Molecular mechanism of lineage choice instruction by hematopoietic cytokines

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1 Abbreviations

AB	add-back
APC	allophycocyanin
BF	bright field
BM	bone marrow
bZip	basic leucine zipper
CA	constitutive active
$CaCl_2$	calcium chloride
Cat. Nr.	catalogue number
CD	cluster of differentiation
C/EBP	CCAAT-enhancer-binding protein
CFC	colony forming cell
CFP	cyan fluorescent protein
CFU	colony forming unit
CLP	common lymphoid progenitor
CML	myelogenous leukemia
CMP	common myeloid progenitor
CSF	colony stimulating factor
DAG	diacylglycerol
DAVID	database for annotation, visualization, and integrated discovery
DC	dendritic cell
DEG	differentially expressed gene
DMEM	Dulbecco's modified eagle medium
DN	dominant negative
DNA	deoxyribonucleic acid
E	erythrocyte
E14.5	embryonic day 14.5
EDTA	ethylenediaminetetraacetic acid
EPO	erythropoietin
EPOR	EPO receptor
ERK	extracellular signal-regulated kinase

ESC	embryonic stem cell
ETP	early thymic progenitor
FACS	fluorescent activated cell sorting
FAK	focal adhesion kinase
FCS	fetal calf serum
FSC	forward scatter
Flt3	fms-related tyrosine kinase 3
Flt3L	Flt3 ligand
FMIP	fms-interacting protein
FOG-1	friend of GATA-1
FRET	fluorescence resonance energy transfer
G	granulocyte
GATE	grid analysis of time series expression
Gab	GRB2-associated-binding protein
GCSFR	G-CSF receptor
GePS	Genomatix Pathway System
GFP	green fluorescent protein
GMCSFR	GM-CSF receptor
GMLP	GM-lymphoid committed progenitors
GMP	granulocyte macrophage progenitor
GO	gene ontology
Grb	growth factor receptor-bound protein
HEK	human embryonic kidney
HSCs	hematopoietic stem cell
HSPCs	hematopoietic stem and progenitor cells
ICSBP	interferon consensus sequence binding protein
IFN	interferon
IKK	I kappa B kinase
IL	interleukin
IL5Rα	interleukin five receptor alpha
IL7Rα	interleukin seven receptor alpha
IRES	internal ribosomal entry site

IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ivGMP	*in vitro* derived GMP
JNK	c-Jun N-terminal kinase
KLF	Krueppel-like factor
LB	luria broth
Lin	lineage
LMPP	lymphoid-primed multipotent progenitor
LSK	lineage-negative Sca-1-positive c-kit-positive
LTR	long terminal repeat
M	monocyte/macrophage
MAPK	mitogen activated protein kinase
MCSFR	M-CSF receptor
MDP	macrophage dendritic cell progenitor
Meg	megakaryocyte
MEK	mitogen-activated kinase kinase
MEP	megakaryocyte erythrocyte progenitor
Mona	monocytic adapter
MOI	multiplicity of infection
MPP	multipotent progenitor
mRNA	messenger RNA
NaN_3	sodium azide
NBD	nemo binding domain
NK	natural killer
OHT	hydroxy-tamoxifen
ORF	open reading frame
PB	Pacific Blue
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PH	pleckstrin homology

PI3K	phosphatidylinositide 3 kinase
PIP_3	phosphatidylinositol (3,4,5) trisphosphate
PKC	protein kinase C
PLCγ2	phospholipase C gamma 2
PTEN	phosphatase and tensin homolog
RANKL	receptor activator of nuclear factor kappa-B ligand
Rb	retinoblastoma
RFP	red fluorescent protein
RNA	ribonucleic acid
RSN	robust spline normalization
Sca-1	stem cell antigen 1
SCF	stem cell factor
SEM	standard error of the mean
SFK	Src family kinase
SFEM	serum-free expansion medium
SFFV	spleen focus forming virus
SH2	Src homology domain 2
SHIP	Src homology region 2 domain-containing inositol 5'-phosphatase
SHP	Src homology region 2 domain-containing phosphatase
SOCS	suppressor of cytokine signaling
SPF	specific pathogen free
SSC	side scatter
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase
TAE	TRIS acetate EDTA
TNF-α	tumor necrosis factor alpha
TPO	thrombopoietin
TRIS	tris-(hydroxymethyl)-aminomethane
VST	variance stabilizing transformation
VSV-G	vesicular stomatitis virus glycoprotein
Y	tyrosine
YFP	yellow fluorescent protein

2 Abstract

Hematopoiesis - the generation of all mature blood cell types of the body - relies on tightly controlled lineage decision as cells differentiate from hematopoietic stem cells (HSCs) towards the different lineages. To a large part, lineage differentiation is controlled by hematopoietic cytokines. It was recently shown by continuous live cell imaging that the two cytokines M- and G-CSF, which *in vivo* are the principal regulators of monocyte/macrophage and granulocyte differentiation, respectively, can instruct the lineage choice of uncommitted bipotent granulocyte macrophage progenitors (GMPs).

M- and G-CSF activate a multitude of signaling pathways that mediate their pleiotropic actions, which include survival, proliferation, and ultimately differentiation. However, the involvement of specific signaling pathways in controlling different cell fates remains poorly understood. Specifically, pathways orchestrating lineage choice instruction remain elusive. The M-CSF receptor (MCSFR) carries eight functional tyrosine residues that transmit M-CSF-evoked signaling. Studies on individual MCSFR tyrosine residue-activated signaling and its concomitant influence on cell fate have mainly relied on myeloid cell lines and/or MCSFR chimeras, often resulting in contradictory conclusions.

In this study we established a system allowing the analysis of M-CSF-induced signaling in uncommitted primary progenitor cells. Combining MCSFR loss of function studies to dissect M-CSF-activated signaling pathways with novel bioimaging technologies allowing long-term quantification of single cell behavior, we investigated the molecular mechanism orchestrating M-CSF-instructed lineage differentiation. Our results show that MCSFR signals mediated by tyrosine residue 559 (Y559) are sufficient for macrophage differentiation from uncommitted progenitors and that overexpression of constitutively active members of the Src family of kinases (SFKs), which bind Y559, recapitulates this effect. Downstream of SFKs, we identify PI3K/Akt and NFκB signaling as putative mediators. Furthermore, we analyzed M- and G-CSF-induced gene expression during lineage commitment of GMPs and found that the overall response is surprisingly similar between the two different cytokines, suggesting that differentiation into macrophages and granulocytes is regulated by a small set of genes.

3 Introduction

3.1 Hematopoiesis

Hematopoiesis is the tightly controlled process of constant regeneration of all mature blood and immune cells throughout life (Orkin & Zon 2008). This process starts from a rare population of mainly quiescent multipotent hematopoietic stem cells (HSCs), which predominantly resides in specialized microenvironments (niches) within the bone marrow (BM) (Schofield 1978, Wilson et al. 2008). A hallmark of HSCs, and stem cells in general, is their ability to self-renew, i.e. to generate progeny with HSC potential. It is believed that they may divide asymmetrically, giving rise to another HSC and a more differentiated multipotent progenitor (MPP) cell that has lost self-renewing capacity. MPPs differentiate towards the different hematopoietic lineages through several lineage-restricted progenitor populations, ultimately generating all the mature blood cell types. Because hematopoiesis is a process of step-wise loss of multipotency, it is often regarded as a relatively linear hierarchy with HSCs being on top and mature cells at the bottom. *In vitro* culture analyses, immunophenotyping, and experimental BM transplantations have led to the classical view of hematopoiesis, in which HSCs/MPPs either differentiate towards the lymphoid lineage (B-cells, T-cells, and natural killer (NK)-cells) through a common lymphoid progenitor (CLP) or towards the myeloid lineage (granulocytes (G), monocytes/macrophages (M), erythrocytes (E), megakaryocytes (Meg), and mast cells) through a common myeloid progenitor (CMP). Dendritic cells (DCs) can be of lymphoid or myeloid origin (Liu & Nussenzweig 2010). Many of the different multipotent progenitor populations have been immunophenotypically defined during the last decades and can now be prospectively isolated for functional studies using fluorescence activated cell sorting (FACS) (Figure 3-1). Due to improvement in FACS technology and the combinatorial use of surface markers along with stage- and/or lineage-specific transgenic expression of reporter genes in mice, new sub-progenitor populations continue to be described, constantly refining and/or challenging the view of the developmental landscape of hematopoiesis. For example, the classical binary view of myeloid versus lymphoid branching has been challenged by the description of the lymphoid-primed multipotent progenitor (LMPP) within the primitive lineage marker negative (lin^{neg}) Sca-1^{pos} c-kit^{pos} (LSK) fraction, which contains HSCs and MPPs. The LMPP is restricted to

the macrophage, T-cell, and B-cell lineage, but lacks MegE potential (Adolfsson et al. 2005), which implies that GM cells could be generated by two different means: through the classical CMP and the LMPP (Iwasaki & Akashi 2007). Subsequent studies reassessing the LMPP population suggested that LMPPs possess residual MegE potential *in vivo* (Forsberg et al. 2006) yet not refuting the existence of true strictly GM-lymphoid committed progenitors (GMLPs).

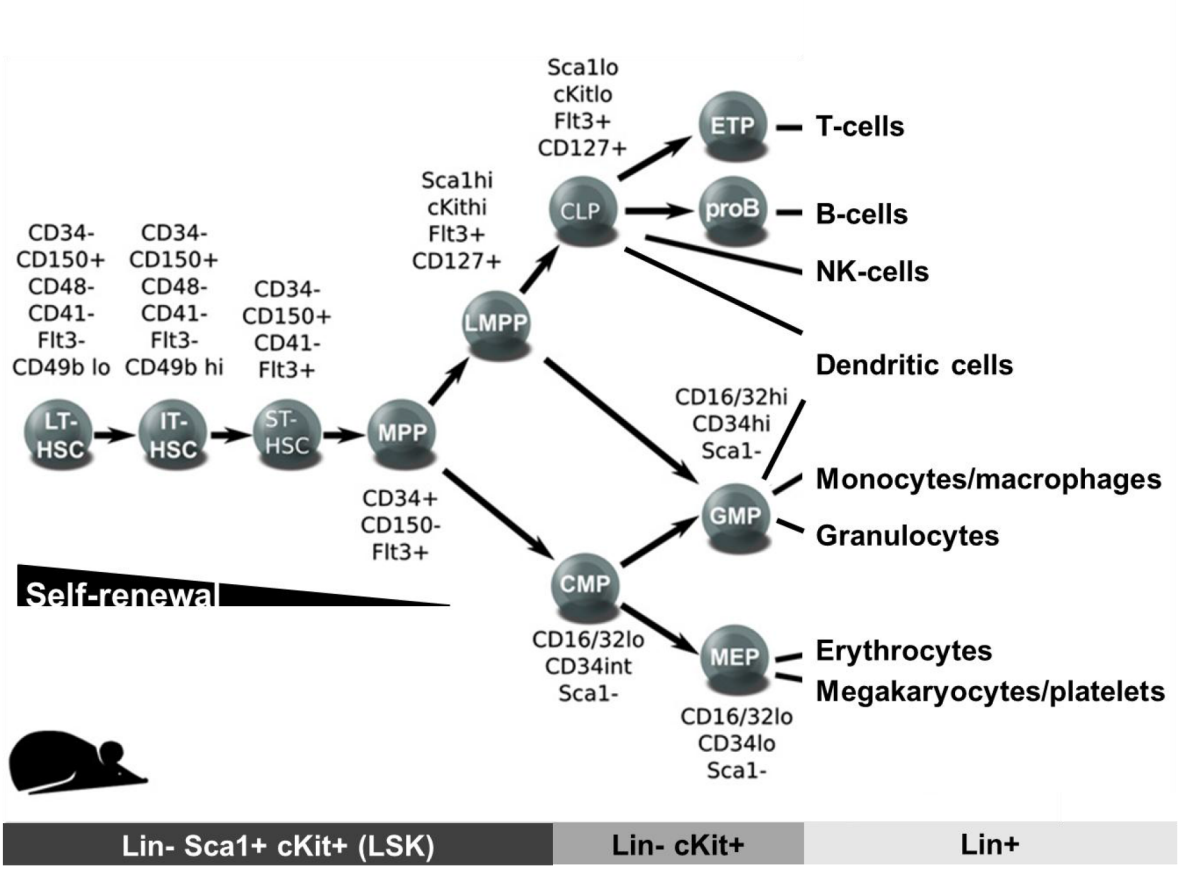

Figure 3-1: The hematopoietic lineage hierarchy.
All hematopoietic cells originate from self-renewing hematopoietic stem cells (HSCs), which progressively give rise to more committed downstream progenitors devoid of self-renewal. Ultimately, lineage-restricted progenitors generate the respective mature cells of their associated lineage. In the murine system, most hematopoietic stem and progenitor cells can be prospectively isolated using a combination of antibodies against the depicted cell surface markers. Note: some intermediate progenitor populations are not depicted. LT-HSC: long-term HSC; IT-HSC: intermediate term HSC; ST-HSC: short-term HSC; MPP: multipotent progenitor; LMPP: lymphoid primed multipotent progenitor; CLP: common lymphoid progenitor; ETP: early thymic progenitor; NK-cells: natural killer cells; CMP: common myeloid progenitor; GMP: granulocyte macrophage progenitor; MEP: megakaryocyte erythroid progenitor; Lin: lineage marker; Flt3: fms-related tyrosine kinase 3; Sca1: stem cell antigen 1. Adapted from (Doulatov et al. 2012).

3.2 Myelopoiesis

Myelopoiesis, the production of cells belonging to the myeloid lineage, makes up the majority of hematopoiesis. For instance, 1-2 x 10^{11} neutrophils (a subset of granulocytes) have to be generated in a normal adult human per day (Dancey et al. 1976). This huge demand of cells is met with high proliferative potential of the intermediate multipotent progenitor populations rather than the mostly quiescent HSCs (Wilson et al. 2008), demonstrating that the progenitor pool has a significant level of control over mature blood cell production.

Cells of the monocyte/macrophage and granulocyte lineages are primarily responsible for innate immunity – the first line of defense against a variety of pathogens – and inflammatory responses. They are phagocytic cells, ingesting foreign material and organisms, and some can act as antigen presenting cells for lymphocytes.

Macrophages are large cells residing mainly in peripheral tissues (Wynn et al. 2013). They are responsible for tissue maintenance by engulfing cellular debris and apoptotic cells that result from tissue injury or remodeling (e.g. during development). Moreover, macrophages are involved in immune regulation by coordinating lymphocyte function through antigen presentation, cytokine secretion, and the recognition and ingestion of cellular pathogens. The prevalent view that all tissue-resident macrophages are derived from circulating monocytes has recently been challenged by lineage tracing experiments showing that macrophages in most tissues are derived from yolk sac or fetal liver progenitors (Ginhoux et al. 2010, Hoeffel et al. 2012, Schulz et al. 2012, Yona et al. 2013).

Granulocytes are a group of cells that can be subdivided into three classes: neutrophils, eosinophil, and basophils. Neutrophils, the predominant class of granulocytes, function in killing bacteria and fungi and are the main effectors of inflammatory responses (Borregaard 2010). Eosinophils are pro-inflammatory cells that tend to reside in tissues exposed to external environment such as skin, gastrointestinal tract, and lungs. Basophils are the least common type of granulocytes in peripheral blood and mediate allergic reactions. Mast cells, unlike mature granulocytes that are found in the peripheral blood and whose nuclei are segmented, do not have a segmented nucleus and do not complete maturation until they home to tissue. The developmental origin of mast cells is still under debate (see below).

Granulocytes and monocytes/macrophages arise from a common progenitor, the granulocyte macrophage progenitor (GMP). The differentiation of the myeloid lineage is

orchestrated through the coordinated integration of intrinsic and extrinsic signals through a network of complex and finely tuned regulatory pathways that results in an overall response and specific gene expression signatures, as will be discussed in detail below.

3.2.1 The myeloid lineage

Soon after the identification of a progenitor population restricted to the lymphoid lineage, known as the CLP (Kondo et al. 1997), a myeloerythroid-restricted equivalent, the CMP, was described (Akashi et al. 2000). This population was identified within a population that lacks markers of mature blood cells (lineage negative), highly expresses c-kit (also known as CD117), and is negative for interleukin seven receptor alpha (IL7Rα) and stem cell antigen 1 (Sca-1). Based on expression of CD16/32 and CD34 this population can be further subfractioned into CMPs ($CD16/32^{lo}$ $CD34^{pos}$), which were suggested to clonally give rise to both GMPs ($CD16/32^{hi}$ $CD34^{pos}$) and megakaryocyte-erythroid progenitors (MEPs) ($CD16/32^{neg/lo}$ $CD34^{neg}$) (Akashi et al. 2000). However, using the additional surface markers CD105 and CD150, recent studies showed that the CMP population largely consists of already lineage-segregated preGM and preMegE progenitors and that only very few cells clonally give rise to mixed myeloid populations (Pronk et al. 2007).

GMPs mainly generate neutrophils and monocytes/macrophages but also dendritic cells, eosinophils, basophils, and mast cells, which collectively are effector cells cooperating in mounting a variety of allergic and innate immune responses. Progenitors restricted to respective lineages downstream of GMPs have been identified and prospectively isolated. Eosinophil-committed progenitors in the BM could be isolated based on interleukin 5 receptor alpha (IL5Rα) expression (Iwasaki et al. 2005a), while a common basophil/mast cell progenitor was identified in the spleen based on the expression of ß7-integrin (Arinobu et al. 2005). However, there is still debate on the true origin of mast cells: While some place them into the GM lineage (Arinobu et al. 2005), others argue that mast cells are generated independently of the GM lineage, but directly from MPPs (Chen et al. 2005, Franco et al. 2010). Restricted basophil progenitors in the BM and mast cell progenitors mainly found in the intestine could be isolated using surface markers FcεR1α and FcγRII/III, respectively (Arinobu et al. 2005).

Likewise, downstream of GMPs, a population restricted to the M and DC lineages was identified using a Cx3cr1:GFP reporter mouse and consequently called macrophage dendritic cell progenitor (MDP) (Fogg et al. 2006). However, DCs can also be generated from lymphoid progenitors (Manz 2001). Downstream of the MDP, common DC progenitors have been identified (Liu et al. 2009, Naik et al. 2007, Onai et al. 2007), which give rise to classical DCs and plasmacytoid DCs but not monocytes. Recently, a Ly6C^{pos} monocyte/macrophage restricted progenitor was identified downstream of the MDP (Hettinger et al. 2013).

3.2.2 Regulation of myeloid cell fate commitment

What are the factors influencing hematopoietic fate choice and what constitutes lineage commitment? Lineage commitment of multipotent cells could be induced either by extrinsic factors such as cytokines and cell-cell interactions or by intrinsic mechanisms, including stochastic upregulation of transcription factors, microRNAs, or other regulatory molecules. Both extrinsic and intrinsic factors may actively induce lineage commitment ('instruct') or alternatively be permissive ('select') for one lineage. The instructive vs. selective model in orchestrating hematopoietic fate has been intensely debated, especially regarding the role of cytokines in this process. In the selective model, lineage commitment occurs independently of cytokines by a stochastic (i.e. random) process, such as spontaneous upregulation of a lineage-determining transcription factor. The cytokines' function is then to provide survival and/or proliferation signals that select for a given lineage (Enver et al. 1998). Quite the opposite, instructive models postulate that cytokines actively drive multipotent cells toward a particular fate (Metcalf 1998). The instructive and selective models, which typically are regarded as competing models, will be discussed in more detail below.

3.2.2.1 Stochastic gene expression in hematopoietic stem and progenitor cells (HSPCs) and its implications for lineage commitment

As mentioned above, hematopoietic differentiation can be seen as a gradual loss of self-renewal potential and a stepwise acquisition of lineage identity. Thus, as HSCs

differentiate towards committed progenitors, one would expect an orchestrated activation of lineage-specific genes and simultaneous silencing of HSC-affiliated genes (e.g. genes involved in self-renewal). Vice versa, lineage-specific genes would be expected to be silenced in HSCs. However, these orderly gene expression patterns do not seem to be the rule in hematopoietic differentiation: Studies have shown that single multipotent cells express genes associated with divergent lineages at a low level compared to differentiated cells. Among them are genes encoding for transcription factors, cytokine receptors, and proteins having lineage-exclusive functions, such as globins and myeloperoxidase (Billia 2001, Hu et al. 1997, Månsson et al. 2007, Miyamoto et al. 2002). It is believed that this 'lineage priming' maintains differentiation flexibility of multipotent cells, before being specified into each lineage. However, the concept of lineage priming is founded almost exclusively on RNA expression data, which might equally reflect random gene expression noise with no functional relevance.

Providing a mechanistic explanation for this apparently stochastic behavior of HSPCs, it has been proposed that low level expression of lineage-associated transcription factors may undergo random fluctuations, leading to self-reinforcing gene expression and stochastic lineage commitment (Chang et al. 2008, Cross & Enver 1997). A paradigmatic example for how such positive feedback loops in a metastable balance of cross-antagonistic transcription factors could lead to stable cell fate decisions is the transcription factor pair PU.1 and GATA-1 (Enver et al. 2009, Graf & Enver 2009). MegE-affiliated GATA-1 and GM-affiliated PU.1 regulate their own expression (Chen et al. 1995, Tsai et al. 1991) and inhibit each other's transcriptional activity (Stopka et al. 2005, Zhang et al. 1999) (see below for details). Furthermore, it has been shown that forced expression of these factors can change lineage identity, demonstrating their potential to instruct lineage commitment (Heyworth et al. 2002, Nerlov & Graf 1998). Therefore, subtle changes in the balance of PU.1 and GATA-1 would lead to one of the factors' downstream program being amplified, while the other factor's program would be shut down, thereby instructing cells towards the GM (PU.1 up) or MegE (GATA-1 up) lineage. Similarly, it has been shown that the balance of transcription factors MafB and PU.1 is involved in controlling macrophage vs. dendritic cell fate (Bakri et al. 2005). Levels of C/EBPα and PU.1 have been proposed to determine macrophage vs. granulocyte differentiation (see below) (Dahl et al. 2003). Integrating known positive and negative regulations of hematopoietic transcription factors into large networks has provided models on how cell fate decisions are executed and stabilized (Laslo et al. 2006, Moignard et

al. 2013, Palani & Sarkar 2009, Soneji et al. 2007). However, whether the cause of initial fluctuations in the metastable state of multipotent HSPCs is of stochastic origin or triggered by external cues (e.g. by cytokines) remains elusive, raising the question what role cytokines play in the lineage commitment process: Do they select or instruct lineage choice?

3.2.2.2 Hematopoietic cytokines: selective vs. instructive functions

Hematopoietic cytokines are small soluble regulators that are produced by a variety of different cell types. They include interleukins (ILs), colony stimulating factors (CSFs), interferons (IFNs), erythropoietin (EPO), and thrombopoietin (TPO). The CSFs, consisting of M-CSF, G-CSF, GM-CSF, and IL-3 (initially called multi-CSF), are a group of cytokines central to the differentiation of hematopoietic cells and will be discussed in more detail in section 3.3. Cytokines bind to and activate a family of structurally and functionally conserved cytokine receptors, eliciting pleiotropic biological responses in target cells. Since their discovery, cytokines have been known to stimulate survival and proliferation of HSPCs and to strongly influence their lineage outcome (Metcalf 2008). Yet, whether this results from a selective function on already committed cells, promoting their proliferation and survival, or from an instruction of lineage choice, has been under debate for decades (Figure 3-2) (Enver et al. 1998, Metcalf 1998).

Studies supporting selective cytokine function

In studies supporting the idea that fluctuations in transcription factor levels are the cause for lineage commitment, cytokine function is usually regarded as secondary, selectively allowing the amplification and survival of cells that upregulated cytokine receptors upon stochastic lineage commitment (Cross & Enver 1997). Indeed, there are numerous studies showing that cytokine receptors are under the transcriptional control of key transcription factors. GATA-1 has been shown to activate transcription of the EPO receptor (EPOR) (Zon et al. 1991), while PU.1 can activate the promoters of the cytokine receptors for M-CSF (MCSFR), Flt3L (Flt3), and in cooperation with C/EPBα the receptors for G-CSF (GCSFR) and GM-CSF (GMCSFR) (Carotta et al. 2010, Hohaus et al. 1995, Smith et al. 1996, Zhang

et al. 1994). However, these studies cannot rule out that the initial variation in gene expression level leading to lineage commitment is not stochastic but deterministic: The primed state of multipotent HSPCs could allow them to rapidly respond to external signals, leading to the same stable scenario via feedback mechanisms as through stochastic fluctuation.

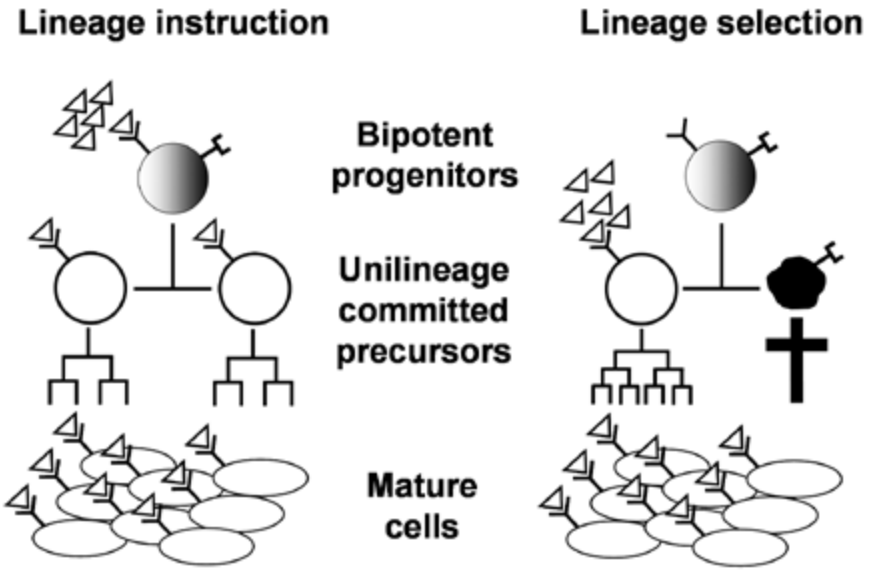

Figure 3-2: Models of hematopoietic lineage differentiation.
In the instructive model, the lineage choice of uncommitted progenitor cells is directly influenced by cytokines, resulting in progeny belonging only to the instructed lineage. In the selective model, lineage choice occurs through random mechanisms (e.g. fluctuating transcription factor networks) independently of cytokines. The function of cytokines is subsequently to select for a specific lineage by selective survival signals, while cells randomly committed to other lineages die. Importantly, both models have the same in- and output and therefore cannot be distinguished by snapshot analyses. Figure taken from (Rieger & Schroeder 2009).

Further data indicating selective cytokine function comes from mice deficient for cytokines or their receptors. In virtually all cases, loss of function of a single cytokine/receptor does not completely abrogate its associated lineage, although minor to severe reductions in progenitors and/or mature cells have been reported. This is true for mice deficient for M-CSF/MCSFR (Dai et al. 2002, Naito et al. 1991, Yoshida et al. 1990), G-CSF/GCSFR (Lieschke et al. 1994, Liu et al. 1996), GM-CSF (Stanley et al. 1994), Flt3L/Flt3 (Mackarehtschian et al. 1995, McKenna et al. 2000), IL7/IL7R (Carvalho et al. 2001, Miller et al. 2002), EPO (Lin et al. 1996, Wu et al. 1995), and TPO (De Sauvage et al. 1996).

Transgenic approaches with receptor chimeras consisting of extracellular and cytoplasmic domains of different receptors were also used to clarify the role of cytokines in lineage commitment. In a knock-in approach, the cytoplasmic domain of the GCSFR was

fused to the extracellular domain of c-Mpl (the receptor for TPO), creating mice bearing a chimeric receptor that binds TPO, but signals through GCSFR. These mice have normal megakaryocyte and platelet counts, demonstrating that the intracellular GCSFR part can functionally replace c-Mpl signaling in these mice. Moreover, granulocyte numbers are normal, collectively indicating GCSFR-mediated non-specific survival and/or proliferation signaling and arguing against a lineage-instructive function (Stoffel et al. 1999). A similar study created knock-in mice bearing a chimeric receptor consisting of the extracellular part of GCSFR and the cytoplasmic signaling domain of EPOR. These mice are able to support the production of morphologically mature neutrophils, and treatment with G-CSF does not affect the number of myeloid or erythroid progenitors in the BM, indicating that G-CSF-specific signaling is not required for granulocytic differentiation or lineage commitment and that EPOR signaling has unspecific survival and/or proliferation function (Semerad et al. 1999).

Studies using bcl-2 overexpression as a mean to suppress apoptosis have shown that in the absence of cytokines, differentiation of a multipotent cell line (Fairbairn et al. 1993), T-cell development in IL-7 deficient mice (Akashi et al. 1997), and development of monocyte/macrophages in M-CSF-deficient mice (Lagasse & Weissman 1997) can be rescued, again indicating an important role of cytokines for survival, but not for commitment/differentiation.

Collectively, these studies have often concluded that lineage commitment can occur independently of cytokines, thus attributing cytokines only a selective function. However, *in vivo* studies investigating cytokine function have to be regarded with caution, as same lineages can be produced by several different cytokines, compensating for the deficiency of a single cytokine. Furthermore, alternative ligands may bind the receptor of the knocked out cytokine, as is the case with IL-34, which was discovered to bind the MCSFR (Lin et al. 2008). Generally, the fact that lineage commitment can occur in absence of a cytokine, does not exclude that cytokines can have an instructive function on uncommitted cells.

