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## **The role of Cathepsin K in haematopoiesis**

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## Abbreviations

°C	degree Celsius
AGM	aorta-gonado-mesonephros
BFU-E	burst-forming unit-erythroid
BM	bone marrow
Bp	base pair
BSA	bovine serum albumin
cDNA	coding DNA
CFU-C	colony-forming unit-culture
CFU-E	colony-forming unit-erythroid
CFU-GM	colony-forming unit-granulocyte macrophage
CFU-GEMM	colony-forming unit-granulocyte erythroid macrophage megakaryocyte
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
cpm	counts per minute
Ctsk	Cathepsin K
DMEM	Dulbecco`s modified eagle medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dTTP	deoxythymine triphosphate
E.coli	Escherichia coli
EDTA	ethylene diamintetraacetate
et al.	et alia
FACS	fluorescence activated cell sorting
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
g	acceleration due to gravity
g	gram
GM-CSF	granulocyte macrophage - colony stimulating factor
GMP	granulocytes macrophages progenitors
HBSS	Hank's balanced salt solution
HSC	haematopoietic stem cells
Kb	kilo base pair
kDa	kilo Dalton
KO	knock-out
l	liter
LB	Luria-Bertami
Lin-	Lineage <sup>negative</sup>
LT-HSC	long time HSC
LSK	Lin-, Sca1+ and Kit+ cells
m	milli
M	molarity
MEP	megakaryocyte erythroid progenitors
min	minute
MPP	multipotent progenitors
MSC	multipotent stromal cells
mRNA	messenger RNA
n	number of probes
NK	cells natural killer cells
nr.	Number

OBC	osteoblastic cells
PB	peripheral blood
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rounds per minute
RT-PCR	reverse transcriptase-PCR
sh	short-hairpin
SDS	sodium dodecyl sulfat
sec.	second
ST-HSC	short time HSC
Tris	trishydromethylaminoethan
U	unit
WT	wild type
μ	micro

The scientific terms are written in italics. The abbreviations of genes correspond to the nomenclature of the International Committee of Standardised Genetics. Nomenclature for mice is written in italics. The proteins are abbreviated like the genes, but not in italics. Chemical elements are abbreviated corresponding to the standard IUPAC symbols.



# 1 Introduction

## 1.1 Haematopoiesis

### 1.1.1 General introduction

Haematopoiesis is the process by which all blood cells develop. It is a hierarchical system of cells initiated by long-term haematopoietic stem cells ((LT)-HSC), which differentiate into a series of multipotent progenitors, losing progressively their self-renewal ability and finally differentiating into mature blood cells (Majeti, Park, and Weissman 2007). Haematopoiesis in adults is mainly located in the bone marrow, but can also be displaced to spleen and liver in cases of injury. Blood cells can be divided into the lymphoid and the myeloid compartment, which both develop from HSCs. Lymphoid cells are responsible for the adaptive immune response while myeloid cells challenge the oxygen transport and are in charge of the innate immune system. HSCs are characterised by two key properties:

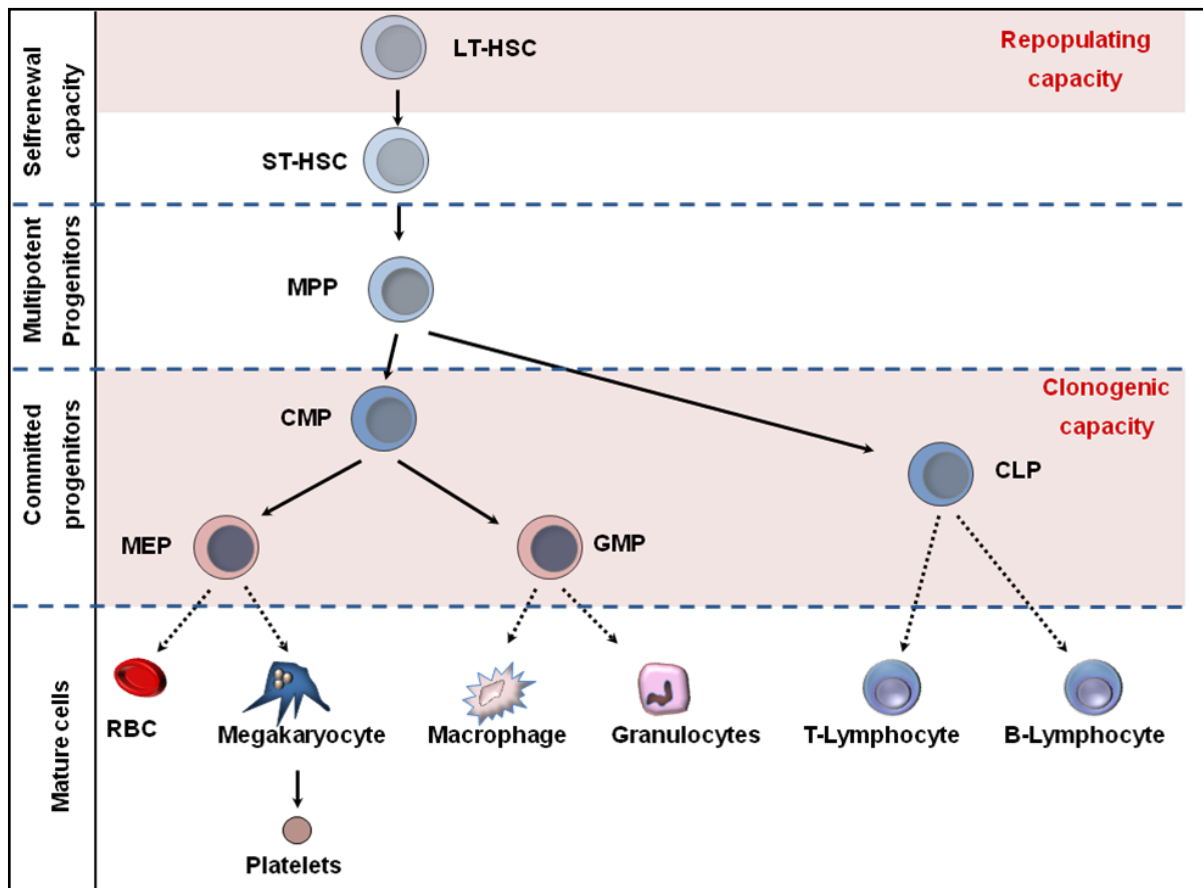
- (1) multipotency, defined as the ability to form all differentiated blood cells, and
- (2) long-term self-renewal, defined as the lifelong ability to give rise to progeny with identical functional properties as the parent through cell division.

To maintain these properties, strict controlled balance exists between the generation of mature blood cells and the maintenance of the number of undifferentiated HSCs, this balance is at least in part regulated by the surrounding bone marrow cells, which form the so called stem cell niches (Wilson et al. 2007). The cells in the niche are heterogenic and the signals are complex (Calvi and Link 2015).

### 1.1.2 The haematopoietic hierarchy

The haematopoietic system is organised in a developmental hierarchy, where the pluripotent cells are at the origin which give rise to progressively more differentiated progenitors cells and further to all mature blood cells (Bryder, Rossi, and Weissman 2006).

Long-term repopulating cells (LT-HSC) are the earliest detectable haematopoietic cells that give rise to short-term repopulating cells (ST-HSC), which will further develop into multipotent progenitor cells (MPP). The self-renewal capacity distinguishes HSC from multipotent progenitors. MPP give rise to committed progenitor cells, like the common lymphoid progenitors (CLP) and the multipotent myeloid progenitors (MMP), which further develop into common myeloid progenitors (CMP). CLPs develop into the cells of the lymphoid compartment and finally will give rise to mature B-cells, T-cells and natural killer (NK) cells. CMP differentiate first into megakaryocytes and erythroid progenitors (MEP) and granulocyte-macrophage progenitors (GMP) which eventually will give rise to mature cells of the myeloid compartment: erythrocytes, platelets, macrophages and granulocytes (Reya et al. 2001) (**Figure 1**). Newer studies indicate that they may be more complex ways in the hierarchy but not much is known about that now (Brown et al. 2015).



**Figure 1 Overview of the haematopoietic hierarchy.** During haematopoiesis, LT-HSC and ST-HSC with self-renewal capacity, can divide and differentiate into MPPs, which can further differentiate into committed myeloid and lymphoid progenitors. CLPs differentiate to mature T- and B-lymphocytes. CMPs differentiate into MEP, the precursor of red blood cells, megakaryocytes and platelets and into GMP, the precursor of macrophages and granulocytes. LT-HSCs show repopulating capacity. The committed progenitor cells show clonogenic capacity.

### 1.1.3 Characterisation of the haematopoietic cells

Different *in vitro* and *in vivo* functional assays have been developed, which allow the characterisation and quantification of the different (sub)populations of HSCs and progenitor cells.

*In vitro* assays are based on the co-culture of HSC together with a supportive layer of stromal cells, HSCs are then harvested in appropriate media and their clonogenic capacity is analysed. The *in vitro* assay closest to characterising HSC is the long-term culture-initiating cell (LTC-IC) assay (Miller, Dykstra, and Eaves 2008; Sutherland et al. 1990), where cells are seeded after 5-8 weeks to analyse their progenitor content in colony-forming unit-culture (CFU-C) assay (Metcalf and Nicola 1984). The CFU-C assay allows the characterisation of the different committed progenitors by analysing the different types of colonies formed in methylcellulose

supplemented with cytokines and growth factors. The colonies formed can be classified in erythroid colonies (formed by MEPs), myeloid colonies (formed by GMP) and mixed colonies (formed by CMPs). The recent progress in the description of HSC-specific combinations of surface markers allows the characterisation of single HSCs both *in vitro* and *in vivo*. Markers such as CD34 (Osawa et al. 1996; Kiel, Yilmaz, and Morrison 2008) CD150 (Kiel, Yilmaz, and Morrison 2008), and the EPC-receptor (Kent et al. 2009) allow the isolation of single HSCs which can be studied in single cell cultures (Wohrer et al. 2014), in immunofluorescence studies, and in transplantations (Dykstra et al. 2007). Although *in vitro* assays, show the potential of haematopoietic activity, definitive knowledge about quality and number of HSC also requires the assessment of repopulating ability in *in vivo* assays.

One of the hallmark properties of an HSC is its ability to repopulate the entire blood cell system *in vivo*. This property is studied in *in vivo* assays, where LT-HSC are analysed by transplanting cells from an LT-HSC-containing donor cell population into lethally irradiated recipients and analysing donor cells several months afterwards. The property of self-renewal distinguishes LT-HSC and ST-HSC from multipotent progenitors and it is experimentally demonstrated by the ability to generate successful secondary transplants from the primary recipients (Majeti, Park, and Weissman 2007).

The different haematopoietic populations are characterised by their differential expression of surface proteins, which can be stained with fluorescent dye-labelled antibodies and sorted by fluorescence-activated cell sorting (FACS). Different combination of antibodies are required to characterise the haematopoietic cells and their enrichment (Spangrude et al. 1991).

In order to show the presence of early haematopoietic cells, mature cells are excluded by using so-called lineage-committed markers: Cd4/8a, Cd3 (T-cells), B220 (B-cells), Gr-1 (granulocytes) and Ter119 (erythroid cells). HSC don't express these lineage-committed markers, but express the stem-cell antigen 1 (Sca-1) and the c-Kit receptors (Lin<sup>-</sup> Sca1<sup>+</sup> Kit<sup>+</sup> (LSK) cells). True repopulating cells are found in a subpopulation of LSK cells which don't express the Cd34 surface marker (Osawa et al. 1996), or the committed marker Cd48 (Slamf2), but do express Cd150 (Slamf1) (Kiel et al. 2005). Dormant Cd34<sup>-</sup> HSCs are at the top of the haematopoietic

hierarchy, they don't contribute to the daily progenitors production. Cd34 expression on the cell surface is one of the earliest events during activation of HSC from the dormant state (Wilson et al. 2007; Sato et al. 2001). Short-term repopulating HSC (ST-HSC) express Cd34 and differentiate into multipotent progenitors (MPP), which are Lin<sup>-</sup> Sca1<sup>-</sup> Kit<sup>+</sup> and can be further differentiated into CMPs, MEPs and GMPs, by their different expression of Cd34 surface marker and Fcγ (CD16/CD32) Receptors (Akashi et al. 2000). Lymphoid progenitors can be enriched by their middle expression levels of Kit and IL7-Receptor (Kondo, Weissman, and Akashi 1997).

#### *1.1.4 The haematopoietic niche*

In adult mice HSC are principally located in the bone marrow, the balance between dormancy, self-renewal and differentiation activity is at least in part controlled by the haematopoietic inductive microenvironment, called stem cell niche (Schofield 1978). Two different types of niches have been proposed, which perform different influences on the HSCs. The first type contains mainly quiescent HSCs, is called quiescent-storage niche and is located in the endosteum (Ohlstein et al. 2004) (Wilson et al. 2007), these niche cells suppress proliferation and differentiation. The endosteum is defined as the inner cellular lining of the bone cavities, interfacing with the bone marrow. The second type of niche is the self-renewing niche (Murphy, Wilson, and Trumpp 2005), it is essential for self-renewal and differentiation of HSCs and it is in contact with the micro vascular sinusoids of the bone marrow. It is widely assumed that the regulation of the balance between dormancy, self-renewal and differentiation of HSC depend on the interactions between the cells comprising the niches and the HSCs. However, the exactly interactions and the molecular mechanism are not fully understood yet.

The endosteal niche consists of various cell types including the bone-forming osteoblasts and the bone-resorbing osteoclasts (Aicher et al. 2008). Osteoblasts are shown to regulate haematopoiesis (Calvi et al. 2003) (Zhang et al. 2003), while osteoclasts seem to influence mesenchymal stem cells and osteoblasts and are required to maintain the integrity of the endosteal niche (Kylmaja, Nakamura, and Tuukkanen 2015). Like HSCs, different subpopulations of niche cells can be isolated using combinations of surface markers. These studies allowed the isolation of endothelial cells (CD31<sup>+</sup>) mesenchymal progenitors, such as osteoblastic cells

(OBC) as well as the multipotent stromal cells (MSC) (Nakamura, Arai, et al. 2010). Osteoclast activation is required for stem cell homing, most probably through secretion of proteolytic enzymes, including the major bone resorbing enzyme Cathepsin K (Ctsk) (Aicher et al. 2008) (Kollet et al. 2006).

#### *1.1.5 HSC supporting cell lines (UG26-1B6, EL08-1D2) and their gene expression*

During embryonic development in mice, haematopoiesis begins in the blood islands of the yolk sac where first primitive erythroid cells appear in embryonic day 7.5 (E7.5). In further embryonic development, definitive HSC detected in the intra-embryonic region of the aorta-gonado-mesonephros (AGM) region and the viteline and umbilical arteries at E10.5. Thereafter haematopoietic cells arrive in the urogenital ridges and the embryonic liver (Oostendorp et al. 2002). The microenvironment of the embryonic HSCs is supported by cell types also found in the bone marrow niches in adults, therefore it is interestingly to use embryonic HSC supporting cell stroma lines in order to analyse HSC's microenvironment *in vitro*. Oostendorp et al., 2002 generated around 50 stroma cell lines out of the AGM region the embryonic liver, as well as the embryonic gut and over 100 clones were established from these sites. Numerous supporting cell lines were found to maintain haematopoiesis in co-culture. Two of these also supported the long-term maintenance of repopulating HSC. One of these was from the urogenital-ridge (which is part of the AGM region) and is called UG26-1B6. Another cell line is from the embryonic liver, called EL08-1D2.

To find out which factors might be involved in HSC maintenance, the gene expressions profiles of EL08-1D2 and UG26-1B6 were compared to the gene expression in four non-supportive clones. 21 genes were detected by the Affymetrix array as higher expressed in the supportive EL08-1D2 and UG26-1B6 cell lines compared to the non-supportive stroma cell lines (Oostendorp, Harvey, and Dzierzak 2005; Ledran et al. 2008). One of the genes identified as highly expressed in both supportive stromal cell lines relative to non-supportive cell lines was the cysteine protease Cathepsin K (**Figure 7**). In this thesis, I will study the role of this cysteine protease in haematopoiesis.

## 1.2 Cathepsin K

### 1.2.1 General introduction

Cathepsin K (*Ctsk*) is a potent collagenase that exhibits bone-specific degradative activity (Li et al. 2002). The protease is abundantly expressed in a specific type of monocytic cell, the osteoclast (Drake et al., 1996), where it is secreted into the ruffled border and has an essential role in normal bone resorption (Zhao, Jia, and Xiao 2009).

### 1.2.2 The protein

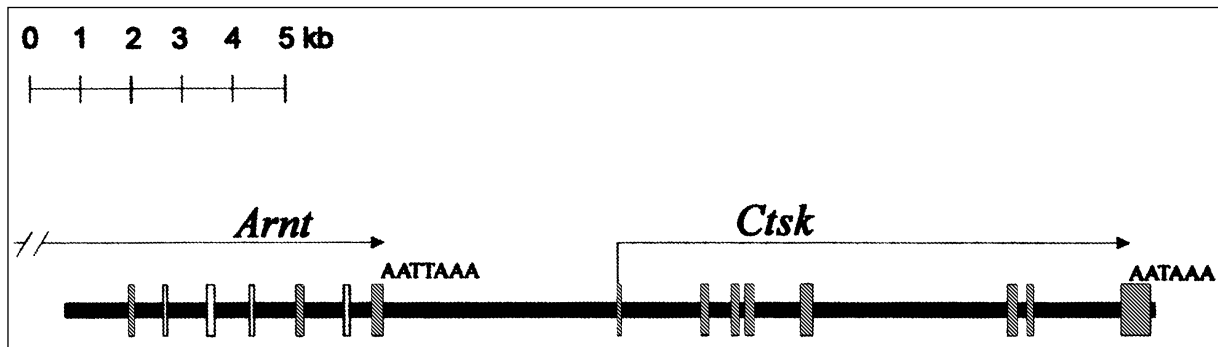
Mouse *Ctsk* is a 46 kDa polypeptide, which belongs to the papain cysteine proteinase family (Rantakokko et al. 1999), which also includes *Ctsb*, *Ctsc*, *Ctsf*, *Ctsh*, *Ctsl1*, *Ctsl2*, *Ctsl3*, *Ctso*, *Ctss*, *Ctsw*, *Ctsx*, *Ctsz*, *TINAG*, *TINAG1*. The cysteine endopeptidase activity consists in catalysis of the hydrolysis of internal alpha-peptide bonds into a polypeptide chain, by a mechanism in which the sulfhydryl group of a cysteine residue at the active centre acts as a nucleophile. *Ctsk* is capable of degrading triple helical type I collagen and osteonectin, and has strong elastase and gelatinase activities (Bossard et al. 1996). *Ctsk* has two functional domains, the protease inhibitor I29, which prevents the access of the substrate into the active site (the zymogen can be activated by removal of the N-terminal inhibitor domain) and the peptidase-C1A papain C-terminal, which is responsible for the proteolysis. The mouse *Ctsk* amino acid sequences are highly similar to the human *Ctsk* (Gelb et al. 1996).

### 1.2.3 The gene

The mouse *Ctsk* gene is located on chromosome 3 at locus 47.9 and consists of 10.1 kb, encoding for 8 exons and 7 introns (Rantakokko et al. 1999). The sizes of the coding exons 2-7, as well as the pattern of intron sizes are conserved between the human and mouse *Ctsk* genes (**Figure 2**).

Analysis of genomic clones extending further upstream of the *Ctsk* gene identified the 3' end of the gene coding for arylhydrocarbon-receptor nuclear translocator (*Arnt*) (Rantakokko et al. 1999). Interestingly, transcripts may extend through the *Arnt-Ctsk* intergenic region and progress into the *Ctsk* gene. Overlapping expression of *Arnt*

and *Ctsk* in many tissue, suggests that *Arnt* read-through may have a negative impact on *Ctsk* transcript levels by interfering with *Ctsk* expression (Giraudeau et al. 2005).



