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MOLECULAR MECHANISMS OF THE DEVELOPMENT AND REGULATION OF HEMATOPOIETIC CELLS

In vitro study

Thesis for the doctoral degree

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ABSTRACT

Hematopoiesis is the best-characterized differentiation process in the body. Hematopoietic stem cells (HSC) are multipotent stem cells that have the ability to self-renew and to differentiate into all the functional blood cell type and they are the only routine clinical used stem cells for treatment of variety of blood cell disease. Even though HSC transplantation has been applied in the clinic for decades, regulation of HSC self-renewal, differentiation, and development is still a major challenge. In order to understand the regulatory mechanisms of HSC, it is important to comprehend the origin and development of HSC. The embryonic stem cells (ESCs) are pluripotent and under specific condition can give rise to cells from all three germ layers. The process of differentiation of ESCs recapitulates the embryogenesis and it gives us a unique insight into developmental processes. Various transcriptional factors which regulate the self-renewal and differentiation of HSC were elucidated but the fully understanding of signaling cascades and origins of specific lineages is still considerable.

In this thesis, we focus on the p38α mitogen activated protein kinase (MAPK) and its involvement in establishment and regulation of the hematopoiesis. First of all is the examination of cell fate of ESCs with depletion of p38α (p38α−/−) in comparison to their wild type cells (p38α+/+). The one of distinction of p38α−/− is higher production of reactive oxygen species (ROS), which could influence the differentiation potential into specific germ layer. However, the overall ESC potential seems to be unchanged. Further, we revealed that p38α kinase is involved in the three steps in the process of hematopoietic differentiation. Firstly, the absence of p38α kinase led to the inhibition of hemangioblast formation during the first step of hematopoiesis. During differentiation, the mutation of p38α kinase led to the impairment of erythrocyte maturation. Concurrently, p38α kinase plays an important role in the differentiation of common myeloid precursor cells into granulocyte lineages.

Finally, we analyzed the role of p38α kinase in the differentiation of the hematopoietic cells from the fetal liver and bone marrow. The role of p38α varies between the hematopoietic progenitors from bone marrow and fetal liver and the differences differences exist in the hematopoietic differentiation from the ESCs. Thus, it is evident; that the p38α is involved in the hematopoietic differentiation at all levels of development.
ABSTRACT

Hematopoiesis is the best-characterized differentiation process in the body. Hematopoietic stem cells (HSC) are multipotent stem cells that have the ability to self-renew and to differentiate into all the functional blood cell type and they are the only routine clinical used stem cells for treatment of variety of blood cell disease. Even though HSC transplantation has been applied in the clinic for decades, regulation of HSC self-renewal, differentiation, and development is still a major challenge. In order to understand the regulatory mechanisms of HSC, it is important to comprehend the origin and development of HSC. The embryonic stem cells (ESCs) are pluripotent and under specific condition can give rise to cells from all three germ layers. The process of differentiation of ESCs recapitulates the embryogenesis and it gives us a unique insight into developmental processes. Various transcriptional factors which regulate the self-renewal and differentiation of HSC were elucidated but the fully understanding of signaling cascades and origins of specific lineages is still considerable.

In this thesis, we focus on the p38\(\alpha\) mitogen activated protein kinase (MAPK) and its involvement in establishment and regulation of the hematopoiesis. First of all is the examination of cell fate of ESCs with depletion of p38\(\alpha\)\((p38\alpha)^{-/-}\) in comparison to their wild type cells (p38\(\alpha^+/+\)). The one of distinction of p38\(\alpha^{-/-}\) is higher production of reactive oxygen species (ROS), which could influence the differentiation potential into specific germ layer. However, the overall ESC potential seems to be unchanged. Further, we revealed that p38\(\alpha\) kinase is involved in the three steps in the process of hematopoietic differentiation. Firstly, the absence of p38\(\alpha\) kinase led to the inhibition of hemangioblast formation during the first step of hematopoiesis. During differentiation, the mutation of p38\(\alpha\) kinase led to the impairment of erythrocyte maturation. Concurrently, p38\(\alpha\) kinase plays an important role in the differentiation of common myeloid precursor cells into granulocyte lineages. Finally, we analyzed the role of p38\(\alpha\) kinase in the differentiation of the hematopoietic cells from the fetal liver and bone marrow. The role of p38\(\alpha\) varies between the hematopoietic progenitors from bone marrow and fetal liver and the differences exist in the hematopoietic differentiation from the ESCs. Thus, it is evident; that the p38\(\alpha\) is involved in the hematopoietic differentiation at all levels of development.
ABSTRAKT


V této práci, se zaměřuji na p38α mitogen aktivovanou proteinkinázu (MAPK) a její zapojení v regulaci a vzniku hematopoézy. Nejprve jsme ověřili vlastnosti embryonálních kmenových buněk s mutací genu p38α a porovnali je s tzv. divokým typem těchto ES buněk. Jedním z rozdílů byla vyšší produkce reaktivních kyslíkových metabolitů (ROS) u buněk p38α-/-, což by mohlo mít vliv na diferenciční potenciál těchto buněk do buněk různých zárodečných listů. Na druhou stranu, obecné vlastnosti embryonálních kmenových buněk se zdají být u p38α-/- buněk nezměněné. Dále, jsme poodhalili zapojení p38α kinázy během hematopoetické diferenciace, a to ve třech krocích. Nepřítomnost funkce p38α kinázy vedla k inhibici tvorby hemangioblastů v prvním kroku hematopoetické diferenciace. Mutace p38α dále vedla k porušení tvorby a zrání erytrocytů. Současně pak p38α kináza hraje důležitou roli v diferenciaci společného myeloïdního progenitoru směrem do linii granulocytů. Nakonec jsme se rozhodli analyzovat úlohu p38α kinázy v průběhu hematopoetické diferenciace z buněk fetálních jater a kostní dřeně. Zapojení p38α MAPK se liší jak v průběhu diferenciace hematopoetických progenitorů z buněk fetálních jater a kostní dřeně, tak také v průběhu diferenciace z ES buněk. Je tedy zřejmé, že p38α kináza je zapojena do hematopoetické diferenciace na všech úrovních vývoje.
Krvetvorba neboli hematopoéza je nejlépe charakterizovaný proces diferenciace v těle. Hematopoetické kmenové buňky (HSC) jsou multipotentní kmenové buňky, které mají schopnost sebeobnovy a diferenciace do všech krevních buněčných typů a jsou zároveň jediné kmenové buňky využívané rutinně v medicíně pro léčbu řady krevních onemocnění. Ačkoli jsou transplantace HSC používány v klinice již po desetiletí, stále je velkou výzvou pochopit proces regulace jejich sebeobnovy a diferenciace. Abychom mohli těmto regulačním mechanismům porozumět, je důležité pochopit také vývoj hematopoetických kmenových buněk a jejich původ.

Embryonální kmenové (ES) buňky jsou pluripotentní buňky, které za určitých specifických podmínek mohou dát vznik buňkám všech tří zárodečných listů. Proces diferenciace ES buněk rekapituuluje embryogenezi a tím nám dává jedinečný model umožňující nahlédnout do vývojových procesů. Mnohé transkripční faktory, které regulují sebeobnovu a diferenciaci HSC již byly objeveny, ale k pochopení vzniku a vývoje hematopoetických buněk nám stále zbývá odhalit značné množství signálních dráh a molekul zde zapojených.

V této práci, se zaměřuji na p38α mitogen aktivovanou proteinkinázu (MAPK) a její zapojení v regulaci a vzniku hematopoézy. Nejprve jsme ověřili vlastnosti embryonálních kmenových buněk s mutací genu p38α a porovnali je s tzv. divokým typem těchto ES buněk. Jedním z rozdílů byla vyšší produkce reaktivních kyslíkových metabolitů (ROS) u buněk p38α-/-, což by mohlo mít vliv na diferenciační potenciál těchto buněk do buněk různých zárodečných listů. Na druhou stranu, obecné vlastnosti embryonálních kmenových buněk se zdají být u p38α-/- buňek nezměněné. Dále, jsme poodhalili zapojení p38α kinázy během hematopoetické diferenciace, a to ve třech krocích. Neprítomnost funkce p38α kinázy vedla k inhibici tvorby hemangioblastů v prvním kroku hematopoetické diferenciace. Mutace p38α dále vedla k porušení tvorby a zrání erytrocytů. Současně pak p38α kináza hraje důležitou roli v diferenciaci společného myeloidního progenitoru směrem do linií granulocytů. Nakonec jsme se rozhodli analyzovat úlohu p38α kinázy v průběhu hematopoetické diferenciace z buněk fetálních jater a kostní dřeně. Zapojení p38α MAPK se liší jak v průběhu diferenciace hematopoetických progenitorů z buněk fetální ch jater a kostní dřeně, tak také v průběhu diferenciace z ES buněk. Je tedy zřejmé, že p38α kináza je zapojena do hematopoetické diferenciace na všech úrovních vývoje.
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Total contribution estimated as 80%


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1. Introduction

The first notions about cell which can differentiate into various lymphoid cells, was presented more than 100 years ago (Dantschakoff, 1908; Pappenheim, 1908; reviewed in Ramalho-Santos and Willenbring, 2007) but it was not until the mid-twentieth century, when Till & McCulloch (Till and McCulloch, 1961) introduce the concept of multipotent progenitors that can be found in adult bone marrow and are responsible for the constant production of blood. Progress in the phenotypic and functional characterization of these precursors led to the isolation of hematopoietic stem cells (HSCs) and has made the hematopoietic system a paradigm in stem cell biology. HSCs have been characterized by two main properties, multipotency, and self-renewal, at the single-cell level. HSCs were first described as clonogenic bone marrow cells that can reconstitute the hematopoietic system in primary and secondary recipients (Becker et al., 1963; Lemischka et al., 1986). HSC pool maintenance and concomitant lineage differentiation are facilitated either by asymmetric selfrenewal in which specific cell fate determinants are redistributed unequally to the two-daughter cell, one of them stay so-called long-term HSC (LT-HSC) and the second one becomes short term HSC (ST-HSC). ST-HSCs have limited self-renewal capacity and can differentiated into all blood cell types (Fig. 1 – The scheme of hematopoiesis).

The original pool of HSC is formed during embryogenesis in a complex developmental process where several anatomical sites are involved. During postnatal life, a steady state of HSC pool size is maintained by their self-renewal and differentiation. During vertebrate ontogenesis, hematopoiesis takes place in the various organs. The yolk sac (YS) is the first source of blood cells (mainly primitive erythrocytes), then the hematopoiesis moves into aorta-gonad-mesonephros (AGM) area followed by the migration to a fetal liver (FL) which is the main hematopoietic organ of the fetus. The placenta and spleen are also considered as the hematopoietic organs during prenatal development (Dzierzak and Robin, 2010). Shortly before birth and the first days of life, the hematopoietic stem cells migrate to bone marrow in mice. The bone marrow represents the main site of adult hematopoiesis, although during times of stress hematopoiesis may also occur in the spleen and liver (Kim, 2010).

During the past several decades, hematopoietic stem cell transplantation has been used as a standard treatment for various hematological disorders. Even though HSC transplantation has been applied in the clinic for decades, regulation of HSC self-renewal, differentiation, and development is still a major challenge. In vitro differentiation of embryonic stem (ES) cells
offers a unique approach to examine events that occur during embryonic development. In particular, when combined with the availability to manipulate their genome, ESCs provide a unique model of stem cell differentiation.

In the first, theoretical, part of this thesis, I sum up the existing knowledge about molecular mechanisms, which are involved in the hematopoietic establishment, especially in mice. In the next section, I outline the aims of my doctoral work. Results and discussion section provides a short summary of the published articles and discussion and comments in the context of other studies follow.

Figure 1. The scheme of hematopoietic differentiation from the hematopoietic stem cell. Long term HSC (LT-HSC); short term HSC (ST-HSC); multipotent progenitor cells (MPP); lymphoid-primed multipotent progenitors (LMPP); common myeloid progenitor (CMP); common lymphoid progenitor (CLP); granulocyte-macrophage progenitor (GMP). (Adapted from (Dzierzak and Philipsen, 2013)).
1.1. Ontogenesis of hematopoiesis

1.1.1. Yolk sac

Hematopoietic cells in the yolk sac were described in mice in the late 19th century. The yolk sac (YS), is derived from mesoderm and provides early alimentation for embryonic development. During the process of gastrulation, the mesodermal cells migrate from the primitive streak and take an intermediate position between the primitive ectoderm and visceral endoderm; this begins at E6.5 and the mesoderm cells together with visceral endoderm form the yolk sac. The first wave of hematopoiesis takes place just here in the structures called blood islands at an early embryonic stage E7 (Fig. 2).

Figure 2. The rise of blood islands in the yolk sac (dotted line – the transverse section in panel B). A) Mesodermal precursors (hemangioblasts) migrate from the primitive streak and take an intermediate position between the primitive ectoderm and visceral endoderm (brown arrow), where they give rise to blood islands (the red arrow shows the migration of other mesodermal cells). B) – primitive erythrocytes are surrounded by endothelial precursor cells (angioblasts). Adapted from (Dzierzak and Philipsen, 2013; Fernandes et al., 2012).

The blood islands are clusters of primitive hematopoietic cells surrounded by endothelial precursor cells (angioblasts) within the mesodermal layer of the yolk sac (Ferkowicz and Yoder, 2005; Risau, 1991). The close spatial and temporal association between the
development of these two cell lineages has led to the hypothesis that they have a common precursor, the “hemangioblast”. Thus, hemangioblast can differentiate into endothelial and/or hematopoietic lineage, but in vitro studies also show the potential of differentiation into muscle cells (Choi et al., 1998; Kennedy et al., 2006; Lu et al., 2007). Early hematopoietic and endothelial cells express a number of common genes, e. g. Flt1, Flk1, Tal1, Tie2. Moreover, mutation of Flk1 caused a complete absence of hematopoietic and endothelial cells in vivo (Shalaby et al., 1995). Flk1 is considered as the marker of hemangioblast and as was proved, is required for migration of this progenitors to the yolk sac (Shalaby et al., 1997).

A role for the visceral endoderm in the yolk sac is to provide the proper environment for the induction hematopoietic and endothelial cells. This affirmation is encouraged by the observation that embryoid bodies formed from Gata4-deficient ES (transcription factor GATA4 is an earliest marker for lateral mesoderm and endoderm) cells lacked visceral endoderm and showed a reduction in primitive erythropoiesis (Belaoussoff et al., 1998). Moreover, visceral endoderm activates hematopoietic and vascular development via Ihh (Indian hedgehog) signal, which regulates expression of bone morphogenetic protein 4 (BMP4) in the mesodermal cells. The next, Ihh and vascular endothelial growth factor (VEGF) from primitive endoderm can stimulate the expansion of primitive erythrocytes (Fig. 3)(Artus et al., 2012; Baron, 2001; Dyer et al., 2001).

This first wave of hematopoiesis is denoted primitive hematopoiesis because during this time primitive erythrocytes arise in the YS. Primitive erythrocytes are bigger, nucleated cells with an embryonic type of hemoglobin. The primitive erythrocytes enucleate in the embryonic circulation (Kingsley et al., 2004; Kingsley et al., 2006). Macrophages and megakaryocytes are also produced in the YS. The primary function for the primitive erythrocytes is to cover an immediate oxygen needs of the developing embryo. Embryonic macrophages are required for tissue remodeling and defense and primitive megakaryocytes function is vascular maintenance (Bertrand et al., 2005; Kingsley et al., 2004; Palis, 2014). The first wave of hematopoiesis does not produce lymphoid cells or HSCs (de Bruijn et al., 2000; Yamane et al., 2013). The hematopoietic production from the YS appeared to be transient until the definitive hematopoiesis leading to adult hematopoietic cell production is established. But it was also shown that, in fact, the majority of adult tissue-resident macrophages (in liver, brain, epidermis, and lung) originates from erythro-myeloid progenitors that develop in E8.5 YS (Gomez Perdiguero et al., 2015). Moreover, the resident macrophage population of the adult central nervous system, named microglia, derives from myeloid progenitors that were initially generated in the YS (before E8; (Ginhoux et al., 2009)). The production of this definitive
development of these two cell lineages has led to the hypothesis that they have a common precursor, the “hemangioblast”. Thus, hemangioblast can differentiate into endothelial and/or hematopoietic lineage, but in vitro studies also show the potential of differentiation into muscle cells (Choi et al., 1998; Kennedy et al., 2006; Lu et al., 2007). Early hematopoietic and endothelial cells express a number of common genes, e.g. Flt1, Flk1, Tal1, Tie2. Moreover, mutation of Flk1 caused a complete absence of hematopoietic and endothelial cells in vivo (Shalaby et al., 1995). Flk1 is considered as the marker of hemangioblast and as was proved, is required for migration of this progenitors to the yolk sac (Shalaby et al., 1997). A role for the visceral endoderm in the yolk sac is to provide the proper environment for the induction hematopoietic and endothelial cells. This affirmation is encouraged by the observation that embryoid bodies formed from Gata4-deficient ES (transcription factor GATA4 is a n earliest marker for lateral mesoderm and endoderm) cells lacked visceral endoderm and showed a reduction in primitive erythropoiesis (Belaoussoff et al., 1998). Moreover, visceral endoderm activates hematopoietic and vascular development via Ihh (Indian hedgehog) signal, which regulates expression of bone morphogenetic protein 4 (BMP4) in the mesodermal cells. The next, Ihh and vascular endothelial growth factor (VEGF) from primitive endoderm can stimulate the expansion of primitive erythrocytes (Fig. 3)(Artus et al., 2012; Baron, 2001; Dyer et al., 2001).

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Figure 3. The scheme of the activation of primitive hematopoiesis and vasculogenesis in the developing yolk sac. Ihh is secreted from visceral endoderm to the target extraembryonic (yolk sac) mesoderm, where it activates expression of Bmp4. Adapted from (Baron, 2001).
In 1970, Moore and Metcalf hypothesized that since the first blood cells are produced in the YS, the first HSCs are also generated in the YS (Moore and Metcalf, 1970), nevertheless the fundamental data argues against this theory. Some studies show that explant of the early yolk sac does not have the potential to generate definitive reconstituting HSC. Cumano and colleagues specified that precirculation yolk sac hematopoietic progenitors do not have the capacity to reconstitute adult hematopoiesis. They injected precirculation whole yolk sac cells into adult Rag2/ge/ immunodeficient mice and observed only short-term myeloid reconstitution, but not lymphoid reconstitution (Cumano et al., 2001). By contrast, Matsuoka and colleagues observed that E8.5 yolk sacs cocultured with E10.5 AGM stromal cells resulted in the acquisition of long-term reconstituting capacity of the yolk sac cells (Matsuoka et al., 2001). From these confirmations is apparent that progenitor cells in the yolk sac have the potential to development into multipotent progenitors when are exposed to the correct environment. It is still more obvious, that in this context the understanding of niche/microenvironment has the same importance as the molecular mechanisms in the cells to insight processes in the body.

After the onset of circulation (E9 – E10), yolk sac c-Kit+CD34+ hematopoietic cells repopulate all hematopoietic compartments in neonatal mice, but lack the capacity to repopulate conditioned adult mice (Yoder and Hiatt, 1997; Yoder et al., 1997). Furthemore, Kumaravelu et al. proved that yolk sac explant from embryo E12.5 can support HSC expansion in vitro (Kumaravelu et al., 2002). The conclusion consequent on these observations can be interpreted that HSCs appeared in the YS after starting of circulation, but it is not clear, whether HSCs migrate here from AGM or/and they arise in the YS de novo.

### 1.1.2. Aorta-Gonade-Mesonephros area

The AGM is an embryonic tissue derived from the mesodermal germ layer that contains the dorsal aorta, the genital ridge (which gives rise to the gonads), and the mesonephros (which gives rise to the kidneys), and that extends from the anterior limbs to the posterior limbs of the E9.5 to E12.5 mouse embryo.

At the end of the 1970s come out that HSCs are developed within embryo instead of the yolk sac, as was hypothesized (Dieterlen-Lièvre, Beaupain, and Martin, 1976; Beaupain, Martin and Dieterlen-Lièvre, 1979; Lassila et al., 1979). Intra-embryonic HSCs have been documented in multiple species (reviewed in Thierry Jaffredo et al. 2005). In all species described, the intra-embryonic HSCs develop in close association with the ventral wall of the
dorsal aorta, or in adjacent vitelline and umbilical arteries. The third wave, which takes place in the embryo, is characterized by the production of definitive hematopoietic cells and HSCs, that are needed for the long-term establishment of the adult hematopoietic system (AGM is the first area where definitive hematopoietic stem cells were observed at E10.5). In this case, as definitive HSCs are considered stem cells capable of repopulating adult recipients (Muller et al., 1994). The definitive HSCs are also detectable in two other main blood vessels in the mouse embryo, the vitelline and umbilical arteries, concurrently with HSC generation in the AGM (de Bruijn et al., 2000). Definitive hematopoietic progenitors (the cells without repopulating capability) were found earlier at somite-pair stages, the paraaortic splanchnopleure (P-Sp; area, which later becomes the aorta-gonade-mesonephros region) and slightly later in the AGM area at E8.5 (Cumano and Godin, 2007; Godin et al., 1995). HSCs are generated from hemogenic endothelium within the AGM as a result of endothelial-to-hematopoietic transitions (EHT; (Bertrand et al., 2010; Boisset et al., 2010; de Bruijn et al., 2002; Jaffredo et al., 1998; Kissa and Herbomel, 2010)). Hemogenic endothelium is described as a small, specialized subset of vascular endothelium that acquires hematopoietic potential and can give rise to multilineage hematopoietic stem and progenitor cells (HSPC). The EHT appears to exist only transiently and is characterized by changes in gene expression and shape in ventral aortic endothelial cells as HSC precursors emerge and then enter to a circulation (Fig. 4).

The developmental origins of HSCs are closely associated with endothelial cells (Jaffredo et al., 1998; Nishikawa et al., 1998a; Nishikawa et al., 1998b; Oberlin et al., 2010). Indeed, the coexpression of early hematopoietic (Runx1, Sca1, c-Kit, CD34) and endothelial (VE-cadherin, CD31) markers in the dorsal aorta endothelium and intraluminal clusters of cells attached to this endothelium suggests an endothelial origin of HSCs (Boisset et al., 2010; Chen et al., 2009; Jaffredo et al., 1998; North et al., 2002; Taoudi et al., 2005; Yvernogean and Robin, 2017; Zovein et al., 2010). Single cell lineage tracing studies have demonstrated multipotent HSPC with cell surface markers CD31 (endothelial cell marker), CD41 (blood cell marker), c-Kit (stem cell growth factor receptor), and SCA-1 (stem cell antigen, also known as Ly6A) arising directly from the ventral wall of the murine dorsal aorta and these cells can be traced to the fetal liver (Boisset et al., 2010). The research from the last years suggested a ventral subendothelial origin for definitive HSCs.

The prevailing hypothesis was that intra-aortic clusters are a morphological manifestation of HSCs budding from the endothelial lining of the dorsal aorta (Chen et al., 2009; North et al., 1999; Zovein et al., 2010). Aortic hematopoietic clusters have been described in many
species, including mouse and human (Jaffredo et al., 2005b). In the mouse, hematopoietic clusters appear at E10 and the first adult-repopulating HSCs are autonomously generated in the aorta at E10.5 (>34 somite pairs; (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994)). Although clusters are found both dorsally and ventrally, in vitro modeling of HSC development has revealed that the AGM region exhibits a dorsal-ventral polarity, with HSCs emerging predominantly from the ventral region (Souilhol et al., 2016; Taoudi and Medvinsky, 2007). Indeed, the ventral domain of the E10.5 dorsal aorta provides an immediate supportive environment for HSC generation. This process is supported by the dorsal domain of the dorsal aorta and urogenital ridges. Thus, we can see a significant complexity of the niche formed by long- and short-range cross signaling (Souilhol et al., 2016). Required hematopoietic transcription factors are expressed in cells of the ventral clusters (Minegishi et al., 1999; North et al., 1999; North et al., 2002). Peeters and colleagues showed the antagonize role of ventral and dorsal tissue in the AGM area. Meanwhile, ventral tissues enhance the growth of AGM HSCs and progenitors in vitro, the dorsal tissues inhibit this growth. Hedgehog proteins were identified as major effectors of induction the early HSC arising. This proteins are produced by the gut which is localized by the ventral part of the AGM (Peeters et al., 2009). The Hedgehog is important in more stages of hematopoietic establishment. First, the Hedgehog is required during the gastrulation stage for the medial migration of dorsal aorta precursors (most likely Flk1⁺) and later plays a role in the production of HSC in AGM. Studies in zebrafish have shown a clear requirement for Hedgehog signaling in the generation of the definitive hematopoietic system (Gering and Patient, 2005). In zebrafish embryos, activation of both the arterial and adult hematopoietic programs is under the control of a signaling cascade that involves Hedgehog, VEGF and Notch. In the mouse, Bmp4 and Shh have been reported to expand HSC numbers in the AGM region (Durand et al., 2007; Peeters et al., 2009). Bmp4 is responsible for the polarization of HSC formation from the ventral side of the dorsal aorta and is considered as a direct upstream of runx1. (Pouget et al., 2014). The transcription factor Runx1 is a key regulator of HSC development in vertebrate species (Burns et al., 2005; Kalem-Zylinska et al., 2002; Swiers et al., 2010). The Runx1-null mouse embryos lack clonogenic progenitors and HSCs and die by E12.5 (Okuda et al., 1996; Yokomizo et al., 2008). The lack of intra-aortic clusters in Runx1 /− embryos led to a hypothesis that Runx1 regulates endothelial-hematopoietic transition in the dorsal aorta (North et al., 1999; Yokomizo et al., 2001). In vitro studies proved that Runx1 deficiency prevents the differentiation of hematopoietic cells from ESC-derived hemogenic endothelium (Lancerin et al., 2009). A stem cell factor (SCF, also known as a Kit ligand) is
considered to be a major HSC maturation factor during early embryonic development from E9 to E10 (Rybtsov et al., 2014). This finding is consistent with the HSC deficiency phenotype reported for the Scf knockout mouse (Barker, 1994; Ding et al., 2012).

Figure 4. Extraembryonic and intraembryonic sites of hematopoiesis in mouse embryo. A) The aorta (AGM region), yolk sac, umbilical and vitelline arteries, placenta, and liver, (dotted line – the transverse section in panel B). (B) Transverse section showing the AGM region of a mouse embryo. A hematopoietic cluster on the ventral wall of the aorta is shown. (C) The rise of the HSC in the AGM - endothelio-hematopoietic transition (EHT); Non-hemogenic endothelial cells (blue) and mesenchymal cell (yellow). Adapted from (Dzierzak and Philipsen, 2013).

1.1.3. Fetal liver, bone marrow, and spleen

The main generation of HSCs is connected with the AGM but the fetal liver (FL) is the predominant embryonic niche for HSC expansion. HSCs are released into circulation and
migrate into the mouse fetal liver, which becomes the major site of the hematopoiesis. Fetal liver seeding by hematopoietic cells, possibly from the yolk sac, the placenta, or the AGM, starts at E9.5–10.5. Unlike adult bone marrow HSCs, which are mostly quiescent, fetal liver HSCs are highly proliferative (Bowie et al., 2006b; Bowie et al., 2007; Morrison et al., 1995). It is not until E11.5 that the first long term LT-HSCs are detected in the liver (Christensen et al., 2004). The number of HSCs dramatically increases in the FL, more than doubling in number from approximately 1000 at E12.5 to more than 5000 at E14.5 (Ema and Nakauchi, 2000). However, this increase is only transient; by E15.5–16.5, the number of FL HSCs declines as they emigrate to seed the spleen and the bone marrow (Christensen et al., 2004).

Mounting evidence has demonstrated that hepatic progenitors, stromal cells, pericytes and endothelial cells in mouse fetal liver are the crucial niche cells for the expansion of HSCs (Khan et al., 2016; Swain et al., 2014). Stromal cells constitute the primary component of the fetal liver niche for hematopoietic cells (Moore et al., 1997; Wineman et al., 1996). In addition, stromal cells in fetal liver show an epithelial-to-mesenchymal transition and their hematopoietic supportive capacity is abrogated upon hepatocytic maturation (Chagraoui et al., 2003). However, there exist cells (SCF^+ DLK^+ fetal liver hepatic progenitors), which were identified as an important stromal cell population that supports HSC expansion in the fetal liver. These cells were reported to express growth factors important for supporting HSC activity, including angiopoietin-like 3 (Angptl3 - cytokine that supports HSC activity in the fetal liver), insulin-like growth factor 2 (IGF2), SCF and thrombopoietin (TPO; (Chou and Lodish, 2010; Chou et al., 2013; Zhang and Lodish, 2004)). Activating transcription factor 4 (ATF4) is another important transcription factor in fetal liver. Stromal cells from Atf4^-/- fetal liver exhibited abrogated capacity to support HSC repopulating activity ex vivo. ATF4 contributes to HSC maintenance by controlling the transcription of cytokines, such as Angptl3 (Zhao et al., 2015). HSCs can be maintained ex vivo with the addition of combinations of growth factors, including SCF, Flt3 ligand and interleukin (IL) family (Yonemura et al., 1996; Yonemura et al., 1997). SCF is a widely used growth factor to promote HSC expansion. Its receptor, c-Kit, resides at the membrane of stem cells (Yonemura et al., 1996; Yonemura et al., 1997).