Studies supporting instructive cytokine function

Support for an instructive role of cytokines comes from studies ectopically overexpressing cytokine receptors in cell types normally not expressing them and then exposing the cells to the respective cytokine. Overexpression of the GMCSFR alpha-chain

instructs myeloid lineage conversion of CLPs and pro-T cells, but not of pro-B cells and MEPs (Iwasaki-Arai et al. 2003, King et al. 2002, Kondo et al. 2000). Instruction of myeloid DC fate has been achieved by overexpressing Flt3 in MEPs (Onai et al. 2006). MCSFR expression instructs myeloid fate in pro-B, pro-T, and multipotent cell lines (Borzillo et al. 1990, Bourette et al. 2007, Pawlak et al. 2000). Yet, in other similarly conducted studies, the overexpressed cytokine receptor only leads to a proliferative signal in absence of lineage conversion, as in the case of MCSFR expression in primary erythroid progenitor cells (McArthur et al. 1994) and EPOR expression in myeloid or multipotent progenitors (McArthur et al. 1995, Pharr et al. 1994). This indicates that the target cells' cellular context - in terms of available intracellular signaling components, transcription factors, and other molecular components - might be relevant for the outcome of ectopic cytokine signaling.

Proving or disproving cytokine-mediated lineage instruction on uncommitted cells and to distinguish it from stochastic lineage commitment followed by selective survival is technically demanding. It requires following the fates of uncommitted cells and their progeny over time until cells commit to one lineage. Initial studies therefore cultured individual GM colony forming cell (GM-CFC)-derived daughters in either M- or GM-CSF. Analysis of the downstream progeny compared with the estimation of lineage potential of input cells suggested an instructive function of these cytokines (Metcalf & Burgess 1982).

More recently, time-lapse imaging at the single cell level was utilized to investigate if M- and G-CSF can instruct the lineage choice of bipotent GMPs. If progenitors in cultures containing only one cytokine would randomly commit to a lineage not supported by the cytokine, single daughter branches of pedigrees should discontinue due to the lack of survival signals, while other branches should differentiate towards the supported lineage. This would indicate a selective function of the cytokine. If the cytokine is instructive, all progeny of single progenitor cells should differentiate into the instructed lineage, without early apoptotic events of single daughter cells (Figure 3-2). It was shown that M- and G-CSF mainly follow the latter scenario, demonstrating that these factors can instruct lineage choice (Rieger et al. 2009). Moreover, using PU.1 upregulation as a read out for myeloid commitment, it was recently suggested that M-CSF can instruct HSCs and that this is controlled by the transcription factor MafB (Mossadegh-Keller et al. 2013, Sarrazin et al. 2009).

In summary, these studies indicate that some cytokines are able to instruct lineage choice, but that it is probably cell type and context dependent.

3.2.2.3 Myeloid transcription factors and mechanisms of lineage differentiation

Regardless of whether one favors the selective or instructive model of cytokine function, it is evident that transcription factors play key roles in the determination of the ultimate cell fate of a differentiating cell by driving lineage-characteristic gene expression. The molecular basis of transcription factor-mediated lineage differentiation depends on protein levels, mutual antagonistic regulation of lineage-specific proteins, and the chromatin/epigenetic state of the cell. Two critical myeloid transcription factors are PU.1 and C/EBPα.

PU.1 is a member of the large Ets family of transcription factors and is expressed at low levels in early HSPCs. As cells differentiate and mature, PU.1 expression is downregulated in the erythroid, megakaryocytic, and T-cell lineages, but increases in the monocytic, granulocytic, dendritic, and B-cell lineages (Carotta et al. 2010, Hromas et al. 1993). Almost all myeloid-affiliated genes contain PU.1 binding sites in their promoters (e.g. those encoding CD11b, MCSFR, GMCSFR, GCSFR) (Hohaus et al. 1995, Smith et al. 1996, Zhang et al. 1994). Mice deficient for PU.1 do not produce macrophages, granulocytes, or lymphoid cells during fetal hematopoiesis and die in late gestation or shortly after birth (McKercher et al. 1996, Scott et al. 1994). Conditional deletion of PU.1 in adult mice showed that adult PU.1$^{-/-}$ HSCs do not give rise to detectable CLPs, CMPs, or GMPs (Dakic et al. 2005, Iwasaki et al. 2005b). Intriguingly, the most pronounced consequence of the conditional inactivation of PU.1 in adult mice is greatly expanded granulopoiesis, suggesting that expression of PU.1 restricts differentiation of GMPs into granulocytes (Dakic et al. 2005). Furthermore, deletion of PU.1 leads to drastically reduced differentiation of dendritic cells (Carotta et al. 2010). Overexpression of PU.1 activates myeloid gene expression and causes irreversible myeloid differentiation with concomitant suppression of other lineage fates (Nerlov & Graf 1998).

The mechanism by which PU.1 specifies the GMP from the CMP and/or the LMPP from the MPP is believed to involve antagonizing the function of GATA-1. GATA-1 is a zinc finger transcription factor expressed in the erythroid, megakaryocytic, mast cell, and eosinophilic lineage and their progenitors (Martin et al. 1990, Zon et al. 1993). Mice deficient for GATA-1 show a block in erythroid and megakaryocyte development (Pevny et al. 1991, Shivdasani et al. 1997). Forced expression of GATA-1 in GM committed primary cells reprograms them into the MegE lineage, while inhibiting normal GM differentiation

(Heyworth et al. 2002). PU.1 and GATA-1 can bind to each other and thereby antagonize each other's transcriptional activity. PU.1 does so by recruiting the retinoblastoma (Rb) protein to GATA-1, which in turn leads to recruitment of a transcriptional repression complex (Rekhtman et al. 2003, Stopka et al. 2005). Conversely, GATA-1 can bind to the DNA-binding domain of PU.1, displacing the critical coactivator c-Jun and thereby decreasing PU.1-mediated transcription (Zhang et al. 1999). Furthermore, both GATA-1 and PU.1 upregulate their own expression (Chen et al. 1995, Tsai et al. 1991).

C/EBPα is a basic leucine zipper (bZip) transcription factor, which functions either as homo- or heterodimer. C/EBPα is expressed predominantly in the granulocytic and monocytic lineages and their precursors (Scott et al. 1992). Mice lacking C/EBPα have no granulopoiesis, impaired monopoiesis, and a decrease in GMP frequency (Heath et al. 2004, Zhang et al. 1997). In line with this, conditional deletion of C/EBPα in adult mice results in block of the CMP to GMP transition (Zhang et al. 2004). In fetal livers of C/EBPα$^{-/-}$ mice, erythroid development is significantly increased, and forced expression of C/EBPα in primary MEPs and an erythroleukemic cell line promotes myeloid over erythroid differentiation (Suh et al. 2006), suggesting a role of C/EBPα in the GM vs. MegE lineage choice. Forced expression of C/EBPα in a bipotential cell line of myeloid origin leads to granulocytic differentiation and suppression of the monocytic differentiation program (Radomska et al. 1998). Intriguingly, using CLPs, MEPs, B-cells, or pre-T cells, several studies have shown that overexpression of C/EBPα (and in some studies C/EBPß) in these cells leads to macrophage rather than granulocyte transdifferentiation (Fukuchi et al. 2006, Laiosa et al. 2006, Xie et al. 2004). These different lineage outcomes might depend on varying C/EBPα interaction partners present in the target cells. In line with this, mutations in the leucine zipper domain of bZip proteins that allow controlling the partnering of bZip proteins showed that different C/EBPα heterodimers have distinct effects on hematopoietic differentiation when expressed in hematopoietic progenitors (Cai et al. 2008). While homodimers of C/EBPα only modestly increase monocyte differentiation, heterodimers of C/EBPα and c-Jun are potent in doing so. This suggests that different bZip interaction partners of C/EBPα might control monocytic vs. granulocytic cell fate. Depending on which C/EBPα interaction partners are available in the target cells used, overexpression of C/EBPα might then either lead to granulocytic or monocytic differentiation.

Moreover, it was shown that the amount of PU.1 directs lineage outcome: High concentrations of PU.1 leads to monocytic rather than to granulocytic differentiation and vice

versa in a PU.1$^{-/-}$ cell line, in which PU.1 activity was restored by introducing a 4-hydroxy-tamoxifen(OHT)-inducible and -tunable PU.1-ER fusion (PUER cell line) (Dahl et al. 2003). This was suggested to be regulated by the PU.1 vs. C/EBPα ratio. Consistent with this hypothesis, it was shown that C/EBPα can bind PU.1 leading to dissociation of c-Jun and blocking PU.1 transactivation (Reddy et al. 2002). However, a converse mechanism of PU.1 directly antagonizing C/EBPα has not been described and might be regulated through further downstream factors.

Analyzing the transcriptomes of single cells after activating PU.1 using the above mentioned 4-OHT-inducible cell line showed that the transcriptional regulators Egr-2 and Nab-2 are upregulated within 24 hours of PU.1 activation and remain expressed during monocyte differentiation (Laslo et al. 2006). Egr-2 and Nab-2 form a repressive transcription complex, which was shown to mediate repression of granulocyte-affiliated genes and simultaneously activate macrophage-associated genes, such as the one encoding the MCSFR (Krysinska et al. 2007, Laslo et al. 2006). Furthermore, Egr-1 was shown to induce monocytic differentiation at the expense of granulocytes *in vivo* (Krishnaraju et al. 2001). A candidate for a transcription factor that inhibits the expression of macrophage-associated genes and promotes granulocytic gene expression is Gfi-1. Gfi-1 deficient mice are severely neutropenic (Hock et al. 2003, Karsunky et al. 2002) and Gfi-1 overexpression in progenitors directs granulocytic differentiation at the expense of macrophages through direct interaction with and repression of PU.1 (Dahl et al. 2007). Differentiation of PUER cells into macrophages is accompanied by downregulation of Gfi1, and Egr-2 and Nab-2 were shown to directly bind the Gfi1 promoter to repress its transcription. Conversely, forced expression of Gfi1 in PUER cells inhibits differentiation into macrophages and decreases expression of Egr-2 (Laslo et al. 2006). A model was suggested, in which PU.1 and C/EBPα are primary cell-fate determinants that upregulate Egr-2/Nab-2 and Gfi1, respectively. Cross-antagonism between Egr-2/Nab-2 and Gfi1 then stabilizes a tilted PU.1:C/EBPα ratio, leading to either macrophage differentiation in the case of dominant PU.1 or granulocyte differentiation in the case of dominant C/EBPα.

Collectively, these studies demonstrate the important role of transcription factors in stabilizing lineage decisions by executing lineage-specific gene expression programs, which lead to lineage differentiation. However, whether the initial upregulation of transcription factors is based on their randomly fluctuating expression levels or on external cues remains unknown. Similarly, signaling pathways induced by external cues and potentially involved in

activating already present lineage-specific transcription factors in HSCPs are also not well described.

3.2.2.4 Epigenetic regulation of hematopoietic differentiation

Expression of lineage-specific gene programs requires more than the availability of the necessary transcription factors. The accessibility of genes to transcription factors and other regulatory proteins is regulated through chromatin modifications, such as acetylation, methylation, phosphorylation, ubiquitination, and other collectively termed epigenetic modifications. As cells differentiate, chromatin remodeling occurs until an irreversible state is achieved that is specific for a terminally differentiated lineage. Recently, comprehensive DNA methylation maps for different HSCPs have been provided, revealing lineage-specific programs of DNA methylation changes during hematopoietic differentiation (Ji et al. 2010). Moreover, DNMT1, a DNA methyltransferase, was shown to be important for HSC self-renewal and commitment to lymphoid vs. myeloid differentiation (Bröske et al. 2009, Trowbridge et al. 2009). Identification of genome-wide changes in gene expression and histone modifications during hematopoiesis revealed that developmentally regulated genes are epigenetically primed in HSCs for subsequent activation or repression during lineage commitment (Cui et al. 2009, Weishaupt et al. 2010). Furthermore, transcription factors such as GATA-1 themselves bind factors that can induce epigenetic changes (Blobel 2002, Gregory et al. 2010, Miccio et al. 2010).

3.2.2.5 miRNAs in myeloid development

An emerging group of putative lineage determinants are microRNAs (miRNAs), small non-coding regulatory RNA molecules that bind target sequences in messenger RNA (mRNA), thereby inhibiting their expression either by induction of their degradation or by inhibition of their translation. Most miRNAs exerting effects on myelopoiesis were discovered by profiling studies, revealing that many miRNAs are expressed at various stages of myeloid development. Several miRNAs have now been analyzed in gain and loss of function studies and are confirmed as being important in myeloid biology. It has been shown

that several myeloid transcription factors can induce the expression of miRNAs, thereby changing the transcriptional profile of a cell.

miR-223 was one of the first miRNAs to be discovered to be highly expressed in myeloid cells and was later attributed a functional role in myelopoiesis itself (Chen et al. 2004). Specifically, miR-223 is highly expressed in granulocytes and its expression is becoming incrementally higher with granulocytic maturation. Loss of miR-223 results in an expanded granulocyte compartment and to neutrophilic hypersensitivity in response to activating stimuli (Johnnidis et al. 2008). Expression of miR-223 is regulated by several factors, including C/EBPα and PU.1 (Fazi et al. 2005, Fukao et al. 2007). Increasing levels of C/EBPα during granulocytic differentiation competitively displaces the transcriptional repressor NFI-A from the miR-223 promoter and induces miR-223 expression. This molecular switch leads to a negative feedback loop in which miR-223 represses NFI-A translation, which enables exit from the progenitor cell state and initiates granulocytic differentiation (Fazi et al. 2005). Interestingly, it was shown that during macrophage differentiation PU.1 induces expression of miR-424, which also targets NFI-A, and that this is necessary to induce differentiation-specific genes including the MCSFR gene (Rosa et al. 2007). This suggests that NFI-A is a key transcription factor required in maintaining myeloid progenitors in an undifferentiated state. C/EBPα and PU.1 each induce distinct miRNAs, which can downregulate NFI-A expression, leading to the onset of either macrophage or granulocyte differentiation.

M-CSF signaling was shown to induce expression of the transcription factor AML1 and repress the expression of miR-17-5p-20a, which was demonstrated to regulate AML1 protein expression by targeting the AML1 mRNA. This leads to a positive feedback loop resulting in the accumulation of AML1 protein and MCSFR expression (Fontana et al. 2007).

Other examples of miRNAs involved in myeloid development include miR-146a, whose deletion eventually causes an overproduction of myeloid cells (Boldin et al. 2011). Overexpression of miR-155, miR-29a, or miR-125b in BM all result in a bias towards the GM lineage, suggesting that these miRNAs are involved in the regulation of the lymphoid vs. myeloid balance (Bousquet et al. 2010, Han et al. 2010, O'Connell et al. 2008).

It has become evident in recent years that miRNAs are also involved in modulating signal transduction pathways. For example, it has been shown that miR-21 and miR-126 target inhibitors of phosphatidylinositide 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades, thereby leading to the upregulation of these signaling pathways

(Fish et al. 2008, Thum et al. 2008). Similarly, miR-24 has been found to repress MKP-7, a negative regulator upstream of the c-Jun N-terminal kinase (JNK) and p38 MAPK signaling cascades. Overexpression of miR-24 in myeloid progenitor cells results in enhanced MAPK activation and a developmental block in granulocytic differentiation (Zaidi et al. 2009).

Collectively, these studies suggest that miRNAs might play an important role in lineage outcome through regulation and fine-tuning of lineage-specific gene expression and growth factor-induced signaling pathways.

3.3 The colony stimulating factors M-CSF and G-CSF and their receptors: function and regulation of myeloid differentiation

As mentioned earlier, the CSFs are a group of cytokines central to hematopoiesis, the modulation of specific blood cells' functional responses, and overall immune competence. This group consists of M-CSF and G-CSF, which exert lineage-specific functions, playing a role in the survival, proliferation, and differentiation of macrophages and granulocytes, respectively, and their progenitors, and GM-CSF and IL3 (also called multi-CSF), which regulate the expansion and maturation of more primitive multipotent progenitors. Production and degradation of CSFs are strictly controlled and so is their expression and that of their cognate receptors, allowing their coordinated biological function during homeostasis and stress conditions. All CSFs exhibit pleiotropic biological functions on various tissues and cells and show extensive functional redundancy, being able to exert similar or overlapping actions on specific cells (Figure 3-3).

The identification and analysis of the CSFs was made possible primarily by cell culture assays developed from the 1960s to early 1980s (Bradley & Metcalf 1966, Ichikawa et al. 1966, Metcalf & Burgess 1982). These assays in semi-solid medium revealed the biological factor-dependent survival, proliferation, and differentiation of immature hematopoietic cells (colony forming units) and led to the subsequent purifications of CSFs.

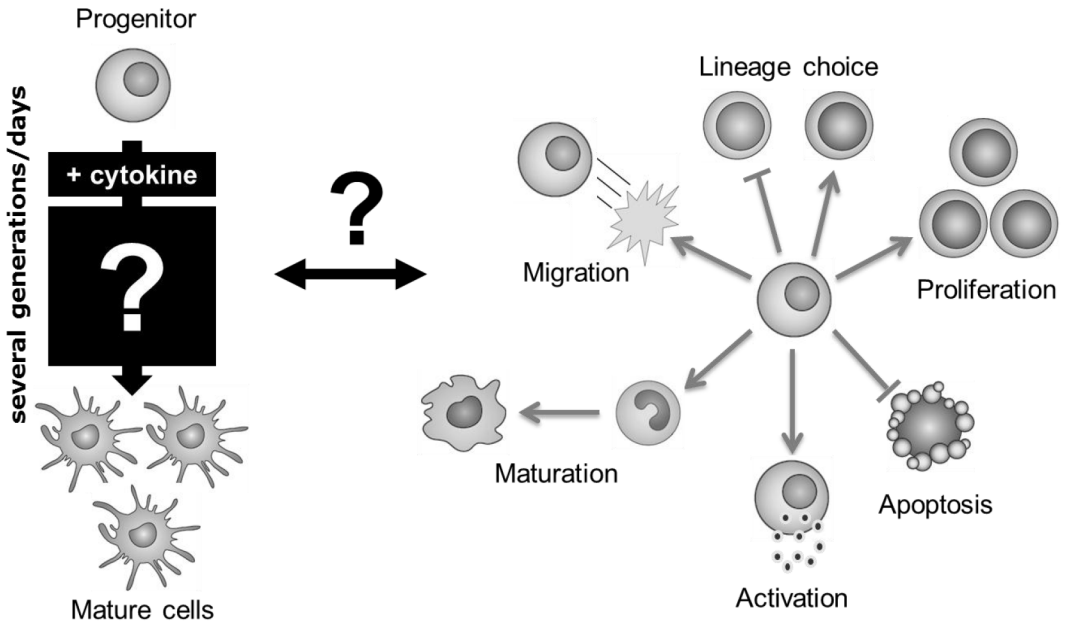

Figure 3-3: Pleiotropic actions of a single cytokine orchestrate lineage differentiation.
Single cytokines can have multiple effects on cells carrying the cognate receptor. The net effect of these cytokine-mediated actions is differentiation towards a specific lineage. Discontinuous analyses cannot detect and/or quantify the contribution of the different cytokine-mediated cell fate effects on differentiation.

3.3.1 M-CSF

M-CSF (also known as CSF-1) was the first CSF to be purified (Stanley & Heard 1977) and was originally found in murine serum and human urine (Bradley et al. 1967, Robinson et al. 1969). M-CSF is a homodimeric sialoglycoprotein and acts as the principal regulator of the survival, proliferation, and differentiation of monocyte/macrophages and their precursors. M-CSF is also a key regulator of mature macrophages, mediating their functional activation and cellular behavior (Pixley & Stanley 2004). M-CSF can synergize with other cytokines, such as IL-6, to induce proliferation of early hematopoietic progenitors (Bot et al. 1989). M-CSF also synergizes with receptor activator of nuclear factor kappa-B ligand (RANKL) to induce osteoclastogenesis (Teitelbaum & Ross 2003). A variety of different cell types can produce and secrete M-CSF. These include endothelial cells, BM stromal cells, fibroblasts, osteoblasts, thymic epithelial cells, keratinocytes, astrocytes, myoblasts, and others. M-CSF production can also be stimulated in monocytes/macrophages, T-cells, B-cells, chondrocytes, and other cell types upon their functional activation through, for example, inflammatory cytokines. In addition to two secreted forms of M-CSF, there is also a membrane-spanning form at the cell surface, which is produced by alternative splicing

(Rettenmier & Roussel 1987). Transgenic expression of the different M-CSF isoforms in M-CSF-deficient mice revealed distinct, but overlapping functions: While the secreted glycoprotein isoform humorally regulates cellular targets, the membrane-bound and the secreted proteoglycan isoforms are suggested to be involved in local regulation (Dai et al. 2004, Nandi et al. 2006, Ryan et al. 2001). The main mechanism to negatively regulate M-CSF activity is through internalization and degradation of ligand-receptor complexes by cells expressing the MCSFR (Bartocci et al. 1987).

The overall biological response of cells to M-CSF is dependent on cell types and co-stimulating signals from additional extracellular or intracellular events. Although cells of the monocytic lineage are considered the main target population, the action of M-CSF is not limited to these cells, as reflected by M-CSF-deficient mice. M-CSF deficiency in mice carrying a null-mutation in the coding region of the M-CSF gene (op/op mice) leads to a severe reduction of osteoclasts and macrophages, absence of teeth, abnormal bone remodeling and osteopetrosis, abnormal breast and brain development, decreased fertility, and an overall shortened life-span (Michaelson et al. 1996, Pollard & Hennighausen 1994, Wiktor-Jedrzejczak & Gordon 1996, Yoshida et al. 1990). Many of these defects can be rescued by injection of recombinant M-CSF into neonatal mice (Wiktor-Jedrzejczak et al. 1991).

3.3.2 M-CSF receptor structure/function

The effects of M-CSF are mediated through the MCSFR (also known as CD115), a member of the class III receptor tyrosine kinases, also including c-kit, Flt3, and the PDGF receptor (Sherr et al. 1985). The MCSFR has an N-terminal glycosylated extracellular part containing five immunoglobulin-like domains, a short single transmembrane domain, a juxtamembrane domain, and a split kinase domain in the C-terminal cytoplasmic portion. Binding of M-CSF promotes receptor dimerization and activation of the intrinsic tyrosine kinase activity, leading to transphosphorylation of specific tyrosine residues in the intracellular part of the receptor (Bourette & Rohrschneider 2000). The phosphorylated tyrosine residues then act as docking sites for a variety of intracellular adaptor proteins containing src-homology 2 (SH2) domains. This leads to activation of downstream signaling pathways and eventual cellular response, in form of cytoskeletal remodeling and increased

adhesion, as well as increased transcription and translation required for growth, proliferation, and differentiation. Stimulation of downstream signaling cascades is followed by polyubiquitination of the cytoplasmic MCSFR domain, kinase inactivation, tyrosine dephosphorylation, internalization, targeting to lysosomes, and destruction of the receptor-ligand complex (Pixley & Stanley 2004).

MCSFR-deficient mice show a more severe phenotype than op/op mice, with further reduction in tissue macrophage numbers, reduced survival, and increased osteopetrosis (Dai et al. 2002). This suggested that there is another MCSFR ligand, which was recently identified as IL34 (Lin et al. 2008). M-CSF and IL34 expression in embryonic and adult mouse tissues revealed different spatiotemporal expression patters. Most notably, IL34 is expressed early in the brain, when MCSFR, but no M-CSF expression is apparent (Nandi et al. 2012, Wei et al. 2010). IL-34-deficient mice selectively lack Langerhans cells and microglia, which are present in op/op mice, but absent in $MCSFR^{-/-}$ mice (Wang et al. 2012b).

Of the 19 tyrosines in the cytoplasmic portion of the MCSFR, six (Y559, Y697, Y706, Y721, Y807, Y974) have been shown to be phosphorylated upon M-CSF stimulation (Tapley et al. 1990, van der Geer & Hunter 1990, Wilhelmsen et al. 2002). Two more tyrosines (Y544 and Y921) have been demonstrated to be phosphorylated in the constitutively active, oncogenic form of the MCSFR (Joos et al. 1996, Mancini et al. 1997). As mentioned above, most of the phosphorylated tyrosine residues form docking sites for signaling proteins that initiate a series of signaling cascades, which results in specific gene expression and cellular responses (Figure 3-4). Studies examining the role of individual receptor tyrosine residues in initiating specific signaling pathways and how these are connected to cellular fates have produced conflicting results depending on the cellular system and methods used. Nevertheless, despite their limitations, ectopic and chimeric MCSFR expression studies have provided significant functional knowledge about single MCSFR tyrosine residues and their associated signaling molecules.

The juxtamembrane region of the MCSFR harbors Y544 and Y559. Y544 has so far not been demonstrated to be phosphorylated in the wild type receptor but only in the oncogenic form, where it binds an as yet unidentified protein of 55k-Da in size (Joos et al. 1996). Y559 has been shown to bind Src family kinases (SFKs) when phosphorylated (Rohde et al. 2004). Furthermore, Y559 was shown to participate in autoinhibition of the MCSFR in absence of M-CSF: Mutation of Y559 significantly reduces receptor tyrosine phosphorylation and inhibits its kinase activity. This was observed in several cellular systems, including a

mature macrophage cell line (Xiong et al. 2011, Yu et al. 2012), a myeloid progenitor cell line (Rohde et al. 2004), and as a chimeric receptor in primary macrophages (Takeshita et al. 2007). Phosphorylated Y559 activates a SFK and c-Cbl dependent pathway that leads to MCSFR ubiquitination and possibly to a further change in conformation, permitting increased receptor phosphorylation (Xiong et al. 2011). Therefore, Y559 seems to be a critical tyrosine residue inducing MCSFR phosphorylation and activation. However, which particular SFK associates with Y559 is still unknown. There are at least six SFKs expressed in macrophages: Hck, Fgr, Lyn, Src, Fyn, and Lck (Yu et al. 2012). Association of MCSFR with Src, Fyn, and Yes was demonstrated in transduced NIH3T3 fibroblasts or myeloid cell lines using GST-SH2 pulldown assays (Courtneidge et al. 1993, Marks et al. 1999, Rohde et al. 2004). There are several contradictory reports on the cellular consequences if Y559 is mutated. Analyzing the effects of mutated Y559 in a myeloblastic leukemia cell line suggested that Y559 is not required for M-CSF-mediated proliferation (Marks et al. 1999). However, expressing the same mutant in another myeloblast-like cell line results in a hyperproliferative response to M-CSF (Rohde et al. 2004). In contrast, expressing Y559-mutated MCSFR in primary macrophages substantially reduces proliferation (Takeshita et al. 2007, Yu et al. 2008). These contradicting results exemplify the importance of studying M-CSF responses in primary cells of interest rather than in cell lines.

The split kinase insert of the MCSFR harbors three tyrosine residues phosphorylated upon M-CSF stimulation: Y697, Y706, and Y721. Phospho-Y697 binds growth factor receptor-bound protein 2 (Grb2), monocytic adaptor (Mona), and suppressor of cytokine signaling (SOCS)1 (Bourette et al. 1998, 2001; Geer et al. 1993). Grb2 is an adaptor protein known to bridge receptor tyrosine kinases to the Ras/Raf/MEK/ERK MAPK signaling cascade. MAPK signaling regulates many fundamental processes including survival, proliferation, differentiation, and cellular behavior. ERK targets include transcription factors such as c-fos, c-jun, and c-myc which mediate transcription of early M-CSF response genes (e.g. cell cycle genes). Hematopoietic-specific protein Mona was also described to activate the MAPK pathway and thereby induce monocyte differentiation in a myeloid progenitor cell line (Bourgin et al. 2000). SOCS1 is a known negative regulator of cytokine signaling and has been shown to downregulate M-CSF-mediated proliferation (Bourette et al. 2001). Mutation of Y697, however, shows only a mild defect on proliferation of primary macrophages transduced with a chimeric receptor (Faccio et al. 2007). Likely, this is because other phosphorylated tyrosine residues can also bind Grb2 (Y921) and SOCS1 (Y721) and

thereby compensate Y697 mutation (Bourette et al. 2001, Mancini et al. 1997). In line with this, combined mutation of Y697 and Y921 greatly accentuates the proliferative defect as compared to the single Y697 mutation (Faccio et al. 2007), further suggesting that Y921 may also be phosphorylated in the wild type receptor and not only in the oncogenic form. Phosphorylated Y706 has so far not been shown to directly bind signaling molecules. However, it has been shown that phospho-Y706 is required for full activation of signal transducer and activator of transcription (STAT)1 (Novak et al. 1996). The most important function of Y721 is to activate PI3K signaling via binding of PI3K's catalytic subunit p85, resulting in production of phosphatidylinositol (3,4,5) trisphosphate (PIP_3) (Reedijk et al. 1992, Sampaio et al. 2011). Many of the immediate M-CSF-induced cytoskeletal changes (e.g. membrane ruffling) in mature macrophages are regulated through Y721-mediated PI3K activation and subsequent PIP_3 production (Sampaio et al. 2011). Small GTPases including Rac, RhoA, and cdc42 act downstream of PI3K to induce M-CSF-mediated cytoskeletal remodeling and motility (Pixley 2012). Immortalized macrophages carrying MCSFR mutated at Y721 exhibit significantly reduced adhesion, spreading, and mobility (Sampaio et al. 2011). PIP_3 production also activates Akt, which can trigger a multitude of downstream effectors involved in cell survival, proliferation, and motility. Activation of PI3K has also been shown to occur through SFKs bound to phosphorylated Y559 or c-Cbl bound to phospho-Y974. Using yeast two hybrid screening, phospho-Y721 was also found to bind phospholipase Cγ2 (PLCγ2) in a myeloid progenitor cell line (Bourette et al. 1997). However, in a mature macrophage context, PLCγ2 binding to MCSFR was shown to be independent of Y721 (Sampaio et al. 2011). SOCS1, besides binding Y697, has also been shown to associate with Y721 when phosphorylated (Yu et al. 2008).

Y807, located in the MCSFR activation loop, has been implicated in MCSFR function along with Y559 (Takeshita et al. 2007, Yu et al. 2008). To date, no protein has been described to directly interact with activated Y807. In macrophages carrying a mutated Y807 MCSFR, receptor phosphorylation is severely affected, suggesting an autoinhibitory role for this residue in MCSFR activity in absence of M-CSF. Using a rat-derived fibroblast cell line, it was shown that mutation of Y807 reduces the proliferative response to M-CSF (van der Geer & Hunter 1991). In contrast, the same mutant expressed in an immature myeloid cell line increases proliferation of these cells in the presence of M-CSF (Bourette et al. 1995). Yet another study analyzed the Y807mutant in primary macrophages using a receptor chimera consisting of the extracellular part of the EPOR and the cytoplasmic part of the MCSFR. This

study found that mutation of Y807 suppresses M-CSF-mediated macrophage proliferation (Takeshita et al. 2007). These studies further demonstrate the importance of studying M-CSF-elicited effects in a correct cellular context.

