The effect of *Dlg7* overexpression and silencing on mouse embryonic stem cell differentiation.

Níels Árni Árnason.

Líf- og umhverfisfræðideild
Háskóli Íslands
2010

The effect of *Dlg7* overexpression and silencing on mouse embryonic stem cell differentiation.

Níels Árni Árnason.

120 eininga ritgerð sem er hluti af
Magister Scientiarum gráðu í líffræði

Leiðbeinandi/endur
Ólafur Eysteinn Sigurjónsson.
Kristbjörn Orri Guðmundson.

Prófdómari / Fulltrúi deildar
Unnur Þorsteinsdóttir.
Guðmundur Hrafn Guðmundsson.

Líf- og umhverfisfræðideild
Verkfræði- og náttúruvísindasvið
Háskóli Íslands

Reykjavík, 30. december 2010

Abstract.

The genetic program controlling stem cell self-renewal and differentiation is yet to be elucidated although significant contributions have been made. Recently, *in vitro* and *in vivo* studies have identified several genetic regulatory programs such as various transcription factors, cell cycle inhibitors, genes implicated in chromosomal rearrangements, essential developmental proteins, and signalling pathways that have all been implicated in the process of stem cell self-renewal and differentiation.

Dlg7, a novel cell cycle regulator gene, is expressed in stem cells, including haematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and mouse embryonic stem cells (mESC). *Dlg7* is expressed in human haematopoietic stem cells (CD34+CD38-) but much less expression was detected in haematopoietic progenitor cells (CD34+38+) or in fully mature blood cells. *Dlg7* is expressed in many leukemic cell lines and in several tumours including bladder, colon and liver, while not being found in healthy adjacent normal tissue. In addition, it is a potential oncogenic target of Aurora-A, and is associated with invasiveness of hepatocellular carcinoma.

The aim of this project was to study the role of *Dlg7* in the differentiation of mESC with special emphases on haematopoiesis. We use lentiviral based vectors to silence and overexpress the *Dlg7* gene in mESC. Transgenic mESC were differentiated to Embryo Bodies and haematopoietic colony forming units. Effects of genetic changes were monitored using a colony forming unit (CFU) assay, flow-cytometry and Q-PCR.

Dlg7 overexpression and silencing significantly reduces the number of EBs that form in Primary differentiation media compared to scrambled control cell population. Of the EBs that formed a significant larger portion of EB from the scrambled control acquired hematopoietic morphology at later stage of differentiation compared to mESC with *Dlg7* overexpression or silencing. BFU-E where in grater numbers with in the CFU population formed from cells with *Dlg7* overexpression then in the scrambled control cells.

We can however not say with full certenty that the effect of *Dlg7* in hematopoiesis is a direct effect or rather a general effect in differentiation due to the role that *Dlg7* plays in the cell cycle.

Útdráttur.

Þó að mikill árangur hafi náðst á undandförnum árum hefur ekki verið að fullu útskýrt hvaða þættir það eru sem stýra sjálfsendurnýjun og sérhæfingu stofnfruma. Nýlega hafa *in vitro* og *in vivo* rannsóknir skýrt frá fjölda ferla, umritunarþátta og annara prótiena sem hafa áhrif á sjálfsendurnýjun og sérhæfingu. Eitt af þessum próteinum sem möguelga hefur þar áhrif er *Dlg7*.

Dlg7 er hugsanlegt stjórngen í frumuhringnum, það er tjáð í stofnfrumum, þar á meðal blóðmyndandi stofnfrumum, bandvefsstofnfrumum og stofnfrumum úr fósturvísum músa. *Dlg7* er tjáð í blóðmyndandi stofnfrumum í mönnum (CD34+CD38-) en mun minni tjáning er í forverafrumum (CD34+CD38+) eða þroskuðum blóðfrumum. *Dlg7* er tjáð í mörgum hvítblæði frumulínum og ýmsum krabbameinsæxlum eins og úr þvagblöðru, ristli og lifur en er ekki að finna í sama mæli í aðlægum eðlilegum vef. *Dlg7* er hugsanleg meingen og markgen Auroru A og tengist ífarandi lifrarkrabbameini.

Markmið þessa verkefnis var að rannsaka þátt *Dlg7* í sérhæfingu stofnfruma úr fóstuvísum músa með sérstaka áherslu á blóðmyndun. Við notum veiru-vektora byggða á lentiveiru til þess að þagga niður og yfirtjá *Dlg7* í stofnfrumum úr fósturvísum músa. Stofnfrumur voru sérhæfðar yfir í frumuþyrpingar (embryo body) og blóðmyndandi frumur. Áhrif þöggunar og yfritjáningar *Dlg7* varu könnuð með greiningu á frumuþyrpingum í smásjá, flæðismásjáar og rauntíma PCR.

Bæði þöggun og yfirtjáning á *Dlg7* olli minnkun á fjölda sérhæfðra frumuþyrpinga sem mynduðust miðað við viðmiðs fósturstofnfrumur sem fengu brenglað DNA. Af þeim sérhæfðu frumuþyrpingum sem mynduðust var stærra hlutfall fumuþyrpinga með blóðmyndandi svipgerð hjá brenglaða viðmiðinu en hjá frumuþyrpingum sem mynduðust út frá fósturstonfnfrumum með *Dlg7* þöggun eða yfirtjáningu. Rauðkorna forverafrumur voru stærra hlutfall af heildar blóðmyndandi frumuþyrpingum sem mynduðust út frá fóstrustofnfrumum með *Dlg7* yfirtjáingu en hjá brenglaða viðmiðinu.

Við getum ekki sagt með vissu að þessar breytingar sem við greindum í erfðabreyttu frumunum séu vegna beinna áhrifa *Dlg7* á blóðmyndun eða vegna almennra áhrifa á sérhæfingu sem tengjast hlutverki *Dlg7* í frumuhringnum.

Acknowledgment.

This project was carried out at the Blood Bank Landsspítali-University Hospital.
This project would not have been possible with out the help and support of some willing and open minded people.

Dr. Ólafur Eysteinn Sigurjónsson for choosing me for this project and believing in me. He gave me freedom to express my self, come up with my own ides and make my own mistakes while always giving me excellent guidance to reach my final goal.

Dr. Sveinn Guðmundsson MD, for making it possible for me to study at the Blood Bank. Sveinn embraced me with open arms from the first day and made me feel welcome. His pep talks made me believe more in my self and that any thing was possible. I would also like to think him for valuable comments during presentation of my work.

Dr. Kristbjörn Orri Guðmundsson for good comments and support during the writing of this thesis and for help in designing the project.

Dr. Guðmundur Hrafn Guðmundsson for being my contact at the Life science department at the University of Iceland, and for good conversations and comments at our status meetings.

My colleague Sigríður Þóra Reynisdóttir for guidance, especially in the first part of this project. I could always reach out to her for help and advice. For good comments at group meetings.

Dr. Leifur Þorsteinsson, with whom I sheer my workspace for being of a grate assistance when ever I needed, especially with the flow cytometry technology. For interesting conversations, whether they revolved around our work or other worldly matters.

My colleague Helga Eyja Hrafnkellsdóttir for over all assistance and good comments at group meetings.

My colleague on the third floor Kristjana Bjarnadóttir and Kristín Sigríður Sigurðardóttir. For giving me access to their molecular lab and being willing to assist me when ever I needed.

My fellow student Ramona Leider for assistance, especially with the Q-PCR work.

My colleague Steinunn Matthíasardóttir for good moral support, interesting conversations and for giving me access to her own work.

Dr. Gestur Viðarsson for reading the thesis and giving me valuable comments.

Dr. Þorbjörn Jónsson for valuable comments during presentation of my work

My fellow student Sævar Ingþórsson for help with microscopic imaging.

I would like to think all the staff and students of the Blood Bank for creating a grate atmosphere for working and studying in.

At last but not least I would like to think my family especially my parents for giving me the opportunity to peruse my dreams and supporting me threw my studies.

My beautiful girlfriend for putting up with me and supporting me for these last few years. I think your amazing.

This project was founded by Rannsóknarsjóður Rannsóknarmiðstöðvar Íslnads (Rannís) and Vísindasjóður Landspítala University Hospital.

Table of contents.

Figures and tables.

Abbreviations.

AML	Acute myeloid leukaemia.
AGM	Aorta-gonad mesonephros.
APC/C	Anaphase Promoting Complex.
CSC	Cancer stem cells.
CD	Cluster of Differentiation.
CFU-S	Colony Forming Unit – Spleen.
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor.
D-box	Destruction boxes.
Dlgap5	Disc large associated protein 5.
Dlg1	Discs large-1
Dlg7	Disc large homolog 7.
DMEM	Dulbecco's Modified Eagle Medium.
EGFP	Enhanced Green fluorescent protein
EB	Embryoid body
ECC	Embryonic carcinoma cells.
EGC	Embryonic germ cells.
ESC	Embryonic stem cells.
FCS	Fetal calf serum.
FBS	Fatal Bovine Serum.
G-CSF	Granulocyte colony-stimulating factor.
GFP	Green fluorescent protein.
GK	Guanylate kinasa
HSC	Hematopoietic stem cells.
HCC	Hepatocellular carcinoma.
HURP	Hepatoma up regulated protein.
hESC	Human ESC.
hEpo	Human Erythropietin.
hIL6	Human interleukin 6.
iPS	Induced pluripotent cells.
ICM	Inner cell mass.
IVF	*In vitro* fertilization.
IMEM	Iscov´s Modified Eagle Medium.
LIF	Leukaemia Inhibitory Factor.
LT-HSC	Long term HSC.
MAGUK	Membrane associated guanylate kinase.
MSC	Mesenchymal stem cells.
MAP	Microtubule Associated Protein.
MTG	Monothioglycerol.
mESC	Mouse ESC.
mIL3	Mouse interleukin-3
MPP	Multi Potent Progenitor.
mSCF	Murine Stem Cell Factor
NES	Nuclear export signal.
PDZ	Postsynaptic density and Zho.
SCF	Skp1 Cdc53/Cullin1 F-box protein.
SH3	Src Homology 3
shRNA	Short hairpin RNA.
siRNA	Small interfering RNAs

ST-HSC	Short term HCS.
SSC	Somatic stem cells.
RNAi	RNA interference.
TCC	Transitional cell carcinoma.

I-Introduction

1 - Stem Cells.

All cells of the body originate from embryonic stem cells. These include hematopoietic stem cells that differentiate from the mesoderm during early embryogenesis. Stem cells can be defined as cells that can both self-renew and differentiate (figure 1). Stem cells are therefore uncommitted and remain so until called upon [1].

Stem cells may be classified into two groups depending on their differentiation potency. Somatic stem cells (SSC) have lesser potency and generally only differentiate into the tissue they reside in and embryonic stem cells (ESC) are more potent and can differentiate into cells of all three germ layers of the body (figure 1) [2]. In addition other categories of stem cells have been named such as embryonic germ cells (EGC) that differentiate during embryogenesis into stem cells that form the reproductive gametes, cancer stem cells (CSC) that may be the origin of at least some cancers [3, 4] and induced pluripotent cells (iPS), somatic cells that can be reprogrammed to mimic ESC [5-7]. The development of ESC and their differentiation potential is depicted in figure 1. [2]

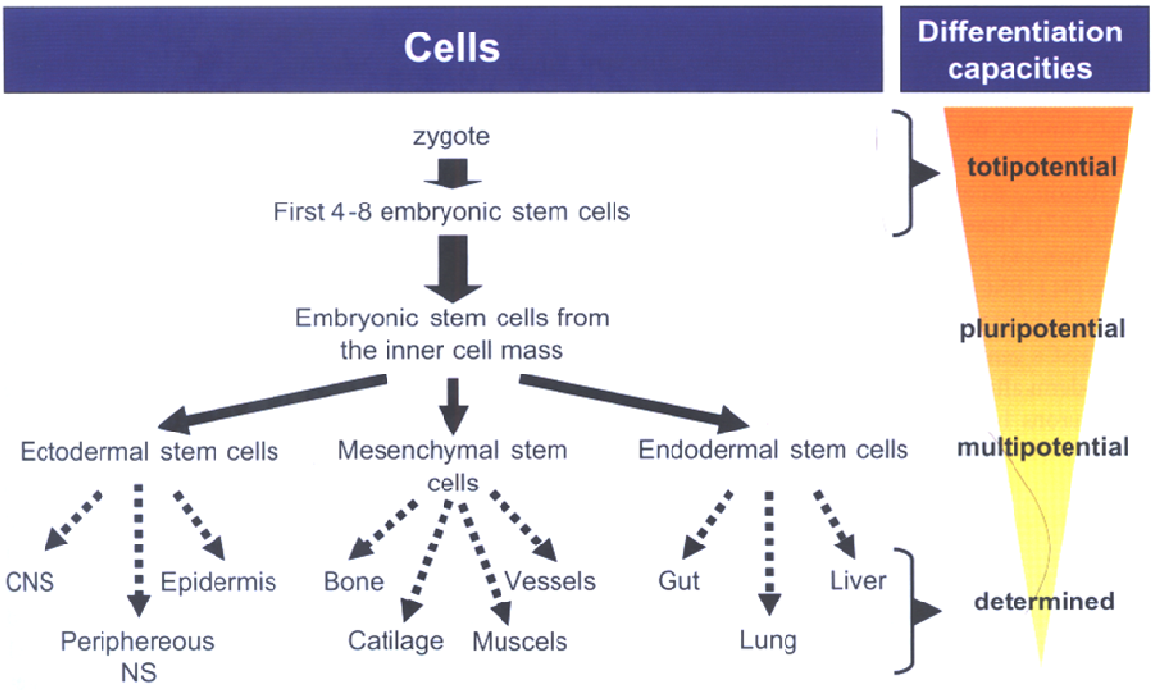


Figure 1. Differentiation cascade of stem cells. The zygote and 4-8 first cells that divide from it are totipotent, embryonic stem cells from the inner cell mass are pluripotent. Stem cells or progenitor cells of each germ layer are multipotent and generate determined cell of various tissues [2].

1.1 Embryonic stem cells.

Embryonic stem cells can be isolated from the inner cell mass (ICM) of a blastocyst in early embryogenesis. Embryonic stem cells have an active telomerase and *in vitro* maintain their ability to differentiate into cells of three germ layers, namely mesoderm, ectoderm and endoderm [8, 9]. This ability makes ESC an important and popular research material as they have the potential to grow into any cell type of the body. A lot of effort has been put in to finding out which factors control the fate of these cells. This knowledge may enable scientists to direct the development of ESC and control the outcome of their differentiation.

The first ESC where isolated from a mouse blastocyst in 1981 by Evans and Kaufman [10]. Seventeen years later a team lead by Jamie Thomson isolated ESC from a human blastocyst [11]. A turning point in stem cell research history came in 1964 when scientists isolated a special kind of cancer cells called Embryonic Carcinoma Cells (ECC). Embryonic carcinoma cells were originally isolated from teratocarcinomas. [12]. Kleinsmith and Pierce demonstrated the stem cell capabilities of these cells when they transplanted a single ECC in to a mouse host which then regenerated a whole tumour [12]. ECC are similar to ESC in there form, growth factors and markers. It was noted that ECC showed enhanced growth ability in the presence of differentiated cells but grew poorly on their own. These findings prompted the usage of feeder cells which proved to be an important tool in growing ECC. Feeder cells secrete factors that are necessary for the survival of ECC and ESC. Fibroblast from mouse embryos seemed to be ideal for this purpose [13]. Feeder cells have been and are still used to grow ESC. Feeder cells can be difficult to standardize and to avoid growing the ESC on feeder cells, Leukaemia Inhibitory Factor (LIF) is used in a mouse ESC culture. LIF is a member of the IL-6 family and interacts with the gp130 receptor which then activates the transcription activator STAT3. It should be noted, that it is necessary to include serum in the culture media if you want to exclude feeder cells from your culture, indicating that additional factors are required. STAT3 has been linked to the undifferentiated state of ESC. Transcription factors like Oct 4 and Nanog have also been shown to play a role in the undifferentiated state of ESC [14]. In the development of a mouse embryo, cells that are identified as being pluripotent require their genome to express *Oct4* [15] and *Nanog* [16]

The procedure to isolate ESC from the ICM for the production of a new ESC line is fairly standardized. Embryos at the blastocyst stage can be used, in some cases even the whole blastocyst, or more commonly the ICM is isolated from the blastocyst. The ICM is isolated from the trophoblast cells using immunosurgery [17,18]. The ICM is then cultured on feeder cells in a standard cell medium enriched with 10–20% fetal calf serum (FCS) and 2-

mercaptoephenol. After a few days the ICM is dissolved and the cell mass is cultured on a new batch of feeder cells. The new culture is made up of both differentiated cells and undifferentiated pluripotent cells. Cells that display an undifferentiated phenotype are picked and transferred into yet another culture. If more undifferentiated cells are generated from their previous mother cells a new stable ESC line has been prepared [18].

There are three criteria's that a true ESC line must fulfil. Can the cell line differentiate in to the three germ layers endoderm, ectoderm and mesoderm? Can it produce teratocarcinomas? Are the cells able to take part in the making of all types of tissues in a chimera including the germ cells [18]. Chimeras can be prepared by injecting ESC in to a host blastocyst and then the blastocyst continues to develop in its natural environment (surrogate mother). In live offspring markers like coat colour can indicate a chimera. Special markers like *β-galactosidasa* and *green fluorescent protein* (GFP) can be integrated into the ESC to allow the researcher to see in what tissues the specific cells have taken part in making throughout development [18].

It is not uncommon that ESC are referred to as being totipotent because they can generate gametes. This definition is only true for a fertilized egg and the germ cells that it generates. In the case of mouse ESC (mESC) these cells are not capable of generating trophoectoderm which is the most outer layer of the embryo. Mouse ESC cannot generate a blastocyst *de novo* and therefore cannot make a complete embryo. The proper term for ESC is to say that they are pluripotent [18]

In the last few years there have been studies that show that many things can go wrong in an ESC culture that is maintained for a long period of time. In a study where human ESC (hESC) were used, eight of the nine late passage cell lines showed changes in their genome which have been linked to cancer [19]. The cells lines were passaged 22 – 175 times. These genomic changes were for example discrepancy in chromosome copy number, mitochondrial DNA and gene promoter methylation. This information makes it important to monitor ESC that are cultured *in vitro* and make sure they are in perfect condition be fore they are used *in vivo*. It is possible that some cell lines that have been maintained for a long time can not be used *in vivo* [19].

ESC have been the topic of moral debate discussing whether it is ethically right to use those cells in research. The discussion has mostly revolved around hESC. In the year 2003 there were 70 stem cell lines from embryos around the world [20]. The embryos used to obtain these cell lines were excess embryos from *in vitro* fertilization (IVF) donated by their owners. To avoid the ethical issues of using ESC, different research groups have come up with

different solutions. As an example Chung *et.al.* managed to isolate ESC from a mouse embryo without destroying it, leaving the embryo to develop into a normal adult [21]. This might also be possible for the isolation of hESC. It is highly unlikely that any potential parent would agree to a procedure like that to be preformed on their embryo. That same year Yamanaka S. *et al.* reported on induced pluripotent stem (iPS) cells [5]. These iPS cells were reprogrammed by transducing them with four transgenes *Oct4, Klf4, Sox2* and *c-Myc*. These four factors induce the reprogramming of the cells which then display typical stem cell attributes. Cells used for this procedure are usually embryonic or epidermis fibroblasts. The biggest benefit of iPS cells is that they eliminate the use of cells of embryonic origin, and could therefore provide a relative easy way to produce patient and disease specific cells that could differentiate into any tissue [7, 22-24].

The debate about the ethical issues concerning ESC research will possibly go on for a long time, but there is no doubt about the endless possibilities and importance concerning ESC research.

1.2 - Somatic stem cells.

Somatic stem cells (SSC) are organ-derived cells, usually with limited differentiation potency that can give rise to more mature cell types. Somatic stem cells are thought to be a part of a natural system for tissue repair. There is an indication that SSC can be found in every organ of the body including bone marrow [25,26], dental pulp [27] skeletal muscle [28], liver [29] and the epidermis of the skin [30,31] to name few. Somatic stem cells usually differentiate through a lineage of progenitor cells before forming a specialized functional cell. Hematopoietic stem cells are the best characterized SSC and have been used as a support treatment for certain haematological cancers for decades [32, 33]. Other SSC that have been much investigated are mesenchymal stem cells. They can be isolated from bone marrow, adipose tissue and other organs and can be differentiated into osteocytes, chondrocytes and adiposities making them an attractive source for tissue engineering [34].

SSC taken out of their niche often lose their stem cell potency and can therefore be difficult to expand and maintain *in vitro*. However, they can still be differentiated as progenitor cells and are therefore important in clinical therapy and basic research [35, 36].

2 - Gene targeting in mouse embryonic stem cells ESC.

Embryonic stem cells isolated from the inner cell mass can grow indefinitely *in vitro* without losing their differentiation capability. These cells can be genetically manipulated *in vitro* and the effects on development studied [37]. Various methods have been used for gene targeting both in introducing transgenes for overexpression or gene silencing.

2.1 - Overexpression

In order to achieve overexpression of genes within a mammalian cell, multiple copies of the desired genes can be introduced into the host cell with various methods. These excess copies are called transgenes [38]. A transient overexpression where the transgenes are expressed via plasmids within the cells is often used. Various methods exist for introducing the foreign DNA in to the cell. One of these methods is to use Electroporation, where an electric current is used to shock the host cells and make their membrane accessible to foreign protein and DNA [39]. This method has some draw backs which include a low efficiency of transfection, especially in cells differentiated from the transfected ESC [40], cytotoxic effects that lead to a large cell death [39] and transient transgene expression [41]. New methods using viral vectors for integration of transgens in to the cell are now available. This new viral based method overcomes some of the problems surrounding electroporation [37]. Adenoviral based vectors can promote efficient transgene expression but do not integrate into the host genome and mostly support transient gene expression [42]. Adenoviral based vectors tend to elicit a strong host immune response [37]. Adeno-associated virus integrates in to the host cell genome and mediates stable transgene expression [43]. Retroviral vectors based on e.g. the HIV-1 Lentivirus have a less defined integration site than the Adeno-associated virus vectors but a higher stable transducing efficiency. Lentivirus based vectors have the following advantages over other systems: high transduction efficiency where up to 70% transduction efficiency has been achieved in Human ESC [44], the transgene is permanently integrated in to the host cell genome and is inherited to the daughter cells [45], host cell immune response is minimal compared to other vectors [46], and that lentiviral vectors are capable of transducing non-dividing cells [47]. Lentivirus based gene delivery promotes integration of multiple copies of the transgene to achieve overexpression of the desired gene or silencing with the use of RNA interference (RNAi). One major draw back in using this system is that the transgens integrate randomly into the genome with unknown effect on cell behaviour [48].

2.2 - RNA interference.

Lentivirus vectors are often used to integrate genes that encode short hairpin RNA's (shRNA) that correspond to mRNA products of a specific gene. The shRNA genes express double stranded RNA that activates the RNAi pathway which reduces the expression of that specific gene drastically [49]. Cells use RNAi to silence genes. In eukaryotic cells this pathway is an ancient evolutionarily defence mechanism that degrades foreign RNA [50]. In plants, these mechanisms are used for protection against RNA viruses. In other organisms it is thought to protect the cell from the proliferation of transposable elements that replicate by producing RNA intermediates. Many of these RNA viruses and transposable elements produce double stranded RNA [50]. Double stranded RNA attracts a RNase complex containing a nuclease called Dicer that degrades the double stranded RNA into small 23 nucleotide RNA fragments termed small interfering RNAs (siRNA). The RNase complex holds on to the RNA fragment and when faced with a complementary RNA nucleotide sequence it binds to it and degrades that RNA as shown in figure 2. By introducing a gene that produces double stranded RNA and is complementary to a known endogenous gene, the endogenous gens mRNA is degraded [50]. RNAi can also direct heterochromatin formation in association with RNA induced transcriptional silencing complex to prevent further initiation of transcription. The cell uses this mechanism for keeping transposable elements in a transcriptional silent state and formation of heterochromatin at centromers which is necessary for segregating chromosomes accurately during mitosis [50].