**Figure 2** Genomic organization of the *Arnt* and *Ctsk* loci on mouse chromosome 3. Exons are shown as boxes. The scale is shown above (Rantakokko et al. 1999)

#### 1.2.4 *Ctsk* expression in osteoclasts

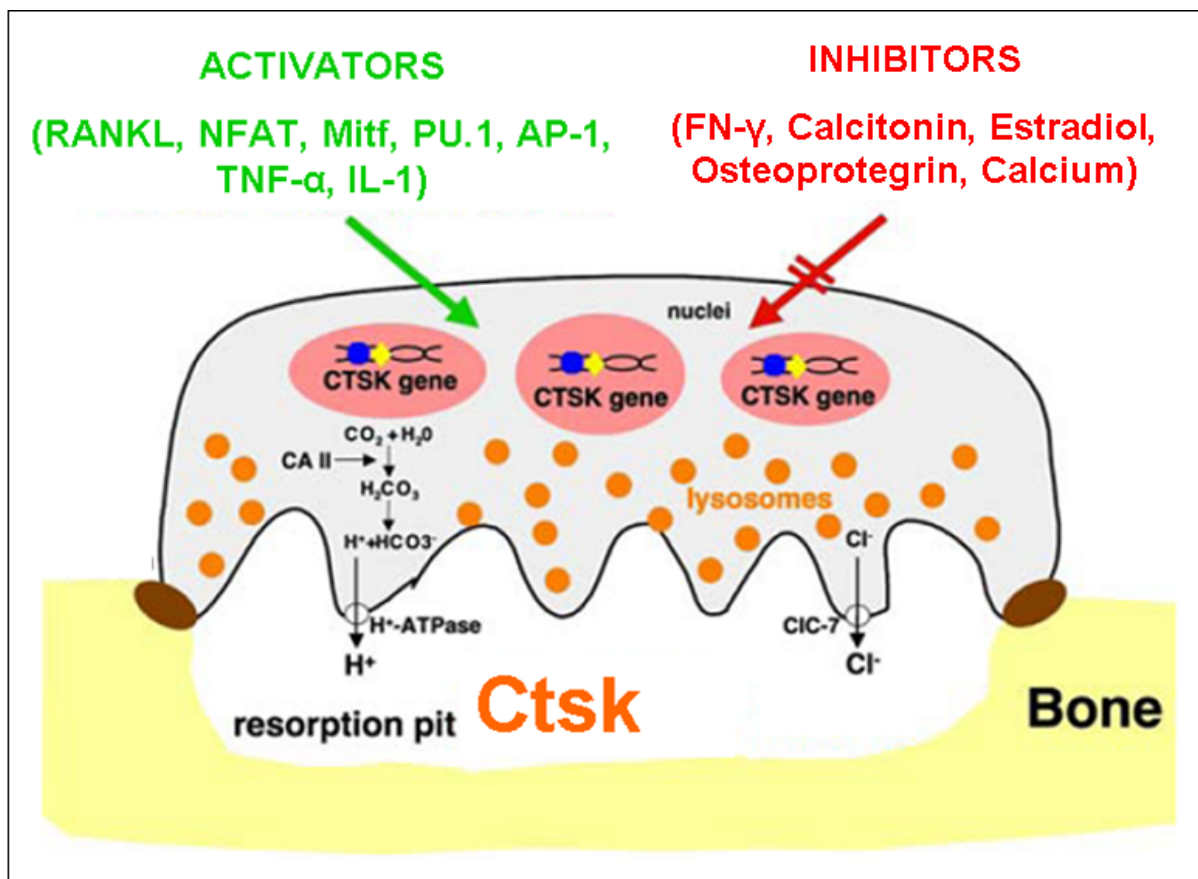
Osteoclasts are multinucleated members of the monocyte/macrophage family and their main function is the bone resorption. In stress situations osteoclasts are activated and the secretion of proteolytic enzymes is induced (Teitelbaum 2007). It has been shown that the activation of osteoclasts also causes a mobilisation of haematopoietic cells (Kollet et al. 2006). Osteoclasts have been shown to be essential for normal bone resorption. When initiating bone resorption, osteoclasts become polarised and attach themselves firmly to the underlying bone surface by using actin-rich podosomes. The essential event for bone resorption is the secretion of *Ctsk* and other proteinases, like the matrix metalloprotease-9 (MMP-9). The organic bone matrix is subsequently degraded and the bone minerals are dissolved. *Ctsk* deficient osteoclasts seem to be capable of demineralizing the extracellular matrix but are unable to adequately remove the demineralized bone (Gowen et al. 1999).

*Ctsk* <sup>-/-</sup> mice show increased extra medullar haematopoiesis, caused by reduced bone cavities indicating the importance of osteoclasts and *Ctsk* in haematopoiesis (Gowen et al. 1999; Troen 2004).



### 1.2.5 Cathepsin K regulation mechanisms

Although *Ctsk* is essential for bone remodelling, not much is understood about the regulation mechanisms of *Ctsk* mRNA and protein expression, either in osteoclasts or in other cell types. The regulation pathways of *Ctsk* expression in osteoclasts are complicated, involving a set of hormonal and cytokine interactions (**Figure 3**).



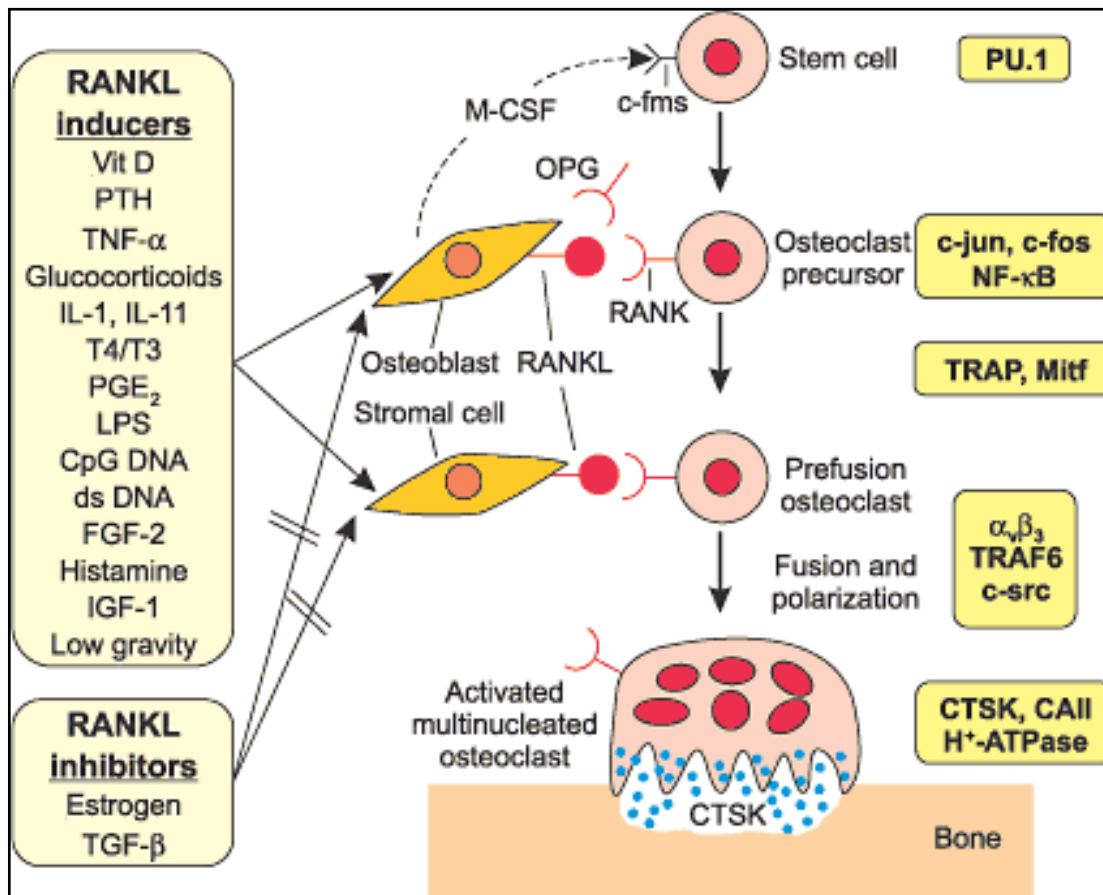
**Figure 3 *Ctsk* expression by osteoclasts.** The osteoclasts adhere tightly to the bone and form the resorption pit. This extracellular compartment is acidified by  $H^+$  and *Ctsk* and other matrix metalloproteinases are secreted into the resorption pit. Agents that stimulate osteoclasts to produce increased amounts of *Ctsk* include: RANKL, NFAT, Mitf, PU.1, AP-1, TNF- $\alpha$ , IL-1. Inhibitors of *Ctsk* expression include: IFN- $\gamma$ , calcitonin, estradiol, osteoprotegrin and calcium (Modified from Troen 2004).

Initially osteoblasts and stromal cells are stimulated to elaborate different factors, which will then finally interact with the osteoclasts (Katagiri and Takahashi 2002; Troen 2006).

Physiological activators of osteoclasts, like receptor activator of NF- $\kappa$ B ligand (RANKL), NFAT, PU.1, IL-1, Mitf and AP-1 enhance *Ctsk* expression. Whereas

inhibitors of the bone resorption, like IFN- $\gamma$ , calcitonin, estradiol, osteoprotegrin and calcium reduce *Ctsk* expression (Troen 2006, 2003).

RANKL plays an essential role in osteoclasts differentiation and activation. RANKL is a membrane-bound factor, which is expressed by osteoblasts and stroma cells in response to a variety of signals. RANKL binds to receptor activated NF- $\kappa$ B (RANK), a cytoplasmic membrane receptor, which is a member of the tumor necrosis factor (TNF) receptor superfamily and is expressed by the osteoclasts, where it can induce *Ctsk* mRNA and protein expression (Kostenuik and Shalhoub 2001). Tumor necrosis factor (TNF)- $\alpha$  is another ligand of RANKL, which has also shown an important ability to stimulate *Ctsk* mRNA (Boyce and Xing 2007; Komine et al. 2001). The RANKL signalling pathway activates TRAF6 (TNF receptor-associated factor 6), which further activates the *Ctsk* promotor. More distally in the signalling pathway, RANKL leads to the phosphorylation of NAFT2 by p38, also causing a stimulation of *Ctsk* promotor activity and expression (Macian 2005). Also, the MAP kinase p38 induces the phosphorylation of microthalmia transcription factor (Mitf), which directly regulates *Ctsk* gene transcription (Motyckova et al. 2001). In addition Mitf and PU.1, an ETS family transcription factor, synergistically influence the stimulation of the transcriptional *Ctsk* regulator NFTA2 (Matsumoto et al. 2004) (**Figure 4**)



**Figure 4 Schematic overview of the regulation mechanisms of osteoclasts.** (Modified from Troen 2004)

### 1.2.6 Cathepsin K in human diseases

In humans, *Ctsk* has shown to be higher expressed in several bone remodelling diseases, like osteoporosis, osteolytic bone metastasis and rheumatoid arthritis, as well as in other tissue remodelling processes.

Mutations in the *Ctsk* gene cause pycnodysostosis, a rare autosomal recessive inherited lysosomal storage disease of the bone. Pycnodysostosis is characterised by severe bone fragility resulting from increased bone density (osteopetrosis), short stature, reduced length of the distal phalanges (acro-osteolysis) and delays in the normal closure of the skull sutures in the infancy (cleidocranial dysplasia) (Alves Pereira, Berini Aytes, and Gay Escoda 2008). The patients have increased incidence of pituitary hypoplasia and cerebral demyelination. The mutation responsible of the typical clinical features seems to be situated in exon 5 of the human *Ctsk* gene (Wen et al. 2015; Li et al. 2009). Administration of growth hormone (GH) is a possible

therapy, improving the linear growth, by increasing IGF-I secretion (Karamizadeh, Ilkhanipoor, and Bagheri 2014).

Osteoporosis has a high prevalence worldwide and is considered a serious public health concern. The disease is characterised by a misbalance between bone resorption and formation, causing a loss of the total bone mass. Especially in elder patients, osteoporosis is an important morbidity cause, increasing the bone fracture risk and the hip fracture mortality. The regulation of *Ctsk* is the most important mechanism for bone remodelling (Yasuda, Kaleta, and Bromme 2005). Completely *Ctsk* *-/-* mice always develop osteoporosis (Troen 2006). The role of *Ctsk* in human osteoporosis has an important significance in the clinic, there are results suggesting that the serum level of *Ctsk* could serve as a marker for bone fracture prediction and bone-mineral density (Holzer et al. 2005). *Ctsk* inhibitors, such as odanacatib, show potential in the treatment of osteoporosis (Stoch et al. 2013; Verbovsek, Van Noorden, and Lah 2015; Srivastava et al. 2008).

Rheumatoid arthritis is a chronic, systemic inflammatory disorder that affects different tissues and organs, but principally causes synovitis of the joints and often progresses to destroy the articulation cartilage and cause ankylosis. It has been evidenced, that the radiological destruction of the bones, correlates significantly with *Ctsk* levels in serum (Skoumal et al. 2005; Podgorski 2009; Skoumal et al. 2008). Newer results show, that *Ctsk* is involved in the rheumatoid process, by cleaving type II collagen, which suggests that *Ctsk* may not only play a role in the pathophysiology of RA, but also in the cartilage degradation of the normal aging process (Dejica et al. 2008; Vinardell et al. 2009; Podgorski 2009).

Osteosarcoma is a highly malignant primary skeletal tumour with a striking tendency to rapidly destroy the surroundings bone and frequently metastasising. New investigations have shown that *Ctsk* expression seems to correlate in this tumour with metastasis and survival rate (Avnet et al. 2008). Therefore, *Ctsk* expression appears to be an useful prognostic value for patients with high-grade cancer diagnosis (Husmann et al. 2008). In other osteolytic bone tumours, similar results have been found. In multiple myeloma, a bone destroying cancer, an up-regulation of *Ctsk* expression in the osteoclasts was demonstrated (Hecht et al. 2008). Radiolabelled cathepsin K inhibitors may even have potential in *in vivo* imaging to

detect changes of osteoclast numbers in osteolytic diseases (Rodnick et al. 2014). In giant cell tumour of the bone, a neoplasm causing osteolytic lesions, *Ctsk* seems to be the main responsible protease (Lindeman et al. 2004).

Worldwide, breast cancer is the second most common type of cancer in woman and therefore of substantial interest for the cancer research. The most common haematogenous metastasis of this cancer is the bone metastasis, lowering the expectation of life significantly. Investigations of possible new targets for breast cancer therapy have shown that *Ctsk* may have a role in breast cancer bone metastasis and that *Ctsk* inhibitors may have a clinical role in decreasing tumour risk (Kleer et al. 2008; Razaq 2013).

Lung fibrosis is a pulmonary disease characterised by tissue remodelling, resulting from an imbalance between synthesis and degradation of extracellular organic matrices in the lung. It has been shown, that *Ctsk* is higher expressed in lung specimens of patients with pulmonary fibrosis and that activation of human pulmonary fibroblasts in primary cell cultures led to an increased activity of *Ctsk* (Buhling et al. 2004; Srivastava et al. 2008).

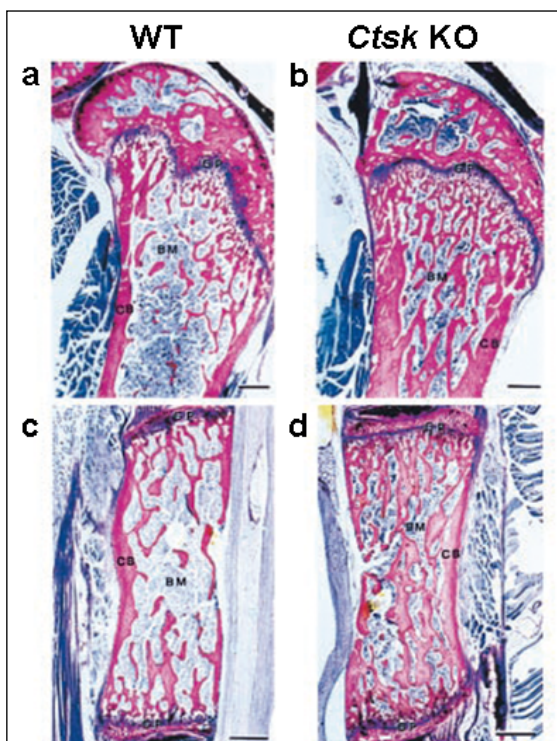
Thyroid hormone suppression is an important part of the treatment of thyroid cancer. Some studies found a deleterious of bone mass density in patients treated with this therapy, interestingly in this patient group increased *Ctsk* levels in serum were found (Mikosch et al. 2008). Possibly inhibitors of *Ctsk* can relieve this side effect.

The atherosclerotic disease is a multifactorial, inflammatory and degenerative disorder of the arterial walls with progressive vascular obstruction, with a great worldwide socio-economic importance. There are findings suggesting that *Ctsk* may be involved in arterial wall remodelling and atherosclerosis (Cheng et al. 2011). Newer studies suggest that circulating *Ctsk* may be a biomarker for coronary artery diseases (Li et al. 2014; Cheng et al. 2013) and *Ctsk* levels are shown to be higher in patients with chronic heart failure (Zhao et al. 2015).

Summarising, although *Ctsk* is involved in many different human diseases, little is known about the pathological mechanisms.

### 1.2.7 The Cathepsin K $-/-$ mouse

In order to analyse the effects of *Ctsk* in mice, *Ctsk*  $-/-$  models have been generated by targeted disruption of this proteinase and analysed. The heterozygous mutants seem not to show any difference to wild-type mice (Gowen et al. 1999). Saftig et al., 1998 developed a *Ctsk*  $-/-$  mouse and showed an osteopetrotic phenotype, with excessive trabeculation of the bone marrow (**Figure 5**). The ultra structural appearance is modified, with a poorly defined resorptive surface and a broad demineralised matrix fringe, containing undigested fine collagen fibrils and their ruffled borders showed a lack of crystal-like inclusions (Saftig et al. 1998). This strongly suggests that *Ctsk* deficient osteoclasts are capable of demineralising the extracellular matrix, but are unable to adequately remove the demineralised bone.



**Figure 5 Osteopetrosis in *Ctsk* deficient mice** (Saftig et al. 1998). (A and B) Histological sections of the meta- and epiphysis of control (A) and *Ctsk* (B) femora embedded in methyl methacrylate and stained with McNeil's tetrachrome. Note the unresorbed primary spongiosa in B. (C and D) Histological sections (sagittal plane) through the ventral half of thoracic vertebrae derived from control (C) and *Ctsk* deficient (D) mice. Bars: 5400 mm.

Newer studies on the *Ctsk*  $-/-$  mice show that the higher cortical bone quantity is associated with a smaller marrow cavity and increased retention of non-lamellar bone, both signs of decreased endocortical resorption (Pennypacker et al. 2009).

Interestingly, also in the haematopoietic compartments alterations were found, like a reduced number of bone marrow cavities, a decreased cellularity in bone marrow and splenomegalie (Gowen et al. 1999). Current investigations even indicate a possible

link between bone remodelling and haematopoiesis, showing an involvement of osteoclasts in selective progenitor recruitment, as part of homeostasis and host defence mechanisms (Kollet et al. 2006). As *Ctsk* is not only expressed in osteoclasts, the mice mutants were also analysed in order to find other functions of the protease. The relevance of *Ctsk* in pulmonary homeostasis was studied, showing that *Ctsk* over-expression improves lung function parameters in bleomycin-induced lung fibrosis (Srivastava et al. 2008). It was also shown, that *Ctsk* mediates proteolytic processing of thyroglobulin, which is essential for thyroid function (Friedrichs et al. 2003). There are evidences of an implication of *Ctsk* in adipogenesis. Researches have shown that *Ctsk* could play a role in the adipocyte differentiation by degradation of collagen Type I and may so be involved in the pathogenesis of obesity (Xiao et al. 2006). *In vivo* knockout studies revealed that *Ctsk* KO mice have reduced atherosclerosis (Lutgens et al. 2007) and recent studies have shown that *Ctsk* control injury-related vascular repair in mice (Hu et al, 2014) and *Ctsk* knockout mice show a reduced age-related decline of the cardiac function (Hua et al. 2015).

## 2 Objectives

Cathepsin K is a cysteine protease, which is predominantly expressed by osteoclasts and plays an essential role in normal degradation of the organic phase, during bone resorption.

Considering that

(1) HSC supportive cell lines express higher levels of *Ctsk* compared to the non-supportive cell lines (Oostendorp, Harvey, and Dzierzak 2005), and

(2) osteoclasts activation induces *Ctsk* secretion and at the same time mobilisation of HSC (Kollet et al. 2006),

we hypothesised that *Ctsk* might play an important role in haematopoiesis.

Aim of this work is to identify a possible role of *Ctsk* in normal mouse haematopoiesis. We investigate how haematopoiesis is influenced by the loss of *Ctsk* and which level of the haematopoietic hierarchy is affected most.

In order to investigate the effects of *Ctsk* we first generated stable *Ctsk* knock-downs of the supportive stroma cell lines for *in vitro* assays. To further understand the effects of *Ctsk* in the blood building system, we studied *Ctsk* *-/-* mice for *in vivo* assays



## **3 Methods**

### **3.1 Working with bacterial cells**

#### *3.1.1 Storage of the bacterial cultures*

The bacterial cultures were stored in glycerine stocks (50% glycerine: 50% bacterial culture). Therefore one bacterial colony of each clone was grown overnight in LB-medium with 1:1000 ampicillin, then 0.5 ml of the culture was added to 0.5 ml of 80% sterile glycerine and stored at 80°C.

