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A NOVEL SHORT HAIRPIN RNA (SHRNA) EXPRESSION SYSTEM PROMOTES SOX9-DEPENDENT GENE SILENCING

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Abstract

Cartilage development and function are dependent on a temporally integrated program of gene expression. With the advent of RNA interference (RNAi), artificial control of these complex programs becomes a possibility, limited only by the ability to regulate and express the RNAi. Using existing methods for production of RNAi's, we have constructed a plasmid-based short hairpin RNA (shRNA) expression system under control of the human pol III H1 promoter and supplemented this promoter with DNA binding sites for the cartilage-specific transcription factor Sox9. The resulting shRNA expression system displays robust, Sox9-dependent gene silencing. Dependence on Sox9 expression was confirmed by electrophoretic mobility shift assays. The ability of the system to regulate heterologously expressed Sox9 was demonstrated by Western blot, as a function of both Sox9 to shRNA ratio, as well as time from transfection. This novel expression system supports auto-regulatory gene silencing, providing a tissue-specific feedback mechanism for temporal control of gene expression. Its applications for both basic mechanistic studies and therapeutic purposes should facilitate the design and implementation of innovative tissue engineering strategies.

Keywords

Sox9; shRNA; tissue-specific; regulated expression; inducible gene silencing

Introduction

Cartilage function and development depends upon a complex pattern of temporally regulated gene expression (Dunn and Kinston, 2007; Lefebvre and Smits, 2005). Cartilaginous differentiation initiates in response to expression of the master transcriptional regulator Sox9, which promotes the transition of mesenchymal stem cells into pre-hypertrophic chondrocytes. Chondrocyte hypertrophy and terminal differentiation, conversely, require the subsequent

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silencing of Sox9. The need for both events in cartilage development has been demonstrated in gene therapy studies which have shown constitutive over-expression of Sox9 to result in an arrested phenotype at the hypertrophic border. We reasoned that this overexpression and silencing of Sox9 could be achieved through an integrated expression/RNAi system.

RNAi silencing occurs through assembly of the RNA-induced silencing complex (RISC) around a 21–25 base single-stranded guiding RNA (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000), followed by RISC binding its transcript and subsequent degradation by cellular exonucleases (Schwarz et al., 2003). Recognition of the components needed for RISC assembly has resulted in plasmid and virally-based expression systems using short hairpin RNAs (shRNA) (Chang et al., 2006). shRNA expression systems have relied almost exclusively on compact, constitutive pol III promoters derived from human H1 RNA or U6 pol III promoters to drive shRNA expression (Bannister et al., 2007; Cheng and Chang, 2007). The H1 RNA promoter contains a region (positions -31/-1) adjacent to the TATA box (positions -32/-28) that allows neutral substitution mutations (Myslinski et al., 2001). The promoter includes known upstream transcriptional regulatory sites, including a distal sequence element (DSE), composed of Oct-1 (positions -97/-90) and STAF (positions -88/-69) recognition sequences, and a proximal sequence element (PSE; positions -68/-51) (Hannon et al., 1991; Myslinski et al., 2001). These sequences are thought to play pivotal roles in both basal and inducible gene expression.

Based on this information, regulated pol III expression systems have been designed that usually depend on insertion of the tet or lac operator, where co-expression of the tet or lac repressor suppresses shRNA expression (Higuchi et al., 2004; Hosono et al., 2004; Kappel et al., 2006; Lin et al., 2004; Ohkawa and Taira, 2000). Similarly, use of ecdysone or Cre-lox mediated stuffer reporter deletions have been incorporated into viral gene delivery systems to permit stable, inducible gene silencing (Gupta et al., 2004; Heinonen et al., 2005; Rangasamy et al., 2008; Szulc et al., 2006; Tiscornia et al., 2004; Yu and McMahon, 2006). Although highly effective for *in vitro* purposes, the *in vivo* application of these expression systems is limited due to their dependence on heterologous, non-mammalian proteins and due to their requirement for systemic administration of pharmacological inducing agents.

We theorized that we could create a regulated shRNA expression system that was based on the H1 promoter and that could autoregulate Sox9 expression as required in cartilage differentiation and maturation. We therefore constructed an H1-driven shRNA expression plasmid and evaluated the effects of replacement of the STAF binding site with binding sites that recognize the Sox9 transcription factor. Herein, we describe the construction and characterization of such a Sox9-regulated shRNA expression system.

