

Clusterin (CLU) and Melanoma Growth: CLU is Expressed in Malignant Melanoma and 1,25-Dihydroxyvitamin D₃ Modulates Expression of CLU in Melanoma Cell Lines *In Vitro*

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Abstract. *Background:* The glycoprotein clusterin (CLU) has two known isoforms generated in human cells. A nuclear form of CLU protein (nCLU) is pro-apoptotic, while a secretory form (sCLU) is pro-survival. CLU expression has been associated with tumorigenesis and the progression of various malignancies. *Materials and Methods:* The expression of CLU was studied immunohistochemically in paraffin sections of primary cutaneous malignant melanomas, metastases of malignant melanomas and acquired melanocytic naevi. Using PCR and Western blotting, the expression of CLU was also investigated in various vitamin D-responsive (MeWo, SK-MEL-28) and vitamin D-resistant melanoma cell lines (SK-MEL-5, SK-MEL-25), as well as in normal human melanocytes (NHM), along with 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] treatment. *Results:* In contrast to acquired melanocytic naevi, CLU immunoreactivity was found in primary cutaneous malignant melanomas and metastases of malignant melanomas in situ. Both CLU protein and RNA were detected in melanoma cell lines and NHM. Treatment with 1,25(OH)₂D₃ modulated CLU's expression in vitamin D-responsive but not in -resistant melanoma cell lines. *Conclusion:* CLU may be of importance for the progression of malignant melanoma. The growth regulatory effects of

1,25(OH)₂D₃ in melanoma cell lines may, at least in part, be mediated via modulation of CLU expression.

Clusterin/apolipoprotein J (CLU) is a glycoprotein that is secreted by several cell types and is present in all human body fluids analyzed to date (1, 2). It is implicated in various cell functions involved in carcinogenesis and tumour progression, including cell adhesion, tissue remodelling, lipid transportation, membrane re-cycling, immune system regulation, DNA repair and apoptosis (1). There are two separate but related CLU protein isoforms, a glycosylated as well as an apparently non-glycosylated form, that are produced in the cell by alternative splicing (3, 4). The secreted form of the CLU protein (sCLU) is a glycosylated protein of 76-80 kDa. Depending on the degree of glycosylation, it usually appears as two separate protein bands on Western blot analysis: one full length uncleaved 60 kDa protein as well as another ~40 kDa α and β protein smear as observed by SDS-PAGE (1-4). The other CLU isoform (~55 kDa) is a nuclear form (nCLU), originating from a 49 kDa primary product that is translated from an alternatively spliced CLU transcript (1-3). CLU expression was shown to be associated with the progression of various malignancies, including tumours of the colon (5), prostate (6-9), bladder (10), breast (3, 11), lung (12) and kidney (13).

Although the role of CLU in apoptosis has been closely investigated, the data are still conflicting. The cause for this discrepancy may be related to the different, sometimes even opposing, functions of CLU in the various cell types and tissues analysed (14). For example, in some studies (4, 6, 8, 10, 15-17) CLU was shown to protect against the effects of apoptosis-inducing agents, while in various other studies (2, 3, 14, 15, 18) it either did not provide any protection against apoptosis-inducing agents or even exerted a pro-apoptotic function (2, 14, 17-19). For example, CLU overexpression was reported to increase the accumulation of cells in the

Abbreviations: CLU, clusterin; nCLU, nuclear clusterin; sCLU, secretory clusterin; 1,25-dihydroxyvitamin D₃, 1,25(OH)₂D₃; SI, staining intensity; PP, percentage of positive cells; IRS, immunoreactivity score; FCS, foetal calf serum; NHM, normal human melanocytes; β 2- μ glob, beta-2-microglobulin.

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G₀/G₁-phase of cell cycle in different cell lines, accompanied by a decrease in DNA synthesis (8).

Others demonstrated that CLU gene overexpression resulted in DNA damage accumulation in LNCaP prostate cells, helped accelerate prostate cancer development (6) and reduced the treatment efficacy of chemotherapeutic agents in human androgen-dependent LNCaP prostate cells (7, 16). Consequently, treatment with antisense oligonucleotides targeting CLU resulted in an increase in chemosensitivity in prostate cancer (19, 20). It was also suggested that CLU may be a potential contributor to sets of biomarkers for the early detection of some other cancers (e.g., colon cancer) (18).

