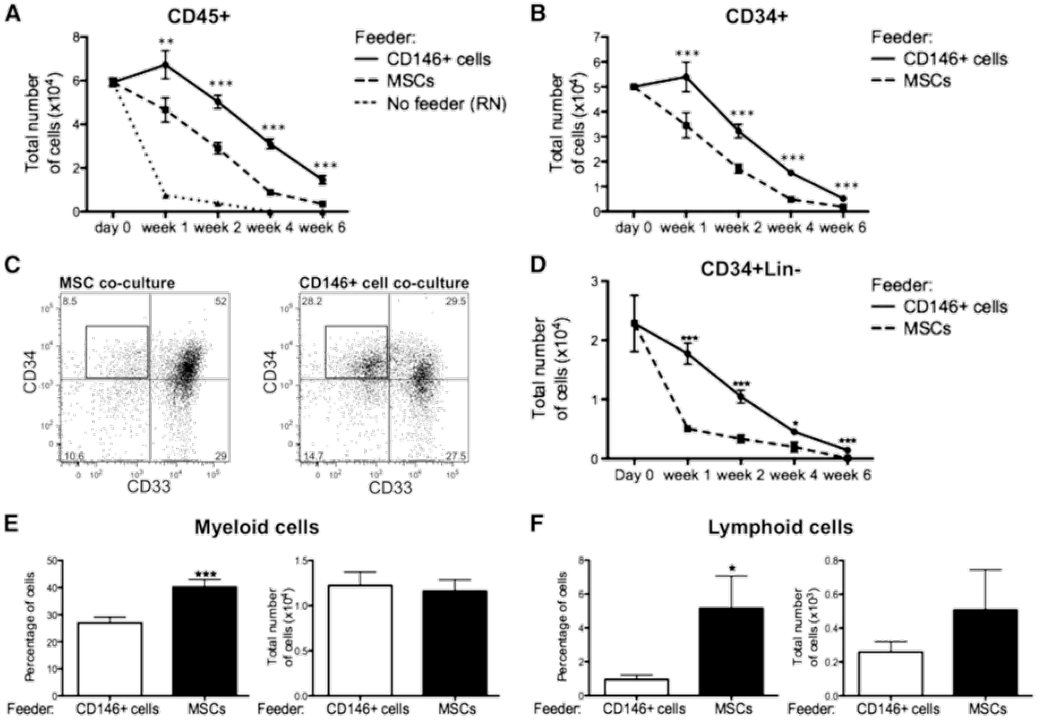

Figure 4. CD146$^+$ perivascular cells promote ex vivo maintenance of undifferentiated HSPCs. (A) In the absence of cytokines and stromal cell feeder layer (No feeder), CD45$^+$ hematopoietic cells cultured in RN-treated wells rapidly died within the first 2 weeks of culture. At any time of culture, the total number of CD45$^+$ cells recovered from CD146$^+$ cell cocultures was significantly higher when compared with MSC cocultures (n = at least 5 independent experiments for each time point, each experiment was performed in triplicate; $^{**}P < .01$, $^{***}P < .001$). (B) A similar pattern was observed for the total number of CD34$^+$ cells (n = at least 5 independent experiments for each time point, each experiment was performed in triplicate; $^{***}P < .001$). (C) Representative FACS analysis after 2 weeks of coculture of CB CD34$^+$ cells with MSCs or CD146$^+$ cell cocultures. After gating on CD45$^+$CD10$^-$CD19$^-$ cells, CD34$^+$33$^-$ cells were defined as CD34$^+$Lin$^-$ cells (black box). (D) The absolute number of CD34$^+$Lin$^-$ cells was significantly higher in CD146$^+$ cell cocultures, compared with MSC cocultures, at any time of culture (n=at least 5 independent experiments for each time point, each experiment was performed in triplicate; $^{**}P < .01$, $^{***}P < .001$). (E-F) Coculture of CB CD34$^+$ cells with MSCs led to a significantly higher frequency of CD14$^+$ myeloid cells after 2 weeks (E) (40.24% ± 2.723% vs 26.67% ± 2.075%. n = 10 independent experiments, each experiment was performed in triplicate; $^{***}P < .0001$) and a higher frequency of CD10$^+$/CD19$^+$ lymphoid progenitors or mature cells after 4 weeks of coculture (F) (5.155% ± 1.918% vs 0.9541% ± 0.2564%, n = 8 independent experiments, each experiment was performed in triplicate; $^{*}P < .05$). No difference in the absolute numbers of myeloid and lymphoid cells was observed between CD146$^+$ cell and MSC cocultures. All data are presented as mean ± SEM.

commitment in vitro through cell-to-cell interaction and partially through Notch activation.

