MicroRNA-21 regulates TGF-β-induced myofibroblast differentiation by targeting PDCD4 in tumour-stroma interaction

Qin Yao [1,2], Siyu Cao [1], Chun Li [1], Asferd Mengesha [1], Beihua Kong[3,*] ,Mingqian Wei [1,*]

[1] *Division of Molecular Medicine and Gene Therapies, School of Medical Science, Griffith University, Gold Coast campus, Southport, Qld, Australia*

[2] *Department of Obstetrics and Gynaecology, Affiliated Hospital of Qingdao University Medical Collage, Qingdao, Shandong, P.R. China*

[3] *Department of Obstetrics and Gynaecology, Qilu Hospital, Shandong University, Ji'nan, Shandong, P.R. China*

Running title: miR-21 regulates myofibroblast differentiation in tumor stroma

[*] Corresponding authors:

Professor Mingqian Wei, Division of Molecular Medicine and Gene Therapies, School of Medical Science, Griffith University, Gold coast campus, Southport, Qld 4222, Australia,

Fax: +61 7 55528908, E-mail address: m.wei@griffith.edu.au (MQ Wei).

Professor Beihua Kong, Department of Obstetrics and Gynaecology, Qilu Hospital, Shandong University, 107 Wenhuaxi Road, Ji'nan 250012, Shandong, P.R. China,

Fax: +86 531 82949225, E-mail address: kongbh2006@yahoo.com.cn (B. Kong).

Abbreviations: CLIC4, chloride intracellular channel 4; CM, conditioned media; miR-21,

microRNA-21; miRNAs, microRNAs ; MMPs, metalloproteinases ; MARCKS, myristoylated alanine-rich protein kinase c substrate; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homolog; α-SMA, α-smooth muscle actin; SMCs, smooth muscle cells; SPRY2, Sprouty2; TGF-β, transforming growth factor-β; TGF-β1 transforming growth factor-β1; TPM1, tropomyosin 1; VSMCs, vascular smooth muscle cells.

Article category: Cancer Cell Biology

Statement describing the novelty and impact of the paper

The paper addresses several important aspects of myofibrobalst transdifferentiation:

(1) Confirming transforming growth factor-β1 (TGF-β1) induced fibroblasts to myofibroblasts conversion;

(2) Detailing the involvement of microRNAs (miRNAs), ie.: miR-21 in myofibroblast transdifferentiation;

(3) Identifying miR-21 directly targets and down-regulates programmed cell death 4 (PDCD4) gene in myofibroblast differentiation.

Abstract

Transforming growth factor-β1 (TGF-β1) induces stromal fibroblast-to-myofibroblast transdifferentiation in the tumour-stroma interactive microenvironment via modulation of the expression of multiple phenotypic and functional genes and this plays a critical role in tumour progression. Up to now, the involvement of microRNAs (miRNAs) and the roles of these miRNAs in TGF-β1-induced myofibroblast differentiation in tumour-stroma interaction are unclear. Using quantitative real time RT-PCR, we demonstrated that the expression of microRNA-21 (miR-21) was up-regulated in activated fibroblasts after treatment with TGF-β1 or conditioned medium (CM) from cancer cells. To determine the potential roles of miR-21 in TGF-β1-mediated gene regulation during myofibroblast conversion, miR-21 expression was down-regulated by miR-21 inhibitor and up-regulated by miR-21 mimic. Interestingly, down-regulation of miR-21 with the inhibitor effectively inhibited TGF-β1-induced myofibroblast differentiation while up-regulation of miR-21 with a mimic significantly promoted myofibroblast differentiation. miR-21 directly targeted and down-regulated programmed cell death 4 (PDCD4) gene, which in turn acted as a negative regulator of several phenotypic and functional genes of myofibroblasts. Taken together, these results suggested that miR-21 participated in TGF-β1-induced myofibroblast transdifferentiation in cancer stroma by targeting PDCD4.

Key word: cancer; myofibroblasts; microRNA-21 (miR-21); programmed cell death 4 (PDCD4); transforming growth factor-β1 (TGF-β1)

Introduction

Increasing evidence has indicated that cancer development is facilitated by continuing interaction between tumour cells and activated stromal cells.[1] Fibroblasts and myofibroblasts are the major cell types in various human tumour stroma. These cells are receiving increasing attention because of their participation in tumour progression, including invasion,[2, 3] metastasis,[2] angiogenesis[4, 5] and the response to therapy[6, 7]. Myofibroblasts modulate the stroma in physiology and pathology via direct cell-cell contacts and/or secretion of a range of phenotypic and functional proteins. In general, stromal fibroblasts that are adjacent to cancer cell nests express various differentiation markers, such as the well-known differentiated marker α-smooth muscle actin (α-SMA), and thus are termed as myofibroblasts.[8, 9] α-SMA positive myofibroblasts do stimulate tumour invasion and angiogenesis whereas α-SMA negative fibroblasts do not promote such activities.[2, 5]