The migration of HSCs into the spleen and BM follows after HSC emergence in the FL. Active seeding of HSCs begins in the mouse spleen at E15.5; HSC activity increases daily in the spleen from E15.5 to E17.5, whereas it declines in the fetal liver after E15.5 (Christensen et al., 2004; Morita et al., 2011). HSC-repopulating activity can be first detected in the bone marrow at E17.5 (Christensen et al., 2004). The repopulating activity of HSCs in the spleen is
the same as the repopulating activity of HSCs in the bone marrow. However, the number of HSCs in the spleen is lower than in the BM and the HSC activity in the spleen is detectable up to a few weeks after birth (Ikuta and Weissman, 1992; Wolber et al., 2002). HSCs proliferate markedly during the first 3 weeks in the neonatal bone marrow and then become quiescent (Bowie et al., 2006a). The next proliferation of these quiescent HSCs is regulated/induced in the connection with the need of organism for the renewal of the blood elements.

1.1.4. Bone marrow niche for HSC

In the physiological state, the predominant site for HSC maintenance and differentiation in adulthood is the bone marrow. The molecular and cellular components of the environment, where the HSC seed is described for the understanding of the development of particular cell lineages from HSCs. The niche of HSCs is composed of distinct cell types with different regulatory functions (Birbrair and Frenette, 2016). In 1978 Ray Schofield formulate the first theory of the HSCs niche. His findings and findings of his colleagues at the University of Manchester led to the hypothesis that bone might regulate hematopoiesis (Dexter et al., 1977; Lord et al., 1975; Schofield, 1978). A niche is defined by anatomy and function – a local tissue microenvironment that directly maintains and regulates a kind of stem cells or progenitor cells. Current data suggest that in the bone marrow, there are specialized niches for distinct types of hematopoietic stem and progenitor cells and that each niche may be created by multiple cell types that contribute to the niches in unique as well as redundant ways (Ding and Morrison, 2013). We can distinguish the microenvironment formed by osteoblasts, endothelial cells, and reticular cells (Fig. 5).

Osteoblastic cells were the first cell population shown to influence hematopoietic stem/progenitor cell (Calvi et al., 2003; Taichman and Emerson, 1994). Osteoblasts line the bone surface and are involved in synthesis, deposition, and mineralization of the extracellular matrix of the bone, and play a central role in bone development. Subsequently, it was reported that treatment leading to increased numbers of osteoblasts in culture (e.g.) parathyroid hormone (PTH), increases HSC numbers in vivo as well (Calvi et al., 2003). Moreover, activated osteoblasts can further produce osteopontin, angiopoietin-1 (Ang-1), vascular cell adhesion molecule-1 (VCAM-1) and TPO, that contribute to HSC quiescence (Arai et al., 2004; Stier et al., 2005; Yoshihara et al., 2007). HSCs are found in contact with a population of osteoblasts, termed spindle-shaped N-cadherin’CD45’ osteoblastic cells (SNO). Niche made by bone-lining osteoblasts, including SNO cells is called osteoblastic or endosteal
niches. Zhang and colleagues observed that an increased number of SNO within this niche cells lead to HSCs enrichment (Zhang et al., 2003).

The signaling lymphocytic activation molecule (SLAM) cell surface markers (CD150^+CD48^-) have identified HSCs in proximity to sinusoidal blood vessels. Perivascular cells known as (C-X-C motif chemokine ligand 12) CXCL12-abundant reticular (CAR) cells are reported to interact with HSCs primarily near sinusoids. The most CD150^+CD48^-CD41^+HSCs (97%), earliest B cell precursors, plasma cells, plasmacytoid dendritic cells, and natural killer cells were found in contact with the processes of CAR cells in the marrow. Thus CAR cells are considered to be another possible candidate that function as a niche for HSCs and all immune cells produced in bone marrow (so-called reticular niches; (Kohara et al., 2007; Sugiyama et al., 2006; Tokoyoda et al., 2004)). HSCs from the CAR cell-depleted mice were reduced in number (about 2-fold) and cell size, more quiescent, and had increased expression of genes involved in myeloid fate decision and differentiation. Furthermore, the numbers of cycling B cell and erythroid progenitors were reduced in the CAR cell-depleted mice. Beside, CAR cells do not express the pan-endothelial marker platelet/endothelial cell-adhesion molecule 1 (PECAM-1)/CD31 or the smooth muscle cell marker and smooth muscle α-actin (SMαA), suggesting that they are different from endothelial cells and smooth muscle cells (Tokoyoda et al., 2004).

Endothelial cells that line the sinusoidal blood vessels in the bone marrow may also contribute to HSC regulation. Kiel and co-workers demonstrated that primitive HSCs defined by SLAM markers (CD150^+, CD48^-) preferentially localize in close proximity to sinusoidal endothelial cells, identifying sinusoidal blood vessels as HSC niche (so-called perivascular niche; (Kiel et al., 2005)). Disruption of Flk1 and VE-cadherin–dependent angiogenic signaling pathways shows that sinusoidal endothelial cells expand the HSC pool, support self-renewal, and prevent exhaustion of HSCs in both serum-free coculture assays and in vivo through Notch signaling (Butler et al., 2010). More recently, perivascular nestin^+ mesenchymal stem cells have been identified as central components of the HSC niche that regulate HSCs via the expression of stem cell factor, CXCL12, Ang-1, VCAM-1; Mendez-Ferrer et al., 2010). Local neurotransmitters, delivered by the sympathetic nervous system, tightly associated with the vasculature, regulate HSC release from the bone marrow. This innervation regulates HSC mobilization through the circadian release of noradrenaline, which modulates CXCL12 expression in the bone marrow. The reticular niche and vascular niche can be denoted as a perivascular niche. There is strong evidence that HSCs reside in this perivascular region of the
BM and that mesenchymal stem cells (MSCs), endothelial cells (ECs), and pericytes regulate HSC maintenance and differentiation through soluble factors and cell contact-dependent signals. Recent studies described the transforming growth factor-β (TGF-β) as a niche factor that controls HSC dormancy. Nonmyelinating Schwann cells that are located around the blood vessels in the BM induce HSC dormancy by secreting TGF-β, suggesting that HSC quiescence is maintained in the vascular niche (Yamazaki and Nakauchi, 2014). Recently, these findings have been further elaborated by conditional deletion of CXCL12 in different cell types of the HSC niche (Ding et al., 2012). HSC maintenance and self-renewal in the BM was primarily regulated by CXCL12 secreted from immature mesenchymal stem and progenitor cells and to a lesser extent from endothelial cells. In addition, CXCL12 secreted from osteoblasts was dispensable for HSC function. (Riether et al., 2015).

**Figure 5. Scheme of HSC niches in bone marrow.** HSCs in the endosteal niche are in close contact with OBs and MSCs, both of which express HSC maintenance and quiescence factors, including CXCL12, SCF, Ang-1, VCAM-1, and TPO, and cooperate to retain HSCs in their niche. The perivascular niche is more distant from the endosteum and includes perivascular CAR cells that secrete factors that promote self-renewal of active HSCs, which are significantly more abundant than dormant HSCs. Both niches contain perivascular MSCs as a key component. They also induce the expression of HSC maintenance factors. The SNS inhibits MSC proliferation and induces circadian oscillations of CXCL12 expression. Adapted from (Ehninger and Trumpp, 2011).
1.1.5. Placenta

Placenta is essential for fetal development as it is responsible for feto-maternal exchange from midgestation and production of important hormones and cytokines (Cross et al.; Rossant and Cross, 2001). Although the placenta has not been considered to be a hematopoietic organ, early reports suggested that the mouse placenta may exhibit hematopoietic activity (Dancis et al., 1968; Dancis et al., 1977; Till and McCulloch, 1961). In mice, the allantois forms the umbilical vessels and the mesodermal parts of the fetal placenta (Downs and Harmann, 1997). It develops after gastrulation as an extension of the posterior primitive streak and fuses with the chorionic plate by E8.5, whereafter the allantoic mesoderm interdigitates into the trophoblast layer of the placenta and gives rise to the fetal vasculature in the placental labyrinth. Hematopoiesis in the mouse placenta is evident from E9.0, when definitive multi-lineage progenitors appear (Alvarez-Silva et al., 2003), whereas mature HSCs are found 1.5-2.0 days later (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

There are several possible explanations for the presence of HSCs in the placenta. The early onset of HSC activity in the placenta, which is evident before HSCs are found in the fetal circulation or liver, suggests that placental HSCs may be generated in situ. Second, HSC in the placenta can originate from the AGM. The placenta is the main vascular route through which the blood cells from the dorsal aorta circulate into the fetal liver. It is also the most likely route for AGM-derived HSCs take. Thus, it is possible that AGM HSCs are nurtured temporarily in the placental niches prior to seeding the fetal liver. After E13.5, the number of HSCs in the placenta decreases, while the liver HSC pool expands (Gekas et al., 2005).

Placental HSCs have a similar surface phenotype to fetal liver HSCs, both of which express Cd34, c-Kit and Sca1 at E12.5 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). On the other hand, there is not found a number of lineages progenitors as in the fetal liver (Gekas et al., 2005). The other purpose of placental hematopoiesis is that the placenta could be a fetal lymphoid organ as it has been shown that B-cell precursors appear in the mouse placenta before they are found in the fetal liver. The B-cell precursors were found in the placenta at E9.5 and their number increased until E12.5. Then the number of these progenitors declines, which can be in connection with the pool of HSCs (Melchers, 1979).

1.1.6. Hemangioblast vs. hemogenic endothelium

During the development of hematopoiesis, there is a close association between endothelial precursors and primitive hematopoietic cells in the blood islands in the yolk sac. A similar
close association is also seen in later intraembryonic regions where definitive precursors arise. The close relationship leads to a hypothesis that endothelial and hematopoietic cells have the same precursor hemangioblast. This hypothesis was announced already at the beginning of the 20th century by Sabin (Sabin, 1917). Sabin, and later Murray (Murray, 1932), are credited for the concept that both the endothelial and hematopoietic lineages are derived from a common precursor, the “hemangioblast,” in the blood islands of the yolk sac. Murray described the newly formed lateral and posterior mesodermal cell migration toward the yolk sac, where they can differentiate to endothelial cells and to hematopoietic cells of the blood islands. Sabin studied the migration of these precursors under the light microscope and could distinguish the hemangioblastic aggregates from the remaining mesodermal cells. The peripheral cells of these aggregates subsequently flatten and differentiate to endothelial cells, while the centrally located cells differentiate to hematopoietic cells. Notably, other hypotheses were presented. Jordan described the clusters of specialized endothelial cells (so called hemogenic), which can give rise to hematopoietic cells (Jordan, 1917). Both theories highlight the close relationship between hematopoietic and endothelial cells.

Since the theory of bipotential precursors of endothelial and hematopoietic cells has been formulated, several lines of the genetic and functional evidence appeared. Experimental support for the hemangioblast came from studies of differentiating mouse and human ESCs and from mouse embryos. The existence of a clonal precursor that has both hematopoietic and vascular potential was first demonstrated in vitro by differentiating mouse ESCs (Choi et al., 1998; Kennedy et al., 2006). This precursor has been characterized by co-expressing mesodermal markers Brachyury and Flk-1. Flk-1 deficient embryos lack blood and endothelium (Shalaby et al., 1995). Likewise, Flk1-/- ESCs fail to contribute to blood and endothelial lineages in chimeras, which is contributed to the existence of Flk1+ hemangioblast cells (Shalaby et al., 1997). Also, the blast colony-forming cells (BL-CFC), identified during mouse ESC differentiation, are able to generate cells of both endothelial and hematopoietic lineages (Choi et al., 1998). Moreover, such bipotential precursors, most likely corresponding to the hemangioblast, can be detected in gastrulating mouse embryos (Huber et al., 2004). They arise in the primitive streak before migrating to the YS, where they will differentiate in vivo into hematopoietic and endothelial cells (Huber et al., 2004; Vogeli et al., 2006).

The other theory results from the observation of hematopoietic cells grouped together in clusters of cells (Intra-Aortic Hematopoietic Clusters, IAHCs), tightly attached to the endothelial layer of the vessels, which are fated to be hematopoietic cells (Jordan, 1917). The presence of IAHCs has been described in many vertebrate species (Dieterlen-Livre et al.,
umbilical vessels and the mesodermal parts of the fetal placenta (Downs and Harmann, 1997). In mice, the allantois forms the Placenta is essential for fetal development as it is responsible for feto-maternal exchange from hematopoietic cells in the blood islands in the yolk sac. A similar progenitors appear (Alvarez-Silva et al., 2003), whereas mature HSCs are found 1.5 -2.0 days after the onset of HSC activity in the placenta, which is evident before HSCs are found in the fetal liver. There are several possible explanations for the presence of HSCs in the placenta. The early lymphoid organ as it has been shown that B-cell precursors appear in the mouse placenta to the hemangioblast, can be detected in gastrulating mouse embryos (Huber et al., 2004). Moreover, such bipotential precursors, most likely corresponding lineages (Choi et al., 1998). Also, the blast colony-forming cells (BL-CFC), identified during mouse ESC differentiation, are able to generate cells of both endothelial and hematopoietic cells (Boisset and Robin, 2012; Eilken et al., 2009; Jaffredo et al., 1998; Kissa and Herbomel, 2010). The live imaging was used to study hematopoietic development in the mouse embryo. The CD31+ cells from the ventral endothelium bud into the lumen of the aorta. These cells either expressed Sca1 or started to express CD41 and after emergence, they start to co-express c-Kit with CD31. Sca1, CD41, and c-Kit are considered as markers of hematopoietic progenitor/stem cells (Boisset and Robin, 2012; Boisset et al., 2010). It is clear, that adult HSC are generated via an endothelial step at an earlier stage during embryonic development. However, there is an obvious discrepancy between the low number of HSCs (< 2 per aorta at E11.5) (Gekas et al., 2005; Kumaravelu et al., 2002; Robin et al., 2006) and the number of cells that make up the IAHCs (between 400 and 600 c-Kit+ cells at E11.5) (Yokomizo and Dzierzak, 2010). Moreover, IAHCs appeared approximately at E9.5 while HSCs about E10.5. The total number of HSCs present in the entire embryo increases from 11 to 152 HSCs in 24 h (from E11.5 to E12.5) (Gekas et al., 2005). All these observations led to the hypothesis, that there must exist an intermediate cell stage between the hemogenic endothelium and the HSCs. These cells are referred to as the pre-HSCs, HSC precursors or pre-definitive HSCs. The first evidence of pre-HSCs came from experiments showing that YS cells or circulating blood cells from E9 mouse embryos could long-term reconstitute hematopoiesis when injected in utero into E11–E15 fetuses (Toles et al., 1989). Medvinski and his team described the precursors for definitive HSCs, pro-HSCs and pre-HSCs type I and type II, which may originate from subendothelial layers of the dorsal aorta (Bertrand et al., 2005; North et al., 1999; Rybtsov et al., 2014; Taoudi et al., 2008b). HSC maturation from the endothelial compartment occurs
through a four-step process: pro-HSC → Type I pre-HSC → Type II pre-HSC → HSCs (Fig. 6). In which case every precursor type is defined by a specific surface set of markers. Pro-HSCs were defined as VE-cad^+ CD41^+ CD43^- CD45^- cells, Type I pre-HSCs as VE-cad^+ CD41^low CD43^+ CD45^- cells and Type II pre-HSCs as VE-cad^+ CD41^low CD43^high CD45^- cells. dHSCs have the same phenotype as Type II pre-HSCs (VE-cad^+ CD41^-low CD43^high CD45^-) but can be assayed by direct transplantation into adult irradiated recipients. All four types of pre-HSCs have been identified morphologically in the area of the dorsal aorta (Chen et al., 2009; Medvinsky et al., 2011; Taoudi et al., 2008a). Moreover, these precursors of HSCs express the principal hematopoietic marker CD45, thus they cannot be considered as endothelial cells (Taoudi et al., 2008a). Therefor, question arises if the cells from hemogenic endothelium are already committed to producing blood or whether any endothelial cells are influenced in particular environment to differentiate into hematopoietic progenitors.

Figure 6. The scheme of HSC maturation in dorsal aorta through a four-step process: pro-HSC → Type I pre-HSC → Type II pre-HSC → HSCs. (Adapted from Rybtsov et al., 2014).
1.2. Transcription factors and molecular mechanisms during hematopoietic development

Several transcription factors, receptors and proteins critically involved in regulation of hematopoiesis have been identified. Here we briefly review notable examples.

1.2.1. Flk1, Tal1, Etv2, GATA2, Runx1 – transcription factors involved in induction and maintenance of hematopoiesis/HSC

Flk1, also known as the Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) and Brachyury are the best-known markers of the hemangioblast (Huber et al., 2004). While no single absolute marker has yet been identified for the hemangioblast, both blood and endothelium cells arise from cells expressing FLK1 (Shalaby et al., 1995). Flk1-/- embryos die between E8.5 and E9.5 due to defects in blood and endothelial development, including a lack of yolk sac blood islands. Further, Flk1-/- ESCs do not contribute to the hematopoietic system in chimeras (Shalaby et al., 1997), indicating a very early defect in hematopoiesis.

Etv2-/- mouse embryos die during gestation with severe blood vessel defects like the Flk1 phenotype, and Flk1 expression is reduced. Moreover, Etv2-/- ESCs, like embryos, do not make blood progenitors (Ferdous et al., 2009; Lee et al., 2008). Over-expression leads to the induction of Flk1+ mesoderm and can rescue its formation when normal induction is blocked by the inhibition of bone morphogenetic protein, Notch and Wnt signaling. Via deletion of Etv2 in the Flk1+ cells (Flk1-Cre-mediated deletion of Etv2) was proposed that E9.5 embryos contained CD41+ cells and hematopoietic progenitors at comparable levels to wild type littermates. In addition, Etv2-/- cells could initiate the formation of CD41+ cells in both Flk1+ and Flk1- cells isolated from embryoid bodies (EBs), when going beyond the reexpression of Etv2 (Wareing et al., 2012).

The further study revealed that Etv2 is not necessary for generating of primitive mesoderm (Flk1+PDGFRA+), but is important for its specification to hemogenic mesoderm (Flk1+PDGFRA+; (Kataoka et al., 2011)). Comparison of Flk1+ mesoderm from wild type and Etv2-/- ESCs indicated that many key hematopoietic genes have greatly reduced expression in the absence of Etv2, including Tal1 (SCL). These findings implicate Etv2 as a key regulator of hemangioblast formation (Moignard et al., 2013).
Loss-of-function of Tal1 (SCL) impairs yolk sac hematopoiesis (Robb et al., 1995; Shivdasani et al., 1995), HSC formation and subsequent hematopoietic lineage differentiation (Porcher et al., 1996; Robb et al., 1995), as well as the vascular remodeling of the yolk sac endothelium (Visvader et al., 1998). Tal1 is not expressed in Etv2-/- or Flk1-/- embryos (Kataoka et al., 2011), but Tal1 overexpression in Etv2-/- mouse cells can restore hematopoiesis. Etv2 binds directly to the Tal1 locus, which advert to Tal1 as a direct target downstream of Etv2 during hematopoietic development (Ema et al., 2003). The Tal1-/- ESCs, which are induced to form hematopoietic cells in vivo, can differentiate into hemangioblastic cells. However, these hemangioblasts are unable to generate endothelial and hematopoietic cells, instead they form colonies of vascular smooth muscle cells (D'Souza et al., 2005). This places the function of Tal1 downstream of the hemangioblast specification and/or the involvement in inhibiting the potential of the hemangioblast to form vascular smooth muscle. These results were found in vivo where Tal1-/- embryos initiated a cardiac transcriptional program in the yolk sac, with CD31+PDGFRα+ cardiac progenitors emerging and generating beating cardiomyocytes (Van Handel et al., 2012). It seems that Tal1, as well as Etv2, affect hematopoietic development in a temporal window.

Endoh and colleagues established Tal1 null ESC lines in which Tal1 expression is rescued by tamoxifen-inducible Cre recombinase loxP site-mediated recombination. In these mutant ESCs, no hematopoietic cells were detected until they reactivated the Tal1 gene. This reactivation leads to rescue of both primitive and definitive hematopoiesis, but only 2–4 days after the initiation of differentiation of ESCs on immortalized stromal cell line OP9. Tal1 reactivation at later phases was ineffective (Endoh et al., 2002). The second study confirming this theory use Tie2-Cre deleting of Tal1. Tie2 is expressed shortly after Tal1 in hemogenic and vasculogenic sites. After inactivation of Tal1 fetal liver hematopoietic cells was still detected, indicating that ablation of Tal1 in the later time of development, has no effect on hematopoietic development, at least not in a non-redundant fashion (Schlaeger, 2005; Souroullas et al., 2009).

Mice deficient for the transcription factor Runx1 do not have any HSCs or progenitors and are also devoid of IAHCs (embryos die around E11.5; (Cai et al., 2000; North et al., 1999; Okuda et al., 1996)). In addition, over-expression of Runx1 in a Runx1-/- ESC line resulted in an increased number of CD41+ hematopoietic cells (Lancrin et al., 2009). Cai and colleagues described the importance of the level of Runx1 expression during differentiation. In this work, Runx1+/— heterozygotes have reduced the production of HSCs in the AGM (Cai et al., 2000). To identify the temporal window of Runx1 activity VE-cadherin-Cre or Vav1-Cre mediated
The ablation of Runx1 at different stages of hematopoietic development was employed. At E8.5, VE-cadherin is expressed in endothelial cells of the yolk sac, dorsal aorta, and heart, and Runx1 ablation at this stage caused severe defects in hematopoiesis. However, when Cre was under the Vav1 control, which is restricted to hematopoietic cells and implicated as a Runx1 target, fetal and adult viability were unaffected, demonstrating that Runx1 expression is no longer required after the endothelial-hematopoietic transition (Chen et al., 2009).

Moignard and colleagues tried to reveal a sequence of activation of the key hematopoietic transcription factors in ESC differentiation. They described that process of hematopoietic differentiation begins with the activation of Etv2 (Moignard et al., 2013). Etv2 subsequently activates the core circuit including Tal1, GATA2, and Fli1 (friend leukemia integration 1), and suggested that the primary role of Etv2 is activation of this self-regulating triad. At the hemangioblast stage, the Tal1-GATA2-Fli1 triad forms a positive feedback loop that is independent of further activation by Etv2. Among the others, Runx1 expression is activated by this three transcription factors. In the hemogenic endothelium, there is close cooperation of Runx1 with Tal1, Fli1, and GATA2 to activate downstream factors (Moignard et al., 2013). For instance, it was reported that Runx1 activates GATA1, which in turn can disable GATA2 auto-regulation by binding to the GATA2 regulatory region. This mechanism could deactivate the autoregulatory loop of Tal1, Fli1, and GATA2 as preparation for differentiation. Thus the Runx1 activation of GATA1 could be involved in priming early hematopoietic progenitors for an erythroid fate (Grass et al., 2003; Pimanda et al., 2007).

1.2.2. PU.1, C/EBPα – transcription factors for myeloid specification

C/EBPα (CCAAT/enhancer-binding protein alpha) plays a nonredundant role in early granulocyte development. Tenen's group reported that C/EBPα null neonates lacked mature granulocytes, but had normal erythrocytes, megakaryocytes lymphocytes, and monocytes/macrophages. Zhang et al. introduced that granulocytic differentiation is blocked along with an accumulation of immature myeloid blasts during C/EBPα−/− fetal liver hematopoiesis (Zhang et al., 1996). Thus, disruption of C/EBPα in mice resulted in an early and specific differentiation block of granulocytes, indicating its essential role in early granulocytic commitment. On the other hand, disruption of C/EBPα in adult hematopoietic cells blocks the transition from the common myeloid progenitor to the granulocyte/monocyte progenitor. However, once myeloid maturation has reached the granulocytic-myeloid progenitor (GMP) stage, C/EBPα is no longer required (Zhang et al., 1996). In addition, the
enhanced expression of C/EBPα in common lymphoid progenitors (CLPs) induces granulocyte/monocyte development, indicating that C/EBPα can activate silenced myeloid programs to convert CLPs into GMPs (Zhang et al., 2004). Zhang et al. also discovered that C/EBPα plays a role in self-renewal and repopulating activity of HSCs. C/EBPα-deficient HSCs have increased expression of Bmi-1 and enhanced competitive repopulating activity (Iwama et al., 2004; Zhang et al., 2004).

PU.1 disruption causes embryonic lethality between E16.5 and E18.5. Morphological analysis and flow cytometry revealed no detectable myelocytes or lymphocytes and the loss of myeloid progenitors in PU.1-/- fetal liver. These studies indicated an early differentiation block on both myelopoiesis and B lymphopoiesis (Scott et al., 1994). Further, PU.1-/- pups were born with the expected Mendelian ratio, but they suffered septicemia within 2 days. Mutant pups could survive up to 2 weeks with antibiotic therapy and flow cytometry on bone marrow and fetal liver cells of those neonates detected no mature granulocytes or macrophages. A small number of aberrant neutrophils and macrophages was found in the bone marrow and spleen of the older pups, indicating a partial, yet severe impairment in myelopoiesis. These mice also exhibited a complete block in B cell differentiation and a delay in T cell maturation (McKercher et al., 1996). PU.1-/- ESCs fail to express a variety of characteristic myeloid genes, including CD11b and CD18, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) (Anderson et al., 1998; Iwama et al., 1998; McKercher et al., 1996). Interestingly, hematopoietic cells derived from PU.1-/- neonates and fetal liver can generate neutrophil colonies. However, they generate cells that exhibit some characteristics of monocytes, such as phagocytosis and expression of characteristic antigens, but they failed to develop into mature monocytes. Reintroduction of PU.1, but not granulocyte colony-stimulating factor–receptor (G-CSF-R) or macrophage colony-stimulating factor-receptor (M-CSF-R), into PU.1 -/- hematopoietic progenitors cells by retrovirus-mediated expression rescued myeloid and lymphoid development in vitro (Anderson et al., 1998; DeKoter and Singh, 2000).

In contrast to C/EBPα, studies of PU.1 expression and its genetic disruption suggest that hematopoietic cells can commit to the myeloid lineages in the absence of PU.1, but they fail to mature in a normal manner (Bjerregaard et al., 2003; Panopoulos et al., 2002).

PU.1 and the erythroid transcription factor, GATA-1, mutually antagonize their respective transcriptional activities. PU.1 can bind to the DNA binding domain of GATA-1 and block its ability to bind to DNA. Conversely, GATA-1 binds to PU.1 and displaces c-Jun, thereby
reducing expression of target genes. PU.1 and GATA-1 autoregulate their own expression functional effects in lineage commitment and gene expression (Rekhtman et al., 1999). In addition, the expression of PU.1 in erythroleukemia cells downregulated GATA-1 expression and caused a switch in lineage fate toward myeloid differentiation (Yamada et al., 2001).

1.2.3. GATA1, KLF1 – transcription factors for erythropoietic specification

GATA1 is essential for normal erythropoiesis and is expressed in primitive and definitive erythroid cells, megakaryocytes, eosinophils, and mast cells (Fujiwara et al., 1996; Pevny et al., 1991). GATA1 null mouse embryos die from severe anemia between E10.5 and E11.5. Studies with the chimeric mice and ESCs deficient for GATA1 have shown that these mutant cells are able to contribute to all different tissues in chimeric mice, with the exception of the mature red blood cells. More detailed, GATA1 null erythroid cells fail to mature beyond the proerythroblast stage (Pevny et al., 1991; Pevny et al., 1995). GATA1-/- ESCs are also unable of formation of mature erythrocytes and they arrest at the proerythroblast stage, in vitro. This finding holds for both primitive and definitive erythropoiesis (Weiss et al., 1994). The fate of common myeloerythroid progenitors (CMPs) to become megakaryocyte-erythroid progenitors or myeloid progenitors is thought to be determined by GATA1 and PU.1. Overexpression of GATA1 in myeloid precursors led to reprogramming resulting in erythroid and megakaryocytic differentiation (Iwasaki et al., 2003; Pevny et al., 1991; Pevny et al., 1995). Forced expression of PU.1 represses erythropoiesis and promotes myeloid differentiation in erythroid cell lines (Yamada et al., 2001).

Krüppel-like factor 1 (KLF1) is an erythroid-specific transcription factor. KLF1 null mice are embryonic lethal due to a defect in definitive erythropoiesis and subsequent severe anemia. The production of the first nucleated red cells in the yolk sac (primitive erythropoiesis) was not affected but the generation of enucleated erythrocytes in the fetal liver (definitive erythropoiesis) was severely impaired (Philipsen and Suske, 1999). Moreover, these embryos have a defect in hemoglobin accumulation, which might be explained by the KLF1 requirement for β-globin expression (Armstrong, Bieker, and Emerson, 1998). Wijgerde and colleagues identified that KLF1 is responsible for switching between embryonic (γ-globins) and fetal (β-globins; (Wijgerde et al., 1996)).

GATA1 can physically interact and functionally synergize with KLF1. KLF1 can recognize GC and/or CACC motifs, which are found in the close proximity to GATA motifs in several promoters, enhancers, and locus control regions suggest functional cooperation between these
proteins. It was demonstrated that GATA1 transcriptional activity can be synergistically increased by these interactions (Gregory et al., 1996). KLF1 is also a GATA1 target gene. Forced expression of GATA1 can activate the KLF1 promoter in nonerythroid cells, and KLF1 expression is downregulated in the absence of GATA1 (Whyatt et al., 2000) and restored upon its reintroduction.

