Investigating the Role of OCIAD2 in Mouse Hematopoiesis

A Thesis submitted for the degree of

Doctor of Philosophy

By

Wulligundam Praveen

Molecular Biology and Genetics Unit (MBGU)

Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR)

Bangalore- 560064 (India)

September 2022

DECLARATION

I hereby declare that this thesis entitled **'Investigating the role of OCIAD2 in mouse hematopoiesis'** is an authentic record of research work carried out by me under the guidance of Prof. Maneesha S. Inamdar at the Laboratory of Stem Cell and Vascular Biology, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore.

In keeping with the norm of reporting scientific observations, due acknowledgement has been made whenever work described here has been based on the findings of other investigators. Any omission owing to oversight or misjudgment is regretted.

Wulligundam Praveen

Place: Bangalore

Date: 06th September 2022

CERTIFICATE

This is to certify that the work described in this thesis entitled **'Investigating the role of OCIAD2 in mouse hematopoiesis'** is the result of investigations carried out by **Mr. Wulligundam Praveen** in the Laboratory of Stem Cell and Vascular Biology, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my guidance and that the results presented here have previously not formed the basis for the award of any other degree, diploma or fellowship.

Prof. Maneesha S. Inamdar

Place: Bangalore

Date: 06th September 2022

Acknowledgements

The work presented in this thesis would not have been possible without my close association with several people. I take this opportunity to extend my sincere gratitude and appreciation to all those who made my Ph.D. thesis possible.

First and foremost, I gratefully acknowledge the guidance, encouragement, and support of my guide, Prof. Maneesha S. Inamdar, during my Ph.D. She has been a tremendous mentor for me, and I could not have imagined having a better advisor for my Ph.D. study! I can never forget her words of wisdom and inspiration at all the needed times during this journey. Her experience and foresight have always helped and inspired me during perplexing situations. I am very much thankful to her for teaching and strengthening the fundamental concepts of life, the patience, perseverance, hard work, and kindness in me. During this amazing, adventurous, and unmatchable journey, I have learned many things from her, and I thank her for giving me so many wonderful opportunities. I am indeed blessed and privileged to be her student. Limited by words to express my gratitude, I thank you for everything, Ma'am!

I thank all the faculty members of MBGU and NSU for the discussions and critical comments- Prof. M.R.S. Rao, Prof. Anuranjan Anand, Prof. Ranga Uday Kumar, Prof. Hemalatha Balaram, Prof. Tapas K. Kundu, Prof. Namita Surolia, Prof. Kaustuv Sanyal, Dr. Ravi Manjithaya, Dr. Kushagra Bansal, Dr. Sheeba Vasu, and Dr. James C. Chelliah. I am thankful to the Animal Facilities of JNCASR and NCBS, especially Dr. Prakash, Mr. Ambareesh, and Mr. Vasanth. I thank the Central Instrumentation Facility of MBGU, JNCASR and NCBS. I thank Dr. H. Krishnamurthy, NCBS, for training me in flow cytometry and confocal microscopy. I am very thankful to Suma Ma'am, Sunil, and Keerthana for helping me with confocal microscopy and Dr. Narendra Nala for flow cytometry, who have been very kind in providing the slots as per the need of the hour. I extend my special thanks to all my teachers and professors who have taught me at different stages of my education. Their hard work and kindness made it possible for me to reach a stage where I could write my thesis.

I am indeed fortunate to be in the company of excellent lab mates who maintain a lively and energetic lab atmosphere and make lab another home. I thank all the past lab members- Dr.

Abhishek, Dr. Sudhir, Dr. Rohan, Dr. Ronak, Dr. Simi, Dr. Deeti, Dr. Suman, Dr. Manju, Dr. Divyesh, Dr. Saloni, Diana, Dr. Anand, Aksah, Shruti, Ridim, Jasper, Amogh, Abarna, Chetan, Anudeep, Kaustubh, Zeenat, Aditi, Bornika, Tirath, Roja, Ankit, Priyamvada, Divya, Sumedha, and Vishal. I am thankful to my present lab mates- Dr. Arindam, Preeti, Rajarshi, Kajal, Alice, Prathamesh, Aishwarya, Yashashwinee, Arghakusum, Anjitha, Pratiksha, Pavithra, and Dr. Subha. I am very much thankful to Dr. Saloni Sinha and Ms. Alice Sinha for being a great help and support for completing this thesis, without which this would not have been possible. I thank Dr. Arindam and Alice for their critical reading and suggestions on this thesis. I am thankful to the lab assistants Nagaraj, Vijay, and Vanitha, who ensure that our work goes smoothly.

I am very much thankful to my close friends Dr. Deeti, Dr. Sashank, Kaustubh, and Preeti for being there for me whenever I needed any help related to work or personal life and playing critical roles in preventing me from being lost during the turbulent times. Your friendship and support have been my strength, and I am grateful for it and will cherish the warmth shown by you till the end.

I thank my batchmates, Dr. Aparna, Dr. Rima, Dr. Suchismita, Dr. Krishnendu, and Dr. Aditya, for fruitful discussions. I thank the timely and friendly support extended by the staff in the Complab, Canteen, Academic, Stores & Purchase and Administrative Units, and Students' Residence. I thank the Union Grants Commission (UGC), Govt. of India, for providing the monthly fellowship. I thank JNCASR for various facilities and the monthly fellowship and salary.

Finally, I thank my parents, wife, and other family members for being by me at all times. I thank their unconditional and unbelievable love, support, encouragement, and understanding. I thank my mother, who believed in me like nobody else and gave me endless support. Without her help, I would not have been able to complete what I have done and become who I am. Thank you, Amma! I am grateful to my wife, the surprise of my life, for her unconditional love, support, and trust in me. I am thankful to my maternal uncle, who identified my interest in biology, directed me towards biological research, and stood by me at all times needed.

Contents

List of figures

Chapter 4. *Ociad2*^{pdsn} KO mice show defective erythropoiesis and JAK/STAT signaling with increased Asrij levels in the bone marrow

Chapter 5. Discussion

List of abbreviations:

7-AAD:	7-Aminoactinomycin D
aa:	Amino acid
AGM:	Aorta-gonad mesonephros
ANOVA:	Analysis of variance
ARF1:	ADP-ribosylation factor-1
BFU:	Burst forming unit
bp:	base pairs
BM:	Bone marrow
CDK:	Cyclin-dependent kinase
cDNA:	Complementary DNA
CFU:	Colony forming unit
CMP:	Common myeloid progenitor
CSF:	Colony stimulating factor
CLP:	Common lymphoid progenitor
CSN:	COP9 signalosome
EHT:	Endothelial to hematopoietic transition
EMH:	Extramedullary hematopoiesis
EMP:	Erythro-myeloid progenitor
Epo:	Erythropoietin
EpoR:	Erythropoietin receptor
FACS:	Fluorescent activated cell sorting
5-FU:	5-fluorouracil
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GMP:	Granulocyte/macrophage progenitor
HCT:	Hematocrit
HRP:	Horse radish peroxidase
HSC:	Hematopoietic stem cell
HSPC:	Hematopoietic stem and progenitor cell
JAK:	Janus kinase

KO:	Knockout
Lin:	Lineage
LMPP:	Lymphoid-primed multipotent progenitor
LPS:	Lipopolysaccharide
LSK:	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$
MACS:	Magnetic activated cell sorting
MCHC:	Mean cellular hemoglobin concentration
MCV:	Mean corpuscular volume
MDM2:	Mouse double minute 2 homolog
MEP:	Megakaryocyte/erythrocyte progenitor
MFI:	Median fluorescence intesntiy
MLP:	Multipotent lymphoid progenitor
MPP:	Multipotent Progenitor
MPV:	Mean platelet volume
OCIA:	Ovarian carcinoma immunoreactive antigen
PAGE:	Polyacrylamide gel electrophoresis
PB:	Peripheral blood
PBS:	Phosphate-buffered saline
PHZ:	Phenylhydrazine
PI3K/Akt:	Phosphoinositide 3-kinase-Akt
P-LCR:	Platelet – large cell ratio
ROS:	Reactive oxygen species
RT-PCR:	Reverse transcription-polymerase chain reaction
Sca1:	Stem cell antigen-1
SCF:	Stem cell factor
SDS:	Sodium dodecyl sulfate
SEM:	Standard error of mean
STAT:	Signal transducers and activators of transcription
TGFβ:	Transforming growth factor beta
WT:	Wild type

Synopsis of the thesis entitled

Investigating the Role of OCIAD2 in Mouse Hematopoiesis

Submitted by

Wulligundam Praveen

Molecular Biology and Genetics Unit,

Jawaharlal Nehru Centre for Advanced Scientific Research,

Jakkur, Bangalore- 560064, India.

Thesis Advisor: **Prof. Maneesha S. Inamdar**

Hematopoiesis in vertebrates is a complex and tightly regulated process that ensures controlled production of the hematopoietic stem, progenitor, and differentiated cells. Loss of this balance leads to disease conditions like leukemia and anemia. Despite considerable progress in deciphering mechanisms of vertebrate hematopoiesis, our ability to control it is limited. Regulation of blood production in normal and pathological situations requires the identification of additional factors required to maintain hematopoietic homeostasis. Earlier work in our laboratory established the role of Ovarian Carcinoma Immunoreactive Antigen Domain containing protein 1 (OCIAD1), also referred to as Asrij, as a novel regulator of hematopoiesis and blood cell homeostasis in both *Drosophila* (Kulkarni, Khadilkar, et al., 2011; Sinha et al., 2013) and mouse (Sinha et al., 2019). Asrij is essential for preserving mouse bone marrow hematopoietic stem cell (HSC) quiescence, and its depletion leads to a myeloproliferative disorder due to uncontrolled p53 ubiquitination and degradation (Sinha et al., 2019). Previous studies from our laboratory showed that *ociad2,* the other member of the OCIAD family, is a genomic neighbor of *asrij* in most vertebrates. OCIAD2 localizes to mitochondria and endosomes, interacts with Asrij, and promotes STAT3 activation in cultured HEK293 cells (Sinha et al., 2018). Also, OCIAD2 is implicated in several diseases and misexpressed in various hematological malignancies (Bagger et al., 2016; Praveen et al., 2021). Nevertheless, the physiological role of OCIAD2 in vertebrate hematopoiesis is not known. Hence, I studied the role of OCIAD2 in mouse hematopoiesis.

To investigate the role of OCIAD2 in murine hematopoiesis, firstly, we studied its expression in various bone marrow hematopoietic cells. Flow cytometry analysis revealed the expression of OCIAD2 in hematopoietic stem cells, progenitors, and differentiated cells of C57BL/6J mouse bone marrow, with its levels decreasing along the differentiation hierarchy. This suggested a possible role of OCIAD2 in mouse hematopoiesis.

Mice knockout for *ociad2* (*ociad2*$^{fl/fl;\ CMV\text{-}Cre^{+}}$; KO) were generated earlier in our laboratory and found viable and fertile, showing no gross developmental abnormalities. As the *ociad2* gene neighbors *asrij* and affects its protein levels (see Chapter-4), we name the knockout allele as *ociad2*^{padosan} (Hindi: *padosan* means neighbor), abbreviated to *ociad2*^{pdsn} (approved by Mouse Genome Informatics). To explore the role of OCIAD2 in mouse hematopoiesis, I undertook a detailed analysis of *ociad2*^{pdsn} KO mice. *Ociad2*^{pdsn} KO mice showed no change in body weight but had mild splenomegaly and kidney atrophy. The abundance of hematopoietic stem and progenitor cells (HSPC) was unaffected across different hematopoietic compartments of the KO mice. However, the frequency of BM myeloid and B cells was increased, with their counts and apoptosis unaffected. *In vitro* and molecular analyses showed no change in the hematopoietic differentiation potential to early progenitors and expression of various lineage specification markers in KO BM, indicating an insignificant role of OCIAD2 in the maintenance of HSPCs and their capacity of lineage specification.

Interestingly, the KO mice showed anemia in peripheral blood and defective steady-state erythropoiesis in BM, indicating that OCIAD2 plays a role in maintaining erythropoiesis in mice.

To understand more about the role of OCIAD2 in mouse erythropoiesis, we subjected the KO mice to hemolytic stress. We found that their ability to respond to stressors was *on par* with the control mice. Also, the KO mice showed increased sensitivity to stress-mediated splenomegaly and albuminuria, indicating that OCIAD2 is required for protection against stress-erythropoiesis and acute kidney injury. Despite impaired kidney function under stress, intriguingly, erythropoietin (*Epo*) transcript levels were unaffected in steady-state KO mouse kidney, indicating possible defective signaling downstream of Epo receptor (EpoR).

JAK/STAT signaling plays a predominant role downstream of EpoR in regulating steady-state and stress erythropoiesis. Molecular analysis of steady-state KO mice showed decreased STAT5 activation and increased STAT3 activation in the lineage-committed BM and increased Asrij protein levels in unfractionated BM. This suggests that steady-state erythropoiesis is deregulated in the KO mice. Based on these results, we propose that there may be functional compensation by Asrij in the KO cells, extenuating the effects of loss of OCIAD2.

OCIAD2 executes cell-specific functions in adult hematopoiesis, particularly in erythropoiesis, probably by regulating STAT activation in association with Asrij and the physiology of extra-medullary tissues like the spleen and kidney. Also, OCIAD proteins might play a vital role in the cross-talk between organelles like mitochondria, endosomes, and lipid rafts in regulating erythropoiesis. Bone marrow Asrij levels decrease upon aging (Sinha et al., 2022), indicating a possibility of severe phenotypes in aged OCIAD2 KO mice. This opens new possibilities to understand the mutual regulation of OCIAD paralog proteins at the levels of post-translational modifications and protein-protein interactions and their effects on vertebrate hematopoiesis.

In summary, we show that OCIAD2 is a novel endosomal and mitochondrial regulator of mouse adult hematopoiesis. We propose that a balance of OCIAD proteins is essential for normal hematopoiesis through regulating cell signaling. The mutant mouse that we have generated will be a valuable tool for understanding the regulation of hematopoietic homeostasis by inter-organ communication and studying anemia, stress erythropoiesis, and kidney function.

Chapter 1. Introduction

Hematopoiesis in the bone marrow (BM) is a continuous process producing nearly a trillion new blood cells every day in humans. The mammalian hematopoietic system is a complex orchestra of different cell types derived from a group of adult stem cells called hematopoietic stem cells (HSCs). Constituting the apex of the differentiation hierarchy, HSCs undergo asymmetric cell divisions to self-renew and differentiate into lineage-committed progenitors. These progenitors ultimately give rise to the terminally differentiated mature blood cells (Zhang et al., 2018). A balance between the abundance of hematopoietic stem and progenitors (HSPCs) and differentiated cells is strictly maintained to ensure normalcy, referred to as hematopoietic homeostasis, loss of which leads to disease conditions like leukemia, anemia, etc.

Studies on hematopoiesis in invertebrates like *Drosophila* and lower vertebrates like zebrafish, *Xenopus,* and chick embryos, led to the discovery of multiple molecular factors governing hematopoietic homeostasis. Several basic principles regulating early hematopoietic development are conserved through evolution across the invertebrates and vertebrates (Gritz and Hirschi, 2016). Also, new molecules have emerged through gene duplication, providing more control over the physiology and safeguarding the robustness of the organism, albeit adding to the complexity of hematopoiesis. Therefore, despite considerable knowledge gained, the identification and characterization of novel molecular regulators of vertebrate hematopoiesis is required.

Being evolutionarily distant from humans, the acquired knowledge from invertebrates and lower vertebrates needs verification in appropriate model systems to understand and treat disease conditions. The house mouse (*Mus musculus*) has been extensively used as a mammalian model system to overcome the ethical and technical limitations of *in vivo* studies in humans. The mouse model approximates anatomical, physiological, and genetic aspects of humans. Also, factors like small size, phylogenetic relatedness, ease of genetic and chemical manipulation, and ease of breeding in large numbers due to a high breeding capacity make the mouse model most suitable to study various aspects of mammalian physiology (Lee et al., 2017). Hence, we aimed to characterize novel molecules in vertebrate hematopoiesis using mouse models.

1.1 Ontogeny of vertebrate hematopoiesis

1.1.1 Primitive wave

In mice, the first wave of hematopoiesis starts around E7.0 in the yolk sac blood-islands of mesoderm-derived specialized cells, giving rise to endothelial and hematopoietic cells. This wave is primitive and transient and produces primitive nucleated erythrocytes, megakaryocytes, and macrophages, essential for embryo growth and organogenesis. (Sankaran and Orkin, 2013).

1.1.2 Pro-definitive wave

Around E8.25, during the pro-definitive wave, the yolk sac vasculature hemogenic endothelial cells produce hematopoietic cells through endothelial to hematopoietic transition (EHT). EHT gives rise to lymphoid-restricted progenitors and multipotent lympho-myeloid progenitors around E9.5, which eventually colonize the fetal liver to generate the innate and adaptive B and T cells (Hoeffel and Ginhoux, 2018). Erythro-myeloid progenitors (EMPs) are generated around E10.5, circulate to the embryo, and colonize the fetal liver to sustain hematopoiesis until birth. EMPs give rise to enucleated RBCs with fetal hemoglobin, megakaryocytes, and monocytes which later develop into tissue-resident macrophages that extend into adult life (Frame et al., 2013).

1.1.3 Definitive wave

Between E9.5 and E11.5, the hemogenic endothelial cells in the aorta-gonad-mesonephros (AGM) initiate the definitive wave of hematopoiesis through EHT and RUNX1 upregulation. Around E9.5, the AGM generates pro-HSC and pre-HSC with multi-lineage and short-term repopulating capacity. The first *bona fide* HSCs arise around E10.5 from the ventral endothelium of the dorsal aorta, subsequently home to the fetal liver, where they undergo further maturation and expansion (Ivanovs et al., 2011). In mice, HSCs also arise from non-AGM regions like the placenta, vitelline veins, umbilical arteries, and arteries in the embryonic head and colonize the fetal liver. This HSC pool in the fetal liver maintains hematopoiesis until around birth, after which it occurs in the BM (Ottersbach and Dzierzak, 2005; Li et al., 2012).

1.2 Medullary hematopoiesis

Adult hematopoiesis predominantly takes place in the medullary region of the bone, homing the quiescent HSCs in a hypoxic microenvironment. Identification, isolation, and characterization of HSCs were accelerated by the advent of antibodies against cell surface markers and fluorescence-activated cell sorting (FACS) (Oguro et al., 2013). Subsequently, multipotent progenitor (MPP) populations were identified between the HSCs and terminally differentiated cells. Weissman's group presented the classical, stepwise, immunophenotype-based tree-like hierarchy model (Kondo et al., 1997; Manz et al., 2002), which places HSCs with long-term repopulating potential (LT-HSCs) at the top of the hierarchy, giving rise to HSCs with short-term repopulating potential (ST-HSCs). ST-HSCs differentiate to MPPs, producing oligopotent and unipotent progenitors and the terminally differentiated cells, as shown in Figure-1.1A.

Shortcomings of the classical stepwise model, like the over-simplification of the complexity and the unaccountability of heterogeneity in the HSCs and progenitors (Lu et al., 2011), were made evident from studies with genetic mouse models and single-cell technology. Identification of myeloid-biased, balanced, and lymphoid-biased HSCs (Muller-Sieburg et al., 2002) and detection of intermediate-term HSCs (IT-HSCs) (Benveniste et al., 2010) are some of the findings that challenge the classical model. Comprehensive analysis of human BM HSPCs using scRNA-seq coupled with computational methods suggested that lineage specification is a continuous process where low-primed undifferentiated HSPCs directly differentiate to unilineage restricted cells without any transition stages (Velten et al., 2017). Additionally, another study on human cord blood cells showed that downstream of stem cells, a continuum of bi- and oligolineage progenitors like the lymphoid-primed multipotent progenitors (LMPPs), granulocyte/monocyte progenitor (GMPs), and multipotent lymphoid progenitors (MLPs) produce terminally differentiated lymphoid and myeloid cells, instead of only unilineage progenitors (Karamitros et al., 2018). These studies show that hematopoiesis is a continuous process rather than a stepwise hierarchy and suggest that no discrete boundaries exist among stem cells and progenitors (Figure-1.1B).

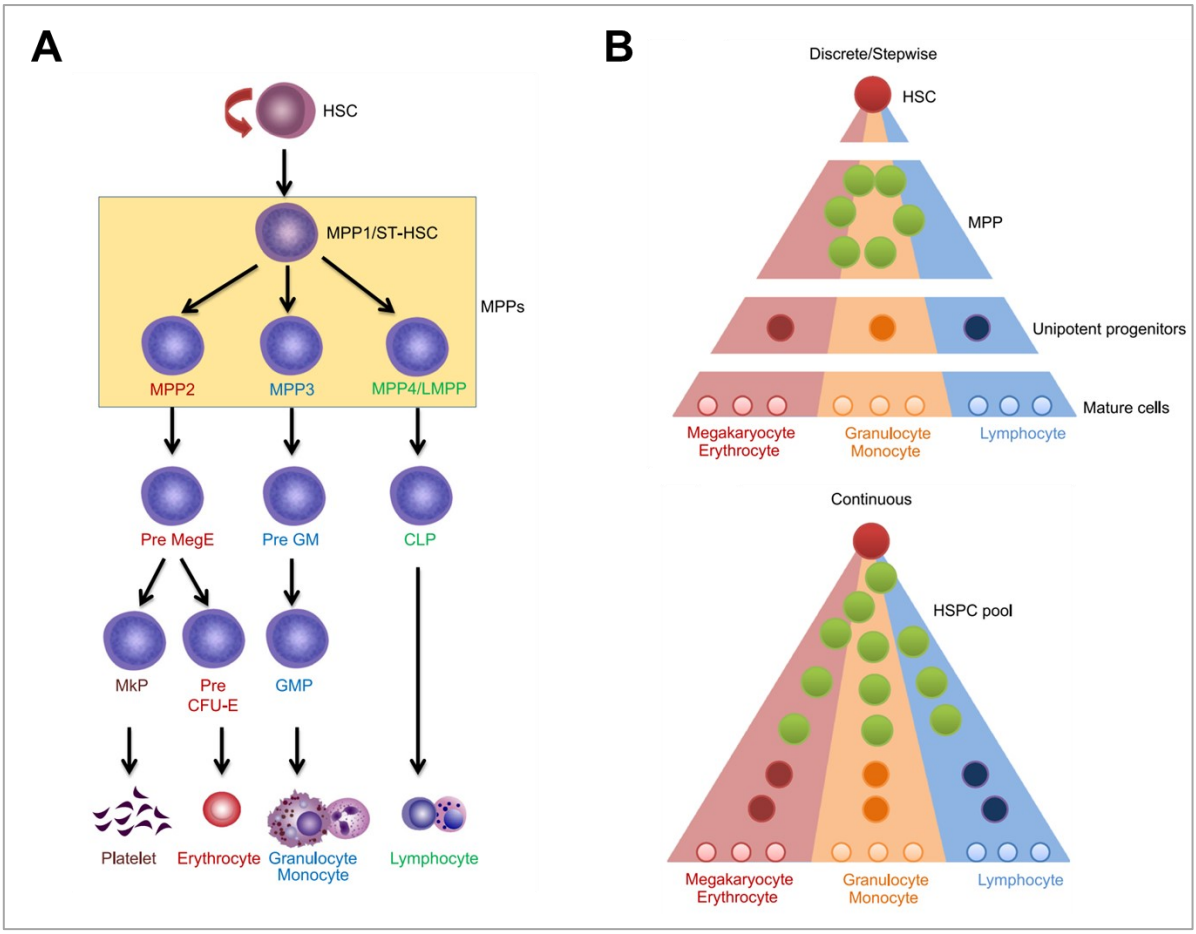


Figure-1.1: Hematopoietic differentiation is a continuous process rather than a discrete one. **(A)** Schematic showing the hierarchy model for hematopoietic differentiation of HSCs to mature terminal cells. According to this model, the HSC or the long-term repopulating HSCs (LT-HSCs) are a *bona fide* group of quiescent cells, which give rise to short-term repopulating HSCs (ST-HSCs), also considered as multipotent progenitors (MPP-1), which further give rise to MPP2, MPP3, and MPP4. While MPP2 gives rise to platelets and erythrocytes, MPP3 gives rise to granulocytes, monocytes, and MPP4, also called lymphomyeloid-primed progenitors (LMPP) differentiate to lymphocytes. Pre MegE: pre-megakaryocyte-erythroid progenitors; Pre GM: pre-granulocyte-monocyte progenitors; CLP: common lymphoid progenitors; MkP: Megakaryocyte progenitors; Pre CFU-E: pre-colony-forming unit-erythroid; GMP: granulocyte-monocyte progenitors. **(B)** Schematic comparing the hematopoietic differentiation between the classical stepwise model and the continuous model (*Adapted from Cheng et al., Protein Cell, 2020).*

1.3 Extramedullary hematopoiesis

The formation of blood cells in spaces other than the BM is called extramedullary hematopoiesis (EMH). The embryonic hematopoiesis is considered as developmental EMH. Post-natal, in humans, EMH is not observed under normal physiological conditions. However,

disease conditions like BM failure, myelostimulation, tissue inflammation, injury, repair, and abnormal systemic or local chemokine production are known to induce EMH (Yang et al., 2020). Contrastingly, mice show persistent EMH throughout their adult life in the spleen and to a certain extent in the liver (Dutta et al., 2015).

HSPCs circulate in the peripheral blood (PB) and lymph regulated by the interaction between the CXCR4 receptor and CXCL12 ligand and circadian rhythms (Garcia-Garcia et al., 2019). In steady-state conditions, adult spleen, liver, gut, gingiva, and lung contain rare HSPC pools, albeit with short residence time and low contribution to hematopoiesis (Chiu et al., 2015). Parabiosis studies in mice show that most PB HSCs under steady-state conditions are not LT-HSCs and fail to return to the BM niche (Mende and Laurenti, 2021). Also, the extramedullary HSCs of both mice and humans show lower reconstitution and colony-forming capacity (Fu et al., 2019).

Stress conditions alter the BM niche and increase the mobilization of HPSC, supporting EMH (Yang et al., 2020). Spleen is the most common site of EMH driven by the HSPCs residing in its red pulp. Studies have identified multiple stages of erythroid differentiation, myeloid cells, and megakaryocytes in the splenic cords and intrasinusoidal spaces of the splenic red pulp. The CXCL12 ligand expressed by the sinus endothelial cells is expected to play a significant role in homing the circulating HSCs to the splenic red pulp (Yang et al., 2020). EMH-driven splenomegaly is observed during disease states like myelofibrosis, anemia, and thalassemia to compensate for the defective BM erythropoiesis via upregulation of the JAK-STAT pathway (Gilles et al., 2017; Song et al., 2018). Apart from the vital role of the hematopoietic site during embryogenesis, the liver can act as an EMH site in adult life during the neo-natal stage and disease conditions like sepsis, anemia, hepatoblastoma, hepatocellular carcinomas, etc., when the homeostasis of the hematopoietic system is disrupted (Yamamoto et al., 2016).

1.4 Hematopoietic homeostasis

Maintaining a steady-state number of different blood cells is defined as hematopoietic homeostasis. During homoeostatic conditions, the quiescence, survival, and self-renewal capacity of HSCs are maintained with balanced hematopoiesis. Therefore, hematopoietic homeostasis is required for sustained hematopoiesis and maintenance of normal physiology.

Loss of this homeostasis leads to increased susceptibility of HSCs to DNA damage, apoptosis, senescence, increased myeloid skewing, and exhaustion of the HSC pool (Termini and Chute, 2020), which has been a common phenomenon underlying various disease conditions like leukemia, anemia, etc. This emphasizes the essentiality of maintaining hematopoietic homeostasis, which is critically regulated by various factors.

1.4.1 Inter-organ communication

Cross-talk between hematopoietic and non-hematopoietic tissues plays a crucial role in governing hematopoietic homeostasis. For example, in erythropoiesis, the formation of red blood cells occurs primarily in the BM and spleen under stress conditions. However, other organs like the kidney, liver and intestine play critical roles in the regulation of erythropoiesis. The kidney is the primary source of erythropoietin, a peptide hormone essential for erythropoiesis in BM and spleen. Enterocytes of duodenum and macrophages of splenic red pulp ensure absorption of dietary iron and reuse of physiological iron, respectively, ensuring the availability of iron in the circulation for the synthesis of hemoglobin in erythroblasts, under the influence of erythroferrone and erythropoietin. This physiological process is kept under check by the action of another peptide, called hepcidin, secreted by the liver. Hepcidin obstructs iron absorption through binding to ferroportin of gut enterocytes and macrophages, leading to lysosomal degradation (Liang and Ghaffari, 2016). Secretory molecules constitute an essential fraction of cell-extrinsic factors regulating steady-state and stress hematopoiesis. Maturation of B cells, removal of old red blood cells, and stress erythropoiesis in the spleen (Shahaf et al., 2016), and T cell development in the thymus (Owen et al., 2019) are well-known examples for the cross-talk between BM and extramedullary tissues in maintaining hematopoietic homeostasis.

1.4.2 Other cell-extrinsic factors

The biology of HSPCs is intricately linked with and regulated by the extracellular cues received from their niche comprising of the following conserved central elements.

a) <u>Stromal cells promote cell-cell adhesion and secrete cytokines for paracrine signaling</u>

The N-Cadherin-expressing BM stromal progenitors that give rise to osteoblasts, chondrocytes, and adipocytes, secrete various cytokines, including stem cell factor (SCF), to maintain the reserved HSC pool in the BM and its recovery upon stress (Zhao et al., 2019). Disease conditions reduce the ability of niche cells to support

HSCs, as seen in the case of mesenchymal stem cells in β-thalassemia (Crippa et al., 2019).

b) Extracellular matrix (ECM) provides anchorage

Adhesion of HSCs to fibronectin is essential for maintaining their long-term hematopoiesis and proliferation and association with laminin for homing and cell cycling (Susek et al., 2018). Also, ECM sense changes in physiological conditions and can sequester or release growth factors and morphogens, therefore indirectly governing stem cell fate (Ahmed, 2016).

c) Blood vessels provide nutrition and systemic signals

Distinct network of arterial blood vessels formed by BM endothelial cells, with less permeability, maintain HSCs with low reactive oxygen species (ROS), whereas the sinusoids with more permeability help in HSPC activation, apart from supporting the trafficking of the leukocytes to and from the BM (Itkin et al., 2016; Chen et al., 2020). Endothelial Notch signaling is crucial in expanding the HSC niche and its secretion of SCF (Kusumbe et al., 2016).

d) Neural inputs integrate signals from different organ systems

The sympathetic nervous system regulates the daily migration of HSCs and leukocytes, with the coordination between the parasympathetic nervous system, epinephrine, and beta-adrenergic receptors (García-García et al., 2019). Hypothalamic-pituitary-adrenal axis controls the granulocyte colony-stimulating factor (G-CSF) induced mobilization of HSCs in the BM through the involvement of muscarinic receptor type 1 (Pierce et al., 2017). Also, cholinergic receptor muscarinic 4 (CHRM4) regulates early erythroid progenitors' self-renewal in BM (Trivedi et al., 2019).

1.4.3 Cell-intrinsic factors

Apart from the inter-organ cross-talk and other external factors, various cell-intrinsic factors like cell cycle, transcription factors, epigenetic modifications, signaling, etc., affect HSPC biology.

a) Cell cycle

Cell cycle machinery comprising of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) regulate cell cycle activity and the abundance of HSPCs. Cyclins show differential effects as deletion of cyclin B1, E1/E2 leads to embryonic lethality (Parisi et al., 2003), and cyclin A deletion leads to reduced abundance of HSCs and CMPs (Kalaszczynska et al., 2009). Deleting both CDK4 and CDK6 leads to embryonic lethality (Malumbres et al., 2004). CKIs also exert differential effects on hematopoiesis, as seen in the case of p21, p27, and p57 (Cheng et al., 2000; Matsumoto et al., 2011). Cyclin D1 regulates HSC expansion via the FGFR3-FRGFR1-ELK1-Cyclin D1 axis (Ran et al., 2021). The role of CDK1, 6, 8, and 19 in various malignancies, including that of hematopoiesis, are known (Aleem and Arceci, 2015).

b) Transcription factors

Transcription factors regulate cell biology by controlling gene expression. Key transcription factors like GATA, Lmo2, Scl, RUNX1, etc., play a critical role in hematopoiesis. Scl regulates quiescence and long-term potential of HSCs, proper development of megakaryocytes and erythroid cells (Porcher et al., 2017). Lmo2 regulates cell development and growth by controlling gene transcription through DNA-binding complexes (Morishima et al., 2019). RUNX1 and GATA2 regulate cell cycle progression and proliferation of HSCs (Bresciani et al., 2021). Additionally, many other transcription factors like p53, PU.1, Erg, Gfi1, etc., also play essential roles in regulating HSPC maintenance. Chromatin remodeling molecules aid transcriptional regulation of gene expression to control HSPC maintenance and differentiation (Prasad et al., 2015; Han et al., 2019).

c) Cell signaling

Signaling molecules act as connecting links between extracellular stimuli and intracellular genetic changes. Signaling pathways like PI3K/Akt, JAK/STAT, Wnt signaling, etc., play crucial roles in maintaining HSPCs and differentiated cells. Hyperactivation of PI3K/Akt signaling leads to myeloproliferative disorder in mice (Sinha et al., 2019). Its negative regulators like PTEN are required to maintain HSC quiescence and self-renewal ability (Ghosh and Kapur 2016). Deletion of STAT5 leads to compromised cell survival, whereas abnormal STAT5 activation is implicated in several cancers (Dorritie et al., 2014). Wnt/β-catenin signaling is essential in the

implantation of fetal HSPCs (Kwarteng et al., 2018). Adhesion of HSCs to Notch expressing osteoblasts suppresses cycling and enhances the retention of HSCs in BM independent of *RBP-J_K*-mediated canonical Notch signaling (Wang et al., 2015). Also, deregulated Wnt and Notch signaling play a critical role in leukemia (Kang et al., 2019).

Additionally, metabolic regulation (Hu and Wang 2019), post-transcriptional, translational, and post-translational mechanisms (de Rooij et al., 2019 and Kidoya et al., 2019) also play an essential role in regulating hematopoiesis. Of late, organelles' role in regulating hematopoiesis is being recognized.

1.4.4 Organellar regulation of hematopoiesis

Macromolecular interactions on the surface of or within the organelles play an important role in cell fate decisions (Julian and Stanford, 2020). Of late, lysosomes are gaining importance in this regard. Lysosomes are known as degradation centers of cellular contents and also serve as major signaling sites for molecules like mTORC1, AMPK, GSK3, and the inflammasome (Lawrence and Zoncu, 2019), which sense, integrate, and facilitate cross-talk among various signaling events and enable responses like autophagy, cell growth, membrane repair, and microbe clearance. Recent reports show an asymmetrical inheritance of lysosomes, autophagosomes, mitophagosomes, NOTCH1, and NUMB proteins between the daughter cells of HSC's asymmetric cell division and are expected to affect lineage fate (Loeffler et al., 2019). Also, pharmacological inhibition of lysosomal v-ATPase enhances mouse HSC quiescence and engraftment potential (Liang et al., 2020; Ghaffari, 2021). A recent study reports reduced lysosomal activity leading to loss of quiescence and mitotic activation of human LT-HSCs and loss of balance in myeloid/erythroid fate regulated by MYC and TFEB. This report strengthens the involvement of endosomes and lysosomes in HSC fate determination (García-Prat et al., 2021).

Mitochondria play a crucial role in HSC expansion. The clearance of damaged mitochondria, mediated through fatty acid oxidation, is essential for maintaining *Tie2*$^+$ HSCs (Ito et al., 2016). Also, during asymmetric cell division of mammary epithelial stem cells, older mitochondria are transferred to daughter cells poised for differentiation (Katajisto et al., 2015), indicating a potential role of mitochondria in the maintenance and differentiation of stem cells. Maintenance of high glycolytic metabolism and low mitochondrial unfolded protein stress is necessary for the quiescence of HSCs (Mohrin et al., 2018). Interestingly, mitochondrial

turnover plays a role in erythropoiesis. Defective mitophagy blocks erythroid differentiation at terminal erythrocyte maturation, whereas an enhanced mitophagy blocks erythroid progenitor differentiation (Jin et al., 2018).

1.5 OCIAD proteins

Despite considerable progress in understanding the maintenance of hematopoietic homeostasis, the role of organelles in hematopoiesis regulation is unclear. Hence, we aimed at unraveling the same by studying the role of endosomal and mitochondrial proteins in hematopoiesis. We focused on the Ovarian Carcinoma Immunoreactive Antigen (OCIA)-domain family of proteins, consisting of two members, namely, Asrij/OCIAD1 and OCIAD2.

1.5.1 Asrij/OCIAD1:

Asrij is a conserved endocytic and mitochondrial protein with the characteristic OCIA domain towards its N-terminal. The OCIA domain is predicted to harbor two transmembrane helices and the C-terminal region with a disordered structure. In *Drosophila,* Asrij is expressed in pole cells, trachea, and hemocytes (Inamdar, 2003). Asrij maintains the normalcy of the primary and secondary lobes of the lymph gland and hemocyte proliferation and differentiation (Kulkarni and Khadilkar et al., 2011) by interacting with trafficking protein ADP-Ribosylation Factor-1 (ARF1) (Khadilkar et al., 2014), regulating the endosomal trafficking of Notch-intracellular domain (Kulkarni et al., 2011), and STAT3 activation (Sinha et al., 2013) (Figure-1.2A). Asrij regulates Cactus ubiquitination, Toll and IMD pathways to maintain immune homeostasis in *Drosophila* (Khadilkar and Ray et al., 2017) (Figure-1.2B). Recently, our lab showed that Asrij maintains lymph gland progenitor balance by regulating the mitochondrial dynamics (Ray et al., 2021) (Figure-1.2C).

The role of Asrij in maintaining stemness is conserved across *Drosophila* and mice (Kulkarni, Khadilkar, et al., 2011; Sinha et al., 2013). Asrij initially identified in a gene-trap screen, maintains pluripotency of mouse embryonic stem cells through its OCIA domain by enhancing the activation of STAT3 (Sinha et al., 2013) (Figure-1.3A). Upon mESC differentiation, expression of Asrij is restricted to cardiovascular and blood lineages (Mukhopadhyay et al., 2003). Also, Asrij/OCIAD1 is essential in maintaining the pluripotency of human embryonic

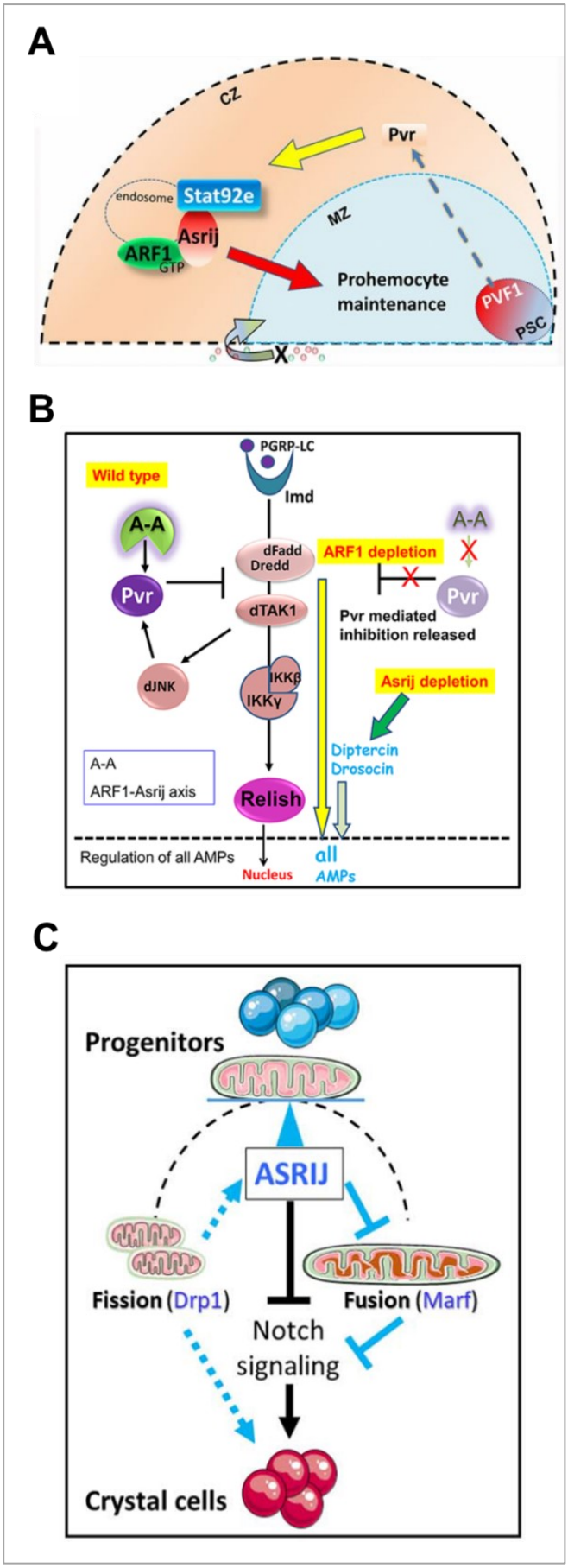


Figure-1.2: Asrij is an essential regulator of *Drosophila* hematopoiesis and immunity. (A) Schematic representation Asrij's regulatory role in the JAK/STAT, Notch, PDGF-VEGF signaling pathways in the *Drosophila* lymph gland (*Modified from Khadilkar et al., 2014*). **(B)** Schematic representation of the suggested role of the ARF1-Asrij endocytic axis in regulating the Imd pathway of *Drosophila (Adapted from Khadilkar and Ray et al., 2017).* **(C)** Schematic representing the role of Asrij in maintaining the balance between mitochondrial fission and fusion to regulate progenitor maintenance and crystal cell differentiation in *Drosophila (Adapted from Ray et al., 2021)*.

stem cells, where its loss leads to increased mitochondrial respiration and propensity towards differentiation in hESCs (Shetty et al., 2018) (Figure-1.3B).

Asrij is expressed in the mouse yolk sac, embryonic blood islands, and blood vessels, preceding and overlapping with vascular markers like VEGF-RII/Flk-I and PECAM/CD31 (Mukhopadhyay et al., 2003). Also, Asrij localizes to $Rab4^{+}$ and $Rab5^{+}$ endosomes and mitochondria in wild-type mouse HSCs, maintaining HSC quiescence by regulating p53 ubiquitin-mediated degradation via the CSN5-MDM2 axis (Sinha et al., 2019). Loss of Asrij leads to increased levels of

MDM2 and thus an increased degradation of p53, resulting in myeloproliferative disorder (Figure-1.3C). Mice knockout for Asrij shows enhanced expansion and mobilization of HSPCs,

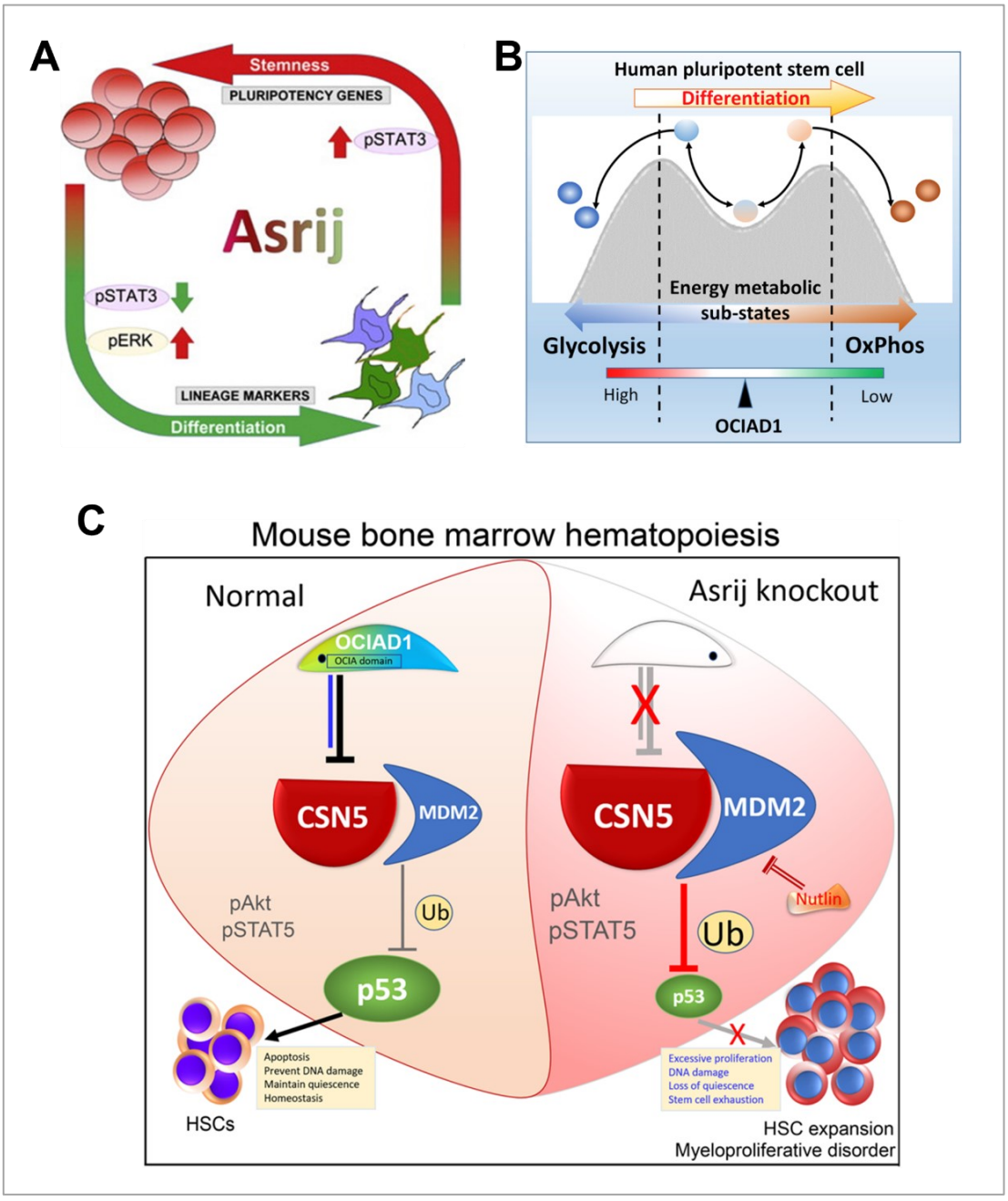


Figure-1.3: Role of Asrij/OCIAD1 in mammalian stem cells and hematopoiesis. (A, B) Schematic representing the role of Asrij in maintaining stemness and pluripotency in (A) mouse ESCs through enhanced STAT3 activation *(Adapted from Sinha et al., 2013)* and (B) in human ESCs through preventing an increase in oxidative phosphorylation *(Adapted from Shetty et al., 2018)*. **(C)** Schematic representing the role of Asrij in mouse BM hematopoiesis in maintaining the HSC quiescence by preventing aberrant degradation of p53 via the CSN5-MDM2 axis *(Adapted from Sinha et al., 2019)*.

myeloid expansion, altered RBC counts in PB, and splenomegaly (Sinha et al., 2019). Also, Asrij is essential for hematopoietic recovery from stress conditions like 5-FU treatment and exposure to gamma radiation. Asrij regulates aberrant activation of AKT and STAT5 in mouse BM HSPC pool (Sinha et al., 2019). This indicates the importance of endosomal and mitochondrial proteins in maintaining mouse hematopoietic homeostasis.

1.5.2 OCIAD2:

Ociad2, the other member of the OCIAD family, was initially identified through sequence similarity to *ociad1* in a revised draft of the human genome (Strausberg et al., 2002). *ociad2* is absent in *Drosophila* and is predicted to have risen from an invertebrate *ociad* gene in early jawed vertebrates, due to a tandem gene duplication, sometime during the Ordovician and Silurian eras. *ociad2* neighbors *ociad1* in both humans (chromosome 4) and mice (chromosome 5), but on the opposite strand in a tail-to-tail orientation, separated by a short intergenic stretch (Sinha et al., 2018) (Figure-1.4A). Mouse *ociad2* gene consists of 7 exons, in which the 1st, part of the 2nd, and 7th exons are non-coding. Multiple splice variants of *ociad2* were reported at transcript level but not at the protein level, with higher expression in the brain, liver, and kidney (Sinha et al., 2018).

OCIAD2 harbors the characteristic OCIA domain coded by exon 3 to exon 6 of the *ociad2* gene, with high sequence similarity to Asrij. The double-helical region of the OCIA domain is sufficient for OCIAD2 localization to mitochondria and endosomes (Sinha et al., 2018) (Figure-1.4B). OCIAD2 also localizes to mitochondria-associated ER membrane (MAM) (Han et al., 2014) (Figure-1.4C). A recent report shows that OCIAD2 is critical for maintaining mitochondrial morphology and numbers by regulating cytochrome C release into the cytosol (Hong et al., 2021). Another report suggests a possible role of OCIAD2 in the mitochondrial electron transport chain (Le Vasseur et al., 2021). OCIAD2 interacts with Asrij and STAT3 through its double-helical region of the OCIA domain. Many other interactions were predicted as per BIOGRID, STRING, and STITCH databases (Praveen et al., 2020). OCIAD2 is essential for STAT3 activation and migration but not for proliferation in cultured HEK293 cells (Sinha et al., 2018) (Figure-1.4D). However, overexpression of OCIAD2 did not affect the migration or proliferation of HEK293.

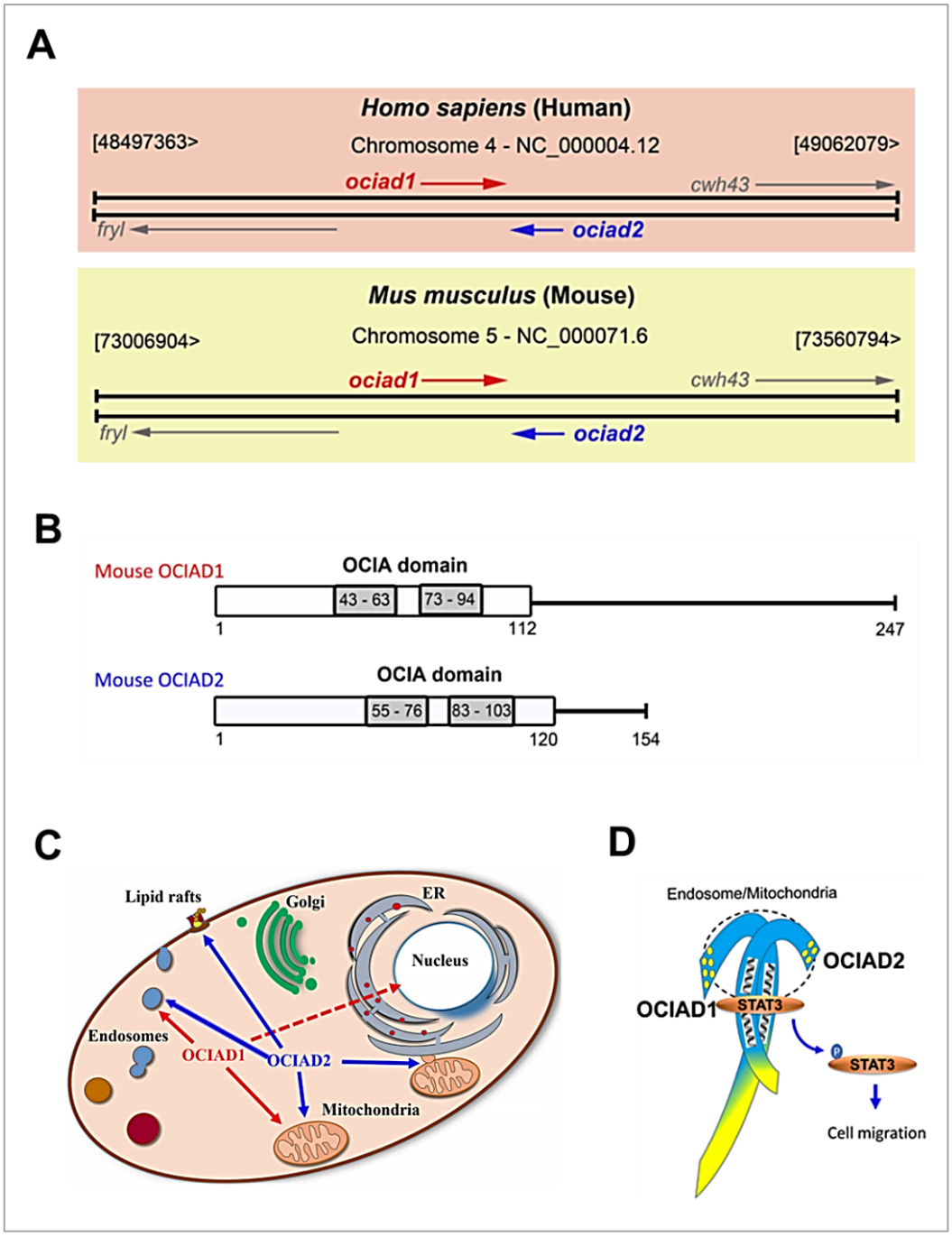


Figure-1.4: Gene location, domain organization, localization, and interaction of OCIAD2. (A) *ociad2* neighbors *asrij/ociad1* in mice and humans *(Adapted from Sinha et al., 2018)*. **(B)** Schematic representation comparing the protein length of Asrij and OCIAD2 with the OCIA domain indicated by rectangle box with double helices indicated by grey-shaded rectangles *(Adapted from Sinha et al., 2018)*. **(C)** Schematic showing the cellular localization of Asrij and OCIAD2 *(Adapted from Praveen et al., 2020)*. (D) Schematic representing the interaction between Asrij and OCIAD2 and their molecular action of STAT3 activation *(Adapted from Sinha et al., 2018)*.

Ociad2 is expressed in lower levels in various human tissues at steady-state conditions relative to *ociad1* (Sinha et al., 2018). However, unlike *ociad1*, the expression levels of *ociad2* increase during cancer conditions (Sakashita et al., 2018; Hong et al., 2021). OCIAD2 is implicated in several cancers and neurodegenerative disorders (Nagata et al., 2012; Han et al., 2014; Wu et al., 2017). Decreased expression of OCIAD2 leads to increased invasion of hepatocellular carcinoma (HCC) cells by downregulating FAK, AKT, and MMP9 signaling (Wu et al., 2017). OCIAD2 enhances the activity of gamma-secretase but does not interact with Nicastrin and C99 but does not affect Notch signaling (Han et al., 2014).