Materials and Methods

Plasmid Construction

The SV40 origin of amplification was PCR amplified and was cloned into the Sma I digested backbone of LITMUS38i (New England Biolabs, Ipswich, MA) to produce pLITMUS-ori. The annealed H1-tata primer pair was cloned into the Nae I-Bam HI backbone of pLITMUS-ori to create pL.H1-tata. Annealed H1 and Sox9 m1 and were cloned into the Afl II-Apa I backbone of pL.H1-tata to create pL-H1 and pL-m1, respectively. All shRNA coding sequences, including shRNAs against GFP, human Sox9, and a randomized control, were cloned into the Bam HI-Bsr GI backbone of the preceding constructs. Oligonucleotide pairs used for these purposes are summarized in Table I. The randomized shRNA sequence was designed by arbitrarily replacing nucleotides within the Sox9-targeted shRNA while retaining 25–30% homology with the Sox9 target sequence and an identical %GC content.

The human Sox9 cDNA (accession number NM_000346O) was kindly provided by Dr. Masahiro Iwamoto (Thomas Jefferson University, Philadelphia, PA). The GFP cDNA was obtained from the plasmid phrGFP (Stratagene, La Jolla, CA). Both cDNA sequences were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) using standard cloning methods to generate pcDNA3-Sox9 and pcDNA3-GFP, respectively.

Cell culture and microscopy

The human embryonic kidney 293-T cell line was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and 100 µg/mL penicillin/streptomycin. The evening prior to transfection, cells were seeded at a density of 2×10^5 cells/mL in either 6 well plates or 75 cm² tissue culture flasks. HEK 293-T cells were transfected by the calcium phosphate co-precipitation method (Jordan et al., 1996). Transfected cultures were visualized in situ within tissue culture plates using an Olympus IX70 inverted confocal microscope (Center Valley, PA) with a long working distance lens.

Electrophoretic mobility shift assay

Nuclear extracts were prepared according to the method of Andrews and Faller (1991). Briefly, 5×10^6 293-T cells were transfected with 10 µg pcDNA-Sox9. Forty-eight hr post-transfection, cells were harvested and were resuspended in 0.4 mL low salt extraction buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) and were incubated on ice for 10 min. Swollen cells were centrifuged briefly for 10 sec and the supernatant was discarded. The pellet was resuspended in high salt extraction buffer (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and was incubated on ice for 20 min. Cellular debris was removed by centrifugation for 2 min at 4°C and nuclear extracts were stored at -70°C prior to use. Mobility shift assays were performed using Pierce Biotechnology's LightShift Electrophoretic Mobility Shift Assay (EMSA) kit (Rockford, IL). Nuclear extracts were diluted 1:10 in the final volume of the binding reaction, ensuring a low salt buffer for the binding assay. As per the supplier's protocol, binding reactions were performed in a 20 µL volume containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 mM KCl, 50 ng/µL poly (dI•dC), 2.5% glycerol, 0.5% NP-40, 2 mM MgCl₂, +/- 20 fmol biotinylated target oligonucleotide, +/- 4 pmol cold competing oligonucleotide, +/- 4–5 µg nuclear extract for 30 min at ambient temperature prior to electrophoresis through a 8% acrylamide gel containing 0.5X TBE. Electrophoretic transfer and chemiluminescent detection were performed according to the manufacturer's protocol. Preparation of nuclear extracts and EMSAs were independently performed three times, and representative findings are shown.

Protein isolation and western blotting

Adherent cultures were washed with excess PBS and recovered by incubation in ice-cold PBS containing 5 mM EDTA with gentle pipetting. Cell suspensions were centrifuged for 5 min at $1,500 \times g$ and cell pellets were lysed in 0.2 mL RIPA lysis buffer (50 mM Tris-HCl, [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1 mM PMSF on ice for 15 min (Harlow and Lane, 1988). Cell lysates were micro-centrifuged for 15 min at $12,000 \times g$ at 4°C and the resulting supernatants were transferred to fresh tubes. Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL). One hundred µg aliquots of protein were boiled for 5 min in 1x SDS loading buffer (10% glycerol, 2% SDS, 50 mM Tris HCl [pH 6.8], 0.001% bromophenol blue, 50 mM dithiothreitol) and were fractionated through 8% denaturing polyacrylamide gels using the discontinuous gel system of Laemmli (1970). Proteins were transferred to Hybond nitrocellulose membranes at 250 mA for 4 hr. Immunological detection was achieved using the ECL detection system (Amersham Life Sciences, Piscataway, NJ) and the supplier's

recommended protocol. Sox9 primary antibody and HRP-conjugated secondary antibodies were obtained through Santa Cruz Biotechnology (San Diego, California) and were used at dilutions of 1:1,000 in 1X PBS containing 0.5% (w/v) Carnation Nonfat Dried Milk and 0.1% (v/v) Tween 20. Incubations were performed for 1 hr with gentle agitation and were followed by sequential washes in excess 1X PBS containing 0.1% Tween 20 prior to both incubation in secondary antibody and prior to detection. Protein isolation and western analysis were independently performed a total of four times.

