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Activation of Protein Kinase C to modulate retroviral gene transfer to human hematopoietic progenitor cells

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Title (English)

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Abstract

Hematopoietic stem cells (HSCs) are the origin of all other blood cells in the body and are attractive targets for gene therapy. Transduction can be accomplished through the use of oncoretroviral vectors. A barrier to successful oncoretroviral transduction of HSCs has been low levels of viral receptors used by vector particles for cell entry. Activation of Protein kinase C (PKC) has been observed to up-regulate expression of mRNA for viral receptor GLVR1 used by Gibbon Ape Leukemia Virus (GALV). We have treated human hematopoietic progenitor cells with PKC stimulating agents bryostatin-1, ingenol 3-angelate (PEP005) and phorbol 12-myristrate 13-acetate (PMA). The aim was to elevate mRNA coding for viral receptors GLVR1 and RDR, used by GALV and feline leukemia virus (RD114) respectively, thus modulating the level of retroviral gene transfer. A minor increase of gene transfer was seen for GALV vector upon exposure to PEP005 and PMA, and in addition mRNA for GLVR1 was elevated. Bryo-1 showed no effect. Transduction with RD114 was not altered by PKC stimulation, nor was the expression of mRNA coding for RDR.

Keywords: Hematopoietic stem cells, Protein Kinase C, Gibbon Ape Leukemia Virus, Feline leukaemia virus, phorbol 12-myristrate 13-acetate, bryostatin-1, ingenol 3-angelate

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Sammanfattning

Hematopoietiska stamceller (HSC) utgör ursprunget till alla blodceller i kroppen. Den unika förmågan till självförnyelse tillsammans med att de kan återskapa ett fullt funktionellt hematopoietiskt system efter transplantation gör dessa stamceller till attraktiva mål för genterapi. Överföring av genetiskt material till HSC kan genomföras med hjälp av retrovirala vektorer, en process kallad transduktion.
Tidigare har observerats att aktivering av protein kinase C (PKC) kan leda till ökad transduktion av primitiva celler då man använt vektorer med hölje från oncoretrovirus Gibbon Ape Leukemia Virus (GALV). Orsaken är troligen att PKC stimulerar uttryck av mRNA som kodar för den virala receptorn GLVR1 vilken GALV utnyttjar för att ta sig in i målcellen. För att aktivera PKC har man behandlat hematopoietiska stamceller med phorbol 12-myristrate 13-acetate (PMA) vilket fungerar som ligand till enzymet. Dock är PMA cancerogent och går därmed inte att använda terapeutiskt. Därför har andra substanser, som liksom PMA är kända ligander till PKC, identifierats. Två av dessa som också använts kliniskt är bryostatin-1 och ingenol 3-angelate (PEP005).
I detta projekt utvärderas substansernas inverkan på effektiviteten vid transduktion av primitiva hematopoietiska celler med avseende på tid och procentuell celluttryck av integrerade markörgen GFP. Två vektorer med virushöljen från GALV och Feline leukemia virus RD114 har jämförts. Därutöver har nivån av mRNA för respektive virusreceptor undersökts efter att celler utsatts för låga koncentrationer av bryostatin-1, PEP005 respektive PMA. Transduktion med GALV verkar ökas något av behandling med PEP005 samt PMA även om effekten inte är signifikant. Även nivån av mRNA för GALV receptor ökas efter att cellerna behandlats med PEP005 och PMA. Bryo-1 verkar inte ha någon inverkan. I fallet RD114 så påverkas varken transduktionsnivå eller mängd receptor mRNA. Detta kan tyda på att antalet ytreceptorer redan är tillräckligt för effektiv transduktion, alternativt att PKC inte reglerar uttrycket för virusreceptorn.

Examensarbete 20p

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Abbreviations

BM	Bone marrow
CB	Umbilical cord blood
CD	Cluster of differentiation
CFU	Colony forming unit
c-kit	Receptor for kit ligand stem cell factor (SCF)
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DAG	Diacylglycerol
Env	Retroviral envelope protein
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf (bovine) serum
FeLV	Feline leukemia virus
FLt3-L	FMS-like tyrosine kinase 3 ligand
GALV	Gibbon Ape Leukemia Virus
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GLVR1	GALV receptor
GOI	Gene of interest
HSC	Hematopoietic stem cell
HT1080	Human fibrosarcoma cell line
IL	Interleukin
PCR	Polymerase Chain Reaction
PEP005	Ingenol 3-angelate
PG13	Packaging cell line producing GALV pseudotyped vectors
PKC	Protein kinase C
PMA	phorbol 12-myristrate 13-acetate
Q-RT-PCR	Quantitative reverse transcriptase PCR
RD114	Feline endogenous retrovirus
RDR	RD114 receptor
Sca-1	Stem cell antigen
SCF	Stem cell factor (same as Kit ligand)
TPO	Thrombopoietin
VCM	Vector containing medium

1. Introduction

1.1 Hematopoiesis

In an adult person the blood represents about $1/12^{th}$ of the body weight. This corresponds to 5-6 liters of blood, composed of 55% plasma and 45% formed elements or cells. Within the blood system a myriad of collaborations is taking place between various types of blood cells, signaling compounds and transporting elements. To maintain the efficiency of the activities comprised by the hematopoietic system around 2×10^{11} erythrocytes and 10^{10} leukocytes must be produced every day [1]. The continuously ongoing generation of new blood cells, termed hematopoiesis, occurs in the bonemarrow. Fully matured cells cannot self-renew, and thus the homeostasis of the blood system depends on the more primitive cells with self-renewing ability: the hematopoietic stem cells (HSCs).

1.2 Hematopoietic stem cells

A stem cell is a cell that is undifferentiated and can undergo unlimited divisions to form other cells and/or self-renew. The daughter cells either remain as stem cells or differentiate and become more specialized. There are essentially two types of stem cells: the embryonic and the somatic. An embryonic stem cell is pluripotent and can differentiate into all cells in all the germ layers in the body, whereas a somatic stem cell is multipotent meaning it can differentiate into cells within a specific tissue, e.g. blood- or neuronal cells.
The hematopoietic stem cell is a somatic stem cell with some unique properties shared with no other cells in an adult person. It can produce all blood lineages resulting in at least eleven functional types (neutrophils, monocytes, basophils, eosinophils, erythrocytes, platelets, osteoclasts, mast cells, dendritic cells, B-cells and T-cells). HSCs can self-renew (produce daughter cells with the same properties as the original) thus maintaining the stem cell-pool. Furthermore they are able to give rise to a fully reconstituted hematopoietic system after transplantation from one individual to another. Despite their importance HSC are of a rare kind of cell and only one per ten thousand cells or even fewer in the bone marrow is an actual stem cell. In addition most of these HSC are quiescent and do not actually participate in the ongoing hematopoiesis at a given moment in time.

The different hematopoietic cells can be described as a hierarchical process where the cells higher up in the hierarchy possess a higher self-renewal capacity compared to the ones further down

(Fig.1). In the top section of the tree there are two sorts of stem cells: the long-term repopulating cells (LT-HSCs) and the short-term repopulating cells (ST-HSCs). LT-HSCs exhibit the highest self-renewing capacity and can produce all other blood cells throughout the entire lifetime of the host. However these LT-HSCs are very slow-dividing and cannot cure a patient in need of transplantation by themselves since they do not provide any immediate hematopoietic protection. The ST-HSCs have limited self-renewing ability but can on the other hand reconstitute myeloid/lymphoid compartments much more rapidly than LT-HSCs [1].

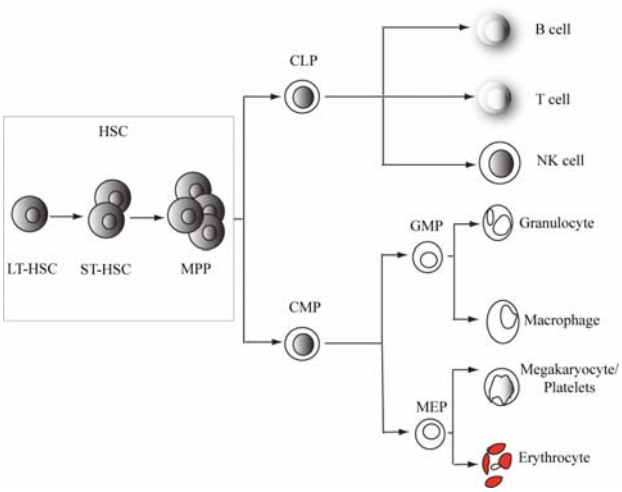

Figure 1. Hematopoiesis as a hierarchical process. The HSCs are capable of self-renewal whereas the more differentiated progenitor cells are restricted in their developmental fate.