Although the origin of stromal myofibroblasts remains controversial, fibroblasts and bone marrow progenitor cells are considered to be the main precursor cells,[5, 10, 11] which are transdifferentiated into myofibroblasts by paracrine signals generated by cancer cells. Among these signals, transforming growth factor-β (TGF-β) is thought to be the most potent. TGF-β induced myofibroblast differentiation constitutes an important niche for tumour development through pro-invasion and pro-angiogenesis.[2-4] In three-dimensional co-culture model of human fibroblasts with human colon cancer cells HCT-8/E11, TGF-β is the dominant factor to mediate induction of myofibroblast differentiation, which then provides pro-invasive signals to cancer cells.[2] Moreover, it was found that cancer cells-derived TGF-β regulates angiogenesis

through the release of pro-angiogenic vascular endothelial growth factor A (VEGFA) from the activated fibroblasts in esophageal squamous cell carcinoma.[4]

MicroRNAs (miRNAs) are a class of endogenous, short (about 20-22-nucleotide), noncoding single-stranded RNA molecules, which negatively regulate gene expression through mRNAs degradation and/or translational inhibition of their target genes.[12] miRNAs involve in regulation of gene expression that control diverse physiological and pathological processes, such as development, cell differentiation, proliferation and apoptosis, migration, and carcinogenesis.[13-17]

miRNAs represent a new layer of gene expression regulators at the post-transcriptional levels,[18, 19] but the involvement of miRNAs and the roles of these miRNAs in TGF-β1-induced myofibroblast differentiation in tumour-stroma interaction are uncertain. In the current study, we found that miR-21 was up-regulated when transdifferentiation from fibroblasts to myofibroblasts was induced by TGF-β1 and conditioned medium (CM) from cancer cells. Gain-of-function and loss-of-function were applied to demonstrate that miR-21 played a critical role in the TGF-β1-induced myofibroblast transdifferentiation process by its target gene, programmed cell death 4 (PDCD4).

Material and methods

Cell culture and treatment

Ovarian cancer cell line OVCAR3, cervical cancer cell line Hela, Colon cancer cell line HCT116 and human fetal lung fibroblast cell line MRC-5 were originally purchased from ATCC (American Tissue Culture Collection), and are currently our own stock at Griffith Institute for Health and Medical Research, School of Medical Science, Griffith University. Human Ovarian cancer tissue specimens were collected from patients who underwent surgery at the Department of Obstetrics and Gynaecology, the affiliated hospital of Qingdao University Medical Collage, with informed consent and approval from the local Human Research Ethics Committee. Primary fibroblasts were isolated from the Ovarian cancers and cultured in DMEM (Invitrogen, San Diego, CA, USA) as described previously.[20] Briefly, following surgical resection, ovaries were washed twice with phosphate buffered saline (PBS) containing 100 units/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Ovarian surface epithelium (OSE) cells were scraped from the surface of the ovary. The layers of stromal cells adjacent to the OSE were isolated from areas of the ovary devoid of developing follicles. The stromal tissue pieces were minced and then digested with 0.1 mg/ml collagenase III (Invitrogen, San Diego, CA, USA) for 2 hours at 37°C. The purity of stromal cells were more than 98% with positive vimentin staining, whereas less than 2% positive keratin.[21] All experiments were performed on passages 3-8 primary fibroblasts. These cells were cultured in DMEM (Invitrogen, San Diego, CA, USA) containing 10% heat-inactivated fetal bovine serum (Invitrogen, San Diego, CA, USA), 100 units/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37°C in an atmosphere of 5% CO_2.

The conditioned medium (CM) was produced by culturing cancer cells in serum-free medium for 48h, which was then collected, centrifugated and stored at −20°C for future use.

When MRC-5 cells or human primary ovarian fibroblasts reached subconfluence, the medium was replaced by serum-free medium, human recombinant TGF-β1 with different

concentrations (PeproTech EC Ltd., London, UK) or CM from cancer cells.