#### *3.1.2 Preparation of the competent cells*

For preparation of competent cells 20 µl of *Escherichia coli* (E.-coli) DH5α-bacteria were inoculated in 20 ml LB-media and harvested at 37°C under 190 U/min shaking till OD600 = max 0.3. The cell suspension was centrifuged at 4°C by 1000 x g for 10 min. The cell pellet was re-suspended gently in 2 ml of ice cold TSS-buffer (Chung, Niemela, and Miller 1989), shock frozen in 50 µl aliquots in liquid nitrogen and stored at 80°C until use.

#### *3.1.3 Chemical Transformation*

50 µl of competent cells were slowly thawed on ice and carefully mixed with 25 – 100 ng of plasmid DNA. After 30 min of incubation on ice cells were heat-shocked for 90 sec at 42° C and harvested in 250 µl of LB-media for 60 min at 37°C under shacking at 180 U/min. After incubation, 100-150 µl of the transformed cells were spread on the selection plate with Ampicilin and incubated overnight at 37°C.

### **3.2 Working with nucleic acids**

#### *3.2.1 Isolation of the recombinant DNA from bacterial cells*

For the isolation of recombinant plasmid DNA the Qiagen® Spin Miniprep Kit was used following manufacturers protocol. The method is based on alkali lyses of bacterial cells (Birnboim and Doly 1979) and selective binding of plasmid DNA on the silica membrane in the presence of high salt concentrations. Plasmid inserts were verified by sequencing and plasmid DNA was frozen in small aliquots at -20°C.

### *3.2.2 Restriction of the plasmid DNA with endonuclease*

For the restriction, 1 µg of the plasmid DNA and 1-10 U of appropriate restriction enzyme were used. The reaction was carried out under supplied conditions.

### *3.2.3 DNA agarose gel electrophoresis*

The DNA fragments were electrophoretically separated according to their molecular size on agarose gels (0.5 - 2%). The agarose was melted for 2 - 3 min in 1x TBE and cooled down till ~55°C. Before cooked agarose was poured into a gel caster and 0.05 µg/ml ethidium bromide were added. The DNA loading buffer (0.2 - 0.5 of the sample volume) was added to the DNA samples and 1x TBE was used as running buffer. The DNA samples were loaded into the wells of the gel and electrophoresis was carried out at steady voltage (100 - 150 V or 3 - 4 V/cm<sup>2</sup>). Under these conditions the negatively charged DNA molecules moved towards the cathode. The DNA fragments were observed and photographed under UV light at a λ of 254 nm or 312 nm due to the intercalation of the fluorescent dye ethidium bromide into the double strand of DNA. Limitations of the method are 5 – 10 ng DNA.

### *3.2.4 Isolation of total RNA*

Total RNA was isolated using RNAeasy kit (as described by manufacturer) from confluent grown stroma cells on a 10 cm dish. The RNA concentration was determined by measuring the OD at 260/280 nm. RNA samples were stored in small aliquots at -80°C.

### *3.2.5 cDNA amplification*

1 µg of isolated total RNA was reverse transcribed with Omniscript ® Reverse Transcription Kit as described by manufacturer. cDNA was used for amplification in quantitative and qualitative PCR after determining concentration of the samples, by measuring the OD at 260/280 nm.

### *3.2.6 Quantitative real-time PCR (QRT-PCR)*

Gene expression level was determined with QRT-PCR. All primers were used at 10 µM concentration. The following Quantitative PCRs were performed using the Power

SYBR® Green PCR Master Mix (Applied Biosystems) according to manufacturer instructions, and analysed on an Applied Biosystems 7900HT machine.

PCR mix:

- 1 µl of each Primer
- 1 µl cDNA (~0.5 µg/ml)
- 10 µl Sybr-Green Mix (10x PCR Buffer, 10mM dNTP mixture, 50µM MgCl<sub>2</sub>, 2.5 units Taq DNA Polymerase)
- 7.6 µl H<sub>2</sub>O

Primer sequences for the genes analysed can be found in materials (4.5).

PCR reaction was conducted under following conditions

	Temperature	Duration
Hot start denaturation	95°C	5 min
Denaturation	95°C	15 sec
Annealing	58°C	20 sec
Extension	72°C	30 sec

} 40 cycles

### 3.3 Working with protein

#### 3.3.1 Isolation of the total protein

The total protein was isolated from confluent grown stroma cells on 10 cm dishes. Cells were centrifuged at 1.800 rpm at 4°C for 5 min and resuspended in 200 µl of lysis buffer supplemented with proteinase inhibitors (Complete Mini) prior to use. After 20 min of incubation on ice, samples were homogenised by ultrasound treatment for 1-2 min. Protein concentration was determined by Bradford assay.

Total protein samples were stored in aliquots at -20°C.

### 3.3.2 SDS-polyacrylamide-gel electrophoresis

The discontinuous SDS-polyacrylamide-gel electrophoresis for separation of denaturised total protein was carried out after Laemmli (1970) and Gallagher (1999). The voltage gradient in the electrophoresis system was generated by the different contents and pHs of the buffers. According to the molecular weight of Ctsk (46 kDa) and  $\beta$ -Actin (42 kDa), resolving gel with an acrylamide concentration of 10 % was used. Electrophoreses was carried out on a Mini-Protean® 3 Apparatus, using a tris-glycine as running buffer. 5  $\mu$ l total protein denaturated for 2 min at 95°C in Laemmli buffer, was applied on the gel. Electrophoresis was carried out at 20-40 mV. Molecular weight of the separated proteins was determined by comparison with a standard weight ladder.

### 3.3.3 Western blot

Proteins separated on the SDS-Polyacrylamid-Gel were transferred on the BioTrace™PVDF membrane. Western blotting (Burnette 1981; Towbin, Staehelin, and Gordon 1992; Sasse and Gallagher 2008) was performed in Criterion Blotter under 250 mA for 3 hours at 4°C. For blocking of unspecific binding sites, the membrane was incubated in 5 % non-fat milk in PBS-Tween (0.1%) solution over night at 4°C under smooth shaking. After 5 x 10 min washing in PBST, the membrane was incubated with primary antibody diluted in 5% milk in PBST for 12 hours at 4°C. The rests of the non coupled antibody solution were washed out with PBST under permanent shaking. After washes membrane was incubated in secondary antibody (horse-reddish peroxidase coupled) diluted in PBST for one hour at room temperature. Membrane was washed 2 times for 5 min in PBST and for 10 min in PBS. After incubation in the Chemo luminescent developing solution Super Signal West Substrate, enzymatic activity of the horseradish peroxidase causes an energy release in form of chemo luminescence. The chemoluminiscent, immobilised proteins on the membrane are detected by an X-ray film. Afterwards, membrane bounded secondary antibodies were detached by incubation with Amido Black Destain for 15 min. On the next step, blocked membranes were incubated with  $\beta$ -actin antibody and processed as described above.  $\beta$ -actin, a cytoplasmatic protein, is an integral part of all cells and was used as marker for total protein load of every sample.

### **3.4 Working with mouse stroma cells**

#### *3.4.1 Culturing of stroma cell lines*

The embryo-derived stroma cell lines UG26-1B6 and EL08-1D2 were cultured on 0.1% gelatine coated wells in stroma media and incubated at 33°C, 5% CO<sub>2</sub> and ≤95% humidity.

#### *3.4.2 The principals of RNA interference*

RNA interference (RNAi) is an established method to silence gene expression in a variety of organisms, it is exhibited by most eukaryotic cells, as a response to foreign double-stranded RNA (dsRNA), resulting in a sequence-specific gene silencing.

DsRNA, when introduced to cells, interferes with the expression of homologous genes, disrupting their normal function (Hannon and Conklin 2004). RNAi was first discovered in an animal cell by Fire in the nematode worm *Caenorhabditis elegans* (Fire et al. 1998). The progress in the understanding of RNAi has led to applications of this process in studies of gene function as well as in therapeutic applications for the treatment of diseases. RNAi can be triggered by two different pathways, (1) an RNA-based approach, using very short dsRNAs (approximately 21-22 bp), such as short interfering RNAs (siRNAs), or (2) via DNA-based strategies in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin transcripts (short hairpin (sh)RNA) (Hannon and Rossi 2004; Kim and Rossi 2008; Scherer and Rossi 2003). By using shRNAs endogenous non-specific antiviral responses are avoided, compared to the longer dsRNAs (Paddison et al. 2004). In order to introduce shRNA into the cells, different methods can be used, like standard vectors as well as retroviral or lentiviral expression systems. Exogenous dsRNA initiates RNAi by activating RNase-III-like ribonuclease protein, called Dicer, which binds and cleaves the dsRNAs to produce double-stranded fragments (Tijsterman and Plasterk 2004). These short RNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary mRNAs. The cleaving of the RNA by RISC mediates a translational repression or induces chromatin modification.

In summary, using RNAi, allows the effective creation of continuous cell lines, in which suppression of a target gene is stably maintained.

### *3.4.3 Lentiviral transfection and infection*

Phoenix E cells harvested in Phoenix medium were transfected with shRNAmir constructs using Lipofectamine 2000 after manufacturer instructions. pMD2.G plasmid expressing envelope gene (*env*) and psPAX2 packaging plasmid (*pax*) were co-transfected in equal concentration for the generation of retroviral particles. Eight hours post transfection Phoenix cells were cultured in stroma medium. After 12 hours stroma medium enriched with lentiviruses was collected, filtered through 0.45 µg filters and added with 8 µg/ml Polybrenne to the semi-confluent UG26-1B5 and EL08-1D2 stroma cells. After 36 h of cultivation, stroma cells were selected with 5 µg/ml puromycin for clones expressing shRNAmir. Knockdown cells were cultured with puromycin during the expansion. For experiments, puromycin was removed and cells were cultured in stroma medium for at least 3 days prior to experimental manipulations.

## **3.5 Functional studies**

### *3.5.1 Isolation of the Lineage negative cells*

Lin<sup>-</sup> cells were separated from the bone marrow of Ly5.1 mice on the MACS Separator Columns using Lineage Cell Depletion Kit, according to the manufacturer protocol. Lineage positive (Lin<sup>+</sup>) cells were indirectly magnetic labelled using a cocktail of biotin-conjugated monoclonal antibodies (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4 and Ter119) conjugated to Anti-Biotin Micro Beads as secondary labelled reagent. The magnetically labelled Lin<sup>+</sup> cells were retained in the magnetic field, while the Lin<sup>-</sup> cells passed through. Cells were kept on ice during the experiment to prevent capping of antibodies on the cell surface and non-specific cell labelling. In order to evaluate the purity of separated cell populations, the cell suspensions were analysed by FACS, using the following antibodies: biotinylated lineage cocktail, PE-Cy5-labelled Sca-1 (Ly-6A/E) and PE-labelled CD117 (c-Kit). Alexa Fluor® 488 anti streptavidin antibody was used as a secondary antibody.

### *3.5.2 Co-culturing of Lin-cells on shCtsk and primary stroma cells*

For the co-cultures, stroma cell lines or primary stroma were grown till 90 % confluence and immortalised by irradiation at 30 Gy. After one week of culturing in LT- media supplemented with 0.1% hydrocortisone, 10000 Lin- cells isolated from the bone marrow of 129xL5.1 mice were added and co-cultured for 2 or 4 weeks in a 33°C, 5% CO<sub>2</sub> and ≤95% humidity. LT- medium was refreshed every week by replacing of the half with fresh LT- medium.

### *3.5.3 Haematopoietic colony assay*

Colony forming ability of the bone marrow cells was tested by colony forming assay on methylcellulose (Metho Cult GF 3434). The  $2.5 \times 10^4$  bone marrow cells,  $1 \times 10^4$  Lin-cells or fractions of cell cultures were seeded in 2.5 ml of methylcellulose and applied on two 3.5 mm dishes. After ten days cultivation at 37°C, 5 % CO<sub>2</sub> and ≤95% humidity, the colonies formed were counted under the microscope.

Following classes of mouse haematopoietic progenitors were counted: colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte macrophage (CFU-GM), colony-forming unit- granulocyte erythroid macrophage megakaryocyte (CFU-GEMM).

### *3.5.4 FACS analysis*

Single cell suspensions were stained in 100µl FACS buffer with antibodies for 15 minutes on ice in the dark, after labelling cells were washed with 3ml FACS buffer and centrifuged, prior to analysis cells were resuspended in 0.5 ml FACS with 1:1000 Propidiumiodide (PI) (1 µg/ml).

The mature and immature haematopoietic populations were stained with the following antibodies: biotinylated Gr-1, phycoerythrin (PE)-labelled Cd4, Cd8 and Cd117 (c-Kit), PE-Cy5-labelled B220, fluorescein isothiocyanate (FITC)-labelled Cd34, Pacific Blue®-labelled Cd16/32 (FcγR), Alyophycocyanin (APC)-labelled Cd117 (c-Kit) and IL7R, and PE-Cy7-labelled anti Ly-6A/E (Sca1). Lineage stains were performed with biotinylated lineage cocktail, supplemented with biotin-labelled CD3 and IL7R antibodies.

The blood samples were erylysed in ammonium chloride, prior to stain with surface markers. The following antibodies and conjugates were used: Biotinylated Gr-1 labelled with PECy5 streptavidin, PECy5 - CD4, PECy5 - CD8a, PECy5 - B220 or PECy5 - Ter119. To distinguish the origin of the cells, donor or recipient, we used a system involving CD45.2 - FITC, CD45.1 - PE antibodies. Following antibodies were used for the stains of: erythroid cells: FITC - Cd71 and PeCy5 - Ter119; myeloid cells: PECy5 - Gr1 and FITC - Cd31; B-cells: PeCy5 - B220, FITC - IgM and PE - Cd43; T-cells: PeCy5 - Cd4 and PE - Cd8a; Natural killer (NK) cells: APC - Nk1.1, PeCy7 - Cd11b and FITC - Cd49b. FACS analyses were performed on a Coulter EPICS XL or CyAn ADP Lx P8 Flow cytometers. FACS data were analysed using Flowjo software package (*Treestar*). Gating strategies for the primitive haematopoietic cells are shown in Results (5.1.4).

### **3.6 Working with mice**

#### *3.6.1 Preparation of mouse tissues*

Blood samples were taken from the retro orbital plexus with 0.8 mm capillaries and collected in the heparinised S-Monovette®. The blood cells were counted on Scil Vet ABC.

Mouse bone marrow cells were flushed from femurs and tibias with HF2+ buffer using a 27 Gauge needle.

Spleens, thymii and lymph nodes were filtered through a 70 µm nylon Cell Strainer.

All cells were passed through a 30 µm nylon mesh to remove cell clumps. Before manual counting of white blood cells the erythroid cells were first lysed using an Ammonium Chloride solution. Viable cell counts were obtained by dilution in Trypan Blue and counting in a haemocytometer.

#### *3.6.2 Transplantation assay*

The 8-10 weeks old recipient mice were irradiated with 9 Gy using a Mevatron KD2 and injected into the tail vein with donor cells the same day. Initially, 10000 Lin- bone marrow cells were co-cultured on the immortalised stroma. After 3 weeks cells were harvested, filtered through a 30 µm nylon mesh and transplanted with  $2 \times 10^5$  of helper



cells into recipients. Helper cells were isolated from spleens of 129XLy5.1 mice. After transplantation mice received 1:10 antibiotics (10.08 g/dm<sup>3</sup> neomycin sulphate and 5x10<sup>6</sup> Units/dm<sup>3</sup> Polymyxin B) in the drinking water during the first 4 weeks. 5 weeks post transplantation (Tx) mice were bled for FACS analysis. At week 16 post Tx mice were sacrificed, bone marrow-, spleen-, thymus-, and blood cells were used for FACS analysis.

## **4 Materials**

### **4.1 Bacterial strains**

*Escherichia coli* (*E.-coli*) DH5 $\alpha$ , for cloning K-12 strain, F-  $\Phi$ 80lacZ $\Delta$ M15  $\Delta$  (LacZYA-argF) U169 deoR recA1 endA1 hsdR17 (rk -, mk+) phoA supE44;  $\lambda$ - thi-1 gyrA96 relA1.

### **4.2 Mouse strains**

*Cathepsin K* deficient (ECK $^{-/-}$ ) mice were generated on 129XC57BL/6.J (129XB6), described previously by Saftig et al., 1998.

Age and sex matched 129B6, C57BL/6.Pep3b.Ptpcr (Ly5.1), and (129xLy5.1) F1 (129Ly 5.1) mice were used as controls in all experiments.

The 129B6, C57BL/6.Pep3b.Ptpcr (Ly5.1) bone marrow cells were used for co-cultures and transplantation experiments.

Mice were kept in Microisolators under SPF conditions according to FELASA recommendations.

### **4.3 Cell lines**

The embryo-derived stroma cell lines UG26-1B6 and EL08-1D2 cultured in stroma medium were described previously by Oostendorp et al., 2002.

The retroviral producer cell line  $\Phi$ NX-Eco (Phoenix E), cells were a gift from Dr. G. Nolan (Stanford, CA, USA), (Stanford Register Nr.; SBR-422) and were cultured in Phoenix E cells medium.

Stable knockdown cells for *Cathepsin K* gene (sh*Ctsk*) were generated by lentiviral shRNAmir transduction into the stroma cell lines (as described in 3.4.3) and cultured in stroma medium.

#### 4.4 Primary stroma

Primary stroma was obtained from the bone marrow of wt and *Ctsk* deficient mice and cultured in stroma medium

#### 4.5 Media

Long- term (LT) media	<ul style="list-style-type: none"> <li>- MyeloCult M5300</li> <li>- 1% Penicillin/Streptomycin</li> <li>- 1% Glutamax</li> </ul>
Luria Broth (LB)-Media	<ul style="list-style-type: none"> <li>- 2% Pepton</li> <li>- 1% Yeast extract</li> <li>- 0.5% NaCl</li> </ul> <p>dissolved in H<sub>2</sub>O</p>
Phoenix E cells media	<ul style="list-style-type: none"> <li>- DMEM 4,5 g/l glucose</li> <li>-10% Fetal bovine/calf serum</li> <li>- 1% Penicillin/Streptomycin</li> </ul>
Stroma media	<ul style="list-style-type: none"> <li>- 400 ml MEM alpha medium</li> <li>- 25 ml Horse Serum</li> <li>- 75 ml Fetal bovine/calf serum (FCS)</li> <li>- 5 ml Penicillin/Streptomycin</li> <li>- 100 µl β-mercaptoethanol</li> </ul>

#### 4.6 Buffers and gels

Amido Black Destain solution	- 10% Methanol - 10% Acetic acid dissolved in H <sub>2</sub> O
FACS buffer	- PBS - 5% Albumin bovine serum
Resolving gel (10%):	- 3.3 ml Acrylamide 30%- bisacrylamide 0.8% solution - 2.5 ml Resolving gel buffer (4 x) (0.5 M Tris/HCl, 0.4% SDS, pH 8.8) - 0.1 ml 10% APS - 0.004 ml TEMED - 4 ml H <sub>2</sub> O
Sample buffer (6x)	- 0.2% bromphenolblue - 60% glycerine dissolved in H <sub>2</sub> O
SDS-running buffer (10x)	- 60 g 125 mM Tris-Base - 288 g Glycine - 20 g SDS - 2000 ml H <sub>2</sub> O

Stacking gel	<ul style="list-style-type: none"> <li>- 0.26 ml Acrylamide30%- bisacrylamide 0.8% solution</li> <li>- 0.505 ml Stacking gel buffer 4 x (0.5 M Tris/HCl, 0.4% SDS, pH 6.8)</li> <li>- 0.01 ml APS 10%</li> <li>- 0.002 ml TEMED</li> <li>- 1.235 ml H<sub>2</sub>O</li> </ul>
TBE (10x)	<ul style="list-style-type: none"> <li>- 1 mM Tris-Base</li> <li>- 0,83 mM Boric acid</li> <li>- 20 mM EDTA</li> </ul>
Transfer Buffer (1x)	<ul style="list-style-type: none"> <li>- 50 ml SDS running buffer (10 x)</li> <li>- 100 ml Methanol</li> <li>- 350 ml H<sub>2</sub>O</li> </ul>
TSS-solution	<ul style="list-style-type: none"> <li>- 50 mM MgSO<sub>4</sub></li> <li>- 10% PEG 3000</li> <li>- 5% DMSO</li> <li>- 100 ml LB-Medium</li> </ul>

#### 4.7 Chemicals and reagents

Acrylamide 30%- bisacrylamide 0.8% solution (*Carl Roth [Karlsruhe, Germany]*)