The extent of the cell division accounted for by ST-HSCs and LT-HSCs is far from enough when it comes to maintaining the amount of blood cells. The expansion and proliferation of the cells is conducted by progenitors further down the hematopoietic tree. After a limited time of self-renewal the ST-HSC develop into multipotent progenitors (MPP) that self-renew for an even more limited time span. From here the differentiation becomes more definite when the cells continue to develop into either of the two lineages common lymphocyte progenitors (CLP) or common myeloid progenitor (CMP). Neither CLP nor CMP possess any self-renewing capacity, and both of them are restricted in differentiation options. CLPs is a committed progenitor to the cell classes T-, B-, natural killer- and dendritic cells, whereas CMP can give rise to granulocytes, monocytes, dendritic cells, erythrocytes and platelets.

1.3 Differentiation vs. self-renewal

HSCs are limited in their developmental decisions. They can virtually self-renew, differentiate, migrate into the blood-system or go into apoptosis (Fig. 3). When a stem cell divides it can do so

in two ways: asymmetric or symmetric division. The first way refers to when one of the resulting daughter cells has preserved stem cell properties whereas the other daughter cell has differentiated towards either of the lineages. The second way, the symmetric division, means that both daughter cells obtain the same features whether it is as stem cells or as differentiated cells.

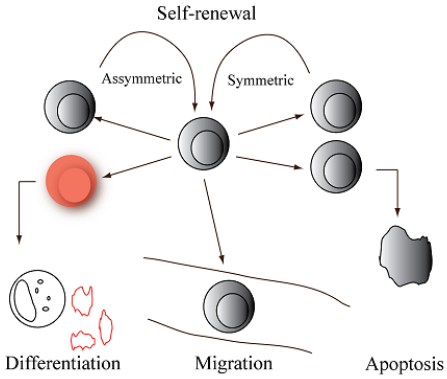

Figure 2. Stem cell fate options. The HSC can undergo symmetric or asymmetric self-renewal, differentiate towards either of the committed progenitors, migrate into the blood or go into apoptosis.

There are two coexisting concepts as for how the stem cell fate is determined: the stochastic and the instructive regulation theory which both involve the idea of impact from cytokines.
The stochastic theory is based on the idea that there is a stem cell specific set of genes that are expressed in a random way thus directing the stem cell towards one of the possible pathways. The cytokines and cell-cell interactions that constitute the surrounding micro environment are considered to have a certain influence in permitting self-renewal or maturation, but not in a conclusive way[2, 3]. On the other hand, in the instructive theory signaling cytokines are believed to be the decisive factor for HSC fate. It has been observed that stem cells and progenitor cells express low levels of various transcription factors and cytokine receptors, and that they upon maturation lose the receptors that are not specific for the lineage the cell has committed itself to [2]. Findings like the one mentioned would speak in favor of the instructive theory. However it seems likely that a mix between random and regulatory pathways governs the HSCs. If a need for one blood-cell lineage would arise e.g. as a result of trauma, it is vital that the body would still produce all other lineages even if the instructive system would stimulate production of the lineage needed at that specific time.

The presence of cytokines for manipulation and regulation of cells is widely used in cell culture *in vitro*. As HSCs are prone to differentiate and hard to keep as stem cells different cytokines have been identified that could stimulate a required development. One goal is being able to expand

stem cells, but at the same time prevent them from maturing into committed progenitors. Nonetheless, although cytokines are beneficial, complementary methods and systems are required in order to regulate cells *in vitro*.

In attempts to manipulate cells a cocktail of some chosen cytokines are often used that suits the purpose of an experiment. Expansion of stem cells has been noticed under the influence of the cytokines stem cell factor (SCF), thrombopoietin (TPO) and Flt3-L[4].
SCF binds to the c-kit receptor protein present on human HSCs and has been shown to stimulate HSC expansion in vitro as is also the case for TPO[5]. Flt3-L has been indicated as a preserver of uncommitted $CD34^{+}$/CD38- progenitor cells during stimulation with SCF and interleukins[6].

1.4 Functional assays

1.4.1 In vivo

The concept of hematopoietic stem cells was conceived in the middle of the 20th century, but it took some time before it became possible to study the actual cells. The first methods used to examine the behavior of stem cells were indirect functional assays. The original *in-vivo* assay was the spleen colony forming (CFU-S) assay developed by Till and McCulloch in the 1960s. Lethally irradiated mice were injected with bone marrow cells, and after a number of days it was possible to detect colonies of proliferating cells in the spleen. They observed that the number of colonies in the spleen increased with the number of cells injected with an almost one-to-one ratio, and it was suspected that each colony consisted of clones derived from one progenitor cell [7].

Even if the techniques for studying HSC have advanced/improved there is still no single optimal stem cell assay available today. Various analyses are needed to complement each other, much due to the fact that the phenotype at a single cell level is yet undetermined. However, widespread and important ways to examine HSC activity are the reconstitution assays like competitive repopulation- and competitive repopulation unit (CRU) assays. These *in-vivo* methods make use of *preconditioned* mice – mice that have undergone lethal irradiation. The preconditioning treatment creates space in the BM of the animal making it possible for transplanted HSCs to repopulate the hematopoietic system.

1.4.2 In vitro

One commonly used *in vitro* assay to examine progenitor cells is the colony-forming unit (CFU)-assay where (human or murine) myeloid cells are generated in methylcellulose cultures thus preventing the cells from moving. The initial cells will give rise to colonies, and by studying these cell clusters one can predict the level of differentiation of the original cell. If the cells show a vast variation it is presumable that the original cell was more primitive than if the colony cells display a similar morphology. Through adding certain cytokines differentiation can be directed and the cells move towards becoming e.g. erythroid or myeloid cells.
A recent breakthrough in the *in vitro* assays is the ability to cultivate HSCs on a stromal cell line in a culture dish: long-term culture-initiating cell assay (LTR-IC). The stromal cells reside in the bone marrow where they make up a natural environment for the stem cells e.g. by excreting cytokines and stimulators as well as offering a structural support. When cultivating myeloid cells

on a stromal cell line the differentiated cells will eventually fade away, whereas the more primitive ones survive. After being cultured on stromal cells the HSCs are transferred onto a methylcellulose culture. After colonies have been formed expansion of the original cells can be evaluated.

1.5 Phenotype & purification

Characterization of hematopoietic cells involves evaluation of surface protein markers present at different stages in the differentiation process. Knowing what markers are typical for HSC activity is a great resource not only for defining a potential HSC but also for enabling purification of these cells. Although the definition of murine HSC has come a long way, characterization of the phenotype of human HSC has proven to be more difficult. However, a few markers have been shown to identify stem cell activity.

The different cell-lineages in the hematopoietic tree display specific lineage markers that reveal the commitment of cells. The more primitive the blood cell, the lower the Lin-expression and the true HSC are negative for lineage markers (Lin^-).

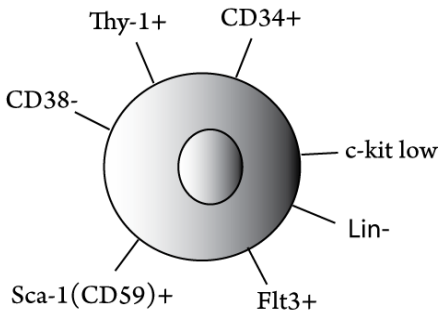

Figure 3. Human HSC markers

It has been found that human hematopoietic stem cells (hHSC) express low levels of c-kit protein, a transmembrane kinase that acts as a receptor for the cytokine stem cell factor (SCF)[8]. Also, hHSCs express the glycoprotein Thy-1, the stem cell associated antigen 1 (Sca-1) and fms-related tyrosine kinase 3 (Flt3) (Fig. 3).

The transmembrane protein CD34 belongs to the sialomucins and is expressed on all progenitor cells, 0,5-5% of human bone marrow cells[1]. It functions as a cell-cell adhesion molecule and is believed to assist HSC in connecting/homing to the stromal cells in the bone marrow. CD34 is used in association with other cell markers to identify subpopulations of purified stem cells like $CD34^+CD38^-$. CD38 is found on more mature cells, but not further up the hematopoietic hierarchy – a characteristics that is utilized for isolation and cell sorting of stem cells.

