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Dual Role of Pituitary adenylate cyclase activating polypeptide (PACAP) in Melanoma: Autocrine and Paracrine Mechanisms on Tumor and Immune cells

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Contents

Abbreviation

aa	amino acid
ABCDE	asymmetry, border irregularity, color variegation, diameter enlargement
AC	adenlyate cyclase
AICD	activation-induced cell death
AJCC	American Joint Committee on Cancer
AKT	v-akt murine thymoma viral oncogene homolog
ALM	acral lentiginous melanoma
APCs	antigen presenting cells
APC	allophycocyanin
ARF	alternate reading frame
BCL-2	B-cell lymphoma 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin fraction
c-Kit	mast/stem cell growth factor receptor/ tyrosine-protein kinase Kit
cAMP	cyclic adenosine monophosphate
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CDK2	cyclin-dependent kinase 2
cDNA	complementary DNA
CRAF	proto-oncogene serine/threonine-protein kinase
CTLA-4	cytotoxic T lymphocytes-associated antigen 4
DAG	diacylglycerol
DC	dendritic cells
DMEN	dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FasL	fas ligand
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fig	figure
FITC	fluorescein isothiocyanate
FoxP3	transcription factor forkhead box P3
FSC	forward scatter
GHRH	growth-hormone-releasing hormone
GPCR	G protein coupled receptor
HGF	hepatocyte growth factor
HRP	horse radish peroxidise

IDO	indoleamine 2,3-dioxygenase
IGF	insulin-like growth factor
IL	interleukin
IP3	inositol 1,4,5-triphosphate
kb	kilobases
LMM	lentigo maligna melanoma
LPS	lipopolysaccharide
MC1R	melanocortin receptor 1
MEK	mitogen-activated protein kinase kinase
MHC	major histocompatibility complex
MIP1/2	MEK1 interacting protein 1
MITF	microphtalamia-associated transcription factor
α-MSH	alpha melanocytes stimulating hormone
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NA	not applicable
NM	nodular melanoma
NRAS	neuroblastoma RAS Viral (V-Ras)
P	phosphate
PAC1	pituitary adenylate cyclase-activating polypeptide type I receptor
PACAP	pituitary adenylate cyclase activating polypeptide
PACE4	paired basic amino acid-cleaving enzyme 4
PAM	peptidyl glycine α-amidating monooxygenase
PBS	phosphate buffered saline
PC	prohormone convertases
PC12	pheochromocytoma
PCR	polymerase chain reaction
PD-L1	programmed death ligand 1
PDK1	phosphoinositide-dependent kinase 1
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PI3K	phosphatidylinositol-3 kinases
PIP2	phosphatidylinositol-4,5-biphospate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PRP	PACAP-related peptide
PTEN	phosphatase and tensin homolog
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
Rb	retinoblastoma
RGP	radial growth phase
RIPA	radio Immuno precipitation assay buffer

RNA	ribonucleic acid
rpm	rotations per minute
RPMI	roswell park memorial institute
RPTKs	receptor protein tyrosine kinases
RT-PCR	reverse transcription PCR
SCF	stem cells factor
SDS	sodium anionic denaturing detergent sodium dodecyl sulphate
SSC	side scatter
SSM	superficial spreading melanoma
Teff	effector T cells
TGF-β	transforming growth factor β
Th1/Th2	helper T cell 1/2
Tis	tumor in situ
TM-region	transmembrane domains
TNF-α	tumor necrosis factor-alpha
TNM	primary tumor/ regional lymph node/distant metastasis
Treg	regulatory T cell
Tresp	responder T cell
UVR	ultraviolet radiation
VEGF	vascular endothelial growth factor
VGP	vertical growth phase
VIP	vasoactive intestinal polypeptide
VPAC1/2	vasoactive intestinal peptide (VIP) receptor type 1/2
WNT	wingless-type MMTV integration site family member

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Abstract

Melanoma arises from the malignant transformation of melanocytes and is responsible for the highest death rates among skin cancers. Advanced melanoma has been regarded as a tumor exhibiting an extreme resistance to therapies, therefore many investigators struggle to develop and to provide appropriate drugs in the area of targeted therapy and immunotherapy. Among the agents for targeted therapy cell signaling inhibitors for MAP kinase pathway like BRAF or MEK inhibitors or tyrosine kinase inhibitors like c-kit inhibitors as well as anti-sense oligonucleotides against BCL-2 to enable apoptosis have been demonstrated to delay tumor progression. Another prominent approach is specific targeting of the immune system, which devotes effective destruction of melanoma cells. In the recent clinical trials, for example, anti-CTLA-4 or anti-PD1 monoclonal antibodies account for prolonged overall survival of melanoma patients. It can be a tempting task to find a novel potent agent that both decreases tumor growth and simultaneously activates antitumor immune cells surrounding the tumor.

Since the discovery of pituitary adenylate cyclase activating polypeptide (PACAP), an immunomodulatory acting neuropeptide, many investigators have demonstrated PACAP expression in various tumor types other than melanoma, and its involvement in functional roles on cell survival and differentiation has been well documented. The effectiveness of PACAP was not restricted to tumor cell growth only but has been established as a regulator for the immune system as well, modulating T cell differentiation, cytokine production, and migration of immune cells. Hence, these previous reports let us presume that PACAP production may promote suppressive immunity and therefore become one of the most potent approaches for targeted therapy in melanoma.

This thesis is based on investigation of PACAP and PAC1R expression and functional roles of PACAP in human melanoma as well as immune cells.

In the first part, the study demonstrates for the first time the expression of PACAP in primary and metastatic melanoma tissues. PACAP is distributed in two distinct subtypes, PACAP 27 and PACAP 38. PACAP 38 expression seems to be more prevalent in primary melanomas but metastatic melanomas profoundly produce PACAP 27. Another very interesting result is the predominant expression of high affinity PACAP receptor, PAC1R, in metastatic melanoma but not in primary melanoma. Co-expression of PACAP 27 and PAC1R can be also detected in metastatic melanoma cell lines. This finding let us consider that PACAP may regulate melanoma cells in an autocrine manner.

PACAP function has been investigated on cell survival, cell growth, and cytokine production. Two melanoma lines were selected, SK-Mel 37 and NW-Mel 450, which express similar tumor-antigens, such as NY-ESO/LAGE-1, and MAGE 3, and have different morphological features and size. PACAP supplement achieved a positive regulation on survival or cell growth in SK-Mel 37, which is clearly susceptible to serum-starvation for reduced survival, in contrast to NW-Mel 450. Gene or protein expressions related to cell survival (BCL-2) and proliferation (Ki67) were induced by PACAP. Moreover, PACAP positively modulated protein expression of cell differentiation (i.e. MITF) and of chemokine receptor (i.e. CCR7) in melanomas. These factors are considered as important checkpoints for

malignancy of melanoma.
In addition, the spontaneous cytokine production of both melanoma lines is different. SK-Mel 37 releases IL-10, an immunosuppressive cytokine, while NW-Mel 450 produces IL-6, a cytokine associated with tumor growth and angiogenesis. Our results show that PACAP up-regulates IL-6 release in NW-Mel 450 but may not change IL-10 production in SK-Mel 37. These observations clarify, that PACAP induced pro-tumor features by different mechanisms.

In the second part of the thesis PACAP-treated immune cells were investigated showing noticeable differences from untreated cells in the aspect of suppressive regulation. Two separated T cell subsets, $CD4^+CD25^+CD127^{low/-}$ T cells (regulatory T cells, Treg) and $CD4^+CD25^-$ T cells (effector or responder T cells, Teff or Tresp) were studied. Both T cell subsets following PACAP supplement showed not only elevated gene transcripts of hallmarks for Treg, FoxP3 and CTLA-4, and of anti-inflammatory cytokines, IL-10 and TGF-β, but also significantly increased chemokine receptors, CCR4 and CCR7, being responsible for migration into dedicated tissue sites. Interestingly, a stronger effectiveness of PACAP on RNA level is observable in Treg cells compared to Teff/Tresp cells. This activity of PACAP may be predominantly mediated by the type II receptor, VPAC1R, which is present on $CD4^+$ T cells. Our study, however, shows for the first time the expression of type I receptor, PAC1R, on T cells following activation with α-CD3/CD28 and particularly PACAP supplement. This finding let us consider, that perpetually provided PACAP by tumor cells may induce PAC1R expression on T cells. In addition in Treg functional assay a stronger suppression of cell growth of Teff/Tresp cells was observed, when co-culturing in the presence of PACAP.

Taken together, for the first time our results show the expression of PACAP and high affinity PACAP receptor, PAC1R, in melanoma and the ability of PACAP to influence not only the cellular activation in cell growth and cytokine production of melanoma but also to enhance the characteristic features of Treg. Therefore, reducing the PACAP effect in melanoma may become valuable to influence the course of the disease. Further investigation of the cellular mechanism triggered by PACAP in melanoma and the PACAP-mediated suppressive regulation of Treg through PAC1R will be required.

Zusammenfassung (German)

Ein Melanom entsteht durch maligne Umwandlung der Melanozyten und ist für die höchste Mortalität unter den Formen von Hautkrebs verantwortlich. Fortgeschrittene Melanome sind extrem widerstandsfähig gegen Therapien, weshalb sich viele Forscher bemühen, geeignete Therapeutika auf dem Gebiet der zielgerichten Therapie sowie der Immuntherapie zu entwickeln und anzubieten. Unter den Medikamenten für die zielgerichtete Therapie haben sich Signalkaskadeinhibitoren wie MEK- oder BRAF-Inhibitoren oder Tyrosinkinaseinhibitoren wie c-kit-Inhibitoren sowie Antisense-Oligonukleotiden gegen Bcl-2 als wirksam gegen Tumorprogression erwiesen. Eine weitere hervorragende Therapieoption ist die gezielte Modulation des Immunsystems, die eine effektive Zerstörung von Melanomzellen hervorruft. Nach neueren klinischen Studien erzielte die Behandlung mit anti-CTLA-4 Antikörper oder die Blockierung von PD-1 mit monoklonalem Antikörper ein verlängertes Gesamtüberleben von Melanompatienten. Es kann eine herausfordernde Aufgabe sein, einen neuen potenten Wirkstoff zu finden, der Tumorwachstum verlangsamt und gleichzeitig Immunzellen gegen diesen Tumor aktiviert.

Seit der Entdeckung von pituitary adenylate cyclase activating polypeptide (PACAP), einem immunmodulatorisch wirksamen Neuropeptid, haben viele Forscher die PACAP-Expression in verschiedenen Tumorarten außer dem Melanom gezeigt und dessen funktionale Rolle auf das Überleben der Zellen und deren Differenzierung untersucht. Die Wirkung von PACAP ist nicht nur auf das Zellwachstum von Tumoren beschränkt, sondern hat sich auch als ein Regulator für das Immunsystem herausgestellt, der sowohl die T-Zell-Differenzierung, die Zytokin-Produktion als auch die Migration von Immunzellen steuern kann. Diese Erkenntnisse lassen uns daher vermuten, dass die Produktion von PACAP mit einer stärker supprimierten Immunität einhergeht und somit eines der wirksamsten Ansätze für eine zielgerichtete Therapie beim Melanom sein könnte.

Die vorliegende Arbeit basiert auf Untersuchungen von PACAP und PAC1R Expression und von den funktionalen Rollen von PACAP beim Melanom und Immunzellen.

Im ersten Teil zeigt unsere Untersuchung die Expression von PACAP in primären und metastatischen Melanomgeweben. PACAP tritt in zwei Subtypen auf, PACAP 27 und PACAP 38. PACAP 38 scheint ausgeprägter in primären Melanomen aufzutreten, während in metastatischen Melanomen eine verstärkte Expression von PACAP 27 vorliegt. Ein weiteres interessantes Ergebnis ist die vorwiegende Expression von hoch-affinem PACAP-Rezeptor, PAC1R, in metastasierenden Melanomen, nicht aber in primären Melanomen. Die Co-Expression von PACAP 27 und PAC1R kann auch in den Zellinien von metastasierenden Melanomen nachgewiesen werden. Anhand dieser Resultate nehmen wir an, dass PACAP das Melanom in autokriner Weise regulieren kann.

Die Wirkung von PACAP wurde bezüglich Zellwachstum und Cytokin-Produktion in Tumorzellinien untersucht. Zwei Melanom-Zellinien, SK-Mel 37 und NW-Mel 450 wurden ausgewählt, welche ähnliche Tumorantigene, wie NY-ESO/LAGE-1 und MAGE- 3 exprimieren, aber unterschiedliche Morphologie in Struktur und Größe aufweisen. Bei SK-Mel 37, deren Überleben deutlich von der Serumkonzentration abhängig ist, bewirkt die Zugabe von PACAP im Gegensatz zu NW-Mel 450, eine positive Regulation des Zellüberlebens oder -wachstums. Die Gen- oder Proteinexpression für Zellüberleben (BCL-2) sowie Zellproliferation (Ki67) wird durch PACAP induziert. Zusätzlich moduliert PACAP auch die

Proteinexpression betreffend Zelldifferenzierung (z.B. MITF) und Chemokinrezeptor (z.B. CCR7) in Melanomen positiv. Die oben genannten Faktoren werden als eine wichtige Schaltstelle für die Malignität des Melanoms betrachtet.
Auch die Zytokinproduction in beiden Melanom-Zellinien unterscheidet sich. SK-Mel 37 setzt IL-10 frei, während NW-Mel 450 IL-6 produziert. PACAP-Zugabe fördert die IL-6 Freisetzung aus NW-Mel 450, nicht aber die IL-10 Produktion aus SK-Mel 37. Diese Beobachtungen erläutern, dass trotz unterschiedlicher Regulation von PACAP auf Melanom-Zellinien, PACAP zur Förderung von Tumor-begünstigenden Funktionen führt.
Im zweiten Teil der Arbeit zeigten PACAP-behandelte Immunzellen im Unterschied zu unbehandelten Zellen einen deutlichen Unterschied in Hinblick auf die immunsuppressive Regulation. Zwei isolierte T-Zell-Untergruppen, $CD4^+CD25^+CD127^{low/-}$ T-Zellen (regulatorische T-Zellen, Treg) und $CD4^+CD25^-$ T-Zellen (Effektor oder Responder T-Zellen, Teff oder Tresp) wurden untersucht. Beide PACAP-behandelten T-Zell-Untergruppen erreichten nicht nur erhöhte Transkription spezieller Gene für die regulatorische Funktion, FoxP3 und CTLA-4, und für anti-inflammatorischen Zytokine, IL-10 und TGF-β, sondern auch eine erhöhte Genexpression von Chemokinrezeptoren, CCR4 und CCR7, die für die Wanderung der T-Zellen in bestimmte Gewebe verantwortlich sind. Interessanterweise kann eine stärkere Wirksamkeit von PACAP jedoch auf Treg im Vergleich mit den Teff/Tresp auf RNA-Ebene beobachtet werden. Diese Aktivität von PACAP wird überwiegend durch den Typ-II Rezeptor, VPAC1R, vermittelt, da auf $CD4^+$ T-Zellen VPAC1R vorhanden sind. Weiterhin zeigt unser Experiment eine Expression von Typ-I Rezeptor, PAC1R, auf $CD4^+$ T-Zellen nach der Aktivierung mit α-CD3/CD28 und PACAP-Zugabe. Dieses Resultat lässt uns vermuten, dass kontinuierlich freigesetztes PACAP aus Tumorzellen die PAC1R-Expression auf T-Zellen induziert. Entsprechend wurde in T-Zell-Suppressionstests ein stärker unterdrücktes Zellwachstum von Teff/Tresp in Co-Kultur mit Treg unter Zusatz von PACAP beoachtet.

Zusammengefasst zeigen unsere Daten erstmals die Expression von PACAP und von hochaffinem PACAP Rezeptor, PAC1R, beim Melanom und die Fähigkeit von PACAP zur zellulären Aktivierung nicht nur des Zellwachstums und der Zytokinproduktion von Melanomzellen, sondern auch zur Verstärkung der charakteristischen Merkmale von Treg-Zellen. Daher könnte die Unterdrückung der PACAP-Wirkung beim Melanom eine wertvolle Strategie zur Beeinflussung des Krankheitsverlaufes werden. Somit ist es erforderlich, die PACAP-induzierten zellulären Mechanismen beim Melanom sowie die PACAP-vermittelte Regulation von Treg durch PAC1R genauer zu untersuchen.
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1. Introduction

1.1. Melanoma

Melanoma is an aggressive skin tumor arising from melanocyte, which resides predominantly at the basal layer of the epidermis in the skin and account for approximately 2 % of epidermal cells (Hoath and Leahy, 2003). Originally, melanocytes are derived from the neural crest, which migrate into the epidermis of skin and developed into melanoblastic precursors (John and Meenhard, 2006). These melanocytes function mainly to protect the neighboring keratinocytes from ultraviolet radiation (UV)-induced DNA damage by production of pigment, so called melanin. A lysosome-like structure, melanosomes in melanocytes produce pigment in response to UV. Mature melanosomes form cap-like structure and localize over nuclei of keratinocytes to protect them from UV-irradiation (Lin and Fisher, 2007). Incidence of malignant melanoma account for 3% of the world's total cancer diagnoses and 1.3% estimated cancer death in the developed and developing regions in 2012 (Ferlay and Bray, 2013). Difficulties of malignant melanoma are the accuracy of diagnosis and the lack of effective treatment.

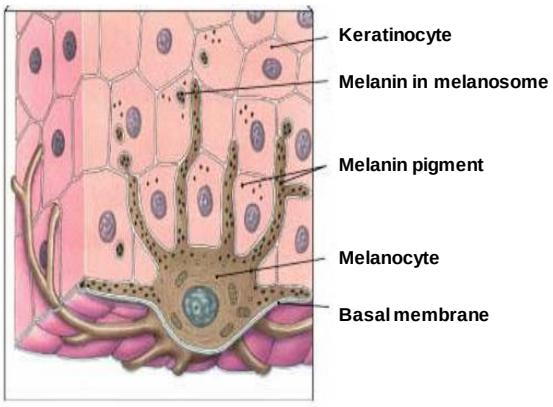


Figure 1. Structure and function of melanoncyte. Melanocytes localize on the basal layer of epidermis and produce pigment, melanin within melanosomes, in response to ultraviolet radiation (UV). These transferred pigments protect the keratinocytes from the UV-induced DNA damage. (Picture adapted from Droual's anatomy course)

1.1.1. Clinical and histopathological features

On the basis of clinical features, melanoma is subdivided into four types: superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM). The first three types of melanoma, SSM, NW and LMM, were classified in 1967 (Clark) and as a new subtype ALM was included in 1979 (Clark, 1969; Arrington, 1977; Coleman, 1980). The majority of malignant melanoma, SSM and NM, account for more than 80 % of all cases cutaneous melanoma. SSM usually arises in sun-exposed skin, such as trunk, back and extremities, and presents as a slowly growing asymmetrical flat lesion with varied pigmentation. Histological feature of SSM was described as large pleomorhphic epithelioid melanocyte. The second common type of melanoma, NM, can occur at any part of skin and presents as a rapid spreading popular form resulting in an intraepidermal growth. LMM occurs mostly on chronic sun exposed skin of face, neck or forearm and its incidence increases with age. The clinical feature of LMM is a flat and large pigmented shape with irregular edges, which is developed from lentigo maligna, non-invasive skin growth.

Histologically, proliferation of dysplastic melanocytes is found at the dermoepidermal junction with extension to adnexal structures, such as hair follicle, sweat glands and sebaceous glands, showing epidermal atrophy. ALM occurs mostly in people with dark skin but is rare in Caucasians and people with lighter skin. This subtype is observed on the palms, soles, digits and subungual sites (under the nails). ALM is characterized histologically by scattered atypical melanocytes near the basal epidermal layer and by associated lymphocytic infiltration (world health organization, 2006).

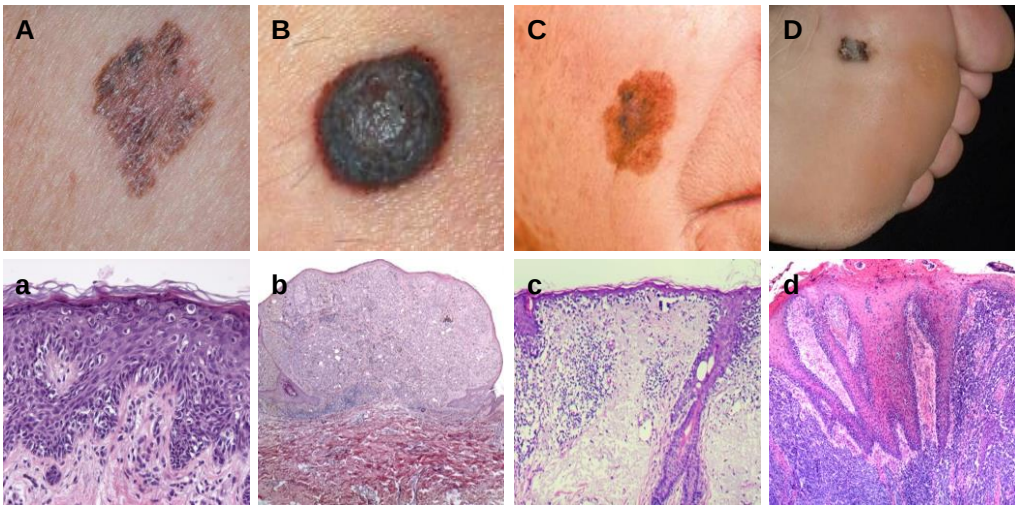


Figure 2. Clinical and histological features of melanoma. (A and a) superficial spreading melanoma (SSM), (B and b) nodular melanoma (NM), (C and c) lentigo maligna melanoma (LMM) and (D and d) acral lentiginous melanoma (ALM). Capital letters and small letters denote clinical and histological presentations, respectively. Pictures are adapted from A: DermIS.net; B: Skintumor.info; C: PCDS.org; BPACnz. (2011). Detecting malignant melanoma; a, b, c and d: MMMP (melanoma molecular map project).

Other melanoma subtypes, such as amelanotic, desmoplastic melanoma or melanoma of mucous membrane, are added to a list following distinguished localization and pathological features and they occur in approximately 5 % of melanoma (Dummer, 2006).

To identify the malignant melanoma, ABCDE score was established: Asymmetry, Border irregularity, Color variegation, Diameter more than 0.5 cm and Enlargement in a short period of time (Abbasi 2004). The effectiveness of ABCDE criteria, however, is limited due to a variety of lesion shapes, irregular boundaries, specula reflection and artifacts, and clinical accuracy of diagnosis rarely exceeds 60% (Houghton, 2002; Abbas, 2013)

1.1.2. Pathological features and staging

Two stages of growth were described in cutaneous melanoma, namely radial and vertical growth. The radial growth phase (RGP) of melanoma is characterized by an irregular plaque with intraepidermal expansion. Cells do not invade the dermis from an expanded nodule. In the next phase, the vertical growth phase (VGP), the lesion invades vertically through the basal layer into dermis, forming a nodule (Clark, 1969; Elder, 1984).

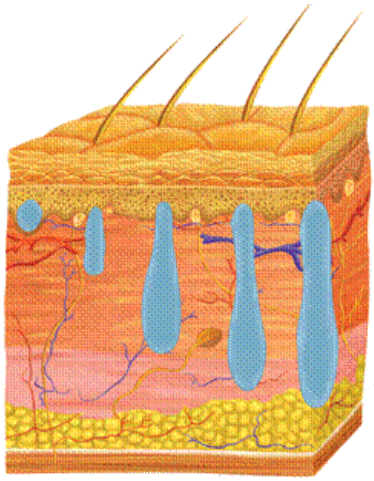
1.1.2.1. Clark's level of invasion

Clark's level indicates the anatomic level of invasion and it is used in correlation with Breslow's thickness. Clark's classification is a helpful prognostic method with high predictive value in thin tumors but less in thicker ones (Breslow's depth <1 mm). Five steps are classified in Clark's level: Level 1 for localization of melanoma in the epidermis (melanoma in situ), Level 2 for the invasive melanoma into the papillary dermis (uppermost layer of dermis), Level 3 for invasive melanoma to the junction of papillary and reticular dermis (lower layer of dermis), Level 4 for invasive melanoma into the reticular dermis and Level 5 for invasive melanoma into the subcutaneous tissue.

1.1.2.2. TNM staging system and Breslow's thickness

The widely used system for the melanoma staging is TNM classification determined by the American Joint Committee on Cancer (AJCC) and it defines cancer stages by following categories: T for the feature of primary melanoma, N for the extension of tumor to regional lymph node and M for the metastasis to distant sites. T staging of melanoma is categorized in three different features: tumor thickness, tumor ulceration and tumor mitotic rate. Five defined subsets of T staging, i.e. Tis, T1, T2, T3 and T4, corresponding to tumor thickness are estimated by the Breslow's technique (0, 1, 2, 4 mm in thickness of tumor). This TNM classification is categorized into five stages based on prognosis. Stage 0 is non-invasive melanoma, in situ melanoma, and stage I and II are the local primary melanoma. Stage III is determined as regional nodal intransit or satellite metastases and stage IV as distant metastases following spreading through blood vessels (Petro, 2002).

Clark' level	Breslow's depth (mm)	AJCC (T)
Ⅰ	Melanoma in situ	Tis
Ⅱ	≤ 1.0 mm	T1
Ⅲ	1.01-2.0 mm	T2
Ⅳ	2.01-4.0 mm	T3
Ⅴ	> 4.0 mm	T4



Adapted from Brunicardi FC et al. 2009

Figure 3. Pathological features and staging. To determine the level of invasion of melanoma, two distinguished criteria, Clark's level and Breslow's depth, are presented. Breslow's' depth measures the melanoma thickness in millimeters and categorized into five stages, Tis, T1, T2, T3 and T4. Clark's level is another classification system corresponding to the anatomical level of melanoma to describe the depth of melanoma in the skin.

TABLE 1 : TNM staging for cutaneous melanoma (Balch et al. 2009)

T	**Primary Tumor**	
Classification	Thickness (mm)	Ulceration status / mitosis
Tis	NA	NA
T1	≤1.0 mm	a) Without ulceration and mitosis ≤ 1 / mm2 b) With ulceration and mitosis ≥ 1 / mm2
T2	1.01-2.0 mm	a) Without ulceration b) With ulceration
T3	2.01-4.0 mm	a) Without ulceration b) With ulceration
T4	>4.0 mm	a) Without ulceration b) With ulceration
N	**Regional Lymph nodes**	
Classification	No. of metastatic nodes	Nodal metastatic burden
N1	1	a)Micrometastases b)macrometastases
N2	2-3	a)Micrometastases b)macrometastases c)in-transit metastases / satellites without metastatic nodes
N3	≥ 4 metastatic lymph nodes, or matted lymph nodes or in-transit metastases/ satellite(s) metastatic lymph nodes	
M	**Distant metastases**	
	site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases Any distant metastases	Normal elevated

NA: not applicable;LDH= lactate dehydrogenase

1.1.3. Important melanoma biology related to cell signaling pathways

Melanoma is characterized as a complex genetic disease and investigation of cellular interaction underlying genetic regulation in melanoma progression will provide understanding of biology of melanoma leading to an improved therapy. The below described three signaling pathways are some of the most critical ones involved in cell fate decision.

1.1.3.1. Ras/Raf/MEK/ERK signaling pathway

One of the crucial pathways for proliferation and cell survival of melanoma is Ras/Raf/MEK/ERK pathway. In melanocytes, this pathway is activated in a cAMP-dependent signaling cascade following, for instance, stimulation of melanocortin-1 receptor with B-RAF. The subsequent ERK signaling

induces cell cycle progression via upregulated gene expression of cyclin D1 (Busca, 2000; Pruitt, 2001). This activated downstream by paracrine growth factors, however, is transient or weak for modest mitogenic effects (Furukawa, 1997; Hsu, 2000). On the other hand, hyperactivated ERK pathway accounts for 90% in melanoma and it is caused by autocrine production of growth factors, SCF or FGF, as well as by mutated activation of growth-factor receptor such as c-kit (Cohen, 2002; Satyamoorthy, 2003). In melanoma, malignant transformation of melanoma is arisen from mutated factors in this pathway. A common mutation in melanoma is gain-of-function mutation in NRAS accounting for 15-30 % of melanomas and displaying an important role for tumor maintenance (Albino, 1998; Tsao, 2004**)**. The most common mutation is in BRAF, which occurs in 50-70 % of melanoma and providing tumor growth and constitutive tumoral functions, such as neoangiogenesis, effects on expression of microphtalamia-associated transcription factor (MITF) and cell cycle regulator cyclin D1 (Wang, 1997).

1.1.3.2. PI3K/AKT/mTOR signaling pathway

PI3K/AKT/mTOR appears to be another important signaling component downstream involved in cell survival and proliferation in melanoma (Vivanco, 2002). Phosphatidylinositol-3 kinases (PI3Ks) are divided into three subclasses, class I, II and III. One of heteromeric class I A PI3Ks are activated by receptor protein tyrosine kinases (RPTKs) and subclass I B PI3Ks are activated by receptors coupled with G proteins. Activated PI3Ks converts phosphatidylinositol-4,5-biphospate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits signaling proteins to the cell membrane and activates downstream targets including AKT (protein kinase B) and protein serin-threonine kinase 3'-phosphoinositide-dependent kinase 1 (PDK1) (Downward, 2004; Meier, 2005). This signaling pathway is terminated by the lipid phosphatase PTEN (phosphatase and tensin homologue) via dephosphorylation of PIP3. In malignant melanoma, hyperactivation of PI3K signaling is found abundantly caused by PI3K mutation (3 % in melanoma) or deleted PTEN function (accounting for 5-20 % in melanoma) and overexpression of AKT (highest amount in melanoma with around 60 %) (Satyamoorthy, 2000; Rizos, 2001). Additionally, PTEN and BRAF mutations are coincident in approximately 20 % of melanoma cases and both are involved in downstream of Ras suggesting cooperative signaling to stimulate the proliferation of melanoma (Soengas, 2001).

1.1.3.3. Microphtalmia-associated transcription factor (MITF)

MITF is a basic helix-loop-helix leucine zipper transcription factor, and is regarded as a master regulator of melanocytes supporting melanoblast survival and melanocyte development (Soengas, 2001). MITF expression is regulated by G protein-coupled receptor melanocortin receptor 1 (MC1R) through cAMP stimulation or by WNT via increasing β-catenin production and phosphorylation of MITF is induced by ERK signaling (Flaherty, 2010). MITF expression is a constitutive factor for melanoma proliferation and survival. The expression level of MITF causes, however, different effects on biology of melanoma cells. In melanoma cells MITF expression was observed at significantly lower level than

in melanocytes, and MITF at high expression levels induced an anti-proliferative effect on melanoma bearing BRAF mutation (Betke, 1998; Soengas, 2001; Welbrock, 2005). Low level of MITF expression, on the other hand, is detected in invasive melanoma cells and is correlated with poor prognosis and disease progression (Salti, 2000; Selzer, 2002; Zhuang, 2007). Moreover, amplified MITF expression was found in 10-15% of melanoma carrying BRAF mutation and MITF cooperates with BRAF to transform melanocytes into immortalized melanocytes, whereby BRAF suppresses MITF protein level through ERK-mediated degradation (Garraway, 2005; Wellbrock, 2005). This effect of MITF on proliferation and survival is associated with gene regulation, such as CDK2 and BCL- 2, respectively (McGill, 2002; Du, 2004).

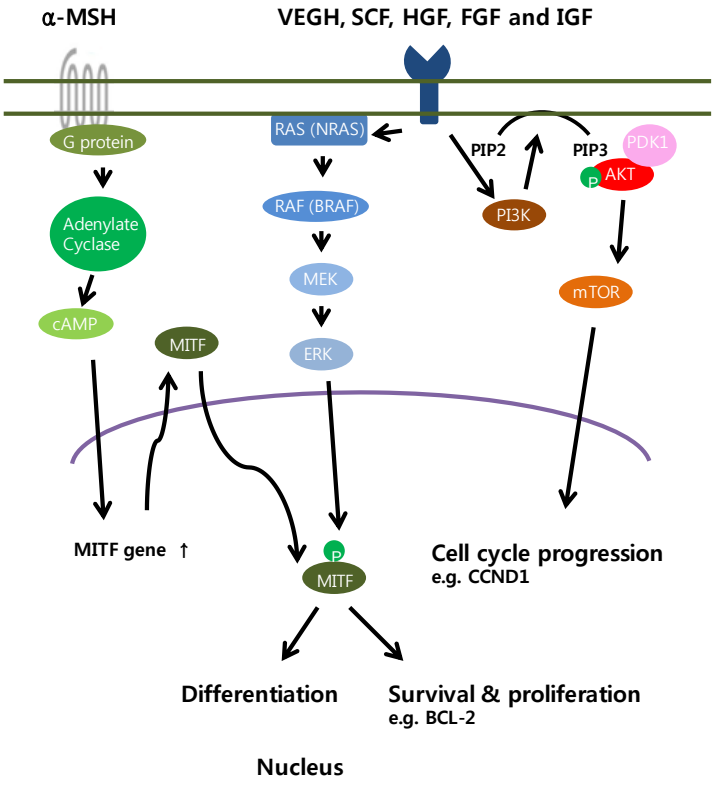


Figure 4. Three main signaling pathways in melanoma: Ras/Raf/Mek/MARK, PI3K/AKT and MITF signaling. VEGF (vascular endothelial growth factor); SCF (stem cells factor); HGF (hepatocyte growth factor); FGF (fibroblast growth factor); IGF (insulin-like growth factor); P (phosphate); PI3K (phosphatidylinositol-3 kinase); PIP2 (phosphatidltinositol-4,5-biphospate); PIP3 (phosphatidylinositol-3,4,5-triphosphate); AKT (v-akt murine thymoma viral oncogene homolog); PDK1 (phosphoinositide-dependent kinase 1); mTOR (mammalian target of rapamycin); Ras (rat sarcoma); Raf (rapidly accelerated fibrosacroma); MEK (mitogen-activated protein kinase kinase); ERK (extracellular signal-regulated kinase); MITF (microphtalamia-associated transcription factor); α-MSH (alpha melanocytes stimulating hormone); adapted from Sekulic, 2008; Jason, 2010; Flaherty, 2012.

1.1.4. Immune system in melanoma

The microenvironment of melanoma is a complex system containing innate immune cells (including macrophages, dendritic cells (DCs), natural killer cells and neutrophils) and adaptive immune cells (such as T and B lymphocytes) in combination with surrounding stroma (fibroblasts, keratinocytes and endothelial cells). These distinct cells communicate with each other via direct contact or stimuli of various cytokines inducing appropriate tumor environment. The common feature of melanoma immunity is characterized by down-regulation of MHC class I and II expression allowing tumor cells to escape from the immune surveillance, such as cytotoxic response of $CD8^+$ T cells and interacting $CD4^+$ T cells (Storkus, 2000; Haque, 2002; Cabrera, 2007) as well as through up-regulation of immunosuppressive cytokines including IL-6, IL-10, TGF-β and TNF-α, which may induce tolerized DCs and T cells (Polak, 2007). Of frequently mentioned interest is the shifting of antitumor immunity to

benefited tumor immunity like enhanced regulatory T cells (Treg), which display self-tolerance and immune homeostasis by means of suppressing immune response.

1.1.4.1. Regulatory T cells in melanoma

Naturally occurring Treg account for 5-10% of $CD4^+$ T cells in peripheral blood and production and formation of Treg occur in the thymus and periphery, respectively. Phenotypes of Treg are various and challenging to determine. However, many studies approve that high expression of the alpha chain of the IL-2 receptor (CD25) and stable expression of transcription factor forkhead box P3 (FoxP3) are the cardinal features of Treg and both markers are often correlated with each other (Kuniyasu, 2000; Fontenot, 2003; Nelson, 2004). On the other hand, interleukin-7 receptor (CD127) is down-regulated in Treg, which distinguish them from activated T cells (Liu, 2006). An additional phenotypes feature of Treg is the constitutive expression of cytotoxic T lymphocytes-associated antigen 4 (CTLA-4), which is an inhibitory molecule suppressing T cell proliferation and effector function and its presentation is dependent on FoxP3 (Hori, 2003; Wing, 2008).

Many studies reported that Treg cells are enriched in the peripheral blood as well as in the tumor microenvironment compared to healthy controls and increased mortality is correlated with the amount of accumulated Treg in malignant melanoma (Curiel, 2004; Ladanyi, 2010; Nizar, 2010). This selective enrichment of Treg in tumor environment can be induced by various processes. Firstly, Treg express specific chemokine receptors (CCR4) and integrins, which attract Treg toward tumors secreting the chemokine (CCL22) and integrin-ligand (Wei, 2006). Locally formation of tolerogenic T cells is another cause for accumulation of Treg in tumor environment. Melanoma produces several immunosuppressive molecules, such as TGF-β, IL-6, and IL-10. These factors can improve the expansion of natural Treg, and generate induced Treg (Nizar, 2010). Moreover, a factor like indoleamine 2,3-dioxygenase (IDO) produced by tumor-infiltrated immune cells, such as macrophages, can cause an anergy of Teff and convert Tconv into Treg (Brody, 2009). At last, survival benefit of Treg is arisen from interaction of melanoma cells with Teff. Melanoma expresses Fas ligand (FasL) and programmed death ligand 1 (PD-L1), which are bound to appropriate receptors expressed by Teff and this interaction causes apoptosis of Teff (Croci, 2007). Additionally, activated Treg attacks Teff via cytotoxin-mediated and receptor-mediated lysis (van Maren, 2008).

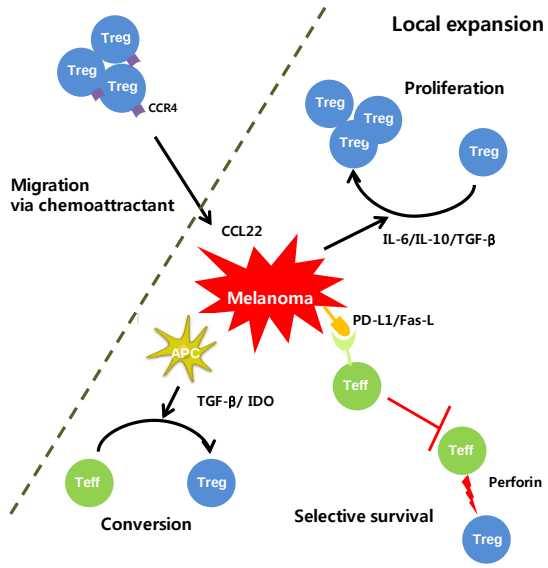


Figure 5. Mechanisms of Treg accumulation. Four distinct processes for expansion of Treg in tumor microenvironment: migration, conversion, selective survival and proliferation. Treg migration is induced by chemoattractants, such as CCL22, produced by melanoma. Conversion from conventional T cells to Treg can be arisen from anti-inflammatory mediators (TGF-β and IL-10) secreted by melanoma as well as by tumor infiltrated antigen-presenting cells (APCs). Negative costimulatory signals, PD1-L and Fas-L expressed by melanoma induce selectively apoptosis of Teff, which results in disruption of cytotoxic activity of Teff against Treg. Lastly, Treg may be proliferated in response to local secreted immunosuppressive factors, i.e. TGF-β by melanoma. CCL (chemokine ligand); CCR (chemokine receptor); IDO (indoleamine 2,3-dioxygenase); PD-L1 (programmed death-ligand); adapted from Jacobs, 2012.

1.1.5. Clinical treatment options for melanoma

Various treatment options are provided to cure advanced melanoma and basically there are five approved applications such as, surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy. These clinical trial methods are performed depending on the stage and the location of melanoma.

1.1.5.1. Surgery

The basic therapy of melanoma is surgery. In early melanoma thinner than 1.0 mm, melanoma is removed by surgery with safety margins up to 1 cm. Only in melanoma thicker than 1.0 mm the primary tumor is removed at first by surgery and additional wide local excision up to 2 cm removing more tissue is performed to reduce the risk of local recurrence of melanoma. Lymph node involvement is screened by performing sentinel lymph node biopsy. Advanced melanoma is defined thicker than 4.0 mm and this metastatic melanoma is treated differently depending on the localization and symptoms (Cancer researchuk.org).

1.1.5.2. Radiotherapy

Radiotherapy is administrated in advanced melanoma, with inoperable tumor, to shrink tumors by using high energy rays. Extensively spread in-transit metastasis or untreatable by isolated limb perfusion can be also controlled by radiotherapy (Garbe, 2008). Especially in brain metastasis, radiotherapy has been performed effectively since high dosage can be applied.

1.1.5.3. Chemotherapy

Chemotherapy is performed in advanced melanoma in combination with other therapies. Drugs, such

as Dacarbazine (DTIC, most common), Temozolomide, Carmustine (BCNU), Vinblastine, Cisplatin, or Fotemustine, are available as mono therapy or in combination with other drugs. Dacarbazine and Carmustine are alkylating agents causing an insertion of alkyl group in DNA of tumor cells, whose DNA replication and transcription is then blocked (Serrone, 2000; Stein, 2002; Quirbt, 2007). Similarly, Cisplatin belongs to the member of platinum-containing drug binding DNA and causing apoptosis by crosslinking of DNA (Legha, 1996). Vinblastine is a vinca alkaloid, which binds tubulin and inhibits the assembly of microtubules raising cell cycle arrest of tumor (Legha, 1996). However, these drugs damage not only melanoma cells but also other cells leading to side effects, such as nausea, vomiting, or kidney damage.

1.1.5.4. Targeted therapy

Recently, application of targeted drugs which are developed to inhibit the molecular pathway/oncogenes of melanoma, such as BRAF, NRAS, MEK and BCL-2 is promising. One of the common mutations in melanoma is BRAF identified in up to 50% of patients and it is correlated with mainly superficial spreading melanoma and nodular melanoma types (Long, 2011). Among the point mutations, the most common mutation is a glutamic acid for valine substitution at position 600 (V600E) accounting for 66-75% of BRAF mutations (Maldonado, 2003; Cheng, 2011). For targeting BRAF mutations, two distinct inhibitors, sorafenib (BAY 43-9006) and vemurafenib (PLX4032) were applied for suppression of activated BRAF V600E mutation. The first one, sorafenib, is the multi-kinase inhibitor targeting BRAF, CRAF and VEGF receptor tyrosine kinases however; its monotherapy is inefficient in melanoma compared to the combination with other drugs (Wilhelm, 2004; Flaherty, 2006). The other one, vemurafenib, is the selective BRAF inhibitor, which blocks the kinase activity of BRAF at low concentrated administration and suppresses proliferation of cells bearing the V600E mutation in vitro at high concentration (Tsai, 2008; Sondergaard, 2010). Meanwhile, a second selective BRAF inhibitor, dabrafenib, has been approved for therapy. Many of MEK inhibitors have been as well investigated for clinical trials and only trametinib is a potent selective inhibitor of MEK kinase 1 and 2 showing clinical efficacy in a phase III trial (Flaherty, 2012). The inhibitory effect of these agents was particularly observable in tumor cell lines bearing mutated BRAF or Ras resulting in suppression of cell growth (Yamaguchi, 2011). An additional potential targets are BCL-2 and c-kit, which are suppressed in tumor by using oblimersen (anti-sense oligonucleotide against BCL-2) and by using Imatinib, respectively (Jansen, 1998; Hodi, 2008).

1.1.5.5. Immunotherapy

One of optimal treatment is the immunotherapy using medicines to regulate the immune system, which leads to an effective destruction of melanoma cells. Among these agents, IL-2 or IFN-α is most common used in adjuvant therapy. As a negative costimulatory molecule, PD-1 is expressed on activated T cells and B cells and its engagement with PD1 ligand expressed by melanoma causes inhibition of T cell activation and apoptosis of Teff (Pilon-thomas, 2010). Monoclonal antibodies

blocking PD-1 (MDX-1106), however, showed anti-tumor activity in a phase I study (Brahmer, 2010) and this antibody disrupts interaction of melanoma with Teff, which attack Treg subsequently (Wang, 2009). The other inhibitory molecule is CTLA-4, which is constitutively expressed on Treg and modulates the reduced proliferation and function of Teff. A monoclonal antibody against CTLA-4, such as Ipilimumab, rescues Teff from suppressed cell growth and various clinical trials proved an increased overall survival in metastatic melanoma following anti-CTLA-4 treatment using Ipilimumab alone or in combination with other drugs (Alexandrescu, 2010; Hodi, 2010).

