miR-200c Prevents TGF-β1-Induced Epithelial-to-Mesenchymal Transition and Fibrogenesis in Mesothelial Cells by Targeting ZEB2 and Notch1

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Peritoneal fibrosis and loss of transport function is a common complication contributing to adverse outcomes in patients on long-term peritoneal dialysis (PD). Epithelial-to-mesenchymal transition (EMT) in mesothelial cells is a salient feature, but its triggering mechanisms remain obscure. Disregulation of microRNA (miR) expression is implicated in EMT and tissue fibrosis. We investigated the role of miR-200c in EMT and fibrogenesis in a murine PD model and in cultured peritoneal mesothelial cells. PD-fluid-treated mice showed peritoneal miR-200c expression reduced by 76.2% compared with PBS-treated mice, and this was accompanied by increased peritoneal miR-200c expression reduced by 76.2% compared with PBS-peritoneal miR-200c contributes to PD-associated peritoneal fibrosis and epithelial-to-mesenchymal transition (EMT) in mesothelial cells, accompanied by mesothelial denudation, increased matrix proteins in the submesothelium, and progressive subendothelial hyalinization of blood vessels with narrowing or obliteration of the vascular lumen. Also, TGF-β1 has been demonstrated as a key mediator of tissue fibrosis and EMT. However, the mechanisms leading to abnormalities in peritoneal mesothelial cells and the peritoneal membrane remain to be fully elucidated.

MicroRNAs (miRs) are a family of endogenous, short non-coding RNAs that regulate gene expression at the post-transcriptional level, either through mRNA destabilization or translational inhibition of their specific targets. The human miRome contains more than 2,500 miRs, each having various expression patterns, ranging from ubiquitous to highly tissue specific. Each miR can recognize over 100 target genes through sequence complementarity between the miR and binding sites in the 3’ UTR of target miRNAs. Dysregulated miR expression has been observed in pathological conditions, such as cancer and tissue fibrosis, and several miRs have been implicated in the regulation of EMT transcription factors, resulting in repression or promotion of EMT and fibrosis. In this respect, miR-21 has been shown to promote peritoneal fibrosis, whereas miR-15a-5p, miR-30a, miR-129-5p, and miR-302c have been shown to suppress peritoneal fibrosis.

The role of miR-200c in cancer and metastasis has been extensively studied, and the results showed that it controls EMT by targeting zinc finger E-box binding homeobox 1 (ZEB1), ZEB2, and Notch1, repressors of E-cadherin transcription that facilitate the dissolution of epithelial cell-cell contacts. The aim of this study was to...
investigate the role of miR-200c in peritoneal fibrosis and peritoneal mesothelial cell EMT, and also its target genes, in the context of PD. Importantly, our results show that conventional glucose-based PD fluid markedly reduced peritoneal miR-200c expression in C57BL/6N mice, and this was accompanied by increased α-smooth muscle actin, fibronectin, and collagen expression. PD fluid and TGF-β1 both reduced miR-200c expression in cultured mesothelial cells, and this was accompanied by E-cadherin downregulation and induction of the EMT transcription factors, fibronectin, collagen I, and collagen III. We also demonstrated that lentivirus-mediated miR-200c overexpression in mesothelial cells attenuated TGF-β1-induced EMT transcription factors and mesenchymal markers, and partially reversed the TGF-β1 effect of reducing E-cadherin expression. Luciferase reporter assays demonstrated that miR-200c directly targeted ZEB2 and Notch1, and indirectly targeted Jagged2. Overall, our results imply that miR-200c has an important role in the pathogenesis of PD-associated peritoneal fibrosis, through its protective effects on EMT and fibrogenesis in the peritoneum, mediated through specific gene targets.