Factors which have been shown to regulate tumour growth may modulate the expression of CLU. 1,25-Dihydroxy-vitamin D₃ [1,25(OH)₂D₃] regulated growth and differentiation in various cell types (21, 22). Additionally, the 1,25(OH)₂D₃-mediated effects on cell death, tumour invasion and angiogenesis make vitamin D analogues promising candidate agents for cancer therapy (23), and some are being used in prostate cancer treatment (24). 1,25(OH)₂D₃ and its analogues inhibit proliferation and induce differentiation and apoptosis in various malignancies, including human malignant melanoma (rev. in 21, 22, 25). However, tumour cell lines that fail to respond to the antiproliferative effects of vitamin D analogues have also been reported. We recently characterized vitamin D-responsive and vitamin D-resistant melanoma cell lines (22). It is well known that 1,25(OH)₂D₃ acts through binding to a corresponding intranuclear receptor (VDR), present in target tissues (26, 27). VDR belongs to the superfamily of trans-acting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors as well as the retinoid-X receptors and retinoic acid receptors (28, 29). 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. It has previously been shown that 1,25(OH)₂D₃ exerts a significant inhibitory effect on the G₁/S checkpoint of the cell cycle and may produce complete arrest of the cell cycle at that point by up-regulating the cyclin dependent kinase inhibitors p27 and p21, and by inhibiting cyclin D1 (23). Indirect mechanisms of 1,25(OH)₂D₃-mediated growth regulation include up-regulation of transforming growth factor-β and down-regulation of the epidermal growth factor receptor (23). 1,25(OH)₂D₃ has the capacity to induce apoptosis, either indirectly through effects on the insulin-like growth factor receptor and tumour necrosis factor-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 (Jun N terminal kinase and p38) (23, 25). Vitamin D-induced apoptosis was previously

Table I. PCR primer sequences and amplification conditions.

Gene	Primers	Primer sequence
CLU	CLUSex6 F (Exon 6)	5'-AAACCTCACgCAAggCgAA-3'
	CLUSex7 A (Exon 7)	5'-CCgCCACggTCTCCATAA-3'
β2-μglob	β2-μglob se	5'-CCAgCAGAgAATggAAAAGTC-3'
	β2-μglob ase	5'-gATgCTgCTTACATgTCTCg-3'

Protocol: 35 (CLU) and 20 (β2-μglob) cycles: 94°C for 1 min, 94°C for 20 sec, 55°C for 30 sec, 72°C for 30 sec, 72°C for 7 min, 4°C cool.

demonstrated in melanoma cell lines (25). The inhibition of tumour invasion and the metastatic potential of 1,25(OH)₂D₃ has also been demonstrated, the mechanisms involved including inhibition of serine proteinases, metalloproteinases and angiogenesis (21, 23).

There are only a few publications concerning the role of CLU or 1,25(OH)₂D₃ in melanoma (22). It was the aim of this study to gain further insights into the importance of CLU for the pathogenesis and progression of malignant melanoma and to investigate whether CLU expression is regulated in melanoma cells by 1,25(OH)₂D₃.

Materials and Methods

Immunohistochemistry. Paraffin sections of primary cutaneous malignant melanomas (n=18), cutaneous metastases of malignant melanomas (n=25) and acquired melanocytic naevi (n=30) were analysed using Clusterin-α/β (H-330) antibody (rabbit polyclonal, working dilution 1:100, Santa Cruz, CA, USA) and a streptavidin-peroxidase technique. Other tissues (basal cell carcinoma n=5, Merkel's cell carcinoma n=5 and cutaneous squamous cell carcinoma n=5) were also tested. The 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, Tauflikirchen, Germany). Incubation with 2% peroxidase in methanol was used to block the endogenous peroxidase activity. The tissues were mounted in permanent Aquatex media (MERCK Biosciences, Schwalbach/Ts., Germany). The immunohistochemical staining was evaluated semi-quantitatively by estimating the staining intensity (SI: 0=no staining, 1=low staining, 2=moderate staining, 3=strong staining) and the 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). The semi-quantitative analysis was performed by two independent observers (B.S. and J. R.).