1.2. Pituitary adenylate cyclase activating polypeptide (PACAP)

Originally, pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from an extract of ovine hypothalamus, based on its potent effect on cAMP stimulation (Miyata et al., 1989). Distribution of PACAP is not restricted to the hypothalamic region. PACAP expression has been found widely in various peripheral tissues. The biological functions of PACAP have been elucidated differently in correlation with expression pattern of PACAP.

1.2.1. Discovery of PACAP

Arimura and colleagues purified a peptide from ovine hypothalamus causing the cAMP stimulation and further sequence analysis of this peptide revealed a 38 amino acid residues peptide, which was named PACAP 38 (Miyata, 1989). Within one year, a side product of PACAP 38 was found and its sequence showed a C-terminally truncated form of PACAP 38. This peptide was a 27 amino acid in length and termed PACAP 27 (Miyata, 1990). The sequence of PACAP peptides showed high homology with vasoactive intestinal polypeptide (VIP) (68 %), categorizing it ordinary member of the VIP-secretin-GHRH-glucagon superfamily (Campbell, 1992; Segre, 1993).

Human PACAP gene was cloned by Hosoya and colleagues, and mapped to the p11 region of chromosome 18 (Hosoya, 1992). The cDNA sequence of human PACAP encodes a 176 amino acids prepro-PACAP, which is cleaved by distinct prohormone convertases (PC) and is generated to produce PACAP 38 and PACAP 27 and PACAP-related peptide (PRP) sharing 22% homology with PACAP 27 (Ohkubo, 1992; Seidah, 1994). The PC in mammals consists of PC1, PC2, PC4, PC5, PC7, furin, and paired basic amino acid-cleaving enzyme 4 (PACE4) (Seidah, 1999). Initial cleavage generates from precursor of PACAP two products, intermediate precursor of PRP (big PRP) and glycine-extended form of PACAP 38. Hydrolysis of the big PRP by carboxypeptidases E, H or M contributes to form PRP (Rouille, 1995) and the latter is further catalyzed by peptidyl glycine α-amidating monooxygenase (PAM) to active α-amidated PACAP 38, which again is generated to produce α-amidated PACAP isoform, PACAP 27 (Eipper, 1992; Okazaki, 1992).

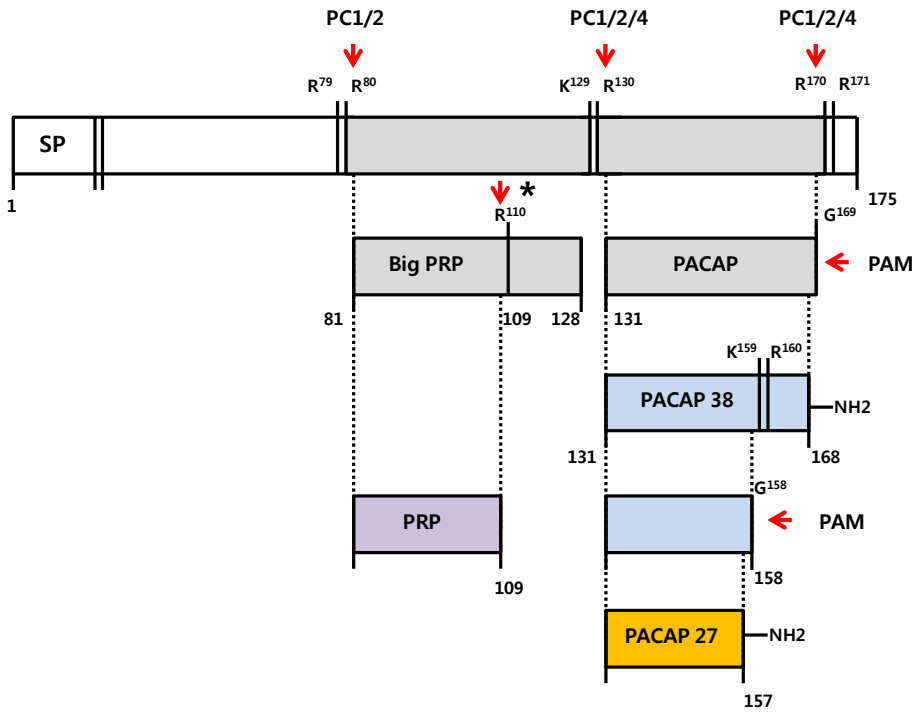


Figure 6. Post-translational process of rat PACAP precursor. PACAP precursor is cleaved by various enzymes and modified into three products, PACAP 38, PACAP 27 and PACAP-related peptide. Enzymes for cleavage of peptide are following: PC 1, 2 and 4 (prohormone convertase), PAM (peptidyl glycine alpha-amidating monooxygenase) and * (Carboxylpeptidases E,H,M). This scheme is adapted from Vaudry, 2009.

1.2.2. Distribution of PACAP in human tumors

In many studies, the expression pattern of PACAP has been investigated in a variety of species and it was found mainly in the brain and the peripheral nerve system, but also in other regions, such as non-neuronal cells and tissues in the periphery. PACAP 38 was defined as the predominant product among the active forms of PACAP throughout the body (Arimura, 1991; Gaytan, 1994). Recent studies have focused on PACAP expression in tumor area. Well-defined PACAP expression in human brain tumor has been investigated by Isobe showing RNA expression of PACAP in human neuroblastomas (Isobe, 2004). Last 15 years, more specific, malignant tumors have been studied to prove the PACAP expression. Odum and his colleagues had detected predominant expression of PACAP 38 in malignant ovarian cancer and its concentration was higher than the other PACAP types, PACAP 27 and PRP (Odum, 1998). Further, HCT8 human colon tumor cells showed PACAP 38 expression in correlation with cell survival (Le, 2002). Two groups working on prostate cancer investigated PACAP expression, which was detected in normal as well as malignant prostate tissues at RNA and protein level. Garcia-Fernandez showed no significant staining intensity but differences in PACAP localization among tissue samples. PACAP was detected mainly in the epithelial cells of healthy tissues, whereas it was expressed in both epithelial cells and stroma of prostate cancer tissues (Garcia-Fernandez, 2002). On the other hand, Moretti and co-workers revealed no clear difference in the distribution pattern among normal, hyperplastic, and tumoral tissues (Moretti, 2006). Subsequently, mRNA and

protein expression of PACAP in breast carcinoma was observed by Garcia-Fernandez, and the distinct pattern of PACAP distribution was demonstrated between normal (in glandular epithelium) and malignant breast tissues (epithelial cells and stroma) (Garcia-Fernandez, 2004). Study in human testis cancer provided as well evidence for PACAP expression in normal testes and seminoma tumor cells, showing more intensive PACAP immunoreactivity compared to embryonal carcinoma cells (Nakamura, 2013).

1.2.3. PACAP receptors

The PACAP receptors are categorized into the family of GPCRs, namely secretin receptor family, containing seven transmembrane domains (7TM-region). Since neuropeptides (VIP and PACAP) were found, receptors for these neuropeptides were identified and cloned in various species. Three defined receptors, VPAC1R, VPAC2R, and PAC1R were investigated to characterize their structures. Initial cloning of VPAC1R was performed from a rat cDNA by Ishihara and two years later, the human homologue was cloned from the HT-29 colon carcinoma cell line (Ishihara 1991; Shreedharan, 1993). In the same year VPAC2R was cloned from a rat pituitary cDNA library and cloning of the human receptor from SUP-T1 cells was performed subsequently (Lutz, 1993; Svoboda, 1994). Several groups succeeded in cloning the rat PAC1R simultaneously from brain or cell lines and the human receptor was cloned in the same year by Ogi et al. (Hashimoto, 1993; Hosoya, 1993; Morrow, 1993; Ogi, 1993; Svoboda, 1993). Based on the pharmacological evaluation, PACAP type II receptors (VPAC receptor), VPAC1R and VPAC2R, showed equal binding-properties to PACAP and VIP. On the other hand, PACAP type I receptor, PAC1R, binds preferentially to PACAP, resulting in 300-1000-fold higher affinity to PACAP compared to VIP (Arimura, 1998; Harmar, 1998).

Over twenty years studies on the receptor variants were performed by many groups and the PAC1R has been known as one of GPCRs demonstrating the most widely alternative splicing forms. So far fourteen different human PAC1R splice variants were revealed (Dautzenberg, 1999; Lutz, 2006). The first identification of PAC1R splicing variants was reported by Spengler showing five distinct PAC1R variants upon presence of two 28 aa cassettes (hip and hop) in the third intracellular loop and they were named null (absence of hip and hop), hip, hop1 (28 residues insertion), hop2 (27 residues insertion) and hiphop1 (Spengler, 1993). Subsequently, alternative splicing forms of PAC1R were reported, such as PAC1-TM4, PAC1-vs, PAC1-short and PAC1-delta5 (Chatterjee, 1996; Pantaloni, 1996; Dautzenberg, 1999; Lutz, 2006).

These variant receptors of VPAC1/2 and PAC1 regulate differently intracellular signaling pathways: Adenlyate cyclase (AC), Phospholipase D (PLD) and Phospholipase C (PLC) (McCulloch, 2000; Dickson, 2006) and it suggested pleiotropic biological activities of PACAP in cells (Vaudry, 2009).

PACAP receptors are widely distributed in various organs, whereas VPAC receptors mainly, were detected in the olifactory bulb, cerebral cortex and thalamus (Masuo, 1992). PAC1R has also been found at high level in the central nervous system, such as the cerebral cortex, thalamus, mesencephalon, hypothalamus and cerebellum (Masuo, 1992; Gonzalez, 1996). Furthermore, a number of tumor cell lines showed predominantly PAC1R expression in neuronal and endocrine

tumors, such as gliomas, neuroblastoma, prostate cancer, and testis (Vertongen, 1996; Reubi, 2000; Lieu, 2006; Muller, 2006; Nakamura, 2013).

In contrast VPAC receptors expressed on thymocytes, peripheral lymphocytes and macrophages, PAC1R expression appears to be limited on immune cells but macrophages showed constitutive PAC1R expression (Delgado, 1996; Pozo, 1997).

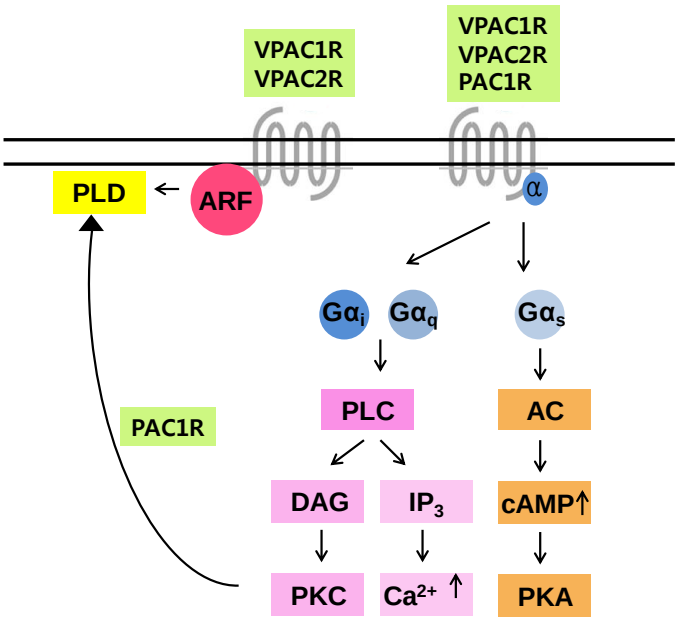


Figure 7. Intracellular signaling pathways stimulated by PACAP receptors. All three receptors are able to coupling to G_a leading to downstream production of cAMP and activate PLC leading to an increase in Ca^{2+} via coupling to $G\alpha_i$ and $G\alpha_q$. PLD activity can be stimulated by three receptors via ARF (VPACR) and PKC (PAC1R) sensitive pathways. ARF (alternate reading frame); PLD (Phospholipase D); PLC (Phospholipase C); DAG (Diacylglycerol); PKC (Protein kinase C); IP3 (Inositol 1.4.5-triphosphate); AC (Adenlyate cyclase); PKA (Protein kinase A); adapted from Langer, 2012.

1.2.4. Biological effects of PACAP

PACAP has been described to have wide range of fundamental functions in CNS and peripheral tissues and these actions of PACAP are not only involved in regulation of cell division, cell cycle arrest, differentiation, and cell death but PACAP affects as a neurotransmitter on vascular system, respiratory system, and digestives system (Nandha, 1991; Uddman, 1991; Hoshino, 1993; Matsumoto, 1993; Gonzalez, 1997; Mirfendereski, 1997; Lu, 1998).

1.2.4.1. Tumor cells

Based on several reports on the effect of PACAP on tumor cells, there are reciprocal functions, namely stimulation or inhibition of proliferation upon tumor cell types. Tumors, such as small cell lung cancer, pancreatic and prostate cancer, showed improved proliferation in the presence of PACAP by regulating gene expression, such as c-fos (Moody, 1993; Draoui, 1996; Schaefer, 1996; Leyton, 1998). An opposite role of PACAP was observed in colonic adenocarcinoma cells showing decreased cell growth (Vertongen, 1996b; Lelievre, 1998). In addition, these mutual effects could be found in a dose- or time-dependent manner in neuroblastoma or prostate tumor, respectively. Low concentration of PACAP enhanced proliferation of neuroblastoma cells, while at high doses of PACAP, cell differentiation was observable (Deutsch, 1993; Monaghan, 2008). Prostate tumor cells showed cell growth following short-term treatment with PACAP while long-term exposure to PACAP induced tumor

cells to differentiate into a neuroendocrine phenotype (Juarranz, 2001; Farini, 2003). In pheochromocytoma (PC12), PACAP protects cells from cell death caused by serum deprivation, glutamate, or rotenone and it stimulates catecholamine production inducing cell differentiation and survival (Tanaka, 1997; Corbitt, 1998; Onoue, 2002; Wang, 2005; Ravani, 2006). PACAP also displays a positive effect on cytokine production in tumor cells. Cytokines, such as IL-6 can be regulated by PACAP in both folliculostella cell line (anterior pituitary population) and prostatic cancer cells (Matsumoto, 1993; Nagakawa, 2005).

1.2.4.2. Immune cells

PACAP effects have been implicated not only in tumor but also in immune cells as a potent anti-inflammatory factor and as a modulator for Th1/Th2 differentiation. Increased pro-inflammatory factors, such as TNF-α, IL-12 and IL-6, which are secreted by macrophages following stimulation with lipopolysaccharide (LPS), are inhibited by PACAP and simultaneously the anti-inflammatory molecule, IL-10, is enhanced (Delgado, 1999a; Martinez, 1998; Delgado, 1999b). Of note, PACAP shows dual roles in the IL-6 production in macrophages as a suppressor and stimulator. Inhibited IL-6 production by PACAP is observed only in macrophages following exposure to high doses of LPS and cells unstimulated or stimulated with low concentrations of LPS produce in the presence of PACAP high level of IL-6 (Martinez, 1998). Moreover, PACAP suppresses distinct chemokine productions by activated macrophages, such as MIP-2 (chemoattractant for leukocytes), MIP-1 and RANTES (chemoattractant for monocytes and T cells) causing failed recruitment of immune cells to sites of inflammation (Delgado, 2001). PACAP interrupts the antigen-specific response of stimulated macrophages through down-regulation of costimulatory factors, B7.1 and B7.2 expression (Delgado, 1999).

In lymphocytes PACAP functions as a survival factor and as a mediator for cell differentiation. Firstly, PACAP acts as a survival factor in reduced activation-induced cell death (AICD) of activated $CD4^+$ T cells through the suppression of Fas ligand expression by stimulated $CD4^+$ T cells and through the decreased cytotoxic activity of $CD8^+$ T cells, whose Fas ligand-mediated lysis is inhibited by PACAP (Delgado, 2000). Regarding immunomodulation of PACAP, various studies proved that PACAP can be a critical factor to determine Th1 and Th2 differentiation. A shift toward a Th2 phenotype may be induced by PACAP by the high production of Th2 type cytokines, such as IL-4 and IL-5 (Delgado, 1999).

2. Aim of the thesis

Since its discovery, PACAP has been considered as one of the challenging neuropeptides in various aspects. The distribution of PACAP in various tumor types has been well documented and PACAP benefits in tumor through displaying as a mediator for tumor survival or proliferation and through modulation of suppressive immunity in an autocrine/paracrine manner (Delgado, 2000; Vaudry, 2000). Since Fabre and colleagues defined a receptor in melanoma cell lines recognizing VIP-related peptides in 1994, the role of VIP/PACAP receptor types and their functions are largely unknown in melanoma (Fabre, 1994). Regarding immunoregulation by VIP/PACAP, the shift of human $CD4^+$ T cells to tolerogenic T cells has been known but it is still not shown, what difference among Treg ($CD4^+CD25^+CD127^{low/-}$) and Teff/Tresp ($CD4^+CD25^-$) can be observed under PACAP treatment. Considering this context the aim of this study is based on the underlying questions:

- Do malignant melanomas express PACAP and PACAP receptor, PAC1R, similarly to other tumor types and does their expression vary in a spatial and temporal pattern?
- What functional effect of PACAP is involved in melanoma biology regarding gene/protein expression related to cell survival or proliferation and other cell functions, such as cytokine production? In addition, does PAC1R function as a specific PACAP receptor to mediate PACAP-induced cell activity of melanoma?
- Furthermore, do two distinct $CD4^+$ T cell subgroups, Treg and Teff/Tresp, respond to PACAP differently? How are the patterns of gene expression and the suppressive capacity of Treg in the presence of PACAP?

To address these questions, initially, PACAP and PAC1R expression in melanoma tissues obtained from patients were defined by immunohistological analysis. Further selected melanoma cell lines were examined and found to produce and express PACAP and PAC1R. Melanoma lines were further investigated on mRNA and protein levels to elucidate functional effectiveness of PACAP on cell growth and cytokine production. Regarding PACAP-induced immune suppression, patterns of gene expression involved in suppressive actions of PACAP were investigated in two distinct $CD4^+$ T cell subgroups.

3. Materials and Methods

3.1. Materials

3.1.1. Clinical and healthy donor samples and melanoma cell lines

Primary melanoma and metastatic melanoma tissues of melanoma patients	Department of Dermatology and Allergology, University Hospital, Marburg
Healthy skin biopsies	Human Tissue and Cell Research (HTCR), University Regensburg
Peripheral blood of healthy donors	Center for Transfusion Medicine and Hemotherapy, University Hospital, Giessen
Melanoma cell lines: NW-Mel 8; SK-Mel 37; NW-Mel 38; NW-Mel 145; NW-Mel 450	Ludwig Institute for Cancer Research, Clinical Trials Center, Northwest Hospitall, Frankfurt

3.1.2. Cell culture media and supplements

DMEN high Glucose (4.5 g/ml)	PAA Laboratories, Cölbe
RPMI 1640, w/o L-Glutamin	PAA Laboratories, Cölbe
Fetal Calf Serum (FCS)	PAA Laboratories, Cölbe
Pooled human AB-Serum (PHS)	PAA Laboratories, Cölbe
L-Glutamin 200mM	PAA Laboratories, Cölbe
Non Essential Amino Acid (50x)	PAA Laboratories, Cölbe
Penicillin / Streptomycin (10.000U/ml)/Streptomycin (10.000µg/ml)	PAA Laboratories, Cölbe
Recombinant human IL-2	Roche Diagnostics, Mannheim

3.1.3. Oligonucleotide primers

Primer/ Melanoma	Sequence (5'-3')	Amplicon (bp)	NCBI ID/Referenz
PAC1R-F PAC1R-R	CTGAGACTGGGGACCAGG GGCCAGTCGCAAACCAGG	635	NM_001118.3 ; Kalmbach (Institue of Anatomy and Cell Biology, University of Marburg)
VPAC1R-F VPAC1R-R	CCTTCTTCTCTGAGCGG AGGCGAACATGATGTAGTG	336	NM_004624.3 ; Lutz, 2006,
VPAC1R_2-F VPAC1R_2-R	ATGGCTAACTTCTTCTGGCTG CACAGTGAGGAGTTGATGGT	202	NM_004624.3

VPAC2R-F VPAC2R-R	ACCTGTTCCTGTCCTTCATC CACCAGCAGCCAGAAGAAGT	176	NM_003382.4
CCND3-F CCND3-R	ACATGATTTCCTGGCCTT TGAGCTCATCCCCGGACA	220	NM_001136017.2 ; Toledo, 1995
CCNE-F CCNE-R	ATAATGCAGTCTGTGCAGAC GTTGTGTGCATCTTCATCAG	887	NM_001238.1 ;Pizzimenti, 1999
BCL-2-F BCL-2-R	CCGCTACCGCCGCGACTTC AAACAGAGGCCGCATGCTG	313	NM_000633.2 ; Karakas, 1998
Ki67-F Ki67-R	GGAAAGTAGGTGTGAAAGAAGAGG GCCTTTATCCTCATCTCCTGGTAC	458	NM_001145966.1;Karakas, 1998,
CCR4-F CCR4-R	AAGAAGAACAAGGCGGTGAAGATG AGGCCCCTGCAGGTTTTGAAG	296	NM_005508.4 ; Yoshie, 2002
CCR7-F CCFR7-R	GTGCCCGCGTCCTTCTCATCAG GGCCAGGACCACCCCATTGTAG	353	NM_001838.3 ; Yoshie, 2002
CD14-F CD14-R	CGTGGGCGACAGGGCGTTCT TAAAGGTGGGGCAAAGGGTT	777	NM_000591.3; Song, 2001
FoxP3-F FoxP3-R	CCCACTTACAGGCACTCCTC CTTCTCCTTCTCAGCACCA	403	NM_014009.3; Kohno, 2005,
CTLA-4-F CTLA-4-R	ATGGCTTGCCTTGGATTTCAGCGGCACAAGG TCAATTGATGGGAATAAAATAAGGCTGAAATTGC	562	NM_001037631.2; Wang, 2001
TGF1-β-F TGF1-β-R	GCCCTGGACACCAACTATTGC GCTGCACTTGCAGGAGCGCAC	336	NM_000660.4; Hering, 2001
IL-10-F IL-10-R	GTGATGCCCCAAGCTGAGA CACGGCCTTGCTCTTGTTTT	138	NM_000572.2; Overbergh, 2003
β-actin-F β-actin-R	GGCATCCTCACCCTGAAGTA AGGGCATACCCCTCGTAGAT	326	NM_001101.3; Gibbings, 2007
GAPDH-F GAPDH-R	AACATCATCCCTGCCTCTACT CTGCTTCACCACCTTCTTGAT	180	NM_002046.3
18sRNA-F 18sRNA-R	CTGAGAAACGGCTACCACAT GACTCATTCCAATTACAGGGC	126	NR_003286.2

3.1.4. Polypeptides and antibodies

3.1.4.1. Polypeptidies

PACAP 27	Calbiochem, Gibbstown, USA
PACAP 38	Calbiochem, Gibbstown, USA
Vasoactive Intestinal Peptide (VIP)	Calbiochem, Gibbstown, USA
acetyl-(D-Phe-Lys, Arg, Leu)-Vip(1-7)-GRF(8-27) trifluoroacetate salt	Bachem, Weil am Rhein

PACAP 6-38 (PACAP 38 trifluoroacetate salt)	Bachem, Weil am Rhein

3.1.4.2. Immunostaining antibodies (IHC/IF/WB/ELISA)

Monoclonal Mouse anti-human HMB 45 (IgG_1, κ)	Dako, Glostrap, Denmark
Monoclonal Mouse anti-human S100 (IgG_1, κ)	Dako, Glostrap, Denmark
Rabbit Serum anti-PACAP 27 Amide (Human, Rat, Ovine) antibody	Phoenix Pharmaceuticals, Burlingame, USA
Rabbit Serum anti-PACAP 38 amide (Human, Rat, Ovine) antibody	Phoenix Pharmaceuticals, Burlingame, USA
Purified Polyclonal Rabbit anti-PACAP receptor Type 1	Milipore, Schwalbach
Mouse IgG_1, κ Isotype control purified	eBioscience, San Diego, USA
Goat anti-Mouse IgG (gamma chain) Peroxidase conjugated	Sigma-Aldrich, St. Louis, USA
Goat anti-Rabbit IgG (whole Molecules) Peroxidase conjugated	Sigma-Aldrich, St. Louis, USA
Biothinlyated anti-Mouse IgG (H+L)	Vector Laboratories, Burlingame, USA
Biotinylated anti-Rabbit IgG (H+L)	Vector Laboratories, Burlingame, USA
Goat anti-Rabbit IgG (H+L), highly cross adsorbed HiLyte Fluor 488-labeled	AnaSpec, Fremont, USA
Streptavidin, HiLyte Fluor TR conjugated	AnaSpec, Fremont, USA
Streptavidin-AKP	BioLegend, San Diego, USA
Monoclonal Mouse anti-BCL-2 (Human, Mouse, Rat) (IgG_1)	Santa Cruz Biotechnology, USA
Monoclonal Mouse anti-MiTF (Human, Mouse, Rat) (IgG_1, κ)	Thermo scientific, Rockford, USA
Polyclonal Rabbit anti-Human CCR7	Thermo scientific, Rockford, USA
Monoclonal Mouse anti-PACAP receptor 1 (Human, Rat) (IgG_1, κ)	Abcam, Cambridge, UK
Monoclonal Mouse anti-PACAP (Human, Rat) (IgG_1, κ)	Invitrogen, Camarillo, USA
Monoclonal Mouse anti-β actin (IgG_1)	Sigma-Aldrich, St. Louis, USA
Monoclonal Rat anti-Human IL-6, capture (IgG_1, κ)	eBioscience, San Diego, USA
Monoclonal Rat anti-Human IL-6, detection (IgG_{2a}, κ)	eBioscience, San Diego, USA
Monoclonal Mouse anti-Human IL-10, capture (IgG_2b)	R&D systems, Abingdon, UK
Polyclonal Goat anti-human IL-10, detection (IgG_1)	R&D systems, Abingdon, UK
Mouse anti-Human IgG HRP	Dako, Glostrap, Denmark
Rabbit anti-Human IgG HRP	Dako, Glostrap, Denmark

3.1.4.3. Flow cytometric antibodies

Specificity	Clone	Species/Isotyp	Conjugat	Company
CD4	RPA-T4	Mouse IgG_1, κ	FITC	BD Pharmingen
CD25	M-A251	Mouse IgG_1, κ	APC	BD Pharmingen
CD127	hIL-7R-M21	Mouse IgG_1, κ	PE	BD Pharmingen
Iso-control	MOPC-21	Mouse IgG_1, κ	FITC	BD Pharmingen
Iso-control	MOPC-21	Mouse IgG_1, κ	APC	BD Pharmingen
Iso-control	MOPC-21	Mouse IgG_1, κ	PE	BD Pharmingen

3.1.4.4. T cell stimulation antibodies

CD28, Mouse IgG1	R&D systems, Abingdon, UK
CD3, Mouse IgG1, κ (UCHT1)	BectonDickinson, Heidelberg

3.1.5. Kits, beads and supplements

3.1.5.1. Immunostaining (IHC/IF/ELISA)

Duoset ELISA human IL-10	R&D systems, Abingdon, UK
Avidin/Biotin Blocking Kit	Vector Lab, Burlingame, USA
Biotin-Streptavidin-HRP	eBioscience, San Diego, USA

3.1.5.2. T cell isolation and stimulation

$CD4^+CD25^+$ Regulatory T cell Isolation Kit	Miltenyi Biotec, Bergisch-Gladbach
$CD4^+CD25^+CD127^{dim/-}$ Regulatory T cell Isolation Kit	Miltenyi Biotec, Bergisch-Gladbach
Dynabeads® Human T-Activator CD3/CD28	Life Technologies**,** Darmstadt

3.1.6. Reagents

Nucleotides

Gene Ruler 100 bp DNA ladder	Fermentas, St Leon-Rot
DNase I	Fermentas, St Leon-Rot
dNTP mix	Fermentas, St Leon-Rot
Ethidiumbromide solution	Roth, Karlsruhe
Gene Ruler 1 kb DNA ladder	Fermentas, St Leon-Rot
Glycogen, RNA grade	Fermentas, St Leon-Rot
M-MulV reverse transcriptase	Fermentas, St Leon-Rot

Oligo(dT)$_{18}$ primer	Fermentas, St Leon-Rot
RNase inhibitor	Fermentas, St Leon-Rot
Taq DNA polymerase	Fermentas, St Leon-Rot
TriFast	Peqlab, Erlangen

Immunostaining (IHC/IF/WB/ELISA)

BCIP/NBT	Dako, Glostrap, Denmark
Fast red	Sigma-Aldrich, Schnelldorf
VECTASHIELD® Mounting Media	Vector Lab, Burlingame, USA
Aqueous Mounting Media	Dako, Glostrap, Denmark
Goat serum	Vector Lab, Burlingame, USA
Protein marker V	Peqlab, Erlangen
Protease/Phosphatase inhibitors	Roche, Mannheim
Chemiluminescent HRP substrate	Merck Milipore, Darmstadt
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich, Schnelldorf
7-AAD	BD Biosciences, Heidelberg
Quick Start™ Bradford	Biorad, CA, USA
TMB	Calbiochem, Darmstadt

3.1.7. Chemicals

Acetic acid ($C_2H_4O_2$)	Merck, Darmstadt
30% ccrylamide solution	Carl Roth, Karlsruhe
3-Amino-9-Ethylcarbazol (AEC; $C_{14}H_{14}N_2$)	Sigma-Aldrich, Schnelldorf
Ammoniumchloride (NH_4Cl)	Mallinckrodt Baker, Griesheim
Bovine serum albumin fraction V (BSA)	Carl Roth, Karlsruhe
Bradford protein assay	Bio-Rad, CA, USA
Citrate	Merck, Darmstadt
Dimethyl sulfoxide (DMSO; C_2H_6OS)	Sigma-Aldrich, Schnelldorf
Dulbecco´s PBS 1x (without Ca^{2+} + Mg^{2+})	PAA Laboratories, Cölbe
Ethidium bromide solution	Carl Roth, Karlsruhe
Ethanol	Sigma-Aldrich, Schnelldorf
Ethylendiamintetraacetic acid (EDTA; $C_{10}H_{16}N_2O_8$)	Sigma-Aldrich, Schnelldorf
FACS Clean solution	Becton Dickinson, Heidelberg
FACS Flow solution	Becton Dickinson, Heidelberg
FACS Rinse solution	Becton Dickinson, Heidelberg
Hematoxylin	Merck, Darmstadt
Hydrogen peroxide 30% (H_2O_2)	Merck, Darmstadt
2-propanol (C_3H_8O)	Sigma-Aldrich, Schnelldorf

N,N-Dimethylformamide (DMF; C_3H_7NO)	Sigma-Aldrich, Schnelldorf
Nonfat dried milk powder	Applichem, Darmstadt
Pancoll, human	PAN-Biotech, Aidenbach
Paraformaldehyde (PFA; $(CH_2O)_n$)	Merck, Darmstadt
Potasium hydrogen carbonate ($KHCO_3$)	Merck, Darmstadt
Saponin	Sigma-Aldrich, Schnelldorf
Sodium acetate ($C_2H_3NaO_2$)	Merck, Darmstadt
Sodium azid (NaN_3)	Merck, Darmstadt
Szintillation solution MicroScint™	PerkinElmer, Rodgau-Jugesheim
TEMED	Carl Roth, Karlsruhe
[methyl-^{3}H]-Thymidine	Amersham Biosciences, Buckinghamshire, UK
Tissue-Tek	Sakura, Leiden, Netherlands
Triton X-100	Sigma-Aldrich, Schnelldorf
Trypanblue solution 0,4%	Sigma-Aldrich, Schnelldorf
Tween® 20	Merck, Darmstadt
Universal Agarose	Peqlab, Erlangen
Xylene	Sigma-Aldrich, Schnelldorf

3.1.8. Consumables

Cell culture dish	Nunc, Roskilde, Denmark
Cell culture Flask	Greiner, Frickenhausen
Cell culture Test Plates (flat bottom: 6, 24, 48, and 96-well; Round-bottom: 96-well)	Nunc, Wiesbaden
Cellfunnels	Tharmac, Waldsolm
Combitips	Eppendorf, Hamburg
Cryotubes	Greiner, Frickenhausen
Disposable pipettes	Greiner, Frickenhausen
FACS-tubes	Becton Dickinson, Heidelberg
Filter Card Cell funnels	Tharmac, Waldsolm
Filter tips (steril)	Starlab, Hamburg
Gel electrophoresis (Mini, Midi)	Peqlab, Erlangen
Glass fiber filter	PerkinElmer, Rodgau-Jugesheim
Glass Pasteur pipettes	Hirschmann Laborgeräte, Eberstadt
Glass Plates (0.75, 1, 1.5 mm)	Bio-Rad, CA, USA
MACS®, MS/LD Columns	Miltenyi Biotec, Bergisch-Gladbach
Microscope slides/coverglass	Menzel, Braunschweig
Neubauer chamber, 20x26 mm	Menzel, Braunschweig
Nitrocellulose blotting membrane	Peqlab, Erlangen

Pipette tips	Kalensee, Gießen
PCR Tubes	Bio-Rad, CA, USA
Steril-filter (0.22 µm)	Millipore, Schwalbach
Tek Chamber slide	Thermo scientific, NY, USA
Tissue culture dish, 100x20 mm polystyrene	Bectondickenson, NJ, USA
Tubes sterile	Greiner, Frickenhausen

3.1.9. Equipment

Analytical balances 770	Gottl. Kern & Sohn, Balingen-Frommern
Axiostar microscop	Carl Zeiss MicroImaging, Göttingen
Casy® Cell Counter(1)	Roche Diagnostic, Mannheim
Centrifuge Megafuge® 1.0	Heraeus Kendro, Langenselbold
Cryostat Microtome Leica CM1850(2)	Leica Biosystems, Vienna, Austria
Electrophoresis Power Supply EPS 3500XL	Pharmacia Biotech, USA,
Electrophoresis, Mini-protean Tetra system	Bio-Rad, CA, USA
FACSCalibur™ flow cytometer	Becton Dickinson, Heidelberg
Heracell™150i CO_2-Incubator	Heraeus Kendro, Langenselbold
Inverted stage microscope ID03	Carl Zeiss MicroImaging, Göttingen
Leica bond (automated IHC)	Leica Biosystems, Vienna, Austria
Microplate absorbance reader, Sunrise™	Tecan, Männedorf, Switzerland
Microplate reader,Infinite® M20(3)	Tecan, Männedorf, Switzerland
Microwave	Philips, Amsterdam, Netherland
MiniMACS™ separator	Miltenyi Biotec, Bergisch-Gladbach
MultiGourmet (Steamer for IHC)	Braun, Kronberg
Multipette® plus	Eppendorf, Hamburg
NanoDrop ND 1000(3)	PeqLab, Erlangen
Olympus BX51TF(2)	Olympus, Tokyo, Japan
Power Supply PAC 300	Bio-Rad, CA, USA
Quantum fluorescence system	PeqLab, Erlangen
Semi-Dry blotter	Biometra, Göttingen
Shaker, HS 250	IKA Laborthechnik, Breisgau
Shandon CytoSpin 2(1)	Thermo Scientific Lab, Waltham, MA, USA
Thermomixer 5436	Eppendorf, Hamburg
TopCount®NXT™ Szintillation Counter	PerkinElmer LAS, Rodgau-Jugesheim
Video printer P93D	Mitsubushi electric, Tokyo, Japan

Note: Departments for facilities:

(1) Institute of Immunology, University of Marburg

(2) Institute for Laboratory Medicine, University of Marburg

(3) Institute of Neurology, University of Marburg

3.1.10. Software

BD CellQuest^{TM}Pro	Becton Dickinson, Heidelberg
XFluor^{TM}, V4.51	TECAN, Männedorf, Switzerland
CellF soft imaging system	Olympus, Tokyo, Japan
Chemicapture 20001	PeqLab, Erlangen
i-control^{TM,} V2.02	TECAN, Männedorf, Switzerland
ImageJ	National Institutes of Health, Bethesda, MD, USA
Microsoft, Excel, 2007	Microsoft, Redmond, USA
Microsoft, Windows XP	Microsoft, Redmond, USA
Microsoft, Word, 2007	Microsoft, Redmond, USA

3.2. Methods

3.2.1. Cell Culture

3.2.1.1. Media and supplements

Tumor cell line Medium

DMEN high Glucose (4.5 g/ml)	50 ml
Fetal bovine serum (FBS)	10 % (v/v)
L-Glutamin	1 % (v/v)
Non-Essential Amino Acid	1 % (v/v)
Penicillin / Streptomycin	1 % (v/v)

Cell detachment solution-Citric saline (pH 7.0)

KCl	1.35 M
Sodium citrate	0.15 M

ddH_2O

Sterilize the solution by autoclaving and by using Steril-filter (0.22 µm).

TC Medium

RPMI 1640, w/o L-Glutamin	50 ml
Pooled human serum (PHS)	10 % (v/v)

L-Glutamin	1 % (v/v)
Penicillin / Streptomycin	1 % (v/v)
Recombinant human IL-2	12.5 U/ml

Freezing Solution

Fetal bovine serum (FBS)	90 % (v/v)
DMSO	10 % (v/v)

3.2.1.2. Cell culture

The melanoma cell lines, NW-Mel 8, SK-Mel 37, NW-Mel 38, NW-Mel 145, and NW-Mel 450 were provided kindly by Dr. Nadette P. Bulgin from the Ludwig Institute for Cancer Research. Cells were maintained in DMEN supplemented with 10 % FBS, 1 % Penicillin / Streptomycin, 1 % non-essential amino acids solution and 1% Sodium pyruvate cultured in a sterile incubator at 37 °C with a CO_2-concentration of 7.5 % (v/v). T cells were cultivated in RPMI as above described and incubated at 37 °C with a CO_2-concentration of 5 % (v/v).

3.2.1.3. Quantification of viable cells

To estimate the number of viable cells in a suspension, a Neubauer counting chamber was used. The cells were diluted 1:2 in 0.4 % (w/v) Trypan Blue stain and placed in the counting chamber. The average number of cells was calculated, whereby counting only live cells in four of nine squares. The total cell number per 1 ml of suspension volume was calculated multiplying the average cell number per square with the dilution factor and the chamber factor 10^4.

3.2.1.4. Passage of adherent cell lines

Melanoma cells were transferred into a new cell culture flask every 3 or 4 days under sterile condition. Cells were rinsed with Dulbecco's PBS twice followed removal of the cell culture medium and were incubated in 1 x Citric Saline solution for 5-10 minutes at 37 °C to detach from the cell flask. The reaction of this solution was terminated adding an equivalent volume of PBS and the detached cells were centrifuged at 1400 rpm for 5 minutes. The pellet was resuspended in DMEM medium and seeded in a new flask.

3.2.1.5. Storage and thawing cells

For cryopreservation, melanoma cells were centrifuged at 1400 rpm for 4 minutes at 4 °C and the pellet was resuspended in freezing medium (FCS containing 10 % DMSO). The suspension was

transferred into cryotubes, and placed in an isopropanol-filled cryo-container allowing slow freezing of cells. This container was kept at -80 °C overnight and subsequently, the cryotubes were replaced in liquid nitrogen for long term storage. For the thawing procedure the frozen cells were thawed in a pre-warmed water bath at 37 °C. Immediately, the thawed cells were diluted in DMEM and centrifuged at 1400 rpm for 4 minutes at 4 °C to completely remove the cryoprotective agent, which causes the cell damage. The pellet was resuspended gently in the fresh DMEM containing 10% FCS and transferred into a new culture flask.

3.2.2. Immunohistochemistry and Immunofluorescence

3.2.2.1. Antibodies and supplements

Reagents

Goat serum
Bovine serum albumin fraction V (BSA)
Fast Red
Dapi solution

Citrate buffer (pH 6.0)

Monohydrate citric acid (A)	0.1 M
Dihydrate sodium citrate (B)	0.1 M

Mix 1.8 % (v/v) of solution (A) and 8.2 % (v/v) of (B) with ddH_2O

Primary antibody

Rabbit Serum anti-PACAP 27 Amide (Human, Rat, Ovine) antibody	1:500
Rabbit Serum anti-PACAP 38 amide (Human, Rat, Ovine) antibody	1:800
Purified Polyclonal Rabbit anti-PACAP receptor Type 1	1:1000
Monoclonal Mouse anti-Human HMB 45	1:80
Monoclonal Mouse anti-Human S100	1:80

Secondary antibody

Biotinylated anti-Rabbit IgG	1:200

Biotinylated anti-Mouse IgG	1:200
Streptavidin-Alkaline Phosphatase	1:200
Goat anti-Mouse IgG (gamma chain) Peroxidase-conjugated	1:200
Goat anti-Rabbit IgG (whole Molecules) Peroxidase-conjugated	1:200
Mouse $IgG_1\kappa$ Isotype control purified	1 ug/ml
Goat anti-Rabbit IgG (H+L), HiLyte Fluor 488-labeled	1:200

3.2.2.2. Tissue specimen of melanoma patients

The historical tissue samples used in this study were collected at the Department of Dermatology and Allergology, Philipps University of Marburg, during clinical treatment. This study was performed on 13 histological samples of melanoma patients classified into two groups: 8 cases of metastatic melanoma and 5 cases of primary melanoma. Detailed information on patients was summarized in the table 1 and 2 (see in IHC part of results, chapter 4.1.1.1). Survivors were asked for consent into scientific evaluation of tissue sample. One patient undergoing diagnostic excision of skin metastasis for a separate clinical trial for melanoma therapy consented the establishment of a melanoma cell culture. For blood leucocyte assays buffy coat preparations from local blood bank were used. In addition, few assays were performed from peripheral blood donated by healthy volunteers.

3.2.2.3. Immunohistochemistry

Manual immunostaining was performed in the following process. Formalin-fixed paraffin-embedded tissues of primary and metastatic melanoma were cut into 3 µm sections, transferred onto slides, and dried overnight at room temperature. To deparaffinise, sections were treated in xylene twice for 10 minutes each and subsequently, hydrated with 100 % ethanol twice for 10 minutes followed by treatment with 97 %, 80 % and 70 % isopropanol for 5 minutes, respectively. The sections were rinsed in the distilled water twice for 5 minutes each. To reconstruct the original structure of antigen, a heat-induced method was utilized boiling of sections with citrate buffer (pH 6) at 95 °C for 27 minutes, and rinsed in PBS for 5 minutes on a shaker. Further, to block endogenous peroxidase the sections were incubated in 0.3 % hydrogen peroxide for 30 minutes at room temperature and were washed three times with PBS for 5 minutes each. To reduce non-specific reaction sections were incubated with 5 % goat serum for 30 minutes at room temperature. The additional blocking of endogenous avidin and biotin was achieved by using Avidin/Biotin Blocking Kit. Avidin or biotin solution was diluted in 1% blocking buffer containing goat serum to make a final concentration of 30 % (v/v) and the sections were immersed in this solution for 20 minutes. Afterwards, sections were incubated with diluted primary antibodies overnight at 4 °C inclusive an additional incubation for 1 hour at room temperature. The sections were washed three times with PBS for 5 minutes each and then were incubated with biotinylated secondary antibody for 45 minutes at room temperature. Following washing with PBS three times for 5 minutes, Streptavidin-Alkaline Phosphatase was applied to the slides and incubated

for 30 minutes at room temperature for polymer detection. After 3 times repeated rinsing with PBS, the sections were developed with chromogen, Fast Red or NBT, for 8-20 minutes. Slides were washed with PBS and stained with diluted Hematoxylin (1:2 in PBS) for 2 minutes and the sections were rinsed in running tap water for 4 minutes. Without dehydration with alcohol, sections were mounted directly using mount media.
The immunostaining with antibody against human HMB 45 and S100 was performed by using the automated IHC system, Leica bond.

