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From solid roots to rustling leaves

E-cadherin and β-catenin across the branches of hematopoiesis in evolution

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Publication date
2024

Link to publication

Citation for published version (APA):
Krimpenfort, R. A. (2024). *From solid roots to rustling leaves: E-cadherin and β-catenin across the branches of hematopoiesis in evolution*. [Thesis, fully internal, Universiteit van Amsterdam].

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Chapter 1

General Introduction

Hematopoiesis

Hematopoiesis (hema=blood, poiein=to create) is the process in which all blood cells are generated. While white blood cells are important for the innate and acquired immune system, red blood cells are responsible for oxygen-carbon dioxide exchange in all tissues in the body. All blood cells derive from hematopoietic stem cells (HSC) in a process that is steered by intrinsic factors (transcription factors) and external factors (growth factors and hormones). During embryonic development, hematopoiesis starts in the yolk sac. This first wave is transient and known as primitive hematopoiesis. Definitive HSC are generated at the aorta-gonad-mesonephros (AGM) region and initially settle in the fetal liver to produce mature blood cells (1–5). Adult hematopoiesis primarily takes place in the bone marrow, but can, in emergency phases, also occur extramedullary in the spleen and liver. During embryogenesis and at early stages after birth, the HSC compartment proliferates. When the organism is full-grown, however, HSCs become quiescent and divide rarely to maintain genetic integrity. When an HSC divides it forms a new stem cell and a daughter cell that develops into a proliferative population of multipotent progenitors (MPP) . The HSC and early progenitors are often indicated as the hematopoietic stem- and progenitor compartment (HSPC). As the word implicates, the MPP has multilineage potential that develops into either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). A CLP ultimately generates NK-cells, B-cells, T-cells, crucial for the adapted immune system (6), whereas a CMP gives rise to granulocyte-macrophage progenitors (GMP's), which play a key role in the innate immune response and megakaryocyte-erythroid progenitor (MEP) (7). Granulocytes are subdivided in neutrophils (important for phagocytosis and chemotaxis), eosinophils (defense against parasites), and basophils and mast cells (both involved for inflammation, parasitic infections and allergic reactions) (8,9). Concurrently, a MEP develops into the megakaryocytic and into the erythroid lineage. Megakaryocytes, in turn, shed platelets, that are important for clot formation upon blood-vessel damage. Erythropoiesis involves expansion of the (pro) erythroblast compartment and differentiation into late erythroblasts that, during maturation, expulse their nucleus to enter the reticulocyte stadium, leaves the bone marrow into the blood stream and there matures into an erythrocyte, more commonly known as red blood cell (RBC) (7,10,11). The fate of a HSC was long seen as a tree with defined side branches, and once committed to a branch, the fate of a HSPC would be determined. However, since the development of modern techniques, such as single cell RNA sequencing, arguments are raised that there is in fact more plasticity during the HSPC programming than initially thought. Nowadays, hematopoiesis is rather seen as a landscape where lineages derive from a central pool of progenitors. The differences between the hematopoietic tree and landscape paradigms are described by Orkin and Zon (2008) (12), Dahlin et al. (2018) (13) and Monga et al. (2022) (14), among many others, and illustrated in figure 1. Additionally, some lineage fates have been revisited to lie closer to one another than previously reported. For example: where initially the basophil/mast cell lineage was hypothesized

to derive from the GMP, multiple single cell RNA studies using both mouse and human progenitor cells have shown the baso-mast cell fate decision to occur more closely to the erythroid lineage commitment (13,15–17)

In this thesis I will zoom into molecular aspects at the decision point between basophil and erythroid fate in mouse hematopoiesis in comparison to human, before focusing on the human differentiation process from MEP to erythrocyte: erythropoiesis and its regulation in response to anemia.

