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Titel der Diplomarbeit

The Influence of the -1639 G>A Variant on the Expression of the Vitamin K Epoxide Reductase Gene in hFOB1 Cells

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A	adenin
A. bidest	Aqua bidestillata
Abmd	areal bone mineral density
ALP	bone specific alkaline phosphatase
ATCC	American Type Culture Collection
BAP	bone specific alkaline phosphatise
BMD	bone mineral density
BMP2	bone morphogenetic protein 2 gene
bp	basepairs
C	cytosin
Ca2+	Calcium
cDNA	complementary DNA
CFU	colony forming units
cOC	carboxylated osteocalcin
CTX	Type I collagen C-telopeptides
ddH$_2$O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPD	deoxypyridioline
DPE	downstream promoter element
EDTA	ethylenediaminetetraacetic
fw	forward
FBS	fetal bovine serum
G	guanine
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gas6	growth-arrest-specific protein
GFP	green flourescent protein
GGCX	gamma glutamyl carboxylase
GLA	gamma carboxy glutamyl

GLU	glutamyl
l	litre
LAR II	luciferase assay reagent
LD	linkage disequilibrium
mg	milligram
min	minute
ml	millilitre
mM	millimol
mRNA	messenger ribonucleic acid
ng	nanogramm
nm	nanometer
NTX	Type I collagen N-telopeptides
OC	osteocalcin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PICP	c-terminal propeptide of type I collagene
PINP	intact n-terminal propeptide of type I collagene
pmol	picomol
PTH	parathyroid hormone
RLU	relative luminescence unit
RNA	ribonucleic acid
rv	reverse
rpm	rounds per minute
sec	seconds
SD	standard deviation
SNP	single nucleotide polymorphism
TRACP5b	TRACP isoform 5b
U	units
ucOC	undercarboxylated osteocalcin
UTR	untranslated region
V	volt

VKCFD	combined deficiency of vitamin k-dependent clotting factors
VKOR	vitamin K epoxide reductase
VKORC1	vitamin k epoxide reductase complex subunit 1
WHO	world health organisation

I. ABSTRACT

Vitamin K epoxide reductase complex subunit 1 (VKORC1) plays a central role in γ-carboxylation – an important biological process. During γ-carboxylation glutamic acid residues are carboxylated to γ-carboxyglutamate (Gla). Several proteins, among them osteocalcin, are only functionally active after γ-carboxylation, a vitamin K dependet process. In the γ-carboxylation process, vitamin K is converted to vitamin K epoxide, which needs to be recycled to vitamin K by reduction. VKOR is centrally involved in this process.

VKORC1 is highly polymorphic, and SNPs have been identified in the promoter, the coding and the 3´-region of the gene. The -1639G>A promoter polymorphism is a functional SNP in VKORC1 and the A allele is associated with a significant decrease of VKORC1 promoter activity and with a 2-fold lower mRNA expression (reported for liver cells). In this study, the influence of the -1639G>A polymorphism in the VKORC1 promoter on the promoter activity in osteoblasts was studied using a dual luciferase reporter gene assay.

Two transfection methods were compared: Lipofectamine 2000 and Metafectene Pro. While transfection with Lipofectamine 2000 was not suitable Metafectene Pro was successful. Following transfection of osteoblasts with Metafectene the reporter gene assay indicated a 43% higher activity of the promoter carrying the G at position -1639 compared to the A variant. This is in agreement with the results reported for liver cells.

An analysis of the impact of the promoter G and A variants on the expression of VKORC1 mRNA in osteoblasts was attempted. However, the experiments were not successful, and further studies will be necessary to answer this question.

II. ZUSAMMENFASSUNG

Vitamin K Epoxid Reduktase Untereinheit 1 (VKORC1) ist ein Gen, dass eine zentrale Rolle bei dem wichtigen biologischen Prozess der γ-Carboxylierung spielt. Die γ-Carboxylierung ist ein Vitamin K abhängiger Vorgang wobei Vitamin K (der Cofaktor der γ-Carboxylierung) zu Vitamin K Epoxid konvertiert wird, welches durch VKOR wieder zu Vitamin K rezykliert werden muss. Während der γ-Carboxylierung werden in Vitamin K abhängigen Proteinen wie Osteocalcin, Glutaminsäurereste zu gamma-Carboxyglutaminsäure (Gla) umgewandelt. Diese posttranslationale Modifikation ist für die biologische Aktivität verschiedener Proteine essenziell. Osteocalcin wird von Osteoblasten gebildet und besitzt im carboxylierten Zustand, die Fähigkeit Ca^{2+} in Knochen einzubauen.

Der -1639G>A Polymorphismus ist ein funktionaler SNP in VKORC1, wobei das A Allel mit einer signifikanten Verminderung der Promoteraktivität und einer 2-fach niedrigeren mRNA Expression assoziiert ist, was für Leber Zellen dokumentiert ist. Diese Studie befasst sich mit dem Einfluss des -1639G>A Polymorphismus auf die Expression von VKORC1 in Osteoblasten. Um dies untersuchen zu können, wurden Plasmide, die den VKORC1 Promoter mit entweder dem G oder dem A Allel an der Position -1639 enthielten, in Osteoblasten transfiziert und die Promoteraktivität mittels einem Dual Luciferase Reporter Gene Assay ermittelt.

Es wurden zwei verschiedene Transfektionsmethoden verglichen – Lipofetamine 2000 und Metafectene Pro, wobei nur die Transfektion mit Metafectene zuverlässige Ergebnisse lieferte.

Die Ergebnisse des Reporter Gene Assays von Metafectene transfizierten Zellen zeigten eine 43% höhere Promoter Aktivität für Promotoren die das G Allel enthielten. Offensichtlich bedingt das A Allel, nicht nur in

Leberzellen sondern auch in Osteoblasten, eine verminderte Promoteraktivität.

Um eine Verbindung zwischen dem Effekt des -1639G>A Polymorphismus und Osteoporose herstellen zu können, wäre es notwendig zu zeigen, dass derselbe Effekt auch dann noch zu beobachten ist, wenn der VKORC1 Promoter das VKOR Gen selbst kontrolliert anstatt des Luciferase Gens. Dafür würde man ein full length Konstrukt des VKORC1 Gens benötigen, doch der Versuch eines herzustellen schlug leider fehl.

III. INTRODUCTION

1. Vitamin K epoxide reductase complex subunit 1 (VKORC1)

The activity of VKOR was first reported in 1970 by Bell R and Matschiner J, but it took more than 30 years until it was possible to purify VKORC1.

Until 2004 several groups, Li et al. among them, tried to purify the VKOR gene, but they were not successful. In 2004, Li et al. started a new attempt to identify the VKOR gene on account of two reports of other research groups: One study reported four markers for warfarin resistance on the mouse chromosome 7 and on human chromosomes 10, 12 and 16 [Kohn and Pelz, 2000]. The second study discovered a gene for combined deficiencies of vitamin K dependent (VKD) proteins located on the human chromosome 16p12-q21 [Fregin et al., 2002]. These findings prompted Li and collegues to focus their search for the VKOR gene on chromosome 16. Out of 190 predicted coding sequences in the region 16p12-q21, 13 genes coding for integral membrane proteins could be identified and were chosen for further analysis. Consequently, the VKOR gene could be identified by specific inhibition of VKOR activity by a single siRNA pool.

At the same time, also in 2004, another group of researchers (Rost et al. from the laboratory of J. Oldenburg) identified the VKORC1 gene independently of Li et al. Rost, used genetic linkage analysis and positional cloning based on sequence similarity between rats and humans [Rost et al., 2004].

In 2004 both, the laboratory of Oldenburg and the laboratory of Stafford were able to identify the VKORC1 gene.

1.1. Structure and Function of the VKORC1 gene

The VKORC1 gene (VKORC1; GenBank accession number AY587020) is located on chromosome 16p 11.2, spans 5126 bp and consists of three exons and two introns. A large family of homologues with significant sequence similarities exists among plants, archaea, bacteria, vertebrates and arthropods [Goodstadt et al. 2004].

The VKORC1 gene codes for a transmembrane protein, located in the mammalian endoplasmatic reticulum [Cain et al. 1997] and consists of 163 amino acids [Li T. et al. 2004]. The protein is composed of three transmembrane domains, the N-terminus is located in the lumen of the endoplasmatic reticulum and the C- terminus is exposed to the cytoplasm [Stafford DW 2005]. Goodstadt and Ponting [2004] found four cysteine residues and one serine/threonine residue, which are absolutely conserved (Cys 43, Cys 51, Cys 132, Cys 135, Ser/Thr 57). They are considered to form the active site of VKORC1 as no other conserved polar residues have been found.
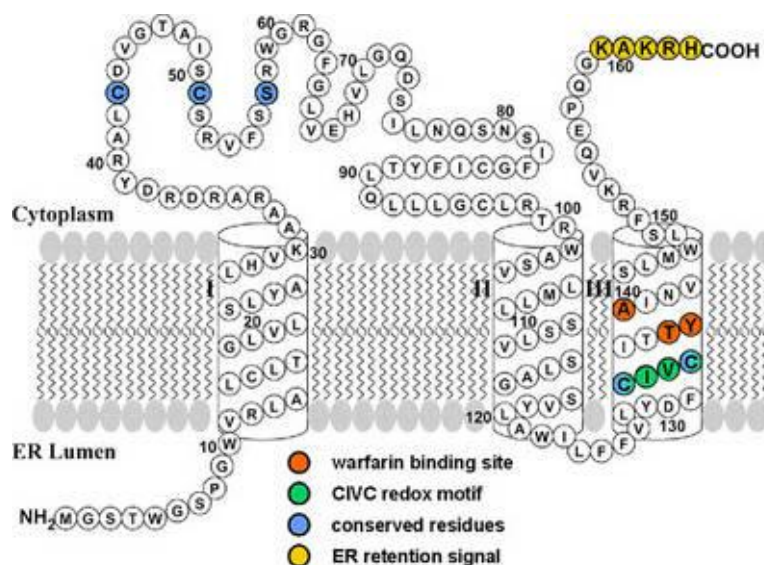
Fig. 1. Proposed membrane topology of VKORC1
[Oldenburg et al. 2007: p. 3, figure 3]]

VKOR is primarily expressed in the liver, kidney, heart and skeletal muscle [Wang Y. et al 2005], and plays a central role in the important biological process of γ-carboxylation. During γ-carboxylation glutamic acid residues are carboxylated to γ-carboxyglutamate (Gla). γ-carboxylation is a vitamin K depending process during which vitamin K hydroquinone, the cofactor for γ-carboxylation, is converted to vitamin K epoxide. The latter form of vitamin K is not active and has to be reduced to vitamin K hydrochinon again. For the reduction VKOR is necessary [Wang et al 2006]. The following Vitamin K-dependent proteins have to be γ-carboxylated: the coagulation factors VII, IX, X, prothrombin, protein S, protein Z and protein C, osteocalcin and matrix Gla-protein, proteins active in bone metabolism, and Gas 6 (growth-arrest-specific protein 6) which is involved in cell proliferation and apoptosis [Rost et al 2005].

1.2. Polymorphisms in VKORC1

In VKORC1 28 SNPs (single nucleotide polymorphisms) were identified by Geisen et al. in 2005. The group found three silent SNPs in the coding region of VKORC1, and 25 SNPs located in non-coding regions: 7 SNPs in the promoter region, 11 in introns, and 5 in 5'UTR (untranslated region) [Geisen et al. 2005].

Six of these SNPs form three main haplotypes (VKORC1*1, VKORC1*2, VKORC1*3) which cover more than 99% of the genetic variability of the VKORC1 gene in Europeans [Geisen et al. 2005]. The VKORC1*2 haplotype comprises the -1639G>A promoter polymorphism, which is the functional SNP in VKOR, and is in perfect linkage disequilibrium (LD) with the 1173C>T polymorphism in intron 1 of the VKORC1 gene [Geisen et al. 2005; Wang et al. 2008].

There are two main diseases caused by mutations in VKORC1: (i) combined deficiency of vitamin K dependent coagulation factors type 2

(VKCFD2) caused by a homozygous missense mutation in the VKORC1 gene, and (ii) the hereditary warfarin resistance as result of a heterozygous missense mutation in VKORC1 [Geisen et al. 2005].