3.2.2.4. Immunofluorescence

Melanoma cell lines, SK-Mel 37 and NW-Mel 450, were washed twice with cold PBS and kept in PBS containing 1 % FCS on ice. For preparation of cytospin, filter cards, slides and cytofunnels were assembled into the metal holder, by which the hole of funnel and the filter should be in proper position for optimal attachment of cells to the slide and were placed in the cytocentrifuge. Aliquots with about 100 µl of suspension, containing $3x10^4$ cells, were distributed onto each slide and samples were centrifuged at 700 rpm for 5 minutes. The filter and funnel were carefully removed from the slide without damaging of cells. The slides were dried overnight at room temperature and kept at -20 °C before using.
For fixation and permeabilization, at first samples were fixed in 4 % paraformaldehyde for 20 minutes at room temperature. After washing with PBS twice for 5 minutes each, for permeabilization the samples were treated with 0.1 % Triton-100 diluted in PBS for 3 minutes at room temperature. These sections were rinsed in PBS for 5 minutes and then were incubated in 5 % goat serum for 30 minutes at room temperature to block the non-specific binding of immunoglobulins. Following double blocking of avidin and biotin, the slides were incubated in diluted primary antibody overnight at 4 °C plus 1 hour incubation at room temperature. The concentration of primary antibody for this method is 10-fold higher than in immunohistochemistry. Sections were washed with PBS three times for 5 minutes and then incubated with biotinylated secondary antibody for 45 minutes at room temperature. After repeated washing in PBS, streptavidin-Fluor 488-labeled antibody was added on sections and sections were incubated for 2 hours at room temperature. Slides were rinsed three times and the cells were counterstained with DAPI containing mount-medium. The covered slides were inspected under the microscope and kept at 4 °C for long term storage.

3.2.3. Nucleotide analysis

3.2.3.1. RNA isolation

The cultured melanoma cells ($5x10^5$) were rinsed with cold PBS twice and after addition of 0.5 ml TriFast, the cell lysate was passed several times through a pipette. To dissociate the nucleoprotein complex this sample was incubated for 5 minutes at room temperature and then 0.1 ml of chloroform

was added. After vigorously shaking for 15 seconds by hands and short incubation at room temperature, the sample was centrifuged at 12,000 x g for 10 minutes at 4 °C. Following centrifugation, the colourless upper aqueous phase, which contains mainly RNA, was transferred into a new tube from the separated various phases.
To precipitate the RNA, 0.25 ml of isopropyl alcohol was added and mixed with the aqueous phase gently. The sample was incubated for 10 minutes at room temperature and then centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was removed completely and the remained RNA pellet was washed with 0.5 ml of 75 % ethanol after good mixing by vortexing. Following centrifugation of the sample at 7,500 x g for 5 minutes at 4 °C, ethanol was discarded completely and the RNA pellet was dried on air. To dissolve the RNA pellet in DEPC-treated water, solution was passed several times through a pipette. The concentration and purity of RNA was determined by the ratio of absorbance at 260 nm and 280 nm using a Nanodrop spectrometer.
To remove genomic DNA from RNA preparation, 1 unit DNase I was added per 1 µg RNA and the sample was incubated at 37 °C for 30 minutes. The reaction was stopped by heating at 65 °C for 10 minutes after adding with 2 µl of 2.5 mM EDTA. Detection of DNA contamination was performed using `no-RT` control in PCR reactions.

3.2.3.2. cDNA synthesis

To generate the first strand cDNA, 0.5 µg (melanoma/10% serum and immune cells) and 1 µg (melanoma/0% serum) RNA was mixed at first with 0.5 µg of OligodT gently and incubated at 65 °C for 5 minutes. The remaining components were added to the sample and incubated at 42 °C for 60 minutes. To terminate the reaction was ended by heating at 70 °C for 10 minutes.

Components (for 20 ul volume)

1x Reaction Buffer (5x)	4 µl
RiboLock RNase Inhibitor	20 Unit
dNTP Mix	1 mM
Reverse Transcriptase	200 Unit

3.2.3.3. PCR amplification of cDNA

Two µl of the synthesized cDNA was mixed with the following components and the process program of PCR was set according to the specificity of each target product. PCR cycle number of genes (CCND3, CCDNE, BLC2, and Ki67) for serum-starved melanoma was run with 33 cycles. For the full-serum system the optimal PCR cycle number of these genes including housekeeping gene (β-actin) was performed by running different cycles to determine the individual optimal cycle numbers for semi-quantitative PCR. The amount of amplified product was detected during successive cycles starting

from 24 to 39 of the PCR portion of the reaction. This detection was performed to establish the cycle numbers for which the product of amplicons stayed exponential for a limited number of cycles, i.e. when logarithmic amplification begins. This established cycle number was used in PCR experiment for melanoma lines under serum-containing condition only.

Targeted gene		CCND3	CCNE	BCL-2	Ki67	β-actin
10 % serum	SK-Mel 37	30	33	33	33	30
	NW-Mel 450	33	33	36	30	30

Components (for 20 µl volume)

10x Taq buffer	2 µl
dNTP mix	0.2 mM
Primer	100 pmol
Taq DNA polymerase	1.25 Unit
$MgCl_2$	2 mM
cDNA	2 µl

General program for PCR

Step	Reaction	Time	Temperature
1	denaturation	5 minutes	94 °C
2	denaturation	3 minutes	94 °C
3	annealing	30-60 seconds	56-65 °C (upon primers)
4	elongation	90 seconds	72 °C
5	elongation	7 minutes	72 °C
6	termination		4 °C

Cycling of step 2-4 for 26-37 times upon primers

TBE buffer

Tris-Base	89 mM
Boric acid	89 mM
0.5 M EDTA	2 mM

To determine the expression of VIP receptors the cDNA coding the VPAC1 and VPAC2 we used as a control, which were kindly provided by Dr. Lutz (Strathclyde Institue of Pharmacy and Biomedical Science University Strathclyde UK).

3.2.4. Protein analysis

3.2.4.1. Buffers, SDS gel, and antibodies

Buffers

RIPA buffer (pH 8.0)	Sodium chloride	150 mM
	Triton X-100	1 % (v/v)
	Sodium deoxycholate	0.5 % (v/v)
	SDS	0.1 % (v/v)
	Tris	50 nM
Laemmli sample buffer	SDS	2 % (v/v)
	Tris (pH 6.8)	50 mM
	Bromphenol blue	0.2 mg/ml
	DTT	0.1 M
	Glycerol	10 % (v/v)
Running buffer	Glycerin	190 mM
	Tris	25 mM
	SDS	0.1%
Transfer buffer (pH 9.0)	Tris	20 mM
	Glycine	150 mM
	Methanol	20 % (v/v)
TBS (pH 7.5)	Tris	0.2 M
	Nacl	1.5 M
TBST	TBS+Tween 20	0.05 % (v/v)
PBST	PBS+Tween 20	0.05 % (v/v)
Blocking buffer	nonfat dry milk in PBS	5 % (w/v)
	or FCS	5 % (w/v)
Stripping buffer (pH 2.2)	Glycine	1.5 % (w/v)
	SDS	0.1 % (w/v)

Tween 20	1 % (v/v)

SDS Gel

Stacking Gel		Resolving Gel (12.5 %)	
Acrylamide	4 %	Acrylamide	12.5 %
Tris (pH 6.8)	60 mM	Tris (pH 8.8)	320 mM
SDS	0.1 %	SDS	0.1 %
APS	0.06 %	APS	0.06 %
TEMED	0.1 %	TEMED	0.1 %

Dilution of antibodies

Primary antibody	Dilution	Secondary antibody	Dilution
anti-PACAP	2.5 µg/ml	HRP-anti mouse-IgG	1:2000
anti-PAC1	1.5 µg/ml	HRP-anti mouse-IgG	1:2000
anti-BCL	1:500	HRP-anti rabbit-IgG	1:2000
anti-MITF	1.5 µg/ml	HRP-anti rabbit-IgG	1:2000
anti-CCR7	1:1000	HRP-anti rabbit-IgG	1:2000
anti-β-actin	1:20000	HRP-anti mouse-IgG	1:2000

3.2.4.2. Lysate preparation

$1.2x10^5$ /ml cells treated with agents, such as PACAP or receptor antagonist, were washed with cold PBS twice and after complete remove PBS the cells were lysed in RIPA buffer containing protease and phosphatase inhibitor. The adherent cells were scrapped off the dish using a scraper, the cell suspension was transferred into a tube. Following gentle agitation of the suspension for 30 minutes on ice, sample was centrifuged at 14,000 x g for 30 minutes at 4 °C. The supernatant was transferred in a fresh tube and the concentration of cell extract was determined by mean of the Bradford assay using BSA for the protein standard. The sample was stored at -20 °C before using in an experiment.

3.2.4.3. SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

SDS separates proteins, whose polypeptide chains were bound to SDS triggering a fractionation corresponding to molecular size during electrophoresis. Before loading the samples, proteins were denatured and reduced by heating at 95 °C for 5 minutes following adding a loading buffer containing DTT (dithiothreitol) to reduce disulphide bridges and SDS. 80 µg total protein of the sample per well was loaded on a prepared 12.5 % SDS-PAGE gel. The gel was run at 110-150 V till the loaded sample reached the bottom of the gel. Molecular weight of proteins was determined using Protein

Marker (High-Range).

3.2.4.4. Western blotting

Western blot is a technique to separate and identify proteins using specific antibodies. The separated proteins were transferred onto nitrocellulose membrane using semi-dry transfer. The papers and the membrane wetted in transfer buffer were formed as a sandwich (papers/membrane/gel/papers), which was placed on the electrodes and was run at 200 mA for 1.5-2 hours. The membrane was blocked at first with 5 % BSA or 5 % milk solution to prevent non-specific background for 1 hour at room temperature. After blocking the membrane was incubated with a primary antibody diluted in 3 % BSA at 4 °C overnight with agitation. Before incubation with secondary antibody, the membrane was washed in TBST three times for 10 minutes each to remove the residual antibody. The blot was further incubated with the HRP conjugated secondary antibody diluted in blocking buffer at room temperature for 1 hour on the shaker. This blot was washed repeatedly three times for 10 minutes in TBST before development of the membrane. To detect the protein, the membrane was immersed in chemiluminescent-HRP substrate for a short time and the fluorescence from the target protein was detected using a detection machine. The quantification of targeted proteins was performed using ImageJ software and β-actin was used as a loading control.

3.2.4.5. Stripping

To strip off antibodies, the membrane was washed in TBST for 10 minutes and submerged in stripping buffer for 5-10 minutes at room temperature. The stripping buffer was discarded and a fresh stripping buffer was added for 5-10 minutes. The membrane was washed twice in PBS for 10 minutes each and subsequently twice in TBST for 5 minutes each. To detect another targeted protein the membrane was blocked with 5 % BSA or 5 % milk solution before incubation with a primary antibody as described above (see. 3.2.4.1)

3.2.5. ELISA (enzyme linked immunosorbent assay)

3.2.5.1. Buffers and antibodies

Reagents

Biotin-streptavidin-HRP	1:250 dilution
3,3',5,5'-Tetramethylbenzidine (TMB)	
Stop solution (sulforic acid, H_2SO_4)	2 mM

Antibodies

Monoclonal Rat anti-Human IL-6, capture	2 ug
Monoclonal Rat anti-Human IL-6, detection	1 ug
Monoclonal Mouse anti-Human IL-10, capture	1:180
Polyclonal Goat anti-human IL-10, detection	1:270

3.2.5.2. Coating antigen to microplate

The microplate was coated with 50 µl/well of the capture antibody against human IL-6 diluted to a final concentration of 2 µg/ml in PBS. The coated plate was covered with a sealer and incubated overnight at 4 °C. The coating solution was removed and the plate was washed five times with PBS-Tween by filling the well with 150 µl/each. The remaining wash buffer was removed by tapping the plate on a paper towel.

3.2.5.3. Blocking

To block the unspecific binding of capture antibody, 150 µl/well of 1 % BSA in PBS was added in the coated wells and incubated for 2 hours at room temperature on the shaker after sealing the plate.
The plate was rinsed five times with PBS-Tween.

3.2.5.4. Incubation with antigen and detection antibody

Following the complete removal of wash solution, 50 µl/well of the culture medium was added onto each well and the plate was sealed and incubated for 2 hours at room temperature. The plate was washed seven times with PBS-Tween and was incubated with a biotin-conjugated antibody against human IL-6 diluted at 1 µg/ml in blocking buffer (1 % BSA in PBS) for 1 hour at room temperature. As the previous steps the plate was washed seven times with PBS-Tween.

3.2.5.5. Detection

For detection of IL-6, 50 µl of HRP with 250-fold dilution was added on each well and incubated for 30 minutes at room temperature. After washing with PBS-Tween seven times, 50 µl TMB was added to each well and incubated for 15 minutes at room temperature. To terminate the enzyme reaction, 25 µl stop solution was added and the plate was measured at 450 nm and 570 nm as a reference wavelength. The final value is calculated by subtracting the value of 570 nm from those of 450 nm.

ELISA assay for IL-10 was performed according to the Duoset ELISA kit instruction (R&D systems, Abingdon, UK).

3.2.6. Cell viability and cell growth

3.2.6.1. Reagents

Thiazolyl Blue Tetrazolium Bromide
Trypan blue solution
^{3}H-thymidine in RPMI

3.2.6.2. MTT

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is one of the methods to determine cell growth by measuring the absorbance of the reduced solution, formazan, at a wavelength 570 nm using a spectrophotometer. The cells were seeded in a 48 well plate with various cell numbers to attach to the plate and on the following day the medium was changed with fresh medium containing agonist or antagonist. At each time point 5 mg/ml MTT (final concentration: 0.5 mg/ml) was added to the culture and the cells were incubated for 90 minutes at 37 °C. The culture medium was removed completely and DMSO was added to solve the formazan crystals. Following incubation for 15 minutes with agitation, the absorbance was measured at 570 nm with a reference of 700 nm**.**

3.2.6.3. Cell count via trypan blue and via Casy®

Melanoma cell lines were seeded with various cell numbers in a 48 well plate for 24 hours to attach the surface. On the following day, the cells were incubated for 48 or 72 hours in serum containing or low serum (3 % or 0.5 %) medium with agonist or antagonist (1 µM, 100 nM, 10 nM). On each time point, the living and dead cells were counted using the trypan blue dye exclusion method.
Cell viability of immune cells was determined using the Casy®. A cell specific setting defined viable cells from dead cells by using the techniques of electric current exclusion and pulse area analysis. Purified 1x10^{5} CD4^{+} T cells/ml were cultured in the various medium supplements, such as 1 µg/ml α-CD3, 1 µg/ml α-CD28 or co-stimulation of α-CD3/CD28 in the presence or absence of 100 nM PACAP 38 for three days. Resuspended cells were diluted in 10 ml Casy ton ready-to-use isotonic saline solution to measure the cell number.

3.2.6.4. ^{3}H-Thymidine incorporation assay

Purified 1.25 x 10^{5} CD4^{+}CD25^{+}CD127$^{low/-}$ (Treg) /ml or CD4^{+}CD25^{-} (Teff/Tresp) was stimulated with CD3/CD28 beads in the presence of 12.5 U/ml IL-2 for four days. PACAP 38 was supplied in a dose-dependent manner from 100 nM/ml to 1 pM/ml. Cell were labeled with ^{3}H-Thymidine (final concentration: 0.2 µCi/ml) for 17 hours before measuring. Cells were transferred on glass fiber filters.

The dried filters following several rinsing steps were placed in a microtiter filterplate and 25 µl liquid scintillation fluid was added to each well. The number of cells, whose DNA was labeled with ^{3}H-Thymidine, was counted by using a scintillating counter (counts per minute, cpm).

3.2.7. Methods of immunology

3.2.7.1. Buffers and media

TC medium	RPMI 1640	50 ml
	Penicillin	100 U/ml
	Streptomycin	100 µg/ml
	L-Glutamin	2 mM
ACK lysis buffer (pH 7.2-7.4)	NH_4Cl	0.15 M
	$KHCO_3$	1 mM
	EDTA	0.1 mM
	ddH_2O	1 L
FACS buffer	Dulbecco´s PBS	50 ml
	BSA	1 % (v/v)
	NaN_30	1 % (v/v)
MACS buffer (pH 7.2)	Dulbecco´s PBS	50 ml
	BSA	0.5 % (v/v)
	EDTA	2 mM
Dynabead buffer (pH 7.4)	Dulbecco´s PBS	50 ml
	BSA	0.1 % (v/v)
	EDTA	2 mM

3.2.7.2. Isolation of fresh PBMCs

To investigate the human immune cells, peripheral blood mononuclear cells (PBMC) were isolated by using different density gradients between mononuclear cells and other elements of the blood.
The blood samples preserved in citrate phosphate dextrose adenine (CPDA: an anticoagulant) tubes were centrifuged at 1.300 rpm (= 350 x g) for 10 minutes at 4 °C to separate the plasma for other tests. The residual blood components were diluted with sterile PBS by 1:2 and the diluted blood was carefully poured into 50 ml centrifuge tubes containing 15 ml Ficoll. The tubes were centrifuged for 30 minutes at 1.500 rpm (= 450 x g) at room temperature (20 °C) with the brake turned off. Using sterile

pasteur glass pipettes the white cell interface was transferred into a new 50 ml tube. Transferred cells were diluted with PBS and following centrifugation at 1.800 rpm (550 x g) for 10 minutes at 4 °C the supernatant was removed. To remove most of platelet the washing step was repeated twice by adding PBS and centrifuging at 1.300 rpm (350 x g) for 10 minutes at 4 °C. The pellet was resuspended in RPMI medium containing 1 % antibiotics, 1 % non-essential amino acids solution and 1 % sodium pyruvate and the cell number was determined by using trypan blue dye.

3.2.7.3. Separation of cell populations by using magnetic cell separation (MACS)

The MACS application bases on the separation of cells, whose targeted surface antigen is bound with a specific antibody coated with magnetic nanoparticles, resulting in attachment to the column, whereas the other cells expressing no targeted antigen flow through. The whole PBMCs were at first incubated in RPMI medium containing 2 % pooled human serum (PHS) for 1.5-2 hours at 37 °C to separate APCs. Adherent cells were discarded and collected non-adherent cells were used further to isolate specific T cell subsets using MACS.

3.2.7.3.1. Isolation of human $CD4^+CD25^-$ T cells (Teff/Tresp)

The isolation of $CD4^+CD25^-$ T cells was performed by using the MACS $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotec, Bergisch-Gladbach). This application consists of two procedures. At first non-$CD4^+$ cells were labeled with a cocktail of biotin-conjugated antibodies, which are coated with magnetic nanoparticles (MicroBeads) and only unlabeled $CD4^+$ T cells pass through the column, while the labeled immune cells attached on the column. Next, collected $CD4^+$ T cells were labeled with $CD25^+$ antibody conjugated with microbeads, which separated labeled $CD4^+CD25^+$ T cells from unlabeled $CD4^+CD25^-$ T cells passing through the column. For the in vitro experiment these unlabeled $CD4^+CD25^-$ T cells were used.

3.2.7.3.2. Isolation of human $CD4^+CD25^+CD127^{dim/-}$ T cells (Treg)

To separate the $CD4^+CD25^+CD127^{dim/-}$ T cells, the MACS $CD4^+CD25^+CD127^{dim/-}$ Treg isolation kit II human (Miltenyi Biotec, Bergisch-Gladbach) was used. This application is more efficient to discriminate between human Treg and activated T cells regarding the specific expression of CD127, the α-chain of the IL-7 receptor, which is expressed on the mature T cells except on Treg. This procedure was performed firstly by isolation of the non-$CD4^+$ and $CD127^{high}$ T cells, which were labeled with a cocktail of biotin-conjugated antibodies and microbeads. The labeled cells were depleted by separation over a column from the unlabeled $CD4^+CD127^{dim/-}$ T cells passing the column. The collected $CD4^+CD127^{dim/-}$ T cells were labeled again with CD25 microbeads and only cells conjugated with CD25 microbeads were attached on the column. The positively selected $CD4^+CD25^+CD127^{dim/-}$ T cells were collected and used in experiments.

For detection of PAC1R monocytes were used that were kindly provided by Dr. Kaufmann (Department of Immunology, University of Marburg). Monocytes were separated by using an elutriation separating cells of different size.

3.2.7.4. Determination of cell population by flowcytometry (FACS)

Fluorescence activated cell sorting (FACS) is an application for measuring and analysing physical characteristics of single cells, such as size, internal complexity, and relative fluorescence intensity. The scattered and fluorescent light on the cells is collected and arranged to the appropriate detectors, which determine the physical character of each individual particle. As one of the detectors, forward-scattered light (FSC) measures the cell surface area or size and side-scattered light (SSC) is correlated with the inner complexity (shape of the nucleus, the type of cytoplasmic granules or the membrane roughness). More specific identifying can be performed by using a fluorescent dye conjugated with a monoclonal antibody by addition different fluorochromes can be used simultaneously in a mixed population due to the respective fluoresecence channels. Commonly, the low-power air-cooled lasers (argon ion), such as FITC and PE, is used in flowcytometry, because this laser excites more than one fluorochrome. Additionally, APC as a diode laser or PerCP can be as well used to label the cells upon the wavelength. Combined with FSC and SSC data, combination of fluorescent markers identifies the cells and evaluates the relative percentages in the population as well.

3.2.7.5. Suppression assay

In this study, suppression assays were performed in two distinct stimulation processes and the suppression of T cell proliferation was measured by using ^{3}H-Thymidine incorporation, in which a radiolabeled base analog, ^{3}H-Thymidine, was inserted into the replicated strands of DNA during cell proliferation. Stimulation processes differed upon the form of stimulus: T cells were stimulated with monoclonal α-CD3 and α-CD28 in one case or with α-CD3/CD28 T cell Expander beads in the other case. In this study antigen-specific stimulation using antigen-pulsed antigen presenting cells (APCs) were not used to avoid bystander effect by APCs via PAC1R, which is expressed constitutively. For the first type of stimulation with monoclonal 1 µg/ml α-CD3 and 1 µg/ml α-CD28, 2 x 10^{4} Teff/Tresp per well were transferred into a 96-well U-bottom plate. The Teff/Tresp were seeded in the absence of or in the presence of Treg in ratios of 1:64, 1:32, 1:16, 1:8, 1:4, and 1:2 (Treg:Teff/Tresp). The completed medium (RPMI + 10 % PHS) containing stimuli and PACAP 27 or PACAP 38 was conducted in duplicate or triplicate in a volume of 200 µl/well. The other suppression assay was performed by adding T cell Activator CD3/CD28 beads at a bead-to-cell ratio of 1:1 to each well. The cells were incubated for five days at 37 °C and 5 % CO_2 and 20 µCi ml ^{3}H-thymidine was added for the last 17 hours before measuring. The quantification of Teff/Tresp proliferation was determined by counting radioactively labelled cells with thymidine. The proliferation was read out with a liquid scintillation

counter following similar process as mentioned above (see in 3.2.6.4).

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3.2.8. Statistic

Data from once or twice performed experiments are presented as the mean ± standard deviation of duplicates and Student's t-test was performed by a paired two tailed calculation for the data for experiments performed more than three times. This analysis was applied for comparison of data derived from two groups. Differences were considered statistically significant at p values of less than (<) 0.05.

4. Results

The potential effects of PACAP on tumor have been explicated by supporting survival and proliferation and by performing the tolerant immune system in tumor area. This study based on understanding dual roles of PACAP in melanoma and immune cells and further considering PACAP as one of the potential targets for tumor therapies.

4.1. Part I: Autocrine effect of PACAP in melanoma

Various types of tumor have shown PACAP expression and in early 1990, investigators reported expression of VIP/PACAP binding receptors on melanoma cell lines (Fouchier, 1992; Fabre, 1993), but studies dealing with the presence of PACAP and PAC1R on melanoma have not yet been published. This study is the first challenge to demonstrate expression of PACAP and PAC1R as well as to reveal the functions of PACAP in melanoma.

4.1.1. PACAP and PAC1R expression in melanoma

4.1.1.1. Primary and metastatic melanoma produced PACAP

The first experiment dealt with the determination of PACAP and PAC1R expression in melanoma tissues obtained from patients and in melanoma cell lines. To examine the expression of PACAP in human melanoma, immunohistochemical studies were performed in 8 metastatic melanomas and in 5 primary melanomas. The sections were incubated with the specific antibody against PACAP 27 or PACAP 38 peptide. The histological analysis of primary melanoma and metastatic melanoma is summarized in table 1 and 2, respectively. To define positive melanoma HMB 45 antibody was used, which recognizes the melanosome-specific glycoprotein gp 100, and the estimation of the non-specific binding of target primary antibodies was assessed by using rabbit whole serum antibody. Some sections that showed negative staining with HMB 45 were proved with another specific antibody against S100 to determine positive areas for melanoma.
The histological evaluation of HMB 45 and PACAP expression was represented following the ratio of positive tumor cells mainly against PACAP 27 or HMB 45 to the total tumor area and additional data on positive immunoreactivity to PACAP 38 were included as well. The analysis consists of four descending orders of positive expression to HMB 45, PACAP 27, or PACAP 38 in melanoma cells: over 30 % positive, 10-30 % positive, less 10 % positive, and negative (see Tab. 1).

Table 1. Summary of selected sections of primary melanoma

	No.	Gender/Age	Localization	HMB 45	PACAP 27	PACAP 38
Primary melanoma	1	M/69	Shoulder blade	nd.	+++	+++
	2	M/65	Lower leg	nd.	+++	+++
	3	M/71	Shoulder blade	+++	+++	nd.
	4	M/78	Lower arm	+++	+	nd.
	5	F/52	Lower leg	-*	-	nd.

Note: Histological grade of immunoreactivity to HMB 45 or PACAP 27
(+++: over 30 %; ++:10-30 %; +: under 10 %; -: no detection)
nd.: staining was not done, due to the lack of materials.
*: positive immunoreactivity to S100

As shown in table 1, three of 5 total cases demonstrated PACAP 27 production in melanoma cells by presenting of PACAP in more than 30 % of tumor cells. Noticeably, two cases of PACAP 27-positive samples were tested additionally with PACAP 38 and they showed high reactivity grades to PACAP 38 as well. One case (No. 4) had immunoreactivity overall in tumor cluster, however, it was evaluated less than 10 % positive expression of PACAP 27 due to low staining intensity (Suppl. 1). The case of No. 5 showed a negative immunoreactivity to HMB 45, whereas strong S 100 expression was detected overall. PACAP 27 expression in case of No. 5 was evaluated as a negative one due to a high background with the rabbit whole serum antibody used as a negative control.

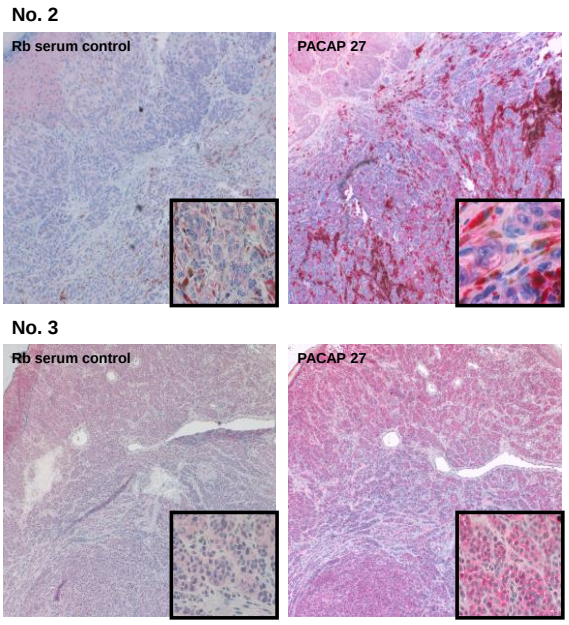

Figure 1. Immunohistochemical analysis of primary melanoma. In primary melanoma (No. 2 and No. 3), the PACAP 27 expression was shown. Anti-PACAP 27 Rb polyclonal antibody was used to detect the target antigen. As negative control for the PACAP 27, Rb whole serum antibody was used. Magnification at 4 x for the background picture and 40 x for small pictures in a boxes, whose image was magnified additionally up to 75 % by using ImageJ.

As shown in figure 1 the incubation of sections from human primary melanoma with PACAP 27 specific antibody gave a clear immunopositive response. The magnified pictures of both sections showed a positive immunoreactivity to PACAP overall, where particularly tumor clusters were found

and detected PACAP 27 was exhibited as a diffuse staining in more or less abundant cytoplasm.
Total evaluated PACAP and HMB 45 expression in selected melanoma sections of primary melanoma were summarized in the table 3. Around 60 % of primary melanoma showed positive immunoreactivity to PACAP 27 in the tumor area with distinct staining intensities. Moreover, sections with a positive immunoreactivity to HMB 45 showed PACAP 27 expression as well, indicating a possibility of co-expression of both factors in primary melanoma (Table 3).

Table 2. summary of selected sections of metastatic melanoma

	No.	Gender/Age	localization	HMB 45	PACAP 27	PACAP 38
Metastatic melanoma	1	M/68	Subcutaneous (Collar)	+++	+	+
	2	M/44	Lymph node (Trunk)	+++	+++	-
	3	F/88	Subcutaneous (Lower leg)	-	+++	+
	4	M/58	Subcutaneous (Trunk)	+++	+++	-
	5	M/80	Subcutaneous (Upper arm)	+++	+++	-
	6	M/62	Lymph node (Trunk)	+++	+	+
	7	M/61	Lymph node (Trunk)	nd.	+++	+
	8	M/68	Lymph node (Collar)	nd.	+++	nd.

Note: Histological grade of immunoreactivity to HMB 45 or PACAP 27
(+++: over 30 %; ++:10-30 %; +: under 10 %; -: no detection)
nd.: staining was not done due to the lack of materials.

Further, five of six cases of metastatic melanoma stained with HMB 45 showed positive immunoreactivity to HMB 45 represented with similar staining grade in cytoplasmic area of melanoma. The expression grade of PACAP 27 was distributed distinctly in sections of metastatic melanoma, but all examined metastatic samples showed positive responses to PACAP 27. An interesting finding was that most metastatic melanoma exhibited negative or weak staining of PACAP 38, whose intensity was much lower than in primary melanoma. Different from primary melanoma, PACAP 27 distribution was observed in all metastatic melanoma sections, although one of samples was negative to HMB 45. In addition, there was no common pattern for intensity of PACAP 27 expression in term of patient's age, gender, or localization of melanoma.

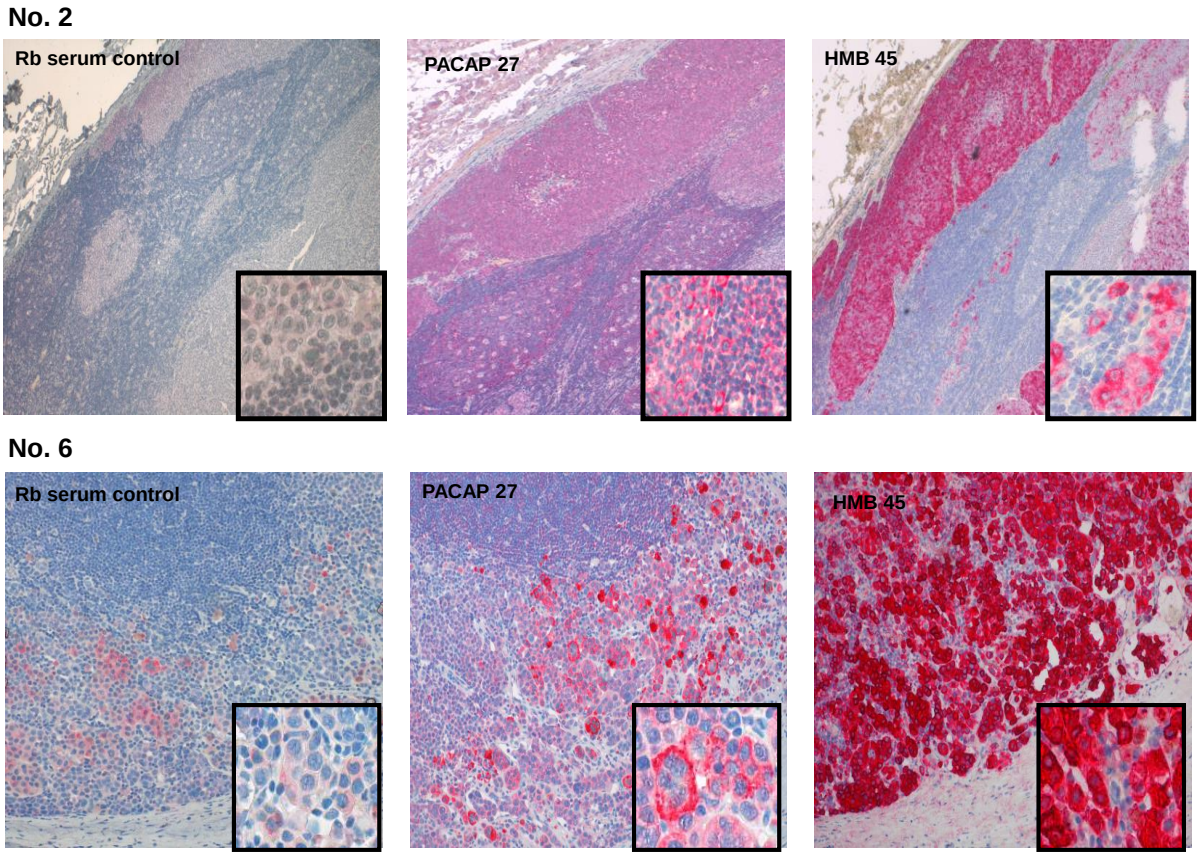

Figure 2. Immunohistochemical analysis of metastatic melanoma. PACAP 27 and HMB 45 expressions were detected in two metastatic melanoma samples in lymph nodes of patients No. 2 and 6. Anti-PACAP 27 Rb polyclonal antibody or anti-HMB 45 mouse monoclonal antibody were used to detect the antigen. As a negative control for the PACAP 27, Rb whole serum antibody was used. Boxed areas are magnified regions where is positive staining. Magnification at 4 x for the background picture and 40 x for small pictures in a boxes, whose image was magnified additionally up to 75 % by using ImageJ.

PACAP 27 in metastatic melanoma was detected in various melanoma cell types (spindle cell, epithelioid) as well as different sizes. Figure 2 presented two selected cases of metastatic melanoma, which had positive PACAP 27 immunoreactivity. Patterns of PACAP immunoreactivity in metastatic melanoma appeared to be different one compared to primary melanoma. Background pictures showed the diffuse staining all over in tumor cells but pictures with high magnification in a box depicted that PACAP 27 was detected in a certain subset of metastatic melanoma cells rather than in all melanoma cells. Compared to primary melanoma the distribution of HMB 45 and PACAP 27 did not completely coincide in metastatic melanoma.

As shown in the table 3, six of eight samples were strongly positive for PACAP 27 expression, which was detected in more than 30 % positive of total tumor cells whereas the remaining samples (two cases) showed a slight positive immunoreactivity with less than 10 % positive of total melanoma cells. Even all metastatic melanoma samples demonstrated a positive response to PACAP 27, the co-expression of PAACAP 27 and HMB 45 was not found in this stage (Table 3). In addition, PACAP 38

seemed to be strongly declined in metastatic melanoma compared to primary melanoma.

Table 3. evaluation of PACAP and HMB 45 in primary and metastatic melanoma

Stage	Staining intensity	HMB 45	PACAP 27	PACAP 38
Primary melanoma	Over 30 % of tumor cells positive	2 (40 %)	3 (40 %)	2
	10-30 % of tumor cells positive			
	<10 % of tumor cells positive	-	1 (20 %)	
	negative	1 (20 %)	1 (20 %)	
	unstained	2		3
Metastatic melanoma	Over 30 % of tumor cells positive	5 (83 %)	6 (75 %)	
	10-30 % of tumor cells positive	-	-	
	<10 % of tumor cells positive	-	2 (25 %)	4
	negative	1 (17 %)	-	3
	unstained	2	-	1

Note: The percentages are shown in brackets.

4.1.1.2. Primary and metastatic melanoma expressed PAC1R

Data on PAC1R expression in the skin from healthy controls showed that melanocytes did not express PAC1R (Suppl. 2). This receptor, however, was detected in the primary and in metastatic melanoma. The summarized data on detected PAC1R in primary and metastatic melanoma is presented in table 4. This analyzed data was based on the ratio of amount of melanoma cells having positive immunoreactivity to PAC1R to the entire tumor area. Different proportions of tumor cells expressing PAC1R were noticeable in this data. Most positively responded immunoreactivity to PAC1R was found in metastatic melanoma rather than in primary melanoma. Clear positive to PAC1R was observable in two cases among the selected sections of primary melanoma stained with PAC1R-specific antibody and the rest showed a negative response to PAC1R. On the other hand, its expression was detected in seven cases of metastatic melanoma with various staining intensities (85 %) and only one case showed a negative immunoreactivity to PAC1R.

Table 4. evaluation of PAC1R in primary and metastatic melanoma

Stage	Staining intensity	PAC1R
Primary melanoma	Over 30 % of tumor cells positive	1 (25 %)
	10-30 % of tumor cells positive	-
	<10 % of tumor cells positive	1 (25 %)
	negative	2 (50 %)
	unstained	1
Metastatic melanoma	Over 30 % of tumor cells positive	2 (25 %)
	10-30 % of tumor cells positive	3 (37.5 %)
	<10 % of tumor cells positive	2 (25 %)
	negative	1 (17 %)
	unstained	-

Note: The percentages are shown in brackets.

Histological pictures of figure 3 demonstrated positive PAC1R expression in metastatic melanoma. The upper panel was a section obtained from lymph node metastasis showing overall a specific positive immunostaining for PAC1R in the cytoplasm and a high magnified picture in a box revealed the localization of PAC1R in melanoma cells including some immune cells, probably APCs. PAC1R expression in another section on the bottom panel was found in a diffuse and scattered pattern as well.

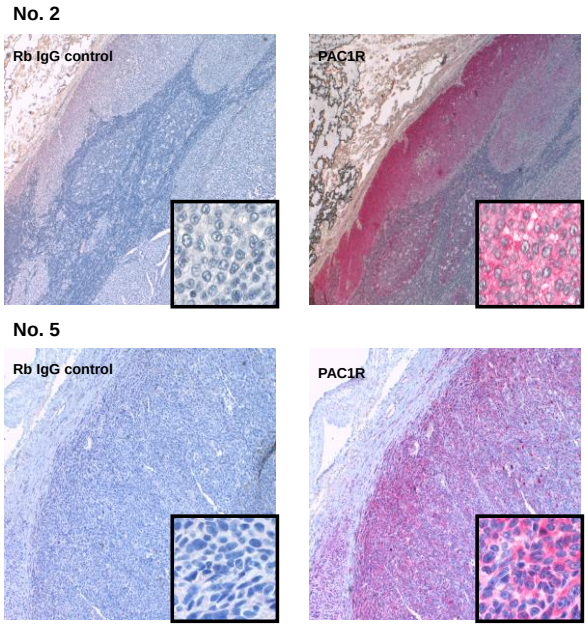

Figure 3. PAC1R expression in metastatic melanoma. Two selected sections obtained from metastatic melanoma patients were stained with a specific antibody against PAC1R. As an isotype control, Rb IgG antibody was used to discriminate the non-specific binding. The upper panel (No. 2) is a section obtained from lymph node metastasis and the bottom one (No. 5) is subcutaneous metastasis in the upper arm. The background picture at 4 x magnification and the boxed picture at 40 x magnification, whose image was magnified up to 75 % by using ImageJ.

4.1.1.3. Melanoma cell lines expressed PACAP and PAC1R

To elucidate a role of PACAP in melanoma, PACAP expression was investigated in the provided five melanoma cell lines, NW-Mel 8, SK-Mel 37, NW-Mel 38, NW-Mel 145, and NW-Mel 450, including melanoma primary cell culture and tissue of lymph node metastasis obtained from melanoma patients by using immunoblotting (Suppl. 3). All examined samples showed the positive expression of precursor PACAP at distinct expression levels. Among the PACAP produced cell lines, two melanoma cell lines, SK-Mel 37 and NW-Mel 450, were selected to further identify, which PACAP subtype was predominantly expressed.

Detection of PACAP subtype expression in melanoma cell lines was assessed by immunohistochemistry and immunofluorescence. The appropriate cell number on the slide performed by cytospin was at first fixed with 4 % PFA and then permeabilized. These prepared cells were stained with specific antibody against human PACAP 27 or PACAP 38. As a negative control rabbit whole serum antibody was assessed.

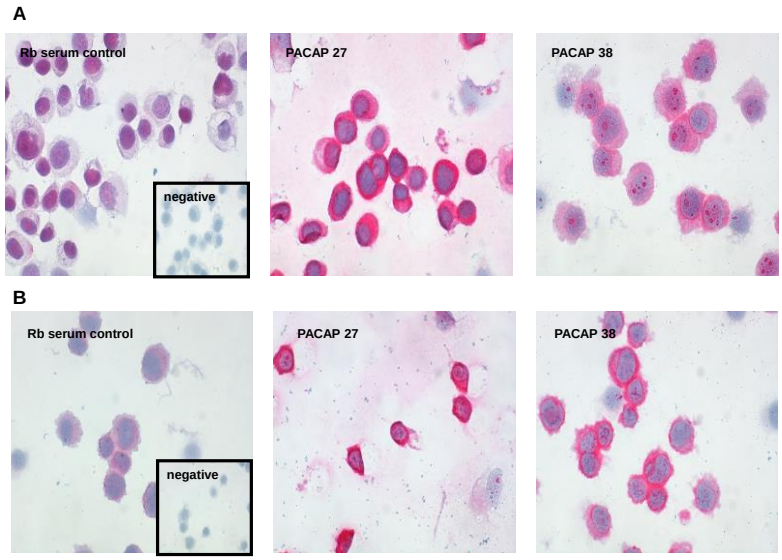

Figure 4. Expression of PACAP 27 or 38 in melanoma cell lines, SK-Mel 37 and NW-Mel 450. (A) SK-Mel 37 and (B) NW-Mel 450 cell lines were stained with a specific antibody against human PACAP 27, PACAP 38, or with the Rb whole serum. Additional negative control was performed by staining without primary antibody following cytospin-preparation of cells. The pictures were captured at 40 x magnification.

The demonstrated immunohistochemical as well as immunofluorescence approaches showed PACAP expression in both melanoma cell lines (Fig. 4; Suppl.4). Positive response to PACAP 27 and PACAP 38 was detected predominantly in cytoplasmic area of both melanoma cell lines but the staining intensity of each PACAP subtype was distinct. SK-Mel 37 expressed both PACAP subtypes, PACAP 27 and PACAP 38, which were however detected with different localization and intensity. The positive signal of PACAP 27 was observed in cytoplasmic area in a high number of cells, whereas PACAP 38 expression presented in or near of nucleic area and this phenomena could be verified by an immunofluorescence approach (Suppl. 4). Similar to SK-Mel 37, NW-Mel 450 expressed both PACAP subtypes and immunofluorescence images elucidated a clear discrimination between cells producing high amount of PACAP 27 and non-producing cells (Fig. 4 B; Suppl. 4). Other than SK-Mel 37, NW-

Mel 450 cells showed distribution of PACAP 38 in cytoplasmic area only.

Further, to detect the VIP/PACAP receptors transcripts at RNA level melanoma cell lines including melanoma primary cell culture were tested with a specific primer for PACAP type I receptor (PAC1R) or type II receptors (VPAC1R and VPAC2R). To prove expression of PACAP receptors SH-SY5Y cells that express constitutively PAC1R or cDNA encoding the human VPAC1R or VPAC2R were used as a positive control. There was no transcript product of VPAC2R in melanoma lines, but all examined melanoma cell lines exhibited VPAC1R and PAC1R at different gene expression level (Suppl. 5).

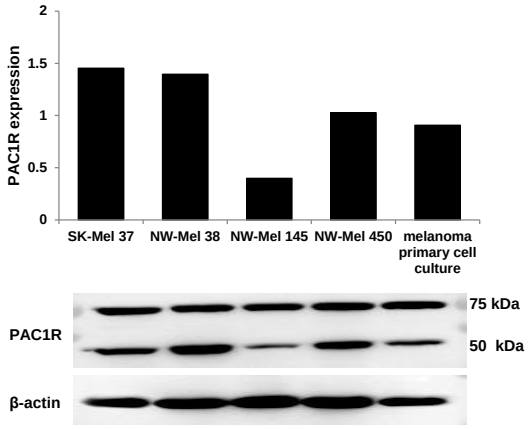

Figure 5. PAC1R expression in melanoma cell lines and melanoma primary cell culture. Immunoblotting was performed to measure PAC1R expression level in selected melanoma cell lines, SK-Mel 37, NW-Mel 38, NW-Mel 145 and NW-Mel 450, including melanoma primary cell culture obtained from one melanoma patient. To detect PAC1R a specific antibody against PAC1R was used and band for PAC1R was detectable at ~50 kDa. As an internal control, β-actin was used. PAC1R expression compared to internal control was analyzed using densitometry and was presented in a bar graph.