RESULTS

PD Fluid Decreased Peritoneal miR-200c Expression

We examined peritoneal expression of 6 miRs in a murine PD model. These miRs were chosen based on their ability to regulate EMT in cancer cells or renal fibrosis, as reported in the literature. In PBS-treated mice, miR-29a, -192, -200b, -200c, -324, and -377 were all detected in peritoneal membrane specimens by real-time PCR, and the levels remained stable throughout the study period (Figure 1). In mice treated with PD fluid, miR-192 was reduced by 29.0% after 1 day, whereas miR-200c was reduced by 76.2% after 3 days of PD (p < 0.05 and p < 0.001 respectively, compared with PBS-treated mice). The suppressive effect of PD fluid on miR-29a, -200b, -324, and -377 was evident after 5 days, with 35.1%, 44.0%, 44.3%, and 39.8% reduction, respectively; 32.9 and 22.7 for miR-200b and U6, respectively; 33.7 and 22.6 for miR-200c and U6, respectively; 29.2 and 22.6 for miR-324 and U6, respectively; and 35.2 and 22.5 for miR-377 and U6, respectively, for PBS-treated mice on day 1. *p < 0.05, **p < 0.01, and ***p < 0.001, PBS versus PD fluid for the same time point; ****p < 0.001, compared to day 1 for the same treatment. Data analyzed by using ANOVA.
expression was increased in the mesothelium, and a marked increase in α-smooth-muscle-actin-positive cells showing an elongated appearance was noted in an intense collagen-rich submesothelium (Figure 2B).

**Effect of Glucose-Based PD Fluid and TGF-β1 on Mesothelial Cell miR-200c Expression**

miR-200c was constitutively expressed in mesothelial cells. Incubation of mesothelial cells with 1.36% glucose-based (Dianeal®) PD fluid for 24 h decreased miR-200c expression by 21.8 ± 4.5% compared to serum-free medium (SFM) (p < 0.001) (Figure 3A). TGF-β1 has been reported to contribute to PD-associated peritoneal fibrosis in mice. Exposure of mesothelial cells to TGF-β1 decreased miR-200c expression in a dose-dependent manner, with 40.7% reduction at 10 ng/mL TGF-β1 compared with SFM (p < 0.001). Repression of miR-200c was accompanied by a significant decrease in E-cadherin and decorin expression and increased expression of mesenchymal markers ZEB2, Notch1, Jagged2, SNAIL, fibronectin, collagen I at transcription and/or translation (Figures 3, 4, 5, and 6). TGF-β1 had no effect on vimentin expression (data not shown). The induction of EMT transcription factors and mesenchymal markers by TGF-β1 was apparent after 24 h, but the effect of TGF-β1 on phenotypic changes in mesothelial cells was observed only after 48 h (Figure 3J). Decorin has been shown to inhibit the profibrotic effects of TGF-β1. Incubating the cells with decorin prior to stimulation with TGF-β1 attenuated the suppressive effect of TGF-β1 on miR-200c expression (Figure 3B).

**Overexpression of miR-200c Represses Mediators of EMT and Fibrogenesis in Mesothelial Cells**

Mesothelial cells transfected with scrambled control hairpin (lenti-scramble) showed expression of miR-200c, E-cadherin, ZEB2, Notch1, Jagged2, SNAIL, fibronectin, collagen I, and collagen III similar to that of non-transfected cells (Figures 5 and 6). Primary human peritoneal mesothelial cells transfected with lentivector-based
E-cadherin