Warfarin is a widespread anticoagulant drug used for the prevention of thromboembolic diseases for patients with mechanical heart valve replacement, deep vein thrombosis and atrial fibrilation [Yuan et al. 2005]. Warfarin sensitivity and the dose requirement for warfarin is variable between patients and differs between different ethnic populations. This variability is associated with a polymorphism in the promoter region of the VKORC1 gene at position -1639. Yuan et al [2005] found out that all warfarin-sensitive patients were homozygous AA at the position -1639 and had lowest dose requirements for warfarin. In contrast, patients who were heterozygous AG or homozygous GG at the position -1639 required an intermediate or high warfarin dose, respectively. By measuring the mRNA expression, using a reporter gene assay, Yuan et al. [2005] detected a 44% higher VKORC1 promoter activity for the -1639 GG genotype compared to the -1639 AA genotype.
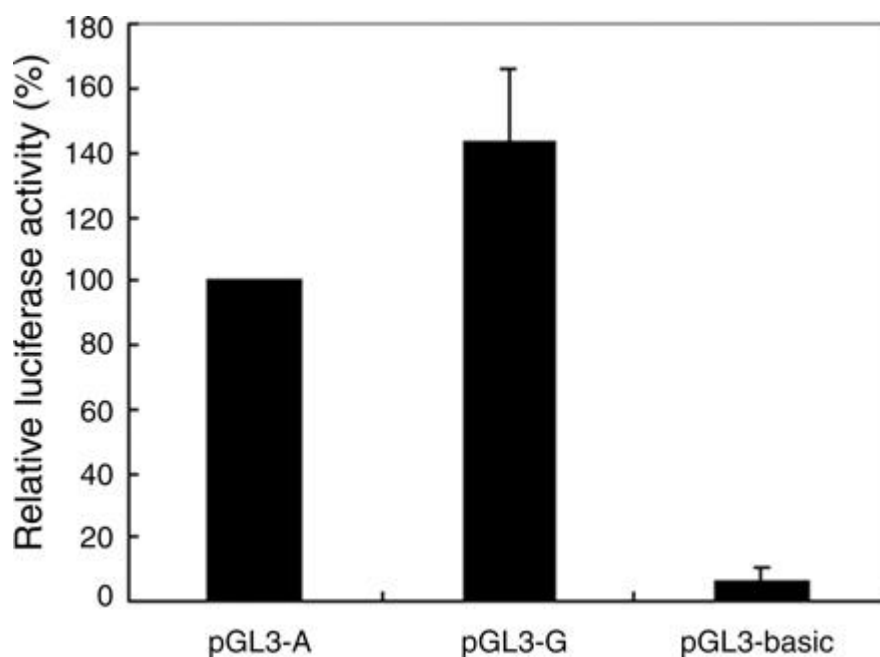
Fig. 2: Promoter activity of the plasmids pGL3-A, pGL3-G and pGL3-basic
[Yuan et al. 2005: p.1749, figure 2]

The VKORC1*2 haplotype with the -1639A allele also explains the differences in warfarin requirement between ethnic populations. Chinese and Malay require lower warfarin dose than Europeans and Indians and in Blacks the overall mean warfarin dose is higher compared to Whites [Wang et al. 2008]. Wang et al [2008] showed that the mean warfarin dose is identical in these two ethnic groups when they are grouped by the -1639G>A and 1173C>T genotype, so the greater overall mean in Blacks is a result of the lower frequency of the minor -1639 A and 1173 T alleles which require reduced dosing of warfarin.

2. Promoters

Transcription involves synthesis of an RNA chain, which is identical to the coding strand and complementary to the template strand of a DNA duplex. Synthesis of RNA is catalyzed by the enzyme RNA polymerase. Transcription starts when the RNA polymerase binds to the promoter, which contains the start point. From this start point, RNA polymerase moves along the DNA molecule synthesizing RNA in 5'→3' direction. Transcription ends when the RNA polymerase reaches a terminator sequence.

2.1. Bacterial promoters

The sequence of a large part of the promoter is not important, but there are some short stretches within the promoter which are conserved and which are very important for its function as a binding site for RNA polymerases as well as other proteins. In bacterial promoters there are four (sometimes five) conserved features: the start point, the -10 sequence, the -35 sequence and the separation between the -10 sequence and the -35 sequence [Knippers R., 2006].

- The startpoint:

    is usually a purine (very often the central base of an CAT triplet).

- The -10 sequence:

    is a 6 bp region directly upstream of the startpoint. The center of the hexamer generally is close to 10 bp upstream of the start point and the consensus of this -10 sequence is TATAAT.

- The -35 sequence:

    is also a conserved hexamer with the center close to -35 bp upstream of the startpoint and the consensus is TTGACA.

- The distance separating -10 and -35:

    in 90% of the promoters the distance between the -10 sequence and the -35 sequence is 16 – 18 bp. It is not important what sequence the actual intervening region is, but holding the appropriate distance between the two sites is critical for the geometry of the RNA polymerase.

- The UP element:

    The UP element is an AT rich sequence further upstream, which is typically found in highly expressed promoters. Its function is to interact with the α - subunit of the RNA polymerase [Knippers R., 2006] .

2.2. Eukaryotic promoters

Like bacterial promoters, eukaryotic promoters also contain conserved sequences which are essential to the function of the promoters as a binding site of RNA polymerases. There are four such elements: (i) the initiator element, (ii) the TATA – box, (iii) the downstream promoter element (DPE) and (iv) the CpG islands. In contrast to bacterial promoters,

eukaryotic promoter elements are not as highly conserved as bacterial ones and the promoters do not necessarily comprise all of the promoter elements [Knippers R., 2006] .

- The initiator element:

  is a pyrimidin-rich sequence containing the start point of eukaryotic transcription which is a purine, usually an adenine.

- The TATA-box:

  The TATA-box is an AT rich stretch of conserved DNA. Its consensus is TATAAA and it is located in the region between -26 bp and -34 bp upstream of the startpoint. This TATA box is similar to the -10 sequence in bacterial promoters. It is rarely found in promoters of housekeeping genes but very frequent in genes which are regulated.

- The downstream promoter element:

  The DPE is located in the range of +28 bp to + 32 bp downstream of the startpoint. Its sequence is not highly conserved as it is in the case of the initiator sequence. Its consensus is A/T. G. A/T. C/T. G/A/C.

- The CpG islands:

  Most of the promoters of housekeeping genes are characterized by stretches of consecutive GC basepairs including CpG islands. CpG islands are special CG elements with the consensus of GGGCGG [Knippers R., 2006].

2.3. The promoter variant -1639G>A of the VKORC1 gene

Promoter polymorphisms are usually point mutations within the promoter sequence. The sequence change may alter binding sites for proteins that

are necessary for the expression of the gene. As a result, the expression of the gene increases or decreases [Murken et al. 2006].

This seems to apply also to the -1639G>A polymorphism in VKORC1. Wang et al [2008] showed that the -1639G>A promoter polymorphism is a functional SNP in VKORC1. This polymorphism is located in the second nucleotide of an E-box with the consensus sequence CA/GNNTG. The SNP changes the second base of the E-box from A to G, which abolishes the E-box consensus sequence and leads to changes of the VKORC1 promoter activity [Yuan et al. 2005].

The A allele of this -1639 SNP is associated with a significant decrease of the VKORC1 promoter activity and therefore with a lower VKORC1 mRNA expression [Geisen et al. 2005]. Wang et al. [2008] confirmed the earlier data indicating that the A allele of this polymorphism decreases promoter activity. They used HepG2 cells (liver cells) and measured the mRNA expression using a reporter gene assay. Like the group around Geisen [2005] they too found that the -1639A allele is associated with a 2-fold lower mRNA expression compared to the -1639G allele, but this decrease of promoter activity is limited to HepG2 cells and could not be observed in other tissues like B-lymphocytes or heart tissue.

3. Bone

3.1. Bone physiology

Bone tissue consists of bone cells, bone matrix, and mineral salts. The bone matrix represents the organic part of bone and includes collagen fibers which are important for the flexibility of bone. Mineral salts, such as hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ and calcium phosphate $[Ca_8H_2(PO_4)_6(H_2O)_5]$, form the anorganic part of bone and make up about

2/3 of the bone weight. These mineral salts are incorporated between the collagenous molecules and guarantee hardness and firmness of the bone [Seibl MJ et al. 2006; Bilezikian JP et al. 2002]

Our skeleton is formed of more than 200 bones which have three important functions: (i) They support posture and motion of the human body and (ii) protect the inner organs, the central nervous system and bone marrow. To be able to fulfil these two tasks, bone has to be hard, flexible and light at the same time. (iii) Third, bone is the biggest calcium reservoir in the human body and about 99% of the calcium is stored in bone [Seibl MJ et al. 2006; Bilezikian JP et al. 2002].

There are two types of bone tissue: spongy (trabecular) bone which is composed of little beams, and compact (cortical) bone which is very dense. Both bone types are solid mineralized matrices with small canals (canaliculi), spaces (lacunae), and bone cells. In trabecular bone, the matrix, lacunae and bone cells are organized in the form of thin interconnecting spicules, whereas cortical bone is organized in Haversian systems or osteons, consisting of a canal containing a blood vessel surrounded by concentric and interstitial lamellae [Seibl MJ et al. 2006; Bilezikian JP et al. 2002].

3.2. Bone formation

Bone consists of the organic matrix, water and different types of bone cells which are osteoblasts, osteoclasts, lining cells and osteocytes. Osteoblasts synthesize and mineralize the collagenous extracellular matrix of bone. Bone is a dynamic tissue that is remodelled continuously throughout life to renew the skeleton and to maintain its structural and anatomical function. Bone remodelling is characterized by constant resorption and formation of bone and it is essential for adapting bone to changing mechanical strain of the skeleton. This process of bone

formation is performed by osteoclasts that remove old bone, and osteoblasts that subsequently form new bone [Manolagas et al. 1995].
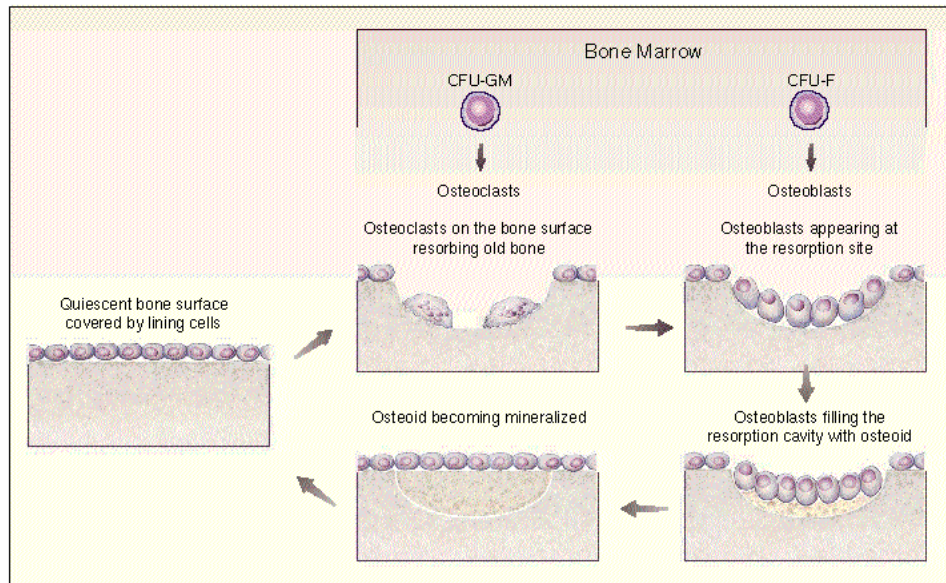
Fig. 3: Schematic illustration of the bone remodelling process
    CFU-GM – hematopoetic progenitor cell, CFU-GM – mesenchymal progenitor cell
    [Manolagas et al. 1995: p.306, figure 1]

Normally, bone remodeling proceeds in cycles. The removal and resorption of old bone is initiated by osteoclasts which lyse small areas of bone by acidification and proteolytic digestion. After resorption of the bone, the osteoclasts leave the resorption site and osteoblasts invade to form new bone by secreting a matrix of collagen and other proteins, called osteoid, which is eventually mineralized. Finally, the surface of the new bone is covered by lining cells [Manolagas et al. 1995].

Osteoblasts as well as osteoclasts originate in the bone marrow, but the marrow progenitors of both cells differ. Osteoclasts derive from the hematopoetic linage, while osteoblasts derive from pluripotent mesenchymal stem cells of the bone marrow [Manolagas et al. 1995, Koshihara et al. 2003].

3.3. Osteoblasts

Pluripotent mesenchymal stem cells as well as hematopoetic stem cells originate in the bone marrow and cooperate through direct cell-to-cell interaction and release of cytokines and growth factors. The progenitor cells of osteoblasts, called fibroblast colony-forming units (CFU), form adherent colonies of stromal cells in marrow cell-cultures, which can become osteoblasts as well as chondrocytes, adipocytes, fibroblasts and muscle cells [Manolagas et al. 1995].

Osteoblasts play an essential role in bone formation and bone remodelling since they synthesize and mineralize the collagenous extracellular matrix of bone. In the beginning of bone formation the new bone is not mineralized yet. Only after deposition of hydroxyapatite, the new bone reaches the mechanical firmness that is needed to fulfil its anatomical and structural function [Pietschmann et al. 1999].

Osteoblasts are also important for the development of osteoclasts as their differentiation of hematopoetic precursers can only be accomplished in the presence of stromal-osteoblastic cells, which mediate the effects of systemic hormones like parathyroid hormone and 1,25-dihydroxyvitamin D_3, and locally produced factors, such as interkeulin-6 and interleukin-7, which stimulate the development of osteoclasts [Manolagas et al. 1995].

3.4. Osteocalcin

Osteocalcin, a low-molecular-weight protein of 49-50 amino acids [Binkley et al. 1995], is produced in osteoblasts during bone matrix formation and accounts for 15-20% of the non-collagen proteins in the bone matrix.

It comprises two domains: the Gla domain and the C-terminal domain. In the Gla domain there are three glutamyl (Glu) residues at the positions 17,

21 and 24, which can be post-translationally modified to γ-carboxyglutamyl (Gla) residues in a γ-carboxylation process [Pearson A. 2007]. These Gla residues have a highly specific affinity to the calcium ion of the hydroxyapatite molecule [Price 1989], and they are able to bind Ca^{2+} ions. Promoting a protein - Ca^{2+}- phospholipid interaction through binding Ca^{2+} ions is essential for the biological activity of osteocalcin [Esmon et al. 1975]. Because of the ability to bind calcium in bone, the concentration of osteocalcin in bone is directly proportional to the amount of calcium [Hauschka and Reid 1978, Lian et al. 1982]. Not all newly synthesized osteocalcin is incorporated into bone, a small amount of osteocalcin is passed into the circulation where it can be measured and used as a marker for bone formation [Delmas et al. 1985].

The C-terminal domain of osteocalcin is important for chemotaxis for osteoclast-like cells [Glowacki et al. 1991, Liggett et al. 1994], because of the ability of this domain to build up a concentration gradient [Vermeer et al. 1996].
Synthesis and posttranslational modification of osteocalcin are dependent on vitamin D and vitamin K. The dependence on vitamin D is due to a vitamin D-responsive element in the promoter of osteocalcin, which is able to stimulate the synthesis of osteocalcin directly [Lian et al. 1989], Vitamin K is important because of its function as a cofactor in the posttranslational γ-carboxylation of the three glutamic acids residues in preosteocalcin.

