Transforming growth factor-β1 selectively inhibits hepatocyte growth factor expression via a micro-RNA-199–dependent posttranscriptional mechanism

Ognoon Mungunsukh and Regina M. Day
Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

ABSTRACT
Hepatocyte growth factor (HGF) is a multipotent endogenous repair factor secreted primarily by mesenchymal cells with effects on cells expressing its receptor, Met. HGF promotes normal tissue regeneration and inhibits fibrotic remodeling in part by promoting proliferation and migration of endothelial and epithelial cells and protecting these cells from apoptosis. HGF also inhibits myofibroblast proliferation. The profibrotic cytokine transforming growth factor β1 (TGF-β1) suppresses HGF expression but not the expression of NK2, an HGF splice variant that antagonizes HGF-induced proliferation. We investigated the mechanism for differential regulation of HGF and NK2 by TGF-β1. TGF-β1 down-regulated HGF in primary human adult pulmonary fibroblasts (HLFb) and increased the expression of miR-199a-3p, a microRNA (miRNA) associated with fibrotic remodeling. HGF and NK2 contain completely different 3′ untranslated regions (UTRs), and we determined that miR-199a-3p targeted HGF mRNA for suppression but not NK2. A pre–miR-199 mimic inhibited the expression of a luciferase reporter harboring the HGF 3′ UTR but not a pmirGLO reporter containing the NK2 3′ UTR. In contrast, an anti-miRNA inhibitor specific for miR-199a-3p prevented TGF-β1–induced reduction of both HGF mRNA and HGF protein secretion. Taken together, these findings demonstrate that HGF is distinctly regulated at the posttranscriptional level from its antagonist NK2.

INTRODUCTION
Hepatocyte growth factor (HGF), also known as scatter factor, is required for embryogenesis; in the adult, HGF plays a key role in tissue homeostasis and repair in many organs, including lung, heart, kidney, liver, skin, and brain (Igawa et al., 1993; Yanagita et al., 1993; Matsuda et al., 1997; Schaper and Kubin, 1997; Azuma et al., 2006; Iwasaki et al., 2006; Doeppner et al., 2011; Panganiban and Day, 2011). The HGF receptor, Met, is a tyrosine kinase receptor that is expressed primarily on epithelial and endothelial cells (Bottaro et al., 1991). On binding to the receptor, HGF induces a variety of signaling events leading to cellular proliferation, migration, morphogenesis, and cell survival (Matsumoto and Nakamura, 1991, 1997; Kagoshima et al., 1992; Aoki et al., 1997; Morishita et al., 1999; Stabile et al., 2006; Ishikawa et al., 2012). In animal models, administration of HGF protein or the use of HGF gene therapy promotes normal tissue repair and prevents fibrotic remodeling in a number of animal models for injury in the lung, liver, kidney, heart, and brain (Ueki et al., 1999; Nakamura et al., 2000; Mizuno et al., 2001; Umeda et al., 2004; Shimamura et al., 2006; Kitamura et al., 2007; Hegab et al., 2008). Accordingly, blockade of HGF signaling by administration of a neutralizing antibody can markedly exacerbate fibrotic tissue remodeling in vivo (Mizuno et al., 2000, 2001; Gong et al., 2003).

Full-length HGF is a multidomain protein, synthesized and secreted primarily by cells of mesenchymal origins as a biologically...
inert 90-kDa precursor that is converted extracellularly into the active form through a single cleavage by a tightly regulated serine protease (Miyazawa et al., 1989; Nakamura, 1989). The active form of HGF is a heterodimer consisting of disulfide-linked α and β chains (55–60 and 32–34 kDa, respectively). The α chain possesses the ability to bind to and activate the receptor and is composed of an amino-terminal hairpin loop (N) domain and four kringle domains (K1–K4). The β chain has strong homology to the protease domain of serine proteases, but does not elicit any biological activity on its own (Rubin et al., 1993).

Several alternatively spliced isoforms of HGF were identified in human cells (Miyazawa et al., 1991a). The NK1 variant contains the N-terminal hairpin and the first kringle domain; the NK2 variant extends through the second kringle (K2) domain. NK1 was demonstrated to induce motility, survival, and proliferation, although its potency is lower than that of full-length HGF (Cioce et al., 1996). In contrast, NK2 antagonizes the mitogenic activity of HGF that is pivotal for its antifibrotic function (Day et al., 1999; Tolbert et al., 2010). Transgenic mice overexpressing NK2 were more sensitive to carbon tetrachloride–induced liver fibrosis compared with wild-type mice (Hagawara et al., 2008).

In humans, the HGF gene is located on chromosome 7q21.1 and spans ~70 kbp of DNA and consists of 18 exons separated by 17 introns (Fukuyama et al., 1991; Miyazawa et al., 1991a). The full-length HGF precursor is encoded by all the exons forming ~3- and 6-kb mature mRNA (Nakamura et al., 1989; Miyazawa et al., 1991a) that differ only in the length of their 3′ untranslated regions (UTRs; 453 and 3575 nucleotides, respectively). The transcript variant 2 (~1.5 kb; Miyazawa et al., 1991b) that encodes NK2 is generated by alternative splicing accompanying the utilization of an alternative 3′ processing site in an extra exon localized within the intron 7–8. Therefore the first 1030 nucleotides (nt) of NK2, including the 5′ UTR, are identical to that of full-length HGF. However, the mature NK2 transcript lacks multiple 3′ exons but, due to the alternative exon splicing, it contains additional sequences encoding the last two carboxy-terminal amino acids accompanied by a totally different 3′ UTR.

The profibrotic cytokine transforming growth factor beta (TGF-β) inhibits HGF secretion by human lung fibroblasts (Matsumoto et al., 1992). TGF-β1 was also demonstrated to inhibit proliferation of pulmonary endothelial and epithelial cells, enhance extracellular matrix synthesis, stimulate fibroblast proliferation, and increase collagen formation (Broekelmann et al., 1991; Border and Noble, 1994; Panganiban and Day, 2011). In a human fetal lung fibroblast cell line, TGF-β1 was demonstrated to drastically down-regulate HGF gene expression without affecting NK2 (Harrison et al., 2000).

