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DISSERTATION

Characterization of microRNA expression in cervical carcinogenesis and cancer stem cells

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ABBREVIATIONS AND ACRONYMS

7-AAD	7-amino-actinomycin D
ABC	ATP binding cassette
ALDH1	Aldehyde dehydrogenase isoform 1
bFGF	Basic Fibroblast Growth Factor
CIN	Cervical intraepithelial neoplasia
CK17	Cytokeratin 17
CSCs	Cancer Stem Cells
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
FK	Foreskin Keratinocyte
HKGS	Human Keratinocyte Growth Supplement
HPV	Human papilloma virus
H-SIL	High-grade squamous intraepithelial lesion
HR-HPV	High-risk human papilloma virus
L-SIL	Low-grade squamous intraepithelial lesion
LR-HPV	Low-risk human papilloma virus
MDCs	Monolayer-derived-cells
miR/miRNA	MicroRNA
Oct3/4	Octamer-binding transcription factor 4
Pap test	Papanicolaou test
PBS	Phosphate Buffered Saline
PDCD4	Programmed Cell Death 4
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction

RNA	Ribonucleic acid
SCs	Stem cells
S.D.	Standard Deviation
SDCs	Spheroid-derived-cells
SNP	Single-Nucleotide Polymorphism
Sox2	Sex-determining region Y-box 2
SP	Side Population
TFs	Transcription Factors
ZEB1	Zinc finger E-box-Binding homeobox 1
ZEB2	Zinc finger E-box-Binding homeobox 2

Zusammenfassung

Hintergrund: Das Paradigma der Krebsstammzellen (CSC) kann die Entstehung von Krebs, und insbesondere Gebärmutterhalskrebs erklären. Dysfunktionale microRNAs wurden kürzlich mit mehreren Krankheiten, darunter Krebs in Verbindung gebracht. Solche microRNAs funktionieren als Hauptschaltstellen der Zellphysiologie und Differenzierung.

Ziel: Untersuchung der Bedeutung von microRNA Dysregulation und HPV-Infektion in Präkanzerosen und Gebärmutterhalskrebs-Stammzellen.

Methoden: In dieser Studie haben wir zunächst vergleichend die oncomir miR-21 und Tumor-Suppressor miR-218 Expression untersucht. Hierzu wurde durch qRT-PCR bei 9 Gebärmutterhalskrebszelllinien (CaSki, HeLa, SiHa, C33A, MRIH186, MRIH215, C4-1, SW756 und ME180) und 4 Vorhaut-Keratinozyten (FK) Primär-zelllinien (FK08-35, FK09-03, FK09-07, FK09-09), die adhärent als Monolayer auf Zellkulturkunststoffplatten gewachsen waren, die Expression verglichen. Zur Anreicherung von CSC wurden alle Gebärmutterhalskrebszelllinien in dreidimonsionalen Sphäroid-Kulturen kultviert. Die Expression stammzellspezifischer microRNAs (miR-34a, miR-200c, miR-203) und Transkriptionsfaktoren (Oct3/4, Sox2, Nanog) wurde mit den entsprechenden, als Monolayer adhärent kultivierten Zellen, verglichen. Die Expression der Stammzellmarker ALDH1, CD44 und CD24 wurde ebenfalls zwischen Sphäroid- und Monolayerkulturen verglichen. Schliesslich wurde die Expression von miRNAs in Pap-Abstrichen von Patientinnen untersucht. Die zytologische und HPV Diagnose wurde zur Expression von miR-21 und miR-218 in den Abstrichproben korreliert.

Ergebnisse: Im Vergleich zu FKs, zeigten die Gebärmutterhalskrebszelllinien Überexpression von miR-21, und verringerte Expression von miR-218. Fünf Zelllinien CaSki, MRIH215, C4-1, ME180 und MARQ bildeten Sphäroide, die sehr kompakt waren. Vier Zelllinien, HeLa, C33A, MRIH186 und SW756-bildeten nur lose Aggregate von Zellen. SiHa bildete nie Sphäroide. Im allgemeinen wurde eine Herabmodulation (1,15-48,64 fach) von miR-34a, miR-200c und miR-203 in Sphäroiden und eine Hochregulierung von Stammzell-spezifischen Transkriptionsfaktoren gemessen. Es wurde ein deutlich höherer Anteil an $ALDH1^{+}$ (2-8 fach), $CD44^{+}$ $CD24^{-}$ (1,5 bis 3,15 fach) und $ALDH1^{+}$ $CD44^{+}$ $CD24^{-}$ (1,14 bis 3,49 fach) positiven

Zellen in Sphäroiden im Vergleich zu Monolayer-kultivierten Zellen gefunden. In Pap-Abstrichen waren miR-21 und miR-218 deutlich dysregulierten. In der HPV-positiven Gruppe im Vergleich zur HPV negativen Gruppe war miR-21 8,22 fach hochreguliert und miR-218 war 98,44 fach herabreguliert. Es wurde keine signifikant unterschiedliche Expression von miR-21 und miR-218 zwischen verschiedenen zytologischen Diagnosegruppen (Einteilung nach Bethesda System) gefunden.

Schlussfolgerungen: Sphäroidzellkultur ist eine effiziente Methode, um Gebärmutterhalskrebsstammzellen anzureichern. Unterschiedliche Expression von miRNAs wurde in normalen Keratinozyten, Gebärmutterhalskrebszelllinien sowie in subkultivierten Sphäroid-abgeleiteten CSCs gefunden. Dies refluktiert, potenziell den Anteil des Stammzell phänotyps. Die Expression von miR-21 und miR-218 wird wahrscheinlich durch Infektion mit HPV in zervikalen Präkanzerosen fehlreguliert.

【Schlüsselworte】: Gebärmutterhalskrebs, Krebs-Stammzellen, HPV, microRNA

SUMMARY

Background: The cancer stem cell (CSC) paradigm is one possible way to understand the genesis of cancer, and cervical cancer in particular. Recently dysfunctional microRNAs have been linked with several diseases, including cancer. Such microRNAs function as master regulators of cell physiology and differentiation.

Objective: To explore the relationship between microRNA dysregulation and HPV infection, cervical precancerous lesions and cervical cancer stem cells.

Methods: In this study, we first comparatively investigated the oncomir miR-21 and tumor suppressor miR-218 expression by qRT-PCR in 9 cervical cancer cell lines (CaSki, HeLa, SiHa, C33A, MRIH186, MRIH215, C4-1, SW756 and ME180) and 4 foreskin keratinocyte (FK) cell lines (FK08-35, FK09-03, FK09-07, FK09-09) grown adherently as monolayer on cell culture plastic. Then three-dimensional cultures were generated from all cervical cancer cell lines enriching CSCs. Stemness-related microRNAs (miR-34a, miR-200c, miR-203) and transcription factor expression (Oct3/4, Sox2 and Nanog) were compared to the corresponding monolayer-derived cells. The expression of stem cell markers ALDH1, CD44 and CD24 was also compared between cervical cancer spheroids and their corresponding monolayer cultures. Expression of miRNAs was investigated in Pap smears from patients. Cytological and HPV diagnosis was correlated to the expression of miR-21 and miR-218 in the smear samples.

Results: As compared to FKs the cervical cancer cells overexpressed miR-21, and had reduced expression of miR-218. Five cell lines, CaSki, MRIH215, C4-1, ME180 and MARQ, formed spheroids which were highly compact. Four cell lines, HeLa, C33A, MRIH186 and SW756, formed only loose aggregates of cells. SiHa never formed any spheroids. There was generally a down-modulation (1.15-48.64 fold) of miR-34a, miR-200c and miR-203 in spheroids formed by cervical cancer cell lines and up-regulation of stemness transcription factors. There was also a significantly higher proportion of $ALDH1^{+}$ (2-8 fold), $CD44^{+}CD24^{-}$ (1.5-3.15 fold) and $ALDH1^{+}CD44^{+}CD24^{-}$ (1.14-3.49 fold) populations in cervical cancer spheroids compared with corresponding monolayers. In Pap smears, miR-21 and miR-218 were found to be significantly dysregulated. In the HPV positive group compared to the HPV free group, miR-21 was 8.22 fold up-regulated and miR-218 was 98.44 fold down-regulated. There was no significantly

different expression of miR-21 and miR-218 between different cytologically diagnosed groups based on the Bethesda system.

Conclusions: Spheroid culture is an efficient method to enrich cervical CSCs. Different expression of miRNAs was found in normal keratinocytes, cervical cancer cell lines and in subcultivated spheroid-derived CSCs potentially reflecting the stemness phenotype of a proportion of cells. The expression of miR-21 and miR-218 was dysregulated by infection of HPV in cervical precancerous lesion.

Key words：Cervical cancer, cancer stem cells, HPV, microRNA

1 INTRODUCTION

Cervical cancer remains a major cause of morbidity and mortality in women worldwide, despite many outstanding advances that have been dedicated to reducing the disease burden in the past three decades. The cancer stem cell (CSC) paradigm might be one possible way to understand cervical carcinogenesis. In the CSC theory, it is hypothesized that not all cancer cells in the bulk tumor cell population are equal. Instead, there is a small stem cell population that replenishes the tumor with proliferating cells of high plasticity. However, the CSC research in cervical cancer is unfortunately at its inception when compared to leukemia and certain solid tumors. Several putative surface markers can be employed for isolation and identification of cervical CSCs. Increasing evidence also indicates that microRNAs (miRNAs) might play an important role in cervical carcinogenesis. Based on the CSC model, novel therapeutic strategies, including CSC targeting chemo- and immunotherapy, as well as miRNA-based molecular therapy, are expected to mitigate the occurrence, metastasis, resistance and recurrence of cervical carcinoma.

1.1 Cancer and cancer stem cells

The processes underlying the etiology of cancer have been the fodder for several theories for a century. The traditional model of carcinogenesis states that any mature somatic cell can evolve into a malignant cell due to the accumulation of multiple mutations and then acquire self-renewal as well as multi-lineage potency to generate an entire tumor [1] . However, based on this model, the complexity of multiple different cell types within a malignant tumor could not be sufficiently explained since the large number of necessary mutations to create such heterogeneity would be rather unlikely to occur [1]. Until the past two decades, the CSC model, first proposed 150 years ago, has re-emerged with the advances in developments of the stem cell theory and research.

Stem cells (SCs) are defined functionally as cells that have the capacity to self-renew as well as to generate differentiated cells. These SCs are characterized by the expression of several signaling pathways (Wnt, TGF-ß, LIF, PI3K/AKT) and genes (Oct3/4, Sox2, Nanog). In the CSC theory, it is pointed out that increased growth potential and/or 'immortalization' are not necessarily characteristics of the majority of cells in primary tumors [2]. Instead, tumors have

only a relatively small CSC subpopulation which is transformed from normal cells or SCs as a result of accumulation of genetic events. These CSC give rise to all other heterogeneous tumor cells [3]. The CSCs were first identified in leukemia and later in solid tumors such as breast, brain, head and neck, colon, ovarian, pancreas and prostate cancers. In cervical cancer, the solid evidence is limited to date (Fig. 1). The concept that there is a small subset of cells with stem-cell like properties in the tumors is widely accepted, although whether the CSCs are truly the only cells with tumorigenic potential remains elusive [4].

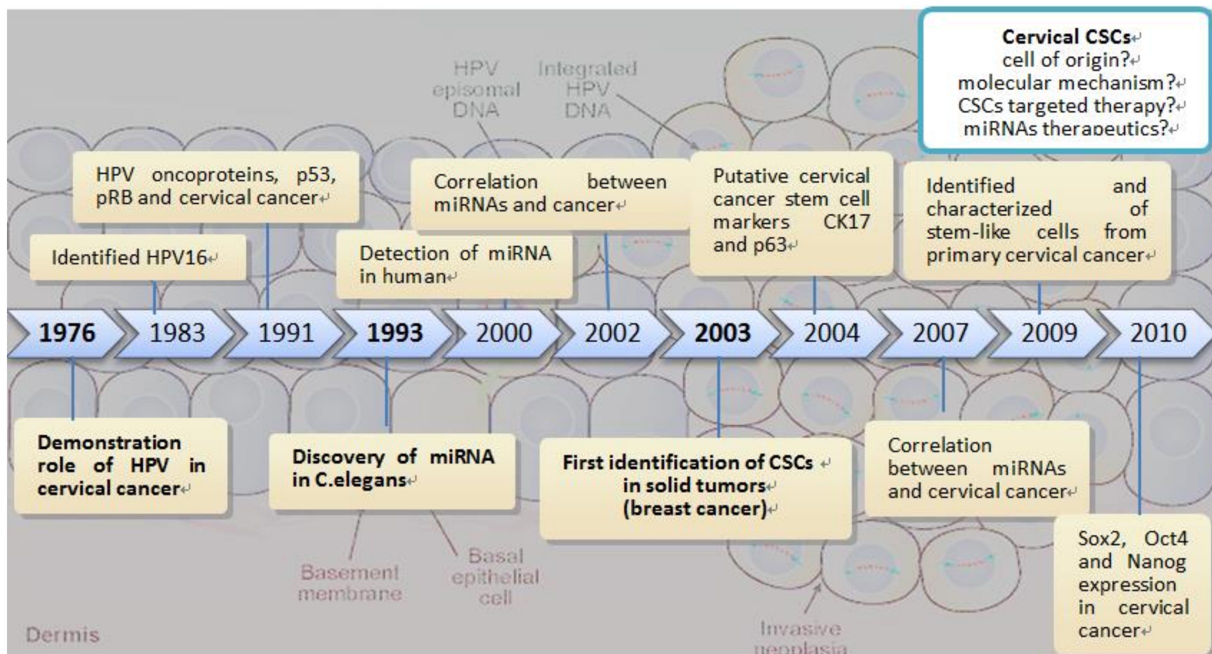


Fig. 1: Breakthrough discoveries of cervical cancer stem cells. Very important discoveries are highlighted in bold.

There are three methods which are commonly employed for the isolation of CSCs. These three methods are (1) the isolation by flow cytometric sorting of a side population (SP) based on Hoechst dye efflux, (2) sorting on the basis of cell surface marker expression, and (3) enrichment by sphere culture. They all lead to an enrichment of the CSC population and each has its advantages and limitation as shown in table 1 [5].

Spheroid culture is a forward straight method to enrich CSC populations from an adherently growing cancer cell line as compared to other methods, such as sorting according to side population or cell surface marker expression. The ability of CSCs to form spheroids was first demonstrated in cells from the central nervous system. In 1992, Reynolds and Weiss

demonstrated that cells isolated from the striatum of adult mouse brain could be clonally expanded by culturing spheres and that these cells could generate both astrocytes and neurons [6]. In humans, CD133+ cells isolated from human fetal brain were shown to form spheres *in vitro* [7]. Furthermore, brain tumours also contain CD133+ cells that are capable of giving rise to neurospheroids [7]. The ability of purified CSCs to form spheres in culture was later demonstrated for breast, prostate, colon, head and neck, pancreatic, and melanoma [5, 8]. Thus, some researchers have used sphere cultures to enrich CSCs.

Tab. 1: Three methods of isolation of CSCs, principles, advantages and limitations

	Principles	Advantages	Limitations
SP side-population	The presence of the SP is a result of inefficient dye uptake as a reflection of the presence of largely quiescent cells, another characteristic of stem cells.	Does not require other special marker, has been successfully used for CSC isolation from a variety of cancers	SP does not represent a homogeneous population of CSCs. In some cases, such as in skin cells [9], the SP does not appear to enrich cells with stem cell characteristics. The method relies on dye staining in which the dye concentration, staining time and temperature are critical. The dye also can have a deleterious effect on the cells [10].
Sorting Based on cell surface markers	Diverse principles depending on the specific markers	Allows for definition of precise populations.	Requires a large number of cells to sort. The choice of specific markers is difficult to make.
Spheroid culture	CSCs have an ability to grow anchorage independently and therefore to form floating spheroids	An easy method to enrich the CSC population	Represents a heterogeneous population, with only a portion of the cells capable of self-renewal. Differences in the enrichment of CSCs in spheres due to different spheroid size, passage, culture medium and technique which is difficult to standardize

1.2 HPV, cervical cancer and cervical cancer stem cells

Cervical cancer is one of the most common types of cancers affecting female reproductive organs. As HPV is detected in 99.7% of cervical cancer cases, it is clearly demonstrated that

certain types of HPV, so-called high-risk HPV, are the etiological agents of cervical cancer [11]. A small percentage of tumors appear to be negative for the presence of HPV DNA, but the possibility that these invasive lesions contain an as yet unidentified HPV type cannot be excluded. HPV infection is a common sexually transmitted infection; however, the majority of persons mount an effective immune response and clear infection within 6-12 months [12]. Approximately 10% of individuals develop a persistent infection that induces the development of low- and/or high-grade cervical intraepithelial neoplasia (CIN), which can regress or progress to an invasive cervical carcinoma after a long period of latency [8]. Studies suggested that the CSC in the cervical carcinogenic lesion is the HPV-target stem cell of the epithelium [13, 14]. The cervical reserve cells (also known as subcolumnar reserve cells), that are progenitor cells of the cervical epithelium that HPV targets for infection, locate in the transformation zone. This tissue undergoes metaplasia and provides a depository for the generation of the mucus-forming epithelium. It emerged to be the candidate for the origin of CSCs as it is the HPV-target and shows stem cell-like properties [15-17].

Infection by HR-HPV is necessary but not sufficient for progression of HPV-transformed cells to cancer. Mutations in cellular genes and chromosomal rearrangements induced by genomic instabilities are important and contribute to tumorigenesis [13]. HR-HPV DNA integration, found in approximately 90% of cervical cancer tissue, is the initiation of genomic impairment, a hallmark of HR-HPV-associated cancer [18-20]. The HPV oncoproteins E6 and E7 are the primary transforming viral proteins. They are expressed at low levels in low-grade intraepithelial neoplasia, whereas they are abundantly expressed in high-grade malignant lesions, with deregulated expression of these viral oncogenes being the key element for neoplastic progression [21]. Cells independently expressing HR-HPV E6 and E7 have an increased ability to integrate foreign DNA as well as higher DNA breakage and mutation frequency [22, 23]. The efficient binding of Rb and E7 can promote cell proliferation through a p53-independent pathway [13]. The HPV E6 oncogenes have evolved to target p53 to degradation [13]. P53 inhibits abnormal cell growth by inducing apoptosis, and cell cycle arrest, thereby preventing the reprogramming of suboptimal cells [24]. Dysfunction of p53 induced by HR-HPV oncoproteins inhibits p53 tumor suppressor activity. Moreover, it also engenders the proteins with an oncogenic ability, such as c-Myc, referred to as a gain of function [25].The combined action of high-risk E6 and E7 proteins in neutralizing the biological functions of Rb and p53 is therefore sufficient to immortalize HPV-infected cells [26].