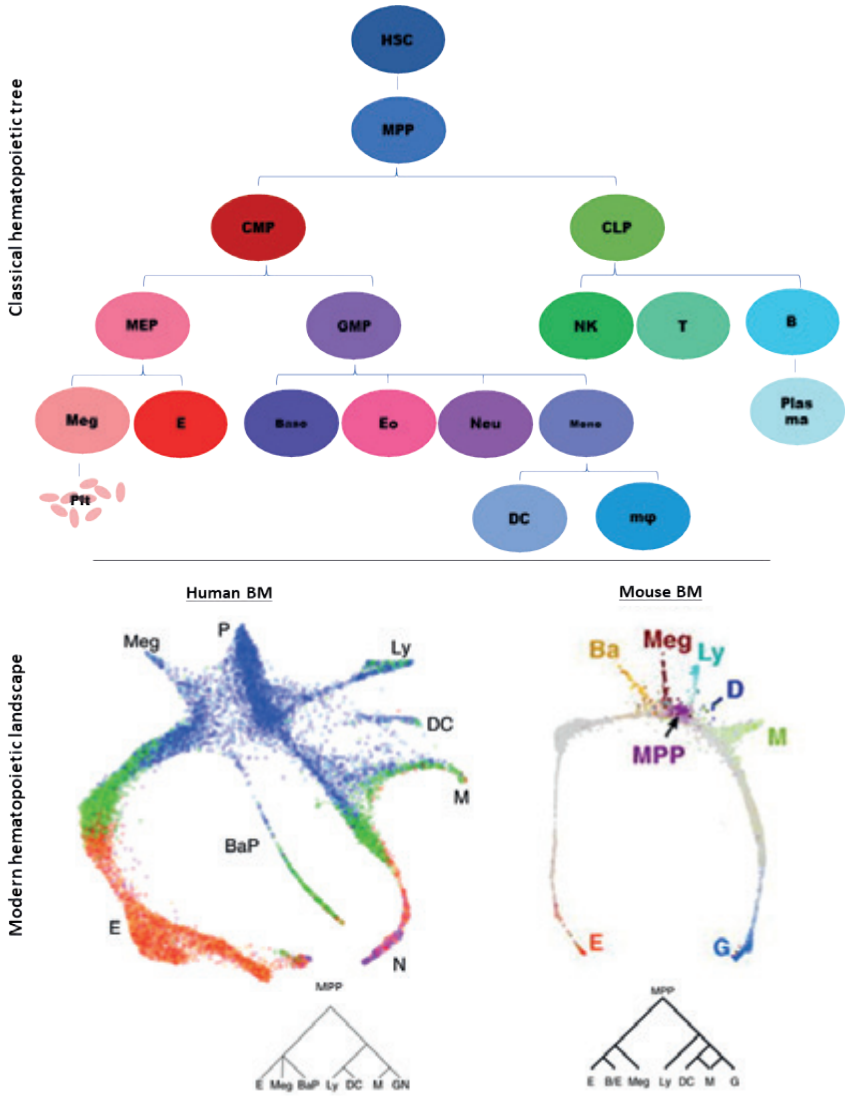

Figure 1. Hematopoiesis according to the classical tree and modern landscape models. Based on illustrations by Monga et al. (2022) (14) and Pellin et al. (2019) (17).

Erythropoiesis

With an estimated 25 trillion cells, RBCs make up for more than 80 % of all cells and are the most abundant cells of a human body (18). During homeostatic conditions, almost 2,5 million RBCs are produced per second in the human bone marrow across a tightly regulated continuum of developmental stages, starting from the hematopoietic stem cell (HSC), differentiating towards erythroid progenitors, which finally mature into erythrocytes (19). The process in which RBCs or erythrocytes are generated is called erythropoiesis. During erythropoiesis, erythroid precursors build up hemoglobin, the carrier of oxygen from the lungs to all tissues of the body and CO2 from the tissues back to the lungs.

Characteristics of the erythroid differentiation stages

The earliest differentiation stages of erythropoiesis are mainly characterized by in vitro colony formation capacity (20–23). In addition, differential phases can be identified using flow cytometry. The first stage is functionally described as Burst Forming Unit-erythroid (BFU-E), because in methylcellulose culture system they generate a large irregularly shaped colony or burst. The next stage is called Colony Forming Unit, which generates much smaller colonies. Upon erythroid commitment, the BFU-E starts downregulating CD34 and upregulating the transferrin receptor (TFR1/TFRC, CD71), essential for iron uptake, and integrin α4 (CD49d), that binds to VCAM on nurse macrophages. CD71 expression peaks from the CFU-E stage up to the basophilic erythroblast stage. When cells proceed to the Pro-Erythroblast (EB) stage, they begin building up hemoglobin protein and start expressing low levels of Glycophorin A (or CD235a). Next, in the Basophilic EB stage, CD235a expression increases and remains high. In the polychromatic stadium, CD71 expression decreases again, the nucleus begins to condensate, the EB become smaller and hemoglobin concentration increases. Lastly, in orthochromatic EB, the cell prepares to enucleate. After enucleation, a reticulocyte is ready to leave the bone marrow and further mature into the typical biconcave shape of the erythrocyte. For a graphical representation of erythroid differentiation, see figure 2 (24–28).