Although many features of an mRNA can contribute to its translation efficiency and half-life, most control elements are located within the UTRs. The 5′ m7GpppG cap and the 3′ poly(A) tail are important determinants of translational efficiency (Wilkie et al., 2003). The 5′ UTRs can contain sequences that function as binding sites for regulatory proteins. Similarly, 3′ UTRs contain numerous cis-acting elements, such as AU-rich elements (ARE) and micro-RNA (miRNA) target sites (Lai et al., 1999; Chen et al., 2001; Jing et al., 2005). Binding of various trans-acting proteins to these elements make a 3′ UTR an important regulatory unit that controls mRNA stability and/or translation efficiency. ARE elements usually target the mRNA for degradation (Chen et al., 2001). miRNAs are highly conserved endogenous regulatory molecules encoded by the genome and expressed in eukaryotic cells. After being processed to a 17- to 24-base, single-stranded mature miRNA, these molecules can bind to specific sites of mRNA and prevent protein expression, either by destabilizing the messenger or by blocking its translation (Jing et al., 2005).

In this study, we addressed the mechanism of TGF-β1 regulation that differentially modulates HGF versus NK2 mRNA levels. Our data provide the first indication that these two isoforms are regulated individually and differentially through their 3′ UTRs via an miR-119–dependent mechanism. Considering the importance of HGF in wound healing and tissue scarring, and the antagonizing activity of NK2 against HGF for epithelial cell proliferation, these results exposing the differential regulatory mechanisms for HGF versus NK2 will likely be important for the understanding molecular events underlying fibrotic remodeling of various tissues.

**RESULTS**

**TGF-β1 regulates HGF expression posttranscriptionally via the 3′ UTR**

Findings by others indicated that TGF-β1 increased the rate of degradation of full-length HGF mRNA without affecting the mRNA half-life of the truncated HGF isoform NK2 in MRC-5 cells, a normal human fetal lung fibroblast culture (Harrison et al., 2000). We investigated the mechanism by which TGF-β1 differentially regulates HGF and NK2 expression in human adult fibroblast culture (HLFb). In HLFb cells, TGF-β1 (10 ng/ml) induced extensive loss of HGF transcript within 6 h (~70% reduction, p < 0.05) that was sustained for at least 24 h (Figure 1, A and B). In contrast, the NK2 transcript level was slightly reduced at 6 h (~20%, p < 0.05) but recovered after 24 h of treatment (Figure 1, A and B). A similar effect was observed in neonatal foreskin fibroblast culture (NFF). In these cells, TGF-β1 reduced HGF mRNA levels by ~60% within 6 h; the reduction of HGF mRNA was sustained through 24 h (~80% reduction, p < 0.05). NK2 mRNA was not significantly affected by TGF-β1 treatment (Figure 1C).

Previous studies in fetal lung fibroblasts indicated that TGF-β1 inhibition of HGF expression did not involve promoter regulation (Harrison et al., 2000). Therefore we investigated mechanisms that govern posttranscriptional regulation. In the human cells, two transcripts (3 and 6 kb) for the full-length HGF were identified by Northern blotting (Harrison et al., 2000). Two splice variants were identified in GenBank (NM_000601, 2820 nt; NM_001010932, 2805 nt) that appeared to belong to the 3-kb transcript. Both variants shared the same 453 nucleotide (nt) 3′ UTR sequence. The 6-kb variant (ENST00000222390, 5989 nt) was the longest splice variant of HGF, containing an extra 3122 nt at the 3′ end of the coding sequence, in addition to the 453 nt 3′ UTR. Interestingly, this long transcript was not identified in adult HLFb after investigation using several PCR techniques utilizing different primer pairs (see Materials and Methods), suggesting that it is not produced in adult HLFb. Two NK2 mRNA variants were identified in GenBank, a 1307 nt transcript and a 1292-nt transcript (NM_001010931 and NM_001010933, respectively). The shorter variant lacked an internal in-frame segment, but both variants shared an identical 252-nt-long 3′ UTR. The HGF and NK2 3′ UTRs differ in their lengths as well as in sequence composition. The HGF 3′ UTR is derived from exon 18 of the HGF gene, and the NK2 3′ UTR is derived from an internal sequence contained within intron 7–8.

To determine the regulatory role of 3′ UTRs, we inserted the 3′ UTR of either HGF or NK2 downstream of a luciferase reporter gene in pmirGLO vector and transiently transfected HLFb. Luciferase activity was examined after TGF-β1 treatment (10 ng/ml, 24 h) and compared with untreated transfected cells (Figure 2). Reporter
A. mRNA level of HGF and NK2

B. mRNA level in HLFβ

C. mRNA level in NFF

FIGURE 1: TGF-β1 blocks expression of HGF, but not NK2. HLFβ were grown to confluence and placed in media containing 0.1% FBS overnight before treatment with TGF-β1 (10 ng/ml) for the indicated times. Untreated cells were used as controls. (A) Agarose (1.5%) gel electrophoresis of RT-PCR. Amplification of α-tubulin in the same sample was used as a loading control. (B and C) mRNA levels were determined by qPCR using the comparative threshold cycle method and expressed as fold differences relative to the untreated (without TGF-β1) samples. Endogenous levels of GAPDH were used for normalization. Data show means ± SEM; n = 3; *, statistical significance from untreated samples, p < 0.05.

TGF-β1 regulation of HGF involves miR-199

To identify regulatory elements responsible for the differential expression of HGF versus NK2 transcript, we first performed sequence analysis of the 3’ UTRs of HGF and NK2. An miRNA target site prediction tool (www.microrna.org) identified numerous potential target sites for various miRNAs. We were interested in miRNAs previously determined to be involved in fibrotic remodeling that might selectively target the 3’ UTR of either HGF or NK2. We identified a target site in the HGF 3’ UTR matching the seed region of an miR-199 (Figure 3A) that was also found to be up-regulated in patients with idiopathic pulmonary fibrosis (Pandit et al., 2010) and liver fibrosis (Murakami et al., 2011). We first investigated the regulation of miR-199a-3p by TGF-β1 in HLFβ. Quantitative PCR (qPCR) analysis showed that TGF-β1 significantly increased expression of miR-199a-3p in HLFβ within 6 h of treatment that was sustained through 24 h (Figure 3B).