1.6 Sources: BM, UCB and blood

The traditional source of hematopoietic stem cells has been the bone (BM) marrow where the frequency lies between 0.01 – 0.001% [9]. Under certain circumstances these HSCs can become mobilized and after stimulation with granulocyte colony-stimulating factor G-CSF or other

cytokines enter the peripheral blood system wherefrom they can be extracted and used in transplants to another person. In the end of the 1980s researchers found that it was possible to isolate quite substantial amounts of hematopoietic stem cells from umbilical cord blood (CB). It also became clear that HSC from CB could be used when treating patients with blood- and bone marrow diseases using cell transplantation to reconstruct the person's hematopoietic system. Since then the knowledge about the use of CB HSC in transplantations has increased, and today there are even CB banks established where patients lacking an HLA-identical donor can look for a match [10, 11]. However, the amount of collected blood cells is most of the time too small to be used in transplants to an adult.

1.7 Gene transfer to HSC

Transfer of genetic material into chosen cells allows for ways of treating patients carrying genetic diseases. Such procedures can be accomplished by utilizing viral vectors; engineered viruses that have the ability to introduce their modified genetic content into cells, but that are unable to infect any secondary cells or organisms. The reasons to why viruses have become successful within gene therapy are the same as to why they are highly capable of causing infection in their natural state. Viruses have developed advanced systems to enter cells and can take over cellular functions in favor of their own replication and spread. By taking advantage of some of these viral properties it is possible to perform controlled gene transfer.

1.7.1 Oncoretrovirus: structure and life cycle

Retroviruses are enveloped RNA viruses of the family *Retroviridae* and belong to group number VI (ssRNA-RT) according to the Baltimore classification. Three genera within the family are oncoretro-, lenti- and spumaviruses. Common for all retroviruses is their content of two strands of ssRNA coding for the essential genes *gag*, *pol* and *env,* and the enzymes protease, reverse transcriptase and integrase. The genetic and enzyme content is embedded in a cone shaped inner capsid that consists out of proteins coded for by the *gag* gene. An outer lipid-layer, perforated by glycoproteins, protects the capsid in its turn. The glycoproteins, also referred to as receptor binding proteins, are encoded by the *env* gene and are the ones that determine the tropism of the virus: depending on the cellular receptor of the glycoprotein the virus can enter different kinds of cells[12].

The enzymes that are encoded for by the *pol* gene empower reverse transcription from RNA to intermediate DNA, and subsequently integration of this DNA into the host's genome.

Oncoretrovirus is a simpler retrovirus compared to others within the family that upon infection eventually may lead to malignant disease. They have a diameter of 70-100 nm and a genome of approximately 7-10kb per RNA strand[12]. Infection by an oncoretrovirus starts with binding of the viral glycoprotein to a receptor at the cell surface whereupon fusion occurs between the viral and cellular membranes releasing the viral capsid into the cytoplasm of the host cell. The receptors are often membrane components that fill essential functions, e.g. amino acid- or phosphate transporters.

Once the capsid has been uncoated the RNA strands are reverse transcribed by the accompanied enzyme into DNA. The resulting DNA is flanked by identical sequences termed long terminal repeats (LTR) which contain powerful enhancer promoter that will ensure integration and successive transcription.

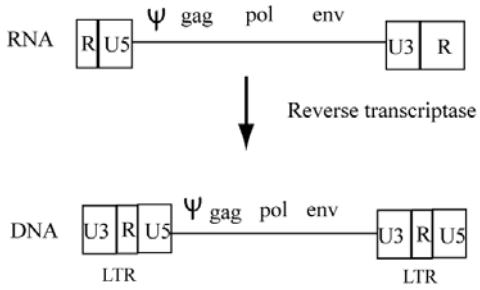

Figure 4. Reverse transcription of viral genome

All along the activities inside the cell the viral genome attracts an assembly of viral proteins, protecting it and ensuring that the replication cycle can be fulfilled.

Oncoretroviruses cannot enter the nucleus by themselves, and thus they are depending on mitosis to occur in order to integrate their DNA, the so-called provirus, into the genome. Once the provirus is inserted into the cellular genome activation of its packaging signal (ψ) attracts cellular transcription factors to make viral RNA and mRNA coding for the structural enzymes. The components are assembled into a new particle that buds off from the cell membrane, thus completing the viral life cycle.

1.7.2 Oncoretroviral vectors

In gene therapy modified viruses known as viral vectors are used as vehicles for transport and integration of genetic material into the DNA of cells, a procedure known as transduction. In the process of generating viruses that are replication incompetent a producer cell line is created. By integration of the genes *gag, pol* and *env* into the genome of the cell line a system is created where

the essential enzymes and structural proteins could be expressed separated from the cis-acting sequences (Ψ and LTR). To hamper recombination events that would risk generation of infectious virus the *gag/pol* genes and the *env* gene are inserted on different plasmids leading to separate integration sites in the DNA of the packaging cell line.

In the production of vector particles the essential genes in the viral genome are replaced with the gene of interest (GOI) whether it is a therapeutic or a marker gene. The GOI that is flanked by LTR and adjoined to Ψ is a replication incompetent viral vector. Subsequently to introduction/integration into a packaging cell line the vector will trigger expression of the viral genes *gag, pol* and *env*, and oncoretroviral vector particles will form with the content of viral enzymes as well as the mRNA from the GOI. The particles bud off from the packaging cell line, competent of integrating their genetic content into target cells but rendered incapable of reassembly and further infectious activities.

It is possible to choose the *env* gene from different virus species depending on what suits the transduction procedure, termed pseudotyping. The envelope proteins displayed on the surface of the outer lipid membrane determines the tropism of the virus and what receptor it uses for entering cells.

In this study two retroviral vectors are used for transduction. Vectors pseudotyped with envelopes from Gibbon Ape Leukemia Virus (GALV) or feline endogenous retrovirus (RD114) are both able to transduce human cells. GALV is produced in packaging cell line PG13 and its cellular receptor is the phosphate transporter GLVR1 that is present in low quantities on $CD34^{+}CD38^{-}$ cells [13]. RD114 is derived from packaging cell line FLYRD18 and binds to receptor RDR: a neutral amino acid transporter present in high levels on human hematopoietic progenitor cells [14].

1.8 Protein Kinase C

1.8.1 Structure and function

Protein kinase C (PKC) is a family of serine/threonine kinases that includes at least ten isoenzymes with different activation patterns. Depending on their biochemical properties and cofactor regulation the isoenzymes are sub-divided into three groups. The conventional PKCs (α,β and γ) are depending on the ligand diacylglycerol (DAG) and Ca^{2+} for activation, the novel PKCs (δ,ε,η and θ) require DAG only and the atypical PKCs (ξ and λ/ι) are independent of ligand interaction [15, 16].

Structurally the PKC enzymes consist of an N-terminal regulatory- and a C-terminal catalytic region connected by a hinge region. In the regulatory region a Cys-rich motif forms the DAG-binding site in conventional and novel PKCs. Connection of ligand to this motif will result in activation of the enzyme. The catalytic half of the protein comprises an ATP-binding site as well as the substrate-binding region (Fig. 5).

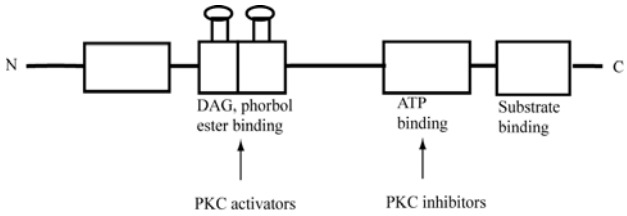

Figure 5. PKC schematic structural sequence: N-terminal regulatory region, and C-terminal catalytic region. Modified from Serova *et.al.* 2006

The roles of PKC are numerous and vary between the different groups and isoforms, but its typical function involves phosphorylation of serine or threonine residues. PKCs also catalyze cofactor-dependent hydrolysis of ATP, and in the case of over-supply of ADP it can act as a phosphatase [15]. Biologically PKC mediates signal transduction of extracellular stimuli that results in release of DAG into the cytosol. The isoforms of PKC are additionally involved in many regulatory processes such as proliferation, differentiation, cell survival, apoptosis and carcinogenesis: features that make them attractive drug targets [17]. In their passive state PKCs reside in the cytosol where a pseudosubstrate occupies the substrate cavity rendering the enzyme incapable of any catalytic activity. When DAG binds to the PKC its membrane-affinity will greatly increase which leads to a translocation of the enzyme from the cytosol to the cell surface.

Due to interactions between enzyme domains and the cellular membrane the pseudosubstrate will get released from its position and the PKC is fully activated as an enzyme [15].