Both *ociad1* and *ociad2* are misexpressed in various hematological malignancies (Bagger et al., 2019). While the role of Asrij in regulating hematopoiesis is well-studied in both *Drosophila* and mouse hematopoiesis (Kulkarni and Khadilkar et al., 2011; Sinha et al., 2013; Khadilkar et al., 2014; Khadilkar and Ray et al., 2017; Sinha et al., 2019; Sinha et al., 2022), little is known about the physiological role of OCIAD2 in vertebrate hematopoiesis.

1.6 Hypothesis

Owing to the established essential role of Asrij in regulating mouse hematopoiesis and the interaction of Asrij with OCIAD2, we hypothesize that OCIAD2 too is essential for maintaining the mouse hematopoietic homeostasis. We tested this hypothesis through a detailed hematopoietic characterization of an *ociad2* null mouse model, at physiological, cellular, and molecular levels.

1.7 Aims of the thesis

The overall aim of this thesis was to decipher further the mechanisms regulating mammalian hematopoietic homeostasis through the identification and characterization of novel molecular regulators. For this, I investigated the role of OCIAD2 in hematopoiesis of the *Mus musculus* (mouse) model. The specific aims of this study were:

1. ***To profile the expression of OCIAD2. (Chapter-2)***
   We studied the expression of OCIAD2 in various hematopoietic populations of C57BL/6J mice using flow cytometry and compared it with the reported transcript levels revealed by high-throughput techniques.

2. ***To elucidate the role of OCIAD2 in mouse steady-state hematopoiesis. (Chapter-3)***
   We characterized the steady-state *ociad2* knockout mice using cellular, molecular, and functional assays.

3. ***To elucidate the role of OCIAD2 in mouse hematopoietic stress response and the molecular mechanisms maintaining hematopoiesis. (Chapter-4)***
   We studied steady-state and stressed *ociad2* knockout mice for various parameters using *in vitro* cell cultures, molecular, and biochemical assays.

1.8 Summary of the thesis

To understand the molecular mechanisms regulating hematopoietic homeostasis during steady-state and stress conditions, we chose to study the function of OCIAD2 using the mouse model. Expression profiling by flow cytometry revealed high levels of OCIAD2 in LT-HSCs, which decreased along the differentiation hierarchy. Using an *ociad2* knockout mouse model, we identified that OCIAD2 is not essential for the maintenance and lineage specification of BM HSPCs, with a tissue-specific effect on splenic HSPCs. We also found that OCIAD2 is essential for normal steady-state erythropoiesis, and its loss enhances stress responses in medullary and extramedullary tissues. Molecular analysis revealed the importance of OCIAD2 in regulating the activation of STAT3 and STAT5 in the lineage-committed mouse BM cells. Further analysis showed that OCIAD2 regulates the levels of Asrij in mouse BM, indicating a possible functional compensation by Asrij during the absence of OCIAD2. Our study puts forth the cell-specific effects of OCIAD2 knockout on mouse hematopoiesis. It pitches the importance of an interplay between the OCIAD proteins in maintaining mouse hematopoietic homeostasis.

Chapter 2. Expression of OCIAD2 in mouse bone marrow hematopoietic cells

2.1 Introduction

The balance across the abundance of hematopoietic stem, progenitor, and differentiated cells is critical for maintaining hematopoietic homeostasis. Despite the progress achieved so far, several molecular details are unknown. This marks the necessity to identify and characterize novel molecules regulating hematopoiesis. High-throughput studies help in the identification of novel molecules. Ovarian Carcinoma Immunoreactive Antigen Domain (OCIAD) – containing protein 1, OCIAD1/Asrij is a novel regulator of mouse HSC quiescence and thence hematopoietic homeostasis (Sinha et al., 2019). However, the physiological role of its interactor, OCIAD2, is unknown in mouse hematopoiesis (Sinha et al., 2018). Before investigating the role of OCIAD2 in mouse hematopoiesis, we aimed to delineate its expression among the various hematopoietic populations. Firstly, we compared and analyzed the available data on the expression of the *ociad* genes in both mouse and human hematopoietic cells. Using flow cytometry, we profiled the expression of OCIAD2 in the various hematopoietic subpopulations of C57BL/6J mouse bone marrow (BM), whose results were in accordance with the high-throughput data, to a certain extent.

2.2 Materials and Methods

2.2.1 *In silico* analysis of *asrij/ociad1* and *ociad2* expression

Expression data of *ociad1* and *ociad2* genes, available from high-throughput databases like single-cell gene expression atlas (http://blood.stemcells.cam.ac.uk/geneMap.html) (Nestorowa et al. 2016), BloodSpot (http://servers.binf.ku.dk/bloodspot/) (Bagger et al., 2016), Gene Expression Commons (https://gexc.riken.jp/) (Seita et al., 2012), and The Human Protein Atlas (https://www.proteinatlas.org/) (Uhlen et al., 2019) were collected. Gene expression values of *ociad1* and *ociad2* available under the Mouse normal hematopoietic system category in the BloodSpot database were averaged and presented as a bar graph. The mean of normalized signal intensity values regarding the expression of *ociad1* and *ociad2,* from the Gene Expression Commons under the Mouse Hematopoiesis and Stroma category, were plotted as bar graphs without any further calculations.

2.2.2 Mice

C57BL/6J mice of both genders and 8-18 weeks of age were maintained in individually ventilated cages (IVCs) at the JNCASR's Animal Facility. Animal experiments were conducted following protocols approved by the Institutional Animal Ethics Committee (IAEC) of JNCASR (Project Number: MSI005).

2.2.3 Isolation of bone marrow cells

Femur and tibia bones were dissected from C57BL/6J mice, and BM was isolated as described previously (Sinha et al., 2019). Briefly, the epiphysis of the bones was removed, and the BM was flushed out using ice-cold sterile PBS. Cells were mixed gently to a homogenous cell suspension and passed through a 70 μm cell strainer to obtain a single-cell suspension.

2.2.4 Sorting of bone marrow cells

Sorting of desired BM cell type was done as described earlier (Sinha et al., 2019). Briefly, mouse BM cells were subjected to magnetic-activated cell sorting (MACS) as per the manufacturer's instructions to sort cell populations that do not express Lineage markers (Lin^-). The single-cell suspensions were incubated with anti-Lineage cocktail microbeads (Miltenyi, Germany) for 1 hour at 4 ºC, followed by washing the cells in ice-cold and sterile MACS buffer (PBS with 0.5% BSA and 2mM β-mercaptoethanol). Cells were then resuspended in MACS buffer and added to the LS column (Miltenyi, Germany), mounted onto the magnetic stand, and equilibrated with the MACS buffer. The Lin^--enriched cells were collected as the run-out suspension. After washing with the MACS buffer, the column was purged to collect the Lin^+-enriched fraction of the BM. The Lin^- cells were further immunomagnetically sorted for c-Kit^+ cells using anti-CD117 microbeads (Miltenyi, Germany) to obtain Lin^- c-Kit^+ BM cells.

2.2.5 Immunostaining

MACS-sorted Lin^- c-Kit^+ BM cells were stained with anti-Sca1 (PE-Cy7-labelled; BD Biosciences, USA), anti-CD150 (FITC-labelled; Invitrogen, USA), and anti-CD48 (APC-labelled; Invitrogen, USA) antibodies, to identify LSK, LT-HSCs, ST-HSCs, MPP2, and MPP3/4 sub-populations through flow cytometry as per marker combinations mentioned in Table-2.1. Simultaneously, the Lin^+ BM cells were stained with anti-CD19 and anti-CD3 (FITC-labelled; BD Biosciences, USA) antibodies to recognize B and T lymphocyte pools, respectively. Myeloid cells were identified by staining the Lin^+ cells with anti-CD11b (BD

Biosciences, USA) antibody, followed by anti-Rat-IgG (conjugated to Alexa-488; Molecular Probes, USA) antibody. This was followed by staining with an anti-OCIAD2 antibody (Sigma, USA). The immunostaining was performed using standard procedures as described previously

Bone marrow cell type	**Cell marker -** Fluorochrome	Marker combination (MACS, Flow cytometry)
HSPC	❖ Lineage (Lin) cocktail - APC • **CD3**-APC (T cell marker) • **CD45R (B220)**-APC (B cell marker) • **Ly6C & Ly6G**-APC (granulocyte marker) • **CD11b**-APC (pan-myeloid marker) • **Ter119**-APC (RBC marker) ❖ **Sca1**-PE-Cy7 ❖ **CD117/c-Kit**-PE	Lin^- $c\text{-}Kit^+$ $Sca1^+$ (LKS)
LT-HSC	❖ **CD150**-FITC ❖ **CD48**-APC	LKS $CD150^+$ $CD48^-$
ST-HSC		LKS $CD150^-$ $CD48^-$
MPP2		LKS $CD150^+$ $CD48^+$
MPP3/4		LKS $CD150^-$ $CD48^+$
Myeloid cells	❖ **CD11b** (primary antibody) ❖ Anti-Rt-IgG-Alexa 488 (secondary antibody)	$CD11b^+$
B cells	❖ **CD19**-FITC	$CD19^+$
T cells	❖ **CD3**-FITC	$CD3^+$

Table-2.1: List of cell surface markers, their respective fluorochromes, and their combinations to identify various hematopoietic cell types of bone marrow. Due to the spectral overlap of fluorochromes conjugated to various antibodies used in this study, a combination of MACS and flow cytometry was used, as indicated in red and blue, respectively, for isolation and analysis of different hematopoietic cells (adapted from Sinha S. Ph.D. thesis, 2019).

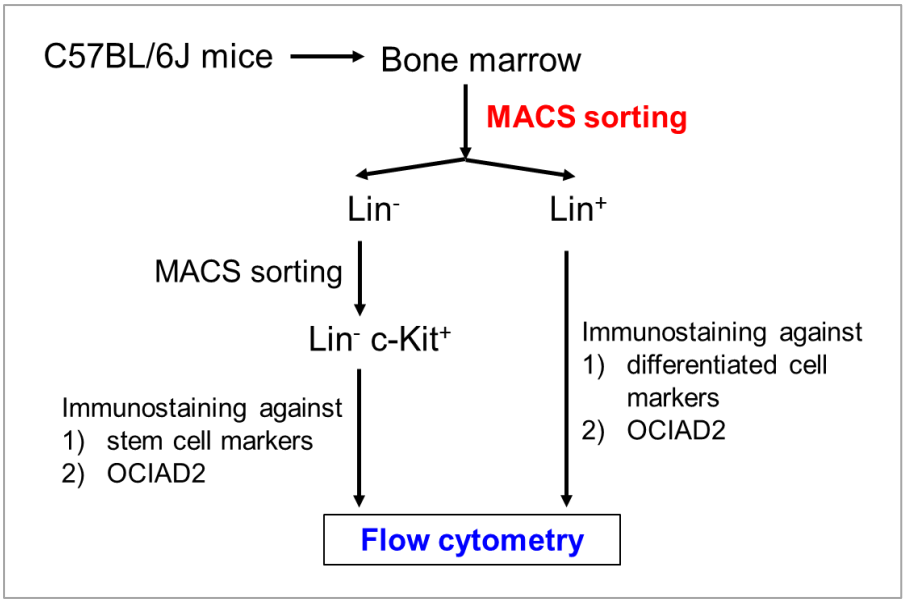
(Sinha et al., 2013, Sinha et al., 2018, Sinha et al., 2019). Briefly, cells either unstained or stained against necessary surface markers were fixed in 2% paraformaldehyde solution for 10 minutes at 4 ºC, followed by two washes in ice-cold sterile PBS. The cells were permeabilized in 0.1% TritonX-100 (Sigma, USA) and washed in PBS before intracellular staining. Cells were then incubated with the anti-OCIAD2 antibody overnight at 4 ºC, followed by staining with anti-Rabbit-IgG (conjugated to Alexa-568; Molecular Probes, USA) for 1 hour, at 4 ºC, in the dark, followed by washes in ice-cold sterile PBS. The staining was checked under a microscope before proceeding for flow cytometry.

2.2.6 Flow cytometry analysis

Stained cells along with appropriate controls like unstained, isotype antibody-stained, single-color stained, no primary control, and fluorescence minus one (FMO) control cells were analyzed using FACS Aria II (SORP) or Aria III (BD Biosciences, USA). About 5×10^4 events were analyzed for undifferentiated cells, and 1×10^5 events were analyzed for differentiated cells. Data was analyzed using FlowJo (version 10.7.1).

2.2.7 Statistical analysis and graphical representation

Statistical significance of gene expression, frequency of cell types, and median fluorescence intensity values was calculated using ANOVA: Single Factor and was indicated by * p<0.05, ** p<0.01, or *** p<0.001. Data Analysis Tool pack in Microsoft Excel 2016 was used for the statistical analysis. Graphs representing the mean ± standard error mean (SEM) were plotted using SigmaPlot 11.0.



Schematic-2.1: Outline of analysis of BM cells from C57BL/6J mice of age 2-4 months, involving a combination of magnetic-activated cell sorting (MACS) and flow cytometry.

2.3 Results

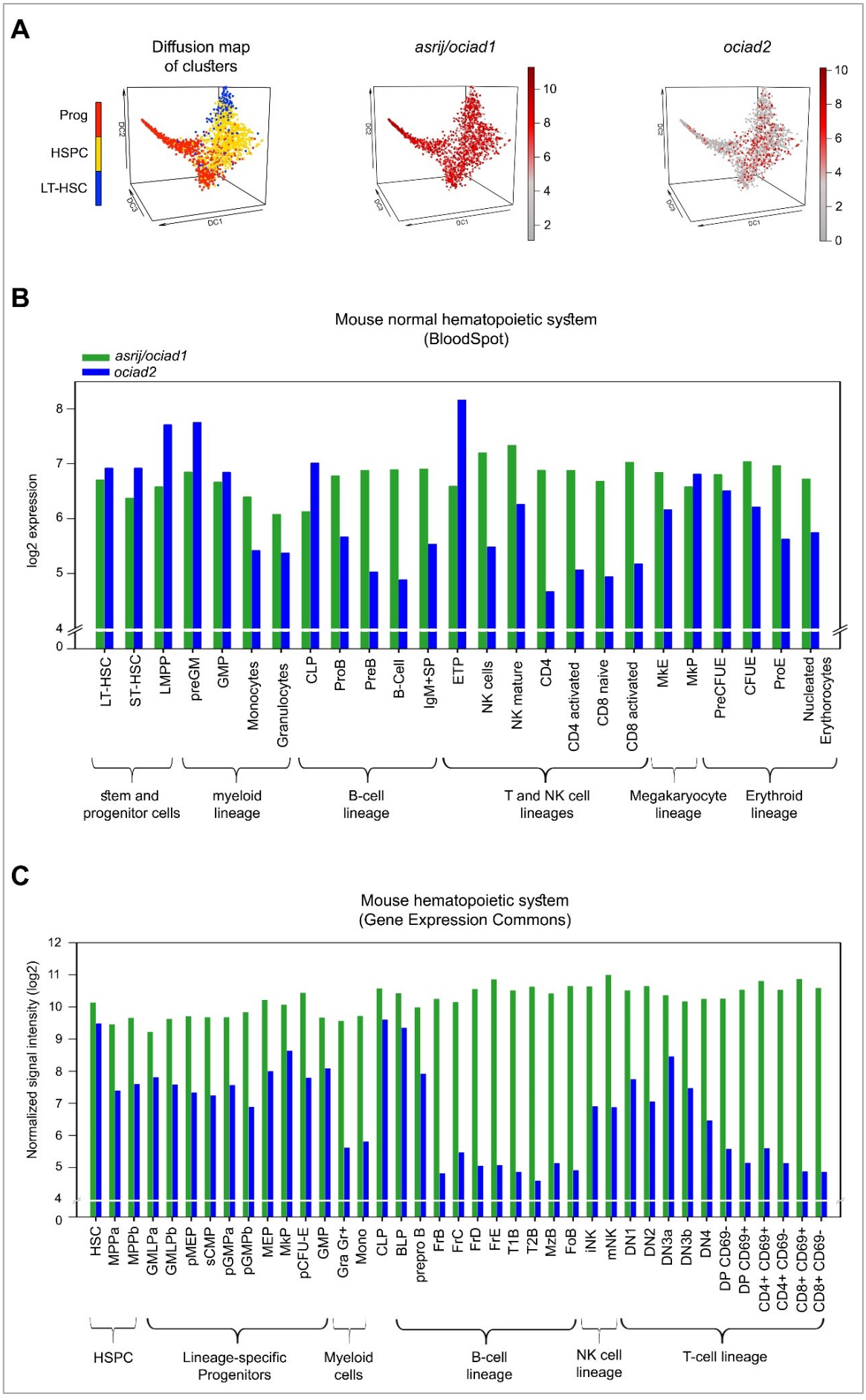
2.3.1 *In silico* analysis of *ociad2* expression in hematopoiesis.

Transcriptome databases provide invaluable insights into the expression pattern of a gene in each physiological situation. These can result from studies at the single-cell level or from a group of cells. Single-cell gene expression study (http://blood.stemcells.cam.ac.uk/geneMap.html) (Nestorowa et al., 2016) shows an expression of *asrij*/*ociad1* in most of the analyzed mouse BM hematopoietic progenitors and nearly half of the LT-HSC. The reported expression of Asrij in nearly 46.8±2.84% of LT-HSC in C57BL/6J mouse BM supports this data (Sinha et al., 2019). Interestingly, only a small fraction of analyzed stem and progenitor cells are detected with high expression of *ociad2* and an overall low level of expression, as per the heatmap scale (Figure-2.1A). This is in accordance with the lower expression of OCIAD2 in BM relative to that of other tissues in C57BL/6J mice (Sinha et al., 2018).

Databases like BloodSpot (Bagger et al., 2016) and Gene Expression Commons (Seita et al., 2012), based on microarray analyses, project a much broader picture of gene expression in mouse hematopoiesis by including both undifferentiated and differentiated cells. These databases show that in BM, the expression of *asrij* does not vary much across different cell types, with a similar scenario in the human hematopoietic cells (Figure-2.1D,E). Interestingly, *ociad2* expression is relatively higher in mouse hematopoietic stem and progenitor cells than in differentiated cells (Figure-2.1B,C). Intriguingly, this pattern is not seen in human hematopoietic cells (Figure-2.1D,E), indicating species-specific differences in the *ociad2* expression.

2.3.2 Expression profiling of OCIAD2 in mouse bone marrow hematopoietic cells.

Asrij and OCIAD2 interact with each other (Sinha et al., 2018), and the protein levels of Asrij are essential for its *in vivo* function (Sinha et al., 2019). Hence, despite the lower expression of OCIAD2 in mouse BM (Sinha et al., 2018), we reasoned that it is worth studying its expression and function in mouse hematopoietic cells. We profiled the expression of OCIAD2 in the unfractionated and various subpopulations of C57BL/6J mouse BM through flow cytometry.


A
Diffusion map of clusters
asrij/ociad1
ociad2
Prog
HSPC
LT-HSC
DC1
DC2
DC3
B
Mouse normal hematopoietic system (BloodSpot)
asrij/ociad1
ociad2
log2 expression
LT-HSC
ST-HSC
LMPP
preGM
GMP
Monocytes
Granulocytes
CLP
ProB
PreB
B-Cell
IgM+SP
ETP
NK cells
NK mature
CD4
CD4 activated
CD8 naive
CD8 activated
MkE
MkP
PreCFUE
CFUE
ProE
Nucleated Erythorocytes
stem and progenitor cells
myeloid lineage
B-cell lineage
T and NK cell lineages
Megakaryocyte lineage
Erythroid lineage
C
Mouse hematopoietic system (Gene Expression Commons)
Normalized signal intensity (log2)
HSC
MPPa
MPPb
GMLPa
GMLPb
pMEP
sCMP
pGMPa
pGMPb
MEP
MkP
pCFU-E
GMP
Gra Gr+
Mono
CLP
BLP
prepro B
FrB
FrC
FrD
FrE
T1B
T2B
MzB
FoB
iNK
mNK
DN1
DN2
DN3a
DN3b
DN4
DP CD69-
DP CD69+
CD4+ CD69+
CD4+ CD69-
CD8+ CD69+
CD8+ CD69-
HSPC
Lineage-specific Progenitors
Myeloid cells
B-cell lineage
NK cell lineage
T-cell lineage

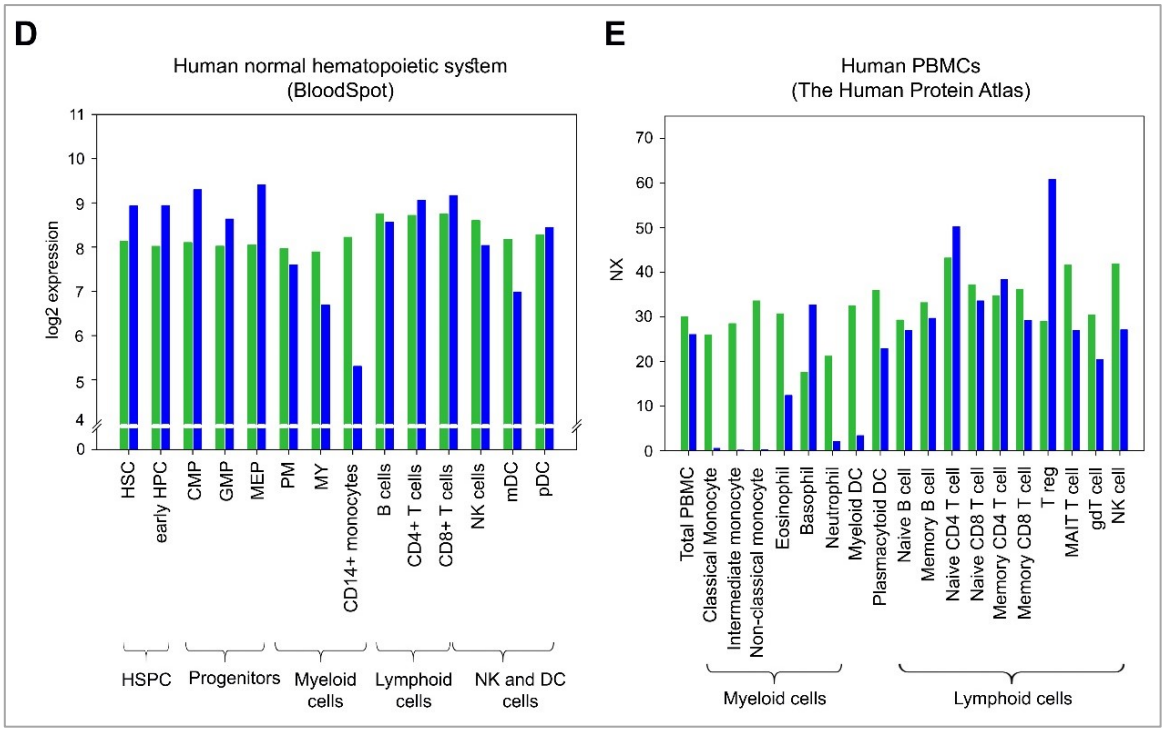


Figure-2.1: *In silico* expression analysis of *ociad* genes in mouse and human hematopoietic cells.

(A) Diffusion map of hematopoietic clusters showing expression of *asrij*/*ociad1* and *ociad2*, in LT-HSC = long-term hematopoietic stem cells; HSPC = hematopoietic stem/progenitor cells; and progenitor subpopulations including LMPP = lymphoid-primed multipotential progenitor, CMP = common myeloid progenitor, MEP = megakaryocyte-erythroid progenitor, and GMP = granulocyte-macrophage progenitor cells obtained from Single-cell gene expression Atlas (Nestorowa et al., 2016). **(B, C)** Graphs depicting the expression pattern of *asrij* and *ociad2* in mouse hematopoietic cells during steady-state hematopoiesis as available from BloodSpot (Bagger et al., 2016) (B) and Gene Expression Commons (Seita et al., 2012) (C) databases. **(D, E)** Graphs comparing the expression of *ociad* genes in human hematopoietic cells as available from BloodSpot (D) and The Human Protein Atlas (Uhlen et al., 2019) (E).

We found the expression of OCIAD2 in 88.8±7.4% of unfractionated/total BM cells, HSPC (98.7±0.75%), LT-HSC (99.1±0.89%), ST-HSC (99.9±0.11%), MPP2 (96.4±3.05%), and MPP3/4 cells (98.7±2.2%). Also, the differentiated cells like myeloid cells (99.3±0.63%), B cells (96.1±2.62%), and T cells (95.1±3.03%) were positive for OCIAD2 (Figure-2.2). Analysis of median fluorescence intensity (MFI) showed significantly decreased levels of OCIAD2 protein, down the differentiation hierarchy, from the LT-HSC (Figure-2.2), in accordance with the *in-silico* analysis from BloodSpot and Gene Expression Commons (Figure-2.1B, C).

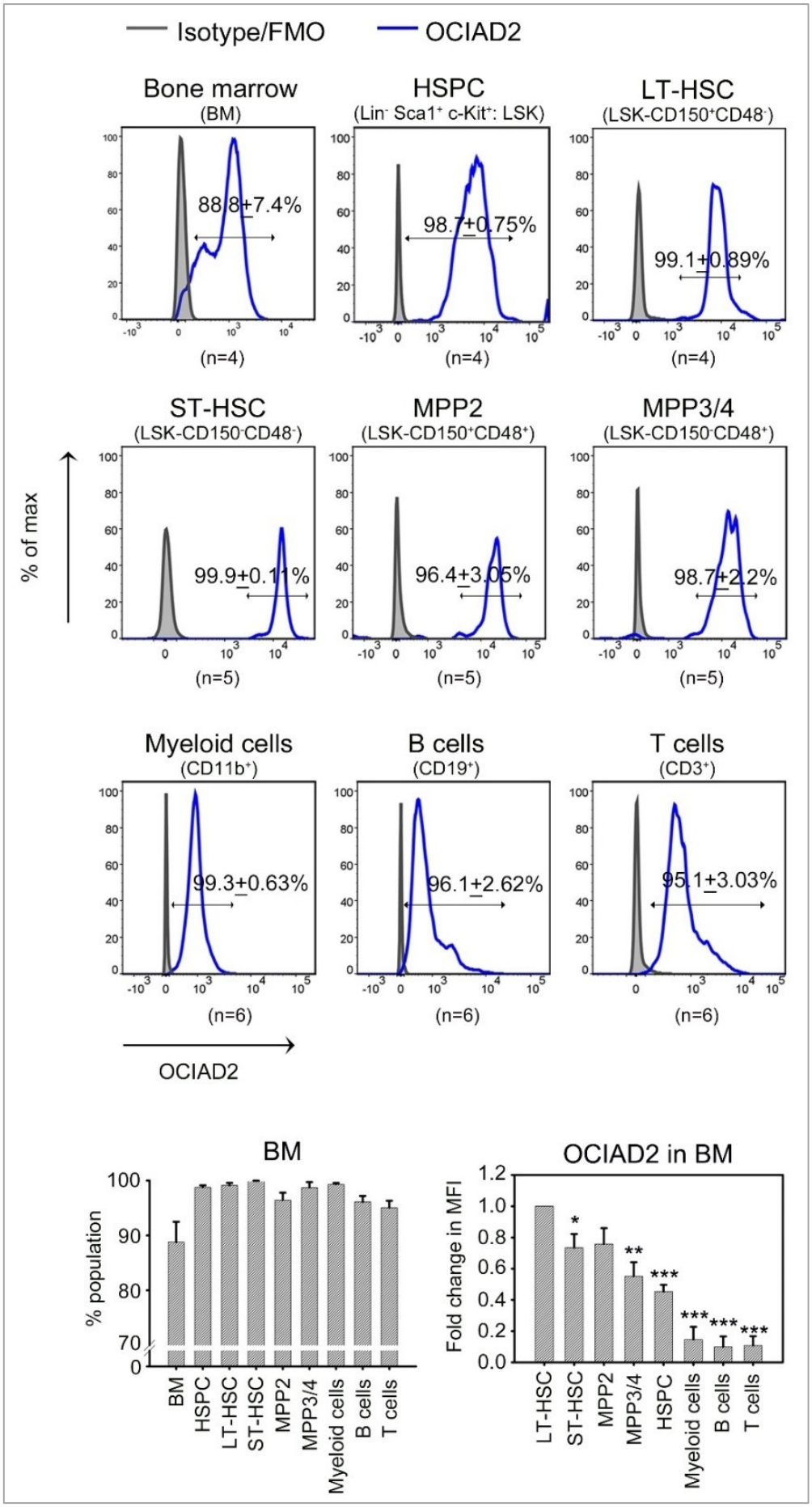

Isotype/FMO
OCIAD2
Bone marrow
(BM)
88.8+7.4%
(n=4)
HSPC
(Lin- Sca1+ c-Kit+: LSK)
98.7+0.75%
(n=4)
LT-HSC
(LSK-CD150+CD48-)
99.1+0.89%
(n=4)
ST-HSC
(LSK-CD150-CD48-)
99.9+0.11%
(n=5)
MPP2
(LSK-CD150+CD48+)
96.4+3.05%
(n=5)
MPP3/4
(LSK-CD150-CD48+)
98.7+2.2%
(n=5)
% of max
Myeloid cells
(CD11b+)
99.3+0.63%
(n=6)
B cells
(CD19+)
96.1+2.62%
(n=6)
T cells
(CD3+)
95.1+3.03%
(n=6)
OCIAD2
BM
% population
BM
HSPC
LT-HSC
ST-HSC
MPP2
MPP3/4
Myeloid cells
B cells
T cells
OCIAD2 in BM
Fold change in MFI
LT-HSC
ST-HSC
MPP2
MPP3/4
HSPC
Myeloid cells
B cells
T cells

Figure-2.2: Mapping expression of OCIAD2 in mouse bone marrow hematopoietic cells.

Histograms representing the flow cytometry analysis for identifying percentage of whole BM cells and its hematopoietic subpopulations, expressing OCIAD2 in wild type mice, accompanied by the graphical representation of the same and the variations in the fold change of median fluorescence intensity of OCIAD2 along the hematopoietic differentiation hierarchy, normalized to LT-HSC. C57BL/6J mice of both genders of age 2-5 months were used. Statistical significance of data was calculated using ANOVA: Single Factor analysis, with $\alpha=0.05$ and were indicated by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

2.4 Discussion

Expression profiling of the gene of interest gives preliminary insights into its possible functions in the target cell or tissue. The advent of high-throughput techniques like microarray and RNA sequencing (RNA Seq) analysis has ramped up the speed and the scale of data collection on the transcriptional status of genes. Nevertheless, these results have to be validated due to errors generated by various technical drawbacks in high-throughput techniques (Russo et al., 2003; Hicks et al., 2018).

The high-throughput databases cited in this study show the expression of *ociad2* in various mouse hematopoietic cells, but there are no reports on its protein expression profile in these cells. Moreover, gene expression levels need not correlate with protein levels (Vogel and Marcotte, 2012). Studying protein levels by immunoblotting is a widely accepted quantitative approach. However, it poses practical difficulties in sampling enough number of rare cell types like HSCs, MPPs, etc. and also does not reveal the heterogeneity of expression in a given population, which is facilitated by flow cytometry, a semi-quantitative technique. Hence, we opted for flow cytometry to profile OCIAD2 expression and showed that OCIAD2 is expressed in most of the differentiated and undifferentiated cells of mouse BM, with its expression decreasing down the differentiation hierarchy. The notable difference between the size of OCIAD2 expressing fractions of LT-HSC and progenitors detected by our flow cytometry analysis and the single-cell gene expression atlas database could be due to the following reasons.

1. The difference in the markers used to identify the LT-HSCs (Lin^- $Sca1^+$ $c\text{-}Kit^+$ $CD34^-$ $Flk2^-$) and progenitors (Lin^- $Sca1^-$ $c\text{-}Kit^+$) (Nestorowa et al. 2016), from the marker combination used in this study (Table-2.1).
2. The effect of gender on the level of gene expression (Kobayashi et al., 2006), as Nestorowa et al., used only female C57BL/6, whereas we used mice of both genders of C57BL/6J strain.
3. The impact of technical drawbacks of single-cell RNA-sequencing, like capture efficiency, library quality, amplification bias, and batch effects (Hicks et al., 2018).

The reported % population of mouse BM and its subpopulations expressing Asrij (Sinha et al., 2019) is lower than that of OCIAD2. While ~99% of the LT-HSC population express OCIAD2, only ~46% express Asrij (Sinha et al., 2019), indicating heterogeneity in expression of OCIAD proteins in the LT-HSC population. Whether this has any relation to the reported heterogeneity of HSCs based on lineage-bias merits investigation (Haas et al., 2018). However, Asrij depletion leads to loss of HSC quiescence and myeloproliferation in mice (Sinha et al., 2019). These observations indicate that OCIAD2 might be playing a cell-specific role, despite its expression in various hematopoietic cells, as the presence of a protein in a cell type does not always essentially reflect its essentiality in the maintenance of that cell (Arndt et al., 2013). Overall, the results described in this chapter hint at a possible role of OCIAD2 in mouse hematopoiesis which merits further investigation.

2.5 Acknowledgements

I thank Dr. Saloni Sinha and Alice Sinha for their kind help in conducting the flow cytometry analysis of hematopoietic stem cells and progenitors.

Chapter 3. Analysis of the effects of OCIAD2 depletion on hematopoietic stem and progenitor cells.

3.1 Introduction

Gene knockout strategies in mice have been of great help for understanding the role of a target gene in a given physiological process. Of the various strategies, the constitutive global knockout is crucial to identify and understand the role of a gene in overall physiology apart from the genetic and molecular processes of interest. The Cre-LoxP recombinase system is routinely used to generate global as well as conditional knockout mouse models (Kim et al., 2018).

OCIAD2 is expressed in various vital organs of C57BL/6J mice, hence we chose a strategy that would allow global as well as conditional knockout, in case the former led to lethality (Sinha et al., 2018). Our study showed that OCIAD2 is expressed in various BM hematopoietic subpopulations (Chapter-2), suggesting a role in mouse hematopoiesis. Hence, we studied the *in vivo* function of OCIAD2, using the *ociad2* null mouse model, generated earlier in our laboratory through Cre-loxP technology (see Materials and Methods) and made available for this study. Since *asrij* null mice show a myeloproliferative disorder-like phenotype with loss of HSC quiescence, splenomegaly, and deregulated PB counts from 6 months of age (Sinha et al., 2019), we examined the *ociad2* KO mice of 6-10 months of age with age- and gender-matched floxed ($ociad2^{fl/fl}$) mice. Wild type ($ociad2^{+/+}$; WT) and heterozygous knockout ($ociad2^{+/-}$; +/-) mice were also included in the study as required.

BM is the primary hematopoietic organ in mice, and spleen acts as the primary extramedullary site (Oda et al., 2018). Underlying the deregulation of hematopoiesis are the cellular and molecular changes in BM, which often manifest as detectable variations in the spleen and peripheral blood (PB) parameters. Hence, we investigated the effects of OCIAD2 KO in the BM, spleen and PB. Flow cytometry has revolutionized the study of hematopoiesis. Based on the detection of cell-surface markers, cell types of various developmental stages can be identified and studied. However, flow cytometry is primarily qualitative (Mittag and Tárnok, 2009; Maecker et al., 2012). Hematopoiesis can be quantitatively assessed by the colony-forming unit (CFU-C) assays, which help score the lineage commitment potential of HSC based on the type, count, and size of progenitor colonies formed in the synthetic culture

medium. Real-time-quantitative PCR (RT-qPCR) and immunoblotting help understand the observed phenotype at the molecular level. Therefore, a combinatorial approach is required to understand the various aspects of hematopoiesis. This chapter will discuss the anatomical, physiological, cellular, and molecular phenotypes concerning hematopoiesis, observed in the OCIAD2 KO mice.

3.2 Materials and Methods

3.2.1 Generation of *ociad2* knockout mice

ociad2$^{fl/+}$ heterozygous recombinant mice with floxed exon 3 of *ociad2* (Chromosome 5: 73332104 – 73332200) were generated using a strategy described earlier (Sinha et al., 2019) (Figure-3.1A, B) and were bred to homozygosity to generate *ociad2*$^{fl/fl}$ (floxed) mice. Exon 3 was targeted for floxing as it, along with exons 4, 5, and 6, codes for the OCIA domain that has been recently reported to be important for the localization and molecular functions of OCIAD2 (Sinha et al., 2018). Floxed mice were crossed with *CMV-Cre*$^{+}$ mice (Schwenk et al., 1995) to generate *ociad2* heterozygous knockout (*ociad2*$^{+/-}$; *CMV-Cre*$^{+}$; +/-) mice, which were bred to generate *ociad2* homozygous knockout (*ociad2*$^{-/-}$; KO) mice. Excision of the exon 3 by Cre recombinase results in frameshift mutation generating a premature stop codon and is predicted to generate a peptide of 28 amino acid residues. KO mice are viable and fertile without any overt phenotype. The floxed, heterozygous knockout and KO mice were generated earlier in our lab and were made available for this study.

3.2.2 Genetic identification and validation of floxed, heterozygous knockout, and KO mice

Floxed, heterozygous knockout, and KO mice were genetically identified by genotyping PCR of genomic DNA. The genomic DNA was isolated from the tail clips either by kit (GenElute Mammalian Genomic DNA MiniPrep Kit, Cat. No. G1N70-1KT, Sigma) or by modified HotSHOT method using 50mM NaOH and 1M Tris (pH 8.0) (Truett et al., 2000). The PCR was conducted using the primers; *ociad2* F1 (5'- CTGCACATCCACAGAGGAGAG-3') and *ociad2* R1 (5'- CGCTTCTGAGCTGAGATGCTGGTG-3'), to amplify the wild type locus (487 bp) and the floxed locus (562 bp). Similarly, the excised copy (689 bp) was identified using *ociad2* F2 (5'-AGAGCCATCTGTGTTTACCCTG-3') and *ociad2* R1 (5'-CGCTTCTGAGCTGAGATGCTGGTG-3') primers (Figure-3.1C).

The mice were further validated by immunostaining, Western blotting, and RT-PCR of the whole BM (Figure-3.2). Similarly, the heterozygous mutant mice were identified by genotyping PCR with the presence of both WT copy and excision copy (Figure-3.3A), further validated by Western blotting (Figure-3.3B).

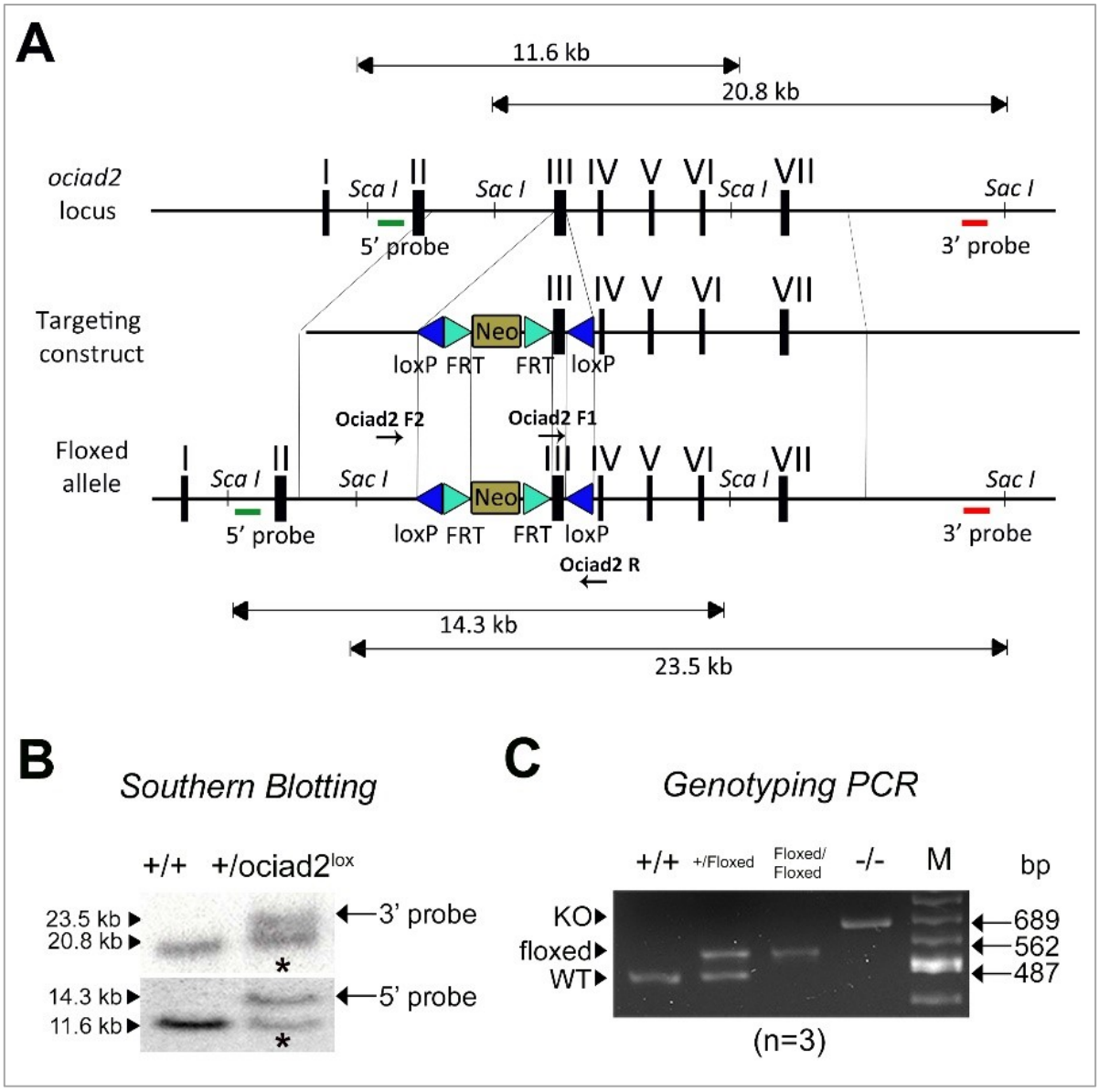


Figure-3.1: Generation and genotyping of *ociad2* floxed, heterozygous knockout (+/-), and KO mice. (A) Schematic depicting strategy for generation of floxed allele of *ociad2* at exon 3. Black rectangles: exons; blue triangles: loxP sites; green triangles: *frt* sites; 5'probe and 3'probe: probes for Southern Blotting; black arrow: genotyping primers (Ociad2 F1, Ociad2 F2, and Ociad2 R1). **(B)** Southern blotting analyses to confirm the

generation of heterozygous floxed *ociad2* mice. Arrows indicate the shifted band due to the insertion, and asterisk marks the positive clone. 5' and 3' probes used for Southern Blotting are indicated in Figure 3.1A. **(C)** Genotyping by PCR analysis to identify control (+/+), floxed heterozygous (+/floxed), floxed homozygous (floxed/floxed), and homozygous *ociad2* knockout (-/-) mice. Homozygous *ociad2* knockout mice are obtained at F2 generation from a cross between *ociad2* floxed homozygous and *CMV-Cre* mice.

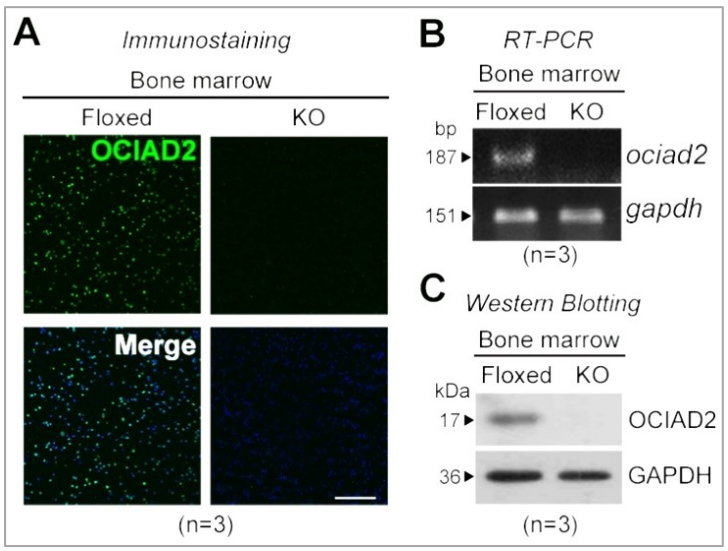


Figure-3.2: Validation of *ociad2* knockout mice in BM cells by immunostaining (A), RT-PCR (B), and immunoblotting (C). Expression levels of *gapdh* and GAPDH were used as loading control in RT-PCR and immunoblotting, respectively (n=3 mice per genotype). In immunostaining, nuclei were marked with DAPI (blue). Scale bar represents 100 µm.

All animal experimental protocols were approved by the Institutional Animal Ethics Committees (IAEC) of JNCASR (Project Number: MSI005) and NCBS (Project Number: KVR-2(2)/2015) and by the Institutional Animal Care and Use Committee (IACUC) of RIKEN Kobe Branch (Approved Number: A2001-03). Maintenance of mouse stocks and their usage for various experiments was performed according to the guidelines of the animal ethics committees of JNCASR, NCBS, and RIKEN BDR. *Ociad2* floxed (Accession No. CDB0696K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html), heterozygous knockout and homozygous knockout mice of both genders and different ages were used for the study.

As the *ociad2* gene neighbors *asrij* and affects its protein levels (see Chapter-4), we named the knockout allele as *ociad2*^{padosan} (Hindi: *padosan* means neighbor), abbreviated to *ociad2*^{pdsn}. The nomenclature was approved by Mouse Genome Informatics as per the details given below.

Name of the KO allele : *Ociad2*$^{tm1.1Msin}$ (MGI:6840945)
Synonyms of the KO allele: *Ociad2*^{pdsn} and *Ociad2*^{Padosan}
Name of the KO mouse strain: B6;CBA-*Ociad2*$^{tm1.1Msin}$/Msin (MGI:6840972)

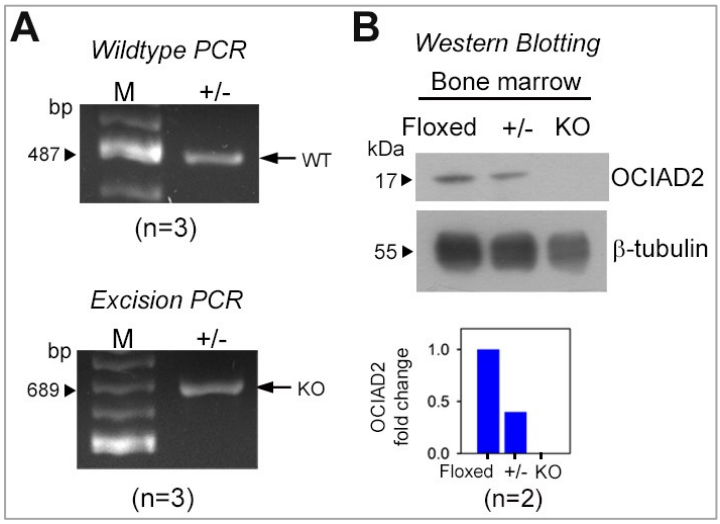


Figure-3.3: Identification and validation of heterozygous knockout (+/-) mice by genotyping PCR (A; n=3 mice per genotype) and immunoblotting analysis (B; n=2 mice per genotype). Expression levels of β-tubulin were used as loading control for immunoblotting.

3.2.3 Body weight and organ relative weight measurement

The body weight and absolute weight of various organs were measured using a weighing balance (Sartorius, Germany). The absolute weight of an organ was divided by the total body weight and multiplied by 100 to get its % body weight or relative weight. Organs from floxed, heterozygous knockout, and KO mice were imaged to scale.

3.2.4 Isolation of bone marrow cells, splenocytes, and peripheral blood cells

BM isolation and cell suspension preparation was done as described in Chapter-2. Single-cell suspension of splenocytes was obtained by mashing the spleen in ice-cold, sterile PBS against a 70 µm cell strainer (Sinha et al., 2019). Cellularity was determined by counting the diluted cell suspensions in a hemocytometer. PB was collected from anesthetized mice by puncturing retro-orbital plexus and was mixed in equal volumes with 0.5M EDTA (aq.) to prevent coagulation. The blood-EDTA mix was RBC lysed before flow cytometry analysis (Sinha et al., 2019).

3.2.5 Flow cytometry

BM, spleen, and RBC lysed-PB cells were stained for various cell surface markers as per the antibody combination tabulated in Table-2.1 of Chapter-2. Flow cytometry analyses of Lin^-, Lin^- $cKit^+$ (LK) and hematopoietic stem and progenitor cells (HSPCs; LSK) cells was performed using the Mouse Hematopoietic Stem and Progenitor Cell Isolation Kit (BD Biosciences, USA). 25×10^5 events were acquired for analyzing HSPCs across BM, spleen, and blood. 5×10^5 events were acquired for differentiated cells in the BM and 5×10^4 events in PB.

FACS Aria II or Aria III (BD Biosciences) was used for the analyses, and the data was analyzed using FlowJo (version 10.7.1).

3.2.6 Cell sorting

Immunomagnetic sorting of LK cells and $CD19^+$ cells from BM was done as described in Chapter-2. Anti-CD19 microbeads (Miltenyi, Germany) were used to sort $CD19^+$ cells.

3.2.7 *In vitro* culture assay

CFU-C assay was performed using Methocult^{TM} GF medium (M3434; STEMCELL Technologies, Canada), as per the manufacturer's instructions. Briefly, about 25000 unfractionated BM cells were thoroughly mixed with ~ 1.1 mL of Methocult and seeded in a 35 mm non-adherent culture dish. Three such dishes were maintained per genotype and were incubated for 12-14 days at 37 ºC, 5% CO2, along with a dish loaded with autoclaved MilliQ water to maintain humidity. Different colonies developed post-incubation were imaged on the Zeiss Lumar V.12 microscope. Colonies were then manually identified and counted. The colony size was calculated using ImageJ software. The experiment was conducted on three independent biological samples.

3.2.8 Apoptosis assay

BM cells either stained with anti-CD11b (BD Biosciences, USA) followed by anti-Rat-IgG antibody (conjugated with Alexa-568; Molecular Probes, USA) or MACS-enriched for $CD19^+$ cells were stained with Anti-Annexin V (FITC-labelled) antibody using Annexin V Apoptosis Detection Kit (Invitrogen, USA), as per manufacturer's protocol. 7-AAD (BD Biosciences, USA) was used to exclude the dead cells. The experiment was conducted on three independent biological samples.

3.2.9 Real-Time – quantitative PCR (RT-qPCR)

Manufacturer's instructions were followed for Trizol® (Invitrogen, USA)-based isolation of RNA from BM and reverse transcription of DNase-treated RNA using Superscript II (Thermo Fisher Scientific, USA). Transcript levels of various genes were detected using the primers mentioned below. Quantification of gene expression was done using EvaGreen-based qPCR reactions in a CFX96 qPCR machine (BioRad, USA), with *gapdh* as the reference gene. All

reactions were performed on three independent biological samples maintaining duplicates per reaction unless otherwise mentioned.

Primer	**Sequence (5'-3')**
ociad2 forward	AGAGCACTCGACGGCAATAG
ociad2 reverse	CTCTCTTCTTGACACTCTCTG
PU.1 forward	GTTTCCTACATGCCCCGGATGTG
PU.1 reverse	CCAAGCCATCAGCTTCTCCATC
Tnfr1 forward	CAACGTCCTGACAATGCAGACC
Tnfr1 reverse	GAAACGCATGAACTCCTTCCAGCG
Ikaros forward	GCAATGTCGCCAAACGTAAGAGC
Ikaros reverse	GTTGATGGCATTGTTGATGGCCTG
E2A forward	GGATCTGAGGTTAATGGCTCGCTC
E2A reverse	CCTGCATCGTAGTTGGGGGATAAG
Ebf1 forward	GCAAAGGGACACCAGGCAGATTC
Ebf1 reverse	GCATCCCATACAGGGCTTCAACC
IL-7r forward	GCAAGGGGTGAAAGCAACTGGAC
IL-7r reverse	GTAGAACTTGGACTCCACTCGCTC
gapdh forward	TGCCCCCATGTTTGTGATG
gapdh reverse	TGTGGTCATGAGCCCTTCC

3.2.10 Immunofluorescence and confocal microscopy

Staining and imaging of cells were done following standard procedures described previously (Sinha et al., 2013; Sinha et al., 2018). BM cells were stained using an antibody against OCIAD2 (SIGMA, USA) followed by the anti-Rabbit-IgG (conjugated to Alexa-568; Molecular Probes, USA). Images were acquired using the Carl Zeiss LSM880 confocal microscope and were processed in ZEN software. Brightness/contrast was adjusted uniformly using Adobe Photoshop CS6. The experiment was conducted on three independent biological samples.

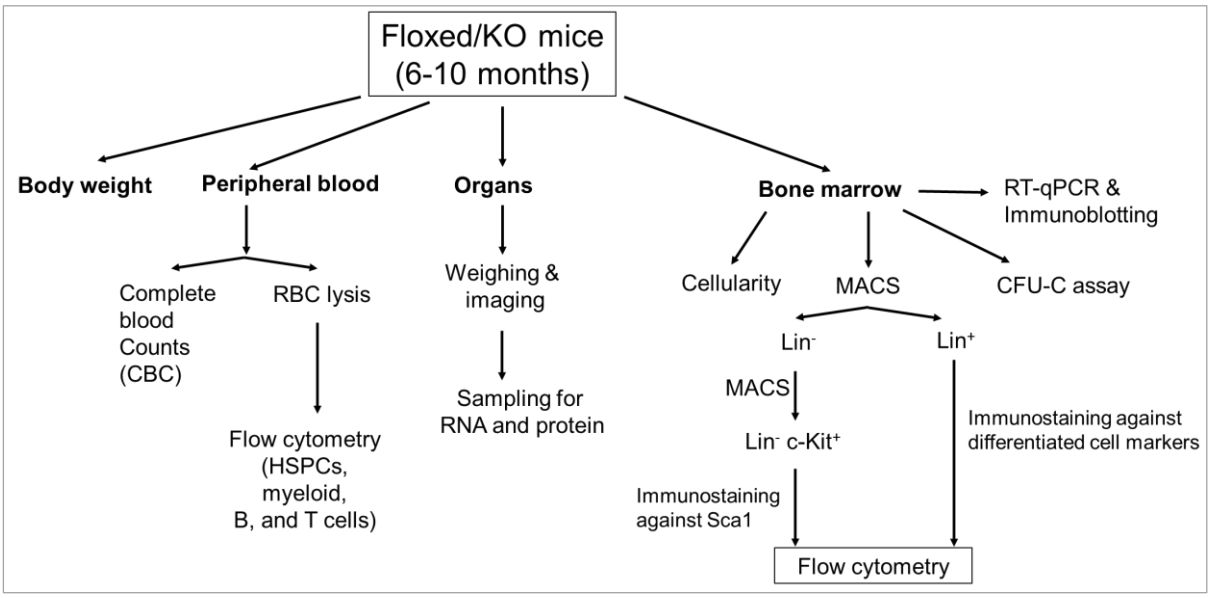
3.2.11 Immunoblotting and densitometry analysis

Immunoblotting of BM cells was done as described previously (Sinha et al., 2013; Sinha et al., 2018). Lysates were probed using an antibody against OCIAD2 (Sigma, USA) and normalized to GAPDH (Sigma, USA) or β-tubulin (Cell Science Technologies, USA). HRP-conjugated anti-Rabbit or anti-Mouse-IgG antibodies (GeNei, India) were used as secondary antibodies. Densitometry-based quantification of the captured enhanced chemiluminescence (ECL) signals

was done using ImageJ, as per standard procedures (Sinha et al., 2013; Sinha et al., 2018). The experiment was performed on at least three independent biological samples unless otherwise mentioned.

