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Syndecan-1 responsive microRNA-126 and 149 regulate cell proliferation in prostate cancer

Tomomi Fujii [a], Keiji Shimada [a], Yoshihiro Tatsumi [a,b], Kiyohide Fujimoto [b], Noboru Konishi [a,*]

[a] Department of Pathology, Nara Medical University School of Medicine, Nara, Japan
[b] Department of Urology, Nara Medical University School of Medicine, Nara, Japan

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ABSTRACT

MicroRNAs (miRNAs) are short (19–24 nt), low molecular weight RNAs that play important roles in the regulation of target genes associated with cell proliferation, differentiation, and development, by binding to the 3′-untranslated region of the target mRNAs. In this study, we examined the expression of miRNA-126 (miR-126) and miR-149 in prostate cancer, and investigated the molecular mechanisms by which they affect syndecan-1 in prostate cancer. Functional analysis of miR-126 and miR-149 was conducted in the prostate cancer cell lines, PC3, Du145, and LNCaP. The expression levels of SOX2, NANOG, Oct4, miR-126 and miR-149 were evaluated by quantitative RT-PCR. After silencing syndecan-1, miR-126, and/or miR-149 in the PC3 cells, cell proliferation, senescence, and p21 induction were assessed using the MTS assay, senescence-associated β-galactosidase (SA-β-Gal) assay, and immunocytochemistry, respectively. Compared to the Du145 and LNCaP cells, PC3 cells exhibited higher expression of syndecan-1. When syndecan-1 was silenced, the PC3 cells showed reduced expression of miR-126 and miR-149 most effectively. Suppression of miR-126 and/or miR-149 significantly inhibited cell growth via p21 induction and subsequently, induced senescence. The mRNA expression levels of SOX2, NANOG, and Oct4 were significantly increased in response to the silencing of miR-126 and/or miR-149. Our results suggest that miR-126 and miR-149 are associated with the expression of syndecan-1 in prostate cancer cells. These miRNAs promote cell proliferation by suppressing SOX2, NANOG, and Oct4. The regulation of these factors by miR-126 and miR-149 is essential for syndecan-1-mediated development of androgen-refractory prostate cancer.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs, about 19–24 nucleotides in length. By binding to the complementary sites in their target gene transcripts, miRNAs cause translational repression or transcript degradation, thereby playing an important role in the regulation of crucial cell processes such as proliferation, differentiation, and development [1–3]. Consequently, miRNAs are involved in the initiation and progression of various human cancers including prostate cancer [4]. Several miRNAs, namely, miRNA (miR)-34a, miR-148a, miR-221, miR-222, miR-143, miR-145, miR-21, and miR-331-3p, are known to be involved in prostate cancer [5–17]. For example, miR-34a negatively regulates the tumor-initiating capacity of prostate cancer stem cells. It also inhibits holoclone formation, clonogenic capacity, and sphere establishment in prostate cancer cell lines by regulating CD44 [7]. The expression of miR-148a is down-regulated in several types of cancers [18,19], while its overexpression suppresses cell growth, migration, and invasion in paclitaxel-resistant PC3 cells, via mitogen- and stress-activated protein kinase, a target of miR-148a [8]. Furthermore, miR-221/222 promotes cancer progression by inhibiting p27, a cell cycle inhibitor [10]. Thus, it is evident that specific miRNAs regulate oncogenic or tumor-suppressive genes, and can be potentially used as biomarkers or therapeutic targets.

Syndecan-1 (CD138, SDC-1), one of the four mammalian heparin sulfate proteoglycans, is highly expressed in many types of normal epithelial cells and their malignant counterparts. Syndecan-1 is involved in cell growth, adhesion, migration, epithelial morphogenesis, and angiogenesis [20]. Syndecan-1 is expressed in a majority of epithelial neoplasms including breast, gastric, lung, colon, hepatocellular, renal cell, bladder, and thyroid carcinomas, and in a variety of nonepithelial neoplasms [21]. Previously, we demonstrated that the expression of syndecan-1 increases in association

* Corresponding author at: Department of Pathology, Nara Medical University School of Medicine, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan. Fax: +81 744 23 5687.
E-mail address: nkonishi@naramed-u.ac.jp (N. Konishi).

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with hormone resistance, and contributes to cell survival by regulating NOX-mediated reactive oxygen species (ROS) generation in androgen-independent prostate cancer cells [22]. On the other hand, in normal prostate tissues, the expression of syndecan-1 is largely limited to basal cells [23]. The mechanistic role of syndecan-1 in the morphogenesis and cell proliferation in the normal or cancerous state of the prostate gland remains unclear.

