

MicroRNA-34a regulates epithelial-mesenchymal transition and cancer stem cell phenotype of head and neck squamous cell carcinoma *in vitro*

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Abstract. MicroRNAs (miRs) are short non-coding single stranded RNAs regulating the translation of target mRNAs in normal and cancer cells in which they are frequently dysregulated promoting tumor progression. Cancer stem cells (CSCs) of head and neck squamous cell carcinoma (HNSCC), identified by aldehyde-dehydrogenase expression (ALDH), are a cell subset within the tumor cell population that takes part in the genesis and progression 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 CSC. CSC from 8 HNSCC lines, 4 of which are human papillomavirus (HPV)-positive, were enriched by spheroid culture (spheroid-derived cells, SDC) and compared to their parental monolayer-derived cells (MDC) to analyze 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. Flow cytometry was used to quantify and enrich ALDH⁺ CSCs. Transfection of miR-34a mimics was used to evaluate its regulatory potential for CSC marker profiles as well as CSC- and EMT-TFs expression in HNSCC-SDC. Invasive, colony-forming and clonogenic capability of the miR-34a mimics transfected SDC 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 SDC derived from HNSCC-lines as compared to parental MDC (-1.6-16.4-fold). For EMT- and CSC-related TF expression, all HNSCC-derived SDC showed a significantly increased level compared to parental MDC (≤ 36.8 -fold). Significantly increased expression of ALDH was found in SDC (2-3-fold). Compared to the HPV⁺, the HPV⁻ group showed a significantly higher mean expression level of EMT-TFs, CSCs-TFs and ALDH (30.3 v.s. 12.8%). Transfection of miR-34a mimics significantly reduced the EMT- and CSC-related TF expression level in UM-SCC9 (HPV⁻) and UM-SCC47 (HPV⁺) SDC. Simultaneously, the ALDH expression was reduced significantly (1.5-2-fold) and the invasive capacity ($\leq 30\%$) and clonogenicity of HNSCC-SDC was also inhibited by transfection of miR-34a mimics compared to controls. Restoration of 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.

Introduction

Head and neck squamous cell carcinoma (HNSCC) with >600,000 newly diagnosed cases per year is the sixth most common cancer in the world (1). In developed countries the average 5-year survival rate is ~50% (2). Improvements during the past decades in radio- and chemotherapy, and recently introduced targeted antibody therapy and improved surgical procedures have resulted in a significantly enhanced quality of life for cancer patients. However, local and distant recurrence is common in HNSCC and during the past years rates of survival have only marginally improved (3).

According to the cancer stem cell (CSC) theory, tumor formation, relapse after therapy and the development of metastases is thought to involve CSC in different phenotypic and functional states. This particular subset of cells was named cancer stem (like) cells (CSCs), similar to that conceptualised in normal tissue as stem cells (4). To date, the existence of

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CSCs have been identified in several human hematologic and solid tumors, including leukemia, bladder cancer, breast cancer, colon carcinoma, HNSCC and pancreatic cancer (5-9). The CSC theory suggests that only a subpopulation of cells in a tumor has the ability to self-renew, differentiate and regenerate similar tumors, as demonstrated *in vitro* (10-13) and by xenotransplantation into immuno-deficient mice (14). These cells display also some resistance to conventional therapy (11,12).

Spheroid culture is an effective and convenient way to enrich the CSC population present in the cancer cell population and is thus increasingly being used, sometimes in combination with further enrichment by specific markers, to evaluate functional stem cell activity in normal tissue and putative CSC. The ability of CSC to form spheroids was first observed in cells derived from the central nervous system in 1992 (15). Todaro *et al* found that cells isolated from the striatum of adult mouse brain could be clonally expanded by culturing spheres, and the cells generated both astrocytes and neurons (16). The ability of isolated cells to form anchorage-independent spheres in culture was demonstrated in breast, colon, HNSCC and melanoma (6,7,17). In HNSCC cell lines, CSC cultured with this method showed a high level of CSC-marker expression such as CD44, CD133 and ALDH (10,18,19).

Epithelial to mesenchymal transition (EMT) is a key event in metastasis and increasing evidence suggests there is a link between EMT and CSCs (20-23) that has also been demonstrated for HNSCC (10). Mani *et al* (22) and Hindriksen and Bijlsma (24) reported that induction of an EMT in human mammary epithelial cells led to an increased expression of CSC markers and in pancreatic cancer the occurrence of EMT is often accompanied by the activation of CSC-related pathways. EMT is a physiological process that plays an important role during embryonic development and morphogenesis (25). 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 (23). 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 (10,25,26). In recent years it has been demonstrated that EMT is involved in the development of metastasis by increased cellular motility and invasiveness (23,25,27) resulting in a more metastatic phenotype (28,29). During the EMT process, several changes in gene expression, such as increased Snail1, Snail2 and Twist transcription factors can be observed (23,30). We and others have demonstrated a link between EMT and CSCs. For example the expression of the EMT-marker Snail1/2 or Twist is increased in CSC, that are involved in the loss of the epithelial phenotype and the acquisition of the mesenchymal phenotype (10,31-33). 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 (21-23,34).

MiRs are a family of 21-25 nucleotide long, non-coding endogenous RNAs, that act by binding to the target mRNA 3'-untranslated region inducing mRNA degradation or repression of mRNA translation (35). 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 (35). 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 (36).

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 (37). 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 found downregulated in HNSCC and -lines (38). However, miR-34a expression and function of in HNSCC is not clear yet.

Materials and methods

Cell lines. Eight HNSCC cell lines were used: UD-SCC1, -2 (gift of Henning Bier, University of Munich, Germany); UM-SCC9, -11B, -47, -104 (University of Michigan, Tom Carey, MI, USA) and UT-SCC33 (University of Turku, Reidar A. Grenman, Finland), 93VU147T (VU Medical Center, John P. de Winter, Amsterdam, The Netherlands). The HPV types were as follows: HPV⁺: UD-SCC2, UM-SCC47, UM-SCC104, 93VU147T; all HPV type 16⁺. HPV⁻: UD-SCC1, UM-SCC9, UM-SCC11B, UT-SCC33. All cell lines were regularly tested for mycoplasma and found free of any contamination.

