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Role of CCR10 in progression and metastasis of malignant melanoma

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1 Abbreviations

Abbreviation	*Meaning*	*Abbreviation*	*Meaning*
AEC	3-Amino-9-Ethyl-carbazol	**Min**	Minute
AJCC	American Joint Committee on Cancer	**MITF**	Microphthalmia-associated transcription factor
ATCC	American Type culture collection	**NRAS**	Neuroblastoma Ras viral oncogene homolog
BCC	basal cell carcinoma	**OD**	Optic density
BRAF	V-RAF murine sarcoma viral oncogene homolog B1	**PBS**	Phosphate buffered saline
CDKN2A	cyclin-dependent kinase inhibitor 2A	**PI3K**	Phosphatidyinositol 3 kinase
CNS	Central nervous system	**PKB or Akt**	Proteinkinase B
DMEM	Dulbeccos´s Modified Eagle Medium	**PTEN**	Phosphatase and tensin homologue
DNA **cDNA**	Deoxyribonucleic acid Complementary DNA	**qRT-PCR**	Quantitative real-time PCR
ERK	Extracellular-related kinase	**RGP**	Radial growth phase
FACS	Flow cytometric analysis	**RNA**	Ribonucleic acid
FCS	Fetal calf serum	**ROS**	Reactice oxygen species
FSC	Forward scatter	**RT**	Room temperature
GPCR	G-protein-coupled receptors	**SCC**	Squamous cell carcinoma
HAbP	biotinylated Hyaluronic Acid binding Protein	**SSC**	Side scatter
HAS	Hyaluronan synthase	**SSM**	Superficial spreading melanoma
HRP	Horseradish peroxidase	**TGF-beta**	Transforming growth factor-beta
HYAL	Hyaluronidase	**TNF-alpha**	Tumour necrosis factor alpha
IL-1	Interleukin 1	**VGP**	Vertical growth phase
MM	Malignant melanoma		

2 Summary

Metastasis remains to be one of the most life limiting factors in the medical attendance of cancer patients. In the past, different concepts have been established to explain its pathogenesis. It has been discussed that mechanical factors and/ or molecular interactions are involved in metastases formation. Similar to most malignancies malignant melanoma (MM) frequently metastasizes to lymph nodes, lung, liver, bone marrow and brain. Next to these major sites of metastasis, a unique feature of MM is the high occurrence of cutaneous metastasis. Recently, it has been shown that the skin-specific chemokine CCL27 directs, via its receptor CCR10, T-cell recruitment to the skin. The aim of the present study was to investigate whether melanoma cells may take advantage of the chemokine system, in particular CCL27 and its receptor CCR10, for tumour progression and organ-specific cutaneous metastasis.

Indeed, primary tumours of MM patients expressed CCR10 in immunohistochemical analyses. *In vitro* observations of MM cell lines revealed strong CCR10 expression. However, further analysis showed that the majority of the CCR10 repertoire is located in the intracellular space. Functional analyses using wound repair and migration assays showed a motility enhancing effect of CCL27 and the capability of CCL27/ CCR10 interactions to induce chemotaxis (directional migration).

Hyaluronans and its degradation products are abundantly present in the tumour microenvironment. Indeed, MM cells do express hyaluronan synthesizing (*HAS),* and degrading enzymes (*HYAL*), leading to the formation of hyaluronic acid fragments. Flow cytometric analyses revealed that these fragments induce a size-dependent shift of CCR10 to the cell surface. However, CCL27 did not induce HAS and HYAL expression.

Taken together, findings of the present study show that melanoma cells express the chemokine receptor CCR10 *in vivo* and *in vitro* and CCR10-signalling is capable of inducing directed migration. Moreover my observations demonstrate that hyaluronic acid fragments, which are an important component of the tumour microenvironment, are able to shift the receptor CCR10 from the intracellular space to the cell-surface. These observations support the hypothesis of CCR10 to play an important role in cutaneous metastasis of malignant melanoma by interacting with its skin-associated ligand CCL27 and that the tumour microenvironment, in particular hyaluronic acid fragments, are involved in receptor regulation of melanoma cells.

3 Zusammenfassung

Das Auftreten von Metastasen bleibt weiterhin einer der wichtigsten lebenszeitverkürzenden Faktoren in der Behandlung von Tumorpatienten. Unterschiedliche Konzepte fanden Einzug in die Literatur mit dem Ziel den Pathomechanismus der Metastasierung zu erklären. Mechanische, sowie molekulare Interaktionen scheinen dabei eine Rolle zu spielen.
Das Maligne Melanom entwickelt, ähnlich wie andere Tumore, häufig Metastasen in Lymphknoten, Lunge, Leber, Knochenmark und Gehirn. Eine Besonderheit ist jedoch das regelhafte Auftreten von Hautmetastasen. Erst kürzlich konnte eine Rekrutierung von T-Lymphozyten in die Haut über Interaktionen des in der Haut spezifisch exprimierten Chemokins CCL27 und seines Rezeptors CCR10 nachgewiesen werden. Das Ziel der vorliegenden Arbeit war die Rolle des Chemokins CCL27 und seines Rezeptors CCR10 im Rahmen der Tumorprogression und Organ-spezifischen kutanen Metastasierung des Malignen Melanoms zu untersuchen.
Tatsächlich ließ sich sowohl in immunhistochemischen Untersuchungen von primären Melanomschnitten, als auch in kultivierten Melanomzelllinien eine deutliche CCR10 Expression nachweisen. Darüber hinaus zeigte sich allerdings auch, dass der Rezeptor vor allem intrazellulär lokalisiert ist. Funktionelle Studien, inklusive Wundheilungs- und Migrationsversuche bestätigten einen motilitätsfördernden Effekt des Chemokins CCL27 auf Melanomzellen, sowie die Fähigkeit der CCL27/ CCR10 Interaktion gerichtete Migration/ Chemotaxis zu induzieren.
Die Matrix des Tumormikromilieus enthält eine große Menge an Hyaluronsäuren und ihren Spaltprodukten (Hyaluronsäure-Fragmente). qPCR-Untersuchungen zeigten, dass Melanomzellen Hyaluronsäure-synthetisierende (*HAS*) sowie degradierende Enzyme (*HYAL*) produzieren und somit an der Bildung solcher Fragmente beteiligt sind. In durchflusszytometrischen Versuchsreihen ließ sich ein Größen-abhängiger Effekt der Fragmente auf die CCR10 Rezeptor-Regulation zur Zelloberfläche nachweisen. Jedoch zeigte sich kein Effekt durch CCL27 auf die Expression von *HYAL* und *HAS*.
Zusammenfassend zeigen die Ergebnisse der vorliegenden Arbeit, dass maligne Melanomzellen *in vitro* und *in vivo* den Chemokinrezeptor CCR10 exprimieren und über diesen gerichtete Migration vermitteln. Außerdem demonstrieren meine Untersuchungen, dass Hyaluronsäure-Fragmente als Komponenten des Tumormikromilieus in der Lage sind CCR10-Zelloberflächenexpression zu induzieren. Die Befunde unterstützen die Hypothese, dass die Interaktion von CCR10 mit seinem hautassoziierten Liganden CCL27 eine Rolle bei der Entwicklung kutaner Metastasen in Patienten mit Malignem Melanom spielt und dass das Tumormikromilieu, insbesondere Hyaluronsäure-Fragmente, an der Rezeptorregulation der Melanomzellen beteiligt ist.

4 Introduction

MM is a skin cancer derived from melanocytes. Among malignancies of the skin it is responsible for the vast majority of disease related death, although it is less frequent than basal cell carcinoma (BCC) or squamous cell carcinoma (SCC)[1]. The poor prognosis is related to the aggressive behaviour of the tumour resulting in fast progression and early metastasis[2,3].

Different concepts, stressing mechanical as well as molecular interactions, have been established to explain the pathogenesis of metastasis. As early as 1889, Stephen Paget hypothesized that metastasis is not a random process and tumour cells do not develop metastases at distant organs by chance[4]. Recently, the interaction of small peptides called chemokines and their receptors could be identified to have key roles in progression and organ-specific metastasis of many malignancies[5–7].

Unlike other malignancies, MM frequently exhibits skin metastases. Homey et al. showed that T-cells, positive for the chemokine receptor CCR10, are directed to the skin by CCL27, which is constitutively produced by keratinocytes[8], during inflammation[9]. Melanoma cells are also known to express the receptor CCR10[6], and CCR10/ CCL27 interactions have been shown to be important in melanoma progression[7]. Due to these findings I sought to investigate the role of CCR10 in organ-specific metastasis to the skin.

4.1 Malignant melanoma

4.1.1 Epidemiology

MM shows rising incidence rates over the last decades in most fair-skinned populations. It is the most rapidly increasing cancer in these populations with an estimated annual increase of between 3 to 7 per cent[10].

In the early 1970s approximately 6 cases per 100.000 inhabitants per year were reported in the USA. At the end of the 1990s, a 3-fold increase was reported with approximately 18 cases per 100.000 inhabitants per year[11]. In the same time a similar increase of incidences was reported in Europe, from 3-4 cases per 100.000 inhabitants per year in the early 70s to 10-15 cases per 100.000 inhabitants per year at the beginning of 2000. However, several studies show stabilizing incidence rates since the 90s[12]. Highest melanoma incidence rates in Europe are reported in Scandinavian countries such as Sweden[13], lowest incidence rates in Mediterranean countries such as Italy[14]. Throughout the world Australia and New Zealand have the highest incident rates, especially in northern equatorial areas such as Queensland, with rates up to 60 cases per 100.000 inhabitants per year[10,15,16].

Mortality rates in fair-skinned populations show similarities to incidence rates, but are generally more than 5-fold lower[10]. Moreover, increase-rates are at a lower level and have stabilized or even declined in younger age-groups since the last twenty years which is related to less sun exposure[12,16].

MM is a cancer, which is diagnosed in a younger age group compared to other solid tumours such as breast cancer or prostate cancer. The median age at diagnosis is approximately 55 years[16]. There is no clearly favoured sex. A predominance for men can be found for example in the USA and Australia[11,16,17], whereas in Great Britain, a country of lower melanoma incidence rates, a predominance for women was shown[18].

Among men 55% of cutaneous melanoma are localised on the trunk (women only 25%). The most common anatomic site among women is the lower limb (42.3% of all cutaneous melanoma, men only 16.5%). Head and neck regions are rare anatomic sites for cutaneous melanoma in both sexes; however, in

patients of older age these regions become more important[16,19].

MM is divided into several subtypes. The most frequent one among the German population is the superficial spreading melanoma, composing approximately 59% of all cases followed by nodular melanoma at 21%, lentigo maligna melanoma at 11% and acrolentiginous melanoma at 4%[16,19]. A similar distribution can be found in other western countries[20,21]. The subtype is an important prognostic factor; however, the tumour thickness is more considerable in non-metastatic melanoma[2,3,22]. In recent decades, thin melanomas (<0.75 mm) were increasingly diagnosed[23,24], even though there is a notable fraction of thick melanomas (>1 mm) in patients at higher age[25].

Studies predict an ongoing increase of melanoma incidences for the next decades[26,27]. However, incidence rates among younger people, for example in Australia, seem to have stabilised, mortality has plateaued and might decline in the future due to early detection[23,28].

4.1.2 Clinic

MMs are mostly dark brown to bluish black, but also completely melanin-free or partly pigment-free tumours do occur. Melanomas present a wide variety in colour, size and secondary changes like crusting, erosion and ulceration[3,29].

90% of primary MM are skin melanoma, the remaining 10% are mucosal melanoma including anorectal melanoma, melanoma of the central nervous system (CNS) and the viscera as well as ocular MM[21,29]. Based on histopathological analysis several subtypes can be defined, although it must be remembered that some types of melanoma cannot be given a definite classification on this basis[3].

In about 30% of cases MM develops from a pigmented nevocytic nevus[3]. But it also arises from lentigo maligna or even from normal skin[3]. Different opinions do exist to which extent melanomas develop de novo, some assuming that this relates to the majority of this tumour entity[30].

The superficial spreading melanoma (SSM) represents the most common melanoma (ca. 59%) and preferentially occurs in middle-aged people on the trunk[16]. The horizontal growth phase lasts usually five to seven years[29]. The

second most frequent melanoma is the nodular melanoma (ca. 21%)[16]. It represents the most aggressive form of MM as it progresses from horizontal to radial growth phase usually within a few months[29]. It usually occurs on sun exposed skin of 40-50 year-old patients[29]. The lentigo maligna melanoma (ca. 11%) develops from lentigo maligna, which represents a melanoma in situ comprising a horizontal growth phase that lasts up to 20 years[16,29]. Therefore, patients are usually of older age. It usually occurs on the face and the back of the hand[29]. A special subtype of MM is the acrolentiginous melanoma due to its preferred anatomic sites. Palms, soles, toes and fingers are commonly affected. It composes 4% of all melanoma[16,29]. The time of the horizontal growth phase is similar to the one of SSM[29]. Melanoma of the mucosa, the viscera and the CNS are rare but important to know because of their poor prognosis and difficult detection in comparison to skin melanoma[3,29]. Physiological pigmented areas like vulva and gingiva are preferred anatomic sites for mucosal melanoma[3] (see **Fig. 1**).

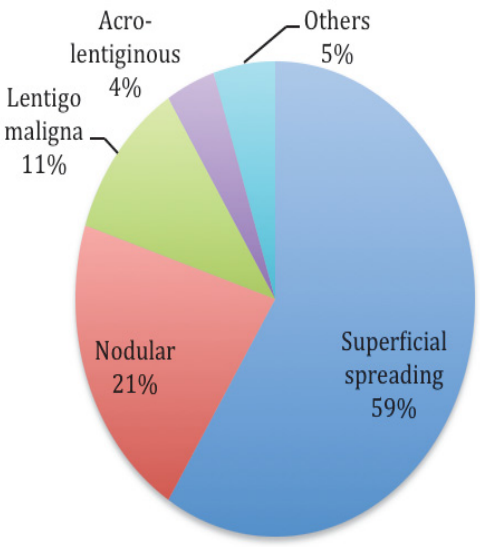

Figure 1 Histopathologic subtypes of melanoma. The analysis is based on 69,962 cases of the German Central Malignant Melanoma Registry database with valid information on melanoma subtype (Updated March 2008).

