Functional analyses of ES cell pluripotency by inducible knockdown of the Polycomb group protein Pcgf6

Functionelle Analysen der ES-Zell-Pluripotenz durch induzierbaren Knockdown des Polycomb group Proteins Pcgf6

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1 Summary

Polycomb group (PcG) proteins are chromatin modifiers involved in heritable gene repression. Two main PcG complexes have been characterized: Polycomb repressive complex (PRC) 2 is involved in the initiation of gene silencing, whereas PRC1 participates in the stable maintenance of gene repression.

Pcgf4 (Polycomb group protein, Bmi1) is one of the most studied PRC1 members with essential functions for embryonic development and adult stem cell self renewal. In embryonic stem cells (ES cells), Pcgf4 is poorly expressed while its paralogs (Pcgf1, Pcgf2, Pcgf3, Pcgf5 and Pcgf6) are expressed at higher levels. The relevance of the Pcgf paralog Pcgf6 for the maintenance of ESC pluripotency has not been addressed so far.

My analyses revealed that Pcgf6 was the most expressed Pcgf paralog in undifferentiated ES cells. When ES cells differentiated, gene expression of Pcgf6 strongly declined. To investigate the functions of Pcgf6 in ES cells, we established a doxycycline (dox) inducible shRNA-targeted knockdown system according to publications by Seibler et al. (Seibler et al. 2005; Seibler et al. 2007). Following dox-induced knockdown (KD) of Pcgf6, we observed decreased ES cell colony formation. In parallel, gene expression of pluripotency markers Oct4, Nanog and Sox2 was reduced upon dox-treatment, wheras the expression of mesoderm genes such as T (Brachyury) were up-regulated. Further, microarray analysis revealed de-repression of several spermatogenesis-specic genes upon Pcgf6-KD, suggesting that Pcgf6 may play a role during spermatogenesis. Upon *in vitro* differentiation, Pcgf6-KD ES cells showed increased hemangioblast formation, paralleled by increased hematopoietic development.

In summary, results of this study suggest that Pcgf6 is involved in maintaining ES cell identity by repressing lineage-specific gene expression in undifferentiated ES cells.

2 Zusammenfassung

Polycomb Gruppe (PcG) Proteine sind Chromatin-Modifikatoren, die an der vererbbaren Genrepression beteiligt sind. Primär wurden bisher zwei PcG-Komplexe charakterisiert: Polycomb-repressiv-Komplex (PRC) 2, der die ersten Schritte des Gen-Silencings übernimmt, und PRC1, der an der stabilen Aufrechterhaltung der Genrepression beteiligt ist.

Pcgf4 (Bmi1) ist das am besten untersuchte PRC1-Mitglied. Pcgf4 hat wichtige Funktionen in der embryonalen Entwicklung und in der Selbst-Erneuerung adulter Stammzellen. In embryonalen Stammzellen (ES-Zellen) wird Pcgf4 kaum exprimiert, während seine Paraloge (Pcgf1, Pcgf2, Pcgf3, Pcgf5 und Pcgf6) höher exprimiert sind. Die Bedeutung des Pcgf-Paralogs Pcgf6 für die Aufrechterhaltung der Pluripotenz von ES-Zellen wurde bislang nicht untersucht.

Meine Analysen zeigten, dass Pcgf6 der am meisten exprimierter Pcgf-Paralog in undifferenzierten ES-Zellen war. Während der Differenzierung von ES-Zellen wurde die Expression von Pcgf6 stark reduziert. Um die Funktionen von Pcgf6 in ES-Zellen zu untersuchen, habe ich ein Doxycyclin (dox)-induzierbares shRNA-Expressionssystem für den gezielten Knockdown (KD) von Pcgf6 nach Seibler et al. (Seibler et al. 2005; Seibler et al. 2007) etabliert. Nach dox-induziertem KD von Pcgf6 beobachtete ich eine Verringerung der ES-Zell-Kolonie-Bildung. Die Expression der Pluripotenzmarker Oct4, Nanog und Sox2 war nach Dox-Behandlung reduziert, während die Expression mesodermaler Gene, wie z.B. T (Brachyury), hochreguliert wurden. Außerdem zeigten Microarray-Analysen eine De-Repression Spermatogenese-spezifischer Gene nach KD von Pcgf6, was darauf hindeutete, dass Pcgf6 eine Rolle in der Spermatogenese spielen könnte. In der *in-vitro*- Differenzierung zeigten Pcgf6-KD-ES-Zellen, neben einer erhöhten Bildung von Hämangioblasten, mehr hämatopoetische Vorläufer.

Zusammenfassend zeigten die Daten dieser Studie, dass das Pcgf-Paralog Pcgf6 an der Aufrechterhaltung der ES-Zell-Identität durch Unterdrücken lineage-spezifischer Geneexpression in undifferenzierten ES-Zellen beteiligt ist.

3 Abbreviations

7AAD	7 amino actinomycin D
%	percent
(v/v)	volume by volume
°C	degree Celsius
ac	acetylation
AP	alkaline phosphatase
BM	bone marrow
bp	base pair
BSA	bovine serum albumine
ChIP	chromatin immunoprecipitation
C-terminus / C-terminal	carboxy terminus
d	day(s)
DAPI	4',6-diamidino-2-phenylindole
ddH2O	double distilled water
DMEM	Dulbecco´s Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Dnmt	DNA methyltransferase
dNTP	desoxy ribonucleotide triphosphate
DTT	dithiotreitol
E. coli	*Escherichia coli*
e.g.	exempli gratia, for example
EB	embryoid body
EDTA	Ethylendiamintetraacetat
eGFP	enhanced green fluorescent protein
ES	embryonic stem
ESC	embryonic stem cell
et al.	et alii (and others)
EtOH	Ethanol
FACS	fuorescence activated cell sorting
FBS	Fetal Bovine Serum
FCS	fetal calf serum
FGF	fibroblast growth factor
g	gram
h	hour
H	histone
HD	hanging drop
HDAC	histone deacetylase
HMT	histone methyltransferase
HPC	hematopoietic progenitor cell
HPSC	hematopoietic progenitor and stem cell

HRP	horseradish peroxidase
HSC	hematopoietic stem cell
ICM	inner cell mass (of blastocyst)
IL	interleukine
IMDM	Iscove's modied Dulbecco's medium
k	kilo
K	lysine
kb	kilo bases
KDM	histone lysine demethylase
KO	knockout
l	liter
LB	Luria-Bertani
LIF	leukemia inhibitory factor
Lin	lineage
m	milli, 10-3
M	Molarity [mol/l]
MC	methylcellulose
me	methylation
MEF	mouse embryonic fibroblast
min	minute
mRNA	messenger RNA
N-terminus / N-terminal	Amino Terminus
OD	Optical density
p	pico,
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Poly-A	poly adenylation
Pcgf	Polycomb group ringfinger protein
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
RNA	Ribonucleic Acid
rpm	revolutions per minute
s	second
SDS	Sodium Dodecyl Sulfate
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TC	tissue culture
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris-(Hydroxymethyl)-Aminoethan
trxG	Trithorax group
U	units
UV	ultra violet
μ	mikro

4 Introduction

4.1 Stem cells

Stem cells, due to their ability to self-renew and their developmental potential, are invaluable tools for basic research such as developmental biology, drug discovery and disease modelling. In addition, the broad and unique differentiation potential makes them promising building blocks for future regenerative strategies.

4.1.1 The stem cell system *in vivo*

In multicellular organisms, cells are organized in hierarchic systems with stem cells at the top of the hierarchy followed by transient amplifying progenitor and down to differentiated effector cells (Potten and Loeffler 1990). A stem cell is able to self-renew, i.e. generating a complete phenocopy that shares the same characteristics with the mother stem cell (Orford and Scadden 2008). During development stem cells with distinct properties arise. According to their developmental potential, stem cells are classified as toti-, pluri- or multipotent. In mammals, live starts with a single totipotent cell, the fertilized egg (zygote). A totipotent cell is capable to develop into a complete organism, including extra-embryonic tissues. Totipotency, however, is lost during progression of embryonic development. At pre-implantation, during the blastocyst stage, cells in the inner cell mass (ICM) are pluripotent. These pluripotent cells can produce all cell types of the 3 germ layers: endoderm, mesoderm and ectoderm, but fail to generate extra-embryonic tissues. In both embryonic and adult tissues, further specialized stem cells, known as somatic or adult stem cells, fulfill cell generation and regeneration following injury to guarantee tissue homeostasis. Adult stem cells are lineage-restricted stem cells that are capable to differentiate into effector cells of a particular stem cell system (Mitsiadis et al. 2007; Voog and Jones 2010). For example, hematopoietic stem cells (HSCs) give rise to all cell types of the blood (Seita and Weissman 2010).

Within the blastocyst ICM, pluripotent cells exist only transiently. As development proceeds, the ICM cells generate differentiated progeny and finally generate the embryo proper. However, pluripotent ICM cells can be isolated and adapted to *in vitro* culture, under which they remain undifferentiated and propagate as pluripotent embryonic stem cells (ES cells)(Evans and Kaufman 1981; Martin 1981).

4.1.2 Embryonic stem cells

ES cells are pluripotent *in vitro* derivates of cells of the ICM of preimplantation blastocyst-stage embryos. In the presence of leukemia inhibitory factor (LIF) and mouse embryonic fibroblasts (MEFs), ES cells remain undifferentiated and self-renewing. In contrast, external differentiation signals, such as withdrawal of LIF, induce ES cells to differentiate into all kinds of somatic cell types including germ cells and many clinically relevant cell types (e.g. hematopoietic, neural)(Evans and Kaufman 1981; Martin 1981; Brook and Gardner 1997; Nichols et al. 1998). Upon ectopic transplantation, ES cells form teratomas that are constituted of cell types of the 3 germ layers.

Under standard ES cell culture conditions, ES cells typically grow in compact and sharp-edged colonies in the presence of serum, LIF and on MEFs. LIF activates the transcription factor STAT3 by binding to a heterodimeric receptor complex consisting of gp130 and LIF receptor (Davis et al. 1993; Niwa et al. 1998; Matsuda et al. 1999). Expression of transcription factors such as Nanog, Sox2 and Klf4 is induced by STAT3, thus LIF-signaling promotes the undifferentiated state of ES cells (Cartwright et al. 2005; Suzuki et al. 2006; Hall et al. 2009). Upon LIF and feeder cell withdrawal, ES cells spontaneously differentiate and form complex-structured cell aggregates, so called embryoid bodies (EBs) (Martin and Evans 1975). Multiple types of tissue-specific progenitor cells arise during EB formation and differentiation. For enrichment of a specific cell population, differentiation conditions can be adjusted by using selected culture medium compositions and by addition of cell lineage-specific growth factors such as mesoderm/hematopoiesis-promoting cytokines. Thus, ES cells provide the basis for establishing an in vitro model of early mammalian development. In addition, because ES cells allow the induction of genetic modifications with relative ease, they offer a widely used platform to study specific gene functions involved in pluripotency and during ES cell differentiation. Furthermore, ES cells represent a potential source of differentiated cell types for cell replacement approaches. Clinical trials based on human ES cells (hES cells) have already been published in 2011(Goldring et al. 2011). In early 2012, Schwartz *et al.* reported the safe engraftment of hESC-derived retinal pigment epithelium cells into patients suffering from macular degeneration. Signs of hyperproliferation, tumorigenicity, ectopic tissue formation or graft rejection were not observed (Schwartz et al. 2012). More progresss in this area can be expected in the near future.

A fascinating and still poorly understood property is that following injection of mouse ES cells into blastocysts, ES cells integrate into the ICM and generate chimeric embryos including the germ line (Bradley et al. 1984). Thus, using genetic manipulation of *in vitro* cultured ES cells the functional characterization of genes of interest during development of a mouse is feasible.

4.1.3 The core pluripotency network

The initiation and the maintenance of the pluripotent state of ES cells require a highly regulated molecular network of pluripotency factors. Among these factors, a limited number of transcription factors (TFs) are of significant importance. As shown by Takahashi and Yamanaka in 2006, the reprogramming of mouse adult fibroblasts to induced pluripotent stem cells (iPS) could be induced by overexpression of just 4 TFs: Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka 2006). This showed that the initiation of pluripotency can be achieved by a small set of TFs. Other factors and mechanism important for pluripotency are chromatin modifiers, factors of the cellular memory system such as polycomb group repressive complexes 1 and 2 (PRC1, PRC2) and extracellular signaling molecules such as LIF (Medvedev et al. 2012).

The pluripotency-associated TFs Oct4, Sox2 and Nanog are highly expressed in the ICM cells, epiblast and in ES cells (Rosner et al. 1990; Pesce and Scholer 2000; Avilion et al. 2003). These transcription factors establish and control the core pluripotency network (Chambers and Smith 2004; Niwa 2007; Silva et al. 2008). But it is not just the presence or absence of these TF important, also the precise expression levels of the POU-family member Oct4 (Pou5f1), plays central roles in blocking differentiation and allowing self-renewal. Repression or overexpression of Oct4 leads to a loss of ES cell characteristics and induces differentiation towards the trophectodermal or meso- and endodermal lineages, respectively (Nichols et al. 1998; Niwa et al. 2000). Oct4 complexes with various TFs including Sox2 and Nanog and modulators of Oct4 function (Pardo et al. 2010). Together with Sox2, Oct4 forms heterodimeric complexes within the promoter regions of genes that regulate pluripotency and embryonic development (Remenyi et al. 2003; Wang et al. 2007). Similar to targeted deletion of Oct4, deletion of Sox2 in ES cells results in trophectodermal differentiation combined with a loss of pluripotency (Avilion et al. 2003; Niwa et al. 2005; Masui et al. 2007; Niwa 2007). In contrast to Oct 4 and Sox2, Nanog is not required for the establishment of ES cell pluripotency but stabilizes the undifferentiated state by maintaining the self-renewal capacity of ES cells (Chambers

et al. 2003; Mitsui et al. 2003; Chambers et al. 2007; Torres and Watt 2008). Oct4 and Sox2 positively regulate their own expression, and also the expression of Nanog (Boyer et al. 2005; Loh et al. 2006). Oct4, Sox2 and Nanog co-occupy a large set of target promoters. The 3 TFs activate genes associated with pluripotency and self-renewal and repress the expression of genes involved in differentiation and lineage commitment at the same time (Boyer et al. 2005; Chew et al. 2005; Okumura-Nakanishi et al. 2005; Rodda et al. 2005; Loh et al. 2006; Wang et al. 2006; Masui et al. 2007; Young 2011). Among the factors that are regulated by the core pluripotency network, polycomb group (PcG) proteins play a central role for the repression of developmental regulators in ES cells ((Boyer et al. 2006; Leeb and Wutz 2007; Cole and Young 2008; Endoh et al. 2008; Kashyap et al. 2009). For instance, double knockout of PcG proteins Ring1A/B (Ring1A/B-dKO) led to de-repression of differentiation associated genes in ES cells and blocked ES cell self-renewal(Endoh et al. 2008). Further analyses revealed a significant overlap of de-repressed genes in Ring1A/B-dKO ES cells and Oct4-KO ES cells. In addition, ChIP and ChIP-on-Chip analyses showed that PRCs require Oct4 to engage their target gene promoters in ES cells and vice versa (Endoh et al. 2008). Together, these data suggested a functional cooperation between PcG proteins and the core pluripotency TFs, and thereby, demonstrated an essential role for PRCs in maintaining ES cell identity.

4.1.4 Chromatin structure in ES cells

The capability of ES cells to differentiate into derivatives of the 3 germ layers requires a molecular flexibility of ES cell-specific gene expression programs. Indeed, a high flexibility of gene expression is characteristic for pluripotent ES cells, e.g. gene expression programs are not permanently silenced but stay accessible for immediate transcription. An unrestricted chromatin architecture is fundamental for ES cells.

Electron microscopy of ES cell nuclei revealed that their chromatin is homogeneous, decondensed and rich in euchromatin. In contrast, differentiated nuclei exhibit frequent, distinct heterochromatin domains (Efroni et al. 2008; Golob et al. 2008). For example, while heterochromatin domains in ES cells are rather diffuse, upon differentiation they become more defined with clear boundaries (Meshorer et al. 2006; Jorgensen et al. 2007). Euchromatin is generally associated with transcriptionally active genes, and indeed, global transcription levels are elevated in ES cells as compared to differentiated cells (Efroni et al. 2008; Golob et al. 2008). The dynamics, i.e. the molecular exchange rates, of chromatin proteins is higher in undifferentiated ES cells than in differentiated

progeny (Meshorer et al. 2006; Jorgensen et al. 2007). By fluorescence recovery after photo-bleaching (FRAP) analyses of GFP-tagged histone proteins such as heterochromatin protein 1 (HP1), histone H1 or core histones, fast and highly mobile chromatin fractions were observed in pluripotent cells (Meshorer et al. 2006).

In addition, global levels of histone modifications that are commonly associated with gene expression are higher in ES cells than in differentiated cells. Histone modifications such as H3K9ac, H3K14ac, H3K4me3 and H3K36me3 were reported to be prevalent in undifferentiated ES cells (Lee et al. 2004; Meshorer et al. 2006; Bartova et al. 2008; Efroni et al. 2008; Bian et al. 2009; Krejci et al. 2009). Activating chromatin modifications increase the accessibility of the chromatin to RNA polymerase, where repressing modifications decrease the accessibility to RNA polymerase. In contrast, levels of repressive histone modifications, like H3K9 methylation, become globally elevated upon differentiation (Aoto et al. 2006; Meshorer et al. 2006; Efroni et al. 2008; Krejci et al. 2009; Wen et al. 2009).

Another regulatory principle that is acting in pluriptent cells is bivalent chromatin. Bivalent chromatin describes histones that carry both activating and repressing modifications at the same time (Bernstein et al. 2006). Usually, both do not occur at the same location, as they are involved in countering effects; however in bivalent chromatin, they are both present. Bivalent chromatin domains are characterized by the simultaneous presence of repressive H3K27me3 and activating H3K4me3 marks at regulatory sites of genes which are involved in lineage commitment, differentiation and tissue development (Surface et al. 2010). As the repressive H3K27me3 modification appears dominant over H3K4me3, bivalent genes are expressed only at low levels in ES cells. However, upon differentiation respective genes can either be immediately activated by removing the H3K27me3 modification via specific histone lysine demethylases (KDMs) or expression can stably be repressed by demethylation of H3K4. This mechanism, which leads to a flexible epigenetic control of gene expression programs upon external or internal stimuli, is not only a feature of pluripotent but also of multipotent hematopoietic progenitor and stem cells (HPSCs)(Cui et al. 2009; Adli et al. 2010; Weishaupt et al. 2010). Thus, bivalent histone modifications have been postulated to be essential for ES cell differentiation into multiple lineages. This view, however, was challenged by a recent study in which two principal states of ES cells, due to different culture conditions, were compared (Marks et al. 2012). In contrast to the standard culture conditions in LIF and serum, the combination of LIF and an inhibitor

cocktail (2i) kept ES cells (2i-ES cells) homogenously in a ground state of pluripotency without lineage priming (Ying et al. 2008; Guo et al. 2010). The authors reported reduced levels of bivalent chromatin modifications and reduced H3K27me3 deposition at repressed promoters in 2i-ES cells, but not in serum-cultured ES cells. This observation indicated that bivalent domains are less essential for the pluripotent state.

4.2 Polycomb factors

The development of multicellular organisms requires the coordinated and selective expression of lineage-specific genes, and the inheritance of active and repressed transcriptional states to daughter cells. As a key component of the cellular memory system, Polycomb factors are involved in gene repression and plays crucial roles for ES cell pluripotency.

4.2.1 Polycomb group repressive complexes

PcG proteins are evolutionary conserved regulatory factors that were originally identified in *Drosophila melanogaster* (Lewis 1978; Duncan 1982; Jürgens 1985). These proteins were found to be essential for the regulation of homeotic (Hox) genes (Lewis 1978; Duncan 1982; Jürgens 1985). Mutations of PcG proteins lead to a lack of Hox gene repression and cause homeotic transformation (Sparmann and van Lohuizen 2006). In mammals, PcG proteins are crucial for many molecular mechanisms involved in stem cell identity and differentiation, as well as in cancer (Pietersen and van Lohuizen 2008; Bracken and Helin 2009; Schuettengruber and Cavalli 2009). As demonstrated in a number of studies in flies and mammals, PcG proteins form multimeric chromatin-binding complexes which can generally be classified in 2 major groups: PRC1 and PRC2.