Relative expression compared to SFM

SFM TGF-β1

P = 0.016

SNAIL

Relative expression compared to SFM

SFM TGF-β1

P = 0.008

FSP-1

Relative expression compared to SFM

SFM TGF-β1

P = 0.016

α-SMA

Relative expression compared to SFM

SFM TGF-β1

P = 0.10

Fibronectin

Relative expression compared to SFM

SFM TGF-β1

P = 0.008

Collagen I

Relative expression compared to SFM

SFM TGF-β1

P = 0.016

Decorin

Relative expression compared to SFM

SFM TGF-β1

P = 0.008

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miR precursor constructs (lenti-miR-200c) showed a 106.7 ± 10.1- and 113.9 ± 4.6-fold increase of miR-200c expression when compared with non-transfected and lenti-scramble-transfected cells, respectively (p < 0.001, for both; Figure 5A); and lenti-miR-200c increased miR-200c expression in Met-5A cells by 414.7 ± 65.5- and 419.8 ± 74.5-fold, respectively (p < 0.001, for both; Figure 5B). miR-200c overexpression had no effect on cell morphology (data not shown) or constitutive gene and protein expression of E-cadherin, decorin, EMT transcription factors, or fibronectin, but significantly decreased collagen I and III expression (Figures 5 and 6; Table 1). In cells stimulated with TGF-β1, miR-200c overexpression attenuated TGF-β1-induced ZEB2, Notch1, Jagged2, SNAIL, fibronectin, and collagen I and III expression and partially reversed the suppression of E-cadherin transcription but not translation, by TGF-β1 (Figures 5 and 6).

miR-200c Directly Targets ZEB2 and Notch1 but not Jagged2 in Mesothelial Cells

To identify putative direct targets of miR-200c, we searched the TargetScan database (ver. 7.2; http://www.targetscan.org/) for miR-200c target sites in mRNA sequences of known repressors of E-cadherin. TargetScan predicted ZEB2, Jagged2, and Notch1 as likely target genes, with their 3’ UTR containing 6, 1, and 1 binding sites, respectively, for miR-200c. To investigate targeting of these transcription factors by miR-200c, their miTarget 3’ UTR miR target cloned downstream of a firefly luciferase reporter gene, were co-transfected with lenti-miR-200c into mesothelial cells. miR-200c overexpression resulted in a 74.0%, 12.8%, and 46.5% reduction in ZEB2, Notch1, and Jagged2 3’ UTR-luciferase activity, respectively (Figures 7A–7C). To determine whether miR-200c regulated ZEB2, Notch1, and Jagged2 through direct interaction with its seed region within the 3’ UTR region of these target genes, luciferase reporter pEZX-MT06 vectors containing 3’ UTR miR targets (wild type) or their mutants were co-transfected with miR-200c mimic oligonucleotides or negative control (Scramble) into mesothelial cells. miR-200c mimic decreased luciferase activity for wild-type 3’ UTR of ZEB2 and Notch1 (44.30% and 20.50% reduction, respectively), whereas mutants in the seed regions attenuated this decrease (Figures 7D and 7E). miR-200c mimic showed similar reduction in luciferase activity in cells transfected with wild-type and mutant 3’ UTR of Jagged2, suggesting that the interaction of miR-200c with Jagged2 in mesothelial cells was indirect (Figure 7F).

DISCUSSION

Little is known about the expression and pathophysiological role of miRs in peritoneal mesothelial cells during PD. In this study, we demonstrated a significant reduction in peritoneal miR-29a, miR-192, miR-200b, miR-200c, miR-324, and miR-377 expression in the murine PD model, when the peritoneal membrane was exposed to conventional glucose-based PD fluids. Peritoneal miR-200c expression was reduced by 80% after 3 days of PD, whereas reduced expression of the other miRs (by 30%–45%) occurred later, but all within 10 days of commencing PD fluid exposure. miR-200b and miR-200c are members of the miR-200 family, and both have been shown to preserve E-cadherin expression in Madin-Darby canine kidney (MDCK) cells, murine mammary gland epithelial cells, and human breast cancer cells.18,27,28 These miRs have been postulated to target various transcription factors related to EMT and mediators of fibrosis. The present report is the first to demonstrate the role of miR-200c in peritoneal fibrosis, and the results suggest that glucose-based PD fluid initiates fibrogenesis through repression of miR-200c. In our study, although we did not detect significant peritoneal membrane thickening after 1 month of PD, marked differences were noted in PD-fluid-treated mice, with an increased number of elongated α-smooth-muscle-actin-positive cells in the collagen-rich submesothelium and increased fibronectin expression in the mesothelium, the latter indicating that mesothelial cells play an important role in peritoneal fibrogenesis. That peritoneal membrane thickening was not apparent after 1 month of PD is in agreement with published data, although other studies have shown that peritoneal thickening can be induced after 1 month.31,32 The mouse strain, volume of PD fluid injected, and frequency of administration per day may account for such differences. It is possible that a longer PD duration would further exacerbate fibrotic processes and induce peritoneal membrane thickening.

