Cancer invasion: Growth factor-induced mechanisms and targeting by image-guided surgery

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Cover design: Julia Odenthal

Cover image: UT-SCC58 spheroid cultured in a 3D collagen matrix and stained for cell nuclei, actin, tubulin and vimentin.

Layout and printing: Gildeprint – the Netherlands ISBN 978-94-92896-81-0 Copyright © by Julia Odenthal 2018

The research presented in this thesis was performed at the Department of Cell Biology, Radboud Institute for Molecular Life Sciences (RIMLS) and the Department of Otorhinolaryngology and Head and Neck Surgery, Radboud University Medical Center, both located in Nijmegen, The Netherlands

Cancer invasion: Growth factor-induced mechanisms and targeting by image-guided surgery

Proefschrift

ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken, volgens besluit van het college van decanen in het openbaar te verdedigen op maandag 10 december 2018 om 16.30 uur precies

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Cancer invasion: Growth factor-induced mechanisms and targeting by image-guided surgery

Doctoral Thesis

to obtain the degree of doctor from Radboud University Nijmegen on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken, according to the decision of the Council of Deans to be defended in public on Monday, December 10, 2018 at 16.30 hours

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Et es wie et es, et kütt wie et kütt un et hät noch immer jot jejange! (Cologne Constitution)

Für meine Großeltern

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CHAPTER 1

General introduction & outline of the thesis



Head and neck squamous cell carcinoma (HNSCC) originates from mucosal surfaces of the upper aerodigestive tract and is the sixth most appearing type of cancer worldwide with approximately 600,000 arising cases each year [1]. The development of new therapeutic strategies are hampered by its effective local invasion in functionally delicate areas. This local invasion is directly associated with high incidences of positive resection margins and poor prognosis of the patient [2]. Consequently, despite recent development in cancer treatment, only 40-50% of HNSCC patients worldwide survive longer than 5 years. Thus, to improve cancer treatment, it is of importance to understand what drives tumor cell invasion in HNSCC.

Infiltrating tumor cells migrate either individually without cell-cell interactions to neighboring cells [3] or collectively as cohesive groups when cell-cell junctions remain intact [4]. Collective cell migration is the dominant migration type in epithelial tumors such as HNSCC, but both cell migration types have been identified in reconstructions of HNSCC samples [5].

Independent of the migration type, cancer invasion occurs in close interaction with the local tissue environment surrounding the tumor cells including growth factors and cytokines secreted by stromal cells, oxygen supply and the composition of the extracellular matrix (ECM) [6]. These influences drive cancer invasion by acting on the cell-intrinsic properties, likely under the control of transcriptional programs such as the epithelial-to-mesenchymal transition (EMT) [7]. Potentially, they force the cell to adapt to the environment, causing an interconversion of the tumor cell dissemination strategies. The ability of cells to change their mechanism of movement is termed plasticity [8]. However, how plasticity is spatiotemporally controlled upon challenge by the tumor microenvironment and whether inhibiting these factors can inhibit the plasticity progress remains unclear.

Chapter 2 provides an overview on growth factors and cytokines and their effects on tumor cell migration. By regulating cell-matrix and cell-cell adhesions, cytoskeletal dynamics and extracellular matrix remodeling, growth factors and cytokines control the reciprocal interactions between tumor cells and their microenvironment. In addition, consequences for the plasticity of invasion programs and metastasis are discussed.

Growth factors are often used at one concentration to stimulate migration in a 2D model system, which may miss potential concentration-dependent effects on cell migration and the role of the 3D environment. Fibroblasts, as part of the tumor stroma, secrete growth factors and cytokines including hepatocyte growth factor (HGF) which induces collective as well as single cell migration. An example of growth factor-induced tumor cell migration plasticity is described in **Chapter 3**. Besides inducing migration, fibroblast-conditioned medium is able to cause a concentration-dependent switch in migration mode, which is recapitulated by single growth factors. To complement biochemical analysis of entire cell populations, a novel 3D single-cell cytometry was developed to detect molecular markers of the EMT in tumor cell subsets. The data show EMT markers randomly expressed with an increasing trend in migrating cells, thus connecting EMT with both collective and single-cell migration modes.

Current treatment of HNSCC includes surgery, radiotherapy and/or systemic therapy. Primary surgery and radiotherapy (with or without addition of systemic therapy) are the most important treatment modalities in curative intent treatment. Additional treatment after surgical resection is often required since surgery often fails to remove the tumor completely [9]. Neurovascular and other tissue structures provide tumor cells with abundant cues to migrate along and infiltrate the tissue which is often non-destructive and subclinical. Thus, regions of deep tissue invasion are difficult if not impossible to detect macroscopically and thereby typically escape surgical removal [10]. As further confounding parameter, surgery is often performed with strict precaution to minimize a loss of vital structures which are required for delicate functions such as tasting, swallowing and speaking, causing the risk of residual disease post-surgery. As a consequence, choosing the resection margin represents a compromise between loss of function of the tissue and complete tumor removal [11]. Strategies to detect tumor invasion include advanced imaging such as MRI, which assists the surgeon by providing information about discriminating tumor from non-tumorous normal tissue. As complementary approach to MRI, molecular imaging by optical near-infrared fluorescence (NIRF) using fluorescent molecular probes to target cancer lesions shows promise to visualize tumor lesions intraoperatively with potential for high sensitivity and selectivity [12]. Advantage of NIRF (700-900 nm) include high tissue genetration to several millimeters and low auto-fluorescence, thereby providing sufficient contrast [13]. In addition, NIR light does not alter the surgical field since it is insensitive to the human eye and several imaging systems have recently become available [14]. During fluorescence-guided surgery (FGS), antibodies with tumor selectivity, conjugated with a near-infrared fluorophore, are injected intravenously, accumulate in the lesion and can be visualized using an intra-operative imaging system [15]. Thereby, tumor regions can potentially be detected and resected more accurately and completely. The identification of epitopes with an expression preferably restricted to the invasion zone is pertinent to selectively visualize this tumor region in HNSCC. In Chapter 4, a literature survey is performed and potential antigens (over-)expressed in HNSCC are tested to identify a reliable epitope in HNSCC which can be used for FGS. Furthermore, the technique of FGS is assessed in a new developed invasive HNSCC mouse model using a monoclonal antibody conjugated to a NIR fluorescence dye. Together, the data identified CD44v6 as a robust epitope present in invasion zones and the accumulation in the preclinical tumor model using a NIR-fluorescent CD44v6 targeting antibody outperforms EGFR, the currently clinically explored detection epitope in HNSCC. For early stage laryngeal as well as oropharyngeal and hypopharyngeal squamous cell carcinomas, carbon dioxide (CO₂) laser surgery has become an established treatment modality, which enables precise resection margins and low complication rates [16, 17]. However, for combining laser surgery with fluorescence guidance, high-power lasers may bleach fluorophores and extinguish the signal. Thereby it is uncertain whether CO_2 laser surgery can be effectively combined with FGS.

In **Chapter 5**, *in vitro* analyses on combining CO₂ laser treatment on fluorescent tumor models show that laser-induced photo-bleaching is negligible since its signal is below the extent of collateral thermal damage.

As summary, **Chapter 6** discusses the relevance of cell invasion strategies for local tumor cell dissemination, the challenges of tumor cell migration plasticity on cancer treatment and perspectives for targeting cancer invasion.

1

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CHAPTER 2

Plasticity of tumor cell invasion – governance by growth factors and cytokines

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Carcinogenesis. 2016;37(12):1117-1128.

Tumor cell migration, the basis for metastatic dissemination, is an adaptive process which depends upon coordinated interaction with the environment, influencing cell-matrix and cell-cell adhesion, cytoskeletal dynamics and extracellular matrix remodeling. Growth factors and cytokines, released within the reactive tumor microenvironment, and their intracellular effector signals strongly impact mechanocoupling functions in tumor cells and thereby control the mode and extent of tumor invasion, including collective and single-cell migration, and their interconversions. Besides their role in controlling tumor cell growth and survival, cytokines and growth factors thus provide complex orchestration of the metastatic cascade and tumor cell adaptation to environmental challenge. We here review the mechanisms by which growth factors and cytokines control the reciprocal interactions between tumor cells and their microenvironment, and the consequences for the efficacy and plasticity of invasion programs and metastasis.

INTRODUCTION

Tumor invasion and resulting metastatic dissemination account for major morbidity and mortality of cancer, through the remodeling, malfunction and eventually destruction of tissues and organs. During metastatic dissemination, tumor cell invasion can employ a range of interconvertible migration strategies, including mesenchymal or amoeboid single migration or collective movements [1-4]. Mesenchymal migration, characterized by fibroblast-like elongation and spindle-shaped morphologies, depends upon integrin-mediated cell-matrix interactions and proteolytic remodeling of the extracellular matrix (ECM), whereby cell movement and matrix remodeling form an integrated program [5, 6]. Amoeboid migration primarily serves cell position change without tissue remodeling, whereby cells lack focal adhesions, intracellular multiprotein assemblies connecting integrin adhesion receptors with the actin cytoskeleton, but move by low adhesion force and high cell deformability and maintain largely rounded, ellipsoid morphology [1, 7]. Collective migration occurs when cells retain cell-cell adhesions and multicellular coordination and move as multicellular clusters, sheets or strands [2, 3, 8].

These basic migration programs can adapt and interconvert in response to a range of cell-intrinsic and extracellular triggers and signals, which control cell-cell and cell matrix interactions and the organization of the cytoskeleton [9]. The transition between mesenchymal and amoeboid cell migration modes depend upon the balance between Rac and Rho signaling and signals which control the strength of cell-matrix interactions [9, 10]. Likewise, individual and collective migration modes are interconvertible, when junctions between cells dissociate or reform again [11, 12]. Consequently, based on engaged cell adhesion and signaling programs, most solid tumor cells use both, collective as well as single cell migration mechanisms in an adaptive manner, as suggested by human pathology and intravital imaging studies [13].

Growth factors and cytokines released by the tumor microenvironment and tumor cells themselves represent central extracellular triggers which control these migration programs and thus regulate adaptive invasion strategies. They act in an autocrine, paracrine or endocrine fashion to orchestrate central cell functions, which besides cell migration further include proliferation, differentiation and apoptosis [14]. Growth factor and cytokine receptors, such as the epidermal growth factor (EGF) receptor, are found to be upregulated in invasive compared to non-invasive tumors, often at the invasive borders of tumors, indicating a link between growth factor signaling and tumor cell invasion [15, 16]. In response to receptor activation, multiple signaling pathways are engaged in a time- and space-controlled manner, including phosphoinositide 3-kinase (PI3K)/ Akt, Mitogen-activated protein kinases (MAPK), Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT), Smad and small RhoGTPase signaling (Figure 1), among others, which cooperate and jointly define the response type, including cell migration [17, 18]. Cytokines and growth factors regulate the function of both tumor cells and non-transformed stromal cells, and induce integrated responses in both the tumor and surrounding tissue. Their function in the induction of proliferation, survival and cell migration is well established, including directionality,



Figure 1 Signaling pathways involved in migration control. Growth factors and cytokines bind to either G protein-coupled receptors (GPCRs), cytokine receptors or receptor tyrosine kinases (RTKs) which can interact with integrins or cadherins. Subsequently, one or more signaling pathways are induced, driving integrated cell functions, including survival and proliferation in addition to migration regulation by influencing cell-matrix and cell-cell adhesion, cytoskeletal dynamics and the extracellular matrix (ECM) structure. Color codes denote downstream pathways that preferentially associate with migration or proliferation and/or survival.

efficiency, and the crossing of tissue boundaries for metastatic evasion [15, 19], yet the multiple roles of growth factors and cytokines in controlling tumor cell adhesion and cytoskeletal kinetics as well as the multiple roles in defining cellular programs to secrete and remodel extracellular matrix generates complex changes in both the tumor cell and the tumor stroma. Here, we review by which mechanisms growth factors and cytokines induce and direct tumor cell migration and discuss how they cooperate to induce plasticity and adaptation of cancer cell invasion and metastatic dissemination.

GROWTH FACTOR AND CYTOKINE REGULATION OF CELL ADHESION

Growth factor and cytokine induced signals modulate cell-matrix and cell-cell adhesion and thereby control substrate specificity, cell polarity and differentiation as well as the ability to migrate.

Cell-matrix adhesion

During adhesive cell migration integrins are the major adhesion receptors which mediate dynamic interactions between the cell and the tissue and determine the binding specificity to diverse ECM components [20, 21]. Cell-matrix adhesions mature by integrin clustering and focal adhesion formation at the leading edge of moving cells and, essential for migration, become dissembled at the rear of the cell [21]. As rule of thumb, optimal cell migration rates are achieved at intermediate integrin adhesion level and require balanced formation and resolution of adhesions. The underlying integrin dynamics depend upon activation, clustering and recycling which are tightly regulated processes. Upon activation, integrins deliver information on cell location, environment and adhesive state through signaling through focal adhesion kinase (FAK) and Src-family kinases followed by engagement of RhoGTPases, Akt and ERK, and further mediate mechanocoupling and signaling underlying cell responses, such as migration, proliferation, survival or differentiation (Figure 1) [22, 23].

Through signaling cross-talk, integrins support growth factor signaling pathways, via FAK, MAPK and Akt, but can also negatively influence growth factor receptor signaling [24]. In turn, growth factors and cytokines regulate integrin adhesiveness and substrate specificity in migrating tumor cells by multiple mechanisms, including integrin expression regulation, integrin activation and focal adhesion turn-over (Figure 2A, B).

Integrin expression regulation

Several growth factors or cytokines control integrin expression and basic adhesion functions, often as part of sustained cell activation and differentiation programs. Stromal cell-derived factor 1 (SDF-1) induces the upregulation of integrins, including $\alpha\nu\beta3$, $\alpha\nu\beta6$ and $\alpha5\beta1$, which enhances adhesiveness and invasion *in vitro* as well as *in vivo* in different tumor models [25-27]. Similarly, transforming growth factor $\beta3$ (TGF- $\beta3$) induces $\beta3$ integrin expression in breast cancer cells which enhances both, collective and single-cell migration on a collagen type I matrix [28]. By directing the substrate recognition repertoire, growth factors and cytokines control the compatibility between cell and tissue environment.

Integrin activation and adhesion

Besides ligand binding, integrins are activated by inside-out signaling pathways including PI3K, diacylglycerol and phospholipase C, which underlie growth factors and cytokines signaling control via the integrin cytoplasmic tails *[29]*. In pancreatic carcinoma cells, EGF induces Src-dependent phosphorylation of the intracellular scaffold protein p130Cas which, via Rap1, activates $\alpha\nu\beta5$ integrin [30]. Engaging this pathway supports invasion on vitronectin and further enhances metastasis *in vivo* [30, 31]. TGF- β stimulation causes Smad-2 and Smad-3 activation, as well as phosphorylation of $\beta1$ integrin which induces invasion in hepatocellular carcinoma (HCC) cells into the stroma and blood vessels in a chicken embryo model [32]. Vascular endothelial growth



Figure 2 Growth factor and cytokine control in cell migration. (**A**) Overview image indicating collective and single cell migration of tumor cells in an extracellular-matrix rich microenvironment including stromal cells. (**B**) Regulation of cell-matrix adhesion, including ① focal adhesion assembly and disassembly, ② recycling of integrins, ③ activation of integrins via inside-out signaling, and ④ expression of integrins. (**C**) Cell-cell adhesion regulation through ① cadherin-associated catenins, ② cadherin endocytosis and degradation, ③ proteolytic cleavage of cadherins by proteases, and ④ expression of cadherins. (**D**) Organization of the cytoskeleton, by ① cell polarization and extension through actin polymerization leading to filopodia/lamellipodia or bleb formation, ② actomyosin contraction, ③ microtubule organization, extension and organization by tumor cells through ① secretion and ② expression of mainly growth factors and cytokines activating stromal cells and matrix degrading proteases. Regulation by stromal cells through ③ secretion and ④ expression of mainly growth factors and cytokines guiding tumor cell migration by haptotaxis/chemotaxis, extracellular matrix proteins, matrix cross-linking enzymes and protease inhibiting proteins leading to remodeling, alignment and stiffening of the ECM.

factor (VEGF), through VEGF receptor 2 signaling, activates the PI3K-Akt pathway, co-engages $\alpha\nu\beta3$, $\alpha5\beta1$, $\alpha2\beta1$ and $\alpha\nu\beta5$ integrins for ligand binding of endothelial and melanoma cells, and thereby enhances both adhesion and migration [33]. Similarly, VEGF signaling through neutrophilin 2 involves protein kinase C (PKC) activation and enhances $\alpha6\beta1$ binding to laminin, which supports the elongation and migration of breast carcinoma cells [34]. Through phosphorylation and dephosphorylation of effector proteins, most integrin activation programs are fast, adaptive and support acceleration and deceleration of migration speed.

Focal adhesion regulation

Interconnected with inside-out signaling, the formation and turnover of focal adhesions are effectively regulated by growth factor and cytokine signaling, resulting in integrin adhesion regulation. TGF- β , insulin-like growth factor 1 (IGF-1), EGF, VEGF-C and hepatocyte growth factor (HGF) all induce actin reorganization and phosphorylation of focal adhesion proteins including paxillin, cortactin, FAK and p130Cas in different tumor cell types, which coincides with cell spreading, turnover of focal adhesions, and migration [35-40]. The regulation of focal adhesions is, however, not a prerequisite for inducing effective cell migration. In prostate cancer cells, HGF induces migration with only little effect on focal adhesions [41]. In highly adhesive and thus immobilized cells, growth factor signaling and migration induction even coincide with the lowering of cell-matrix binding. EGF signaling leads to transient downregulation of FAK activity which weakens attachment to the matrix and favors cell motility in different carcinoma types [41, 42]. Thus, by regulating focal adhesion assembly and disassembly, growth factors and cytokines can "tune" cell adhesion strength and dynamics.

Subcellular integrin distribution and recycling

Endocytic recycling of integrins regulates the level of integrins available at the cell surface, which underlies complex control mechanisms, including growth factor and cytokine signaling. Plateletderived growth factor (PDGF) receptor signaling through protein kinase D induces a short-loop recycling of $\alpha v\beta 3$ integrin to the leading edge of breast cancer cells, which in turn inhibits $\alpha 5\beta 1$ integrin recycling to the cell surface [43]. This promotes $\alpha v\beta 3$ -dependent cell invasion into multiligand ECM [43]. Likewise, SDF-1 α activates diacylglycerol kinase α which is required for $\beta 1$ integrin delivery to cell protrusions, and supports the spreading and invasion of breast cancer cells [44]. As part of a multi-step program, IGF-I stimulation disrupts molecular complexes consisting of αv integrin, IGF-I receptor and E-cadherin at cell-cell contacts, which allows αv integrin localization to substrate binding sites and partly regulates cell-cell contact adhesion, which enables invasion of colon cancer cells in a 3D collagen model [45].

Thus, by regulating the expression and localization kinetics of ECM-binding adhesion receptors, growth factors or cytokine signaling regulates cell adhesion and thereby migration of predominantly individual tumor cells.

Cell-cell adhesion

Cell-cell adhesions are critical for cell-cell cooperation, multicellular polarity, tissue homeostasis, as well as collective cell movements [46, 47]. In epithelial tumor cells, cell-cell cooperation is mainly mediated by adherens junctions consisting of cadherin-cadherin interactions which are connected to actin and microtubule filaments [48, 49]. Adherens junctions are controlled by at least four complementary mechanisms: cadherin expression, altered function of the adapter proteins p120-catenin and α/β -catenin which connect cadherins to the actin cytoskeleton and control junction stability [50, 51]; cadherin delivery and turn-over at the plasma membrane; and proteolytic processing of cadherins by extracellular and intracellular proteases [52]. Each of these regulation mechanisms underlies control by growth factors or cytokines (Figure 2A, C).

Cadherin expression regulation

E-cadherin is highly expressed in most epithelia. Signaling pathways which induce transcription factors which repress E-cadherin expression directly or indirectly, including Snail, Slug, zinc finger E-box-binding homeobox (ZEB) and Twist, can modulate E-cadherin levels and accordingly function of the epithelium [53]. Many growth factors and cytokines induce upstream control of E-cadherin expression, including Akt, MAPK pathways, Smad, Notch, Wnt, hypoxia-inducible factor 1α and STAT3, and thereby regulate cell-cell adhesion [54]. EGF induces Snail, which downregulates E-cadherin in cell-cell junctions and favors mobility induction and cell detachment in different epithelial cancer cell types [55]. Likewise, HGF or C-X-C motif chemokine 5 (CXCL5) induce Snail and thereby lead to cell scattering and migration in HCC and breast cancer cells [56, 57]. Possibly as consequence of escape of detaching cells from the primary site, growth factorinduced repression of E-cadherin is positively associated with metastasis formation in vivo [57-59]. Besides downregulating E-cadherin, growth factors, including bone morphogenetic protein 4 (BMP4), TGF- β or EGF, also upregulate N-cadherin and other cadherins [60-62], which, likely by initiating pro-migratory signaling together with flexiblization of cell-cell adhesion, supports collective migration [63, 64]. The expression regulation of cell-cell adhesion proteins thus can lead to long-lasting reprogramming of cell-cell assemblies and their dynamics.

Intracelluar cadherin degradation

Cadherin levels at the plasma membrane are regulated by phosphorylation of the cytoplasmic tail, which leads to endocytosis of the cadherin complex and reduced cell-cell adhesion [65]. Growth factor receptors can directly interact with and phosphorylate cadherins, followed by internalization within minutes to hours [65], and further may engage Src. EGF and TGF- β signaling activate Cdc42-interacting protein 4 which controls Src and jointly induce E-cadherin endocytosis, followed by motility induction, cell scattering and invasion into 3D matrices and *in vivo* of breast cancer cells [66]. Similarly, HGF induces E-cadherin degradation through Src engagement in breast cancer [67] or canine kidney cells [68]. With cadherin internalization, its adaptors β -catenin

and p120 become released from the cell adhesion complex and activate pro-migratory signaling pathways, including NFKB and the small GTPases RhoA, Rac1 and Cdc42 [69, 70]. Thereby, cadherin downregulation and degradation are canonical routes towards promigratory signaling, which converts a cohesive into dynamic epithelium or detaching and individually migrating cells [55, 71].

Proteolytic processing

Growth factors and cytokines regulate the expression and activation of extracellular proteases which cleave cadherins and thereby control intercellular adhesion strength [72]. EGF increases the release of matrix metalloproteinase (MMP) 9 which cleaves E-cadherin in head and neck squamous cell carcinoma (HNSCC) cells promoting scattering and migration [73]. Similarly, HGF induces the upregulation of MMP7 which cleaves E-cadherin and disrupts the E-cadherin/ β -catenin complex in prostate and gastric cancer cells [74, 75]. By regulating both autocrine and paracrine release of proteases, growth factors and cytokines thus modulate cell-cell adhesions on tumor cells as well as neighboring cells of the tumor stroma [72, 76].