Real-time RT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, San Diego, CA, USA). RNA was reverse transcribed using miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). The primers for mRNAs were designed as follows: α-SMA (accession no. NM_001613), 5′-AGGTAACGAGTCAGAGCTTTGGC-3′ (sense) and 5′-CTCTCTGTCCACCTTCCAGCAG-3′ (antisense); CD248 (accession no. NM_020404), 5'-GCAAGTGGCGAGCACCGCTGGCT-3' (sense) and 5'-GGCAGGCGCCCTCGAAGCCA-3′ (antisense); VEGFA (accession no. NM_001025366), 5′-CCTGGTGGACATCTTCCAGGAGTACC -3′ (sense) and 5′-GAAGCTCATCTCTCCTATGTGCTGGC-3′ (antisense); CLIC4 (accession no. NM_013943), 5′-CACGTAAATTTCTGGATGGCAATG-3′ (sense) and 5′-ATCACTGGGACAGGTATTGGTGAAC-3′ (antisense); SDF-1 (accession no. NM_000609), 5’-AGTCAGGTGGTGGCTTAACAG--3′ (sense) and 5’-GAGGAGGTGAAGGCAGTGG-3’ (antisense); GAPDH (accession no. NM_002046), 5′-CATGAGAAGTATGACAACAGCCT-3′ (sense) and 5′-AGTCCTTCCACGATACCAAAGT-3′ (antisense). The primers for miRNAs used in this study were designed by and purchased from Qiagen (Qiagen, Hilden, Germany). The quantification was performed with SYBR Green (Qiagen, Hilden, Germany). Each sample was analysed in duplicate using iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The comparative threshold cycle (CT) method was used to determine gene relative

expression. GAPDH was chosen as house keeping gene and used as an internal control for mRNA and U6 snRNA for miRNA.

Immunoblot assay

Cell lysates were collected, separated by SDS-PAGE, transferred to polyvinylidene difuoride membrane, and blocked with 5% milk in TBS + 0.05% Tween-20. Membranes were incubated with α-SMA, PDCD4 and α-tubulin (Abcam, Cambridge, MA, UK) monoclonal antibodies followed by secondary antibody conjugated with horseradish peroxidise (HRP) (Abcam, Cambridge, MA, UK). The enhanced chemiluminescence detection kit (Bio-Rad, Hercules, CA, USA) was used and the membranes were exposed to VersaDoc MP System (Bio-Rad, Hercules, CA, USA) for various time.

Immunofluorescence staining

The cells were fixed with ice-cold 1:1 methanol/acetone solution for immunofluorescence analysis. Afterwards, these sections and cells were blocked with 10% normal goat serum, incubated with mouse anti-α-SMA monoclonal antibody (Abcam, Cambridge, MA, UK) at 4°C overnight. After washing with PBS, the slides were incubated with FITC conjugated secondary antibody, nuclei with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). Images were collected on a Zeiss laser-scanning confocal microscope (Olympus, Center Valley, PA, USA).

Transfection of the miRNA mimic, miRNA inhibitor and siRNA

The miRNA mimic and miRNA inhibitor of miR-21, miR-130a used in this study were designed by and purchased from Qiagen (Qiagen, Hilden, Germany). MRC-5 fibroblasts were transfected with a final concentration of 5nM for miRNA mimic and 50nM for miRNA inhibitor using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA). The PDCD4 validated siRNA was purchased from Qiagen (Qiagen, Hilden, Germany). 16 hours after successful transfection, cells were incubated in serum-free medium with or without TGF-β1 or CM. Controls of the miRNA mimic scramble and miRNA inhibitor scramble used in this study were designed by and purchased from Ambion (Applied Biosystems, Foster, CA, USA).

Construction of 3'-untranslated region (UTR) reporter and luciferase assay

3'-UTR of PDCD4 fragments were PCR-amplified from human genomic DNA and inserted into the pGL3 control vector (Promega, Madison, WI, USA) at the XbaI site 3' of the luciferase gene. The primer sequences used for PCR amplification were as follows (restriction sites are underlined): 5'-GGGTCTAGAGACATTTTATAAACCTACAT-3' (sense); 5'-GGGAGATCTAATCAATACTGCTTCACATG-3' (antisense). MRC-5 cells were seeded in 96-well plates and transfected with 10nM miRNA mimic or 100nM miRNA inhibitor, 100ng of luciferase vector (pGL3 constructs), and 25ng of Renilla vector (pRL-TK) using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA). 24h after transfection, cells were harvested and luciferase activity was measured using the Dual-Glo luciferase assay (Promega, Madison, WI, USA).

Statistic analysis

Data were analysed with ANOVA, followed by Tukey's multiple comparison test or by Student's *t*-test, using Prism 5 (GraphPad software Inc., La Jolla, CA, USA). Differences between experimental groups were considered to be significant at *P*-value <0.05.

Results

TGF-β1 and conditioned medium (CM) from cancer cells induced transdifferentiation from fibroblasts to myofibroblasts

Differentiation from fibroblasts to myofibroblasts is induced by paracrine signals generated by cancer cells. TGF-β1 is the most potent factor among these signals, which drives the morphologic, phenotypic and functional changes associated with differentiation process.[22] We used a fibroblast-to-myofibroblast conversion culture model where human fibroblast MRC-5 cells or human primary ovarian fibroblasts were co-cultured with CM from OVCAR3 cancer cells or TGF-β1 to examine the modulation of phenotypic gene expression and morphology during myofibroblast transdifferentiation. As shown in Fig. 1A,B, the real time quantitative RT-PCR and immunoblot data provided evidence that the level of the myofibroblast marker α-SMA expressed in "activated" fibroblasts were up-regulated both at the transcriptional and translational levels compared with the untreated control. In addition, fibroblasts displayed a typical small spindle shape in serum-free medium. After incubation with CM from cancer cell or treatment with 5ng/ml TGF-β1 cells became bigger and polygonal indicating the initiation of myofibroblast transdifferentiation. (Fig. 1C).