The terminal proliferation, differentiation, and maturation of erythroid progenitor cells require Epo - EpoR signaling. Epo- and EpoR-deficient mice die during embryogenesis due to severe anemia because the erythroid progenitor cells cannot differentiate beyond the CFU-E stage. The fetal liver of mice deficient for Epo or EpoR has a normal number of erythroid colonies (BFU-E/CFU-E) but no mature erythroid cells. So, the Epo signaling is not essential for lineage commitment but vital for regulating definitive erythropoiesis by controlling the processes such as proliferation, survival and irreversible terminal differentiation of the late erythroid progenitors (Wu et al., 1995). Erythropoietin stimulation of erythroid precursors leads to phosphorylation and activation GATA1, which is in turn responsible for the induction of critical gene transcription e.g. β-globin or EpoR genes (Zhao et al., 2006).

### 1.2.4. Hematopoietic cytokines

The expression of mentioned transcription factors in hematopoietic development and regulation comes under the influence of extracellular regulatory signals in the form of hematopoietic cytokines. Numerous cytokines are involved in the regulation of hematopoiesis within a complex network of positive and negative regulators. Some cytokines have very narrow lineage specificities of their actions, while many others have rather broad and overlapping specificity ranges. The cytokines can induce cell viability and multiplication and can thus induce the formation of clones. These have been identified as G-CSF, GM-CSF, M-CSF, interleukins, Epo, and TPO (Lotem and Sachs, 2002; Metcalf, 2008).

There is also a number of other cytokines that exert significant roles in modulating hematopoiesis which include stem cell factor (SCF), transforming growth factor-β (TGF-β) or tumor necrosis factor-α (TNF-α) (Broudy, 1997; Dufour et al., 2003; Endele et al., 2014). A network of cytokine interactions allows considerable flexibility. It also allows a ready amplification of response to a particular stimulus such as bacterial lipopolysaccharide or the environmental stresses. It is well known that mitogen activated protein kinase (MAPK) p38α is one of the key signaling pathways, which is involved in the regulation of hematopoiesis by all the mentioned cytokines (Raman et al., 2007).
1.3. MAPK

Cells need to be constantly aware of changes in the extracellular surroundings to adapt their behavior by force of circumstances. Cellular responses to extracellular signals are executed by signaling pathways. Those transmit the signal from the surface of the cell into the nucleus and the consequently cells may react by the change of transcription activity. Mitogen activated protein kinases (MAPKs) seem to be involved in majority of signaling transduction pathways. MAPK belong to a large family of serine-threonine protein kinases activated by a broad range of extracellular stimuli that regulate a variety of critical cellular functions, including proliferation, differentiation, migration, and apoptosis. In mammals, we distinguish four major subfamilies; extracellular signal-regulated kinases (ERKs), p38 MAPK (p38), c-Jun amino-terminal kinases (JNKs) and ERK5/BMK1.

Each group of MAPKs is activated by a distinct kinase cascade (Figure 7) in which a MAP3K or MEKK phosphorylates and activates a downstream dual specificity MAP2K or MEK, which in turn stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues within a conserved tripeptide motif (Thr-X-Tyr; (Raman et al., 2007)).

1.3.1. p38 MAPK

MAPK p38 (also known as CSBP, mHOG1, RK, and SAPK2) is the archetypal member of the MAPK family in mammalian cells. MAPK p38 is activated by a variety of extracellular stimuli, particularly cellular stresses, such as osmotic shock, UV radiation, hypoxia, inflammatory cytokines, and growth factors (Raman et al., 2007). Importantly, p38 kinase can be also activated by oxidative stress, through the production of reactive oxygen species (ROS) (Son et al., 2011). The first member of the p38 MAPK family was isolated as a 38-kDa protein rapidly tyrosine phosphorylated in response to lipopolysaccharide (LPS) stimulation in murine peritoneal macrophages. This protein was found to be the homolog of Saccharomyces cerevisiae Hog1, an important regulator of the osmotic response, and is now referred to as p38α (Han et al., 1995).

Four splice variants of the p38 family have been identified: p38α, p38β, p38γ, and p38δ. While p38α and p38β are expressed ubiquitously, expression of p38δ is restricted to lung, pancreas, kidney, testis, and small intestine. Expression of p38γ is limited to skeletal muscle. Sequence comparisons revealed that the four p38 isoforms share only 60% identity, further suggesting distinct cellular functions (Geest and Coffer, 2009).
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Figure 7. MAPK cascade. Each group of MAPKs is activated by a distinct kinase cascade in which a MAP3K or MEKK phosphorylates and activates a downstream dual specificity MAP2K or MEK. Activated MAPKs phosphorylate various substrate proteins (e.g., transcription factors), resulting in regulation of various cellular activities (e.g., proliferation, differentiation, inflammatory responses, and apoptosis); adapted from (Son et al., 2011).

Stimuli that activate p38 typically activate also JNK, but only p38 is inhibited by the anti-inflammatory drug SB203580, which has been extremely useful in delineating the function of p38. This inhibitor specifically influences p38α and partially p38β isoforms (English and Cobb, 2002). The canonical p38 pathway consists of several upstream kinases MAP3Ks, including MEKK1-4, MLK2 (mixed lineage protein kinase) and -3, DLK (dual leucine zipper kinase), ASK1 (Apoptosis signal-regulating kinase 1), Tpl2 (tumor progression locus 2; also termed Cot), and Tak1. The MAP3KK activates the MAPKKs MEK3 and MEK6 (also termed MKK3 and MKK6). MKK3 and MKK6 (also called SKK3) are highly selective for p38 MAPKs and do not activate JNKs or ERK1/2. MAP3K activation of p38 MAPKs occurs via dual phosphorylation of their Thr–Gly–Tyr motif, in the activation loop. MKK3 and MKK6 show a redundancy in the knockout studies and are the major...
upstream regulators of p38, but in some circumstances e.g. UV radiation MKK4, an upstream of JNK can activate p38. The MKK required for p38 activation may not only be affected by the stimuli, but also by cell type as their level of expression varies. MKK3 has been shown to be p38 activator in mesangial cells stimulated by transforming growth factor (Cuenda and Rousseau, 2007), while MKK6 appears to be the predominant isoform in thymocytes. MAPK p38 regulates the phosphorylation of various proteins, such as the kinase MAP kinase-activated protein kinase 2 and 3 (MAPKAPK-2 and -3), the small 27kDa heat shock protein (Hsp27), and several transcription factors – C/EBPβ, ATF-1/2, CHOP, STAT1, STAT3, and the others (Cuadrado and Nebreda, 2010).

Depletion of p38α MAPK in adult mice resulted in enhanced proliferation and defective differentiation of lung stem and progenitor cells (Ventura et al., 2007). Knockout of p38α is embryonic-lethal, and most p38α−/− embryos die between E10.5 and E12.5 as a result of a defect in placental angiogenesis. However, some mice deficient of p38α survive until E16.5 and they evince anemia as a result of abnormal erythropoiesis (Tamura et al., 2000). MAPK p38α has been also demonstrated to regulate the differentiation and proliferation of various cell types, including myocytes, cardiomyocytes, neurons, adipocytes, and immune cells (Adams et al., 2000; Aouadi et al., 2006; Engel et al., 2005; Geest et al., 2009; Yang et al., 2014; Zhang et al., 2018). In contrast to p38α, mice lacking p38β, p38γ and p38δ are viable and exhibit no pathological changes (Cuenda and Rousseau, 2007). Nevertheless, subsequent studies showed that p38δ and p38γ are at least partially involved in various metabolic diseases, cancer and tissue regeneration (Gillespie et al., 2009; Lluis et al., 2006; Schindler et al., 2009).

MAPKs are considered as major players during the inflammatory response. p38α MAPK was first recognized for its role in inflammation in regulating the biosynthesis of pro-inflammatory cytokines, namely IL-1 and tumor-necrosis factor α (TNFα), in endotoxin-stimulated monocytes. Several lines of evidence suggests that p38 plays also an important role in arthritis and inflammation of the liver, kidney, brain, and lung and that it acts as a critical player in inflammatory diseases mediated by macrophages. Interestingly, with respect to inflammatory responses, several studies have reported p38 regulation in macrophages treated with LPS, endothelial cells stimulated with TNF-α, U1 monocytic cells treated with IL-18, and human neutrophils activated with mediators of inflammatory reaction. p38α is involved in the expression of proinflammatory mediators in macrophages such as IL-1β, TNF-α, PGE2, IL-12, COX-2 (cyclooxygenase), IL-8, IL-6, IL-3, IL-2, and IL-1 (Kang et al., 2008). Moreover,
p38 can regulate the production of endothelial vascular cell adhesion molecule-1 (VCAM-1), which participates in cell proliferation and differentiation of the immune response (Cook-Mills et al., 2011). Furthermore, p38 is associated with various inflammatory diseases, including endotoxin-induced shock, collagen-induced arthritis, granuloma, diabetes, and acute lung inflammation (Eiró and Vizoso, 2012; Provinciali et al., 2011; Wee Yong, 2010; Wyss-Coray and Rogers, 2012).

1.3.2. p38α in hematopoiesis and hematopoietic cells

As mentioned above, in vivo study of p38α knockout proved impairment of angiogenesis and hematopoiesis in embryos which lead to lethality. A large body of evidence indicates that p38α activity is critical for normal immune and inflammatory responses. Furthermore, it was observed that p38α is involved in the maintenance of stemness of hematopoietic stem cells. All these suggestions presume that p38 plays a central role in maintaining the homeostasis of hematopoiesis by balancing, promoting, and inhibiting signals. It is well established that many cytokines and growth factors, including stem cell factor, IL-3, Epo, G-CSF, M-CSF, GM-CSF, and TPO, regulate normal hematopoietic cell proliferation, survival, and differentiation by activating p38α MAPK pathways to generate their effects (Foltz and Schrader, 1997; Miyazaki et al., 2001; Nagata et al., 1997; Okuda et al., 1992).

HSCs are defined by their ability to both life-long self-renew and give rise to all mature blood cell lineages. A tight balance between self-renewal and differentiation is crucial to maintain the integrity of the entire hematopoietic system, preventing exhaustion of the stem cell pool or development of hematopoietic malignancies such as leukemia (Foudi et al., 2009; Takizawa et al., 2011; Wilson et al., 2008). MAPK signaling has been demonstrated to play a key role in the maintenance of HSC quiescence. The activation of p38 MAPK in response to increasing levels of ROS limits the lifespan of HSCs in vivo. This observation was confirmed in a study with mice deficient in the gene encoding Atm, a cell cycle checkpoint regulator that is also thought to be involved in regulating oxidant level. In Atm-/- mice, constitutively activated p38 MAPK pathway resulted in the defects in the maintenance of HSC quiescence (Ito et al., 2006). Notably, aberrant activation of p38α in HSCs has been shown to impair HSC self-renewal and cause HSC premature exhaustion in several human hematological diseases and in the Atm mutant and FoxO3 genes knockout mice (Ito et al., 2006; Miyamoto et al., 2007). Treatment with an antioxidant N-acetyl-l-cysteine (NAC) or p38 MAPK inhibitor rescued ROS-induced defects in HSC repopulating capacity and in the maintenance of HSC
quiescence, indicating that the ROS-p38 MAPK pathway contributes to exhaustion of the stem cell population (Ito et al., 2006). Another work supports the connection between oxidants and stem cell aging and indicates that inactivation of p38 MAPK protects HSCs against loss of self-renewal capacity (Jang and Sharkis, 2007; Jung et al., 2016).

During differentiation of hematopoietic cell, MAPK p38 plays an important role. It has been demonstrated that p38 is implicated in the regulation of TEL (a member of the ETS family of transcription factors). TEL plays a critical role in the establishment of postnatal hematopoiesis for all lineages due to its involvement in transition of hematopoietic activity from fetal liver to bone marrow (Hock et al., 2004; Wang et al., 1998). p38α can phosphorylate TEL and so reduce its transcriptional repression abilities (Arai et al., 2002).

Another critical role has p38 MAPK in erythropoiesis, p38 MAPK is activated by Epo and is suggested that p38 is necessary for the initiation of erythroid differentiation. The inhibition of p38α by SB203580 suppressed differentiation of erythroleukemic SKT6 cells (Nagata and Todokoro, 1999). It has been demonstrated that activation of p38 by Epo, IL-3, G-CSF, and TPO stimulates HSC/HPC proliferation and differentiation (Foltz and Schrader, 1997; Miyazaki et al., 2001; Nagata et al., 1997; Okuda et al., 1992). In contrast, p38 activation by myelosuppressive cytokines such as tumor necrosis factor-α, interferon-γ, and tumor growth factor-β inhibits HSC/HPC proliferation and induces HSC/HPC apoptosis (Kang et al., 2008; Katsoulidis et al., 2005; Verma et al., 2002). These findings indicate that p38 regulates hematopoiesis in a cell context-dependent manner.

MAPK p38 is also involved in myeloid differentiation. Geest et al. observed an important role of p38 in regulation of expansion and differentiation of myeloid progenitors during granulopoiesis where inhibition of p38α increased neutrophil differentiation. The constitutively activated p38α inhibited neutrophil differentiation completely. The suggested molecular mechanisms included inhibition of phosphorylation of C/EBPα on serine 21 in response to p38α activation (Geest et al., 2009). Interestingly, while phosphorylation of C/EBPα caused inhibition of proliferation and differentiation of neutrophils, the number of eosinophils was on the contrary increased. In short, MAPK p38 plays a role in myelopoiesis and its differentiation into a particular type of myelocytes.

The other studies investigating the role of p38 in hematopoiesis were published, focused on the migration of hematopoietic cells. Several authors highlighted that MAPK p38 activity is essential for chemotaxis of mast cells, eosinophils, neutrophils, macrophages and monocytes (Ayala et al., 2000; Kampen et al., 2000; Ogura and Kitamura, 1998; Sundström et al., 2001;
Zu et al., 1998). Somervaille observed that BFU-E migration, but not adhesion to fibronectin, is also p38 MAPK dependent, demonstrating a wide role for p38 MAPK activity in hemopoietic cell movement (Somervaille et al., 2003).

In summary, p38 MAPK signaling is critically involved in different steps in hematopoietic regulation. In the developmental approach, it is important for the establishment of hematopoiesis as well as for the maintenance of hematopoietic stem cells.
1.4. Stem cells

Stem cells are defined by two abilities, one of them is the ability to self-renew themselves and the second is differentiation to another type of cells. This is ensured by asymmetric replication/division, which means that a stem cell divides into one mother cell that is identical to the original stem cell, and another daughter cell that becomes differentiated. According to the number of types of the new arise differentiated cells, we can distinguish pluripotent, multipotent and unipotent cells. Totipotent stem cells are cells that have the capacity to self-renew by dividing and to develop into the three primary germ cell layers of the early embryo and into extra-embryonic tissues such as the placenta. Pluripotent stem cells can give rise to all cell types of the body (but not the placenta). While in vivo totipotent and pluripotent stem cells can be found only in the prenatal age for a very short time, the multipotent and unipotent stem cell can be found in the various tissue also after birth and in the adults. Multipotent stem cells can develop into a limited number of cell types in a particular lineage, e. g. HSC are a multipotent stem cell. Unipotent cells keep the ability of self-renewal but have a very limited ability to differentiate compared to other stem cell. They can differentiate only to one cell type, e. g. limbal epithelial stem cells (Mort et al., 2012). Cultivation of stem cells in vitro introduces a unique model for developmental biology, drug screening, and in the case of human embryonic stem cells a potential source for cell replacement strategies.

1.4.1. Pluripotent stem cells in vitro

Embryonic stem cells are critical tools for genetic engineering, development of stem cell-based therapies, and basic research of pluripotency and early lineage commitment. Embryonic stem cells are cells with pluripotent potential, which are derived from the inner cell mass (ICM) of the blastocyst, approximately from E3.5–4.5 pre-implantation embryos in mice. The inner cell mass is removed from its normal embryonic environment and cultured under appropriate conditions. Mouse embryonic stem (mES) cells were first derived in 1981 by two groups using different protocols (Evans and Kaufman, 1981; Martin, 1981). Both studies generated fully pluripotent cells able to self-renew if maintained on mouse embryonic fibroblasts or in feeder-free conditions supplemented with leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). The cytokine LIF is necessary to maintain cells in their pluripotent state (Pease et al., 1990; Williams et al., 1988).
It was another seventeen years before human embryonic stem (hES) cells derived from the inner cell mass of the human blastocyst (stage 4-5 days post-fertilization) were isolated by Thomson et al. in 1998. In 2006, Yamanaka and colleagues published a new revelation, induced pluripotent stem. Induced pluripotent stem cells show the characteristics of self-renewal and pluripotency as well as ESCs. They can be maintained in culture in an undifferentiated state for several months and can be induced to differentiate into derivates of all three germ layers (Yamanaka and Takahashi, 2006).

Mouse embryonic stem cells have been now studied for several decades and have provided major advances in our understanding of developmental biology and gene function in the adult organism. The single greatest application of mouse embryonic stem cells has been in studies of gene function through homologous recombination (knockout or knockin strategies). These studies were made possible by the remarkable ability of genetically modified embryonic stem cells to incorporate into all tissues of a developing mouse after injection into a blastocyst, followed by the ability to result in chimeric mice to pass the genetic modification via the germline. Embryonic stem cells have also been useful tools for understanding molecular events controlling differentiation into the early germ layers and more distal branches of the developmental tree.

### 1.4.2. Hematopoietic differentiation of ESCs

Strong evidence supports the hypothesis that ESCs differentiation accurately recapitulates the early developmental events occurring during embryogenesis. Detailed analyses have suggested that the kinetics of hematopoietic development from ESCs, including expression of cell surface markers and hematopoietic genes, closely resemble the one of the yolk sac, indicating that ESCs provide an excellent model in which to study the developing hematopoietic system. To date, erythroid, myeloid, and lymphoid lineages have all been reproducibly generated from in vitro differentiating mouse and human ESCs (reviewed in Batta et al., 2016). Nonetheless, the most challenging goal remains the derivation of long-term engrafting HSCs, which has eluded the field for several decades now. Even though there exist studies using mouse ESCs, which shown clearly detectable engraftment of derived HSC/HPC on transplantation in mouse recipients, they use serum-supplemented culture in their protocols (Burt et al., 2004). The serum related factors are specific but unknown. For this reason, it is difficult to determine the specific condition for differentiation of repopulating blood cells. Two studies reported the generation of blood cells with engrafting capacity from
human ESCs. However, they also used serum-supplemented cultures and/or stromal cell lines (Kim et al., 2017; Ledran et al., 2008; Wang et al., 2005). As well as in the studies with mESC, the use of serum and stroma compromises the reproducibility of the differentiation protocols, hindering their validation. The studies, which use serum-free conditions in their protocols are able to derive hematopoietic progenitors with repopulating capacity, but secondary transplantation assays were not successful, suggesting that these culture conditions produced multipotent progenitors with poor self-renewal potential (Lengerke et al., 2008; Nostro et al., 2008; Pearson et al., 2008).

The addition of growth factors and cytokines and exposure to stromal coculture was sufficient to drive ESC differentiation toward many adult cell types. The most common differentiation protocol is based on suspension cultures in which ESCs form aggregates termed embryoid bodies (EBs), containing derivatives of all three germ layers. The two types of protocols are used to stimulate the differentiation of the precursors from EBs into hematopoietic lineages. The first step is disaggregating the EBs to single cell suspension and then to seed into the culture with a specific condition. These aggregated cells could be seeded into semi-solid media with specific cytokines, for the support of all hematopoietic colony forming units or the cytokines could be specific only for certain lineages (e.g. erythroid, myeloid; see in supplements - Material and methods). The second method is to seed the disaggregated cells from EBs on the mitotic inactivated stromal cells (e.g. OP9 lineage).

When ESCs were plated in methylcellulose with vascular endothelial growth factor (VEGF) and Kit ligand, two cytokines important for hematopoietic development, they produced cells with blast morphology, called blast colony-forming cells (BL-CFCs). BL-CFCs could generate cells of multiple hematopoietic lineages that express the hemato-endothelial markers FLK1, CD34, Tal1 and GATA1, but not the pan-mesodermal marker Brachyury (Kennedy et al., 1997; Moignard et al., 2013). Intensive research of hematopoiesis using ESCs as a unique starting cell population has enabled differentiation and isolation of diverse hematopoietic cell lineages. A better understanding of the intrinsic regulators and signaling pathways driving hematopoietic specification of ESCs is highly demanded, as well as elucidation of functional impact of early hematopoietic regulators that more closely mimic the in vivo developmental program of the hematopoietic specification. The MAPK p38 signaling pathway is involved in the transduction of a variety of signals and its downstream targets critically regulate biological responses. Moreover, as highlighted above, p38α is involved during hematopoietic
development. Hence, we decided to study closer the role of p38α MAPK in the establishment of hematopoiesis in differenting ESCs.
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2. **Aims**

1. To analyze the involvement of p38α kinase in the development of hematopoietic progenitors.
   - How the depletion of p38α kinase influences the cell fate of ESCs?
   - What impact has the mutation of p38α kinase during hematopoietic differentiation from ESCs?
   - Is there any difference between differentiation of p38α+/- and p38α-/- cells in specific hematopoietic cell types?

2. To compare inhibitors of MAPK p38α and p38α mutant ESCs and hematopoietic differentiation.

3. To compare the treatment with inhibition of p38α kinase in the culture with cells from bone marrow and fetal liver.

4. To compare the gain data with NAC treatment of the p38α mutant and wild type ESCs, cells from bone marrow and fetal liver.
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3. Results and discussion

In this section, I would like to summarize my work during my doctoral study. The aims specified in the previous section were determined experimentally and results were published or are discussed below. The publications are an integral part of this Dissertation thesis and are attached. Articles are referenced by Roman numerals as presented in the list of publications. The short summary of the articles is provided below following with discussion and comments in the context of other studies.

In my thesis, I focused on the p38α MAPK and its involvement in the hematopoietic differentiation of ESCs (Article I). I was engaged in the cell fate studies of mutant p38α-/− ESCs, especially in comparison to p38α wild type ESCs. The one distinction of p38α-/− is higher production of ROS, which could influence the differentiation potential into specific germ layer (Article II). Further we assessed the overall production of ROS in ESCs (Article III) and demonstrated unexpected prooxidative activity of NOXs inhibitors and their effect of signaling and ESCs maintenance. The next part of my research was dedicated to the difference between the differentiation potential of the hematopoietic progenitor cells from the fetal liver and bone marrow and the involvement of p38α kinase. Finally, we assessed the relevance of antioxidant NAC in the connection with the ROS and hematopoietic differentiation. The results gained from the unpublished experiments are preliminary and require following confirmation. The material and methods, which are not involved in the published articles, are included in the supplementary section.

Article I. MAPK p38alpha Kinase Influences Haematopoiesis in Embryonic Stem Cells.
The results presented in this article show that p38α affects hematopoiesis in at least three different ways. First is the need for p38α kinase during the formation of hemangioblast in vitro. Next is the involvement of p38α in erythropoiesis and erythrocyte maturation. Finally, the third role is regulation the differentiation of common myeloid progenitor (CMP) cells into granulocyte lineages.

Article II. The depletion of p38alpha kinase upregulates NADPH oxidase 2/NOX2/gp91 expression and the production of superoxide in mouse embryonic stem cells.
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Article III. Apocynin and Diphenyleneiodonium Induce Oxidative Stress and Modulate PI3K/Akt and MAPK/Erk Activity in Mouse Embryonic Stem Cells.
In this work analyzed the ROS production in mouse ESCs and demonstrated how NOX inhibitors affected ROS production, proliferation and differentiation of mESCs. We employed inhibitors of NOXs the apocynin (APO) and diphenyleneiodonium chloride (DPI). Our study demonstrates surprising pro-oxidant activity of APO and DPI in undifferentiated mESCs with impairment of cell proliferation. Moreover, APO markedly downregulates activity of Akt and its downstream Nanog and augments Wnt signaling. DPI promotes Akt and Erk (extracellular signal mediated kinase; the member of MAPK) activation. Importantly, based on the results from this article, we further employed NAC as bona fide antioxidant in our future studies.
3.1. Profile of ESCs deficient for p38 MAPK and their wild type.

Intensive research of hematopoiesis using embryonic stem cells as a unique starting cell population has enabled differentiation and isolation of diverse hematopoietic cell lineages. A better understanding of the intrinsic regulators and signaling pathways driving hematopoietic specification of ESCs is highly demanded, as well as the need to study the functional impact of early hematopoietic regulators that more closely mimic the *in vivo* developmental program of the hematopoietic specification.

In this work, we used cells deficient for p38α (p38α--/--) and their wild type (p38α+/+) cells described by Kim et al. 2005 (kindly provided by Dr. Barry P. Sleckman, Washington University School of Medicine at St. Louise; (Kim et al., 2005)). The generation of these cell lines has been described in detail in the given reference. The qRT-PCR and Western blot methods were used for verification of the genotype and phenotype of these cells, as you can see in Fig. 8 A, B. The ESCs are maintained in an undifferentiated state in monolayer with the addition of LIF into media (Fig. 8 D). The ESCs express the markers typical for mouse ESCs, such as Oct3/4 or Nanog (II). The differentiation of the cells is induced spontaneously by seeding the cells on the non-adhesive plastic and removing LIF from the medium. In these conditions the cells aggregate and form EBs, the 3D structure contained cells from all three germ layers (Fig. 8 C, E, F).

It is known that undifferentiated ESCs have low ROS level and that this feature is important for their phenotype. During differentiation, there is an increase in the ROS level and the redox alterations are though to play a role in the balance between self-renewal and differentiation. It was shown that short term increase of the ROS favors the differentiation into cardiomyocytes and into endoderm and mesoderm lineage (Cho et al., 2006; Ji et al., 2010; Sauer et al., 1999). On the other hand, the p38α--/-- mESCs, which have higher level of ROS in comparison to wild type cells, favor the differentiation into cells of neuronal lineage (II.). NADPH oxidase (NOX), dual oxidase (DUOX) and oxidative reaction on the mitochondrial membrane are the main sources of ROS. The regulated ROS production by NOX is linked to regulation of different processes including proliferation, migration, differentiation, immunomodulation and oxygen sensing (Bedard and Krause, 2007). In the article III. Overall ROS level and proliferation and differentiation of mESCs. In our experiments, we observed generally low level of NOXs/DUOXs expression in mESCs (III).
NOX4 expression was the highest in mESCs (III), which is in agreement with other authors Li et al., 2006; Xiao et al, 2009). To further elucidate involvement of NOX in mESCs regulation we used the NOX inhibitors, vanillin derivative 4-hydroxy-3-methoxyacetophenone (trivial name: apocynin, APO) and diphenyleneiodonium chloride (DPI) – treatment with APO and DPI resulted in modulation of intracellular pathways critical for regulation of proliferation and differentiation. APO downregulated activity of Akt and its downstream Nanog and augmented Wnt signaling. DPI promotes Akt and Erk activation (III). Equally important, we have revealed unexpected pro-oxidant activity of both APO and DPI in mESCs and consequently rather employed NAC as *bona fide* antioxidant in our later studies (III).

There is no significant difference in proliferation and/or in the basic pluripotent phenotype between p38α mutant and wild type cells (II). However, in the p38α-/- cells we detected higher production of ROS compared to the p38α+/+ ESCs (II). ROS can induce stress and/or their level might increase as a part of the stress response. MAPK p38 is suggested to play a key role during cell response to various stress stimuli. Thus, it advert to the involvement of p38 in the maintenance of the intracellular redox status and balance. We investigated the role of p38 kinase and the level of ROS during differentiation of ESCs. ESCs exhibit a very low level of ROS (III), however, during the differentiation, the level of ROS increases. The mitochondria of ESCs are immature and the expression levels of NADPH oxidase (NOX) or dual oxidase (DUOX) are insignificant. Beside the mitochondria, the NOX and DUOX enzyme complexes, participate in the generation of intracellular superoxide (III) (Bedard and Krause, 2007; Brand, 2016). We focused on the expression of NOXs, DUOXs, and mitochondrial activity. The mitochondria mass was lower in p38α-/- cells in comparison with p38α+/+ cells. Whereas, the higher ROS production in the mutant cells correlates with the upregulation of NOX2 expression (II). Based on these observations we hypothesized that there exists a possible negative feedback mechanism between ROS, p38α, and NOX2. Briefly, higher ROS induce activation of p38α kinase, which in turn inhibits NOX2 transcription and it leads to restoration of ROS level.
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**Figure 8. The comparison of p38α-/− and p38α+/+ ESCs.** A) Expression level of the p38α in the p38α+/+ and p38α-/− ESCs and 10ds EBs. B) Protein level of the p38α, comparison in the mutant and wild type ESCs. C) The growth of volume (nL) of wt and p38−/− embryoid bodies. Morphology of non-differentiated feeder-free adopted ESCs (D), 6 days old compact EBs (E), and 14 days old EBs with cavities (F).
3.2. Involvement of p38α kinase in the development of hematopoietic progenitors

The MAPK p38 and its downstream targets critically regulate a variety of biological responses, including apoptosis, proliferation, and differentiation. In our work, we investigated the role of MAPK p38 in the hematopoietic commitment during differentiation from ESCs. The in vivo studies engaged in the involvement of MAPK p38 in development observed that majority of p38α null mice embryos dies between E10.5 and E12.5 as a result of defects in placental angiogenesis (Adams et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). However, some of these embryos survived until E16.5. They exhibited a normal morphology but were anemic as a result of stress-induced abnormal erythropoiesis (Tamura et al., 2000). Moreover, Hadjal et al. proved that p38α deficient cells differentiate slower into mesoderm but have enhanced differentiation into ectodermal cell lineages (Hadjal et al., 2013). We observed that the transcription activity of the variety of hematopoiesis markers is inhibited during differentiation of the p38α-/- cells. The markers involved in primitive and definitive hematopoiesis, as well as markers for HSC and the erythroid and myeloid lineages, were studied. Our results are in agreement with these studies. Mutant p38α-/- ESCs have lower expression of the markers specific for mesodermal lineage such as Flk1 (receptor of VEGF), the typical marker for hemangioblast, the common precursor of hematopoietic, endothelial and smooth muscle cells (Choi et al., 1998; Kennedy et al., 2006; Lu et al., 2007; Nishikawa et al., 1998b), but the expression of VEGF is not influenced (I).