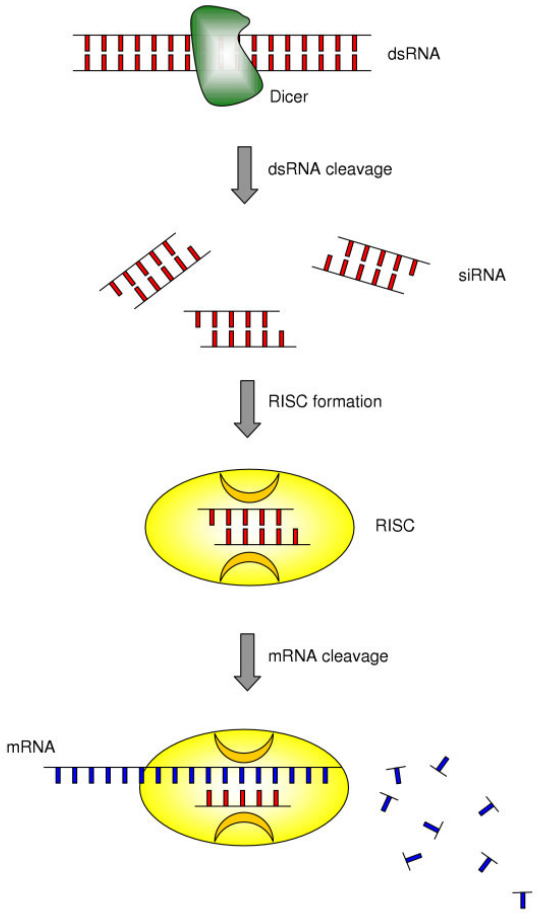


Figure 2. Process of dsRNA degration by RNAi pathway. Dicer nuclease degrades double stranded RNA into small 23 nucleotide RNA (siRNA) that bind to the RISC RNase complex. The RISC complex uses the siRNA to bind to complementary mRNA and degrades it. [51]

3-Hematopoiesis

By the 1960's scientist had carried out experiments to learn more about the lifespan of blood cells by using reinfused thymidine-labeld cells. The data showed that except for some lymphoid cells, blood cells were short lived [52]. From this data it was evident that for maintaining normal levels of mature cells in our adult life their formation had to be continuous [52]. In the early sixties Till and McCulloch injected bone marrow intravenously into irradiated mice and found large hematopoietic colonies formed in the spleen [25]. Further results showed that these colonies were the progeny of a single cell, termed Colony Forming Unit – Spleen (CFU-S) [53]. Cells from these colonies were capable of generating hematopoietic populations of multiple lineages. The most important finding was that these CFU-s-derived colonies contained CFU-S cells indicating the ability of self-generation [53]. A few years later other studies showed that CFU-s could be isolated from other bone marrow cells indicating

that hematopoietic stem cells (HSC) were a subset of marrow cells that were capable of self-renewal and could generate progenitor cells of multiple lineages of the blood tissue [54] [55]. HSC have a very low frequency of 0.01% of total nucleated cells in the bone marrow [56]. Today markers that define the phenotype of a HSC have been defined both mouse and human. These are cell surface markers detectable by monoclonal antibodies. Mouse HSC are positive for the markers Sca-1 and C-kit, they are negative or express low levels of markers found on mature blood cells and are termed Linage negative (Lin-). Mouse HSC are there fore c-Kit+Sca+Lin-, which is commonly called the KSL phenotype [36]. Mouse HSC are negative or have low expression of Cluster of Differentiation (CD) 34 but positive for CD 38 [57] and Thy-1 [58]. Methods for purifying HSC can vary between different labs and not all agree on what combination of markers should be used for that purpose. In the early 1980's cell separation studies showed that hematopoietic cells in the bone marrow could be characterised into three different generations. These were CFU-S, progenitor cells and immature cells [59]. In the following years this simple model would get more complicated. It was shown that CFU-S could not contribute to haematopoiesis for a long period of time. A fourth generation of hematopoietic stem cells was characterized that were even more primitive than CFU-S and could sustain haematopoiesis for a long period of time [60]. As shown in figure 3 HSC are divided into short term HSC (ST-HSC) or long term HSC (LT-HSC) and instead of having a population called CFU-S there is a population called Multi Potent Progenitor (MPP) that can give rise to the Common Myeloid Progenitor (CMP) or the Common Lymphoid Progenitor (CLP) [61].

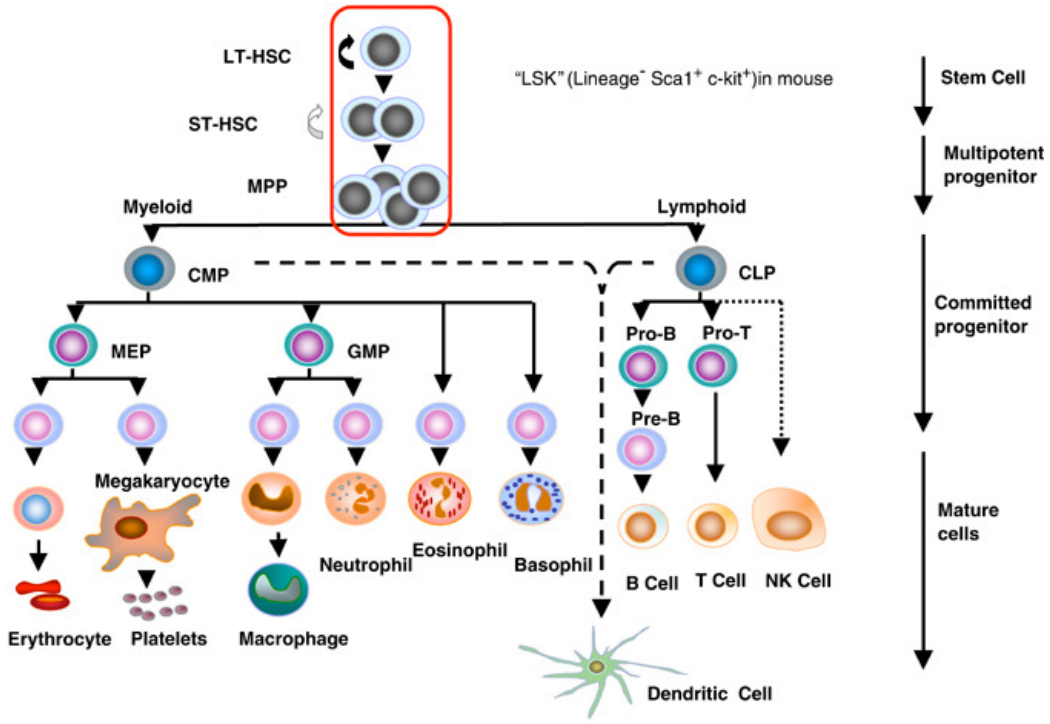


Figure 3. The hematopoietic stem cell hierarchy. Eight specific blood cells that develop from the same common ancestor cell, the HSC. This figure displays a simple diagram of how the blood system works in an adult mouse. LT (long term) ,ST (short term), MPP (Multi Potent Progenitor), CMP (Common Myeloid Progenitor), CLP (Common Lymphoid Progenitor), MEP (Megakaryocyte Erythrocyte Progenitor), GMP (Granulocyte Macrophage Progenitor) [61].

The basis for the haematopoietic family tree shown in figure 3 is derived from the work of Peppenheim and Maximov in the beginning of the 20th century [62]. Not all scientists agree on how the blood tissue develops in a fetus or embryo. The site of the first HSC in development is not clear. Haematopoiesis in development has been studied extensively in the mouse where mouse developing embryo haematopoiesis occurs first in the blood islands of the yolk sack. This blood production is termed primitive and serves mostly for producing red blood cells for tissue oxygenation of the rapidly growing embryo [63]. On embryonic day 8.5 haematopoiesis occurs in the aorta-gonad mesonephros (AGM) region. At a later stage on embryonic day 10 the developing haematopoiesis colonizes the fetal liver, thymus, spleen and ultimately the bone marrow in the new born mouse, as shown in figure 4 [63].

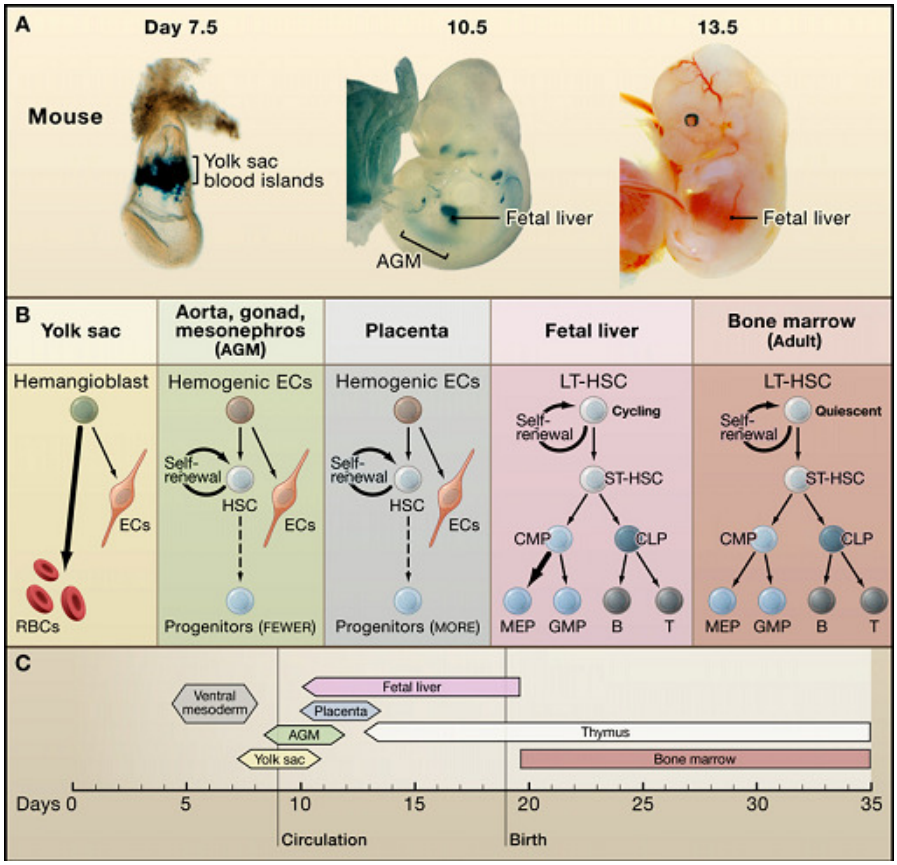


Figure 4. Development of haematopoiesis in the mouse. (A) Yolk sac blood islands are visualized by LacZ staining of transgenic embryo expression GATA-1-driven LacZ. AGM and Fetal Liver are stained by LacZ in Runx1-LacZ knock in mice. (B) Each site of haematopoiesis in development favours specific linage of blood cells. Abbreviations: ECs, endothelial cells; RBCs, red blood cells; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor ;MEP, megakaryocyte/erythroid progenitor;GMP, granulocyte/macrophage progenitor. C: Timescale in days showing at what time each organ is active in haematopoiesis [63].

4 - Haematopoietic stem cells in clinical therapy.

Hematopoietic stem cells have been used as a support therapy for treatment of various blood disorders, e.g. leukaemia and myeloma for more than fifty years. A report on six patients that received intravenous infusion from a healthy individual after irradiation treatment was published in 1957 [64]. This was only one year after a report on treatment of mice with leukaemia with lethal irradiation and marrow transplantation had been published. [65]. In these early days a vast majority of allogeneic transplants failed due to immune rejection to the incompatible bone marrow [66]. In 1959 a patient with advanced leukaemia who was treated with a whole body irradiation treatment received bone marrow from his identical twin. The bone marrow of the recipient was restored and the leukaemia disappeared for four months. [67].

This case showed that it was crucial that the bone marrow of the recipient and the donor were compatible. In the following decades methods for defining compatible donors were developed and allogenic bone marrow transplantation became a routine procedure for patients with blood tissue related cancer or diseases of the immune system. Today, autologus transplantation is a more common procedure used on patients [68]. The patients own cells are collected through aphaeresis after being mobilised from the bon marrow to the blood using G-CSF or similar drugs. The patient is transplanted with his own cells after going through intense chemotherapy [68]. Autologous HSC transplantation has been in practise at the Landspítali University Hospital in collaboration between the Blood Bank and the Department of Hematology since late 2003 with good results. Over 20 patients are treated in this program every year [69].

5 - The mouse as a model system for haematopoiesis.

Over 90% of the human and mouse genome is preserved from their last common ancestor [70]. The whole mouse genome was sequenced in 2002 where it was shown that 40% of the human genome could be directly aligned with the mouse genome and 80% of human genes have a corresponding gene in the mouse genome [71]. Genetic studies have been performed on the mouse for over 100 years which makes the mouse an ideal research model. A variety of mouse strains are available and hundreds of spontaneous mutations have been mapped. Methods for producing controlled and random mutations have been well studied. One of the most useful tools of mouse models are knock-in and knock-out models, where a specific gene is replaced with a human homologue or turned off in the mouse genome [71]. These mouse models provide useful information in the understanding of many human diseases [72]. mESC have been used to study the development of hematopoiesis [73]. *In vitro* mESC have e.g. been differentiated in to megakaryocytes producing functional platelets [74], neutrophils [75] and erythroid progenitors [76]. These mouse models have also been used extensively to study human diseases, e.g. the role of Jun transcription factors in the formation of acute myeloid leukaemia (AML) [77], a cure for sickle cell disease [23], diabetes [78] and advancement in understanding congenital heart disease [79].

6 - Discs large homolog 7 (Dlg7).

The *disc large homolog 7* (*Dlg7)* was first cloned and reported on by Nomura *et.al* in 1994. Nomura and his collueges where using randomly sampled cDNA clones from the Myeloid

cell line KG-1 to identify new human genes. They identified 40 new genes which they assigned to numerical names KIAA0001-KIAA0040. Among these genes was *Dlg7* with the name KIAA0008 [80]. The *Dlg7* gene was characterized in humans in 2001. Northern blot analyzes showed abundant mRNA transcripts in highly proliferating tissues such as thymus, testis and colon [81]. Expression of *Dlg7* mRNA and protein has also been detected in tumour tissue from the breast colon, bladder and liver [81, 82]. Figure 5 shows a *Dlg7* gene expression profile in various human tissues obtained by quantitative RT-PCR. The expression profile showed strong expression in fetal liver, colon, testis, bone marrow and placenta of which are highly proliferating tissues [83]

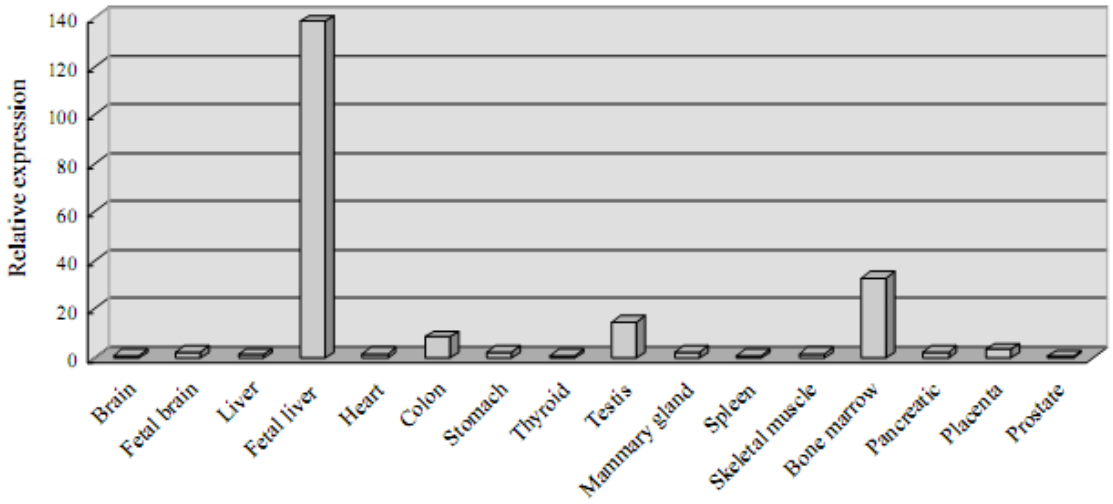


Figure 5. *Dlg7* gene expression profile in human tissue. A quantitative RT-PCR was used to compare expression levels of various human tissues [83].

A more detailed expression profile of the *Dlg7* gene in man and mouse has been assembled and put together in a database by Novarits. This database shows strong expression of *Dlg7* in bone marrow cells, CD71 positive early erythroid, CD34 positive cells, CD105 endothelial cells and leukaemia/lymphoma blastic cells in the human. In the mouse the strongest *Dlg7* expression is found in ESC lines, HSC, hematopoietic progenitors cells, whole bone marrow, osteoclasts and bone tissue [84].

The *Dlg7* gene is located on chromosome 14q22-q23. Investigators found that this novel gene was cell cycle regulated and shared homology with *Drosophila melanogaster discs large-1 (dlg1)* tumour suppressor gene and membrane associated guanylate kinase (MAGUK) protein family members [81]. In the Drosophila the $Dlg1^{v59}$ mutant is lethal due to overgrowth of epithelial cells in the imaginal discs during development [85, 86]. MAGUK's are multi domain proteins consisting of postsynaptic density and Zho (PDZ) domain, a Src

homology domain 3 (SH3) and guanylate kinasa (GK) domain [87]. These proteins are components of tight junctions and septate junctions in insects [88] and synaptic densities in mammalian neurons [89]. Full length cDNA's encoding the human and mouse *Dlg7* gene have been isolated. The human and mouse *Dlg7* share 72 and 61 % identity at the nucleotide and amino acid level, respectively. The mouse *Dlg7* encodes a protein of 808 amino acids and the human one has 846 amino acids. The *Dlg7* gene consist of 19 exons and include a leucine rich nuclear export signal (NES), two destruction boxes (D-box), a Ken box, a coiled-coil domain and a GH1 domain [83].

The official name used for the *Dlg7* gene in databases such as Pubmed. is *Disc large associated protein 5* (Dlgap5) in this thesis we will use the name *Dlg7.* ul

6.1 *Dlg7* and the cell cycle.

In the last decade researchers have studied the role of *Dlg7* in the cell cycle as well as in cancer formation and progression. *Dlg7* mRNA is cell cycle regulated and expressed during the S-phase and maintained at both G2- and M-phases [81]. The *Dlg7* expression rises steadily through the S-phase and the G2/M phase and peaks just as cells exits the mitosis. The *Dlg7* protein product (Hurp) localizes at the spindle poles during mitosis [83]. Hurp has been linked to the Ran-importin β-regulated spindle assembly pathway. Studies showed that Hurp is a direct cargo of importin- β and during the interphase the protein shuttles between cytoplasm and the nucleus and is a novel target of the spindle assembly GTPase Ran. During mitosis Hurp is localized at the kinetochore microtubules in the vicinity of chromosomes. Depletion of Hurp results in less stable K-fibers and chromosome congression is delayed [90, 91]. Ran-GTPase induces the assembly of microtubules into ester like and spindle like structures during cell cycle progression. The Microtubule Associated Protein (MAP) TPX2 is a Ran target in forming microtubules in to ester like structures. Hurp was identified as being a part of a protein complex that consists of two Maps (TPX2, XMAP215), a motor protein (Eg5) and the mitotic kinasa (Aroura A) that is responsible for the transition of the microtubules from ester- to spindle like structures [91]. Ran-GTP is generated via RCC1 a chromatin associated guanine nucleotide exchange factor and promotes the release of spindle assembly factors from inhibitory complexes with importins [92]. Not all experimental models do agree on if Hurp works as a part of this protein complex in stabilizing microtubules or if it acts independently of this complex.

Hurp binds to microtubules through its N-terminal domain and promotes chromosome congression and interkinetochore tension. The C-terminal domain of the Hurp protein binds to the N-terminal and thus blocks the binding of microtubules. The mitotic kinase Aurora A controls the binding affinity of Hurp by phosphorylation of the C-terminal domain with the help of Ran-GTP [93, 94]. Figure 6 shows a simplified drawing of this process. In the case of Hurp overexpression the mitotic spindle is hyper stabilized, tension across sister kinetochores is reduced and microtubule kinetochore attachment is also reduced. Both of these defects activate the spindle checkpoint [94]. Similar problems arise in cells depleted of Hurp [93]. In both overexpression and depletion of Hurp there is persistence of unaligned chromosomes. Despite these defects and the activation of the spindle checkpoint cells eventually go through cytokinesis. These defects can cause genomic instability and aneuploidy which are hallmarks of cancer cells [93, 94].

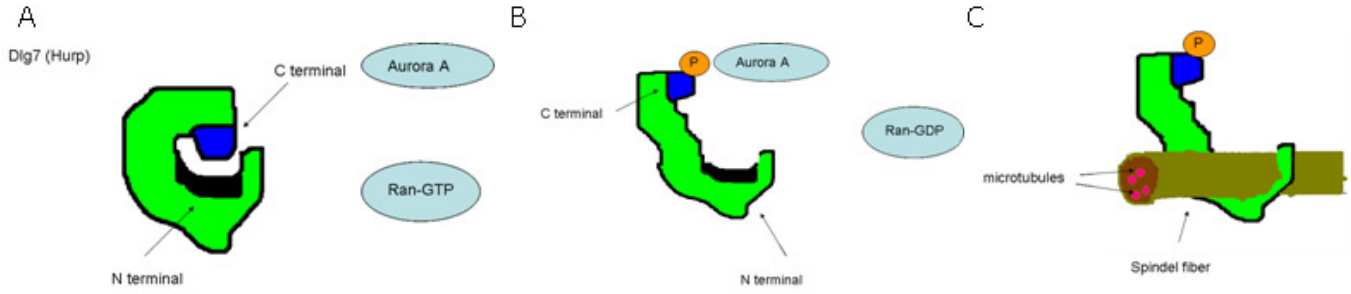


Figure 6. A proposed model for the function of Dlg7. Hurp microtubule binding is controlled by Aurora-A. (A) The C-terminal of Hurp binds to the N-terminal and blocks the binding of microtubules. (B) Aurora-A phosphorylates the C-terminal domain and thus releasing the N-terminal domain with the help of Ran-GTP. (C) The N-terminal domain binds microtubules and promotes the formation of spindle fibres.

Hsu J.M *et.al* reported on the mechanism behind the degration of Hurp. When studying F-box proteins components of the Skp1 Cdc53/Cullin1 F-box protein (SCF) ubiquitin E3 ligase complex, the group recognized Fbx7 a F-box protein to be required for proteosome mediated proteolysis of Hurp. Fbx7 recruits Hurp through its C-terminal prolin rich domain in a Cdk1-cyclin B-phosphorylation dependent manner [95]. In a more recent study another E3 ligase is proposed as being behind the regulation of Hurp amount in the cell. The Anaphase Promoting Complex (APC/C) a E3 ligase is responsible for the degration of Hurp in an Ran dependent manner. APC/C can only promote Hurp proteolysis after Ran-GTP has released Hurp from importins inhibitors. This study also showed that Hurp could be degraded via APC/C during

spindle formation. The correct spindle formation relies on the correct activation and degradation of Hurp which is controlled by the interplay of GTPase Ran and the APC/C E3 ligasa [96]. It is possible that both degradation pathways are responsible for the proteolysis of Hurp but at different stages in the cell cycle. Figure 7 shows a simplified map of Hurp activity and how regulation could look like within the cell.

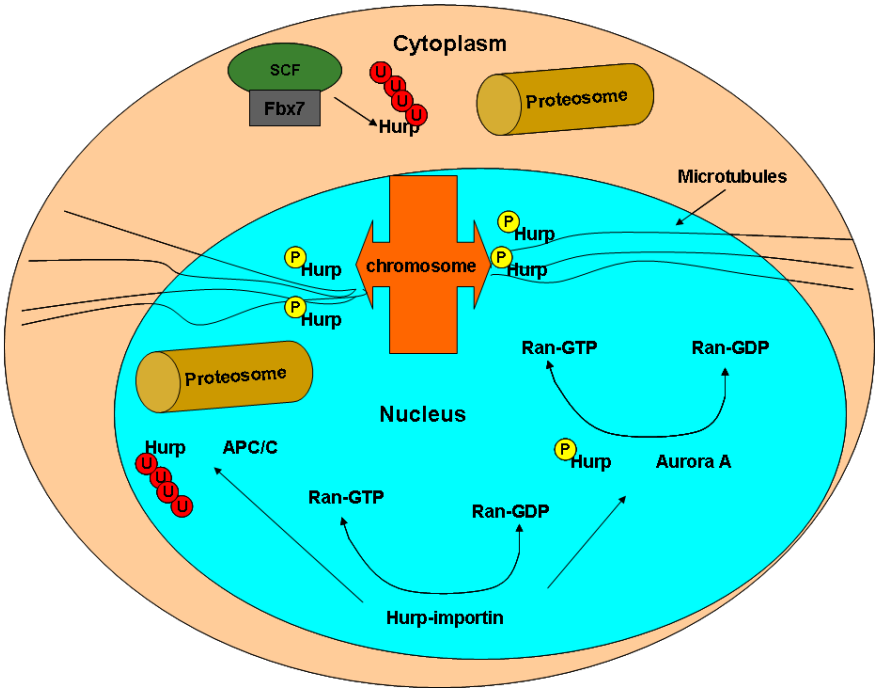


Figure 7. Hurp activity and regulation. Hurp enters the Nucleus attached to a importin inhibitor. Ran-GTP frees Hurp of the importin. A importin free Hurp can be degraded via APC/C pathway or activated by Aurora A which again uses Ran-GTP for this action. Aurora A phosphorylates the C-terminal domain of Hurp which frees the N-terminal that has a binding affinity for microtubules. In this picture Hurp is downgraded by a second pathway via SCF-Fbx7 in the cytoplasm.

6.2 - *Dlg7* and cancer.

In 2002 Chiu A.W. *et.al* reported on the *Dlg7* gene as being a potential molecular marker for detecting transitional cell carcinoma (TCC). TCC tissue samples and urine samples were tested for *Dlg7* detection. 88.8 % of the tissue samples showed *Dlg7* expression and *Dlg7* was detected in 100% of the urine samples from patients with TCC [97]. In a more recent study the expression level of *Dlg7* was used to predict malignancy of adrenocortical tumours and disease free survival of the patient [98].

A group of researchers published data in 2003 identifying *Dlg7* as a novel cell cycle regulated gene that was overexpressed in hepatocellular carcinom (HCC). When performing a

partial hepatectomy in the mouse, elevated expression levels of the *Dlg7* gene where detected during the regeneration of the liver. Overexpression of *Dlg7* in 293T cells resulted in an enhanced cell growth at low serum levels as well as the cells behaving like cancer cells in an anchorage independent growth assay. Immunofluorescence studies showed that the protein product of *Dlg7* localized to the spindle poles during mitosis in HeLa cells. The investigators called the protein Hepatoma up regulated protein (HURP) [83]. Today the name HURP is commonly used to describe the *Dlg7* gene as well as its protein product. The following *Dlg7* was associated with the invasiveness of HCC In this study both HCC tissue samples and HCC cell lines with different levels of invasiveness where evaluated. The *Dlg7* expression level increased gradually with the higher level of invasiveness [99]. Further studies showed that Hurp is detected in liver and colon carcinoma tumors but not in normal adjacent tissues which indicates a role in carcinogenesis [82].

To study the potential of *Dlg7* being an oncogene, one group of investigators used the NIH3T3 embryonic fibroblast cell line overexpressing the gene. The transduced NIH3T3 cells like the *Dlg7* overexpressing 293T cells showed general cancer cell characteristics, with higher growth rate in low serum media and advanced ability of colony formation in an anchorage independent growth assay. In addition transduced NIH3T3 cells showed enhanced sensitivity to deoxycytosine analogs, that are used in chemotherapy [100]. Furthermore *Dlg7* has been reported to be encoding an antigen recognized by autologus antibodies of patients with soft tissue sarcoma.[101].