Regulation of catenins

The stability of surface cadherins is controlled by catenins which define cadherin surface positioning and connection to the actin cytoskeleton. Cadherin-associated catenins are, in turn, regulated by growth factor signaling. For example, TGF- β 1 induces PI3K-dependent tyrosine phosphorylation of β -catenin which causes dissociation of the E-cadherin/catenin complex, reduces cell-cell adhesion and induces migration of pancreatic carcinoma cells [77]. HGF and tumor necrosis factor α (TNF α) signaling stabilize β -catenin in the cytoplasm or nucleus, which weakens E-cadherin complexes at the plasma membrane and cell-cell cohesion, and supports cell migration [78, 79]. If cadherin-based junctions are weakened, but not fully dissolved, growth factor or cytokine signaling supports collective migration. In HNSCC cells EGF-induces predominantly collective migration which requires intact adherens junctions and the stabilizing function of p120 catenin [80]. Thus, growth factors and cytokines differentially regulate cell-cell adhesion stability and control the mode of cell migration in a cell-type dependent manner.

Modulation of other adhesion systems

Besides adherens junctions, growth factors and cytokines promote tumor cell migration by regulating other, parallel cell-cell adhesion systems, particularly tight junctions [81]. EGF, via Src and PKC δ signaling, reduces the expression of the tight junction protein occludin in epithelial tumor cell lines [82] and, via MEK/ERK and PI3K/Akt signaling, induces de-localization and degradation of the tight junction proteins claudin-3 and -4 in ovarian cancer cells [83]. Consistent with cadherin regulation, BMP4 causes a deregulation of the epithelial cell polarization by perturbing the distribution of the tight junction protein zona occludens protein-1 in epithelial carcinoma cells and enhances migration into a type I collagen gel [60]. Altered cadherin and tight junction regulation by growth factors and cytokines likely occurs concurrently and synergizes to reduce cell-cell junctions and facilitated single-cell dissemination. In addition, growth factor stimulation may perturb intercellular communication through gap junction [84], with yet unclear consequences for tumor cell migration.

In summary, by impacting the steady-state cadherin functionality in cell-cell adhesion, growth factors and cytokines control whether tumor cells migrate in groups or as single cells.

CYTOSKELETAL DYNAMICS

The polarization and the mechanical force coupling of cancer cells through adhesive cell-cell and cell-matrix interactions are both mediated by the actin cytoskeleton. In single-cell migration, the cytoskeleton undergoes a cyclic four-step process consisting of (1) protrusion of the leading edge, (2) adhesive cytoskeletal anchorage to substrate via adhesion sites, (3) actomyosin contraction along the cell body and (4) retraction of the cell rear [85]. For force generation, cytoskeletal systems cooperate with adhesion systems to define the type, strength and duration of cell interaction with the ECM, cell polarity and migration. The small GTPases Cdc42 and Rac induce actin polymerization to form cell protrusions, including lamellipodia and filopodia, which push the leading edge forward. Actomyosin contraction, under the control of the small GTPase RhoA, mediates focal adhesion maturation and cell contraction. Microtubules maintain cell polarity, regulate vesicle trafficking and delivery of function proteins to cell-matrix interaction sites, and thereby support adhesion regulation and cell migration [86, 87]. Intermediate filaments including cytokeratins and vimentin, regulate cell shape, cell-cell adhesion via desmosomes, polarity and cell protrusion [88, 89]. Extracellular signals including growth factor and cytokine receptor pathways, through PI3K/Akt, MAPK and guanine nucleotide exchange factors regulating the small Rho GTPases Cdc42, Rac1 and RhoA, direct the dynamics of all three cytoskeletal systems and thereby control cell shape, adhesion and movement (Figure 2A, D).

Cell polarization and leading edge formation

When distributed uniformly in tissues, growth factors and cytokines induce cell polarization and migration non-directionally (chemokinesis); when present as gradients, directional migration is induced (chemotaxis/haptotaxis) [90]. Growth factors and cytokines, including EGF, HGF, TGF- β and TNF ligand, activate the Rho GTPase Cdc42, often in cooperation with Rac, which induces the migration in a range of tumor cell models, including breast, breast adenocarcinoma, glioma, epidermoid and prostate carcinoma cells [91-94]. A subset of growth factors and cytokines further activates RhoA, which favors rounded cell morphology and amoeboid migration in tumor cells, with blebs and small membrane protrusions at the cell front [9]. Examples include TGF- β , HGF and interleukin 6 (IL-6) stimulation in melanoma or breast cancer cells [95-97]. Thus, by regulat-

ing the Rac/Rho balance, growth factors and cytokines determine the type of cell protrusion in migrating tumor cells.

Actomyosin contraction

Cell contractility is regulated by cytokines and growth factors, mainly through regulating the activity of RhoA and the myosin light chain (MLC), which control myosin II to intercalate between and contract actin filaments (Fig. 2C). EGF and HGF activate RhoA through the GTPase ADP-ribosylation factor 1 or the tyrosine kinases AbI and Arg, respectively, followed by enhanced contractility and migration of individual breast cancer cells [98-100]. In cell groups, increasing actomyosin contractility can generate tangential forces and support the dissolution of cell-cell junctions. Accordingly, the scattering of single cells can be induced by TGF- β signalling, by activating MLC phosphorylation and actomyosin contractility alongside with E-cadherin endocytosis in breast cancer cells [66] or by HGF in non-transformed epithelial cells [101].

Microtubule organization and dynamics

Microtubule dynamics underlie the control of RhoGTPases and PI3K/Akt/GSK-3 pathways and upstream regulation by growth factor or cytokine [86]. Growth factors and cytokines further regulate motor proteins required for microtubule-dependent intracellular transport of cargo in vesicles. EGF and HGF control microtubule extension and protein and membrane trafficking along microtubules by kinesins, which supports cell protrusion, adhesion and migration in breast and gastric carcinoma cells [102-106]; TGF-β receptor triggering activates, via Smad7, p38 and GSK-3β signaling, the recruitment of Adenomatous Polyposis Coli to the microtubule in the leading edge, which stabilizes microtubules and enhances polarization and migration in prostate cancer cells [107]. IL-6 induces STAT3 which regulates microtubule stability and stimulates migration and invasion in gastric cancer cells [105]. Thus, although most cells can still migrate when microtubule functions are impaired, microtubule regulation by growth factors and cytokines contributes to an efficient migration cycle.

Expression and function regulation of intermediate filaments

Most intermediate filaments are deregulated in tumor cells, which promotes or inhibits migration [88, 89, 108]. Growth factors and cytokines can influence intermediate filament expression or function. Vimentin, predominantly expressed by mesenchymal cells, is induced by a range of growth factors and cytokines, including TGF- β , EGF, HGF, C-C motif chemokine ligand 20 (CCL20) and TNF- α , in many epithelial cancers, as part of the epithelial-to-mesenchymal transition (EMT) program [73, 109-112]. Vimentin upregulation increases migration in non-transformed epithelial as well as cancer cells, including head and neck, breast, lung, liver and pancreas carcinoma cells, by multiple functions, including cell protrusion formation, destabilization of desmosomes and accelerated focal adhesion dynamics [88].

Unlike vimentin, the link between keratins and tumor cell migration is less well characterized. Growth factors or downstream effectors, including Akt or ERK signaling, regulate the expression and network dynamics of keratins, which is positively associated with enhanced cell migration [113-116]. Growth factors and cytokines thus increase intermediate filament dynamics, either as cause or consequence of enhanced migration.

ECM/MICROENVIRONMENT ARCHITECTURE

In progressing tumor lesions, the stroma undergoes significant structural and molecular remodeling, which leads to increased deposition of ECM molecules as well as tissue compaction with stronger and longer ECM fibers and alignment parallel or perpendicular to the tumor border [117-119]. The ECM strongly impacts the migration type and efficiency by its dual function to (1) provide structural framework for resident and passenger cells and (2) by binding and immobilizing soluble factors which act as reservoir and information cue. Tumor cells sense both, the mechanical and molecular properties of the ECM, by simultaneously integrating adhesion and growth factor receptor signaling. Accordingly, cell migration can be guided by stiffness gradients, with migration towards more stiffer substrate (durotaxis) [120], towards the gradient of an immobilized ligand (haptotaxis), or towards soluble ligand (chemotaxis) [90]. Growth factors and cytokines display multiple, direct and indirect effects on the tissue organization and tissue-derived signaling with important consequences for the movement and function of tumor cells (Figure 2A, D).

ECM synthesis and remodeling

The remodeling of ECM is performed by stromal fibroblasts and other stromal cells which become stimulated by growth factors and cytokines, including EGF, FGF, IL-1 α , PDGF and TGF- β [121]. Upon stimulation with TGF- β or FGF fibroblasts downregulate secretion of matrix degrading proteinases and up-regulate tissue inhibitors of metalloproteinases to inhibit the collagen degradation function of extracellular MMPs, which induces collagen accumulation [122-124]. Growth factor signaling further impacts enzymatic cross-linking of collagen fibers with increased ECM stiffness as outcome [76, 117, 125, 126]. TGF- β is a strong inducer of lysyl oxidase in malignant mammary epithelial cells which cross-links collagen and mediates tumor cell invasion and proliferation [127]. The increased extracellular confinement and higher stiffness of the tumor stroma promotes integrin engagement and elongated movement in different tumor cell models, consistent with mesenchymal features [9, 128]. By guiding cells along confined space, aligned and bundled ECM structures further supports multicellular streaming along and cell jamming within the track, with conversion to collective cell migration [9, 129-131].

ECM degradation

Proteolytic degradation of ECM is an important mechanism for invading cells to widen tissue space. Whereas cell migration in interstitial connective tissue imposes little mechanical barriers to

moving cells, passage through dense tissue and particularly the basement membrane is facilitated by proteolytic degradation of ECM macromolecules [132-134]. A wide range of growth factors and cytokines regulates ECM-degrading enzyme systems, including MMPs, serine proteases and cathepsins [135, 136]. TGF- β , EGF, HGF, TNF- α and IL-1 induce or increase the expression of MT-1 MMP, MMP1, MMP2, MMP9 and the serine protease uPA, all of which support tumor cell migration in 3D models and *in vivo* [28, 137-142]. Besides cleaving ECM proteins directly, degrading enzymes can release growth factors and cytokines which are bound to the ECM and thereby increase their biological function [143].

Thus, growth factors and cytokines have a dual function in controlling ECM composition and organization, by controlling cell activation and ECM deposition, and by directly binding to the ECM scaffold and providing topologic guidance for migrating cancer and stromal cells.

PLASTICITY OF CANCER CELL MIGRATION IN RESPONSE TO GROWTH FACTOR AND CYTOKINE SIGNALING

By simultaneously controlling multiple components of the cell adhesion and cytoskeletal machinery as well as the extracellular structure and composition, growth factors and cytokines activate and modulate tumor cell migration programs in an overarching, integrated manner. Besides the routes and efficiency, they also modulate strategies by which cells invade. Growth factor and cytokine stimulation which weakens cell-cell adhesion favors single cell dissemination over collective invasion; increasing cell-matrix adhesion or ECM ligand density as well as stiffness promotes mesenchymal movement; and lowering cell-ECM adhesion and activating RhoA rather than Rac signaling favors amoeboid migration strategies (Figure 3) [9, 144]. Because each migration strategy conveys particular "abilities" for cells to infiltrate tissue and cross tissue barriers, this induced plasticity response also impacts metastatic efficacy and prognosis.



Figure 3 Growth factor- and cytokine-induced plasticity in tumor cell migration. Growth factors and cytokines have primary effects on tumor and stroma cells influencing cell-matrix and cell-cell adhesion, cytoskeletal dynamics and extracellular matrix (ECM) composition and structure. This leads, depending on cell type and environmental context, to a plastic response in tumor cell migration of collective, mesenchymal or amoeboid migration.

Induction of collective or single-cell migration

Growth factors and cytokines may induce or enhance cell movement, either as collective or single-cell migration modes, which depends upon the cell type and environmental context. HGF enhances collective migration in normal kidney epithelial cells and mammary adenocarcinoma, but stimulates cell detachment and mesenchymal migration in HCC [111, 145, 146]. Similarly, SDF-1a increases E-cadherin stability in cell-cell contacts and supports collective migration of colon cancer cells [147], whereas cells single-cell migration is enhanced in cholangiocarcinoma [148].

Epithelial-to-mesenchymal transition (EMT)

EMT is a molecular program which can be initiated by growth factors or cytokines through pathways including PI3K/Akt/GSK-3β, MAPK, Smad and JAK/STAT and leads to a fundamental reprogramming of epithelial cells [149]. EMT commonly leads to increased expression of transcription factors such as Snail, Slug, Twist or ZEB which repress E-cadherin and support expression of N-cadherin and vimentin [150]. As a consequence, cells which undergo EMT downregulate their cell-cell contacts and increase their capacity for migration and 3D invasion. The strongest evidence for the contribution of EMT in metastasis formation is the ability of multiple EMT regulators to enhance tumor cell migration, together with survival programs [54, 151]. Depending on its strength and duration EMT may induce a spectrum of molecular events, including complete EMT with entire loss of epithelial features and incomplete EMT which yields in a hybrid state with epithelial and mesenchymal features [152]. With induction of complete EMT, cells typically individualize and acquire elongated, spindle-like shapes [152]. However, EMT can result in a spectrum of migration modes, including single cell and collective migration, sometimes in the same cell model indicating a heterogeneous behavior within the same cell population [153, 154]. Moreover, there is no direct correlation between E-cadherin levels and migration mode in response to growth factor or cytokine stimulation. TGF- β reduces expression levels and cell surface localization of E-cadherin, without inducing N-cadherin expression, and enhances collective sheet migration in colon cancer cells [147]. Similarly, TGF- β treatment although inducing complete EMT in murine breast cancer cells, induces a range of migration programs, including collective sheet migration and single cell migration on 2D surfaces and near-exclusive collective invasion into 3D reconstituted basement membrane [155]. Thus, growth factor or cytokine-induced EMT enhances both single-cell and collective migration programs in a context dependent manner.

Collective-to-single cell transition

Little is known whether and how growth factors and cytokines induce collective-to-single-cell dissemination by EMT-independent mechanisms. HGF, by increasing integrin-mediated adhesion without regulating E-cadherin, can disrupt cell-cell adhesion and induce scattering in non-transformed MDCK cells [101]. *In vivo*, TGF- β 1, via Smad4 and Rho/ROCK signaling, induces a

switch from collective to single-cell motility in breast cancer cells [156], with unclear contribution of EMT in this transition. Similarly, TGF- β activates Smad2 and its adaptor CITED1 and promotes detachment of amoeboid-migrating melanoma cells [97]. Likely, similar to EMT, EMT-independent mechanisms induce a spectrum of cancer cell invasion modes defined by the spectrum and magnitude of growth factor and cytokine signaling.

Mesenchymal-to-amoeboid and amoeboid-to-mesenchymal transition (MAT, AMT)

In individually moving tumor cells, growth factors and cytokines can promote mesenchymal or amoeboid migration, and likely transitions between both modes. SDF-1 α activates RhoA and mDia2 which induce the re-organization of filamentous actin towards bleb formation and migration in mesenchymal breast cancer cells [157]. Similarly, IL-6 receptor signaling engages JAK/Stat, Rho signaling and actomyosin contraction which promote a preferentially rounded, amoeboidmoving phenotype in melanoma cells in 3D collagen and *in vivo* [96]. To which extent such single-cell plasticity supports or counteracts tumor cell transition between tissues and metastatic evasion remains to be clarified.

Synergistic and negative effects on cell migration and metastasis formation

Many growth factor and cytokine receptors activate similar, overlapping signaling pathways, or induce cross-talk between pathways to synergize or antagonize their respective downstream signaling. Consequently, growth factors and cytokines can induce synergistic or antagonistic effects in the regulation of cell-cell and cell-matrix interaction, actomyosin contractility and resulting migration in tumor cells. In ovarian cancer cells HGF-induced migration requires Akt and ERK1/2 pathways while EGF-induced migration depends on Akt and p38 pathways [158]; when applied simultaneously, HGF and EGF synergize and enhance invasion through reconstituted basement membrane [158]. IL-6 and EGF receptor signaling both occur via PI3K and MAPK pathways and synergize to induce migration of breast cancer cells [159]. EGF and TNF-a promote respective ERK or Smad signaling pathways and jointly enhance the efficacy of TGF-B in inducing EMT and migration of lung or pancreatic cancer cells [160, 161]. TGF- β and downstream Smad signaling induces the expression of PDGF which in an autocrine manner activates the PI3K/Akt pathway and synergistically enhances an EMT-like phenotype in melanoma cells [162]. Such cooperation not only may enhance the efficacy of an individual growth factor in a given environment; it further represents a likely mechanism of compensation upon targeted therapy against individual receptor systems.

Besides synergism, growth factors and cytokines can also counteract and suppress otherwise ongoing tumor cell migration and metastasis formation in certain experimental models. In HNSCC, CXCL14 strongly activates Rac1 and supports focal adhesion formation and adhesion to collagen, which stabilizes cell adhesion followed by cell immobilization and impaired migration [163]. IL-27 limits EMT, either by antagonizing PGE2 signaling and suppressing vimentin

expression, or by promoting STAT1 pathways, which ultimately maintains epithelial differentiation and reduces the migration of non-small cell lung cancer cells [164, 165]. Furthermore, although TGF-β signaling enhances collective and single cell migration of murine mammary carcinoma cells, interference with the TGF-β receptor, counterintuitively, promotes metastasis formation in chick embryos [154]. In mammary tumor cells, EGF modulates chemotactic migration towards an SDF-1α gradient and either enhances or decreases migration [166, 167], possibly by dosedependent chemokine and EGFR cross-desensitization. Similarly, interferon- γ inhibits TGF-β induced invasion via Smad7, which interferes with R-Smad signaling in a 3D gastric carcinoma model or breast cancer cells [168, 169]. Thus, the effect of growth factors and cytokines on cell migration is context-dependent, resulting in both induction or inhibition and diversity of the cell migration response. Consequently, given the plethora of mechanical and chemical signals present in the tumor microenvironment *in vivo*, growth factors and cytokines likely provide complex orchestration of signaling networks to induce plasticity as well as unexpected outcome in tumor cell migration and metastasis models.

The stimulatory effects of growth factors and cytokines are typically cell-type specific, with varying sensitivity and response profile in dependence of the cell type and tumor model. In lung adenocarcinoma and pancreatic cancer cells, high-dose EGF and TNF- α stimulation fails to induce EMT, whereas TGF- β treatment leads to EMT and enhances migration across 2D substrate [102]. Upon direct comparison of different growth factors, TGF- β is superior and sufficient in inducing complete EMT, including induction of E-cadherin transcription repressors, N-cadherin, vimentin and fibronectin, and enhanced migration in 2D and 3D in epithelial mouse breast cancer cells *in vitro*, while EGF, IGF-1, HGF, FGF, PDGF and IL-6 cause no or partial EMT in the same cells [155]. Conversely, in a panel of HNSCC cell lines TGF- β stimulation and downstream Smad signaling elicit variable levels of EMT ranging from downregulation of E-cadherin and Cytokeratin 18 and upregulation of N-cadherin and vimentin to complete unresponsiveness or even up-regulation of epithelial markers [39]. Such variability likely reflects the intra- and interindividual differences of genetic and epigenetic programs in tumor cells, and contribute to tumor heterogeneity and molecular adaptation programs.

DISCUSSION

Cancer therapies involving inhibition of growth factor or cytokine receptor signaling often show transient efficacy in reducing tumor growth and circulating tumor cell counts, indicating the importance of growth factors and cytokines in tumor progression and, arguably, metastatic progression in patients [170-172]. However, since longitudinal histology from serial biopsies and circulating tumor cell analysis are often missing in clinical trials, the migratory mechanisms underlying the clinical efficacy of growth factor receptor targeting, such as altered tumor cell migration or intra- and extravasation into the blood vasculature, are poorly resolved to date. Furthermore, even though preclinical studies indicate that growth factors can promote migration
and metastasis without influencing primary tumor growth, the relative contribution of tumor cell migration and/or survival programs in response to growth factor signaling remains unknown [30, 31, 173]. Thus, the translation from in vitro studies in defined environments to clinical end-points requires intermediate analysis by mechanistic preclinical studies, such as intravital microscopy in small animal models to monitor every step of the metastatic cascade before and after targeted therapy [174-177]. In addition, since targeting single growth factors or cytokines has been proven insufficient in therapy, a more global approach, such as the use of antibody arrays and (phospho-) proteomic analysis of invasive tumor regions will allow to identify the composition of growth factors and cytokines in the tumor microenvironment and to delineate signaling cross-talk. To this end, mathematical modeling will support multi-parametric integration of complex signaling and effector regulation in tumor biology. This will allow to combine varying degrees of growth factor signaling with the induction of plasticity of tumor cell migration in different tissue contexts to model both deterministic and probabilistic effects of invasion programs [178-181]. Thus, generating an integrated approach of cell-based in vitro analysis, preclinical mouse models, clinical outcome studies and mathematical modeling will enable the dissection of plasticity of both invasion/dissemination and survival programs in response to growth factors and cytokines, and outcome after therapeutic targeting.

ACKNOWLEDGEMENTS

We thank Mirjam Zegers for critical reading and helpful suggestions on the manuscript. We gratefully acknowledge financial support by the European Research Council (617430-DEEP-INSIGHT), NWO-Vici (918.11.626), Horizon 2020 consortium MULTIMOT (634107-2), and the Cancer Genomics Center.

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CHAPTER 3

Growth factor-induced plasticity of invasion induces EMT with amoeboid traits

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Manuscript in preparation

Metastatic cancer progression is mediated by adaptive collective, mesenchymal and amoeboid invasion programs as well as molecular plasticity, including the epithelial-to-mesenchymal transition (EMT). However which cancer invasion strategies associate with EMT, and which not, remains poorly defined. During 3D collagen-based coculture with fibroblasts or exposure to fibroblastconditioned medium stimulating the HGF/c-Met axis, head-and-neck squamous cell carcinoma (HNSCC) spheroids developed collective invasion patterns connected by E-cadherin-positive cell-cell junctions which transited to single-cell dissemination with elongated, mesenchymal-like (<30% of detached cells) or roundish-amoeboid (>70%) movement, downregulated E-cadherin and notable collagenolytic migration track generation. In a cell-line dependent manner, both collective and individual invasion zones expressed the EMT-marker vimentin with approximately 20% (SCC38) up to 80% (SCC58) frequency, detected by 3D single-cell cytometry. Vimentinpositive and -negative cells were competent for collective or amoeboid migration. Neutralizing anti-HGF antibody or pharmacological c-Met inhibitor SU11274 eliminated single cell dissemination and reduced collective invasion, indicating differential sensitivity to targeted intervention. In summary, HGF induces EMT together with collective, proteolytic-amoeboid and, less frequently, mesenchymal invasion, indicating EMT as a probabilistic molecular program associated with any invasion strategy.