The transcription factors, SOX2, NANOG, and Oct4, are involved in the self-renewal, pluripotency, and epigenetic network regulation of embryonic stem cells. In gastric cancer cells, SOX2 inhibits cancer growth through cell cycle arrest and apoptosis [24]. Moreover, exogenous miR-126 transfection decreases the SOX2 protein level in mouse ES cells, suggesting that miR-126 can repress SOX2 expression in various species and cell lineages [24]. HIF, NANOG, and Oct4 are known to be expressed in prostate cancer; in particular, the over-expression of NANOG and Oct4 shows a significant positive correlation with the Gleason score of prostate cancer [25]. miR-126 has several specific functions in multiple cancers. In non-small cell lung carcinoma and oral squamous cell carcinoma, reduced expression of miR-126 is significantly correlated with reduced survival [26–28]. On the other hand, increased expression of miR-126 is associated with neo-angiogenesis in colorectal cancer [29], and promotes metastasis in prostate cancer [30]. Thus, it is unclear whether miR-126 is tumor-suppressive or oncogenic.

In this study, we demonstrate that syndecan-1 regulates cell proliferation by enhancing miR-126- and miR-149-mediated expression of stem cell-related factors in prostate cancer.

2. Materials and methods

2.1. Cell lines

The human prostate cancer cell lines, PC3, Du145, and LNCaP, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 units/mL of penicillin/streptomycin.

2.2. Transfections of siRNA and miRNA inhibitors in PC3 cells

A total of 1×10^4 PC3 cells/well were seeded in a 6-well dish and transfected with 100 ng/L of siRNA against syndecan-1, for 72 h. The transfections of syndecan-1 siRNA and Anti-miR™ miRNA Inhibitors (hsa-miR-126-3p and hsa-miR-146-5p; Life Technologies) were carried out using Lipofectamine RNAiMAX (Life Technologies), in accordance with the manufacturer's protocol.