After HR-HPV infection, diverse cellular changes associated with epithelial-mesenchymal transition (EMT) were observed [27]. EMT is a complex process that shares molecular characteristics with CSCs [28]. Studies involving clinical tissue have shown that EMT is involved in the invasion of cervical cancer and associated with malignant tumor progression [29, 30]. Induction of EMT transcription factor Twist in cervical cancer cell lines is critical for activation of the β-catenin and Akt pathway and morphological changes associated with EMT [31]. Overexpression of Twist also induced significant elevation of cancer stemness abilities, such as tumorsphere formation, expression of ALDH1 and CD44 [31]. This finding suggests that EMT may endow tumor cells with stem cell-like properties. Twist1 positive expression can predict poor clinical survival rates of cervical cancer patients [32]. Twist2 overexpression promotes EMT by affecting expression of E-cadherin and was also associated with malignant transformation of cervical epithelium and histological progression in cervical cancer [33].

Loss or aberrant expression of E-cadherin has been characterized as the major hallmark responsible for the loss of cell-cell contacts during the EMT process [34-36]. Loss of E-cadherin expression is often correlated with the tumor grade and stage [37], because it results in the disruption of cell-cell adhesion and an increase in nuclear β-catenin, thus leading to cell growth and survival [31]. P53 also represses the EMT program by promoting expression of the microRNA-200 family which is a suppressor of E-cadherin repressors ZEB1 and ZEB2 [38]. E-cadherin protein obviously decreases in cervical cancer tissue as compared to normal cervical tissue [29]. This downregulation is promoted by overexpression of epidermal growth factor (EGF) receptor via up-regulated Snail [29]. Several metastasis suppressors such as SFRP1&2 and LMX-1A have also been identified as repressors of EMT in cervical cancer [39, 40].

The cervical tumorigenesis is also regulated by several signaling pathways. The Notch signaling is an important form of direct cell-cell communication involved in cell fate determination and stem cell potential [3]. Interestingly, it has been shown that the function of Notch signaling in tumorigenesis could be either oncogenic or anti-proliferative, and the function could be context dependent [28]. In a limited number of tumor types, including skin cancer, human hepatocellular carcinoma and small cell lung cancer, Notch signaling has been shown to be anti-proliferative rather than oncogenic [3, 41, 42]. In contrast, most of the studies have shown opposite function of Notch in many human carcinomas, including cervical cancer[3]. Notch1 induces anoikis resistance, inhibits p53 activity and upregulates oncogene myc

expression in cervical cancer [43-45]. Activation of the Notch signaling pathway cooperates with HPV-induced cervical carcinogenesis through a mechanism involving an increase in cell survival [44, 46]. This cooperation was shown to be able to transform human keratinocytes [43, 47, 48]. Downstream signals generated by the Notch pathway cooperate to generate and maintain a fully transformed phenotype in the context of HPV oncogenes E6 and E7 [49].This is consistent with the finding that the Notch1 receptor expression is elevated in cervical carcinomas [50, 51]. However, in light of the normal pro-differentiation function of Notch activation in normal keratinocytes [46, 52-55], elevated expression of the Notch1 receptor in cervical cancer could also be interpreted as a marker of differentiation [3]. Inhibition of Notch1 expression in the HPV16 cervical cancer cell line CaSki resulted in a loss of neoplastic potential *in vitro* and *in vivo* [56].

As described above, Notch plays a role in promoting cervical carcinogenesis. Furthermore, Notch1 signaling pathway is also involved in EMT through the phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway [57, 58]. However, Notch is a negative regulator of keratinocyte stem cell potential and inducer of differentiation via a complex cross-talk with p63, one of the putative cervical CSCs markers [54]. It was shown in cervical cancer cell lines that the expression of p63 and Notch are both elevated in cervical cancer, the cross-talk between them might be involved in the balance of cervical CSCs self-renewal, differentiation and the EMT process (Fig.2).

Stem-like genes are overexpressed in cervical cancer cells or tissues. Sox2 is significantly more highly expressed in cervical cancers compared to normal cervix tissue. The level of expression is correlated with cervical tumor pathologic grade [59]. In HPV-positive cervical cancer cell lines CaSki and HeLa, Oct3/4 is over-expressed as compared to HPV-negative cell lines C33A [60]. The expression levels of Nanog are also significantly higher in squamous cervical carcinoma (SCC) patients compared to CIN patients, and also higher in CIN patients compared to those with normal cervical epithelia. Furthermore, the expression levels also vary significantly according to tumor size [61]. One possibility is that Nanog is suppressed by p53 which is deficient in HPV-target cervical cancer cells [62] .

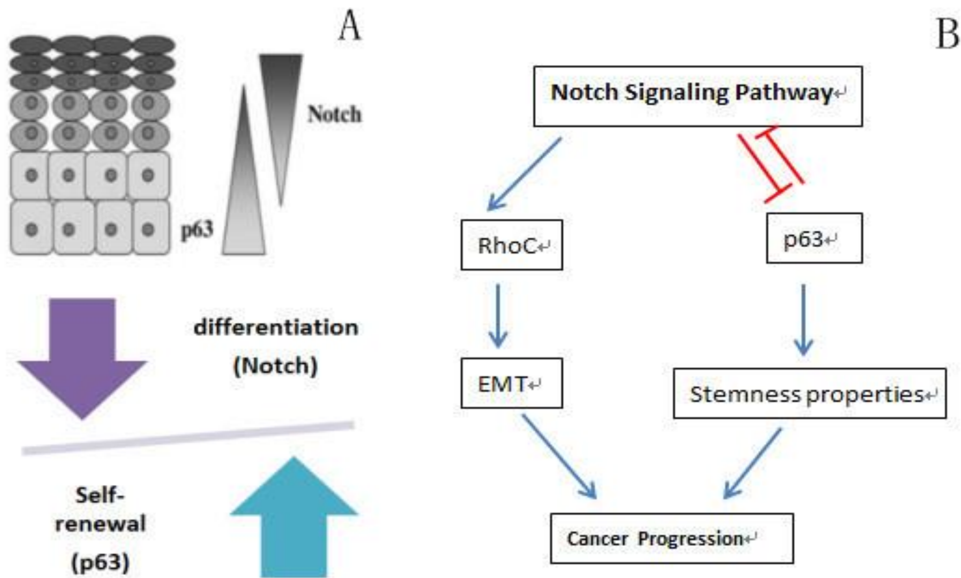


Fig. 2: Role of Notch signaling pathway in cervical CSCs. (A) Diagram of the epidermis illustrating the inverse gradient of p63 expression versus Notch activity in the lower versus upper epidermal layers, which is likely to result, at least in part, from their reciprocal negative regulation [54]. (B) The cross-talk between Notch and p63 regulates the balance of differentiation and self-renewal. Notch inhibits cancer progression via interaction with p63; however, it also promotes the EMT process which is important for tumor invasion and progression.

1.3 Putative cervical cancer stem cell markers

Cytokeratin17 (CK17), one of the acidic cytokeratin members, is expressed in reserve cells and immature metaplastic cells [16]. The expression of CK17 in abnormal cervical tissue is considered a signal of proliferation of cervical reserve cells as well as the possibility to develop into infiltrating carcinoma[63] . CK17 expression also correlates with increasing CIN lesion grade and carcinoma [64]. Therefore, CK17 is regarded as a marker for identification of the progression of high-grade CIN into invasive cancer. It is indicated that those CIN lesions with a full pallet of CK17 positive reserve cells could have a higher malignant potential than those without [65], whereas Regauer et al. considered that CK17 expression in pseudo-stratified epithelia merely reflects a metaplastic process [64].

CD44 is used as an important cell surface marker or evaluating factor in isolating cells with

stemness properties from multiple tumor types, including cervical cancer [66, 67]. CD44 activates many receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (ERBB2) in many cancer types [68]. This leads to increased proliferation and survival of CSCs via activation of the MAPK and PI3K/AKT signaling pathways [69]. Notch signaling pathway is also important to maintain the CD44 function in EMT process. Inhibition of Notch1 reduces CD44 expression and brain metastases formation ability in breast cancer [70]. Recently, it was reported that CD44 regulates the activation of Furin regulator NF-κB. Furin is an extracellular protease implicated in HPV processing and enhances cervical cancer cell motility [71]. Despite a lack of compelling evidence for a central role of CD44 in self-renewal and pluripotency, CD44 can contribute to the activation and also can be a target of SCs regulatory genes [72]. However, it was also reported in head and neck cell lines that CD44-negative cells have stem-cell-like properties [73].

P63 is a member of the p53 family of transcription factors. It accumulates after Rb inactivation induced by HPV E7 [74] and is involved in the maintenance of basal progenitor cell populations. P63 expression guarantees the capacity of tissues to develop and regenerate [75]. It is a proliferation marker that is highly expressed in proliferative undifferentiated basal keratinocytes such as in reserve cells of the transformation zone, but is poorly expressed in differentiated non-proliferative cells such as endo-cervical epithelium [76, 77]. P63 is necessary in HPV late viral function and plays an important role in the development of cancer and CSCs [78]. Its expression is up-regulated along with the malignant level of cancer cells [63]. P63 is important for maintaining the undifferentiated state via Notch, Sonic hedgehog (SHH) and Indian hedgehog (IHH) signaling pathways [79-81]. It also promotes the expression of CD44 by up-regulating the CD44 mRNA expression [82]. As a consequence, P63 became one of the candidate markers in cervical cancer stem-like cells.

The novel marker for CSC, Aldehyde dehydrogenase 1 (ALDH1), is a cytosolic enzyme responsible for oxidizing a variety of intracellular aldehydes to carboxylic acids [83]. The ALDH1 functions mainly in retinoid metabolism and is highly expressed in hematopoietic progenitor cells, intestinal crypt cells and breast tumor cells [84, 85]. The retinoid promotes the terminal differentiation in normal late hematopoietic precursors while displaying an opposite role to enhance self-renewal in immature hematopoietic cell populations which are enriched in stem cells [86]. By regulating the retinoid acid metabolism, ALDH1 maintains the balance of

self-renewal and differentiation corresponding to environmental cues. ALDH1 has been used successfully as a stem cell marker in head and neck squamous cell carcinoma, and in lung, prostate, pancreas, and breast cancer [87]. A recent publication reported that a small subpopulation of tumor and cell line-derived cells expressed ALDH1 in cervical carcinoma [88, 89]. However, ALDH1 is not a universal stem cell marker. It is reported that ALDH1 expression is significantly reduced in malignant ovarian tumor while it was relatively unchanged in benign tumors compared to normal ovary [90].

To date, there is still neither a universal CSC marker nor a specific cervical CSC marker identified. To combine the application of putative CSC markers may be a promising approach. Combined use of different putative CSC markers may provide a more precise definition of stem-like populations. It was recently shown in breast cancer that CSC marker ALDH1 can further divide the $CD44^{+}CD24^{-/low}$ cell population into fractions that are highly tumorigenic: $ALDH1^{+}CD44^{+}CD24^{-/low}$ cells were able to generate tumours from only 20 cells, whereas $ALDH1^{-}CD44^{+}CD24^{-/low}$ were not tumorigenic in this same cell density [91, 92].

1.4 MicroRNAs: new players on the path to CSCs

MicroRNAs are a class of small non-coding RNAs (~20 nt in length) that regulate genome expression post-transcriptionally and control the stability and translation of mRNA by inhibition of translation or mRNA degradation [93, 94]. Aberrant expression of miRNAs are connected to human diseases, including cancer development [95]. One gain of function described for p53-mutants is their ability to interfere with miRNA biogenesis, decreasing the availability of several mature miRNAs involved in the p53 response to DNA damage [96]. The functional disorders of miRNA are caused by gene mutation, epigenetic variation or genetic polymorphism [97]. A case-control study in over 400 cervical cancer cases reported a single-nucleotide polymorphism (SNP) of pre-miR-146a, and pre-miR-218 was associated with increased risk for cervical cancer development [98, 99]. The emergence of the CSC concept has led to a research focus on CSCs and miRNA (Fig.3)[100].

As an oncogene, miRNA miR-21 is over-expressed in breast tumor tissues and correlates with specific breast cancer biopathologic features as well as clinical outcome [101]. Being promoted by stemness genes Nanog and STAT3, miR-21 functions as an oncogene by

modulating tumorigenesis through up-regulating Bcl-2 and down-regulating Programmed Cell Death 4 (PDCD4) expression. These result in increased tumor growth and decreased apoptosis [102, 103]. A recent study indicated that together with EMT, miR-21 played a crucial role in metastasis development in p53-deficient lung cancer [104]. MiR-21 is increased in cervical cancer tissue/cells compared with normal cervical tissue/cells [105]. An experiment in HeLa cells showed that miR-21 targets PDCD4-3'UTR directly [106].

The miRNA203 (miR-203) is expressed specifically in the suprabasal layer of stratified epithelia, as well as in psoriatic plaques, implicating it as a regulator of epithelial maturation [107-109]. The miR-203 is significantly underexpressed in cervical cancer [110]. The primary role of miR-203 is to suppress the proliferative capacity of epithelial cells upon differentiation [109]. In mouse keratinocytes, miR-203 targets the 3'UTR of p63 which regulates the balance between proliferation and differentiation [111]. The expression of miR-203 is increased upon differentiation of normal keratinocytes, leading to suppression of p63 translation in suprabasal epithelia [111]. In HPV-infected epithelia, E7 blocks miR-203 upregulation through the MAP kinase pathway, leading to increased levels of p63. Thus, the cell remains active in the cell cycle and HPV genome amplification is possible [112]. Other studies have shown that expression of miR-203 is dependent on the regulation of p53 by E6 [113].

MiRNA miR-34a, a tumor suppressor which directly targets p53 post-transcriptionally, functions by inducing G1 arrest, apoptosis and senescence by affecting cell cycle regulators [114]. In p53-deficient human gastric and pancreatic cancer cells, restoration of functional miR-34 inhibits cell growth and induces G1 arrest and senescence, indicating that miR-34a may restore p53 function [115, 116]. The mechanism is considered to be related to miR-34a downstream targets Bcl-2, Notch, and HMGA2 which are involved in cancer stem cell self-renewal and differentiation [115, 116]. In prostate cancer, it was indicated that miR-34a directly represses CD44 mRNA [117]. HPV is also involved in reducing miR-34a expression by E6-mediated p53 degradation in raft cultures, CIN and cervical cancer tissues [118-120]. Li et al reported that miR-34a expression was significantly reduced in normal cervical epithelium and CIN with HR-HPV infection as compared to those without. Moreover, there was also a reduction in SiHa cells (cervical cancer cells with HR-HPV16 E6) compared to normal cells, indicating that HR-HPV E6 induced expression of miR-34a in cervical carcinogenesis [118]. It was shown in HeLa cells that miR-34a may suppress tumor invasion by down-regulating Notch1 and

Jagged1, two receptors in the Notch signaling pathway which are associated with tumorigenesis and invasion [121].

MiR-218 can be regulated by HPV E6. It is down-regulated in cell lines that are HPV16-positive or have HPV18 integrated when compared with cervical epithelium and a HPV negative cervical cancer cell line C33A [122]. It is also under-expressed in HPV16 positive epidermal cell lines of head and neck cancer [123]. Introduction of miR-218 into SiHa cells could reduce the levels of laminin 5β mRNA. The laminin 5β protein acts as a transient receptor of HPV and promotes HPV infection of basal cells [122, 124]. Although it is still unknown exactly how tumor suppressor miR-218 interacts with high-risk HPV E6, it is suggested that miR-218 may be a specific cellular target of high-risk HPVs [122].

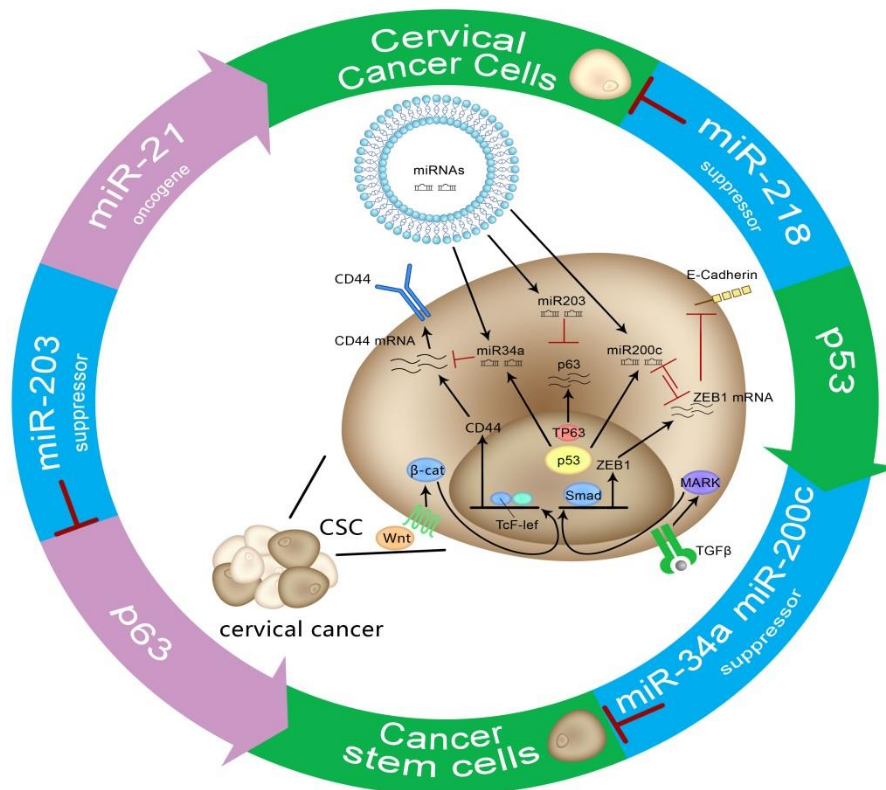


Fig. 3: Potential roles of several miRNAs in cervical cancer and CSCs. The miR-21 is described as oncomir as most of its targets are tumor suppressor genes. The miR-218 is a tumor suppressor which can be regulated by HPV E6. The p53-regulated miR-34a and miR-200c can inhibit the "stemness" properties of CSCs by targeting different mRNAs. The miR-203 targets p63 which is an essential regulator of "stemness" maintenance [125].

