Nkx2.5/Csx Represses Myofibroblast Differentiation

Biao Hu¹, Yue Ming Wu¹, Zhe Wu¹, and Sem H. Phan¹

¹Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

Myofibroblasts are known to play key roles in wound healing and fibrosis. They are thought to arise *de novo* from fibroblasts, and are characterized by induction of α -smooth muscle actin (α -SMA) expression. The homeobox transcription factor Csx/Nkx2.5 is critical in heart development and cardiogenesis by inducing expression of genes associated with cardiomyocyte differentiation. Here, we report a novel repressor activity of Nkx2.5 on myofibroblast differentiation. Because a key marker of myofibroblast differentiation is expression of the α -SMA gene, we first scanned its promoter region for possible *cis*-acting elements. The results show three potential binding sites (designated Nkx2.5 element [NKE]-1, -2, and -3) containing the Nkx2.5/Csx consensus binding motif (5'-TNNAGTG-3'). To determine their functional importance, site-directed mutagenesis directed at these elements individually and in combination indicated that mutation of NKE1 and -3 significantly enhanced α -SMA gene promoter activity, whereas mutation of NKE2 did not have a significant effect. The results of gel shift assays confirmed that Nkx2.5 could bind to both NKE1 and -3, but not to NKE2. Consistent with the mutagenesis studies, ectopically induced expression of Nkx2.5 inhibited α -SMA gene expression. Analysis of *nkx2.5* gene expression indicates that it was significantly induced by basic fibroblast growth factor treatment of isolated lung fibroblasts *in vitro*. *In vivo*, lung Nkx2.5 expression was significantly diminished in bleomycin-induced pulmonary fibrosis. These findings are consistent with a novel function for Nkx2.5 as a repressor of α -SMA gene transcription, which may be of homeostatic significance as a means of suppressing myofibroblast differentiation in the absence of tissue injury.

Keywords: Nkx2.5; myofibroblast; fibrosis

Fibroblasts are mesenchymal cellular constituents of the connective tissue, and are widely distributed in virtually every major organ (1, 2). In the heart, together with endothelial and smooth muscle cells, they represent approximately 70% of noncardiomyocytes (3, 4). In lung and other tissues, they contribute to the deposition of interstitial collagens and other matrix components that typify the fibrotic lesion (5–7). Myofibroblasts can be considered as an activated form of fibroblasts, arising through a differentiation process characterized by expression of α -smooth muscle actin (α -SMA) (2, 7). This α -actin isoform is normally expressed primarily in smooth muscle cells, and transiently during development of cardiac and skeletal muscle, et cetera (8, 9). In wound healing and tissue remodeling, myofibroblasts are a major source of extracellular matrix, as well as fibrogenic cytokines, such as transforming growth factor- β , and have features intermediate between smooth muscle cells and fibroblasts (2, 5–7). They emerge *de novo* in the early stage of pulmonary fibrosis, and their differentiation from fibroblasts is subject to complex regulation by a variety of factors, some of

CLINICAL RELEVANCE

Myofibroblasts are known to play key roles in wound healing and fibrosis. The homeobox transcription factor Csx/Nkx2.5 is critical in heart development and cardiogenesis. We report a novel repressor activity of Nkx2.5 on myofibroblast differentiation, which may be of homeostatic significance as a means of suppressing myofibroblast differentiation in the absence of tissue injury.

which are linked in a sequence-dependent manner. The factors that have been identified include mechanical tension and cytokines, such as transforming growth factor- β , whereas other factors such as IL-1 β and basic fibroblast growth factor (bFGF) are known to down-regulate myofibroblast differentiation (5, 10–13).

Nkx2.5, also named as Csx, is a transcription factor belonging to the natural killer homeobox gene family (14, 15). It is primarily known as a critical regulator of the expression of genes related to cardiac development, and thus is critical for cardiogenesis (16–19). Homozygous *nkx2.5*-null embryos die before completion of cardiac looping and showed poor development of blood vessels and defects in vascular formation and hematopoiesis in the mutant yolk sac (17). Thus, it is primarily known as a positive regulator of cardiac development and inducer of cardiomyocyte differentiation. Nkx2.5 is highly expressed in the adult and embryonic heart and, to a less extent, in lingual muscle, spleen, stomach, and lung as well (14–19). However, little is known about its function outside the heart. In this study, in contrast to its well known role as a promoter of cardiac cell differentiation, we uncovered a novel activity of Nkx2.5 as a suppressor of α -SMA gene expression, and thus myofibroblast differentiation.