INTRODUCTION

The invasion of cancer cells into the adjacent tissue initiates metastasis and thereby contributes to increased mortality of cancer patients. Invading cancer cells apply cytoskeletal activity towards extracellular matrix (ECM) and adapt their migration strategies to accommodate local signaling and tissue signatures, either as single cells without cell-cell interactions to neighboring cells [1] or as multicellular groups with cell-cell junctions and cytoskeletal coordination between neighboring cells retained [2]. Both single-cell and collective invasion modes can interconvert in response to cell-intrinsic or microenvironmental programs [3]. Weakening of cell-cell junctions allows cells to detach from a cell group and migrate individually [4-6]. Modulating cell adhesion and protease activity can support interconversions between single-cell migration modes. Mesenchymal movement, characterized by elongated cell morphologies, strong cell-matrix interactions and proteolytic ECM remodeling, can switch to amoeboid movement, with roundish yet adaptive cell morphology, low adhesion force and diminished proteolytic ECM processing [7-9]. Whereas collective migration depends upon tissue remodeling to form multi-cellular or epithelial structures, mesenchymal and amoeboid migration are considered to enable efficient cancer-cell spread throughout interstitial tissues [4, 8, 10]. Whereas the basic mechanisms underlying individual and collective cell migration modes have been established in vitro and in vivo [11, 12], the microenvironmental stimuli prompting switching of migration modes and their relation to molecular plasticity of cancer disease, including adhesion and cytoskeletal plasticity and the epithelial-tomesenchymal transition (EMT), remain poorly defined [11, 13, 14].

External triggers controlling plasticity of cell migration include ECM topology [15], promigratory factors released by stromal cells [13, 14] and stabilization of HIF-1 α by hypoxia [5]. Activated fibroblasts can induce and guide tumor invasion by remodeling and aligning ECM, and this directs tumor cell migration by providing heterotypic cell-cell contacts and guidance by aligned tissue paths [16-18]. Cytokines and growth factors, including TGF- β , HGF or EGF released by the stroma or tumor cells themselves induces EMT, which destabilizes cell-cell adhesion and causes single-cell dissemination [19]. EMT is a multi-signal molecular program induced by transcription factors snail, slug and/or twist which downregulate E-cadherin dependent adherens junctions, upregulate mesenchymal markers, and stimulate cell migration [20]. Whereas EMT-dependent induction of mesenchymal single-cell invasion is well established [20], a range of EMT-associated other migration modes were predicted by in silico modeling, including collective and amoeboid migration and their interconversion [21, 22], awaiting wet-lab confirmation [23].

Using fibroblast-induced invasion in 3D spheroid models, we here classify the invasion plasticity of epithelial cancer cells from head and neck squamous cell carcinomas (HNSCC) in response to HGF, and identify collective and single cell invasion and their interconversions, together with the induction of EMT at single-cell level. The data identify EMT as overarching program in both collective and single-cell migration modes after stimulation with growth factors, and reveal a previously unappreciated HGF-induced collagenolytic amoeboid migration with EMT signature.

MATERIAL METHODS

Reagents and antibodies

The following antibodies (Ab) and reagents were used for immunofluorescence: monoclonal rat anti-tubulin Ab (Novus Biologicals, YOL 1/34, 1/200), polyclonal rabbit anti-vimentin Ab (Biotrend, rp115, 1/200), polyclonal rabbit Ab directed against the C-terminal cleavage neo-epitope of collagen I (Col3/4, immunoGlobe, 1µg/ml), monoclonal mouse anti-E-cadherin Ab (Zymed, SHE 78-7, 1/100), secondary Alexa Fluor 546- and 647-conjugated pre-absorbed goat anti-mouse or goat anti-rabbit IgG (Invitrogen, 1/200), Alexa Fluor 488-conjugated phalloidin (Invitrogen, 1/200), DAPI (Roche, 1/1000).

For functional live-cell experiments, the following antibodies and reagents were used: monoclonal mouse anti-c-Met Ab (kind gift from Raffaella Albano, University of Turin, DO-24, 5µg/ml), isotypic mouse IgG2a kappa (BioLegend, 1/100), secondary Alexa Fluor 647 goat anti-mouse Ab (Invitrogen, 1/200), propidium iodide (Sigma, 5µg/ml).

The following antibodies were used for immunoblotting: polyclonal rabbit anti-GAPDH Ab (Sigma, G9545, 1/1000), monoclonal rabbit anti-c-Met Ab (Abcam, EP1454Y, 1/2500), monoclonal mouse anti-E-cadherin Ab (BD Biosciences, 36, 1/500), polyclonal chicken anti-vimentin Ab (Abcam, ab24525, 1/100) and secondary IRDye680RD- or 800CW-conjugated goat anti-rabbit, -mouse or –chicken Ab (Licor, 1/10000).

For 3D migration assays, the following reagents were used: epidermal growth factor (EGF, Thermo Fisher Scientific, PHG0311), recombinant human hepatocyte growth factor (HGF; kind gift from Ralph Schwall, Genentech, South San Francisco, CA), monoclonal mouse anti-HGF neutralizing Ab (R&D systems), c-Met inhibitor SU11274 (Selleck Chemicals), GM6001 (ilomastat, Calbiochem, 5µM) and dimethylsulfoxid (DMSO) for spheroid invasion assay.

Cell culture

Human UT-SCC38 cells and UT-SCC58 cells were cultured in complete growth medium consisting of Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Aldrich), 100 U/ml penicillin (PAA) and 100 ug/ ml streptomycin (PAA), 2 mM L-glutamine (Invitrogen/Life Technologies), 20 mM HEPES buffer solution (Invitrogen/Life Technologies) and 1 mM sodium pyruvate (Invitrogen/Life technologies). MRC5 fibroblasts (ATCC) and cancer associated fibroblasts (CAFs) derived from the UT-SCC38 tumor (CAF553) were cultured in DMEM supplemented with 200 nm L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin and 10% FCS. Conditioned medium (CM) from MCR5 fibroblast culture was obtained 4 and again 8 days after reaching 100% confluency. CM was filtered (0.22 µm pore sized filter, Corning) and stored at -20°C.

3D tumor spheroid culture

Sub-confluent cells were detached using trypsin/EDTA (Life Technologies) and aggregated to multicellular tumor spheroids using the hanging-drop method. Cells were suspended in DMEM supplemented with 10% methylcellulose solution (Sigma) and incubated as 25 μ l droplets overnight (500 cells/drop). After overnight incubation, spheroids were harvested, washed with PBS and full growth medium and embedded non-pepsinized rat tail type I collagen lattices (BD Biosciences, final concentration: 2.5 mg/ml) in 12- or 24-well plates. For coculture experiments, MRC5 or CAF553 fibroblasts were added to the collagen solution (15 000 cells/100 μ l) prior polymerization. Spheroids were allowed to invade for 72 h after incubation with full growth medium, fibroblast CM in different dilution and/or the presence of rhHGF, nAb HGF, EGF, SU11274, DMSO and GM6001.

Immunofluorescence and imaging of spheroids

For routine immunofluorescence analysis of invasion plasticity in collagen-embedded 3D spheroids were fixed in 4% in phosphate buffered paraformaldehyde (PFA) at 37°C for 20 min, washed (PBS) and stained with DAPI and phalloidin (30 min, 20°C). For detection of intracellular proteins, fixed and washed samples were incubated in PBS-buffered 5% normal goat serum (NGS) and 0.3% Triton X-100 (Sigma; 90 min, 20°C). All antibodies were dissolved in PBS containing 0.1% bovine serum albumin (BSA) (Aurion) and 0.3% Triton X-100 (Sigma). Unless stated otherwise, primary antibodies were incubated overnight at 4°C and secondary antibodies for 3 h at RT, followed by rigorous washing (6x 5 min) in excess PBS. Col3/4 staining was performed at 4°C. Confocal fluorescence microscopy for up to four channels was performed by sequential confocal scanning keeping an inter-slice distance of 10 μ m (Olympus FV100) using 10x/0.4 NA, 20x/0.5 NA and 40x/0.8 NA water objectives or, for up to five channels, a Leica TCS SP8 scanner equipped with 10x/0.4 NA air and 40x/0.85 NA water objectives.

Assessment of migration mode

The types of invasion including the number of cohesive strands (>50 µm in length) and number of detached single cells and clusters (>50 µm diameter) was counted after 72 h of invasion by bright-field microscopy using 10x NA 0.20 and 20x NA 0.30 air objectives (Sentech CCD camera). Elongation index (length / width) was measured from time-lapse recordings every hour using Fiji/ImageJ (software version 1.51k, http://fiji.sc/Fiji). Cells with an elongation index <3 were considered "rounded" and cells with an elongation index \geq 3 were considered as "elongated". End-point data on the invasion type of detached and moving single cells as well as MMP inhibition were validated by time-lapse microscopy. Spheroids were grown for 64h with 0, 5 or 50% CM and monitored (20x/0.5) under humidified conditions (Oko-lab stage incubator, Ottaviano, Italy; 5% CO₂, 37°C) [24].

Image quantification and data analysis

The fluorescence intensity of E-cadherin and vimentin associated with different migration types were quantified from 3D samples using single slices or stacks composed of 2-3 slides and normalized to tubulin (Fiji/ImageJ software version 1.51k, http://fiji.sc/Fiji; Suppl. Fig. 5B, 6A). For E-cadherin, regions of interest (ROI) were defined manually to represent the non-invading rim of the spheroid, multicellular invasion strands and detached cells. For detecting vimentin at single-cell level, ROIs were drawn around individual cells.

To measure vimentin intensities over time, round ROIs with an area of 345 pixels (SCC38) and 275 pixels (SCC58) were positioned over nuclei based on DAPI signal. Background values from cell-free areas of the same images were subtracted for each specific measurement and fluores-cence channel. and the ratios of specific signal were calculated. Normalized intensity ratios for vimentin/DAPI (>0.0815 = 10% for SCC38 at time point 0h and >0.1225 = 33% for SCC58 at time point 0h) were defined manually as threshold discriminating between vimentin-positive and cell-associated background values. Unspecific IgG / tubulin ratios were below threshold values.

Reverse transcription and qPCR

Total RNA was extracted from tumour spheroids or fibroblasts grown in 3D organotypic 2.5mg/ ml collagen cultures using the RNEasy mini kit (Qiagen, 74104). About 1µg of this total RNA from each sample was used as the input for cDNA synthesis using the RT² First Strand kit (Qiagen, 330401) and the reverse transcription performed according to manufacturer's recommendations. The cDNA thus obtained was mixed with the RT² SYBR Green qPCR Mastermix (Qiagen, 330503) and robotically loaded on to a 96 well Custom RT² Profiler PCR Array (Qiagen, 330131), followed by qPCR cycling and fluorescence data collection on a Bio-Rad CFX96.

Generation of stable c-Met knockdown cells

To create MET knockdown constructs, shMET1 (GTATGTCCATGCCTTTGAA) and shMET2 (GTAT-GTCCATGCCTTTGAA) oligonucleotide heteroduplexes were ligated in pENTR/U6 vector and subsequently Gateway-cloned into pLenti6/BLOCK-iT-DEST according to the manufacturer's protocols (Invitrogen). Generation of lentiviruses was done in HEK293FT cells as described previously [25]. A control group was transfected with a shscramble-EGFP vector (pLenti6/BLOCK-iT-DEST-EGFP) [26]. Cells were selected based on blastocidin resistance and knockdown efficacy was assessed with flow cytometry up to 4 weeks after transfection. Live cells were stained on ice and viability was verified with propidium iodide. Stained cells were evaluated with a FACSCalibur flow cytometer (BD Bioscience) and the data was analyzed with the FCS Express software (De Novo Software).

Immunoblotting

Western blot analysis of protein expression was performed using spheroids cultured in 2.5 mg/ ml collagen gels for 72 h with 0, 5 or 50% CM or whole cell lysates from cells cultured on 2D.

Collagen gels were washed with PBS before lysis with lysis buffer (100 mM Tris HCl pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, bromphenol blue) supplemented with a protease inhibitor cocktail (Roche). Lysates were boiled at 95°C for 10 min and then loaded on an 8% SDS-PAGE gel and transferred by electroblotting in transfer buffer onto PVDF membranes (Milipore). Antibody complexes were imaged with the Odyssey CLx scanner (Licor).

Reversed phase protein assay

The protocol provided by the MD Anderson Cancer Center for preparation of tumor lysate from frozen tissue was used with following modifications: SCC spheroids were cultured in 2.5 mg/ml collagen gels for 72 h with 0, 5 or 50% CM as described previously. Gels were quickly washed with PBS and immediately shock frozen in liquid nitrogen until further use. Cells were lysed in ice-cold lysis buffer using an electric homogenizer and analyzed at the RPPA facility at the MD Anderson Cancer Center.

Normalized linear data was used for the heatmap generation.

Statistical analysis

Unless stated otherwise, statistical analysis was performed using the non-parametric Mann-Whitney test with a post-hoc correction for multiple comparisons with p \leq 0.05/N indicated by *, p<0.001/N indicated by ** and p<0.0001/N by ***. Normalized ratiometric data were statistically analyzed as log transformed data using a paired T test.

RESULTS

Fibroblast-induced plasticity of HNSCC cell invasion

HNSCC SCC38 and SCC58 cells, embedded as multicellular spheroids in 3D fibrillar collagen under FCS-containing medium conditions, developed only low-level spontaneous invasion without notable single-cell detachment (Fig. 1A and Suppl. Movie 3 and 4). To stimulate invasion, cancer associated fibroblasts derived from the same tumor as SCC38 cells (CAF 553) or MRC5 human lung fibroblasts were seeded in the 3D collagen matrix at the time point of spheroid incorporation, and both fibroblast types enhanced the efficacy of invasion (Fig. 1A, C). In contrast to previous evidence of exclusively collective invasion induced by fibroblasts [17, 18], both CAF 553 and MRC5 fibroblasts induced collective invasion (Fig. 1A, black arrowheads) as well as detaching single-cell migration in SCC38 and SCC58 cells (Fig 1A, white arrowheads; Fig. 1C and Suppl. Movie 1 and 2) as seen in 3D reconstructions of HNSCC patient samples [27]. CAF 553 cells grew poorly after passaging and failed to produce conditioned medium in quantities required to stimulate spheroid cultures, therefore conditioned medium from MRC5 cells was used for further analyses. MRC5-conditioned medium was sufficient to induce collective migration at low concentration and an increasing proportion of detaching cell clusters and single cells at higher concentration (Fig. 1B, C; Suppl. Fig. 1; Movie 3 and 4). SCC38 cells responded to



Figure 1 Induction of plasticity of invasion in HNSCC spheroids by fibroblasts. **A** Overview (left) and zoomed (right) brightfield images showing SCC38 and SCC58 spheroids in rat tail collagen after 72 h of incubation with or without the presence of cancer-associated fibroblasts (CAF) or MRC5 lung fibroblasts. **B** Confocal 3D stack images of spheroids after 72 h culture with increasing concentration of MCR5 fibroblast conditioned medium (CM). Black arrowheads denote cohesive strands, white arrowheads detached single cells, and green arrows cortical actin. **C** Quantification of invasion modes originating from SCC spheroids incubated with MRC5 cells or CM. Data show box whiskers, from at least 12 spheroids from 3 independent experiments. Scale bars, 100 µm (overview) and 20 µm (detail).

50% conditioned medium with near-complete individualization, whereas SCC58 cells retained a larger proportion of collective strands and disseminating clusters, alongside detached single cells (Fig. 1B, C; Suppl. Fig. 1). The spectrum of collective and single-cell invasion in response to low concentrations of conditioned medium (1-5%) recapitulated in both HNSCC models closely the spectrum of invasion modes during co-culture with fibroblasts (Fig. 1C), suggesting that soluble factors released by fibroblasts were sufficient to induce invasion plasticity.

HGF as non-redundant mediator of fibroblast-induced invasion plasticity

A quantitative RT-PCR screen of growth factors, cytokines and their respective receptors expressed by SCC38, CAF553 and MRC5 cells identified candidate migration-inducing cytokines and chemokines, including HGF, EGF and CXCL12, expressed by fibroblasts and their respective receptors c-Met, EGFR and CXCR7 but not CXCR4 in SCC38 cells (Fig. 2A and Suppl. Fig. 2A). This confirms HGF and CXCL12/SDF-1 expression in MRC5 cells or CAFs in HNSCC [28-30] and c-Met and CXCR7 expression in HNSCC cells [31, 32]. Both SCC38 and SCC58 cells expressed c-Met protein (Fig. 2B) and developed a spectrum of collective and single-cell migration types when increasing concentrations of recombinant human HGF, at concentrations reached in body fluids of disease models (up 10-77 ng/ml) [33, 34], were added to the culture (Fig. 2C, D and Suppl. Fig. 3A). Thus HGF recapitulated the response to MRC5-conditioned medium and consistently, downregulation of c-Met protein in SCC38 and SCC58 cells by RNA interference (Suppl. Fig. 2B; 60-80% reduction) was sufficient to compromise the induction of invasion by HGF, by inhibiting collective and fully reverting single cell migration (Fig. 2E, F). These data indicate that soluble HGF, through interaction with c-Met, induces plasticity of invasion. Deregulation of the HGF/c-Met signaling pathway is known to stimulate cancer growth, EMT and metastatic lesions in preclinical models and associates with metastatic progression in cancer patients [35, 36]. To test whether other ligand/receptor systems are able to induce similar invasion plasticity, EGF was added to spheroid cultures. EGF induced collective invasion at low concentrations and both collective and single cell migration at high dose (Suppl. Fig. 3B). Thus, HNSCC cells translate the concentration of extracellular HGF, EGF and fibroblast-conditioned medium by first initiating collective invasion

Figure 2 (next page) HGF as dominant inducer of invasion plasticity. **A** Expression of cytokine and growth factor receptors and their ligands measured by RT-qPCR in SCC38, CAF553 and MRC5 cells after 60 h (SCC38) or 36 h (fibroblasts) of culture in rat tail collagen. The horizontal line indicates the threshold cycle. Data represent the mean values and standard deviation from selected molecules from 3 independent experiments (see full list in Suppl. Information, Fig. 2). **B** Western blot analysis of SCC38 and SCC58 cells, representing 2 independent experiments. **C, D** Brightfield images (C) and quantitative analysis (D) of 3D SCC spheroid culture incubated with CM from MRC5 cells or recombinant human hepatocyte growth factor (rhHGF) for 72 h. Data represent at least 9 spheroids from 3 independent experiments. P values compare invasion patterns induced by CM and rhHGF. **E, F** Brightfield images (E) and quantitative analysis of invasion plasticity of SCC control and c-Met shRNA expressing spheroids after stimulation with 50% CM from MRC5 cells for 72 h. Data originated from at least 9 spheroids from 3 independent experiments. Scale bars indicate 100 µm. Black arrowheads denote cohesive strands, white arrowheads detached single cells.





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Figure 3 (previous page) Induction of vimentin expression and association with migration modes. **A** Confocal stack images of SCC spheroids after 72 h invasion in rat tail collagen. Scale bars indicate 100 µm (overview) and 20 µm (detail). Black arrows denote vimentin negative and white arrows vimentin positive cells. **B** Single-cell 3D cytometry of vimentin expression relative to tubulin (see detailed workflow in suppl. Figure 6). Data originate from at least 6 spheroids from 3 independent experiments. Numbers indicate vimentin positive percentage of cells. **C** Elongation index (length/width) of detached single cells plotted against the vimentin/tubulin expression ratio. Numbers in cell images indicate the elongation index as example and fluorescence colors represent tubulin (red) and nuclei (blue). Horizontal lines separate vimentin positive and negative events, vertical lines rounded from elongated cells. Data represent 20-92 cells from 3 independent experiments.

and, with higher concentrations, causing a switch to single-cell dissemination. Although EGF induces similar plasticity in migration and mRNA was expressed in MRC5 fibroblasts, targeted downregulation of c-Met was sufficient to revert both single-cell and collective invasion indicating that HGF was relevant in mediating fibroblast-induced plasticity.

Fibroblast-derived growth factors induce EMT diversity

We next addressed whether fibroblast-conditioned medium induces EMT in SCC38 and SCC58 cells, similar to HGF and other growth factors in other models [37-39]. The regulation of EMT-associated proteins was analyzed in tumor cell lysates after spheroid culture by Reverse Phase Protein Array (Suppl. Fig. 4A). EMT markers including E-cadherin, N-cadherin, Snail and Twist remained unchanged after stimulation by fibroblast-conditioned medium, whereas phosphorylation of effectors downstream of c-Met was induced, including Akt, B-Raf and Stat3 (Suppl. Fig. 4B). Parallel Western blot analysis of E-cadherin and vimentin indicated a mild but inconsistent protein down- or up-regulation, respectively (Suppl. Fig. 4C). Despite the strong pro-invasive effects in cell subsets, we reasoned that the fraction of invading cells in spheroid cultures may be insufficient to significantly impact protein levels in bulk cultures and therefore performed validation using single-cell analysis of EMT-related proteins *in situ*. In control spheroids E-cadherin was mainly present in cell-cell junctions, however after stimulation with conditioned medium E-cadherin re-localized to the cytoplasm in both collective strands and detached single cells

Figure 4 (next page) Proteolytic movement of rounded cells after detachment from spheroid. **A** Stability of roundish cell shape after detachment. False-color representation of the elongation index over time obtained from time-lapse brightfield movies. **B** Confocal stack images of SCC spheroids incubated with 50% CM for 72 h. Yellow arrowheads denote zones positive for collagen degradation epitope associated with detached single cells and white open arrowheads for collective invasion zones. **C**, **D** Brightfield images (C) and quantification of migration modes (D) of DMSO control and GM6001 treated SCC spheroids after 72 h of incubation. Data represent at least 11 spheroids from 3 independent experiments. **E** Confocal 3D stack images of SCC spheroids cultured in the presence of GM6001 for 72 h. For comparison see GM6001-untreated samples in (B). Dotted line marks the spheroid core. **F** Confocal stack images of cell nuclei from detached cells in DMSO control and GM6001 treated spheroid culture and quantitative analysis of the smallest nuclear diameter. Yellow arrows denote locally deformed nuclei. Scale bars indicate 100 μm (overview) and 20 μm (detail). Black arrowheads show cohesive strands, white arrowheads detached single cells.



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Figure 5 Prevention of invasion plasticity by interference with HGF/c-Met signaling. **A**, **B** Brightfield images (A) and quantification of invasion (B) of SCC spheroids incubated with 50% CM for 72 h in the presence of c-Met inhibitor SU211274. Data represent at least 8 spheroids from 3 independent experiments. The horizontal line at 50 µm length indicates the threshold value for cohesive strands. **C**, **D** Brightfield images (C) and quantification of invasion (D) of SCC spheroids treated with 50% CM together with anti-HGF antibody after 72 h. Data originated from at least 7 spheroids from 3 independent experiments. Scale bars indicate 100 µm. Black arrowheads show cohesive strands, white arrowheads detached single cells.

(Suppl. Fig. 5A). Referenced against tubulin staining by semiquantitative analysis (Suppl. Fig. 5B), E-cadherin was gradually down-regulated in collective invasion strands and minimum levels reached in detached cells (Suppl. Fig. 5C).