1.8.2 PKC targeting agents

The natural activator of PKC in signal transduction is diacylglycerol (DAG): a glyceride where two fatty acid chains are covalently bonded to a glycerol molecule. It has been discovered that other substances can substitute DAG in a PKC-activating manner. One such is the diterpene phorbol 12-myristrate 13-acetate (PMA) which is a PKC targeting agent that has been proven to induce the same effects as DAG. Observations have been made of membrane translocations of PKC isotypes -α,-γ,-δ,-ε,-η and -θ upon binding of PMA to the regulatory region [18, 19]. Since phorbol esters are not readily metabolized activation with these agents will have a prolonged influence on cellular systems. This makes PMA a useful substance when it comes to experiments in biological systems. However, PMA is a potent tumor promoter which is a major drawback if the agent is included in research aiming for clinical use. Therefore we have identified other PKC-activating agents that have already been tested in the clinic, and among these we selected bryostatin-1 and ingenol 3-angelate (PEP005) for further experiments (table 1).

Bryostatin-1 is a macrocyclic lactone with proven PKC modulator properties (Fig. 6). It is well tolerated in vivo and has been shown to cause up-regulation of growth factors interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on human bone marrow cells. It is also known that bryo-1 mimics the PKC-activities of PMA in cells of hematological origin [20]. This would indicate a role of bryostain-1 as a stimulator of hematopoiesis in vivo [21]. Bryostatin-1 does not activate as many of the PKC isotypes as does the phorbol esters, but the ones it does activate show a more distinct response. Of importance is that bryostatin-1 inhibits the PCKs it does not induce [17]. Two PKC isotypes that are activated by bryostatin-1 are PKC-γ and -δ [18].

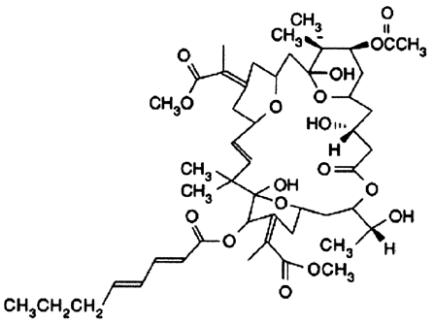

Figure 6. Chemical structure of bryostatin-1 (Figure used with permission from American Association for Cancer Research).

The ingenol derivative PEP005 is structurally more similar to the phorbol esters than bryostatin-1, but has not been as thoroughly investigated in the role as a PKC-mediator. The substance has been found in the plant *Euphorbia peplus* and was used in traditional medicine against warts and skin problems. In modern research it has shown antileukemic effects on oncogenic cells inducing apoptosis, something that, however, is not the case for $CD34^+$ cord blood cells where the effects are of a proliferative kind [22]

PEP005 has proven a potent ligand and activator of PKC in various experiments. Of the PKC isoforms particularly -δ was translocated in response to PEP005, which could have a positive effect tumor inhibition, and also stimulate cell growth and differentiation. Also PKC-α, which is associated with suppression of apoptosis, could be activated by PEP005 but to a lower level than shown by PMA [16, 19].

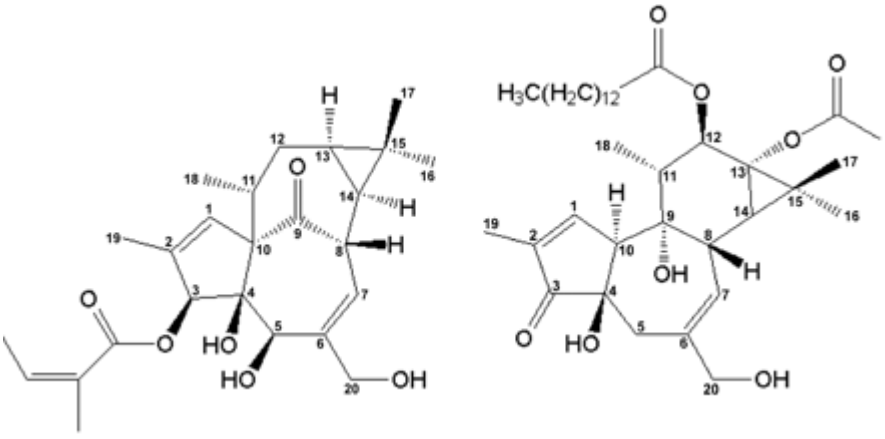

Figure 7. Chemical structures of ingenol 3-angelate (PEP005) to the left and phorbol 12-myristate 13-acetate (PMA) to the right. (Figures used with permission from American Association for Cancer Research).

	α	β	γ	δ	ε	η	θ	λ
PMA	+	-	+	+	+	+	+	?
Bryostatin-1	-	?	+	+	+	+	?	?
PEP005	+	+	+	+	+	?	?	?

Table 1. Activation of PKC by agents PMA, bryostatin-1 and PEP005.

Previous research indicates that activation of PKC by PMA-treatment leads to up-regulated expression of mRNA coding for the cell surface receptor for GALV pseudotyped vectors; GLVR1 [23]. This results in an increased gene transfer to the treated cells. It is believed that retroviral transduction carries the risk that non-functional vector particles inhibit gene transfer by interfering with cell surface receptors. This can be overcome by induced over expression of the receptor mRNA as earlier shown by Relander *et. al.* [15]. The group used a low PMA-dosage to attain up-regulation of GLVR1 mRNA [23]. However PMA is a potent tumor promoter, which prevents any clinical use of the substance. In the present study PMA was again investigated along with two other substances known for their PKC-stimulating ability with potential application in the clinic, bryostatin-1 and PEP005.

1.9 Aim of the project

In this project the aim was to investigate whether activation of Protein Kinase C in hematopoietic progenitor cells would improve gene transfer to these cells. Previous results have shown that treating cells with PMA has the potential of up-regulating the mRNA expression of viral receptors in hematopoietic stem cells (HSC) thus enabling for an increased transduction using viral vectors [23]. However, PMA is a potent tumor promoter, which prevents clinical use of the substance. Thus two other PKC activating agents that have already been tested in the clinic were identified and tested. The current project proposed to treat HSCs with PKC stimulating agents PMA, bryostatin-1 and PEP005, with an effort to improve transduction efficiency.

Retroviral vectors carrying the reporter gene for GFP were used for transduction of prestimulated $CD34^+$ cells isolated from umbilical cord blood. Two oncoretroviral vectors from cell lines PG13 and FLYRD18 pseudotyped with viral envelopes GALV and RD114 respectively were used. At transduction cells were treated with PKC activating agents bryostatin-1, PEP005 or PMA. The transduction efficiency from the different treatments was examined and compared by FACS, and the level of receptor mRNA expression evaluated by Q-RT-PCR analysis.

2. Materials and methods

2.1 Isolation and preparation of $CD34^+$ cells

Umbilical cord blood (CB) was collected from newborn infants at Helsingborg hospital after written consent of the mother under a protocol approved by the local ethical committé. The CB was stored for less than 24h in 4°C in flasks containing Dulbecco's modified Eagles medium (DMEM; GIBCO BRL, Paisley, Scotland), 0.1% detoxified bovine serum albumin (BSA; Stem Cell Technologies, Vancouver, Canada), heparin at 150 IU/ml (Pharmacia AB, Stockholm, Sweden), 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL). Separation of mononuclear cells was performed using Lymphoprep density gradient (Nycomed, Oslo, Norway) in centrifugation at 2000 rpm for 20min. The cells were then enriched for $CD34^+$ using the MidiMACS kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Mononuclear cells were incubated with magnetic MicroBeads coupled to $CD34^+$ antibodies. FcR blocking was added to prevent unspecific binding. The cell suspension was put on a MidiMACS column placed in a magnetic holder and washed with buffer to elute $CD34^-$ cells and retain $CD34^+$ cells. The column was removed from the holder and the remaining $CD34^+$ cells were washed out and collected. Purity was examined by flow cytometry analysis of cells stained with an APC conjugated anti-CD34 antibody (Becton Dickinson, USA). The purity was >70%.

Cells were spun down and resuspended in DMEM medium (GIBCO BRL) containing 20% fetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO) before being frozen and stored in liquid nitrogen.

When taken from storage to cultivation the cells were thawed in DMEM (GIBCO BRL) with 10% FCS and spun down at 1300 rpm before washed, counted and viability-checked. Thereafter the medium was changed into X-vivo15 medium (Bio Whittaker, Walkersville, Maryland, USA) with 1% BSA (Stem Cell Technologies), 2mM L-glutamin (GIBCO BRL), 10^{-4}M 0,22μm filtered 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL) and a cytokine-cocktail of 50 ng/ml SCF (Peprotech, USA), 50 ng/ml Flt3-L (Peprotech) and 10 ng/ml TPO (Peprotech). The above concentrations were used for all experiments using X-vivo15 medium.