MM tends to grow rapidly and present early metastasis during progression[3]. Metastasis can occur through lymphatic or hematogenic dissemination. In early stages, metastasis can be found in the surrounding skin (in-transit metastasis) or regional lymph nodes. Later, hematogenous or lymphogenous metastases at distant sites can be found. Distant metastases can occur at various sites especially the lungs, liver, heart, brain, bones and frequently the skin[3]. The onset of symptoms is usually observed at the stage of metastases. Particularly, lung and brain metastases cause serious complications leading to death[31].

For prognostic propositions on survival, staging is necessary. The most relevant melanoma staging and classification was published by the American Joint Committee on Cancer (AJCC). Important factors are vertical tumour thickness (Breslow depth), lymph node metastases and distant metastases[2]. A more precise division can be made with the help of e.g. mitotic rate and ulceration status[2]. Five-year survival rate in early stages with no detectable metastases is around 90%. Whereas, when metastases are clinically detectable survival rates are low. Primary tumours with distant metastases show five-year survival rates of around 10%, depending on the infiltrated organ[2]. Therefore early detection and correct diagnosis is crucial for survival.

The diagnostic method of choice is excisional biopsy in any suspected case in order to confirm the diagnosis of MM[16,32]. According to the ABCDE rule, every pigmented skin lesion is suspected, when it appears **a**symmetric, with irregular **b**order, very dark or speckled **c**olour, a **d**iameter more than 5 mm or **e**levated and evolving over time[3,33]. Histological studies lead to a precise diagnosis in 90% of cases[3]. When the diagnosis of MM is confirmed, the stage of the disease has to be determined. This requires X-ray, sonography and CT examinations in order to detect metastases.

When the diagnosis is confirmed the primary tumour should be removed surgically with a resection margin of 0.5 cm when dealing with a carcinoma in-situ, 1 cm in melanoma up to a tumour thickness of 2 mm and 2 cm in melanoma of more than 2 mm thickness[16]. Additionally, sentinel lymph node biopsy is recommended in tumours thicker than 1 mm[16]. If the sentinel lymph node is positive, total lymphadenectomy should be performed[34]. Surgical treatment is also indicated in metastatic melanoma, when R0 resection can be

achieved or in palliative intentions[35]. For palliative purposes radiotherapy also might be an option, particularly when dealing with bone or multiple brain metastases[35]. Adjuvant interferon alpha therapy is recommended in tumours of more than 1.5 mm thickness because of the high risk of developing metastatic disease and when lymph node metastases are found[35]. Distant metastases, when R0 resection is not possible, are an indication for systemic chemotherapy[36]. First-line treatment is mono chemotherapy with Dacarbazine[36]. This therapy is performed to shrink the tumour and thus reduce tumour-related symptoms. Recent studies show promising results in the treatment of melanoma at advanced stages. Ipilimumab, an antibody which binds the receptor CTLA-4 on cytotoxic T-lymphocytes, improved survival in metastatic melanoma patients by modulating the anti-tumour response[37]. In patients which also showed V600E mutations in the BRAF gene, treatment with the BRAF-kinase inhibitor Vemurafenib also showed positive effects on survival[38].
A new field of interest are oncolytic viruses which are injected directly in the tumour in order to have lytic effects on the tumour and induce an enhanced anti-tumour response[39].

4.1.3 Ethiopathogenesis

Several risk factors that promote the development of MM have been identified. Via complex biological mechanisms they may lead to the initiation and progression of the tumour. Host risk factors can be distinguished from environmental risk factors. The strongest environmental risk factor seems to be UV-light exposure[40,41]. It plays an important role not only in the initiation of nevi but in all stages of melanoma development[40].
Sunburns particularly contribute to melanoma development, whereas low grade sun exposure may induce protection[42]. Moreover many studies showed a higher risk of melanoma in occupations associated with the electronic and chemical industries[43,44]. In both cases an increased formation of reactive oxygen species (ROS) seems to play an important role leading to deoxyribonucleic acid (DNA) damage[45].

Among host risk factors, a family history of melanoma, multiple benign or atypical nevi and a history of one or more previous melanomas have to be pointed out[46]. These risk factors are understood to different degrees at a molecular level. Different gene mutations have been reported, which are linked to a susceptibility for melanoma[46]. In many melanoma-prone families mutations in tumour suppressor genes like cyclin-dependent kinase inhibitor 2A (CDKN2A) have been found[47,48].

Mutations in regulatory genes are important in melanoma pathogenesis. They allow melanocytes to escape their regulation by keratinocytes leading to the formation of a nevus[46]. A nevus can then progress to radial growth phase (RGP)-melanoma and later on to a vertical growth phase (VGP)-melanoma, a stage in which cells have metastatic potential[49]. But not all phases must be passed, RGP-melanoma or VGP-melanoma can both develop directly from melanocytes or nevi[46].

Molecular changes in the initiation and progression of melanoma often affect the Ras/ Raf/ MEK/ ERK cascade (see **Fig. 2**), which plays a considerable role in cell proliferation[50]. In melanocytes this pathway is activated through growth factors leading to a mitogenic effect[51]. In MM hyperphosphorylation has been observed most commonly due to mutations of genes called V-RAF murine sarcoma viral oncogene homolog B1 (BRAF) and Neuroblastoma Ras viral oncogene homolog (NRAS)[52,53]. Extracellular-related kinase (ERK) hyperactivation can be found in up to 90% of human melanoma[49,54].

Nevertheless, neither BRAF nor NRAS alone are sufficient for malignant transformation[55]. The control of growth is disrupted but additional factors are needed to form malignancy such as phosphatase and tensin homologue (PTEN) deletion or mutations of microphthalmia-associated transcription factor (MITF)[55] .

PTEN is a lipid phosphatase which negatively regulates the phosphatidyinositol 3 kinase (PI3K) pathway (see **Fig. 2**). In response to activated growth factor receptors, PI3K leads to activation of proteinkinase B (PKB), also called Akt. Akt itself induces proliferation and survival through phosphorylation of cellular proteins[56]. Deregulation of the PI3K signalling can be found in a high proportion of melanomas for example by deletion of PTEN[57,58].

MITF is a transcription factor, which regulates differentiation and development in melanocytes[59]. It is a complex molecule, which can function as an inducer or repressor of cellular proliferation depending on its level of expression[49]. Only at intermediate levels proliferation is favoured[49].

Local invasion and metastasis play a crucial role in morbidity and mortality of melanoma patients. Lesions that progress to vertical growth phase show striking changes in the control of cell adhesion[60]. Important changes enabling metastatic spreading are the loss of E-Cadherin and the expression of N-Cadherin[60]. E-Cadherin causes melanocytes to associate with keratinocytes, whereas N-Cadherin interacts with dermal fibroblasts and vascular endothelium[61].

Various molecular changes in the progression of MM are known, however, the Ras/ Raf/ MEK/ ERK and PI3K pathway seems to be of particular importance[46,49].

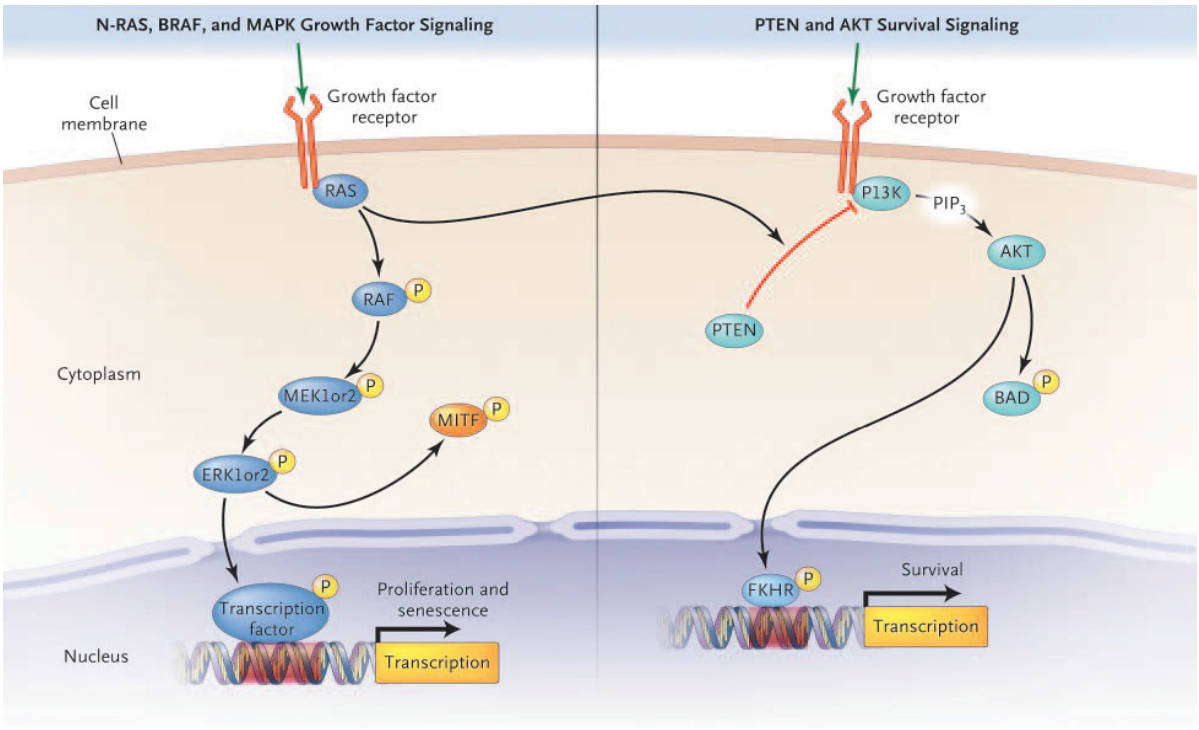

Figure 2 Mechanisms of disease. Figure two shows the Ras/ Raf/ MEK/ ERK pathway (left) and PI3K pathway (right). Mutations of these pathways are frequently involved in melanoma pathogenesis. Adapted from Miller, A. J. & Mihm, M. C.[46].

4.2 Chemokines

Chemokines are small secreted molecules known for the regulation of leukocyte migration[62]. They play an important role in development and organ homeostasis as well as in many disease processes such as inflammation and cancer[9,63,64].

Homey et al. showed that CCL27, preferentially produced by keratinocytes and upregulated during inflammation, attracts T-cells, which express the corresponding receptor CCR10[9].

There are at least 46 chemokine ligands in humans which bind to 18 functionally active and three inactive, so called “scavenger” or “decoy” receptors[65] (see **Fig. 3**). The functionally signalling receptors are a subfamily of class A (rhodopsin-like) G-protein-coupled receptors (GPCRs)[65].

Chemokines are divided into four subclasses based on the position of the first two cysteine residues in their amino acid sequence: in the CC subfamily the first two cysteine residues are adjacent, whereas the CXC subfamily comprises a

single variable amino acid between the first two cysteins[65]. The CX_3C subfamily comprises three amino acids between the first two cysteines and the XC subfamily is devoid of the first and third cysteine[65]. The CC subfamily is the largest group including 26 members[65]. The CXC subfamily includes 17, the CX3C and the XC subfamily include one, respectively two, members[65].

Alternatively, chemokines can be devided into groups by their genomic organization. Chemokines whose genes are located in large clusters at particular chromosomal regions are referred to as “major cluster chemokines”[65]. They usually have the ability to bind multiple receptors and are involved in inflammation processes[9,65]. Those whose genes are located separately or only in small clusters in unique chromsomal locations are known as “non-cluster” or “mini-cluster chemokines”. These chemokines usually do not act on multiple receptors and have an important role in development of the organism[65].

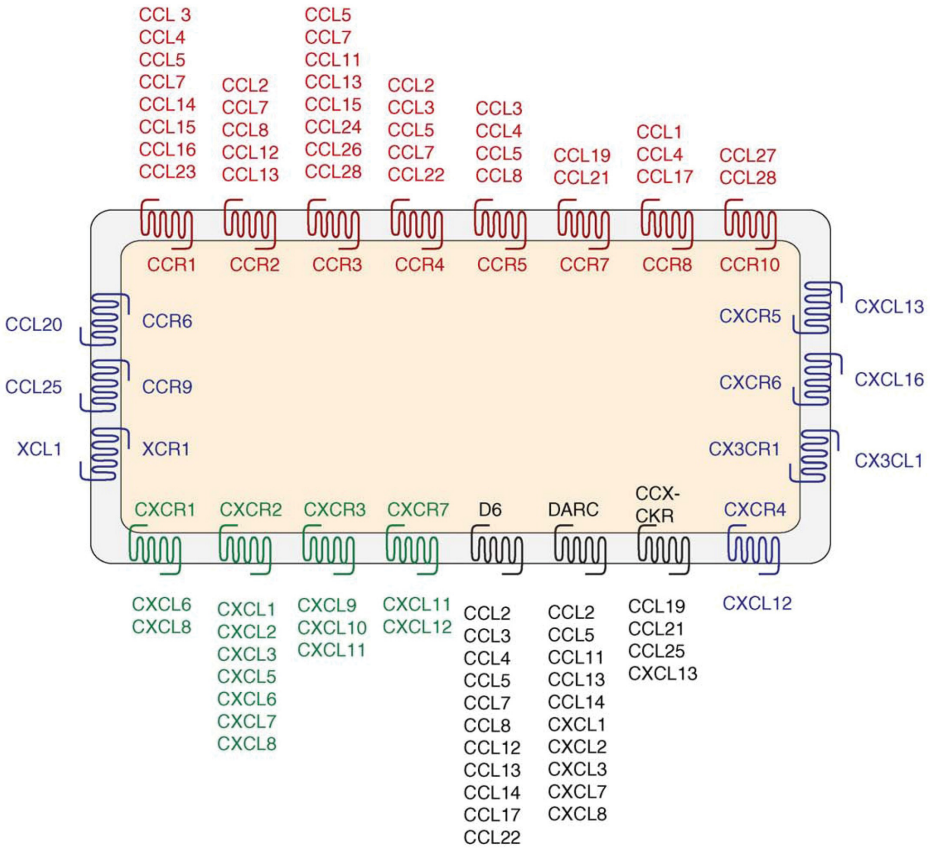

Figure 3 Chemokines and their receptors. CC (red), CXC (green) and decoy (black) receptors usually bind multiple chemokines. Receptors marked blue only bind one ligand. Adapted from Lazennec, G. & Richmond, A.[66].