4. Osteoporosis

Osteoporosis is a multifactorial disorder of bone. It is characterized by a reduced bone mass and bone mineral density (BMD) with an increased fragility of the bone and as a result an increased risk of fractures. The World Health Organisation (WHO) criteria for the diagnosis of osteoporosis are based on comparison with peak adult bone mass measured by bone densitometry. According to these criteria, osteoporosis

is characterized by a bone mineral density of more than 2.5 standard deviations (SD) below the mean value of peak bone mass in young healthy individuals. A moderate decrease of BMD in the range of at least 1 SD to no more than 2.5 SD below the mean value of the peak bone mass is called osteopenia [Weber P. 2001].
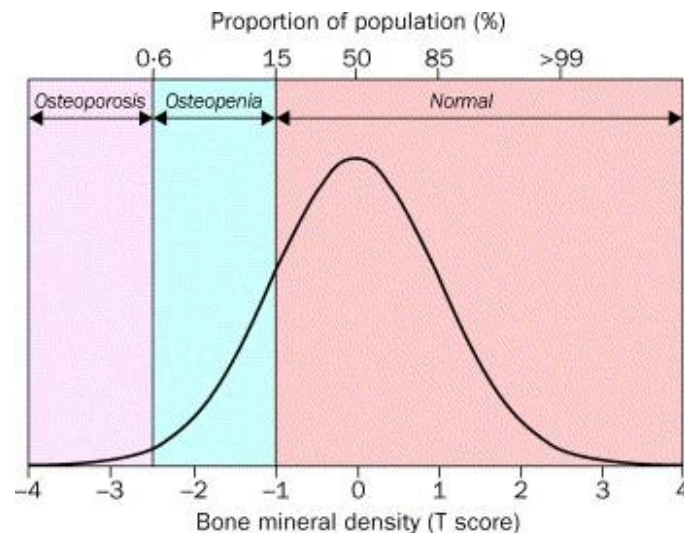
Fig. 4: Distribution of bone mineral density in healthy women aged between 30-40 years. [Kanis JA; 2002]

The loss of bone mass is a result of an imbalance between bone formation and bone resorption. In healthy bone, bone remodeling is necessary to maintain bone strength and to be able to react on changing mechanical strain. An imbalance of bone resorption and bone formation can be responsible for excessive removal of bone and too little bone formation which leads to reduced bone mass [Pietschmann and Peterlik 1999].

Such reduced bone mass usually concerns both cortical and trabecular bone, but not necessarily to an equal extent. The effects on cortical bone are thinning of the cortex [Peacock et al. 1998, Atkinson PJ 1965] and increased intracortical porosity [Atkinson PJ 1965, Bousson et al. 2001], whereas the effects in trabecular bone refer to trabecular thinning [Atkinson PJ 1967, Aaron et al. 1987] and loss of trabecular connectivity [Aaron et al. 2000, Dempster DM 2000].

Two main forms of osteoporosis can be distinguished: (i) primary osteoporosis, including the postmenopausal and the senile osteoporosis, and (ii) secondary osteoporosis - this form is caused by other diseases like diabetes mellitus, diseases of the liver, immobilization, malabsorption, chronic treatment with glucocorticoids, therapy with anticoagulants, and alcohol abuse [Pietschmann et al. 1999].

Postmenopausal osteoporosis:

The major pathophysologic factor for the development of the postmenopausal osteoporosis is estrogen deficiency in women after the menopause. An important determinant thereby is the severity of the estrogen deficiency. Several studies proved that a lack of estrogen leads to increased bone resorption, since estrogen inhibits osteoclastogenesis [Schiller et al. 1997], reduces the resorption activity of osteoclasts [Pacifici et al. 1991] and inhibits the production of different cytokines which improve bone resorption [Pietschmann et al 1999]. Therefore, postmenopausal bone loss is associated with excessive osteoclast activity. The bone loss occurs primarily in trabecular bone [Manolagas et al. 1995].

There are also other factors that play a role in postmenopausal osteoporosis, such as the peak bone mass which is the maximum bone density at the end of the development of the skeleton, the extent of physical exercise, environmental influences, and genetic determinants.

Senile osteoporosis:

Senile osteoporosis develops after 70 to 75 years of life. The main determinant of this type of osteoporosis is a vitamin D deficiency which is common among elderly people, because of reduced exposure to the sun light, imbalanced diet and reduced kidney-activity, especially among patients living in nursing homes. This lack of vitamin D leads to a decrease of serum calcium which is compensated by an increased secretion of

parathyroid hormone. This can keep the serum calcium in the normal range, but still results in increased bone resorption. The bone loss of senile osteoporosis affects mainly cortical bone [Manolagas et al. 1995].

Another reason for bone loss in aging is the fact that in each remodeling cycle the amount of formed bone decreases with age, because there are not enough osteoblasts available in proportion to the demand for them, and the demand for osteoblasts is determined by the frequency of new cycles of bone remodeling [Manolagas et al. 1995].

4.1. Environmental factors for osteoporosis

As mentioned above, osteoporosis is a multifactorial disease with several risk factors including genetic factors, environmental factors such as smoking, alcohol abuse, low calcium intake, delayed menarche, low body weight, and physical inactivity.

Adverse risk factors	Protective factors
Smoking	High phytoestrogen intake
Alcoholism	Sports activity
Low calcium intake	Pregnancy
Vitamin D insufficiency	
Low body weight	
– BMI <18-20 kg/m^2	
Estrogen deficiency	
– Delayed menarche	
– Early menopause	
– Bilateral ovariectomy	
– Premenopausal amenorrhea	
High parathyroid hormone	
History of fracture	
– Personal and in first-degree relative	
Caucasian race	
Advanced age	
Female	
Dementia	

Table 1. Clinical risk factors for osteoporosis [Kung AWC and Huan QY; 2007]

A study of Cornuz et al. [1999], which was running over 12 years and included 116,229 female nurses aged 34-59 at the beginning of the study,

showed that smokers are more likely to have hip fractures than non-smokers. Ten years after stopping of smoking the hip fracture risk decreased. Liu et al. [2003] demonstrated that cigarette smoke extract inhibits the in vitro differentiation of human osteoprogenitor cells into osteoblast-like cells. As a result previous smokers have no increased bone resorption but a lower bone mineral density (BMD) than people who never smoked, because less osteoblast-like cells mean less osteoblasts and therefore less bone formation. This decreased bone formation leads to a lower BMD although bone resorption is on a normal level.

Alcohol also reduces BMD as well as markers of bone turnover and levels of parathyroid hormone, which leads to the suggestion that alcohol may reduce bone remodeling [Rapuri et al. 2000]. A larger study of Hoidrup et al [1999] showed an increased risk of hip fractures depending on the extent of increased alcohol consumption.

Also, inadequate dietary calcium and/or vitamin D levels have negative effects on bone remodeling and BMD, because impaired calcium absorption with a compensatory increase of parathyroid hormone (PTH) levels leads to increased bone resorption [Reginster JY, 2005]. In contrast, BMD and serum $25(OH)D_3$ increase significantly in case of vitamin D and calcium supplementation, whereas serum parathyroid hormone and risk of hip fractures decrease [Freskanich et al., 2003; Grados et al 2003].

Low body weigth (<44kg) was associated with risk of having low BMD at the spine and hip; delayed puberty, characterized with an onset of menstruation beyond 14 years, was also associated with a higher risk for low BMD at the hip. This was also observed for physical inactivity [Kung and Huang 2007].

4.2. Genetic factors for osteoporosis

Despite the key role of environmental factors in osteoporosis, genetic factors seem to have an influence on the development of osteoporosis. Twin- and family studies indicate that 60-85% of the variance in bone mineral density is genetically determined [Krall& Dawson-Hughes 1993; Gueguen et al. 1995]. It is well established that BMD which is widely used as a criterion for the diagnosis of osteoporosis is under genetic control,:

It seems likely that genes which influence BMD are also involved in the development of osteoporosis. However, it is unlikely that there are only few genes with a major influence on BMD. The genetic component of osteoporosis is determined by an assembly of multiple genes, each with a small individual effect. Most likely, each gene is only responsible for less than 5% of the genetic variance in the general population [Kung and Huang 2007].
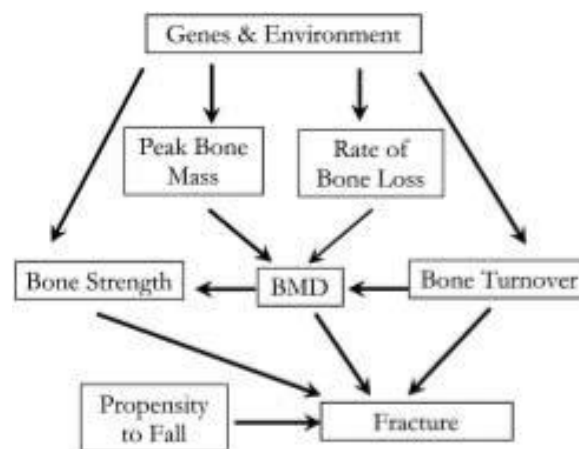
Fig. 5. Common model of susceptibility to osteoporosis and fracture
[Kung AWC and Huang QY, 2007]

Several candidate-gene association studies have been performed. Such studies are powerful instruments for the identification of genes with small effects. By the end of 2005, 63 genes had been associated with BMD and osteoporosis-related phenotypes [Kung and Huang 2007].

Initially, linkage studies were used to identify genes with an effect on BMD: The advantage of linkage studies is their ability to identify both novel genes or pathways and genes with larger phenotypic effects. More than 10 genome-wide linkage scans in humans have been performed and have identified some significant or suggestive linkage regions for BMD [Munafo et al. 2004; Ioannidis et al. 2002; Ioannidis et al. 2004; Efstathiadou et al. 2001; Mann et al. 2001; Gong et al. 1999; Thakkinstian et al. 2004]. A recent meta-analysis of genome-wide linkage scans in osteoporosis revealed that regions on chromosomes 1p, 3p, 6, 10, 16p, 18, 20p and 22q were associated, the region 16p had the strongest association [Lee et al. 2006]

The first gene associated with osteoporosis, was the bone morphogenetic protein 2 gene (BMP2). It was identified by linkage scans in humans. BMP2 is an important regulator of osteoblast differentiation and there are indications that it is significantly associated with osteoporosis risk and BMD [Styrkarsdottir et al. 2003].

4.3. Biochemical markers of bone turnover and fracture risk

The importance of estrogen deficiency in the rapid bone loss around the time of menopause has been recognized for several years [Albright et al. 1941; Lindsay et al. 1980], but circulation estradiol levels explain only a small proportion of the interindividual variance of bone mineral density (BMD) and bone loss [Slemenda et al. 1996]. Measuring areal bone mineral density (aBMD) can predict fractures [Johnell et al. 2005; Stewart et al. 2006], but there is also evidence, that measuring bone metabolism by means of biochemical markers of bone turnover can predict fractures independently of bone mass [Gerdhem et al. 2004; Garnero et al. 1996; Garnero et al. 2000; van Daele et al. 1996]. Markers of bone turnover are biochemical substances in blood and urine that are produced or released

during bone turnover. There are two categories: (i) enzymes or other proteins secreted by osteoblasts or osteoclasts and (ii) substances which are produced during the formation or breakdown of type I collagen, which is the primary protein forming the organic matrix of bone [Looker et al. 2000].

Markers of bone formation:

- Osteocalcin (OC)
- Bone-specific alkaline phosphatase (BAP)
- C-terminal propeptide of type I collagen (PICP)
- Intact N-terminal propeptide of type I collagen (PINP)
- Bone specific alkaline phosphatase (ALP)

Markers of bone resorption:

- Type I collagen N-telopeptides (NTX)
- Type I collagen C-telopeptide (CTX)
- TRACP isoform 5b (TRACP5b)
- Deoxypyridioline (DPD)

Several studies indicate that bone markers are associated with fracture risk. Szulc et al. [1996] showed that total osteocalcin, carboxylated and undercarboxylated osteocalcin (OC) and alkaline phosphatase (ALP) were significantly higher in hip fracture cases than in controls. Other than total OC, cOC and ALP, serum ucOC remained an independent predictor of hip fracture risk when age was included in the model of regression. The age-adjusted odds ratio for hip fracture was three times higher in women with increased undercarboxylated osteocalcin (ucOC) at baseline. Garnero et al. [2000] showed that increased levels of some biochemical markers of bone turnover, including BAP, serum OC, PICP and PINP, are associated with increased risk of hip fractures although only BAP was significant. Moreover, Garnero et al. [1999] were able to show that there is a significantly greater rate of bone loss in postmenopausal women when

these bone formation markers are increased. Thus, it was reported that serum osteocalcin is associated with hip bone loss in elderly women and in perimenopausal women [Chapurlat et al. 2000; Bauer et al. 1999; Dresner-Pollak et al. 1996; Iki et al. 2006]. Lenora et al. [2007] were also able to demonstrate that there is a significant association of serum OC, urinary OC and to some extent serum TRACP5b with bone density loss in hip. Gerdhem et al. [2004] reported a predictive feature of serum TRACP5b and urinary OC for forthcoming (vertebral) fractures.

4.4. Vitamin K

Vitamin K is naturally found in two forms. The primary dietary source of vitamin K is vitamin K_1 (phyllochinone) which is found in high concentrations in leafy, green vegetables and in some oil plants. Vitamin K_2 (menaquinones) is found in animal meats, dairy products and fermented foods (Kalkworf et al. 2004). Vitamin K is a fat-soluble protein and its intestinal absorbtion is dependent on bile-salts that are components of the micelles. These micelles incorporate vitamin K in the small intestine where it is taken up by the intestinal mucosa from which the vitamin is then set free to the lymphatic system and to the circulation [Vermeer et al. 1996].