For the studies focusing on miR-200c, we used two sources of peritoneal mesothelial cells: primary peritoneal mesothelial cells isolated from overnight dialysate effluent from PD patients and Met-5A cells, which are normal mesothelial cells transfected with the pRSV-T plasmid. Met-5A cells are often used in studies related to PD, and the data obtained from Met-5A cells showed concordance with that from primary mesothelial cells.33,34 Our results showed that glucose-based PD fluid and TGF-β1 reduced miR-200c expression in cultured mesothelial cells and that this was accompanied by down-regulation of E-cadherin and decorin and induction of EMT transcription factors and α-smooth muscle actin, fibronectin, collagen I, and collagen III. Co-incubation with decorin attenuated the repression of miR-200c by TGF-β1. Decorin is a dermatan sulfate proteoglycan that possesses anti-fibrotic properties through sequestration of TGF-β1, thus inhibiting its biological activity.30,35 Decorin is the...
Figure 4. The Effect of TGF-β1 on Protein Expression of Mediators of EMT and Fibrosis in Mesothelial Cells
(A) Representative Western blots showing the effect of TGF-β1 on E-cadherin, SNAIL, FSP-1, α-smooth muscle actin (α-SMA), fibronectin, and collagen I expression in Met-5A cells. The intensity of the bands for (B) E-cadherin, (C) SNAIL, (D) FSP-1, (E) α-SMA, (F) fibronectin, and (G) collagen I was normalized to β-actin and expressed as relative expression compared to serum-free medium (SFM). (H) The effect of TGF-β1 on decorin secretion in mesothelial cells. *p < 0.05, **p < 0.01, and ***p < 0.001, with versus without TGF-β1. Data analyzed by using ANOVA.
predominant proteoglycan secreted by cultured human peritoneal mesothelial cells. Reduced peritoneal decorin expression has been observed in patients on long-term PD, and dialysate decorin level declines with increasing time on PD (unpublished data).

TGF-β1 induced mesenchymal markers and transcription factors of EMT in mesothelial cells after 24 h of exposure, but phenotypic changes were evident only after 48 h, showing a loss of epithelial morphology, cell detachment, and acquisition of a fibroblastic appearance. In the absence of TGF-β1, lentivector-mediated miR-200c overexpression did not affect constitutive expression of EMT transcription markers or fibronectin. In contrast, cells with miR-200c overexpression showed less induction of ZEB2, Notch1, Jagged2, SNAIL, fibronectin, collagen I, and collagen III by TGF-β1. miR-200c overexpression was associated with reduced suppression of gene expression, but not protein expression of E-cadherin by TGF-β1. Possible explanations regarding the discrepancy between gene and protein expression of E-cadherin include variation in the duration of experiment, so that the effect of miR-200c becomes statistically significant, and also additional mechanisms that modulate the protein level of E-cadherin in addition to its gene expression. The impact of miR-200c on cellular changes following TGF-β1 exposure was not mediated by decorin, as miR-200c overexpression had no effect on decorin expression.