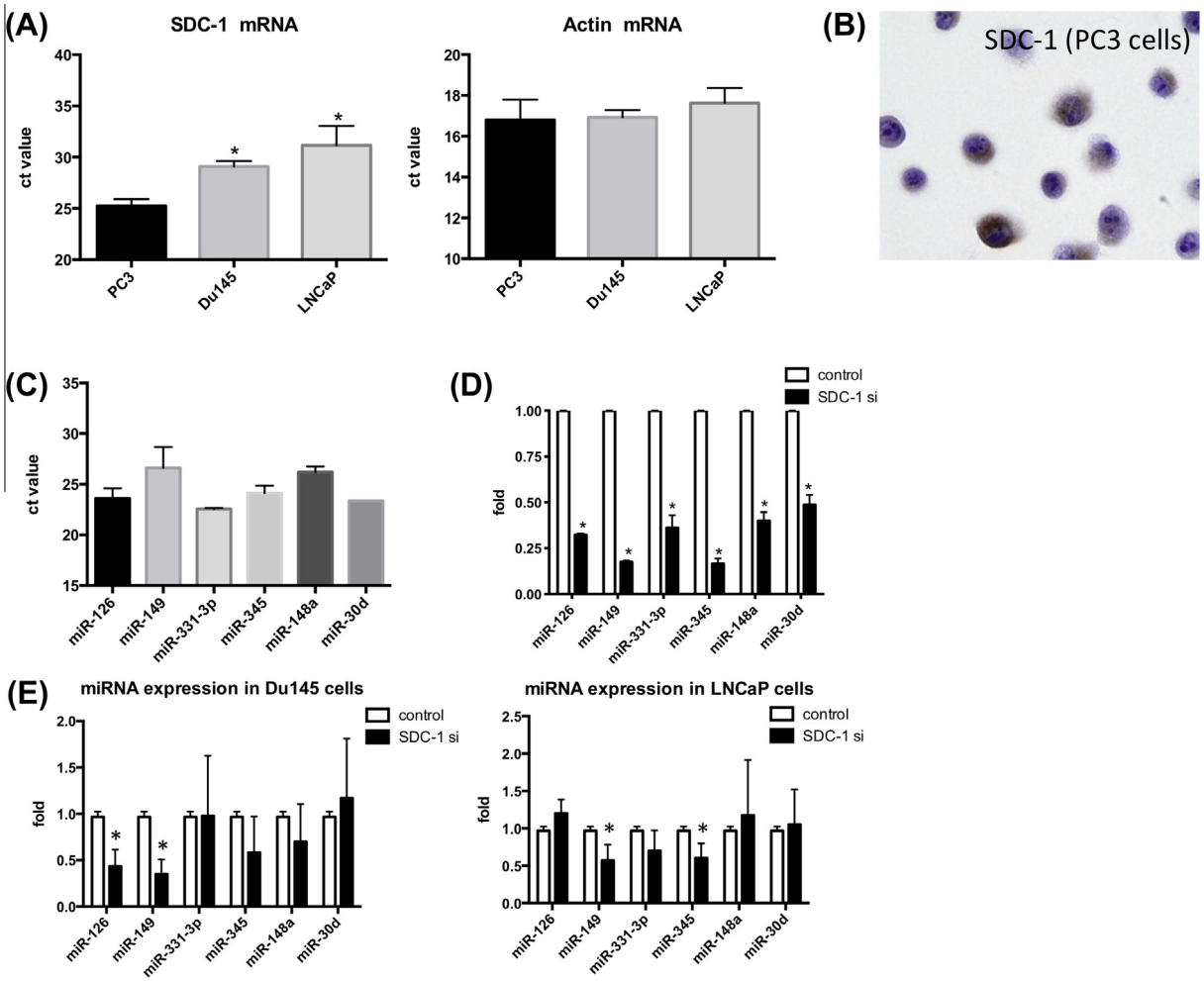

Fig. 1. Expression of syndecan-1 mRNA and miRNAs in the prostate cancer cell lines. (A) The expression of syndecan-1 mRNA in PC3 cells compared to that in the cell lines, Du145 and LnCaP (*$p < 0.05$). (SDC-1; syndecan-1) (B) Immunocytochemistry shows that syndecan-1 expression in the PC3 cells is heterogeneous. (SDC-1; syndecan-1) (C) The expression of some microRNAs in the PC3 cells. (D) miR-126, miR-149, miR-331-3p, miR-345, miR-148a, and miR-30d are suppressed in PC3 cells transiently transfected with syndecan-1 siRNA (SDC-1 si). (E) On the other hand, the miRNA expression pattern under conditions of syndecan-1 inhibition is different in Du145 and LNCaP cells (*$p < 0.05$).

The syndecan-1 siRNA sequence, designed after selecting the appropriate DNA target sequences, is as follows: 5′-TCCGACTGCTTTGGACCTAAA-3′.

2.3. Quantitative RT-PCR (qRT-PCR) analysis of miRNAs and mRNAs

Total RNA, including the miRNA, was purified from the cell lines and paraffin-embedded tissue sections using miRNeasy Mini kit (QIAGEN) and miRNeasy FFPE kit (QIAGEN), respectively. First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT Master Mix (Perfect Real Time) and SYBR Premix Ex Taq II (Tli RNaseH Plus). The qPCR conditions were set at 35–45 cycles of 95 °C for 30 s, followed by 55–63 °C for 30 s. The PCR primers are as follows:

Syndecan-1 sense 5′-GGCTGTAGTCCTGCCAGAAG-3′;
Syndecan-1 antisense 5′-GTTGAGGCCTGATGAGTGGT-3′;
SOX2 sense 5′-GACCAGCTCGCAGACCTACAT-3′;
SOX2 antisense 5′-ATGGAGCCAAGAGCCATGC-3′;
NANOG sense 5′-AACCTCAGCTACAAACAGGTGAAG-3′;
NANOG antisense 5′-CTGCGTCACACCATTGCTATTCT-3′;
Oct4 sense 5′-TTCCCCCTGTCTCCGTCAC-3′;
Oct4 antisense 5′-AGAACTTAATCCCAAAAACCCTGG-3′;
Actin sense 5′-CTCTTCCAGCCTTCCTTCCT-3′;
Actin antisense 5′-AGCACTGTGTTGGCGTACAG-3′.

2.4. Cell proliferation assay

For the cell proliferation assay, a methane thiosulfonate (MTS) reagent was used as previously described [22,26,31]. All the experiments were performed in triplicate.

2.5. Tissue samples

This study examined 15 prostate carcinomas from patients without chemotherapy at radical prostatectomy. Normal healthy prostate tissues were obtained as control samples from autopsies. Histological evaluation of the prostate carcinomas was performed using the Gleason score. Informed consent was obtained from all the patients before collecting the specimens. This study was approved by the Ethics Committee of the Nara Medical University.