The minimal DNA-binding consensus for Nkx2.5 contains a 5'-TNNAGTG-3' sequence motif (20). To better understand the complex regulation of myofibroblast differentiation, we have studied the mechanisms associated with regulation of α -SMA gene expression, the key marker of differentiation. In this study, we first scanned for possible *cis*-acting elements in the promoter of this gene that contain this consensus binding sequence. This analysis led to the identification of three previously unreported regions containing the 5'-TNNAGTG-3' motif in the α -SMA gene promoter, which are referred to as Nkx2.5 elements (NKEs) 1, 2, and 3. The functional significance of these potential binding sites was revealed by site-directed mutagenesis studies, which indicated that only mutation of NKE1 and/or -3 significantly enhanced α -SMA gene promoter activity. Consistent with this, gel shift analysis confirmed that Nkx2.5 could bind to NKE1 and -3, but not to NKE2. Ectopically induced expression of Nkx2.5 inhibited α -SMA gene expression. Analysis of *nkx2.5* gene expression revealed that it could be induced by bFGF in lung fibroblasts *in vitro*, whereas its expression in the lung *in vivo* was significantly reduced in bleomycin-induced pulmonary fibrosis. Because α -SMA expression was induced in this model of pulmonary fibrosis, but suppressed by bFGF, Nkx2.5 expression negatively correlated with α -SMA expression. Thus, a novel function of

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Correspondence and requests for reprints should be addressed to Sem H. Phan, M.D., Ph.D., Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-2200. E-mail: shphan@umich.edu

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Nkx2.5 as a potential homeostatic suppressor of myofibroblast differentiation was suggested.

MATERIALS AND METHODS

Animal and Cells

For induction of pulmonary fibrosis by bleomycin, 7- to 8-week-old female Fisher 344 rats were endotracheally injected with 7.5 U/kg (body weight) of bleomycin (Blenoxane; Mead Johnson, Princeton, NJ) in sterile PBS, as described previously (13). Fibroblasts were isolated from adult rat lungs and cultured as described previously (11–13). For bFGF treatment, the fibroblasts were washed with PBS and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% plasma-derived serum and 50 ng/ml bFGF (R&D systems, Inc., Minneapolis, MN) for the indicated times before harvesting.

Plasmids

The wild-type α -SMA promoter-luciferase reporter construct (pGL3- α -SMA-luc) was made as previously described (11). Analysis of the promoter revealed the presence of three potential Nkx binding elements with the 5'-TNNAGTG-3' consensus binding motif—NKE1, -2 and -3 (Figure 1). The wild-type promoter construct was used as template, together with primer pairs A (5'-AGGGTTAACGCAGTTACGTAGATTCTGACTTCTAAGTTCCTC3') and B (5'-GAGGAACTTAGAAGTCAGAATCTACGTAACGCTTAACCCCT3'), primer pairs C (5'-GAGAGCAGAGCAGAGGAATGCGCAGGAA GAGACCCACGCTCTGG3') and D (5'-CCAGAGCGTGGGTCTC TTCTGCGCATTTCTGCTCTGCTCTC3'), and primer pairs E (5'-TATGGTTTGTTAGATCTAACGCCAGCTTCAGCCTG3')

and F (5'-CAGGCTGAAGCTGGCCGTTAGATCTAACACAAC TATAG3'), respectively, or sequentially, to generate the various NKE-mutated α -SMA promoter constructs (Figure 2A). These were undertaken with the Quick-Change Mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the manufacturer's protocol. The final constructs were sequenced to confirm the intended mutations. The mouse Nkx2.5 expression plasmid, pCDNA3-*nkx2.5*, was a kind gift from Dr. Richard P. Harvey of Victor Chang Cardiac Research Institute, Australia. There are two restriction endonuclease EcoR I sites at the beginning and ending of the *nkx2.5* gene, respectively, in plasmid pCDNA3-*nkx2.5*. Therefore, an antisense *nkx2.5* expression plasmid, pCDNA3-*nkx2.5-Rev*, was made by digesting the plasmid pCDNA3-*nkx2.5* with EcoR I and then religated to form plasmid pCDNA3-*nkx2.5-Rev*, where the *nkx2.5* gene was inserted in an antisense direction under the control of a cytomegalovirus promoter.