Hodges et al. [1991,1993] and Feskanich et al. [1999] were able to show that vitamin K has beneficial effects on bone health, and Kameda et al. [1996] proved that Vitamin K_2 affects osteoclasts directly and as a result inhibits bone resorption.

Studies of Koshihara et al. [1996], Koshihara and Hoshi [1997], and Miyake et al. [2001] showed that the inhibition of osteoclastic bone resorption and the stimulation of osteoblastic bone formation relates to MK-4 (menaquinone-4) and is observed in different assay systems and different species. Moreover, Koshihara et al. [2003] could confirm the

suggestion that there is a predominant effect of Vitamin K_2 on bone formation via osteoblasts, by showing that both types of vitamin K promote the differentiation of bone marrow mesenchymal progenitor cells to osteoprogenitor cells and also inhibit the formation of osteoclasts in human bone marrow culture.

4.5. The vitamin K cycle

The vitamin K-dependent γ-carboxylation system is a multicomponent system of proteins in the endoplasmatic reticulum. The system post-translationally modifies vitamin K-dependent proteins such as the coagulation factors VII, IX, X, prothombin, protein S, protein Z, protein C, and the bone related proteins osteocalcin and matrix Gla protein [Stafford 2005]. During the post-translational modification glutamic (Glu) acid residues of the proteins are converted to γ-carboxyglutamic (Gla) residues which are then able to bind calcium. The above mentioned proteins are only biologically active following gamma-carboxylation [Wallin et al. 2003].

The system consists of the vitamin K-dependent γ-carboxylase and the enzyme vitamin K 2,3-epoxide reductase (VKOR – see 1.1. above). The former requires the reduced hydrochinone form of vitamin K as its cofactor, while the latter produces the cofactor [Wallin et al. 2003]. Because of this role of VKOR in the vitamin K cycle, mutations in VKOR could have an impact on the proper function of this cycle.

Glu

Clotting factor

Gla

Carboxylase

$CO_2 + O_2$

Vitamin K$_1$ hydroquinone

OH

CH$_3$

R

OH

O

CH$_3$

O

R

O

Vitamin K$_1$ epoxide

DT diaphorase

Vitamin K reductase

Vitamin K epoxide reductase

Inhibition

O

CH$_3$

R

O

Vitamin K quinone
R = Phytyl

Inhibition

WHO 95162
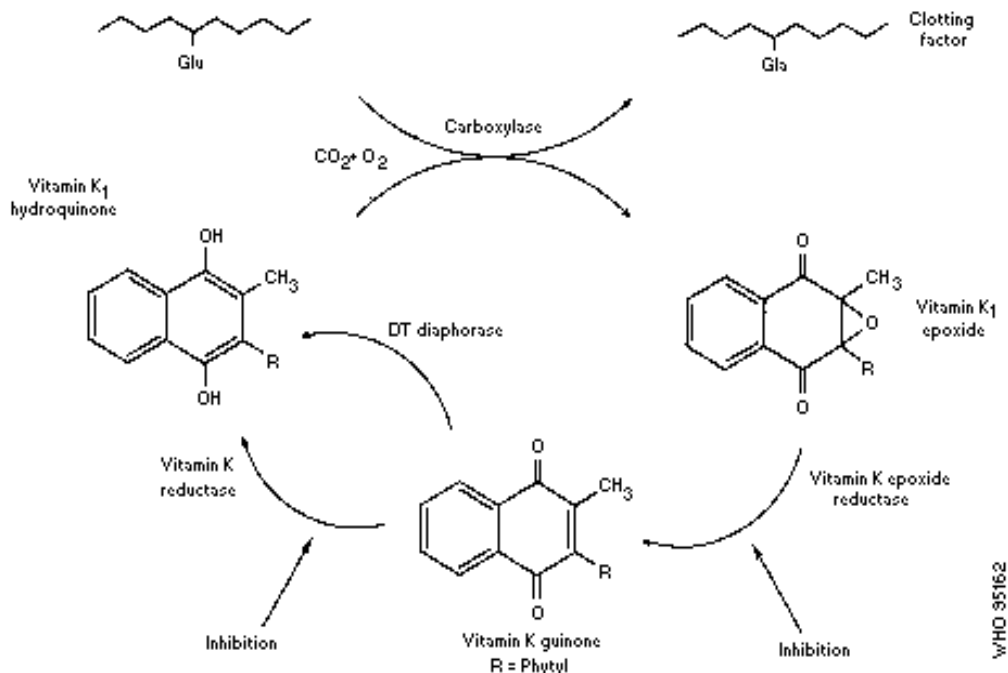
Fig. 6. The vitamin K cycle
Mediated by the enzyme gamma glutamyl carboxylase Glu residues are converted into Gla residues. Thereby vitamin K hydrochinone is turned into vitamin K epoxide, which has to be reconverted to be reusable. In a first step vitamin K epoxide is turned into vitamin K quinone in a reaction catalyzed by the enzyme vitamin K epoxide reductase (VKOR). In a second step vitamin K hydrochinone is regenerated and is then functional again. The regeneration can be inhibited for example by Warfarin [Bell et al., 1972].
(downloaded from http://www.inchem.org/documents/ehc/ehc/ehc175.htm)

4.6. γ-carboxylation

Gamma-carboxylation requires the abstraction of a proton from the 4th carbon of glutamate by reduced vitamin K. This reaction is catalysed by the vitamin K gamma glutamyl carboxylase (GGCX), while CO2, O2 and the reduced form of vitamin K are co-substrates. Through this reaction, for each carboxylated glutamate a molecule of vitamin K epoxide is formed. The generated vitamin K epoxide is converted back to reduced vitamin K by Vitamin K epoxide reductase (VKOR). On account of this ability of VKOR, vitamin K can be reused for several cycles of γ-carboxylation.

4.7. Vitamin K and osteoporosis

As mentioned above, Vitamin K is essential for the γ-carboxylation of osteocalcin which binds calcium ions of hydroxyapatite when carboxylated.

Vitamin K deficiency leads to elevated concentrations of undercarboxylated osteocalcin (ucOC) or a low ratio of serum carboxylated OC (cOC) to serum total OC. The deficiency seems to be common in postmenopausal women [Knapen et al. 1989, Plantalech et al. 1990], and contributes to development of osteoporosis and the increased risk of hip fracture in elderly [Iwamoto et al. 2006]. This was confirmed by several studies associating a high serum concentration of ucOC or low serum concentrations of carboxylated osteocalcin with skeletal turnover [Knapen et al. 1993], low bone mineral density [Szulc et al. 1994], and increased risk of osteoporotic fracture [Szulc et al. 1993; Szulc et al. 1996; Vergnaud et al. 1997; Luukinen et al. 2000]. Clinical use of vitamin K antagonists, like oral anticoagulants (coumarin derivatives), also leads to low bone mineral density [Fiore et al. 1990; Philip et al. 1995; Resch et al. 1991; Sato et al. 1997; Caraballo et al. 1999] and increased fracture risk [Caraballo et al. 1999].

Vitamin K seems to have a positive effect on bone metabolism as vitamin K supplementation increases serum phylloquinone concentration and reduces ucOC [Binkley et al. 2000]. Miki et al. [2003] and Ozuru et al. [2002] came to similar results when they demonstrated that vitamin K rapidly decreases the ucOC level and increases the cOC level.

5. Focus of the study

This study focuses on the examination of the influence of the -1639G>A promoter polymorphism on the expression of VKORC1 in osteoblasts and

addresses the question of whether there are any effects of this polymorphism on bone.

Until now, there have been no investigations on the effect of polymorphisms of the VKORC1 gene on γ-carboxylation of osteocalcin. By testing the influence of the -1639G>A polymorphism of the VKORC1 gene in osteoblasts I hoped to be able to establish a connection between this mutation and osteoporosis.

II. MATERIALS AND METHODS

1. Materials

1.1. Reagents

Agarose
(Sigma Aldrich, Germany)

AmpliTag Gold$^®$ with GeneAmp$^®$ 10x PCR Buffer II
(Roche, USA)

Aqua bidestillata
(Mayrhofer Pharmazeutika, Austria)

Bacteriological Agar
(Sigma Aldrich, Germany)

Big Dye$^®$ Terminator v3.1 Cycle Sequencing Kit with Big Dye$^®$ Terminator v3.1, Sequencing Buffer (5x) and Big Dye$^®$ Terminator Mix
(Applied Bioscience, UK)

Criterion^{TM} Precast Gel, 5% Acrylamid Precast Gel
(Bio-Rad Laboratories, USA)

Dimethyl Sulphoxide (DMSO)
Sigma Aldrich, Germany

D-MEM/F-12 (1x) liquid 1:1
Invitrogen

DMEM+GlutaMax^{TM}-I 1x
(Gibco, UK)

Dual- Luciferase® Reporter Assay System

(Promega Corporation, USA)

Ethanol 96% for analysis

(Merck, Darmstadt, Germany)

Ethidium bromide (10mg/ml)

(Sigma Aldrich, Germany)

FastRuler™DNA Ladder, High Range

(Fermentas International, Canada))

Fetal Bovine Serum (FBS)

(Gibco, UK)

Gel Star

(Invitrogen, USA)

GeneRuler™ 100bp Ladder

(Fermentas International, Canada)

Gentamicin 50mg/ml

(Gibco, Paisly, UK)

Glycerol, 99,5%+% A.C.S. Reagent

(Sigma Aldrich, Germany)

LB Broth

(Becton Dickinson, USA)

Lipofectamine™ 2000 Reagent

(Invitrogen, USA)

Loading Buffer (5x)

(Elchrom Scientific AG, Switzerland)

$MgCl_2$ Solution 25mM

(Roche, USA)

Metafectene™PRO

(Biontex, Germany)

Montage™ SEQ$_{96}$ Sequencing Reaction Cleanup Kit

(Millipore, USA)

OptiMEM$^®$ I- + GlutaMax™-I 1x

(Gibco, UK)

pBR322 DNA-Msp I Digest

(New England BioLabs, USA)

PBS

(Gibco, UK)

PfuUltra™ Hotstart DNA Polymerase, *PfuUltra*™ HF Reaction Buffer (10x)

(Stratagene, USA)

QIAprep$^®$ Spin Miniprep Kit, EndoFree$^®$ Plasmid Maxi Kit

(Qiagen Inc., USA)

Rapid- Load™ PCR Loading Dye

(OriGene Technologies, USA)

Restriction enzymes Hind III (10U/µl), Xho I (10U/µl), Xba I (10U/µl)

(Fermentas International, Canada)

S.O.C. Medium

(Invitrogen, USA)

T4 DNA Ligase (5U/µl) with Ligation buffer (10x)

(Roche Diagnostics, Germany)

5x TBE Buffer

(Eppendorf, Germany)

0.05% Trypsin- EDTA 1x

(Gibco, UK)

1.2. Cells:

DH5α cells

Osteoblasts

ATCC®, Nr.: CRL-11372

1.3. Vectors for cloning:

pGL3 basic vector

Promega Corporation, Madison, WI, USA
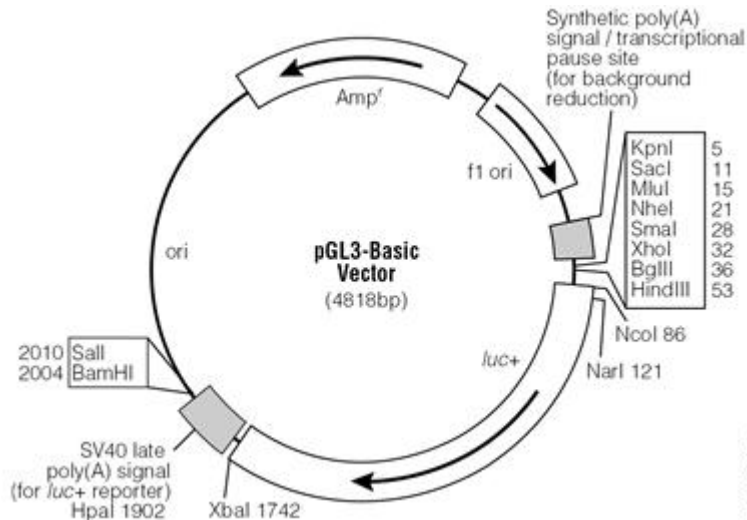
Base pairs	4818 bases
Promoter	(none)
Enhancer	(none)
Multiple cloning site	1-58
Luciferase gene (luc+)	88-1740
Glprimer2 biding site	89-111
SV40 late poly(A) signal	1772-1993
Rvprimer4 biding site	2061-2080
ColE1-derived plasmid replication origin	2318
Beta-lactamase gene (Ampr)	3080-3940
F1 origin	4072-4527
Synthetic (upstream) poly(A) signal	4658-4811
Rvprimer3 binding site	4760-4779

pGL3-G and pGL3-A:

Plasmids composed of the pGL3 vector with and VKORC1 promoter sequences corresponding to the -1639G allele and -1639A allele, respectively, had been produced by a fellow student and were at my disposal.

phRL-TK vector
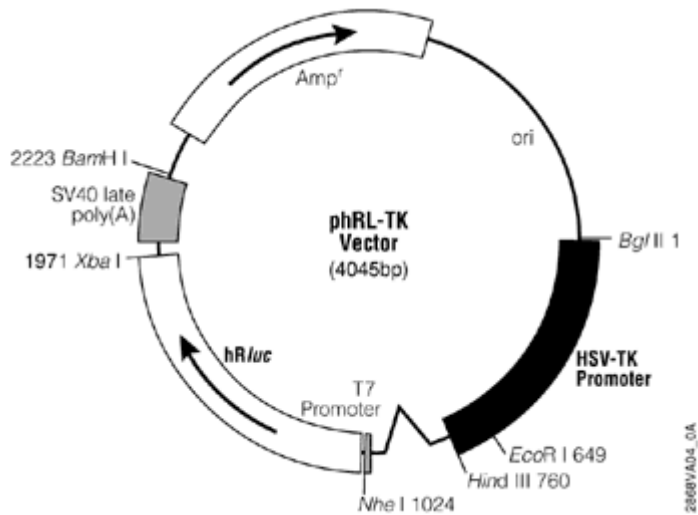
Base pairs	4045
HSV-TK promoter	7-759
Chimeric intron	826-962
T7 RNA polymerase promoter (-17 to +2)	1006-1024
T7 promoter transcription start site	1023
hRluc reporter gene	1034-1969
SV40 late poly(A) region	1991-2212
Beta-lactamase (Ampr) coding region	2359-3219

2. Methods

2.1. Production of the plasmids pGL3-G and pGL3-A

The pGL3-G and pGL3-A plasmids, containing the promoter sequence of VKORC1 variant G or A , had been generated previously and were stored in glycerol at -80°C. To produce fresh plasmid, a small aliquot of the glycerol stock was obtained with a sterile pipette tip, distributed on a LB agar plate containing 50 µg/ml ampicillin and incubated over night at 37°C.