Using luciferase reporter assays, we further demonstrated that miR-200c directly targeted the 3' UTR of ZEB2 and Notch1, whereas the effect of miR-200c on the 3' UTR of Jagged2 appeared to be indirect. The data on ZEB2 is consistent with previous findings in MDCK cells stimulated with TGF-β1 and in murine NMuMG mammary epithelial cells. It has been suggested that miRs with perfect or near-perfect complementarity can induce mRNA degradation, whereas imperfect complementarity is associated with translational repression. In this respect, the perfect complementarity between the seed sequence of miR-200c and the 3' UTR of ZEB2 suggests the former mechanism of regulating gene expression. The ability of miR-200c to target E-cadherin repressors ZEB1 and -2 is well established in a number of epithelial-derived cancer cell types. Independent researchers have also identified Notch1 as a target of miR-200c in pancreatic cancer cells and endothelial cells. Notch with its ligand Jagged has been reported to promote EMT through
such as collagen and laminin. Reducing scaffold for the deposition of other extracellular matrix components, fibroblast deposition during tissue genes. Our results showed that repression of ZEB2 by miR-200c followed SNAIL activation and is associated with repression of epithelial markers and polarity genes, and activation of mesenchymal genes. In addition to mesothelial cells, other cell types, such as cancer cell lines. Fibronectin is one of the first matrix proteins to be deposited during tissue fibrosis, and it serves as a provisional scaffold for the deposition of other extracellular matrix components, such as collagen and laminin. Reducing fibronectin deposition by peritoneal mesothelial cells may have therapeutic implications on peritoneal submesothelial thickening commonly observed in patients on PD.

Other investigators have reported miR-200c expression in dialysate-derived mesothelial cells, which decreased with increasing time on PD, but in another study, investigators using a hybridization array found low signal intensity of miR-200c expression in omentum-derived mesothelial cells that did not change upon stimulation with TGF-β1. The discrepant findings may be related to the source of mesothelial cells used in the experiments, the dose of TGF-β1, and the methods used to detect miRs. miR-200a, a member of the miR-200 family, has also been detected in mesothelial cells and negatively regulates TGF-β1-induced EMT by targeting ZEB1 and -2. In addition to mesothelial cells, other cell types, such as fibroblasts and endothelial cells, may also participate in peritoneal fibrosis during PD through myofibroblast activation and EMT, respectively. The role of miRs in these processes requires further investigation.

To summarize, we have identified six miRs that demonstrate a significant preventive effect on EMT and fibrogenesis in peritoneal mesothelial cells subjected to chemical and metabolic injury inflicted by PD fluids that is mediated through TGF-β1. The expression of these miRs is markedly reduced within 1 week of commencing PD in the animal model tested, with the most profound reduction observed with miR-200c, which targets ZEB2 and Notch1 in mesothelial cells. Mechanistic studies showed that reduction of miR-200c was associated with decreased E-cadherin and decorin expression, and increased expression of EMT transcription factors and mesenchymal markers. The data provides evidence that loss of miR-200c is an important contributor to peritoneal fibrosis in PD, and results from miR-200c overexpression experiments suggest that replenishment of miR-200c could be a means to tackle this common PD complication which leads to adverse patient outcomes, but for which there is a total lack of therapeutic options.

**MATERIALS AND METHODS**

**Reagents, Chemicals, and Assay Kits**

Tissue culture flasks were purchased from Falcon (Becton-Dickinson, Gene Company, Hong Kong), and culture medium (Medium-199) and supplements, Trizol reagent, Lipofectamine, LTX reagent, and PLUS reagent were purchased from Life Technologies (Thermo Fisher Scientific, Hong Kong). Locked nucleic acid (LNA) probes were purchased from Exiqon (Genetex ExCell International Holdings, Hong Kong). Digoxigenin (DIG) labeling kits and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution were purchased from Roche Diagnostics (Hong Kong).