The relationship of *Dlg7* with the serin/threonin kinase Aurora-A gives further evidence on *Dlg7* being involved in cancer formation and progression. *Aurora-A* and *Dlg7* have similar expression patterns in HCC, liver regeneration after partial hepatectomy, in cell cycle progression and across a variety of tissues and cell lines. [93, 102].

6.1 - *Dlg7* in stem cells and differentiation.

Guðmudsson *et.al* have studied *Dlg7* expression in stem cells and differentiating cells of the haematopoietic system. *Dlg7* expression declined during the differentiation of human myeloid cell line KG1. *Dlg7* is expressed in CD34+CD38- progenitors isolated from cord blood but not in the more committed CD34+CD38- population. *Dlg7* was not detected in *in vitro* haematopoietic colony culture. No *Dlg7* expression was detected in purified human T-cells, B-cells and monocytes [82]. In an experiment where *Dlg7* was transiently overexpressed in mESC the cells displayed an increased growth rate but decreased number of EB´s during

differentiation, an indication of inhibition of differentiation. The mESC overexpressing *Dlg7* had elevated expression levels of known stem cell genes and decreased levels of genes associated with differentiation. As in the human cell line KG1 *Dlg7* expression declined during differentiation of mESC (figure 8) [82].

In an experiment to look at the function of *Dlg7 in vivo* a *Dlg7* knock out mouse model was generated. The female knock out mouse proved to be infertile due to a defect in endometrial stroma proliferation that causes implantation failure. This experiment showed that *Dlg7* is necessary for the proliferation of specific tissue in the mouse [103].

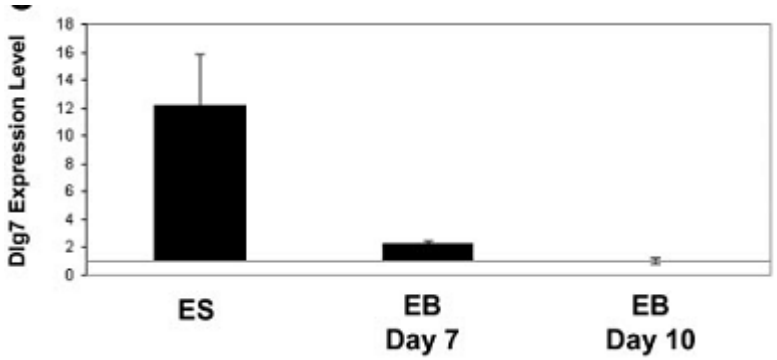


Figure 8. *Dlg7* expression in Embryonic Stem cells. Undifferentiated mouse Embryonic Stem (mESC) cells were differentiated in to EB's. An assessment of *Dlg7* expression was done by Q-PCR at three time points in the differentiaton process, Day 0 ES cells, Day 7 EB and day 10 EB. *Dlg7* expression declines dramatically from day 0 ES cells to day 7 EB and is not detected on day 10 EB.[104].

II - Aim of study

The aim of this study was to analyse the role of *Dlg7* in hematopoietic differentiation of mouse ESC. For that purpose, *Dlg7* was either overexpressed or silenced in using lentiviral based expression systems. The effect of overexpression or silencing on hematopoietic differentiation was assessed using embryoid body (EB) and hematopoietic colony forming culture assays.

To achieve the specific aims, the project was divided into the following steps:

1. Establishment of mESC cultures.
2. Establishing a hematopoietic colony forming assay for mESC.
3. Design and construct a lentiviral *Dlg7* overexpression vector.
4. Generate mESC clones with stable overexpression or silencing of the *Dlg7* gene using lentiviral based methods.
5. Assessment of hematopoietic differentiation potential of the mESC clones using EB and hematopoietic colony forming culture assays.

III - Material and Methods.

1 - Cell lines

Mouse embryonic stem cell line (mESC) was a kind gift from Stem Cells inc (CCE). In this project we used mESC that could be cultured without a feeder layer. Cells were kept in liquid nitrogen in a concentration of 0.5 - 1.0 x 10^6 cells/ mL until used.

2 - Maintenance of mouse embryonic stem cells.

Cells were cultured in 6 well Falcon plates (Becton Dickenson, Franklin Lakes ,NJ, USA). The plates where gelatinised with 0.1% gelatine in PBS prior to the plating of the cells so the cells would adhere to the surface. Cells were maintained in a specific Maintenance medium containing Dulbecco's Modified Eagle Medium (DMEM) with 15% Fatal Bovine Serum (FBS), 1% of sodium pyruvat 100 mM, 1% penicillin G + streptomycin, 1% Glutamine 200 mM, 1% none-essential amino acids 10 mM (Stem Cell Technologies, Vancouver, BC, Canada) and 100 μM of monothioglycerol (MTG) (Sigma, Taufkirchen, Germany). In order to keep cells undifferentiated 10 ng/ml of Leukaemia Inhibitory Factor (LIF) (Stem Cell Technologies, Vancouver, BC, Canada) was added to the media.

Passage of the cells was done every other day or before the cells became confluent or the media acidic. When cells where passaged all media was aspirate from culture vessel and rinsed once with PBS. 1 – 2 ml of trypsin-EDTA (Stem Cell Technologies, Vancouver, BC, Canada) was added to the cell culture and the culture incubated at room temperature for 2 – 3 minutes. A 1000 ul pipette was used to further loosen the cells from the surface and each other. The cells were harvested in a 15 ml tube containing DMEM with 10% FCS and centrifuged for 8 minutes at the speed of 300 *g*. All media was aspirated and the pallet resuspended in 2 ml of Maintenance media. A 1000 ul pipette was used to achieve single cell suspension. Cells where counted using a light microscopy (Leitz,Wetzlar, Germany) and Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany). 1.0 – 2.0 x 10^5 cells where replated to keep the culture going. To ensure enough cells were in stock a low passage of cells was routinely frozen using Freezing media containing 50% FBS, 40% Maintenance medium and 10% DMSO (Impfstoffwerk Dessau-Tornau GmbH IDT ,Tornau, Germany). Cells used in experiments were all ways below passage 24.

2.1 - Embryo body differentiation.

48 hours prior to the formation of EB´s 0.5 - 1.0 x 10^5 cells where plated in a 25 cm^2 flask (Nunc, Roskilde, Denmark) with Predifferentiation medium containing Iscov´s Modified Eagle Medium (IMEM) with 15% FBS, 100 µM of MTG, 10 ng/ml LIF 1% of sodium pyruvat 100 mM, penicillin G + streptomycin, Glutamine 200 mM and none-essential amino acids 10 mM. (Stem Cell Technologies, Vancouver, BC, Canada). After 48 hours of culture and if the cells were below 40-50 % confluence the cells were rinsed with PBS, disrupted with 3 ml of trypsin-EDTA for 3 min, harvested, resuspended and counted as described in the chapter Maintenance of mouse embryonic stem cells. To achieve EB formation the cells were seeded in a methylcellulose based medium called Primary differentiation medium at the density of 1000 – 5000 cells/ml. The Primary differentiation medium contains MTG 150 µM, 15 % FBS, 1% L-Glutamine 200 mM, 40% Basic methylcellulose (MC) (Stem Cell Technologies, Vancouver, BC, Canada) 37,2% IMDM and 0,0044% murine Stem Cell Factor (mSCF) 40 ng/ml (Stem Cell Technologies, Vancouver, BC, Canada). A 12cc syringe and a 16g blunt-end needle (Stem Cell Technologies, Vancouver, BC, Canada) is used to plate the cells in a 35 mm low attachment culture dish (Stem Cell Technologies, Vancouver, BC, Canada) 1 ml/dish. Two 35 mm dishes containing cultures and one 35 mm dish with no lid containing sterile water are placed in a 100 mm culture dish (Nunc, Kamstrupvej, Denmark). The sterile water prohibits the methylcellulose Primary differentiation medium from becoming dry and unviable for cell growth. EB´s start forming as early as day 2-3. EB's are cultured fore 10-14 days, on day 7 a 0,5 ml of feeding medium containing 50% Primary differentiation medium 7.5% FBS, MTG 150 µM, mSCF 160 ng/ml, murine interleukin-3 (mIL3) 30 ng/ml, human interleukin 6 (hIL6) 20 ng/ml, human Erythropietin (hEpo) 3 U/ml (Stem Cell Technologies, Vancouver, BC, Canada) and 40% IMDM is layered over the culture. In the time period between culture day 10 and 12 the EB´s are counted and scored for haematopoiesis using light microscopy.

2.1 - Hematopoietic colony forming unit assay

In the final step the EB´s cultures are flooded with IMDM with 2% FBS and mixed. EB´s are harvested in to a 15 ml Falcon tube (Becton Dickenson, Franklin Lakes, NJ, USA). No more then two ore three 1 ml cultures are transferred in to a 15 ml tube. EB's cultures are

centrifuged for 10 min at a speed of 300 g. The supernatant is removed and replaced with 2 – 3 ml of trypsin/EDTA for disrupting the EB's. The EB's are incubated in the trypsin/EDTA for no more then 2 minutes and then passed threw a 12cc syringe and a 16g blunt-end needle for several times to archive single cell suspension. IMDM with 5% FBS is added to the single cell suspension to inactivate the trypsin/EDTA. The single cell suspension is centrifuged for 8 min at the speed of 300 g. The supernatant is removed and the pallet resuspended in 2 ml of IMDM with 2% FBS. The cells are counted as described before and then 2.5-5 x 10^4 are seeded in 1 ml of Hematopoietic differentiation medium containing 24% IMDM, 40 % Basic methylcellulose, 15% FBS, 20% ,1% BSA -10µg/ml Insulin – 200 µl/ml Transferrin (BIT 9500) (Stem Cell Technologies, Vancouver, BC, Canada), 1% L-Glutamin, MTG 150 µM, mSCF 150 ng/ml, murine interleukin-3 (mIL3) 30 ng/ml, human interleukin 6 (hIL6) 30 ng/ml, human Erythropietin (hEpo) 3 U/ml. As in the EB´s cultures two 35 mm low attachment dishes and one with sterile water are placed in a 100 mm culture dish. The cells are cultured fore 8 – 12 days. In this time period the cells form various colonies of the hematopoietic system. The colonies are identified based on there morphology.

3 – Proliferation.

2,0 x 10^5 mESC cells were seeded in a 25 cm^2 flask and cultured for 48 hours. After the culture period, cells where counted using light microscopy and Neubauer haemocytometer. Their density was calculated as 10^3 cells per cm^2 .

4 - RNA isolation.

RNA purification: Cells where rinsed with PBS, disrupted with 3 ml of trypsin-EDTA for 3 min, harvested, resuspended and counted as described in the chapter Maintenance of Cells. No more than 1 x 10^6 cells where used fore RNA purification. Lysis of cells was done with RNeasy Lysis (RTL) Buffer (QIAGEN, Hilden, Germany). The cell-buffer solution was homogenized by vortexing with vortex mixer (BIBBY STERILIN LTD, Stone, Staffordshiere, UK.). RNA purification was preformed in EZ1 BioRobot using RNA Cell Mini Kit (QIAGEN, Hilden, Germany). RNA was stored at – 20°C.

5 - cDNA synthesis.

High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (QIAGEN, Hilden, Germany) was used for synthesising cDNA from the purified RNA. 10 ul of RNA were mixed with 2.0 µl 10X Reverse Transcriptase Buffer, 0.8 µl 25 X dNTP, 2.0 ul 10X RT Random Primers, 1.0 µl Multiscribe Reverse Transcriptase, 1.0 µl RNase Inhibitor, 3.2 µl Nuclease-free H20 (QIAGEN, Hilden, Germany) . The reverse transcriptasa reaction was done in a 2720 Thermal Cycler (Applied Biosystems (ABI), Foster City, California, USA), cycling conditions were as fallows, 10 min at 25°C for enzyme activation, 120 min 37°C cycling, 5 sec. at 85°C for enzyme deactivation and at last cooled to 4°C. cDNA was stored at – 20°C.

6 - PCR (Reverse transcriptase - polymerase chain reaction)

Table 1. RT-PCR primers.

Primer name.	Sequence	Target	Amount
Dlg-out.F	GTGGAGTTTTCTGTGTTGCGACATT	Mouse	20uM
Dlg-out.R	TAAATTTAAAGCCCTCCTGGTGTT	Mouse	20uM
Dlg-nested1.F	CACCATGCTGGTGTCACGTTT	Mouse	20uM
Dlg-nested1.R	TAGTGGTGAGAAGAGAATGAGGTCA	Mouse	20uM
Dlg-nested2.F	CACCATGGTGGTGTCACGTTTTGCCAGTC	Mouse	20uM
Dlg-nested2.R	TAGTGGTGAGAAGAGAATGAGGTCACCTCCAA	Mouse	20uM
Dlg7det.F	CTGACACCCAATTGTGATTGGAACCAG	Mouse	20uM
Dlg7det.R	CGAGGTCAGCCTGTCAGTTTCTGATTG	Mouse	20uM
Dlg7_mouse_seq.R	CGTCCTTCAAACCGAAGTGT	Mouse	20uM
Dlg7_mouse_seq.F	CCTCACAGAGCAACACCTCA	Mouse	20uM
V5-epitope.R	AGACCGAGGAGAGGGTTAGG	viral	20uM
V5-epitope.2.R	GTAGAATCGAGACCGAGGAGAG	viral	20uM
CMV.1.F	CCTCCATAGAAGACACCGACTC	viral	20uM
CMV.2.F	GATCCACTAGTCCAGTGTGGTG	viral	20uM
Nanoq_mouse.F	AAGTACCTCAGCCTCCAGCA	Mouse	20uM
Nanoq_mouse.R	CGTAAGGCTGCAGAAAGTCC	Mouse	20uM
Oct4_mouse.F	CCAATCAGCTTGGGCTAGAG	Mouse	20uM
Oct4_mouse.R	GCTCCTGATCAACAGCATCA	Mouse	20uM
Epo-R.F	TTCTCCTCGCTATCACCGCATC	Mouse	20uM
Epo-R.R	CCTCAAACTCGCTCTCTGGGCT	Mouse	20uM
Gata-1.F	TAAGGTGGCTGAATCCTCTGCATC	Mouse	20uM
Gata-1.R	ACGTTCTTGGACACCTTGAAGACGG	Mouse	20uM

6.1 – Taq PCR.

2 ul of cDNA mixed with 5 µl 10 x PCR buffer, 1 µl 10mM dNTP and 0,25 µl Taq polymerase (New England Biolabs, Ipswich, MA, USA), 0,5 µl of 20 µM Forward and

Reverse primers (Sigma, Taufkirchen, Germany) where added to the mix along with 40.75 ul of nucleasa free water (Invitrogen, Carlsbad, CA, USA). Taq PCR was done in a 2720 Thermal Cycler (Applied Biosystems (ABI), Foster City, California, USA), cycling conditions were as fallows, 1 cycle 3 min at 94°C denature, 35 cycles of 30 sec at 94°C denature, 50 sec at 58°C annealing, 2 min at 72°C extension and 1 7 min cycle at 72°C for final extension.

6.2 - Proofstart PCR.

2 µl of cDNA or 2 ul Taq PCR product diluted 1:10 were mixed with 2.5 ul 10 x Proofstart buffer, 0.25 µl Proofstart polymerase (QIAGEN, Hilden, Germany) and 0.75 ul 10mM dNTP and (New England Biolabs, Ipswich, MA, USA). 1.25 ul of 20 uM Forward and Reverse primers (Sigma, Taufkirchen, Germany) and 16.25 ul of nucleasa free water (Invitrogen, Carlsbad, CA, USA). Proofstart PCR was done in a 2720 Thermal Cycler (Applied Biosystems (ABI), Foster City, California, USA), cycling conditions were as fallows, 1 cycle 5 min at 95°C denature, 35 cycles of 30 sec at 94°C denature, 30 sec at 58°C annealing, 6 min at 72°C extension and one 7 min cycle at 72°C for final extension.

6.3 - Quantitative revers transcriptase polymerase chain reaction (Q-PCR).

The followint TaqMan Gene Expression Assays wehre used: *Dlg7, GAPDH, Nanog, Oct 4, and Brachyury* (Applied Biosystems (ABI), Foster City, California, USA).

Table 2 TaqMan Expression Assays

Gene.	Reference Sequence
Dlg7	Mm01322961_g1
GAPDH	NM_008084.2
Nanog	Mm02384862_g1
Oct4	Mm00658129_gH
Brachyury_	Mm01318252_m1

For quantitative reverse polymerase chain reaction (Q- PCR) we used Taq-man assays according to the manufactures procedures. Reactions were designed using StepOne softwere V 2.0 (Applied Biosystems (ABI), Foster City, California, USA) according to the

manufactures description. In brief 1 µl of TaqMan Gene Expression Assay was added to 10 µl of TaqMan Gene Expression Master Mix(Applied Biosystems (ABI), Foster City, California, USA) and 9 ul of 1:10 diluted cDNA. The Q-RT-PCR reaction was preformed in a StepOne Real Time PCR system thermal cycling block (Applied Biosystems (ABI), Foster City, California, USA) using MicroAmp 48 well reaction plate (Applied Biosystems (ABI), Foster City, California, USA). Plates were centrifuged for 5 – 10 min. at 3000 round per minute (RPM) before going in to the thermal cycling block. Results where displayed using DELL Latitude D530 and StepOne softwere V 2.0 (Applied Biosystems (ABI), Foster City, California, USA). Data was presented using the comparative Ct method where GAPDH was used as housekeeping gene.

7 - Agarosagel electrophoresis.

A solution of 1% Agarose for routine use (Sigma, Taufkirchen, Germany) in 100 ml of 1 x TBE buffer (Invitrogen, Carlsbad, CA, USA) was prepared. Solution was brought to boiling temperture in a microwave oven Micro-Chef 900w (Scarborough, ON, Canada) . The solution was cooled to 60 °C in a Thermomix 1419 water bath (B.Braun Melsungen, Germany). 10 ul of SYBER safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) was added to the solution. The solution was stirred to disperse of the SYBER safe DNA gel stain and then poured into a gel rack with a comb for making sample wells. The solution became a solid gel in 20 to 30 min. The gel was transferred in to Wide mini-sub cell GT tank (BIO-RAD, Hercules, CA, USA) containing 1x TBE buffer. 5 ul of 100 bp or 1 kb Trackit DNA ladder (Invitrogen, Carlsbad, CA, USA) where pippeted in to the first wells and then the samples. PCR product samples where prepared with 10 x gel loding solution (Ambion, Austin, TX, USA) in the ration 4:1 respectively. Current for the electrophoresis was applied by PowerPac 300 (BIO-RAD, Hercules, CA, USA) at 100 V for 30 to 40 min. DNA fragment where visualized on a GelVue CVM 20 (Syngene, Cambridge, UK) Ultra Violet (UV) light board. DNA fragments where photographed with PhowerShot G3 camera and RemoteCapture softwre (Canon, Ohta-Ku, Tokyo)

8 - Gel Extraction.

QIAEX II Agarose Gel Extraction kit (QIAGEN, Hilden, Germany) was used for the extraction of desired DNA fragments from the agarosegel. The DNA fragment was excised from the gel with a scalpel and put in to a 1.5 ml microfuge tube. The gel slice was weighed

and 3 x this weight was the volume of QX1 buffer added. 30 µl of QIAEX II solution was added and mixed by vortexin for 30 sec. The mixture was incubated at 50 °C for 10 min. and vortexed every 2 min. The sample was centrifuged at 13.000 rpm. for 30 sec. and the supernatant was removed. Two wash steps were preformed, first with 500 µl of QX1 buffer and next with 500 µl of PE buffer. The pellet was air dried for 10 to 15 min. 20 µl of 10 mM Tris-Cl with a pH of 8,5 was added and the pellet resuspended by vortexing. Sample was incubated at 50 °C for 10 min and then centrifuged for 30 sec. The supernatant containing the DNA fragments was moved to a clean tube.

9 - TOPO cloning.

pLenti6/V5 Directional TOPO cloning kit (Invitrogen, Carlsbad, CA, USA). was used for the cloning reaction of *Dlg7* gene in to a vector pLenti6/V5-D- TOPO for ViraPower expression system(Invitrogen, Carlsbad, CA, USA). 2 – 4 µl of PCR. product was mixed with 1 µl of TOPO vector. Salt solution and water was added to a final volume of 6 µl. The vector with the cloned DNA was transformed into One shot Stbl3 chemically competent E.coli. 2 µl of the cloning reaction was added to one 50 µl vial of One shot stbl3 E.coli. Vial was incubated on Ice for 30 min. The E.coli cells were heat shocked for 30 sec. in a 42 °C water bath. The vial was placed on ice for 2 min. Cells were transferred in to a sterile 15 ml falcon tube containing 1 ml of Luria-Bertani (LB) medium. The tubes were placed in a Hedolph Unimax 1010 shaking incubator (Hedolph, Kalheim, Germany) at 37 °C at 225 rpm for one hour. 100 µl of the transformation mix were spread on to pre warmed selective plates that were prepared with Difco LB Agar Lennox (Becton Dickenson (BD), Franklin Lakes, NJ, USA) with or without 100 µg/ml ampicillin (Sigma, Taufkirchen, Germany). Plates were incubated at 37 °C over night. Colonies were picked and placed in a 96 well plate containing 1 ml of Luria Broth (Sigma, Taufkirchen, Germany) in each well. Plates were incubated in a Heidolph Unimax 1010 shakin incubator at 37 °C at 225 rpm over night.

9.1 - Plasmid purification.

For isolation of plasmid from stbl3 E.coli cells Pure Link HQ Mini plasmid purification kit (Invitrogen, Carlsbad, CA, USA) was used. stbl3 overnight culture was centrifuged at 1500 g for 15 min. The pellet was resuspended in 240 ul of resuspension solution containing RNasa A. 240 µl of lysis buffer was added to the suspension and incubated for 3 to 4 min. 340 ul of Neutralization/Binding Buffer was added and the sample centrifuged for 10 min at 15000 g. The sample was moved to a collection tube containing a spin column and centrifuged at

14000 g. 650 µl of Wash buffer with ethanol was added to the column and centrifuged for 1 min at 14000 g. The column was placed in a elution tube and 50 µl of elution buffer (10 mM Tris-HCl, pH 8,5) was added to the column. The column was incubated for 1 min at centrifuged for 1 min at 15000 g. The elution containing the purified plasmid DNA was stored at – 20 °C.

Upscaling 1 µl of plasmid DNA was diluted at 1:500 ratio in TE buffer. 1 µl of the diluted plasmid DNA was retransformed in to stbl3 E.coli cells as described in the chapter cloning procedure page. Positive colonies were picked and cultured in 96 well plate in 1 ml LB medium containing 100 µg/ml ampecillin and cultured for 6-7 hours at 37 °C to obtain a starter culture. The starter culture was inoculated into flasks containing 100 ml of LB medium with 100 µg/ml ampecillin and cultured over night. Plasmid DNA from the 100 ml volume was isolated using S.N.A.P MidiPrep kit. 4 ml. of Resuspension buffer (R3) containing RNase was added to the pellet and homogenized. 4 ml of Lysis buffer (L7) was added and mixed gently before incubated at room temperature for 5 min. 4 ml of Precipitation buffer (N3) and centrifuged for 15 min at 15000 g at room temperature. Supernatant was put threw a Equilibrium column by gravity flow. The column was washed twice with 10 ml of Wash buffer (W8) 5 ml of Elution buffer was added to the column for elution of the DNA. 3.5 ml. of isopropanol was added to the plasmid DNA and centrifuged at 4 °C at 15000 g for 30 min. 70% ethanol was added to the pallet and centrifuged at 4 °C at 15000 g for 5 min. The pellet was airdrayed for 10 min. and resuspended in 100 ul of TE buffer (TE) and plasmid DNA stored at – 20 °C. Sequencing

10 - Production of lentiviral particles.

Twenty four hours before transfection 293FT cell line (Invitrogen, Carlsbad, CA, USA) was plated in 25 cm^2 flask and grown to a 90-95% confluent. A transfection mix was made with 10 ug of Lenti-Combomix and 10 µg of pLenti expression plasmid DNA (Capital Bioscience, Rockwille, MD, USA) in 1 ml of DMEM. 80 ug of Lentifectin (Capital Bioscience, Rockwille, MD, USA) in 1 ml of DMEM and incubated for 5 min. After the 5 min incubation the transfection mix and the Lentifectin where combined in one test tube and incubated for 20 min at room temperature for the formation of Lentifectin/DNA complexes. 4.5 ml of serum free DMEM was added to the complexes. Lentifectin/DNA complexes where added to 293FT cells and incubated for 6 hours at 37 °C and 5% CO_2. 650 ul FBS. was added to the culture and incubated over night at 37 °C and 5% CO_2. After over night incubation the

media containing the Lentifectin/DNA complexes was replaced with Maitenance medium. The virus containing supernant was harvested 52 hours post transfection. The supernant was centrifuged at 1700 g for 15 min. to pellet the debris. The supernant was filtered threw a 0.45 µm PVDF syringe filter (Millipore, Billerica, MA, USA). Viral supernant was divided in to 1 ml vials and stored in liquid N_2.

10.1 - Death Curve.

25.000 mESC where plated in a 12 well culture dish. Various concentrations from 0.5 µg/ml to 10 µg/ml of puromycin and 250 µg/ml to 1 mg/ml of geneticin (G418) where used to perform the death curve. A minimum concentration that killed all cells in 2 – 3 days was used for selection of transfected cells.

10.2 - Infection with ready for use viral-particles.

250.000 mESC where plated in i 6 well plate in maintenance media. The day after the media was replaced with media containing viral-particles and 8 ug/ml of hexadimethrine bromide. Multiplicity of infection (MOI) was 0.5 to 5.0. mESC where infected for 16 to 18 hours. Selection with antibiotics was started 48 to 72 hours post infection depending on cell growth. Change in genexpression was confirmed with Q-PCR. Two controls where used a Green Fluorescent Protein (GFP) insert and a control with a scrambled insert with a sequence corresponding to no known gene. GFP expression was analyzed with Flow cytometry and reverse microscopy. A list of ready to use viral particles is shown in table 3.