Spheroid culture. Adherent monolayer cells were grown in 75 cm² cell culture flasks (BD Science, Franklin Lakes, NJ, 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 μ g/ml, respectively; Biochrom) at 37°C in humidified atmosphere with 5% CO₂, 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 x g for 5 min, cells were resuspended in serum-free Quantum 263 (PAA), supplemented with 10 ng/ml EGF and 10 ng/ml β FGF (Biochrom). To generate spheroids, single cells were plated in cell culture dishes (BD FalconTM, 100x20 mm) with a coated surface with 1% agarose at a specific density of 2x10⁴ cells/ml. Cells were maintained at 37°C in humidified atmosphere with 5% CO₂ content.

Every three to four days, half of the medium was replaced. After 5-7 days, spheroid formation was checked and representative images were taken. For passaging or following experiments, culture medium with spheroids was removed from the dish and filtered through a 40- μ m mesh (BD Biosciences, Heidelberg, Germany) to collect the spheroids. For experiments, typically 2nd or higher generation were used.

Table I. Primer sequences used for RT-PCR (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 | |

Aldefluor assay and FACS sorting. The ALDH activity of SDC and MDC was determined by using the Aldefluor assay kit (Stem Cell Technologies, Durham, NC, USA). For collection of the spheroids a 40 μ m mesh was used. Subsequently cells were dissociated into single cells by trypsin/EDTA digestion for 10 min at 37°C followed by 30 times up and down pipetting using a 1,000 μ l pipette tip. Then, the single-cell suspension was washed twice in PBS without Ca²⁺/Mg²⁺ and suspended in 1 ml Aldefluor assay buffer containing 5 μ l ALDH substrate (1 ml/per 1x10⁶ cells) and incubated for 30-40 min at 37°C in the dark. As a negative control, for each sample, an aliquot was treated with 5 μ l diethylaminobenzaldehyde (DEAB; 50 mmol/l), a specific ALDH inhibitor. Following incubation, all samples were centrifuged for 5 min at 250 x 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 1x10⁷ cells per ml and run on an Aria cell sorter (BD Biosciences). The sorting gates were established, by negative control cells which were treated with the ALDH inhibitor DEAB.

Quantitative real-time PCR. To quantify the expression of mRNA, total RNA was isolated by TRIzol reagent (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions and then cDNA was prepared using the Omniscript First-Strand synthesis system (Qiagen, Hilden, Germany) 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 Bio-Rad Chromo 4 (Bio-Rad, München, Germany). GAPDH was the reference gene.

For the detection 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 Bio-Rad Chromo 4 (Bio-Rad) with Express SYBR GreenER™ qPCR SuperMix Universal (Invitrogen). GAPDH was the reference gene. Reactions were carried out in triplicate with controls; primer sequences are listed in Table I. The data were statistically analyzed by the modified 2^{- $\Delta\Delta$ Ct} value method (39).

Transfection of miRs. For transfection of miR mimics, spheroids were harvested using a 40 μ m mesh and dispersed into single cells as described above. The single cell suspension was plated at a density of 8x10⁴ 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 h, the medium was replaced with spheroid culture medium. Twenty-four hours 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™ Alexa Fluor Red Fluorescent Oligo using fluorescence microscopy.

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

Invasion assay. Warm (37°C) 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 h in a humidified tissue culture incubator, at 37°C in 5% CO₂ atmosphere. After rehydration, the medium was carefully removed without disturbing the layer of Matrigel™ matrix on the membrane. Next, cell suspensions (SDC transfected with miR-34a mimics and controls) were prepared in culture medium containing 5x10⁴ cells/ml for 24-well chambers. DMEM (750 μ l) containing 10% FBS 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 (2.5x10⁴ cells) was added to the 24-well chambers that were then incubated for 24 h in a humidified tissue culture incubator, at 37°C in 5% CO₂ 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 under x200 magnification (Axiovert, Axiovision, Zeiss, Germany).

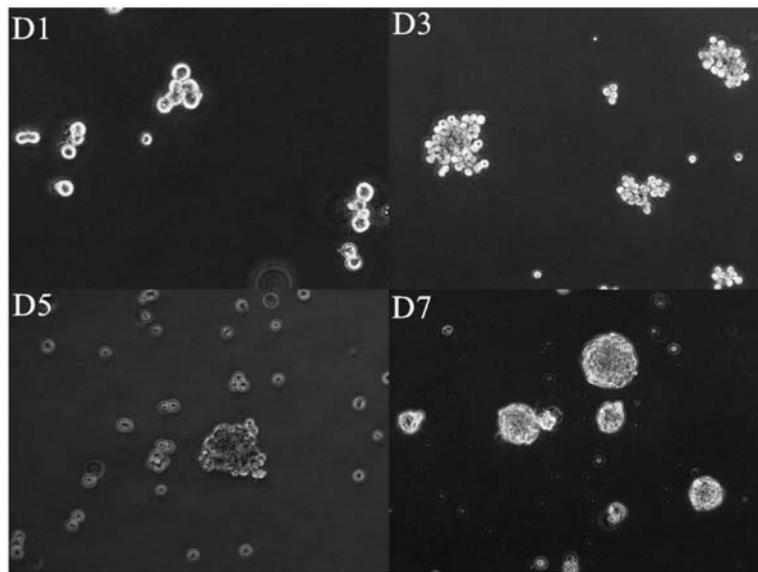


Figure 1. Time course of the spheroid culture (cell line UM-SCC9). Magnification, 100-fold. To determine the self-renewing ability, a single cell suspension generated from spheroids was plated at a clonal density of 1,000 cells per ml. After 7-10 days of subculture, tumor subspheroids (second generation) appeared, which contained 20-40 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 (10). Spheroids maintained in long-term culture up to the 10th generation, still showed this capacity for self-renewing ability that generated the parental HNSCC cells.

Colony formation assay and spheroid formation assay. A colony formation assay was used to assess the clonogenicity of miR-34a mimic-transfected UM-SCC9 cells sorted according to ALDH⁺ and ALDH⁻. After FACS sorting, the ALDH⁺ and ALDH⁻ cells were placed in 6-well plates for transfection with miR-34a mimics.

After FACS sorting, 800 ALDH⁺- and ALDH⁻ cells, respectively, 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), supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom). Fresh medium containing growth factors was added each week for 2 weeks.

