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DISSERTATION

MicroRNA-34a regulates epithelial–mesenchymal transition (EMT) in cancer stem (like) cells (CSCs) of head and neck squamous cell carcinoma (HNSCC) and is a possible molecular target

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von

Zhifeng Sun

aus Liaoning, China

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CONTENTS

ABBREVIATIONS

ALDH	Aldehyde Dehydrogenase
BMI1	B Lymphoma Mo-MLV Insertion Region 1 Homolog
CSC	Cancer Stem Cell
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco Modified Eagles Medium
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
EZH2	Enhancer of Zeste Homologue 2
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
HPV	Human Papilloma Virus
HNSCC	Head and Neck Squamous Cell Carcinoma
MDCs	Monolayer-derived Cells
miR	MicroRNA
Oct3/4	Octamer-binding Transcription Factor 3/4
PDCD4	Programmed Cell Death Protein 4
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
SDCs	Spheroid-derived Cells
Sox2	Sex-determined Region Y-box 2
TF	Transcription Factor
ZEB1/2	Zinc Finger E-box-Binding Homeobox 1/2

MicroRNA-34a regulates epithelial-mesenchymal transition (EMT) in cancer stem (like) cells (CSCs) of head and neck squamous cell carcinoma (HNSCC) and is a possible molecular target

Summary

MicroRNAs (miRs) are short non-coding single-strand RNAs that regulate the translation of target mRNAs in normal and cancer cells. In the latter, they are frequently dysregulated, promoting tumor progression. Cancer stem cells (CSCs) are a small cell subset within a tumor that play a role in the genesis and progress of cancer. The relevance of epithelial-mesenchymal transition (EMT) has recently been recognized for tumor development and metastasis. Several studies have illustrated that miRs regulate EMT of CSCs.

CSC from 8 squamous cell carcinoma of the head and neck (HNSCC) cell lines (4 HPV^+, 4 HPV^-) were enriched by spheroid culture (spheroid-derived cells, SDCs) and compared to their parental monolayer-derived cells (MDCs) to analyze possibly distinct expression patterns of miR-34a, CSC-related transcription factors (CSC-TFs: Sox2, Nanog, Oct3/4) and EMT-related TFs (EMT-TFs: Twist, Snail1, Snail2) by RT-qPCR. FACS and FACS-sorting was used to quantify and enrich for $ALDH^+$ CSCs. Transfection of miR-34a mimics was used to evaluate the regulatory role of miR-34a for CSC marker profiles as well as CSC- and EMT- TF expression in HNSCC-SDCs. The invasion, colony-forming and clonogenic capability of the SDCs, which were transfected with miR-34a mimics after sorting for $ALDH^+$ and $ALDH^-$ cells, was assessed by matrigel invasion, clonogenicity- and spheroid-formation assay, respectively.

miR-34a expression levels were significantly downregulated in the majority of SDCs derived from HNSCC cell lines as compared to parental MDCs (-1.61 to 16.37 fold, $P < 0.05$). For EMT- and CSC-related TF expression, all HNSCC-derived SDCs showed a significantly increased level compared to parental MDCs (1.04 to 36.81 fold, $P < 0.05$). Increased expression of ALDH was found in SDCs (2 to 3 fold, $P < 0.05$). Compared to the HPV^+-, the HPV^- group (n=4) showed a significantly higher expression level of EMT-TFs, CSCs-TFs, and the CSC marker ALDH (30.33% vs. 12.83%, $P < 0.05$). Transfection of miR-34a mimics significantly reduced the EMT and CSC-related TF expression level in UM-SCC9 (HPV^-) and UM-SCC47 (HPV^+) SDCs. Simultaneously, the ALDH expression was reduced (1.5 to 2 fold, $P < 0.05$) and the

invasive capacity and clonogenicity of HNSCC-SDCs was also inhibited by transfection of miR-34a mimics compared to controls.

Restoration miR-34a significantly inhibited the capability for EMT, formation of CSC-phenotype and functionally reduced clonogenic and invasive capacity in HNSCC cell lines. Therapeutic modulation of miR-34a in HNSCC and CSCs may reduce the rate of metastasis and recurrence of tumors after therapy.

Zusammenfassung

MicroRNAs (miRs) sind kurze nicht-kodierende, einzelsträngige RNAs, die die Translation von Ziel-mRNAs in normalen- und Krebszellen regulieren. In letzteren sind sie häufig fehlreguliert und fördern die Tumorprogression. Krebsstammzellen (CSCs) sind eine kleine Untergruppe von Zellen im Tumor, die eine Rolle bei der Entstehung und dem Progress von Krebs spielen. Die Bedeutung der Epithelial-Mesenchymalen-Transition (EMT) für die Tumorentwicklung und Metastasierung wurde vor kurzem erkannt. Mehrere Studien haben illustriert, das miR den EMT von CSC regulieren.

Aus 8 Plattenepithelkarzinom- (HNSCC-) Zelllinien (4 HPV^+, 4 HPV^-) wurden CSC durch Spheroidkulturen angereichert (spheroid-derived cells, SDCs) und mit ihren Mutterzelllinien verglichen, um die möglicherweise unterschiedlichen Expressionsmuster von miR-34a, CSC-Transkriptionsfaktoren (TF; CSC-TFs: Sox2, Nanog, Oct3/4) und EMT-TF (EMT-TF: Twist, Snail1, Snail2) durch RT-qPCR zu analysieren. FACS und FACS-Sortierung wurde zur Quantifizierung und Anreicherung von $ALDH^+$ CSCs verwendet. Die Transfektion von miR-34a-Mimics wurde verwendet, um die regulatorische Rolle von miR-34a auf CSC-Markerprofile und CSC- und EMT-TF-Expression in HNSCC-SDCs zu evaluieren. Die Fähigkeit zur Invasion, Klon- und Koloniebildung von SDCs, die mit miR-34a-Mimics nach Sortierung von $ALDH^+$- und $ALDH^-$ Zellen wurde durch Matrigelinvasion-, Klonogenizitäts- and Spheroidbildungstests geprüft.

Die miR-34a-Expression war in der Mehrheit der SDCs aus HNSCC im Vergleich zu parentalen MDCs signifikant (-1.61 bis 16.37-fach, $P < 0.05$) herabreguliert. Die EMT- und CSC-TF-Expression zeigte ein im Vergleich zu MDCs in allen HNSCC-SDCs ein signifikant erhöhtes Expressionsniveau (1.04 bis 36.81-fach, $P < 0.05$). Eine erhöhte Expression von ALDH wurde in SDCs nachgewiesen (2 bis 3-fach, $P < 0.05$). Verglichen zu der HPV^+- zeigte die HPV^- -Gruppe (n=4) eine signifikant höhere Expression von EMT-TFs, CSCs-TFs und dem CSC-Marker ALDH (30.33% versus 12.83%, $P < 0.05$). Die Transfektion von miR-34a-Mimics reduzierte signifikant die EMT- und CSC-TF-Expression von UM-SCC9 und UM-SCC47 SDCs. Gleichzeitig war die ALDH-Expression reduziert (1.5 bis 2-fach, $P < 0.05$) und die Invasionsfähigkeit und Klonbildungsfähigkeit von HNSCC-SDCs durch Transfektion von miR-34a-Mimics verglichen zu Kontrollen gehemmt.

Eine Wiederherstellung von miR-34a-Spiegeln hemmte signifikant die Fähigkeit zur EMT, Ausbildung des CSC-Phänotyps und reduzierte funktionell die Fähigkeit zu Klonbildung und Invasion in HNSCC-Zellen. Eine therapeutische Modulation von miR-34a in HNSCC und CSC könnte die Rate von Metastasen und die Rezidivrate von Tumoren nach Therapie reduzieren.

1. INTRODUCTION

1.1 Relevance of cancer stem (like) cells (CSC) in cancer

In 2012, 14.1 million new cancer cases were diagnosed and 8.2 million cancer-caused deaths registered world-wide which is a significant increase. Compared to 2008 these numbers were 12.7 million and 7.6 million, respectively[1]. By incidence, head and neck squamous cell carcinoma (HNSCC) is the sixth commonest cancer in the world. More than 600,000 new cases are diagnosed annually worldwide[2]. The average 5-year survival rate is around 50% in developed countries[3]. Development of effective treatment options like radiotherapy, chemotherapy, recently introduced targeted antibody therapy and improved surgical procedures during the past decades have resulted in a significantly enhanced quality of life for cancer patients. However, local and distant recurrence is common in HNSCC, and in recent years rates of survival have improved only marginally[4].

In the 20th century, cancer research was dominated by the clonal evolution theory. According to this theory, it was believed that accumulation of certain cellular mutations leads to the initiation of cancer. Each cell within a tumor has an equal ability to acquire genetic or epigenetic changes, conferring growth advantages and generating new tumors[5,6] (Figure 1). However, this model could not explain adequately the complexity of several different cell types within a malignant tumor, since the large number of mutations necessary to create such heterogeneity would be rather unlikely to occur [7]. In addition, it would be impossible to determine which cell initiated the tumor because each cell would have had an equal ability to be malignant. Therefore, another hypothesis emerged in the 1970s [8]. This hypothesis suggested that only a subpopulation of cells in a tumor has the ability to self-renew, differentiate and regenerate similar tumors, as demonstrated by xenotransplantation into immuno-deficient mice. These cells also display resistance to conventional therapy[9,10]. This particular subset of cells was named cancer stem (like) cells (CSCs), similar to what is conceptualised in normal tissue stem cells[11]. To date, the existence of CSC has been identified in several human solid tumors, including leukaemia, bladder cancer, breast cancer, colon carcinoma, HNSCC, and pancreatic cancer[12-16].

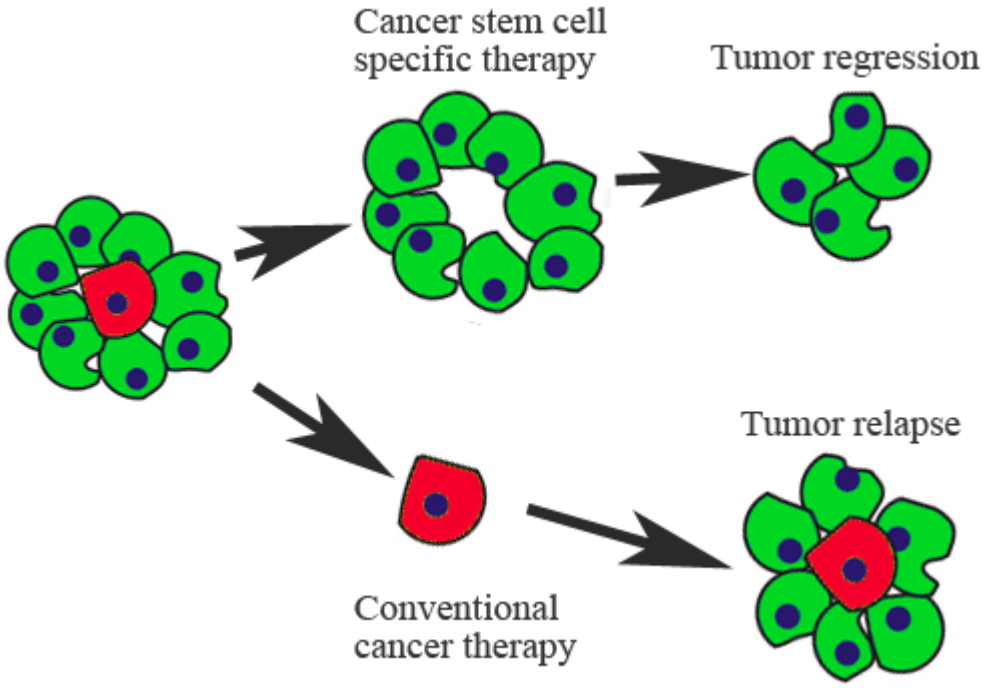


Figure 1: Cancer stem cells induce tumor relapse. Within a tumor (green) only a subpopulation of cells (red) have CSC-properties. These are more resistant to conventional cancer therapy, such as chemotherapy, radiotherapy and surgery. The conventional cancer therapy only reduces the bulk tumor mass but surviving CSCs may lead to tumor recurrence. The targeted CSC therapy should include targeting the molecular signalling pathways, target CSC-markers, target the CSC-niche, and induce CSC-apoptosis and differentiation. Such treatments could eradicate the unique CSC population and in combination with the conventional cancer therapies may eradicate the whole cancer and lead to complete tumor regression.

Properties of CSCs are:

1) To initiate malignant tumors and induce neoplastic proliferation[17].

2) When transplanted into an appropriate host, CSCs may have the ability to regenerate the original tumor[18].

3) CSCs are more resistant to cancer therapies, such as radiotherapy and chemotherapy[19].

4) Different biomarkers could be used to identify and isolate CSCs from the “bulk”-tumor population[5]. However a universal marker has not been identified yet.

The self-renewal and differentiation ability of CSCs can drive tumor formation. In contrast, differentiated cancer cells, lose the self-renewing ability and proliferate extensively, thus they lose the tumorigenic potential[10].

Because CSCs share some of the characteristics of normal tissue stem cells, CSCs were analysed and isolated using strategies comparable to those used for identifying normal tissue stem cells[20]. The main methods that were used to isolate these CSCs were: 1) fluorescence-activated cell sorting (FACS), taking advantage of antibody markers directed at cell-surface structures, 2) functional methods including Aldefluor assay, and side population assay (SP analysis), 3) *in vitro* enrichment of CSCs by spheroid culture for cell-sorting.

Compared to other options, spheroid culture is an easy and convenient way to enrich the CSC population present in the normal cancer cell population. The ability of CSC to form spheroids was first observed in cells derived from the central nervous system in 1992[21]. Reynold and colleagues found that the cells isolated from the striatum of adult mouse brain could be clonally expanded by culturing spheres, these cells could generate both astrocytes and neurons[22]. The ability of isolated cells to form anchorage-independent spheres in culture was demonstrated in breast, colon, HNSCC, and melanoma[13,14,23]. In HNSCC cell lines, CSC cultured with this method showed a high level of CSC-marker expression like CD44, CD133 and ALDH[24-26].

1.2 Putative markers for HNSCC-CSC

The identification of specific markers could help to develop an effective targeted therapy and prevent tumor recurrence. For this the identification and isolation of CSCs from the tumor cell population is important. It is most likely that CSC markers are molecules or cell-surface markers which play roles in particular metabolic or signalling pathways involved in stemness, differentiation and migration.