The mammalian PRC2 contains Ezh1/2 (enhancer of zeste), Eed (embyonic ectoderm development), Suz12 (suppressor of zeste) and the histone binding proteins RbAp46/RbAp48 (Cao et al. 2002; Kuzmichev et al. 2002). The homologs EZH1 and EZH2 represent the executing H3K27 histone methyltransferase (HMT) enzymes. These 2 proteins can partially substitute for each other (Shen et al. 2008). EED exists in 4 different isoforms that are the result of different translational start sites of the EED mRNA (Montgomery et al. 2007). Besides the core subunits of PRC2 further proteins assemble into the multifactorial PRC2 complex, e.g. Dnmt, Suv39h1, HDACs and Jarid2 (van der Vlag and Otte 1999; Kuzmichev et al. 2002; Sewalt et al. 2002; Vire et al. 2006; de la Cruz et al. 2007; Herz and Shilatifard 2010).

In comparison to PRC2, the composition of PRC1 is more complex and heterogeneous. The canonical mammalian PRC1 is composed of homologs of 4 Drosophila proteins: Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) and Sex combs extra (Sce). In addition, a non-canonical PRC1 containing L3mbtl2, Ring1b, Rybp, Pcgf6 and other factors was reported for ES cells (Tab. **1**).
In the canonical model of PRC function, PRC2 and PRC1 act in a hierarchical order. Firstly, PRC2 catalyzes the tri-methylation of histone H3 at lysine residue 27 (H3K27me3) which can be recognized and bound by the chromodomain of the PRC1 component Cbx. Following binding to the chromatin, the Ring1 subunit that possesses E3 ubiquitin ligase activity catalyzes ubiquitination of histone H2 at lysine residue 119 (H2AK119ub) (Cao et al. 2005). H2AK119ub is thought to contribute to transcriptional repression by restraining RNA polymerase II from elongation (Stock et al. 2007). However, several recent studies reported the PRC2-independent recruitment of PRC1 to chromatin and PRC1-associated repression of some loci in absence of H3K27me3 by a so far unknown mechanism (Sauvageau and Sauvageau 2010; Surface et al. 2010; Gao et al. 2012; Tavares et al. 2012).

Tab. 1 PRC1 and PRC2 components in Drophophila and mouse/human

		Drosophila	mouse / human
PRC1	*core*	Sex combs extra (RING)	Ring1A / Ring1B
		Posterior Sex combs (PSC)	*Polycomb group ring finger proteins:* Pcgf1 (Nspc1), Pcgf2 (Mel18), Pcgf3, Pcgf4 (Bmi1), Pcgf5, Pcgf6 (Mblr)
		Polycomb (PC)	*Chromobox homologs:* Cbx2, Cbx4, Cbx6, Cbx7, Cbx8 (associated with PRC1.2 and PRC1.4)
		Polyhomeotic (PH)	*Polyhomeotic homologs*: Phc1, Phc2, Phc3
	associated	Rybp	Rybp or Yaf2
		Sex Comb on Midleg (SCM)	Scmh1, Scml1, Scml2
			BcoR, BcoRL1, Skp1, Kdm28 (associated with PRC1.1)
			Auts2, FBRS, FBRSL1, CKlls (associated with PRC1.3 and PRC1.5)
			L3mbtl2, Hp1, E2F6, Dp-1, Wdr5, Max, Mag, Hdac2/1 (associated with PRC1.6)
PRC2	*core*	enhancer of Zeste (E(z))	Ezh1, Ezh2
		suppressor of zeste 12(Su(z)12)	Suz12
		extra sex-comb	Eed
		Nucleosome remodelling factor 55 (p55 and CAF1)	Rbap48, Rbap46
	associated	Polycomb-like	Pcl1, Pcl2, Pcl3

4.2.2 Heterogeneity of mammalian PRC1

Multiple mammalian homologs of each *Drosophila* PRC1 subunit exist in the mammalian genome (Morey and Helin 2010; Gao et al. 2012). The PRC1 core-subunits have different paralogs (Tab. 1). For example, there are 2 paralogs of Ring1 in the genome (Ring1A and Ring1B), 5 paralogs of Chromobox homologs (Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8), 3 paralogs of Polyhomeotic homologs (Phc1-3) and 6 paralogs of Polycomb group ring finger proteins (Pcgf1-6) (see Tab. 1). Different combinations of these paralogs result in a considerable heterogeneity of PRC1 with potentially divergent functions (Orlando and Paro 1995; Kerppola 2009; Schuettengruber and Cavalli 2009; Surface et al. 2010; Vandamme et al. 2011; Gao et al. 2012). Indeed, recent studies reported that whereas the Ring1 protein is common to all PRC1 complexes, Pcgf, Cbx and Rybp/Yaf2 composition functionally define distinct PRC1 complexes that not only occupy different genomic loci, but also show distinct cell lineage expression patterns (Trojer et al. 2011; Gao et al. 2012; Morey et al. 2012; O'Loghlen et al. 2012; Tavares et al. 2012). Up to date, 6 groups of PRC1 complexes, PRC1.1 – PRC1.6, were identified that can be distinguished by the presence of a different member of the Pcgf family (Gao et al. 2012). In addition, 2 further PRC1 subtypes were defined by the mutually exclusive presence of Cbx or Rypb

(Gao et al. 2012; Tavares et al. 2012). The growing complexity of PRC1 also broadened the canonical view of the PRC targeting mechanisms. For example, Pcgf2 (a part of PRC1.2) and Pcgf4 (a part of PRC1.4) co-localize with Cbx2 within H3K27me3-enriched genomic regions, however, in promoter regions not bound by Cbx2 but bound by PRC1.2 and PRC1.4 no significant H3K27me3 level was detected (Gao et al. 2012). This observation suggested that the classical view of PRC1 recruitment by H3K27me3 recognition is only applicable to a subset of PRC1 complexes and mostly restricted to Cbx-containing PRC1.2 and PRC1.4 (Gao et al. 2012). Thus, targeting mechanisms of PRC1.1, 1.3, 1.5 and 1.6 and PRC1.2 and 1.4 without Cbx are probably H3K27me3-independent. Alternative targeting pathways have been presumed, but still remain largely unknown.
Adding a further layer to the complexity of PRC1-dependent regulation, recent studies demonstrated that the expression and functions of some RPC1 members are dependent on developmental stages. For instance, while Cbx7 is essential for the maintenance of pluripotency and its expression is restricted to pluripotent cells, Cbx2 and Cbx4 are associated with lineage commitment and expressed in differentiated cells (Morey et al. 2012; O'Loghlen et al. 2012). A similar phenomenon was observed in expression of Pcgf paralog Pcgf4. While the expression of Pcgf4 is hardly detectable in undifferentiated ES cells, it is expressed in differentiated cell types (Ding et al. 2012).

4.2.3 PRC1 in ES cells

Polycomb complexes play crucial roles both in undifferentiated and differentiating ES cells by contributing to the repression of pluripotency and lineage-commitment genes (Luis et al. 2012). While knockout of any core subunit of PRC2 was shown to prevent proper differentiation of ES cells and embryo development (Luis et al. 2012), investigations into the functions of PRC1 subunits in ES cells have been more complex due to the heterogeneity of PRC1 family complexes. Analyses of PRC1 subunits function in ES cells has revealed a complex picture as recently published data indicate that different PRC1 may exist with, at least in part, non-overlapping target genes (Vandamme et al. 2011; Gao et al. 2012; Qin et al. 2012). This argues for substantive heterogeneity between different PRC1 complexes. Studies of a concomitant knockout of Ring 1A and Ring 1B demonstrated an essential role of PRC1 for the maintenance of undifferentiated ES cells (Endoh et al. 2008). Ring 1A/B double knockout abrogates PRC1 function. As a consequence, ES cell self-renewal was blocked and differentiation-associated genes

were de-repressed, along with phenotypic changes indicative of differentiation. Ring 1A/B double knockout ES cells further caused reduced DNA binding of PRC2 core factor EED along with decreased H3K27me3 levels at specific promoters by a yet unidentified mechanism, suggesting an interdependence of PRC2 and PRC1 functions in ES cells (Endoh et al. 2008).

In line with the heterogeneity of PRC1, at least two classes of PRC1 were recently reported for ES cells: the canonical PRC1.2 containing Cbx7 or Rybp, and the non-canonical PRC1.6 containing additional components that were previously not detected in 293T-Rex cells. PRC1.2 subtypes are composed of either Rybp, Ring1a/b Pcgf2 or Cbx7, Ring1A/B and Pcgf2 (Tavares et al. 2012), whereas PRC1.6 contains L3mbtl2, Ring1b, Rybp, Pcgf6, some components of the E2F6 and NuRD repressor complexes, as well as the H3K9 dimethyltransferases G9a and GLP (Qin et al. 2012). Insights into the recruiting mechanism and function of Rybp- and Cbx-containing PRC1 in ES cells were provided by several recent studies. While Rybp-containing PRC1 is recruited to chromatin independently of PRC2 and H3K27me3 (Morey et al. 2012; Tavares et al. 2012), Cbx-containing PRC1 requires the presence of H3K27me3 for its genomic localization and therefore follows the canonical model of dependency of PRC1 on PRC2 (Morey et al. 2012; Tavares et al. 2012). The genomic localization of both PRC1 subtypes overlaps in certain genes, it can also be mutually exclusive (Morey et al. 2013). Together, the diversity of PRC1 subtypes in ES cells suggests individual and partially redundant patterns of regulation that contribute to common and non-overlapping aspects of ES cell pluripotency and differentiation. The functional redundancy of PRC1 subtypes would also explain why PRC2 function is dispensable while PRC1 function is crucial for ES cells. Less understood is the non-canonical mechanism of recruiting Rybp-containing PRC1 to chromatin which seems not to be mediated through Rybp (Hisada et al. 2012), but may depend on other PRC1 subunits, e.g. Pcgf paralogs (Gao et al. 2012), transcription factors, e.g. Runx1 (Yu et al. 2012) or other factors. Notably, genome-wide comparison of target genes of canonical PRC1/PRC2 and non-canonical L3mbtl2-containing PRC1 in ES cells revealed a low level of overlap (Qin et al. 2012). This observation emphasizes the complex and versatile regulatory functions of polycomb complexes in ES cells and during early embryonic development.

4.2.4 Paralogs of polycomb group ring finger proteins

Pcgf proteins are mammalian homologs of the *Drosophila* PcG protein Psc. They are characterized by a highly conserved RING finger domain which is probably is involved in the interaction with other PcG proteins (Kyba and Brock 1998). At least 6 different Pcgf paralogs have been found in the mammalian genome (Pcgf1-6). As mentioned earlier, each of them is associated with a distinct PRC1 type that occupies different genomic loci (see **Tab.1** and **chapter 4.2.2**). As shown by several studies, Pcgf1, Pcgf2 and Pcgf4 mediate the ubiquitination of H2AK119 by targeting Ring1B to chromatin (Wu et al. 2008; Wu et al. 2013). In addition, Pcgf6 was shown to directly interact with H3K4me3 demethylase Jarid1d and regulate its enzymatic activity (Lee et al. 2007). Pcgf2 and Pcgf4 are the most characterized Pcgf paralogs that are essential for maintenance of the appropriate expression of Hox cluster genes. Further, Pcgf2 and Pcgf4 regulate differentiation and self-renewal of hematopoietic stem cells (HSCs) as well as carcinogenesis and progression of gastric cancer in a reciprocal manner (Kajiume et al. 2009; Zhang et al. 2010), providing evidence for distinct biological functions of different PRC1 types. Notably, beside Pcgf2 and Pcgf4, Pcgf1 was also shown to regulate differentiation and self-renewal of hematopoietic cells (Ross et al. 2012). Furthermore, while Pcgf4 is hardly detectable in ES cells, its forced expression promoted ES cells to differentiate toward hematopoietic lineages (Ding et al. 2012). These data lead to the question whether other Pcgf paralogs, Pcgf3, Pcgf5 or Pcgf6, also possess regulatory functions during hematopoietic development.

4.2.5 PRC1 components in hematopoiesis

Beside their functions in ES cells, polycomb complexes are required for hematopoietic development. Extensive investigations were made to reveal the roles of PRC2 and PRC1 in hematopoietic stem cells (HSCs). PRC2 and PRC1 components, in particular Pcgf2 and Pcgf4, have opposing regulatory roles in HSCs (Majewski et al. 2010). While PRC2 restricts the activity of HSCs and hematopoietic progenitors, Pcgf2 and Pcgf4 are required for the self-renewal and repopulating capacity of HSCs (Majewski et al. 2010). In addition, as demonstrated by a very recent study, the PRC1 subunit Cbx7 preserves HSC self-renewal by repressing progenitor-specific genes (Klauke et al. 2013). Therefore, Cbx7 is required for the maintaining the balance between self-renewal and differentiation of HSCs (Klauke et al. 2013). In comparison to the knowledge about polycomb functions in HSCs, less information is available about the role of PRC1 during

early embryonic hematopoiesis. As demonstrated in a recent study, loss of the PRC1 core-subunits Ring1a/b in ES cells caused up-regulation of hematopoietic regulators such as Runx1 (Mazzarella et al. 2011). Further, Ring1b was shown to bind to promoters of neural genes in EB-derived cells that carried markers for hemangioblast, a common precursor of hematopoietic and epithelial lineages. Deletion of Ring1b in these cells caused overt expression of neural genes and blocked the *in vitro* generation of hematopoietic colonies (Mazzarella et al. 2011). These results suggest that PRC1 activity is required for the appropriate early hematopoietic development. One of the open questions is whether the loss of other PRC1 components in ES cells, e.g. Pcgf paralogs, would affect ES cell-derived hematopoietic development.

4.3 Scientific aim and strategy

The multi-protein complex PRC1 acts as a key-regulator for transcriptional silencing. Multiple paralogs of each PRC1 core subunit exist in mammals. Various combinations of different subunits lead to the significant diversity of PRC1 subtypes with potentially distinct functions.

As a part of PRC1, Pcgf paralogs are involved in regulating self-renewal and differentiation of stem cells, including ES cells and adult stem cells. The relevance of the Pcgf paralog Pcgf6 for the maintenance of ES cell pluripotency and chromatin biology has so far not been addressed. A molecular analysis of Pcgf6 which is active in ES cells will improve our understanding of PRC1 function in cellular memory formation, ESC pluripotency and differentiation. Here I studied the function Pcgf6 via inducible shRNA-targeted knockdown in ES cells. The data will be instrumental to generate a comprehensive picture of the functional framework of Pcgf paralogs. I expect this analysis to provide important new insight into the regulatory circuitry of ES cells.

5 Results

5.1 The Pcgf paralog Pcgf6 is highly expressed in ES cells

To characterize the tissue- and cell type-specific gene expression of Pcgf paralogs, qRT-PCR analyses on various murine embryonic and adult cell and tissue types were performed. As shown in Figure **1A**, while Pcgf1 and Pcgf4 were highly expressed in murine embryonic fibroblasts (MEFs) and neural stem cells (NSCs), high transcription levels of Pcgf4 (Bmi1), Pcgf5 and Pcgf6 were found in adult testis. In bone marrow (BM), all Pcgf paralogs were detected at low levels. In ES cells (lines: OG2 and V6.5), Pcgf6 was expressed at notably higher expression compared to the other paralogs (Figure **1A**). To determine whether the gene expression pattern of Pcgfs changes upon ES cell differentiation, Pcgf1-6 expression was monitored in undifferentiated and differentiating ES cells. As shown in Figure **1B**, gene expression of Pcgf1 and Pcgf6 decreased with progressing time of differentiation. Noticeable was the expression of Pcgf6 as its expression declined while ES cells differentiate. In contrast, expression of Pcgf4 and Pcgf5 increased up to 6-fold following ES cell differentiation. For Pcgf2 and Pcgf3 no obvious change in gene expression was observed. In parallel, Pcgf6 expression levels were determined in blastocyst. As shown in Figure **1C**, the Pcgf6 transcript level in blastocysts is similar to ES cells. Pcgf6 transcripts were also detected in fetal tissues (Figure **1C**). In adult tissues, Pcgf6 was expressed at low levels, except in testis (Figure **1C**). Taken together, the gene expression profile of Pcgf1-6 suggested specific functions of Pcgf6 in blastocyst stage embryos, in ES cells and testes.

To further investigate whether Pcgf6 contributes to the maintenance of ES cell pluripotency, we specifically knocked down Pcgf6 in murine ES cells by using shRNAs with inducible expression (Figure **2A**). For specific shRNA knockdown, 2 independent shRNA sequences were chosen from the data bank "The RNAi consortium" (http://www.broadinstitute.org/rnai/public/), targeting the protein coding region or the 3´-UTR of the Pcgf6 mRNA (Figure **2D**). For monitoring changes in ES cell pluripotency or differentiation upon gene knockdown, OG2 ES cells (Szabo et al. 2002; Do and Scholer 2005) carrying an Oct4 promoter-driven eGFP transgene derived from OG2 mice were used.