Results

Design of a Sox9-responsive shRNA expression plasmid

To develop a Sox9-responsive shRNA expression system, we initially created a constitutive H1 shRNA expression system based on the parent plasmid LITMUS38i in which we inserted the native H1 promoter as well as the SV40 origin of replication to amplify plasmid expression within HEK 293-T cells (Figure 1: pL-H1). To create a Sox9-responsive system, the STAF element of the native H1 promoter in pL-H1 was replaced by a monomeric Sox9 recognition sequence (Figure 1: pL-m1). Finally, to test gene silencing, a green fluorescent protein (GFP) targeted shRNA was introduced into the Bam HI/Bsr GI backbone of this expression plasmid, as well as within the control pL-H1 plasmid (pL-m1.gfp and pL-H1.gfp, respectively). Sequences for each of these inserts are reported in Table I.

Initial characterization of the Sox9-responsive shRNA expression system

In order to initially assess the Sox9-dependence of the pL-m1 expression plasmid, the SV40 transformed, human epithelial kidney HEK 293-T cell line was transiently co-transfected with (1) either pL-H1.gfp or pL-m1.gfp, and (2) pcDNA3-GFP, which constitutively expresses GFP under control of the CMV promoter. When cells were transfected with pcDNA-GFP alone, fluorescence was readily detected (Figure 2A). Co-transfection with the constitutive pL-H1/gfp control plasmid (Figure 2B) resulted in a marked decrease in fluorescence, presumably due to shRNA-mediated knockdown of GFP levels. Importantly, co-transfection with the experimental Sox-9 regulated pLm1/gfp plasmid (Figure 2D) failed to suppress fluorescence. As HEK-293T cells do not express Sox-9, this latter result was consistent with expression being dependent on Sox-9 expression.

We therefore tested how co-expression of Sox-9 would alter the fluorescence patterns of the different constructs. Cells transfected with pcDNA3-GFP as well as the constitutive pL-H1.gfp shRNA plasmid and/or with pcDNA3-Sox9, a CMV-driver Sox-9 expression plasmid, showed no Sox-9 dependent change in fluorescence (Figure 2C). Co-transfection of pL-m1/gfp with pcDNA3-Sox9 (Figure 2E) resulted in a nearly complete abrogation of GFP expression. To confirm that Sox9 specifically interacted with the m1 promoter sequences, an electrophoretic mobility shift assay was performed (Figure 2F). Nuclear extracts from HEK 293-T cells that had been transiently transfected with pcDNA3-Sox9 were incubated with oligonucleotides representing control sequences or Sox-9 specific sequences. In the absence of extract, only the unbound probe was resolved. In the presence of the Sox-9 transfected extract, a band was present that was absent in both control (P) and untransfected extract (C) and that was absent after competition with excess cold m1-oligonucleotide. Together, the data suggested a Sox9-dependent interaction with its target promoter sequence.

Sox9 expression can be auto-regulated by coordinate expression of inhibitory shRNA

By combining the elements of the system we have described, we hypothesized that we could create an auto-regulatory feed back loop in which Sox9 would promote expression of shRNA targeted against Sox9. In order to address this possibility, HEK 293-T cells were transfected with pcDNA3-Sox9 and pL-m1/Sox9i at ratios of 1:1, 1:3 or 1:5 (w/w). The 293-T cell line

was useful for this purpose as it tolerates large quantities of DNA during transfection, with minimal toxicity (Gavrilescu and Van Etten, 2007; Naldini et al, 1996). Abundant Sox9 expression was evident in cells transfected with pcDNA3-Sox9 alone (Figure 3A). Cells co-transfected with pL-m1-Sox9i (Sox9 shRNA) and pcDNA-Sox9 at ratios of 1:1 and 3:1 exhibited similar levels of Sox9 to those cells transfected with Sox-9 alone. However, when cells were co-transfected with pL-m1-Sox9i and pcDNA3-Sox9 at a ratio of 5:1, Sox9 expression was significantly reduced relative to the positive control. When HEK 293 T cells were co-transfected with pcDNA3-Sox9 and the randomized pL-m1-control shRNA expression plasmid at a ratio of 5:1, Sox9 expression remained comparable to cells transfected with pcDNA3-Sox9 alone.