Cell culture. The human melanoma cell lines SK-MEL-28, SK-MEL-5, SK-MEL-25 (23) and MeWo (24) were cultivated in RPMI medium (10% foetal calf serum [FCS], 37°C, 5% CO₂) using 10-cm plates (Greiner Bio-One GmbH, Frickenhausen, Germany) or 6-well plates (Greiner). The primary normal human melanocytes (NHM) were cultivated in serum-free melanocyte growth medium (PromoCell®,

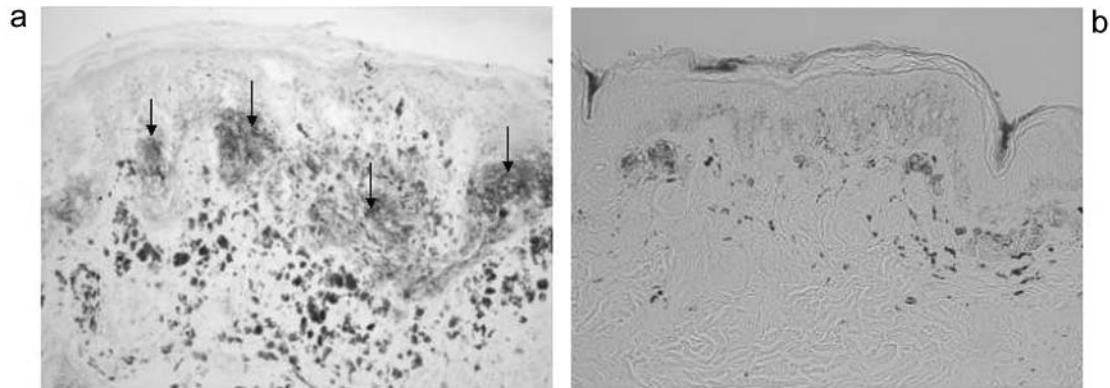


Figure 1. (a) Immunohistochemical detection of CLU in primary cutaneous malignant melanoma. Notice moderate nuclear and cytoplasmic staining in positive tumour cells using antibody H330 directed against clusterin- α/β (arrows). (b) No immunohistochemical detection of CLU in acquired melanocytic naevi. Representative samples are shown.

Heidelberg, Germany), after the addition of the supplement mix (1.0 ng / ml PMA, 0.5 μ g / ml Insulin, 0.5 μ g / ml hydrocortisone, 2 ml BPE and gentamycin/amphotericin B). Around 50% confluent melanoma cell lines were incubated with 1% FCS (charcoal-treated) and treated with 1,25(OH) $_2$ D $_3$ (10^{-7} M) at three time-points (24, 48 and 72 h). 1,25(OH) $_2$ D $_3$ -treated cells were compared with cells in the control group that had been treated with the vehicle (ethanol) alone. The 1,25(OH) $_2$ D $_3$ was kindly provided by Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark). LNCaP prostate cells, stably transfected and overexpressing CLU (16), were used as a positive control in the Western blot experiments.

Proliferation analysis (colony-forming assay). MeWo and SK-MEL-25 cells were seeded in 10-cm plates at 10,000 cells / plate. After 24 h, the cells were treated with 1,25(OH) $_2$ D $_3$ (10^{-6} M, 10^{-7} M, or 10^{-10} M) and cultured in BSA medium for 7 days. Fresh medium and treatment were given daily. After the 7-day treatment, the cells were fixed in ethanol and stained using crystal violet dye. Finally, the number of colonies was counted and the inhibition rate of colony-formation (proliferation) following treatment with 1,25(OH) $_2$ D $_3$ was calculated. The experiments were performed in duplicate and repeated twice.

Western blot analysis. Protein was isolated using a double volume of the pellet in RIPA buffer (extraction buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodiumdeoxycholate, 0.1% SDS and 1% Triton X 100) and complete EDTA-free protease-inhibitor-cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). The proteins were then detected by Western blot analysis using a CLU specific primary mouse monoclonal IgG antibody (Clusterin- β ; B-5, Santa Cruz, working dilution 1:500). The samples included equal volumes of protein (100 μ g) and were separated on an SDS-PAGE gel and transfected to nitrocellulose membranes. The membranes were blocked in TBS (10 mM Tris-HCl / pH 8.0, 150 mM NaCl with 0.05% Tween 20) containing 5% non-fat milk powder for 1 h at room temperature. The primary antibody was then added to the membranes (1 h, room temperature). After a washing step, the secondary antibody labelled with peroxides (goat anti-mouse, Sigma Immunodiagnostics, 1:30,000 dilution) was added. A horseradish peroxidase-based detection reagent, was added for 5 min

(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). Ponceau S, a water soluble dye used for electrophoresis (MERCK Biosciences), was used to stain the membranes. All the blots were repeated in duplicate at least. A human prostate sCLU-overexpressing cell line, which was stably-transfected with CLU (LNCaP), was used as a positive control in the Western blots (16). **RNA extraction.** The cells were harvested and the total RNA was isolated using the RNeasy MiniKit (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. The integrity and amount of extracted total RNA was checked by gel electrophoresis and spectrophotometric analysis.