2.6. Immunohistochemistry

The tissue sections were incubated with the primary antibodies for 1 h at room temperature. The reactions were visualized using a

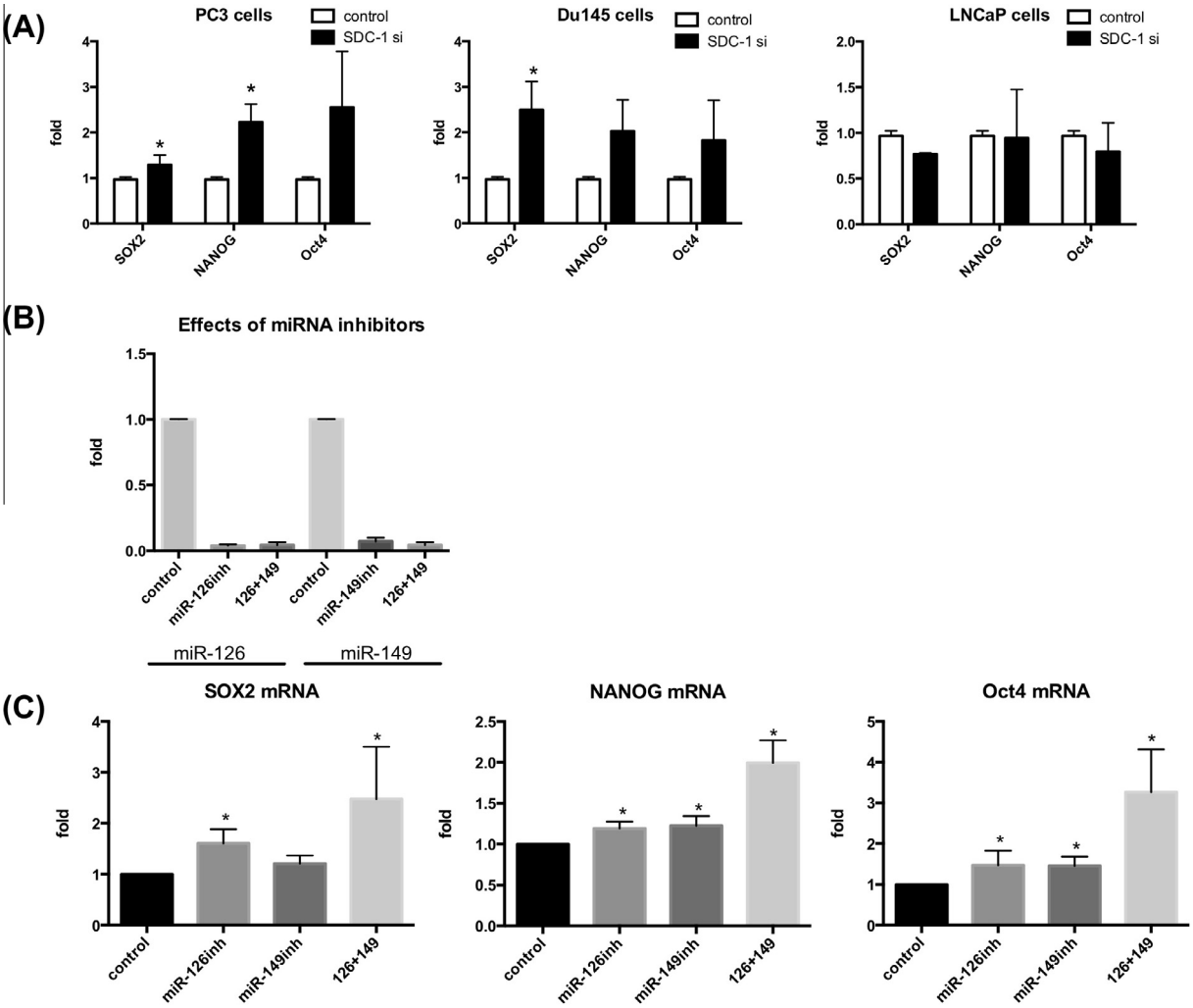

Fig. 2. mRNA expression of SOX2, NANOG, and Oct4 is increased under conditions of syndecan-1 suppression in PC3 cells. (A) The mRNA expression of SOX2, NANOG, and Oct4 increased in the PC3 and Du145 cells but not in LnCaP cells, following transient transfections of syndecan-1 siRNA (SDC-1 si). (B) Effects of transient transfection of miRNA inhibitors. Both miR-126 and miR-149 are significantly suppressed by transfections of the individual miRNA inhibitors ($^*p < 0.05$). (C) Suppression of miR-126 and/or miR-149 elevates the mRNA expression of SOX2, NANOG, and Oct4 ($^*p < 0.05$).