4.2.1 Chemokines and metastasis

Complex molecular transformations lead to metastasis, a process that leads to secondary tumours at distant anatomic sites. One theory of metastasis formation was that metastasis is a mechanical process in which the tumour cells that escape from the primary tumour, reach the circulation and finally get lodged in a small blood vessel from where a secondary focus develops[67].

In contrast, the observation that different tumour entities give rise to metastases at different locations in the body has implicated that metastasis is not only a simple mechanical process. Instead, it is now discussed that specific molecular interactions between circulating tumour cells and the site of metastasis are important for site-specific tumour formation[4,68]. Especially, chemokines, secreted at the site of metastasis, and their corresponding receptors, expressed by tumour cells, are able to induce site-specific metastasis formation[6].

Cancer cells have a characteristic chemokine receptor repertoire and its ligands are highly expressed at frequent sites of metastasis[69]. The most common chemokine receptor expressed in most types of cancer cells is CXCR4[64]. Its ligand CXCL12 is strongly expressed in common sites of metastasis such as lung and liver[6]. Another important and very common chemokine receptor is CCR7, which seems to play an important role to enter lymph nodes, where the homeostatic chemokine CCL21 is expressed by high endothelial venules[6,70]. The interaction between CCR7 and CCL21 seems to be important for lymph node metastasis in various carcinomas such as breast cancer, non-small cell lung cancer and MM[6,71–73].

These findings indicate an important role of chemokine-chemokine receptor interactions in tumour progression and organ-specific metastasis.

4.2.2 Chemokine and chemokine receptor regulation

Molecules at the site of the tumour are involved in the regulation of chemokine and chemokine receptor expression. The importance of the microenvironment in chemokine regulation has been observed in melanoma. CXCL8 is upregulated in melanoma cells by keratinocyte-derived interleukin-1 (IL-1), whereas negative regulation was induced by hepatocyte-derived transforming growth factor-beta (TGF-β)[74]. Moreover tumour necrosis factor-alpha (TNF-α) was observed to induce CCL5 expression in melanoma resulting in a more aggressive behaviour of the tumour[75]. Little is known about the receptor regulation in melanoma. Schutyser et al. showed that the chemokine receptor CXCR4 is upregulated in melanoma cells by hypoxia[76]. However, there is more evidence for chemokine receptor regulation by local factors in other cell types. In B cells, 1,25-dihydroxyvitamin D3 was able to enhance CCR10 surface levels[77].

Furthermore, a role of hyaluronic acid fragments in the regulation of chemokines has been described. CXCL8 was induced especially by lower molecular weight fragments in alveolar macrophages[78]. Hyaluronans are linear chain glycosaminoglycans which are a major component of the extracellular matrix in vertebrate tissues[79]. They are synthesized by enzymes like hyaluronan synthase 1 (*HAS1*), hyaluronan synthase 2 (*HAS2*) and hyaluronan synthase 3 (*HAS3*) and their degradation is mediated via hyaluronidases, particularly hyaluronidase 1 (*HYAL1*) and hyaluronidase 2 (*HYAL2*)[80]. In the course of degradation hyaluronic acid fragments are generated[81].

4.2.3 Chemokines and malignant melanoma

Melanoma cells express a number of chemokines, which have been implicated in tumour growth and progression[69]. Furthermore, they exhibit chemokine receptors on their surface that play a critical role in organ-specific metastasis[5].

Important chemokines, expressed by melanoma cells, seem to be CXCL1, CXCL8 and CCL2 and CCL5. CXCL1 is known as an autocrine growth factor in

melanoma[82], moreover it induces angiogenesis when binding its receptor CXCR2 on endothelial cells[83]. It was reported to be constitutively expressed on mRNA level in cultured nevocytes and melanoma cells, but was undetectable in primary melanocytes[84].

CXCL8, which is constitutively expressed by melanoma cell lines *in vitro*, acts upon CXCR1 and CXCR2 and shows similar effects as CXCL1. An autocrine effect has been demonstrated[85], as well as paracrine enhancement of tumour angiogenesis[86]. Furthermore, CXCL8 promotes tumour cell growth and haptotactic migration[87].

The chemokine CCL5 is only expressed by a subset of melanoma cells[75]. It has received much attention because its receptor CCR5 is one of the human immunodeficiency virus cell entry receptors on CD4+ T cells[88]. CCL5-expressing melanoma cells caused increased tumour formation in nude mice, and in transplantation experiments CCL5 expression favoured tumour progression[75].

CCL2 which binds to CCR2 is a potent monocyte chemoattractant[89,90]. Its role in tumourigenesis has been widely investigated[91,92]. CCL2 expressed by melanoma cells enhances tumour angiogenesis and tumour growth[93].

On the other hand, melanoma cells were found to express high levels of receptors CXCR4, CCR7 and CCR10[94]. Their respective ligands are CXCL12, CCL21, CCL27 and CCL28[94]. CXCL12, the specific ligand of CXCR4, exhibits high levels of expression in lung and liver[6,95]. In contrast, the ligand of CCR7, CCL21, was found at elevated levels in lymph nodes[6,96]. The interaction of these chemokines and their receptors were suggested to play an important role in organ-specific metastasis and tumour progression[5].

CCL27 is predominantly expressed in the skin by keratinocytes[8]. Its expression has been linked to directed migration of CCR10-positive immune cells to the skin[9]. As MM, unlike other malignancies, frequently exhibits skin metastases, I sought to elucidate the role of CCR10/ CCL27 interactions in melanoma metastasis to the skin.

4.3 Aim of thesis

A unique feature of MM is the high frequency of skin metastases. Recently, homing of T-cells to the skin has been observed due to chemokine/ chemokine receptor interactions of CCL27 and CCR10[9]. Observations in the past years also showed that the interaction of chemokines and their corresponding receptors play an important role in tumour progression and metastasis[6,72,95]. Especially the expression of the receptor CCR10 by melanoma cells and its ligand CCL27 by epidermal keratinocytes implicate a pivotal role in melanoma metastasis to the skin[6,8].

Furthermore, the importance of the tumour microenvironment in chemokine and chemokine receptor regulation has been demonstrated before[74]. However, little is known about the regulation of CCR10 on melanoma cells.

Hence, the following questions were addressed:

- Is CCR10 expressed on MM cells *in vivo* and *in vitro*?
- Does CCR10 signalling in melanoma cells induce motility/ chemokinesis and directional cell migration/ chemotaxis?
- Do factors of the tumour microenvironment, such as hyaluronic acid fragments, regulate CCR10 expression on melanoma cells?

5 Material and Methods

5.1 Cell culture and biopsy samples

The following melanoma cell lines were obtained from the American Type Culture Collection (ATCC) and cultured as recommended by the provider: SK-Mel-2, SK-Mel-28. MV3 melanoma cells were a gift from D. J. Ruiter (Department of Pathology, University Medical Center Nijmegen, The Netherlands). Cells were cultured in Dulbeccos´s Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany). UKRV-Mel-4 melanoma cells were a gift from D. Schadendorf (Department of Dermatology, Venereology and Allergology, University Medical Center and Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany) and were grown in RPMI-1640 medium (Lonza) supplemented with 10% FCS (Biochrom AG).
All melanoma cells were cultured in an incubator at 37°C with 95% humidity and 5% CO_2 (INCO 2, Memmert, Schwabach, Germany) and all media were supplemented with 10% FCS (BiochromAG) and 1% of a mixture of antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) (PAA, Pasching, Austria).
Biopsy samples were acquired from melanoma patients after obtaining informed patients consent. All melanoma tissue samples were collected by the Department of Dermatology (Department of Dermatology, University Hospital Düsseldorf, Germany). Tissue samples were either fixed in paraffin or shock frozen in liquid nitrogen and stored at -80°C. The study was approved by the local ethics committee (Study-No. 1928).

5.2 Chemotactic cell migration

Chemotactic cell migration assays were performed using chemotaxis µ-slides with physical surface modification for improved cell adhesion according to the µ-slides chemotaxis manual (ibidi, Munich, Germany) (see **Fig. 4**). UKRV-Mel4 melanoma cells were seeded into observation chambers at a density of 1 x 10^6 cells/ml. CCL27 (R&D Systems. Minneapolis, MN, USA) gradients were

generated at 1,000 to 10 ng/ml. In controls no chemokine gradient was generated and only RPMI-1640 medium (Lonza) supplemented with 10% FCS (Biochrom AG) was added. Cells were observed for 22 hours with a Zeiss Cell Observer (Zeiss, Göttingen, Germany). Movement of at least 79 cells, and an average of 94 cells, were analysed each experiment using ImageJ 1.37c tracking software (Wayne Rasband, National Institute of Health). Analysis of melanoma cell migration was performed as previously described by Fabro et al.[97]. Starting and end points of cell migration trajectories were set to the origin of a coordinate system and x- and y-values were used for chemotactic migration analysis. ΔY, the distance of cells travelled along the chemokine gradient (Y-axis), and the percentages of melanoma cells travelled in the direction of chemokine gradients (Y-axis) $\Delta Y < 0$ were calculated. Values above 50% show a tendency of cell movement in direction of higher chemokine concentrations. Furthermore melanoma cells traveling a longer distance in the direction of chemokine gradients (Y-axis) than in the direction orthogonal to the gradients (X-axis) $\Delta Y / |\Delta X| < -1$ were determined. Values higher than 25% indicate a directed migration of cells to higher concentrations of the chemokine.

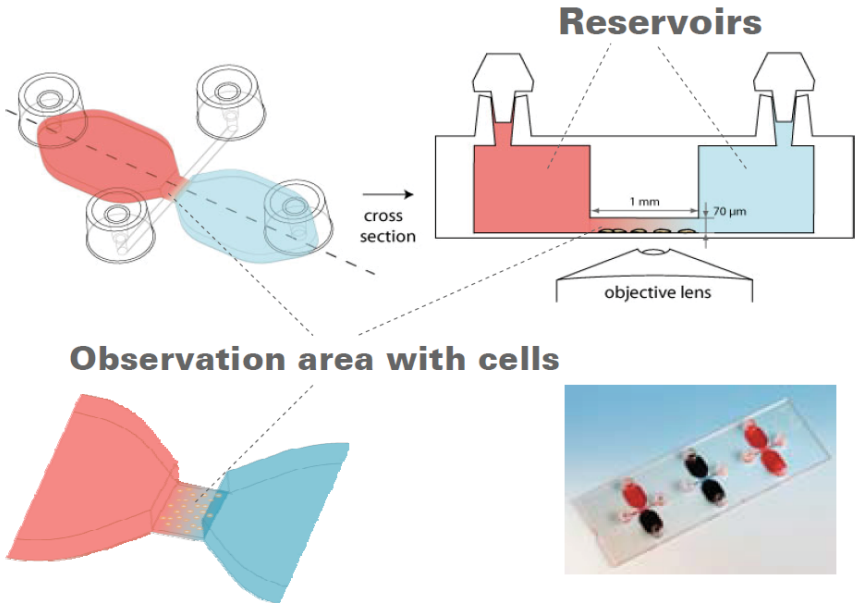

Figure 4 Chemotaxis µ-slides. Reservoirs are filled with either medium alone or with medium containing CCL27. Cells are seeded on observation area and cell migration is filmed. Adapted from ibidi GmbH.

5.3 *In vitro* monolayer wound healing assay

UKRV-Mel4 melanoma cells were cultured to confluence in 6-well plates (Cellstar, Greiner Bio-One, Kremsmünster, Austria). Scratch wounds were introduced in the monolayers by using a sterile 100 µl pipette tip. After washing away suspended cells, cultures were supplied with RPMI-1640 medium (Lonza) containing 0 or 500 ng/ml recombinant CCL27 (R&D Systems). Images of the same area were taken right after adding the chemokine or medium only and 15 hours later using a Zeiss Cell Observer (Zeiss). Wound repair was quantified by subtracting the area free of melanoma cells in mm^2 after 15 hours from the initial area in mm^2.

5.4 Flow cytometric analysis (FACS)

Melanoma cells were analysed using flow cytometry. Therefore cells were harvested by treatment with accutase (PAA) for 5 min at 37°C and centrifuged (1200 rpm, 8 min, 4°C) (Rotixa RP, Hettich Zentrifugen, Mülheim an der Ruhr, Germany). After two washing steps with PBS (PAA), 10^6 cells were stained with monoclonal anti-human CCR10 (R&D Systems) for 25 min on ice in the dark. After a wash step with PBS cells were fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany) and subsequently analysed with a FACSCalibur flow cytometer and CellQuest software (all BD Biosciences, San Jose, CA, USA).

When intracellular staining was performed, cells were fixed and permeabilized by adding 500 µl fixation/ permeabilization solution (BD Biosciences) and then incubated for 20 min in the dark at room temperature after antibody incubation. After centrifugation, cells were incubated with 2 ml BD wash/ perm buffer (BD Biosciences) for 10 min in the dark at room temperature. Cells were now centrifuged again and then incubated with monoclonal anti-human CCR10 (R&D Systems) for 25 min on ice in the dark for intracellular staining. After staining a wash step with 2 ml BD perm/ wash buffer (BD Biosciences) was performed. Then samples were fixed in 2% paraformaldehyde (Merck) and analysed.

At least 15.000 events were counted. The acquired data was analysed by a histogram plot (counts over fluorescence). Cells were gated using forward scatter (FSC) and side scatter (SSC) characteristics to exclude dead cells from analysis. To determine non-specific staining, the PE-labelled Rat IgG2a kappa antibody (BD Biosciences) was used.

5.5 Immunofluorescence

Melanoma cells were seeded on Lab-Tek® 2 well glass chamber slides (NalgeNunc) and cultured to 80% confluence. Cells were fixed in 4% paraformaldehyde (Sigma) for 15 min. After cells were washed twice with PBS (PAA), an incubation step with PBS containing 0.03% Triton X 100 (Carl Roth GmbH & Co., Karlsruhe, Germany) for 10 min followed to permeabilise the cell membrane and to make intracellular receptor staining possible.