Albumin bovine serum (Sigma-Aldrich Chemie [München, Germany])

Ammonium Chloride solution (Stem Cell Technologies [Köln, Germany])

Ampicillin (Invitrogen GmbH [Karlsruhe, Germany])

APS (Ammoniumpersulfate) (Sigma-Aldrich Chemie [München, Germany])

Bradford assay (Bio-Rad Laboratories [Hercules, CA, USA])

Bromphenol blue (Carl Roth [Karlsruhe, Germany])

Chemoluminiscent developing solution Super Signal West Pico/Dura/Femto Extended Duration Substrate (*Pierce [Rockford, IL, USA]*)

Complete Mini (Roche [Mannheim, Germany])

DMEM (Dulbecco's modified Eagles Medium) 4,5 g/l glucose (*Gibco BRL [Karlsruhe, Germany]*)

DMSO (Dimethylsulfoxide) (Sigma-Aldrich Chemie [München, Germany])

DTT (Dithiothreitol) (Sigma-Aldrich Chemie [München, Germany])

Dual Colour Precision Assay (*BioRad [München, Germany]*)

EDTA (Sigma-Aldrich Chemie [München, Germany])

Eosin Y stain solution (Sigma-Aldrich Chemie [München, Germany])

FCS (Fetal bovine/calf serum) (PAN Biotech [Aidenbach, Germany])

Flow Count Beads (Beckman Coulter [Krefeld, Germany])

Glutamax (Gibco BRL [Karlsruhe, Germany])

Haemalaun solution (Sigma-Aldrich Chemie [München, Germany])

HBSS (Hank's Balanced Salt Solution) 10x (*Gibco BRL [Karlsruhe, Germany]*)

HEPES buffer solution 1 M (Gibco BRL [Karlsruhe, Germany])

Horse Serum (Stem Cell Technologies [Köln, Germany])

Lipofectamine 2000 (Invitrogen GmbH [Karlsruhe, Germany])

MEM alpha medium (Gibco BRL [Karlsruhe, Germany])

MethoCult<sup>®</sup> M3434 (Stem Cell Technologies, [Köln, Germany])

MicroBeads (Miltenyi Biotec [Bergisch Gladbach, Germany])

mSDF-1 $\alpha$  (Mouse stroma cell derived factor) (*R&D Systems [Mineapolis, USA]*)

MyeloCult M5300 (Stem Cell Technologies, [Köln, Germany])

Neomycin sulphate (Sigma-Aldrich Chemie [München, Germany])

Omniscript<sup>®</sup> Reverse Transcription Kit (*Qiagen, [Hilden, Germany]*)

Opti-MEM Reduced Serum Medium (Gibco BRL [Karlsruhe, Germany])

Paraformaldehyde (PFA) Accustain<sup>®</sup> (*Sigma Aldrich [München, Germany]*)

PBS (Phosphate Buffered Saline) (Gibco BRL [Karlsruhe, Germany])

PEG (Polyethylenglycole) 3000 (Sigma-Aldrich Chemie [München, Germany])

Penicillin/Streptomycin (Gibco BRL [Karlsruhe, Germany])

peqGOLD PCR Master Mix Y (PEQLAB Biotechnologie GMBH [Erlangen, Germany])

PI (Propidiumiodide) (Sigma-Aldrich Chemie [München, Germany])

Polybrenne<sup>®</sup> (Sigma-Aldrich Chemie [München, Germany])

Polymyxin B (Sigma-Aldrich Chemie [München, Germany])

Power SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems [Darmstadt, Germany])

Precision Plus Protein Standards (*BioRad [München, Germany]*)

Puromycin (Sigma-Aldrich Chemie [München, Germany])

Resolving gel buffer (4 x) (0.5 M Tris/HCl, 0.4% SDS, pH 8.8) (*Carl Roth [Karlsruhe, Germany]*)

RNAeasy Kit (Qiagen, [Hilden, Germany])

SDS (Sodiumdodecylsulfate) (Carl Roth [Karlsruhe, Germany])

Sodiumdodecylsulfate (SDS) (Carl Roth [Karlsruhe, Germany])

$\beta$ -mercaptoethanol (Invitrogen GmbH [Karlsruhe, Germany])

Stacking gel buffer (4 x) (0.5 M Tris/HCl, 0.4% SDS, pH 6.8) (*Carl Roth [Karlsruhe, Germany]*)

TEMED (Carl Roth [Karlsruhe, Germany])

Tris base [1 M] (Carl Roth [Karlsruhe, Germany])

Tris HCl (pH 8.0) (Carl Roth [Karlsruhe, Germany])

Tris-HCl gels (10%, 4-10% and 4-20%) (*BioRad [München, Germany]*)

Triton X 100 (Sigma-Aldrich Chemie [München, Germany])

Trypan Blue (Invitrogen GmbH [Karlsruhe, Germany])

Tween 20 (Fluka Chemie [Buchs, Switzerland])

#### **4.8 Equipment**

Applied Biosystems 7900HT machine (Applied Biosystems [Foster city, USA])

BioTrace™ PVDF membrane (Pall, [Dreieich, Germany])

Capillaries 0,8mm (Neolab [Heidelberg, Germany])

Cellulose-acetate-filter 0.45  $\mu$ m (Schleicher & Schuell BioScience [Keene, USA])

Coulter EPICS XL (Beckman Coulter GMBH [Krefeld, Germany])

Criterion Blotter (BioRad [München, Germany])

CyAn ADP Lx P8 (Beckman Coulter [Krefeld, Germany])

Flowjo software package (Treestar [Olten, Switzerland])

Gauge needle (27 mm) (B. Braun Melsungen [Melsungen, Germany])

ImageJ (<http://rsb.info.nih.gov/ij/index.html>, *National Institute of Health*)

-Psq nitrocellulose/polyvinylidenefluoride transfer membrane (1.45 µm/ 0.2 µm pore size) (*Amersham-Pharmacia Biotech Europe [Frankfurt, Germany]*)

MACS Separator Columns (Miltenyi Biotec [Bergisch Gladbach, Germany])

Mevatron KD2 (Siemens [Munich, Germany])

Mini-Protean® 3 Apparatus (BioRad [München, Germany])

Monoject blunt needles (Sherwood-Davis & Geck [Gosport, UK])

Nylon Cell Strainer (30µm) (BD Biosciences [Erembodegem, Belgium])

Optical well plate and Adhesive Cover (Applied Biosystems [Darmstadt, Germany])

Scil Vet ABC (Scil Animal Care Company [Viernheim, Germany])

S-Monovette® (Sarsdedt [Nümbrecht, Germany])

Transwell polycarbonate membrane (8 µm pore) (Costar, Corning Incorporated [Wiesbaden, Germany])

Whatman blotting paper (BioRad [München, Germany])

X-ray film Reflection NEF-496 (NEN Dupont [Bad Homburg, Germany])

#### **4.9 Plasmids**

The Expression Arrest™ microRNA-adapted shRNA (shRNAmir) constructs were designed by Open Biosystems [*Huntsville, USA*].



	TYPE	CATALOG NUMBER	CLONE ID	ACCESSION	CLUSTER	VECTOR	HOST
CTSK	shRNAs	RMM3981-9597997	TRCN0000030589	NM_007802	Mm.272085	TRC mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9597998	TRCN0000030590	NM_007802	Mm.272085	TRC mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9597999	TRCN0000030591	NM_007802	Mm.272085	TRC mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9598000	TRCN0000030592	NM_007802	Mm.272085	TRC mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9598001	TRCN0000030593	NM_007802	Mm.272085	TRC mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9621822	TRCN0000054623	NM_007802	Mm.272085	TRC2 mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9621823	TRCN0000054624	NM_007802	Mm.272085	TRC2 mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9621824	TRCN0000054625	NM_007802	Mm.272085	TRC2 mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9621825	TRCN0000054626	NM_007802	Mm.272085	TRC2 mouse shRNA Lenti	pLKO.1
CTSK	shRNAs	RMM3981-9621826	TRCN0000054627	NM_007802	Mm.272085	TRC2 mouse shRNA Lenti	pLKO.1

pMD2.G- envelope plasmid (Addgene Inc. [Cambridge, USA])

psPAX2- packaging vector (Addgene Inc. [Cambridge, USA])

All shRNAmir constructs were cloned into the lenti-viral based *pLKO.1* vector (Addgene Inc. [Cambridge, USA]).

#### 4.10 PCR Primers

Description	Sequence
<i>Arnt</i> 3' UTR	Forward: 5' GAGAGAGGGGAAGAGTCGGGA 3'
<i>Ctsk</i> exon1	Reverse: 5' CTGCTGATGGAAATCTGTTGTCT 3'
<i>Ctsk</i> exon 2	Forward: 5' GCTCAAGGTTCTGCTGCTACCT 3' Reverse: 5' CTCCCAGTGGGTGTCCAGTATC 3'
<i>Ythdf1</i>	Forward: 5' CAGGACAGGGTGGGATTCTA 3' Reverse: 5' TTGGTCCAGCCACAGTATCA 3'

All primers were provided from Sigma Genosys (*Sigma-Aldrich Chemie [München, Germany]*).

#### 4.11 Western Blot Antibodies

	Antibody	Manufacturer
Primary Antibodies	Cathepsin K (N-20), sc-6507, goat polyclonal IgG	Santa Cruz Biotechnology [Heidelberg, Germany]
	Anti $\beta$ -Actin mouse anti human antibody	R&D Systems [Wiesbaden, Germany]
Secondary Antibodies	Donkey Anti-Goat IgG HRP Affinity Purified PAb, Donkey IgG	R&D Systems [Wiesbaden, Germany]
	ECL Anti mouse IgG, Horseradish peroxidase linked, AB from sheep	GE, Healthcare [UK]

#### 4.12 FACS Antibodies

Antibody	Manufacturer
Cd117 (c-Kit) , phycoerythrin (PE)-labelled, anti-mouse	eBioscience [San Diego, USA]
Cd117 (c-Kit), allophycocyanin (APC)-labelled, anti-mouse	eBioscience [San Diego, USA]
CD117 (c-Kit), Allophycocyanin (APC)-Alexa Fluor® 750-labelled anti-mouse	eBioscience [San Diego, USA]
CD11b (Integrin $\alpha_M$ , Mac-1 $\alpha$ ), Phycoerythrin-Cy7 (PE-Cy7)-labelled, anti-mouse	eBioscience [San Diego, USA]
Cd127 (IL7R), allophycocyanin (APC)-labelled, anti mouse	eBioscience [San Diego, USA]

Cd127 (IL7R), biotin-labelled, anti mouse	eBioscience [Sandiego, USA]
CD135 (Flk-2), Phycoerythrin-Cy5 (PE-Cy5)-labelled anti-mouse	eBioscience [Sandiego, USA]
Cd16/32 (FcγR), Pacific Blue®-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD16/32 (FcγR), Phycoerythrin (PE)-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD3 (CD3e, epsilon subunit), Pacific Blue®-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD31 (PECAM-1) Fluorescein isothiocyanate (FITC)-labelled anti-mouse	eBioscience [Sandiego, USA]
CD34, fluorescein isothiocyanate (FITC)-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD3e, biotin-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD4 (L3T4) , phycoerythrin (PE)-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD4 (L3T4), phycoerythrin-Cy5 (PE-Cy5)-labelled, anti-mouse	BD Pharmigen [Heidelberg, Germany]
CD43 (Leukosialin), Phycoerythrin (PE)-labelled, anti-mouse	eBioscience [Sandiego, USA]
CD45.1 (Ly5.1), phycoerythrin (PE)-labelled, anti-mouse	BD Pharmigen [Heidelberg, Germany]
CD45.2 (Ly5.2), fluorescein isothiocyanate (FITC)-labelled, anti-mouse	BD Pharmigen [Heidelberg, Germany]

CD45R (B220), phycoerythrin-Cy5 (PE-Cy5)-labelled, anti-mouse	eBioscience [San Diego, USA]
CD71 (Transferrin Receptor) Fluorescein isothiocyanate (FITC)-labelled anti-mouse	eBioscience [San Diego, USA]
CD8a (Ly-2), phycoerythrin (PE)-labelled, anti-mouse	eBioscience [San Diego, USA]
CD8a (Ly-2), phycoerythrin-Cy5 (PE-Cy5)-labelled, anti-mouse	eBioscience [San Diego, USA]
IgM, Fluorescein isothiocyanate (FITC)-labelled, anti-mouse	eBioscience [San Diego, USA]
Lineage negative Cocktail, biotinylated	Miltenyi Biotec [Bergisch Gladbach, Germany]
Ly-6G (Gr-1), biotinylated anti-mouse	eBioscience [San Diego, USA]
NK1.1, Allophycocyanin (APC)-labelled, anti-mouse	eBioscience [San Diego, USA]
pan-NK cells (CD49b, $\alpha_2$ integrin, very late antigen-2), Fluorescein isothiocyanate (FITC)-labelled, anti-mouse	eBioscience [San Diego, USA]
Sca-1 (Ly-6A/E), phycoerythrin-Cy7 (PE-Cy7)-labelled, anti-mouse	eBioscience [San Diego, USA]
Sca-1 (Ly-6A/E), phycoerythrin-Cy5 (PE-Cy5)-labelled, anti-mouse	eBioscience [San Diego, USA]
Streptavidin, Alexa Fluor® 488 conjugated	Invitrogen GmbH [Karlsruhe, Germany]
Ter119 (Ly-76) , phycoerythrin-Cy5 (PE-Cy5)-labelled, anti-mouse	eBioscience [San Diego, USA]

$\alpha$ -Streptavidin, phycoerythrin-Cy5 (PE-Cy5)-labelled

Invitrogen GmbH  
[Karlsruhe, Germany]

## 5 Results

### 5.1 Ctsk may be involved in the maintenance of haematopoiesis *in vitro*

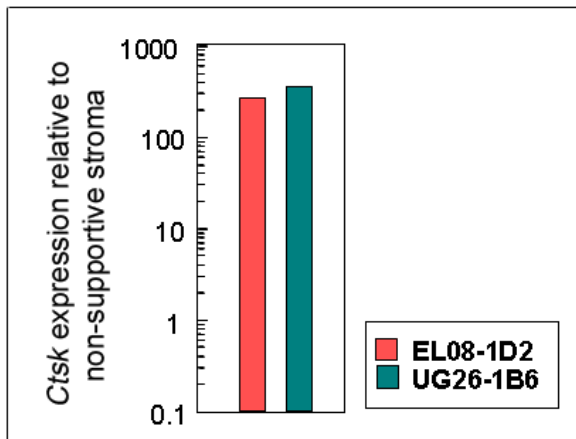
In previous studies done by our team, we generated several stroma cell lines out of midgestation mouse embryos. Two of these, UG26-1B6 (derived from the urogenital ridge) and EL08-1D2 (derived from the embryonic liver) support the long-term maintenance of HSC in culture (Oostendorp et al. 2002) (**Figure 6**).

		Cell line	Repopulated/ Transplanted	Repopulation
Supportive	EL08-1D2		9/27	(33%)
	UG26-1B6		9/22	(41%)
non-Supportive	EL08-2C2		1/18	( 6%)
	AM20-1B4		0/ 8	( 0%)
	AM30 -3F4		0/12	( 0%)

**Figure 6 Long-term repopulation by HSCs after co-culture with stroma clones.** HSC were co-cultured for four weeks on the supportive and non-supportive stroma and then transplanted into lethally irradiated mice. Repopulation capacity was up to 33% for EL08-1D2 and 44% for UG26-1B6, compared to the non-supportive stroma (EL08-2C2, AM20-1B4 and AM30-3F4), where repopulation was only between 6% and 0%.

In order to identify factors secreted by the supportive stroma cells, gene expression profiles of UG26-1B6 and EL08-1D2 were compared with those of non-supportive cell lines (UG15-1B7, EL28-1B3, AM30-3F4 and AM20-1B4) on ClonTech macroarrays (Oostendorp, Harvey, and Dzierzak 2005) as well as Affymetrix microarrays (Ledran

et al. 2008). Interestingly, these comparisons identified high expression of *Ctsk* gene in supportive stroma cell lines compared to the non-supportive ones (**Figure 7**).



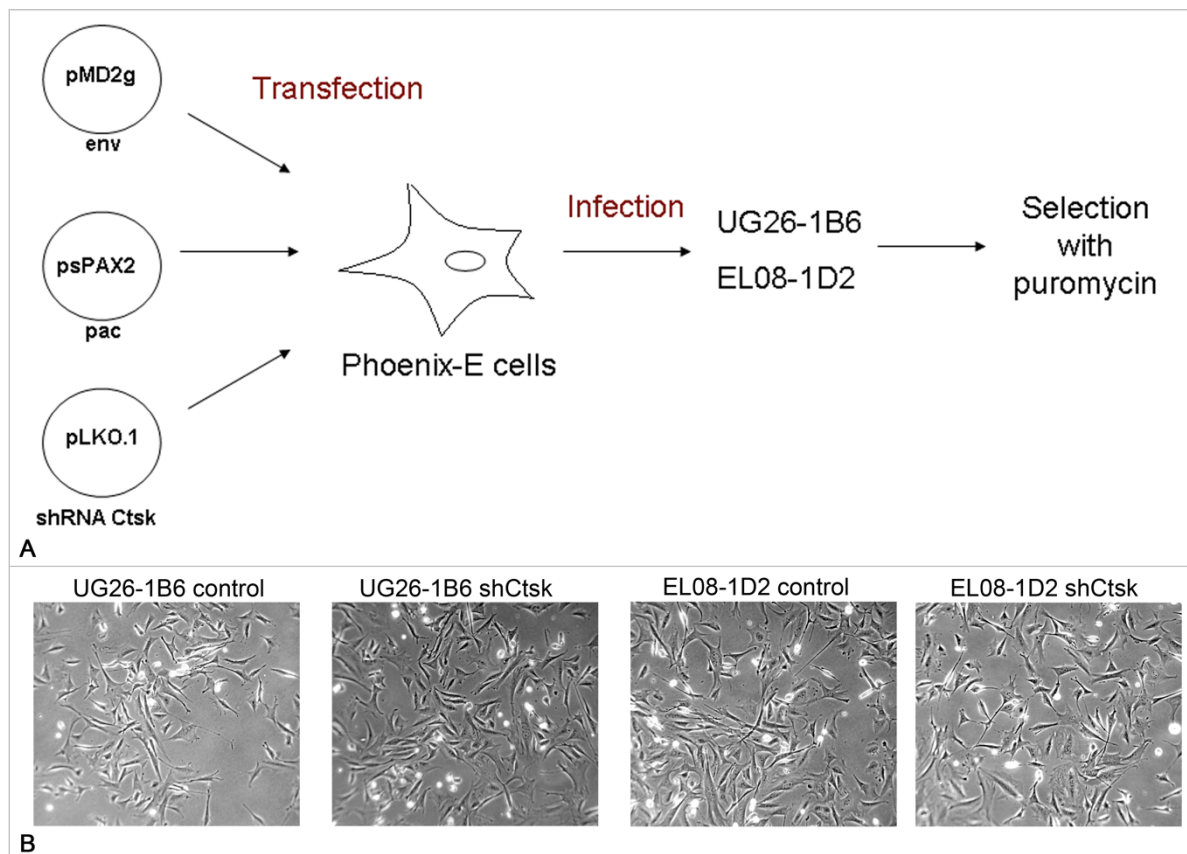
**Figure 7 Increased expression of *Ctsk* by supportive stroma.** Relative expression of *Ctsk* in EL08-1D2 and UG26-1B6 compared to non-supportive cell lines (mix of UG15-1B7, EL28-1B3, and AM30-3F4) detected by Affymetrix array (Oostendorp, Harvey, and Dzierzak 2005).

Considering these data, we hypothesised a possible role for *Ctsk* in haematopoiesis. In order to study this hypothesis, we generated stable *Ctsk* knockdown of EL08-1D2 and UG26-1B6 for further experiments.

#### 5.1.1 Generation of stable *Ctsk* knockdown stroma cell line using shRNA constructs

To lower the expression of *Ctsk*, we decided to silence the *Ctsk* gene by RNA interference (**Figure 8A**), using ten different short hairpin RNAs (shRNAs) constructs described in materials (4.9). This method consists in transfecting Phoenix Eco cells (Nolan et al.) with shRNA constructs, which were embedded in the retroviral expression vector pLKO, which also included the puromycin-resistance gene. The retroviral particles in the supernatant of the transfected Phoenix cells, were then used to infect the UG26-1B6 and EL08-1D2 stroma cells, as described in methods (3.4.3).

Then cell lines were selected for expression of the retroviral vector using puromycin (**Figure 8B**) and seven stable *Ctsk* knockdowns for UG26-1B6 and eight knockdowns for EL08-1D2 were generated. These sub-cell lines were verified for their *Ctsk* expression by real-time PCR and Western Blot.