The defect in the transcription of factors GATA2, Etv2, SCL/Tal-1 lead to embryonic lethality because of impaired hematopoiesis or/and vasculogenesis. The depletion of Gata2 results in embryonic lethality at E11.5, in part, due to anemia (Tsai et al., 1994). Etv2 mutant embryos are nonviable after E9.5 and these embryos lack hematopoietic and vascular lineages (Kataoka et al., 2011). Tal1-/- embryos die at around E9.5 with an absence of hematopoietic cells (Robb et al., 1995). The similar effect was observed in the knockout of p38α mice (Tamura et al., 2000). Our results show that the expression levels of GATA2 and Etv2 are influenced by the depletion of p38α MAPK (I). The expression of Etv2, which is considered to play a role in the differentiation of hemangioblast, is decreased in 6, 10 and 14days of differentiation of p38α-/- ESCs. While the expression level of GATA2 is not influenced before 14days of differentiation. This supports the previous statement that Etv2 is important in the earlier mesodermal differentiation and GATA2 plays a role later in the development (Shi et al., 2014). Interestingly, the level of Tal1 is not altered, but when we then focused on
the expression of Runx1, the direct downstream factor of Tal1, we observed downregulation of Runx1 in p38α-/- cells. The transcription factor Etv2 and Tal1 are involved in the early steps of hematopoiesis, particularly Etv2 in hemangioblast differentiation, Tal1 in erythropoiesis in the yolk sac and differentiation of hematogenic endothelium. The Runx1-/- mice die in utero at E12.5 and their fetal liver contains only primitive erythroblasts. Chen et al. demonstrated that Runx1 is crucial in the endothelio-hematopoietic transition (Chen et al., 2009). Moreover, deletion of Runx1 gene blocks both erythro-myeloid hematopoietic progenitors (CFU-C) and HSC formation (Cai et al., 2000), which leads to severe anemia and embryonic death by E12.5. Consequently, summarizing of our observation of expression levels of transcription factors important for hematopoiesis indicate that the p38α MAPK plays a role in the certain stages of the hematopoietic development.

When we focused on the expression level of markers connected with hematopoietic stem/progenitor cells, such as CD38, CD34 or CD150, we observed that the level of expression was also reduced in p38α-/- differentiating cells. However, the expression of markers Sca1 and c-Kit, which are presented as typical markers for hematopoietic cells, were enhanced. Additionally, when we tested the origination of hematopoietic progenitor via colony forming assay, we observed the reduced number of these progenitors in EBs formed from p38α-/- ESC in comparison to the wild type (I). These findings led us to investigate if this increase can be caused by differentiation of the p38α-/- into some other lineages. Withal, p38α-/- ESCs differentiated to a neural lineage with higher frequency in contrast to wild type (Aouadi et al., 2006; Duval et al., 2006). The levels of three transcripts representing neural markers (Sox1, Nestin, MASH1) were determined and we observed that Sox1 and MASH1 had higher expression levels in p38α-/- cells in comparison to wild type cells, while the expression level of Nestin was unchanged (II). The increased level of neurogenesis markers could be in part explained by the higher transcription level of c-Kit accordingly to study of Keshet and colleagues which described the expression of c-Kit transcripts on the dorsal aspect of the neural tube during embryonic development (Keshet et al., 1991). Moreover, expression of the c-Kit transcript is found in some neurons in the adult brain (Matsui et al., 1990; Motro et al., 1991; Zhang and Fedoroff, 1997).

Barruet et al. in their work show that p38α MAPK controls the mesodermal commitment of the embryonic stem cells. They observed that deficiency of p38α in ESCs lead to defect in differentiation into some of the mesodermal lineages such as smooth muscle cells, cardiac cells, skeletal muscle or endothelial cells (Barruet et al., 2011). On the other hand, Guo et al. demonstrated that p38α is not essential for ESC differentiation to endothelial or smooth
muscle cells in vitro (Guo et al., 2007). The data from in vivo experiments show certain control of angiogenesis in early p38α/- embryo, but if these embryos survived until E16.5 they embodied normal morphology but were anemic (Tamura et al., 2000). Besides, the studies with endothelial progenitor cells (EPCs) demonstrated that the proliferation of EPCs was significantly augmented after treatment of EPCs with the p38 inhibitor SB203580 (Seeger et al., 2005). Therefore, the expression levels of Nkx2.5, CD31 (PECAM), Tie2 and VE-Cadherin were investigated (Fig. 9). In agreement with previous work of Aoudi et al. (Aouadi et al., 2006) Nkx2.5, a marker of cardiomyogenesis, was moderately decreased in p38α/- cells (Fig. 9 A). On the contrary, the level of endothelial markers CD31, Tie2 and VE-Cadherin were elevated in 10 and 14 days EBs from p38α/- ESC (SFig. 9 B, C, D).

Accordingly, our results suggest, that the elevated expression of c-Kit and Sea1 in p38α/- cells is induced by differentiation of these cells into endothelial lineage rather than hematopoietic lineage. However, when we focused on the phenotype of these cells and analyze the expression of CD31 on the cell surface, the FACS analyzes did not show any difference between p38α+/+ and p38α/- (Fig. 9 E). Conclusively, these findings lead to the assumption that p38α MAPK is crucial for differentiation of endothelial lineage in the early mesodermal commitment, but when they reach to later phase of the development, it becomes more important for hematopoietic differentiation.

Embryoid bodies derived from mouse ESCs have been shown to be useful in vitro model for the study of a variety of differentiation programs, including hematopoiesis. A large number of studies have documented the development of various erythroid, myeloid and, to a lesser extent, lymphoid lineages within EBs (reviewed in Keller, 2005). As the p38α is known to regulate the proliferation of myeloid and erythroid progenitor cells (Kapur et al., 2002), we aimed to compare the hematopoietic differentiation of p38α/- ESCs and their wild type, in particular with regard to its role in the regulation of erythropoiesis and myelopoiesis. For the analyses of the formation of specific hematopoietic progenitors in embryoid bodies, we used the semisolid medium with specific cytokines for differentiation of erythropoietic colonies, or colonies of granulocytes and monocytes/macrophages.
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It was proposed that anemia during development in p38α deficient mice embryos was connected with diminished Epo expression (Tamura et al., 2000). Wu et al. demonstrated that for the formation of early erythroid progenitors, burst forming unit - erythrocyte and colony forming unit - erythrocyte (BFU-E and CFU-E), Epo is not important, but plays the role in regulating definitive erythropoiesis by controlling processes such as proliferation, survival, and irreversible terminal differentiation of the late progenitors of CFU-E (Wu et al., 1995).

![Figure 9](image-url)

**Figure 9.** Analyzes of expression of markers of mesodermal lineage other than hematopoietic cell line – qRT-PCR. A) Nkx2.5 is the marker of cardiomyogenesis, B, C, D) Tie2, CD31, and VE-cad – are markers of endothelial differentiation. E) FACS analyze of marker CD31. The comparison of expression of these markers during the differentiation of p38α+/+ and p38α−/− cells in the 6, 10, and 14 days old EBs.
Our results indicate that the deficiency of MAPK p38α also plays another role in erythroid commitment (I). The total number of BFU-E and CFU-E colonies formed from erythroid progenitors was decreased in EBs from p38α-/- ESCs. Additionally, the transcription levels of mRNA of erythropoietic markers (hemoglobins, GATA1, Klf1, and EpoR) were decreased in the mutant cells. GATA1 has an effect on the maturation of erythrocytes. As demonstrated by Pevny et al., GATA1 null erythroid cells fail to mature beyond the proerythroblast stage (Pevny et al., 1991; Pevny et al., 1995). Furthermore, the formation of mature erythrocytes in vitro in GATA1/- ESCs is also severely impaired (Tsai et al., 1994). We observed a decline in expression of GATA1 in p38α-/- cells during differentiation, which also highlights the anemic phenotype of the p38α mutant embryos and ESCs. These results strongly suggest that the absence of p38α results in a block to erythroid differentiation/maturation.

Geest et al. in their work demonstrated the involvement of MAPK p38α in the myelopoiesis. Their results show that p38 MAPK activity is detrimental for neutrophil development but required for eosinophil differentiation (Geest et al., 2009). Moreover, they focused on the transcription factor C/EBPα, which plays a critical role in myelopoiesis. Expression of C/EBPα is detectable in early myeloid precursors and is upregulated on a commitment to granulocytes (Radomska et al., 1998). The similar results we observed in the case of myeloid markers PU.1 and M-CSFR (CSFR1 – colony stimulating factor receptor 1), as their expression levels were also decreased in the p38α-/- EBs. Thus, the MAPK p38α is involved also in the early development of myeloid progenitors (I).
3.3. Effect of inhibitors of p38 MAPK on hematopoietic differentiation of ESC.

In the above-described experiments, we showed that p38α depletion affects ESCs differentiation to the hematopoietic cell types. To further confirm this conclusion, the differentiation process was repeated with ESC in the presence or absence of SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole) and SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) 1H-imidazole) (Davies et al., 2000), which are widely used as a selective inhibitors of p38α (and partially p38β). The transcription activity of the markers connected with hematopoiesis after using the p38α inhibitors was influenced in the similar manner as the depletion of p38α kinase in ESCs. Except for the expressions of c-Kit and Sca1 RNA transcripts, wt cells adopt the phenotype of p38α null cells in the presence of p38α kinase inhibitors (I). The total amount of colonies per dish was significantly declined in the case of hematopoietic differentiation in 6ds old EBs cultivated with inhibitor, whereas in 10 days old EBs inhibition of p38 had no effect on a number of colonies (I). When we analyse the formation of specific hematopoietic progenitors in EBs after treatment with p38α inhibitors, we also have not observed the differences (I).

These results are generally in agreement with the results obtained by analyses of differentiation of p38α ESCs. Aforementioned discrepancies might be caused by the non-specificity of the inhibitors because some works state that these p38α inhibitors can also influence the activity of some other signaling molecules such as p38β or JNK (Chuang et al., 2000).
3.4. The use of NAC in hematopoietic differentiation and the connection to the p38 MAPK pathway

When we characterized the ESCs phenotype of p38α+/+ and p38α−/−, we observed that mutation of p38α kinase had no effect on the expressions of the main pluripotent markers. However, the p38α−/− ESCs had a higher level of intracellular ROS compared to the to wild type and increase in ROS might be reduced by the pre-treatment of the cells with NAC (II). NAC is a glutathione (GSH) precursor and direct antioxidant. As a potent antioxidant, NAC scavenges hydrogen peroxide (H2O2), hydroxyl free radicals (•OH), and hypochloric acid (HOCI) in vitro (Aruoma et al., 1989). NAC also decreases free radical levels by increasing GSH synthesis (Neuschwander-Tetri et al., 1996). Importantly, we have shown that unlike the other antioxidants such as APO or DPI, NAC effectively works in ESC which are characterized by relatively low intrinsic ROS production (III).

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**Figure 10. Formation of hematopoietic CFU in wild-type and mutant p38 EBs** with or without NAC treatment (1 mM) on days 6 and 10 of differentiation. Single-cell suspensions were seeded into complete hematopoietic selective media for 14 days. The overall frequency of all types of hematopoietic colonies (A) and the frequencies of particular CFU-G, CFU-GM, CFU-M, CFU-E, and CFU-GEMM on days 6 (B), 10 (C) are shown.
As ROS can affect cell fate through the regulation of various signals responsible for cell differentiation, we investigated, whether the elevated production of ROS in p38α-/- could play a role in hematopoietic differentiation. Remarkably, the use of NAC decreased the total number of colonies from p38α+/+ ESCs in comparison to non-treated p38α+/+ ESCs (Fig. 10) but the NAC treatment has no effect on the p38α-/- cell differentiation an/or expression of hematopoietic markers (Fig. 10, 11, 12). Thus, we hypothesized, that hematopoiesis is not influenced by the level of ROS, but the treatment of NAC act in a different manner. The treatment the cells with NAC cause the similar effect in colony forming assay as depletion of p38α kinase and this is in agreement with studies, where they present the attenuation of activated p38α kinase after treatment with NAC in other cell lines (Hashimoto et al., 2001; Horst et al., 2014; Sato et al., 2014; Usatyuk et al., 2003). On the other hand, when we focused on the expression of the genes connected to hematopoiesis, the NAC treatment has no effect on the expression of overall hematopoietic markers. However, we noticed a certain effect in erythropoiesis. MAPK p38α influences the stabilization of Epo and as a consequence, p38 null mice embryos, which survive until E16.5 are anemic (Tamura et al., 2000). In addition, also evidence from the different models suggest the Epo/p38 interconnection. For example, lack of p38α activity was found to interfere with stabilization of Epo mRNA in human hepatoma cells undergoing hypoxic stress, resulting in diminished Epo gene expression (Adams et al., 2000; Tamura et al., 2000). Despite that Epo is added into the methylcellulose media in the in vitro condition, the number of erythroid colonies is decreased in the mutant cells or when inhibitor SB203580 is employed in the ESCs (I). Treatment with NAC has a similar effect as depletion of p38α in the analyze of colony assay. We observed a lower number of erythroid colonies in p38α +/+ cells after treatment these cells with NAC in 6ds EBs (Fig. 10 B). Additonaly the expression levels of transcription factors connect to erythropoiesis are downregulated after addition of NAC into culture media for cultivation of p38α+/+ ESCs (Fig. 12). Nevertheless, it should be emphasized that the observed effect of NAC treatment on the myeloid differentiation needs to be further clarified.
CFU-GEMM on days 6 (B), 10 (C) are shown. hematopoietic colonies (A) and the frequencies of particular CFU-G, CFU-GM, CFU-M, CFU-E, and complete hematopoietic selective media for 14 days. The overall frequency of all types 3.4.

NAC treatment (1mM). on days 6 and 10 of differentiation. Single-cell suspensions were seeded into α However, the p38 mutation of p38 kinase had no effect on the expressions of the main pluripotent markers. -/- ESCs had a higher level of intracellular ROS compared to the to wild-type (HOCl). NAC is a glutathione (GSH) precursor and direct antioxidant. As a potent antioxidant, NAC other antioxidants such as APO or DPI, NAC effectively works in ESC which are the connection to the p38 MAPK pathway. The use of NAC in hematopoietic differentiation and the activation of p38 in influenced by the level of ROS, but the treatment of NAC act in a different manner. The observed effect of NAC treatment on the myeloid differentiation needs to be further clarified. We observed a lower number of erythroid colonies in p38α-/- cells with NAC in 6ds EBs (Fig. 10 B). Adittionaly the expression levels of transcription factors connect to erythropoiesis are downregulated after addition of NAC into culture media condition, the number of erythroid colonies is decreased in the mutant cells or when inhibitor SB203580  is employed in the ESCs (I), of Epo mRNA in human hepatoma cells undergoing hypoxic stress, resulting in diminished activity was found to interfere with stabilization of Epo and as a consequence, p38 null mice embryos, which survive until E16.5 are anemic (Tamura et al., 2000). In addition, also evidence from the different models suggest the Epo/p38 interconnection. For example, lack of p38α-/- and p38α+/+ ESCs (Fig. 12). Nevertheless, it should be emphasized that the observed effect of NAC treatment on the myeloid differentiation needs to be further clarified.

Figure 11. Expression of the genes connected to hematopoiesis – qRT-PCR. The comparison of the expression level of markers in p38α+/+ and p38α-/- after treatment with NAC (1mM). A) HoxB4, B) CD34, C) VEGF, D) Flk1, E) c.Kit, F) Scal and G) CD38.
Figure 12. Expression of erythropoiesis-regulating transcription factors and markers determined by qRT-PCR. The levels of transcription factors Klf1 (A) and GATA1 (B) and the transcripts of hemoglobin Hbb-b1 (C), Hbb γ (D), Hbb ζ (E), and are shown in wild-type and mutant p38α−/− cells after treatment with NAC (1mM).
3.5. Involvement of p38 in the hematopoiesis of cells from fetal liver and bone marrow

During development, particularly in the FL, the main role of HSCs is to rapidly produce sufficient numbers of stem cells for the adult life while also giving rise to homeostatic levels of differentiated blood progeny. Thus, in the FL HSCs undergo multiple rounds of symmetrical self-renewing cell divisions to prepare the pool of stem cells required for the lifetime of the organisms. However, in adults, the most primitive HSCs (the LT-HSCs, that repopulate the hematopoietic system long-term following transplantations), are quiescent, dividing only every 140–180 days in mouse (Foudi et al., 2009; Wilson et al., 2008). BM HSC divisions are asymmetrical generating one HSC and one committed progenitor/ST-HSC (Takano et al., 2004). Nevertheless, quiescent LT-HSCs can respond very rapidly to stress or damage, and quickly exit from quiescence to regenerate the blood system (Cheng et al., 2000). Manesia and his colleagues hypothesized that in contrast to adult bone marrow HSCs, which use glycolysis for energy production, fetal liver HSCs may use alternative metabolic pathways to fuel their ability to expand extensively, such as an oxidative phosphorylation pathway.

Fetal liver HSCs contain more mitochondria than bone marrow HSCs, which results in increased levels of oxygen consumption and ROS production. Moreover, there is also increased expression of the genes connected to oxidative phosphorylation in the fetal liver compared to BM HSCs (Manesia et al., 2017). In the bone marrow HSC production of ROS, as the main result of the mitochondrial oxidation, induces HSC differentiation and aging, ultimately abrogating engraftment potential of adult BM HSCs (Ito et al., 2006). Ito et al. in their work suggested that oxidative stress induces the HSC-specific phosphorylation of p38 MAPK, and this activation of p38 MAPK leads to the defect in the maintenance of HSC self-renewal capacity in the BM. Activation of p38α in BM HSCs has been shown to impair HSC self-renewal and cause BM HSC premature exhaustion in several human hematological diseases and in ATM mutant and FoxO3 genes knockout mice (Ito et al., 2006; Miyamoto et al., 2007). Although it is well accepted that significant differences exist between the ability of the fetal liver and adult bone marrow HSCs to undergo symmetrical self-renewing cell divisions without stem cell exhaustion, only a few studies have addressed underlying molecular mechanisms. Previously, we investigated the role of p38α kinase in the hematopoietic differentiation from
ESCs. Next, we decided to use similar model for analyzing of hematopoietic progenitors from BM and/or FL and we aimed to compare the involvement of the MAPK p38α in the hematopoiesis from FL and BM. Wang and his colleagues observed that inhibition of p38 MAPK in the hematopoietic stem/progenitor cells promotes ex vivo expansion of these cells. Notably, this effect was not caused by the increased number of the HSC/HPC cells. Authors suggest that inhibition of p38 promotes ex vivo HSC expansion through the suppression of HSC apoptosis and senescence rather than by HSC differentiation impairment (Wang et al., 2011). Our findings are in agreement with their data (Wang et al., 2011). We also observed that treatment of BM HSC/HPC with the p38α inhibitor has no effect on the formation of overall number of CFU as well as individual colonies (CFU-G, CFU-M, CFU-GM, CFU-GEMM and CFU-E/BFU-E) from the progeny of BM HSCs in vitro (Fig. 13 A, C).

Figure 13. Comparison of colony forming assay after treatment of hematopoietic cells from FL and BM with SB203580. The absolute number of colonies from the progenitors from FL or BM (A). The number of individual colonies from FL (B) and the number of individual colonies from BM (C).

On the other hand, the use of inhibitor with the FL HSC/HPCs lead to the difference in the number of formed colonies. The number of CFU was decreased after the treatment of cells
with SB203580 in general (Fig. 13 A) and when we focus on the types of colonies, we revealed that the treatment with p38α inhibitor cause decreased in the myeloid CFUs. The number of erythropoietic colonies remained unchanged (Fig. 13 B).

Next, we analyzed the expression of markers connected to hematopoiesis. Inhibition of p38α in BM HSC/HPC cells increased expression levels of HoxB4 and Flk1 (Fig. 14 A, D), but the expression level of VEGF was decreased (Fig. 14 C). The same increased level of HoxB4 was observed after treatment of cells from FL, as in the cells treated with inhibitor from BM (Fig. 9 A). It has been well established that HoxB4 is a very important homeobox transcription factor that regulates HSC self-renewal. Overexpression of HoxB4 or incubation of HSCs with HoxB4 promotes HSC expansion in vivo and ex vivo (Wang et al., 2011). In contrast to BM HSC, the expression levels of marker Flk1 was unchanged after treatment of FL HSC/HPC (Fig. 14 D). Interestingly, we detected the decreased expression level of VEGF in both the cells from fetal liver and the cells from bone marrow (Fig. 14 C). A reduction in survival, colony formation and in vivo repopulation rates of HSCs were observed after ablation of the vegf gene in mice (Gerber et al., 2002). Moreover, studies, which claiming that VEGF promotes the formation of myeloid colonies from lineage commited progenitors were published (Dikov et al., 2001; Gabrilovich et al., 1998). This is partially in agreement with the decreased number of colonies after the treatment of the hematopoietic progenitors from the fetal liver (Fig. 13 B). However, the number of colonies from bone marrow remained unchanged (Fig. 13 C). It could be explained by the increased expression level of Flk1 (VEGFR-2) in the hematopoietic cells from the bone marrow after the treatment with the SB203580 (Fig. 14 D) what is in agreement with Gerber and colleagues. They show that the Flk1 restored survival and differenatiation in VEGF-deficient HSCs in vitro (Gerber et al., 2002). Next, we observed the elevated level of c.Kit expression after the SB203580 treatment of FL progenitors in comparison to untreated cells (Fig. 14 E). The expression level of c.Kit in BM cells was unchanged (Fig. 14 E) when we compare treated and nontreted BM progenitors. By contrast, we detected the attenuation of Sca1 expression level after the treatment of BM progenitors, but not in Sca1 level in FL progenitors. Dalmas and colleagues demonstrated that the expression level of Sca1 is reduced or increased depending on the p38α inhibitor concentration (Dalmas et al., 2008). This could explain the distinction expression of markers from BM and FL progenitors after the p38α inhibition. The expression of the markers CD34 and CD38 were not changed in the cells isolated from BM and/or FL after incubation with the inhibitor of MAPK p38α (Fig. 14 B, G).
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Interestingly, we detected the decreased expression level of VEGF in both the cells from fetal liver and the cells from bone marrow (Fig. 14 C). A reduction in survival, colony formation and in vivo repopulation rates of HSCs were observed after ablation of the vegf gene in mice (Gerber et al., 2002). Moreover, studies, which claiming that VEGF promotes the formation of myeloid colonies from lineage committed progenitors were published (Dikov et al., 2001; Gabrilovich et al., 1998). This is partially in agreement with the decreased number of colonies after the treatment of the hematopoietic progenitors from the fetal liver (Fig. 13 B). However, the number of colonies from bone marrow remained unchanged (Fig. 13 C). It could be explained by the increased expression level of Flk1 (VEGFR-2) in the hematopoietic cells from the bone marrow after the treatment with the SB203580 (Fig. 14 D) what is in agreement with Gerber and colleagues. They show that the Flk1 restored survival and differentiation in VEGF-deficient HSCs in vitro (Gerber et al., 2002). Next, we observed the elevated level of c.Kit expression after the SB203580 treatment of FL progenitors in comparison to untreated cells (Fig. 14 E). The expression level of c.Kit in BM cells was unchanged (Fig. 14 E) when we compare treated and nontreated BM progenitors. By contrast, we detected the attenuation of Sca1 expression level after the treatment of BM progenitors, but not in Sca1 level in FL progenitors. Dalmas and colleagues demonstrated that the expression level of Sca1 is reduced or increased depending on the p38α inhibitor concentration (Dalmas et al., 2008). This could explain the distinction expression of markers from BM and FL progenitors after the p38α inhibition. The expression of the markers CD34 and CD38 were not changed in the cells isolated from BM and/or FL after incubation with the inhibitor of MAPK p38α (Fig. 14 B, G).

**Figure 14. Expression of the genes connected to hematopoiesis – qRT-PCR.** The comparison of the expression level of markers during differentiation of progenitors from FL or BM with or without treatment with inhibitor of p38α kinase (SB203580, 5μM for 72h). Expression of A) HoxB4, B) CD34, C) VEGF, D) Flk1, E) cKit, F) Sca1 and G) CD38 is shown.

The p38α is involved in the erythropoiesis in mice, thus we analyzed the expression of the markers connected with erythropoiesis. We did not observe any changes in the level of
expression of Klf1 or genes for embryonic globins (Hbbγ, Hbbζ; Fig. 15 A, D, E), in the cells from both bone marrow and fetal liver after treatment with SB203580. However, the cultivation of cells from the fetal liver with SB203580 caused a modest increased level of GATA1 and Hbb-b1 expression (Fig. 10 B, C) unlike the cells from the bone marrow, where the expression levels of these genes were the same in control and in the inhibitor-treated cells. The discrepancy in the expression of genes and the colony forming assay can be caused by the involvement of GATA1 in the erythropoiesis. GATA-1 null proerythroblasts undergo apoptosis and reduced GATA-1 levels inhibit proerythroblast differentiation, ESCs clones overexpressing GATA-1 generate erythroid colonies that are inhibited in terminal differentiation (Weiss et al., 1994). Nevertheless, the role of GATA-1 later in erythroid differentiation remains obscure. The effect of inhibitors on the FL and BM progenitors and myeloid differentiation need to be elucidated subsequently and the obtained data need to be confirmed.

Conclusively, evident differences exist between hematopoietic progenitors from bone marrow and fetal liver. Hematopoiesis from the ESCs is also distinct.
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Conclusively, evident differences exist between hematopoietic progenitors from bone marrow and fetal liver. Hematopoiesis from the ESCs is also distinct.

Figure 15. Expression of erythropoiesis-regulating transcription factors and markers determined by qRT-PCR. The levels of transcription factors Klf1 (A) and GATA1 (B) and the transcripts of hemoglobin Hbb-b1 (C), Hbb\(\gamma\) (D), Hbb\(\zeta\) (E), and shown. The comparison of expression level of these markers in progenitors from FL or BM with or without inhibitor of 72h).
3.6. The use of N-acetylcysteine in differentiation of cells from FL and BM

The activation of p38α in HSC is mainly connected with inflammatory cytokines or oxidative stress (Raman, 2007). As discussed above, oxidative stress induces the HSC-specific phosphorylation of p38 MAPK, and this activation of p38 MAPK leads to the defect in the maintenance of HSC self-renewal capacity in the bone marrow HSC (Ito et al., 2006). In their work, Ito and his colleagues also use the antioxidant NAC for the influencing of HSC expansion. They observed that NAC, as well as the inhibition of p38α kinase, blocked ROS-induced increase of p16 Ink4a and p19 Arf, which is connected with hematopoietic stem/progenitor cells ex vivo expansion of these cells (Ito et al., 2006; Shao et al., 2011).

Figure 16. Formation of hematopoietic CFU from progenitor cells from FL or BM with or without NAC (1mM; 72h) treatment. Single-cell suspensions were seeded into complete hematopoietic selective media for 14 days. The overall frequency of all types of hematopoietic colonies (A) and the frequencies of particular CFU-G, CFU-GM, CFU-M, CFU-E, and CFU-GEMM from FL or BM are shown.
Finally, we analyze the effect of NAC on the cells from BM and FL. As the FL HSCs contain more mitochondria than bone marrow HSCs, what results in increased levels of oxygen consumption and ROS production. However, the hematopoietic cells from FL were not influenced after NAC treatment in the number of colonies (Fig. 16 A, B). On the contrary, the number of colonies from the cells derived from the BM was increased following the NAC treatment (Fig. 16 A) and when we focus on the individual colonies, there was more CFU-G and CFU-M (Fig. 16 C). Further, we analyze the expression of hematopoietic markers, the only difference was in the level of cKit expression (SFig. 17 E), which was decreased after treatment of hematopoietic cells from FL with NAC. The expression of the other markers remains unchanged before and after treatment with NAC in both the hematopoietic cells from BM and FL (SFig. 17 A – D, F ). No important difference was observed during analyzing of the transcription level of markers connected with erythropoiesis (Fig. 18). The effect of NAC treatment on the myeloid differentiation need to be elucidated subsequently and the obtained data need to be confirmed.
Frequencies of particular CFU-G, CFU-GM, CFU-M, CFU-E, and CFU-GEMM from FL or BM are selectived media for 14 days. The overall frequency of all types of hematopoietic colonies (A) and the without NAC (1mM; 72h) treatment. Single-cell suspensions were seeded into complete hematopoietic

Figure 16. Formation of hematopoietic CFU from progenitor cells from FL or BM

The activation of p38 stress (Raman, 2007). As discussed above, oxidative stress induces the HSC-specific maintenance of HSC self-renewal capacity in the bone marrow HSC (Ito et al., 2006). In their work, Ito and his colleagues also use the antioxidant NAC for the influencing of HSC

Finally, we analyze the effect of NAC on the cells from BM and FL. As the FL HSCs contain more mitochondria than bone marrow HSCs, what results in increased levels of oxygen consumption and ROS production. However, the hematopoietic cells from FL were not influenced after NAC treatment in the number of colonies (Fig. 16 A, B). On the contrary, the number of colonies from the cells derived from the BM was increased following the NAC treatment (Fig. 16 A) and when we focus on the individual colonies, there was more CFU-G and CFU-M (Fig. 16 C). Further, we analyze the expression of hematopoietic markers, the treatment of hematopoietic cells from FL with NAC. The expression of the other markers remain unchanged before and after treatment with NAC in both the hematopoietic cells from FL and BM (SFig. 17 A–D). No important difference was observed during analyzing of

Figure 17. Expression of the genes connected to hematopoiesis – qRT-PCR. The comparison of the expression level of markers during differentiation of progenitors from FL or BM with or without treatment with NAC (1mM for 72h). Expression of A) HoxB4, B) CD34, C) VEGF, D) Flk1, E) cKit, F) Sca1 and G) CD38 is shown.