3.2.12 Statistical analysis and graphical representation

Statistical significance of absolute weight, relative weight, cell frequencies, absolute cell counts, colony numbers, colony size, signal intensity measurements, and transcript levels was calculated using ANOVA: Single Factor and was indicated by * $p<0.05$, ** $p<0.01$, or *** $p<0.001$. Data Analysis Tool pack in Microsoft Excel 2016 was used for the statistical analysis. Graphs representing the mean ± standard error mean (SEM) were plotted using SigmaPlot 11.0.



Schematic-3.1: Outline of various analyses conducted on floxed and KO mice, at organismal, tissue, cellular, and molecular levels, under steady-state conditions.

3.3 Results:

3.3.1 KO mice show mildly larger spleen and smaller kidneys

Loss of OCIAD2 did not affect mouse body weight (Figure-3.4). Gross abnormalities were absent in both heterozygous knockout and KO mice. However, both heterozygous knockout and KO mice showed mild splenomegaly, while only the KO mice showed smaller kidneys than floxed mice. The relative weight of other vital organs like the brain, thymus, heart, liver, and testis was unchanged upon deleting OCIAD2 (Figure-3.4).

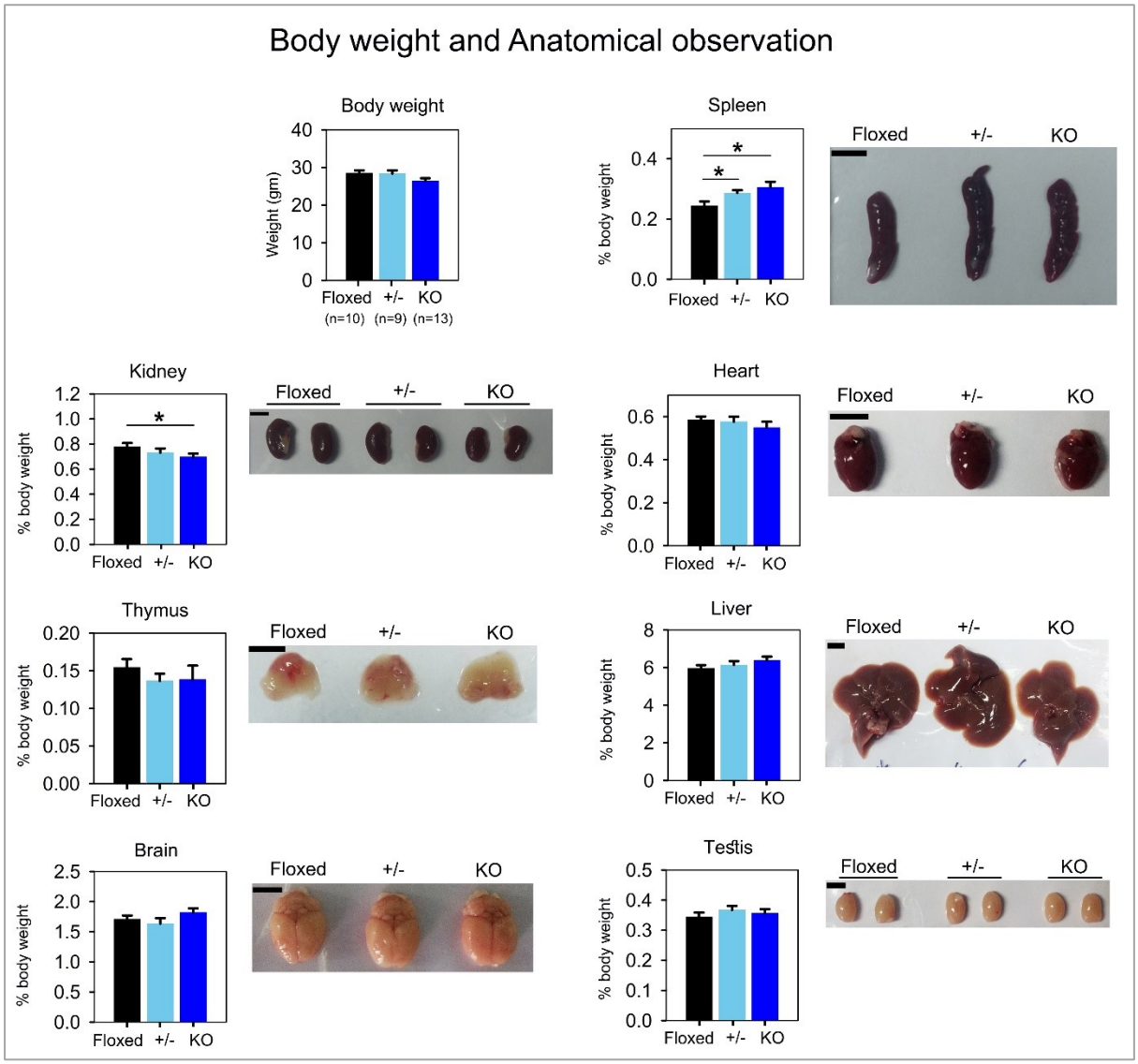


Figure-3.4: Loss of OCIAD2 leads to splenomegaly and kidney atrophy in mice. Graphs representing variation in the body weight and relative weight of organs accompanied by their representative images across floxed (n=10), +/- (n=9), and KO (n=13) mice. Scale bar (black) represents 0.5 cm. Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05.

3.3.2 OCIAD2 KO leads to increased frequency of bone marrow myeloid and B cells

To check for the role of OCIAD2 in mouse hematopoiesis, we firstly analyzed the BM, the primary site of adult hematopoiesis. Cellularity of BM was unchanged in both heterozygous knockout and KO mice (Figure-3.5A). Through flow cytometry analysis, we observed that the abundance of the total undifferentiated pool (Lin^-), early myeloid progenitors (Lin^- $c\text{-}Kit^+$; LK), and HPSCs (LSK) was unaffected in both heterozygous knockout and KO mice (Figure-3.5B). This indicated a possibly dispensable role of OCIAD2 in maintaining hematopoietic progenitors.

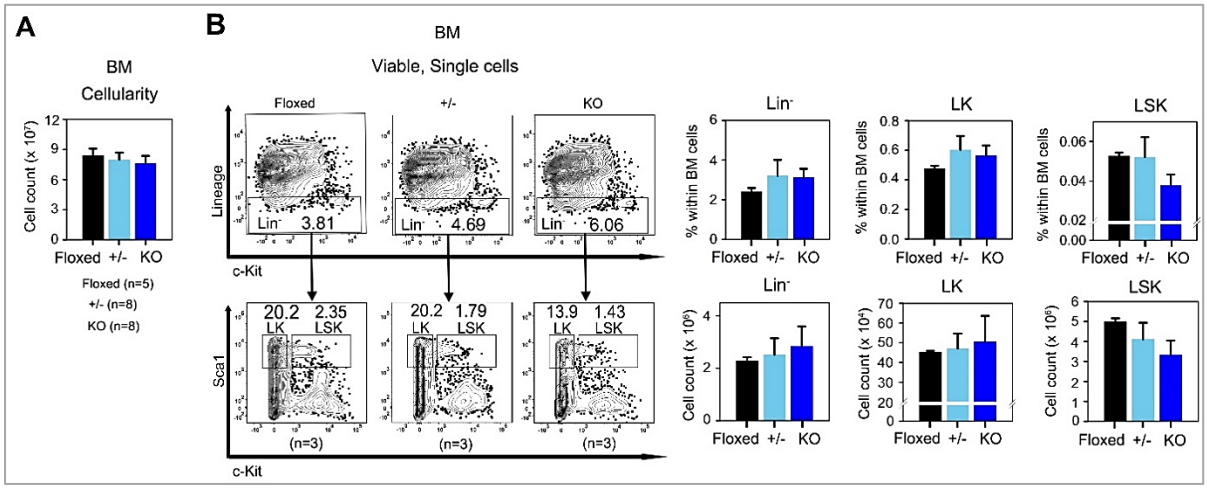


Figure-3.5: OCIAD2 depletion does not alter total cellularity of mouse bone marrow and its abundance of hematopoietic progenitors. (A) Graph representing variation in the total cellularity of bone marrow across floxed (n=5), +/- (n=8), and KO (n=8) mice. **(B)** Representative flow cytometry plots showing the frequency of bone marrow Lin^-, LK, and LSK cells, accompanied by graphs quantitating the variation in their frequency and counts across floxed, +/-, and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean.

Further, we looked into the status of myeloid, B, and T cells, which make up most of the BM cellularity (Nombela-Arrieta and Manz, 2017). Interestingly, we observed an increase in the frequency of myeloid cells, only in the KO mice (Figure-3.6A), and an increase in the frequency of B cells in both heterozygous knockout and KO mice (Figure-3.6B). However, this phenotype did not reflect in their cell counts (Figure-3.6A, 3.6B). On the other hand, the T cell pool was unaffected (Figure-3.6C).

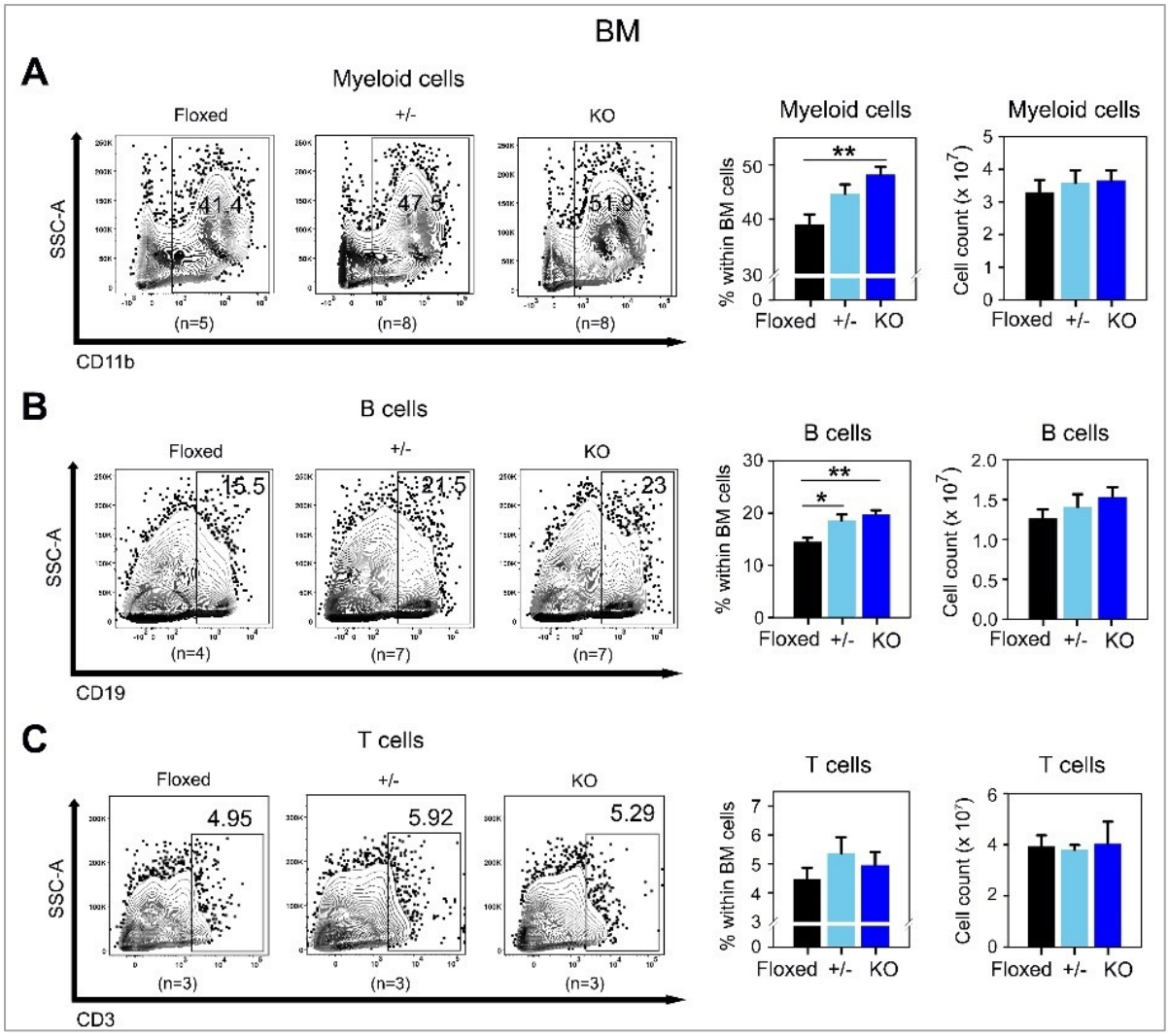


Figure-3.6: KO mice show increased frequency of the bone marrow myeloid and B cells. Representative flow cytometry plots showing the frequency of BM myeloid (A), B cells (B), and T cells (C), accompanied by graphs quantitating the variation in their frequency and counts across floxed, +/-, and KO mice. Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05 and ** p<0.01.

3.3.3 Loss of OCIAD2 does not affect differentiation potential and lineage specification towards myeloid and B cells in mouse bone marrow.

Hematopoietic stem cells and progenitors are lineage-biased (Muller-Sieburg et al., 2004; Pietras et al., 2015; Pinho et al., 2018; Montecino-Rodriguez et al., 2019). The observed increase in the frequency of myeloid and B cells indicates a possibly altered lineage specification of KO BM towards these lineages.

To check for the same, we performed the CFU-C assay using Methocult (M3434) medium containing recombinant human insulin, human transferrin (iron-saturated), recombinant mouse stem cell factor (SCF), recombinant mouse interleukin 3 (IL-3), recombinant human interleukin 6 (IL-6), and recombinant human erythropoietin (EPO), which supports the development of burst forming unit-erythroid (BFU-E; primitive erythroid progenitor), granulocyte and macrophage colony-forming unit (CFU-GM; myeloid progenitor), and granulocyte, erythroid, macrophage, and megakaryocyte progenitors (CFU-GEMM; multipotent progenitor) (https://www.stemcell.com/methocult-gf-m3434.html). This *in vitro* culture system does not help in enumerating the HSCs but helps to find out any change in their differentiation potential in terms of the number of colonies (Purton and Scadden, 2007) and any change in the proliferative capacity of progenitors in terms of colony size (Sinha et al., 2019).

We observed no change in either the count or the size of the CFU-GM and CFU-GEMM colonies formed from the KO mice relative to that of floxed mice (Figure-3.7A), inferring that loss of OCIAD2 does not affect the differentiation potential of BM HSCs and proliferative capacity of BM myeloid and multipotent progenitors.

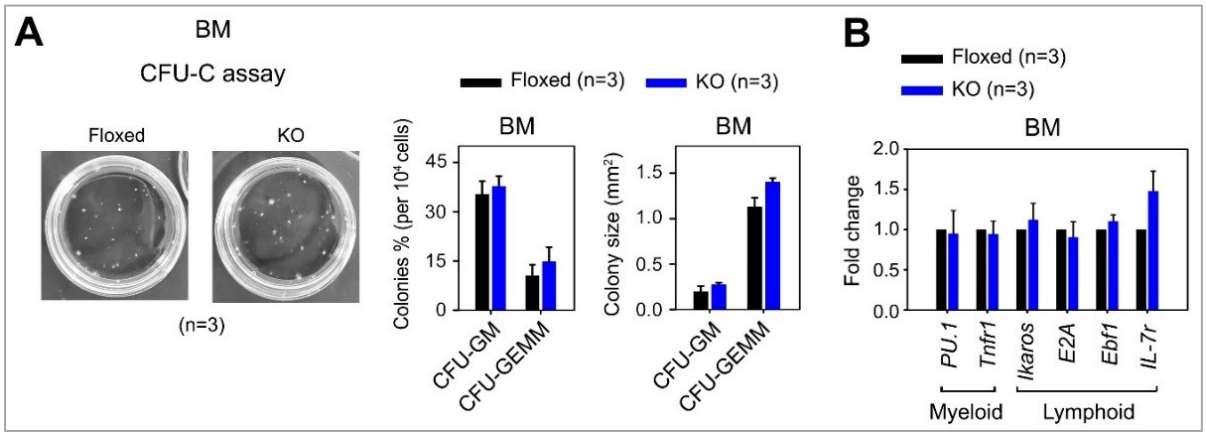


Figure-3.7: Effect of OCIAD2 KO on the progenitor colony formation and expression of genes regulating lineage specification in mouse bone marrow. (A) Images of Methocult dishes seeded with BM cells of floxed or KO mice followed by graphs showing variation in the frequency and size of myeloid (CFU-GM) and multipotent (CFU-GEMM) colonies across the genotypes (n=3 mice per genotype). **(B)** Graphs representing variation in the expression of key genes regulating the specification of myeloid and lymphoid lineages in the bone marrow of floxed and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean.

Further, we analyzed the KO BM and found unchanged transcript levels of genes known to regulate myeloid (*PU.1*, *Tnfr1*) and lymphoid (*Ikaros*, *E2A*, *Ebf1*, and *IL-7r*) lineage specification (Imperato et al., 2015; Wajant et al., 2019; Glimcher et al., 1999; Elliott et al., 2014; Goldfarb, 2007), relative to the floxed BM (Figure-3.7B). These observations indicate that OCIAD2 does not regulate the lineage specification in mouse BM.

3.3.4 Loss of OCIAD2 does not affect apoptosis of myeloid and B cells in mouse bone marrow

Since the lineage-committed progenitor specification was unaltered, the increased frequency of myeloid and B cells in the KO BM could be due to deregulated apoptosis, cell proliferation, or migration. To check the effect of OCIAD2 KO on apoptosis, we conducted Annexin-V-based flow cytometry analysis. We found both early and late apoptosis unchanged in the BM myeloid (Figure-3.8A) and B-cells (Figure-3.8B) of both heterozygous knockout and KO mice.

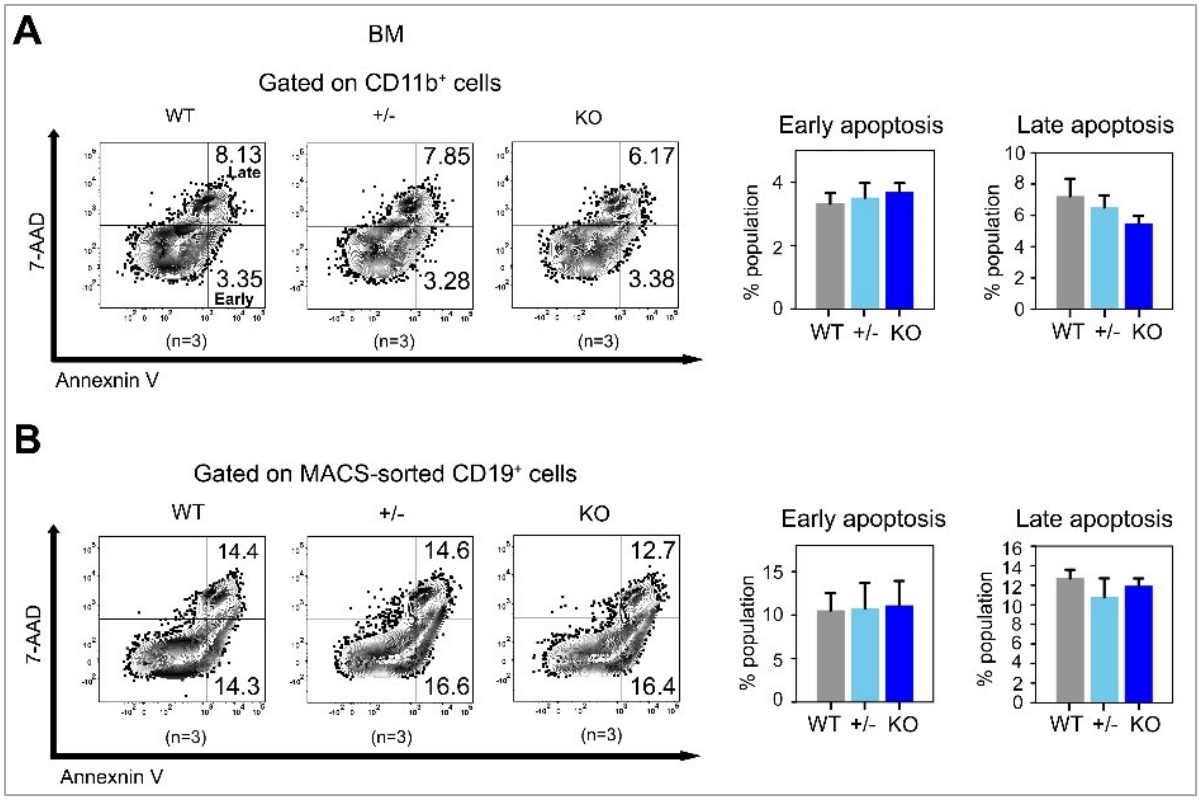


Figure-3.8: Effect of OCIAD2 KO on the apoptosis of mouse bone marrow myeloid and B cells. (A, B) Representative flow cytometry plots showing the frequency of both early (7-AAD$^-$ Annexin V$^+$) and late (7-AAD$^+$ Annexin V$^+$) apoptotic stages of BM myeloid (CD11b$^+$; A) and B lymphoid (CD19$^+$; B) cells, accompanied by graphs depicting the variation in their frequency and total counts across *ociad2* WT, +/-, and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean.

OCIAD2 is essential for the migration but not for proliferation in cultured HEK293 cells (Sinha et al., 2018). Hence, the increased frequency of myeloid and B cells could be due to decreased migration, which merits further investigation.

3.3.5 OCIAD2 KO leads to decreased frequency of mouse splenic HSPCs

Spleen is the primary extramedullary lymphoid organ in mice, known for its role in stress-mediated hematopoiesis, associated with mild to severe splenomegaly (Oda et al., 2018; Wu et al., 2018; Sinha et al., 2019). Since both the heterozygous knockout and KO mice show enlarged spleen than the floxed mice, we examined the spleen for any abnormality in the hematopoietic progenitors. The total cellularity of the spleen was unchanged in both heterozygous mutant and KO mice (Figure-3.9A). Through flow cytometry analysis, we found significantly decreased HSPC frequency only in the KO mice (Figure-3.9B), hinting at possible altered hematopoiesis in the spleen, which needs further investigation. Since splenic HSPCs originate in the BM and migrate to the spleen via circulation (Wu et al., 2018), a possible defect in the KO BM HSPC migration ability needs to be tested.

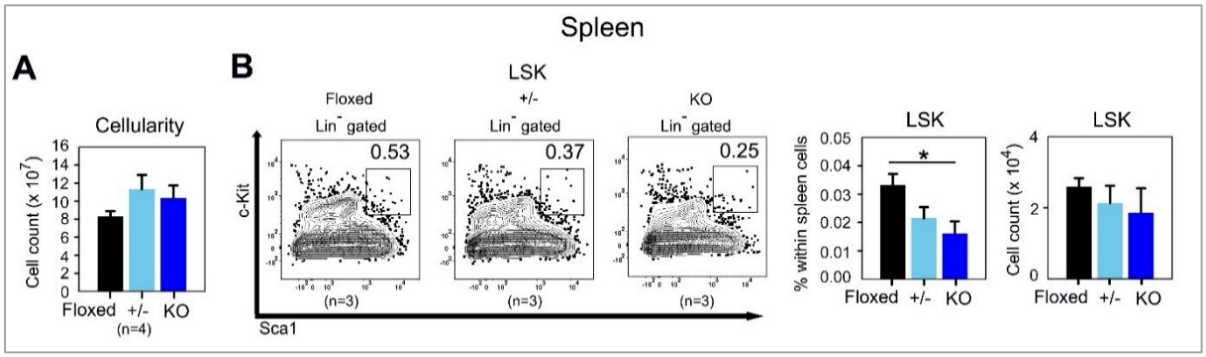


Figure-3.9: Effect of OCIAD2 KO on the cellularity and the abundance of HSPCs in mouse spleen. (A) Graph showing the variation in the total cellularity of mouse spleen across floxed, +/-, and KO mice (n=4 mice per genotype). **(B)** Representative flow cytometry plots showing the frequency of splenic HSPCs (LSK cells), accompanied by graphs quantitating the variation in their frequency and total counts across floxed, +/-, and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05.

3.3.6 Loss of OCIAD2 does not affect the abundance of circulating HSPCs and leukocytes

Blood circulation interlinks various hematopoietic organs during steady-state and stress conditions (Paulson et al., 2020). Besides terminally differentiated and effector cells, HSPCs are also detected at a relatively lower frequency (Sinha et al., 2019). Hematopoietic cells circulate in the body between BM and other tissues via blood circulation. As mentioned earlier,

the BM HSPCs, myeloid, and B cells migrate out of BM to peripheral tissues like the spleen. To understand more about the decreased frequency of HSPCs in the spleen and increased frequency of myeloid and B cells in the BM of the KO mice, we checked the RBC-lysed PB for any fluctuations in the frequency of these cells and found it to be unchanged (Figure-3.10A, B).

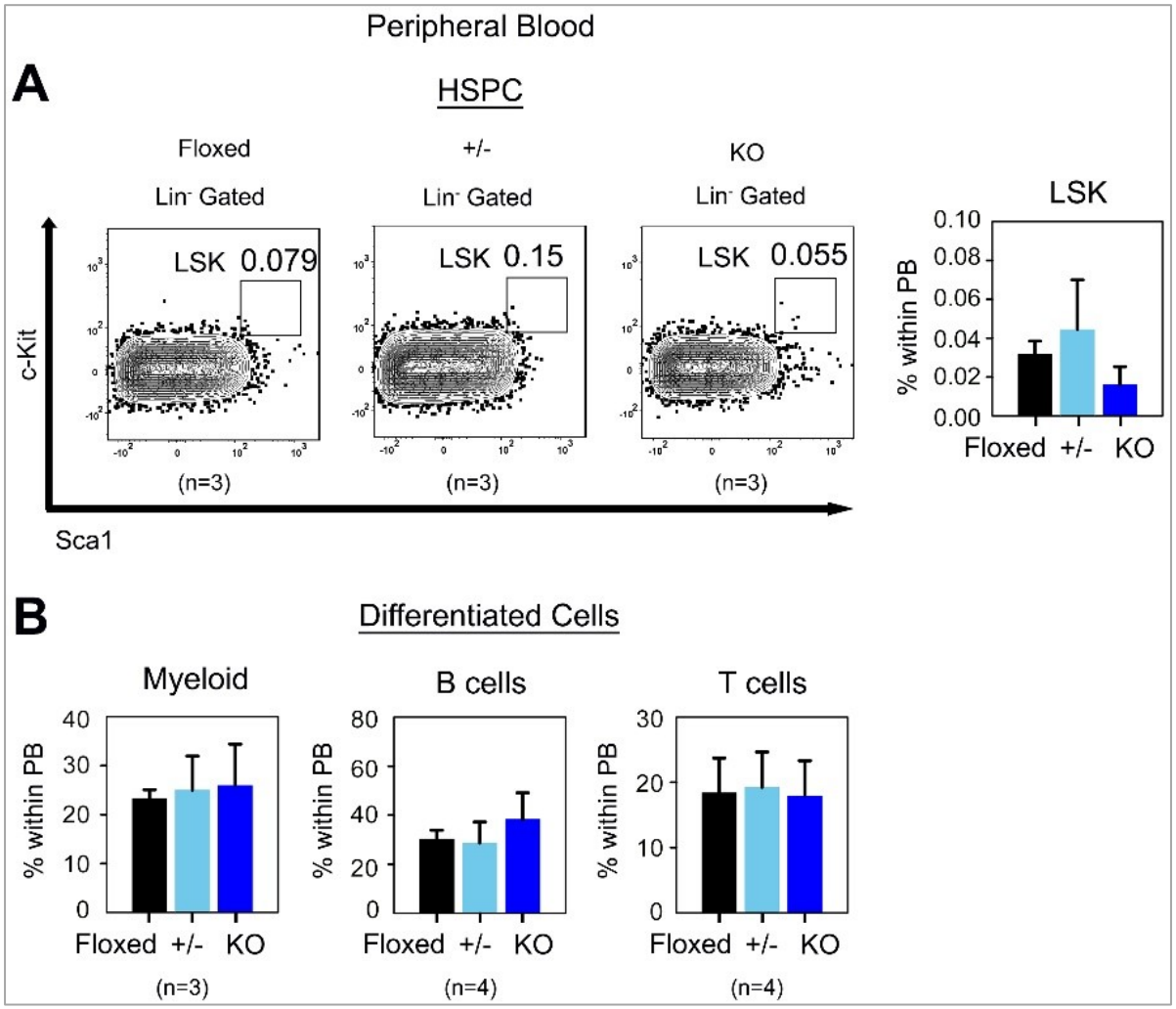


Figure-3.10: Loss of OCIAD2 leads to splenomegaly and kidney atrophy in mice. (A) Representative flow cytometry plots showing the frequency of PB HSPCs (LSK cells), accompanied by graphs quantitating the variation in their frequency and total counts across floxed, +/-, and KO mice (n=3 mice per genotype). (B) Graphs quantitating the variation in the frequency of myeloid, B, and T cells in PB of floxed (n=3 mice), +/- (n=4 mice), and KO (n=4 mice) mice. Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean.

3.4 Discussion:

Loss of OCIAD2 might not affect the overall growth of mice, as can be inferred from the unchanged body weight in both heterozygous knockout and KO mice. OCIAD2 is expressed in various vital organs of mice (Sinha et al., 2018). Nevertheless, its loss led to noticeable morphological changes only in a few organs, suggesting tissue-specific functions of OCIAD2 in governing homeostasis. Supporting this notion is the observation of unchanged brain relative weight in KO mice, despite the higher transcript and protein levels of OCIAD2 (www.ncbi.nlm.nih.gov; Sinha et al., 2018) and its reported implication in Alzheimer's disease (Han et al., 2014), known for causing brain atrophy (Chan et al., 2003). Adding to this is the observed splenomegaly and smaller kidney in KO mice, with respect to the lower expression levels in the spleen than the kidney of C57BL6/J mice (Sinha et al., 2018). However, the presence of splenomegaly in the heterozygous knockout mice contrasts the notion. Examining the size of the spleen and kidneys at a younger age, like 2 months and postnatal stages (P0-P1) would help to know whether the phenotypes are due to any developmental defect (Cain et al., 2010) or acquired with age (Habib, 2018). If they are developmental defects, then the loss of OCIAD2 could be affecting the expression of genes regulating the development of the spleen (*Hox11*, *Pbx1*, *Nkx3.2* (*Bapx1*), and *Pod1* (*capsulin*, *Tcf21*) (Kanzler et al., 2001; Brendolan et al., 2005) and kidney (*Hox11*, *Lim1*, *Osr1*, *Pax2/8*, and *Eya1*) (Khoshdel et al., 2020) which needs to be tested.

The unchanged HSPC abundance across hematopoietic compartments, unaltered hematopoietic differentiation and lineage specification potential of KO BM suggest an insignificant role of OCIAD2 in the maintenance and functions of BM HSPCs. While this is in contrast to the higher expression of OCIAD2 in hematopoietic progenitors (Chapter-2), it supports an earlier report showing that a high expression of a gene/protein does not essentially mark its necessity to maintain a given cell type (Arndt et al., 2013). Alternatively, this also indicates compensatory and/or gene redundancy mechanisms to maintain hematopoietic homeostasis in the KO mice, which needs further investigation. The increase in the frequency of KO BM myeloid and B cells with no change in T cell frequency indicates cell-specific functions of OCIAD2 like migration and proliferation, if not lineage specification and apoptosis. Adding to this, the unchanged relative weight of the thymus in KO mice suggests a

dispensable role of OCIAD2 in T cell development, a process predominantly taking place in the thymus and regulating its size (Prockop and Petrie, 2004; Koch and Radtke, 2011).

The insignificant increase in total cellularity of the KO spleen might be due to its mild enlargement. Apart from defective development, stress erythropoiesis triggered by factors like anemia, infection, leukemia, etc., can cause splenomegaly (Paulson et al., 2020) and are to be tested in the KO mice. During stress erythropoiesis, short-term-repopulating hematopoietic stem cells in the spleen, which initially migrate from the mouse BM, commit to stress erythroid fate under the influence of Hedgehog and BMP4 signaling presented by splenic niche (Perry et al., 2009; Harandi et al., 2010). An accelerated commitment to the stress erythroid fate could be another reason, additional to the anticipated migratory defect, explaining the lower frequency of HPSC in the KO spleen, which needs further investigation. While the flow cytometry-assessed parameters in PB are unaltered, analyzing other parameters like counts of RBC, platelets, hemoglobin levels, etc., provide more insights into the effect of OCIAD2 KO on hematopoietic homeostasis.

Overall, the study presented in this chapter reports an insignificant, probably a dispensable role for OCIAD2 in maintenance and lineage specification of BM HSPCs and an insight into its tissue-specific effects of OCIAD2 KO.

3.5 Acknowledgements:

I thank Dr. Saloni Sinha and Alice Sinha for their kind help. Dr. Saloni Sinha helped in validating *ociad2* mutant mice. Dr. Saloni Sinha and Alice Sinha helped in the flow cytometry analysis of HSPCs. Alice Sinha helped in dissections, weighing, and imaging organs in some experiments.

Chapter 4. *Ociad2*^{pdsn} KO mice show defective erythropoiesis and JAK/STAT signaling with increased Asrij levels in the bone marrow.

4.1 Introduction

As described in Chapter-3, OCIAD2 has a non-essential role in maintaining HSPCs and the majority of the differentiated cell pools across hematopoietic compartments. However, a significantly enlarged spleen was observed in both the heterozygous knockout and KO mice (Chapter-3), indicating the possibility of atypical physiology. Different factors like anemia, leukemia, etc., can cause splenomegaly (Paulson et al., 2020). With respect to the phenotypes described so far, leukemic conditions are unlikely in the OCIAD2 KO mice. RBC lysis and reduced hemoglobin causing hypoxia also can cause splenomegaly apart from inflammation or infection (Wang et al., 2021). Since *asrij* KO mice show abnormal RBC counts and splenomegaly (Sinha et al., 2019), we reasoned to check the possibility of any change in the RBC counts and hemoglobin parameters of the *ociad2*^{pdsn} KO mice. To address this, we conducted complete blood counts (CBC) analysis of the peripheral blood (PB) from *ociad2* floxed, heterozygous knockout, and KO mice of age 6-10 months. Further, we employed multiple approaches to understand more about the effect of OCIAD2 KO on mouse hematopoiesis. This chapter will discuss the effect of OCIAD2 KO on mouse steady-state erythropoiesis, response to stress conditions, and molecular erythropoietic regulators.

4.2 Materials and Methods

4.2.1 Complete blood counts

Mouse PB was acquired as described in Chapter-3. The blood-EDTA mix was analyzed using Sysmex XP-100 (Japan) hematology analyzer for complete blood counts. The obtained values of blood cell counts and related parameters were corrected for their dilution before further analysis.

4.2.2 Isolation of bone marrow cells and *in vitro* culture assay

Isolation of BM cells and *in vitro* culture assay were done as described in Chapter-3.

4.2.3 Real-Time – quantitative PCR (RT-qPCR)

RNA isolation, cDNA synthesis, and quantitative PCRs were done as described in Chapter-3. Following primers were used to detect the transcript levels of genes of interest with *gapdh* as the reference gene.

Primer	Sequence (5'-3')
Gata-1 forward	GGATGCAGCATCTTCTTCCACTTC
Gata-1 reverse	CATAAGGTGAGCCCCCAGGAATTC
Epor forward	GGGCTCCGAAGAACTTCTGTGC
Epor reverse	CATGACTTTCGTGACTCACCCTC
Nf-e2 forward	GAGTTGTTGGCACAGTATCCGC
Nf-e2 reverse	CTCTTGCGACAGTTTTGGGCTG
asrij forward	GGAGATCTCGAGATGAATGGGAGGGCTGATTTT
asrij reverse	GTGGTGGCGGCCGCTCACTCATCCCAAGTATCTCC
gapdh forward	TGCCCCCATGTTTGTGATG
gapdh reverse	TGTGGTCATGAGCCCTTCC

4.2.4 Cytospin smear preparation and Giemsa staining

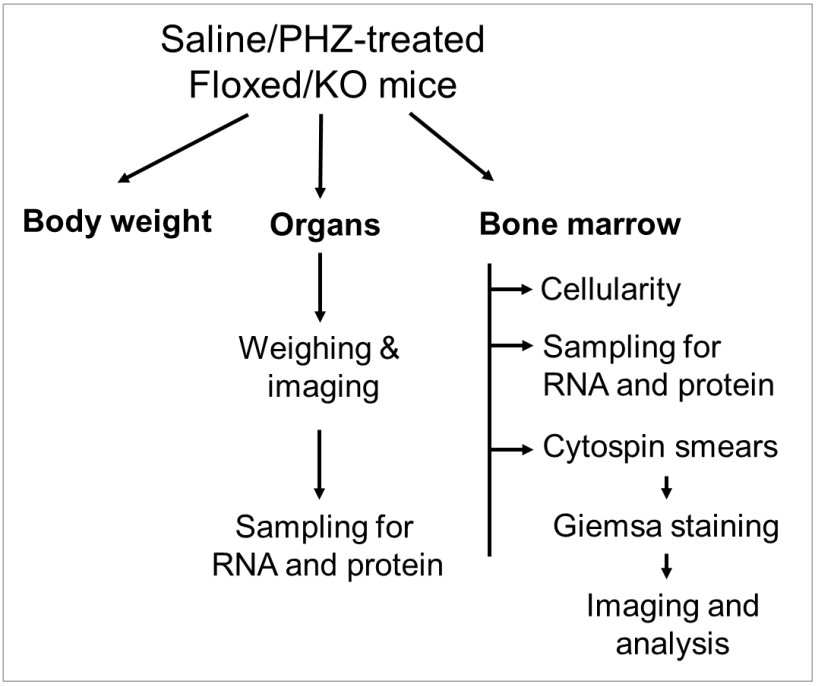
Around 5×10^4 BM cells were harvested from each mouse and were deposited onto a glass slide at 1000 rpm for 5 minutes, at room temperature, using Cytospin 4 centrifuge (Thermo Scientific, USA). The cytospin smears were air-dried, followed by fixation in methanol for 5 minutes. The smears were then stained with Giemsa solution (Sigma, USA) diluted in a 1:1 ratio with double distilled (MilliQ) water for 5 minutes, followed by a wash with distilled water. The stained smears were air-dried and mounted with glass coverslips using D.P.X (Merck, USA). Three smears were made from each mouse.

4.2.5 Imaging of cytospin smears and quantification of reticulocytes

Giemsa-stained cytospin smears were imaged on Axio Scan.Z1 slide scanner (Carl Zeiss AG, Germany), in brightfield using 40x objective. The images were analyzed using Zen 3.3 software. RBCs and reticulocytes were identified as per the Giemsa staining (McGarry et al., 2010). They were counted at five different positions - center, 2 o'clock, 4 o'clock, 8 o'clock, and 10 o'clock, of a region towards the center of the cytospin smear, to a total of 500. Reticulocyte count per 100 RBCs was represented as reticulocyte frequency. Two to three smears were analyzed per mouse.

4.2.6 Phenylhydrazine (PHZ)-mediated stress assay

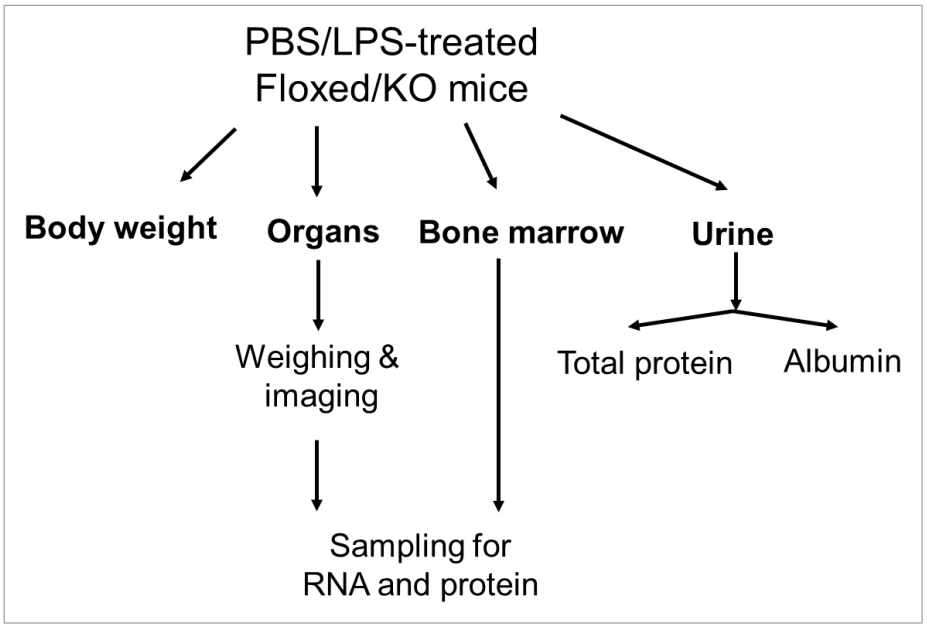
Mice were intraperitoneally injected with PHZ (Sigma, USA) in saline at a dose of 40 mg/kg body weight on day-0 (t=0 hrs) and twice on day-1 (t=16 and 24 hrs) (Moreau et al., 2012). Body weight was measured on days- 0, 3, 5, 7, and 8. Mice were dissected on day-8, or earlier if deceased, to harvest BM and for anatomical examination of various organs (Schematic-4.1). Analysis was performed on four independent biological sets.



Schematic-4.1: Outline of various analyses conducted on floxed and KO mice treated with saline or PHZ.

4.2.7 Lipopolysaccharide (LPS)-mediated stress assay

Mice were intraperitoneally injected with LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich, USA) in PBS at a dose of 10 mg/kg body weight on day-0 (t=0 hrs) (Su et al., 2018). Body weight was measured, and spot urine was collected on day-0, 1 (t=24 hrs), and 2 (t=48 hrs). Mice were dissected on day-2 for anatomical examination of various vital organs (Schematic-4.2). Analysis was performed on three independent biological sets.



Schematic-4.2: Outline of various analyses conducted on floxed and KO mice treated with PBS or LPS.

4.2.8 Body weight and organ relative weight measurement

The body weight and relative weight of various vital organs of mice were measured as described in Chapter-3.

4.2.9 Assessment of total urinary protein

Total protein of mouse urine was estimated by colorimetry using Bradford reagent (Bio-Rad, USA). 5 µL of diluted mouse urine samples were mixed with 100 µL of 1X Bradford reagent. Following 30 minutes of incubation, the optical density was measured at 595 nm. Protein concentration was calculated from the optical density values using the standard curve derived from BSA (HiMedia, India) (Prakash et al., 2008).

4.2.10 Assessment of albuminuria

The presence of albumin in the mouse urine samples was qualitatively assessed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (Sigma-Aldrich, USA). 5 µg of BSA was used as a positive control to identify the albumin band in the urine samples accurately. Stained gels were imaged using the Chemidoc system (BioRad, USA), and the albumin band intensity was quantified using ImageJ (version 1.53f51). The analysis was performed on three independent biological samples.

4.2.11 Immunoblotting and densitometry analysis

Immunoblotting and densitometry analysis of unfractionated and Lin^+ BM cells of floxed and KO mice was done as described in Chapter-3. Primary antibodies against Asrij/OCIAD1 (Abcam, USA), STAT3, pSTAT3, STAT5, and pSTAT5 (Cell Signaling Technology, USA) were used for detecting their levels in protein lysates. Lysates were normalized to the loading control, using antibodies against β-tubulin (Abcam, USA) or Vinculin (SIGMA, USA). The secondary antibodies used were anti-rabbit and anti-mouse IgG HRP-conjugated (GeNei, India). Analysis was performed on three independent biological samples.

4.2.12 Immunostaining and flow cytometry analysis

Immunostaining and flow cytometry analysis of BM was done as described earlier in Chapter-2. BM cells were stained using an antibody against Asrij/OCIAD1 (Abcam, USA) followed by the anti-Rabbit-IgG (conjugated to Alexa-488; Molecular Probes, USA).

4.2.13 Statistical analysis and graphical representation

Graphs representing the mean ± SEM were plotted using SigmaPlot11.0. Statistical significance values for blood parameters, body weight, relative weight (% body weight) of organs, cell frequencies, signal intensity measurements, and total protein content were calculated using ANOVA: Single Factor. Unpaired t-test: Two-sample Assuming Unequal Variances was used for data across treatment groups and genotypes from stress assays, while t-test: Paired Two Sample for Means was used for data across different time points within a given treatment group. Data Analysis Tool pack in Microsoft Excel 2016 was used for statistical analysis. Significant difference between the data from two different cell types, genotypes, or treatment groups was indicated by * $p<0.05$, ** $p<0.01$, or *** $p<0.001$. Significant difference between the data from two different time points of a given treatment group was indicated by \$ $p<0.05$ or \$\$ $p<0.01$.

4.3 Results

4.3.1 Loss of OCIAD2 leads to macrocytic hyperchromic anemia

Complete blood count analysis revealed decreased RBC counts, hematocrit, and total hemoglobin values in KO mice, more robust than the heterozygous knockout (Figure-4.1A-C). However, only KO mice showed an increased mean corpuscular volume (MCV) and mean cellular hemoglobin (MCH, MCHC) values, indicating macrocytic, hyperchromic anemia (Figure-4.1B). The counts of white blood cells (WBC) and platelets did not change upon partial or complete loss of OCIAD2 (Figure-4.1A). However, the mean platelet volume (MPV) and platelet-large cell ratio (P-LCR) values increased only in the KO mice (Figure-4.1D), indicating the presence of platelets larger than usual.

4.3.2 OCIAD2 is essential for steady-state erythropoiesis in mouse bone marrow

Erythropoiesis, the formation of RBCs, happens predominantly in the BM during steady-state conditions (Myneni et al., 2021). HSPCs give rise to RBCs through multiple intermediate cell types in the order of burst forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E), erythroblasts, and then reticulocytes, which give rise to RBCs (An and Mohandas, 2011). Decreased RBC counts in the PB of KO mice indicated a possibly defective steady-state erythropoiesis. To check this, we analyzed the KO BM using different approaches. Since the *ociad2* heterozygous knockout mice showed an overall mild hematopoietic phenotype, we conducted our further analysis with floxed and KO mice only.

Through the CFU-C assay, we observed no change in the potential of KO BM to give rise to primitive erythroid (BFU-E) colonies in the Methocult medium (Figure-4.2A). Also, the transcript levels of essential genes regulating erythroid lineage specification and maintenance were unchanged (Suzuki et al., 2003; Gothwal et al., 2016; Hidalgo et al., 2021) (Figure-4.2B), indicating an insignificant role of OCIAD2 in the early-erythroid development. Further, we checked the terminal stages of erythropoiesis by Giemsa staining of cytospin smears from the BM of floxed and KO mice. Interestingly, the reticulocyte frequency was severely decreased in the KO BM (Figure-4.2C), indicating defective erythropoiesis downstream of BFU-E cells.

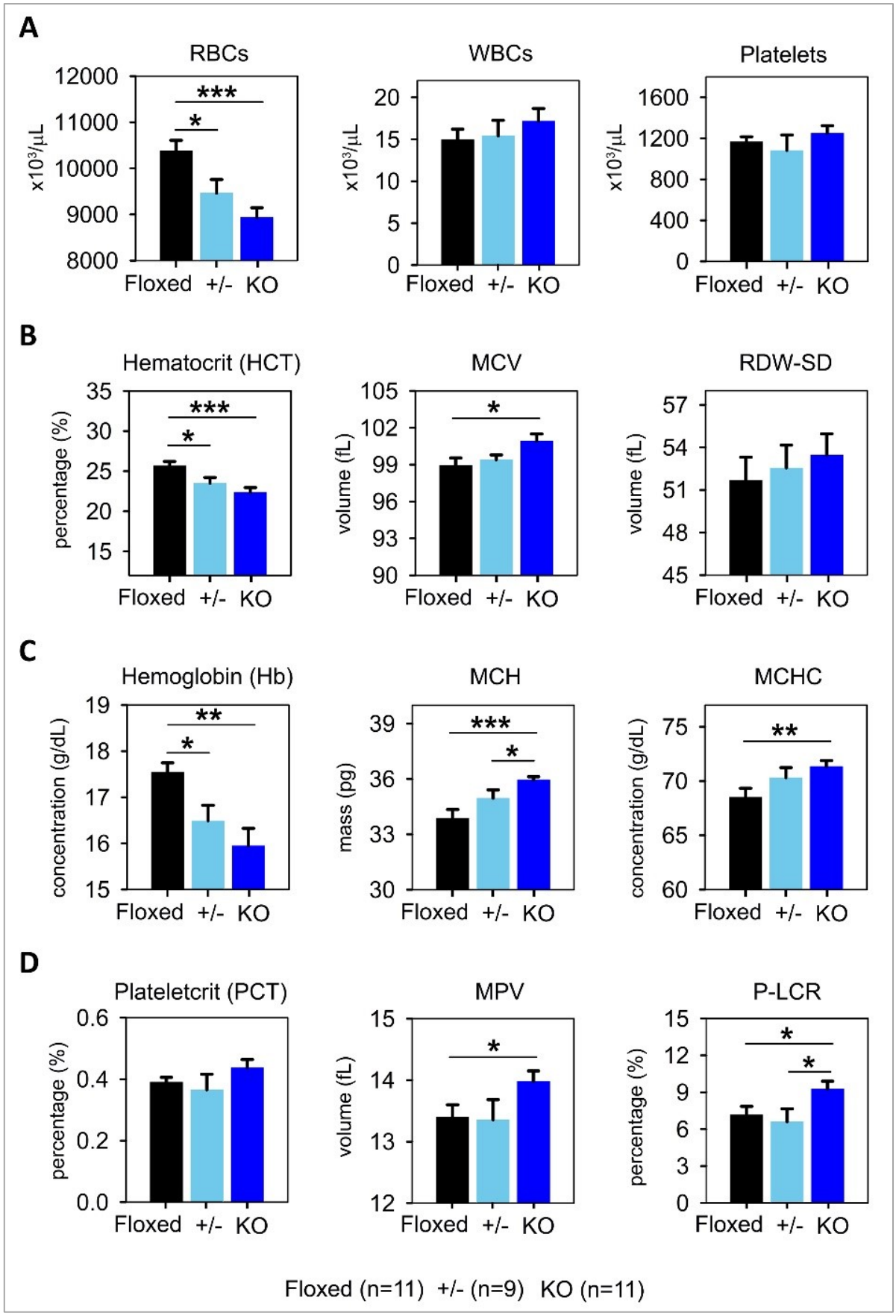

A
RBCs
x10³/µL
12000
11000
10000
9000
8000
***
*
Floxed +/- KO
WBCs
x10³/µL
20
15
10
5
0
Floxed +/- KO
Platelets
x10³/µL
1600
1200
800
400
0
Floxed +/- KO
B
Hematocrit (HCT)
percentage (%)
35
30
25
20
15
***
*
Floxed +/- KO
MCV
volume (fL)
105
102
99
96
93
90
*
Floxed +/- KO
RDW-SD
volume (fL)
57
54
51
48
45
Floxed +/- KO
C
Hemoglobin (Hb)
concentration (g/dL)
19
18
17
16
15
**
*
Floxed +/- KO
MCH
mass (pg)
39
36
33
30
***
*
Floxed +/- KO
MCHC
concentration (g/dL)
75
70
65
60
**
Floxed +/- KO
D
Plateletcrit (PCT)
percentage (%)
0.6
0.4
0.2
0.0
Floxed +/- KO
MPV
volume (fL)
15
14
13
12
*
Floxed +/- KO
P-LCR
percentage (%)
15
12
9
6
3
0
*
*
Floxed +/- KO
Floxed (n=11) +/- (n=9) KO (n=11)

Figure-4.1: *Ociad2*^{pdsn} KO mice develop anemia with larger RBCs and platelets. Graphs showing variation in multiple parameters of PB. **(A)** Counts of RBCs, WBCs, and platelets. **(B)** RBC parameters like hematocrit (HCT), mean corpuscular volume (MCV), and standard deviation of RBC distribution width (RDW-SD). **(C)** Total hemoglobin content (Hb), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). **(D)** Plateletcrit (PCT), mean platelet volume (MPV), and platelet-large cell ratio (P-LCR), across floxed (n=11), +/- (n=9), and KO (n=11) mice. Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05, ** p<0.01 and *** p<0.001.