Electrophoresis Mobility Shift Assay

³²P-labeled double-stranded oligonucleotide probe spanning the α -SMA promoter's NKE1 with sequence 5'-TAACGCAGTTACAGTGATT CTGACTTCTA3', NKE2 with sequence 5'-AGAGCAGAGCAG AGGAATGCAGTGGAAGAGACCCACGCT3', or NKE3 with sequence 5'-ATGGTTGTGTTAGAGTGAACGGCCAGCTTCA3' was incubated with nuclear extracts from rat lung fibroblasts in gel shift binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.05 mg/ml poly [dI-dC]) at 25°C for 20 minutes. They were then applied to 4% nondenaturing polyacrylamide gels in 1× tris-borate-EDTA and electrophoresed for analysis of NKE-binding complexes. To confirm specificity of binding, in select samples, as indicated, the nuclear extracts were preincubated with anti-Nkx2.5 antibodies, nonimmune goat IgG, or the respective excess

		NKE1	
RASMAP	TAAGGGTT-AACGCAGT	TACAGTG	ATTCTGACTTCTAAGTTCCTCTTAGG -619
HASMAP	AAAAAATGAATGTAGT	TATAGTA	ATGCTAACATCCAAATTCCTCTTTGT -624
		Cons: TNNAGTG	
RASMAP	GTAACATAGGCTGGTGAATCCTGATTACATACTTCCATATGTAATACATA -569		
HASMAP	AAGACATAGGCTGTCAACCTTGTCTCCATACTTCAATTCTATTTC -A -575		
RASMAP	CAGACTTCATTGATACTACACAGACTCCAGACTACATACAATGTGGCT -519		
HASMAP	CTCACCTCC-----CTCAAGAACTTGA-TTTATAAACAGTGTGCCT -535		
RASMAP	TCCATAAAATGATCACTCC-TCTGCAGATTTCGAGGTGACCCAAG---CA -473		
HASMAP	ACCATAAAATCATCACTCCCTCTATGTATTATAGACGACTGAAGGAATA -485		
RASMAP	TCTTTTGTATAG---GCTACCTTTTGCAACAGTGTGTCCTTAAAGTCCC -426		
HASMAP	TCTTTCTCTTTGCATGCTACCGTG GTAGAAGGGTT--TTAAAGTCCG -437		
RASMAP	AGCTAGTCAGAGACAGGCCCTTC-----CTCATCTCAAGCCCTTAG -385		
HASMAP	TGCTAGGCAGAGGCAGCCCTTCTGCCCCCTTCTGTTCTCAGTTATTAG -387		
RASMAP	CTAATGGACCCAAAGGCTAGCCTGACAGGAAGAGCTGGCATCTTCTGAGG -335		
HASMAP	GAAATGGCCTGAAATTCAGCATGATAGCAAG--CTGGCATCCTCTGTGG -339		
RASMAP	AATGTGCAAACCATGCCTGCGTCTGCTCCATGGCACTAGCCAGTGTCT- -286		
HASMAP	AATGTGCAAACCATGCCTGCATCTGC-CCATTACCCTAGCTCAGTGTCTC -290		
RASMAP	-GGGCATTTGAGCAGTTGTTCTGAGGGCTCAGGATGTTTATC-CCCATAA -238		
HASMAP	TGGGCATTTCTGCAGTTGTTCTGAAGGCTTGCGTGTTATCTCCCACAG -240		
		NKE2	
RASMAP	GCAGCTGAAGTGCCTCCTGTTTCGAGAGCAGAGCAGAGGAA	TGCAGTG	GGA -188
HASMAP	GCGGCTGAACCGCCTCCCGTTTCATGAGCAGACCAAGTGGAA	TGCAGTG	GGA -190
		Cons: TNNAGTG	
RASMAP	AGAGACCCACGCTTCTGGCCACCCAGATTAGAGAGTTTGTGCTGAGGTC -138		
HASMAP	AGAGACCCAGCCTCCGGCCACCCAGATTAGAGAGTTTGTGCTGAGGTC -140		
		NKE3	
RASMAP	CCTATATGGTTGTGT	TAGAGTG	AACGGCCAGCTTCAGCCTGTCTTTGCTC -88
HASMAP	CCTATATGGTTGTGT	TAGACTG	AACGACAGGCTCAAGTCTGTCTTTGCTC -90
		Cons: TNNAGTG	
RASMAP	CTTGTTTGGGAAGCGAGTGGGAGGGGATCAGACCAGGGGGCTATATAACC -38		
HASMAP	CTTGTTTGGGAAGCAAGTGGGAGGAGAGCAGGCCAAGGGGCTATATAACC -40		
		*	
RASMAP	CTTCAGCATTACGCCTCCCCAGACACCCACCCAGAGTGAGAGAAGCCC +13		
HASMAP	CTTCAGCTTTACGTTCCCTGAACACCACCCA---GTGTGGAGCAGCCC +7		