Plasmid DNA was isolated from the bacteria with either the QIAgen Spin Miniprep Kit. or the EndoFree Plasmid Maxiprep Kit for the transfection of cells (osteoblasts). The concentration and quality (optical density = ratio of 260nm/280nm) of the DNA was measured with a NanoDrop® ND- 1000 Spectrophotometer.

2.2. Miniprep

The QIAgen Spin Miniprep Kit contains following reagents: Buffer P1 + RNase A, Buffer P2, Buffer N3, Buffer PE + ethanol, Buffer EB, Collection tubes and QIAprep Spin Columns.

Single bacterial colonies were picked, inoculated in 2ml LB-medium containing 100 µg/ml ampicillin and incubated with shaking at 37°C. After 16 hours, 1.5 ml of the culture were transferred into a 1.5ml tube (Eppendorf) and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and the pelleted bacterial cells were resuspended in 250µl Buffer P1. 250µl Buffer P2 was added to lyse the cells and to denature chromosomal and plasmid DNAs as well as proteins. By adding 350 µl Buffer N3 the lysate was neutralized and adjusted to the high-salt binding

conditions, which causes precipitation of denatured proteins, chromosomal DNA and cell debris, while the plasmid DNA renatured and stayed in solution. The solution was centrifuged at 13000 rpm for 10 min at room temperature and the supernatant containing the plasmid DNA was applied to the QIAprep spin column and again centrifuged for 1 min at 13000 rpm. The plasmid DNA bound to the QIAprep silica membrane, the flow-through was discarded. To remove endonucleases and salts, the spin column was washed with 500µl Buffer PB and 750µl Buffer PE, the flow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual wash buffer. Then the plasmid DNA was eluted with 50µl ddH$_2$O by incubating at room temperature and centrifuging.

To examine the size and the amount of the eluted plasmids, the plasmids were applied on a 0.8% agarose gel (0.32 g agarose and 40 ml of 1x TBE).
Running conditions for agarose gels were generally 100V, 50 min, at room temperature.

2.3. Restriction digestion

To test, whether the plasmids still contained the right insert after the long storage period at –80°C, the plasmid DNA was digested with the restriction enzymes HindIII and XhoI. The mastermix for each sample contained 2µl 10x Puffer (R), 1µl HindIII, 1µl XhoI, 11µl ddH$_2$O and 5µl plasmid DNA. The final reaction volume was 20µl. Then the reaction mix was incubated for 2 hours at 37°C and afterwards the plasmid DNA was again examined on an 0.8% agarose gel.

2.4. Maxiprep

For the large prep 100µl bacterial culture were inoculated in 4 ml LB-medium containing 100 µg/ml ampicillin and incubated on a shaker at 37°C for 16 hours. From this culture 100 µl were inoculated in 100 ml LB-medium in an Erlenmeyerkolben and incubated on a shaker at 37°C at 125 rpm. After 16- 18 hours the bacterial culture was pelleted at 6000 x g for 15 min at 4°C and the supernatant was discarded. The pellet was resuspended to homogeneity in 10 ml Buffer P1, 10 ml Puffer P2 was added and incubated at room temperature for 5 min. Then 10 ml Buffer P3 was added, the lysate was poured into the barrel of the QIAfilter cartridge, incubated at room temperature for 10 min and then filtered through the cartridge into a 50 ml tube. Buffer ER (2.5 ml) was added to the filtered lysate and incubated on ice for 30 min. A QIAGEN- tip 500 was equilibrated by applying 10 ml Buffer QBT and the column was allowed to empty by gravity flow. The filtered lysate was applied to the QIAGEN- tip and allowed to enter the resin by gravity-flow. Afterwards the QIAGEN- tip was washed two times with 30 ml Buffer QC, then DNA was eluted with 15 ml Buffer QN. The DNA was precipitated by adding 10.5ml isopropanol at room-temperature, following centrifugation at 15000 x g for 30 min at 4°C. The supernatant was removed carefully and discarded. The DNA pellet was washed with 5ml endotoxin-free 70% ethanol, centrifuged at 15000 x g for 10 min and the supernatant was discarded again. At last, the pellet was air-dryed for 5- 10 min and redissolved in 100 µl of 1 x TE.

The DNA was quality controlled on a 0.8% agarose gel, aliquoted and stored at −20°C.

Glycerol stock

Fresh glycerol stocks were prepared for long time storage. 600µl of the bacterial culture and 300µl glyerol were homogeneously mixed and stored at -80°C.

2.5. Sequencing

To ensure, that the plasmids contained the right insert with the right sequence of the promoter, the constructs were sequenced with the following primers:

Name	Position	Sequence 5' – 3'
VKORC1 5'UTRi fw	3514- 3539	CCG CTC GAG TAG ATG TGA GAA ACA GCA TCT GG
VKORC1 5'UTRi rv	5277- 5260	CCC AGG CTT AAA CCA GCC ACG GAG CAG
VKORC1 5'UTR1 rv	4080- 4961	CTT CCT CAC TTC TTC CTT GC
VKORC1 5'UTR4 rv	5025- 5006	GGG AAA TGA AGT CTC CAC AG
VKORC1 5'UTR2 fw	3924- 3938	CTG GGC GAC AGA GTG
VKORC1 5'UTR3 fw	4225- 4243	AGT GTA GAT GGG GAG GAT G

Sequencing was performed with the Big Dye® Terminator v3.1 Cycle Sequencing Kit with Big Dye® Terminator v3.1 Sequencing Buffer (5x) and Big Dye® Terminator Mix. The Big Dye Terminator Mix contains terminating nucleotides labelled with four different dyes, which makes a differentiation between A, T, C and G possible.

For each sample 2 µl Sequencing Buffer (5x), 0,4µl Big Dye Terminator Mix, 5pmol primer, 25ng plasmid DNA and ddH$_2$O to reach a final volume of 10µl, were pipetted into a 96- well reaction plate. The sequencing reaction was carried out using a 9700 Perkin Elmer PCR Cycler. (Cycling conditions: initial denaturation of 1 min at 96°C; 25 cycles with 10 sec 96°C, 5 sec 50°C and 4 min 60°C; storage at 4°C)

Because of the length of the sequence of interest and the fact that the sequence cannot be read immediately after the primer, six different primers were used, which generate overlapping fragments. This way the whole sequence of the plasmid could be read.

Purification of the sequencing reaction products

The sequencing reaction products were diluted with 25µl of injection solution and transferred into a SEQ 96- well plate. Unused wells were covered with sticky tape to avoid contaminations, so the other wells of the same plate could still be used afterwards. The plate was put into a vacuum exhaust and the injection solution was sucked off with a pressure of 20 mmHg by a vacuum pump until the wells were empty. 25µl injection solution was added and the procedure was repeated. After this second washing step, the purified sample was dissolved in 25µl injection solution and transferred into a 96-well sequencing plate, which was covered with a septum. Sequencing was performed on an ABI PRISM 3100 Genetic Analyser, using a 3100 POP-6 polymere. The sequence was analyzed with the SeqScape program.

2.6. Cell culture

2.6.1. Culture of osteoblasts

The cell line hFOB1 was delivered in a tube containing 7×10^6/ml. It was processed according to the recommendations of the American Type Culture Collection (ATCC). Originally, an incubation temperature of 34°C was chosen because of the recommendations of ATCC.

Cells were thawed in a water bath at 37°C; 500µl of the cells were mixed with 5 ml FBS and centrifuged at 1200 rpm for 5 min. The supernatant was

discarded, the pellet was resuspended in 5 ml FBS, centrifuged again, resuspended in 10 ml of complete growth medium (DMEM/F12 1:1 with 2.5mM L- glutamine, 15mM HEPES, 0.5mM sodium pyruvate, 1.2g/L sodium bicarbonate, 0.3mg/ml G418 and 10% FBS without phenol red) and cultivated in cell-culturing flasks at 34°C and 5% CO_2. The medium was changed every 2-3 days. As contaminations were observed when using this medium, the composition of the medium was changed. The new medium contained: DMEM/F12 1:1 without phenol red (with 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate, 1.2 g/L sodium bicarbonate), 10% FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml fungizone.]

Cells were splitted depending on the confluence. The cells were washed with ~ 5ml PBS followed by incubation with trypsin until all cells detached from the bottom of the flask. By adding complete growth medium, the trypsin was inactivated through FBS and the cells were centrifuged, resuspended in complete growth medium and split into new flasks.

Freezing Cells for Storage

The medium was removed from the flask and the cells were washed with PBS. By adding trypsin-EDTA the cells were detached from the bottom of the flask, medium with FBS was added to inactivate trypsin, the cells were centrifuged for 5 minutes at 1200rpm and the supernatant was removed. The cells were then resuspended in icecold DMSO, placed at -80°C over night and then put in liquid nitrogen for long term storage.

2.7. Transfection of osteoblasts hFOB1

2.7.1. Transfection with Lipofectamine 2000

Osteoblasts hFOB1 were seeded into a 24- well plate with each well containing 4×10^5 cells in 0.5 ml of medium without antibiotics. The cells were incubated 24 hours prior to transfection at 34°C.

After 24 hours the Co-Transfection was carried out. For each sample 2µl Lipofectamine 2000 was diluted with 48µl Opti-MEM and incubated at room temperature for 5 min. Then, 25ng phRL-TK vector and 750ng pGL3 vector (basic, G or A) were diluted in 50µl Opti-MEM. The DNA and the Lipofectamine solution were mixed to a total volume of 100µl and incubated at room temperature for 20 min. The medium of the osteoblasts seeded into a 24-well plate was renewed, 100µl of the DNA complex was added to each well and the plate was incubated at 34°C for 48 hours. After the transfection, the medium was removed and 100µl passive lysis buffer per well were added. The cells were lysed under shaking for 15 min, centrifuged at 13000 rpm for 1 min and stored at -20°C.

2.7.2. Transfection with Metafectene Pro

Metafectene Pro:

Metafectene Pro is a new transfection reagent, which was specifically developed for moderately hard- or hard-to-transfect mammalian cell lines.

Its novel composition combines the RMA-Technology ("Repulsive Membrane Acidolysis") with a new TOP-Technology (Toxicity-Optimization Module).

Basic mechanism of intracellular gene transfer using cationic lipids:

When the cationic liposomes come into contact with DNA, a DNA-lipid complex (=lipoplex) is formed. These lipoplexes are transferred through the outer cell membrane by endocytosis and as a result lipoplex-containing endosomes are formed within the endoplasm. These endosomes possess H+-pumps, so the pumps are no longer able to acidificate the endosomal lumen. This results in a relative low pH value and high osmotic pressure, which breaks the endosomal membrane and releases the lipoplexes into the cytosol. In case of plasmid DNA the released genetic material must reach the nucleus and pass the nucleus membrane. This is only possible during cell division when the nuclear membrane opens, since plasmid DNA is not able to penetrate the membrane by itself.

Repulsive Membrane Acidolysis (RMA):

This technology uses the acidic environment of the late endosomes to weaken the membrane structure of the lipoplexes, which is achieved by a protonable basic position near the lipophilic part of the cationic lipids. Among these positively charged parts of the lipids, repulsive forces ease the disruption of the endosomal membrane and thus the release of the genetic material, so a high concentration of naked DNA is present in the cytosol.

Toxicity Optimization Module (TOP):

The toxicity optimization module moderately destabilizes the DNA-lipid-complex and potentiates the access for the RMA. Furthermore, it decreases the toxic effects in transfection technology, which is essential for in-vivo applications.

The combination of both techniques -RMA and TOP- ensures the required release of genetic material in the cytoplasma and the availability of enough genetic material for uptake into the cell nucleus.

Transfection:

For each well 100µl of Opti-MEM and 1µl of Metafectine Pro were mixed and incubated for 15 min at room temperature.

The osteoblasts were seeded in a 24-well plate. Each well contained 4x 10^5 cells in 0.5 ml of media without antibiotics and serum.

Per well 1µg of the pGL3 constructs (empty vector, G or A plasmid), 25ng phRL-TK vector or 0.5µg GFP were mixed with the diluted lipid reagent and were incubated for 15 min at room temperature. phRL-TK was added as a negative control and GFP as a control for the transfection. 100µl of the lipid - DNA mix was added to each well of the plate containing the cells and incubated at 37°C. After 4 hours 0.5 ml of media with 20% FBS were added per well, so the final concentration of FBS in one well was 10%. After 16-24 hours the fluorescence of GFP was examined, in order to see if the transfection has worked.

After 48 hours the transfection medium was removed and the cells were lysed under shaking with 100µl passive lysis buffer per well. After 15 min the lysate was centrifuged at 13000 rpm for 1 min and stored at -20°C.