Based on these findings, we examined the kinetics of Sox9 expression and silencing under these same conditions. For this purpose, HEK 293 T cells were cotransfected with pcDNA3-Sox9 and pL-m1-Sox9i at a ratio of 1:5. Protein extracts were prepared every 12 hr through 48 hr post-transfection and were analyzed by Western blotting for Sox9 (Figure 3B). Mock transfected 293 T control cells showed undetectable Sox9 expression, while Sox9 expression was present, but faint, at 12 and 24 hr post-transfection. Sox9 expression became abundant at 36 hr post-transfection and a lower band became apparent that could be unphosphorylated Sox 9. Sox-9 expression subsequently dropped to undetectable levels at 48 hr post-transfection.

Discussion

RNAi provides a powerful tool for achieving the sequence-dependent silencing of cellular genes. The fidelity of the silencing machinery and the amenability with which it lends itself to both transient and stable applications has spurred interest in its use in both basic research and clinical settings. Recent efforts to expand upon the versatility of RNAi, particularly by means of shRNA expression, have focused primarily on incorporating regulatory elements derived from a variety of well established regulated, RNA polymerase II-based mammalian expression systems (Higuchi et al., 2004; Hosono et al., 2004; Kappel et al., 2006; Lin et al., 2004; Ohkawa and Taira, 2000). In this study, we have described a regulated shRNA expression system for which expression is dependent on the cartilage-specific Sox9 transcription factor. We tested this system using green fluorescent protein. Co-expression of GFP, Sox9, and a GFP-targeted shRNA under the control of the m1 promoter resulted in a significant decline in GFP expression. In the absence of Sox9, however, GFP expression remained abundant, comparable to that observed in positive controls. The purpose of this system was to test if pL.m1-Sox9i expression plasmid exerted auto-regulatory control on Sox9 expression. The ability of this expression system to foster autoregulatory feedback gene silencing was further demonstrated to be dependent on the ratio of Sox9 to shRNA, and to be sequence specific as indicated by the inability of a randomized control shRNA to affect Sox9 expression under identical conditions. As an aside, Sox9 expression increased when 293-T cells were co-transfected with the randomized control shRNA. This unexpected effect is presumably a poorly understood consequence frequently attributed to non-specific upregulation of gene expression resulting from randomized shRNA controls (Bot et al, 2009; CR Chapman, 2006; Hsieh et al, 2004). The kinetics of Sox9-silencing was assessed over a 48 hr period in transiently transfected 293-T cells and an initial burst in Sox9 protein expression was observed to precede its subsequent silencing, further supporting the Sox9-dependence of the expression system. The specificity of the interaction between Sox9 and the m1, but not control H1, promoter was verified by electrophoretic mobility shift assay. In short, this shRNA expression system is the first to be dependent on endogenous, rather than heterologous or synthetic transcription factors.

Importantly, the design of the shRNA expression system described in this study is fundamentally different from conventional regulated shRNA expression systems. We chose to precisely delete the STAF transcriptional enhancer from the constitutive H1 promoter,

presumably debilitating basal expression. This region was replaced with the m1 oligonucleotide which contains a single consensus Sox9 binding site. Multimeric Sox9 binding sites were similarly introduced in place of the STAF enhancer element, but multimerization was not found to significantly improve upon the Sox9-dependence of the system (data not shown). Of particular note, a similar design strategy was recently employed in the production of an ecdysone-responsive shRNA expressing retroviral vector, replacing the Oct and STAF enhancer elements of the U6 promoter with a multimeric GAL4 binding motif (Gupta et al., 2004). Expression from this system was found to be tightly regulated, reversible, and highly induced in the presence of muristerone. More conventional, and generally applicable strategies have instead relied almost entirely upon shRNA suppression through steric hindrance by introducing heterologous operator sequences in the intervening region between the PSE and the TATA box, the promoter region determined to be most tolerant of sequence substitutions (Myslinski et al., 2001; Higuchi et al., 2004; Hosono et al., 2004; Kappel et al., 2006; Lin et al., 2004). Co-expression of the appropriate repressor in these models, suppressed shRNA expression; conversely, shRNA expression is permitted in the presence of the inducing agent (typically, IPTG or doxycycline). One alternative to this general strategy was recently described, in which the tetO sequence was cloned immediately upstream of the H1 promoter. Co-expression of a tetR-Krüppel-associated box fusion protein suppressed shRNA expression. Again, in this model, shRNA suppression was alleviated through the administration of doxycycline (Szulc et al., 2006).