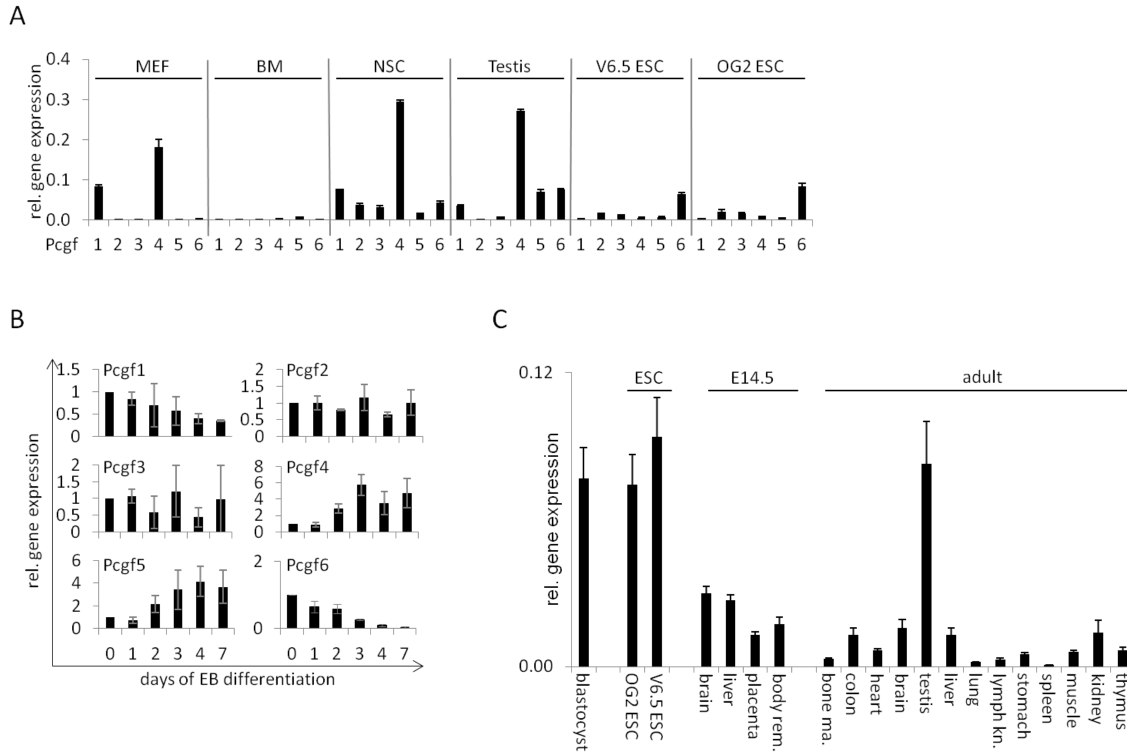


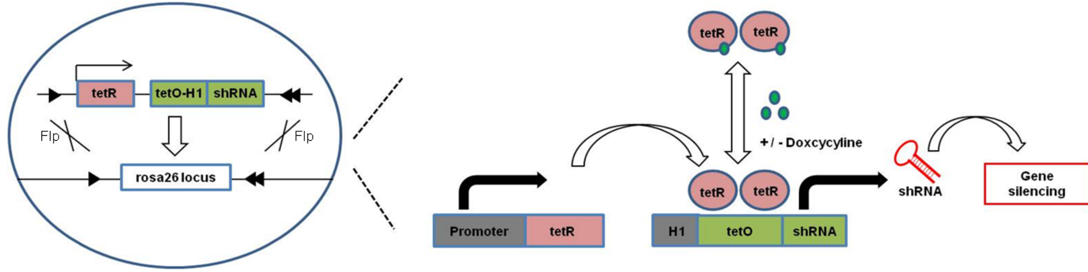
Figure 1: qRT PCR analyses for the expression of Pcgf paralogs. Relative expression levels of Pcgf1-6 **A)** in different cell types and **B)** in undifferentiated ES cells and during ES cell differentiation. Expression level in undifferentiated ES cells was set to 1. **C)** Relative expression levels of Pcgf6 in different murine tissues. Expression was assayed by qRT-PCR. Shown are quantitative comparisons relative to Hprt expression, using the $2^{-\Delta\Delta T}$ method. n=3

5.2 Generation of inducible Pcgf6 shRNA ES cells

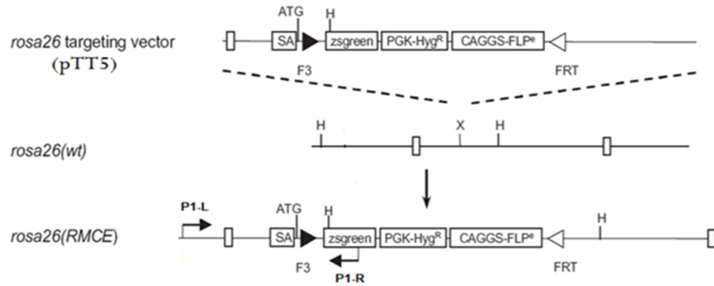
To generate cells with specific and inducible knockdown of Pcgf6 in ES cells, we used the RMCE (recombinase-mediated cassette exchange) vector approach with site-directed integration of a shRNA expression cassette in a previously modified genomic locus. In short: the rosa26 locus was modified by the acceptor vector pTT5 that is than exchanged by the donor vector pINV7 via Flp-mediated RMCE. The pINV7 vector contains a shRNA expression cassette that can be induced by doxycyclin, and is silenced in absence of doxycyclin (Figure **2A**)(Seibler et al. 2005; Seibler et al. 2007). In this two-step process, the pTT5 vector was first inserted into the wild type rosa26 locus (rosa26(wt)) of OG2 ES cells via homologues recombination (Figure **2B**, top). Following hygromycin selection, 60 ES cell clones were isolated and expanded. 3 positive clones (pTT5 clone #A2, #A7, #A15) carrying a modified rosa26 locus (rosa26(RMCE)) were identified by PCR using genomic DNA and primers spanning genomic and pTT5 vector sequences (Figure **2B**, bottom). Based on ES cell morphology and overall appearance, the pTT5 ES cell clone #A2 was selected for further experimentation.

In a second step shRNA carrying pINV7 vectors for dox-mediated shRNA gene expression were inserted into the modified rosa26 locus of pTT5 ES cell clone #A2 via Flp-mediated RMCE (Figure **2C**, top). After 6 days of G418 selection, clones were isolated and expanded. To confirm successful RMCE, clones carrying shRNA sequences (shRNA ES cell clones) were analyzed by PCR using genomic DNA and primers spanning genomic and pINV7 vector sequences. All analyzed shRNA ES cell clones were positive for the exchanged gene cassette (rosa26(RMCE exchanged). Examples from PCR analysis of a clone carrying shRNA1 (pTT5A2-pINV7-Pcgf6_2 clone 2) or shRNA2 (pTT5A2-pINV7-Pcgf6_3 clone 1) are shown in Figure **2C**. To assess specific knockdown, a scrambled control shRNA sequence that is not complementary to any known transcript was used for RMCE. Using this sequence scrambled shRNA ES cell clones (pTT5A2-pINV7-Scrambled clone 1 or scrambled shRNA ES cell clone) were generated following the same procedure as described for the Pcgf6 shRNA ES cell clones.

A



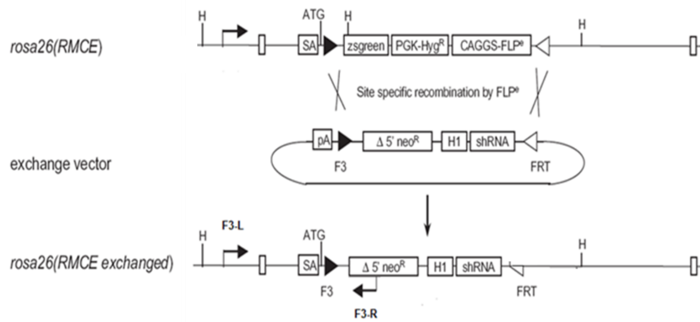
B



PCR with primer pair P1-L/P1-R

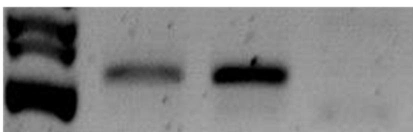
Marker | pTT5 clone #A2 | pTT5 clone #A7 | pTT5 clone #A15 | pTT5A2-pINV7-Pcgf6 | OG2 wt

C



PCR with primer pair F3-L/F3-R

Marker | pTT5A2-pINV7-Pcgf6 a | pTT5A2-pINV7-Pcgf6 b | pTT5 clone #A2



D

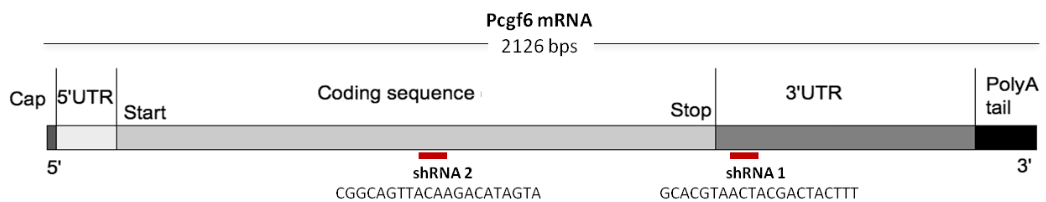


Figure 2: Inducible shRNA knockdown in OG2 ES cells. A) Left: overall structure of Flp-mediated RMCE (recombinase-mediated cassette exchange). Right: principle of doxycycline (dox)-induced shRNA expression. **B)** Upper panel: insertion of pTT5 vector (rosa26 targeting vector) into the wild type rosa26 locus (rosa26(wt)) via homologous recombination. Lower: PCR on genomic DNA to indentify positive clones (pTT5 clone #A2, 7 and 15). zsgreen: fluorescence protein zsgreen; PGK-Hyg: PGK promoter-driven hygromycin transgene; CAGGS-FLP: CAGGS promoter and Flp gene for constitutive expression; F3/FRT: Flp recognition sites; 'X': insertion point within the rosa26 locus. P1-L, P1-R: PCR primers, **C)** Upper panel: insertion of pINV7 exchange vector into the rosa26(RMCE) locus via Flp-mediated RMCE. After G418 selection, positive clones (pTT5-A2PINV7-Pcgf6a, pTT5-A2PINV7-Pcgf6b) carrying the exchanged gene cassettes (rosa26RMCE (exchanged)) were identified by PCR. H1: H1 promoter; shRNA: shRNA sequences; Δ5´neo^{R}: truncated neo^{R} gene; pA: polyA signal; SA: splice acceptor site; F3-L, F3-R: PCR primers. **(D)** Structure of Pcgf6 mRNA and target sits of shRNAs.

5.3 Knockdown efficiency of Pcgf6-shRNAs

To identify shRNA ES cell clones with maximum knockdown, 6 clones carrying shRNA1 or shRNA2 were isolated and treated with dox for 6 days or left untreated. Subsequently, knockdown efficiencies were quantified using qRT-PCR analyses. As shown in Figure **3A**, pTT5A2-pINV7-Pcgf6_2 clone 2 carrying shRNA1 (further on referred to as ES cell clone shRNA1) and pTT5A2-pINV7-Pcgf6_3 clone 1 carrying shRNA2 (further on referred to as ES cell clone shRNA2) possessed the highest knockdown efficiency and were therefore chosen for subsequent analysis.

To study the shRNA-induced knockdown kinetics, Pcgf6 transcript levels were assessed upon dox treatment. In addition, the reversibility of knockdown upon dox withdrawal was tested. For these purposes, undifferentiated ES cell clones shRNA1, shRNA2 and Scrambled shRNA were treated with dox for 0, 1, 3, 5 or 6 days or left untreated for the corresponding time points. For a fraction of cells dox treatment was withdrawn after 6 days and cells were left untreated for additional 6 days to monitor reversibility of the knock down (see also schematic representation of work flow in Figure **3B**). As shown in Figure **3C**, both dox-treated shRNA1 and shRNA2 ES cells showed up to 80% reduction of Pcgf6 transcript levels while Pcgf6 transcript levels in untreated or scrambled shRNA ES cells remained unaltered. Further, 6 days after withdrawal of dox, the dox induced knockdown of Pcgf6 was reversed (Figure **3C**).

Because Pcgfs share conserved domains in their protein sequences, potential cross-targeting of Pcgf6 shRNAs was assessed by qRT-PCRs specific for Pcgf paralogs. As shown in Figure **3D**, expression levels of Pcgf1 – 5 remained unaltered following Pcgf6 knockdown, indicating that the Pcgf6 shRNAs did not cross-react with paralogs of Pcgf6. Taken together, these results indicate successful generation of specific, inducible and reversible knockdown of Pcgf6 in OG2 ES cells by 2 independent shRNAs.

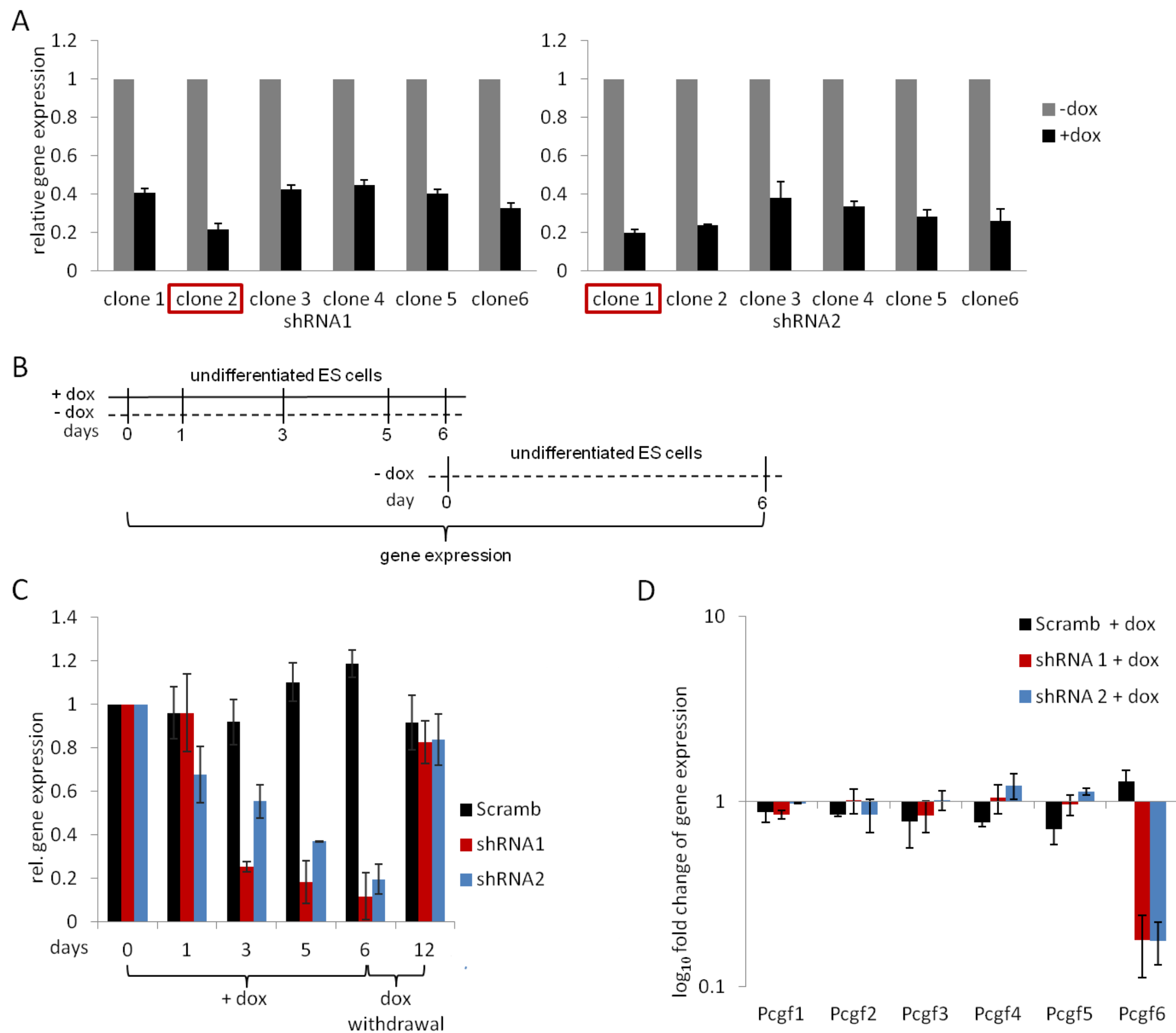


Figure 3: Knockdown of Pcgf6: efficiency and kinetics. A) Relative gene expression of Pcgf6 in shRNA1 (left panel) and shRNA2 (right panel) ES cell clones, treated with dox for 6 days or in untreated cells, assayed by qRT-PCR. Red boxes indicate the ES cell clones chosen for subsequent analyses. Shown are relative Hprt-normalized quantitative comparisons, using the $2^{-\Delta\Delta T}$ method. Expression levels in untreated shRNA-ES cells were set to 1. n=3. **B)** Experimental design for analyzing knockdown kinetics: undifferentiated ES cells were treated with dox for different days or were left untreated. Clones that were treated with dox for 6 days were further cultured for 6 days following dox withdrawal. **C)** Knockdown kinetics of both shRNAs or of scrambled shRNA (Scramb) ES cells assayed by qRT-PCR. Shown are relative Hprt-normalized quantitative comparisons using the $2^{-\Delta\Delta T}$ method. Expression levels in untreated shRNA-ES cells (day 0) were set to 1. n=3. **D)** Relative gene expression of Pcgf1 – 6 in untreated or dox-treated shRNA 1, shRNA2 and scrambled shRNA (Scramb) ES cell cultures. Shown are relative Hprt-normalized quantitative comparisons, using the $2^{-\Delta\Delta T}$ method. Expression levels in untreated shRNA1 ES cells were set to 1. n=3.

5.4 Pcgf6 knockdown reduces ES cell colony forming ability

Upon Pcgf6 knockdown, ES cells could be maintained in culture without showing evidence of morphological changes unless the colonies of dox-treated shRNA1 and shRNA2 ES cells were smaller (Figure **5B** upper panel). However, dox-treated shRNA1 and shRNA2 ES cells showed a reduction in cell number increase. To quantify this observation, $5x10^5$ ES cells (shRNA1, shRNA2 and scrambled) were plated +/- dox

treatment. After 2 days, cell numbers were determined and $5x10^5$ cells were plated into new cultures. This procedure was repeated for 18 days as illustrated in Figure **5A**. Cell numbers were determined by counting and plotted against time. As shown in Figure **5B** (lower panel), reduced cell numbers in shRNA1 and shRNA2 ES cell cultures were observed upon dox treatment. Compared to untreated or treated scrambled ES cell cultures treated shRNA 1, shRNA2 ES cell cultures slowed down cell number increase over a period of 6 days when shRNA 1, shRNA2 ES cell cultures reached a stable plateau of reduced cell numbers. To determine whether the reduction in cell numbers was paralleled by changes in ES cell pluripotency, dox-treated or untreated shRNA1, shRNA2 and scrambled shRNA-ES cell cultures were stained for alkaline phosphatase (AP) activity (Fig5**A**). Under standard ES cell culture conditions, undifferentiated ES cells grow in uniform and tightly compacted multicellular colonies with high expression of membrane alkaline phosphatase (AP). AP activity is considered to be a marker for pluripotency (Wobus et al. 1984). As shown in Figure **5C** (upper panel), both dox-treated and untreated ES cells formed AP-positive colonies with ES cell-typical morphology. However, in dox-treated shRNA1 or shRNA2 ES cell cultures, reduced total colony numbers were counted. In contrast, colony numbers in untreated cultures remained unchanged compared to scrambled shRNA cultures (Figure 5**C**, lower panel), indicating a reduction in ES cell colony forming ability upon Pcgf6 knockdown.

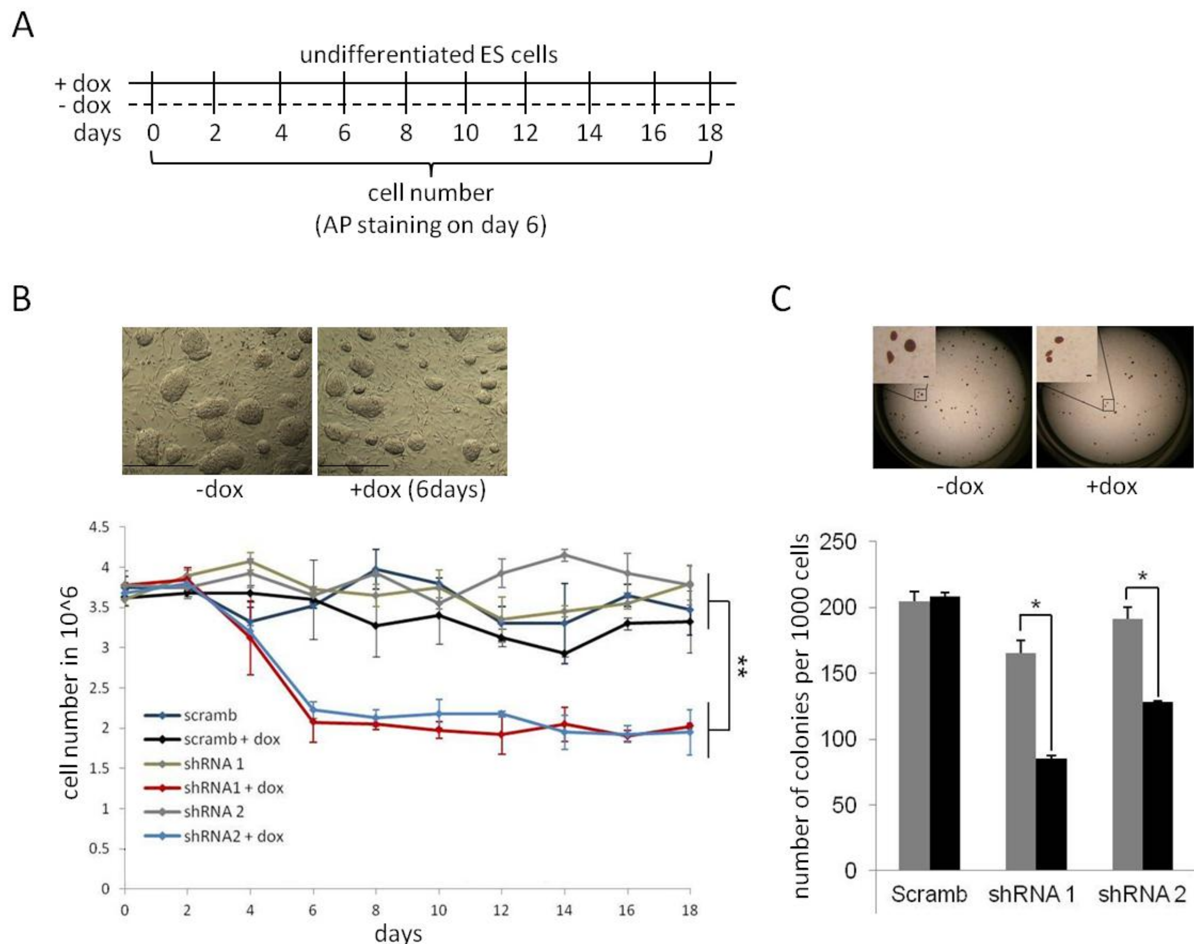


Figure 4. Reduced ES cell colony formation upon Pcgf6 knockdown. A) Experimental design: ES cells were plated at a density of 5x10^5 cells per 6cm-plate, treated with dox or left untreated. Life cell numbers were determined by Trypan blue staining every 2 days for a period of 18 days. AP-staining was performed on day 6 +/-dox. **B)** Representative colony morphology of shRNA1-ES cell cultures on day 6 +/-dox (upper panel). Numbers of shRNA1, shRNA2 and Scrambled shRNA-ES cell cultures +/- dox are shown in the diagram (lower panel). n=3. **C)** Numbers of AP$^+$ colonies in undifferentiated ES cell cultures. *, p<0.01; **, p<0.001; n=3. Representative pictures of AP$^+$ colonies in shRNA1 ES cell cultures (+ or – dox). Light microscopy. Scale bare: 200µm. n=3.