In solid tumors no CSC marker shared by all tumors has been identified yet. One possible reason is that CSC markers are unique to a particular tumor, because they depend on the source of the tissue or where the tumor grows (also called microenvironment) and its specific origin. Table 1 gives a summary of currently accepted specific markers of HNSCC and several other solid tumors. Tissue stem cells (TSCs) and CSCs share many properties, for example the ability of self-renewal and differentiation. Therefore, they also share some specific markers. Comparative studies showed that the Wnt and Bmi1 signalling pathways play similar roles in self-renewal for

CSCs and TSCs which are derived from the same tissues, suggesting that both populations are regulated by common molecular pathways[27].

At first, $CD44^{+}/CD24^{-/low}$ expression was considered as CSC marker in breast cancer, because these cells exhibit CSC properties, including self-renewal and differentiation[13]. In HNSCC, the first report about CD44 is a study by Prince et al[15] that first identified CD44 as CSC marker that defined a subset of tumor cells which had higher tumor formation ability than the CD44 negative tumor cells. Later, in brain tumors [28], colorectal carcinoma, pancreatic carcinoma[29,30] and HNSCC[31], CD133 was reported to identify CSCs. Recently, high aldehyde-dehydrogenase (ALDH) expression was shown to be expressed in a subpopulation of a certain number of solid tumors including HNSCC. This subpopulation showed high tumorigenic potential after transplantation in mice and other characteristic features for CSCs[32-35]. As few as 500 $ALDH^{+}$ cells injected into mice could generate tumors in 24/25 mice, while a transplantation of $ALDH^{-}$ cells from the same tumor initiated a new tumor only in 3/37 mice. Moreover, it was demonstrated that $ALDH^{+}$ cells have a high ability for sphere formation, invasion and are more resistant to radiotherapy. Oct3/4, Sox2 and Nanog are transcriptional factors which are involved in maintaining pluripotency and self-renewal in embryonic stem cells[36,37]. In poorly differentiated tumors they show higher levels of expression[38]. Recently, they were shown to be upregulated also in HNSCC-derived CSC[39].

Table 1: Overview of important CSC markers in HNSCC and other solid tumors.
(Modified according to [40] **)**

CSC marker	Origin	Function/physiological role	Reference
$CD44^+$ /$CD24^-$	breast, lung, HNSCC, hepatoma	CD44: A cell surface glycoprotein, plays roles in cell to cell interaction, cell migration, and adhesion	15,32,33
		CD24: A cell adhesion molecule, which is expressed at the surface of differentiating neuroblasts and also on most B cells.	
$ALDH^+$	HNSCC, breast	ALDH: ALDH regulates the conversion of retinol to retinoic acid, which is an important process for these TSC and CSC properties, such as proliferation, differentiation and survival.	32,35,41
$CD133^+$	HNSCC, CNS, colon, pancreas	CD133: a penta-span transmembrane glycoprotein domain is expressed in several stem cell populations and cancers.	16,25,28,42,43
Otc-3/4	HNSCC, embryonic stem cells	Oct-3/4: It is a transcription factor found in germ cells and pluripotent embryonic stem cells. Normally, Oct-4 mRNA is expressed in totipotent and pluripotent stem cells of embryos. Their function is a master switch during differentiation processes, and regulates the stem cell pluripotency potentials.	25,44
Sox2	embryonic stem cells	Sox2: In ES cells, the transcription factor Sox2 is important for retaining the pluripotent phenotype and is a partner of Oct3/4 in regulating several ES cell-related genes.	45
Nanog	HNSCC, embryonic stem cells	Nanog is a transcription factor, and essential to maintaining the pluripotent embryonic stem cell phenotype. Through the cooperative interaction, Sox2 and Oct4 have been demonstrated to drive pluripotent specific expression of a number of genes.	25,44,45

1.3 Epithelial-mesenchymal transition (EMT) and CSCs

Recent studies have demonstrated that the CSC phenotype is linked to tumor cells via EMT. Mani et al. reported that induction of an EMT in human mammary epithelial cells led to an increased expression of stem cell markers and in pancreatic cancer, occurrence of EMT is often accompanied by the activation of CSC-related pathways[46,47]. EMT is a physiological process that plays an important role during embryonic development and morphogenesis[48]. Important characteristics of EMT are a loss of polarity of the epithelial cells, loss of intercellular contacts, acquisition of mesenchymal features and phenotype along with an increased cellular motility. Therefore, during EMT the expression of epithelial markers, such as E-cadherin decreases while the expression of mesenchymal markers, such as N-cadherin, vimentin, and α-smooth muscle actin increases[48,49] (Figure 2). In recent years, it has been demonstrated that EMT is involved in the development of metastasis by increased cellular motility and invasiveness[48,50] resulting in a more metastatic phenotype[51,52]. During the EMT process, several changes in gene expression, such as increased Snail1, Snail2 and Twist transcription factors can be observed[53,54]. Transforming growth factor-β (TGF-β) also has been demonstrated to be capable of inducing EMT. In malignant tumors, TGF-β may be expressed by inflammatory cells, but also other stimuli may induce its expression [55].

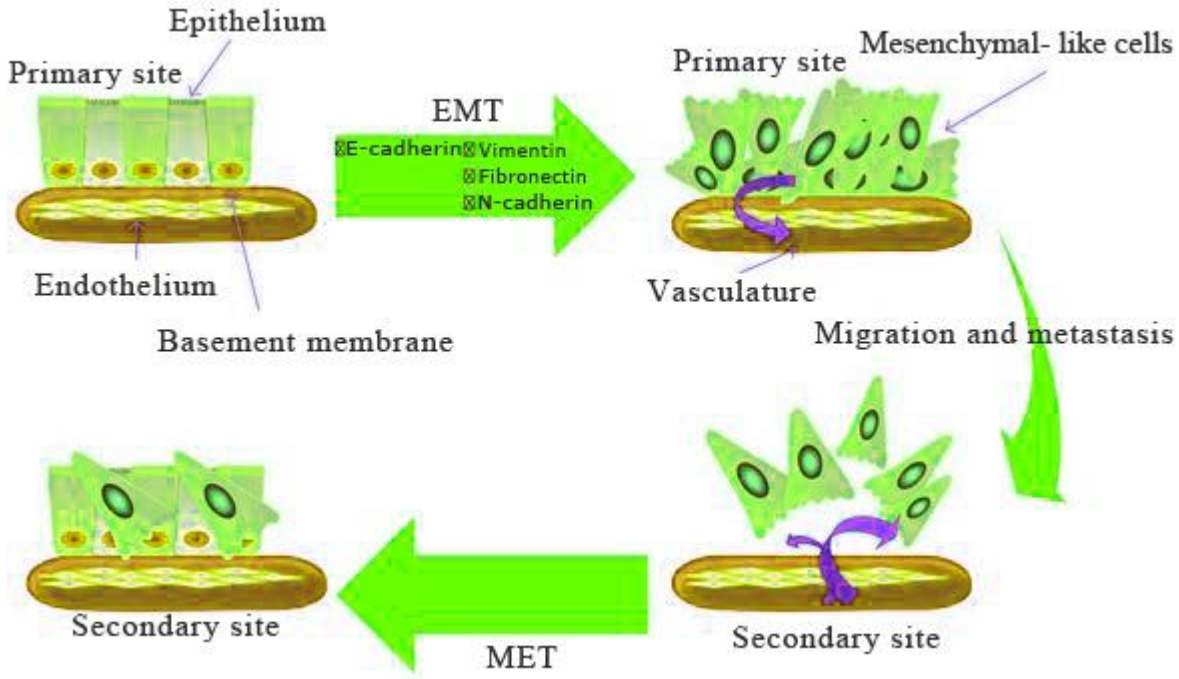


Figure 2: Contribution of EMT to the formation of metastasis. The epithelial to mesenchymal transition (EMT) is a physiological program characterized by a loss of cellular polarity and acquisition of a mesenchymal phenotype, intravasation, extravasation at the site of metastasis, invasion and reacquisition of an epithelial phenotype and formation of a metastasis. (MET= mesenchymal-epithelial transition). (Modified from[49]).

Several reports have demonstrated a link between EMT and CSCs stemness properties. This is supported by the expression of the EMT-marker Snail1/2 or Twist expression in CSC, that are involved in the loss of the epithelial phenotype and the acquisition of the mesenchymal phenotype[56,57]. In the past few years, it became evident that the induction of EMT not only promotes tumor cell metastasis and invasion, but it is also involved in drug resistance and enrichment of CSCs [46,58,59]. A summary of molecules or markers which play roles in metastasis/EMT and CSC phenotype and function is provided in Table 2.

In our previous research, we found in HNSCC cell lines that spheroid cell cultures enriched for $ALDH^+$ CSCs with EMT properties and those cells had increased invasiveness and colony forming ability[60]. In order to successfully metastasize, EMT-CSCs required the $ALDH^+$ phenotype. In the body, HNSCC-CSCs are able to move to other locations by using the EMT process, and the $ALDH^+$ cell population is able to revert back into the non-EMT phenotype by

the process of MET (mesenchymal to epithelial transition). These MET cells can then grow out into the metastasis by their proliferative activity[61,62].

Table 2: Molecules linking CSC phenotype and the EMT process.

Name	Function	Reference
ALDH1A1	CSC marker, participates in EMT process, migration, invasion	26,60,63,64
Bmi-1	Involved in EMT, invasion, metastasis, self-renewal	15,65,66
CD44	CSC marker, play roles in migration, invasion, EMT	15,67,68
CD133	CSC marker and EMT process	69,70
EZH2	Maintaining the CSC property and supports to self-renewal, migration by repression of E-cadherin	71-73
Nanog	Self-renewal, EMT, motility, migration, invasion, proliferation	25,60,69,74
Notch	CSC phenotype, EMT	75-77
Oct3/4	Self-renewal, EMT, metastasis	25,60,78
Snail1/2	Invasion, migration, self-renewal and EMT	60,74,79
Twist	EMT, invasion, migration, metastasis, regulation of CD24	60,80-84
Vimentin	Increased in CSCs and EMT	46,60

1.4 HPV in HNSCC

Human papilloma virus (HPV) infection is the commonest sexually transmitted infection in the world [85]. HPV is a small DNA virus, formed by a double-stranded DNA and a capsid which includes two structural proteins. The viral genome includes early (E) genes and late (L) genes. The oncoproteins of high risk (HR) HPV which are involved in carcinogenesis are encoded by E6 and E7 genes, and structural proteins of the capsid are encoded by late genes L1 and L2[86].

Nowadays, over 120 HPV types are fully recognized; they are divided into cutaneous and mucosal types. On the basis of their oncogenic ability, they are classified as low risk (LR) and HR. LR (e.g. HPV-6 and HPV-11 are the most commonly detected types) are usually associated with benign lesions like genital and perianal warts or papillomatosis of the larynx. HR-types (e.g. HPV-16, -18, -31, -33, and 35) are associated with cervical cancer, some other tumors of the genitoanal region and a subgroup of HNSCC[87].

In HNSCC, HPV infection is considered as a major etiologic factor. There are some differences between HPV^+ and HPV^- HNSCC, including anatomical site[88], histology[89], age, risk factors, incidence[90], stage and survival[91] (Table 3). Compared to HPV^- cancers, HPV^+ HNSCC seem to be a more distinct epidemiologic entity. Importantly, compared to HPV^- patients, HPV^+ HNSCC patients have a substantially better prognosis[87].

Table 3: Differences between HPV^+ and HPV^- HNSCC

	HPV^+ HNSCC	HPV^- HNSCC	Reference
Anatomical site	Base of tongue and tonsil	All sites	88
Histology	Non-keratinised	Keratinised	89
Age	Younger people	Older people	90
Stage	Tx, T1–2	Variable	91
Risk factors	Sexual behaviour	Abuse of tobacco and alcohol	90
Incidence	Increasing	Decreasing	90
Survival	Improved	Unchanged	91

1.5 miRs involved in the regulation of CSCs and EMT

MiRs are a family of 21-25 nucleotide long, non-coding endogenous RNAs, that act by binding to the target mRNAs' 3′-untranslated region and induce mRNA degradation or repress mRNA translation [92]. A single miR may regulate several mRNAs, and one mRNA can be targeted by dozens of miRs. Although small, miRs play an essential role in biological processes, such as development, metastasis, proliferation and apoptosis [92]. Early investigations demonstrated that miRs control embryonic stem cell (ESC) properties including self-renewal and differentiation. Later studies have shown that abnormal expression/functions of miRs are involved in tumorigenesis [93].

1.5.1 Regulation of CSC properties by miRs

Recently, studies identified a regulatory circuitry between miRs and "pluripotency" genes. These pluripotency genes are required for maintaining embryonic stem cell (ESC) stemness. For example, Oct-4, Nanog and Sox2, all bind to ESC-specific miRs promoter regions and directly regulate these miRs. They were therefore called the master regulators of stem cell pluripotency[94]. On a post-transcriptional level, miRs also regulate pluripotency genes, including miR-134, miR-296, and miR-470 by binding to their coding regions which suppresses the expression of these pluripotency genes[95].

miR expression levels in cancer cells often have similarity to those in ESCs. For example, Let-7 expression is decreased in ESCs and is also lost in breast, lung and ovarian cancers[96]. These expression features of cancer-specific miRs may be very important to the purposes of diagnosis and prognosis. Functional studies indicated that dysregulated miRs regulate molecular pathways in tumors via targeting different oncogenes or tumor suppressors. More recent evidence suggests that miRs are involved in tumor development via regulation of CSCs[96].

The expression of miRs was first investigated in breast CSCs. Yu and colleagues enriched for self-renewing breast cancer cells[97]. Consecutively, the isolated cells were cultured in suspension to generate mammospheres .These cells contained a high proportion of $CD44^{+}CD24^{-/low}$ cells and showed high capacity to generate mammospheres *in vitro* and *in vivo*. Most importantly, these enriched breast CSC showed much lower expression of let-7 and many other miRs, including miR-16, miR-107, miR-128 and miR-20b than the parental cells and differentiated progeny *in vitro*[97]. Afterwards, Shimono et al. reported that 37 miRs showed different expression

levels in $CD44^{+}CD24^{-/low}$ breast CSCs, in which three clusters, i.e. miR-200c-141, miR-200b-200a-429, and miR-183-96-182 were significantly downregulated. These miRs are also significantly decreased in progenitor cells and normal mammary stem cells[98]. In pancreatic CSCs, the $CD44^{+}CD133^{+}$ cells showed tumor sphere forming ability, tumor initiating ability and loss of miR-34. Restoration of the expression of miR-34 significantly reduced the tumor initiating cell population, inhibited sphere formation *in vitro* and tumor regeneration *in vivo* [99]. Regulation of CSCs properties by miRs were also demonstrated in other tumors, such as colon cancer[100], ovarian cancer[101], glioma[102], prostate cancer[103], pancreatic cancer[104] and HNSCC.