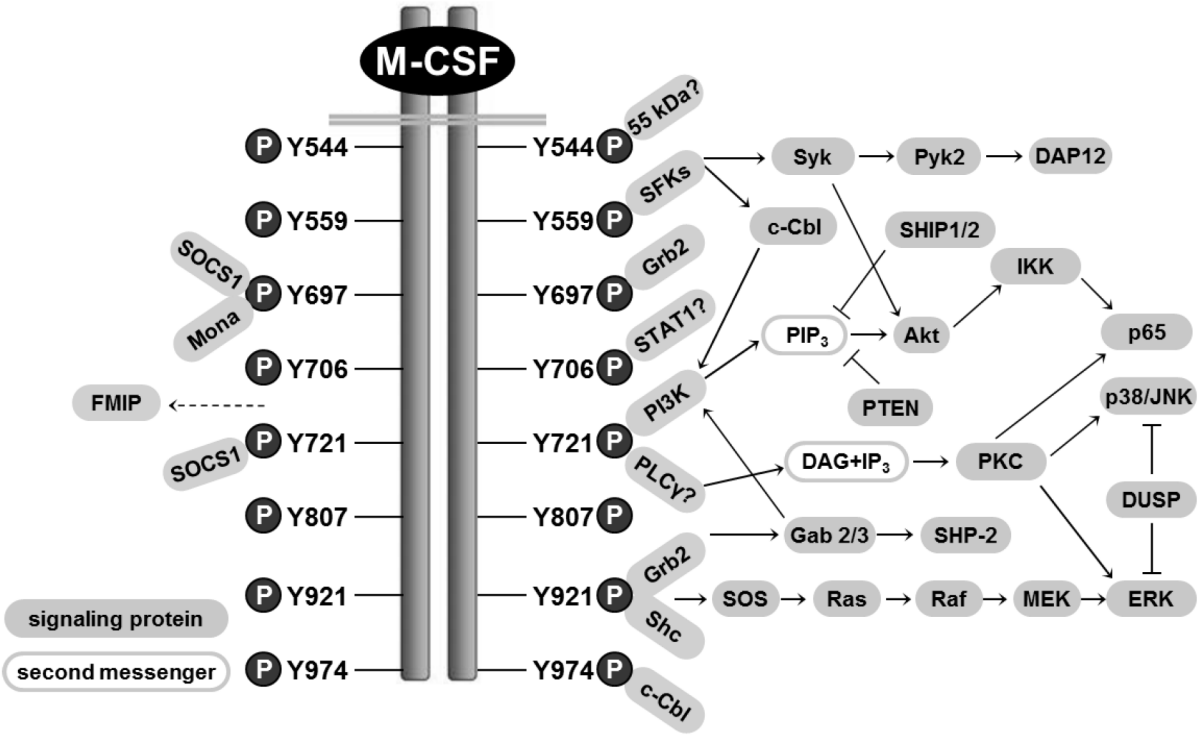

Figure 3-4: Major MCSFR-activated signaling pathways.
The MCSFR has eight functional tyrosine residues that upon binding of M-CSF to the receptor are transphosphorylated and subsequently act as docking sites for a number of SH2-containing adaptor proteins. As a result, a variety of different downstream signaling pathways are activated that mediate the pleiotropic actions of M-CSF. How each signaling pathway contributes to the effects elicited by M-CSF is not well understood. SOCS: suppressor of cytokine signaling; FMIP: fms-interacting protein; SFK: Src family kinases; Grb: Growth factor receptor-bound protein; STAT: signal transducer and activator of transcription; Gab: GRB2-associated-binding protein; PIP_3: phosphatidylinositol (3,4,5)-triphosphate; DAG: diacylglycerol; Syk: spleen tyrosine kinase; SHIP: Src homology region 2 domain-containing inositol 5'-phosphatase; IKK: I kappa B kinase; PTEN: phosphatase and tensin homolog; PKC: protein kinase C; SHP: Src homology region 2 domain-containing phosphatase; MEK: mitogen-activated kinase kinase; ERK: Extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; Mona: monocytic adapter; SOS: son of sevenless; Shc: Src homology 2 domain containing protein.

The C-terminus of the receptor carries two more tyrosines: Y921 and Y974. As mentioned above, Y921 is mainly associated with the oncogenic form of MCSFR, where it was found to bind Grb2 when phosphorylated (Mancini et al. 1997). Y974 is associated with the E3 ubiquitin ligase c-Cbl, which ubiquitinates MCSFR (and receptor tyrosine kinases in general) and targets it for degradation (Mancini et al. 2002, Wilhelmsen et al. 2002). However, c-Cbl can also be indirectly activated through the Y559-SFK and Y721-PI3K axes. Additionally, c-Cbl is required for full phosphorylation and activation of the receptor (Xiong

et al. 2011). Another protein interacting with MCSFR, fms-interacting protein (FMIP) (also known as THOC5), has not been associated with a specific tyrosine residue yet, and its specific role in MCSFR signaling remains to be elucidated (Carney et al. 2009, Mancini et al. 2004, Tamura et al. 1999).

Mutation of all eight tyrosines discussed above has been shown to result in a receptor incapable to transmit survival and/or differentiation cues (Yu et al. 2008). Other tyrosine, serine, or threonine residues phosphorylated in the MCSFR and activating other signaling pathways cannot be excluded to have additional functions but have so far not been described.

3.3.3 G-CSF

G-CSF is central to differentiation, proliferation, survival, and functional activation of granulocytes, of which relatively short-lived neutrophils are the most abundant. It was first purified from murine lung-conditioned medium (Nicola & Metcalf 1983). G-CSF can be generated by a variety of cells and production can be triggered under stress conditions by several cytokines, including IL-3, IL-17, GM-CSF, M-CSF, tumor necrosis factor alpha (TNF-α), and IFN-γ, which can lead to a dramatic increase in G-CSF concentration and up to a 10-fold increase in neutrophil number. Because of its effects on the modulation of granulocyte production and its ability to mobilize HSCs from the BM to circulation, G-CSF is being used clinically as a therapeutic agent. Furthermore, G-CSF treatment accelerates hematopoietic recovery after transplantation and chemotherapy and ameliorates neutropenia in patients with severe congenital or chronic neutropenia. Unlike M-CSF, excess G-CSF level has relatively few negative side effects, as shown in mice (Chang et al. 1989). Mice deficient for G-CSF or the GCSFR have a marked decrease in peripheral blood neutrophil counts (15-30% of wild type mice), as well as a significant decrease of progenitors and mature granulocytes in the BM (Lieschke et al. 1994, Liu et al. 1996). As a result, G-CSF$^{-/-}$ mice are significantly impaired in fighting infections. Similarly to M-CSF, circulating G-CSF is actively cleared by ligand-receptor internalization and degradation (Ericson et al. 1997).

3.3.4 G-CSF receptor structure/function

The GCSFR is a member of the type I cytokine receptors. Unlike the MCSFR, the GCSFR does not have an intrinsic tyrosine kinase domain, but relies on cytoplasmic kinases for activation of downstream signaling pathways. The GCSFR is primarily expressed in granulocytic progenitors and mature neutrophils, but can also be found on other hematopoietic (Nicola & Metcalf 1985) and non-hematopoietic cells, including neurons (Schneider et al. 2005). It consists of an extracellular portion that harbors a conserved cytokine receptor homologous (CRH) domain, an Ig-like domain, and three fibronectin type III-like domains. The transmembrane domain is followed by a cytoplasmic domain containing two regions of sequence homology shared with other cytokine receptors and referred to as Box 1 and Box 2. Moreover, the cytoplasmic domain contains a more distal Box 3 motif and four functional tyrosine residues (Y703, Y728, Y743 and Y763). Upon G-CSF binding, GCSFR homodimerizes and receptor-associated Janus protein tyrosine kinases (JAKs) undergo trans-phosphorylation and full kinase activation. JAKs in turn phosphorylate the four functional tyrosine residues of the receptor, which recruit intracellular SH2-containing proteins to mediate downstream signaling (Figure 3-5).

Early structure/function studies of the GCSFR revealed that the cytoplasmic portion contained regions with distinct signaling functions. The membrane-proximal region, including the Box 1 and 2 motifs required for JAK binding, is essential for G-CSF-induced proliferation, while the distal region containing the four functional tyrosines and the Box 3 motif, regulates granulocytic differentiation and specific gene induction (Dong et al. 1993, Fukunaga et al. 1993, Ziegler et al. 1993). The precise role of individual tyrosine residues in terms of signaling activation and downstream cellular response has also been examined. Intriguingly, at least *in vitro*, and in contrast to the MCSFR, the GCSFR tyrosine residues appear to be dispensable for G-CSF signaling in saturated G-CSF concentration (Ward et al. 1999).

The pivotal signaling mechanism of the GCSFR (and other receptors of its family) is through the JAK/STAT pathway. JAK/STAT signaling components activated through the GCSFR include JAK1, JAK2, TYK2, STAT1, STAT3, and STAT5 (Nicholson et al. 1994, Shimoda et al. 1997, Tian et al. 1994, 1996). The specific roles of JAK1, JAK2, and TYK2 in G-CSF signaling are still unclear. Using a JAK-deficient human fibrosarcoma cell line, it was suggested that JAK1, but not the other members, is critical for receptor phosphorylation and

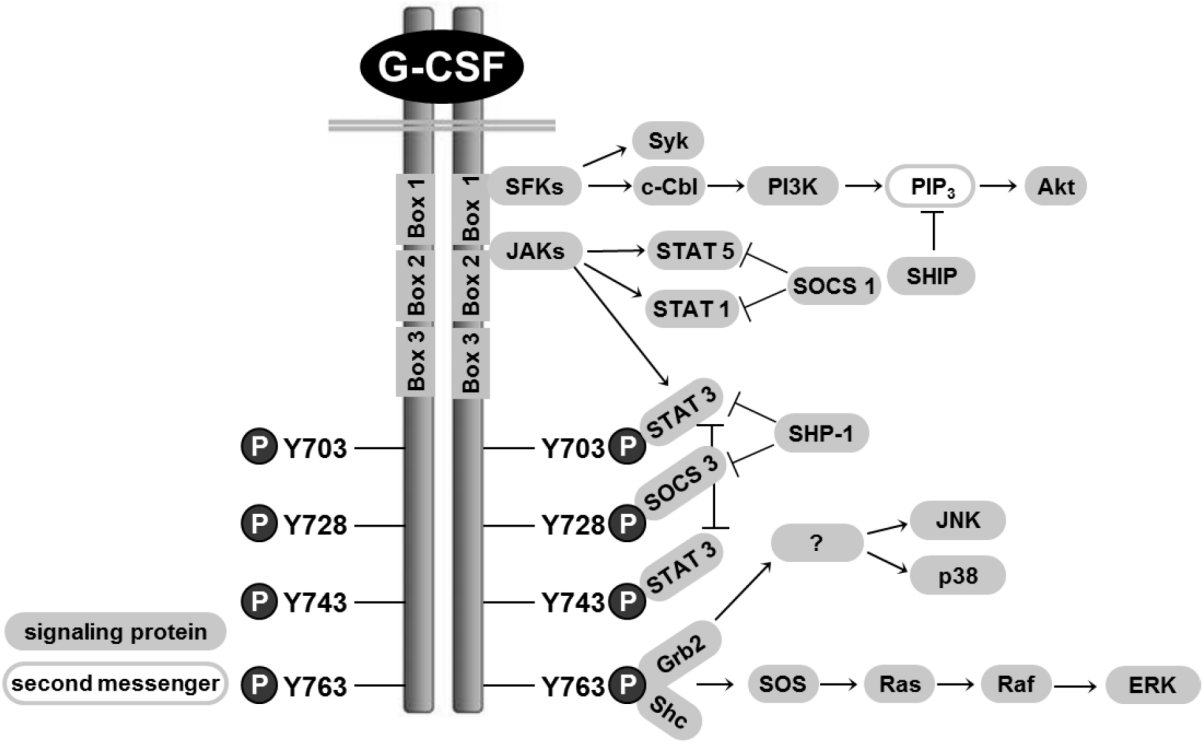

Figure 3-5: Major GCSFR-activated signaling pathways.
The GCSFR has three conserved domains known as Box 1-3 and four functional tyrosine residues. JAKs are constitutively associated with the GCSFR's Box motives and are transphosphorylated and activated upon G-CSF binding and homodimerization of the receptor. The JAKs then phosphorylate the intracellular GCSFR tyrosine residues, which in turn act as docking sites for STATs and other SH2-containing adaptor proteins. This results in activation of several signaling pathways that mediate G-CSF-induced effects.

STAT activation (Shimoda et al. 1997). However, dominant negative forms of either JAK1, JAK2, or TYK2 coexpressed with wild type GCSFR in COS cells blocked STAT5 activation (Dong & Larner 2000). Additionally, JAK1-deficient mice have a normal neutrophil count, speaking against a major non-redundant role for JAK1 (Rodig et al. 1998). STAT1 does not seem to be crucial: it is only weakly and transiently activated by G-CSF, and STAT1-deficient mice do not show a defect in granulopoiesis (de Koning et al. 1998, Durbin et al. 1996). STAT3, however, is robustly activated and docks on phosphorylated tyrosines Y704 and Y744 of the human GCSFR (Chakraborty et al. 1999). Yet, conditional knockout mice with selective deletion of STAT3 in hematopoietic progenitors results in neutrophilia with a specific increase in late stage neutrophils, suggesting that STAT3 has a limiting function in late granulopoiesis, but is not required for the production of functional neutrophils *in vivo* (Lee et al. 2002). More recently, using mice with conditional deletion of STAT3 in BM, it was shown that STAT3 is required for stress-induced granulopoiesis by driving expression of C/EBPß (Zhang et al. 2010). STAT5 is activated independently of tyrosine residues, most likely through direct recruitment to JAKs (Dong et al. 1998, Fujitani et al. 1997). G-CSF-

mediated activation of STAT5 is only transient and has been implicated in survival and proliferation (Dong et al. 1998). STAT5A and STAT5B double knockout mice show a 50% decrease in peripheral neutrophils, but this appears to be independent of G-CSF, indicating that STAT5 is required for granulocyte maintenance *in vivo*, but not for G-CSF-induced granulopoiesis (Kimura et al. 2009).

Besides JAK/STAT, MAPK and PI3K/Akt signaling pathways are activated through G-CSF, and both were found to contribute to G-CSF-mediated survival and proliferation (de Koning et al. 1998, Dong & Larner 2000). Y764 of human GCSFR was found to have a major role in proliferation signaling in cell lines as well as in primary myeloid progenitors: Mutation of Y764 reduces proliferation of myeloid progenitors, while adding it back to a tyrosine null receptor background greatly increases proliferation (Akbarzadeh et al. 2002, de Koning et al. 1998, Hermans et al. 2003). Once phosphorylated, human Y764, as well as the murine equivalent Y763, was shown to bind Grb2 and SH2 domain containing protein (Shc), which are signaling intermediates of the MAPK pathway (De Koning et al. 1996, Rausch et al. 1997). Downstream of Y763/764, several MAPKs have been shown to be activated, including ERK, p38, and JNK (Bashey et al. 1994, Kendrick et al. 2004, Rausch & Marshall 1999, Rausch et al. 1997). Activation of Akt signaling was found to be possible independent of JAKs and involves SFKs (Dong & Larner 2000, Zhu et al. 2006).

The phosphatases SHP-1 and SHIP, and SOCS proteins seem to play a major role in the negative regulation of G-CSF signaling. SOCS1 and SOCS3 are both able to attenuate G-CSF signaling by blocking JAK-induced STAT activation and possibly STAT ubiquitination (Geijn et al. 2004, Zhuang et al. 2005). G-CSF strongly induces transcription of SOCS3 via STAT3 as a negative feedback loop, while SOCS1 remains at a relatively low and constant level (Geijn et al. 2004). SOCS3 was shown to bind the activated human GCSFR on Y729 (Hörtner et al. 2002). Conditional SOCS3 knockout mice, somewhat resembling the $STAT3^{-/-}$ phenotype, react hyperproliferative to G-CSF injection and cells show prolonged STAT3 activation upon G-CSF stimulation *in vitro* (Croker et al. 2004). Besides blocking JAK/STAT signaling, SOCS proteins might also affect other pathways. Recently, SFKs Hck and Lyn, which have been shown to be activated by the GCSFR, were implicated in the negative regulation of G-CSF signaling and granulopoiesis (Mermel et al. 2006).

In summary, many signaling pathways have been described to be activated upon M- and G-CSF stimulation. However, if and/or to what extent the activated signaling cascades are involved in orchestrating the different cytokine-mediated cell fates is less clear and can vary between the cellular model systems used. Specifically, signaling pathways involved in transmitting lineage choice instruction remain elusive. Moreover, the identity of signaling pathways leading to lineage-specific transcription factor expression and/or activation is not well understood. Of note, signaling pathways activated by M-CSF and G-CSF are extensively overlapping, suggesting that other parameters (e.g. duration and strength of signaling) are also important.

3.4 The need for single cell analysis in HSPC research

Clonal assays allowing the readout of all known differentiated blood cell types was a prerequisite for the discovery of hematopoietic stem cells by Till and McCulloch (Becker et al. 1963, Till & McCulloch 1961). Similarly, establishing cell culture assays that allowed the growth of colonies derived from single HSPCs led to the discovery of cytokines (Bradley & Metcalf 1966). Since then, single cell based assays have continued to contribute to the elucidation of many fundamentally important cellular and molecular aspects of HSPCs and their control. For example, single cell transplantations recently revealed the functional heterogeneity of the immunophenotypically homogenous HSC population (Dykstra et al. 2007, Müller-Sieburg et al. 2002, Sieburg et al. 2006). This heterogeneity could not have been detected if not one, but several cells were transplanted, as in this case average output would have been read out. The same holds true for conventional biochemical approaches, which usually read out population averages and mask information on heterogeneity. Although flow cytometry gives single cell resolution and can unravel heterogeneous populations, it reflects only a snapshot in time and lacks information on changing cellular or molecular properties over time. Moreover, these classical methodologies either kill the cells of interest during sample preparation or lose the cells' future identities. Therefore, relationships between current signaling state and future cell fate cannot be inferred by these methods. Fluorescent biosensors, which can visualize and quantify signaling activity in living cells, circumvent

these issues and in combination with continuous time-lapse imaging allow to link cellular behavior and molecular dynamics to future cell fate (Figure 3-6) (Endele & Schroeder 2012).

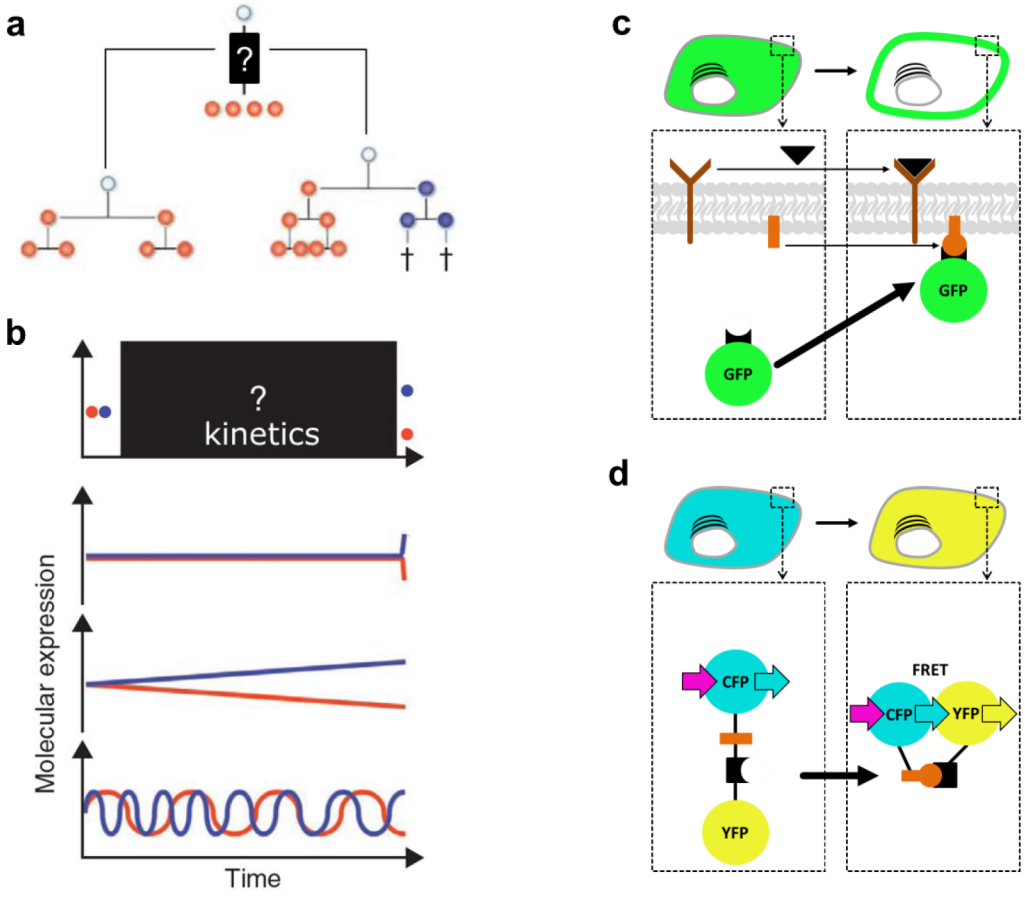

Figure 3-6: Continuous single cell analysis allows detection of cellular genealogy and changing molecular properties obscured by population or snapshot analyses.
(a) Only single cell analysis can detect how a single white cell gives rise to four red cells. Two possible scenarios are depicted that could not be distinguished by population and/or snapshot analyses. (b) Similarly, molecular behavior leading to upregulation (blue) or downregulation (red) of a given factor or signaling pathway cannot be detected by snapshot analysis of bulk cultures. Three possible scenarios are depicted. (c+d) Biosensors can detect signaling kinetics in real time within living cells and allow quantification of molecular behavior over time. (c) Translocation-based biosensors typically consist of a minimal protein binding domain fused to a fluorescent protein. External stimulation leads to the transient accumulation of a second messenger in a specific subcellular localization (e.g. a phospholipid in the plasma membrane), which is detected and bound by the sensor. This results in the translocation of the sensor to the membrane, thereby indicating signaling activation. (d) Fluorescence resonance energy transfer (FRET)-based biosensors contain a sensing domain, which is modified by the signaling activity of interest (e.g. through a kinase). Modification of the sensing domain leads to a conformational change of the sensor inducing FRET and thereby indicating signaling activity. Colored arrows indicate light of different wavelengths. Figure adapted from (Endele & Schroeder 2012, Schroeder 2011). GFP: green fluorescent protein; YFP: yellow fluorescent protein; CFP: cyan fluorescent protein.

Live cell imaging could recently provide evidence that M- and G-CSF can instruct the lineage choice of bipotent uncommitted GMPs (Rieger et al. 2009). Similarly, live cell imaging can be used to dissect the pleiotropic actions M- and G-CSF exert on their target cells. Both cytokines affect many different cell fates that can be impossible to distinguish

when snapshots of bulk cultures are analyzed. In contrast, live cell imaging allows simultaneous detection and quantification of single cytokine-influenced parameters at the single cell level (Figure 3-3). For example, previous studies determined M-CSF-mediated proliferation effects by looking at population cell counts at several timepoints. However, in contrast to live cell imaging, these approaches cannot distinguish whether altered cell numbers resulted from changed cell death frequencies or from modulated cell cycle times, whose individual effect on total cell output cannot be determined by discontinuous, snap-shot analysis (Figure 3-6a). Moreover, live cell imaging allows quantification of the heterogeneous response of cells to cytokines (e.g. regarding the timepoint of commitment, differentiation, or morphological changes), generating valuable information that can be used to e.g. predict cell fates.

4 Aim of Thesis

Aim of this thesis was to elucidate signaling pathways involved in transmitting M-CSF-instructed macrophage differentiation. To screen for candidate pathways, genetically modified MCSFRs lacking individual or several tyrosine residues ought to be analyzed in primary progenitor cells for their capability to transmit lineage-instructive signals. In order to simultaneously assess M-CSF-affected cellular parameters, continuous time-lapse imaging at the single cell level should be applied during these analyses. Complementing and confirming the MCSFR screening, small molecule inhibitors and loss and gain of function mutants of signaling molecules should be used to manipulate MCSFR downstream signaling. Finally, microarray analysis should be carried out to detect differences in M- and G-CSF-mediated gene expression during lineage commitment, potentially revealing differential mechanisms of lineage instruction between the two cytokines.

5 Material and Methods

5.1 Molecular biology

5.1.1 Plasmids

Construct(s)	Origin
MCSFR mutants	(Yu et al. 2008, 2012)
Akt constitutive active (CA)	(Orsulic et al. 2002)
Akt dominant negative (DN)	(Zhou et al. 2000)
Fyn CA, Lyn CA, c-Src CA	(Cai et al. 2011)
Hck CA	(Scholz et al. 2000)
Src DN	Plasmid 13657 (www.addgene.org)
p65	(Lee et al. 2009)
STAT3 CA, DN	(Bromberg et al. 1999)
pRRL.PPT.SFFV.IRES.VENUSnucmem.PRE (lentiviral backbone)	(Schambach et al. 2006)

5.1.2 Cloning strategies

Cloning strategies were pre-designed *in silico* using Clone Manager Professional 9 software (Scientific & Educational Software, Cary, USA) based on complete sequences of available plasmids.

5.1.3 Restriction digests and ligations

Restriction digests, ligations, generation of blunt-ended DNA fragments, and DNA dephosphorylation were conducted using enzymes and suitable buffers from either New England Biolabs (Ipswich, USA) or Thermo Fisher Scientific (Waltham, USA) according to manufacturer's instruction.

5.1.4 Polymerase Chain Reaction (PCR)

PCR primers and strategy including annealing and melting temperatures were designed with Clone Manager Professional 9 software. PCR was conducted according to manufacturer's instruction using either Taq polymerase from Thermo Fisher Scientific (Cat. Nr. EP0072, Waltham, USA) for animal genotypings or Advantage Polymerase 2 from Clontech (Cat. Nr. 639206, Mountain View, USA) for cloning purposes.

5.1.5 Agarose gel electrophoresis

DNA fragments from restriction digests and PCR products were separated on 0.7% to 1.5% agarose (Cat. Nr. 840004, Biozym, Oldendorf, Germany) gels prepared with 1xTAE-buffer composed of 40mM tris-(hydroxymetyhl)-aminomethane (TRIS) (Cat. Nr. 5429.3, Roth, Karlsruhe, Germany), 20mM acetic acid (Cat. Nr. 1000632511, Merck, Darmstadt, Germany) and 1mM ethylenediaminetetraacetate (EDTA) (Cat. Nr. 8043.2, Roth, Karlsruhe, Germany). Agarose gels were stained with 1% ethidium bromide solution (Cat. Nr. 2218.2, Roth, Karlsruhe, Germany) at a concentration of 6µl per 100ml agarose solution. DNA fragments were separated with voltages between 80V and 150V in 1xTAE-buffer.

5.1.6 Purification of DNA fragments

DNA fragments were excised from agarose gels and purified using the QIAquick Gel Extraction Kit (Cat. Nr. 28704, Qiagen, Hilden, Germany) according to manufacturer's

instruction. Purification of PCR products was carried out using the QIAquick kit (Cat. Nr. 28104, Qiagen, Hilden, Germany) according to manufacturer's instruction. DNA fragments were resuspended in H_2O bidest and DNA concentration was measured using a NanoDrop spectrophotometer nd-1000 (Thermo Scientific, Waltham, USA).

5.1.7 Transformation of bacteria

Chemical competent DH5α *Escherichia coli* bacteria were thawed on ice. 1-100ng of plasmid or ligation cocktail was added to the bacteria suspension followed by incubation on ice for 30min. After heatshock treatment of bacteria for 90s at 42°C and cooling on ice for 2min, bacteria were incubated in LB-medium at 37°C for 1h. Different dilutions of the bacteria suspension were plated on LB agar (Cat. Nr. 244520, Becton Dickinson, Franklin Lakes, USA) plates containing adequate antibiotics for selection of successfully transformed bacteria. LB agar plates were incubated overnight at 37°C.

5.1.8 Isolation of plasmid DNA

High copy plasmids from DH5α *Escherichia coli* bacteria were purified from 4ml overnight cultures using the QIAprep Spin Miniprep Kit (Cat. Nr. 27104, Qiagen, Hilden, Germany) according to manufacturer's instruction or a modified protocol replacing columns with an isopropanol (Cat. Nr. 6752.2, Roth, Karlsruhe, Germany) precipitation step. Purification from 200ml overnight cultures was performed using Qiagen Plasmid Maxi Kit (Cat. Nr. 12165, Qiagen, Hilden, Germany) according to manufacturer's instruction.

5.1.9 DNA ethanol precipitation

DNA solutions were mixed with 3M sodium acetate (10% of sample volume) (Cat. Nr. 6773.1, Roth, Karlsruhe, Germany), followed by addition of 100% ethanol (2x sample volume). Solutions were incubated at -20°C overnight and then centrifuged in a table-top centrifuge at maximum speed for 15minutes at 4°C. Supernatants were removed and DNA

pellets washed with ice-cold 70% ethanol. After another centrifugation step and removal of supernatants, pellets were air-dried and resolved in H_2O.

5.1.10 DNA sequencing

PCR reactions for sequencing were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Cat. Nr. 4337455, Applied Biosystem, Foster City, USA) according to manufacturer's instruction. After DNA ethanol precipitation, capillary sequencing was performed in the in-house sequencing facility on a 3730 DNA Analyzer (Applied Biosystem, Foster City, USA).