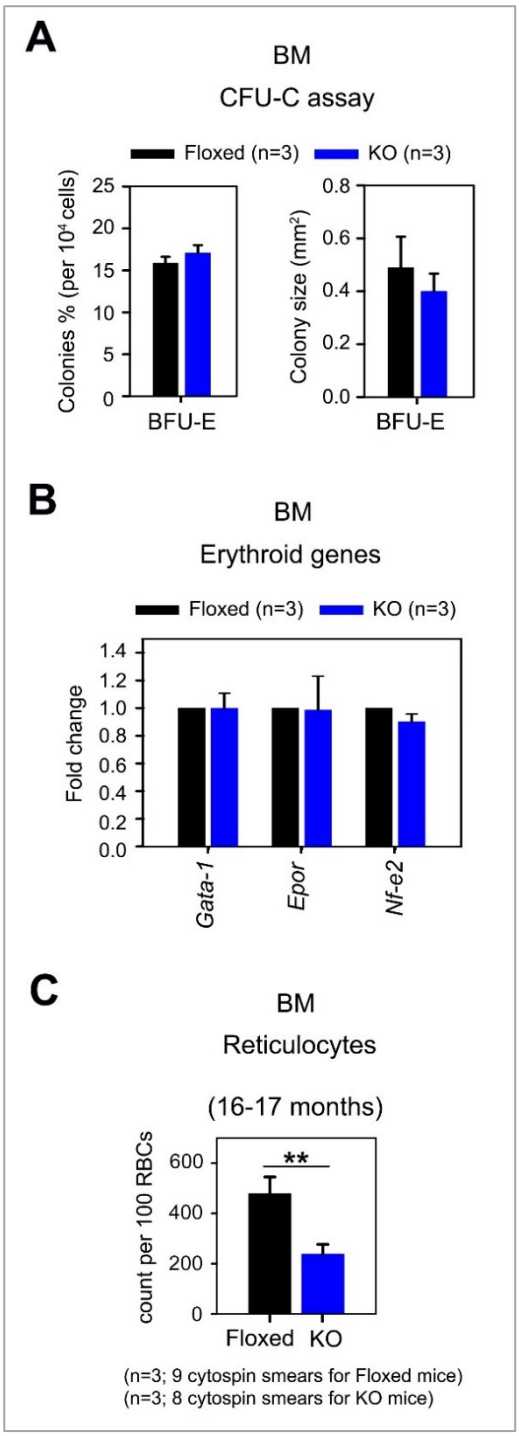


Figure-4.2: *Ociad2*^{pdsn} KO mice show defective erythropoiesis. Graph showing variation in **(A)** the counts and size of primitive erythroid (BFU-E) colonies from the BM of floxed and KO mice (n=3 mice per genotype), **(B)** transcript levels of erythroid lineage specification genes (n=3 mice per genotype), and **(C)** frequency of BM reticulocytes across floxed (n=3 mice; 9 cytospin smears) and KO (n=3 mice; 8 cytospin smears) mice. Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05 and ** p<0.01.

4.3.3 Loss of OCIAD2 does not limit the response to hemolytic stress in mice

To understand the physiological relevance of defective erythropoiesis in the KO mice, we subjected them to hemolytic stress mediated by phenylhydrazine (PHZ; 40 mg/kg body weight) (Figure-4.3A). PHZ precipitates hemoglobin leading to the lysis of erythroid cells (Goldberg et al., 1977). Free heme in the circulation acts as a pro-inflammatory signal, elicits

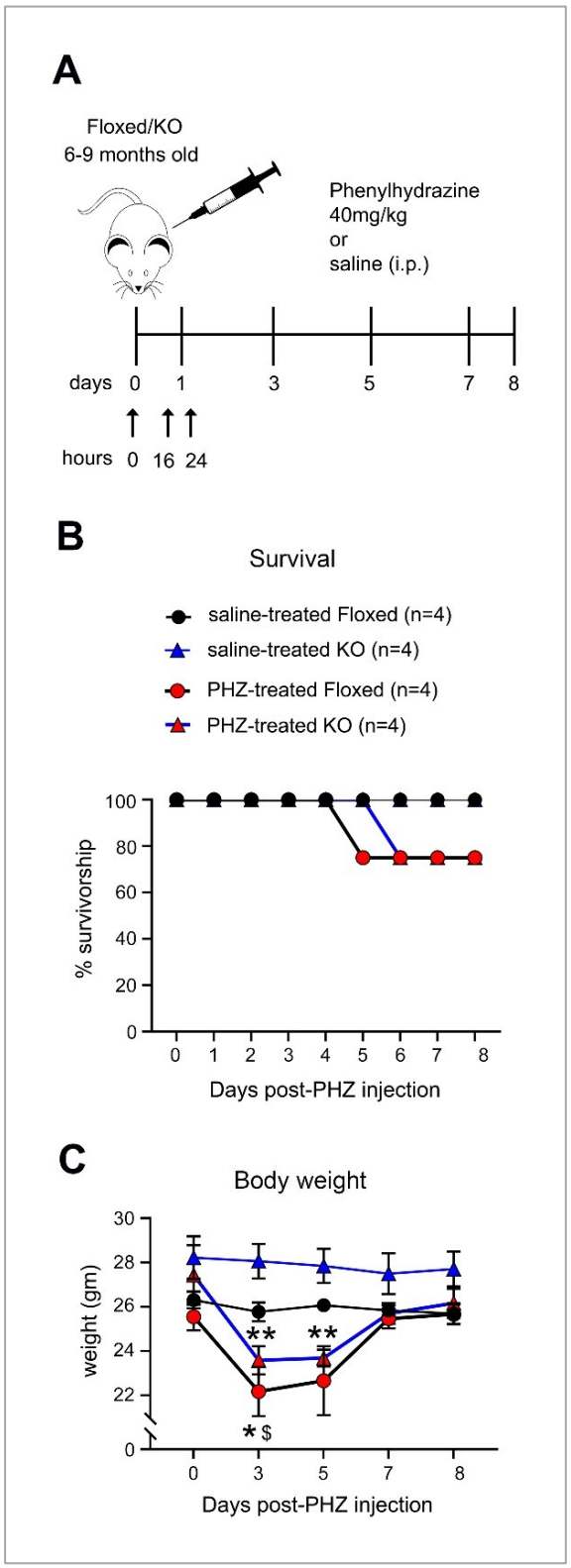


Figure-4.3: *Ociad2*^{pdsn} KO mice show similar survival but greater body weight loss under hemolytic stress. (A) Schematic outlining the treatment of floxed and KO mice with saline or phenylhydrazine (PHZ; 40 mg/kg body weight) for 8 days (three doses from day-0 to day-1). **(B)** Kaplan-Meier survival analysis curve of floxed and KO mice treated with saline or PHZ (n=4 mice per genotype). **(C)** Graph showing variation in the body weight of floxed and KO mice treated with saline or PHZ (n=4 mice per genotype). Statistically significant differences identified using unpaired t-test: Two-sample Assuming Unequal Variances and t-test: Paired Two Sample for Means as applicable were indicated. Error bars denote standard error of mean. * $p<0.05$ and ** $p<0.01$. $ $p<0.05$ represent statistical significance within a treatment group with respect to day-0.

inflammation, and obstructs steady-state erythropoiesis. Inflammation parallelly promotes stress erythropoiesis in the spleen leading to splenomegaly (Moreau et al., 2012; Bennet et al., 2019).

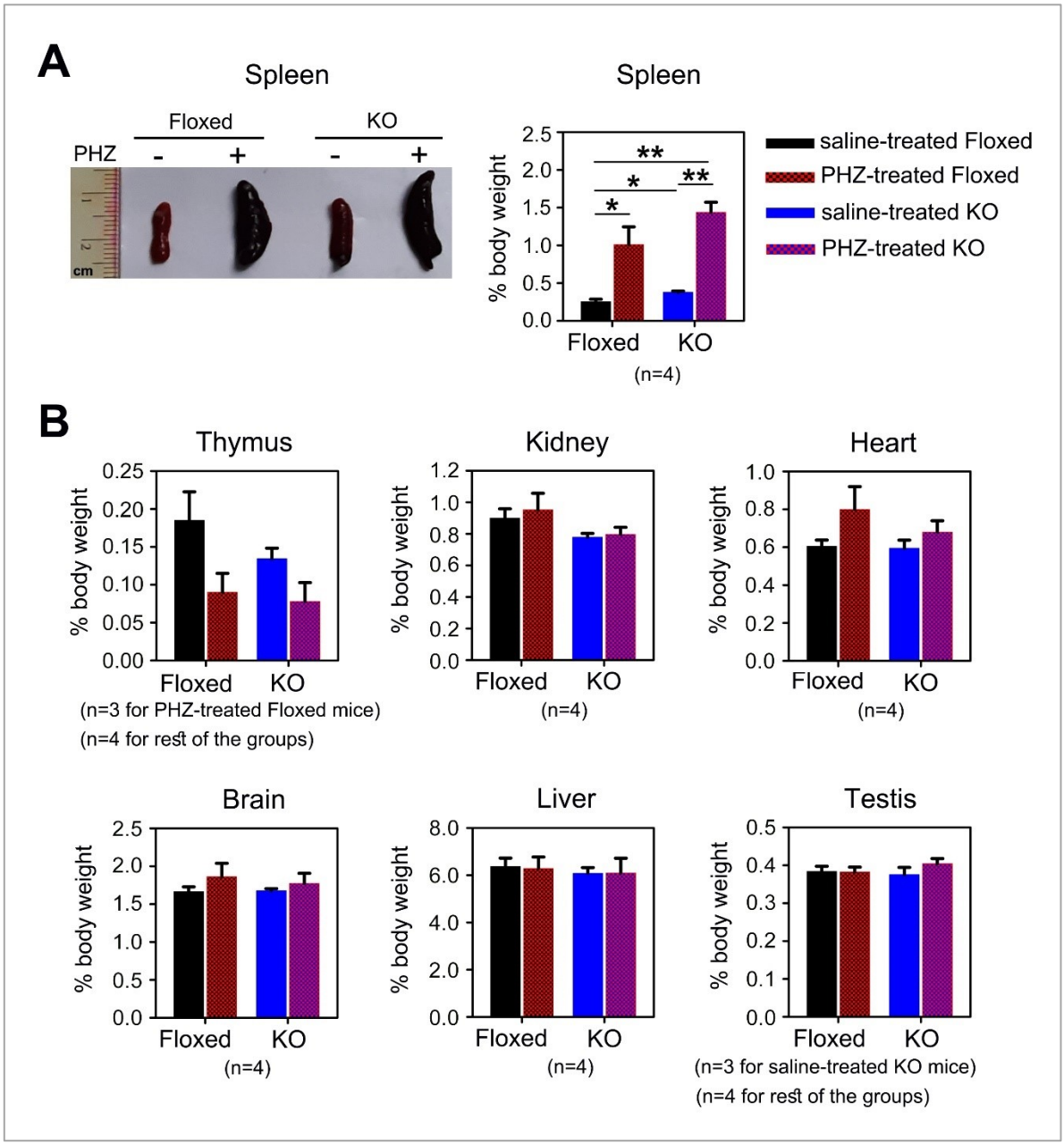


Figure-4.4: *Ociad2*^{pdsn} KO mice develop hemolytic stress-mediated splenomegaly on par with floxed mice. **(A)** Representative images of spleens from floxed and KO mice on day-8 of treatment with saline or phenylhydrazine (PHZ), followed by graph quantitating the variation in the relative of weight of the spleens. (B) Graphs showing the variation in the relative weight of other vital organs like the thymus, kidney, heart, brain, liver, and testis, from floxed and KO mice post-treatment (n=3-4 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05 and ** p<0.01.

Upon PHZ-treatment, the KO mice survived on par with the floxed mice (Figure-4.3B). However, the KO mice showed more significant loss and a trend of inadequate recovery by day-8 of the treatment than the floxed mice (Figure-4.3C), indicating increased susceptibility of the KO mice to stress response. Post-treatment, both floxed and KO mice showed significant splenomegaly relative to their saline-treated control mice. The splenomegaly in the KO mice was comparable to that of floxed mice (Figure-4.4A), indicating an intact stress-response mechanism. Other organs like the thymus, kidney, heart, brain, liver, and testis were unaffected (Figure-4.4B).

PHZ-mediated stress reduces BM cellularity and causes reticulocytosis in mice (Moreau et al., 2012). Upon PHZ-treatment, only the floxed but not the KO mice showed a decreased BM cellularity (Figure-4.5A). Saline-treated KO mice showed decreased BM reticulocyte frequency (Figure-4.2C), similar to the observations made in steady-state mice (Figure-4.5B). However, upon PHZ-treatment, the KO BM showed a significant increase in reticulocyte frequency, bringing it on par with the floxed mouse BM (Figure-4.5B). This suggests that the KO BM retains the capacity of erythropoietic response to stress but may have dysregulated signaling or an inability to sense the right cues, leading to defective steady-state erythropoiesis.

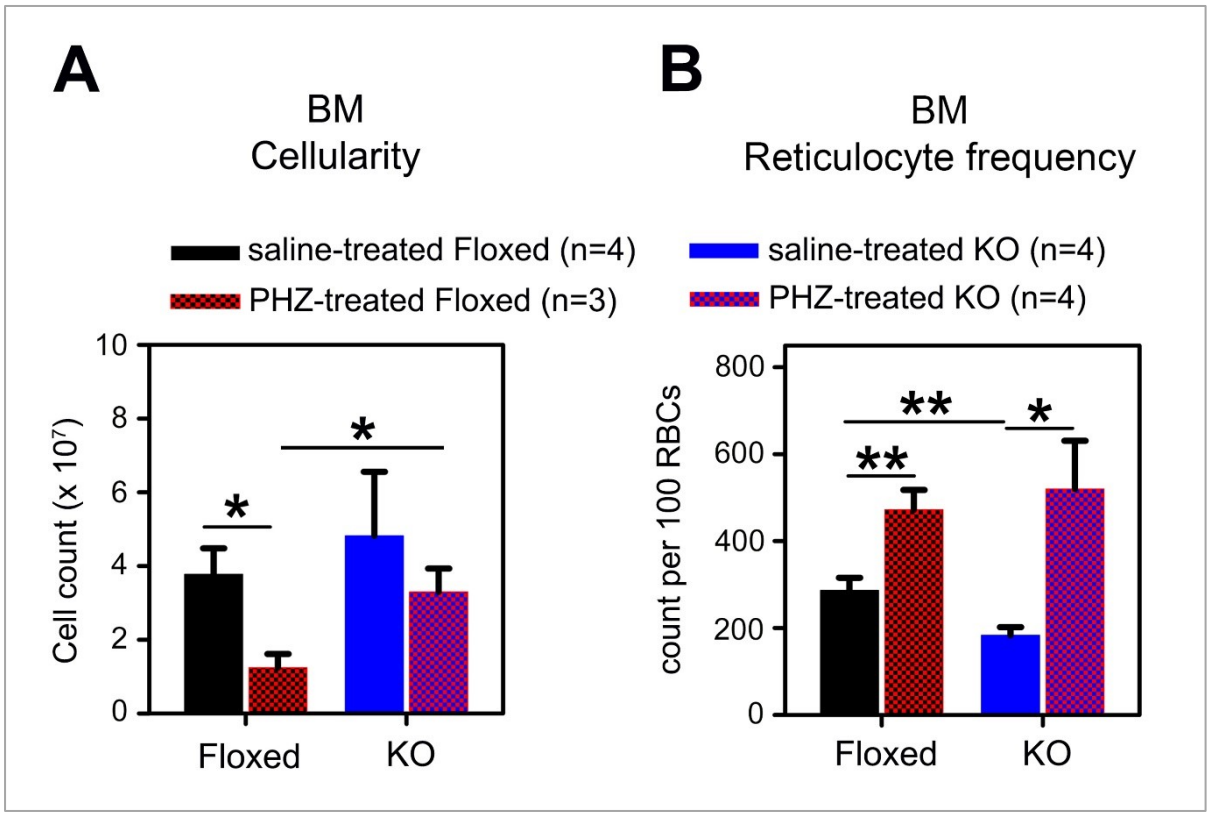


Figure-4.5: *Ociad2*^{pdsn} KO mouse BM responds on par with floxed BM, under PHZ-mediated stress. **(A)** Graph quantitating the variation in BM cellularity of floxed (n=3-4) and KO (n=4) mice post-treatment. **(B)** Graph showing the variation in the BM reticulocyte frequency across floxed and KO mice post-treatment (9-12 cytospin smears per treatment group). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * $p<0.05$, ** $p<0.01$ and ***

4.3.4 OCIAD2 KO leads to increased susceptibility towards stress erythropoiesis and acute kidney injury in mice

Splenomegaly in the KO mice during steady-state and stress conditions indicated a possibility of increased susceptibility towards stress-mediated splenomegaly. To test the same, we subjected the floxed and KO mice to a low dose of lipopolysaccharide (LPS; 10 mg/kg body weight) (Figure-4.6A), which is known for its effect of eliciting inflammation and stress erythropoiesis-mediated splenomegaly (Seeman et al., 2017).

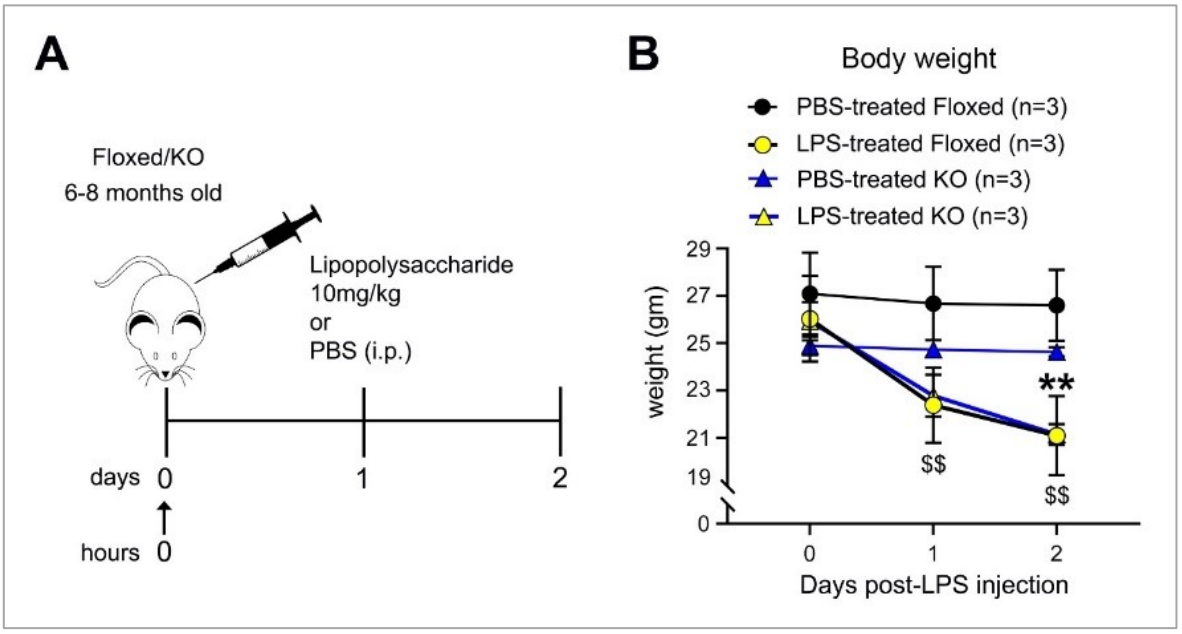


Figure-4.6: *Ociad2*^{pdsn} KO mice show greater body weight loss due to LPS-mediated stress. (A) Schematic outlining the treatment of floxed and KO mice with PBS or lipopolysaccharide (LPS; 10 mg/kg body weight) for 1 day (single dose on day-0). **(B)** Graph showing variation in the body weight of floxed and KO mice across the treatment period (n=3 mice per genotype). Statistically significant differences identified using unpaired t-test: Two-sample Assuming Unequal Variances and t-test: Paired Two Sample for Means as applicable were indicated. Error bars denote standard error of mean. * $p<0.05$ and ** $p<0.01$. $ $p<0.05$ and $$ $p<0.01$ represent statistical significance within a treatment group with respect to day-0.

LPS led to decreased body weight as expected (Hsu et al., 2017), but more significant in the KO mice than the floxed mice (Figure-4.6B). Anatomical examination revealed thymic atrophy as expected (Hick et al., 2006), with other organs like the kidney, heart, brain, liver, and testis unaffected. More importantly, an enlarged spleen was observed only in the KO mice treated with LPS (Figure-4.7A,B), inferring that the loss of OCIAD2 makes mice more susceptible to stress-induced splenomegaly.

KO mice show smaller kidneys (Chapter-3), and kidney size correlates to protein filtration as seen in chronic kidney disease (Andrassy et al., 2013; Singh, 2018). Protein filtration is governed by PDGFRβ^{+} renal pericytes, which secrete the erythroid hormone, erythropoietin (Epo) (Millot et al., 2010; Lemos et al., 2016; Suzuki et al., 2018). Thus, renal protein filtration and erythropoiesis are interlinked. Hence, to check for any effect of kidney atrophy on renal filtration in the KO mice, we assessed the total protein content of urine from floxed and KO mice and found it unaltered upon OCIAD2 KO (Figure-4.8A). Apart from triggering inflammation and stress erythropoiesis, LPS also disrupts renal filtration (Nakano et al., 2020). Surprisingly, upon LPS-treatment, KO mice showed increased albumin in the urine (Figure-4.8B), inferring an impaired renal function and a protective role of OCIAD2 against stress-induced acute kidney injury.

Kidney is the primary source of Epo (Lacombe et al., 1991). Despite impaired renal function, the transcript levels of *Epo* were unchanged in the KO kidney (Figure-4.8C). Also, we observed no change in the transcript levels of *Epor* in the KO BM (Figure-4.2B). These observations indicate a possibility of a defective signaling mechanism in the BM downstream of the EpoR, leading to decreased reticulocytes during steady-state.

4.3.5 *Ociad2^{pdsn}* KO mice show deregulated STAT activation in bone marrow

Epo hormone is required for BM erythropoiesis. Erythroid lineage development from HSCs to BFU-E and colony-forming unit-erythroid (CFU-E) does not need the hormone erythropoietin (Epo). However, it is essential to develop different erythroblasts from CFU-E cells that express the receptor for Epo (Epor) (Wu et al., 1995; Paulson et al., 2020). Upon Epo binding with EpoR, the complex activates JAK/STAT signaling, leading to the nuclear translocation of STAT molecules and the expression of anti-apoptotic genes (Elliott and Sinclair, 2012). Thus, Epo mainly prevents apoptosis of erythroid cells and ensures their continuation of the erythroid

differentiation to reticulocytes. Epo is also essential for stress-erythropoiesis in the spleen (Millot et al., 2010; Chen et al., 2020).

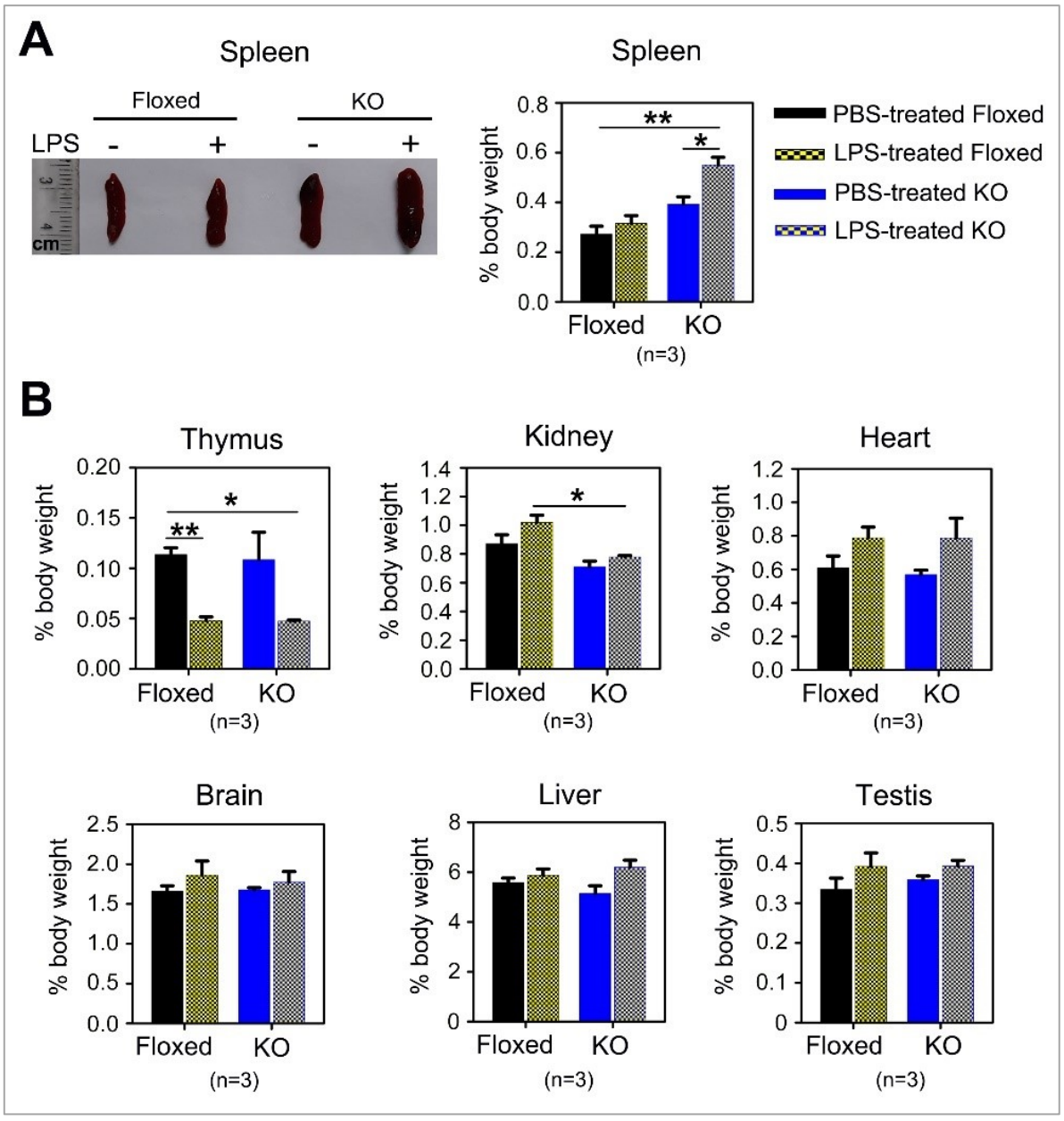


Figure-4.7: *Ociad2*^{pdsn} KO mice are more sensitive to stress-mediated splenomegaly than floxed mice. **(A)** Representative images of spleens from floxed and KO mice on day-2 of treatment with PBS or lipopolysaccharide (LPS), followed by graph quantitating the variation in the relative weight of the spleens. (B) Graphs showing the variation in the relative weight of other vital organs like the thymus, kidney, heart, brain, liver, and testis, from floxed and KO mice post-treatment (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05 and ** p<0.01.

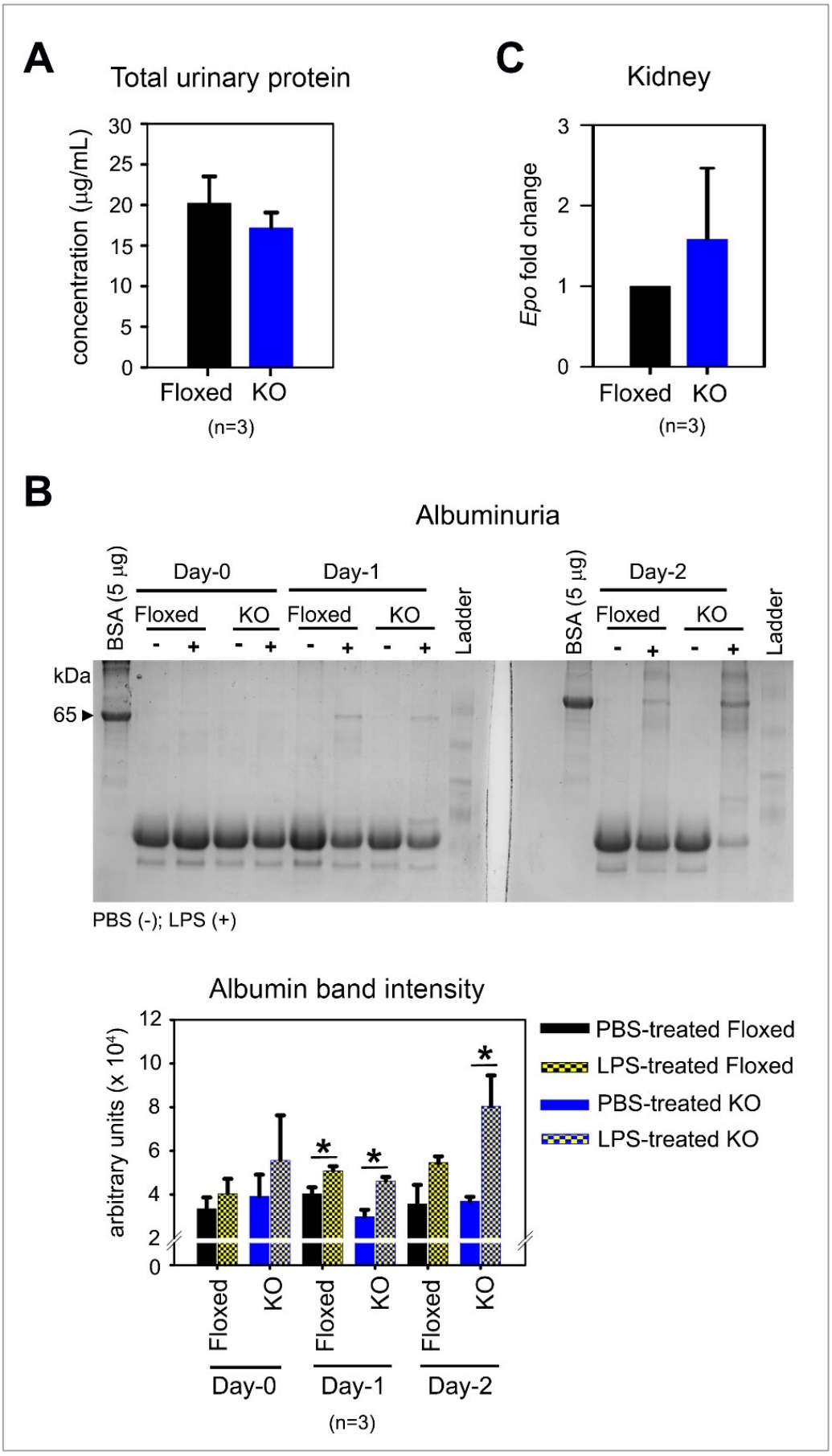

A
Total urinary protein
concentration (µg/mL)
30
25
20
15
10
5
0
Floxed
KO
(n=3)
C
Kidney
Epo fold change
3
2
1
0
Floxed
KO
(n=3)
B
Albuminuria
BSA (5 µg)
Day-0
Day-1
Floxed
KO
Floxed
KO
Ladder
BSA (5 µg)
Day-2
Floxed
KO
Ladder
- + - + - + - +
kDa
65
PBS (-); LPS (+)
Albumin band intensity
arbitrary units (x 10⁴)
12
10
8
6
4
2
0
*
*
*
PBS-treated Floxed
LPS-treated Floxed
PBS-treated KO
LPS-treated KO
Floxed
KO
Floxed
KO
Floxed
KO
Day-0
Day-1
Day-2
(n=3)

Figure-4.8: *Ociad2*^{pdsn} KO mice more susceptible to LPS-mediated acute kidney injury. (A) Graph quantitating the variation in the total urinary protein of untreated floxed and KO mice (n=3 mice per genotype). **(B)** Representative Coomassie-stained SDS-polyacrylamide gel showing proteinuria at various time points of the LPS treatment and graph comparing the variation in the albumin band intensity. (C) Graph quantitating the variation in the transcript levels of erythropoietin (*Epo*) in the kidney of floxed and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * $p<0.05$.

STAT5 is a critical intracellular signaling intermediate in the Epo-EpoR signaling of erythropoiesis (Grebien et al., 2008; Tóthová et al., 2021). Adult mice lacking STAT5 were reported with a significantly reduced erythropoiesis under PHZ-mediated stress and showed decreased expression of BCL-X_L antiapoptotic protein (Socolovsky et al., 2001). Though the phenotype of erythroid dysplasia was reported in STAT3$^{-/-}$ mice a while ago (Mantel et al., 2012), more evidence of STAT3 in erythropoiesis is surfacing late. Contrary to STAT5, a gain of function of STAT3 is associated with anemia and erythropoiesis defects (Mauracher et al., 2020).

Hence, we checked the activation of both STAT3 and STAT5 in the lineage-committed fraction of floxed and KO mice BM. Indeed, the STAT3 activation was increased (Figure-4.9A), and that of STAT5 was decreased (Figure-4.9B), confirming a deregulated JAK/STAT signaling in the absence of OCIAD2, explaining the defective erythropoiesis.

4.3.6 *Ociad2*^{pdsn} KO mice show increased levels of Asrij in the bone marrow

Despite the unchanged expression of *Epo* and *Epor*, the altered activation of STAT proteins in the KO BM indicated a possible intracellular molecular change. JAK proteins activated by the transmembrane receptors activate STAT molecules. Earlier reports show Asrij as an intracellular modulator of STAT3 activation, upstream to JAK1 in mESCs (Sinha et al., 2013). Also, Asrij prevents aberrant STAT5 activation in mouse HSPCs (Sinha et al., 2019). Moreover, Asrij is an interactor of OCIAD2 (Sinha et al., 2018) and is essential for mouse erythropoiesis during steady-state as the *asrij* KO mice show abnormal RBC counts in PB across different age groups. Also, radiation-stressed *asrij* KO mice show poor recovery of RBC counts (Sinha et al., 2019).

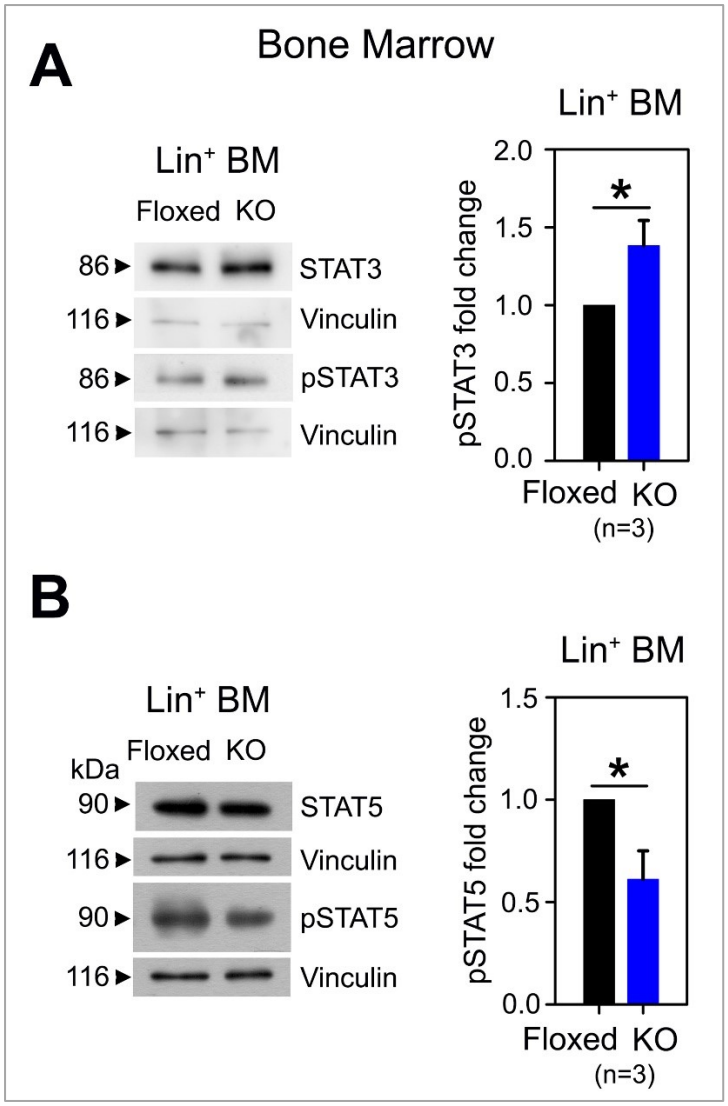


Figure-4.9: Loss of OCIAD2 leads to deregulated STAT activation in mouse BM. (A, B) Immunoblot analysis for STAT3/pSTAT3 (A), STAT5/pSTAT5 (B) in Lin^+ BM cells of floxed and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * $p<0.05$.

Owing to the increase in the levels of OCIAD2 in the *asrij* KO BM (Sinha S. Ph.D. thesis, 2019) and the observed mild hematopoietic phenotype in the *ociad2*^{pdsn} KO mice, we expected an increase in the levels of Asrij. Therefore, we reasoned to check for any change in the transcript and protein levels of Asrij in the OCIAD2 KO mouse BM. Interestingly, the loss of OCIAD2 did not affect the transcript levels of *asrij* in mouse BM (Figure-4.10A). However, it increased its protein levels to a several-fold higher (Figure-4.10B) without affecting the frequency of $Asrij^+$ cells (Figure-4.10C). This suggests that the loss of OCIAD2 leads to a loss of translational or post-translational compensation by Asrij, which leads to altered STAT3 and STAT5 activation leading to defective erythropoiesis in mouse BM.

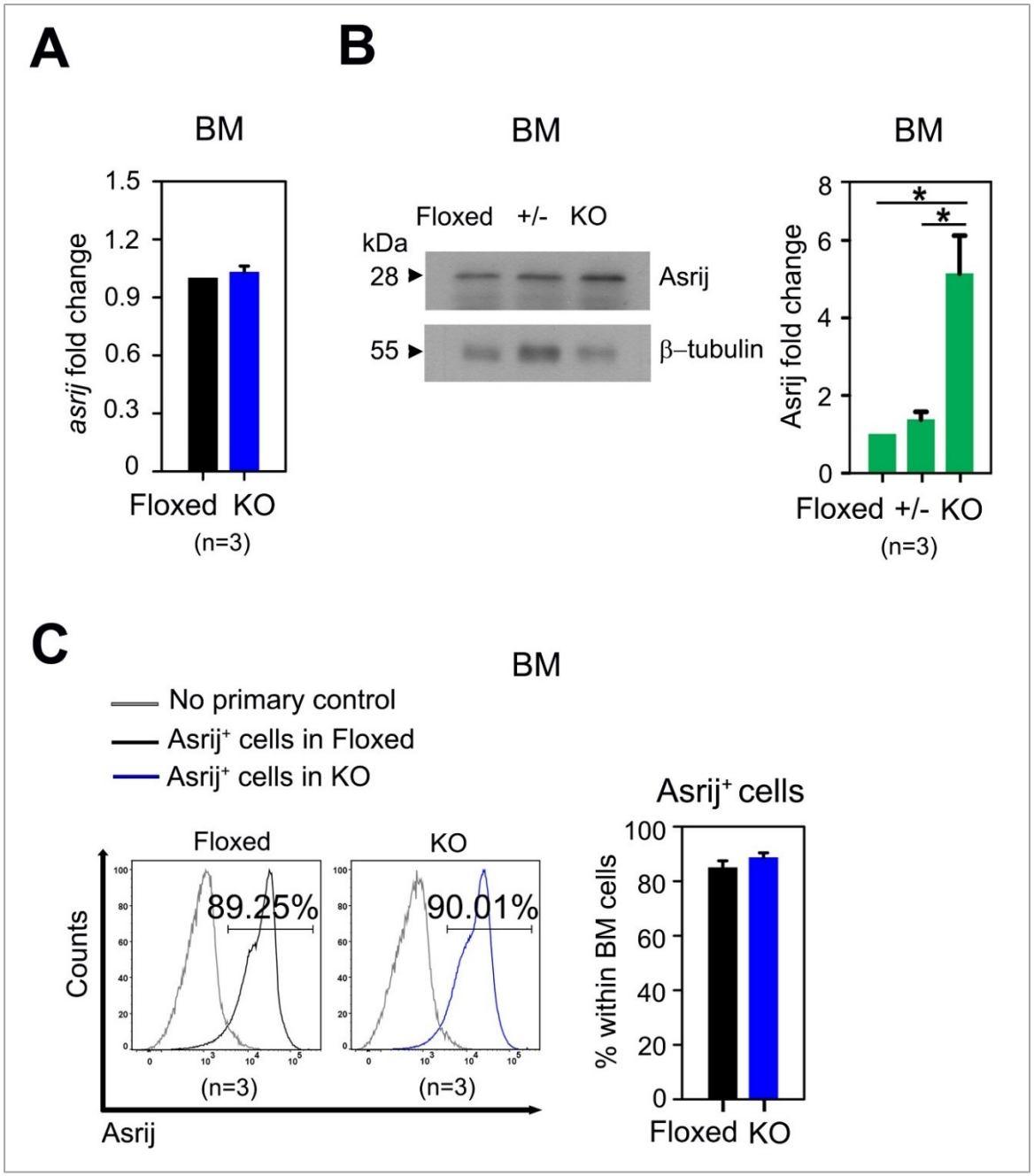


Figure-4.10: Loss of OCIAD2 leads to increased levels of Asrij in mouse BM. (A) Graph quantitating the variation in the transcript levels of asrij in the unfractionated BM of floxed and KO mice (n=3 mice per genotype). **(B)** Immunoblot analysis for Asrij (green) in unfractionated BM of floxed and KO mice (n=3 mice per genotype). **(C)** Representative flow cytometry histograms showing the frequency of Asrij$^+$ cells in the unfractionated BM of floxed and KO mice (n=3 mice per genotype). Statistically significant differences identified using ANOVA: Single Factor analysis are indicated. Error bars denote standard error of mean. * p<0.05.

4.4 Discussion

Despite its insignificance in maintaining the HPSC pool, loss of OCIAD2 led to reduced RBCs in circulation, confirming cell-specific functions of OCIAD2. Anemia, decreased RBC counts, and MCV can be associated with runting in mice (Sharma et al., 2019). Despite anemia and decreased RBC counts, *ociad2*^{pdsn} KO mice showed no overt phenotype. In this regard, whether the increased RBC volume and per cell concentration of hemoglobin are compensatory effects is unknown. Hemoglobin is synthesized in the erythroblasts till the reticulocyte stage, from heme, an iron (Fe) containing molecule and globin proteins (Singh, 2018; Koury and Ponka, 2004). Heme levels are dependent on Fe levels and positively regulate the globin mRNA levels (Tahara et al., 2004). Severe Fe loss decreases only hemoglobin production but not RBCs, resulting in smaller RBC size (Koury and Ponka 2004). Also, Fe levels were shown to effectively regulate Epo production in mouse kidneys (Suzuki et al., 2018). A defect in Fe levels is not expected in the *ociad2*^{pdsn} KO mice as we observe increased values of the MCV, MCH, and MCHC in RBCs and unchanged expression of *Epo* in kidneys. Therefore, testing whether or not the loss of OCIAD2 affects the expression of globin genes is required. Platelets are formed from their precursor cells called megakaryocytes seated in the BM (Machlus and Italiano, 2013). The increased MPV and P-LCR values in the *ociad2*^{pdsn} KO mice indicate an apparent increase in the fraction of more giant cells in circulation. They could be due to a defect in dissociation of platelets from their precursors, which needs further investigation.

CFU-E cells formed from BFU-E give rise to proerythroblast and basophilic erythroblast, which express the EpoR. The basophilic erythroblasts give rise to polychromatophilic and orthochromatophilic erythroblasts, which do not express the EpoR (Shih et al., 2018). The orthochromatophilic erythroblasts give rise to reticulocytes. Since the KO mice show decreased reticulocytes and deregulated JAK/STAT signaling in the BM, analyzing the abundance, survival, and proliferation of CFU-E and the various erythroblasts help in understanding further the stage-specific effects of OCIAD2 KO on BM erythropoiesis. PHZ-treatment is known to cause inflammation which causes myeloid bias in the BM (Pietras et al., 2016). KO BM cellularity was not affected to the extent of the floxed mice under PHZ-mediated stress. It is unknown whether it is due to any stress-induced increase in the abundance of the myeloid cell pool.

LPS mediates renal filtrate leakage via Toll-like receptor 4 (TLR4)-dependent pathway (Nakano et al., 2020). Since OCIAD2 KO mice show increased albuminuria with LPS-

treatment, it suggests that OCIAD2 might be a negative regulator of the LPS-TLR4 signaling axis, which merits further study to unravel the protective role of OCIAD2 against acute kidney injury. Expression of *Epo* was unchanged in the KO kidneys. However, the regulation of Epo production was reported even at the translational level (Barbosa et al., 2014). Hence, checking the serum levels of Epo in the floxed and KO mice can help understand the actual effect of OCIAD2 KO on Epo production.

An increase in the Asrij levels but not the *asrij* transcript hints at possible translational or post-translational compensatory mechanisms in the OCIAD2 KO BM (Wang et al., 2018). Also, this indicates a possibility of functional compensation by Asrij, mitigating the actual effects of OCIAD2 KO on the maintenance of hematopoietic homeostasis. This study also shows the indispensable role of OCIAD2 in maintaining erythropoietic homeostasis and kidney function under stress.

4.5 Acknowledgements

I thank Dr. Saloni Sinha for her kind help in the immunoblotting study of Asrij levels. I thank Alice Sinha for her kind help in dissections, weighing and imaging organs, and cell counting. I extend my thanks to Prathamesh Dongre for his kind help in the day-8 analysis of a repetition of the PHZ-mediated stress assay.

Chapter 5. Discussion

Hematopoiesis is regulated at multiple levels like signal transduction (de Roo and Staal, 2020), transcription (Liu et al., 2018[a,b]), chromatin remodeling (Xu et al., 2018), RNA processing, translation, and stability (de Rooij et al., 2019), protein synthesis (Lv et al., 2021) and protein degradation (Xu et al., 2020), and post-translational regulation (Sinha et al., 2019[b]). However, details on coordination of these various factors and their functional consequences on blood cell homeostasis remain underexplored. Hence it is crucial to expand our current understanding of this intricate and complex regulation and its implications on normal and malignant hematopoiesis. In this regard, investigation of novel molecules is necessary.

Asrij/Ovarian Carcinoma Immunoreactive Antigen Domain-containing protein 1 (OCIAD1) is an essential regulator of hematopoiesis and its loss leads to a leukemia-like phenotype in *Drosophila* (Kulkarni, Khadilkar et al., 2011; Sinha et al., 2013; Khadilkar et al., 2014; Khadilkar, Ray et al., 2017). Also, Asrij is a critical interlinking molecule between metabolism, immune response, cell signaling, protein ubiquitination, and vesicle-mediated transport in *Drosophila* (Sinha et al., 2019[a]). Interestingly, the aberrant hematopoiesis in *asrij* null *Drosophila* can be rescued by mouse *asrij* (Sinha et al., 2013), depicting the conserved role of Asrij in maintaining hematopoiesis. Supporting this, a recent study proves the essential role of Asrij in preserving the mouse bone marrow (BM) HSC quiescence by regulating p53 stability via the CSN5-MDM2-p53 axis (Sinha et al., 2019[b]). Asrij is essential for maintaining normal RBC counts in peripheral blood (PB) during steady-state and stress conditions. Its loss leads to a myeloproliferative disorder in mice (Sinha et al., 2019[b]).

Mouse BM cells express both Asrij and OCIAD2. OCIAD2 is absent in *Drosophila* and other invertebrates. OCIAD2 shares evolutionary relatedness, structural and sequence homology, and interacts with Asrij in vertebrates (Sinha et al., 2018). OCIAD2 localizes to endosomes and mitochondria, promotes STAT3 activation, supports the migration of HEK293 cells (Sinha et al., 2018) and regulates the invasion of various cancers (Wu et al., 2017; Sakashita et al., 2018). Misexpression of OCIAD2 is detected in various hematological malignancies (Bagger et al., 2016). However, the *in vivo* role of OCIAD2 in mouse hematopoiesis has not been reported. Hence, in this study, we investigated the role of OCIAD2 in maintaining mouse hematopoietic homeostasis. We find that OCIAD2 is essential for maintaining steady-state

erythropoiesis, hemoglobin levels in circulation, protection against stress erythropoiesis, and acute kidney injury in mice. Also, we report the importance of OCIAD2 in regulating JAK/STAT signaling and levels of Asrij in mouse BM. Through this study, we propose an interplay between the OCIAD proteins in maintaining blood cell homeostasis, which upon further investigation may aid in developing therapeutics for several hematological conditions.

5.1 Structure-function relationship of OCIAD2

The structure of OCIAD proteins is yet to be determined. According to the structure predictions, the OCIA domain located towards the N-terminal has a double helical transmembrane structure, and the C-terminal region is intrinsically disordered, extended either into the cytosol (as in Asrij) or extracellular space (as in OCIAD2) (Praveen et al., 2020). Like Asrij, the OCIA domain of OCIAD2 is crucial for its localization and molecular interactions (Sinha et al., 2013; Sinha et al., 2018). The double-helical region of the OCIA domain in OCIAD2 is sufficient for its localization and interaction with Asrij and STAT3 (Sinha et al., 2018). The exact mechanism of OCIAD2 in regulating erythropoiesis and the role of the double-helical region in the same is an interesting aspect to be studied.

Proteins like p53 and RELT, which play critical roles in hematopoietic homeostasis, harbor intrinsically disordered regions (Uversky, 2016; Cusick et al., 2020) known to adopt conformations in an interactor-specific manner (Uversky, 2013). Of late, OCIAD2 has been predicted to harbor an additional helix in its C-terminal region (https://www.uniprot.org/uniprot/Q9D8W7). However, these predictions are to be verified by X-ray crystallography, and detailed analyses are required to know the biochemical nature of Asrij and OCIAD2, which help in understanding their structure-function relationship.

5.2 OCIAD2 isoforms and their role in mouse hematopoiesis

Protein isoforms can play differential roles in mouse hematopoiesis (Corrigan et al., 2018). Different isoforms of specific proteins show distinct temporal expression patterns, like in the case of RUNX1c, which is expressed only during the emergence of HSCs in the fetal stage, but not in adult hematopoiesis (Challen and Goodell, 2010). OCIAD2 has a verified shorter isoform of 103 amino acids, and it differs from the full-length isoform of 154 amino acids at its C-terminal (https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=433904).

Though the transcripts of full-length and the shorter isoforms of *ociad2* were detected in all major organs of C57BL/6J adult mice, protein detection showed only the full-length isoform at higher levels in the kidney, liver, and brain, relative to other tissues (Sinha et al., 2018). Since the shorter isoform harbors the OCIA domain (https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=433904), it might display functions similar to that of its full-length isoform, at least those governed by the domain, which needs to be verified. Also, it is unknown whether the shorter isoform shows preferential expression and function in embryonic hematopoietic tissues, which merits investigation. This analysis is essential in the light of studies reporting the indispensable role of certain genes in embryonic but not in adult hematopoiesis like the *Cdx4* and *Runx1* in mouse and zebrafish, respectively (Koo et al., 2010; Sood et al., 2010).

5.3 Tissue-specific functions of OCIAD2

Despite the expression of OCIAD2 across many vital organs of mice (Sinha et al., 2018), its deletion results in macroscopic changes only in the spleen and kidney. Splenomegaly reported in *asrij* KO mice (Sinha et al., 2019[b]) is more severe than that of *ociad2*^{pdsn} KO mice presented in this study. It indicates a diversified or a less critical function for OCIAD2 in maintaining splenic relative weight and homeostasis. While the loss of *ociad2* leads to kidney atrophy (Chapter-3), the effect of *asrij* KO on mouse kidneys is not reported. Nevertheless, studying Asrij expression in OCIAD2 KO kidney may give important insights into the potential role of Asrij in kidney function. OCIAD2 expression is higher in mouse liver relative to BM (Sinha et al., 2018). However, its loss did not affect the relative weight of the liver (Chapter-3), indicating a tissue-specific function of OCIAD2, which merits further investigation.

5.4 Defective erythropoiesis and anemia of *ociad2*^{pdsn} KO mice

Decreased PB RBC counts and BM reticulocyte frequency in *ociad2*^{pdsn} KO mice, despite the increased levels of Asrij in BM (Chapter-4), indicate the indispensable role of OCIAD2 in the maintenance of erythropoiesis. However, Asrij is also important for normal erythropoiesis as *asrij* KO mice also show decreased PB RBC counts (Sinha et al., 2019[b]). Further, identifying the specific erythroid differentiation stages responsible for the lowered production of reticulocytes in the *ociad2*^{pdsn} KO mice and analyzing the status of JAK/STAT, PI3K/AKT, and Erk1/2 signaling in the effector cells help in deciphering the cell-specific role of OCIAD2 in steady-state erythropoiesis.

Though our study shows reticulocytopenia in BM as the possible reason behind decreased RBC counts in the KO mice, other factors of RBCs like the reduced half-life and increased clearance in the spleen merits investigation. The reduced half-life of RBCs can lead to anemia and is associated with chronic kidney disease (Li et al., 2019) and chronic heart failure (Manabe et al., 2016). Aged RBCs lose their ability to deform and pass through the intricate mesh of splenic red pulp leading to their subsequent lysis and phagocytosis by the red pulp macrophages (Klei et al., 2020). Increased RBC filtration can lead to splenomegaly and anemia (Lv et al., 2016). OCIAD2 KO increases RBC volume, and whether this decreases RBCs' deformability is unknown. Histological analysis of the *ociad2*^{pdsn} KO spleen may help in observing any changes in the distribution of red and white pulp and flow cytometry for cellular composition.

Renal pericytes produce erythropoietin (Epo) upon transcriptional activation by HIF2α, a molecule induced by lower hemoglobin levels in the blood (Suzuki et al., 2018). Since *ociad2*^{pdsn} KO mice show anemia, any possibility of hypoxia and its effect on HIF2α expression in the kidney is to be checked. However, the KO kidney shows unchanged transcript levels of *Epo* (Chapter-4). Possibilities of defective hemoglobin production in the KO BM or its loss due to an excess RBC clearance in the KO spleen leading to anemia need to be tested. The mouse liver regulates the iron homeostasis in the body through hepcidin and thus the hemoglobin production (Liang and Ghaffari, 2016) and acts as an extra-medullary organ during acute anemia involving erythroblasts and macrophages (Sonoda et al., 2012). The effect of *ociad2*^{pdsn} KO on this role of the liver is unknown.

5.5 Functional significance of OCIAD2 localization to mitochondria

The essential role of mitochondria in hematopoiesis is well documented. Increased mitochondrial metabolism is coupled with the elongation of mitochondria and the progression of HSPCs towards differentiation (Diebold and Chandel, 2016). Mitochondrial dynamics vary across HSCs primed towards different lineages. Loss of mitofusin specifically affects the self-renewal and repopulating capacity of lymphoid-biased HSCs with minimal effects on myeloid-biased HSCs (Luchsinger et al., 2016). Asrij localizes to mitochondria (Shetty et al., 2018, Sinha et al., 2019[b]). Asrij is important for regulating mitochondrial dynamics in the lymph gland progenitor pool. Its loss leads to elongation of mitochondria and increased differentiation of progenitors towards crystal cells in *Drosophila* (Ray et al., 2021). Asrij is localized to the

inner mitochondrial membrane and interacts with electron transport chain complex I components in hESCs (Shetty et al., 2018) and other human cells (Le Vasseur et al., 2021). Reduced Asrij levels lead to increased oxidative phosphorylation and enhanced differentiation in hESCs (Shetty et al., 2018). Recently loss of Asrij was shown to reduce the mitochondrial junction dynamics in hESCs (Ray et al., 2021). Asrij maintains hematopoietic progenitors and stemness of hESCs through modulating mitochondrial dynamics and metabolism.