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

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, the expression analysis was performed using the modified $\Delta\Delta C_t$ value method. Expression analysis and statistical evaluations was carried out by using the pair-wise fixed re-allocation randomization by Qiagen REST 2009 software (version 2.0.13).

Results

HNSCC cell lines contain cells with self-renewing capacity and the ability to form spheroids. Cells from 8 HNSCC cell

lines were cultured in suspension for 7-10 days at a specific density of 2×10^4 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 did not change in size anymore, but the number of the spheroids in culture still continued to increase and the cell clusters became more compact (Fig. 1).

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

The CSCs marker ALDH shows a higher expression level in SDC than in MDC. 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 (40). We measured ALDH enzymatic activity of SDC of the eight HNSCC cell lines and their matched MDC to identify and quantify the stem cell-like population (Fig. 2A).

All HNSCC-derived SDC showed a significant increase in the number of ALDH⁺ cells compared to parental MDC ($P < 0.05$) (Fig. 2B). In the HPV⁺ group, the cell line with the highest ALDH content was UM-SCC47 SDC ($25.62 \pm 0.50\%$) compared to the parental MDC ($11.05 \pm 0.16\%$). In the HPV⁻ group, the highest proportion of ALDH⁺ cells was found in SDC derived from UM-SCC11b ($45.05 \pm 0.22\%$) which was 3.3-fold higher than in the corresponding parental MDC ($13.38 \pm 0.11\%$). Interestingly, ALDH expression was higher in SDC and MDC cultured from HPV⁻ than from HPV⁺ cell lines. The average percentage of ALDH⁺ in the MDC cell population derived from the four HPV⁻ cell lines was $10.64 \pm 1.37\%$ while in the four HPV⁺ HNSCC cell lines on average $5.74 \pm 0.92\%$ of cells were ALDH⁺ ($P < 0.01$). In SDC

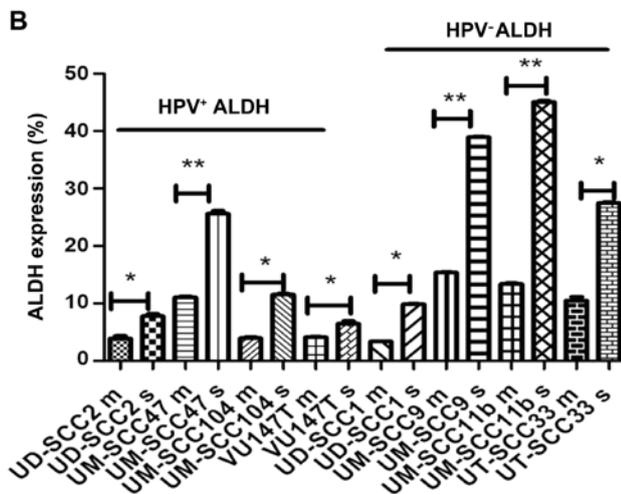
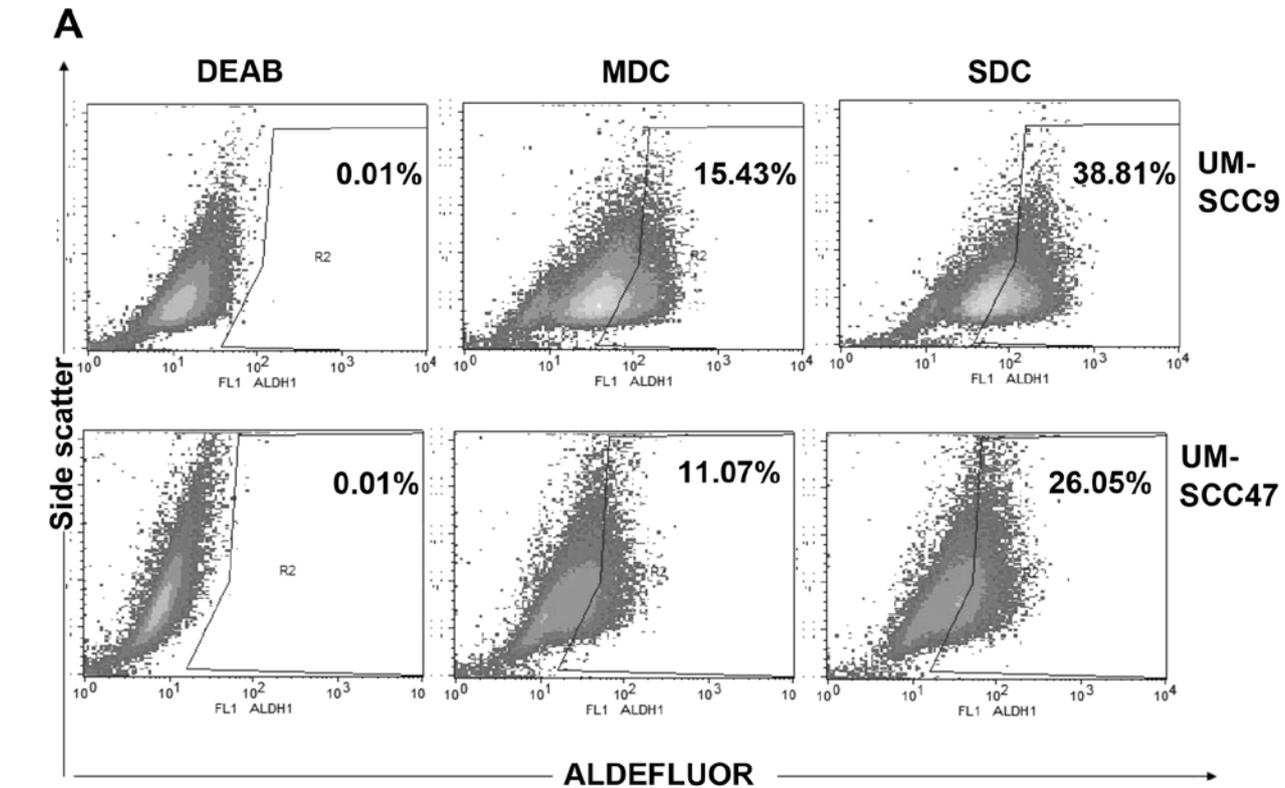


Figure 2. Expression of ALDH in HNSCC cell lines. (A) Representative example of a flow cytometric analysis of the expression of ALDH in SDC compared to MDC, and control SDC and MDC treated with DEAB, a specific inhibitor of ALDH. Region R2 depicts fluorescence intensity of ALDH⁺ cells. The number in the region represents the respective percentage of CSC in relation to all cells. (B) Mean percentage of ALDH⁺ cells contained in the population of SDC and MDC of the investigated cell lines relative to their HPV status. The bars represent mean percentage \pm SD of three independent determinations (* P <0.05, ** P <0.01). m, MDC; s, SDC.

the average percentage of ALDH⁺ in the HPV⁻ cell lines was 30.33 ± 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* (41) and may also reflect the poor success rate with HPV⁺ HNSCC tumor specimen in a xenograft mouse model due to a relatively low CSC content (42). Despite the fact that UD-SCC2 did not form compact spheroids, the SDC still showed an increased expression frequency of ALDH⁺ as compared to its MDC.

