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

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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 beta 1 (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.**

Monitoring Editor
William P. Tansey
Vanderbilt University

Received: Jan 8, 2013
Revised: Apr 2, 2013
Accepted: May 2, 2013

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

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-01-0017) on May 8, 2013.

The authors have no conflicts of interests to report.

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Abbreviations used: ARE, AU-rich elements; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HGF, hepatocyte growth factor; HLFb, human adult fibroblast culture; miRNA, micro-RNA; NFF, neonatal foreskin fibroblast culture; nt, nucleotides; PBS, phosphate-buffered saline; qPCR, quantitative PCR; TGF-β, transforming growth factor-β; UTR, untranslated region.

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 (Hagiwara *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 accompanied by 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 matrices 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′ m^7GpppG 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 isomers 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 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

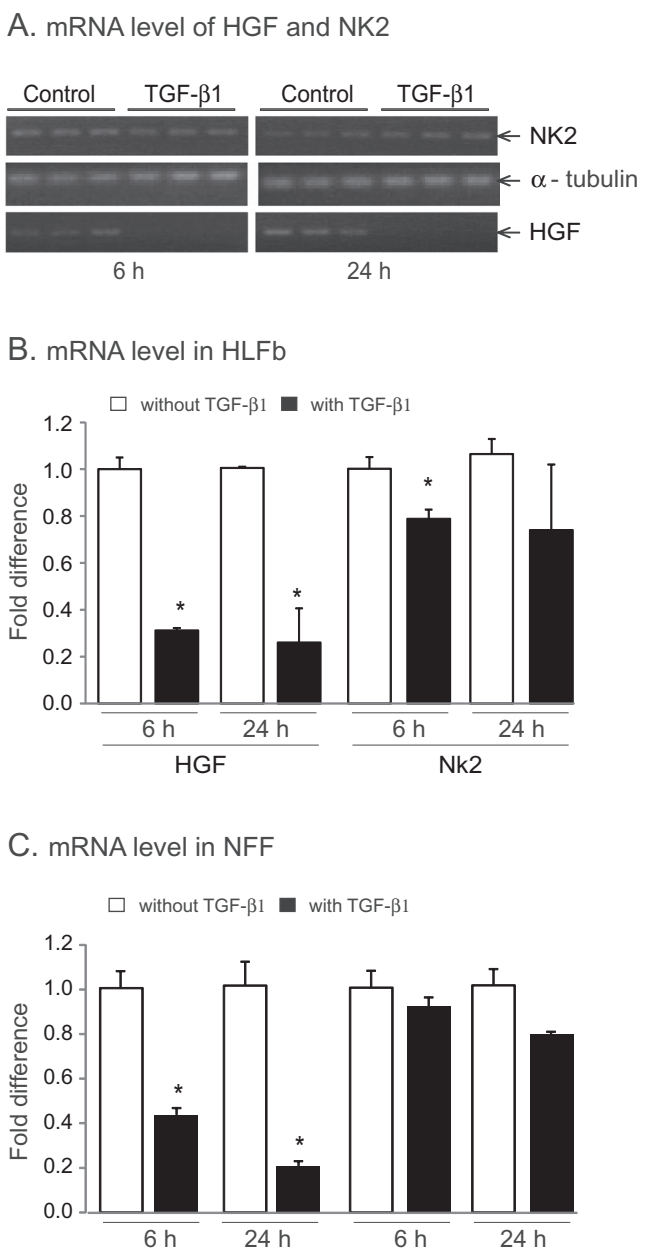

FIGURE 1: TGF-β1 blocks expression of HGF, but not NK2. HLFb were grown to confluence and placed in media containing 0.1% FBS overnight before treatment with TGF-β1 (10 ng/ml) for the indicated times. Untreated cells were used as controls. (A) Agarose (1.5%) gel electrophoresis of RT-PCR. Amplification 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, $p < 0.05$.

constructs without any 3′ UTR or constructs harboring the 3′ UTR of the NK2 were not responsive to TGF-β1 treatment. However, in TGF-β1–treated cells, the 453-nt 3′ UTR of HGF provided more than 60% repression of the reporter activity ($p < 0.05$), suggesting that the TGF-β1 regulation is specific for the full-length HGF in a mechanism that involves 3′ UTR elements.