Besides miRNAs mentioned above, there are certain miRNAs with aberrant expression in cervical lesions and cancer, including miR-17-92 as oncogenes and miR-15a, miR-16-1, miR-23b, miR-124 and miR-214 etc. as tumor suppressors [126]. Abnormal expression of miRNAs is connected to the dysregulation of CSCs. Therefore, further research would provide a better understanding of the role of carcinogenesis and therapeutic potential of miRNAs.

1.5 Therapeutic potential targeting putative cervical CSCs

One of the major obstacles in cancer chemotherapy is drug resistance. According to the CSC model this may be due to the small subset of CSC-like populations with low turnover and infrequent cell cycling [127]. Although the mechanism underlying chemo-resistance is poorly understood, various types of stem-like tumor cells often overexpress drug resistance proteins such as ATP binding cassette (ABC), drug transporters which can reduce intracellular drug concentration, and have increased levels of these proteins that may contribute to the refractoriness of metastatic cancer to chemotherapy [128]. Hence, ABC proteins are considered as one of the candidate markers for CSCs [129]. Multidrug-resistance gene1 (MDR1) and its product Pgp, a member of the ABC super family, are significantly over-expressed in cervical cancer tissues as compared to normal tissues [130]. Moreover, instead of clinical staging and histological types, the expression of MDR1 and Pgp are in pace with cytological classification. The higher cytological class of the tumor (the lower grade of differentiation) and the higher MDR1 and Pgp are expressed, the worse are the clinical outcomes [131]. In a recent study, the ABC family was also shown to be over-expressed when used for evaluation of stemness after isolation of cervical CSC-like populations from cervical cancer tissues [66]. Potentially, a study on chemo-resistance would provide new drug targets, such as Pgp reversal agents, Pgp inhibitor peptides and antibody [132].

In addition to surgery, radio- and chemotherapy are conventional treatment in cervical cancer. However, radio- and chemotherapy display severe side-effects and have limitations in clinical benefits. Immunotherapy is another potential alternative or complementary treatment. Current efforts to develop cervical cancer immunotherapy have been dedicated to improving therapeutic HPV vaccines, targeting to enhance HPV-specific immune responses [133]. The CSC model provides a strategy in immunotherapy by targeting the CSCs instead of bulk tumor cells. Though related research is sparse in cervical cancer currently, it is available in head and neck as

well as prostate cancer [134-136]. The goal is to bring about the specific destruction of the CSCs while sparing normal cells [137]. This calls for specific markers of the CSCs which could be a CSC specific antigen as well as molecules or cells that support the CSCs in their niche [137]. The signaling pathways Wnt, Hedgehog and Notch play crucial roles in CSC biology as well as ALDH1 which is increased in the CSCs. They facilitate resistance to cyclophosphamide and cisplatin and therefore are novel potential targets [1]. However, to target the CSCs is not as simple as it seems. One of the reasons is that CSCs are typically present at very low levels and specific markers for certain CSCs are absent [137].

MiRNAs are very promising as new targets in anti-cancer molecular therapy. There are two approaches: antagonists and mimics. Antagonists are used to block the effects of the oncomir. In contrast, the mimics restore the tumor suppressor's natural potential [138]. An anti-miR-21 oligonucleotide was transfected into breast cancer MCF-7 cells and it suppressed both cell growth in vitro and tumor growth in a xenograft mouse model [138]. Delivery of a miR-34a mimic using a neutral lipid emulsion, either by direct injections into the tumor or by systemic tail vein injections, prevented the outgrowth of viable subcutaneous lung tumor xenografts [139]. A similar result is also found in pancreatic cancer [115]. In gastric cancer cells, transfection of miR-34 mimics block the cell cycle in G1 phase as well as inhibit cancer cell growth and sphere formation [116]. However, the transfection of mimics can only last for a couple of days [116]. Like with other therapeutic oligonucleotides, the main challenge remains the successful delivery of the therapeutic miRNAs to the target without compromising their integrity [140]. The miRNA therapy in cervical cancer targeting at HPV-related CSCs holds a promising prospect, though the literature on this is still limited.

In conclusion, although CSCs in cervical carcinoma remain poorly proven, accumulating evidence has enhanced the understanding on the CSC model in cervical cancer biology. Identification of a cervical CSC population and investigation of its biological features, including specific cervical CSC markers and miRNA expression profiles, may provide potential opportunities to develop strategies in anti-cancer therapy that might mitigate the occurrence, progress, metastasis, resistance and recurrence of cervical carcinoma and its CSC.

2 AIMS OF THE STUDY

1. To investigate the expression of miR-21 and miR-218 in cervical cancer cell lines compared to foreskin keratinocyte cell lines,

2. To compare the expression of “stemness”-related transcription factors (Sox2, Oct3/4 and Nanog) and miRNAs (miR-34a, miR-200c and miR-203) between monolayer-derived cervical cancer cells and their corresponding spheroid-derived cells,

3. To characterize the expression of ALDH1, CD44 and CD24 by flow-cytometric analysis in monolayer-derived cervical cancer cells and their corresponding spheroid-derived cells,

4. Transfer of findings in cell lines to clinical material: Evaluate the expression of miR-21 and miR-218 in Pap smears and investigate a potential correlation of microRNA dysregulation and the presence of human papillomavirus (HPV) infection and cytological change of cervical tissue.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Laboratory equipment

BD FACSCalibur System	BD Sciences, 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
Vortexer	Scientific Industries, N.Y., USA

3.1.2 Chemicals, reagents, kits, media and PCR primers

Chemicals and reagents

7-AAD	BD Pharmingen, CA, USA
Agarose	Bio & Sell, Nürnberg, Germany
APC mouse Anti-human CD44	BD Pharmingen, CA, USA
BD FACSFlow™	Biozym, Oldendorf, Germany
Chloroform	BD Sciences, Franklin Lakes, USA
DEPC-treated water	Merck KgaA, Darmstadt, Germany
Dimethyl Sulphoxide (DMSO)	Ambion, Carlsbad, CA, USA
Ethanol, 70%	Sigma, Steinheim, Germany
Epidermal Growth Factor (EGF)	Sigma, Deisenhofen, Germany
Fetal bovine serum (FBS)	Biochrom, Berlin, Germany

Fibroblast Growth Factor-basic (bFGF)	Gibco BRL, Karlsruhe, Germany
PE mouse anti-human CD24	BD Pharmingen, CA, USA
Penicillin/streptomycin	Biochrom, Berlin, Germany
Phosphate-buffered saline (PBS)	Biochrom, Berlin, Germany
RNase AWAY^{TM}	Carl Roth GmbH, Karlsruhe, Germany
Trizol Reagent	Invitrogen, Carlsbad, CA, USA
Trypsin/EDTA Solution (T/E)	Biochrom, Berlin, Germany

Cell culture media

Dulbecco's Modified Eagles Medium with GlutaMAXTM-I (DMEM)	Invitrogen, Heidelberg, Germany
Quantum 263 medium	Biochrom, Berlin, Germany
RPMI 1640	Invitrogen, Heidelberg, Germany
MEPICF	Gibco, Carlsbad, CA, USA

PCR primers (table 2)

Table 2: qRT-PCR-Primers (5´→ 3´)

Primer	Forward primer sequence	Reverse primer sequence
Nanog	AATACCTCAGCCTCCAGCAGATG	TGCGTCACACCATTGCTATTCTTC
Oct3/4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
ACTB (reference)	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
Hsa-miR-21	TAGCTTATCAGACTGATGTTGA	
Hsa-miR-34a	TGGCAGTGTCTTAGCTGGTTGT	
Hsa-miR-200c	TAATACTGCCGGGTAATGATGGA	
Hsa-miR-203	GTGAAATGTTTAGGACCACTAG	
Hsa-miR-218	TTGTGCTTGATCTAACCATGT	

Kits and other materials

Aldefluor assay Kit	StemCell Technologies, NC, USA
BD Falcon™ Cell Culture Flasks	BD Sciences, Franklin Lakes, USA
BD Falcon™ Cell Strainer (40 μm)	BD Sciences, Franklin Lakes, USA
BD Falcon™ Polypropylene Conical Tubes (15 ml, 50 ml)	BD Sciences, Franklin Lakes, USA
BD Falcon™ Polystyrene Round-Bottom Tubes	BD Sciences, Franklin Lakes, USA
BD Falcon™ Tissue Culture Dish (100*20 mm)	BD Sciences, Franklin Lakes, USA
Cluster Tubes, Polypropylene (1,2 ml)	Corning, NY, USA
Genomic DNA mini kit	Qiagen, Hilden, Germany
Human Keratinocyte Growth Supplement (HKGS)	Gibco, Carlsbad, CA, USA
PureLink™ RNA Mini Kit	Ambion, Carlsbad, CA, USA
NCode VILO miRNA cDNA Synthesis Kit and EXPRESS SYBR GreenER miRNA qRT-PCR 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, US

3.1.3 Cell lines

Cervical cancer cell lines

CaSki (HPV16), HeLa (HPV18), SiHa (HPV18), C33A (mutation of p53, HPV-), MRIH186 (HPV16), MRIH215 (HPV45), ME180 (HPV68), SW756 (HPV18) and C4-1 (HPV18). All cell lines were regularly tested for mycoplasma and found to be free of any contamination.

Human Foreskin Keratinocytes

FK08-35, FK09-03, FK09-07 and FK09-09

3.1.4 Pap smears

Pap smears from 115 patients were taken under colposcopic control at the Clinic for

Gynecology, Campus Benjamin Franklin, Charite-Universitätsmedizin Berlin, Germany. For each patient, one diagnostic Pap smear was taken and a slide for Pap staining was prepared. The residual material on the cytobrush was extracted into RNAlater buffer for HPV genotyping and RNA extraction.

3.2 Methods

3.2.1 Cell Lines and Cell Culture

Nine cervical cancer cell lines and four foreskin keratinocyte cell lines were used in the experiments. The nine cervical cancer cell lines were CaSki, SiHa, HeLa, MARQ, C33A, MRIH215, MRIH186, ME180, C4-1 and SW756. Four foreskin keratinocytes were FK08-35, FK09-03, FK09-07 and FK09-09. CaSki, HeLa, SiHa and C33A were cultured in DMEM with 10% FBS and 1% penicillin and streptomycin. MRIH186, MRIH215, ME180, SW756 and C4-1 were cultured in RPMI with 10% FBS head inactivated (20min, 56℃) and 1% penicillin and streptomycin. All foreskin keratinocyte cell lines were cultured in MEPICF supplemented with Human Keratinocyte Growth Supplement (HKGS) Kit.

3.2.2 Spheroid Cell Formation and Culture

Spheroids of nine cervical cancer cell lines were generated in 3-D non-adherent culture with cytokines supporting their growth. Adherent monolayer cells were grown in 75 cm^2 tissue culture flasks until 70-75% density before spheroid culture setup.

The first passage of spheroid formation culture from monolayer was performed by the following protocol: culture medium was removed and cells washed with PBS without Mg^{2+}/Ca^{2+}. PBS was aspirated and 1 ml T/E was added. TE was swirled gently for 1 min before removing. The cells were incubated at 37 ℃ with 5% CO_2 content. Cells were shaken off by tapping the plate, resuspended in 5 ml Quantum 263 medium and washed twice with PBS without Mg^{2+}/Ca^{2+} by sedimentation at 1500 rpm for 5 min. Cells were resuspended in serum-free Quantum 263 medium, supplemented with 10ng/ml EGF and 10 ng/ml b-FGF. To generate spheroids, single cells were plated in Corning* Ultra-Low Attachment flasks at a specific density of 2×10^4 cells/ml. Cells were kept in the incubator at 37 ℃ in humidified

atmosphere with 5% CO_2 content.

Half of the medium was replaced every three days. To do this, culture medium with spheroids was removed from the flask to a 50 ml tube. The tube was kept upright at room temperature for 15 min. The cell sediment deposited to the bottom of the tube. Half of the medium was aspirated from the top of the liquid level so that the lower half of the medium with most of the cells remained in the tube. One volume of fresh medium was added to the tube and the sedimented cells resuspended carefully by pipetting. This suspension was placed back into the flask.

The cervical cancer cell line SiHa never formed any spheroids and was cultured by replacing medium instead of passaging.

The spheroids were passaged in 7-14 days depending on their ability in forming spheroids. To passage the spheroids into next generations, a 40 µm mesh filter was used for collecting the cells. The cells were centrifuged at 1500 rmp for 5 min, 2 ml TE was added and resuspended. After incubation at 37 ℃ at 5% CO_2 the cells were washed with PBS without Mg^{2+}/Ca^{2+} twice before resuspending them again in fresh culture medium. The cell culture was continued in Corning* Ultra-Low Attachment flasks at a specific density of 2×10^4 cells/ml and kept in the incubator at 37 ℃ in humidified atmosphere with 5% CO_2. At least the second generation of spheroids was generated and applied in the following experiments.

The morphology of spheroids was assessed and recorded using a HBO50 Microscope and a 5× objective. Pictures were taken with an AxioCam MRC Zeiss Camera using the AxioVision Rel.4.8 Software.

3.2.3 Flow Cytometry analysis

The ALDH1, CD24 and CD44 expression of the monolayers and spheroids of cervical cancer cell lines were evaluated by flow cytometry assay. 7-AAD was used to discriminate between the living and dead cells.

Cell collection

The monolayer cells were collected by using Trypsin/EDTA and washed by PBS buffer once. The spheroids were collected using a 40 μm mesh and disaggregated into single cells by T/E incubated in 37 ℃ for 5 min followed by up and down pipetting 20 times using a 1000 μm pipette tip. Then the cell suspension was centrifuged at 1500 rpm for 5 min and washed with PBS buffer once.

Quadruple staining

For quadruple staining, an amount of $2x10^5$ cells was suspended in 200 μl per test of ALDEFLUOR™ assay buffer to adjust the sample to a concentration of 1×10^6 cells /ml.

Each sample was labelled “control” or “test”. 200 μl of the adjusted cell suspension was placed into each “test” sample tube. One μl of ALDEFLUOR™ DEAB Reagent (diethylaminobenzaldehyde, a specific ALDH inhibitor) was added into the “control” tube and the tube recapped immediately. One μl of the activated ALDEFLUOR™ Reagent was added into the sample “test” tube and mixed, then 100μL of the mixture in the “test” tube was immediately transferred into the DEAB “control” tube. Five μl 7-AAD was added into the control tube. Ten μl PE Mouse Anti-Human CD24, 10 μl APC Mouse Anti-Human CD44, and 5 μl 7-AAD were added into the test tube (Fig. 4).

All samples and controls were incubated for 45 min at 37 ℃ in the dark. Following incubation, all tubes were centrifuged for 5 minutes at 250 x g and the supernatant removed. Cell pellets were suspended in 0.1 ml of ALDEFLUOR™ Assay Buffer and stored on ice or at 4 ℃ and were analysed by flow cytometry immediately.

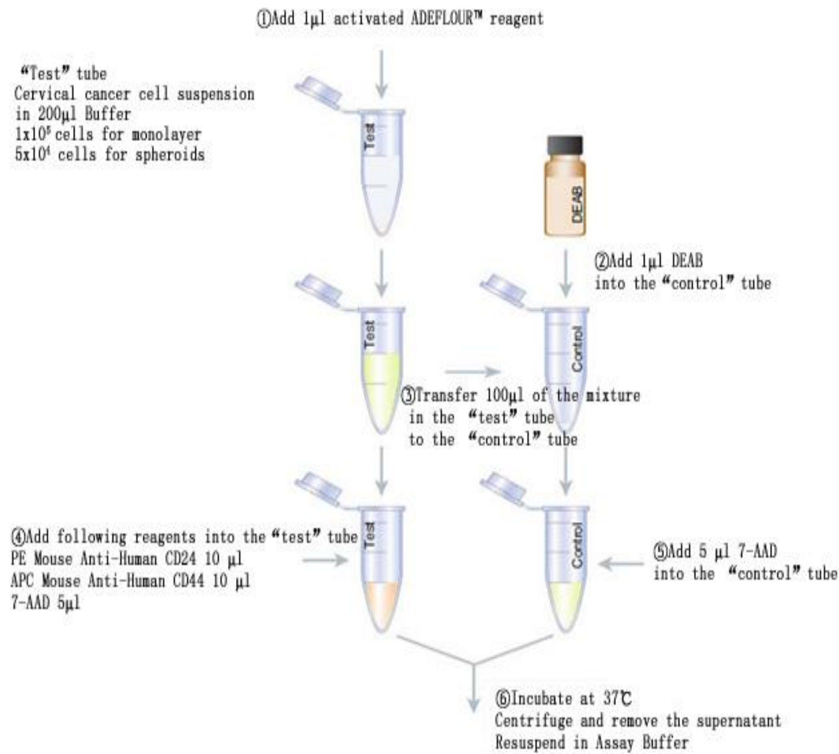


Fig. 4: Quadruple staining for FACS analysis

Flow cytometer data acquisition

The Flow Cytometry Analysis was performed on a BD FACSCalibur System using CellQuest Pro software. Compensation was performed for every cell line to correct for the spectral overlap of different fluorochromes. The percentage of ALDH1+, CD44+CD24-, ALDH1+CD44+CD24- and ALDH1-CD44+CD24- populations in monolayer and spheroid cultures of cervical cancer cell lines was acquired for data analysis. These assays were performed three times independently and analysed using the mean values of the data.

The gating strategies for data acquisition were performed as follow:

a. Create a Forward Scatter (FSC) vs. Side Scatter (SSC) dot plot. Create a region "R1" that will encompass the nucleated cells based on scatter and gate out debris (Fig. 5a).

b. Create a Fluorescence Channel 3 (FL3, 7-AAD) vs. SSC dot plot, gated on “R1”. Create a region “R2” to encompass the cell population that is 7-AAD negative (alive cells) (Fig. 5b).

c. Create a Fluorescence Channel 1 (FL1, ALDH1) vs. SSC dot plot, gated on “R1 and R2”. Open the data of the “control” sample (ALDH1-stained population in the DEAB). Create “R3” that will gate out all the nucleated ALDH1^{dim} cells but will encompass the cell population that is ALDH bright (ALDH^{br}). Open the data of the “test” sample (ALDH1-stained population without the DEAB), the cell population in the “R3” represents the ALDH1 positive population (Fig. 5c).

d. Create a Fluorescence Channel 2 (FL2, CD24) vs. Fluorescence Channel4 (FL4, CD44) dot plot, gated on “R1 and R2”. Create a quad region (Quad location 10, 10) that will encompass the cell population that is CD44+CD24- (Quad UL) (Fig. 5d).

e. Create another Fluorescence Channel 2 (FL2, CD24) vs. Fluorescence Channel 4(FL4, CD44) dot plot, gated on “R1 and, R2 and R3”. Create a quad region (Quad location 10, 10) that will encompass the cell population that is ALDH1+CD44+CD24- (Quad UL) (Fig. 5e).

f. Statistical evaluation was made using SPSS.20 software. The graphs and data in the result section represent the mean values for three independent biological replicates.