miRNA-21 was up-regulated in TGF-β1- and CM-activated fibroblasts

We investigated the involvement of miR-21 in TGF-β1- and CM-stimulated myofibroblast conversion by comparing the relative abundance of miR-21 expression in vehicle-, CM- or TGF-β1-treated fibroblasts. A range of miRNAs expression levels in MRC-5 fibroblasts were measured by quantitative RT-PCR after 24h in the presence of 5ng/ml TGF-β1 or CM from OVCAR3 ovarian cancer cells (Fig. 2A). 4.1-and 5.8-fold increase of the level of miR-21 in fibroblasts was showed, respectively, after stimulation with TGF-β1 and CM from OVCAR3 ovarian cancer cell. Similarly, miR-21 expression in MRC-5 fibroblasts was significantly up-regulated after treatment with CM from HCT116 and Hela cancer cells (3.5- and 2.6-fold, respectively) (Fig. 2B). In addition, the expression of miR-21 in human primary ovarian fibroblasts increased 7.4- and 5.5- fold in the presence of CM from OVCAR3 ovarian cancer cells or TGF-β1 (Fig. 2B).

We next tested whether TGF-β1- or CM-induced miR-21 up-regulation was time- and dose-dependent. We observed a remarkable induction of mature miR-21 2h after stimulation, which peaked at the 24h after stimulation (Fig. 2C). Furthermore, TGF-β1 increased expression of miR-21 was dose-dependent in the range of from 0.5ng/ml to 5ng/ml (Fig. 2D). These time- and dose-dependent data convincingly showed that miR-21 was up-regulated in “activated” fibroblasts.

Regulation of miR-21 expression in fibroblasts

We used both gain-of-function and loss-of-function approaches to determine whether the

expression of miR-21 in fibroblasts was regulated. As shown in Fig. 3, miR-21 mimic significantly increased, but miR-21 inhibitor decreased miR-21 expression in fibroblasts. In contrast, there were no effects of their control scrambled oligos on miR-21 expression. Moreover, we did not observe the effects on other miRNAs such as miR-130a, demonstrating that the effects of both miR-21 mimic and inhibitor on miR-21 expression were specific.

The functional effect of miR-21 on TGF-β1-induced myofibroblast transdifferentiation

TGF-β1 and CM promote stromal myofibroblast differentiation, which plays an important role in tumour invasion,[2, 3] metastasis[2] and pro-angiogenesis[4, 5]. The function of miR-21 in myofibroblast conversion was investigated by transfecting fibroblasts with miR-21 inhibitor, a single-strain RNA oligonucleotide, which is complementary to miR-21 sequence and modified with 2'-O-methyl. Our results showed that miR-21 inhibitor specifically decreased miR-21 expression (Fig. 3B), and miR-21 inhibitor significantly reduced both TGF-β1- and CM-induced expression of myofibroblast phenotypic marker α-SMA (Fig. 4A, B). In gain-of-function experiments, miR-21 mimic-transfected fibroblasts induced the expression of miR-21 which up-regulated the level of α-SMA mRNA and protein expression (Fig. 4D,E).

In the current study, we also investigated whether miR-21 regulates the expression of other myofibroblast markers. Our results showed that a range of phenotypic and functional markers of cancer stromal myofibroblasts, including chloride intracellular channel 4 (CLIC4),[23, 24] CD248[25] and VEGFA[26], were induced to different extents at the transcriptional level. Moreover, the up-regulation could be inhibited by pre-treatment with miR-21 inhibitor (Fig. 4C).

Moreover, we further examined whether miR-21 regulated TGF-β-induced myofibroblast differentiation in ovarian tumor-stroma interaction using primary ovarian fibroblasts. As shown in Fig. 5A, the expression of miR-21 in human primary ovarian fibroblasts increased 7.4- and 5.5- fold after 24h in the presence of CM from OVCAR3 ovarian cancer cells or 5ng/ml TGF-β1. As expected, the use of specific miR-21 inhibitor could prevent the up-regulation of α-SMA expression in ovarian fibroblasts induced by CM from OVCAR3 ovarian cancer cells or TGF-β1 (Fig. 5B,C). In addition, the increased expression of other tumor stromal myofibroblast functional markers, VEGFA, CLIC4 and CD248, were also down-regulated by pre-treatment with miR-21 inhibitor (Fig, 5D). Taken together, these results suggested that miR-21 participated in the stromal myofibroblast conversion by negatively regulating the expression of phenotypic and functional genes of myofibroblasts.

