Antisense and Short Hairpin RNA (shRNA) Constructs Targeting PIN (Protein Inhibitor of NOS) Ameliorate Aging-Related Erectile Dysfunction in the Rat

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A B S T R A C T

Introduction. Over-expression of penile neuronal nitric oxide synthase (PnNOS) from a plasmid ameliorates aging-related erectile dysfunction (ED), whereas over-expression of the protein inhibitor of NOS (PIN), that binds to nNOS, increases ED.

Aim. To improve this form of gene therapy for ED by comparing the electrical field response of short hairpin RNA (shRNA) for PIN with that of antisense PIN RNA.

Main Outcome Measure. Both shRNA and antisense RNA gene therapy vectors increased intracavernosal pressure in aged rats.

Methods. PIN small interfering RNA (siRNA), and plasmid constructs for cytomegalovirus promoter plasmid vector (pCMV-PIN), pCMV-PIN antisense RNA, pSilencer2.1-U6-PIN-shRNA; and pSilencer2.1-U6-randomer-shRNA were prepared and validated by transfection into HEK293 cells, determining the effects on PIN expression by Western blot. Plasmid constructs were then injected, followed by electroporation, into the penile corpora cavernosa of aged (20-month-old) Fisher 344 rats and, 1 month later, the erectile response was measured by intracavernosal pressure increase following electrical field stimulation (EFS) of the cavernosal nerve. PIN was estimated in penile tissue by Western blot and real-time reverse transcriptase–polymerase chain reaction. Cyclic guanosine monophosphate (cGMP) measurements were conducted by competitive enzyme immunoassay (EIA). Immunohistofluorescence detected PIN in corporal tissue sections.

Results. In cell culture, PIN siRNA and plasmid-expressed pU6-PIN-shRNA effectively reduced PIN expression from pCMV-PIN. pSilencer2.1-U6-PIN-shRNA corrected the impaired erectile response to EFS in aged rats and raised it above the value for young rats, more efficiently than pCMV-PIN antisense RNA. PIN mRNA expression in the penis was decreased by >70% by the shRNA but remained unaffected by the antisense RNA, whereas PIN protein expression was reduced in both cases, particularly in the dorsal nerve. PIN antisense increased cGMP concentration in treated tissue by twofold.


Key Words. Erectile Dysfunction; Gene Therapy; siRNA Technology

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Introduction

Erectile dysfunction (ED) in the human is strongly associated with aging [1–5], and in the laboratory rat, this is evidenced by a marked impairment of spontaneous and induced erections either by electrical stimulation of the cavernosal nerve or by pharmacologically induced relaxation of the corpora cavernosa smooth muscle [6–10]. This is presumably due to an inadequate activation of neuronal nitric oxide synthase (nNOS, NOS I), and thus a defective production of nitric oxide, the physiological mediator of neurotransmission controlling corpora smooth muscle relaxation [11–13]. Besides nNOS, endothelial nitric oxide synthase (eNOS) has been shown to be important in the maintenance of erection, possibly by shear stress activation [14].

The protein inhibitor of NOS (PIN) is an 89-amino-acid protein that inhibits nNOS activity by binding to this enzyme by impeding its dimerization [15–21]. This is thought to occur by PIN binding to a region (nNOS amino acids 224–240 [22]), between the amino terminal nNOS PDZ domain comprised of post synaptic density protein, drosophilia disc large tumor suppressor, and z0-1 protein [23] and the oxygenase domain [18], thereby destabilizing the nearby homodimer contact region in the oxygenase domain [24]. Truncated protein splice variants of both of penile nNOS (PnNOS) and the brain-type nNOS arise from alternative splicing that bypasses exon 2 [25], and conceptually, they should be refractory to modulation by PIN. Several lines of evidence suggest, however, that this may not be the mechanism or there may be several mechanisms by which PIN interferes with NOS activity. PIN has also been shown to inhibit the other NOS isoforms eNOS and inducible nitric oxide synthase (iNOS) [17–19]. Additionally, PIN is a light-chain dynein [21,26–29], a component of microtubules, and it has been proposed that PIN may be involved in nNOS association with the neuronal cytoskeleton during axonal transport [21,26], rather than acting solely as a NOS inhibitor.