**Table 1. Effect of miR-200c Overexpression on Mediators of EMT and Fibrosis in Mesothelial Cells**

| Marker | SFM | TGF-β1 |
|--------|-----|--------|
|        | Non-transfected Mesothelial Cells | Transfected with Lenti-Scramble | Transfected with Lenti-miR-200c |
| E-cadherin | 1.01 ± 0.02 | 0.96 ± 0.07 | 1.09 ± 0.07 |
| ZEB2 | 1.06 ± 0.07 | 0.99 ± 0.1 | 1.08 ± 0.06 |
| Notch1 | ND | ND | ND |
| Jagged2 | ND | ND | ND |
| Fibronectin | 1.0 ± 0.01 | 1.07 ± 0.09 | 0.96 ± 0.04 |
| Collagen I | 1.37 ± 0.19*** | 1.26 ± 0.15*** | 0.96 ± 0.06 |

Data expressed as mean ± SEM from 3 separate experiments. ND, not detected. ***p < 0.001, SFM versus TGF-β1 for the same transfection; ***p < 0.01, non-transfected or lenti-scramble versus lenti-miR-200c for the same stimulation. Data analyzed by ANOVA.
Position 455-462 of ZEB2 3'-UTR
hsa-miR-200c-3p

Position 777-783 of JAG2 3'-UTR
hsa-miR-200c-3p

Position 736-743 of Notch1 3'-UTR
hsa-miR-200c-3p

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Kong). Pre-hybridization buffer, PerfectHyb Plus Hybridization buffer, mouse anti-human fibronectin, mouse anti-human z-smooth muscle actin, and mouse anti-human β-actin antibodies were purchased from Sigma Aldrich Co. (Tin Hang Technology, Hong Kong). Met-5A cells were purchased from the American Type Culture Collection (Tin Hang Technology, Hong Kong). Lentivector-based miR precursor constructs expressing miR-200c (PMIRH200cPA-1) and scrambled control hairpin (PMIRH000PA-1), the pPACKH1 lentivector packaging kit, PureFecction reagent, PEG-it Virus Precipitation Solution, Global UltraRapid Titer kits, and Transdux reagent were purchased from System Biosciences (Genet-imes ExCell International Holdings, Hong Kong). miTarget 3’ UTR miR target clones for ZEB2 (HmiT054256-MT01), Notch1 (HmiT011873-MT01), and Jagged2 (HmiT067341-MT01), pEZX-miR target clones for ZEB2 (HmiT054256-MT01), Notch1 (Hs00195591_m1), FSP-1 (Hs00243202_m1), Notch1 (Hs01062011_m1), Jagged2 (Hs00171432_m1), SNAIL probes for E-cadherin (Hs01023894_m1), ZEB2 (Hs00207691_m1), Transcription kits, Taqman microRNA assay, and Taqman from GenePharma (Shanghai, China). Taqman MicroRNA Reverse 200c-3p mimic and negative control (Scramble) were purchased chased from Beijing Genomics Institute (Hong Kong). Hsa-miR-200c panels (D–F) highlights the putative miR-200c binding sites that were deleted in mutant-type target gene 3 scrambled versus miR-200c mimic;#p < 0.05 and##p < 0.01, wild-type versus mutant for miR-200c mimic. Data analyzed by using ANOVA.

Peritoneal membrane histology obtained from PBS- and PD-fluid-treated mice was assessed by H&E, Masson’s trichrome, and cytochemical staining for z-smooth muscle actin and fibronectin. Peritoneal membrane thickening was determined at six different locations along the length of each specimen using Axiovision software (Carl Zeiss Far East, Hong Kong).