Figure 1. Sequence alignment of rat and human α -smooth muscle actin (α -SMA) gene promoter region. The Nkx2.5 elements (NKEs) are numbered sequentially, boxed, shaded, and bolded. The Nkx2.5 binding consensus is indicated as "Cons." The transcriptional start site is bold, underlined, and indicated with an asterisk.

Figure 2. Effects of NKE mutations on α -SMA gene transcription. (A) A cartoon drawing shows the various individual and combined NKE-mutated α -SMA promoter-luciferase constructs and their nomenclature. The individual, intact NKEs are shown as indicated, and are absent when mutated. (B) The indicated wild-type and NKE-mutated α -SMA gene promoter-luciferase constructs were transfected into rat lung fibroblast and the promoter activity was measured as luciferase activity. After normalizing to the internal transfection efficiency control (*Renilla* luciferase activity driven by SV40 promoter in plasmid pRL-SV40), the relative light units were expressed as means ($\pm$ SE). Experiments with each construct were repeated two to four times with comparable results. *Statistical significance ($P < 0.001$) when compared with wild-type control mean value.

unlabeled probe on ice for 30 minutes before addition of the 32 P-labeled DNA probe. After electrophoresis, the gels were dried and exposed to X-ray film for visualization of any bound complex.

Transfection and Reporter Gene Assay

All transient transfections of cells were performed using the FuGENE6 reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions, as previously described (11). Supercoiled DNA was isolated with an endotoxin-free Qiagen column kit (Qiagen Inc., Valencia, CA). Unless otherwise indicated, 2 μ g DNA of the α -SMA promoter-luciferase construct of interest and 100 ng plasmid pRL-SV40 control vector (used for normalization) were cotransfected per culture into rat lung fibroblast in serum-free DMEM medium. At 4 hours after the transfection, the medium was replaced with DMEM containing 10% plasma-derived serum. In experiments to examine the effects of Nkx2.5 on α -SMA promoter activity, 2 μ g of Nkx2.5 expression plasmid (pCDNA3-nkx2.5) or the empty vector, pCDNA3, was cotransfected with the α -SMA promoter-luciferase construct. The cells were harvested 48 hours after transfection, and the activity of firefly or *Renilla* luciferase was measured using the dual luciferase assay system from Promega Corporation (Madison, WI). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of *Renilla* luciferase. Experiments with each construct were repeated two to four times, and relative light units were expressed as means ($\pm$ SE).

Western Blot

Western blot was conducted as previously described (11). The anti-Nkx2.5 antibody and the mouse monoclonal anti- α -SMA antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma-Aldrich Co. (St. Louis, MO), respectively. For quantitative analysis, the blots were digitized by scanning, and band intensities were determined using Kodak Molecular Imaging software (Carestream Health Inc., New Haven, CT).

Real-Time RT-PCR

Semiquantitative real-time RT-PCR was conducted as previously described (13). With this method, a cycle threshold (CT) value reflects the cycle number at which DNA amplification is detected. The amount of target, normalized to endogenous reference and relative to a calibrator,

is given by $2^{-\Delta\Delta CT}$ (21). Total RNA extract (250 ng) from either rat lung fibroblast or lung tissue by Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) was deposited into each well of a 96-well plate as the template, and the quantity of GAPDH was used as internal control. The primer and probe set to detect the housekeeping gene, GAPDH, was purchased from Applied Biosystems (Foster City, CA). The primer and probe set to detect the rat Nkx2.5 was: forward primer, CTTCAAGCAACAGCGGTACCT; reverse primer, CGCTGTCGC TTTCATTGTGTA; probe, 6FAMAGCTCACGTCCACGCAGGTCA AGATAMRA3'. Samples from saline-treated control lung tissue or untreated cells were used as calibrator for the tissue and cellular analyses, respectively. Results are expressed as $2^{-\Delta\Delta CT}$.