2.2 Retroviral vectors

The Phoenix amphotropic cell line was transfected with $CaPO_4$ and the vector plasmid containing the marker gene for green fluorescence protein (GFP). Vector containing medium was harvested

and used to transduce the stable packaging cell lines PG13 and FLYRD18 containing the *env* genes for GALV and RD114 respectively. The transduced packaging cells were cultivated in 10 cm dishes at 37°C in 10 ml DMEM with 10% FCS until the point of 80-90% confluency. The medium was then switched to an amount of 6 ml and the cells were put in 33°C for another 24h before the vector containing medium (VCM) was harvested. The VCM was filtered through a 0.45 μm low-protein binding filter (Millipore, Bedford, MA, USA), aliquoted and stored at -80°C until use.

Viral envelope	Receptor	Packaging cell line	Reference
GALV	GLVR1	PG13	American Type Culture Collection (ATCC); Rockville, MD, USA
RD114	RDR	FLYRD18	Provided by Yasu Takeuchi, London

Table 2. Retroviral envelopes, corresponding receptor and packaging cell lines used in the current project.

2.3 Titration of retroviral vectors

Cells from the human fibrosarcoma cell line HT1080 (ATCC) were used for titration of VCM. 50.000 cells in 1ml DMEM with 10% FCS were plated in 4 wells of a 24 well plate. This was repeated for each vector. Cells were grown over night, and the medium was then changed to 1ml fresh medium/well with 10% FCS and 4μg/ml protamin sulphate. VCM was added at amounts of 30μl, 10μl, 3μl and 1μl to each well in a row. Cells were grown and transduced overnight and the medium was changed on day 3. On day 4 the medium was aspirated and the cells were washed with PBS prior to trypsinization to make the cells detach from the bottom. The cells were washed and resuspended in PBS before analysis by FACS with respect to GFP expression. The titre was calculated from the well that resulted in GFP expression closest to 10% with respect to the dilution factor valid for that specific well.

Volume of VCM	Dilution factor
30μl	33
10μl	100
3μl	333
1μl	1000

Table 3. Dilution factors used in calculations of viral titer.

2.4 Transduction and analysis of $CD34^+$ cells

Freshly thawed cells enriched for $CD34^+$ were prestimulated in X-vivo15 medium (Bio Whittaker) with a cytokine cocktail containing 50 ng/ml SCF, 50 ng/ml Flt3-L and 10 ng/ml TPO (Peprotech) for 24h and 48h at a cell concentration of $3*10^5$cells/ml. Transduction was

performed after 24h or 48h of prestimulation. Both 48-well and 96-well plates were used at separate occasions, and the cell concentration at transduction was 1 – 1.5 $*10^5$ cells/ml.

Prior to transduction non-tissue culture-treated plates were coated with 10.5μg/cm^2 Retronectin for 2h before they were blocked with 2% BSA (Stem Cell Technologies) in PBS for another 30 min at room temperature. Next, virus-containing medium was loaded onto the plates and incubated in 37°C for 30-60 min. GALV pseudotyped vector with at titre of $2*10^5$ was loaded undiluted whereas RD114 pseudotyped vector with titre $1.45*10^6$ was diluted 5-20%. In dilution of VCM DMEM with 10% FCS was used.

Prestimulated cells were washed and resuspended in X-vivo15 (Bio Whittaker) with cytokines and distributed to the wells of the pre-loaded plate at a cell concentration of 1 – 1.5 $*10^5$ cells/ml. In the transductions where VCM was used in addition to preloading with Retronectin the cells were resuspended in DMEM with 10% FCS and the same cytokine cocktail as described above.

At start of transduction the PKC stimulating agents bryostatin-1 (Sigma-Aldrich, Missouri, USA), PEP005 (Sigma-Aldrich) and PMA (Sigma-Aldrich) were added to wells pre-loaded with GALV or RD114 viral vector. Concentrations of agents were as follows: bryostatin-1: 1nM, PEP005: 20nM and PMA: 10nM. After 24h transduction the cells were washed and transferred to FACS tubes with fresh X-vivo15 medium supplemented with cytokines for another 24h. Cells were then washed and resuspended in PBS with 1μg/ml 7-amino-actinomycin D (7AAD). GFP expression was examined with FACS Calibur instrument (Becton Dickinson) and the results evaluated in software.

2.5 Analysis of GLVR1- and RDR expression by quantitative PCR

The expression levels of mRNA for GLVR1 and RDR were analyzed using Quantitative reverse transcriptase PCR (Q-RT-PCR) in a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). CB cells enriched for CD34 were thawn, washed in DMEM with 10% FCS and resuspended in X-vivo 15 medium (Bio Whittaker) with added cytokines. After exposure to PKC stimulating agents bryostatin-1, PEP005 and PMA cells were harvested, washed and frozen to -80°C. After at least 24h in frozen condition the cells were thawn and the mRNA extracted using RNeasy micro kit (QIAGEN, GmbH, Germany). cDNA was reverse transcribed using Superscript III (GIBCO BRL) according to the manufacturer's instructions. The cDNA was used in Q-RT-PCR with SybrGreenI (Sigma-Aldrich) for detection of PCR products. An amount of 2 μl cDNA was used per reaction together with another 13 μl of PCR-mix containing

10 U/ml Platinum *Taq* DNA Polymerase (Invitrogen), 1×buffer (provided with the enzyme), 0,8 mM dNTP, 2-2,5 mM $MgCl_2$, 5 mg/ml BSA, 5% DMSO, 0,5 μM forward primer, 0,5 μM reverse primer (table x) and 1:20000 dilution of SybrGreenI. The PCR mix for GALV did not contain DMSO.

The Q-RT-PCR in the LightCycler was programmed to initially denaturate the samples at 92°C for 3 minutes prior to a number of amplification cycles enough to build up sufficient amount of PCR product (30-55 cycles). Each cycle consisted of denaturation at 92°C for 5 s, annealing at 58°C for 5 s and extension at 72°C for 5 s, and the amount of PCR product was measured at the end of each extension step. The GALV and RDR expression levels were normalized against expression of the housekeeping gene GADPH.

GADPH	fw 5' GGC ACC GTC AAG GCT GAG AA 3' rev 5' CCA GTG GAC TCC ACG ACG TA 3'
GALV	fw 5' GCA TAG ATA GCA CCG TGA ATG G 3' rev 5' GCT GAC GGC TTG ACT GAA CTG 3' fragment size, 74 nt
RDR	fw 5' CAA GCA GGT TGG CTC GAA GG 3' rev 5' GCG GGC AAA GAG TAA ACC C 3' fragment size, 92 nt

Table 4. Primer sequences used in Q-RT-PCR assay. All primers were products from Invitrogen custom primers, USA

2.6 Statistical analysis

Mean values and standard deviations were computed using Excel (Microsoft, Redmond, WA).

3. Results

3.1 Transduction of PKC stimulated CD34+ cells

Transductions with viral vectors pseudotyped with GALV or RD114 (GALV-GFP and RD-GFP) were performed on $CD34^+$ cells after 24h and 48h of prestimulation with cytokines. At the start of transduction PKC activating agents bryostatin-1, PEP005 and PMA were added to the cells. At 48h after start of transduction the genetically modified cells were quantified for expression of the GFP marker gene by FACS.

Previous studies have been made where cells were prestimulated for 48h followed by transduction with GALV-GFP in the presence of PKC stimulating agent PMA. This lead to an increased transduction efficiency compared to untreated cells. It was also shown that the mRNA level for GALV-GFP receptor GLVR1 was up-regulated upon treatment with PMA which was believed to explain the increased level of transduction [23]. Here, we decreased the prestimulation period to 24h before transduction with a view to investigate whether activation of PKC would compensate for the shorter time of cell culture, hereby allowing for earlier gene-transfer. Parallel to all experiments control samples were prepared without treatment of PKC activating agents. Neither of the vectors GALV-GFP nor RD-GFP showed any difference in transduction level between the stimulated cells and the control (Fig. 8A-B).