Erythroid development from the progenitor cell to a mature erythrocyte requires the activation of several transcriptional programs. Transcription factors that are essential for erythroid commitment include GATA1 and Kruppel like factor 1 (KLF1) (29–31). GATA1 in HSPC is low, but important for priming into the erythroid lineage. Towards the BFU-E stage, GATA1 expression increases at the cost of myeloid transcription factor PU.1, and its expression further increases until the basophilic erythroblast stage. GATA1 controls expression of many erythroid specific genes among which *KLF1*, *α- and β-globins*, and *EPO Receptor* expression that are essential for erythroid development (32–34). KLF-1 is also essential for erythropoiesis and is uniquely expressed in committed

erythroid cells. KLF1 controls expression of genes that are essential during the maturation of erythroblasts such as genes encoding α- and β-globins and several blood group antigens including Rh-cde, Duffy, Lutheran and P1 (35).

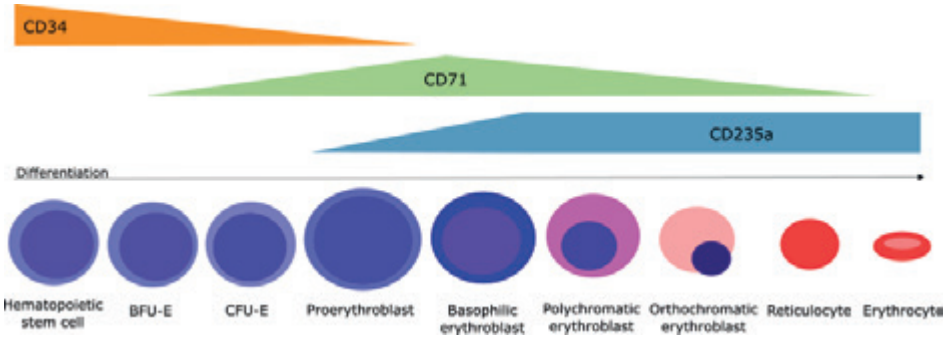

Figure 2. Differential stages from hematopoietic stem cell to mature erythrocyte.

Extracellular signals stimulating erythroid cell fate

During erythroid commitment and early erythroid development, hematopoietic progenitors are dependent on Thrombopoietin (TPO), interleukin 3 (IL3) and most importantly Stem Cell Factor (SCF), but not yet EPO (36–38). Binding of SCF to its cognate receptor cKIT (CD117), initiates a signaling cascade involving LYN and PI3 kinase, in turn activating mTOR and MAP kinase (39). Following erythroid commitment, progenitors become more and more dependent on EPO which is produced in the kidneys an can be increased upon HIF1a signaling during hypoxia (40). The EPO receptor (EpoR) is constitutively associated with the tyrosine kinase JAK2. Upon EPO binding, JAK2 is activated by cross phosphorylation, which results in phosphorylation of the EPO receptor and downstream signaling molecules including Stat5, important for cell survival and differentiation. Phosphorylated EpoR also recruits SHC, SOS, and GRB that together initiate the Ras-Raf-Mek1/2-Erk1/2 signaling cascade, as well as PI3kinase. The concerted signaling pathways stimulate proliferation and differentiation (41). There is a large overlap between EPO and SCF signaling, and the two signals collaborate and amplify each other, which improves cell survival and proliferation. However, while proliferation is dependent on both EPO and SCF, terminal differentiation relies on EPO signaling only.

Aside EPO and SCF signaling, a key signaling molecule during stress erythropoiesis is the glucocorticoid receptor (42). Glucocorticoids are expressed during hypoxia and stress and bind in the cytoplasm to their receptor which can transmigrate to the nucleus and activate transcription. Altogether, the GR cooperates with EPO and SCF to enhance erythroid cell survival and delay of differentiation, enabling progenitors to undergo more cell divisions before entering terminal

differentiation (43,44). For the most efficient erythroblast in vitro cultures, it is therefore beneficial to add all EPO, SCF and Dexamethasone (45).