The role of PIN in erectile function may be multifactorial. PIN has been detected in the rat brain [15,25,30,31], kidney, and skeletal muscle [32–35]. In the brain, PIN is present specifically in the hypothalamus in regions involved in the control of penile erection, and it colocalizes with PnNOS in oxytocinergic neurons [36]. However, the role of PIN in erectile function is unclear, because in hypothalamic tissue extracts it exhibited marginal inhibitory activity on NOS [36]. In the rat, PIN is expressed in the pelvic ganglia and the cavernosal nerves that control penile erection. By immunohistochemistry, PIN has been localized to both the cavernosal and dorsal nerves [37], where it has been shown by dual-fluorescence labeling to colocalize with PnNOS. Despite the colocalization of PIN with PnNOS, in aged rats, the mRNA levels of PnNOS or PIN in these nerves were not affected. Furthermore, in the adult rat, the intracavernosal injection of PIN cDNA into the intracavernosal tissue reduced the erectile response to electrical field stimulation (EFS) of the cavernosal nerve [37]. Notwithstanding this indirect evidence, the functional significance of PIN/PnNOS interactions for penile erection remains to be elucidated.

Several groups, including ours, have used the gene therapy of NOS isoforms to the corpora cavernosa as a therapeutic approach for aging-related ED. It is known that antisense and short hairpin RNA (shRNA) plasmid constructs silence gene expression by inhibiting mRNA translation and/or inducing its degradation [38–40]. In this current study, we have extended our previous observations on the effect of PIN on penile erections by determining whether the reduction of PIN levels by treatment with antisense or shRNA PIN constructs, which would presumably increase nNOS activity in the penis, can ameliorate aging-related ED.

Materials and Methods

Plasmid Constructs Encoding the PIN Antisense RNA and the PIN shRNA

The preparation of the antisense (AS) construct pCMV-PIN has been described previously [36,37]. Briefly, the full-length rat PIN cDNA was prepared and then subcloned in both forward and reverse orientations into the TA cloning, mammalian expression vector pCR3.1 (Invitrogen, Carlsbad, CA), thereby originating the antisense construct, as well as the sense construct named pCMV-PIN. Both inserted cDNAs were sequenced, and PIN protein expression was verified by transfection of HEK293 cells and Western blotting of PIN expression.

For the construction of the PIN siRNA, the rat PIN gene sequence (GenBank Accession No. NM_053319) was analyzed for a potential siRNA target using the web-based siRNA target finder and design tool provided on the Ambion website (Ambion, Inc., Austin, TX). Four regions were
identified as having the most likely inhibitory activity by siRNAs, defined by: (nucleotide position) and [target #]: 126 [2], 210 [8], 294 [15], and 372 [17]. Double-stranded siRNAs were transcribed “in vitro” using the Silencer siRNA construction kit (Ambion) as per the manufacturer’s instructions. In addition, a control siRNA targeting glyceraldehyde phosphate dehydrogenase (GAPDH) and provided with the kit was also synthesized. Each siRNA was tested for inhibitory activity at 0.1-, 2-, 10-, and 200-nM concentrations by cotransfection with pCMV-PIN and the individual siRNAs into HEK293 cell cultures grown in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) +10% fetal bovine serum using Lipofectamine 2000 (BD Biosciences, Palo Alto, CA). A nucleic acid (1-µg pCMV-PIN and appropriate concentration of siRNA) to liposome ratio of 1:1 was applied to six-well plates, and cells were incubated for 48 hours. Cell lysates were collected in mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL), and Western blots were performed (detailed below).

The best inhibitory siRNA (#2, 5′-AAGGCGGTGATCAAAAAATGCA-3′, nucleotide position 126) was then cloned into the pSilencer 2.1 U6-neo plasmid vector (2nd version, Ambion) as a short hairpin DNA sequence (5′ sense strand: 5′-GATCCGGCGGTGATCAAA AATGCAAGAGATGCTTTTGTGA CCGGCTTTTTTTGGAAA-3′) according to the manufacturer’s instructions, in order to generate the shRNA. The DNA sequence consists of a BamHI DNA restriction site, sense strand, nine nucleotide loop, antisense strand, RNA polymerase III terminator, and HindIII DNA restriction sites 5′ to 3′. In addition, an shRNA “randomer”, provided with the pSilencer kit and known not to block any mammalian mRNA, was also prepared. To test for silencing, the pSilencer 2.1-U6 neo-PIN shRNA plasmid construct or pSilencer 2.1-U6 neo-randomer plasmid was cotransfected with pcDNA3.1-PIN (each 1 µg per well, six-well plate) into HEK293 cell cultures using Lipofectamine 2000 (1:1 DNA/liposome ratio) as before for 48 hours and assayed by Western blotting. Inhibition of PIN by pCMV-PIN (AS) was performed in a similar manner.