Table 3. Ready to use viral-particles

Viral-particles.	Manufacture.
p-Lenti-III-Ubc-Dlg7.	Capital Bioscience
p-Lenti-III-Ubc-Scrambled.	Capital Bioscience
p-Lenti-III-Ubc-EGFP.	Capital Bioscience
pLenti-III-CMV-EGFP	Capital Bioscience
Mission-shRNA-133-Dlg7.	Sigma.
Mission-shRNA-134-Dlg7.	Sigma
Mission-shRNA-135-Dlg7.	Sigma
Mission-shRNA-136-Dlg7.	Sigma
Mission-shRNA-137-Dlg7.	Sigma
Mission-Scrambled-shRNA.	Sigma
Mission-Turbo-GFP control.	Sigma

11 - Statistical analysis.

Statistical calculations were made using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA). Significance tests were performed using a paired Student's *t*-test. The difference was considered significant if $p<0.05$. The results are presented as mean $\pm$ standard error of the mean or standard error of deviation.

IV - Results

1-Establishment of mESC cultures.

In order to analyze the role of *Dlg7* in hematopoiesis we used mESC as a model system. We used low passage cells for all experiments (P18-24). We confirmed the quality of the mESC with microscopic analysis demonstrating that the cells showed typical morphology of undifferentiated mESC (Figure 9 A, B and C.). In addition, we analyzed the cells for the expression of *Oct4* and *Nanog*, well known mESC genes (figure 9 D) and found that the mESC expressed high levels of *Oct4* and *Nanog* (figure 9 D). We therefore concluded that the cells used were undifferentiated mESC.

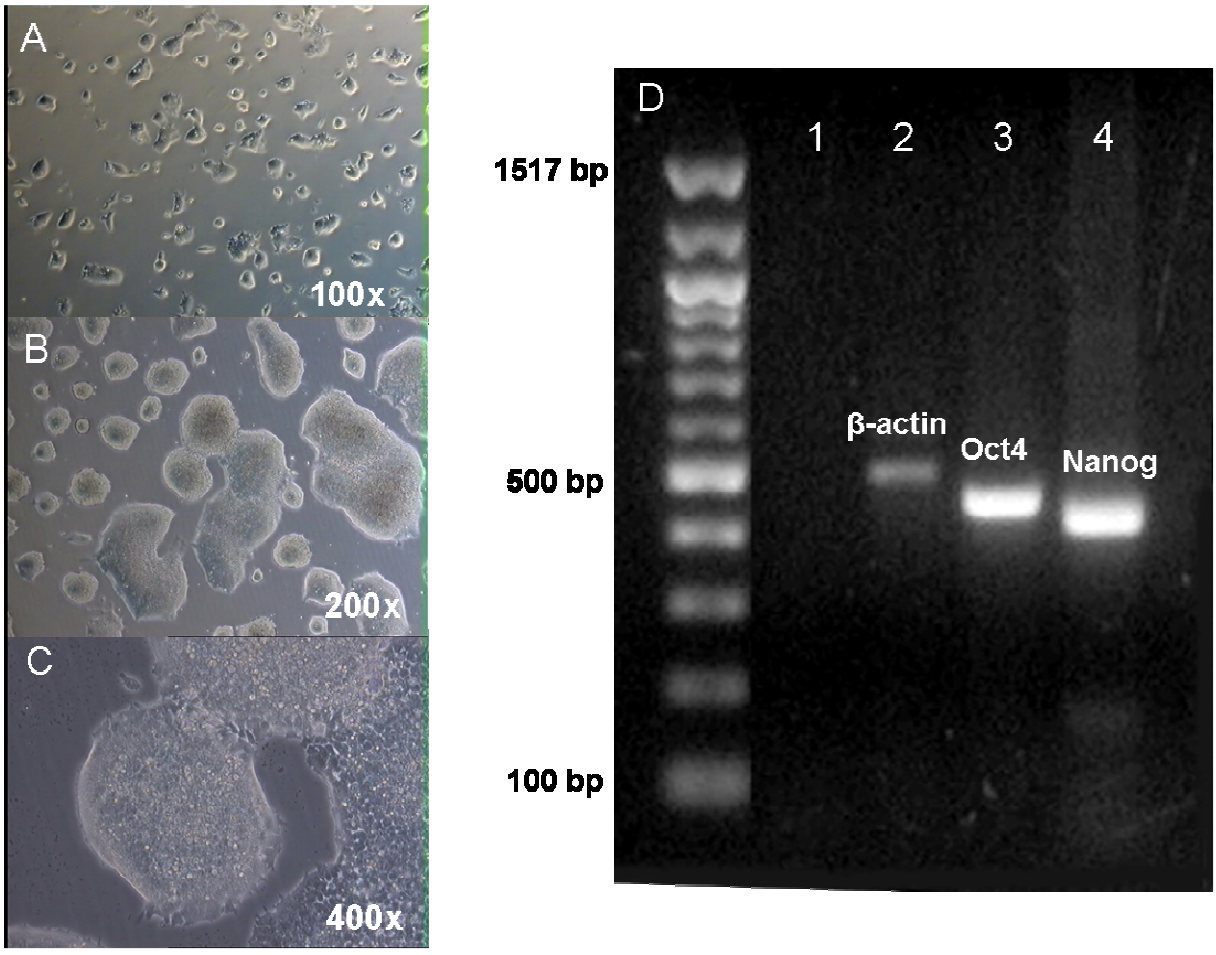


Figure 9. mESC morphology and expression of stem cell genes. (A-C) Morphology of mESC colonies at various magnifications (100X, 200X and 400X). (D) RT-PCR analysis of the stem cell genes Oct4 and Nanog. in undifferentiated mESC .

2 - Establishment of hematopoietic colony forming unit assays.

In order to analyse the hematopoietic differentiation efficiency of the mESC lines, we set up a two step *in vitro* hematopoietic differentiation assay: 1) Primary differentiation. mESC were plated in Primary differentiation media containing SCF but without LIF to promote embryoid body differentiation. 2). Hematopoietic differentiation. Around days 10-14, EB's with hematopoietic potential were harvested and dissociated into single cells that are cultured for 8-12 days in Hematopoietic differentiation medium containing EPO, IL-3, IL-6 and SCF, to stimulate hematopoietic differentiation. EB's with hematopoietic potential and hematopoietic colonies derived from the EB's were scored according to guidelines provided by StemCell Technologies Inc. (*"Identification and scoring of embryoid bodies and haematopoetic colonies").* After culturing mESC for two to three days in Primary differentiation culture without LIF, the cells started to differentiate and small EB´s became visible that grew in size over the next days (Figure 10 A-C,).

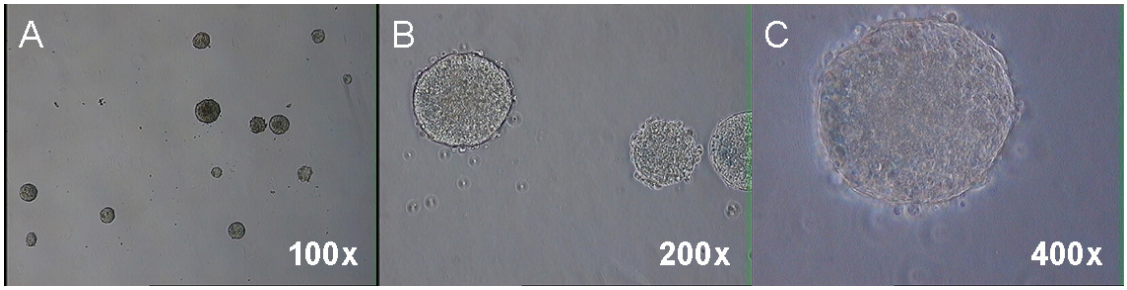


Figure 10. EB formation in Primary differentiation medium. A, B and C show 6 day old EB's at various magnifications (100-400x).

At day 10 to 14 of culture, EB's with distinct morphology (hematopoietic EB's) started to appear. These EB's, are red in colour (hemoglobinized) and surrounded by hematopoietic cells. Figure 11 B and C shows 12 day old EB's witch where categorized as being hematopoietic. Figure 11 A shows an 12 day old EB from the same culture with no visible characteristics of hematopoiesis that was categorized as being a none hematopoietic EB. EB's were harvested, dissociated and induced for differentiation in a CFU assay.

.

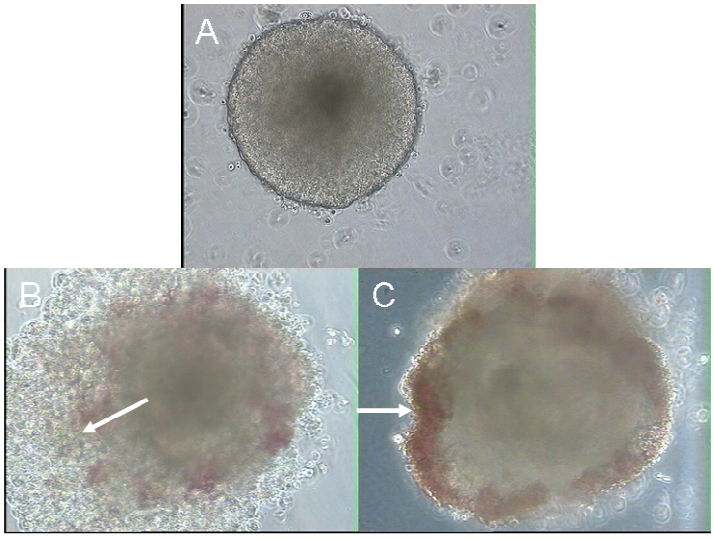


Figure 11. Hematopoietic EB's at day 12 of culture. mESC were culture in Primary differentiation media without LIF for 10-14 days. (A) None hematopoietic EB. (B) Hematopoietic EB containing hemoglobinized cells surrounded by macrophages. White arrow point to macrophages cells scattered around the EB (C) Hemoglobinized EB. White arrow points to red color in EB caused by hemoglobulin with in the cells of the EB's

EB's are dissociated and made in to a single cell solution and the cells seeded in to Hematopoietic differentiation medium. After 8 to 12 days of haematopoietic differentiation, various types of colonies started to emerge. Colonies are scored based on morphology and can be categorized as: primitive erythrocytes or erythroblast (BFU-E), colonies consisting of monocyte-macrophage and/or granulocytes and mast cells (CFU-GM) and colonies with mixed population of granulocytes, macrophages, erythroid cells and megakaryocytes (CFU-GEMM). In this project all colonies originating from erythrocyte progenitors where categorized as BFU-E (BFU-E and CFU-E). The various kinds of colonies obtained in the cultures are depicted in figure 12.

.

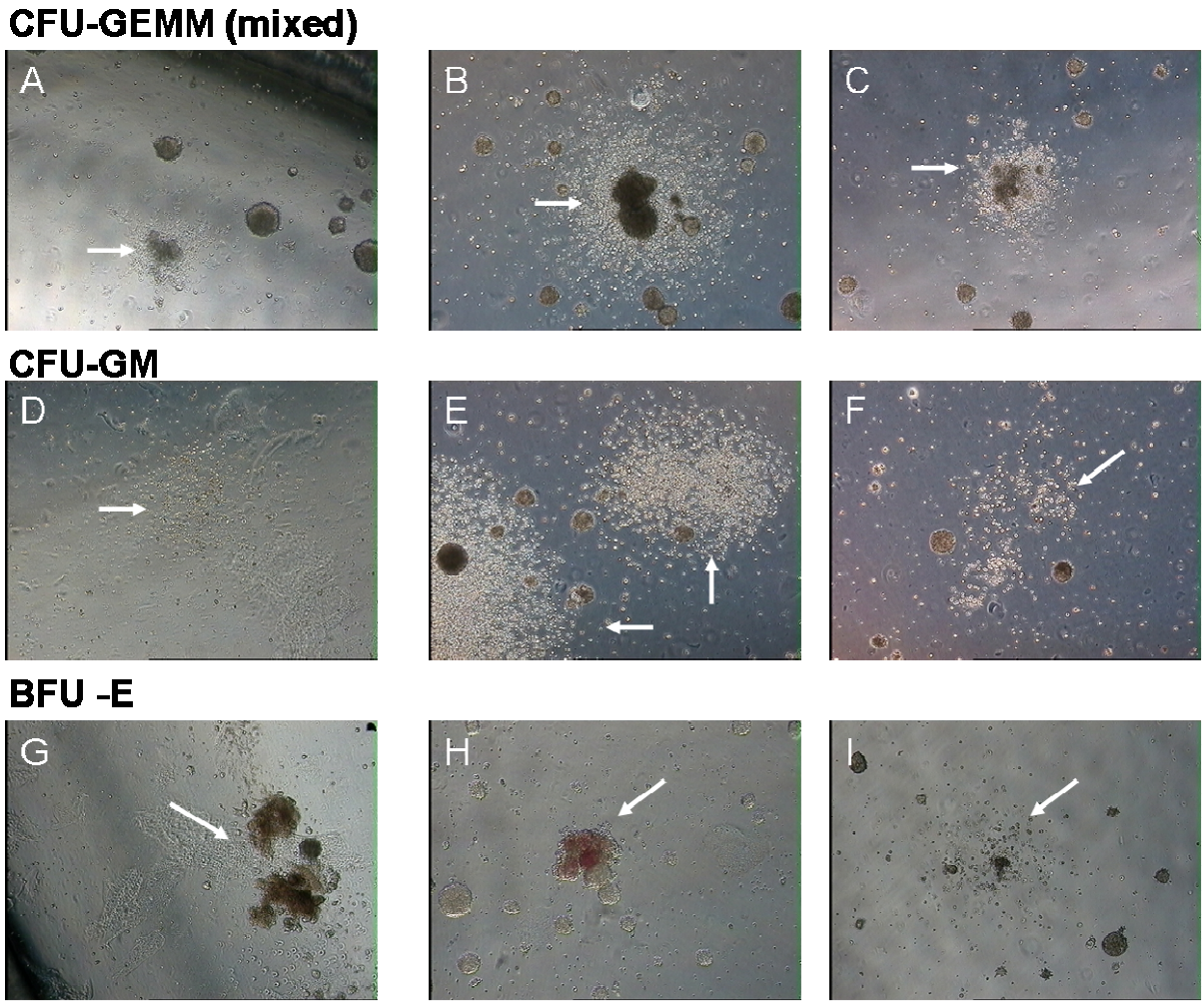


Figure 12. Hematopoietic colonies (CFU) differentiated from day 10-14 EBs. A, B and C. Mixed colonies consisting of granulocytes, macrophages, erythroid cells and megakaryocytes (CFU-GEMM). D, E and F. Colonies consisting of granulocytes and/or monocyte-macrophags (CFU-GM). G, H and I. erythroblast colonies (BFU-E). White arrows point to colonies.

Taken together, we were able to efficiently differentiate mESC cells into hematopoietic cells and could therefore use that as model to analyze the role of *Dlg7* in hematopoiesis.

.

2.1 - Transduction efficiency of lentiviral vectors in mouse embryonic stem cells.

To get an indication of the efficiency by which lentiviral vectors infect mESC, the cells were infected with viral particles containing a pLenti-III-CMV-EGFP vector. These viral particles integrate the *EGFP* gene, which is under the control of a CMV promoter, into the host genome. Cells were infected with the EGFP viral particles at a MOI of 0.8 and 2.0 for 16-18 hours and exposed to an antibiotic (G418) for 144 hours. Cells were then analyzed for viability and EGFP expression. Cells exposed to a higher MOI show better growth (Figure 13

C) compared to cells exposed to lower MOI (Figure 13 B) and cells that were not infected (Figure 13 A).

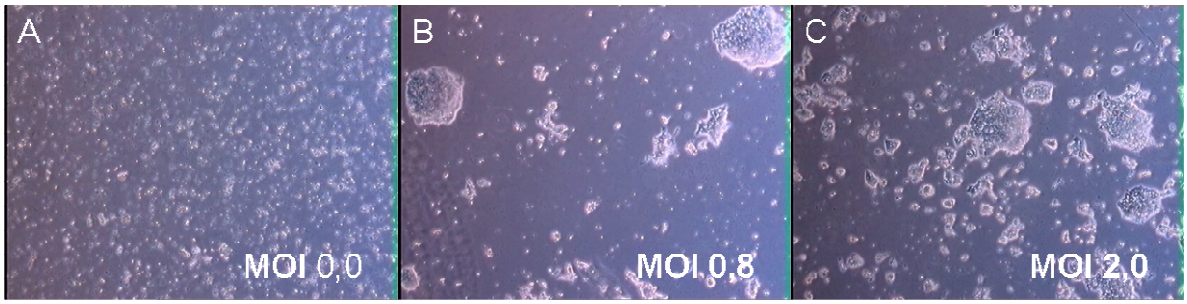


Figure 13.Effect of MOI on mESC infection efficiency. Cells were infected with pLenti-III-CMV-EGFP particles at a MOI of 0.8 and 2.0. After 48 hour growth period cells were exposed to 350 µg/ml of G418 for 144 hours. (A) Morphology of mESC that were not infected. (B) Morphology of mESC cells infected with MOI of 0.8. (C) Morphology of mESC cells infected with MOI of 2.0.

Cells exposed to MOI 2.0 were further analyzed for the expression of EGFP with a transmission fluorescent microscope. Clear expression was visible in cells infected with the pLenti-III-CMV-EGFP vector with a good co-localization of EGFP and the cell bodies indicating a successful infection (figure 14 A-I). To quantify the EGFP expression, cells were analyzed by flow cytometry and the percentage of GFP positive cells compared to non-infected cells. Approximately 56% of mESC expressed EGFP following antibiotic treatment (Figure 15 A and B and Table 4).

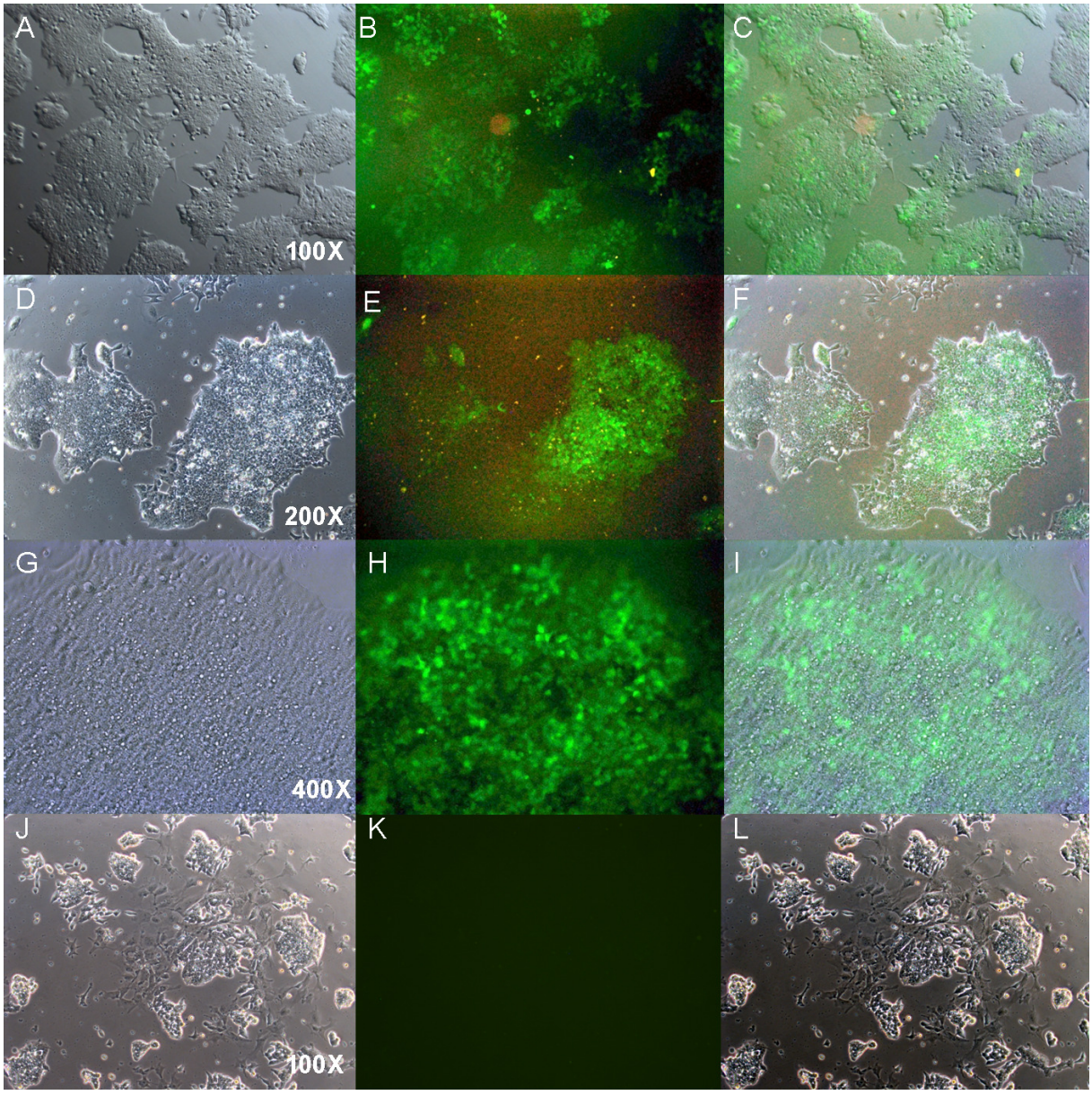


Figure 14. EGFP expression in mESC cells infected with a pLenti-III-CMV-EGFP vector. mESC cells were analyzed using a transmission light microscope with a fluorescence filter (A, D, G) Transmission light microscope images of infected mESC at different a magnification (100X, 200X, 400X). (J) Transmission light microscope images of none infected mESC at 100X magnification(B, E, H) fluorescence images of infected mESC at different magnifications (100X, 200X, 400X). (K) fluorescence images of none infected mESC at 100X magnifications (C, F, I and L) Overlay of transmission light microscope images and fluorescence images.

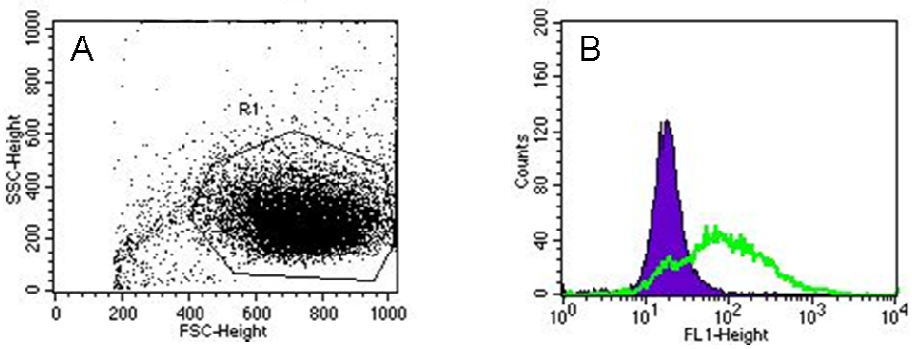


Figure 15. Flow cytometric analysis of mESC infected with pLenti-III-CMV-EGFP vector. (A) Dot plot showing forward scatter, side scatter and gating strategy. (B) Histogram showing EGFP expression in non-infected cells (purple filled line) and cell infected with the pLenti-III-CMV-EGFP vector (green line).

Table 4. Percentage of control or infected mESC cells expressing EGFP

Cell population	% EGFP
mESC-cont.	0.31
mESC-EGFP	56.5

Taken together, these results demonstrate that the mESC can be efficiently infected with lentiviral vectors. In addition, they provided information on the minimum amount of MOI for the infection of mESC with the pLenti-III-Ubc-Dlg7 and the Mission-shRNA lentiviral vectors for overexpressing or knocking down the *Dlg7* gene respectively.

3 – Effect of overexpression of Dlg7 on hematopoietic differentiation.

To analyse the functional role of *Dlg7* on mesodermal differentiation, specifically hematopoiesis, we overexpressed *Dlg7* in mESC using lentiviral particles containing either the *Dlg7* gene or a scrambled control DNA. The cells were differentiated into EB's and subsequently hematopoietic cells as described above. As we have previously shown *Dlg7* is expressed in mESC but is downregulated upon differentiation (figure 8) [104]

3.1 - Puromycin death curve

To achieve overexpression of the *Dlg7* gene, pLenti-III-Ubc-Dlg7 viral particles were used to infect mESC. As a control, mESC were infected with pLenti-III-Ubc-Scrambled viral

particles containing a scrambled DNA sequence corresponding to no known mouse gene. Following infection, resistance to antibiotics (puromycin) was tested in order to expand only mESC clones that contained the *Dlg7* transgene or the scrambled DNA. Cells that where not infected and only received hexadimethrine bromide tratment where used as a control (Hexamdi control) for resistanse to puromicyn. A death curve was performed to find the appropriate amount of puromycin that would kill all cells that were not infected within 48 hours. Two µg/mL of puromycin were found to be sufficient to kill all cells that were not infected within 48 hours (figure 16). This concentration of puromycin was used in all subsequent experiments. mESC cells that survived the antibiotic selection were expanded, frozen down and stored for later use (figure 17).