Then, cells were washed with PBS (PAA) three times and incubated with PBS containing 10% donkey serum (AbD Serotec) to block unspecific binding of the secondary antibodies. Afterwards cells were stained against human CCR10 (Abcam Cambridge Science Park, Cambridge, UK; Tab. 1) in 2% donkey serum (AbD Serotec) in a humidified chamber for 1 hour at room temperature in the dark. After being washed three times with PBS (PAA), cells were incubated with the secondary antibody conjugated with fluorochrome NL557 (R&D Systems; Tab. 1) (1:200) in 2% donkey serum (AbD Serotec) for 1 hour at room temperature in the dark. Cell nuclei were stained with DAPI (Invitrogen) (1:400) for 10 min at room temperature, after cells have been washed with PBS (PAA) for three times.

Slides were mounted with Fluoromount-G (Southern Biotech) and covered with cover slips to provide a semi-permanent seal. Immunofluorescent reactions were detected by use of a microscope (Axiovert 200M) (Zeiss) using the software Axiovision 4.7 (Zeiss).

Table 1: List of different antibodies, isotype controls and corresponding dilutions.

Antibody	***Dilution / Incubation time***	***Company***
Rabbit polyclonal to GPR2/ CCR10	1:20, 60 min at RT	Abcam
NorthernLights anti-rabbit IgG-NL557	1:200, 60 min at RT	R&D Systems
Isotype control, rabbit Immunoglobulin negative control	1:300, 60 min at RT	Dako

5.6 Immunhistochemistry

Tissue samples were cut into 8 µm (frozen sections) or 5 µm (paraffin) transversal sections. Frozen sections were cut by use of a cryomicrotome (-30°C) (2800 Figocut E, Reichert-Jung, Wetzlar, Germany); paraffin sections were cut using a microtome (1150/Autocut, Reichert-Jung, Wetzlar, Germany). All sections were mounted on adhesive microscope slides (Thermo Scientific) and either stored at RT (paraffin) or at -80°C (frozen sections). Images were generated by using a Zeiss Cell Observer (Zeiss Axiovision 4.6 software, Carl Zeiss Microimaging, Göttingen, Germany).

5.6.1 Frozen sections

Tissue sections of frozen tumour tissues of melanoma patients were air dried and fixed in acetone (Roth, Karlsruhe, Germany) for 5 min. Afterwards, samples were pretreated with 0.6% hydrogen peroxide (Merck) in PBS (PAA) for 10 min at room temperature to quench endogenous peroxidases. Sections were then incubated with Avidin D solution (Avidin/ Biotin Blocking Kit; Vector Laboratories, Inc., Burlingame, CA, USA) for 10 min and a biotin solution (Avidin/ Biotin Blocking Kit) for 10 min to prevent unspecific binding of the antibodies. Before and in between incubation sections were washed with PBS

(PAA). After another three washing steps with PBS (PAA), sections were either stained against human CCR10 (Schering Plough Research Institute, Dardilly, France) (1:6) or human Melan-A (Dako, Glostrup, Denmark) (1:40) (Tab. 2). After incubation of the primary antibody sections were washed with PBS (PAA) three times and incubated with a biotinylated secondary anti-mouse antibody (Vectastain ABC Kit, Vector Laboratories; Tab. 2) (1:400) for 45 min. Again sections were washed three times with PBS (PAA) and subsequently incubated in Streptavidin horseradish peroxidase-(HRP) conjugate (Dako). Bound antibody was visualized using the ABC reagent (Vector Laboratories) with 3-Amino-9-Ethyl-carbazol (AEC) (Vector Laboratories) as a substrate for 30 min in case Melan-A (Dako) was used as primary antibody and 50 min when CCR10 (supernatant) was used. Before visualization sections were washed three times with PBS. Afterwards slides were counterstained with hematoxylin (Sigma-Aldrich) for less than one min at RT and rinsed in water. Sections were then contrasted in saturated lithium-carbonate solution, rinsed in water and permanently mounted in Ultramount Plus (Labvision Products, Fremont, CA, USA).

5.6.2 Paraffin sections

Paraffin embedded sections were first heated at 60°C over night and thereafter heated at 70°C for 20 min to improve adhesion of the sections on the slides.
Sections were then deparaffinized in xylene (Merck) and rehydrated in decreasing series of ethanol (Merck) and subsequently unmasked by heat-induced epitope retrieval using high pH solution (Dako) for 20 min. Staining was performed against Melan-A. Antibodies were incubated for one hour at room temperature. Afterwards sections were washed three times with PBS (PAA). After incubation of the primary antibody the following steps were performed as described in the staining procedure of frozen sections.

HA staining was performed in cooperation with the Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University Düsseldorf.

Melanoma tissue sections were deparaffinised and then blocked with 10% fetal calf serum and 1% bovine serum albumin for one hour at room temperature. Afterwards the slides were incubated with biotinylated HAbP (2 g/ml; Seikagaku, Tokyo, Japan) at 4°C overnight. The slides were then washed with PBS three times and subsequently incubated with avidin-biotin peroxidase (Sigma, St. Louis, MO). The colour was developed with 3,3-diaminobenzidine (DAB; Sigma) containing 0.03% H_2O_2. Staining of the nuclei was performed with hemalaun solution (Merck, Darmstadt, Germany). Negative controls were incubated with Streptomyces hyaluronidase (ICN, Costa Mesa, CA) at a concentration of 2 U/ml (1 hour, 37°C) before HA staining. HA staining was performed as described before by Dai et al.[98].

Table 2: *List of different antibodies, isotype controls and corresponding dilutions. Antibodies are diluted in PBS (PAA).*

Antibody	***Dilution / Incubation time***	***Company***
Mouse anti-human CCR10 supernatant	1:6, 60 min at RT	Schering
Monoclonal mouse anti-human Melan-A	1:40, 60 min at RT	Dako
Isotype control, negative control mouse IgG1	1:10/ 1:40, 60 min at RT	Dako
Biotinylated anti-mouse IgG	1:400, 45 min at RT	Vector
Biotinylated hyaluronic acid binding protein (HAbP)	2 g/ml, over night at 4°C	Seikagaku
Streptomyces hyaluronidase	2 U/ml, 60 min at 37°C	ICN

5.7 Hyaluronic acid fragment stimulation

Melanoma cells (UKRV-Mel4) were seeded on 6-Well cell culture plates (Cellstar) and cultured to 70% confluence. Cells were incubated with hyaluronic acid fragments (5 µg/ml) (Tab. 3) or with RPMI-1640 medium (Lonza) only for 24h. All cells were incubated at 37°C with 95% humidity and 5% CO_2.

Three different hyaluronic acid fragments were used: fragment18, which comprises 13-16 disaccharides, fragment38, comprising eight disaccharides, and fragment64, comprising four disaccharides. All fragments were a kind gift by Jonathan Sleeman (Karlsruhe Institute of Technology, Karlsruhe, Germany) and were provided dissolved in PBS (PAA). In order to use the fragments at a certain concentration, an isolation procedure had to be performed. Therefore the needed amount of fragments was transferred into a new tube and supplemented with DEPC-treated H_2O (Roth) to 100 µl. The solution was diluted (1:5) by adding 400 µl ethanol (Merck) and afterwards centrifuged (30 min, 16.100 rcf, 4°C) (Centrifuge 5415R, Eppendorf, Wesseling-Bezdorf, Germany). Ethanol abrogates the hydrogen bonding between water molecules and hyaluronans, thereby the precipitation of hyaluronic acids is achieved. After removal of supernatant, fragments were visible as a clear pellet on the bottom of the tube. The isolated fragments were diluted with PBS (PAA) to the desired concentration and finally added to the 6-Wells. After incubation melanoma cells were analysed for CCR10 (R&D Systems) by using flow cytometry (BD Biosciences) according to the FACS protocol mentioned before.

Table 3: *Number of disaccharides of hyaluronic acid fragments used in stimulation experiments*

	Fragment18	**Fragment38**	**Fragment64**
Disaccharides (ds)	13-16 ds	8 ds	4 ds

5.8 Total RNA isolation

In order to isolate ribonucleic acid (RNA) from melanoma cells, 1 ml of TRIzol® Reagent (Invitrogen) was added to each well of a 6-well plate. TRIzol® Reagent is a monophasic solution of guanidine isothiocyanate and phenol. Guanidine isothiocyanate is used for cell lysis and phenol to remove proteins from nucleic acid samples during isolation.

Addition of $^1/_5$ volume chloroform (Merck) and subsequent vortexing and centrifugation (15 min, 12.000 rcf, 4°C) (Biofuge 13R, Heraeus Sepatech GmbH, Osterode, Germany) separates the RNA containing aqueous phase from the organic phase. The aqueous upper phase was then transferred into a new tube. To achieve RNA precipitation ½ volume isopropanol (Merck) was added. The solution was vortexed and incubated overnight at -20°C. After centrifugation (16.100 rcf, 30 min, 4°C) (Centrifuge 5415R, Eppendorf, Wesseling-Bezdorf, Germany) the RNA precipitate was visible as a pellet on the bottom of the tube. Residual salt was removed by washing the pellet with 1 ml of 80% ethanol (Merck) after supernatant had been carefully removed. After another centrifugation step (16.100 rcf, 30 min, 4°C) (Centrifuge 5415R, Eppendorf) the supernatant was again removed and the pellet was dried for 10 min at room temperature. Finally the pellet was dissolved in an appropriate volume (usually 50 µl) of DEPC-treated H_2O (Roth) and samples were stored at -80°C.

5.9 OD measurement

Nano Drop™ 2000 (Thermo Scientific, Wilmington, DE, USA) photometer was used to determine the RNA yield. Throughout all experiments the RNA concentration was measured according to the manufacturer's manual of Nano Drop. The optical density of the RNA samples (OD) was measured at 260 nm (relative absorbance maximum of RNA) and 280 nm (relative absorbance maximum of aromatic residues of proteins). An A_{260}/A_{280} absorbance ratio between 1.8 to 2.1 indicated that extracted RNA was devoid of any appreciable

protein, salt or solvent contamination and therefore qualified the samples for further analysis and interpretation.

5.10 Complementary DNA (cDNA) synthesis

cDNA was synthesized from messenger RNA (mRNA) templates using reverse transcriptase enzyme Superscript II (Invitrogen).

To remove possible genomic DNA contaminants, a DNase digestion was performed as a first step. DNA digestion was achieved by mixing 40 U RNasin (Promega, Madison, WI, USA), 10 U recombinant DNase I (Roche, Basel, Switzerland) and 5x first strand buffer (Invitrogen) to 4 µg RNA. DEPC-treated H_2O (Roth, Karlsruhe, Germany) was added to a final volume of 16 µl and prepared mixes were incubated for 20 min at 37°C, 10 min at 70°C, and afterwards placed at 4°C.

As a second step RNA was treated with 1 µg of anchored oligo$(dt)_{12\text{-}18}$ (Invitrogen) and 0.2 µg random hexamer primers (Promega). DEPC-treated H_2O was added to a final volume of 20 µl. Thereafter, samples were incubated for 10 min at 70°C in order to reduce RNA secondary structures.

Thirdly, 40 U RNasin (Roche), 3.57 mM DTT (Invitrogen), 1.61 mM dNTP mix (Bioline USA Inc., Tauton, MA, USA), 5x first strand buffer (Invitrogen) and DEPC-treated H_2O were added to a final sample volume of 29 µl. Samples were carefully mixed and incubated for 2 min at 42°C for optimal primer annealing.

Finally, cDNA synthesis was performed by adding of 400 U Superscript II resulting in a final volume of 30 µl. Samples were incubated for 50 min at 42°C and 10 min at 70°C to inactivate the enzyme (Trio-Thermoblock, Biometra, Göttingen, Germany). Thereafter, samples were stored at -80°C.

5.11 Quantitative real-time PCR (qRT-PCR) analysis

To quantify mRNA expression, qRT-PCR was performed. Synthesized cDNA was filled in wells of an Optical 96-Well Reaction Plate (Invitrogen) and gene specific primer mixes were added (Table 3). The reaction plate was sealed with Optical Adhesive Film (Microam) and centrifuged (2 min, 1000 rcf, RT) (Labofuge 400, Heraeus). During 40 cycles of qRT-PCR a specific sequence of the gene was amplified. To detect gene specific amplification, either SYBR® Green (Applied Biosystems Inc., Foster City, CA, USA) or TaqMan® (Roche) was used. SYBR® Green is a dye, which binds to double stranded DNAs with high affinity. The resulting DNA-dye-complex emits green light (λ_{max} = 522 nm), which can be detected with the Applied Biosystem 7000 System (Applied Biosystems) after each cycle (real time). An increase in fluorescence indicates a higher amount of qRT-PCR products. For further analysis a fluorescence threshold is set. High expressed genes cross this threshold at an early cycle, low expressed genes at later cycles. The cycle at which the threshold is being crossed is called C_t-value (threshold cycle). Gene expression analysis of 18S rRNA was used as an internal control since it is expressed at constant levels in a variety of cells. The 18S rRNA expression was analysed by a TaqMan®qRT-PCR system. This system comprises a 5' to 3' exonuclease activity (TaqMan polymerase), which is able to cleave phosphodiester bonds of nucleotide sequences. During the assay the TaqMan polymerase degrades a short nucleotide sequence called probe, which is linked with a fluorescent dye at the 5' end (VIC™) and a quencher dye at the 3' end (TAMRA™). The probe binds to the target gene in between the primers during the annealing stage of the PCR. When the probe is degraded the fluorescent dye separates from the quencher dye and the resulting fluorescence signal can be measured by the Applied Biosystem 7000 System (Applied Biosystems Inc.).