5.2 Generation of lentivirus

5.2.1 Virus production

Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentivirus was produced in human embryonic kidney (HEK) 293T cells. HEK cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Cat. Nr. 41966-029, Invitrogen, Karlsruhe, Germany) with 10% fetal calf serum (FCS) (Cat. Nr. S1900-500, Lot Nr. S05130S1900, Biowest SAS, Nuaillé, France). For each virus four 10cm dishes (Cat. Nr. 150350, Thermo Scientific, Waltham, USA) were seeded with $5x10^6$ HEK cells each. The next day plates were cotransfected with viral packing plasmids (2.5µg pRSV_rev, 5µg pMDLg_pRRE, and 1µg pMD2.VSV-g) and 10µg the plasmid containing the gene of interest (derived from pRRL.PPT.SFFV.GFP.PRE (Figure 5-1) (Schambach et al. 2006)) in 0.5ml 250mM calcium chloride ($CaCl_2$) HEPES-buffered saline (HBS) (Cat. Nr. CAPHOS, Sigma-Aldrich, Taufkirchen, Germany). Prior to transfection, the medium was replaced with DMEM, 10% FCS, 0.1mM non-essential amino-acids (Cat. Nr. 11140-035, Invitrogen, Karlsruhe, Germany), 1mM sodium pyruvate (Cat. Nr. S8636, Sigma-Aldrich, Taufkirchen, Germany), 20mM HEPES (Cat. Nr. 15630-056, Invitrogen, Karlsruhe, Germany), and 100U/ml and 100µg/ml penicillin/streptomycin (Cat. Nr. 15140122, Invitrogen, Karlsruhe, Germany). Two

days after transfection, the virus-containing medium from 4 dishes was collected, centrifuged at 400g at 4°C for 5min, and the supernatant filtered through a 0.2µm filter (Cat. Nr. 17805, Sartorius, Göttingen, Germany). Supernatant was then centrifuged at 50000g at 4°C for at least 1h. The supernatant was aspirated and pellets were resuspended in 200µl StemSpan serum free expansion medium (SFEM) (Cat. Nr. 09650, Stem Cell Technologies, Vancouver Canada), aliquoted in 10µl vials, and frozen at – 80 °C until further use.

Figure 5-1: Basic map of lentiviral backbone used.
The expression of the inserted gene of interest is driven by the spleen focus forming virus (SFFV) promoter. The fluorescent protein Venus serves as a reporter and can be used to FACS purify positively transduced cells. LTR: long terminal repeat; IRES: internal ribosomal entry site.

5.2.2 Virus titration

Viral titers were determined by infecting NIH-3T3 cells and quantifying transduced cells by flow cytometry via a fluorescent transduction marker. NIH-3T3 cells were cultured in DMEM/10% FCS and seeded at a density of $1.5x10^4$ cells per well of a 24-well plate (Cat. Nr. 131068, Thermo Scientific, Waltham, USA). The next day a 1:10 dilution series of lentivirus was prepared and cells were infected with each dilution in triplicates. Two days later, medium was removed, cells were trypsinized (Cat. Nr. 25300-054, Invitrogen, Karlsruhe, Germany), counted, and analyzed on a FACSCalibur or FACSAriaIII (Beckton Dickinson, San Jose, USA). The percentage of fluorescent marker positive cells was used for titer calculation.

5.2.3 Virus infection of preGMPs

Cells were sorted, counted, and cultured overnight in 100µl SFEM containing 100ng/ml stem cell factor (SCF) (Cat. Nr. 250-03), 100ng/ml Flt3L (Cat. Nr. 250-31L, both PeproTech, Hamburg, Germany) in a well of a 96-well plate. Lentivirus was then added at a

multiplicity of infection (MOI) of 50 – 100. Cells were incubated at 37°C for 48h before further usage.

5.3 Isolation and transplantation of primary murine cells

5.3.1 Mouse lines

All mice were housed in a specific pathogen free (SPF) environment and sacrificed by CO_2 asphyxiation. All mice used for experiments were 12-16 weeks old and included C57BL/6J wild type mice from the in-house breeding facility, LysM:EGFP mice (Faust et al. 2000), $MCSFR^{-/-}$ mice (Dai et al. 2002), $c\text{-}kit^{W41/W41}$ mice (Geissler et al. 1981), bcl2-overexpressing mice (Ogilvy et al. 1999), and intercrosses of those.

5.3.2 Isolation and transplantation of fetal liver cells

Fetal livers from $MCSFR^{-/-}$ E14.5 day embryos were isolated, pooled, and singularized by vigorous pipetting in PBS. Adult W41 mice were tail vein-injected with $2x10^7$ fetal liver cells resuspended in 200-400µl of PBS using insulin syringes (Cat. Nr. 9151133, B.Braun, Melsungen, Germany). Contribution of donor cells was analyzed in recipients' peripheral blood (collected from the tail vein) using flow cytometry.

5.3.3 Isolation of bone marrow cells

For analysis and sorting of mononuclear cells from murine bone marrow, femurs, tibiae, humeri, hip bones, and vertebrae from adult mice were isolated. Bones were crushed using mortar and pestle in cold PBS/2% FCS and filtered through a 40µm filter (Cat. Nr. 352340, Becton Dickinson, Franklin Lakes, USA) in a total volume of 50ml. The cell suspension was centrifuged at 400g at 4°C for 5min. The pellet was then resuspended in ACK erythrocyte lysis buffer (500µl/mouse) (Cat. Nr. 10-548E, Lonza, Basel, Switzerland)

and incubated at 4°C for 2min. After washing with PBS, cells were counted using a hemocytometer.

Alternatively, femurs and tibiae of adult mice were isolated, flushed with 6ml cold PBS and isolated from the interphase of a Histopaque-1083 Ficoll gradient (Cat. Nr., 10831, Sigma-Aldrich, Taufkirchen, Germany) after centrifugation for 15min at room temperature at 490g with minimal acceleration and no brake.

5.3.4 Staining of primary cells for flow cytometry

Freshly isolated cells were resuspended in FACS buffer (PBS, 10% FCS, 0.1% sodium azide (NaN_3) (Cat. Nr. S2002, Sigma-Aldrich, Taufkirchen, Germany), and 10mM EDTA) to a concentration of 10^8 cells per ml. For analysis and sorting of BM-HSPCs, 1µl per biotinylated lineage-antibody was added per 10^7 cells followed by incubation on ice for 20min. Lineage-antibodies included CD3 (clone: 17A2, Cat. Nr. 13-0031), CD19 (clone: eBio1D3 (1D3), Cat. Nr. 13-0193), B220 (clone: RA3-6B2, Cat. Nr. 13-0452), Gr1 (Cat. Nr. 13-5931), MacI (clone: M1/70, Cat. Nr. 13-0112), Ter119 (clone: TER-119, Cat. Nr. 13-5921) and CD41 (clone: eBioMWReg30 (MWReg30), Cat. Nr. 13-0411, all eBioscience, San Diego, USA). If cells were prepared for sorting, a magnetic depletion of lineage positive cells was performed: Cells were washed, centrifuged, and resuspended in FACS buffer to a concentration of 10^8 cells per ml. Streptavidin-coated magnetic beads (Cat. Nr. HP57.1, Roth, Karlsruhe, Germany) were added to the cells (2µl per 10^7 cells) and incubated on ice for 10min. Cells were then incubated on an EasySep magnet (Cat. Nr. 18001, Stem Cell Technologies, Vancouver, Canada) for 10min and lineage-negative cells were decanted. Cells were washed, counted, centrifuged, and resuspended in FACS buffer (100µl per mouse). Antibodies and streptavidin labeled with fluorescent dyes were added to the cells (2-3µl/10^7 cells) and incubated on ice for at least 30min. If the cells to be stained were already in culture, no lineage depletion was performed. If CD16/32 was stained, the antibody was added to the cells first to block unspecific binding of Fc fragments. Antibodies used for staining were CD105-PE (clone: MJ7/18, Cat. Nr. 12-1051-82), CD34-eFluor660 (clone: RAM34, Cat. Nr. 50-0341-82), c-kit-PE-Cy7 (clone: 2B8, Cat. Nr. 25-1171-82), Ter119-APC-eFluor780 (clone: TER-119, Cat. Nr. 47-5921-82), MacI-eFluor450 (clone: M1/70, Cat. Nr. 48-0112-82), F4/80-eFluor450 (clone: BM8, Cat. Nr. 48-4801-82, all eBiocience, San Diego,

USA), CD16/32-APC (clone: 93, Cat. Nr. 101326), CD150-PE (clone TC15-12F12.2, Cat. Nr. 115904), Sca-1-PB (clone: D7, Cat. Nr. 108120), Ly6G (clone: 1A8, Cat. Nr. 127608, all Biolegend, San Diego, USA), CD16/32-FITC (clone: 2.4G2, Cat. Nr. 553144), and CD16/32-PE (clone: 2.4G2, Cat. Nr. 553145, all Becton Dickinson, Franklin Lakes, USA). APC-eFluor780-labeled streptavidin (Cat. Nr. 47-4317-82, eBioscience, San Diego, USA) was used to stain biotin-labeled lineage markers.

After staining, cells were washed, centrifuged, resuspended in FACS Buffer (200-400 µl per mouse), and filtered through a 35µm filter (Cat. Nr. 352235, Becton Dickinson, Franklin Lakes, USA) into polypropylene round-bottom tubes (Cat. Nr. 352063, Becton Dickinson, Franklin Lakes, USA).

5.3.5 Flow cytometry and cell sorting

Flow cytometry and sorting was performed on a FACSAriaIII (Becton Dickinson, Franklin Lakes, USA) equipped with 405nm, 488nm, 561nm, and 633nm lasers using a 70µm nozzle and manually adjusted compensations required for multi-color staining. Cells were sorted in 'purity' or '4-way-sort' mode into 4°C cold SFEM. A sorting purity of ≥95% was confirmed by reanalysis of sorted populations. Flow cytometry data was analyzed using FACSDiva software version 6.1.3 (Becton Dickinson, Franklin Lakes, USA).

5.4 Cell culture of primary HSCPs

5.4.1 Liquid culture

FACS-purified HSPCs were cultured in SFEM containing either a permissive cytokine cocktail (100ng/ml SCF, 10ng/ml IL-3 (Cat. Nr. 213-13), 100ng/ml TPO (Cat. Nr. 315-14, all PeproTech, Hamburg, Germany), 2U/ml EPO (Cat. Nr. C-60023, Promokine, Heidelberg, Germany), 10%FCS) or instructive cytokines (20ng/ml M-CSF (Cat. Nr. 315-02) ± 20ng/ml G-CSF (Cat. Nr. 250-05, all PeproTech, Hamburg, Germany) ± 10%FCS). For inhibitor experiments, cells were starved in presence of inhibitors for 1h before adding

cytokines. Inhibitors used were Ly294002 (Cat. Nr. 440204, Merck, Darmstadt, Germany), SU6656 (Cat. Nr. 572636-500UG Merck Millipore, Darmstadt, Germany), PD0325901 (Cat. Nr. 04-0006, Stemgent, Cambridge, USA), SB203580 (Cat. Nr. 5633S, Cell Signaling Technology, Beverly, USA), and Nemo binding domain (NBD) inhibitory peptide (Cat. Nr. IMG-2000, Biomol, Hamburg, Germany).

5.4.2 Colony assay

FACS-purified cells were counted and seeded at suitable densities in 35mm dishes (Cat. Nr. 430165, Corning, Amsterdam, Netherlands) with MethoCult GF M3434 colony assay medium (Stem Cell Technologies, Vancouver, Canada) containing cytokines SCF, IL3, IL6, and EPO according to manufacturer's instruction. After 5-7 days, colonies with at least 30 cells were scored according to morphology of cells and colonies and live in-culture antibody staining.

5.4.3 Live in-culture antibody staining

Live antibody staining in liquid cultures or colony assays was performed as described (Eilken et al. 2011). Either commercially available (MacI-PE) or self-labeled antibodies were used (F4/80-AlexaFluor647). Antibodies were labeled using the AlexaFluor 647 Labeling Kit (Cat. Nr. A-20186, Invitrogen, Karlsruhe, Germany) according to manufacturer's protocol. Antibodies were added to cell cultures at a final concentration of 10 - 50ng/ml.

5.5 Cytospin

5.5.1 Cytospin

Up to 1ml of cell suspension was transferred to object slides captured with Hettich cytospin equipment (Cat. Nr 1662, 1668, 5280, Hettich, Tuttlingen, Germany). Slides were

centrifuged at 270g (Rotanta 460 R centrifuge (Cat. Nr. 5660, Hettich, Tuttlingen, Germany)) and room temperature for 3min. Supernatants were aspirated, the cyto-chamber removed, and slides centrifuged at 1100g for 1min.

5.5.2 Cytospin staining and analysis

Cytoplasm and granulae of cytospun cells were stained with May-Gruenwald-Solution (Cat. Nr. T863.1, Roth, Karlsruhe, Germany) for 4min. Cells were washed twice with H_2O bidest, and subsequently nuclei were stained with a 5% Giemsa-Solution (Cat. Nr. 1.09204.0500, Merck, Darmstadt, Germany) for 16min. Cells were washed 3 times with H_2O bidest, air-dried, and covered with Pertex mounting medium (Cat. Nr. PER20000, Medite, Burgdorf, Germany). Cytospins were analyzed using a 63x oil-immersion objective on an Axiovert 200M inverted microscope.

5.6 Immunofluorescence

For immunofluorescent staining, cells were seeded on poly-lysine (Cat. Nr. P8920, Sigma-Aldrich, Taufkirchen, Germany)-coated slides, fixed with 2% paraformaldehyde (Cat. Nr. 158127, Sigma-Aldrich, Taufkirchen, Germany) in PBS at room temperature for 10min and subsequently permeabilized by 0.2% Triton-X (Cat. Nr. T8787, Sigma-Aldrich, Taufkirchen, Germany) in PBS. Next, cells were incubated with blocking buffer (PBS/10% donkey serum (Dianova, Hamburg, Germany)) at room temperature for 1h. Incubation with primary antibodies was performed overnight in blocking buffer. After washing, cells were incubated with secondary antibody at room temperature for 1h. Slides were mounted with Vectashield containing DAPI (Cat. Nr. H-1200, Vector laboratories, Burlingame, USA) and analyzed using a confocal microscope (TCS SP5, Leica, Wetzlar, Germany). Cells were stained with an anti-phospho-Src antibody (1:100) (clone: 9A6, Cat. Nr. 05-677, Merck Millipore, Darmstadt, Germany) detected with a donkey-anti-mouse AlexaFluor555-conjugated secondary antibody (Cat. Nr. A-31570, Life Technologies, Carlsbad, USA).

5.7 Time-lapse imaging

5.7.1 Movie acquisition

Time-lapse movies were acquired on an inverted epifluorescence microscope (Axio Observer, Zeiss, Hallbergmoos, Germany) using AxioVision Software 4.9. A self-written macro (TAT) was used to control hardware equipment in AxioVision. Microscopes were enclosed in plexiglass housings, allowing cells to be constantly kept at 37°C through a Heating Unit XL S (Cat. Nr. 411857-9030-000, Zeiss, Hallbergmoos, Germany). Cells were directly supplied with pre-mixed gas consisting of 5% CO_2, 5% O_2 and 90% N_2 (Praxair, Düsseldorf, Germany). Images were acquired with a 10x Fluar objective (Cat. Nr. 440135-0000-000, Zeiss, Hallbergmoos, Germany) and a 0.5-1x TV-Adapter (Cat. Nr., 426113-0000-000 Zeiss, Hallbergmoos, Germany). The brightfield lightsource was a halogen lamp and images were acquired every 2-3min with an Axiocam HRm (Cat. Nr. 426511-9901-000, Zeiss, Hallbergmoos, Germany) camera at a resolution of 1388 x 1040 pixels in lossless TIF or PNG format. Fluorescent light was provided either by a HXP 120 light source (Cat. Nr. 423013-9010-000, Zeiss, Hallbergmoos, Germany) with an Osram HXP-R 120W/45C VIS bulb (Cat. Nr. 882772, Osram, Munich, Germany) or a SPECTRA X light engine (Lumencore, Beaverton, USA). Filters for excitation/emission of different fluorophores were 46 HE (Cat. Nr. 489046-9901-000, Zeiss, Hallbergmoos, Germany) for VENUS, 43 HE (Cat. Nr. 489043-9901-000, Zeiss, Hallbergmoos, Germany) for PE, and AHF Cy5 (Cat. Nr. F46-006, AHF Analysentechnik, Tübingen, Germany) for AlexaFluor 647. Excitation times were between 20 and 500ms. Hardware autofocus (Cat. Nr. 410133-0506-000, Zeiss, Hallbergmoos, Germany) was applied on brightfield images every 1-2h. Fluorescent images were acquired every 2-3h.

5.7.2 Tracking software

Time-lapse data was retrospectively analyzed using self-written tracking software TTT on Fujitsu Siemens (Munich, Germany) workstations. The software allows manual tracking of individual colonies at the single-cell level generating cell genealogies with exact information about several parameters, including division frequencies, cell cycle length, cell

death frequencies, or cell movement. Furthermore, onsets of marker expression as detected via live antibody staining as well as appearance of morphological features (e.g. adherence) can be manually annotated. Any cell with insecure identity (loss of single cell data) was excluded from analysis. For statistical analysis of TTT data, self-written software StaTTTs was used. StaTTTs allows the generation of tree and cell filters to quantify parameters annotated in TTT.

5.8 Gene expression analysis

5.8.1 Reverse transcriptase real-time PCR

Cells in culture were washed once with PBS and then lysed. Total RNA extraction and cDNA synthesis was performed using the RNeasy Plus Micro kit (Cat. Nr. 74034, Qiagen, Hilden, Germany) including digestion of remaining genomic DNA and the SuperScript III kit (Cat. Nr. 18080-051, Invitrogen, Karlsruhe, Germany), respectively, according to manufacturer's instruction. The PCR reaction was carried out using SYBR Green Master Mix (Cat. Nr. 4367659) on a QuantStudio 12k Flex system (both Applied Biosystems, Foster City, USA). Primers used were:

Gene:	Forward primer:	Reverse primer:
Hck	5-GTCCAGGTTCCTCCGAGATG-3	5-CCATCTGGTCTCCCTTCTGG-3
Fgr	5-ATTCGTCGCCCTGTACGACT-3	5-CTTTGGTGGTCTCGCTTTCC-3
Lyn	5-GCAAAGGCCAGTTCCTGAAT-3	5-CAGAAGCTGTCGCTCTGCAT-3
Src	5-CAGCAACAAGAGCAAGCCCA-3	5-TATTGACAATCTGCAGCCGC-3
Yes	5-TGGAGGAGCGTCTTCCTCAT-3	5-CATTGTCACCCCTCACCTCA-3
Fyn	5-GAGAGCGAAACCACCAAAGG-3	5-AGCCACACTTCAGCGAAACA-3
Yrk	5-CTCAAGCCCGAGAACATCCT-3	5-CAGATGTCGATGGCCAGGT-3
Lck	5-CCAGAACCAGGGAGAAGTGG-3	5-TGCAGCTGCTTCATGAGGTT-3
Blk	5-GGTCAGTGAGAAGGGCAAGG-3	5-CACAAAGTTGCTGGGCACAT-3
ß-actin	5-CGTGGGCCGCCCTAGGCACCA-3	5-TTGGCCTTAGGGTTCAGGGGGG-3

5.8.2 Microarray analysis

Microarray analyses were performed by Dr. Martin Irmler (Institute of Experimental Genetics, Helmholtz Center Munich) and analyzed with the help from Dr. Stavroula Skylaki (Research Unit Stem Cell Dynamics, Helmholtz Center Munich).

Total RNA was isolated employing the RNeasy Plus Micro kit (Cat. Nr. 74034, Qiagen, Hilden, Germany) including digestion of remaining genomic DNA. The 2100 Bioanalyzer (Agilent Technologies, Oberhaching, Germany) was used to assess RNA quality and only high quality RNA (RIN>7) was used for microarray analysis.

Total RNA (about 2ng) was amplified using the Ovation PicoSL WTA System V2 (Cat. Nr.: 3302) in combination with the Encore BiotinIL Module (both NuGEN, San Carlos, USA). 750ng of amplified cDNA was hybridized to Mouse Ref-8 v2.0 Expression BeadChips (Cat. Nr.: BD-202-0202, Illumina, San Diego, USA). Staining and scanning were done according to the Illumina expression protocol. Data was processed using the GenomeStudio V2010.1 software (gene expression module version 1.6.0) (Illumina, San Diego, USA) in combination with the MouseRef-8_V2_0_R3_11278551_A.bgx annotation file. The numbers of microarrays utilized for data analysis were as follows: no cytokine (2), M-CSF 2h (3), M-CSF 24h GFP_lo (3), M-CSF 24h GFP_hi (3), M-CSF 48h GFP (3), G-CSF 2h (3), G-CSF 24h GFP_lo (4), G-CSF 24h GFP_hi (3), G-CSF 48h GFP (2).

Microarray data analysis was performed in the R statistical environment using the lumi package (Du et al. 2008). Data pre-processing was performed using background subtraction, variance stabilizing transformation (VST) and robust spline normalization (RSN). Differentially expressed genes were identified using the limma package with fold-change (FC) ≥ 1.5 and FDR ≤ 0.05 (Smyth 2005). Differentially expressed genes (DEGs) in any two subpopulation comparison (2906 DEGs in total) were used for further analysis. Hierarchical clustering and K-means clustering was performed using Cluster 3.0 (de Hoon et al. 2004) and Java Treeview (Saldanha 2004) was used for visualization.

Gene lists were subjected to standard gene ontology (GO) term enrichment analysis through Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov) (Huang et al. 2009). For pathway finding, gene lists were analyzed with Genomatix Pathway System (GePS) (http://www.genomatix.de). To visualize gene expression changes over time, data was loaded into Grid Analysis of Time series Expression (GATE) software (MacArthur et al. 2010).

5.9 Statistical analysis

All statistical analyses were determined with Prism software (GraphPad Software Inc., La Jolla, USA). When data were assumed to meet normal distribution, an unpaired two-sided Student's t-tests was performed to determine the significance of the difference between means of two groups. When data was not normally distributed, a non-parametric Mann-Whitney U test was performed to detect significant differences in the distributions of two unmatched groups. The variance was similar between groups that were statistically compared. All data are plotted as mean±standard error of the mean (SEM) unless differently stated. The SEM indicates the precision of an estimated mean. The number (n) of biological repeats (samples obtained from experiments repeated on different days and starting from different mice) and single colonies/cells (in case of time-lapse movies) included in the final statistical analysis is indicated in each figure legend.

6 Results

6.1 Establishment of a system to study the role of individual MCSFR tyrosine residues in primary MCSFR-deficient GMPs

Numerous studies over the last decades have attempted to elucidate the functional role of individual MCSFR tyrosine residues using a variety of cellular systems and experimental approaches. Many of these studies have led to contradictory results regarding the function of single tyrosine residues and concomitant consequences on downstream cellular fates (Hamilton 1997a,b). These discrepancies most likely arose from the different, often artificial, model systems used and exemplify the importance to study MCSFR function in the correct cellular context. Therefore, we set out to establish a system allowing the study of single MCSFR tyrosine function in primary cells physiologically responding to M-CSF. GMPs are a bipotent, BM-resident progenitor population that during hematopoiesis gives rise to granulocytes and macrophages (Akashi et al. 2000), which is physiologically controlled by the cytokines G-CSF and M-CSF, respectively. Therefore, GMPs represent the appropriate cellular model system to study MCSFR structure/function. As GMPs endogenously express the MCSFR, we sought out to use MCSFR-deficient mice to set up the experimental system. However, the described MCSFR knock-out mouse model is neonatal lethal when bred onto the C57BL/6 mouse genetic background, which is the preferred background in hematologic research (Dai et al. 2002, Li et al. 2006). In order to obtain adult BM-derived $MCSFR^{-/-}$ cells of C57BL/6 background, we took advantage of White Spotting mice (Geissler et al. 1981). These lines of mice bear different spontaneous point mutations in the gene encoding for c-kit (the receptor for the cytokine SCF), resulting in partial loss of c-kit function and concomitant defects in sustained HSC self-renewal (Miller et al. 1997). Due to these defects, c-kit mutant mice accept syngenic wild type HSCs, allowing long term engraftment and amplification of donor cells without prior irradiation (Capel & Mintz 1989, Migliaccio et al. 1999). In this study we used $c\text{-}kit^{W41/W41}$ (W41) mice, bearing a c-kit mutation of milder phenotype, which in contrast to more severe mutations allows homozygous breeding.

To test W41 mice as a potential recipient for $MCSFR^{-/-}$ cells, we transplanted W41 mice intravenously with $2x10^7$ unfractionated E14.5 fetal liver cells (corresponding to one fetal liver) of $MCSFR^{-/-}$ or wild type (littermate control) genotype. Engraftment was

measured bi-weekly in peripheral blood by flow cytometry. Donor cells (CD45.2) can be distinguished from recipient cells (CD45.1) by detecting the allelic variants CD45.1/CD45.2 in flow cytometry using variant-specific antibodies (Figure 6-1a).

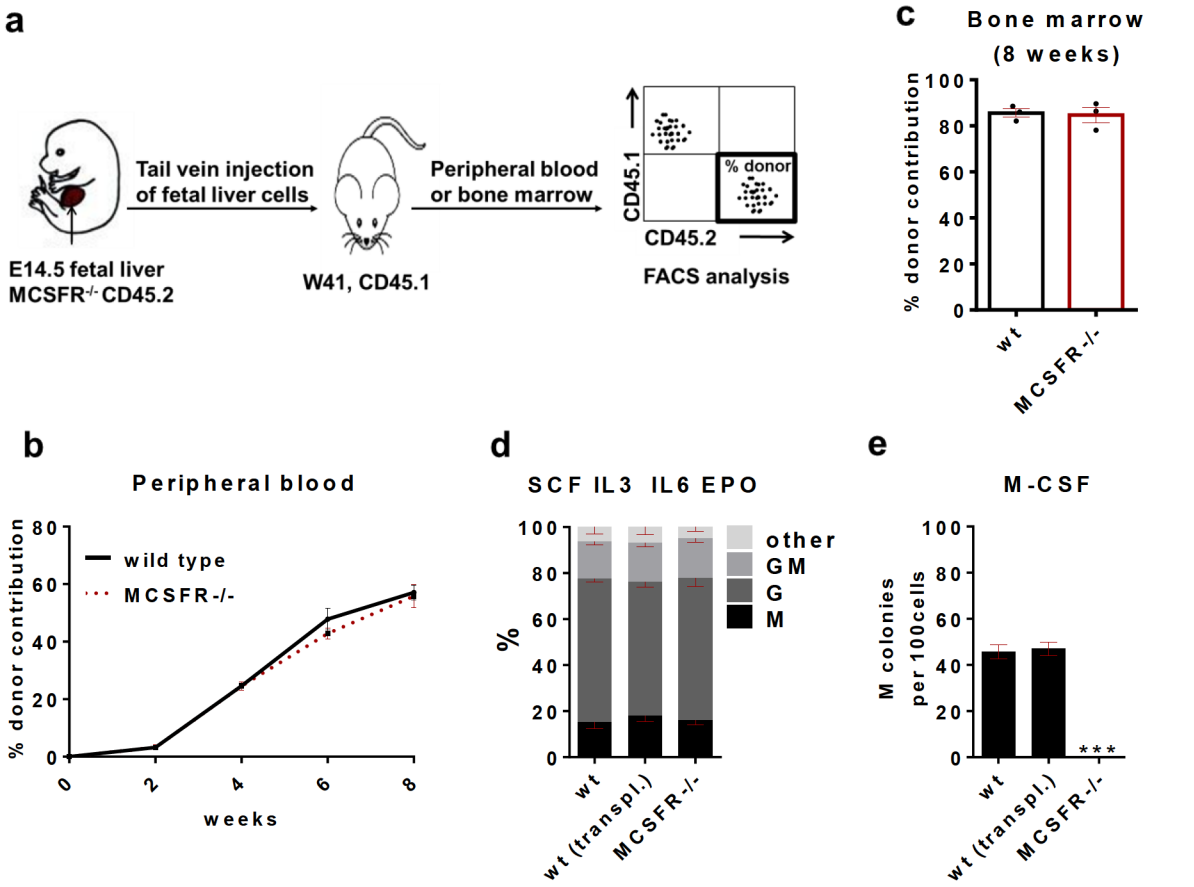

Figure 6-1: Transplantation strategy to obtain BM-derived MCSFR$^{-/-}$ progenitors.
(a) Experimental scheme. W41 (CD45.1) mice were transplanted intravenously with 2x10^7 E14.5 fetal liver cells of wild type or MCSFR$^{-/-}$ (CD45.2) genotype. Donor contribution was checked at various timepoints in recipients' peripheral blood or bone marrow via flow cytometry. (b) Donor contribution to peripheral blood shows no difference between wild type and MCSFR$^{-/-}$ donor cells, regarding both degree and kinetics (n=3-6 per timepoint per genotype). Data points represent mean±SEM. (c) Donor contribution to bone marrow myeloid progenitors 8 weeks post transplantation shows no difference in the degree of contribution between wild type and MCSFR$^{-/-}$ cells (n=3 per genotype). (d+e) Freshly isolated GMPs from C57Bl/6 (wt), W41 transplanted with wild type fetal liver cells (wt (transpl.)), or W41 transplanted with MCSFR$^{-/-}$ fetal liver cells (MCSF$^{-/-}$) were cultured in colony assay medium containing either (d) SCF, IL3, IL6, and EPO (M3434) or (e) M-CSF. After 5-7 days, colonies were enumerated and identified according to morphology (n=3 per genotype per condition). Bars represent mean±SEM. An unpaired Student's t-test was used to determine the significance of the difference between means of two groups. *<0.05, **<0.01, ***<0.001 compared to wt.