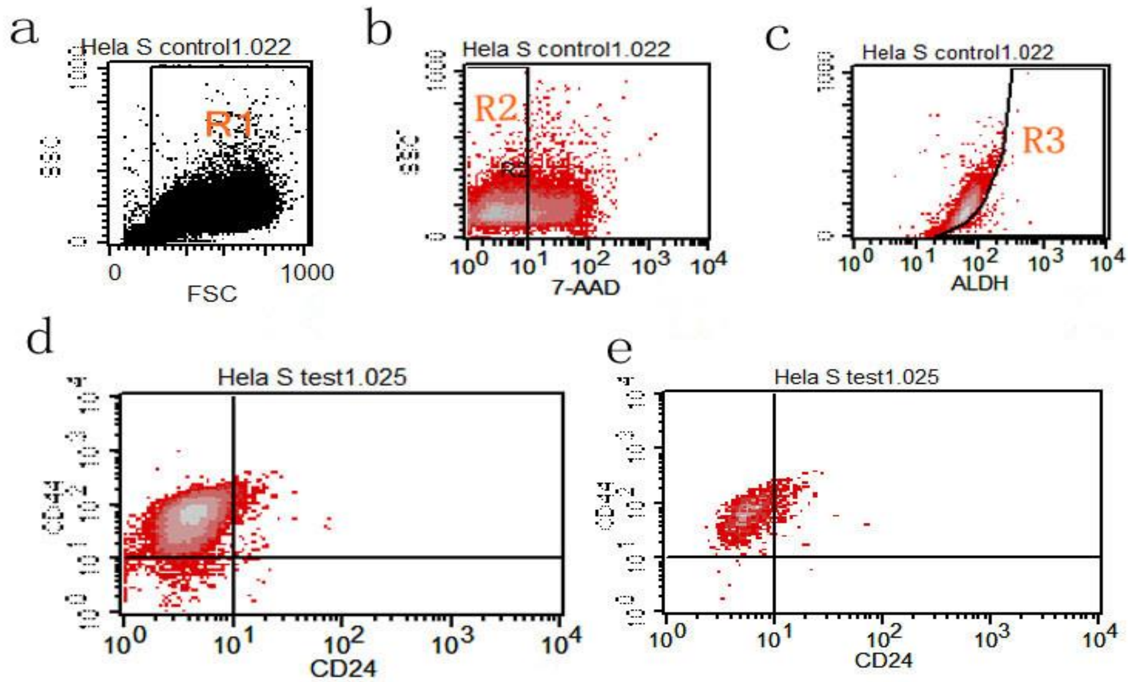


Fig. 5: Gating strategies for FACS analysis. The aim is to encompass the live cell population with $ALDH1^{+}$, $CD44^{+}CD24^{-}$, $ALDH1^{+}CD44^{+}CD24^{-}$, and $ALDH1^{-}CD44^{+}CD24^{-}$.

3.2.4 Quantitative RT-PCR analysis

The miR-21, miR-218, miR-34a, miR-200c, and miR-203 were selected for characterization of the miRNA expression in this experiment. The expression of oncomir miR-21 and tumor suppressor miR-218 were comparatively investigated in monolayer-derived cells of 9 cervical cancer cell lines and 4 foreskin keratinocyte (FK) cell lines. After three-dimensional cultures, generated spheroids were isolated from all cervical cancer cell lines to enrich for CSCs. Stemness-related microRNAs (miR-34a, miR-200c, miR-203) and transcription factors (Oct3/4, Sox2, and Nanog) expression was compared to the corresponding monolayer-derived cells.

Quantitative RT-PCR analysis was also performed in Pap smears. However, only miR-21 and miR-218 were investigated in Pap smear samples.

3.2.4.1 RNA extraction

Total RNA was extracted by Trizol reagent following the protocol below.

a. Cell homogenization

For monolayer: Growth media was removed from culture dish; 1ml Trizol reagent was added directly to the cells in the culture dish per 10 cm^2 of culture dish surface area; the cells were lysed directly in the culture dish by pipetting up and down several times. The lysed cells were transferred into a new eppendorf tube.

For spheroids: The cells were harvested by 40 µm mesh. Then the cells were centrifuged at 1500 rpm for 5 min. Two ml TE were added to the cell pellet and the cells were resuspended. The cells were incubated at 37 ℃ with 5% CO_2. Then the cells were washed with PBS without Mg^{2+}/Ca^{2+} twice. After counting the cells, they were centrifuged at 1500 rpm for 5min. The supernatant was removed and 1 ml per 10^6 cells Trizol was added. The cells were lysed by pipetting up and down several times before being transferred into an eppendorf tube.

For Pap smears: Cervical smears were taken and the cyto-brushes were immediately immersed and stored in RNA-later, after removing the brush head the materials in RNA-later were transferred to -20℃ within 24h. For extraction of the RNA, the samples in RNA-later were centrifuged at 12000 rpm after adding one volume of DEPC-treated water. The pellet was lysed by 1 ml trizol by pipetting up and down several times and the lysate transferred into a new eppendorf tube.

b. RNA isolation by Trizol

Chloroform (0.2 ml per 1 ml of Trizol reagent) was added. The tube was capped securely and shaken vigorously by hand for 15 sec. Then the tube was incubated on ice for 15 min. The tube was centrifuged at 12000xg for 15 min at 4 ℃. The aqueous phase of the sample was removed by angking the tube at 45 ° and pipetting the solution out. Intaking of any interphase or organic layer into the pipette when removing the aqueous phase was carefully avoided. The aqueous phase was placed into a new tube.

Per 1 ml used for homogenization 0.5ml of 100% isopropanol was added into the new tube with aqueous phase. The tube was incubated on ice for 10 min and then centrifuged at 12000×g for 10 min at 4 ℃.

The supernatant from the tube was removed, leaving only the RNA pellet. The pellet was washed with 1 ml 75% ethanol per 1 ml of Trizol Reagent used in the initial homonization. The sample in the tube was vortexed briefly then centrifuged at 7500×g for 5 min at 4 ℃. The supernatant was discarded. The RNA pellet was air dried for 5-10 min.

c. RNA resuspension and storage

The RNA pellet was resuspended in DEPC-treated water by passing the solution up and down several times by pipetting. The Nanodrop was used to determine the concentration of RNA before storing at -80 ℃.

3.2.4.2 Poly A tailing and cDNA synthesis

Poly A tailing and cDNA synthesis were performed by using NCode™ VILO™ miRNA cDNA synthesis kit. The following components were combined in a tube on ice: 4 µl of 5x Reaction Mix, 2 µl of 10x Superscript Enzyme Mix, 1µg of template RNA, adjusted to 20µl of DEPC-treated water. The tube was capped and gently vortexed to mix. Then the tube was centrifuged briefly to collect the contents. The reaction was incubated using a PCR cycler at 37 ℃ for 60 min and the reaction terminated at 95 ℃ for 5 min. The reaction was held at 4 ℃ until use. For long-term storage, the cDNA was stored at -20 ℃.

3.2.4.3 qRT-PCR analysis

Quantitative RT-PCR was performed on a Chromo4 machine with Express SYBR GreenER™ qPCR SuperMix Universal. The β-actin (ACTB) expression was used as a reference gene. The following components were combined in a tube on ice: 10 µl of Express SYBR GreenER™ qPCR SuperMix Universal, 0.4 µl of 10µM miRNA-specific forward primer, 0.4 µl of 10µM Universal qPCR Primer, 1 µl of cDNA, 8.2 µl of DEPC-treated water. No-template control (NTC) was prepared to test for DNA contamination of the enzyme/primer mixes. No RT control (with 1 µl RNA instead of DNA) was prepared to test for DNA contamination in RNA

preparations. The PCR tubes were capped and gently mixed. The reaction was placed on the real-time instrument Chromo4 and programmed as described below. For ACTB and transcription factors: 95 ℃ for 15 min, 40 cycles of 95 ℃ for 30 sec, 60 ℃ for 1 min and 72 ℃ for 30sec. For miRNAs: 50 ℃ for 2 min, 95 ℃ for 2 min, 40 cycles of 95 ℃ for 15 sec and 60 ℃ for 1 min. Analysis was performed using the Opticon real-time PCR detection system (Bio-Rad).

For all qRT-PCR analyses, the expression levels were assessed in triplicate and normalized to ACTB levels. The data were statistically analysed using the modified delta delta Ct method following the Michael P et al. protocol reported method [141]. Statistical evaluation was performed using Qiagen Rest software (Relative expression software tool). The graphs in the result represent the mean value of at least three independent biological replicates.

3.2.5 HPV detection and typing

Genomic DNA from cervical Pap smears was extracted with Genomic DNA Mini Kit (Qiagen, Hilden, Germany) according to the standard protocol and eluted in 160µl TE buffer. A β-globin PCR was made and analyzed on a 2% Agarose gel, to monitor sufficient DNA quality and amount [142]. The generic GP5+/bio6+ primer set was used to amplify a sequence from the L1 gene of a large number of different HPV types [142]. After PCR amplification, HPV sequences were genotyped by MPG Luminex suspension array technology [143].

3.2.6 Clinical data

The patients' clinical data including age, cytological diagnosis, clinical findings and diagnosis was extracted from the databank of electronic medical record (SAP, Mannheim, Germany) of the clinic for Gynecology, Campus Benjamin Franklin, Charite-Universitätsmedizin Berlin, Germany and were anonymized.

3.2.7 Statistical analysis

For statistical comparison in the Flow Cytometry analysis, the SPSS software (version 20; SPSS, Chicago, IL, USA) was applied. Student's t-test was used to analyse statistical significance of the data.

For all qRT-PCR analyses, the expression levels were statistically analysed using the modified delta delta Ct method. Expression analysis and statistical evaluation was made using the pair-wise fixed re-allocation randomization test by the Qiagen REST 2009 Software (version 2.0.13).

4 RESULTS

4.1 Spheroid formation of cervical cancer cell lines

Spheroid culture is widely used as it provides an *in vitro* 3-dimensional (3D) model to study proliferation, cell death, differentiation, and metabolism of cells derived from tumours and the response of tumour cells to radiotherapy and chemotherapy [144]. Furthermore, it is also easier to enrich CSC populations when compared to other methods. The ability of purified CSCs to grow anchorage independently and to form spheres in cell culture has been demonstrated for breast, prostate, colon, head and neck, pancreatic, and melanoma derived CSCs [5, 8]. Here, we selected spheroid culture as an essential step to enrich for CSC-like populations from our cervical cancer cell lines.

Cells from nine cervical cancer cell lines were grown in suspension at low density in defined serum-free medium with b-FGF and EGF for 7-14 days. The spheroid formation usually started at the first day after starting suspension culture and the size became progressively larger. At days 3-5 an initial spheroid formation could be observed. After 4-7 days the morphology of spheroids would not change in size, but the number of spheroids still continued to grow (Fig. 6).

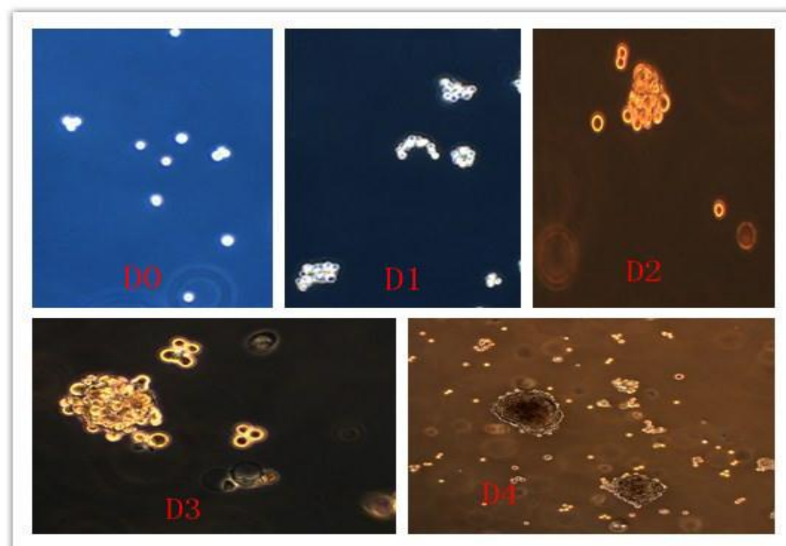


Fig. 6: Time course of spheroid culture over 4 days (cell line CaSki). The micrographs show representative cell aggregates in low density cultures. The magnification is 400-fold.

Nine cervical cancer cell lines showed different ability of spheroid formation. CaSki, MRIH215, C4-1 and ME180, formed spheroids which were highly compact. Four cell lines, namely, MRIH186, C33A, HeLa and SW756, formed only loose aggregates of cells. The cell-cell contacts established by these cultures were weak, and the aggregates could easily be dispersed mechanically by pipetting. SiHa never formed any spheroids. This morphology observed initially did not change within 4 weeks of cultivation or in subsequent passages (generation) (Fig. 7).

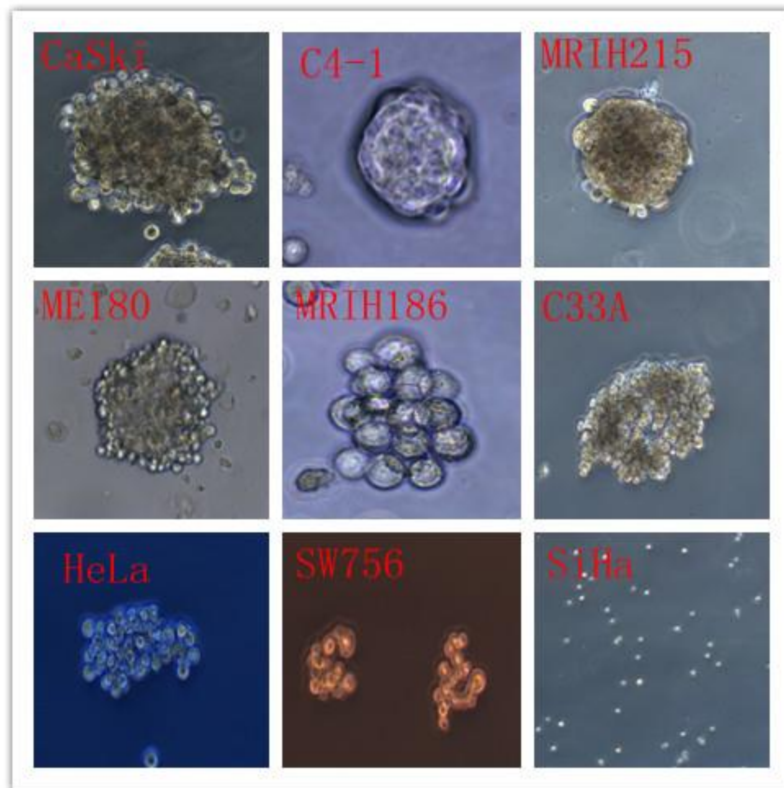


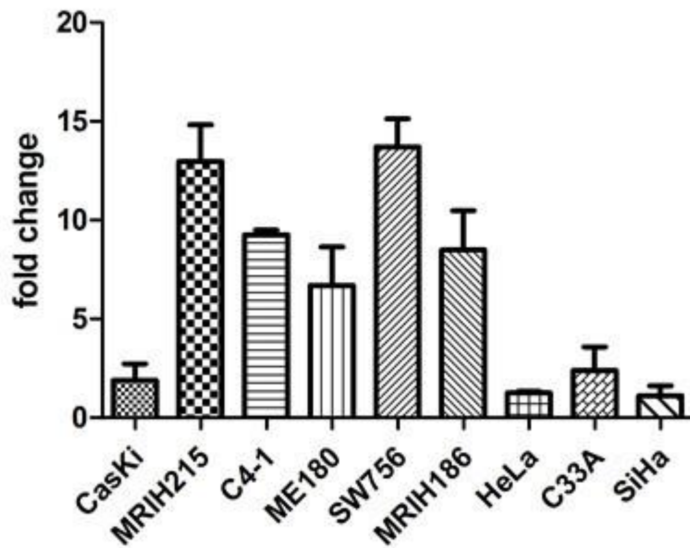
Fig. 7: Formation of spheroids or aggregates by different cervical cancer cell lines. Starting density was 2×10^4 cells/ml and culture period was 7-14 days**.** The magnification is 400 fold. CaSki, MRIH215, C4-1 and ME180, formed spheroids which were highly compact. MRIH186, C33A, HeLa and SW756, formed only loose aggregates of cells. SiHa never formed any spheroids.

4.2 Expression of miR-21 and miR-218 in cervical cancer cell lines compared to foreskin keratinocyte cell lines

The miR-21 is considered an oncomir. It is over-expressed in tumor tissues and correlated with specific cancer biopathologic features as well as clinical outcome [101]. In this experiment, nine cervical cancer cell lines generally overexpressed oncomir miR-21 relative to keratinocyte cell lines (Fig. 8). When compared to human foreskin keratinocytes, the nine cervical cancer cell lines had reduced expression of miR-218, a tumor suppressor miRNA. The cell line C33A, which is a cervical cancer cell line with mutated p53 and without detectable HPV infection, also shows a decrease of miR-218 (Fig. 8).

These descriptive results did show a trend in upregulation of miR-21 (1.06-13.1 fold) when the four FK cell lines were compared to the nine cervical cancer cell lines. However, the selected four FKs showed a highly variable expression of miR-21 (Fig. 9). A significantly different expression of miR-218 (9.14-78.15 fold) between the FKs and cervical cancer cell lines was observed ($p<0.05$).

No correlation between HPV type and the dysregulation of miR-21 and miR-218 was found in cervical cancer cell lines in this experiment. There was no significant difference in the up- or down-regulation level of miR-21 and miR-218 between cervical cancer cell lines which were HPV16 positive (CaSki and MRIH186) or HPV 18 positive (HeLa, SiHa, SW756 and C4-1) ($p>0.05$). The same finding was shown between eight HPV positive cell lines and C33A, the only cervical cancer cell line without HPV-infection ($p>0.05$).