For a final reaction volume of 25 µl per well, the following gene specific mixes were prepared:

	Primer forward	***Primer reverse***	***Target probe***	***Detection mix***	***cDNA***
***HAS1,2,3*; *HYAL1,2* (SYBR® Green)**	200 nM	200 nM		½ vol.	2.5 ng/µl
Eukaryotic 18S rRNA (TaqMan®)	60 nM	60 nM	60 nM	½ vol.	2.5 ng/µl

The following PCR program was applied:

Temperature	Time	
50°C	2 min	
95°C	10 min	
95°C	15 s	} 40 cycles
60°C	1 min	

To analyse the specificity of SYBR® Green products, a dissociation protocol was carried out after termination of the PCR program to detect nonspecific amplification. By increasing the temperature DNA double strands separate, resulting in a decrease of fluorescence. PCR products obtained with the same primer pair should have similar melting points. For absolute quantification, standard curves were prepared from 0.01 pg/µl DNA to 1,000 pg/µl DNA. Quantity of gene expression was calculated automatically by the Applied Biosystem 7000 System (Applied Biosystems, Inc.):

Quantity [pg] = m x + b

Where m is the slope and b is the Y-axis intercept of the standard curve line. The x is the C_t-value obtained for specific gene in a specific sample.

Quantity was standardized to the gene expression of 18S rRNA:

$Quantity_{standardized}$ [fg] = Quantity [pg] · $2^{(Ct18SrRNA - \underline{Ct}18SrRNA)} \cdot 10^3$

Where $\underline{C_t}$18SrRNA is the mean of all C_t-values obtained for the 18S rRNA of all samples.

Gene expression was illustrated as mean values ± standard deviation.

5.12 Statistical analysis

Statistical analyses were calculated using nonparametric Mann-Whitney U test. Variations were considered to be statistically significant at values of $P<0.05$. P-values < 0.05 are indicated by *.

6 Results

6.1 Analysis of CCR10 expression in vivo

To investigate a potential role of CCR10/ CCL27 interactions *in vivo,* I analysed the CCR10 expression in immunohistochemical stainings of MM tissue samples. In order to distinguish melanoma tissue from healthy tissue, I also performed Melan-A staining. Melan-A is a cytoplasmic protein of mature melanocytes, which can be used to identify melanoma cells[99–101].

In cryo-sections of MM strong CCR10 expression could be observed in Melan-A-positive regions (**Fig. 5 A, C** and **E, G**) indicating that melanoma cells express CCR10 *in vivo*. In Figure 5, representative stainings of consecutive tissue sections of a patient with histopathologically confirmed lentigo-maligna melanoma show CCR10 detection in Melan-A-positive regions within the dermal (**A, C**) and the epidermal (**E, G**) compartment.

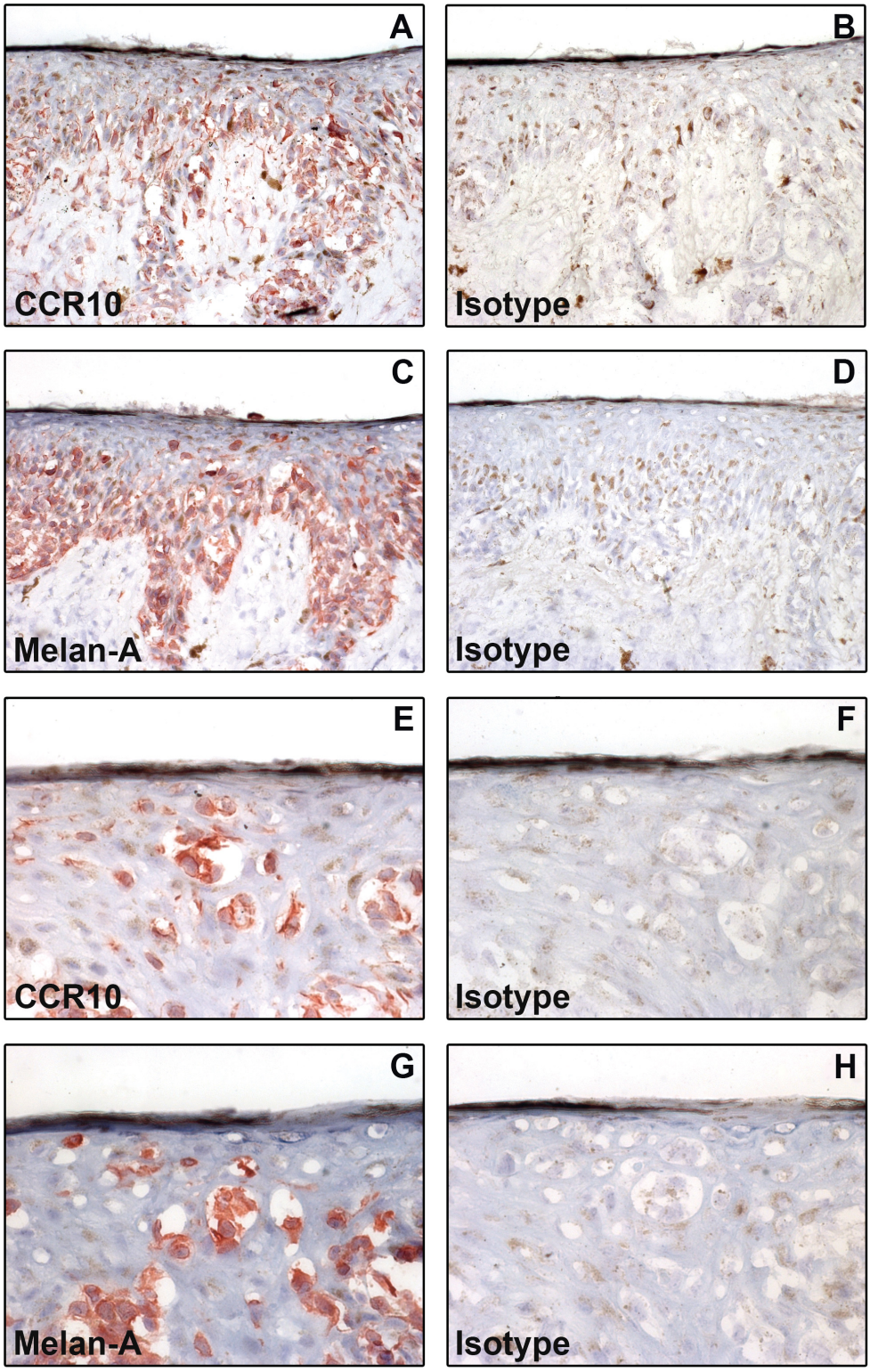

Figure 5 Representative immunohistochemical analysis of CCR10 expression (red) in Melan-A-positive regions (red), indicating melanoma cells within the primary tumour of a melanoma patient. Corresponding isotypes are in the right column (B, D, F, H). A-D magnification 200x, E-H magnification 400x.

6.2 Analysis of CCR10 expression in vitro

I also analysed the CCR10 expression of MM cells *in vitro* as a prerequisite for successful planning of assays that investigate the role of CCR10/ CCL27 interactions. FACS analyses of four different well established melanoma cell lines showed a distinct but differential cell surface expression of the receptor CCR10. UKRV-Mel4, MV3 and SK-Mel28 showed high CCR10 expression (9-10% positive cells), whereas SK-Mel2 showed low expression (3.8% positive cells) (**Fig. 6**). The detected cells, positive for CCR10, formed a subpopulation of cells with high CCR10 expression. Especially, UKRV-Mel4 cells showed a distinct subset of highly CCR10-expressing cells. Even though MV3 and SK-Mel28 cells showed similar amounts of cells positive for CCR10, the subset of cells, positive for CCR10, showed lower CCR10 expression compared to UKRV-Mel4 cells. Among all analysed melanoma cell lines, UKRV-Mel4 cells presented with the highest cell surface expression of CCR10. Additionally, strong intracytoplasmic staining for CCR10 in UKRV-Mel4 could be detected using immunofluorescence (**Fig. 7**).

Intracellular protein expression of CCR10 was also analysed by flow cytometry of permeabilised UKRV-Mel4 cells (**Fig. 8**). Here, I showed that all analysed melanoma cells were positive for CCR10, indicating that the majority of the receptor is localised within the cytoplasm.

The observed receptor expression raises the question of a potential role of CCR10 in the biology of malignant melanoma.

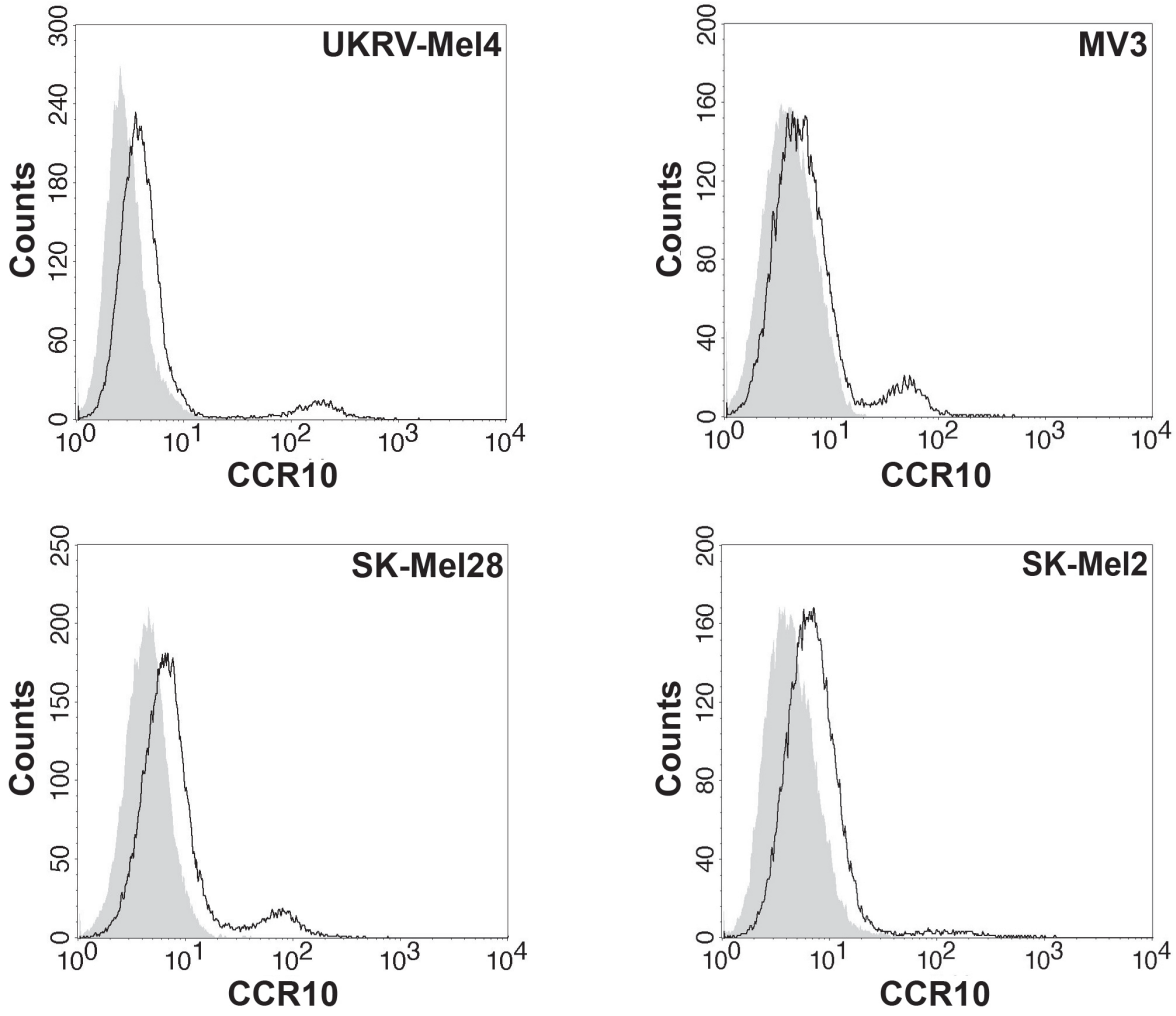

Figure 6 Representative flow cytometric analysis of CCR10 surface expression on different melanoma cell lines (UKRV-Mel4, MV3, SK-Mel28, SK-Mel2). CCR10 surface expression (black line). Filled histogram shows isotype controls.

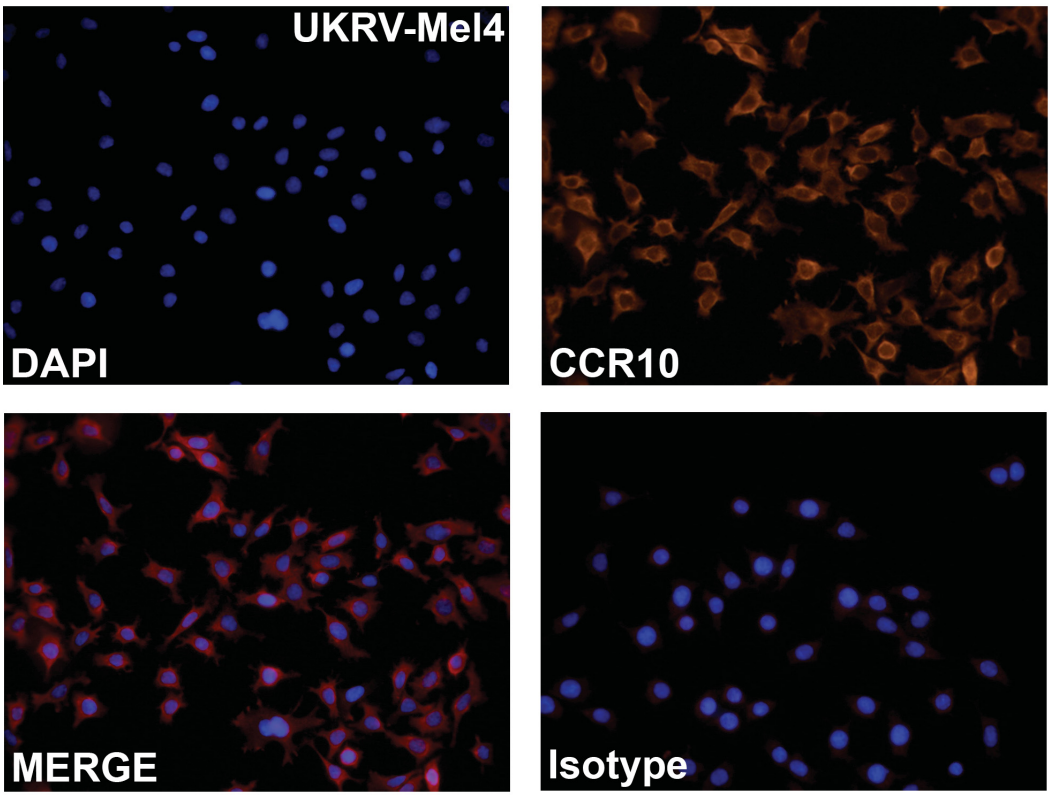

Figure 7 Representative immunofluorescence analysis of CCR10 (red) expression of melanoma cells (UKRV-Mel4). Cell nuclei were stained with DAPI (blue). Representative images out of three independent experiments. (magnification 400x).