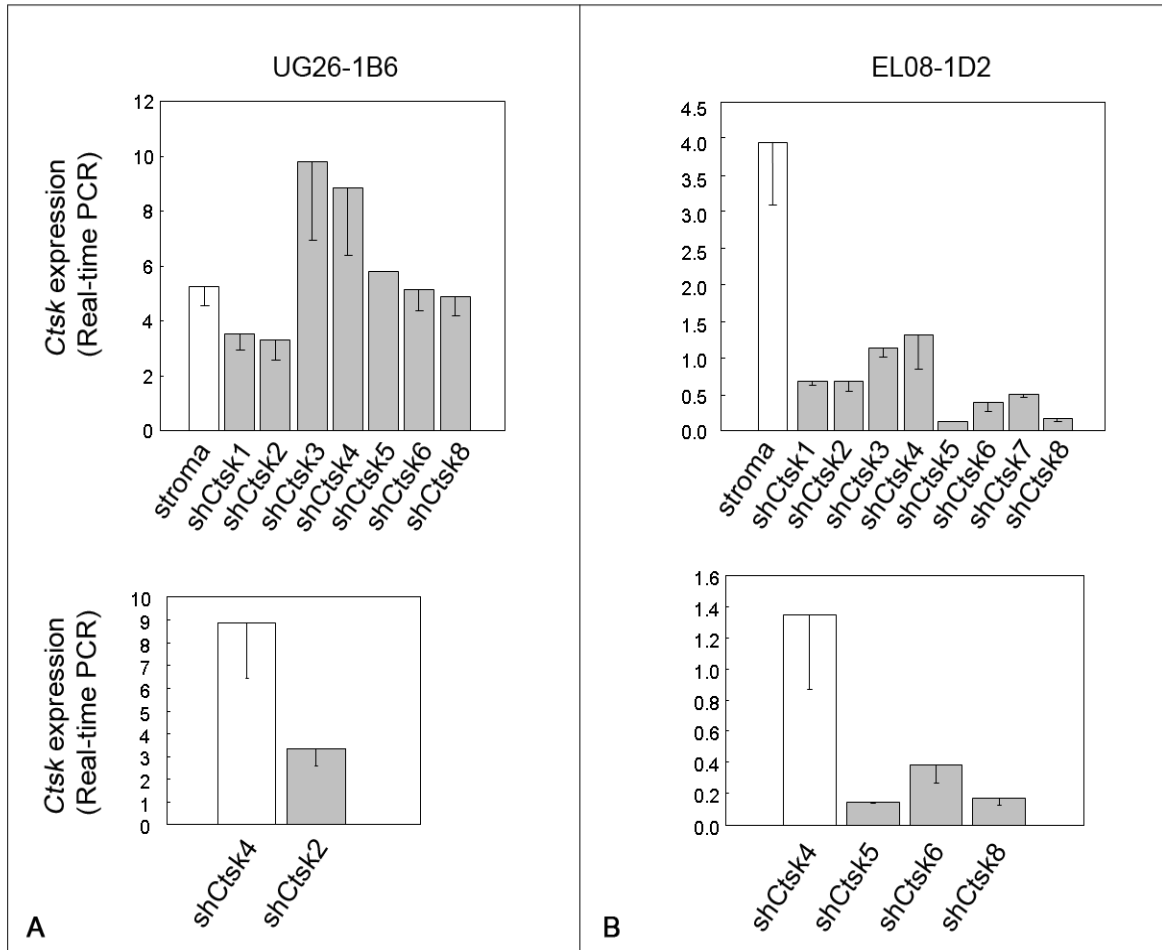


**Figure 8 Generating the stable shCtsk knock-downs by shRNA interference.** (A) Scheme of principles of shRNA interference. ShRNA *Ctsk* constructs, pMD2.G plasmid expressing envelope gene (*env*) and psPAX2 packaging plasmid (*pax*) were co-transfected for the generation of retroviral particles into the Phoenix E cells and the stroma cell lines UG26-1B6 and EL08-1D2 were then infected with this viruses. In order to select the knockdowns puromycin was added. (B) Pictures of the morphology of the knockdown stroma after puromycin selection and the wild type stroma.

### 5.1.2 Verification of shCtsk knockdowns

We verified efficiency of *Ctsk* down regulation by qPCR and by Western blot analysis (see Materials and Methods). Where some of the shRNAs showed a clear knockdown, others did not seem to affect *Ctsk* expression. Since shCtsk4 did not affect *Ctsk* expression, we choose to use *shCtsk4* transfected cells as a negative control for both cell lines. Cells transduced with shCtsk4 still expresses *Ctsk* up to 80% compared to the wild type at the RNA level (**Figure 9**).

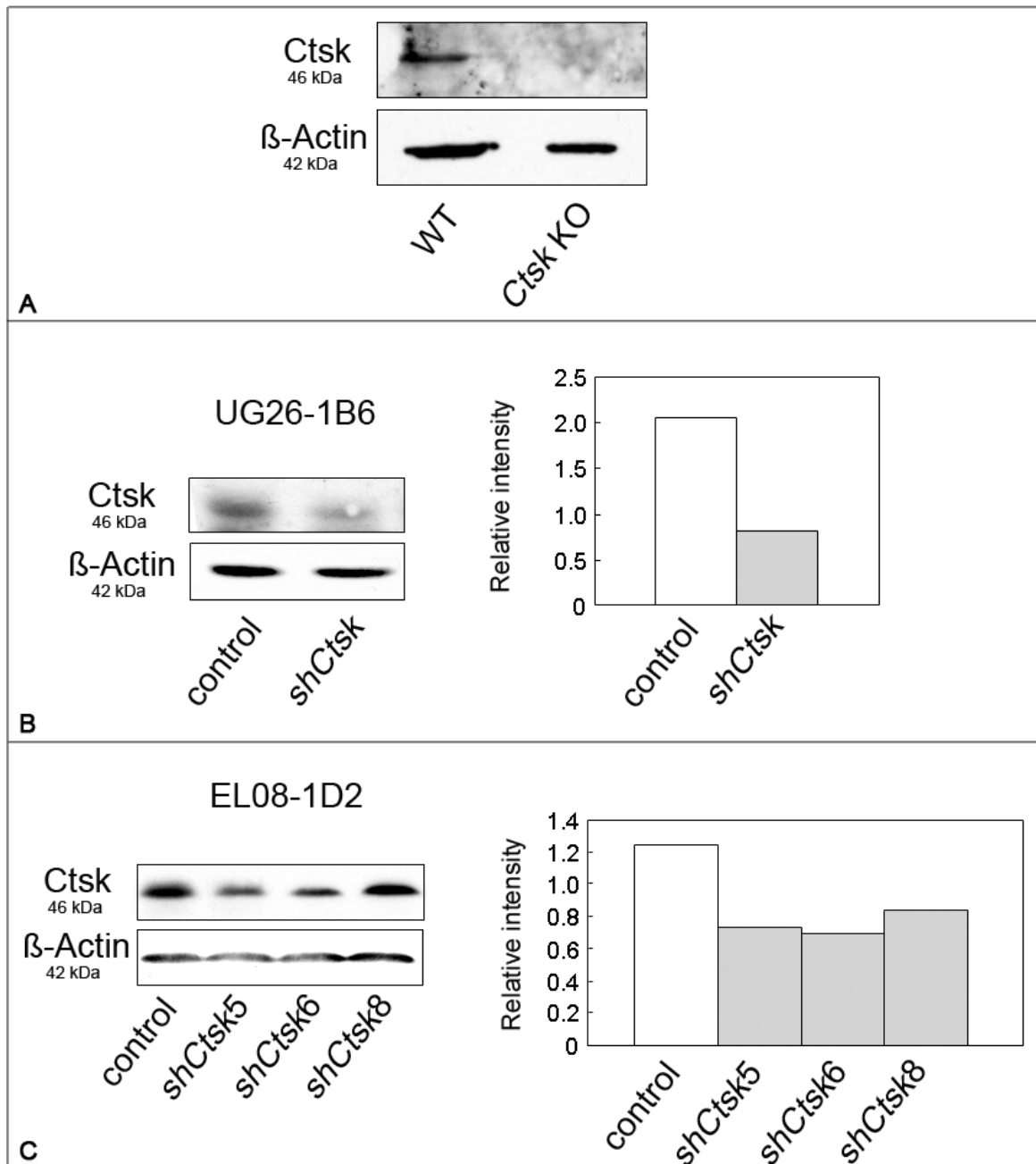




**Figure 9** *Ctsk* expression measured by real-time PCR relative to housekeeping gene *Ythdf1*. (A) Upper graph: *Ctsk* expression of the seven *shCtsk* UG26-1B6 knockdowns compared to the wild type UG26-1B6 stroma. Lower graph: Relative *Ctsk* expression of *shCtsk4* compared to *shCtsk2* UG26-1B6 knockdown. (B) Upper graph: *Ctsk* expression of the eight *shCtsk* EL08-1D2 knockdowns compared to the wild type EL08-1D2 stroma. Lower graph: Relative expression of *shCtsk4* compared to *shCtsk5*, *shCtsk6* and *shCtsk8* EL08-1D2 knockdowns. Grey bars represent control and black bars represent the different *shCtsk* knockdowns. All values are shown as mean  $\pm$  SEM.

The *shCtsk2* UG26-1B6 knockdown displayed the highest expressional repression (80% down-regulation compared to wild type). For EL08-1D2 we selected three knock downs: *shCtsk5*, *shCtsk6* and *shCtsk8* with 92%, 80% and 95% down regulation respectively.

In the next step we analysed *Ctsk* expression at the protein level by Western Blot (3.3.3). First of all, we the tested *Ctsk* antibody (materials 4.11) on the total protein isolated from the brain of *Ctsk*  $-/-$  (Saftig et al. 1998) and wild type mice (**Figure 10A**).



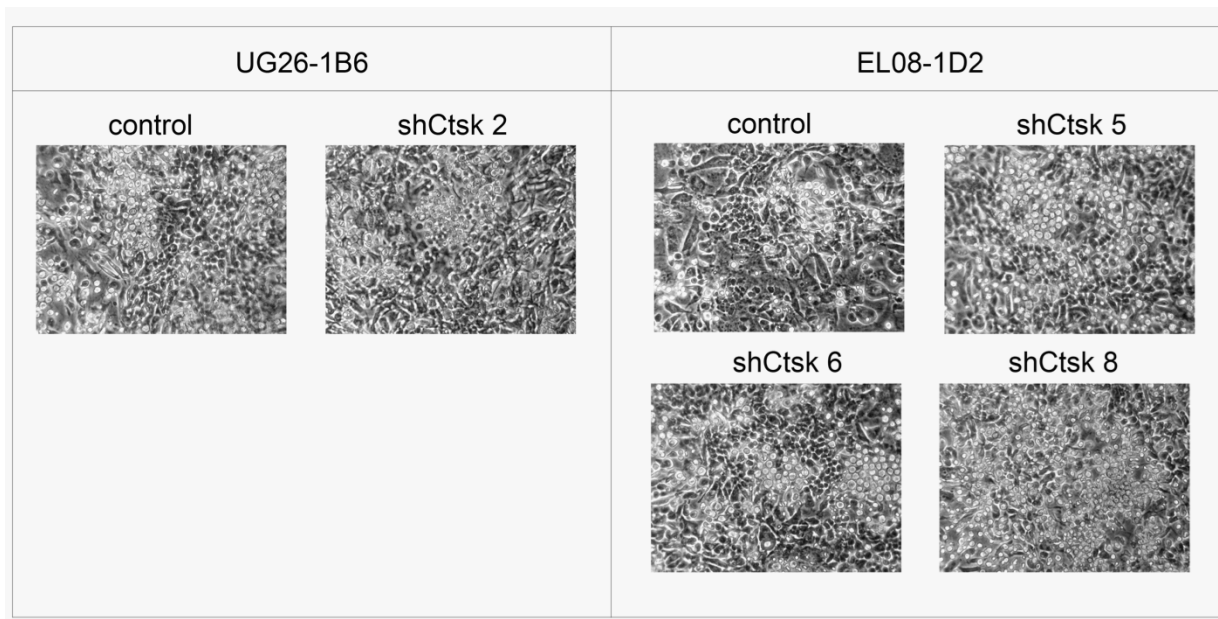
**Figure 10 Western Blot of the Ctsk expression relative to β-Actin.** (A) Test of α-Ctsk using total protein isolated from the brain of *Ctsk* KO (Saftig et al.) and wild type mice. The antibody detected a 46kDa protein. (B) + (C) *Ctsk* expression measured by Western Blot and analysis of the signal relative intensity using ImageJ, white bars represent controls and grey bars represent knockdowns. (B) *Ctsk* protein expression of *shCtsk2* UG26-1B6 knockdown cell lysat compared to the negative control, showing a decreased secretion *Ctsk* protein by the knockdown. (C) *Ctsk* protein expression of *shCtsk5*, *shCtsk6* and *shCtsk8* EL08-1D2 knockdowns cell lysat compared to the negative control, showing as expected a decreased secretion of *Ctsk* protein by the knockdowns.

We measured relative signal intensity and quantitated it by using ImageJ program see materials (4.8), as an internal control for quantitation we used  $\beta$ -Actin. As expected we identified decreased expression of Ctsk protein in knock downs compared to the negative control.

Also, lowered Ctsk levels were found in all knocked down cells, whereas Ctsk in shCtsk4 cells was similar to wild type control cells. Thus, in further experiments we used one knockdown of UG26-1B6 (*shCtsk2*) and three knockdowns of EL08-1D2 (*shCtsk5* *shCtsk6* and *shCtsk8*) for the functional analyses.

### 5.1.3 Decreased clonogenic capacity of wild type *Lin*<sup>-</sup> cells after co-culturing on Ctsk knock-down stroma

To study the effect of decreased Ctsk levels on haematopoiesis in culture, we assessed the ability of the generated Ctsk knockdowns to support haematopoiesis in co-cultures with *Lin*<sup>-</sup> wild type mouse bone marrow cells. Co-cultures were established for 2 and 4 weeks, as described in methods (3.5.2). The representative pictures of co-cultures with cobblestone areas are presented in **Figure 11**. We did not observe morphological differences between the cultures set up with knockdown stromal cells and the controls.



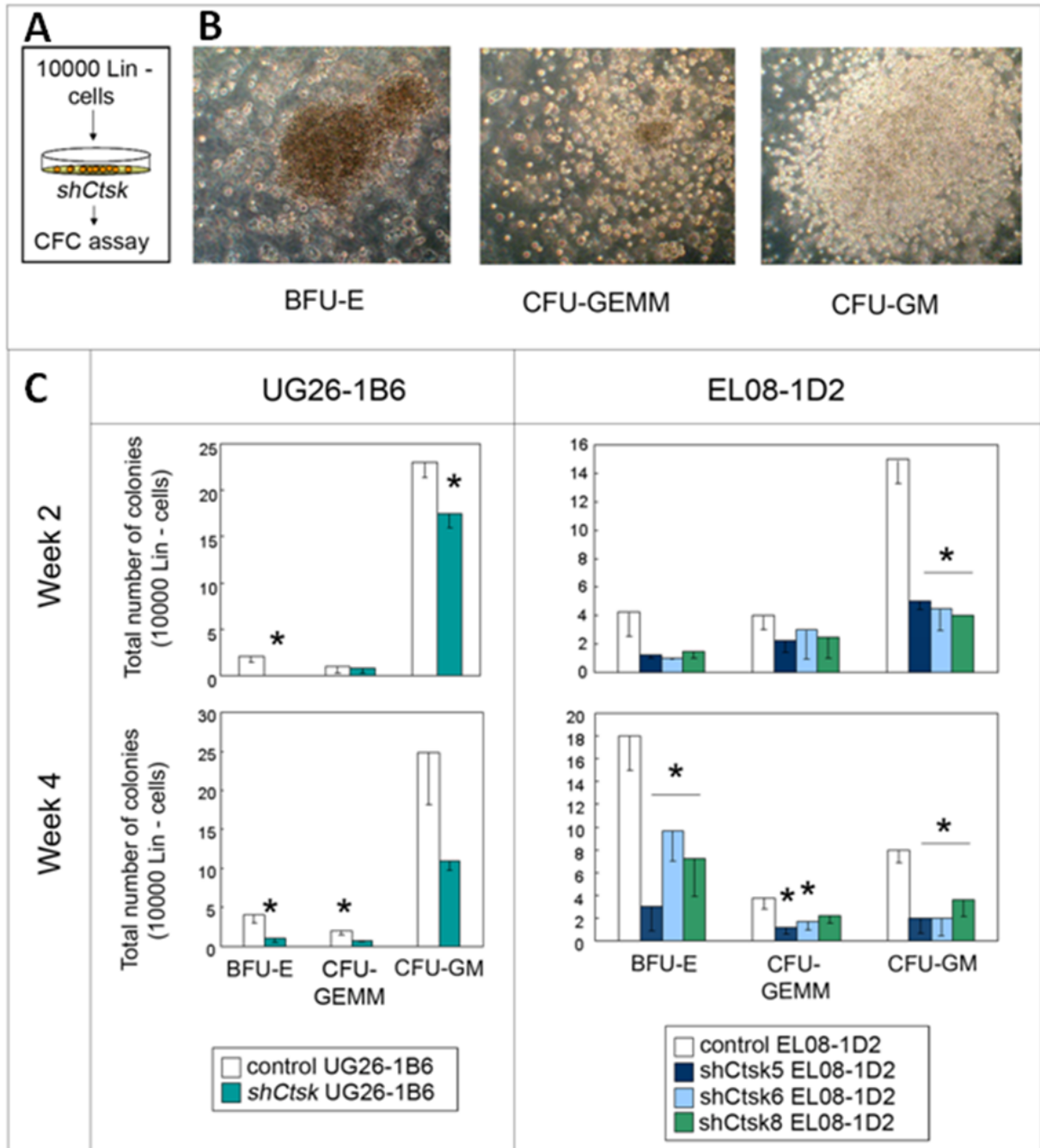
**Figure 11 Pictures of *Lin*<sup>-</sup> cells co-cultured on the knockdown and control stroma, after 4 weeks.** Cobblestone areas formed by the *Lin*<sup>-</sup> cells can be observed in all pictures.

After co-culture, cells were seeded into semi-solid methylcellulose and cultured as described in methods (3.5.3) to determine the number of colony-forming cells in the culture. Formed colonies were analysed after their morphology using a microscope and counted (as described in 3.5.3). Representative pictures of the colonies formed are shown in **Figure 12B**.

Interestingly, the number of erythroid (BFU-E) and myeloid (CFU-GM) and mixed (CFU-GEMM) colonies produced after two and four weeks co-culturing on *shCtsk* UG26-1B6 and EL08-1D2 stroma was significantly decreased (**Figure 12**). The results indicate, that the decrease of *Ctsk* affects the clonogenic capacity of the committed progenitor cells. After 4 weeks of co-culturing, we also observed a significant decrease in mixed colonies (CFU-GEMM) (**Figure 12**).

Having a closer look at the results, we find for the UG26-1B6-*shCtsk2* cells a significant decrease of erythroid colonies after two and four weeks, as well as after two weeks of the myeloid colonies and after four weeks of the mixed colonies.

We observed a similar effect on the three EL08-1D2*shCtsk* knockdown cell lines (*shCtsk5*, *shCtsk6* and *shCtsk8*), with significant decrease in myeloid colonies after two weeks of co-culturing and in all colony types after four weeks.