Discussion

Blood formation in vertebrates is an opportunistic phenomenon that does not take place exclusively in specialized, hematopoiesis-restricted sites such as the bone marrow, thymus, spleen and avian bursa of Fabricius. Blood cells are also produced transiently in organs assuming other functions, such as the yolk sac, placenta, allantois and embryonic aorta-gonad-mesonephros, and liver. Moreover, extramedullary hematopoiesis can be resumed in pathologic conditions of the adult. Such anatomic diversity in blood-forming ability implies that developmentally and structurally distinct cellular environments can sustain hematopoiesis. Different blood-forming tissues may therefore share stromal cell subsets involved in blood formation. Although HSPCs have been characterized in detail and purified to homogeneity, the identity and function of the stromal cells involved in hematopoiesis have remained largely unknown. Although stroma-dependent hematopoiesis has been recapitulated in vitro for more than 3 decades using primary stromal cells or stromal cell lines,[9-11] the nature of the stromal cells involved has been elusive. As an obstacle to characterization, native stromal cells involved in supporting hematopoiesis are infrequent: Wineman et al[41] found that only a rare subpopulation of clonal fetal liver stromal cells is able to maintain HSPCs.

MSCs are cultured, multipotent adherent cells that can support hematopoiesis.[19-24] We hypothesized that MSCs contain distinct subsets of cells with different roles in the regulation of HSPCs. Based on recent descriptions of (1) a key contribution of murine perivascular cells to the medullary hematopoietic "niche,"[35-37] and (2) a pericyte ancestry for human MSCs,[29] we directly addressed whether cultured human perivascular cells can sustain human HSPCs. Conventionally derived, heterogeneous MSCs and CD146$^+$CD34$^-$CD45$^-$ perivascular cells can be obtained from virtually all human vascularized tissues.[29] In the present study, we derived MSCs and CD146$^+$ perivascular cells from FBM and human adipose tissue, which is commonly used as a convenient