1.5.2 miR regulation of metastasis and EMT

Initially, miRs have been identified to regulate several cellular processes, including metastasis and EMT[92]. Later, they were associated with cancer and progression because they served as tumor suppressors or oncogenes[96]. For example, in lung adenocarcinoma and melanoma, ectopic expression of miR-200 inhibited EMT through upregulation of E-cadherin and decreased invasion and metastasis[105,106]. Several studies have found that the miR-200 family and miR-205 regulate EMT by affecting ZEB1/2 expression[107]. Lo et al. reported that an increased Bmi-1 expression significantly decreased expression of miR-200c in HNSCC lymph node metastases, and this phenomenon was also found in $ALDH^{+}/CD44^{+}$ HNSCC CSCs. Conversely, transfection of the mature miR-200c downregulated the expression of Bmi-1 in $ALDH^{+}/CD44^{+}$ HNSCC CSCs, accompanied by decreased ZEB1 and increased E-cadherin expression, and finally repressed the metastatic ability of these cells. The authors therefore concluded that increased expression of miR-200c could be a therapeutic opportunity in targeting HNSCC and CSCs[108].

The miR-34 family, miR-203 and Snail1/2 form epithelial regulatory loops[104]. Interestingly, the miR-34 family seed sequence targets a highly conserved 3′ UTR sequence of Snail/2, ZEB1, and also of the stemness markers Bmi1, CD44, CD133. Conversely, the miR-34 family are also directly inhibited by Snail1/2 and ZEB1[104], and miR-200 family promoters[109]. Thus, a tight crosstalk between the Snail1/miR-34 and ZEB/miR-200 pathways can be observed.

For HNSCC, several reports have demonstrated that the miR-21, miR-31 and miR-155 are increased and miR-34a, miR-26b, miR-107, miR-133b are decreased[110-114]. In several cancer types, including HNSCC, the up–regulation of miR-21 has been associated with tumor progression and EMT via the inhibition of the tumor suppressor protein programmed cell death (PDCD) 4 and Rho-A[114-116]. Moreover, the authors reported that miR-25 induced invasion and

migration of HNSCC by directly targeting E-cadherin[117]. Recently, it was reported that the downregulation of miR-138 inhibited EMT in HNSCC. The downregulation was caused by increased expression of E-cadherin and suppressed expression of vimentin[118]. In addition to vimentin, miR-138 also targets other genes, such as ZEB2 and EZH2 (enhancer of zeste homologue 2). In nasopharyngeal cancer, EZH2 was recently identified to inhibit E-cadherin expression (and to lead to EMT) via repression of Snail[119].

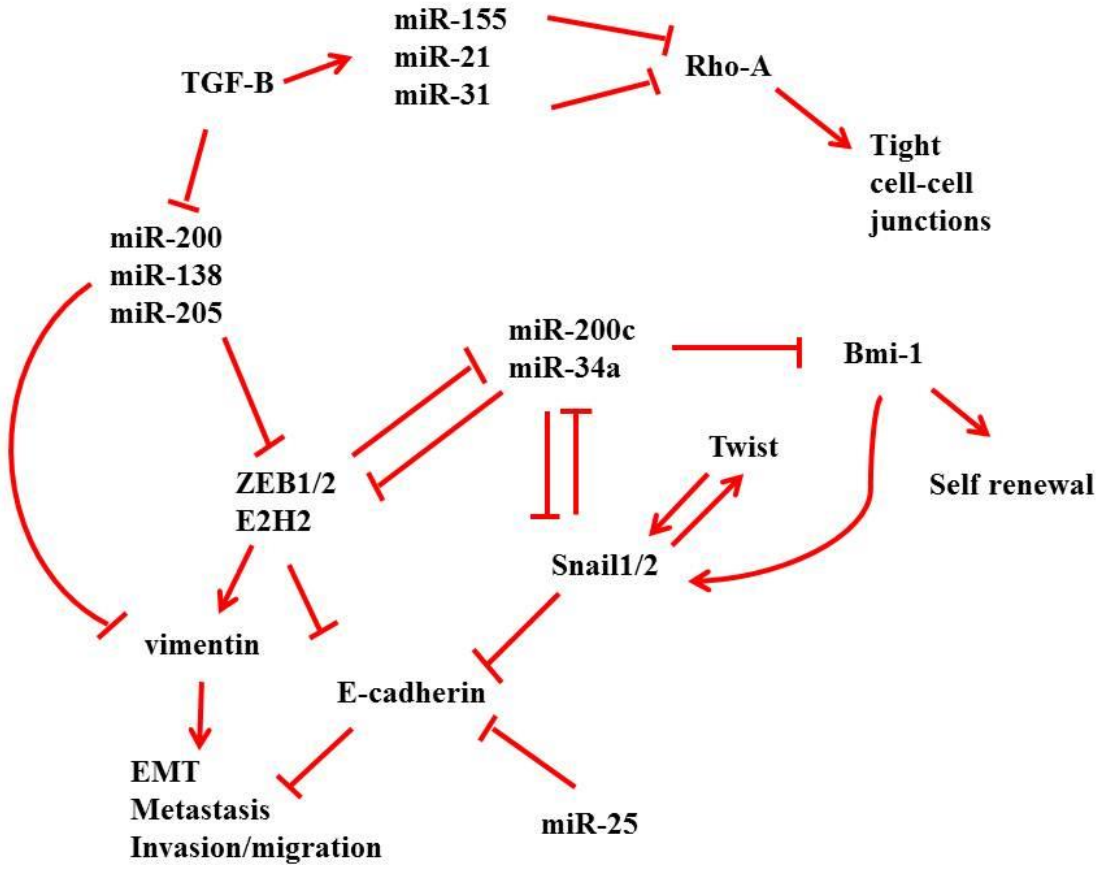


Figure 3: MiRs are key regulators in cellular activities. The functions of miRs in these central EMT/CSC-related processes imply possible roles as therapeutic tools. (Modified according to[120]).

1.6 miRs as therapeutic target against CSCs

In non-surgical tumor therapy, the major obstacles are cellular resistance to chemo- and radiotherapy. According to the CSCs theory, this resistance may be due to the small subgroup of CSC. Because CSC are different from the non-CSCs on a molecular level, methods or drugs able to eliminate or at least to reduce the number of CSCs are sought [121]. A therapy against CSCs is a challenging development because CSCs must be successfully identified and eliminated.

MiRs may be providing one key to this dilemma, because studies have demonstrated that miRs are involved in the regulation of human CSC-characteristics, such as self-renewal, differentiation, or chemoresistance. MiRs may also suppress the function of CSCs in many ways, such as inhibition of proliferation, migration, invasion and induction of differentiation (Table 4). These results suggest that using miRs to suppress the properties of CSCs is a promising method to complement the current therapeutic regimen. Several studies reviewed here have shown that miRs can function as tumor suppressors and play important roles in regulating these properties of CSC. In this regard, miRs could be considered to serve as the functional markers of CSCs. Therefore, understanding more details of the function of miRs in CSC properties may ameliorate cancer therapies, and possibly miRs may be used in clinical applications in cancer diagnosis, treatment, and prediction.

One of the most promising methods is the regulation of miR expression. The currently available miR regulators include antisense oligonucleotides, miRs masking or miRs sponges, and miR mimics (Figure 4)[122].

Table 4: Overview of miRs that regulate CSCs in cancer (Modified according to[123]).

miRs	Type	Cell line/tissue sample	Expression in CSC	Function
miR-93	Breast (basal)	HCC1954, SUM159	Downregulation	Inhibits proliferation and metastasis [124]
	Breast (luminal)	MCF7, primary tumor	Upregulation	Increases proliferation [124]
	Colon	SW116	Downregulation	Inhibits proliferation [100]
miR-200a	Ovarian	OVCAR3	Downregulation	Inhibits migration and invasion [101]
	Breast	Breast cancer tissue	Downregulation	Not known [98]
miR-199a	Ovarian	Primary tumors	Downregulation	Reduces tumor growth and invasion, increases expression of stemness genes [125]
miR-34 family	Glioma	Stem cell line0308 and 1228	Downregulation	Induces differentiation [102]
	Prostate	Primary tumor	Downregulation	Inhibits metastasis and proliferation [103]
	Pancreatic	MIA PaCa-2, BxPC3	Downregulation	Reduces tumorsphere formation [99]
	HNSCC	UM-SCC9, UM-SCC47	Downregulation	Reduces tumor invasion, tumorsphere formation and colony formation

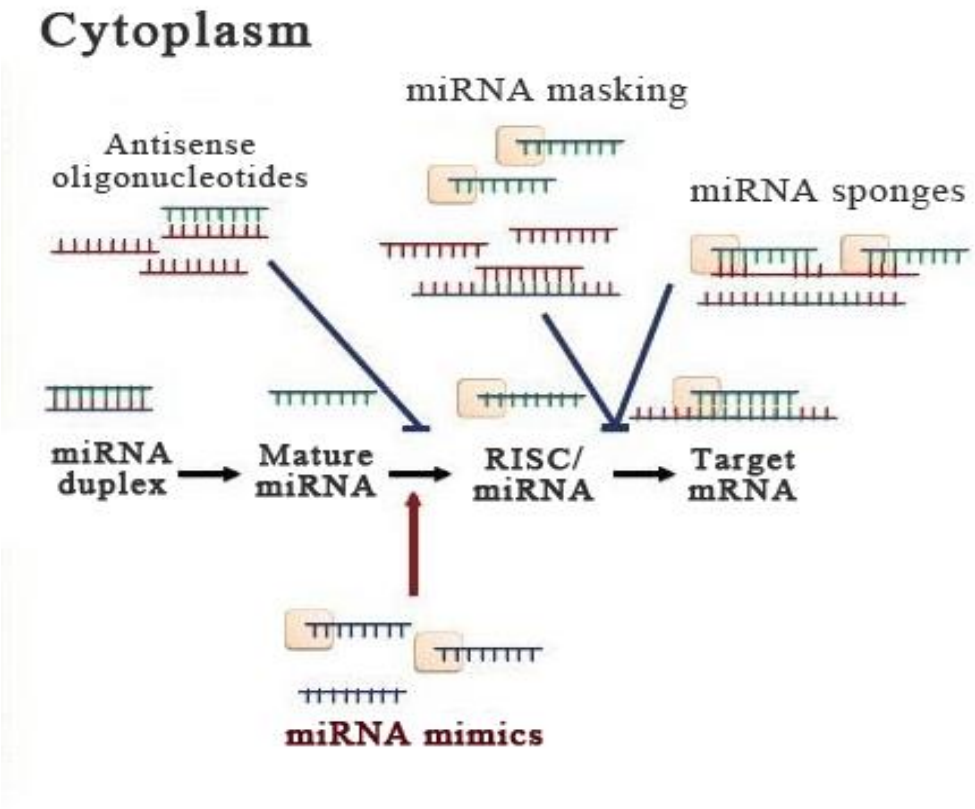


Figure 4: Potential therapeutic strategies against cancer involving miR. The miR-based cancer therapies include antisense oligonucleotides (anti-miRs), miR sponges, miR masking and miR mimics. (Modified according to[122]).

The function and use of miR regulators are as follows:

1) Antisense oligonucleotides: Bind to miRs by complementary sequences and induce formation of duplex and degradation of miR. They have been used to inhibit the oncogenic miRs.

2) MiR masking: 3′-UTR of the target miR binds to complementary molecules resulting in an inhibition of the downstream target effects. They have been used to inhibit oncogenic miRs.

3) MiR sponges: oligonucleotide constructs with several complementary miR binding sites to the target miR. They have been used to capture and inhibit oncogenic miRs.

4) MiR mimics: The function of these tumor suppressor miRs can be recovered by adding systemic miRs or inserting miR coding genes into viral constructs [122]. They have been used to enhance the anti-oncogenic miRs.

MiR34a is a tumor suppressor which directly targets p53 on a post-transcriptional level. In p53-deficient human pancreatic cancer cells, overexpression of miR-34a inhibited cell proliferation, cell cycle progression, self-renewal, EMT and invasion, indicating that miR-34a may restore p53 function, potentially directly via the downstream targets Notch and Bcl-2 which are involved in CSC differentiation and self-renewal[99]. In prostate cancer, miR-34a was reported to directly repress CSC properties and metastasis. In breast cancer, ectopic miR-34a expression reduced CSC properties and enhanced sensitivity to chemical treatment. Thus, miR-34a is considered a tumor suppressor which represses "stemness" features and function. In HNSCC cell lines, miR34a was downregulated in CSC, similar to the reports mentioned above, while the miR-34a function in HNSCC-CSC is not clear yet.

2. Hypothesis and aims

Hypothesis: miR-34a is involved in the regulation of phenotype and function on HNSCC-CSC and may therefore be a potential therapeutic target

Aims

1. To quantitatively compare the stemness-related transcription factor (Sox2, Nanog, and Oct3/4) and miR-34a expression between MDCs and their corresponding SDCs in HNSCC.

2. To quantitatively compare the expression of EMT-related TFs (Twist, Snail1, and Snail2) and CSCs-related TFs (Sox2, Nanog, and Oct3/4) level in HNSCC-MDCs and -SDCs.

3. To characterize and quantify the expression of ALDH by flow cytometry analysis in HNSCC-SDCs and their parental MDCs.

4. To assess phenotypically expression patterns of stemness-, EMT- and CSC-related transcription factors and functionally the role of miR-34a in regulating HNSCC EMT- and CSC properties as a possible molecular target by transfection of miR-34a mimics.