Peripheral blood contribution increased steadily over time irrespective of donor genotype. Eight weeks post transplantation, donor contribution reached 57.1±2.6% for wild type and 55.8±4% (mean±SEM) for MCSFR-/- (Figure 6-1b). Furthermore, there was no difference in the kinetic of contribution when comparing wild type and MCSFR$^{-/-}$ donor fetal liver cells, demonstrating that the lack of the MCSFR is not detrimental for the engraftment

and differentiation of HSPCs. Recipients were sacrificed after eight weeks to check donor contribution in the BM. Similar to donor contribution in peripheral blood, no difference was detected when comparing wild type (85.5±1.9%) with MCSFR$^{-/-}$ (84.7±3.4%, mean±SEM) donor contribution to myeloid progenitors in the BM (Figure 6-1c). Contribution to BM populations at timepoints later than eight weeks only increased marginally (data not shown). MCSFR$^{-/-}$ GMPs purified from W41 mice had the same lineage potential as wild type GMPs derived from either C57BL/6 mice or from W41 transplanted with wild type fetal liver cells, as assessed by permissive (i.e. all lineages a given cell can potentially differentiate to are supported) colony assays (M3434-medium, containing SCF, IL3, IL6, and EPO) (Figure 6-1d). This demonstrates that the transplantation *per se* has no influence on the generation of GMPs. As expected, MCSFR$^{-/-}$ GMPs purified from W41 were unable to form colonies in M-CSF colony assays (Figure 6-1e).

In summary, transplantation of W41 mice with MCSFR$^{-/-}$ fetal liver cells proved to be a suitable approach to obtain BM-derived MCSFR$^{-/-}$ progenitors of C57BL/6 genetic background.

GMPs are a transient BM population and quickly differentiate into monopotential cells. This makes it difficult to genetically manipulate GMPs before differentiation to study the biological effects of ectopically expressed genes. We therefore set out to establish an *in vitro* differentiation system that would give rise to GMPs and at the same time allow time for transgene expression. To this end, preGMPs, an immediate precursor population of GMPs (Pronk et al. 2007), were FACS purified and cultured in SCF and Flt3L (Figure 6-2a, b) to allow time for genetic manipulation during the generation of GMPs *in vitro*. As a control, GMPs were cultured in the same cytokine conditions. When reanalyzing the cells after three days in culture, preGMPs (originally sorted as CD16/32^{lo} MacI^{neg}) gave rise to a CD16/32^{hi} MacI^{neg} population, which is immunophenotypically identical to BM-derived GMPs (Figure 6-2b). In contrast, cultured GMPs retained high CD16/32 expression, and virtually all cells expressed MacI (CD11b), an early marker for myeloid differentiation. Repeating the experiments with LysM:EGFP mice (Faust et al. 2000) revealed that 30% of preGMP-derived CD16/32^{hi} MacI^{pos} cells expressed the lineage commitment marker lysozyme M (Rieger et al. 2009), while none of the CD16/32^{hi} MacI^{neg} cells did (Figure 6-2b). CD16/32^{hi} MacI^{neg} cells therefore represent uncommitted progenitors. Consequently, we decided to exclude MacI^{pos} cells in order to minimize the risk of having already pre-committed cells in the sorted

population. The CD16/32^{hi} MacI^{neg} population was termed "in-vitro derived GMPs" (ivGMPs).

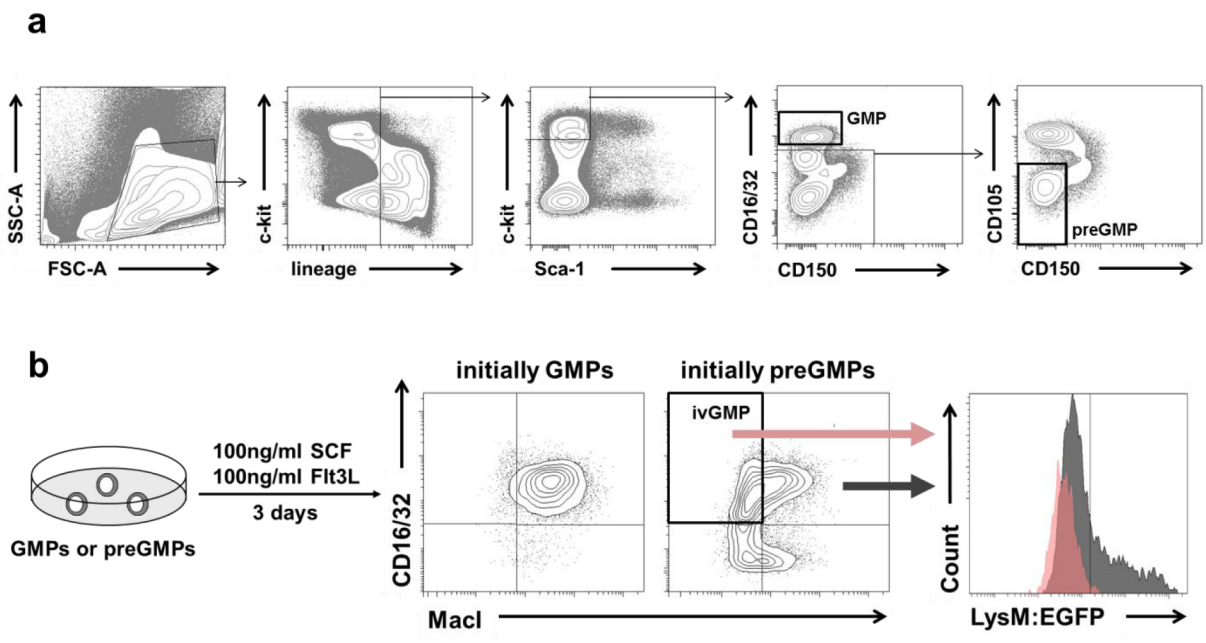

Figure 6-2: In vitro derivation of GMPs (ivGMPs) from BM-derived preGMPs.
(a) FACS gating scheme to sort BM-derived GMPs and preGMPs. GMPs are gated as lin^{neg} c-kit^{pos} Sca-1^{neg} CD16/32^{hi}, preGMPs are gated as lin^{neg} c-kit^{pos} Sca-1^{neg} CD16/32$^{neg/lo}$ CD105^{neg} CD150^{neg}. (b) GMPs or preGMPs were cultured in presence of 100ng/ml SCF and Flt3L for 3 days and then analyzed for CD16/32 and MacI expression via flow cytometry. CD16/32^{hi} MacI^{neg} ivGMPs are LysM-negative and therefore uncommitted. Representative FACS plots are shown.

To assess the lineage potential of ivGMPs, cells were FACS purified and compared side by side with freshly isolated BM-derived GMPs in liquid or semi-solid cultures with different cytokine mixtures. Lineage output was determined after four to five days by flow cytometry (Figure 6-3a, macrophage (M) = MacI^{pos} F4/80^{pos} Ly6G^{neg}, granulocyte (G) = MacI^{pos} F4/80^{neg} Ly6G^{pos}) or by scoring colonies (Figure 6-3b). In permissive liquid culture condition, containing serum, SCF, IL3, TPO, and EPO, GMPs and ivGMPs produced similar proportions of M (22±0.4% and 24.9±1.6%, respectively) and G (47.4±7.2% and 43.8±6.4%, respectively (mean±SEM)). Likewise, lineage output of GMPs and ivGMPs cultured in serum, M-CSF, and G-CSF was similar (M: 34.6±0.9% and 37.1±3.6%, respectively; G: 37.7±3.3% and 35.1±1.6%, respectively (mean±SEM)) (Figure 6-3a). Additionally, lineage output in permissive colony assays was comparable for GMPs and ivGMPs (M: 15.6±2% and 17±5%, respectively; G: 60.5±2.3% and 54.5±6.1%, respectively; GM: 14.2±0.8% and 17±1%, respectively (mean±SEM)) (Figure 6-3b). Collectively, the lineage potential of ivGMPs and GMPs was comparable in all conditions tested, indicating that ivGMPs are

identical to GMPs in all relevant aspects tested. This makes ivGMPs an appropriate cellular model system to study effects of overexpressed proteins in a normal bipotent cellular context.

Successfully setting up the transplantation approach to obtain BM derived C57BL/6 MCSFR$^{-/-}$ progenitors in combination with the *in vitro* generation of GMPs was a prerequisite to properly analyze MCSFR function described in the following section.

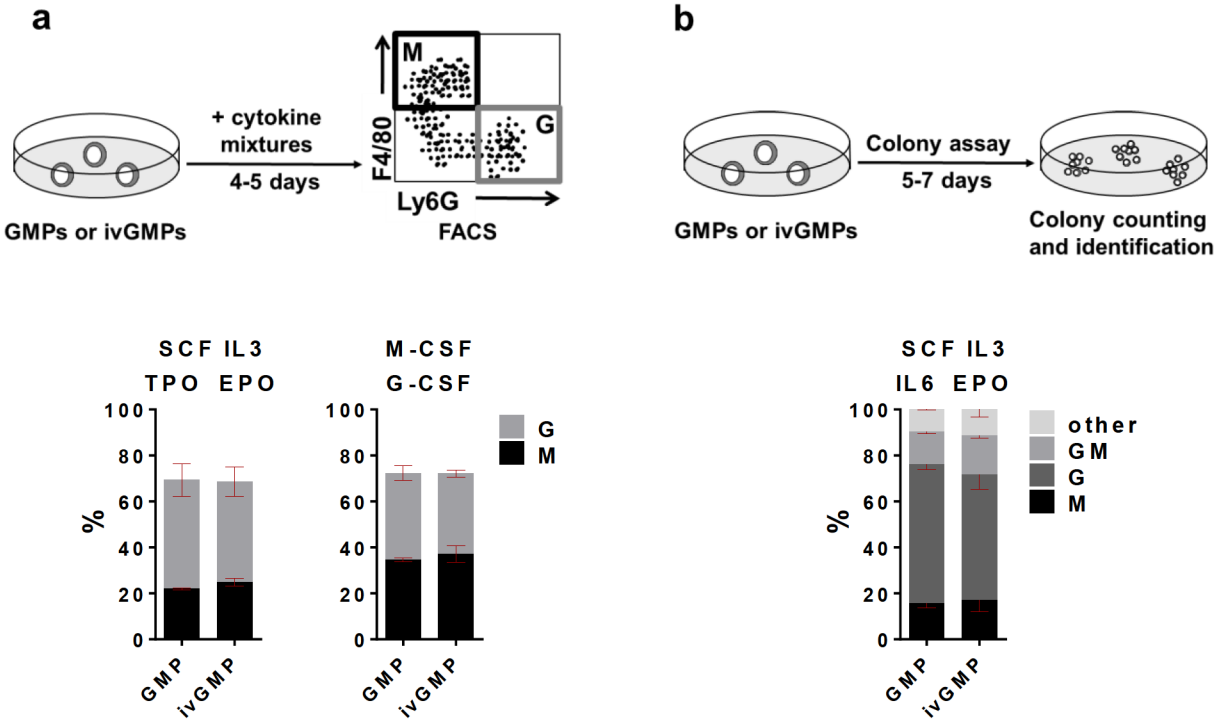

Figure 6-3: GMPs and ivGMPs have similar lineage potential.
(a) Freshly isolated GMPs ivGMPs were cultured in either SCF, IL3, TPO, EPO, 10%FCS or M-CSF, G-CSF, 10%FCS and analyzed for macrophage (MacI^{pos} F4/80^{pos} Ly6G^{neg}) and granulocyte (MacI^{pos} F4/80^{neg} Ly6G^{pos}) output via flow cytometry after 4-5 days (n=3-6). (b) Fresh BM-derived GMPs and ivGMPs were cultured in colony assay medium (M3434 containing SCF, IL3, IL6, and EPO). After 5-7 days, colonies were enumerated and identified according to morphology (n=3). Bars represent mean percentage±SEM. An unpaired Student's t-test did not detect a significance difference between means of the two groups.

6.2 Investigating the role of individual MCSFR tyrosine residues in M-CSF-mediated cell fates

The MCSFR has eight described functional tyrosine residues that upon M-CSF binding and receptor dimerization are auto-transphosphorylated to initiate several downstream signaling pathways that mediate the pleiotropic actions of M-CSF. Other phosphorylated sites in the MCSFR activating signaling pathways have so far not been

described, which is why we focused on the eight known functional tyrosine residues. To gain insight into the function of individual MCSFR tyrosines, we re-introduced MCSFR constructs lacking individual or several tyrosine residues (Yu et al. 2008) into MCSFR$^{-/-}$ preGMPs by lentiviral transduction and analyzed how the lack of tyrosines affects M-CSF-mediated differentiation of ivGMPs (Figure 6-4a). We chose to do so by time lapse imaging, which allows simultaneous detection and quantification of several cell fate parameters influenced by M-CSF, including cell death, proliferation, and macrophage differentiation.

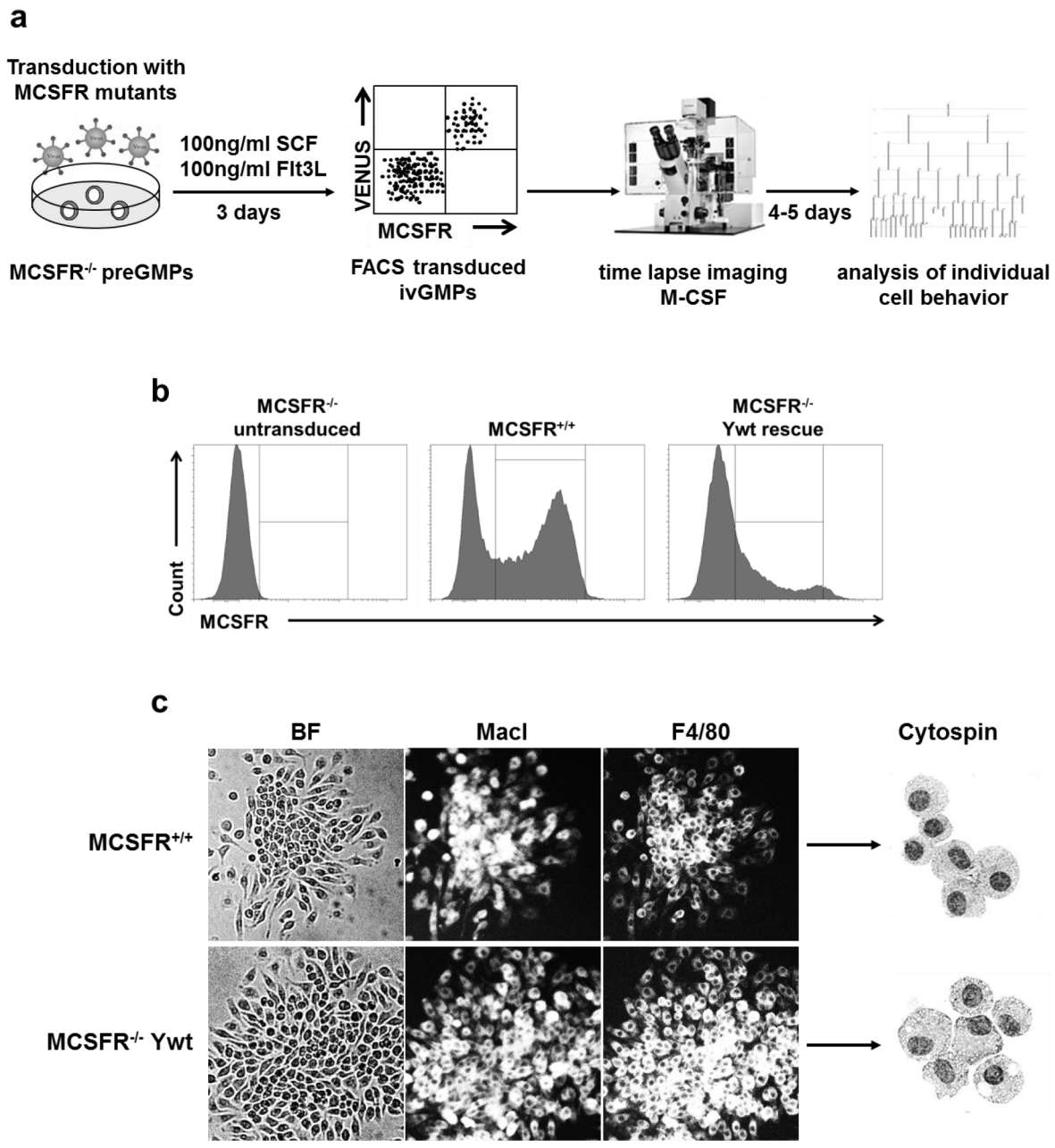

Figure 6-4: Experimental strategy to produce and analyze MCSFR$^{-/-}$ ivGMPs rescued with MCSFR mutants.

(a) Eight weeks post transplantation of W41 mice with MCSFR$^{-/-}$ fetal liver cells (according to Figure 6-1), donor-derived MCSFR$^{-/-}$ preGMPs were FACS purified and transduced with lentiviruses (Figure 5-1) encoding MCSFR mutants lacking none (Ywt) or individual to all (YEF) tyrosine residues. Three days post infection, successfully transduced ivGMPs were isolated by FACS by means of an anti-MCSFR (CD115) antibody and the lentiviral Venus reporter. Sorted cells were then time-lapsed imaged during M-CSF-mediated differentiation and retrospectively analyzed using self-developed software. (b) Representative FACS plots showing MCSFR expression on untransduced MCSFR$^{-/-}$ (used for setting the negative gate), wild type (used for setting the positive gate according to endogenous levels), and MCSFR$^{-/-}$ cells rescued via lentiviral transduction. (c) MCSFR$^{+/+}$ cells and MCSFR$^{-/-}$ cells rescued with the wild type receptor (Ywt) generate MacI^{pos} F4/80^{pos} macrophage colonies in response to M-CSF (20ng/ml). Pictures are representative snapshots from a time-lapse experiment. Cytospins confirmed macrophage morphology (representative pictures are shown).

MCSFR$^{-/-}$ ivGMPs were generated according to Figure 6-1 and 6-2. Successfully transduced ivGMPs were FACS purified using an anti-MCSFR antibody and a fluorescent marker included in the lentiviral construct. To avoid potential artifacts caused by receptor overexpression, only transduced cells with a similar MCSFR expression level as littermate-derived MCSFR$^{+/+}$ cells were sorted (Figure 6-4b). Transducing MCSFR$^{-/-}$ cells with the wild type MCSFR (Ywt) restored their capability to form M-CSF-induced macrophage colonies in liquid culture and methylcellulose, demonstrating that M-CSF-mediated survival, proliferation, and differentiation could be rescued (Figure 6-4c, 6-5, and data not shown). Of note, the high survival and differentiation rates of C57BL/6 ivGMPs (Figure 6-5b, c, d) demonstrate that the MCSFR antibody (clone AFS98 (Sudo et al. 1995)) used for sorting transduced ivGMPs carrying endogenous levels of MCSFR is neither detrimental to cell survival nor colony formation.

Next, MCSFR$^{-/-}$ ivGMPs rescued with different MCSFR single tyrosine mutants (Figure 6-5a) were exposed to M-CSF and time-lapse imaged for several days during their differentiation. Using self-developed software, colonies were retrospectively tracked and their M-CSF-induced behavior analyzed at the single cell level (Figure 6-4a).

First, we looked at the contribution of individual MCSFR tyrosine residues to M-CSF-mediated survival and proliferation. As GMPs are usually committed after two consecutive divisions (Rieger et al. 2009), we quantified the percentage of dying and proliferating cells in the first two generations (Figure 6-5b, c). As expected, virtually all cells reconstituted with a MCSFR lacking all eight tyrosine residues (YEF) died within the first two generations. In contrast, cells rescued with the wild type MCSFR (Ywt) were not significantly different in terms of survival and proliferation rates as compared to C57BL/6 wild type control cells (n.s., Student's t-test). This demonstrates that the rescue of MCSFR$^{-/-}$ cells by reintroducing MCSFR-variants is principally feasible and functionally efficient during a time window in which lineage commitment usually occurs. Mutation of single

tyrosine residues had varying effects on colony survival, but only those of Y706, Y721, and Y807 resulted in significantly lower survival and proliferation as compared to Ywt ($p<0.05$, Student's t-test) (Figure 6-5b, c). Although being quite heterogeneous in general, none of the single tyrosine mutated receptors drastically influenced the average cell cycle times of non-adherent (i.e. non-macrophages) cells as compared to Ywt and C57BL/6 controls (Figure 6-5e). Moreover, the frequency distribution of cell cycle times was not significantly different compared to Ywt (n.s., Mann-Whitney U test).

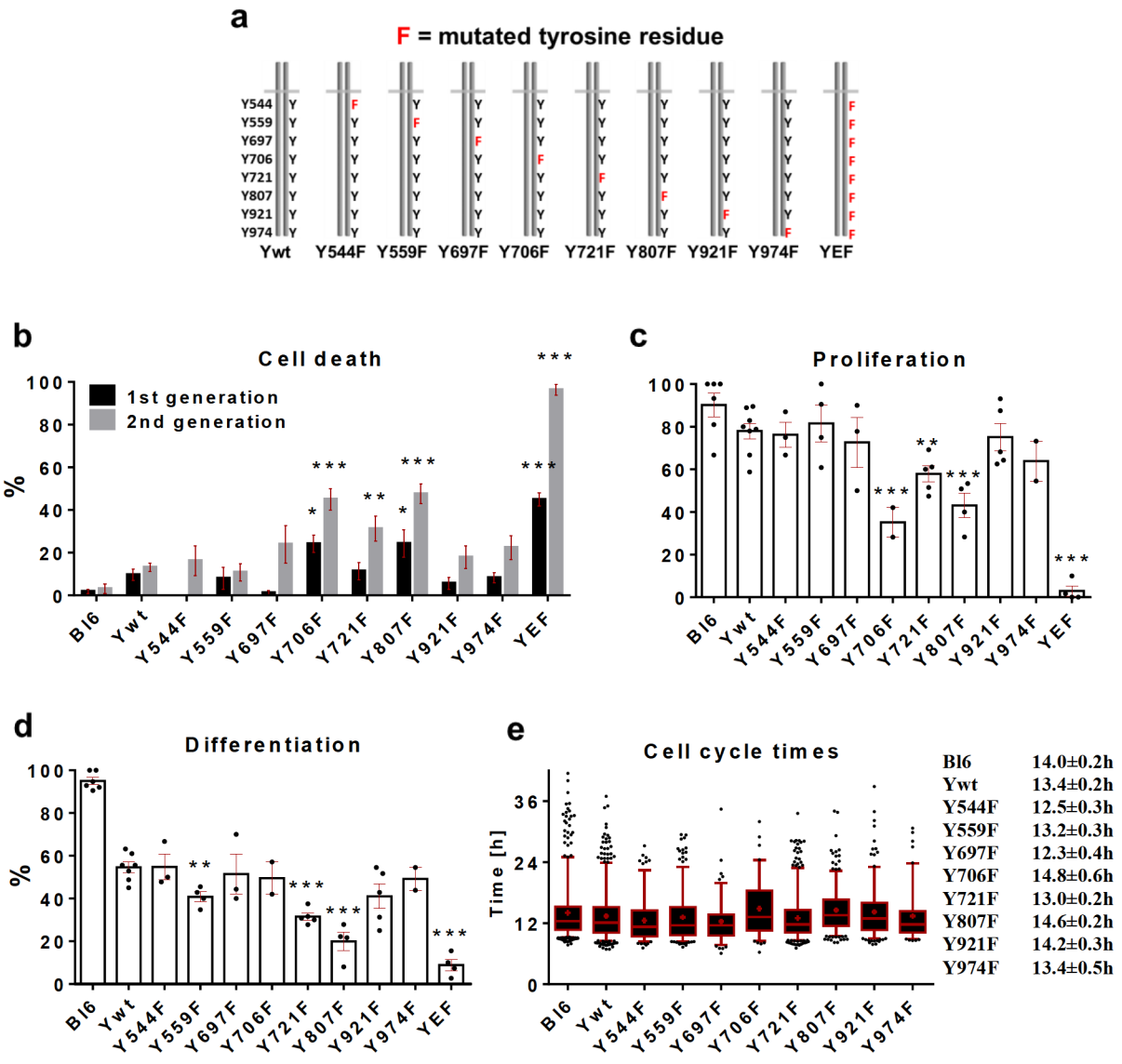

Figure 6-5: Time lapse imaging reveals contribution of single MCSFR tyrosines to M-CSF-mediated effects.
(a) MCSFR mutants analyzed. Ywt served as a positive control, YEF as a negative control. Other Y→F mutants lack indicated single tyrosine residues. (b) Percentage of cells dying within the first two generations, after which cells are usually committed. (c) Proliferating colonies were quantified as starting cells that underwent at least two consecutive cell divisions. (d) Differentiating colonies were scored as colonies that gave rise to $MacI^{pos}$ $F4/80^{pos}$ adherent cells. Bars represent mean±SEM. Each data point represents one movie. Total movies/colonies analyzed: Bl6 (6/194), Ywt (7/246), Y544F (3/57), Y559F (4/77), Y697F (3/54), Y706F (2/49), Y721F (5/156),

Y807F (4/178), Y921F (5/131), Y974F (2/58), YEF (4/147). An unpaired Student's t-test was used to determine the significance of the difference between means of two groups. *<0.05, **<0.01, ***<0.001 compared to Ywt. (e) Cell cycle times of non-adherent cells. The 1st generation was omitted due to unknown cell cycle length before the start of imaging. Box plots represent 5-95% confidence intervals (whiskers), population medians (horizontal line), and population means (+). Average cell cycle times (hours±SEM) are written. Total movies/cells analyzed: Bl6 (6/602), Ywt (7/616), Y544F (3/155), Y559F (4/292), Y697F (3/113), Y706F (2/101), Y721F (5/453), Y807F (4/314), Y921F (5/263), Y974F (2/104). A Mann-Whitney U test did not detect significant differences in the frequency distribution of cell cycle times compared to Ywt.

Next, we looked at the efficiency of single tyrosine-mutated MCSFRs to rescue macrophage differentiation. Macrophages were scored as cells that adhered and expressed the markers MacI and F4/80 as detected by live in-culture antibody staining (Eilken et al. 2011) (Figure 6-4c). Notably, all $MCSFR^{-/-}$-derived ivGMPs produced less macrophage colonies than the C57BL/6 control, irrespective of which receptor was re-introduced (including Ywt) (Figure 6-5d). Colonies that did not form macrophages either died prior to differentiation, or produced cells that were not classified as macrophages according to the above mentioned criteria (data not shown). Compared to Ywt, the mutation of Y559, Y721, and Y807 resulted in significantly fewer macrophage colonies (75%, 58%, and 37% of Ywt level, respectively ($p<0.05$, Student's t-test)).

As mutation of Y807 led to a marked reduction of survival, it is difficult to tell whether a potential differentiation defect might be masked through cells dying before being able to differentiate. To circumvent this, we used a bcl2-overexpressing mouse line (Ogilvy et al. 1999) that we crossed into the MCSFR-deficient background. Bcl2 is an anti-apoptotic protein whose overexpression can prolong cytokine-independent survival of cells (Ogilvy et al. 1999). Bcl2-overexpressing $MCSFR^{-/-}$ ivGMPs rescued with the Y807F mutant survived comparably to the Ywt rescue. However, most cells carrying the Y807F MCSFR were still incapable to differentiate into macrophages, indicating that Y807 is indeed required for both survival and differentiation (data not shown). Furthermore, Y807-transduced cells mainly produced colonies that resembled granulocytic cells (data not shown).

In summary, the varying effects single MCSFR Y→F mutants had on survival, proliferation, and differentiation indicate that individual tyrosines are functionally involved in regulating these cell fates in primary GMPs. However, none of the individual Y→F mutants totally abrogated survival or differentiation, pointing towards high functional overlap among the single tyrosine residues.

In similar experiments, YEF add-back (AB) mutants were analyzed. In these mutants, all eight tyrosine residues are mutated *but* the one or two indicated in the name (Figure 6-6a). Single tyrosine AB mutants partially rescued M-CSF-mediated survival when compared to

the full mutant YEF. Yet, in all cases, over 50% of cells died within the second generation (Figure 6-6b). Compared to the levels of Ywt, adding back single Y544, Y721, or Y807 partially rescued M-CSF-mediated proliferation to 12%, 37%, or 26% of Ywt, respectively (Figure 6-6c). However, adding back single Y544, Y721, or Y807 did not significantly rescue macrophage differentiation compared to YEF (n.s., Student's t-test) (Figure 6-6d). Adding back single Y559 improved proliferation and differentiation to 64% and 50% of Ywt level, respectively, indicating that this tyrosine residue plays a major role in transmitting proliferation- and differentiation-inducing signaling.

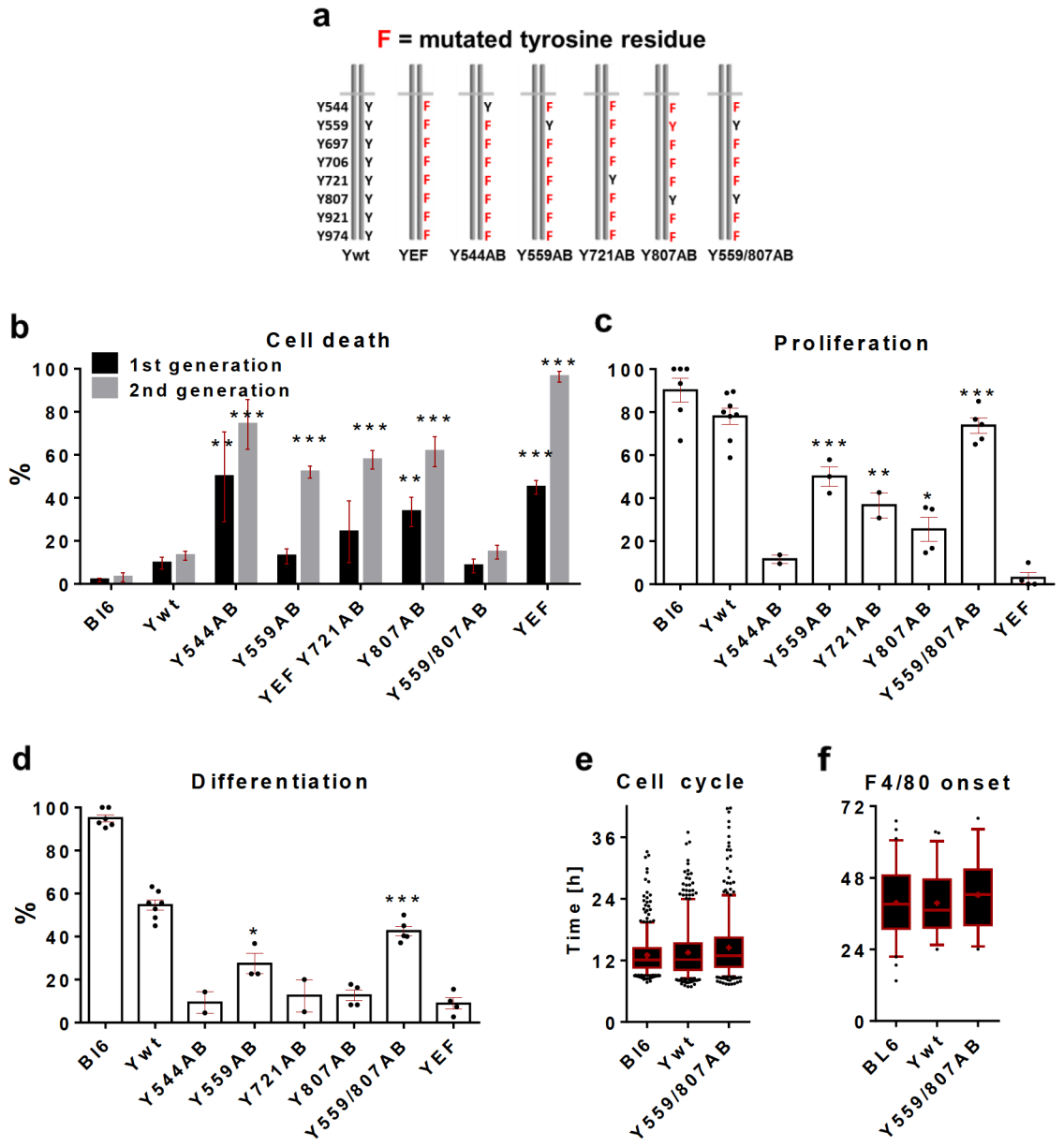

Figure 6-6: Time-lapse imaging of add-back mutans reveals Y559 to be sufficient for M-CSF-mediated effects.