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

Conventional PCR. Initially, polymerase chain reaction protocols were established with specific oligonucleotide primers for CLU, which is a low-expressed gene (primer synthesis by TIB MOLBIOL, Berlin, Germany), and β 2-microglobulin (β 2- μ glob, housekeeping gene; TIB MOLBIOL) in separate approaches: 2 μ l cDNA were added to a master mix containing 1.5 mM MgCl $_2$, 4 nM dNTP, 10 pmol of each specific oligonucleotide (β 2- μ glob), 10 pmol (CLU), 5 U Taq polymerase (Eppendorf) and 1 x reaction buffer in a total volume of 50 μ l. The primer sequences for β 2- μ glob and CLU are shown in Table I. The semi-quantitative mRNA expression analysis of the different cell lines was based on consecutively performed co-amplifications of CLU with β 2- μ glob. To co-amplificate CLU and β 2- μ glob, 35 and 20 PCR cycles were performed, respectively, under the conditions described in Table I. The PCR products were run on a 2.5% agarose gel containing 1 μ g/ml ethidium bromide and visualized by UV-light at 366 nm. The gel image was documented and processed using a digital documentation system (Gel Print, 2000i, MWG-Biotech, Ebersberg, Germany). Semi-quantitative analysis was performed

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

Clusterin- α/β (H-330)		Malignant melanoma (n=18)	Metastases of malignant melanoma (n=25)	Acquired melanocytic naevi (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 was detected in a proportion of malignant melanomas and metastases of malignant melanoma, but not in acquired melanocytic naevi (n= number of samples examined).

using optical densitometry and 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 duplicate and repeated at least twice.

Results

CLU was detected immunohistochemically in a minority of cases of primary cutaneous malignant melanomas and metastases of malignant melanoma, but not in acquired melanocytic naevi. Positive cases of primary malignant melanomas (n=5/18) revealed a low or moderate CLU immunoreactivity for the H-330 antibody used (Figure 1a). The staining was homogeneous (in the nuclei and cytoplasm of positive cells). There were also no apparent differences on 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=5/25) revealed homogeneously low nuclear and cytoplasmic immunoreactivity for CLU. All cases of acquired melanocytic naevi analysed (n=30) were negative for CLU (Figure 1b). Table II provides a semi-quantitative analysis of CLU immunoreactivity in melanocytic skin tumours (values for SI, PP and resulting IRS). Other tissues tested (basal cell carcinoma, Merkel's cell carcinoma, squamous cell carcinoma) showed negative staining for CLU (data not shown). Table III provides the histological data of the tissues analysed.

Treatment with $1,25(\text{OH})_2\text{D}_3$ inhibited proliferation in vitamin D-responsive (MeWo) but not in vitamin D-resistant melanoma cells (SK-Mel-25). The antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$

were investigated in MeWo (vitamin D-responsive) and SK-MEL-25 (vitamin D-resistant) cells using a colony-forming assay (Figure 2). At 10^{-6} and 10^{-7} M, $1,25(\text{OH})_2\text{D}_3$ inhibited the proliferation of MeWo but not of SK-MEL-25 cells (Figure 2). The antiproliferative effect of $1,25(\text{OH})_2\text{D}_3$ was dose-dependent and was most pronounced at 10^{-6} M. Interestingly, after $1,25(\text{OH})_2\text{D}_3$ treatment at a concentration of 10^{-10} M, the proliferation of MeWo cells was increased.

CLU was expressed in melanoma cell lines and primary cultured normal melanocytes. Using conventional PCR, CLU mRNA was detected in all the melanoma cell lines analysed (SK-MEL-28, SK-MEL-25, SK-MEL-5, MeWo) and NHM (Figure 3). Both the CLU and β_2 - μ glob bands were of the expected size, corresponding to 200 bp and 250 bp, respectively. Western blot analysis, using a specific CLU antibody, revealed bands at ~80 kDa and ~40 kDa in all the melanoma cell lines and NHM analysed, representing sCLU where the cleaved α - and β - protein smears were apparent, respectively (Figure 4a, b).