Parallel to E-cadherin downregulation, vimentin protein content referenced against tubulin staining was measured in individual cells (Suppl. Fig. 6A). In untreated spheroids of SCC38 and SCC58 cells, vimentin was randomly expressed with a positive trend in invading cells, gradually increasing from collective strands to detached single cells (Fig. 3A, B). Conditioned medium intensified this trend and migrating cells developed consistently higher intensity compared to non-migrating cells (Fig. 3A, white arrowheads; B, center vs. rim/detached cells). Notably, the majority of migrating cells in SCC38 including tip cells in multicellular strands and detached cells and a minority in SCC58 cells remained vimentin-negative (Fig. 3A, C). Elongation of cells was uncorrelated with vimentin expression (Fig. 3C). To assess the kinetics and variability of vimentin induction spheroids at different time points after embedding were analyzed. In both, SCC38 and SCC58 cells, vimentin-positive subsets were present immediately after collagen polymerization and before migration was initiated (Suppl. Fig. 6B, C). This indicates that vimentin expression in cell subsets was intrinsic and did not depend upon contact to collagen or fibroblast-conditioned medium. With progressing invasion, the frequency of vimentin-positive cells remained largely unaffected in SCC38 cells but increased over time in SCC58 cells (Suppl. Fig. 6B, C). Thus, at single cell level, fibroblast-conditioned medium induces a spectrum of vimentin-positive, EMT-like, and vimentinnegative subsets collective and single-cell invasion compartments.

Proteolytic amoeboid movement induced by fibroblast-conditioned medium

In 2D epithelial cancer cell cultures EMT induction associates with cell elongation and produces individualized fibroblast-like spindle-shaped morphologies in moving cells [37, 40, 41], consistent with EMT-mediated mesenchymal migration [42]. To invade high-density 3D fibrillar collagen with pore dimensions of ~ 2-10 μ m², as used here, moving cells require the ability to proteolytically degrade the ECM [43], a typical feature of mesenchymal migration [44]. However, 70% (SCC38) or 90% (SCC58) of detached cells, irrespective of vimentin expression, maintained a rounded morphology (Fig. 3C; elongation index < 3). To verify whether cells with a rounded morphology developed occasional spindle-shaped periods, elongation was measured over time from time-lapse recordings. Both cell lines retained predominantly round morphology (Fig. 4A) with visible cortical actin structures (Fig. 1B, yellow arrowheads) and occasional short-lived elongation periods (duration below 1 h; Fig. 4A; Movie 3 and 4).

Unlike amoeboid migration in leukocytes or tumor cells treated matrix metalloproteinase inhibitors [43, 45] or dependence on Rho/ROCK functions [46], individually moving SCC38 and SCC58 cells caused collagen degradation, visualized as cleavage epitope in vicinity /associated with multicellular strands and single cells and linear tissue tracks left behind by detached cells (Fig. 4B). When treated with broad-spectrum matrix metalloproteinase inhibitor GM6001, both collective and single-cell migration were significantly reduced (Fig. 4C and Movie 5 and 6), and extracellular collagen degradation strongly diminished (Fig. 4E). Albeit diminished, residual single-cell migration was associated with increased deformation of cell nuclei in time-lapse recordings of detached cells (Movie 5 and 6) and fixed samples (Fig. 4F), similar to moving leukocytes and tumor cells after MMP inhibition [43].

Thus, beyond its known stimulating effects on mesenchymal and collective invasion, fibroblastconditioned medium induces amoeboid moving cells which remodel ECM by pericellular proteolysis.

Antagonization of invasion plasticity by HGF inhibitors

To test whether fibroblast-induced plasticity, and particularly amoeboid dissemination, can be prevented by pharmacologic HGF-targeting strategies, a HGF-scavenging antibody and the c-Met inhibitor SU11274 were applied to spheroid invasion cultures together with fibroblast-conditioned medium. At low to moderate dosing, both HGF and c-Met inhibition reverted single cell detachment, whereas collective invasion remained largely intact (Fig. 5). With at maximum dose, single-cell invasion was abrogated and collective invasion strongly reduced in SCC58 cells with HGF blocking while in SCC38 spheroids only single cell but not collective invasion was diminished (Fig. 5). Notably, in contrast with direct HGF targeting, SU11274 was insufficient to reduce the initiation of collective invasion in both cell types, but both treatments reduced the prolongation of existing invasion strands (Fig. 5B, D). These data indicate that both HGF neutralization and downstream signaling inhibition abrogate single-cell detachment and amoeboid dissemination, whereas collective invasion is largely insensitive to pharmacological inhibition.

DISCUSSION

Using single-cell 3D cytometry of HNSCC spheroid invasion cultures, we here shed initial light on the interdependence of EMT-associated molecular transitions and plasticity of collective and single-cell cancer invasion modes in response to microenvironmental stimulation. HGF, a clinically relevant growth factor to mediate EMT, induces both plasticity of invasion strategies and molecular diversity with EMT and non-EMT subsets. Whereas mesenchymal-like HNSCC cell invasion was infrequent, HGF induced notable collective invasion and, at higher concentration, amoeboid single-cell dissemination, and both invasion types contained cells expressing the EMT-marker vimentin. These findings indicate that, in addition to previous notions associating EMT with mesenchymal movement [20], an EMT signature is compatible with further migration programs, including collective and previously unappreciated collagenolytic-amoeboid migration.

Squamous cell carcinoma invasion involves collective migration as primary strategy [27, 42], with fibroblasts as prominent inducers [17, 18]. Similar to HNSCC cells tested here, HGF enhances collective migration in normal kidney epithelial cells, liver progenitor cells and mammary adenocarcinoma across 2D surfaces *in vitro* [47-49] and of mouse mammary tumor cells and normal kidney

epithelial cells in ECM-based 3D models [50, 51]. Serum levels of HGF are elevated in body fluids of cancer patients (up to 10 ng/mL) [52] reaching concentrations which induce collective invasion from 3D spheroids, and local HGF levels in tumor tissue are likely higher [53]. Thus, besides its established function in inducing single-cell scattering [39, 54], at low concentration HGF is a potent inducer of collective invasion by inducing cell migration without complete downregulation of E-cadherin based cell-cell interactions.

Besides the type of stimulus, additional cell migration plasticity is caused by the strength of environmental signals. At higher HGF concentrations (above 10 ng/ml), HNSCC cells transit from collective to amoeboid migration, with a minor mesenchymal population in SCC38 cells, likely as consequence of downregulated E-cadherin combined with actin regulation. Cell rounding results from high actomyosin contractility and can be induced by growth factors, cytokines or ligand-independent receptor activation including c-Met via Rho/ROCK [13, 55]. In 3D HNSCC spheroid culture, initially collective invasion strands released proteolytic-amoeboid moving cells with diminished E-cadherin levels, cortical actin cytoskeleton without focalization to the ECM but collagenolytic capacity, which led to the generation of collagenolytic tracks. This differs from amoeboid movement of melanoma cells invading 3D collagen type I which is mediated by non-catalytic function of MMP9 [56]. Thus, in the current model, proteolytic-amoeboid movement combined the features of rounded migration and MMP-dependent degradation of collageneous ECM environment, which deviate from otherwise non-proteolytic amoeboid migration detected in leukocytes and other cancer models [46, 57].

The transition from collective to amoeboid migration modes occurred as a probabilistic process which depended on the HNSCC type and increasing HGF/c-Met signaling strength. Similarly, the induction of EMT by HGF, which is well established for other cell types and mostly 2D culture conditions [38, 58, 59], was cell-type dependent and probabilistic when assessed by single-cell cytometry in situ. SCC58 cells, which are invasive and metastatic [60], developed high frequencies of vimentin-positive cells which invaded collectively or individually in response to HGF, whereas invading but non-metastatic SCC38 cells [5, 60] contained low frequency of vimentin-positive cells in non-invading as well as collectively and individually moving cells and no further upregulation was induced by HGF. This indicates vimentin induction by HGF varies with cell type and that any invasion mode can harbor EMT-like and EMT-independent signaling states [61]. In collective invasion strands, both vimentin-positive and -negative cells retained somewhat lowered and/or delocalized E-cadherin along cell-cell junctions, consistent with invasive or metastatic HNSCC phenotype in primary tumors of patients exhibiting multicellular invasion patterns, reduced Ecadherin content and positivity for vimentin [62-64]. The vimentin and E-cadherin double-positive state is consistent with partial EMT, retaining both epithelial and mesenchymal features in the same cell [65]. After detachment, vimentin-positive amoeboid phenotypes developed yet another molecular fingerprint in situ, with diminished E-cadherin but retained MMP activity and ability to degrade fibrillar collagen to clear the migration path. These data indicate that, besides mesenchymal migration, a spectrum of collective and amoeboid migration strategies can be associated with EMT and further include a variable fraction of alternative, EMT-independent states.

Clinically, a subset (19%) of HNSCC patients develops an EMT phenotype with decreased Ecadherin and increased vimentin expression in the primary lesion which increases the risk for distant metastasis by 2-fold compared to patients without signs of EMT [63]. This suggests that, with lower probability, EMT-negative cells which locally invade tissue may retain the ability metastasize as in ductal or lobular breast cancer invading collectively from the primary site [66], e.g. by developing transient or locally very confined EMT-positive states [67, 68]. Because EMT- and non-EMT states coexist in collective and individual migration modes, a critical progression-enhancing effect of HGF may result from broadening the range of migration modes and molecular diversity, thereby diversifying the microenvironmental coping abilities of disseminating cancer cells. Consistently, the presence of single cells is detected in 3D reconstructions of HNSCC samples [27], and disseminated rounded single cells emanate in preclinical models of breast tumors [69] and, with upregulated carboanhydrase-IX, in the invasion zone of clinical HNSCC samples [5]. Thus, constitutive or environment-induced amoeboid migration may contribute to discrete steps of cancer cell invasion and metastasis.

In preclinical mouse models for epithelial cancer progression, targeting the HGF/c-Met signaling pathway inhibits tumor growth and metastatic dissemination [70-73]. However, no clinically relevant overall survival benefit has been reported in clinical phase II and III trials in patients with late-stage or recurrent metastatic HNSCC disease [31, 73]. Our data suggest that both HGF neutralization and c-Met inhibition diminish particularly single-cell dissemination with comparable efficiency, whereas collective invasion retains limited ability to resist targeted intervention. This indicates particular resilience of HGF-induced collective processes to withstand pharmacological intervention or, in a cell-context dependent manner, the function of other soluble factors released by MRC5 cells, such as EGF. In summary, HGF-induced molecular and functional diversification of migratory strategies may enhance the ability of invading cells to adapt to complex microenvironments, increase the probability for escape from the primary site [74] and further differentially resist to pharmacotherapy.

ACKNOWLEDGEMENTS

We gratefully acknowledge Jan Schepens, William Leenders and Anneke Navis for producing and providing the RNAi constructs and Cindy Dieteren for expert assistance in image analysis. P.F. was supported by NWO-VICI (918.11.626), the European Research Council (ERC-CoG DEEPINSIGHT, Project No. 617430), EU Horizon 2020-MULTIMOT (634107-2) and the Cancer Genomics Center, The Netherlands.

AUTHORS CONTRIBUTION

J.O. designed and carried out the experiments, analyzed the data, supervised the work and wrote the manuscript. M.R., H.M. and S.S. performed experiments and analyzed data. R.G. isolated UT-SCC38 and UT-SCC58 cells and provided clinical background information. R.T. supervised the work. P.F. designed the experiments, supervised the work and wrote the manuscript.

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SUPPLEMENTARY FIGURES



Supplementary figure 1 Induction of plasticity of invasion in HNSCC spheroids by MRC5 fibroblast conditioned medium. **A** Overview (left) and zoomed (right) brightfield images showing SCC38 and SCC58 spheroids in rat tail collagen after 72 h of incubation with or without MRC5 CM. **B** Quantification of invasion modes originating from SCC spheroids incubated with MRC5 CM. For images see figure 1A. Data from at least 12 spheroids from 3 independent experiments. Black arrowheads denote cohesive strands, white arrowheads detached single cells, and gray arrowheads detached cell clusters. Scale bars, 100 µm (overview) and 20 µm (detail).


Supplementary figure 2 Expression of cytokines, growth factors and receptors and c-Met knockdown. **A** Expression of cytokine and growth factor receptors and their ligands measured by RT-qPCR in SCC38, CAF553 and MRC5 cells after 60 h (SCC38) or 36 h (fibroblasts) of culture in rat tail collagen. The horizontal line indicates the threshold cycle. Data represent the mean values and standard deviation from selected molecules from 3 independent experiments. **B** Flow cytometry detection of extracellular c-Met in control and c-Met shRNA expressing SCC cells. Data represents 3 independent experiments.



Supplementary figure 3 HGF and EGF induced plasticity of invasion. **A** Confocal 3D stack images of SCC spheroids incubated with recombinant human HGF for 72 h. **B**, **C** Brightfield images (B) and quantitative analysis (C) of 3D SCC spheroid culture incubated with recombinant human epidermal growth factor (EGF) for 72 h. Data represent at least 8 spheroids from 2 independent experiments. P values compare invasion patterns induced by CM and EGF. Scale bars indicate 100 μm (overview) and 20 μm (detail). Black arrowheads show cohesive strands, white arrowheads detached single cells.



Supplementary figure 4 Whole spheroid analysis of EMT markers. **A** Workflow of lysate generation from 3D spheroids. **B** Reversed phase protein assay (RPPA) results and **C** Western blot analysis of SCC spheroids in collagen gel incubated with increasing concentrations of MRC5 fibroblast CM.; RPPA results represent data from 3 independent experiments and western blot data from 4 independent experiments.



Supplementary figure 5 E-cadherin regulation by CM from MRC5 fibroblasts. **A** Confocal single slice images of SCC spheroids after 72 h invasion in rat tail collagen. Scale bars indicate 100 μ m (overview) and 20 μ m (detail). Black arrows denote membranous E-cadherin and white arrows cytoplasmic E-cadherin in SCC cells. **B, C** Workflow of region 3D analysis of E-cadherin expression relative to tubulin (B) and quantitative analysis (C) of 3D SCC spheroid culture incubated with CM from MRC5 cells. Data originated from at least 9 spheroids from 3 independent experiments.

A

Vimentinanalysis







Supplementary figure 6 Vimentin expression in SCC spheroids. **A** Workflow of single-cell 3D cytometry of vimentin expression relative to tubulin. See corresponding images and results in figure 3. **B**, **C** Confocal 3D stack images of SCC spheroid culture incubated with 50% CM from MRC5 cells at different time points during an incubation of 72 h (B) and quantitative analysis (C) by single-cell 3D cytometry. Scale bar indicates 100 µm. Green line separates vimentin positive and negative cells and numbers show percentage of positive cells. Data represent 2 spheroids from one experiment.

CHAPTER 4

Targeting CD44v6 for fluorescence-guided surgery in head and neck squamous cell carcinoma

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Sci Rep. 2018;8(1):10467

Head and neck squamous cell carcinoma (HNSCC) is an often highly invasive tumor, infiltrating functionally important tissue areas. Achieving complete tumor resection and preserving functionally relevant tissue structures depends on precise identification of tumor-free resection margins during surgery. Fluorescence-guided surgery (FGS), by intraoperative detection of tumor cells using a fluorescent tracer, may guide surgical excision and identify tumor-positive resection margins. Using a literature survey on potential surface molecules followed by immunohistochemical validation, we identified CD44 variant 6 (CD44v6) as a constitutively expressed antigen in the invasion zone of HNSCC lesions. The monoclonal anti-CD44v6 antibody BIWA was labeled with both a near-infrared fluorescent dye (IRDye800CW) and a radioactive label (Indium-111) and dual-modality imaging was applied in a locally invasive tumor mouse model. BIWA accurately detected human HNSCC xenografts in mice with a tumor uptake of $54 \pm 11\%$ ID/g and invasion regions with an accuracy of 94%. When dissected under clinical-like conditions, tumor remnants approximately 0.7 mm in diameter consisting of a few thousand cells were identified by fluorescence imaging, resulting in reliable dissection of invasive microregions. These data indicate that CD44v6 is a suitable target for reliable near-infrared detection and FGS of invasive HNSCC lesions in vivo.

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) are invasively growing tumors in a functionally delicate and important area with an overall five-year survival rate below 60%¹. Surgical treatment of HNSCC aims to completely remove both tumor and marginal invasion zones, and tumor-free resection margins represent a critical prognostic parameter for reducing tumor recurrence and improving overall survival². In functionally critical areas, such as the head and neck region, maximizing surgical margins has to be weighed against loss of functional tissue resulting in compromised guality of life post surgery ³. Therefore, developments such as intraoperative histology and image-guided surgery aim to more accurately delineate the tumor border during surgery, enabling more accurate tumor resection. As complementary strategy fluorescence-guided surgery (FGS) aims to intraoperatively detect a fluorescent tracer after selective accumulation in tumor tissue and enables preclinical and clinical detection of cancer lesions eventually resulting in improved progression-free survival after surgery ⁴. Improved signal detection has been achieved by conjugating fluorophores in the near-infrared (NIR) range of 650-900 nm with targeting antibody binding extracellular epitopes preferentially expressed on tumor cells, allowing macro- and microscopic detection of even small tumors and tumor subregions⁵. This resulted in a range of cancer-targeting antibodies developed for FGS surgery to validate molecular targets, establish conjugate safety and develop sensitive imaging devices ⁶⁻⁸. In clinical trials safe tracer administration and subsequent identification of cancers in the sub-millimeter resolution have been demonstrated ^{4,9-11}. Potential shortcomings of FGS, however, include inhomogeneous antigen expression and/or tissue distribution of the antibody as well as non-specific antibody uptake in peritumor tissue and limited tumor detection due to high background fluorescence 4,12.

First-generation FGS of HNSCC focused on the epidermal growth factor receptor (EGFR), based on its prominent expression in HNSCC and efficient *in vivo* detection of tumors and metastasis in preclinical studies ¹³⁻¹⁵. A fluorescently-labeled anti-EGFR antibody (cetuximab) is clinically well tolerated and efficiently differentiates tumor from normal tissue ^{9,10}. However, reliable cetuximabbased FGS is hampered by uncertain sensitivity and specificity, as a consequence of variable antigen expression in tumors and high binding of cetuximab to normal tissues (tumor stroma, liver, skin, a.o.) ¹⁶. Thus, identifying antigens with a more tumor restricted expression remains pertinent to reliably and selectively visualize HNSCC tumor regions.

To reliably detect the invasion zone of HNSCC, we performed a literature survey and tested the presence of a range of potential antigens (over-)expressed in HNSCC including c-Met, CD44 variant 6 (CD44v6), E-cadherin, epidermal growth factor receptor (EGFR), extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) and epithelial cell adhesion molecule (EpCAM). We identify CD44v6 as candidate and apply anti-CD44v6 antibody BIWA for sensitive detection of the invasion margins in HNSCC in a preclinical mouse model.

RESULTS

CD44v6 expression in invasive HNSCC

To identify surface markers in HNSCC patient material which reliably detect the margin of invasion and, hence, might be suitable for FGS, we applied comparative immunohistochemistry on human tumor samples. Candidate cell surface proteins, including c-Met, CD44v6, E-cadherin, EGFR, EMMPRIN and EpCAM, were identified based on a literature survey focusing on the percentage of positive tumors, the homogeneity of expression within the same tumor, and whether the protein was expressed on the epithelium or the tumor stroma (Suppl. Table 1). As further criteria for marker selection, extracellular cell-surface localization and expression level and variability in HNSCC were considered. Additionally, the availability of a monoclonal antibody with established low toxicity profile and imaging application in clinical trials was taken into consideration. Approximately 97% of HNSCCs were positive for CD44v6 followed by EGFR (85%) and lower frequencies for the other markers. CD44v6 was consistently present throughout the tumor with defined membrane staining, but reduced expression in keratinized or necrotizing areas in the tumor core (Fig. 1). EGFR and c-Met showed a strong expression throughout the tumor similar to CD44v6 (Fig. 1; Suppl. Fig. 1). Likewise, EMMRPIN showed reliable expression throughout the lesion albeit with lower intensity (Suppl. Fig. 1), whereas E-cadherin and EpCAM expression



Figure 1. Expression of CD44v6 and EGFR in primary human HNSCC samples. Tumor (T), normal epithelium (E), stroma (S). Dotted lines mark the tumor edge. Representative samples from 7 (CD44v6) or 5 (EGFR) independent tumors. Scale bars indicate 1000 μ m (overview) and 100 μ m (zoom).

were less reliable with notable inter-individual variability (Suppl. Fig. 1). Whereas for CD44v6 and EMMPRIN the signal was near-exclusively tumor cell specific with only weak background staining from the desmoplastic stroma and adjacent epithelial structures, particularly epidermis and hair follicles, E-cadherin positivity resulted from both tumor-derived and non-transformed epithelial structures (Fig. 1; Suppl. Fig. 1). EpCAM, c-Met and EGFR were also expressed by stromal cells resulting in a high peri-tumor background signal (Fig. 1; Suppl. Fig. 1). The reliable immunohistochemical staining together with published evidence indicated CD44v6 as epitope with abundant expression throughout HNSCC lesions including the invasion zone. For application in FGS, CD44v6-targeting antibodies were previously shown to macroscopically identify CD44v6 expressing epithelial xenograft tumors in mice, including HNSCC (Suppl. Table 1) ¹⁷⁻¹⁹, whereas its suitability for identifying the tumor margin and disseminated invasion zones remain untested. We therefore selected anti-CD44v6 antibody BIWA, of which the humanized form demonstrated safe administration in clinical trials and reliably visualized HNSCC lesions by nuclear imaging ^{20,21}.

Expression of CD44v6 in HNSCC cell lines and invasive xenograft tumors in mice

To establish an invasive HNSCC mouse model for FGS, a range of HNSCC cell lines was analyzed for expression of CD44v6 and growth pattern *in vivo*. Consistent with reliable CD44v6 expression in human samples, all HNSCC cell lines expressed CD44v6 at high and homogeneous levels in contrast to two melanoma cell lines serving as negative controls based on previous findings (Suppl. Fig. 2A)²². UT-SCC58 showed a low-to-medium level of cell surface expression (50-fold CD44v6/lgG1 signal-to-background ratio; total range between cell lines 30 to 181), which we considered as a representative cell model reflecting non-overstated CD44v6 availability for realistic in vivo antibody targeting. As xenograft lesions in mice, UT-SCC58 cells grew within 3-4 weeks to macroscopically visible tumors (Fig. 2A, B) and showed invasive growth pattern characteristics of differentiated HNSCC, including nest-like dissemination in the interstitial tissue (Fig. 2C1) and invasion along nerve fibers (Fig. 2C2). Co-staining of CD44v6 and pan-cytokeratin as epithelial reference marker indicated a relatively uniform expression of CD44v6 throughout the lesion (Fig. 2D). Sub-region analysis of the tumor core, border and invasive cells, as well as scoring of CD44v6 intensity at single cell level revealed constant expression in all tumor areas without significant variation (Fig. 2E, F; Suppl. Fig. 2B). Thus, UT-SCC58 xenografts recapitulate the invasive growth and CD44v6 expression patterns of patient samples and represent a suitable preclinical model for FGS of invasive and moderately CD44v6-positive HNSCC tumors.