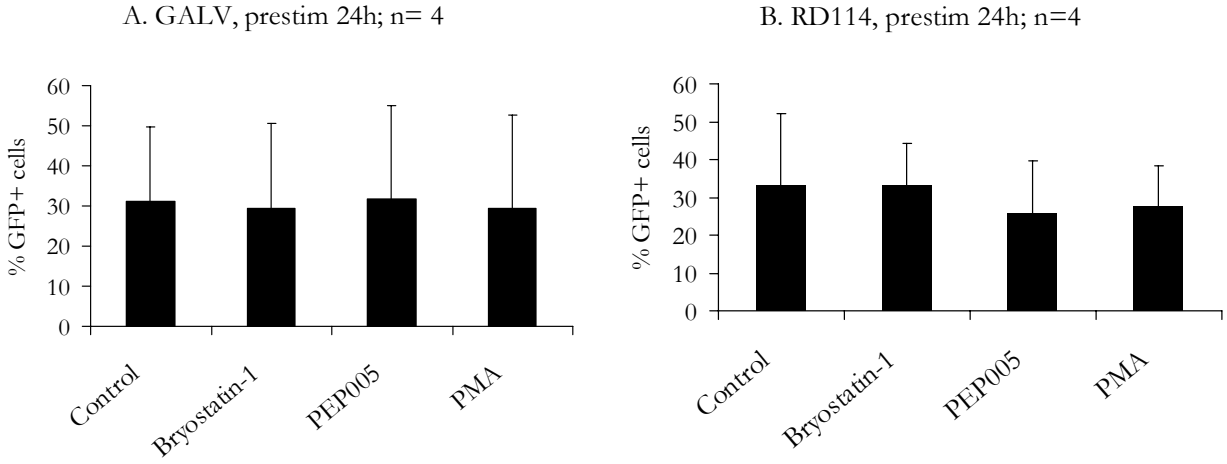

Figure 8. Level GFP+ cells upon transduction using GALV-GFP(A) or RD-GFP(B). Cells were prestimulated 24h and transduction performed with pre-load of vector onto Retronectin. Where indicated, PKC-stimulating agent was added at the initiation of transduction. FACS analysis was performed after another 48h.

Next we prestimulated the cells for 48h followed by transduction in the presence of PKC stimulating agents. Transduction efficiency was not significantly different between cells that were PKC activated and cells that were transduced in the absence of agent (Fig. 9A-B).

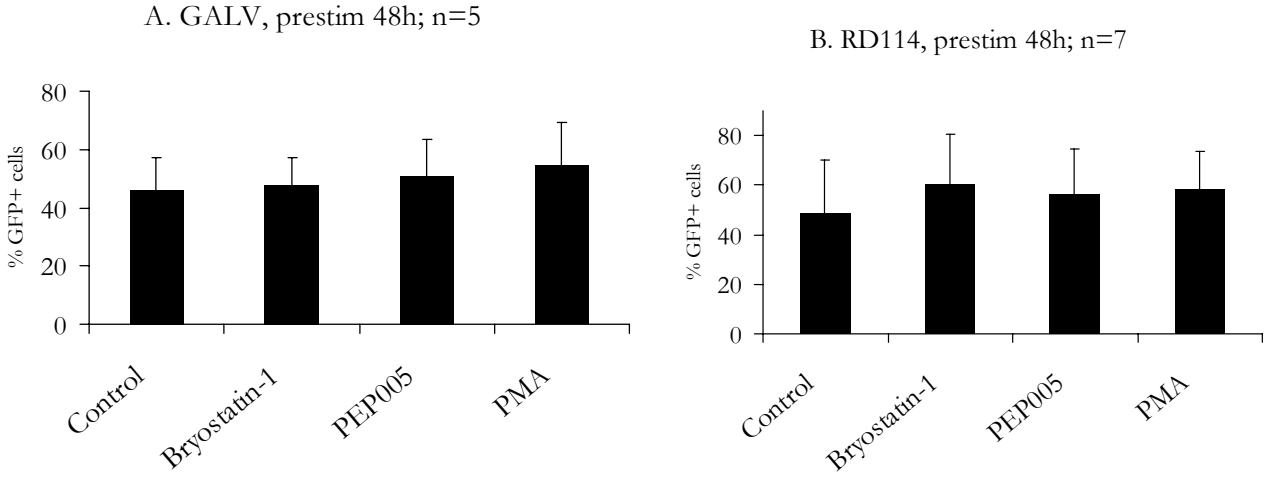

Figure 9. Level GFP+ cells upon transduction using GALV-GFP(A) or RD-GFP(B). Cells were prestimulated 48h and transduction performed with pre-load of vector onto Retronectin. Where indicated, PKC-stimulating agent was added at the initiation of transduction. FACS analysis was performed after another 48h.

The effect of PKC activation on transduction was evaluated further in dose-response experiments (Fig. 10) The increased concentration of each agent is showed separately (table 5). Before transduction cells enriched for $CD34^+$ were prestimulated for 48h.

In the case of GALV-GFP bryostatin-1 did not show any effect on the level of GFP+ cells whereas PEP005, and even more so PMA, appeared to have a slightly positive influence on the transduction efficiency (Fig. 10A). Transduction with RD-GFP showed similar tendencies where bryostatin-1 had no effect and treatment with PEP005 and PMA lead to slightly increased transduction level.

	Conc. 1 (nM)	Conc. 2	Conc. 3	Conc. 4	Conc. 5
Bryo-1	0,33	0,99	3	9	-
PEP005	6,67	20	60	180	540
PMA	3,33	10	30	90	-

Table 5. Concentrations of PKC-stimulating agents used for dose-response experiments.

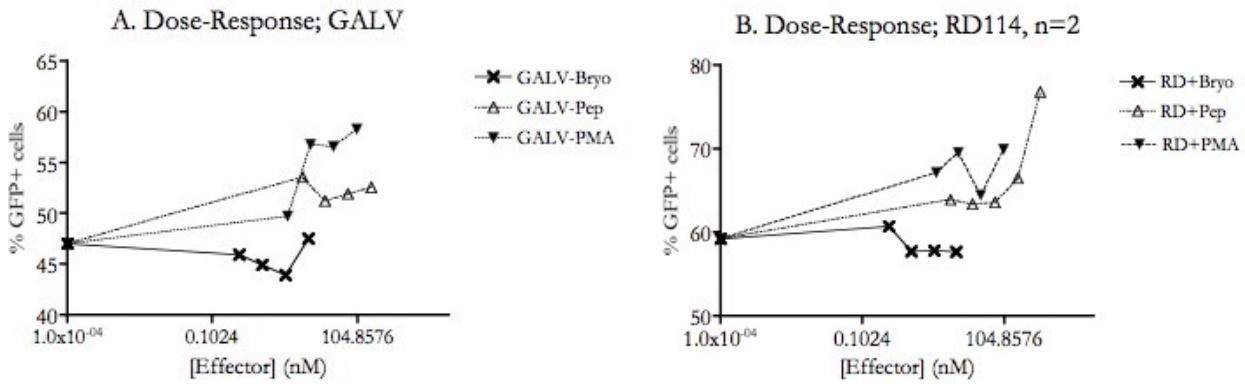

Figure 10. Dose-response curves. Level of GFP+ cells measured against increased concentrations of agents bryostatin-1 (bryo), PEP005 and PMA. Cells were prestimulated for 48h prior to transduction with vector preloaded onto Retronecin. FACS analysis was performed 48h after initiation of transduction.

Earlier experiments made by Relander *et. al.* lead to the observation that gene transfer to $CD34^+$ cells on Retronectin plus high-titer GALV-GFP VCM is impeded due to that noninfectious vector particles (e.g. empty vectors or vector fragments) interfere with and block the viral receptors. When comparing transduction with preload on Retronectin only with cells that are transduced on Retronectin plus VCM, the latter showed a decreased level of GFP+ cells. This inhibitory effect was abolished upon PKC mediated up-regulation of mRNA for GLVR1, the viral receptor for GALV [23, 24].

We transduced enriched $CD34^+$ cells with GALV-GFP and RD-GFP vectors under exposure to the PKC activating agents PMA, bryostatin-1 and PEP005 and compared preload of vector onto Retronectin with preload plus VCM. It was observed that the transduction level was inhibited when GALV-GFP VCM was used in addition to Retronectin on untreated cells, but the inhibition was not abolished subsequent to treatment with PMA as indicated by previous research [23]. The standard deviation for transduction with GALV-GFP VCM in the presence of PMA, however, is high enough to undermine any definite conclusion regarding the level of gene transfer. Treatment with bryostatin-1 as well as PEP005 at transduction resulted in abolishment of the inhibitory effect believed to be caused by receptor-interference (Fig. 11).