3. Materials

3.1 Laboratory equipment

Table 5: Laboratory equipment

Axiovert Microscope	Carl Zeiss, Jena, Germany
BD FACSCalibur System	BD Science, Heidelberg, Germany
Biological safety cabinet	NUNC™, Wiesbaden, Germany
BioRad Chromo 4	BioRad, München, Germany
Freezer, -80°C	Heraeus, Hanau, Germany
Incubator, HERA cell 150	Heraeus, Hanau, Germany
Multicentrifuge	Heraeus, Hanau, Germany
Nanodrop	Peqlab, Erlangen, Germany
Pipettes	Eppendorf AG, Hamburg, Germany
Thermocycler	Eppendorf AG, Hamburg, Germany
Vortex mixer	Scientific Industries, N.Y, USA

3.2 Chemicals, reagents, media and RT-qPCR primers

Table 6: Chemicals and reagents

Agarose	BD Pharmingen, CA, USA
Chloroform	BD sciences, Franklin Lakes, USA
DEPC-treated water	Merck KgaA, Darmstadt, Germany
Dimenthyl Sulphoxide(DMSO)	Ambion, Carlsbad, CA, USA
Ethanol 70%	Sigma, Steinheim, Germany
Epidermal Growth Factor (EGF)	Biochrom, Berlin, Germany
Fetal bovine serum (FBS)	Biochrom, Berlin, Germany
Fibroblast Growth Factor (FGF)	Biochrom, Berlin, Germany
Penicillin/streptomycin	Biochrom, Berlin, Germany
Phosphate-buffered saline	Biochrom, Berlin, Germany
Trizol Reagent	Invitrogen, Carlabad, CA, USA
Trypsin/EDTA solution(T/E)	Biochrom, Berlin, Germany

Cell culture media

Quantum 263	PAA, Paching, Austria
RPMI 1640	Invitrogen, Heidelberg, Germany
Opti-MEM	Invitrogen, Carlsbad, CA,

Table 7: Primer sequences used for RT-qPCR (5'→3')

Transcript name	Forward primer sequence	Reverse primer sequence
Nanog	AAT ACC TCA GCC TCC AGC AGA TG	TGC GTC ACA CCA TTG CTA TTC TTC
Oct3/4	GAC AGG GGG AGG GGA GGA GCT AGG	CTT CCC TCC AAC CAG TTG CCC CAA AC
Sox2	GGG AAA TGG GAG GGG TGC AAA AGA GG	TTG CGT GAG TGT GGA TGG GAT TGG TG
Snail1	GGC GCA CCT GCT CGG GGA GTG	GCC GAT TCG CGC AGC A
Snail2	GGG GAG AAG CCT TTT TCT TG	TCC TCA TGT TTG TGC AGG AG
Twist	GGA GTC CGC AGT CTT ACG AG	TCT GGA GGA CCT GGT AGA GG
GAPDH (reference)	AGC TCC CAA AAA TAG ACG CAC	TTC ATA GCA GTA GGC ACA AAG G
Hsa-miR-34a	TGG CAG TGT CTT AGC TGG TTG T	

3.3 Kits and other materials

Table 8:

Aldefluor assay Kit	StemCell Technologies, NC, USA
BD Falcon™ Cell Culture Flasks	BD Science, Franklin Lakes, USA
BD Falcon™ Cell Strainer (40 µm)	BD Science, Franklin Lakes, USA
BD Falcon™ Polypropylene Conical Tubes (15 ml, 50 ml)	BD Science, Franklin Lakes, USA
BD Falcon™ Polypropylene	BD Science, Franklin Lakes, USA
BD Falcon™ Tissue Culture Dish (100*20 mm)	BD Science, Franklin Lakes, USA
Cluster Tubes, Polypropylene (1, 2 ml)	Corning, NY, USA
Corning® BioCoat™ Matrigel® Invasion Chambers	Corning, NY, USA
Ncode VILO miR cDNA Synthesis Kit and Expression SYBR GreenER miR RT-qPCR Kits	Invitrogen, Carlsbad, CA, USA
Venor GeM Myoplasma Detection Kit for PCR with Gel evaluation	Minerva Biolabs GmbH, Berlin, Germany
Ultra-Low Attachment Cell Culture Flask (75 cm^2)	Corning, NY, USA
Lipofectamine RNAiMAX reagent	Invitrogen, Carlsbad, CA,

HNSCC-lines

HPV^+: UD-SCC-2, UM-SCC-47, UM-SCC-104, 93VU147T

HPV^-: UD-SCC-1, UM-SCC-9, UM-SCC-11B, UT-SCC-33

4. Methods

4.1 Cell lines

Eight HNSCC cell lines were used: UD (gift of Henning Bier, University of Munich formerly Düsseldorf, Germany) -SCC-1, -2; UM (University of Michigan, Tom Carey, USA) -SCC-9, -11B, -47, -104 and UT (University of Turku, Reidar A. Grenman, Finland) -SCC-33, 93VU147T (VU Medical Center, John P. de Winter, Amsterdam, Holland). The HPV types were as follows: HPV^+: UD-SCC-2, UM-SCC-47, UM-SCC-104, 93VU147T, they are all HPV 16 type. HPV^-: UD-SCC-1, UM-SCC-9, UM-SCC-11B, UT-SCC-33. All cell lines were regularly tested for mycoplasma and found free of any contamination.

4.2 Spheroid culture

Adherent monolayer cells were grown in normal 75 cm^2 cell culture flasks (BD Science, Franklin Lakes, USA) in DMEM (Invitrogen, Heidelberg, Germany) supplemented with 10% heat inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany), and 1% Penicillin/Streptomycin (10,000 U/ml and 10,000 µg/ml, respectively; Biochrom) at 37 ℃ in humidified atmosphere with 5% CO_2, until 70-80 % confluence. Cells were washed with PBS twice and detached using Trypsin/EDTA solution (T/E; Biochrom). The reaction was stopped by adding complete culture medium. After centrifugation at 200 × g for 5 minutes, cells were resuspended in serum-free Quantum 263 (PAA), supplemented with 10 ng/ml EGF and 10 ng/ml βFGF (Biochrom). To generate spheroids, single cells were plated in cell culture dish (BD FalconTM,100*20 mm) coated surfaces with 10% agarose at a specific density of 2×10^4 cells/ml. Cells were maintained at 37 ℃ in humidified atmosphere with 5% CO_2 content.

Every three to four days, half of the medium was replaced. After 5 to 7 days, spheroid formation was checked and representative pictures were taken. For passaging or following experiments, culture medium with spheroids was removed from the dish and filtered with a 40 µm mesh to collect the spheroids.

4.3 Aldefluor assay and FACS sorting

The ALDH activity of SDCs and MDCs was determined by using the Aldefluor assay Kit (StemCell Technologies, Durham, NC, USA). For collection of the spheroids, a 40 μm mesh was used. Subsequently cells were dissociated into single cells by Trypsin/EDTA digestion for 10 min at 37 ℃ followed by 30 times up and down pipetting using a 1,000 μl pipette tip. Then, the single-cell suspension was washed twice in PBS without Ca^{2+}/Mg^{2+} and suspended in 1 ml ALDEFLUOR assay buffer containing 5 μl ALDH substrate (1 ml/per 1×10^6 cells) and incubated for 30-40 min at 37 ℃ in the dark. As a negative control, for each sample, an aliquot was treated with 5 μl diethylaminobenzaldehyde (DEAB; 50 mmol/l), a specific ALDH inhibitor. Following incubation, all samples were centrifuged for 5 min at $250 \times g$ and the supernatant was removed. After washing twice with buffer, cells were maintained in ALDH buffer on ice during all subsequent procedures.

For FACS sorting, cells were resuspended in PBS buffer at 1×10^7 cells per ml and run on an Aria cell sorter (BD Biosciences, Heidelberg, Germany). The sorting gates were established by negative control cells which were treated with the ALDH inhibitor DEAB.

4.4 Quantitative real-time PCR

To quantify the expression of mRNA, total RNA was isolated by Trizol reagent according to the manufacturer's instructions, and then cDNA was prepared the Omniscript First-Strand synthesis system (Qiagen, Hilden, Germany) using random primers (Qiagen). RT-qPCRs were carried out using ABI Power SYBR Green mix (ABI, Applied Biosystems Inc, Foster City, CA, USA) and run on a BioRad Chromo 4 (BioRad, München, Germany). Reference gene was GAPDH.

For detecting of miR-34a, Trizol reagent was used to extract total RNA according to the manufacturer's instructions. Poly A tailing and cDNA synthesis were performed by using the Ncode VILO miR cDNA (Invitrogen, Carlsbad, CA, USA) synthesis kit. The RT-qPCR analysis was performed on a BioRad Chromo 4 (BioRad) with Express SYBR GreenERTM qPCR SuperMix Universal (Invitrogen). Reference gene was GAPDH. Reactions were carried out in triplicate with controls; primer sequences are listed above. The data were statistically analyzed by the modified $2^{\Delta\Delta Ct}$ value method[126].

4.5 Transfection of miRs

For miR mimics transfection, spheroids were harvested using a 40 μm mesh and dispersed into single cells as describe above. The single cell suspension was plated at a density of 8×10^4 per well in 6-well ultra-low attachment plates with complete medium. Then cells were transfected with 50 nmol/L miR-34a mimic, negative control (NC) or MOCK control using the Lipofectamine RNAiMAX reagent (Invitrogen) in antibiotic-free Opti-MEM (Invitrogen) according to the manufacturer's instructions. After 6 hours, the medium was replaced with spheroid culture medium. 24 h after transfection, cells were harvested and processed for further analysis. The transfection efficiency was determined by cell counting after visualization of transfected cells with a BLOCK-iT^{TM} Alexa Fluor Red Fluorescent Oligo using fluorescence microscopy.

The expression of the CSCs marker ALDH was assessed by FACS as described above at 24-, 48-, and 72 hour intervals after transfection. Quantification of EMT- and CSC- related TFs expression by transfected SDCs was carried out by RT-qPCR as described before.

4.6 Invasion assay

Warm (37 ℃) culture medium was added to the interior of the inserts and bottom of BD BioCoat Matrigel Invasion Chambers (BD) and allowed to rehydrate for 2 hours in a humidified tissue culture incubator at 37 ℃ in 5% CO_2 atmosphere. After rehydration, the medium was carefully removed without disturbing the layer of Matrigel™ Matrix on the membrane. Next, cell suspensions (SDCs transfected with miR-34a mimics and controls) were prepared in culture medium containing 5×10^4 cells/ml for 24-well chambers. 750 μl of DMEM containing 10% FCS serving as chemoattractant was added to the wells of the plate. Sterile forceps were used to transfer the chambers to the wells. Immediately 0.5 ml of cell suspension (2.5×10^4 cells) was added to the 24-well chambers that were then incubated for 24 hours in a humidified tissue culture incubator at 37 ℃ in 5% CO_2 atmosphere.

After incubation, the non-invading cells were removed from the upper surface of the membrane by gentle scrubbing, and the cells on the lower surface of the membrane were stained with Giemsa. Cell counting was facilitated by photographing the membrane through the microscope and 3 fields per membrane of triplicate membranes were counted at 200 × magnification (Axiovert, Axiovision, Zeiss, Germany).

4.7 Clone formation assay and spheroid formation assay

A colony formation assay was used to assess the clonogenicity of UM-SCC9 sorted cells transfected with miR-34a mimics in $ALDH^+$ and $ALDH^-$ cells. After FACS sorting, the $ALDH^+$ and $ALDH^-$ cells were placed in 6-well plates for transfection with miR-34a mimics. After transfection, cells were maintained in complete medium for 2 weeks. The initial cell density was 1000 cells/ml in DMEM medium supplemented with 10% FBS.

A spheroid formation assay was used to assess the spheroid formation ability of UM-SCC9 sorted cells transfected with miR-34a mimics in $ALDH^+$ and $ALDH^-$ cells. After FACS sorting, 800 $ALDH^+$- and $ALDH^-$ cells were inoculated into Ultra-low attachment 24-well plates (Corning, NY, USA) for transfection with miR-34a mimics, respectively. Cells were resuspended in serum-free Quantum 263 medium (Biochrom AG, Berlin, Germany) supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom). Fresh medium containing growth factors was added every week.

Two weeks later, colonies were visualized by staining with Giemsa and viable colonies that contained >50 cells or were >0.1 mm were counted with an ocular micrometer. The clone formation rate was calculated according to the following formula: Clone formation rate = number of formed colonies / number of seeded cells $\times 100\%$.

4.8 Statistical analysis

For statistical evaluation of flow-cytometric results, SPSS software (version 22; SpSS, Chicago, IL, USA) was used. Student's t-test was used to analyze statistical significance of the data.

For all RT-qPCR data, expression analysis was performed using the modified delta delta Ct value method. Expression analysis and statistical evaluations was carried out by using the pair-wise fixed re-allocation randomization by the Qiagen REST 2009 Software (version 2.0.13).

5. Results

To approach our hypothesis and experiment aims, we developed an experimental strategy detailed in figure 5.

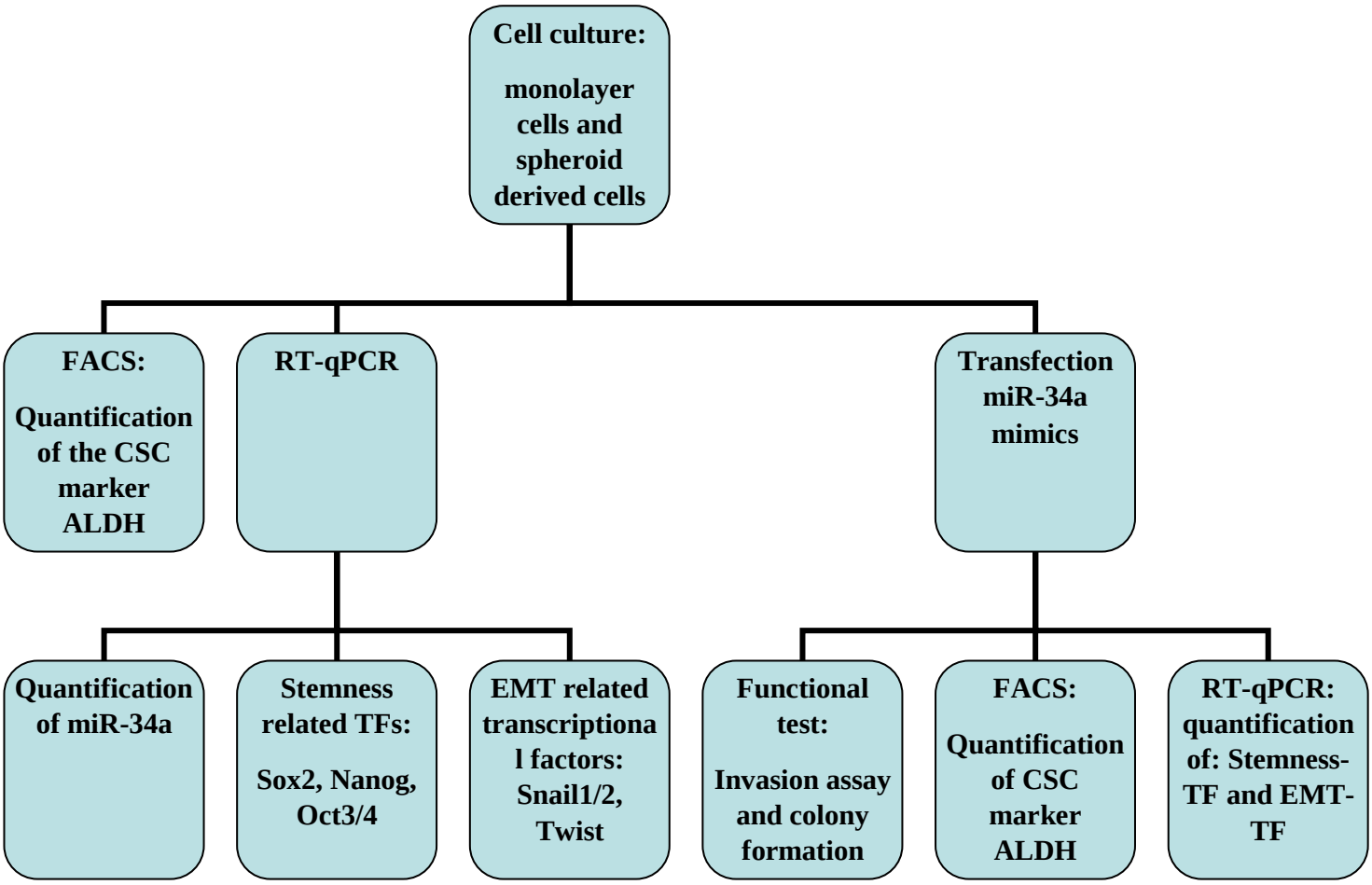


Figure 5: Synopsis of experimental strategy

5.1 HNSCC cell lines contain cells with self-renewing capacity and the ability to form spheroids

Cells from 8 HNSCC cell lines were grown in suspension for 7-10 days at a specific density of 2 $\times 10^4$ cells/ml. The spheroid formation typically started at the first day after plating suspension cultures and the spheroid size became progressively larger. After 4-7, days the morphology of the spheroids would not change in size any more, but the number of the spheroids in culture still continued to increase and the cell clusters became more compact (Figure 6).