BIWA detects CD44v6-expressing tumors in vivo

To detect small metastases and assess the biodistribution of fluorescent tracers, a cohort of UT-SCC58 tumor-bearing mice with a lesion volume of 10-42 mm³ received ¹¹¹In-DTPA-BIWA-IRDye800CW or ¹¹¹In-DTPA-IgG1-IRDye800CW isotype-matched control antibody intravenously. Antibody uptake by UT-SCC58 tumors using NIR whole-body fluorescence detection reached a maximum three days



Figure 2. Invasive HNSCC mouse xenograft model for *in vivo* detection of CD44v6. **A** Macroscopic detection of a representative small and large tumor in the left cheek of two different mice. Arrowhead indicates the tumor location. **B** Growth curve of UT-SCC58 tumors. Data show the means of 12 mice. **C** Central section of an UT-SCC58 tumor, showing multifocal invasive tumor islands in the interstitium (1) and in perineural location (2). Scale bars indicate 500 μ m (overviews) and 100 μ m (zooms). **D** Expression of CD44v6 and pan-cytokeratin (CK) in UT-SCC58 tumors. Dotted lines mark the tumor border and tumor islands. Scale bars indicate 500 μ m (overviews) and 100 μ m (zooms). **E** Whole-region analysis of (D). Staining intensity of CD44v6 in different areas analyzed at day 28 after implantation. Data show medians from 12-38 analyzed images per tumor from 4 independent tumors. **F** Single cell analysis of (D). Fluorescence intensities after background substraction for CD44v6 and CK of the same tumors as in (E). 50 cells were analyzed per subregion and geometric symbols represent 5 different tumors. Core of the tumor (C), border of the tumor (B), distant cells (DC), skin (S).

after injection (data not shown), similar to the bioavailability kinetics reported for other antibodies and HNSCC tumor models ^{19,23}. ¹¹¹In-DTPA-BIWA-IRDye800CW showed a most prominent signal originating from the tumor and weaker signal originating from the left cervical lymph node and the liver (Fig. 3A). In comparison, fluorescent signals from ¹¹¹In-DTPA-IgG1-IRDye800CW were detected in the liver and weakly in the tumor (Fig. 3A). SPECT/CT images confirmed this biodistribution profile, whereby one mouse also showed weak signal enrichment in the left cervical lymph node (Fig. 3B). Biodistribution analysis revealed a mean tumor uptake of $54 \pm 11\%$ ID/g for ¹¹¹In-DTPA-BIWA-IRDye800CW compared to $5 \pm 2\%$ ID/g for IgG1 control antibody conjugate (Fig. 3C). Uptake of dual-labeled BIWA in other organs was considerably lower, quantified as total amount (Fig. 3C) and



Figure 3. Biodistribution of ¹¹¹In-DTPA-BIWA-IRDye800CW and ¹¹¹In-DTPA-IgG1-IRDye800CW control antibody in mice carrying HNSCC tumors in the left cheek for 28 days and 72 h after i.v. administration. **A, B** Near infrared fluorescent (NIRF) whole body images of mice n=9 (A) and corresponding micro SPECT/CT images n= 4 (B). Tumor (T), lymph node (LN), liver (L). **C** Biodistribution data shown as percentage injected dose per gram of tissue of both dual-labeled antibodies. **D** Tumor-to-blood ratios obtained by ratios from (C).

tumor-to-blood ratio (Fig. 3D). Despite yielding a positive signal (Suppl. Fig. 3A) the cervical lymph nodes lacked pan-cytokeratin positive tumor nests (Suppl. Fig. 3B, C) and thus were considered tumor-free. Most likely, false-positive detection in lymph nodes resulted from uptake of BIWA by macrophages via Fc-receptors, as described for other tumor models ²⁴. Thus, systemically applied dual-labeled BIWA accumulates in CD44v6-expressing tumors with high selectivity.

BIWA detects invasive tumor regions

To determine whether dual-labeled BIWA is able to detect the invasive tumor margin, we performed sub-region immunohistochemistry and NIR fluorescence scans after systemic administration of ¹¹¹In-DTPA-BIWA-IRDve800CW. Both, the tumor core and invasion zones were macroscopically positive for the near-infrared signal, whereas the surrounding stroma showed a low background signal (Fig. 4A; Suppl. Fig. 4). Microscopic analysis revealed cell surface localization of BIWA label on tumor cells (Fig. 4A, white arrowheads), consistent with antigen-dependent accumulation after in vivo administration. Sub-region co-localization analysis of the CD44v6 NIRF signal after in vivo administration and pan-cytokeratin immunofluorescence showed that BIWA detected > 85% of the CK-positive tumor cell nests (Fig. 4A, black arrowheads: Fig. 4B). Notably, occasional BIWA-negative tumor clusters were frequently located in direct vicinity (below ~ 250 µm distance) of BIWA-positive regions (Fig. 4A, open arrowheads), and thus would colocalize when viewed by intraoperative microscopic surgery with a sub-millimeter resolution. Including such co-localized events, 94% of the invasion regions were detected by ¹¹¹In-DTPA-BIWA-IRDye800CW (Fig. 4B). No signal from IgG1 control antibody was detected in both tumor cells or the tumor stroma by histological scoring (Suppl. Fig. 4). Taken together, systemically applied dual-labeled BIWA detects all tumor regions and > 90% of invasive zones.

Preclinical FGS: sensitive detection of tumor cells

FGS experiments were performed using systemically applied BIWA-IRDye800CW detected by the Intraoperative QMI Spectrum fluorescence imaging system (Quest Medical Imaging), to approximate signal intensity and detection of the tumor margin under clinical-like conditions. To simulate incomplete tumor resection and estimate the size of minimal remnants of the resection margin based on the BIWA-IRDye800CW label, step-wise FGS was performed until a minimal-residual fluorescent signal was left *in situ* (Fig. 5A). Subsequently, remnants were detected by high-sensitivity NIRF imaging followed by high-resolution immunohistochemistry to validate the presence and amount of tumor cells (Fig. 5A). Post-operative immunohistochemistry showed that NIR fluorescence-positive regions also stained positively for pan-cytokeratin (Fig. 5B), confirming high selectivity of CD44v6 for the detection of tumor cells. The estimated residual tumor masses, which could still be detected by BIWA-IRDye800CW in minimal lesions, were 0.7 – 2 mm in size and contained few thousand to hundred thousand cells, based on NIR scans and immunohistochemistry of the residual tissue.



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l i	Tur	nor	Invasive cells		
No	Main mass	Border	Single nest	Multiple nests	
1*	2/2	2/2	0/0	0/0	
2**	2/2	2/2	8/9	5/5	
3	2/2	2/2	5/6	2/3	
4	2/2	2/2	8/9	4/4	
5	2/2	2/2	6/7	5/5	
Sum	10/10 (100%)	10/10 (100%)	27/31 (87%)	16/17 (94%)	

Figure 4. Detection of invasive tumor regions by dual-labeled BIWA 3 days after systemic administration. **A** Central, serial sections of a tumor (H&E staining, CK staining and NIRF signal). Images show invasive tumor islands (1, 2, dotted line) adjacent to the main tumor (3, dotted line). Tumor (T), normal epithelium (E), stroma (S). Black arrowheads indicate fluorescence positive islands, the transparent arrowhead a negative/weakly stained island. White arrowheads highlight membranous staining. Scale bars indicate 1 mm (overview) and 100 µm (zoom). **B** Scoring of NIRF signal intensity for tumor core, border and invasive cells including isolated nests and clusters of adjacent islands. Data represent scoring of two sections per tumor in 5 mice. *Example shown in Suppl. Fig. 4, ** Example shown in Fig. 4A.



Figure 5. Preclinical FGS using BIWA-IRDye800CW and post-resection validation. **A** Experimental procedure of step-wise tumor resection, *in situ* detection and post-fixation analysis. **B** (1) UT-SCC58 tumors 28 days after implantation into mice were monitored by intraoperative fluorescence imaging (QMI Spectrum) followed by step-wise FGS. Dotted lines mark the mouse and tumor tissue and the arrow indicates weak fluorescent signal from tumor remnant *in situ*. Numbers show tumor-to-background ratios. (2) Post-operative high-sensitivity NIR fluorescence analysis of excised tissues and *in situ* remnant using the Odyssey CLx flatbed scanner. The scale bar indicates 1 mm. Further processing and re-scanning of the tumor remnant as 400- and then 7-µm thick sections. Immunofluorescence of a NIRF-positive 7 µm section of the remnant. Scale bars indicate 500 µm (overview) and 100 µm (zoom). The total estimated remnant tumor cells and size are shown for four experiments.

DISCUSSION

We here show that systemically applied BIWA-IRDye800CW provides sensitive detection of HN-SCC lesions under clinical fluorescence imaging conditions and allows a precise step-wise removal of the tumor margin with a detection limit in the submillimeter range. CD44v6 is expressed in HNSCC across all tumor stages, with varying trends in T stage and/or nodal status²⁵⁻²⁸. The robust expression of CD44v6 across independent HNSCC lesions and most, if not all, HNSCC tumor cells and the availability of clinically applicable anti-CD44v6 antibody Bivatuzumab emphasize CD44v6 as a promising antigen for reliable FGS in HNSCC cancer, enabling visualization of tumor margins and minimal residual lesions. Besides in primary tumors, CD44v6 is expressed in recurrent tumors and lymph node metastases ^{27,29,30}, therefore administration of ¹¹¹In or IRDve800CW labeled Bivatuzumab should enhance detection of primary as well as secondary and metastatic HNSCC disease. When directly compared, CD44v6 expression is approximately 8-fold higher in HNSCC samples compared to EGFR ³¹. CD44v6 is expressed only in a subset of adjacent normal epithelia and absent or only weakly expressed in other, non-tumor tissues ³⁰, which minimizes background signal, systemic uptake and potential off-target effects. In addition, radiolabeled Bivatuzumab, which targets the same epitope as BIWA, was successfully applied for scintigraphic in vivo imaging of HNSCC tumors in mouse models and patients with low toxicity and immunogenicity^{19,32,33}. Tumor uptake of the dual-labeled BIWA in UT-SCC58 tumors with moderate CD44v6 expression was comparable to that of radiolabeled anti-CD44v6 antibodies, while tumor-to-blood ratios even exceeded previously reported results ^{17,19}.

Using detailed immunohistochemical analysis of CD44v6 in patient material and by *in vivo* targeting of HNSCC xenografts by BIWA, we demonstrate that CD44v6 is strongly expressed in the invasion margin of tumor lesions and thus suitable for precise microscopic FGS mapping of invasive HNSCC. Using minimal lesion analysis, we identify high detection sensitivity of BIWA-IRDye800CW using a clinical fluorescence imaging system detecting as little as sub-millimeter sized residues containing only a few thousand cells. The staining pattern was predominantly cellsurface associated with heterogeneous targeting in the main tumor mass, potentially caused by heterogeneous antibody distribution ³⁴. However, the consistent labelling of tumor borders and invasive cells indicated that Bivatuzumab may enable reliable intraoperative fluorescent imaging of both, invasion zone and small tumor remnants.

Besides HNSCC, CD44v6 is expressed in most carcinoma types, including lung, skin, cervix, breast and colon ³⁰. Thus, beyond HNSCC, CD44v6 may serve for probing resection margins in a range of other invasive tumor types. CD44v6 targeting antibodies have achieved reliable NIR fluorescence enrichment *in vivo* in breast cancer xenografts ¹⁸, and immuno-PET imaging in a thyroid carcinoma model ³⁵. Likewise, ¹⁸⁶Re-labeled Bivatuzumab was safely administered to patients with early-stage breast cancer, but failed to reliably detect small lesions with low CD44v6 expression ³⁶. Thus, further studies will be required to establish the application in small lesions with clinically variable CD44v6 expression. Besides application as a single agent, CD44v6 detection may be combined with EGFR detection, the expression of which, in contrast to CD44v6, increases in poorly differentiated carcinomas ^{16,30}. Thus, combining CD44v6 and EGFR targeting may increase overall sensitivity for the detection of poorly differentiated carcinomas. Additional epitopes for dual- or multi-targeted FGS may include c-Met and EMMPRIN, based on high expression in HNSCC samples and their presence in the invasive front (Suppl. Table 1). In conclusion, based on its beneficial signal-to-noise ratio and low side effects ³² Bivatuzumab, as either NIR-fluorescence- or dual-labeled conjugate, shows potential to improve the outcomes of surgical treatment in HNSCC.

MATERIAL AND METHODS

Literature survey

A systematic PubMed search was performed to identify potential cell surface markers expressed in the invasion region of HNSCC. The following search strategies were used for a publication period from 1985 to September 2015: protein name + immunohistochemistry + head and neck squamous cell carcinoma; protein name + imaging + head and neck squamous cell carcinoma. As indicator, the percentage of patients positive for the protein of interest was averaged from retrieved articles and, where available, differential expression within different tumor regions was taken into account (tumor core, tumor border, surrounding tumor stroma).

Immunohistochemistry of patient material

Tissue samples from 7 HNSCC patients were obtained from the Department of Pathology, Radboudumc, Nijmegen. Tumor samples were encrypted and analyzed in an anonymized manner, as approved by the institutional review board and according to national law. Formalin-fixed and paraffin-embedded tissues were cut into sections of 4 µm, mounted onto SuperFrost slides (Thermo Scientific) and dried overnight at 37°C. Sections were deparaffinized in xylene, rehydrated in graded alcohols to water, quenched of endogenous peroxidases (0.3% H₂O₂, 30 min), boiled in citrate buffer for antigen retrieval (pH 6, 13 min), pre-incubated with normal goat serum (20%, 30 min), washed, and incubated with primary antibody (4°C, overnight). The following antibodies were used: monoclonal mouse anti-human E-cadherin (SPM471, 1/300; Thermo Scientific), monoclonal mouse anti-human EpCAM (VU1D9, 1/200; Thermo Scientific), monoclonal rabbit anti-human c-Met (EP1454Y, 1/200; Epitomics), polyclonal rabbit anti-human EGFR (sc-03, 1/200; Santa Cruz); monoclonal mouse anti-human EMMPRIN (sc-21746, 1/200; Santa Cruz) and monoclonal mouse anti-human CD44v6 (VFF-18, 1/1000; AbD Serotec). After washing, sections were incubated with biotinylated secondary goat anti-mouse or anti-rabbit antibody and subsequently with the VECTASTAIN ABC reagent (Vector Laboratories) according to the manufacturer's protocol. The signal was developed with diaminobenzidine and counterstained with hematoxylin. Sections were dehydrated in ethanol and xylene and mounted with Pertex (Histolab). Negative controls (buffer only) were included in each analysis. Sections were imaged with an automated slide scanner with a 20x, 0.243 μ m/pixels objective (Pannoramic 250 Flash II, 3DHITECH).

Cell culture

HNSCC cell lines from primary human SCCs of cutaneous SCC (UT-SCC12A), tongue (UT-SCC16A and UT-SCC40), glottic larynx (UT-SCC38), supraglottic larynx (UT-SCC42B) as well as from metastases of SCCs of cutaneous SCC (UT-SCC12B), tongue (UT-SCC16B) and transglottic larynx (UT-SCC58) were established at the University of Turku, Finland ³⁷. Other cells used were FaDu cells (kind gift by the Dept. of Radiation Oncology, University of Technology Dresden) and the human melanoma cell lines A3755M (kind gift from Dr. Menashe Bar-Eli/Dr. Michael Davis, MD Anderson Cancer Center, USA) and MV3 ³⁸. All cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (both PAA), 2 mM L-glutamine, 1 mM sodium pyruvate (both Invitrogen/Life technologies) and 20 mM HEPES (Gibco).

Flow cytometry

2 x 10⁵ HNSCC and melanoma cells were seeded in 6-well plates two days prior to the experiment. Cells were detached with 4 mM ethylenediamine tetraacetic acid (EDTA) and stained on ice with murine anti-human CD44v6 antibody (BIWA), the humanized form of which has been applied clinically (bivatuzumab; BIWA-4) ^{19,39} or a human IgG1 istotypic control (Alpha Diagnostic Intl. Inc.) and a secondary goat anti-mouse/human 647 antibody (ThermoFisher Scientific). CD44v6 expression and cell viability co-registered by lack of propidium iodide uptake were obtained by flow cytometry (FACS Caliber) and analyzed using the FCS Express software (version 5).

Mouse model

The animal experiments were performed in accordance with the guidelines and rules of the Dutch Act on animal experiments (WOD) and was approved by the Animal Welfare body of the Radboud University, Nijmegen (RU-DEC 2014-142). 9×10^5 UT-SCC58 cells suspended in 3 mg/ ml Matrigel (total volume 50 µl; BD Biosciences) were injected into the floor of mouth of BALB/c nu/nu female mice (6-8 weeks old; Charles River Laboratories). Tumors were allowed to grow for 4-6 weeks. Tumor growth over time was measured using a caliper.

Antibody conjugation

Antibodies were used as either fluorescence-only or dual-labeled conjugates carrying fluorescence and the radioactive isotope ¹¹¹In. Human IgG1 isotypic control antibody (Alpha Diagnostic Intl. Inc.) was dialyzed against phosphate buffered saline (PBS) using the 10,000 Da molecular weight cutoff Slide-A-Lyzer MINI Dialysis device (Thermo Scientific) to remove azide and other additives. To generate NIR fluorescent conjugates, BIWA (1 mg) was incubated with a 3-fold excess of

IRDye800CW-N-hydroxysuccinamide (NHS) in 0.1 M sodium carbonate buffer (pH 8.5) for 1 h at room temperature (RT). For fluorescence guided surgery experiments, BIWA was only labeled with IRDye800CW-NHS. The final concentration of the antibody and the molecular substitution ratio were determined spectrophotometrically (Ultrospec 2000 spectrophotometer, Pharmacia Biotech) yielding an antibody:fluorophore ratio of 1:2. BIWA-IRDye800CW was stored in the dark at 4°C.

For dual-labeling, IRDye800CW-labeled antibodies were conjugated with the chelator *p*isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (ITC-DTPA) with a 10-fold excess in 0.1 M sodium carbonate buffer (pH 9.5) for 1 h at RT. The solution was dialyzed for 1 week against 0.25 M ammonium acetate (pH 5.5) using a Slide-A-Lyzer cassette with a molecular weight cutoff of 20,000 Da (Thermo Scientific). The final concentration of the antibody and the molecular substitution ratio of the fluorescent dye were determined spectrophotometrically (Ultrospec 2000 spectrophotometer, Pharmacia Biotech) yielding a ratio of 1.4. DTPA-BIWA-IRDye800CW and DTPA-IgG1-IRDye800CW were stored in the dark at 4°C until further use.

For radiolabeling, DTPA-BIWA-IRDye800CW and DTPA-IgG1-IRDye800CW were incubated with 0.05 MBq of ¹¹¹In (Mallinckrodt) per microgram of antibody in two volumes of 0.5 M 2-(N-morpholino)ethansulfonic acid (MES) buffer (pH 5.5). For SPECT/CT studies antibodies were labeled with 1.5 MBq of ¹¹¹In per microgram of antibody. After incubation of 20 min at RT 50 mM EDTA was added to a final concentration of 5 mM to chelate unincorporated ¹¹¹In. Labeling efficiency was determined by instant thin-layer chromatography on silica gel strips (Agilent Technologies) using 0.15 M citrate buffer, pH 6.0 as the mobile phase. For all preparations, the radiochemical purity of ¹¹¹In-DTPA-BIWA-IRDye800CW and ¹¹¹In-DTPA-IgG1-IRDye800CW exceeded 95%.

Unperturbed immunoreactivity of the antibody conjugates was confirmed as described ⁴⁰ with minor modifications. A serial dilution of UT-SCC58 cells was incubated with radiolabeled antibody conjugate (333 Bq equivalent to 6.7 ng antibody per dilution in DMEM medium) for 30 min at 37°C. To determine nonspecific binding, an excess of unlabeled antibody conjugate was added to a duplicate of the lowest cell concentration. Unbound antibody was removed by washing with DMEM and samples were analyzed in a γ -counter (Wizard; Pharmacia-LKB). For all preparations, the immunoreactive fraction of available antibody exceeded 85%.

SPECT/CT and fluorescence whole body imaging

Mice bearing UT-SCC58 tumors in the left cheek were injected into the tail vein with either 10 µg of the dual-labeled antibody ¹¹¹In-DTPA-BIWA-IRDye800CW or ¹¹¹In-DTPA-IgG1-IRDye800CW. Three days after injection the distribution of the tracer was determined with a U-SPECT II SPECT/ CT scanner (MILabs, Utrecht, The Netherlands) in four mice using a 1.0-mm-diameter multipinhole mouse/rat collimator (2x 25 min with 44 bed positions). Images were reconstructed by ordered-subset maximization expectation using the MILabs reconstruction software (U-SPECT-Rec, Milabs, Utrecht, The Netherlands) with the following settings: selection of the lower ¹¹¹In photopeak (152-183 keV), corrected for two backgrounds (135-151 keV and 184-211 keV), pixel based OSEM, voxel size 0.4 mm³ and 1 iteration over 16 subsets. After SPECT imaging, all mice were euthanized and whole-body fluorescence images were acquired (IVIS Lumina imaging system, Caliper Life Science) using the following settings: F/Stop - 2, excitation filter - 745 nm, emission filter - ICG, field of view - C, 675 nm autofluorescence and background correction, recording time of 1 min and medium binning factor. Images were processed with the IVIS Lumina software.

Biodistribution analysis

Three days after intravenous administration of the antibodies and whole-body imaging, the biodistribution of ¹¹¹In-DTPA-BIWA-IRDye800CW and ¹¹¹In-DTPA-IgG1-IRDye800CW in body fluids and excised organs of mice were determined. Blood was collected by heart puncture and tissue samples of the tumor lesion, superficial cervical lymph nodes, muscle, heart, lung, spleen, pancreas, kidney, liver, stomach and duodenum were collected, weighed and the radioactivity was measured in a well type γ counter (Wallac 2480 wizard, Perkin Elmer). Radioactivity uptake in each tissue was calculated as the percentage of the injected dose per gram of tissue.

Immunohistochemistry of mouse tumors and cervical lymph nodes

Frozen tumor sections (7 µm) were fixed in ice-cold methanol (10 min), washed and pre-incubated with normal goat serum (5%, in 1% BSA, 1 h) followed by incubation with primary antibody (overnight, 4°C). Formalin-fixed cervical lymph nodes and tumors were embedded in paraffin, sectioned (7 µm), mounted onto SuperFrost slides (Thermo Scientific), dried overnight (37°C), deparaffinized in xylene and rehydrated in graded alcohols to water. Sections were stained with hematoxylin eosin (H&E) or boiled in EDTA/Tris buffer (pH 9, 15 min) for antigen retrieval and preincubated with normal goat serum (5%, in 1% BSA, 1h) followed by incubation with antibody (overnight, 4°C). The following antibodies were used: polyclonal rabbit anti-human and mouse pan-cytokeratin (CK) (ab9377, 1/100; Abcam); monoclonal murine anti-human CD44v6 (BIWA, 5µg/ml; kind gift from V. Orian-Rousseau, Karlsruhe Institute of Technology, Germany). After washing, sections were incubated with secondary goat anti-rabbit Alexa Fluor 546 and goat anti-mouse/human Alexa Fluor 647 antibody (Invitrogen) for 2h at RT and counter stained with DAPI (Sigma). Negative controls were obtained by IgG isotype staining and tumor tissue served as positive controls for lymph node staining. Fluorescence on tissue sections was imaged using a 10x NA 0.25 objective (DMI6000B slide scanner, Leica) and H&E staining using a 20x 0.243 µm/ pixels objective (Pannoramic 250 Flash scanner, 3DHITECH).