5.5 Apoptosis rate, cell cycle distribution and proliferation were not altered upon Pcgf6 knockdown

To investigate whether the reduction of cell numbers, shown in the previous chapter, was a consequence of altered apoptosis rate, cell cycle phase distribution or due to a proliferative defect in dox-treated shRNA ES cells, these parameters were analyzed (Figure **5A**). The number of apoptotic cells was quantified by AnnexinV/7AAD staining. No significant differences in the frequencies of AnnexinV positive cells were observed in dox-treated or untreated shRNA ES cell cultures (Figure **5B**). Further, cell cycle phase distributions was analyzed by propidium iodide (PI) staining which showed no differences in dox-treated or untreated shRNA ES cell cultures (Figure **5C**). Finally, cell proliferation in dox-treated or untreated shRNA ES cell cultures was quantified based on

BrdU incorporation during DNA synthesis in replicating (cycling) cells. As shown in Figure **5D**, no significant differences in BrdU incorporation rate was detected, indicating no changes in cell proliferation. In summary, Pcgf6 knockdown caused no significant changes in apoptosis rate, cell cycle distribution or cell proliferation.

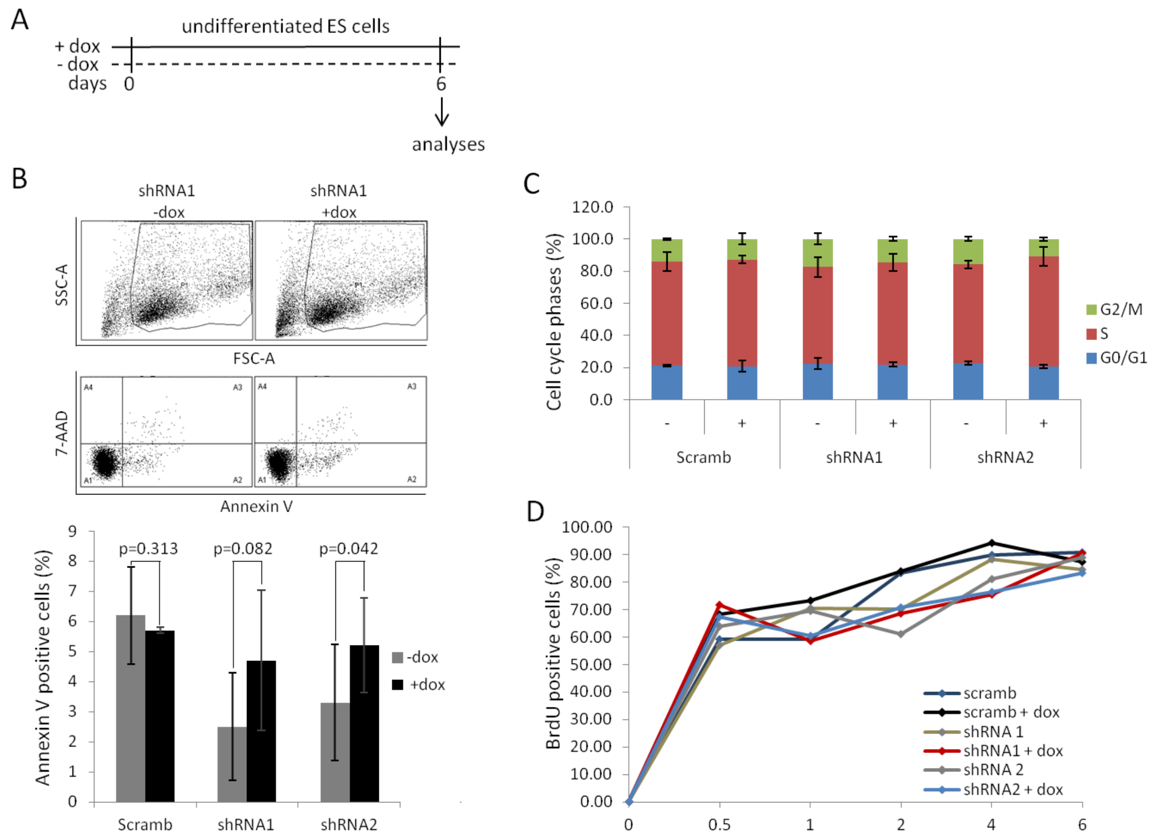


Figure 5. Analyses for apoptosis, cell cycle distribution and proliferation. A) Experimental design. ES cells were treated with dox for 6 days or left untreated before analyses. **B)** Apoptosis rate in shRNA-ES cell cultures +/- dox was evaluated by AnnexinV/7AAD staining followed by flow cytometry (FACS) analysis. Representative FACS profiles (upper panel) show the analyzed cell populations, and AnnexinV$^+$ cells in quadrant A2. Diagram (lower panel) shows the percentages of AnnexinV$^+$ cells in shRNA1, shRNA2 and Scrambled shRNA-ES cell cultures +/- dox. Statistical significance from three independent experiments is expressed in p-values. **C)** Cell cycle distribution by propidium iodide (PI) staining followed by FACS analysis. Data were analyzed with ModFit software. Diagram shows the percentages of cells in different phases of the cell cycle in shRNA1, shRNA2 and Scrambled shRNA-ES cell cultures +/- dox. n=3. **D)** Flow cytometric analysis of BrdU incorporation in shRNA1, shRNA2 and Scrambled shRNA-ES cell cultures +/- dox. Cells were incubated with BrdU for different times (up to 6 h). Percentages of BrdU$^+$ cells were determined by FACS using an anti-BrdU antibody. n=3.

5.6 Global Histone modifications in ES cells upon Pcgf6 knockdown

All Pcgf paralogs directly interact with the PRC1 core-enzyme Ring1B that catalyzes the ubiquitination of histon H2A (H2Aub) (Gao et al. 2012). On the functional level, loss of Pcgf4 leads to global reduction of H2Aub, suggesting a critical role for Pcgf4 in H2A ubiquitination (Cao et al. 2005). To address the consequences of Pcgf6 knockdown on the global level of H2Aub or other histone modifications in ES cells, Western blot analyses were performed using specific antibodies. I further was interested in the global

level of H3K4me3 because Pcgf6 interacts with Jarid1d in somatic cells, and it was shown to enhance its H3K4 demethylase activity (Lee et al. 2007). Also, global levels of known Polycomb associated histone modifications, H3K9me3 and H3K27me3, were analyzed. In addition, because histone deacetylases 1 (Hdac1) and 2 (Hdac2) were reported being interaction partners of Pcgf6 (Gao et al. 2012), global levels of H3- (H3ac) and H4-acetylation (H4ac) were determined. For Western blot analyses, whole cell lysates were collected from shRNA1, shRNA2 and scrambled shRNA ES cell cultures +/- dox for 6 days (Figure **6A**). As shown in Figure **6B**, no alterations in global levels of H2Aub, H3ac, H4ac, H3K4me3, H3K9me3 or H3K27me3 histone modifications were observed.

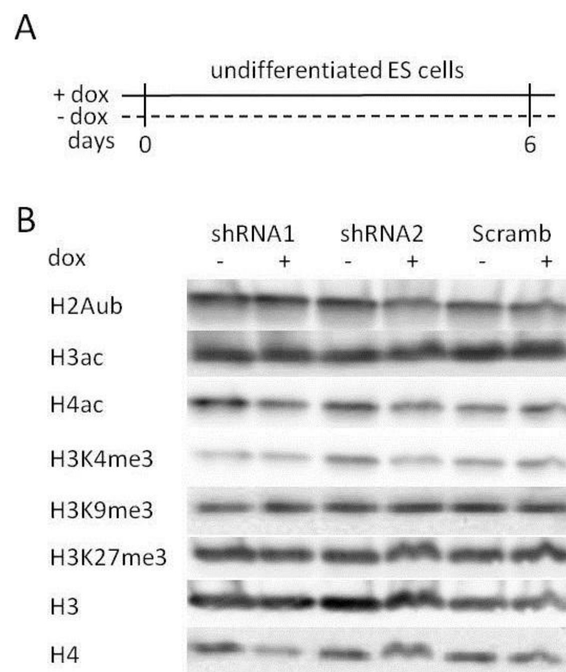


Figure 6. Global levels of selected histone modifications. A) Experimental design: ES cells were treated with dox for 6 days or left untreated. Whole cell lysates were prepared. **B)** Representative Western blot analysis for global levels of histone modifications in undifferentiated shRNA-ES cells +/- dox. Loading controls: histone H3 and H4. n=3.

5.7 Pcgf6 knockdown increases expression of developmental and testis specific genes in ES cells

PRC1 plays essential roles in maintaining ES cell pluripotency. Loss of PRC1 functions causes de-repression of differentiation-associated genes (see in Introduction). As a part of PRC1.6 complex, Pcgf6 was shown to directly interact with Ring1B, the core enzyme of PRC1 (Gao et al. 2012). Together with the observation that Pcgf6 is highly expressed in undifferentiated ES cells (Figure **1A**, **C**), global changes of gene expression in ES cells was assumed upon Pcgf6 knockdown. To address this assumption, global gene

expression patterns were compared between shRNA1, shRNA2 and scrambled ES cells +/- dox treatment using cDNA microarray analysis (Affymetrix) (Figure **7A, B**). Gene expression levels were normalized with the RMA algorithm (Irizarry et al. 2003). Expression values from the microarray analysis confirmed that Pcgf6 was knocked down in dox-treated shRNA1 or shRNA2 ES cell cultures, and as expected, not in dox-treated scrambled shRNA cultures (Figure **7B**). Further, the expression values indicated that the most of the differentially expressed transcripts after Pcgf6 knockdown showed up-regulation (Figure **7B**). Compared to untreated shRNA1 or shRNA2 or scrambled shRNA ES cell cultures, microarray analysis revealed 290 transcripts hereunder 59 annotated genes with greater than 2-fold altered expression levels in dox-treated shRNA1 ES cell culture, 402 transcripts hereunder 71 annotated genes in dox-treated shRNA2 ES cell culture, and 190 transcripts hereunder 22 annotated genes in dox-treated scrambled shRNA ES cell culture (Figure **7C**). Among these differentially expressed transcripts, 36 annotated genes in dox-treated shRNA1 and shRNA2 ES cell cultures overlapped (Figure **7C**). Except for Pcgf6 which was knocked down, all other 35 genes showed up-regulated expression (Data not shown). None of the 35 genes was found differentially expressed in dox-treated scrambled shRNA cultures (Figure **7C**). Gene ontology (GO) analysis of the 35 overlapping genes was performed using the DAVID classification tool (Huang da et al. 2009b; Huang da et al. 2009a). The analysis revealed, besides categories like "developmental protein" or "differentiation", strong associations with "reproduction" or "spermatogenesis" (Figure **7D**). To access whether the altered gene expression following Pcgf6 knockdown overlaps with those after the loss of PRC1 function or the loss of the PRC1.6 subunit L3mbtl2, up-regulated or de-repressed genes in ES cells were compared between Pcgf6 knockdown, Ring1A/B double knockout (dKO) (Endoh et al. 2008) or L3mbtl2 knockout (KO)(Qin et al. 2012) (Figure **7E**). In total, 12 de-repressed genes were shared by Pcgf6 knockdown and Ring1A/B dKO; and 16 de-repressed genes were shared by Pcgf6 knockdown and L3mbtl2 KO. In parallel, 6 genes were found in overlap of all 3 phenotypes. Notably, all 6 genes are involved in spermatogenesis (Dazl, Mael, Piwil2 and Taf7l) or possess testis-specific expression (Stk31, Tex13) (Figure **7E**).

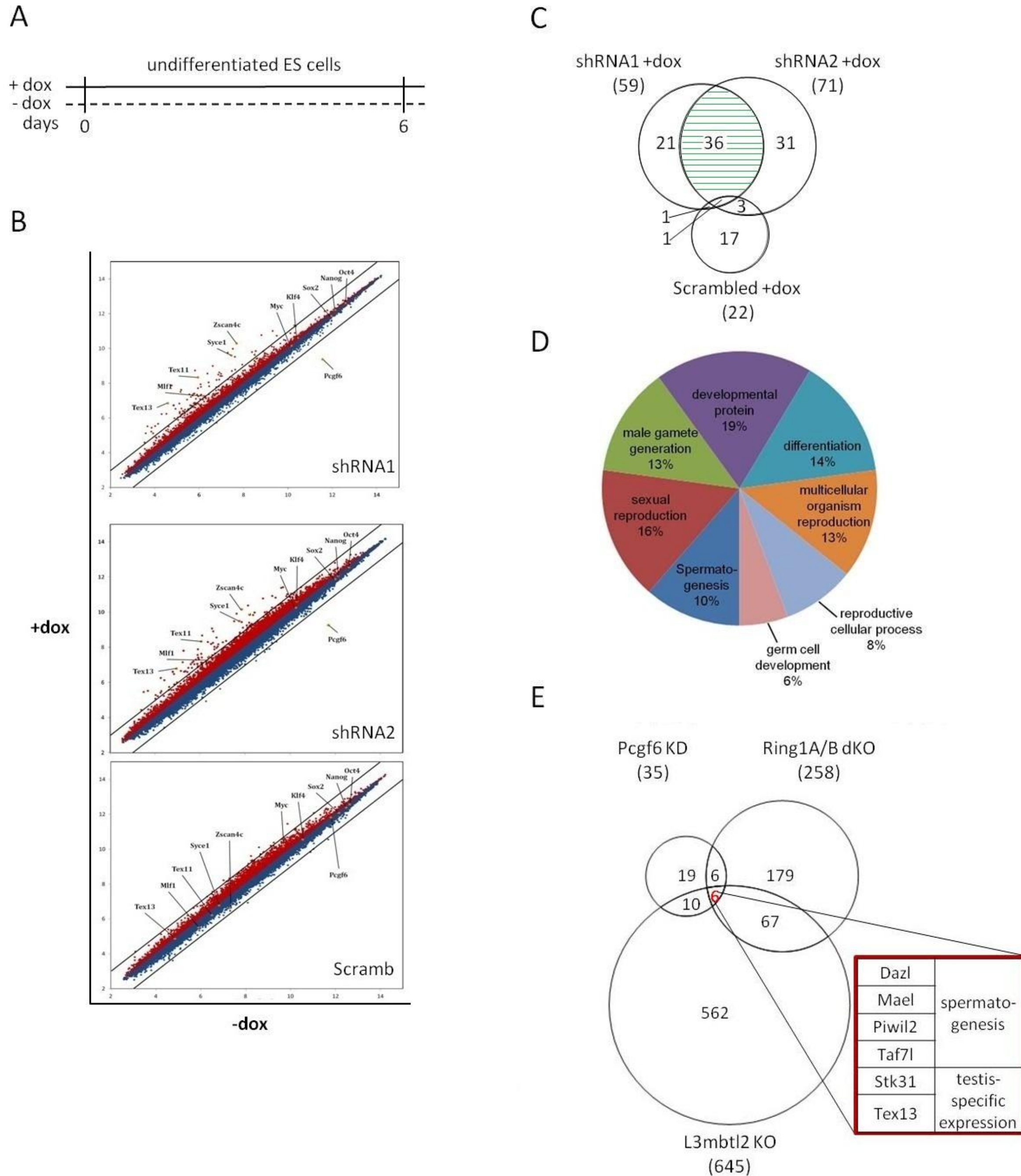


Figure 7: Global gene expression analysis following Pcgf6 knockdown. A) Experimental design. ES cells were treated with dox for 6 days or left untreated before cDNA microarray analysis (Affymetrix). **B**) Pair-wise scatter plot of expression values from the microarray analysis. shRNA1 +dox *versus* –dox (top), shRNA2 +dox *versus* –dox (middle) and Scrambled +dox vs –dox (bottom). Black lines indicate 2-fold changes in expression values. Over-expressed transcripts are indicated by red and under-expressed transcripts by blue. **C)** Venn diagram shows the overlap of annotated genes with altered expression (fold change ≥2) in shRNA1, shRNA2 and Scrambled shRNA ES cell cultures +/- dox. **D)** Gene ontology (GO) categories associated with the overlapping and up-regulated genes in shRNA1 and shRNA2 ES cell cultures +/- dox. GO classification was performed with the DAVID classification tool. **E)** Venn diagram shows the overlap of up-regulated genes in Pcgf6-knockdown (KD), Ring1A/B double knockout (dKO), and L3mbtl2 KO ES cells. Red box shows the overlapping genes.

Pcgf6 knockdown in undifferentiated and differentiating ES cells increases the expression of mesodermal marker genes

Pcgf paralogs Pcgf1, Pcgf2 and Pcgf4 are involved in the regulation of hematopoietic development (Park et al. 2003; Kajiume et al. 2004; Ross et al. 2012). To address whether Pcgf6 also possesses regulatory functions during hematopoietic development, the consequences of Pcgf6 knockdown on ES cell mesodermal and hematopoietic differentiation were analyzed. For this purpose, gene expression of pluripotency markers, lineage markers including markers for early mesoderm, hematopoietic development, endoderm and ectoderm, and also PRC1 and PRC2 members were characterised following Pcgf6 knockdown using qRT-PCR. Analyzed were ES cell cultures (shRNA1, shRNA2 and scrambled shRNA), dox-treated for 6 days or left untreated, and EBs at day 3.5 of differentiation, a time point which approximately marks the *in vitro* onset of early mesodermal gene expression (Figure **8A**) (Pearson et al. 2010).

Upon Pcgf6 knockdown, gene expression of pluripotency markers Oct4, Nanog and Sox2 showed slight down-regulation in undifferentiated ES cells (Figure **8B**, left), but no differences after 3.5 days of differentiation in EBs (Figure **8B**, right). In contrast, transcripts of early mesodermal genes such as T and Tie-2 were up-regulated in both shRNA ES cells and in day 3.5 EBs. Gene expression of early mesoderm markers such as Runx1 and Flk1 showed upon knockdown no alterations in ES cells, but were up-regulation in day 3.5 EBs. In addition, HoxA3, HoxA7 and HoxA9, homeobox genes that previously have been shown to be involved in hematopoietic development (So et al. 2004; Lebert-Ghali et al. 2010; Mahdipour et al. 2011) also showed elevated transcription in day 3.5 EBs upon Pcgf6 knockdown. In contrast, expression of endodermal gene Sox17 and ectodermal genes Nestin and Sox21 were down regulated in both ES cells and in day 3.5 EBs. In addition, gene expression of PRC1 subunits including Pcgf1-5, and subunits that were shown to interact with Pcgf6, such as Ring1B and L3mbtl2, were analyzed. In both undifferentiated ES cells and day 3.5 EBs, the expression of these genes remained unaltered following Pcgf6 knockdown. Also, alterations in gene expression of PRC2 components were not observed.