Histofine kit (Nichirei, Tokyo, Japan), using diaminobenzidine as the chromogen, with hematoxylin counterstaining.

2.7. Statistical analyses

Differences in the measurement of continuous variables were analyzed using ANOVA or nonparametric tests (Mann–Whitney and Kruskal–Wallis tests). All the experimental results were analyzed using the 1-way analysis of variance and Tukey's post-hoc test. The 2-tailed student's *t*-test was used to compare 2 data points. The results were considered to be statistically significant if $p < 0.05$.

3. Results

3.1. Syndecan-1 mRNA is expressed in prostate cancer cell lines

We previously showed that knockdown of syndecan-1 reduces cell growth and induces apoptosis in PC3 and DU145 cells, which are less androgen-dependent, malignant, and metastatic prostate cancer cell lines. On the other hand, LNCaP cells are androgen-dependent and less malignant. In this study, we first examined the mRNA expression of syndecan-1 in the 3 prostate cancer cell lines – PC3, DU145, and LNCaP – using qRT-PCR, and found that the PC3 cells showed the strongest expression of syndecan-1 (Fig. 1A). Immunocytochemical analysis demonstrated that the PC3 cells are heterogeneously positive for syndecan-1 (Fig. 1B).

3.2. Syndecan-1 regulates the expression of miR-331-3p, miR-126, miR-149, miR-345, miR-148a, and miR-30d

In order to explore novel miRNAs that are regulated by syndecan-1 in prostate cancer cells, we performed miRNA array analysis of the PC3 cells using Human miRNA Oligo chip (3D-Gene DNA chip, TORAY). To determine which miRNAs are regulated by syndecan-1, we selected some candidate miRNAs predicted to be factors in the progression of several cancers, and analyzed their expression in the PC3 cells, using qRT-PCR. As shown in Fig. 1C, the expression of miR-126, miR-149, miR-331-3p, miR-148a, miR-345, and miR-30d was detected between the ct values of 20–30. In addition, the transient suppression of syndecan-1 significantly decreased the expression of these miRNAs (Fig. 1D). Similarly, the expression of miR-126 and miR-149 in Du145 cells and that of miR-149 and miR-345 in LNCaP cells was significantly reduced under conditions of syndecan-1 suppression (Fig. 1E). These results indicate that miR-126 and miR-149, along with syndecan-1, are involved in the progression of androgen-independent prostate cancer.

3.3. Suppression of syndecan-1 or miRNA-126 and miR-149 significantly increases the expression of SOX2, Oct4, and NANOG in PC3 cells

In normal and prostate cancer cells, syndecan-1 is not or seldom expressed with the exception of basal cells (see Fig. 4B). PC3 cells exhibited a significantly increased expression of syndecan-1, sug-