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Figure 17. Expression of the genes connected to hematopoiesis – qRT-PCR. The comparison of the expression level of markers during differentiation of progenitors from FL or BM with or without treatment with NAC (1mM for 72h). Expression of A) HoxB4, B) CD34, C) VEGF, D) Flk1, E) cKit, F) Sca1 and G) CD38 is shown.

Figure 18. Expression of erythropoiesis-regulating transcription factors and markers determined by qRT-PCR. The levels of transcription factors Klf1 (A) and GATA1 (B) and the transcripts of hemoglobin Hb-b1 (C), Hbγ (D), Hbζ (E), and shown. The comparison of expression level of these markers in progenitors from FL or BM with or NAC (1mM for 72h).
### Figure 17. Expression of the genes connected to hematopoiesis – qRT-PCR. The comparison of the expression level of markers during differentiation of progenitors from FL or BM with or without treatment with NAC (1mM for 72h). Expression of A) HoxB4, B) CD34, C) VEGF, D) Flk1, E) cKit, F) Sca1 and G) CD38 is shown.

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Transcription Level relative to GAPDH</th>
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<tbody>
<tr>
<td>FL</td>
<td>BM</td>
</tr>
<tr>
<td>HoxB4</td>
<td>0.0000</td>
</tr>
<tr>
<td>CD34</td>
<td>0.0000</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.0000</td>
</tr>
<tr>
<td>Flk1</td>
<td>0.0000</td>
</tr>
<tr>
<td>cKit</td>
<td>0.0000</td>
</tr>
<tr>
<td>Sca1</td>
<td>0.0000</td>
</tr>
<tr>
<td>CD38</td>
<td>0.0000</td>
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</tbody>
</table>

### Figure 18. Expression of erythropoiesis-regulating transcription factors and markers determined by qRT-PCR. The levels of transcription factors Klf1 (A) and GATA1 (B) and the transcripts of hemoglobin Hbb-b1 (C), Hbb\(\gamma\) (D), Hbb\(\zeta\) (E) shown. The comparison of expression level of these markers in progenitors from FL or BM with or NAC (1mM for 72h).
4. Conclusion

During my doctoral study, I tried to improve our understanding of the process of hematopoiesis establishment. In conclusion, the p38α kinase is involved in the hematopoietic differentiation at all levels of development and its role could be summarized as follows:

- The depletion of p38α results in the up-regulated expression of NOX2 and elevated level of ROS - we hypothesized about a possible negative feedback process regulating the relationship between p38α and NOX2.
- Kinase p38α is involved in three steps of differentiation process of development of hematopoietic cells from ESCs – in vitro.
- Erythropoiesis and myelopoiesis (especially granulopoiesis) is impaired in the p38α-/- differentiation
- The results from analyzing of p38α-/- and p38α+/+ cells are in agreement with the results gained from analyzing of the cells treated with p38α inhibitor.

The following conclusions are based on the preliminary data and demand following confirmation.

- The p38α kinase influences distinctly the hematopoietic differentiation of the hematopoietic progenitors from the bone marrow and from the fetal liver.
- NAC treatment during the differentiation of ESCs has a similar effect in the followed hematopoietic markers as the use of p38α inhibitor.

Taken together, our results described that p38α kinase is important in the regulation of multiple processes involved in blood cell production. Using of the ESCs with depletion of p38α kinase give us an important insights into the molecular mechanisms of the development and regulation of hematopoiesis. The understanding of these processes could result in better development of hematopoietic cell in vitro and gene therapies.
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- The depletion of p38 $\alpha$ results in the up-regulated expression of NOX2 and elevated level of ROS—we hypothesized about a possible negative feedback process regulating the relationship between p38 $\alpha$ and NOX2.
- Kinase p38 $\alpha$ is involved in three steps of differentiation process of development of hematopoietic cells from ESCs—in vitro.
- Erythropoiesis and myelopoiesis (especially granulopoiesis) is impaired in the p38 $\alpha$-/- differentiation.
- The results from analyzing of p38 $\alpha$-/- and p38 $\alpha$+/- cells are in agreement with the results gained from analyzing of the cells treated with p38 $\alpha$ inhibitor.

The following conclusions are based on the preliminary data and demand following confirmation.

- The p38 $\alpha$ kinase influences distinctly the hematopoietic differentiation of the hematopoietic progenitors from the bone marrow and from the fetal liver.
- NAC treatment during the differentiation of ESCs has a similar effect in the followed hematopoietic markers as the use of p38 $\alpha$ inhibitor.

Taken together, our results described that p38 $\alpha$ kinase is important in the regulation of multiple processes involved in blood cell production. Using of the ESCs with depletion of p38 $\alpha$ give us an important insights into the molecular mechanisms of the development and regulation of hematopoiesis. The understanding of these processes could result in better development of hematopoietic cell in vitro and gene therapies.
5. Acknowledgement

Completion this thesis could not have been possible without the participation and assistance of many people whose names may not all be enumerated. Their contributions are sincerely appreciated and gratefully acknowledged. No words can express my gratitude what I feel to everybody of you.

First and foremost, I would like to thank my PhD supervisor Dr. Jiří Pacherník for giving me a guidance and valuable suggestion through each stage of my doctoral study. Thanks goes also to Dr. Lukáš Kubala and his lab for collaboration and help with experiments and articles. Further I would like to thank Honza and Katka for psychological assistance and text corrections.

I would also like to expand my gratitude to all those who directly or indirectly inspired and encouraged me during my studies: to my classmates Katka, Pavel, Libor, Honza Karolina and Katarina; to my labmates Marketa, Marta, Kasia, Iva, Hanka; and to whole Department of experimental biology especially from Animal physiology and Immunology.

Last but not least, I also would like to thank my parents and my family ( Jára – my soulmate, and Kryšťufek, Rozárka, and Apolenka – the source of my infinite happiness) for their constant support and endless belief in me.
Aknowledgement

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### 6. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGM</td>
<td>aorta-gonad-mesonephros</td>
</tr>
<tr>
<td>Ang-1</td>
<td>angiopoietin-1</td>
</tr>
<tr>
<td>Angptl3</td>
<td>angiopoietin-like 3</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ATF4</td>
<td>activating transcription factor 4</td>
</tr>
<tr>
<td>BFU-E</td>
<td>burst forming unit - erythrocyte</td>
</tr>
<tr>
<td>BL-CFC</td>
<td>blast-colony forming cell</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMP4</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>C/EBPa</td>
<td>CCAAT/enhancer-binding protein alpha</td>
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<td>CAR</td>
<td>CXCL12-abundant reticular cells</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CFU-C</td>
<td>colony forming unit cell</td>
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<td>CFU-E</td>
<td>colony forming unit - erythrocyte</td>
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<td>CFU-G</td>
<td>colony forming unit - granulocyte</td>
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<td>CFU-GEMM</td>
<td>colony forming unit granulocyte-erythrocyte-monocyte-makrophage</td>
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<td>CFU-GM</td>
<td>colony forming unit granulocyte-macrophage</td>
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<td>CFU-M</td>
<td>colony forming unit - macrophage</td>
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<tr>
<td>c-Kit - (CD117)</td>
<td>mast/stem cell growth factor receptor</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
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<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
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<td>cyklooxygenase</td>
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<td>colony stimulating factor receptor 1</td>
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<td>DLK</td>
<td>dual leucine zipper kinase</td>
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<td>extracellular signal-regulated kinase</td>
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<td>ETS Variant 2</td>
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<td>GMP</td>
<td>granulocyte-myeloid progenitor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>AGM</td>
<td>aorta-gonad-mesonephros</td>
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<tr>
<td>Ang-1</td>
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<tr>
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<td>ATF4</td>
<td>activating transcription factor 4</td>
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<td>BFU-E</td>
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<td>BL-CFC</td>
<td>blast-colony forming cell</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<td>BMP4</td>
<td>bone morphogenetic protein 4</td>
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<tr>
<td>C/EBPa</td>
<td>CCAAT/enhancer-binding protein alpha</td>
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<tr>
<td>CAR</td>
<td>CXCL12-abundant reticular cells</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU-C</td>
<td>colony forming unit cell</td>
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<tr>
<td>CFU-E</td>
<td>colony forming unit - erythrocyte</td>
</tr>
<tr>
<td>CFU-G</td>
<td>colony forming unit - granulocyte</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>colony forming unit granulocyte-erythrocyte-monocyte-makrophage</td>
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<tr>
<td>c-Kit</td>
<td>mast/stem cell growth factor receptor</td>
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<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
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<td>CMP</td>
<td>common myeloid progenitor</td>
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<td>cyklooxygenase</td>
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<td>CXCL12</td>
<td>C-X-C Motif Chemokine Ligand 12</td>
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<td>extracellular signal-regulated kinase</td>
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<td>ESC</td>
<td>embryonal stem cell</td>
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<td>Etv2</td>
<td>ETS Variant 2</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FL</td>
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<td>friend leukemia integration 1</td>
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<td>fetal liver kinase 1</td>
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<td>Fms related tyrosine kinase</td>
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<td>inner cell mass</td>
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<td>insulin-like growth factor 2</td>
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<td>indian hedgehog</td>
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<td>leukemia inhibitory factor</td>
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<td>MAPK</td>
<td>mitogen activate protein kinase</td>
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<td>MASH1</td>
<td>Achaete-scute complex homolog-1 in rodents</td>
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<td>M-CSF</td>
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<td>MLK</td>
<td>mixed-lineage protein kinase</td>
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<td>PECAM-1</td>
<td>platelet/endothelial cell-adhesion molecule</td>
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<td>paraaortic splanchnopleure</td>
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<td>parathyroid hormone</td>
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<tr>
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<td>reactive oxygen species</td>
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<td>Runx1</td>
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<td>stem cell antigen 1</td>
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<td>stem cell factor</td>
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<td>Shh</td>
<td>sonic hedgehog</td>
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<tr>
<td>SLAM</td>
<td>signaling lymphocytic activation molecule</td>
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<td>SM\alpha\alpha</td>
<td>smooth muscle \alpha-actin</td>
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<tr>
<td>SNO</td>
<td>spindle shaped N-cadherin+CD45- osteoblastic cells</td>
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<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
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<tr>
<td>ST-HSC</td>
<td>short term hematopoietic stem cell</td>
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<tr>
<td>Tal1/SCL</td>
<td>stem cell ligand</td>
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<td>Tgf-\beta</td>
<td>transforming growth factor \beta</td>
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<td>Thr</td>
<td>threonin</td>
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<td>TNF\alpha</td>
<td>tumor necrosis factor \alpha</td>
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<td>tumor progression locus 2</td>
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<td>thrombopoietin</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>Tyr</td>
<td>tyronin</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<td>vascular endothelial growth factor</td>
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<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2</td>
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<td>YS</td>
<td>yolk sac</td>
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References:


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dependent, IL-3-independent CD43(-) embryonic precursor. Stem cell reports 3, 489–501.


Immunity 20, 707–718.


Supplements

Material and methods:

Isolation of fetal liver and bone marrow

Fetal liver were dissected from E13.5 embryo. On E13.5 the fetal liver is clearly visible as a red distinctive area in the center of the embryo. Fetal liver was placed into fresh ES media and resuspended by pippeting up and down until the whole liver tissue were homogenized. Lysis buffer for remove of erythrocytes was added to suspension of cells from fetal liver in media in ratio 1:4 and put on ice for 10 min. After 10min of incubation with lysis buffer the cells were centrifuged 200 x g for 5 min in 25°C. The pellet was rinsed two times with PBS. The cells were incubated on culture dish, previously coated with 0,1% gelatine, in ES medium without LIF.

The femurs and tibias were separated aseptically from euthanized mice. The ends of bones were cut and bone marrow was expelled by flushing ES through the medullary cavities using a 25-G needle and 2-ml syringe into 15-ml tube. Lysis buffer for remove of erythrocytes was used as above. The cells were incubated on culture dish, previously coated with 0,1% gelatine in ES medium without LIF.

Cells from fetal liver and from bone marrow were treated with inhibitor of p38α MAPK SB203580 (…)in concentration 5μM and DMSO was added to the cells as a control. Or the cells were treated by N-acetylcysteine (NAC; concentration 1 mM). The cells with or without treatment were cultivated for 72 hours and then they were used for colony forming assay or analysed for expression of hematopoietic markers (Sfig. 1).
Supplements

**Material and methods:**

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**Figure 1.** Scheme of cultivation and treatment of the cells derived from bone marrow or fetal liver.

**Treatment of the ESCs with NAC**

The ESCs were maintained in an undifferentiated state in a monolayer on a gelatinized dish (by 0.1% water solution of porcine gelatin) in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, 100 mM nonessential amino acids (all Gibco-Invitrogen, UK), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-Invitrogen, UK), and 1000 U/ml recombinant leukemia inhibitory factor (LIF) (Chemicon International, USA). The differentiation of the cells was induced spontaneously through the formation of embryoid bodies (EBs), floating cell aggregates, and LIF depletion. The formation of embryoid bodies was achieved by the direct culture of ESCs on bacteriological dishes coated with agar (0.5% agar diluted in water, 5 ml per 90 mm in diameter dish) in complete ES medium without LIF (5 × 10^6 cells per 90 mm in diameter dish). The medium was replaced every two days and the 1 mM NAC was added into the culture with the fresh new medium. The control dishes were without NAC. The following analyzes were performed as described in the article I (Scheme – SFig. 2).
SFigure 1. Scheme of cultivation and treatment of the cells derived from bone marrow or fetal liver.

Treatment of the ESCs with NAC

The ESCs were maintained in an undifferentiated state in a monolayer on a gelatinized dish (by 0.1% water solution of porcine gelatin) in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 100 mM nonessential amino acids (all Gibco-Invitrogen, UK), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-Invitrogen, UK), and 1000 U/ml recombinant leukemia inhibitory factor (LIF) (Chemicon International, USA). The differentiation of the cells was induced spontaneously through the formation of embryoid bodies (EBs), floating cell aggregates, and LIF depletion. The formation of embryoid bodies was achieved by the direct culture of ESCs on bacteriological dishes coated with agar (0.5% agar diluted in water, 5 ml per 90 mm in diameter dish) in complete ES medium without LIF (5 × 10^6 cells per 90 mm in diameter dish). The medium was replaced every two days and the 1 mM NAC was added into the culture with the fresh new medium. The control dishes were without NAC. The following analyzes were performed as described in the article I (Scheme – SFig. 2).

SFigure 2. Scheme of cultivation and treatment of the embryoid bodies from the ESCs p38α−/− and p38α+/+. 
Figure 1. Scheme of cultivation and treatment of the cells derived from bone marrow or fetal liver. Treatment of the ESCs with NAC

The ESCs were maintained in an undifferentiated state in a monolayer on a gelatinized dish (by 0.1% water solution of porcine gelatin) in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 100 mM nonessential amino acids (all Gibco - Invitrogen, UK), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco - Invitrogen, UK), and 1000 U/ml recombinant leukemia inhibitory factor (LIF) (Chemicon International, USA). The differentiation of the cells was induced spontaneously through the formation of embryoid bodies, floating cell aggregates, and LIF depletion. The formation of embryoid bodies was achieved by the direct culture of ESCs on bacteriological dishes coated with agar (0.5% agar diluted in water, 5 ml per 90 mm in diameter dish) in complete ES medium without LIF (5 × 10⁶ cells per 90 mm in diameter dish). The medium was replaced every two days and the 1 mM NAC was added into the culture with the fresh new medium. The control dishes were without NAC. The following analyzes were performed as described in the article I (Scheme SFig. 2).

Figure 2. Scheme of cultivation and treatment of the embryoid bodies from the ESCs p38α-/- and p38α+ [+].
The activation of p38alpha kinase mediates cell response to various extracellular factors including many interleukins and growth factors important for haematopoiesis. The role of p38alpha kinase was previously analysed in particular haematopoietic cells. In this study and for the first time, the role of p38alpha kinase in haematopoiesis was studied using a model of continuous haematopoietic development in pluripotent embryonic stem cells in vitro. The expression of transcripts associated with haematopoiesis and the potential for the formation of specific haematopoietic cell colonies were compared between wild-type and mutant p38alpha gene-depleted cells. The absence of p38alpha kinase led to the inhibition of hemangioblast formation during the first step of haematopoiesis. Later, during differentiation, due to the lack of p38alpha kinase, erythrocyte maturation was impaired. Mutant p38α−/− cells also exhibited decreased potential with respect to the expansion of granulocyte colony-forming units. This effect was reversed in the absence of erythropoietin as shown by colony-forming unit assay in media for colony-forming unit granulocytes/macrophages. p38alpha kinase thus plays an important role in the differentiation of common myeloid precursor cells into granulocyte lineages.

1. Introduction

Embryonic stem (ES) cells are derived from pluripotent cells of the inner cell mass of a blastocyst and have the potential to turn into cells of all three germ layers in the body. The differentiation of ES cells therefore represents a unique in vitro model for the analysis of developmental processes. The hematopoietic specification of ES cells has been shown to recapitulate embryonic haematopoiesis [1, 2]. Haematopoiesis in embryonal development represents a complex of developmental process that involves several anatomical sites, after which HSCs that have finally arisen colonise bone marrow at birth. The first wave of haematopoiesis takes place in the yolk sac, the extraembryonic organ of the embryo, and is called primitive haematopoiesis. Nucleated so-called primitive erythrocytes, which have the embryonal type of hemoglobin, appear in the yolk sac along with some myeloid precursors. The second wave, already referred to as definitive, is rapidly followed by the emergence of erythromyeloid progenitors (EMP) and lymphocytes also in the yolk sac. The third wave occurs in the intraembryonic AGM (aorta-gonad-mesonephros) area, and definitive HSCs originate here from hemogenic endothelium [3–5]. Then, the HSCs migrate into the fetal liver, which serves as the main hematopoietic organ of the fetus [6].

Mitogen-activated protein kinases (MAPK) are a superfamily of protein kinases that are the key players in numerous signaling events in cells from yeast to mammals. The MAPK superfamily comprises at least four families, namely, extracellular signal-related kinases 1 and 2 (ERK1 and ERK2), ERK5, Jun amino-terminal kinases (JNKs), and p38 kinases. Kinase p38 has been characterised as a protein kinase which is activated in mammalian cells in response to lipopolysaccharide, toxins, radicals, and extracellular
changes in osmolarity, linking the p38 kinase pathway to a stress-induced response. Moreover, it has been shown that p38 kinase is involved in many other cellular responses including cell proliferation, differentiation, development, and apoptosis.

Four isoforms of p38 kinase have been identified so far: p38α, p38β, p38δ, and p38γ [7, 8]. In the majority of cell types, p38α is the most abundant p38 family member. Kinase p38α has a key role in the regulation of developmental processes as has been demonstrated in animal models. It is known that in adults, p38α kinase is required for HSC activation as well as for the specification and maturation of hematopoietic cell lineages [9]. During mouse embryogenesis, the depletion of p38α kinase leads to embryonic mortality at around E10.5 due to defects in vascularisation and in the formation of vessel structures in placenta [10–13]. In one study, it was also found that if p38α−/− embryos survived up to 16.5 dpc, they were also anemic due to abnormal erythropoiesis, which is caused by the insufficient production of erythropoietin [14]; however, in this case, other hematopoietic cell lineage developments were not investigated. In addition, studies performed with p38α knockout ES cells or with the biochemical inhibition of p38α kinase showed that p38 controls mesodermal commitment during ES cell differentiation [15]. Therefore, we hypothesized that p38α kinase plays a role in the development of hemangioblast and its differentiation into hematopoietic lineages.

Our results show that p38α affects haematopoiesis in at least three different ways. Firstly, p38α is required for hemangioblast formation in vitro. Secondly, p38α is required for erythropoiesis and erythrocyte maturation. Finally, p38α regulates the differentiation of common myeloid progenitor (CMP) cells into granulocyte lineages.

2. Materials and Methods

2.1. Culture and Differentiation of ES Cells. In this study, cells deficient in p38α kinase (p38α−/−) and their wild-type counterpart (p38α+/+) were used (kindly provided by Dr. Barry P. Sleckman, Washington University School of Medicine at St. Louis). The generation of these cell lines is described in detail by Kim and coworkers [16]. The ES cells were maintained in an undifferentiated state in a monolayer on a gelatinized dish (by 0.1% water solution of porcine gelatin) in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, 100 mM nonessential amino acids (all Gibco-Invitrogen, UK), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-Invitrogen, UK), and 1000 U/ml recombinant leukemia inhibitory factor (LIF) (Chemicon International, USA). The differentiation of the cells was induced spontaneously through the formation of embryoid bodies (EBs), floating cell aggregates, and LIF depletion. The formation of embryoid bodies was achieved by the hanging drop technique (400 cells/drop) or by the direct culture of ES cells on bacteriological dishes coated with agar (0.5% agar diluted in water, 5 ml per 90 mm in diameter dish) in complete ES medium without LIF (5 × 105 cells per 90 mm in diameter dish). The medium was replaced every two days.

2.2. Quantitative RT-PCR Analysis of Gene Expression. In order to identify hematopoietic processes, the expressions of selected transcription factors associated with haematopoiesis were analysed. The expressions of the following genes were used: (i) VEGF, Flik-1, Etv2, GATA2, Tal1, Runx1, Sca1, c-Kit, and Tie2 as markers of hemangioblast/early haematopoiesis, (ii) HoxB4, CD34, CD38, and CD150 as markers of haematopoiesis, (iii) GATA1, Klf1, Epor, Hbbβ, Hbb-α, and Hbbγ as markers of erythroid lineage, (iv) PU.1, C/EBPβ, G-CSF-R (v1, v2), and csf1r as markers of myeloid lineage, and (v) the key hematopoietic cytokines IL6, IL3, and EPO (Table 1).

Total RNA was extracted by UltraClean® Tissue & Cells RNA Isolation Kit (MO BIO Laboratories, USA). cDNA was prepared using Mu-MLV reverse transcriptase kit (Sigma-Aldrich, USA). qRT-PCR was performed in a Roche LightCycler using the following program: an initial activation step at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, an annealing temperature (see Table 1) for 10 s, and a temperature of 72°C for 10 s.

2.3. Immunoblot Analysis. Immunoblot analysis and cell sample harvesting were performed as presented previously [40]. Briefly, ES cells and/or EBs were washed twice with PBS (pH 7.2) and lysed in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCL, pH 7.5; 1% SDS; 10% glycerol). Protein concentrations were determined using the DC protein assay kit (Bio-Rad, USA). Lysates were supplemented with bromophenol blue (0.01%) and β-mercaptoethanol (1%) and incubated for 5 min at 95°C. Equal amounts of total protein (10 µg) were subjected to 8 or 10% SDS-PAGE. After being electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore, USA), proteins were immunodetected using appropriate primary and secondary antibodies and visualized by ECL Plus reagent (Amersham Pharmacia Biotech, USA) according to manufacturer’s instructions. We used the following primary antibodies: rabbit polyclonal antibodies against p38alpha kinase, Oct4 (Santa Cruz Biotechnology, USA), phospho p38 kinase, and GAPDH (Cell Signaling Technology, USA). After immunodetection, each membrane was stained by Amido black to confirm equal protein loading. The total level of β-actin was detected as loading control.

2.4. Colony-Forming Assay. The formation of ES cells into EBs was achieved by the direct culture of ES cells on bacteriological dishes, as described above. After 3 (for hemangioblast colonies), 6, 10, or 14 (for CFU colonies) days of differentiation, EBs were dissociated into single cells and 3 × 10^6 cells were replated into 1.0% methylcellulose-based medium containing a cocktail of hematopoietic cytokines (MethoCult; STEMCELL Tech., Canada). After 3 (for hemangioblast colonies) or 14 (for CFU colonies) days of cultivation, hematopoietic colonies were scored and their morphologies were documented by photography [41].

To analyse the efficiency of the differentiation into hematopoietic progenitors, we used colony-forming assay in various MethoCult media. The cells were seeded into methylcellulose with cytokines specific to erythroid and
β (all Gibco-Invitrogen, UK), 0.05 mM with 15% fetal calf serum, 100 mM nonessential amino acids

Stem Cells International
diluted in water, 5 ml per 90 mm in diameter dish) in com-
ES cells on bacteriological dishes coated with agar (0.5% agar
international, USA). The di-
recombinant leukemia inhibitory factor (LIF) (Chemicon
streptomycin (Gibco-Invitrogen, UK), and 1000 U/ml
atin) in Dulbecco
were maintained in an undi-
described in detail by Kim and coworkers [16]. The ES cells

2.1. Culture and Di-
erentiation of the cells was
p38 kinase is involved in many other cellular responses
changes in osmolarity, linking the p38 kinase pathway to a

2.2. Quantitative RT-PCR Analysis of Gene Expression. In

2.3. Western Blot Analysis. After cells were

2.4. Morphological and Immunocytochemical Changes. After

2.5. Flow Cytometry. Flow cytometry was performed using
cells were replated into 1.0% methylcellulose-based
methylcellulose with cytokines speci-
fi

Table 1: Primers for the particular markers of haematopoiesis used in the study.

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myeloid differentiation, indicated as full medium (MethoCult GF M3344, containing SCF, IL-3, IL-6, and EPO), erythroid differentiation (MethoCult GF M3344, containing EPO, indicated as erythroid media), or granulocyte and macrophage differentiation (MethoCult GF M3344, containing SCF, IL-3, and IL-6, indicated as GM media). For blast colonies, cells were seeded into full media supplemented with 5 ng per ml of mouse VEGF (PeproTech, USA). The numbers and morphologies of formed colonies were examined by means of light microscopy.

2.5. Identification of Hemoglobin-Positive Cells. The formation of ES cells into EBs was achieved by the hanging drop technique. After 5 days of differentiation, EBs were transferred into 24-well plates previously coated with 0.1% gelatin (1 EB per 1 well). As described above, the EBs were then cultured in DMEM-F 12 (1:1) supplemented with insulin, transferrin, selenium (ITS; Gibco-Invitrogen, UK), and antibiotics, which was then used as serum-free medium. The medium was replaced every two days. EBs with red cell islands were scored under a microscope. The 6-, 10-, and 14-day-old adhesive EBs were stained with 2,7-diaminoﬂuorescein (DAF) (Sigma-Aldrich, USA) (0.1% DAF, 0.1% H₂O₂, and 200 mM Tris-HCl pH 7). Due to the pseudoperoxidase activity of hemoglobin, erythroid cells oxidise DAF, which catalyzes the formation of a blue compound (fluorescent blue). Blue cells were then observed under the microscope.

2.6. Quantitative Staining of Hemoglobin by DAF. EBs were prepared in suspension as described earlier. After 6, 10, or 14 days of differentiation, the EBs were washed twice with cold PBS. Then, the EBs were lysed in NP-40 lysis buffer and frozen at -20°C. 50 μl of lysates and 150 μl of assay buffer (0.1% DAF, 0.06% H₂O₂, 100 mM Tris phosphoric acid buffer pH 7, and 6 M Urea) were pipetted into a 96-well plate and absorbance was measured at 620 nm.

2.7. Statistics. Data are expressed as mean ± SD. Statistical analysis was performed using ANOVA or the Kruskal-Wallis test with post hoc Bonferroni or Dunn’s test. Values of P < 0.05 were considered to be statistically significant.

3. Results

3.1. p38α MAPK Regulates Haematopoiensis in ES Cells. The general/overall hematopoietic potential of wt and mutant p38α−/− ES cells was analysed both by an assay of the potential to form hematopoietic colony-forming unit (CFU) cells and by determination of the expression of transcripts which are associated with haematopoiensis (see Materials and Methods).

The full medium for the CFU, which supports all expected types of hematopoietic CFU, was used in this study. When 6-day-old EBs were used, p38α−/− cells formed a lower number of hematopoietic CFU than their wt counterparts. In 10- and 14-day-old EBs, we did not observe any difference in the formation of hematopoietic CFU (Figure 1(a)). In detail, five types of CFU were recognized [42]: CFU-M (macrophage) and CFU-G (granulocyte; Figure 1(e)), CFU-GM (granulocyte, macrophage; Figure 1(f)), CFU-E/BFU-E (erythrocyte/burst-forming unit erythroid; Figure 1(g)), and CFU-GEMM (granulocyte, erythrocyte, macrophage, and megakaryocyte; Figure 1(h)). CFU-G and CFU-E/BFU-E were formed at a higher frequency in about 5% of colonies. CFU-GM was formed in about 1% of colonies derived from 6- and 10-day-old EBs. The count of CFU-M and CFU-GEMM was under 0.5% in all cases. An overall higher hematopoietic CFU capacity/potential was observed in 10-day-old EBs (Figure 1(a)). Not only does the absence of p38α in cells lead to a decrease in overall hematopoietic CFU capacity (the count of all CFU mentioned above) but also 6-day-old p38α−/− EBs have a lower number of CFU-G, CFU-GM, and CFU-E in contrast to their wt counterparts (Figure 1(b)). No difference was observed between p38α+/+ and p38α−/− in the number of particular hematopoietic CFU in 10- and 14-day-old EBs; however, there was a decrease in CFU-G in 14-day-old p38α−/− EBs (Figures 1(c) and 1(d)). Hemangioblast progenitor/blast cell colonies were also determined. Mutant p38α−/− cells formed a lower number of blast colonies than their wt counterparts (Figure 1(i)).