PDCD4 was a functional target of miR-21 during myofibroblast conversion

Computer analysis shows that PDCD4 is a potential target gene of miR-21 (Fig. 6A). Previous reports showed that miR-21 targeted tumour suppressor gene PDCD4 and decreased its expression in cancer cells, thus promoted cell proliferation and invasion.[27, 28] Furthermore, miR-21 down-regulated PDCD4, which in turn negatively regulated smooth muscle contractile genes in human vascular smooth muscle cells.[29] Thus we hypothesised that PDCD4 might be the target gene of miR-21 in TGF-β1-induced myofibroblast transdifferentiation. Since TGF-β1 up-regulated miR-21 expression in fibroblasts, PDCD4 expression should be down-regulated after treatment with TGF-β1. To test the hypothesis, we incubated fibroblasts with TGF-β1 or vehicle control for 48h, the level of PDCD4 protein was determined by immunoblot. As

expected, treatment with TGF-β1 decreased the expression of PDCD4 protein (Fig. 5B). Next, we determine the function of PDCD4 in fibroblasts by knocking down the PDCD4 expression using specific PDCD4 siRNA. When the expression of PDCD4 was knocked down by 72%, there is an increased expression of myofibroblastic phenotypic and functional markers α-SMA and VEGFA by 5.1- and 1.1-fold, respectively (Fig. 6C). These results suggested that PDCD4 might be the potential target gene of miR-21 in TGF-β1-stimulated myofibroblast differentiation.

To further confirm PDCD4 as a target gene of miR-21 during TGF-β1-stimulated myofibroblasts transdifferentiation, we performed the miR-21 loss- and gain-of-function experiments in fibroblasts. As shown in Fig. 6D, reduction of miR-21 by the miR-21 inhibitor and enhanced expression of miR-21 by the miR-21 mimic increased or decreased PDCD4 protein expression, respectively. When TGF-β1 stimulated the up-regulation of miR-21 the PDCD4 expression was reduced in the translation level; however miR-21 inhibitor abolished the effect. To further investigate whether miR-21 directly bound to 3'-UTR of PDCD4 and inhibited PDCD4 expression in fibroblasts, the fragment of 3'-UTR of PDCD4 mRNA with the putative miR-21 binding sequence was cloned into a pGL3 vector at the downstream of the luciferase gene and co-transfected the pGL3 construct with either miR-21 mimic or miR-21 inhibitor into MRC-5 fibroblasts using Lipofectamine 2000. As expected, the miR-21 mimic increased the expression of miR-21 and inhibited the luciferase activity. On the contrary, the miR-21 inhibitor down-regulated the expression of miR-21, which in tern, up-regulated the luciferase activity (Fig. 6E). These results implied that miR-21 could bind to

3’-UTR of PDCD4 and inhibit its expression in fibroblasts.

Discussion

In the present study, we found that the up-regulation of miR-21 in fibroblasts was induced by TGF-β1 and CM from cancer cells, which is known to lead to myofibroblast transdifferentiation. Knockdown of miR-21 inhibited TGF-β1-induced myofibroblast transdifferentiation. The ectopic expression of miR-21 regulated the expression of several genes related to TGF-β1-induced myofibroblast transdifferentiation. Moreover, PDCD4 was negatively regulated by miR-21 in myofibroblast conversion. Thus, we demonstrated for the first time that miR-21 was regulated by TGF-β1 and played a pivotal role in myofibroblast transdifferentiation through targeting PDCD4.

miR-21 is the most consistently up-regulated microRNA in many cancer types. The oncogenic potential of miR-21 lies in its ability to regulate multiple cancer-associated pathways probably via multiple cellular targets. miR-21 can inhibit apoptosis through targeting both phosphatase and tensin homolog (PTEN)[30] and PDCD4[27, 31]. Additionally, miR-21 may target important tumour suppressor genes including tropomyosin 1 (TPM1)[32] and maspin (a serpin peptidase inhibitor)[33], Sprouty2 (SPRY2),[34] RECK (a membrane-anchored inhibitor of metalloproteinases (MMPs),[35] and MARCKS (myristoylated alanine-rich protein kinase c substrate),[16] suggesting that miR-21 also plays an important role in tumour invasion and metastasis. Interestingly, high level of miR-21 is not only a characteristic of cancer cells but also represents a common feature of pathological cell stress. miR-21 is up-regulated in

cardiac hypertrophy in vascular walls after balloon injury and in a variety of other human proliferative disorders, implying a function in regulating cell growth. miR-21 mediates induction of a contractile phenotype by TGF-β or bone morphogenetic proteins (BMPs)[29] and participates in ROS-mediated gene regulation in vascular smooth muscle cells (VSMCs)[36]. In our study, we found the miR-21 expression was very sensitive to TGF-β1 or CM stimulation. Two hours after treatment, miR-21 up-regulation was shown in a time-dependent manner. Moreover, TGF-β1 (0.5-5ng/ml) also increased the expression of miR-21 in a dose-dependent manner. We further tested whether miR-21 participated in gene modulation during myofibroblast conversion. Down-regulation of miR-21 expression inhibited TGF-β1-mediated myofibroblast conversion marker expression. In contrast, up-regulation of miR-21 promoted myofibroblast conversion. These results suggested that miR-21 had a pro-differentiational effect in TGF-β1-mediated myofibroblast conversion.