OCIAD2 also localizes to mitochondria (Sinha et al., 2018), and a recent report suggests that OCIAD2 is associated with mitochondrial membrane in lung adenocarcinoma cells (Hong et al., 2021). An earlier report showed OCIAD2 localization to the inner mitochondrial membrane (Da Cruz et al., 2003). It is crucial to know whether the reported interaction of Asrij and OCIAD2 occurs in the mitochondrial fraction and whether OCIAD2 is a part of the interaction complex of Asrij, which can provide the first line of evidence for the role of OCIAD2 in mitochondrial biology. Based on the phenotypes observed in the *ociad2*^{pdsn} KO mice, the unperturbed HSPC abundance and their lineage specification capacity, OCIAD2 might play a minimal role in mitochondrial dynamics in HSPCs. However, the possibility of increased levels of Asrij in OCIAD2 KO HSPCs and its effect on their mitochondrial dynamics is an interesting aspect to be tested. Mitochondria play an essential role in the terminal differentiation of erythroid cells. Loss of p32/C1qbp protein impairs mitochondrial oxidation and inactivates mTORC1 signaling, hampering erythrocyte differentiation leading to anemia in mice (Gotoh et al., 2020). While *ociad2*^{pdsn} KO mice show defective reticulocyte production and anemia (Chapter-4), studies on mitochondrial localization of OCIAD2 in erythroid progenitors and any possible relation with p32/C1qbp and mitochondrial oxidation merit investigation.

5.6 Functional significance of OCIAD2 localization to endosomes and lipid rafts

Rab5 protein plays an essential role in vertebrate hematopoiesis, especially during the emergence of HSPCs from the hemogenic endothelium (Heng et al., 2020). Notch and AKT signaling are essential for normal hematopoiesis (Jafari et al., 2019). Rab5c is indispensable for proper vesicular transport of Notch and AKT signaling molecules involved in the emergence of HPSC. Rab5c-Notch relation is important for HSPC specification, while the AKT signaling is essential for survival. Also, overexpression of Rab5c leads to excessive endocytic trafficking and decreased production of HSPCs, indicating the necessity of a

threshold in Rab5c levels to maintain normalcy (Heng et al., 2020). Asrij localizes to $Rab5^+$ endosomes (Sinha et al., 2013; Sinha et al., 2019[b]), regulates the trafficking of the notch intracellular domain (NICD) (Kulkarni and Khadilkar et al., 2011) and PI3K/AKT signaling (Khadilkar et al., 2014; Sinha et al., 2019[b]) in multiple contexts. This indicates a possible role of Asrij in Rab5 mediated regulation of Notch and AKT signaling and might give insights into the role of OCIAD2, which also localizes to $Rab5^+$ endosomes and interacts with Asrij (Sinha et al., 2018). Interestingly, canonical Notch signaling is dispensable for myelo-erythropoiesis during steady-state and stress conditions (Duarte et al., 2018), indicating cell-specific functions of signaling pathways.

Human OCIAD2 localizes to lipid rafts (Han et al., 2014), highly ordered plasma membrane regions with high cholesterol and sphingolipids (Morgan et al., 2020). Maintaining normal cholesterol levels is important as increased cholesterol levels lead to HSPC differentiation, migration, skewing towards myeloid lineages and thrombocytosis, affecting various inflammatory cytokine receptors concentrated in the lipid rafts (Murphy et al., 2011; Murphy et al., 2013). Interestingly, lipid rafts are essential for the conduction of Epo-EpoR signaling as cholesterol depletion attenuates Epo-induced STAT5 phosphorylation in primary erythroid progenitors (McGraw et al., 2012). It is an intriguing future aspect to check whether mouse OCIAD2 also localizes to lipid rafts and regulates Epo-EpoR signaling in mouse erythroid cells.

5.7 Role of OCIAD2 in modulating STAT activation

STAT proteins are essential and common signal transducers for various upstream signaling molecules like EGF, hepatocyte growth factor, interleukin (IL)-6, insulin-like growth factor, colony-stimulating factor-1, platelet-derived growth factor, hormones (growth hormone, insulin, Epo), and downstream of some G-protein coupled receptors (Loh et al., 2019). While persistent activation of STAT3 and STAT5 is known to cause chronic inflammation and carcinogenesis, their decreased levels cause cell death, indicating a critical regulation of their levels and activation to maintain homeostasis. Not all known STAT molecules behave alike. For example, STAT1 suppresses tumor growth by exerting pro-apoptotic and anti-proliferative effects (Loh et al., 2019). Concerning hematopoiesis, STAT molecules exert cell-specific effects. STAT5 is a well-known regulator of differentiation, proliferation, and survival of erythroid progenitors, while STAT1 antagonizes STAT5 activation in the splenic erythroblasts,

post-hemolytic stress. Interestingly, loss of STAT1 decreases the CFU-E cells in the BM but increases the number of BFU-E and CFU-E cells in the spleen with increased response to Epo (Halupa et al., 2005), indicating a context-dependent function of STAT1. The inverse relation between STAT3 activation and anemia has been recently reported (Mauracher et al., 2020).

While both Asrij and OCIAD2 promote STAT3 activation (Sinha et al., 2013; Sinha et al., 2018), the *ociad2*^{pdsn} KO mouse Lin^{+} BM showed increased STAT3 activation (Chapter-4), indicating a context-dependent function of OCIAD2. However, the deregulated STAT activation seems to be tissue-specific as constitutive activation of STAT3 leads to tumors and metastasis (Tolomeo et al., 2021), and no such overt abnormalities were found in the *ociad2*^{pdsn} KO mice. Increased activation of STAT3 in Lin^{+} BM might render the myeloid and B cells with proliferation advantage, leading to their increased frequency (Huang, 2007). We show that OCIAD2 is required to activate STAT5 (Chapter-4). The binding of STAT5 to effector DNA elements is epigenetically regulated (Sollinger et al., 2017). Hence, different erythroid stages with specific chromatin organization (Schulz et al., 2019) can show differential responses to Epo-EpoR signaling. Therefore, cell-specific effects of deregulated STAT activation are more likely to mediate the effect of OCIAD2 KO on mouse hematopoiesis and the homeostasis of other tissues, which merits further investigation.

Asrij colocalizes with STAT3 on Rab5^{+} endosomes (Sinha et al., 2013). Interestingly, OCIAD2 also localizes to Rab5^{+} endosomes (Sinha et al., 2018). Also, OCIAD proteins (Sinha et al., 2018; Sinha et al., 2019^{b}), STAT3 (Wegrzyn et al., 2009), and STAT5 (Zhang et al., 2021) localize to mitochondria. Activation of STAT3 at Y705 (pY705) regulated by Jak is essential for mitochondrial localization to the inner mitochondrial membrane (IMM), whereas pS727 is regulated by MAPK signaling and governs mitochondrial transcription, independent of nuclear transcription (Peron et al., 2021). Interestingly, both Asrij and OCIAD2 promote STAT3 activation on Y705, indicating a possible interplay involving endosomes and mitochondria in maintaining tissue homeostasis (Sinha et al., 2013; Sinha et al., 2018). However, this requires a thorough investigation, as a recent report claims that the actual localization of STAT3 is not the pure mitochondria but the mitochondria-associated endoplasmic reticulum membrane (MAM) (Su et al., 2020). Therefore, deciphering the precise molecular mechanism of OCIAD proteins regulating STAT activation widens the knowledge on both the spatial regulation of JAK/STAT signaling and any possible role of inter-organelle crosstalk in hematopoiesis,

especially as organelle dynamics are gaining importance in this regard (Heng et al., 2020; Julian et al., 2020).

5.8 Role of OCIAD2 in cell signaling under stress conditions

In contrast to the steady-state erythropoiesis in BM, stress erythropoiesis in the spleen is dependent on the BMP-4 and Hedgehog signaling and the splenic macrophages (Paulson et al., 2020). Since the *ociad2*^{pdsn} KO mice are more susceptible to stress-induced splenomegaly (Chapter-4), the potential role of OCIAD2 in regulating splenic cellular changes like stress-erythroid progenitors (SEPs), stress-BFU-E, and splenic macrophages, and molecular changes like downregulation of Wnt signaling, upregulation of Epo-EpoR signaling, etc., causing stress erythropoiesis needs to be investigated (Paulson et al., 2020). Also, the Epo-EpoR signaling activates PI3K/AKT and MAPK pathways (Tothova et al., 2021), marking the necessity to study the effect of OCIAD2 KO on these signaling pathways.

Stress mediated by LPS increases the susceptibility of *ociad2*^{pdsn} KO mice to kidney injury apart from splenomegaly (Chapter-4). LPS damages kidney podocytes' structural and functional integrity by upregulating the Wnt/β-catenin pathway (Senouthai et al., 2019) and expression of Angptl4 (Shen et al., 2020), indicating a possible role of OCIAD2 in modulating these signaling pathways. Concomitant analysis of Asrij levels in spleen and kidney of *ociad2*^{pdsn} KO mice help to understand the possible implications of OCIAD proteins' interplay in the manifestation of splenomegaly and kidney malfunction.

5.9 Asrij and OCIAD2 equilibrium in hematopoietic homeostasis

Asrij is expressed in nearly 77% of whole BM, 48% of LT-HSCs, 98% of HSPC, 93% of CD11b^{+} cells, and 80% of CD19^{+} cells in mouse BM with higher transcript levels in hematopoietic stem cells and progenitors than the differentiated cells (Sinha et al., 2019^{b}). In this study, we show the presence of OCIAD2 in nearly 89% of whole BM and >92% of undifferentiated and differentiated BM hematopoietic cells in C57BL/6J mice. OCIAD2 protein levels decrease down the differentiation hierarchy (Chapter-2).

Molecular analyses show increased Asrij levels in the whole BM of *ociad2*^{pdsn} KO mice (Chapter-4), indicating a possible compensatory effect of Asrij. Though Asrij levels increased in the unfractionated BM, it is unknown whether the increase is a pan-hematopoietic effect or

restricted to specific cell types. Interestingly, loss of *asrij* increases the OCIAD2 levels in mouse BM (Sinha S. Ph.D. thesis, 2019). However, the effects of OCIAD2 KO on mouse hematopoiesis are notably different from those of Asrij KO in mice, indicating diversified and cell-specific functions of the paralogs and an interplay between them in regulating hematopoietic homeostasis. Although the expression of OCIAD proteins in mouse BM and its sub-populations is known from individual studies, details regarding their coexpression are unclear. Appending this is equally essential to know the expression levels of Asrij in *ociad2*^{pdsn} knockout hematopoietic cells and vice versa, which will help decipher the regulation of the equilibrium. Interestingly, protein levels of Asrij decrease with age in mouse BM (Sinha et al., 2022). Questions like how the expression of OCIAD2 changes with age in mice; Does Asrij levels decrease in aged *ociad2*^{pdsn} KO mouse BM, and what effect do these changes show on mouse hematopoiesis, are crucial in not only understanding the interplay between the OCIAD proteins but also the impact of aging on hematopoietic regulation.

5.10 Compensatory mechanisms between *asrij* and *ociad2*

Genetic compensation maintains genetic robustness in the absence of a gene by enhancing the expression of its paralog/functional equivalent genes. *Runx* and *Hox* genes are well-known for genetic compensation. RUNX1 regulates HSC differentiation (Hsu et al., 2020). Increased protein levels of RUNX2 and RUNX3 compensate upon inhibition of RUNX1 in leukemic cells due to the loss of mutual suppression of transcription. *Runx* family genes carry the characteristic Runx-binding sequences in their promoter region, by which each other family member regulates their expression. This regulation is lost when a given RUNX protein is knocked out or inhibited, leading to the overexpression of the other *Runx* family genes (Morita et al., 2017).

HOXA proteins play an essential role in fetal hematopoiesis. HOXA5 and HOXA7 are essential for *in vitro* expansion and engraftment of HPSC from the fetal liver, while HOXA9 is implicated in fetal HSC self-renewal. Loss of the Hoxa5 gene does not lead to strong HSC phenotypes, while depleting the entire cluster of Hoxa severely reduces adult HSC activity, indicating a compensatory mechanism between individual Hoxa genes (Dou et al., 2016). However, HOXA proteins are no exception to the deviation from the functional equivalence as HOXA5 inhibits cell differentiation whereas HOXB5 promotes differentiation (Winnik et al., 2009).

Caudal-related homeobox (*Cdx*) genes regulate Hox gene expression. Knockdown of *Cdx1a* in zebrafish is associated with a mild phenotype and is expected due to compensation by *Cdx4* and other *Cdx* family members. However, the knockdown of *Cdx4* leads to severe axial phenotype, further emphasized by the knockdown of *Cdx1a*, indicating the loss of functional equivalence (Davidson and Zon, 2006). Similarly, transcriptional regulators of the cohesin family, *Stag1* and *Stag2,* show compensation at transcript and protein levels (Viny et al., 2019). However, the compensation is unidirectional, with increased *Stag1* transcription and protein level in *Stag2* KO hematopoietic cells but not vice versa. Also, *Stag2* and *Stag1* play a non-redundant role in hematopoiesis, with a specific role for Stag2 in regulating the balance between hematopoietic differentiation and self-renewal (Viny et al., 2019).

The exact mechanism of genetic compensation is still unclear. However, based on experimental evidence from various model systems, multiple mechanisms were postulated, mainly categorized into DNA damage responses and mutant RNA responses (El-Brolosy et al., 2017). In the DNA damage response mechanism, damage to the loci of the target gene might cause reorganization and increased accessibility of the chromatin around the compensating gene, resulting in its enhanced transcription. Mutations in genes leading to the formation of premature termination codon (PTC) undergo nonsense-mediated decay (NMD). Evidence from a zebrafish study shows that the genetic compensation is positively correlated to the degree of NMD of mRNA. This has been hypothesized that the stable RNA fragments resulting from the NMD, capable of forming dsRNA duplexes comprised of antisense transcripts from the compensating locus, can lead to transcriptional upregulation of the compensating gene (Schuermann et al., 2015). Enhanced expression of the compensating gene was also found at the translation level involving RNA-binding proteins (RBPs) (Kuwano et al., 2009). Increased availability of the RBPs due to the unavailability of the target gene might lead to increased binding to and hence the stability of the mRNA of the compensating gene leading to its increased translation (El-Brolosy et al., 2017). Also, miRNAs under starvation conditions were reported to bind to 5'-UTR of the target gene and upregulate its expression (Jacobsen et al., 2013). Other possible mechanisms involving mRNA could be the pseudouridylation or N6-methylation of adenosines (m6A)-mediated increased stability and upstream open reading frames (uORFs)-mediated translational repression (El-Brolosy et al., 2017).

The scenario of mutual genetic suppression like in the case of *Runx* family genes is possible too as Asrij is reported in the nucleus under some contexts (Sinha et al., 2013). However as

there is no information about whether OCIAD proteins have transcriptional activity or promoter binding sites this possibility is difficult to test. Bioinformatic analyses too did not suggest OCIAD protein factor binding sites, as per the data from the TF2DNA database (Pujato et al., 2014; www.fiserlab.org/tf2dna_db/cite_us.html; Praveen et al., 2020). The strategy of knocking out OCIAD genes in mice, as reported earlier (Sinha et al., 2019[b]), and in this study, are predicted to produce truncated mRNA with PTC. Loss of OCIAD2 did not affect the transcript levels of *asrij* but caused an increase in the protein levels, indicating fewer chances of DNA damage response-mediated and NMD-mediated compensation, but probably due to RBP-mediated increased translation which needs to be tested.

Mice double knockout for both the *ociad* genes is the standard approach to test the redundancy between the OCIAD paralogs. However, the double knockout mice cannot be produced by the conventional crossing strategies due to the proximity between the two genes. However, mice double heterozygous knockout for both *asrij* and *ociad2* are possible and can help in revealing the actual effect of OCIAD2 on HSPC biology to a certain extent.

5.11 Role of physical interactions in governing relative abundance and functional output of OCIAD proteins

Apart from the genetic and translational level, compensation can also occur through protein-protein interactions (PPIs). Studies show that paralogous genes opt for different functions for evolutionary success. A significant fraction of paralogous proteins may be functionally dependent on each other through PPIs, generally by forming heterodimers. Recent studies have shown that heterodimers of paralogous proteins may maintain a stricter balance in their levels, increasing the risk of loss of homeostasis upon loss of any monomer (Dandage and Landry, 2019). Also, a recent meta-analysis study indicates that two paralogs might have overlapping binding partners or interactors. Their relative binding affinity and abundance can dictate the balance between the paralogs and thus their functional outcome (Sousa et al., 2019). Interestingly, Asrij and OCIAD2 seem to have different functions in maintaining mouse hematopoietic homeostasis, interacting with each other and STAT3 as a common interactor (Sinha et al., 2018, Sinha et al., 2019[b]). This study adds strength to the earlier reports proposing the heterodimerization of Asrij and OCIAD2 to execute their functions (Sinha et al., 2018) and opens new venues to study the possible role of physical interactions governing the relative abundance and functions of OCIAD proteins.

5.12 Role of OCIAD2 in cytoskeletal dynamics

Ociad2^{pdsn} KO mice show larger platelets in the blood (Chapter-4) sequentially derived from the BM megakaryocytes. Proplatelets, the cytoplasmic extensions of BM megakaryocytes formed in the blood vessel sinuses, break further into preplatelets (~10 μm in diameter) and finally into platelets of 1 to 3 μm in diameter (Machlus and Italiano, 2013). This process involves endomitosis, cytoplasmic remodeling, and fission. The fission of proplatelets is majorly dependent on the dynamics and stability of microtubules, and expression levels of Integrin $\alpha_2\beta_1$, as α chains regulate the size of platelets in a direct manner (Kunicki et al., 2012[a,b]; Habart et al. 2013), suggesting a possible role of OCIAD2 in microtubule dynamics and stability, supporting its reported role in cell migration (Sinha et al., 2018).

5.13 Inflammation in *ociad2*^{pdsn} KO mice

The presence of splenomegaly, kidney atrophy, impaired renal function, and decrease in RBC counts and hemoglobin levels in blood suggest a possibility of IL-6-mediated inflammation in *ociad2*^{pdsn} KO mice (Chen et al., 2013; Akchurin et al., 2019). IL-6-mediated hyperactivation of JAK/STAT3 signaling in hematological malignancies and solid tumors is well-known (Johnson et al., 2018). Hence, the increased activation of STAT3 could also be due to a rise in IL-6 levels in *ociad2*^{pdsn} KO mice. While STAT3 activation is Asrij-dependent, transcript levels of *asrij* are governed by the LIF-LIFR-GP130 signaling axis where LIF belongs to the IL-6 cytokine family (Sinha et al., 2013). This leads to an intriguing question about the possible role of IL-6 in regulating Asrij levels, which needs to be investigated. Evidence indicates that the internalization of IL-6 and its receptor can lead to the activation of STAT molecules at early endosomes (Schmidt-Arras and Rose-John, 2021). Owing to the localization to endosomes and the regulation of STAT3 activation, the OCIAD proteins may interact with IL-6 signaling components on endosomes (Sinha et al., 2013; Sinha et al., 2018; Schmidt-Arras and Rose-John, 2021), which merits further investigation.

5.14 Future directions

Additional to the discussed prospective aspects of research based on this thesis, a few more potential research avenues are listed below.

i. Transplanting *ociad2*^{pdsn} KO BM HSCs into lethally-irradiated syngeneic wild-type mice helps to test whether the observed hematopoietic phenotype in the KO mice is due to the cell-intrinsic property of HSCs.

ii. Transcript levels of *ociad2* are elevated in certain leukemia (Bagger et al., 2016), indicating that a threshold of its expression might be important to maintain normal hematopoiesis. This hypothesis can be tested through hematopoietic characterization of *ociad2*-overexpressing transgenic mice or irradiated mice transplanted with HSCs overexpressing *ociad2*.

iii. Phenotypic rescue following vector-mediated delivery of *ociad2* gene in *ociad2*^{pdsn} KO BM HSPCs help to test whether OCIAD2 acts upstream of STAT3 and STAT5 in mouse BM cells. A similar approach, but to downregulate the expression of *asrij* in *ociad2*^{pdsn} KO BM HSPCs will be helpful to test the effect of increased Asrij levels on the observed phenotype of *ociad2*^{pdsn} KO mice.

5.14 Concluding remarks

Although Asrij and OCIAD2 belong to the same family of proteins, share structural, and sequence homology, and interact with each other (Sinha et al., 2018) and are expressed in both HSPCs and differentiated cells of mouse BM (Sinha et al., 2019[b]), the effects of their knockout on mouse hematopoiesis are different and most probably play a cell-specific role in maintaining hematopoietic homeostasis (Figure-5.1). Our work identifies OCIAD2 as a novel endosomal and mitochondrial regulator of mouse adult hematopoiesis, especially the steady-state erythropoiesis with possible implications in stress erythropoiesis and underlines the importance of inter-organ coordination in the regulation of erythropoietic homeostasis (Figure-5.2). It provides new insights into JAK/STAT signaling regulation in mouse BM. Our work strengthens the proposition of the essentiality of balance between the OCIAD proteins in maintaining hematopoietic homeostasis and marks the importance of synchronous analysis of these paralogs. Also, our study gives new insight into the organelle crosstalk that controls the hematopoietic response to stress. The *ociad2* mutant mice reported in this study will be a

valuable tool to understand hematopoiesis regulation and especially to study anemia, stress erythropoiesis, and kidney function.

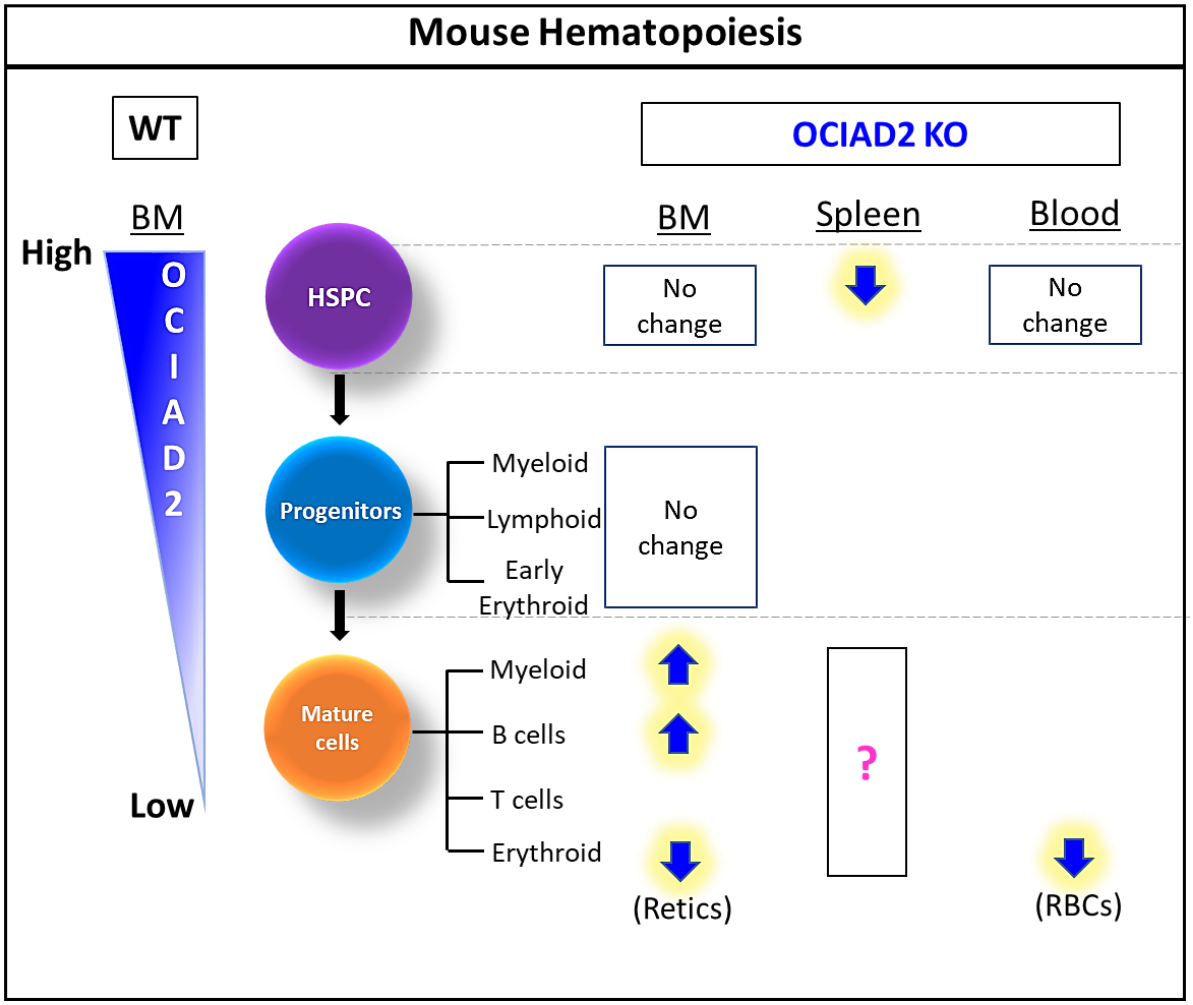


Figure-5.1: Schematic summarizing the expression profile of OCIAD2 in mouse bone marrow and the hematopoietic phenotype observed upon its loss. Expression of OCIAD2 decreases with hematopoietic differentiation (blue gradient map). Loss of OCIAD2 leads to a prominent phenotype of defective reticulocyte (retics) production in the BM and is most likely translated into the decreased RBC counts in the PB. Interestingly, OCIAD2 KO does not affect the overall abundance of HSPCs and their lineage specification ability, probably due to the hypothesized functional compensation by the increased levels of Asrij in the KO BM. This thesis reports the importance of OCIAD2 in maintaining mouse hematopoietic homeostasis and strengthens the possibility of an interplay between the OCIAD proteins in the same. Dashed lines demarcate the simplified three different categories of hematopoietic cells. Upward blue arrows indicate an increase and downward blue arrows indicate a decrease. Aspects to be studied are indicated by '?' in pink.

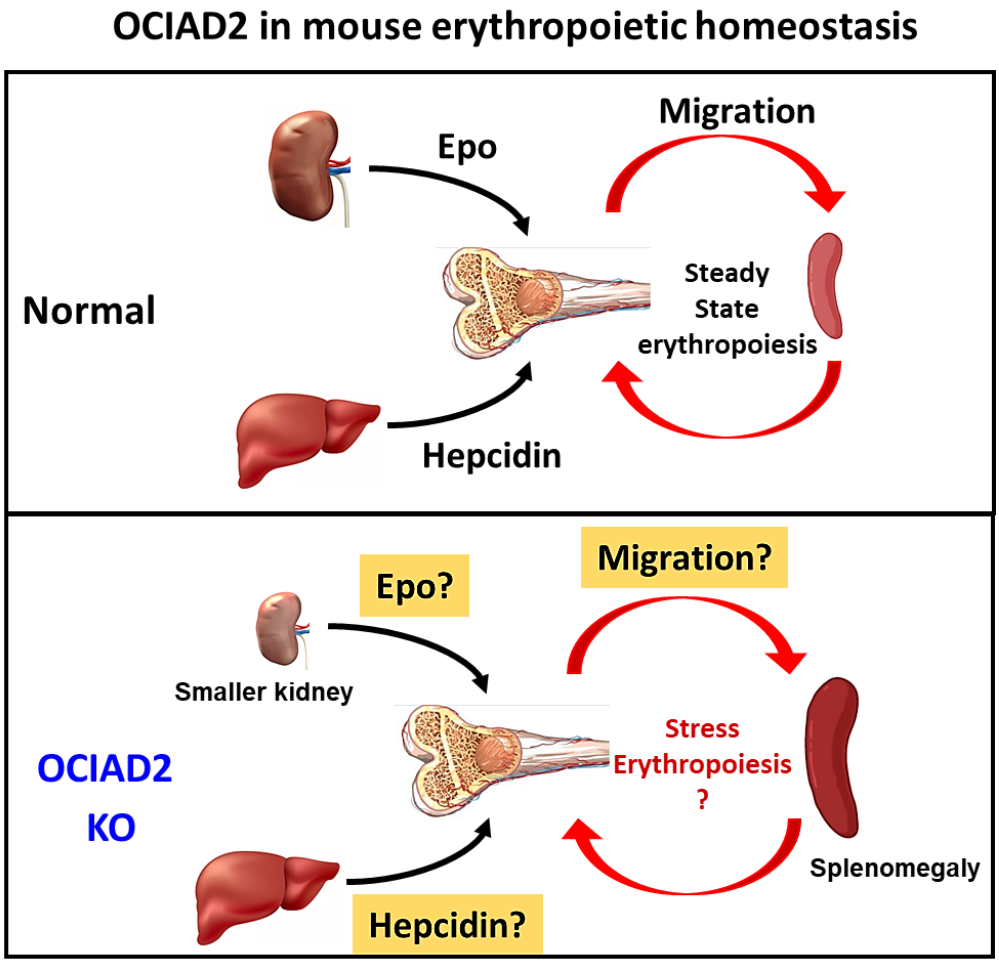


Figure-5.2: Schematic representing the key anatomical changes in mice upon deletion of OCIAD2 and possible implications on erythropoietic homeostasis. Factors like erythropoietin (Epo) and hepcidin secreted by the spleen and liver (indicated by black angular arrows), respectively, are the primary regulators of the steady-state erythropoiesis in the BM and the stress erythropoiesis in the spleen. Loss of OCIAD2 leads to kidney atrophy and splenomegaly in mice, indicating impaired erythropoietic regulation and stress erythropoiesis. Cellular analyses reported in this study indicate an affected cell migration (indicated by red angular arrows) probably playing a role in the splenomegaly. This thesis hints at a possible role of OCIAD2 in multiple tissues coordinating RBC production and provides a new mouse model to study inter-organ communication in maintaining erythropoietic homeostasis.

References

Ahmed, M., 2016. Extracellular matrix regulation of stem cell behavior. *Current stem cell reports*, *2*(3), pp.197-206.

Akchurin, O., Patino, E., Dalal, V., Meza, K., Bhatia, D., Brovender, S., Zhu, Y.S., Cunningham-Rundles, S., Perelstein, E., Kumar, J. and Rivella, S., 2019. Interleukin-6 contributes to the development of anemia in juvenile CKD. *Kidney international reports*, *4*(3), pp.470-483.

Aleem, E. and Arceci, R.J., 2015. Targeting cell cycle regulators in hematologic malignancies. *Frontiers in cell and developmental biology*, *3*, p.16.

Andrassy, K.M., 2013. Comments on 'KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease'. *Kidney international*, *84*(3), pp.622-623.

Arndt, K., Grinenko, T., Mende, N., Reichert, D., Portz, M., Ripich, T., Carmeliet, P., Corbeil, D. and Waskow, C., 2013. CD133 is a modifier of hematopoietic progenitor frequencies but is dispensable for the maintenance of mouse hematopoietic stem cells. *Proceedings of the National Academy of Sciences*, *110*(14), pp.5582-5587.

Bagger FO, Sasivarevic D, Sohi SH, et al. BloodSpot: a database of gene expression profiles and transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Res*. 2016;44(D1):D917-924.

Barbosa, C. and Romão, L., 2014. Translation of the human erythropoietin transcript is regulated by an upstream open reading frame in response to hypoxia. *Rna*, *20*(5), pp.594-608.

Benveniste, P., Frelin, C., Janmohamed, S., Barbara, M., Herrington, R., Hyam, D. and Iscove, N.N., 2010. Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. *Cell stem cell*, *6*(1), pp.48-58.

Bhoopalan, S.V., Huang, L.J.S. and Weiss, M.J., 2020. Erythropoietin regulation of red blood cell production: From bench to bedside and back. *F1000Research*, *9*.

Brendolan, A., Ferretti, E., Salsi, V., Moses, K., Quaggin, S., Blasi, F., Cleary, M.L. and Selleri, L., 2005. A Pbx1-dependent genetic and transcriptional network regulates spleen ontogeny. Development, 132(13), pp.3113-3126.

Bresciani, E., Carrington, B., Yu, K., Kim, E.M., Zhen, T., Guzman, V.S., Broadbridge, E., Bishop, K., Kirby, M., Harper, U. and Wincovitch, S., 2021. Redundant mechanisms driven independently by RUNX1 and GATA2 for hematopoietic development. *Blood Advances*, *5*(23), pp.4949-4962.

Bröske, A.M., Vockentanz, L., Kharazi, S., Huska, M.R., Mancini, E., Scheller, M., Kuhl, C., Enns, A., Prinz, M., Jaenisch, R. and Nerlov, C., 2009. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nature genetics*, *41*(11), pp.1207-1215.

Brown, G. and Ceredig, R., 2019. Modeling the hematopoietic landscape. *Frontiers in cell and developmental biology*, *7*, p.104.

Brown, G., Tsapogas, P. and Ceredig, R., 2018. The changing face of hematopoiesis: a spectrum of options is available to stem cells. *Immunology and cell biology*, *96*(9), pp.898-911.

Cain, J.E., Di Giovanni, V., Smeeton, J. and Rosenblum, N.D., 2010. Genetics of renal hypoplasia: insights into the mechanisms controlling nephron endowment. Pediatric research, 68(2), pp.91-98.

Canu, G. and Ruhrberg, C., 2021. First blood: the endothelial origins of hematopoietic progenitors. *Angiogenesis*, pp.1-13.

Challen, G.A. and Goodell, M.A., 2010. Runx1 isoforms show differential expression patterns during hematopoietic development but have similar functional effects in adult hematopoietic stem cells. *Experimental hematology*, *38*(5), pp.403-416.

Chan, D., Janssen, J.C., Whitwell, J.L., Watt, H.C., Jenkins, R., Frost, C., Rossor, M.N. and Fox, N.C., 2003. Change in rates of cerebral atrophy over time in early-onset Alzheimer's disease: longitudinal MRI study. *The Lancet*, *362*(9390), pp.1121-1122.

Chen, J., Hendriks, M., Chatzis, A., Ramasamy, S.K. and Kusumbe, A.P., 2020. Bone vasculature and bone marrow vascular niches in health and disease. *Journal of Bone and Mineral Research*, *35*(11), pp.2103-2120.Chasis, J.A. and Mohandas, N., 2008. Erythroblastic islands: niches for erythropoiesis. Blood, The Journal of the American Society of Hematology, 112(3), pp.470-478.

Chen, Y., Xiang, J., Qian, F., Diwakar, B.T., Ruan, B., Hao, S., Prabhu, K.S. and Paulson, R.F., 2020. Epo receptor signaling in macrophages alters the splenic niche to promote erythroid differentiation. *Blood*, *136*(2), pp.235-246.

Chen, Y.F., Wu, Z.M., Xie, C., Bai, S. and Zhao, L.D., 2013. Expression level of IL-6 secreted by bone marrow stromal cells in mice with aplastic anemia. *International Scholarly Research Notices*, *2013*.

Cheng, H., Zheng, Z. and Cheng, T., 2020. New paradigms on hematopoietic stem cell differentiation. *Protein & cell*, *11*(1), pp.34-44.

Cheng, T., Rodrigues, N., Shen, H., Yang, Y.G., Dombkowski, D., Sykes, M. and Scadden, D.T., 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science*, *287*(5459), pp.1804-1808.

Chiu, S.C., Liu, H.H., Chen, C.L., Chen, P.R., Liu, M.C., Lin, S.Z. and Chang, K.T., 2015. Extramedullary hematopoiesis (EMH) in laboratory animals: offering an insight into stem cell research. *Cell Transplantation*, *24*(3), pp.349-366.

Chotinantakul, K. and Leeanansaksiri, W., 2012. Hematopoietic stem cell development, niches, and signaling pathways. *Bone marrow research*, *2012,* 16 pages.

Chute, J.P., Muramoto, G.G., Fung, J. and Oxford, C., 2005. Soluble factors elaborated by human brain endothelial cells induce the concomitant expansion of purified human BM CD34+ CD38–cells and SCID-repopulating cells. *Blood*, *105*(2), pp.576-583.

Clark, M.R., Mandal, M., Ochiai, K. and Singh, H., 2014. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nature Reviews Immunology*, *14*(2), pp.69-80.

Classen, S., Muth, C., Debey-Pascher, S., Eggle, D., Beyer, M., Mallmann, M.R., Rudlowski, C., Zander, T., Pölcher, M., Kuhn, W. and Lahn, M., 2010. Application of T cell-based transcriptomics to identify three candidate biomarkers for monitoring anti-TGFβR therapy. *Pharmacogenetics and genomics*, *20*(3), pp.147-156.

Corrigan, D.J., Luchsinger, L.L., de Almeida, M.J., Williams, L.J., Strikoudis, A. and Snoeck, H.W., 2018. PRDM16 isoforms differentially regulate normal and leukemic hematopoiesis and inflammatory gene signature. *The Journal of clinical investigation*, *128*(8), pp.3250-3264.

Craig, C.E.H., Quaglia, A. and Dhillon, A.P., 2004. Extramedullary haematopoiesis in massive hepatic necrosis. *Histopathology*, *45*(5), pp.518-525.

Crippa, S., Rossella, V., Aprile, A., Silvestri, L., Rivis, S., Scaramuzza, S., Pirroni, S., Avanzini, M.A., Basso-Ricci, L., Hernandez, R.J. and Zecca, M., 2019. Bone marrow stromal cells from β-thalassemia patients have impaired hematopoietic supportive capacity. *The Journal of clinical investigation*, *129*(4), pp.1566-1580.

Cusick, J.K., Alhomsy, Y., Wong, S., Talbott, G., Uversky, V.N., Hart, C., Hejazi, N., Jacobs, A.T. and Shi, Y., 2020. RELT stains prominently in B-cell lymphomas and binds the hematopoietic transcription factor MDFIC. *Biochemistry and Biophysics Reports*, *24*, p.100868.

Da Cruz, S., Xenarios, I., Langridge, J., Vilbois, F., Parone, P.A. and Martinou, J.C., 2003. Proteomic analysis of the mouse liver mitochondrial inner membrane. *Journal of Biological Chemistry*, *278*(42), pp.41566-41571.

Dandage, R. and Landry, C.R., 2019. Paralog dependency indirectly affects the robustness of human cells. *Molecular systems biology*, *15*(9), p.e8871.

Davidson, A.J. and Zon, L.I., 2006. The caudal-related homeobox genes cdx1a and cdx4 act redundantly to regulate hox gene expression and the formation of putative hematopoietic stem cells during zebrafish embryogenesis. *Developmental biology*, *292*(2), pp.506-518.

de Bruin, A.M., Voermans, C. and Nolte, M.A., 2014. Impact of interferon-γ on hematopoiesis. *Blood, The Journal of the American Society of Hematology*, *124*(16), pp.2479-2486.

de Pater, E., Kaimakis, P., Vink, C.S., Yokomizo, T., Yamada-Inagawa, T., van der Linden, R., Kartalaei, P.S., Camper, S.A., Speck, N. and Dzierzak, E., 2013. Gata2 is required for HSC generation and survival. *Journal of Experimental Medicine*, *210*(13), pp.2843-2850.

de Roo, J. and Staal, F., 2020. Cell signaling pathway reporters in adult hematopoietic stem cells. *Cells*, *9*(10), p.2264.

de Rooij, L.P., Chan, D.C., Keyvani Chahi, A. and Hope, K.J., 2019. Post-transcriptional regulation in hematopoiesis: RNA binding proteins take control. *Biochemistry and Cell Biology*, *97*(1), pp.10-20.

Diebold, L.P. and Chandel, N.S., 2016. HSC fate is tethered to mitochondria. *Cell stem cell*, *18*(3), pp.303-304.

Dore, L.C., Amigo, J.D., Dos Santos, C.O., Zhang, Z., Gai, X., Tobias, J.W., Yu, D., Klein, A.M., Dorman, C., Wu, W. and Hardison, R.C., 2008. A GATA-1-regulated microRNA locus essential for erythropoiesis. *Proceedings of the National Academy of Sciences*, *105*(9), pp.3333-3338.

Dorritie, K.A., McCubrey, J.A. and Johnson, D.E., 2014. STAT transcription factors in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention. *Leukemia*, *28*(2), pp.248-257.

Dou, D.R., Calvanese, V., Sierra, M.I., Nguyen, A.T., Minasian, A., Saarikoski, P., Sasidharan, R., Ramirez, C.M., Zack, J.A., Crooks, G.M. and Galic, Z., 2016. Medial HOXA genes demarcate haematopoietic stem cell fate during human development. *Nature cell biology*, *18*(6), pp.595-606.

Duarte, S., Woll, P.S., Buza-Vidas, N., Chin, D.W.L., Boukarabila, H., Luís, T.C., Stenson, L., Bouriez-Jones, T., Ferry, H., Mead, A.J. and Atkinson, D., 2018. Canonical Notch signaling is dispensable for adult steady-state and stress myelo-erythropoiesis. *Blood, The Journal of the American Society of Hematology*, *131*(15), pp.1712-1719.

Dutta, P., Hoyer, F.F., Grigoryeva, L.S., Sager, H.B., Leuschner, F., Courties, G., Borodovsky, A., Novobrantseva, T., Ruda, V.M., Fitzgerald, K. and Iwamoto, Y., 2015. Macrophages retain hematopoietic stem cells in the spleen via VCAM-1. *Journal of Experimental Medicine*, *212*(4), pp.497-512.

El-Brolosy, M.A. and Stainier, D.Y., 2017. Genetic compensation: A phenomenon in search of mechanisms. *PLoS genetics*, *13*(7), p.e1006780.

Elliott, S. and Sinclair, A.M., 2012. The effect of erythropoietin on normal and neoplastic cells. *Biologics: targets & therapy*, *6*, p.163.

Feng, Y., Yang, Y., Ortega, M.M., Copeland, J.N., Zhang, M., Jacob, J.B., Fields, T.A., Vivian, J.L. and Fields, P.E., 2010. Early mammalian erythropoiesis requires the Dot1L methyltransferase. *Blood, The Journal of the American Society of Hematology*, *116*(22), pp.4483-4491.

Flores-Figueroa, E., Varma, S., Montgomery, K., Greenberg, P.L. and Gratzinger, D., 2012. Distinctive contact between CD34+ hematopoietic progenitors and CXCL12+ CD271+ mesenchymal stromal cells in benign and myelodysplastic bone marrow. *Laboratory investigation*, *92*(9), pp.1330-1341.

Frame, J.M., McGrath, K.E. and Palis, J., 2013. Erythro-myeloid progenitors: ""definitive"" hematopoiesis in the conceptus prior to the emergence of hematopoietic stem cells. *Blood Cells, Molecules, and Diseases*, *51*(4), pp.220-225.

Fu, J., Zuber, J., Martinez, M., Shonts, B., Obradovic, A., Wang, H., Lau, S.P., Xia, A., Waffarn, E.E., Frangaj, K. and Savage, T.M., 2019. Human intestinal allografts contain functional hematopoietic stem and progenitor cells that are maintained by a circulating pool. *Cell Stem Cell*, *24*(2), pp.227-239.

García-García, A., Korn, C., García-Fernández, M., Domingues, O., Villadiego, J., Martín-Pérez, D., Isern, J., Bejarano-García, J.A., Zimmer, J., Pérez-Simón, J.A. and Toledo-Aral, J.J., 2019. Dual cholinergic signals regulate daily migration of hematopoietic stem cells and leukocytes. *Blood, The Journal of the American Society of Hematology*, *133*(3), pp.224-236.

García-Prat, L., Kaufmann, K.B., Schneiter, F., Voisin, V., Murison, A., Chen, J., Chan-Seng-Yue, M., Gan, O.I., McLeod, J.L., Smith, S.A. and Shoong, M.C., 2021. TFEB-mediated endolysosomal activity controls human hematopoietic stem cell fate. *Cell Stem Cell*, *28*(10), pp.1838-1850.

Ghaffari, S., 2021. Lysosomal regulation of metabolism in quiescent hematopoietic stem cells: more than just autophagy. *Cell Stem Cell*, *28*(3), pp.374-377.

Ghisi, M., Corradin, A., Basso, K., Frasson, C., Serafin, V., Mukherjee, S., Mussolin, L., Ruggero, K., Bonanno, L., Guffanti, A. and De Bellis, G., 2011. Modulation of microRNA expression in human T-cell development: targeting of NOTCH3 by miR-150. *Blood, The Journal of the American Society of Hematology*, *117*(26), pp.7053-7062.

Ghosh, J. and Kapur, R., 2016. Regulation of hematopoietic stem cell self-renewal and leukemia maintenance by the PI3K-mTORC1 pathway. *Current stem cell reports*, *2*(4), pp.368-378.

Gilles, L., Arslan, A.D., Marinaccio, C., Wen, Q.J., Arya, P., McNulty, M., Yang, Q., Zhao, J.C., Konstantinoff, K., Lasho, T. and Pardanani, A., 2017. Downregulation of GATA1 drives impaired hematopoiesis in primary myelofibrosis. *The Journal of clinical investigation*, *127*(4), pp.1316-1320.

Goldberg, B. and Stern, A., 1977. The mechanism of oxidative hemolysis produced by phenylhydrazine. *Molecular pharmacology*, *13*(5), pp.832-839.

González-García, S., García-Peydró, M., Alcain, J. and Toribio, M.L., 2012. Notch1 and IL-7 receptor signalling in early T-cell development and leukaemia. *Notch Regulation of the Immune System*, pp.47-73.

Gothwal, M., Wehrle, J., Aumann, K., Zimmermann, V., Gründer, A. and Pahl, H.L., 2016. A novel role for nuclear factor-erythroid 2 in erythroid maturation by modulation of mitochondrial autophagy. *Haematologica*, *101*(9), p.1054.

Gotoh, K., Kunisaki, Y., Mizuguchi, S., Setoyama, D., Hosokawa, K., Yao, H., Nakashima, Y., Yagi, M., Uchiumi, T., Semba, Y. and Nogami, J., 2020. Mitochondrial protein synthesis is essential for terminal differentiation of CD45–TER119–erythroid and lymphoid progenitors. *Iscience*, *23*(11), p.101654.

Grebien, F., Kerenyi, M.A., Kovacic, B., Kolbe, T., Becker, V., Dolznig, H., Pfeffer, K., Klingmüller, U., Müller, M., Beug, H. and Müllner, E.W., 2008. Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2. *Blood, The Journal of the American Society of Hematology*, *111*(9), pp.4511-4522.

Gritz, E. and Hirschi, K.K., 2016. Specification and function of hemogenic endothelium during embryogenesis. *Cellular and Molecular Life Sciences*, *73*(8), pp.1547-1567.

Haas, S., Trumpp, A. and Milsom, M.D., 2018. Causes and consequences of hematopoietic stem cell heterogeneity. *Cell stem cell*, *22*(5), pp.627-638

Habart, D., Cheli, Y., Nugent, D.J., Ruggeri, Z.M. and Kunicki, T.J., 2013. Conditional knockout of integrin α2β1 in murine megakaryocytes leads to reduced mean platelet volume. *PLoS One*, *8*(1), p.e55094.

Habib, S.L., 2018. Kidney atrophy vs hypertrophy in diabetes: which cells are involved?. Cell Cycle, 17(14), pp.1683-1687.

Hall, M.A., Curtis, D.J., Metcalf, D., Elefanty, A.G., Sourris, K., Robb, L., Göthert, J.R., Jane, S.M. and Begley, C.G., 2003. The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. *Proceedings of the National Academy of Sciences*, *100*(3), pp.992-997.

Halupa, A., Bailey, M.L., Huang, K., Iscove, N.N., Levy, D.E. and Barber, D.L., 2005. A novel role for STAT1 in regulating murine erythropoiesis: deletion of STAT1 results in overall reduction of erythroid progenitors and alters their distribution. *Blood*, *105*(2), pp.552-561.

Han, J., Jung, S., Jang, J., Kam, T.I., Choi, H., Kim, B.J., Nah, J., Jo, D.G., Nakagawa, T., Nishimura, M. and Jung, Y.K., 2014. OCIAD2 activates γ-secretase to enhance amyloid β production by interacting with nicastrin. *Cellular and molecular life sciences*, *71*(13), pp.2561-2576.

Han, L., Madan, V., Mayakonda, A., Dakle, P., Woon, T.W., Shyamsunder, P., Nordin, H.B.M., Cao, Z., Sundaresan, J., Lei, I. and Wang, Z., 2019. Chromatin remodeling mediated by ARID1A is indispensable for normal hematopoiesis in mice. *Leukemia*, *33*(9), pp.2291-2305.

Harandi, O.F., Hedge, S., Wu, D.C., Mckeone, D. and Paulson, R.F., 2010. Murine erythroid short-term radioprotection requires a BMP4-dependent, self-renewing population of stress erythroid progenitors. *The Journal of clinical investigation*, *120*(12), pp.4507-4519.

Heng, J., Lv, P., Zhang, Y., Cheng, X., Wang, L., Ma, D. and Liu, F., 2020. Rab5c-mediated endocytic trafficking regulates hematopoietic stem and progenitor cell development via Notch and AKT signaling. *PLoS biology*, *18*(4), p.e3000696.

Hick, R.W., Gruver, A.L., Ventevogel, M.S., Haynes, B.F. and Sempowski, G.D., 2006. Leptin selectively augments thymopoiesis in leptin deficiency and lipopolysaccharide-induced thymic atrophy. *The Journal of Immunology*, *177*(1), pp.169-176.

Hicks, S.C., Townes, F.W., Teng, M. and Irizarry, R.A., 2018. Missing data and technical variability in single-cell RNA-sequencing experiments. *Biostatistics*, *19*(4), pp.562-578.

Hidalgo, D., Bejder, J., Pop, R., Gellatly, K., Hwang, Y., Maxwell Scalf, S., Eastman, A.E., Chen, J.J., Zhu, L.J., Heuberger, J.A. and Guo, S., 2021. EpoR stimulates rapid cycling and larger red cells during mouse and human erythropoiesis. *Nature Communications*, *12*(1), pp.1-17.

Hoeffel, G. and Ginhoux, F., 2018. Fetal monocytes and the origins of tissue-resident macrophages. *Cellular immunology*, *330*, pp.5-15.

Hong, J., Shiba-Ishii, A., Kim, Y., Noguchi, M. and Sakamoto, N., 2021. Ovarian carcinoma immunoreactive antigen domain 2 controls mitochondrial apoptosis in lung adenocarcinoma. *Cancer Science*, *112*(12), p.5114.

Hsu, J., Huang, H.T., Lee, C.T., Choudhuri, A., Wilson, N.K., Abraham, B.J., Moignard, V., Kucinski, I., Yu, S., Hyde, R.K. and Tober, J., 2020. CHD7 and Runx1 interaction provides a braking mechanism for hematopoietic differentiation. *Proceedings of the National Academy of Sciences*, *117*(38), pp.23626-23635.

Hsu, M.F., Bettaieb, A., Ito, Y., Graham, J., Havel, P.J. and Haj, F.G., 2017. Protein tyrosine phosphatase Shp2 deficiency in podocytes attenuates lipopolysaccharide-induced proteinuria. *Scientific reports*, *7*(1), pp.1-13.

Hu, M. and Wang, J., 2019. Mitochondrial metabolism and the maintenance of hematopoietic stem cell quiescence. *Current opinion in hematology*, *26*(4), pp.228-234.

Huang, S., 2007. Regulation of metastases by signal transducer and activator of transcription 3 signaling pathway: clinical implications. *Clinical Cancer Research*, *13*(5), pp.1362-1366.

Inamdar, M.S., 2003. Drosophila asrij is expressed in pole cells, trachea and hemocytes. *Development genes and evolution*, *213*(3), pp.134-137.

Irvine, D.A. and Copland, M., 2012. Targeting hedgehog in hematologic malignancy. *Blood, The Journal of the American Society of Hematology*, *119*(10), pp.2196-2204.

Itkin, T., Gur-Cohen, S., Spencer, J.A., Schajnovitz, A., Ramasamy, S.K., Kusumbe, A.P., Ledergor, G., Jung, Y., Milo, I., Poulos, M.G. and Kalinkovich, A., 2016. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature*, *532*(7599), pp.323-328.

Ito, K., Turcotte, R., Cui, J., Zimmerman, S.E., Pinho, S., Mizoguchi, T., Arai, F., Runnels, J.M., Alt, C., Teruya-Feldstein, J. and Mar, J.C., 2016. Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science*, *354*(6316), pp.1156-1160.

Ivanovs, A., Rybtsov, S., Welch, L., Anderson, R.A., Turner, M.L. and Medvinsky, A., 2011. Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. *Journal of Experimental Medicine*, *208*(12), pp.2417-2427.

Jacobsen, A., Silber, J., Harinath, G., Huse, J.T., Schultz, N. and Sander, C., 2013. Analysis of microRNA-target interactions across diverse cancer types. *Nature structural & molecular biology*, *20*(11), pp.1325-1332.

Jafari, M., Ghadami, E., Dadkhah, T. and Akhavan-Niaki, H., 2019. PI3k/AKT signaling pathway: erythropoiesis and beyond. *Journal of cellular physiology*, *234*(3), pp.2373-2385.

Jin, G., Xu, C., Zhang, X., Long, J., Rezaeian, A.H., Liu, C., Furth, M.E., Kridel, S., Pasche, B., Bian, X.W. and Lin, H.K., 2018. Atad3a suppresses Pink1-dependent mitophagy to maintain homeostasis of hematopoietic progenitor cells. *Nature immunology*, *19*(1), pp.29-40.

Johns, J.L. and Christopher, M.M., 2012. Extramedullary hematopoiesis: a new look at the underlying stem cell niche, theories of development, and occurrence in animals. *Veterinary pathology*, *49*(3), pp.508-523.

Johnson, D.E., O'Keefe, R.A. and Grandis, J.R., 2018. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nature reviews Clinical oncology*, *15*(4), pp.234-248.

Julian, L.M. and Stanford, W.L., 2020. Organelle cooperation in stem cell fate: Lysosomes as emerging regulators of cell identity. *Frontiers in cell and developmental biology*, *8*, p.591.