Among the well-established effectors of both EPO and SCF is FOXO3, which expression increases towards the basophilic EB stage. FOXO3 is important for regulation of oxidative stress and driving autophagy of mitochondria and the nucleus at terminal differentiation (46,47). FOXO3 deficient erythroblast show hampered enucleation and reticulocyte maturation (48). FOXO3 mediated transcriptional activation is tightly regulated by EPO and SCF, which both activate Akt (also known as protein kinase B (PKB)), that phosphorylates FOXO3, anchoring it to 14-3-3 to retain it in the cytoplasm. Interestingly, there are specific FOXO target genes, such as Cited2 that are not inhibited but activated by a FOXO3-Stat5 complex when only EPO, but not SCF signals are transduced (49). Interestingly, FOXO proteins have been reported to interact with Wnt signal transducer β-catenin and compete with its canonical binding partner TCF for its transcriptional activation capacity (50,51). On the other hand, β-catenin accumulation and interaction with FOXO3 can, compared to the presence of FOXO3 alone, enhance transcription of FOXO target genes (52). It would be of great interest to unravel the relevance of this interaction during erythropoiesis.

Wnt/β-catenin signaling is an evolutionary conserved pathway that is important for regulation of differentiation and proliferation in many tissues. However, the role of Wnt/β-catenin signaling during erythropoiesis is largely unknown. It has been suggested to be important for regulation of proliferation and differentiation during stress erythropoiesis (53). Additionally, a dark side of Wnt/β-catenin has been revealed as unregulated signaling has been reported to be associated with the transformation of preleukemic condition Myeloid Dysplastic Syndrome (54,55). In chapter 2, I dedicated a textbox to an overview of the molecular events that occur in the canonical Wnt pathway. One should keep in mind that the molecular outcome is highly context and tissue dependent. In chapter 2, we addressed in a literature study how Wnt/b-catenin can be involved in healthy and malignant erythropoiesis.

Erythropoietic niche

In terms of the erythropoietic niche, two stages can be distinguished: early and terminal differentiation. Little is known about the early erythropoietic niche, from erythroid commitment by the MEP up until approximately the pro-EB stage. HSPCs depend on SCF, most predominantly produced by mesenchymal stem cells (MSCs) and/or endothelial cells. Comazetto et al (2019) suggest in a mouse study it is Leptin Receptor+ (LepR) cells which surround vascular endothelium and produce SCF (56). In humans, mesenchymal stem cells (MSCs) have been reported to express LepR and to give rise to LepR+ adipocytes, just like mouse LepR+ MSCs (57). Adipocytes have been reported to be an important source of SCF in human BM (58). Interestingly, SCF is produced as

membrane-bound and as soluble form. The Steel-dickie mouse model, which only produces the soluble but not membrane bound SCF, is anemic, indicating that erythroid progenitors are at least partially dependent on membrane-bound SCF (39). Therefore, it is likely that MEPs, BFU-E reside in close proximity to SCF producing cells. After the CFU-E stage, erythroid maturation is supported by a so-called nurse macrophage (59,60). The nurse macrophage, or central macrophage is characterized by expression of specific adhesion molecules among which CD169 (Siclec-1), VLA-α5 integrin and VCAM-1 (61). From approximately the CFU-E stage, during the terminal differentiation until after the enucleation, progenitors express Integrin α4 (CD49d). One nurse macrophage provides residence to a multitude of erythroblasts altogether forming the so-called erythroblast island (figure 3). The exact role of the nurse macrophage is still under investigation, but several interactions exist between the central macrophage and the erythroblasts. First, the central macrophage excretes several cytokines and signaling molecules such as IGF1, IL-6, TNF-α and Wnt ligands, that regulate EB proliferation, as well as factors such as TGF-β, prostaglandin J2 and E2 that stimulate erythroblast terminal differe5ntiation (62,63). Additionally, the macrophage supports iron uptake which is essential for hemoglobin synthesis during the differentiation of erythroblasts (64). Lastly, the orthochromatic erythroblasts is assisted during enucleation, resulting in the endocytosis of the expulsed pyrenocyte by the macrophage (65,66). The in vivo relevance of the EB-island becomes clear in animal models. Depletion of the CD169+ macrophages in mice on the long term results in mild anemia and elevated EPO levels (67). Moreover, support by the central macrophage shows to be of crucial relevance during stress, such as bleeding and hemolytic anemia, as mice lacking the essential integrins supporting EB-island interactions fail to enhance erythropoiesis and restore RBC levels (68). Paradoxically, depletion of CD169 macrophages in mouse models of defective erythropoietic diseases β-thalassemia and polycythemia vera significantly reverses pathologic phenotype by modulating erythropoiesis (67,69).