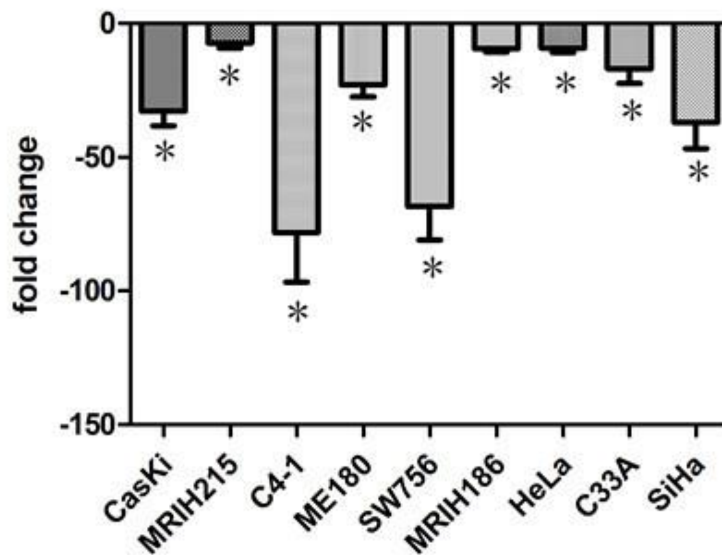


Fig. 8: Quantitative PCR analysis of mRNA expression of miR-21 and miR-218. The ratio in differences of expression in cervical cancer cells as compared to FKs is given. (a) Up-regulation of oncogene miR-21 in cancer cell lines compared to foreskin keratinocytes. (b) Down-regulation of tumor suppressor miR-218 in cancer cell lines compared to foreskin keratinocytes. Significant differences were * $p<0.05$.

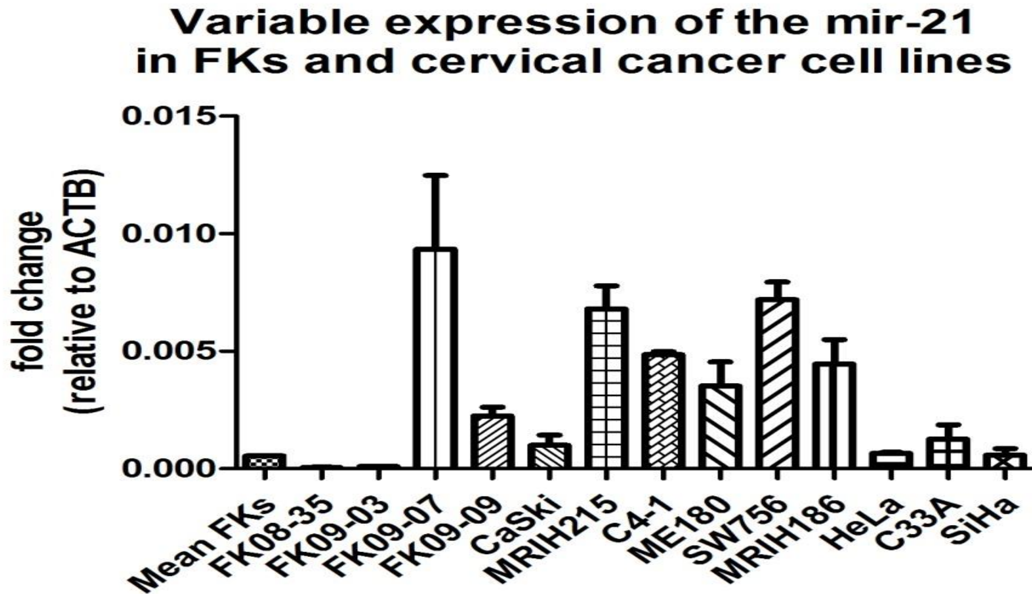


Fig. 9: Variable expression of the miR-21 in FKs and cervical cancer cell lines. The given fold of expression was the relative expression to ACTB in the same cell line. Here the FK09-07 showed a higher fold change level than all cervical cancer cell lines. This might contribute to the absence of a significantly different expression of miR-21 expression between FKs and cervical cancer cell lines.

4.3 Expression of "stemness"-related transcription factors in spheroid-derived cells compared to monolayer-derived cells in cervical cancer cell lines

It has been reported that Sox2, Oct3/4 and Nanog are master transcriptional factors that are essential in maintaining the stemness features such as self-renewal and pluripotency capacity of human embryonic stem cells [145, 146]. In our previous studies, we demonstrated that they were also up-regulated in stem-like cell populations in head and neck cancer cell lines [147]. Here we quantitatively compared the mRNA expression of these TFs between cervical cancer cell lines spheroid-derived cells (SDCs) and monolayer derived-cells (MDCs).

The mRNA levels of TFs Sox2, Oct3/4 and Nanog were generally significantly increased in the SDCs as compared to MDCs. However, the TFs expression showed a small and not significant increase (less than 2 fold) or even a small decrease (3.61 fold at the most) in some

cell lines, including cell line ME180 which showed a good capacity in spheroid formation. Interestingly, cervical cancer cell line SiHa which never formed any spheroids also showed a significant increase of expression of the three "stemness"-related TFs when cultured under the conditions for spheroid formation (Fig. 10).

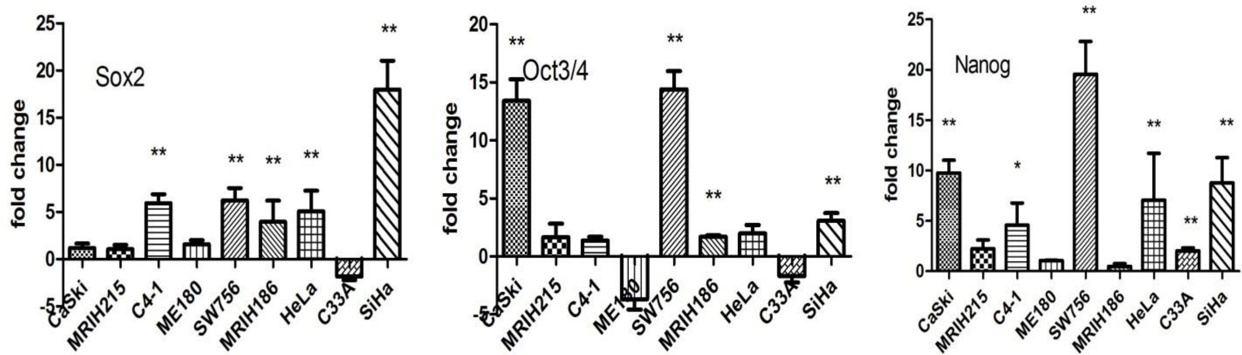


Fig. 10: Quantitative PCR analysis of mRNA expression of "stemness"-related transcription factors. The fold changes in the cervical cancer cell line SDCs to corresponding MDCs are given. The CSC-specific transcription factors Sox2, Oct3/4 and Nanog were generally up-regulated in spheroid cultures. Significant differences were * $p<0.05$; ** $p<0.01$.

4.4 Expression of "stemness"-related microRNAs in cervical cancer cell lines

The miRNAs can regulate normal and malignant stem cells by simultaneously regulating the expression of potentially hundreds of genes [117]. These miRNAs may therefore regulate multiple pathways involved in stem cell fate decisions, including self-renewal, proliferation and differentiation pathways frequently altered in cancer [125]. In the following experiment, the expression level of tumour suppressors miR-34a, miR-200c, and miR-203, selected "stemness"-related miRNA, was investigated. The p53-regulated miR-34a and miR-200c can inhibit the "stemness" properties of CSCs by modulating different targets, while the miR-203 targets p63 which is an essential regulator of "stemness" maintenance.

In our experiment, the expression of all three selected "stemness"-related microRNAs was down-regulated in SDCs when compared to MDCs in all cervical cancer cell lines, though some of them didn't show significant differences ($P>0.05$). No correlation between spheroid

formation ability and the level of down-regulation of the miRNAs was found. Cervical cancer cell line CaSki that forms spheroids which are highly compact showed a low level of decrease of expression of the three tumour suppressor microRNAs (P>0.05). SiHa, that never forms spheroids, showed a significant difference in miRNAs expression level between cells cultured in condition for SDCs and MDCs, respectively, in miR-34a and miR-200c (Fig. 11).

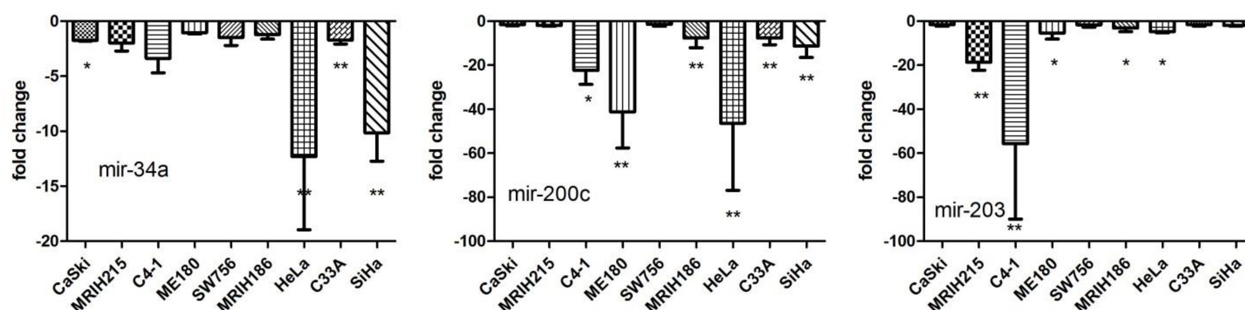


Fig. 11: Quantitative PCR analysis of RNA expression of “stemness”-related microRNAs. The ratio of fold changes in the cervical cancer cell line SDCs to corresponding MDCs is given. Significant differences were * $p<0.05$; ** $p<0.01$. The selected “stemness”-related microRNAs were down-regulated in SDCs compared to MDCs in all cervical cancer cell lines.

4.5 ALDH1 expression in cervical cancer cell lines

We measured ALDH1 enzymatic activity of the SDC of 9 cervical cancer cell lines and their matched MDC to verify the presence of a stem cell-like population. As control cells incubated with ALDEFLUOR substrate (BAAA) together with the specific ALDH inhibitor (DEAB) were used to establish the background fluorescence and to define the ALDH1 positive population.

As it is shown in Fig. 12, a highly variable expression of a proportion of ALDH1+ cells was found (0.94% - 66.29%) in the nine cervical cancer cell lines. In seven of nine cell lines, a low proportion of ALDH1 expression was found in their MDCs (0.94%-10.62%) and SDCs showed an increase of 2-8 fold when compared to MDCs. All seven cervical cancer cell lines had a significantly different expression ($p<0.05$). In the other two cervical cancer cell lines, namely MRIH215 and SW756, a high proportion of ALDH1+ cells, was present in their MDC population (58.57% and 66.29%) and a relative decrease of the $ALDH1^{+}$ cells was seen in their SDCs when compared to MDCs. The SDCs of the cancer cell line MRIH215 which still showed

a high capacity in spheroids formation contained less $ALDH1^+$ cells than its MDCs ($p<0.01$). In the cell line MRIH215, ALDH1 expression in SDCs was 38.03% lower which was a significantly different CSC frequency, while the other cell line SW756 had a decrease by 15.11% which did not reach significance to its MDCs. Conversely, the cells cultured under SDC conditions of cell line SiHa, which never formed spheroids, showed an increased expression frequency of ALDH1 as compared to its MDCs.

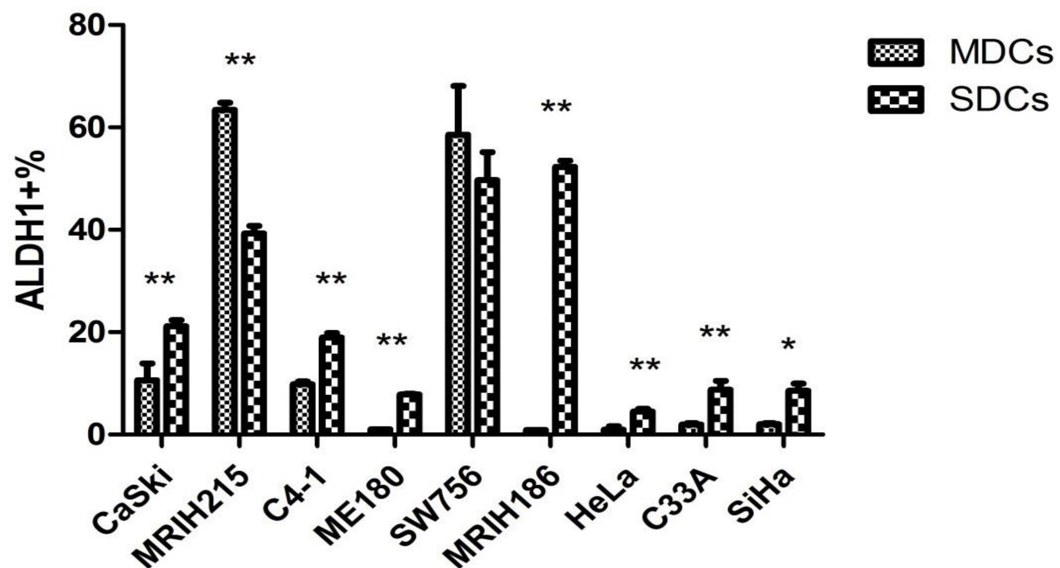


Fig. 12: Comparison of proportion of $ALDH1^+$ cells in MDC and SDC populations from 9 cervical cancer cell lines. A highly variable expression of the proportion of ALDH1+ was found in the nine cervical cancer cell lines. Significant differences were * $p<0.05$; ** $p<0.01$. The SDCs of seven of the nine cell lines showed a higher proportion of ALDH1+ cell population when compared to their MDCs. The other two cell lines expressed high ALDH1 in their MDCs and showed a low level or decrease of ALDH1 expression in their SDCs.

4.6 $CD44^+CD24^-$ cell population in cervical cancer cell lines

Tumor cells with a $CD44^+/CD24^-$ phenotype were shown to have CSC properties in a variety of solid tumors [148]. We therefore also investigated comparatively the CD44 and CD24 expression in the cervical cancer cell lines.

A highly variable expression of $CD44^+CD24^-$ cells was found (0.35% - 63.98%) in the nine cervical cancer cell lines as shown in Fig. 13. In seven of the nine cell lines, the SDCs

showed an increase in the $CD44^{+}/CD24^{-}$ phenotype of 1.5-3.15 fold when compared to MDCs. Four of the seven cervical cancer cell lines had a significantly different proportion ($p<0.05$ or $p<0.01$). In the SDCs of cell lines SW756 and HeLa, an increase as high as 1.5 and 2.04 fold was still pronounced as compared to their MDCs, although their MDCs contained already a high proportion of the $CD44^{+}CD24^{-}$ population (60.8% and 48.29%).

In the cell line CaSki that formed spheroids with highly compact morphology, a 32.4% decrease of the $CD44^{+}CD24^{-}$ expression was seen in its SDCs when compared to MDCs. Although a very low (0.55%) proportion of $CD44^{+}CD24^{-}$ population was found in the MDCs of cell line C33A, a decrease of -74.55% of the cell population was found in the corresponding SDCs ($p<0.01$). In the cell line SiHa that never formed spheroids, an 1.84 fold increase of the $CD44^{+}CD24^{-}$ population was found in cells cultured under non-adherent conditions as compared to its MDCs ($p<0.01$).

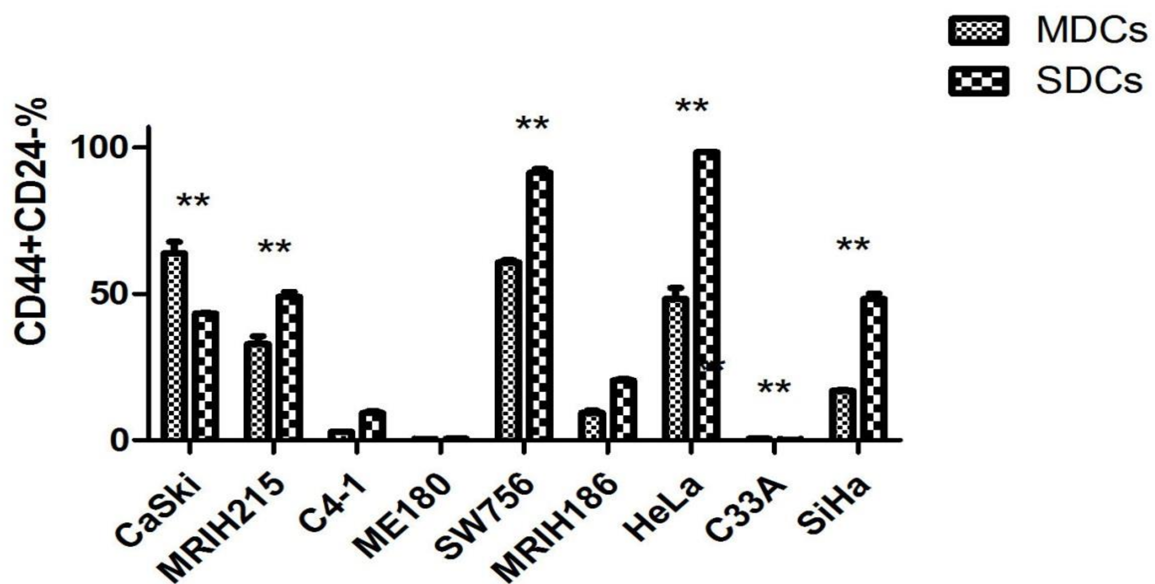


Fig. 13: Comparison of $CD44^{+}CD24^{-}$ cells in MDC and SDC populations from 9 cervical cancer cell lines. A highly variable proportion of $CD44^{+}CD24^{-}$ cells was found in the nine cervical cancer cell lines. The SDCs of seven of the nine cell lines showed a higher proportion of $CD44^{+}CD24^{-}$ cell population when compared to their MDCs. The other two cell lines showed a decrease of the $CD44^{+}CD24^{-}$ cell population in their SDCs. Significant differences were * $p<0.05$; ** $p<0.01$.

4.7 ALDH1$^+$CD44$^+$CD24$^-$ cell population in cervical cancer cell lines

The combined use of different putative CSC markers may provide a more precise definition of stem cell-like populations. In our experiment, the MDCs from all nine cervical cell lines showed a low proportion (0-3.36%) of the ALDH1$^+$CD44$^+$CD24$^-$ cell population except for two cell lines MRIH215 (8.7%) and SW756 (37.76%) (Fig. 14). No ALDH1$^+$CD44$^+$CD24$^-$ cells were found in the MDCs of cell lines C4-1 and ME180 (the number of MDCs to be tested in the experiment was $1x10^5$). When compared to their MDCs, the SDCs in all nine cervical cancer cell lines showed an increase (1.14-3.49 fold) of the proportion (range 0.02%-43.39%) of this population. Five of them showed a significant difference in the proportion of the cell population between their MDCs and SDCs ($P<0.05$ and $P<0.01$, respectively). The SDCs from the cell lines C4-1 and ME180 also showed a low proportion of ALDH1$^+$CD44$^+$CD24$^-$ cells in the population. The increase of the investigated cell population of the cell lines C4-1 and SiHa was the highest (292 and 349 fold, respectively). These cell lines had a very low ALDH1$^+$CD44$^+$CD24$^-$ cell frequency in their MDCs population (0.01% and 0.02%, respectively). Conversely, the lowest increase (1.14% fold) was in cell line SW756 where the proportion in the MDCs cell population was already the highest (37.76%).