Taken together, upon Pcgf6 knockdown, gene expression analyses revealed altered expression of lineage markers in undifferentiated ES cells and during differentiation. In Pcgf6 knockdown ES cells, expression of early mesoderm genes such as T were up-

regulated whereas endoderm genes, e.g. Sox17, or ectoderm genes, e.g. Sox21, were down regulated.

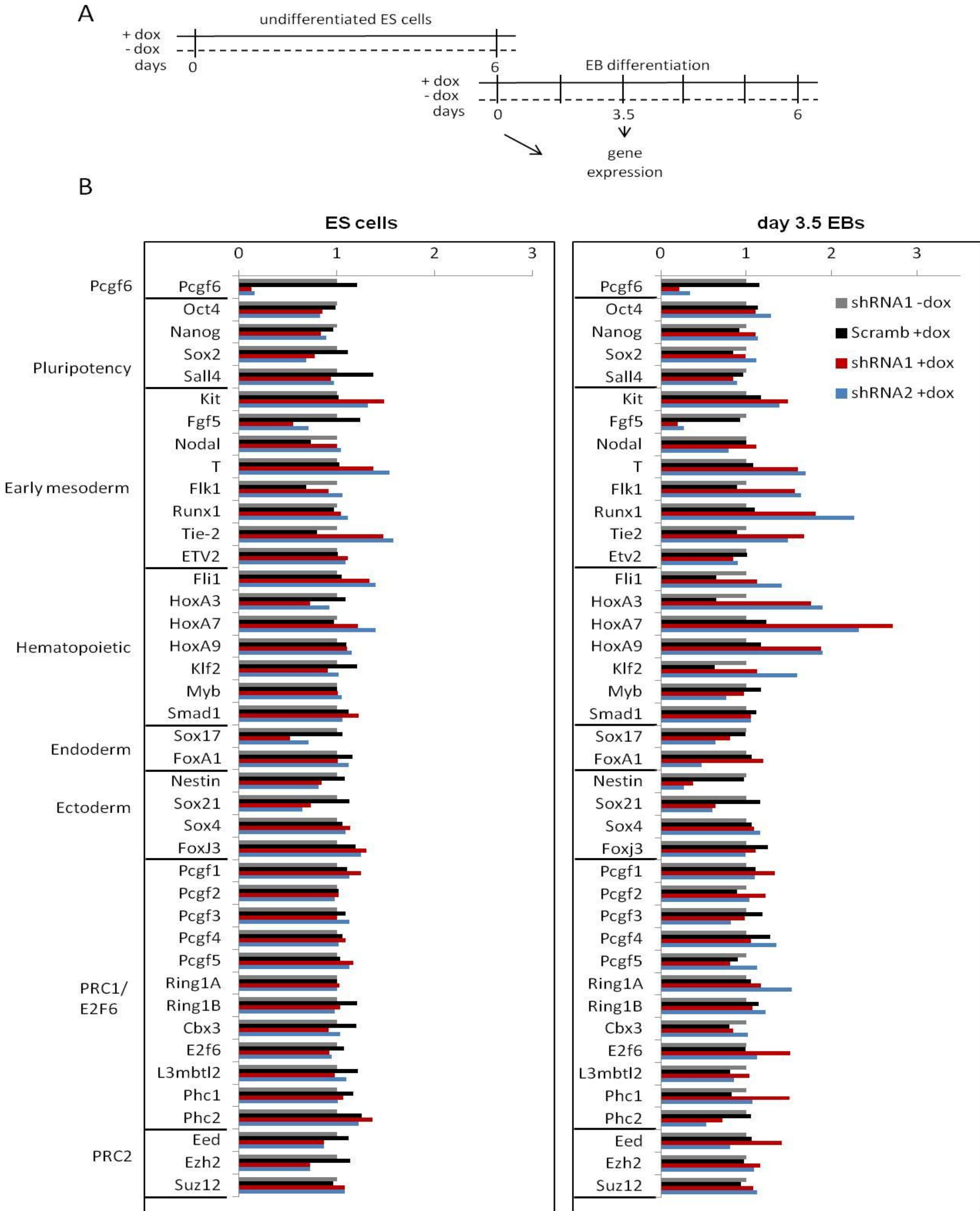


Figure 8. qRT PCR analyses for the expression of pluripotency markers, lineage markers, PRC1 and PRC2 members. A) Experimental design. ES cells were treated with dox for 6 days or left untreated before EB differentiation. Dox-treatment proceeded during EB differentiation. **B)** Relative gene expression levels of pluripotency markers, lineage markers, PRC1 and PRC2 members in undifferentiated ES cells and in day 3.5 RBs. Shown are quantitative comparisons relative to Hprt and Rpl113a expression, using the $2^{-\Delta\Delta T}$ method. Untreated shRNA1 cultures (shRNA -dox) were set to 1. n=1.

5.8 Pcgf6 knockdown in ES cells caused transiently increased up-regulation of early mesodermal/hematopoietic marker genes during ES cell differentiation

As Pcgf6 is down-regulated during ES cell differentiation (Figure **1B**), the next question was whether the differentially expressed genes in day 3.5 EBs, caused by Pcgf6 knockdown, retained their altered expression levels during ES cell differentiation. To answer this, expression levels of Pcgf6, Oct4 and 3 early mesodermal/hematopoietic marker genes were analyzed at different time points of ES cell differentiation +/- dox (Figure **9A**). As expected, Pcgf6 transcript levels were knocked down in dox-treated shRNA1 and shRNA2 ES cell cultures (Figure **9B**). In addition, while Oct4 transcript levels in dox-treated cultures showed no alterations compared to untreated and scrambled shRNA cultures, expression of early mesodermal markers T, Fkl1 and Runx1 were temporally increased in dox-treated shRNA1 and shRNA2 cultures at day 3.5 of differentiation. On day 6 of differentiation, expression of these markers in dox-treated and untreated cultures was similar. Taken together, early mesodermal markers T, Flk1 and Runx1 were temporally up-regulated during ES cell differentiation.

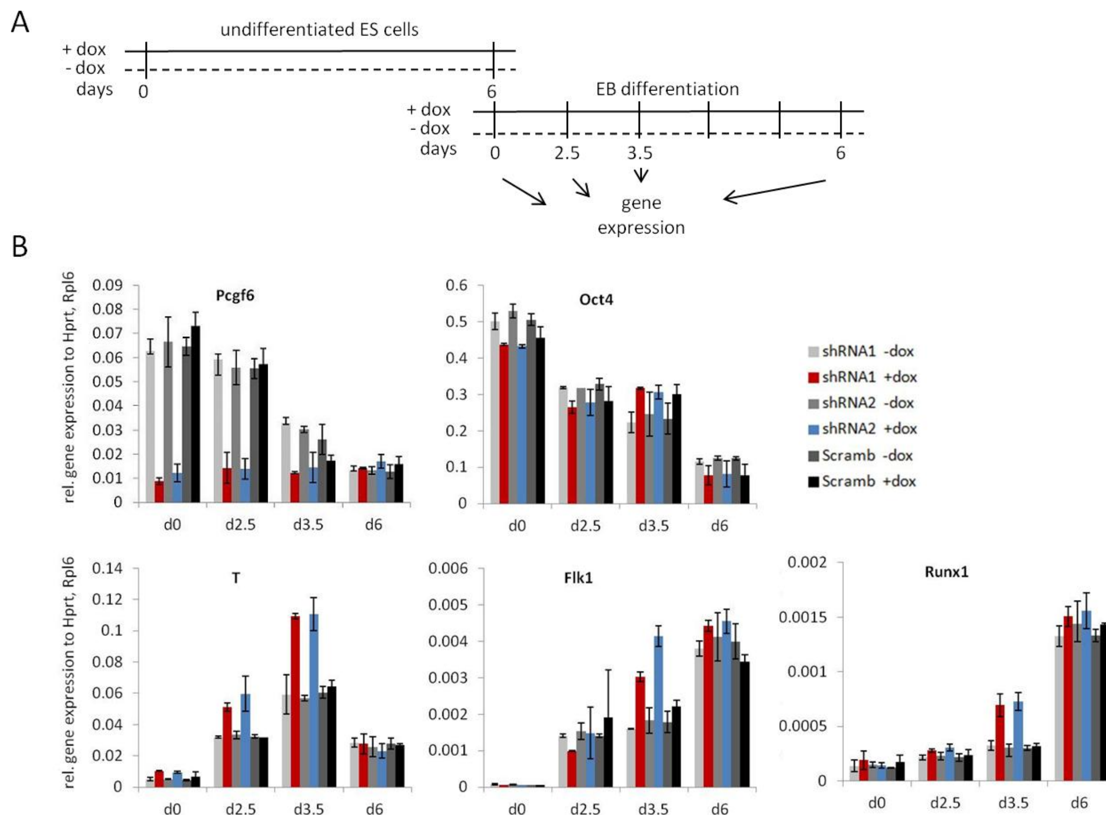


Figure 9. qRT PCR analyses for the expression of early mesodermal/ hematopoietic markers A) Experimental design. ES cells were treated with dox for 6 days or left untreated before set into EB differentiation. Dox-treatment proceeded during EB differentiation. **B)** Relative expression levels of Pcgf6, Oct4 and early mesodermal/hematopoietic markers in undifferentiated ES cells and in EBs. Expression was assayed by qRT-PCR. Shown are quantitative comparisons relative to Hprt expression, using the $2^{-\Delta\Delta T}$ method. n=3.

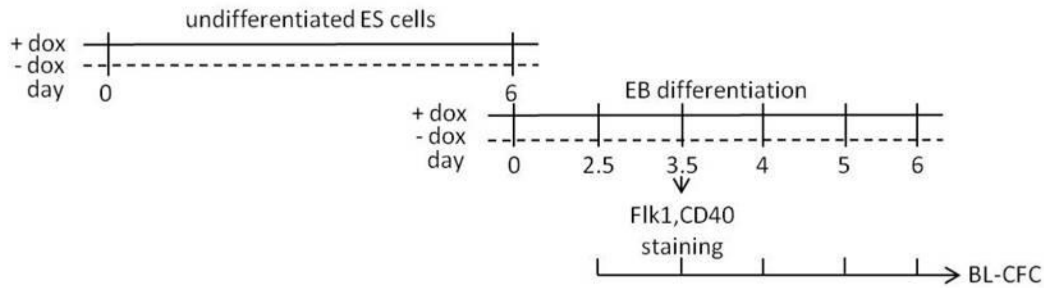
5.9 Pcgf6 knockdown increases ES cell hemangioblast differentiation

To address whether upon Pcgf6 knockdown the altered gene expression affects lineage outcomes in differentiating ES cell cultures, the development of hemangioblasts, which are common precursors of both hematopoietic and endothelial lineages, was probed. During development of hemangioblasts, the vascular endothelial growth factor (VEGF) receptor Flk-1 plays a key role (Shalaby et al. 1997). *Flk1*$^{+}$ cells rise after 3 to 4 days of EB differentiation. These cells were shown to be the *in vitro* equivalents of hemangioblasts (Kennedy et al. 1997; Choi et al. 1998; Faloon et al. 2000). In methylcellulose cultures (MC) these cells can generate blast colonies (blast colony-forming cells, BL-CFC). Before the onset of Flk1 expression, the early mesoderm marker CD40 is expressed on mesodermal precursor cells. At the hemangioblast stage, Flk1 and CD40 are co-expressed (Pearson et al. 2010). To monitor ES cell-derived hemangioblast development under Pcgf6 knockdown, I analyzed cell surface expression of Flk1 and CD40 in day 3.5 EB cells (shRNA1, shRNA2 and scrambled shRNA ES cells) +/- dox (Figure **10A**). As shown in Figure **10B**, of day3.5 EBs the frequency of *Flk1*$^{+}$ cells and *CD40*$^{+}$ *cells* increased upon dox-induced Pcgf6 knockdown. To monitor the exit from pluripotency, ectopic expression of an Oct4-eGFP transgene in OG2 ES cells was measured using FACS (Figure **10B**). As expected, *Flk1*$^{+}$ cells or *CD40*$^{+}$ cells were Oct4-eGFP negative.

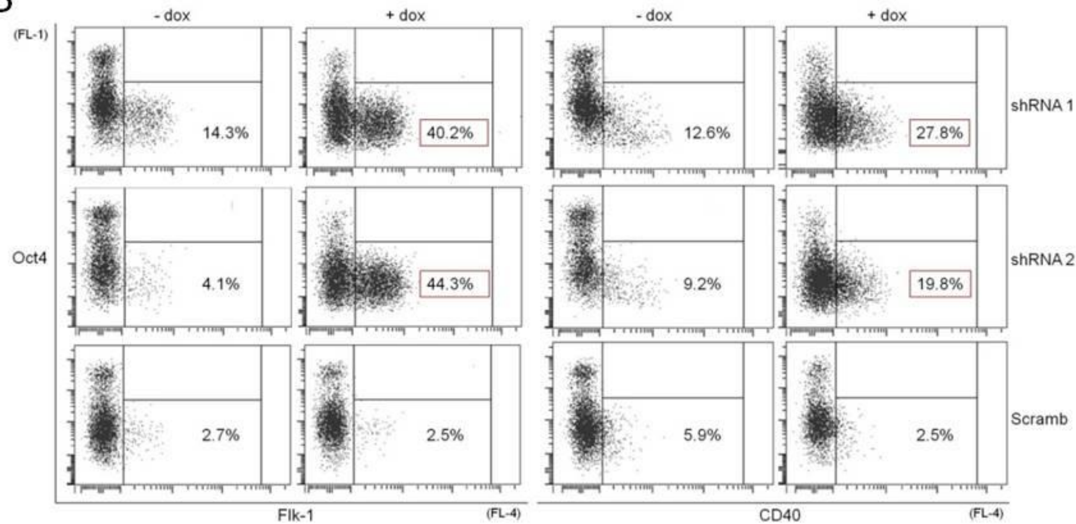
To analyze BL-CFC frequencies, single cell suspensions of EBs at different time points of differentiation were cultured in MC cultures supplemented with growth factors that favor endothelial and hematopoietic differentiation (Figure **10A**). As shown in Figure **10C**, both dox-treated and untreated EBs differentiating for 4 days showed the highest numbers of BL-CFCs. However, dox-treated shRNA1 and shRNA2 EB cells differentiating for 2.5, 3.5 or 4 days generated significantly elevated BL-CFC numbers compared to dox-treated scrambled control. BL-CFC frequencies in dox-treated shRNA cultures ranges from 600 to 800 per $5x10^4$ cells derived from 4 days old EBs while untreated day 4 EBs generated on average 500 BL-CFCs per $5x10^4$ cells.

In summary, Pcgf6 knockdown in ES cells caused increased frequencies of *Flk1*$^{+}$ and *CD40*$^{+}$ cells at day 3.5 of EB differentiation that was associated with elevated levels of BL-CFC formation. These results suggest a negative regulatory role of Pcgf6 for ES cell hemangioblast formation.

A



B



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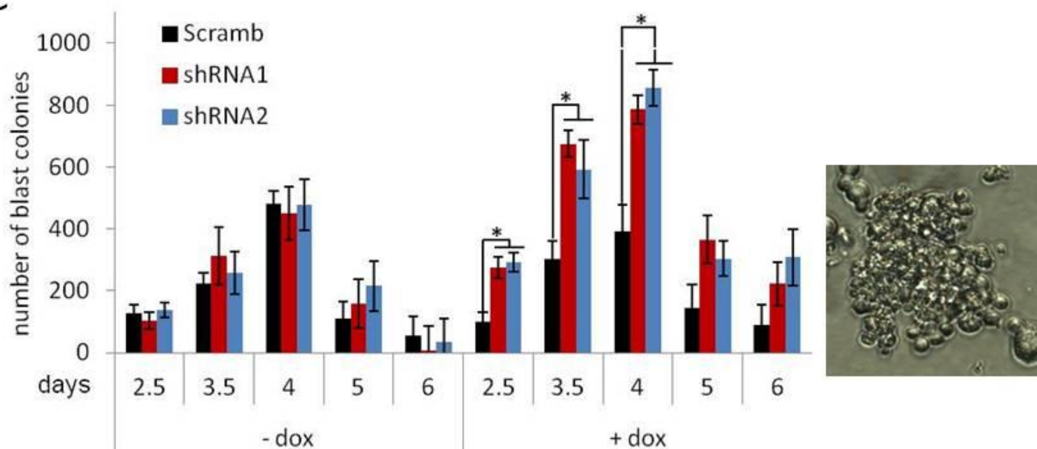

Figure 10. Pcgf6 knockdown increases blast colony formation. A) Experimental design. ES cells were dox-treated for 6 days or left untreated before differentiation into EBs was induced. After 3 days of differentiation, hemangioblast markers Flk1 and CD40 were analyzed, or EB cells were seeded into methylcellulose cultures containing growth factors for hemangioblast development. After 3-5 days blast colonies were counted. **B)** Flk1 and CD40 expression in day 3.5 EB cultures +/- dox. Analysed were shRNA1, shRNA2 and scrambled ES cells. Blotted are Flk1/Oct4-eGFP and CD40/Oct4-eGFP signals. Indicated are Oct4-eGFP$^-$/Flk1$^+$ and Oct4$^-$eGFP-/CD40$^+$ cell frequencies. A representative analysis is shown. n=3. **C)** Numbers of blast colonies generated by day 2 to day 6 EBs (shRNA1, shRNA2 and scrambled shRNA ES cells +/- dox). n=3. Insert shows a typical blast colony. *, p<0.01

5.10 Pcgf6 knockdown enhances ES cell differentiation towards hematopoietic lineages

The augmented development of hemangioblasts upon Pcgf6 knockdown prompted me to analyze the hematopoietic capacity of EB cultures that derived from Pcgf6 knockdown-ES cells. Hematopoietic progenitors develop sequentially within EBs. A first step is represented by transient BL-CFCs that are followed by a primitive erythroid wave at day 4 to day 8 of differentiation (Keller et al. 1993). Definitive erythroid and myeloid progenitors develop shortly after primitive erythroid progenitors. To determine whether knockdown of Pcgf6 promotes hematopoietic progenitor cell development, cells from 6, 7 or 9 days old EBs (shRNA1, shRNA2 and scrambled ES cells) +/- dox were replated into MC medium containing a cocktail of growth factors for hematopoietic differentiation (Figure **11A**). As shown in Figure **11B**, significantly increased numbers of primitive erythroid (BFU-E) and multipotential colonies *(CFU-GM) were* observed in dox-treated shRNA1 and shRNA2 *cultures*. Representative morphology of colony types is shown in Figure **11C**.