**LNA In Situ Hybridization**

Paraffin-embedded peritoneal specimens (5 μm) from control and PD-fluid-treated mice were used to determine miR-200c expression, using LNA in situ hybridization with a DIG-labeled LNA probe complementary to the miR-200c mature miR sequence. Briefly, deparaffinized sections were permeabilized by incubation with proteinase K (15 μg/mL) for 2 min, washed three times with diethyl pyrocarbonate (DEPC)-treated PBS, and acetylated for 10 min in 0.1 M triethanolamine (pH 8), containing 0.25% acetic anhydride. Sections were washed three times with DEPC-treated PBS, incubated in pre-hybridization buffer for 1 h at 42°C, and hybridized in PerfectHyb Plus Hybridization buffer containing DIG-labeled LNA probes (0.5 μM) overnight at 42°C. Sections were subsequently washed twice with 2× saline sodium citrate (SSC) buffer for 30 min at 42°C, twice with 1× SSC buffer for 10 min at 42°C, and twice with 0.5× SSC buffer for 10 min at 42°C. Sections were then blocked with 3% fetal bovine serum (FBS) in 0.1 M Tris (pH 7.5), containing, 0.15 M NaCl, and 0.1% Tween-20 (NTT) for 1 h and incubated with anti-DIG antibody (dilution 1:500) in NTT overnight at 4°C. The slides were washed three times with NTT followed by three washes for 5 min each with 0.1 M Tris (pH 9.5), containing 50 mM MgCl₂, 0.1 M NaCl, and 0.2% Tween-20, and the sections were developed by incubation with NBT/BCIP solution over 3 days.

**Collection of Spent PD Fluid**

Overnight PD fluid (1.35% glucose-based Dianee®) was collected from three stable PD patients who had been free of peritonitis for...
at least 6 months. PD fluid was centrifuged at 3,000 rpm at 4°C for 10 min, and the supernatant was collected, filter sterilized, and stored at −80°C until analysis. The collection of PD fluid was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. All patients were recruited from the PD Unit at Queen Mary Hospital.

Cell Culture
Immortalized human mesothelial cells (Met-5A cells) were cultured in Medium-199 supplemented with penicillin (100 IU/mL), streptomycin (100 mg/mL), insulin (5 μg/mL), transferrin (5 μg/mL), and 10% FBS (Complete Medium). Primary human peritoneal mesothelial cells were isolated from overnight 1.36%-glucose-based Dianeal® PD fluid obtained from patients who had been on PD for less than 2 years. Briefly, cells were centrifuged at 1,000 × g for 5 min, and pelleted cells seeded in 25 cm² flasks in Complete Medium. Only primary mesothelial cells with a polygonal, cobblestone appearance of the second passage were used, and mixed cell populations or senescent mesothelial cells were discarded.

miR-200c Target Analysis and Luciferase Reporter Assay
Using TargetScan version 7.2 (http://www.targetscan.org/), ZEB2 (target gene accession: NM_001171653.1), Notch1 (NM_017617.5), and Jagged2 (NM_002226.4) were predicted to be target genes of miR-200c. Mesothelial cells were transfected with miTarget ZEB2, Notch1, or Jagged2 3’ UTR constructs cloned downstream of the secreted Gaussia luciferase gene (1 μg), with or without lenti-scramble or lenti-miR-200c (1.4 μg), using Lipofectamine 2000 according to the manufacturer’s instructions. These miTarget constructs also contained the secreted embryonic alkaline phosphatase gene driven by a cytomegalovirus (CMV) promoter used as the internal control. After 5 h, the medium was replaced with Complete Medium and the cells incubated for 48 h, after which the supernatants were collected for analysis of secreted Gaussia luciferase and secreted embryonic alkaline phosphatase activities using the Luc-Pair miR Luciferase Assay kit according to the manufacturer’s instructions.

In a separate study, wild-type and mutant 3’ UTR of ZEB2, Notch1, and Jagged2 were cloned into the EcoRI and XhoI sites downstream of Renilla luciferase in a CMV-driven firefly/Renilla Duo-Luciferase reporter vector (pEZX-MT06). To investigate whether miR-200c directly targeted the 3’ UTR of ZEB2, Notch1, and Jagged2, 85%–95% confluent mesothelial cells were co-transfected with pEZX-MT06 reporter plasmids (0.4 μg) and either miR-200c mimic or negative control (0.4 μg) using Lipofectamine 2000 for 48 h, after which time, luciferase activities were determined using the Dual Luciferase Reporter Assay System. For ZEB2 mutant, seed sequences at positions 455–462, 805–812, 898–905, 1,029–1,036, and 1,363–1,369 were deleted. For Notch1 and Jagged2 mutants, seed sequences at positions 736–743 and 777–783, respectively, were deleted.