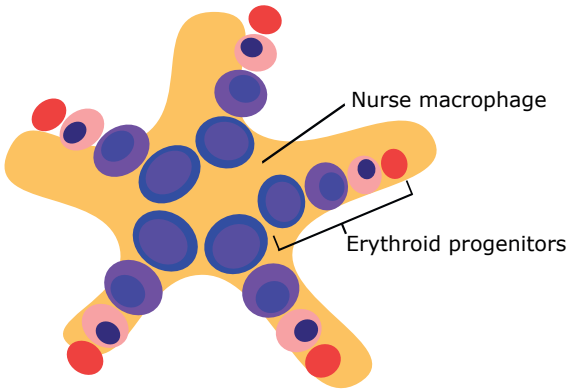

Figure 3. An erythroblast island consists of a central macrophage in support of surrounding erythroid progenitors.

Anemia

Anemia is a medical condition defined by a deficit of red blood cells (RBCs) or hemoglobin (70). This deficit results in shortage of oxygen transported to all tissues in the body. Patients suffering from anemia cope with a decreased oxygen delivery to all tissues resulting in acute to chronic symptoms with mild to fatal outcome. Symptoms of anemia vary from fatigue, weakness, lightheadedness to dyspnea, heart failure, and cognitive dysfunction. With a prevalence of approximately 1 in 5 persons worldwide, anemia is additionally a major burden on society (71).

Anemia is a symptom that can develop from several underlying conditions, such as active bleeding, poor RBC production or a shortened RBC life span. Iron deficiency is a major cause of anemia resulting in decreased synthesis of RBC that are smaller in size (microcytic anemia). Blood loss through trauma or due to chronic vascular damage of, for instance, the intestinal tract is also a major cause of anemia that may go hand-in-hand with iron deficiency. Alternatively, RBC production can be hampered due to nutritional deficiencies: vitamin B12, folate (causing macrocytic anemia), inflammation, kidney disease, bone marrow conditions, medication (e.g. cancer treatment) or hemoglobinopathy. Furthermore, defective RBC production may have a genetic background. Many hereditary forms of anemia are known, of which sickle cell disease and thalassemia are the most common monogenic disorder in the world. Lastly, shortened RBC life span as a consequence of hemolysis can cause anemia. This can be caused by non-immune mediated mechanisms (e.g. medication, enzyme deficiencies and many more) and auto- or allo-immune responses to blood group antigens.

Treatment of anemia will aim to eliminate the cause, for instance by iron supplementation and cessation of bleeding. Bone marrow transplantation and gene therapy are used for some patients with hereditary or acquired anemia. Despite the progress in curative therapies, many patients still require blood transfusions. All these treatments come with complications. Recurrent blood transfusion could result in iron overload as well as allo-immunisation. Bone marrow or stem cell transplantation may result in graft-versus-host-disease.

More modern treatment strategies include erythropoiesis stimulating agents (ESAs), such as EPO, and TGF-β ligand traps, such as Luspatercept and Sotatercept. These interventions stimulate the bone marrow to circumvent ineffective erythropoiesis and exploit the restored capacity to generate RBCs. ESAs emerged as alternative for blood transfusions in oncology patients and for Myeloid Dysplastic Syndrome (MDS) and Chronic Kidney Disease (CKD) (72–74). However, ESAs have been described to be limited to specific types of anemia and efficiency is often temporary due to occurrence of therapy resistance. Additionally, ESAs are suggested to come with elevated risk of thrombotic events and the stimulation of tumor growth in for example head and neck cancer (73,75,76). Hence, there is a need to further develop anemia treatment in order to reduce complications and improve response rate in anemia patients (77). Lastly, TGF-β ligand traps are

indicated for patients with for example low-risk MDS and thalassemia. These molecules aim to reverse overactivation of the Smad2/3 pathway, ultimately resulting in improvement of cell survival and erythroid maturation (78).