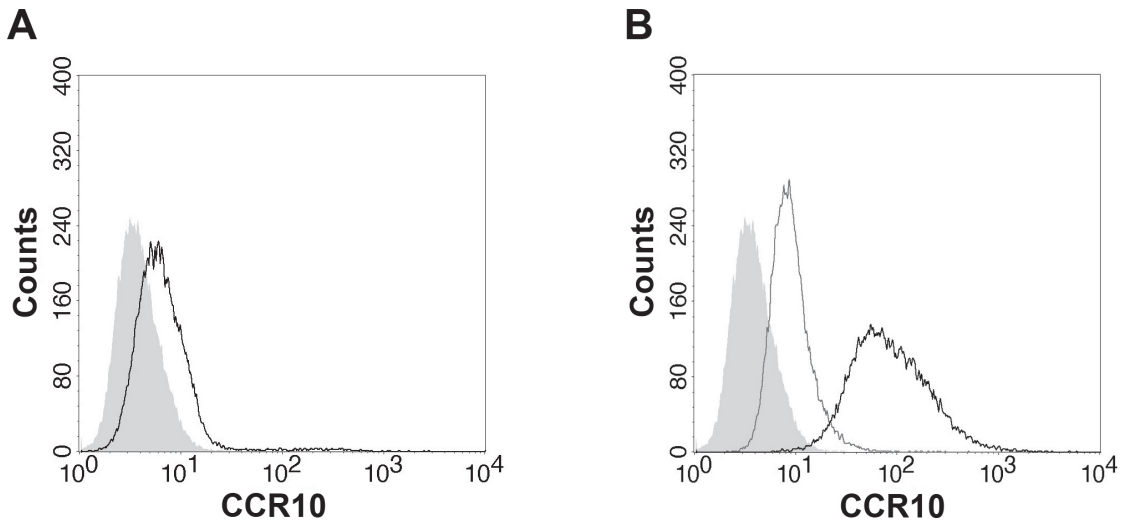

Figure 8 Representative flow cytometric analysis of CCR10 expression on melanoma cells (UKRV-Mel4). (A) CCR10 surface expression (black line). Filled histogram shows isotype control. (B) Intracellular CCR10 expression (black line). Filled histogram shows isotype control extracellular, grey line shows isotype control intracellular.

6.3 Functional analysis of CCR10 in melanoma

To investigate a potential role of CCR10/ CCL27 interaction in cutaneous metastasis, I observed the effect of CCL27 on motility/ chemokinesis and directional migration/ chemotaxis in melanoma cells (UKRV-Mel4, MV3) *in vitro*. I was able to demonstrate a significant motility enhancing effect of CCL27 on melanoma cells in a wound-repair assay. In medium-treated controls, melanoma cells migrated into the scratch wound and induced a 1.39 mm^2 (mean value) wound-healing response after 15 hours. In contrast, melanoma cells incubated with 500 ng/ml CCL27 for 15 hours showed a significantly enhanced wound closure of 1.75 mm^2 (mean value) (P=0.0286; Mann-Whitney U test) (**Fig. 9**).

Chemotaxis assays were performed using IBIDI µ-chemotaxis chamber. All slides were analysed by computer-assisted videomicroscopic motion modules to obtain trajectories made by motile melanoma cells (**Fig. 10**). Afterwards melanoma cell trajectories were statistically analysed relative to the direction of the chemical gradient using an established directionality-based assay, independent of melanoma cell motility speed and pattern of movement and therefore measuring chemotaxis only (**Fig. 10 C-E**). Incubation with CCL27 showed significant stronger chemotactic responses of melanoma cells when compared to medium-treated controls ($P \leq 0.05$; Mann-Whitney U test). A significantly higher percentage of melanoma cells travelling in the direction of chemokine gradients and a higher percentage of melanoma cells travelling a longer distance in the direction of chemokine gradients than in the direction orthogonal to the gradients were observed. Moreover the mean net distance of cells travelled along the chemokine gradient was also significantly increased.

These findings suggest that CCL27 represents a capable chemoattractant for melanoma cells and enhances directional migration/ chemotaxis. Consequently, chemokine ligand-receptor interactions of CCL27 and CCR10 seem to play an important role in cutaneous metastasis.

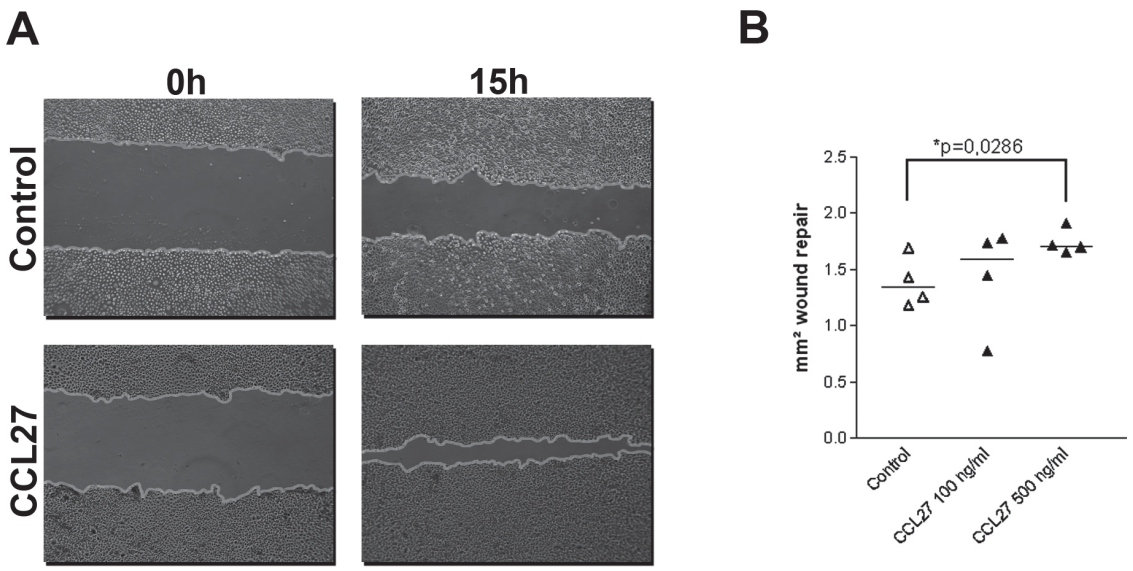

Figure 9 *In vitro* wound repair assays, CCL27 was able to induce significant wound repair and migration of melanoma cells when compared to corresponding controls (*, P=0,0286; Mann-Whitney U test). (A) Microscopic images of scratch wounds at t=0h and after incubation of 15h. Medium-treated controls were compared with CCL27 incubation. (B) Scatter graph displaying wound-healing response (wound repair in mm^2) of melanoma cells incubated with different CCL27 concentrations compared to controls.

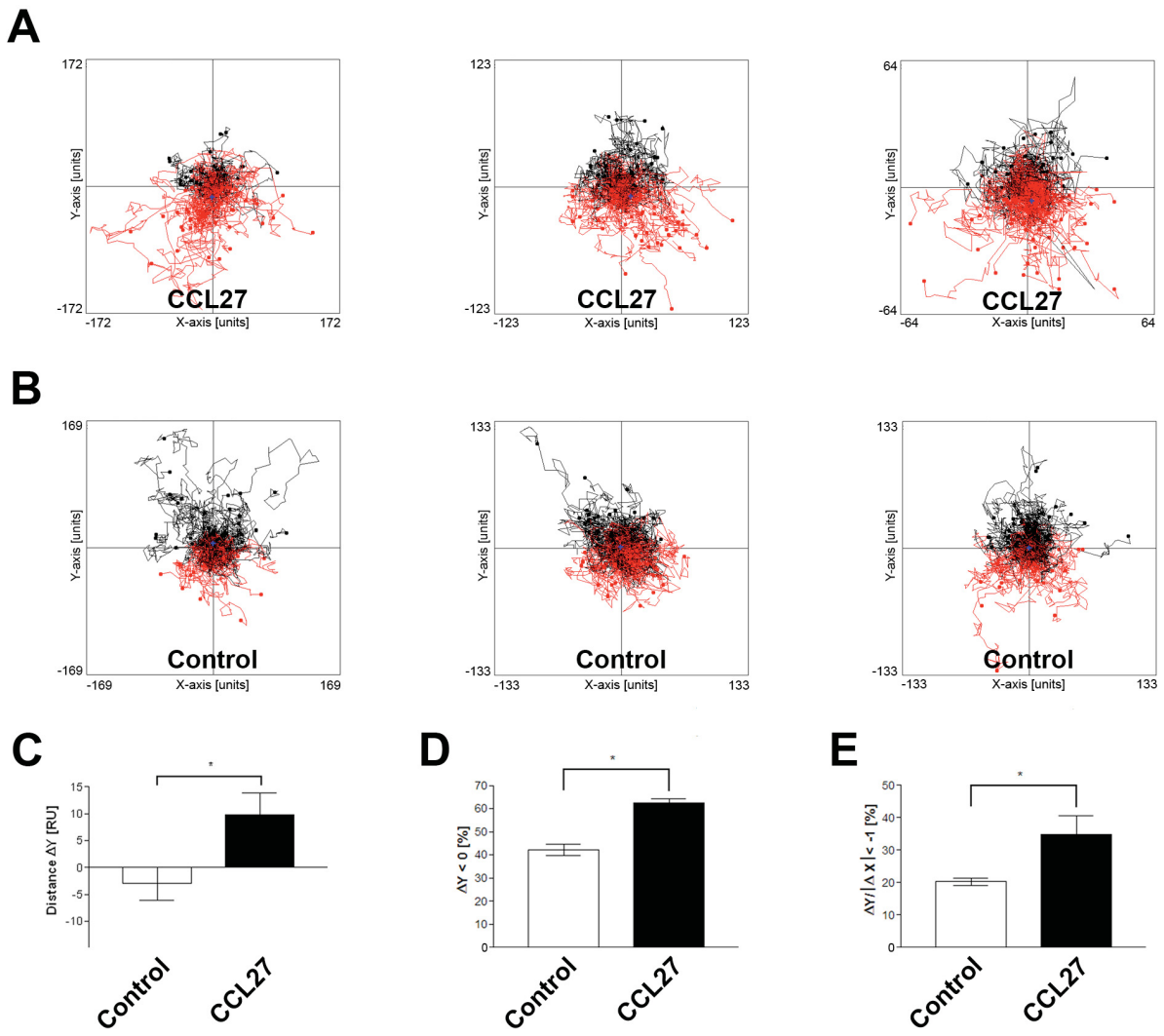

Figure 10 Trajectories of melanoma cells (UKRV-Mel4) cultured inside an IBIDI µ-chemotaxis chamber containing chemokine gradients of CCL27 (0 to 1,000 ng/ml)(A) or medium only as control (B). (C-E**)** Statistical analysis of melanoma cell trajectories. (C) Average ΔY, mean net distance (RU) of cells travelled along the chemokine gradient (Y-axis). (D) ΔY < 0, Percentages of melanoma cells traveling in the direction of chemokine gradients (Y-axis). Values above 50% show a tendency of cell movement in direction of higher chemokine concentrations (*, P≤0.05; Mann-Whitney U test). (E) ΔY / lΔXl < -1, Percentages of melanoma cells traveling a longer distance in the direction of chemokine gradients (Y-axis) than in the direction orthogonal to the gradients (X-axis). Values higher than 25% indicate a directed migration of cells in direction to higher concentrations of the chemokine gradient (*, P≤0.05; Mann-Whitney U test).

6.4 Regulation of CCR10 on malignant melanoma cells

Hyaluronans are a major component of the extracellular matrix in the skin and can be detected in the tumour microenvironment[79,103]. In immunohistochemical analysis strong hyaluronan staining can be detected all over the epidermis and the cutis (**Fig. 11 A, E**). In Melan-A-positive regions (**Fig. 11 C**) even stronger staining of hyaluronans (**Fig. 12 A**) can be detected, whereas in tumour margins (**Fig.11**; **E, G**) and Melan-A-negative regions weaker staining is visible (**Fig. 11 E**).

Flow cytometric analyses of cultured UKRV-Mel4 melanoma cells showed that the receptor CCR10 is located intracellular in a majority of cells. Here I show that the hyaluronic acid fragment18 strongly induces the shift of the receptor to the surface of melanoma cells (**Fig. 12**), whereas fragments of different sizes like fragment38 and fragment64 failed to induce this shift or only showed weak induction (**Fig. 12 C**). In detail, incubation of UKRV-Mel4 melanoma cells with 5 µg/ml of fragment18 for 24 hours induced a shift of CCR10-positive cells from 8.41% to 13.46% in one experiment. In further experiments similar shifts were observed regarding different base expression of CCR10 before the stimulation (12.08% to 22.22%, 2.1% to 3.4%, 2.68% to 4.87%). In contrast, incubation with fragment38 induced only a weak shift of 8.41% to 9.37%, respectively, 12.08% to 15.77%, 2.1% to 2.69% and 2.68% to 4.69%. Overall a mean fold change of 71.25% was observed when using fragment18, respectively 36% when using fragment38 and 16.52% when using fragment64.