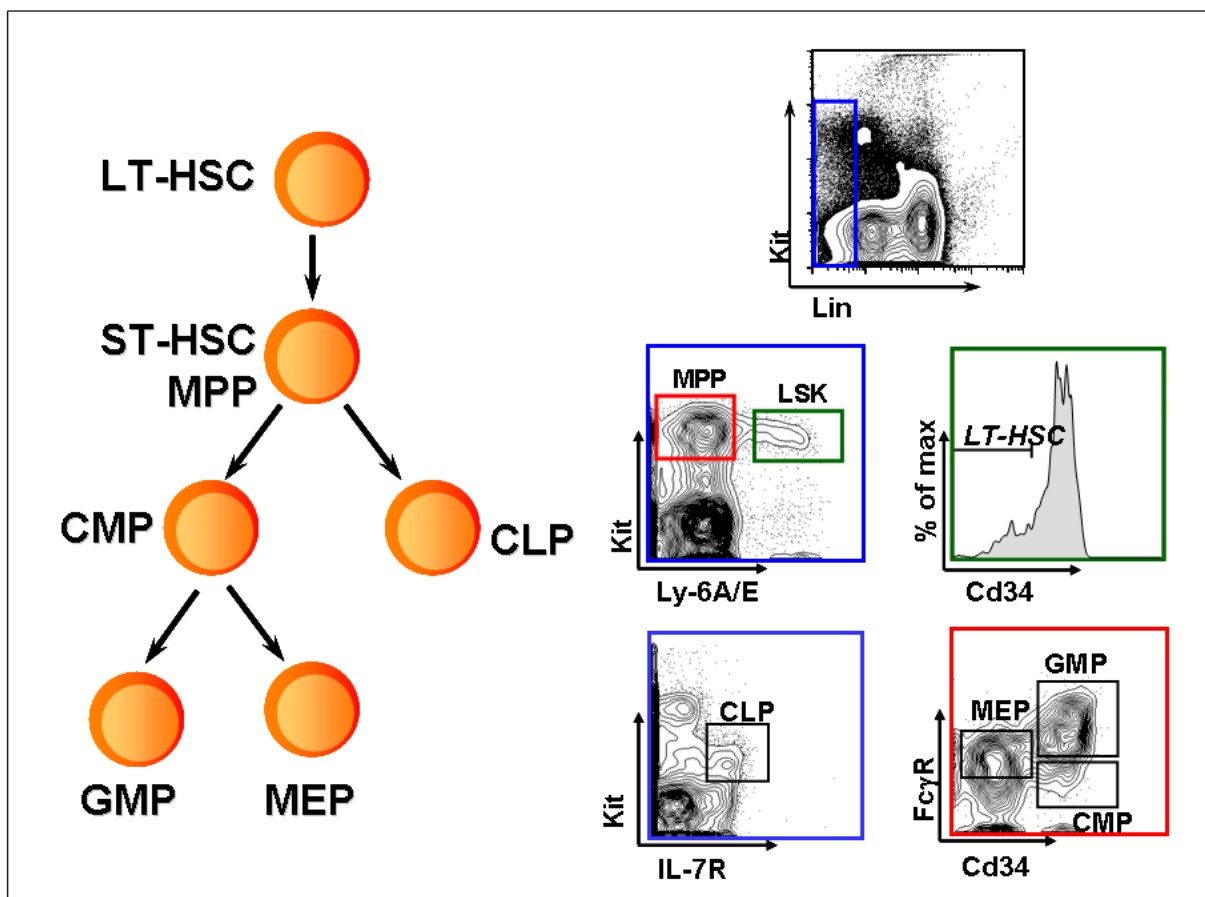


**Figure 12 Clonogenic activity of Lin<sup>-</sup> wild type cells after co-culturing on the knockdown and control stroma.** (A) Scheme of the experiment, 10000 Lin<sup>-</sup> wild type were co-cultured on the knockdown and control stroma for two and four weeks and were then seeded on methylcellulose in order to count the colonies formed. (B) Pictures of the morphology of the BFU-E, CFU-GEMM and CFU-GM colonies, formed by the Lin<sup>-</sup> cells co-cultured on the knockdown stroma. (C) Total number of colonies formed from 10000 Lin<sup>-</sup> cells seeded on methylcellulose, after two and four weeks. White bars represent control stroma and colored bars represent the *shCtsk* stroma. Results for UG26-1B6 stroma are shown on the left graphs and results for EL08-1D2 on the right graphs. All values are shown as mean ± SEM. \* p<0.05.

In summary, the results indicate strongly decreased clonogenic activity of wild type progenitor cells after co-culturing with *Ctsk* deficient stroma and suggest that *Ctsk* is required for optimal supportive ability of the UG26-1B6 and EL08-1D2 stromal cells.

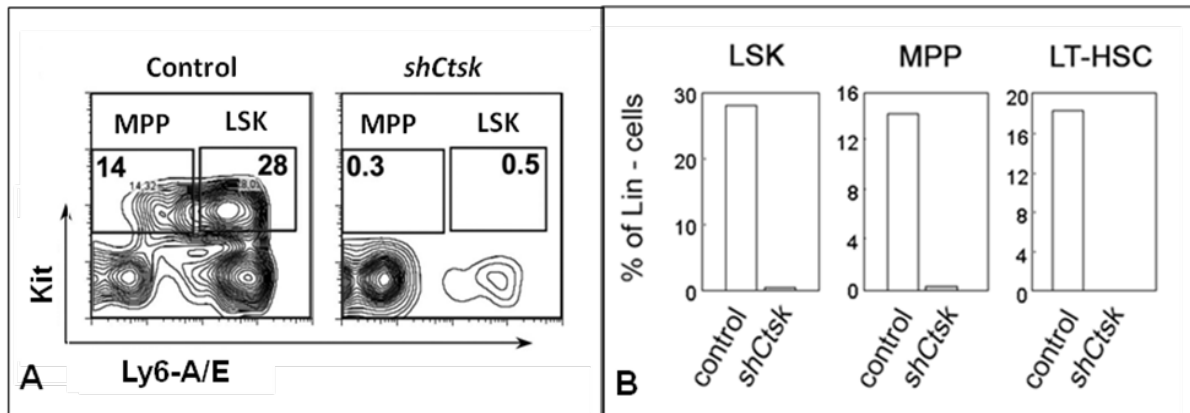
#### 5.1.4 Reduced number of the early haematopoietic subsets in wild type *Lin*<sup>-</sup> cells after co-culturing on the *Ctsk* knockdown stroma

After three weeks of co-culture, haematopoietic cells were analysed by flow cytometry, and stained as described in methods (3.5.4). The used gating strategy for primitive haematopoietic cells is shown in (Figure 13) and described in introduction (1.1.3).



**Figure 13 FACS gating strategy for analysis HSC and progenitors.** First mature cells are excluded as they express Lineage committed markers: Cd4/8a, Cd3 (T-cells), B220 (B-cells), Gr-1 (granulocytes) and Ter119 (erythroid cells). Further, HSC don't express lineage-committed markers, but express Sca-1 and the c-Kit receptor and are called *Lin*<sup>-</sup> Sca1<sup>+</sup> Kit<sup>+</sup> (LSK) cells. LT-HSCs are LSK cells which don't express Cd34 surface markers. ST-HSCs are CD34<sup>+</sup> and differentiate into MPP, which are *Lin*<sup>-</sup>, Sca1<sup>-</sup> and Kit<sup>+</sup>. They further differentiated into CMPs, MEPs and GMPs and can be selected by their different expression of Cd34 surface marker and Fcγ-Receptor, as well as into CLPs, which can be enriched by their middle expression level of Kit and IL7-Receptor.

We found interesting results in the total number of MPPs and LSKs and LT-HSCs, which are dramatically decreased in the co-cultures of Lin<sup>-</sup> cells on the knockdown stroma compared to the control (**Figure 14**).

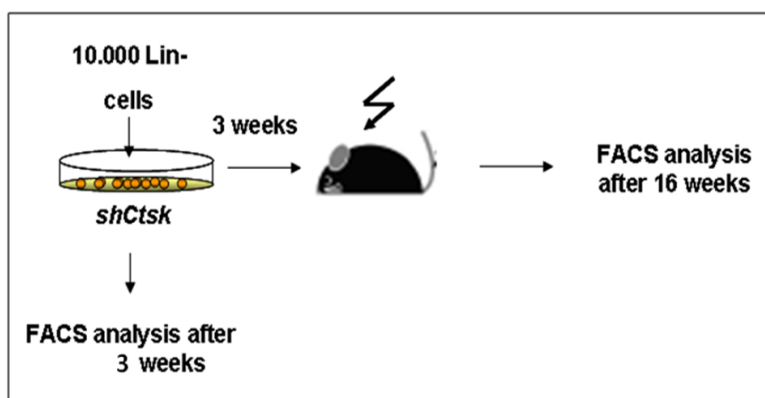


**Figure 14 Percentages LSK, MPP and LT-HSC of the Lin<sup>-</sup> cells co-cultured for three weeks on the knockdown and control stroma.** (A) Representative FACS plots of WT Lin<sup>-</sup> cells co-cultured on the control and the *Ctsk* knockdown stroma, gated for LSK and MPP. LSK are separated as Lin<sup>-</sup>Ly6A/E+cKit<sup>+</sup> population. MPP are separated as Lin<sup>-</sup>Ly6A/E-cKit<sup>+</sup> population. (B) LSK, MPP and LT-HSC percentages of total WT Lin<sup>-</sup> cells co-cultured on the control and the *Ctsk* knockdown stroma. LSK are gated as Lin<sup>-</sup> Sca1<sup>+</sup> Kit<sup>+</sup> cells, MPP as Lin<sup>-</sup> Sca1<sup>-</sup> Kit<sup>+</sup> cells and LT-HSC as Cd34<sup>-</sup> cells in the LSK compartment. The results show highly reduced number of the early haematopoietic subsets in WT Lin<sup>-</sup> cells co-cultured on the *Ctsk* knockdowns. White bars represent the co-cultures on control stroma and grey bars represent the co-cultures on *shCtsk* stroma.

### 5.1.5 Engraftment of HSC after co-culturing with *Ctsk* knockdown stroma cells

The results of the *in vitro* experiments, suggest that down regulation of *Ctsk* causes reduced maintenance of committed progenitor cells. Thus, in the next experiments we wanted to investigate the possible role of *Ctsk* in earlier steps of haematopoiesis.

In order to find out whether decreased *Ctsk* affects the maintenance of repopulating capacity of LT-HSC, we co-cultured Lin<sup>-</sup> wild type cells on knockdown and control UG26-1B2 stroma for three weeks and transplanted the cultured cells into wild type recipient mice as described in methods (3.6.2) as well as performed flow-cytometric analyses by FACS (**Figure 15**).



**Figure 15 Scheme of primary transplantation experiment.** Lin- wild type cells were co-cultured on *shCtsk* UG26-1B6 stroma and on control stroma. After three weeks of co-culturing, half of the cells were analysed by FACS and the other half were transplanted into lethally irradiated wild type mice, which were sacrificed after sixteen weeks for further FACS analysis.

### 5.1.5.1 FACS analyses of the transplanted mice tissues

To find out whether repopulating activity was affected by a decrease of *Ctsk* in stromal cells, lethally irradiated recipient mice were transplanted with Lin- cells, which had been co-cultured for three weeks on the knockdown, and the control UG26-1B6 stromal cells. After 16 weeks mice were sacrificed for FACS analyses of the different tissues, as described in methods and materials.

#### 5.1.5.1.1 FACS analyses of the blood samples

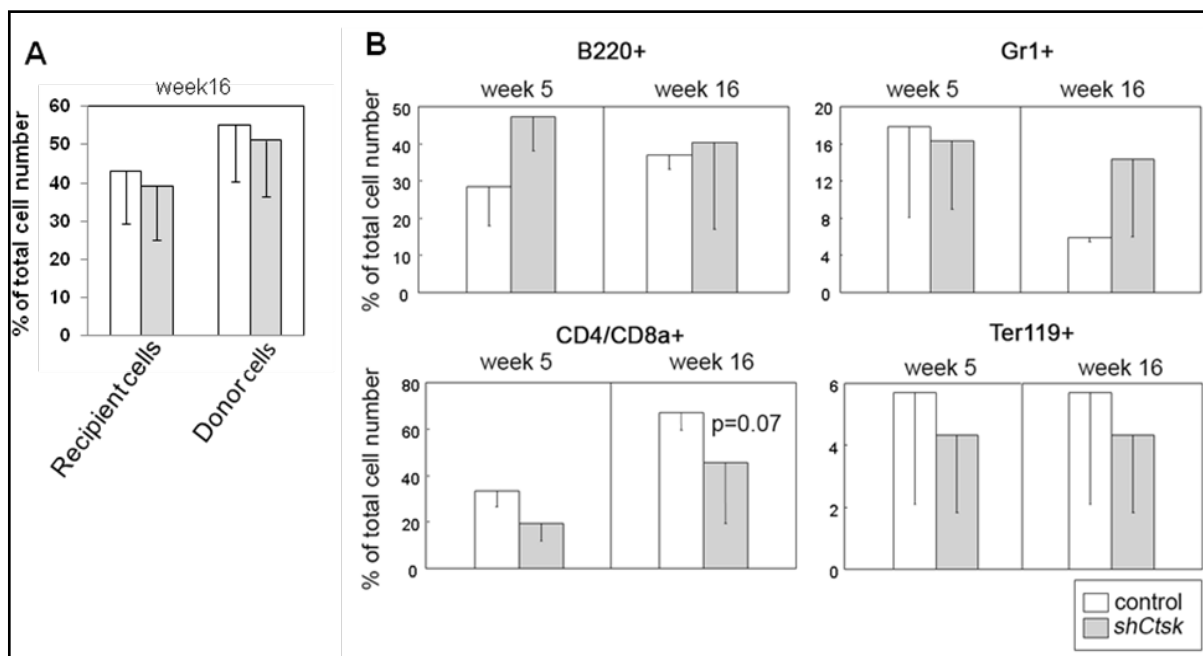
Firstly the different blood components of the transplanted mice were counted on the Scil Vet ABC blood counter. We observed significant increased numbers of white blood cells, lymphocytes and platelets (**Figure 16**).

	Control	<i>shCtsk</i>
WBC ( $10^3/\mu\text{l}$ )	1.9 $\pm$ 0.18	4.2 $\pm$ 0.7 *
Lymph ( $10^3/\mu\text{l}$ )	1.4 $\pm$ 0.15	3.2 $\pm$ 0.6 *
Mono ( $10^3/\mu\text{l}$ )	0.1 $\pm$ 0	0.1 $\pm$ 0.04
Gran ( $10^3/\mu\text{l}$ )	0.5 $\pm$ 0.05	0.9 $\pm$ 0.2
Eos ( $10^3/\mu\text{l}$ )	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
RBC ( $10^3/\mu\text{l}$ )	8.2 $\pm$ 0.7	7 $\pm$ 1.4
PLT ( $10^3/\mu\text{l}$ )	210 $\pm$ 134	1032 $\pm$ 169.4 **

**Figure 16 Blood cell counts in 16-weeks-old transplanted mice.** PLT, WBC and Lymph are significant increased in mice transplanted with Lin- cells co-cultured on the *Ctsk* knockdown stroma compared to control stroma. Average values are shown with Standard Error of Mean (SEM). (WBC, white blood cell; Lymph, lymphocytes; Mono, monocytes; Gran, granulocytes; Eos, eosinophiles; RBC, red blood cells; HCT haematocrit; PLT, platelets)



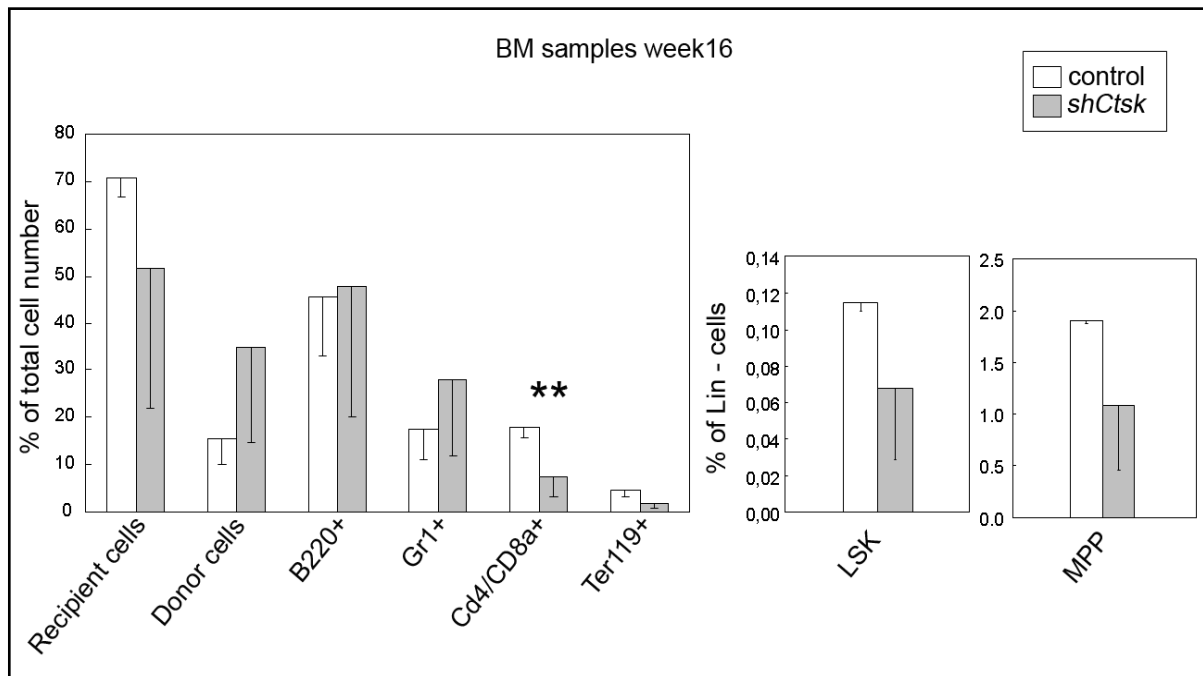
In order to verify the level of engraftment, recipient mice were bled after five weeks, as well as when scarified at week sixteen. The donor and recipient cells were distinguished by flow cytometric analyses of mature blood cell lineages, after their expression of Ly5.1 (donor mice) and Ly5.2 (recipient mice) markers as describe in materials (4.12). In peripheral blood, engraftment is not significantly decreased in mice transplanted with Lin<sup>-</sup> cells co-cultured on the *Ctsk* knockdown stroma cells compared to recipient mice receiving cells co-cultured with control stromal cells (**Figure 17A**). Having a closer look, we found a decreased recovery also for T cells (Cd4/CD8a<sup>+</sup> cells) and erythroid cells (Ter119<sup>+</sup> cells), while we couldn't observe changes on the B220<sup>+</sup> cells, but a highly increased amount of donor derived myeloid cells (Gr1<sup>+</sup> cells) (**Figure 17B**).



**Figure 17 FACS analyses of the engrafted cells peripheral blood.** (A) Primary transplantation engraftment levels in peripheral blood at week sixteen. We can observe a slightly decreased recovery in mice receiving Lin<sup>-</sup> cells co-cultured on the knockdowns. (B) Relative numbers of engrafted B (B220<sup>+</sup> cells), T (Cd4<sup>+</sup>and/or Cd8a<sup>+</sup> cells), myeloid (Gr1<sup>+</sup> cells) and erythroid (Ter119<sup>+</sup> cells) cells in peripheral blood, five and sixteen weeks after mice transplantation. The results show, a decreased engraftment of HSC after co-culturing on *Ctsk* knockdown stroma for the CD4/CD8a<sup>+</sup> and the Ter119<sup>+</sup> cells, while there are no on the B220<sup>+</sup> cells and there is even an increase on the Gr1<sup>+</sup> cells. White bars represent engrafted cells in the transplants with the co-cultures on control stroma and grey bars represent engrafted cells in transplants with the co-cultures on *shCtsk* knock-down stroma. All values are shown as mean  $\pm$  SEM.

### 5.1.5.1.2 FACS analyses of the BM samples

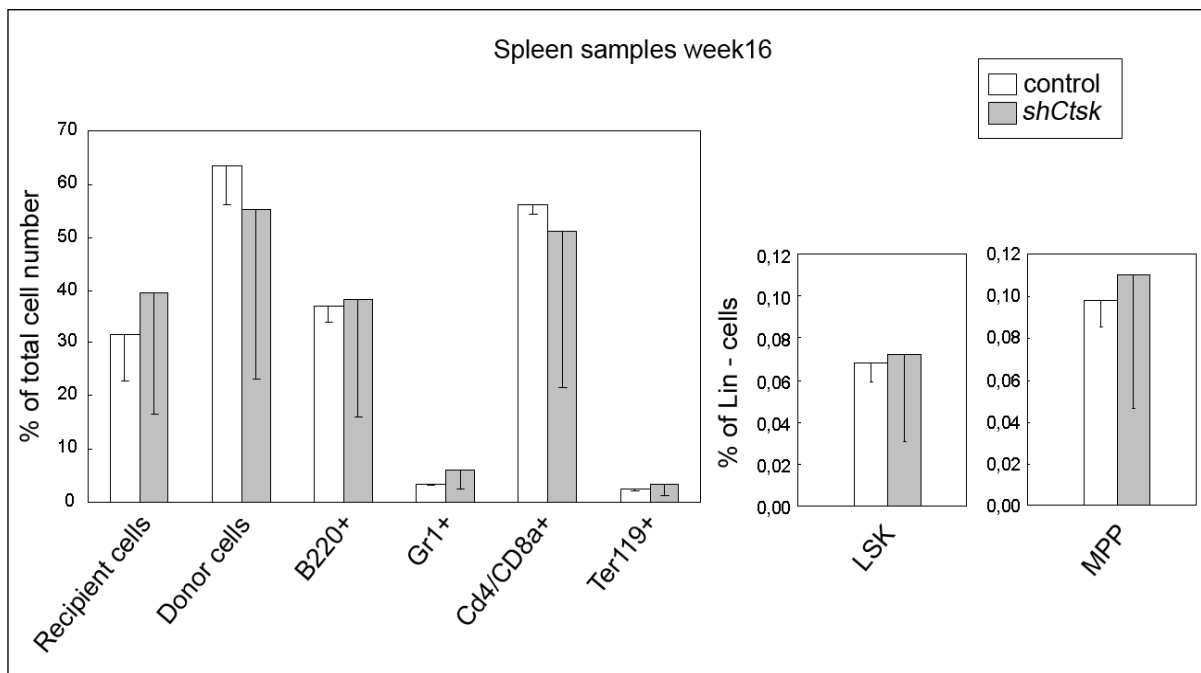
For the analysis of the level of engraftment of mature cell types in BM, a similar flow-cytometric analysis of BM isolated at week sixteen post-transplantation was performed. Although the total level of engraftment seems to be increased in mice transplanted with Lin<sup>-</sup> cells co-cultured on the *Ctsk* knockdown stromal cells, we observed a strongly significant diminished recovery of T cells (Cd4/Cd8a<sup>+</sup> cells), as well as the early LSK and MPP cell subpopulations, while we observed an increased of engrafted of myeloid cells (Gr1<sup>+</sup> cells) and no differences for the B cells (B220<sup>+</sup> cells) (**Figure 18**). Our findings of the BM analyses agree with the results we obtained by analysing the peripheral blood at week sixteen (**Figure 16**).