In vivo Injection and Electroporation of Plasmid Constructs
Plasmids were grown up in E. coli strain DH5-α and purified using an Endo-free Maxi kit (Qiagen, Valencia, CA). Plasmids were quantitated by spectrophotometry and prepared in 0.9% saline solution at a concentration of 2 µg/µL. Male Fisher 344 rats, 20 months old, were maintained under controlled temperature and lighting, and treated according to National Institutes of Health regulations. The protocol was approved by the LA BioMed Animal Care and Use Review Committee. Animals were divided in four groups, anesthetized, and maintained during the procedure with 3% isoflurane gas, and treated by intracavernosal injection (100 µL) as follows: (1) saline; (2) pCMV-PIN (AS), 200 µg; (3) randomer shRNA, 200 µg; (4) PIN-shRNA 200 µg; (n = 6/group). Immediately after injection, electroporation was performed as described [11,41], using a 0.5-cm platinum needle electrode coupled to an electro-square porator (model ECM830; Gentronix, San Diego, CA). Settings were: 100 V (voltage); 40 msec (duration); 8 pulses per second (frequency); 1 second (interval); and unipolar (polarity).

Measurement of Erectile Response to Electrical Field Stimulation
At the indicated periods, animals were used for determinations of erectile response to EFS as described previously [11]. Briefly, after induction of general anesthesia, a midline surgical approach was used to obtain exposure of the cavernous nerve. Platinum electrodes were then applied to the cavernous nerve, and arterial and intracavernosal pressure measurements were obtained by simultaneous direct intrafemoral artery and cavernosal catheterization, respectively. The EFS was applied at 10 V and a frequency of 15 Hz for pulses of 30 seconds, separated for 2-minute intervals, with a Grass Stimulator (Grass Instruments, Quincy, MA). A data acquisition system (Biopac Systems, Santa Barbara, CA) connected to a personal computer simultaneously recorded arterial blood pressure (femoral artery) and intracavernosal pressure, and values were expressed as mm Hg (mean ± SEM). The ratios between the maximal intracavernosal pressure (MIP) and the mean arterial pressure (MAP) obtained at the peak of erectile response were calculated to normalize for variations in blood pressure.

Estimation of PIN mRNA by Real-Time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)
Total RNA was extracted from aliquots of penile shaft tissue (denuded shaft containing all components; i.e., urethra, connective tissue, smooth muscle cells, and endothelium) by the Trizol pro-
cedure (Gibco BRL, Gaithersburg, MD), and residual DNA was eliminated using TURBO DNA-free solution (Ambion). In total, 0.2 μg of RNA sample was reverse transcribed using Superscript III RNase H- reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) and random hexamers (0.25 μg) following the manufacturer’s protocol. Real-time polymerase chain reaction (PCR) was done with rat PIN-specific primers (forward, nucleotide position 224–243: TATCGCGGCC CCGTT -3, reverse, nucleotide position 304–324: TGGTCTCGTGTCGTCAGTGCC) to generate a 100-base pair PCR fragment (GenBank Accession No. BC059110). GAPDH was used as the control reference gene. Reaction conditions were based on the qPCR Mastermix Plus for SYBR Green I kit protocol (Applied Biosystems, Warrington, UK). Reaction was amplified in triplicate and ratio results were calculated based on the 2^-ΔΔC_T method. Real-time RT-PCR was conducted in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK).

**Estimation of PIN by Quantitative Western Blot**

Homogenates of frozen penile tissue (100 mg) or cells were obtained in boiling lysis buffer (10-mM Tris pH 7.4, 2% sodium dodecyl sulfate (SDS), and 1-mM Na ortho-vanadate) or M-PER (Pierce Biotechnology, Inc., Rockford, IL) and protease inhibitors (3-μM leupeptin, 1-μM pepstatin A, and 1-mM phenyl methyl sulfonyl fluoride), and centrifuging at 10,000 × g for 5 minutes. Equal amounts of supernatant protein (30 μg) were run on 15% polyacrylamide gels, and submitted to Western blot immunodetection with monoclonal antirat PIN IgG (whole-length 89 amino acids), at 1:500 dilution (BD Transduction), detecting the 10–11 kDa PIN band [36,37], followed by an antimouse goat IgG linked to horseradish peroxidase (BD Transduction). Visualizable of the bands was performed by a luminol enhancer solution reaction (Pierce Biotechnology, Inc.). Negative controls were performed without primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, GAPDH (1:3,000 dilution; Chemicon, Temecula, CA), upon reprobing.