Independent of HPV-association, all HNSCC cell lines except UD-SCC2 formed highly compact spheroids. Only UD-SCC2 formed loose aggregates of cells easily to be dispersed by pipetting.

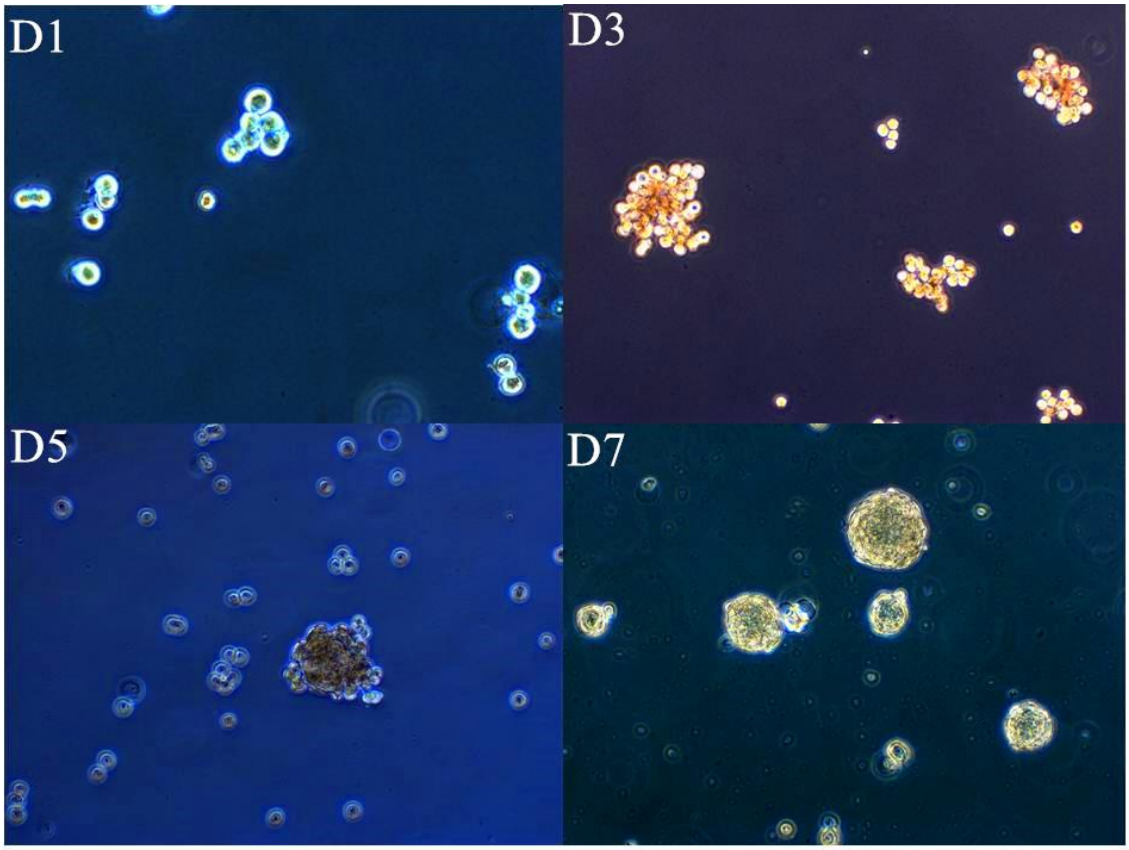


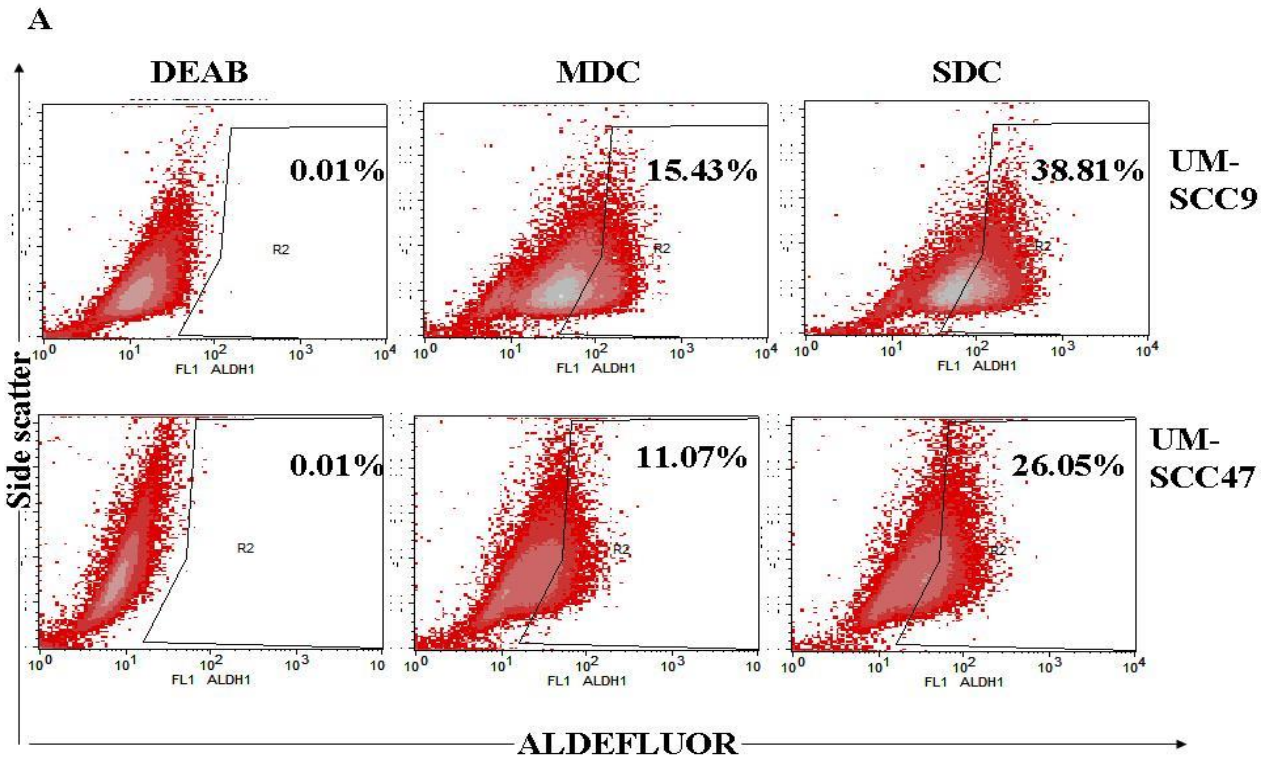
Figure 6: Time course of spheroid culture over (cell line UM-SCC9). Magnification: 100-fold.

To measure self-renewing ability, we collected spheroids and dissociated them into signal cell suspension, and plated at a clonal density of 1,000 cells per ml. After 7-10 days of subculture, tumor subspheroids (second generation) appeared, which contained 20 to 40 cells. When the spheroids were transferred back to a regular tissue culture flasks coated for monolayer cell culture, the spheroids adhered to the flask and cells migrated out from the spheroid and formed a confluent monolayer. The phenotype of these cells was identical to the parental cell lines as demonstrated before[26]. Spheroids maintained in long-time culture up to the 10^{th} generation still showed this self-renewing ability that generated the parental HNSCC cell.

5.2 The CSCs marker ALDH shows a higher expression level in SDCs than MDCs in HNSCC cell lines

Recently, the ALDEFLUOR assay has been successfully applied to detect ALDH-expression in CSCs from primary tumors or established cancer cell lines in various solid tumors, including HNSCC[127]. We measured ALDH enzymatic activity of SDCs of the eight HNSCC cell lines and their matched MDCs to identify and quantify the stem cell-like population (Figure 7A).

All HNSCC-derived SDCs showed a significant increase in the number of $ALDH^+$ cells compared to parental MDCs ($P < 0.05$) (Figure 7B). In the HPV^+ group, the cell line with the highest ALDH content was UM-SCC47 SDCs ($25.62 \pm 0.50\%$) compared to the parental MDCs ($11.05 \pm 0.16\%$). In the HPV^- group, the highest proportion of $ALDH^+$ cells was found in SDCs derived from UM-SCC11b ($45.05 \pm 0.22\%$) which was 3.3 fold higher than in the corresponding parental MDCs ($13.38 \pm 0.11\%$). Interestingly, ALDH expression was higher in SDCs and MDCs cultured from HPV^- than from HPV^+ cell lines. On average, in the four HPV^- cell lines the MDCs cell population contained $10.64 \pm 1.37\%$ of $ALDH^+$, while in the four HPV^+ HNSCC cell lines on average $5.74 \pm 0.92\%$ of cells were $ALDH^+$ ($p < 0.01$). In SDCs the average percentage of $ALDH^+$ in the HPV^- cell lines was $30.33 \pm 4.03\%$ vs. $12.83 \pm 2.29\%$ in the HPV^+ cell lines ($p < 0.01$). These data correspond to our findings in HNSCC *in vivo*[128]. Despite the fact that UD-SCC2 did not form compact spheroids, the SDCs still showed an increased expression frequency of $ALDH^+$ as compared to its MDCs.


A
DEAB
MDC
SDC
0.01%
15.43%
38.81%
UM-SCC9
0.01%
11.07%
26.05%
UM-SCC47
R2
FL1 ALDH1
Side scatter
ALDEFLUOR

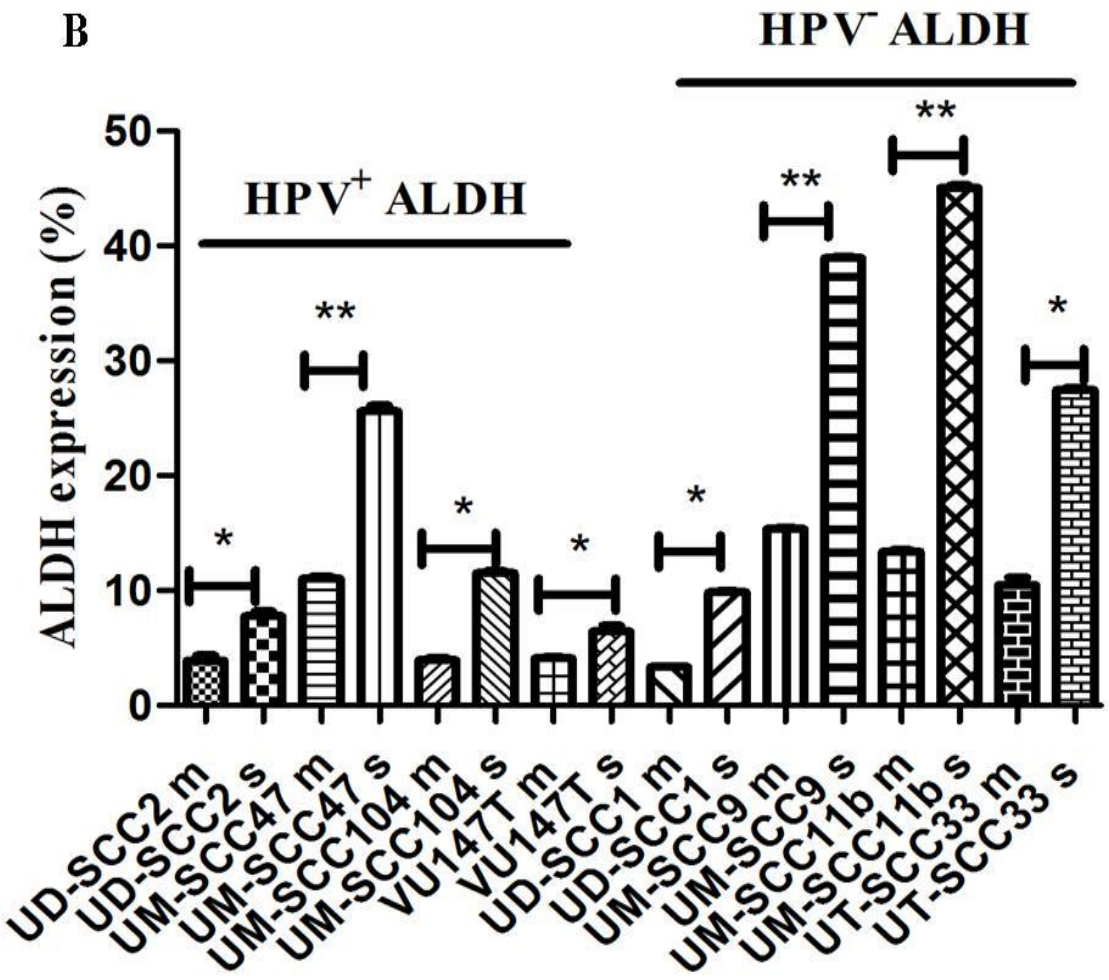


Figure 7: Expression of ALDH in HNSCC cell lines. (A) Representative example of a flow cytometric analysis of the expression of ALDH in SDCs compared to MDCs, and control cells treated with DEAB, a specific inhibitor of ALDH. Region R2 depicts fluorescence intensity of $ALDH^+$ cells. The number in the region represents the respective percentage of CSC. In relation to all cells (B) Mean percentage of $ALDH^+$ cells contained in the population of SDCs and MDCs of the investigated cell lines relative to their HPV^- status. The bars represent mean value ± SD of three independent determinations. *=P < 0.05, **=P < 0.01). m, MDCs, s, SDCs.