**Figure 18 FACS analyses of the engrafted cells in bone marrow sixteen weeks after transplantation.** On the left graph: primary transplantation engraftment levels, engrafted B (B220<sup>+</sup> cells), T (Cd4<sup>+</sup>and/or Cd8a<sup>+</sup> cells), myeloid (Gr1<sup>+</sup> cells) and erythroid (Ter119<sup>+</sup> cells) cells. On the right graphs: engraftment of LSK and MPP in relative numbers to total Lin<sup>-</sup> cells. The results show, an increased engraftment in mice transplanted with cells co-cultured on the *Ctsk* knockdowns, a highly decreased engraftment of Cd4/Cd8a<sup>+</sup> cells, as well as of LSK and MPP. Ter119<sup>+</sup> are slightly lower when co-cultured with *shCtsk* knockdown stroma, while there are no differences on the B220<sup>+</sup> cells and there is an increase on the Gr1<sup>+</sup> cells. White bars represent engrafted cells in the transplants with the co-cultures on control stroma and grey bars represent engrafted cells in transplants with the co-cultures on *shCtsk* knock-down stroma. BM: bone marrow. All values are shown as mean  $\pm$  SEM. \*\* p<0.03.

### 5.1.5.1.3 FACS analyses of the spleen samples

The spleen was extracted of the transplanted mice at week sixteen post-transplantation and analysed by FACS, as described in methods (3.5.4 and 3.6.1). The level of donor engraftment in spleen is unchanged in recipients receiving cells from co-cultures of *shCtsk* stromal cells or control stroma. Interestingly, recovery of LSK and MPP is increased in the mice recovered with the *shCtsk* co-cultures (**Figure 19**). We didn't observe significant differences in engraftment of B, T, myeloid and erythroid cells.

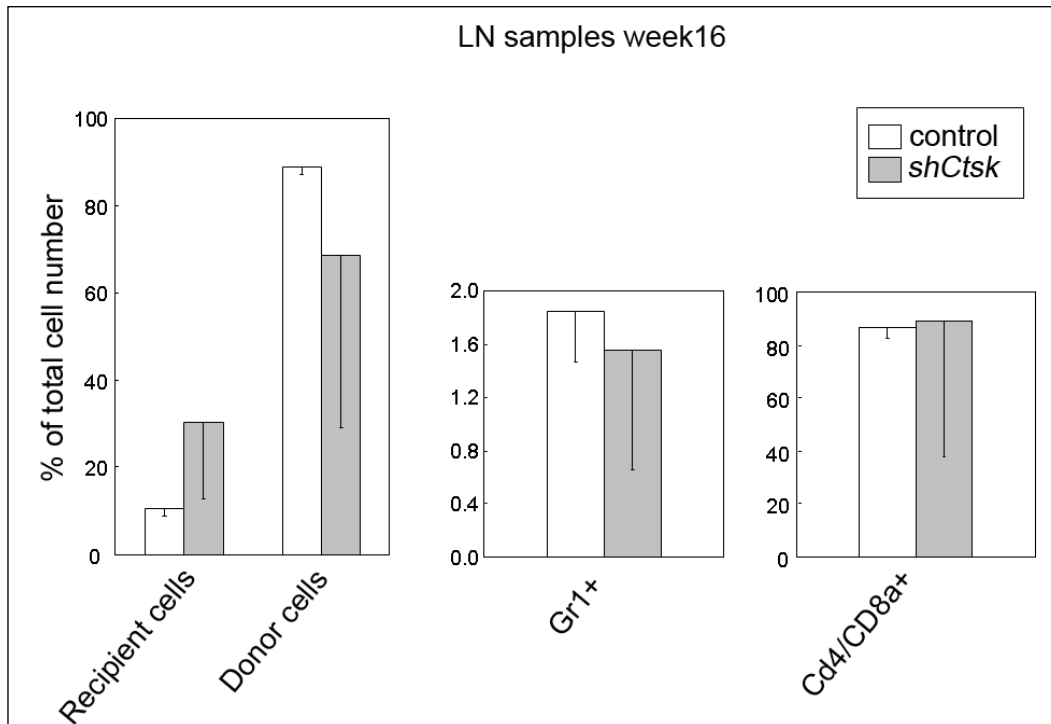


**Figure 19 FACS analyses of the engrafted cells in the spleen sixteen weeks after transplantation.** On the left graph: primary transplantation engraftment levels, engrafted B (B220+ cells), T (Cd4+and/or Cd8a+ cells), myeloid (Gr1+ cells) and erythroid (Ter119+ cells) cells. On the right graphs: engraftment of LSK and MPP in relative numbers to total Lin- cells. The results do show an decrease total recovery in spleen, while LSK and MPP engraftment is slightly higher in mice transplanted with Lin- cells co-cultured on *shCtsk* knock-down stroma. White bars represent engrafted cells in the transplants with the co-cultures on control stroma and grey bars represent engrafted cells in transplants with the co-cultures on *shCtsk* knock-down stroma. All values are shown as mean  $\pm$  SEM.

### 5.1.5.1.4 FACS analyses of the lymph node samples

Considering the decreased T cell engraftment in mice transplanted with Lin- cells co-cultured on UG26-1B6-*shCtsk2*, we additionally analysed lymph nodes of the transplanted mice and processed as described in methods (3.5.4 and 3.6.1).

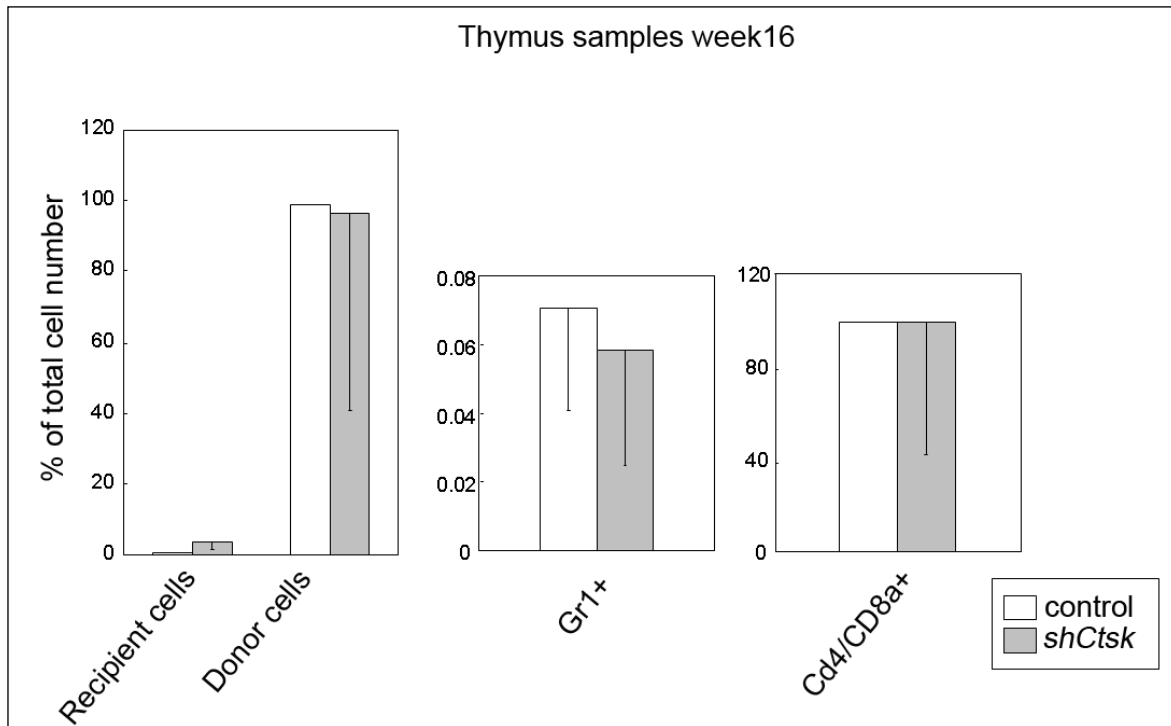
However, we did not detect a difference on the engraftment of myeloid cells (Gr1+ cells) or lymphoid B or T cells (Cd4/Cd8a+ cells), between mice transplanted with control co-cultures and mice transplanted with the *shCtsk* stroma co-cultures (**Figure 20**), suggesting that *Ctsk* deficiency only depletes for marrow T cells.



**Figure 20 FACS analyses of the engrafted cells in the lymph nodes sixteen weeks after transplantation.** On the left graph: Transplantation engraftment levels. On right graphs: Relative numbers of myeloid (Gr1+ cells) and T (Cd4+and/or Cd8a+ cells) cells. The results do not show significant differences in the engraftment behaviour of Gr1+ cells and Cd4/Cd8a+ cells in lymph nodes between mice recovered with Lin- cells co-cultured on control stroma and the ones recovered with Lin- cells co-cultured on *shCtsk* knock-down stroma. White bars represent engrafted cells in the transplants with the co-cultures on control stroma and grey bars represent engrafted cells in transplants with the co-cultures on *shCtsk* knock-down stroma. LN: Lymph nodes. All values are shown as mean  $\pm$  SEM.

#### 5.1.5.1.5 FACS analyses of the thymus samples

The thymus was extracted at week sixteen post-transplantation and further analysed by FACS, as described in methods (3.5.4 and 3.6.1). The results show no significant alterations on engraftment caused transplantation with *shCtsk* co-cultures, compared to control co-cultures (**Figure 21**).



**Figure 21 FACS analyses of the engrafted cells in the thymus, sixteen weeks after transplantation.** On the left graph: Transplantation engraftment levels. On right graphs: Relative numbers of myeloid (Gr1+ cells) and T (Cd4+and/or Cd8a+ cells) cells. The results do not show significant discrepancies between mice transplanted with WT Lin- cells co-cultured on the *Ctsk* knockdowns and WT recipient mice. White bars represent engrafted cells in the transplants with the co-cultures on control stroma and grey bars represent engrafted cells in transplants with the co-cultures on *shCtsk* knock-down stroma. All values are shown as mean  $\pm$  SEM.

## 5.2 *Ctsk* may be involved in the maintenance of haematopoiesis *in vivo*

In summary, the results from co-cultures demonstrate that decreased expression of *Ctsk* causes decreased maintenance of haematopoietic progenitors and also affects *in vivo* engraftment. To study the role of *Ctsk* in more detail *in vivo*, we decided to study the *Ctsk* deficient mice (Saftig et al. 1998), described in introduction (1.2.7).

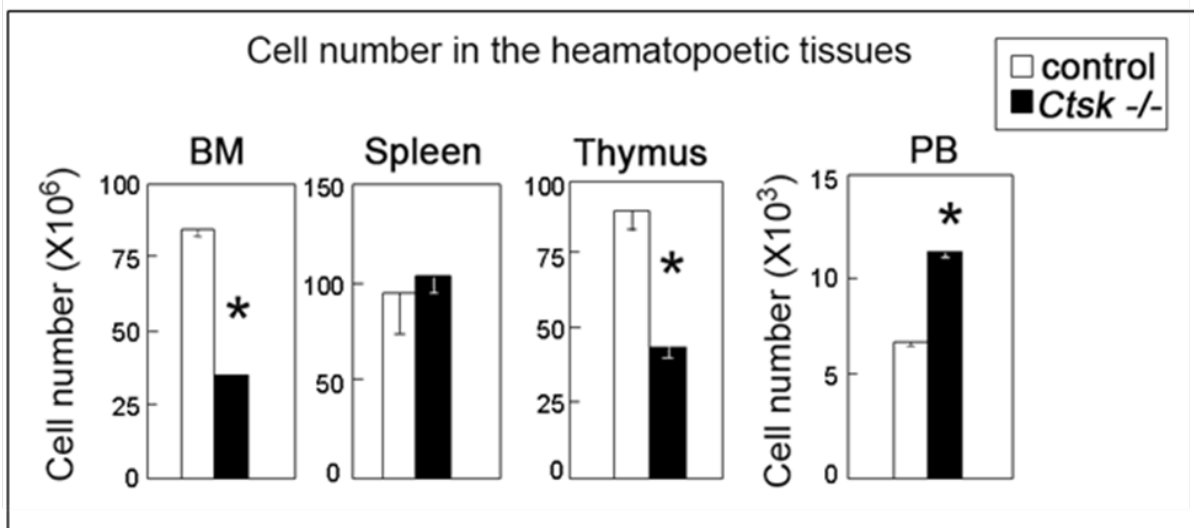
Considering the increased trabecular surface in *Ctsk* <sup>-/-</sup> mice, and the importance of trabecular bone in haematopoiesis (Ellis et al. 2011) we hypothesized that haematopoiesis in the *Ctsk* deficient mice would be affected. Hence, we first investigated the phenotype and assessed haematopoietic subpopulations in these mice.

### 5.2.1 Redistribution of haematopoietic cells in the *Ctsk* <sup>-/-</sup> mice

Firstly we characterised in detail the haematopoietic organs and subpopulations of the *Ctsk* <sup>-/-</sup> mice during “steady-state” conditions, by phenotyping bone marrow, spleen, thymus and peripheral blood.

Interestingly we found differences in the number of viable cells (trypan blue exclusion assay) between the different haematopoietic tissues, what can be an indicator of possible alterations in haematopoiesis. In the spleen viable cells were unchanged, while in the BM and the thymus the cells are reduced and in the blood they are increased (**Figure 22**).

This alteration suggests a redistribution of haematopoietic cells in the *Ctsk* <sup>-/-</sup> mouse, which might be related to the trabecular alterations described in this mouse mutant (Gowen et al. 1999). Another possibility could be that homing properties of haematopoietic cells could be different from those in wild-type mice.



**Figure 22 Total cell numbers of the haematopoietic tissues**, from bone marrow (BM, four long bones: two femurs and two tibia per mouse), spleen, thymus, and peripheral blood (PB, 103 cells/ml). White bars represent control and black bars represent *Ctsk* <sup>-/-</sup>. PB: peripheral blood. All values are shown as mean ± SEM. \* p<0.05.

Another way of assessing blood cell numbers is using the Scil Vet ABC blood counter. When we counted blood cells of the control and the *Ctsk*<sup>-/-</sup> mice, we found that the numbers of white blood cells, lymphocytes, eosinophiles, red blood cells, haematocrit and platelets were within the normal range. However, the main contribution to the increased cell number in peripheral blood was found to be attributable to increased numbers of mature myeloid cells: monocytes and granulocytes).

	<b>Control</b>	<b><i>Ctsk</i><sup>-/-</sup></b>
WBC (10 <sup>3</sup> /μl)	<b>6.5 ± 1.46</b>	<b>9.4 ± 1.66</b>
Lymph (10 <sup>3</sup> /μl)	<b>5.5 ± 1.36</b>	<b>7.6 ± 1.06</b>
Mono (10 <sup>3</sup> /μl)	<b>0.1 ± 0.04</b>	<b>0.5 ± 0.04 *</b>
Gran (10 <sup>3</sup> /μl)	<b>0.9 ± 0.09</b>	<b>2.3 ± 0.14 *</b>
Eos (10 <sup>3</sup> /μl)	<b>0.04 ± 0.02</b>	<b>0.1 ± 0.03</b>
RBC (10 <sup>6</sup> /μl)	<b>9.1 ± 1.39</b>	<b>10.1 ± 0.16</b>
PLT (10 <sup>3</sup> /μl)	<b>1123.5 ± 289</b>	<b>688.2 ± 167</b>

**Figure 23 Blood cell counts in Control and *Ctsk*<sup>-/-</sup> mice.** Counts show significant increased numbers of Mono and Gran. Average values are shown with Standard Error of Mean (SEM). (WBC, white blood cell; Lymph, lymphocytes; Mono, monocytes; Gran, granulocytes; Eos, eosinophiles; RBC, red blood cells; HCT haematocrit; PLT, platelets)

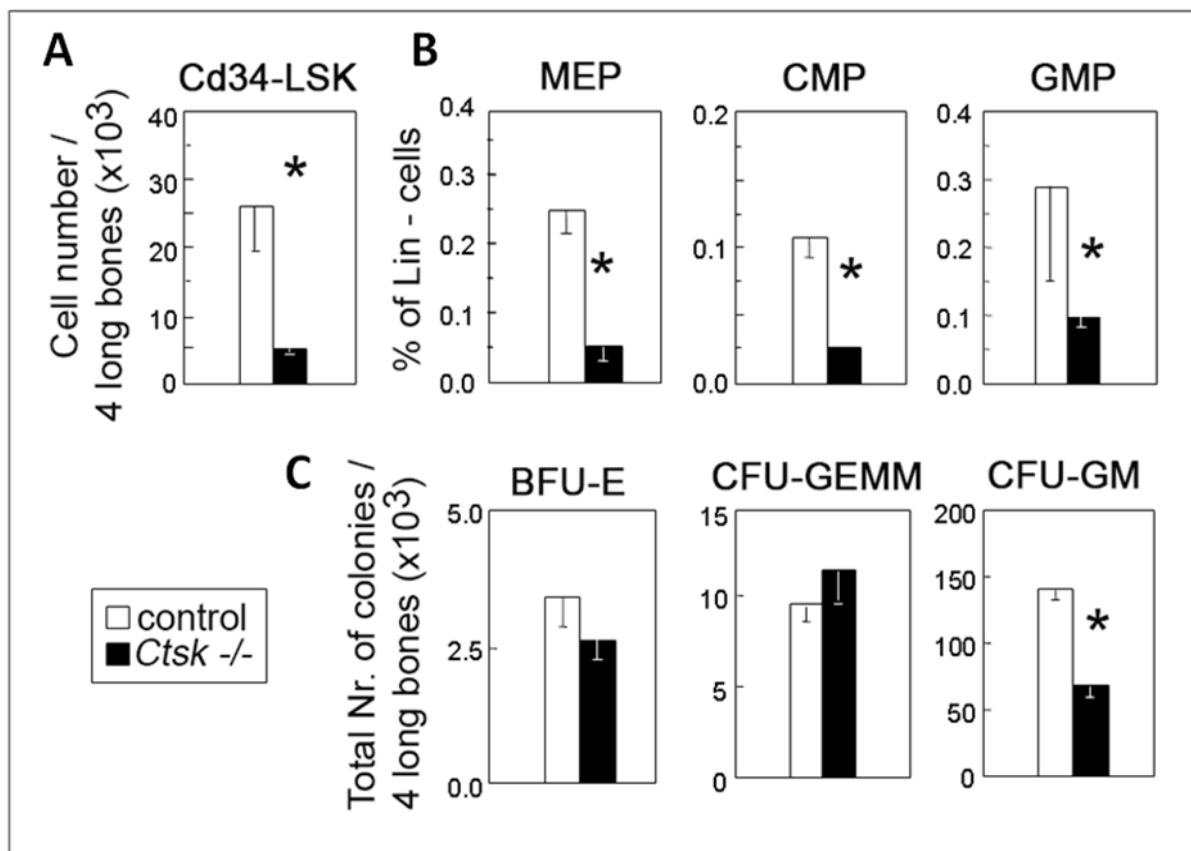
### 5.2.2 Alterations in early haematopoietic cells in *Ctsk*<sup>-/-</sup> mice

To find out whether the observed changes in *Ctsk*<sup>-/-</sup> mice result from alterations in early haematopoiesis, we then analysed the primitive cell populations by FACS. For this purpose, cells labelled with antibodies and analysed by flow cytometry. Flow-cytometric profiles were gated, as shown in **Figure 13** and described in methods (3.5.4) in order to separate early cell subsets of the haematopoietic hierarchy.