Stemness-related TFs and EMT-related TFs are overexpressed in SDC. 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 (43). In line with previous findings (10), we confirmed as prerequisite for further work, that the mRNA level of Sox2, Oct3/4 and Nanog were significantly increased in SDC of the eight investigated HNSCC cell lines (Fig. 3). The highest increase was observed in UM-SCC9 SDC, where a 36.81 ± 5.66 -fold increase in Oct3/4 expression was found over the expression present in the parental MDC. 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⁻ SDC/MDC was 8.21 ± 0.79 - vs. 1.83 ± 0.36 -fold in HPV⁺ cell lines (P <0.01). The mean Oct3/4 expression ratio in HPV⁻ SDC/MDC was 13.32 ± 4.38 - vs. 4.07 ± 1.34 -fold in HPV⁺ cell lines ($P=0.07$). Finally, the mean Nanog expression ratio was in HPV⁻ SDC/MDC 11.93 ± 2.80 - vs. 1.80 ± 0.52 -fold in HPV⁺ cell lines (P <0.01).

Snail1, one of the key TFs involved in EMT, was significantly increased in all SDC generated from the 8 tested HNSCC cell lines as compared to MDC 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⁻ SDC/MDC 20.72 vs. HPV⁺ 2.90-fold, P <0.05) and of Twist (HPV⁻ 9.87 SDC/MDC vs. HPV⁺ 2.72-fold, P <0.05) was significantly higher in HPV⁻ SDC/MDC than HPV⁺ cell lines.

miR-34a is downregulated in HNSCC-derived SDC. We selected miR-34a due to its role as a tumor suppressor. We found that the expression of miR-34a was consistently and significantly

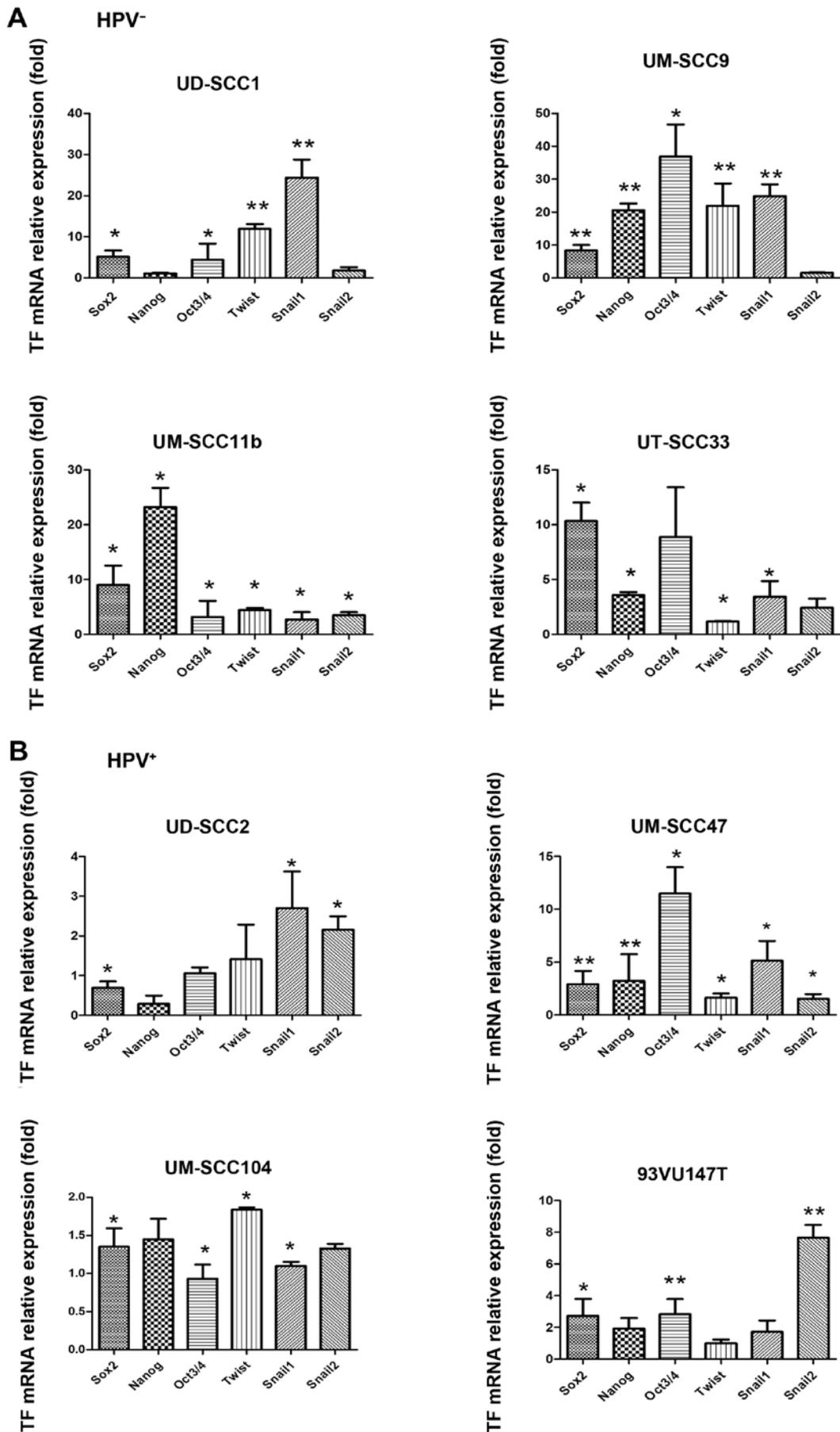


Figure 3. RT-qPCR analysis of mRNA expression of stemness-related TF and EMT-related TF. mRNA isolated from SDC and MDC was quantified for expression of the indicated panel of TF. The ratio of expression between SDC and MDC is represented by columns in the diagram (fold increase in SDC vs. MDC). Mean value \pm SD of three independent determinations (* P <0.05, ** P <0.01).