PAC1R expression at protein level was verified by Western blotting. Receptor expression was examined on the extracts from four melanoma cell lines and melanoma primary cell culture obtained from one melanoma patient by using a PAC1R specific antibody, which presented two bands at 50 kDa and 75 kDa (Fig. 5). The PAC1R specific band was at 50 kDa, showing varied amount of detected PAC1R expression in the melanoma lines. Analyzed level of PAC1R compared to β-actin was presented in a bar graph indicating a different grade of PAC1R expression in melanoma lines. There was a similar expression pattern between gene and protein expression of PAC1R particularly in SK-Mel 37 and NW-Mel 450, whereby lower expressed PAC1R in NW-Mel 450 than SK-Mel 37 was noticed. On the other hand, stronger gene expression of VPAC1R was observed in NW-Mel 450 rather than SK-Mel 37.

4.1.2. Functions of PACAP in melanoma

To investigate functions of PACAP, two melanoma cell lines, SK-Mel 37 and NW-Mel 450, were selected among the available cell lines. Both melanoma lines express common cancer/testis antigens, NY-ESO/LAGE-1 and MAGE 3, but have different morphological features and sizes. The alteration of morphology was presented in fibroblast-like shape of SK-Mel 37 or epithelial-like shape of NW-Mel 450. SK-Mel 37 cell line has an elongated bipolar shape, while a feature of NW-Mel 450 the regular polygon (Suppl. 6). Additionally, distinct cell sizes of these melanoma cell lines were observed under

the microscope with a more than fivefold larger size of SK-Mel 37 compared to NW-Mel 450. However, both cell lines demonstrated a large nucleus, scarce cytoplasm and expressed simultaneously PACAP and its receptor, PAC1R (Fig. 4 and 5).

4.1.2.1. PACAP displays a cytoprotective and a proliferative role

PACAP has been shown to attenuate cell death induced by various agents in nerve and tumors as well as to be involved in proliferative regulation of mouse primordial germ cells (Chang, 1996; Said, 1998; Gutiérrez-Cañas, 2009; Pesce, 1996). This protective or proliferative effect of PACAP has been implicated in PACAP deficient-mice experiments as well, which showed an increasing apoptosis of cells from ethanol or hydrogen peroxide-induced toxicity compared to the wild type mice (Vaudry, 2005). The following study pointed out that PACAP induced a cytoprotective as well as a proliferative effect in melanoma and these dual effects are dependent on serum conditions.

4.1.2.1.1. SK-Mel 37 is susceptible to serum-induced cell survival

At first, the effect of serum in culture medium on cell viability of the melanoma cells was investigated. SK-Mel 37 and NW-Mel 450 were cultured in serum-containing or serum-deprived culture medium for five days. At each time point viable cells were measured by using MTT. In addition, cell growth pattern was examined in both melanoma lines, which were seeded with five different initial cell seeding numbers (starting from $2x10^5$ cells/ml of SK-Mel 37 and $4x10^5$ cells/ml of NW-Mel 450), and cultured under four different amounts of serum (10, 3, and 0.5 %, or without serum) for three days. Cell viability was measured on day 2 and day 3 by using MTT.

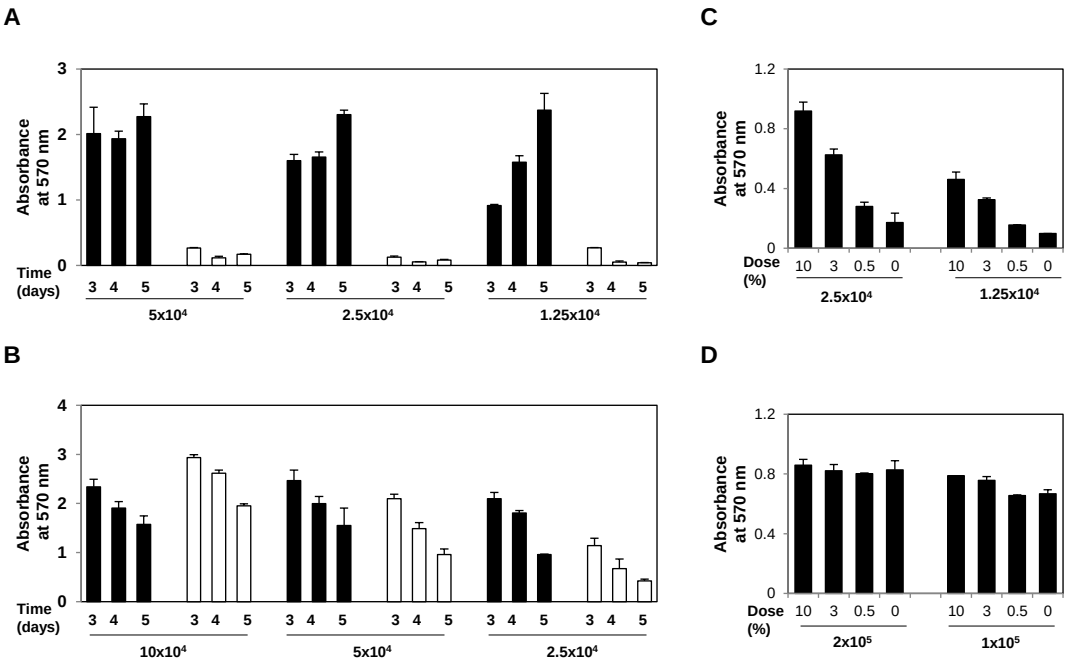

Figure 6. Cell viability of melanoma cell lines under various serum contents. (A) SK-Mel 37 and (B) NW-Mel 450 were cultured in 10 % serum-containing (black bar) or serum-deprived (white bar) medium for five days. Three different initial cell seeding numbers of each melanoma lines were set up and cells were seeded in 96 well plate. Viable cells were measured at each time points (on day 3, 4, or 5) by using MTT. The culture medium was refreshed after every two days. The diagram represents the mean ± SD of duplicates from one or two independent experiments. Survival of melanoma cell lines, (C) SK-Mel 37 and (D) NW-Mel 450, cultured under various serum concentrations was tested. Melanoma cells were seeded in 48 well plate and following changing with fresh medium containing various concentrations of serum (10, 3, 0.5, or 0 %), cell viability was measured on day 3 by using MTT. Cell numbers of each cell line were set up differently according to previous test: SK-Mel 37 with 2.5x or $1.25x10^4$ cells/ml and NW-Mel 450 with 2x or $1x10^5$ cells/ml. The diagram represents the mean ± SD of duplicates from one experiment.

As shown in figure 6, different patterns of cell growth depending on the serum concentration were observed in both melanoma lines. Viable cells of SK-Mel 37 increased in the presence of tropic factor, serum, while in the absence of serum their survival reduced down to less than 10 %. A similar result of cell growth pattern was observed in this cell line seeded with different initial cell seeding numbers as well (Fig. 6 A). On the other hand, serum did not affect much on cell growth of NW-Mel 450. In the presence of serum viable cells decreased instead of increasing and this tendency was shown also in other case with different initial cell seeding numbers. Moreover, cell growth of this cell line was not suppressed even following serum withdrawal. Only in the low initial cell seeding numbers, an increased cell death following serum deprivation was observed (Fig. 6 B). However, the level of survival in serum-deprived NW-Mel 450 cells was not dramatically low like SK-Mel 37. In the next test of cell viability following culturing under various serum contents, distinct serum-induced cell viabilities were observed in both melanoma lines. The more serum amounts in culture medium the higher number of viable cells resulted in SK-Mel 37 and this serum-dependent cell growth became clear in low initial cell seeding numbers and on day 3 compared to high initial cell seeding numbers and on day 2, when showing a slight difference of cell viability among the various serum levels (Fig. 6 C; Suppl. 7 A and C). On the other hand, NW-Mel 450 maintained constant level of viability despite low-serum concentration or different initial cell seeding numbers. Slightly increased cell growth was observed under the full-serum condition (10 %) on day 3, however, NW-Mel 450 did not show a discernible difference in the rate of viable cells (Fig. 6 D; Suppl. 7 B and D). A serum-dependent cell growth was shown in SK-Mel 37 rather than NW-Mel 450 following exposure to four different concentrations of serum (10 %, 3 %, 0.5 %, and 0 %), indicating possibly that mitochondrial activities of both melanoma lines were regulated differently by serum.

4.1.2.1.2. Short-term PACAP treatment displayed a proliferative effect in SK-Mel 37

Next we examined, whether PACAP supplement could regulate viability of melanoma cells. Both melanoma cell lines were cultured in medium containing four different serum concentrations (10 %, 3 %, 0.5 %, or without serum) with 100 nM PACAP 27 for three days and cell viability was measured by MTT assay.

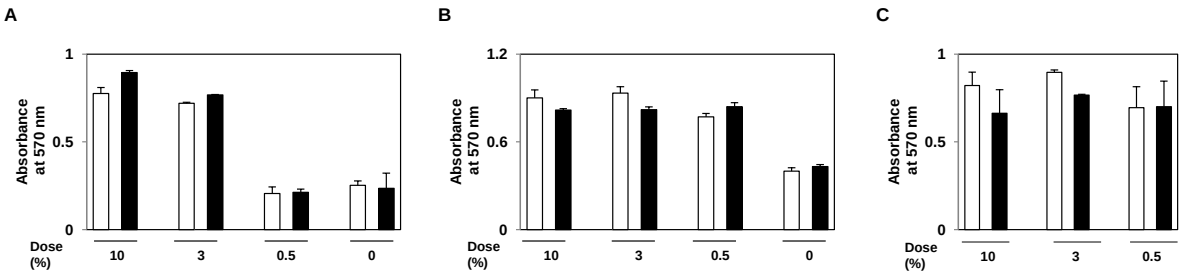

Figure 7. Cell viability after short-term culture with PACAP 27 in medium containing various serum levels. (A) SK-Mel 37 seeded at $5x10^4$ cells/ml or (B) NW-Mel 450 seeded at $1x10^5$ cells/ml were cultured in medium containing various serum amounts (10, 3, 0.5, or 0 %) and 100 nM/ml PACAP 27 for three days. (C) Additional cell growth test of NW-Mel 450 was set up with a high cell seeding number ($2x10^5$ cells/ml) and under three distinct medium conditions (10, 3, or 0.5 % serum in the presence of 100 nM PACAP 27).Their proliferation was analyzed by using the MTT assay and representative data was shown as mean ± SD of duplicates from one or two independent experiments. White bar: control cells; Black bar: PACAP 27-treated cells.

Figure 7 demonstrated the level of viability of SK-Mel 37 and NW-Mel 450 cultured in different media containing various serum concentrations and 100 nM PACAP 27. Firstly, PACAP did not improve survival of SK-Mel 37 under low-serum (0.5 %) as well as deprived-serum conditions. Particularly, cells cultured in 10 % or 3 % serum containing medium showed a slight increase in cell growth in the presence of PACAP, suggesting that PACAP 27 supported cell proliferation as an additional trophic factor in SK-Mel 37, but not as a cytoprotective agent (Fig. 7 A). In contrast, in NW-Mel 450, PACAP suppressed cell growth regardless of serum concentrations. 10 % less and 13 % less cell growth in the presence of PACAP was detected in cells cultured in full-serum and 3 % serum containing medium, respectively (Fig. 7 B). This PACAP-mediated reduction in cell growth was observed in another test with a high cell seeding number (20 % less in full-serum and 15 % less in 3 % serum) as well (Fig. 7 C). In full-serum or 3 % serum containing condition, PACAP exerted a proliferative effect in SK-Mel 37 but an anti-proliferative effect in NW-Mel 450. Furthermore, short-term PACAP treatment (three days) did not protect cell survival of both melanoma lines from serum starvation.

4.1.2.1.3. Long-term PACAP treatment induced dual effects, proliferative and cytoprotective in SK-Mel 37

It was considered that the long-term PACAP treatment could induce different effects on cell growth. Therefore, both melanoma lines were treated with PACAP for four days under deprived-serum condition (0 %) or up to six days under full-serum (10 %) and low-serum condition (0.5 %). Simultaneously, various concentrations of PACAP were added in the culture medium and viable cells were measured by MTT assay at different time period. The initial cell seeding numbers for the long-term culture were set up differently according to previous data, which showed various absorbance levels per culture well depending on cell seeding numbers and serum concentration. A value of

absorbance, which was smaller than 1, was determined as an optimal one, since an expected result was observed by absorption below 1 rather than above 1.5 (Suppl. 7 A). Therefore, an initial cell seeding number for the long-term culture was started with small cell number in order to obtain the final absorbance value below 1. A long-term serum starvation of SK-Mel 37 for more than four days was no more reliable due to an increased detachment of cells in spite of using the attachment factor, Poly(ethyleneimine) (PEI).

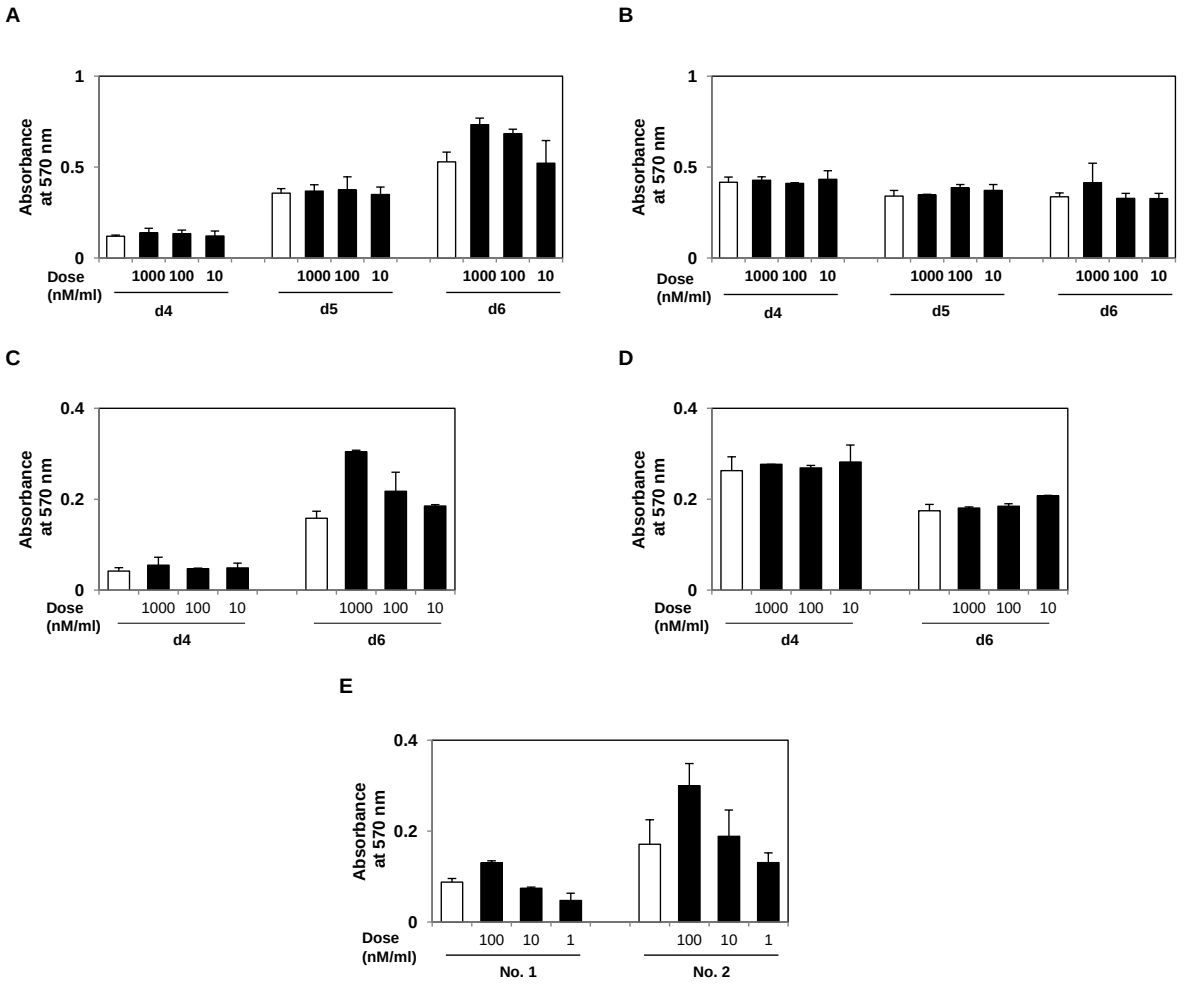

Figure 8. Cell viability of SK-Mel 37 following long-term culturing with PACAP 27 in medium containing three different serum concentrations. Effect of PACAP 27 on the cell growth of SK-Mel 37 measured in the presence of two different serum concentrations. Cells were seeded at $1.25x10^4$ cells/ml (A) or at $0.625x10^4$ cells/ml (C) in full-serum (10 %), and at $1x10^5$ cells/ml (B) or $5x10^4$ cells/ml (D) in low-serum condition (0.5 %) in the presence of three different PACAP concentrations (1 µM, 100 nM, and 10 nM/ml) for 6 days. Cell viability was measured by MTT at different time points. (E) PACAP-induced cell survival was investigated in cells following serum withdrawal. Cells seeded at $5x10^4$ cells/ml (No. 1: low cell seeding number) or $1x10^5$ cells/ml (No. 2: high cell seeding number) were treated with various amounts of PACAP 27 (100 nM, 10 nM, and 1 nM/ml) for 4 days. Quantification of live cells was performed by using MTT and the results are shown in the absorbance (570 nm-700 nm). The data on (A) and (E) represent the mean of two independent experiments with ± SD performed in duplicates. The other data on (B, C, and D) was derived from one experiment with ± SD performed in duplicates. White bar: control cells; black bar: PACAP 27-treated cells.

The effect of PACPA 27 on SK-Mel 37 proliferation was shown in Figure 8. An increase in cell numbers was seen following a long-term PACAP treatment, particularly on day 6. This effect of PACAP was concentration-dependent and the highest level reached on day 6 with 1.4-fold increased cell growth in the presence of 1 µM PACAP 27 (Fig. 8 A). A similar proliferative effect of PACAP was observed in other approach performed with a low cell seeding number. Under same culture condition, SK-Mel 37 showed a 1.9-fold increased cell growth following PACAP supplement (Fig. 8 C). This proliferative effect of PACAP was maintained for 8 days regardless of PACAP concentration. Interestingly, cell viability on day 8 at highest PACAP dose, however, showed a lower increase than the other medium conditions, supposing that the long-term exposure to high concentrated PACAP could induce the occurrence of early cell exhaustion, which caused a cell death or cell cycle arrest (Suppl. 8 A). This proliferative PACAP effect was not restricted in cells under full-serum system. Cells cultured in 3 % serum containing medium with PACAP 27 resulted as well in an elevated cell growth (Supp. 8 B). On the other hand, neither supplement with high dose of PACAP nor long-term PACAP treatment was capable to improve cell growth of SK-Mel 37 under low-serum condition (0.5%), which was seen in another test with different initial cell seeding number as well (Fig. 8 B and D). It should be noticed here that PACAP is a tropic factor helping cell growth rather than being a substitute for serum. An interesting observation in the presence of PACAP 27 was an increased survival cells when serum-deprived after few days of culture (Fig. 8 E). The quantification of viable cells from both approaches, in which two different initial cell seeding numbers were set up, showed a similarly increased cell viability and an approximately 1.8-fold enhanced cell growth was observed in the PACAP-treated group with a high cell seeding number. This result implies that PACAP is protective against serum starvation and a long-term PACAP treatment is required for the prevention from cell death. To sum up, long-term PACAP treatment might have dual effects, a proliferative as well as a cytoprotective in SK-Mel 37 depending on serum conditions.

4.1.2.1.4. PACAP was neither involved in proliferative nor in cytoprotective effect in NW-Mel 450

Previous data on the short-term PACAP treatment (3 days) showed that PACAP suppressed the proliferation of NW-Mel 450 cultured in 10 % or 3 % containing medium, but improved cell growth under low-serum (0.5 %) or serum-deprived condition (0 %) slightly (Fig. 7 B). To examine cell viability following a long-term PACAP treatment, NW-Mel 450 was treated with three different concentrations of PACAP (1 µM, 100 nM, or 10 nM) in 10 % or 0.5 % serum-contained medium for six days as well as in serum-deprived medium for four days. The viable cells were measured by MTT assay in a time-dependent manner.

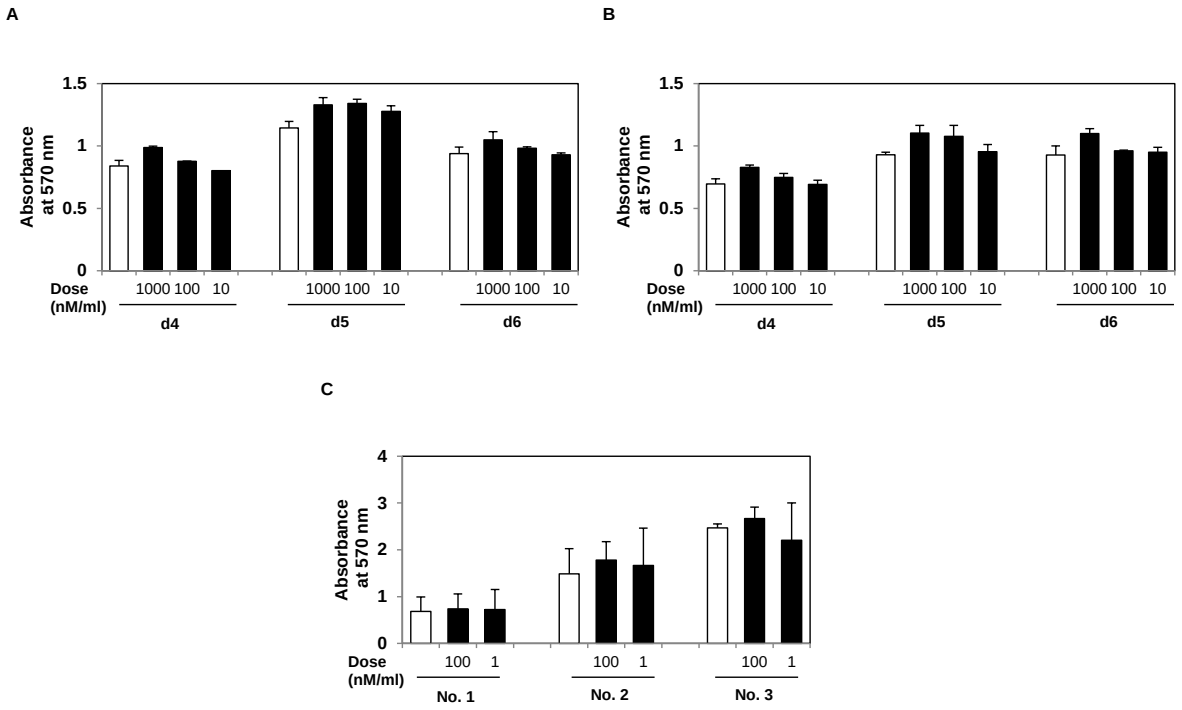

Figure 9. Cell viability of NW-Mel 450 following long-term culturing with PACAP 27 in medium containing three different serum concentrations. NW-Mel 450 was seeded at 2.5x10^4 cells/ml in 10 % serum (A) and at 5x10^4 cells/ml in 0.5 % serum-containing medium (B) and cells were incubated in different concentrations of PACAP 27 (1 µM, 100 nM and 10 nM/ml) for 6 days. Cell growth was measured by using MTT in a time-dependent manner. (C) To investigate the PACAP effect on cell survival, NW-Mel 450 was seeded at three different cell seeding numbers (No.1: 2.5x10^4 cells/ml; No.2: 5x10^4 cells/ml; No.3: 1x10^5 cells/ml) in serum-withdrawal culture medium containing 100 nM or 1 nM/ml PACAP 27. After four days viable cells were measured by MTT assay. The results are shown in the absorbance (570 nm-700 nm). The data is presented as a mean ± SD of duplicates from one experiment (A and B) or two independent experiments (C). White bar: control cells; black bar: PACAP 27-treated cells.

Analyzed data showed a slightly improved cell growth under both serum-containing conditions (1.2-fold for 10 % and 0.5 % serum) during 6 days-culture in the presence of 1 µM PACAP 27 and a similar growth rate was shown regardless of serum contents, or culture duration (Fig. 9 A and B). Another set of experiments, in which cell growth under 10 % or 0.5 % serum condition was examined for 8 days following PACAP 27 supplement, showed a similar potency of PACAP on cell growth (1.2-fold increased) regardless of serum concentration (Suppl. 8 C and D).

Furthermore, the rate of cell survival under serum starvation also was improved up to 1.2-fold in the presence of 100 nM PACAP compared to the control (Fig. 9 C). PACAP 27-mediated effect on cell growth of NW-Mel 450 led to a similar result in all tested culture systems, supposing that PACAP did not display a critical role in cell growth regulation.

To sum up, the viability level of NW-Mel 450 did not reduce despite serum withdrawal, while SK-Mel 37 was susceptible to serum starvation with a dramatic decrease in cell viability. Secondary, PACAP showed different effects in both melanomas cell lines: long-term PACAP treatment in SK-Mel 37 caused proliferative as well as cytoprotective effects in full-serum and serum-deprived condition,

respectively, whereas such effects did not appear in NW-Mel 450.

4.1.2.1.5. PAC1 receptor is major receptor involved in regulation of cell viability in SK-Mel 37

We observed that exogenous PACAP administration particularly in SK-Mel 37 provided proliferative and cytoprotective effects. It was considered that disrupting the binding of PACAP by using PACAP receptor antagonists could inversely reduce cell viability. Therefore, melanoma cells were treated with VPAC1or PAC1 antagonist to confirm the PACAP effect on viability and to determine the selectivity of PACAP receptors being involved in regulation of cell growth. Both melanoma cell lines, SK-Mel 37 and NW-Mel 450 were incubated in full-serum medium containing PACAP receptor antagonist, VPAC1R antagonist or PAC1R antagonist, for four days and then cell viability of each line was analyzed by using the MTT assay. Appropriated cell numbers per well were selected from experiments described in previous chapters.

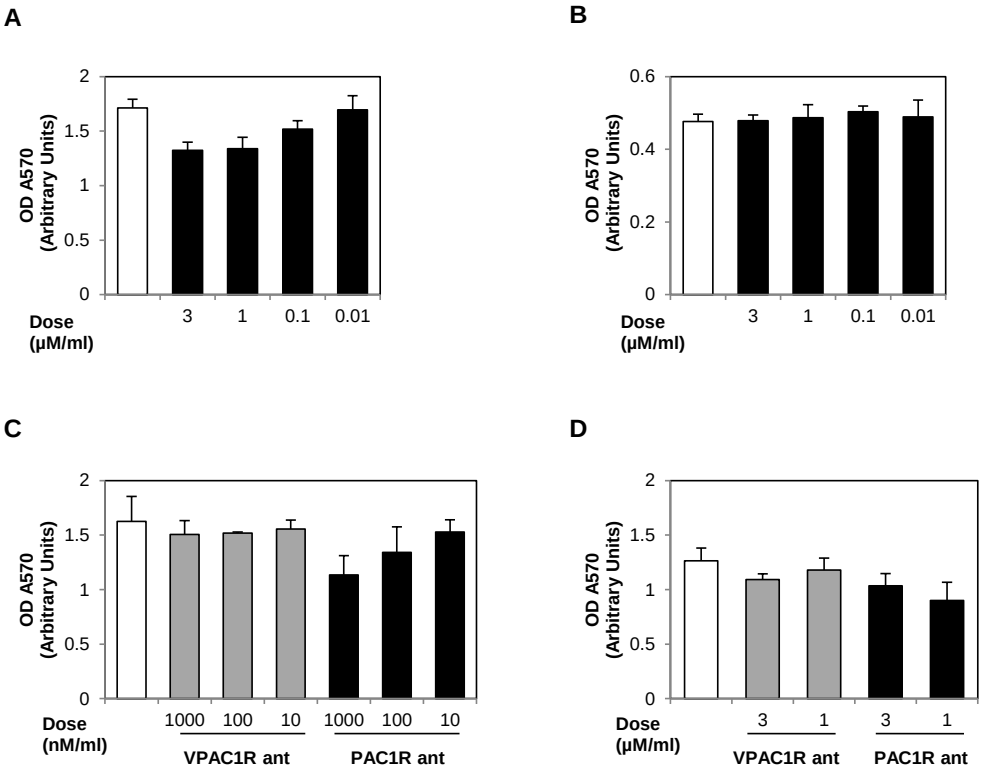

Figure 10. Suppression of cell survival following treatment with PACAP receptor type I or II antagonists in melanoma cell lines, SK-Mel 37 and NW-Mel 450. (A) SK-Mel 37 ($1x10^5$ cells/ml) and (B) NW-Mel 450 ($2.5x10^4$ cells/ml) were cultured in serum containing medium (10 %) including PACAP receptor 1 antagonist, PAC1R antagonist, for three days. Cell viability was assessed in an antagonist dose-dependent manner (3 μM, 1 μM, 100 nM and 10 nM/ml). To estimate the main receptor responding to PACAP, (C) SK-Mel 37 ($5x10^4$ cells/ml) and (D) NW-Mel 450 ($2.5x10^4$ cells/ml) were incubated in the presence of each receptor antagonist, VPAC1R or PAC1R antagonist for four days. MTT measuring showed the different regulation of PACAP antagonism for cell survival in both melanoma cell lines. Results shown are presented as a mean ± SD of duplicates from one experiment. White bar: control cells; Grey bar: VPAC1R antagonist-treated cells; Black bar: PAC1R antagonist-treated

cells.

Blocking the PACAP binding via PAC1R antagonist caused a decreased cell survival only in SK-Mel 37, whereas there was no clear difference in cell viability between antagonist-treated and untreated NW-Mel 450 cells in spite of exposure to high concentration of PAC1R antagonist, (3 µM) (Fig. 12 A and B). Cell growth of SK-Mel 37 decreased in a receptor antagonist dose-dependent manner and approximately 27 % decreased cell growth was shown at 1 µM PAC1R antagonist. An interesting observation was that VPAC1R antagonist did not suppress cell growth and an approach with low cell seeding numbers led to a similar result on cell viability (Fig. 12 C and Suppl. 9). This observation indicated that PAC1R displayed a main role for the regulation of cell viability in SK-Mel 37. On the other hand, neither PAC1R antagonist nor VPAC1R antagonist showed substantially decreased cell growth of NW-Mel 450, which reinforced the previous observations.

4.1.2.2. PACAP up-regulated gene expression involved in survival and proliferation of melanoma cells

PACAP prevents cell death by up-regulating BCL-2 expression or by regulating cell cycle compartments and it improves on the other hand cell proliferation through enhanced cAMP stimulation shown by others in different tumor types (Erhardt, 2004; Onoue, 2008; Sokolowska, 2008). Previous data on PACAP effects causing improved cell survival posed the suggestion that elevated cell viability following PACAP treatment in serum-deprived culture medium could be associated with the expression of gene related to cell survival (BCL-2). Furthermore, other gene expressions involved in cell proliferation were investigated such as, cyclin D, cyclin E, and Ki67, whose highly expressed genes have been found in most malignant melanomas and have been regarded as a mark for metastatic melanoma (Henrique, 2000; Bales, 2005). In particular, expression of cyclin D3 was detected in 96 % of the primary melanomas and 97 % of the metastatic melanomas (Flørenes, 2000). To reveal whether PACAP is involved in regulation of cell survival or proliferation in melanoma, four selected genes were examined in this study.

4.1.2.2.1. PACAP up-regulated gene expression related to the cell cycle regulators, cyclin D3 and cyclin E in serum-containing medium

Distinct effects of PACAP were observed on cell viability of the two melanoma cell lines, SK-Mel 37 and NW-Mel 450. PACAP displayed in SK-Mel 37 as a proliferative or cytoprotective role depending on serum concentration, while PACAP-mediated cell growth regulation in NW-Mel 450 was not observed (Fig. 8; Fig. 9). To understand the distinct effects of PACAP on cell viability of both cell lines, the involvement of PACAP in gene expression related to cell cycle regulators, cyclin D3 and cyclin E was examined.

Both melanoma lines were cultured in full-serum (10 %) medium containing 100 nM PACAP 27 for four days and SK-Mel 37 or NW-Mel 450 were collected on each day. To evaluate the expression level, RT-PCR analysis was performed using specific primers for the two cell cycle regulators, cyclin D3 and cyclin E.

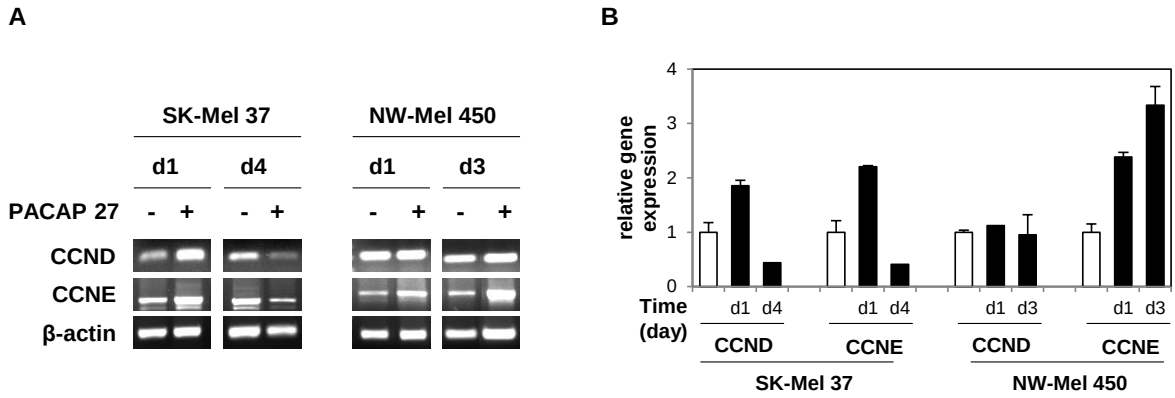

Figure 11. Gene expression of cell cycle regulators in SK-Mel 37 and NW-Mel 450 cultured in serum-containing medium in the presence of PACAP 27. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.5x10^5$ cells/ml in full serum-medium (10 %) and after 24 hours cells were cultured in a new medium containing 100 nM/ml PACAP 27 and 10% serum as indicated. RNA was isolated from treated and control cells every day and the gene expression level of cell cycle regulators, cyclin D3 (CCND) and cyclin E (CCNE), was analyzed by using PCR. (B) Quantification of each band for SK-Mel 37 (on day 1 and 4) and NW-Mel 450 (on day 3) was demonstrated as the ratio of each targeted gene expression of treated or control cells relative to individual β-actin of each sample by using densitometric analysis. The analyzed results represent mean ±SD of two independent experiments. To avoid nonspecific background PCR production (plateau effect) or low product yield, the optimal cycle number for each primer was estimated by choosing the exponential phase for PCR product amplification. White bar: control; black bar: PACAP 27-treated cells.

Amplified gene expression of cyclin D3 and cyclin E was observed in both melanoma cell lines following PACAP 27 treatment and their expressions were regulated in a time-dependent manner. Figure 11 A exhibited that transcripts of cyclines were highly up-regulated in PACAP-treated cells on the first day, whose level however dropped drastically on day 4. This difference is more obvious in the diagram showing PACAP-induced cyclin D3 expression (1.9-fold) and cyclin E (2.2-fold) on day 1, but dramatically decreased gene expressions (60 % less) on day 4 (Fig. 11 B). This finding suggests that PACAP induces transcripts for cell cycle regulators in SK-Mel 37 at early time point causing early cell exhaustion, while PCR products from control cells still increased on day 4. NW-Mel 450 demonstrated a different pattern of both gene expressions. The gel images presented nearly constant cyclin E expression levels in PACAP-untreated cells relative to the control gene, β-actin, and a slightly decreased cyclin D3 on day 3. PACAP-treated cells, in contrast, showed a highly increased cyclin E expression on day 3 only, which was presented in the diagram with up to 3.3-fold increased cyclin E but with a constant cyclin D expression (Fig. 11 A and B).

4.1.2.2.2. PACAP induced gene expression related to apoptosis protection and proliferation marker, BCL-2 and Ki67, in serum-contained medium

Further, both melanoma cell lines were examined to validate the involvement of PACAP in gene expressions of BCL-2 or Ki67 by PCR.

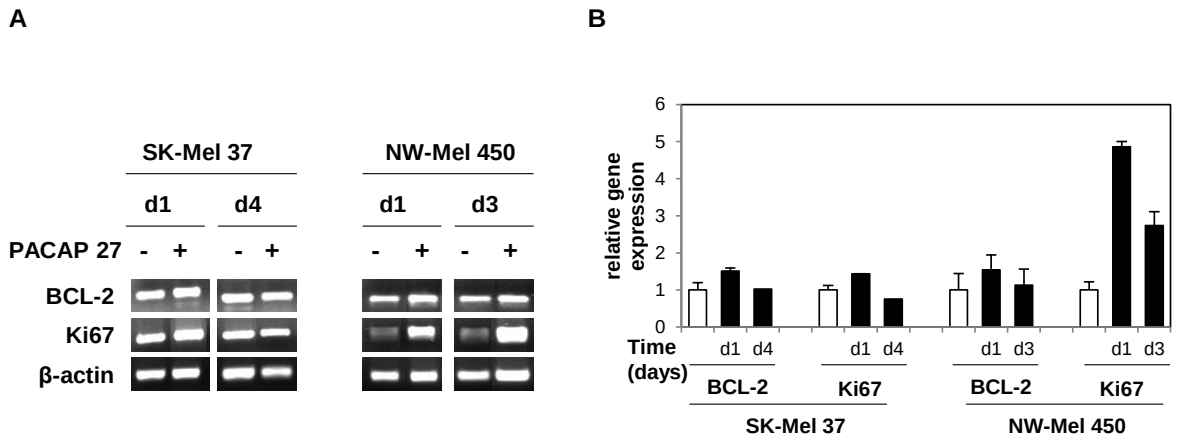

Figure 12. Gene expression of cell survival and proliferation in SK-Mel 37 and NW-Mel 450 cultured in serum-containing medium in the presence of PACAP 27. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.5x10^5$ cells/ml in full serum-medium and after 24 hours cells were cultured in a new medium containing 100 nM/ml PACAP 27 and 10 % serum as indicated. RNA was isolated from treated and control cells every day and the gene expression level of the cell survival (BCL-2) and proliferation marker (Ki67) was analyzed using PCR. (B) Quantification of each band for SK-Mel 37 (on day 1 and 4) and NW-Mel 450 (on day 3) was demonstrated as the ratio of each targeted gene expression of treated or untreated cells relative to individual β-actin of each sample using densitometric analysis. The analyzed data was presented as mean ±SD of two independent experiments. To avoid nonspecific background PCR production (plateau effect) or low product yield, the optimal cycle number for each primer was estimated by selecting the exponential phase for PCR product amplification. White bar: control; black bar: PACAP 27-treated cells.

The gel image showed that PACAP up-regulated gene expressions of BCL-2 and Ki67 in SK-Mel 37 on day 1, but their levels decreased on day 4. On the other hand, the control cells maintained a constant level of gene expression of BCL-2 and Ki67 relative to β-actin for 4 days (Fig. 12 A). The bar graph demonstrated a 1.5-fold and 1.4-fold increase in BCL-2 and Ki67 expression in SK-Mel 37 following PACAP supplement, respectively, which however decreased on day 4 and particularly Ki67 expression level was under the control (25% less) (Fig. 12 B). NW-Mel 450 showed a strongly increased gene expression of BCL-2 and Ki67 in the presence of PACAP on the gel photo, which represented in the bar graph with a 1.5-fold or 4.9-fold increase in BCL-2 or Ki67, respectively (Fig. 12 A and B). A noticeable observation in NW-Mel 450 was the long-term PACAP effect on Ki67 expression, whose level remained still high on third day (2.7-fold) (Fig 12 B).

4.1.2.2.3. PACAP affected gene expression of cell cycle regulators under serum deprivation, particularly in NW-Mel 450

Cell viability studies showed the cytoprotective effect of PACAP in SK-Mel 37 under serum-deprived conditions (Fig. 8 E). To define the involvement of this effect in gene expression related to the cell cycle regulators or proliferation, both melanoma cell lines, SK-Mel 37 and NW-Mel 450, were cultured in serum-deprived medium containing 100 nM PACAP 27 for 5 days and mRNA expressions of cyclin D3 and cyclin E from the collected cells were tested by using PCR method. The results of the PCR levels on the diagram were evaluated in relation to the control.

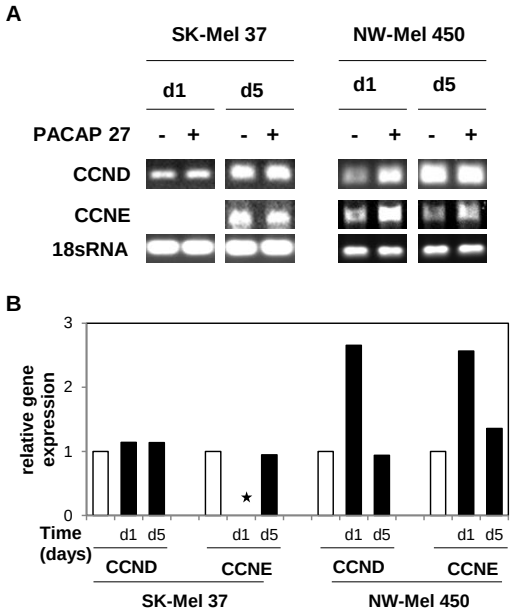

Figure 13. Gene expression of cell cycle regulators in SK-Mel 37 and NW-Mel 450 cultured in serum-deprived medium in the presence of PACAP 27. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.5x10^5$ cells/ml in full serum-medium and after 24 hours cells were cultured in serum-deprived medium containing 100 nM/ml PACAP 27 as indicated. RNA was isolated from treated and control cells on day 1, 3, and 5 and the gene expression level of cell cycle regulators (cyclin D3/CCND and cyclin E/CCNE) was analyzed by using PCR method. (B) Quantification of each band for SK-Mel 37 and NW-Mel 450 (on day 1 or 5) was demonstrated as the ratio of each targeted gene relative to individual 18sRNA of treated cells and untreated using densitometric analysis from one experiment. *: no PCR product on a gel. White bar: control; black bar: PACAP 27-treated cells.

SK-Mel 37 did not show a noticeable difference in RNA expression of both cell cycle regulators, cyclin D3 and cyclin E, between treated and untreated cells under serum withdrawal. Almost similar expression level of cyclin D3 in PACAP 27-treated SK-Mel 37 to the control cells could be observed on both days 1 and 5 (1.1-fold). PCR product of cyclin E was not detectable on the gel on the first day and its relative level on day 5 was slightly decreased compared to the control level on day 5 (Fig. 13 A and B). NW-Mel 450 treated with PACAP induced a 2.7-fold and 2.6-fold up-regulated gene expression of cyclin D3 and cyclin E on day 1, respectively. However, PACAP did not appear to support the maintenance of highly elevated level of cyclin D3 and cyclin E in the long-term serum-free culture (Fig. 13 A and B).

4.1.2.2.4. PACAP affected gene expression of apoptosis protection and proliferation marker, BCL-2 and Ki67, under serum deprivation in a time-dependent manner

Further, RNA expression of apoptosis protection and proliferation marker, BCL-2 and Ki67, in both

melanoma lines was checked in the absence of serum for elucidating PACAP efficacy.