Statistical Analysis

Statistical analysis was undertaken as before using ANOVA, followed, where appropriate, by *post hoc* testing using Scheffé's test (12). A P value of less than 0.05 was used as a criterion for statistical significance in comparisons between any two groups.

RESULTS

Identification and Function of NKEs in α -SMA Gene Promoter

De novo genesis of myofibroblasts in wound healing and tissue fibrosis is thought to occur via differentiation of fibroblasts (5–10), but the mechanism remains unclear. A key marker of differentiation is the expression of α -SMA (7–13). Hence, analysis of the molecular regulation of its expression may provide insight into this mechanism. In an attempt to find out how the α -SMA gene is regulated in response to different stimuli, we scanned the rat α -SMA gene promoter for potential *cis*-acting elements. This analysis has previously identified a number of potential regulatory elements (10–12) and, in this study, further analysis revealed the presence of three previously unreported Nkx2.5 binding elements (NKE1, -2, and -3) with the 5'-TNNAGTG-3' consensus binding motif (Figure 1). The most upstream or distal to the transcriptional start site was NKE1 at -651, whereas NKE2 was at -196, and the most

Figure 3. Gel shift analysis of Nkx2.5 binding to NKEs. Double-stranded oligonucleotide probes containing either NKE1, -2, or -3 sequences in the α -SMA promoter were incubated with nuclear extracts from rat lung fibroblasts and then separated by nondenaturing polyacrylamide gel electrophoresis. A representative experiment with NKE1 or -2 probes is shown A, whereas an NKE3 probe was used in a separate experiment (B). In select samples as indicated, the nuclear extracts were preincubated with anti-Nkx2.5 antibody, nonimmune goat IgG, or 100-fold excess of the respective unlabeled probe before addition of the indicated 32 P-labeled DNA probe. The DNA-protein complexes are indicated by solid arrows and the Nkx2.5 antibody super-shifted complex is shown by the open arrows.

proximal NKE3 was at -122 in the rat α -SMA gene promoter. When aligned with the corresponding human sequence, all three elements are conserved. To determine if these sites are functionally important with respect to transcription of the α -SMA gene expression, site-directed mutagenesis was conducted to selectively alter the nucleotide sequence at each of these putative binding sites individually, or in the indicated combinations (Figure 2A). After transfection into rat lung fibroblasts, the activities of the wild-type and mutant α -SMA gene promoter constructs were then determined to analyze the impact of these various mutations. The results show that the wild-type α -SMA promoter expressed the expected activity, which was not significantly affected by mutation of the NKE2 (NKE2m in Figure 2B). However, the mutant constructs with mutated NKE1 (NKE1m) or NKE3 (NKE3m), or both (NKE1m3), showed significantly enhanced promoter activity. Combined mutations of all three elements (NKE1m2m3) did not further enhance the activity compared with combined mutations of NKE1 and -3. The enhancement was particularly substantial in NKE1m (>twofold increase over the wild-type promoter), and NKE1m3 or NKE1m2m3 (>threefold increase) mutant promoters. Combinations of mutations in NKE2 plus NKE1 (NKE1m2), or NKE2 plus NKE3 (NKE2m3) failed to increase the promoter activity beyond that caused by NKE1 or NKE3 mutation alone, respectively. The effect of NKE3 mutation was essentially additive to that of NKE1 mutation in the combined NKE1 plus NKE3 (NKE1m3) mutant construct. These results suggest that only NKE1 and -3 identified in the α -SMA promoter are important for repression of α -SMA gene expression, with NKE1 playing a predominant role.