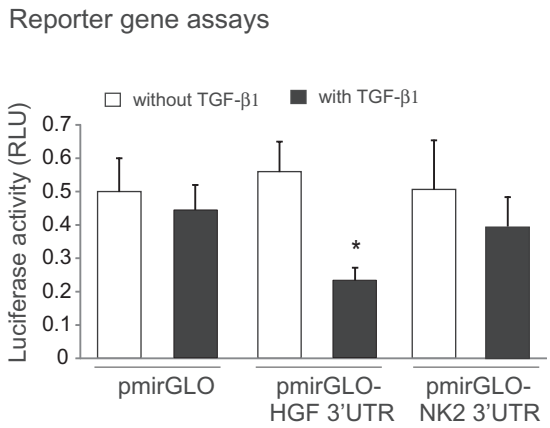

FIGURE 2: TGF-β1 regulates HGF expression through the 3′ UTR of the mRNA. HLFb were transfected with either vector control (pmirGLO) or pmirGLO harboring 3′ UTR of HGF or NK2. At 24 h posttransfection, the medium was changed to media containing 0.1% FBS. Next day, cells were treated with TGF-β1 (10 ng/ml) for 24 h prior to dual luciferase activity measurements. Results were normalized to *Renilla* activity. Data show means ± SEM; $n = 4$; *, statistical significance from basal levels of the same construct, $p < 0.05$.

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 HLFb. Quantitative PCR (qPCR) analysis showed that TGF-β1 significantly increased expression of miR-199a-3p in HLFb 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 HLFb (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

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

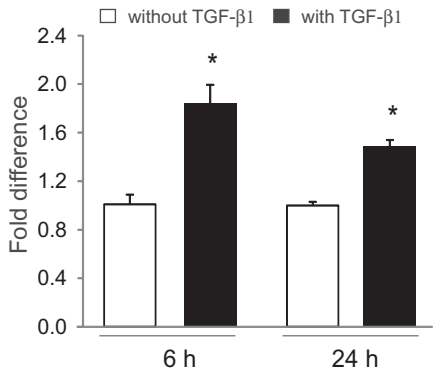

FIGURE 3: Mir-199a-3p is a possible regulatory link between TGF-β1 signaling and suppression of HGF expression. (A) The HGF 3′ UTR sequence was analyzed for miRNA target sites. The miR-199a-3p site is printed in bold and underlined. (B) HLFb were treated with TGF-β1 (10 ng/ml) for the indicated times. 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 ($p \leq 0.05$). 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, motogenic, 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 TGFβ-1 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 degradation 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 (Patel and Noureddine, 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,