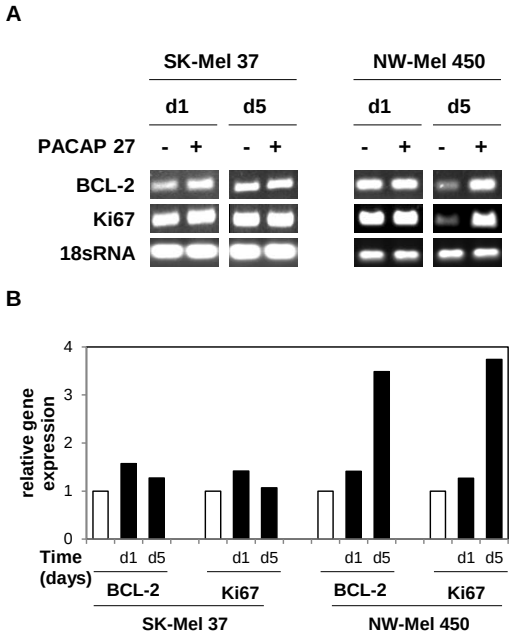

Figure 14. Gene expression of cell survival and proliferation in SK-Mel 37 and NW-Mel 450 cultured in serum-deprived medium in the presence of PACAP 27. (A) SK-Mel 37 or NW-Mel 450 was seeded at $1.5x10^5$ cells/ml in full serum-medium and after 24 hours cells were cultured in serum-deprived medium containing 100 nM/ml PACAP 27 as indicated. RNA was isolated from treated and control cells on day 1, 3, and 5 and the gene expression level of cell survival (BCL-2) and proliferation (Ki67) was analyzed using PCR. (B) Quantification of each band for SK-Mel 37 and NW-Mel 450 (on day 1 or 5) was demonstrated as the ratio of each targeted gene relative to individual 18sRNA of treated cells and control cells using densitometric analysis from one experiment. White bar: control; black bar: PACAP 27-treated cells.

PACAP induced a 1.7-fold and 1.4-fold increased gene expression of BCL-2 and Ki67 in SK-Mel 37, respectively. However, its effect did not remain till the fifth day, when both expression levels of BCL-2 (1.3-fold) and Ki67 (1.1-fold) decreased (Fig. 14 A and B). On the other hand, the gel photo showed almost similar transcripts amount of BCL-2 and Ki67 in PACAP-untreated and -treated NW-Mel 450 on day 1. Surprisingly, high amount of PCR product of both genes in PACAP-treated cells maintained till the fifth day, while these genes in control cells declined. This greater difference could be observed in a densitometry diagram showing a high amount of transcripts reaching almost 4-fold enhanced BCL-2 and Ki67 on day 5. A long-term PACAP treatment in NW-Mel 450 seems to induce more strong effects in both gene expression (Fig. 14 A and B).

To sum up, PACAP improved gene expression of BCL-2 and Ki67 in both culture systems in the presence or absence of serum and the constant elevated level of BCL-2 could be observed in long-term culture under serum starvation as well, corresponding to cell viability. In addition, PACAP-induced regulation of gene expressions was shown to differ depending on serum contents, culture duration, or melanoma cell types.

4.1.2.2.5. Blocking PACAP binding caused a reduction in gene expression

The regulation of exogenous PACAP in the four targeted gene expressions was proved in previous study (Fig. 11, 12, 13, and 14). Further, it was examined, how the expression of the four selected genes, cyclin D3, cyclin E, BCL-2, and Ki67, changed following interrupting PACAP binding. Melanoma lines were treated with VPAC1R or PAC1R antagonist and these gene expressions were

analyzed by PCR.

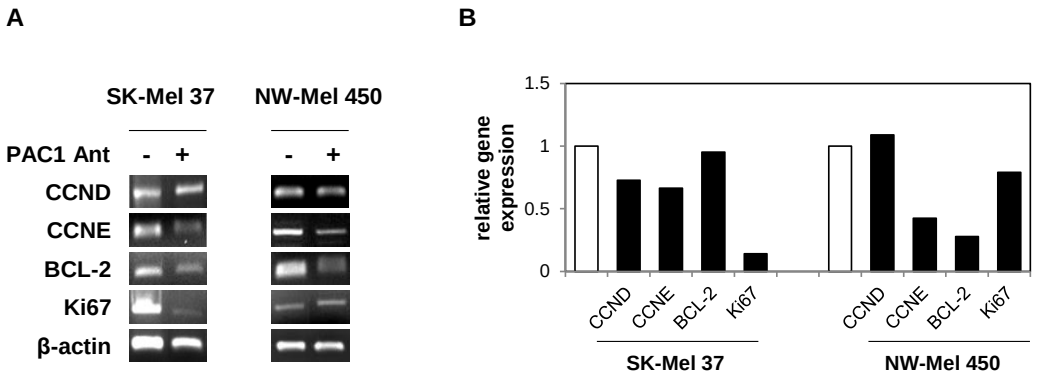

Figure 15. Suppressed gene expression in melanoma cell lines by blocking of PAC1R. (A) SK-Mel 37 and NW-Mel 450 were seeded at 1.5x10^5 cells/ml in full serum-medium and after 24 hours cells were cultured in new medium containing 1 µM/ml PAC1R antagonist and 10 % serum for 17 hours. RNA was isolated from treated and control cells and the gene expression levels of cell cycle regulators, cyclin D3 (CCND) and cyclin E (CCNE) and of anti-apoptotic regulator (BCL-2) as well as proliferation marker (Ki67) were analyzed by using PCR. (B) Quantification of each band was demonstrated as the ratio of each targeted gene relative to individual β-actin of treated cells to control cells from one experiment using densitometric analysis. White bar: control; black bar: PAC1R antagonist-treated cells.

The binding of endogenous PACAP to PAC1R was blocked by PAC1R antagonist, which induced a prominent reduction, especially in cyclin E in both melanoma cell lines, SK-Mel 37 and NW-Mel-450 (40 % and 60 % less, respectively). Corresponding to the previous results showing enhanced cyclin E by PACAP, the blocking of PACAP binding caused a substantial decrease in cyclin E level. In contrast, both cell lines showed differently regulated gene expression of cyclin D3 following blocking receptor: 30% less expression of cyclin D3 in SK-Mel 37, but 1.1-fold slightly enhanced transcripts in NW-Mel 450 (Fig. 15 A and B). A strongly reduced gene expression of Ki67 in SK-Mel 37 (90 %) and BCL-2 in NW-Mel 450 (70 %) was observed in the presence of PAC1R antagonist as well. On the other hand, the level of BCL-2 in SK-Mel 37 and of Ki67 in NW-Mel 450 was decreased only 10% and 20% less following PAC1R blocking, respectively (Fig. 15 A and B). VPAC1R antagonist showed a similar suppressive effect on gene expression in SK-Mel 37, but in NW-Mel 450 VPAC1R did not seem to be involved in gene expression except for cyclin E (Suppl. 11). This finding indicated that at first VPAC1R as well as PAC1R might be involved in regulation of gene expression, however, a critical regulation of gene expression of Ki67 and BCL-2 may be differently influenced via PAC1R in SK-Mel 37 and NW-Mel 450, respectively.

4.1.2.3. PACAP improved BCL-2 protein production in melanoma cells

Previous approaches elucidated that PACAP was involved in regulation of four cell growth-related gene expressions in melanoma lines. In particular, elevated gene expression of BCL-2 was observed

in both melanoma cell lines in the presence of PACAP corresponding to protection of cell viability from serum starvation. Next, to confirm previous data on enhanced gene expression by PACAP, BCL-2 expression was tested at the protein level in SK-Mel 37 and NW-Mel 450 following treatment with PACAP.

4.1.2.3.1. BCL-2 protein level slightly increased in SK-Mel 37 following PACAP treatment under serum-free condition

At first, BCL-2 protein expression was evaluated in both melanoma cell lines cultured in serum-free medium. Cells were treated with 100 nM PACAP 27 under serum-free condition for four days. Cell lysates were collected on day 2 and 4 and immunoblotting was performed by using a specific antibody against BCL-2.

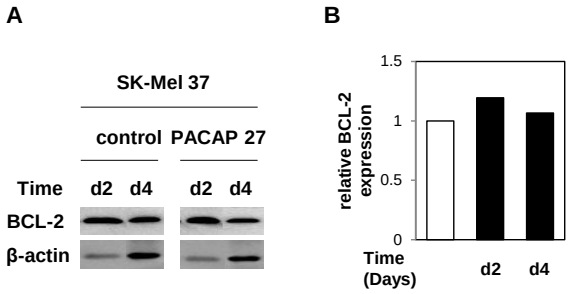

Figure 16. PACAP effect on BCL-2 protein expression in SK-Mel 37 under serum-free condition. (A) SK-Mel 37 was seeded at $1.9x10^5$ cells/ml in full serum-medium and after 24 hours cells were cultured in new changed serum-deprived medium containing 100 nM/ml PACAP 27 for four days. Collected cell lysates on day 2 or 4 were used to analyze the protein level of BCL-2 of treated and control cells by western blot. (B) Quantification of each band was demonstrated as the ratio of BCL-2/β-actin of treated cells from one experiment by using densitometric analysis. White bar: control; black bar: PACAP 27-treated cells.

PACAP induced a slight increased BCL-2 protein expression in SK-Mel 37 (1.2-fold) on day 2, while its level on day 4 was similar to the control despite PACAP supplement (Fig. 16). This result corresponded to the gene expression of BCL-2, whose level was up-regulated in the presence of PACAP 27 (Fig. 14). Evaluation of BCL-2 in NW-Mel 450 was difficult under serum starvation due to weak detected β-actin expression especially on day 2 and analyzed BCL-2 expression from the fourth day showed much lower protein level in PACAP-treated NW-Mel 450 than in control, while PACAP-treated cells resulted in much higher β-actin product but lower expression of BCL-2 than in control (Suppl. 12).

4.1.2.3.2. BCL-2 protein level increased in both melanoma lines under PACAP supplement In a dose- and time-dependent manner

Under serum deprivation the expression of housekeeping protein, β-actin, was disrupted substantially in both melanoma cell lines, which caused the difficulty to analyze the PACAP-mediated BCL-2 expression in our study. Therefore, we examined melanomas cultured in full-serum medium (10%) to define PACAP-mediated regulation of BCL-2 expression. Cells were treated with PACAP in a dose-

dependent manner starting from 100 nM to 1 nM and after 48 hours incubating, collected cell lysates were used for quantification of BCL-2 protein level.

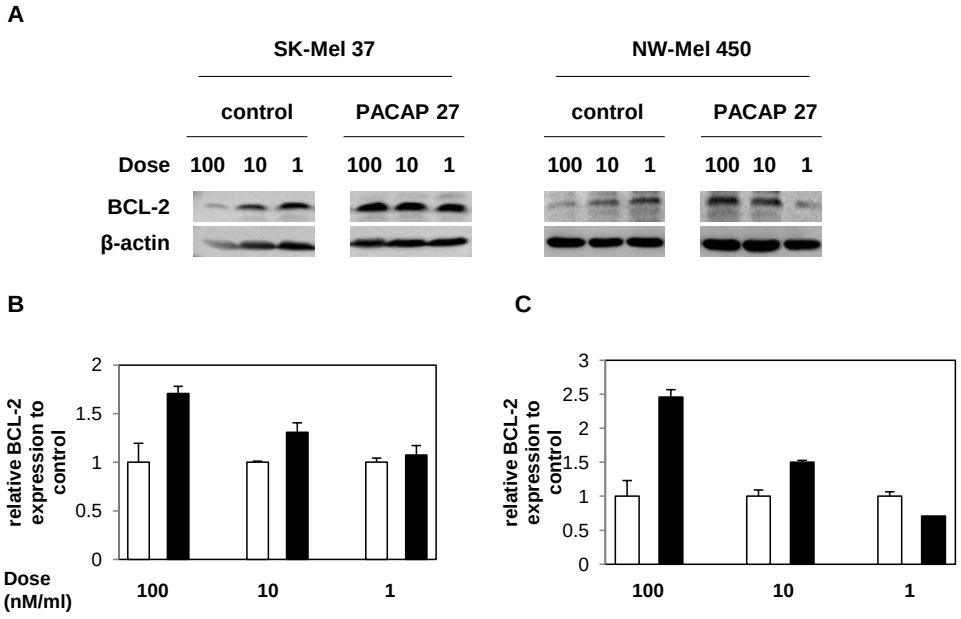

Figure 17. PACAP effect on BCL-2 protein expression in a dose-dependent manner in SK-Mel 37 and NW-Mel 450 cultured in full-serum condition. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.2x10^5$ cells/ml and $2.4x10^5$ cells/ml, respectively and cells were cultured for 24 hours. The next day culture media were renewed with fresh serum-containing medium including three different concentrations of PACAP 27 starting from 100 nM to 1 nM/ml and cell were cultured for 48 hours. Collected cell lysates were subjected to Western blot with anti-BCL-2 antibody and quantification of each band for SK-Mel 37 (B) and NW-Mel 450 (C) was analyzed by using densitometry. The data was shown as a ratio of BCL-2/β-actin of treated cells compared to the control samples. Data on the lower panel are representing the mean ± SD of two independent experiments. White bar: control cells; black bar: PACAP 27-treated cells.

Both melanoma cell lines showed a PACAP dose-dependent induction in BCL-2 level. BCL-2 in SK-Mel 37 was enhanced at 100 nM PACAP 27 (1.7-fold) but low PACAP concentration was much less effective to up-regulate BCL-2, showing an effect on BCL-2 regulation only at the highest concentration used. Similarly, up-regulation of BCL-2 product in NW-Mel 450 was detectable, seen even at low concentration of 10 nM PACAP (1.5-fold) and at the highest PACAP concentration showed 2.6-fold increase in BCL-2 level (Fig. 17). These data showed that PACAP improved the protein level of BCL-2 in both melanoma lines corresponding to an elevated BCL-2 at transcriptional level (Fig. 12) particularly in NW-Mel 450.

Next, it was tested how long elevated BCL-2 expression could be maintained constantly following PACAP exposure in both melanoma lines. Cells were treated with 100 nM PACAP 27 or PACAP 38 for five days and BCL-2 expression level was analyzed from collected cell lysates by immunoblotting.

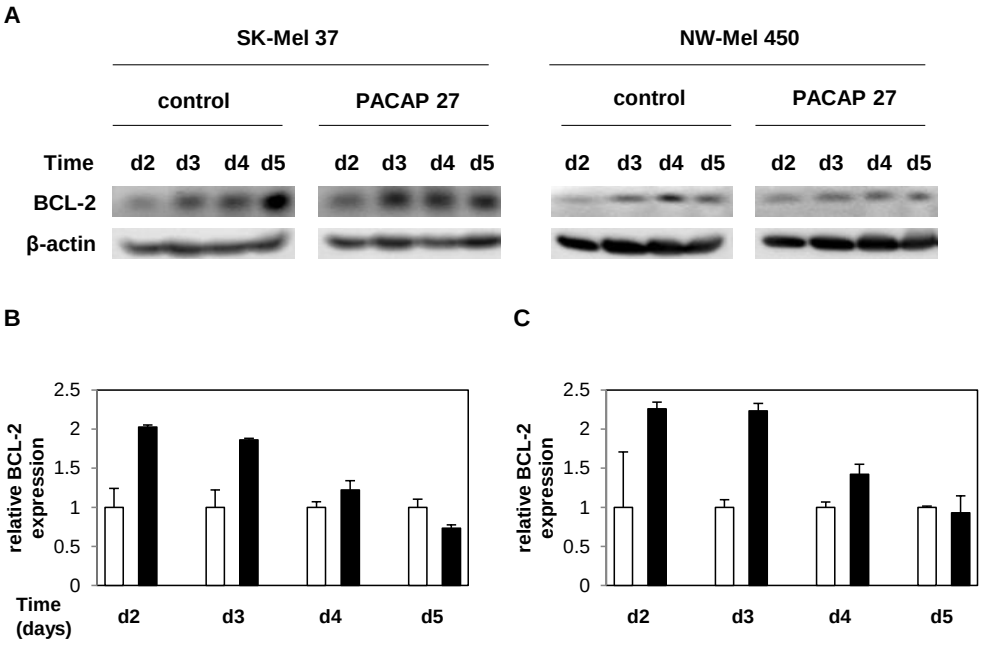

Figure 18. PACAP effect on BCL-2 production by SK-Mel 37 and NW-Mel 450 in long-term culture. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.2x10^5$ cells/ml for 24 hours and after rinsing cells were cultured in serum-containing medium in the presence of 100 nM/ml PACAP 27 for five days. Medium was refreshed every two days and cell lysates were collected at each time point. Prepared lysates were analyzed for BCL-2 level from treated and control cells by immunoblotting. The graph for SK-Mel 37 (B) or NW-Mel 450 (C) showed the ratio of BCL-2/β-actin of treated cells compared to the control samples and the represented data was the mean ± SD of two independent experiments. White bar: control cells; black bar: PACAP 27-treated cells.

Figure 18 demonstrated that an up-regulated BCL-2 in both cell lines maintained over 4 days in the presence of PACAP 27 and a time-dependent decrease in BCL-2 protein level in both cell lines was seen over time. The highest increase in BCL-2 production of both melanoma cell lines was on day 2 (more than 2-fold for both cell lines) and it was consistent on following day (1.9-fold for SK-Mel 37 and 2.2-fold for NW-Mel 450). Noteworthy, on day 4 a minute augmentation of BCL-2 production occurred in both melanoma lines despite PACAP supplement and a further decrease in BCL-2 was observed on day 5 even below vehicle control (27% less in SK-Mel 37 and 7% less in NW-Mel 450 compared to the control). Similar results were observable with elevated BCL-2 level following PACAP 38 supplement as well (Suppl. 13).

4.1.2.3.3. BCL-2 production was suppressed following blocking PAC1R

In the previous approach, different patterns of RNA expression of BCL-2 in both cell lines were shown following PAC1R antagonist treatment: less effect of PAC1R antagonist on suppression of BCL-2 gene expression in SK-Mel 37 but a remarkable decrease in BCL-2 expression in NW-Mel 450 following PAC1R blocking (Fig. 15). To confirm the involvement of PAC1R in the regulation of BCL-2 expression, melanoma cell lines were exposed to 1 µM antagonist for four days and BCL-2 level was

quantified by Western blot.

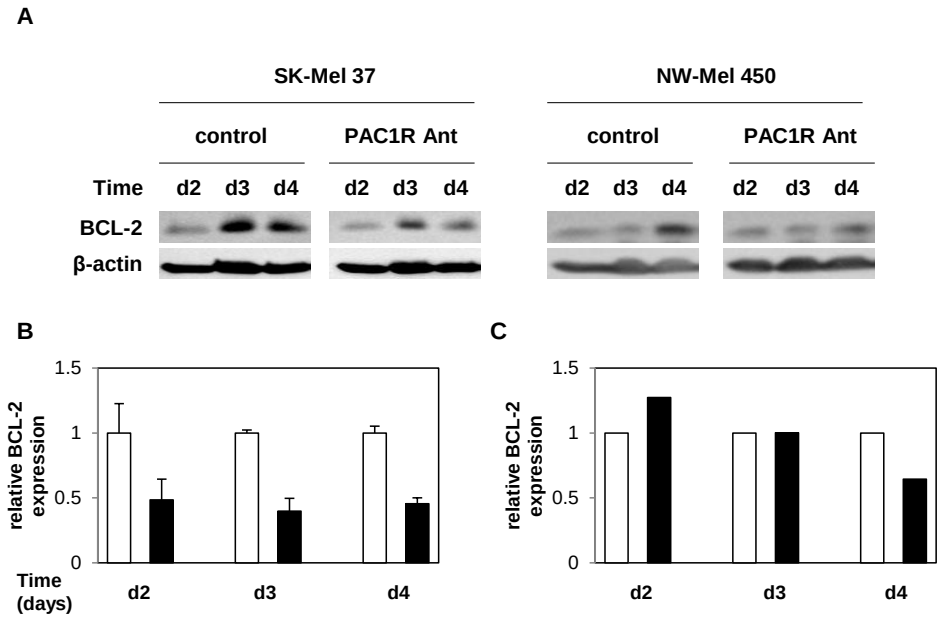

Figure 19. Reduced BCL-2 expression in SK-Mel 37 and NW-Mel 450 following treatment with PAC1R antagonist. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.2x10^5$ cells/ml for 24 hours and after rinsing cells were cultured in serum-containing medium in the presence of 1 µM/ml PAC1R antagonist for four days. Medium was refreshed every two days and cell lysates were collected at each time point. BCL-2 protein levels were assessed by immunoblotting. The graph for SK-Mel 37 (B) and for NW-Mel 450 (C) showed the ratio of BCL-2/β-actin of treated cells compared to the control samples and the represented data was the mean ± SD of two independent experiments. White bar: control cells; black bar: PAC1R antagonist-treated cells

Interestingly, figure 19 demonstrated different results in BCL-2 protein expression in both cell lines following exposure to PAC1R antagonist. A strong decrease in BCL-2 level was observable in SK-Mel 37 and its substantial reduced expression continued for four days (50 % less expression compared to the control), while NW-Mel 450 showed virtually unchanged BCL-2 level for three days and lately on day 4 the suppressive effect of PAC1R antagonist on BCL-2 production (36 % less production) was detected. Since PACAP acts in part via VPAC1R, VPAC1R antagonist was applied in few experiments (Suppl. 14). This VPAC1R antagonist caused a similar result in declined BCL-2 expression in both melanoma lines on the fourth day only (Suppl. 14 A and B). Moreover, treatment with combination of both VPAC1R and PAC1R antagonists as well induced a decrease in BCL-2 production in SK-Mel 37, but not in NW-Mel 450 (Suppl. 14 C and D). These experiments indicated that PAC1 receptor and VPAC1R in SK-Mel 37 were involved in regulation of BCL-2 expression and particularly PAC1R seemed to be a pivotal receptor for BCL-2 expression. NW-Mel 450 showed suppressive effect of both antagonists but only following long-term treatment.

4.1.2.4. PACAP was involved in the regulation of MITF protein expression in melanoma

One of factors inducing up-regulation of BCL-2 is MITF, which regulates development and differentiation of melanocytes (Gaël, 2002). To reveal whether PACAP was involved in the regulation of MITF expression, both melanoma lines were cultured in presence of PACAP or PAC1R antagonist for four days. MITF protein level analyzed from collected cell lysates by immunoblotting.

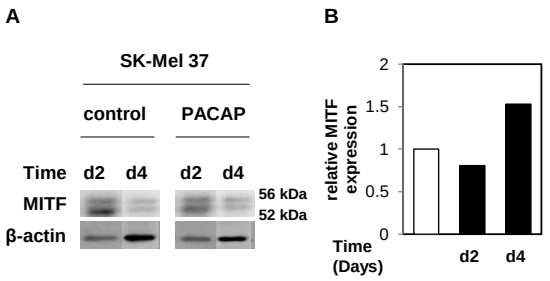

Figure 20. MITF expression in melanoma lines following treatment with PACAP 27. (A) SK-Mel 37 was seeded at $1.9x10^5$ cells/ml and was cultured in serum-contained medium for 24 hours. After rinsing on the following day cells were incubated for four days in serum-free medium containing 100 nM/ml PACAP 27. (B) The evaluation of MITF level was assessed by blotting and the ratio of MITF/β-actin from one experiment was presented in a diagram. White bar: control cells; black bar: PACAP 27-treated cells.

SK-Mel 37 showed a short form of MITF with two bands at 52 kDa and 56 kDa, while MITF with only one band at 56 kDa was detected in NW-Mel 450 (Fig. 20; Suppl. 15). MITF expression in PACAP-treated SK-Mel 37 was lower than the control on day 2, but increased slightly (1.5-fold) on the fourth day (Fig. 20). An additional approach with PACAP 38 as well showed an enhanced MITF on the fourth day in SK-Mel 37 particularly (Suppl. 16 A and B). On the other hand, NW-Mel 450 weakly expressed MITF in untreated and treated cells with PACAP and a relatively low β-actin expression led to difficulties in analysis of MITF expression in NW-Mel 450 (Suppl. 15). Similar to BCL-2 expression under serum-deprived conditions, PACAP did not seem to contribute much to the regulation of MITF expression.

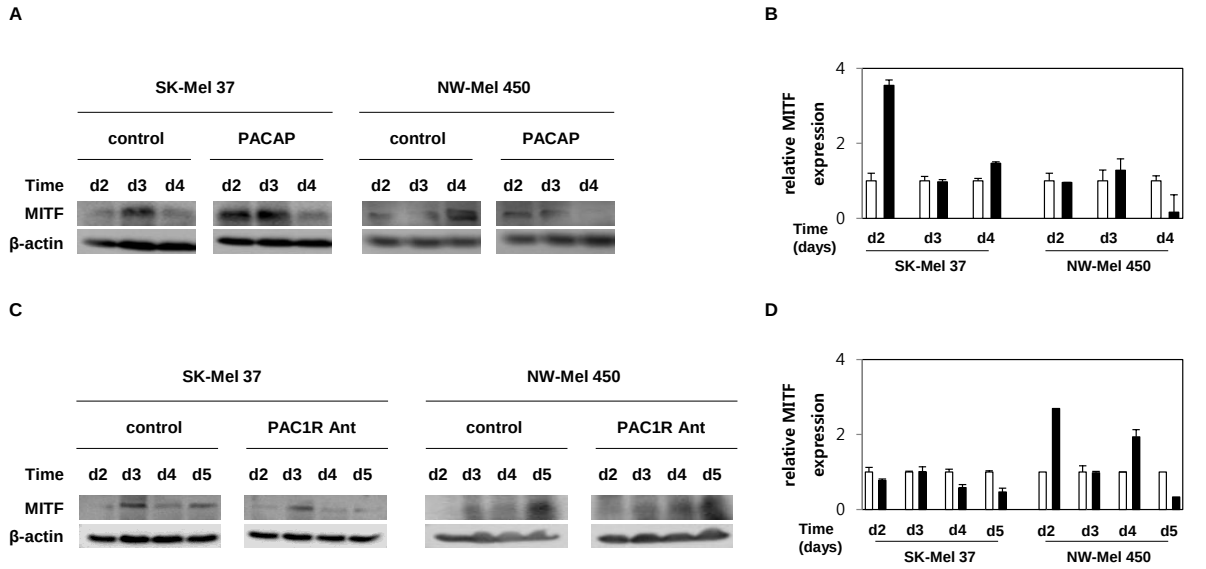

Figure 21. MITF expression in melanoma cells following treatment with PACAP 27 or PAC1R antagonist. (A) Both melanoma lines were incubated at $1.2x10^5$ cells/ml for four days in full-serum medium (10 %) containing 100 nM/ml PACAP 27. (C) Melanoma lines were seeded at $1.2x10^5$ cells/ml and treated with 1 µM/ml PAC1R antagonist under full-serum condition for five days. Medium was refreshed every two days and cell lysates were collected at each time point. MITF expression level was assessed by immunoblotting and the graph for the melanoma treated with PACAP (B) or treated with antagonist (D) showed the ratio of MITF/β-actin of treated cells compared to the control samples. The represented data was the mean ± SD of two independent experiments. White bar: control cells; Black bar: PACAP or PAC1R antagonist-treated cells. White bar: control cells; black bar: PACAP or PAC1R antagonist-treated cells.

However, up-regulated MITF expression was observed in SK-Mel 37 cultured in serum-containing medium. Under full-serum condition (10 %) both melanoma cell lines produced a single low band at 52 kDa of MITF only and showed a remarkable difference in the MITF level in the presence of PACAP 27 (Fig. 21 A and B). Induction of MITF in PACAP-treated SK-Mel 37 was much higher on the second day (3.5-fold increased MITF) than the other days, when MITF expression dropped steeply (Fig. 21 B). In contrast, MITF expression in NW-Mel 450 was almost similar to the control and only on day 3 its level was slightly elevated (1.3-fold). A similar pattern of MITF expression could be observed in both melanoma lines following treatment with PACAP 38 peptide and particularly PACAP-treated NW-Mel 450 showed a 4.1-fold up-regulated MITF expression on third day (Suppl. 16 C and D). This observation indicates that PACAP-mediated regulation of MITF appears earlier in SK-Mel 37 than in NW-Mel 450.

Controversially, i.e. a declined MITF expression was shown following blocking the PAC1R (Fig. 21 C and D). The MITF levels were reduced by long-term PAC1R blocking in SK-Mel 37, which demonstrated a decreasing MITF level over time (4.2-fold less on day 4 and 5.4-fold less on day 5), except on the third day when showing a similar level of MITF to the control. PAC1R blocking in NW-Mel 450 seems to induce more MITF expression rather than suppress it and a suppressive effect of PAC1R antagonist on MITF appeared only the day after refreshing the medium with antagonist, supposing that its action could be achieved only after short-treatment with PAC1R antagonist (Fig. 21

C and D).

4.1.2.5. Effect of PACAP on chemokine receptor CCR7 of melanoma cells

Metastatic melanoma is occurring when primary tumors invade into various organs via blood vessels and chemokine receptors play a critical role for the invasion of tumor (Wiley, 2001; Murakami, 2002; Kim, 2006). Previous investigations by others indicated that cAMP stimulation was implicated in an increased chemokine receptor expression (Gagliardi, 2003; Zhu, 2013). It would be interesting to define whether PACAP as a known cAMP stimulator is involved in chemokine receptor regulation to support the invasiveness of melanoma. One of chemokine receptors playing a critical role in invasive metastatic melanoma is the C-C chemokine receptor 7 (CCR7) for homing to lymph nodes during metastasis of melanoma (Emmett, 2011; Van den Bosch, 2013). To address this question, CCR7 expression in melanoma cell lines was investigated under PACAP supplement. PCR or immunoblotting was conducted to analyze mRNA or protein expression of CCR7 in melanoma cells, respectively. SK-Mel 37 and NW-Mel 450 were cultured for 17 hours in the presence of 100 nM PACAP 27 and the purified RNA from collected cells was used to evaluate the level of CCR7 gene expression. For immunoblotting cells were treated with three different doses of PACAP 27 (100, 10, and 1 nM) for 48 hours under full serum conditions (10 %) and quantitative CCR7 expression from PACAP-treated or control cells was analyzed.

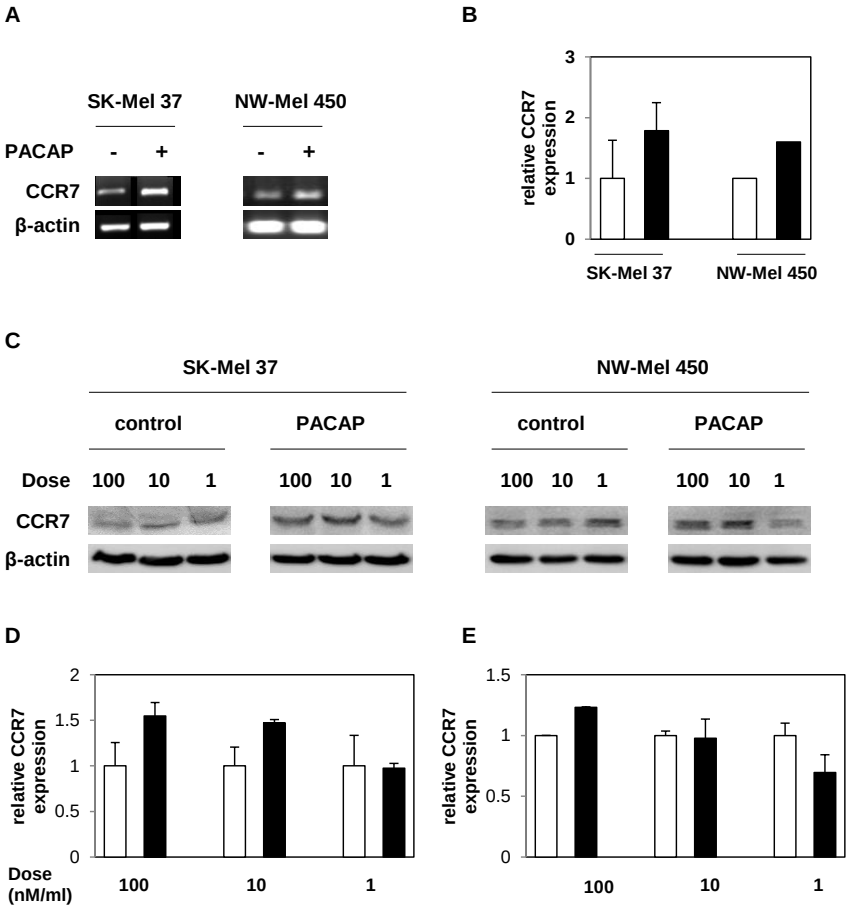

Figure 22. CCR7 expression in SK-Mel 37 and NW-Mel 450 following treatment with PACAP 27. (A) SK-Mel 37 and NW-Mel 450 were seeded at $1.5x10^5$ cells/ml and cultured in new changed medium containing 100 nM/ml PACAP 27 and 10 % serum for 17 hours. Isolated RNA from treated and control cells was used for analyzing CCR7 gene expression level. (B) Quantification of each band is demonstrated as the ratio of each targeted gene/β-actin of treated cells to control cells using densitometric analysis. The represented data on SK-Mel 37 was the mean of two independent experiments. (C) SK-Mel 37 or NW-Mel 450 cells were seeded at $1.2x10^5$ cells/ml for 24 hours and after rinsing on the following day, cells were incubated for 48 hours in serum-containing medium with various concentrations of PACAP 27 (100, 10, 1 nM/ml). The graph for SK-Mel 37 (D) or for NW-Mel 450 (E) showed the ratio of CCR7/β-actin protein expression of treated cells compared to the control samples and the represented data is the mean ± SD of two independent experiments. White bar: control cells; black bar: PACAP 27-treated cells.

PACAP-treated melanoma lines induced an increase in gene expression of CCR7 and analyzed transcript levels of CCR7 were slightly higher in SK-Mel 37 (1.8-fold) than in NW-Mel 450 (1.6-fold) (Fig. 22 B). This result corresponded to CCR7 expression at the protein level in melanoma exposed to PACAP (Fig. 22 D and E). At the highest PACAP concentration tested, i.e., 100 nM, CCR7 expression reached up to 1.5 fold or 1.2-fold increased level in SK-Mel 37 or in NW-Mel 450, respectively and this PACAP-evoked CCR7 expression was dose-dependent. At the low PACAP dose, however, NW-Mel 450 showed a lower expression level (30 % less) than the control.

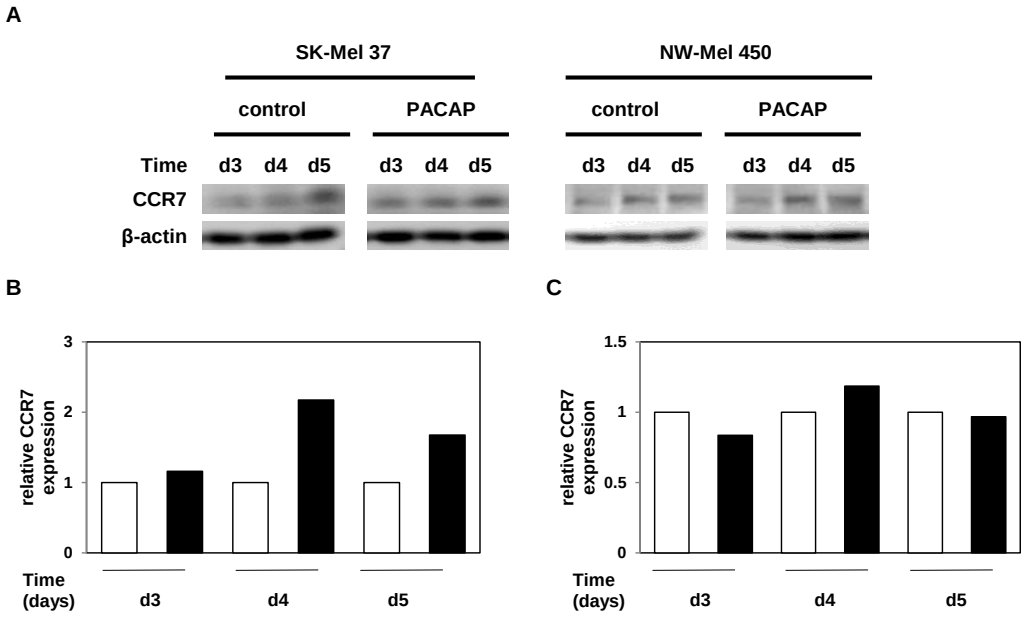

Figure 23. CCR7 expression following long-term treatment with PACAP 27. (A) SK-Mel 37 or NW-Mel 450 cells were incubated at 1.2x10^5 cells/ml for five days as indicated. Medium containing 10 % serum and 100 nM/ml PACAP 27 was refreshed every two days and cell lysates were collected at each time point for Western blotting. The evaluation of CCR7 level for SK-Mel 37 (B) or for NW-Mel 450 (C) was assessed by blotting and represented bar graph was the ratio of CCR7/β-actin of treated cells compared to the control samples from one experiment. White bar: control cells; black bar: PACAP 27-treated cells.

In the long-term PACAP treatment, an increased CCR7 expression was seen only in SK-Mel 37 and CCR7 regulation in NW-Mel 450 did not seem to be under influence of PACAP (Fig. 23). The graphs elucidated that PACAP-induced CCR7 expression in SK-Mel 37 gradually increased during four days, which slightly regressed on day 5 but its level still remained higher than the control (Fig. 23 B). On the other hand, NW-Mel 450 showed a similar level of CCR7 to the control over the culture time except on day 4, when PACAP led to a slight increase in CCR7 (Fig. 23 C). These results including previous data indicated that a noticeable PACAP-mediated CCR7 regulation at the transcriptional as well as at translational level appeared in SK-Mel 37 rather than in NW-Mel 450 (Fig. 22 B and D; Fig. 23 B)

4.1.2.6. Effect of PACAP on cytokine production in melanoma cells

Many studies proved the correlation of the elevated levels of cytokines, IL-6 and -10, in serum with progressed stage of metastatic melanoma by autocrine or paracrine regulation of these cytokines in the tumor environment (Lázár-Molnár, 2000; Moretti, 2001). The production of cytokines, IL-6 and IL-10 was regulated by cAMP stimulators, such as neuropeptides. VIP or PACAP, as one of the potent cAMP stimulators, showed a pivotal role in regulation of various cytokine productions in cells (Grimaldi, 1994; Seki, 2006; Delgado, 2013).

The initial immunostaining results showed endogenous PACAP 27 expression in metastatic melanoma cell lines, SK-Mel 37 and NW-Mel 450 (Fig. 4), which could be involved in the regulation of cytokine production. To prove this assumption, the production of IL-6 or IL-10 released by metastatic melanoma cells into supernatant under various culture conditions was estimated by ELISA and the effect of PACAP treatment on these cytokine productions was tested.

4.1.2.6.1. SK-Mel 37 or NW-Mel 450 predominantly produced IL-10 or IL-6, respectively

To define the type of produced by melanoma cells, at first ELISA assays were performed. The supernatants of the melanoma lines were collected after 2 days of culture and the amount of IL-6 and IL-10 was measured. An interesting observation was that SK-Mel 37 or NW-Mel 450 produced overwhelming IL-10 or IL-6 in the culture medium, respectively, and inversely only a small amount of IL-10 in NW-Mel 450 or IL-6 in SK-Mel 37 was detected (Fig. 24).

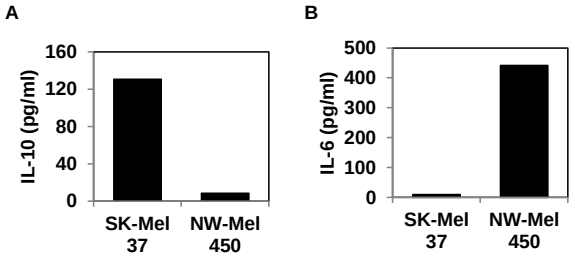

Figure 24. IL-6 and IL-10 production by various melanoma cell lines. The supernatants from metastatic melanoma cell lines, SK-Mel 37 and NW-Mel 450 were collected after two days culturing and the IL-10 (A) or IL-6 (B) production was analyzed by using ELISA. The cell number of each melanoma cell line was $1x10^5$ cells/ml of SK-Mel 37 and $5x10^5$ cells/ml of NW-Mel 450. The cytokine amount is presented as average of duplicates from one test.

4.1.2.6.2. Both cytokines, IL-6 and IL-10 were increased under full-serum but not under serum-deprived condition

Serum starvation can induce cells to produce a distinct cytokine compared to full-serum conditions. To prove whether the pattern of cytokine secretion in melanoma cells could be changed under serum withdrawal, SK-Mel 37 and NW-Mel 450 were cultured under two different serum conditions, full-serum (10%) or serum starvation (0%). The culture medium was collected at each time point (2, 4, 8, 12, 24, and 48 hours) and the level of cytokine production was measured by ELISA.

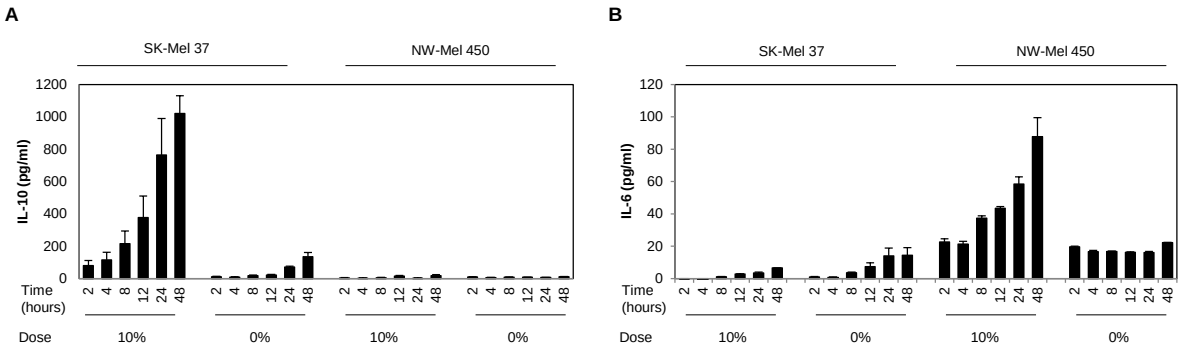

Figure 25. IL-10 or IL-6 secretion by SK-Mel 37 and NW-Mel 450 under distinct serum conditions. 9x10^4 cells/ml of the melanoma cell lines, SK-Mel 37 (left panel) or NW-Mel 450 (right panel) were cultured in DMEM with 10 % serum or 0 % serum for 2 days. At indicated time points, the culture medium was collected and the level of IL-10 (A) or IL-6 (B) was measured using ELISA. The average of duplicates in two independent experiments is represented as mean ± SD.

Firstly, under full-serum condition only, a substantial release of IL-10 by SK-Mel 37 or IL-6 by NW-Mel 450 was observed and their levels were up-regulated in a time-dependent manner. Secondly, under serum deprivation IL-10 production by SK-Mel 37 was almost undetectable and after 12 hours this marginal level of IL-10 remained, which then elevated slightly up to 48 hours (Fig. 25 A). Similarly, NW-Mel 450 showed a serum deprivation-induced decrease in IL-6, but maintained constitutive production of small amount of IL-6 for 48 hours (Fig. 25 B). Third, serum withdrawal causing metabolic stress was not involved in regulation of IL-10 in NW-Mel 450 (Fig. 25 A). SK-Mel 37, in contrast, released more IL-6 production than the cells cultured in serum-containing medium (Fig. 25 B). Taking these data, serum is a critical factor for both cytokine productions and constant production of IL-6 by NW-Mel 450 in the absence of serum suggests that IL-6 production might cause a protective effect on serum deprivation-induced cell death in accord with the observation in cell viability (Fig. 6 D).

4.1.2.6.3. PACAP up-regulated IL-6 production in NW-Mel 450 but not IL-10

Several studies showed the effect of neuropeptides, such as VIP on the elevated IL-6 or -10 production in immune cells via cAMP stimulation. Therefore, it was assumed that an up-regulated IL-6 or IL-10 production by melanoma cells could be induced by supplement with PACAP. Both melanoma cell lines, SK-Mel 37 and NW-Mel 450, were cultured for two days in variable medium conditions: 10% serum-enriched DMEM with or without 100 nM PACAP 27 or PACAP 38 and serum-deprived DMEM with or without PACAP. The supernatants were collected in time-dependent manner (2, 4, 8, 12, 24, and 48 hours) and the amount of IL-6 or IL-10 was measured by using ELISA.