Our results show, that the earliest, most HSC-enriched cells, the CD34<sup>-</sup> LSK cells are severely depleted in *Ctsk*<sup>-/-</sup> mice. In addition, multipotent progenitors (Lin<sup>-</sup>, Sca1<sup>-</sup>, Kit<sup>+</sup> and Cd34<sup>-</sup>) were significantly reduced in BM in *Ctsk*<sup>-/-</sup> mice, in accord with this result is, that committed progenitors, which make up the MP population (CMPs, GMPs, MEPs) are significant reduced as well (**Figure 24A and B**).

As next experiment, we investigated whether the changes found could be caused by the inability of the *Ctsk*-deficient environment, to properly maintain haematopoiesis. Thus, we studied the capacity of primary *Ctsk*<sup>-/-</sup> stroma to maintain haematopoiesis in co-cultures with Lin<sup>-</sup> negative cells. On a functional level, we found that the number of colony-forming cells, such as BFU-E and CFU-GEMM, were unchanged after 2 weeks of co-culture, but the number of CFU-GM was significantly decreased (**Figure 24C**), which is in accordance with the earlier findings with the *Ctsk* knockdown stroma cell lines UG26-1B6 and EL08-1D2 (**Figure 12**).

Thus, loss of *Ctsk* results in a decreased progenitor cell activity.



**Figure 24 Alterations in early haematopoiesis in *Ctsk*<sup>-/-</sup> mice.** (A) Cd34-LSK cells from four long bones (2 tibia and 2 femurs), presented in absolute cell number. (B) MEP, CMP and GMP, presented in percentage of the Lin<sup>-</sup> cells. (C) Total number of colonies formed from four long bones (2 tibia and 2 femurs). White bars represent control and black bars represent *Ctsk*<sup>-/-</sup>. All values are shown as mean ± SEM. \* p<0.05.



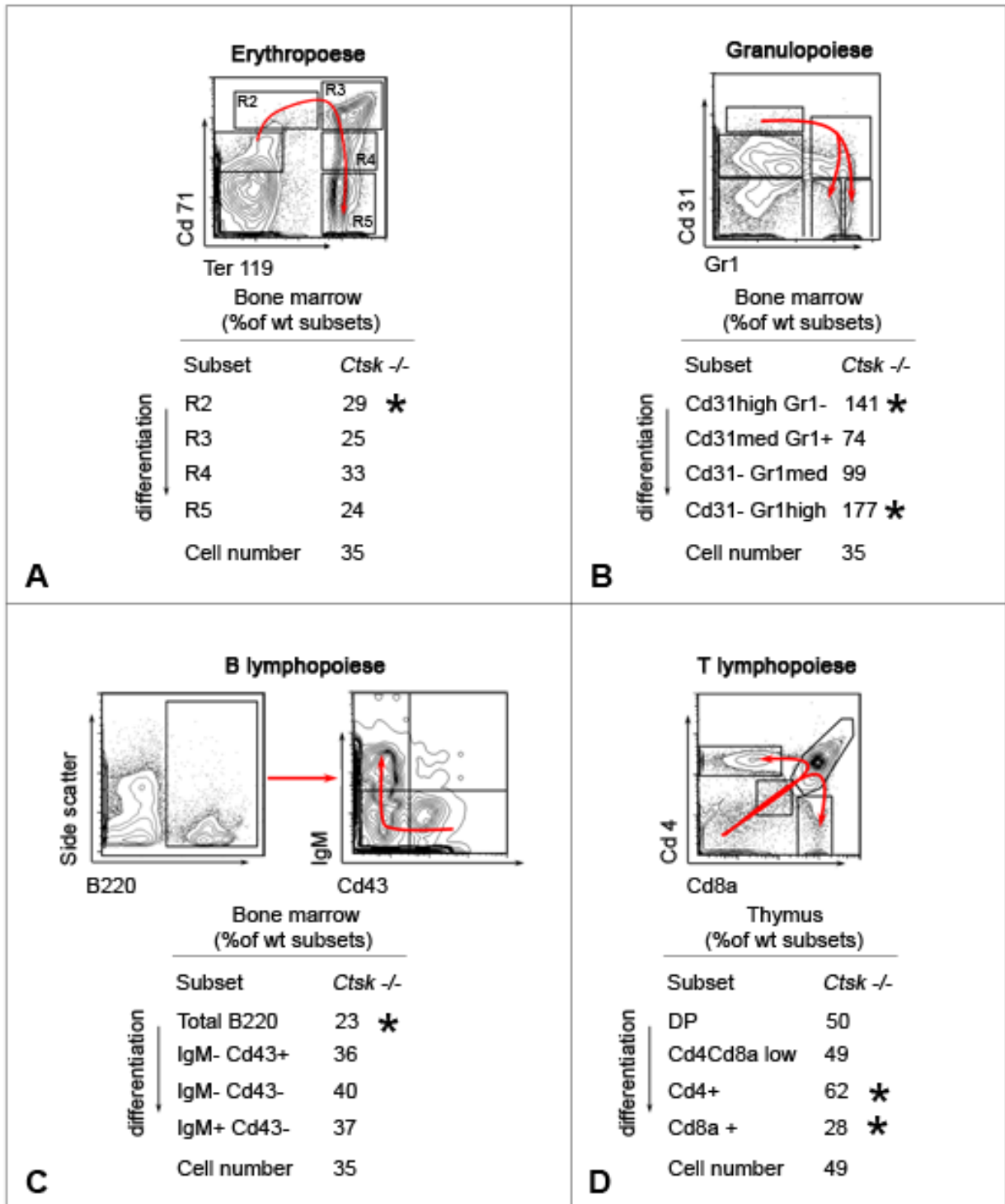
### 5.2.3 Alterations in the maturation stages of the erythroid, myeloid and lymphatic cells in *Ctsk*<sup>-/-</sup> mice

On the next step, we were interested in the effects of *Ctsk* on the different maturation stages of the four haematopoietic columns, erythropoiesis, granulopoiesis, B-lymphopoiesis and T-lymphopoiesis. Analyses were carried out by FACS, *Ctsk* mutant mice were compared to wild type mice, as described in methods (3.5.4). Cells for the erythropoiesis, granulopoiesis and B-lymphopoiesis analysis were taken from the bone marrow and cells for T-lymphopoiesis analysis from the thymus. In order to analyse the erythropoiesis, cell subsets were gated after their Cd71 and Ter119 surface marker expression. Early cells show a high expression of Cd71, during maturation cells begin to express also Ter119 and finally mature cells express mainly Ter119, shown by the red arrow in **Figure 25A**. We differentiate four different cell subsets by the surface marker expression, called R2, R3, R4 and R5. Interestingly, FACS results show decreased cell subsets in *Ctsk*<sup>-/-</sup> mice in all stages of erythropoiesis, when compared to the wild type mice. A significant decrease was shown for the Cd71<sup>+</sup> and Ter119<sup>-</sup> subsets, the R2 (**Figure 25A**).

In order to analyse myelopoiesis, cells were gated for their expression of Cd31 and Gr1 surface markers (de Bruijn et al. 1996). Early cells show a high expression Cd31 and are Gr1<sup>-</sup>, during maturation cells lose Cd31 expression and are Gr1<sup>+</sup>, shown by the red arrow in **Figure 25B**. We differentiated four different cell subsets, Cd31<sup>high</sup> Gr1<sup>-</sup>, Cd31<sup>med</sup> Gr1<sup>+</sup>, Cd31<sup>neg</sup> Gr1<sup>med</sup> and Cd31<sup>neg</sup> Gr1<sup>high</sup>. In the FACS results, we observed an increase of all granulopoietic cell subsets in the *Ctsk*<sup>-/-</sup> mice compared to the wild type mice. A significant increase was shown Cd31<sup>high</sup> Gr1<sup>-</sup> and Cd31<sup>neg</sup> Gr1<sup>high</sup> cell subsets (**Figure 25B**), suggesting altered myelopoiesis.

When we analysed B and T lymphopoiesis, we found a significant decrease of 23% in the B220<sup>+</sup> cell population (B cells), which propagated into the earlier IgM<sup>-</sup> Cd43<sup>+</sup>, IgM<sup>-</sup> Cd43<sup>-</sup>, and IgM<sup>+</sup> Cd43<sup>-</sup> subsets (**Figure 25C**). In order to analyse T lymphopoiesis, cells were gated for expression of Cd4 and Cd8a surface markers. Early cells in lymphopoiesis express both surface markers (double positive=DP), differentiate then to cells with a lower expression of both markers, then into highly Cd4<sup>+</sup> cells and highly Cd8a positive cells shown by the red arrows in **Figure 25D**. We differentiated four cell subsets, DP, Cd4Cd8a low, Cd4<sup>+</sup> and Cd8a<sup>+</sup>. The results

of the flow cytometry show a decrease in all T cell populations, which was significant for both mature Cd4+ and Cd8a+ cells in *Ctsk*<sup>-/-</sup> mice compared to the wt (**Figure 25D**).



**Figure 25 Maturation stages of mature haematopoietic lineages.** (A) Erythropoiesis. (B) Granulopoiesis. (C) B- lymphopoiesis. (D) T-lymphopoiesis. Red arrows show the differentiation stages on the FACS plots. Cell subsets of the *Ctsk*<sup>-/-</sup> mice are presented in percentages of the corresponding subsets in the wild type mice. \**p*<0.05

## 6 Discussion

The secreted protein *Ctsk* is known to be involved in several cellular functions in various cell types, however not much is known about the role of *Ctsk* in early adult haematopoiesis. It is known that *Ctsk* is secreted by osteoclasts in the haematopoietic niche, where it is essential for bone remodelling and degradation of type I collagen (Goto, Yamaza, and Tanaka 2003). In addition, *Ctsk* expressed by osteoclast has been shown to play an essential role in the breakdown of the niche, required for optimal G-CSF-mediated mobilization (Aicher et al. 2008). Since we found that *Ctsk* was over expressed in stromal cell lines supporting HSC in long-term cultures (**Figure 7**) (Oostendorp et al. 2002), we were anticipating that the loss of *Ctsk* in the niche would have a negative effect on HSC behaviour. In this thesis, we effectively demonstrated that this is indeed the case. As such, we show that *Ctsk* plays a novel, previously non-recognized role as a niche factor, which regulates adult BM haematopoiesis and the maintenance of early HSCs.

### 6.1 *Ctsk* Knockdowns using RNA interference

In this thesis, I generated stromal cells which show a knockdown in the expression of *Ctsk* by lentiviral shRNA interference, an established method for gene silencing (Abbas-Terki et al. 2002). Both stroma cell lines were transfected with ten different vectors. In order to determine which vector most effectively decreases expression of *Ctsk*, we verified *Ctsk* expression at the RNA level (**Figure 9**). From these results, I selected the *shCtsk* stroma cell lines with the lowest *Ctsk* expression for further experiments. As control stromal cells, I took the knockdowns with the highest *Ctsk* expression, since they passed through the same processes but still produced near normal levels of *Ctsk*. At RNA level, the *Ctsk* expression was up to 80% lower compared to the controls.

The ten shRNA vector constructs did not knock down the expression of *Ctsk* at the same level. Whereas *shCtsk2* was most effective in UG26-1B6 stromal cells, expression of *Ctsk* in EL08-1D2 was most effectively knocked down by the *shCtsk5*, 6 and 8 constructs. It is unclear why different *shCtsk* constructs show different results in the two stromal cell lines. One reason could be that perhaps the *Ctsk* gene is transcribed in different transcript variants, which lack perhaps the sequence bound by

the different shCtsk-expressed shRNA<sup>mir</sup> sequences. Given that Arnt and Ctsk are expressed in an overlapping range of tissues, Arnt read-through may have a negative impact on Ctsk transcript levels by interfering with Ctsk expression.

In subsequent experiments, we confirmed the down-regulation of Ctsk at the protein level using Western Blot of supernatants from the stromal cell lines (**Figure 10**). These experiments showed that in stromal cells in which Ctsk mRNA was decreased, also a decline in Ctsk protein expression, even though the extent of the decline was less.

## **6.2 The role of stromal Ctsk in the regulation of early haematopoiesis**

### *6.2.1 Loss Ctsk accelerates haematopoiesis*

In co-culture experiments of stromal cells with lineage-negative (Lin<sup>-</sup>) haematopoietic cells, we found that the down-regulation of *Ctsk* in the embryo-derived stroma cell lines UG26-1B6 and EL08-1B2 led to a reduced production of haematopoietic progenitors (**Figure 12**). In later experiments, we obtained similar results in Lin<sup>-</sup> cells co-cultured on the *Ctsk* <sup>-/-</sup> primary stroma (**Figure 24C**), indicating that stromal Ctsk is required to maintain colony-forming cells in co-cultures.

Since it is known that Ctsk is secreted by osteoclasts in the haematopoietic niche, where it is essential for bone remodelling and enhances bone formation (Goto, Yamaza, and Tanaka 2003; Lotinun et al. 2013) and results have showed that Ctsk can cleave cytokines which regulate stem cell proliferation survival and mobilization (Kollet et al. 2006), we hypothesize an important role of Ctsk in the stromal compartment of the niche in the regulation of haematopoiesis. In order to explain the lack of colony building progenitors caused by the deficiency of stromal Ctsk, we formulated two possibilities:

- (1) Differentiation of HSC may be decelerated, so that the number of colony building progenitors is decreased.
- (2) Differentiation of HSC may be accelerated, so that the haematopoietic hierarchy is skewed towards mature cells, rapidly exhausting the more primitive cells.

Hence, we decided to analyse the behaviour of earlier haematopoietic cells in the co-cultures. Our FACS analyses of the *Ctsk* knockdown co-cultures after three weeks showed dramatically decreased number of LSKs, MPPs and CD34- LSK (LT-HSC, **Figure 13**) in *shCtsk* stromal co-cultures. Since results from *in vitro* experiments do not always predict *in vivo* results, we performed transplantation of the co-cultures and showed that the loss of *Ctsk* causes an early recovery of myeloid cells (**Figure 17 - Figure 21**). Similar to the *in vitro* results, in the *Ctsk* *-/-* mice we observed severely decreased numbers of the earliest progenitors: CD34- LSK, LSK, CMP, GMP, and MEP (**Figure 24A**). These observations suggest an essential role of *Ctsk* in the maintenance of self-renewal in the *Ctsk* *-/-* niche. As we showed that early HSC are reduced in the absence of micro-environmental *Ctsk*, our results support the hypothesis (2), indicating that differentiation of haematopoietic cells is accelerated and skewed toward mature myeloid cells. We also found increased total cell number in peripheral blood in *Ctsk* *-/-* mice (**Figure 22**). One implication of this would be that in aged *Ctsk* *-/-* mice, HSC would be severely reduced, possibly affecting response to infections or lifespan. I found no reports specifically addressing the effects of *Ctsk* deletion on haematopoiesis in aged mice. Unfortunately, due to time constraints, I was unable to test this hypothesis.

### *6.2.2 Loss of Ctsk leads to reduced BM cellularity and concomitant reduction of LSK and progenitor cell subsets*

The majority of HSC are located within the BM, whereas a small population is constitutively released into circulation. This event involves proteolytic enzymes such as *Ctsk*, which cleaves molecules important in the interaction between HSC and the BM microenvironment, such as SDF-1 (CXCL12). *Ctsk* is known to have a pivotal role in bone remodelling and studies attest *Ctsk* to be involved in progenitor mobilisation (Kollet et al. 2006).

Our results in *Ctsk* *-/-* mice showed a decreased BM cellularity and HSC content, unchanged numbers of spleen cells, while total cell number was increased in blood (**Figure 24**). In keeping with our results, a reduced number of BM cellularity, as well as a reduced number of HSC, was also recently shown by Jacome-Galarza (Jacome-Galarza et al. 2014). In a more detailed analysis, we found no difference in the number of HSC between the spleens of control and *Ctsk* deficient mice. Our results

are in accordance with those of Jacome-Galarza and co-workers, who showed a reduction of LSK cells in BM. These investigators additionally found an increased number of LSK in spleens of *Ctsk*<sup>-/-</sup> mice. These results show that the LSK and progenitor compartments are reduced in *Ctsk*<sup>-/-</sup> mice. Whether the long-term repopulating ability of the CD34<sup>+</sup> LSK is also affected by *Ctsk* deficiency is another question that has not been answered yet. Since the mice we studied were of mixed 129 x C7BL6/J origin, we were unable to perform congenic transplants to address this tissue.

### 6.2.3 Increased myelopoiesis *Ctsk* deficient mice

As we showed that the number of HSC is reduced due to the loss of *Ctsk*, we also analysed the different stages of all mature haematopoietic lineages. Interestingly we find that granulopoietic cell types are increased, while erythropoiesis, B- and T-cells lymphopoiesis are decreased. The observation of increased granulopoiesis is not shared by Jacome-Galarza and co-workers (2014), who found a decrease in absolute numbers of several monocyte subtypes. As the strain and age of the mice in our studies were similar, the source of the disparities remains unclear and can be due to several factors. First of all, myelopoiesis was studied using different markers. Whereas we looked into different subpopulations using the CD31 and Gr1 markers, Jacome-Galarza and co-workers looked into many different markers and we can't rule out that we may have missed a decreased subpopulation of myeloid cells using our analysis. Since both mature subsets were increased in our study, we cannot exclude the possibility that there was perhaps an underlying condition, leading to secondary increased inflammatory myelopoiesis in our mice. However it seems unlikely that these is indeed the case, since the transplantations from cells co-cultured on sh*Ctsk* stromal cells also show increased myelopoiesis in the BM (**Figure 17**) and these co-culture transplants also agree with the decreased LSK and MP content, indicating the transplantation results closely mimic the *in vivo* situation.

### 6.2.4 A role for *Ctsk* in the localisation of haematopoietic cells?

We found that in *Ctsk*<sup>-/-</sup> mice, the total cell number was increased in PB, while decreased in BM. This suggests that loss of *Ctsk* causes a redistribution of different cell populations over different organs. Similar effects were described in mice with a

deletion of the Ca<sup>2+</sup>-sensing receptor (Casr) (Adams et al. 2006), and mice lacking early gene response factor 1 (Egr1) (Min et al. 2008), which both also show an increase in circulating HSCs.

The similarity in phenotypes between these mice suggests that these molecules are all involved in similar biological processes. At the moment, I can only speculate about the underlying process. Studies have revealed that calcium triggers the osteogenic differentiation or reproduction of HSCs niche microenvironment (Nakamura, Matsumoto, et al. 2010). The fact that calcium level is triggered by the pH (Silver, Murrills, and Etherington 1988) and that activated osteoclasts secrete protons together with Ctsk (**Figure 3**) suggests a possible relation between calcium and Ctsk in the niche. Like the Casr, Egr1 has also been implicated in bone biology, particularly in bone repair (Reumann et al. 2011) where Egr1 drives proliferation of osteogenic progenitors. Interestingly, Ctsk, Casr, and Egr1 are all involved in TGFbeta signalling, where Egr1 up regulates TGFbeta transcription (Dhaouadi et al. 2014), Casr inhibits Smad2-relay of TGFbeta signals (Organista-Juarez et al. 2013) and Ctsk expression is down-regulated by TGFbeta (van den Brule et al. 2005) suggesting that the phenotype in Ctsk-deficient mice may be caused in part by upregulated TGFbeta activity. We thus speculate that the alterations in LSK localisation in Ctsk<sup>-/-</sup> mice is caused at least in part by dysregulated TGFbeta signalling.

### 6.3 Conclusion

In this work, the role of Ctsk in the haematopoiesis was analysed using *in vitro* and genetic models of haematopoiesis. We found that Ctsk<sup>-/-</sup> mice effectively showed a reduced number of BM cellularity and LSK when compared to Ctsk<sup>+/+</sup> WT mice. Furthermore, cellularity was increased in PB. We also found a higher number of mature myeloid cells in these mice. In co-culture experiments of WT HSCs using stromal cells in which expression of Ctsk was knocked down, we found that HSC activity was diminished and that this was also accompanied by an increase in myelopoietic cells. These results strongly suggest that the alterations of haematopoiesis in Ctsk<sup>-/-</sup> mice are caused by an underlying defect in niche cell function. Further experiments would be necessary to clarify the mode of action of the decrease in haematopoietic activity on shCtsk stromal cells and in Ctsk<sup>-/-</sup> mice.

Taken as a whole, the work described in this thesis shows that stromal Ctsk is required for maintaining HSC homeostasis.



## 7. References

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## 8 Appendix

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