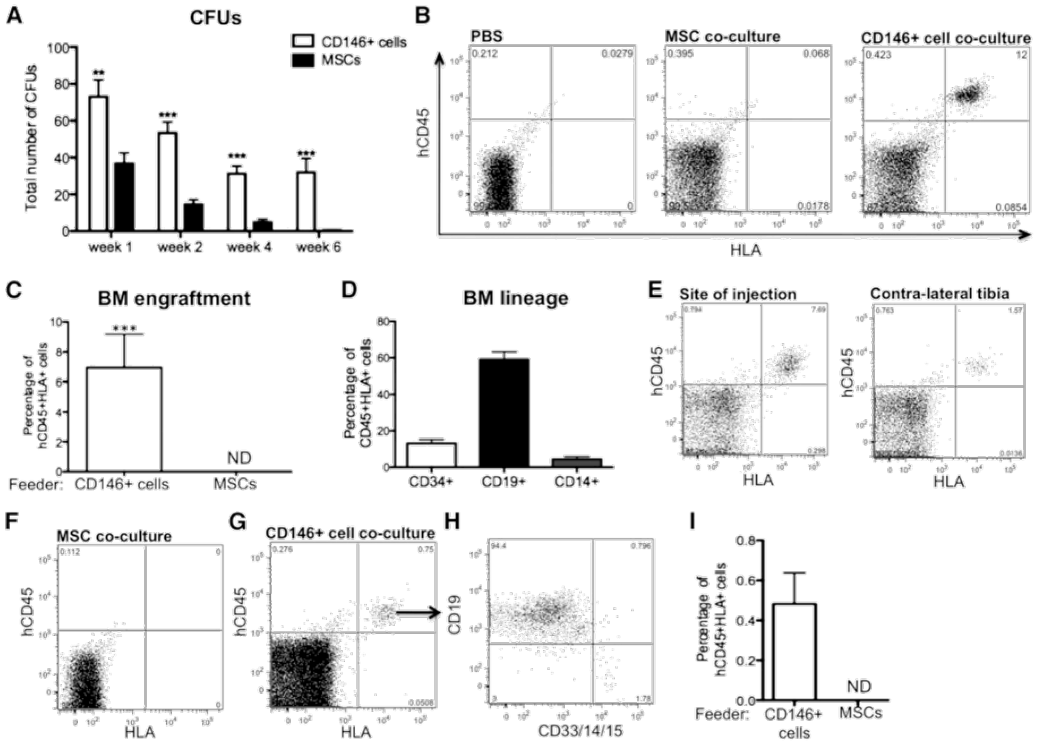

Figure 5. CD146$^+$ perivascular cells but not MSCs sustain functional HSPCs with engraftment potential and self-renewal ability. (A) CFU assay revealed significantly higher number of CFUs in CD146$^+$ cell cocultures after 1, 2, 4, and 6 weeks of coculture as compared with MSC cocultures (n = 3 independent experiments, each experiment was performed in triplicate; $^*P < .05$, $^{***}P < .001$). (B) Representative flow cytometry analysis for the detection of human CD45$^+$HLA$^+$ cells in bone marrow of NSG mice 6 weeks posttransplantation with phosphate-buffered saline, or with the same number of CD45$^+$ cells (10^5) harvested after 2 weeks of CB CD34$^+$ cell coculture with MSCs or CD146$^+$ cells. (C) All mice injected with CD45$^+$ cells obtained from CD146$^+$ cell cocultures showed human engraftment whereas no engraftment was ever detected (ND) in mice that received MSC cocultures (n = 3 independent experiments, n = 11 mice per group; $^{***}P < .0001$). (D) Frequency of CD34$^+$ progenitors, CD19$^+$ lymphoid, and CD14$^+$ myeloid cells within the CD45$^+$HLA$^+$ population of cells in the bone marrow of chimeric mice. (E) Human CD45$^+$HLA$^+$ hematopoietic cells were also detected 6 weeks posttransplantation in the contralateral tibia of mice injected with HSPCs cocultured with CD146$^+$ perivascular cells. (F-I) Representative flow cytometry analysis of secondary host bone marrow. (F) Bone marrow from primary hosts transplanted with MSC coculture was injected in secondary hosts as a negative control. (G) Human engraftment was observed 4 weeks after secondary transplantation of bone marrow from chimeric mice transplanted with CD146$^+$ cell coculture (n = 3 engrafted mice of 4). (H) Both CD19$^+$ lymphoid and CD33/CD14/CD15$^+$ myeloid cells were detectable within the human CD45$^+$ engrafted hematopoietic cells in secondary hosts. (I) Quantification of the level of chimerism in secondary mice. All data are presented as mean ± SEM.

and abundant source of MSCs. Interestingly, the sustained presence of hematopoietic cells within adipose tissue has been recently reported.[42,43]

CD146$^+$ perivascular cells expressing nestin, CXCL12, and Lep-R were found in situ in the hematopoietic FBM as well as in adipose tissue. Sorted CD146$^+$ perivascular cells homogeneously expressed in culture CD146 and higher levels of nestin, CXCL12, and Jagged-1 compared with unfractionated MSCs or to CD146$^-$ cells. CD146$^+$ perivascular cells therefore appear to represent the human counterpart of the CAR cells or nestin$^+$ cells recently described in the mouse.[35-37] A similar cell population has been documented in human bone marrow, where CD146$^+$ perivascular cells expressing CXCL12 and Jagged-1 can clonally recapitulate an ectopic hematopoietic microenvironment when implanted into mice.[44] Human bone marrow reticular stromal cells, including CD146$^+$nestin$^+$VCAM$^+$ cells, regulate HSPC homing through the secretion of CXCL12.[45] Pericyte-like cells from the human placenta have been also suggested to support hematopoietic cells in culture.[46] However, direct evidence for the ability of prospectively purified human perivascular cells to sustain primitive hematopoietic cells in long-term culture has not been provided. Several studies have investigated the ability of MSCs to maintain HSPCs in coculture systems, but these have routinely used cytokine supplementation either by direct addition or through transgene expression in MSCs.[19-24,47,48] In most cases the decisive assays, primary and secondary transplantations of cocultured hematopoietic cells into immunodeficient mice, have not been used to document the maintenance of primitive self-renewing stem cells. Most importantly, the identity of the specific subset of MSCs directly involved in the interaction with HSPCs is still unknown. In the present study, culture of CD34$^+$ cells with MSCs or CD146$^+$ perivascular cells without the addition of exogenous cytokines allowed us to define the intrinsic properties of these stromal populations in terms of hematopoietic cell support. Remarkably, unfractionated MSCs and purified CD146$^+$ perivascular cells derived from the same specimen exhibited profound differences in the ability to sustain HSPCs. Both stromal cell populations improved the survival of hematopoietic cells compared with stroma-free, cytokine-free cultures.