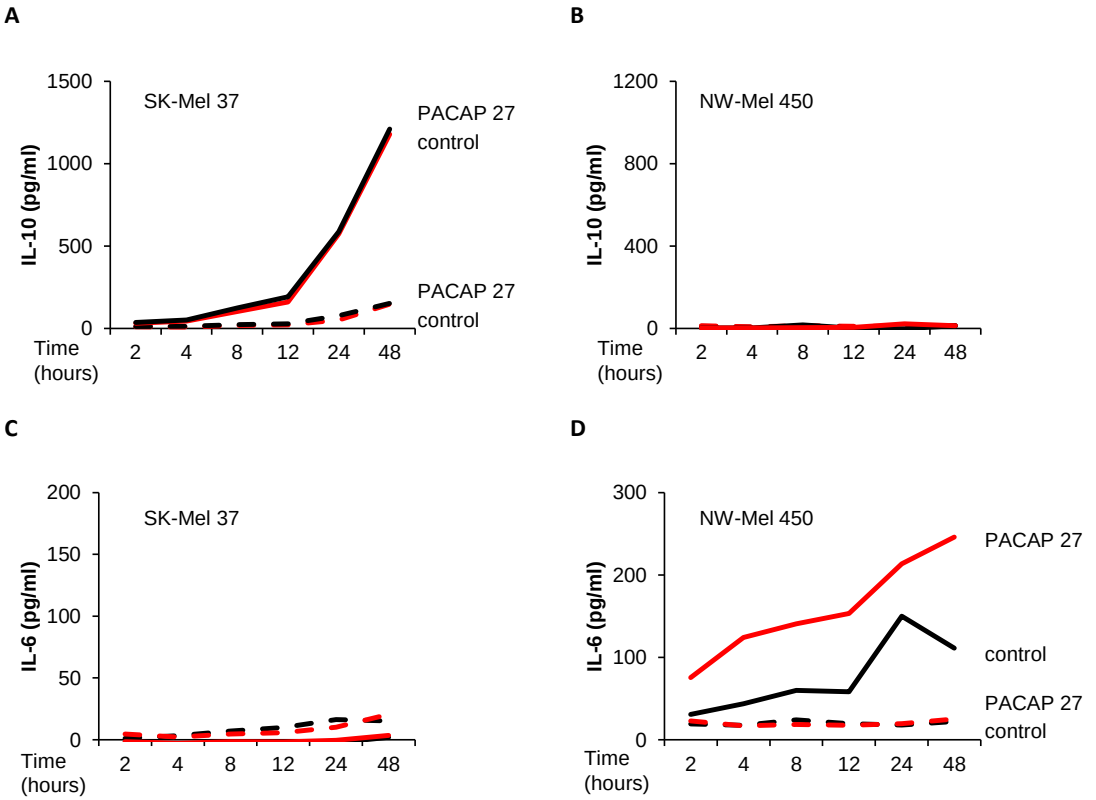

Figure 26. Effect of exogenous PACAP 27 on cytokine production, IL-10 and IL-6, in SK-Mel 37 and NW-Mel 450. The supernatants of PACAP27 (100 nM/ml) treated melanoma lines, SK-Mel 37 and NW-Mel 450, were collected at various time points (2, 4, 8, 12, 24, and 48 hours) and the amount of cytokine, IL-10 or IL-6, was measured by using ELISA. The amounts of IL-10 in SK-Mel 37 (A) or NW-Mel 450 (B) and the level of IL-6 production in SK-Mel 37 (C) or NW-Mel 450 (D) were measured after collecting the supernatant in a time-dependent manner. Each cytokine level in a graph from one experiment was presented upon two different serum conditions: solid lines for serum-containing conditions (10 %) and dashed lines for serum-free conditions (0 %). (A) A steadily increased IL-10 level in SK-Mel 37 cultured in serum-containing medium over time was shown and the value of IL-10 in serum-deprived medium was presented on the bottom. PACAP peptides did not further influence the production of IL-6. (B) No efficacy of PACAP on IL-10 production in NW-Mel 450 was presented regardless of serum conditions. (C) A very small amount of IL-6 was observed in SK-Mel 37 under serum starvation (dashed lines) but not under full-serum condition. (D) Increasing IL-6 production by NW-Mel 450 over time was observed by co-culturing with PACAP in full serum condition. Under serum withdrawal, however, IL-6 production was not affected by exogenous PACAP supplement and it remained constant during the course of culture. Black: control, red: PACAP 27 treated cells.

As mentioned above, SK-Mel 37 produced high amounts of IL-10, particularly under full-serum condition (Fig. 25). However, the amount of IL-10 in cells treated with either exogenous PACAP 27 or PACAP 38 was not changed and its level was similar to control cells (Fig. 26 A; Suppl. 18 A). This finding suggested that IL-10 production in SK-Mel 37 was not modulated by PACAP. As expected, a small amount of IL-6 production was detectable only under serum-deprived conditions in SK-Mel 37 but PACAP did not improve this cytokine secretion in any medium condition (Fig. 26 C; Suppl. 18 C). NW-Mel 450, on the other hand, showed a substantial difference in IL-6 production between PACAP-treated and -untreated cells cultured in serum-containing medium. In the absence of PACAP, the peak

of IL-6 production was reached after 24 hours and then its level decreased, whereas cells treated with PACAP 27 or PACAP 38 showed a steady increase in IL-6 production over time (Fig. 26 D; Supp. 18 D). The rate of IL-6 production was more than twice higher in cells cultured with PACAP 27 or PACAP 38 than the control. After 48 hours an interesting observation on this analysis was that PACAP 27 affected more the IL-6 release than PACAP 38. In addition, PACAP did not up-regulated either IL-6 in serum-deprived medium or IL-10 production by NW-Mel 450. Taking together, PACAP regulated IL-6 release in NW-Mel 450 only in 10% serum-enriched medium, but was not involved in IL-10 production in SK-Mel 37.

4.1.2.6.4. Cytokine production following blocking PACAP receptor, VPAC1R or PAC1R

The prior data revealed that melanoma cell lines, SK-Mel 37 and NW-Mel 450, produced distinct cytokines, IL-10 and IL-6, respectively, and that PACAP was involved in regulation of IL-6 release in NW-Mel 450 but not IL-10 in SK-Mel 37 (Fig. 24; Fig. 26). Next, it was examined, whether the blocking of PAC1R could down-regulate cytokine production in these melanoma lines. Both melanoma lines were cultured for 2 days in a full serum medium containing receptor antagonist, VPAC1R or PAC1R, or the neuropeptide, PACAP or VIP, as a positive control. The culture medium was collected and the amount of cytokine, IL-6 or IL-10, was analyzed by ELISA.

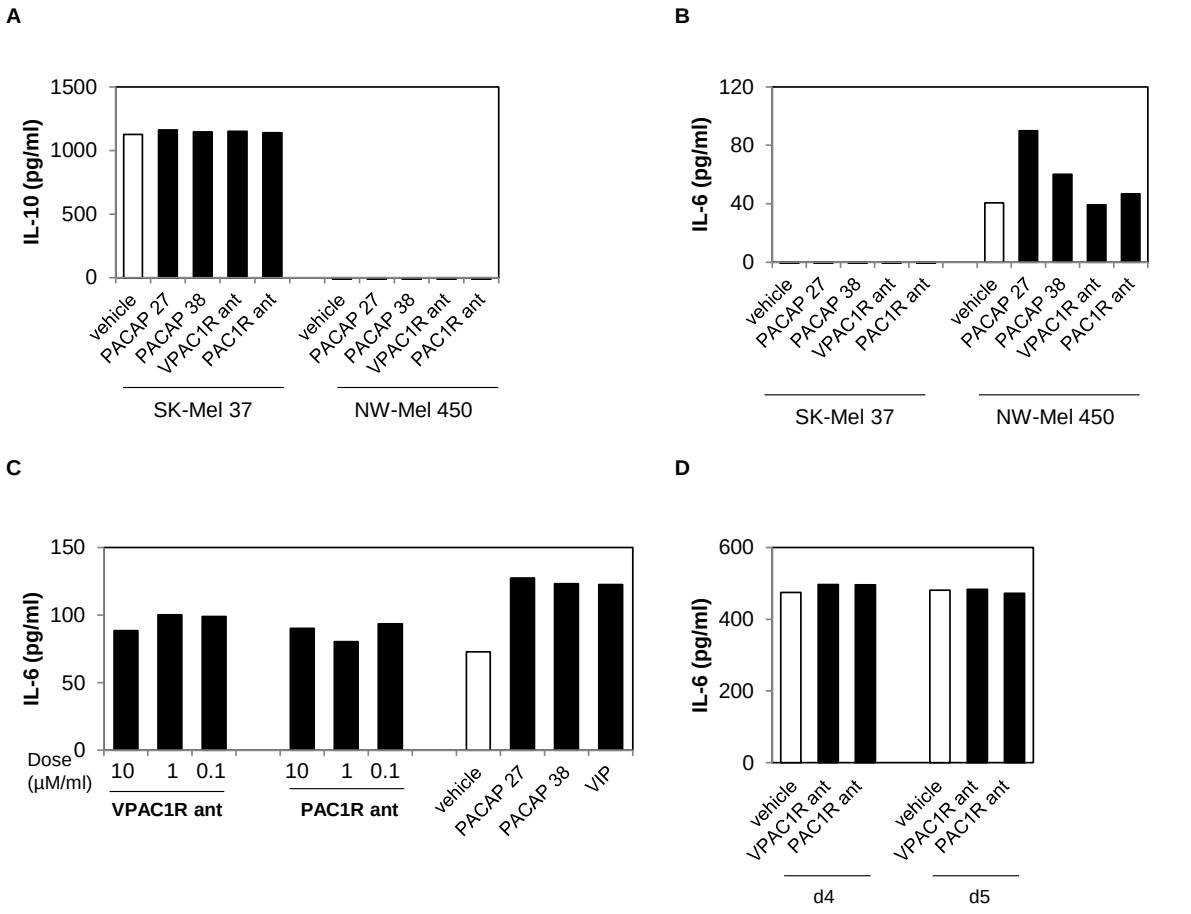

Figure 27. The level of IL-6 following addition of PACAP receptor type I or II antagonist, PAC1R or VPAC1R antagonist. (A) IL-6 level or (B) IL-10 level in SK-Mel 37 and NW-Mel 450 was measured after co-culture with 1 μM/ml PACAP receptor antagonist, VPAC1R or PAC1R antagonist or 100 nM/ml PACAP 27, PACAP 38, or VIP in full serum medium(10 %) for 2 days. (C) To confirm the effect of receptor antagonist on the IL-6 production, NW-Mel 450 was treated with receptor antagonist in a dose-dependent manner (starting from 10 μM to 100 nM/ml) and supernatant obtained on day 2 was used to analyze the amount of IL-6. Additional data on the IL-6 release from cells exposed to PACAP or VIP are shown as a positive control. (D) To prove the time-dependent effect of receptor antagonist, NW-Mel 450 was co-cultured with PACAP receptor antagonist for long-term culture course (day 4 or 5) and the amount of IL-6 in culture medium was analyzed by ELISA. Medium was refreshed every two days in the presence of antagonist. Data are presented as mean of duplicates or triplicates from one experiment.

At first, the amount of IL-10 in SK-Mel 37 remained unaffected by PACAP or by receptor antagonists, while IL-6 levels were elevated in NW-Mel 450 cultured with PACAP 27 or PACAP 38 as shown before (Fig. 26; Fig. 27). However, blocking of PACAP binding in NW-Mel 450 cell line either with antagonists for VPAC1 R or PAC1R partially diminished IL-6 release (Fig. 27 B). Furthermore, the level of IL-6 was not completely abolished by supplement with high concentration of either one of the receptor antagonists supposing that the regulation of IL-6 release in NW-Mel 450 may be regulated not by just one receptor, but possibly by a combination of both (Fig. 27 C). To ascertain whether receptor antagonists affect IL-6 production in a time-dependent manner, NW-Mel 450 was cultured in medium containing receptor antagonist for 4 or 5 days and then the collected supernatant was analyzed. The graph shows the unchanged amount of IL-6 in cells treated with receptor antagonist for five days culturing (Fig. 27 D). The long-term treatment with receptor antagonists supplemented on day 1 and day 3 of culture as well did not suppress the IL-6 release. Low concentration of endogenous PACAP produced by melanoma might be involved in regulation of IL-6 secretion, since only a certain additional dose of PACAP (100 nM) could up-regulate the IL-6 production and single receptor blocking did not suppress IL-6 production.

4.2. Part II: Paracrine effect of PACAP in immune cells

The previous study was focused on the autocrine effect of PACAP on melanoma cells and the involvement of PACAP in biological activities of melanoma cells was investigated with various aspects such as, cell survival, cytokine production, and chemokine receptor expression. Next, the paracrine effect of PACAP on immune cells was studied, particularly Treg, which have been found around tumor area with a significantly high amount published by others (Miracco, 2007; Mourmouras, 2007; Lagouros, 2009; Mougiakakos, 2010). Several reports have demonstrated an effect of PACAP or VIP on the regulation of immune cells, which raises a question about the involvement of PACAP production by melanoma cells in tumor-induced immune tolerance (Delgado, 2002; Delgado, 2004). To address this question, Treg and Teff/Tresp were tested in various aspects of cellular function affected by PACAP.

4.2.1. PACAP induced the expression of PAC1R on stimulated CD4$^+$ T cells

Expression of PACAP receptor type II, VPAC1R and VPAC2R, in human immune cells has been shown in recent reports (Goetzl, 2001; Lara-Marquez, 2001; Voice, 2002). Firstly, the gene expression of PACAP receptors was proved in CD4$^+$ T cells and non-CD4$^+$ T cells following purifying lymphocytes from healthy donor peripheral blood.

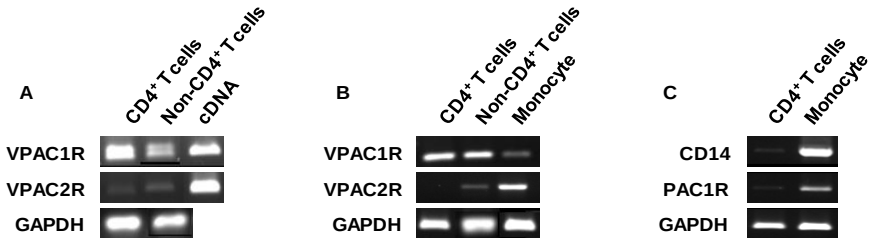

Figure 28. Gene expression of PACAP receptor types I and II, PAC1R, VPAC1R, and VPAC2R on T cells. (A) Isolated CD4$^+$ T cells ($4x10^6$) and non-CD4$^+$ T cells ($5.8x10^6$) were used to prove the expression of VIP receptors, VPAC1, VPAC2 and as a positive control cDNA for VIP receptor was used. (B) A similar approach including monocytes ($8.5x10^6$) was performed to detect the VIP receptors. (C) CD4$^+$ T cells and monocytes were tested to evaluate expression of PACA1R. Purified total RNA was subjected to PCR to evaluate the targeted gene expression. Three different approaches were represented in gel photos.

An expression of VPAC1R was detected in the unstimulated CD4$^+$ T cells and non-CD4$^+$ T cells in peripheral blood corresponding to recent studies (Lara-Marquez, 2001). On the other hand, the VPAC2R expression was comparatively weak in both subpopulations (Fig. 28 A). Another approach including monocytes showed a similar result in a relatively strong VPAC1R expression on T cells compared to VPAC2R. Unexpected founding was a difference in expression of PACAP receptor type II on untouched monocytes isolated by counter flow centrifugation (elutriation), whose VPAC2R transcript was much higher compared to VPAC1R (Fig. 28 B). Unlike the report by Lara-Marquez and colleagues, demonstrating that resting monocytes expressed only VPAC1R but not VPAC2R, PCR test showed more VPAC2R expression on monocytes than VPAC1R (Lara-Marquez, 2001).

Further, expression of PAC1R was investigated in isolated CD4$^+$ T cells. According to previous reports CD4$^+$ T cells did not express PAC1R, whose sustaining expression was detectable in monocytes. To exclude contamination with monocytes in purified CD4$^+$ T cells following MACS isolation, CD14 transcripts were additionally examined in all experiments. Figure 28 C showed that isolated CD4$^+$ T cells were not contaminated with monocytes and macrophages, as CD14 mRNA expression was not detected in T cells, and that PAC1R expression was observable only in monocytes but not in isolated CD4$^+$ T cells.

Unlike expression of PACAP receptor type II on T cells, to our best knowledge, PAC1R expression has never been reported on T cells, as is shown here following stimulation with α-CD3/CD28. Considering whether stimulation with α-CD3 alone or with co-stimuli of α-CD3/CD28 could induce T cells to express PAC1R, two purified T cell subsets, CD4$^+$CD25$^+$CD127$^{low/-}$ (Treg) and CD4$^+$CD25$^-$

(Teff/Tresp) were cultured with stimulus and PACAP in various combinations. Approximately 17 hours later RNA was isolated and the PAC1R transcripts were analyzed by using RT-PCR. To achieve an accurate result on the PAC1 expression, CD14 transcription was tested in each sample.

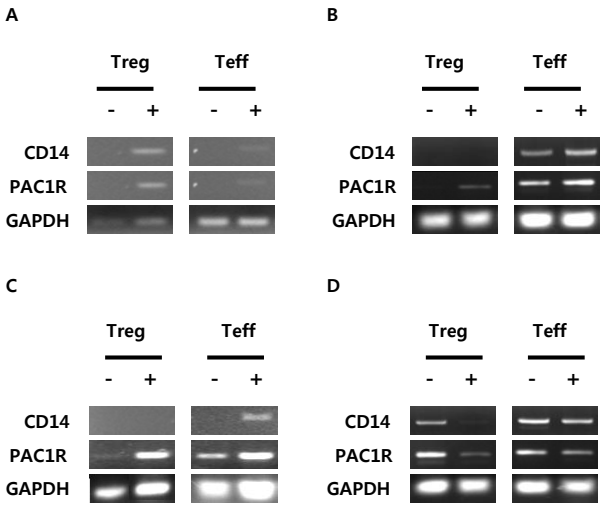

Figure 29. PAC1R expression in purified Treg and Teff/Tresp. $CD4^+CD25^+CD127^{low/-}$ (Treg) and $CD4^+CD25^-$ (Teff/Tresp) T cells were cultured in various combinations of stimulator in the presence of PACAP 27 or PACAP 38 and the total RNA was purified after 17 hours incubation to test the PAC1R expression. Additionally, CD14 transcripts were detected as a control for contamination of monocytes. (A) Stimulation with 1 ug/ml α-CD3 +/- 100 nM/ml PACAP 38, (B) stimulation with 1 ug/ml α-CD3/CD28 +/- 100 nM/ml PACAP 27, (C) stimulation with 1 ug/ml α-CD3/CD28 +/- 100 nM/ml PACAP 38, (D) stimulation with 1 ug/ml α-CD3/CD28 and 12.5 U/ml IL-2 +/- 100 nM/ml PACAP 27; (+) in the presence ; (-) in the absence. Four different approaches were shown.

Figure 29 showed the PAC1R expression on RNA level of purified Treg and Teff following PACAP treatment. Positive PAC1R on T cells was evaluated after testing with CD14 gene expression for monocyte contamination, since monocytes express CD4 at low level. One difficulty in this study was the evaluation of gene transcript of PAC1R in T cells particularly that were contaminated with monocytes and macrophages, which showed a positive expression for CD14. Barring contamination of T cells by monocytes this study indicated that Treg could express PAC1R in the presence of PACAP (Fig. 29). Activation with α-CD3 alone did not induce PAC1R expression in Treg and the detected PAC1R in the presence of PACAP was not confirmed, since it could be a positive one due to the contamination with monocytes and macrophages (Fig. 29 A). Treg stimulated with co-stimuli of α-CD3/CD28 monoclonal antibodies alone also did not express PAC1R. However, PAC1R expression appeared in Treg following combined stimulation of α-CD3/CD28 in the presence of PACAP 27 or PACAP 38 (Fig. 29 B and C). These PAC1R transcripts could be detected as well in Treg cultured in the medium containing α-CD3/CD28, exogenous IL-2, and PACAP 27 (Fig. 29 D). Teff/Tresp even showed PAC1R expression co-stimulation with α-CD3/CD28 alone but PAC1R expression could not be evaluated from the other cases due to contamination with monocytes and macrophages. To sum up, PACAP could induce PAC1R gene expression in Treg and it can be supposed that PACAP produced by melanoma might affect similarly on PAC1R expression and further on cellular functions of Treg via PACAP receptors, VPAC1R or PAC1R.

4.2.2. PACAP suppressed the proliferation of T cells

Rodriguez and colleagues demonstrated that increased cAMP by forskolin stimulation in human T cells caused a decreased proliferation via suppressed cell cycle regulatory genes (Rodriquez, 2013). This finding led to the consideration whether PACAP as a cAMP stimulator could suppress the cell proliferation of T cells. To reveal this PACAP effect, purified $CD4^+$ T cells were activated with α-CD3 or α-CD28 alone or in combination of both stimuli in the absence or presence of PACAP 38 and after three days of incubation cell numbers were counted. In another approach, purified T cell subsets, Treg and Teff/Tresp cells, from PBMC of healthy donors were cultured in medium containing α-CD3/CD28 beads, 12.5 U/ml of IL-2 and PACAP for four days. The rate of proliferation of cells was measured by ^{3}H-thymidine incorporation assay.

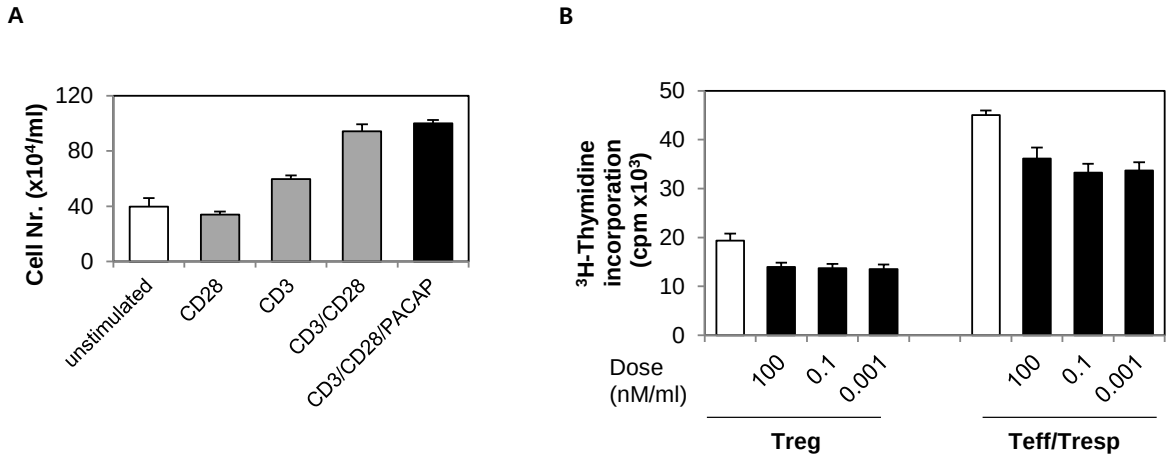

Figure 30. Proliferation of T cell subsets during PACAP treatment. (A) Purified $CD4^+$ T cells were cultured under various medium conditions (with stimulator α-CD3, α-CD28 alone or combination of both stimuli or in the additional presence of 100 nM/ml PACAP 38). After three days cell were counted by using Casy cell counter. Data are presented as mean ± SD of duplicates. (B) $CD4^+CD25^+CD127^{low/-}$ (Treg) and $CD4^+CD25^-$ (Teff/Tresp) T cells were cultured at 1.25 x10^5 cells/ml under stimulation with α-CD3/CD28 beads and 12.5 U/ml of IL-2 for four days. PACAP 38 was supplied in a dose-dependent manner from 100 nM to 1 pM. Cell proliferation was measured by using ^{3}H-thymidine incorporation assay. The presented data were results from two independent experiments with ± SD. White bar: control cells; black bar: PACAP 38-treated cells.

At first, cell growth of $CD4^+$ T cells was enhanced following with α-CD3 alone or with combination of α-CD3/CD28 on day 3. T cells activated with α-CD3 alone showed 1.5-fold up-regulated cell growth, whereas α-CD3/CD28 co-stimulation achieved 2.4-fold increased cell yield. However, compared to the cells stimulated with α-CD3/CD28, PACAP 38 did not affect much on the proliferation, whose level was nearly similar to that in the absence of PACAP (Fig. 30 A). Afterwards, the PACAP effect on the proliferation of Treg and Teff/Tresp cells was examined separately. Survival cell numbers were counted 17 hours later following stimulation with α-CD3/CD28 in the presence of IL-2 or PACAP 38. A similar proliferative pattern was observed in both T cell subsets, whose cell growth was enhanced

slightly following stimulation with α-CD3/CD28 in the presence of IL-2 compared to the control in the absence of IL-2. However, PACAP 38 caused a suppressive effect on cell growth rather than increased cell numbers (Suppl. 19 A). More pronounced reduction in cell growth could be observed in T cells cultured in combination of α-CD3/CD28, IL-2 and PACAP (Suppl. 19 B).

To verify this suppressive effect of PACAP a long-term cell proliferation was monitored in the presence of PACAP 38. Treg and Teff/Tresp cells were cultured in medium containing CD3/CD28 beads and IL-2 for four days (Fig. 30 B). In a dose-dependent manner, both T cell subsets were treated with PACAP 38 starting from 100 nM to 1 pM. The clonal expansion was not observable in Treg and its level was lower than the control without PACAP. Teff/Tresp as well showed a decreased cell growth and less difference in reduced cell growth was observed in high or low PACAP concentration tested. A declined proliferation rate could also be observed in both Treg and Teff/Tresp following exposure to PACAP 27 and the decreased level was much lower in Treg than it in Teff/Tresp (Supp. 19 C). PACAP 38 triggered a decline in proliferation rate (approximately 28% less Tregs and 20% less Teff/Tresp) and in a limited series of experiments, similarly PACAP 27 caused 43% and 20% reduced cell growth in Treg and Teff/Tresp, respectively.

4.2.3. PACAP enhanced FoxP3 and CTLA-4 gene expression in T cells

Next, to investigate the involvement of PACAP in regulation of Treg-related gene expression, FoxP3 and CTLA-4, the isolated T cell subsets, Treg and Teff/Tresp, were cultured in various culture systems, such as unstimulated or stimulated with α-CD3/CD28 or IL-2 supplement and T cells were treated additionally with PACAP 27 or PACAP 38 for 17 hours. RNA was purified from each collected sample and the targeted gene expression was analyzed by RT-PCR, which presented by densitometry in a bar graph. Every sample was tested in addition for CD14 transcripts to exclude contamination with monocytes and macrophages.

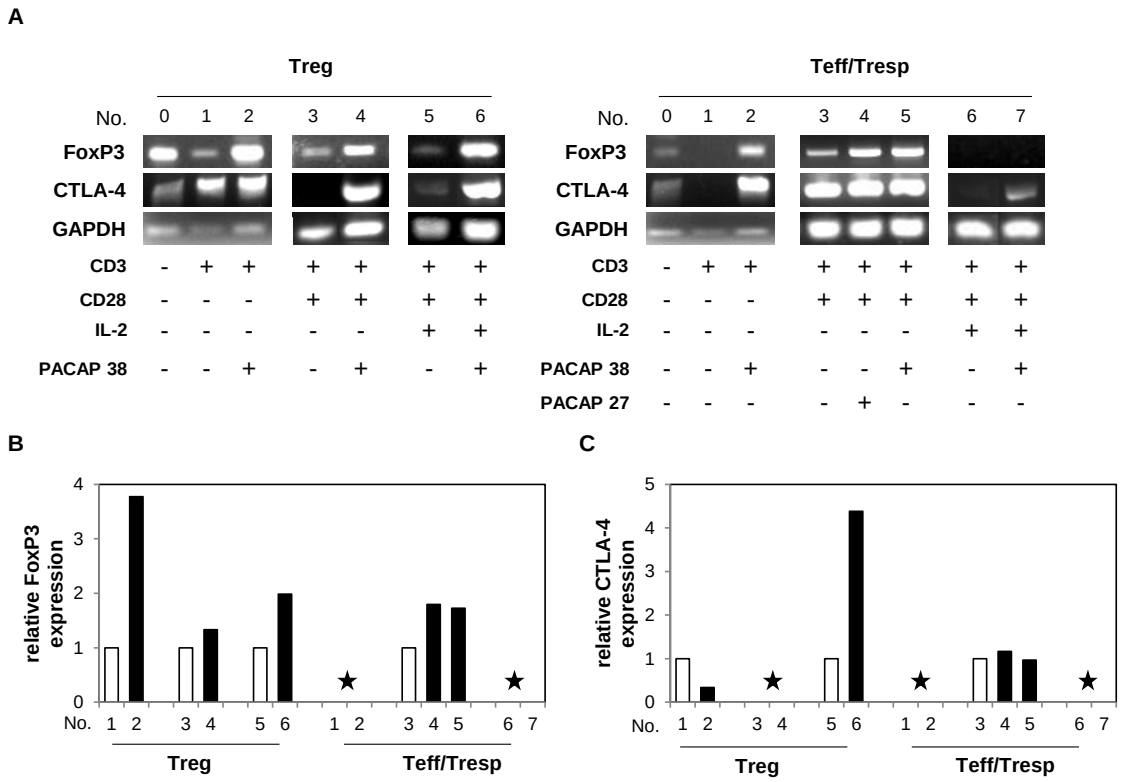

Figure 31. PACAP effect on Treg-related gene expressions, FoxP3 and CTLA-4. (A) $CD4^+CD25^+CD127^{low/-}$ (Treg) and (B) $CD4^+CD25^-$ T cells (Teff/Tresp) were isolated from PBMC and they were cultured in various medium conditions: α-CD3 or α-CD3/CD28 or CD3/CD28 including exogenous IL-2 at 12.5 U/ml. These cells were additionally treated with 100 nM/ml PACAP 27 or PACAP 38 for 17 hours and then cells were collected to purify RNA. By using the specific primers each targeted gene expression, FoxP3 or CTLA-4, was analyzed and quantification of expression level was demonstrated as the ratio of targeted gene/ GAPDH of treated cells compared to the control by using densitometric analysis. Three different approaches were shown. (*:no analyzed data due to the lack of amplification of targeted gene in one control panel without PACAP 38).

Firstly, different expression level of the housekeeping gene, GAPDH, was observable on the gel photos particularly in Treg. Stimulated Treg with α-CD3 alone showed much lower GAPDH expression compared to the unstimulated or the treated cells with PACAP 38. Treg stimulated with α-CD3/CD28 or in the presence of IL-2 showed as well a weak GAPDH expression, which however was enhanced by PACAP treatment supposing that GAPDH expression might be differently regulated in Treg by stimuli and that PACAP 38 improved GAPDH expression. GAPDH transcripts in Teff/Tresp, on the other hand, maintained constantly regardless of PACAP supplement.

A noticeable recognition of different FoxP3 expression levels was observable in unstimulated Treg and Teff/Tresp, showing mostly highly expressed FoxP3 transcript in Treg compared to Teff/Tresp (Fig. 31). A PACAP-triggered increase in FoxP3 was observed in both T cell subsets, if evaluable, and the most significant increase in FoxP3 was detected in Treg stimulated with α-CD3 alone in the presence of PACAP 38 and its expression level reached at 3.8-fold. Combination of co-stimuli and IL-2 as well caused an enhanced FoxP3 (2-fold) following PACAP 38 supplement. Teff/Tresp cells showed as well a slight rise in FoxP3 expression, when cells were stimulated with α-CD3/CD28 in the presence of

PACAP 27 or PACAP 38 (1.1-fold or 1.3-fold increased FoxP3, respectively). However, there was no outcome of FoxP3 from Teff/Tresp activated with α-CD3/CD28 including IL-2, although a strong GAPDH expression was shown in these cells. Taking together PACAP regulated positively FoxP3 gene expression in both T cell subsets supposing that PACAP reinforces a tolerant status of the immune.

Another specific gene expression, CTLA-4, was further investigated in both T cell subsets following PACAP treatment (Fig. 31 A and C). Similar to FoxP3 expression, a difference in the expression level of CTLA-4 could be observed between Treg and Teff/Tresp and the exposure to PACAP caused a high increase in CTLA-4 expression as well. Among various stimulation conditions, the combination with α-CD3/CD28 and IL-2 triggered the highest effectiveness of PACAP on CTLA-4 expression in Treg, reaching a 4.4-fold enhanced level. Exceptionally, Treg activation with α-CD3 in the presence of PACAP 38 showed much lower amplified CTLA-4 expression than cells activated with α-CD3 alone. An amplified gene expression of CTLA-4 was observed in Tregs stimulated with α-CD3/CD28 and PACAP 38 on the gel photo, but a lack of CTLA-4 PCR product in control cells caused an impaired analysis to evaluate PACAP-mediated CTLA-4 gene expression (Fig. 31 A). As shown on gel pictures, an analysis of CTLA-4 expression in Teff/Tresp was not available, since PCR product of CTLA-4 was not detected in control cells. Teff/Tresp stimulated with α-CD3/CD28 and PACAP 27 only showed a slight elevated transcript level (Fig. 31 A and C). This data on gene expression demonstrated that PACAP was involved in the CTLA-4 regulation particularly in Treg.

4.2.4. PACAP induced an increase in gene expression of cytokines, IL-10 and TGF-β.

Gonzalez-Rey and colleagues suggested that the neuropeptide, VIP, might induce the generation of naïve $CD4^+$ T cells to produce IL-10/TGF-β, although there was no expansion of preexisting IL-10/TGF-β producing cells in the presence of VIP (Gonzalez-Rey, 2006). This suggestion provided a possibility that PACAP as another structurally-related peptide to VIP could regulate the IL-10/TGF-β production. To reveal the involvement of PACAP in this regulation purified Treg and Teff/Tresp were cultured in distinct medium conditions, such as stimulation with α-CD3/CD28 or IL-2 supplement in the presence of PACAP. Each band on the gel was analyzed by using densitometric analysis.

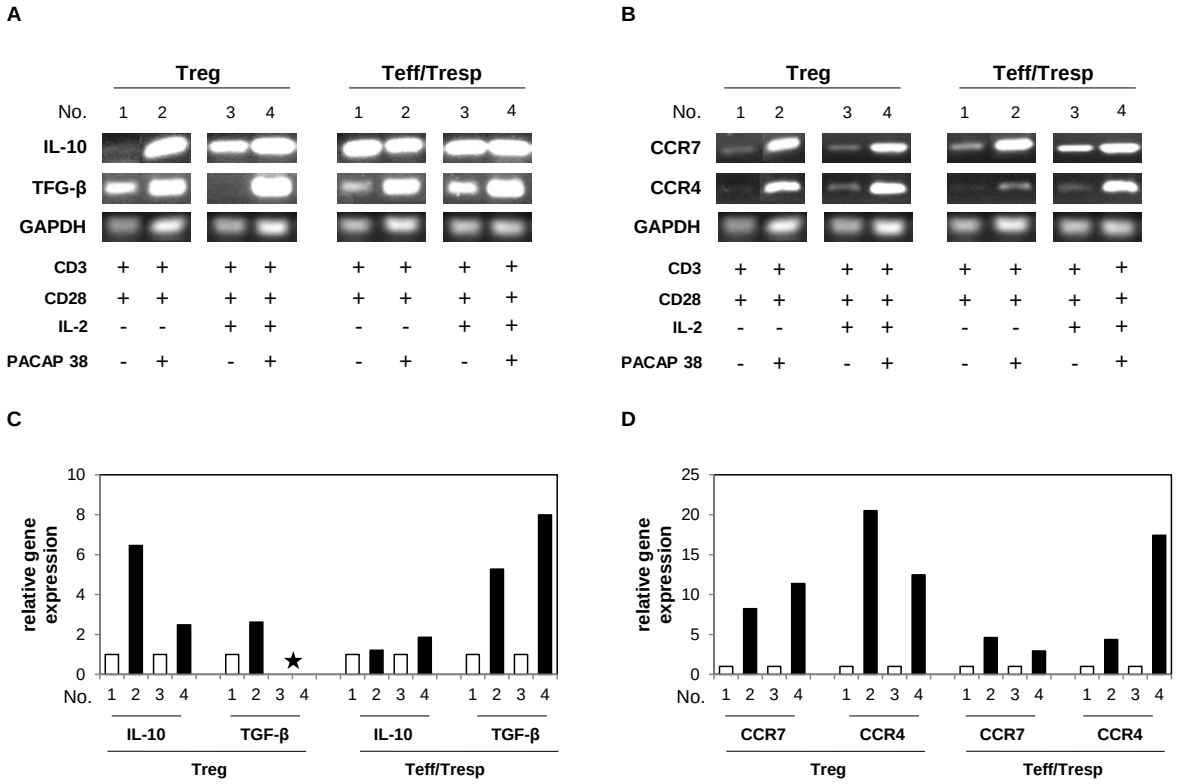

Figure 32. Gene expression of cytokines, IL-10 and TGF-β, and of chemokine receptors, CCR7 and CCR4, following PACAP supplement. $CD4^+CD25^+CD127^{low/-}$ T cells (Treg) and $CD4^+CD25^-$ (Teff/Tresp) were isolated from PBMC and they were cultured in various medium conditions: α-CD3/CD28 or α-CD3/CD28 including exogenous IL-2. These cells were additionally treated with 100 nM/ml PACAP 38 for 17 hours and then collected RNA was purified. PCR was performed by using the specific primers to detect a targeted gene expression. The amplified PCR product of (A) cytokines, IL-10 and TGF-β or (B) chemokine receptor, CCR7 and CCR4 was demonstrated on the gel. The quantification of expression level of (C) cytokine or (D) chemokine receptor was presented as the ratio of targeted gene/GAPDH of treated cells compared to the control by using densitometric analysis. Two different approaches were shown. (white bar: control; black bar: PACAP treatment; *: no presented data due to absence of PCR production in the control).

The gel picture showed strongly amplified expression of targeted genes, IL-10 and TGF-β, following PACAP treatment in both T cell subsets. Quantification of these gene expressions in a diagram demonstrated a positive involvement of PACAP in the regulation of cytokine gene expressions. An interesting finding was the different regulation of cytokine release by PACAP in both T cell subsets (Fig. 32 A and B). IL-10 expression was strongly up-regulated in Treg activated with co-stimuli of α-CD3/CD28 and PACAP compared to Teff, whose IL-10 transcript was not affected much by addition of PACAP. On the other hand, PACAP-induced amplification of TGF-β expression was observed in Teff/Tresp activated with α-CD3/CD28 rather than in Treg.

Yagi and colleagues showed that FoxP3-transduced $CD4^+CD25^-$ naïve T cells up-regulated the expression of CCR4 and acquired the functions of $CD4^+CD25^+$ Treg cells (Yagi, 2006). These reports gave rise to the question about the possibility that PACAP could up-regulate CCR4 as well as CCR7.

This might support infiltration of T cells into tumor area and further enhance a suppressive immunity. Unstimulated Treg as well as Teff/Tresp showed a weak PCR product of chemokine receptors, which however, highly increased following PACAP treatment (Fig. 32 B and D). An up-regulation of both chemokine receptors, CCR7 and CCR4, by PACAP reached at 11-fold and 21-fold increased CCR7 and CCR4 level in Treg, respectively. Similarly, enhanced receptor expressions were observed in Teff/Tresp but their levels did not appear as remarkable like those in Treg. Only Teff/Tresp stimulated with α-CD3/CD28 and IL-2 achieved the highest CCR4 expression at 17-fold level in the presence of PACAP (Fig. 32 D). These data indicated that PACAP may be a crucial factor favoring an immunosuppressive state through an increased gene expression of immune suppressive cytokines or chemokine receptors.

4.2.5. PACAP improved the suppressive function of regulatory T cells

Since PACAP could promote immune tolerance by regulating gene expression of immune suppressive molecules shown in previous approaches, the suppressive capacity of Treg in the presence of PACAP was investigated (Fig. 31; Fig. 32). Isolated Treg and Teff/Tresp cells from PBMC were co-cultured following PACAP supplement and in this study two distinct systems, coated α-CD3 and soluble α-CD28 or α-CD3/CD28-beads, in the absence of antigen presenting (APCs) were used to avoid bystander effects of PACAP on APCs that are capable to bind PACAP and to induce the suppressive immune factors.

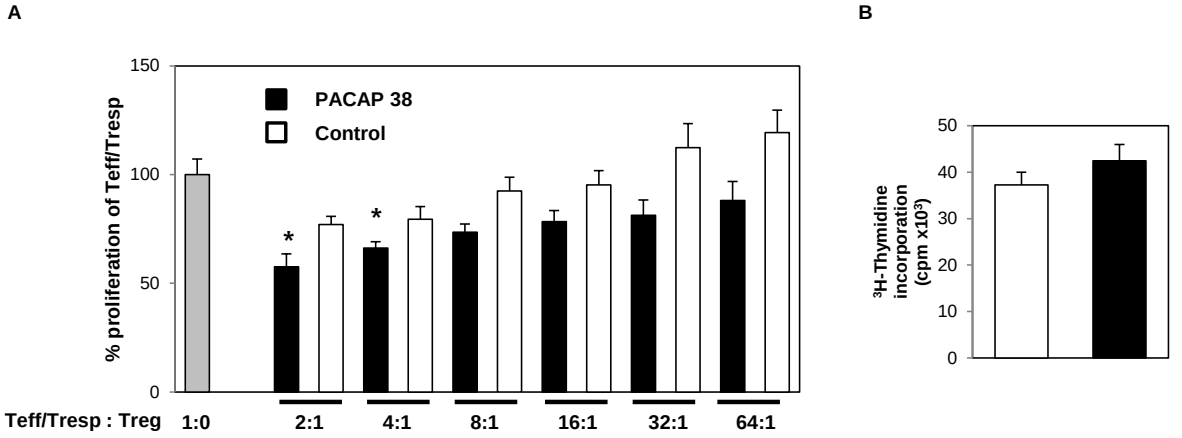

Figure 33. Suppressed proliferation of Teff/Tresp cells in the presence of PACAP 38. (A) $CD4^+CD25^+CD127^{low/-}$ T cells (Treg) and $CD4^+CD25^-$ (Teff/Tresp) were isolated from healthy PBMC and Tregs were titrated into a Teff/Tresp cell proliferation assay starting at a 2:1 Teff/Tresp:Treg ratio. Cells were incubated with α-CD3/CD28 beads in the presence or in the absence of 100 nM/ml PACAP 38 for 5 days. The proliferation of Tresp was determined by ^{3}H-Thymidine incorporation. The diagram showed the ratio of the proliferative level following PACAP treatment compared to the control. This data was represented as mean ± S.E.M from four independent experiments. Statistical analyses were performed by unpaired two tailed t-test. *: $p<0.05$. (B) Absolute numbers of Tresp cultured in the presence or in the absence of 100 nM/ml PACAP 38 were demonstrated. White: control (in the absence of PACAP) ; black: PACAP 38 containing medium.

An immune suppressive effect of PACAP could be observed in this approach, in which PACAP 38 caused a decreased cell growth of Teff/Tresp. Compared to proliferation of Teff/Tresp alone, approximately 43% (p=0.028) and 23% (p=0.094) less proliferation were resulted in the presence or in the absence of PACAP 38, respectively, when Teff/Tresp:Treg were co-cultured at a ratio of 2:1. This observation indicated that PACAP 38 promoted a suppressive effect on cell proliferation of Teff/Tresp (Fig. 33 A). This inhibiting effect of PACAP on cell growth could be detected as well in Teff/Tresp co-cultured with low numbers of Treg up to a ratio of 64:1. Such suppressive effect on Teff/Tresp was shown following co-culture with Treg in the presence of PACAP only. A diagram demonstrated a slightly increase in cell number of PACAP 38-treated Teff/Tresp suggesting that PACAP supported suppressive quality of Treg while not suppressing cell growth of Teff/Tresp themselves in this particular set of experiments without addition of exogenous IL-2 (Fig. 33 B). Furthermore, the α-CD3/CD28 beads system showed a more clear suppressive effect of PACAP 38 on cell growth between PACAP-treated and -untreated cells compared to another system, in which Treg and Teff/Tresp were co-cultured with coated α-CD3 and soluble α-CD28 in the presence of PACAP 38 (Suppl. 20 A). A result in suppressed cell growth of Teff/Tresp was also achieved in the presence of PACAP 27 in the co-culture of Treg and Teff/Tresp compared to the control (56% less in the presence of PACAP 27 and 41% less in the absence of PACAP 27) (Suppl. 20 B).