Central to the function of all of these systems is the requirement for co-expression of heterologous *trans*-acting proteins and the need for pharmacological induction. In contrast, the Sox9-dependent shRNA expression system we have described is presented as “proof of principle,” providing an important model system for achieving gene silencing through tissue-specific transcription factors. Importantly, this approach eliminates the need for *trans*-acting components, vastly simplifies and streamlines system design and implementation, and provides for a finer degree of shRNA regulation within the specific target tissue depending upon cell cycle and/or differentiation status. This finer degree of control, we anticipate, will allow for more definitive and accurate characterization of gene silencing effects than might be obtained through pharmacological induction. Moreover, the precision with which gene silencing may be achieved suggests an array of therapeutic and tissue engineering strategies which may evolve through the use of “designer” shRNA expression systems intended for tissue-specific applications.

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Figure 1.

Schematic to depict the H1 promoter, core transcription elements, and derivative plasmids used in this study. Oct= Octamer-1 binding site, STAF= STAF enhancer, PSE= proximal sequence element, TATA= TATA box, shRNA depicts the relative cloning site for all shRNAs used in this study.

Figure 2.

Sox9 suppresses GFP expression through its recognition of the m1 promoter. Top Panel: 293-T cells were transiently co-transfected with pcDNA3-GFP (A) and either pL-H1/gfp or pL-m1/gfp (B and D, respectively) +/- pcDNA3-Sox9 (C and E, respectively). Fluorescence was assessed 48 hr post-transfection using a scanning laser confocal microscope at 40X magnification. (F) Representative results of electrophoretic mobility shift assay results using a m1 oligonucleotide and nuclear extracts prepared from 293-T cells transiently transfected with the constitutive Sox9 expression plasmid pcDNA-Sox9. P= probe alone, without extract. C= Probe with untransfected extract. E= Probe with Sox9-transfected extract. I= Probe with Sox9-transfected extract and a 50 molar excess of cold competing oligonucleotide.

Figure 3.

Auto-regulatory silencing of Sox9 expression is both time and dose dependent. (A) 293-T cells were transiently co-transfected with the amounts and plasmids indicated in the table above the figure. (B) 293-T cells were transiently co-transfected with pcDNA3-Sox9 and pL-m1/Sox9i at a ratio of 1:5. Protein samples were isolated at the indicated time points and were analyzed by Western blot to detect Sox9. The band below Sox9 may represent unphosphorylated Sox9 detected by our antibody. (C) Densitometry of western blots representing three independent transfections, showing expression levels as a function of time.

Table I

Oligonucleotide pairs used for the construction of plasmids described in this study.

Oligonucleotide	Sequence (5' 3')
ori 5'	5'-ctgtggaatgtgtcagta-3'
ori 3'	5'-agctttttgcaaaagcctagg-3'
H1-tata 5'	5'-gtcttaagatttgggaatcttaagtctgtatgagaccactcttccg-3'
H1-tata 3'	5'-gatccgggaaagagtggtctcatcagaacttataagattcccaaatcttaagac-3'
H1-wt 5'	5'-caatatttgcattgctgctatgtttctgggaatcaccataaacgtgaaatgtc-3'
H1-wt 3'	5'-ttaagacatttcacgtttatggtgatttccagaaacacatagcgacatgcaaatattgggcc-3'
Sox9 m1 5'	5'-caatatttgcattgctgctatgtttctgggaatcaccataaacgtgaaatgtc-3'
Sox9 m1 3'	5'-ttaagacatttcacgtttatggtgatttccagaaacacatagcgacatgcaaatattgggcc-3'
GFP F	5'-gatccgcaagctgaccctgaagttcaccacctgaacttcaggtcagcttcttttt-3'
GFP R	5'-gtacaaaaaagcaagctgaccctgaagttcaggtggtgaacttcaggtcagcttcttcg-3'
Sox9 533 F	5'-gatccggaggaagtcggtgaagaaccaccttcttcaccgacttctcttttt-3'
Sox9 533 R	5'-gtacaaaaaaggaggaagtcggtgaagaaccaccttcttcaccgacttctcttcg-3'
Control F	5'-gatccgactgtcacctgacgatgctccaccaagcatcgtcaggtgacagctcttttt-3'
Control R	5'-gtacaaaaaagactgtcacctgacgatgctccaccaagcatcgtcaggtgacagctc-3'

A summary of oligonucleotide pairs used during plasmid construction within this study. Those above the heavy black line contributed to the essential plasmid backbones described. Consensus Sox9 recognition sequences are presented in upper case. Oligonucleotide pairs below the line identify shRNA sequences used to target GFP, Sox9, and as control oligonucleotides used as a negative control, respectively. **The corresponding siRNA sequences expressed from these duplex molecules are 21, 19 and 21 nucleotides in length, respectively.**