5.3 Overexpression of stemness-related TFs and EMT-related TFs in SDCs

Sox2, Oct3/4, and Nanog were reported as important TFs in maintaining stemness characteristics such as self-renewal and pluripotency in human embryonic stem cells and cancer cells[129]. In line

with previous findings[26], we confirmed as prerequisite for further work, that the mRNA levels of Sox2, Oct3/4, and Nanog were significantly increased in SDCs of the eight investigated HNSCC cell lines (Figure 8). The highest increase was observed in UM-SCC9 SDCs, where a 36.81 ±5.66- fold increase in Oct3/4 expression was found over the expression present in the parental MDCs. The smallest difference was observed in UD-SCC2, where the Nanog expression showed a 0.28 ±0.12-fold decrease. With regard to HPV-association, the mean Sox2 expression ratio in HPV^- SDCs/MDCs was 8.21 ±0.79-fold vs. 1.83 ±0.36-fold in HPV^+ ($P < 0.01$). The mean Oct3/4 expression ratio in HPV^- SDCs/MDCs was 13.32 ±4.38-fold vs. 4.07 ±1.34-fold in HPV^+ ($P=0.07$). Finally, the mean Nanog expression ratio was in HPV^- SDCs/MDCs 11.93 ±2.80-fold vs. 1.80 ±0.52-fold in HPV^+ ($P < 0.01$).

Snail1, one of the key TFs involved in EMT, was significantly increased in all SDCs generated from the 8 tested HNSCC cell lines as compared to MDCs independent of their HPV status (1.04-27.28 fold, $P < 0.01$). Interestingly, we also found that the mean expression ratio of Snail1 (HPV^- SDCS/MDCs 20.72 vs. HPV^+ 2.90 fold, $P < 0.05$) and of Twist (HPV^- 9.87 SDCs/MDCs vs. HPV^+ 2.72 fold, $P < 0.05$) were significantly higher in HPV^- SDCs/MDCs than HPV^+.

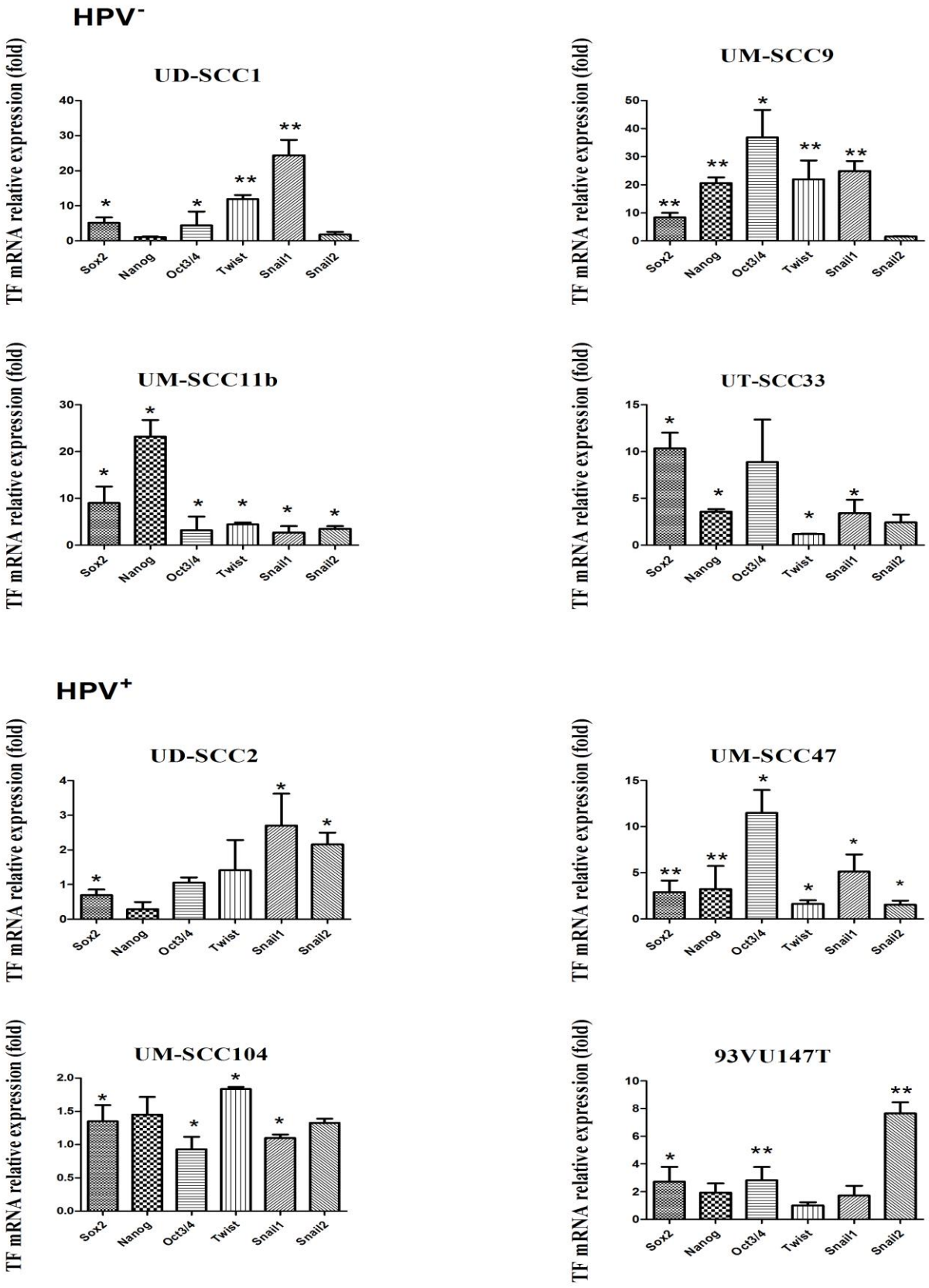


Figure 8: RT-qPCR analysis of mRNA expression of stemness-related TF and EMT-related TF. mRNA isolated from SDCs and MDCs was quantified for expression of the indicated

panel of TF. The ratio of expression between SDCs and MDCs is represented by bars in the diagram (fold increase in SDCs vs. MDCs). Mean value ± SD of three independent determinations (*=P < 0.05, **=P < 0.01).

5.4 miR-34a is downregulated in HNSCC-derived SDCs

MiRs regulate normal and malignant stem cells by simultaneously regulating the expression of hundreds of potentially genes[130]. These miRs may therefore regulate multiple pathways involved in stem cell fate decisions, including self-renewal, proliferation and differentiation pathways frequently altered in cancer [131]. The expression of the tumor suppressor miR-34a was selected for its known role as stemness-related miR. We found that the expression of miR-34a was consistently and significantly down-regulated in SDCs compared to the parental MDCs in all tested HNSCC cell lines (-1.61 to -16.37 fold, P < 0.05) (Figure 9).

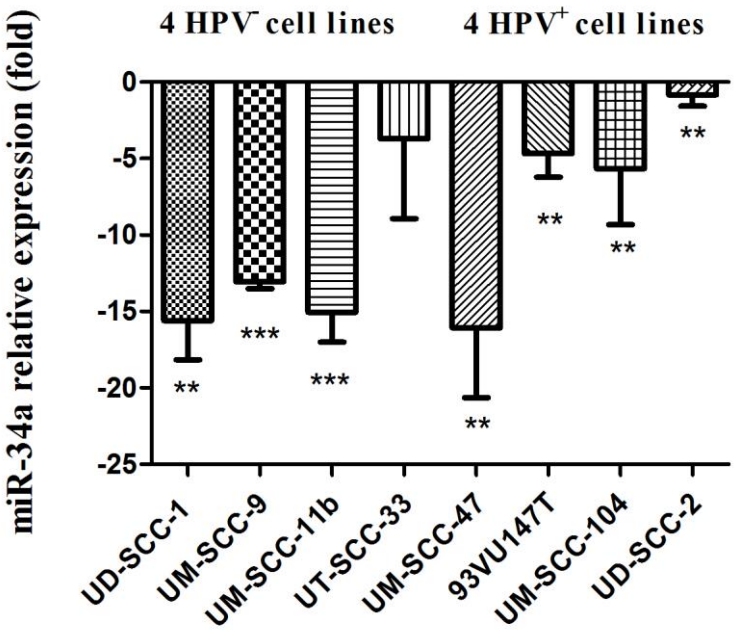


Figure 9: RT-qPCR analysis of miR-34a expression in HNSCC cell lines. The relative fold changes in the HNSCC cell lines SDCs to corresponding MDCs is given. Mean value ± SD of three independent determinations. *=P < 0.05, **=P < 0.01, ***=P < 0.001.

5.5 Over-expression of miR-34a reduces stemness and EMT properties in HNSCC-SDCs

According to the literature described in section 5.4, it is likely that miR-34a serves as a possible link between EMT and CSCs, including the possibility of regulating EMT in HNSCC-SDCs. In order to test whether miR-34a expression was sufficient to downregulate EMT and CSCs marker expression, ectopic miR-34a mimics were transiently transfected into UM-SCC9 and UM-SCC47 cells.

The transfection efficiency of UM-SCC9 was 94.33 ±0.57% and of UM-SCC47 93.66 ±1.52%. We also measured the transfection efficiency by measuring relative expression level of miR-34a mRNA after the transfection with miR-34a mimics and found that miR-34a mRNA level significantly increased after 24 h, 48 h and 72 h, while the mock transfected group showed no differences in expression levels in comparison to the normal control (NC group) (Figure 10A and B). The peak of miR-34a level was found 48h post-transfection (UM-SCC9 4.49 fold, UM-SCC47 9.1 fold, $P < 0.01$).

With respect to the regulation of CSC marker, we observed a downregulation of ALDH expression after miR-34a mimics transfection after 24 h, 48 h and 72 h (Figure 10C and D). The strongest change in $ALDH^+$ cell frequency was found in correspondence to the highest miR-34a levels 48h post-transfection (UM-SCC9 1.47 fold, UM-SCC47 1.93 fold, $P < 0.01$). Furthermore, transfection of miR-34a mimics resulted in a decrease in CSC- and EMT-related TFs mRNA which was tested 48h post-transfection (Figure 10E and F). Nanog, Oct3/4 and Snail1 were significantly decreased over controls in the two transfected HNSCC cell lines and Sox2 and Twist showed a significant down-regulation in UM-SCC47. While Snail 2 showed a decreased expression in UM-SCC9 and an increase in expression in UM-SCC47 cell line.

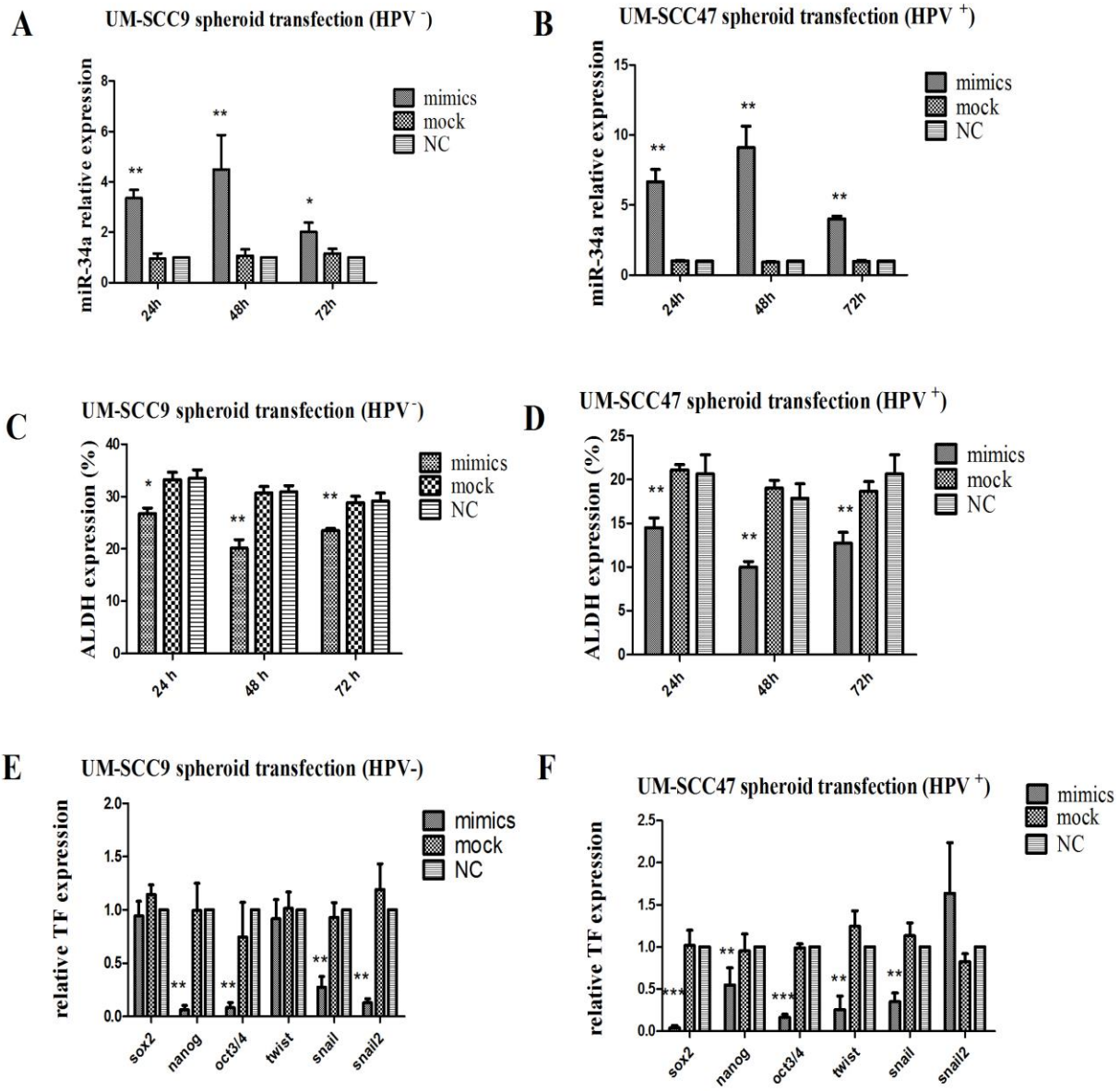
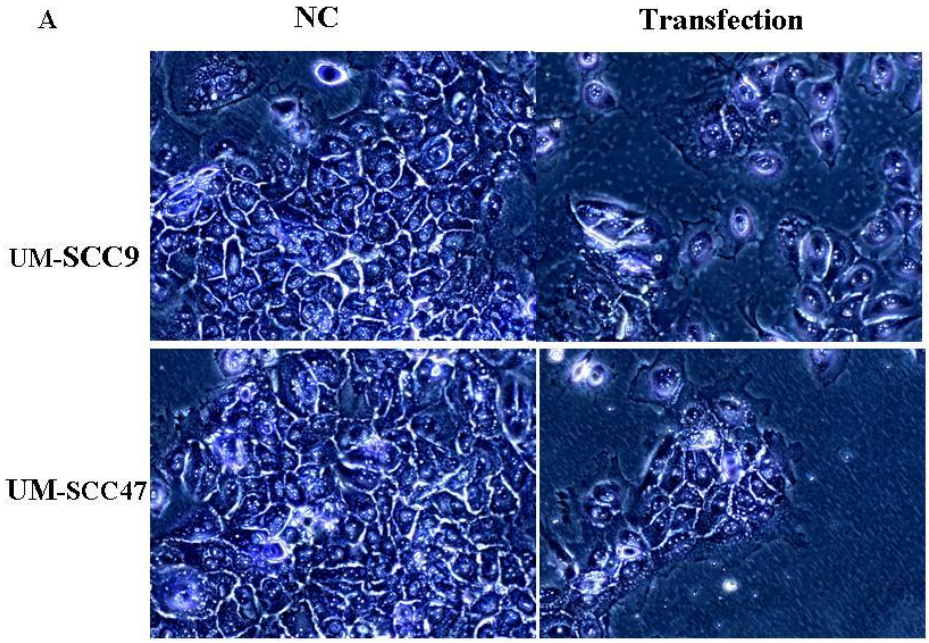


Figure 10: Overexpression of miR-34a reduced stemness and EMT properties in HNSCC-SDCs. (A-D): Change of miR-34a and ALDH expression levels in SDCs after transfection with miR-34a mimics over time (24 h, 48 h and 72 h). (E-F): Change in the expression of CSC- and EMT- related TFs expression levels in SDCs after transfection 48h. Mean value ± SD of three independent determinations. Significant differences were *=P **<** 0.05, **=P **<** 0.01 and ***=P **<** 0.001.