Nkx2.5 Binding to NKEs in α -SMA Gene Promoter

In view of the importance of NKEs in repressing α -SMA promoter activity, the ability of their cognate transcription factor, Nkx2.5, to bind to these elements was investigated. Double-stranded oligonucleotide primers with sequences corresponding to the indicated NKEs in the α -SMA gene promoter were synthesized and used together with nuclear extracts from rat lung fibroblast in a gel mobility shift assay. The results revealed the generation of two retarded bands when the radio-labeled probes were incubated with the nuclear extracts, except for the NKE2 probe (Figures 3A and 3B). The specificity of the binding to NKE1 and -3 probes was confirmed by abolition of complexes when 100-fold excess of the respective unlabeled or cold probe was added to the incubation mixture. Preincubation of the nuclear extract with anti-Nkx2.5 antibodies caused a super-shifted band to appear with both NKE1 (Figure 3A) and NKE3 (Figure 3B) probes. As a control, no super-shifted band was identified in protein samples preincubated with non-immune IgG. This suggests that Nkx2.5 in the nuclear extracts could bind to the NKE1 and -3 probes, but not to the NKE2 probe. This would be consistent with the site-directed mutagenesis studies showing the insignificant functional importance of NKE2 in repression of α -SMA promoter activity nuclear extract, which was in contrast to the importance of NKE1 and -3. These data suggest that Nkx2.5 could bind to both NKE1 and -3 in the α -SMA promoter to suppress transcription.

Effect of Ectopically Induced Nkx2.5 Expression on α -SMA Gene Expression

To confirm that Nkx2.5 could regulate α -SMA gene expression, the effects of ectopically induced Nkx2.5 expression or suppression of its expression using an antisense Nkx2.5 expression plasmid were examined. Fibroblasts were transfected with an Nkx2.5 expression plasmid, an antisense Nkx2.5 expression plasmid, or the empty vector. The effects on a cotransfected

Figure 4. Effects of basic fibroblast growth factor (bFGF) and Nkx2.5 expression levels on α -SMA gene expression. (A) Wild-type α -SMA gene promoter-luciferase construct was cotransfected into rat lung fibroblasts with pCDNA3-*nkx2.5* (Nkx2.5), pCDNA3-*nkx2.5-Rev* (Nkx2.5-Rev), or pCDNA3, as indicated. The cells were then treated without (None) or with bFGF. The α -SMA promoter activity was then measured in terms of luciferase activity. After normalization with the internal transfection efficiency control (*Renilla* luciferase activity driven by SV40 promoter in plasmid pRL-SV40), the relative light units are expressed as means ($\pm$ SE). Experiments with each construct were repeated two to four times with similar results. (B) Rat lung fibroblasts were transfected with pCDNA3-*nkx2.5*, pCDNA3-*nkx2.5-Rev*, or pCDNA3, as indicated. At 4 hours after transfection, the cells were treated without (None) or with bFGF, as indicated. At the end of incubation, the cells were lysed and equal amounts of total cell lysate protein were loaded onto gels for Western blotting analysis to assess α -SMA protein levels. To confirm equal loading, the membrane was stripped and reblotted with anti-GAPDH antibodies. The blots were digitized by densitometric scanning, and band intensity quantitated using Kodak Molecular Imaging software. Results are expressed as mean α -SMA:GAPDH ratio ($\pm$ SE) of triplicates. (A and B) *Statistical significance ($P < 0.001$) when compared with untreated control vector group (pCDNA3); #indicated significance ($P < 0.001$, except for group transfected with Nkx2.5 in A, where $P < 0.02$) when compared with the bFGF-treated control vector group.

α -SMA gene promoter reporter construct (luciferase assay) and α -SMA protein expression were determined. The results show that ectopically induced Nkx2.5 expression caused a significant reduction in α -SMA promoter activity (Figure 4A). Treatment with bFGF also caused a significant reduction in promoter activity, which was further inhibited by the Nkx2.5 plasmid. In contrast, treatment of cells with the antisense Nkx2.5 plasmid caused almost a doubling of promoter activity. These effects on α -SMA transcription were reflected also in terms of α -SMA protein expression. Thus, Nkx2.5 overexpression caused inhibition of α -SMA protein expression, whereas the antisense Nkx2.5 construct caused a marked induction of protein expression (Figure 4B). This pattern was repeated in cells also treated with bFGF, but at a significantly lower level of expression. The combination of bFGF and Nkx2.5 plasmid caused the most inhibition to a virtually undetectable level. These findings indicate a constitutive inhibition of α -SMA gene expression by Nkx2.5 in rat lung fibroblasts, which could be enhanced by bFGF treatment. These results also confirm that Nkx2.5 regulated α -SMA gene expression by repressing its transcription.