There was no correlation of the capacity to form spheroids with the proportion or the increase of the ALDH1$^+$CD44$^+$CD24$^-$ cell population in the MDCs/SDCs of the nine cervical cancer cell lines. The proportion of the targeted stem cell-like population in MDCs or SDCs were low in the cancer cell lines C4-1 and ME180 which have a strong capacity for forming spheroids. Instead, in the nine cancer cell lines, the highest percentage of the stem-cell-like populations either in MDCs or SDCs was found in cell line SW756 which could form spheroids as loose aggregates. When compared to its MDCs, the cells cultured under non-adherent conditions from the cell line SiHa showed the highest increase of the ALDH1$^+$CD44$^+$CD24$^-$ population proportion in the nine cervical cancer cell lines.

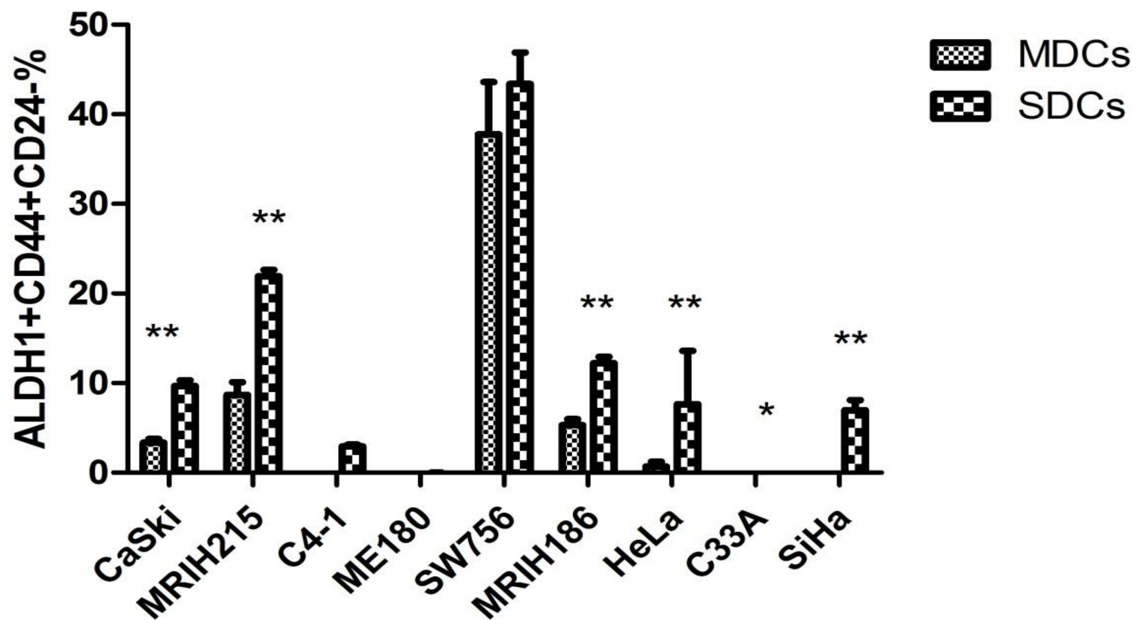


Fig. 14: Comparison of ALDH1+CD44+CD24- expression in MDC and SDC populations from 9 cervical cancer cell lines. The SDCs of all nine cervical cancer cell lines showed a higher proportion of an $ALDH1^{+}CD44^{+}CD24^{-}$ cell population when compared to their MDCs. $ALDH1^{+}CD44^{+}CD24^{-}$ cell population was not visible in MDCs of the cell lines ME180 and C33A. Significant differences were * $p<0.05$; ** $p<0.01$.

4.8 Detection and genotyping of HPV in Pap smears

For all Pap smears, HPV detection and typing was performed and the results were summarized as low risk-HPV and/or high risk-HPV. The information on HPV detection is shown in the Table 3. HPV infection was not found in 57 of the 115 (49.57%) Pap smears, while the other (58, 50.43%) samples were HPV positive. The age of HPV positive patients is lower than of the HPV negative patients ($p<0.05$), probably because of a higher HPV prevalence in young women.

In the HPV free group, 93% (n=53) patients were diagnosed as cytologically normal according to the Bethesda system, while only 7% (n=4) were diagnosed as abnormal. This was lower than in the HPV positive group (44.83%, n=26).

The HPV positive group was divided into subgroups according to the risk of the HPV types present. Patients infected with various HR-HPV types were classified into a single HR-HPV

group, without considering multiple infections. The same classification was used in the LR-HPV group. In the HPV positive group, only 6.9% (n=4) were infected only by LR-HPV, most of them (n=54, 93.1%) were infected by HR-HPV.

Table 3: HPV detection and typing result in patients

HPV Status	Bethesda system diagnosis			Total	Median Age (range)
	Normal	LSIL	HSIL		
HPV- (n=57)	53	3	1	57	45.4 (26-79)
HPV+ (n=58)	32	12	14	58	38.95 (19-77)
LR-HPV+ (n=4)	3	1	0	4	
HR-HPV+ (n=54)	29	11	14	54	
Total (n=115)	85	15	15	115	42.15 (19-79)

Abbreviations: LR, low risk; HR high risk; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade intraepithelial lesion.

4.9 Correlation of Age and miRNA expression in Pap smears

As the age of the HPV-infected group is lower than the HPV-negative group (P<0.05), we wanted to test whether the expression of the investigated miRNAs was age-related. Patients in the HPV negative group whose Bethesda diagnosis was normal (n=53) were divided into two groups of upper and lower median age 42 (Tab. 4) and analysed for miR-21 and miR-218 expression (Tab. 5).

Table 4: Stratification for age of patient with HPV-negative and cytological normal results

Groups	N	HPV	TBS	Mean age (range)
Younger normal women	27	negative	normal	34.19(26-42)
Older normal women	26	negative	normal	58.81(43-79)

As shown in the table 5, the level of the microRNAs miR-21 and miR-218 was only marginally up-regulated (1.64 and 1.91 fold, respectively) in older cytologically normal women compared to younger cytologically normal women. The expression was not significantly different between the two groups (p>0.05).

Table 5: Relative expression of microRNAs in relation to age in women with normal cytology

Control	Case	Transcript	N-fold	P
Younger normal women	Older normal women	miR-21	1.64	>0.05
Younger normal women	Older normal women	miR-218	1.91	>0.05

4.10 HPV infection and microRNA expression in Pap smears

As described above, the microRNA miR-21 is an oncomiRNA which is up-regulated in cancer tissues or cells, while miR-218 is a tumor suppressor which is reported to be related to HPV infection by being regulated by HPV E6 mRNA. Its down-regulation has been confirmed in cell lines of cervical cancer origin (see Fig. 8). In this experiment, we comparatively investigated the microRNA miR-21 and miR-218 expression in different patient groups according to HPV status, regardless the cytological diagnosis result. The group information is shown in table 6.

Table 6: Patient stratification by HPV status

Group	N	Bethesda system diagnosis		
		normal	LSIL	HSIL
HPV-	57	92.98 %	5.26%	1.75%
HPV+	58	55.17%	20.69%	24.14%
HR-HPV+	54	53.7%	20.37%	25.93%
LR-HPV+	4	75%	25%	0

The expression of the oncomiRNA miR-21 was significantly up-regulated in all HPV-infected groups when compared to the HPV-negative group ($p<0.01$) (Tab. 7). There was no statistically significant difference of miR-21 expression shown between LR-HPV and HR-HPV groups ($p>0.05$).

Table 7: Expression of miR-21 and miR-218 in smears stratified for HPV infection status.

Control	Case	Transcript	N-fold	P
HPV- n=57	HPV+ n=58	miR-21	8.22	<0.01
		miR-218	-98.94	<0.01
HPV- n=57	LR-HPV+	miR-21	7.96	<0.01
	n=4	miR-218	-72.73	<0.05
HPV- n=57	HR-HPV+	miR-21	9.62	<0.01
	n=54	miR-218	-147.43	<0.01
LR-HPV+	HR-HPV+	miR-21	1.21	>0.05
n=4	n=54	miR-218	-2.03	>0.05

When compared to the HPV-negative group, the expression of the putative HPV-related microRNA miR-218 was significantly down-regulated in the HPV+ group, LR-HPV+ group and HR-HPV+ group. When compared to the HPV-negative group, the down-regulation of miR-218 in the HR-HPV+ group was over 70 fold more reduced than in the LR-HPV group (-147.43 fold compared to -72.73 fold). However, there was no significant difference of miR-218 expression shown between LR-HPV+ and HR-HPV+ groups in this experiment ($p>0.05$). This might indicate that the two microRNAs were only HPV-related without discriminating LR- from HR-HPV infections. However, the low sample number in the LR-HPV+ group (n=4) might potentially compromise the result.

4.11 Cytological diagnosis and microRNA expression in Pap smears

To observe the relationship between cytological diagnosis and microRNA expression, we compared the miR-21 and miR-218 expression in patients with different cytological diagnosis without considering their HPV result (Tab. 8).

Table 8: Patients stratification according to cytological result

Group	N	HPV+	HR-HPV+
normal	85	37.65%	34.12%
LSIL	15	80%	73.33%
HSIL	15	93.33%	93.33%

After grouping by cytological results, the proportions of HPV+ and HR-HPV+ were different. In the cytologically normal group, the HPV+ and HR-HPV+ rates were lower when compared to the LSIL and HSIL group, as expected. The HPV+ and HR-HPV+ infection rate in HSIL was the highest in the three groups.

For the miR-21 expression, there was no statistically significant difference found between the different cytological diagnosis groups ($p>0.05$) (Tab. 9). For the miR-218 expression, significantly lower expression was found for both LSIL and HSIL groups when compared to the cytologically normal group. However, there was no significant difference in miR-218 expression found between LSIL and HSIL group ($p>0.05$) (Tab. 9).

Table 9: Expression of miR-21 and miR-218 in smears with different cytological diagnosis

Control	**Case**	**Transcript**	**N-fold**	**P**
Normal	LSIL	miR-21	1.17	>0.05
		miR-218	-3.17	**<0.05**
Normal	HSIL	miR-21	3.46	>0.05
		miR-218	-12.04	**<0.01**
LSIL	HSIL	miR-21	2.95	>0.05
		miR-218	-3.79	>0.05

4.12 Correlation of HPV status, cytological diagnosis and miRNA expression in Pap smears

As shown above, the HPV-negative group had a significantly different level of miRNA expression when compared to the HPV-positive groups. The same situation was found between the cytologically normal group and the LSIL/HSIL groups. Correspondingly, the proportion of cytologically normal patients was highest in the HPV-negative group when the groups were based on HPV status, while the percentage of HPV-negative was also highest in the cytologically normal group when the groups were based on cytological diagnosis. To investigate whether the dysregulation of miR-21 and miR-218 was associated with HPV status or cytological diagnosis, we further grouped the patients by considering both the HPV status and cytological diagnosis at the same time. The patient stratification information is shown in table 10. The results are shown in table 11.

Table 10: Patients stratified according to HPV status and cytological diagnosis

		HPV status				Bethesda system diagnosis		
Group	**n**	HPV-	HPV+	LR-HPV+	HR-HPV+	cyto-normal	LSIL	HSIL
HPV- cyto- normal	53	53 (100%)	0	0	0	53 (100%)	0	0
HPV- LSIL/HSIL	4	4 (100%)	0	0	0	0	3 (75%)	1 (25%)
HPV+ cyto- normal	32	0	32 (100%)	3 (9.4%)	29 (90.6%)	32 (100%)	0	0
HPV+ LSIL	14	0	14 (100%)	3 (21.4%)	11 (78.6%)	0	14 (100%)	0
HPV+ HSIL	14	0	15 (100%)	1 (6.7%)	14 (93.3%)	0	0	15 (100%)
LR-HPV+ cyto- normal	3	0	3 (100%)	3 (100%)	0	3 (100%)	0	0
HR-HPV+ cyto- normal	29	0	29 (100%)	0	29 (100%)	29 (100%)	0	0
HR-HPV+ LSIL	11	0	11 (100%)	0	11 (100%)	0	11 (100%)	0
HR-HPV+ HSIL	14	0	14 (100%)	0	14 (100%)	0	0	14 (100%)

As shown in the table 11, a significantly different expression of miR-21 and miR-218 was found only between the HPV-/cytologically normal group and the HPV+/cytologically normal group ($p<0.01$). This suggested that the miR-21 and miR-218 modulation was HPV-related. In the LR-HPV+/cytologically normal group compared to the HR-HPV+/cytologically normal group, there was no significant difference in either miR-21 or miR-218 found ($p>0.05$), this might suggest that there was no different miR-21 or miR-218 dysregulation level between LR- and HR-HPV infection in cervical tissue. However, it is also possible that this was due to the low number of samples (in LR-HPV+/cytologically normal group n=3). Between groups with the same HPV status but different cytological diagnosis, no significantly different expression of

miR-21 or miR-218 was found (p>0.05).

Table 11: Expression of miR-21 and miR-218 in cytological samples with different HPV type and cytological diagnosis

Control	Case	Transcription	N-Fold	P
HPV-, cyto- normal	HPV-, LSIL/HSIL	miR-21	1.06	>0.05
n=53	n=4	miR-218	-1.1	>0.05
HPV-, cyto- normal	HPV+, cyto- normal	miR-21	6.05	**<0.01**
n=53	n=32	miR-218	-118.95	**<0.01**
HPV+, cyto- normal	HPV+, LSIL	miR-21	-1.6	>0.05
n=32	n=14	miR-218	-4.34	>0.05
HPV+, cyto- normal	HPV+, HSIL	miR-21	1.65	>0.05
n=32	n=14	miR-218	-2.11	>0.05
HPV+, LSIL	HPV+, HSIL	miR-21	2.63	>0.05
n=14	n=14	miR-218	2.06	>0,05
LR-HPV+, cyto-	HR-HPV+, cyto-	miR-21	1.07	>0.05
normal n=3	normal n=29	miR-218	-1.48	>0.05
HR-HPV+, cyto-	HR-HPV+, LSIL	miR-21	0.61	>0.05
normal n=29	n=11	miR-218	-6.52	>0.05
HR-HPV+, cyto-	HR-HPV+, HSIL	miR-21	1.64	>0.05
normal n=29	n=14	miR-218	-2.02	>0.05
HR-HPV+, LSIL	HR-HPV+, HSIL	miR-21	2.69	>0.05
n=11	n=14	miR-218	3.23	>0.05

5 DISCUSSION

5.1 Spheroid culture and cervical CSC isolation

The spheroid formation assay is widely used to define CSC subpopulations. The spheroid-forming ability was found to correspond to expression of established CSC markers. However, it is reported that the spheroid-forming ability was not always reflected in tumour-initiating properties in vivo [149]. In our experiments, we cultured spheroids from nine cervical cancer cell lines. The nine cell lines showed varying ability to form spheroids. We then evaluated the “stemness” characteristics of the spheroids by flow cytometry and qRT-PCR. We found that in SDCs compared with corresponding MDCs, the “stemness”-related transcription factors Sox2, Oct3/4 and Nanog were up-regulated, the anti-“stemness” microRNAs were down-regulated and the proportion of $ALDH1^{+}$, $CD44^{+}CD24^{-}$ and $ALDH1^{+}CD44^{+}CD24^{-}$ cell population was generally increased to different extent. This finding reveals that spheroid culture is an efficient method to enrich CSC-like cell populations from cervical cancer cell lines.

Enrichment of CSCs by spheroid culture also has limitations. The most important is that the spheres still represent a heterogeneous population, with only a portion of the cells capable of self-renewal [150, 151]. In our experiments, although the spheroids of nine cervical cancer cell lines showed an increase of the $ALDH1^{+}CD44^{+}CD24^{-}$ cell population compared to MDCs, most of the spheroids contained less than 10% CSCs in the population. To purify the CSC population from SDCs might call for further steps. Another drawback is that the ability of spheroid formation is not related to the expression level of the “stemness” characteristics. The cervical cancer cell line SiHa that never formed any spheroids, also showed, after anchorage independent growth under culture conditions for spheroids, an increase of “stemness”-related transcription factor expression, decrease of “stemness” suppressor miRNAs and increase of an $ALDH1^{+}CD44^{+}CD24^{-}$ population when compared with adherently grown MDCs. When compared to the SDCs of C4-1 and ME180 cell lines, which can form highly compact spheroids, SiHa-derived cells from “spheroid conditions” contained a larger $ALDH1^{+}CD44^{+}CD24^{-}$ cell population and showed a higher level of “stemness”-related transcription factor up-regulation. The SDCs of the cervical cancer cell line CaSki, which showed a high capacity for spheroid formation, did not show a significant difference in down-regulation of “stemness” suppressor

miRNAs when compared to the corresponding MDCs. Thus, spheroid culture is an essential method to isolate and enrich CSC populations, but it cannot provide a precise population of CSCs and might not entirely reflect “stemness” properties.

5.2 ALDH1 and CD44 as markers of cervical CSCs

Cell surface markers have been used as a means of identification and isolation. By using cell surface markers, more precise populations can be isolated. Most of the markers utilized for CSC isolation to date are based on knowledge of tissue development or are derived from hematopoietic or embryonic stem cells [5]. The information obtained from CSCs isolated from one type of cancer was not always applicable to other cancer, type. Therefore, the choice of the markers used for isolation represents the biggest challenge in the method. These markers often come from what is known about the development of the tissue and from markers of stem cells from systems in which a hierarchy of differentiation has been clearly established [5].

CSC identification, especially from cervical cancer cell lines, is still in its infancy. What has been hypothesized in other types of cancer is not transferrable directly to cervical cancer. The two markers ALDH1 and CD44, mainly used in these experiments have not been initially established in cervical cancer cell lines. In our experiments, the proportions of $ALDH1^{+}$ and $CD44^{+}CD24^{-}$ populations were highly variable in cervical cancer MDCs and SDCs. A decrease of $ALDH1^{+}$ or $CD44^{+}CD24^{-}$ populations in SDCs compared to MDCs was even observed in cell lines CaSki and MRIH215 which could form spheroids with high compactness. This reveals that $ALDH1^{+}$ and $CD44^{+}CD24^{-}$ alone might not be the best choice of CSC markers for cervical cancer cell lines. In fact, it has been reported that ALDH1 expression is significantly reduced rather than increased in malignant ovarian tumors while it was relatively unchanged in benign tumors compared to normal ovary [90] and CD44-negative cells also have stem-cell like traits in head and neck cell lines [73].