5.6 Overexpression of miR-34a reduces invasive capacity

For evaluation of the invasive capacity of tumor cells, we used the Matrigel invasion assay. Cells were transfected with miR-34a mimics and NC. After 24 h of culture, the number of miR-34a transfected HNSCC-SDCs that migrated through the Matrigel, coated chamber was significantly decreased ($P < 0.001$) compared with NC and mock-transfected cells (Figure 10). The miR-34a levels after transfection were significantly increased as described above. (UM-SCC9: 29.75 ± 1.08%vs12.87 ± 0.61%, UM-SCC47: 30.25 ± 1.23%vs13.12 ± 0.66 %). Transfection of miR-34a significantly decreased the migration/invasion of UM-SCC9 and UM-SCC47 SDCs by approximately 50%.



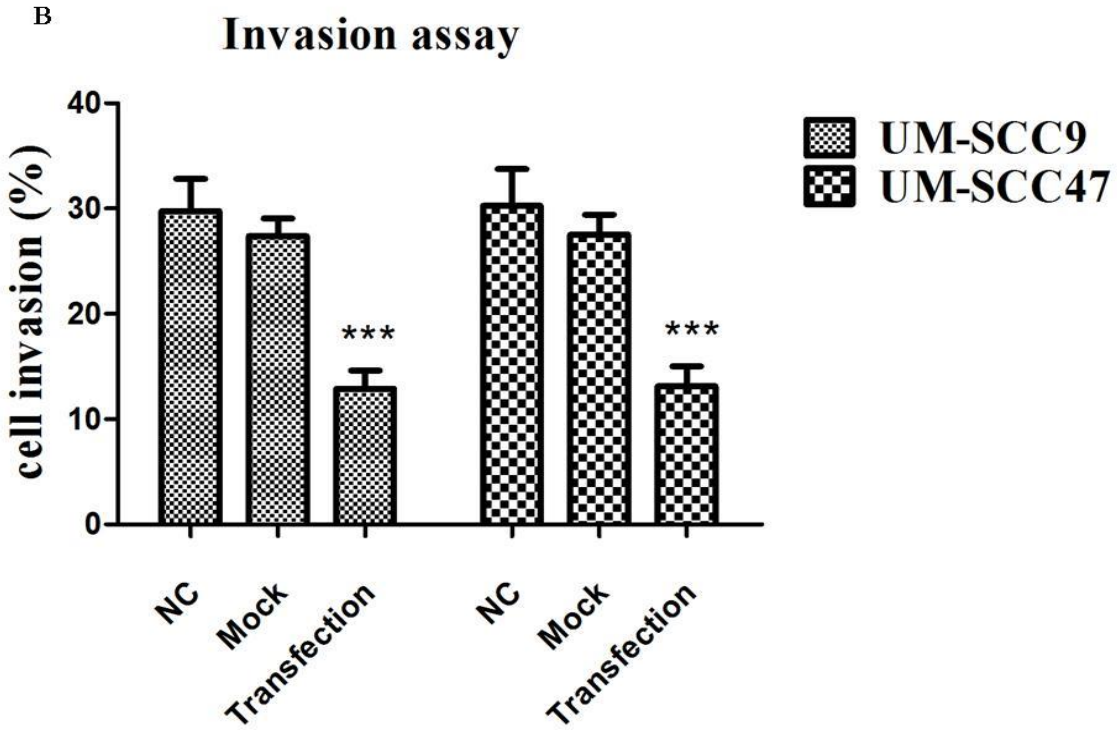


Figure 11: miR-34a transfection into HNSCC-SDCs reduces invasion capacity. Cell invasion was measured by using a Matrigel, coated system with the pore size of 8 µm. (A): Representative picture of NC cells and miR-34a transfected cells after migration through the membrane. (B): NC and mock-transfected cells crossed the membrane significantly more frequently than miR-34a transfected cells. ***=P < 0.001. Magnification: 200-fold.

5.7 Decreased ability of spheroid and colony formation as well as anchorage-independent growth after miR-34a transfection

After flow-cytometric sorting of UM-SCC9 for ALDH-positivity, the role of miR-34a in spheroid and colony formation and anchorage-independent growth was assessed.

Before transfection of miR-34a, the sorted ALDH+ cells showed a higher spheroid formation (Figure 12A) and colony formation ability than ALDH- cells (Figure 13A). Transfection with miR-34a mimics reduced the ability for anchorage-independent growth of UM-SCC9 cells significantly. Analysis of spheroid formation showed that cells transfected with miR-34a mimics displayed much fewer and smaller spheroids than the control transfected cells (Figure 12B-G). Regarding colony number and size, both decreased significantly after the transfection of miR-34a mimics in $ALDH^+$ cells.

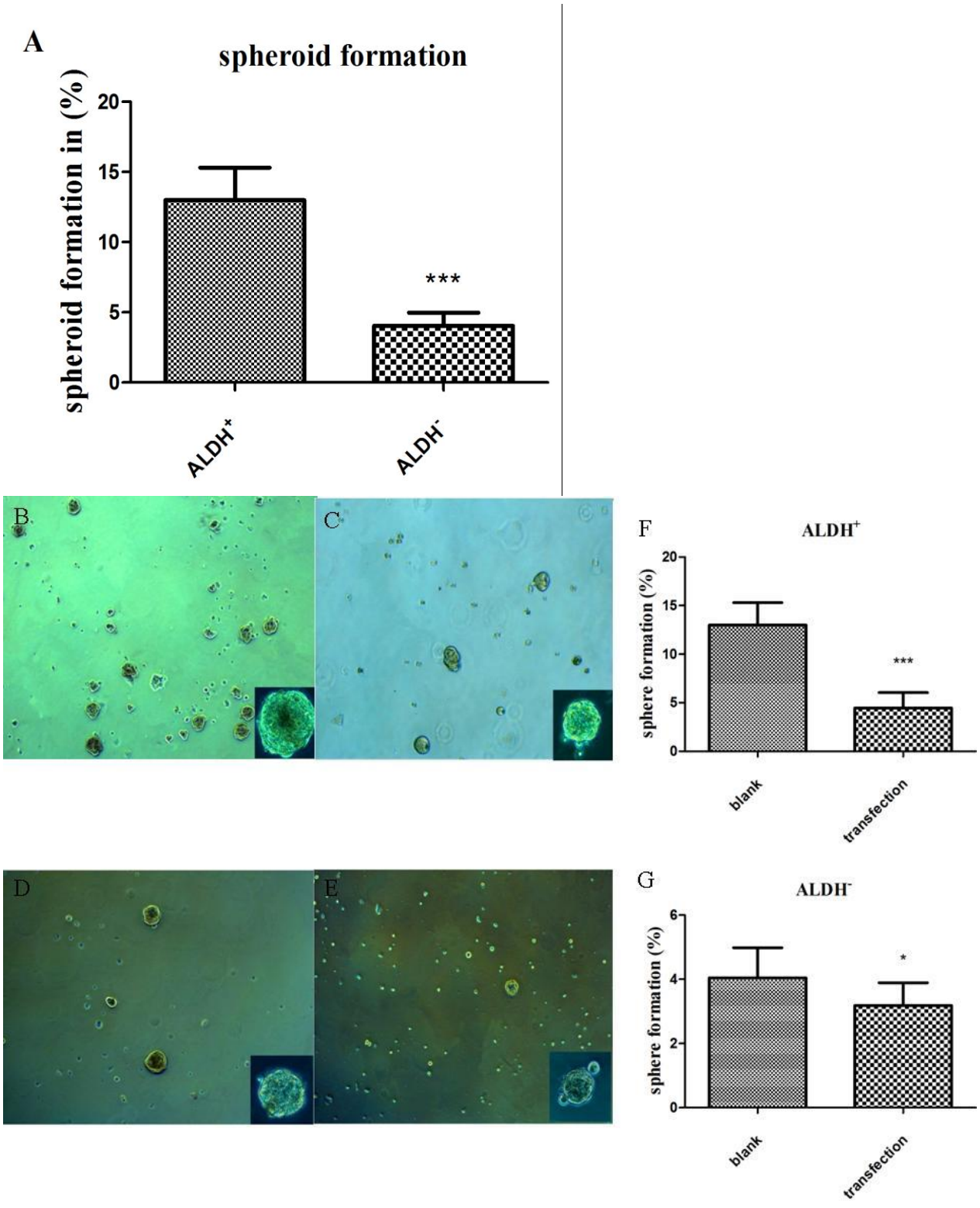


Figure 12: Representative results of anchorage-independent growth in UM-SCC9. (A) $ALDH^+$ cells formed significantly more spheroids than $ALDH^-$ cells. (B) Sphere formation of $ALDH^+$ cells, (C) sphere formation in $ALDH^+$ cells after transfection of miR-34a mimics, (D) sphere formation of $ALDH^-$ cells and (E) sphere formation of $ALDH^-$ cells after transfection of

miR-34a mimics. The number of spheroids and their size were significantly decreased in miR-34a mimics transfected samples as compared with NC. (F, G) The quantitative analysis indicated that miR-34a mimics could markedly inhibit anchorage-independent cell growth in $ALDH^+$ and $ALDH^-$ cells. The mean ± SD from three independent experiments are shown. *=P<0.05, ***=P<0.001. Magnification of insert representative sphere: 200-fold.

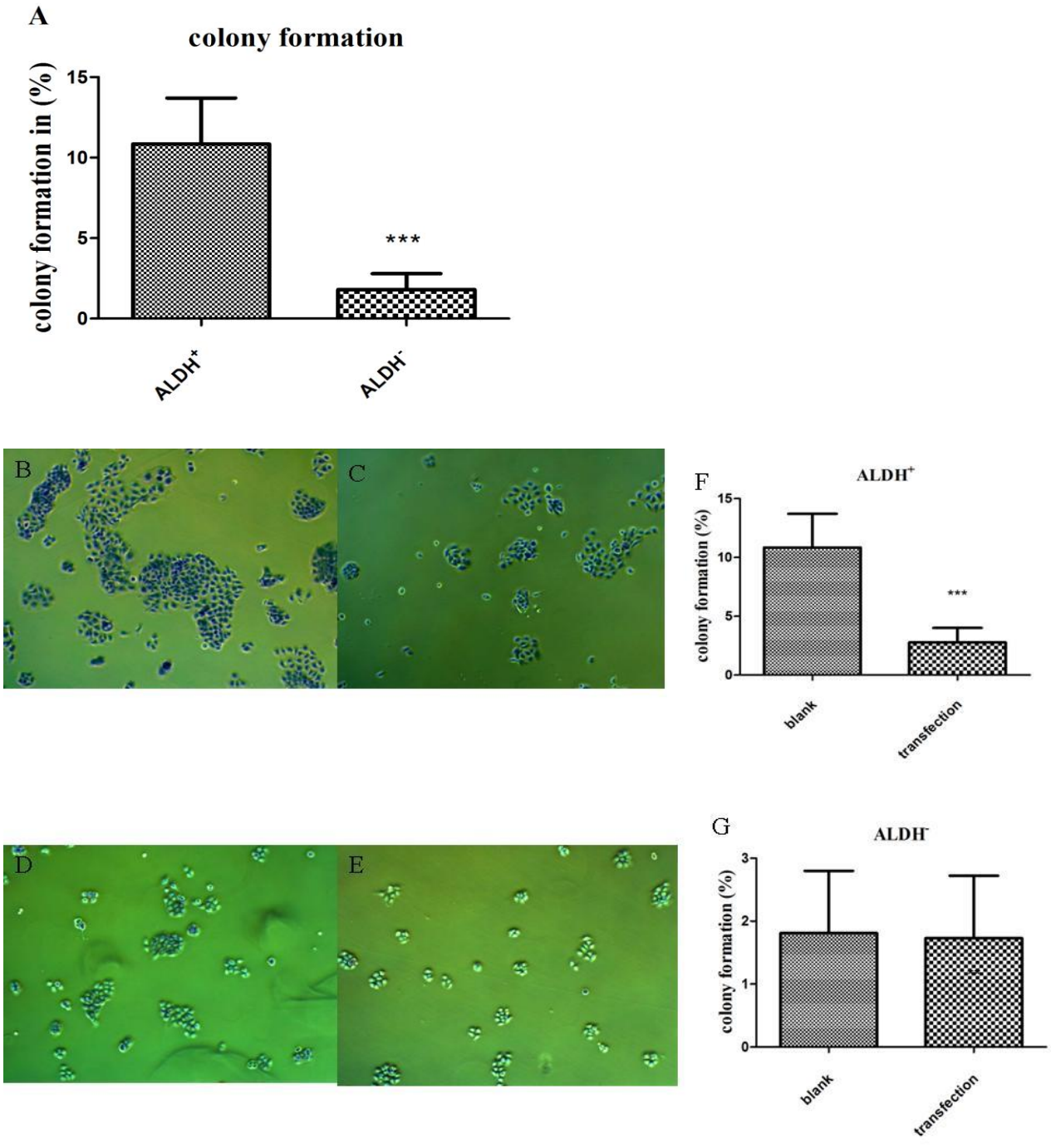


Figure 13: **Representative results of colony formation in UM-SCC9.** (A) $ALDH^+$ cells formed significantly more colonies than $ALDH^-$ cells. (B) Colony formation of $ALDH^+$ cells, (C) colony formation of $ALDH^+$ cells after transfection of miR-34a mimics. (D) $ALDH^-$ cell colony formation and (E) colony formation of $ALDH^-$ cells after transfection of miR-34a mimics. The number of colonies and their size were significantly reduced in miR-34a mimics-transfected samples compared with control transfected cells. (F, G) Columns indicate that miR-34a mimics could significantly inhibit colony formation in $ALDH^+$ cells. The mean $\pm$ SD from three independent experiments are shown. ***=P<0.001.