miRNAs regulate their biological functions through their multiple target gene expression. Target gene must be experimentally verified in experimental cells as the miRNA targets and functions are cell specific.[37] Computer analysis has indicated that PCDC4 is a miR-21 target gene (Fig.5A). Recently, miR-21 has been shown to target PDCD4 gene to promote cell proliferation in cancer cells.[31, 38] PDCD4 also is a target gene of miR-21 in VSMC, where it acts as a negative regulator of smooth muscles contractile genes.[29] In our study, knockdown of PDCD4 resulted in up-regulation of the myofibroblastic phenotypic and functional genes, α-SMA and VEGFA. To test whether PDCD4 is a miR-21 target gene during TGF-β1-induced myofibroblast transdifferentiation, we firstly showed that TGF-β1 decreased PDCD4

expression. In addition, using both gain-of-function and loss-of-function expression, we showed that the expression of PDCD4 in fibroblasts could be regulated by miR-21. Moreover, miR-21 inhibitor could inhibit TGF-β1-induced expression of myofibroblast marker α-SMA. Finally, miR-21 was able to decrease PDCD4 expression in fibroblasts directly using a construct which includes the putative miR-21 binding sequence in the 3'-untranslated region (UTR) of PDCD4 mRNA. These results convincingly indicated that PDCD4 was most likely a functional target gene of miR-21 that participated in myofibroblast conversion stimulated by TGF-β1 or CM from cancer cells.

In summary, this study demonstrated that miR-21 in fibroblasts was sensitive to the stimulation of paracrine factors from cancer cells, and participated in the myofibroblast transdifferentiation induced by TGF-β1 via its target gene PDCD4. Our results further contributed to the current knowledge on oncogenic potential of miR-21, which might not only involve in proliferation and mobility in cancer cells by its direct regulation of multiple genes associated with tumour suppression, pro-apoptosis and anti-invasion, but also promote the stomal myofibroblast transdifferentiation through tumour-stroma interaction reported here.

Acknowledgements

This study was supported by a Dr. Jian Zhou Smart State Fellowship (project No. 37274) from the Queensland Government, a grant from the Australian National Health and Medical Research Council/Cancer Council Australia (Queensland) (No. 401681) to MQW.

References

1. Tlsty TD, Coussens LM. Tumor stroma and regulation of cancer development. Annual review of pathology 2006;1:119-50.

2. Denys H, Derycke L, Hendrix A, Westbroek W, Gheldof A, Narine K, Pauwels P, Gespach C, Bracke M, De Wever O. Differential impact of TGF-beta and EGF on fibroblast differentiation and invasion reciprocally promotes colon cancer cell invasion. Cancer Lett 2008;266:263-74.

3. Casey TM, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Harlow S, Bunn JY, Weaver D, Muss H, Plaut K. Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-beta 1) increase invasion rate of tumor cells: a population study. Breast Cancer Res Treat 2008;110:39-49.

4. Noma K, Smalley KS, Lioni M, Naomoto Y, Tanaka N, El-Deiry W, King AJ, Nakagawa H, Herlyn M. The essential role of fibroblasts in esophageal squamous cell carcinoma-induced

angiogenesis. Gastroenterology 2008;134:1981-93.

5. Guo X, Oshima H, Kitmura T, Taketo MM, Oshima M. Stromal fibroblasts activated by tumor cells promote angiogenesis in mouse gastric cancer. J Biol Chem 2008;283:19864-71.

6. Chometon G, Jendrossek V. Targeting the tumour stroma to increase efficacy of chemo- and radiotherapy. Clin Transl Oncol 2009;11:75-81.

7. Sonnenberg M, van der Kuip H, Haubeis S, Fritz P, Schroth W, Friedel G, Simon W, Murdter TE, Aulitzky WE. Highly variable response to cytotoxic chemotherapy in carcinoma-associated fibroblasts (CAFs) from lung and breast. BMC Cancer 2008;8:364.

8. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nature reviews 2004;4:839-49.

9. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332-7.

10. Cat B, Stuhlmann D, Steinbrenner H, Alili L, Holtkotter O, Sies H, Brenneisen P. Enhancement of tumor invasion depends on transdifferentiation of skin fibroblasts mediated by reactive oxygen species. J Cell Sci 2006;119:2727-38.

