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THE ROLE OF CLUSTERIN FOR GROWTH REGULATION IN MELANOMA AND PROSTATE CANCER CELLS *IN VITRO*

DISSERTATION FOR AWARDING A DOCTORAL DEGREE

IN NATURAL SCIENCES (Dr. rer. nat.)

University of Saarland

2006

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Born in Damascus, Syria 05.02.1982

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SUMMARY

Clusterin (CLU), also known as Apolipoprotein J, is a glycoprotein that has been implicated in various cell functions involved in carcinogenesis and tumour progression. There are two known CLU protein isoforms generated in human cells. The nuclear form of the CLU protein (nCLU) is proapoptotic, whereas the secretory form (sCLU) has pro-survival functions. sCLU up-regulation appears to be a general molecular stress response. CLU expression has been associated with tumorigenesis of various malignancies, including human tumours of the prostate, colon, and breast. CLU expression is modulated by many factors that are involved in carcinogenesis or that regulate tumour growth and/or apoptosis, including 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], transforming growth factor beta-1, ultraviolet radiation, and ionizing radiation. However, the contribution of CLU signalling pathways for the UV-induced photocarcinogenesis of malignant melanoma, and for its progression and therapeutic outcome are still unknown. Presently, preliminary results in prostate cancer indicate that therapeutic modalities targeting CLU may be effective in cancer treatment. It was the aim of this study to gain further insights in the role of CLU in malignant melanoma and prostate cancer.

First, CLU expression was studied immunohistochemically in paraffin sections of primary cutaneous malignant melanomas (n= 18), metastases of malignant melanoma (n= 25), and acquired melanocytic nevi (n= 30). We developed an immunoreactivity score (IRS) to characterize the intracellular localization of CLU and the expression pattern of CLU during the progression of malignant melanoma.

In contrast to acquired melanocytic nevi, CLU immunoreactivity was detected in human primary cutaneous malignant melanomas (5 cases out of 18) and metastases of malignant melanomas (3 cases out of 20) *in situ*. The staining of the tissues was homogeneous (in the nuclei and cytoplasm of positive cells). The IRS score for malignant melanoma tissues (0.18) was higher than that for metastases of malignant melanomas (0.014).

Data reported in the literature indicate that expression of CLU may be directly regulated by 1,25$(OH)_2D_3$, an agent that induces apoptosis in melanoma cells and that is discussed to be of therapeutic use in the treatment of malignant melanoma. We therefore investigated CLU expression along with 1,25$(OH)_2D_3$ treatment in vitamin D-sensitive (MeWo, SK-MEL-28) and -resistant melanoma cell lines (SK-MEL-5, -25), as well as in normal human melanocytes (NHM), using conventional and real-time RT-PCR, and western blot. It was important to test for any differences in CLU expression depending on the kind of the melanoma cell line.

Both the CLU protein and the corresponding mRNA were detected in all melanoma cell lines and NHM investigated, with varying expression levels. Real-time RT-PCR showed that vitamin D-resistant human melanoma cell lines had a higher basal level of CLU expression (7.5×10^{-3} of normalized ratio) as compared to -sensitive human melanoma cell lines (2.8×10^{-4} of normalized ratio). NHM had an expression level similar to that of vitamin D-sensitive melanoma cell lines (2×10^{-4} of normalized ratio). In western blot analyses, only sCLU was detected in all melanoma cell lines and NHM analyzed. This sCLU protein expression was comparable to that of the mRNA where vitamin D-resistant melanoma cell lines showed stronger bands compared to -sensitive melanoma cell lines. We confirmed that vitamin D-sensitive cell lines revealed anti-proliferative effects when treated with 1,25$(OH)_2D_3$ (60% inhibition of cell proliferation in MeWo after 7 days of 1,25$(OH)_2D_3$ treatment, 10^{-6} M). Following 1,25$(OH)_2D_3$ treatment, one out of the two vitamin D-sensitive human melanoma cell lines analyzed (MeWo), showed an up-regulation in CLU mRNA expression after 96 hrs (1.5 fold increase at 10^{-6} M, $p < 0.1$). In contrast, the vitamin D-resistant melanoma cell line investigated (SK-MEL-5) showed no regulation of CLU expression along with 1,25$(OH)_2D_3$ treatment (10^{-6} M, $p > 1$).

Next, we analyzed whether changes in the expression of CLU modulate proapoptotic or anti-proliferative effects of 1,25$(OH)_2D_3$ or UV-B. Treatment with 1,25$(OH)_2D_3$ is discussed as a potential therapy for metastasizing melanoma, while solar UV-B radiation is involved in pathogenesis of malignant melanoma. To address this question, we used a cell culture model

with human LNCaP prostate cancer cells. These cells were either stably transfected by exogenous transfection and over-express sCLU (LNT-1), or were stably transfected with the vector alone (LN/C) and express sCLU at a similar level as compared to the isogenic LNCaP cells. The antiproliferative effects of $1,25(OH)_2D_3$ (10^{-6}, 10^{-7}, or 10^{-10} M) or UV-B (0, 100, or 500 J/m^2) on LNT-1 as compared to LN/C were evaluated using colony forming ability assays. Apoptotic responses following treatment with different doses of $1,25(OH)_2D_3$ or UV-B were measured using TUNEL assay.

sCLU over-expression slightly protected LNCaP prostate cancer cells dose-dependently against both anti-proliferative and proapoptotic effects of $1,25(OH)_2D_3$. This effect was most significant at a $1,25(OH)_2D_3$-concentration of 10^{-7} M (30% protection, $p< 0.1$, against the anti-proliferative effects and approximately 12% protection, $p< 0.1$, against the proapoptotic effects). Interestingly, sCLU over-expression protected against the proapoptotic (approximately 16%, $p= 0.038$) but not the anti-proliferative (approximately 0%, $p> 0.1$) effects following UV-B treatment. As investigated by the FACS analysis, sCLU over-expression did not affect the cell cycle of LNCaP cells following treatment either with $1,25(OH)_2D_3$ or UV-B.

CLU constitutive expression, and changes in expression after cell stress, could be of great importance for the growth regulation of human malignant melanoma and human prostate cancer, and could be involved in $1,25(OH)_2D_3$ apoptotic pathways. Moreover, our findings indicate that CLU may be of importance for the pathogenesis, progression and therapeutic outcome in these malignancies.

ZUSAMMENFASSUNG

Das Glykoprotein Clusterin (CLU), auch bekannt als Apolipoprotein J, reguliert zahlreiche Zellfunktionen, die an Tumorentstehung und Tumorprogression beteiligt sind. In menschlichen Zellen sind zwei unterschiedliche Isoformen des CLU Proteins bekannt. Die nukleäre Form des CLU Proteins (nCLU) vermittelt proapoptotische Eigenschaften, wogegen die sekretorische Form (sCLU) zytoprotektive Funktionen ausübt. Die vermehrte Expression von sCLU ist wahrscheinlich ein wichtiger molekularer Mechanismus der zellulären Stressantwort. Die Expression von CLU wurde mit der Pathogenese zahlreicher Malignome, einschließlich Prostata-, Kolon- und Mammakarzinom assoziiert. Die Expression von CLU wird durch zahlreiche Faktoren, die auch für Tumorentstehung, Tumorprogression oder Apoptose von Bedeutung sind, moduliert. Hierzu zählen z.B. 1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], Transforming growth factor beta-1, ultraviolette und ionisierende Strahlung. Die Beteiligung von CLU Signaltransduktionswegen an der Photokarzinogenese des malignen Melanoms, sowie an dessen Progression und therapeutischem Ansprechen, ist bislang jedoch noch völlig ungeklärt. Aktuell deuten erste Untersuchungsergebnisse beim Prostatakarzinom darauf hin, dass CLU eine interessante Zielstruktur zur Etablierung neuer Therapiemodalitäten darstellt. Die Zielsetzung dieser Arbeit bestand darin, neue Erkenntnisse über die Bedeutung von CLU beim malignen Melanom und beim Prostatakarzinom zu gewinnen

Hierzu untersuchten wir zunächst immunhistochemisch die Expression von CLU in Paraffinschnitten von primär kutanen malignen Melanomen (n= 18), von Melanommetastasen (n= 25), und von erworbenen melanozytären Nävi (n= 30). Um die intrazelluläre Lokalisation von CLU und dessen Expressionsmuster während der Progression des malignen Melanom zu charakterisieren entwickelten wir einen Immunreaktivitätsscore (IRS).
Im Gegensatz zu gutartigen melanozytären Nävi konnten wir eine CLU Immunreaktivität in primär kutanen malignen Melanomen (5 von 18 untersuchten Präparaten) und in Melanommetastasen (3 von 20 Präparaten)

in situ nachweisen. In positiven Zellen zeigte sich eine homogene Markierung im Kern und im Zytoplasma. Bei den malignen Melanomen war der Immunreaktivitätsscore (0,18) höher, verglichen mit Melanommetastasen (0.014).

Literaturdaten deuten darauf hin, daß die CLU Expression möglicherweise durch $1,25(OH)_2D_3$ direkt reguliert wird, eine Substanz die in Melanomzellen Apoptose induziert und deren therapeutischer Einsatz beim malignen Melanom diskutiert wird. Deshalb untersuchten wir die CLU Expression in $1,25(OH)_2D_3$-behandelten Vitamin D-sensitiven (MeWo, SK-MEL-28) und -resistenten Melanomzelllinien (SK-MEL-5, -25), und in normalen humanen Melanozyten (NHM). Hierzu wurden neben der konventionellen PCR auch die sogenannte real-time PCR sowie der Western Blot eingesetzt.
Sowohl das CLU Protein als auch die korrespondierende mRNA konnten in allen untersuchten Melanomzelllinien und in NHM in unterschiedlicher Intensität nachgewiesen werden. In der real-time RT-PCR zeigte sich, daß Vitamin D-resistente Melanomzelllinien eine höhere basale CLU Expression (7.5×10^{-3} des normalisierten Quotienten) aufweisen, verglichen mit –sensitiven Melanomzelllinien (2.8×10^{-4} des normalisierten Quotienten). Die CLU Expression in NHM (2×10^{-4} des normalisierten Quotienten) war vergleichbar mit der in Vitamin D-sensitiven Melanomzelllinien. Im Western Blot wurde ausschließlich sCLU in Melanomzelllinien und in NHM nachgewiesen. Diese sCLU Protein Expression war vergleichbar mit der mRNA Expression. Vitamin D-resistenten Melanomzelllinien zeigten im Western Blot stärkere Banden als Vitamin D-sensitive Zelllinien. Wir bestätigten die antiproliferative Wirkung von $1,25(OH)_2D_3$ auf Vitamin D-sensitive Zelllinien (60%ige Hemmung der Proliferation von MeWo nach 7 tägiger Behandlung mit $1,25(OH)_2D_3$, 10^{-6} M). Eine 96-stündige Behandlung mit $1,25(OH)_2D_3$ (10^{-6} M) führte in einer der beiden untersuchten Vitamin D-sensitiven Melanomzellinien (MeWo) zu einer vermehrten CLU mRNA Expression (1.5 facher Anstieg, $p< 0.1$). Dieser Anstieg der CLU Expression nach 96-stündiger Behandlung mit $1,25(OH)_2D_3$ (10^{-6} M) fand sich nicht ($p > 1$) in SK-MEL-5, der einzigen untersuchten Vitamin D-resistenten Melanomzelllinie.

Danach analysierten wir, inwieweit eine Veränderung der CLU Expression die Apoptose-induzierende oder antiproliferative Wirkung von $1,25(OH)_2D_3$ oder UV-B auf Zielzellen beeinflußt. $1,25(OH)_2D_3$ wird als mögliches Therapeutikum zur Behandlung des malignen Melanoms diskutiert, die UV-B-Strahlung ist an dessen Pathogenese beteiligt. Zur Untersuchung dieser Fragestellung verwendeten wir ein uns zur Verfügung stehendes Zellkulturmodell mit humanen LNCaP-Prostatakarzinomzellen die entweder sCLU nach stabiler Transfektion vermehrt exprimieren (LNT-1), oder die nach Transfektion des Vektors keine Veränderungen in der Expression von sCLU aufweisen (LN/C). Die Effekte der Behandlung mit $1,25(OH)_2D_3$ in unterschiedlicher Dosierung (10^{-6}, 10^{-7}, oder 10^{-10} M) oder mit UV-B (0, 100, oder 500 J/m^2) auf die Proliferation von LNT-1- und LN/C-Zellen wurde unter Verwendung des sogenannten "colony forming ability assays" verglichen, die Apoptoserate unter Verwendung des sogenannten TUNEL Assays.
Die vermehrte Expression von sCLU schützte LNCaP Prostatakarzinomzellen dosisabhängig sowohl gegen die antiproliferativen als auch gegen die proapoptotischen Effekte von $1,25(OH)_2D_3$. Dieser Effekt war bei einer Konzentration von 10^{-7} M $1,25(OH)_2D_3$ am stärksten ausgeprägt (30%ige Protektion gegen antiproliferative Wirkung, $p< 0.1;$ ~12%ige Protektion gegen proapoptotische Wirkung, $p< 0.1$). Interessanterweise schützte die vermehrte Expression von sCLU gegen proapoptotische (~16%, $p= 0.038$), aber nicht gegen antiproliferative Wirkung (~ 0 %) ($p> 0.1$) der UV-B Bestrahlung. In der FACS Analyse zeigten sich in Abhängigkeit von der sCLU Expression in LNCaP Zellen weder nach Behandlung mit $1,25(OH)_2D_3$ noch mit UV-B Veränderungen in der Zellzyklusregulation.

Die konstitutive CLU Expression, und Veränderungen in deren Expression unter Stress können für die Regulation des Tumorwachstums beim malignen Melanom und beim Prostatakarzinom von großer Bedeutung sein. Unsere Ergebnisse sprechen für eine Bedeutung von CLU für Pathogenese, Progression und Therapie dieser Malignome.

LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitmain D_3
APS	amonium persulphate
ASO	antisense oligonucleotide
BPE	bovine pituitary extract
BSA	bovine serum albumin
CFA	colony forming ability assay
CLU	clusterin
DNA-PK	DNA-dependent protein kinase
DSB	double strand break
EDTA	ethelyne-diamine-tetra-acetic acid
EGFR	epidermal growth factor receptor
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
IGF-1R	insulin-like growth factor receptor
IHC	immunohistochemistry
IR	ionizing radiation
IRS	immunoreactivity score
nCLU	nuclear clusterin
NHM	normal human melanocytes
NLS	nuclear localization signal
PEs	plating efficiencies
PMA	phorbol myristate acetae
pnCLU	precursor nuclear clusterin
PP	percentage of positive cells
RXR	retinoid X receptor
sCLU	secretory clusterin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SI	staining intensity
SiRNA	short interfering RNA
TEMED	tetramethylethylenediamine
TGF-ß	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl-mediated dUTP-biotin nick end-labelling
UV	ultraviolet
VDR	vitamin D receptor
VDREs	vitamin D response elements

THE SCOPE OF THE STUDY

Human malignant melanoma is a highly aggressive skin cancer, compromising only 10% of all skin cancers. Nevertheless, human melanocytes are responsible for 85% of skin cancer deaths. Unlike the rest of the skin, melanomas are divided from neural crest cells pinched off during development. When melanocytes become malignant they invade the surrounding healthy skin. Melanoma may stay in the skin, but more often it metastasizes through the blood or the lymph system to bones and other organs. The standard cytotoxic therapy, dacarbazine, has a response rate between 15% and 20% in more optimistic studies and rarely leads to long-lasting responses. Drug resistance is a major problem for malignant melanoma therapy (Namkoong *et al.*, 2006).

Chances are 100%, that if a man lives long enough he will get prostate cancer. Prostate cancer kills 1 in 4 men who get the disease, making it one of the deadliest forms of cancer. The growth of human cancerous cells in the prostate is stimulated by male hormones, especially, testosterone. Prostate cancer cells can metastasise to lymph nodes, various bones (especially those of the hip and lower back), lungs, and occasionally to the brain. Common treatment methods include prostatectomy, chemo- and/or hormonal (e.g., anti-testosterone) therapies, though drug-resistance occurs more often than not (Costello *et al.*, 2006).

It is therefore, extremely important to find new therapies to overcome drug resistance for the treatment of human malignant melanoma, as well as human prostate cancer. 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is a promising agent to induce apoptosis in human prostate cancer and melanoma cancers. However, due to their hypercalcaemic effects, new vitamin D analogues with little or no hypercalcaemic side-effects were introduced. Many of these compounds are still under investigation in clinical trials, and the need for a new agent in the therapy of human prostate and melanoma cancers is still unfulfilled. Secretory clusterin (sCLU), a glycoprotein with antiapoptotic

properties, has emerged lately as a target for cancer therapy. Various studies showed that silencing sCLU expression led to more efficient therapy outcome. We hypothesize that CLU may be related to the pathogenesis and progression of certain phenotypes of malignant melanoma, and that CLU has a role in UV-B-induced apoptosis. Thus, this study aimed at exploring CLU expression in human malignant melanoma cell lines and tissues. A major goal of our study was to test whether CLU expression was modulated by $1,25(OH)_2D_3$ exposure, and to determine the extent of protection afforded by increased sCLU expression in human prostate cancer cell lines following various apoptosis-inducing agents. We demonstrated that sCLU is over-expressed in some of the human melanoma cells used, and expression of this gene could be of importance for the pathogenesis and/or progression of certain malignant melanoma phenotypes.

INTRODUCTION

1.1. PROSTATE CANCER AND MALIGNANT MELANOMA

Prostate cancer is one of the leading causes of death in men in most western countries. The exact causes of prostate cancer are unknown, but both diet and genetic inheritance are known to play role in the onset and progression of the disease. A hallmark of human prostate cancer cells is their slow growth, which can be stimulated by male hormones, especially testosterone. Androgen withdrawal, prostatectomy, radiation, and chemotherapy are some of the common treatments for prostate cancer (Singh *et al.*, 2006). Unfortunately, many of these current anticancer agents are most effective against rapidly growing cells, making slow growing prostate cancer cells inherently resistant to their effects.

It is accepted that malignant melanoma represents the most dangerous type of skin cancer (Millen *et al.*, 2004). UV radiation of normal melanocytes has been shown to induce apoptosis and other biologic responses that are involved in carcinogenesis (Millen *et al.*, 2004). Therefore and from epidemiological studies, ultraviolet (UV) radiation has been discussed as a main risk factor in the pathogenesis of malignant melanoma. It is known that malignant melanoma has a very low intrinsic level of apoptosis and different proteins in the apoptotic pathway can be defective by mutation or loss of expression (Hoeller *et al.* 2005). Treatment of malignant melanoma is difficult and varies. According to the stage of its progression, a combination of surgery and chemo- or radiotherapy may be needed (Millen *et al.*, 2004).

Due to its anti-proliferative effects, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and its analogues have been used for prostate cancer and melanoma treatment. Thus, extensive research was done to find certain agents that would increase the efficiency of chemotherapy in these two malignancies.

1.2. CLUSTERIN, A HISTORICAL OVERVIEW

Clusterin (CLU), also known as Apolipoprotein J (Apo J), testosterone-repressed prostate message-2 (TRPM-2), x-ray-induced transcript leading to protein-8 (xip8), sulphated protein 40-40 (SP 40-40), complement lysis inhibitor, glycoprotein 80 (gp80), glycoprotein III, tumour protein 64 (T64), or sulphate glycoprotein-2 (SG-2), is a secretory heterodimeric disulphide-linked glycoprotein (449 amino acids) that is expressed in virtually all tissues and found in all human fluids analyzed so far (Trougakos *et al.,* 2002, 2004; Pucci *et al.,* 2004; Gleave and Miyake, 2005; Pajak *et al.,* 2006). It was first isolated from rat testes fluid in 1986 by Montpetit *et al*., and has been implicated in various cell functions involved in carcinogenesis and tumour progression, including cell adhesion, tissue remodelling, lipid transportation, membrane re-cycling, immune processes and cell cycle regulation, DNA repair, and in both pro- as well as anti-apoptotic cell death processes (Trougakos *et al.,* 2002, 2004; Pucci *et al.,* 2004). It functions as an extracellular chaperone that stabilizes stressed and unfolded proteins in a folding-component state (Trougakos *et al*., 2002). CLU transcription can be regulated by heat shock factor-1 and because it binds to a variety of biologic ligands acting to potentially inhibit stress-induced protein precipitation, CLU is considered to function similarly to a small heat shock protein to chaperone and stabilize conformations of proteins at times of cell stress (Gleave *et al*., 2005).

Increased CLU mRNA and protein levels have been detected in various tissues undergoing stress, including heart, brain, liver, kidney, and retinal tissues both *in vivo* and *in vitro*. CLU expression was shown to be associated with the progression of various human malignancies, including tumours of bladder (Miyake *et al*., 2001), colon (Pucci *et al*., 2004), prostate (Miyake *et al*., 2000, 2004; Zellweger *et al*., 2002; Bettuzzi *et al*., 2002), breast (van Weelden *et al*., 1998; Leskov *et al*., 2003), lung (July *et al*., 2004), and kidney (Hara *et al*., 2001). However, the role of CLU in the progression of malignant melanoma is yet to be defined.

1.3. CLUSTERIN ISOFORMS

CLU, which is coded by a single gene located on human chromosome 8 (Pajak *et al.*, 2006), has been attributed two distinct and opposing functions by many recent observations, either cell survival, tumour progression, treatment resistance *in vivo*, or apoptosis (Trougakos *et al.*, 2004; Gleave and Miyake, 2005). In fact, these functions seem to be attributed to the existence of two different, but related, CLU protein isoforms, a glycosylated and non-glycosylated form that are produced in the cell by alternative splicing (Leskov *et al.*, 2003). These two forms of the CLU protein can be immunologically distinguished (Leskov *et al.*, 2003, reviewed in Shannan *et al.*, 2006, b). However, little is known about CLU isoforms in malignant melanoma, and in this study we investigated this issue further.

sCLU: The secreted form of the CLU protein (sCLU) is a glycosylated protein of ~80 kDa. Depending on the degree of glycosylation, it usually appears as two different protein bands by western blot analyses: one full-length non-cleaved 60 kDa protein, and another rather smeared band appearing as ~40 kDa α and β proteins as detected by SDS-PAGE (Leskov *et al.*, 2003). The expression of sCLU is initiated by the translation from full-length CLU mRNA, where the first AUG codon is translated into the 49 kDa sCLU precursor protein (Figure 1.2.a) (Leskov *et al.*, 2003; Trougakos *et al.*, 2004, reviewed in Shannan *et al.*, 2006 b). This protein is directed to the endoplasmic reticulum by a leader signalling peptide, which is cleaved from the protein during transportation. While the protein is being transported towards the golgi, this pre-cursor polypeptide is cleaved at its α and β site and subsequently heavily glycosylated. The result is a secreted protein of ~80 kDa that consists of α and β heavily glycosylated peptides, linked by five disulphide bonds, Figure. 1.2.b.

nCLU: In contrast, the nuclear form of the CLU protein (nCLU) is an initially synthesized 49 kDa protein, referred to as a precursor form of nCLU (i.e. pnCLU). In human cells, the pnCLU protein was shown to be synthesized from a second in-frame AUG codon from an alternatively spliced nCLU, which eliminated exon II that would otherwise encode for the sCLU start AUG and

signalling leader peptide. It is currently unclear as to how nCLU is expressed in rodent cells, since the same splice junction is not conserved. One theory is that there is alternative reading from the second AUG start site by intervening IRIS sequence-like sequences (Leskov *et al.*, 2003). Interestingly, once made, the 49 kDa pnCLU protein does not undergo any α and β cleavage, nor does it appear to be heavily glycosylated. pnCLU appears to be localized to the cytoplasm of normal human and rodent cells (Figure 1.2.) (Leskov *et al.*, 2003).