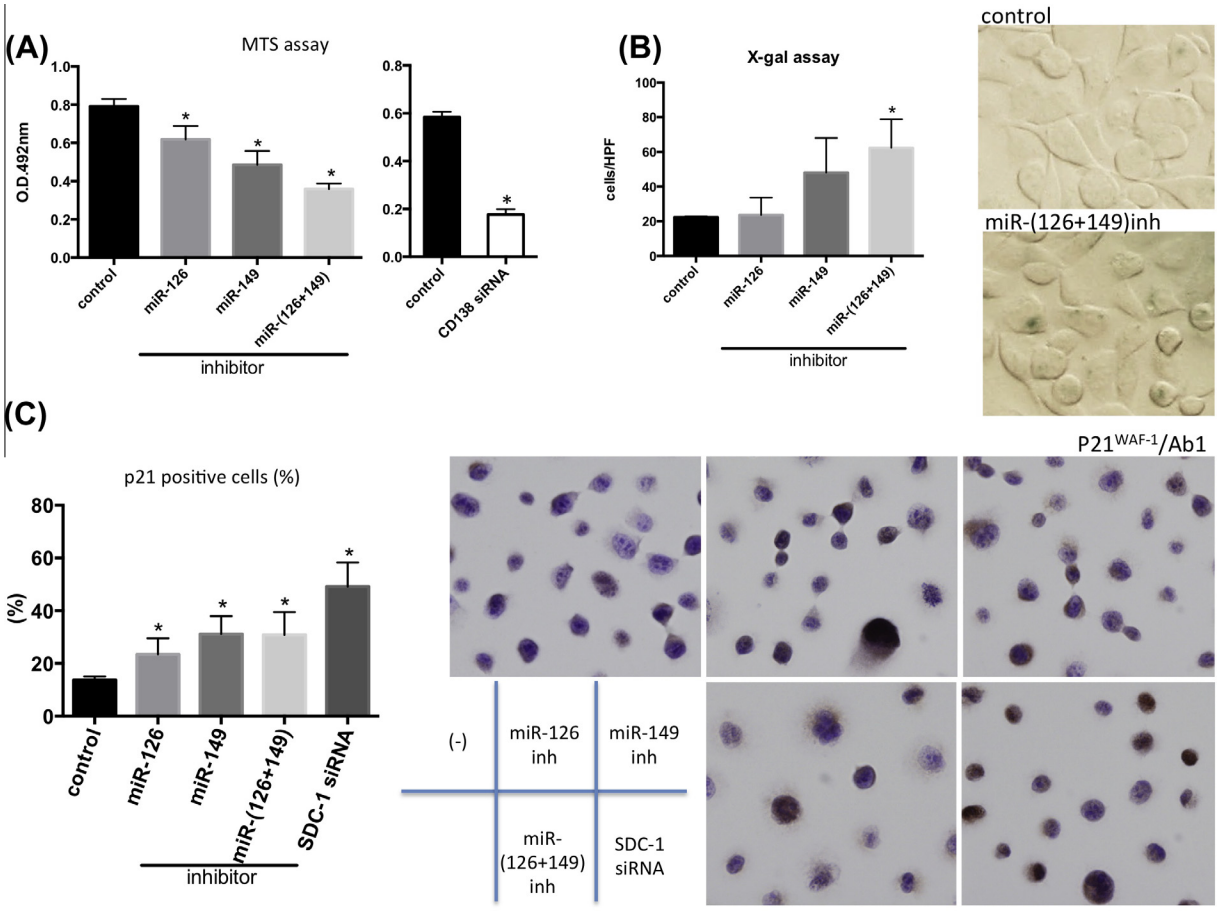

Fig. 3. Cell proliferation assay in the PC3 cells. (A and B) MTS assay and X-gal assay. Cell proliferation was suppressed and senescence was induced by transient transfections of inhibitors of miR-126 and/or miR-149, and syndecan-1 siRNA (*$p < 0.05$). (C) Cells transfected with syndecan-1 siRNA, inhibitors of miR-126, or miR-149 exhibit significant increase in the expression of p21.

gesting that syndecan-1 may be involved in the progression of androgen-independent prostate cancer cells. We evaluated the effect of the suppression of syndecan-1 on the expression of SOX2, NANOG, and Oct4 in the PC3, Du145, and LNCaP cells. As shown in Fig. 2A, PC3 and Du145 cells transfected with syndecan-1 siRNA overexpressed mRNAs of SOX2, NANOG, and Oct4, but partially not statistically significant. On the other hand, syndecan-1 inhibition in LNCaP cells did not affect the expression of SOX2, NANOG, and Oct4. To determine how miRNAs regulate syndecan-1 in the prostate cancer cells, we transfected inhibitors of miR126 and/or miR-149 into the PC3 cells (Fig. 2B). As shown in Fig. 2C, inhibition of miR-126 and miR-149 significantly increased the expression of SOX2, NANOG, and Oct4. However, the inhibition of miR-331-3p, miR-345, miR-148a, and miR-30d did not affect the expression of these 3 factors (data not shown). Moreover, compared to the inhibition of miR-126 or miR-149 alone, simultaneous inhibition of both the miRNAs significantly increased the expression of SOX2, NANOG, and Oct4. Taken together, these results indicate a correlation between miR-126, miR-149, and syndecan-1 in the PC3 cells.

3.4. miR-126 and miR-149 contribute to syndecan-1-dependent cell growth in prostate cancer cells

The suppression of syndecan-1 significantly decreases tumor growth in prostate cancer (Fig. 3A). Therefore, we examined whether miR-126 and miR-149 affect syndecan-1-dependent cancer cell growth. The inhibition of miR-126 and miR-149 suppressed cell proliferation through induction of senescence as assessed by MTS and SA-β-gal assays (Fig. 3A and B). Moreover, PC3 cells transfected with syndecan-1 siRNA, or inhibitors of miR-126 or miR-149 showed a significant increase in p21 expression (Fig. 3C), indicating that the inhibition of syndecan-1, miR-126, and miR-149 suppresses cell proliferation and viability through cellular senescence.