$1,25(\text{OH})_2\text{D}_3$ increased CLU expression in a time-dependent manner in vitamin D-responsive but not in vitamin D-resistant melanoma cell lines. Using conventional PCR, the time-dependent modulation of CLU mRNA expression was analysed in the melanoma cell lines upon treatment with $1,25(\text{OH})_2\text{D}_3$ (10^{-7} M). The CLU mRNA level was increased 2.5-fold (as assessed by optical density measurement) 48 h after treatment with $1,25(\text{OH})_2\text{D}_3$ in SK-MEL-28 (Figure 5a) and 3-fold in MeWo (Figure 5b). We have previously shown that both cell lines were vitamin D-

Table III. Histopathological data of malignant melanomas, metastases of malignant melanomas and acquired melanocytic naevi.

Case number	Patient initials	Age	Gender	Tumour thickness	Type	Localisation
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
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
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 naevi (n=30)						
45	FB	39	M	-	Intradermal melanocytic naevus	Left breast
46	RR	43	M	-	Compound melanocytic naevus	Shoulder blade
47	PS	18	M	-	Intradermal melanocytic naevus	Left arm
48	KB	46	M	-	Compound melanocytic naevus	Stomach
49	FB	40	M	-	Junctional melanocytic naevus	Left breast
50	FP	53	M	-	Compound melanocytic naevus	Left shoulder
51	MS	36	M	-	Intradermal melanocytic naevus	Left neck bone
52	MW	38	M	-	Intradermal melanocytic naevus	Right rib cage

continued

Table III. *continued*

Case number	Patient initials	Age	Gender	Tumour thickness	Type	Localisation
53	PD	32	M	-	Intradermal melanocytic naevus	Penis
54	KL	23	F	-	Intradermal melanocytic naevus	Neck
55	VM	30	F	-	Dermal melanocytic naevus	Left arm
56	SW	40	F	-	Compound melanocytic naevus	Third right finger
57	MP	19	F	-	Intradermal melanocytic naevus	Neck
58	HB	61	M	-	Compound melanocytic naevus	Abdomen
59	MA	64	M	-	Junctional melanocytic naevus	Back
60	JM	39	M	-	Junctional melanocytic naevus	Back
61	LA	45	M	-	Compound melanocytic naevus	Back
62	IS	32	F	-	Compound melanocytic naevus	Right foot
63	NW	26	F	-	Intradermal melanocytic naevus	Right ear
64	DP	31	F	-	Junctional melanocytic naevus	Abdomen
65	JD	23	F	-	Compound melanocytic naevus	Abdomen
66	KZ	35	F	-	Compound melanocytic naevus	Right breast
67	LB	28	F	-	Compound melanocytic naevus	Thorax
68	HP	31	F	-	Compound melanocytic naevus	Abdomen
69	AW	26	F	-	Compound melanocytic naevus	Back
70	UF	39	M	-	Junctional melanocytic naevus	Lower left thigh
71	VF	45	M	-	Compound melanocytic naevus	Face
72	KB	46	M	-	Compound melanocytic naevus	Right breast
73	MD	51	M	-	Compound melanocytic naevus	Right arm
74	MB	85	F	-	Intradermal melanocytic naevus	Right eyebrow

*SSM: superficial spreading melanoma.

responsive (22). The melanoma cell lines that we had previously characterized as vitamin D-resistant cell lines showed no up-regulation of CLU expression following treatment with $1,25(\text{OH})_2\text{D}_3$ (SK-MEL-5 and SK-MEL-25, data not shown).

Discussion

An increasing body of evidence points to the contribution of CLU to the progression of several cancers including kidney carcinoma (30, 31), anaplastic large cell lymphoma (32), ovarian cancer (33), breast tumours (3, 11), prostate cancer (6-9) and colon tumours (5). However, the exact roles of CLU in the pathogenesis and progression of cancer are still not well defined, as is the case for malignant melanoma.

In this paper, it was demonstrated that CLU was present in melanoma tissues and melanoma cell lines. The presence of CLU in a proportion of malignant melanocytic tumours, but not in benign melanocytic naevi, indicated that CLU may be of importance for the pathogenesis and progression of malignant melanoma (Figure 1). While no cases of acquired melanocytic naevi and only a minority of malignant melanomas and melanoma metastases were immunoreactive for the CLU protein, the presence of CLU (mRNA and protein) was demonstrated in all the melanoma cell lines and NHM tested (Figures 3, 4). This discrepancy may be

due to the differing sensitivities of the techniques used for the detection of CLU *in situ* (immunohistochemistry) as compared to *in vitro* (Western blotting and PCR). Another reason may be the difference in the cell proliferation rate in the tissues as compared to the cell cultures analysed. Analysing both NHM and melanoma cell lines, the Western blot results showed two bands at ~80 and 40 kDa, indicating that sCLU represents the abundant isoform in cultured normal and malignant melanocytes (Figure 4).