In response to severe cell stress (i.e., exceeding 1 Gray IR), pnCLU is post-translationally modified and a mature ~55 kDa pro-apoptotic protein form (nCLU) is generated and induces apoptosis. pnCLU is induced and translocated from the cytoplasm to the nucleus following certain cytotoxic events (Leskov *et al.*, 2003). Despite the fact that the structure/function relationship of pnCLU is not completely understood, it appears to contain at least two nuclear localization signals that target the nCLU protein to the nucleus. Previous studies demonstrated that over-expression of nCLU protein with both a C-terminal coiled-coil domain and a functional nuclear localization signal (NLSs) only caused cell death. Some experiments have shown a mechanism by which the inert pnCLU could be activated to a nCLU 'death' protein. Recent data indicate that regulation of nCLU into the nuclei of irradiated cells occurs through both nuclear localization as well as nuclear export sequences. Importantly, mutation of the NLS in the C-terminus of nCLU appears to abrogate nCLU functions (Leskov *et al.*, 2003), suggesting that nuclear accumulations are essential for the protein's pro-apoptotic functions.

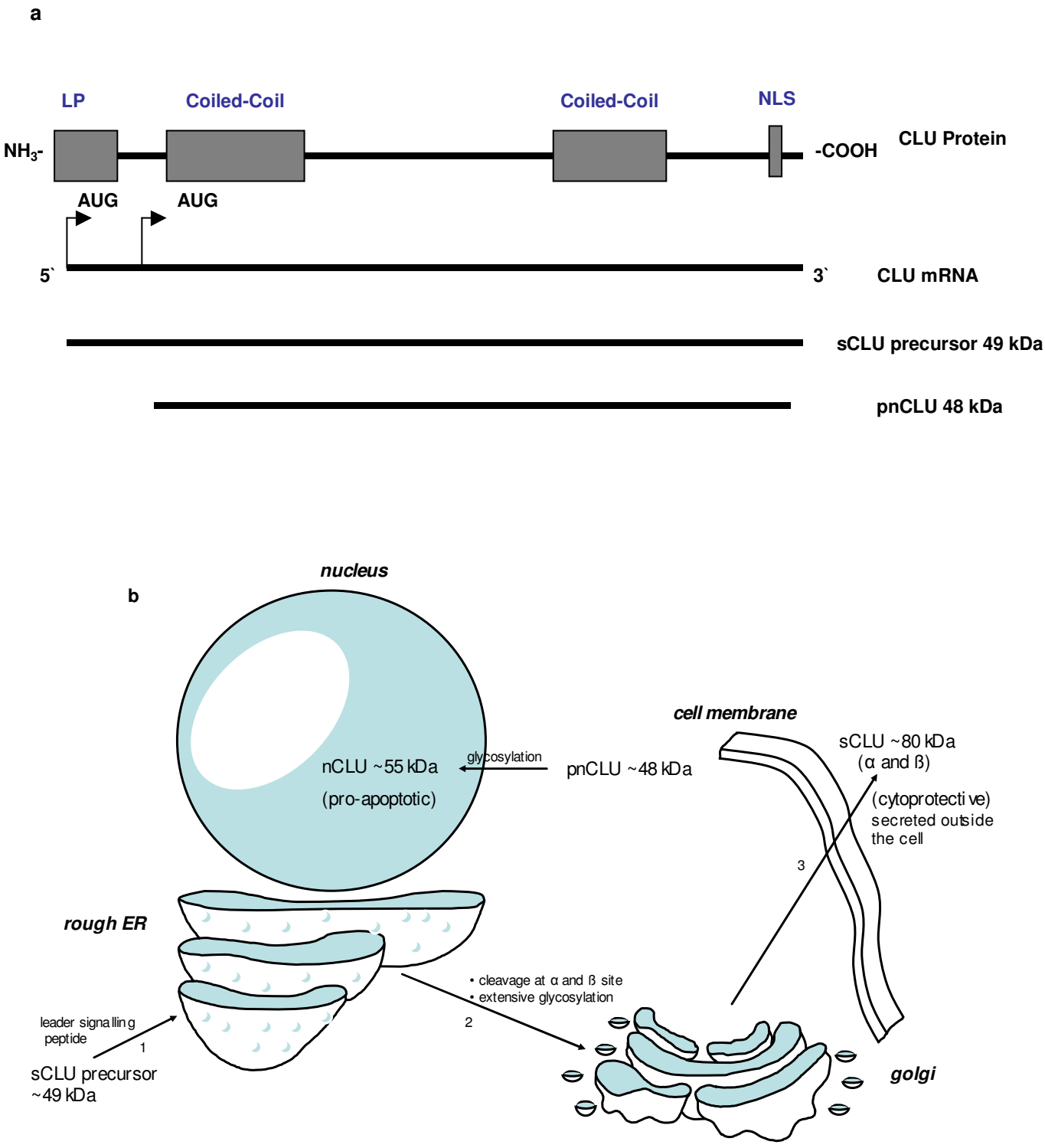


Figure 1.2. Generation of sCLU and nCLU. (a) sCLU precursor is translated from the first AUG codon of the full-length CLU mRNA, while nCLU precursor (pnCLU) is obtained by alternative splicing from the second in-frame AUG codon of the full-length CLU mRNA. LP: leader peptide; NLS: nuclear localization signal. (b) sCLU persursor is transported into the rough ER by leader signalling peptide (1), and then undergoes cleavage and extensive glycosylation while being transported to the golgi apparatus (2). The result is a secreted protein of ~80 kDa with five disulphide bonds between the α- and β-subunit that is secreted

outside the cell (3). pnCLU does not undergo any cleavage nor extensive glycosylation, and resides in the cytoplasm of unstressed cells. It becomes the mature form (~55 kDa) once it is transported to the nucleus.

1.4. *IN VIVO* FUNCTIONS AND CLUSTERIN

1.4.1. APOPTOSIS

Although it has been proposed that CLU up-regulation represents a marker of apoptotic response, the precise relationship between CLU gene expression and programmed cell death has not yet been elucidated clearly (Yang *et al.*, 1999). Elucidation of CLU function in cells after stress is clearly complicated by the presence of two different forms of the protein that have apparently two separate functions in the cell.

A number of studies have demonstrated that over-expression of the full-length CLU mRNA that would produce (depending on where the translation would start from and in this case) nCLU that acts as a pro-death signal, inhibiting cell growth and survival (Lakins *et al.*, 1998; Jin and Howe, 1999; Trougakos *et al.*, 2002).

However, other studies have shown that CLU over-expression may exert cytoprotective properties (Zellweger *et al.*, 2001, 2002, 2003; Miyake *et al.*, 2002, 2003, 2004; Criswell *et al.*, 2003; Trougakos *et al.*, 2004). Furthermore, it has been proposed that tumour cell survival is connected with over-expression of sCLU and the loss of nCLU (Pucci *et al.*, 2004). This theory has been supported by recent data which suggest that cells must suppress sCLU to stimulate cell death. One proposed mechanism for this death process is through the activation of the p53 tumour suppressor gene (Criswell *et al.*, 2003). This would explain how progression towards high-grade and metastatic carcinoma leads to a shift from nCLU to sCLU expression, and that the role of CLU for tumour growth maybe related to a pattern shift in its isoform production (Pucci *et al.*, 2004).

It is well known that the apoptotic pathways are regulated via an orchestrated interplay of multiple activators (e.g. tumour necrosis factor, Fas ligand, Bax, and p53) and suppressors (e.g. various growth factors and Bcl-2) (Osborne and Hutchinson, 2002). In this context, it is interesting that p53, an activator of the apoptotic cascade, can suppress basal as well as IR (ionizing radiation)-

induced sCLU expression in both MCF-7 (breast cancer) and HCT116 (colon cancer) cells by repressing CLU promoter activity and transcription (Criswell *et al.*, 2003).

A new report has recently described how CLU may exert an anti-apoptotic or pro-apoptotic function by interacting with different proteins depending on its distribution. CLU has an essential role in chemoresistance by interacting with activated Bax, thereby inhibiting cytochrome C release and apoptosis, and in order to induce apoptosis in cancer cells, the CLU-Bax interaction should be disturbed (Zhang *et al.*, 2005). It has also been proposed that Ca^{2+} depletion causes pnCLU activation leading it to the nucleus and thus leading to cell death (Pajak *et al.*, 2006). In another study, it was also shown that sCLU induction is triggered by changes in Ca^{2+} homeostasis, where a release in Ca^{2+}, causes an induction of sCLU (Araki *et al.*, 2005).

1.4.2. CELL CYCLE REGULATION

The cell cycle is an ordered set of events resulting in cell growth and division into two cells. The stages are G_1, S, G_2, and M. G_0 is when cells stop cycling after division, entering a state of quiescence. It is still a matter of debate as to what extent is CLU expression cell cycle dependent. It was shown that in human dermal fibroblasts CLU acts as a cell cycle-related gene, controlled by serum starvation, and that CLU protein accumulation occurred in the G_0 phase (Bettuzzi *et al.*, 1999). On the other hand, another study proved that the effect of p53 on IR-inducible sCLU expression was not dependent on the cell cycle, but was delayed in its induction, requiring at least 48 hrs post IR in MCF-7 breast and HCT116 colon cancer cells (Criswell *et al.*, 2003).

The overall function of CLU expression in cell cycle regulation remains unclear, and so is the case for CLU expression in UV-induced cell cycle changes, which will be discussed in this work. Use of short interfering RNA (siRNA) specific to each form of the CLU protein should allow future studies to define cause-effect relationships and reveal whether sCLU or nCLU directly affect cell cycle regulation with or without cell stress.

CLU expression was reported to be directly regulated by B-MYB, a transcription factor involved in the regulation of cell survival, proliferation, and

differentiation (Cervellera *et al.*, 2000). Another transcription factor that was reported to be regulated by CLU is NF-κB. Modulation of the NF-κB activity is believed of importance for cell survival, cell motility, proliferation, and transformation (Sankilli *et al.*, 2003). CLU expression could be required to suppress excessive NF-κB activation, which determines inflammation and cardiomyocyte apoptosis in autoimmune myocarditis in the rat. Loss of CLU expression in cells that depend on NF-κB activity for proliferation or chemoresistance could lead to tumour progression (Sankilli *et al.*, 2003).

In a recent study, sCLU expression was shown to be regulated by IGF-1 receptor (IGF-1R) in MCF-7 breast cancer cells. IGF-1 is secreted by the lymph nodes and is thought to be a factor in tumorigenesis of neighbouring breast tissues (Criswell *et al.*, 2005). In this study, IGF-1R activation resulted in mitogenic growth and cell survival, and a selective inhibitor of IGF-1R blocked sCLU induction after IR. The authors stated that the specific cytoprotective role of sCLU after IR strongly suggests that activation of IGF-1/IGF-1R signalling is an essential survival pathway that can be targeted for radiotherapy (Criswell *et al.*, 2005).

1.5. CLUSTERIN AND DNA REPAIR

In addition to the function of CLU in mediating apoptosis (Lakins *et al.*, 1998; Yang *et al.*, 1999; Trougakos *et al.*, 2002; Criswell *et al.*, 2003; Leskov *et al.*, 2003), increasing evidence supports an important function for CLU in DNA repair (Bettuzzi *et al.*, 1999). Interestingly, CLU has been identified as the first stress-inducible protein that is associated with the DNA-dependent protein kinases (DNA-PK) double-strand break (DSB) repair complex (Yang *et al.*, 1999).

Two types of DSB repair occur after induction of DNA lesions; homologous and non-homologous. At least two processes for non-homologous end joining of DSBs are thought to occur in mammalian cells, one involving the DNA-PK complex, and the other a combination of proteins. nCLU protein binds to Ku70, forming a trimeric protein complex with Ku80; Ku70 and Ku80 are two components of the DNA-PK complex (Yang *et al.*, 1999, 2000). DNA-PK is a

nuclear serine/threonine kinase that requires DNA ends for catalytic activity. It is selectively activated by the binding to the ends of linear double-strand breaks.

The exact mechanism by which DNA-PK takes part in physical resealing of a DSB is not fully understood. Data showing that over-expression of truncated CLU mRNA reduces the binding activity of Ku70/Ku80 to DNA ends in whole-cell extracts suggests that nCLU may directly affect DSB repair. However, the importance of nCLU in DNA repair *versus* the induction of apoptosis remains unclear. Recent data indicate that Ku70 plays an important dual role in binding Bax and protecting cells from Bax-mediated cell responses (Suzuki *et al.*, 2003). Since nCLU and Bax bind Ku70 at the same site (Yang *et al.*, 2000; Leskov *et al.*, 2001), it has been proposed that these proteins compete for Ku70 binding and that nCLU may free Bax from Ku70. Thus, it is possible that nCLU may both free Bax and bind Ku70, thereby inducing apoptosis and inhibiting Ku70/Ku80 end binding activity. It is not surprising that a cell committing itself to death would perform such functions, since DNA-PK itself is a substract for apoptosis-related activated caspase 3 (Mandal *et al.*, 1999). Indeed Bax-deficient cells appear to be resistant to nCLU-induced apoptotic cell death (Leskov and Boothman *et al.*, unpublished data).

1.6. REGULATION OF CLU EXPRESSION IN CANCER CELLS

A major phenomenological observation is that sCLU levels are altered in many cancers at an early stage, however, the exact mechanisms by which this gene is permanently up-regulated remains unknown. One hint in the process of the gene's regulation is that a wide variety of different compounds involved in the modulation of tumour growth or in cancer therapy can regulate CLU expression in both normal and cancer cells. Since vitamin D_3 analogues (e.g., 1,25-dihydroxyvitamin D_3, [1,25$(OH)_2D_3$]) have been used to treat some tumours, a major goal of my studies was to investigate the role of this agent in the up-regulation of sCLU in normal versus tumour cells, and then to explore the role of this protein in responses to 1,25$(OH)_2D_3$ exposure.

1,25$(OH)_2D_3$ and/or its analogues inhibit proliferation and induce apoptosis in a wide variety of cell lines, including cancer cells (James *et al.*, 1996; Narvaez

et al., 1996; Simboli-Campbell *et al.*, 1997; Osborne and Hutchinson, 2002; Leskov *et al.*, 2003). An increase in sCLU was observed in MCF-7 breast cancer cells following $1,25(OH)_2D_3$ treatment as compared to control cells (Simboli-Campbell *et al.*, 1996). Increased expression of both sCLU and prostatic atrophy markers were observed after treatment of benign prostate hyperplasia cells with the $1,25(OH)_2D_3$ analogue, BXL-628 (Crescioli *et al.*, 2004). BXL-628 inhibited prostate cell proliferation *in vivo* and *in vitro*. Since, sCLU over-expression in LNCaP cells (some LNCaP clones express very low levels of sCLU) may accelerate prostate cancer development through the inhibition of membrane damage induced by H_2O_2 (Miyake *et al.*, 2004).

Other factors that have been shown to affect the expression of CLU in tumour cells include various growth factors that mediate proliferation and differentiation signals that are important for tumour cell growth. CLU mRNA was induced and activated in PC12 prostate cancer cells by nerve growth factor and epidermal growth factor (Gutacker *et al.*, 1999). Transforming growth factor beta (TGF-β), which is involved in a wide variety of biological processes, ranging from embryonic development to neoplastic transformation, induces CLU gene expression in various cell types. Transforming growth factor beta-1 (TGF-β1), which is a potent inhibitor of the proliferation of many cell types and one that is involved in cell cycle control and apoptosis, enhanced sCLU protein and mRNA expression in thyroid epithelial cells. Up-regulation of CLU expression may be a marker for TGF-β1 mediated thyrocyte differentiation (Wegrowski *et al.*, 1999).

In rodent cells, CLU gene expression was proposed to occur via a consensus AP-1 binding site located in the promoter region of this gene (Jin and Howe, 1999; Criswell *et al.*, 2003). Further, TGF-β1 induced CLU expression in CCL64 mink lung cancer cells was repressed by over-expression of the c-Fos proto-oncogene, and it was speculated that TGF-β1 could exert effects on c-Fos protein synthesis and/or stability, resulting in the repression of c-Fos, and thus, increased CLU gene expression. Another important cell survival pathway in prostate and other cancers involves IGF-1/IGF-1R signalling pathways that converge to block apoptosis. Activated IGF-1R stimulates Ras-Mek-Erk signal

transduction to transactivate the early growth response-1 transcription factor, which increases sCLU expression (Criswell *et al.*, 2005).

1.7. EXPRESSION OF CLU IN MALIGNANT TUMOURS

1.7.1. ROLE OF CLU AS A PROGNOSTIC MARKER

Changes in sCLU expression have been documented in a variety of different malignancies, including in human prostate, skin, pancreatic, breast, lung, and colon tumours, as well as in oesophageal squamous cell carcinoma, and neuroblastoma (Trougakos *et al.*, 2004). The primary function of sCLU in distinct backgrounds of cancer cells appears to be anti-apoptotic (Trougakos *et al.*, 2004). On the other hand, higher CLU levels were seen in prostate and kidney carcinomas when compared with normal tissues. Whether increased expression of anti-apoptotic sCLU is a common feature of tumorigenesis, thereby protecting cancer cells against apoptotic stimuli that might cause cell death, is still a matter of debate. Moreover, the question of whether anti-apoptotic sCLU is the only form of CLU expressed in cancer, or whether pro-apoptotic forms of nCLU are down-regulated in distinct tumour entities, has not been definitely answered at this time.

Recent data indicate that progression towards a high-grade and metastatic carcinoma leads to elevated sCLU levels and altered intracellular distribution of nCLU. Thus, the function of CLU in tumours may be related to a pattern shift in its isoform production (Pucci *et al.*, 2004). As discussed above, over-expression of sCLU was shown in the majority of tumours investigated including prostate cancer (Miyake *et al.*, 2003, 2004; Zellweger *et al.*, 2003; Scaltriti *et al.*, 2004), breast carcinoma (van Weelden *et al.*, 1998; Redono *et al.*, 2002), lung (July *et al.*, 2004), bladder (Miyake *et al.*, 2001), and colon (Pucci *et al.*, 2004) cancers. Full-length CLU mRNA and sCLU protein were both involved in intestinal tumours (Chen *et al.*, 2003). In prostate cancer, sCLU expression was associated with the development of androgen resistance and it (i.e., sCLU) is thought to act as a protective factor against treatment-induced cell death (Redono *et al.*, 2002; Miyake *et al.*, 2004). A few reports, do, however, suggest decreased sCLU levels in specific cancers, including nonmelanoma skin cancer (Thomas-Tikhonenko *et al.*, 2004),

oesophageal squamous cell carcinoma (Zhang *et al.*, 2003), neuroblastoma (Sankilli *et al.*, 2003), prostate (Bettuzzi *et al.*, 2002; Scaltriti *et al.*, 2004), and pancreatic cancer (Xie *et al.*, 2002). In addition, in human prostate LNCaP cells, CLU appeared to be associated with tumour progression and resistance to chemotherapy *in vivo* (Zellweger *et al.*, 2003). Table 1.7. summarizes the expression of sCLU in various tumours.

In one study, CLU expression was analyzed during progression of prostate cancer (Scaltriti *et al.*, 2004). While expression levels of sCLU protein and full-length CLU mRNA were non-detectable in prostate carcinoma cells, high expression levels of CLU protein and mRNA were found in stromal peritumoral fibroblasts. In different stages of tumour progression, sCLU accumulated in specific, well-defined areas of the stromal compartments near these tumours.

In advanced prostate cancer, staining of remnants of the extracellular matrix suggested a role for sCLU in the process of dismantling the stromal organization caused by prostate cancer (Scaltriti *et al.*, 2004). CLU expression was induced in pancreatic cancer and its down-regulation may be associated with disease progression (Xie *et al.*, 2004). It is not clear, however, in these studies if sCLU *versus* nCLU expression levels were clearly separable. There is a need for improved methods to detect nCLU in cases like this. Thus, although the majority of studies indicate that sCLU expression is up-regulated along with the progression of distinct malignancies, its role as a prognostic factor is still questionable and more research examining expression levels of sCLU *versus* nCLU in these cancers is needed (Miyake *et al.*, 2000, 2001, 2004; Chen *et al.*, 2003; Zellweger *et al.*, 2003; Pucci *et al.*, 2004).

Table 1.7. Summary of CLU expression in various tumours as observed by various studies

Tumour	**CLU expression**	**Reference**
Prostate cancer	*↑ mRNA and protein	Lakins, 1998; Miyake 2002; Zellweger, 2002, 2003
Breast carcinoma	↑ mRNA	Van Weelden 1998; Leskov, 2003
Lung cancer	↑ mRNA	July, 2004
Colon cancer	↑ mRNA and protein	Seiberg, 1995; Pucci, 2004
Skin cancer	**↓ mRNA	Redono, 2002
Oesophageal squamous cell carcinoma	↓ Protein	Chen, 2003
Neuroblastoma cancers	↓ Protein	Thomas-Tikhonenko, 2004
Pancreatic cancer	↓ mRNA	Zhang, 2003
Prostate cancer	↓ mRNA	Bettuzzi, 2002; Scaltriti, 2004

*↑: increase in expression; **↓: decrease in expression

In contrast to prostate and pancreatic cancers, sCLU levels have been implicated as having prognostic value in specific cancers, such as colon and breast. sCLU was reported to be a prognostic indicator in tumorigenesis and progression of human breast carcinoma (Redono *et al.*, 2002). Furthermore, sCLU has been proposed to be a potentially new prognostic and predictive marker for colon carcinoma aggressiveness, since over-expression of sCLU is reported or observed in highly aggressive tumours as well as metastatic nodules (Pucci *et al.*, 2004). Thus, as with overall basal levels of sCLU, the notion of whether changes in sCLU expression may be an indicator of treatment response is still to be elucidated.

1.7.2. ROLE OF sCLU AS A DIAGNOSTIC MARKER

The sCLU antigen has been characterized as a sensitive and stable histological marker for murine and human intestinal tumours, where elevated levels of sCLU were found in normal human colon crypts adjacent to adenomas or adenocarcinomas, but not in normal crypts far removed from the tumours or tumour-free crypts (Chen *et al.*, 2003). This property may indicate that sCLU is a potential biomarker for diagnosis of human colorectal cancer using immunohistochemical or molecular biology techniques. Furthermore, the elevated production of sCLU antigen, if secreted from tumour cells, as proposed by the authors, may be detected in body fluids, such as serum (Chen *et al.*, 2003). Consequently, it was speculated that markers derived from sCLU may be used as part of a set of indices for early detection of human colorectal cancer (Chen *et al.*, 2003). sCLU was identified as a marker of anaplastic large-cell lymphoma (Saffer *et al.*, 2002) and it has been recommended that sCLU should be added as a useful biomarker to antibody panels designed to distinguish systemic anaplastic cell lymphoma from classical Hodgkin's lymphoma (Saffer *et al.*, 2002). Thus, an increasing body of evidence now indicates that the specific expression patterns of sCLU and its correlation with cellular events during tumorigensis make it a promising diagnostic tool in anaplastic cell large-cell lymphoma, as well as in other malignancies. Hence, sCLU is a potential contributor to the set of biomarkers for early detection of a variety of cancers, especially for colon cancer.