2.8. Dual Luciferase Reporter Gene Assay

The Dual Luciferase Reporter Assay was carried out with the Luminometer Lucy2 (Fa Anthos). The Dual Luciferase Reporter Assay is based on the simultaneous expression and measurement of two individual reporter

enzymes within a single system. The "experimental" reporter is correlated with the specific conditions of the experiment, while the co-transfected "control" reporter represents the baseline response. The activity of the experimental reporter is normalized to that of the internal control, which minimizes experimental variability, caused by differences in cell viability or transfection efficiency. Moreover variability through cell lysis efficiency and assay efficiency can be eliminated

The VKORC1- promoter in the plasmid- constructs pGL3-G and pGL3-A induces the activity of the Firefly luciferase, which is normalized by the *Renilla* luciferase coded in the phRL-TK vector. In my experiments, the pGL3-basic vector was used as a negative control. It does not contain the promoter sequence and as a consequence shows no luciferase activity.

The luciferase assay reagent (LAR II) and the Stop & Glo solution were prepared according to the introductions of the manufacturer (Promega).

For the assay 20µl of each sample were transferred into a 96-well plate, which was placed into the luminometer. The luciferase activity of each well was measured by firstly adding 100µl Luciferase assay reagent II (LAR II), measuring the firefly luciferase activity and then immediately adding 100µl Stop & Glo reagent to measure the *Renilla* luciferase activity. By adding the Stop & Glo reagent, the luminescence signal of the firefly luciferase is quenched and the *Renilla* luciferase reaction is initiated simultaneously.

2.9. Cloning of VKORC1 full length constructs

To produce a full length construct of VKORC1 the plasmids pGL3-G and pGL3-A, containing the promoter sequence G and A, respectively, were used. The luciferase gene located directly behind the promoter was replaced by the coding sequence of the VKOR gene. In a first step, the

plasmids were digested with the restriction enzymes Hind III and Xba I to release the luciferase gene. Then, the coding sequence of VKORC1 was amplified with primers containing restriction sites for Hind III and Xba I. The PCR product was treated with Hind III and Xba I and ligated with the pGL3 plasmids. At last, the full length constructs were cloned into DH5α cells which were produced by a fellow student, who provided them for my diploma thesis.

2.9.1. RNA- Isolation

To obtain VKORC1 mRNA, the liver cell line HepG$_2$ was used.

The RNA isolation was carried out with a RNeasy Mini Kit (QIAGEN).

HepG$_2$ cells were lysed in PLB (passive lysis buffer) and 700 µl of this cell lysate were spun through a QIAshredder spin column to homogenize the cell lysate. After that, 700µl 70% ethanol was added and mixed by pipetting. 700µl of this solution was transferred into a RNeasy spin column placed in a 2 ml collection tube, centrifuged for 30 sec at 13200 rpm, so the total RNA was bound to the membrane of the spin column. The flow-through was discarded. To wash the spin column membrane, 700µl of buffer RW1 was added, centrifuged for 30 sec at 13200 rpm and the flow-through was discarded again. Following this, the spin column was washed twice with 500µl of buffer RPE for 30 sec at 13200 rpm.

To eliminate any possible carryover of residual buffer RPE or flow-through, the spin column placed in a 2ml tube was centrifuged for additional 2 min at 13200 rpm and transferred into a new 1.5ml tube. 50µl RNase free water was added to the spin column, incubated for 10 min at room temperature and centrifuged for 1 min at 13200 rpm to elute the RNA, which was immediately put on ice and stored at –80°C.

To determine the amount of isolated RNA, the optical density was measured with NanoDrop® ND- 1000 Spectralphotometer (PEQLAB)

2.9.2. cDNA- Synthesis

An aliquot containing 1ng RNA was incubated for 10 min at 70°C and immediately put on ice. A mastermix was prepared as follows: 10.75µl of the Premix containing 5µl 5x Buffer, 1.25 µl dNTP Mix (20 mM/dNTP), 2µl Oligo- desoxy- Thymin Primer (= oligo dT; 10 pmol/µl)) and 2.5µl DTT (100mM) per sample were mixed with 1µl RNA Guard (23.7 U) and 1µl MMLV- RT (8 U/µl). 12.5 µl of this master mix were added to the RNA and incubated for 10 min at 22°C, followed by 45 min at 37°C and at finally for 3 min at 99°C. The samples were cooled down on ice and 37µl of ddH$_2$O were added.

2.9.3. Quality control of cDNA by control gene PCR

As a quality control of cDNA synthesis, the housekeeping gene GAPDH was amplified by PCR. This PCR allows to verify, that a suitable amount and of cDNA in appropriate quality was generated.

The Mastermix contained the following reagents per sample: 2µl 10x PCR-Buffer II without MgCl$_2$ (25mM), 0.8µl Rapid Load PCR Loading Dye, 0.2 µl dNTP Mix (20 mM/base), 0.17 µl AmpliTaq Gold (5 U/µl), 1 µl primer GAPDH fw (10 pmol/µl), 1 µl primer GAPDH rv (10 pmol/µl) and 14.8 µl A. bidest. To 20 µl of the master mix 2 µl of undiluted DNA (=cDNA) were added.

Gene	Primer	Sequence 5' – 3'
GAPDH	Gap- f	GAA GGT GAA GGT CGG AGT C
	Gap- r	GGG GAT GGT GAT GGG ATT TC

Temperature protocol:

Initial denaturation	95°C	7 min
38 cycles	95°C	30 sec
	60°C	30 sec
	72°C	30 sec
Final elongation	72°C	10 min
Storing	4°C	-

Electrophoresis:

Seven µl of the PCR products and 3.5 µl marker (1x TBE marker) were loaded on a 5 % criterion gel.

Running conditions: 1x TBE buffer at 175 V for 45 minutes.

2.9.4. Generation of cDNA with restriction sites

To be able to ligate the cDNA of VKORC1 into a vector, which contains restriction sites for the endonucleases Hind III and XbaI, these restriction sites have to be attached to the ends of the cDNA. The Hind III restriction site was attached to the 5' end of the forward primer, directly in front of the start codon and the restriction site of XbaI was placed directly behind the stop codon of the coding sequence of the VKORC1 gene.

Primer	Sequence 5' – 3'	Length
#182 VKORC1cdsfw	AAG CTT ATG GGC AGC ACC TGG G	21 bp
#183 VKORC1cdsrv	TCT AGA TCA GTG CCT CTT AGC CTT GCC	27 bp

A master mix was prepared, containing the following amounts of reagents per sample: 2µl PfuUltra HF reaction buffer (10x), 1µl VKORC1cds fw (10pmol/µl), 1µl VKORC1cds rv (10 pmol/µl), 0.2µl dNTP (20mM), 0.5µl PfuUltra Hotstart DNA Polymerase and 100ng of cDNA. In order to reach the final reaction volume of 20µl, an appropriate amount of ddH$_2$O was added.

Temperature protocol:

Initial denaturation	94°C	10 min
	94°C	45 sec
35 cycles	60°C	45 sec
	72°C	2 min
Final elongation	72°C	7 min
Storing	4°C	-

Electrophoresis:

Sample: 3.5 µl of a 1x TBE marker and 7 µl of the PCR product

Gel: 5% criterion polyacrylamide gel

Running conditions: 1x TBE at 175V for 45 minutes.

The chosen 5'-primer was able to bind not only at the 5' site of the cDNA, but also within the cDNA sequence. Thus, a PCR product 120bp shorter than the full length coding sequence of the VKOR gene was generated in addition to the full length fragment. As it was not possible to design a primer with no homology, it was necessary to separate both fragments by electrophoresis followed by elution from the gel.

Isolation of the full length VKOR cDNA construct

The two different products were separated by electrophoresis on an agarose gel (0.8% agarose gel; 1x TBE, 100V, 60min). The band, which corresponds to the PCR product of full length cDNA of VKORC1, was cut out of the gel and the DNA was extracted using a QIAGEN gel extraction kit.

The QIAquick Gel Extraction Kit contains following reagents: Buffer QG, Buffer PE and Buffer EB. The excised gel slice was weighed, 3 volumes of buffer QG were added to 1 volume of gel slice (100mg ~ 100µl) and incubated at 50°C for 10 minutes until the gel slice had completely dissolved. Then, 1 gel volume of isopropanol was added and mixed. To bind the DNA to the spin column, the sample was applied to a QIAquick spin column and centrifuged for 1 min at 13000 rpm. The flow-through was discarded. To wash the bound DNA, 750µl of Buffer PE were added to the spin column and again centrifuged for 1 min at 13000 rpm. To remove residual ethanol from buffer PE, the spin column was centrifuged at 13000 rpm for an additional 1 min. To elute the DNA, the column was placed in a 1.5 ml tube, 30 µl of Buffer EB were added to the center of the membrane. After 1min incubation at room temperature, the column was centrifuged for 1 min at 13000 rpm.

2.9.5. Restriction digestion of the plasmids pGL3-G, pGL3-A and the VKORC1 PCR product

To linearise the plasmids and to remove the luciferase gene from the plasmids prior to ligation with the VKORC1 full length construct, the plasmids pGL3-G and pGL3-A were double digested with the restriction enzymes HindIII and XbaI. The full length PCR fragment of the VKORC1 coding sequence was also digested with the same enzymes.

For the restriction digestion, an enzyme mix containing 25µl Buffer B (10x), 1µl HindIII (1U/µl) and 1µl XbaI (1U/µl) was prepared. Then, 2.5µl of the enzyme mixwere mixed with 1µg pGL3-G or pGL3-A or 120ng PCR product and an appropriate amount of A. bidest to reach a final reaction volume of 25µl. The restriction mix was incubated at 37°C for 1 hour and the results were examined on a 0.8% agarose gel (running conditions: 1x TBE, 100V, 60min). The linearised plasmids were excised of the gel and extracted with the QIAquick gel extraction kit.

2.9.6. Ligation of the VKORC1 PCR product into the plasmids

The double digestion of the vector plasmids and the insert PCR product of the VKORC1 gene should support insertion of the sequence of interest into the vector in the correct orientation.

The ligation was performed using the rapid DNA ligation & transformation kit (Fermentas).

For the ligation reaction 70 ng plasmid DNA, 40ng VKORC1 PCR product, 4µl 5x Rapid Ligation Buffer and 1µl T4 DNA Ligase (5U) were mixed and incubated for 5 minutes at 22°C.

The ligation product was examined on an 0.8% agarose gel at 100V for 90 min and stored at -20°C.

2.9.7. Transformation of the VKORC1 full length construct into DH5α cells

The ligation product and the cells were thawed on ice. Then, 5µl of the ligation product were added to 50µl of cells and incubated under shaking

at 37°C for 10 minutes. Afterwards the cells were plated on prewarmed LB agar plates containing 100µg/ml ampicillin and incubated for 12 – 16 hours at 37°C.

Of each construct (pGL3-A and pGL3-G) five colonies were picked, inoculated in 2 ml LB media and again incubated under shaking at 37°C over night. Of 1.5ml of the liquid culture plasmid DNAs were isolated with the QIAgen Spin Miniprep Kit according to the recommendations of the manufacturer and as described in section II.2.2.

2.10. Analysis of the isolated VKORC1 full length plasmids

2.10.1. Restriction digestion

To verify, that the isolated plasmids contained the insert, three different digestion reactions were carried out. One with the enzymes Hind III and Xba I to release the insert, one with the enzyme Hind III and one with the enzyme Xba I only.

For the double digestion 2.5µl enzyme mix (25µl 10x Buffer B + 10 U Hind III + 10 U Xba I) and ~ 800ng plasmid DNA were mixed. Water was added to reach the final reaction volume of 25µl.

For the reactions with only one enzyme, 1µl of Hind III or Xba I, respectively, ~800ng plasmid DNA and water were mixed. The final reaction volume was 25µl.

The three samples were incubated at 37°C for one hour and analyzed on 0.8% agarose gel. Running conditions: 1x TBE, 100V, 60 minutes.

2.10.2. PCR

Three PCR reactions were carried out with primer pairs listed below. (a) for the full length fragment (fragment of interest) containing the promotor and the insert, which should be located adjacently (b) for the insert fragment and (c) for the promotor sequence only.

Used primers:

Primer	Sequence 5'-3'
VKORC1 cds fw	AAG CTT ATG GGC AGC ACC TGG G
VKORC1 cds rv	TCT AGA TCA GTG CCT CTT AGC CTT GCC
VKOR -1639 C-F	ACA GTA AGG GAT CCC TCT GGG AAG TC
VKOR -1639 W-R	AGG CGT GAG CCA CCG CAA CC
VKOR -1639 M-R	AGG ATT ATT AGC GTG AGC CAC CGC TCC T

The mastermixes were prepared as follows:

a) 2µl 10x PCR Buffer II without MgCl$_2$, 1.5µl MgCl$_2$ (25mM), 0.8µl Rapid Load PCR Loading Dye, 0.2µl dNTP Mix (20 mM/base), 0.1µl AmpliTaq Gold (5U/µl), 0.4µl primer VKOR -1639C-F (10pmol/µl), 1µl primer VKORC1 cds rv (10pmol/µl), 1µl plasmid DNA and 12.4µl A. bidest.

b) 2µl 10x PCR Buffer II without MgCl$_2$, 1.5µl MgCl$_2$ (25mM), 0.8µl Rapid Load PCR Loading Dye, 0.2 µl dNTP Mix (20 mM/base), 0.1 µl AmpliTaq Gold (5U/µl), 1µl primer VKORC1 cds fw (10pmol/µl), 1µl primer VKORC1 cds rv (10pmol/µl), 1µl plasmid DNA and 12.4µl A. bidest.

c) 2.5µl 10x PCR Buffer II without MgCl$_2$, 1.5µl MgCl$_2$ (25mM), 0.8µl Rapid Load PCR Loading Dye, 0.25µl dNTP Mix (20mM/base), 0.1 µl AmpliTaq Gold (5U/µl), 0.5µl primer VKOR -1639W-R (10

pmol/µl), 0.4µl primer VKOR -1639C-F (10pmol/µl), 0.08µl VKOR -1639 M-R (10pmol/µl), 1µl plasmid DNA and 12.4 µl A. bidest.