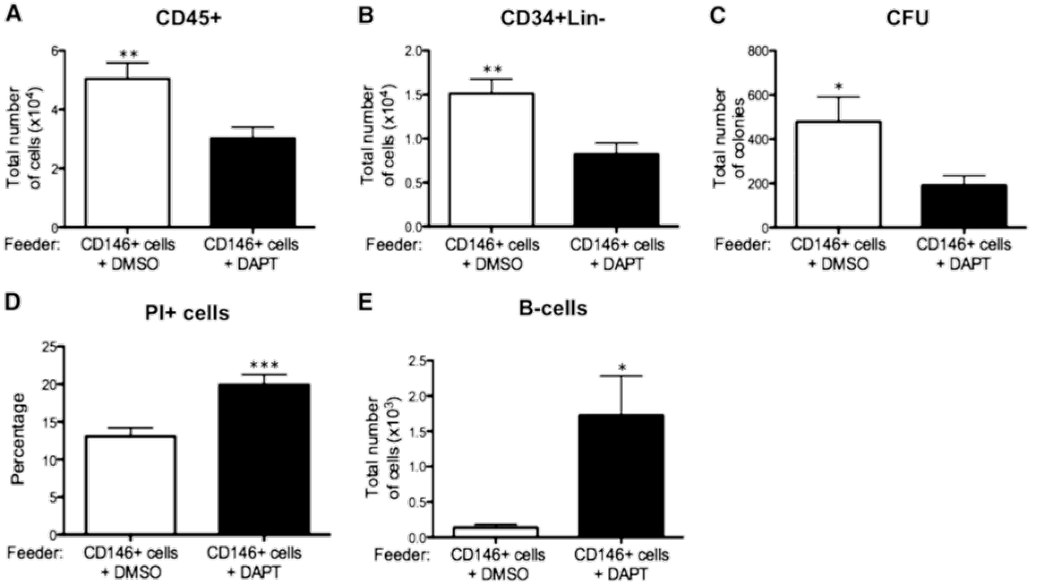

Figure 7. Notch inhibition affects survival and B-cell differentiation of HSPCs. Inhibition of Notch was achieved by addition of 10μM DAPT to CD146$^+$ perivascular cells and CB CD34$^+$ cell coculture every other day. Vehicle (DMSO) was added to control cocultures. (A-B) Total number of CD45$^+$ cells and CD34$^+$Lin$^-$ cells was significantly reduced after 2 weeks of coculture with DAPT (5.03 ± 0.54 × 10^4 vs 3.02 ± 0.37 × 10^4 CD45$^+$ cells, n = 4 independent experiments, each experiment was performed in triplicate, $^{**}P < .01$; 1.5 ± 0.16 × 10^4 vs 0.82 ± 0.12 × 10^4 CD34$^+$Lin$^-$ cells, n = 4 independent experiments, each experiment was performed in triplicate, $^{**}P < .01$). (C) Similarly, the total number of CFUs was significantly reduced after 4 weeks of coculture with DAPT (478.3 ± 112.4 vs 191.0 ± 43.28, n = 3 independent experiments, each experiment was performed in triplicate; $^{*}P < .05$). (D) Flow cytometry viability analysis revealed a significantly higher frequency of PI$^+$ dead cells in coculture performed in the presence of DAPT (13.08% ± 1.13% vs 19.94 ± 1.31, n = 4 independent experiments, each experiment was performed in triplicate, $^{***}P < .0001$). (E) Notch inhibition also significantly increased B-cell development (0.13 ± 0.04 × 10^3 vs 1.72 ± 0.55 × 10^3 of lymphoid cells, n = 3 individual experiments, each experiment performed in triplicate; $^{**}P < .01$). Data are presented as mean ± SEM.

express α-SMA,[29] therefore these findings also support a myofibroblastic identity for human hematopoietic stromal cells.[51] Besides a functional ability to support hematopoietic cells following dissociation, purification, and culture, human CD146$^+$ perivascular cells from nonhematopoietic tissues share a similar phenotype with the perivascular niche cells recently described in murine bone marrow.[35-37] Perivascular cells are ubiquitous[52] and may therefore represent the key stem cell support shared by all blood-forming organs. It remains to be determined whether and how this ability to sustain HSCs is repressed in situ in nonhematopoietic tissues, and may be reactivated in pathologic conditions, as in the course of extramedullary hematopoiesis or leukemic dissemination.

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Authorship

Contribution: M.C. designed and performed research, analyzed and interpreted data, and wrote the manuscript; C.P., A.S., W.W., S.G., D.E., and C.J.C. performed research; X.W. performed statistical analysis; E.M. and L.L. analyzed and interpreted data and contributed to writing the manuscript; and G.M.C and B.P. designed research, analyzed and interpreted data, and wrote the manuscript.

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Correspondence: Bruno Péault, 615 Charles E. Young Dr South, Room 410, Los Angeles, CA 90095; bpeault@mednet.ucla.edu.

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