The combined use of different putative CSC markers may provide a more precise definition of stem cell-like populations. It was shown in breast cancer that CSC marker ALDH1 can further divide the $CD44^{+}CD24^{-/low}$ cell population into fractions that are highly tumorigenic: $ALDH1^{+}CD44^{+}CD24^{-/low}$ cells were able to generate tumours from only 20 cells, whereas $ALDH1^{-}CD44^{+}CD24^{-/low}$ were not tumorigenic in this same cell density [91, 92]. When we

defined the CSC-like cells as ALDH1^{+}CD44^{+}CD24^{-} in this study, the percentage of the CSC-like population was higher in SDCs compared to MDCs in all cell lines. Thus, ALDH1^{+}CD44^{+}CD24^{-} cells were considered a better defining parameter for CSC populations for cervical cancer cell lines than using them individually. Our further aim was to characterize the “stemness”-related transcription factors and miRNAs in the ALDH1^{+}CD44^{+}CD24^{-} and ALDH1^{-}CD44^{+}CD24^{-} cell population.

5.3 The miR-21, miR-218 and cervical cancer

The microRNA miR-21 was one of the earliest mammalian microRNAs identified. It is known as an oncomir. In cervical cancer, it was demonstrated that miR-21 is increased in cervical cancer tissue/cells as compared to normal cervical tissue/cells [105]. One potential mechanism that was shown in the cervical cancer cell line HeLa is that miR-21 targets directly the PDCD4-3’UTR and silences the programmed cell death process of the cancer cells [106]. Although we could not prove a significant difference, there was a trend that the miR-21 was generally up-regulated in cervical cancer cell lines when compared to the FKs. The reason might be that the amount of different FKs was too small and the selected four FKs showed a highly variable expression of the miR-21. A further aim is to enlarge the number of different FKs in order to develop a better control.

The miR-218 is a miRNA which is HPV-related, although it is still unknown exactly how tumor suppressor miR-218 interacts with high-risk HPV. It was reported that the miR-218 is encoded in SLIT2 and infection with HPV16 reduces the expression of the miR-218 in cervical cancer cell lines, cervical lesions and cancer tissues [122]. It was also reported that miR-218 was under-expressed in tissues infected with HR-HPV, and miR-218 in patients with CIN2 and CIN3 was lower than in patients with CIN1 [152]. In this experiment, a significantly different expression of the miR-218 between the FKs and cervical cancer cell lines was observed. However, the 8 cervical cancer cell lines that were HPV positive did not show a significant change of miR-218 expression when compared to C33A, the only HPV-negative cervical cancer cell line in this study. There are no other HPV negative cervical cancer cell lines available and no further comparison was possible to us.

Further changes of miR-21 and miR-218 expression were shown in Pap smears. Patients

who were HPV-positive showed a significantly higher level of miR-21 and miR-218 dysregulation as compared to patients without HPV infection.

Apoptosis is a key program in carcinogenesis. The miR-21 up-regulates PDCD4 and silences the programmed cell death process in cancer cells, while p53 plays an opposite role by promoting the programmed cell death in cancer cells [106]. In fact, the p53 is reported to be one of the miR-21 targets. In a Glioblastoma cell line the miR-21 suppressed the p53-mediated apoptosis [153]. In HPV positive cervical cancer cell lines, HPV E6 impairs the p53 function, while in C33A cell line the p53 is mutated which also results in impaired function. The impaired p53 function in the cell lines would all lead to the over-expression of miR-21.

The laminin 5β protein is one of the miR-218 targets which is regarded a marker of invasiveness of cervical lesions. It can be used by HPV as a transient receptor to help the virus during infection of basal cells and promotes carcinogenesis in collaboration with its ligand α6β4-integrin [122, 124]. Our studies in Pap smears showed that the miR-218 expression has a close association with HPV infection. However, the miR-218 expression was also down-regulated in the HPV-free cell line C33A with its p53 mutation. As a correlation between the p53 and miR-218 or laminin 5βprotein is still absent to date, clarification of this issue calls for further understanding of the miR-218 target network.

While microRNA expression profiling has been shown to be highly specific for cancer typing, tumor diagnosis and identifying tumors of unknown origin, it is not yet clear if microRNAs can perform as markers of disease progression. This requires access to large numbers of clinical samples which cover the range of histologically defined tissue types involved in disease progression [154]. Previous studies in cervical cancer tissues demonstrated that the levels of mir-21 and mir-218 expression were also correlated with different staging of the cancer tissues [152, 154]. However, in our experiments, no significant difference was found between different cytological diagnoses in HPV-positive samples. One possible reason could be that as yet a small number of samples in the specific groups (in LSIL and HSIL groups, n=15) was compared. Another possibility is that the miR-21 and miR-218 are only HPV-related are not involved in pathogenesis in cervical cancer. Furthermore, cytological diagnosis is an essential method in cervical cancer screening and early diagnosis, but it is relatively unreliable because of the risks in inappropriate sampling, processing, or erroneous reading of the cytology samples.

Therefore, the cytological diagnosis is not a final diagnostic criterion for cervical cancer.

5.4 The significance of miRNAs and cervical CSCs

The expression profile of microRNAs in stem cells and cancer provides evidence of their significance in carcinogenesis and in maintaining the "stemness" features of the cancer progeny cells. However, the regulatory miRNA levels are always low in cancer stem cells [155]. In this study, we investigated the expression levels of three key "stemness"-related miRNAs identified from the literature in cervical SDCs compared to MDCs.

The miR-34a is a tumor suppressor which directly targets p53 post-transcriptionally [114]. In prostate cancer, the miR-34a also directly represses CD44 mRNA which is a common CSC marker [117]. Thus, the miR-34a is considered a tumor suppressor which represses "stemness" features and function. The miR-200c, a member of the miR-200 family, is a down-stream target of the p53 and plays a role in the EMT process by interacting with ZEB1, an EMT promoter [156]. The miR-203 suppresses the "stemness" features by interacting with p63 function which regulates the balance between proliferation and differentiation [111].

In the present study, the down-regulation of expression of these three selected "stemness"-related miRNAs was shown in SDCs compared to MDCs of all cervical cancer cell lines. This might suggest that these miRNAs also play a role in "stemness"-feature regulation in cervical CSCs. In our investigation, no correlation between spheroid formation and the dysregulation level of the "stemness"-related miRNAs was found. Nor was there a correlation between the change of proportional $CD44^{+}$ cells and miR-34a down-regulation. This finding might reveal that the "stemness" regulation is a network with redundant factors. It is not easy to determine which individual microRNA contributes crucially to stem cell-like features. This would be the most important challenge when it comes to the selection of microRNAs for therapeutic purposes.

5.5 Conclusion

Varying spheroid formation ability was found in 9 cervical cancer cell lines. When compared to their corresponding MDCs, the SDCs from the 9 cancer cell lines showed higher "stemness" properties including up-regulation of "stemness"-related transcription factors,

down-regulation of “stemness” suppressor miRNAs and expression of CSC markers. This indicated that spheroid culture is an essential method for enriching a CSC subpopulation. The miRNA expression also reflects the “stemness” feature of cervical CSC.

When compared to FKs, cervical cancer cell lines showed a trend in up-regulation of oncomir miR-21 and a significant down regulation of tumor suppressor miR-218. This finding was confirmed in Pap smear samples. The HPV positive group showed significant up-regulation of miR-21 and down-regulation of miR-218. These data reveal that dysregulation of miRNA expression is also related to HPV infection and cervical carcinogenesis.

6 LIMITATIONS OF THE STUDY

This study focused on the characterization of cervical CSCs and miRNA expression. We characterized the cervical cancer cell line monolayer-derived and spheroid-derived cells by comparing their “stemness” features, including the ability of spheroid formation, the content of defined CSC sub-populations, the expression of “stemness”-related transcription factors and microRNAs. We also investigated the miRNA dysregulation in Pap smears with or without HPV infection. However, there are some limitations in this study.

The four Foreskin Keratinocyte cell lines which were applied as controls in miR-21 and miR-218 expression measurements showed a highly variable expression of the two miRNAs. This resulted in failure to demonstrate a significant difference in miRNA expression in FKs versus cervical cancer cell lines. A further aim was to develop a better control material like true primary cells isolated directly from the tissue.

A large number of miRNAs has been described to date. It is difficult to determine which one would be the most crucial in cervical carcinogenesis and stemness feature development. Depending on our manpower, budget and time fund, we selected several key miRNAs reported in the literature. However, there certainly are some key miRNAs which are not included. A more ubiquitous method for selection is miRNA arrays which could screen a large number of miRNAs.

In the present study, we found that the miR-21 and miR-218 were significantly dysregulated in HPV positive samples. However, we could not demonstrate a significantly different expression level of the two microRNAs between samples which are HR or LR-HPV positive and between different cytological diagnoses with the same HPV condition. Small sample size in some groups may be the cause. In the future, we would aim to enlarge the number of the samples tested.

The expression of the “stemness”-related TFs and miRNAs is a method for characterizing of “stemness”-features of SDCs in our study. However, the expression of the “stemness”-related TFs and miRNAs was only investigated in MDCs and SDCs without FACS sorting. To investigate the genes and miRNA expression after FACS sorting of subpopulations might add

strong evidence to the reliability of the selected surface markers.

7 REFERENCES

1. Bohl, S.R., A. Pircher, and W. Hilbe, *Cancer stem cells: characteristics and their potential role for new therapeutic strategies.* Onkologie, 2011. **34**(5): p. 269-74.
2. Merlo, L.M., et al., *Cancer as an evolutionary and ecological process.* Nat Rev Cancer, 2006. **6**(12): p. 924-35.
3. Dotto, G.P., *Notch tumor suppressor function.* Oncogene, 2008. **27**(38): p. 5115-23.
4. Gupta, P.B., C.L. Chaffer, and R.A. Weinberg, *Cancer stem cells: mirage or reality?* Nat Med, 2009. **15**(9): p. 1010-2.
5. Farrar, E.M.H.a.W.L., *CANCER STEM CELLS*2009, UK: Cambridge University Press.
6. Todaro, M., et al., *Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4.* Cell Stem Cell, 2007. **1**(4): p. 389-402.
7. Singh, S.K., et al., *Identification of human brain tumour initiating cells.* Nature, 2004. **432**(7015): p. 396-401.
8. Tommasino, M., et al., *The role of TP53 in Cervical carcinogenesis.* Hum Mutat, 2003. **21**(3): p. 307-12.
9. Triel, C., et al., *Side population cells in human and mouse epidermis lack stem cell characteristics.* Exp Cell Res, 2004. **295**(1): p. 79-90.
10. Ho, M.M., et al., *Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells.* Cancer Res, 2007. **67**(10): p. 4827-33.
11. Walboomers, J.M., et al., *Human papillomavirus is a necessary cause of invasive cervical cancer worldwide.* J Pathol, 1999. **189**(1): p. 12-9.
12. zur Hausen, H., *Papillomaviruses and cancer: from basic studies to clinical application.* Nat Rev Cancer, 2002. **2**(5): p. 342-50.
13. Moody, C.A. and L.A. Laimins, *Human papillomavirus oncoproteins: pathways to transformation.* Nat Rev Cancer, 2010. **10**(8): p. 550-60.
14. Pierce, G.B., *Neoplasms, differentiations and mutations.* Am J Pathol, 1974. **77**(1): p. 103-118.
15. Elson, D.A., et al., *Sensitivity of the cervical transformation zone to estrogen-induced squamous carcinogenesis.* Cancer Res, 2000. **60**(5): p. 1267-75.
16. Martens, J.E., et al., *Cytokeratin 17 and p63 are markers of the HPV target cell, the cervical stem cell.* Anticancer Res, 2004. **24**(2B): p. 771-5.
17. Martens, J.E., et al., *Reserve cells in human uterine cervical epithelium are derived from mullerian epithelium at midgestational age.* Int J Gynecol Pathol, 2007. **26**(4): p. 463-8.
18. Melsheimer, P., et al., *DNA aneuploidy and integration of human papillomavirus type 16 e6/e7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri.* Clin Cancer Res, 2004. **10**(9): p. 3059-63.
19. Wentzensen, N., S. Vinokurova, and M. von Knebel Doeberitz, *Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract.* Cancer Res, 2004. **64**(11): p. 3878-84.
20. Duensing, S. and K. Munger, *Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins.* Int J Cancer, 2004. **109**(2): p. 157-62.
21. zur Hausen, H., *Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis.* J Natl Cancer Inst, 2000. **92**(9): p. 690-8.
22. Liu, X., et al., *HPV-16 oncogenes E6 and E7 are mutagenic in normal human oral keratinocytes.* Oncogene, 1997. **14**(19): p. 2347-53.
23. Kessis, T.D., et al., *Expression of HPV16 E6 or E7 increases integration of foreign DNA.* Oncogene, 1996. **13**(2): p. 427-31.
24. Tapia, N. and H.R. Scholer, *p53 connects tumorigenesis and reprogramming to pluripotency.* J Exp Med,

2010. **207**(10): p. 2045-8.
25. Brosh, R. and V. Rotter, *When mutants gain new powers: news from the mutant p53 field.* Nat Rev Cancer, 2009. **9**(10): p. 701-13.
26. Halbert, C.L., G.W. Demers, and D.A. Galloway, *The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells.* J Virol, 1992. **66**(4): p. 2125-34.
27. Geiger, T., et al., *Anomalous features of EMT during keratinocyte transformation.* PLoS One, 2008. **3**(2): p. e1574.
28. Wang, Z., et al., *The role of Notch signaling pathway in epithelial-mesenchymal transition (EMT) during development and tumor aggressiveness.* Curr Drug Targets, 2010. **11**(6): p. 745-51.
29. Lee, M.Y., et al., *Epithelial-mesenchymal transition in cervical cancer: correlation with tumor progression, epidermal growth factor receptor overexpression, and snail up-regulation.* Clin Cancer Res, 2008. **14**(15): p. 4743-50.
30. Hsu, Y.M., et al., *KCl cotransporter-3 down-regulates E-cadherin/beta-catenin complex to promote epithelial-mesenchymal transition.* Cancer Res, 2007. **67**(22): p. 11064-73.
31. Li, J. and B.P. Zhou, *Activation of beta-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters.* BMC Cancer, 2011. **11**: p. 49.
32. Shibata, K., et al., *Twist expression in patients with cervical cancer is associated with poor disease outcome.* Ann Oncol, 2008. **19**(1): p. 81-5.
33. Li, Y., et al., *Correlation of TWIST2 up-regulation and epithelial-mesenchymal transition during tumorigenesis and progression of cervical carcinoma.* Gynecol Oncol, 2012. **124**(1): p. 112-8.
34. Yang, J., et al., *Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis.* Cell, 2004. **117**(7): p. 927-39.
35. Bolos, V., et al., *The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors.* J Cell Sci, 2003. **116**(Pt 3): p. 499-511.
36. Cano, A., et al., *The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.* Nat Cell Biol, 2000. **2**(2): p. 76-83.
37. Cowin, P., T.M. Rowlands, and S.J. Hatsell, *Cadherins and catenins in breast cancer.* Curr Opin Cell Biol, 2005. **17**(5): p. 499-508.
38. Chang, C.J., et al., *p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs.* Nat Cell Biol, 2011. **13**(3): p. 317-23.
39. Chung, M.T., et al., *SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway.* Gynecol Oncol, 2009. **112**(3): p. 646-53.
40. Liu, C.Y., et al., *Characterization of LMX-1A as a metastasis suppressor in cervical cancer.* J Pathol, 2009. **219**(2): p. 222-31.
41. Kopan, R. and M.X. Ilagan, *The canonical Notch signaling pathway: unfolding the activation mechanism.* Cell, 2009. **137**(2): p. 216-33.
42. Dufraine, J., Y. Funahashi, and J. Kitajewski, *Notch signaling regulates tumor angiogenesis by diverse mechanisms.* Oncogene, 2008. **27**(38): p. 5132-7.
43. Rangarajan, A., et al., *Activated Notch1 signaling cooperates with papillomavirus oncogenes in transformation and generates resistance to apoptosis on matrix withdrawal through PKB/Akt.* Virology, 2001. **286**(1): p. 23-30.
44. Nair, P., K. Somasundaram, and S. Krishna, *Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway.* J Virol, 2003. **77**(12): p. 7106-12.
45. Klinakis, A., et al., *Myc is a Notch1 transcriptional target and a requisite for Notch1-induced mammary tumorigenesis in mice.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9262-7.
46. Rangarajan, A., et al., *Notch signaling is a direct determinant of keratinocyte growth arrest and entry into*

differentiation. EMBO J, 2001. **20**(13): p. 3427-36.