Real-Time PCR
Total RNA from peritoneal membrane specimens or mesothelial cells was extracted by using Trizol reagent according to the manufacturer’s instructions, and dissolved in RNase-free water. The yield and purity of the total RNA was determined with the Nanodrop system. To determine the expression of miR-29a, -192, -200b, -200c, -324, -1,036, and 1,363, total RNA (50 ng) was reverse transcribed with Taqman MicroRNA Reverse Transcription kits, according to the manufacturer’s instructions followed by real-time PCR, using Taqman microRNA assays. miRs expression was normalized to endogenous U6 snRNA expression.

To determine mRNA expression of mediators of EMT and fibrosis in mesothelial cells, total mRNA was extracted with RNeasy mini kits according to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed into cDNA with Moloney murine leukemia virus (MMLV) reverse transcriptase, by using the random hexamer method. The effect of TGF-β1 and miR-200c overexpression on E-cadherin, SNAIL, Notch1, ZEB2, Jagged2, FSP-1, α-smooth muscle actin, fibronectin, collagen I, collagen III, and decorin was assessed by quantitative real-time PCR on a Lightcycler 480 II real-time PCR system (Roche Diagnostics, DKSH Hong Kong, Hong Kong). All
samples were analyzed in triplicate, and mRNA expression of the aforementioned mediators of fibrosis was calculated using the delta Ct ($2^{-\Delta\Delta Ct}$) method, normalized to GAPDH.

**Western Blot**

Whole-cell lysates (20 µg total protein) were electrophoresed under denaturing conditions on 8% polyacrylamide gels to determine fibroconnectin and collagen I expression; on 12% polyacrylamide gels to determine E-cadherin, ZEB2, Notch1, Jagged2, vimentin, α-smooth muscle actin, and β-actin expression; and on 15% polyacrylamide gels to determine SNAIL expression. Proteins were transferred onto nitrocellulose membranes and immunoblotted with the relevant primary antibodies followed by the addition of secondary antibodies, as previously described. Bands were visualized with enhanced chemiluminescence (ECL), semi-quantitated by densitometry using ImageJ (NIH, USA), and expressed as arbitrary densitometric units (DU). All proteins of interest were normalized to β-actin.

**Measurement of Decorin in Culture Supernatant**

Decorin secretion in mesothelial cells cultured under control and experimental conditions was measured in culture supernatant using decorin DuoSet ELISA (R&D Systems, Bio-Technne Hong Kong, Hong Kong). The detection range was 31.2–2,000 pg/mL.

**Statistical Analysis**

Data are presented as means ± SEM of results from at least three separate experiments, unless otherwise mentioned. Data were analyzed by ANOVA with Bonferroni’s multiple-comparison post hoc test, Mann-Whitney or Kolmogorov-Smirnov test as appropriate, using GraphPad Prism version 6 for Windows, (GraphPad Software, San Diego, CA, USA). A two-tailed p < 0.05 was considered statistically significant.

**AUTHOR CONTRIBUTIONS**

Conceptualization: S.Y. and T.M.C.; Methodology: S.Y., J.Y.S.C., and A.C.P.T.; Investigation: J.Y.S.C., M.K.M.C., C.C.Y.C., A.C.P.T., and K.F.C.; Validation: J.Y.S.C., A.C.P.T., and S.Y.; Formal Analysis: A.C.P.T.; Investigation: J.Y.S.C., M.K.M.C., C.C.Y.C., A.C.P.T., and S.Y.; Funding Acquisition: S.Y. and T.M.C.; and Supervision: S.Y. and T.M.C.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

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