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Small Interfering Peptide (*siP*) for In Vivo Examination of the Developing Lung Interactome

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Abstract

To understand the role of reactive oxygen species in mechanosensory control of lung development a new approach to interfere with protein-protein interactions via a short interacting peptide was developed. This technology was used in the developing rodent lung to examine the role of NADPH oxidase (NOX), CK2 (casein kinase 2), and the cystic fibrosis transmembrane conductance regulator (CFTR) in stretch induced differentiation. Interactions between these molecules was targeted in an *in utero* system with rAAV containing inserted DNA sequences that express a control peptide or small interfering peptides (*siPs*) specific for subunit interaction or phosphorylation predicted to be necessary for multimeric enzyme formation. In all cases only *siPs* with sequences necessary for a predicted normal function were found to interfere with assembly of the multimeric enzyme. A non-interfering control *siP* to non-essential regions or reporter genes alone had no effect. Physiologically, it was shown that *siPs* that interfered with the NOX-CFTR-CK2 complex that we call an “interactome” affected markers of stretch induced lung organogenesis including Wnt/ β -catenin signaling.

Background

Growth and development of the fetal lung require mechanosensory regulation of gene expression (Sanchez-Esteban et al., 1998; Ingber, 2006). The consequent changes in pressure in the fluid filled lung lead to stretch induced differentiation through established pathways involving CFTR (Cohen and Larson, 2006) as well as PTHrP and canonical Wnt signaling (Torday et al., 1998; Torday, 2003; Rehan and Torday, 2007; Torday and Rehan, 2007a; Cohen et al., 2008). Although recent work by our laboratory suggested that CFTR may act as a stretch sensor to convert the mechanical forces into activation of developmental pathways (Cohen et al., 2008), the interactions between the genes that regulate stretch verses those involved in differentiation are not known.

Examination of genes essential to developmental processes usually involves disruption of function either through genomic mutation or inhibition of translation. In eukaryotes, the knockout mouse (Mikkola and Orkin, 2005) is the most established method for the inactivation

of genes. Combining this technology with knockin of genes under inducible or tissue specific promoters (Kwan, 2002; Mauvais-Jarvis et al., 2002; Giuriato et al., 2004) refines the control of gene expression. Both methods, however, lack the ability to transiently modify gene expression in the relatively small numbers of cells involved in mechanosensory control of development. In previous studies we have used transient in utero knockout of gene expression with viral vectors expressing anti-sense gene constructs to disrupt stretch-induced differentiation and associated pathways (Cohen et al., 2004; Cohen and Larson, 2005; Cohen et al., 2008).

The interactomics of proteins can be examined in vitro using interfering peptides (Pagano et al., 2008). In vivo developmental protein-protein interactions, however, can not be defined directly, thus knockout and knockin systems of expression must be used to infer the underlying interactomics. However, these methods affect all cells in the target tissue and thus do not permit isolation of only the stretch dependent effects.

NADPH oxidase (NOX) and the production of reactive oxygen species (ROS) was shown recently to be involved in regulation of smooth muscle contraction (Ritsick et al., 2007). In addition, NOX and the related dual oxidase (DUOX) are known to be expressed in the lung and regulated developmentally (Geiszt et al., 2003; Fischer et al., 2007; Pacquelet et al., 2008; van der Vliet, 2008). In this paper a technique is developed to examine the underlying interactions which we define as the interactome of the developing lung. This method utilizes *in utero* gene transfer (Sekhon and Larson, 1995; Garrett et al., 2003) which allows direct targeting of the lung via intra-amniotic injection and stage specific effects via gestation timing. Using this novel technique the interaction between NOX components (p47 and gp91), muscle contraction (myosin light chain), lung development (wnt signaling), protein kinase CK2 (formerly casein kinase 2), and CFTR was examined. This work was precipitated by the striking hypothesis that CFTR might act allosterically to control CK2, the first ion channel shown to control a developmentally essential kinase with divergent biology (Pagano et al., 2008).

Methods

Recombinant Adeno-Associated Virus (rAAV) production

DNA sequences for selected pHOX 47 amino acid sequences (Morozov et al., 1998) (Table 1) were synthesized and cloned into the Helper Free rAAV cloning system, pAAV-IRES-hrGFP from the Stratagene (La Jolla, CA, USA). The multiple cloning site uses a CMV promoter and an internal ribosome entry site (IRES) for the independent translation of hrGFP (green fluorescent protein) as a validating marker of recombinant gene expression. Virus was produced in 293 cells and rAAV purified using BioVantage Inc. purification method. Infectious units were estimated based on the formula $1 \text{ OD}_{280} = 10^{12} \text{ rAAV particles/ml}$.

BAT-gal Wnt/ β -Catenin Reporter Mice

Generation and characterization of the BAT-gal mice have been previously described (Maretto et al., 2003). E1 was defined as the day that the vaginal plug was observed

In utero gene and inhibitor transfer

Rat or mouse fetuses are treated with 10^{10} rAAV particles/ml amniotic fluid at 16 days gestation (E16) as described previously (Garrett et al., 2003; Garrett et al., 2004). Briefly, the fetuses were exposed following laparotomy and rAAV was injected directly into the amniotic fluid surrounding each fetus. Fetuses spontaneously breathe and swallow amniotic fluid thereby resulting in primary infection of the lungs and intestines (Sekhon and Larson, 1995). All fetuses were harvested at 24 or 48 hours post gene transfer. Casein kinase 2 (CK2) inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB; 10 μ M) and P2 receptor antagonist

suramin (100 μ M) were introduced into the amniotic fluid in an identical manner and with DMEM carrier.