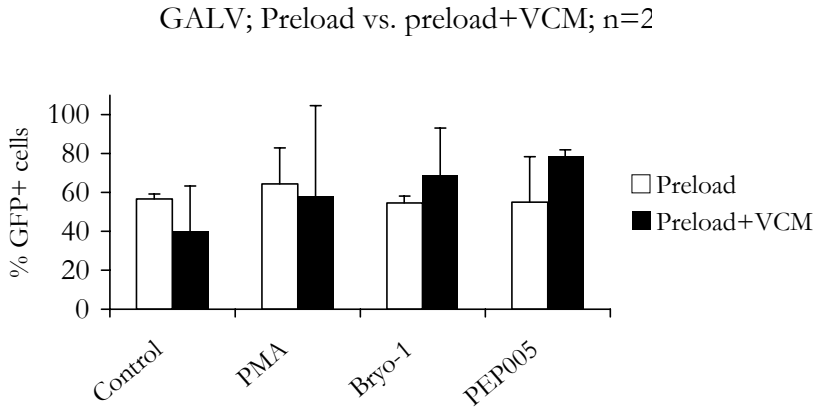

Figure 11. Bryostatin-1 (bryo-1) and PEP005 reverse the inhibition of transduction from GALV-GFP VCM. Cells were prestimulated for 48h and transduced with preload of vector on Retronectin only, or with preload of vector plus VCM.

Using oncoretroviral vector RD-GFP in the same experimental set-up gave no apparent difference with or without PKC activating agent present. In all cases investigated the gene transfer to $CD34^+$ cells was more efficient where transduction was carried out on preload on Retronectin and additional VCM (Fig. 12).

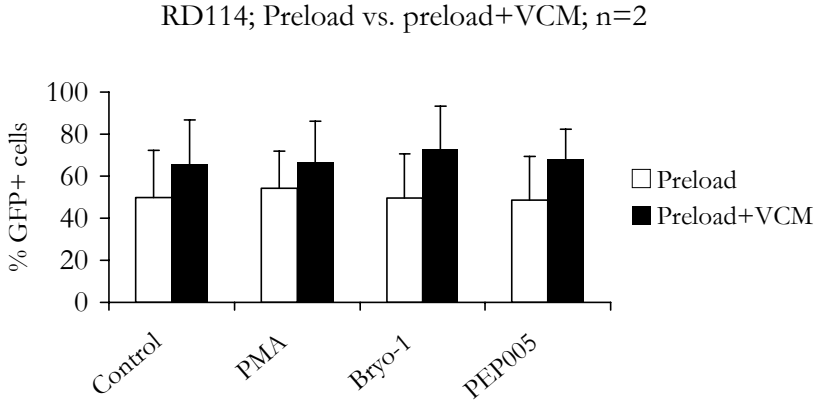

Figure 12. PKC-stimulation does not affect the transduction efficiency with vector RD-GFP. Cells were prestimulated for 48h and transduced with preload of vector on Retronectin only, or with preload of vector plus VCM.

3.2 Expression of mRNA for GLVR1 and RDR

Activation of PKC in $CD34^+$ cells could increase the expression of mRNA for certain surface receptors. We aimed to investigate the effect of PKC stimulating agents PMA, bryostatin-1 and PEP005 on mRNA levels for receptors GLVR1 and RDR.

Two experimental set-ups were performed. First PKC stimulating agents were added at the start of cultivation at the following concentrations: bryostatin-1 1nM, PEP005 20nM and PMA 10nM. The cells were harvested after 3h, 6h and 24h (Fig. 13A).

Second the cells were prestimulated with cytokines for 48h. At 3h, 6h and 24h prior to harvest PKC stimulating agents were added to the wells (Fig. 13B). The cells were harvested, the mRNA was extracted and Q-RT-PCR performed.

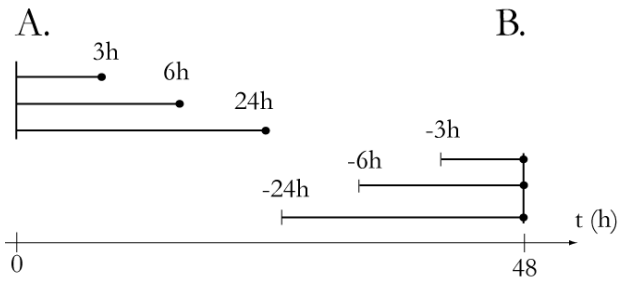

Figure 13. Experimental set-ups for evaluation of influence of PKC-stimulating agents on level of mRNA coding for GLVR1 or RDR. Agents were either added at the initiation of culture and harvested after indicated time spans (A), or prestimulated until agents were added 24h, 6h or 3h before harvest (B).

For each sample the level of mRNA was normalized against the assumably constant expression of the housekeeping gene GADPH.

Treatment as described in Fig. 13B gave Q-RT-PCR results showing that the mRNA for GALV-GFP receptor remained unchanged after treatment with bryostatin-1 compared to cells grown in the absence of agent. PEP005 and PMA displayed a time-dependent increase of mRNA level for GLVR1. The most distinct increase was noticed after 24h of exposure to PEP005 and PMA respectively. Treatment with PEP005 for 24h lead to a 7,7-fold increase (standard deviation=2,69, n=2), and for PMA a 16,7-fold increase (SD=2,49, n=2) (Fig. 14).

The mRNA receptor level in thawed, uncultivated $CD34^+$ cells was higher than in cultivated, untreated cells. This phenomenon has earlier been seen for amphotropic retrovirus receptor (amphoR) where freshly thawed $Lin^-CD34^+CD38^-$ cells from UCB have a significantly higher level of receptor mRNA compared to cultivated cells [13].

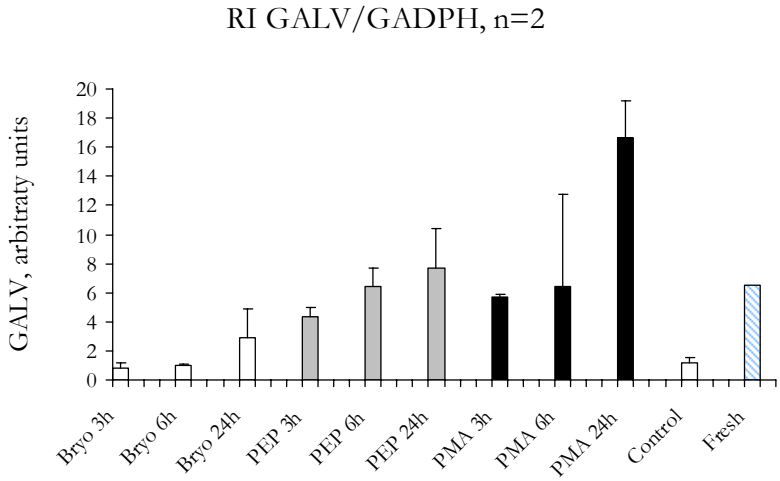

Figure 14. Level of mRNA coding for GALV receptor in CD34+ CB cells. Cells were cultivated for 48h. PKC-activating agents were added before harvest as indicated. mRNA was isolated from harvested cells, concerted into cDNA and quantified by Q-RT-PCR and normalized to GADPH as described in the Materials and methods section.

The receptor for retrovirus RD114, RDR, was equally evaluated in Q-RT-PCR. The mRNA for RDR did not show any increase as a response to PKC stimulation. All of the samples evaluated, including fresh and control cells, showed mRNA levels between 2,4 – 4,8 times higher compared to GADPH (SD = 0,07 – 1,8; n=2)(Fig. 15).

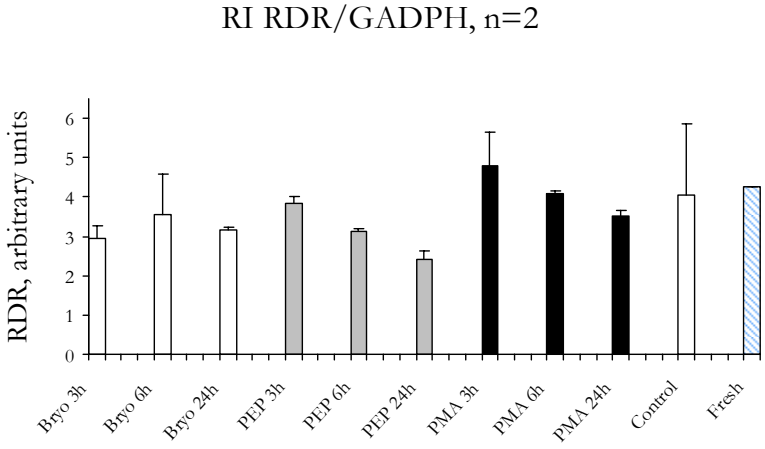

Figure 15. Level of mRNA coding for RD114 receptor in CD34+ CB cells. Cells were cultivated for 48h. PKC-activating agents were added before harvest as indicated. mRNA was isolated from harvested cells, concerted into cDNA and quantified by Q-RT-PCR and normalized to GADPH.