Stress erythropoiesis

Upon bleeding or hemolysis, hematopoietic tissue itself is still able to enact an emergency response and enhances erythropoiesis in order to restore hemoglobin levels in a process called stress-erythropoiesis. Stress erythropoiesis has been mostly been studied in mice which regulate erythrocyte turnover in the spleen. The Paulson lab uncovered in mice the SCF, hypoxia (HIF1α) and the BMP4 (bone morphogenetic protein 4), Hedgehog and Wnt signaling pathways to activate stress erythroid progenitors residing in the spleen upon acute anemia (53,79–85).

In contrast to mice, humans are able to regulate erythrocyte turnover in BM up to 20 times as compared to homeostatic erythropoiesis (86). Human BM largely consists of adipocytes which, in case of compensated anemia, are able to decrease, which space can then occupied by erythropoiesis (87). However, adipocytes are not as abundantly present in mouse BM. this could be a reason why mice predominantly regulate stress erythropoiesis extramedullary. Only in cases of severe and/or prolonged anemia, humans upregulate erythropoiesis in the spleen. Because of this difference between mice and humans in terms of stress erythropoiesis, molecular mechanisms regulating BM erythrocyte turnover are still poorly understood in man. Interestingly, a recent study by Zhang et al., (2019) introduced rats as superior models of stress erythropoiesis as they have hypocellular, adipocyte rich BM like humans, which undergoes major architectural changes during anemia as they primarily enhance erythropoiesis in the BM, before addressing the spleen (87). Additionally they reveal that some pathways, including the BMP4 pathway, do not overlap between the mouse spleen and rat BM during stress erythropoiesis (88). As the rat model for (stress) erythropoiesis is in its infancy, we are on the brink of the discovery of molecular mechanisms regulating erythroid turnover in human and rat BM.

E-cadherin

Because there are multiple erythroblasts present per single macrophage, it is not unlikely that EBs have specific interactions among themselves. Although more and more details are revealed about the interactions between macrophage and EB, little is known about the associations between the erythroblasts. Interestingly, in 1995, in an in vitro study with human EBs, adhesion molecule Epithelial cadherin (E-cadherin, CDH1) has been suggested to be involved in erythroid maturation (89). Since, there have been a handful of clinical reports using E-cadherin marker to distinguish erythroid from myeloid progenitors in bone marrow immunohistochemistry

sections (90,91). This is interesting, because E-cadherin is known to form homomultimers with neighboring cells. Through its molecular properties (described below) E-cadherin plays a crucial role in embryogenesis, tissue maintenance and wound healing (92–95). Additionally, E-cadherin is known as an important regulator of tumor progression in the mammary gland and many other types of cancer (96–100). Its loss is a hallmark for epithelial-to-mesenchymal transition (EMT), contributing to proliferation, motility, cell survival and ultimately metastasis. The question remains whether E-cadherin could play such a role for the erythroblast Island. In the section below, molecular properties of E-cadherin will be discussed in more detail.

E-cadherin is an evolutionary conserved transmembrane molecule that is the key role player in adherens junctions. Interestingly, E-cadherin is known to regulate cell behavior though several mechanisms (101). Extracellularly, E-cadherin forms homophilic interactions in both cis and trans that maintain rigidity of the cells within the tissue. Intracellularly, E-cadherin is bound by p120, α and β catenin. By binding α- and β-catenin, E-cadherin associates with the cytoskeleton, thereby directly regulating cell behavior. Several studies are devoted to changes in cadherin tension and tissue rigidity. E-cadherin clusters at the site of tension, and α-catenin dissociates from the E-cadherin/β-catenin complex. Simultaneously, the released α-catenin regulates Arp2/3 mediated polymerization of cytoskeleton protein F-actin (102,103).

B-catenin is the key signaling molecule of the canonical Wnt pathway. Therefore, E-cadherin has the potential to directly cross talk with the Wnt signaling cascade. B-catenin signaling is well-established to regulate stemcell-ness and proliferation during development, tissue recovery and tumor progression in many tissues including the bone marrow and erythroid progenitors (53,104–106). Molecular mechanisms by which E-cadherin can cross talk with the Wnt pathway have mainly been studied in epithelia. These studies paradoxically address both agonistic and antagonistic roles for E-cadherin. On one hand, E-cadherin sequesters β-catenin at the cytoplasmic membrane, where it is prevented from translocation into the nucleus. Previous studies have shown that loss of E-cadherin in breast cancer cells results in β-catenin/TCF mediated transcription and thereby contributes to cell motility and proliferation (107–111). In contrast, other studies describe that β-catenin stability and mediated transcription activation depends on E-cadherin. Here, E-cadherin functions as a vehicle, but also as a shield, protecting β-catenin from the destruction complex (112). Alternatively, E-cadherin has been shown to promote nuclear accumulation of b-catenin in response to mechanical tension (113). Upon mechanical tension, E-cadherin regulates activation of Yes-associated protein 1 (YAP1) and β-catenin, and thereby mediates cell cycle entry (114,115). Weis and Nelson elegantly reviewed past discoveries according the E-cadherin-catenin collaboration (116). Additionally, other studies demonstrate in several epithelial cell types that E-cadherin endocytosis and shuttling to and from the cytoplasmic membrane facilitates β-catenin as part of the complex to lift as cargo and translocate to the nucleus (112,117).