Additionally, the effect of $1,25(\text{OH})_2\text{D}_3$ treatment on CLU expression in melanoma cell lines was analysed. $1,25(\text{OH})_2\text{D}_3$ has been shown to modulate growth and apoptosis in melanoma cells (34, 35). Using different methodology (colony-forming assay), here we confirmed our previous studies, in which we had characterised various melanoma cell lines to be vitamin D-responsive or vitamin D-resistant (22). We showed that at higher $1,25(\text{OH})_2\text{D}_3$ concentrations (10^{-6} M or 10^{-7} M), the proliferation of vitamin D-responsive cells (e.g., MeWo) was inhibited. These effects were not observed in vitamin D-resistant melanoma cells (e.g., SK-MEL-25). Interestingly, $1,25(\text{OH})_2\text{D}_3$ -treated vitamin D-responsive cells (SK-MEL-28 and MeWo), that were shown to reveal no functional defect in vitamin D receptor-mediated gene transcription (22), showed an up-regulation (2.5-fold increase in SK-MEL-28 and 3-fold increase in MeWo, as measured by optical density) of the CLU mRNA level (Figure 5a,b). In contrast, vitamin D-resistant cells (SK-MEL-5 and SK-MEL-

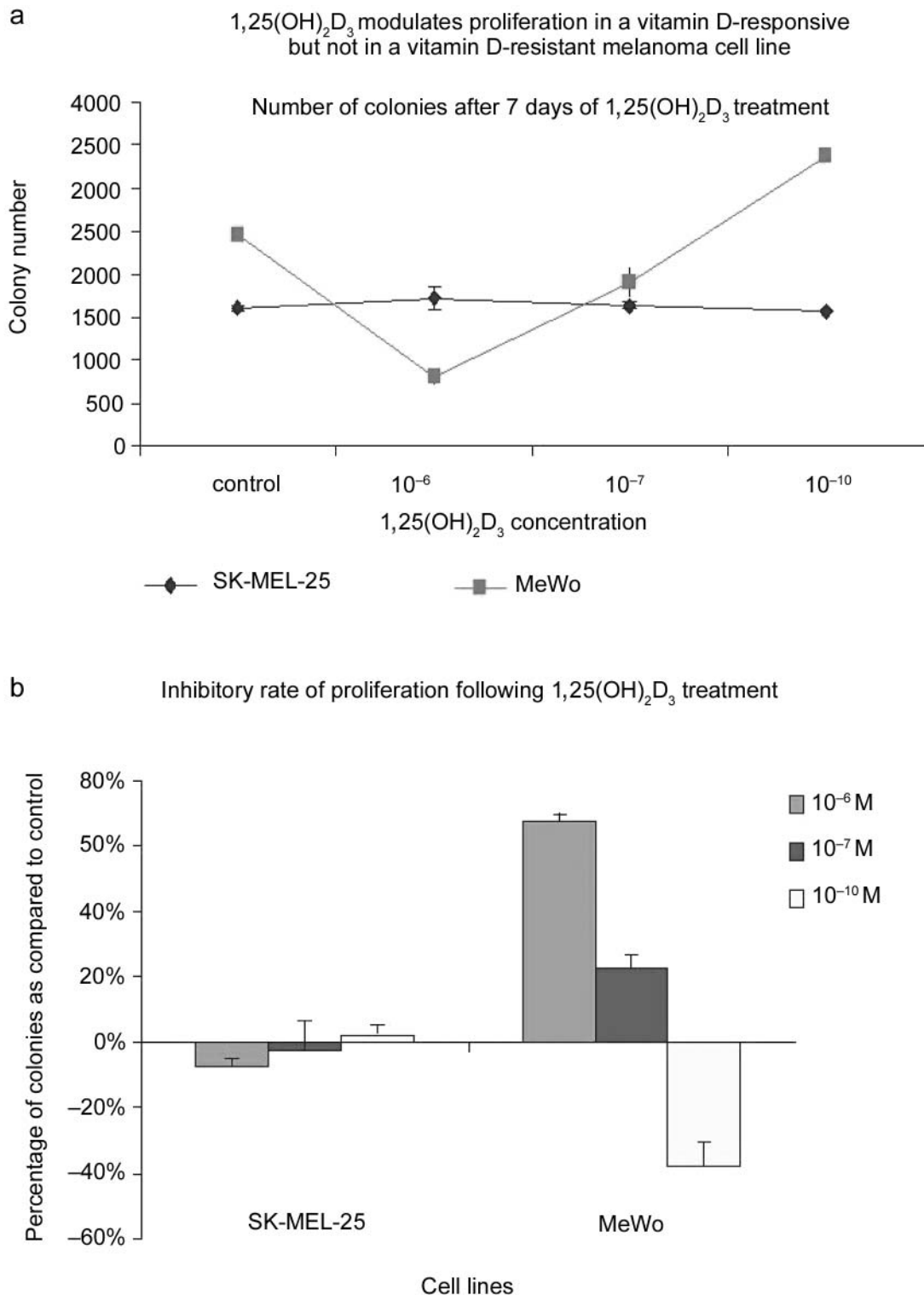