Because of the ability of hyaluronic acid fragments to induce CCR10 surface expression, I investigated the expression of hyaluronan synthases and hyaluronidases in several melanoma cell lines, as they are necessary for the synthesis and degradation of hyaluronans and hyaluronic acid fragments[81,104,105]. Indeed, melanoma cells show high expression for hyaluronidases and hyaluronan synthases on mRNA level (**Fig. 13**). Especially *HYAL1*, *HYAL2* and *HAS3* were expressed at high levels by UKRV-Mel4 melanoma cells. However, there are differences in mRNA expression between melanoma cell lines. My observations showed that all melanoma cell lines

express HYAL1 and HYAL2. However, HYAL2 is expressed at much higher levels by all melanoma cells. Looking at HAS enzymes I found a more differentiated expression pattern. All melanoma cells express *HAS3*, most express *HAS2*, but only some melanoma cells express *HAS1*. Furthermore, *HAS1* is only expressed at very low levels. Compared to the enzyme expression of melanocytes, we have mostly higher mRNA levels in melanoma cells. Melanocytes do not express *HAS1* and *HAS2*, but do express *HYAL1* and *HYAL2* in similar levels to some melanoma cell lines; however, most melanoma cell lines show higher expression. *HAS3* is also expressed by some melanocytes at rather low levels (see **Fig. 13**).

These results indicate that melanoma cells might form a microenvironment conductive to metastasis through the generation of CCR10-inducing small hyaluronic acid fragments.

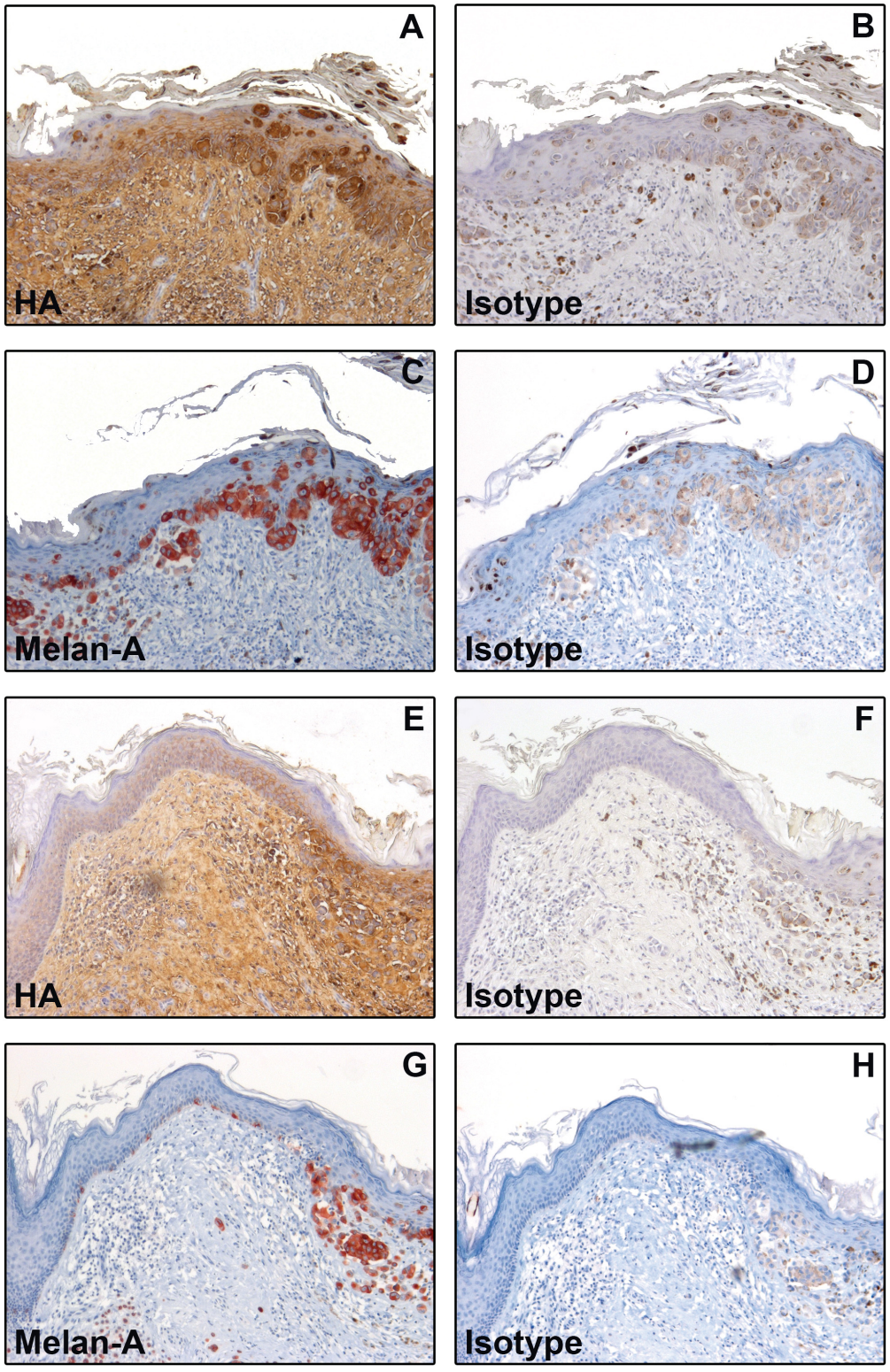

Figure 11 Representative immunohistochemical analysis of hyaluronic acid expression in melanoma tissue samples. Corresponding tissue samples are stained for Melan-A to indicate melanoma cells (A, C and E, G). Isotypes are displayed in the right column (B, D, F, H). (Magnification 200x).

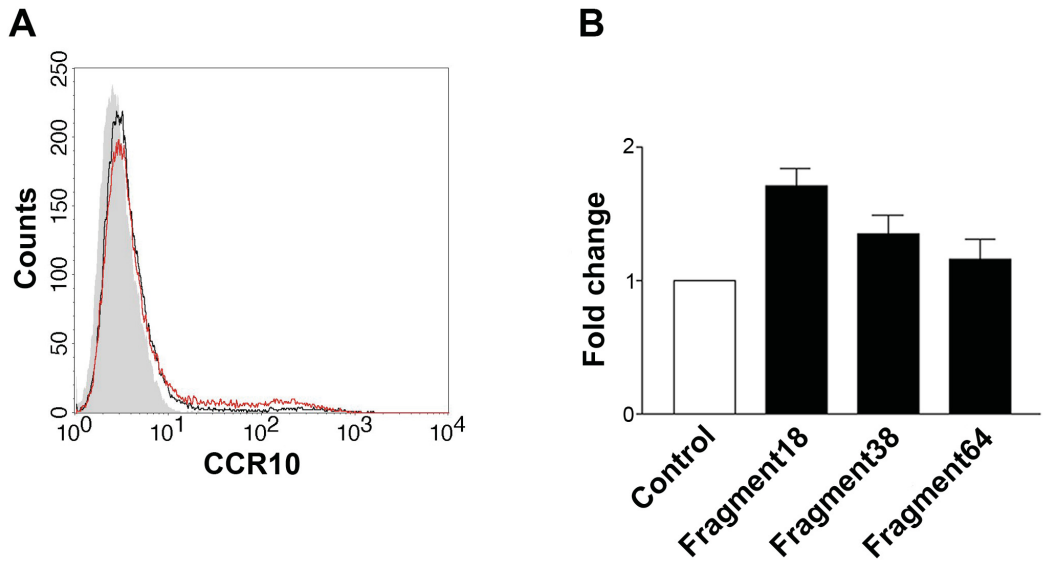

Figure 12 Representative flow cytometric analysis of CCR10 expression on melanoma cells (UKRV-Mel4) after stimulation with hyaluronic acid fragments. (A) CCR10 expression of control (black line) compared to CCR10 expression after stimulation with hyaluronic acid fragment18 (5 µg/ml for 24h) (red line). Filled histogram shows isotype control. (B) Fold change of CCR10 expression after stimulation with three different hyaluronic acid fragments. Values represent the mean ± SD of four independent experiments.

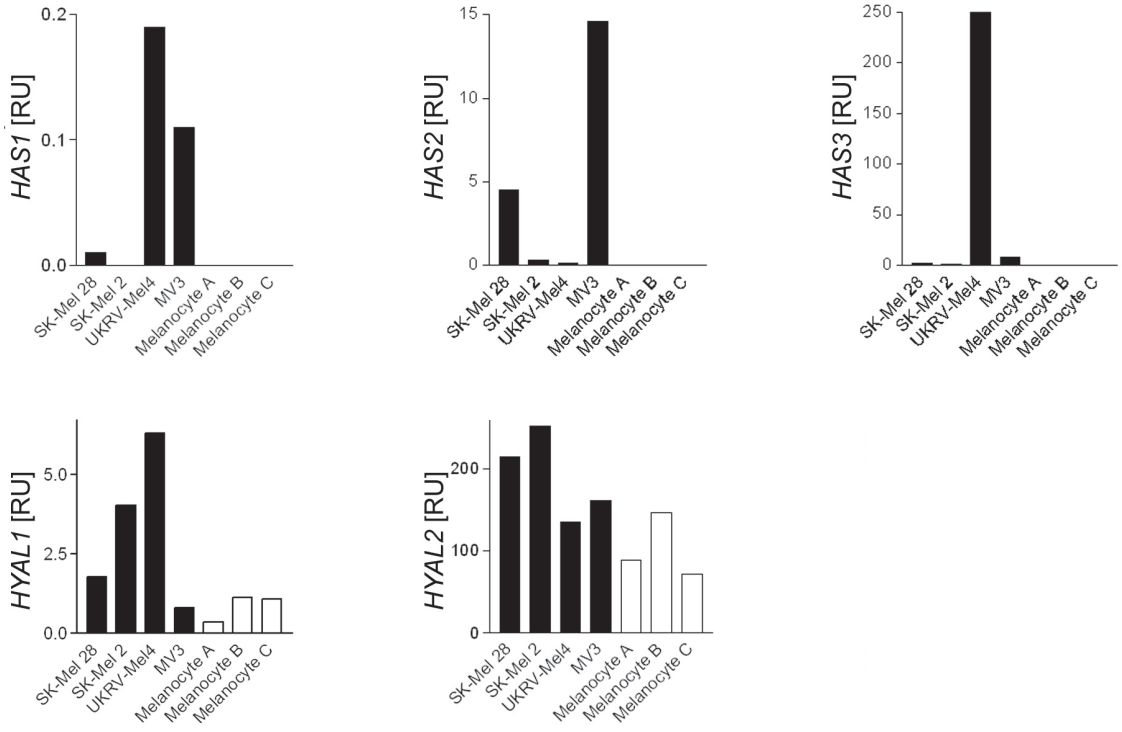

Figure 13 *HAS1, HAS2, HAS3, HYAL1 and HYAL2* mRNA expression of different melanoma cell lines and three different donors of human primary melanocytes was analysed by qRT-PCR. Values are expressed as relative expression.

To investigate a possible paracrine function of CCL27 on the expression of hyaluronan synthesizing or degrading enzymes, I stimulated UKRV-Mel4 melanoma cells with CCL27 and analysed the hyaluronan synthases and hyaluronidases mRNA expression by qRT-PCR (**Fig. 14**). However, no significant differences between CCL27-stimulated and medium-only incubated melanoma cells concerning HYAL and HAS expression have been observed, indicating that CCL27 does not function as an inducer of HYAL and HAS in melanoma cells.

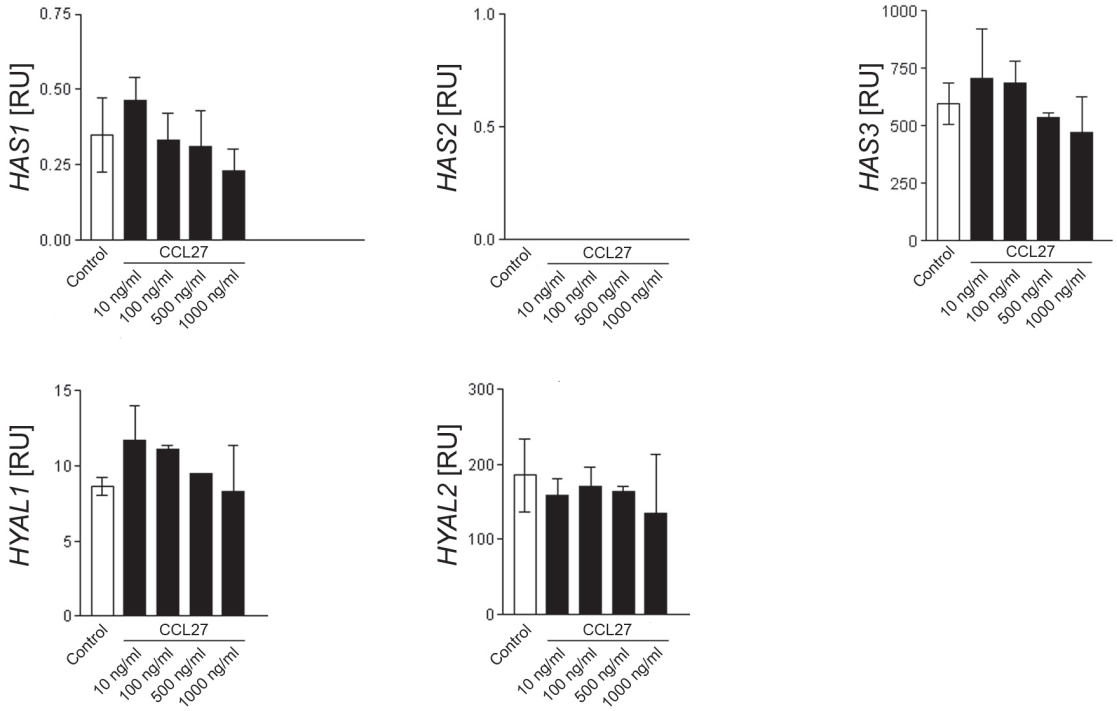

Figure 14 *HAS1, HAS2, HAS3, HYAL1 and HYAL2* mRNA expression of melanoma cells (UKRV-Mel4) was analysed by qRT-PCR. Values are expressed as relative expression and represent the mean ± SD of two independent experiments. Cells were either incubated with different CCL27 concentrations (10, 100, 500, 1000 ng/ml) or with medium alone as control for 24h.