Kalaszczynska, I., Geng, Y., Iino, T., Mizuno, S.I., Choi, Y., Kondratiuk, I., Silver, D.P., Wolgemuth, D.J., Akashi, K. and Sicinski, P., 2009. Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells. *Cell*, *138*(2), pp.352-365.

Kang, Y.A., Pietras, E.M. and Passegué, E., 2020. Deregulated Notch and Wnt signaling activates early-stage myeloid regeneration pathways in leukemia. *Journal of Experimental Medicine*, *217*(3), [jem.20190787].

Kanzler, B. and Dear, T.N., 2001. Hox11 acts cell autonomously in spleen development and its absence results in altered cell fate of mesenchymal spleen precursors. Developmental biology, 234(1), pp.231-243.

Karamitros, D., Stoilova, B., Aboukhalil, Z., Hamey, F., Reinisch, A., Samitsch, M., Quek, L., Otto, G., Repapi, E., Doondeea, J. and Usukhbayar, B., 2018. Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells. *Nature immunology*, *19*(1), pp.85-97.

Katajisto, P., Döhla, J., Chaffer, C.L., Pentinmikko, N., Marjanovic, N., Iqbal, S., Zoncu, R., Chen, W., Weinberg, R.A. and Sabatini, D.M., 2015. Asymmetric apportioning of aged mitochondria between daughter cells is required for stemness. *Science*, *348*(6232), pp.340-343.

Khadilkar, R.J., Ray, A., Chetan, D.R., Sinha, A.R., Magadi, S.S., Kulkarni, V. and Inamdar, M.S., 2017. Differential modulation of the cellular and humoral immune responses in Drosophila is mediated by the endosomal ARF1-Asrij axis. *Scientific reports*, *7*(1), pp.1-14.

Khadilkar, R.J., Rodrigues, D., Mote, R.D., Sinha, A.R., Kulkarni, V., Magadi, S.S. and Inamdar, M.S., 2014. ARF1–GTP regulates Asrij to provide endocytic control of Drosophila blood cell homeostasis. *Proceedings of the National Academy of Sciences*, *111*(13), pp.4898-4903.

Khoshdel Rad, N., Aghdami, N. and Moghadasali, R., 2020. Cellular and molecular mechanisms of kidney development: from the embryo to the kidney organoid. *Frontiers in cell and developmental biology*, *8*, p.183.

Kidoya, H., Muramatsu, F., Shimamura, T., Jia, W., Satoh, T., Hayashi, Y., Naito, H., Kunisaki, Y., Arai, F., Seki, M. and Suzuki, Y., 2019. Regnase-1-mediated post-transcriptional regulation is essential for hematopoietic stem and progenitor cell homeostasis. *Nature communications*, *10*(1), pp.1-16.

Kim, H., Kim, M., Im, S.K. and Fang, S., 2018. Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. *Laboratory animal research*, *34*(4), pp.147-159.

Klei, T.R.L., Dalimot, J., Nota, B., Veldthuis, M., Mul, F.P.J., Rademakers, T., Hoogenboezem, M., Nagelkerke, S.Q., van IJcken, W.F.J., Oole, E. and Svendsen, P., 2020. Hemolysis in the spleen drives erythrocyte turnover. *Blood*, *136*(14), pp.1579-1589.

Kobayashi, S., Isotani, A., Mise, N., Yamamoto, M., Fujihara, Y., Kaseda, K., Nakanishi, T., Ikawa, M., Hamada, H., Abe, K. and Okabe, M., 2006. Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. *Current Biology*, *16*(2), pp.166-172.

Koch, U. and Radtke, F., 2011. Mechanisms of T cell development and transformation. *Annual review of cell and developmental biology*, *27*, pp.539-562.

Kondo, M., Weissman, I.L. and Akashi, K., 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*, *91*(5), pp.661-672.

Koo, S., Huntly, B.J., Wang, Y., Chen, J., Brumme, K., Ball, B., McKinney-Freeman, S.L., Yabuuchi, A., Scholl, C., Bansal, D. and Zon, L.I., 2010. Cdx4 is dispensable for murine adult hematopoietic stem cells but promotes MLL-AF9-mediated leukemogenesis. *Haematologica*, *95*(10), p.1642.

Kopp, H.G., Avecilla, S.T., Hooper, A.T. and Rafii, S., 2005. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology*, *20*(5), pp.349-356.

Koury, M.J. and Ponka, P., 2004. New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. *Annu. Rev. Nutr.*, *24*, pp.105-131.

Kulkarni V, Khadilkar RJ, Magadi SS, Inamdar MS. Asrij maintains the stem cell niche and controls differentiation during Drosophila lymph gland hematopoiesis. *pLoS One*. 2011;6(11):e27667.

[a]Kunicki, T.J., Williams, S.A., Diaz, D., Farndale, R.W. and Nugent, D.J., 2012. Platelet adhesion to decorin but not collagen I correlates with the integrin α2 dimorphism E534K, the basis of the human platelet alloantigen (HPA)-5 system. *haematologica*, *97*(5), p.692.

[b]Kunicki, T.J., Williams, S.A., Nugent, D.J. and Yeager, M., 2012. Mean platelet volume and integrin alleles correlate with levels of integrins αIIbβ3 and α2β1 in acute coronary syndrome patients and normal subjects. *Arteriosclerosis, thrombosis, and vascular biology*, *32*(1), pp.147-152.

Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mäe, M.A., Langen, U.H., Betsholtz, C., Lapidot, T. and Adams, R.H., 2016. Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature*, *532*(7599), pp.380-384.

Kuwano, Y., Rabinovic, A., Srikantan, S., Gorospe, M. and Demple, B., 2009. Analysis of nitric oxide-stabilized mRNAs in human fibroblasts reveals HuR-dependent heme oxygenase 1 upregulation. *Molecular and cellular biology*, *29*(10), pp.2622-2635.

Kwarteng, E.O., Hétu-Arbour, R. and Heinonen, K.M., 2018. Frontline Science: Wnt/β-catenin pathway promotes early engraftment of fetal hematopoietic stem/progenitor cells. *Journal of Leukocyte Biology*, *103*(3), pp.381-393.

Lacombe, C., Da Silva, J.L., Bruneval, P., Casadevall, N., Camilleri, J.P., Bariety, J., Tambourin, P. and Varet, B., 1991. Erythropoietin: sites of synthesis and regulation of secretion. *American journal of kidney diseases: the official journal of the National Kidney Foundation*, *18*(4 Suppl 1), pp.14-19.

Lawrence, R.E. and Zoncu, R., 2019. The lysosome as a cellular centre for signalling, metabolism and quality control. *Nature cell biology*, *21*(2), pp.133-142.

Le Vasseur, M., Friedman, J., Jost, M., Xu, J., Yamada, J., Kampmann, M., Horlbeck, M.A., Salemi, M.R., Phinney, B.S., Weissman, J.S. and Nunnari, J., 2021. Genome-wide CRISPRi screening identifies OCIAD1 as a prohibitin client and regulatory determinant of mitochondrial Complex III assembly in human cells. *Elife*, *10*, p.e67624.

Lee, Y.T., Lin, H.Y., Chan, Y.W.F., Li, K.H.C., To, O.T.L., Yan, B.P., Liu, T., Li, G., Wong, W.T., Keung, W. and Tse, G., 2017. Mouse models of atherosclerosis: a historical perspective and recent advances. *Lipids in health and disease*, *16*(1), pp.1-11.

Lemos, D.R., Marsh, G., Huang, A., Campanholle, G., Aburatani, T., Dang, L., Gomez, I., Fisher, K., Ligresti, G., Peti-Peterdi, J. and Duffield, J.S., 2016. Maintenance of vascular integrity by pericytes is essential for normal kidney function. *American Journal of Physiology-Renal Physiology*, *311*(6), pp.F1230-F1242.

Li, J.H., Luo, J.F., Jiang, Y., Ma, Y.J., Ji, Y.Q., Zhu, G.L., Zhou, C., Chu, H.W. and Zhang, H.D., 2019. Red blood cell lifespan shortening in patients with early-stage chronic kidney disease. *Kidney and Blood Pressure Research*, *44*(5), pp.1158-1165.

Li, Z., Lan, Y., He, W., Chen, D., Wang, J., Zhou, F., Wang, Y., Sun, H., Chen, X., Xu, C. and Li, S., 2012. Mouse embryonic head as a site for hematopoietic stem cell development. *Cell stem cell*, *11*(5), pp.663-675.

Liang, R. and Ghaffari, S., 2016. Advances in understanding the mechanisms of erythropoiesis in homeostasis and disease. *British journal of haematology*, *174*(5), pp.661-673.

Liang, R., Arif, T., Kalmykova, S., Kasianov, A., Lin, M., Menon, V., Qiu, J., Bernitz, J.M., Moore, K., Lin, F. and Benson, D.L., 2020. Restraining lysosomal activity preserves hematopoietic stem cell quiescence and potency. *Cell Stem Cell*, *26*(3), pp.359-376.

Lilly, A.J., Johnson, W.E. and Bunce, C.M., 2011. The haematopoietic stem cell niche: new insights into the mechanisms regulating haematopoietic stem cell behaviour. *Stem cells international*, *2011*, 10 pages.

Lin, J., Zhu, Z., Xiao, H., Wakefield, M.R., Ding, V.A., Bai, Q. and Fang, Y., 2017. The role of IL-7 in Immunity and Cancer. *Anticancer research*, *37*(3), pp.963-967.

[a]Liu, L., Wan, X., Zhou, P., Zhou, X., Zhang, W., Hui, X., Yuan, X., Ding, X., Zhu, R., Meng, G. and Xiao, H., 2018. The chromatin remodeling subunit Baf200 promotes normal hematopoiesis and inhibits leukemogenesis. *Journal of hematology & oncology*, *11*(1), pp.1-16.

[b]Liu, T., Kong, W.X., Tang, X.Y., Xu, M., Wang, Q.H., Zhang, B., Hu, L.D. and Chen, H., 2018. The transcription factor Zfp90 regulates the self-renewal and differentiation of hematopoietic stem cells. *Cell death & disease*, *9*(6), pp.1-12.

Loeffler, D., Wehling, A., Schneiter, F., Zhang, Y., Müller-Bötticher, N., Hoppe, P.S., Hilsenbeck, O., Kokkaliaris, K.D., Endele, M. and Schroeder, T., 2019. Asymmetric lysosome inheritance predicts activation of haematopoietic stem cells. *Nature*, *573*(7774), pp.426-429.

Loh, C.Y., Arya, A., Naema, A.F., Wong, W.F., Sethi, G. and Looi, C.Y., 2019. Signal transducer and activator of transcription (STATs) proteins in cancer and inflammation: functions and therapeutic implication. *Frontiers in oncology*, *9*, p.48.

Lu, R., Neff, N.F., Quake, S.R. and Weissman, I.L., 2011. Tracking single hematopoietic stem cells in vivo using high-throughput sequencing in conjunction with viral genetic barcoding. *Nature biotechnology*, *29*(10), pp.928-933.

Luchsinger, L.L., de Almeida, M.J., Corrigan, D.J., Mumau, M. and Snoeck, H.W., 2016. Mitofusin 2 maintains haematopoietic stem cells with extensive lymphoid potential. *Nature*, *529*(7587), pp.528-531.

Lv, K., Gong, C., Antony, C., Han, X., Ren, J.G., Donaghy, R., Cheng, Y., Pellegrino, S., Warren, A.J., Paralkar, V.R. and Tong, W., 2021. HectD1 controls hematopoietic stem cell regeneration by coordinating ribosome assembly and protein synthesis. *Cell Stem Cell*, *28*(7), pp.1275-1290.

Lv, Y., Lau, W.Y., Li, Y., Deng, J., Han, X., Gong, X., Liu, N. and Wu, H., 2016. Hypersplenism: history and current status. *Experimental and therapeutic medicine*, *12*(4), pp.2377-2382.

Machlus, K.R. and Italiano, J.E., 2013. The incredible journey: From megakaryocyte development to platelet formation. *Journal of Cell Biology*, *201*(6), pp.785-796.

Maecker, H.T., McCoy, J.P. and Nussenblatt, R., 2012. Standardizing immunophenotyping for the human immunology project. *Nature Reviews Immunology*, *12*(3), pp.191-200.

Malumbres, M., Sotillo, R., Santamaría, D., Galán, J., Cerezo, A., Ortega, S., Dubus, P. and Barbacid, M., 2004. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. *Cell*, *118*(4), pp.493-504.

Manabe, E., Sasaki, N., Ohno, Y., Tanaka, T., Ito, S., Masuyama, T. and Tsujino, T., 2016. Reduced Half-Life of Red Blood Cells Causes Anemia in the Model of Hypertensive Chronic Heart Failure. *Journal of Cardiac Failure*, *22*(9), p.S206.

Mantel, Charlie, Steven Messina-Graham, Akira Moh, Scott Cooper, Giao Hangoc, Xin-Yuan Fu, and Hal E. Broxmeyer. "Mouse hematopoietic cell–targeted STAT3 deletion: stem/progenitor cell defects, mitochondrial dysfunction, ROS overproduction, and a rapid aging–like phenotype." *Blood, The Journal of the American Society of Hematology* 120, no. 13 (2012): 2589-2599.

Manz, M.G., Miyamoto, T., Akashi, K. and Weissman, I.L., 2002. Prospective isolation of human clonogenic common myeloid progenitors. *Proceedings of the National Academy of Sciences*, *99*(18), pp.11872-11877.

Matsumoto, A., Takeishi, S., Kanie, T., Susaki, E., Onoyama, I., Tateishi, Y., Nakayama, K. and Nakayama, K.I., 2011. p57 is required for quiescence and maintenance of adult hematopoietic stem cells. *Cell stem cell*, *9*(3), pp.262-271.

Mauracher, A.A., Eekels, J.J., Woytschak, J., van Drogen, A., Bosch, A., Prader, S., Felber, M., Heeg, M., Opitz, L., Trück, J. and Schroeder, S., 2020. Erythropoiesis defect observed in STAT3 GOF patients with severe anemia. *Journal of Allergy and Clinical Immunology*, *145*(4), pp.1297-1301.

McGraw, K.L., Fuhler, G.M., Johnson, J.O., Clark, J.A., Caceres, G.C., Sokol, L. and List, A.F., 2012. Erythropoietin receptor signaling is membrane raft dependent. *PloS one*, *7*(4), p.e34477.

Mende, N. and Laurenti, E., 2021. Hematopoietic stem and progenitor cells outside the bone marrow: where, when, and why. *Experimental Hematology*, *104*, pp.9-16.

Mendelson, A. and Frenette, P.S., 2014. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nature medicine*, *20*(8), pp.833-846.

Millot, S., Andrieu, V., Letteron, P., Lyoumi, S., Hurtado-Nedelec, M., Karim, Z., Thibaudeau, O., Bennada, S., Charrier, J.L., Lasocki, S. and Beaumont, C., 2010. Erythropoietin stimulates

spleen BMP4-dependent stress erythropoiesis and partially corrects anemia in a mouse model of generalized inflammation. *Blood, The Journal of the American Society of Hematology*, *116*(26), pp.6072-6081.

Mittag, A. and Tárnok, A., 2009. Basics of standardization and calibration in cytometry–a review. *Journal of biophotonics*, *2*(8-9), pp.470-481.

Mohrin, M., Widjaja, A., Liu, Y., Luo, H. and Chen, D., 2018. The mitochondrial unfolded protein response is activated upon hematopoietic stem cell exit from quiescence. *Aging Cell*, *17*(3), p.e12756.

Moreau R, Tshikudi Malu D, Dumais M, et al. Alterations in bone and erythropoiesis in hemolytic anemia: comparative study in bled, phenylhydrazine-treated and Plasmodium-infected mice. *PLoS One.* 2012;7(9):e46101.

Morgan, P.K., Fang, L., Lancaster, G.I. and Murphy, A.J., 2020. Hematopoiesis is regulated by cholesterol efflux pathways and lipid rafts: connections with cardiovascular diseases: Thematic Review Series: Biology of Lipid Rafts. *Journal of lipid research*, *61*(5), pp.667-675.

Morishima, T., Krahl, A.C., Nasri, M., Xu, Y., Aghaallaei, N., Findik, B., Klimiankou, M., Ritter, M., Hartmann, M.D., Gloeckner, C.J. and Stefanczyk, S., 2019. LMO2 activation by deacetylation is indispensable for hematopoiesis and T-ALL leukemogenesis. *Blood, The Journal of the American Society of Hematology*, *134*(14), pp.1159-1175.

Morita, K., Suzuki, K., Maeda, S., Matsuo, A., Mitsuda, Y., Tokushige, C., Kashiwazaki, G., Taniguchi, J., Maeda, R., Noura, M. and Hirata, M., 2017. Genetic regulation of the RUNX transcription factor family has antitumor effects. *The Journal of clinical investigation*, *127*(7), pp.2815-2828.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M. and Weissman, I.L., 1995. The purification and characterization of fetal liver hematopoietic stem cells. *Proceedings of the National Academy of Sciences*, *92*(22), pp.10302-10306.

Mukhopadhyay, A., Das, D. and Inamdar, M.S., 2003. Embryonic stem cell and tissue-specific expression of a novel conserved gene, asrij. *Developmental dynamics: an official publication of the American Association of Anatomists*, *227*(4), pp.578-586.

Murphy, A.J., Akhtari, M., Tolani, S., Pagler, T., Bijl, N., Kuo, C.L., Wang, M., Sanson, M., Abramowicz, S., Welch, C. and Bochem, A.E., 2011. ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. *The Journal of clinical investigation*, *121*(10), pp.4138-4149.

Murphy, A.J., Bijl, N., Yvan-Charvet, L., Welch, C.B., Bhagwat, N., Reheman, A., Wang, Y., Shaw, J.A., Levine, R.L., Ni, H. and Tall, A.R., 2013. Cholesterol efflux in megakaryocyte progenitors suppresses platelet production and thrombocytosis. *Nature medicine*, *19*(5), pp.586-594.

Myneni, V.D., Szalayova, I. and Mezey, E., 2021. Differences in Steady-State Erythropoiesis in Different Mouse Bones and Postnatal Spleen. *Frontiers in Cell and Developmental Biology*, *9*, p.1237.

Nagata, C., Kobayashi, H., Sakata, A., Satomi, K., Minami, Y., Morishita, Y., Ohara, R., Yoshikawa, H., Arai, Y., Nishida, M. and Noguchi, M., 2012. Increased expression of OCIA domain containing 2 during stepwise progression of ovarian mucinous tumor. *Pathology international*, *62*(7), pp.471-476.

Nakano, D., Kitada, K., Wan, N., Zhang, Y., Wiig, H., Wararat, K., Yanagita, M., Lee, S., Jia, L., Titze, J.M. and Nishiyama, A., 2020. Lipopolysaccharide induces filtrate leakage from renal tubular lumina into the interstitial space via a proximal tubular Toll-like receptor 4–dependent pathway and limits sensitivity to fluid therapy in mice. *Kidney international*, *97*(5), pp.904-912.

Nestorowa S, Hamey FK, Pijuan Sala B, et al. A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. *Blood*. 2016;128(8):e20-31.

Nie, Y., Han, Y.C. and Zou, Y.R., 2008. CXCR4 is required for the quiescence of primitive hematopoietic cells. *Journal of Experimental Medicine*, *205*(4), pp.777-783.

Oda A, Tezuka T, Ueno Y, et al. Niche-induced extramedullary hematopoiesis in the spleen is regulated by the transcription factor Tlx1. *Sci Rep.* 2018;8(1):8308.

Oguro, H., Ding, L. and Morrison, S.J., 2013. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell stem cell*, *13*(1), pp.102-116.

Orkin, S.H. and Zon, L.I., 2008. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*, *132*(4), pp.631-644.

Otsuka, H., Yagi, H., Endo, Y., Nonaka, N. and Nakamura, M., 2011. Kupffer cells support extramedullary erythropoiesis induced by nitrogen-containing bisphosphonate in splenectomized mice. *Cellular immunology*, *271*(1), pp.197-204.

Ottersbach, K. and Dzierzak, E., 2005. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Developmental cell*, *8*(3), pp.377-387.

Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G., 1999. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development*, *126*(22), pp.5073-5084.

Parisi, T., Beck, A.R., Rougier, N., McNeil, T., Lucian, L., Werb, Z. and Amati, B., 2003. Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells. *The EMBO journal*, *22*(18), pp.4794-4803.

Paulson, R.F., Ruan, B., Hao, S. and Chen, Y., 2020. Stress erythropoiesis is a key inflammatory response. *Cells*, *9*(3), p.634.

Peron, M., Dinarello, A., Meneghetti, G., Martorano, L., Betto, R.M., Facchinello, N., Tesoriere, A., Tiso, N., Martello, G. and Argenton, F., 2021. Y705 and S727 are required for

the mitochondrial import and transcriptional activities of STAT3, and for regulation of stem cell proliferation. *Development*, *148*(17), p.dev199477.

Perry, J.M., Harandi, O.F., Porayette, P., Hegde, S., Kannan, A.K. and Paulson, R.F., 2009. Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. *Blood, The Journal of the American Society of Hematology*, *113*(4), pp.911-918.

Pierce, H., Zhang, D., Magnon, C., Lucas, D., Christin, J.R., Huggins, M., Schwartz, G.J. and Frenette, P.S., 2017. Cholinergic signals from the CNS regulate G-CSF-mediated HSC mobilization from bone marrow via a glucocorticoid signaling relay. *Cell stem cell*, *20*(5), pp.648-658.

Pietras, E.M., Mirantes-Barbeito, C., Fong, S., Loeffler, D., Kovtonyuk, L.V., Zhang, S., Lakshminarasimhan, R., Chin, C.P., Techner, J.M., Will, B. and Nerlov, C., 2016. Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. *Nature cell biology*, *18*(6), pp.607-618.

Porcher, C., Chagraoui, H. and Kristiansen, M.S., 2017. SCL/TAL1: a multifaceted regulator from blood development to disease. *Blood, The Journal of the American Society of Hematology*, *129*(15), pp.2051-2060.

Prakash, M., Shetty, J.K., Dash, S., Barik, B.K., Sarkar, A. and Prabhu, R., 2008. Determination of urinary peptides in patients with proteinuria. *Indian journal of nephrology*, *18*(4), p.150.

Prasad, P., Lennartsson, A. and Ekwall, K., 2015. The roles of SNF2/SWI2 nucleosome remodeling enzymes in blood cell differentiation and leukemia. *BioMed Research International*, *2015*, 17 pages.

Praveen, W., Sinha, S., Batabyal, R., Kamat, K. and Inamdar, M.S., 2020. The OCIAD protein family: comparative developmental biology and stem cell application. *Int. J. Dev. Biol*, *64*, pp.223-235.

Prockop, S.E. and Petrie, H.T., 2004. Regulation of thymus size by competition for stromal niches among early T cell progenitors. *The Journal of Immunology*, *173*(3), pp.1604-1611.

Pujato, M., Kieken, F., Skiles, A.A., Tapinos, N. and Fiser, A., 2014. Prediction of DNA binding motifs from 3D models of transcription factors; identifying TLX3 regulated genes. *Nucleic acids research*, *42*(22), pp.13500-13512.

Ran, Q., Guo, C., Sun, C., Liu, Q., He, H., Zhao, W., Zhang, J. and Xiao, Y., 2021. Loss of fgfr3 accelerates bone marrow suppression-induced hematopoietic stem and progenitor cell expansion by activating fgfr1-elk1-cyclin d1 signaling. *Transplantation and Cellular Therapy*, *27*(1), pp.45-e1.

Ray, A., Kamat, K. and Inamdar, M.S., 2021. A conserved role for Asrij/OCIAD1 in progenitor differentiation and lineage specification through functional interaction with the regulators of mitochondrial dynamics. *Frontiers in Cell and Developmental Biology*, *9*:643444.

Rehn, M., Olsson, A., Reckzeh, K., Diffner, E., Carmeliet, P., Landberg, G. and Cammenga, J., 2011. Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. *Blood, The Journal of the American Society of Hematology*, *118*(6), pp.1534-1543.

Rivella, S., 2012. The role of ineffective erythropoiesis in non-transfusion-dependent thalassemia. *Blood reviews*, *26*, pp.S12-S15.

Robbins, C.S., Chudnovskiy, A., Rauch, P.J., Figueiredo, J.L., Iwamoto, Y., Gorbatov, R., Etzrodt, M., Weber, G.F., Ueno, T., van Rooijen, N. and Mulligan-Kehoe, M.J., 2012. Extramedullary hematopoiesis generates Ly-6Chigh monocytes that infiltrate atherosclerotic lesions. *Circulation*, *125*(2), pp.364-374.

Russo, G., Zegar, C. and Giordano, A., 2003. Advantages and limitations of microarray technology in human cancer. *Oncogene*, *22*(42), pp.6497-6507.

Sakashita, M., Sakashita, S., Murata, Y., Shiba-Ishii, A., Kim, Y., Matsuoka, R., Nakano, N., Sato, Y. and Noguchi, M., 2018. High expression of ovarian cancer immunoreactive antigen domain containing 2 (OCIAD2) is associated with poor prognosis in lung adenocarcinoma. *Pathology international*, *68*(11), pp.596-604.

Sankaran, V.G. and Orkin, S.H., 2013. The switch from fetal to adult hemoglobin. *Cold Spring Harbor perspectives in medicine*, *3*(1), p.a011643.

Schmidt-Arras, D. and Rose-John, S., 2021. Endosomes as Signaling Platforms for IL-6 Family Cytokine Receptors. *Frontiers in Cell and Developmental Biology*, *9*, p.1298.

Schuermann, A., Helker, C.S. and Herzog, W., 2015. Metallothionein 2 regulates endothelial cell migration through transcriptional regulation of vegfc expression. *Angiogenesis*, *18*(4), pp.463-475.

Schulz, V.P., Yan, H., Lezon-Geyda, K., An, X., Hale, J., Hillyer, C.D., Mohandas, N. and Gallagher, P.G., 2019. A unique epigenomic landscape defines human erythropoiesis. *Cell reports*, *28*(11), pp.2996-3009.

Schwenk, F., U. Baron, and K. Rajewsky. 1995. 'A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells', Nucleic Acids Res, 23: 5080-1.

Seemann, S., Zohles, F. and Lupp, A., 2017. Comprehensive comparison of three different animal models for systemic inflammation. *Journal of biomedical science*, *24*(1), pp.1-17.

Seita J, Sahoo D, Rossi DJ, et al. Gene Expression Commons: an open platform for absolute gene expression profiling. *PLoS One*. 2012;7(7):e40321.

Senouthai, S., Wang, J., Fu, D. and You, Y., 2019. Fractalkine is involved in lipopolysaccharide-induced podocyte injury through the Wnt/β-catenin pathway in an acute kidney injury mouse model. *Inflammation*, *42*(4), pp.1287-1300.

Sharma, D., Malik, A., Guy, C., Vogel, P. and Kanneganti, T.D., 2019. TNF/TNFR axis promotes pyrin inflammasome activation and distinctly modulates pyrin inflammasomopathy. *The Journal of clinical investigation*, *129*(1), pp.150-162.

Shen, X., Weng, C., Wang, Y., Wang, C., Feng, S., Li, X., Li, H., Jiang, H., Wang, H. and Chen, J., 2020. Lipopolysaccharide-induced podocyte injury is regulated by calcineurin/NFAT and TLR4/MyD88/NF-κB signaling pathways through angiopoietin-like protein 4. *Genes & Diseases*.

Shetty, D.K., Kalamkar, K.P. and Inamdar, M.S., 2018. OCIAD1 controls electron transport chain complex I activity to regulate energy metabolism in human pluripotent stem cells. *Stem cell reports*, *11*(1), pp.128-141.

Shih, H.M., Wu, C.J. and Lin, S.L., 2018. Physiology and pathophysiology of renal erythropoietin-producing cells. *Journal of the Formosan Medical Association*, *117*(11), pp.955-963.

Signer, R.A., Magee, J.A., Salic, A. and Morrison, S.J., 2014. Haematopoietic stem cells require a highly regulated protein synthesis rate. *Nature*, *509*(7498), pp.49-54.

Singh, Ajay. 2018. *Textbook of nephro-endocrinology* (Elsevier/Academic Press: London, United Kingdom).

Sinha S, Bheemsetty VA, Inamdar MS. A double helical motif in OCIAD2 is essential for its localization, interactions and STAT3 activation. *Sci Rep*. 2018;8(1):7362.

Sinha, A., Khadilkar, R.J., Vinay, K.S., Sinha, A.R. and Inamdar, M.S., 2013. Conserved regulation of the Jak/STAT pathway by the endosomal protein asrij maintains stem cell potency. *Cell reports*, *4*(4), pp.649-658.

[a]Sinha, S., Ray, A., Abhilash, L., Kumar, M., Sreenivasamurthy, S.K., Prasad, T.K. and Inamdar, M.S., 2019. Proteomics of Asrij Perturbation in Drosophila Lymph Glands for Identification of New Regulators of Hematopoiesis*[S]. *Molecular & Cellular Proteomics*, *18*(6), pp.1171-1182.

Sinha, S., Sinha, A., Dongre, P., Kamat, K. and Inamdar, M.S., 2022. Organelle dysfunction upon asrij depletion causes aging-like changes in mouse hematopoietic stem cells. *Aging Cell*, p.e13570.

[b]Sinha, S., T. R. Dwivedi, R. Yengkhom, V. A. Bheemsetty, T. Abe, H. Kiyonari, K. VijayRaghavan, and M. S. Inamdar. 2019. 'Asrij/OCIAD1 suppresses CSN5-mediated p53 degradation and maintains mouse hematopoietic stem cell quiescence', *Blood*, 133: 2385-400.

Socolovsky, M., Nam, H.S., Fleming, M.D., Haase, V.H., Brugnara, C. and Lodish, H.F., 2001. Ineffective erythropoiesis in Stat5a−/− 5b−/− mice due to decreased survival of early erythroblasts. *Blood, The Journal of the American Society of Hematology*, *98*(12), pp.3261-3273.

Sollinger, C., Lillis, J., Malik, J., Getman, M., Proschel, C. and Steiner, L., 2017. Erythropoietin signaling regulates key epigenetic and transcription networks in fetal neural progenitor cells. *Scientific reports*, *7*(1), pp.1-13.

Song, M.K., Park, B.B. and Uhm, J.E., 2018. Understanding splenomegaly in myelofibrosis: association with molecular pathogenesis. *International journal of molecular sciences*, *19*(3), p.898.

Sonoda, Y. and Sasaki, K., 2012. Hepatic extra-medullary hematopoiesis and macrophages in the adult mouse: histometrical and immunohistochemical studies. *Cells Tissues Organs*, *196*(6), pp.555-564.

Sood, R., English, M.A., Belele, C.L., Jin, H., Bishop, K., Haskins, R., McKinney, M.C., Chahal, J., Weinstein, B.M., Wen, Z. and Liu, P.P., 2010. Development of multilineage adult hematopoiesis in the zebrafish with a runx1 truncation mutation. *Blood, The Journal of the American Society of Hematology*, *115*(14), pp.2806-2809.

Sousa, A., Gonçalves, E., Mirauta, B., Ochoa, D., Stegle, O. and Beltrao, P., 2019. Multi-omics characterization of interaction-mediated control of human protein abundance levels. *Molecular & Cellular Proteomics*, *18*(8), pp.S114-S125.

Stanley, E.R. and Chitu, V., 2014. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harbor perspectives in biology*, *6*(6), p.a021857.

Strausberg, Feingold, Grouse, Derge, Klausner, Collins, et al., (2002). Mammalian Gene Collection Program Team. Generation and initial analysis of more than 15 000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci USA 99: 16899–16903.

Su, Y., Huang, X., Huang, Z., Huang, T., Xu, Y. and Yi, C., 2020. STAT3 localizes in mitochondria-associated ER membranes instead of in mitochondria. *Frontiers in cell and developmental biology*, *8*, p.274.

Su, Z., Yu, P., Sheng, L., Ye, J. and Qin, Z., 2018. Fangjifuling ameliorates lipopolysaccharide-induced renal injury via inhibition of inflammatory and apoptotic response in mice. *Cellular Physiology and Biochemistry*, *49*(6), pp.2124-2137.

Susek, K.H., Korpos, E., Huppert, J., Wu, C., Savelyeva, I., Rosenbauer, F., Müller-Tidow, C., Koschmieder, S. and Sorokin, L., 2018. Bone marrow laminins influence hematopoietic stem and progenitor cell cycling and homing to the bone marrow. *Matrix Biology*, *67*, pp.47-62.

Suzuki, N., Matsuo-Tezuka, Y., Sasaki, Y., Sato, K., Miyauchi, K., Kato, K., Saito, S., Shimonaka, Y., Hirata, M. and Yamamoto, M., 2018. Iron attenuates erythropoietin production by decreasing hypoxia-inducible transcription factor 2α concentrations in renal interstitial fibroblasts. *Kidney international*, *94*(5), pp.900-911.

Suzuki, N., Suwabe, N., Ohneda, O., Obara, N., Imagawa, S., Pan, X., Motohashi, H. and Yamamoto, M., 2003. Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels. *Blood*, *102*(10), pp.3575-3583.

Tahara, T., Sun, J., Nakanishi, K., Yamamoto, M., Mori, H., Saito, T., Fujita, H., Igarashi, K. and Taketani, S., 2004. Heme positively regulates the expression of β-globin at the locus

control region via the transcriptional factor Bach1 in erythroid cells. *Journal of Biological Chemistry*, *279*(7), pp.5480-5487.

Taichman, R.S., Reilly, M.J., Verma, R.S., Ehrenman, K. and Emerson, S.G., 2001. Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *British journal of haematology*, *112*(2), pp.438-448.

Termini, C.M. and Chute, J.P., 2020. Hematopoietic Stem Cell Stress and Regeneration. *Current Stem Cell Reports*, *6*(4), pp.134-143.

Tolomeo, M. and Cascio, A., 2021. The Multifaced Role of STAT3 in Cancer and Its Implication for Anticancer Therapy. *International Journal of Molecular Sciences*, *22*(2), p.603.

Tóthová, Z., Šemeláková, M., Solárová, Z., Tomc, J., Debeljak, N. and Solár, P., 2021. The Role of PI3K/AKT and MAPK Signaling Pathways in Erythropoietin Signalization. *International Journal of Molecular Sciences*, *22*(14), p.7682.

Tóthová, Z., Tomc, J., Debeljak, N. and Solár, P., 2021. STAT5 as a key protein of erythropoietin signalization. *International Journal of Molecular Sciences*, *22*(13), p.7109.

Trivedi, G., Inoue, D., Chen, C., Bitner, L., Chung, Y.R., Taylor, J., Gönen, M., Wess, J., Abdel-Wahab, O. and Zhang, L., 2019. Muscarinic acetylcholine receptor regulates self-renewal of early erythroid progenitors. *Science translational medicine*, *11*(511), p.eaaw3781.

Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A. and Warman, M.L., 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques, 29(1), pp.52-54.

Tsamandas, A.C., Jain, A.B., Raikow, R.B., Demetris, A.J., Nalesnik, M.A. and Randhawa, P.S., 1995. Extramedullary hematopoiesis in the allograft liver. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*, *8*(6), pp.671-674.

Uhlen, M., Karlsson, M.J., Zhong, W., Tebani, A., Pou, C., Mikes, J., Lakshmikanth, T., Forsström, B., Edfors, F., Odeberg, J. and Mardinoglu, A., 2019. A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. *Science*, *366*(6472).

Uversky, V.N., 2013. Intrinsic disorder-based protein interactions and their modulators. *Current pharmaceutical design*, *19*(23), pp.4191-4213.

Uversky, V.N., 2016. p53 proteoforms and intrinsic disorder: an illustration of the protein structure–function continuum concept. *International journal of molecular sciences*, *17*(11), p.1874.

Vassiliou, V., Papamichael, D., Lutz, S., Eracleous, E., Kountourakis, P., Polyviou, P., Michaelides, I., Shoukris, M. and Andreopoulos, D., 2012. Presacral extramedullary hematopoiesis in a patient with rectal adenocarcinoma: report of a case and literature review. *Journal of gastrointestinal cancer*, *43*(1), pp.131-135.

Velten, L., Haas, S.F., Raffel, S., Blaszkiewicz, S., Islam, S., Hennig, B.P., Hirche, C., Lutz, C., Buss, E.C., Nowak, D. and Boch, T., 2017. Human haematopoietic stem cell lineage commitment is a continuous process. *Nature cell biology*, *19*(4), pp.271-281.

Viny, A.D., Bowman, R.L., Liu, Y., Lavallée, V.P., Eisman, S.E., Xiao, W., Durham, B.H., Navitski, A., Park, J., Braunstein, S. and Alija, B., 2019. Cohesin members Stag1 and Stag2 display distinct roles in chromatin accessibility and topological control of HSC self-renewal and differentiation. *Cell Stem Cell*, *25*(5), pp.682-696.

Vogel, C. and Marcotte, E.M., 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature reviews genetics*, *13*(4), pp.227-232.

Wang, H., Liu, D., Song, P., Jiang, F., Chi, X. and Zhang, T., 2021. Exposure to hypoxia causes stress erythropoiesis and downregulates immune response genes in spleen of mice. *BMC genomics*, *22*(1), pp.1-14.

Wang, S.H., Hsiao, C.J., Khan, Z. and Pritchard, J.K., 2018. Post-translational buffering leads to convergent protein expression levels between primates. *Genome biology*, *19*(1), pp.1-15.

Wang, W., Yu, S., Zimmerman, G., Wang, Y., Myers, J., Yu, V.W., Huang, D., Huang, X., Shim, J., Huang, Y. and Xin, W., 2015. Notch Receptor-Ligand Engagement Maintains Hematopoietic Stem Cell Quiescence and Niche Retention. *Stem Cells*, *33*(7), pp.2280-2293.

Wegrzyn, J., Potla, R., Chwae, Y.J., Sepuri, N.B., Zhang, Q., Koeck, T., Derecka, M., Szczepanek, K., Szelag, M., Gornicka, A. and Moh, A., 2009. Function of mitochondrial Stat3 in cellular respiration. *Science*, *323*(5915), pp.793-797

Westerterp, M., Gourion-Arsiquaud, S., Murphy, A.J., Shih, A., Cremers, S., Levine, R.L., Tall, A.R. and Yvan-Charvet, L., 2012. Regulation of hematopoietic stem and progenitor cell mobilization by cholesterol efflux pathways. *Cell stem cell*, *11*(2), pp.195-206.

Wielockx, B., Grinenko, T., Mirtschink, P. and Chavakis, T., 2019. Hypoxia pathway proteins in normal and malignant hematopoiesis. *Cells*, *8*(2), p.155.

Winnik, S., Klinkert, M., Kurz, H., Zoeller, C., Heinke, J., Wu, Y., Bode, C., Patterson, C. and Moser, M., 2009. HoxB5 induces endothelial sprouting in vitro and modifies intussusceptive angiogenesis in vivo involving angiopoietin-2. *Cardiovascular research*, *83*(3), pp.558-565.

Wu, D., Yang, X., Peng, H., Guo, D., Zhao, W., Zhao, C. and Zhou, X., 2017. OCIAD2 suppressed tumor growth and invasion via AKT pathway in Hepatocelluar carcinoma. *Carcinogenesis*, *38*(9), pp.910-919.

Wu, H., Liu, X., Jaenisch, R. and Lodish, H.F., 1995. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*, *83*(1), pp.59-67.

Xu, B., Cai, L., Butler, J.M., Chen, D., Lu, X., Allison, D.F., Lu, R., Rafii, S., Parker, J.S., Zheng, D. and Wang, G.G., 2018. The chromatin remodeler BPTF activates a stemness gene-expression program essential for the maintenance of adult hematopoietic stem cells. *Stem cell reports*, *10*(3), pp.675-683.

Xu, L., Liu, X., Peng, F., Zhang, W., Zheng, L., Ding, Y., Gu, T., Lv, K., Wang, J., Ortinau, L. and Hu, T., 2020. Protein quality control through endoplasmic reticulum-associated degradation maintains haematopoietic stem cell identity and niche interactions. *Nature cell biology*, *22*(10), pp.1162-1169.

Yamamoto, K., Miwa, Y., Abe-Suzuki, S., Abe, S., Kirimura, S., Onishi, I., Kitagawa, M. and Kurata, M., 2016. Extramedullary hematopoiesis: Elucidating the function of the hematopoietic stem cell niche. *Molecular medicine reports*, *13*(1), pp.587-591.

Yang, X., Chen, D., Long, H. and Zhu, B., 2020. The mechanisms of pathological extramedullary hematopoiesis in diseases. *Cellular and Molecular Life Sciences*, *77*(14), pp.2723-2738.

Zhang, L., Zhang, J., Liu, Y., Zhang, P., Nie, J., Zhao, R., Shi, Q., Sun, H., Jiao, D., Chen, Y. and Zhao, X., 2021. Mitochondrial STAT5A promotes metabolic remodeling and the Warburg effect by inactivating the pyruvate dehydrogenase complex. *Cell Death & Disease*, *12*(7), pp.1-12.

Zhang, Y., Gao, S., Xia, J. and Liu, F., 2018. Hematopoietic hierarchy–an updated roadmap. *Trends in cell biology*, *28*(12), pp.976-986.

Zhao, M., Tao, F., Venkatraman, A., Li, Z., Smith, S.E., Unruh, J., Chen, S., Ward, C., Qian, P., Perry, J.M. and Marshall, H., 2019. N-cadherin-expressing bone and marrow stromal progenitor cells maintain reserve hematopoietic stem cells. *Cell reports*, *26*(3), pp.652-669.

Appendix 1: Details of the antibodies

I. Antibodies and kits used for fluorescence activated cell sorting (FACS):

S. No.	Antibody/Kit	Catalog Number, Company	Dilution (flow cytometry)	Dilution (immuno-fluorescence)
1.	Annexin V Apoptosis Detection Kit I: Annexin-FITC	556547, eBioscience	 1:100	 -
2.	BD Hematopoietic Stem and Progenitor Cell Isolation Kit: -Lineage cocktail (APC) -c-Kit/CD117 (PE) -Sca1 (PE-Cy7) -CD34 (FITC) -7-AAD	560492, BD Biosciences	 1:100 1:100 1:100 1:100 1:100	 - - - - -
3.	CD48 (APC)	17-0481-82, eBioscience	1:100	-
4.	CD150 (FITC)	11-1502-82, eBioscience	1:100	-
5.	CD11b (FITC)	553308, BD Biosciences	1:40	-
6.	CD19 (FITC)	553785, BD Biosciences	1:40	-
7.	CD3 (FITC)	561798, BD Biosciences	1:100	-

II. Antibodies and kits used for magnetic activated cell sorting (MACS):

S. No.	Antibody/Kit	Catalog Number, Company	Dilution (MACS)
1.	c-Kit/CD117 microbeads	130-091-224, Miltenyi	1:5
2.	Direct Lineage Cell Depletion Kit -Lineage microbeads	130-110-470, Miltenyi	 1:4
3.	Sca1 microbead kit: -Sca1 (FITC) -FITC microbeads	130-092-529, Miltenyi	 1:5 1:5

III. Antibodies and kits used for immunofluorescence and Western Blotting:

S. No.	Antibody	Catalog Number, Company	Dilution (immuno-fluorescence)	Dilution (Western Blotting)
1.	Asrij	ab91574, Abcam	1:500	1:2000
2.	GAPDH	G9545, SIGMA	-	1:4000
3.	OCIAD2	SAB3500119, SIGMA	1:100	1:2000
4.	pSTAT3 (Y705)	9145, CST	-	1:2000
5.	pSTAT5 (Y694)	9359, CST	-	1:2000
6.	STAT3	9139, CST	-	1:2000
7.	STAT5	9363, CST	-	1:2000
8.	Vinculin	V4505, SIGMA	-	1:2000
9.	β-Tubulin	T4026, SIGMA	-	1:500

Appendix 2: Details of the reagents

S. No.	Reagent	Cat. No., Company	Working concentration
1.	Methocult	M334, STEMCELL Technologies	-
2.	MG-132	M7499, SIGMA	10 μM
3.	Protease Inhibitor (PI) cocktail	P8340, SIGMA	1:100
4.	MACS columns	130-042-401, Miltenyi	-
5.	Giemsa stain solution	48900, SIGMA	1:2 in water

List of Publications:

Articles

1- **Wulligundam, P**. and Inamdar, M.S., 2017. To D (e) rive or Reverse: The Challenge and Choice of Pluripotent Stem Cells for Regenerative Medicine. In *Regenerative Medicine: Laboratory to Clinic* (pp. 99-111). Springer, Singapore.

2- Praveen, W., Sinha, S., Batabyal, R., Kamat, K. and Inamdar, M.S., 2020. The OCIAD protein family: comparative developmental biology and stem cell application. *Int. J. Dev. Biol*, *64*, pp.223-235.

Conference Papers

3- Inamdar, M., **Wulligundam, P**., Sinha, S., Sinha, A., Abe, T., Kiyonari, H. and VijayRaghavan, K., 2020. 3087–THE ASRIJ INTERACTOR OCIAD2 IS ESSENTIAL FOR MOUSE HEMATOPOIETIC HOMEOSTASIS. *Experimental Hematology*, *88*, p.S64.

To D(e)rive or Reverse: The Challenge and Choice of Pluripotent Stem Cells for Regenerative Medicine

6

Praveen Wulligundam and Maneesha S. Inamdar

Abstract

The immense potential of pluripotent human stem cells in transforming modern medicine is undeniable. Less than two decades since human embryonic stem cells (hESCs) were first derived, several clinical trials with hESC derivatives are underway. Though human-induced pluripotent stem cell (iPSC) lines are accepted by a wider community for use in research and therapy, issues of maintaining stem cell potency and achieving efficient differentiation are common to hESCs and iPSCs. While iPSCs are considered more accessible and acceptable, it is increasingly clear that iPSCs will be of limited use in autologous therapy. Hence haplobanks are being established for use in regenerative medicine. The additional cost of reprogramming to and characterizing iPSCs compared to deriving hESCs brings into question their suitability for regenerative applications in the Indian scenario, given the limited facilities and resources available. Here we discuss the importance of making an informed choice for the Indian context.

Keywords

Haplobanking • Human ESCs • Human iPSCs • Human embryos • Stem cell therapy

P. Wulligundam
Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Jakkur, Bengaluru, India

M.S. Inamdar, Ph.D. (✉)
Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Jakkur, Bengaluru, India

Institute for Stem Cell Biology and Regenerative Medicine (InStem), Bengaluru, India
e-mail: inamdar@jncasr.ac.in

A. Mukhopadhyay (ed.), *Regenerative Medicine: Laboratory to Clinic*,
DOI 10.1007/978-981-10-3701-6_6

Abbreviations

ART	Assisted reproductive technology
BAC	Bacterial artificial chromosome
CAS9	CRISPR-associated protein 9
cGMP	Current good manufacturing practices
ChIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
EBNA	Epstein-Barr virus nuclear antigen
ESC	Embryonic stem cells
ES-like	Embryonic stem cell-like
HLA	Human leukocyte antigen
iPSC	Induced pluripotent stem cell
iPSCs	Induced pluripotent stem cells
IVF	In vitro fertilization
miRNA or mir	MicroRNA
mRNA	Messenger RNA
OKSM	Oct3/Oct4, Sox2, Klf4, and c-Myc
OriP	Plasmid origin of replication

6.1 Introduction

The challenge of human pluripotent stem cells is the choice between moving forward or in reverse. We have an abundant natural resource in human embryos – a system that inherently knows how to make pluripotent cells. Is it then worth the time and effort, not to mention money, required to reinvent the wheel for regenerative purposes? While there are obvious reasons that support efforts to reprogram somatic cells for research purposes, their utility for regenerative purposes, especially for India, is not clear at this point.

6.2 Human Embryonic Stem Cells and Applications

Human embryonic stem cells (hESCs) were first derived in 1998 [1], and the first clinical trials with hESC-derived progenitors were an unbelievable 10 years later [2]. This was astounding not only because of the immense potential that stem cell-derived therapies offered but also the speed with which the new science of human pluripotent stem cells had progressed to clinical trials. This is reflected in the intense research that is being carried out during the last two decades, to develop hESC-based therapies (Fig. 6.1). In contrast, most drugs that make it to a comparable stage of clinical application require investments possibly larger than that required for hESCs and often much longer periods of time to understand and manipulate the molecule of interest. In this regard, the fact that hESC research

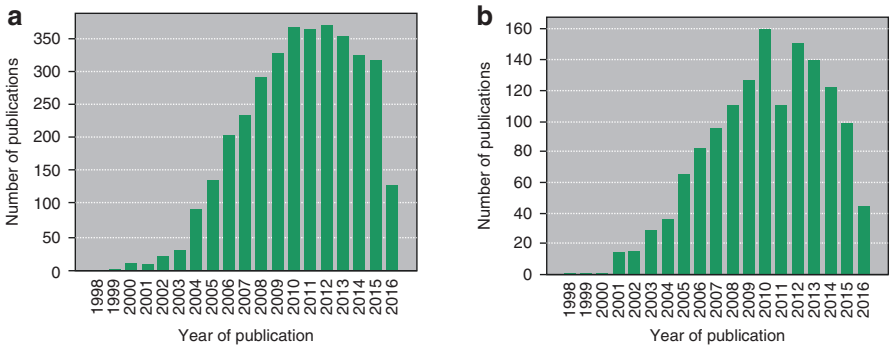


Fig. 6.1 Number of publications reporting (**a**) Application of hESCs in non-therapeutic/basic research (total number = 5864). (**b**) Application of hESCs in therapeutic research (total number = 1409) between 1998 and 2016 (Source: Web of Science, retrieved July 10, 2016, from http://apps.webofknowledge.com/; Key search terms for (**a**) human embryonic stem cells, not therapy and (**b**) human embryonic stem cells and therapy; refined the search for article, correction, retraction, letter, and clinical trial)

was backed by a whole new community of talented scientists applying wisdom of basic research helped.

6.3 Induced Pluripotent Stem Cells and Applications

The discovery of induced pluripotent stem cells (iPSCs) similarly gave birth to a new breed of stem cell biologists, several times larger than the hESC community. This discovery allowed new ideas, research models, and technology to flourish, and no doubt has made stem cell research accessible to all in the scientific community. This is reflected in the greatly increased number of iPSC-based publications compared to hESC (Fig. 6.2). An appraisal of this field provides an idea of how easily iPSC technology can be adapted and applied to different fields of biological research (Fig. 6.3).

Being able to ride upon the shoulders of about a decade of hESC research, iPSC research has progressed rapidly. Several new and improved methods of reprogramming to pluripotency or to intermediates were discovered. This also helped develop newer approaches such as directed differentiation. However, as the discovery of iPSCs completes a decade, the road to the clinic seems longer. Contrary to expectations, iPSCs have not made it to clinical trials faster than hESCs. This is not to say that their potential is any less or that efforts are insufficient. In fact, the possibilities that open up when starting with somatic cells are far more than with pluripotent stem cells. However, it must be acknowledged that this is just the nature of the beast – it requires much more time and money than hESC research to reach a comparable stage of having bona fide pluripotent cells. This is reflected in the number of different analyses required for characterizing iPSCs compared to hESCs (Table 6.1).