Figure 2. Analysis of cell proliferation (colony-forming assay as described in Materials and Methods) in a vitamin D-responsive (MeWo) and in a vitamin D-resistant (SK-MEL-25) melanoma cell line following treatment with 1,25(OH)₂D₃. (a) Number of colonies after treatment of MeWo and SK-MEL-25 melanoma cells with different concentrations of 1,25(OH)₂D₃ for 7 days. (b) The inhibitory rate of proliferation in 1,25(OH)₂D₃-treated cells as compared to the control (cells treated with vehicle alone). Note that MeWo cells showed a dose-dependent inhibition (10⁻⁶ M and 10⁻⁷ M) or an increase (10⁻¹⁰ M) of proliferation as compared to the controls treated with vehicle (ethanol) alone. These effects were not observed in SK-MEL-25 cells. Error bars represent the mean \pm SD.

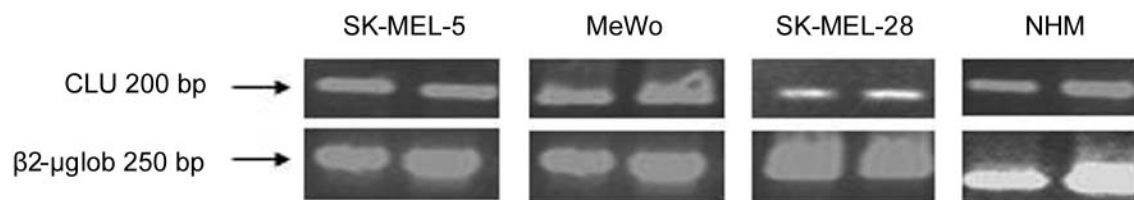


Figure 3. Expression of *CLU* mRNA in melanoma cell lines: PCR gel image showing *CLU* expression in SK-MEL-5, MeWo, SK-MEL-28 and NHM using *CLU* and β 2- μ glob primers, as described in Materials and Methods. The PCR product sizes were 200 bp and 250 bp, respectively. Each sample was tested in duplicate.