Aside interference with the Wnt cascade, E-cadherin homodimers can cross talk with receptor tyrosine kinases (RTKs), such as EGFR and IGF-1R. Here, E-cadherin binds and immobilizes the RTK within the cytoplasmic membrane, and thereby inhibits downstream signaling (118,119). Additionally, E-cadherin has been reported to directly interact with the PI3K/AKT axis (120,121) and thereby may control cell proliferation and survival. There can therefore be many parallels found with erythroid signaling pathways. Despite the extensive functional knowledge of E-cadherin in epithelial tissue, its molecular function in the erythroid lineage is unknown. However, if there are parallels between epithelia and EB islands in terms of E-cadherin and its association with the cytoskeleton and cross talk with signaling cascades such as the Wnt pathway, but also the SCF receptor c-KIT as an RTK, E-cadherin could be crucial for erythroid tissue regulation. It can be of great interest to learn more about its function during homeostatic, stress and ineffective erythropoiesis and to exploit its properties for treatment of anemia and/or erythroid neoplasms.

Scope of this thesis

In this thesis I will investigate the role of E-cadherin and β-catenin signaling in hematopoiesis in vivo and in vitro. First, the paradoxical role of the Wnt/β-catenin pathway in erythropoiesis will be addressed. On one hand, β-catenin signaling is essential for tissue development and recovery. On the other hand, β-catenin dysregulation is often associated with tumorigenesis and metastasis. A large number of studies have investigated the role of Wnt/β-catenin signaling pathway in embryonic stem cells, epithelia, among many other tissue types.

First, I investigate which animal model would be suitable for functional studies of the E-cadherin-β-catenin complex in erythropoiesis.

A straightforward animal model to investigate most biological processes in vivo, is the mouse. It is cheap and highly similar to humans. The E-cadherin- β-catenin complex is highly evolutionary conserved in epithelia from drosophila to mammals. Therefore, I address the evolutionary conservation of E-cadherin in the mouse bone marrow and potential phenotype of mouse hematopoietic knock out *in vivo* **(chapter 2)**. Interestingly, however, rats were recently indicated as preferable in vivo model for human stress erythropoiesis. Therefore, I also investigate E-cadherin and β-catenin expression and function in rat erythroid progenitors during steady state and anemia (**chapter 3)**. Besides these *in vivo* analyses, I examine the function of E-cadherin and in human EB *in vitro* cultures using CRISPR-Cas9 technology **(chapter 4)**. Subsequently, expression of Wnt pathway components in human erythroid progenitors is analyzed in order to assess their capability to process Wnt signals and regulate β-catenin signaling. Finally, I explore current opinions on Wnt/β-catenin and its regulation on healthy primitive and adult erythropoiesis, but also during ineffective erythropoiesis **(chapter 5)**.

Next, I look more closely at molecular mechanisms by which β-catenin can controls erythropoiesis **(chapter 6)**. Hereby we are interested in both canonical transcription factor TCF/LEF, but also non-canonical binding partner FOXO3. Therefore we will observe expression during steady-state and stress erythropoiesis in rat BM. In addition we will perform functional studies in erythroblast cultures using CRISPR-Cas9 generated FOXO3 KO erythroblasts.

In the last chapter (**chapter 7**), I discuss the technical challenges that are faced when modelling E-cadherin functions in hematopoiesis *in vivo* and *in vitro*. Additionally, a species comparison in terms of evolutionary conservation of E-cadherin expression in hematopoiesis in mouse, human and rat. Additionally, I speculate about E-cadherin and Wnt interaction and its potential implications for healthy and pathologic erythropoiesis.

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