6. Discussion

6.1 Spheroid culture and enrichment of HNSCC-CSCs

Spheroids are spherical three-dimensional tumor cell clusters that are grown from one or several cell clones. As compared to cells cultured in monolayer, measured culture cell doubling time and the rate and pattern of spheroid growth *in vitro* better matches that observed in tumors *in vivo*[132]. Spheroid cell growth has also been identified to be a property of normal tissue cells which display stem cell properties[133]. Spheroid-enriched CSCs can be derived from a panel of different solid malignancies such as HNSCC[26], melanoma[134], breast cancer[135], and gliosarcoma[130]. Cells from these entities could be propagated by anchorage independent growth and displayed the phenotype of non-adherent spheroids. The spheroid-forming ability was found to correspond to the expression of established CSC markers[136].

The ability of a single cell to regenerate a malignant tumor consisting of cells with heterogeneous phenotypes is one characteristic of CSCs that may help to explain some of the differences which discriminate tumor cells from differentiated somatic cells like immortality, quiescence, invasion, metastasis, and relapse after treatment. Initial studies identified $CD44^+$ HNSCC cells that could generate new tumors *in vivo* (<5,000 cells injected into mice)[15], and concluded that CD44 is a CSC marker. Recently, ALDH was demonstrated to be another useful CSC marker to identify CSCs in many epithelial cancers including HNSCC[32,33,41]. In HNSCC, Chen et al. showed in immunocompromised mice that 500 injected $ALDH^+$ HNSCC cells resulted in visible tumors in all cases after 6 weeks, while 10^4 $ALDH^-$ cells failed to produce tumors[41]. According to our own results and in line with the observations mentioned previously, $ALDH^+$ cells showed increased CSCs properties compared to $ALDH^-$ cells derived from HNSCC cell lines[26]. We and others could demonstrate that in HNSCC there is a varying overlap of ALDH and CD44 populations[127].

In our experiments, we cultured spheroids from eight HNSCC cell lines. The eight cell lines showed varying ability to form spheroids. Sox2, Nanog and Oct3/4 were chosen as the stemness related TFs in this project, because they were expressed at high levels, both in normal stem cells and CSC as demonstrated by Chickarmane et al.[137] and for HNSCC by us[26].We evaluated the content of SDCs positive for the HNSCC-CSC marker ALDH by flow cytometry and CSC-related TFs by RT-qPCR. We found that in SDCs compared with corresponding MDCs, the CSC- related transcription factors Sox2, Oct3/4 and Nanog were significantly upregulated as was the proportion of $ALDH^+$ cells.

These findings reveal that the spheroid culture assay is a useful and efficient method for enriching cells with CSC characteristics from HNSCC cell lines and that sorting for $ALDH^+$ cells even allows for further enrichment of cells with spheroid- and clone-forming abilities. However, there was also a limitation in the spheroid culture assay to enrich for CSCs. The most important limitation was that the spheres still represent a heterogeneous population, with only a part of the cells having the ability of self-renewal[138]. To efficiently purify the CSC population from SDCs might require further more specific procedures. Therefore, it would be advantageous for cells to be cultured in spheroids followed by a purification step according to the following experiment such a FACS-sorting as demonstrated e.g. for ALDH.

6.2 miR-34a regulates the stemness and EMT properties in HNSCC CSCs

MiR-34a has been considered a tumor suppressor which represses stemness–related features and functions in prostate cancer[103], breast cancer[139] and pancreatic cancer[99]. An important finding of this thesis was that our data support the observation that miR-34a acts as tumor stemness repressor in HNSCC. Recent publications indicate a downregulation of miR-34a in HNSCC cell lines and tumor samples that may promote tumor growth and tumor angiogenesis[140]. Our own data confirm the observation of dysregulated miR-34a expression in HNSCC cell lines. We could demonstrate that the expression in stem cell-enriched HNSCC-SDCs was significantly lower than in parental HNSCC-MDCs. Furthermore, the frequency of $ALDH^+$ CSC was increased in SDCs compared to MDCs. However, transfection of miR-34a mimics into the HNSCC-SDCs resulted in a significant increase of miR-34a while simultaneously the expression of ALDH along with the stemness-related TFs Sox2, Nanog and Oct3/4 decreased. After upregulation of miR-34a, we found the colony and sphere formation ability and invasive capacity decreased in $ALDH^+$ cells compared to controls.

Although the direct effects of miR-34a have been studied in a wide range of different cancers, relatively few studies have investigated other possible cellular functions of miR-34a. Nam et al. reported that the p53/miR-34a axis regulates Snail1-dependent EMT. Among the proto type EMT regulators (ZEB1/2, Snail1, Slug), also miR-34a directly targets Snail1[141]. According to a study by Siemens et al. [104] miR-34a and Snail forms a double-negative feedback loop to regulate EMT. Activated p53 downregulates the EMT-inducing transcription factor Snail1 by upregulation of miR-34a. On the other hand, the TF Snail1 binds to the E boxes in miR-34a promoters to repress its expression. However, an indirect downregulation of ZEB1/2 and Slug,

which are all miR-200 targets, may occur via de-repression of the Snail-regulated miR-200 promoters after miR-34a activation[104].

In this study we compared EMT-properties of SDCs to MDCs after transfection with miR-34a mimics to further characterize the role of this molecule in EMT. We were able to demonstrate that the transfection of miR-34a mimics led to a downregulation of EMT-related TFs in SDCs. In line with these findings, the invasive capacity of these cells was reduced, indicating an implication of miR-34a in the regulation of EMT in HNSCC. This observation may be of future clinical relevance since, in a prostate cancer mouse model, systemic delivery of miR-34a inhibited formation of metastasis[103]. Therefore the mechanisms of miR-34a action and regulation presented here may have diagnostic and therapeutic implications in the future.

In our experiments, the EMT-related TF Snail1 and CSC-related TFs showed a decreased expression in HNSCC cell lines after transfection of miR-34a mimics, indicating that the miR-34a may be play multiple roles in suppressing mesenchymal traits and inhibiting stemness properties. A similar phenomenon was also reported in pancreatic cancer, where a restoration of miR-34a reduced CSC properties and inhibited the EMT[142]. In breast cancer cells, it was demonstrated that re-expression of miR-200 suppressed EMT-related genes and stemness properties[143] . C-MYC and CD44 represent direct miR-34a targets[103,144-146] whereas the effect on the other stemness markers/factors is presumably indirect. (Figure 14)

Taken together, we were able to demonstrate that miR-34a is involved in the regulation of EMT and invasive properties of CSC by quantifying TFs involved in the regulation of EMT (Snail and Twist) and by conducting functional assays displaying colony forming and invasive capacities. Previously, we demonstrated as a property of HNSCC-CSC an increased invasive capacity and expression of EMT markers such as α-smooth muscle actin and Vimentin, while at the same time the expression of the adhesion molecule E-Cadherin was significantly reduced [26]. Our experiments demonstrated that this can be reversed by transfer of miR34a-mimics in HNSCC in vitro. Evidence for an accumulation of the formulated miR34a mimics was reported in the spleen, lung and kidney[147], suggesting a potential opportunity for cancer therapeutic development.

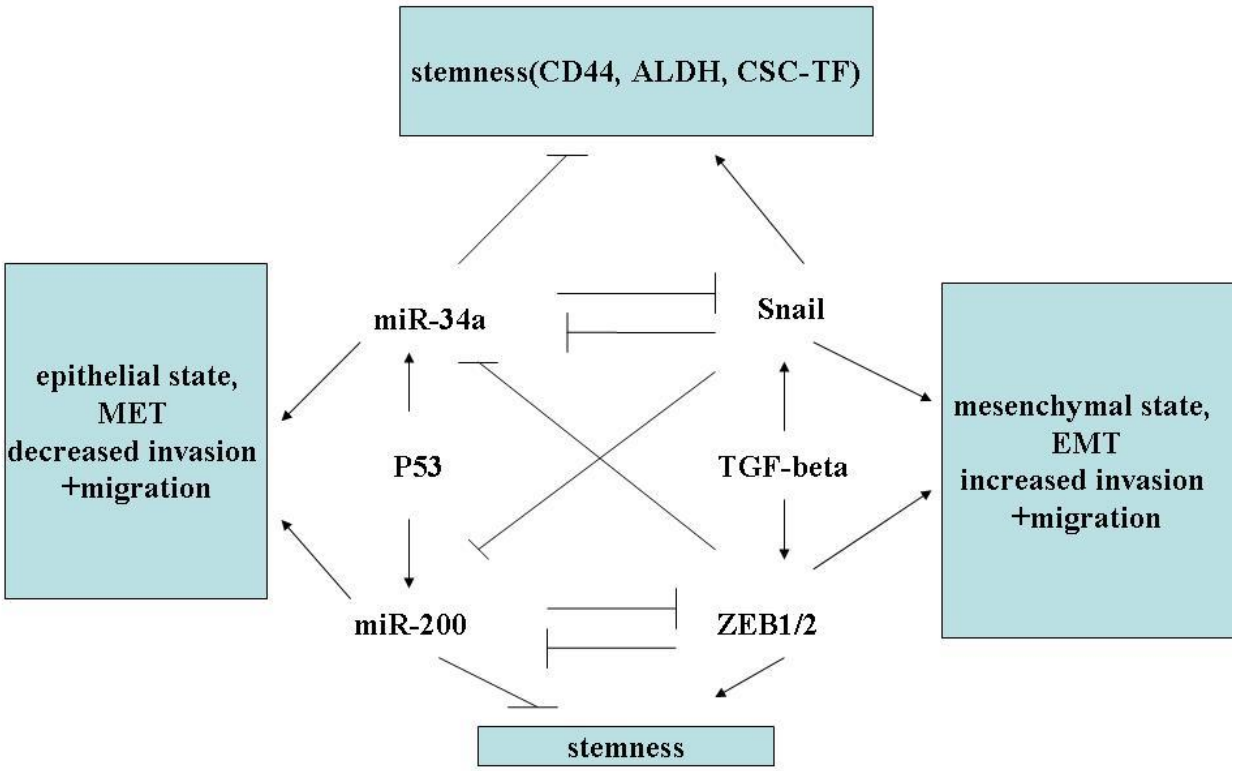


Figure 14: Summary of the regulatory roles of miR-34a in cancer. Putative interactions of miR-34a regulation by p53 and miR-200 found in this study were combined with findings from previous studies.

6.3 Influence of the HPV on HNSCC-CSC characteristics

HPV^+- and HPV^- HNSCC show distinct characteristics in its biological and clinical behavior. Xu et al. found in primary oropharyngeal squamous cell carcinoma, that HPV-DNA^+ tumors had a lower ALDH1A1 expression and the HPV-DNA^- group had higher expression as measured by immunohistochemistry[128]. ALDH1A1 is not only a CSC marker but it also serves as a prognostic biomarker in HNSCC[148,149]. A recent study assessed the utility of ALDH1A1 staining-intensity as a measure for its expression as a prognostic biomarker in surgically treated HNSCC patients. In this study, a significant correlation between ALDH1A1 staining intensity and prognosis was demonstrated. HNSCC patients with ALDH1A1 strong expression, had a significantly worse 5-year overall survival compared with those HNSCC patients who had weak ALDH1A1 expression[150]. On the basis of these observations, we hypothesized that the clinically proven

improved outcome of patients with HPV^+ HNSCC may be due to the fact that HPV^+ HNSCC has a lower expression level of ALDH than HPV^- HNSCC and a lower CSC content. Our data support these clinical results, as also in cell culture where HPV^+ HNSCC cell lines have shown a lower expression level of ALDH compared to the HPV^- group. However the number of tested cell lines was limited and may represent characteristics specific to HNSCC that have the ability to grow in-vitro.

In conclusion, in this study, we found that increased miR-34a can suppress CSC-like properties of HNSCC and EMT. This was demonstrated by the downregulation of CSC- and EMT-related TFs, colony- and spheroid-forming abilities and last not least decreased invasive capacity. These findings suggest that miR-34a may play important roles in these processes and may therefore have a potential in a novel therapeutic regimen or in combination with existing treatments of HNSCC a) by reducing the frequency of the CSC phenotype which is believed responsible for therapy resistance and b) by reducing the formation of novel metastases by inhibiting EMT. However, routes to apply this molecule in a therapeutic setting in human remain to be explored.

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8. Affidavit

“I, [Zhifeng, Sun] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [MicroRNA-34a regulates epithelial–mesenchymal transition (EMT) in cancer stem (like) cells (CSCs) of head and neck squamous cell carcinoma (HNSCC) and is a possible molecular target] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interests in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

<u>Declaration of any eventual publications</u>

[Zhifeng Sun] had the following share in the following publications:

1. **Sun Z**, Li S, Kaufmann AM, Albers AE. miR-21 increases the programmed cell death 4 gene-regulated cell proliferation in head and neck squamous carcinoma cell lines. Oncol Rep. 2014 Nov; 32 (5):2283-9. doi: 10.3892/or.2014.3456. Epub 2014 Sep 1. Responsible for drafting the manuscript

2. Coordes A, **Zhifeng Sun**, Sangvatanakul V, Qian X, Lenary M, Kaufmann AM, Albers AE**.** Cancer stem cell phenotypes and miRNA: therapeutic targets in head and neck squamous cell carcinoma. HNO. 2014 Dec; 62(12):867-72. doi: 10.1007/s00106-014-2931-4.Responsible for data collection.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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9. Curriculum Vita and Publications

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publications

1. Sun Z, Li S, Kaufmann AM, Albers AE. miR-21 increases the programmed cell death 4 gene-regulated cell proliferation in head and neck squamous carcinoma cell lines. Oncol Rep. 2014 Nov; 32 (5):2283-9. doi: 10.3892/or.2014.3456. Epub 2014 Sep 1.

2. Coordes A, **Zhifeng S**, Sangvatanakul V, Qian X, Lenarz M, Kaufmann AM, Albers AE. Cancer stem cell phenotypes and miRNA: therapeutic targets in head and neck squamous cell carcinoma. HNO 2014 Dec; 62(12):867-72. doi: 10.1007/s00106-014-2931-4.

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