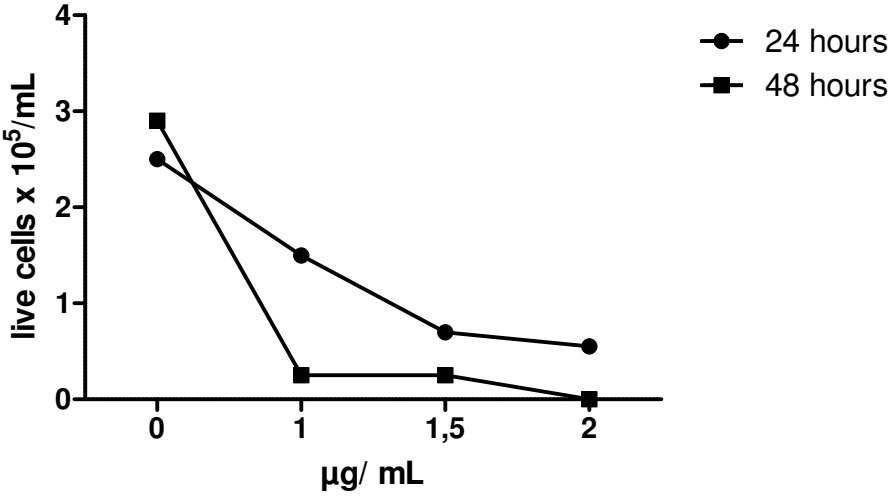


Figure 16. Puromycin death curve. mESC where treated with three different concentrations of puromycin (1,0, 1,5, 2,0 ug/ml). Cells that received no puromycin where used as a control. All non-infected cells were killed within 48 hours using 2,0 ug/ml of puromycin.

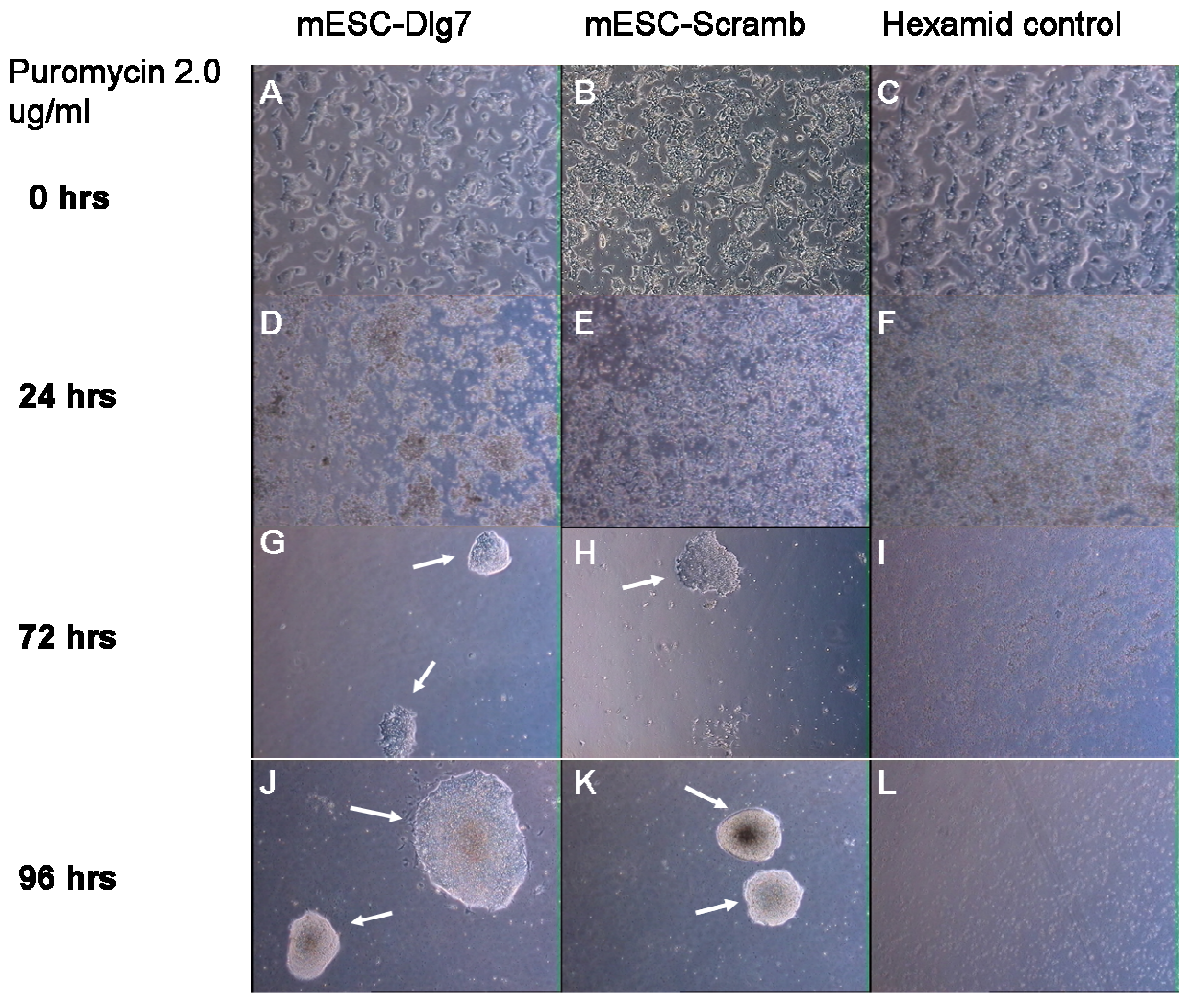


Figure 17. Puromycin resistant cells infected with either mESC-Dlg7 or mESC-Scrambled lentiviral vectors. mES where infected with pLenti-III-Ubc-Dlg7 (mES-Dlg7) A,D,G and J or pLenti-III-Ubc-Scramb (mES-Scramb) B,E,H and K. Non-infected cells where used as a control (Hexamid control) C,F,I and L. Arrows point to puromycin resistant colonies.

3.2 - Overexpression of *Dlg7* in mouse embryonic stem cells

Quantitative RT-PCR (Q- PCR) was performed on selected resistant mESC-Dlg7 clones and on mECS-Scrambled clones in order to compare the fold changes in expression of *Dlg7*. mESC-Dlg7 clones showed a 2,17 fold increase in expression compared to the mECS-Scrambled control cells (figure 18). In order to determine if the *Dlg7* overexpression was maintained during differentiation, *Dlg7* expression was analyzed in 4 day old and 12 day old embryoid bodies differentiated from mESC-Dlg7 and mESC-Scrambled cells. Four day old EB's showed 1,7 fold more *Dlg7* expression compared to 4 day old EB's differentiated from mESC-Scrambled clones (figure 18). Twelve day old EB's mESC-Dlg7 clones showed 1,2

fold more *Dlg7* expression compared to 12 day old EB's differentiated from mESC-Scrambled clones (figure 18).

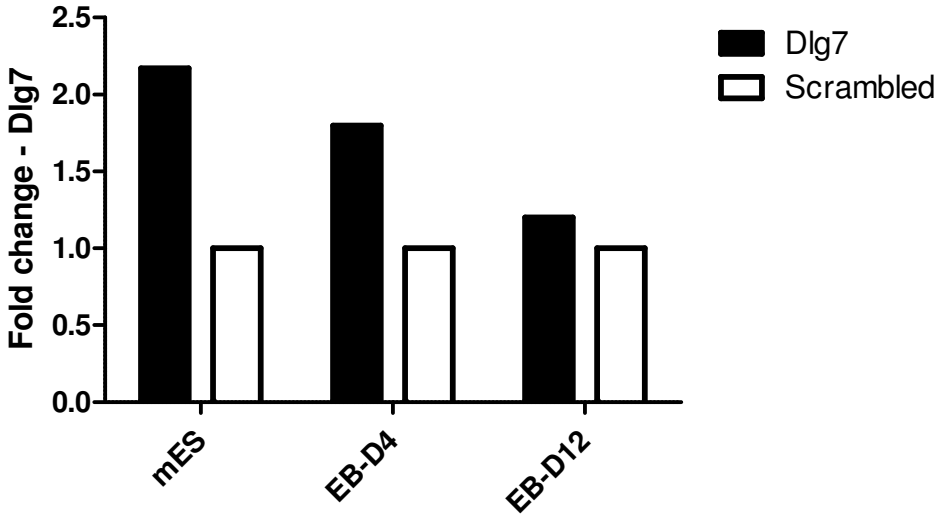


Figure 18. Q-PCR analysis of *Dlg7* expression in mESC and 4 and 12 day old EB's. mESC were infected with a lentiviral vectors containing the *Dlg7* gene or a scrambled DNA sequence. EBs were differentiated from infected mESC. Expression was analyzed by Q-PCR and data presented as an average of two experiments using the ΔΔCT method (n=2).

To determine whether the overexpression of the *Dlg7* gene was effecting the expression of stem cell genes or genes expressed in differentiating mESC, we compared the expression levels of *Nanog* and *Brachyury* (expressed in differentiating ES cells) in 4 and 12 days old EB's differentiated from mESC-Dlg7 and mESC-Scrambled cells [82]. EB's differentiated from mESC-Dlg7 cells showed higher expression of *Nanog* both in 4 day old and 12 day old EB's, 1.2 fold vs. 2.4 fold respectively compared to mESC-Scrambled cells (figure 19A). Conversely, EB's differentiated from mESC-Dlg7 cells showed a lower expression of *Brachyury* both in 4 day old and 12 day old EB's, 1.1 fold vs. 1.35 fold compared to mESC-Scrambled cells (figure 19B).

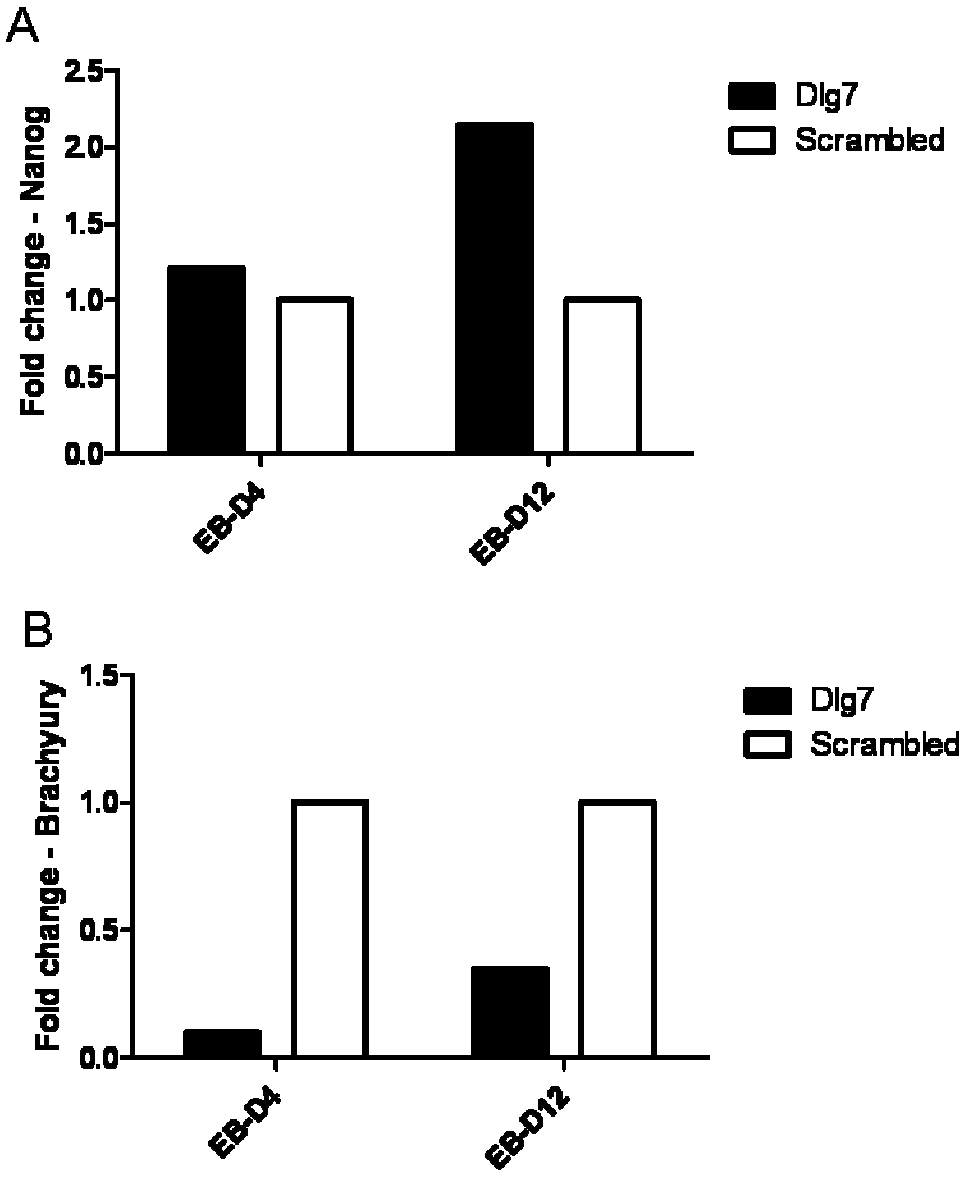


Figure 19. Expression of Nanog and Brachyury in EB's differentiated from mESC overexpressing Dlg7 or scrambled DNA sequence. (A) Expression of *Nanog* in 4 day and 12 day old EB's differentiated from mESC infected with mESC-Dlg7 or mESC-Scrambled constructs. (B) Expression of Brachyury in 4 day and 12 day old EB's differentiated from mESC infected with mESC-Dlg7 or mESC-Scrambled constructs. Expression was analyzed by Q-PCR and data presented as an average of two experiments using the ΔΔCT method (n=2).

3.3 – Embryoid body formation.

Next, we analyzed the effect of overexpressing the *Dlg7* gene on EB differentiation. mESC clones infected with the *Dlg7* gene or Scrambled DNA sequence were differentiated for 13 days and counted. mESC overexpressing *Dlg7* produced fewer EB's (161±22.6 EB's/ 2500 cells) compared to cells containing the scrambled DNA (237±32.7 EB's/ 2500 cells). The difference was statistically significant, (p=0.0008, power=0,35)(figure 20).

.

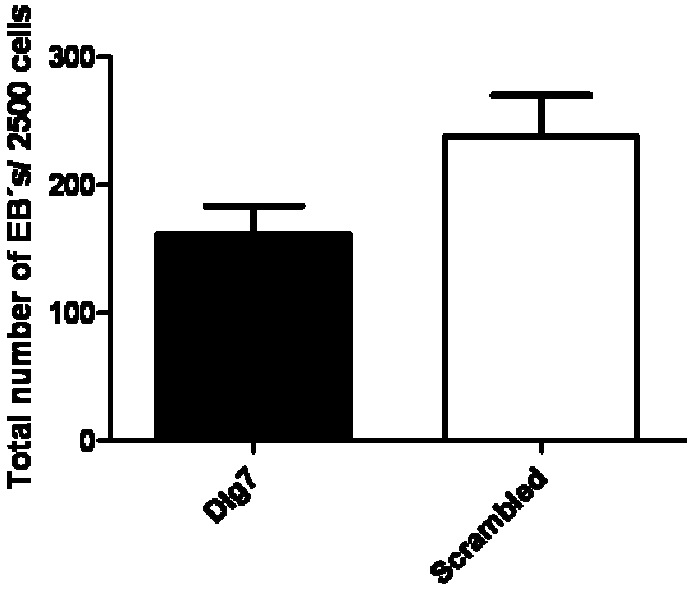


Figure 20. The effect of *Dlg7* overexpression on the production of EB's. The bar chart shows number of EB's formed per 2500 mESC plated. Black bar represents mESC overexpressing *Dlg7* (161±22.6 EB's/ 2500 mESC). The white bar represents mESC infected with the scrambled DNA sequence (237±32.7 EB's/ 2500 mESC). The difference was statistically significant, p=0.008, power=0.35 n=6. Data are presented as average ±SD.

EB's where kept in differentiation for 12-14 days to establish their haematopoietic potential and scored based on their morphology. The percentage of EB´s considered haematopoietic was compared between the two cell populations (mESC-Dlg7 and mESC-Scrambled) (figure 21). EB's derived from mESC clones that overexpress the *Dlg7* gene produced significantly lower number of hematopoietic EB's compared to EB's derived from the scrambled control (36.3±15,70 vs. 73±10.82 respectively). (n=3, p=0.0029, power=0.203) (Figure 21). In addition, the percentage of hematopoietic EB's was significantly lower in mESC overexpressing *Dlg7* (22.65±11.41 % vs. 43.72±6.94 %).

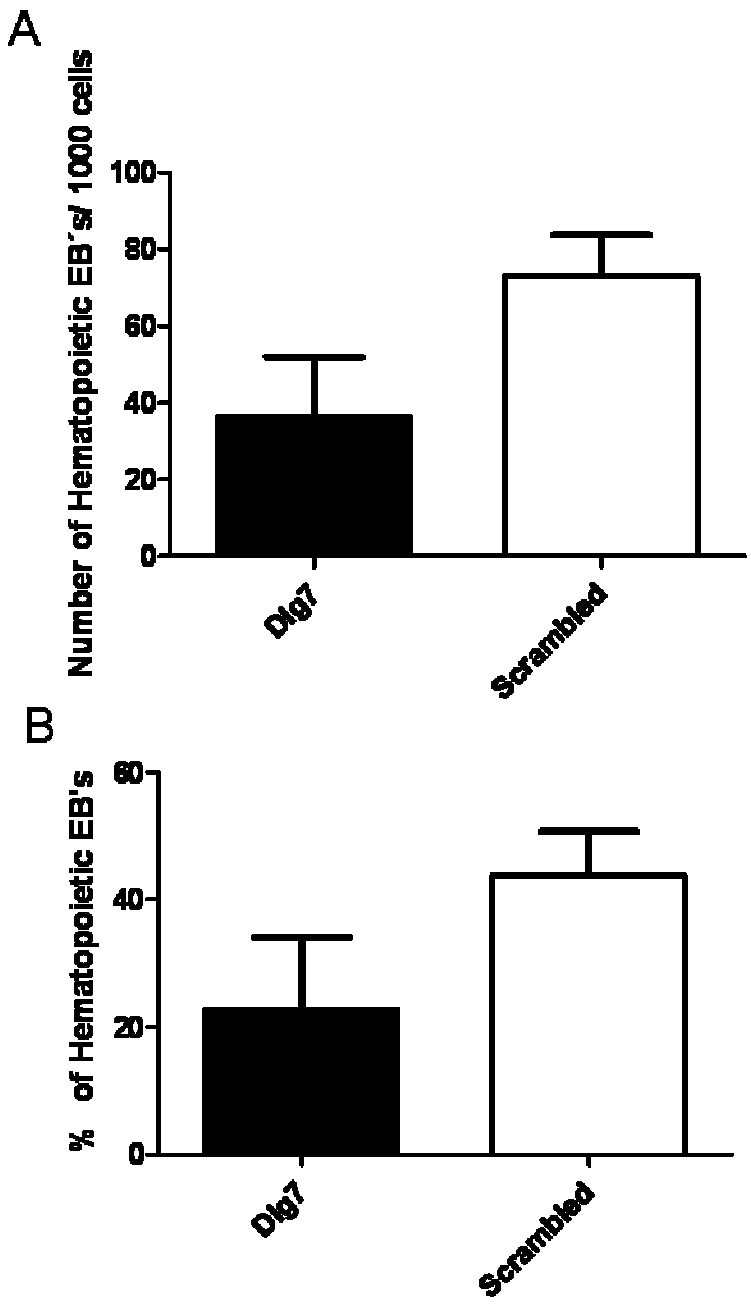


Figure 21. Effect of *Dlg7* overexpression on hematopoietic EB formation. (A) Total number of haematopoietic EB's formed /1000 cells plated. The black bar representing the mESC-Dlg7 population has an average of 36.3±15.7 haematopoietic EB's and the white bar representing the mESC-scrambled control population has an average of 73±10.7 haematopoietic EB's (n=3, *P*=0.029, Power=0.203). (B) The percentage of EB's showing hematopoietic morphology. The black bars represents the mESC-Dlg7 population with 22.65±11.41 % of EB's with hematopoietic morphology. The white bars represents the mES-scrambled population with 43.72±6,94 % of EB's with hematopoietic morphology. Data are presented as average ±SD.

Taken together, these data show that overexpression of *Dlg7* in mESC affects the differentiation of EB's both in regards to total numbers and by promoting the production of non-hematopoietic EB's.

3.4 - Haematopoietic colony forming units.

To determine if EB's differentiated from mESC overexpressing *Dlg7* had the capability to form colonies in a hematopoietic colony forming assay, 12 day old EB's cultured in a primary differentiation media were dissociated and seeded in methylcellulose containing hematopoietic cytokines. Lower number of CFU were produced from EB's derived from mESC overexpressing *Dlg7* compared to cells infected with a scrambled DNA, 32.5±4.32 CFU vs. 61.1±31.44 CFU respectively. The difference was not statistically significant (p=0.051, Power=0.23, n=6) (figure 22).

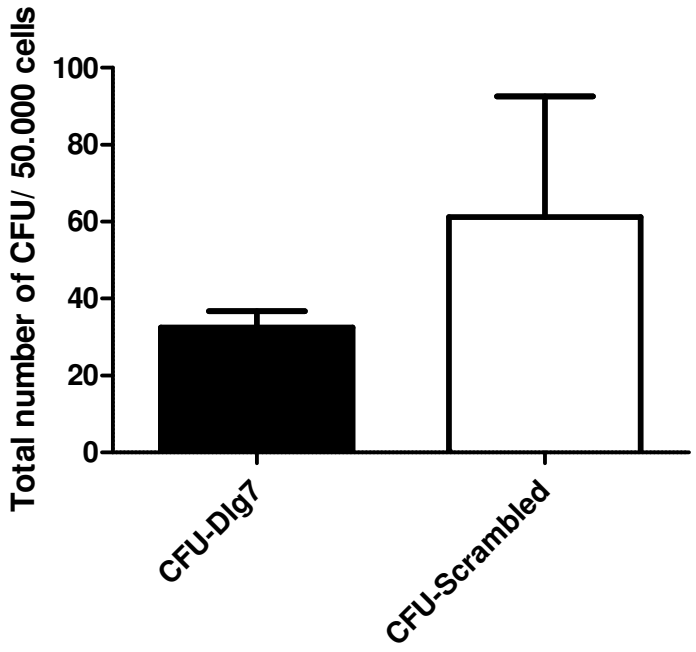


Figure 22. Effect of *Dlg7* overexpression in mESC on the formation of hematopoietic CFUs. The bars show total colonies / 50.000 EB derived cells seeded. The black bar represents mESC overexpressing *Dlg7* with an average CFU number of 32.5±4.32 , while the white bar represent mESC infected with scrambled control DNA population with an average CFU number of 61.1±31.44 (*p*= 0,051, power=0.23, n=6). Data are presented as average ±SD.

Next, CFUs were scored based on their hematopoietic differentiation capacity and categorized into BFU-E (includes CFU-E and BFU-E), CFU-GM or CFU-GEMM (mixed) colonies. No significant difference was found between BFU-E from mESC-Dlg7, averaging 25.6±6.89 BFU-E/ 50.000 EB cells seeded compared to 30.5±14.95 BFU-E from mESC-Scrambled (p=0.49; power=0.059) (figure 23A). Significant difference was found in number of CFU-GEMM from mESC-Dlg7, averaging 1.5 ±1.38 CFU-GEMM / 50.000 cells seeded compared to 6.1±2.48 CFU-GEMM mESC-Scrambled (figure 23A) (p=0.0024; power=0.608). Significant difference was also found in the number of CFU-GM from mESC-Dlg7, averaging 5.3 ±1.63 CFU-GM / 50.000 cells seeded compared to 24.5±14.31 CFU-GM from mESC-Scrambled (p=0.0086; power=0.44) (figure 23A). Interestingly, we found that mESC overexpressing *Dlg7* were skewed towards the BFU-E phenotype compared to mESC infected with the scrambled DNA, 77.78±10.96% vs. 50.56±2.26% respectively. Conversely, the percentage of CFU-GM and CFU-GEMM was lower, being 5.12±4.83% vs. 10.79±1.96% for CFU-GEMM and 38.64±3.64% vs. 17.01±7.10% for CFU-GM (figure 23B).