Effect of bFGF on Nkx2.5 Expression

bFGF is a well known key regulator of cell proliferation and differentiation (22, 23). It is also an inhibitor of myofibroblast differentiation and α -SMA expression (13), which was confirmed in the preceding experiments. To study if Nkx2.5 was

involved in the down-regulation of α -SMA gene expression by bFGF, we analyzed *nkx2.5* gene expression in fibroblasts after bFGF treatment by Western blot and real-time PCR analyses. The results show that treatment of cells with bFGF significantly induced Nkx2.5 protein expression in a dose-dependent manner (Figure 5A). This induction was also observed in terms of mRNA levels by real-time PCR (Figure 5B). These results suggest that Nkx2.5 may play a role in mediating the bFGF regulation of α -SMA gene expression.

To confirm that Nkx2.5 mediated the bFGF signal, the wild-type α -SMA promoter and Nkx2.5 binding site-mutated α -SMA promoter (NKE1m3) were transfected into rat lung fibroblasts, and their responses to bFGF treatment examined. As shown in Figure 6, bFGF treatment caused a significant reduction (>50% inhibition) in the wild-type α -SMA promoter activity. In contrast, bFGF caused less than a 20% inhibition in the activity of the NKE mutant promoter. Thus, mutation of the NKE1 and -3 made the α -SMA promoter essentially unresponsive to bFGF-induced repression. Because these two *cis* elements were found to be required for Nkx2.5-mediated repression, these findings confirm the importance of Nkx2.5 in mediating bFGF inhibition of α -SMA gene expression.

nkx2.5 Gene Expression in Pulmonary Fibrosis

Endotracheal injection of bleomycin causes lung injury, inflammation, and fibrosis that is associated with significant induction of α -SMA expression due to *de novo* genesis of

Figure 5. Effect of bFGF on Nkx2.5 expression. Rat lung fibroblasts were treated with bFGF at the indicated doses and then harvested for Nkx2.5 protein levels by Western blotting (A). Equal amounts of protein were loaded, and confirmation of comparable loading was undertaken by stripping the membrane and reblotting with anti-GAPDH antibodies. The blots were quantitated by densitometry, and the results shown as means ($\pm$ SE) of triplicates after normalization to GAPDH. *Statistical significance when compared with the untreated control mean value ($P < 0.001$). (B) Cells were similarly treated with or without bFGF, as indicated, and total cellular RNA was extracted and analyzed for α -SMA mRNA levels by real-time PCR. Results were expressed as $2^{-\Delta\Delta CT}$ with GAPDH used as the endogenous control and the buffer-treated (None) group used as reference. Data are shown as the mean ($\pm$ SE) from triplicate samples. *The effect of bFGF treatment was statistically significant ($P < 0.05$).

myofibroblasts (24, 25). To examine if this induction of α -SMA expression could be due to derepression from homeostatic or constitutive Nkx2.5-mediated repression of this gene, the level of lung Nkx2.5 mRNA was examined in this animal model. Consistent with previous reports, bleomycin injection caused

a greater than twofold induction of lung α -SMA gene expression compared with that in the saline-treated control animals (data not shown). In contrast, the expression of Nkx2.5 in lungs of bleomycin-treated animals was inhibited by more than 60% relative to that in lungs of saline-treated control animals (Figure

Figure 6. Effect of bFGF on α -SMA promoter activity. Wild-type or NKE1m3 mutant α -SMA promoter-luciferase constructs were transfected into rat lung fibroblasts, as indicated. The cells were then treated with either buffer only (untreated control) or with bFGF. The promoter activity was then measured as luciferase activity and normalized to the endogenous transfection efficiency control, as described in the legend to Figure 2. The normalized activities in bFGF-treated cells were then expressed as a percentage of the mean activity in untreated control cells, and shown as the mean ($\pm$ SE) from triplicate experiments. *The activity of the wild-type promoter was significantly different ($P < 0.05$) from that of the mutant NKE1m3 promoter.

Figure 7. *nkx2.5* Gene expression in the bleomycin model. Total RNA extracted from lungs (A) or lung fibroblasts (B) of either bleomycin- or saline-treated rats was analyzed for Nkx2.5 mRNA levels by real-time PCR 7 days after treatment. The results are expressed as $2^{-\Delta\Delta CT}$ using GAPDH mRNA as endogenous control, and the values from the saline-treated controls as reference. Data are shown as the mean ($\pm$ SE) ($n = 5$). *Reduction in lung tissue or lung fibroblast Nkx2.5 mRNA in bleomycin-treated animals was statistically significant ($P < 0.05$) when compared with corresponding saline-treated controls.