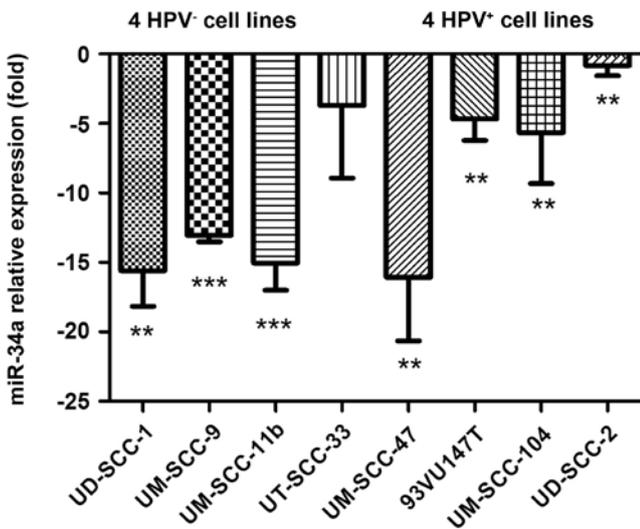


Figure 4. RT-qPCR analysis of miR-34a expression in HNSCC cell lines. The relative-fold changes in the HNSCC cell lines SDC to corresponding MDC is given. Mean value \pm SD of three independent determinations (* P <0.05, ** P <0.01, *** P <0.001).

downregulated in SDC compared to the parental MDC in all tested HNSCC cell lines (1.61-16.37-fold, P <0.05) (Fig. 4).

Overexpression of miR-34a reduces stemness- and EMT properties in HNSCC-SDC. To investigate the possibility that miR-34a serves as a link between EMT and CSCs by regulating EMT in HNSCC-SDC the following experiments were performed. First, 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 $94.33 \pm 0.57\%$ and of UM-SCC47 $93.66 \pm 1.52\%$. We also measured the transfection efficiency by verifying 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, 48 and 72 h, while the mock transfected group showed no differences in expression levels in comparison to the normal control (NC group) (Fig. 5A and B). The peak of miR-34a level was found 48 h post-transfection (UM-SCC9 4.49-fold, UM-SCC47 9.1-fold, P <0.01).

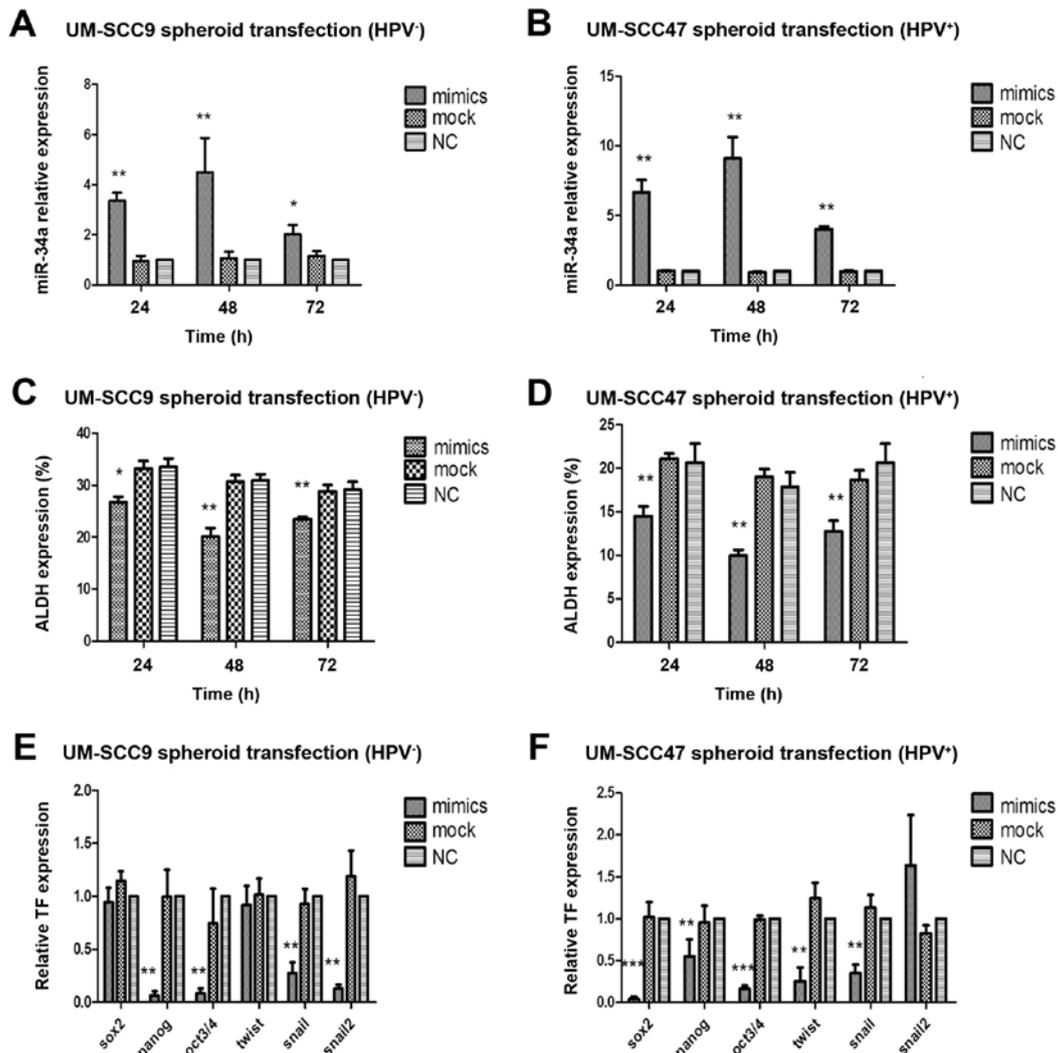


Figure 5. Overexpression of miR-34a reduces stemness- and EMT properties in HNSCC-SDC. (A and B) Change of miR-34a levels in SDC after transfection with miR-34a mimics over time (24, 48 and 72 h). (C and D) Change in the ALDH expression and (E and F) expression of CSC- and EMT-related TFs in SDC 48 h after transfection. Mean value \pm SD of three independent determinations. Significant differences were * P <0.05, ** P <0.01 and *** P <0.001.