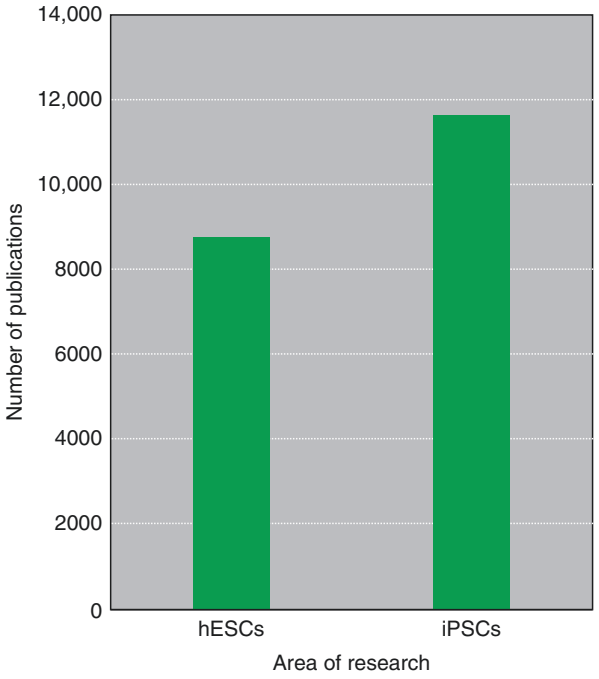


Fig. 6.2 Number of publications in the research areas of hESCs (1998–2016; total number = 8738) and iPSCs (2006–2016; total number = 11,620) (Source: Web of Science, retrieved July 29, 2016, from http://apps.webofknowledge.com/; Key search terms: human embryonic stem cells OR hESCs and induced pluripotent stem cells OR iPSCs; Refined the search for article, correction, report, letter, case report, and clinical trial)

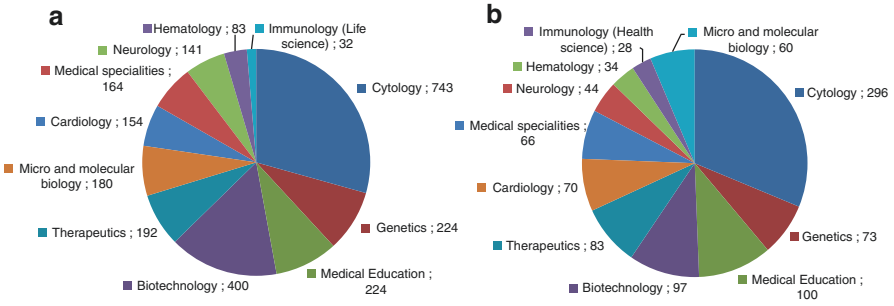


Fig. 6.3 Overview of adaptation of (**a**) iPSCs in general and (**b**) only human iPSCs, across various branches of biological research along with the number of publications during the last decade (Source: J-Gate, retrieved July 16, 2016, from http://jgateplus.com/; Key search terms for (**a**) induced pluripotent stem cells and (**b**) induced pluripotent stem cells and human)

Table 6.1 Techniques for characterization of derived hESCs and iPSCs, indicating the approximate number of cells required and time line

Cell type	Method	No. of cells required (approximately)	Time line (approximate values)	References
hESCs and iPSCs	Alkaline phosphatase staining	$1.5–3 \times 10^6$	2 h	Singh et al. [3]
	Immunofluorescence staining	$1.5–3 \times 10^6$	2 days	Peura et al. [4] Rasmussen et al. [5]
	Gene expression/transcriptome analysis	$1.5–3 \times 10^6$	2–4 days	Oldershaw et al. [6] Hibaoui et al. [7]
	Karyotyping	$3.7–7.5 \times 10^6$	2 days	Campos et al. [8]
	CGH-ChIP analysis	$1.5–3 \times 10^6$	4 days	Cell line genetics www.clgenetics.com
	Telomerase activity	$1.5–3 \times 10^6$	2 days	Weinrich et al. [9]
	Embryoid body formation	2×10^5 cells/mL	6 days	Outten et al. [10]
	Teratoma formation	$2–5 \times 10^6$	6–10 weeks	Hentze et al. [11] Gutierrez-Aranda et al. [12]
	Genome profiling	$7.5–11.5 \times 10^6$	5–6 days for sample preparation and weeks for bioinformatics analysis	Liu et al. [13] Elliott et al. [14]
	HLA typing	$1.5–3 \times 10^6$	2 days	TBG Biotechnology corporation http://www.tbgbio.com/
iPSCs only	Bisulfite genomic sequencing	$1.5–3 \times 10^6$	3–4 days	Pappas et al. [15]
	Histone modifications (ChIP)	5×10^7	3 days	Guenther et al. [16]
	X-chromosome inactivation status	1.0×10^6	2–3 days	Kiedrowski et al. [17]
	PluriTest	$7.5–11.5 \times 10^6$	5–6 days for sample preparation and weeks bioinformatics analysis	Muller et al. [18]
	Chimera formation	50–100	20–30 days	Boland et al. [19]

6.4 Comparison Between hESCs and iPSCs

hESC derivation involves harvesting naïve pluripotent cells of the morula or inner cell mass of the human blastocyst by one of several means and coaxing them to grow in culture with chosen protocols [20, 21] (Fig. 6.4). Pluripotent cells that can adapt to and grow in the *in vitro* conditions can easily be distinguished from

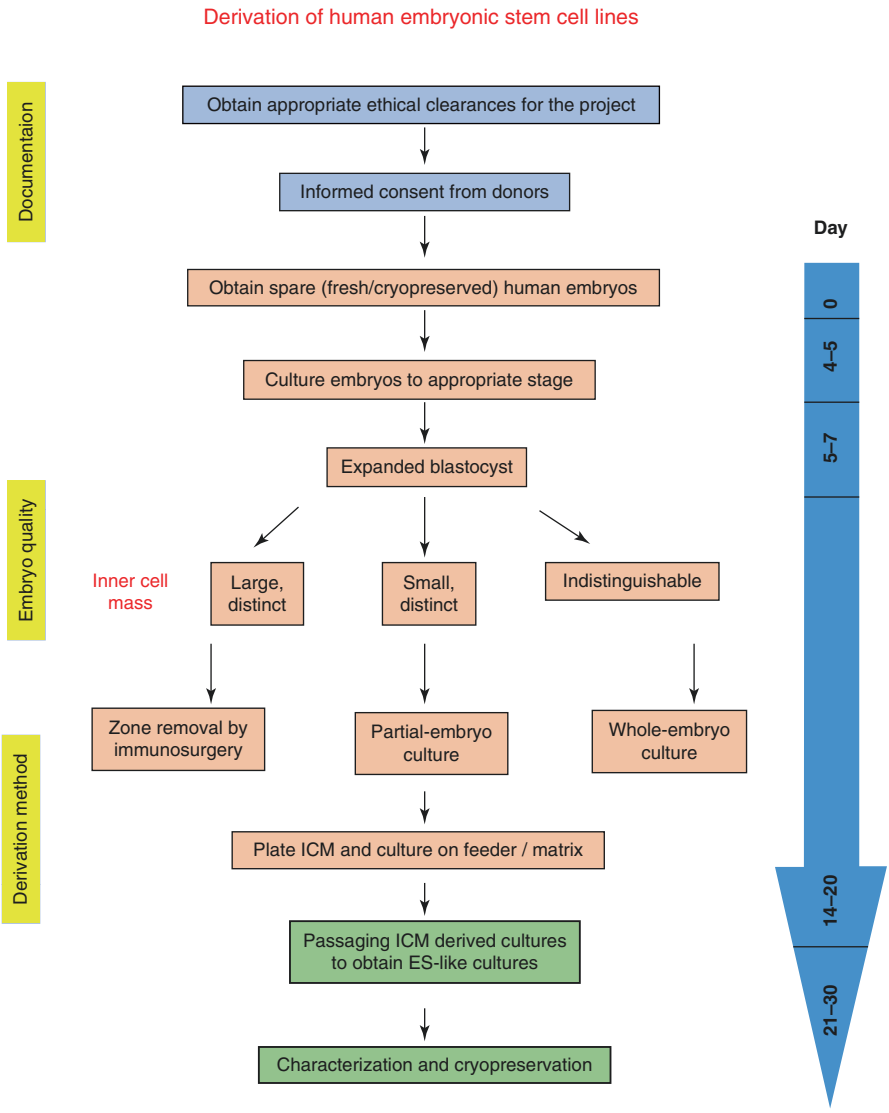


Fig. 6.4 Flowchart depicting key steps involved in and factors to be considered for derivation of hESCs. The time taken for generating sufficient cells for cryopreservation and characterization is 21–30 days

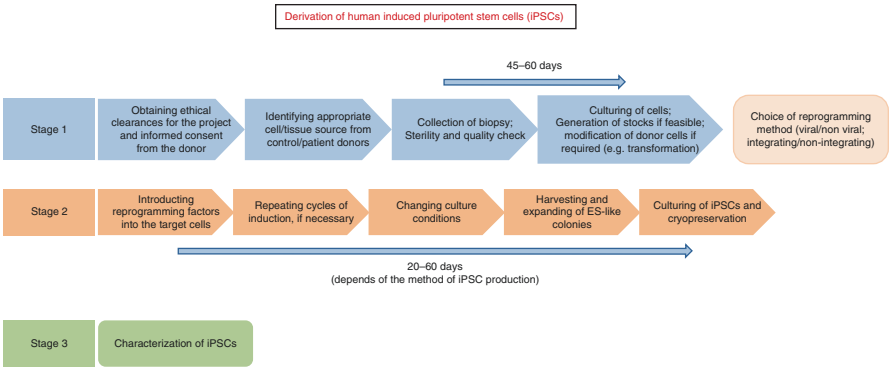


Fig. 6.5 Schematic showing the general outline of deriving human iPSCs. Various methods of characterizing iPSCs are given in Table 6.1

imposters on the basis of gene expression profiles and also by their ability to proliferate indefinitely and capability of differentiating to derivatives of the three primary germ layers in vitro or in vivo. In contrast, several factors are to be considered before reprogramming of somatic cells to pluripotency. The source of cells is a primary concern, as availability, viability, and ease of accessing the tissue are important factors in determining the success of the reprogramming protocol. Derivation of somatic cells from the tissue, reprogramming, and characterizing them take longer compared to hESCs (Fig. 6.5).

Further, given the low efficiency of well-established reprogramming methods available to date, cell number available and the ability to expand the desired primary cells in culture are also major factors. Few recent reports of high-efficiency reprogramming are promising [22, 23]. However, it is not clear whether they apply to all cell types and ages, indicating that several parameters of reprogramming remain to be defined and optimized. Table 6.2 provides an overview of commonly used techniques, cell source, and efficiency of reprogramming.

Progenitors or lineage-specific stem cells have shown better efficiency of reprogramming compared to terminally differentiated cells [38]. A couple of recent reports of reprogramming with nearly 95–100% efficiency are promising [22, 23]. It is hoped that these methods could be generally applicable to other cell types. Rapid reprogramming could also significantly reduce associated costs and make iPSC a more feasible option for use in regenerative medicine. However, several safety issues and the extra cost of additional characterization remain. It should also be noted that only derivation methods for generating research grade hESC and iPSC lines are compared here. cGMP level derivation for clinical or therapeutic application requires several additional procedures and parameters like maintenance of xeno-free conditions, regular checking for genomic alterations and pathogens, etc. These are to be controlled in both cases – but especially for iPSC.

Additionally, the risk of undesired somatic mutations present in the donor cells is a serious consideration. Choice of reprogramming method, genetic changes introduced during reprogramming, and epigenetic inconsistencies are also of concern.

Table 6.2 List of various methods, reprogramming factors used for producing human iPSCs, and the possible efficiencies

Reprogramming method		Factors	Cell type	Efficiency (%)	Duration of iPSCs production (days)	References
Integrating methods	Lentiviral	OKSM	Fibroblasts	0.1–1.0	30	Yu et al. [24]
	Inducible lentiviral	OKSM	Fibroblasts	0.1–2.0	>31	Maherali et al. [25]
Non-integrating methods	Sendai virus	OKSM	Fibroblasts	1	30	Fusaki et al. [26]
			Circulating human T cells	0.1	~30	Seki et al. [27]
	Single cassette lentiviral vector with lox sites	OKSM	Adult human dermal fibroblasts	0.1–1.5	30	Somers et al. [28]
	Adenovirus	OKSM	Fibroblasts	0.0002	25–30	Zhou et al. [29]
	PiggyBAC	OKSM	Human mesenchymal stem cells	0.02	12–24	Mali et al. [30]
	Minicircle vectors	Oct4, Sox2, Nanog and Lin28	Human adipose stromal cells	0.005	~28	Narsinh et al. [31]
	Episomal vectors (OriP-/EBNA-based plasmids)	Oct4, Sox2, c-Myc, and Lin28	Human foreskin fibroblasts	0.0003–0.0006	~20	Yu et al. [24]
		Mix of Yamanaka factors + Lin28	CD34+ cord blood cells	0.02	14	Cheng et al. [32]
			CD34+ peripheral blood cells	0.009	14	
			CD34+ bone mononuclear cells	0.005	14	
Methods free of exogenous DNA material	RNA modified synthetic mRNA	OKSM	Fibroblasts	4.40	21	Warren et al. [33]
	Proteins	OKSM proteins	Human newborn fibroblasts	0.001	56	Kim et al. [34]
	miRNAs	mir-369s, Mir-302s, and mir-200c	Fibroblasts and adipose stromal cells	0.002	20	Miyoshi et al. [35]
	Extracellular matrix modification	Fibromodulin	Human newborn foreskin fibroblasts	0.03%	21–24	Zheng et al. [36]
	Xeno-free method	miRNA + mRNA cocktail	Human adult dermal fibroblasts	0.2–0.3	11	Lee et al. [37]

Note: OKSM refers to Oct3/Oct4, Klf4, Sox2, and c-Myc

Genetic changes can be easily detected by whole genome sequencing; however this is expensive and time-consuming and requires access to bioinformatics expertise. Epigenetic changes also could vary depending on tissue source, age of individual, etc. Finally, multiple clones need to be tested, though some methods claim that testing a single colony is sufficient.

6.5 Pluripotent Stem Cell Generation in the Indian Context

It must be mentioned that all of the above factors have been investigated thoroughly by several eminent groups, and a few have been chosen and validated by consensus from consortia or large groups like the International Stem Cell Initiative (ISCI). However, these are feasible, both economically and technically, only in groups endowed with significant funding and access to core facilities with all the required expertise. Such environments are hard to come by in India. While serious and successful efforts are underway to create the requisite setup for iPSC or somatic cell reprogramming such that it will be reliable and rapid. This promises to remain in the domain of very few academic and commercial institutes in India, given the limited expertise and funding available. It should be noted that the primary driving force for these projects has been the desire to aid fundamental research and not toward using iPS cells for therapy. This is rightly so, given the complexity of choosing tissue source, additional time required as compared to hESC derivation, cost of reprogramming, and cost of analysis.

6.6 Stem Cell Choice

The case of regenerative medicine is quite different from that of basic research. As with hESCs, successfully reprogrammed somatic cells that are pluripotent, i.e., iPSCs, are also expected to be capable of generating all cell types. However, all pluripotent human stem cells presently come with the challenge of being able to differentiate them successfully and efficiently to the desired lineage and functional cell type. Research conducted in the last 5 years showed that this may not be a concern at least for deriving cardiomyocytes [39], erythroid cells [40], and neuronal cells [41] to some extent. Further, it is not clear whether differentiation to a pure population or a set of related or cooperating cell types is preferable for regenerative purposes. In the case of iPSCs, this is further complicated by the fact that differentiation ability to a given lineage is greatly influenced by the source of cells in terms of tissue type, presence of stem cells or precursors, and age of the donor. Thus one has to question whether the time and money spent making somatic cells pluripotent are worth it when the starting material, i.e., pluripotent hESCs can be generated easily, quickly, efficiently, and cost-effectively.

A major application of iPSCs has been in research, especially the ability to generate patient-derived iPSCs that allow one to model various diseases in vitro. The genetic changes that accompany the disease can thus be identified, analyzed, and

corrected as desired. However the recent advent of quick, efficient, and low-cost genome-editing technologies, especially the CRISPR/CAS9 system, allows multiple desired changes to be incorporated in the human genome quickly and efficiently, at a significantly lower cost [42–45]. Thus the flexibility and reliability of human genome manipulation are greatly increased and can be applied to several contexts. While the risk of off-target changes and safety concerns regarding the cells generated still exist, these concern fewer parameters for hESCs than for iPSCs. An important consideration is that epigenetic changes accompanying the patient-generated iPSCs may be missed. However, while these are likely to be important for research, targeted genome manipulation of wild-type hESCs allows the researcher to control changes and possibly deal with fewer unknowns than for iPSCs.

6.7 Haplobanks

Given the prohibitive cost of autologous iPSC generation for personalized regenerative medicine, several efforts are underway to generate clinical grade iPSC banks which represent all HLA types – a haplobank. HLA genes are inherited in a Mendelian dominant manner, and in humans they are located on chromosome 6, an autosome. Hence offspring are haplo-identical to their parents, and siblings may have 50% or less similarity between each other [46]. This, along with conditions like ethnic diversity, demographics, etc., allows one to arrive at the minimum number of homozygous HLA-typed lines/donors for iPSC and hESC lines, so as to cover a given population [47–49]. India has around 55 endogamous populations with a high level of genetic and haplotype diversity, which is clustered mostly on the basis of ethnicity and language [50]. In such a scenario, a single bank of cell lines cannot serve the needs of the entire Indian population. Establishing multiple banks of HLA-typed ESCs or HLA-typed iPSCs could be a feasible solution [46].

Conclusion

The need for haplobanks defeats the argument that iPSCs will allow autologous stem cell therapy, as was hoped. In light of this, it is clear that a hESC haplobank will be far more cost-effective and easier to generate. Surplus embryos are the only source of hESCs. Depending on the quality of the embryos and age of the female recipient, a maximum of two embryos are implanted in any of up to three attempts [51]. The rest are frozen for future attempts or given for research purpose. In 2014, it was reported that in India, nearly 85,000 ART (assisted reproductive technology) cycles are done per year. As per the above information, there can exist a large surplus resource of spare embryos available for research [52].

Similarly, the large number of surplus embryos from in vitro fertilization (IVF) available the world over should make this very feasible. It will also cut down the cost of sample acquisition and donor trauma often experienced in obtaining cells for reprogramming. However, the resistance to this route seems to be rooted in religious opposition to hESC rather than scientific rationale. Fortunately, this is not a bias that is promoted in India.

Acknowledgments We thank Ronak K. Shetty, Ph.D., and Ms. Deeti Shetty, JNCASR, for critical reading of the manuscript.

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.
2. Alper J. Geron gets green light for human trial of ES cell-derived product. Nat Biotechnol. 2009;27:213–4.
3. Singh U, Quintanilla RH, Grecian S, et al. Novel live alkaline phosphatase substrate for identification of pluripotent stem cells. Stem Cell Rev. 2012;8(3):1021–9.
4. Peura TT, Bosman A, Stojanov T. Derivation of human embryonic stem cell lines. Theriogenology. 2007;67(1):32–42.
5. Rasmussen MA, Hjermind LE, Hasholt LF, et al. Induced pluripotent stem cells (iPSCs) derived from a patient with frontotemporal dementia caused by a R406W mutation in microtubule-associated protein tau (MAPT). Stem Cell Res. 2016;16(1):75–8.
6. Oldershaw RA, Baxter MA, Lowe ET, et al. Directed differentiation of human embryonic stem cells toward chondrocytes. Nat Biotechnol. 2010;28(11):1187–94.
7. Hibaoui Y, Grad I, Letourneau A, et al. Data in brief: transcriptome analysis of induced pluripotent stem cells from monozygotic twins discordant for trisomy 21. Genome Data. 2014;2:226–9.
8. Campos PB, Sartore RC, Abdalla SN, et al. Chromosomal spread preparation of human embryonic stem cells for karyotyping. J Vis Exp. 2009;31:1512.
9. Weinrich SL, Pruzan R, Ma L, et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat Genet. 1997;17(4):498–502.
10. Outten JT, Gadue P, French DL, et al. High-throughput screening assay for embryoid body differentiation of human embryonic stem cells. Curr Protoc Stem Cell Biol. 2012;Chapter 1:Unit 1D 6.
11. Hentze H, Soong PL, Wang ST, et al. Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. Stem Cell Res. 2009;2(3):198–210.
12. Gutierrez-Aranda I, Ramos-Mejia V, Bueno C, et al. Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless the site of injection. Stem Cells. 2010;28(9):1568–70.
13. Liu Y, Shin S, Zeng X, et al. Genome wide profiling of human embryonic stem cells (hESCs), their derivatives and embryonal carcinoma cells to develop base profiles of U.S. Federal government approved hESC lines. BMC Dev Biol. 2006;6:20.
14. Elliott AM, Hohenstein Elliott KA, Kammesheidt A. High-resolution genomic profiling of chromosomal abnormalities in human stem cells using the 135K StemArray. Stem Cells Int. 2012;2012:431534.
15. Pappas JJ, Toulouse A, Bradley WE. A modified protocol for bisulfite genomic sequencing of difficult samples. Biol Proced Online. 2009;11:99–112.
16. Guenther MG, Frampton GM, Soldner F, et al. Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. Cell Stem Cell. 2010;7(2):249–57.
17. Kiedrowski LA, Raca G, Laffin JJ, et al. DNA methylation assay for X-chromosome inactivation in female human iPS cells. Stem Cell Rev. 2011;7(4):969–75.
18. Muller FJ, Schuldt MB, Williams R, et al. A bioinformatic assay for pluripotency in human cells. Nat Methods. 2011;8(4):315–7.
19. Boland MJ, Hazen JL, Nazor KL, et al. Generation of mice derived from induced pluripotent stem cells. J Vis Exp. 2012;69:e4003.
20. Kim HS, Oh SK, Park YB, et al. Methods for derivation of human embryonic stem cells. Stem Cells. 2005;23:1228–33.

21. Hasegawa K, Pomeroy JE, Pera MF. Current technology for the derivation of pluripotent stem cell lines from human embryos. Cell Stem Cell. 2010;6:521–31.
22. Rais Y, Zviran A, Geula S, et al. Deterministic direct reprogramming of somatic cells to pluripotency. Nature. 2013;502:65–70.
23. Di Stefano B, Sardina JL, van Oevelen C, et al. C/EBPalpha poises B cells for rapid reprogramming into induced pluripotent stem cells. Nature. 2014;506:235–9.
24. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007;318(5858):1917–20.
25. Maherali N, Ahfeldt T, Rigamonti A, et al. A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell. 2008;3(3):340–5.
26. Fusaki N, Ban H, Nishiyama A, et al. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci. 2009;85(8):348–62.
27. Seki T, Yuasa S, Oda M, et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell. 2010;7(1):11–4.
28. Somers A, Jean JC, Sommer CA, et al. Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. Stem Cells. 2010;28(10):1728–40.
29. Zhou W, Freed CR. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells. 2009;27(11):2667–74.
30. Mali P, Chou BK, Yen J, et al. Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. Stem Cells. 2010;28(4):713–20.
31. Narsinh KH, Jia F, Robbins RC, et al. Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors. Nat Protoc. 2011;6(1):78–88.
32. Cheng L, Hansen NF, Zhao L, et al. Low incidence of DNA sequence variation in human induced pluripotent stem cells generated by nonintegrating plasmid expression. Cell Stem Cell. 2012;10(3):337–44.
33. Warren L, Manos PD, Ahfeldt T, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell. 2010;7(5):618–30.
34. Kim D, Kim CH, Moon JI, et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell. 2009;4(6):472–6.
35. Miyoshi N, Ishii H, Nagano H, et al. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell. 2011;8(6):633–8.
36. Zheng Z, Jian J, Zhang X, et al. Reprogramming of human fibroblasts into multipotent cells with a single ECM proteoglycan, fibromodulin. Biomaterials. 2012;33(24):5821–31.
37. Lee KI, Lee SY, Hwang DY. Extracellular matrix-dependent generation of integration- and xeno-free iPS cells using a modified mRNA transfection method. Stem Cells Int. 2016;2016:6853081.
38. Ohmine S, Dietz AB, Deeds MC, et al. Induced pluripotent stem cells from GMP-grade hematopoietic progenitor cells and mononuclear myeloid cells. Stem Cell Res Ther. 2011;2:46.
39. Lian X, Hsiao C, Wilson G, et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci U S A. 2012;109:E1848–57.
40. Olivier EN, Marenah L, McCahill A, et al. High-efficiency serum-free feeder-free erythroid differentiation of human pluripotent stem cells using small molecules. Stem Cells Transl Med. 2016;5(10):1394–405.
41. DuZW CH, Liu H, et al. Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. Nat Commun. 2015;6:6626.
42. Krentz NA, Nian C, Lynn FC. TALEN/CRISPR-mediated eGFP knock-in add-on at the OCT4 locus does not impact differentiation of human embryonic stem cells towards endoderm. PLoS One. 2014;9:e114275.

43. Liang P, Xu Y, Zhang X, et al. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell. 2015;6:363–72.
44. Shetty DK, Inamdar MS. Generation of a heterozygous knockout human embryonic stem cell line for the OCIAD1 locus using CRISPR/CAS9 mediated targeting: BJNhem20-OCIAD1-CRISPR-20. Stem Cell Res. 2016;16:207–9.
45. Shetty DK, Inamdar MS. Generation of a heterozygous knockout human embryonic stem cell line for the OCIAD1 locus using CRISPR/CAS9 mediated targeting: BJNhem20-OCIAD1-CRISPR-39. Stem Cell Res. 2016;16:308–10.
46. Solomon S, Pitossi F, Rao MS. Banking on iPSC—is it doable and is it worthwhile. Stem Cell Rev. 2015;11:1–10.
47. Gourraud PA, Gilson L, Girard M, et al. The role of human leukocyte antigen matching in the development of multiethnic "haplobank" of induced pluripotent stem cell lines. Stem Cells. 2012;30:180–6.
48. Taylor CJ, Bolton EM, Pocock S, et al. Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet. 2005;366:2019–25.
49. Andrews PW, Cavagnaro J, Deans R, et al. Harmonizing standards for producing clinical-grade therapies from pluripotent stem cells. Nat Biotechnol. 2014;32:724–6.
50. Indian Genome Variation Consortium. Genetic landscape of the people of India: a canvas for disease gene exploration. J Genet. 2008;87:3–20.
51. National Institute for Health and Care Excellence, UK. Fertility problems: assessment and treatment. Clinical guidelines 2013. London: National Institute for Health and Care Excellence, UK; 2013.
52. Mehta RH. Sourcing human embryos for embryonic stem cell lines: problems & perspectives. Indian J Med Res. 2014;140(Suppl):S106–11.

Int. J. Dev. Biol. 64: 213-225 (2020)
https://doi.org/10.1387/ijdb.190038mi

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The OCIAD protein family: comparative developmental biology and stem cell application

WULLIGUNDAM PRAVEEN[1], SALONI SINHA[1], RAJARSHI BATABYAL[1], KAJAL KAMAT[1] and MANEESHA S. INAMDAR*[,1,2]

[1]*Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore and*
[2]*Institute for Stem Cell Biology and Regenerative Medicine, Bangalore, India*

ABSTRACT Over the last two decades, an exponential growth in technologies and techniques available to biologists has provided mind-boggling quantities of data and led to information overload. Yet, answers to fundamental questions such as "how are we made?" and "what keeps us ticking?" remain incomplete. Developmental biology has provided elegant approaches to address such questions leading to enlightening insights. While several important contributions to developmental biology have come from India over the decades, this area of research remains nascent. Here, we review the journey in India, from the discovery of the *ociad* gene family to decoding its role in development and stem cells. We compare analysis *in silico, in vivo* and *ex vivo*, with developmental models such as *Drosophila,* mouse and stem cells that gave important insight into how these clinically significant genes function.

KEY WORDS: *OCIAD1/2, stem cell, hematopoiesis, development, disease*

Introduction

Application of approaches from the natural and computational sciences, engineering as well as mathematics allow deep analysis at the single cell as well as single molecule resolution, thus helping one build models and theories of biological processes at the systems level. Yet, how a single unit of life, the cell, co-exists and functions in concert with millions or billions of others in complex multicellular organisms, seems indiscernible. Biologists continue to seek more information to understand principles of development and dysregulation in disease. Here, we use the example of the *ociad* gene family to discuss how a comparative approach using invertebrate and vertebrate developmental models revealed new modes of cell fate regulation and gave insight into gene function.

Navigating uncharted territory: identification of a new gene family

The first member of the *ociad* gene family was discovered about twenty years ago as an expressed sequence tag (EST) identified in a gene trap screen in mouse embryonic stem cells (mESCs), aimed at finding regulators of cardiovascular development (Stanford *et al.*, 1998). Subsequently, the first draft of the human genome sequence in 2000, identified a corresponding human-expressed sequence, suggesting gene conservation. A cDNA library generated from ascites fluid of human ovarian cancer patients subsequently showed the presence of this gene sequence, leading to the name Ovarian Carcinoma Immunoreactive Antigen 1 (*ocia1*) (Luo *et al.*, 2001). Its expression in mice was tracked by the reporter beta-galactosidase and subsequently confirmed by transcript and protein analysis in mESCs (Mukhopadhyay *et al.*, 2003). High level of expression of this gene in undifferentiated pluripotent cells declined rapidly during their differentiation and persisted predominantly in early mesoderm and blood vascular precursors *in vitro*. This led the gene to be named as *asrij* (Sanskrit: blood). Meanwhile, a revised draft of the human genome and homology searches revealed the presence of another gene (1810027I20Rik), initially reported by Strausberg *et al.,* in 2002, that shared sequence similarity to *ocia1* (found through the National Institutes of Health Mammalian Gene Collection project), hence named *ocia2*. Later, the expression of this gene was reported in a proteomic study of the mitochondrial inner membrane of mouse liver cells and also verified by immunostaining

Abbreviations used in this paper: ARF1, ADP-ribosylation factor 1; hESC, human embryonic stem cell; JAK/STAT, Janus kinase/signal transducer and activator of transcription; KO, knockout; LPA, lysophosphatidic acid; mESC, mouse embryonic stem cell; OCIAD, ovarian carcinoma immunoreactive antigen domain; PTM, post-translational modification; TSS, transcription start site.

***Address correspondence to:** Maneesha S. Inamdar. Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560064, India.
Tel: 080-22082818. Fax: 080-22082766. E-mail: inamdar@jncasr.ac.in -Web: http://www.jncasr.ac.in/inamdar/ - https://orcid.org/0000-0002-8243-2821

Submitted: 4 March, 2019; *Accepted:* 28 October, 2019.

ISSN: Online 1696-3547, Print 0214-6282

Printed in Spain

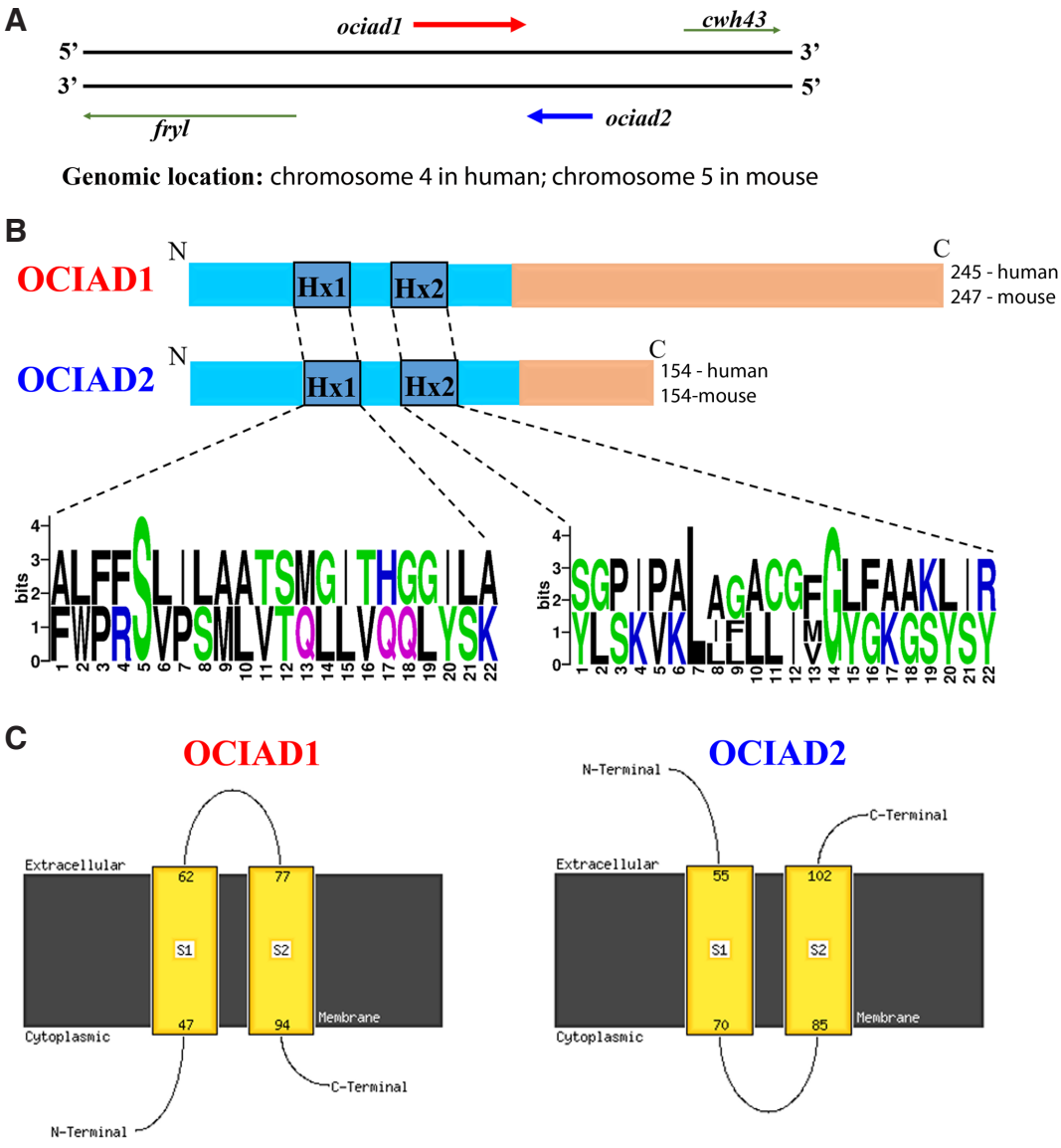


Fig. 1. A schematic overview of the *ociad* (ovarian carcinoma immunoreactive antigen domain) genes and OCIAD proteins. (A) *Genomic organization of* ociad *genes in mice and humans.* **(B)** *Schematic showing comparison of OCIAD protein organization. Numbers indicate amino acids at the beginning and end of the protein or domain. Blue: OCIA domain; peach: non-domain C-terminal region. The sequence similarity between the helices of the N-terminal region between OCIAD1 and OCIAD2 of mouse and human, generated by Weblogo software (weblogo.berkeley.edu) is represented below the proteins.* **(C)** *Predicted orientation of the proteins using PHYRE2 program (www.sbg.bio.ic.ac.uk/~phyre2/). OCIAD1 and OCIAD2 are predicted to harbor two hydrophobic helices and are oriented in opposite direction.*

(Da Cruz *et al.*, 2003). Almost a decade later, its association with ovarian cancer was reported (Nagata *et al.*, 2012). Interestingly, these two genes were identified to be neighbors and syntenic in human and mouse, raising several possibilities regarding their provenance. Sequence conservation was restricted to within 120 amino acids of the N-terminal of the two proteins and this region was conserved in vertebrates as well as the newly sequenced invertebrate genomes such as that of *Drosophila melanogaster*. Hence, the names were edited to Ovarian Carcinoma Immunoreactive Antigen Domain containing 1 and 2 (*ociad1* and *ociad2*).

What's in a name?

The general convention in biology to name genes and cells based on their deciphered aberrant phenotype aids analysis yet leaves a lot of unknowns. Where, when and how this identity arose and whether it is relevant in the context of a tissue or organism, often remains a mystery. Subsequent reports in the literature revealed that *ociad* genes were associated with several diseases, including a variety of carcinomas as well as neurological disorders (Han *et al.*, 2014; Nagata *et al.*, 2012; Sengupta *et al.*, 2008; Wu *et al.*, 2017). We chose to address the genes by their more informative names- *asrij*, meaning blood, and for want of functional information, its neighbor *ociad2* was named *padosan* (Hindi: neighbor). While *Drosophila* and mouse *asrij* were named earlier and are accepted, convention dictates that the human orthologs be referred to as *ociad1* and *ociad2*, though this gives no insight into gene function.

All in the family: OCIAD1 and OCIAD2

Although there was increasing information regarding the expression of *ociad1/2* genes in human carcinoma tissues, their function remained a mystery. The lack of homology of the OCIAD proteins to any known protein domain and absence of predicted structures apart from the two short helices led to classifying these proteins as a two-member family. The OCIA domain bearing two helical stretches is the most distinctive and highly conserved feature of this family. OCIAD1/Asrij, the first protein of this family to be discovered, is predominantly expressed in the blood vessels (Inamdar, 2003; Mukhopadhyay *et al.*, 2003), and plays important

roles in regulation of blood stem cell maintenance, hematopoiesis and immunity (Khadilkar, Ray *et al.*, 2017; Khadilkar *et al.*, 2014; Kulkarni, Khadilkar *et al.*, 2011; Sinha *et al.*, 2013). OCIAD2, the other member of the family, which is implicated in several cancers and certain neurodegenerative disorders (Han *et al.*, 2014; Nagata *et al.*, 2012; Wu *et al.*, 2017), was predicted to have risen from a tandem gene duplication event from an invertebrate *ociad* gene, sometime during the Ordovician and Silurian eras, as detailed elsewhere (Sinha *et al.*, 2018).

In most vertebrates, the *ociad1* and *ociad2* genes are located on opposite strands in a tail-to-tail orientation, mapping to chromosome 4 in humans and chromosome 5 in mice. Moreover, the gene synteny is strongly conserved across vertebrate species with the flanking genes *fryl* and *cwh43* or *dcun1d4* (Sinha *et al.*, 2018) (Fig. 1A). In mice and humans, the *ociad1* gene has 9 exons while *ociad2* has 7, the first exon being non-coding. The terminal codon of *ociad1* is also non-coding, whereas that of *ociad2* is partially non-coding. Multiple isoforms have been reported for both *ociad1* and *ociad2*, suggesting complex regulation of these genes. Interestingly, a brain-enriched shorter isoform of *ociad1* lacking exon 8 has also been reported in mice (Mukhopadhyay *et al.*, 2003) and humans (Luo *et al.*, 2001) whose importance in neuronal development and disease remains unexplored.

Although the structures of the OCIAD proteins have not been elucidated, the N-terminal OCIA domain has a high degree of sequence conservation (Fig. 1B) and is essential for regulation of multiple signaling pathways, cellular processes and localization (Sinha *et al.*, 2019a; Sinha *et al.*, 2018; Sinha *et al.*, 2013). This domain is predicted to harbor two transmembrane helices that likely have opposing orientation in the membrane (Fig. 1C). On

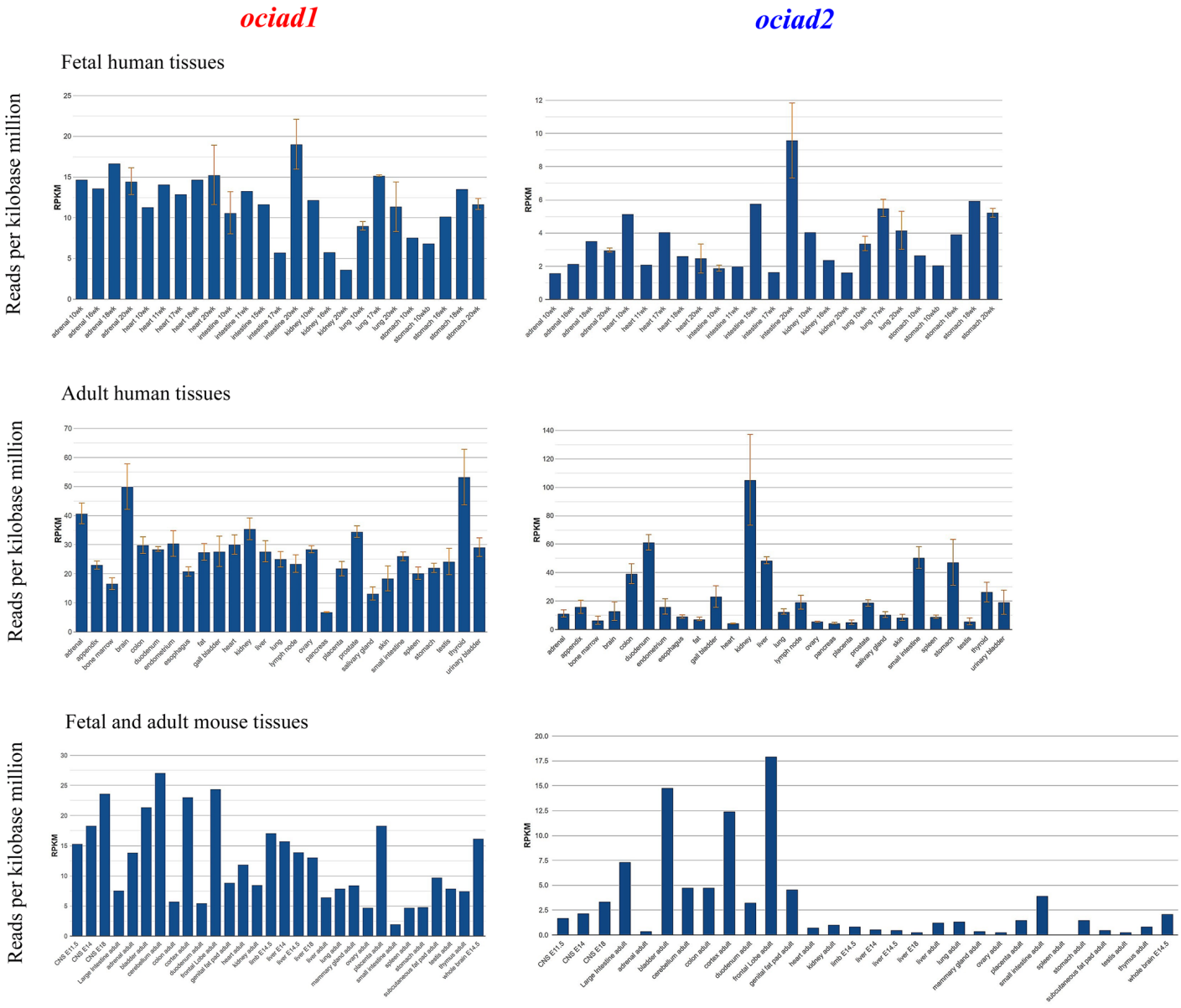


Fig. 2. Comparison of the RNA expression of *ociad1* and *ociad2* in fetal and adult tissues of humans and mice as represented in the NCBI database (www.ncbi.nlm.nih.gov).

the other hand, the C-terminal non-OCIA domain region of these proteins is predicted to be intrinsically disordered (Sinha *et al.*, 2018; Sinha *et al.*, 2013), functionally dispensable and to have a dominant negative function in *Drosophila* and mESCs (Sinha *et al.*, 2018; Sinha *et al.*, 2013).

Humble beginnings - the fruit fly

An early hint of the normal *in vivo* role for Asrij came from analysis of the *asrij* expression pattern during *Drosophila* embryogenesis. Asrij is expressed in anterior and mesodermal regions of the embryo and its expression preceded that of known hematopoietic transcription factors. Further, a chromosomal deficiency that removed *asrij* abolished embryonic blood cells (hemocytes) (Inamdar, 2003). The gene expression pattern coupled with the fact that it is expressed in all hemocytes (Kulkarni, Khadilkar *et al.*, 2011) pointed to an apparent role for Asrij in *Drosophila* hematopoiesis. Asrij depletion from the lymph gland, the primary site of *Drosophila* larval hematopoiesis, led to a reduction in Collier^{+} niche cells and domeless^{+} prohemocytes accompanied by increased numbers of P1^{+} plasmatocytes and Lozenge^{+} crystal cells as well as hyperproliferation of lymph gland lobes (Kulkarni, Khadilkar *et al.*, 2011). Consistent with the aberrant hematopoietic phenotypes observed in *asrij* mutant lymph glands, Asrij contributes to regulation of blood cell homeostasis through the control of multiple signaling pathways such as the Notch, JAK/STAT, Pvr and insulin signaling, mostly via its interaction with the ubiquitous trafficking molecule, ADP-ribosylation factor 1 (ARF1) (Khadilkar *et al.*, 2014; Kulkarni, Khadilkar *et al.*, 2011; Sinha *et al.*, 2013). More recent work showed that Asrij differentially modulates humoral and cellular immunity and that loss of Asrij or ARF1 leads to reduced survival and lifespan upon infection (Khadilkar, Ray *et al.*, 2017). While Asrij does not affect plasmatocyte-mediated phagocytosis, it is essential for anti-microbial peptide production through the Imd pathway and regulates crystal cell melanization and phenoloxidase activity. Moreover, upon infection, Asrij expression is perturbed,

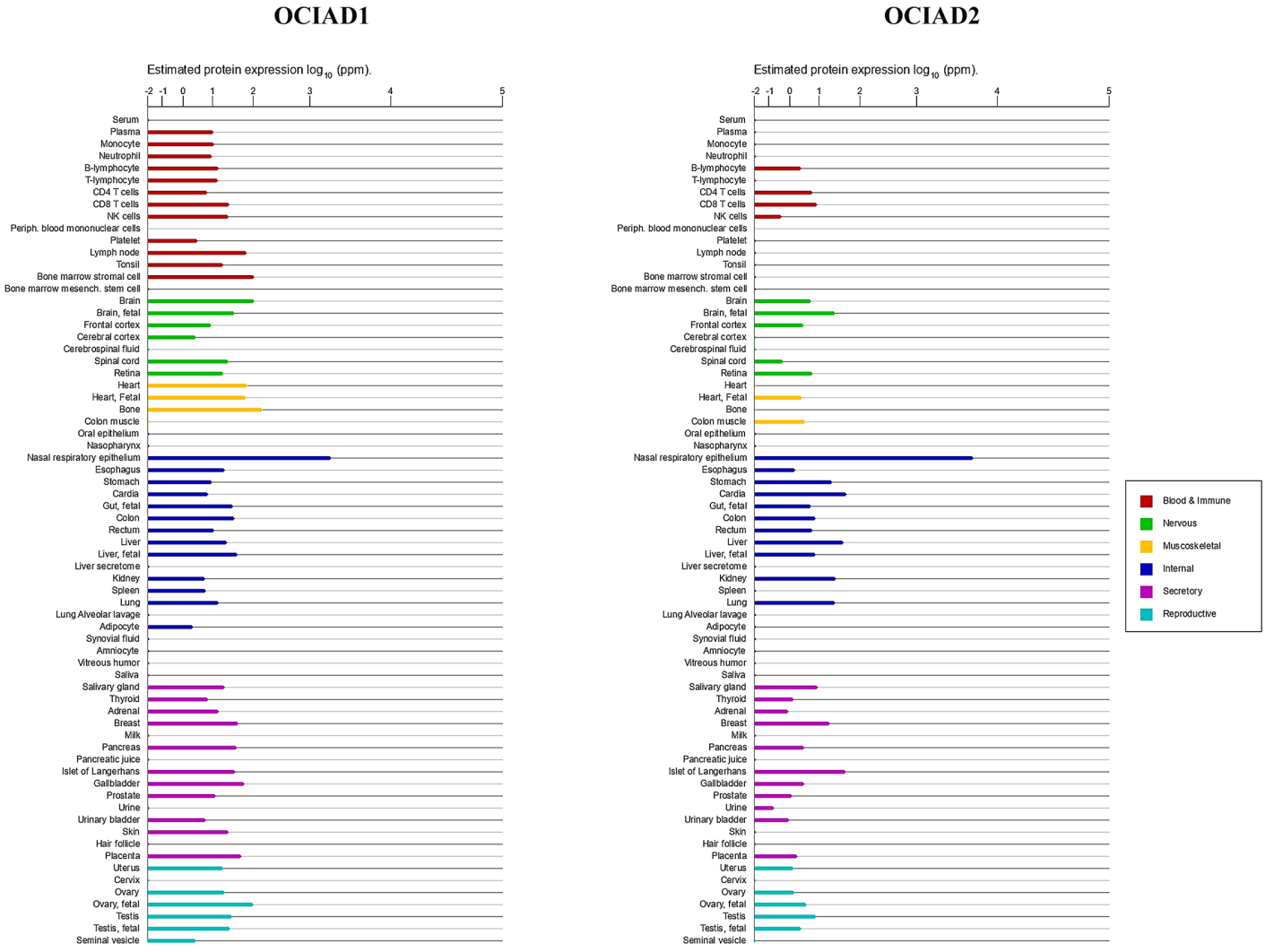


Fig. 3. Comparison of expression of OCIAD (ovarian carcinoma immunoreactive antigen domain) proteins in various human tissues, as shown in the GeneCards database (www.genecards.org). *Both proteins have a similarity in their expression pattern. OCIAD1 expresses in a greater number of tissues and cell types compared to OCIAD2, which is more lineage-restricted, especially in the circulatory system.*

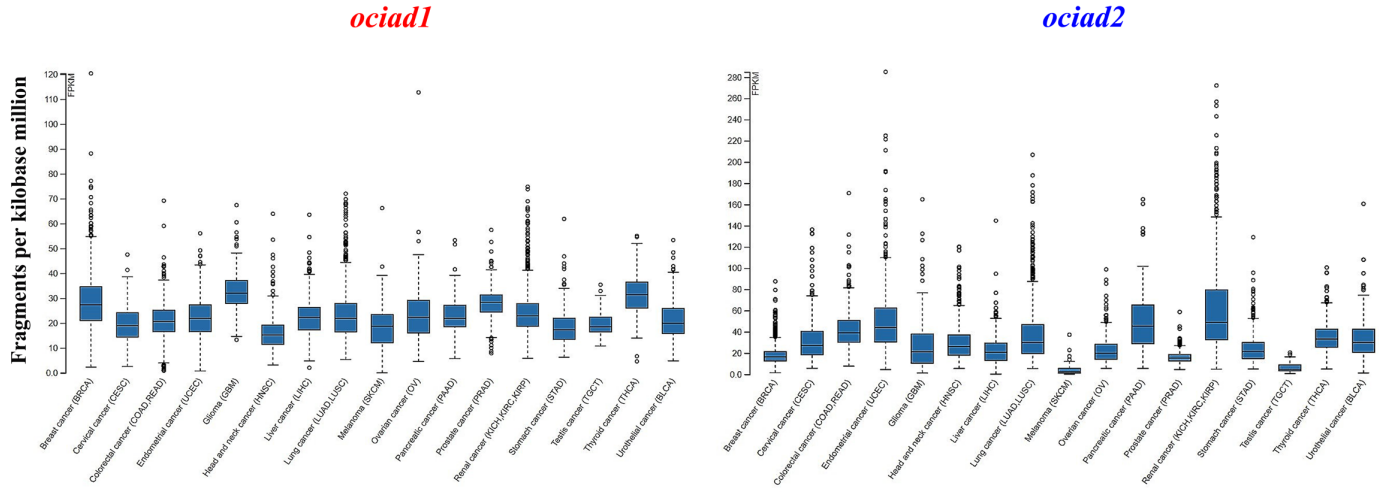


Fig. 4. Comparison of the RNA expression of *ociad* (ovarian carcinoma immunoreactive antigen domain) genes in various human cancers as depicted in The Cancer Genome Atlas database (www.cancergenome.nih.gov). *In contrast to the lower expression relative to* ociad1 *in normal human tissues,* ociad2 *seems to be expressed at higher levels in some cancer tissues.*

indicating that it is required for mounting immune responses in *Drosophila* (Khadilkar *et al*., 2017). In agreement with previous reports, analysis of the larval lymph gland proteome showed that Asrij affects expression of several known regulators of *Drosophila* hematopoiesis, immunity and lymph gland development (Sinha *et al*., 2019b).

Interestingly, while insects express only one copy of the OCIAD protein (Asrij/OCIAD1), vertebrates express two (Asrij/OCIAD1 and OCIAD2). Mammalian OCIAD1 and OCIAD2 are expressed in blood stem cells (Nestorowa *et al*., 2016; Phillips *et al*., 2000) and implicated in several blood cell disorders (Arai *et al*., 1987; Bagger *et al*., 2019; Jundt *et al*., 2008; Nestorowa *et al*., 2016; Nigrovic *et al*., 2008; Wolf *et al*., 2019), which suggests these proteins may function in vertebrate hematopoiesis. Recently, we showed that Asrij is necessary for maintaining quiescence of bone marrow hematopoietic stem cells in mice (Sinha *et al*., 2019a).

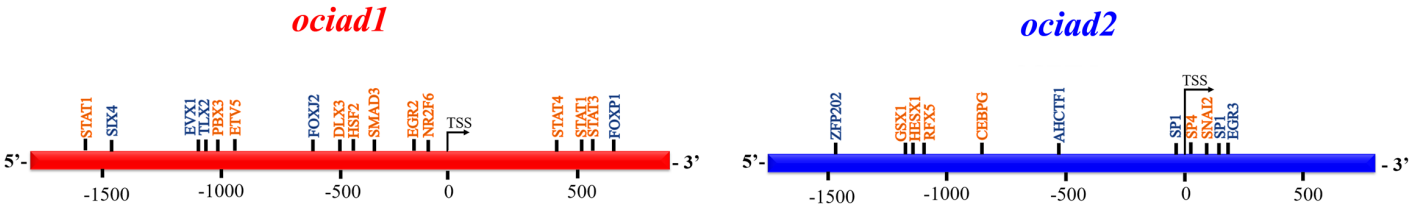
Mammalian *ociad1/2* genes

In vitro analysis of mouse early embryonic development using differentiation of stem cells, showed high OCIAD1/Asrij expression levels in mESCs, hinting at a role in stem cell maintenance. Nevertheless, Asrij expression is dynamic and prominent in early hematopoietic cells, like blood islands, and to a certain extent in the early vasculature. Interestingly, Asrij expression is detected earlier to that of Flk1, suggesting a probable role in the specification of Flk1^{+} cells, which needs to be investigated further (Mukhopadhyay *et al*., 2003). Reporter mice revealed a detailed expression pattern of Asrij in fetal mouse, corroborating the *in vitro* results (Mukhopadhyay *et al*., 2003). Asrij is expressed at multiple stages, predominantly in the cardiovascular system. As mentioned earlier, an isoform lacking the coding exon 8 is enriched in the adult mouse brain (Mukhopadhyay *et al*., 2003). Additionally, databases also provide a quantified expression of Asrij in various fetal and adult tissues of both mice and humans (www.ncbi.nlm.nih.gov/gene/54940#gene-expression; www.ncbi.nlm.nih.gov/gene/68095#gene-expression), as shown in Fig. 2. Similar expression pattern of Asrij across species, suggests a probable conserved function.

Like *ociad1*, *ociad2* is also expressed in mESCs (Tapial *et al*., 2017), but at a relatively lower level. Reminiscent of the dynamic expression pattern of *ociad1*, as described above, *ociad2* is expressed in various tissues (www.ncbi.nlm.nih.gov/gene/433904#gene-expression; www.ncbi.nlm.nih.gov/gene/132299#gene-expression) and may play a tissue-specific role, which warrants further investigation (Fig. 2). Brain, liver and kidney show higher protein expression than heart, bone marrow, spleen and testis (Sinha *et al*., 2018). A comparison of the expression pattern of OCIAD proteins in humans is given in Fig. 3, which shows that OCIAD2 is expressed in a more lineage-restricted pattern as compared to OCIAD1.

OCIAD proteins are highly expressed in various human cancers, as depicted in Fig. 4. Interestingly, while *ociad2* is expressed at lower levels compared to *ociad1* in normal tissues, its level often exceeds that of *ociad1* in several cancers. The role of OCIAD1/2 proteins in cancers and other diseases is discussed in greater detail in the later part of this review. In spite of the diverse expression of *ociad1/2* genes in various tissues and cancers, very little is known about their regulation. While the regulation of *ociad1* is yet to be reported, expression of *ociad2* is regulated by survival motor neuron (SMN) protein and erythropoietin in a context-dependent manner (Mille-Hamard *et al*., 2012; Zhang *et al*., 2013). Additionally, *ociad2* expression can also be regulated by DNA methylation in various cancers, as discussed in the later part of this review.