We investigated the effect of miR-199a-3p on the luciferase reporter constructs using transient cotransfections of a control synthetic pre-miRNA or a synthetic mimic of the miR-199a-3p precursor molecule (pre-miR-199a-3p) in HLFβ (Figure 4). The control pre-miRNA had no significant effect on any of the reporter constructs. The pre-miR-199a-3p caused a ∼40% decrease in the activity of the reporter harboring the HGF 3’ UTR (Figure 4B), but the 3’ UTR activity of NK2 was not significantly affected by the control pre-miRNA or the pre-miR-199a-3p (Figure 4C). Interestingly, the synthetic pre-miR-199a-3p increased the activity of the control luciferase vector by ∼20%. Because a single microRNA can target multiple transcripts (Krek et al., 2005), it is possible that the pre-miR-199a-3p could indirectly increase transcription of the luciferase reporter by regulating other regulatory proteins. The specific mechanism for the observed increase is not known.

Through sequence analysis, we identified a heptamer sequence within the HGF 3’ UTR with perfect complementarity to the seed region (positions 2–8 at the 5’ end) of miR-199a-3p and sequences that support compensatory pairing to the 3’ end of this miRNA (Figure 5A). To confirm that this site is responsible for regulation of the HGF 3’ UTR, we replaced four of the base-pairing nucleotides in the HGF 3’ UTR with noncomplementary nucleotides using site-directed
A. 3’UTR sequence of full length HGF

[Sequence not visible]

B. Level of mature miR-199a-3p

![Graph showing fold difference for miR-199a-3p with and without TGF-β1](image)

FIGURE 3: Mir-199a-3p is a possible regulatory link between TGF-β1 signaling and suppression of HGF expression. (A) The HGF 3’ UTR sequence was analyzed for miRNA target sites. The miR-199a-3p site is printed in bold and underlined. (B) HLFB were treated with TGF-β1 (10 ng/ml) for the indicated times. miRNA-enriched total RNA was isolated, and the level of mature miR-199a-3p was determined by qPCR using TaqMan assays. Data are expressed as fold changes relative to the untreated controls. Endogenous levels of 18sRNA were used for normalization. *, statistical significance from untreated controls, p < 0.05; n = 3.

mutagenesis (Figure 5A). The pmirGLO reporter harboring the mutant 3’ UTR was then transfected into HLFB cells with the pre-miR-199a-3p, and luciferase activities of the reporter were measured. As expected, the pre-miR-199a-3p inhibited the activity of the reporter harboring the wild-type (HGF 3’ UTR WT) by -40% compared with the transfection with the pre-miRNA control (p ≤ 0.05). In contrast, reporter genes containing the mutant (HGF 3’ UTR mut) were not responsive to the miR-199a-3p mimic (Figure 5B), suggesting that the predicted miR-199a-3p site is functional.

Anti-miR-199a-3p prevents TGF-β1 inhibition of HGF expression

To verify the role of miR-199a-3p that leads to selective inhibition of full-length HGF by TGF-β1, we transfected HLFB with anti-miR-199a-3p and measured protein levels of HGF and NK2 secreted into the medium. As shown in Figure 6, transfection of anti-miR-199a-3p reversed TGF-β1 inhibition of HGF protein expression almost to the control level (Figure 6A). Commercially available enzyme-linked immunosorbent assays (ELISAs) are specific for detection of full-length HGF. We therefore used Western blotting to detect NK2 production, using an antibody that recognizes the N-terminal portion of HGF (N-17). In agreement with our mRNA findings, the level of NK2 protein was not affected by TGF-β1, and no significant changes were observed between transfected cells (Figure 6B).

Semiquantitative PCR results showed that TGF-β1 selectively inhibited HGF but not NK2 expression. This inhibition was reversed in cells transfected with anti-miR-199a-3p (Figure 6C). Neither mock transfection nor transfection with an anti-miR negative control altered the TGF-β1-induced decrease of HGF mRNA level. In contrast, and in agreement with our other results, NK2 mRNA level remained unchanged after TGF-β1 treatment. However, transfection of anti-miR-199a-3p slightly reduced NK2 mRNA level, but the degree of reduction was similar to that obtained by transfection with the nonspecific, anti-miR negative control (Figure 6D).

DISCUSSION

HGF is a multipotent factor that is produced and secreted by fibroblasts and acts as a mitogenic, morphogenic, and anti-apoptotic factor on cells expressing the Met receptor, including the endothelial and epithelial cells of most tissues. HGF expression is required for normal tissue repair and has been demonstrated to block fibrotic remodeling in the heart, lung, kidney, liver, and brain (Matsuda et al., 1995; Ueki et al., 1999; Ahmet et al., 2002; Watanabe et al., 2005; Doeppner et al., 2011). In contrast, NK2, a naturally occurring isoform of HGF, has been shown to antagonize the mitogenic activity of HGF (Day et al., 1999; Tolbert et al., 2010). In vivo, overexpression of NK2 was demonstrated to antagonize normal tissue repair and caused increased fibrosis (Hagiwara et al., 2008). The profibrotic cytokine TGF-β1 is dysregulated in fibrotic remodeling and specifically inhibits HGF expression with little or no effect on the expression of NK2 (Harrison et al., 2000). Thus, under conditions in which TGF-β1 down-regulates HGF expression, increased production of NK2 can prevent any remaining proliferative signaling of HGF that is pivotal for tissue regeneration after injury. In this paper, we provide evidence that TGF-β1 selective inhibition of HGF expression occurs through up-regulation of miR-199a-3p, an miRNA that binds to the HGF mRNA 3’ UTR, leading to degrada
tion of HGF mRNA. NK2 mRNA has an alternative 3’ UTR sequence not regulated by miR-199a-3p.