Further, we determined the expression of various transcripts associated with hematopoietic differentiation in samples of 6-, 10-, and 14-day-old EBs by means of qRT-PCR. A wide range of genes related to this process, from genes linked to mesoderm/hemangioblast to genes which play a role in primitive and definitive haematopoiesis, was chosen for analysis. The expression of Flk1 increased during differentiation and was reduced in mutant p38α−/− cells (Figure 2(a)). Flk1 ligand VEGF expression was also increased during differentiation, but no difference between wt and mutant cells was observed (Figure 2(b)). In contrast to Flk1 and VEGF, Etv2 is expressed transiently in a narrow developmental context.
Data are expressed as mean ± SD from a minimum of four independent experiments. Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk “*” indicates a statistical significance of $P < 0.05$. **Table 1:** Continued.

![Figure 1](image-url)

**Figure 1:** Formation of hematopoietic CFU in wild-type and mutant p38α−/− EBs on days 6, 10, and 14 of differentiation. Single-cell suspensions were seeded into complete hematopoietic selective media for 14 days. The overall frequency of all types of hematopoietic colonies (a) and the frequencies of particular CFU-G, CFU-GM, CFU-M, CFU-E, and CFU-GEMM on days 6 (b), 10 (c), and 14 (d) are shown. Representative morphologies of the determined hematopoietic CFU colonies, CFU-M and CFU-G (e), CFU-GM (f), CFU-E (g), and CFU-GEMM (h) are presented. The formation of hemangioblast progenitor/blast cell colonies and their representative morphology are also shown (i). Data are presented as mean ± SEM from a minimum of four independent experiments. Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk “*” indicates a statistical significance of $P < 0.05$. **Table 1:** Continued.
The expression of Etv2 was higher in 10-day-old EBs, compared to 6- and 14-day-old EBs (Figure 2(c)). Compared to mutant p38α−/− cells, wt cells exhibited a higher level of Etv2 transcripts at all determined times of differentiation. Next, we analysed the expression of key regulators of hematopoietic development. The level of HoxB4 transcription was similar for each time and for both genotypes (Figure 2(d)). Interestingly, the expressions of c-Kit and Sca1 transcripts were higher in mutant p38α−/− cells, except for the level of c-Kit in 10-day-old EBs, where it was lower and comparable in both cell types (Figures 2(e) and 2(f)). The expression of GATA2 was higher in 14-day-old wt EBs. In mutant p38α−/− cells as well as in 6- and 10-day-old wt EBs, the GATA2 level was low and comparable to that in wt cells in 6- and 10-day-old EBs at all times of analysis (Figure 3(a)). Tα1 expression was higher in mutant p38α−/− than in wt 6-day-old EBs. In older EBs, its level in wt was identical to

**Figure 2:** The expressions of genes required for and/or marking the development of hemangioblast and early hematopoietic development determined by qRT-PCR. The levels of transcripts of key components of the Flk1 signaling axis, Flk1 (a), VEGF (b), and Etv2 (c) and the levels of transcripts of hemangioblast/hematopoietic markers of HoxB4 (d), c-Kit (e), and Sca1 (f) in 6-, 10-, and 14-day-old EBs, are shown. Data are presented as mean ± SEM from a minimum of four independent experiments. Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk “*” indicates a statistical significance of P < 0.05.
expression of Etv2 was higher in 10-day-old EBs, compared to mutant EBs. The levels of transcripts of key components of the Flk1 signaling axis, Flk1 (a), VEGF (b), and Etv2 (c) and the transcript level relative to GAPDH is shown in Figure 2(d). Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk ** indicates a statistical significance of P < 0.05.

In mutant EBs, wt cells exhibited a higher level of Etv2 transcripts at all determined times of differentiation, we did not observe differences in the levels of these transcripts between wt and p38α−/− cells. The expression levels of IL6 and EPO transcripts at these times were lower in p38α−/− cells compared to their wt counterparts (Figures 3(g)–3(i)). Thus, p38α kinase is required for regular haematopoiesis. Pluripotent p38α−/− ES cells have an attenuated potential for the formation of hematopoietic CFU. This corresponds with the low expressions of transcripts Flk1, Etv2, and Runx1, which are key regulators of haematopoiesis.

3.2. p38α Kinase Is Required for Erythropoiesis. Erythropoiesis itself was also studied in detail. The overall number of erythroid colonies in erythroid-specific CFU media was not different in wt cells derived from 6-, 10-, or 14-day-old EBs. Mutant p38α−/− cells formed a lower number of CFU-E in comparison with their wt counterparts when cells were
expression of Etv2 was higher in 10-day-old EBs, compared levels of transcripts of hemangioblast/hematopoietic markers of HoxB4 (d), cKit (e), and Sca1 (f) in 6-, 10-, and 14-day-old EBs, are determined by qRT-PCR. The levels of transcripts of key components of the Flk1 signaling axis, Flk1 (a), VEGF (b), and Etv2 (c) and the accordingly, the expressions of c-Kit and Sca1 transcripts were cells, wt cells exhibited a higher level of Etv2 trans-

![Erythroid colonies in medium selective for erythrocytes](image)

![Hemoglobin staining](image)

FIGURE 4: Analyses of erythropoiesis. Single-cell suspensions from 6-, 10-, and 14-day-old EBs were seeded into erythroid CFU selective media for 14 days. The frequency of CFU-E/BFU-E in wild-type and mutant p38α−/− EBs is shown (a). We also determined the hemoglobin level based on their pseudoperoxidase activity (b) and the frequency of EBs with visible erythroid clusters in wild-type and mutant p38alpha cells (c). A representative EB with red-coloured clusters of erythroid cells is also shown (d). Data are presented as mean ± SEM from a minimum of four independent experiments. Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk “*” indicates a statistical significance of P < 0.05.

seeded from 6-day-old EBs. We observed no difference in the number of CFU-E/BFU-E between wt and p38α−/− cells derived from 10- and 14-day-old EBs (Figure 4(a)). The reduced erythropoietic potential of p38α−/− cells was further confirmed by the low hemoglobin level determined by staining for pseudoperoxidase activity. The hemoglobin level was near to the assay background in 6-day-old EBs but strongly increased in 10- and 14-day-old EBs (Figure 4(b)), which correlates with the observable clusters of erythroid cells in differentiating EBs. The first rare red-coloured erythroid cell clusters were observed from day 8 or day 9 of differentiation (not shown). In wt p38α+/+ cells, about 50% of EBs were contained from 1 to 3 red-coloured clusters of erythroid cells. They were clearly visible up to days 12 or 13 of differentiation. Later, these clusters disappeared. They were present in only 20% of 14-day-old differentiating EBs. In mutant p38α−/− cells, no more than 10% and 2% of EBs were positive for these erythroid clusters on days 10 and 14 of differentiation, respectively (Figures 4(c) and 4(d)).

We also determined the expressions of transcripts which play a key role in erythropoiesis (GATA1 and Klf1) and of globin transcripts (Hbb-b1, ζ-globin, and γ-globin). wt p38α+/+ cells exhibited higher levels of GATA1 and Klf1 transcripts than mutant p38α−/− cells (Figures 5(a) and 5(b)). The analysis of hemoglobin transcript expressions showed similar results: lower levels of Hbb-b1, γ-(gamma-) globin, and ζ- (zeta-) globin transcripts were observed in p38α−/− cells compared to p38α+/+ cells (Figures 5(c)–5(e)). Interestingly, there was no difference in the levels of EpoR transcripts between wt and mutant cells, except on day 14, when its expression was higher in wt cells (Figure 5(f)).

The erythroid lineage, CFU-E, was significantly reduced in p38α−/− cells at the early phase of haematopoiesis, which corresponds to the reduction in hemangioblast progenitors (see above). Later, the number of CFU-E was the same in both cell lineages but p38α−/− erythrocytes did not mature, as is shown by the reduced levels of GATA1, hemoglobin transcripts, and hemoglobin protein.

3.3. Involvement of p38α in Myeloid Differentiation. Next, the differentiation into myeloid lineages was studied in detail. First, cells from 6-, 10-, and 14-day-old EBs were tested
for granulocyte and monocyte colony-forming potential in granulocyte-monocyte- (GM-) selective media. CFU-G represented about 8% of all colonies formed from wt 6- and 10-day-old EBs. Its proportion decreased to 4% in wt 14-day-old EBs.

Except for p38α−/− cells from 6-day-old EBs, the number of CFU-G in mutant p38α−/− EBs was similar to that in their wt counterparts. p38α−/− cells isolated from 6-day-old EBs exhibited 20 times lower potential to form granulocyte colonies than wt cells (Figure 6(a)).

Colonies of CFU-GM and CFU-M were formed with frequencies of about 1% and 0.5%, respectively. Mutant p38α−/− cells formed a lower number of CFU-GM in 6-day-old EBs, but there was no difference in 10- and 14-day-old EBs compared to wt cells. Generally, CFU-M were formed at very low frequencies, which slightly increased up

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**Figure 5:** Expression of erythropoiesis-regulating transcription factors and markers determined by qRT-PCR. The levels of transcription factors GATA1 (a) and Klf1 (b) and the transcripts of hemoglobin Hbb (c), Hbb (d), Hbb-b1 (e), and EpoR (f) are shown in wild-type and mutant p38α−/− cells. Data are presented as mean ± SEM from a minimum of four independent experiments. Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk *** indicates a statistical significance of P < 0.05.
to 0.5% in 10- and 14-day-old EBs (Figures 6(b) and 6(c)). The role of p38α in this process remains to be clarified.

In contrast to the results for G/GM/M colony-forming assay, the levels of key myeloid transcription factors changed due to p38α depletion. The expression of PU.1 transcript increased during wt EB differentiation in a time-dependent manner. The highest level was determined in 14-day-old EBs. This was not observed in mutant p38α−/− EBs, where the expression of PU.1 was similar at all times of analysis and lower in 10- and 14-day-old EBs than in wt EBs (Figure 7(a)). In contrast to PU.1, the level of C/EBPα transcript was not elevated in EBs in either a time- or genotype-dependent manner (Figure 7(b)). Also, we did not detect any elevation in G-CSF-R1 or G-CSF-R2 transcripts, although M-CSF-R1 had a higher expression in 14-day-old wt EBs compared to 6- and 10-day-old wt EBs (Figures 7(c)–7(e)).

Kinase p38α is required for the regular expression of the PU.1 transcript. Excluding the early phase of haematopoiesis, the depletion of p38α kinase did not significantly affect the formation of CFU-G, CFU-GM, or CFU-M in GM media, which is EPO free, in contrast to full media. Therefore, p38α kinase and EPO are able to regulate the fate of myeloid progenitors.

4. Discussion

The first step in haematopoiesis is the formation of primitive hematopoietic cells from hemangioblasts. The formation of hemangioblast is induced by the VEGF/Flk1/Etv2 signaling axis. The expression of Flk1 ligand VEGF transcripts was unresponsive to the absence of p38α kinase, but cells without p38α had lower expressions of both Flk1 and its downstream signaling target Etv2. The Flk1-mediated signaling pathway is attenuated not only by low Flk1 transcription but also by the fact that the binding of VEGF to Flk1 leads to the activation of p38α kinase, which is responsible for the induction of Etv2 expression [23]. Hemangioblast is thus formed in a p38α kinase-dependent manner. The loss of hemangioblast leads to the attenuation of primitive haematopoiesis within the early stage of development. In the next step, Etv2 continuously induces the formation of hemangioblast and its subsequent transformation into angioblast and hematoblast [43]. In hematoblast, Etv2 induces the expression of Tal1 (Scl),
Runx1, GATA1, and GATA2, key regulators of hematopoiesis [27, 44, 45]. Defects in the transcription of Flk1, GATA2, Etv2, and Tal1 lead to embryonic lethality due to defective hematopoiesis and vasculogenesis [46–48]. The depletion of GATA2 results in embryonic lethality at E11.5, in part due to anemia [49]. Etv2 mutant embryos are nonviable after E9.5, and these embryos lack hematopoietic and vascular lineages [50]. Our results showed that the expression levels of Flk1, GATA2, and Etv2 were influenced by the depletion of p38α MAPK in ES cells. This also correlated with the low number of hemangioblast colonies in p38α−/− cells. Tal1 plays an important role in primitive and definitive hematopoiesis and is a direct upstream regulator of Runx1 [39, 51]. The transcription factors Etv2 and Tal1 are involved in the early steps of hematopoiesis in vivo; Etv2 is important for hemangioblast differentiation [27] while Tal1 is responsible

**Figure 7:** Expression of transcription factors and marker transcripts associated with the regulation of myelopoiesis. Levels of selected transcripts were determined by qRT-PCR in 6-, 10-, and 14-day-old wild-type and mutant p38α−/− EBs. The effect of p38α depletion on the expressions of transcription factors PU.1 (a) and C/EBPα (b) and the receptors coding the transcripts of key myeloid cytokines M-CSF-R1 (c), G-CSF-R1 (d), and G-CSF-R2 (e) is shown. Data are presented as mean ± SEM from a minimum of four independent experiments. Statistical significance was determined by ANOVA with post hoc Bonferroni’s multiple comparison test; asterisk “∗” indicates a statistical significance of P < 0.05.
for erythropoiesis in the yolk sac and the differentiation of hemogenic endothelium [52–54]. Runx1−/− mice die in utero at E12.5, and their fetal liver contains only primitive erythroblasts. Moreover, it has been demonstrated that Runx1 is crucial in endothelial-hematopoietic transition [36–38, 55]. Tal1 cooperates with other factors and increases the expression of GATA1, Runx1, and itself. Landry and colleagues observed that Runx1 expression in the yolk sac is directly regulated by Tal1 [51]. Moreover, the disruption of Runx1 in mice leads to the decreased expression of GATA1 and Klf1 [56]. While the expression of Tal1 was not affected in p38α−/− cells in our study, the expression level of Runx1 and thus the expression level of GATA1 were p38α dependent and correlated well with Etv2 expression; that is, both the expression levels of Runx1 and GATA1 decreased in a p38α-dependent manner. This could indicate a so-far unknown Etv2-independent mechanism behind Tal1 regulation and/or that Tal1 induces Runx1 expression in a p38α-dependent manner.

The expression of HoxB4 was independent of both p38α kinase and the level of Etv2. HoxB4 is also a key regulator of the hematopoietic lineage and is essential for the maintenance of hematopoietic stem cells [31, 32]. Therefore, we analysed the expression of other markers which can be associated with hematopoietic stem/progenitor cells. Unexpectedly, the expressions of Sca1 and c-Kit were increased in contrast to the overall decrease in haematopoiesis in p38α−/− cells but this could be explained as a delay in the differentiation process [14, 57]. In contrast, lower expressions of CD34 transcripts in mutant cells mark a decrease in hematopoietic potential. However, we did not observe a significant difference in the overall potential for hematopoietic CFU formation between wt and mutant cells derived from 10- and 14-day-old EBs. Thus, our results show that p38α is important for hemangioblast formation but not for the further formation of EMP, HPC/HSC, or the hematopoietic progenitors themselves, excluding the CFU-G lineage. Subsequently, on the basis of our results, it should be hypothesized that when haematopoiesis is established, p38α signaling is a necessary factor in both erythropoietic maturation and the direction of CMP fate. On the basis of our results and the previously published data discussed above, we propose that p38α kinase plays a key role in haematopoiesis through the regulation of Etv2 expression. VEGF, through Flk1, induces the expression and activity of Etv2 via p38α kinase. Etv2 is an inducer and a crucial regulator of hemangioblast formation and regulates the expressions of other transcription factors that are necessary for hemangioblast and early hematopoietic development. Moreover, the Etv2-induced expression of Flk1 represents a source of positive feedback in hematopoietic mesoderm formation [58]. The absence of p38α prevents the induction of Etv2 by VEGF/Flk1 and inhibits the initial process of haematopoiesis.

When we looked at erythropoiesis itself, the formation of CFU-E/BFU-E was affected in 6-day-old p38α−/− EBs. The overall reduction in CFU in 6-day-old p38α−/− EBs could be caused by the general delay in mesodermal/hemangioblast formation, which is in agreement with Barruet et al. [15]. In addition, we hypothesized that the decreased number of erythroid progenitors in 6-day-old EBs is connected to a reduction in the GATA1 expression level. Weiss and his colleagues observed that GATA1-deficient ES cells are unable to give rise to primitive erythrocytes, while in definitive erythropoiesis, GATA1 plays a role in the maturation of proerythroblast [59, 60]. The formation of CFU-E/BFU-E was not affected by the depletion of p38α kinase in 10- and 14-day-old EBs, although GATA1 and Klf1 transcript levels remained continuously low. In addition, GATA1 and Klf1 factors mediated the maturation of erythrocytes in coordination with EPO, when EPO induced phosphorylation and the transcription activity of GATA1 [61]. Previously, it was shown that EPO activity is mediated by p38α kinase and that p38α kinase is required for EPO mRNA stability and hemoglobin synthesis. Moreover, the inhibition of p38α leads to the blocking of the EPO-dependent accumulation of mouse globin chains in erythroid precursors [14, 57]. Also, erythrocyte maturation is impaired not only by defects in EPO/EpoR downstream signaling but also by a low level of EpoR transcript, the expression of which is induced by GATA1 [61]. This can explain our observations that p38α attenuated haematopoiesis and that p38α−/− erythroblasts did not mature, although EPO was present in the culture media (full media and erythroid media for CFU) and the expression of EPO was not different in wt and p38α−/− cells in 10- and 14-day-old EBs. Thus, it seems that the maturation of erythroblast is not connected only with the expression and/or level of EPO in a p38α-dependent manner. We conclude that p38α should be necessary for erythrocyte maturation but not for the formation of CFU-E/BFU-E during definitive waves of haematopoiesis, which is in agreement with the observation by Tamura and colleagues [14]. The GATA1 level is regulated through Etv2 and Runx1, whose expression is also decreased in a p38α-dependent manner, as discussed above and shown in our results. Erythropoiesis is thus regulated by Etv2, Runx1, and GATA1 signaling in a p38α-dependent manner. The depletion of p38α leads to the attenuation of Etv2 expression due to the insufficient expression of GATA transcription factors, followed by a decline in erythroblast production. Further, the maintenance of GATA factors and their phosphorylation, which is partially mediated by both p38α kinase and the presence of EPO, induces the expression of hemoglobin and erythrocyte maturation. Altogether, the depletion of p38α leads to the formation of a low number of immature erythrocytes.

Myelopoiesis is driven by PU.1 and C/EBPα transcripts, the expressions of which are induced by Runx1 [36]. Both myeloid lineages, granulocytes, and monocytes, are induced by increasing PU.1 and C/EBPα expressions [62]. A balanced ratio of PU.1 and C/EBPα leads to granulopoiesis, but a higher expression ratio of PU.1 to C/EBPα leads to monocytopenia [63]. The ratio of PU.1 to C/EBPα increased continuously during the differentiation of wt cells. However, we did not observe the increased formation of CFU-M in wt, nor, conversely, an increase in G-CFU in mutant cells, as could be expected. CFU-G formation was decreased in p38α−/− cells expanded in complete CFU full media. However, the expression of M-CSF-R1 was higher in wt compared to mutant cells in 14-day-old EBs, which could indicate a higher
population of promonocytic progenitors. There was no difference in G-CSF-R1 or G-CSF-R2 expression or in the expression of their upstream regulator C/EBPα. Nevertheless, the frequency of CFU-M was very low in both cell types. Analyses of the potential role of p38α kinase in monocytopenesis would require a different type of experiment.

Interestingly, when we tested the capacity to form CFU in CFU media without EPO (G/M selective media—GM medium), the frequencies of CFU-G and CFU-GM were affected in 6-day-old EBs only. In 10- and 14-day-old EBs, we did not observe any significant difference in myeloid CFU formation between wt and mutant cells. This is in contrast to CFU-G expansion in complete CFU media, i.e., the full medium (methylcellulose medium with SCF, IL-3, IL-6, and EPO), where we observed a low frequency of CFU-G in mutant cells compared to wt cells. The full medium and GM medium differed only in the presence of EPO. The absence of EPO (GM medium) thus reverses the effect of p38α kinase depletion on impaired granulopoiesis.

If granulopoiesis is compensated to a normal level in p38α−/− cells due to EPO depletion, we hypothesize that common myeloid progenitors (CMP) and/or their early ancestors undergo transformation into CFU-G. However, in the presence of EPO, they develop normally into CFU-E/BFU-E. This demonstrates that the balance of the EPO level and p38α activity determines whether CMP differentiates into erythroid or myeloid lineages. Importantly, this data also suggests a probable difference in EPO signaling. It seems that EPO enables the induction of CFU-E/BFU-E formation in a p38α-independent manner but that the maturation of erythroblast is p38α dependent. Further detailed study would be required to explain this phenomenon.

The effect of p38 kinase signaling inhibitors (SB203580 and SB202190 [64]) on haematopoiesis in ES cells was also determined. Except for the expressions of c-Kit and Sca1 RNA transcripts, wt cells adopt the phenotype of p38α−/− cells in the presence of p38α kinase inhibitors (Supp. 3 and 4).

Finally, the potential role of p38α in the specification of other germ layers and their progeny should also be taken into consideration. P38α−/− ES cells are pluripotent, as are their wt counterpart, and they are able to form all germ layers [10–13, 65]. However, their potential to form some cell lineages appears to be limited. It was reported that the depletion of p38α in vivo leads to defects in placentoid development and the fetus being anemic [14]. Notably, when cells in vitro are induced to differentiate by EB formation, p38α−/− cells more frequently adopt neuronal phenotypes and the development of mesoderm lineages is attenuated [15, 66]. In agreement with our study, dysregulated mesoderm development might at least partially correlate with the reduced level of Etv2 (expressed in mesoderm progeny) and the low number of hematopoietic CFU observed in 6-day-old EBs. Later, when haematopoiesis is already established and the overall number of hematopoietic CFU is identical in wt and mutant EBs, haematopoiesis might still be affected by p38α-dependent phosphorylation and the expression of relevant hematopoietic genes.

In conclusion, in this work, we describe the involvement of p38α in a model of the continuous development of haematopoiesis from pluripotent embryonic stem cells in vitro. On the basis of our observations, we hypothesized that the depletion of p38α regulates the balance in the hematopoietic developmental program by means of several mechanisms. Firstly, we demonstrated that p38α is required for the establishment of hemangioblast as a part of Flk1 signaling. Secondly, we showed that p38α regulates both the direction of CMP differentiation into the granulocyte lineage and erythroblast maturation. We found that the action of p38α is associated with the regulation of the expressions of key transcription factors of haematopoiesis, such as GATA1, Klf1, Runx1, and PU.1, which has also been described previously within particular hematopoietic cell lines or lineages. The most interesting result, the role of p38α in the fate of CMP, will require further detailed analysis. Two frequently used inhibitors of p38 kinase signaling also closely mimicked the effect of p38α kinase depletion on haematopoiesis, which confirmed that active kinase is required for the regular process. We suggest that, taken together, these findings could help us to understand the role of p38α and its importance as a therapeutic target within some leukemic illnesses, as discussed recently [67–70].

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This research was supported by the Czech Science Foundation (Project 17-05466S) and by the Faculty of Science of Masaryk University (MUNI/A/1145/2017). LK was supported by a grant from the Ministry of Education, Youth and Sports of the Czech Republic (MEYS CR; project no. 1Q1605).

Supplementary Materials

Supp. 1. Suggested scheme of haematopoiesis both in in vivo (a) [1–3] and in in vitro (b) [4, 5] models of ES cell differentiation in mice. The scheme of the potential role of p38 kinase in haematopoiesis based on previous published results and those observed in our work (c). VEGF induces expression of Etv2 in a p38 kinase-dependent manner, which leads to hemangioblast formation and development. EPO/EpoR-induced activation of p38 kinase and phosphorylation (green arrow) of its targets GATA1 and GATA2, in which transcriptional activity is required for erythropoiesis. Haematopoiesis in our experiments with ES cells. The capability of forming hematopoietic colony-forming unit (CFU) progenitors (d), CFU-G, CFU-GM, CFU-M, CFU-E, and CFU-GEMM in differentiating wt ES cells (e). The expression of haematopoiesis-specific transcripts in our model of in vitro haematopoiesis in wt ES cells (f). Details appear as Materials and Methods and Results in our manuscript. Supp. 2.
Mutated p38α−/− cells did not express p38α protein, in contrast to their wt counterparts. The expressions of key pluripotent protein Oct4 and generally abundant GAPDH were equal in both cell lines (a). When cells were differentiated by means of EB techniques, the overall level of p38α transcript RNA did not change (b). The levels of p38α kinase protein as well as its phosphorylated form were also unchanged. This was in contrast to the level of Oct4 protein, a marker of undifferentiated pluripotent cells, that decreased continuously with differentiation time. The protein level of GAPDH, which was used as a reference gene, is also shown (c). The phosphorylated form of p38α kinase when wt cells were treated by inhibitors of the p38 kinase pathway SB203580 or SB202190 (5 μM) for 1 hour and subsequently by 200 μM H2O2 for 1 hour (d). Data are presented as representative Western blot from two experiments and RNA level as mean + SEM from four independent experiments. Supp. 3. The effect of the p38 kinase pathway inhibitor (SB203580 or SB202190) on the formation of CFU and selected RNA transcripts through EB-differentiating ES cells. The number of CFU and the level of selected RNA transcripts in mutant p38α−/− cells are also shown. For details, see Materials and Methods. The concentration of inhibitors was 5 μM. Cells were exposed to inhibitors for the full duration of the differentiation of EBs. Inhibitors were always added to the medium when old medium was replaced by new medium, which was every two days of the culture. The number of all hematopoietic CFU and particular CFU in 6-, 10-, and 14-day-old EBs (a). The expression of RNA transcripts regulating and marking haematopoiesis (b, c) and the expression of RNA transcripts associated with erythropoiesis (d). Data are presented as mean + SEM from a minimum of three independent experiments. Supp. 4. The table summarizes data of both the effect of p38α−/− depletion and the effect of p38 kinase signaling inhibitors on the expression of selected RNA transcripts that are associated with haematopoiesis and that are presented in Figure 3 (for details, see Materials and Methods). It shows that the upregulation (↑), downregulation (↓), and no-change (−) of the mentioned RNA transcript expressions compare to the levels of the same RNA transcripts in wt cells. The c-Kit and Sca1 (both marked in bold in the table) RNA transcripts levels only have an opposite trend in expression in p38α−/− cells compared to wt cells treated by p38 kinase inhibitors. (Supplementary Materials)

References


References


Supplements

Supp. 1

Suggested scheme of hematopoiesis both in vivo (a) [1, 2, 3] and in in vitro model of ES cells differentiation (b) [4, 5] in mice. The scheme of potential role of p38 kinase in hematopoiesis based on previous published results and observed in our work (c). VEGF induces expression of Etv2 in p38 kinase-dependent manner, what leads to hemangioblast formation and development. EPO/EpoR induced activation of p38 kinase and phosphorylation (green arrow) of its targets GATA1 and 2, which transcriptional activity is required for erythropoiesis. Hematopoiesis in our experiments with ES cells. The capability forming hematopoietic colony forming unit (CFU) progenitors (d), CFU-G, -GM, -M, -E, and -GEMM in differentiating wt ES cells (e). The expression of hematopoiesis specific transcripts in our model of in vitro hematopoiesis in wt ES cells (f). For details, see Materials and Methods, and Results in our manuscript.

Supp. 2

Mutated p38α−/− cells did not express p38α protein, in contrast to their wt counterparts. The expression of key pluripotent protein Oct4 and general abundant GAPDH was equal in both cell lines (a). When cells were differentiated by means of EB techniques, the overall level of p38α kinase RNA did not change (b). The level of p38α kinase protein as well as its phosphorylated form were also unchanged. This was in contrast to the level of Oct4 protein, a marker of undifferentiated pluripotent cells, that decreased continuously with differentiation time. The protein level of GAPDH, which was used as a reference gene, is also shown (c). The phosphorylated form of p38α kinase when wt cells were treated by inhibitors of p38 kinase pathway SB203580 or SB202190 (5μM) for 1 hour and subsequently by 200 μM H2O2 for 1 hour (d). Data are presented as representative western blot from two experiments, RNA level as mean ± SEM from four independent experiments.
Supp. 3

The effect of p38 kinase pathway inhibitor (SB203580 or SB202190) on the formation of CFU and selected RNA transcripts through EB-differentiating ES cells. The number of CFU and the level of selected RNA transcripts in mutant p38α-/- cells are also shown. For details, see Materials and Methods. The concentration of inhibitors was 5 μM. Cells were exposed to inhibitors for the full duration of the differentiation of EBs. Inhibitors were always added to the medium when old medium was replaced by new medium, which was every two days of the culture. The number of all hematopoietical CFU and particular CFU in 6-, 10-, and 14-day-old EBs (a). The expression of RNA transcripts regulating and marking hematopoiesis (b, c) and the expression of RNA transcripts associated with erythropoiesis (d). Data are presented as mean + SEM from a minimum of three independent experiments.