7A). To confirm that this reduction in Nkx2.5 was occurring in lung fibroblasts, and thus relevant to the induction of myofibroblast differentiation in the injured lung, the level of Nkx2.5 mRNA was also measured in cells isolated from saline- or bleomycin-treated animals. The results paralleled the reduction in Nkx2.5 mRNA seen in lung tissues (Figure 7B). Thus, *in vivo* during pulmonary fibrosis, α -SMA gene expression negatively correlated with Nkx2.5 expression in the lung, consistent with the postulated role of Nkx2.5 as a homeostatic repressor of α -SMA gene expression.

DISCUSSION

As the homologous counterpart of *Drosophila tinman* in vertebrates, Nkx2.5 is required for the dorsal mesoderm specification (14–19). To date, its functions in cardiac development are well documented (14–19), although the specific molecular mechanisms remain incompletely understood. However, its function is not exclusive to heart development, as, recently, its expression has been found to be significant in lingual muscle, spleen, stomach, and in the lung (14–19). In addition, the Nkx2.5 binding elements have been identified in a number of genes that are expressed in noncardiac cells, and suggest their importance in the regulation of expression of these genes. For instance, Nkx2.5 binding elements are known to be important in

the regulation of the collagen I gene in smooth muscle cells (26), CC10 in lung alveolar epithelial cells (27), and MMP13 (28). Therefore, identification of additional Nkx2.5 downstream target genes and upstream signaling pathways may be useful for a more complete definition of the function of this homeobox protein in the adult animal.

Myofibroblast differentiation, characterized by induction of α -SMA expression, is one of the major events during pulmonary fibrosis that is thought to be crucial to the abnormal deposition of collagen and propagation of fibrosis in the lung (5–9). It is also the key feature of cardiac fibrosis, which is associated with both cardiac hypertrophy and heart failure (29). In this study, isolated rat lung fibroblasts were investigated as a model of myofibroblast differentiation in an attempt to evaluate a potential novel role for Nkx2.5. Analysis of the rat α -SMA promoter identified three putative Nkx2.5 binding elements based on the known consensus binding sequence for this homeobox protein. Of these three elements, two—namely, NKE1 and -3, were found to be functionally important for repression of α -SMA gene expression. Thus, mutations at these two elements caused significant additive enhancement of α -SMA promoter activity, consistent with their role as repressive elements. Confirmation that this repression was due to actual binding of Nkx2.5 was obtained by gel shift analysis, which revealed binding to NKE1 and -3, but not to NKE2. Moreover, overexpression of Nkx2.5,

by transfection of an expression plasmid or by treatment with bFGF, resulted in significant inhibition of α -SMA gene expression. Conversely, suppression of endogenous Nkx2.5 expression using an antisense construct caused marked enhancement of α -SMA gene expression. Thus, our data indicate that Nkx2.5 inhibited α -SMA gene expression through binding to the two identified NKEs in the α -SMA gene promoter. This study provides evidence, for the first time, that Nkx2.5 is involved in the regulation of myofibroblast differentiation, which may have a bearing on the pathogenesis of fibrotic diseases. At least in the bleomycin model of lung injury and fibrosis, down-regulation of Nkx2.5 expression may be key to induction of myofibroblast differentiation. In addition, it suggests that Nkx2.5 may serve as an important homeostatic controller of cell differentiation, in this case functioning as a repressor of myofibroblast differentiation under normal conditions. With injury and associated stimuli, its down-regulation may serve to derepress α -SMA gene expression, and thus induce myofibroblast differentiation.

bFGF is a heparin-binding mitogenic protein that mediates the formation of new blood vessels (30) and enhances proliferation of a wide variety of cell types under serum-free or serum-reduced conditions (31). bFGF is also known as a repressor of myofibroblast differentiation (13, 31–34), which would be consistent with its effects on stimulation of Nkx2.5 expression and consequent repression of α -SMA gene expression. This is also consistent with the concept of bFGF as a homeostatic controller of cell differentiation, and its utility in preventing precursor or stem cell differentiation or maintenance of stemness (35). Furthermore, bFGF is a well known promoter of angiogenesis, which would have relevance for tissue repair and remodeling. It is unknown, however, whether bFGF regulation of angiogenesis is mediated by Nkx2.5, but this possibility certainly suggests additional relevance to cardiac repair. Additional studies are necessary to explore fully these interrelated possibilities with respect to Nkx2.5 as a mediator of these bFGF biological activities.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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