**Immunohistochemical Detection of PIN Expression by Immunofluorescence**

After EFS was performed, rats were perfused with saline, and the penises were excised and denuded, and the penile shaft dissected. Small portions of tissue were fixed overnight in 10% formalin, washed in phosphate buffer solution (PBS) and stored at 4°C in 25% sucrose until sinking, embedded in optimal cutting temperature compound (OCT) and frozen onto dry ice. The remainder nonfixed tissue was frozen in liquid nitrogen and stored at -80°C. Tissue detection of PIN was carried out on cryostat 6-μm sections from the OCT-frozen tissue and refixed for 10 minutes at 4°C with 4% paraformaldehyde in Sorensen solution (0.1-M phosphate buffer) [36,37]. Sections were washed with PBS, permeabized with 0.3% Triton X-100, blocked with 10% normal goat serum in PBS with 0.3% Triton X-100, and incubated overnight in a humidified chamber at 4°C with the antirat PIN, described above (1:100 [w/v]). Fluorescence labeling was performed with biotinylated antirabbit secondary antibody followed by incubation with streptavidin Texas Red (13 μg/mL Vector Laboratories). Sections were mounted in Prolong Anti-Fade (Molecular Probes, Eugene, OR) and were examined using an Olympus BH-2 equipped with Spot-RT digital camera (Diagnostic Instruments Inc, Sterling Heights, MI, USA). Images were taken at 200 and 400× magnification, and were not adjusted for background intensity. Negative controls were done by replacing the first antibody with nonimmune IgG. Quantitative evaluation of stained sections for PIN staining was performed using the ImagePro 4.01 software (Media Cybernetics, Silver Spring, MD), coupled to an Olympus BHS microscope equipped with a Spot-RT digital camera, calibrated for spatial measurement and intensity. PIN was determined by measuring the area of staining per a fixed area. Four sections were examined per sample, with 6–8 fields assessed per tissue section.

**Determination of cGMP Concentration in Tissue Sections by Elisa Immunoassay**

Penile tissues (4 control and 4 PIN antisense) remaining following OCT tissue sectioning were removed from blocks, weighed, and homogenized in 1 mL of 5% TCA solution on ice [43]. Each
sample was centrifuged for 10 minutes, 1,500 × g, and the supernatant was recovered. TCA was removed by three extractions with a five-times volume of ether. Each sample’s cGMP concentration was determined in duplicate using a cyclic GMP EIA kit according to the manufacturer’s protocol (Cayman Chemical Co., Ann Arbor, MI). In brief, 500 µL of each sample was acetylated along with the cGMP standard dilution series. The samples and standard dilution (50 µL each) were applied to the plate containing a rabbit antibody specific for cGMP that is in turn bound by wells coated with mouse antirabbit IgG. Binding is determined by competition with a cGMP tracer. After overnight incubation, the wells were washed extensively, followed by incubation with Ellman’s reagent. The enzymatic reaction product was determined by spectrophotometry at 405-nm absorbance and expressed as pmol/mL.

**Statistical Analysis**

Values were expressed as mean ± SEM. The normality distribution of the data was established using the Wilk-Shapiro test, and the outcome measures between two groups were compared by unpaired *t*-test with Welch’s correction. Multiple comparisons among the different groups were analyzed by a single-factor ANOVA or a Kruskal–Wallis nonparametric test, followed by post-hoc comparisons with the Newman-Keuls or Dunn’s test, respectively, according to the GraphPad Prism 4.1 statistical program (San Diego, CA, USA). Differences among groups were considered significant at *P* < 0.05.

**Results**

When transfected into HEK293 cells, both the double-stranded siRNA and the shRNA plasmid constructs against PIN, inhibited considerably (70% siRNA at 10 nM; 60% shRNA) in vitro PIN protein expression from the respective PIN cDNA plasmid construct, as determined by Western blot (Figure 1, top and middle panel). In this case, cotransfections were performed in the presence of the PIN cDNA to generate detectable amounts of PIN protein, because the untreated control cells express PIN at an extremely low level. The antisense PIN construct, which consists of the entire antisense RNA for the PIN transcript, was also tested, and it reduced PIN protein expression by ~50% (Figure 1, bottom panel). No significant change was seen in the expression of the housekeeping gene, GAPDH for each experiment (Figure 1 and data not shown).