5. Discussion

The incidence of cutaneous melanoma continues to rise yearly and melanoma causes a main contribution to high mortality rate among skin related cancers, although it accounts for 3% of skin cancers. For dermatologist and oncologist, melanoma has been remained a controversial and challengeable area due to extreme therapy-resistance. To date, prospective and promising treatments in the area of targeted therapy and immunotherapy have been developed and resulted in improved overall survival of patients. In the targeted therapy signaling pathways, whose genetic aberration drives consistent cell survival and cell proliferation, are considered as a crucial checkpoint to treat melanomas. Interestingly, this study for the first time shows that metastatic melanoma, primary melanoma, and melanoma cell liens produce the neuropeptide PACAP, which has been known as an important factor for enhancing tumor cell growth and anti-inflammatory immune function (Moody, 1993; Delgado, 2000). The examples from other studies led to the speculation that PACAP might also be involved in the regulation of melanoma cell growth as well as melanoma specific immunity in the surrounding tumor environment and this dual interaction through PACAP could support melanoma progression. Therefore, PACAP may be considered as a potential and major checkpoint for targeted tumor therapy, since down-regulating PACAP signaling may impair melanoma cell survival, while unblocking anti-tumor immune responses.

5.1. Part I: Autocrine effect of PACAP on melanoma

Preliminary studies revealed the expression of PACAP and its high affinity receptor, PAC1R, in various human tumor types, such as breast cancer, prostate cancer, colonic tumor cells, and testis cancer as well as in immune cells (Garcia-Fernandez, 2002; Le, 2002; Garcia-Fernandez, 2004; Muller, 2006; Nakamura, 2013). Furthermore, PACAP functions as a regulator for cell survival, which is associated with enhanced expression of anti-apoptotic protein, BCL-2, via cAMP stimulation (Gutiérrez-Cañas, 2003; Dickson, 2006). However, it is not known, whether melanomas express PACAP as well as PAC1R, and whether PACAP contributes to melanoma cell growth or regulates other functions including specific gene expression involved in cell cycle regulators or cytokine production. Considering the possibility that melanoma produces PACAP and/or expresses PAC1R similar to other tumor types, has prompted us to investigate a role of PACAP in melanoma.

5.1.1. Occurrence of PACAP and PAC1R in human melanoma

This study is the first comparative analysis of the presence of PACAP and its high affinity receptor, PAC1R, in human melanocytic lesions, including primary melanoma and metastatic melanoma. Investigation of PACAP expression in melanoma was assessed by immunohistochemical evaluation with melanoma tissues obtained from various melanoma patients. For detection of PACAP expression, two specific rabbit serum anti-PACAP 27 and PACAP 38 antisera for known splice variant peptide

PACAP 27 and PACAP 38, and for PAC1R expression a specific rabbit polyclonal anti-human PAC1R antibody were used, respectively. For the consecutive series, PACAP staining was compared in side-by-side fashion with HMB-45. All 8 cases of metastatic melanoma and 4 out of 5 cases of primary melanoma showed positive PACAP 27 expression at different levels and only one case of primary melanoma was observed with PACAP 27-negative result. The total rate of more than 30% of the melanoma area staining positive for PACAP 27 in metastatic melanoma was almost twice higher than it in primary melanoma. On the other hand, positive immunoreactivity to PACAP 38 was consistently found in primary melanoma rather than in metastatic melanoma. Although more cases of primary and metastatic melanoma are needed to be evaluated, to investigate which of the PACAP subtypes will predominantly be produced in each melanoma stage, observations in this study showed that there are differential PACAP subtype expressions upon melanoma stages and that in the late phase of melanoma PACAP 38 is reduced. The pattern of distribution and the intensity of PACAP 27 staining differ in both melanoma stages as well. A strong PACAP 27 expression in metastatic melanoma was observed in a certain subsets of melanoma cells rather than in all melanoma cells, whereas primary melanoma cells exhibited diffuse staining of PACAP 27 overall and the total rate of PACAP 27 expression is still higher than it in primary melanoma. The present result reflects various subclasses of melanoma cells in a tumor cluster, which might help to understand the progression of melanoma. Garcia-Fernandez et al reported PACAP expression in breast or prostate cancer, in which epithelial cells and stromal cells both showed an immunohistochemical staining with PACAP antibody, while PACAP was detected only in epithelial cells in normal breast and prostate tissues (Garcia-Fernandez, 2002 and 2004). Stromal cells in metastatic melanoma area, such as immune cells also showed PACAP immunoreactivity but it should be elucidated, whether infiltrated immune cells produce PACAP themselves or bind to PACAP produced by melanoma via PACAP receptors. Different from other healthy tissues, in healthy skin containing melanocytes in epithelial layer no expression of PACAP 27 or PACAP 38 was observed (data not shown, tested by immunofluorescence method). In addition, in metastatic melanoma a co-localization of PACAP 27 or PACAP 38 with melanoma marker HMB 45 was not found, whereas PACAP distribution is better associated with HMP 45 expression in primary melanoma. Furthermore, PACAP expression was not related to age and gender of patients or anatomical location of melanoma occurrence.

PACAP expression was detected in metastatic melanoma cell lines as well. Immunoblotting analysis, using specific mouse anti-PACAP monoclonal antibody to detect PACAP protein was performed in five established melanoma cell lines, a sample of melanoma metastasis in lymph node, and melanoma primary cell culture obtained from one patient. The results provided evidence of PACAP expression corresponding to the molecular size at 23 kDa, which accounts for around 200 amino acids and is slightly higher than the expected band (18 kDa; 176 amino acids). One of the reasons for a higher molecular weight of PACAP may be a different post-translational modification of the protein in melanoma. Using an inappropriate percentage of gel can be as well responsible for poor separation of protein, but the latter reason can be excluded because a prestained protein marker spanned a range

even down to 15 kDa on the membrane. Additionally, denaturation of protein can be a possible cause for the different size of targeted protein. However, PACAP expression in melanoma lines was confirmed by immunohistochemical analysis. Two melanoma lines, SK-Mel 37 and NW-Mel 450 were selected for detailed studies. These lines produced both PACAP-subtypes, PACAP 27 and PACAP 38. Similar to metastatic melanoma tissues of patients, melanoma lines showed a stronger positive immunoreactivity for PACAP 27 rather than PACAP 38, supposing that PACAP 27 might be a main product in both melanoma lines. PACAP 27 or PACAP 38 was observed in cytoplasmic area in both melanoma lines, except the localization of PACAP 38 in SK-Mel 37. Another approach using an immunofluorescence showed again that SK-Mel 37 presented PACAP 38 in or near of the nucleic area but not in the cytoplasmic area. The localization of PACAP 38 in the cytoplasma has been demonstrated by Hegg et al (2003). The synthesis of each PACAP subtype might be separately regulated in a time dependent manner.

Further, immunohistochemical staining with specific PAC1R antibody demonstrated that healthy melanocytes did not express PAC1R, which however was detected for the first time in primary and metastatic melanoma with diverse distribution. Only two out of five cases of primary melanoma responded positively to specific PAC1R antibody, whereas seven out of eight cases of metastatic melanoma showed PAC1R expression with variable staining intensities, supposing that PAC1R expression in metastatic melanoma seems to be an important mediator for biological activity. Furthermore, PAC1R gene transcripts could be observed in melanoma lines and melanoma primary cell culture and its expression was verified by Western Blot using a monoclonal mouse anti-human PAC1R antibody yielding ~50 kDa peptide. In 1994 Fabre showed that the VIP-related peptide recognized receptor in the melanoma cell line IGR37 and six years later Moody and his colleagues reported the VPAC1R expression in melanoma cell lines (Fabre, 1994; Moody, 2000). Accordingly, also in this study metastatic melanoma cell lines including primary melanoma cell culture were shown to express VPAC1R at different mRNA expression levels but not VPAC2R. Despite lacking in information with regard to VPAC1R expression at protein level, preferential expression of PACAP receptor types on mRNA level was noticeable in melanoma cell lines, especially VPAC1R expression in NW-Mel 450 and PAC1R expression in SK-Mel 37. PACAP receptor variants result in differential signal transduction responses, coupling to cAMP or PI (McCulloch, 2000; Dickson, 2006). This different expression level of receptors in both melanoma lines leads to the speculation that each melanoma line might have a preferential expression of VPAC1R or PAC1R, which connects with different characteristic features. In addition, expression of PAC1R splice variant was examined with specific primer encoding hip or hop area and only hop area was detected in melanoma cell lines (PCR product of hip area; data not shown). Although identifying splice variants of PAC1R on melanoma cell lines was not the purpose of this study, it should be interesting to reveal, which PAC1R splice variant could be expressed in melanoma cells besides hop area because it may explain the different characters of melanoma lines.

As reported by Le and the colleagues showing an autocrine or paracrine effect of PACAP on breast

cancer cells expressing PAC1R, co-expression of PACAP and PAC1R in melanoma cell lines suggested the involvement of PACAP in the biological regulation of melanoma in an autocrine or paracrine manner (Le, 2002).

5.1.2. Functions of PACAP in melanoma

PACAP is involved in a variety of biologic actions, including regulation of cell division, neuronal differentiation and neuronal survival. These pleiotropic activities of PACAP have been investigated in several cancer cell lines in extensive studies (Gozes, 1999; Vaudry, 2000), but its biological activities have not been defined in melanoma yet. For detailed studies two melanoma lines were selected, SK-Mel 37 and NW-Mel 450, which have common features, such as expression of cancer/testis antigens, NY-ESO/LAGE-1 and MAGE 3, or co-expression of PACAP and PAC1R. In contrast, morphological features and size of cells are different between two melanoma lines and a positive immunoreactivity to HMB45 was observed only in SK-Mel 37 (data not shown). In this study PACAP-induced cellular activities were investigated in melanoma cell lines with following aspects: cell survival, proliferation, and cytokine production.

5.1.2.1. PACAP has a cytoprotective and a proliferative effect in melanoma

Studies of the PACAP effect on the proliferative or cytoprotective activity of tumor cells have demonstrated a differential influence on cell growth, depending on a culture system, duration of culture, or the PACAP concentration used (Moody, 1993; Farini, 2003; Lelievre, 1998). Therefore, the dose-dependent effect of PACAP on the melanoma cell lines was examined in the short-term (3 days) and in the long-term (4-8 days) incubation. Firstly, PACAP 27 showed two diverse effects in melanoma cells as a cytoprotective and proliferative effect depending on serum conditions. Cell viability studies showed a differential response of SK-Mel 37 and NW-Mel 450 to serum deprivation. SK-Mel 37 is more susceptible to serum starvation and cell growth is strongly associated with the serum content in medium. In contrast, NW-Mel 450 showed a similar cell growth rate regardless of either low serum-content (3% or 0.5%) or serum withdrawal, supposing that serum may not be a pivotal factor for maintaining cell vitality. This distinct character of both cell lines was more obvious following PACAP 27 treatment. Short-term exposure of PACAP 27 (3 days) to SK-Mel 37 increased cell growth slightly, which occurred only in 10 % or 3 % serum conditions, but not under low serum (0.5 %) or serum-deprived condition. A noticeable efficacy of PACAP on cell proliferation of SK-Mel 37 was shown after long-term exposure to a serum-enriched environment (10 %) with PACAP 27 (4-8 days), reaching at 1.4-fold or 1.9-fold increased cell yield with high or low cell seeding number, respectively. This increased cell growth of SK-Mel 37 was exerted by PACAP 27 in the absence of serum as well. PACAP 27 partly restored cell growth or survival of SK-Mel 37 damaged by serum starvation, and contributed for nearly 1.8-fold increased cell viability level. However, this cytoprotective effect in SK-Mel 37 was shown only following prolonged (4 days), but not short-term

exposure to PACAP 27 (3 days). Journot et al (1998) described that PACAP 38 protected cerebellar neurons from cell death induced by serum and potassium withdrawal. Our data is in line with this report, suggesting that a cytoprotective effect of PACAP similarly occurs in melanoma cells following serum starvation. Reversely, anti-proliferative activity was induced by a PACAP receptor antagonist. The suppressed cell growth was observed after exposure to peptide PACAP6-38, which has been used as a selective PAC1R antagonist, causing a 27 % decrease in cell viability of SK-Mel 37. This inhibitory effect, however, was exerted only by PAC1R antagonist, but not VPAC1R antagonist, indicating the involvement of autocrine loops in regulating cell viability of SK-Mel 37 via PAC1R but not VPAC1R. The effect of PAC1R antagonist on melanoma cell growth has not yet examined but our result resembles to data reported by Leyton (1998) and Sokolowsk (2008), showing a suppressed cell growth of breast cancer and glioma cells by peptide PACAP6-38.

In contrast, PACAP 27 decreased cell growth of NW-Mel 450 cultured in serum-containing medium (10 % and 3 %), while cell growth in low-serum (0.5 %) or serum-deprived culture systems slightly increased rather than reduced. This tendency to suppress cell growth changed under long-term exposure to PACAP, resulting in a minute increased proliferation in serum-enriched environment (1.2-fold), suggesting that PACAP 27 did not strongly involve in a proliferative as well as cytoprotective action for this melanoma line. Another investigation of cell proliferation following receptor blockage by using VPAC1R or PAC1R antagonist confirmed a minimal influence of PACAP on cell growth of NW-Mel 450. Most studies demonstrated PACAP effect on cell survival after induced cell death by various agents and the results in SK-Mel 37 correspond to these previous investigations (Gutierrez-Canas, 2003; Casz, 2005; Castorina, 2008; Onoue, 2008), supposing that a markedly proliferative or cytoprotective PACAP effect may occur only in cells that are susceptible to serum starvation. Moreover, Meyer and colleagues showed an antiproliferative effect of PACAP 38 on astroglial cell growth through inhibition of the small GTPase RhoA that promotes cell cycle entry from G1 into S phase (Meyer, 2005). Concerning this report cell cycle of NW-Mel 450 may be differently regulated by PACAP depending on serum concentration and time of incubation.

Taken together, this finding supports the evidence that PACAP exerts a potent mitogenic role in SK-Mel 37 at first and its action is melanoma cell-type specific. Therefore, PACAP might support melanoma cell survival even under low nutritional condition.

5.1.2.2. PACAP regulation in gene expression involved in survival and proliferation

The initial approaches outlined in this study demonstrated that PACAP 27 has proliferative or cytoprotective effect in SK-Mel 37 depending on serum contents. These effects of PACAP have been defined in several studies on other tumor types through the regulation of signaling pathways or certain gene expressions involved in cell survival or proliferation (Villalba, 1997; Obara, 2007; Aubert, 2006; Botia, 2007). To investigate the relationship between PACAP and gene expression involved in survival or proliferation in melanoma the four following genes were selected in this study: cyclin D3, cyclin E, Ki67, and BCL-2, since these have been well investigated in melanoma progression and were defined

as useful biomarkers (Bales, 1999; Florenes, 2000; Pearl, 2007; Prasad, 2012). In melanoma cell lines, incubation with PACAP 27 for 4 days affects the mRNA expression of these genes depending on serum concentration, time for incubation with PACAP 27, and melanoma cell type. In full-serum culture system (10 %), SK-Mel 37 responded to PACAP 27 with increased express four genes, and particularly cell cycle regulators, cyclin D3 and cyclin E, were highly induced. This amplified gene expression decreased over time on day 4 compared to other genes. In serum-deprived culture of SK-Mel 37, PACAP-mediated increase in gene expression could be observed especially BCL-2 and Ki67 rather than for cell cycle regulators. To verify specificity and to define the involved PACAP receptors an antagonist for high affinity receptor PAC1R was used with SK-Mel 37 line demonstrating the reverse observation with much less of the four gene expressions. Differently to this result an investigation by D'Amico (2013) on protein level demonstrated an increased cyclin D1 and BCL-2 in glioma cells cultured in serum containing medium with PACAP or VIP but low yield of both proteins in serum-deprived condition in the presence of neuropeptide. SK-Mel 37 melanoma cells instead showed a positive response to PACAP 27 with all four expressions on mRNA level regardless of serum contents. Although mRNA expression levels do not necessarily correlate with protein levels, our results in gene expression were supported by MTT assay in cell growth of PACAP-treated SK-Mel 37 showing an increased cell proliferation or cell survival both under serum-containing or serum-deprived condition.

PACAP 27 also induced an increase in the four gene expressions of NW-Mel 450 regardless of serum contents. Ki-67 expression was much higher than it in SK-Mel 37 under full-serum condition and its expression under serum starvation increased after long-term exposure to PACAP 27 rather than decreased like in SK-Mel 37. Secondly, cyclin D3 was not much up-regulated by PACAP 27 in full-serum system (10 %), while it increased highly under serum starvation. In addition, cyclin D3 was slightly induced despite PAC1R antagonist treatment in NM-Mel 450 in contrast to SK-Mel 37 line, where cyclin D3 in SK-Mel 37 was regulated positively with PACAP 27 or negatively with PAC1R antagonist supplement. The involvement of cyclin D3 in cell proliferation upon mitogenic stimulation is not clearly defined, but some studies suggested that cyclin D3 may display a role in differentiation and growth arrest in certain cell types (Bartkova, 1998). In another study, on the other hand, investigators showed that cyclin D3 activation may be dependent on forskolin-mediated mTOR activation leading to cell proliferation (Anderson, 2010). Differently regulated cyclin D3 of both PACAP-treated melanoma lines might be a factor involved in different PACAP-mediated regulation of cell growth. Cyclin D3 level in NW-Mel 450 was constant regardless of PACAP or PAC1R antagonist treatment, which resembles to the minimal effect of both peptides on cell growth in the full-serum condition. A discrepancy with less proliferation of NW-Mel 450 despite highly enhanced gene expression of cyclin E and Ki67 by PACAP 27 might be explained by differently regulated cyclin D3, therefore, supposed to be an important checkpoint in cell growth regulation. Furthermore, the characteristic difference between SK-Mel 37 and NW-Mel 450 stands out prominently through the additional experiment with PACAP 38 or VPAC1R antagonist. In SK-Mel 37 line PACAP 38 less affected gene expressions compared to PACAP 27, while in NW-Mel 450 a similar outcome of gene expressions regardless of PACAP

subtypes was fund, except for cyclin E. Blocking the low affinity receptor VPAC1R with antagonist reduced gene transcripts in SK-Mel 37, which however induced gene expressions in NW-Mel 450, again except for cyclin E and the latter has not been further evaluated.

5.1.2.3. PACAP regulation in protein expression involved in survival

PACAP has been reported to be a regulator of BCL-2 expression involved in the prevention from cell death either by up-regulating BCL-2 expression or by regulating cell cycle components. In addition, PACAP was found to induce a proliferative effect of cells through enhanced cAMP stimulation (Erhardt, 2004; Onoue, 2008; Sokolowska, 2008). Our finding of enhanced mRNA expression of BCL-2 by PACAP 27 treatment in both melanoma cell lines regardless of serum contents is in accordance with our Western blot results showing an up-regulated BCL-2 protein product in PACAP-treated melanoma lines. Moreover, the enhanced level of BCL-2 was lasting for 4 days in the serum-containing culture system. A similar result could be observed after PACAP 38 treatment in NW-Mel 450, with a higher BCL-2 level yielded by PACAP 27 than by PACAP 38. The present result is in line with a before mentioned study by D'Amico showing an increased BCL-2 protein level by PACAP in glioma cells grown in the presence of serum (D'Amico, 2013). On the other hand, serum-starved glioma cells displayed a reduced BCL-2 expression despite PACAP treatment in this report. In another study, VIP and PACAP were shown to increase BCL-2 protein synthesis in a serum-starved human prostate cancer cell line PC-3 (Gutierrez-Cana, 2003). Like PC-3 cells, serum-starved SK-Mel 37 increased BCL-2 protein slightly in the presence of PACAP 27. Therefore, it seems likely that not only the type of cancer cell line examined but also the individual source of metastasis for the cell lines could determine how PACAP regulates anti-apoptotic protein expression, BCL-2. A positive regulation of PACAP in BCL-2 expression of melanoma cells could be verified by another experiment with receptor antagonists. As expected a receptor antagonist caused a reduced BCL-2 protein expression in SK-Mel 37. In one setting, PAC1R antagonist increased BCL-2 expression of NW-Mel 450 in the first three days and a declined BCL-2 level occurred only following long-term exposure to PAC1R antagonist (on the fourth day). NW-Mel 450 treated with antagonist for low affinity receptor VPAC1R showed as well suppressed BCL-2 expression in similar time window like PAC1R antagonist. Another approach with combined-blockage of both receptors, VPAC1R and PAC1R, was shown also to reduce BCL-2 level of NW-Mel 450 on day 3. Based on this result it is supposed that autocrine released or exogenously supplemented PACAP may act via both receptors in regulating BCL-2 expression.

As reported in several studies, the regulation of BCL-2 expression in melanoma was associated with MITF up-regulation modulated by cAMP-stimulating agents, such as forskolin or α-MSH (Bertolotto, 1998; Price, 1998; McGill, 2002; Zuhang, 2007). Therefore it is noteworthy to consider the involvement of PACAP in the regulation of both BCL-2 and MITF. Western blot analysis for MITF protein synthesis in both melanoma cell lines under serum-starved condition revealed MITF-specific bands as a doublet migrating at approximately 52 and 56 kDa, while cells under full-serum condition

expressed only one band at 56 kDa. Previous study by Hemesath (1998) has investigated these MITF bands by using two-dimensional phosphotryptic analyses and identified these isoforms, which differently appeared in the presence of a MAP kinase-mediated phosphorylation at serine 73 in the upper migrating species. The detected band at 56 kDa in melanoma lines seems likely to be corresponding to this serum-induced phosphorylated MITF band. Under full serum the exposure to PACAP 27 induced an increase or under PAC1R antagonist a decrease in MITF expression of SK-Mel 37 grown under full-serum condition, while a slightly reduced MITF protein in serum-starved cells was resulted following PACAP supplement. An up- or down-regulated MITF expression is in accordance with the immunoblotting result in BCL-2 expression in serum-containing system but the reason for the discrepancy between enhanced BCL-2 and reduced MITF expression by PACAP in serum-deprived cells could not be explained in this study. One possible explanation for low level of MITF could be an inappropriate measuring time for MITF expression regulated by PACAP. A study in modulation of MITF expression by Lin (2002) demonstrated a profound effect of treatment with acid agents on MITF expression between 6 and 24 hours, suggesting the differential kinetic regulation of MITF. In our study, NW-Mel 450 produced relatively weak and smear band of MITF protein compared to SK-Mel 37. Selzer et al reported (2002) that MITF-M expression was associated with a transition from an epitheliod towards a more spindle-shaped cellular morphology indicating a role of MITF in phenotype of human melanoma. This report might explain the differential MITF expression level in melanoma lines upon distinct morphological features. Furthermore, the detected MITF protein level in PACAP or PAC1R antagonist treated NW-Mel 450 was not clearly different from the control. It is still unexplained why elevated BCL-2 level by PACAP was not linked to MITF expression in NW-Mel 450.

5.1.2.4. PACAP regulation in protein expression of migration chemokine receptor

Invasiveness of malignant melanoma is a crucial step in melanoma progression and expression of chemokine receptors on melanoma contributes to migrate into a specific site. One of chemokine receptors, which are strongly correlated with lymphatic metastasis of melanoma, is CCR7 (Mori, 2005; Shields, 2007; Takeuchi, 2004). Overexpression of CCR7 has been implicated in primary and metastatic melanoma and studies in a murine melanoma model demonstrated that injection of B16 melanoma cells bearing CCR7 into mice induced 701-fold enhanced metastasis to the draining lymph node (Emmett, 2011; Willey, 2001). Therefore, CCR7 is considered as a critical factor in lymphatic metastasis of melanoma. Known cAMP stimulators, such as forskolin, were published to induce the chemokine receptor CCR7 and CXCR4 in DCs (Gagliardi, 2002). And, PACAP has been described as a cAMP stimulator. Our study now demonstrated CCR7 expression under PACAP treatment in both melanoma lines, SK-Mel 37 and NW-Mel 450, on both CCR7 mRNA (17 hours) and protein level (48 hours) in a PACAP dose-dependent manner. Further, the long-term PACAP treatment accounted for consistent CCR7 expression over 5 days, especially on SK-Mel 37, but not that high on NW-Mel 450. A study by Sancho (2006) showed CCR7 expression on thyroid tumor cell line TPC-1, with cell proliferation following stimulation with CCL21, one of the two described ligands for CCR7. This might

resemble to the cell growth of PACAP-treated melanoma lines at least for SK-Mel 37. Another study by Kim (2005) reported the correlation of CCR7 expression on $CD8^+$ T cells with BCL-2 expression conveying the protection from cell death. In present study CCR7 expression in serum-starved melanoma cells was not investigated, however SK-Mel 37 cultured in full-serum medium showed an increased protein expression of CCR7 and BCL-2 in short- and long-term exposure to PACAP, supposing PACAP might be involved in cell survival. This speculation is in accordance with the result of NW-Mel 450 as well, whose cell proliferation and CCR7 expression was impaired by long-term PACAP treatment. Taking together PACAP can up-regulate the CCR7 expression in melanoma cells, suggesting being capable support migration of melanoma cells into the draining lymph node and possibly to enhance melanoma cell survival through this receptor.

5.1.2.5. PACAP regulation of cytokine production in melanoma

Finally, the two selected melanoma cell lines showed distinct spontaneous cytokine production of IL-10 in SK-Mel 37 or IL-6 or NW-Mel 450, however in serum containing medium only. Moreover, this study showed positive effect of PACAP on cytokine production in melanoma. Several studies had shown that PACAP modulates secretion or production of some cytokines from various immune cells (Vaudry, 2009). Especially, PACAP was shown to suppress the production of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α, whereas it promotes the release of anti-inflammatory molecule IL-10 (Ganea, 2002). This data suggested that PACAP displays an anti-inflammatory regulator in immune system. In other studies, PACAP stimulates positively IL-6 secretion in mammals (Kageyama, 2007; Seki, 2006) and it induces pro-inflammatory cytokines (IL-1β and TNF-α) in leukocytes of fish with unchanged IL-10 level (Wang, 2013). Corresponding to previous studies PACAP stimulated the IL-6 release in NW-Mel 450 cultured in serum-containing medium but not in the absence of serum. Unlike immune cells, in SK-Mel 37 regulation of already high IL-10 production did not show an additional positive response to PACAP. This result indicates firstly that PACAP regulates IL-6 in NW-Mel 450 but not IL-10 in SK-Mel 37. Secondarily, exogenous PACAP could not rescue IL-6 production in NW-Mel 450 under serum starvation. Furthermore, blocking the PACAP binding with receptor antagonists for VPAC1R and PAC1R for 48 hours did not inhibit either IL-6 or IL-10 production, supposing that these receptors did not seem to be associated with modulation of cytokine production directly. However, it is needed to investigate, whether receptor antagonist has a too short lasting mode of inhibitory effect on the cytokine release in melanoma. IL-6 has been reported to inhibit serum starvation-induced apoptosis in myeloma cells and this inhibitory mechanism was not involved in BCL-2 expression (Lichtenstein, 1995). Although released IL-6 level was much lower than it in the presence of serum, NW-Mel 450 constantly produced some IL-6 for 48 hours in the serum-free medium and cell viability studies showed that cell growth of this melanoma line was stabile after serum starvation. Based on these results constant cell survival of serum-deprived NW-Mel 450 might be supported by IL-6 production. An inhibitory effect of IL-6 on cell proliferation of primary or early stage of melanoma (Florenes, 1999; Lazar-Molnar, 2002) as well as melanoma cell line (Morinaga,

1989) has been reported in several studies. These findings give a possible explanation for the suppressed cell growth of PACAP-treated NW-Mel 450 despite increased IL-6. Taken together, PACAP was involved in regulation of IL-6 production but not of IL-10.
The study on two melanoma cell lines, SK-Mel 37 and NW-Mel 450, suggests PACAP may be considered as a cell type specific differentially modulating peptide in melanoma.

5.2. Part II: Paracrine effect of PACAP on T cells

The present study showing PACAP production by melanoma with possible paracrine effects led to the consideration that PACAP might also account for the accumulation of tolerant immune cells surrounding the tumor area. In other words, immune tolerance to melanoma may be enforced by PACAP produced by melanomas. Many other investigators have proved that neuropeptides, such as VIP or PACAP, display a crucial role for the regulation of immunity and inflammation (Tan, 2009; Waschek, 2013, Tan 2013). As mentioned in the introduction, PACAP is capable to promote a Th1/Th2 cell differentiation via highly and constitutively expressed VPAC1R on T cells with enhanced release of anti-inflammatory factors (Delgado, 1999; Martinez, 1998; Goetzl, 2001). In addition, PACAP was observed in lymphocytes to act as a survival agent through reduced activation-induced cell death of activated $CD4^+$ T cells (Delgado, 2000). Considering the involvement of PACAP production by melanoma in the regulation of immune system, it was worthy to examine the effect of PACAP on $CD4^+$ T cells, particularly on Treg.

5.2.1. Occurrence of PACAP receptors in human T cell

As shown for PACAP type II receptors, VPAC1R expression was clearly detectable in resting $CD4^+$ T cells and non-$CD4^+$ T cells of peripheral blood from healthy donors on mRNA level tested by PCR while relatively small amount of VPAC2R gene transcripts were found. This result is in line with the reports showing the constitutively expressed VPAC1R, marginally expressed VPAC2R, and no expression of PAC1R in resting T cells (Delgado, 1996; Johnson, 1996; Lara-Marquez, 2001). On the other hand, expression of type I receptor PAC1R was detected on monocytes only, but not on unstimulated $CD4^+$ T cells. Resting monocytes as well were published to express VPAC1R rather than VPAC2R, getting up-regulated by activation, as has been demonstrated by Lara-Marquez et al (2001) or Martinez et al (2002). In our study, resting monocytes expressed higher amount of transcripts for VPAC2R compared to VPAC1R, which is in accordance with the report by Dewit et al (1998), but in contrast to Lara-Marquez et al, who did not detect VPAC2R expression in resting monocytes. There are different isolation methods used to enrich for monocytes, i.e. plate-adherence method (Dewit et al) or a magnetic beads method with conjugation of CD14 antibody (Lara-Marquez et al), and the latter suggested that plate-adherence might active monocytes including VPAC2R. Based on this argument, in our study monocytes isolated by countflow centrifugal elutriation might be activated (not further investigated).

In mouse splenic T cells and thymocytes no expression of PAC1R was observed before or after stimulation (Delgado, 1996; Pankhaniya, 1998; Voice, 2001). Current results, however, for the first time showed PAC1R expression on $CD4^+$ T cells upon different stimuli together with PACAP supplement. Although some cases showed a contamination with monocytes and macrophages, pure Treg expressed PAC1R following stimulation with α-CD3/CD28 in the presence of either PACAP 27 or PACAP 38 regardless of additional IL-2 administration. Although PAC1R gene transcripts were also detectable in Teff/Tresp under most medium conditions, it was not necessarily a T cell result, but most likely due to monocyte contamination, demonstrated by positive expression for CD14 transcripts. In this study particularly PACAP 38 seems to account for stronger PAC1R transcripts in Treg compared to PACAP 27, while VPAC1R has been published to share similar affinity for VIP, PACAP 27, and PACAP 38 (Malendowicz, 1998). Another consideration may be the different kinetics of peptide degradation. A study reported that degradation of VIP and PACAP 27 was caused by neutral endopeptidase but PACAP 38 was not sensitive to this peptidase (Banks WA, 1993; Gourlet, 1997). In our experimental setting, PACAP 38 might therefore remain longer active in T cell culture than PACAP 27.

5.2.2. Suppression of T cell growth in the presence of PACAP

Many investigators demonstrated that suppression of T cell growth was mediated by cAMP stimuli, such as forskolin, phosphodiesterase inhibitors, or adenosine (Elliott, 1992; Zhang, 2004; Rodriguez, 2013). Our observation showed that total $CD4^+$ T cells stimulated with α-CD3/CD28 showed a similar cell amount within 3 days of culture regardless of PACAP 38 supplement. In another approach, separated $CD4^+$ T cells of $CD4^+CD25^+CD127^{low/-}$ (Treg) and $CD4^+CD25^-$ (Teff/Tresp) T cells were counted short-term incubation for 17 hours with different stimuli and both T cell subsets resulted in decreased cell growth in the presence of PACAP 38, but slightly increasing with exogenous IL-2 supply, which however did not completely recover the PACAP 38-mediated suppressive effect. A similar result was obtained following long-term exposure to PACAP 38 (over four days), showing an approximately 25 % reduced cell growth of both T cell subsets, even when stimulated with α-CD3/CD28 and cultured in the presence of IL-2. This suppressed cell growth by PACAP 38 is likely to be caused by cell cycle arrest, as has been investigated by Anderson et al (2010). These authors were showing that VIP and PACAP induced cell cycle arrest of activated T cells via blockage of G1/S transition and inhibition of cyclin D3 and E synthesis (Anderson, 2010). The cAMP/PKA pathway, which is influenced by VIP or PACAP might play a critical role for the regulation of cell growth, since other studies reported cell cycle arrest of T cells induced by cAMP/PKA-mediated agents (Boussiotis, 2000; Grader-Beck, 2003; Heijink, 2003). IL-2 supplement did not change cell growth under PACAP 38, meaning this neuropeptide might inhibit the IL-2-induced signaling pathway. Rodriguez et al (2013) reported that the adenylate cyclase activator forskolin reduced human T cell growth via negatively regulated IL-2 signaling in Jak/Stat5 activation via uncoupled IL-2R complex formation. There is a lack

of data in this study to define the mechanism for PACAP 38 involvement in cell proliferation, but several approaches with the two purified T cell subsets confirmed a suppressive effect on cell growth. This might on the other hand result in promotion of cell differentiation and better specific function.

5.2.3. PACAP induces in gene expression related to Treg

With cAMP stimulation, an up-regulation of hallmarks for regulatory T cells, namely FoxP3 and CTLA-4 expression, in naïve T cells have been reported in several studies (Baratelli, 2005; Vendetti, 2006; Jen, 2007). In our investigation a positive interaction between PACAP, a known cAMP stimulator, and the regulation of FoxP3 and CTLA-4 expression was demonstrated. In the presence of PACAP 27 or PACAP 38, both T cell subsets, Treg and Teff/Tresp yielded higher amounts of transcripts of both genes. The highest FoxP3 expression was observed in Treg activated with α-CD3 in the presence of PACAP. The expression level in Treg stimulated with α-CD3/CD28 did not further increased despite of PACAP supplement. A positive regulation in FoxP3 expression, however, was observed as well in Treg by PACAP following treatment with a combination of α-CD3/CD28 and IL-2. Although the expression level of FoxP3/β-actin was generally low in Teff/Tresp compared to Treg, FoxP3 transcript was prompted by PACAP as well. In the literature, a constant FoxP3 expression in naïve and activated Treg has been reported in several studies (Fontenot, 2003; Hori, 2003, Khattri, 2003). On the other hand, the optimal strength of co-stimulation is critical for FoxP3 expression in the periphery (Hsieh, 2006).

Similar to FoxP3 expression, PACAP 27 or PACAP 38 caused an enhanced CTLA-4 gene transcript in both T cell subsets. PACAP 38 induced increased PCR products of CTLA-4 in Treg stimulated either with α-CD3/CD28 or with combination of α-CD3/CD28 and IL-2, except Treg activated with α-CD3 alone, which already highly expressed CTLA-4 compared to Treg treated with α-CD3 and PACAP 38. On the other hand, Teff/Tresp treated with α-CD3/CD28 and PACAP 27 only showed a slightly up-regulated level of transcripts and in the remaining cases no PCR product of CTLA-4 was observed in control cells. FoxP3 expression was generally enhanced following PACAP 27- or PACAP 38-treatment in both T cell subsets but PACAP-mediated regulation of CTLA-4 expression seemed to be restricted in Treg. Little is known for the molecular regulation of both genes encoding FoxP3 and CTLA-4 by PACAP which does not necessarily exclude the possibility that PACAP could modulate gene expression differently in T cell subtypes.

In three separate approaches it could be observed that there are different expression levels of GAPDH in Treg upon distinct stimuli, although RNA quality and techniques for RNA isolation were subjected to controlled steps. This difference in GAPDH expression was not observed however in Teff/Tresp. Common results for the increased GAPDH expression in the presence of PACAP may be explained through the enhanced cellular activity. This suggesting was supported by a report from Mozdziak and colleagues showing changed GAPDH expression upon nutrition status (Mozdziak, 2003). Accurate analysis for the participation of PACAP in controlling GAPDH gene expression may be achieved by quantitative real-time RT-PCR (Q-RTPCR) technique (not done).

The effect of PACAP was not limited to the regulation of FoxP3 and CTLA-4 gene expressions. Our study showed that IL-10 and TGF-β transcripts were positively regulated by PACAP as well. This observation was supported by the report from Anderson and colleague. They demonstrated the generation of naïve $CD4^+$ T cells to IL-10/TGF-β producing cells in the presence of VIP (Anderson, 2010). An interesting observation in this study was, however that two distinct anti-inflammatory cytokines, IL-10 and TGF-β, were not regulated similarly by PACAP in Treg and Teff/Tresp. In general, PACAP caused an increase of both gene expressions in the two T cell subsets, but a strong up-regulation of IL-10 expression could be observed in Treg stimulated with α-CD3/CD28 in the presence of PACAP compared to its level in Teff/Tresp under same condition. In contrast, an increased TGF-β expression was observed in Teff/Tresp rather than in Treg under same conditions with stimuli and PACAP supplement. Despite limited results in gene expression, it can be suggested that predominantly up-regulated cytokine type by PACAP was distinct upon T cell subsets. Unexpected were the high transcripts of IL-10 in Teff/Tresp activated with α-CD3/CD28 alone. As some studies demonstrated that antigen-specific activated $CD4^+$ T cells in the acute and chronic inflammatory condition can produce IL-10 displaying roles in anti-apoptosis and suppression of inflammation, it can be supposed that stimulation with α-CD3/CD28 can induce high amount of IL-10 gene transcripts in Tconv particularly (Jankovic, 2007; Anderson, 2007).

In addition to regulation of PACAP in gene expression of cytokines, the expression of chemokine receptors, CCR4 and CCR7 was investigated. Other investigators have found a correlation of these chemokine receptor expressions with a suppressive immunity (Yagi, 2006; .Zhou, 2013). Treg and Teff/Tresp stimulated with α-CD3/CD28 or addition of IL-2 induced a slightly increased gene expression of both chemokine receptors, which was boosted in the presence of PACAP 38. Even these gene expressions should be verified at the protein level again. In one study, $Foxp3^-$ transduced $CD4^+CD25^-$ naïve T cells have been shown to up-regulate the expression of CCR4 and to gain the functions of $CD4^+CD25^+$ Treg cells, indicating a relationship between Foxp3 and CCR4 expression (Yagi, 2005). A similar finding by Ishida (2004) demonstrated a reduced level of Foxp3 gene expression in parallel with the CCR4 in PBMC. Expression and function of CCR7 has been as well investigated in $Foxp3^+$ Treg by Schneider et al (2007), who demonstrated CCR7 knock out Treg fail to localize to lymph node and to inhibit specific effector response. Indeed, an upregulated Foxp3 expression by PACAP-treated T cells showed a highly increased CCR4 and CCR7 in this study. Another study showed that CCR7 expression increased during macrophage differentiation following adrenergic receptor activation via elevation of cAMP level (Damas, 2007). Therefore, Treg under PACAP influence released from melanoma might negatively regulate the tumor-specific immune reaction in the draining lymph node via CCR7 in T cell as well.

This result indicated that each T cell subset responded distinctly to PACAP through diverse cytokine production or chemokine receptor expression. It is still necessary to confirm the present data by other

methods or settings and to answer how PACAP modulates gene transcripts in Treg and Teff/Tresp triggering tolerogenic immune action. Further studies of PACAP-regulated genes and pathways will be helpful to understand melanoma biology and to develop better clinical therapies for patients. In addition, considering improved gene expression of FoxP3 or CTLA-4 and anti-inflammatory cytokines, such as IL10 or TGF-β, by PACAP leads to the assumption that this neuropeptide is capable of supporting suppressive function of Treg as well as converting Teff/Tresp into tolerogenic T cells.

Moreover, with the preferential protein expression of PACAP 38 in primary melanoma, T cells might newly express PAC1R in the early phase of disease, promoting a tolerogenic pro-tumor microenvironment.

5.2.4. Improved suppressive function of Treg following PACAP supplement

The molecule cAMP has been known as a general inhibitor of T cell growth resulting in a suppressive immunity (Bodor, 2001). Bopp and his colleagues demonstrated enhanced suppressive function of nTreg cells in the presence of the PDE4 inhibitor rolipram, which blocks cAMP degradation (Bopp. 2009). Other investigators verified that cAMP up-regulated the expression of CTLA-4 and its cognate B7 ligands and CTLA-4 expression in the absence of cAMP did not induce effective suppression (Vendetti, 2002). These findings support our current results in the suppression of cell growth or in enhanced gene expressions involved in the suppressive function. Moreover, the examined suppressive capacity of Treg in the presence of PACAP, a cAMP stimulator, in addition showed a suppressed cell growth of Teff/Tresp in co-culture assay. Following PACAP 38 supplement around 43% declined cell growth of responding T cells was shown, while 23% less proliferation was achieved at a ratio of 2:1 (Teff/Tresp:Treg) in the absence of PACAP 38. This inhibition of proliferation could be still observed in Teff/Tresp co-cultured with low numbers of Treg at a ratio of 64:1. Similarly, PACAP 27 showed decreased cell growth as well, but the effectiveness was less than with PACAP 38. The differential effects of PACAP 27 and PACAP 38 might be caused by distinct kinetics for degradation of peptide in T cell culture medium mentioned above with proposed longer activity for PACAP 38. One controversial result in this study is an increased cell number of Teff/Tresp following PACAP 38 treatment in a specific setting. Previous experiments for some donors with regard to cell proliferation of Teff/Tresp showed a suppressed cell growth in the presence of PACAP 38, which did not recover despite IL-2 supplement. This discrepant result might be caused by different kinetic. The experimental setting for proliferation was a measuring of cell growth within 17 hours or four days of culture and control cells were cultured in the medium containing α-CD3/CD28 and exogenous IL-2 inducing a boost of cell growth. In the suppression assay Teff/Tresp were stimulated with α-CD3/CD28 in the absence of exogenous IL-2, and cultured in the absence or presence of PACAP for 5 days. VIP and PACAP have been indicated in a study by Anderson (2010) to induce cell cycle arrest of α-CD3/CD28-stimulated human T cells and based on this report it can be hypothesized that an early supplement of PACAP can delay cell growth compared to the PACAP-untreated T cells which were stimulated with α-

CD3/CD28 first, triggering an elevated cell proliferation and an early apoptosis. In contrast, in a long-term culture a single PACAP supply at the beginning could not maintain this delayed cell growth, so that cells could proliferate later as we observed in suppression assays. More studies have to be done to elucidate the distinct kinetics and order of supplement actions.

5.3. Concluding remarks

In conclusion, the present studies indicate for the first time the presence of neuropeptide PACAP and its high affinity receptor PAC1R in human melanoma, including melanoma tissues, melanoma primary cell culture, and melanoma cell lines, as shown by immunohistochemistry, immunofluorescence, and Western blot. The distinct distribution of PACAP subtypes, PACAP 27 and PACAP 38, in melanoma tissues suggests a predominant expression of PACAP subtypes dependent on melanoma stages. Furthermore, functional studies showed that exogenous PACAP promotes melanoma cell growth and gene expression involved in cell proliferation or cell survival, which were verified by immunoblotting for some survival factors. PACAP effect further appears to positively regulate the expression of chemokine receptor and cytokine production. Although the effect of the individual substances varies between two melanoma cell lines, PACAP-mediated outcomes may favor pro-tumor microenvironment. Supporting this pro-tumor microenvironment was also found for PACAP effects on $CD4^+$ T cells shown in these studies. A positively regulated gene expression involved in suppressive character and an improved suppressive function for the interaction of $CD4^+$ T cell subsets suggest that PACAP may strengthen the function of Tregs and modulate Teff/Tresp towards tolerogenic action. Taken together, these findings indicates that PACAP is an important factor for an altered status of melanoma microenvironment, however there are still questions about the PACAP-mediated mechanism in melanoma and T cells, particularly Treg.