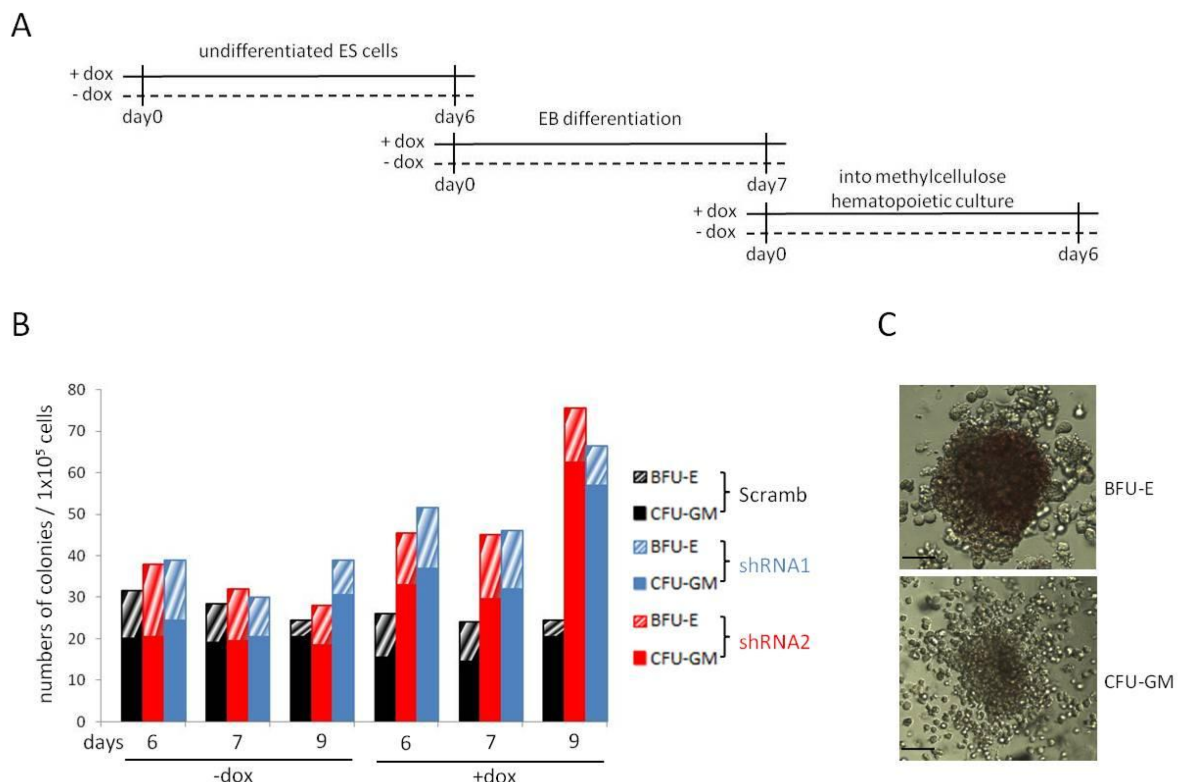

Figure 11. Hematopoietic development upon Pcgf6 knockdown. A) Experimental design: ES cells were treated with dox for 6 days or left untreated before differentiation into EBs. After 7 days of differentiation, EB cells were seeded into MC cultures containing hematopoietic growth factors (mSCF, mTPO, hEPO, mIL-3, mIL-6 and mGM-CSF). After 5-6 days hematopoietic colonies were counted. **B)** Numbers of hematopoietic colonies per 10^5 day 7 EB cells. n = 3. *, p<0.01. **C)** Representative pictures of CFU-GM- and BFU-E-type colonies under phase contrast microscopy.

6 Discussion

The aim of this thesis was to study the function of the Pcgf paralog Pcgf6 via inducible shRNA-targeted knockdown in ES cells. In summary, consequences of Pcgf6 knockdown include reduced ES cell colony formation, increased expression of differentiation associated genes, in particular spermatogenesis-specific and mesodermal genes, and increased *in vitro* ES cell hemangioblast formation and hematopoietic differentiation.
The results will be further discussed in the following paragraphs.

6.1 Differential gene expression of Pcgf paralogs suggests paralog-specific functions

Paralogs of the Pcgf gene family encode for a group of proteins (Pcgf1-Pcgf6) that directly interact with Ring1A and Ring1B, the E3 ubiquitin ligases and subunits common to all forms of PRC1 (Gao et al. 2012). At the functional level, Pcgf paralogs define distinct types of PRC1 (PRC1.1–PRC1.6) that bind to different target genes (Gao et al. 2012). In undifferentiated ES cells, several different PRC1 types were reported recently: PRC1.1 (PRC1 containing Pcgf1, Rybp, Fbxl10) (Wu et al. 2013), 2 subtypes of PRC1.2 (PRC1 containing Pcgf2, Rybp or Pcgf2, Cbx7) (Morey et al. 2012; O'Loghlen et al. 2012; Tavares et al. 2012) and PRC1.6 (PRC1 containing Pcgf6, Rybp, L3mbtl2) (Qin et al. 2012). Among these PRC1 types, PRC1.2 binding sites are marked by H3K27me3 which is catalyzed by PRC2 (Tavares et al. 2012). In contrast, PRC1.1 and PRC1.6 were shown to be functionally independent from the activities of PRC2 (Wu et al. 2013). While PRC1.1 requires its co-factor Fbxl10 for target binding (Qin et al. 2012; Wu et al. 2013), PRC1.6 shares subunits with the E2F6-complex (Qin et al. 2012), and is likely to interact with its target sites through L3mbtl2 (Qin et al. 2012). The existence of multiple PRC1 types in undifferentiated ES cells points to complex and combinatorial PRC1-dependent regulation. Notably, ES cell differentiation is accompanied by alterations in the PRC1 repertoire. For example, PRC1.2 (containing Pcgf2, Cbx7) is substituted by PRC1.4 (containing Pcgf4, Cbx2 and Cbx4) (Tavares et al. 2012). Findings in this study confirmed this observation by showing alterations in expression levels of Pcgf paralogs upon ES cell differentiation (Figure **1B**). A down-regulation of Pcgf2 transcripts was not observed in this study although a slight reduction on Pcgf2 protein level upon differentiation was published (Tavares et al. 2012). This could be explained by cell type variance or predominant regulation of Pcgf2 on the translational level. Gene expression

analysis of Pcgf paralogs in this study argues for their developmental stage-depended functions. In addition, the co-expression of Pcgf paralogs appears to be cell type-specific. For example, high expression levels of Pcgf4, Pcgf5 and Pcgf6 were found in adult testis, while this combination was not observed in ES cells (Figure **1A**). Taken together, expression of Pcgf paralogs is cell type-specific and depends on the developmental stage.

6.2 shRNA-based Pcgf6 knockdown in ES cells was inducible and reversible

To study the functions of Pcgf6 in ES cells stable and specific knockdown of Pcgf6 transcripts via shRNAs was established in this study. Using a published 2-step-RMCE system (Seibler et al. 2005; Seibler et al. 2007), Pcgf6 transcript level was knocked down via doxycycline-induced shRNA expression, and it could be restored after withdrawal of doxycycline. These results showed that the shRNA-knockdown system was working as expected. The controllable knockdown of Pcgf6 via RNA interference (RNAi) in ES cells was not only suitable for the *in vitro* applications in this study, but will provided a basis for reproducible *in vivo* applications via blastocyst injection (Seibler et al. 2007) in future studies. In particular, the site-specific insertion of shRNAs into the rosa26 locus excluded any unique and irreproducible shRNA expression which can be generated by e.g. lentiviral infection (Bahi et al. 2005; Seibler et al. 2007).

6.3 Pcgf6 knockdown led to reduced ES cell colony formation, but did not affect ES cell morphology, proliferation, cell cycle distribution or cell survival

PRC1 is required for the maintenance of ES cell identity. ES cells lacking Ring1A and Ring1B (Ring1A/B-dKO ES cells) exhibited decreased proliferation and gradual loss of typical ES cell morphology (Endoh et al. 2008). These phenotypes, however, were not observed in Ring1B-KO ES cells, indicating compensation by Ring1A and functional overlap between the Ring1 paralogs (de Napoles et al. 2004; Endoh et al. 2008). Such functional overlap was also observed between different Pcgf paralogs. For example, while knockout of Pcgf2 or Pcgf4 in mouse led to partially overlapping phenotypes (van der Lugt et al. 1994; Akasaka et al. 1996; van der Lugt et al. 1996; Akasaka et al. 2001), mice double deficient for Pcgf2 and Pcgf4 exhibited unique phenotypes (Akasaka et al. 2001). In this study, knockdown of Pcgf6 did not affect ES cell morphology, proliferation or cell cycle phase distribution (Figure **5C**, **5D**). Only a weak trend towards an increased apoptosis rate upon Pcgf6 knockdown was observed (Figure **5B**). Considering these results together with a possible compensation by other active Pcgf paralogs, Pcgf6 is at

least not essential for the regulation of ES cell proliferation, cell cycle distribution or cell survival.

Notably, Pcgf6 knockdown in ES cells resulted in significantly reduced colony numbers in this study, paralleled by decreased cell numbers (Figure **4B**, **C**). This was surprising because ES cell proliferation, cell cycle phase distribution or apoptosis after Pcgf6 knockdown did not show significant changes. As shown in several previous studies, regulation of intercellular adhesion and colony formation of ES cells involves the Ca^{2+}-dependent cell-cell-adhesion protein E-cadherin (Larue et al. 1996; Dang et al. 2004; Redmer et al. 2011). However, gene expression of E-cadherin in ES cells was not affected after Pcgf6 knockdown (Microarray analysis, data not shown), suggesting that Pcgf6 is not essential for E-cadherin regulation. Therefore, Pcgf6 may be involved in the regulation of ES cell colony formation through other yet unknown mechanisms.

6.4 Pcgf6 is not required for gene expression of other Pcgf paralogs, PRC1 or PRC2 core subunits in ES cells

Loss-of-function studies of Pcgf2 and Pcgf4 in mouse revealed important functions of Pcgf paralogs in mammalian development. As Pcgf2 was shown to regulate cell proliferation and senescence as an up-stream repressor of Pcgf4 (Guo et al. 2007), Pcgf paralogs may regulate each other's expression. Although there is no evidence so far that Pcgf4 directly targets its paralogs, loss of Pcgf4 in MEFs led to up-regulation of Pcgf2, Pcgf3, Pcgf5, Pcgf6 but down-regulation of Pcgf1 (Global gene expression analysis in: (Kallin et al. 2009)). In this study, altered gene expression of Pcgf1-Pcgf5 were not observed upon Pcgf6 knockdown in undifferentiated ES cells (Figure **3D**). Further, expression of PRC1 or PRC2 core subunits, such as Ring1A and Ring1B or Eed, Ezh2 and Suz12, were also not affected in Pcgf6 knockdown ES cells or EBs. In summary, these results indicate that Pcgf6 is not involved in the regulation of other Pcgf paralogs or PRC core subunits.

6.5 Pcgf6 is involved in regulating expression of pluripotency genes Oct4, Sox2 and Nanog

In this study, shRNA mediated knockdown of Pcgf6 led to reduced expression of the pluripotency genes Oct4, Sox2 and Nanog in undifferentiated ES cells (Figure **8B**, **9B**). The link between Pcgf6 and pluripotency genes was first reported in a genome-wide RNAi screen in mES cells that was setup to identify novel pluripotency factors. In this

RNAi screen, Pcgf6 knockdown via 2 different siRNAs led to decreased transcript levels of Oct4, Sox2 and Nanog, paralleled by a tendency of knockdown-ES cells to spontaneously differentiate (Hu et al. 2009). However, the tendency to spontaneous differentiation of Pcgf6 knockdown-ES cells was not observed in this study. Taken into account that the knockdown efficiencies of Pcgf6 transcript level were similar in both studies (Hu et al. 2009)(Figure **3D**), this phenotypic difference may be explained by variations in cell culture conditions. For example, unlike in the RNAi screen, ES cells were always cultured on MEFs in this study. Taken together, results of this study confirmed that Pcgf6 plays a role in ES cell pluripotency by being involved in transcriptional regulation of pluripotency genes such as Oct4, Sox2 and Nanog.

6.6 Pcgf6 is required for the repression of differentiation-associated genes and spermatogenesis-specific genes

PRC1 function is essential for maintaining the undifferentiated state of ES cells by repressing the expression of differentiation-associated genes (Boyer et al. 2006; Leeb and Wutz 2007; Stock et al. 2007; Endoh et al. 2008; van der Stoop et al. 2008; Leeb et al. 2010). Knockdown of Pcgf6 in ES cells resulted in altered expression of a set of genes which were mostly up-regulated or, considering the repressive functions of PRC1, de-repressed (Figure **7B**). The expression pattern of Pcgf6 knockdown-ES cells only partially overlapped with expression patterns that were previously described for ES cells lacking other PRC1 subunits. For example, ES cells lacking both Ring1A and Ring1B (Ring1A/B-dKO ES cells) showed de-repression of several lineage-specific genes. Among these genes, only a small set of testis/spermatogenesis genes were also found to be de-repressed after Pcgf6 knockdown (Figure **7E**). Notably, one of these testis/spermatogenesis genes, Tex13, was also de-repressed in ES cells lacking the PRC2 subunit Eed (Schoeftner et al. 2006; Leeb et al. 2010). Eed-KO led to loss of H3K27 mono-, di- and trimethylation (Montgomery et al. 2005; Montgomery et al. 2007). Because PRC1.6 (contains Pcgf6) was reported to act independently of PRC2 (Qin et al. 2012; Tavares et al. 2012), it is possible that certain genes like Tex13 are targeted by multiple PRC1 subtypes.

Overall the expression pattern overlap between L3mbtl2 KO-ES cells and Pcgf6 knockdown-ES cells was relatively small (Figure **7E**). L3mbtl2 is a PRC1.6 subunit that is required for PRC1.6 target binding in ES cells. Loss of L3mbtl2 in ES cells caused de-repression of genes involved in development, differentiation, spermatogenesis and cell

growth (Gao et al. 2012; Qin et al. 2012). Both Pcgf6 and L3mbtl2 were previously identified as members of the PRC1.6, it was therefore unexpected that only less than 50% of de-repressed genes in Pcgf6 knockdown-ES cells overlap with de-repressed genes in L3mbtl2KO ES cells. Also the comparison between the de-repressed genes in Ring1A/B-dKO ES cells (Endoh et al. 2008), in L3mbtl2 KO-ES cells (Qin et al. 2012) or in Pcgf6 knockdown-ES cells revealed only a small overlap which included 6 spermatogenesis- or testis-specific genes (Figure **7E**). These data suggest that Pcgf6 may have non-overlapping functions which are independent from L3mbtl2 or Ring1A/B.

Interestingly, loss of Pcgf4 in MEFs (Kallin et al. 2009) led to up-regulation of 3 of these genes (Dazl, Mael and Tex13) and down-regulation of the other 3 (Piwil2, Stk31 and Taf7l). These observations suggest that Pcgf6 may be involved in repressing several testis-specific genes in undifferentiated ES cells possibly in concert with other PRC1 members.

Another notable observation was the partially opposing alterations in expression of pluripotency or lineage-specific genes in L3mbtl2 KO-ES cells and in Pcgf6 knockdown-ES cells. First, loss of L3mbtl2 did not affect the expression of Oct4 or Nanog in undifferentiated ES cells (Qin et al. 2012). Unlike in L3mbtl2 KO-ES cells, the expression of Oct4, Nanog and Sox2 was slightly reduced in undifferentiated Pcgf6 knockdown-ES cells. Second, endoderm marker Sox17 was down-regulated in both undifferentiated Pcgf6 knockdown ES cells and EBs. In contrast, increased expression of Sox17 was observed in undifferentiated L3mbtl2 KO-ES cells and Ring1B KO-ES cells (Leeb et al. 2010; Qin et al. 2012). Third, upon Pcgf6 knockdown, expression levels of mesodermal markers T, Flk1 and Runx1 were increased in both ES cells and day 3 EBs. In day 6 EBs, T was down-regulated to a similar level as in scrambled control cultures. Also in Ring1A/B dKO-ES cells, up-regulation of T was observed (Endoh et al. 2008). In contrast, T was down-regulated in undifferentiated L3mbtl2 KO-ES cells, and in day 12 L3mbtl2 KO-EBs T was aberrantly up-regulated (Qin et al. 2012). Overlapping effects were observed on reduced expression of some ectoderm genes in ES cells and EBs, albeit different genes were affected upon L3mntl2 KO (e.g. Sox11) or Pcgf6 knockdown (e.g. Sox21). Taken together, these data again suggest that Pcgf6 may also function in ES cells independently form L3mbtl2 or Ring1A/B. This idea has been supported by the following findings: first, Pcgf6 directly interacts with Jarid1d, an H3K4me3 demethylase not being reported as a part of PRC1 complexes, in human somatic cells (Lee et al. 2007); and second, in contrast to Pcgf6 knockdown-ES cells, the expression of pluripotency

genes such as Oct4, Nanog or Sox2 was not down-regulated in Ring1B KO or Ring1A/B dKO or L3mbtl2 KO-ES cells (Endoh et al. 2008; Qin et al. 2012).

6.7 Pcgf6 is not required for maintaining the global level of H2AK119 histone marks

A number of studies have demonstrated that mono-ubiquitination of histone H2A at lysine 119 is important in PcG-mediated silencing, with Ring1A/B as the E3 ligase in this process (de Napoles et al. 2004; Wang et al. 2004; Endoh et al. 2008; Luis et al. 2012). Pcgf paralogs Pcgf1, Pcgf2 and Pcgf4 were shown to be involved in Ring1A and Ring1B-dependent H2AK119 ubiquitination. For example, knockdown of Pcgf1 in ES cells (Wu et al. 2013) or knockout of Pcgf4 in somatic cells caused global reduction of H2AK119ub levels (Cao et al. 2005). In addition, mutations of Pcgf2 protein sequence led to less efficient ubiquitination of nucleosomes *in vitro* (Elderkin et al. 2007). In this study, global alterations of the H2AK119ub levels, as well as levels of other known polycomb-associated histone modifications, such as H3K4me3, H3K9me3 or H3K27me3 were not detected upon Pcgf6 knockdown. One possible explanation is that the detection method (Western blot) was probably not sensitive enough because other PRC1 complexes were still active while Pcgf6 was knocked down. Nevertheless, it is possible that local H2AK119ub levels at promoter regions of Pcgf6 target genes changed after Pcgf6 knockdown. Future ChIP analysis will address this issue.

6.8 Pcgf6 is involved in maintaining ES cell identity by preventing mesodermal/ hematopoietic differentiation

Pcgf paralogs are involved in self-renewal and differentiation of hematopoietic cells (Kajiume et al. 2009; Konuma et al. 2010; Ding et al. 2012; Kajiume et al. 2012). Mutant animal models suggested essential functions of Pcgf2, Pcgf4 and Ring1B in adult hematopoiesis (van der Lugt et al. 1994; Park et al. 2003; Iwama et al. 2004; Kajiume et al. 2004; Oguro et al. 2006; Cales et al. 2008). In addition, Loss of PRC1 subunits Pcgf2, Pcgf4, Phc1, Phc2, Ring1A, Ring1B, and Cbx2 cause impaired hematopoietic stem cell (HSC) function (Sauvageau and Sauvageau 2010). However, the roles of Pcgf paralogs during the initiation of the embryonic hematopoiesis still remain largely unknown. The initiation of hematopoiesis in the mouse embryo was believed to take place in the yolk sac where blood islands consisting of primitive erythroid cells surrounded by a layer of angioblasts develop around day 7.5 of gestation (Moore and Metcalf 1970; Haar and

Ackerman 1971). The hypothesis that these progenitors arise from a common precursor, the hemangioblast (Sabin 1917; Murray 1932), could be corroborated in later studies (Kennedy et al. 1997). New insights in the origins of the hemangioblast were added by another study, as hemagioblasts were detected at maximum frequency in the posterior streak region of the neural plate-stage mouse embryo (Huber et al. 2004), indicating that the initial stages of hematopoietic and vascular commitment take place in the posterior primitive streak mesoderm before blood island development in the yolk sac. During *in vitro* differentiation of ES cells, a precursor can be transiently identified within 2 – 4 days of EB differentiation that generates blast colonies containing both primitive hematopoietic and endothelial progenitors (Choi et al. 1998). Because this so-called blast colony forming cell (BL-CFC) expresses T-box transcription factor T and Flk-1, and shares the same differentiation potential with the hemangioblast, it is considered to be the *in vitro* equivalent of the common precursor (Kennedy et al. 1997; Choi et al. 1998; Nishikawa et al. 1998; Ogawa et al. 2001). With progression of differentiation, primitive/definitive hematopoietic progenitors appear within 6 to 10 days old EBs. A hint that Pcgf paralogs could play roles in the initiation of hematopoiesis was given by a recent study (Ding et al. 2012).The authors demonstrated that ectopic expression of Pcgf4 in ES cells promoted ES cell derived hematopoietic cell development (Ding et al. 2012). Using Pcgf6 knockdown-ES cells, regulatory functions of Pcgf6 during ES cell derived hematopoietic development were demonstrated in this study. In concert with the up-regulation of early mesoderm/hematopoietic genes, directed differentiation of Pcgf6 knockdown-ES cells showed enhanced blast colony formation and increased number of hematopoietic colonies (Figure **10**, **11**). Altogether, results of this study suggest that Pcgf6 is involved in maintaining ES cell identity by repressing mesodermal/hematopoietic development in undifferentiated ES cells. Therefore, Pcgf6 may play a role in regulating the initiation of embryonic hematopoiesis.