In order to determine whether blocking PIN protein expression in vivo by either the antisense or the shRNA technology is effective in stimulating erectile function, aged rats injected with the respective DNA constructs were subjected to EFS after 1 month. Figure 2 shows that in the aged rats injected with the PIN-AS vector, the
low MIP/MAP ratio indicative of ED, as seen in the saline injected control group, was significantly increased, reaching close to the control value (0.80) that we have routinely obtained in previous studies in young, adult rats of the same strain (e.g., [11]). The PIN shRNA was even more effective, increasing the EFS response slightly above the level seen in the young, adult rats. No significant effect of the PIN-AS or PIN shRNA vectors on unstimulated, basal intracavernosal pressure was detected (MIP : MAP values in mm Hg; saline 7.02 ± 0.47; PIN-AS 7.58 ± 0.53; randomer shRNA 7.51 ± 0.20; PIN shRNA 7.22 ± 0.46). No side effects resulting from blocking PIN were evident in terms of inducing priapism, or the activity, alertness, and general health status of the treated rats.

Determination of PIN mRNA by real-time RT-PCR showed that, in the penis of the rats treated with PIN-AS, there were no effects on PIN mRNA levels as compared with control rats in injected with saline (Figure 3). This was expected, because a full-length antisense RNA acts predominantly on translation and not necessarily on mRNA breakdown [38]. In contrast, PIN shRNA treatment led to a significant 50% reduction in PIN mRNA as compared with the rats treated with random shRNA, concordant to the postulated mechanism of action of shRNAs that leads to the respective mRNA degradation [39,40].

Quantitative Western blot analysis confirmed the interpretations regarding mRNA levels, because the treatment of aged rats with PIN-AS did inhibit the expression of the 10–11 kDa PIN protein, and this was virtually similar to the inhibition obtained with PIN shRNA (Figure 4). In agreement with the direct or indirect effects on translation of the antisense and shRNA constructs, the blockade of protein expression was very considerable, irrespective of the lack of effect on PIN mRNA levels by the antisense construct.

When PIN was visualized in penile sections by immunohistofluorescence, the downregulation of its expression in the dorsal nerve by PIN-AS appeared to be lower than the one seen by Western blot (Figure 5). The identification of the dorsal nerve was based on localization in the neurovascular bundle, as has been previously confirmed with dual staining with synaptophysin [37]. A similar situation was seen in the cavernous nerve terminals around the trabecular smooth muscle. This difference in the degree of inhibition of PIN expression by antisense PIN as assessed by the two procedures may reflect regional differences in PIN expression and the...
distribution of the injected plasmid along the penis which disappears when total shaft tissue extracts are analyzed by Western blot. In the case of the PIN shRNA, the obliteration of PIN expression in the dorsal nerve and the cavernous nerve terminals was very considerable, in line with the mRNA and Western blot results. No quantitative determination with image analysis was attempted because of the difficulty in applying it to measure variations in fluorescence intensity.

Although the action of PIN in erectile function is undoubtedly complex based on its multifaceted proposed activity, we examined whether blocking PIN would increase cGMP in the gene therapy-treated penile shaft tissues. PIN blocking by the antisense vector increased cGMP concentration approximately twofold (saline control: $0.072 \pm 0.011$ pmol/mL vs. antisense treated: $0.173 \pm 0.032$ pmol/mL; $P < 0.05$). PIN shRNA was not examined.

**Discussion**

To our knowledge, this is the first demonstration that inhibiting PIN expression by gene transfer to the penile corpora cavernosa of DNA constructs for the antisense and shRNA against PIN corrects, for at least 1 month, the ED that occurs in the aged rat. This opens up the possibility of strengthening the effects of nNOS gene therapy on erectile function.

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**Figure 4** Long-term effect of PIN antisense and PIN shRNA plasmid constructs on the expression of PIN protein in the penile tissue of aged rats subjected to EFS assayed by Western blot. Total protein homogenates were prepared from another aliquot of the penile tissue of the rats assayed for Figure 2 and were analyzed by Western blot as in Figure 1 ($N = 6$). *$P < 0.05$; **$P < 0.01$. AS = antisense; GAPDH = glyceraldehyde phosphate dehydrogenase; PIN = protein inhibitor of nitric oxide synthase; shRNA = short hairpin RNA.