Open questions:

1. What types of high affinity PACAP receptors, namely PAC1R splice variants were expressed in the melanoma and how does the distribution differ between melanoma lines SK-Mel 37 and NW-Mel450? In this present study the expression of PAC1R splice variant of hop area but not hip area was detected in both melanoma lines. It is necessary to investigate PAC1R splice variants in different signaling pathways, which might be involved in PACAP-mediated outcomes.

2. What second messenger systems are involved in the response to PACAP in melanoma? Measurement of cAMP level in PACAP-treated melanoma lines have not yet yielded any obvious result compared to neuroblastoma cell line, SY5Y (data not shown). Further investigation may focus on a signal transduction pathways coupled to PACAP stimulation in melanoma.

3. Does PACAP affects melanoma morphological changes after serum starvation? Serum starvation has been investigated by other to change morphology and invasiveness of melanoma. Although

morphological analysis of melanoma was not a primary goal in our studies, serum-deprived SK-Mel 37 in the presence of PACAP yielded a high amount of viable cells which in part detached from culture flask or strengthened dendritic morphology in adherence to culture flask. This observation was more obvious in the long exposure to PACAP.

4. Are there chances for relevant inhibitory effects in melanoma proliferation following blockage of PACAP production by using siRNA techniques? The blockage of PACAP receptors, PAC1R or VPAC1R, on melanoma lines showed suppression of cell activities but these effects were not very strong. Therefore, it is considerable to investigate further down-regulation of PACAP production, which can help to control melanoma.

5. Do $CD4^+$ T cells including Treg and Teff/Tresp express different PAC1R splice variants following long exposure to PACAP? In our studies, mRNA expression of PAC1R in hop area was demonstrated following stimulation with α-CD3/CD28 in the presence of PACAP and it is needed to investigate other splice forms of PAC1R in $CD4^+$ T cells. Moreover, the co-operative regulation between PACAP type 1 and 2 receptor on Treg should be studied in the future in more detail, providing the understanding for molecular intracellular mechanism in Treg, since it can be a potential therapeutic application in melanoma.

Finally, to assess therapeutic potential preclinical studies as well are needed to prove the effectiveness of PACAP inhibition on lowering aggressiveness of melanoma and on improving anti-tumor immunity.

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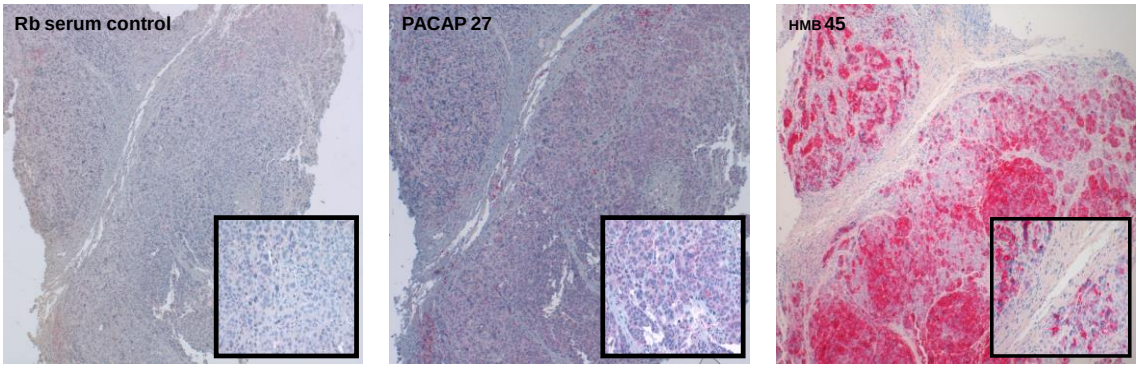
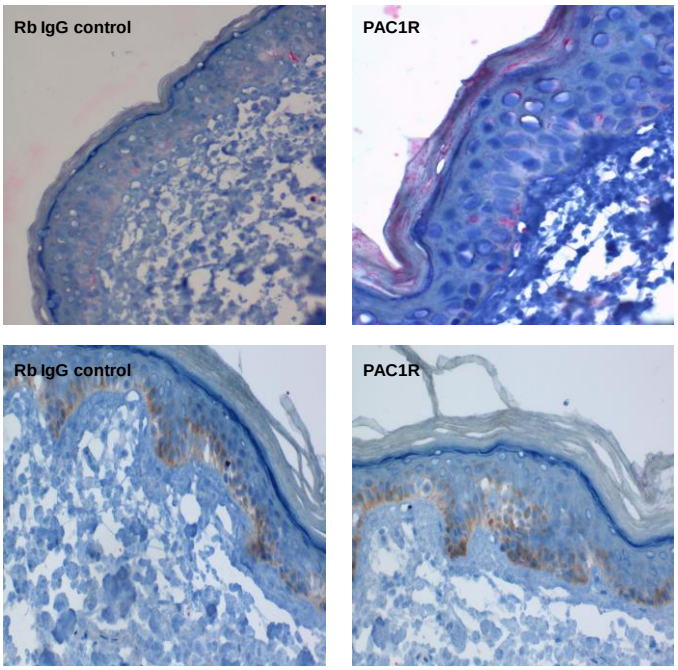
7. Supplementary data

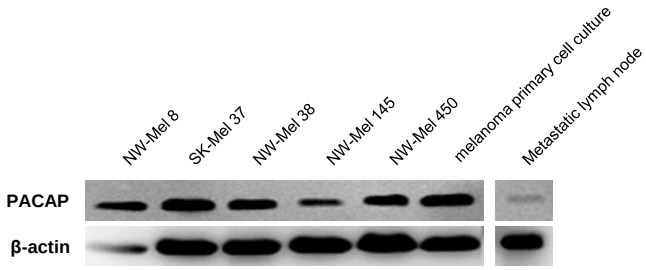
The following data are additional and directly relevant to the contents but they are separated in this section for reasons:
-additional data for understanding particular aspects of the contents,
-extended data sets, such as PACAP 38-treated melanoma or PACAP 27-treated T cells
-individual experimental data yet unconfirmed



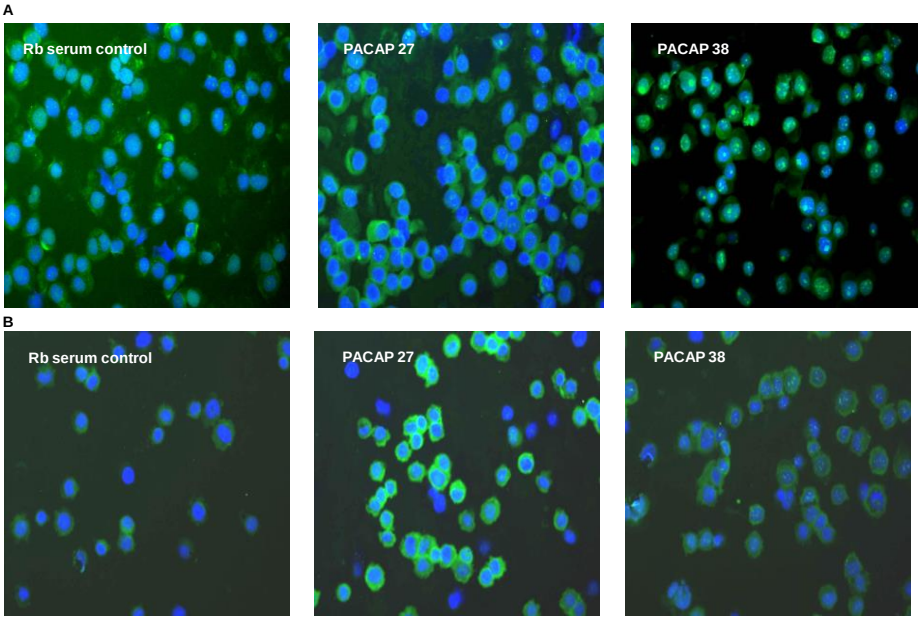
Supplement 1. PACAP expression in human primary melanoma. No.4 of primary melanoma was tested with a specific Rb serum anti-PACAP 27, whose staining intensity was very low but a weak positive response could be observed overall in tumor cells compared to the Rb serum control.



Supplement 2. Expression of PAC1R in healthy skin tissues. The upper panel is a healthy skin of the breast and the bottom one is a sample of the upper arm. These sections were stained with a Rb antibody against human PAC1R and as a rabbit IgG antibody was used as a isotype control. The pictures were captured at 10 x magnification.

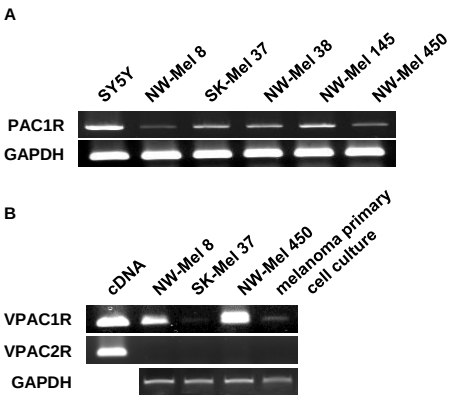


Supplement 3. PACAP expression in melanoma. Immunoblotting was performed to detect PACAP expression in various melanoma cell lines, in melanoma primary cell culture, and in tissue of lymph node metastasis. To detect PACAP a specific monoclonal mouse antibody against precursor PACAP was used and a band for PACAP was detectable at ~23 kDa (~18 kDa should be detected for precursor PACAP according to the data sheet). As an internal control β-actin was used.

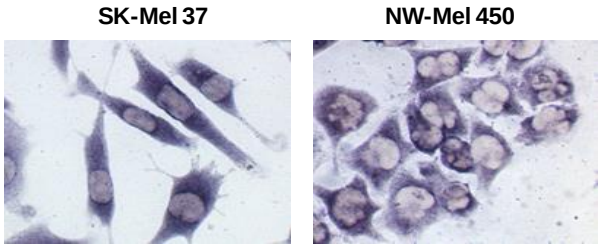


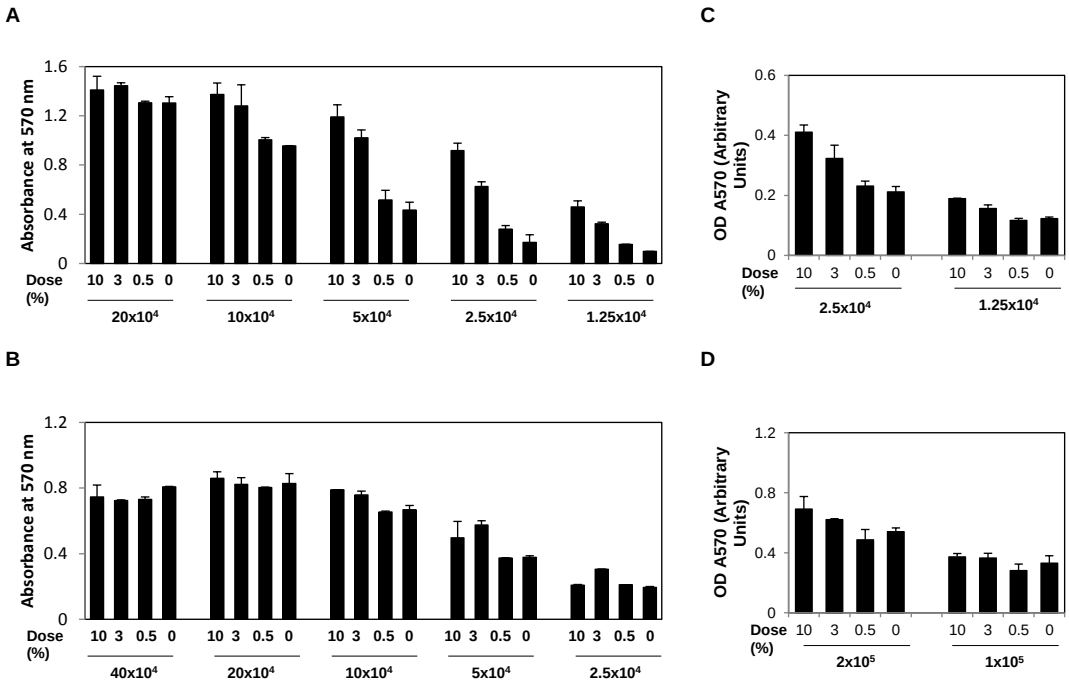
Supplement 4. Expression of PACAP 27 or 38 in melanoma cell lines, SK-Mel 37 and NW-Mel 450. (A) SK-Mel 37 and (B) NW-Mel 450 cell lines following cytospin-preparation of cells were stained with a specific Rb serum for PACAP 27, PACAP 38, or with the Rb control serum. Additional negative control was performed by staining without primary antibody. The pictures were captured at 40x magnification. Blue: Dapi staining a nucleus; Green: staining PACAP positive cells.

Supplement 5. Expression of PACAP receptor types I or II in melanoma. PCR was performed to detect expression of PACAP receptor type I (PAC1R) (A) or PACAP receptor type II (VPAC1R, VPAC2R) (B) in melanoma cell lines, melanoma primary cell culture, and SH-SY5Y (neuroblastoma cell line). SY5Y cells or cDNA was used to control the positive PACAP receptors expression. All five melanoma cell lines expressed the PAC1R with different transcripts level, but VPAC2 receptor was not detected in tested cell lines. The amount of VPAC1R transcripts showed differences in each melanoma line. Strong expression of VPAC1R was observed in NW-Mel 8 and NW-Mel 450, but weak expression was detected in SK-Mel 37 and melanoma primary cell culture.

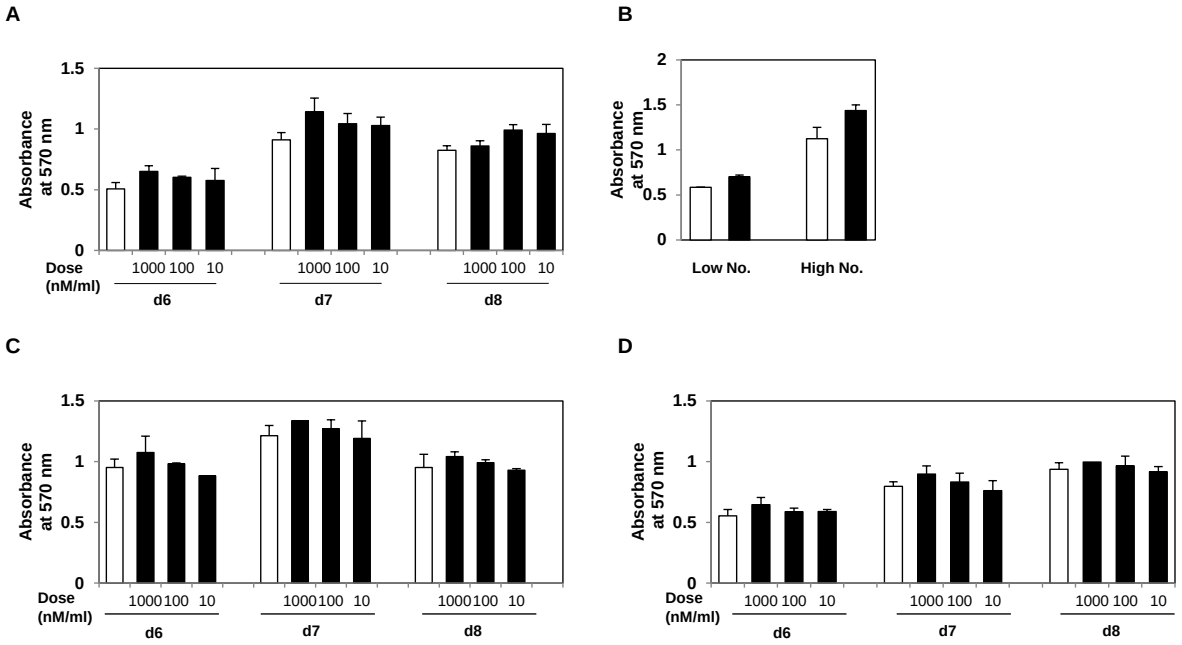


Supplement 6. Expression of PACAP 27 in SK-Mel 37 and NW-Mel 450. SK-Mel 37 and NW-Mel 450 cell lines were seeded on the chamber slides and stained with a specific Rb serum anti-PACAP 27. To detect PACAP 27 nitro blue tetrazolium (NBT) was used as a chromogenic staining. The pictures were captured at 40x magnification. These pictures showed a difference in the features of both melanoma lines.



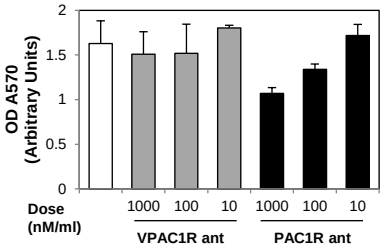


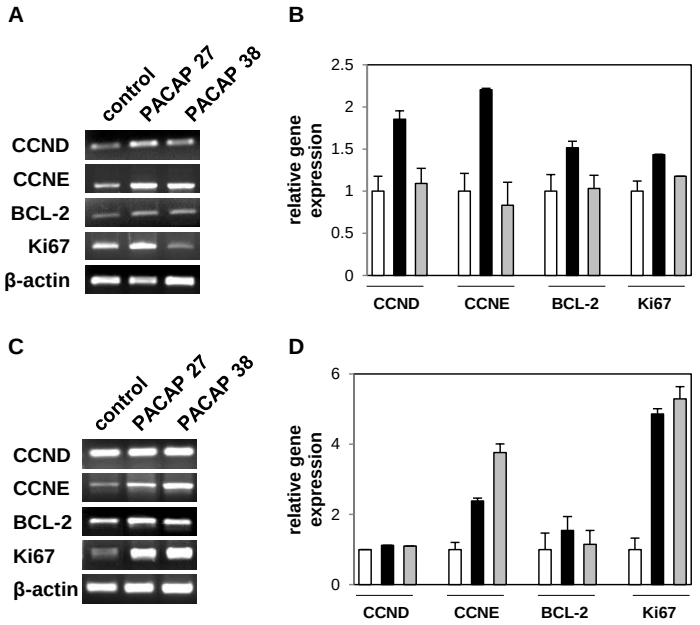
Supplement 7. Cell viability of melanoma cell lines under various serum contents. (A) SK-Mel 37 and (B) NW-Mel 450 were seeded at different cell seeding numbers in a 48 well plate. To determine the optimal initial cell seeding numbers each cell line was set up differently based on different size: SK-Mel 37 with initial cell seeding numbers starting from $2x10^5$ cells/ml and NW-Mel 450 with seeding numbers starting from $4x10^5$ cells/ml. Cells were cultured for 3 days following changing with fresh medium after 18-24 hours containing various concentrations of serum (10, 3, 0.5, or 0 %) and cell viability was measured by using MTT. The diagram represents the mean ± SD of duplicates from one experiment. Survival of melanoma cell lines, (C) SK-Mel 37 and (D) NW-Mel 450, cultured under various serum concentrations was tested. Melanoma cells were seeded in a 48 well plate and following changing after 18-24 hours with fresh medium containing various concentrations of serum (10, 3, 0.5, or 0 %), cell viability was measured on day 2 by using MTT. The diagram represents the mean ± SD of duplicates from one experiment.



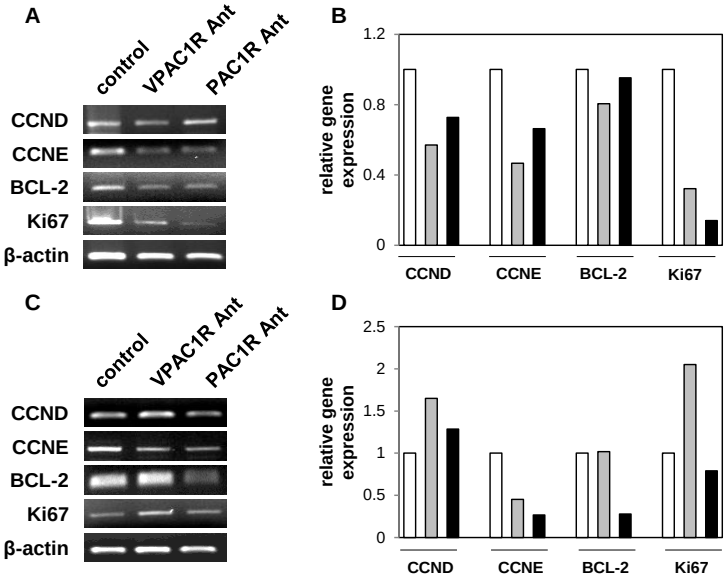
Supplement 8. Cell viability of melanoma cell lines following long-term culturing with PACAP 27 in medium containing various serum levels. (A) Time-dependent cell viability of SK-Mel 37 was investigated with $6.25x10^3$ cells/ml cells, which were incubated for 8 days under full serum condition (10 %) with various contents of PACAP 27 (1 µM, 100 nM, and 10 nM). (B) SK-Mel 37 seeded at $3x10^4$ cells/ml (Low No) or at $5x10^4$ cells/ml (High No.) were cultured in 3 % serum containing medium with 100 nM PACAP for four days. (C) NW-Mel 450 seeded at $2.5x10^4$ cells/ml in serum-containing medium (10 %) or (D) cells seeded at $5x10^4$ cells/ml in 0.5 % serum-containing medium were cultured for 8 days with the three distinct concentrations of PACAP 27 (1 µM, 100 nM, and 10 nM). The level of viable cells at each time point was measured using MTT and the average of duplicated ± SD from one experiment was represented in this diagram. White bar: control cells; black bar: PACAP-treated cells.

Supplement 9. Suppression of cell survival following treatment with PACAP receptor antagonists in melanoma line, SK-Mel 37 (A) SK-Mel 37 ($1.25x10^4$ cells/ml) was cultured in serum containing medium (10 %) following PACAP receptor antagonist, VPAC1R or PAC1R antagonist, for 4 days. Cell viability was assessed in an antagonist-dose-dependent manner (1 µM, 100 nM and 10 nM). MTT measuring showed the different regulation of PACAP receptor in the cell survival in melanoma. Results shown are presented as a mean of duplicates. White bar: control cells; black bar: receptor antagonist-treated cells.



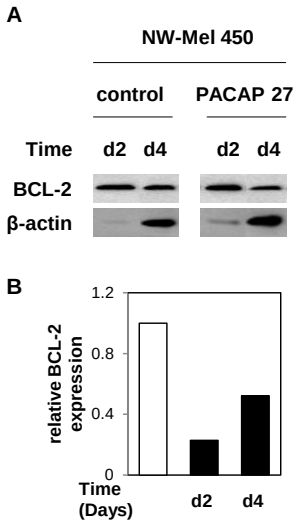


Supplement 10. Gene expression in SK-Mel 37 and NW-Mel 450 cultured in serum-containing medium in the presence of PACAP. (A) SK-Mel 37 and (C) NW-Mel 450 were seeded at 1.5x10^5 cells/ml in full serum-medium and after 24 hours cells were cultured in new changed medium containing 100 nM PACAP 27 or PACAP 38 and 10 % serum for 17 hours. RNA was isolated from treated and control cells and the gene expression (CCND, CCNE, BCL-2, and Ki67) was analyzed using PCR. Quantification of each band for SK-Mel 37 (B) and NW-Mel 450 (D) was demonstrated as the ratio of each targeted gene/β-actin of treated cells to control cells using densitometric analysis. The analyzed data with ±SD was presented by means of two independent experiments. White bar: control cells; black bar: PACAP 27-treated; grey bar: PACAP 38-treated cells.

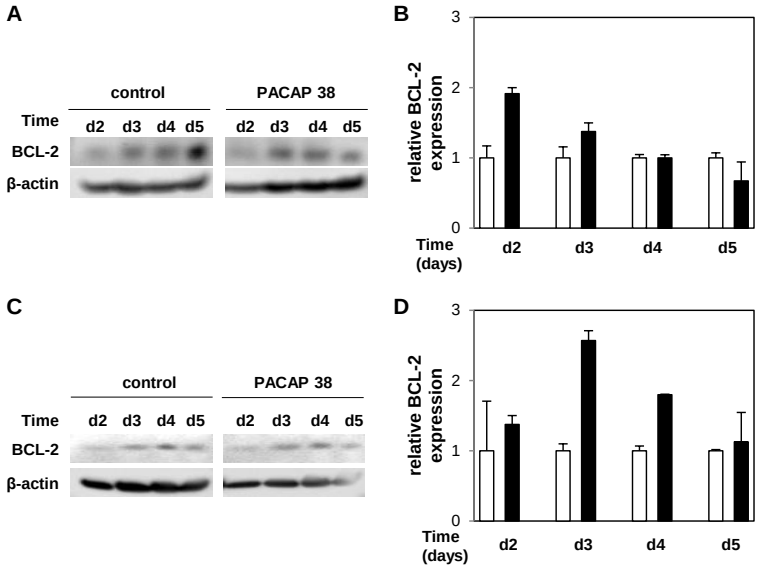


Supplement 11. Gene expression in SK-Mel 37 and NW-Mel 450 following blocking of PACAP receptor, VPAC1R or PAC1R. (A) SK-Mel 37 or (C) NW-Mel 450 were seeded at 1.5x10^5 cells/ml, and cultured in new changed medium containing 1 μM VPAC1R or PAC1R antagonist and 10 % serum for 17 hours. Gene expression level of cyclin D3 (CCND), cyclin E (CCNE),

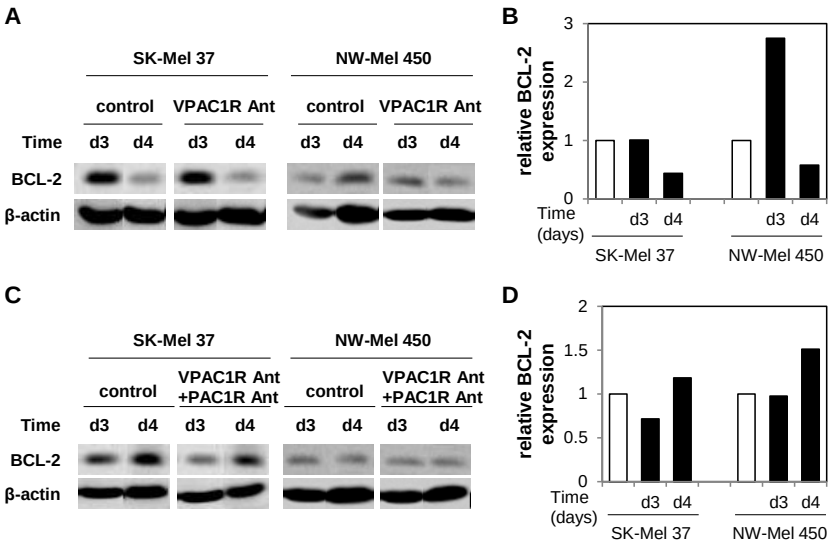
BCL-2, and Ki67 were analyzed by using PCR. Quantification of each band for SK-Mel 37 (B) or for NW-Mel 450 (D) was demonstrated as the ratio of each targeted gene/β-actin of treated cells to control cells using densitometric analysis. White bar: control cells; black bar: PAC1R ant-treated; grey bar: VPAC1R ant-treated cells.



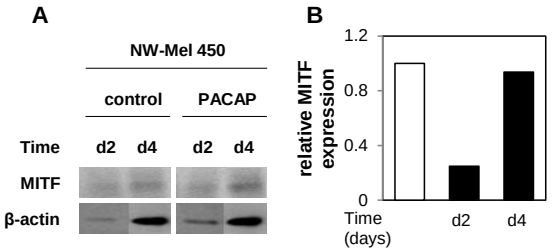
Supplement 12. PACAP effect on BCL-2 protein expression in NW-Mel 450 under serum-free condition. (A) NW-Mel 450 were seeded at $1.9x10^5$ cells/ml in full serum-medium and after 24 hours cells were cultured in new changed serum-deprived medium containing 100 nM PACAP 27 for 4 days. Collected cell lysates on day 2 or 4 were used to analyze the protein level of BCL-2 of treated and control cells by Western blot. (B) Quantification of each band was demonstrated as the ratio of BCL-2/β-actin of treated cells by using densitometric analysis. White bar: control cells; black bar: PACAP 27-treated cells.



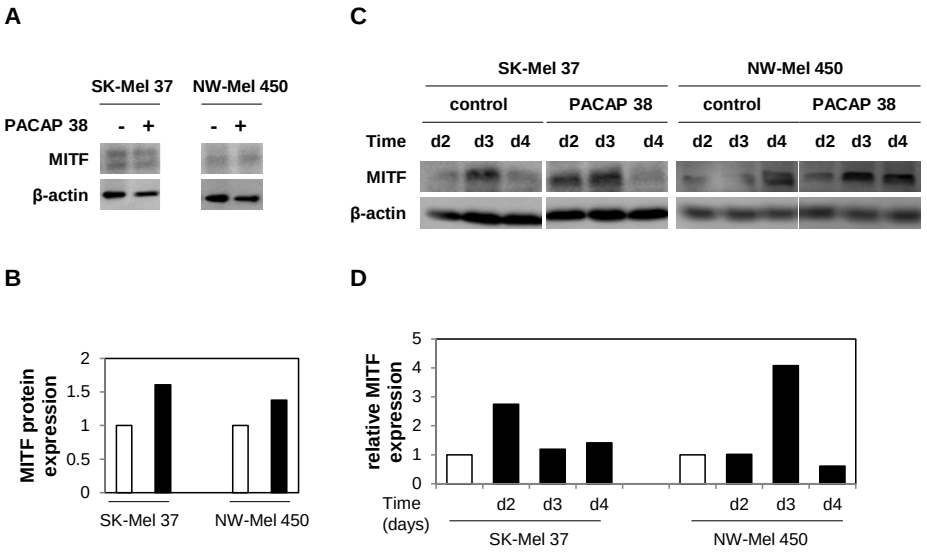
Supplement 13. PACAP 38 effect on BCL-2 production by SK-Mel 37 and NW-Mel 450 in long-term culture. SK-Mel 37 (A) or NW-Mel 450 (C) were seeded at $1.2x10^5$ cells/ml and cells were cultured in serum-containing medium in the presence of 100 nM PACAP 38 for 5 days. Medium was refreshed every two days and cell lysates were collected at each time point. Prepared lysates were used for evaluation of BCL-2 level from treated and control cells by immunoblotting. The graph for SK-Mel 37 (B) or NW-Mel 450 (D) showed the ratio of BCL-2/β-actin of treated to the control cells and the represented data was the mean of two independent experiments. White bar: control cells; black bar: PACAP 38-treated cells.



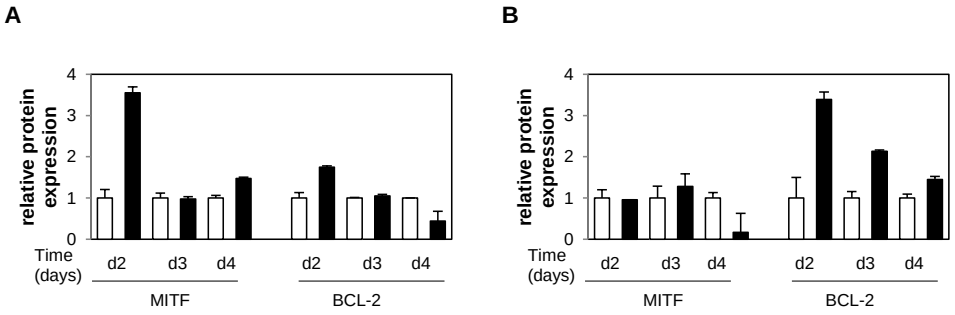
Supplement 14. Reduced BCL-2 expression in SK-Mel 37 and NW-Mel 450 following treatment with PACAP receptor antagonists. (A) SK-Mel 37 or NW-Mel 450 was seeded at $1.2x10^5$ cells/ml and cells were cultured in serum-containing medium in the presence of 1 µM VPAC1R antagonist for 4 days. (C) Same numbers of both melanoma lines were treated with 1 µM VPAC1R and PAC1R antagonist simultaneously. Medium was refreshed every two days and cell lysates were collected at each time point. The evaluation of BCL-2 levels was assessed by blotting. The graph for VPAC1R antagonist (B) and for combination of VPAC1R and PAC1R anatgonist (D) showed the ratio of BCL-2/β-actin of treated cells to compared the control sample. White bar: control cells; black bar: receptor antagonist-treated cells.



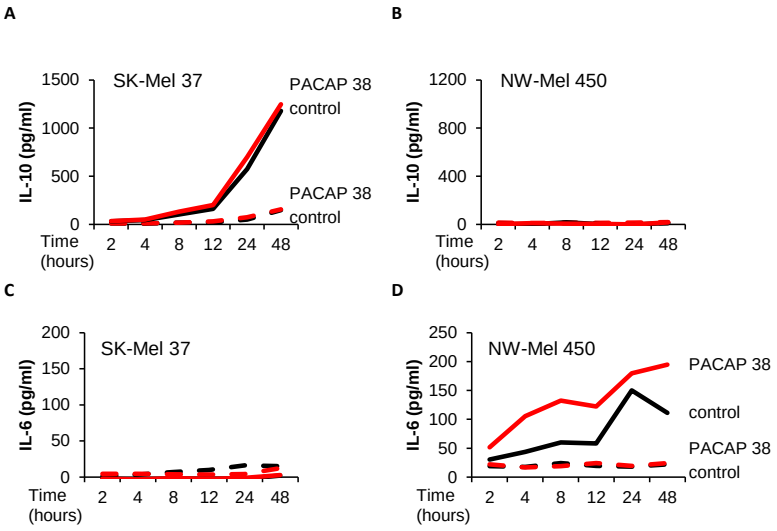
Supplement 15. MITF expression in NW-Mel 450 following treatment with PACAP 27. (A) NW-Mel 450 was seeded at $1.9x10^5$ cells/ml for 24 hours and after rinsing cells were cultured in serum-contained medium and next day cells were incubated for 4 days in serum-free medium containing 100 nM PACAP 27. (B) The evaluation of MITF level was assessed by blotting and the ratio of MITF/β-actin was presented in a diagram. White bar: control cells for each day (set 1,0); black bar: PACAP 27-treated cells.



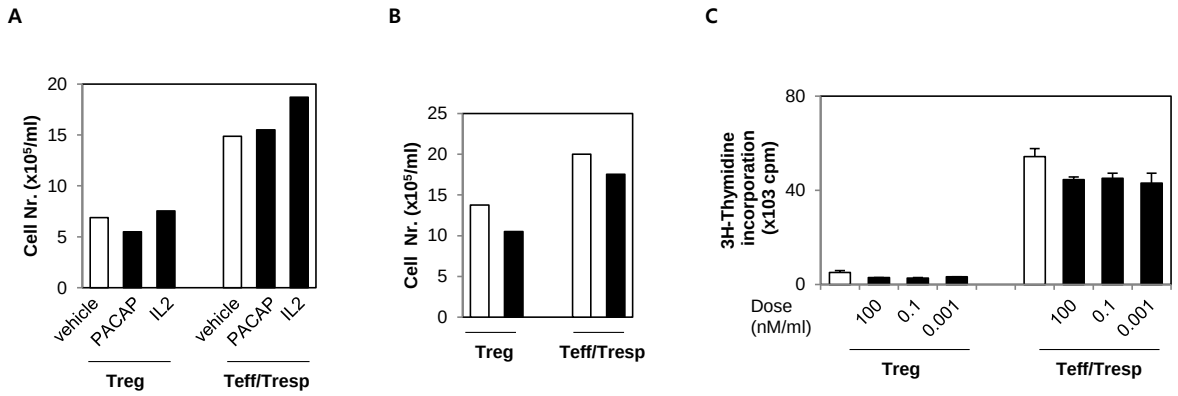
Supplement 16. MITF expression in melanoma lines following treatment with PACAP 38. (A) Both melanoma lines were seeded at 1.9x10^5 cells/ml for 24 hours and after rinsing cells were cultured in serum-containing medium and next day cells were incubated for 4 days in serum-free medium containing 100 nM PACAP 38 and medium containing PACPA 38 was refreshed every 2 days. To detect the MITF expression cells were collected on day 4 and blotting was performed. (C) 1.2x10^5 cells/ml of melanoma lines were cultured in serum-containing medium with 100 nM PACAP 38 for 4 days. Cells were collected on each time point and MITF level was analysed by using Western blot. (B) and (D) The ratio of MITF/β-actin was presented in a diagram. White bar: control cells for each day; black bar: PACAP 38-treated cells



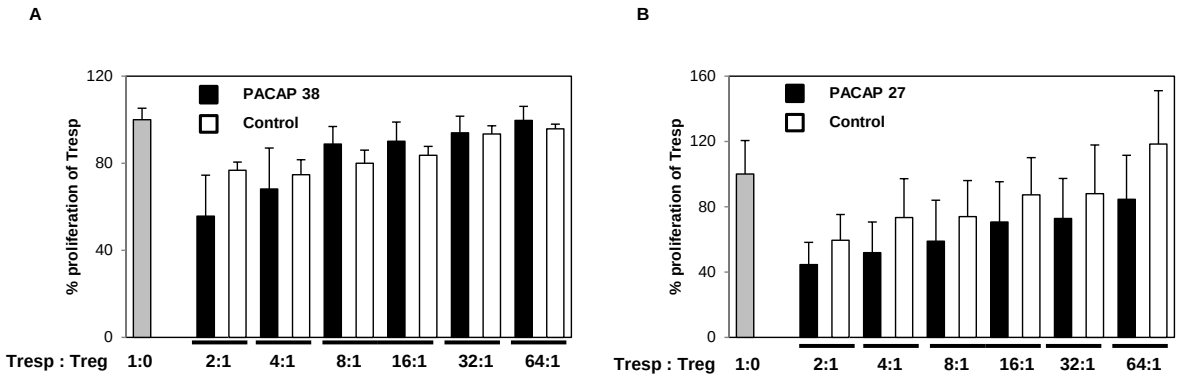
Supplement 17. Comparison of MITF and BCL-2 expression in melanoma lines following treatment with PACAP 27. (A) SK-Mel 37 and (B) NW-Mel 450 showed both protein expressions, MITF and BCL-2 I,n the diagram. The relative value of each protein expression was calculated from previous data and was presented in a graph to compare. White bar: control cells; black bar: PACAP 27-treated cells.



Supplement 18. Effect of exogenous PACAP 38 on cytokine production, IL-10 and IL-6 in SK-Mel 37 and NW-Mel 450. The supernatants of melanoma lines, SK-Mel 37 and NW-Mel 450, treated with 100 nM PACAP38 were collected at various time points and IL-10 or IL-6 was measured by using ELISA assay. Each amount of IL-10 in SK-Mel 37 (A) or NW-Mel 450 (B) and of IL-6 production in SK-Mel 37 (C) or NW-Mel 450 (D) were presented from one experiment in a graph. There were two different serum conditions: solid line for 10 % serum or dashed line for 0 % serum condition. Black: control cells; red: PACAP 38-treated cells.



Supplement 19. Proliferation of T cell subsets under PACAP treatment. (A) Purified $CD4^+CD25^+CD127^{low/-}$ (Tregs) and $CD4^+CD25^-$ (Teff/Tresp) T cells seeded at $3.5x10^5$ cells/ml were stimulated with α-CD3/CD28 beads in the presence of 100 nM PACAP 38 or 12.5 U/ml IL-2 and cells were incubated for 17 hours. Cells were collected and live cells counted by using trypan blue. White bar: control cells (CD3/CD28 stimulated cells); black bar: IL-2 or PACAP 38 treated cells. (B) Isolated Treg and Teff/Tresp cells seeded at $4.1x10^5$ cells/ml were activated with α-CD3/CD28 beads in the presence of IL-2. In the absence or presence of 100 nM PACAP 38 cells were cultured for 17 hours and cell numbers were determined by counting viable cells via trypan blue. White bar: control cells; black bar: PACAP 38-treated cells. (C) Purified Tregs and Teff/Tresp cells seeded at 1.25 $x10^5$ cells/ml were stimulated with α-CD3/CD28 beads with 12.5 U/ml IL-2 for 4 days. PACAP 27 was supplied in dose-dependent manner starting from 100 nM/ml to 1 pM/ml. Cell proliferation was measured by using ^{3}H-Thymidine incorporation assay. White bar: control cells; black bar: PACAP 27-treated cells.



Supplement 20. Suppressed proliferation of Teff/Tresp cells in Treg inhibitory assay in the presence of PACAP. $CD4^+CD25^+CD127^{low/-}$ T cells (Treg) and $CD4^+CD25^-$ (Teff/Tresp) were isolated from healthy donor peripheral blood (PBMC). (A) Both T cell subsets were stimulated with coated α-CD3 and soluble CD28. These cells were additionally treated with 100 nM/ml PACAP 38 for 5 days and then the proliferation of Teff/Tresp was determined by ^{3}H-Thymidine incorporation. (B) Both T cell subsets were cultured in medium containing α-CD3/CD28 beads and 100 nM/ml PACAP 27. Five days later the proliferation of Teff/Tresp was measured by ^{3}H-Thymidine incorporation. The diagram showed the ratio of proliferative level following PACAP treatment compared to the control. This data was represented by two independent experiments. White bar: control cells; black bar: PACAP 27 or PACAP 38-treated cells; grey bar: Teff/Tresp alone.

8. Appendix

8.1 List of academic teachers

The following persons were my teachers at University of Cologne and at Philipps-University Marburg:
University of Cologne: Abken, Arndt, Baumann, Berkessel, Brüning, Dohmen, Döring, Eckart, Flügge, Fußwinkel, Gehring, Griesbeck, Howard, Hülskamp, Kallies, Klein, Kloppenburg, Korsching, Langer, Leptin, Lichtenberg, Meyer, Naumann, Praefcke, Rajewsky, Roth, Schnetz, Tautz, Wiehe, Wilcken-Bergmann, Wünschiers.
Philipps-University Marburg: Bauer, Hertl, Huber, Lohoff, Müller, Schäfer, Weihe.

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8.2 Acknowledgements

I would like to acknowledge and express my heartfelt gratitude to the following persons who have given me support to complete this dissertation and because of whom I will cherish my gradulate expeience.

My deepest gratitude at first is to my supervisor **Dr. Armin Bender**. I owe him thanks for his support, patience, and supervision. I have learnt fortunately from his profound knowledge which broadened my view on melanoma involved in immunology and his constructive criticism that expanded my scientific way of thinking. I also thank him for providing a motivational working atmosphere which helped me to confront and to get through tough situations.

I also feel very honoured to express my sincere appreciation to **Prof. Dr. Michael Hertl** who researched from his heart and devoted his life to immunotherapy regarding skin diseases. I am deeply grateful to him for his insightful and precious comments during my study.

I would like to acknowledge the supportive cooperation with **Prof. Dr. Eberhard Weihe** and **Dr. Martin Schäfer** from Anatomy University Marburg. Also, I would like to express my special respects to the passion for neuropeptide.

I am sincerely grateful to **Prof. Dr. Rolf Müller** and **Dr. Abdo Konur** for giving me the possibility to join in one of LOEWE Projects and for allowing me to pursue some ideas in this study.

I want to appreciate **Ludiwig Institue for Cancer Research** for providing melanoma cell lines, which are the essential materials for this study. Also, I would like to thank **Dr. Eve Lutz** from Strathclyde Institue of Pharmacy and Biomedical Science University Strathclyde UK for generous providing with

cDNA for PACAP receptors. I would like to denote my respects to her brilliant working on PACAP receptors.

I sincerely thank **Dr. Andreas Kaufmann** from Immunology University Marburg for kindly providing me the access to their facilities and experimental materials which generated good data for this thesis.

My very special thank goes to a **Dr. Marianne Nain** who encouraged me with her precious advice and supported me with helpful suggestions on this study.
I am grateful to the other member of our group for the various help they offered me. I admire excellent experimental skills of **Dr. Maria Llamzares** and a strict self-management of **Dr. Karlin Karlmark** and sharing valuable expertise on T cells of **Dr. Christian Möbs**. Without their supports this thesis would not be completed.

Lastly, I would not be reaching this without unrelenting love and care of my **family** and **friends**. I like to thank them for their support and encouragement in all my pursuits that made me overcome crisises and focuse on my study. Thank you.