6.9 Future perspectives

6.9.1 Overexpression of Pcgf6 in ES cells will provide more insight into the roles of Pcgf6 in maintaining ES cell identity

On certain developmental stages, factors with key regulatory functions act in a dosage-dependent manner. For example, repression or overexpression of Oct4 leads to a loss of ES cell characteristics and induces differentiation towards the trophectodermal or meso- and endodermal lineages, respectively (Nichols et al. 1998; Niwa et al. 2000).

Analysis on Ring1A mutant mice offered an example for dose-dependent PcG gene function. In both Ring1A$^{-/-}$ and Ring1A$^{+/-}$ mice, anterior transformations and abnormalities of the axial skeleton were observed indicating the importance of Ring1A gene dosage for axial skeleton patterning (del Mar Lorente et al. 2000). Therefore, overexpression of Pcgf6 in ES cells or sustained expression of Pcgf6 during ES cell differentiation will provide more insight into the roles Pcgf6.

6.9.2 Proteomic analysis will reveal interaction partners of Pcgf6

In this study, several questions still remain open due to the lack of information about Pcgf6 interaction partners. For example, is Pcgf6 involved in any PRC1-independent pathways or complexes that play a role in ES cells? We currently are working on ectopic expression of Pcgf6 in ES cells via an Avi-tagged Pcgf6-ORF expression vector (http://www.genecopoeia.com/tech/avitag-biotinylation-tag/). The Avi-tagged Pcgf6 protein will be biotinylated *in vivo*, and subsequently purified *in vitro* by specific binding of streptavidin. Using mass spectrometry, Pcgf6 binding partners will be identified.

6.9.3 The role of Pcgf6 in embryonic spermatogenesis will be addressed via ES cell-derived primordial germ cell like cell (PGCLC) differentiation

Analysis of Pcgf6 knockdown in ES cells revealed regulatory functions of Pcgf6 on expression of testis/spermatogenesis genes. In addition, Pcgf6 is highly expressed in mouse testis. To functionally address the roles of Pcgf6 during embryonic spermatogenesis, we currently are establishing a stable differentiation assay that drives ES cells to differentiate into PGCLCs (Hayashi et al. 2011). Using this assay, the Pcgf6 function for PGCLCT development will be analyzed.

7 Material and Methods

7.1 Material

7.1.1 Mouse ES cell lines

Name	Genetic background	Specific feature	Source
V6.5	129/Sv X C57BL6	wild type	open biosystems
OG2	CBA X C57BL6	GFP under transgenic Oct4 promoter control	kindly provided by Prof. Schoeler

7.1.2 Cell culture media and supplements

Standard ES cell medium

Ingredient	Concentration	Volume	Distributor
DMEM high glucose	fill to 100%	400 ml	PAA
FCS, ES cell tested	15%	75 ml	PAA
Sodium pyruvate	1 mM	5 ml	PAA
Penicillin, Streptomycin	100 U/ml, 0.1 mg/ml	5 ml	PAA
L-Glutamine	2 mM	5 ml	PAA
Non-essential aminoacids	1x	5 ml	PAA
Beta-Mercaptoethanol	0.1 mM	3.5 µl in 5 ml Hepes	Sigma, PAA
LIF-conditioned medium		500 µl	own production

ES cell primary differentiation medium (EB medium)

Ingredient	Concentration	Volume	Distributor
IMDM high glucose	fill to 100%	425 ml	PAA
FCS, cell culture tested	10%	50 ml	Gibco
Sodium pyruvate	1 mM	5 ml	PAA
Penicillin, Streptomycin	100 U/ml, 0.1 mg/ml	5 ml	PAA
L-Glutamine	2 mM	5 ml	PAA
Non-essential aminoacids	1x	5 ml	PAA
beta-Mercaptoethanol	0.1 mM	3.5 µl in 5 ml Hepes	Sigma, PAA

Methylcellulose-based medium for blast colony formation

Ingredient	Concentration	Distributor
IMDM	fill to 100%	PAA
Methylcellulose	1%	Stem Cell Technologies
FCS, cell culture tested	10%	Gibco
MTG	4.5 x10^{-4} M	Sigma
mouse SCF	100ng/ml	PeproTech
mouse VEGF	5 ng/ml	PeproTech
human IL-6	10ng/ml	PeproTech
Holo-transferrin	200µg/ml	Sigma
Ascorbic acid	25 µg/ml	Sigma
L–Glutamine	2 mM	PAA
Penicillin, Streptomycin	1:100 dilution	PAA
Thrombopoietin (TPO)	25ng/ml	PeproTech

Methylcellulose-based medium for hematopoietic differentiation

Ingredient	Concentration	Distributor
IMDM	fill to 100%	PAA
Methylcellulose	1%	Stem Cell Technologies
FCS, cell culture tested	10%	Gibco
MTG	4.5 x10^-4 M	Sigma
L–Glutamine	2 mM	PAA
BIT9500 (1% BSA, 10µg/ml Insulin, 200µg/ml Transferrin)	20%	Stem Cell Technologies
mouse SCF	150 ng/ml	PeproTech
human IL-3	30 ng/mL	PeproTech
human IL-6	30 ng/ml	PeproTech
GM-CSF	50 ng/ml	PeproTech
Epo	3 Units/ml	kindly provided by Prof. Sirén

MEF medium

Ingredient	Concentration	Volume	Distributor
IMDM low glucose	fill to 100%	500 ml	PAA
FCS, cell culture tested	10%	50 ml	PAA
Sodium pyruvate	1 mM	5 ml	PAA
Penicillin, Streptomycin	100 U/ml, 0.1 mg/ml	5 ml	PAA
L-Glutamine	2 mM	5 ml	PAA

7.1.3 Antibodies

Primary antibodies

Target	Species	Distributor
H2AK119ub	rabbit, monoclonal	Cell Signaling
H2AK119ub	mouse, monoclonal	Upstate
H2A	rabbit, polyclonal	Upstate
H3	rabbit, polyclonal	Abcam
H3K4me3	rabbit, polyclonal	Diagenode
H3K9me1	rabbit, polyclonal	Diagenode
H3K9me2	rabbit, polyclonal	Diagenode
H3K9me3	rabbit, polyclonal	Upstate
pan acH3	rabbit, polyclonal	Upstate
H4	rabbit, polyclonal	Upstate
pan acH4	rabbit, polyclonal	Upstate
Flk1	rat, monoclonal	eBioscience
CD40	rat, monoclonal	eBioscience
Pcgf6	rabbit, polyclonal	abcam
Pcgf6	goat, polyclonal	Santa Cruz
Pcgf6	mouse, polyclonal	Abnova
ß-Actin	rabbit, polyclonal	Santa Cruz

Secondary antibodies/reagents

Target	Label	Distributor
mouse	HRP	Amersham
rabbit	HRP	Amersham
biotin (streptavidin)	APC	Dianova

7.1.4 Enzymes

Enzymes	Distributor
BamH1	Fermentas
Bbs1	Fermentas
Bfi1	Fermentas
EcoR1	Fermentas
Hind3	Fermentas
Hinf1	Fermentas
I-Sce1	Fermentas
Mlu1	Fermentas
Xho1	Fermentas
rDNAse1	Invitrogen
Super-Taq-DNA polymerase	Invitrogen
T4-DNA-Ligase	Fermentas

7.1.5 Primers

PCR primers for identification of vector integration

Vector	Target region	Sequence	Size of the product	Name of the primer pair
pTT5	flanking the 5´-homologe sequence of the pTT5 vector	CCTGCAGGGATATCGGTTAC (genomic seq.) CGCCTAAAGAAGAGGCTGTG (pTT5 vector seq.)	1218 bp	P1-L, P1-R
pINV7	genomic sequence and pINV7sequence	AGGGAGCTGCAGTGGAGTAG (genomic seq.) TCGTCCTGCAGTTCATTCAG (pINV7 vector seq.)	422	F3-L, F3-R

Gene expression primers

Target	Sequence
Pcgf1	AGATGGACCCACTACGGAAC
	GCTGCGTCTCGTGGATCTT
Pcgf2	CGGACCACACGGATTAAAATCA
	CGATGCAGGTTTTGCAGAAGG
Pcgf3	CAGGTAAGCATCTGTCTGGAATG
	GTAACAACCACGAACTTGAGAGT
Pcgf4	AATTAGTCCCAGGGCTTTTCAA
	TCTTCTCCTCATCTGCAACTTCTC
Pcgf5	GTAAGACCTGTATTGTCCAGCAC
	TCTCGTAGTCCAGGCACTAATTT
Pcgf6	GATGCAACCACCATTACAGAGT
	ACTGCCGGTCCAACCTTATATT
Pcgf6	GGTTGGACCGGCAGTTACAA
	CGAAACACTGACTCTAGGACCT
ß-actin	GATATCGCTGCGCTGGTCGTC
	ACGCAGCTCATTGTAGAAGGTGTGG
Oct4	CCGTGAAGTTGGAGAAGGTG
	GAAGCGACAGATGGTGGTCT
Nanog	TCTTCCTGGTCCCCACAGTTT
	GCAAGAATAGTTCTCGGGATGAA
Sox2	GCGGAGTGGAAACTTTTGTCC
	CGGGAAGCGTGTACTTATCCTT
T	CAGCCCACCTACTGGCTCTA
	GAGCCTCGAAAGAACTGAGC
HoxA7	TATGTGAACGCGCTTTTTAGCA
	GGGGGCTGTTGACATTGTATAA
GSC	CAGATGCTGCCCTACATGAAC

	TCTGGGTACTTCGTCTCCTGG
Nodal	ACTTTTCTGCTCGACTGGACA
	CAGATGCTGCCCTACATGAAC
Flk1	AGGGGAACTGAAGACAGGCTA
	GATGCTCCAAGGTCAGGAAGT
Gata6	TTGCTCCGGTAACAGCAGTG
	GTGGTCGCTTGTGTAGAAGGA
Sox17	GATGCGGGATACGCCAGTG
	CCACCACCTCGCCTTTCAC
Nestin	CAGAGAGGCGC GGAACAGAGATT
	AGACATAGGTGGGATGGGAGTGCT
Runx1	TTTCGCAGAGCGGTGAAAGA
	GCACTGTGGATATGAAGGAA

Sequencing primer

For identification of successful cloning of shRNA oligos into the pINV7 vector, the multiple cloning site (MCS) of pINV7 vector was sequenced using the following primer: 5'-TGTGTTCTGGGAAATCAC-3'.

7.1.6 Plasmid vectors

RMCE requires the FLP recombinase that is carried by the pINV7 vector. pCAGGS-FLPe vector which was always co-transfected with the pINV7 vector provided an extra source for FLP recombinase. Because the gene cassettes carried by pCAGGS-FLPe vector were no longer needed after the RMCE, no specific drug selection was done for genomic integration of this vector. For detailed plasmid maps see the section Attachment.

Plasmid vector	Drug resistance	Source
pTT5	ampicillin, hygromycin	All 3 plasmid vectors were kindly provided by Dr. J. Seibler.
pINV7	ampicillin, G418	
pCAGGS-FLPe	ampicillin, puromycin	

7.1.7 shRNA sequences

shRNA	sequence	Target region
shRNA1 (Pcgf6_2)	GCACGTAACTACGACTACTTT	1166-1186 (3´UTR)
shRNA2 (Pcgf6_3)	CCTGAACTTGATATGTCTTTA	780-800 (CDS)
Scrambled shRNA	CCTAAGGTTAAGTCGCCCTCG	non

7.1.8 Buffers and solutions

PBS

137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4

FACS-buffer

PBS, 0.3 % BSA, 0.1% NaN3, pH 7.4

Protein sample buffer

100 mM Tris HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol

Trypsin

0.05% trypsin in PBS, commercially purchased from PAA

Gelatin

0.1% in PBS

Western blot stripping solution

100 mM Beta-Mercaptoethanol, 2% SDS, 62.5 mM Tris HCl pH 6.7

Gey's Solution

20% Stock A:

NH_4Cl 35.0 g, KCl 1.85 g, Na_2HPO_4-12.H_2O 1.5 g, KH_2PO_4 0.12 g, Glucose 5.0 g,

Phenol red 50 mg, bring to 1 L

5% Stock B:

$MgCl_2$.6-H20 0.42 g, $MgSO_4$-7-H20 0.14 g, $CaCl_2$ 0.34 g, bring to 100 ml

5% Stock C:

$NaHCO_3$ 2.25 g, bring to 100 ml

70% water

7.1.9 Commercail kits and reagents

Kit / Reagent	Distributor
M-MLV Reverse transcriptase kit	Invitrogen
Alkaline Phosphatase kit	Sigma
ABsolute SybrGreen Mix	ThermoFisher
PeqGold RNAPure	Peq Lab
RNeasy Mini kit	Qiagen
AnnexinV-FITC Apoptosis detection kit	BD
Roche Realtime Ready 96well qRT-PCR	Roche Applied Science
Mouse embryonic stem cell Nucleofection kit	Lonza (Amaxa)
RNase and DNase free water	Sigma
Protease inhibitor cocktail	Applichem
QIAquick Gel Extraction Kit	Qiagen

7.1.10 Plastic consumables, devices and software

Plastic consumables	Distributor
2 ml cryotubes	PAA
3.5 cm TC plates	Greiner
3.5 cm suspension plates	Sarstedt
6 cm TC plates	PAA
10 cm TC plates	Greiner
10 cm petri dish	Greiner
15 cm petri dish	Sarstedt
75 cm2 TC flasks, red cap	Sarstedt
24 well plates TC	Nunc
24 well plates, suspension	Greiner
48 well plates TC	Greiner
15 ml tubes	Greiner
50 ml tubes	Greiner
70 µm cell strainer	Greiner
96 well qRT-PCR plates	Roche

Devices	Distributor
Nucleofector I	Lonza (Amaxa)
FACS Canto I	BD
Real time Cycler Rotor Gene 3000	Corbett
Roche Light Cycler 480	Roche Applied Science
Cell culture microscope	EVOS AMG

Software	Distributor
ImageJ	NIH
FlowJo	Tree Star, Inc.
FACS Diva	BD
Rotor Gene 6	Corbett
Roche Light Cycler 480 software	Roche Applied Science
Micron	EVOS AMG
ModFit	Verity software house
ApE (A plasmid Editor)	M. Wayne Davis

7.2 Methods

7.2.1 Cell culture and differentiation

All cells were cultured under the same condition: at 37°C and 5% CO2 in a humidified atmosphere. Cells were frozen in 10% DMSO in FCS.

Establishing and culturing of primary DR4-murine embryonic fibroblasts (DR4 MEFs)

DR4 MEFs derived from 13.5 day old DR4 mouse embryos that display resistance to G-418, hygromycin, puromycin and 6-thioguanin were used for the culture of ES cells under drug selection (Tucker et al. 1997). Pregnant females were sacrificed and embryos were freed from residual extraembryonic tissue. After removal of heads and inner organs, remaining embryonic tissue was incubated in trypsin solution over night at 4°C allowing diffusion of the enzyme inside the tissue. The enzymatic activity was enhanced by 30 min incubation at 37°C and stopped by adding MEF medium. Further, the tissue was disrupted by thorough pipetting, and large tissue particles were then allowed to settle down. The supernatant containing single MEF suspension was spun down at 200x g and cells were resuspended and plated in MEF medium at a density of 1/3 embryo per 10 cm tissue culture (TC) plate. One or two days later, adherent fibroblasts were designated passage 0.

Primary DR4-MEFs were either used directly upon isolation or thawed and were used for no more than 6 passages. Immortalized DR4-MEFs, in contrast, could be maintained in culture for at least 3 months. Longer terms of culturing were not tested. DR4-MEF were maintained in TC flasks (T75 or T175) with MEF medium and split twice a week by washing them once with PBS, incubating them with minimum volume of trypsin at 37°C for maximal 5 min, washing them out with MEF medium and distributing them at equal amounts into three new flasks. For mitotic inactivation, proliferating DR4-MEFs were

treated with Mitomycin C (Mito C) (10 µg/ml) for 2 to 3 hours, washed at least 2 times with PBS, trypsinized and counted. Inactivated DR4-MEFs were plated at a density of $1x10^6$ cells per 6 cm TC plate. After some hours or the next day MEFs had attached and were read as feeder cells for subsequent ES cell culture.

Murine embryonic stem (ES) cell culture

ES cells were and cultured in standard ES cell medium on inactivated DR4-MEFs in 6 cm TC plates. For antibiotic treatment, antibiotics were added to the medium at required concentration. Medium was changed daily. For passaging, ES cells were washed twice with 5 ml of PBS and incubated with 1 ml trypsin at 37°C for 5 min. Trypsin reaction was stopped by adding 5 ml of ES cell medium and cells were dissociated by pipetting. After centrifugation at 90x g, the cell pellet was resuspended in ES cell medium. Desired number of ES cells was given to a new culture dish with feeder cells in presence of ES cell medium. For inducible knockdown of Pcgf6, 1µg/ml dox in final concentration was given into the medium. The medium was changed every day.

To obtain feeder-free ES cells, cell suspension containing ES cells and MEFs was given to a gelatin coated 10 cm TC plate for 45 min. This allows MEFs to strongly attach to the bottom of the dish while ES cells only loosely adhere and dead cells as well as cell clusters remain in suspension. ES cells are isolated by removing the suspension phase and washing off the plate with ES cell medium or 1xPBS.

ES cells colony formation assay

To analyze the potential of cells to form colonies, defined numbers of feeder-free ES cells or differentiated cells were transferred onto a MEF feeder layer in standard ES cell medium. After four days the colonies were fixed and the activity of their AP was visualized by applying the Alkaline Phosphatase Kit according to manufacturer (see Material). Only AP-positive colonies were enumerated by light microscopy.

ES cell differentiation

To induce differentiation, ES cells were cultured in the absence of LIF and MEFs with reduced FCS concentration. In this study, ES cell differentiation was achieved by the formation of embryoid bodies (EBs). For inducible knockdown of Pcgf6, 1µg/ml dox in final concentration was given into the medium. EBs were generated by clustering 1,000 ES cells in 30 µl EB medium as “hanging drops” at the lids of 15 cm petri-dish. 1x PBS

was added to the petri-dish for humidification. After two days, the formed EBs from one 15 cm lid (usually 120 EBs) were transferred into 10 cm petri-dishes in 10 ml of fresh EB medium and cultured for desired days. For further analyses, EBs were dissociated into single cells. For doing this, floating EBs were harvested, allowed to settle down by gravity, washing twice in PBS and incubated with trypsin at 37°C for 3 to 10 min. After stopping the trypsin reaction with EB medium, single cell suspension was made by gentle pipetting.

Blast colony-forming cell (BL-CFC) assay

EBs were collected at different days post differentiation, washed in 1xPBS, and treated with trypsin at 37ºC for 3 minutes. Dissociated single cells were plated at 5×10^4 cells in 1ml of BL-CFC methycellulose medium containing a cocktail of growth factors (see chapter 7.1). Cells were maintained at 37ºC in a humidified 5% $CO2$ incubator. After 4-6 days, developing BL-CFCs were counted.

ES cell derived hematopoietic differentiation

On day 6, 7 or 9 of ES cell differentiation, EBs were collected, washed in 1xPBS, and treated with trypsin at 37ºC for 5-7 minutes. Dissociated single cells were plated at a density of 1×10^5 cells/ml in hematopoietic methycellulose medium containing a cocktail of hematopoietic growth factors (see chapter 7.1). Hematopoietic colonies appeared after 6-12 days of culturing at 37°C.

Generation of inducible Pcgf6 shRNA knockdown in ES cells

OG2 ES cells (carrying an Oct4 promoter-driven GFP transgene) were used for shRNA knockdown in this study. $4\text{x } 10^6$ ES cells were transfected with 2µg plasmid DNA by electroporation using Lonza (Amaxa) mouse ES cell Nucleofection Kit according to manufacturer's guide. Prior to transfection, pTT5 vector was linearized using the restriction enzyme I-SceI. Antibiotic selection with hygromycin (200µg/ml final concentration) for stable positive clones began 24h after transfection. ES cell medium containing hygromycin was changed every day. After 3-5 days of hygromicin treatment, ES cell colonies appeared which were picked and clonally expanded. ESC clones carry pTT5 gene cassettes in the rosa26 locus were confirmed by PCR using site-specific primers. The best suitable ES cell clone was transfected with the pINV7 vector which carries the shRNA expression cassettes. The pINV7 vector was co-transfected with the

same amount of CAGGS-vector for enhanced flipase activity to achieve more efficient recombinase mediated gene cassette exchange. Positive clones carry exchanged shRNA expression cassettes were selected by G418 treatment (800µg/ml final concentration). ES cell medium containing G418 was changed every day.