47. Chakrabarti, O., et al., *Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signaling and oncogenic Ras*. J Virol, 2004. **78**(11): p. 5934-45.
48. Lathion, S., et al., *Notch1 can contribute to viral-induced transformation of primary human keratinocytes.* Cancer Res, 2003. **63**(24): p. 8687-94.
49. Subramanyam, D. and S. Krishna, *c-Myc substitutes for Notch1-CBF1 functions in cooperative transformation with papillomavirus oncogenes.* Virology, 2006. **347**(1): p. 191-8.
50. Zagouras, P., et al., *Alterations in Notch signaling in neoplastic lesions of the human cervix.* Proc Natl Acad Sci U S A, 1995. **92**(14): p. 6414-8.
51. Ramdass, B., et al., *Coexpression of Notch1 and NF-kappaB signaling pathway components in human cervical cancer progression.* Gynecol Oncol, 2007. **104**(2): p. 352-61.
52. Lowell, S., et al., *Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters.* Curr Biol, 2000. **10**(9): p. 491-500.
53. Nickoloff, B.J., et al., *Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma.* Cell Death Differ, 2002. **9**(8): p. 842-55.
54. Nguyen, B.C., et al., *Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation.* Genes Dev, 2006. **20**(8): p. 1028-42.
55. Lefort, K., et al., *Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases.* Genes Dev, 2007. **21**(5): p. 562-77.
56. Weijzen, S., et al., *HPV16 E6 and E7 oncoproteins regulate Notch-1 expression and cooperate to induce transformation.* J Cell Physiol, 2003. **194**(3): p. 356-62.
57. Srivastava, S., et al., *Notch1 regulates the functional contribution of RhoC to cervical carcinoma progression.* Br J Cancer, 2010. **102**(1): p. 196-205.
58. He, X., et al., *The effect of RhoC siRNA on the invasiveness and proliferation of human cervical cancer cell line SiHa cells.* J Huazhong Univ Sci Technolog Med Sci, 2008. **28**(6): p. 665-9.
59. Ji, J. and P.S. Zheng, *Expression of Sox2 in human cervical carcinogenesis.* Hum Pathol, 2010. **41**(10): p. 1438-47.
60. Liu, D., et al., *Differential expression of Oct4 in HPV-positive and HPV-negative cervical cancer cells is not regulated by DNA methyltransferase 3A.* Tumour Biol, 2011. **32**(5): p. 941-50.
61. Ye, F., et al., *Stem-cell-abundant proteins Nanog, Nucleostemin and Musashi1 are highly expressed in malignant cervical epithelial cells.* BMC Cancer, 2008. **8**: p. 108.
62. Moon, J.H., et al., *Nanog-induced dedifferentiation of p53-deficient mouse astrocytes into brain cancer stem-like cells.* Biochem Biophys Res Commun, 2011. **412**(1): p. 175-81.
63. Ikeda, K., et al., *Coordinate expression of cytokeratin 8 and cytokeratin 17 immunohistochemical staining in cervical intraepithelial neoplasia and cervical squamous cell carcinoma: an immunohistochemical analysis and review of the literature.* Gynecol Oncol, 2008. **108**(3): p. 598-602.
64. Regauer, S. and O. Reich, *CK17 and p16 expression patterns distinguish (atypical) immature squamous metaplasia from high-grade cervical intraepithelial neoplasia (CIN III).* Histopathology, 2007. **50**(5): p. 629-35.
65. Smedts, F., F.C. Ramaekers, and P.G. Vooijs, *The dynamics of keratin expression in malignant transformation of cervical epithelium: a review.* Obstet Gynecol, 1993. **82**(3): p. 465.
66. Feng, D., et al., *Identification and characterization of cancer stem-like cells from primary carcinoma of the cervix uteri.* Oncol Rep, 2009. **22**(5): p. 1129-34.
67. Prince, M.E., et al., *Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 973-8.
68. Sheridan, C., et al., *CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis.* Breast Cancer Res, 2006. **8**(5): p. R59.

69. Omara-Opyene, A.L., et al., *Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18.* Lab Invest, 2004. **84**(7): p. 894-907.
70. McGowan, P.M., et al., *Notch1 inhibition alters the CD44hi/CD24lo population and reduces the formation of brain metastases from breast cancer.* Mol Cancer Res, 2011. **9**(7): p. 834-44.
71. Kumar, V., et al., *p38 kinase is crucial for osteopontin-induced furin expression that supports cervical cancer progression.* Cancer Res, 2010. **70**(24): p. 10381-91.
72. Zoller, M., *CD44: can a cancer-initiating cell profit from an abundantly expressed molecule?* Nat Rev Cancer, 2011. **11**(4): p. 254-67.
73. Oh, S.Y., et al., *CD44-negative cells in head and neck squamous carcinoma also have stem-cell like traits.* Eur J Cancer, 2012.
74. Sano, T., et al., *Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions.* Am J Pathol, 1998. **153**(6): p. 1741-8.
75. Watt, F.M., *Epidermal stem cells: markers, patterning and the control of stem cell fate.* Philos Trans R Soc Lond B Biol Sci, 1998. **353**(1370): p. 831-7.
76. Truong, A.B., et al., *p63 regulates proliferation and differentiation of developmentally mature keratinocytes.* Genes Dev, 2006. **20**(22): p. 3185-97.
77. Witkiewicz, A.K., et al., *Microglandular hyperplasia: a model for the de novo emergence and evolution of endocervical reserve cells.* Hum Pathol, 2005. **36**(2): p. 154-61.
78. Nekulova, M., et al., *The role of p63 in cancer, stem cells and cancer stem cells.* Cell Mol Biol Lett, 2011. **16**(2): p. 296-327.
79. Okuyama, R., et al., *p53 homologue, p51/p63, maintains the immaturity of keratinocyte stem cells by inhibiting Notch1 activity.* Oncogene, 2007. **26**(31): p. 4478-88.
80. Caserta, T.M., et al., *p63 overexpression induces the expression of Sonic Hedgehog.* Mol Cancer Res, 2006. **4**(10): p. 759-68.
81. Li, N., et al., *Reciprocal intraepithelial interactions between TP63 and hedgehog signaling regulate quiescence and activation of progenitor elaboration by mammary stem cells.* Stem Cells, 2008. **26**(5): p. 1253-64.
82. Boldrup, L., et al., *DeltaNp63 isoforms regulate CD44 and keratins 4, 6, 14 and 19 in squamous cell carcinoma of head and neck.* J Pathol, 2007. **213**(4): p. 384-91.
83. Ma, I. and A.L. Allan, *The role of human aldehyde dehydrogenase in normal and cancer stem cells.* Stem Cell Rev, 2011. **7**(2): p. 292-306.
84. Kastan, M.B., et al., *Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells.* Blood, 1990. **75**(10): p. 1947-50.
85. Sladek, N.E., et al., *Cellular levels of aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) as predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer: a retrospective study. Rational individualization of oxazaphosphorine-based cancer chemotherapeutic regimens.* Cancer Chemother Pharmacol, 2002. **49**(4): p. 309-21.
86. Purton, L.E., *Roles of retinoids and retinoic Acid receptors in the regulation of hematopoietic stem cell self-renewal and differentiation.* PPAR Res, 2007. **2007**: p. 87934.
87. Douville, J., R. Beaulieu, and D. Balicki, *ALDH1 as a functional marker of cancer stem and progenitor cells.* Stem Cells Dev, 2009. **18**(1): p. 17-25.
88. Bortolomai, I., et al., *Tumor initiating cells: development and critical characterization of a model derived from the A431 carcinoma cell line forming spheres in suspension.* Cell Cycle, 2010. **9**(6): p. 1194-206.
89. Yao, T., et al., *The expression of ALDH1 in cervical carcinoma.* Med Sci Monit, 2011. **17**(8): p. HY21-26.
90. Penumatsa, K., et al., *Differential expression of aldehyde dehydrogenase 1a1 (ALDH1) in normal ovary and serous ovarian tumors.* J Ovarian Res, 2010. **3**: p. 28.
91. Ginestier, C., et al., *ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome.* Cell Stem Cell, 2007. **1**(5): p. 555-67.

92. Croker, A.K., et al., *High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability.* J Cell Mol Med, 2009. **13**(8B): p. 2236-52.
93. Ambros, V., *The functions of animal microRNAs.* Nature, 2004. **431**(7006): p. 350-5.
94. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
95. Calin, G.A. and C.M. Croce, *MicroRNA signatures in human cancers.* Nat Rev Cancer, 2006. **6**(11): p. 857-66.
96. Suzuki, H.I., et al., *Modulation of microRNA processing by p53.* Nature, 2009. **460**(7254): p. 529-33.
97. Jazdzewski, K., et al., *Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma.* Proc Natl Acad Sci U S A, 2008. **105**(20): p. 7269-74.
98. Yue, C., et al., *Polymorphism of the pre-miR-146a is associated with risk of cervical cancer in a Chinese population.* Gynecol Oncol, 2011. **122**(1): p. 33-7.
99. Zhou, X., et al., *Polymorphisms involved in the miR-218-LAMB3 pathway and susceptibility of cervical cancer, a case-control study in Chinese women.* Gynecol Oncol, 2010. **117**(2): p. 287-90.
100. DeSano, J.T. and L. Xu, *MicroRNA regulation of cancer stem cells and therapeutic implications.* AAPS J, 2009. **11**(4): p. 682-92.
101. Yan, L.X., et al., *MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis.* RNA, 2008. **14**(11): p. 2348-60.
102. Bourguignon, L.Y., et al., *Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells.* Oncogene, 2012. **31**(2): p. 149-60.
103. Asangani, I.A., et al., *MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer.* Oncogene, 2008. **27**(15): p. 2128-36.
104. Bornachea, O., et al., *EMT and induction of miR-21 mediate metastasis development in Trp53-deficient tumours.* Sci Rep, 2012. **2**: p. 434.
105. Lui, W.O., et al., *Patterns of known and novel small RNAs in human cervical cancer.* Cancer Res, 2007. **67**(13): p. 6031-43.
106. Yao, Q., et al., *MicroRNA-21 promotes cell proliferation and down-regulates the expression of programmed cell death 4 (PDCD4) in HeLa cervical carcinoma cells.* Biochem Biophys Res Commun, 2009. **388**(3): p. 539-42.
107. Sonkoly, E., M. Stahle, and A. Pivarcsi, *MicroRNAs: novel regulators in skin inflammation.* Clin Exp Dermatol, 2008. **33**(3): p. 312-5.
108. Sonkoly, E., et al., *MicroRNAs: novel regulators involved in the pathogenesis of psoriasis?* PLoS One, 2007. **2**(7): p. e610.
109. Yi, R., et al., *A skin microRNA promotes differentiation by repressing 'stemness'.* Nature, 2008. **452**(7184): p. 225-9.
110. Lee, J.W., et al., *Altered MicroRNA expression in cervical carcinomas.* Clin Cancer Res, 2008. **14**(9): p. 2535-42.
111. Lena, A.M., et al., *miR-203 represses 'stemness' by repressing DeltaNp63.* Cell Death Differ, 2008. **15**(7): p. 1187-95.
112. Melar-New, M. and L.A. Laimins, *Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins.* J Virol, 2010. **84**(10): p. 5212-21.
113. McKenna, D.J., et al., *MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6.* J Virol, 2010. **84**(20): p. 10644-52.
114. He, L., et al., *A microRNA component of the p53 tumour suppressor network.* Nature, 2007. **447**(7148): p. 1130-4.
115. Ji, Q., et al., *MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells.* PLoS One, 2009.

4(8): p. e6816.
116. Ji, Q., et al., *Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres.* BMC Cancer, 2008. **8**: p. 266.
117. Liu, C., et al., *The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44.* Nat Med, 2011. **17**(2): p. 211-5.
118. Li, B., et al., *Reduced miR-34a expression in normal cervical tissues and cervical lesions with high-risk human papillomavirus infection.* Int J Gynecol Cancer, 2010. **20**(4): p. 597-604.
119. Zheng, Z.M. and X. Wang, *Regulation of cellular miRNA expression by human papillomaviruses.* Biochim Biophys Acta, 2011. **1809**(11-12): p. 668-77.
120. Wang, X., et al., *Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6.* RNA, 2009. **15**(4): p. 637-47.
121. Pang, R.T., et al., *MicroRNA-34a suppresses invasion through downregulation of Notch1 and Jagged1 in cervical carcinoma and choriocarcinoma cells.* Carcinogenesis, 2010. **31**(6): p. 1037-44.
122. Martinez, I., et al., *Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells.* Oncogene, 2008. **27**(18): p. 2575-82.
123. Wald, A.I., et al., *Alteration of microRNA profiles in squamous cell carcinoma of the head and neck cell lines by human papillomavirus.* Head Neck, 2011. **33**(4): p. 504-12.
124. Culp, T.D., et al., *Keratinocyte-secreted laminin 5 can function as a transient receptor for human papillomaviruses by binding virions and transferring them to adjacent cells.* J Virol, 2006. **80**(18): p. 8940-50.
125. Wicha, M.S., *Stemming a tumor with a little miR.* Nat Med, 2011. **17**(2): p. 162-4.
126. Wang, X., et al., *Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth.* PLoS One, 2008. **3**(7): p. e2557.
127. Hermann, P.C., et al., *Cancer stem cells in solid tumors.* Semin Cancer Biol, 2010. **20**(2): p. 77-84.
128. Dean, M., *ABC transporters, drug resistance, and cancer stem cells.* J Mammary Gland Biol Neoplasia, 2009. **14**(1): p. 3-9.
129. Ou, Y. and X.L. Guo, *[Tumor stem cells and drug resistance].* Sheng Li Ke Xue Jin Zhan, 2007. **38**(2): p. 115-9.
130. Riou, G.F., et al., *Expression of multidrug-resistance (MDR1) gene in normal epithelia and in invasive carcinomas of the uterine cervix.* J Natl Cancer Inst, 1990. **82**(18): p. 1493-6.
131. Liu XP, W.S., Han SP et al., *Expression of resistance gene MDR1 and GST-π in cervical cancer tissues.* J Xi'an Jiaotong University, 2000. **21(6)**: p. 563-564.
132. Fojo, T. and S. Bates, *Strategies for reversing drug resistance.* Oncogene, 2003. **22**(47): p. 7512-23.
133. Su, J.H., et al., *Immunotherapy for cervical cancer: Research status and clinical potential.* BioDrugs, 2010. **24**(2): p. 109-29.
134. Pellegatta, S., et al., *Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas.* Cancer Res, 2006. **66**(21): p. 10247-52.
135. Visus, C., et al., *Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck.* Cancer Res, 2007. **67**(21): p. 10538-45.
136. Garcia-Hernandez Mde, L., et al., *Prostate stem cell antigen vaccination induces a long-term protective immune response against prostate cancer in the absence of autoimmunity.* Cancer Res, 2008. **68**(3): p. 861-9.
137. Deonarain, M.P., C.A. Kousparou, and A.A. Epenetos, *Antibodies targeting cancer stem cells: a new paradigm in immunotherapy?* MAbs, 2009. **1**(1): p. 12-25.
138. Krutzfeldt, J., et al., *Silencing of microRNAs in vivo with 'antagomirs'.* Nature, 2005. **438**(7068): p. 685-9.
139. Wiggins, J.F., et al., *Development of a lung cancer therapeutic based on the tumor suppressor*

microRNA-34. Cancer Res, 2010. **70**(14): p. 5923-30.

140. Whitehead, K.A., R. Langer, and D.G. Anderson, *Knocking down barriers: advances in siRNA delivery.* Nat Rev Drug Discov, 2009. **8**(2): p. 129-38.
141. Pfaffl, M.W., G.W. Horgan, and L. Dempfle, *Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR.* Nucleic Acids Res, 2002. **30**(9): p. e36.
142. de Roda Husman, A.M., et al., *The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR.* J Gen Virol, 1995. **76 (Pt 4)**: p. 1057-62.
143. Schmitt, M., et al., *Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers.* J Clin Microbiol, 2008. **46**(3): p. 1050-9.
144. Ivascu, A. and M. Kubbies, *Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis.* J Biomol Screen, 2006. **11**(8): p. 922-32.
145. Boyer, L.A., et al., *Core transcriptional regulatory circuitry in human embryonic stem cells.* Cell, 2005. **122**(6): p. 947-56.
146. Loh, Y.H., et al., *The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells.* Nat Genet, 2006. **38**(4): p. 431-40.
147. Chen, C., et al., *Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma.* PLoS One, 2011. **6**(1): p. e16466.
148. Visvader, J.E. and G.J. Lindeman, *Cancer stem cells in solid tumours: accumulating evidence and unresolved questions.* Nat Rev Cancer, 2008. **8**(10): p. 755-68.
149. Kuch, V., et al., *Tumor-initiating properties of breast cancer and melanoma cells in vivo are not invariably reflected by spheroid formation in vitro, but can be increased by long-term culturing as adherent monolayers.* Int J Cancer, 2013. **132**(3): p. E94-105.
150. Yuan, X., et al., *Isolation of cancer stem cells from adult glioblastoma multiforme.* Oncogene, 2004. **23**(58): p. 9392-400.
151. Jensen, J.B. and M. Parmar, *Strengths and limitations of the neurosphere culture system.* Mol Neurobiol, 2006. **34**(3): p. 153-61.
152. Li, Y., et al., *High-risk human papillomavirus reduces the expression of microRNA-218 in women with cervical intraepithelial neoplasia.* J Int Med Res, 2010. **38**(5): p. 1730-6.
153. Papagiannakopoulos, T., A. Shapiro, and K.S. Kosik, *MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells.* Cancer Res, 2008. **68**(19): p. 8164-72.
154. Deftereos, G., et al., *Expression of mir-21 and mir-143 in cervical specimens ranging from histologically normal through to invasive cervical cancer.* PLoS One, 2011. **6**(12): p. e28423.
155. Garg, M., *MicroRNAs, stem cells and cancer stem cells.* World J Stem Cells, 2012. **4**(7): p. 62-70.
156. Schubert, J. and T. Brabletz, *p53 Spreads out further: suppression of EMT and stemness by activating miR-200c expression.* Cell Res, 2011. **21**(5): p. 705-7.

8 Affidavit

"I, [Voramon, Sangvatanakul] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Characterization of microRNA expression in cervical carcinogenesis and cancer stem cells] 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 interest 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 09092013 Signature Voramon Sangvatanakul

Declaration of any eventual publications

[Voramon Sangvatanakul] had the following share in the following publications:

Liao, T., Kaufmann AM, Xu Q, **Voramon Sangvatanakul** et al., Susceptibility to cytotoxic T cell lysis of cancer stem cells derived from cervical and head and neck tumor cell lines. J Cancer Res Clin Oncol. Responsible for qRT-PCR analysis.
Lin, J., J. Qin, and **V. Sangvatanakul**, Human epididymis protein 4 for differential diagnosis between benign gynecologic disease and ovarian cancer: a systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol. Responsible for statistical analysis and language check.

Signature, date and stamp of the supervising University teacher

_Andreas Kaufmann________

Signature of the doctoral candidate

__Voramon Sangvatanakul____

9 Curriculum Vitae and Publications

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

Publications

- Liao, T., Kaufmann AM, Xu Q, **Voramon Sangvatanakul** et al., Susceptibility to cytotoxic T cell lysis of cancer stem cells derived from cervical and head and neck tumor cell lines. J Cancer Res Clin Oncol, 2013. 139(1): p. 159-70.
- Lin, J., J. Qin, and **V. Sangvatanakul**, Human epididymis protein 4 for differential diagnosis between benign gynecologic disease and ovarian cancer: a systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol, 2012.

Conference abstracts

- **Voramon Sangvatanakul (Tairong Ye)**, Tina Kube, Helmut von Keyserling, Andreas E. Albers, Andreas M. Kaufmann. Characterization of microRNAs in cervical cancer stem cells. Emerging concepts in cancer. Berlin, Germany. 15-16, June 2012
- Tian Liao, **Voramon Sangvatanakul (Tairong Ye)**, Andreas M. Kaufmann and Andreas E. Albers. Immunogenicity of cervical and HNSCC-derived putative cancer stem-like cells. The 27th International Papillomavirus Conference. Berlin, Germany. 17-25, September 2011.

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