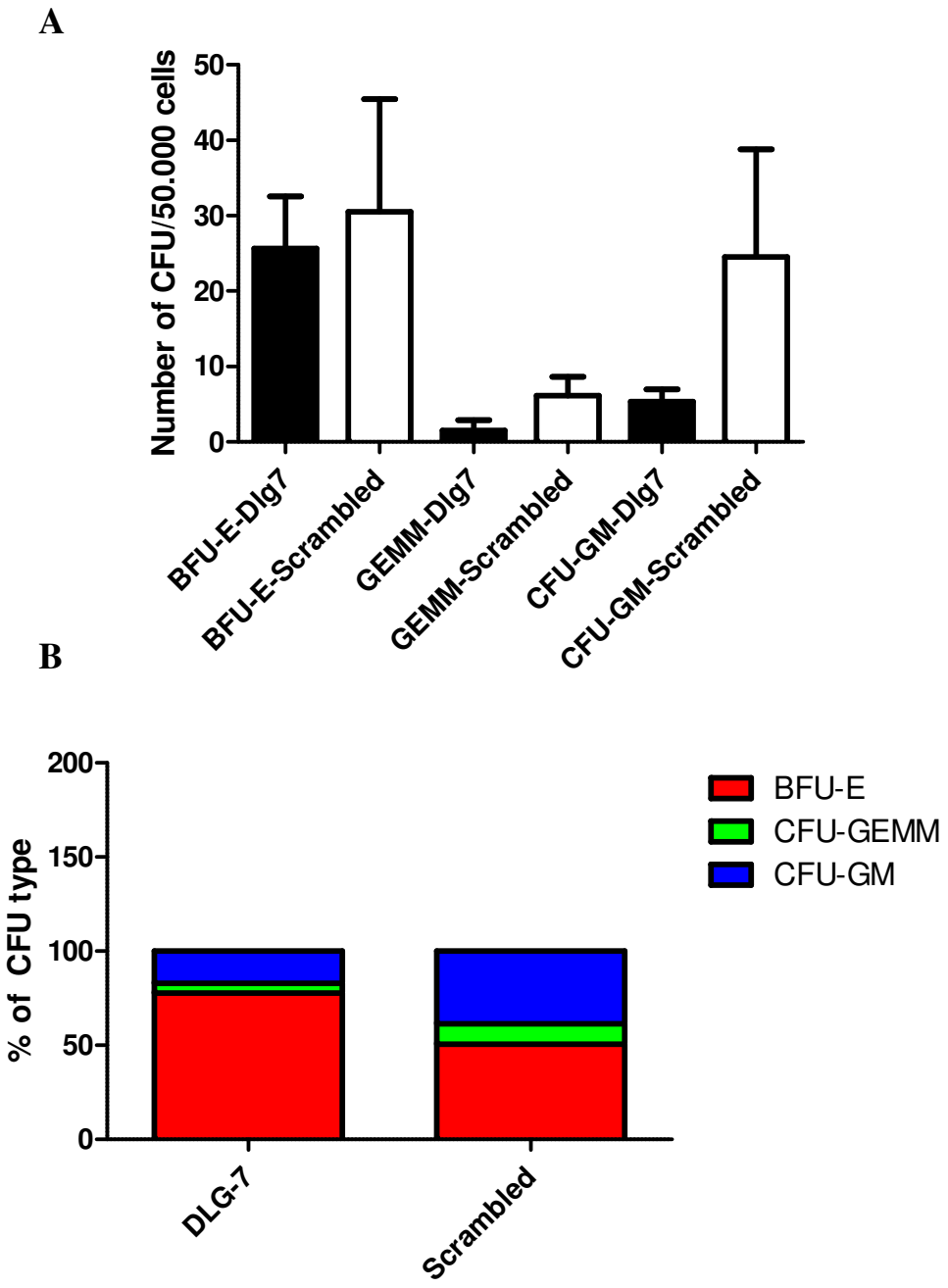


Figure 23. Effect of Dlg7 overexpression in mESC on the percentage of hematopoietic differentiation capacity. (A) Colony forming units were categorized based on their morphology in to three different groups BFU-E, CFU-GMM, CFU-GM. In average BFU-E colonies emerging from the mESC-Dlg7 population were 25. 6±6.89 compared to 30.5±14.95 mESC-scrambled (n=6 p=0.0024; power=0.608), CFU-GEMM mECS-Dlg7 1.5 ±1.38 compared to 6.1±2.48 in the scrambled control (n=6 *P*=0.0024, Power=0.608) and CFU-GM mESC-Dlg7 5.3 ±1.63 compared to mESC-scrambled 24.5±14.31 (n=6, p=0.0086; power=0.44) . (B) In the mESC-Dlg7 population 77.78±10.96 % of CFUs where BFU-E, 5.12±4.83 % CFU-GEMM and 17.01±7.10 % CFU-GM. In the mESC-Scramb population 50.56±2.26 % colonies where BFU-E, 10.79%±1.96% was CFU-GEMM and 17.01±7.10 % CFU-GM Data are presented as average ±SD

3.5 - Proliferation.

To determine if the overexpression of *Dlg7* had an effect on the proliferation of mESC, 2.0 x 10^5 cells were seeded in a 25 cm^2 flask and cultured for 48 hours. After the culture period, cells where counted and their density calculated. Overxpression, of *Dlg7* lowered significantly the proliferation rate of mESC, 252.000±31.749/cm^2 in the mESC-Dlg7 population and 450.667±75.719/cm^2 in cells infected with scrambled DNA (p=0.0138, Power=0.29) (figure 24).

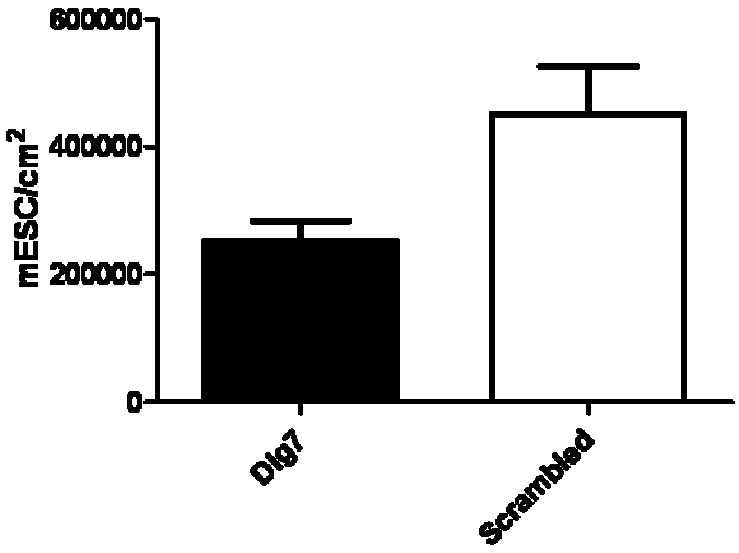


Figure 24. Effect of *Dlg7* overexpression on proliferation of mESC. 2,0 x 10^5 mESC were seeded in a 25 cm^2 flask and cultured for 48 hours. After the culture period, cells were counted and their density per cm^2 calculated. Overxpression of *Dlg7* lowered significantly the proliferation rate of mESC, with 252.000±31.749/ cm^2 in the mESC-Dlg7 population vs. 450.667±75.719/ cm^2 in cells infected with scrambled DNA (p=0.0138, Power=0.29, n=3). Data are presented as average ±SD.

These data clearly show that overexpression of *Dlg7* in mESC cells decreases the proliferation rate of mESC.

4 - Silencing of Dlg7 in mouse embryonic stem cells.

To further analyse the functional role of *Dlg7* in hematopoietic differentiation we knocked the gene down in mESC using lentivrial particles containing short hairpin RNA (shRNA) for the *Dlg7* gene. As a control we used a shRNA for a scrambled DNA corresponding to no known mouse gene. The mESC infected with these constructs were differentiated into EB's and hematopoietic cells as before.

4.1 – Puromycin resistant cells.

Cells that integrate the shRNA construct for *Dlg7* or the scrambled DNA become resistant to puromycin (figure 25). We found that puromycin concentration of 2.0 μg/ml, killed 100% of non-infected mESC (Hexamid control) within 48 hours (Figure 25 I, L) while mESC infected with the *Dlg7* or scrambled shRNA constructs survived the treatment (Figure 25 G, H).After 72 hours large puromycin resistant cell colonies where growing in the *Dlg7* and scrambled shRNA cultures (figure 25 J, K). Those colonies were picked and expanded.

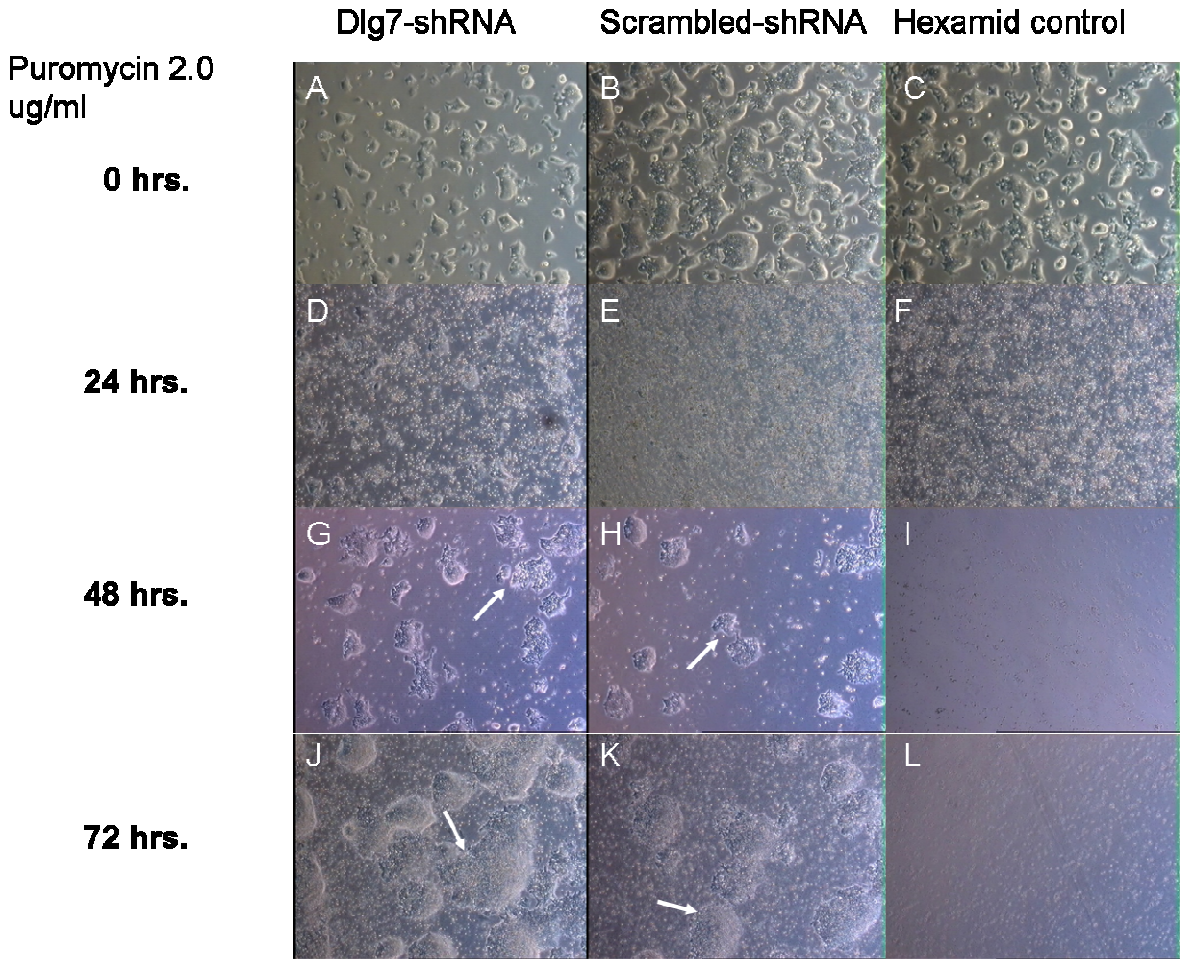


Figure 25. mESC-Dlg7-shRNA and mESC-Scrambled-shRNA puromycin resistant cells. mES were infected with Mission-shRNA-Dlg7 (Dlg7-shRNA) A,D,G and J or Mission-shRNA-Scrambled (Scrambled-shRNA) lentiviral particles B,E,H and K. Non-infected cells where used as a control (Hexamid control) C,F,I and L. Cells were exposed to 2.0 ug/ml puromycin. Total cell death was achieved in the control population with in 48 hours. After 72 hours large resistant colonies were apparent in the Dlg7-shRNA and Scrambled-shRNA culture. Arrows point to puromycin resistant colonies.

4.2 – Silencing of *Dlg7* in mouse embryonic stem cells

Quantitative RT-PCR (Q- PCR) was preformed on selected resistant mESC that where transduced with Mission-shRNA-Dlg7 (Dlg7-shRNA) and Mission-shRNA-Scrambled (Scrambled-shRNA) viral particles in order to compare the changes in expression of *Dlg7* between the two populations. One out five Dlg7-shRNA hairpins achieved more than 80% silencing in all three infection experiments (figure 26A). Only clones with more than 70% silencing of *Dlg7* expression were used for further experiments (figure 26B).

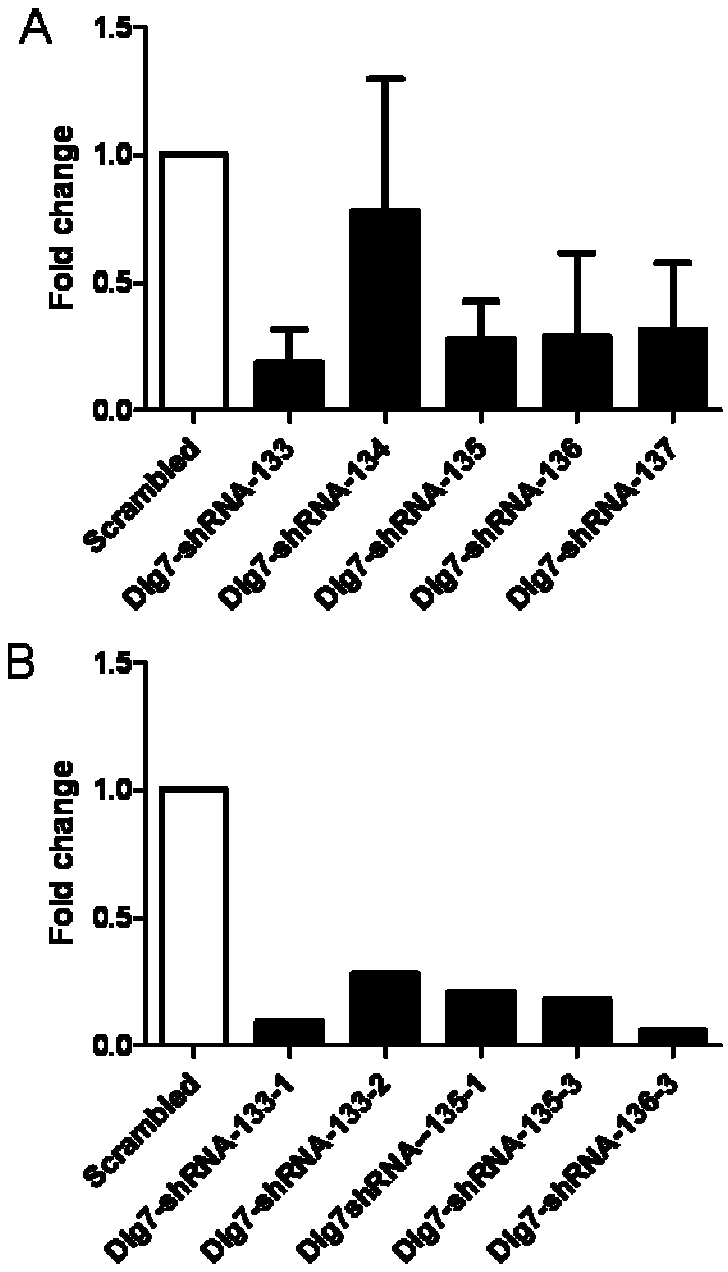


Figure 26. Silencing of *Dlg7*. (A) Q-PCR of the expression of *Dlg7* using different Dlg7-shRNA and a Scrambled-shRNA. The white bar indicates the expression in the Scrambled-shRNA population and is set at 1. The black columns represents the silenced Dlg7-shRNA clones (n=3). (B) shRNA-Dlg7 clones that expressed less then 0.3 fold *Dlg7* compared to the shRNA-Scrambled clones were used for further experiments. Data are presented as average ±SD. Calculations were made using the ΔΔCT method.

These data demonstrate the effective silencing of the *Dlg7* gene in mESC and the establishment of five clones with less then 0.3 fold expression of *Dlg7* compared to the shRNA scrambled population that where used in further experiments .

To see if the *Dlg7* silencing continued in to the differentiation process, *Dlg7* expression was analysed by Q-PCR in 6 day old EB's. EB's from the Dlg7-Scrambled population had 1.83 fold more *Dlg7* expression then EB's from Dlg7-shRNA (figure 27).

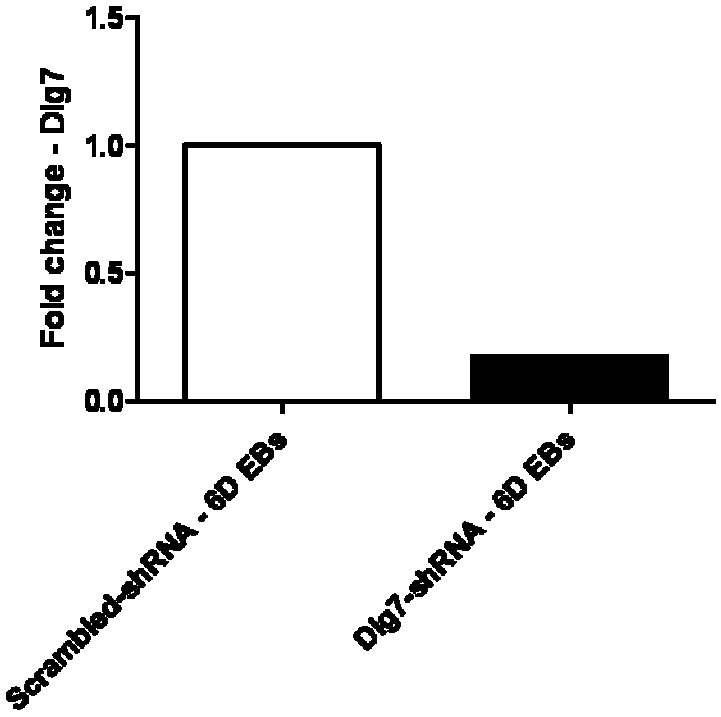


Figure 27. Silencing of Dlg7 during EB differentiation. Q-PCR of the expression of *Dlg7* using Dlg7-shRNA and a Scrambled-shRNA. The white bar indicates the expression in the Scrambled-shRNA population and is set at 1. The black columns represents the silenced Dlg7-shRNA clones (n=1). Calculations were made using the ΔΔCT method.

The expression of two genes where looked at to assess the state of the Dlg7-shRNA and Scrambled-shRNA in the differentiation process, *Nanog* and *Oct4* known stem cell markers [82]. Day 6 EB's where used in this experiment. In shRNA-Dlg7 a stronger expression of *Nanog* and *Oct4* was detected (figure 28).

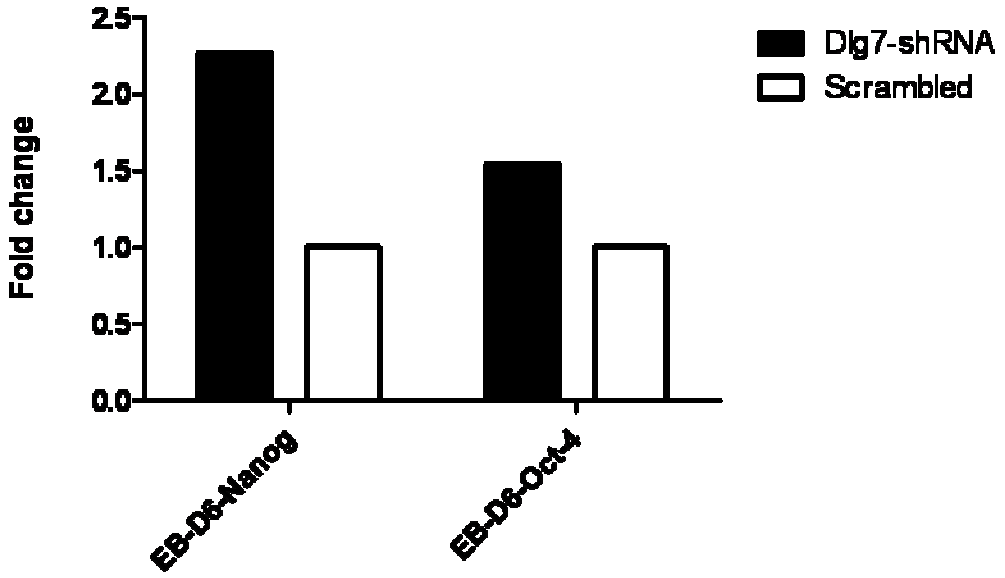


Figure 28. Nanog and Oct4 expression in 6 day EB. A Q-PCR was preformed on c-DNA generated from whole RNA extracts from 6 day old EB's. The expression of *Nanog* and *Oct4* was compared between the two populations shRNA. There was a 2.2 fold more expression of *Nanog* in 6 day old EB's from the Dlg7-shRNA population compared to the Scrambled-shRNA population. A 1.5 fold increase in *Dlg7* expression was detected in 6 day EB's from the Dlg7-shRNA compared to the Scrambled-shRNA.

4.3- Embryoid body formation.

To analyze the functional effect of silencing of the *Dlg7* gene on EB differentiation, mESC cells infected with a Dlg7-shRNA or a Scrambled-shRNA were differentiated to EB's. Embryoid bodies from both populations were counted using a reverse microscope and presented as number of EB's / 2500 mES cells plated. *Dlg7* silencing lead to a significant decrease (120.3±8.33 EB's/ 2500 cells) in the number of EB's compared to cells containing the scrambled transgene (232.3±27.15 EB's/ 2500 cells) ($p=0.0024$, power=0.637, (figure 29).

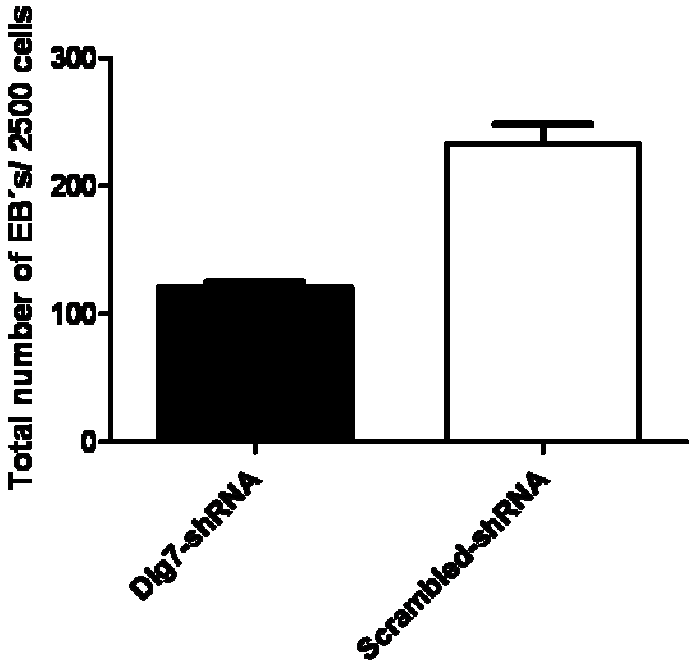


Figure 29. Effect of Dlg7 silencing on EB numbers. The bar chart shows the number of EB's formed per 2500 cells plated. Black bar represents the mESC-Dlg7-shRNA population where 120.3±8.33 EB's were formed per 2500 cells plated. The white bar represents the mESC-Scrambled-shRNA population with 232.3±27.15 EB's per 2500 cells. This difference was statistically significant, p=0.0024, Power=0.637, n=3. Data are presented as average ±SD.

EB's were kept in differentiation for 12-14 days to establish their haematopoietic potential and scored based on their morphology. The percentage of EB's considered haematopoietic were compared between the two populations (figure 30A). Embryoid bodies derived from Dlg7-shRNA cells showed significantly lower number of hematopoietic EB's, 72.75±15.76 compared to 170.7±48.01 in the cells transduced with the scrambled DNA (p=0.0112, Power=0.203). The same was true for the percentage of hematopoietic EB's, where EB's derived from Scrambled-shRNA cells showed a higher percentage (72.66±12.22%) compared to EB's from Dlg7-shRNA cells (55.33±2.81%).

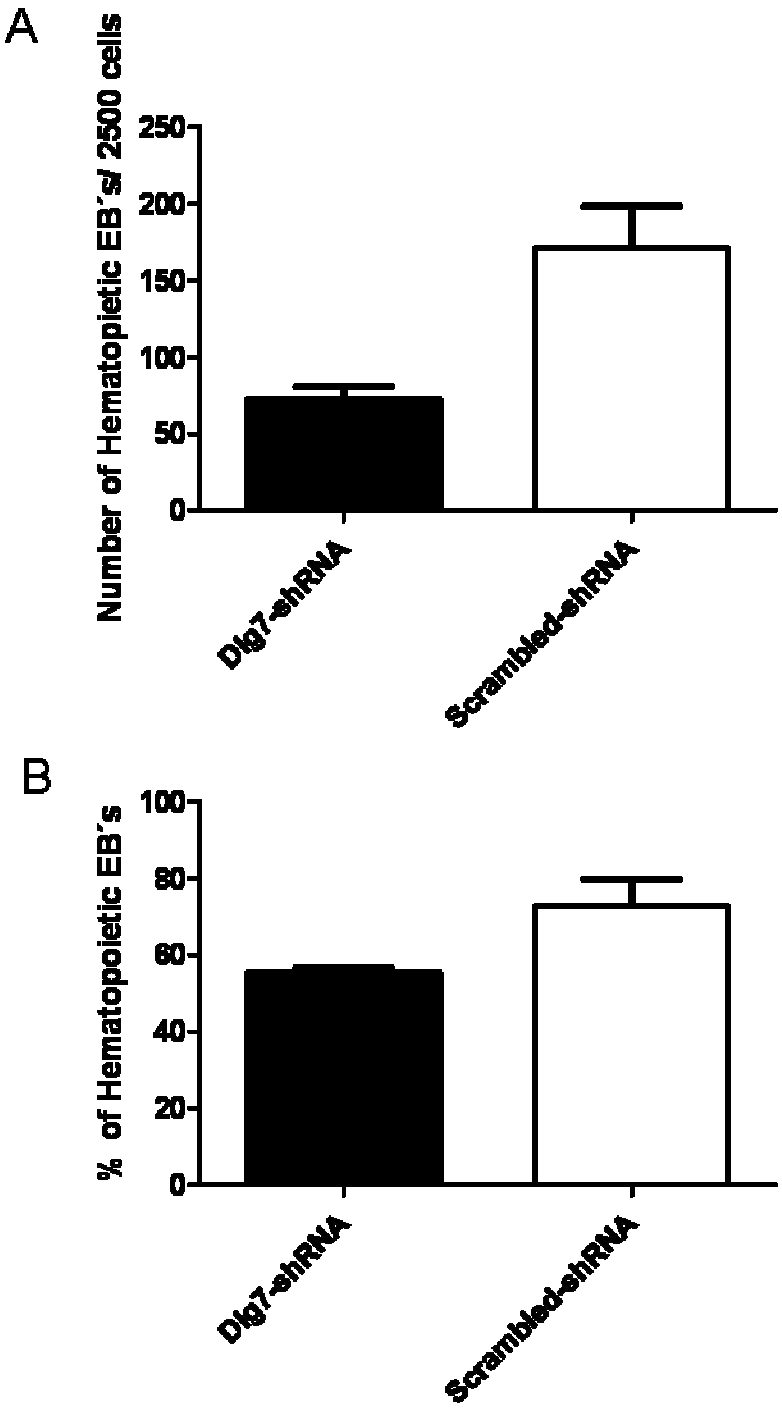


Figure 30. Effect of Dlg7 silencing on hematopoietic EB formation. (A) Total number of hematopoietic EB's formed /2500 cells plated. The black bar representing the Dlg7-shRNA population has an average of 72.75±15.76 hematopoietic EB's and the white bar representing the Scrambled-shRNA control population has an average of 170.7±48.01 hematopoietic EB's (n=3, *P*=0.0112, Power=0.20). (B) Percentage of EB's formed showing hematopoietic morphology. The black bar shows the Dlg7-shRNA population with 55.33±2.81% of EB's with hematopoietic morphology. The white bar shows the Scrambled-shRNA population with 72.66±12.22 % of EB's with hematopoietic morphology. Data are presented as average ±SD (n=3).