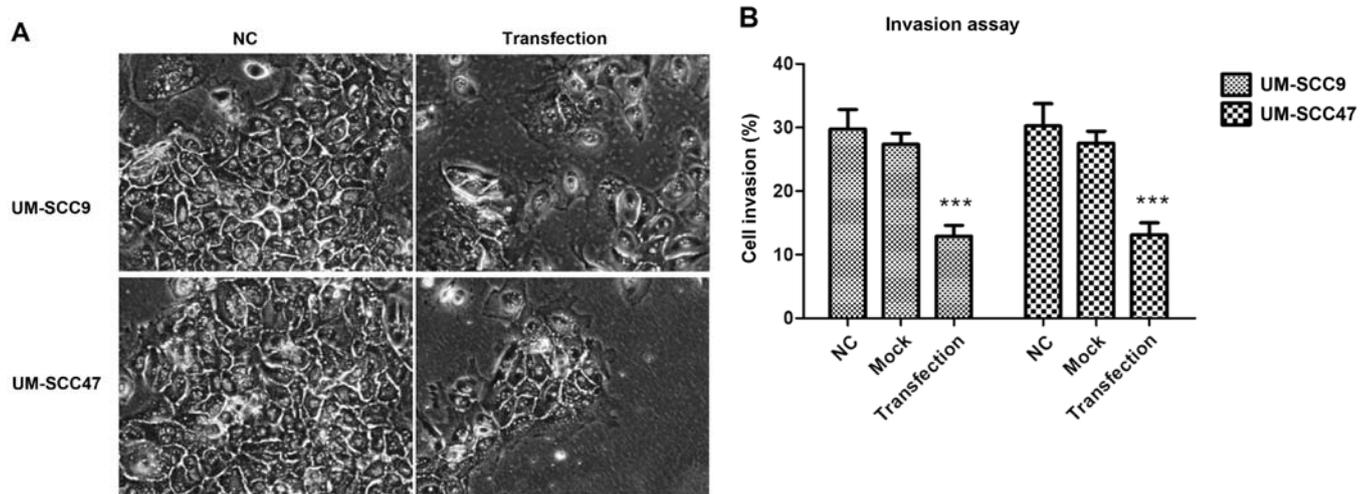


Figure 6. miR-34a transfection into HNSCC-SDC reduces invasion capacity. Cell invasion was measured by using a Matrigel coated system with the pore size of 8 μm . (A) Representative image of NC cells and miR-34a transfected cells after migration through the membrane. (B) NC and mock-transfected cells crossed significantly more frequently the membrane than miR-34a transfected cells. *** $P < 0.001$. Magnification, 200-fold.

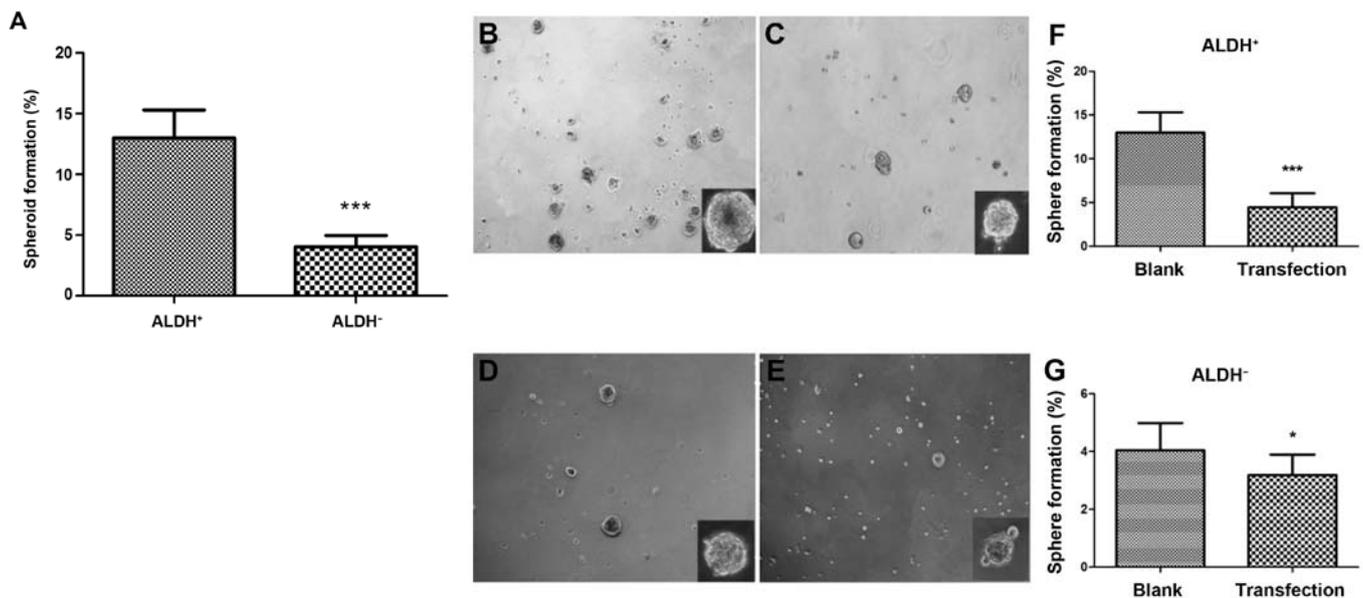


Figure 7. Representative results of anchorage-independent growth of UM-SCC9. (A) ALDH⁺ cells formed significantly more spheroids than ALDH⁻ cells. (B) Sphere formation of ALDH⁺ cells, (C) sphere formation in ALDH⁺ cells after transfection of miR-34a mimics, (D) sphere formation of ALDH⁻ cells and (E) sphere formation of ALDH⁻ cells after transfection of miR-34a mimics. (F and G) The quantitative analysis indicated that miR-34a mimics markedly inhibited anchorage-independent cell growth in ALDH⁺ and ALDH⁻ cells. The mean \pm SD from three independent experiments is shown. * $P < 0.05$, *** $P < 0.001$. Magnification of the inserted representative sphere, 200-fold.