3.5. Expression analysis of syndecan-1, miR-126, and miR-149 in human prostate cancer tissues

Next, we examined the relation between the expression of miR-126 and miR-149 with that of syndecan-1 in tissue samples of primary prostate cancer. First, we classified the cancerous prostate tissues pathologically, based on the Gleason patterns (Fig. 4A). We evaluated the expression of syndecan-1 in the normal and cancerous prostate tissues by immunohistochemistry. Syndecan-1 was expressed in the basal cells of the normal prostate cancer glands. In the cancerous prostate tissue, syndecan-1 was undetected or expressed at a low level in Gleason pattern 3 or 4, and highly expressed in Gleason pattern 5 (Fig. 4B). Total RNA was isolated from the cancer foci. The expression levels of miR-126 and miR-149, determined by qRT-PCR analysis, were significantly higher in the normal prostate compared with those in the prostate cancer lesions (data not shown). Although the expression of miR-126 or miR-149 appeared to increase in the Gleason pattern 5, it

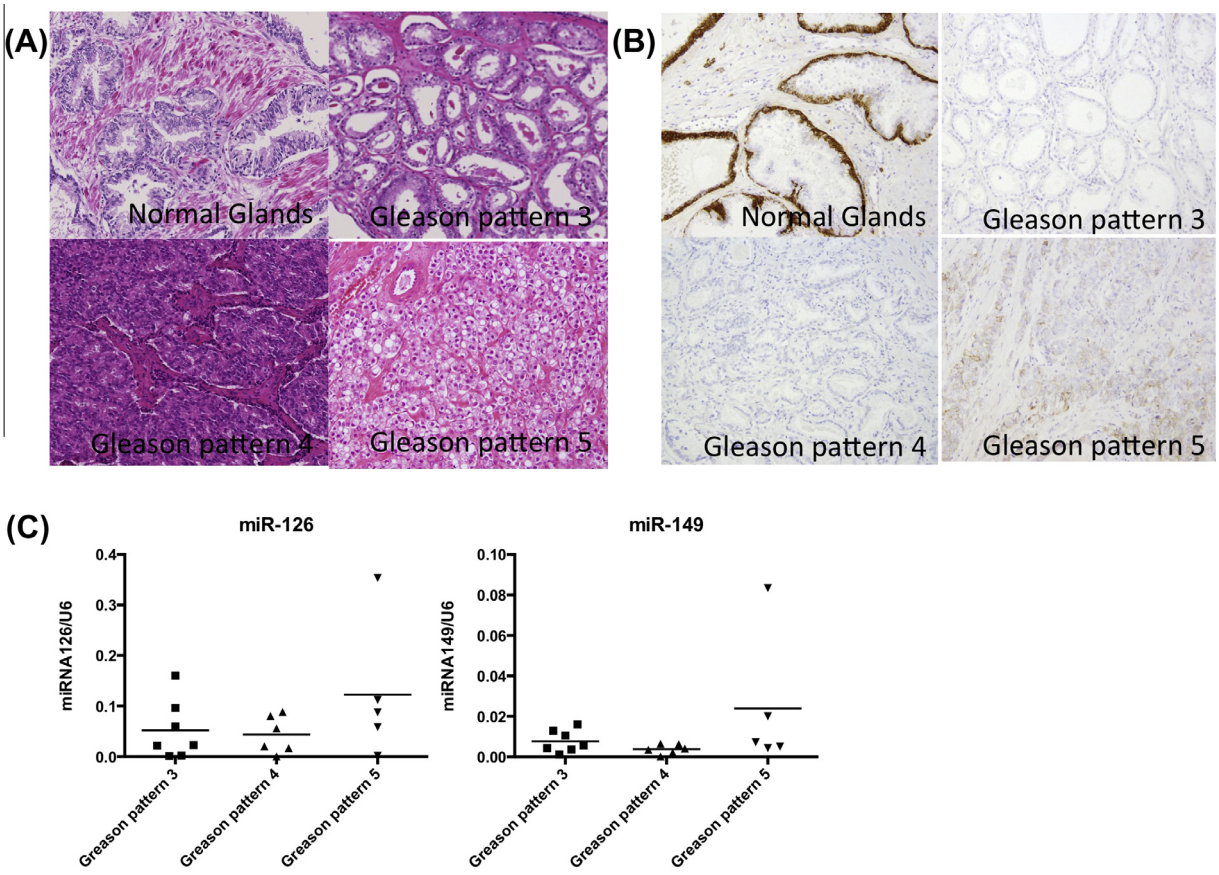

Fig. 4. Expression of syndecan-1, miR-126, and miR-149 in prostate cancer tissues. (A) Normal glands and adenocarcinoma of the prostate tissues. Adenocarcinoma was histologically classified in to Gleason patterns (Hematoxylin–eosin staining). (B) Immunohistochemistry of syndecan-1 demonstrates that the normal glands are positive for basal cells. The adenocarcinoma tissues were negative for the well-differentiated cancer, denoted by Gleason pattern 3, and positive for the poorly differentiated cancer, denoted by Gleason pattern 5. (C) qRT-PCR analysis of the adenocarcinoma tissues showed that miR-126 and miR-149 are highly expressed in Gleason pattern 5.