1.8. CLUSTERIN AS A THERAPEUTIC TARGET

1.8.1. ANTISENSE OLIGONUCLEOTIDES

Recent scientific findings demonstrate that drugs targeting sCLU expression, including CLU silencing using antisense oligonucleotides (ASO) or short interfering RNA (siRNA), may become promising tools for cancer therapy (Zellweger *et al.*, 2001; Criswell *et al.*, 2003; Gleave and Miyake, 2005). It is now accepted that the primary function of the 76-80 kDa sCLU protein form is cytoprotective. Resistance to cancer treatment is mediated, at least in part, by enhanced expression of cell survival proteins that help facilitate tumour progression (Miyake *et al.*, 2000). Thus, it has become increasingly clear that

in most cells, sCLU is a stress-associated cytoprotective protein that is up-regulated by various apoptotic triggers. Furthermore, sCLU confers resistance by some unknown mechanism when over-expressed (Miyake *et al.*, 2000). The novel therapeutic strategy of silencing sCLU expression to overcome resistance to cancer therapy is of interest for the treatment of cancers that over-express sCLU (reviewed in Gleave and Monia, 2005).

It has been hypothesized that sCLU over-expression may influence the apoptotic response of prostate tumour cells to IR and that the up-regulation of CLU after various apoptotic signals represents an adaptive cell survival mechanism, thereby affecting a potential target for down-regulation to improve the results of radiation therapy for patients with prostate cancer (Zellweger *et al.*, 2003). Thus, sCLU over-expression helps to confer a chemoresistant phenotype through inhibition of apoptosis in human renal cell carcinoma cells (Hara *et al.*, 2001). Furthermore, sCLU was supported as a valid therapeutic target in strategies employing novel multimodality therapy for advanced prostate cancer, as sCLU is up-regulated following androgen withdrawal therapy, and this may represent an adaptive cell survival response following apoptotic signals like androgen withdrawal (July *et al.*, 2002).

1.8.2. SHORT INTERFERING RNA

It has been hypothesized that sCLU gene silencing using siRNA or other techniques may ultimately develop into attractive anti-tumour therapeutics (Trougakos *et al.*, 2004). It has recently been shown that sCLU knockdown in human cancer cells, using siRNA-mediated CLU gene silencing, induces significant reduction of cellular growth and higher rates of spontaneous apoptosis (Trougakos *et al.*, 2004). sCLU provides cytoprotection for IR-exposed MCF-7 breast cancer cells that is not observed after siRNA to silence CLU gene (Criswell *et al.*, 2003). The most common delivery method of siRNA for *in vitro* application is the use of cationic lipids. This system provides reasonable transfection efficiencies in cell culture *in vitro,* as these cationic lipids are too toxic *in vivo*. Thus, the use of cationic polymers (mainly polyethylenimine, PEI) grafted with polyethylene glycol (PEG), which reduces the toxicity of the cationic polymers, was introduced (Sutton *et al.*, 2006).

Thus, PEG-grafted PEI was shown to bind to siRNA and form nanometer-sized complexes. These nanocomplexes inhibited sCLU expression as well as its IR-induced up-regulation in MCF-7 cells, resulting in increased IR lethality. These results validate the concept of using polymer delivered siRNA to specifically down-regulate sCLU as an aid to cancer radiotherapy (Sutton *et al*., 2006).

However, great care in reducing sCLU levels without affecting nCLU levels must be taken using siRNA targeted against a specific CLU isoform. siRNA knockdown CLU levels enhances cytotoxic agent efficiency, whereas siRNA specific to sCLU enhances the efficacy of cytotoxic agents, whereas siRNA against nCLU confers cytoprotection against a variety of chemotherapeutics.

1.9. 1,25-DIHYDROXYVITAMIN D_3

1.9.1. BACKGROUND

Vitamin D is a generic designation for a group of fat-soluble, structurally similar sterols, several of which are important in calcium and phosphorus metabolism, and bone formation. The two most important sterols are vitamin D_2 (ergosterol), and vitamin D_3 (calcitriol). Vitamin D_3 is found in certain animal tissues and products. It is also synthesised in the skin by ultraviolet (UV) irradiation of 7-dehydrocholesterol. Vitamin D_3 is transported to the liver, where it undergoes hydroxylation to produce 25-hydroxyvitamin D_3 ($25(OH)D_3$). Although $25(OH)D_3$ has limited biological activity, it is the major circulating metabolite of vitamin D_3. In the kidney, $25(OH)D_3$ undergoes further hydroxylation to form dihydroxy metabolites: $1,25(OH)_2D_3$, $23,25(OH)_2D_3$, and $24,25(OH)_2D_3$. The 23-metabolite is biologically inert and its function is still unknown. The production of $1,25(OH)_2D_3$, the active metabolite of vitamin D_3, is regulated by a negative feedback mechanism depending on the need for calcium and phosphorus in circulation (Holick, 2003) (Figure 1.9).

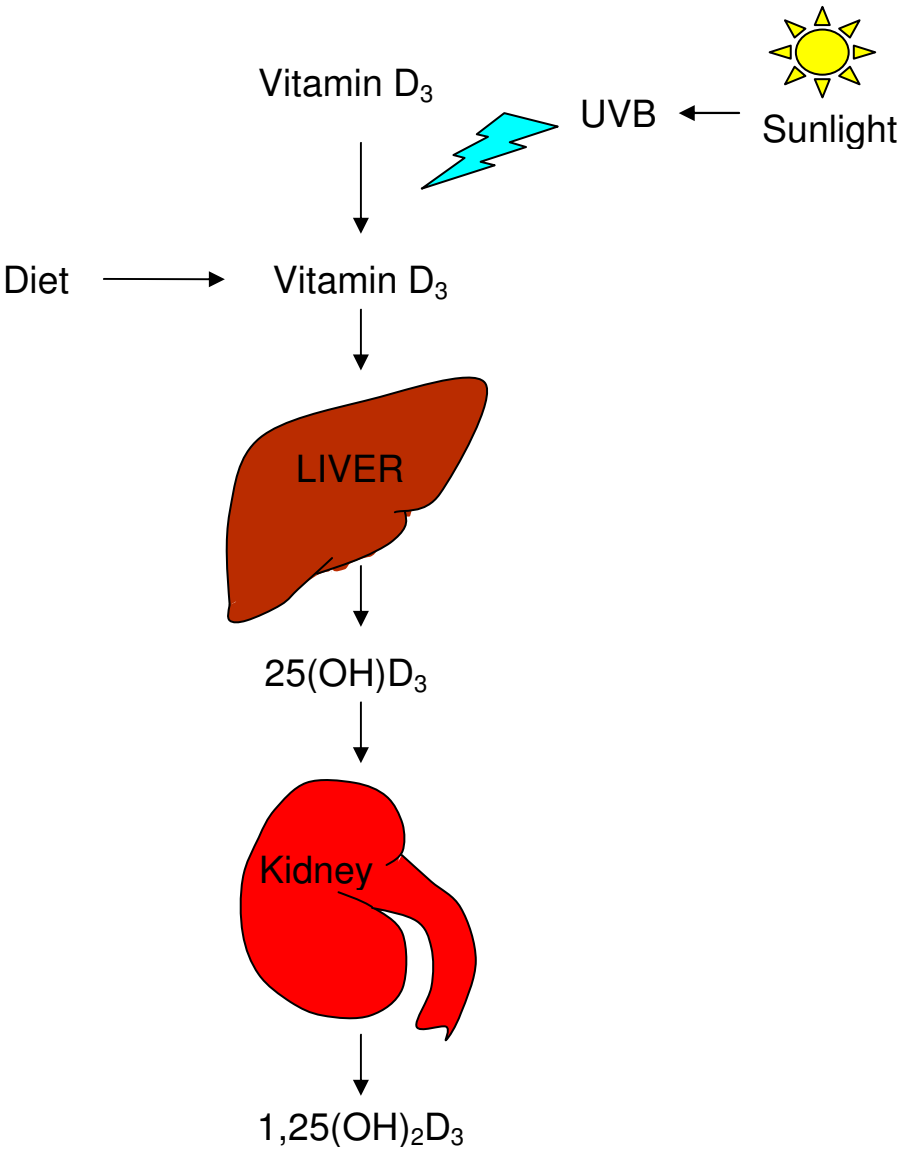
$1,25(OH)_2D_3$ has also been shown to have significant anti-proliferative activity when administered to many cancer cells *in vitro*. $1,25(OH)_2D_3$ regulates

growth and differentiation in various cell types (Hansen *et al.*, 2001; Seifert *et al.*, 2004). $1,25(OH)_2D_3$ acts by binding to a corresponding intra-nuclear receptor (VDR), present in target tissues (Stumpf *et al.*, 1979; Baker *et al.*, 1988). VDR belongs to a superfamily of trans-acting transcriptional regulatory factors that includes steroid and thyroid hormone receptors as well as retinoic acid and retinoic-X (RXR) receptors (Kliewer *et al.*, 1992; Evans *et al.*, 1988).

VDR functions as a ligand-activated transcription factor that binds to vitamin D-responsive elements (VDREs) in the promoter regions of vitamin D-responsive genes (Bao *et al.*, 2004). VDR binds with high affinity to VDREs by forming a heterodimeric complex with the RXR. The VDR/RXR heterodimers function as transcriptional factors in regulating vitamin D-mediated gene expression (Bao *et al.*, 2004). The effects of VDR-mediated genomic pathways include inhibition of cellular growth and invasion. Non-genomic cytoplasmic signalling pathways are increasingly being recognized, which similarly may regulate not only cellular growth and differentiation but also apoptosis.

$1,25(OH)_2D_3$ can exert a significant inhibitory effect on the G_1/S checkpoint of the cell cycle and may produce complete arrest by up-regulating the cyclin dependent kinase inhibitors, p27 and p21. $1,25(OH)_2D_3$ can also inhibit cyclin D1 (Osborne and Hutchinson, 2002). Indirect mechanisms of $1,25(OH)_2D_3$-mediated growth regulation include up-regulation of TGF-ß1 and down-regulation of the epidermal growth factor receptor (EGFR) (Osborne and Hutchinson, 2002). $1,25(OH)_2D_3$ has the capacity to induce apoptosis either indirectly through effects on the insulin-like growth factor receptor (IGF-1R) and tumour necrosis factor-α (TNF- α) or more directly via the Bcl-2 family system, the ceramide pathway, the death receptors (e.g. Fas) and the stress-activated protein kinase pathways (i.e., Jun N terminal kinase and p38) (Danielsson *et al.*, 1998; Osborne and Hutchinson, 2002).

(a)


Vitamin D_3
UVB
Sunlight
Diet
Vitamin D_3
LIVER
25(OH)D_3
Kidney
1,25(OH)$_2$$D_3$

(b)

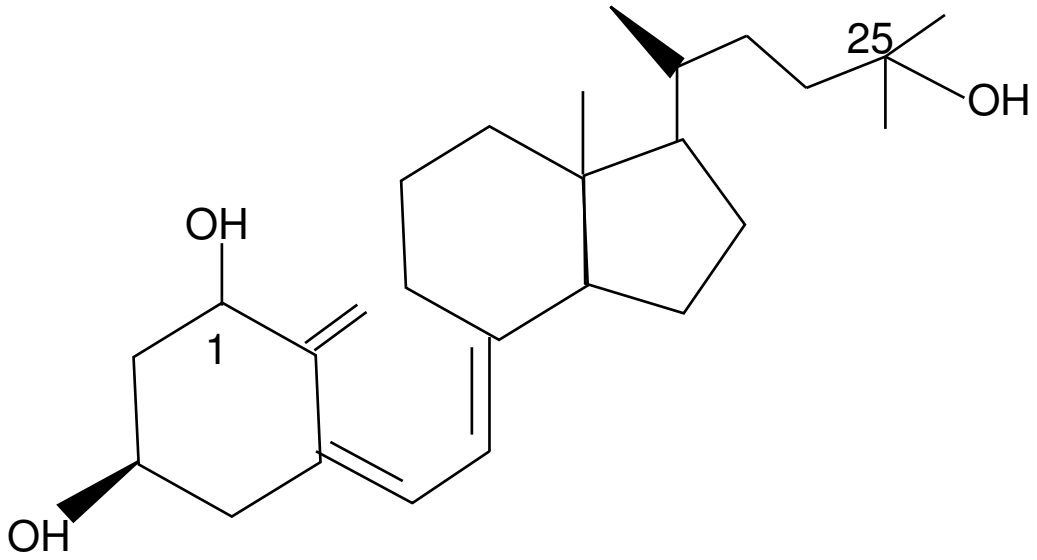


Figure 9.1.1. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). (a) $1,25(OH)_2D_3$ is obtained either from the diet or from skin exposure to UV-B. The compound is then transported to the liver and undergoes hydroxylation to produce 25-hydroxyvitamin D_3. In the kidney, 25-hydroxyvitamin D_3 undergoes further hydroxylation to produced dihydroxy metabolites, and one of them is 1,25-dihydroxyvitamin D_3, the active metabolite. (b) The chemical structure of $1,25(OH)_2D_3$.

1.9.2. ROLE OF VITAMIN D_3 IN MALIGNANCIES

The $1,25(OH)_2D_3$-mediated effects on cell death, tumour invasion and angiogenesis make vitamin D analogues promising candidate agents for cancer therapy (Osborne and Hutchinson, 2002). $1,25(OH)_2D_3$ and its analogues inhibit proliferation and induce differentiation and apoptosis in various malignancies, including human malignant melanoma, and prostate cancer (Danielsson *et al.*, 1998; Hansen *et al.*, 2001; Seifert *et al.*, 2004).

Unfortunately resistance to vitamin D analogue treatment remains a serious problem. Tumour cell lines that fail to respond to the anti-proliferative effects of vitamin D analogues have also been reported. Vitamin D-induced apoptosis was demonstrated in melanoma cell lines (Danielsson *et al.*, 1998). Inhibition of tumour invasion and metastasis by $1,25(OH)_2D_3$ has been demonstrated as

well, and the mechanisms involved include inhibition of serine proteinases, metallo-proteinases and angiogenesis (Osborne and Hutchinson, 2002; Seifert *et al*., 2004). Vitamin D analogues exposure can reduce autocrine TGF-ß1 activity by increasing expression of TGF-ß isoforms and/or TGF-ß receptors in non-malignant and malignant breast cells. Further, Smad 3, a TGF-ß1 signalling mediator, co-activates VDR to further enhance TGF-ß and vitamin D signalling (Masuda and Jones, 2006).

In several prostate cancer models, $1,25(OH)_2D_3$ can induce apoptosis, and this often involves down-regulating the anti-apoptotic protein, Bcl-2. Reduced expression of Akt, a kinase that regulates an important survival pathway that involves down-regulation of p53 (Mayo *et al*., 2005) may also contribute to the pro-apoptotic activity of $1,25(OH)_2D_3$. p53 is thought to be involved in $1,25(OH)_2D_3$-induced apoptosis, however, there is conflicting information about its role in this $1,25(OH)_2D_3$-induced apoptosis.

One role for vitamin D analogues could be in their use as synergistic agents for the treatment of cancer. $1,25(OH)_2D_3$ has been shown to produce additive or synergistic activities when combined with several classes if anti-neoplastic agents that are relevant for the treatment of human prostate cancer (Beer *et al*., 2005).

In malignant melanoma, $1,25(OH)_2D_3$ or its analogues represent a palliative therapy for the metastasis of malignant melanoma. However, as mentioned above, it has been shown that certain melanoma cell lines fail to respond to the anti-proliferative effects of vitamin D analogues. The reason is thought to be due to a defect in VDR-mediated transcription (Seifert *et al*., 2004), but more research in this area is warranted. Since CLU has important functions in apoptosis, it is essential to examine a possible relation between $1,25(OH)_2D_3$ and CLU signalling pathways.

This research was focused on examining any differences in CLU expression in malignant melanoma cells, both untreated and $1,25(OH)_2D_3$ treated. The results from this work would bring further understanding to the progression and pathogenesis of malignant melanoma.

2. MATERIALS AND METHODS

2.1. DETECTION OF CLU *IN VIVO*

2.1.1. TISSUES

We analyzed immunohistochemically paraffin sections of primary cutaneous malignant melanomas (n= 18), metastases of malignant melanomas (n= 25) and acquired melanocytic nevi (n= 30). Other tissues (basal cell carcinoma n= 5, Merkel's cell carcinoma n= 5, and cutaneous squamous cell carcinoma n= 5) were also tested. Table 2.1. summarizes the histological data of the tissues analyzed. The tissues were obtained from the Saarland University Hospital, Dermatology Department, 66421 Homburg/Saar. The diagnoses of the cases were made by certified histopathologists in the Dermatology Department.

Table 2.1. Histopathological data of malignant melanomas, metastases of malignant melanomas and acquired melanocytic nevi

Case number	Patient initials	Age	Gender	Tumour thickness	Type	Localization
Primary cutaneous malignant melanomas (n = 18)						
1	AB	48	F	0.75 mm	SSM*	Left arm
2	TB	75	F	4.20 mm	Nodular melanoma	Lower left foot
3	SM	41	M	1.00 mm	SSM	Shoulder blade
4	LT	59	M	0.90 mm	SSM	Left foot
5	HW	67	M	1.50 mm	SSM	Right arm
6	BK	75	F	Tumour scrapes	-	Left thigh
7	EK	82	F	4.10 mm	Nodular melanoma	Left calf
8	WS	72	M	1.30 mm	SSM	Forehead
9	LN	81	F	3.50 mm	Ulcerative melanoma	Right heel
10	AW	61	F	0.80 mm	Subcutaneous melanoma	Groin
11	GH	55	F	2.25 mm	Nodular melanoma	Right knee
12	GA	47	F	0.80 mm	SSM	Right calf
13	FD	67	M	1.60 mm	Ulcerative nodular melanoma	Left thigh
14	BA	43	F	1.50 mm	SSM	Lower left thigh
15	RC	73	M	0.40 mm	Nodular melanoma	Back
16	EM	37	F	1.00 mm	SSM	Left calf
17	BB	41	F	2.10 mm	SSM	Lower right thigh
18	MS	34	F	0.80 mm	SSM	Right chest
Metastases of malignant melanomas (n = 25)						
19	KHD	77	M	-	Cutaneous melanoma metastasis	Neck
20	JR	79	M	-	Cutaneous melanoma metastasis	Chin
21	HL	35	F	-	Melanoma lymph node metastasis	Neck
22	HS	66	F	-	Cutaneous melanoma metastasis	Abdomen
23	NS	65	F	-	Subcutaneous melanoma metastasis	Head

Case number (continued)	Patient initials	Age	Gender	Tumour thickness	Type	Localization
24	EF	92	F	-	Cutaneous melanoma metastasis	Upper right thigh
25	HPM	54	M	-	Cutaneous melanoma metastasis	Upper right thigh
26	HL	39	M	-	Cutaneous melanoma metastasis	Neck
27	GB	71	M	-	Melanoma lymph node metastasis	Right scapula
28	HR	70	F	-	Cutaneous melanoma metastasis	Lower left thigh
29	HB	70	F	-	Cutaneous melanoma metastasis	Left shoulder
30	NR	63	F	-	Cutaneous melanoma metastasis	Lower left thigh
31	UM	40	M	-	Melanoma lymph node metastasis	Right calf
32	OK	69	M	-	Cutaneous melanoma metastasis	Upper right thigh
33	OG	74	M	-	Melanoma lymph node metastasis	Neck
34	WK	58	M	-	Cutaneous melanoma metastasis	Left lumbar
35	PM	58	M	-	Cutaneous melanoma metastasis	Thorax
36	JA	96	F	-	Subcutaneous melanoma metastasis	Head
37	HL	35	F	-	Melanoma lymph node metastasis	Neck
38	RH	78	F	-	Cutaneous melanoma metastasis	Lower left thigh
39	GB	92	M	-	Melanoma lymph node metastasis	Back
40	KS	84	M	-	Subcutaneous melanoma metastasis	Neck

Case number (continued)	Patient initials	Age	Gender	Tumour thickness	Type	Localization
41	MA	65	M	-	Cutaneous melanoma metastasis	Thorax
42	BT	55	M	-	Cutaneous melanoma metastasis	Left arm
43	HF	66	M	-	Cutaneous melanoma metastasis	Thorax
Benign acquired melanocytic nevi (n = 30)						
45	FB	39	M	-	Intradermal melanocytic nevus	Left breast
46	RR	43	M	-	Compound melanocytic nevus	Shoulder blade
47	PS	18	M	-	Intradermal melanocytic nevus	Left arm
48	KB	46	M	-	Compound melanocytic nevus	Stomach
49	FB	40	M	-	Junctional melanocytic nevus	Left breast
50	FP	53	M	-	Compound melanocytic nevus	Left shoulder
51	MS	36	M	-	Intradermal melanocytic nevus	Left neck bone
52	MW	38	M	-	Intradermal melanocytic nevus	Right rib cage
53	PD	32	M	-	Intradermal melanocytic nevus	Penis
54	KL	23	F	-	Intradermal melanocytic nevus	Neck
55	VM	30	F	-	Dermal melanocytic nevus	Left arm
56	SW	40	F	-	Compound melanocytic nevus	Third right finger
57	MP	19	F	-	Intradermal melanocytic nevus	Neck
58	HB	61	M	-	Compound melanocytic nevus	Abdomen

Case number (continued)	Patient initials	Age	Gender	Tumour thickness	Type	Localization
59	MA	64	M	-	Junctional melanocytic nevus	Back
60	JM	39	M	-	Junctional melanocytic nevus	Back
61	LA	45	M	-	Compound melanocytic nevus	Back
62	IS	32	F	-	Compound melanocytic nevus	Right foot
63	NW	26	F	-	Intradermal melanocytic nevus	Right ear
64	DP	31	F	-	Junctional melanocytic nevus	Abdomen
65	JD	23	F	-	Compound melanocytic nevus	Abdomen
66	KZ	35	F	-	Compound melanocytic nevus	Right breast
67	LB	28	F	-	Compound melanocytic nevus	Thorax
68	HP	31	F	-	Compound melanocytic nevus	Abdomen
69	AW	26	F	-	Compound melanocytic nevus	Back
70	UF	39	M	-	Junctional melanocytic nevus	Lower left thigh
71	VF	45	M	-	Compound melanocytic nevus	Face
72	KB	46	M	-	Compound melanocytic nevus	Right breast
73	MD	51	M	-	Compound melanocytic nevus	Right arm
74	MB	85	F	-	Intradermal melanocytic nevus	Right eyebrow

*SSM: superficial spreading melanoma.

2.1.2. IMMUNOHISTOCHEMISTRY

The tissues were fixed in 4% formalin overnight and cut into 5 mm sections using a Reichert-Jung 2030 microtome (Heidelberg, Germany). After the tissue sections were attached to slides and dried, they were deparafinized in xylene and hydrated using graded alcohols and finally washed in distilled water. The tissues were then incubated in PBS and analyzed using Clusterin-α/ß (H-330) antibody for 1 hr at room temperature (rabbit polyclonal, working dilution 1:100, Santa Cruz, CA, USA) and a streptavidin-peroxidase technique. The primary antibody detects both CLU isoforms (Dr. Gleave, University of British Colombia, Vancouver General Hospital, Canada, personal communication). Primary antibody binding was detected by a two-step biotin/streptavidin based antibody detection system employing a peroxidase-mediated 3-Amino-9-Ethylcarbazol staining (Sigma Immunodiagnostics, Taufkirchen, Germany). Incubation with 2% peroxidase in methanol was used to block endogenous peroxidase activity. The tissues were mounted in permanent Aquatex media (MERCK Biosciences, Schwalbach/Ts., Germany). Immunohistochemical staining was evaluated semi-quantitatively, estimating staining intensity (SI: 0= no staining, 1= low staining, 2= moderate staining, 3= strong staining) and percentage of positive cells (PP: 0= no positive cells, 1= 1-50% positive cells, 2= 51<75% positive cells, 3= > 75% positive cells) and calculating a resulting immunoreactivity score (IRS = SI x PP). Semi-quantitative analyses were performed by two independent observers (B.S. and J. R.) (Shannan *et al.*, 2006, a).