Supp. 4

The table summarizes data both the effect of p38α-/- depletion and the effect of p38 kinase signaling inhibitors on the expression of selected RNA transcripts that are associated with hematopoiesis and that are presented on Fig. 3 (For details, see Material and Methods). It shows that the up-regulation (↑), down-regulation (↓) and no-change (-) of the mentioned RNA transcript expressions compare to the levels of the same RNA transcripts in wt cells. The cKit and Sca1 (both marked in bold in the table) RNA transcripts levels only have an opposite trend in expression in p38α-/- cells compared to wt cells treated by p38 kinase inhibitors.

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<td>VEGF</td>
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References


In vivo

Supplement 1.

- Blastocyst → Inner cell mass (pluripotent stem cell)
- Primitive streak → Mesoderm (Hemangioblast)
- Yolk sac → Blood islands (Primitive erythrocyte)
- AGM/fetal liver (Hematogenic endothelium) → EMP (Erythro/myeloid progenitor)
- Dorsal aorta (Hematogenic endothelium) → Pre-HSC
- E 3.5-4
- E 6.5
- E 6.5
- E 7.5-9
- E 8.5
- E 11.5
- E 14.5

In vitro

- Embryonic stem cell → Mesoderm (Hemangioblast)
- Primitive erythrocyte
- EMP → Definitive erythrocyte
- Myleoid progenitor
- Pre-HSC → HPC/HSC
- EPO / EpoR >> p38
- VEGF / Flk >> p38 >> Etv2
- Tal1
- GATA2
- GATA1
- Runx1
- P-GATA2
- P-GATA1
- Klf1
- Hemoglobins
- Erythropoiesis

(a) (b) (c)
Supplement 1.

(d) Relative number of hematopoietic colonies

(e) Relative number of colonies in full medium

(f) Transcript level relative to GAPDH

Days of EB differentiation
Supplement 2.

(a) p38α (+/+ -/-)

(b) Transcript level relative to GAPDH

(c) (a) (b) (c) (d)
Supplement 3.

Hematopoietic colonies

Relative number of colonies in full medium (6ds old EBs)

Relative number of colonies in full medium (10ds old EBs)

Relative number of colonies in full medium (14ds old EBs)

Type of CFU

(a)
Supplement 3.

(a) Hematopoietic colonies

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(b) Transcript level relative to GAPDH

- **Fik1**
- **VEGF**
- **HoxB4**
- **Runx1**
- **GATA2**
- **PU.1**
Supplement 3.

(a) Hematopoietic colonies

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Relative number of colonies in full medium (6ds sold EBs)

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(b) Transcript level relative to GAPDH

V E G F

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(c) Transcript level relative to GAPDH

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The depletion of p38alpha kinase upregulates NADPH oxidase 2/NOX2/gp91 expression and the production of superoxide in mouse embryonic stem cells

Lucia Binó a,b, Iva Veselá a, Iva Papežíková b, Jiřina Procházková c, Ondřej Vašiček b, Kateřina Štefková b, Jan Kučera n, Markéta Hanáčková b, Lukáš Kubala b, Jiří Pacherník a,n

ABSTRACT

P38alpha kinase plays an important role in the regulation of both cell stress response and cell fate. In this study, we report that p38alpha kinase-deficient embryonic stem cells exhibit a higher production of reactive oxygen species (ROS) in contrast to their wild-type counterpart. Analysis of the expressions of NADPH oxidases (NOXs) and dual oxidases, crucial enzymes involved in intracellular ROS formation, shows NOX2/gp91phox is overexpressed in p38alpha deficient cells. The particular increase in superoxide formation was confirmed by the specific detection of hydroethidine derivat 2-hydroxyethidium. ROS formation decreased when the level of NOX2 was silenced by siRNA in p38alpha deficient cells. These data suggest the importance of p38alpha kinase in the regulation of ROS metabolism in embryonic stem cells and the significance of the observed phenomena of cancer cell-like phenotypes, which is discussed.

1. Introduction

P38 (mainly p38α) kinase, which is member of the mitogen-activated protein kinase (MAPK) superfamily of protein kinases that are key players in numerous signaling events in cells from yeast to mammals, has been suggested to play a key role during cell response to various stress stimuli. Different reactive oxygen species (ROS) can induce stress and/or their level can increase during stress response. This reaction leads to the activation of p38 kinase, which has been characterized as a protein kinase activated in mammalian cells in response to lipopolysaccharide and extracellular changes in osmolality. Chemical stressors and toxins, similarly to extracellular and intracellular radicals, also induce the activity of p38 kinases. Moreover, it has recently been shown that p38 kinase is involved in many other cellular responses, including cell proliferation, differentiation, development, and apoptosis [1–3]. Four isoforms of p38 kinase have been identified so far: p38α, p38β, p38γ, and p38δ. The isoforms p38α and p38δ are widely expressed in several tissues. In contrast to p38α and p38γ, the expressions of the p38β and p38δ isoforms are tissue specific [4,5]. In the majority of cell types, the most abundant p38 family member is p38α.

We hypothesize that p38 kinases, as ROS targets, can play a negative feedback role in ROS formation. Thus, p38α deficiency should mediate an increase in intracellular ROS formation.

A cell's response to stress is in many cases associated with the elevation of ROS, which leads to p38 kinase activation, as mentioned above. P38 kinase is thus integrated into processes connected with intracellular redox status and balance. Here, we focus on the ROS level in cells with depleted p38α kinase. As a model, the undifferentiated pluripotent embryonic stem (ES) cell line DB (p38α−/+−) and its p38α kinase-deficient subclone (p38α−−/+−) were employed [6]. We detected higher ROS production in p38α−−/+− cells, which correlates with superoxide production and the upregulation of NOX2 expression in these cells. A possible role of p38α kinase in the regulation of NOX2 expression is suggested.
Table 1
List of primers for the determination of NOXs, DUOXs and neural-specific transcript levels using qRT-PCR employed in our study.

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</table>

* Universal Probe Library (Roche).

2. Material and methods

2.1. Cell culture, growth, and differentiation

P38α +/- and p38β +/- ES cells derived in Dr. Barry P. Skeelxman’s laboratory [6] were propagated in an undifferentiated state by culturing on gelatinized tissue culture plastic in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum, 100 mM nucleosides, 0.05 mM β-mercaptoethanol, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (referred to as complete DMEM), supplemented with 1000 U/ml of leukemia inhibitory factor (LIF, Chemicon). Both p38α +/- and p38β +/- ES cells were differentiated in the form of floating colonies calling embryoid bodies (EBs) for 5 and 10 days on non-adherent 0.5% agar coated dishes. This approach further prevented any EB adhesion to this kind of low adherent plastic surface [7].

2.2. Measurement of ROS production in live cells

Cells were washed and incubated with 2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) or hydroethidine (HE, both compounds from Sigma-Aldrich) in serum-free, phenol red-free DMEM/F12 medium at 37°C for 30 min. Then, the cells were harvested and the cell suspension placed on ice. Before DCFH-DA (40 μM) and HE (20 μM) incubation, the cells were preloaded with N-aceetyl cysteine (NAC) (10 mM) for 15 min in order to scavenge ROS. NAC had been employed as pan-specific ROS scavenger and antioxidant here [8,9]. At least ten thousand cells were analyzed using a FACSCalibur flow cytometer (BD Bioscience); the whole series of measurements took no more than 20 min to complete. The geometric mean in relative fluorescent units (RFU) was quantified for each sample [10]. Alternatively, cells (5 × 10⁴ per well) growing in a fluorescent 96-well plate were washed, then incubated with 30 μM Amplex Red (10-acetyll-3,7-dihydoroxphenoxyxavone, Invitrogen) and 0.1 U horse-radish peroxygenase (HRP, Sigma-Aldrich) in serum-free and phenol red-free DMEM medium in a tissue culture incubator in 5% CO₂ at 37°C for 3 h. Fluorescence was measured by a Hidex Sense microplate reader with an excitation maximum at 565 nm and an emission maximum at 590 nm at time points 5, 30 and 180 min [11].

2.3. High-performance liquid chromatography (HPLC) analysis of 2-hydroxyethidium

Superoxide determination was based on the HPLC detection of the specific product 2-hydroxyethidium (2-OH-E⁺), which is formed in the reaction of O₂⁻ with HE [12]. Besides specific 2-OH-E⁺, also a non-specific product of hydride acceptors with HE, ethidium (E⁺), was also detected. For our experiment we modified the protocols introduced by Zielonka [13] and Cia [14], as described previously [15]. The cells were seeded in a 6-well plate. Thirty minutes before the end of the experiment, HE in a final concentration of 20 μM was added. After centrifugation, the medium was stored for optional HPLC analysis. To extract the HE products, ice cold methanol was added to the cells for 15 min in the dark at 4°C. The supernatant was transferred to an Eppendorf tube and centrifuged. A 75 μl sample was injected into the HPLC system (Agilent series 1100) equipped with fluorescence and UV detectors (Agilent series 1260) in order to separate the 2-OH-E⁺ product. Fluorescence was detected at 510 nm (excitation) and 595 nm (emission). The mobile phase consisted of H₂O/CH₃CN. A Kromasil C18 (4.6 mm × 250 mm) column was used as the stationary phase. The amount of 2-OH-E⁺, E⁺ and HE were adjusted to the level of protein measured by Bio-RAD Protein kit (Bio-RAD, USA).

2.4. Detection of SH groups

The detection of SH groups was performed as described previously [10]. Briefly, cells were washed twice with PBS containing 600 μM deferoxamine and 10 mM ethylene diamine tetraacetic acid (EDTA), lysed by 2% SDS with 10 mM EDTA and 600 μM deferoxamine, and sonicated 2 times for 10 s on ice. 0.1 ml of 0.01 M 5,5-dithio-bis-(2-nitrobenzoic acid was added to 9.9 ml 0.2 M Tris pH 8.2. 230 μl of this buffer was mixed with 20 μl of sample and incubated for 30 min at room temperature. The absorbance was measured at 412 nm on a Spectra Rainbow microplate reader (Tecan, Austria). The total level of SH groups was adjusted to the level of protein measured by DC Bio-RAD Protein kit (Bio-RAD, USA), as described above.

RT-PCR analysis of NOX expression and the expressions of markers of ES and neural phenotypes.

Total RNA was extracted from each cell preparation by Qiagen RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized according to the manufacturer’s instructions for M-MLV reverse transcriptase (Sigma). For quantification of the gene expressions of NOXs, DUOXs and neural specific genes by real-time PCR, we employed the Universal ProbeLibrary Mouse system (Roche Applied Science). Primers and probes for NOXs, DUOXs, and neural-specific transcripts are listed in Table 1. The annealing temperature was 60°C. Ribosomal protein L13A (RPL13A) was selected as the reference gene. Real-time PCR of ES cell phenotype markers was performed in a Roche 480 Light-Cycler using LightCycler® 480 DNA SYBR Green I Master (Roche, Switzerland). Primers and annealing conditions are listed in Table 2. GAPDH was used as a reference gene. Data are presented as the differences between control and treatment groups or as the differences between wild-type and mutated cells after normalization to the reference gene by the 2⁻^ΔΔCq method [15,16].

2.5. Measurement of mitochondria and mitochondria membrane potential

Cells were incubated with mitochondria fluorescence probes
according to the manufacturer’s instructions in a tissue culture incubator for 20 min at 37 °C and in 5% CO2 and 95% humidity. Cells were stained with the following probes: DioC6 (3) (3,3′-Dihexyloxocarbocyanine iodide; 0.5 μM) for mitochondria; JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide, 0.25 μg per ml) and/or TMRE (Tetramethylrhodamine ethyl ester perchlorate, 100 nM) for mitochondria membrane potential (all probes from Invitrogen). Samples were washed in HBSS and analyzed using FACSscalibur (Becton Dickinson, Franklin Lakes, NJ, USA). An argon laser (488 nm) was used to excite the fluorescence of the dyes. Fluorescence emission was then measured with a 530/30 (FL-1) filter (JC1 and DioC 6 (3)) and a 585/42 BP filter (JC-1 and TMRE) [17].

2.6. NOX2 silencing by siRNA

For the small interfering RNA (siRNA) knockdown of NOX2, cells were transfected with Lipofectamine RNAi/MAX (Invitrogen) according to the manufacturer’s instructions. SiRNA supplied by Santa Cruz Biotechnology (Cat. No. sc-355604) was used against NOX2 (gp91phox).

2.7. Western-blot analysis

Immunoblot analysis and cell sample harvesting were performed as presented previously [15]. We used the following primary antibodies: rabbit polyclonal antibodies against p38α, Oct-4 (Santa Cruz Biotechnology), Nanog and NOX2/gp91phox (Abcam), cytochrome c, COX IV (Cytochrome c oxidase subunit IV), VDAC (Voltage-dependent anion-selective channel) and GAPDH (Cell Signaling Technology). Following immunodetection, each membrane was stained by amido black to confirm an equal protein loading.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Groups were compared using a paired Student’s t-test or ANOVA with A Bonferroni posttest. Values of p less than 0.05 were considered significant.

3. Results

3.1. Depletion of p38α kinase partially affects the ES cell phenotype

First, we characterized the p38α+/+ and p38α−/− ES cell phenotype. The depletion of p38α kinase had no effect on the expressions of the main pluripotent markers, the Oct4 and Nanog protein levels (Fig. 1A). P38α+/+ and p38α−/− ES cells also expressed the same level of Oct4, Nanog, Zfp42 and TNAP mRNA transcript. Interestingly, p38α−/− cells exhibited a higher level of Fgf5 transcript (Fig. 1B).

3.2. ROS production and the level of –SH groups in p38α +/+ and p38α −/− ES cells

ROS production was determined in p38α+/+ and p38α−/− cells by flow-cytometry analysis employing DCFH and HE as ROS-sensitive fluorescence probes and by fluorometric analysis using Amplex Red. P38α−/− cells had a higher level of intracellular ROS determined by HE and DFCF than their wild-type counterparts. When cells were pretreated with NAC, a decrease in ROS level in mutant cells was observed (Fig. 2A and B). P38α−/− cells also had a higher level of extracellular ROS than their wild-type counterparts, this determined by Amplex Red assay (Fig. 2C). To better characterize the formed ROS, we determined the superoxide-mediated formation of 2-OH-E′−, and intracellular E′− and HE by means of HPLC analysis. Interestingly, p38α−/− cells generated more 2-OH-E′− (a specific product of superoxide) than p38α+/+ cells. We did not observe a difference in E′− and HE levels between p38α+/+ or p38α−/− cells (Fig. 2D).

To further characterize the redox status of the cells, the cells were
lysed 24 h after seeding and the level of intracellular -SH groups was evaluated together with the level of non-protein-SH groups. P38α−/− cells had a lower total level of -SH groups but there was no difference in the levels of non-protein -SH groups compared to their wild-type counterparts. In parallel, the effect of NAC on the level of -SH groups in cells was also determined. In this experiment, NAC increased the level of non-protein -SH groups, but the total level of -SH groups was not affected (Fig. 3).

3.3. Level of NOXs in p38α+/+ and p38α−/− ES cells

Using real-time PCR analysis of the relevant mRNA level, we showed a significantly higher level of mRNA for NOX2 in p38α mutated cells compared to their wild-type counterparts. We also showed a significantly higher level of NOX2/gp91 phox protein expression in p38α mutated cells compared to their wild-type counterparts. The expression of mRNA for NOX1, NOX3, NOX4, DUOX1 and DUOX2 was generally very low in both tested cell lines and we did not observe a difference in the transcription levels of these enzymes between p38α−/− and wild-type p38α+/+ ES cells (Fig. 4A and B). Similarly, there were no differences in the levels of the transcripts of the NOX catalytic subunits p47phox and p67phox (Fig. 4C).

Further, changes in the expressions of NOXs and DUOXs during the spontaneous differentiation of ES cells mediated through EB formation for 5-days were also analyzed. Interestingly, only NOX4 transcript expression increased during differentiation in both p38α+/+ and
3.4. Mitochondria level and mitochondria membrane potential in p38α+/+ and p38α−/− ES cells

Since mitochondria contribute to ROS formation in cells and affect the overall redox status of cells, the level of mitochondria and mitochondria membrane potential, which correspond with mitochondria activity, were determined in p38α+/+ and p38α−/− cells by means of flow-cytometry analysis employing DioC6 (3), JC-1 and TMRE probes. P38α−/− exhibited a lower level of fluorescence signal in all three assays compared to their wild-type counterpart (Fig. 5A, B, C). The level of expression of mitochondria-specific proteins was also evaluated by western-blot in both cell lines. P38α−/− exhibited a lower level of cytochrome c protein compared to p38α+/+ cells, but COX IV and VDAC protein levels were comparable in both cell lines (Fig. 5D, E, F).

3.5. NOX2 mediated ROS production in p38α−/− ES cells

The effect of NOX2 depletion by siRNA on ROS production was analyzed. siRNA against NOX2/gp91phox decreased the level of NOX2 transcript in p38α−/− cells, but not in p38α+/+ cells (Fig. 6A). Cells with silenced NOX2 produced a lower level of both 2-OH-E+ and E+, which was not observed in p38α+/+ cells. The level of intracellular HE was identical in both cell lines (Fig. 6B, C, D).

3.6. NAC had no effect on the p38α-mediated shift in ES cell differentiation to neural lineage

The depletion of p38α kinase potentiates ES cell differentiation to...
neural lineages [18,19]. Thus, to investigate the importance of ROS formation in this phenomenon, the levels of three transcripts representing neural markers were determined during spontaneous ES cell differentiation through EBs. The EBs derived from p38α−/− ES cells had higher levels of Sox1 and Mash1 transcripts compared to EBs from wild-type counterparts. We did not observe a difference in the expression of Nestin transcript between p38α+/+ and p38α−/− cells. The presence of NAC had no effect on the expressions of Sox1 and Mash1. Interestingly, NAC enhanced the Nestin transcript level in p38α−/− cells higher than the level found in p38α+/+ cells (Fig. 7A, B, C).

4. Discussion

In this work we analyzed ROS production in wild-type (p38α+/+) and mutant (p38α−/−) ES cells. In the first step, we used the broadly employed ROS-sensitive probes DCFH-DA, HE and Amplex Red [20]. Importantly, mutant p38α−/− cells exhibited higher ROS levels than p38α+/+ cells. A decrease in cellular SH groups, which buffer intracellular ROS, was also observed in p38α depleted cells. This therefore suggests a change in overall redox status in these cells.

To cover the wide spectrum of ROS, we employed different fluorescent probes with different specificity. DCFH-DA is supposed to react

Fig. 5. Determination of mitochondria membrane and its potential in p38α+/+ and p38α−/− ES cells by means of fluorescence probes DioC6 (A), JC-1 (B), and TMRE (C), and analyzed by FACS. Below, representative histograms from analyses are also shown. Determination of expressions of mitochondria proteins cytochrome c (D), COX (E) and VDAC (F) in p38α+/+ and p38α−/− ES cells analyzed by Western blot. GADPH was employed as a reference for sample loading. Bars represent the mean ± standard deviation from three independent experiments. Asterisks mark statistically significant differences in expression level between cell lines, P < 0.05 (*) or P < 0.01 (**).
Interestingly, NAC enhanced the Nestin transcript level in the presence of NAC without affecting the expression of Nestin transcript between p38α+/+ and p38α−/− wild-type counterparts. We did not observe a difference in the expression of Sox1 and Mash1 transcripts compared to EBs from differentiating p38α+/+ and p38α−/− ES cells. The presence of neural markers were determined during spontaneous ES cell differentiation through EBs. The EBs derived from p38α+/+ and p38α−/− cells (Fig. 7A, B, C).

Determination of mitochondria membrane potential and its potential in p38α+/+ and p38α−/− ES cells is shown in Fig. 7. The intracellular HE (D) in p38α+/+ and p38α−/− cells. The differences in expression level between cell lines, P < 0.05.

Importantly, mutant p38α−/− ES cells exhibited higher ROS levels than p38α+/+ cells. A decrease in cellular SH-groups, which buffers the cellular redox status, was also observed in p38α−/− ES cells. This indicates a change in overall redox status in these cells. Fore suggests a change in overall redox status in these cells. Different proteins with different specifities were employed ROS-sensitive probes DCFH-DA, HE and Amplex Red [20]. Following Zhao et al., the HPLC-mediated determination of 2-OH-E+ (B) products of HE oxidation and level of intracellular E+ (C) were determined using qRT-PCR. Results from three independent experiments are presented as means and standard deviation. A statistically significant difference is indicated in the graph.

with a wide range of oxidants, in contrast to HE, which is at least semispecific to superoxide, and also in contrast to Amplex Red, whose oxidation is dependent on hydrogen peroxide driven peroxidase activity [20,21]. Following Zhao et al., the HPLC-mediated determination of 2-OH-E+, the specific adducts of HE reaction with superoxide, was also employed in this study to precisely analyze superoxide [12,15]. P38α−/− cells produced a higher amount of 2-OH-E+ and thus superoxide than p38α+/+ cells. We did not observe a difference in the production of oxidized HE, E+ between these two cell lines. This shows that mainly superoxide is produced due to the depletion of p38α kinase. Intracellular superoxide is predominantly generated in mitochondria [21] or by NOX and DUOX enzyme complexes [22]. ES cells exhibit a very low level of ROS because their mitochondria are immature and the expression of NOXs or DUOXs is negligible [15,23]. During differentiation, the intracellular level of ROS increases [24,25]. It is mediated by more mature mitochondria as well as by upregulated NOXs in more mature cells. Neither we nor other authors observed a difference in the basic pluripotent phenotype between wild-type and p38α−/− cells.
excluding the expression of FGF-5. The upregulation of FGF-5 is associated with the conversion of the inner cell mass from embryoblast-like to epiblast-like stem cells [26]. It is likely that ROS, as well as p38 kinases, participates in stem cell maintenance [27–29]. Increased ROS production is associated with the induction of proliferation and differentiation in stem cells [30]. Neither we (data not shown) nor others [31] determined a significant difference in proliferation between p38a mutant and wild-type ES cells. Importantly, p38α−/− cell self-renewal is still LIF-dependent in contrast to authentic epiblast-like stem cells, which are Activin/Nodal-dependent [32,33]. Thus, we suggest no significance of such a difference in the overall differentiation/maintenance of these cells. Therefore, we focused on the expression of NOXs, DUOXs and mitochondria activity in detail. Particular NOXs/DUOXs are expressed in a cell type-specific manner [15,22]. During the spontaneous differentiation of ES cells, where all three germ layers appear, the expression of NOX4 is increased [25], which we also confirm here. However, in our study, the expression of NOX4 was independent of the absence or presence of p38a kinase. Nevertheless, p38α−/− cells exhibit a higher expression of NOX2.

The analysis of mitochondria suggested that p38α−/− cells in comparison to p38a+/+ cells had lower mitochondria mass and activity, as was demonstrated by the evaluation of mitochondrial membrane potential by DioC6 (3) and mitochondria membrane potential by JC-1 and TMRE. P38α−/− cells also expressed less protein cytochrome c, which was confirmed by the abovementioned analysis of mitochondria membrane level and potential. On the basis of these results, we suggest that mitochondria, with high probability, are not responsible for increased superoxide production in p38α−/− cells. When we downregulated NOX2 expression using siRNA, the cell production of 2-OH-E+ declined. Thus, we conclude that NOX2 is the source of the higher production of superoxide in p38α-deficient cells.

The increased production of ROS in cells lacking p38a kinase may imply a possible negative feedback mechanism in which increased ROS activates p38 kinase, which in turn may inhibit NOX2 transcription and thus restore the normal ROS level. The activation of p38 kinase by ROS is well documented [34,35], but the connection between p38 kinase and the regulation of NOX2 expression has not been described. Thus, our recent results together with recent data presented by Liu et al. [36] show for the first time the role of p38α in the regulation of NOX2. However, it remains unknown whether the connection between p38 kinase and NOX2 expression is direct, due to p38 kinase substrate/downstream transcription factors, or indirect. The molecular background of the relationship between p38 kinase and NOX2 expression or activity is also unknown. However, Liu et al. showed that the depletion of p38 kinase in skin tumor cell lines increased the overall production of NOX2 expression and ROS production in a p35+/− background [36]. ES cells expressed p53 and responses to various stress or apoptotic stimuli in the same manner as somatic cells. However, it seems that the regulation and role of p53 is not the same as in somatic cells. In ES cells, p53 is abundant in the cytoplasm and not in the nucleus. Various stress responses in ES cells are also independent of p53, in contrast to somatic cells [37–39]. Thus, our and Liu’s results fit a scenario suggesting the specific interaction of p38a kinase and p53 activity in the regulation of NOX2 expression. Nevertheless, further research will be required to elucidate the mechanism of this regulation as well as how it plays a regulatory role in normal somatic cells. The induction of ROS by p38 depletion was very modest, but statistically significant in the investigated system. In fact, the level of 2-OH-E+ is increased only when the data are normalized to the total cellular protein level. The biological significance of such effects should be further studied and in the future and similar studies should be performed in other cell types, of higher background ROS production.

ROS can also affect cell fate through the regulation of various signals responsible for cell differentiation. It has been shown that p38α−/− ES cells develop into cells of all three germ layers in vitro, similarly to their wild-type counterpart [40,41]. Nevertheless, p38α−/− ES cells differentiated to a neural lineage with higher frequency in contrast to wild-type cells [18,19]. Neurogenesis and neural differentiation are sensitive to the intracellular level of ROS [8,42]. Therefore, we asked whether the preferred neurogenesis in p38α−/− cells is based on their higher production of ROS. When p38αa mutant and wild-type cells were left to differentiate spontaneously, superior neurogenesis was observed in p38α−/− cells. However, we did not observe the inhibition of neurogenesis in our system, when cells were differentiating in the presence of the ROS scavenger NAC.

To conclude, the present findings show that the depletion of p38a kinase in ES cells results in the up-regulated expression of NOX2 and thus the production of superoxide. Accordingly, we hypothesized about a possible negative feedback process regulating the relationship between p38α and NOX2. We suggest that this linkage may play an important role in the maintenance of stem or cancer cells, as well as in the regulation of cell differentiation and cell responses to stress stimuli. Nevertheless, overall data indicate a fine control of cellular superoxide production in ES cells.

Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgement

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References

p53 is abundant in the cytoplasm and not in the nucleus. Various ES cells expressed p53 and responses to various stress or apoptotic of p38 activity is also unknown. However, Liu et al. showed that the depletion ground of the relationship between p38 kinase and NOX2 expression or inhibition of neurogenesis in our system, when cells were differentiated in the presence of the ROS scavenger NAC. When p38−/− cells were left to differentiate/ma-
derentiation and cell responses to stress stimuli. It has been shown that p38 α−/− cells expressed less protein cytochrome c interaction of p38 kinase stimulated skin tumorigenesis through the induction of proliferation and di-
erentiation. It has been shown that p38−/− cell self-renewal is well documented [34,35], but the connection between p38 kinase and NOX2 transcription and production is associated with the induction of proliferation and di-
erentiation.
stimuli in the same manner as somatic cells. However, it seems that the background ROS production.

Depletion was very modest, but statistically significant in the induction of ROS by p38 kinase and NOX2 expression. Nevertheless, further research will be required to determine a significance of such effects should be further studied and in the future.

The activation of p38 kinase by ROS implies a possible negative feedback mechanism in which increased ROS may contribute to ROS production. The upregulation of FGF-5 is associated with the expression of NOX4 and is well documented [34,35], but the connection between p38 kinase activity is still LIF-dependent in contrast to authentic epiblast-like stem cells, [31] determined a significance of such effects should be further studied and in the future.
Research Article

Apocynin and Diphenyleneiodonium Induce Oxidative Stress and Modulate PI3K/Akt and MAPK/Erk Activity in Mouse Embryonic Stem Cells

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Reactive oxygen species (ROS) are important regulators of cellular functions. In embryonic stem cells, ROS are suggested to influence differentiation status. Regulated ROS formation is catalyzed primarily by NADPH-dependent oxidases (NOXs). Apocynin and diphenyleneiodonium are frequently used inhibitors of NOXs; however, both exhibit uncharacterized effects not related to NOXs inhibition. Interestingly, in our model of mouse embryonic stem cells we demonstrate low expression of NOXs. Therefore we aimed to clarify potential side effects of these drugs. Both apocynin and diphenyleneiodonium impaired proliferation of cells. Surprisingly, we observed prooxidant activity of these drugs determined by hydroethidine. Further, we revealed that apocynin inhibited PI3K/Akt pathway with its downstream transcriptional factor Nanog. Opposite to this, apocynin augmented activity of canonical Wnt signaling. On the contrary, diphenyleneiodonium activated both PI3K/Akt and Erk signaling pathways without affecting Wnt. Our data indicates limits and possible unexpected interactions of NOXs inhibitors with intracellular signaling pathways.