Our data indicate that the pre-miR-199a-3p induced a -40% decrease in the activity of the luciferase reporter via HGF 3’ UTR. This amount of decrease is in contrast with the amount of suppression observed for the endogenous HGF mRNA in response to TGF-β1 treatment in HLFB cells (≈70% suppression). One explanation for the difference between suppression observed in these two types of experiments is a potential limitation to the amount of down-regulation that can be generated in the luciferase reporter system; such a limitation could be due to transfection efficiency of the synthetic miR-199 relative to the amount of luciferase mRNA produced in our primary cell cultures. Alternatively, the endogenous HGF mRNA could be regulated by additional mechanism(s) by TGF-β1. A previous study in fetal lung fibroblasts indicated that TGF-β1 inhibition of HGF expression did not involve promoter regulation (Harrison et al., 2000).

In humans, altered miRNA expression has been demonstrated in fibrotic disorders of the lung, liver, and kidney (Papat et al. and Noveddine, 2012; Vettori et al., 2012). These changes have also been demonstrated in several animal models of fibrotic remodeling diseases (Thum et al., 2008; van Rooij et al., 2008; Liu et al., 2010). Of note, increased expression of miR-199a-3p was shown to occur in fibrotic tissues of lung and liver (Pandit et al., 2010; Murakami et al., 2011). TGF-β1 is a primary profibrotic factor, driving the transdifferentiation of normal fibroblasts to myofibroblasts (Willis and Borok, 2007; Biernacka et al., 2011). TGF-β1 induces increased proliferation,
migration, and the increased synthesis of extracellular matrix proteins, including collagens I and III of myofibroblasts (Desmouliere et al., 1993; Lijnen and Petrov, 2002; Willis and Borok, 2007).

TGF-β1–induced alterations in gene expression have been demonstrated to occur through transcriptional and posttranscriptional mechanisms. Most recently, TGF-β1–induced gene expression in pulmonary artery smooth muscle cells (PASMC) was shown to occur via regulation of specific miRNA maturation (Davis et al., 2008). The stem region of the primary transcripts of some microRNAs, including miR-199a, contain a conserved sequence similar to Smad-binding elements found in the promoters of TGF-β1–regulated genes (Davis et al., 2010). In PASMC, the binding of TGF-β1–activated Smad proteins to these sites facilitated recruitment of Drosha to these microRNAs and thereby promoted their maturation (Davis et al., 2008, 2010). We hypothesize that TGF-β1–induced increase in the level of mature miR-199a-3p may occur via this same mechanism in human adult fibroblasts.

In general, it is believed that complete complementation between miRNA and target mRNA sequence leads to mRNA degradation. In contrast, incomplete complementation between the miRNA and target mRNA sequence triggers silencing by prevention of translation (Bagga et al., 2005; Brennecke et al., 2005). We identified an miR-199a target site within the 3′ UTR of a full-length HGF transcript that contains a heptamer with perfect complementarity with the miRNA seed region. We demonstrated that an anti–miR-199a-3p effectively inhibited down-regulation of HGF mRNA by TGF-β1. On the basis of these findings, we hypothesize that regulation of the mRNA half-life is the primary function of miR-199a-3p on the HGF 3′ UTR.

Unlike the full-length HGF isoform, the truncated NK2 isoform remains protected from TGF-β1–induced down-regulation by miR-199a-3p. Others have also shown that transcript variants of a gene possessing nonoverlapping 3′ UTRs are regulated differentially at the posttranscriptional level. For example, neurotrophin-3 receptor NTRK3 has two isoforms, a full-length kinase-active form and a truncated noncatalytic form, each having different 3′ UTRs. The two

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**FIGURE 4:** An miRNA mimic of pre–mir-199a-3p inhibits 3′ UTR activity of HGF but not that of NK2. HLFb were transfected with either vector control (A) pmirGLO, (B) pmirGLO constructs possessing the 3′ UTR of HGF, or (C) 3′ UTR of NK2. Cells were either mock transfected (without miRNA) or transfected with a negative control pre-miRNA or with pre–miR-199a-3p. At 24 h posttransfection, cells were assayed for dual luciferase activity. Results were normalized to Renilla activity. Data show means ± SEM; n = 4; *, statistical significance from the controls (without RNA), p < 0.05.

**FIGURE 5:** The miR-199a-3p binding site in the HGF 3′ UTR is functional. (A) Predicted interaction of miR-199a-3p and human HGF 3′ UTR (HGF 3′ UTR WT) sequences. Point mutations (*) were introduced by site-directed mutagenesis to destroy the base-pairing with the miRNA. (B) HLFb were transfected with pre–miR-199a-3p 24 h prior to transfection of pmirGLO vectors, either possessing the wild-type (HGF 3′ UTR WT) or the mutant (HGF 3′ UTR mut) sequence. Next day, cells were harvested and assayed for dual luciferase activity. As controls, cells were either mock transfected (without miRNA) or transfected with a pre-miRNA negative control. Results were normalized to Renilla activity of the same vector. Data show means ± SEM; n = 4; *, statistical significance from controls, p < 0.05.
transcripts are targeted by different miRNAs, which results in different patterns of regulation (Guidi et al., 2010). Mayoral et al. (2009) also demonstrated that, in the mouse, the cell cycle inhibitor p27Kip1 exists as two different transcript variants containing alternatively spliced 3' UTRs, only one of which has the potential to be regulated by miR-221–222 (Mayoral et al., 2009). The NK2 isoform of HGF was first described in human cells. Interestingly, the murine gene does not contain the splice sites required for the generation of NK2 (Figure 7). Furthermore, rapid amplification of cDNA-3’ ends techniques in our laboratory were not able to isolate a murine NK2 orthologue (unpublished data). Sequence analysis using the Basic Local Alignment Search Tool (BLAST), revealed that only primate HGF sequences contain the splice sites necessary for the generation of NK2 variants, suggesting that the differential regulation of NK2 is an event unique to primate systems. To our knowledge, this is the first report of the primate-specificity of NK2 splicing. The biological function of NK2 that would convey an advantage for primates is currently unknown.