was not statistically significant (Fig. 4C). Thus, it is possible that poorly differentiated adenocarcinoma in the prostate cancer cells contains syndecan-1, miR-126 or miR-149 positive cells.

4. Discussion

miRNAs can act as cancer growth and invasion factors in the manner of tumor inducer and suppressor on the expression of factors related to growth and invasion. Some proteins are known to regulate the expression of miRNAs. In the present study, we show, for the first time, that miRNAs are involved in syndecan-1-related cell proliferation in prostate cancer. Syndecan-1 is expressed in various types of cancers including breast, gastric, and head/neck. It is also associated with poor prognosis of patients with malignancies [32–35]. In this study, syndecan-1 was weakly expressed in poorly differentiated cancer cells, but was undetected in well-differentiated cancer cells (Fig. 4). Recently, we found that the holoclone obtained from single cell culture of PC3 cells developed high expression of syndecan-1, and promoted the growth of cancer cells through p21 induction [36]. Thus, syndecan-1 plays an important role in the initiation and progression of prostate cancer cells.

Among the miRNAs controlled by syndecan-1, miR-126 is reported as an oncogenic miRNA, because it induces the proliferation of gastric cancer cells by suppressing the expression of SOX2 [24]. miR-126 is specific to endothelial cells and usually promotes angiogenesis by targeting SPRED1 and PIK3R2, which in turn inhibit VEGF signaling [37–39]. On the other hand, miR-126 has also been reported to be tumor-suppressive, because it inhibits tumor cell growth by targeting p85β in colon cancer cell lines and IRS-1 in HEK293 and MCF-7 cells [40,41]. Here, we demonstrate that miR-126 acts as an oncogenic miRNA by targeting SOX2 in prostate cancer cells. The expression of miR-149 is also correlated with that of syndecan-1 in the PC3 cells. Similar to miR-126, miR-149 also acts as an oncogene, and is elevated in progressive nasopharyngeal carcinoma [42]. The inhibition of syndecan-1 or miR-126 and miR-149 induced the expression of the stem cell-related factors, NANOG, Oct4, and SOX2, in the PC3 cells. The control of miR-126 and miR-149 through syndecan-1 did not affect the expression of other stem cell markers. SOX-2, NANOG, and Oct4 are expected to suppress cell proliferation, but not maintain the survival of stem cells in cancer. Although these factors are important in the maintenance of normal stem cells, they are regulated by syndecan-1 and miRNAs to control negatively in cell proliferation. We found that syndecan-1, miR-126, and miR-149 are expressed in the stage of stem cells and regulate cell proliferation. In addition, the inhibition of syndecan-1, miR-126, and/or miR-149 suppresses cell proliferation, induces cellular senescence, but not cell differentiation, through p21 expression. Therefore, it is evident that SOX2, NANOG, and Oct4 do not participate in the cell differentiation potency of the androgen-independent prostate cancer cells.

Syndecan-1 is up-regulated in prostate cancer cell lines, especially the androgen-independent PC3 cell line, which switched from being androgen-dependent to independent and acquired hormonal resistance [22]. Syndecan-1 expression was significantly higher in the PC3 cells than in the LNCaP cells, which are androgen-dependent. We used the PC3 cell line in this study, because it exhibited the highest expression of syndecan-1 among the three prostate cancer cell lines. Du145 cells also showed a similar expression pattern of SOX2, NANOG, Oct4, and the miRNAs, under conditions of syndecan-1 suppression. Although the precise association between the androgenic effects and miRNAs is unclear, it is possible that they may be common factors associated with the role of syndecan-1 in androgen-independent cells.

In this study, we identified one of the mechanisms involved in prostate cancer progression, which is mediated by syndecan-1 that acts in conjunction with miR-126 and miR-149, to regulate cell proliferation by controlling the expression of SOX2, NANOG, and Oct4. The present data demonstrates that these factors control cell proliferation through cellular senescence and not through stem cell maintenance.

Disclosure statement

The authors declare no conflict of interest.

Acknowledgments

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