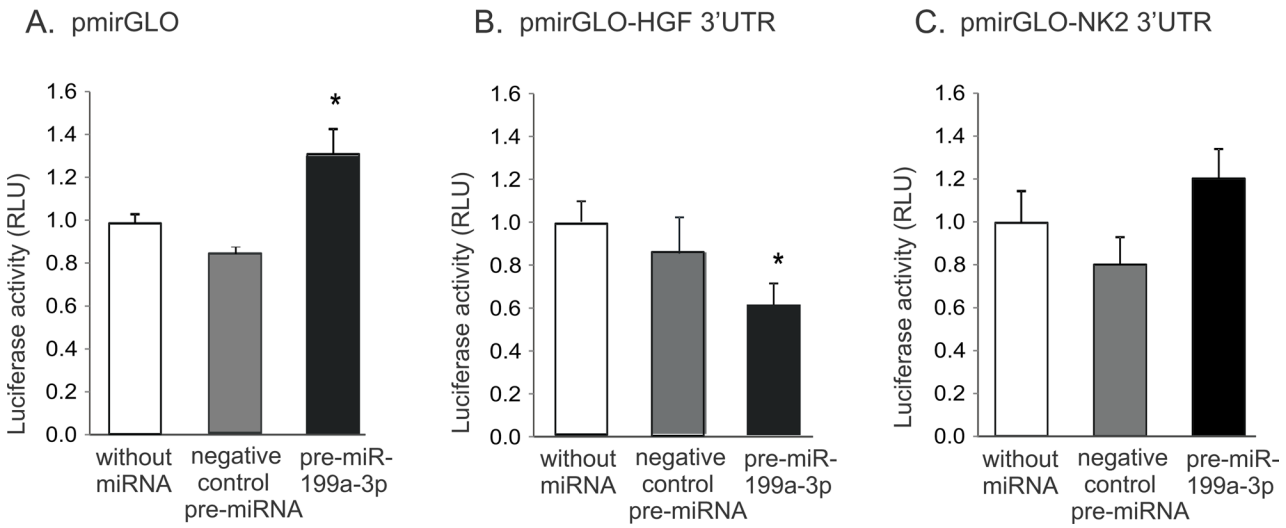

FIGURE 4: An miRNA mimic of pre–mir-199a-3p inhibits 3′ UTR activity of HGF but not that of NK2. HLFb were transfected with either vector control (A) pmirGLO, (B) pmirGLO constructs possessing the 3′ UTR of HGF, or (C) 3′ UTR of NK2. Cells were either mock transfected (without miRNA) or transfected with 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), $p < 0.05$.

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 via 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

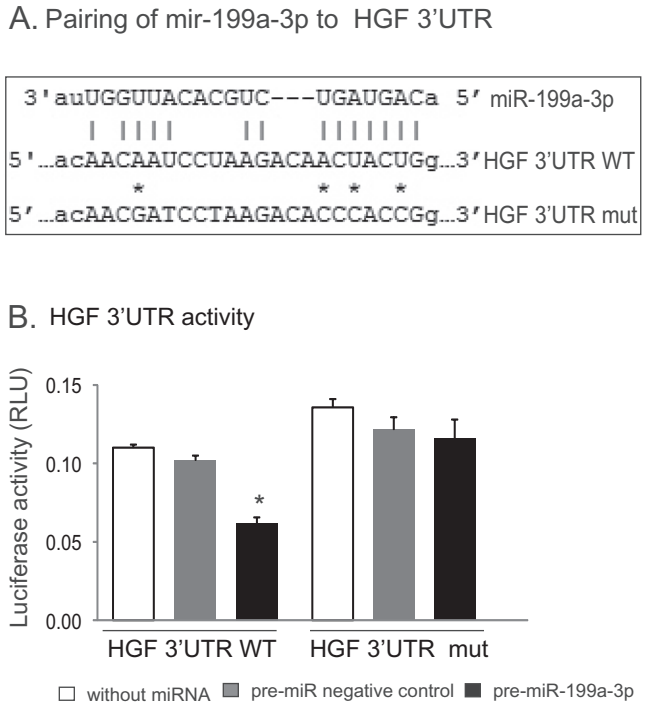

FIGURE 5: The miR-199a-3p binding site in the HGF 3′ UTR is functional. (A) Predicted interaction of miR-199a-3p and human HGF 3′ UTR (HGF 3′ UTR WT) sequences. Point mutations (*) were introduced by site-directed mutagenesis to destroy the base-pairing with the miRNA. (B) HLFb were transfected with 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, $p < 0.05$.