In conclusion, our findings show that HGF and its naturally occurring NK2 isoform are differentially regulated by a posttranscriptional mechanism involving an miR-199a microRNA. This finding extends the understanding of the molecular mechanisms underlying the fibrotic remodeling.

**MATERIALS AND METHODS**

**Reagents**

Fetal bovine serum (FBS; 100–106) was purchased from Gemini Bio-Products (Woodland, CA). Minimum essential medium (MEM), DMEM, Opti-MEM I reduced serum medium (Opti-MEM I), nonessential amino acids, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). Human TGF-β1 was purchased from Cell Signaling Technology (Danvers, MA) and was activated and stored in accordance with the manufacturer’s instructions.

**Cell culture**

Apparently healthy nonfetal HLFb were purchased from Coriell Cell Repositories (Camden, NJ). HLFb were cultured in MEM with 10% FBS and 1× nonessential amino acids. NFF were isolated from

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**FIGURE 6:** Effect of an miR-199a-3p inhibitor on HGF and NK2 expression. HLFb were transfected either with an anti-miRNA negative control (anti-mRNA NC) or with anti-miR-199a-3p. After 24 h, cells were placed in starvation media containing 0.1% FBS. Next day, cells were treated with TGF-β1 (10 ng/ml, 24 h). Nontreated cells were included as controls (without TGF-β1). (A) The level of HGF protein released into the medium was measured by HGF-specific ELISA. (B) Representative Western blot with anti-HGF (N-17) antibody. (C and D) HGF and NK2 mRNA levels were determined by semiquantitative RT-PCR and analyzed by agarose gel electrophoresis using band densitometry. Results were normalized to endogenous level of α-tubulin. Data show means ± SEM; n = 4; *, statistical significance from the anti-miRNA negative controls without TGF-β1; ‡, statistical significance from the anti-miRNA negative control with TGF-β1; p < 0.05.

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**TABLE 1**

| Condition                  | HGF Protein Level | NK2 Protein Level | HGF mRNA Level | NK2 mRNA Level |
|----------------------------|-------------------|-------------------|----------------|----------------|
| Anti-miRNA-NC              |                  |                   |                |                |
| Anti-miR-199-3p            |                  |                   |                |                |
| TGF-β1                     |                  |                   |                |                |
| Anti-miRNA-NC              |                  |                   |                |                |
| Anti-miR-199-3p            |                  |                   |                |                |
| TGF-β1                     |                  |                   |                |                |

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**A. HGF protein level**

**B. NK2 protein level**

**C. mRNA level of HGF**

**D. mRNA level of NK2**
foreskins of unidentified normal neonates. Briefly, tissues were treated overnight with dispase at 4°C. Epidermal sheets were separated from dermis, and the dermal layers were cut into small pieces and placed in DMEM with 10% FBS in culture dishes. Medium was changed twice weekly until the fibroblasts migrated out to cover the dishes. Cells were then harvested for passage. Passage 6–9 cells were used for all experiments. Cells were grown in 5% CO₂ at 37°C in a humidified atmosphere cell culture incubator.

**RNA isolation**

Total RNA was isolated from cells using an RNeasy Kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNasefree DNase set (Qiangen). RNA was quantified spectrophotometrically (ND-1000 Spectrophotometer; NanoDrop, Wilmington, DE), and integrity was assessed by capillary electrophoresis (Experion, Bio-Rad, Hercules, CA).

**Isolation of 3′ UTR sequences for human HGF transcript variants**

We amplified 3′ UTRs by PCR using the GC-rich PCR system according to the manufacturer’s protocol (Roche, Indianapolis, IN) with HLFb cDNA. To isolate the 3575-bp 3′ UTR of the 6-kb transcript, we used the following primer pairs: HGF3′F and HGF3′R (5′-gggctcgagCCTGAAGTAAGTGTGTCTGAAGCAC and 5′-gggctcagAGCATTAGTACGTTAAAAATTAGTTTATTTGTA, with uppercase bases corresponding to the human NK2 transcript and lowercase bases indicating 5′ extensions with restriction enzyme sites [bold] for Nhel and Xhol, respectively) were used to isolate 3′ UTR of NK2 transcript (252 bp). Thirty-five cycles of PCR with elongation time of 2 min per 1 kb at 58°C were performed to obtain 3′ UTR sequences. PCR products were analyzed by 1–2% agarose gel electrophoresis.

**Reporter gene constructs**

The 453-bp HGF or 252-bp NK2 3′ UTR were amplified by PCR, as described above; purified; and cloned into the Nhel and Xhol sites of pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI). Insertions were confirmed for accuracy by sequencing of both DNA strands.

**mRNA quantitation**

RNA (250 ng) was reverse-transcribed using GeneAmp RNA PCR kit, according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA).

For semiquantitative RT-PCR analysis, 2 μl of cDNA (5 ng RNA) was used for 20 μl PCR with either of the primer pairs HGF3′F and HGF3′R or NK2-3′F and NK2-3′R. Forty cycles of PCR with elongation time of 2 min at 58°C were performed using TaqDNA Polymerase (New England Biolabs, Beverly, MA). As loading control, an α-tubulin fragment was amplified by 29 cycles of PCR using the primers 5′-ctccatctcctacccaccaac and 5′-agggttacatttccacatttccacattt. Ten microliters of PCR was analyzed by 1.5% agarose gel electrophoresis, and subsequent band densitometry was performed using Wright Cell Imaging Facility (WCIF) ImageJ software (www.uhnresearch.ca/facilities/wcif/index.htm).