2.2. DETECTION OF CLU *IN VITRO*

2.2.1. CELL CULTURE

Human melanoma cell lines SK-MEL-28, SK-MEL-5, SK-MEL-25 (23), and MeWo (24), were cultivated in RPMI 1640 media (10% foetal calf serum [FCS], 37°C, 5% CO_2) (PAA Laboratories GmbH, Coelber, Germany) using 10-cm plates (Greiner Bio-One GmbH, Frickenhausen, Germany) or 6-well plates (Greiner). Melanoma cell lines were previously characterized as vitamin D-resistant and -sensitive (Seifert *et al.*, 2004). Vitamin D-resistant melanoma cell lines are thought to have a functional defect in VDR-mediated gene

transcription. Primary normal human melanocytes (NHM) were isolated from human foreskin and cultivated in serum free melanocyte growth media (PromoCell®, Heidelberg, Germany), after addition of supplement mix (10 ng/ml PMA, 0.5 µg/ml Insulin, 0.5 µg/ml hydrocortisone, 2 ml BPE, and 1.0 ng/ml gentamycin/amphotericin B). Human LNCaP prostate cells that were trasfected with a control vector alone (LN/C) and stable transfected sCLU over-expressing prostate cells (LNT-1) (Zellweger *et al*., 2003) were cultivated in RPMI 1640 medium (5% FCS, 300 µg/ml G-418 to maintain stable transfected sCLU over-expression, 37°C, 5% CO_2) (PAA) using 10-cm plates (Greiner). The LNCaP cells were kindly provided by Dr. David Boothman (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). Around 50% confluent cells were incubated with 1% bovine serum albumin [BSA] dissolved in RPMI 1640 medium and treated with $1,25(OH)_2D_3$ (10^{-10} M, 10^{-7} M, or 10^{-6} M). Various experiments were then performed on the cell lines (PCR, RT-PCR, colony forming assay, cell cycle analysis, and TUNEL). An ethanol (vehicle) control was used due to the fact that $1,25(OH)_2D_3$ is water-insoluble and is dissolved in ethanol. $1,25(OH)_2D_3$ was kindly provided by Dr. Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark).

2.2.2. ULTRAVIOLET-B IRRADIATION

Human LNCaP prostate cells were irradiated with UV-B to evaluate the differences between LNT-1 and LN/C. The ultraviolet-B (UV-B) irradiation source was a fluorescent lamp that emitted an energy peak at 306 nm (Waldmann, Villingen-Schwenningen, Germany). The emitted dose was calculated using a UV-B radiometer photodetector. Cells were plated on 10-cm plates (Greiner) and left overnight to attach. The cells were then washed once with phosphate buffered saline (PBS) before being irradiated using UV-B lamp. The UV-B doses were as follows: 0, 100, and 500 J/m^2 for colony forming ability assays, cell cycle analyses, and TUNEL analyses. Only LNCaP prostate cells were irradiated with UV-B to test for the difference between the sCLU transfected (LNT-1) and the empty vector transfected (LN/C) cell lines.

2.2.3. RNA ANALYSES

2.2.3.1 RNA EXTRACTION

Cells were harvested and total RNA was isolated using RNAeasy MiniKit (QIAGEN®, Hilden, Germany) according to the manufacturer's recommendations. The integrity and amount of extracted total RNA were monitored by gel electrophoresis and spectrophotometric analyses. The integrity of RNA was checked by mixing 1µl of the isolated RNA, 3µl of gel loading solution (Sigma-Aldrich, Taufkirchen, Germany), and 9µl distilled water. The mixture was applied to a 2% agarose gel (run at 70 Volt for 1hr). Bands were then visualized using a Biometra TI 2 transilluminator (230 Volt). The amount of RNA was checked by measuring (260-280λ) the isolated RNA (1:50 dilution) using a spectrophotometer (Ultraspec 1000, Pharmacia Biotech, Germany)

2.2.3.2. RNA REVERSE TRANSCRIPTION

RNA was reverse-transcribed into cDNA either with a method using Omniscript reverse transcription (QIAGEN®), or using M-MLV RT H(-) point with poly-dT-primer (Promega GmbH, Mannheim, Germany). The existence of genomic DNA contamination was excluded via reverse transcriptase-minus reaction.

2.2.3.3. POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) protocols were established with specific oligonucleotide primers for CLU, which is a low expressed gene (primer synthesis by TIB MOLBIOL, Berlin, Germany), and ß2-microglobulin (ß2-µglob, housekeeping gene; TIB MOLBIOL); in separate approaches: 2 µl cDNA were added to a master mix containing 1.5 mM $MgCl_2$, 4 nM dNTP, 10 pmol of each specific oligonucleotide (ß2-µglob), 10 pmol (CLU), 5 U Taq polymerase (Eppendorf, Hamburg, Germany) and 1 x Taq buffer advanced (Eppendorf) in a total volume of 50 µl. The primer sequences for ß2-µglob and CLU are shown in Table 2.2.3.3.

Semi-quantitative mRNA expression analysis of different cell lines was based on consecutively performed co-amplifications of CLU with ß2-µglob mRNA isolates. To co-amplify CLU and ß2-µglob transcripts, 35 and 20 PCR cycles were performed, respectively, with the conditions listed in Table 2.4.3. PCR products were separated on 2.5% agarose gels containing 1 µg/ml ethidium bromide and visualized by UV-light at 366 nm. A step ladder 50bp marker was run with the samples (Sigma). The gel image was documented and processed using a digital documentation system (Gel Print, 2000i, MWG-Biotech, Ebersberg, Germany).

Semi-quantitative analysis were performed using optical densitometry and NIH Image J, a public domain Java image processing programme that was used to create density histograms and line profile plots. All PCR runs were performed in duplicates and experiments were repeated at least twice. To enable us to obtain quantitative results, we developed a real-time RT-PCR technique, as indicated, for the detection of CLU in human melanoma cell lines.

Table 2.2.3.3. PCR primer sequences and amplification conditions

Primer	**Sequence**
CLUSex6 F (Exon 6)	5´-AAACCTCACgCAAggCgAA-3´
CLUSex7 A (Exon 7)	5´-CCgCCACggTCTCCATAA-3´
ß2-µglob se	5´-CCAgCAgAgAATggAAAgTC-3´
ß2-µglob as	5´-gATgCTgCTTACATgTCTCg-3´

Protocol: 35 (CLU) and 20 (ß2-µglob) cycles: 94°C for 1 min, 94°C for 20 secs, 55°C for 30 secs, 72°C for 30 secs, 72°C for 7 min, 4°C cool.

2.2.3.4. Real-time RT-PCR

Two microliters each of a 1:5 dilution of RT reaction mixture were used as the template for real-time PCR, containing 5 and 4 mmol/L Mg Cl_2 for CLU gene and β2 microglobulin (β2-µglob) respectively; and 0.5 mmol/L primers as shown in Table 2.2.3.4. After adding 10 µl mix from the SYBER Green I kit (Qiagen), the volume was adjusted to 20 µl with nuclease-free distilled water.

Table 2.2.3.4. Real-time RT-PCR primer sequences and amplification conditions

Primer	Sequence
ß2-µglob se	5̀- CCA gCA gAg AAT ggA AAg TC -3́
ß2-µglob as	5̀- gAT gCT gCT TAC ATg TCT Cg -3́
Hs_CLU_1_SG	Purchased from Qiagen QuantiTech, Catalogue number QT00054460

Protocol: 95°C for 15 min, 60 cycles: 95°C for 20 secs, 55°C for 20 secs, 72°C for 20 secs. Melting curve: 65°C for 15 secs, 95°C, cool.

The samples were amplified in the LightCycler System and PCR was performed by an initial amplification step at 95ºC for 15 min followed by 60 cycles with a denaturation step at 95ºC for 20 secs, primer annealing at 55ºC for 20 secs, and an extension phase at 72ºC for 20 secs. A melting curve was generated after 60 cycles for the final PCR product for all genes investigated by decreasing the temperature to 65ºC for 15 secs followed by a slow increase in the temperature to 95ºC. During the slow heating process the fluorescence was measured at 0.2ºC increments. To further insure specificity, gel electrophoresis was conducted for selected samples of each specific product. In all cases bands of the expected size were consistently observed.

Construction of a standard curve for CLU was achieved using cDNA from cultured prostate LNCaP cells (LNT-1) that over-express CLU (as mentioned in section 2.3.). Total RNA was prepared from 1 x 10^6 LNT-1 cells and used for cDNA synthesis. Five 10-fold serial dilutions of the retrieved cDNA were used as external standards to determine PCR efficiency. The same was performed to achieve a standard curve for β2-µglob, used as a housekeeping control gene, only the cDNA was obtained from a cultured melanoma cell line, MeWo.

To generate a calibrator for CLU, RNA from LNT-1 cells was used for cDNA synthesis in several reaction tubes. The reaction mixture was diluted 1:10 and kept in aliquots at -20ºC. Two microliters each were used per LightCycler run.

The relative amount of target gene mRNA (CLU) and of reference gene (β2-µglob) was determined for each sample and the calibrator (LNT-1), integrated

into each LightCycler run. The relative ratio of target to reference for each sample and for the calibrator was calculated first. This corrects for sample-to-sample variations caused by differences in initial quality and quantity of the nucleic acid-the basic concept of each relative quantification (Roche, technical note LC 13/2001). The target/reference ratio of each sample was finally divided by the target/reference ratio of the calibrator. The calculation of the relative amount of any target or reference gene is based on the crossing point of the PCR reaction. PCR efficiency is expressed as $E = 10^{-1/slope}$.

The calculations of normalized ratio are made according to the following formula:

$$\text{Normalized ratio} = E_T^{CpT(C)-CpT(S)} \times E_R^{CpR(S)-CpR(C)}$$

Where E = efficiency, T = target, R = reference, S = unknown sample, C = calibrator, and Cp = crossing point.

This procedure was used to compensate for different detection sensitivities of target and reference amplifications, providing a constant calibrator point between PCR runs.

2.2.4. WESTERN BLOT ANALYSES

After 1,25$(OH)_2D_3$ or UV-B treatment, the cells (melanoma and LNCaP prostate cell lines) were harvested and protein was isolated using a double volume of the pellet in RIPA buffer, and complete EDTA (ethelyne-diamine-tetra-acetic acid) free protease-inhibitor-cocktail tablets (Roche Diagnostics). The proteins were then detected by western blot analyses using a CLU specific primary mouse monoclonal antibody (clone 41D, Upstate, Hamburg, Germany) with a working dilution of 1:1000. The samples included equal volumes of protein (150 µg) and were separated on an SDS-PAGE gel using electrophoresis buffer. The SDS-PAGE gel was run for 1.5 hrs at 65 mAmp, and consisted of a separation gel (12.5%) and a collection gel (5%). A prestained protein molecular weight marker was run with the samples

(Fermentas, St. Leon-Rot, Germany). The protein samples were then transfected to nitrocellulose membranes using transfection buffer in a buffer tank for 16 hrs at 45 mAmp. The membranes were blocked in TBS containing 5% nonfat milk powder for 1 hr at room temperature. The primary antibody was added to the membranes (1 hr, room temperature). After a washing step, the secondary antibody labelled with peroxides (goat anti-mouse, Sigma Immunodiagnostics, 1:30000 working dilution) was added. A horseradish peroxidase-based detection reagent was added for 5 minutes (Amersham Biosciences, Buckinghamshire, UK). The membranes were then washed several times using TBS. The immunoblots were detected afterwards by chemoluminescence according to the manufacturer's instructions (Roche Molecular Biochemicals) using Lumi-films chemiluminescent detection films (Roche). The membranes were then stripped using the following stripping buffer, and actin antibody (goat polyclonal IgG, working dilution 1:500, Santa Cruz) was used as a loading control. The buffers and solutions used in the western blot analyses are summarized in Table 2.2.4.

Table 2.2.4. The buffers and solutions used in the western blot analyses

Collection Gel (5%)	0.375 mL Solution A, 0.8 mL Solution C, 2.125 mL distilled water, 2.7 µl TEMED, 33.3 µl APS (10%)
Electrophoresis buffer	0.025 M Tris-HCl/ pH 8.3, 0.132 M glycine, 0.1% SDS
RIPA Buffer (extraction buffer)	50 mM Tris-HCl/ pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X 100
Separation Gel (12.5%)	3.15 mL Solution A, 2.5 mL Solution B, 4.35 mL distilled water, 8.3 µl TEMED, 100 µl APS (10%)
Solution A	40% Acrylamid, 1.04% Bis-acrylamid
Solution B	1.5 M Tris-HCl/ pH 8.8, 0.4% SDS
Solution C	0.5 M Tris-HCl/ pH 6.8, 0.4% SDS
Stripping Buffer	25 mM glycine, 2% SDS, pH 1.0
TBS	10 mM Tris-HCl/ pH 8.0, 150 mM NaCl with 0.05% Tween 20
Transfection Buffer	20 mM Tris-HCl/ pH 8.3, 150 mM glycine

2.2.5. CELL VIABILITY ASSAYS

2.2.5.1. COLONY FORMING ASSAY (CFA)

MeWo, SK-MEL-25 and LNCaP cells were seeded in 10-cm plates with 10,000 cells/plate (for melanoma cells) and 15,000 cells/plate (for LNCaP cells). The number of cells was determined following a separate CFA assessing the platting efficiency of these cells. After 24 hrs, cells were treated with $1,25(OH)_2D_3$ (10^{-6}, 10^{-7}, or 10^{-10} M), and cultured in BSA medium for 7 days continually. Fresh medium and treatment were given daily. Then the cells were fixed in ethanol and stained using crystal violet dye. Finally, the sum of colonies was counted and the inhibitory rate of colony-forming (proliferation) following treatment with $1,25(OH)_2D_3$ was calculated. Experiments were performed in duplicate and repeated at least twice.

2.2.5.2. CELL CYCLE ANALYSES

LNCaP cells were seeded into 10-cm plates, left to attach overnight and treated the next day with $1,25(OH)_2D_3$ or UV-B. Following various time points, cells were harvested and fixed in 70% methanol in PBS at RT for 30 min. Cells were then pelleted and resuspended in 1 mL PBS. 100 µL of 1 mg/mL RNase A (DNase-free) and 100 µL of propidium iodide were added. The cells were incubated at 37°C for 30 min. Cell cycle analysis was then measured using the FACscan flow cytometry, as described below (section 2.2.6.). Experiments were performed in duplicates and repeated at least twice.

2.2.5.3. TUNEL ASSAY

Detection of DNA fragmentation *in situ* in LNCaP cells following $1,25(OH)_2D_3$ treatment was detected by TUNEL (terminal deoxynucleotidyl-mediated dUTP-biotin nick end-labelling) assays using the Cell Death Detection Kit (Roche Diagnostics®, Mannheim, Germany). This assay relies on fluorescent *in situ* labelling of DNA strand breaks. An equal number of cells was seeded in duplicate on 10-cm plates, allowed to attach overnight, and treated with BSA medium and $1,25(OH)_2D_3$ daily. After 72 hrs of treatment, cells were harvested and cell density adjusted to $2x10^5$, and DNA fragmentation was detected by TUNEL according to the manufacturer's instructions. Apoptosis

was measured using the FACscan flow cytometry (Beckman Coulter, Krefeld, Germany). Experiments were repeated three times each in duplicate.

2.2.6. FLOW CYTOMETRIC ANALYSES (FACS)

Flow cytometric analyses were performed using fluorescence activated cell sorting (FACS) device. It is a method for quantifying components or structural features of cells primarily by optical means. Although it is limited to measurements on single-cell basis, it can process thousands of cells in a few seconds. Laser-based flow cytometry includes a cell transporter system, a laser light source, a flow chamber, mono-chromatic filters, lenses, dictoric mirrors, photomultiplier tubes, and a computer for data analysis. The cell suspension, to be analyzed, is introduced into the flow chamber by air pressure. As cells pass through the flow chamber, they are surrounded by a low-pressure sheath fluid. The outer fluid stream creates a laminar flow forcing the specimen to the centre, and results in a single-file alignment of individual cells. This process is known as hydrodynamic focusing. Every cell sample is then intersected by a laser beam. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The forward light scatter is proportional to the cell size and the 90º or right angle scatter is related to the cell granuality and density. These two scatters build a specific programme for each assay/cell line (Henry, 1996). The cells were prepared as mentioned in sections 2.2.5.2. and 2.2.5.3.

2.2.7. STATISTICAL ANALYSES

Statistical significance was evaluated using the SPSS statistical programme. One way analysis of variance (ANOVA) and post-hoc Bonferroni tests were used for multiple comparisons. Student's T test was used for comparing means of two independent groups. Results at $p \leq 0.05$ were considered statistically significant.

The following table summarizes the most important reagents used in this study

Reagents	Company
1,25-dihydroxyvitamin D_3	Leo Pharmaceutical
3-Amino-9-Ethylcarbazol	Sigma-Aldrich
Actin antibody	Santa Cruz
Cell culture plates	Greiner Bio-One
Cell Death Detection Kit (TUNEL)	Roche
CLU 41D Clone antibody	Upstate
Clusterin-α/ß (H-330) antibody	Santa Cruz
Conventional PCR primers (CLU, ß2µglob)	Tib Mol Biol
Detection reagent	Amersham Biosciences
FCS	PAA Laboratories GmbH
G-418 Sulphate	PAA Laboratories GmbH
Goat anti-mouse antibody	Sigma-Aldrich
Melanocyte growth medium	Promocell
M-MLV RT H (-) Point	Promega
Omniscript	Qiagen
Permanent Aquatex media	MERCK Biosciences
Real-time RT-PCR primer (CLU: HS_CLU_1_SG)	Qiagen QuantiTech
Real-time RT-PCR primer (ß2µglob)	Tib Mol Biol
RNAeasy MiniKit	Qiagen
RPMI 1640	PAA Laboratories GmbH
SYBER Green kit	Qiagen
UV-B lamp	Waldmann
Western blot films	Roche
Western blot membranes	Roche

All other chemicals were of analytical grade

3. RESULTS

3.1. CLUSTERIN EXPRESSION IN MELANOMA TISSUES AND CELL LINES

Immunohistochemistry detection of CLU:

It was the aim of this study to characterize the expression of CLU in melanoma tissues and cell lines in order to test whether CLU expression is connected to malignant melanoma progression and/or pathogenesis. Hence, patient tissues, as described in Materials and Methods, were immunohistochemically stained. Immunohistochemistry (IHC) revealed CLU staining in a proportion of the malignant melanoma and the metastasis of malignant melanoma tissues, but not in acquired melanocytic nevi tissues. Positive cases of primary malignant melanomas (n= 5/18) revealed a low or moderate CLU immunoreactivity for the H-330 antibody used (Figure 3.1.1.a). The staining was homogeneous (in the nuclei and cytoplasm of positive cells). There were also no apparent differences comparing the labelling pattern for CLU in primary malignant melanoma according to histological type (superficial spreading or nodular melanoma) or histological grading. Positive cases of metastases of malignant melanoma (n= 3/25) revealed homogeneously low nuclear and cytoplasmic immunoreactivity for CLU. All cases of acquired melanocytic nevi analyzed (n= 30) were negative for CLU (Figure 3.1.1.b). Table 3.1. provides a semi-quantitative analyses of clusterin immunoreactivity in melanocytic skin tumours (values for SI, PP, and resulting IRS). The epidermal keratinocytes showed no staining of CLU, and neither did the hair follicles nor the sebaceous glands. Next, we were interested in the question of whether CLU was expressed in melanoma cell lines in the same pattern as in the IHC.

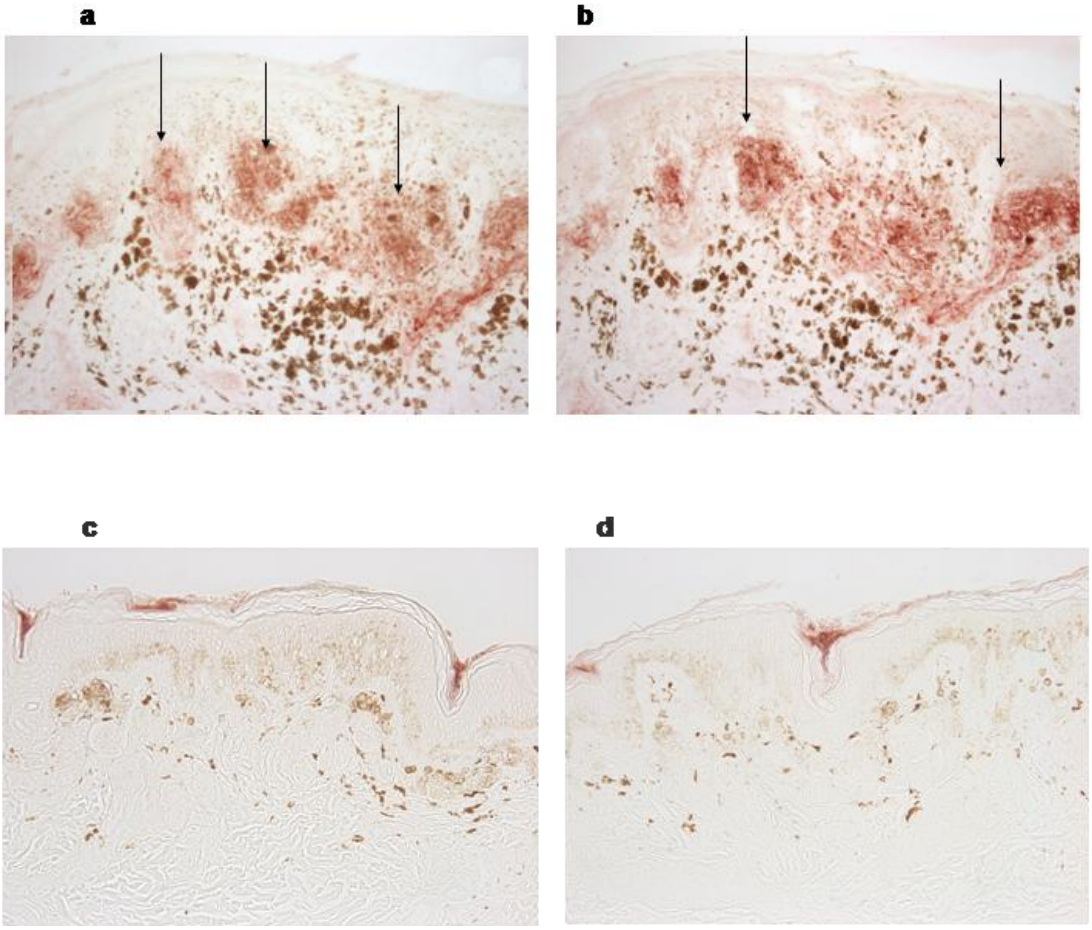


Figure 3.1.1. (a,b) Immunohistochemical detection of CLU in primary malignant melanoma (type: superficial spreading melanoma, case 1 in Table 2.1.). Notice moderate nuclear and cytoplasmic staining in positive tumour cells using antibody H330 directed against clusterin-α/ß (the arrows show the positive CLU staining). (c,d) Immunohistochemical detection of CLU was not apparent in acquired melanocytic nevi. Representative images are shown with a magnification of 1:100.