Temperature protocol for all three PCR reactions:

Cycling conditions		
Time	Grade	Cycles
10'	94	1
45"	94	
45"	60	35
2'	72	
7'	72	1
~	4	

Electrophoresis:

3.5µl MspI marker and 7µl of the PCR product were loaded on a 5% criterion gel. Running conditions: 1x TBE, 175V, 45 minutes.

2.10.3. Sequencing

To verify the sequence of the plasmid construct, the plasmid DNA was sequenced with three different primers. A primer complimentary to the 5'-end of the promotor, to verify that the coding sequence of VKORC1 was inserted adjacent to the promoter. Two primers complementary to the 3' and 5' end of the insert to test whether the correct sequence of the insert was present.

Name	Sequence 5' – 3'
VKORC1 cds fw	aag ctt atg ggc agc acc tgg g
VKORC1 cds rv	tct aga tca gtg cct ctt agc ctt gcc
VKORC1 5'UTR 5fw	tgg ctg ttt tcc taa ctc

For each primer a mastermix was prepared as follows:

2µl Sequencing Buffer (5x), 0.4µl Big Dye Terminator Mix, 1µl primer (5pmol/µl), 25ng plasmid DNA and water to reach a final volume of 10µl. The sequencing reaction was carried out in a 9700 Perkin Cycler. The purification of the sequencing reaction products and the analysis were performed as described in 2.6. and 2.7.

PCR conditions:

1'	96°C	1x
10"	96°C	
5"	50°C	25
4'	60°C	
Hold	4°C	~

2.10.4 Digestion with EXO SAP-it

To remove unspecific products and reduce back ground noise during sequencing the PCR products were digested with EXO SAP-it prior to sequencing.

Two µl EXO SAP-it were added to 10µl PCR product followed by incubation for 30 minutes at 37°C and 15 minutes at 80°C.

The sequencing reaction was carried out with the same primers and temperature protocol as described in 2.10.3. Purification and analysis of the sequencing reaction products were performed as described in 2.6. and 2.7.

III. RESULTS

1.Restriction digestion of the plasmids pGL3-G and pGL3-A

Some of the plasmid DNAs isolated from the glycerol stocks showed an insert with double the length than expected. This may indicate that in some vectors two promoter fragments were inserted in tandem fashion.

These bacterial cells were discarded. For all further experiments only plasmids with the correct length of the insert were used.

2. Sequencing of the plasmids pGL3-G and pGL3-A

Resequencing of the promoter constructs, pGL3-A and pGL3-G, showed the correct sequence of the promoter with an A nucleotide and a G nucleotide, respectively, at the position -1639.

3. Dual Luciferase Reporter Assay

The results of the dual luciferase reporter gene assay, either of Lipofectamine 2000 or Metafectene Pro transfections, are listed in table 1 and 2. Table 1 shows the results of the transfection of the osteoblasts hFOB1 with Lipofectamine 2000. The transfections were performed on two different days with four replicas per construct. The results of the first transfection shows an average luciferase activity of 8.9 for the pGL3-G construct and 14.0 for the pGL3-A construct. During the second transfection much higher luciferase activity values were obtained with an average luciferase activity of 14.4 for the pGL3-G construct and 159.9 for the pGL3-A construct.

The results of the transfections with Metafectene Pro are listed in table 2. These transfections were performed on four different days with four

replicas per construct. The average luciferase activity for the pGL3-G construct was 3.3, 3.8, 3.5 and 5.7. For the pGL3-A construct the average luciferase activity was 2.5, 2.7, 2.7 and 3.4. The variation between transfections was much lower than observed for Lipofetamine.

Table 3 shows the mean values of the four transfection experiments with Metafectene Pro for the pGL3-G and pGL3-A constructs, with a mean value of 4.1 for pGL3-G and 2.85 for pGL3-A.

3.1. Results of the dual luciferase reporter gene assay

Transfection with Lipofectamine 2000:

No treatment	pGL3 basic	pGL3-G	pGL3-A
-0,009	0,033	8,823	9,501
-0,008	0,021	7,438	14,462
-0,013	-0,006	11,873	17,096
-0,021	0,025	7,475	15,088
		8,90225	*14,0368*
-0,005	1,578	20,327	84,109
0,002	0,694	9,624	188,151
0	2,191	5,084	142,261
0,005	0,822	22,502	225,309
		14,3843	*159,958*

Table 1. Results of the Dual luciferase reporter gene assay
Transfection of osteoblasts with Lipofectamine 2000

The results of the reporter gene assay following Lipofectamine 2000 transfection of osteoblasts were poorly reproducible and were considered

inadequate for both constructs. The luminescence values differed highly between transfections, as shown in figure 7.

Transfection with Lipofectamine
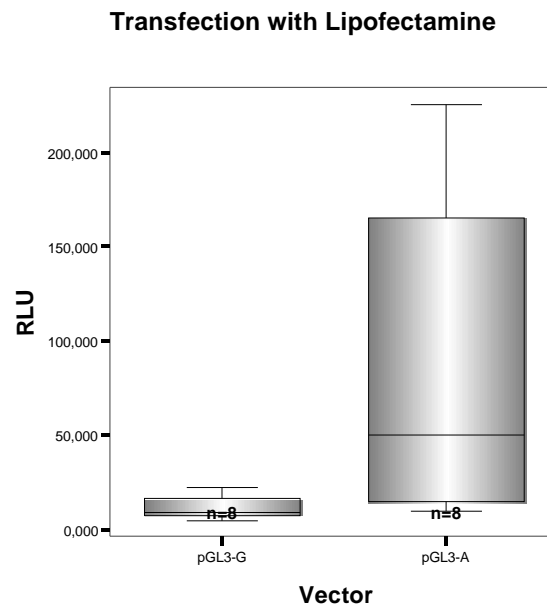
Fig. 7. Transfection with lipofectamine shows inconsistent efficiency

Figure 7 illustrates the high variation in the transfection efficiency with lipofectamine 2000. The values for pGL3-G vary between 7 RLU (relative luminescence unit) and 22 RLU, in contrast the values of pGL3-A vary between 9 RLU and 225 RLU. In one experiment, the difference between luciferase activity induced by the promoter element pGL3-A was comparable to the activity induced by pGL3-G, in the second experiment pGL3-A induced much higher luciferase activity.
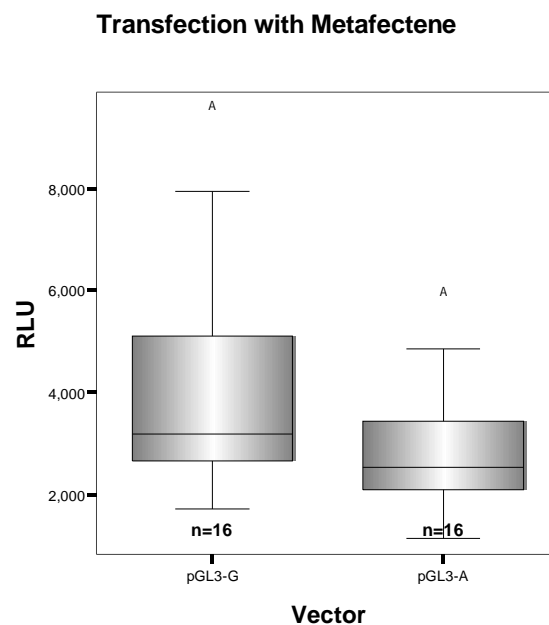
Transfection with Metafectene Pro

| no treatment	pGL3 basic	pGL3-G	pGL3-A
-0,001	0,026	4,052	2,426
0,005	0,025	2,792	2,62
-0,002	0,035	2,694	2,971
-0,001	0,015	3,655	2,035
		3,29825	*2,513*
0	0,04	5,122	2,919
0,003	0,014	5,091	2,627
0,001	0,018	1,712	1,285
0,004	0,004	3,466	3,931
		3,84775	*2,6905*
0,001	0,011	2,636	1,78
0,003	0,015	1,879	4,85
0,003	0,008	6,758	2,176
0,004	0,013	2,938	2,25
		3,55275	*2,764*
-0,001	0,026	7,942	2,468
0,002	0,012	2,596	4,233
0,002	0,023	2,744	1,159
-0,002	0,011	9,522	5,9
		5,701	*3,44*

Table 2. Results of the Dual luciferase reporter gene assay
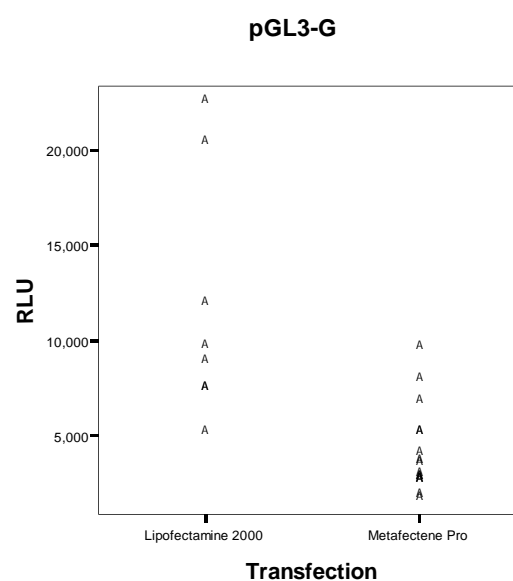Transfection of osteoblasts with Metafectene Pro

The table shows results of transfection of osteoblasts with Metafectene Pro on 4 different days with four replica per construct. The values of the average luciferase activity lie between 3.3 RLU and 5.7 RLU for the pGL3-G construct and between 2.5 RLU and 3.4 RLU for the pGL3-A construct. In contrast to transfection with Lipofectamine the luciferase activity was higher in the pGL3-G promoter construct than in the pGL3-A construct in each transfection experiment, also shown in figure 8.

Transfection with Metafectene

**Fig. 8. Results of the dual luciferase repoter gene assay
Transfection of osteoblasts with Metafectene Pro**
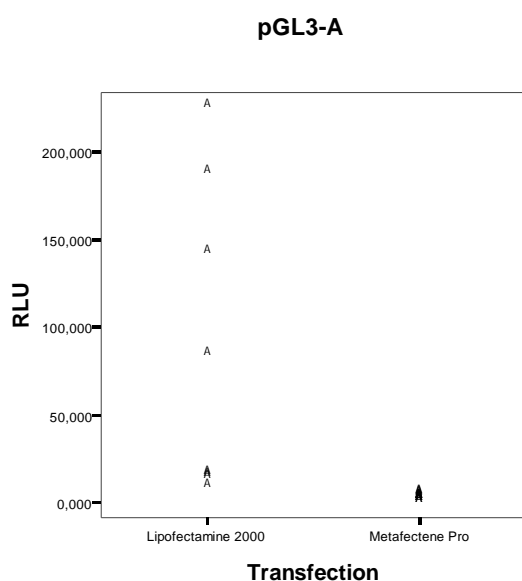
pGL3-A

pGL3-G

Fig. 9. Comparison between the two trans-
fection methods in osteoblasts with the pGL3-
G plasmid

Fig. 10. Comparison between the two trans-
fection methods in osteoblasts with the pGL3-A
plasmid

Figures 9 and 10 demonstrate the high variation of the luminescence
following transfections with Lipofectamine 2000 compared to transfections
with Metafectene Pro.

Plasmids	Mean	Median
pGL3-G	4,0999	3,2020
pGL3-A	2,8519	2,5440

**Table 3. Median and mean values for the pGL3-G and pGL3-A plasmids of all
transfections with Metafectene Pro**

The mean values of the transfection with Metafectine Pro demonstrate a
43% higher expression in osteoblasts containing the pGL3-G plasmid
compared to osteoblasts containing the pGL3-A vector, illustrated in figure
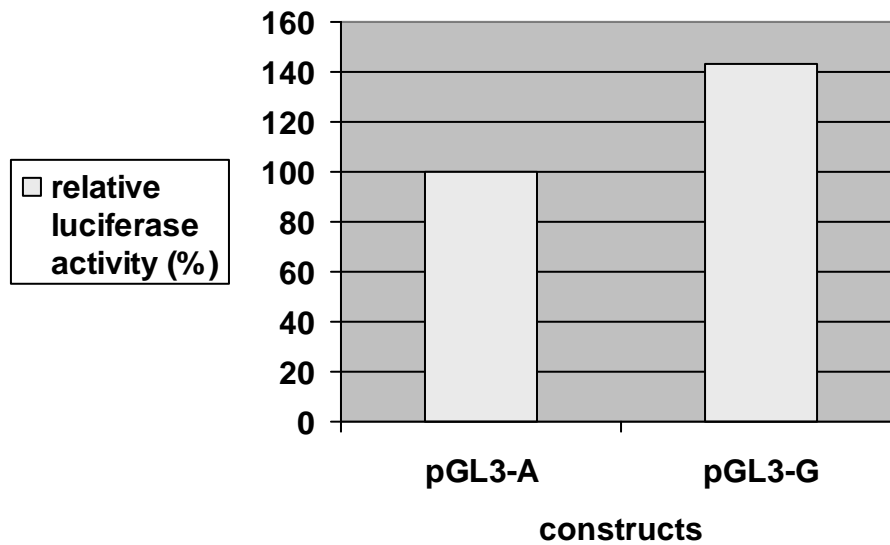11.