**Figure 5** Long-term effect of PIN antisense and PIN shRNA plasmid constructs on the expression of PIN protein in the penile tissue of aged rats subjected to EFS around the site of injection assayed by immunofluorescence. Penile tissue cross-sections (4 sections per animal and 6 fields per section) were assayed for PIN expression by Texas red-streptavidin-biotin-linked secondary antibody, and representative pictures were taken around the dorsal nerve or the cavernosal trabecular smooth muscle. Control: animal injected with saline; sections from the randomer shRNA had a virtually identical appearance (data not shown). Penile sections from a second set of animals were examined and shown to replicate the representative pictures shown above. PIN = protein inhibitor of nitric oxide synthase; shRNA = short hairpin RNA.
response by also blocking the inhibitor of nNOS activity. Even more significant is the implication that PIN may regulate nitrergic neurotransmission, and that the other roles of PIN as a light-chain dynein in the maintenance of cell cytoskeleton do not seem to be essential because no toxic effects were visually evident in this animal model.

We had previously shown that in the rat, PnNOS, which is presumably responsible for the central and peripheral nitrergic neurotransmission for triggering erection during sexual stimulation, colocalizes with PIN in the dorsal and cavernosal nerves [37]. This same colocalization was also found in the hypothalamic regions of the rat, mainly the paraventricular nucleus (PVN), that control penile erection [36]. The improvement of erectile response achieved by the blockade of PIN expression in the penis in the current work provides indirect evidence that PIN physiologically may be inhibiting nitric oxide production, and this stimulation of erection is likely due to the removal of an inhibitory restraint on the activation of the full-length α form of nNOS. The β form of nNOS, which is present in both the rat and mouse [25], is insensitive to PIN because of the absence of exon 2.

Both of the gene therapy modalities used in this study to inhibit PIN expression appeared to be effective, but the PIN shRNA appears to be more effective, because it was superior not only in down-regulating PIN expression in vivo but also in stimulating the MIP/MAP ratio. In addition, the shRNA technology has in general replaced the earlier antisense cDNA approaches, because shRNA degrades the mRNA itself rather than just interfering with its translation as is seen with the antisense technology [39,40].

In another previous study, we showed that gene therapy of the penis with a replication-deficient adenoviral construct of PnNOS at low viral loads in combination with electroporation of the penile tissue can correct aging-related ED in the rat, but the effect of this treatment began to subside after approximately 4 weeks [11]. Because it has been reported that very little, if any, immune rejection occurs with this type of “gutless” adenoviral vectors [44], or that transcription activity would be gradually silenced in the case of the strong CMV promoter, it may be possible to extend the effectiveness of this form of gene therapy to longer periods by simply increasing the viral load. However, the current demonstration that simply blocking PIN expression via plasmid constructs restored erectile function for at least 1 month, opens up a new strategy that deserves further investigation (i.e., gene therapy with the combination of PnNOS and PIN shRNA). Whether replication-defective adenovirus or plasmids are the vectors of choice also needs to be determined.

Perhaps the most surprising result is the fact that no obvious deleterious effects were evident in the rat by markedly reducing the penile levels of a relatively abundant member of the cytoskeleton (dynein light chain LC8-type 1 [Dynll1]) [21,26–29]. PIN is highly conserved across species (100% protein homology between rat, mouse, and human, and over 90% with Caenorhabditis and Drosophila), and mutational inactivation of PIN in Drosophila causes multiple morphological abnormalities while its complete deletion is lethal [45]. Although PIN antisense or shRNA expression in other organs, such as the testis, where the dynein complex is vital for sperm motility [46,47], may be deleterious to fertility, it is still puzzling to consider that PIN may be of less importance as a component of the microtubule motor in the penis. Our PIN shRNA both in vitro and in vivo was a relatively weak shRNA vector. All other cloned PIN shRNAs tested were even weaker (data not shown). It may be plausible that a more effective shRNA would actually cause pleiotrophic damage to cells in culture or to the animals. This points out that future use of PIN shRNA may require a regulated expression or restricted tissue specificity.

PIN is an unusual protein in that it interacts with multiple partners through a degenerate binding motif [22]. This may at least partially explain its pleiotrophic effects. Future studies should determine whether PIN ablation on potential molecular and cellular targets, such as axonal transport, or even smooth muscle contractility, could be segregated. However, from the current study it appears that partial PIN inhibition is a viable approach to restore normal erectile function, possibly in combination with PnNOS therapy, and suggests that gene therapy with the PIN-insensitive PnNOS β form is another potential strategy.

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References