Table 3.1. Semi-quantitative analysis of clusterin immunoreactivity in melanocytic skin tumours

Clusterin-α/ß (H-330)		Malignant melanoma (n=18)	Metastases of malignant melanoma (n=25)	Acquired melanocytic nevi (n=30)
Staining Intensity (SI)*	0	13	22	30
	1	3	3	0
	2	2	0	0
	3	0	0	0
Mean SI		0.39	0.12	0
Percentage of positive cells (PP)**	0	13	22	30
	1	2	3	0
	2	3	0	0
	3	0	0	0
Mean PP		0.45	0.12	0
Mean Immunoreactivity Score (IRS)***		0.18	0.014	0

* 0= no staining, 1= low staining, 2= moderate staining, 3= strong staining

** 0= no positive cells, 1= 1-50% positive cells, 2= 51<75% positive cells, 3= >75% positive cells

*** IRS= SI x PP

Note that CLU is detected in a proportion of malignant melanomas and metastases of malignant melanoma, but not in acquired melanocytic nevi (n= number of samples examined).

Conventional PCR detection of CLU:

To test whether CLU mRNA expression in melanoma cell lines was comparable to IHC, conventional PCR was used. CLU mRNA was detected in all melanoma cell lines analyzed (SK-MEL-28, SK-MEL-25, SK-MEL-5, MeWo) and NHM (Figure 3.1.2.). Both CLU and ß2 µglob bands were at the expected size corresponding to 200 bp and 250 bp, respectively. However, these results were partially in agreement with the IHC results, where nevi tissues were negative for CLU expression, and only some cases of malignant melanoma showed positive CLU staining. Thus, we sought a quantitative technique to assess CLU mRNA levels.

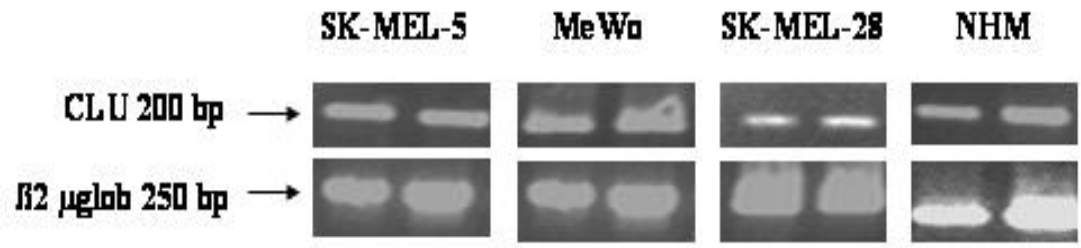


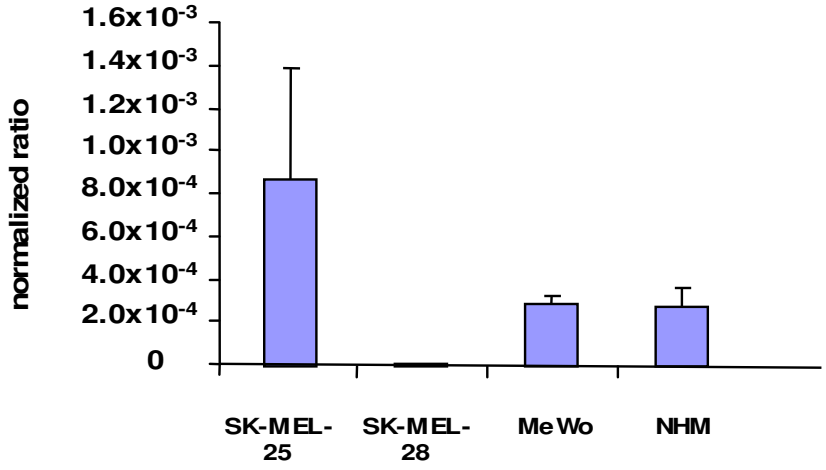
Figure 3.1.2. Expression of CLU mRNA in melanoma cells: Conventional PCR gel images showing CLU expression in SK-MEL-5, MeWo, SK-MEL-28, and NHM human melanoma cells using CLU and β2-µglob primers as described in 'Materials and Methods'. PCR product sizes were 200 bp and 250 bp, respectively. The bands' sizes were determined by using a step ladder 50bp marker in each run. Experiments were done in duplicates.

Real-time RT-PCR detection of CLU:

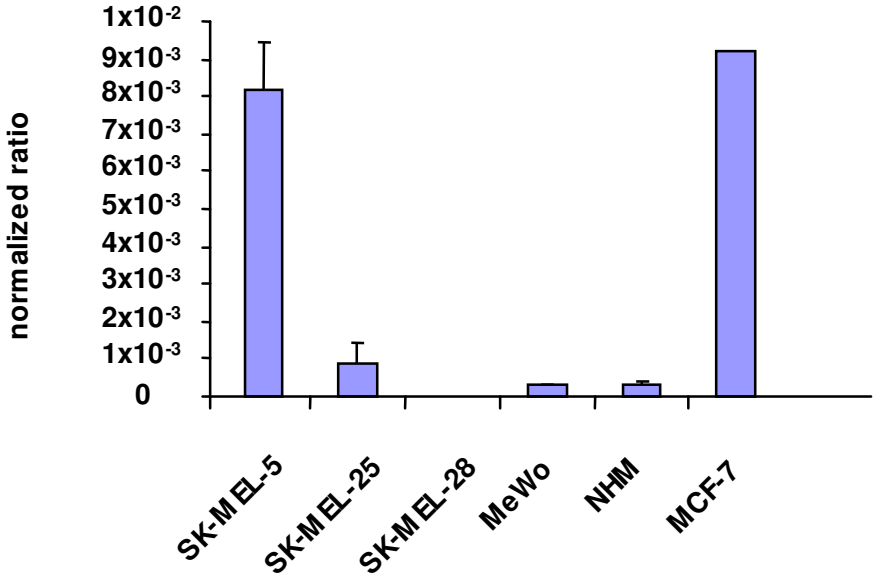
To answer the question of whether CLU mRNA expression is different in melanoma cell lines compared to their $1,25(OH)_2D_3$ sensitivity, two different groups of human melanoma cell lines were used, vitamin D-resistant and -sensitive melanoma cell lines. These cell lines were used to test whether CLU expression varies between the cell lines, and also because $1,25(OH)_2D_3$ is an agent that is discussed to be of therapeutic use in the treatment of malignant melanoma. CLU mRNA was also quantitatively detected using real-time RT-PCR in all the melanoma cell lines analyzed at various expression levels. Vitamin D-resistant melanoma cell lines, SK-MEL-5 and SK-MEL-25 had the highest levels of CLU (8×10^{-3} and 8×10^{-4} of normalized ratio, respectively), while vitamin D-sensitive melanoma cell lines, MeWo, SK-MEL-28 and normal human melanocytes (NHM) had significantly lower levels of CLU (3×10^{-4}, 1×10^{-5} and 2×10^{-4} of normalized ratio, respectively), Figure 3.1.3. The normalized ratio represents the ratio between the target gene, in this case, CLU, and the corresponding reference gene, and in this case, ß2-µglob. NHM had an expression level similar to vitamin D-sensitive melanoma cell lines. These results are in agreement with the conventional PCR results, but again

only partially in agreement with the IHC staining, where nevi tissues were negative for CLU staining.

(a)



(b)



(c)

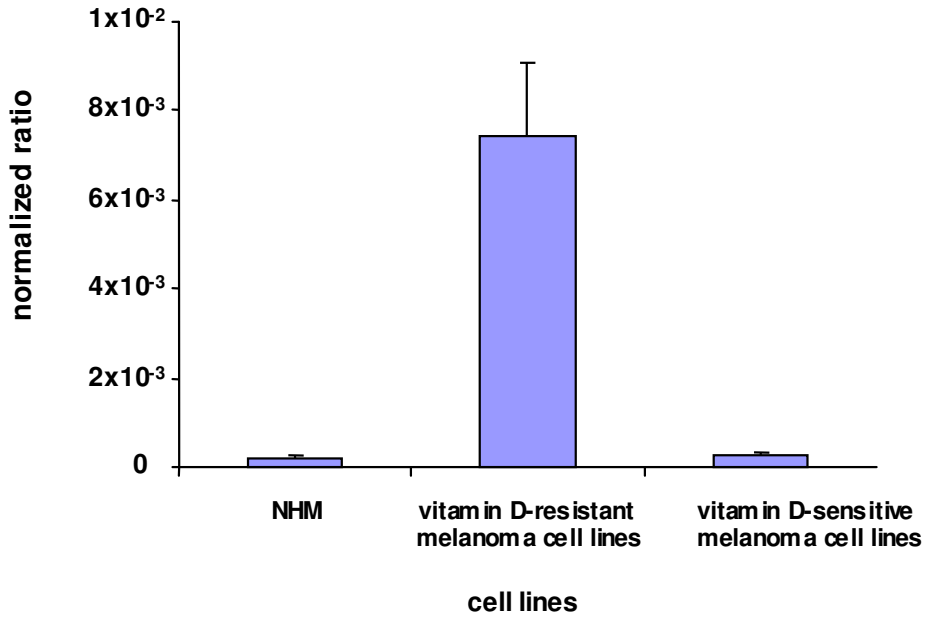


Figure 3.1.3. Expression of CLU mRNA in various melanoma cell lines: Real-time RT-PCR quantitative results showing levels of CLU expression in the various melanoma cell lines used. SK-MEL-5, -25 (vitamin D-resistant melanoma cell lines), and MCF (breast cancer cell lines) had the highest levels of CLU, while MeWo and SK-MEL-28 (vitamin D-sensitive melanoma cell lines) had the lowest levels of CLU expression (a, b). CLU expression is higher in vitamin D-resistant as compared to -sensitive melanoma cell lines and NHM (c). The normalized ratio represents the ratio between the target gene, in this case, CLU, and the corresponding reference gene, and in this case, ß2-µglob. Experiments were performed in duplicate and repeated at least twice. Error bars represent the SD.

Protein analysis:

We then sought to confirm the differences in CLU expression on the protein level among the melanoma cell lines used. Thus, western blot analyses, using a specific CLU antibody, revealed bands at ~80 and ~40 kDa in all melanoma cell lines and NHM analyzed; these polypeptide bands represent, sCLU protein forms, where cleaved α- and ß- protein smears are apparent and mature sCLU protein was also noted, respectively (Figure 3.1.4.). The western blot images are in agreement with the real-time RT-PCR results, where the sCLU protein bands vary between strong visible bands, to moderate or even faint bands in MeWo and SK-MEL-28 (notice the ~ 80 kDa band), respectively. Both CLU mRNA and protein are expressed in lower amounts in vitamin D-sensitive melanoma cell lines compared to vitamin D-resistant

melanoma cell lines. Hence, it was important to elucidate whether CLU expression is regulated by $1,25(OH)_2D_3$ treatment.

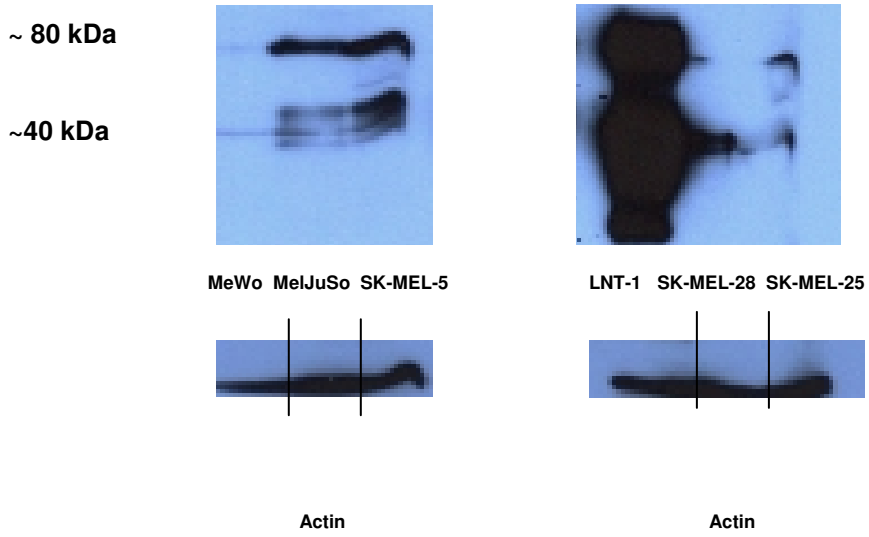


Figure 3.1.4. Western Blot analysis showing a double band (80 and 40 kDa) indicating the presence of sCLU in the upper panel. CLU expression was detected at various levels, where vitamin D-sensitive melanoma cell lines (SK-MEL-28 and MeWo) had lower CLU expression as compared to –resistant melanoma cell lines (SK-MEL-5, -25). The lower panels show actin levels as a loading control. LNT-1 prostate cell lines were used as a positive control as they over-express sCLU. The bands' sizes were determined by using a prestained protein marker in each gel. Experiments were repeated at least twice, and representative images are shown.

3.2. TIME-DEPENDENT CLU REGULATION IN MELANOMA CELL LINES BY 1,25-DIHYDROXYVITAMIN D_3

Different experiments were performed to answer the question of whether CLU expression is regulated by $1,25(OH)_2D_3$ treatment due to the fact that $1,25(OH)_2D_3$ treatment represents a palliative therapy for the metastasis of malignant melanoma. The first set of experiments was a colony forming ability assay (CFAs). The plating efficiencies (PEs) of cell lines to be analyzed were determined before a CFA with $1,25(OH)_2D_3$ were performed. To determine PEs, 1,000, 5,000, and 10,000 cells were platted onto 10-cm plates and the medium was changed daily for 7 days. Cells were then fixed and stained with

crystal violet and counted. Optimal PEs for each cell line was the determined and reported in graphic form in Figure 3.2.1. The different melanoma cell lines used had different PEs. MeWo had the highest PE. Following that, the PE for melanoma cell lines following 1,25$(OH)_2D_3$ treatment had to be determined to be able to compare with CLU expression.

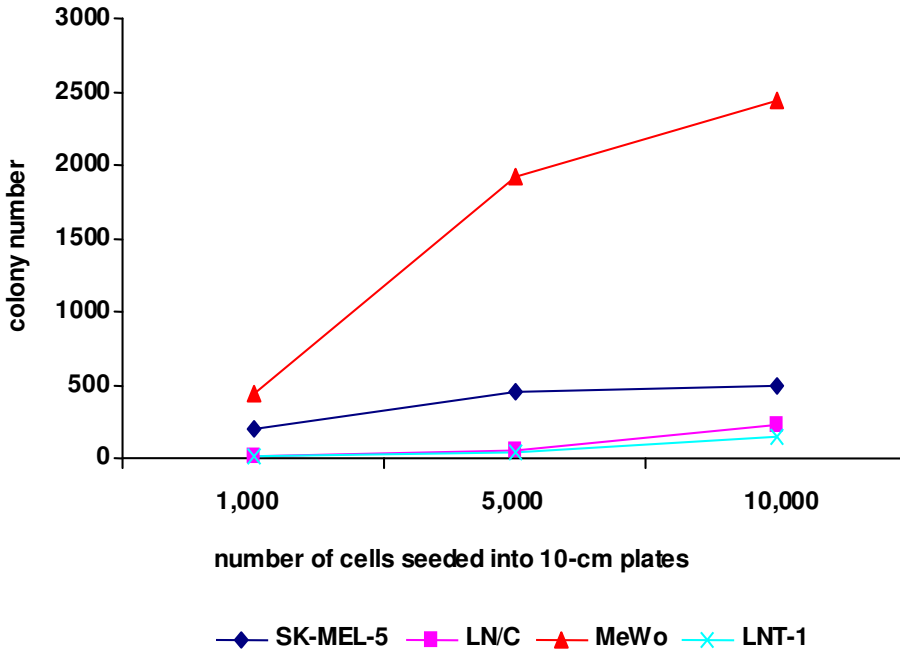
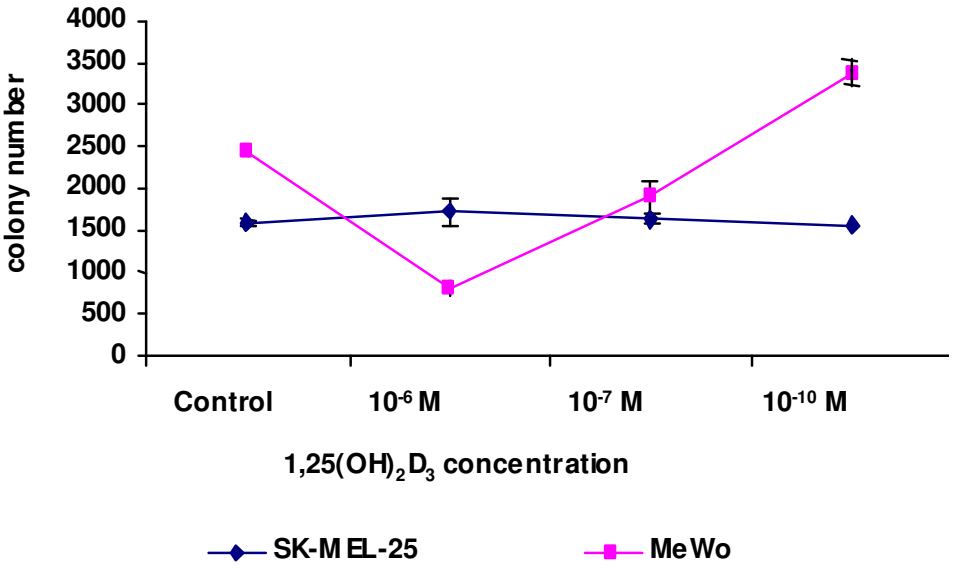


Figure 3.2.1. Plating efficiencies for various cell lines. Analysis of cell proliferation (CFA) in some of cell lines used (described in Materials and Methods), showing the number of colonies counted after 7 days without any treatment. The various cell lines tested had different PEs.

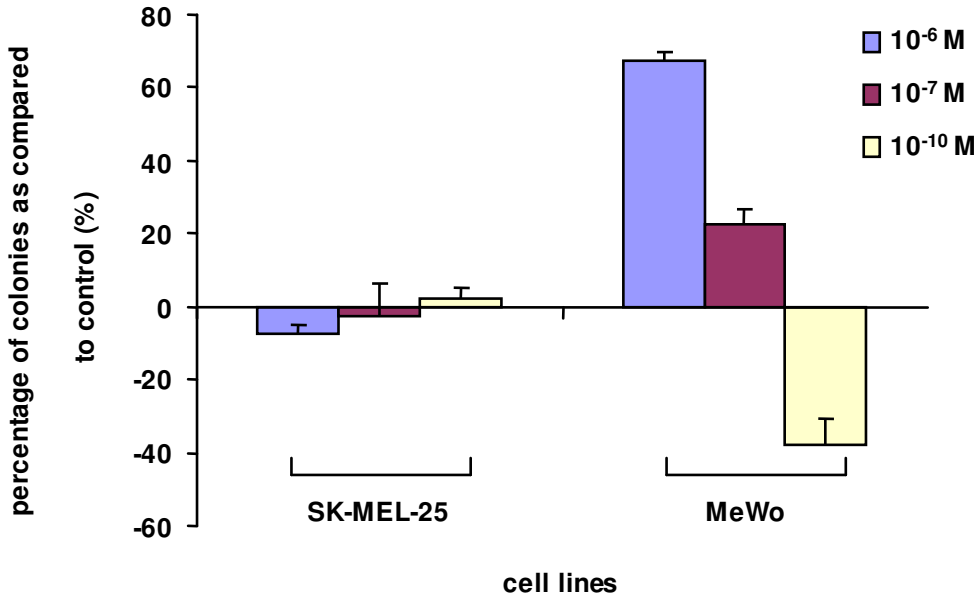
Anti-proliferative effects of 1,25$(OH)_2D_3$ were investigated in MeWo (vitamin D-sensitive) and SK-MEL-25 (vitamin D-resistant) cells using CFAs (Figure 3.2.2.a). At 10^{-6} and 10^{-7} M, 1,25$(OH)_2D_3$ inhibited the growth and proliferation of MeWo (70% and 20%, respectively) but not of SK-MEL-25 cells (Figure 3.2.2.b). The anti-proliferative effects of 1,25$(OH)_2D_3$ were dose- and time-dependent and most pronounced at 10^{-6} M. Interestingly, after 1,25$(OH)_2D_3$-treatment at a concentration of 10^{-10} M, the proliferation of MeWo cells was increased (~ 40%). This experiment confirms that the human melanoma cell

lines we have in culture were indeed either vitamin D-sensitive or -resistant melanoma cell lines, as described in the literature (Seifert *et al.*, 2004).

(a)



(b)



(c)

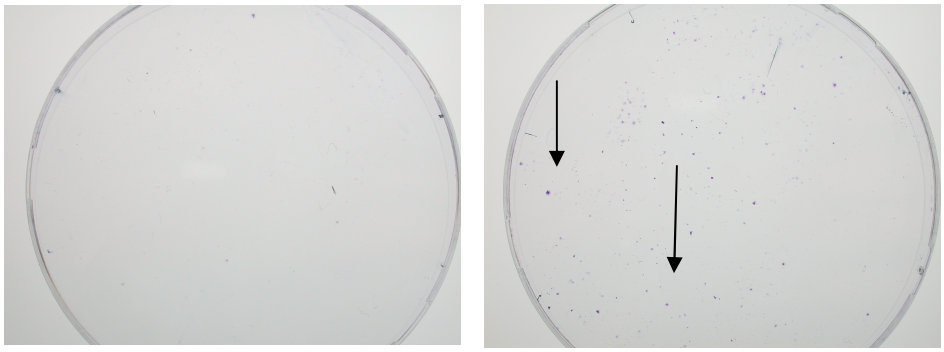


Figure 3.2.2. Analysis of cell proliferation (colony forming ability assays (CFAs) as described in 'Materials and Methods') in a vitamin D-responsive (MeWo) and -resistant (SK-MEL-25) melanoma cell line following treatment with $1,25(OH)_2D_3$. (a) Number of colonies after treatment of MeWo and SK-MEL-25 melanoma cells with different concentrations of $1,25(OH)_2D_3$ for 7 days. (b) Inhibition of cell proliferation following $1,25(OH)_2D_3$ treatment. Individual columns represent the inhibitory rate of colony forming, calculated from percentage of colonies counted following $1,25(OH)_2D_3$ treatment as compared to control. Note that MeWo cells show dose-dependently an inhibition (10^{-6} M and 10^{-7} M) or an increase (10^{-10} M) of proliferation as compared to controls treated with vehicle (ethanol) alone. These effects are not observed in SK-MEL-25 cells. Shown are means plus standard deviation, where error bars represent SD. (c) Two plates showing the colonies observed following crystal violet staining. The left-side plate is MeWo cells treated with $1,25(OH)_2D_3$ 10^{-6} M, while the right-hand one shows those treated with 10^{-10} M. Note that there are more colonies in right-side plate (arrows point to some of the colonies on the plates). Experiments were done in duplicates and repeated twice.

3.2.1. CLU REGULATION IN VITAMIN D-SENSITIVE BUT NOT IN VITAMIN D-RESISTANT HUMAN MELANOMA CELL LINES

The second set of experiments to determine whether CLU expression is regulated by $1,25(OH)_2D_3$ treatment were performed by measuring CLU mRNA expression in treated melanoma cell lines using real-time RT-PCR. Real-time RT-PCR showed an increase in CLU mRNA expression after 96 hrs of $1,25(OH)_2D_3$ treatment in MeWo, Figure 3.2.1.1.a. The increase was most prominent when compared to the expression level at 24 hrs and 72 hrs. This increase was not significant ($p \leq 0.1$), however, a trend was noted, suggesting that an additional analysis at longer times may reveal a slowly increasing

expression pattern. In SK-MEL-28 and SK-MEL-5, no differences were observed in CLU-mRNA levels following $1,25(OH)_2D_3$ treatment, Figure 3.2.1.1.b,c. CLU does not seem to be directly regulated by $1,25(OH)_2D_3$ treatment, nor does CLU expression depend on the PE of the cell lines.