With respect to the regulation of CSC marker, we observed downregulation of ALDH expression after miR-34a mimics transfection after 24, 48 and 72 h (Fig. 5C and D). The strongest change in ALDH⁺ cell frequency was found to correlate to the highest miR-34a levels 48 h 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 TF mRNA expression which was tested 48 h post-transfection (Fig. 5E and F). Nanog, Oct3/4 and Snail1 were significantly decreased over controls in the two transfected HNSCC cell lines and Sox2 and Twist showed significant downregulation in UM-SCC47. While Snail 2

showed decreased expression in UM-SCC9 and an increase in expression in UM-SCC47.

Overexpression of miR-34a reduces the invasive capacity. For evaluation of the invasive capacity of tumor cells we used the Matrigel invasion assay. After 24 h of culture, the number of miR-34a mimic-transfected HNSCC-SDC that migrated through the Matrigel coated chamber was significantly decreased ($P < 0.001$) compared with NC and mock-transfected cells (Fig. 6). The miR-34a levels after transfection were significantly increased as described above (UM-SCC9: 29.75 ± 1.08 vs. $12.87 \pm 0.61\%$, UM-SCC47: 30.25 ± 1.23 vs. $13.12 \pm 0.66\%$).

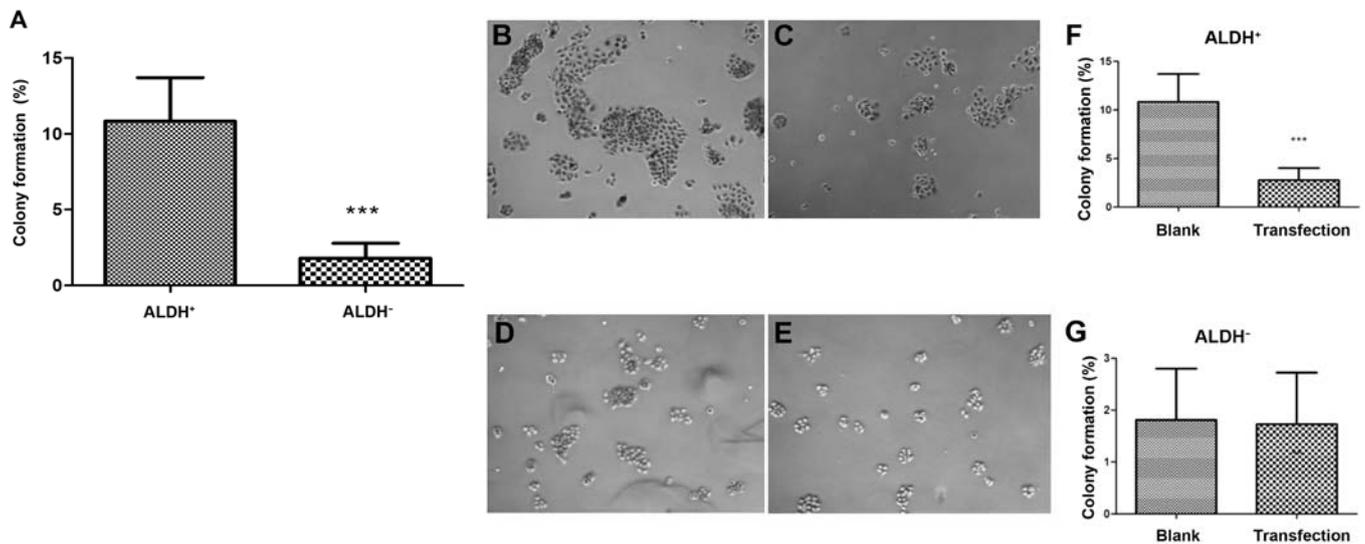


Figure 8. Representative results of colony formation in UM-SCC9. (A) ALDH⁺ cells formed significantly more colonies than ALDH⁻ cells. (B) Colony formation of ALDH⁺ cells, (C) colony formation of ALDH⁺ cells after transfection of miR-34a mimics. (D) ALDH⁻ cell colony formation and (E) colony formation of ALDH⁻ cells after transfection of miR-34a mimics. The number of colonies and their size was significantly reduced in miR-34a mimic-transfected samples compared with control-transfected cells. (F and G) Columns indicate that miR-34a mimics significantly inhibited colony formation in ALDH⁺ cells. The mean \pm SD from three independent experiments is shown. ***P<0.001.