For qPCR analysis, 2 μl of cDNA (5 ng RNA) was used for 20 μl PCR using 6 μM of each primer and 10 μl of SybrGreen PCR master mix (Applied Biosystems). Primers for qPCR were designed using the ProbeFinder software, version 2.35 (Roche Applied Sciences, Indianapolis, IN). HGF3′-qF (5′-gATTTGATCGAGAACCATG TGAT) and

**FIGURE 7:** A schematic comparison of the human and mouse HGF genes. Bars and numbers indicate exons. Compared with the human gene, the murine orthologue has an extra noncoding exon (1) at the 5′ end. The human HGF gene contains an alternative exon located within the intron 7–8 (403 nt downstream of exon 7). The murine equivalent (intron 8–9) does not possess this exon. Sequence regions containing the splice donor (GT) and acceptor (AG) sites for NK2 splicing are shown in a magnified section. The carboxy-terminal amino acids of NK2 and the stop codon (*) are shown below the coding sequence.
HGF3′-qR (5′-CCATTCTCATTTATgTTgCTCA) primers were used for HGF-specific amplification. The nature of sequence identity (> 99%) between HGF and NK2 limited the options for NK2-specific primer design. Therefore we used the NK2-3′ UTR primers, NK2-3′F and NK2-3′R, for NK2-specific amplification. As an internal control, mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined using the primer pair 5′-agccacatcgctcataatgg or 5′-caattaaaacgtgcgctcacag. For quantification, the comparative threshold cycle method was used to assess relative changes in mRNA levels between the untreated and the TGF-β1-treated samples.

Site-directed mutagenesis

The 3′ UTR sequence of HGF was subcloned into pCR2.1-TOPO (Invitrogen) and used as template for the PCR. For introduction of point mutations, two complementary, mutagenic primers were designed: 5′-CACATTGACgcATCTAAcACAcCgCCAGGAGGCATGTTGGTTTG (forward) and 5′-CAAACAAACTGACTTCCgGTGGTGTTTAGcGTGGTAAgTG (reverse, with bold lowercase bases indicating mutant nucleotides). Ten nanograms of plasmid DNA and 125 ng of each primer were used for PCR (50 μl) with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer’s protocol. PCR conditions: an initial denaturation for 1 min at 95°C, followed by 18 cycles of 95°C for 50 s, 55°C for 50 s, and 68°C for 6 min, with a final extension of 7 min at 68°C. PCR product was visible after electrophoresis of 10 μl reaction using an agarose gel (1.5%). One microliter (10 μl) DpnI restriction enzyme was added directly to the PCR and incubated for 1 h at 37°C to digest parental DNA. One microliter of the DpnI-treated DNA was used to transform XL1-Blue supercompetent cells (Stratagene) according to the manufacturer’s protocol. Plasmids were isolated from positive colonies and sequenced to confirm mutations introduced.

Cell transfection and dual-luciferase assay

HLFb were seeded onto 12-well plates at 30% confluence. The next day, the medium was changed to Opti-MEM I, and cells were transfected with pmirGLO-3′ UTR constructs (0.5 μg per well) using the FuGENE 6 Transfection Reagent (3 μl per well), according to the manufacturer’s instructions (Roche). After 6 h, medium was replaced by MEM containing 10% FBS. For cell starvation, 24-h posttransfection medium was changed to MEM containing 0.1% FBS for overnight and treated with 10 ng/ml TGF-β1 (Cell Signaling Technology) for the indicated times or left untreated as control. Transfected cells were washed twice with cold PBS, lysed using 1× passive lysis buffer (Promega), and assayed for firefly and Renilla luciferase activities by the dual-luciferase assay (Promega), according to the manufacturer’s instructions, in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). At least three independent experiments were performed for each assay, each time with a minimum of n = 4. For cotransfection, cells were grown on 12-well plates. For each well, a transfection mixture (100 μl) consisting of 0.5-μg reporter construct, 30-pmole anti-miR miRNA inhibitor (AM11779, has-mir-199a-3p; Ambion, Austin, TX) or pre-miRNA miRNA precursor (AM11779, has-mir-199a-3p; Ambion), and 2 μl Lipofectamine 2000 Reagent (Invitrogen) was prepared. Opti-MEM I was used as diluent.

miRNA quantitation

Total RNA was isolated using mirVana miRNA Isolation Kit (Ambion). RNA (0.1 μg) was reverse-transcribed using hsa-miR-199a-3p–specific RT primer (TaqMan MicroRNA assay ID: 002304) and MuLV reverse transcriptase according to the TaqMan MicroRNA assays protocol (Applied Biosystems). The reaction was diluted subsequently five times with water, and 6.65 μl was subjected to qPCR (20 μl). PCR was performed in triplicate using TaqMan MicroRNA assay for hsa-miR-199a-3p (assay ID: 002304; Applied Biosystems). As internal control, levels of eukaryotic 18S RNA were determined using a TaqMan Endogenous Control Assay (4352930E; Applied Biosystems). For quantification, the comparative threshold cycle method was used to assess relative changes in miRNA levels between the untreated (control) and the drug-treated samples.

Western blots

For detection of cellular NK2, whole-cell lysates were prepared from cells grown on 35-mm dishes. Cells were washed once with cold PBS and resuspended in 60 μl of 1× SDS buffer (62.5 mM Tris-HCl, pH 6.8; 2% wt/vol SDS; 10% glycerol; and 0.01% bromophenol blue). The cell suspension was ultrasonicated for 5 s at 4°C using a Sonic Dismembrator at 50% output control (Model 500; Fisher Scientific, Pittsburgh, PA). Subsequently, samples were separated by SDS–PAGE (12%) and electrobolotted onto polyvinylidene fluoride membrane. Membrane was blocked with 1× PBS supplemented with 2.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Membranes were incubated with the primary antibody, anti-HGF (N-17, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA). For normalization of the results, membranes were rebotted for α-tubulin. Proteins were detected with horseradish peroxidase–linked secondary antibodies (anti-goat, 1:5000; R&D Systems, Minneapolis, MN) and SuperSignal West Dura Chemiluminescent Substrate (Pierce, Rockford, IL). WCIF ImageJ software was used for densitometry analysis.

Detection of HGF protein level

HLFb were seeded onto 6-well plates at 50% confluence. The next day, medium was changed to Opti-MEM I, and cells were transfected either with anti–miR-199a-3p (60 nM per well) or with anti-miR negative control using Lipofectamine RNAiMax (6 μl per well), according to the manufacturer’s instructions (Invitrogen). After 6 h, medium was replaced by MEM containing 10% FBS. For cell starvation, 24 h posttransfection medium was changed to MEM containing 0.1% FBS for overnight, and cells were treated with 10 ng/ml TGF-β1 for 24 h. Medium (50 μl) was assayed for HGF level using Human HGF Quantikine ELISA Kit according to the manufacturer’s instruction (R&D Systems). Nontreated cells were included as controls.