1. Introduction

Reactive oxygen species (ROS) play multiple roles in the biology of the cell [1]. NADPH oxidase (NOX) and oxidative reactions on the mitochondrial membrane are the main sources of ROS, although it can also be produced by other enzymatic and nonenzymatic sources [2, 3]. NOX is a membrane-bound protein complex generating superoxide anion ($O_2^{-}$) from molecular oxygen which initiates the cascade of free radical reactions in response to various stimuli. Production of ROS by NOX family has long been considered a unique property of phagocytic cells, which utilize this enzyme as a part of host defense immune system. Currently, the regulated ROS production in nonphagocytic cells by NOX was linked to regulation of different processes including proliferation, migration, differentiation, immunomodulation, and oxygen sensing, and therefore, its expression and activity are tissue specific and are tightly controlled [4, 5]. On the basis of the homology to the catalytic subunit of the original phagocytic NOX (gp91phox or preferably NOX2), other NOX isoforms—NOX1, NOX3, NOX4 and NOX5—have been identified in nonphagocytic cells. In parallel, two other members of the NOX family were discovered, namely, dual oxidases 1 and 2 (DUOX 1, 2), initially also referred to as thyroid oxidases [6]. Despite studies suggesting the importance of NOXs in general, the involvement of individual NOX family members in specific function is still not completely understood. One of the reasons is a lack of highly specific inhibitors that would
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ROS production in nonphagocytic cells by NOX was linked to cell proliferation. Further, we show that APO enhances both PI3K and Erk activity. Interestingly, APO strengthens Wnt activity, pointing out another unknown mechanism of APO-mediated changes in signaling cascades regulating mES. In contrast to PI3K and Erk, we did not observe any effect of APO or DPI on Stat3 phosphorylation, which is considered to play the major role in mES maintenance.

2. Material and Methods

2.1. Cell Culture and Treatment. A feeder-free adapted mES line RI was propagated as described previously [29] in an undifferentiated state by cell culturing on tissue culture plastic coated by gelatin (0.1% porcine gelatin solution in water) in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (FBS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1x nonessential amino acid (all from Gibco-Invitrogen, UK), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), supplemented with 5 ng/mL of leukemia inhibitory factor (LIF, Chemicon, USA) referred to here as the complete medium.

APO, DPI, hydrogen peroxide (H2O2), N-acetylcysteine (NAC), and LY294002 (LY) were provided from Sigma-Aldrich. Stock solutions of APO (0.4 M), DPI (10 mM), and LY (10 mM) were prepared by dissolving the compounds in dimethyl sulfoxide. Aliquots were stored at −20°C. NAC was prepared as a 0.5 M stock solution in serum-free DMEM medium, pH was adjusted to 7.4, and filter-sterilized aliquots were stored at −20°C. Drugs were added directly to the incubation medium or freshly prediluted in sterile phosphate-buffered saline (PBS) to desired concentration.

2.2. Cell Proliferation. The cell proliferation was determined by estimation of overall cellular protein mass in whole cell lysates that reflects the cell number as demonstrated previously [34]. ES cells were seeded to 24-well plate in complete media at density 5 000 cells per cm². Next day, the cells were treated with drugs for further 48 hours. Finally, the cells were washed twice with PBS and lysed in SDS buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1% SDS, 1 mM EDTA). Protein concentration was determined using DC protein assay (Bio-Rad, USA) kit according to manufacturer’s instructions.

2.3. Determination of NOX Expression. Total RNA was extracted using the UltraClean Tissue & Cells RNA Isolation Kit (MO BIO Laboratories, USA) for ESC and RNAzol RT (Molecular research center Inc., USA) for mouse tissues according to manufacturer’s instructions. 1 μg of total RNA was used for cDNA synthesis with DyNAmo cDNA Synthesis Kit (Finnzymes, Finland) according to the manufacturer’s instructions. qPCR was performed in LightCycler 480 instrument using LightCycler Probes Master with Universal Probe library probes (all from Roche, Germany) according to manufacturer’s instructions. Ribosomal protein L13A (RPL13A) was used as reference gene; data are presented as
impairment of cell proliferation. Further, we show that APO affected by NOX or other ROS modulating agents, although modified by ROS production [5, 32, 33] and therefore might be of evidence indicates that Wnt/\(^\beta\) negatively regulates the self-renewal in mES [28, 29]. Further, was demonstrated in many studies where its inhibition was found to interfere with other flavoenzymes, including nitric oxide derived inflammatory mediators [13].

idant stimulating ROS production [10–12]. Further, APO was identified by NOX or acetovanillone, APO) and diphenyleneiodonium chloride (DPI). Both of these drugs were applied in numerous experiments. The most frequently used inhibitor employed in experiments is 4-vanillin derivative 4-(4-hydroxy-3-methoxyphenyl)benzylamine (HBOC-203). This compound reliably blocks particular NOX. The above mentioned nonspecific effect was thought to be responsible for contradictory results obtained using these inhibitors. Further studies are needed to determine specific effects are thought to be responsible for contradictory results obtained using these inhibitors. Further studies are needed to determine specific effects are thought to be responsible for contradictory results obtained using these inhibitors.

2.4. Analysis of ROS Production in Live Cells by Automated Time-Lapse Image (Live Imaging Fluorescent Microscopy). The cells were seeded to 96-well plate designed for live imaging fluorescence microscopy (Greiner Bio-one, Germany). After 24 hours, cells were pretreated with tested drugs for 15 minutes and loaded with hydroethidine (HE, 5\(\mu\)M, Sigma-Aldrich). Live imaging of prepared samples was performed at 37°C and 5% CO\(_2\) atmosphere using the high-content screening microscope ImageXpressMicroXL (Molecular Devices, USA). Seven images per well were acquired during 120 minutes with scanning interval of 10 minutes. By Gaussian thresholding of the HE fluorescence images, mask area of the viewfield covered with metabolizing cells was detected using the Otsu method of fitting individual objects. The 10640 images for every measured plate were analyzed with CellProfiler [35], running on processor cluster provided by MetaCentrum (The National Grid Infrastructure). Intensity of fitted regions was lowered by minimal intensity of an appropriate image to subtract the background and the mean intensity per individual well was then calculated and visualized with Kinevo [36], using HCS tools and R Statistics Integration extensions [37].

2.5. High-Performance Liquid Chromatography (HPLC) Analysis of ROS Production. The HPLC detection of O\(_2^-\) was based on the detection of a specific product 2-hydroxymethylidium 2-OH-E(+) which is formed in the reaction of O\(_2^-\) with HE [38, 39]. Besides specific 2-OH-E(+), also a nonspecific product of hydride acceptors with HE – ethidium (E(+)) was detected. The cells were seeded to 6-well plate and before the treatment the medium was changed for DMEM (without phenol red and sodium pyruvate) with 1% FBS. The cells were treated with APO and DPI for 120 minutes in total; 30 minutes before the end of the experiment HE in final concentration 10\(\mu\)M was added. The medium after centrifugation was stored for optional HPLC analysis. To extract the HE products, ice cold methanol was added to the cells [40] for 15 minutes at 4°C in dark, shaking. The supernatant was transferred to an Eppendorf tube and centrifuged. A 75\(\mu\)L sample was injected into the HPLC system (Agilent series 1100) equipped with fluorescence and UV detectors (Agilent series 1260) to separate the 2-OH-E(+) product. Fluorescence was detected at 510 nm (excitation) and 595 nm (emission). The mobile phase consisted of H\(_2\)O/CH\(_3\)CN. Kromasil C18 (4.6 mm \(\times\) 250 mm) column was used as the stationary phase. Elution conditions for the analysis of HE and its products were used from Nature Protocols [38].

2.6. Western Blot Analysis. Western blot analysis, cell sample harvesting, and preparation were performed by a standard procedure as presented previously [29]. We used the following primary antibodies against Nanog, \(\beta\)-actin (Abcam, USA), p-Akt (S473), Akt, p-Stat3 (Y705), Stat3, Erk1/2, p-Erk1/2 (T202/Y204), and p-GSK3\(\beta\) (S9) (all from Cell Signaling Technology, USA). Following immunodetection, each membrane was stained by amido black to confirm the transfer of the protein samples. The total level of \(\beta\)-actin was detected as loading control.

2.7. Cell Transfection and TOPflash Luciferase Reporter Assay. Cells were transiently transfected using polyethyleneimine in a stoichiometry of 4 \(\mu\)L per 1 \(\mu\)g of DNA. Superb8X TOPflash construct, Renilla luciferase construct, and expression vector for mutant nondegradable \(\beta\)-catenin, and S33-\(\beta\)-catenin (codon 33 substitution of Y for S, generously provided by Professor Korswagen) were used in concentration of 0.5 \(\mu\)g per well in a 24-well plate 24 hours after seeding. 6 hours after transfection medium was changed and cells were treated with NOX inhibitors or LY for 24 hours. For cell stimulation Wnt3a or control conditioned medium [41] was added 8 hours before harvest. Dual-Luciferase assay kit (Promega, USA) was used according to the manufacturer’s instructions for the evaluation of luciferase activity. Relative luciferase units were measured on a plate luminometer Chameleon V (Hidex, Finland) and normalized to the Renilla luciferase expression.

2.8. Statistical Analysis. Data are expressed as mean ± standard error in the mean (SEM). Statistical analysis was assessed by \(t\)-test or by one-way analysis of variance ANOVA and Bonferroni’s Multiple Comparison posttest. The values of \(P < 0.05\) were considered statistically significant (\(* P < 0.05\), \(* * P < 0.01\), and \(* * * P < 0.001\)).

3. Results

3.1. Expression of NOXs in mES Is Relatively Low. To confirm the assumption of the negligible presence of NOX and DUOX homologues in the undifferentiated mES, the gene expression was compared to the selected mouse tissues. Particular organs were chosen based on the described presence of NOX homologues by various authors [4, 6]. In agreement with the literature, all determined NOX homologues NOX1, NOX2, NOX3, NOX4, DUOX1, and DUOX2 showed two to three orders lower expression in undifferentiated mES compared to selected tissues (Figures 1(a)–1(f)). The comparison of the NOXs relative expression in mES revealed the NOX4 expression to be the highest, approximately 10 times compared to other NOXs (Figure 1(g)), altogether, it can be concluded that the expression of all NOXs except NOX4 is very low.

3.2. APO and DPI Affect mES Proliferation. Both APO and DPI affected growth of mES in dose-dependent manner in the concentration range 0.1, 0.25, 0.5, 1.0, and 2.0 \(\mu\)M for APO (Figure 2(a)) and 10, 20, 40, 80, 160, and 320 \(\mu\)M for DPI (Figure 2(b)). Data showed decrease in cell proliferation
impairment of cell proliferation. Further, we show that APO and DPI in undifferentiated mES together with the importance of this phenomenon in mES remains elusive. 

ROS production 

The catenin signaling pathway was demonstrated in many studies where its inhibition is critical for the regulation of pluripotency is controlled by Stat3 together with PI3K/Akt [24]. 

It was shown that short-term differentiated mES have several times lower ROS level in comparison with differentiating mES [21]. It was shown that short-term differentiated mES have several times lower ROS level in comparison with differentiating mES [21].

On the other hand, DPI induces O2.

mediated apoptosis [13], inhibits cell redox metabolism, and promotes general oxidant stimulating ROS production [10–12]. Further, APO was also shown to act directly as ROS scavenger [9]. Contrarily to this finding, other studies suggest that APO is rather a pro-oxidant derivative inflammatory mediators [13].

Results obtained using these inhibitors. Further studies are needed to better understand the actions of APO and DPI not only in terms of their effects on ROS levels, but also in terms of the specific effects are thought to be responsible for contradictory results obtained using these inhibitors. Further studies are needed to better understand the actions of APO and DPI not only in terms of their effects on ROS levels, but also in terms of the specific effects that are thought to be responsible for contradictory results obtained using these inhibitors.

Drug Administration 

Both of these drugs were applied in numerous studies and although their effect was attributed to ROS production [5, 32, 33] and therefore might be identified by ROS production [5, 32, 33] and therefore might be identified by ROS production [5, 32, 33]

Intracellular formation of ROS leading to overall redox imbalance [17], inhibits cell redox metabolism, and promotes general oxidant stimulating ROS production [10–12]. Further, APO was also shown to act directly as ROS scavenger [9]. Contrarily to this finding, other studies suggest that APO is rather a pro-oxidant derivative inflammatory mediators [13].

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Figure 1: Continued.
Figure 1: Relative expression of NOX1 (a), NOX2 (b), NOX3 (c), NOX4 (d), DUOX1 (e), and DUOX2 (f) mRNA in mES and selected tissues. Comparison of relative expression of individual NOXs and DUOXs mRNA within mES cells is also shown (g). Data are presented as mean ± SEM from at least two independent experiments.

3.3. APO and DPI Do Not Inhibit but Potentiate Formation of ROS in mES. Despite the very low expression of NOXs enzymes in undifferentiated mES, the ROS production was detectable in our mES lines. Contrary to the expectations, treatment by APO or DPI significantly induced generation of ROS in mES cells detected by live imaging analysis of HE fluorescence, continuously for up to 120 minutes (Figures 3(a), 3(b), and 3(c)). To confirm the specificity of this determination, the formation of specific product of HE reaction with \( \text{O}_2^- \), the 2-OH-E(+), and also nonspecific product, ethidium (E+), were determined by HPLC (Figures 3(d) and 3(e)). This analysis shows potentiation of nonspecific HE oxidation rather than \( \text{O}_2^- \) formation in DPI treated cells, but not in APO treated cells.

3.4. Effect of APO and DPI on Stat3, Akt, and Erk Phosphorylation in mES. To investigate the effect of short term treatment, mES were serum and LIF starved for 12 hours and treated by APO and DPI for 20 minutes followed by 20-minute stimulation with FBS or LIF. APO treatment resulted in decrease of Akt phosphorylation in every condition without effect on Erk. DPI had no significant effect on Akt and Erk kinases signaling. Level of p-Stat3 remained unchanged by NOX inhibitors (Figure 4). To further clarify the dose-dependent effect of tested drugs, we employed the same experimental design with serum starvation and FBS activation. Moreover, glutathione
Figure 1: Relative expression of NOX1 (a), NOX2 (b), NOX3 (c), NOX4 (d), DUOX1 (e), and DUOX2 (f) mRNA in mES and selected tissues.

Figure 2: Effect of APO (a) and DPI (b) on mES proliferation after 48 h treatment based on total cellular protein mass. Data represent mean ± SEM from at least two independent experiments. Statistical significance was determined by ANOVA post hoc Bonferroni’s Multiple Comparison test.

Figure 3: ROS production in mES cells treated by 1 mM APO and 100 nM DPI computed from the automated time-lapse image acquisition of HE fluorescence for 120 minutes (a, b); selected single time point 60 minutes (c). HPLC determination of specific 2-OH-E(+) and nonspecific product E(+) of HE oxidation in the presence of 1 mM APO and 100 nM DPI (d, e). Data are presented as mean ± SEM from four independent experiments. Statistical significance was determined by ANOVA post hoc Bonferroni’s Multiple Comparison test, $P < 0.05$. The groups marked by an asterisk are statistically significantly different from control.
precursor NAC and H$_2$O$_2$ were used as a *bona fide* antioxidant and a prooxidant, respectively, to distinguish between effects mediated via redox changes in cultivated mES and ROS independent actions of APO and DPI.

APO abolished phosphorylation of Akt in a dose-dependent manner with supportive effect on phosphorylation of Erk in higher concentrations. In contrast, DPI slightly increased phosphorylation of Akt and also upregulated phosphorylation of Erk in the highest concentration. Consistent with expectations, H$_2$O$_2$ increased both Akt and Erk phosphorylation. NAC had no effect on signaling in this setup (Figure 5(a)). To test whether the effect on signaling was also preserved during cultivation in complete medium, mES were treated by the same concentration of drugs for 1 hour. In this case, similarly to previous treatment, APO inhibited Akt phosphorylation in a dose-dependent manner. Erk phosphorylation was impaired in the presence of the highest concentration of APO. Notably, in this setup 100 µM concentration of DPI strongly induced both Akt and Erk phosphorylation. Contrary to the previous setup, NAC decreased phosphorylation of both pathways (Figure 5(b)).

Finally, we examined the impact of APO and DPI on Akt and Erk activity after 24 hours in absence or presence of NAC (Figure 6). Contrary to the effect observed after a short term treatment, Akt phosphorylation was upregulated when APO was employed. Phosphorylated form of Akt was also increased following the DPI treatment. This activation could be prevented by addition of NAC. Erk phosphorylation was slightly decreased by APO treatment and augmented by DPI even in the presence of NAC. APO strongly downregulated

Nanog protein level in mES, independently of NAC supplementation (Figure 6). Level of Stat3 phosphorylation was modulated in the above mentioned experimental condition by neither APO nor DPI treatment (data not shown). We did not observe any effect of H$_2$O$_2$ on the evaluated signaling pathways after 24 hours (data not shown).

3.5. APO Augments Canonical Wnt Activity in mES. To assess the effects of APO and DPI supplementation on activity of Wnt pathway, we used TCF/LEF reporter gene assay (TOPflash) to determine the level of canonical Wnt activation mediated by β-catenin which specifically induces the transcriptional activity of TCF/LEF [42]. Firstly, we examined our system with addition of Wnt3a conditioned media or exogenous nondegradable β-catenin, both known agonists, to promote its activation. These interventions induced transcription activity of reporter gene 45 and 25 times, respectively (Figure 7(a)).

PI3K inhibitor LY294002 (LY) and both NOXs inhibitors APO and DPI did not change the spontaneous β-catenin mediated transcription activity of TCF/LEF (Figure 7(b)). In contrast, when the cells were treated by Wnt3a conditioned media, presence of LY and APO significantly augmented the TCF/LEF transcription activity (Figure 7(c)). On the other hand, DPI had no effect (Figure 7(c)). However, all tested drugs did not have any effect in the presence of exogenous nondegradable β-catenin (Figure 7(d)).

To further clarify the effects of LY, APO, and DPI on Wnt/β-catenin signaling, the GSK3β S9 phosphorylation, allowing accumulation of β-catenin, was evaluated [43]. Treatment with both APO and LY but not DPI decreased GSK3β S9 phosphorylation in this setup as shown by the western blot analysis (Figure 7(e)).

4. Discussion

APO and DPI are the most commonly used inhibitors of NOX, involved in numerous studies despite the increasing evidence questioning their specificity. We employed mES as a model to analyze effects of these compounds that might not be directly mediated through impairment of NOXs, because of their generally negligible expression in undifferentiated mES. We aimed to investigate modulation of ROS production by APO and DPI in mES as well as interactions with signaling pathways important for stem cell regulation.

Although NOXs were attracting attention as an important source of intracellular ROS production for a long time, their role in stem biology remains poorly understood. Previously, it was demonstrated that NOXs expression is precisely regulated during embryonic stem cell differentiation into cardiomyocytes [44, 45] and vascular smooth muscle lineage [46].

In our experiments, we observed generally low level of NOXs/DUOXs expression close to the limit of detection, which we concluded both from real Cq values of PCR amplification and from comparison of NOXs/DUOXs expression in several employed tissues. As such control sample, tissues with well described NOXs/DUOXs expression and activity were
used [4, 6]. Among NOXs and DUOXs, NOX4 expression was the highest in mES, which is in agreement with other authors [46, 47]. It may also correspond to relative abundant expression of this enzyme across other tissues [48]. However, it should be emphasized that level of NOX4 transcript in mES was still nearly three orders below expression in selected control tissue (kidney). NOX4 was shown to be constitutively active, and hence its activity is mostly regulated by the level of its expression [49]. Concerning other potential sources of intracellular ROS, it is also noteworthy that favored reliance on anaerobic glycolysis in undifferentiated mES leads to reduced mitochondrial biogenesis and activity, manifested by declined ROS production [50, 51]. Thus mES can be considered not only NOX-low, but also overall ROS-low model.

Next, we aimed to assess effect of APO and DPI on proliferation of undifferentiated mES. Selection of used concentrations was based on comprehensive literature search. APO as NOX inhibitor was used in range 30–1200 μM [9, 52] with the upper range of these concentrations corresponding to APO supplementation preferably employed in our experiments. Regarding DPI, many authors used concentrations ranging 1–100 μM [summarized in [53]] which is approximately one order of magnitude higher than doses used in our experiments, as we observed significant growth impairment even when concentrations as low as 20 mM were applied. Impact on cell proliferation after NOX inhibitors treatment was described earlier, for both normal and transformed cell lines. The observed effects of DPI and APO were suggested to be attributed to the various mechanisms including changes in NOX mediated ROS production, downregulation of integrin expression, cell cycle arrest, or modulation of mitogenic-signaling pathways [54–57]. Therefore, we can assume that direct modulation of ROS production could contribute to the observed decrease of mES proliferation. At the same time, the effects of inhibitors employed in this study can also be related to their direct effects on the other promitogen cell signaling pathways as discussed later. The potential of APO and DPI to inhibit cell proliferation could also be beneficial in the context of anticancer agents. A recent publication showed that APO suppressed prostate cancer and that the reduction of Rac1 and NfκB phosphorylation was involved [58].

Further, we assessed how NOX inhibitors affected ROS production in our model. Due to their natural short half-life and high reactivity, precise detection of ROS represents
a tremendous challenge, especially in nonphagocytic cells. We were aware of limits in ROS measurement with respect to specificity and generation of possible artefacts; therefore, we used two different assays, live imaging fluorescent microscopy and HPLC, both utilizing ROS-sensitive probe HE. The reaction between $\text{O}_2^-$ and HE generates a highly specific red fluorescent product 2-hydroxyethidium 2-OH-E(+). However, in the intracellular milieu, the presence of redox metal ions or hemeproteins with peroxidase activity or other one-electron oxidants can oxidize HE to several nonspecific products, including the ethidium E(+) and dimeric products [38, 59, 60]. Despite the expectations, the live imaging assay showed significant prooxidant activity of APO and DPI when continuous nonscillating probe oxidation was determined by live imaging. On the other hand, HPLC analysis used for determination of specific HE derivatives did not confirm $\text{O}_2^-$ production after APO and DPI treatment. The only observed effect was significant DPI-mediated elevation of E(+). APO did not induce generation of HE oxidation products [both 2-OH-E(+) and E(+)] which is partially in contrast with the results from live imaging fluorescent microscopy where all nonspecific HE oxidation
fluorescent products are summarized [60]. This suggests that other oxidants, rather than \( \text{O}_2^- \), are responsible for increase in fluorescence.

Although NOX inhibitors should generally relieve oxidative stress, several lines of evidence for APO and DPI exist demonstrating the opposite. It was reported that DPI inhibits pentose phosphate pathway (PPP) responsible for the synthesis of NADPH, a redox cofactor important for many antioxidant enzymes, thus making the cells more prone to oxidative stress [18]. Suggested mechanism included direct inhibition of NADP-dependent enzymes such as glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and lactate dehydrogenase. In agreement with our data, evidence from different group exists, showing that DPI is exerting prooxidative effects in given cell types and conditions [17]. Further, it was reported that APO, contrary to DPI, stimulates PPP which is known to be a subsequent step following oxidative stress exposure. This was prevented by addition of GSH into medium, further suggesting that APO-induced GSH oxidation might be involved in observed PPP activation [11]. However, in a different study APO opposingly increased the synthesis of the GSH through activation of transcription factor AP-1. Notably, levels of GSSG were not altered, implying that APO itself does not cause oxidative stress [61]. Many studies are highlighting that APO is a prodrug that must be first metabolized through oxidation to its oligomeric form; thus certain redox environment must be present in order to achieve full APO activation [62] narrowing its function preferably to cells with strong ROS production like stimulated endothelial cells [63] or professional phagocytes [64]. In compliance with these findings, it was demonstrated that APO can act both as an antioxidant and as a prooxidant, depending on the cell type and its oxidizing potential [9, 10]. This is in agreement with our experiments performed on nonphagocytic low-level ROS cells where APO might rather contribute to oxidative stress as demonstrated by live imaging. Interestingly, different modifications of APO are suggested for in vivo experiments [65], further revealing the complexity of this issue.

Critical features of mES, pluripotency, self-renewal, and unlimited proliferation, are predominantly exerted through actions of cytokine LIF, which is routinely added to the culture medium. Binding of LIF to its receptor triggers the activity of three major intracellular signaling cascades: JAK/Stat3, PI3K/Akt, and MAPK/Erk. These pathways converge to regulate the gene expression pattern typical for mES [27]. To clarify possible effects of APO and DPI on selected signaling pathways, the modulation of Stat3, Akt, and Erk activation status was analyzed in mES cells. Firstly, mES were serum and LIF depleted in order to increase cellular response, as cultivation in complete medium leads to a constant activation of those pathways. Stimulation by LIF and FBS was employed to reactivate signaling [25, 66]. As expected, LIF addition induced preferably Stat3 response while FBS, containing growth factors and cytokines, augmented Akt and Erk signaling. To further analyze effect of drugs on selected kinases, we examined their phosphorylation status also in complete medium after 1-hour and 24-hour treatment, to elucidate early changes and later cell response in signaling pathways. In agreement with other studies, both Akt and Erk kinases responded in ROS-sensitive manner [67, 68], which we demonstrated by \( \text{H}_2\text{O}_2 \)-induced phosphorylation. Similarly, we can assume that DPI-mediated ROS production is responsible for the observed effects on Akt and Erk activation which is in contrast to the response to APO. Properties of general antioxidant NAC in sense of attenuation of kinase phosphorylation were more profound during treatment in complete medium. Notably, we did not observe changes of Stat3 phosphorylation in presence of any drugs tested in our study. The most striking effect was detected downstream of PI3K on the level of S473 Akt phosphorylation when treatment was employed. In serum starved cells, in FBS or LIF activated cells, and also in the presence of complete medium, APO decreased Akt phosphorylation when short term impact was studied. It was earlier reported that vanillin and some of its derivatives, including APO, readily inhibited PI3K in lung adenocarcinoma cell line [69]. These authors are also suggesting, in agreement with our data, that radical scavenging or other antioxidant properties of those compounds are not responsible for the observed effect. Thus, the mode and mechanism of inhibition needs to be further clarified.

Remarkably, critical role of PI3K was also described for the process of NOX activation [70]; thus it can be hypothesized that some of the APO inhibitory actions towards NOXs might be also mediated via PI3K inhibition. To further elucidate impact of APO-induced PI3K inhibition, we examined the level of homeodomain transcription factor Nanog, which was suggested as a downstream target of PI3K and is also recognized as an important intrinsic regulator of stem cell pluripotency [28]. After 24 hours of APO treatment, the level of Nanog was dramatically reduced. Interestingly Akt phosphorylation was upregulated by APO after 24 hours compared to untreated cells, and this increase could be reverted by addition of NAC, although Nanog levels remained diminished, suggesting that this impairment is perhaps not related to APO-induced modulation of ROS levels in the cell. Phosphorylation of both Akt and Erk was augmented by DPI treatment that may be attributed to its above described prooxidative effect, as it was reduced by NAC supplementation in the case of Akt.

Next, we examined the impact of NOX inhibitors on canonical Wnt signaling, as it also plays a distinctive role in regulation of embryonic stem cells [31, 71] and the modulator of this pathway, GSK3, is a known PI3K downstream [72]. GSK3 regulates Wnt pathway by phosphorylating \( \beta \)-catenin on multiple sites that enhances its subsequent degradation [43]. GSK3 is active in resting cells but is readily inhibited through the PI3K/Akt-mediated phosphorylation of N-terminal serine residues (S9 in GSK3\( \beta \) and S21 in GSK3\( \alpha \)) [72]. Therefore, in the conventional view, it is assumed that activity of PI3K/Akt should promote canonical Wnt signaling. In contrast to this, experimental evidence argues against this simplified scenario, as Akt activation failed to promote Wnt/\( \beta \)-catenin signaling when insulin and constitutively active Akt were administered [73]. Moreover, it was reported that Axin-associated GSK3, responsible for mediating \( \beta \)-catenin degradation, represents just a minor fraction of GSK3 cellular content and this complex is not accessible to Akt
phosphorylation, thus preventing PI3K/Akt/Wnt cross-talk [74]. In our experiments, we did not see any effect on basal Wnt transcription activity, when NOX inhibitors or LY was employed. Moreover, treatment of both APO and LY decreased GSK3β S9 phosphorylation, further questioning the simplified model of PI3K/Wnt cross-talk mediated solely by level of GSK3 inhibition. However, after Wnt3a induction, both LY and APO augmented Wnt transcription activity. Notably, PI3K inhibition was recently shown to increase the amount of active nuclear β-catenin and to promote induction in TOPFlash assay in epithelial cell culture system [75]. In agreement with other studies [76, 77], authors were suggesting the pivotal role of bidirectional loop between receptor tyrosine kinase-driven MAPKs and Wnt/β-catenin signaling. Although Erk kinase was not dramatically affected by APO treatment in our system, we are not excluding the possibility of a different MAPK involvement in observed phenomena, as numerous convergence points were described between PI3K and MAPKs [78].

5. Conclusions

Altogether, our study suggests prooxidant activity of APO and DPI in mES. Moreover, treatment with those drugs results in different modulation of intracellular pathways critical for regulation of proliferation and differentiation. APO markedly downregulates activity of Akt and its downstream Nanog and augments Wnt signaling. DPI promotes Akt and Erk activation. Taking into account described negligible NOXs levels in mES we suggest that actions of drugs observed in our experiments are rather independent of their typical function as NOX inhibitors. Therefore, caution should be taken to potential applications of these NOX inhibitors and interpretation of obtained results, especially in studies focused on the stem cell biology and intracellular and redox signaling.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. The authors alone are responsible for the content and writing of the paper.

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References


other oxidants, rather than O2. Oxidative Medicine and Cellular Longevity

be present in order to achieve full APO activation [62].

Further, it was reported that APO, contrary to hydrogenase, and lactate dehydrogenase. In agreement with in HE fluorescence.

complete medium after 1-hour and 24-hour treatment, to elucidate early changes and later cell response in signaling pathways.

To further analyze effect of drugs on selected targets, containing growth factors and cytokines, augmented Akt and LIF addition induced preferably Stat3 response while FBS, as cultivation in complete medium leads to a constant level of Stat3 activation of those pathways. Stimulation by LIF and FBS -catenin signaling when insulin and constitutively induced Wnt-beta-catenin signalling through dishevelled.