Taken together these data show that silencing of *Dlg7* results in fewer numbers of EB's and both fewer numbers and lower proportion of hematopoietic EB's.

4.3 - Haematopoietic colony forming units

For the final analysis of the effect of *Dlg7* silencing on hematopoietic differentiation 12 day old EB's where dissociated into a single cell population and seeded in a semisolid Hematopoietic differentiation media containing hematopoietic growth factors for the differentiation into hematopoietic colony forming units.

First, the total number of CFUs with no regard to the cell type was compared between the two populations. No difference was found between CFUs derived from Dlg7-shRNA clones compared to cells derived from Scrambled-shRNA, 23.75±8.75 CFU and 28.25±3.75 CFU respectively (figure 31).

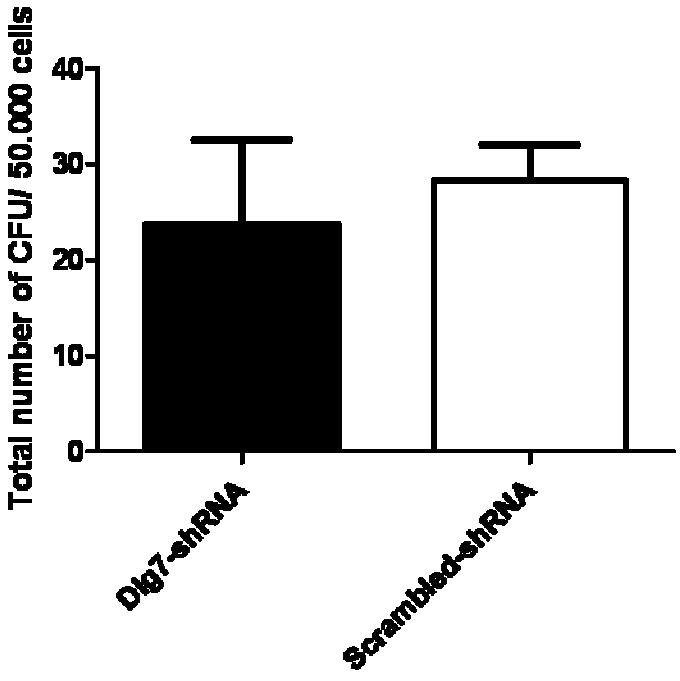


Figure 31. Effect of *Dlg7* silencing on total number of hematopoietic CFUs. The bars show total CFUs / 50.000 EB derived cells seeded. The black bar shows the Dlg7-shRNA population with an average number of CFUs of 23.75±8.75 , while the white bar shows the Scrambled-shRNA population with an average number of CFUs of 28.2±3.75. Data are presented as average ±SEM (n=2).

Next, CFUs were scored based on their hematopoietic differentiation capacity and categorized into BFU-E, CFU-GM or CFU-GEMM (mixed). Interestingly, lower number of BFU-E

colonies were produced from Dlg7-shRNA cells, averaging 13.75±0.25 BFU-E/ 50.000 cells seeded compared to 20.75±3.25 BFU-E from Scrambled-shRNA cells (figure 32A). In addition, the number of CFU-GEMM colonies produced from Dlg7-shRNA cells was higher, averaging 7.00 ±6.00 CFU-GEMM / 50.000 cells seeded compared to 1.50±0.50 CFU-GEMM from Scrambled-shRNA cells (figure 32A) whereas, little difference was found in number of CFU-GM colonies generated from Dlg7-shRNA cells, averaging 3.00±3.00 CFU-GM / 50.000 cells seeded compared to 5.50±0.71 CFU-GEMM from Scrambled-shRNA cells (figure 32A).

When looking at the percentage of different types of CFUs we found that a lower proportion of BFU-E colonies were produced from the Dlg7-shRNA cells compared to Scrambled-shRNA control cells, 67.44±25.90 vs. 73.21±1.78 respectively, and higher proportion of CFU-GEMM colonies compared to the control cells 23.33±16.67 vs. 7.20±0.96 (figure 32B).

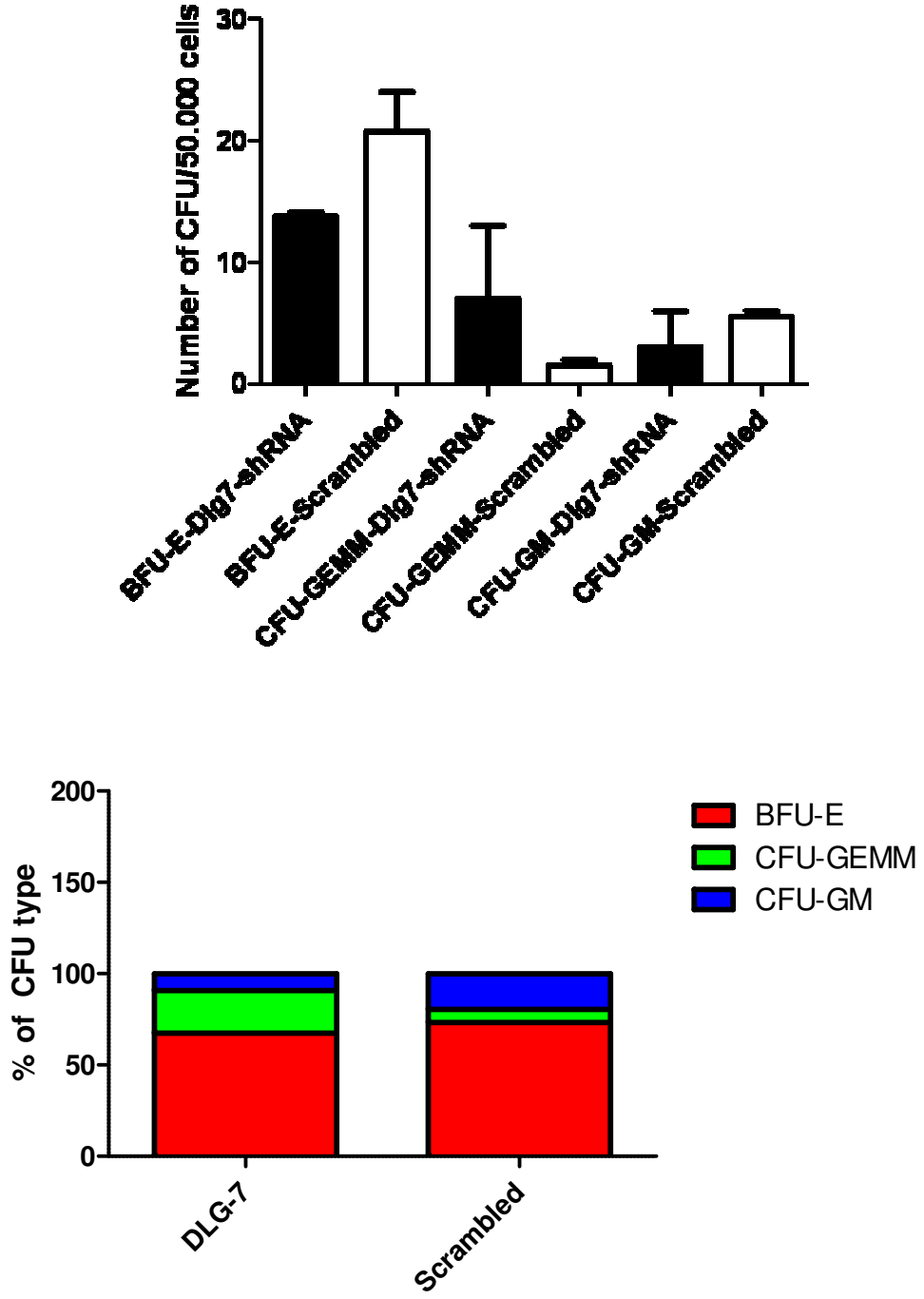


Figure 32. Effect of Dlg7 silencing on hematopoietic differentiation. (A) Colony forming units were categorized based on their morphology into three different groups: BFU-E, CFU-GEMM, CFU-GM. The total number of BFU-E colonies emerging from the Dlg7-shRNA population/ 50000 EB cells were 13.75±0.25 compared to 20.75±3.25 from Scrambled-shRNA control, 7.00 ±6.00 CFU-GEMM compared to 1.50±0.50 from the Scrambled-shRNA control and 3.00±3.00 CFU-GM compared to 5.50±0.71 from the Scrambled-shRNA control. (B) The proportion of BFU-E colonies was 67.44±25.90 % in the Dlg7-shRNA population compared to 73.21±1.78 in the Scrambled-shRNA control, the proportion of CFU-GM was 9.23±9.23 % compared to 19.58±0.83 % in the Scrambled-shRNA control and the proportion of CFU-GEMM was 23.33±16.67 % compared to 7.20±0.9% in the Scrambled-shRNA control. Data are presented as average ±SEM (n=2).

Taken together, the data indicate that silencing *Dlg7* in mESC results in lower numbers of cells with the capability of differentiating into the erythroid lineage but increase in the number of cells that have the capability to differentiate into multiple hematopoietic lineages.

4.4 - Proliferation.

To determine if silencing of *Dlg7* had an effect on the proliferation of mESC, 2.0 x 10^5 cells where seeded in a 25 cm^2 flask, cultured for 48 hours and counted. Our data show that silencing of *Dlg7* did not have a significant effect on the proliferation rate of mESC (379.333±84.198 cells/ cm^2 from the Dlg7-shRNA population vs. 338.000±121.705 cells/ cm^2 in from the Scrambled-shRNA control (figure 33)(p=0.65, Power=0.036).

.

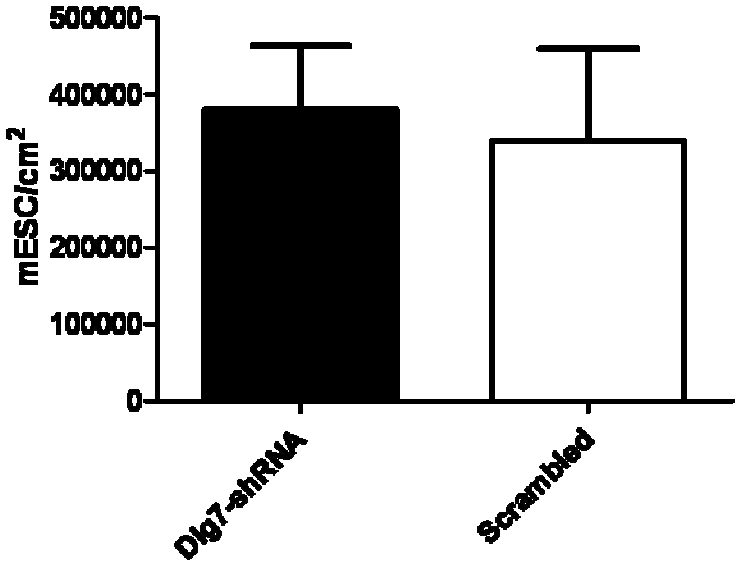


Figure 33. Effect of *Dlg7* silencing on proliferation of mESC. 2,0 x 10^5 mESC where seeded in a 25 cm^2 flask and cultured for 48 hours. After the culture period cells where counted and their density per cm^2 calculated. Silencing of *Dlg7* did not have a significant effect on the proliferation rate of mESC (379.333±84.198/ cm^2 from the Dlg7-shRNA population vs. 338.000±121.705/ cm^2 in the Scrambled-shRNA control (p=0.65, Power=0.036, n=3). Data are presented as average ±SD.

No clear difference was found in the proliferation rate of the mESC transduces with *Dlg7* shRNA and scrambled shRNA.

V - Discussion.

1- Summary

This project is a part of a bigger project aiming to deduce the functional role of *Dlg7* with a specific focus on hematopoiesis. This has included an attempt to create a mouse knock out model for the gene that has so far not given a *Dlg7* KO phenotype. The original goal of this project was to analyze the effect of overexpression of the *Dlg7* gene in mESC on hematopoietic differentiation from these cells. Our initial plans included the design and construction of lentiviral based vector carrying the *Dlg7* gene to overexpress the gene in mESC. In the final step of sequencing the expression plasmid the results showed that only parts of the gene had been cloned. We therefore turned to commercially available overexpression lentiviral based vector to achieve the set aims and added silencing experiments to the project.

The main results from this project show that overexpression of the *Dlg7* gene remains throughout the differentiation process, although it decreases during differentiation. Overexpression leads to a decrease in proliferation of the mESC as well as a decrease in the number of EB's. There was a clear effect on hematopoiesis where both the number and the proportion of hematopoietic EB's where significantly fewer from mESC cells where the *Dlg7* gene had been overexpressed. When looking at the hematopoietic CFU it was clear that over expression also lead to a reduction in the number of CFUs and to an increase in the proportion of BFU-E (not significant) and reduction in the number of CFU-GM (significant). The data from the silencing experiments were not quite as conclusive. There was a significant reduction in the number of EB's differentiated from mESC with silencing of the *Dlg7* gene but we did not see this in the proliferation of these cells. There was a significant effect in the reduction of hematopoietic EB's. On the other hand there was no clear difference in the total number of CFU formed.

2 – Dlg7 as a stem cell gene

An indication for a role of the *Dlg7* gene in stem cell growth and differentiation is the high expression of the gene in mESC and downregulation during EB differentiation [82]. *Dlg7* is also expressed in other stem cell populations including msenchymal stem cells, CD133+ neural

stem cells and CD34+CD38- hematopoietic stem cells (Gudmundsson KO and Sigurjonsson OE unpublished results). Transient overexpression in mESC showed impaired EB formation as well as elevated or downregulaton of expression of genes (*Oct4, Rex-1, Bmp4, Nanog and Brachyury*) associated with the pluripotency of ESC [82]. These data led us to further analyze whether *Dlg7* may play a role in stemness of mESC and their hematopoietic differentiation.

We were able to get a overexpression and silencing of the *Dlg7* gene that effected both the proliferation and the differentiation potential of the mESC as well as the expression of a known stem cell gene (*Nanog*) and differentiation gene (*Brachyury*). Previous studies by Tsou et al. had indicated that overexpression of *Dlg7* in 293T cells resulted in an enhanced cell growth at low serum levels, suggesting a role for the gene in promoting survival [83]. Our data did not show this but rather that overexpression leads to lower proliferation rate of mESC. The difference may lie in different cell types used and different ways of getting overexpression. Another explanation for this may be that due to random integration of the lentiviral vector into genes that may effect the proliferation of the mESC. Genes that are downregulated during differentiation may give important clues on how the stem cell phenotype is maintained and differentiation is prevented. Our data do not demonstrate completely that *Dlg7* is a stem cell gene, but are more an indication that the gene may be important in highly proliferative cells and tissue such as colon, bone marrow, testis and thymus.

3 – Dlg7 and hematopoiesis

Previous results (Gudmundsson KO et.al. 2007) showed that the *Dlg7* is more highly expressed in the more primitive CD34+CD38- stem cell population compared to the CD34+CD38+ progenitor population and not expressed in myeloid or erythroid CFUs. In addition *Dlg7* is not found in blood cells except to some extent in CD14+ cells [82]. The fact that we see an decrease in hematopoietic EB's differentiated from mESC that had *Dlg7* overexpressed or silenced is of interest. There are no previous results showing that *Dlg7* may influence hematopoiesis. We have no clear evidence on how *Dlg7* is influencing hematopoiesis. To further investigate if *Dlg7* overexpression or silencing has an direct effect on hematopoiesis the expression of transcription factors important in commitment of HSC like GATA1 and PU.1 could be looked at [105]. In this project a stable, although diminished overexpression of *Dlg7* at day 12 EB's was achieved. In future experiments the assessment of the expression level of *Dlg7* in the CFU formed from cells from these EB´s would be of

interest, and scanning of individual CFU for known linage specific markers in flow cytometry. The attempt to transduce more committed cells that have gone further in the differentiation process then mESC e.g. HSC [106, 107] for the purpose of overexpressing *Dlg7* could give more inside in to whether *Dlg7* has an effect on hematopoiesis.

It is quite possible that the *Dlg7* overexpression and silencing is not having a direct effect on hematopoiesis and the difference we see is due to defects in the cell cycle where the gene is thought to play substantive role.

4 – Dlg7 and the cell cycle

It has been shown that abundance of Hurp in HeLa cells can cause hyper stabilization of spindle fibres where the spindle becomes much more stretched then normal [94]. It has also been reported on that Hurp depletion in HeLa cells causes spindle fibres not to attach to kinetochores and the spindle looks unorganized [108]. Both the depletion and the abundance of Hurp with in HeLa cells causes unaligned chromosomes, reduction of tension across sister kinetochores and an insensitive spindle checkpoint. All these defects can cause genomic instability and aneuploidy that can cause tumour formation [94, 108].

It is possible that in our experiment using mESC to differentiate in to EB's and then CFU these defects also accumulate in the cells where there is overexpression or silencing of the *Dlg7* gene. mESC that have chromosome missagregation and genomic instability could be less pluripotent and thus not behave as expected in differentiation protocols [19]. Elevated expression of *Nanog* in the *Dlg7* transgene clones could be a indication of inhabitation of differentiation and that these cells are trying to hold on to there stem cell origin. It should be noted that the expression of *Nanog* has been linked to various cancers. [109].

5 - Future experiments and limitations of the thesis

In this project two population of mESC transduced with viral-particles containing scrambled DNA where used as a control. One for the mESC-Dlg7 *Dlg7* overexpression populations and one for the Dlg7-shRNA *Dlg7* silenced population. The benefits of using the scrambled populations as controls is the exultation of the transduction method having an effect on a difference seen between the control and the mESC-Dlg7 or Dlg7-shRNA populations. Using lentiviral based viral-particles that integrate multiple copies of their DNA in to the host genome, it is possible that foreign DNA is integrated at host genomic sides that have an effect on the outcome of our experiments.

An RT-PCR was used to show the expression of *Nanog* and *Oct4* in undifferentiated mESC. Q-PCR as well as histological or flow cytometry analyses could give further evidence of plurioptentcy of the cells but where not done in this project. We have now acquired a human and mouse pluripotent stem cell kit for analysis in flow cytometry that will be used in further experiments. In figures 17 J and K some of the colonies have dark spots in the middle witch could indicate differentiation. This is due to the extended time the colonies where in the culture to achieve enough cells for expansion. It is there fore possible that the cell population was heterogenic. In future experiments we will work on the transduction efficiency with higher MOI and altered culture conditions. Only one time point is displayed for the expression of *Dlg7* EB´s formed from mESC with *Dlg7* silencing, this is due to lack of material and unsuccessful extraction RNA from EB from other time points. A Q-PCR was preformed to calculate the fold difference between the *Dlg7* overexpressed and silenced clones and the scrambled controls. We have no evidence of the actual physical effect of the overexpression and silencing on protein production. One of our future experiments is westen-blott analysis of our clones to address this matter. Other future experiments that could be of interest is differentiation of our clones in to different cells then hematopoietic cells such as cardiomyocytes or even cells originating from a different germ layer then mesoderm such as neural precursors or early endoderm cells. It is possible that *Dlg7* overexpression and silencing would also impair the differentiation in to these cells types witch would give us an indication of general inhabitation of differentiation due to gene manipulation of *Dlg7*. Another experiment would let mESC differentiate randomly with out trying to direct them to any specific cell type and see if cells with overexpression ore silencing of *Dlg7* are more prone to form one cell type then the other. One of the most exiting and important issues of revelling how *Dlg7* works, is it behaviour in some tumours where elevated expression of the gene has been detected. It is important to do further experiments in to this field to expand our understanding of caner and tumour genesis.

VI - Conclusion

We have been successful in the differentiation of mESC in to CFU of the hematopoietic system. Overexpression and silencing of the *Dlg7* gene was achieved, at least on the mRNA level, in mESC using viral-particles for gene delivery in to the host genome.

Dlg7 overexpression and silencing significantly reduces the number of EB's that form in Primary differentiation media compared to scrambled control cell population. Of the EB's that formed a significant larger portion of EB from the scrambled control acquired hematopoietic morphology at later stage of differentiation compared to mESC with *Dlg7* overexpression or silencing. BFU-E where in grater numbers with in the CFU population formed from cells with *Dlg7* overexpression then in the scrambled control cells.

We can however not say with full certainty that the effect of *Dlg7* in hematopoiesis is a direct effect or rather a general effect in differentiation due to the role that *Dlg7* plays in the cell cycle.

VII – Appendix

In this part of the thesis we show data from experiments that were not vital to the final conclusion of the thesis but were important in the training of the M.Sc. student.

1 - Designing and construction of a *Dlg7* lentiviral vector.

In order to overexpress *Dlg7* in mESC we made an attempt to design and construct a lentiviral vector containing the *Dlg7* cDNA. This would give us a long term and stable overexpression of the gene during expansion and differentiation of the cells. The data that is presented in this chapter are the design parameters and our attempt of constructing this vector using the pLenti6/V5 Directional TOPO cloning system. The first part was to design the primers for the amplification of the mouse *Dlg7* coding sequence, see table 1 in materials and methods.

For the amplification of the *Dlg7* cDNA, RNA extracted from mESC was used. Three different sets of primers were used to amplify a 2427 bp segment of the *Dlg7* gene (*Dlg7*-Out, *Dlg7*-nested-1 *Dlg7*-nested-2) (Figure 34). *Dlg7*-Out amplifies the whole cDNA plus a few base pairs (bp) upstream and downstream. That product is subsequently used in a reaction using the *Dlg7* nested primers which amplify a blunt end product of the whole cDNA. The nested primers produce a gene product with a CACC overhang that insures the correct orientation of the gene product when used in cloning procedure.

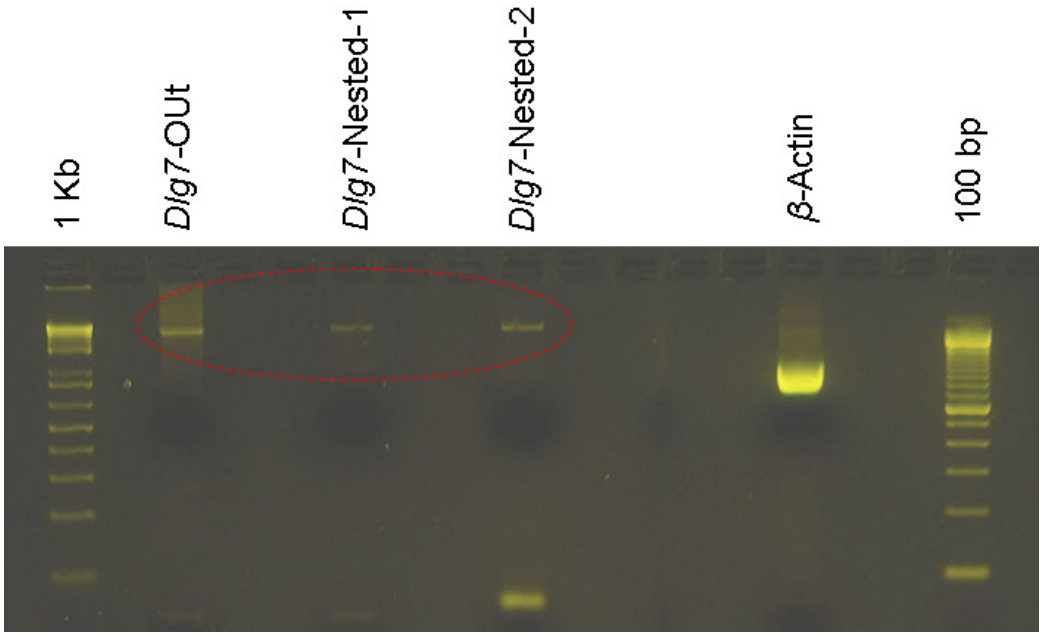


Figure 34. Taq-Polymerase PCR. Three different primer pairs were used: *Dlg7*-Out lane 1, *Dlg7*-Nested-1 lane 2, *Dlg7*-Nested-2 lane 3 to amplify the 2427 bp *Dlg7* cDNA. A red dashed line is shown around the correct sized bands. Size of DNA fragments was estimated by using 1 Kb and 100 bp ladders. β-actin-1 and 2 where used for assessment of PCR.

In order to achieve a blunt end PCR product for use in the cloning procedure a ProofStart PCR was performed on the Taq PCR product. The Proof start polymerase is a proof reading polymerase with 10 times more accuracy than the Taq polymerase [110]. A low error rate in the DNA is essential for use in cloning experiments. Figure 35 shows PCR products in an agarose gel achieved with a Proof start PCR. Amplified segments have the same molecular mass as the segments from the Taq polymerase PCR.

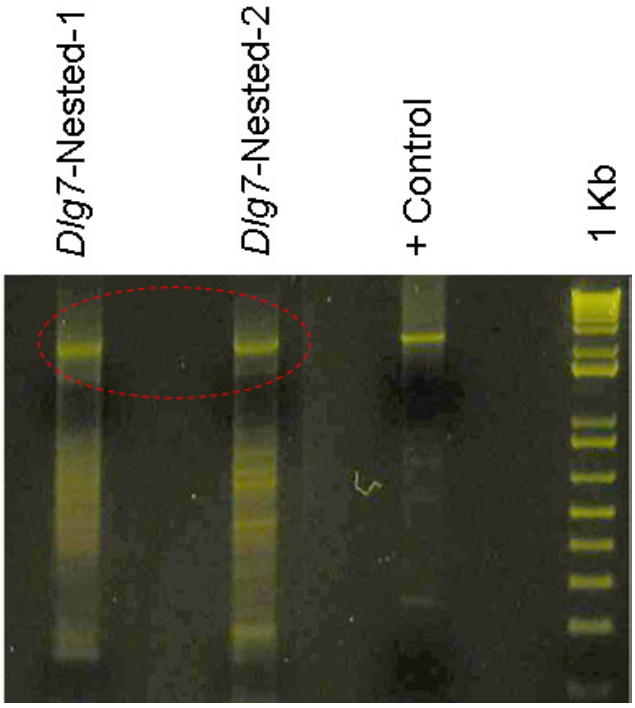


Figure 35. Proof start Polymerase PCR. Two primer pairs were used, *Dlg7*-Nested-1 lane 1, *Dlg7*-Nested-2 lane 2 to amplify the 2427 bp Dlg7 cDNA.. A red dashed line is formed around the correct size bands. Size of DNA fragments was estimated using a 1 Kb ladder. A Taq polymerase PCR product was used as a positive control.