(a) MCSFR add-back (AB) mutants analyzed. Ywt and YEF served as positive and negative control, respectively. AB mutants lack all but the indicated tyrosine residue(s). (b) Percentage of cells dying within the first two generations, after which cells are usually committed. (c) Proliferating colonies were quantified as starting cells that underwent at least two consecutive cell divisions. (d) Differentiating colonies were scored as colonies that gave rise to $MacI^{pos}$ $F4/80^{pos}$ adherent cells. Bars represent mean±SEM. Each data point represents one movie. Total movies/colonies analyzed: Bl6 (6/194), Ywt (7/246), Y544AB (2/69), Y559AB (3/139), Y721AB (2/67), Y807AB (4/168), Y559/807AB (5/179), YEF (4/147). An unpaired Student's t-test was used to determine the significance of the difference between means of two groups. *<0.05, **<0.01, ***<0.001 compared to Ywt (b) or YEF (c+d). (e) Cell cycle times of non-adherent cells. The 1st generation was omitted due to unknown cell cycle length before the start of imaging. Total movies/cells analyzed: Bl6 (6/602), Ywt (7/616), Y559/807AB (5/502). (f) Onset of F4/80 expression as detected via live in-culture antibody staining. Total movies/cells analyzed: Bl6 (4/79), Ywt (4/54), Y559/807AB (3/35). Box plots represent 5-95% confidence intervals (whiskers), population medians (horizontal line), and population means (+). A Mann-Whitney U test did not detect significant differences in the frequency distribution of cell cycle times and F4/80 onsets compared to Ywt.

Adding back Y807 in addition to Y559 (Y559/807AB) almost completely restored Ywt levels of survival, proliferation (to 95%), and differentiation (to 78%). Furthermore, cells rescued with Y559/807AB had similar cycling times as cells rescued with Ywt (13.4±0.2 for Ywt and 14.5±0.3h for Y559/807AB (mean±SEM)) (Figure 6-6e) and the macrophage differentiation kinetics, read out by onset of F4/80 expression, did not differ significantly (n.s., Mann-Whitney U test) (Figure 6-6f).

Using the aforementioned bcl2 overexpressing mouse background to prolong cytokine-independent survival confirmed that Y807 or Y721 alone were not sufficient for M-CSF-mediated differentiation (data not shown).

Taken together, while single mutations proved to be limited in assessing the function of individual tyrosine residues, adding back tyrosines to a complete mutant background revealed Y559 to be sufficient for M-CSF-mediated survival and macrophage differentiation. The addition of Y807 alongside Y559 added to this effect, while by itself Y807 had little rescue capacity, which is in line with its proposed role in structural activation of the MCSFR rather than activating cell fate-mediating signaling pathways.

6.3 Manipulating MCSFR signaling using small molecules

The above described receptor mutant screening indicated an important role for Y559 in M-CSF-induced differentiation and hence indirectly suggests that the lineage-instructive signal can be transmitted via the same tyrosine. Y559 is well described to activate members of the SFKs, which in turn can activate Akt and MAPK signaling. With this insight, we

sought out to manipulate signaling downstream of the MCSFR by using small molecule inhibitors in order to identify pathways involved in lineage instruction and differentiation. To that end, we cultured wild type BM-derived GMPs in instructive or permissive conditions, either in presence or absence of pathway inhibitors. A list of inhibitors and concentrations used can be found in Table 6-1. Cultures of cells were either analyzed by flow cytometry after 4-5 days (Figure 6-7a) or imaged continuously by time-lapse microscopy during their differentiation.

Table 6-1: Used inhibitor concentrations

Inhibitor	Target	Concentration
SU6656	SFK	2µM
Ly294002	PI3K	50µM
SB203580	p38	10µM
PD0325901	MEK	1µM
NBD	IKK	50µM

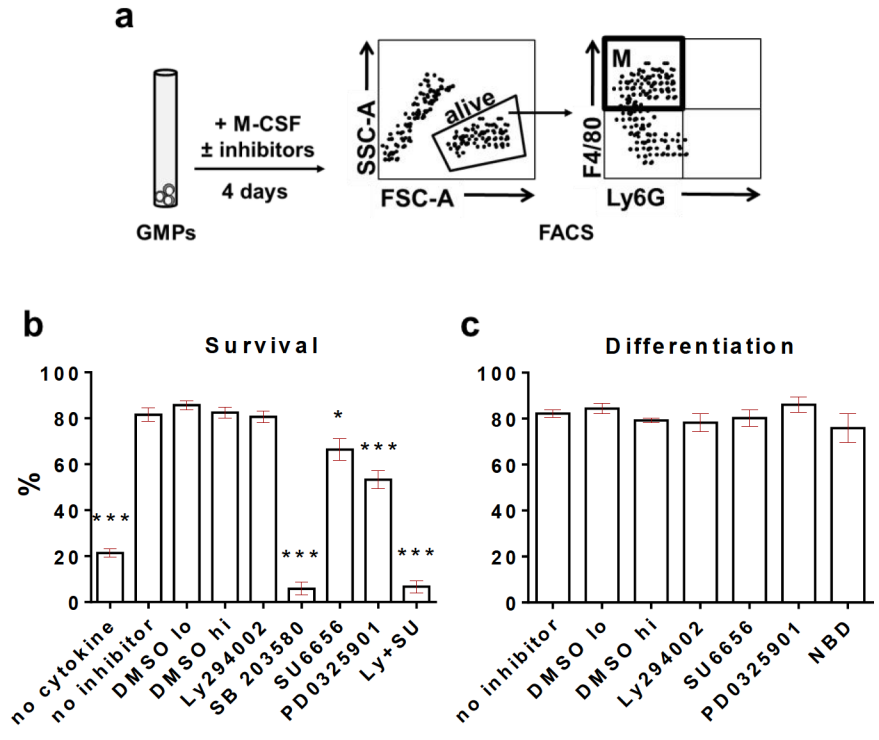

Figure 6-7: Inhibiting various signaling pathways does not block M-CSF-instructed differentiation.
(a) Experimental strategy. Freshly isolated GMPs were starved in presence of signaling pathway inhibitors for 1h before adding M-CSF. After 4 days of culture in 5ml FACS tubes, cells were analyzed by flow cytometry. The FSC/SSC gate was used to determine cell survival. Macrophage output was determined using macrophage marker F4/80. (b) Fraction of living cells after 4 days of culture, as determined via FSC/SSC gating. Note: survival of cells in presence of NBD could not be determined due to inhibitor precipitates falsifying the

FSC/SSC gate used to assess survival. (c) Fraction of F4/80^{pos} macrophages after 4 days of culture. Bars represent mean±SEM. (n=3-8 experiments). An unpaired Student's t-test was used to determine the significance of the difference between means of two groups. *<0.05, **<0.01, ***<0.001 compared to DMSO vehicle control.

Compared to M-CSF (no inhibitor) or DMSO vehicle control cultures, significantly fewer cells survived in presence of SFK (SU6656) or ERK (PD0325901) inhibitor (81% or 65% survival of DMSO vehicle control, respectively) ($p<0.05$, Student's t-test) (Figure 6-7b). Yet, in presence of these inhibitors surviving cells formed equal portions of macrophages compared to control cultures, indicating that lineage decision was not affected. Presence of a PI3K inhibitor (Ly294002) had no effect on M-CSF-mediated survival and differentiation. Inhibition of MAPK p38 abrogated cell survival, preventing assessment of macrophage differentiation. Virtually no cells survived when inhibiting PI3K and SFKs simultaneously. Blocking of NFκB signaling, a potential candidate pathways downstream of Akt, via NBD seemed to have no effect on differentiation.

To more directly assess the effect of cell signaling inhibitors on lineage choice, we made use of aforementioned LysM:EGFP mice, which express EGFP when cells commit to either the M or G lineage. Time-lapse movies of LysM:EGFP GMPs cultured in M-CSF in absence or presence of inhibitors SU6656 (SFKs) or NBD (NFκB) revealed no differences in cell survival or the fraction of cells committing and differentiating into macrophages, when compared to the DMSO vehicle control. Similarly, long-term imaging of LysM:EGFP GMPs cultured in both M-CSF and G-CSF either containing DMSO (vehicle control) or SU6656 (SFKs) revealed no inhibitor-dependent defect in survival frequency, lineage commitment, or differentiation into either macrophages or granulocytes (data not shown).

We repeated the inhibitor experiments in permissive culture conditions (Figure 6-8a). None of the tested inhibitors showed effects on the survival of GMPs in permissive cytokine condition (Figure 6-8b). Notably, this also held true for the p38 inhibitor and the PI3K/SFK inhibitor combination, demonstrating that the survival defects observed with these inhibitors were M-CSF specific. Inhibition of PI3K reproducibly led to a ~50% decrease of immunophenotypically defined macrophages as compared to the DMSO control (Figure 6-8c, d). However, the output of granulocytes was unaffected. Cultures containing p38 inhibitor led to a decrease in granulocytic (down by 28% compared to control) and an increase in macrophage (up by 12%) output. Inhibiting ERK led to an increase in the macrophage compartment by 34% and to a decrease of granulocytic output by 9% compared to controls. The SFK inhibitor SU6656 had no effect on differentiation by itself, but seemed to decrease

granulocytic output in combination with the PI3K/Akt inhibitor (by 18% compared to control) while no further decrease in macrophage differentiation was observed as compared to the PI3K inhibitor alone (Figure 6-8c).

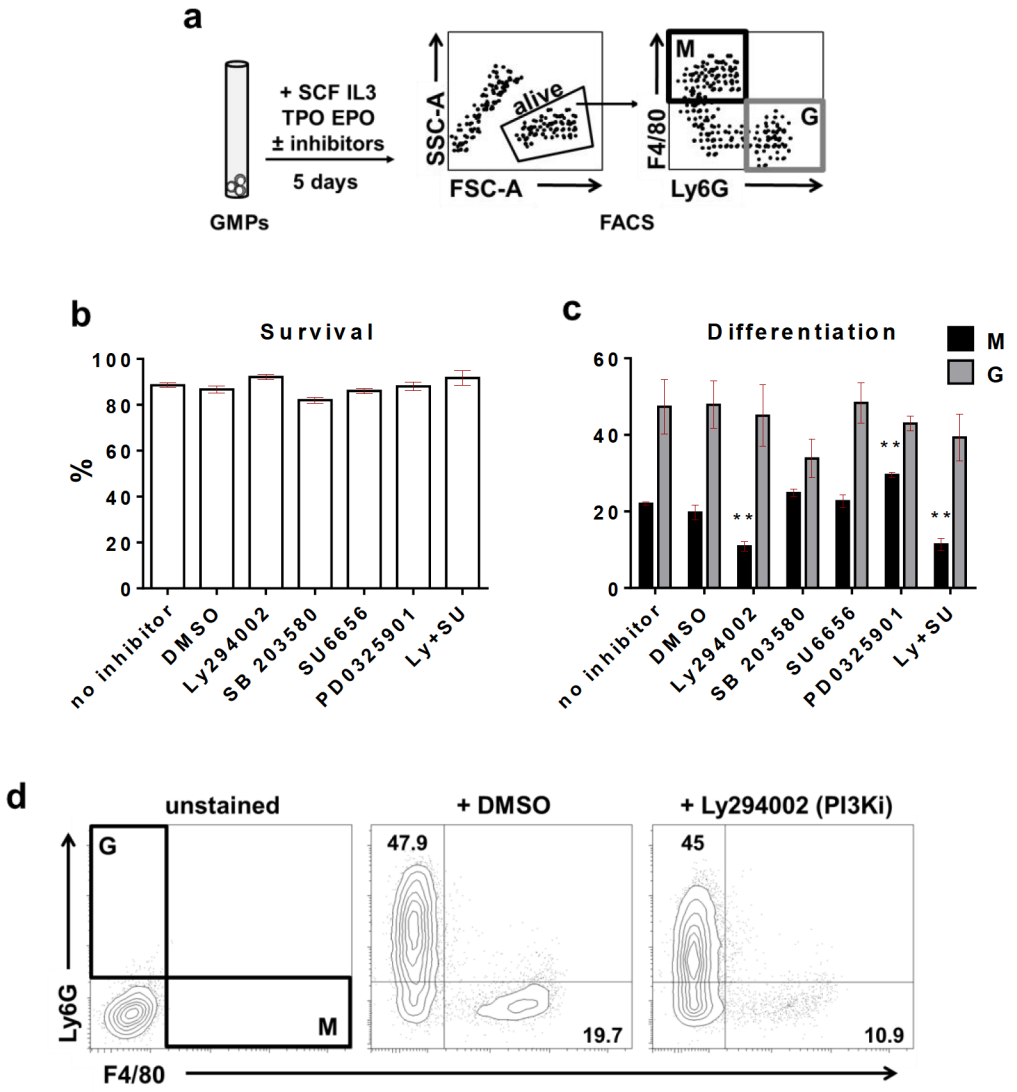

Figure 6-8: Inhibiting various signaling pathways in permissive cytokine conditions has varying effects on M vs. G output.
(a) Experimental strategy. Freshly isolated GMPs were starved in presence of signaling pathway inhibitors for 1h before adding cytokines (SCF, IL3, TPO, EPO, and 10% FCS). After 5 days of culture in 5ml FACS tubes, cells were analyzed by flow cytometry. The FSC/SSC gate was used to determine cell survival. Macrophage and granulocyte output was determined using lineage specific markers F4/80 and Ly6G, respectively. (b) Fraction of living cells after 5 days of culture, as determined via FCS/SSC gating. (c) Fraction of $F4/80^{pos}$ $Ly6G^{neg}$ macrophages and $F4/80^{neg}$ $Ly6G^{pos}$ granulocytes after 5 days of culture. Bars represent mean±SEM (n=3-6 experiments). An unpaired Student's t-test was used to determine the significance of the difference between means of two groups. *<0.05, **<0.01 compared to no inhibitor control. (d) Representative FACS plot showing reduction of macrophage output in the presence of PI3K inhibitor Ly294002. Numbers reflect average percentage from 5-6 experiments.

In summary, none of the tested inhibitors had an effect on the M-CSF-instructed differentiation towards macrophages. Blocking p38 signaling resulted in cell death, indicating that M-CSF-induced survival is mediated via p38, but simultaneously rendering it impossible to reveal potential effects on M-CSF differentiation. Simultaneous inhibition of PI3K and SFKs synergistically prevented cell survival. Both survival defects seem to be specific for M-CSF, as cells survived normally in presence of the same inhibitors in permissive conditions. A variety of effects on the macrophage vs. granulocyte output could be observed in permissive conditions with the inhibitors tested, with Ly294002 being the most notable by leading to a decrease in macrophage output by 50%. Yet, none of the inhibitors tested totally abrogated the differentiation of GMPs to either one of the two lineages they can differentiate into.

As the MCSFR residue Y559 proved to be sufficient for M-CSF-instructed macrophage differentiation, we next cultured MCSFR-deficient ivGMPs rescued with Ywt or the Y559/807AB mutant in M-CSF in presence of the SFK inhibitor SU6656 and serum. In control cultures (M-CSF and serum only), cells carrying Ywt or Y559/807AB were able to differentiate into macrophages as shown earlier. In contrast, virtually no cells transduced with the Y559/807 MCSFR differentiated into macrophages in presence of the SFK inhibitor, while cells rescued with Ywt were still able to do so despite the presence of SFK inhibitor, similarly to what we observed with wild type GMPs (Figure 6-9). While the survival of cells

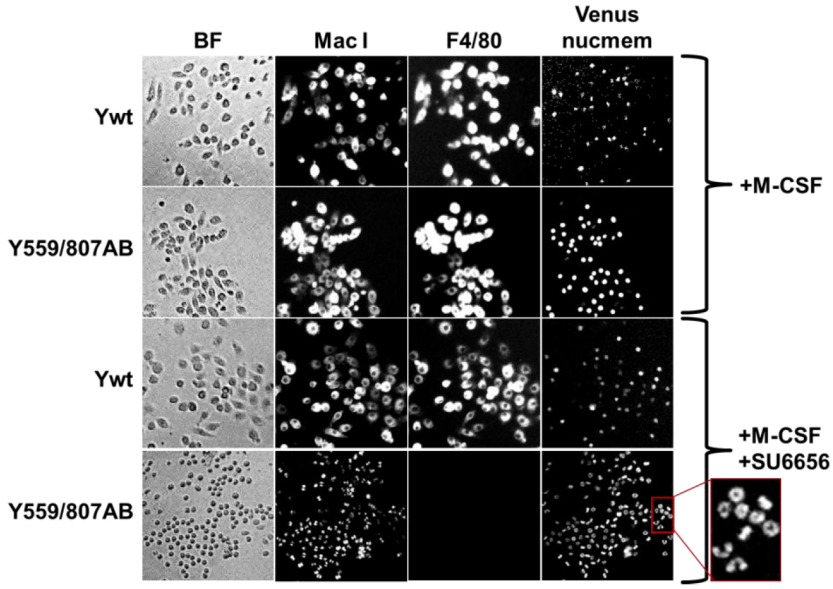

Figure 6-9: Inhibition of SFKs prevents MCSFR Y559/807AB-mediated macrophage differentiation.
MCSFR$^{-/-}$ ivGMPs rescued with either Ywt or Y559/807AB were cultured in M-CSF and 10% FCS in absence or presence of SFK inhibitor SU6656 (2µM). Representative images are stills (~day 4) from a time-lapse imaging experiment with live antibody staining against MacI and F4/80. The nuclear membrane-tagged Venus stems from the virus used to transduce MCSFR$^{-/-}$ cells. Y559/807AB cells cultured in presence of SU6656 mainly generate MacI^{pos} F4/80^{neg} cells with ring/horseshoe-shaped nuclei (zoom), resembling granulocytes.

transduced with the Y559/807AB MCSFR decreased ~20% in presence of SU6656 compared to Ywt and control cultures, most surviving colonies seemed to differentiate into MacI^{pos} F4/80^{neg} cells with granulocyte-typical ring/horseshoe-shaped nuclei (Figure 6-9 and data not shown). This demonstrates that Y559 can transmit the M-CSF lineage-instructive signal through SFKs.

6.4 Manipulating MCSFR signaling through loss and gain of function mutants

Complementary to the small molecule approach, we sought out to manipulate MCSFR downstream signaling pathways by overexpressing either dominant-negative (DN) or constitutive-active (CA) variants of signaling proteins. With regards to the MCSFR mutant screening, which indicated a role of SFKs in the transmission of MCSF-induced lineage instruction, we chose to investigate different SFK members (c-Src, Fyn, Lyn, and Hck) and their potential downstream targets (Akt, NFκB). Furthermore, we included STAT3 for being a major mediator of G-CSF-induced signaling. To this end, we made use of the previously established *in vitro* differentiation system, generating ivGMPs from preGMPs (Figure 6-2). Similarly to the screening of MCSFR mutants, preGMPs were transduced with DN or CA signaling molecules via lentiviral vectors, while differentiating into ivGMPs. Transduced ivGMPs were then sorted and cultured in permissive cytokine conditions. After five days, cultures were analyzed for skewed lineage output by flow cytometry (Figure 6-10a).

None of the analyzed CA or DN mutants had a detrimental effect on cell survival (Figure 6-10b). Untransduced and empty vector controls (Venus control) were indistinguishable in their lineage output with ~25% M and ~50% G. All mutant proteins tested led to a decrease in granulocyte output. In sharp contrast, only overexpression of constitutive active Akt, constitutive active c-Src, and wild type p65 resulted in significantly increased macrophage output, raising it by 1.8-, 2.1-, and 2.7-fold compared to Venus control, respectively ($p<0.05$, Student's t-test) (Figure 6-10c, d). As survival rates were not affected by the overexpression of these signaling proteins, the observed lineage skewing points towards an instructive effect on the lineage decision, rather than a selective mechanism. Alternatively, altered proliferation could also cause an increase in lineage output.

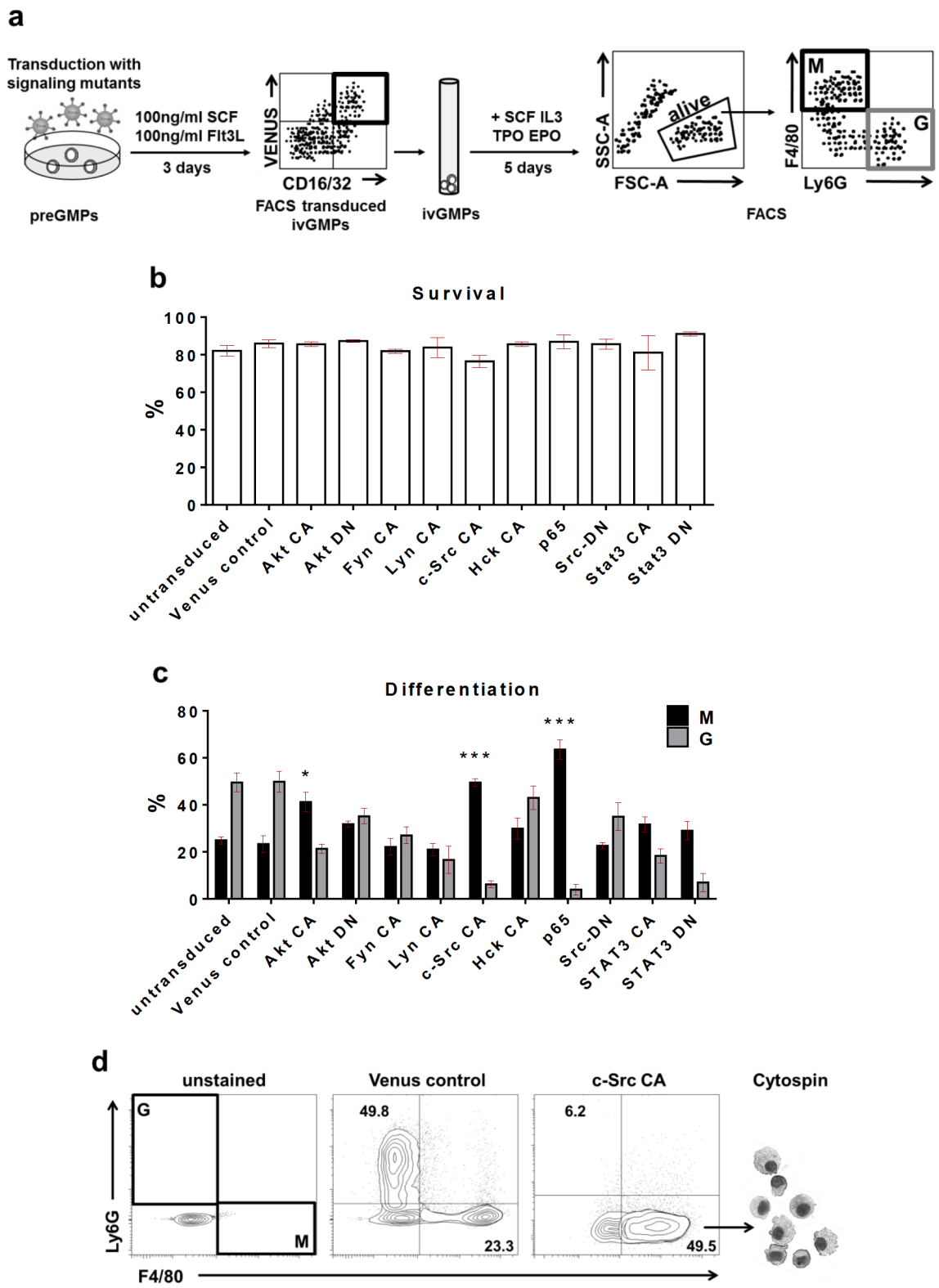

Figure 6-10: Overexpression of signaling mutants in ivGMPs indicates involvement of c-Src and NFκB in macrophage differentiation *in vitro*.

(a) Experimental strategy. preGMPs were freshly isolated, cultured in 100ng/ml SCF and Flt3L while being transduced with lentiviruses encoding variants of signaling molecules (constitutive active (CA) or dominant negative (DN)). After 3 days transduced ivGMPs were sorted and cultured in permissive cytokine conditions (SCF, IL3, TPO, EPO, and 10% FCS). After 5 days in culture, cells were analyzed for macrophage and granulocyte output by flow cytometry using lineage specific markers F4/80 and Ly6G, respectively. The

FSC/SSC gate was used to determine cell survival. (b) Fraction of living cells after 5 days of culture, as determined via FSC/SSC gating. (c) Fraction of F4/80^{pos} Ly6G^{neg} macrophages (M) and F4/80^{neg} Ly6G^{pos} granulocytes (G) after 5 days of culture. Bars represent mean±SEM. (n=3-7 experiments). An unpaired Student's t-test was used to determine the significance of the difference between means of two groups. *<0.05, **<0.01, ***<0.001 compared to Venus control. Note: significance for differences in G output is not annotated, as all overexpressed signaling proteins seemed detrimental to it. (d) Representative FACS plot showing increase in macrophage output when cells were transduced with constitutive active (CA) c-Src. Numbers reflect average percentage from 6 experiments. Cytospin of sorted F4/80^{pos} Ly6G^{neg} cells revealed macrophage morphology (representative picture).

Noteworthy, constitutive active mutants of other SFKs Fyn, Lyn, and Hck did not lead to significantly increased macrophage output and neither did STAT3 variants. Unexpectedly, dominant negative variants of Akt and c-Src had no effect on M output.

In order to have a more quantitative readout regarding the M vs. G output, transduced ivGMPs were alternatively cultured in permissive colony assays (Figure 6-11a). To make colony scoring easier, lineage-specific live antibody staining was successfully applied to these cultures (Figure 6-11b). Complementing the results obtained from liquid culture assays, overexpression of constitutive active Akt, constitutive active c-Src, and wild type p65 resulted in an increase in macrophage colonies and a concomitant decrease in granulocyte colonies (Figure 6-11c). In the case of p65, virtually no G colonies could be detected. SFK members Fyn, Lyn, and Hck, as well as STAT3 variants, had no major effect on colony types and frequencies as compared to controls. Of note, compared to liquid cultures (Figure 6-10c), a general decrease in G colonies was not observed. Furthermore, colony sizes did not seem to differ between the different overexpressed proteins tested, indicating that proliferation times were not affected (data not shown).

In conclusion, overexpression of constitutive active c-Src, constitutive active Akt, and wild type p65 resulted in skewing of the lineage output of bipotent ivGMPs towards macrophages at the expense of granulocytes. As survival and proliferation rates did not seem to be affected, this suggests that the effect occurred at the level of lineage decision.

6.5 Detecting activity of signaling pathways in single living cells

Besides monitoring cellular behavior, there is also the need to continuously quantify dynamic molecular behavior, such as signaling activity, at the single cell level over time.