11. Mishra PJ, Humeniuk R, Medina DJ, Alexe G, Mesirov JP, Ganesan S, Glod JW, Banerjee D. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. Cancer Res 2008;68:4331-9.

12. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-97.

13. Morton SU, Scherz PJ, Cordes KR, Ivey KN, Stainier DY, Srivastava D. microRNA-138 modulates cardiac patterning during embryonic development. Proc Natl Acad Sci U S A 2008;105:17830-5.

14. Hino K, Fukao T, Watanabe M. Regulatory interaction of HNF1-alpha to microRNA-194 gene during intestinal epithelial cell differentiation. Nucleic Acids Symp Ser (Oxf) 2007:415-6.

15. Corney DC, Flesken-Nikitin A, Godwin AK, Wang W, Nikitin AY. MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res 2007;67:8433-8.

16. Li T, Li D, Sha J, Sun P, Huang Y. MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells. Biochem Biophys Res Commun 2009;383:280-5.

17. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007;449:682-8.

18. Makeyev EV, Maniatis T. Multilevel regulation of gene expression by microRNAs. Science 2008;319:1789-90.

19. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. Cell 2008;132:9-14.

20. Parrott JA, Nilsson E, Mosher R, Magrane G, Albertson D, Pinkel D, Gray JW, Skinner MK. Stromal-epithelial interactions in the progression of ovarian cancer: influence and source of tumor stromal cells. Molecular and cellular endocrinology 2001;175:29-39.

21. Vigne JL, Halburnt LL, Skinner MK. Characterization of bovine ovarian surface epithelium and stromal cells: identification of secreted proteins. Biology of reproduction 1994;51:1213-21.

22. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer 2008;123:2229-38.

23. Yao Q, Qu X, Yang Q, Wei M, Kong B. CLIC4 mediates TGF-beta1-induced fibroblast-to-myofibroblast transdifferentiation in ovarian cancer. Oncol Rep 2009;22:541-8.

24. Suh KS, Crutchley JM, Koochek A, Ryscavage A, Bhat K, Tanaka T, Oshima A, Fitzgerald P, Yuspa SH. Reciprocal modifications of CLIC4 in tumor epithelium and stroma mark malignant progression of multiple human cancers. Clin Cancer Res 2007;13:121-31.

25. Christian S, Winkler R, Helfrich I, Boos AM, Besemfelder E, Schadendorf D, Augustin HG. Endosialin (Tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells. Am J Pathol 2008;172:486-94.

26. Shao J, Sheng GG, Mifflin RC, Powell DW, Sheng H. Roles of myofibroblasts in prostaglandin E2-stimulated intestinal epithelial proliferation and angiogenesis. Cancer Res 2006;66:846-55.

27. Hiyoshi Y, Kamohara H, Karashima R, Sato N, Imamura Y, Nagai Y, Yoshida N, Toyama E, Hayashi N, Watanabe M, Baba H. MicroRNA-21 regulates the proliferation and invasion in esophageal squamous cell carcinoma. Clin Cancer Res 2009;15:1915-22.

28. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008;27:2128-36.

29. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 2008;454:56-61.

30. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer.

Gastroenterology 2007;133:647-58.

31. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 2008;283:1026-33.

32. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007;282:14328-36.

33. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 2008;18:350-9.

34. Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H, Yan L, Malhotra A, Vatner D, Abdellatif M. MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. Mol Biol Cell 2008;19:3272-82.

35. Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS, Krichevsky AM. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 2008;28:5369-80.

36. Lin Y, Liu X, Cheng Y, Yang J, Huo Y, Zhang C. Involvement of MicroRNAs in hydrogen peroxide-mediated gene regulation and cellular injury response in vascular smooth muscle cells. J Biol Chem 2009;284:7903-13.

37. Zhang C. MicroRNomics: a newly emerging approach for disease biology. Physiol Genomics 2008;33:139-47.

38. Chen Y, Liu W, Chao T, Zhang Y, Yan X, Gong Y, Qiang B, Yuan J, Sun M, Peng X. MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G. Cancer Lett 2008;272:197-205.

Figure legends

Fig. 1 Phenotypic and morphological changes in TGF-β1 or CM from cancer cells induced fibroblast-to-myofibroblast transdifferentiation.

A,B. The expression of α-SMA at the transcriptional and translational levels after stimulating fibroblasts with 5ng/ml TGF-β1 or CM from OVCAR3 cancer cells for 48h. The expression of α-SMA was examined by real time RT-PCR and western blots in MRC-5 or human primary ovarian fibroblasts. (*$P<0.05$, compared with vehicle control, n=3)

C. Morphological alterations following stimulation of fibroblasts with TGF-β1 or CM from OVCAR3 cancer cells for 48h. Bar, 100μm.