Digital image analysis was performed using Fiji (software version 1.51k, https://imagej.net/Fiji). Fluorescence intensity of CD44v6 and CK were coregistered for tissue subregions from images containing skin, local invasion, the core or the border of the tumor and each subregion was manually segmented based on topology, DAPI and CK signal. Mean CD44v6 and CK fluorescence intensities in individual cells were obtained using same region of interest, using an area of 51

 μ m² to sufficiently represent the size of single cells. Fluorescence intensities were normalized by subtracting the mean intensity from five background measurements from tumor-free tissue regions.

NIR imaging and analysis of mouse tumors and lymph nodes

Fresh-frozen tumor tissue from mice after i.v. administration of ¹¹¹In-DTPA-BIWA-IRDye800CW or ¹¹¹In-DTPA-IgG1-IRDye800CW were sectioned (7 μ m) and two positions with at least 400 μ m distance were scanned for NIR fluorescence intensity (settings: slide, intensity 2, resolution 21 μ m, highest quality; Odyssey CLx, LI-COR Biosciences). Regions of interest identified by fluorescence detection from the Odyssey scans were re-sampled with high resolution by NIR imaging (20x NA 0.8; mono setting, 5 sec acquisition time; Nuance-XR, PerkinElmer). Further subsampling and validation of fluorescence-positive regions obtained by Odyssey scanning were obtained by H&E and CK staining.

Fluorescence-guided surgery

UT-SCC58 tumor-bearing mice after 28 days of growth in the left cheek received 10 µg of BIWA-IRDye800CW by tail vein injection. Three days later whole-body fluorescence was detected under isoflurane anesthesia followed by euthanasia. For intraoperative fluorescence detection, the tumor-containing cheek including surrounding fluorescence-negative tissue was surgically isolated and monitored in real-time using the Spectrum camera system (Quest medical imaging, Wieringerwerf) under clinically used imaging conditions. To match step-wise tumor resection conditions, sequential removal of the lesion guided by fluorescence was performed, until a weak but nonetheless positive signal in situ was obtained, reflecting a minimal tumor residue. Tumorto-background signals were analyzed using Fiji (software version 1.51k, https://imagej.net/Fiji). For verification of fluorescence, all freshly excised tissue samples including the residual lesion in the cheek in situ were subjected to high-sensitivity NIR scanning (instrument settings as described above; Odyssey CLx, LI-COR Biosciences) as native whole-mounts, followed by fixation (10% formalin, over night, RT, in the dark). Images were used to estimate the remnant tumor size. To detect both antibody distribution and topology of the resected lesions, the cheeks were sliced into 400 µm thick sections (Vibratome VT 1000 S, Leica), scanned again for fluorescence (Odyssey CLx), embedded in paraffin, sliced into 7 µm thick sections and scanned for NIR fluorescence (Odyssey). Subsequently, samples were stained for CK and DAPI or H&E and the amount of tumor cells in the remnant was estimated by counting the tumor cells of one central slide multiplied by the volume of cytokeratin positive sections, taking into account that each nucleus is present on every 1.7 section based on a mean diameter of a tumor cell nucleus of 12.3 µm (averaged from 50 UT-SCC58 cell nuclei).

Statistics

Statistical analysis was performed using the non-parametric Mann-Whitney test with a post-hoc correction for multiple comparisons ($p \le 0.05/N$).

ACKNOWLEDGEMENTS

We gratefully acknowledge Marlène Hekman, Gert-Jan Bakker, Irene Otte-Holler, Piet Slootweg and Manon Vullings for expert technical assistance, sample selection and helpful discussions; Véronique Orian-Rousseau for providing BIWA and Bianca Lemmers-van de Weem and co-workers at the Central Animal Facility for assistance with animal experiments. P.F. is supported by NWO-VICI (918.11.626), the European Research Council (ERC-CoG DEEPINSIGHT, Project No. 617430) and the Cancer Genomics Center, The Netherlands.

AUTHORS CONTRIBUTION

J.O. designed and carried out the experiments, analyzed the data, supervised the work and wrote the manuscript. M.R. designed and supervised *in vivo* experiments. D.B. generated ¹¹¹In-BIWA-IRDye800CW and ¹¹¹In-IgG1-IRDye800CW and performed SPECT/CT and biodistribution experiments. E.W. performed FGS experiments. H.C. performed IHC experiments. R.G. contributed to the UT-SCC58 cell isolate and the relevant clinical background information. O.B. designed and supervised *in vivo* experiments. R.T. supervised the work. P.F. designed the experiments, supervised the work and wrote the manuscript. All authors read and corrected the manuscript.

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Supplementary table 1. [iterature surve	y on proteins expressed in HNSCC and ap	pplication for medical or intra	operative imagi	ng.	
Marker	Prevalence (%) in HNSCC	Expression level and distribution in tumor cells	Pattern in non-tumor tissue	lmaging application	Used antibody [#]	References
C-Met / Hepatocyte Growth Factor Receptor (HGFR)	~ 62%	Upregulated in primary tumor; upregulated in invasive front; pos. in metastasis, strong cytoplasmic signal	Weak expression mainly in basal layers of epithelium; mainly neg. in stroma	1		[1-9]
Carcinoembryonic antigen (CEA)	~ 69%	Heterogeneous; stronger staining in differentiated and keratinized cells	Neg	ı	ı	[1, 10-13]
CD44	~ 77%	Slightly diminished in poorly differentiated carcinomas; decreased in tongue carcinoma; predominantly found at invasive front of tumor; higher in metastasis	Expression in normal epithelium and lymphocytes			[14-17]
CD44v6	~ 97%	Pos in metastasis; no sig. difference to invasive front; slightly increased, but diminished in poorly differentiated carcinomas	Expression mainly in basal layers of epithelium and endothelium	Preclinical [18-23] Clinical [24- 26]	U36/ Bivatuzumab	[17, 27-32]
E-cadherin	~ 74%	Higher expression at tumor centre; neg in poor differentiated tumors	Strong expression in normal epithelium	ı	ı	[33-41]
Epidermal Growth Factor Receptor (EGFR)	~ 85%	Pos in paired metastasis; stronger at invasive front	Expression mainly in basal layers of epithelium	Preclinical [42-49]* Clinical [50]*	Cetuximab, Panitumumab	[51-60]
Epithelial Cell Adhesion Molecule (EpCAM)	~ 61%	Heterogeneous; diminished in metastasis	Neg in normal squamous epithelium; pos in hyperplasia	I		[61-64]
Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) / CD147 / Basigin	~ 76%	Higher in metastatic tumors; partially higher at invasive front	Low in mucosa	Preclinical [65, 66]		[67-69]

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SUPPLEMENTARY MATERIAL

Supplementary table 1.	Literature surve	y on proteins expressed in HNSCC and ap	pplication for medical or intra	aoperative imagi	ng. (continued)	
Marker	Prevalence (%) in HNSCC	Expression level and distribution in tumor cells	Pattern in non-tumor tissue	lmaging application	Used antibody [#]	References
Insulin-like growth factor receptor (IGFR)	~ 58%			. 1	1	[70-73]
Mucin-1	~ 74%	Heterogeneous; pos in metastasis; strong cytoplasmic staining	Low expression in mucosa	I	I	[74-77]
Vascular Endothelial Growth Factor (VEGF) (-A)	~ 78%	Heterogeneous	Expressed in epithelium, endothelium and immune cells	Preclinical [78]	Bevacizumab	[79-83]
* Only fluorescence imagi	na included					

* Only fluorescence imaging included # For epitope-based preclinical or clinical targeting, used antibodies are mentioned.

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В

	C-Met	CD44v6	E-cadherin	EGFR	EMMPRIN	EpCam
	Strong	Strong	Strong (4/5)	Strong	Strong (3/4)	Strong (1/5)
Tumor	(5/5)	(7/7)	-	(5/5)	-	Weak (2/5)
			Weak (1/5)		Weak (1/4)	Neg (2/5)
Tumor border	Positive	Positive	Positive	Positive	Positive	Positive
Epithelial tissue	Positive	Positive	Positive	Positive	Positive	Positive
Background stroma	Strong	Weak	Weak	Strong	Weak	Strong

Supplementary figure 1. *In situ* detection of candidate targets for FGS in human HNSCC tumors. **A** Representative primary HNSCC lesion showing expression of c-Met, EMMPRIN (weak expression), E-cadherin and EpCAM. Tumor (T), normal epithelium (E), stroma (S). Scale bars indicate 1000 µm (overview) and 100 µm (zoom). **B** Scoring of expression level in 4-7 primary HNSCC lesions based on immunohistochemical staining.



Supplementary figure 2. Expression of CD44v6 in HNSCC and other cell lines cultured *in vitro* and UT-SCC58 tumors *in vivo*. **A** Surface expression of CD44v6 in 9 HNSCC and 2 melanoma cell lines maintained in liquid culture, detected by flow cytometry for IgG1 control (black lines) and CD44v6 (red lines). Numbers indicate the geometric mean. **B** Immunofluorescence whole-region analysis of Fig. 4D. Co-localization of CD44v6 and pan-cytokeratin (CK) staining in different areas in UT-SCC58 tumors analyzed at day 28 after implantation. Data show the medians from 12-38 analyzed images per tumor from 4 independent tumors.



Supplementary figure 3. Radioactive and fluorescence detection in cervial lymph nodes. **A** Biodistribution expressed as percentage injected dose of ¹¹¹In-DTPA-BIWA-IRDye800CW and ¹¹¹In-DTPA-IgG1-IRDye800CW in right and left cervical lymph nodes (LN). **B** Central section and NIRF signal of a left cervical LN of a mouse injected with dual-labeled BIWA. Dotted line marks the LN edge. Scale bars indicate 500 μm (overview) and 50 μm (zoom). **C** Detection of CK in left cervical LNs. Scale bars indicate 500 μm.


Supplementary figure 4. HE staining and NIRF signals in tumors injected with dual-labeled BIWA and IgG1 isotypic control. Central, serial sections of mouse cheeks (H&E staining and NIRF signal) indicating the tumor border (dotted line; T), normal epithelium (E) and stroma (S); Left zoomed images: (1) tumor border and (2) false positive NIRF signal. Right zoomed images: (1) positive signal from tumor cells and (2) negative back-ground from tumor stroma. Arrowheads indicate membranous staining. Scale bars indicate 2 mm (overview) and 100 µm (zoom).

CHAPTER 5

Compatibility of CO₂ laser surgery and fluorescence detection in head and neck cancer cells

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Accept with minor revision

Surgical treatment of cancer requires tumor excision with emphasis on function preservation which is achieved in (early stage) laryngeal cancer by transoral carbon dioxide (CO₂) laser surgery. Whereas conventional laser surgery is restricted by the surgeon's visual recognition of tumor tissue, new approaches based on fluorescence-guided surgery (FGS) improve the detection of the tumor and its margin. However, it is unclear whether fluorophores are compatible with high-power laser application or whether precision is compromised by laser-induced bleaching of the dye. We applied topology-controlled 3D laser resection of fluorescent tumors *in vitro* and laser-induced autofluorescence analysis ex vivo. Laser-induced bleaching of fluorescent dyes in the visible and near-infrared light spectrum (650-900 nm) ranges below the resolution range of operation microscopes. Furthermore, specific fluorescent signals in an FGS mouse model is 10⁴ higher than laser induced autofluorescence in mouse tissue. Laser-induced lateral photobleaching is negligible indicating a path forward for fluorescence-guided laser surgery in head and neck cancer.

INTRODUCTION

The use of the carbon dioxide (CO_2) laser in transoral surgery of the larynx was first described in 1972 ⁽¹⁾. During the following decades it has become an established treatment modality for the transoral resection of early stage laryngeal as well as oropharyngeal and hypopharyngeal squamous cell carcinomas. With comparable oncological outcomes, its advantage over open partial laryngectomy is considered to result from lower complication rates and faster recovery ⁽²⁻⁴⁾.

The objective of surgical oncological treatment is to achieve adequate surgical margins which are free of tumor cell infiltration. In an anatomically and functionally delicate structure such as the larynx this is challenging to achieve, due to the choice of small margins to preserve as much tissue as possible. In the search of techniques that could be helpful in optimizing surgical outcomes, a new, evolving technique is the use of fluorescence as guidance during surgery with the potential use for all areas in head and neck squamous cell carcinoma (HNSCC) ⁽⁵⁾. During this fluorescence-guided surgery (FGS), autofluorescence of the tumor in the visible light range can be used as guidance ⁽⁶⁾, and much improved signals have been achieved by conjugating fluorophores in the near-infrared (NIR) range of 650-900 nm to targeting antibodies which bind extracellular epitopes preferentially expressed on tumor cells ⁽⁵⁾. NIR-FGS allows biomarker-based demarcation of the tumor margin, potentially detecting tumor residues and invasive regions which remain undetected otherwise.

 CO_2 laser surgery is usually applied through a microscope and this type of surgery could potentially be combined with FGS with NIR fluorophores. However, when performing laser surgery, thermal damage and photobleaching is caused to the tissue which also might interfere with the fluorophore signal ^(7, 8). To combine NIR-guided tumor detection with transoral laser surgery (TOLS), the compatibility of both approaches has to be established, particularly whether CO_2 laser ablation may negatively affect fluorophore signals. Thus, the purpose of the current study was to test whether CO_2 laser ablation has an effect on fluorophore signals, potentially hampering its application in TOLS.

MATERIAL AND METHODS

Cell culture

Mouse mammary tumor (MMT) cells expressing nuclear EGFP coupled to histone-2B (H2B) ⁽⁹⁾ were used as a proxy for an epithelial solid tumor type. The cells were maintained in RPMI 1640 growth medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA).

HNSCC cell line UT-SCC58 was a kind gift from Reidar Grenman, University of Turku, Finland ⁽¹⁰⁾ and cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate (both Invitrogen/Life technologies) and 20 mM HEPES (Gibco).

To generate SCC58-H2B-GFP cells, lentiviral particles, produced in HEK293T cells using the ViralPower expression system (Life technologies, containing pLenti6.2v5-H2B-GFP (Clontech) were used followed by blasticidin selection (5 µg/ml) and cell sorting for GFP-expressing cells (FACS Aria SORP, Becton Dickinson).

Antibody conjugation

NIR fluorescent conjugation to murine anti-CD44v6 IgG1 antibody (BIWA), the humanized form of which (Bivatuzumab, BIWA-4) has been applied in human clinical trials was performed. In short, BIWA was incubated with a 3-fold excess of IRDye800CW-N-hydroxysuccinamide (NHS) in 0.1 M sodium carbonate buffer (pH 8.5) for 1 h at room temperature (RT). The final concentration of the antibody and the molecular substitution ratio were determined spectrophotometrically (Ultrospec 2000 spectrophotometer, Pharmacia Biotech) yielding an antibody:fluorophore ratio of 1:2. BIWA-IRDye800CW was stored in the dark at 4°C.

Unperturbed immunoreactivity of the antibody conjugates was confirmed as described ⁽¹¹⁾.

3D tumor spheroid culture and CO₂ laser treatment

Sub-confluent cells were detached using trypsin/EDTA (SCC58-H2B-GFP) or EDTA (MMT-H2B-GFP) (Life Technologies) and aggregated to multicellular tumor spheroids using the hanging-drop method. Cells were suspended in DMEM or RPMI medium supplemented with 10% methylcellulose solution (Sigma) and incubated as 30 µl droplets (50,000 cells/drop). After overnight incubation, spheroids were harvested, washed with PBS, washed again in full growth medium, and embedded in non-pepsinized rat tail type I collagen lattices (BD Biosciences, final concentration: 5 mg/ml) on a layer of rat tail type I collagen in 12-well plates. Spheroids were allowed to invade 3D collagen scaffolds *in vitro* for 72 h. To reach equilibrium saturation in SCC58-H2B-GFP spheroids, 5 μ g/ml of the antibody BIWA-IRDye800CW was added to the growth medium at time point 0. After removal of the medium, spheroids were burned with a CO₂ laser (SHARPLAN, Laser vision) using a single pulse, 2W for 0.1, 0.5 or 1 sec (MMT-H2B-GFP), for 0.5 sec (SCC58-H2B-GFP) or cut with a scalpel.

Spheroids imaging and analysis

After partial laser dissection of spheroids, which aimed to remove approximately one third to half of the tumor mass, the remaining portion was analyzed by epifluorescence or confocal microscopy. MMT-H2B-GFP spheroids were imaged by sequential confocal scanning keeping an inter-slice distance of 10 μ m (Olympus FV100) using a 20x/0.5 NA water objective. NIR and GFP imaging of SCC58 spheroids was performed with the Nuance XR (PerkinElmer; 20x NA 0.8; mono setting, 5 sec acquisition time for NIR).

Digital image analysis was performed using Fiji (software version 1.51k, https://imagej.net/Fiji). Rectangle regions of interest crossing the resection margin were defined (blue rectangle in figure 1B) and the intensities of the reflectance and fluorescent signals were obtained in perpendicular direction to the resection margin (figure 1C, arrow). Resection margins from laser cuts were irregular due to debris. Thus, the slope of reflectance and fluorescent intensity signal were gradual over distances of 100 µm and varied from sample to sample. Therefore, intensities were normalized to define cutting edges for inter-sample comparison, as follows: the edge between tissue-free background and the onset of signal was defined as inner margin (0% value). Then the region approximating maximum fluorescence intensity was identified to denote unperturbed tissue (100% value). To avoid errors caused by debris, the tissue region representing 50% of the maximum of the fluorescence channel was defined as reference point, thus representing the zone of clearly detectable fluorescence (figure 1D, blue dotted line). The distance of bleaching was obtained as distance between the inner margin of the reflectance channel to the location of 50% GFP intensity (MMT-H2B-GFP). Because IR-Dye800CW detection using the Nuance imaging system did not enable reflection signals, the H2B-GFP signal was used as reference for SCC58 cells, as H2B-GFP largely withstood exposure to laser irradiation and showed only negligible bleaching below 50 µm in extension.

Animal experiments

The animal experiments were performed in accordance with the guidelines and rules of the Dutch Act on animal experiments (WOD) and was approved by the Animal Welfare body of the Radboud University, Nijmegen (RU-DEC 2014-142). A laryngeal tumor mouse model is ethically and logistically not possible to perform, thus 9 x 10^5 UT-SCC58 cells suspended in 3 mg/ml Matrigel (BD Biosciences; total volume 50 µl) were injected via an intraoral approach into the left floor of mouth of BALB/c nu/nu female mice (Charles River Laboratories; 6-8 weeks old). Tumors were allowed to grow for 4-6 weeks which led to tumor extension in the floor of mouth and the left cheek. Tumor growth over time was measured using a caliper. UT-SCC58 cells were chosen as they resemble the Immunohistochemical invasion and growth pattern of human HNSCC.

Mice bearing UT-SCC58 tumors in the left cheek were injected into the tail vein with 10 µg of BIWA-IRDye800CW. Three days after injection mice were euthanized and whole-body fluorescence images were acquired (IVIS Lumina imaging system, Caliper Life Science) using the following settings: F/Stop - 2, excitation filter - 745 nm, emission filter – ICG (810-875 nm), field of view - C, 675 nm autofluorescence and background correction, recording time of 1 min and medium binning factor.

To further test whether laser application could create false-positive events by autofluorescence induction, mouse skin was burned with a CO₂ laser (SHARPLAN, Laser vision) using the following settings: single pulse, 2 or 5W for 0.1, 0.5 or 1 sec. Mouse floor-of-mouth tissue was burned with a single pulse, 2W for 0.5 sec and the wound was washed with PBS. Subsequently, the fluorescence was detected by whole-body imaging (IVIS Lumina; settings as described above). Images were processed with the IVIS Lumina software. For NIR intensities, regions of interest (ROI) were defined manually to represent laser-treated tissue. Background values from tissue free areas of the same images were subtracted for each specific measurement.

Statistics

Statistical analysis was performed using GraphPad Prism (version 5). Non-parametric Mann-Whitney test with a two-tailed p value and a conficence interval of 95% was chosen. In addition, a post-hoc correction for multiple comparisons ($p \le 0.05/3$) was used for data shown in figure 1.

RESULTS

Stability of fluorophores after CO₂ laser treatment

The effects from lasers on tissues have been well-described, causing a very fine carbonization zone, a necrotic zone and cell debris including a denaturing of collagen ⁽⁷⁾. To mimic this effect and study the influence of CO₂ laser application on fluorescence in a tumor-like model, GFP fluorescent tumor spheroids were embedded in a 3D collagen lattice (Figure 1A). Spheroids were allowed to invade into the collagen for 72 h and subsequently treatment was performed with a single pulse of a CO₂ laser (Figure 1B) or cut in half with a scalpel as control. Analysis of the cutting edge (Figure 1 C, D) indicated a non-significant shift of the genetically encoded, histone-bound GFP fluorescent signals to the reflection signal caused by fibrillar collagen and with lower intensities cells (Figure 1E). This shift was independent of the duration of the single CO₂ laser shot. Thus, the ablation of genetically encoded fluorescence in the green visible light range by a CO₂ laser occurred within the spatial range of collateral thermal damage in patients which lies on average between 150 - 160 μ m ^(12, 13).

To analyze a fluorophore in the NIR range, spheroids were stained with the anti-CD44v6 antibody BIWA conjugated to the NIR dye IRDye800CW. Spheroids were then cut with a scalpel or burned with a CO₂ laser (Figure 2A). Fluorescent images showed an equal distribution of the antibody throughout the spheroid prior to laser ablation (Figure 2B). This distribution was not influenced after cutting with a scalpel or a CO₂ laser, with identical position of NIR fluorescence to the histone-tagged GFP signal, which has been shown to be the same as the collagen signal (Figure 1E), in either cutting method (Figure 2C). Thus, CO₂ laser application does not lead to significant ablation of cell-surface bound NIR fluorescence.

Interference of CO₂ laser-dependent autofluorescence and NIR fluorescence

Burning wounds caused by lasers induce autofluorescence of the tissue ⁽¹⁴⁾. Consequently, we tested whether the resulting autofluorescence can produce false-positive results during surgery (Figure 3). Single pulse shots with 2 or 5 W causing roundish wounds in the mouse skin caused autofluorescence in the NIR fluorescent range (810-875 nm) with increasing intensities depending on laser power and pulse duration (Figure 3A). However, the *in vivo* fluorescent signal from an oral tumor targeted with BIWA-IRDye800CW was approximately 10⁴ times higher as the caused autofluorescence (Figure 3B). Since autofluorescence is tissue-specific ⁽¹⁵⁾, autofluorescence originating from the floor-of-mouth was also compared. A treatment with 2W for 0.5 sec was not



Figure 1 Stability of genetically encoded H2B-EGFP and DsRed2 after CO₂ laser treatment. **A** Experimental setup. **B** Brightfield image of MMT-H2B-GFP spheroids incubated in rat tail type I collagen for 72h before (left) and after (right) treatment with a CO₂ laser. **C** Confocal image showing the burned edge of an MMT-H2B-GFP spheroids marked in A. Numbers indicate time of CO₂ laser shot. **D** Plot profiles show analysis of area in (B). The blue dotted line indicates the edge according to reflection signal. **E** Graph shows analysis of plot profiles from 1-8 spheroids. Scale bars indicate 100 µm.