Rapid amplification of cDNA-3′ ends

For isolation of the 3′ UTR of NK2 in mice, total RNA was isolated from mouse lung using the RNeasy Kit (Qiagen) and reverse-transcribed as described above. The cDNA (5 μl) was subjected to 50 μl PCR using the FirstChoice RLM-RACE Kit according to the manufacturer’s protocol (Invitrogen) and the GC-rich PCR system (Roche). Mouse sequence–specific primers used were 5′-gaatgcatgacctg-caatgg or 5′-caattaaaacgtgcgctcagag. For quantification, the comparative threshold cycle method was used to assess relative changes in mRNA levels between the untreated (control) and the drug-treated samples.

Statistics

Means ± SEMs were calculated, and statistically significant differences between two groups were determined by Student’s t test. For three or more groups, statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Bonferroni postanalysis, as appropriate; p < 0.05 was considered statistically significant.
ACKNOWLEDGMENTS

We thank Thomas N. Darling (Department of Dermatology and Department of Anatomy, Physiology, and Genetics, Uniformed Services University of the Health Sciences, Bethesda, MD) and Rajesh Thangapazham (Department of Dermatology, Uniformed Services University of the Health Sciences) for providing human neonatal foreskin fibroblasts. This work was supported by a Uniformed Services University of the Health Sciences research grant to R.M.D. The authors are employees of the U.S. government, and this manuscript was prepared as part of their official duties. Title 17 U.S.C. §105 provides that “Copyright protection under this title is not available for any work of the United States Government.” Title 17 U.S.C §101 defined a U.S. that “Copyright protection under this title is not available for any work of the United States Government.”

REFERENCES

Ahner T, Sawa Y, Iwata K, Matsuda H (2002). Gene transfer of hepatocyte growth factor attenuates cardiac remodeling in the canine heart: a novel gene therapy for cardiomyopathy. J Thorac Cardiovasc Surg 124, 957–963. Aoki S, Takahashi K, Matsumoto K, Nakamura T (1997). Activation of Met tyrosine kinase by hepatocyte growth factor is essential for inherent organogenesis in Xenopus embryo. Biochem Biophys Res Commun 234, 8–14.

Azuma J, Taniyama Y, Takeya Y, Iekush K, Aoki M, Dosaka N, Matsumoto K, Nakamura T, Oghira T, Moshita R (2006). Angiogenic and antiﬁbrotic actions of hepatocyte growth factor improve cardiac dysfunction in porcine ischemic cardiomyopathy. Gene Therapy 13, 1206–1213.

Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122, 553–563.

Biernacka A, Dobaczewski M, Frangogiannis NG (2011). TGF-beta signaling in fibrosis. Growth Factors 29, 196–202.

Border W, Noble N (1994). Transforming growth factor β in tissue fibrosis. N Engl J Med 10, 1286–1292.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronsen SA (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 251, 802–804.

Brennan L, Stoff A, Rajka A, Cohen SM (2005). Principles of microRNA-target interaction. PLoS Biol 3, e85.

Broekelmann T, Limper A, Colby T, McDonald J (1991). Transforming growth factor-β occurs in scalp hair follicle tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122, 103–111.

Doepner TR, Kaltwasser B, ElAli A, Zechariah A, Hermann DM, Bahr M (2011). Acute hepatocyte growth factor treatment induces long-term neuroprotection and stroke recovery via mechanisms involving neural precursor cell proliferation and differentiation. J Cereb Blood Flow Metab 31, 1251–1262.

Fukuyama R, Ichijyo Y, Minoshima S, Kitamura N, Shimizu N (1991). Regional localization of the hepatocyte growth factor (HGF) gene to human chromosome 7 band q21.1. Genomics 11, 410–415.

Gong R, Rifai A, Tolbert EM, Centracchio JN, Dworkin LD (2003). Hepatocyte growth factor modulates matrix metalloproteinases and plasminogen activator/plasmin proteinolytic pathways in progressive renal interstitial ﬁbrosis. J Am Soc Nephrol 14, 3047–3060.

Guidi M, Munos-Gimeno K, Kagerbauer B, Marti E, Estvili X, Espinosa-Parrilla Y (2010). Overexpression of miR-128 speciﬁcally inhibits the truncated isoform of NTRK3 and upregulates BCL2 in SH-SY5Y neuroblastoma cells. BMC Mol Biol 11, 95.

Hagisawa S, Otsuka T, Yamazaki Y, Kosone T, Sohara N, Ichikawa T, Sato K, Kikizaki S, Takagi H, Mori M (2008). Overexpression of Nk2 promotes liver ﬁbrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 28, 126–131.

Harrison P, Bradley L, Bomford A (2000). Mechanism of regulation of HGF/SF gene expression in fibroblasts by TGF-beta1. Biochem Biophys Res Commun 271, 203–211.

Hegab AE, Kubo H, Yamada M, Asada M, He M, Fujino N, Mizuno S, Nakamura T (2008). Intranasal HGF administration ameliorates the physiological and morphologic changes in lung emphysema. Mol Ther 16, 1417–1426.

Igawa T, Matsumoto K, Kanda S, Saito Y, Nakamura T (1993). Hepatocyte growth factor may function as a renotopic factor for regeneration in rats with acute renal injury. Am J Physiol 265, F61–F69.

Ichikawa T, Factor VM, Marquardt JU, Raggi C, Seo D, Kitade M, Conner EA, Thorgerisson SS (2012). Hepatocyte growth factor/c-met signaling is required for stem-cell-mediated liver regeneration in mice. Hepatology 55, 1215–1226.

Iwasi T, Imado T, Kitanó S, Sano H (2006). Hepatocyte growth factor ameliorates dermal sclerosis in the tight-skin mouse model of scleroderma. Arthritis Res Ther 8, R161.