7 Discussion

Metastasis occurs in the course of malignant diseases and significantly determines its prognosis[2]. Therefore, early detection of metastases is an important element in the medical attendance of tumour patients. Different hypotheses have been suggested to explain its pathogenesis in order to develop successful and practicable therapeutic concepts[68].

Apart from the hypothesis that postulates metastasis to be a mechanical process in which tumour cells get lodged in the vasculature at a distant destination[67], other theories have been established in which the molecular phenotype of the tumour cells and the destination of metastasis are highlighted[68]. For many years concepts stress the idea that metastasis is a specific, non-random process[5]. According to the “seed and soil“ concept local growth factors at the site of metastasis favour the settling of appropriate tumour cells[4]. The “adhesion theory“ suggests that organ-specific adhesion molecules, expressed by endothelial cells, mediate the invasion of tumour cells[106].

More recently, the discovery of chemokines and their corresponding receptors in the tumour environment led to the hypothesis that organ-specific metastasis is mediated by its interaction[5]. In many malignancies specific receptor-profiles have been determined and corresponding chemokines have been found at common sites of metastasis like lymph nodes and lung[69]. The most widely expressed chemokine receptor by tumour cells is CXCR4[107]. Its role in organ-specific metastasis in breast cancer was demonstrated for the first time by Müller et al. in 2001[6].

In addition to CXCR4, melanoma cells show high expression levels of the chemokine receptor CCR10 on the mRNA level[6]. This receptor is also expressed by melanocytes, dermal fibroblasts, dermal microvascular endothelial cells and skin-homing T cells[8]. Recent observations have shown the ability of the skin specific chemokine CCL27 to direct CCR10-positive T cells to cutaneous sites[9]. As MM frequently exhibits skin metastases, in contrast to other malignancies, a potential role of CCR10 in cutaneous metastasis has been suggested.

Findings of the present study underline the importance of CCR10 as it shows strong expression on melanoma cells *in vivo* and *in vitro.* However, the observed melanoma cell lines exhibit the receptor on their surface to a different extent indicating a differing aggressiveness and invasiveness of those cell lines. Similar to my observations several studies describe differences in metastatic capacity of melanoma cell lines[108,109]. MV3 for example, a cell line with highly aggressive characteristics also shows higher CCR10 expression.
Moreover, my findings depict that the vast majority of the receptor is located inside the cell and not on the cell surface (see **Fig. 8**) suggesting that local factors are provided at the site of the tumour to shift the receptor to the surface. A role of the microenvironment in receptor regulation has been described before and will be discussed in detail.

In 1999, Morales et al. discovered the chemokine CTACK, which was renamed CCL27 according to the systematic nomenclature of chemokines[62,110]. This chemokine is predominantly expressed in the skin by keratinocytes[110]. Further studies showed that this chemokine is able to bind the receptor CCR10[8]. Next to CCL28, which is known to be expressed by mucosal tissue in the gut[111], CCL27 is the only identified chemokine which binds CCR10[65]. These findings raise the question, if the interaction of CCR10 and its skin-specific ligand CCL27 plays a potential role in organ-specific metastasis of MM, a tumour entity known for the development of skin metastases unlike other malignancies[3].
Several studies showed that chemokines induce directional migration/ chemotaxis of various cell types. For example, interactions of chemokines with their corresponding receptors are involved in the guidance of embryonic stem cells to sites of organogenesis[63], in the recruitment of leukocytes to lymphatic tissue or endothelial cell migration and angiogenesis[112,113]. CCR10 and CCL27 are known to play a key role in the recruitment of T cells to skin-associated inflammatory sites[9]. In keratinocyte-derived skin tumours, a downregulation of CCL27 was observed, which led to decreased immune response and enhanced primary tumour growth[114].
Furthermore an important role of chemokines in organ-specific metastasis in

various malignancies has been described[107]. In a mouse model, Kawada et al. showed that CXCR3 expression on melanoma cells and the expression of its ligands CXCL9 and CXCL10 in lymph nodes are crucial in melanoma cell metastasis to lymph nodes[115]. Blocking of CXCL9 and CXCL10 resulted in decreased lymph node metastasis[115].

My aim was to investigate, if the skin-specific chemokine CCL27 is also able to induce directional migration/ chemotaxis in melanoma cells as a possible explanation for the high frequency of cutaneous metastasis in melanoma patients.

Here, I demonstrated for the first time that CCL27 induced *in vitro* wound repair in melanoma cells indicating that CCL27-CCR10 interactions mediate melanoma cell migration. Furthermore chemotaxis assays showed CCL27 to be a capable chemoattractant for melanoma cells as well as it was able to induce directed migration. These observations support recent findings about the functional role of CCL27-CCR10 interactions. Apart from its chemotactic and motility-enhancing characteristics presented in this work, CCL27 is also known to modulate the host immune response in MM. Observations showed that the expression of CCR10 enables melanoma cells to evade FAS-triggered apoptosis[116]. Simonetti et al. showed that CCR10 expression in MM is directly correlated with tumour thickness and inversely with T-lymphocyte density[7]. Taken together, an important role of CCR10 and its ligand CCL27 in melanoma progression and metastasis in particular to the skin has to be assumed. However, further research is necessary to prove the hypothesis of chemokine-driven metastasis to be applicable *in vivo.*

Flow cytometric analyses have shown that cultured melanoma cells exhibit the majority of CCR10 intracellular (see **Fig. 7**). This raises the question how the shift of the receptor to the surface is obtained *in vitro* and might give indications of receptor regulation *in vivo*.

In plasmablasts, IL-21 in combination with TGFβ-1 was able to induce CCR10 surface expression[117]. Moreover 1,25-dihydroxyvitamin D3 has been shown to enhance CCR10 surface levels of B cells[77]. However, little is known about the regulation of CCR10 from the intracellular compartment to the surface in

melanoma cells. CCR10 appears to be upregulated on mRNA level upon induction with IL-1β and TNF-α in melanocytes[8], which might also give indications on melanoma receptor regulation.

The occurrence of high amounts of hyaluronans in the skin and the tumour microenvironment make them a perfect candidate for a novel inductor of chemokine receptor surface expression. Hyaluronic acids are a major component of the extracellular matrix and are located in connectives tissues such as the skin, synovial fluid, as well as in kidney, lung, brain or muscle[79]. They play an important role in the preservation of the integrity of different tissues. Its ability to bind proteoglycans and aggrecans appears stabilizing[118].

Besides many other functions hyaluronans are also known to work as signalling molecules. Especially the role of smaller fragments of hyaluronic acid polymers have been described in e.g. inflammation and cancer[80]. Hyaluronic acid fragments are generated by the degradation of high-molecular-size hyaluronic acids[81].

Degradation of hyaluronic acids is obtained by enzymes called *HYAL1* and *HYAL2*[81]. They are produced by various cells including melanoma cells[119]. High-molecular-weight hyaluronic acids themselves are synthesized by enzymes called *HAS1*, *HAS2* and *HAS3*[80]. These enzymes are expressed by e.g. fibroblasts but also again by melanoma cells[119].

Various and to some extent opposing functions are obtained depending on the fragment's size[81]. Interestingly, Sugahara et al. showed that hyaluronic acid fragments enhance tumour cell motility in human pancreatic carcinoma cells[105,120].

Contrary to these observations, Zeng et al. showed an inhibitory effect on tumour growth of hyaluronan oligomers in subcutaneously injected melanoma cells[119]. However, Zeng et al. used fragments of smaller size, mainly composed of five to six disaccharide units[121], whereas Sugahara showed a main motility enhancing effect with fragments mainly composed of 36 units[105,120].

This work demonstrates a strong expression of hyaluronic acids all over the epidermis and cutis in immunohistochemical analyses of melanoma tissues. In Melan-A positive regions even an elevated amount of hyaluronans was detectable in comparison to Melan-A negative regions (see **Fig. 11**), indicating

that melanoma cells might form a microenvironment conductive to metastasis through the generation of CCR10-inducing small hyaluronic acid fragments.

HYAL2 and *HAS3* are the most abundant expressed enzymes by melanoma cells in hyaluronan metabolism suggesting a key role of these enzymes, though there are some hyaluronidases I did not observe (*HYAL3*, *HYAL4*, *PH-20*, *HYALP1*). My observations show a predominant expression of these enzymes by malignant cells compared to human primary melanocytes underlining the importance of hyaluronans in the tumour microenvironment and in tumour progression.

The expression of hyaluronidases and hyaluronan synthases is crucial for synthesizing and degrading hyaluronans and therefore essential to provide hyaluronic acid fragments. An increased expression of these enzymes in melanoma cells compared to melanocytes leads to a higher turnover of hyaluronans and potentially to a higher amount of hyaluronic acid fragments. However, I could not detect an upregulation of hyaluronidases and hyaluronan synthases when incubating melanoma cells with CCL27 (see **Fig. 14**). These observations indicate that CCL27 does not function as an inducer of HAS and HYAL enzymes in MM.

Indeed, incubation of melanoma cells with hyaluronic acid fragments induced a strong shift of CCR10 to the surface depending on the size of the fragments (see **Fig. 12**). These observations underline their importance in tumour biology and prove their ability to work as signalling molecules in a size-dependent manner as described before by Sugahara et al. and Zeng et al.[120,121], but additionally demonstrate hyaluronic acid fragments to work as receptor regulators. Especially larger fragments (13-16 disaccharides) were highly capable of shifting the receptor to the surface. Decreasing induction of receptor expression was observed with eight disaccharide fragments and four disaccharide fragments. However, due to different base expressions and small number of experiments (n=4) in this work, the results showed no significance in statistical analyses, but regarding strong fold changes this work still underlines the importance of hyaluronic acid fragments in the tumour microenvironment and their ability to induce chemokine receptor shift to the surface.

This might show how melanoma cells create a favourable tumour environment

by the production of hyaluronic acid fragments, which again positively influence melanoma cells e.g. by induction of CCR10 expression. Both the interaction of CCL27 and CCR10, and certain fragments themselves promote tumour motility. However, it is not known how the production of fragments of specific size is regulated. Depending on the size of the fragments different functions are obtained[81,105,120].

Collectively, my results suggest a role of CCR10 and CCL27 in the pathogenesis of progression and metastasis of MM. Constant expression of CCR10 by melanoma cells and the ability of CCR10/ CCL27 interactions to induce motility and directed migration, as well as resistance from apoptosis could be an explanation for frequently observed skin metastases.

Furthermore, the ability of CCL27 to recruit CCR10-positive leukocytes to provide immune surveillance and enhance motility in other cell populations might depict that CCL27 is a more general growth and motility-enhancing factor than previously assumed.

Findings of the present study have also shown that local factors of the tumour environment influence the receptor expression of melanoma cells and are therefore essential for a more effective chemokine interaction. High amounts of hyaluronans in the skin and high expression of hyaluronan synthesizing and degrading enzymes by melanoma cells make hyaluronic acid fragments a perfect candidate for tumour modulation.

These findings are complement to other theories. Adhesion molecules, local growth factors and mechanical factors may also play important roles in the pathogenesis of metastasis and add to the concept of chemokines. Even though, my findings suggest a key role of chemokines in progression and organ-specific metastasis of melanoma (see **Fig. 15**).

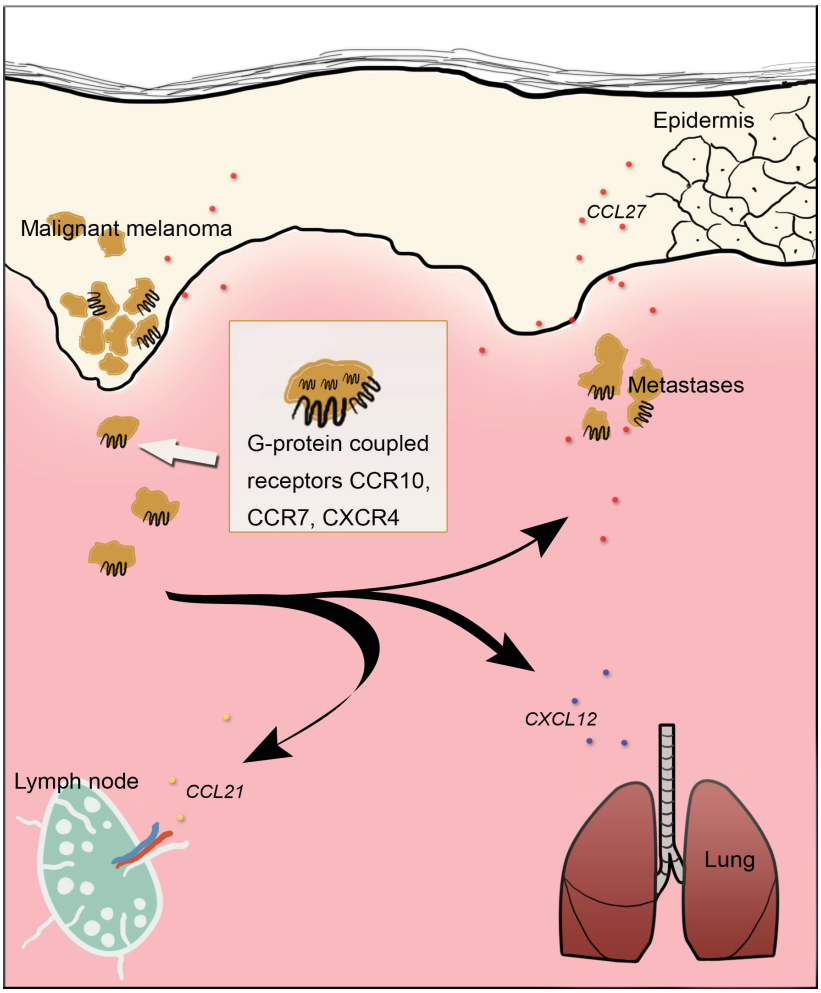

Figure 15 Metastasis in malignant melanoma. Interactions of chemokines produced at sites of metastasis (CCL27, CCL21, CXCL12) and their corresponding receptors on melanoma cells (CCR10, CCR7, CXCR4) are involved in organ-specific metastases formation in the skin, lymph nodes or lungs.

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9 Declaration

Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbständig und ohne unzulässige fremde Hilfe erstellt und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

Datum, Vor- und Nachname

Unterschrift