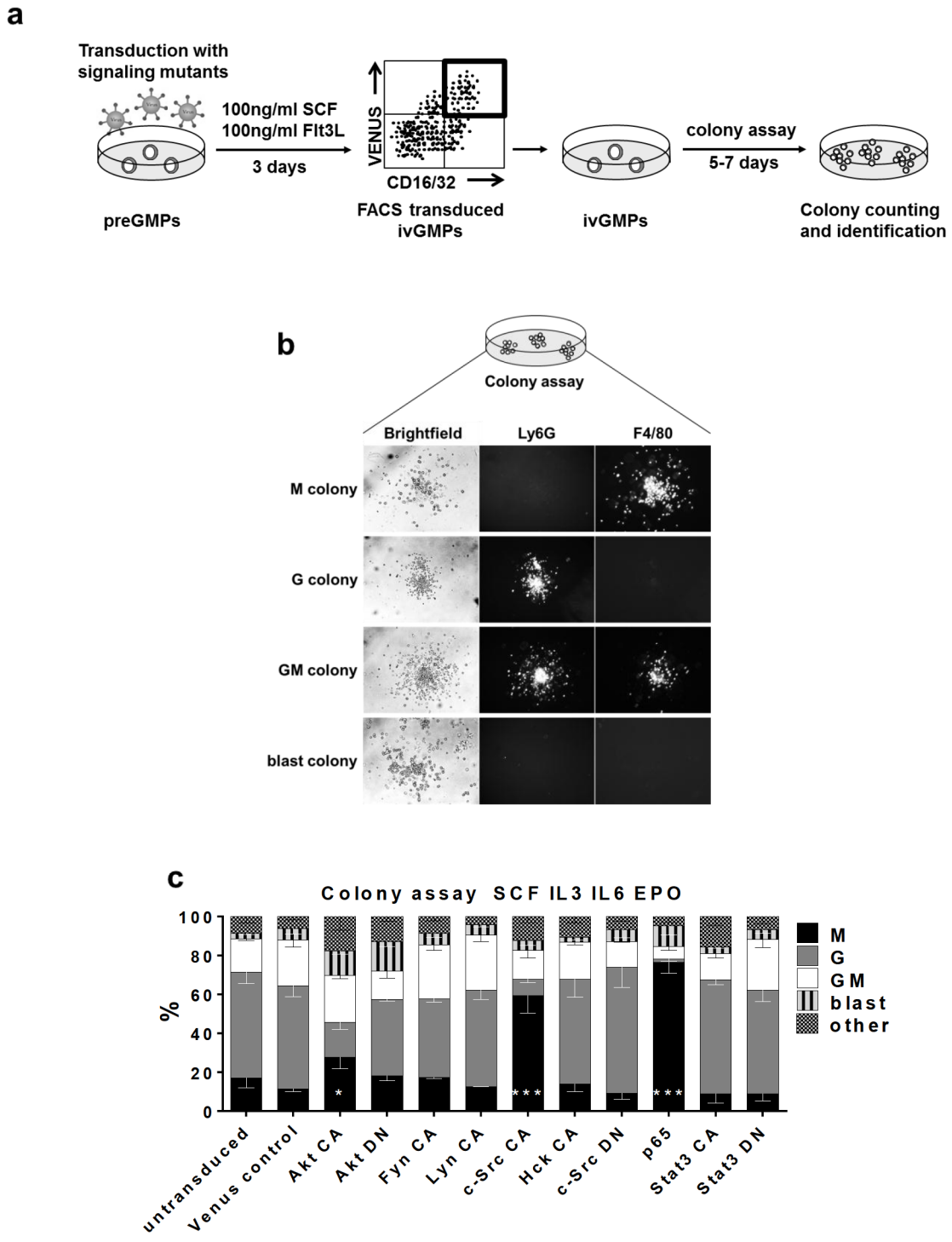

Figure 6-11: Overexpression of signaling mutants in ivGMPs confirms involvement of c-Src and NFκB in macrophage differentiation *in vitro*.
(a) Experimental strategy. preGMPs were freshly isolated, cultured in 100ng/ml SCF and Flt3L while being transduced with lentiviruses encoding variants of signaling molecules (constitutive active (CA) or dominant negative (DN)). After 3 days transduced ivGMPs were sorted and cultured in permissive colony assay medium (M3434, containing SCF, IL3, IL6, and EPO). After 5-7 days in culture, colonies were enumerated and identified using lineage specific markers (F4/80 for macrophages and Ly6G for granulocytes). (b) Representative examples of colony types identified with the help of live in-culture antibody staining. (c) Quantification of colony types after 5-7 days in culture. Bars represent mean±SEM (n=3-6 experiments). An unpaired Student's t-test was used to determine the significance of the difference between means of two groups (only shown for M). *<0.05, **<0.01, ***<0.001 compared to Venus control.

Strength and timing of signaling events are important to fully understand their functional consequence on cell fate. Fluorescent biosensors visualize signaling activity in living cells and therefore allow to link dynamic signaling behavior to future cell fate. To detect M-CSF-induced signaling events in live cells, we sought out to establish the use of fluorescent biosensors in primary myeloid progenitors.

Biosensors detecting different signaling pathways were gathered and cloned into lentiviral vectors (Table 8-3, 8-4 in *Appendix*). To test the general applicability of biosensors with M-CSF, we first utilized a sensor for PI3K signaling (Haugh et al. 2000), which is well described to be activated by M-CSF. The PI3K sensor is based on the pleckstrin homology (PH) domain of Akt, which specifically binds PI3K-produced PIP_3 in the plasma membrane. As a result of PIP_3 production, the PI3K sensor translocates to the plasma membrane, which is visualized by the fluorescent protein Venus fused to the PH domain. We transduced monocytic RAW 264 cells, which express the MCSFR endogenously, with the PI3K sensor. Stimulation of the cells with M-CSF resulted in a rapid and transient translocation of the sensor from the nucleus and cytoplasm to the plasma membrane, demonstrating that M-CSF activates PI3K signaling in this cell line (Figure 6-12a). Next, we repeated the experiment using freshly isolated GMPs. Similar to the monocytic cell line, the sensor rapidly and transiently translocated to the plasma membrane (Figure 6-12b), confirming that M-CSF activates PI3K signaling in primary myeloid progenitors. Another translocation-based sensor tested consists of the C1 domain of protein kinase C (PKC), which detects diacylglycerol production in the plasma membrane and thereby indirectly activity of PLC (Oancea et al. 1998). In GMPs the sensor translocated upon chemical stimulation with a phorbol ester (a diacylglycerol analogue), but not upon M-CSF stimulation (data not shown), suggesting that PLC is not activated by M-CSF.

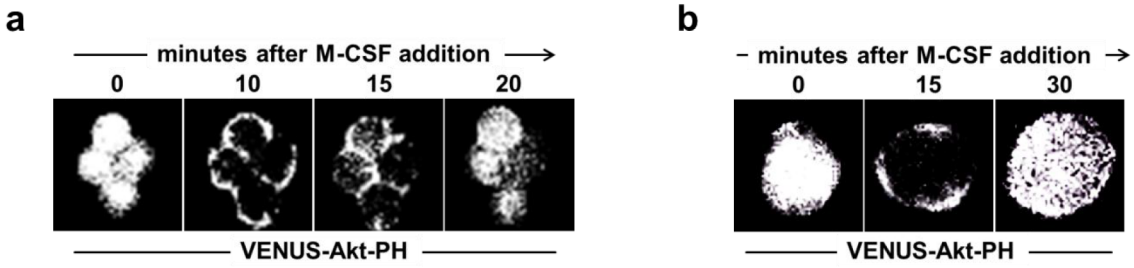

Figure 6-12: Detection of rapid and transient activation of M-CSF-induced signaling in living cells.
(a+b) Time-lapse microscopy of (a) live monocytic RAW 264 cells and (b) primary GMPs transduced with a biosensor for PI3K activation. Stimulation with M-CSF results in rapid and transient translocation of the sensor from cytosol and nucleus to the plasma membrane, indicating activation of the pathway. Time lapse pictures were taken every 1-2 minutes. Representative timepoints are shown.

Collectively, this demonstrates the feasibility of using biosensors to detect intracellular signaling events in primary progenitors at single cell sensitivity and high temporal resolution without losing single cell identity.

6.6 Transcriptional program activated by M and G-CSF

In order to get insight into the transcriptional program activated by M- and G-CSF, we carried out microarray analyses. We took advantage of aforementioned mouse model, which expresses EGFP under the lysozyme M promoter. It has been shown previously, that cells expressing GFP in this model are unilineage committed either to macrophages or granulocytes (Rieger et al. 2009). We FACS-purified GMPs from these mice and cultured them with either M- or G-CSF for different lengths of time (Figure 6-13a). Samples were collected at two, twenty-four, and forty-eight hours after cytokine addition in order to capture and discriminate between early and late target genes. Twenty-four and forty-eight hour samples were sorted according to LysM:EGFP expression (low and high) by FACS to distinguish between recently committed (GFP low) and further differentiated cells (GFP hi) (Figure 6-13b). RNA from all samples was isolated and subjected to microarray analysis.

Figure 6-13c shows the global changes in gene expression along the two time series and differences between the corresponding timepoints. Very early (2h timepoint) genes activated by M- and G-CSF seem to extensively overlap. Gene expression after 48h of stimulation with M- or G-CSF was more similar than after 24h of stimulation, which suggests that the biggest differences between M- and G-CSF gene expression patterns exist during lineage commitment. Noteworthy, it has been shown before that on a transcriptional level, granulocytes (neutrophils) and macrophages are relatively similar (Sasmono et al. 2007). Indeed, many genes associated with macrophage differentiation were also upregulated upon G-CSF stimulation, such as *Klf4*, *Emr1* (F4/80), and *Csf1r* (MCSFR) (Figure 6-14a). Genes specifically upregulated by M-CSF included *Dab2* and *Dok2*, two negative regulators of SFK and MAPK signaling, respectively (Shinohara et al. 2005, Zhou et al. 2003) (Figure 6-14b). G-CSF-specific genes included *Stat3* and *Socs3*, two well known molecules involved in G-CSF signaling (Figure 6-14c). Moreover *Socs3* is known to be upregulated upon G-CSF stimulation (Geijn et al. 2004).

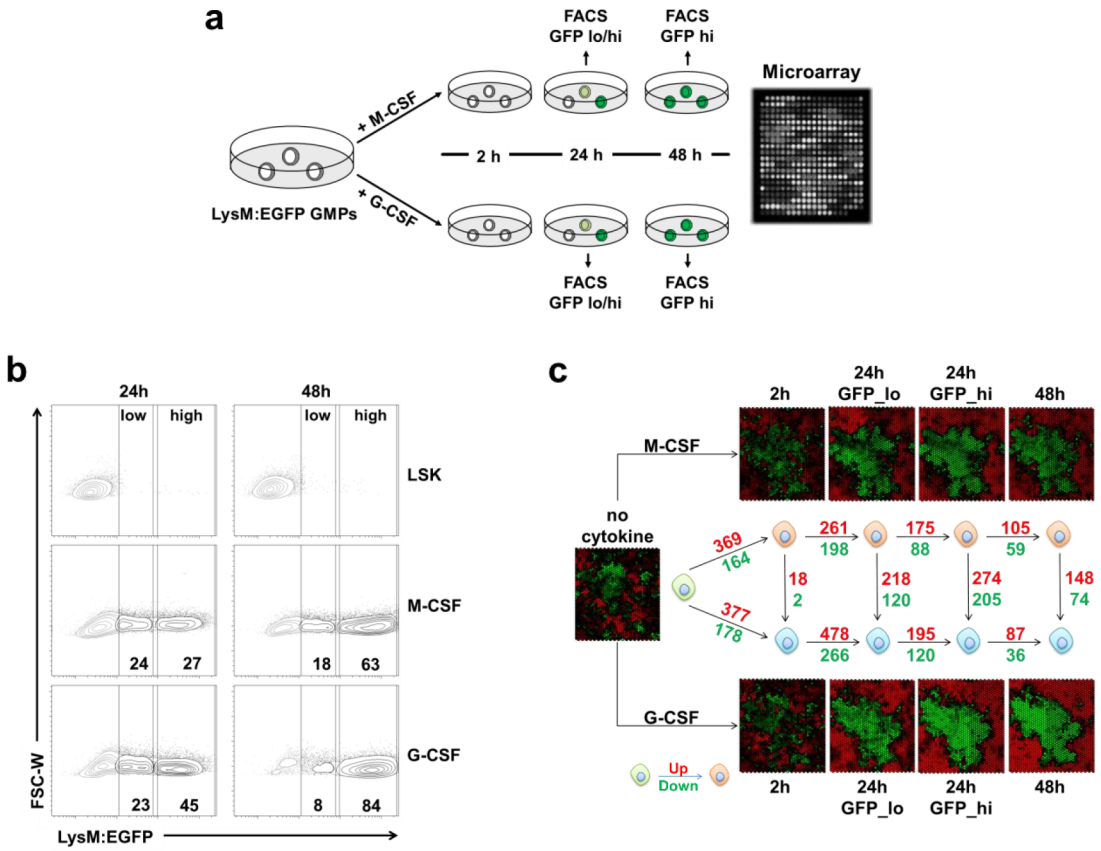

Figure 6-13: LysM:EGFP mice as a tool to dissect M- and G-CSF-mediated gene expression involved in lineage instruction.
(a) Experimental scheme. GMPs were sorted from LysM:EGFP mice and cultured either in M-CSF or G-CSF. After 2 h cells were directly collected for RNA isolation and subsequent gene expression analysis. 24h and 48h samples were FACS purified according to GFP expression (low and high), subjected to RNA extraction and subsequent gene expression analysis. (b) Representative FACS plots of 24h and 48h samples sorted according to low or high GFP expression. LSK cells served as negative gating control. Numbers represent average percentage from 3 experiments. (c) Global gene expression changes along M- and G-CSF mediated differentiation. Numbers indicate genes expressed at least 1.5-fold higher or lower than in the compared condition. Heat maps were generated with GATE (MacArthur et al. 2010).

Next, we clustered differentially expressed genes according to their expression kinetic. This resulted in seven distinct patterns. For M-CSF stimulation, patterns I-III included genes upregulated throughout the time series, while patterns V-VII consisted of downregulated genes. Pattern IV included genes that were early up- and then downregulated (Figure 6-15a). For G-CSF stimulation, patterns I-IV consisted of genes being upregulated, while patterns V-VII consisted of genes being downregulated (Figure 6-15b). GO-term analysis of the different clusters using the online platform DAVID (Huang et al. 2009) revealed that most genes within all clusters were associated with metabolic processes (Table 8-1 and 8-2 in *Appendix*). To reveal potential gene signatures associated with specific signaling pathways, we analyzed the different clusters using Genomatix pathway system (GePS), an online-based platform to identify enriched gene sets in gene lists.

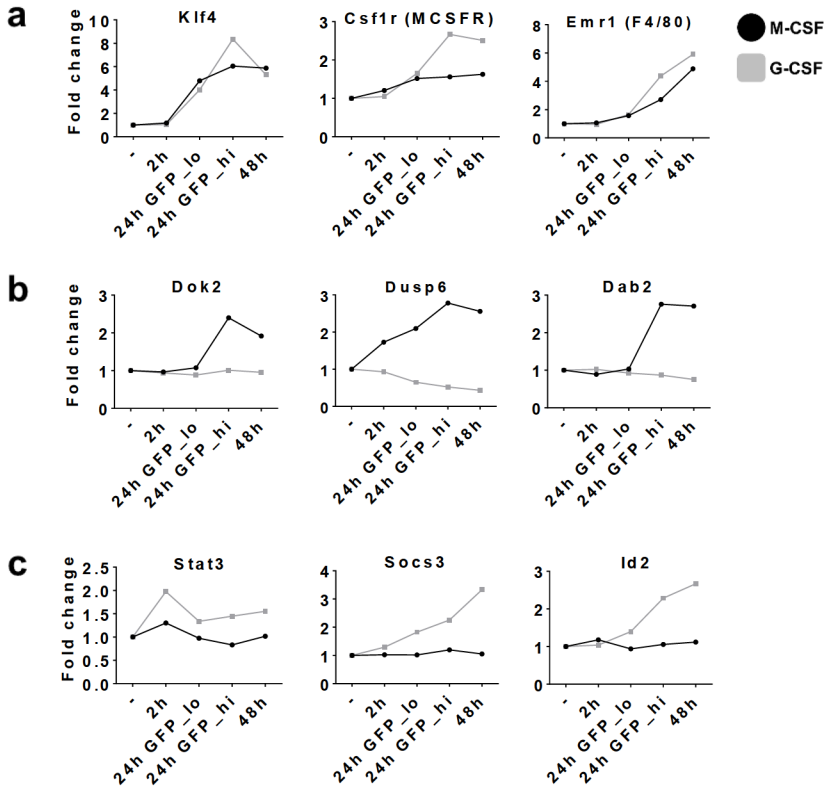

Figure 6-14: Many macrophage-associated genes are also upregulated by G-CSF stimulation.
(a) Examples of genes typically associated with macrophage differentiation that are also upregulated by G-CSF. (b) Examples of genes specifically upregulated by M-CSF. (c) Examples of genes specifically upregulated by G-CSF.

M-CSF-regulated genes within pattern I and II were significantly enriched in Syk, NFκB, and MAPK gene sets and others (Figure 6-15c). M-CSF patterns III-VII did not include signaling pathway-associated gene sets. G-CSF gene expression patterns included, amongst others, STAT, Syk, Hck, Src, and NFκB (Figure 6-15d).

Analyzing all M-CSF-regulated genes (up- and downregulated) that fell within the Genomatix gene sets for Syk and NFκB revealed that a fraction of Syk-associated genes is upregulated already after two hours. Most genes within the Syk gene set, however, were upregulated at the 24h GFP_low timepoint and continued to be upregulated at later timepoints. Only few genes in this gene set were downregulated (Figure 6-16a). Similarly, most M-CSF-regulated genes falling into the NFκB gene set were up- or downregulated upon the 24h GFP_ low timepoint, while some were already upregulated after two hours (Figure 6-16a). Analyzing only M-CSF-upregulated (and not downregulated) genes furthermore revealed that most genes belonging to the Src gene set were upregulated at the 24h GFP_lo timepoint, which represents cells shortly after lineage commitment (Figure 6-16b).

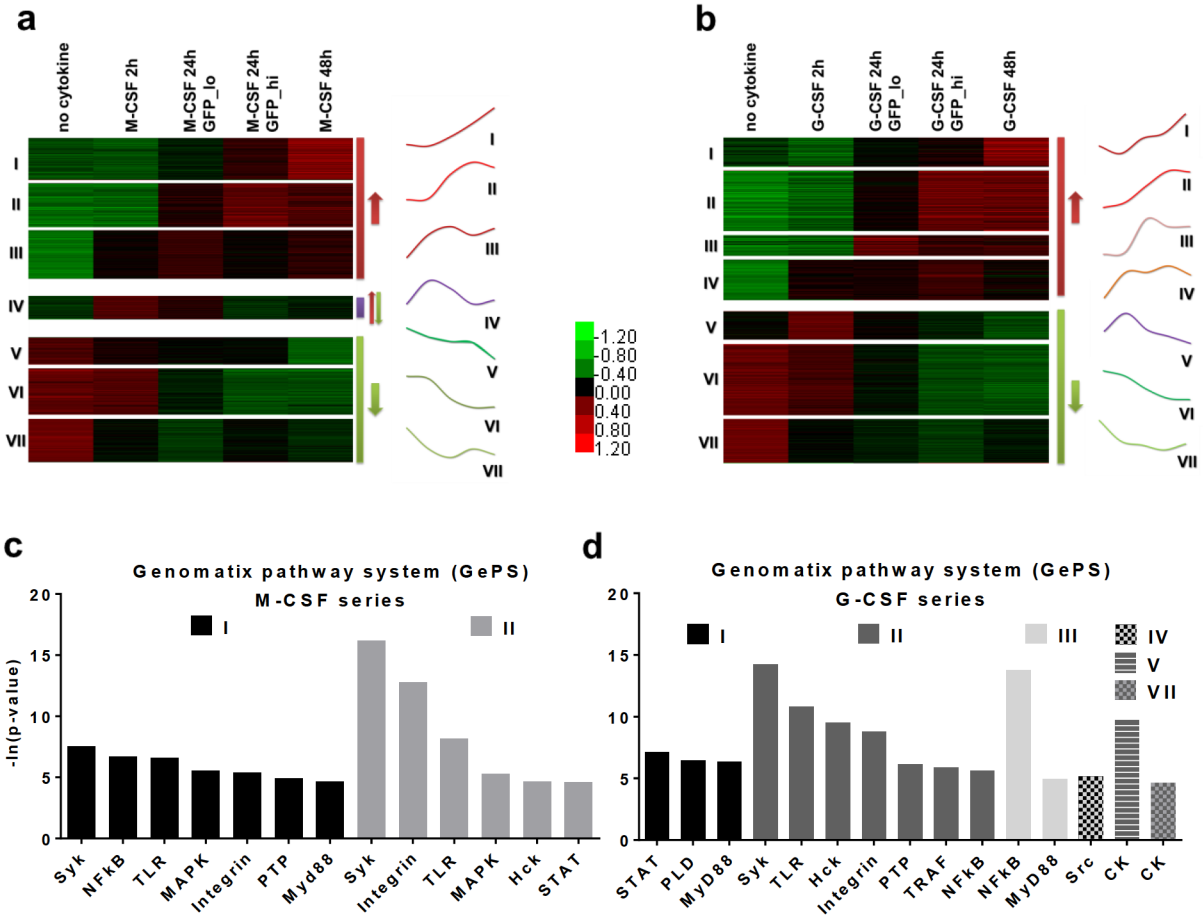

Figure 6-15: Transcriptional profiling of M-CSF- and G-CSF-mediated lineage instruction.
(a+b) Clustering of genes according to their expression kinetics revealed 7 distinct patterns for (a) M-CSF stimulation and (b) G-CSF stimulation. (c+d) Analysis of patterns using online-based platform Genomatix pathway system (GePS) for (c) M-CSF and (d) G-CSF. Shown are only significantly enriched ($p<0.05$) gene sets associated with signaling pathways. Note: clusters not shown were not enriched in signaling pathway-associated gene sets. Plotted are -ln(p-value) values. Syk: spleen tyrosine kinase; TLR: toll like receptor; MAPK: mitogen- activated protein kinase, PTP: protein tyrosine phosphatase non receptor type; Myd88: myeloid differentiation primary response 88; Hck: hemopoietic cell kinase; STAT: signal transducer and activator of transcription; PLD: phospholipase D; TRAF: TNF receptor associated factor; CK: casein kinase.

Collectively, microarray analysis revealed that the overall M- and G-CSF-induced gene expression is very similar. Enrichment analysis of regulated genes indicate that SFK/Syk- and NFκB-signaling pathways are prominently activated upon M- and G-CSF stimulation.

Because Syk and Src gene sets were prevalent among M- and G-CSF-regulated genes, and because constitutive active c-Src drove macrophage differentiation, we performed RT-qPCR to identify which SFK members are expressed in freshly isolated GMPs. Surprisingly, we were unable to detect c-Src. Expressed members were Hck, Fgr, Lyn, Fyn, Yrk, and Lck (Figure 6-17a). Immunostaining against p-SFK revealed that SFKs are expressed and activated at the protein level in freshly isolated steady-state GMPs (Figure 6-17b).

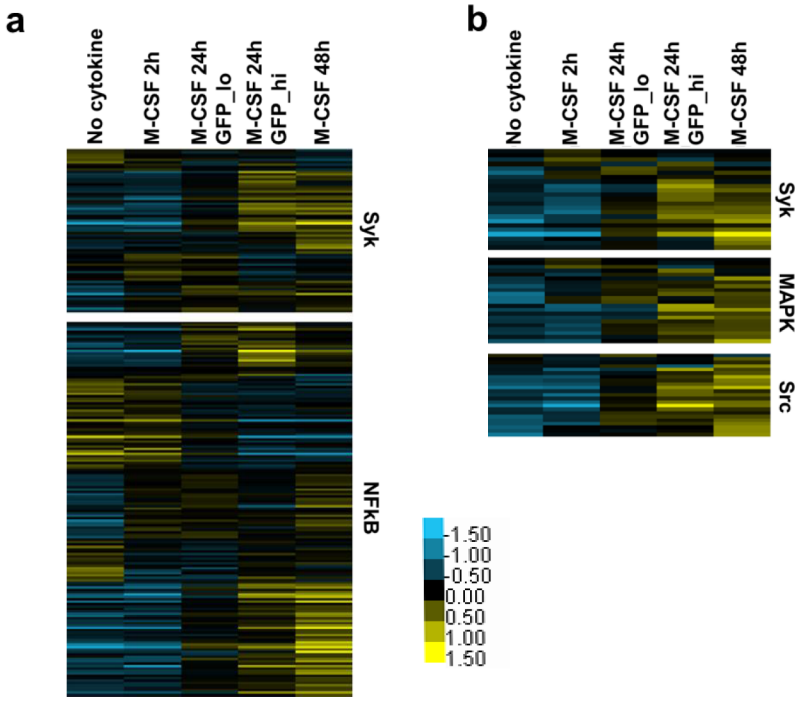

Figure 6-16: Most M-CSF-regulated genes associated with Src/Syk, MAPK, and NFκB signaling are upregulated after lineage commitment.
(a) All genes regulated by M-CSF (up and down) or (b) genes only upregulated by M-CSF were analyzed in GePS to extract significant gene sets. The corresponding genes within the gene sets were extracted and their expression plotted over time.

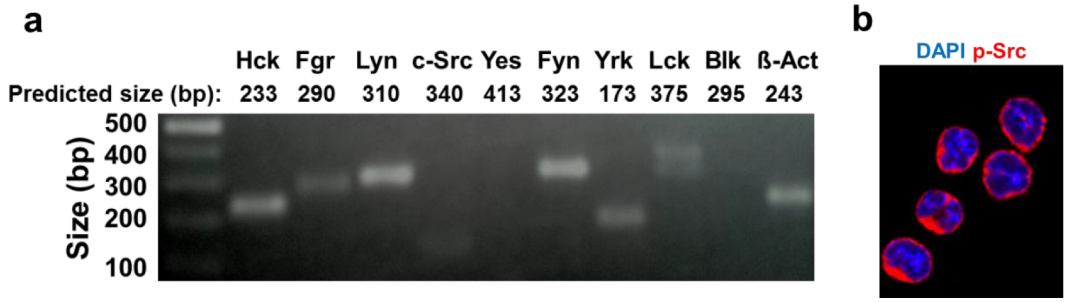

Figure 6-17: Expression of SFKs in primary GMPs.
(a) RT-qPCR against all known mammalian SFK members. (b) Immunostaining against active p-Src (representative picture showing a single optical section from confocal microscopy). Note: The antibody used is not SFK member-specific.

7 Discussion

GMPs can be instructed to differentiate into macrophages or granulocytes by stimulation with the hematopoietic cytokines M- and G-CSF, respectively (Rieger et al. 2009). M- and G-CSF activate specific signaling pathways that mediate the pleiotropic actions of these cytokines and ultimately drive lineage differentiation. However, the abundance of activated signaling cascades makes it difficult to functionally link specific pathways to specific biological effects. The goal of this study was to better understand the involvement of individual activated signaling pathways in transmitting M-CSF-mediated responses, including survival, proliferation, lineage choice, and ultimately differentiation in primary myeloid progenitors.

7.1 Analyzing MCSFR function in the correct cellular context

Studies examining the role of individual receptor tyrosine residues in initiating specific signaling pathways and how these are connected to cellular fates have produced conflicting results. For example, depending on the cellular system and methods used, mutation of MCSFR Y559 was shown to enhance cell proliferation (Rohde et al. 2004), decrease proliferation (Takeshita et al. 2007, Yu et al. 2008), or have no effect on proliferation (Marks et al. 1999). Similarly, mutation of Y807 was shown to reduce cell proliferation (Takeshita et al. 2007, van der Geer & Hunter 1991), or enhance it (Bourette et al. 1995). Previous approaches investigating MCSFR structure/function often depended on myeloid cell lines or fibroblasts devoid of endogenous MCSFR expression (Bourette et al. 1995, 1997; Bourgin et al. 2002, Liu et al. 2001, Mancini et al. 1997, van der Geer & Hunter 1991, Wilhelmsen et al. 2002, Wolf et al. 2002) or on chimeric receptors consisting of extracellular and intracellular parts of different cytokine receptors (Faccio et al. 2007, Takeshita et al. 2007). Chimeric receptors were used when the experimental cells endogenously express the MCSFR, such as in the case of primary BM-derived macrophages. The extracellular domain of these chimeric receptors then allows triggering MCSFR signaling using a different ligand. However, a chimeric receptor may not behave exactly like a wild type full-length MCSFR: e.g. binding of a different ligand might not activate the cytoplasmic part of the MCSFR to an extent as the original ligand M-CSF would. Myeloid

cell lines are easy to cultivate and can be grown indefinitely, but their intracellular milieu is often (pre)leukemic and hardly reflects that of primary cells. Yet, cytokine-induced effects may only be initiated if the cell has the intracellular and molecular context required to correctly interpret the external stimulus. Cells that do not endogenously express the MCSFR might not express signaling molecules and/or transcription factors that are normally recruited or activated by the receptor. Similarly, chromatin structures of target genes and miRNAs might not be in the required configuration to elicit effects reflecting physiological responses. Moreover, expression levels of cytokine receptors might affect cytokine-mediated cellular outcomes, and molecular machineries mediating different cytokine effects may only be active or present during specific time windows of differentiation. All of these points likely explain the conflicting results obtained for single tyrosine signaling activation and concomitant consequences on cell fate. It is therefore critical to examine M-CSF-evoked signaling in the specific cell of interest, and if possible in cells of primary origin. On the contrary to myeloid cell lines, primary progenitor cells, which physiologically respond to and differentiate upon M-CSF exposure and therefore represent the appropriate cellular model system to study M-CSF-induced signaling, are rare in numbers, difficult to obtain, and cannot be kept in culture for a great length of time, as isolated cells start differentiating as soon as they are placed in culture.

Here, we established a system that allowed us to analyze the role of single MCSFR tyrosine-evoked signaling in transmitting M-CSF-mediated effects in uncommitted, primary BM-derived progenitors that normally express endogenous MCSFR, avoiding the use of chimeric receptors. To this end, we utilized a MCSFR-deficient strain (Dai et al. 2002). Because the MCSFR$^{-/-}$ genotype is neonatally lethal, we isolated fetal liver cells at E14.5 and transplanted them into W41 recipient mice. Donor-derived MCSFR$^{-/-}$ hematopoietic precursors isolated from W41 recipient mice were subsequently lentivirally transduced with MCSFR transgenes carrying the desired tyrosine mutations. The *in vitro* differentiation step starting with BM-derived preGMPs generated GMPs that were identical to fresh BM-derived GMPs in their lineage output and also in cell cycling times as determined in time lapse movies. We therefore assume that the *in vitro* generated GMPs closely resemble their primary BM-derived equivalent and that M-CSF-mediated effects observed in *in vitro*-derived GMPs hold also true for primary GMPs. To analyze individual MCSFR tyrosine residues in this cellular model system, we re-introduced MCSFR mutants lacking individual or several tyrosines and then exposed the cells to M-CSF. Because cells are lacking endogenous wild

type MCSFR due to the genetic knock out background, M-CSF-induced effect can only be initiated via the re-introduced mutant MCSFR. To our knowledge, no one has analyzed MCSFR function in a system as close to physiological background before. A MCSFR-deficient, GM-CSF-dependent BM-derived immortalized macrophage cell line has been used to characterize different MCSFR mutants (Yu et al. 2008). However, as these cells are in a mature stage, they were not suitable for our purpose of investigating M-CSF-mediated differentiation and lineage choice of uncommitted progenitor cells.