**Fig. 11. Relative luciferase activity measured in osteoblasts transfected with Metafectine Pro**

4. Cloning of VKORC1 full length constructs

4.1. Generation of cDNA of the VKORC1 coding region containing restriction sites

The results – generation of two PCR products instead of one - indicated that the selected VKORC1 cds primers bind not only to the expected binding site but also to another sequence within the cDNA. The longer fragment with 506 bp length represents the cDNA containing the two restriction sites. The second fragment with a length of approximately 400bp was identified as a product corresponding to a fragment of the cDNA generated by the binding of the VKORC1 cds fw primer to a homologous sequence located approximately 120 bp downstream of the start codon within the cDNA.

Several modifications regarding temperature and primer concentrations did not eliminate the generation of the second PCR product (Fig.12). To

proceed with cloning, the correct band had to be separated by gel electrophoresis and the DNA had to be eluted from the gel. This DNA extraction product was used for all following experiments.
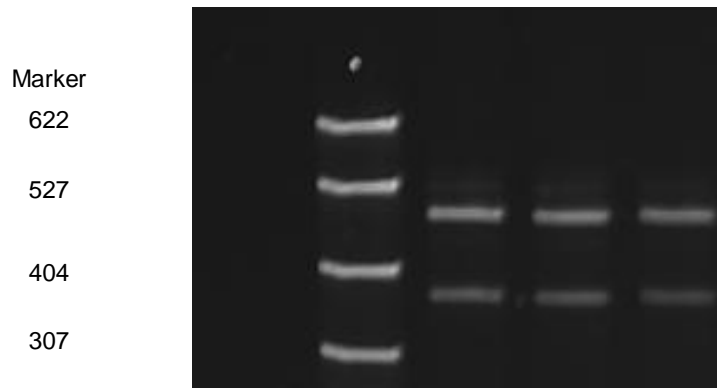
Fig. 12. Coding region of VKORC1 with restriction sites
Slot 1: Marker pBR322 DNA MspI-digest, Slot 2+3+4: Insert with restriction sites

4.2. Introduction of the VKORC1 coding region into the pGL3-G and pGL3-A plasmids

Figure 13 shows the results of the ligation of the VKORC1 coding region into the pGL3-G and pGL3-A plasmids. Slot 2 and slot 6 depict the full length constructs containing either pGL3-G or pGL3-A. The full length constructs were compared to the digested full length plasmids pGL3-G and pGL3-A (digestion with both endonucleases XbaI and HindIII). This double digestion was performed to cut out the inserted coding region of VKORC1, giving 2 fragments on the gel. A longer fragment which represents the linearised pGL3-G or pGL3-A and a shorter fragment representing the VKORC1 coding region (slot 3 and 7 of figure 13). The shorter fragment, which should correspond to the VKORC1 coding region, migrated with approximately 1800bp – which is 3 times longer than the actual length of 506bp (PCR fragment corresponding to the coding region). This indicated that the insertion of the coding region segment of VKORC1 into the plasmids did not occur correctly.
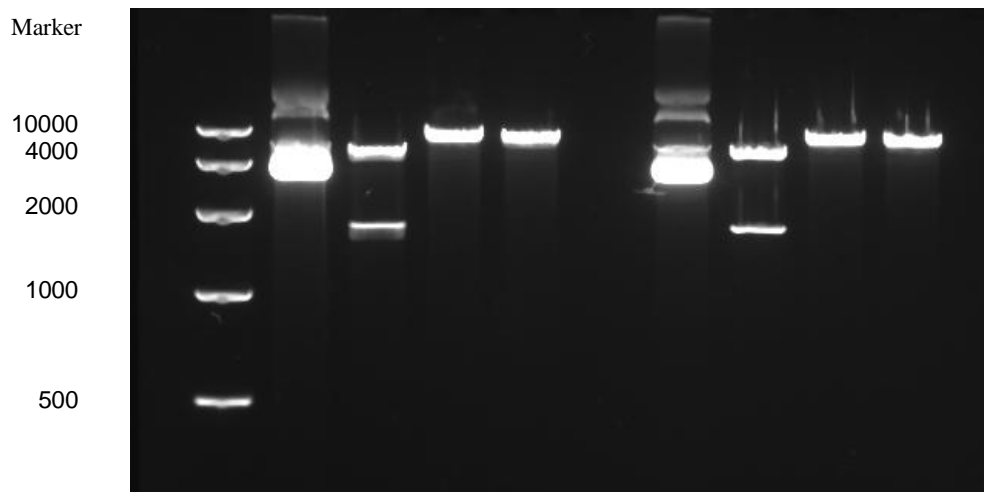
Fig. 13. Control of the full length plasmids pGL3-G and pGL3-A
Slot 1: Marker: FastRuler™DNA Ladder High Range, *Slot 2:* undigested pGL3-G plasmid, *Slot 3:* plasmid restricted with XbaI and HindIII, *Slot 4+5:* linearised pGL3-G digested with either XbaI or HindIII, *Slot 6:* undigested pGL3-A plasmid, S*lot 7:* plasmid restricted with XbaI and HindIII, *Slot 8+9:* pGL3-A linearised with either XbaI or HindIII

5. Analysis of the VKORC1 constructs

The generated full length plasmids were examined by PCR and sequencing.

5.1. PCR

The results of the PCRs are shown in figure 14, 15 and 16.

All three PCR amplifications worked and generated products. In figure 15 and 16 the bands corresponding to the promoter and the insert are shown to migrate with the correct length. In contrast, with primers spanning the promoter and the cDNA sequence (figure 14), the expected fragment of ~2.2 kb was not generated.
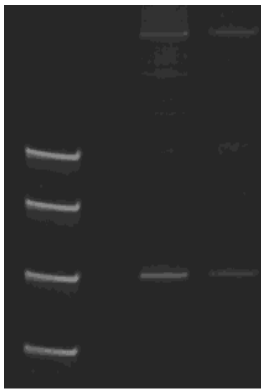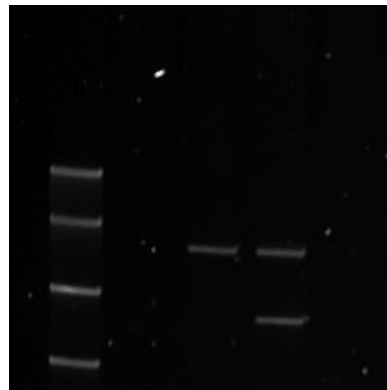
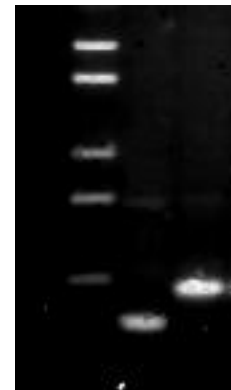**Fig. 14**   **Fig. 15**   **Fig.16**

Figure 14: Lane 1 shows the marker pBR322 DNA MspI-digest with fragment length of 622bp, 527bp, 404bp and 307bp. Lane 2 and 3 show PCR products containing two fragments. One at the very top of the picture that is not in the range of the marker anymore and a second fragment of approximately 300bp. According to the position of the primers, one fragment of 2.2kb should have been generated. This fragment was supposed to contain the promoter and the coding region of the VKORC1 gene and should demonstrate that the coding region of VKORC1 was integrated into the plasmid directly behind the promoter. According to these results, this was obviously not the case.

Figure 15 shows the PCR product corresponding to the coding region of VKORC1 with ~500bp. The first lane contains the marker (pBR322 DNA MspI-digest with fragments of 622bp, 527bp, 404bp and 307bp). Lane 2 and 3 show the PCR product. The longer fragment represents the coding region of VKORC1 and the shorter fragment corresponds to the shorter cDNA fragment generated by the annealing of the primer to the homologous cDNA sequence (explained in III.4.1).

Figure 16 shows the PCR products of the promoter variants -1639G and -1639A. Lane 1 shows the marker pBR322 DNA MspI-digest with

fragments of 160bp, 147bp, 123bp, 110bp, 90bp. Lane 2 shows the fragment corrsponding to the G allele fragments with a length of 82bp and lane 3 shows the fragment corrsponding to the A allele fragment with a length of 88bp.

The results indicate that the full length plasmids pGL3-G and pGL3-A contain the promoter as well as the inserted coding region of VKORC1 as intended, but also another sequence element.

5.2. Sequencing

The sequences obtained with the 5' UTR fw primer showed, that the promoter sequence was not followed by the coding region of VKORC1 as was planned, but by segments of the luciferase gene. However, the sequence of the luciferase gene was incomplete. The sequence segment of the luciferase gene was followed by a sequence that could not be identified using the chosen sequencing programm NCBI mega BLAST. Surprisingly, the VKORC1 coding region was not found in the plasmid construct by sequencing although a PCR product was generated when using cDNA specific primers**.**

IV. DISCUSSION

Osteocalcin is a protein which needs to be carboxylated to be active. The carboxylation is vitamin K dependent and is carried out by γ-carboxylase, the enzyme that post translationally modifies the Gla-residues of several proteins, among them osteocalcin. Following this modification osteocalcin is activated and is then able to bind Ca^{2+}. Bound Ca^{2+} is incooperated in bone, which confers bone its hardness and stability.

The -1639G>A polymorphism of the VKORC1 gene is the functional SNP in VKORC1 [Wang et al 2008]. The A allele is associated with a decrease of the promoter activity, resulting in lower mRNA expression compared to the G allele [Geisen et al 2005]. This difference in mRNA expression has been shown to partly explain the variability of warfarin sensitivy and dose requirement of warfarin between different patients and different ethnic populations. Patients with the genotype -1639AA are more warfarin-sensitive and require the lowest doses of warfarin, whereas patients with the genotype -1639AG or -1639GG require intermediate and high warfarin doses, respectively [Yuan et al. 2005]. The variability of warfarin dose requirement between different ethnic populations is due to different frequencies of the genotype -1639AA in different ethnic populations [Wang et al. 2008].

To my knowledge, the -1639G>A polymorphism was never investigated in association with bone and osteoporosis. The aim of this study was to collect information on the effect of the -1639G>A polymorphism on the expression of VKORC1 in osteoblasts,.

In my thesis I investigated whether the -1639AA genotype of the -1639G>A polymorphism decreases the promoter activity of VKOR in osteoblasts as has been shown by Geisen et al. [2005] for liver cells.

The transfection of the osteoblasts was performed with Lipofectamine 2000 as well as Metafectene Pro. The transfection with Lipofectamine was performed on two different days with four replica in each experiment, but the dual luciferase gene assay did not show stable results. This could be due to the fact, that the transfection with lipofectamine was performed at 34°C, but the temperature at which luciferase is most active is 37°C. It is possible that the low transfection temperature of 34°C influenced the efficiency and reproducability of transfection with Lipofectamine followed by the luciferase reporter gene assay. As only Wang et al. but no other research groups reported difficulties in transfection of cells including osteoblasts with Lipofectamine, a general problem of transfection with Lipofectamine can be excluded. Wang transfected HepG2 cells with Lipofectamine and measured the mRNA expression of the -1639G>A polymorphism in a dual luciferase reporter gene assay, but got no reliable results. The authors [2008] suggested that the transfection method with Lipofectamine for the reporter gene assay is an inappropriate method for detecting -1639G>A effects. Thus, it is possible that the problem is not the transfection method with Lipofectamine but the detection of this particular polymorphism with this method.

The results of the reporter gene assay of Metafectene transfected osteoblasts were much more consistent, than with Lipofectamine transfected cells, with significantly less variation within one transfection experiment and only slight variation between transfections performed on different days. The results of the reporter gene assay, following transfection with Metafectene, showed that in osteoblasts the promoter activity of the pGL3-G plasmid is 43% higher than the promoter activity of the pGL3-A plasmid.

This suggests that in osteoblasts, the A allele of the -1639G>A polymorphism is also associated with lower promoter activity of VKORC1 as reported for HepG2 cells. The results are in line with the data

reported by Yuan et al. [2005] who found a 44% higher promoter activity for the -1639GG allele compared to the -1639AA allele. These results are also in agreement with the studies of Geisen et al. [2005] and Wang et al. [2008] who both tested the effect of the -1639G>A polymorphism in liver cells.

In contrast, following lipofection with Lipofectamine the -1639A allele carrying constructs induced a higher promoter activity, albeit these results could only be observed in one of two experiments. Also, the differences between the quadruplicates were highly variable and not reliable. I assume, that the transfection method influences the results of the reporter gene assay.

To find an association, if any, between the effect of the -1639G>A polymorphism and osteoporosis, it would be necessary to prove that this polymorphism has the same effect when the promoter drives the VKORC1 gene instead of the luciferase gene.

After I found that the -1639G>A polymorphism in VKORC1 also has an effect in osteoblasts, I wanted to know if and how the promoter genotype influences the VKORC1 expression in osteoblasts. I planned to investigate a construct containing the VKORC1 promoter as well as the coding sequence. The strategy that I chose was based on cloning the coding region of VKORC1 into the plasmids that I already had which contained the promoter of VKORC1 with either the G allele or A allele at the position -1639. This strategy was not successful, because of problems during the preparation of the construct. The first indication was the observation of fragments with incorrect lengths as indicated by electrophoretic analyses of the construct. Analysis of the construct by sequencing showed that the promoter of VKORC1 is not followed by the coding region of VKORC1 but by a segment of the luciferase gene. The VKORC1 coding sequence could not be found by sequencing, instead, a sequence that was not assignable

was found directly following the luciferase fragment. Obviously the double digestion of the pGL3 plasmids, to cut out the luciferase gene, failed and that prevented the insertion of the coding region of VKORC1 into the pGL3-A and pGL3-G plasmids.

Due to time constraints, it was not possible for me to try another method to successfully produce a full length construct, which would be needed to be able to analyse the effect of the -1639G>A polymorphism on osteocalcin in osteoblasts.

The research group around Wang [2008] managed to produce a full length construct of VKORC1, proceeding differently. In contrast to my study, they cloned the whole VKORC1 gene into the pGL3-G and pGL3-A vector, and this approach was successful. Thus, the strategy chosen by Wang et al maybe the appropriate way to obtain a full length construct.

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