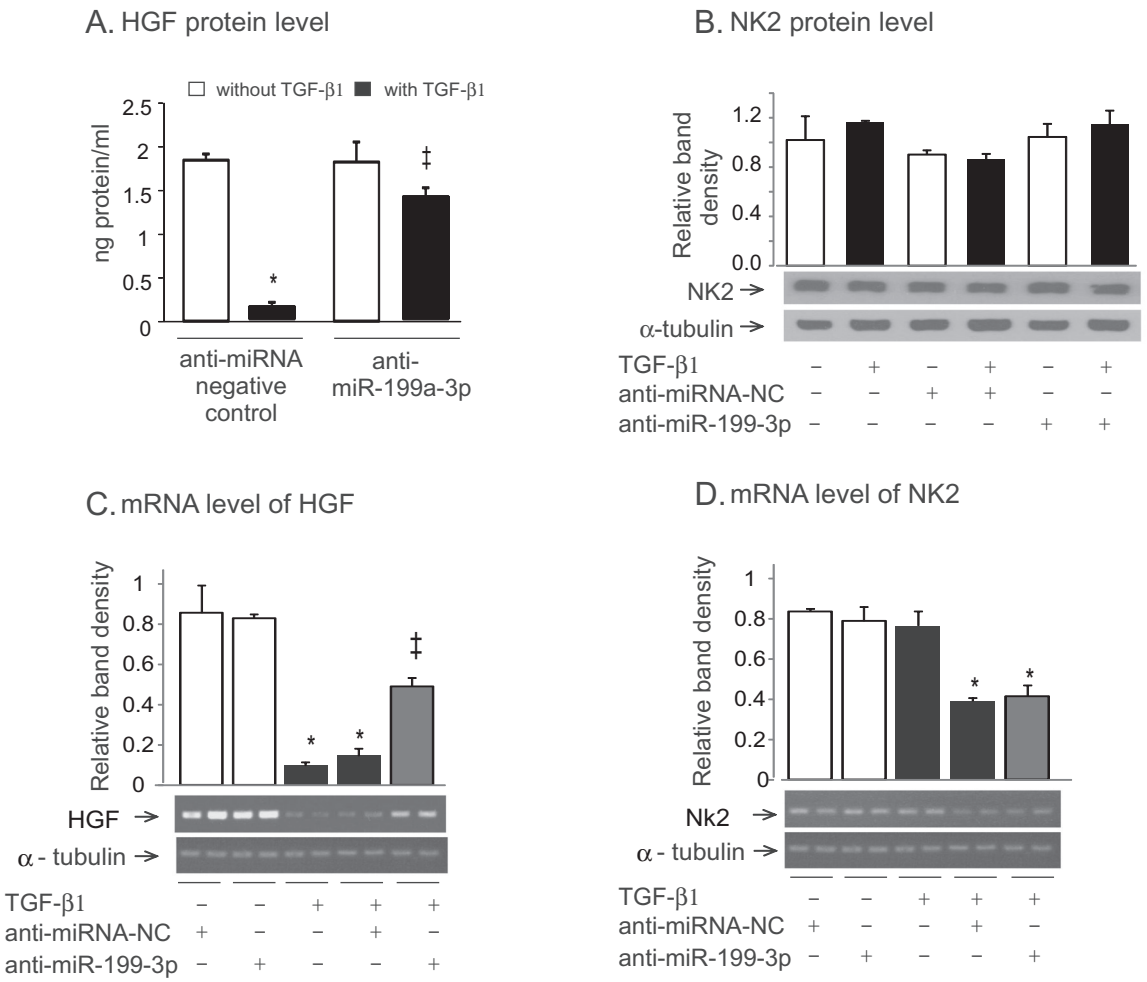

FIGURE 6: Effect of an miR-199a-3p inhibitor on HGF and NK2 expression. HLFb were transfected either with an anti-miRNA negative control (anti-miRNA NC) or with anti–mir-199a-3p. After 24 h, cells were placed in starvation media containing 0.1% FBS. Next day, cells were treated with TGF-β1 (10 ng/ml, 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; $p < 0.05$.

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 $p27^{Kip1}$ 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