We noticed that reintroducing the wild type MCSFR into the MCSFR-deficient background did not rescue M-CSF-induced survival and differentiation to levels of endogenous MCSFR expression, although we sorted for endogenous MCSFR expression levels after transduction. Cell death triggered by stress through cell sorting and lentivral transduction *per se* can be mainly excluded as sorted ivGMPs carrying endogenous MCSFR and transduced with a Venus control virus survived and differentiated comparably to fresh BM-derived GMPs. One explanation might involve the lentivirus-mediated random integration of the MCSFR transgene, which is inherent to the viral transduction. The random insertion prevents genetic regulation by the endogenous MCSFR locus regulatory elements. Therefore, feedback mechanisms might fail to regulate MCSFR expression, which could be crucial for proper differentiation. Furthermore, there is a risk that the integrated promoter driving the MCSFR transgene is silenced during differentiation as is the case in other cell types (Herbst et al. 2012). This would lead to cells losing the receptor and therefore cell death due to lack of survival signals. Indeed, we often saw relatively late cell death events (data not shown), which is not the case in cells expressing endogenous MCSFR. The MCSFR antibody used for sorting transduced cells has been described to be blocking and to result in reduced and smaller colonies when used for sorting $MCSFR^{pos}$ MDPs and then culturing them in M-CSF-containing medium (Auffray et al. 2009). However, sorting $MCSFR^{pos}$ ivGMPs derived from wild type animals did not have a detrimental effect on survival and differentiation in our hands and is therefore unlikely the cause for less differentiating cells in the rescue experiments. Of note, a recent study describing $MCSFR^{pos}$ monocyte-restricted progenitors used the MCSFR antibody for sorting and also did not report any detrimental effect (Hettinger et al. 2013). Despite reduced survival and differentiation compared to endogenous MCSFR expression, more than 50% of $MCSFR^{-/-}$ cells transduced with the MCSFR were rescued in their M-CSF-mediated differentiation to macrophages. This was sufficient to detect contributions of different tyrosine residues to this process.

7.2 Analysis of individual MCSFR tyrosine function

Former studies analyzing MCSFR tyrosine functions not only often relied on myeloid cell lines, but also used classical cellular assays to read out M-CSF-mediated effects on bulk cultures. Besides losing information on cell-to-cell variability, these assays can also obscure contributions of individual M-CSF-affected cell fates to an observed effect. Importantly, and in contrast to previous studies, we are able to simultaneously analyze individual cell fate parameters influenced by M-CSF at the single cell level by using live cell imaging. Therefore, our data can reveal, for example, if increasing cell number is due to modulated cell death frequencies, altered cell cycle times, or a combination of both.

Mutation of individual MCSFR tyrosines revealed varying effects on M-CSF-mediated cell survival, proliferation, and differentiation, indicating that each site differentially contributes to these responses. As previously described (Yu et al. 2008), mutation of all eight tyrosine residues results in a receptor incapable to transmit survival and/or differentiation signals, which we confirm to be the case in primary progenitors in this study.

To look at contributions of individual tyrosines to progenitor proliferation and survival, we determined how many starting cells would at least make two consecutive cell divisions - after which cells are usually committed (Rieger et al. 2009) - or die before making a third consecutive division. Data from almost 2000 individually tracked colonies showed that none of the single MCSFR tyrosine mutations totally abrogated cell survival and proliferation, which corresponds to findings previously reported using mature macrophages (Yu et al. 2008). We find that mutation of Y706, Y721, and Y807 significantly compromised survival and proliferation, which is in line with previous studies in other cellular systems reporting proliferation defects if these sites are mutated (Takeshita et al. 2007, van der Geer & Hunter 1991, Yu et al. 2008). In our system, mutation of Y807 had the strongest defect on cell survival, which is also in line with a study conducted in mature macrophages, although the survival defect observed by Yu et al. was not as drastic (Yu et al. 2008). Individual mutation of Y559 or the remaining tyrosines did not have an effect on early proliferation or cell cycle times. This is somewhat surprising, as there are reports indicating a major role of Y559 in proliferation (Takeshita et al. 2007, Yu et al. 2008). However, these studies were done in a mature macrophage context, which divide infrequently (approximately every 24h). In contrast, the primary progenitors we used are highly proliferative, and it is possible that in

these cells, different pathways activated by different residues induce proliferation. Likewise, a hyperproliferative response to M-CSF when mutating Y559, as was reported for a myeloid cell line, was not observed in our model (Rohde et al. 2004). Surprisingly, cell cycle times, although being quite heterogeneous in general, were not greatly affected by mutation of single MCSFR tyrosine residues.

Individual mutation of Y559, Y721, and Y807 resulted in compromised M-CSF-mediated macrophage differentiation. However, it is difficult to tell if decreased macrophage differentiation is due to a true differentiation defect, lack of survival, or a maturation defect. Moreover, it is not known if cells that have a block in differentiation would die or stay in an undifferentiated, proliferating state. For tyrosine residue Y807, which had a prominent effect on cell survival, we tackled this issue with a bcl2-overexpressing mouse model (Ogilvy et al. 1999) crossed into the $MCSFR^{-/-}$ background. Cells of this background have prolonged survival in absence of cytokines due to the anti-apoptotic properties of bcl2. Indeed, we were able to rescue survival by that strategy independently of M-CSF and could confirm that Y807 is also involved in M-CSF-mediated differentiation. However, due to complex mouse breedings, this strategy was so far not applied to other tyrosine residues involved in survival signaling. Worth mentioning, rescuing survival of cells transduced with the fully mutated receptor (YEF) by bcl2 overexpression did not restore M-CSF-induced proliferation and differentiation. This demonstrates that these M-CSF-mediated effects are indeed transmitted through the tyrosine residues studied here.

A previous study using the myeloid progenitor cell line FDCP1 showed that mutation of Y807 abrogated M-CSF-induced differentiation and at the same time enhanced proliferation (Bourette et al. 1995). Although we can confirm the contribution of Y807 to differentiation, we do not detect hyperproliferation. The same study showed that Y721 was not required for differentiation. We also find that Y721 is not essential for differentiation, yet its mutation decreases the number of differentiating cells. Also in line with our results, mutation of Y559 has been previously linked to defects in M-CSF-induced differentiation of myeloid cell lines (Marks et al. 1999; Bourgin-Hierle et al. 2008).

Strikingly, adding back Y559 to a fully mutated MCSFR background was sufficient to partially restore M-CSF-mediated survival, proliferation, and macrophage differentiation. This was further improved by additionally adding back Y807, rescuing survival and differentiation almost to Ywt levels and confirming the suggestion of previous studies that Y559 and Y807 play a central role in the major responses to M-CSF. In macrophages,

mutation of Y559 and Y807 significantly compromises M-CSF-dependent proliferation and maturation (Takeshita et al. 2007, Yu et al. 2008). In a later study, adding back only Y559 or Y807 was sufficient to restore M-CSF-mediated survival but not maturation of macrophages, as determined via MacI expression (Yu et al. 2012). In contrast to Yu et al., adding back only Y807 was not sufficient to restore survival in our system, nor could we confirm cytokine-independent proliferation of cells rescued with Y807AB. Adding back only Y544, Y721, or Y807 partially restored M-CSF-induced survival and proliferation, but was not sufficient to rescue macrophage differentiation, which is in line with what has been observed in macrophages (Yu et al. 2012). Interestingly, it has been shown that besides Y559 and Y807, Y544 is required to fully restore receptor kinase activation and the capacity to proliferate in response to M-CSF (Yu et al. 2012). Thus, additionally adding back Y544 to Y559 and Y807 might improve M-CSF-induced survival and differentiation in our rescue experiments even more. A summary of the different cell fates that have been linked to individual MCSFR tyrosine residues by mutation analysis in this study is depicted in Figure 7-1.

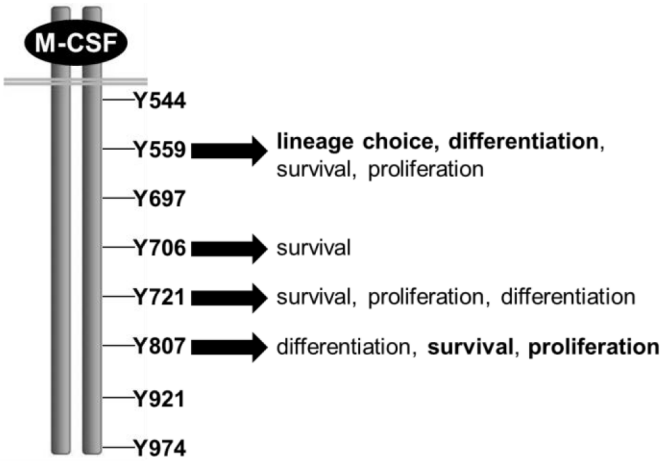

Figure 7-1: Summary of the role of individual MCSFR tyrosine residues in M-CSF-mediated progenitor cell fates.
Only significant results are summarized. Bold terms were most striking.

In order to directly address the role of MCSFR tyrosine residues in lineage choice, we crossed the LysM:EGFP mouse model, in which EGFP is expressed once cells are committed to the M or G lineage, into the MCSFR$^{-/-}$ background. However, transplantation of W41 mice with LysM:EGFP x MCSFR$^{-/-}$ fetal liver cells never resulted in donor contribution, potentially caused by the lack of lysozyme protein. However, as Y559 rescues macrophage differentiation, it indicates that it is also sufficient for M-CSF-mediated lineage instruction. It

would be interesting to assess how rescued MCSFR$^{-/-}$ progenitors would differentiate *in vivo* following transplantation and whether the monocyte/macrophage vs. granulocyte output would be shifted upon mutation of single tyrosine residues. However, *in vivo*, cells are exposed to a diversity of cytokines that can be functionally redundant and might mask altered signaling from a single receptor.

7.3 M-CSF-induced SFK signaling is sufficient for macrophage differentiation

Screening of individual MCSFR tyrosine residues revealed that Y559 was sufficient to rescue M-CSF-induced survival, proliferation, and differentiation. Y559 is the first tyrosine to be phosphorylated and has been proposed to be a switch residue, being critical for MCSFR kinase activity and receptor phosphorylation and at the same time activating a SFK/c-Cbl ubiquitination pathway that leads to full receptor activation on the one hand, and to ligand-induced receptor internalization and degradation on the other hand (Rohde et al. 2004, Takeshita et al. 2007, Xiong et al. 2011, Yu et al. 2008). Several reports have described that Y559 is the only MCSFR SFK binding site in NIH3T3 fibroblasts and macrophages (Alonso et al. 1995, Courtneidge et al. 1993, Faccio et al. 2007). Other molecules binding Y559 have not been described. Interestingly, besides its role in receptor activation, Y807 has also been suggested to be involved in SFK activation (but not direct binding) (Courtneidge et al. 1993) and adding back Y807 together with Y559 improved M-CSF-elicited responses in our hands. Therefore, we reasoned that SFKs are sufficient to initiate and transmit M-CSF-mediated actions. The fact that Y559 on its own is sufficient for M-CSF-mediated survival and differentiation but single mutation of Y559 does not have a drastic negative effect, indicates that further downstream signaling pathways activated via SFKs, can also (directly or indirectly) be activated through other MCSFR tyrosine residues.

SFKs are highly redundant cytoplasmic tyrosine kinases that initiate or modulate the response of many blood cells to extracellular stimuli. They often operate together with other cytoplasmic tyrosine kinases, such as Syk and those of the Tec family to activate further downstream signaling cascades (Lowell 2011). Classically, in immune cells carrying immunoreceptors (such as the T-cell/B-cell receptors or Fc receptors), SFKs phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs), which associate with the

activated receptor and function as docking sites for Syk. Activated Syk then phosphorylates downstream effectors, leading to activation of signaling pathways including PI3K/Akt and PLCγ signaling (Kurosaki et al. 2010, Smith-Garvin et al. 2009). SFKs also phosphorylate signal transduction proteins directly (e.g. focal adhesion kinase (FAK)) or other adaptor proteins (e.g. c-Cbl or Shc) that link SFKs to specific signaling molecules (e.g. PI3K or Ras, respectively) (Lowell 2011). Furthermore, SFKs can also elicit inhibitory signals in a similar fashion through phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Munitz 2010). The SFK Lyn is primarily responsible for the phosphorylation of ITIMS, which then serve as docking sites for different types of phosphatases, such as SHP-1/2 or SHIP-1 (Scapini et al. 2009). Phosphatases then down-modulate signaling responses by dephosphorylating downstream substrates. SFKs also play important roles in pathways where ITAMs/Syk are not involved, as is the case for G-CSF and GM-CSF for instance. In these cases, SFKs have been found to be directly associated to the growth factor receptors (Perugini et al. 2010, Sampson et al. 2007). Due to the high functional redundancy of SFKs, approaches to study their isotype-specific functions are cumbersome. The study of SFKs is furthermore hampered by the unavailability of SFK member-specific phospho-antibodies.

In line with our observation that Y559/SFK signaling is sufficient for M-CSF-mediated effects, overexpression of constitutive active c-Src, but not SFK members Fyn, Lyn, and Hck, in wild type ivGMPs resulted in a marked shift of progenitor differentiation towards macrophages at the expense of granulocytes in permissive conditions. We did not observe cytokine-independent proliferation of cells transduced with constitutive active c-Src, arguing against a myeloid transformation-reflecting artifact. Inhibition of c-Src, or SFKs in general, via a dominant negative mutant of c-Src or the small molecule inhibitor SU6656, respectively, did not affect macrophage differentiation of wild type GMPs. This again indicates that pathways activated through c-Src can also be directly or indirectly activated through other, Y559/SFK-independent mechanisms. Of note, since SFKs are functionally highly redundant, targeting c-src alone with a dominant negative construct is probably compensated for by other SFKs. However, using a small molecule inhibitor against SFKs, we were able to block M-CSF-instructed macrophage differentiation of $MCSFR^{-/-}$ cells rescued with the Y559/807AB MCSFR. This demonstrates that the instructive signal can be transmitted through a SFK-dependent signaling axis. Unexpectedly, qPCR revealed that c-Src does not seem to be expressed in primary GMPs. The constitutive active c-Src might

therefore be phenocopying another SFK member, such as Fgr or Lck; two SFK members expressed in GMPs, but not studied further in this project.

In summary, our results indicate that MCSFR Y559/SFK-mediated signaling is sufficient for M-CSF-orchestrated macrophage differentiation. However, in wild type GMPs other MCSFR tyrosine residues and signaling pathways may be additionally involved.

What lies downstream of SFKs? Upon M-CSF induction, SFKs have been reported to activate several major downstream signaling pathways, including MAPKs (ERK1/2 and p38), PI3K/Akt, PLCγ, and NFκB (Bourgin-Hierle et al. 2008, Lee & States 2000, Takeshita et al. 2007, Wang et al. 2012a, Yu et al. 2012). SFKs have also been described to have a negative regulatory role in M-CSF signaling by downregulating PI3K/Akt activity through recruitment of the phosphatase SHIP-1 (Baran et al. 2003). Recently, it was found that M-CSF-activated SFKs can also signal via ITAM-containing adaptor protein DAP12 in osteoclasts and macrophages, similar to classic SFK signaling involving immunoreceptors (Otero et al. 2009, Zou et al. 2008). This suggests that M-CSF-mediated SFK activation may act through Syk and/or possibly other kinases, such as those of the Tec family (Melcher et al. 2008). Of note, both M- and G-CSF led to upregulation of DAP12 as detected by microarray analysis. Our microarray data further suggests that Syk signaling is among the most prevalent pathways activated upon M-CSF stimulation, with most Syk-associated genes being upregulated shortly after lineage commitment.

Overexpression of constitutive active Akt, although not as pronounced as compared to c-Src, also led to an increased macrophage output, while inhibiting PI3K/Akt via a small molecule reduced macrophage differentiation. Moreover, mutation of Y721, the major MCSFR residue mediating direct PI3K/Akt activation, led to a decrease in macrophage differentiation. Noteworthy, in mice, constitutive active Akt has been shown to induce myeloid expansion in the spleen *in vivo* and was suggested to play a role in human myeloid lineage decisions (Buitenhuis 2008, Kharas et al. 2010). Interestingly, overexpression of p65 also resulted in a strong increase in macrophage output at the expense of granulocytes. Activation of the NFκB pathway by Akt is well described (Dan et al. 2008, Madrid et al. 2001). In response to M-CSF, it has also been suggested that p65 is directly activated via PKC (Wang et al. 2011).

Collectively, our results suggest that M-CSF-instructed differentiation of GMPs to macrophages can occur through SFK-activated signaling, possibly involving downstream activation of Syk, PI3K, and/or NFκB signaling (Figure 7-2). However, it is not clear whether

such a mechanism alone regulates M-CSF differentiation signaling. Other pathways downstream of SFKs may also be involved and might further be activated through other tyrosine residues independently of SFKs. These include, for example, MAPK or PLCγ/PKC signaling. Indeed, studies associating loss of Y559/SFK signaling with M-CSF-mediated differentiation of myeloid cell lines and BM cells suggested involvement of STATs and PLCγ/MAPK signaling downstream of SFKs (Bourgin-Hierle et al. 2008, Marks et al. 1999). Mice deficient for individual SFKs do not show obvious phenotypes in general, demonstrating that there is substantial functional overlap among the different SFKs. Macrophage development in mice lacking single or multiple SFK members is normal (Hibbs et al. 1995, Lowell et al. 1994, 1996). Interestingly, c-Src-deficient mice, similar to M-CSF-deficient mice, develop severe osteopetrosis due to malfunctioning osteoclasts (Soriano et al. 1991). However, this seems to be independent of the kinase function of c-Src (Schwartzberg et al. 1997). Nevertheless, it would be interesting to analyze whether SFK-deficient GMPs would still differentiate normally upon M-CSF exposure *in vitro* and whether GM progenitor cells expressing a constitutive active c-Src would also produce more monocytes/macrophage upon transplantation and exposure to *in vivo* conditions.

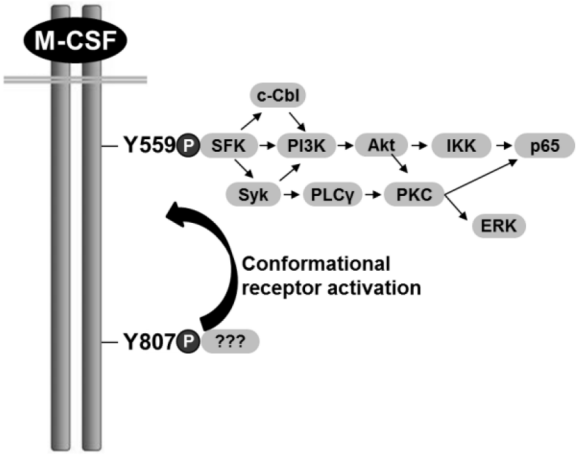

Figure 7-2: Proposed minimal M-CSF-triggered signaling sufficient for macrophage lineage instruction and differentiation.
Our results demonstrate that Y559/SFK signaling is sufficient for M-CSF-mediated lineage instruction and differentiation. Downstream of SFKs our results suggest involvement of PI3K/Akt and/or p65 (NFκB) signaling. However, we cannot exclude involvement of additional signaling pathways such as ERK (MAPK) signaling.

7.4 M- and G-CSF signaling are highly overlapping

The receptors for M-CSF and G-CSF are fundamentally different and belong to different receptor classes. Nevertheless, both receptors activate a common set of downstream signaling pathways inducing proliferation and differentiation to produce distinct lineages from the same progenitor cells. Among the shared signaling components are SFKs, PI3K/Akt, and MAPKs. One possibility how the same signaling pathway could lead to different lineage outputs from a common progenitor population might be through activation strength and duration (Figure 7-3). It has been suggested that persistent and potent MAPK activation is required for M-CSF-mediated macrophage differentiation and that reduced MAPK signaling leads to granulocytic differentiation (Bourgin-Hierle et al. 2008, Gobert Gosse et al. 2005, Jack et al. 2009). One explanation why overexpression of constitutive active c-Src resulted in enhanced macrophage differentiation at the expense of granulocytes might therefore be the strong and persistent activation of a downstream signaling pathway, such as PI3K, NFκB, or MAPK.

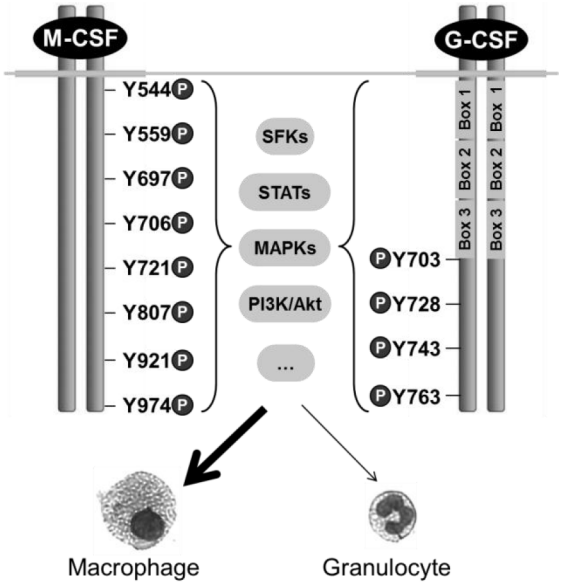

Figure 7-3: M- and G-CSF signaling extensively overlaps.
Although being two different types of receptors, MCSFR and GCSFR activate common signaling pathways to produce cells of different lineages. The different outcomes might be explained by different duration or strength of signaling activity (indicated by the bold vs. thin arrow).

To address how signaling strength and length affect lineage outcome, methods are required that allow continuous detection and quantification of signaling activity in primary HSPCs. The low number of available primary progenitors renders the use of classical

biochemical approaches (e.g. Western blot or flow cytometry) to investigate activation of signaling pathways impractical or impossible. Furthermore, these methodologies are static and do not allow investigating relationships between current molecular state and future cell fates. Therefore, combining live cell imaging with sensors of signaling activity could link strength and length of signaling to future cell fates, which would help to understand above mentioned differentiation mechanisms (Endele & Schroeder 2012).

In this study, we show that the application of biosensors in primary myeloid cells is principally feasible by demonstrating PI3K signaling activation in single live GMPs using a translocation-based sensor. Biosensors based on translocation-events are particularly useful to get insight into signaling dynamics. Due to the wide range of fluorescent hues, multiple pathway activations can be monitored within a single cell using different biosensors simultaneously. Other sensors are based on FRET. These sensors harbor a specific substrate whose modification by the molecule of interest (e.g. a kinase or GTPase) results in a conformational change and FRET, thereby visualizing protein activity. Moreover, as they are genetically encoded, FRET-based sensors can be targeted to distinct intracellular compartments via specific genetic localization sequences, allowing to measure signaling activity from functionally distinct organelles within the cell. This has provided insight into how intracellular compartmentalization can affect signaling (Gallegos et al. 2006, Kajimoto et al. 2010). Our effort to integrate FRET-based biosensors into lentiviruses as a mean to transduce difficult-to-transfect HSPCs never resulted in viral particles harboring the correct sensors (data not shown). This was most likely due to the high sequence similarity of the genes for CFP and YFP typically used as FRET pairs in these sensors, leading to homologous recombination during reverse transcription in the production of lentivirus. Novel fluorescent proteins without sequence similarities to the GFP family of fluorescent proteins could help to overcome this issue.

Despite their advantages over classical biochemistry, biosensors have some caveats that need to be carefully considered. Most importantly, it has to be ensured that cellular physiology is not disrupted through potential competition of the sensor with natural intracellular ligands. To avoid this, the strength of biosensor expression should not exceed levels of endogenous products, while simultaneously being high enough to reliably detect it. Another issue is phototoxicity. Detecting highly dynamic intracellular processes often requires a temporal resolution (i.e. imaging frequency) in the seconds to minutes range. To

minimize phototoxic effects, fluorescent proteins with longer excitation wavelengths and thus lower energy are preferred over ones excited by blue or near-ultraviolet wavelengths.

In conclusion, combining long-term single cell imaging with the detection of live signaling activity through biosensors could help identifying the individual roles of cytokine-triggered signaling cascades in controlling HSPC fates.

7.5 M- and G-CSF-induced gene expression

To learn about differences in the response of primary progenitor cells to M- and G-CSF-stimulation we carried out microarray analysis. Considering the fact that many signaling pathways are shared by M- and G-CSF, it might not be surprising that the overall gene expression induced by M- and G-CSF was relatively similar. Even genes associated with macrophage differentiation were upregulated by G-CSF stimulation, such as the genes for macrophage markers F4/80 or MCSFR. This does not necessarily mean that all these genes are ultimately translated into protein, as has been known for many years (Jack & Fearon 1988). However, there have also been reports demonstrating that terminally differentiated neutrophils can be transdifferentiated into macrophages by M-CSF and other cytokines, suggesting that they do functionally express the MCSFR (Araki et al. 2004, Sasmono et al. 2007).

Due to the high overlap of gene expression changes upon M- and G-CSF stimulation but the higher number of G-CSF-specific genes (that are not regulated by M-CSF), it is tempting to speculate that macrophages are the default myeloid cell type and that granulocytes arise through induction of a few additional lineage-determining genes, as has been suggested before (Sasmono et al. 2007). Such genes might be the ones for transcription factors Id2 and STAT3, which in our microarray were specifically upregulated by G-CSF and not M-CSF. Indeed, it has been shown in previous studies that inhibition of Id2 expression blocks differentiation to granulocytes (Buitenhuis et al. 2005). Furthermore, STATs are extensively described to be involved in G-CSF signaling. However, expression of constitutive active STAT3 in ivGMPs did not seem to have an effect on granulocyte output in our hands, which is in line with the generation of functional granulocytes in STAT3 knockout mice (Lee et al. 2002).

7.6 Outlook

Tightly controlled lineage decisions are essential for steady-state hematopoiesis and their perturbation can lead to severe blood disorders and leukemia. Therefore, understanding the molecular mechanisms underlying hematopoietic lineage choices is of high clinical interest and could lead to applications aiming at manipulating lineage decisions in order to enhance lineages that are diminished due to disease. In this study, we show that M-CSF-mediated lineage instruction of GMPs towards macrophages can be transmitted via a SFK-initiated signaling axis. It remains to be identified, which exact signaling pathways activated downstream of SFKs are involved in propagating the lineage-instructive signal. Our results suggest PI3K and/or NFκB signaling as possible mediators downstream of SFKs. Similarly, transcription factors involved in ultimately carrying out lineage commitment by activating lineage-specific gene expression remain to be elucidated. Finally, whether M-CSF and G-CSF utilize differential or same signaling axes to instruct different lineage choices from the same progenitor is still unknown. Early target gene expression in GMPs was very similar between M- and G-CSF stimulation, suggesting that at least the initially activated signaling pathways are shared. How same signaling pathways activated by two different stimuli lead to two different lineages could be resolved using fluorescent biosensors that allow the quantification of signaling dynamics and kinetics in single living cells.

8 Appendix

Table 8-1: GO term analysis of M-CSF time series clusters

Pattern (M-CSF)	GO terms (top 3)	p-value (Benjamini)
I	immune response	2,5E-5
	regulation NFκB cascade	1,8E-3
	chemotaxis	1,6E-2
II	sterol biosynthetic process	9,8E-11
	cholesterol biosynthetic process	1,2E-9
	sterol metabolic process	1,4E-9
III	cellular protein catabolic process	6,2E-5
	macromolecule catabolic process	7,5E-5
	proteolysis	9,0E-5
IV	cell cycle	2,8E-4
	mRNA metabolic process	3,1E-3
	RNA processing	3,7E-3
V	DNA metabolic process	6,1E-11
	RNA processing	2,9E-9
	DNA replication	2,0E-7
VI	transcription	5,3E-5
	regulation of transcription	1,9E-2
VII	cofactor metabolic process	1,1E-3
	coenzyme metabolic process	2,1E-3
	transcription	4,5E-2

Table 8-2: GO term analysis of G-CSF time series clusters

Pattern (G-CSF)	GO terms (top 3)	p-value (Benjamini)
I	immune response	6,8E-5
	regulation of cell death	4,7E-4
	regulation of programmed cell death	5,3E-4
II	immune response	1,6E-6
	regulation of actin polymerization	3,2E-4
	regulation of actin filament length	2,6E-4
III	sterol biosynthetic process	1,4E-7
	cholesterol biosynthetic process	2,1E-7
	steroid biosynthetic process	1,2E-5
IV	DNA packaging	2,1E-7
	nucleosome assembly	7,8E-7
	cellular macromolecular complex assembly	5,7E-7
V	ribonucleoprotein biogenesis	3,2E-13
	ribosome biogenesis	7,1E-12
	RNA processing	2,7E-11
VI	DNA metabolic process	1,2E-14
	RNA processing	1,8E-10
	ncRNA metabolic process	3,3E-10
VII	transcription	3,8E-4
	regulation of transcription	6,6E-3
	negative regulation of macromolecule biosynthetic process	4,4E-2

Table 8-3: List of acquired translocation-based biosensors for signaling activation

Biosensor	Detection	Reference
PH-Akt	$PI(3,4)P_2$ and $PI(3,4,5)P_3$, PI3K activation	(Haugh et al. 2000)
PLCδ-PH	$PI(4,5)P_2$ and IP_3, PLC activation	(Stauffer et al. 1998)
PKCγ-C1	DAG, PLC activation	(Oancea et al. 1998)
p65-GFP	NFκB activation	(Tay et al. 2010)
PKC-RFP	PKC activation	(Kajimoto et al. 2010)

Table 8-4: List of acquired FRET-based biosensors for signaling activity

Biosensor	Detection	Reference
Raichu-cdc42	cdc42 activity	(Itoh et al. 2002)
Raichu-RhoA	RhoA activity	(Yoshizaki et al. 2003)
Akind	Akt activity	(Yoshizaki et al. 2007)
Miu2	ERK2 MAPK	(Fujioka et al. 2006)
CKAR	PKC activity	(Violin et al. 2003)
KCP-1	PKC activity	(Schleifenbaum et al. 2004)
KCAP-1	PKA, PKC	(Brumbaugh et al. 2006)

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