(a) MeWo

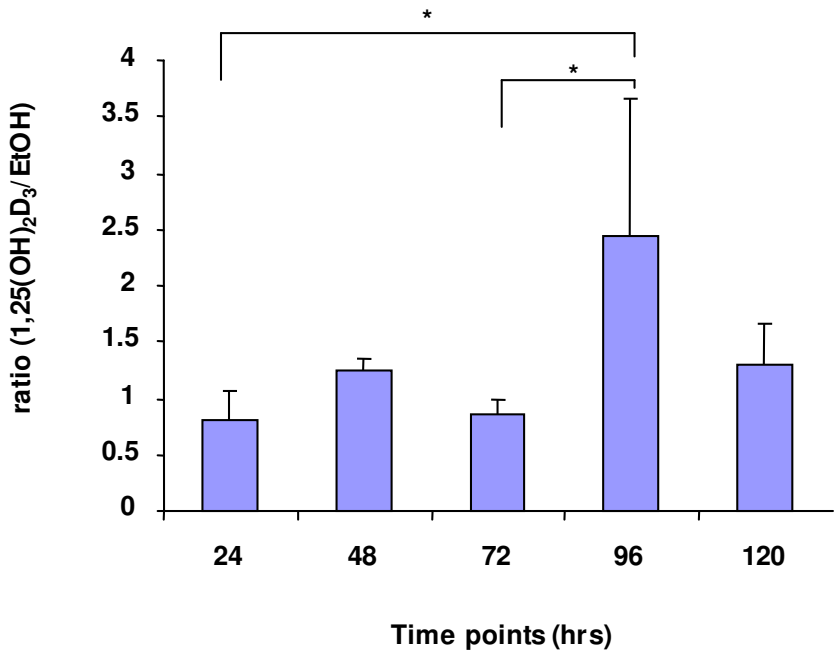


Figure 3.2.1.1. CLU mRNA expression in melanoma cell lines (RT-PCR). (a) CLU expression in MeWo cells. The expression is shown as a ratio between $1,25(OH)_2D_3$ and EtOH (vehicle) treated cells, and the ethanol (control) ratio was adjusted to 1. Note an increase in CLU mRNA expression after 96 hrs of treatment. Experiments were performed in duplicates and repeated at least twice. Error bars represent SD, and *p* value ≤ 0.1 is marked with an asterisk.

(b) SK-MEL-28

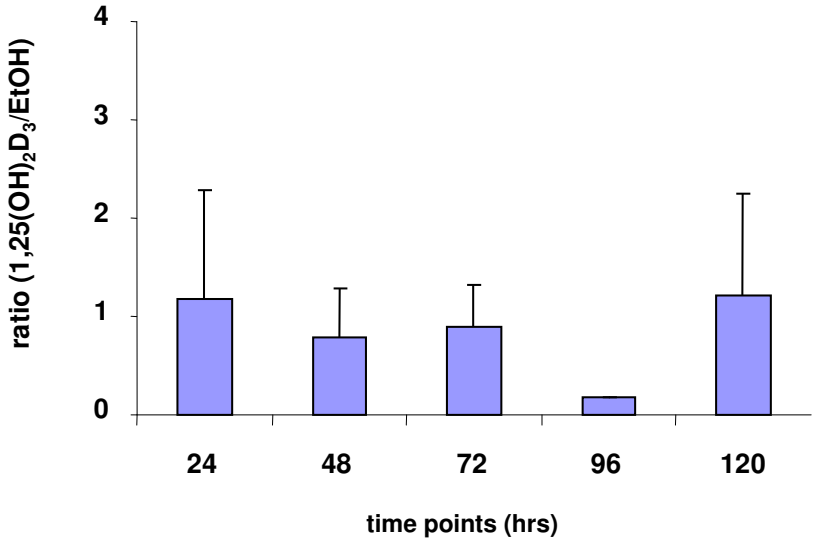


Figure 3.2.1.1. CLU mRNA expression in melanoma cell lines (RT-PCR). (b) CLU expression in SK-MEL-28. The expression is shown as a ratio between $1,25(OH)_2D_3$ and EtOH (vehicle) treated cells, and the ethanol (control) ratio was adjusted to 1. CLU mRNA expression was not affected following $1,25(OH)_2D_3$ treatment. Experiments were performed in duplicates and repeated at least twice.Error bars represent SD.

(c) SK-MEL-5

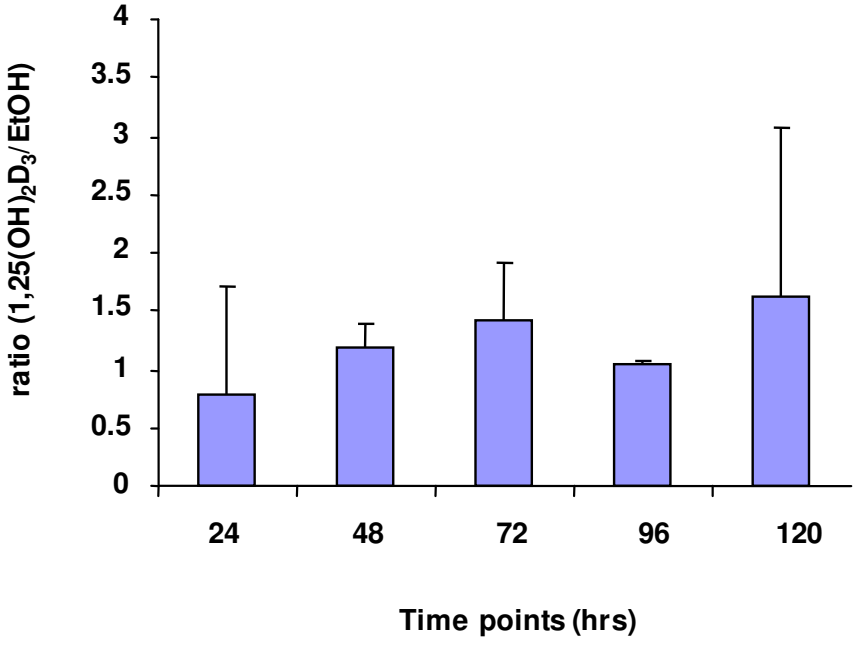


Figure 3.2.1.1. CLU mRNA expression in melanoma cell lines (RT-PCR). (c) CLU expression in SK-MEL-5. The expression is shown as a ratio between $1,25(OH)_2D_3$ and EtOH (vehicle) treated cells, and the ethanol (control) ratio was adjusted to 1. CLU mRNA expression was

not affected following $1,25(OH)_2D_3$ treatment. Experiments were performed in duplicates and repeated at least twice. Error bars represent SD.

However, a comparison between the three cell lines (MeWo, SK-MEL-28, and -5) at 96 hrs showed a statistically significant ($p \leq 0.05$) difference between CLU mRNA expression, Figure 3.2.1.2.

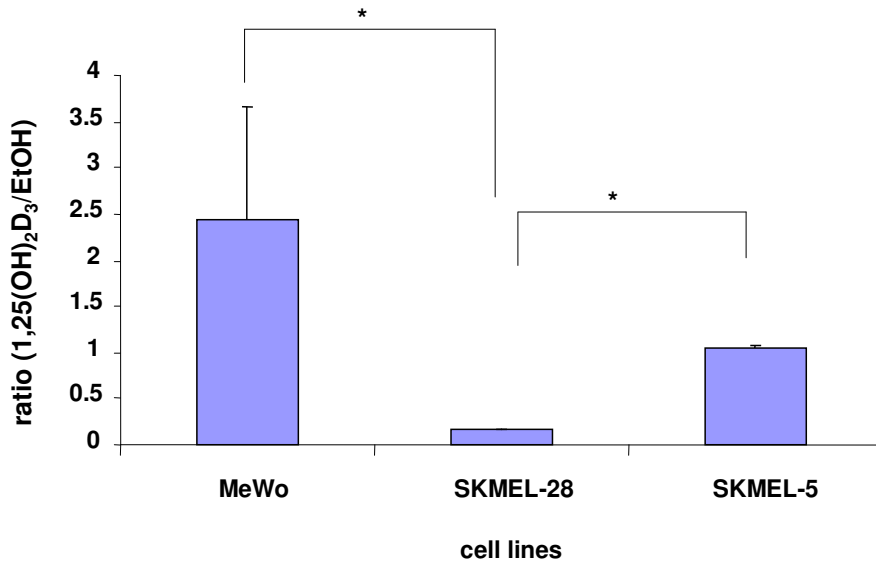


Figure 3.2.1.2. Comparison between the CLU mRNA expression (shown in an x-fold expression ratio of $1,25(OH)_2D_3$ to ethanol) in MeWo, SK-MEL-28, and –5 melanoma cell lines after 96 hrs of $1,25(OH)_2D_3$ treatment.

3.3. CLU EXPRESSION IN LNCaP PROSTATE CELL LINES

To better understand the role of CLU for proliferation, cell cycle, and apoptosis, we required a cell line that would stably over-express either sCLU or nCLU. We were able to attain a suitable cell culture model system using LNCaP human prostate cancer cells that over-expressed a stably transfected sCLU plasmid vector (LNT-1) as compared to its isogenic counterparts (LN/C). Before any experiments could be done and conclusions drawn out, the over-expression in LNCaP cells had to be confirmed. The stable transfection of sCLU in LNT-1 cells and the presence of sCLU in LN/C cells were confirmed using western analysis. As shown in Figure 3.3., two bands were obtained at ~80 kDa and 40 kDa, which have been reported previously to be characteristic for sCLU. Both bands were much stronger in LNT-1 cells

as compared to LN/C cells, clearly demonstrating the over-expression of sCLU in LNT-1 cells. In both cell lines used, no bands characteristic for nCLU were observed. These findings are in agreement with results reported previously (Miyake *et al.*, 2004).

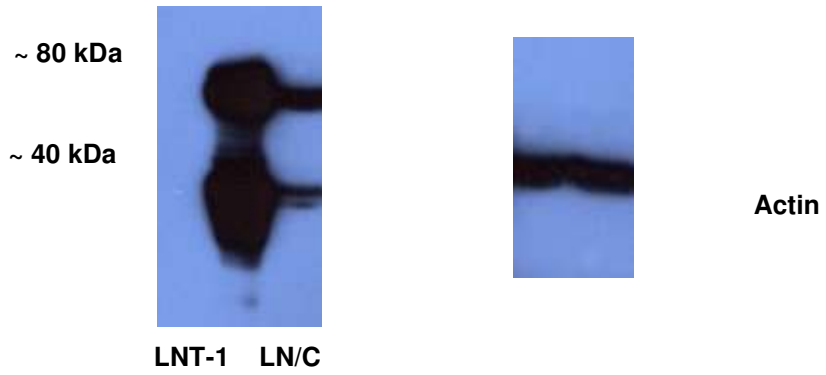


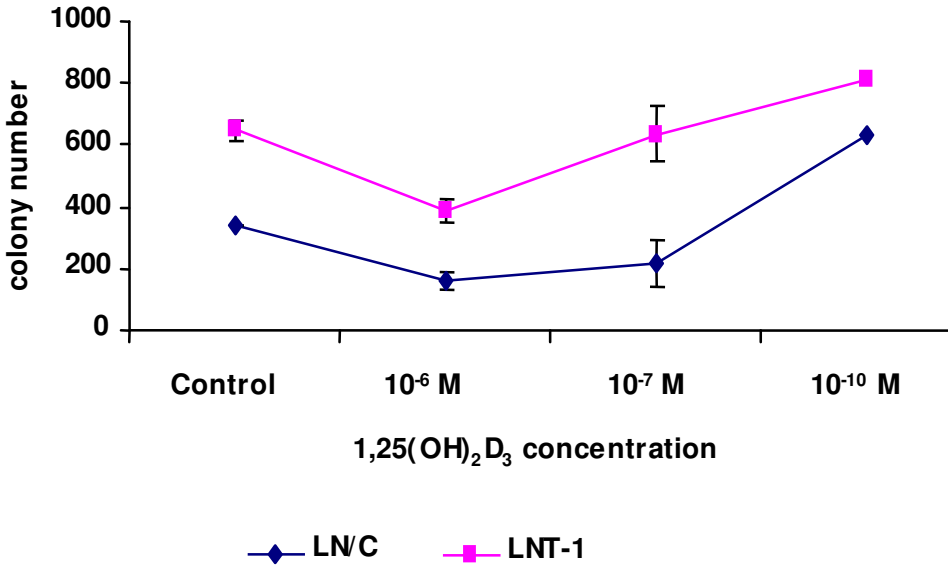
Figure 3.3. Western blot showing expression of sCLU protein in LNCaP cells stable transfected with sCLU (LNT-1) as compared to empty vector alone-transfected LNCaP cells (LN/C). The bands' sizes were determined by using a prestained protein marker in each gel. The over-expression of sCLU is apparent in LNT-1. Actin levels are shown as loading controls.

3.4. OVER-EXPRESSION OF sCLU MODULATES THE ANTI-PROLIFERATIVE EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3 IN HUMAN LNCaP PROSTATE CANCER CELL LINES

To assess whether sCLU over-expression would protect the cell lines used against anti-proliferative effects of $1,25(OH)_2D_3$, a number of experiments were carried out. First, CFA was performed on LNT-1 and LN/C cells to evaluate whether sCLU over-expression modulates the anti-proliferative effects of $1,25(OH)_2D_3$. LNCaP cells were plated on 10-cm plates, then medium and $1,25(OH)_2D_3$ were given daily for 7 days. The colonies were then stained and counted. $1,25(OH)_2D_3$ inhibited the growth and proliferation of LN/C cells and LNT-1 cells at doses of 10^{-6} and 10^{-7} M (53% and 40%, respectively) (36% and 2%, respectively), while at 10^{-10} M, it induced slightly pronounced proliferation in both cell lines (the induction of proliferation was

87% in LN/C and 25% in LNT-1 cells) (Figure 3.4.a). Differences comparing both cell lines were most prominent after treatment with $1,25(OH)_2D_3$ at a dose of 10^{-7} M ($P = 0.081$), although this effect was not statistically significant. sCLU over-expression also protected slightly against the proliferation-inducing effects of $1,25(OH)_2D_3$ at the 10^{-10} M in LNT-1 cells. This effect was also not statistically significant, but rather a trend. Thus, sCLU over-expression does not protect against the anti-proliferative effects of $1,25(OH)_2D_3$.

(a)



(b)

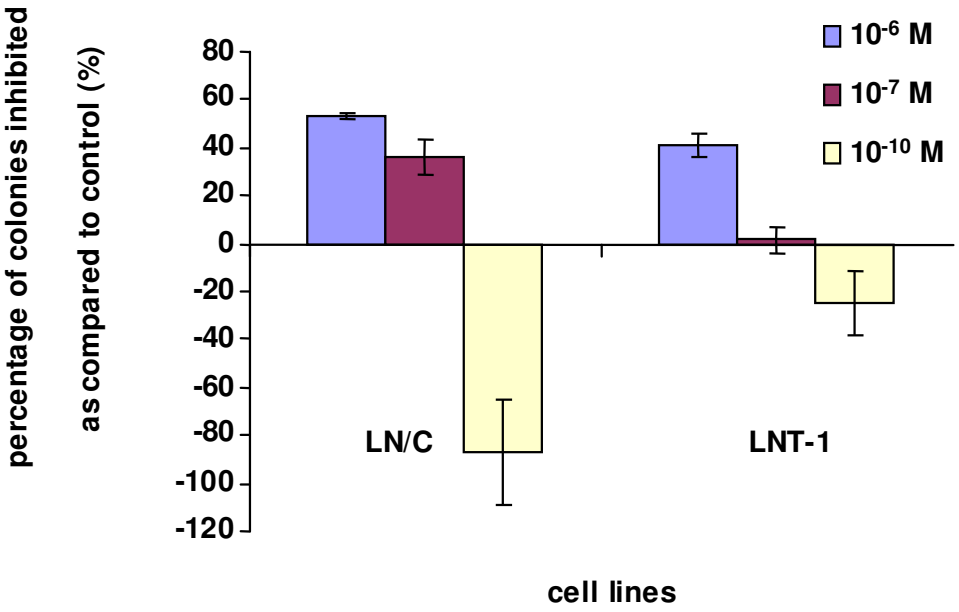


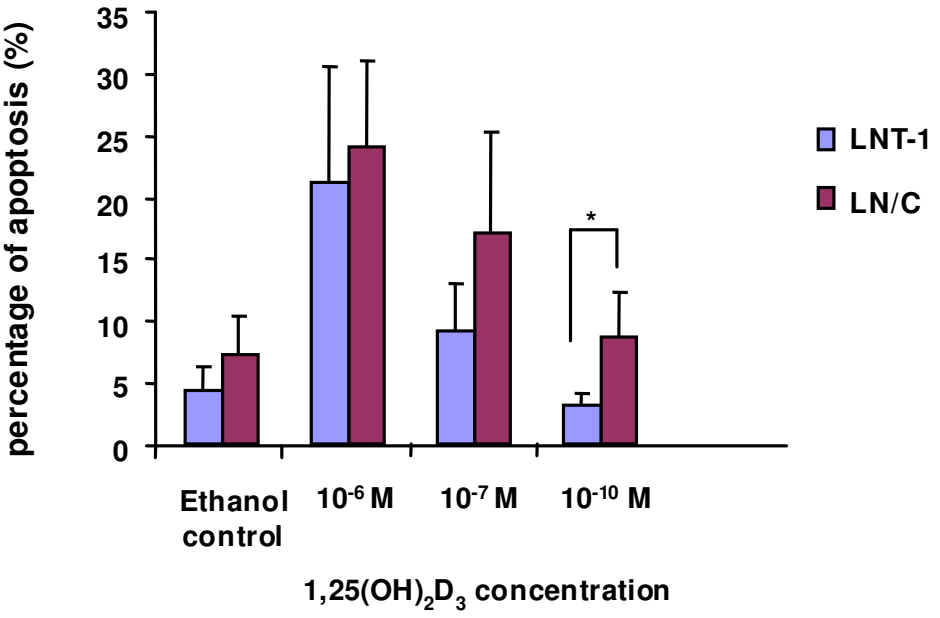
Figure 3.4. $1,25(OH)_2D_3$ modulates proliferation in prostate cancer cells (LNCaP) that are stable transfected with and over-express sCLU (LNT-1) and in control vector-transfected cells (LN/C) (CFA). (a) Number of colonies after treatment of LNT-1 and LN/C with different doses of $1,25(OH)_2D_3$ after 7 days (10^{-6} M, 10^{-7} M, and 10^{-10} M). (b) Inhibition of cell proliferation following $1,25(OH)_2D_3$ treatment. Individual columns represent the inhibitory rate of colony forming, calculated from percentage of colonies counted following $1,25(OH)_2D_3$ treatment as compared to controls. Error bars correspond to SD. *p* value ≤ 0.1 is marked with an asterisk.

3.5. OVER-EXPRESSION OF sCLU MODULATES THE PROAPOPTOTIC EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3 IN HUMAN LNCaP PROSTATE CANCER CELL LINES

Next, we tested if sCLU over-expression would protect against $1,25(OH)_2D_3$-induced apoptosis. TUNEL analysis was used to compare apoptosis in LNT-1 *versus* LN/C following treatment with $1,25(OH)_2D_3$ at different doses (10^{-6} M, 10^{-7} M, 10^{-10} M) according to the manufacturer's instructions, and apoptosis was measured using FACscan. As shown in Figure 3.5., daily $1,25(OH)_2D_3$ treatment induced more apoptosis in LN/C as compared to LNT-1 after 72 hrs in all doses used. The effect was most prominent at 10^{-7} M (p= 0.093), (10% apoptosis induction in LN/C as compared to 5% in LNT-1 cells). However, this effect was statistically not significant. The following question was whether sCLU over-expression would protect against the effects of other apoptosis-

inducing agents, since it has been reported that sCLU over-expression protects against H_2O_2, for example (Miyake *et al.*, 2004).

(a)



(b)

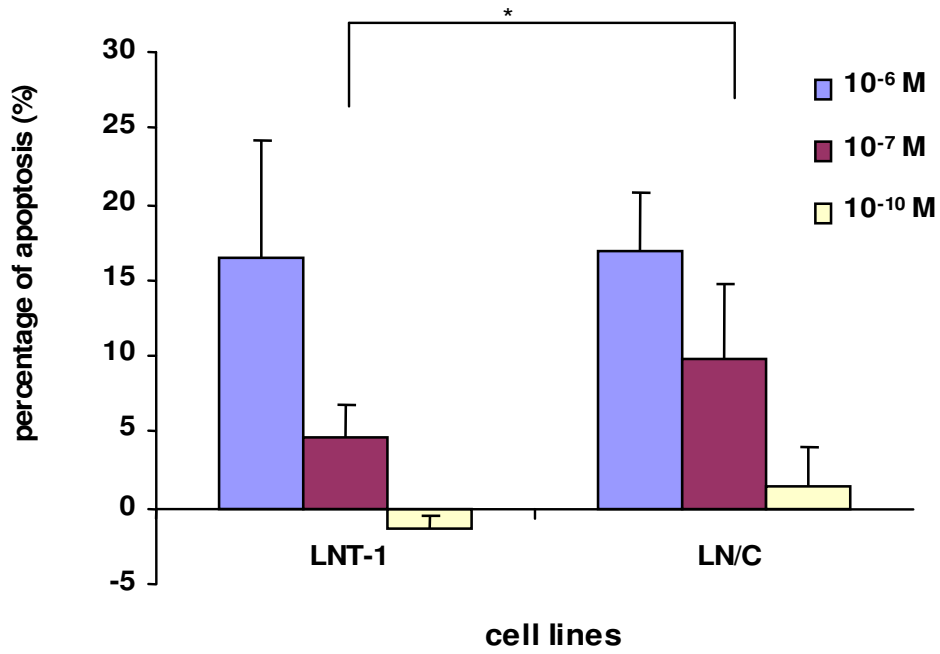


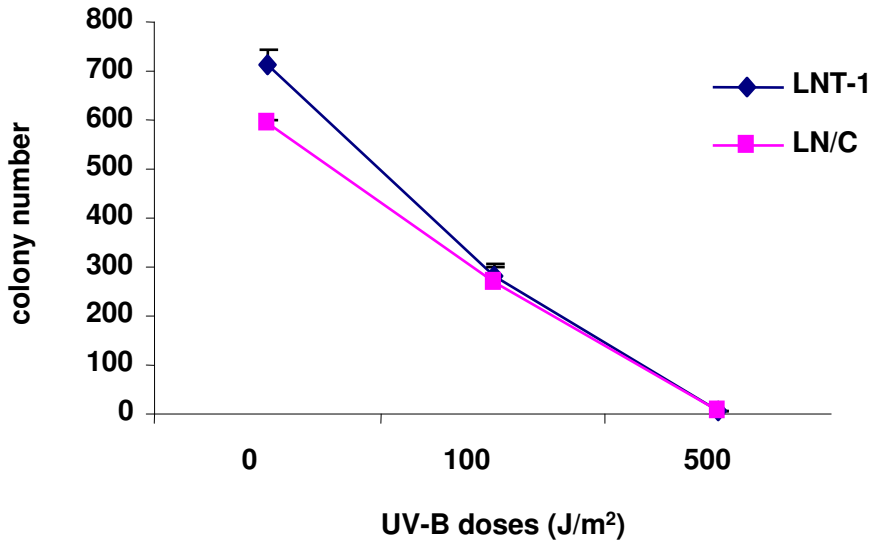
Figure 3.5. LN/C cells are more sensitive to $1,25(OH)_2D_3$-induced apoptosis as compared to LNT-1 cells (TUNEL assay) (a) Percentage of apoptotic cells (LNT-1 and LN/C cells) following 72 hrs of $1,25(OH)_2D_3$ treatment (10^{-6} M, 10^{-7} M, and 10^{-10} M). (b) The percentage of apoptotic cells (LNT-1 and LN/C cells) in the control group is subtracted from percentage of apoptotic

cells following treatment with $1,25(OH)_2D_3$. Error bars correspond to standard deviation, and *P* value ≤ 0.1 is marked with an asterisk.