7.2.2 FACS staining

Cell surface marker staining

For the detection of surface antigens on living cells, fluorochrome or biotin conjugated antibodies were used. Single cell suspensions were prepared from ES cells or EBs. $2x10^5$ cells were washed once and resuspended in FACS buffer, incubated with antibody of interest at 4°C for 20 min. After 2 washing steps with FACS buffer, cells stained with were fluorochrome conjugated antibodies were resuspended in 100µl FACS buffer and ready for analysis. Cells stained by biotin conjugated antibodies were incubated with Streptavidin-APC (1µl/1ml) in 100 µl FACS buffer at 4°C for 20 min, washed twice with FACS buffer and resuspended in 100µl FACS buffer for FACS analysis. Approximately 10,000 events were gated for each sample. The working dilutions of the antibodies are listed below:

Flk1: 1µl/50µl
CD40: 0.5µl/50µl

Cell cycle analysis by propidium iodide (PI) staining

To indentify the phases of the cell cycle, DNA content in cells was measured by staining with the DNA binding dye propidium iodide. The measurement occurred by using FACS. Data were analyzed with Modfit software. Approximately $2x10^5$ cells were fixed in ice-cold 70% ethanol at -20°C for at least 30 min, or stored at -20°C for up to one week. Cells were washed twice in 1xPBS, resuspended in 200µl 1xPBS and incubated with 100 µg/ml RNase and 50 µg/ml PI at 37°C for 30 min. Cells were immediately analyzed.

Apoptosis analysis by combined AnnexinV and 7AAD staining

Approx. $2x\ 10^5$ freshly harvested cells were washed once in 1x binding buffer and incubated with 5µl AnnexinV-PE and 2.5µl 7AAD in 100 µl 1x binding buffer for 15min at 37°C. Immediately cells were analyzed.

Cell proliferation analysis by bromodeoxyuridine (BrdU) incorporation

ES cells on inactivated MEFs were incubated with 10 µM BrdU in ES cell medium for various times (0.5–6 h). ES cells were harvested without being separated from MEFs to avoid losing apoptotic cells. Cell were washed once with PBS and fixed in 70% ethanol overnight. 2 N HCl/0.5% Triton X-100 solution was used to denature DNA then neutralized with 0.1 M $Na_2B_4O_7 \cdot 10\ H_2O$, pH 8.5 solution. During these procedures, EGFP proteins in OG2 ES cells were denatured and no longer detectable. Cells were incubated with FITC-conjugated anti-BrdU antibody for 60min at room temperature. After 2 washing steps in FACS buffer, the cells were ready for analysis.

7.2.3 Isolation of mouse tissues

BM isolation

Female C57BL6 mice at an age of 8 to 12 weeks were sacrificed, femur and tibia bones were isolated and the BM was flushed out with 0.3% BSA in PBS by using needle and syringe. Single cell suspension was obtained by thorough pipetting and the pass though a 70 µm cell strainer. Erythrocytes were depleted by incubation in hypotonic Gey's solution for 5 min on ice and the BM cells were subsequently collected in a phase of FCS by centrifugation.

Testis isolation

Male C57BL6 mice at an age of 3 days to 12 weeks were sacrificed. Isolated testes were immediately given into liquid nitrogen to prevent RNA degradation.

7.2.4 Molecular biology

Bacterial transformation

Calcium sulfate competent DH5α-Escherichia coli (E. coli) were thawed at room temperature and immediately transferred onto ice. 50µl of the competent DH5α were mixed with 100pg – 100ng plasmid DNA and incubated on ice for 30 minutes. After heat shock at 42°C in water bath for 30 seconds, the bacteria were transferred into ice bath for 2 min before 500µl of LB medium was added. The bacteria suspension was vigorously shaken at 37°C for 1 hour.

For selecting the positive clones the transformed cells were plated onto LB agar plates containing antibiotics and incubated at 37°C over night. One exception here was the

culturing of E. coli carrying pTT5 vector that required a lower temperature of 30°C to avoid mutations.

Isolation of plasmid DNA from E. coli

Plasmid DNA was amplified in 150 ml E. coli culture (LB medium) under vigorous shaking overnight at 37°C. Amplification of pTT5 vector in E. coli required shaking at 30°C for 24 hours. Plasmid isolation from E. coli was done with MaxPrepKit from Qiagene according to the manufacturer´s guide. Plasmid DNA were solved in tris-buffer (PH 7.8) and stored at -20°C. One exception here was the culturing of E. coli carrying pTT5 vector that required shaking at 30°C for 24 hours to avoid mutations.

Linearization and purification of pTT5 vector

40µg of pTT5 vector was linearized by 40 units of I-Sce1 in 1x Tango-buffer at 37°C over night. Digested plasmid DNA was separated by agarose gel electrophoresis, and subsequently isolated from the gel by using Plasmid Gel Purification Kit of Qiagen according to the manufacturer´s guide.

Annealing of shRNA oligos

shRNAs were ordered as forward (F) and reverse (R) DNA oligos. Annealing of the shRNA oligos was performed as following: 10 µM of each F and R oligos were mixed with in 1X ligation buffer (Fermentas) and H_2O in a total volume of 25 µl. The mixture was put into a PCR thermocycler, and sequentially incubated at 95°C for 10 min, at 75°C for 10 min and ramp cooled down to 25°C. Annealed oligos were never heated again and stayed always on ice or frozen.

Cloning of shRNA oligos into pINV7 vector

Before ligation, pINV7 vector was digested with Bbs1 and Mlu1 and purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen). 50ng of digested and purified pINV7 plasmid DNA was mixed with 0.05 pmol of annealed shRNA oligos, 1µl of 10x ligase buffer, 1µl of T4 ligase (Fermentas) and brought to a final volume of 10µl with nuclease-free water. The ligation took place at 22°C for 1 hour. T4 ligase was inactivated by incubation in 65°C water bath for 10 min, before the pINV7-shRNA vector was transformed into E. coli.

DNA sequencing

The successful ligation of pINV7 vector and shRNA oligos was confirmed by DNA sequencing. 1µg DNA was mixed with 10pM sequencing primer and was send to the company SeqLab. Sequencing data were analyzed using the software ApE.

RNA isolation

Total RNA of cells was purified by using peqGOLD RNA Pure Kit, an optimized guanidine isothiocyanate/phenol reagent for RNA extraction, or by the Qiagen spin column RNeasy Mini Kit. RNA isolation was performed as recommended by the manufacturer. The concentration and purity of the RNA was determined using photospectrometry.

cDNA synthesis

Purified RNA was reverse transcribed by the M-MLV reverse transcriptase. DNase was used to remove the remaining genomic DNA. Briefly, 1 µg of total RNA were treated with DNase at 37°C for 30 min. The reaction was stopped by adding 7% v/v 25 mM EDTA and incubating at 65°C for 10 min. Oligo dT primers (5' TTT TTT TTT TTT TTT T 3') were allowed to anneal to poly-A RNA 65°C for 5 min, followed by rapid chill down on ice. Subsequently, RNA was incubated with 5 mM dNTPs, reverse transcriptase and first-strand buffer at 37°C for 1 hour. cDNA was further diluted with RNase and DNase free water before qRT-PCR.

Quantitative realtime PCR

qRT-PCRs were performed using Syber-Green Mix or taq-man master mix in Roche Light Cycler 480. For general gene expression analyses, 1 µl cDNA (~50ng) was mixed with 10 µl 2x Sybr-Green Mix, 8.5 µl water and each 0.25 µl of 100 pM primers. Analyses were referred to beta-actin and Hprt by the delta-delta Ct calculation. All reactions were carried out as duplicates with the following PCR program: 95°C for 15 min, 40x (95°C for 10 s, 60°C for 20 s, 72°C for 30 s, 80°C for 20 s), 50°C for 1 min, 67°C to 95°C with 0.5°C temperature increase per 5 s. Ct values were determined by Roche Light Cycler software.

The Roche Realtime Ready 96-well PCR array was used with cDNA which was synthesized from Qiagen purified RNA. After dilution (1:5) each 1 µl cDNA together with water and Light Cycler 480 Probes Master Mix was placed per well, as recommended by

the manufacturer. Roche Light Cycler software determined Cp values automatically and results were normalized to ß-Actin, Hprt and Rp113a.

Global gene expression analysis (Microarray)

RNA was isolated using RNeasy Mini Kit (Qiagen). The RNA quality was checked by agarose gel electrophoresis and the UV spectroscopy (A260/280 ratio). Microarray and determination of gene expression levels were performed by Prof. M. Zenke, Qiong Lin and colleagues. Briefly, sample preparation was performed according to the Expression Analysis TechnicalManual (Affymetrix). Gene Chip One-cycle Target Labeling Kit (Affymetrix) and 1 mg total RNA were used. Biotin-labeled cRNA was hybridized on Affymetrix Mouse Genome 430 2.0 GeneChip arrays. Arrays were stained, washed, and scanned according to the manufacturer's protocols. Gene expression levels were determined by GCRMA algorithm in R/Bioconductor. Hierarchical clustering was performed using Pearson correlation coefficient and the average linkage method and represented by dendrogram and heat-map (Data not shown). Differential expression between 2 conditions was analyzed using Student's t-test. The transcripts with fold change >2 and P-values < 0.05 were considered as being differentially expressed. Raw P-values were adjusted by Benjamini and Hochberg's method. Subsequent data-mining was done by me.

Western blot

Protein samples were prepared from 1 x 10^6 cells which were lysed in 100 µl protein lysis buffer and sheared by passing through a 21 gauge needle for several times. Samples were heated up to 95°C for 5 min and proteins were separated by SDS-PAGE in 12% SDS gels. Proteins were next blotted onto nitrocellulose membranes followed by blocking of unspecific binding sites in 5% milk in PBS with 0.1% Tween20 at room temperature for 30 min. Subsequently, membranes were incubated with primary antibodies in 1x PBS containing1% milk and 0.1% Tween20 at 4°C over night. After three washes secondary HRP-coupled antibodies were incubated with the membranes in 1x PBS containing 0.1% Tween20 at room temperature for 2 h. Proteins were detected by ECL addition and chemiluminescence measurement, either by X-ray film or by BioRad Imaging system. Membranes were stripped from antibodies incubation with stripping solution at 56°C for 30 min.

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10 Affidavit

I, Xiaoli Li, hereby declare that my thesis entitled

Functional analyses of ES cell pluripotency by inducible knockdown of the Polycomb group protein Pcgf6

is the result of my own work. I did not receive any help or support from commercial consultants.

All sources and / or materials used are listed or specifed in the thesis.

Furthermore, I verify that this entire thesis or any part of its contents has not been submitted or is not under consideration for another examination process, neither in identical nor in similar form.

Place, Date ……………………………… Signature ……………………………….

Hereby it is confirmed that Xiaoli Li has worked on his own on the following parts of his doctoral thesis:

Generation of inducible Pcgf6 shRNA-ES cells, ES cell culture, analysis of apoptosis, cell cycle distribution and proliferation, qRT-PCR-based gene expression analyses, and characterisation of hemangioblast and hematopoietic differentiation outcomes.

Prof. Dr. Albrecht Müller

Place, Date .... *Signature* ....

Figure 7: Xiaoli Li prepared RNA samples. The following co-authors performed the cDNA microarray analysis (Affymetrix) and characterised the global gene expression changes.

Qiong Lin

Place, Date .... *Signature* ....

Prof. Dr. Martin Zenke

Place, Date .... *Signature* ....

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11 Curriculum Vitae

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1999 – 2001	Abitur at Main-Taunus-Schule in Hofheim am Taunus
1994 – 1999	Middle school at Schulzentrum an der Gottfried-Menken-Str. in Bremen
1986 – 1994	Grammar school and middle school in Dalian, China

Place, Date ……………………………… Signature ………………………………

12 Publications

2013 *Functional analyses of ES cell pluripotency by inducible knockdown of the Polycomb group protein Pcgf6*
Li X, Lin Q, Zenke M, Becker M, Müller AM (Manuscript in preparation)

2013 *PRC1-mediated gene silencing in pluripotent ES cells: function and evolution*
Becker M, Mah N, Zdzieblo D, Li X, Mer A, Andrade M, Müller AM (Under review)

2009 Book Chapter *Möglichkeiten und Chancen der Stammzellforschung: Stammzellen für Alle?* in *Forschung contra Lebensschutz? Der Streit um die Stammzellforschung* Hilpert K (Ed.)
Müller AM, Obier N, Choi SW, Li X, Dinger TC and Brousos N.
Herder Verlag, ISBN: 978-3-451-02233-3

Poster Presentations

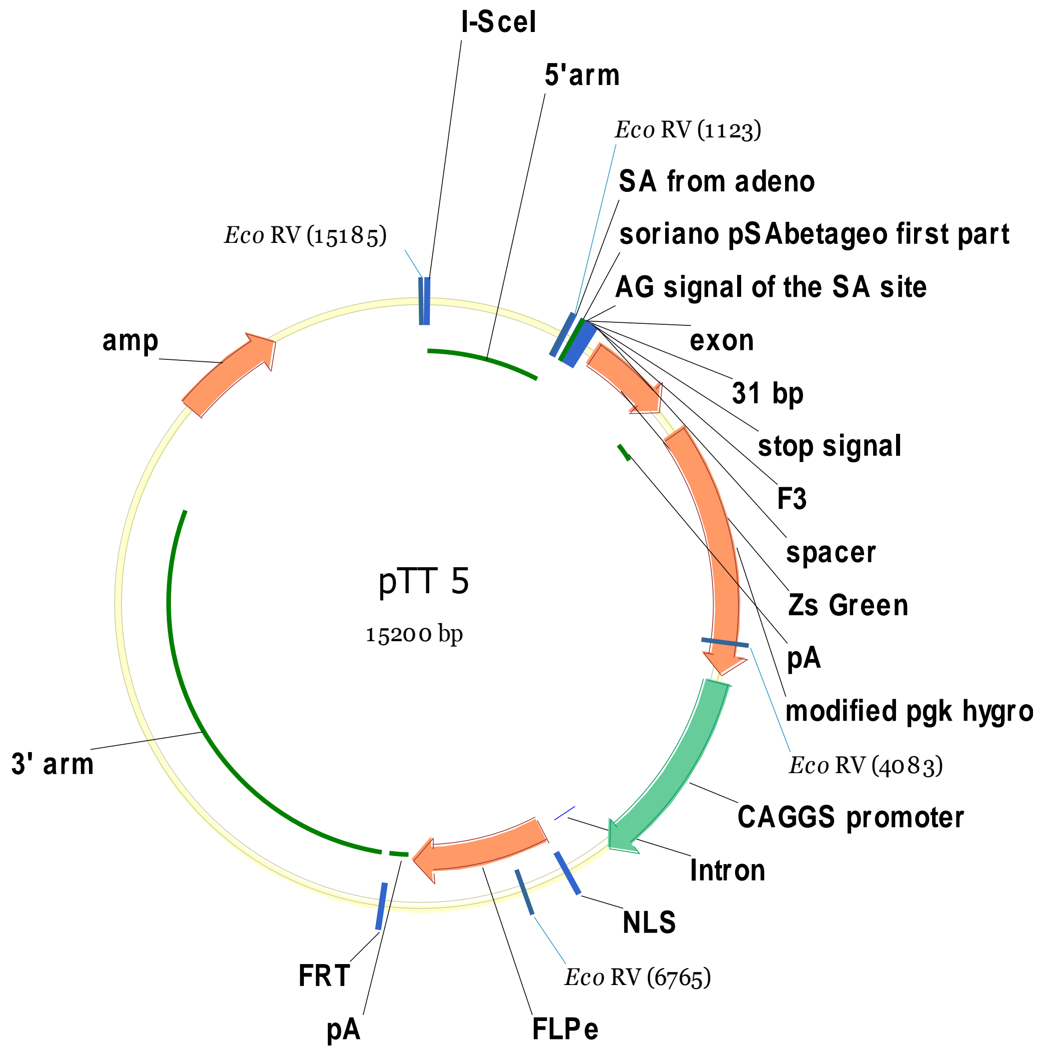
2011 *Functional analyses and chromatin studies of ES cell pluripotency by reversible knockdown of Bmi1 paralog Pcgf6*
Li X, Obier N, Kampka J, Becker M, Meisterernst M, Müller AM
The 6th International Meeting of the Stem Cell Network NRW, Essen (poster number 89)

2010 *Functional analyses of mouse embryonic stem cell pluripotency by reversible knockdown of selected ESC-specific genes*
Li X, Xu J, Obier N, Albert T, Meisterernst M, Müller AM
The 3rd International Congress on Stem Cells and Tissue Formation, Dresden (poster number P-5.40)

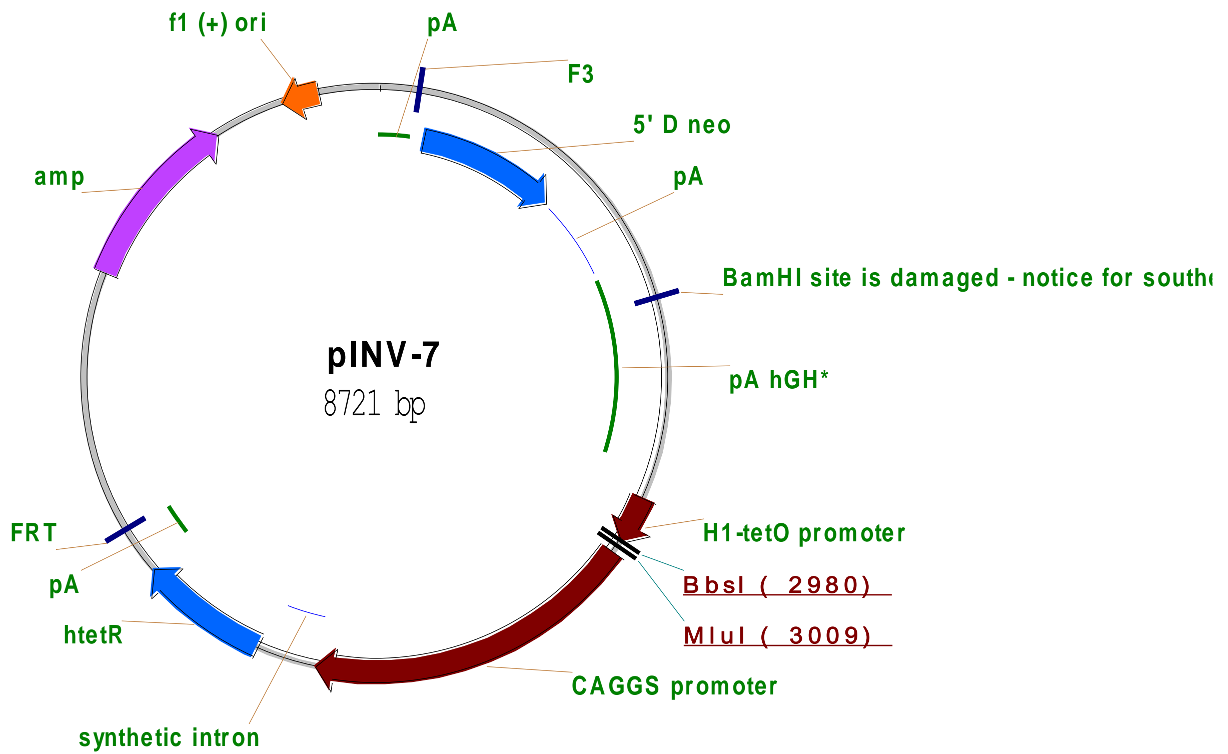
13 Attachment

Plasmid maps

pTT5 vector



pINV7 vector



pCAGGS-FLPe vector