Binding of transcriptional regulators to the promoters, upstream and downstream elements of the target genes is a prerequisite for gene regulation. Reports indicate that *ociad1/2* genes are bound by various transcriptional regulators both upstream and downstream to their transcription start sites (TSSs), as summarized in Fig. 5. Signal transducer and activator of transcription (STAT) family proteins such as STAT1, STAT3 and STAT5 bind to regulatory elements upstream to *ociad1* TSS. Transcription factor activity of STAT proteins plays important roles in mediating many aspects of cell proliferation, apoptosis, migration, immunity and differentiation (Calo *et al*., 2003; Levy *et al*., 2002). On the other hand, transcription factors such as RFX5, CEBPG, SP4 *etc.* bind



Promoter location (genomic): 48831743 - 48833822

Organism	Transcription Factor	Position from TSS	Binding strength
Human	STAT3	521	1283.91
	STAT1	519	1335.48
	STAT4	414	1147.78
	NR2F6	-83	1116.21
	EGR2	-100	1142.58
	SMAD3	-395	1154.17
	HSF2	-438	1156.7
	DLX3	-500	1248.2
	PBX3	-1033	1725.22
	ETV5	-880	1170.96
	STAT1	-1524	1233.82
Mouse	FOXP1	668	1027.31
	FOXJ2	-667	1179.35
	TLX2	-1289	1211.09
	EVX1	-1299	916.42
	SIX4	-1437	718.9

Promoter location (genomic): 48908345 - 48910345

Organism	Transcription Factor	Position from TSS	Binding strength
Human	SNAI2	64	1193.64
	SP4	19	1221.83
	CEBPG	-838	1194.57
	RFX5	-1121	1274.54
	HESX1	-1187	1097.15
	GSX1	-1197	1024
Mouse	EGR3	165	561.65
	SP1	159	1302.9
	SP1	-2	1468.41
	AHCTF1	-513	1019.15
	ZFP202	-1431	869.72

Fig. 5: Schematic showing the binding of various transcription factors to the *ociad1* (red) and *ociad2* (blue) gene sequences, both upstream and downstream of the transcription start site (TSS). *Names in orange represent the human proteins and in purple represent the mouse proteins. Tables show binding position and strength and the promoter location [(Pujato* et al.*, 2014); www.fiserlab.org/tf2dna_db/cite_us.html].*

to regulatory elements upstream of *ociad2* (Pujato *et al.*, 2014) and these may play important roles in modulating its expression (Fig. 5). Although studies to elucidate the transcriptional regulatory networks of *ociad1/2* genes remain to be performed, it is likely that these networks strongly dictate the expression profiles of these genes under normal as well as diseased conditions. Further studies will help in dissecting the regulation of *ociad1/2* genes.

While gene expression is primarily regulated by a transcriptional network, protein function, localization and turnover can also be regulated by post-translational modifications (PTMs) such as phosphorylation, methylation, ubiquitylation, *etc.* Several high-throughput studies show multiple PTMs of OCIAD1/2 (Hornbeck *et al.*, 2015). Interestingly, while most of the OCIAD1 PTMs are serine phosphorylation, the majority of OCIAD2 PTMs are ubiquitylation (Fig. 6). Although the effect of the various PTMs is yet to be explored, these have been reported in a diverse spectrum of cell types such as hESCs, certain hematopoietic and cancer cell lines, suggesting that these modifications may play important roles in regulating the multifarious array of functions performed by OCIAD1/2 proteins (Hornbeck *et al.*, 2015).

Modeling early mammalian development

An important component missing from *in vitro* analyses is time! Development and its principles are evident only to the keen and patient observer of seemingly ordinary events that could give extraordinary insights. Building a complex system successfully is only a part of the challenge- keeping it going and maintaining it requires a whole new set of rules and toolkit. Increased complexity requires more time for analysis, which is a luxury one cannot generally afford. Use of stem cells as a model provides a partial, yet crucial solution to this.

The early stages of mammalian development are characterized by the maintenance of a developmentally plastic, pluripotent stem cell population that eventually gives rise to all types of cells present in a mature organism through the process of differentiation. While this journey from a pluripotent stem cell to a cell with a differentiated fate encompasses a series of events, studying the first few stages of maintenance to loss of pluripotency can help in predicting the role of genes during development. The OCIAD family of proteins are clinically important, yet, their normal function remains unknown. Early studies showed their importance in stem cell maintenance, suggesting a developmentally important role. OCIAD1/Asrij function has been closely associated with the maintenance of pluripotency of mESCs, which asserts its significance in mammalian development. Overexpression of OCIAD1 in mESCs increases proliferation, with a greater percentage of cells in the S-phase of the cell cycle, compared to OCIAD1-depleted mESCs (Sinha *et al.*, 2013). Further, OCIAD1 levels directly correlate with stem cell self-renewal capacity. Leukemia inhibitory factor (LIF)-mediated activation of JAK-STAT signaling is absolutely essential for the maintenance of pluripotency in mESCs (Van Oosten *et al.*, 2012; Williams *et al.*, 1988). In the absence of exogenous LIF, *ociad1/asrij* transcript levels decreased as mESC differentiation proceeded, shedding light on the involvement of the protein during pluripotent stages of development. OCIAD1, through its endocytic localization, could facilitate STAT3 phosphorylation at tyrosine 705 in a LIF-independent manner, leading to STAT3 activation and further transcription of key pluripotency markers - *oct3/4*, *sox2*, *nanog*, thereby promoting the state of pluripotency in these cells. Moreover, OCIAD1 negatively regulates phospho-Erk levels in mESCs, which, in turn, is essential to maintain ground-state plu-

ripotency (Sinha *et al.*, 2013).

Human embryonic stem cells (hESCs) represent the epiblast stage and hence differ from mESCs in their requirement of LIF-induced JAK-STAT signaling. JAK-STAT pathway activation via LIF induction is not sufficient to maintain hESCs in an undifferentiated state, highlighting the differences in molecular mechanisms that maintain pluripotency across these two systems (Daheron *et al.*, 2004). Hence, unsurprisingly, modulating OCIAD1 levels in hESCs showed no apparent effect on hESC pluripotency. However, OCIAD1-depleted hESCs were more poised towards differentiating into mesodermal lineages upon receiving external cues when compared with wild type (WT) and OCIAD1 overexpressing (OV) hESCs. Interestingly, OCIAD1 resides primarily in the mitochondria in hESCs, underpinning its association with mitochondrial function. OCIAD1 negatively regulates complex I activity of the electron transport chain, thereby pushing the hESCs to depend more on glycolysis than on oxidative phosphorylation, an established property of most stem cells (Shyh-Chang *et al.*, 2017). As most aspects of mitochondrial biology such as energy metabolism, morphology, reactive oxygen species (ROS) production are interrelated, OCIAD1 modulated hESCs also exhibit altered signatures of morphology and ROS levels. Additionally, OCIAD1 regulates hESC differentiation towards the mesodermal lineage by regulating various aspects of mitochondrial biology (Shetty *et al.*, 2018).

Signaling and cellular phenotype of OCIAD1/2 proteins

Cell signaling and cellular phenotypes are intricately connected and unraveling their regulation is crucial for understanding biological processes. OCIAD1/2 proteins are evolutionarily conserved and are also implicated in several pathological conditions. Hence, they regulate multiple signaling pathways in different species in a context-dependent manner. As mentioned earlier, Asrij regulates the Notch, JAK/STAT, Pvr and insulin signaling pathways, in order to maintain the hemocyte progenitor pool in *Drosophila* (Khadilkar *et al.*, 2014; Kulkarni, Khadilkar *et al.*, 2011; Sinha *et al.*, 2013). Asrij is also essential for mounting and modulating immune responses in *Drosophila* by regulating the Imd and Toll pathways, in association with ARF1 (Khadilkar, Ray *et al.*, 2017). A recent proteome analysis of the *asrij* mutant *Drosophila* lymph glands revealed additional pathways and processes that are under the control of the Asrij-ARF1 axis. Interestingly, 27% of the affected proteins have human homologs implicated in diseases (Sinha *et al.*, 2019b).

The relevance of insight gained from studies on *Drosophila* Asrij to human development and disease was further strengthened by the recent analysis of *asrij* null (knockout; KO) mice. Just as in *Drosophila* mutants of *asrij*, KO mice too develop a blood disorder (Sinha *et al.*, 2019a). Hematopoietic stem cell (HSC) quiescence is lost leading to increased stem and progenitor cell numbers and a myeloproliferative disorder. Though *Drosophila* lack true blood stem cells, the similarity of phenotypes such as hyperproliferation of posterior lobe progenitors and increased myeloid differentiation suggests homologous pathways and functions that can now be tested. In support of this, we found that the *Drosophila* lymph gland proteome indicated a key role for the ubiquitin-proteasome system in regulating hematopoiesis. Interestingly, *asrij* KO mice also showed increased accumulation of polyubiquitinated proteins. Importantly, this had a direct outcome on blood cell homeostasis. We showed that the COP9 signalosome subunit 5 (CSN5)-MDM2-p53 axis is deregulated in *asrij* KO HSCs resulting in accelerated degradation of p53, increased proliferation, reduced apoptosis and loss of HSC quiescence. Additionally, Akt and STAT5 signaling are hyperactivated (Sinha *et al.*, 2019a).

Mouse Asrij/OCIAD1 positively regulates JAK/STAT pathway by promoting STAT3 activation and negatively regulates Erk signaling pathway to maintain pluripotency in mESCs (Sinha *et al.*, 2013). However, similar studies are yet to be

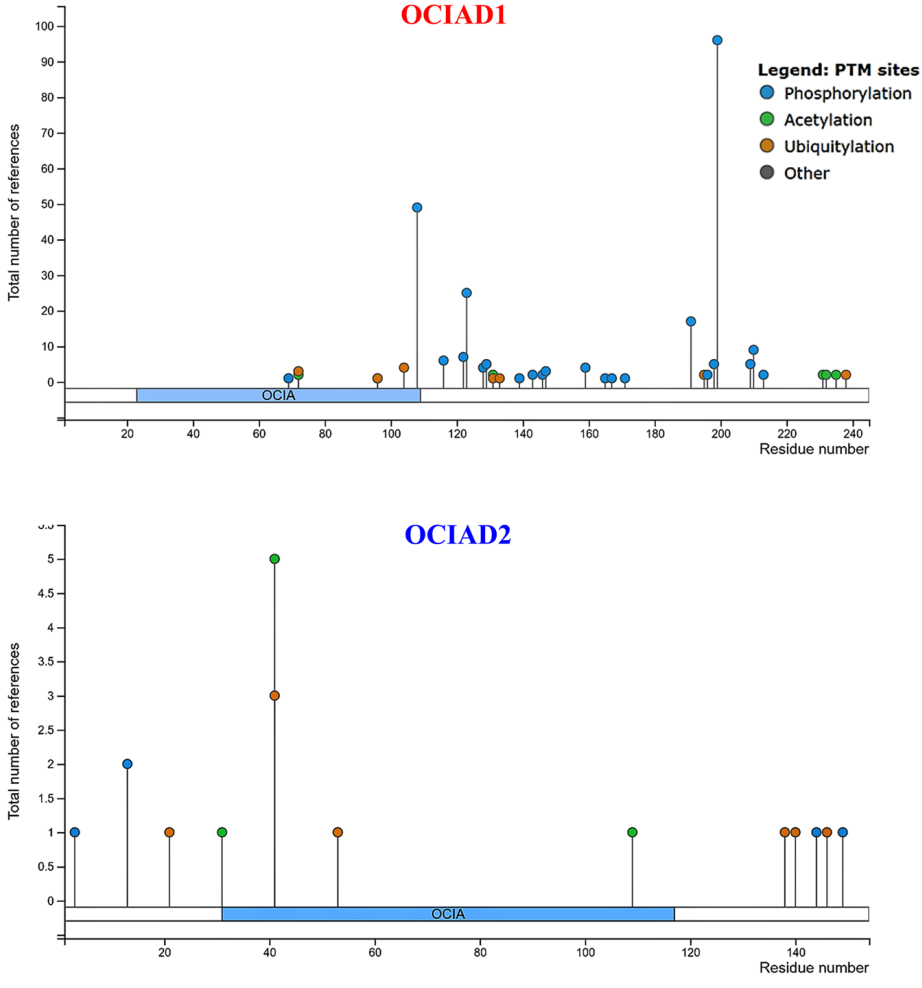


Fig. 6. Experimentally identified post-translational modifications of OCIAD1/2 proteins along their protein sequence as available in PhosphositePlus database (Hornbeck *et al.*, 2015). *The color code for the types of modifications is represented in the legend.*

conducted in hESCs. Lysophosphatidic acid (LPA)-mediated signaling acts upstream to OCIAD1/Asrij in mESCs, hESCs and ovarian cancer cells (Sengupta *et al.*, 2008; Shetty *et al.*, 2018; Sinha *et al.*, 2013). OCIAD1 affects cellular adhesion through components of the extracellular matrix in an LPA-dependent manner, while increasing the functional implication of β1-integrin in the above process. OCIAD1 phosphorylation on certain serine residues was also suggested to be a downstream effect of LPA stimulation, although the importance of this is unclear (Wang *et al.*, 2010).

OCIAD2 also promotes activation of STAT3 of JAK/STAT pathway and is required for migration and invasion but not proliferation in HEK293 cells. Nevertheless, overexpression of OCIAD2 did not lead to increased cell proliferation or migration (Sinha *et al.*, 2018). This may be a cell- and context-specific function of OCIAD2, since it shows the opposite effect in hepatocellular carcinoma (HCC) cells and patients, by downregulating Akt, FAK and MMP9 mediated signaling (Wu *et al.*, 2017). This suggests differential roles of OCIAD2 based on its level of expression, as in general, its expression is higher in cancerous tissues. OCIAD2 enhances the activity of gamma-secretase complex to produce amyloid-β fibrils from Aβ precursor proteins in Alzheimer's disease, by interacting with Nicastrin and C99 proteins. It does not regulate Notch signaling. Interestingly, OCIAD1 does not interact with Nicastrin and C99 proteins (Han *et al.*, 2014). TGF-β and BMP signaling pathways act upstream to OCIAD2 as shown in peripheral blood mononuclear cells of mouse and humans (Classen *et al.*, 2010), and in hair follicles of Liaoning Cashmere goat (Jin *et al.*, 2018). Thus, OCIAD1/2 proteins control and are controlled by cross-regulatory signaling pathways. Further studies will help elucidate the mechanistic role of these proteins, which can be tailored or adapted in a context-specific manner. The known cellular effects of OCIAD1/2 proteins are summarized in Fig. 7.

The detailed mechanism of how OCIAD1/2 proteins affect STAT3 activation is not clear. OCIAD1/Asrij was shown to colocalize with STAT3 on Rab5⁺ endosomes. Since OCIAD1 is not known to have any enzymatic activity but has an unstructured region, it likely acts as a scaffold to facilitate the activation of STAT3. Recently, OCIAD2 was also shown to be essential for STAT3 activation. Both OCIAD1 and OCIAD2 interact with each other and localize to Rab5⁺ early endosomes suggesting a possible scaffolding function for OCIAD2 as well (Sinha *et al.*, 2018; Sinha *et al.*, 2013).

Location, location, location! Endosome-mitochondria crosstalk

An important outstanding question in biology is how intracellular organelles coordinate molecular processing, signal generation and regulation of the cell state. While our understanding of this process continues to evolve, unraveling how the intricate yet dynamic and overlapping molecular signaling networks generated at various intracellular locations coordinate specific developmental processes remains a challenge. It is becoming increasingly clear that endogenous, intracellular signaling regulates organelle function, dynamics and replication; and additionally, organelles also play an active role in initiating and relaying signals (Gough, 2016). Recent studies show that control of signaling networks extends beyond soluble cytosolic proteins and transcription factors and that membrane-bound organelles such as endosomes, mitochondria, endoplasmic reticulum (ER), Golgi body and nucleus along with components of the vesicular transport machinery can affect spatial and temporal control of cell signaling (Ghibelli *et al.*, 2012).

Several databases (www.genecards.org/, www.uniprot.org/, compartments.jensenlab.org/) and previous reports on OCIAD1 (Kulkarni, Khadilkar *et al.*, 2011; Mukhopadhyay *et al.*, 2003; Shetty *et al.*, 2018; Sinha *et al.*, 2013) show that this molecule is primarily localized to early (Rab5⁺) endosomes and mitochondria. In hESCs, OCIAD1 shows high colocalization with mitochondria and little or no colocalization with endosomes (Rab5⁺ and Rab11⁺),

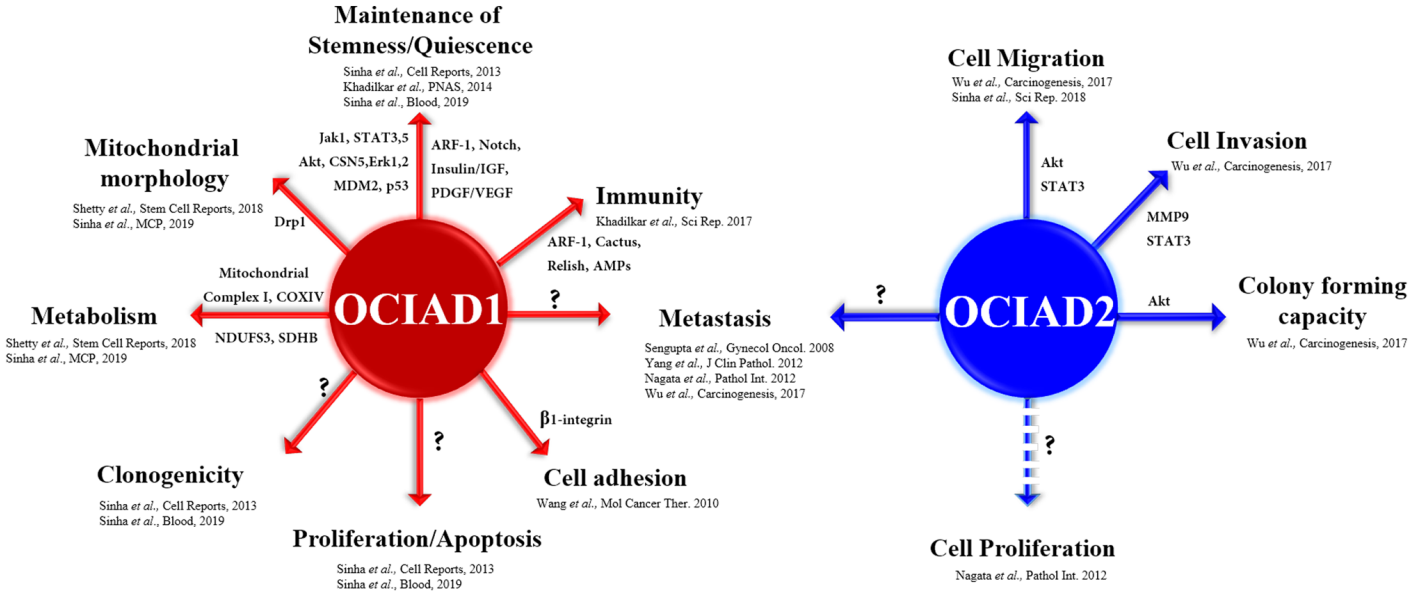


Fig. 7. Cellular processes mediated by OCIAD1 (red) and OCIAD2 (blue). *Key proteins involved in mediating the cellular processes are indicated, where known, along with references. '?' indicates that the molecular mediators are yet to be identified. Solid lines represent the data with evidence, dotted line represents speculation or data to be validated.*

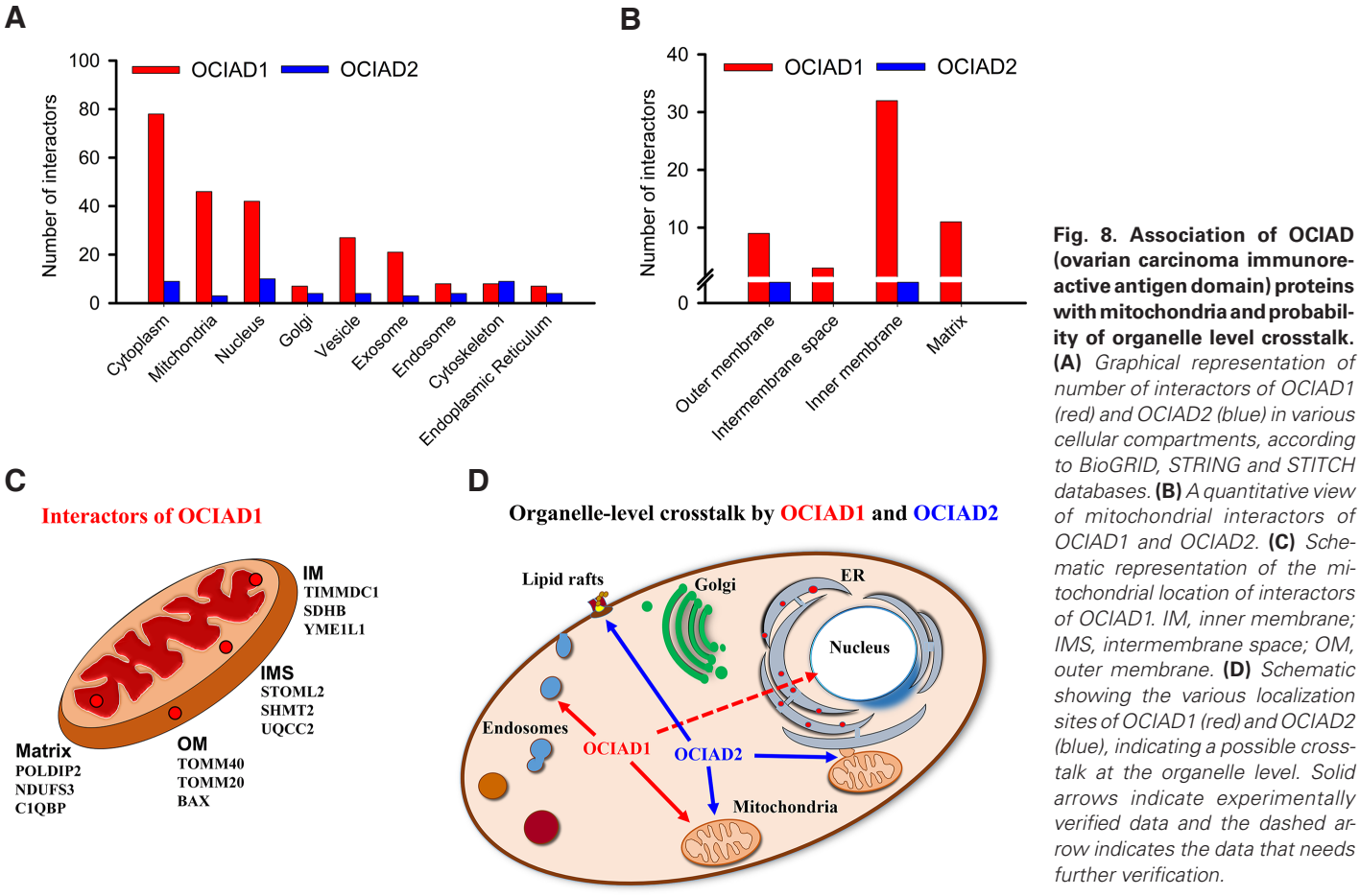


Fig. 8. Association of OCIAD (ovarian carcinoma immunoreactive antigen domain) proteins with mitochondria and probability of organelle level crosstalk. **(A)** *Graphical representation of number of interactors of OCIAD1 (red) and OCIAD2 (blue) in various cellular compartments, according to BioGRID, STRING and STITCH databases.* **(B)** *A quantitative view of mitochondrial interactors of OCIAD1 and OCIAD2.* **(C)** *Schematic representation of the mitochondrial location of interactors of OCIAD1. IM, inner membrane; IMS, intermembrane space; OM, outer membrane.* **(D)** *Schematic showing the various localization sites of OCIAD1 (red) and OCIAD2 (blue), indicating a possible crosstalk at the organelle level. Solid arrows indicate experimentally verified data and the dashed arrow indicates the data that needs further verification.*

Golgi bodies and lysosomes (Shetty *et al.*, 2018).

OCIAD2 is also predicted to localize to mitochondria and endosomes, which was verified in HeLa (Han *et al.*, 2014), HEK293 (Sinha *et al.*, 2018) and A549 cells (Sakashita *et al.*, 2018). OCIAD2 localizes to lipid rafts during the process of forming active gamma-secretase complex and also to mitochondria-associated ER membrane (MAM) (Han *et al.*, 2014). Recently, OCIAD1/2 proteins were shown to colocalize and interact with each other via their OCIA domain (Sinha *et al.*, 2018) and this explains the overlapping subcellular localization.

Localization of a protein, when coupled to the knowledge of its interacting proteins can aid in understanding its function. OCIAD1/2 proteins, as mentioned above, localize mainly to early endosomes or mitochondria, in a context-dependent manner and interact with specific proteins at their location. In hESCs, OCIAD1 interacts with mitochondrial proteins such as TIMMDC1, NDUFS3, COXIV and ATP5A (Shetty *et al.*, 2018). Interestingly, the *Drosophila* lymph gland proteome revealed mitochondrial metabolism and oxidative phosphorylation as key deregulated categories in *asrij* mutants (Sinha *et al.*, 2019b). Thus, further investigation of the role of Asrij in *Drosophila* blood cell mitochondria is likely to shed light on its metabolic role in mammalian systems too.

In HEK293 cells, OCIAD1/Asrij, interacts with STAT3 on Rab5^{+} endosomes (Sinha *et al.*, 2013). In mouse CD150^{+} HSCs, Asrij colocalizes with Rab4, Rab5, Cox4 and CSN5, but not with Rab11 (Sinha *et al.*, 2019a). Further, Asrij interacts with and sequesters CSN5 via its N-terminal domain and controls the ubiquitin-mediated degradation of p53 (Sinha *et al.*, 2019a). On the other hand, OCIAD2 also interacts with STAT3 (Sinha *et al.*, 2018) and also with mitochondrial proteins (Tables 1 and 2). Further, it interacts with and regulates the stability of Nicastrin, one of the four components of the gamma-secretase enzyme, via the peptide sequence ^{134}CEXCK^{138}, located in its hydrophilic C-terminal region (Han *et al.*, 2014). Since OCIAD1 and OCIAD2 interact with each other via their OCIA domain, this raises the possibility that they might have highly conserved roles and their functions may be cooperative or redundant with common regulators (Sinha *et al.*, 2018).

Apart from the above-reported studies, databases like BioGRID, STRING and STITCH suggest that OCIAD proteins may interact with many other proteins and play a versatile cellular role. The various interactors of these proteins, segregated as per the cellular compartment using g:Profiler (https://biit.cs.ut.ee/gprofiler/), are listed in Tables 1 and 2, which is quantitatively represented in Fig. 8A. Since both OCIAD proteins localize to mitochondria, we quantitatively represent the number of mitochondrial interactors (Fig. 8B) and their spatial distribution within the mitochondria (Fig. 8C). A comparative analysis shows that fewer interactors are reported for OCIAD2 as compared to OCIAD1, both in humans and mice.

The importance of organelle level crosstalk in the regulation of cell biology is being increasingly recognized. Very recently, the "kiss and run" interaction between endosomes and mitochondria has been reported, confirming a direct interaction between these

subcellular compartments (Das *et al.*, 2016). Since OCIAD proteins localize to these subcellular compartments and interact with proteins that localize dynamically (e.g. STAT3), it is highly likely that OCIAD1/2 proteins might be involved in mediating organelle crosstalk and in regulation of signaling pathways, probably by their scaffolding function (Sinha *et al.*, 2013) (Fig. 8D).

Implications in disease

Although the developmental role of OCIAD1/2 proteins has come to light from studies on mESCs and hESCs, the association with various diseases is also intriguing. OCIAD1 owes its name to the fact that it was identified in patients suffering from metastatic ovarian cancers, contemporary to the studies in the developmental context. Expression of OCIAD1 is higher in metastatic ovarian cancer tissues as compared to their benign counterparts. Additionally, it plays a role in secondary recurrence of the disease in an LPA-dependent manner and also promotes metastasis (Sengupta *et al.*, 2008; Wang *et al.*, 2010). In contrast, in thyroid cancer, OCIAD1 does not promote metastasis (Yang *et al.*, 2012).

Asrij/OCIAD1 also regulates immune function in *Drosophila* (Khadilkar, Ray *et al.*, 2017) and interestingly is associated with multiple infectious diseases (Cheng *et al.*, 2012; Kammula *et al.*, 2012). A brain transcriptome study, identified OCIAD1 as a key interactor of the HIV-Nef protein, along with other membrane proteins (Kammula *et al.*, 2012). Upon infection by *Toxoplasma gondii*, OCIAD1 interacts with Rop18 kinase to facilitate pathogenesis (Cheng *et al.*, 2012). Analysis of the lymph gland proteome indicated that several human diseases may be implicated upon Asrij perturbation such as mitochondrial disorders, myelination dysregulation, hypertrophic cardiomyopathy, among others (Sinha *et al.*, 2019b). In mice, absence of Asrij triggers loss of quiescence and myeloid-biased proliferation of HSCs. While Asrij is required for the regulated production of common lymphoid progenitors (CLPs) and B lymphocytes in mice, its role in immune homeostasis remains to be explored. However, importantly, *asrij* KO mice are

TABLE 1

LIST OF INTERACTORS OF OCIAD1 AND OCIAD2 IN HUMANS AS PER BIOGRID, STRING AND STITCH DATABASES

Cytoplasm		Mitochondria		Nucleus		Golgi		Vesicle		Exosome		Endosome		Cytoskeleton		Endoplasmic reticulum	
OCIAD1	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**
ABCD3	APP	ABCD3	ILF3	ATAD1, 3A	APP	ARF1, 3, 4, 5	APP	ARF1, 3, 4, 5	APP	ARF1, 3, 4, 5	APP	ARF1	APP	AKAP14	APP	ATP2A2	APP
AFG3L2	ARFIP1	AFG3L2	NDUFC1	ATF7	COMMD8	CLU	ARFIP1	ATP2A2	MMGT1	BAX	NCSTN	EXOC8	MMGT1	CAPZB	MCM2	BAX	MMGT1
AKAP14	COMMD8	ATAD3A	UBC	ATP2A2, 5H, 5O	GABRB1	GNAS	MMGT1	BAX	NCSTN	BLVRA	UBC	FLOT1	UBC	CCT4	SYNPO2	CLU	NCSTN
ARF1, 3, 4, 5	ILF3	ATP5H, 5J, 50		BAX	ILF3	UST	NCSTN	BLVRA	UBC	CAPZB		LPAR1		CLU		CTDNEP1	UBC
ATAD1, 3A	MMGT1	BAX		C1QBP	MCM2			CAPZB		CCT4		RAB5C		FLOT1		CYB5R3	
ATF7	NCSTN	C1QBP		CCT4	MMGT1			CCT4		CHCHD3		UBC		SHMT2		TMEM67	
ATP2A2, 5H, 5J, 5O	NDUFC1	CHCHD3		CHCHD3	RNF2			CHCHD3		CLU		VPS25		STOML2		UBCA	
BAX	SYNPO2	CISD1		CLU	SYNPO2			CLU		CPVL				TMEM67			
BLVRA	UBC	CLPB		CTDNEP1	TIGD4			CPVL		CYB5R3							
C1QBP		CLU		EGLN3	UBC			CYB5R3		ECH1							
CAPZB		COX6A1, C, 7A2, 18		ELAVL1				ECH1		EXOC8							
CCT4		CYB5R3		FLOT1				EXOC8		FLOT1							
CHCHD3		ECH1		FUNDC2				FLOT1		GNAS							
CISD1		FUNDC2		GNAS				GNAS		PCBP1							
CLPB		IMMT		IPPK				LPAR1		RAB5C							
CLU		MTCH2		MOV10				PCBP1		RALB							
COX6A1,6C, 7A2, 18		MTX1		MTCH2				RAB5C		SHMT2							
CTDNEP1		NDUFA4, A9, B1,		NDUFA9, B1, S2, S3				RALB		SLC25A1							
CYB5R3		NDUFS2, S3, S8, V1		NXF1				RGL4		UBC							
ECH1		PMPCB		PCBP1				SHMT2		VPS25							
EGLN3		POLDIP2		POLDIP2				SLC25A1									
ELAVL1		SCO1		SDHB				TMEM67									
EXOC8		SDHB		SHMT2				UBC									
FLOT1		SHMT2		SLC25A1				VPS25									
FUNDC2		SLC25A1		STOML2													
GNAS		STOML2		SYCE3													
GNAT2		TIMM44, 50, DC1		TIMM50, DC1													
IMMT		TOMM5, 20, 40		UBC													
IPPK		UBC		UNK													
LANCL1		UQCC2		UQCC2													
LPAR1		USMG5		VPS25													
MOV10		YME1L1		XPO1													
MTCH2				YME1L1													
MTX1				ZNF629, 682													
NDUFA4, A9, B1,																	
NDUFS2, S3, S8, V1																	
NXF1																	
PCBP1																	
PMPCB																	
POLDIP2																	
RAB5C																	
RALB																	
RGL4																	
SCO1																	
SDHB																	
SHMT2																	
SLC25A1																	
STOML2																	
TIMM44, 50, DC1																	
TMEM67																	
TOMM5, 20, 40																	
UBC																	
UNK																	
UQCC2																	
USMG5																	
UST																	
VPS25																	
XPO1																	
YME1L1																	

'?' indicates the necessity of further validation to confirm the cellular compartment of interaction.

TABLE 2

LIST OF INTERACTORS OF OCIAD1 AND OCIAD2 IN MICE AS PER BIOGRID, STRING AND STITCH DATABASES

Cytoplasm		Mitochondria		Nucleus		Golgi		Vesicle		Exosome		Endosome		Cytoskeleton		Endoplasmic reticulum	
OCIAD1	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**	**OCIAD1**	**OCIAD2**
ARF1	ARFIP1	COX18	ETFDH	ATRAID	GABRB1	ARF1	ARFIP1	ARF1	NCSTN	CAPZB	---	ARF1	---	CAPZB	SYNPO2	---	NCSTN
ARF2	DDIT4L	TIMMDC1	LIAS	CSN5 (?)	SYNPO2	ARF2	NCSTN	ARF2				VPS25					
ARF3	ETFDH	VPS25	NDUFC1	TIMMDC1	TIGD4	ARF3		ARF3									
ARF4	GABRB1			VPS25		ARF 4		ARF4									
ARF5	LIAS			ZFP629		ARF 5		ARF5									
ARF5	NCSTN					UBAC1		ATRAID									
ATRAID	NDUFC1							CAPZB									
CAPZB	SYNPO2							UBAC1									
COX18								VPS25									
CSN5 (?)																	
TIMMDC1																	
UBAC1																	
VPS25																	

'?' indicates the necessity of further validation to confirm the cellular compartment of interaction.

viable, fertile and do not show any gross abnormalities, thereby providing a good model for understanding human myeloproliferative neoplasms (Sinha *et al*., 2019a).

OCIAD2 is expressed prominently in the areas of papillary proliferation, infiltration and stromal invasion of ovarian mucinous cancer (Nagata *et al*., 2012) and could be a better marker for malignancy detection, as compared to OCIAD1 and carcinoembryonic antigen (CEA). Patients of Alzheimer's disease (AD) and PDAPP mice (model for AD) show elevated expression of OCIAD2 in their brain tissues (Han *et al*., 2014). Interestingly, a recent study shows a positive correlation between the expression levels of OCIAD1 and disease severity in vulnerable brain areas and dystrophic neurites of the AD mouse model via the Aβ/GSK-3β-OCIAD1-BCL-2 axis (Li *et al.,* 2020). Increased OCIAD2 expression leads to blood vessel and lymphatic invasion, lymph node metastasis and directly correlates with clinical outcome in patients suffering from invasive lung adenocarcinoma (Sakashita *et al*., 2018). Nevertheless, this correlation of OCIAD2 expression seems to be cancer-specific. Low expression of OCIAD2 has also been linked to tumor malignancy. Aberrant DNA methylation coupled to downregulation of *ociad2* expression is implicated in the pathogenesis of paediatric hepatoblastoma (Honda *et al*., 2016). In general, aging and chronic inflammation are known to cause aberrant DNA methylation and lead to cancers such as gastric cancer, colitic cancer and hepatocellular carcinoma (HCC) (Chiba *et al*., 2012). *Ociad2* is hypermethylated leading to lower expression and associated with metastasis in hepatoblastoma, ovarian cancer, glioblastoma and HCC (Kim *et al*., 2010; Matsumura *et al*., 2012; Noushmehr *et al*., 2010). Additionally, in hepatoblastoma patients, lower expression of *ociad2* also causes hepatic vein invasion, poor prognosis and survival (Honda *et al*., 2016). *Ociad2* mRNA is overexpressed in gliomas with poor prognosis (Nikas, 2014, 2016; Zhang *et al*., 2014). The significant downregulation of *ociad2* in HCC tissues compared to that of the surrounding non-tumor tissues is due to the hypermethylation of TSS 200, TSS 1500, 5'-UTR, first exon and gene body of *ociad2* (Wu *et al*., 2017). On the other hand, hypomethylation of *ociad2* is detected in chronic lymphocytic leukemia (Kulis *et al*., 2012). Malignant pleural mesothelioma, triple-negative breast cancer mediated brain-metastasis and BRAF WT melanoma are some more clinical situations with lower expression of OCIAD2 (Gueugnon *et al*., 2011; Su *et al*., 2012). Varying expression of OCIAD1/2 proteins in different cancers indicates their context-specific expression and probably their function.

Blood relatives: of mice, women and fruit flies

When examined in the context of evolution, the comparative developmental analysis provides a potent mix of information that helps analysis of genes and their function. Here we review the journey from discovery to decoding that helped decipher the roles of the OCIAD family that is conserved in function and sequence across multiple species and to understand their clinical relevance. Mutations that affect both alleles or parents are likely to be lethal at pre-implantation or very early in development and hence untractable. In *Drosophila*, *asrij*/*ociad1* expression is restricted to tissues that are not essential for viability- as there is only one gene (Inamdar, 2003). Interestingly, mice depleted of *ociad1/asrij* are not lethal, in spite of its requirement for maintaining pluripotency in mESCs (Sinha *et al*., 2019a; Inamdar *et al*., 2018; Sinha *et al*., 2017). However, Asrij plays a critical role in maintaining quiescence of HSCs and balancing their differentiation to the myeloid and lymphoid lineages (Sinha *et al*., 2019a). Further studies will shed light on the conserved function of OCIAD proteins and elucidate their role in hematopoiesis across species. The Leukemia MILE study (Bagger *et al*., 2019) shows that myeloid expansion disorders (e.g. Acute myeloid leukemia (AML), Chronic myelocytic leukemia (CML) and myelodysplastic syndrome (MDS)) are associated with extremely low levels of p53 and OCIAD1 expression, whereas CSN5 expression is upregulated in these disorders. This resemblance to the mouse KO phenotypes highlights the utility and significance of our evo-devo approach to study the OCIAD family of proteins.

Summary: looking ahead

Initially identified in ovarian cancers, OCIAD family proteins, were later found to be misexpressed in many other human cancers and diseases. It is very interesting to note that the expression of *ociad2* is low in normal human tissues compared to *ociad1*, however, this is not the case in many cancers. A tightly regulated balance between expression levels of *ociad1* and *ociad2* may be essential for the prevention of cancers. While mechanisms regulating this balance are being addressed, *ociad1* and *ociad2* could nevertheless serve as biomarkers for cancer classification leading to better prognosis and treatment. While vertebrate *ociad* genes are contiguous, their opposite orientation suggests that their proximal regulatory sequences are independent, though they may share common enhancers and epigenetic regulators. Despite the fact that both OCIAD1 and OCIAD2 localize to mitochondria, the protein-protein interaction databases show fewer interactors for OCIAD2. A deeper analysis of their individual and mutual regulation is essential for understanding their importance in development and disease.

A large number of diseases are now being recognized as having links to subtle developmental aberrations- especially neurological and behavioral disorders (Inzitari *et al*., 2008). This emphasizes the importance of encouraging research in developmental biology, for a better understanding of various pathological conditions. This is especially important for and feasible in India, where clinical studies in conjunction with basic research benefit from the availability of a large cohort of patients at various early and advanced stages of diseases. Vertebrate developmental biologists are a rare species in the country and in danger of becoming extinct unless actively propagated.

Acknowledgements

This work was funded by grants from the Indo-French Centre for the Promotion of Advanced Research, the Department of Biotechnology, Govt. of India, the Science and Engineering Research Board, the Department of Science and Technology, Govt. of India and the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore.

Competing interests

The authors declare no competing or financial interests.

References

ARAI, EMSON, MOUNTJOY, CARASSCO, & HEIZMANN. (1987). Loss of parvalbumin-immunoreactive neurones from cortex in Alzheimer-type dementia. *Brain Res* 418: 164-169.

BAGGER, KINALIS, & RAPIN. (2019). BloodSpot: a database of healthy and malignant haematopoiesis updated with purified and single cell mRNA sequencing profiles. *Nucleic Acids Res* 47: D881-D885.

CALO, MIGLIAVACCA, BAZAN, MACALUSO, BUSCEMI, GEBBIA*, et al.,* (2003). STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 197: 157-168.

CHENG, CHEN, CHEN, SHEN, SHEN, AN*, et al.,* (2012). Interactions between the ROP18 kinase and host cell proteins that aid in the parasitism of Toxoplasma gondii. *Acta Trop* 122: 255-260.

CHIBA, MARUSAWA, & USHIJIMA. (2012). Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. *Gastroenterology* 143: 550-563.

CLASSEN, MUTH, DEBEY-PASCHER, EGGLE, BEYER, MALLMANN*, et al.,* (2010). Application of T cell-based transcriptomics to identify three candidate biomarkers for monitoring anti-TGFbetaR therapy. *Pharmacogenet Genomics* 20: 147-156.

DA CRUZ, XENARIOS, LANGRIDGE, VILBOIS, PARONE, & MARTINOU. (2003). Proteomic analysis of the mouse liver mitochondrial inner membrane. *J Biol Chem* 278: 41566-41571.

DAHERON, OPITZ, ZAEHRES, LENSCH, ANDREWS, ITSKOVITZ-ELDOR*, et al.,* (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* 22: 770-778.

DAS, NAG, MASON, & BARROSO. (2016). Endosome-mitochondria interactions are modulated by iron release from transferrin. *J Cell Biol* 214: 831-845.

GHIBELLI, & GRZANKA. (2012). Organelle cross-talk in apoptotic and survival pathways. *Int J Cell Biol* 2012: 968586.

GOUGH. (2016). Signaling proteins in the spotlight. *Sci Signal* 9: eg8.

GUEUGNON, LECLERCQ, BLANQUART, SAGAN, CELLERIN, PADIEU*, et al.,* (2011). Identification of novel markers for the diagnosis of malignant pleural mesothelioma. *Am J Pathol* 178: 1033-1042.

HAN, JUNG, JANG, KAM, CHOI, KIM*, et al.,* (2014). OCIAD2 activates gamma-secretase to enhance amyloid beta production by interacting with nicastrin. *Cell Mol Life Sci* 71: 2561-2576.

HONDA, MINATO, SUZUKI, FUJIYOSHI, MIYAGI, HARUTA*, et al.,* (2016). Clinical prognostic value of DNA methylation in hepatoblastoma: Four novel tumor suppressor candidates. *Cancer Sci* 107: 812-819.

HORNBECK, ZHANG, MURRAY, KORNHAUSER, LATHAM, & SKRZYPEK. (2015). PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res* 43(Database issue): D512-D520.

INAMDAR. (2003). *Drosophila* asrij is expressed in pole cells, trachea and hemocytes. *Dev Genes Evol* 213: 134-137.

INAMDAR, SINHA, VENKATA, ABE, KIYONARI, & VIJAYRAGHAVAN. (2018). Loss of Asrij/OCIAD1 in Mice Promotes Hematopoietic Stem and Progenitor Cell Expansion Due to Increased CSN5-Mediated P53 Degradation. *Exp. Hematol.* 64: S73.

INZITARI, POZZI, FERRUCCI, CHIARANTINI, RINALDI, BACCINI*, et al.,* (2008). Subtle neurological abnormalities as risk factors for cognitive and functional decline, cerebrovascular events, and mortality in older community-dwelling adults. *Arch Intern Med* 168: 1270-1276.

JIN, ZHANG, CHU, PIAO, PIAO, & ZHAO. (2018). Cashmere growth control in Liaoning cashmere goat by ovarian carcinoma immunoreactive antigen-like protein 2 and decorin genes. *Asian-Australas J Anim Sci* 31: 650-657.

JUNDT, ACIKGOZ, KWON, SCHWARZER, ANAGNOSTOPOULOS, WIESNER*, et al.,* (2008). Aberrant expression of Notch1 interferes with the B-lymphoid phenotype of neoplastic B cells in classical Hodgkin lymphoma. *Leukemia* 22: 1587-1594.

KAMMULA, MOTTER, GORGELS, JONAS, HOFFMANN, & WILLBOLD. (2012). Brain transcriptome-wide screen for HIV-1 Nef protein interaction partners reveals various membrane-associated proteins. *PLoS One* 7: e51578.

KHADILKAR RJ, RAY A, CHETAN DR, *et al.*(2017). Differential modulation of the cellular and humoral immune responses in *Drosophila* is mediated by the endosomal ARF1-Asrij axis. *Sci Rep* 7: 118.

KHADILKAR, RODRIGUES, MOTE, SINHA, KULKARNI, MAGADI*, et al.,* (2014). ARF1-GTP regulates Asrij to provide endocytic control of *Drosophila* blood cell homeostasis. *Proc Natl Acad Sci USA* 111: 4898-4903.

KIM, WATKINSON, VARADAN, & ANASTASSIOU. (2010). Multi-cancer computational analysis reveals invasion-associated variant of desmoplastic reaction involving INHBA, THBS2 and COL11A1. *BMC Med Genomics* 3: 51.

KULIS, HEATH, BIBIKOVA, QUEIROS, NAVARRO, CLOT*, et al.,* (2012). Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet* 44: 1236-1242.

KULKARNI, KHADILKAR, MAGADI, & INAMDAR. (2011). Asrij maintains the stem cell niche and controls differentiation during *Drosophila* lymph gland hematopoiesis. *PLoS One* 6: e27667.

LEVY, & DARNELL. (2002). Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3: 651-662.

LI, WANG, CYKOWSKI, HE, LIU, CHAKRANARAYAN, *et al.* (2020). OCIAD1 contributes to neurodegeneration in Alzheimer's disease by inducing mitochondria dysfunction, neuronal vulnerability and synaptic damages. *EBioMedicine* 51:102569.

LUO, SOOSAIPILLAI, & DIAMANDIS. (2001). Molecular cloning of a novel human gene on chromosome 4p11 by immunoscreening of an ovarian carcinoma cDNA library. *Biochem Biophys Res Commun* 280: 401-406.

MATSUMURA, IMOTO, KOZAKI, MATSUI, MURAMATSU, FURUTA*, et al.,* (2012). Integrative array-based approach identifies MZB1 as a frequently methylated putative tumor suppressor in hepatocellular carcinoma. *Clin Cancer Res* 18: 3541-3551.

MILLE-HAMARD, BILLAT, HENRY, BONNAMY, JOLY, BENECH*, et al.,* (2012). Skeletal muscle alterations and exercise performance decrease in erythropoietin-deficient mice: a comparative study. *BMC Med Genomics* 5: 29.

MUKHOPADHYAY, DAS, & INAMDAR. (2003). Embryonic stem cell and tissue-specific expression of a novel conserved gene, asrij. *Dev Dyn* 227: 578-586.

NAGATA, KOBAYASHI, SAKATA, SATOMI, MINAMI, MORISHITA*, et al.,* (2012). Increased expression of OCIA domain containing 2 during stepwise progression of ovarian mucinous tumor. *Pathol Int* 62: 471-476.

NESTOROWA, HAMEY, PIJUAN SALA, DIAMANTI, SHEPHERD, LAURENTI*, et al.,* (2016). A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. *Blood* 128: e20-31.

NIGROVIC, GRAY, JONES, HALLGREN, KUO, CHALETZKY*, et al.,* (2008). Genetic inversion in mast cell-deficient (Wsh) mice interrupts corin and manifests as hematopoietic and cardiac aberrancy. *Am J Pathol* 173: 1693-1701.

NIKAS. (2014). A mathematical model for short-term vs. long-term survival in patients with glioma. *Am J Cancer Res* 4: 862-873.

NIKAS. (2016). Independent validation of a mathematical genomic model for survival of glioma patients. *Am J Cancer Res* 6: 1408-1419.

NOUSHMEHR, WEISENBERGER, DIEFES, PHILLIPS, PUJARA, BERMAN*, et al.,* (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* 17: 510-522.

PHILLIPS, ERNST, BRUNK, IVANOVA, MAHAN, DEANEHAN*, et al.,* (2000). The genetic program of hematopoietic stem cells. *Science* 288: 1635-1640.

PUJATO, KIEKEN, SKILES, TAPINOS, & FISER. (2014). Prediction of DNA binding motifs from 3D models of transcription factors; identifying TLX3 regulated genes. *Nucleic Acids Res* 42: 13500-13512.

SAKASHITA, SAKASHITA, MURATA, SHIBA-ISHII, KIM, MATSUOKA*, et al.,* (2018). High expression of ovarian cancer immunoreactive antigen domain containing 2 (OCIAD2) is associated with poor prognosis in lung adenocarcinoma. *Pathol Int* 68: 596-604.

SENGUPTA, MICHENER, ESCOBAR, BELINSON, & GANAPATHI. (2008). Ovarian cancer immuno-reactive antigen domain containing 1 (OCIAD1), a key player in ovarian cancer cell adhesion. *Gynecol Oncol* 109: 226-233.

SHETTY, KALAMKAR, & INAMDAR. (2018). OCIAD1 Controls Electron Transport Chain Complex I Activity to Regulate Energy Metabolism in Human Pluripotent Stem Cells. *Stem Cell Reports* 11: 128-141.

SHYH-CHANG, & NG. (2017). The metabolic programming of stem cells. *Genes Dev* 31: 336-346.

SINHA, BHEEMSETTY, & INAMDAR. (2018). A double helical motif in OCIAD2 is essential for its localization, interactions and STAT3 activation. *Sci Rep* 8: 7362.

SINHA, DWIVEDI, YENGKHOM, BHEEMSETTY, ABE, KIYONARI, VIJAYRAGHAVAN, & INAMDAR. (2019). Asrij/OCIAD1 suppresses CSN5-mediated p53 degradation and maintains hematopoietic stem cell quiescence. *Blood, 133*: 2385-2400.

SINHA, KHADILKAR, S, ROYCHOWDHURY SINHA, & INAMDAR. (2013). Conserved regulation of the Jak/STAT pathway by the endosomal protein asrij maintains stem cell potency. *Cell Rep* 4: 649-658.

SINHA, RAY, ABHILASH, KUMAR, SREENIVASAMURTHY, KESHAVA PRASAD*, et al.,* (2019). Proteomics of Asrij perturbation in *Drosophila* lymph glands for identi-

fication of new regulators of hematopoiesis. *Mol Cell Proteomics* 18: 1171-1182.

SINHA, VENKATA, ABE, KIYONARI, VIJAYRAGHAVAN, & INAMDAR. (2017). Asrij deficiency in mice allows enhanced CSN5-mediated p53 degradation causing aberrant hematopoietic stem cell expansion and a myeloproliferative disorder. *Mech. Dev.* 145: S172-S172.

STANFORD, CARUANA, VALLIS, INAMDAR, HIDAKA, BAUTCH*, et al.,* (1998). Expression trapping: identification of novel genes expressed in hematopoietic and endothelial lineages by gene trapping in ES cells. *Blood* 92: 4622-4631.

STRAUSBERG, FEINGOLD, GROUSE, DERGE, KLAUSNER, COLLINS, *et al.,* (2002). Mammalian Gene Collection Program Team. Generation and initial analysis of more than 15 000 full-length human and mouse cDNA sequences. *Proc Natl Acad Sci USA* 99: 16899–16903.

SU, VILGELM, KELLEY, HAWKINS, LIU, BOYD*, et al.,* (2012). RAF265 inhibits the growth of advanced human melanoma tumors. *Clin Cancer Res* 18: 2184-2198.

TAPIAL, HA, STERNE-WEILER, GOHR, BRAUNSCHWEIG, HERMOSO-PULIDO*, et al.,* (2017). An atlas of alternative splicing profiles and functional associations reveals new regulatory programs and genes that simultaneously express multiple major isoforms. *Genome Res* 27: 1759-1768.

VAN OOSTEN, COSTA, SMITH, & SILVA. (2012). JAK/STAT3 signalling is sufficient and dominant over antagonistic cues for the establishment of naive pluripotency. *Nat Commun* 3: 817.

WANG, MICHENER, BELINSON, VAZIRI, GANAPATHI, & SENGUPTA. (2010). Role of the 18:1 Lysophosphatidic Acid-Ovarian Cancer Immunoreactive Antigen Domain Containing 1 (OCIAD1)-Integrin Axis in Generating Late-Stage Ovarian Cancer. *Molec. Cancer Therap.* 9: 1709-1718.

WILLIAMS, HILTON, PEASE, WILLSON, STEWART, GEARING*, et al.,* (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336: 684-687.

WOLF, HAMEY, PLASS, SOLANA, DAHLIN, GÖTTGENS, *et al.,* (2019). PAGA: graph abstraction reconciles clustering with trajectory inference through a topology preserving map of single cells. *Genome Biol.* 20: 59.

WU, YANG, PENG, GUO, ZHAO, ZHAO*, et al.,* (2017). OCIAD2 suppressed tumor growth and invasion via AKT pathway in Hepatocelluar carcinoma. *Carcinogenesis* 38: 910-919.

YANG, CHEN, & LEE. (2012). Expression of NCAM and OCIAD1 in well-differentiated thyroid carcinoma: correlation with the risk of distant metastasis. *J Clin Pathol* 65: 206-212.

ZHANG, PINTO, WAN, WANG, BERG, OLIVA*, et al.,* (2013). Dysregulation of synaptogenesis genes antecedes motor neuron pathology in spinal muscular atrophy. *Proc Natl Acad Sci USA* 110: 19348-19353.

ZHANG, ZHAO, XIONG, & ZHOU. (2014). Pathway bridge based multiobjective optimization approach for lurking pathway prediction. *Biomed Res Int* 2014: 351095.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Definitive human and mouse hematopoiesis originates from the embryonic endothelium: a new class of HSCs based on VE-cadherin expression
Estelle Oberlin, Bouchra El Hafny, Laurence Petit-Cocault and Michèle Souyri
Int. J. Dev. Biol. 54: 1165 - 1173 (2010)
https://doi.org/10.1387/ijdb.103121eo

Embryonic origin of human hematopoiesis
Manuela Tavian, Katia Biasch, Lidia Sinka, Judith Vallet and Bruno Péault
Int. J. Dev. Biol. 54: 1061 - 1065 (2010)
https://doi.org/10.1387/ijdb.103097mt

Dissecting hematopoietic differentiation using the embryonic stem cell differentiation model
Tara L. Huber
Int. J. Dev. Biol. 54: 991 - 1002 (2010)
https://doi.org/10.1387/ijdb.103065th

Developmental hematopoiesis: historical background and perspectives. An interview with Nicole Le Douarin
Charles Durand and Thierry Jaffredo
Int. J. Dev. Biol. 54: 951 - 954 (2010)
https://doi.org/10.1387/ijdb.103154cd

Embryonic stem cells and transgenic mice in the study of hematopoiesis
SH Orkin
Int. J. Dev. Biol. 42: 927 - 934 (1998)
http://www.intjdevbiol.com/web/paper/9853823