3.6. OVER-EXPRESSION OF sCLU MODULATES THE CYTOTXIC EFFECTS OF UV-B IN HUMAN LNCaP PROSTATE CANCER CELL LINES

To address the question of whether sCLU over-expression protects LNCaP cell lines against other apoptosis-inducing agents, for example, UV-B; CFA was performed. Anti-proliferative effects of UV-B were investigated in LNT-1 and LN/C cells using CFA, as described before. Following 100 J/m^2 irradiation, the growth of both cell lines was inhibited (~ 60%). However, following higher doses of irradiation, 500 J/m^2, the cells' growth was almost 100% inhibited. There were no differences between the two cell lines with regard to sCLU over-expression, Figure 3.6.1. sCLU over-expression did not protect against UV-B-induced antiproliferative effects.

(a)



(b)

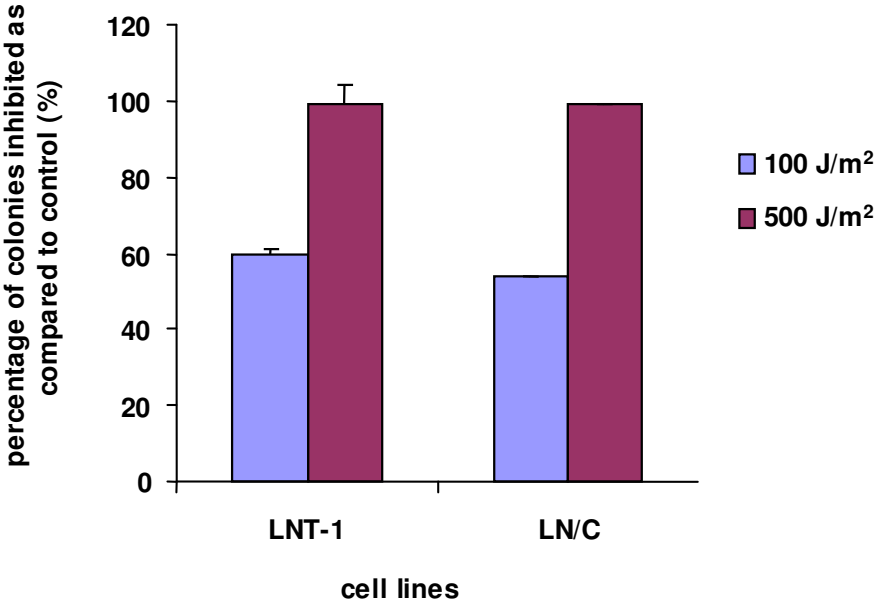
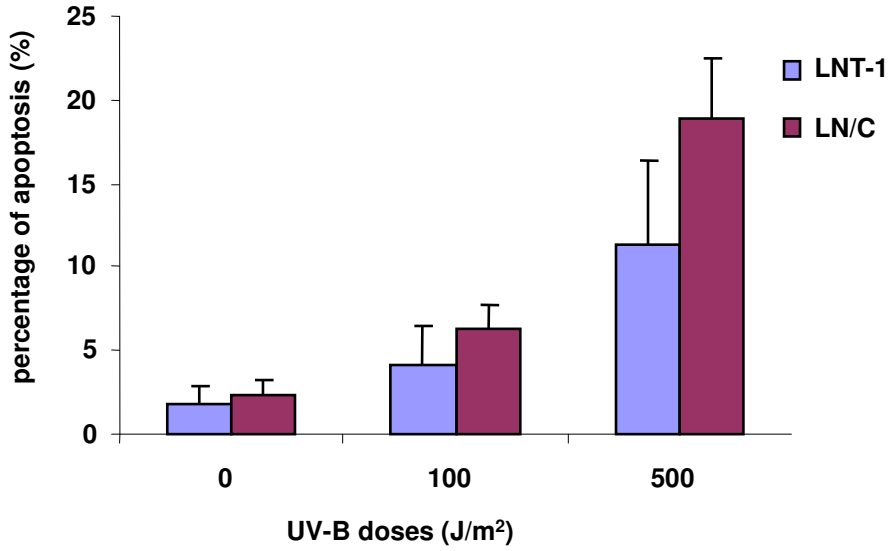


Figure 3.6.1. UV-B modulates proliferation in prostate cancer cells, LNT-1 and LN/C. (a) Number of colonies after UV-B exposure with different joules after 7 days. (b) Inhibition of proliferation following UV-B exposure. Individual column represent the inhibitory rate of colony forming, calculated from the percentage of colonies counted following UV-B exposure as compared to controls. Error bars represent SD.

Then, we checked the apoptotic effects of UV-B on LNCaP cell lines, and how would sCLU over-expression change the outcome. TUNEL analysis was used to compare apoptosis in LNT-1 *versus* LN/C following treatment with UV-B at different doses (0 J/m^2, 100 J/m^2, 500 J/m^2). As shown in Figure. 3.6.2., UV-B treatment induced more apoptosis in LN/C as compared to LNT-1 after 72 hrs in all doses used. The effect was statistically significant in the percentage of apoptotic cells at 500 J/m^2 (p= 0.038), (~7% apoptosis induction in LNT-1 as compared to ~17% in LN/C cells). sCLU over-expression protected LNT-1 cells against UV-B-induced apoptosis. Finally, we sought to analyze whether sCLU over-expression has any effects on the cell cycle regulation, as this issue is a controversial one in the literature (Bettuzzi *et al*., 2002, Criswell *et al*., 2003).

(a)



(b)

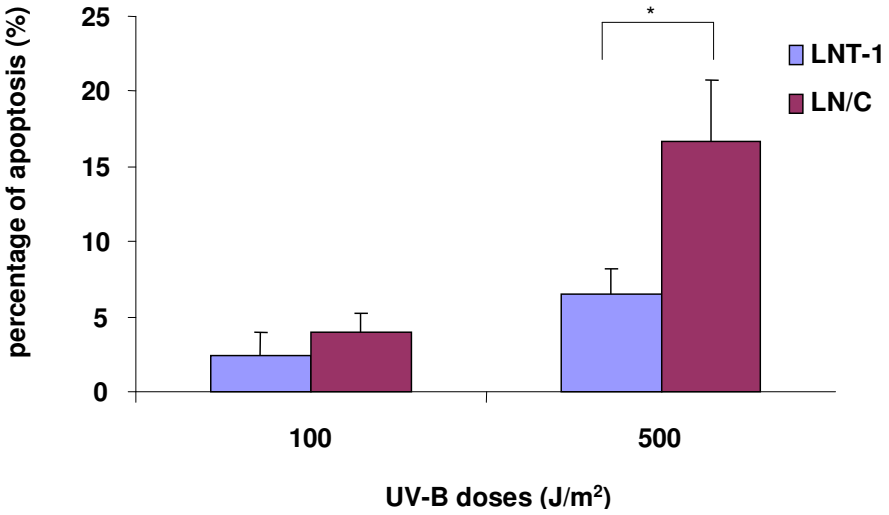
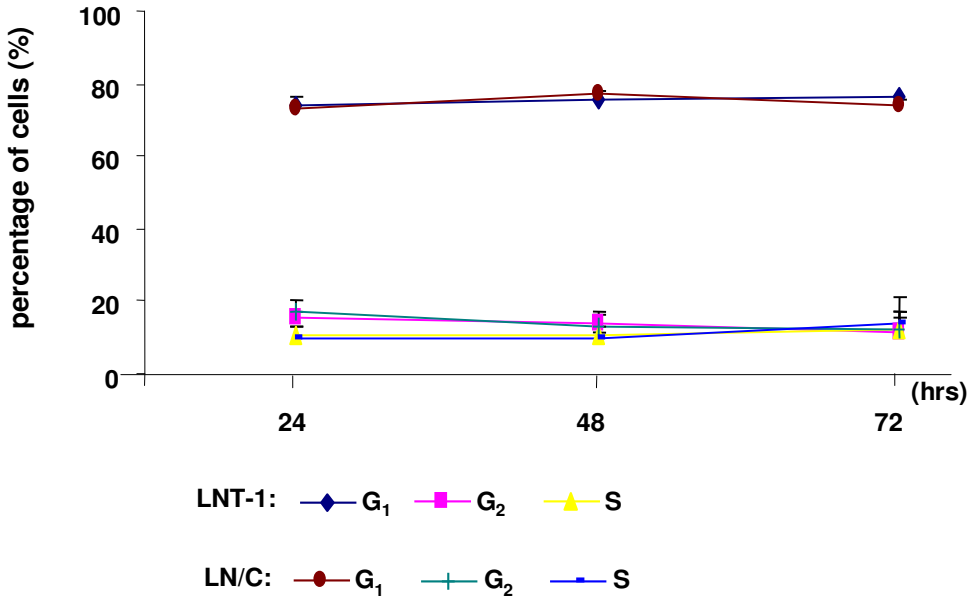


Figure 3.6.2. LN/C cells are more sensitive to UV-B induced apoptosis as compared to LNT-1 cells (TUNEL assay). (a) Percentage of apoptotic cells following 72 hrs of UV-B exposure (100 J/m^2, 500 J/m^2). (b) The percentage of apoptotic cells in the control group is subtracted from the percentage of apoptotic cells following UV-B exposure. Error bars represent the SD, and *p* value ≤ 0.05 is marked with an asterisk.

3.7. OVER-EXPRESSION OF sCLU DOES NOT AFFECT THE CELL CYCLE FOLLOWING 1,25-DIHYDROXYVITAMIN D_3 TREATMENT OR UV-B IRRADIATION

Following cell cycle analysis on LNCaP cells that were either treated with $1,25(OH)_2D_3$ (vehicle ethanol, 10^{-6} M, 10^{-7} M) or with UV-B (0 J/m^2, 100 J/m^2, 500 J/m^2), no changes were noted in the cell cycle. There were no effects on the cell cycle regulation with respect to sCLU over-expression. The cell cycle was analyzed after 24, 48, and 72 hrs of $1,25(OH)_2D_3$ treatment, and 9, 12, 15, 18, 21, and 24 hrs of UV-B irradiation. The difference between the time points is due to the fact that cells respond to the effects of UV-B earlier than those of $1,25(OH)_2D_3$ Figures 3.7.1 and 3.7.2. These results show that sCLU over-protection does not affect the cell cycle regulation following either $1,25(OH)_2D_3$ or UV-B exposures.

(a)



(b)

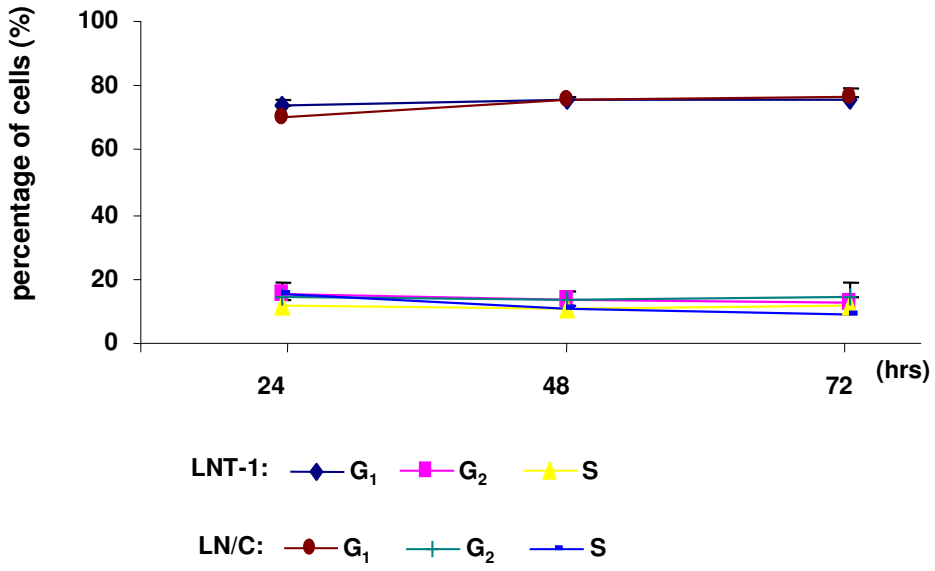
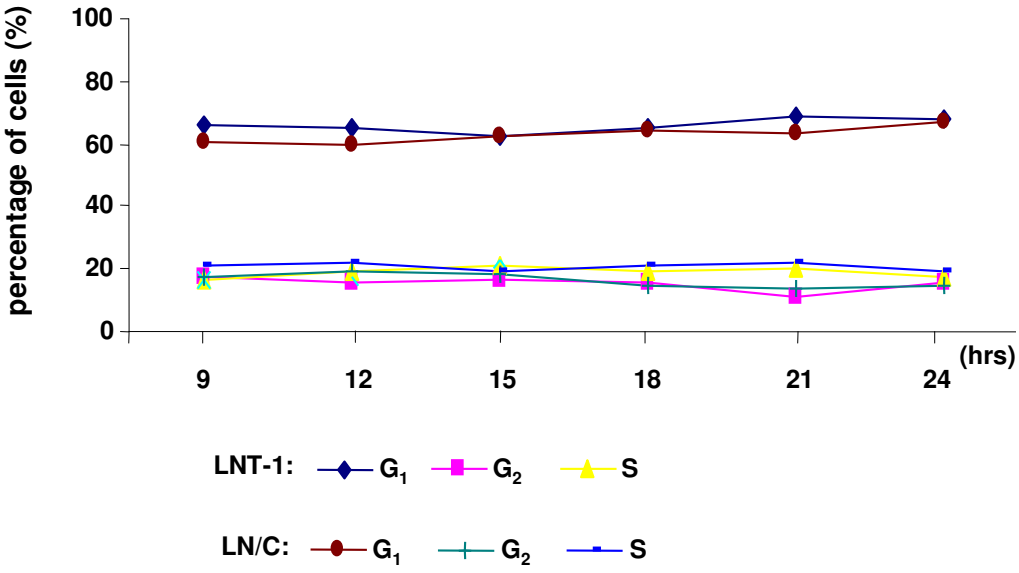


Figure 3.7.1. Cell cycle analysis of LNCaP cells following $1,25(OH)_2D_3$ treatment. (a) sCLU over-expression did not affect the cell cycle regulation in stable transfected cells, LNT-1, compared to the isogenic cell, LN/C, when treated with $1,25(OH)_2D_3$ [10^{-6} M] (b) sCLU over-expression also did not affect the cell cycle regulation in stable transfected cells, LNT-1, compared to the isogenic cell, LN/C, when treated with $1,25(OH)_2D_3$ [10^{-7} M]

(a)



(b)

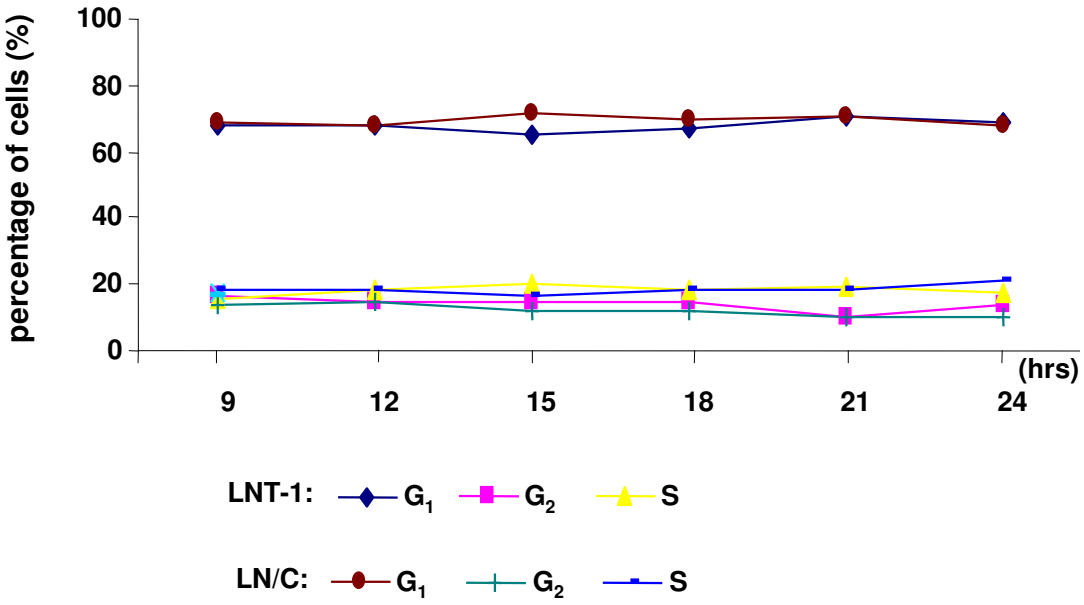


Figure 3.7.2. Cell cycle analysis of LNCaP cells following UV-B irradiation. (a) sCLU over-expression did not induce any changes in cell cycle in the stable transfected cells, LNT-1, compared to the isogenic cell, LN/C, when exposed to a UV-B does of 100 J/m^2 (b) sCLU over-expression did not induce any changes in cell cycle in the stable transfected cells, LNT-1, compared to the isogenic cell, LN/C, when exposed to a UV-B does of 500 J/m^2.

4. DISCUSSION

An increasing body of evidence points to a contribution of CLU to the progression of several cancers, including kidney carcinoma (Evans *et al.*, 1988; Matuda *et al.*, 2003), anaplastic large cell lymphoma (Seiberg *et al.*, 1995), ovarian cancer (Thomas-Salgar *et al.*, 1994), breast tumours (van Weelden *et al.*, 1998; Leskov *et al.*, 2003; July *et al.*, 2004), prostate cancer (Miyake *et al.*, 2000, 2004; Bettuzzi *et al.*, 2002; Zellweger *et al.*, 2002) and colon tumours (Chen *et al.*, 2003; Pucci *et al.*, 2004). However, the exact roles of CLU in pathogenesis and progression of cancer are still not well defined. For example, the role of CLU in malignant melanoma has yet to be defined. UV-B-induced apoptosis has been discussed to represent an important factor for the pathogenesis of malignant melanoma, and as described in the introduction section, CLU is a key regulator of apoptosis. Thus, one goal of our study was to examine the role of CLU in the sensitivity of malignant melanoma cells to cytotoxic agents, UV-B and $1,25(OH)_2D_3$ exposures.

In this work, we show that CLU is expressed in melanoma tissues and melanoma cell lines. The presence of CLU in a proportion of primary cutaneous malignant melanomas, but not in benign melanocytic nevi, indicates that CLU may be of importance in the pathogenesis of malignant melanoma. Expression of CLU in melanoma has been analyzed recently by another group using a different immunohistochemical technique in a much lower number of cases as compared to our study (Hoeller *et al.*, 2005). Antigen retrieval by cooking in citrate buffer, and staining using the LSAB kit were among the differences in the immunohistochemical techniques used by Hoeller *et al.*, compared to our study. These researchers found low levels of CLU expression in all their nevi tissues examined, except for 18% of the cases where high CLU expression was noted. In contrast, 40% of the primary melanomas analyzed in their study exhibited high CLU expression levels (Hoeller *et al.*, 2005). Thus, their study supports our results where we demonstrated, with a significant number of patient samples, that CLU protein was significantly increased in primary cutaneous malignant melanomas compared to benign nevi. However, the mechanisms that underlie this

increased expression of CLU in melanomas as compared to nevi are presently unknown, as will be discussed below.

In our study we also assessed whether changes in CLU expression may be associated with progression from primary cutaneous malignant melanoma to cutaneous or lymph node metastases. Our immunohistochemical studies did not reveal differences comparing CLU immunoreactivity in primary cutaneous malignant melanomas (IRS = 0.18) and metastases (IRS = 0.014). These data could suggest that sCLU expression is elevated early in melanoma carcinogenesis, and thereby is not elevated as cells progress to metastatic states. Nevertheless, these data do not support the concept, that CLU expression may be associated with the progression from primary cutaneous malignant melanoma to cutaneous or lymph node metastases. Our results do, however, conflict with the work of Hoeller *et al.* (Hoeller *et al.*, 2005). These researchers found elevated CLU expression in 40% of cases with primary melanomas and in 60% of melanoma metastases and concluded that CLU expression was elevated in the course of melanoma disease progression (Hoeller *et al.*, 2005). Partial discrepancies between our results and the study of Hoeller *et al.*, may be explained by differences in the immunohistochemical techniques used or by differences in the evaluation of the CLU staining (Table 3.1. was used in our staining evaluation). Finally, differences in our results may be related to the much higher case numbers in our study as compared to the study of Hoeller *et al.*, wherein a statistical power advantage favours our results.

Expression of certain genes (e.g., CLU) might, generally, be the result of elevated rate of transcription, stability of mRNA, elevated protein synthesis or increased protein stability. These features can not be analyzed in tumour tissues, and therefore, cell culture models are used. When then examined CLU mRNA expression in cultured melanocytes *in vitro*, our real-time RT-PCR data revealed increased levels of CLU mRNA expression in melanoma cell lines (that were derived from metastases of malignant melanomas) as compared to NHM. CLU protein expression was also documented using western blot techniques, where two bands representing the sCLU were observed. No bands showing the ~ 55 kDa nCLU isoform were observed in

any of the melanoma cell lines (the antibody used here detects both CLU isoforms. Personal communications, Prof. Bettuzzi, Dipartimento di Medicina Seperimentale, Universita di Parma, Italy). To our knowledge, there are no studies comparing CLU mRNA expression in between melanoma cells, however, our western blot results are in agreement with Hoeller *et al.*, where no nCLU protein isoform was seen in the western blot performed by these researchers in the melanoma cell lines used, but the band thickness/visibility (protein) varied according to the cell line tested (Hoeller *et al.*, 2005). Since it is not possible to culture cells from primary melanomas (the whole tissue is in general needed for histopathological analyses) and human melanoma cell lines can be altered by growth in tissue culture and the selection of growth advantages in this environment, these results do not allow a conclusion whether increased CLU mRNA levels in melanoma cell lines compared to NHM are the result of an up-regulation of CLU during the development from nevi to primary melanoma. Nor so they offer insight in understanding the role of sCLU during the progression of primary melanoma to metastasizing melanoma *in vivo*. However, our results are in agreement when compared to other studies assessing CLU mRNA expression (mostly determined using real-time RT-PCR). CLU mRNA levels were increased in tumour cells when compared to their normal counterparts in prostate cancer (Miyake *et al.*, 2002, 2004; Zellweger *et al.*, 2002, 2003), breast carcinoma (Van Weelden *et al.*, 1998; Leskov *et al.*, 2003), lung cancer (July *et al.*, 2004) and colon cancer (Pucci *et al.*, 2004). Some of these researchers were able to draw conclusions supporting the theory that increased CLU mRNA levels are associated with the progression of certain tumours like prostate cancer (Miyake *et al.*, 2004) and colon cancer (Pucci *et al.*, 2004). Furthermore, increases CLU protein levels correlated with the aggressiveness of breast tumours (Trougakos *et al.*, 2004). Some studies have highlighted the importance of CLU as a prognostic factor in breast (Redono *et al.*, 2002) and colon (Pucci *et al.*, 2004) cancers. Our results do not support the hypothesis that CLU may be an important prognostic marker for malignant melanoma.