Fig. 2 The expression of miR-21 was up-regulated in "activated" fibroblasts after treatment with TGF-β1 or CM from cancer cells.

A. The levels of miRNAs expression in MRC-5 fibroblasts treated with vehicle, TGF-β1 or CM from ovarian cancer cell OVCAR3 for 24h. (*$P<0.05$, compared with vehicle control, n=3)

B. Fibroblasts were treated with vehicle, 5ng/ml TGF-β1 or CM from ovarian cancer OVCAR3, cervical cancer Hela, and colon cancer HCT-116 for 24h. miR-21 levels in MRC-5 (left) and human primary ovarian fibroblasts (right) were determined by qRT-PCR. (*$P<0.05$, compared with vehicle control, n=3)

C. Time course of miR-21 expression in fibroblasts stimulated with TGF-β1 or CM from cancer cells OVCAR3.

D. Dose-dependent effect of TGF-β1 on miR-21 expression in fibroblasts stimulated with vehicle or TGF-β1 (0.1-10ng/ml) for 24h. (*$P<0.05$, compared with vehicle control, n=3)

Fig. 3 Modulated miR-21 expression in fibroblasts.

Fibroblasts were treated with vehicle, miR-21 inhibitor (50nM), miRNA inhibitor scramble (50nM), miR-21 mimic (5nM), or miRNA mimic scramble (5nM) for 8h. The levels of miR-21 expression were determined 24h later by qRT-PCR. The expression of miR-130a modulated by its mimic and inhibitor acted as control to value specific regulating effect. (*$P<0.05$, compared with vehicle control, n=3)

Fig. 4 The effect of miR-21 on TGF-β1- or CM- induced fibroblast-to-myofibroblast transdifferentiation.

A, B miR-21 inhibitor prevented up-regulation of myofibroblast marker α-SMA during fibroblast-to-myofibroblast conversion. (A) MRC-5 cells transfected with miR-21 inhibitor (50nM) or miRNA inhibitor scramble (50nM). Following treatment with TGF-β1 or CM for 48h, cells were stained by anti-α-SMA primary antibody and FITC-conjugated secondary antibody. Nuclei were visualized by DAPI staining. Bar, 50μm. (B) Total RNAs prepared from MRC-5 cells transfected with miR-21 inhibitor (50nM) or miRNA inhibitor scramble (50nM) were examined by qRT-PCR of α-SMA. (*$P<0.05$, compared with vehicle control, n=3)

C. MiR-21 inhibitor blocked the up-regulation of several myofibroblast markers during myofibroblast conversion. The expression levels of these markers were examined by qRT-PCR in fibroblasts treated with 5ng/ml TGF-β1 or treated with miR-21 inhibitor prior to TGF-β1.

D,E. miR-21 mimic up-regulated the expression of myofibroblast marker α-SMA in fibroblasts. (D) MRC-5 cells transfected with miR-21 mimic (5nM) or miRNA mimic scramble (5nM) was stained 48h later by anti-α-SMA primary antibody and FITC-conjugated secondary antibody. Nuclei were visualized by DAPI staining. Bar, 50μm. (E) Total RNAs prepared from MRC-5

cells transfected with miR-21 mimic (5nM) or miRNA mimic scramble (5nM) was examined by qRT-PCR of α-SMA. (*$P<0.05$, compared with vehicle control, n=3)

Fig. 5. ……..

Fig. 6. PDCD4 was a target gene of miR-21 during fibroblast-to-myofibroblast transdifferentiation.

A. Computer analysis indicated the PDCD4 might be a target gene of miR-21.

B. TGF-β1 decreased PDCD4 expression in fibroblasts determined by western blots. Three independent experiments were performed.

C. MRC-5 fibroblasts were transfected with control siRNA or specific PDCD4 siRNA. Following treatment with vehicle or TGF-β1 for 48h, the expression of PDCD4, α-SMA and VEGFA was measured by qRT-PCR. (*$P<0.05$, compared with vehicle control, n=3)

D. miR-21 inhibitor increased PDCD4 expression, whereas miR-21 mimic decreases PDCD4 expression in fibroblasts treated with vehicle or TGF-β1 determined by western blots. Three independent experiments were performed.

E. miR-21 was able to bind to PDCD4 in MRC-5 cell. A construct containing a fragment of the 3'-UTR of PDCD4 mRNA with the putative miR-21 binding sequence was cloned into a luciferase reporter construct (pGL3 construct) and transfected into MRC-5 cells with vehicle, oligo control (50nM), miR-21 mimic (10nM) or miR-21 inhibitor (100nM) and 100ng of

luciferase vector and 25ng of *Renilla* vector (pRL-TK). miR-21 mimic significantly inhibited luciferase activity, but miR-21 inhibitor increased luciferase activity in fibroblasts treated with serum-free medium or TGF-β1. (*$P<0.05$, compared with vehicle control, n=3)