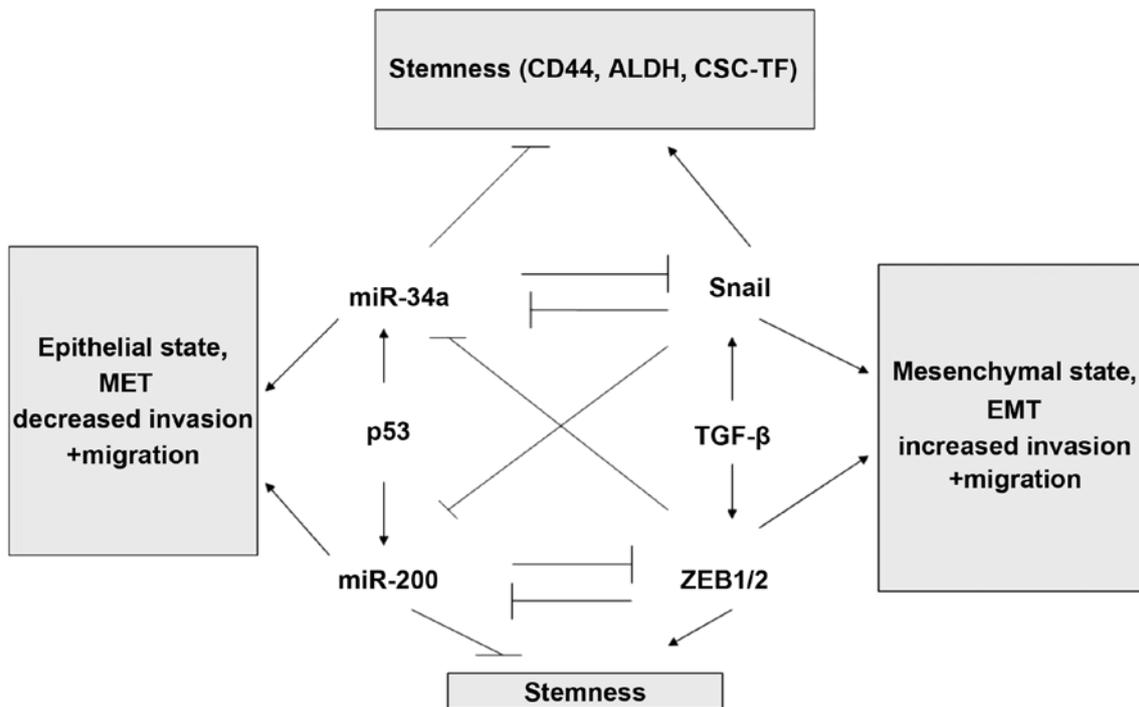


Figure 9. Summary of the regulatory roles of miR-34a in cancer. Putative interactions of miR-34a regulation by p53 and miR-200 found in this study were combined with findings from previous studies as described in the text.

Transfection of miR-34a significantly decreased the migration/invasion of UM-SCC9 and UM-SCC47 SDC by ~50%.

Decreased ability of spheroid and colony formation as well as anchorage-independent growth after miR-34a transfection. Following 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 (Fig. 7A) and colony formation ability (Fig. 8A) than ALDH⁻ cells. Transfection with miR-34a mimics reduced significantly the ability for anchorage-independent growth of UM-SCC9 cells. Analysis of spheroid formation showed that cells transfected with miR-34a mimics displayed much fewer and smaller spheroids than the control-transfected cells (Fig. 7B-G). The number of spheroids

and their size were significantly decreased in miR-34a mimic-transfected samples as compared with NC.

Discussion

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, cell doubling time and the rate and pattern of spheroid growth *in vitro* better matches that observed in tumors *in vivo* (44). Spheroid cell growth has also been identified to be a property of normal tissue cells which display stem cell properties (45). Spheroid enriched CSCs can be derived from a panel of different solid malignancies such as HNSCC (10), melanoma (46), breast cancer (47) and gliosarcoma (14). 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 (48).

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 such as 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) (8) and concluded that CD44 is a CSC marker. ALDH has been demonstrated to be another useful CSC marker to identify CSCs in many epithelial cancers including HNSCC (10,33,41,49-51). In HNSCC, Chen *et al* showed in immunocompromised mice that 500 injected ALDH⁺ HNSCC cells resulted in visible tumors in all animals after 6 weeks, while 10⁴ ALDH⁻ cells failed to produce tumors (51). According to our own results and in line with the above mentioned observations, ALDH⁺ cells showed increased CSCs properties compared to ALDH⁻ cells derived from HNSCC cell lines (10). We and others have demonstrated a varying overlap of ALDH and CD44 populations in HNSCC (10,40).

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 predicted by Chickarmane and Peterson (52) and demonstrated for HNSCC by us (10). We validated the content of SDC positive for the HNSCC-CSC marker ALDH by flow cytometry and CSC-related TFs by RT-qPCR. We found that in SDC compared with corresponding MDC, 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 to enrich 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 (53).

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 (54), breast cancer (55) and pancreatic cancer (37). An important finding of this study was that the data support the observation that miR-34a acts as tumor stemness repressor in HNSCC. Recent reports indicate downregulation of miR-34a in HNSCC cell lines and tumor samples that may promote tumor growth and tumor angiogenesis (38). Our own data confirm the observation of dysregulated miR-34a expression in HNSCC cell lines. We demonstrated that the expression in stem cell-enriched HNSCC-SDC was significantly lower than in parental HNSCC-MDC. Furthermore, the frequency of ALDH⁺ CSC was increased in SDC compared to MDC. However, transfection of miR-34a mimics into the HNSCC-SDC replenishing miR-34a levels simultaneously decreased the expression of ALDH along with the stemness-related TFs Sox2, Nanog and Oct3/4. 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. Kim *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 (56). According to a study by Siemens *et al* (57) 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, indirect downregulation of ZEB1/2 and Slug, which are miR-200 targets, may occur via de-repression of the Snail-regulated miR-200 promoters after miR-34a activation (57).

In this study, we compared EMT-properties of SDC to MDC 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 downregulation of EMT-related TFs in SDC. 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 (54). Therefore, the mechanisms of miR-34a action and regulation presented here may have diagnostic and therapeutic relevance in the future.

In our experiments, the EMT-related TF Snail1 and CSC-related TFs showed decreased expression in HNSCC cell lines after transfection of miR-34a mimics, indicating that the miR-34a may 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 (58). In breast cancer cells, it was demonstrated that re-expression of miR-200 suppressed EMT-related genes and stemness properties (59). C-MYC and CD44 represent direct miR-34a targets (54,60-62), whereas, the effect on the other stemness markers/factors is presumably indirect (Fig. 9).

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 (10). 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 (63), suggesting a potential opportunity for cancer therapeutic development.

Influence of the HPV on HNSCC-CSC characteristics. HPV⁺- and HPV⁻ HNSCC show distinct characteristics in their biological and clinical behavior. We 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 (41). ALDH1A1 is not only a CSC marker but it also serves as a prognostic biomarker in HNSCC (33,64). Moreover, we have assessed the utility of ALDH1A1 staining-intensity as measure for its expression as a prognostic biomarker in surgically treated HNSCC patients. We were able to demonstrate a significant correlation between ALDH1A1 staining intensity and prognosis (33). HNSCC patients with strong ALDH1A1 expression, had a significantly inferior 5-year overall survival compared with those HNSCC patients who had weak ALDH1A1 expression (65). On the basis of these observations, we hypothesized that the improved clinical 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 HPV⁺ HNSCC cell lines have 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-lines 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 decreased invasive capacity. These findings suggest that miR-34a may play important roles in these processes and may therefore have a potential in novel therapeutic regimen or in combination with existing treatments of HNSCC: a) by reducing the frequency of the CSC-phenotype which is held 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 humans remain to be explored.

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