Exposure to PKC stimulating agents directly at the initiation of culture (Fig. 13A) gave similar results as for the ones prestimulated for 48h. The samples within one set showed the same trend: mRNA expression of RDR was at its highest after 3h cultivation and subsequently decreased at 6h and 24h (Fig. 16).

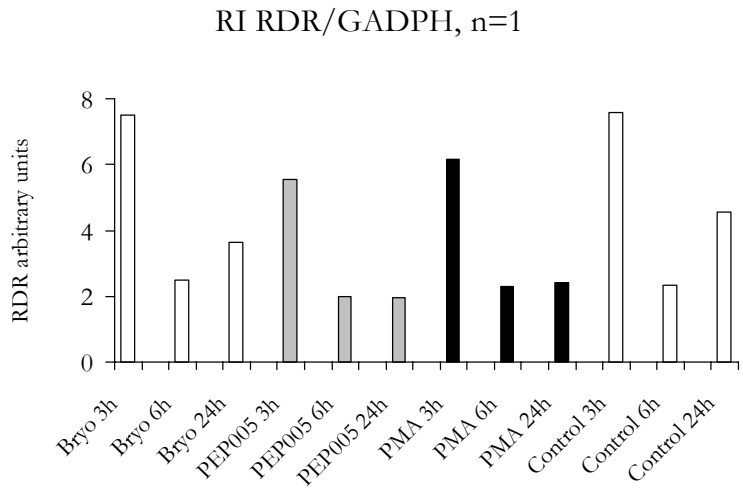

Figure 16. Level of mRNA coding for RD114 receptor in $CD34^+$ CB cells. At initiation of cell culture PKC-stimulating agents were added as indicated. After 3 – 24h cells were harvested. mRNA was isolated from harvested cells, concerted into cDNA and quantified by Q-RT-PCR and normalized to GADPH as described in the Materials and methods section.

4. Discussion

Gene transfer to HSCs has been limited by the relative quiescence of the cells, and also by low expression of viral receptors [25, 26]. While it is possible to use certain specific cytokines to push cells into mitosis, the insufficient receptor-level remain a barrier to hematopoietic stemcell targeted gene-transfer. However, activation of PKC has been observed to up-regulate mRNA levels of viral receptor for GALV resulting in increased transduction efficiency [23].

In this project we have evaluated the effect of PKC-activation on transduction efficiency and level of mRNA for two viral receptors. We chose to compare the PKC-activating compounds PMA, bryostatin-1 and PEP005 and their influence on transduction level on human hematopoietic progenitor cells. Vectors containing the marker gene for GFP and pseudotyped with GALV or RD114 envelopes were used in the transduction experiments.

Cells that were prestimulated with cytokines 24h prior to transduction on preload of Retronectin did not show any difference between the various treatments with PMA, bryostatin-1 or PEP005. Moreover, percentage of GFP+ cells was generally lower than for the cells prestimulated 48h. Thus, activation of PKC does not seem to allow for a shortened time of pretreatment for neither of the vectors used. In contrast to these observations RD-GFP has earlier shown to efficiently transduce cord blood $CD34^+$ cells after only 24h of prestimulation with cytokines [26].

Prestimulation for 48h and subsequent transduction on preload of Retronectin gave an over all higher percentage of GFP+ cells, but none of the treatments resulted in higher or lower transduction level compared to the control. Increased concentration of the agents PEP005 and PMA lead to a slight elevation of transduction-level with both vectors. Bryostatin-1 did not have any positive effect on the gene transfer, but on the contrary showed weak tendencies to hamper the transduction at higher concentrations. It is known that bryostatin-1 is a potent activator of some of the PKC isozymes, but it is also a fact that the substance has inhibitory effects on the isozymes it does not stimulate and can even act as an antagonist of PKC-mediated effects [27]. The isozymes activated by bryostatin-1 might not be the particular ones that induce an up-regulation of mRNA for the viral receptors studied in this project.

To further compare the effect of PKC stimulation, comparison was made between human progenitor cells transduced on Retronectin only and cells transduced on Retronectin with supplement of vector containing medium. This experiment was made with GALV pseudotyped vector and under exposure to PMA by Relander *et. al.*. The observation was made that high-titer VCM does supress the gene transfer and it was thought to be caused by interference by nonfunctional vector particles. When producing viral vectors from packaging cell lines only 0,5-

1% of the particles are transduction competent [28]. The rest consist out of e.g. empty envelopes or fragments with no function, but that are still able to interfere with the viral receptors upon transduction. This means that the ratio between vector particles and cellular receptors can become a limiting factor if the titer is too high, and a way to overcome this negative effect would be increase of receptor expression, something that could be done by activation of PKC. Our results show the same inhibitory effect of GALV-GFP VCM compared to transduction on preload only as was observed by Relander *et.al.* However, exposure to PMA did not reverse the inhibition even if the difference between preload and preload plus VCM was decreased with PMA present. This is somehow contradictory when considering that GALV receptor mRNA (GLVR1) was shown to be up-regulated after 6-24h exposure to PMA. It might be worth to take into consideration the notably high standard deviation of transduction on preload plus VCM in presence of PMA.

Treatment with PEP005 led to increased mRNA expression for GLVR1, and in this case the same substance abolished the inhibition by VCM at transduction which would support the hypothesis that increased receptor expression sustains entry of viral particles even in the cases of high titer. Bryostatin-1 also showed to abolish the inhibition of gene transfer even though it did not induce any elevation of mRNA. On the other hand, as has previously been noted, treatment with bryostatin-1 didn't lead to any significant improvement in transduction on preload only.

RD-GFP vector caused a higher percentage of transduced cells compared to GALV-GFP in all experiments that were carried out during this project. Even when compensating for the higher titer of RD-GFP vector ($1{,}4x10^6$) by diluting to 5-20% the transduction was notably higher than GALV-GFP (titer $2x10^5$). Recent evidence has shown that the levels of mRNA coding for viral receptor RDR (identified neutral amino acid transporter) is significantly higher than mRNA levels for GLVR1 in human cells enriched for $Lin^-CD34^+CD38^-$ [14]. In addition RD114 pseudotyped vectors have proven to be quite efficient when it comes to gene transfer to human progenitor cells and were shown to be more efficacious than both amphotropic- and GALV viral particles in this respect [26]. The results we have seen after transduction with RD114 pseudotyped vectors are compatible with these findings. Apart from the fact that RD-GFP gave the best over-all transduction levels, no influence from PKC activation was observed for either substance that was tested. Hence, it seems like the receptor level for RDR is abundant or in any event doesn't bring about any hindrance of viral entry. Quantification of mRNA coding for viral receptor RDR showed that the level did not change upon stimulation with PKC. Compared to the control and to the freshly thawed human progenitor cells, all other samples had an amount of RDR mRNA

within the similar range. Thus, the level of receptor mRNA does not seem to be affected by PKC activation, which could be another proof of the efficiency of RD-GFP vectors. Albeit the unchanged mRNA for RDR receptor the vector still transduces progenitor cells to a much higher extent than does GALV-GFP, and high titer VCM does not have any inhibitory effect on RD-GFP.

To summarize, bryostatin-1 does not increase transduction efficiency in the way treatment with PMA is capable of. It was observed to abolish the inhibitory effect caused by GALV-GFP VCM, but for the experiments with preload alone of vector onto Retronectin no increase was detected.
PEP005 may have a positive effect on transduction efficiency as it could reverse the inhibition from VCM, up-regulate mRNA expression of GLVR1 and showed a slight increase of transduction at vector preload on Retronectin. However, the elevated levels of GFP+ cells are still not statistically significant to be conclusive in the context.
Moreover, RD-GFP is a potent vector with high ability to integrate proviral DNA into progenitor cells. The level of viral receptor RDR on human progenitor cells seems sufficient and does not cause any inhibitory events. In addition, according to our observations, the mRNA expression was not governed by any PKC activities and did not change upon treatment with either of PMA, bryostatin-1 or PEP005.

In conclusion, the objective of the present project was to evaluate the effect of PKC-activation on gene transfer to human hematopoietic progenitor cells. Being that the previously used PKC-activator PMA is tumor promoting we set out to investigate the effect of two other substances with known PKC stimulatory properties, both of which have previously been used in the clinic: bryostatin-1 and ingenol 3-angelate (PEP005). Neither of the substances increased the gene transfer to the transduced cells. However, stimulation of PKC is known to induce higher susceptibility of transduction, and other stimulating agents may prove to be able of replacing PMA. Such studies could be the object for future investigations.

5. Acknowledgements

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