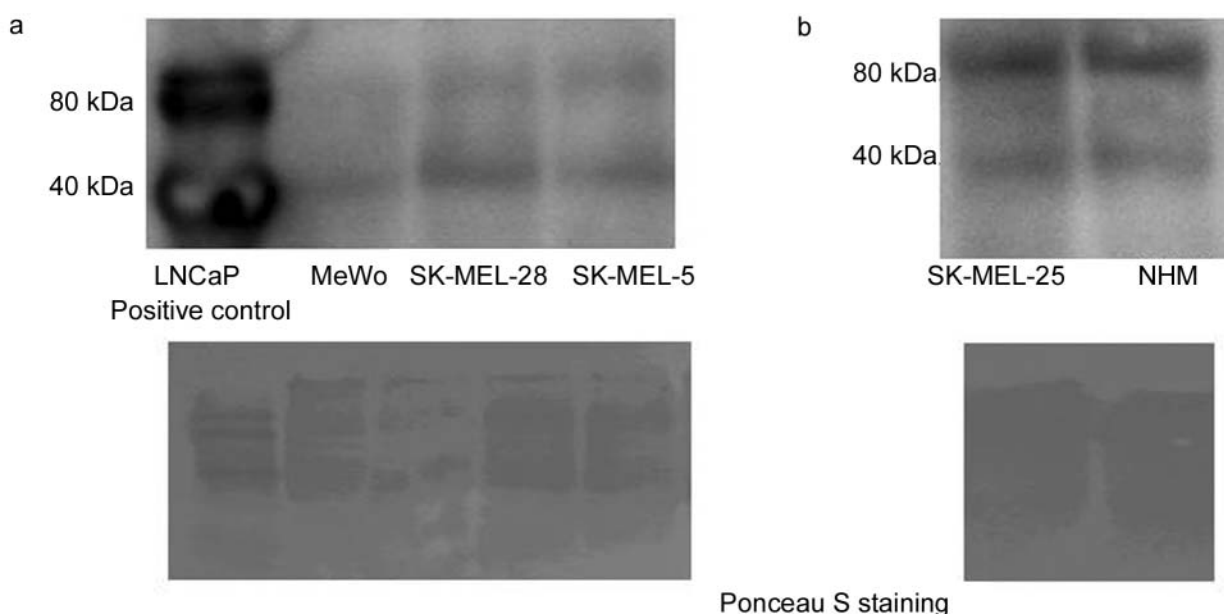


Figure 4. Expression of *CLU* protein in melanoma cells. Western analysis showed a double band (40 and 80 kDa) indicating the presence of sCLU (the cleaved α - and β -subunits) in LNCaP (positive control), MeWo, SK-MEL-28 and SK-MEL-5 (a), and in SK-MEL-25 and NHM cells (b). Ponceau S staining revealed the amount of protein used in each well (100 μ g; except LNCaP 20 μ g).

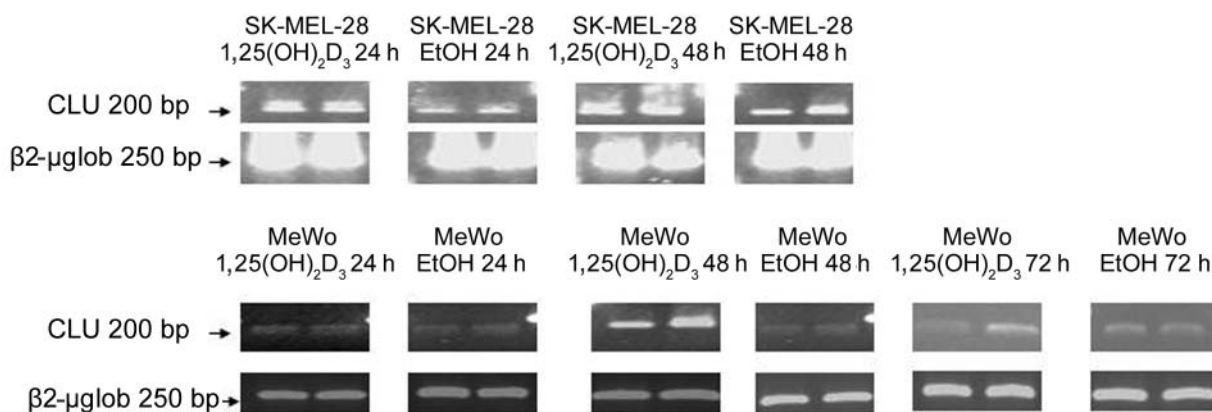


Figure 5. Up-regulation of *CLU* mRNA in vitamin D-responsive melanoma cell lines: (a) PCR gel picture showing SK-MEL-28 upon treatment with $1,25(\text{OH})_2\text{D}_3$ (10^{-7} M) as compared to an ethanol control after 24 and 48 h. Notice the increase in *CLU* expression upon $1,25(\text{OH})_2\text{D}_3$ treatment after 48 h (2.5-fold). (b) PCR gel picture showing MeWo upon treatment with $1,25(\text{OH})_2\text{D}_3$ as compared to an ethanol control after 24, 48 and 72 h. Notice the increase in *CLU* expression upon $1,25(\text{OH})_2\text{D}_3$ treatment after 48 h (3-fold) (compared to ethanol). Each sample was tested in duplicate.

25) showed no such up-regulation after 1,25(OH)₂D₃ treatment. It was concluded that 1,25(OH)₂D₃ treatment increased the CLU mRNA and protein levels in a time-dependent manner in vitamin D-responsive melanoma cell lines. It was speculated that this increase in CLU expression may be of importance for 1,25(OH)₂D₃-mediated biological responses in melanoma cells, including antiproliferative effects and apoptosis. These findings indicated that the up-regulation of CLU might be related to 1,25(OH)₂D₃-mediated biological responses in melanoma cells (25). Additionally, 1,25(OH)₂D₃ was shown to inhibit malignant cell invasion and tumour-induced neo-angiogenesis by inducing apoptosis (e.g., in breast, prostate and colon cancers) (23). It remains to be elucidated whether the modulation of CLU expression may be involved in the antitumour effects of 1,25(OH)₂D₃ as well.

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