Figure 2 Stability of near infrared fluorescent (NIRF) antibody (BIWA-IRDye800CW) after CO₂ laser treatment. **A** Scheme of experimental setup. Arrowheads indicate cutting edge, blue rectangle marks area of analysis. **B** Brightfield and fluorescent images showing SCC58-H2B-GFP spheroids before and after treatment. BIWA-IRDye800CW (red) and H2B-GFP (green) signals were detected by epifluorescence microscopy. Scale bar indicates 100 µm, arrowheads mark cutting edge. **C** Analysis of B as shown in figure 1 from 4 spheroids. Blue rectangle marks area of analysis.



Figure 3 Autofluorescence of burned wounds. **A** Brightfield and NIR fluorescence image of a mouse skin burned with a CO₂ laser. **B** NIR fluorescence whole body image of a mice with a growing HNSCC tumor in the left cheek 72 h after i.v. administration of BIWA-IRDye800CW (left). Tumor (T), liver (L). Images with the same settings of a mouse floor-of-mouth burned with a CO₂ laser (right). Arrowheads indicate burning wounds. **C** The graphs show an analysis of autofluorescence signals before and after washing the wound; n=3. Fl. Int. – fluorescence intensity in (p/sec/cm²/sr)/(μ W/cm²).

detected at the same image settings as the oral tumor (Figure 3B) and washing the wound did not influence the autofluorescence signal. Differences in the autofluorescence signals from the mouse skin and the floor-of-mouth are likely due to variations in tissue structure^(15, 16) and tissue preparation. In conclusion, CO_2 laser-dependent autofluorescence is unlikely to interfere with strong NIR signals.

DISCUSSION

In this study we provide proof of principle for a combination of NIR fluorescence imaging and CO_2 laser ablation in a 3D *in vitro* system. The occurring error range of fluorescent signal in the visible light range after laser ablation ranges within resolution limits of state-of-the-art operation microscopes. Furthermore, near-infrared fluorescence was not damaged by the CO_2 laser which, however, requires confirmation under clinical conditions. Taken together, the bleached corridor ranges below the extent of collateral thermal damage induced by a CO_2 laser which ranges between 0.15 and <0.3 mm^(12, 13, 17), thus enabling a combination of both CO_2 laser and fluorescence-guided surgery.

Fluorescence-guided surgery using NIR dyes has recently been introduced in HNSCC patients showing efficient tumor detection ⁽¹⁸⁾ and permitting visualization of microscopic lesions ⁽¹⁹⁾, thus potentially assisting the surgeon in more accurately defining tumor deposits and borders. Indeed, a first approach of fluorescent-guided laser surgery has been applied in a soft tissue sarcoma model using a Nd:YAG laser system which ablated far-red fluorescent tumor tissue with minimal disruption of adjacent tissue improving recurrence-free survival in mice ⁽²⁰⁾.

In addition, we confirm that laser wounds induce autofluorescence ⁽¹⁴⁾ in the NIR light range, but show that the unspecific fluorescent signal was 100 - 10,000 times below the specific signal emitted by the tumor after *in vivo* application of an IRDye800CW conjugated anti-CD44v6 antibody. Direct autofluorescence of tumor tissue has been used in TOLS using a D-light system to identify positive superficial margins in patients without mentioning difficulties with laser-induced autofluorescence ⁽⁶⁾. Thus, although autofluorescence-induced false-positive signals are unlikely in a fluorescence-guided laser surgery approach, caution should be taken for weak signals. Such weaker signals are expected for smaller tumors or tumors expressing less of the target epitope. Taking together, first *in vitro* and preclinical data suggest that fluorescence-guided laser surgery is an effective and promising technique in improving transoral laser surgery of laryngeal cancer but potentially of other tumors as well.

AUTHORS CONTRIBUTION

J.O. designed and carried out the experiments, analyzed the data and wrote the manuscript. P.F. designed the experiments, supervised the work and wrote the manuscript. R.T. supervised the work and wrote the manuscript.

ACKNOWLEDGEMENTS

We gratefully acknowledge Silvia van Vugt-Eijkhout and Corien Janssen for expert technical assistance and Véronique Orian-Rousseau for providing the BIWA antibody and Bianca Lemmers-van de Weem and co-workers at the Central Animal Facility for assistance with animal experiments. P.F. was supported by NWO-VICI (918.11.626), the European Research Council (ERC-CoG DEEP-INSIGHT, Project No. 617430) and the Cancer Genomics Center, The Netherlands.

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CHAPTER 6

General discussion and future directions



Head and neck squamous cell carcinomas (HNSCCs) are located in a functionally critical region including the naso-, oro- and hypopharynx, larynx, oral and nasal cavity. This heterogeneous group of tumors are characterized by intense local invasion which hampers effective treatment, consequently leading to a high frequency of local recurrence [1, 2]. In this thesis, new mechanism of local invasion and strategies to target this invasion are provided. Using an innovative 3D *in vitro* model for HNSCC invasion, growth factors have been identified as regulators of tumor cell migration plasticity. This finding may have a predictive value in targeting growth factor signaling in tumor progression. Collective invasion, as one type of growth factor-induced tumor cell migration, has been visualized using intraoperative fluorescence imaging in an HNSCC mouse model potentially contributing to the improvement of radical surgery.

COLLECTIVE MIGRATION AS HNSCC INVASION MECHANISM

Migrating cells can move either individually or as cohesive groups. During collective migration, cadherin-based junctions enable the maintenance of collective polarization, mechanocoupling and cytoskeletal kinetics of cohesive groups [3]. In cancer development, collective invasion differs from single cell migration which includes pro-survival signals due to cell-cell contacts and local growth factor or cytokine secretion, protection against immune responses and a passive movement of non-migratory cells. Highlighting the importance of collectivity is the growing evidence that epithelial cancer metastasis involves the traveling of tumor cell clusters into blood vessels followed by collective organ colonization [4, 5].

The primary strategy of squamous cell carcinoma invasion is collective migration [3]. Three-dimensional reconstruction of human oral tongue SCCs, multi-photon imaging of an oral tongue SCC mouse model and immunohistochemistry results of the oral HNSCC mouse model, as presented in Chapter 4, show multicellular groups at the invasive front [6, 7]. These findings support the presence of collective invasion strategies in HNSCC. Furthermore, buds of primary tumor masses have been observed in immunohistochemical analysis to frequently penetrate lymphatic vessels [6, 8], and these nests coincide with similar immunohistochemically detected collective invasion zones shown in this thesis. As further clinically relevant complication, local collective invasion in the soft tissue of the head and neck area accounts for difficult-to-detect tissue contamination with tumor cells and hence incomplete tumor removal. As a consequence, collective migration is one aspect of invasion which needs more examination in HNSCC.

Fibroblasts have been shown to guide collective invasion in epithelial cells [9-11]. However, in the HNSCC models used for this thesis, fibroblast guided invasion is negligible, as low concentrations of both hepatocyte growth factor (HGF) and epidermal growth factor (EGF) efficiently stimulate collective migration despite the absence of fibroblasts. Both growth factors signal via MAPK and Akt signaling pathways [12], which are also activated by other growth factors and cytokines. Thus, low levels other growth factors or cytokines likely induce collective invasion as well [13]. In conclusion, comparable to breast and colon cancer [14, 15], HNSCC predominantly invades by collective strategies likely stimulated by growth factors and cytokines.

AMOEBOID DISSEMINATION AS HNSCC INVASION MECHANISM

On the basis of morphology, kinetics and function, amoeboid and mesenchymal movement have been defined *in vivo* as the two types of single cell migration. Amoeboid dissemination is an emerging avenue in cancer research [15] and although it is difficult to appreciate in histological sections, live cell culture and intravital imaging reveal amoeboid migration in carcinoma cells [16, 17]. In HNSCC, rounded cells with amoeboid-like nuclear deformation were detected in hypoxic regions of clinical samples, suggesting a collective-to-amoeboid transition under oxygen deprivation [18]. Furthermore, amoeboid migration has been detected *in vivo* in breast cancer mouse models by intravital microscopy [19], indicating that this migration type is a frequent mode across tumor types.

In Chapter 3, multicellular spheroid culture stimulation with high growth factors concentrations downregulates E-cadherin and stimulates disseminating single migrating cells with a roundish-amoeboid movement. As particular subtype identified in this thesis, amoeboid moving HNSCC cells were proteolytic and generated migration tracks in the collagen. Furthermore, migration was largely dependent on proteinases since matrix metalloproteinase inhibition almost blocked migration. Thus, amoeboid cancer cell movement may occur as cell-intrinsic, hypoxia- or growth factor-induced variants, including HGF and transforming growth factor- β (TGF- β) in melanoma, breast and HNSCC cells [18, 20, 21].

In addition, whereas collective migration depends upon tissue remodeling to form multi-cellular or epithelial structures, mesenchymal and amoeboid migration are considered to enable efficient cancer-cell spread throughout interstitial tissues with amoeboid migration being the fastest migration type [22-24]. Consistently, in HNSCC, reduced expression or loss of the epithelial marker E-cadherin is associated with lymph node metastasis, tumor stage, poor differentiation and infiltrative margin [25-27]. Together, these findings lead to the concept that detachment of tumor cells is more efficient in migration and leads to poorer prognosis. However, the relevance of amoeboid migration in driving metastasis warrants further exploration [15, 17].

PLASTICITY OF HNSCC INVASION

Cell migration plasticity is defined as the conversion from one migration mode to another. It can be triggered *in vitro* by several mechanism including matrix confinement as shown in transition from mesenchymal to collective migration with increasing collagen density [28] or the transition from collective to amoeboid migration under hypoxic conditions [18]. Mechanisms by which growth factors or cytokines induce plasticity are summarized in Chapter 2. In Chapter 3, HGF released by fibroblasts is identified as potent inducer of simultaneous collective and single cell migration in HNSCC spheroids. Furthermore, fibroblast conditioned medium induces a concentration-dependent transition from collective to single cells with single cells or clusters detaching from collective strands.

Given that each migration mode has unique advantages and potential shortcomings for tumor cell spreading, plasticity induction is thought to facilitate the possibilities for escape in a heterogeneous tumor microenvironment. The concept that tumor cell migration plasticity might be the most efficient strategy *in vivo* is supported by mathematical models. The ECM is characterized by structural heterogeneity [29] while chemical gradients are present simultaneously [30, 31]. In such heterogeneous conditions, *in silico* amoeboid-mesenchymal migration plasticity is most efficient [32]. Accordingly, amoeboid cells can benefit from the presence of a small number of paths created by slower migrating proteolytic cells [32]. Thus, as also shown in this thesis, *in vitro* systems studying tumor invasion should take into account the complexity of the microenviroment by combining 3D structural and chemical heterogeneities.

Similar to the models described in this thesis, patient samples of HNSCC show detached heterogeneous groups of single cells and clusters reaching hundreds of cells at the invasive zone [6]. These findings highlight remarkable variance of invasion mechanisms present in the same tumor, including differences in cluster size and heterogeneous morphology including spheroidal, amoeboid, branching or stretching features [6]. In conclusion, migration plasticity is part of tumor heterogeneity resulting in a major challenge to overcome in cancer treatment.

COOPERATION OF MOLECULAR PLASTICITY AND INVASION PLASTICITY

The results of this thesis indicate that amoeboid movement can be associated with epithelialmesenchymal transition (EMT), a well-established molecular program driving cancer progression, metastasis and therapy resistance [33]. EMT inducers such as Twist or Snail can initiate invasion and metastasis in animal models and correlate with poor prognosis [18, 34-36]. E-cadherin, as epithelial marker, decreases at invasive regions in HNSCC patient samples [25] as well as other cancers. Cells expressing EMT markers have been localized to the periphery of the tumor where they are in closer contact to growth factors of the tumor environment [37, 38]. This phenotype is reflected in SCC58 cells in our spheroid model in which E-cadherin was gradually down-regulated in collective invasion strands and reached minimum levels in detached cells while vimentin was more prominent in invasive cells. These similarities suggest that during cancer development the transition from an epithelial to a mesenchymal state must not be absolute and independent from one another, but that it is instead a continuous spectrum of both properties. Thus, plasticity of invasion programs and EMT may partially overlap, likely in response to environmental cues [25, 35, 38]. As outcome, this epithelial plasticity might then lead to single cell and/or collective cell migration and each migration mode may harbor EMT-like and EMT-independent signaling states. However, besides induction by defined triggers, EMT may also occur as 'random' process in small cell subsets, as reflected in the SCC38 cell line in Chapter 3. Similarly, vimentin or snail are often found in subsets of tumor cells in HNSCC patient samples [39, 40], reflecting EMT as a 'random' process.

EMT induction in cancer appears to have clinical significance, potentially offering new drug targets, e.g. by neutralizing factors which induce EMT. As seen in Chapter 3, HGF induces cell migration, destabilizes cell-cell adhesions and regulates vimentin expression. Inhibition of HGF signaling *in vitro* readily reduces single cell migration and markedly reduced collective migration as outcome. Likewise, TGF- β is known to induce EMT and migration in several tumor models, and inhibition of the signaling in a rat mammary carcinoma model inhibited single cell migration whereas collective migration and subsequent lymphatic metastasis were maintained [23]. Together, these results suggest that growth factor induced collective migration is particularly resistant to treatment, asking for complementary targeted approaches for collective processes in cancer.

FLUORESCENCE-GUIDED SURGERY

The importance of resection margins varies between cancer types. While positive surgical margins in kidney cancer patients are not associated with an increased risk for local recurrence or metastasis [41], incomplete resection is an important risk factor limiting the prognosis of HNSCC [42, 43] and pancreatic cancer patients [44]. This indicates the importance of radical surgery in HNSCC. Thus, new technologies, such as intraoperative fluorescence imaging, show the potential to improve tumor detection and outcomes of curative surgery. Indeed, animal models of pancreatic and colorectal cancer have shown better tumor resections, detection of small satellite tumors and longer disease-free survival [45, 46]. Chapter 4 describes the dissection of orthotopic HNSCC tumors under clinic-like conditions using a commonly used operation microscope. Here, tumor remnants of approximately 0.7 mm in diameter consisting of a few thousand cells were reliable detected by near-infrared fluorescence, allowing precise dissection of invasive microregions. Other fluorescence imaging systems were able to visualize and resect tumors of comparable sizes in vivo and in patients which were otherwise not detected under standard conditions [47, 48]. Thus, fluorescence-guided surgery (FGS) shows promise to detect the invasion zones and improve outcomes of HNSCC surgery. However, a significant part of HNSCC invasion consists of small cluster and even single cells [6]. Although small strands or clusters were detected by the CD44v6 targeting antibody fluorescence imaging, this method may be insufficiently sensitive to detect such small lesions under clinical conditions. Thus, despite efficient detection of the collective invasion zone, single cell dissemination beyond the resection margin may remain a potential threat for optimal results of surgical procedures. Importantly, although successfully applied in animal models, FGS still has to prove its contribution to recurrence-free and overall survival in patients.

CD44V6 AS TARGET IN HNSCC

The consistent expression of CD44v6 across heterogeneous HNSCC lesions and most, if not all, HNSCC tumor cells emphasize CD44v6 as a promising antigen for reliable FGS in HNSCC cancer. In addition, the availability of clinically applicable anti-CD44v6 antibody Bivatuzumab enables visualization of tumor margins and minimal residual lesions (Chapter 4). Targeting CD44v6 in

other cancer types has been shown to inhibit proliferation and migration *in vitro* and *in vivo* and seems to have a role in c-Met and vascular endothelial growth factor receptor signaling [49-51]. Thus CD44v6 could potentially be used in cancer therapy after surgery. However, despite its high and homogeneous expression in HNSCC patients, the association with CD44v6 and disease outcome is contradictory and depends on the tumor site [52-54]. In addition, inhibiting CD44 and its variants shows only a minor function in migration and invasion in cancer cells in 3D *in vitro* models [55, 56]. Thus, besides its use for intraoperative detecting of the tumor margin, therapeutic targeting of CD44v6 in HNSCC might require careful selection among patient groups.

CONCLUSION

The work presented in this thesis identifies collective invasion as important target for reducing the emergence of local tumor infiltration into healthy tissue and improving the determination of the optimal resection margin for curative surgery. Fluorescence imaging, because of its high sensitivity and real-time capabilities has great potential to improve surgical outcomes. Strategies include the targeting of proteins such as CD44v6 which are highly expressed in collective invasion which is characteristic for HNSCC. Subsequently, since growth factors or cytokines are major contributors in driving collective tumor invasion as well as migration plasticity with cell detachment, they form an interesting target in post-surgical treatment (Figure 1). Thus, FGS could improve surgical outcomes and reduce the need for adjuvant treatment with its additional toxicity and costs. In this way it can add to better oncologic as well as functional outcomes and therefore also



Figure 1. Growth factor induced cell migration plasticity and potential targets for fluorescence-guided surgery and/or interference of cell migration processes.

more cost-effective treatment. As FGS cannot detect small volumes of detaching cells it could be combined with other novel treatments such as immunotherapy (Figure 1). Such combinations have been shown to decrease metastatic recurrence in animal models of pancreatic cancer, breast cancer or soft tissue sarcoma [57-59] and are informed by mechanistic cell biological studies as in this thesis. This dual approach could enable minimally invasive, function-preserving surgery and destruction of microscopic tumor deposits.

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CHAPTER 7

Summary



Head and neck squamous cell carcinoma (HNSCC) arises from the mucosal surfaces of the upper aerodigestive tract. Despite efforts in improving both surgical and non-surgical treatment, outcomes have barely improved with 40-50% of patients developing lethal recurrences and metastasis. Characteristic for HNSCC is its local invasion in the often functionally important soft tissues of the head and neck. Therefore, in case of surgical treatment, the resection margins are often inadequate and/or the functional consequences of treatment are significant. Inadequate margins require further surgery or post-operative (chemo-) radiotherapy. This additional oncologic treatment adds to the toxicity and impact on quality of life. Thus, to improve cancer treatment, it is important to understand the basis of HNSCC tumor cell invasion and to optimize surgical outcomes.

Analysis of HNSCC tumors have identified growth factor and cytokine signaling as potential targets which contribute to carcinogenesis and invasion. By acting on the cell intrinsic properties, likely under the control of transcriptional programs such as the epithelial-to-mesenchymal transition (EMT), growth factors and cytokines can induce all known forms of cell migration. These are individual cell movements without cell-cell interactions to neighboring cells or collective migration, where cells move as cohesive groups with cell-cell junction remaining intact. In addition to the induction of cell migration, growth factors and cytokines are also able to cause an interconversion of the tumor cell dissemination strategies, termed plasticity of invasion. However, how invasion plasticity is spatiotemporally controlled upon challenge by the tumor microenvironment and whether inhibiting these factors can prohibit the plasticity progress remains unclear. A general introduction and outline of the thesis are given in **Chapter 1**.

In **Chapter 2** we group current concepts and functional consequences of growth factor and cytokine signaling control in key "modules" of single-cell and collective invasion programs. By focusing on the best-documented examples that support the concepts, influences on cell-matrix adhesion, cell-cell interaction, cytoskeletal dynamics and remodeling of the extracellular matrix are described. Individual and combined effects of growth factors and cytokines contribute to the reprogramming and plasticity of cancer invasion programs as typically detected in the tumor microenvironment. To better understand the mechanisms of invasion plasticity and potential intervention points, combining cell-based *in vitro* analysis, preclinical mouse models, clinical outcome studies and mathematical modeling is necessary to dissect the cooperation of growth factors and cytokines and improve outcome by therapeutic targeting.

Fibroblasts, as part of the tumor stroma, secrete growth factors and cytokines. In **Chapter 3**, the presence of fibroblasts induce collective as well as single cell migration at the same time in HNSCC cells in a 3D collagen spheroid invasion assay. Hepatocyte growth factor (HGF) was identified as main contributor of fibroblast conditioned medium to induce this invasion. In addition, increasing concentrations of fibroblast conditioned medium, HGF or epidermal growth factor (EGF) promoted collective-to-single cell transition. The majority of detaching cells developed amoeboid migration and, uncharacteristically for amoeboid migration, degraded the collagen

which was dependent on matrix metalloproteinases. To complement biochemical analysis of entire cell populations, novel 3D single-cell cytometry was developed to detect molecular markers of the EMT in tumor cell subsets. Both collective and individual invasion zones expressed the EMT-marker vimentin with approximately 20% up to 80% frequency, depending on the cell line. Inhibition of HGF signaling eliminated single cell dissemination and reduced collective invasion, indicating differential sensitivity to targeted intervention. Thus, HGF induces EMT together with collective, proteolytic-amoeboid and, less frequently, mesenchymal invasion. This indicates EMT as a probabilistic molecular program, in association with any invasion strategy.

As most promising strategy to improve surgical outcomes, image-guided surgery (FGS) aiming to identify the tumor margin shows great potential to guide and improve the precision of surgical excision. For FGS, antibodies with tumor selectivity become conjugated with a near-infrared fluorophore, are injected intravenously and accumulate in the lesion of the patient. They can be visualized subsequently by using an intra-operative imaging system. However, the type of biomarkers and detection strategies are under development and by combining fluorescence guidance with other types of surgery, such as CO₂ laser surgery, high-power lasers may bleach fluorophores and extinguish the signal. Thereby, it is uncertain whether CO₂ laser surgery can be effectively combined with FGS.

In **Chapter 4** we demonstrate the suitability for FGS in a preclinical model of invasive HNSCC. Based on a literature survey on potential surface molecules followed by immunohistochemical and functional validation, we here identify CD44v6 as antigen reliably expressed in the invasion zone of HNSCC lesions. The monoclonal anti-CD44v6 antibody BIWA was used for near-infrared fluorescent detection in a series of human HNSCC cells and a locally invasive tumor mouse model. BIWA accurately detected human HNSCC xenografts in mice with excellent signal-to-noise ratio and low uptake in the tumor-free margin and other organs. This was sufficient to resolve tumor portions below 1 mm consisting of a few thousand cells under clinical-like resection conditions. The data indicate CD44v6 as a suitable target for reliable near-infrared detection of invasive margins in HNSCC lesions *in vivo*.

In **Chapter 5** we demonstrate that the irradiation by a CO_2 laser does not interfere significantly with the fluorescence signal in the visible and near-infrared light spectrum *in vitro*. Furthermore, specific fluorescent signals in a FGS mouse model was 1000-times stronger than laser induced auto-fluorescence in mouse tissue. These findings indicate a compatibility of CO_2 laser surgery and FGS.

Chapter 6 discusses the relevance of cell invasion strategies for local tumor cell dissemination, the challenges of tumor cell migration plasticity on cancer treatment and the perspectives for targeting cancer invasion by surgical and molecular therapy.