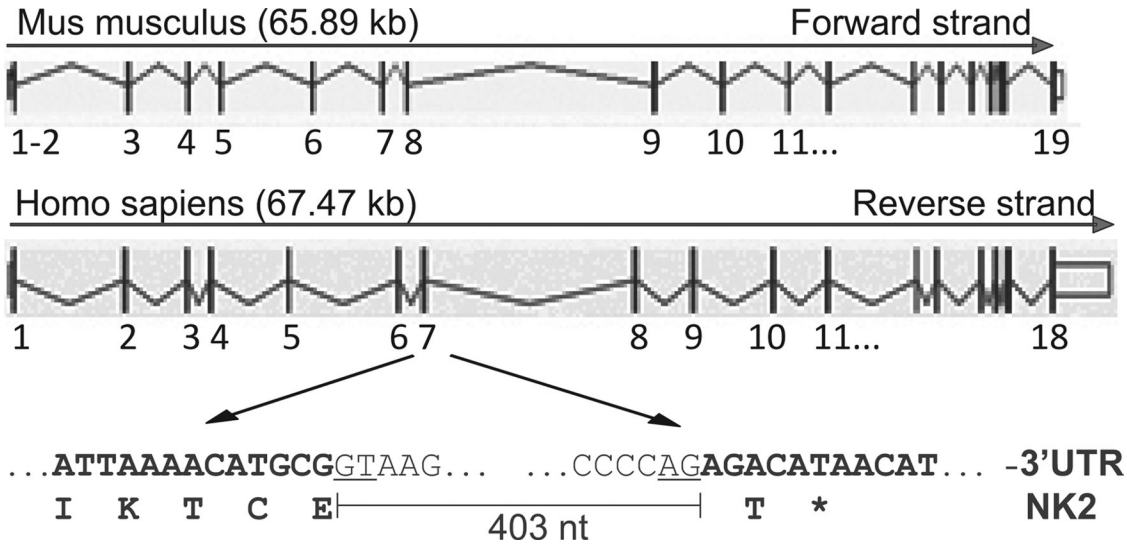

FIGURE 7: A schematic comparison of the human and mouse HGF genes. Bars and numbers indicate exons. Compared with the human gene, the murine orthologue has an extra noncoding exon (1) at the 5′ end. The human HGF gene contains an alternative exon located within the intron 7–8 (403 nt downstream of exon 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.

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% CO_2 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 (Qiagen). RNA was quantified spectroscopically (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′LR (5′-ggg**gctagC**-CTGAAGTAAGTGTGTCTGAAGCAC and 5′-ggg**tctaga**AGCATTTT-GATAAAATATTTTATTAATAATAA, with uppercase bases corresponding to the human 6-kb HGF transcript and lowercase bases indicating 5′ extensions with restriction enzyme sites [bold] for *Nhe*I and *Xba*I, respectively); HGF-3′F and HGF-3′NdeR (5′-gtta**catatg**GCATTAG-GAGTGAAC) or HGF-3′NdeF (5′-CCTAATGC**CATATG**TAACAGACA) and HGF3′LR were used to obtain two shorter fragments (1395 and 2180 bp, respectively) that can be joined using the *Nde*I (bold) sites. To obtain the 453-bp 3′UTR of the 3-kb transcript, we used HGF-3′F and HGF-3′R (5′-ggg**ctcgag**TGAAATGTAATATAATTTAATATAGG-GCT, with a *Xho*I site [bold]) primers. Primer pairs NK2-3′F and NK2-3′R (5′-ggg**gctagc**CATGGGCTCTCAACTGATGGTGAA and 5′-ggg**ctcgag**TCACGTTAAAAAATAGTTTTTATTGTA, with uppercase bases corresponding to the human NK2 transcript and lowercase bases indicating 5′ extensions with restriction enzyme sites [bold] for *Nhe*I and *Xho*I, 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 *Nhe*I and *Xho*I 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 *Taq*DNA Polymerase (New England Biolabs, Beverly, MA). As loading control, an α-tubulin fragment was amplified by 29 cycles of PCR using the primers 5′-ctccatcctcaccacccacac and 5′-cagggtcacattt caccatct. 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′-gATTGGATCAggACCATgTgA) and

HGF3′-qR (5′-CCATTCTCATTTTATgTTgCTCA) 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′-agccacatcgctcagacac and 5′-ggatttggtcgtattgggc. 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′-CACTTACAAC**g**ATCCTAAGACA**c**C**c**AC**c**GGAGAGTCATGTTTGTTG (forward) and 5′-CAACAAACATGACTCTCC**g**GT**g**G**g**TGTCTTAGGAT**c**GTTGTAAGTG (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 QuikChange 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 U) *Dpn*I restriction enzyme was added directly to the PCR and incubated for 1 h at 37°C to digest parental DNA. One microliter of the *Dpn*I-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 *mir*Vana 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 rRNA 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 mRNA 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 electroblotted 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 reblotted 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′-gaatgcatgacctgcaatgg or 5′-caattaaaacgtgcgctcacag.

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. government work as a work prepared by a military service member or employees of the U.S. government as part of that person's official duties. The views in this article are those of the authors and do not necessarily reflect the views, official policy, or position of the Uniformed Services University of the Health Sciences, Department of the Navy, Department of Defense, or the U.S. Federal Government.

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