A PCR band representing a DNA segment of the correct size (for the *Dlg7* gene) was excised from the agarose gel and purified using a QIAEX II Agarose Gel Extraction kit. The purified DNA segment was used in the cloning procedure. The DNA segment was cloned into a TOPO expression plasmid (pLenti6/V5-D- TOPO) and the plasmid transformed into stbl3 E.coli cells for expansion. Transformed bacterial cells were plated out and cultured on agar containing 100 μg/ml ampicillin in order to detect ampicillin resistant clones (Figure 36).

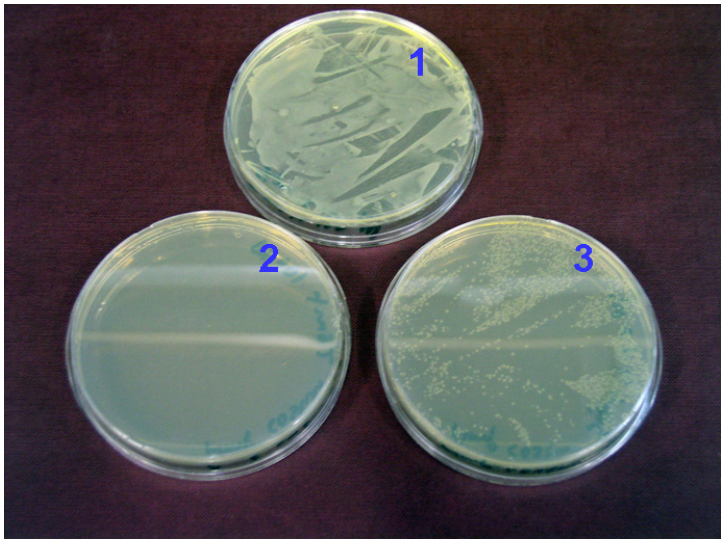


Figure 36. Detection of ampicillin resistant clones. Stbl3 E. Coli cells were transformed with pLenti6/V5-D-TOPO vector containing the *Dlg7* cDNA and cultured on basic agar containing 100 µg/ml ampicillin. Plate 1. Transformed cells cultured on an agar plate without ampicillin. Plate 2. Un-transformed cells cultured on an ampicillin plate. Plate 3. E. Coli cells transformed with the pLenti6/V5-D- TOPO vector containing the *Dlg7* cDNA cultured on an ampicillin plate.

Positive colonies where picked and grown in a large volume (100 ml) of medium containing100 µg/ml ampicillin. The plasmids were isolated from the E.coli cells and RT-PCR with *Dlg7*-detect primers was performed to establish the presence of the *Dlg7* cDNA. The *Dlg7*-detect primers amplify a 296 pb segment of the *Dlg7* gene. A host of vector clones were scanned with the *Dlg7*-detect primers (Figure 37). The clones demonstrating the strongest amplification were selected for sequencing to detect the correct orientation of the *Dlg7* gene within the plasmid.

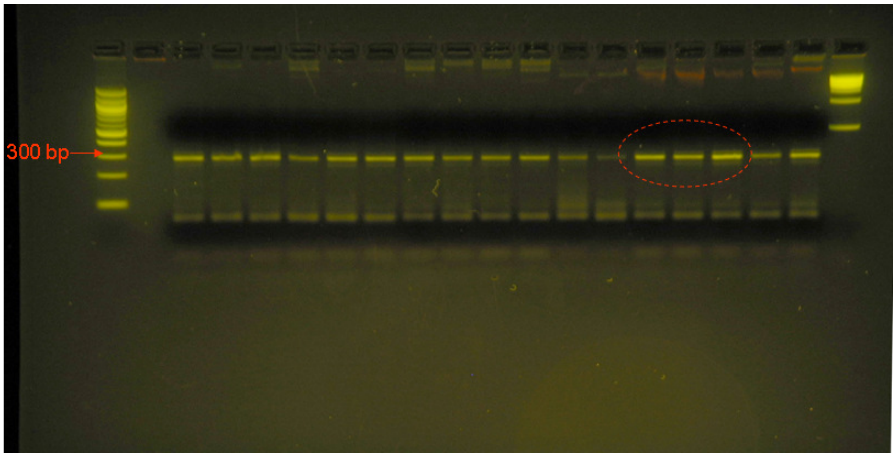


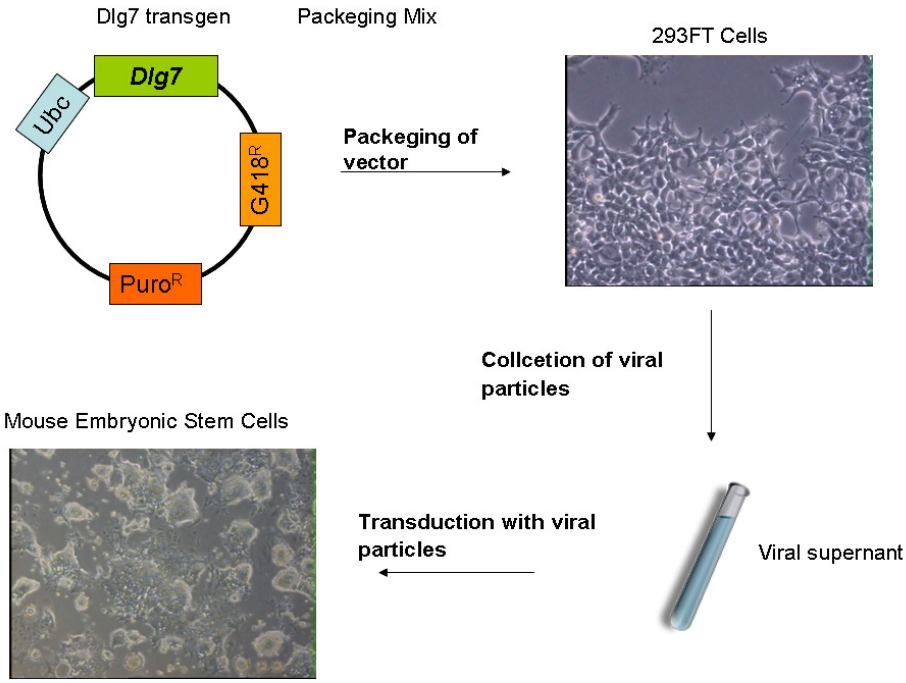
Figure 37. Scanning of library of clones with *Dlg7*-detect primers. A library of 18 clones was scanned with *Dlg7*-detect primers for establishing the presences of the *Dlg7* cDNA within the TOPO vector. Circled with a red dashed line are three bands representing three clones sent for sequencing.

Results from sequencing where inconclusive. In all three clones large parts of the *Dlg7* cDNA was missing and even parts of the TOPO vector appeared to missing in some instance.

2- Lentiviral preparation and infection.

A ready to use vector (pLenti-III-Ubc) containing the *Dlg7* gene was packaged with a packaging mix containing necessary viral genes for the production of viral particles. 293FT packaging cell line was used for this purpose. Viral particles where collected and used in the transduction of mESC figure 38A. Transduced cells where exposed to puromycin for 96 hours in figure 38B left and middle pictures show mESC colonies resistant to the puromycin, the picture on the right shows negative control mESC that received no vector and showed no resistant to the puromycin figure 38B.

A.



B.

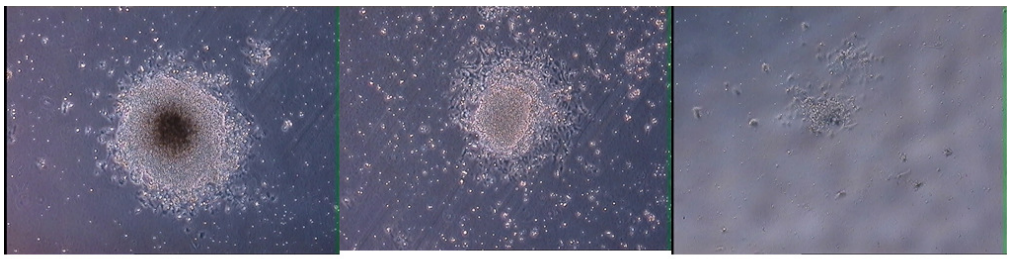


Figure 38. Production of viral particles and puromycin resistance. (A) viral vector was packaged in 293FT cells, viral particles where collected and used in the transduction of mESC. B two pictures far left and the middle one show puromycin resistant colonies, the picture to the far right shows negative control. (B) Cells where exposed to 1.5 ug/ml of puromycin for 96 hours. All three pictures show 100 x magnifications.

IX – References.

References

1. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet. 2000; 1(1):57-64.

2. Meyer U, Meyer T, Handschel J, Weisman HP (eds). Fundamentals of Tissue Engineering and Regenerative Medicine. Springer., 2009.

3. Bomken S, Fiser K, Heidenreich O, Vormoor J. Understanding the cancer stem cell. Br J Cancer. 2010; 103(4):439-445.

4. De Miguel MP, Fuentes-Julian S, Alcaina Y. Pluripotent stem cells: Origin, maintenance and induction. Stem Cell Rev. 2010; 6(4):633-649.

5. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126(4):663-676.

6. Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol. 2007; 25(10):1177-1181.

7. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131(5):861-872.

8. Chambers I, Smith A. Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene. 2004; 23(43):7150-7160.

9. Varga AC, Wrana JL. The disparate role of BMP in stem cell biology. Oncogene. 2005; 24(37):5713-5721.

10. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292(5819):154-156.

11. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282(5391):1145-1147.

12. KLEINSMITH LJ, PIERCE GB,Jr. Multipotentiality of single embryonal carcinoma cells. Cancer Res. 1964; 24:1544-1551.

13. Massague J. How cells read TGF-beta signals. Nat Rev Mol Cell Biol. 2000; 1(3):169-178.

14. Valdimarsdottir G, Mummery C. Functions of the TGFbeta superfamily in human embryonic stem cells. APMIS. 2005; 113(11-12):773-789.

15. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998; 95(3):379-391.

16. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003; 113(5):631-642.

17. Solter D, Knowles BB. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci U S A. 1975; 72(12):5099-5102.

18. Smith AG. Embryo-derived stem cells: Of mice and men. Annu Rev Cell Dev Biol. 2001; 17:435-462.

19. Maitra A, Arking DE, Shivapurkar N, et al. Genomic alterations in cultured human embryonic stem cells. Nat Genet. 2005; 37(10):1099-1103.

20. Draper JS, Fox V. Human embryonic stem cells: Multilineage differentiation and mechanisms of self-renewal. Arch Med Res. 2003; 34(6):558-564.

21. Chung Y, Klimanskaya I, Becker S, et al. Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. Nature. 2006; 439(7073):216-219.

22. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007; 448(7151):313-317.

23. Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science. 2007; 318(5858):1920-1923.

24. Park IH, Arora N, Huo H, et al. Disease-specific induced pluripotent stem cells. Cell. 2008; 134(5):877-886.

25. TILL JE, McCULLOCH EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961; 14:213-222.

26. Lemoli RM, Bertolini F, Cancedda R, et al. Stem cell plasticity: Time for a reappraisal? Haematologica. 2005; 90(3):360-381.

27. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000; 97(25):13625-13630.

28. Zammit P, Beauchamp J. The skeletal muscle satellite cell: Stem cell or son of stem cell? Differentiation. 2001; 68(4-5):193-204.

29. Lemire JM, Shiojiri N, Fausto N. Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol. 1991; 139(3):535-552.

30. Niemann C, Watt FM. Designer skin: Lineage commitment in postnatal epidermis. Trends Cell Biol. 2002; 12(4):185-192.

31. Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell. 1990; 61(7):1329-1337.

32. Thomas ED. Landmarks in the development of hematopoietic cell transplantation. World J Surg. 2000; 24(7):815-818.

33. Blume KG, Thomas ED. A review of autologous hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2000; 6(1):1-12.

34. Wagner J, Kean T, Young R, Dennis JE, Caplan AI. Optimizing mesenchymal stem cell-based therapeutics. Curr Opin Biotechnol. 2009; 20(5):531-536.

35. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A. 1997; 94(10):5320-5325.

36. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988; 241(4861):58-62.

37. Hong S, Hwang DY, Yoon S, et al. Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells. Mol Ther. 2007; 15(9):1630-1639.

38. Thomson JA, McWhir J (eds). Gene Targeting and Embryonic Stem Cells. BIOS, 2004.

39. Furuya M, Yasuchika K, Mizutani K, Yoshimura Y, Nakatsuji N, Suemori H. Electroporation of cynomolgus monkey embryonic stem cells. Genesis. 2003; 37(4):180-187.

40. Chung S, Andersson T, Sonntag KC, Bjorklund L, Isacson O, Kim KS. Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. Stem Cells. 2002; 20(2):139-145.

41. Lakshmipathy U, Pelacho B, Sudo K, et al. Efficient transfection of embryonic and adult stem cells. Stem Cells. 2004; 22(4):531-543.

42. Kawabata K, Sakurai F, Yamaguchi T, Hayakawa T, Mizuguchi H. Efficient gene transfer into mouse embryonic stem cells with adenovirus vectors. Mol Ther. 2005; 12(3):547-554.

43. Monahan PE, Samulski RJ. Adeno-associated virus vectors for gene therapy: More pros than cons? Mol Med Today. 2000; 6(11):433-440.

44. Xiong C, Tang DQ, Xie CQ, et al. Genetic engineering of human embryonic stem cells with lentiviral vectors. Stem Cells Dev. 2005; 14(4):367-377.

45. Cockrell AS, Kafri T. HIV-1 vectors: Fulfillment of expectations, further advancements, and still a way to go. Curr HIV Res. 2003; 1(4):419-439.

46. Kordower JH, Emborg ME, Bloch J, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of parkinson's disease. Science. 2000; 290(5492):767-773.

47. Kosaka Y, Kobayashi N, Fukazawa T, et al. Lentivirus-based gene delivery in mouse embryonic stem cells. Artif Organs. 2004; 28(3):271-277.

48. Prelle K, Zink N, Wolf E. Pluripotent stem cells--model of embryonic development, tool for gene targeting, and basis of cell therapy. Anat Histol Embryol. 2002; 31(3):169-186.

49. Voorhoeve PM, Agami R. Knockdown stands up. Trends Biotechnol. 2003; 21(1):2-4.

50. Alberts B, Jhonson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. In: Fifth ed. New York USA: Garland Science, 2008:495-497.

51. Mocellin S, Provenzano M. RNA interference: Learning gene knock-down from cell physiology. J Transl Med. 2004; 2(1):39.

52. Leonard I. Zon. Hematopoiesis A Developmental Approach. Oxford New York: Oxford university press, 2001.

53. BECKER AJ, McCULLOCH EA, TILL JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963; 197:452-454.

54. Trentin JJ (ed). Handbook of the Hematopoietic Microinvironment. Human press, 1989.

55. Curry JL, Trentin JJ. Hemopoietic spleen colony studies. I. growth and differentiation. Dev Biol. 1967; 15(5):395-413.

56. Challen GA, Boles N, Lin KK, Goodell MA. Mouse hematopoietic stem cell identification and analysis. Cytometry A. 2009; 75(1):14-24.

57. Tajima F, Deguchi T, Laver JH, Zeng H, Ogawa M. Reciprocal expression of CD38 and CD34 by adult murine hematopoietic stem cells. Blood. 2001; 97(9):2618-2624.

58. Haeryfar SM, Hoskin DW. Thy-1: More than a mouse pan-T cell marker. J Immunol. 2004; 173(6):3581-3588.

59. Metcalf D. The hemopoietic colony stimulating factors. 1984; .

60. Szilvassy SJ, Cory S. Phenotypic and functional characterization of competitive long-term repopulating hematopoietic stem cells enriched from 5-fluorouracil-treated murine marrow. Blood. 1993; 81(9):2310-2320.

61. Larsson J, Karlsson S. The role of smad signaling in hematopoiesis. Oncogene. 2005; 24(37):5676-5692.

62. Metcalf D. Concise review: Hematopoietic stem cells and tissue stem cells: Current concepts and unanswered questions. Stem Cells. 2007; 25(10):2390-2395.

63. Orkin SH, Zon LI. Hematopoiesis: An evolving paradigm for stem cell biology. Cell. 2008; 132(4):631-644.

64. THOMAS ED, LOCHTE HL,Jr, LU WC, FERREBEE JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med. 1957; 257(11):491-496.

65. BARNES DW, CORP MJ, LOUTIT JF, NEAL FE. Treatment of murine leukaemia with X rays and homologous bone marrow; preliminary communication. Br Med J. 1956; 2(4993):626-627.

66. Bortin MM. A compendium of reported human bone marrow transplants. Transplantation. 1970; 9(6):571-587.

67. THOMAS ED, LOCHTE HL,Jr, CANNON JH, SAHLER OD, FERREBEE JW. Supralethal whole body irradiation and isologous marrow transplantation in man. J Clin Invest. 1959; 38:1709-1716.

68. Appelbaum FR, Forman SJ, Negrin RS, Blume KG (eds). Thomas' Hematopoietic Cell Transplantation Fourth Edition. 4th ed. Southern Gate-Chichester-West Sussex: Wiley-Blackwell, 2009.

69. Matthíasdóttir SJ. Vinnsla stofnfrumna fyrir háskammtalyfjameðferð á íslandi 2003-2006. Tímarit Lífeindafræðinga. 2008; 3(1):9-16.

70. Miller W, Makova KD, Nekrutenko A, Hardison RC. Comparative genomics. Annu Rev Genomics Hum Genet. 2004; 5:15-56.

71. Mouse Genome Sequencing Consortium, Waterston RH, Lindblad-Toh K, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002; 420(6915):520-562.

72. Rossant J, McKerlie C. Mouse-based phenogenomics for modelling human disease. Trends Mol Med. 2001; 7(11):502-507.

73. Willey S, Ayuso-Sacido A, Zhang H, et al. Acceleration of mesoderm development and expansion of hematopoietic progenitors in differentiating ES cells by the mouse mix-like homeodomain transcription factor. Blood. 2006; 107(8):3122-3130.

74. Fujimoto TT, Kohata S, Suzuki H, Miyazaki H, Fujimura K. Production of functional platelets by differentiated embryonic stem (ES) cells in vitro. Blood. 2003; 102(12):4044-4051.

75. Lieber JG, Webb S, Suratt BT, et al. The in vitro production and characterization of neutrophils from embryonic stem cells. Blood. 2004; 103(3):852-859.

76. Carotta S, Pilat S, Mairhofer A, et al. Directed differentiation and mass cultivation of pure erythroid progenitors from mouse embryonic stem cells. Blood. 2004; 104(6):1873-1880.

77. Steidl U, Rosenbauer F, Verhaak RG, et al. Essential role of jun family transcription factors in PU.1 knockdown-induced leukemic stem cells. Nat Genet. 2006; 38(11):1269-1277.

78. Alipio Z, Liao W, Roemer EJ, et al. Reversal of hyperglycemia in diabetic mouse models using induced-pluripotent stem (iPS)-derived pancreatic beta-like cells. Proc Natl Acad Sci U S A. 2010; 107(30):13426-13431.

79. Kamp A, Peterson MA, Svenson KL, et al. Genome-wide identification of mouse congenital heart disease loci. Hum Mol Genet. 2010; 19(16):3105-3113.

80. Nomura N, Miyajima N, Sazuka T, et al. Prediction of the coding sequences of unidentified human genes. I. the coding sequences of 40 new genes (KIAA0001-

KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. DNA Res. 1994; 1(1):27-35.

81. Bassal S, Nomura N, Venter D, Brand K, McKay MJ, van der Spek PJ. Characterization of a novel human cell-cycle-regulated homologue of drosophila dlg1. Genomics. 2001; 77(1-2):5-7.

82. Gudmundsson KO, Thorsteinsson L, Sigurjonsson OE, et al. Gene expression analysis of hematopoietic progenitor cells identifies Dlg7 as a potential stem cell gene. Stem Cells. 2007; 25(6):1498-1506.

83. Tsou AP, Yang CW, Huang CY, et al. Identification of a novel cell cycle regulated gene, HURP, overexpressed in human hepatocellular carcinoma. Oncogene. 2003; 22(2):298-307.

84. Novartis Research Foundation. BioGPS <http://biogps.gnf.org/#goto=welcome>. .

85. Woods DF, Bryant PJ. Molecular cloning of the lethal(1)discs large-1 oncogene of drosophila. Dev Biol. 1989; 134(1):222-235.

86. Stewart M, Murphy C, Fristrom JW. The recovery and preliminary characterization of X chromosome mutants affecting imaginal discs of drosophila melanogaster. Dev Biol. 1972; 27(1):71-83.

87. Kornau HC, Seeburg PH. Partner selection by PDZ domains. Nat Biotechnol. 1997; 15(4):319.

88. Woods DF, Bryant PJ. The discs-large tumor suppressor gene of drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell. 1991; 66(3):451-464.

89. Cho KO, Hunt CA, Kennedy MB. The rat brain postsynaptic density fraction contains a homolog of the drosophila discs-large tumor suppressor protein. Neuron. 1992; 9(5):929-942.

90. Sillje HH, Nagel S, Korner R, Nigg EA. HURP is a ran-importin beta-regulated protein that stabilizes kinetochore microtubules in the vicinity of chromosomes. Curr Biol. 2006; 16(8):731-742.

91. Koffa MD, Casanova CM, Santarella R, Kocher T, Wilm M, Mattaj IW. HURP is part of a ran-dependent complex involved in spindle formation. Curr Biol. 2006; 16(8):743-754.

92. Sanderson HS, Clarke PR. Cell biology: Ran, mitosis and the cancer connection. Curr Biol. 2006; 16(12):R466-8.

93. Yu CT, Hsu JM, Lee YC, Tsou AP, Chou CK, Huang CY. Phosphorylation and stabilization of HURP by aurora-A: Implication of HURP as a transforming target of aurora-A. Mol Cell Biol. 2005; 25(14):5789-5800.

94. Wong J, Lerrigo R, Jang CY, Fang G. Aurora A regulates the activity of HURP by controlling the accessibility of its microtubule-binding domain. Mol Biol Cell. 2008; 19(5):2083-2091.

95. Hsu JM, Lee YC, Yu CT, Huang CY. Fbx7 functions in the SCF complex regulating Cdk1-cyclin B-phosphorylated hepatoma up-regulated protein (HURP) proteolysis by a proline-rich region. J Biol Chem. 2004; 279(31):32592-32602.

96. Song L, Rape M. Regulated degradation of spindle assembly factors by the anaphase-promoting complex. Mol Cell. 2010; 38(3):369-382.

97. Chiu AW, Huang YL, Huan SK, et al. Potential molecular marker for detecting transitional cell carcinoma. Urology. 2002; 60(1):181-185.

98. de Reynies A, Assie G, Rickman DS, et al. Gene expression profiling reveals a new classification of adrenocortical tumors and identifies molecular predictors of malignancy and survival. J Clin Oncol. 2009; 27(7):1108-1115.

99. Zhao L, Qin LX, Ye QH, et al. KIAA0008 gene is associated with invasive phenotype of human hepatocellular carcinoma--a functional analysis. J Cancer Res Clin Oncol. 2004; 130(12):719-727.

100. Wang YC, Lee YH, Huang GC, et al. Enhanced transformation and chemosensitivity of NIH3T3 cells transduced with hepatoma up-regulated protein. Biochem Biophys Res Commun. 2006; 340(1):244-249.

101. Segal NH, Blachere NE, Shiu HY, et al. Antigens recognized by autologous antibodies of patients with soft tissue sarcoma. Cancer Immun. 2005; 5:4.

102. Wong J, Lerrigo R, Jang CY, Fang G. Aurora A regulates the activity of HURP by controlling the accessibility of its microtubule-binding domain. Mol Biol Cell. 2008; 19(5):2083-2091.

103. Tsai CY, Chou CK, Yang CW, et al. Hurp deficiency in mice leads to female infertility caused by an implantation defect. J Biol Chem. 2008; 283(39):26302-26306.

104. Gudmundsson KO, Thorsteinsson L, Sigurjonsson OE, et al. Gene expression analysis of hematopoietic progenitor cells identifies Dlg7 as a potential stem cell gene. Stem Cells. 2007; 25(6):1498-1506.

105. Zhu J, Emerson SG. Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene. 2002; 21(21):3295-3313.

106. Barrette S, Douglas JL, Seidel NE, Bodine DM. Lentivirus-based vectors transduce mouse hematopoietic stem cells with similar efficiency to moloney murine leukemia virus-based vectors. Blood. 2000; 96(10):3385-3391.

107. Barrette S, Douglas J, Orlic D, et al. Superior transduction of mouse hematopoietic stem cells with 10A1 and VSV-G pseudotyped retrovirus vectors. Mol Ther. 2000; 1(4):330-338.

108. Wong J, Fang G. HURP controls spindle dynamics to promote proper interkinetochore tension and efficient kinetochore capture. J Cell Biol. 2006; 173(6):879-891.

109. Chiou SH, Yu CC, Huang CY, et al. Positive correlations of oct-4 and nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. Clin Cancer Res. 2008; 14(13):4085-4095.

110. Quiagen. ProofStart DNA polymerase a new concept for high-fidelity PCR. 2001; 2001(2):20-21.