APPENDIX

Dutch summary (samenvatting)

German summary (Zusammenfassung)

Acknowledgements

List of publications

Curriculum Vitae

SAMENVATTING

Het hoofd-hals plaveiselcelcarcinoom (HHPCC) ontstaat uit de mucosale oppervlakte van de bovenste aerodigestieve kanaal. Ondanks recente verbeteringen in chirurgische technieken en doelgerichte behandelmethodes, zijn de behandelresultaten nauwelijks verbeterd en 40-50% van de patiënten ontwikkelt lethale recidieven en/of metastasen. Kenmerkend voor HHPCC is de lokale invasie in het, vaak functioneel belangrijke, zachte weefsel van het hoofd en de hals. Daarom zijn de resectiemarges na een chirurgische behandeling vaak ontoereikend en / of zijn de functionele gevolgen van de behandeling aanzienlijk. Ontoereikende marges vereisen extra chirurgie of postoperative (chemo-) radiotherapie, wat bijdraagt aan de toxiciteit van oncologische behandelingen en de impact ervan op de kwaliteit van leven. Om de behandeling van deze kanker te verbeteren is het dus van belang de basis van tumorcel invasie in HHPCC te begrijpen en om de uitkomst van de chirurgische behandelingen te verbeteren.

Analyse van HHPCC tumoren heeft de signaaltransductie van groeifactoren en cytokines geidentificeerd als potentieel doelwit die bijdragen aan carcinogenese en invasie. Ze werken op transcriptionele programma's zoals de epitheliale-mesenchymale transitie (EMT) en kunnen zo alle bekende vormen van celmigratie induceren. Dit zijn zowel de individuele celbewegingen, waarbij er geen interactie is met naburige cellen, als collectieve migratie, waarbij cellen zich verplaatsen als cohesieve groepen met intacte cel-cel contacten. Naast het induceren van invasie, kunnen groeifactoren en cytokinen ook een conversie van de strategie van tumorcel verspreiding veroorzaken, hetgeen de plasticiteit van invasie wordt genoemd. Hoe de plasticiteit van invasie echter spatiotemporeel wordt gecontroleerd door de micro-omgeving van de tumor en of het remmen van deze factoren de voortgang van de plasticiteit kan remmen, blijft onduidelijk.

Een algemene inleiding en schets van de scriptie worden gegeven in Hoofdstuk 1.

In **Hoofdstuk 2** worden de huidige concepten en functionele consequenties van de signaaltransductie van groeifactoren en cytokines gegroepeerd in functionele "modules" die bepalend zijn in de keuze tussen een individueel, of een collectief invasieprogramma. We onderscheiden hier de invloed op celmatrix adhesie, cel-cel interactie, cytoskelet dynamiek en remodellering van de extracellulaire matrix op basis van goed gedocumenteerde voorbeelden. Dit hoofdstuk beschrijft verder hoe individuele en gecombineerde effecten van groeifactoren en cytokines cellen kunnen herprogrammeren en zo de plasticiteit van kankerinvasie veroorzaken. Om de mechanismen van de plasticiteit van invasie en potentiële interventiepunten beter te begrijpen, is het combineren van op cellen gebaseerde *in vitro* analyse, preklinische muismodellen, klinische studies en mathematische modellering noodzakelijk. Zo kan in de toekomst door specifieke therapie de uitkomst verbeterd worden.

Groeifactoren en cytokines worden geproduceerd door fibroblasten die zich in de tumor omgeving bevinden. **Hoofdstuk 3** beschrijft hoe er met behulp van een 3D-collageen-spheroïde invasie test wordt gedemonstreerd dat de aanwezigheid van fibroblasten zowel collectieve als enkelvoudige celmigratie in HHPCC cellen bewerkstelligt. We identificeren Hepatocyte groeifactor (HGF) als de belangrijkste factor waarmee fibroblasten de migratie van HHPCC cellen reguleert en laten zien dat toenemende concentraties van fibroblast geconditioneerd medium, HGF of epidermale groeifactor (EGF), een overgang van collectief naar individuele celmigratie induceert. De meeste individueel migrerende cellen ontwikkelden amoeboide migratie. Echter, deze cellen waren nog steeds in staat om collageen af te breken, en waren voor migratie afhankelijk van matrix-metalloproteïnasen, wat ongebruikelijk is voor amoeboide migratie. Om de biochemische analyse van volledige celpopulaties aan te vullen, werd een nieuwe 3D-celcytometrie ontwikkeld om moleculaire markers van EMT in subsets van tumorcellen te detecteren. Zowel collectieve als individuele invasiegebieden brachten de mesenchymale marker vimentine tot expressie met een frequentie van ongeveer 20 tot 80%, afhankelijk van de cellijn. Remming van de HGF signaaltransductie route elimineerde de verspreiding van individuele cellen en verminderde collectieve invasie, wat een verschil in gevoeligheid voor gerichte interventie aangeeft. HGF induceert dus EMT samen met collectieve, proteolytisch-amoeboïde en somsmesenchymale invasie, wat EMT aanduidt als een mogelijk moleculair programma dat betrokken is bij elke invasiestrategie.

Fluorescentie gestuurde chirurgie (FGS) met als doel de tumormarge te identificeren is de meest veelbelovende strategie ter verbetering van routine klinische chirurgie. Tijdens FGS worden tumor- specifieke antilichamen geconjugeerd met een nabij-infrarood fluorofoor en intraveneus geïnjecteerd. Zij accumuleren in de tumor laesie en kunnen worden gevisualiseerd met behulp van een intraoperatief beeldvormingssysteem. Tumor-specifieke antilichamen en detectiestrategieën zijn echter nog in ontwikkeling en door het combineren van fluorescentie met bijvoorbeeld CO₂ laserchirurgie, kunnen fluoroforen verbleken en het signaal uitdoven. Daardoor is het belangrijk de effectiviteit van CO₂ laserchirurgie in combinatie met FGS te onderzoeken.

Op basis van een literatuuronderzoek naar potentiële oppervlaktemoleculen gevolgd door immunohistochemische en functionele validatie, identificeren wij in **Hoofdstuk 4** CD44v6 als antigeen dat op betrouwbare wijze tot expressie wordt gebracht in de invasiezone van HHPCC tumoren. Monoklonaal anti-CD44v6 antilichaam BIWA werd gebruikt voor nabij-infrarood fluo-rescentiedetectie in een reeks menselijke HHPCC cellen en een lokaal invasief tumor muismodel. BIWA detecteerde nauwkeurig menselijke HHPCC xenotransplantaten in muizen met een uitstekende signaal - ruis verhouding en lage opname in het weefsel rond de tumor en andere organen. Dit was voldoende om tumorgedeelten kleiner dan 1 mm, bestaande uit een paar duizend cellen, onder klinische condities zichtbaar weer te geven. De gegevens duiden aan dat CD44v6 een geschikt doelwit is voor betrouwbare detectie van invasieve regio's in HHPCC tumoren in vivo.

In **Hoofdstuk 5** laten wij zien dat de bestraling door de CO₂ laser *in vitro* niet significant interfereert met het fluorescentiesignaal in het zichtbare en nabij-infrarode lichtspectrum. Verder waren specifieke fluorescentiesignalen in een FGS muismodel 1000 keer sterker dan door laser geïnduceerde auto-fluorescentie in muizenweefsel. Deze bevindingen duiden op compatibiliteit van CO₂ laserchirurgie en FGS.
Hoofdstuk 6 bespreekt de relevantie van celinvasie strategieën voor de verspreiding van lokale tumorcellen, de uitdagingen van de plasticiteit van tumorcelmigratie bij de behandeling van kanker en perspectieven voor het doelgericht bestrijden van kanker door chirurgische en moleculaire therapie.

ZUSAMMENFASSUNG

Kopf-Hals-Plattenepithelkarzinome (head and neck squamous cell carcinoma, HNSCC) sind eine Gruppe von Tumoren, die ihren Ursprung in den Schleimhautoberflächen des oberen Aerodigestivtraktes haben. Trotz jüngster Bemühungen entwickeln noch immer 40 – 50% der Patienten tödliche Rezidive und Metastasen. Eine Ursache dafür ist die hohe lokale Invasion in das umliegende Gewebe, was zur Behandlung oft radikale Operationen erfordert. Diese führen häufig zu einem Verlust von gesundem, funktionalem Gewebe mit klinisch relevanten Komplikationen und/ oder einer unvollständigem Entfernung des Tumors. Um die Krebsbehandlung zu verbessern ist es daher wichtig, die Grundlagen der Tumorzellinvasion von Kopf-Hals-Plattenepithelkarzinomen zu verstehen und die chirurgischen Eingriffe zu optimieren.

Studien von Kopf-Hals-Plattenepithelkarzinomen haben Signalwege von Wachstumsfaktoren und Zytokinen identifiziert die zur Karzinogenese und Invasion beitragen. In der Tumorzelle beeinflussen sie Transkriptionsprogramme wie die Epithelial-mesenchymale Transition (EMT) und können so alle bekannten Formen von Zellmigration induzieren. Dazu gehört die Wanderung von Einzelzellen, die keine Verbindung mit benachbarten Zellen eingehen oder kollektive Wanderung, bei der sich Zellen als zusammenhängende Gruppen mit Zell-Zell-Adhäsionen fortbewegen. Zusätzlich zu der Induktion von Invasion können Wachstumsfaktoren und Zytokine eine Interkonversion der Tumorzellwanderung verursachen, die sogenannte Plastizität der Invasion. Wie jedoch diese Invasionsplastizität durch die Umgebung des Tumors räumlich und zeitlich kontrolliert wird und ob die Hemmung von Wachstumsfaktor- und Zytokinsignalwegen die Plastizität hemmen kann, bleibt ungeklärt.

Kapitel 1 gibt eine Einführung in die Thematik und stellt die Gliederung der Arbeit vor.

In **Kapitel 2** werden die aktuellen Konzepte und funktionalen Konsequenzen der Signaltransduktion von Wachstumsfaktoren und Zytokinen in funktionelle "Module" gruppiert, die entscheidend sind für die Wahl zwischen einem individuellen oder kollektiven Invasionsprogrammes. Anhand gut dokumentierter Beispiele, die diese Konzepte unterstützen, werden Einflüsse auf Zell-Matrix-Adhäsion, Zell-Zell-Interaktion, Zytoskelettdynamik und die Veränderung der extrazellulären Matrix beschrieben. Um die Mechanismen der Invasionsplastizität und möglicher Interventionsziele besser zu verstehen, ist eine Kombination aus zellbasierten *In-vitro*-Studien, präklinischen Mausmodellen, klinischer Studien und mathematischen Modellen notwendig. Dann kann das Zusammenwirken von Wachstumsfaktoren und Zytokinen analysiert werden und spezifische Therapie zur Krebsbehandlung herausgearbeitet werden.

Fibroblasten sezernieren als Teil des Tumorstromas Wachstumsfaktoren und Zytokine. **Kapitel 3** beschreibt die gleichzeitige Induzierung kollektiver Migration sowie Einzelzellmigration durch Fibroblasten in HNSCC-Zellen in einem 3D Kollagen-Sphäroid-Invasionsmodell. In Fibroblasten-konditioniertem Medium verursacht hauptsächlich Hepatozyten-Wachstumsfaktor (hepatocyte growth factor, HGF) diese Invasion. Darüber hinaus sorgt eine ansteigende Konzentration von

Fibroblasten-konditioniertem Medium, HGF oder dem epidermalen Wachstumsfaktor für einen Übergang von kollektiver zur Einzelzellwanderung. Die Mehrheit der sich ablösenden Zellen entwickelte eine runde, amöboide Wanderung. Jedoch untypisch für amöboide Wanderung degradierten diese Zellen das Kollagen und waren somit abhängig von Matrix-Metalloproteinasen. Um die biochemische Analyse ganzer Zellpopulationen zu vertiefen, wurde eine neuartige 3D-Einzelzellzytometrie entwickelt. So konnten molekulare Marker der EMT in Tumorzellsubpopulationen nachgewiesen werden. Der EMT-Marker Vimentin wurde sowohl in kollektiv als auch die individuell wandernde Zellen exprimiert mit einer Häufigkeit von etwa 20% bis zu 80% in Abhängigkeit von der verwendeten Zelllinie. Die Hemmung von HGF beseitigte die Einzelzellwanderung und konnte die kollektive Invasion jedoch nur reduzieren. Dies deutet auf einen Unterschied in der Empfindlichkeit gegenüber einer gezielten Intervention hin. HGF induziert daher EMT zusammen mit kollektiver, proteolytisch-amöboider und weniger häufig mesenchymaler Invasion, was darauf hindeutet, dass EMT ein molekulares Programm ist, das mit jeder Invasionsstrategie assoziiert ist. Als eine vielversprechende Strategie zur Verbesserung der klinischen Routine der Krebsbehandlung erweist sich die Fluoreszenz-gesteuerte Chirurgie, mit dem Ziel, den Tumor und Tumorrand zu identifizieren und sichtbar zu machen. Sie hat damit ein großes Potenzial, die Präzision der chirurgischen Exzision zu steuern und zu verbessern. Während der Fluoreszenz-gesteuerten Chirurgie werden tumorgerichtete Antikörper, die mit einem Nahinfrarot-Fluorophor konjugiert sind, intravenös injiziert, welche sich dann in der Läsion ansammeln und unter Verwendung eines intraoperativen Bildgebungssystems sichtbar gemacht werden können. Die Art der Biomarker und Detektionsstrategien sind jedoch in der Entwicklung und bei Kombinationen von Fluoreszenzgesteuerter Chirurgie mit anderen Operationsarten, wie die CO₂-Laserchirurgie, könnte der Laser Fluorophore bleichen und das Signal schwächen. Daher ist es unsicher, ob CO₂-Laserchirurgie effektiv mit Fluoreszenz-gesteuerter Chirurgie kombiniert werden kann.

Basierend auf einer Literaturstudie zu möglichen Oberflächenmolekülen, gefolgt von einer immunhistochemischen und funktionellen Validierung, wird in **Kapitel 4** CD44v6 als Antigen identifiziert, welches in der Invasionszone von HNSCC-Tumoren zuverlässig exprimiert wird. Der monoklonale Anti-CD44v6 Antikörper BIWA wurde für den nahinfrarot-Fluoreszenznachweis in einer Reihe von humanen HNSCC-Zellen und einem lokal invasiven präklinischen Krebsmodell verwendet. BIWA konnte humane HNSCC-Xenotransplantate in Mäusen mit ausgezeichnetem Signal-Rausch-Verhältnis und geringer Aufnahme im krebsfreien Gewebe und anderen Organen genau nachweisen. Das Signal war ausreichend um Tumorgewebe unter 1 mm Durchmesser, welches aus einigen tausend Zellen bestand, unter klinisch ähnlichen Bedingungen zu detektieren. Diese Daten deuten somit darauf hin, dass CD44v6 für eine zuverlässige Nahinfrarotdetektion von invasiven Rändern von HNSCC-Tumoren ein geeignetes Zielobjekt ist.

In **Kapitel 5** wird gezeigt, dass die Bestrahlung mit einem CO₂-Laser das Fluoreszenzsignal im sichtbaren und nah-infraroten Lichtspektrum *in vitro* nicht signifikant stört. Darüber hinaus war das spezifische Fluoreszenzsignal in einem Fluoreszenz-gesteuerten Chirurgie Mausmodell tausendmal

stärker als die Laser-induzierte Autofluoreszenz im Mausgewebe. Diese Ergebnisse weisen darauf hin, dass die CO₂-Laserchirurgie mit Fluoreszenz-gesteuerter Chirurgie kompatibel ist.

Die Bedeutung von Zellinvasionsstrategien für die lokale Tumorzelldissemination, die Herausforderungen der Tumorzellmigrationsplastizität in der Krebsbehandlung und Perspektiven für die gezielte Behandlung von Krebs durch chirurgische und molekulare Therapie werden in **Kapitel 6** diskutiert.

ACKNOWLEDGEMENTS

When I first came to Nijmegen I only intended to get a Masters in Biomedical Sciences but somehow it turned out that I should stay a little while longer. Now, finally, my PhD work is done and I would like to express my gratitude to everyone who supported me throughout this time.

Prof. dr. P. Friedl, dear Peter. Thank you for giving me the opportunity to perform my PhD in your lab. In the past seven years I learnt a lot and I greatly improved my scientific skills. Starting three projects from scratch, convincing clinicians to collaborate with us and writing papers and this thesis across two or more countries was not always easy and demanded patience and good communication skills from both of us. I am impressed by your positive attitude towards our research and your talent to raise its level. Finally this thesis is complete!

Dr. R.P. Takes, dear Robert, thank you for giving me the opportunity to do my PhD in your Department. Your clinical input helped me to understand the problems HNSCC patients and clinicians face and allowed me to experience our research from a different perspective. Our meetings provided me with a highly valuable source of motivation.

My time in the lab of Cell Biology was greatly enhanced thanks to my amazing colleagues and guest researchers from abroad. I really appreciate your support during work and the great social time I had in Nijmegen. No matter if big (dagje uit, Christmas, Easter) or small (lunch, sports, coffee), I hope you enjoyed it as much as I did. Many of the fun moments also included the rest of the 6th floor and later the newcomers from the 5th floor as well. Thank you all for creating a friendly atmosphere in the labs and the common rooms. Not to forget our team at Houston with a big thank you to Michael and Liying for taking care of the RPPA experiments.

Mr. Khalil, dear Antoine. When I entered the lab you were the first person I talked to and you shared my journey through the Friedl lab until the end. In key moments you were there for me and knew what to say. Thank you for your advise and support throughout this time (not to mention the nutritional support in terms of drinks).

Dear Cornelia, we shared similar problems in the lab but we connected even more through many great moments outside the lab. Thank you for your enthusiasm, your support and many funny memories. I hope you find some last energy from this thesis to finish yours.

Dear Mirjam, I don't know how often I turned around to ask for your advice. You never hesitated to make time to answer my endless questions. Thank you for your support in all kind of ways!

Dear Jan-Hendrik, my neighbor-chat-choir-GilmoreGirls-lunch-buddy. I don't know where to start, thank you for your help, discussions and so many good memories.

Dear Marina and Samuel, thank you for your help in the lab and many (mainly funny) conversations in Nijmegen or Kleve.

Dear Esther, Manon and Huib. Thank you for helping me with the last data for this thesis and (hopefully) the last data for the final paper.

Dear Moniek and Sebastian. I was lucky to have two great students during my PhD and I enjoyed working together with you. Thank you for your efforts, I am happy your data is part of this thesis.

Dear Cindy, thank you for your creative ideas, an open ear and sharing my love for coffee.

Dear Gert-Jan, thank you for your infinite peace of mind to teach me the techniques in the field of microscopy. This actually applies to all the people from MIC.

Dear Harsha and Angela, thank you for welcoming me into the lab and teaching me the 'tricks' behind making collagen gels. Harsha, it is great that our data could be combined in the end.

Furthermore, I would like to thank everybody with whom I have worked with and who provided us with the material for this PhD project. In particular Otto, Desirée and Mark. Without you we couldn't have done the fluorescence-guided surgery paper as it is. Thank you for your great support, help and input. Also a big thanks goes to the teams in PRIME, the OR and the KNO team at the CDB department.

A special thanks goes to my family and friends. Many couldn't quite understand what I was doing all this time, but still listened to my complains. Others shared my journey in different departments or countries.

Dear Rike, many thanks for your open ear and your contagious good mood! It is great having you as my paranimf! And thank you for introducing Judith ;-) I really enjoyed our time in Nijmegen. With some delay, we all finished our PhD!

Mijn 'nieuwe' familie, Anja, Gerard, Michiel, Peter, Geert-Willem en Lise. Bedankt voor jullie ondersteuning, babysitten en afleiding.

Liebe Mama, lieber Papa. Vielen Dank, dass ihr mich meinen Weg habt gehen lassen, immer an mich geglaubt und mich unterstützt habt! Es bedeutet mir sehr viel.

Mein großer Bruder. Vielen Dank fürs Zuhören und ,Vormachen'. Denn was du kannst, kann ich schon lange! Liebe Steffi und Johanna, danke für eure süße Ablenkung und Unterstützung.

Gert, du kleiner Sonnenschein. Danke, dass du so bist wie du bist und mir beim Schreiben Gesellschaft geleistet hast.

Lieber Harm, ich weiß diese Arbeit hat unser Leben mehr beeinflusst als ich wollte. Danke für deine Liebe, Geduld und Gelassenheit. Das Leben mit dir wird immer schöner!

LIST OF PUBLICATIONS

- J. Odenthal, B.W. van Heumen, H.M. Roelofs, R.H. te Morsche, B. Marian, F.M. Nagengast, W.H. Peters. The influence of curcumin, quercetin and eicosapentaenoic acid on the expression of phase II detoxification enzymes in the intestinal cell lines HT-29, Caco-2, HuTu 80 and LT97. Nutr Cancer. 2012;64:856-63
- D.A. Janssen, P.J. Geutjes, J. Odenthal, T.H. van Kuppevelt, J.A. Schalken, W.F.J. Feitz, J.F. Heesakkers. A new, straightforward ex vivo organoid bladder mucosal model for preclinical research. J Urol. 2013;190:341-9
- 3. J. Odenthal, R.P. Takes, P. Friedl. *Plasticity of tumor cell invasion governance by growth factors and cytokines*. Carcinogenesis 2016;37:1117-1128
- S. Lehmann, V. te Boekhorst, J. Odenthal, R. Bianchi, S. van Helvert, K. Ikenberg, O. Ilina, S. Stoma, J. Xandry, L. Jiang, R. Grenman, M. Rudin, P. Friedl. *Hypoxia induces a HIF-1-dependent transition from collective-to-amoeboid dissemination in epithelial cancer cells.* Curr Biol 2017;27:392-400
- J. Odenthal, M. Rijpkema, D. Bos, E. Wagena, H. Croes, R. Grenman, O. Boerman, R.P. Takes, P. Friedl. *Targeting CD44v6 for fluorescence-guided surgery in head and neck squamous cell carcinoma*. Sci Rep. 2018;8(1):10467.
- 6. J. Odenthal, P. Friedl and R.P. Takes. Compatibility of CO2 laser surgery with fluorescence detection in head and neck cancer cells. Manuscript accepted with minor revisions.
- 7. J. Odenthal, M. Raaijmakers, H. Mahabaleshwar, S. Sommer, R. Grenman, R.P. Takes, P. Friedl. *Growth factor-induced plasticity of invasion induces EMT with amoeboid traits.* Manuscript in preparation.

CURRICULUM VITAE

Julia Odenthal was born on August 9th, 1986 in Langenfeld (Rhineland), Germany. In 2006 she began her Bachelor study in Applied Biology at the Bonn-Rhein-Sieg University of Applied Sciences in Rheinbach, Germany and concluded it at the University of Aberdeen, Scotland. After graduating from both institutions in 2009, she continued her study in Biomedical Science at the Radboud University Nijmegen where she graduated in 2011. Subsequently, she started her PhD studies at the Department of Otorhinolaryngology and Head and Neck Surgery at the Radboud University Medical Center in collaboration with the Department of Cell Biology at the Radboud Institute of Molecular Life Sciences (RIMLS) under the supervision of Prof. Dr. Peter Friedl and Dr. Robert Takes. The results of her research entitled "Cancer invasion: growth factor-induced mechanisms and targeting by fluorescence-guided surgery" are described in this thesis. In 2018 she began to work at hict, consulting in healthcare in Bruges, Belgium.