Jing Q, Huang S, Guth S, Zarubin T, Motosayá A, Chen J, Di Padova F, Lin SC, Gram H, Han J (2005). Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell 120, 623–634.

Kagoshima M, Kinoshita T, Matsumoto K, Nakamura T (1992). Developmental changes in hepatocyte growth factor mRNA and its receptor in rat liver, kidney, and lung. Eur J Biochem 210, 375–380.

Kitamura K et al. (2007). Hepatocyte growth factor promotes endogenous repair and functional recovery after spinal cord injury. J Neurosci Res 85, 2332–2342.

Krek A et al. (2005). Combinatorial microRNA target predictions. Nat Genet 37, 495–500.

Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, Blackshear PJ (1999). Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylon and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol 19, 1431–1433.

Lijnen P, Petrov V (2002). Transforming growth factor-beta1-induced collagen production in cultures of cardiac ﬁbroblasts is the result of the appearance of microﬁbroblasts. Methods Find Exp Clin Pharmacol 24, 333–344.

Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, Abraham E (2010). miR-21 mediates fibrogenic activation of pulmonary ﬁbroblasts. Methods Find Exp Clin Pharmacol 32, 55, 1215–1226.

Matsuda Y, Matsumoto K, Ichida T, Nakamura T (1995). Hepatocyte growth factor suppresses the onset of liver cirrhosis and abrogates lethal hepatic dysfunction in rats. J Biochem 118, 643–649.

Matsuda Y, Matsumoto K, Yamada A, Ichida T, Asakura H, Komoriya Y, Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects in rats of hepatocyte growth factor infusion on liver ﬁbrosis/cirrhosis. Hepatology 26, 81–89.

Metab 31, 1251–1262.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of hepatocyte growth factor on liver fibrosis after carbon tetrachloride-induced chronic liver injury. Hepatology 26, 81–89.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor. J Biol Chem 272, 13110–13115.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor on liver fibrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 17, 1417–1426.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor on liver fibrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 17, 1417–1426.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor on liver fibrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 17, 1417–1426.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor on liver fibrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 17, 1417–1426.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor on liver fibrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 17, 1417–1426.

Nishiyama E, Nakamura T (1997). Preventive and therapeutic effects of the human hepatocyte growth factor on liver fibrosis in carbon tetrachloride-induced chronic liver injury. Liver Int 17, 1417–1426.
Miyazawa K, Kitamura A, Naka D, Kitamura N (1991b). An alternatively processed mRNA generated from human hepatocyte growth factor gene. Eur J Biochem 197, 15–22.

Miyazawa K et al. (1989). Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem Biophys Res Commun 163, 967–973.

Mizuno S, Matsumoto K, Kurosawa T, Mizuno-Horikawa Y, Nakamura T (2000). Reciprocal balance of hepatocyte growth factor and transforming growth factor-β1 in renal fibrosis in mice. Kidney Int 57, 937–948.

Mizuno S, Matsumoto K, Nakamura T (2001). Hepatocyte growth factor suppresses interstitial fibrosis in a mouse model of obstructive nephropathy. Kidney Int 59, 1304–1314.

Morishita R et al. (1999). Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. Hypertension 33, 1379–1384.

Murakami Y, Toyoda H, Tanaka M, Kuroda M, Harada Y, Matsuda F, Tajima A, Kosaka N, Ochiya T, Shimotohno K (2011). The progression of liver fibrosis is related with overexpression of the miR-199 and 200 families. PloS One 6, e16081.

Nakamura T (1989). Molecular characterization of hepatocyte growth factor (HGF) [article in Japanese]. Seikagaku [J Japanese Biochem Soc] 61, 1243–1247.

Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T (2000). Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest 106, 1511–1519.

Pandit KV et al. (2010). Inhibition and role of let-7d in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 182, 220–229.

Rubin JS, Bottaro DP, Aaronson SA (1993). Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product. Biochim Biophys Acta 1155, 357–371.

Schaper W, Kubin T (1997). Is hepatocyte growth factor a protein with cardioprotective activity in the ischemic heart? Circulation 95, 2471–2472.

Shimamura M, Sato N, Waguri S, Uchiyama Y, Hayashi T, Iida H, Nakamura T, Ogihara T, Kaneda Y, Morishita R (2006). Gene transfer of hepatocyte growth factor gene improves learning and memory in the chronic stage of cerebral infarction. Hypertension 47, 742–751.

Stabile LP, Lyker JS, Land SR, Dacic S, Zamboni BA, Siegfried JM (2006). Transgenic mice overexpressing hepatocyte growth factor in the airways show increased susceptibility to lung cancer. Carcinogenesis 27, 1547–1555.

Thum T et al. (2008). MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456, 980–984.

Tolbert WD, Daugherty-Holtrop J, Gherardi E, Vandé Woude G, Xu HE (2010). Structural basis for agonism and antagonism of hepatocyte growth factor. Proc Natl Acad Sci USA 107, 13264–13269.

Ueki T et al. (1999). Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med 5, 226–230.

Umeda Y et al. (2004). Skeletal muscle targeting in vivo electroproporation-mediated HGF gene therapy of bleomycin-induced pulmonary fibrosis in mice. Lab Invest 84, 836–844.

van Rooij E, Sutherland LB, Thatcher JE, D’Maio JM, Naseem RH, Marshall WS, Hill JA, Olson EN (2008). Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105, 13027–13032.

Vettori S, Gay S, Distler O (2012). Role of microRNAs in fibrosis. Open Rheumatol J 6, 130–139.

Watanabe M et al. (2005). Hepatocyte growth factor gene transfer to alveolar septa for effective suppression of lung fibrosis. Mol Ther 12, 58–67.

Willke GS, Dickson KS, Gray NK (2003). Regulation of mRNA translation by 5’- and 3’-UTR-binding factors. Trends Biochem Sci 28, 182–188.

Willis BC, Borok Z (2007). TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 293, L525–L534.

Yanagita K, Matsumoto K, Sekiguchi K, Ishibashi H, Niho Y, Nakamura T (1993). Hepatocyte growth factor may act as a pulmotrophic factor on lung regeneration after acute lung injury. J Biol Chem 268, 21212–21217.