The discrepancy between the immunohistochemistry (no CLU staining was detected in nevi) and the PCR as well as real-time RT-PCR results (CLU

mRNA was detected in NHM), may be due to the differing sensitivities of the techniques used for the detection of CLU expression *in situ* (IHC) as compared to *in vitro* analyses (i.e., using PCR and real-time RT-PCR). Another reason may be the difference in the cell proliferation rates in tissues compared to the melanoma cells grown *in vitro*.

An isoform shift from the expression of nCLU to sCLU was reported and theorized to be important for progression of various malignancies, including colon cancer (Pucci *et al.*, 2004). Also, over-expression of CLU was closely associated with disease progression in bladder cancer (Miyake *et al.*, 2001) and prostate cancer acceleration (Miyake *et al.*, 2004). While nCLU is mostly found in the nucleus, sCLU is present in the cytoplasmic cell compartment (reviewed in Shannan *et al.*, 2006, b). Therefore, we evaluated the immunohistochemical staining pattern in primary cutaneous malignant melanomas and metastases to assess any differences between nuclear and cytoplasmic staining patterns in the progression of malignant melanoma and whether these differences could be detected. There were no detectable differences between nuclear (nCLU) and cytoplasmic (sCLU) CLU staining. Thus, our immunohistochemical results do not support the hypothesis that a pattern shift of CLU isoforms may be of importance during progression of malignant melanoma. A major caveat in our interpretation of these data is that we did not have a positive control for nCLU expression and therefore we can not be completely certain we were detecting nuclear *versus* secretory CLU expression in the samples examined in our study. Based on the above, we document that both CLU mRNA and protein expressions vary in human melanomas and melanoma cell lines, however, unlike other tumours, CLU expression does not seem of importance for the progression and/or pathogenesis of malignant melanoma.

Nuclear CLU expression (nCLU) has been shown to be involved in DNA repair, at least via Ku70-dependent mechanisms (Yang *et al.*, 1999), where nCLU binds to Ku70 forming a trimeric complex with Ku80, and directly affects DSB repair. It is well known that up-regulation of DNA repair pathways is associated with the pathogenesis of various malignancies, including malignant melanoma (Shpitz *et al.*, 2005). Therefore, increased CLU expression in

malignant melanoma may be related to the genetic instability in these tumour cells and that may stimulate DNA repair pathways. Other authors have speculated whether CLU expression in malignant tumours may be related to the proliferative activity of tumour cells (Pucci *et al.*, 2004). Therefore, increased expression of CLU in malignant melanomas may be related to increased proliferative activity. However, when the proliferative activity of malignant melanomas was analyzed immunohistochemically, data reported were conflicting (Li *et al.*, 2000; Gimotty *et al.*, 2005; Ilmonen *et al.*, 2005). Most studies did not report a strong increase in the expression of markers associated with cellular proliferation, including Ki-67 and proliferating cell nuclear antigen (PCNA) (Ilmonen *et al.*, 2005). Thus, when we compared CLU expression in NHM and melanoma cell lines, no correlation of CLU expression with proliferative activity was found, as evaluated using CFA, where a fast proliferating cell line did not express higher CLU mRNA levels in the real-time RT-PCR than lower replicating cell lines examined. This is in agreement with the data of Criswell *et al.*, where the expression of sCLU was not related to proliferation (Criswell *et al.*, 2003). These data were confirmed in all melanoma cells examined in our study, where we also showed that the proliferative activity of human melanoma cell lines did not correlate with CLU mRNA expression. In summary, the results of our study (IHC and real-time RT-PCR) and data reported in the literature do not support the hypothesis that increased CLU expression in malignant melanoma may be related to the increased proliferative activity in these tumours. A limitation to our elucidation of these data is, again, the differences in techniques used between IHC and cell culture, where CLU levels of expression are, simply, different.

UV-B radiation is recognized as a well known external risk factor for the development of melanoma, especially lentigo maligna melanoma that affects the chronically sun-exposed facial area. UV-B plays a critical role in the aetiology of human melanoma, and is involved in initiation and promotion of the multi-step carcinogenesis in melanoma (Zhang *et al.*, 2003). UV-B exposure induces apoptosis in human melanoma cells via down-regulation of bcl-2 and activation of caspase-mediated apoptotic pathways (Zhang *et al.*, 2003). These two opposing functions of UV-B (risk factor for melanoma, and

apoptosis-inducing agent in melanoma cells) could be explained by the doses of UV-B, the exposure time, and the frequency of exposure. Although both cell cycle and apoptosis changes have been suggested to play essential roles in the carcinogenesis of melanoma, the basic mechanisms behind the photocarcinogenesis of UV-induced melanomas are still not fully understood (Zhang *et al.*, 2003; Yamazaki *et al.*, 2005).

To better understand the role of CLU in UV-induced changes in cancer progression and apoptosis, we required a cell line that would stably over-express sCLU, the cytoprotective isoform. We were able to obtain a suitable cell culture model system using LNCaP human prostate cancer cells that over-expressed a stably transfected sCLU plasmid vector (LNT-1) as compared to its isogenic counterparts (LN/C). Using these cells, we were able to asses the general response mechanism following exposure to either $1,25(OH)_2D_3$ or UV-B, as discussed below. Proliferation rate, apoptosis, and cell cycle changes were evaluated in LNT-1 *versus* LN/C following UV-B exposure. CFA analyses showed that proliferation in both cell lines was reduced following UV-B irradiation. However, sCLU did not provide any protection against the anti-proliferative effects of UV-B, since both cell lines showed comparable responses against the anti-proliferative effects of thus type of radiation. UV-B induced anti-proliferative effects have been linked to damage-induced cell cycle checkpoint responses (Reagan-Shaw *et al.*, 2006). We also demonstrated that UV-B induced dramatic increases in sub G_1 cells both in LNT-1 and LN/C cells. However, cell cycle analyses of LNT-1 and LN/C following various doses of UV-B irradiation showed no significant differences between the two cell lines. It is important to note here that the higher dose of UV-B used was 500 J/m^2, and at this dose, floating/dead cells were seen in the medium before the cells were handled. This could explain the similar responses of the cell lines at 500 J/m^2 in CFA, and cell cycle analysis. The role of CLU expression in cell cycle regulation has been controversial. CLU over-expression in immortalized human prostate epithelial cells resulted in an increased accumulation of the cells at the G_0/G_1 phases of the cell cycle, accompanied by a slow down of the cell cycle progression and a reduction of DNA synthesis (Bettuzzi *et al.*, 2002). CLU over-expression

also resulted in DNA damage accumulation in human LNCaP prostate cells, and helped accelerate prostate cancer development. This is thought to have occurred, at least in part, through the inhibition of membrane damage induced by H_2O_2 (Miyake *et al.*, 2004). High levels of nCLU were reported to cause G_1 cell cycle arrest in distinct cell types (Yang *et al.*, 1999).

Other groups denied an important role of CLU for cell cycle regulation (Criswell *et al.*, 2003). Where Criswell *et al.*, found that sCLU was transcriptionally repressed by functional p53 independent of the cell cycle, and loss of functional p53 appeared to relieve negative regulation on the IR-induction responses of the CLU gene in a variety of cell types. It can be speculated, that either the effects of sCLU over-expression on the cell cycle are not a general mechanism, but mediated through certain pathways depending on the apoptosis-inducing agent used, or that sCLU does not regulate the cell cycle. Our results favour the last speculation, and do not support the hypothesis that sCLU exerts profound changes on cell cycle regulation in melanoma or in human prostate cancer cells before or after UV-B irradiation.

In contrast to its lack of affects on cell cycle regulation and proliferation, sCLU over-expression dramatically protected cells against UV-B-induced apoptosis (TUNEL analyses). The protection was statistically significant ($p \leq 0.05$) as measured 72 hrs following a 500 J/m^2 dose. High doses of UV-B induce blockage of DNA replication which causes DSBs in the cells, and as mentioned above, nCLU is important for DNA-PK. It had been proposed that nCLU induces apoptosis by freeing Bax protein, which would normally bind to Ku70, then nCLU would bind to Ku70 and inhibit Ku70/Ku80 binding activity (Yang *et al.*, 1999; Leskov *et al.*, 2001). This nCLU/Ku70 interaction may undergo changes when sCLU is over-expressed, making it possible for cells to repair themselves rather than go into apoptosis. This would explain the 'lower-than-expected' percentage of apoptosis in TUNEL analyses. Another reason could be, as mentioned previously, that cells were already apoptotic upon measurement following a 500 J/m^2 UV-B dose. sCLU-mediated protection against apoptosis has been reported in the literature against many different stimuli (Miyake *et al.*, 2004). Also, it has been speculated that tumour

cell survival is associated with over-expression of sCLU and loss of nCLU (Pucci *et al.*, 2004). CLU was also proposed to exert anti-apoptotic or pro-apoptotic functions by interacting with different proteins depending on its distribution (Zhang *et al.*, 2005). The mechanisms that underlie this function are not well understood and mainly include: CLU regulation by B-MYB (Cervellera *et al.*, 2000), NF-κB (Sankilli *et al.*, 2003), IGF-1 (Criswell *et al.*, 2005) and p53 (Criswell *et al.*, 2003). Our results point to an important role of sCLU in protecting cells against UV-B-induced apoptosis.

CLU has been shown to be of importance for the therapeutic outcome and the response to various treatment modalities in a broad range of different malignancies (Beer *et al.*, 2005). In this respect, the expression of sCLU in tumour cells and its putative counteracting affects against the apoptosis-inducing capacity of chemotherapeutics has been suggested to be of particular importance. Therefore, we investigated the role of CLU on the cell death responses of human prostate and melanoma cells to $1,25(OH)_2D_3$, an agent that has been shown to induce apoptosis in tumour cells and that is at the moment under clinical investigation for the treatment of various types of cancers (Osborne and Hutchinson, 2002; Beer *et al.*, 2005). $1,25(OH)_2D_3$ and its analogues have been shown to inhibit cell proliferation in a variety of preclinical models of cancer, including human prostate cancer. These preclinical findings have promoted the clinical use of $1,25(OH)_2D_3$ and its analogues for cancer treatment (Beer *et al.*, 2005). Additionally, as pointed out in the introduction, preliminary data reported in the literature indicate that CLU expression may be directly up-regulated in response to $1,25(OH)_2D_3$ exposure (Simboli-Campbell *et al.*, 1996).

It was previously demonstrated that the introduction of full-length CLU cDNA into LNCaP cells increases resistance to apoptosis induced by TNF-α treatment (Miyake *et al.*, 2000). Increased expression of CLU in prostate cancer is closely correlated with higher Gleason score and cancer progression (Miyake *et al.*, 2000). Thus, silencing CLU has been considered a promising target for prostate cancer treatment. In several studies, CLU antisense oligonucleotides increased the cytotoxic effects of paclitaxel, reducing the IC_{50}

of PC3 cells by 75%-90% compared to paclitaxel alone (reviewed in Shannan *et al.*, 2006, b).

To analyze whether CLU expression in melanoma cell lines was regulated by $1,25(OH)_2D_3$, we tested various human melanoma cell lines that were previously characterized to be resistant or sensitive against the anti-proliferative effects of vitamin D analogues (Seifert *et al.*, 2004). Real-time RT-PCR revealed different levels of CLU mRNA in the human melanoma cell lines tested. The vitamin D-sensitive melanoma cell lines, MeWo and SK-MEL-28, had the lowest levels of CLU mRNA as compared to vitamin D-resistant, SK-MEL-5 and SK-MEL-25, melanoma cell lines. Thus, the basal levels of CLU mRNA expression were higher in the vitamin D-resistant melanoma cell lines compared to non-isogenic sensitive cell lines. Furthermore, NHM expressed similar CLU mRNA expression levels as vitamin D-sensitive melanoma cell lines (mainly MeWo). Vitamin D-resistant cell lines were reported to have a functional defect in vitamin D receptor-mediated gene transcription (Seifert *et al.*, 2004), while -sensitive cell lines and NHM cells showed no such vitamin D receptor defect. We speculate that a functional vitamin D system acts either directly on CLU expression and suppresses its levels as compared to a defect one, or indirectly by suppressing cell proliferation and thus CLU expression.

Additionally, we analyzed the affects of $1,25(OH)_2D_3$ treatment on CLU expression in melanoma cell lines. Previous studies demonstrated that $1,25(OH)_2D_3$ inhibited malignant cell invasion and tumour-induced neo-angiogenesis by inducing apoptosis (*e.g.*, in breast, prostate and colon cancers) (Osborne and Hutchinson, 2002), and modulated growth and apoptosis in melanoma cells (Danielsson *et al.*, 1998; Osborne and Hutchinson, 2002). Using CFAs, we demonstrated that at high $1,25(OH)_2D_3$ concentrations (10^{-6} M or 10^{-7} M), the proliferation of vitamin D-sensitive cells (MeWo) was inhibited. These effects were not observed in vitamin D-resistant melanoma cells (SK-MEL-25). Interestingly, using real-time RT-PCR, $1,25(OH)_2D_3$-treated (10^{-6} M) vitamin D-sensitive cells (MeWo) showed up-regulation of CLU mRNA level (~1.5 fold induction) after 96 hrs. This up-regulation was a trend ($p \leq 0.1$) compared to the CLU mRNA levels after 24

hrs. In contrast, vitamin D-resistant cells (SK-MEL-5) showed no such up-regulation after 1,25$(OH)_2D_3$ treatment. Comparison between the three cell lines tested (MeWo, SK-MEK-28, and -5), a statistically significant difference in CLU mRNA expression following 96 hrs of 1,25$(OH)_2D_3$ treatment was observed.

Nevertheless, based on real-time RT-PCR results, 1,25$(OH)_2D_3$ treatment does not appear to induce up-regulation of CLU mRNA expression (in two melanoma cell line out of three used), and increases in the levels of expression appear to be independent of the basal CLU mRNA expression. In western blot analyses, the up-regulation could not be confirmed due to the low expression levels of CLU (as shown in real-time RT-PCR). This leaves us with two hypotheses: (1) Real-time RT-PCR primers measure total CLU mRNA (both isoforms, sCLU and nCLU), and since nCLU was not detected in the western blot for unstressed melanoma cell lines, it can be assumed that the basal levels of CLU mRNA are those of sCLU. However, when the cells are under stress (e.g. after 1,25$(OH)_2D_3$ treatment), and based on previous studies, nCLU might be produced. Thus, the increase of CLU mRNA in MeWo could be that of nCLU rather than sCLU, especially that after 48 hrs of 1,25$(OH)_2D_3$ treatment where floating cells were observed in the cell culture indicative of apoptotic responses. However, nCLU was not up-regulated in SK-MEL-28 and SK-MEL-5 cells, and the reason behind this remains to be elucidated.

(2) It is known that more than half of all human tumours contain p53 mutations, and in most malignancies, mutations in p53 lead to a shutdown of p53 tumour suppressor signalling pathway (Ho and Li, 2005). Previous studies have shown that MeWo has a single p53 point mutation in codon 341 (Ho and Li, 2005). SK-MEL-28 also contains a single p53 point mutation in codon 145, and over-expresses mutant p53 (Girnita *et al.*, 2000). SK-MEL-5 exhibits no p53 mutations, but it has a functionally aberrant p53, due to the absence of $p21^{Waf1}$, that is also over-expressed (Girnita *et al.*, 2000). As mentioned in the introduction, p53 can suppress basal as well as ionizing radiation-induced sCLU expression in breast and colon cell lines by repressing CLU promoter activity and transcription (Criswell *el al.*, 2003). Also, sCLU expression was

shown to be regulated by IGF-1 signalling, and since IGF-1 is thought to be a factor in tumorigenesis, it is possible that melanomas over-express IGF-1. Thus, based on our results and what is reported in the literature, it is possible that CLU up-regulation depends on the type and place of the point mutation in the investigated cell lines through the IGF-1 signalling pathway. Since these melanoma cell lines express either mutations in p53, or have a functionally aberrant p53 and it (p53) regulates IGF-1 ligand expression, which in turn, regulates sCLU expression (Dr. Boothman, personal communications), it can be hypothesized that the increase in sCLU expression in malignant melanoma might be due to p53 mutations. Thus, in this context, tumour formation might depend on the distorted interaction between p53 and sCLU.

However, it is still possible that the increase in CLU expression may be of importance for $1,25(OH)_2D_3$-mediated biological responses in melanoma cells, including anti-proliferative effects and apoptosis. It remains to be elucidated whether the modulation of CLU expression may be involved in the anti-tumour effects of $1,25(OH)_2D_3$ as well. Furthermore, it has been previously reported that MeWo is characterized as a high invasive melanoma cell lines, while SK-MEL-28 as a low invasive cell line (Billion *et al.*, 2006), and since different levels of CLU mRNA were reported in non-treated melanoma cell lines, our results point to the fact that CLU expression may be related to the aggressive and invasive status of melanoma, and thus to the pathogenesis of melanomas.

To evaluate the effects of $1,25(OH)_2D_3$ on sCLU over-expression, the LNCaP human prostate cancer cells (LNT-1 and LN/C) were treated with $1,25(OH)_2D_3$ to test for differences in the anti-proliferative and apoptotic effects exerted by over-expression of sCLU. CFA and TUNEL analyses revealed that both cell lines show anti-proliferative and apoptotic effects following $1,25(OH)_2D_3$ treatment, respectively. Nevertheless, sCLU over-expression did not protect against the anti-proliferative and apoptotic effects of $1,25(OH)_2D_3$, ($p \leq 0.1$ representing a trend). Cell cycle analyses showed no differences between the two cell lines before and after $1,25(OH)_2D_3$ exposure.

In conclusion, following UV-B exposure, sCLU only provides protection against UV-B-induced apoptosis, but not against -induced anti-proliferative

effects. On the other hand, sCLU provided no significant protection (only a trend with $p< 0.1$) against $1,25(OH)_2D_3$-induced apoptosis and anti-proliferative effects.

Future research regarding CLU and malignant melanoma should include silencing sCLU expression and then assessing how melanoma cell lines would respond to various cytotoxic agents, screening and sequencing the CLU gene for any mutations in human melanoma cell lines, and assessing the IGF-1 signalling pathway in melanoma cell lines (since it regulates sCLU expression). Furthermore, to overcome the problem in our immunohisochemical analyses of the positive control of nCLU, this truncated form (nCLU) could be over-expressed in human melanoma cell lines then used as a positive control.

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6. ACKNOWLEDGMENTS

A new country, a new language, new friends, and hopefully a new degree. The first few months in Homburg weren't easy. However, we learn to cope and make the best of what we have. These three years would not have been possible without the help and support of many people. I would like to thank:

Prof. Reichrath, for making this possible, and for his help and patience in writing manuscripts and work in the lab.

Prof. Boothman and Prof. Dr. Montenarh for their valuable comments on my work.

Dr. Seifert, for his help in the lab.

Prof. Hermann and Prof. Tilgen for their help in getting my scholarship.

Also, a big thank-you to my mum and dad for providing me with the opportunity to come here and supporting me through out the way. My sisters, Shaza and Lujein, there are no friends like sisters. You are the best!

My friends, Anne, Rima, and Annett, thank you for being there when I needed someone to talk and nag to, for your continuous help in German. Thank you for everything.

Last, to my co-workers in the lab, Heike, Alex, and Sonja.

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I am pursuing a career in the clinical, the research, or the educational field, and would like to build upon the foundation of my experience in both routine clinical and research work. My particular areas of interest include haematology, transplantation sciences, and molecular genetics.

Education

PhD fellow in Biological Sciences (Dr. rer. nat), March 2004-present date. Department of Dermatology, Saarland University Hospital, Homburg/Saar, Germany. Expected date to finish, October 2006.

MSc in Clinical Laboratory Sciences, September 2002-September 2003. University of Newcastle-upon-Tyne, Newcastle, United Kingdom. Course selection focused on basic mechanisms of diseases, pathology, immunology, transplantation, molecular biology of cancer, and clinical biochemistry. Plus practical work in the haematology department. Dissertation title: 'Polymorphisms of Interleukin-8 and its association with Graft-Versus-Host Disease'.

BSc in Medical Laboratory Technology, October 1999-June 2002. American University of Beirut, Beirut, Lebanon.

Work Experience

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Working on my PhD project in the Department of Dermatology, Saarland University Hospital.

- Important experience in a research laboratory working on cell culture, flow cytometric analysis, DNA, RNA, and protein analysis
- Knowledge in writing research papers for journal publications

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Research Technician in the Molecular Haematology Department in the University of Newcastle-upon-Tyne.

- Essential practice in tissue culture and aseptic techniques

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Working on my MSc project in the Molecular Haematology Department in the University of Newcastle-upon-Tyne.

- Valuable experience in molecular haematology, especially using PCR, acrylamide gels, and silver stain analysis
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Monitor and Receptionist in the American University of Beirut, Boustani Students‘ Dormitory.

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Trainee at the American University of Beirut Hospital clinical laboratories (part of the BSc programme).

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Presented Abstracts

1. Poster presentation: **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J. Expression of clusterin is upregulated in vitamin D-sensitive but not in vitamin D-resistant melanoma cells following $1,25(OH)_2D_3$ treatment. Tübingen (Germany) 22-24 September 2005: 35^{th} Annual ESDR Meeting.

2. (a) Oral presentation: : **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J. Clusterin over-expression protects against 1,25-dihydroxyvitamin D_3-induced apoptosis in LNCap cells *in vitro*. Villars-sur-Ollon (Switzerland) 16-18 June 2005 : 4^{th} Workshop on Clustein/APOJ.
   (b) Oral presentation: : **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J. Clusterin is expressed in benign and malignant melanocytes and modulates dihydroxyvitamin D_3-induced apoptosis in melanoma cells *in vitro*. Villars-sur-Ollon (Switzerland) 16-18 June 2005 : 4^{th} Workshop on Clustein/APOJ.

3. Poster presentation: **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J. 1,25dihydroxyvitamin D_3 regulates clusterin expression in melanoma cells. Lübeck (Germany) 7-8 May 2005: 2^{nd} Symposium on Vitamin D_3.

4. Poster presentation: **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J. Clusterin is differentially expressed in malignant melanoma as compared to acquired melanocytic nevi and modulates UV-B-induced apoptosis *in vitro*. Glasgow (UK) 7-12 May 2005: 16^{th} IFCC-FESCC European Congress of Clinical Chemistry and Laboratory Medicine.

5. Poster presentation: **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J. Implications for an important function of clusterin for pathogenesis and progression of malignant melanoma. Innsbruck (Austria) 2-5 May 2005: Arbeitsgemeinschaft Dermatologische Forschung.

Publications

1. **Shannan B,** Seifert M, Leskov K, Willis J, Boothman D, Tilgen W, Reichrath J (2006) Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. **Cell Death Differ. 13:12-19**

2. **Shannan B,** Seifert M, Leskov K, Boothman D, Tilgen W, Reichrath J (2006) Clusterin and melanoma growth: clusterin is expressed in malignant melanoma and 1,25-dihydroxyvitamin D_3 modulates clusterin expression in melanoma cell lines *in vitro*. **Anticancer Res. 26:2407-2716**

3. **Shannan B,** Seifert M, Boothman D, Tilgen W, Reichrath J. Clusterin and DNA repair: a new function in cancer for a key player in apoptosis and cell cycle control. **Journal of Molecular Histology. In press.**

4. **Shannan B,** Seifert M, Boothman D, Tilgen W, Reichrath J. Clusterin over expression protects against 1,25-dihydroxyvitamin D_3-induced apoptosis in LNCaP prostate cells *in vitro*. **J Steroid Biochem Mol Biol. Submitted.**