Tissue processing and immunohistochemistry

Fetuses were fixed in 4% phosphate buffered, methanol-free paraformaldehyde for at least 24 hours. Cryopreserved fetuses were then sectioned (8 microns), washed, immunohistochemistry performed as described previously (Cohen and Larson, 2006). Species-specific, fluorescently labeled Alexa (568nm or 647nm; Invitrogen, USA) antibodies were used. Primary antibodies for NADPH oxidase subunits gp91 and p47 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent microscopy was performed on an Olympus microscope with Xenon light source SlideBook software data acquisition, deconvolution, and 3-dimensional imaging. Nuclei were stained with DAPI. Fetal lungs were examined in all sections for cells expressing gp91, p47, and hrGFP using 4 color imaging.

Results

Specificity of peptides in disrupting assembly of NADPH oxidase gp91/p47 complex

Fetuses from separate litters were infected with rAAV containing either β -galactosidase (*lacZ*) or *siPs* (*sipNOX1-3*; Table 1) at E16 for harvest 24 hours later. Normally, the NADPH oxidase subunits gp91 and p47 form a complex that is membrane associated (DeLeo et al., 1995). As shown in Figure 1, *lacZ* and *sipNOX-1*, which is a peptide from a region of p47 not known to have any function in forming this complex, did not have any significant effect on the association of gp91 and p47 in the membrane. Co-localization to form membrane associated complex is indicated by both the merged colors (yellow) and the 3D localization (surface 3D does not merge colors, but red lies adjacent to green). That these cells were actually infected with the *sipNOX-1* rAAV is indicated by the hrGFP positive pink signal. Thus, expression of a non-specific peptide had no effect on the normal association of subunits, similar to that obtained with reporter, *lacZ*, gene (see also Figure 2).

In contrast, expression of *sipNOX-2*, which is specific for a gp91-binding region in p47 (Table 1), had a significant effect on complex formation and there was now only marginal evidence of any association between p47 and gp91 in either the merge or 3D reconstructions (third row). Additionally, the uniform green staining suggests that p47 remained in the cytoplasm which was confirmed by serial video sectioning represented by the 3-D reconstructions. Again, hrGFP expression in these cells confirmed infection by the rAAV.

The NOX complex must be phosphorylated for activation of the membrane associated complex (Decoursey and Ligeti, 2005). Thus, the question arises as to whether one can use *siP* technology to effectively compete with the phosphorylating enzyme. The peptide *sipNOX-3* was specific for the phosphorylation site on p47. Because a CMV promoter was used, one would expect sustained, high level expression of the *siP*. Cells expressing *sipNOX-3* (Fig. 1; lowermost panel; hrGFP positive) exhibited a phenotype similar to that seen with *sipNOX-2*, in which p47 remained cytoplasmic with little evidence from the merge or 3D reconstruction of complex formation. However, one difference between *sipNOX-2* and *sipNOX-3* was noted. In *sipNOX-3* expressing cells the p47 staining appears to form aggregates within the cytoplasmic space of the cell demonstrating that inappropriate assembly of p47 was occurring.

The ability of *sipNOX-2* and *-3* to interfere with the formation of the membrane associated complex demonstrates the utility of this methodology. In addition, different phenotypes were readily recognized upon microscopic examination increasing its utility in analyzing structural versus biochemical modifications necessary for function.

Partial homology *siPs* in dissecting amino acids essential for function

The *siP* technology potentially could be used to determine those amino acids either essential for subunit association or involved in post-translation modification and activation. For these analyses one would produce *siP* sequences with only partial homology to the original protein. To test the utility of *siPs* in defining functional amino acids, *sipNOX-4* was produced which had 61.5% homology with *sipNOX-2* region of p47 (Table 1). For these experiments a reporter gene, β -galactosidase (*lacZ*) was used. Because this rAAV does not include hrGFP, it was not visualized in this analysis. As expected the rAAVCMV*lacZ* treated animals showed cells that exhibited co-localization of p47 and gp91 in the membrane of the cell (Figure 2). In contrast, *sipNOX-4*, completely blocked p47 association with gp91. Once again, as with *sip-NOX-3*, a significant aggregation of p47 in the cytoplasm occurred (Fig. 1).

Physiologic effect of *siP*-dependent disruption of NOX

This laboratory has examined contraction of smooth muscles in the developing lung using phosphorylation of regulatory myosin light chain 20kda (MLC₂₀) as a marker (Cohen and Larson, 2006). NOX activity was shown previously by others to alter muscle contraction (Lyle and Griendling, 2006). To determine if the *siP*-dependent disruption of NOX had any effect on the physiology of this tissue related to stretch induced differentiation, the expression and phosphorylation of MLC₂₀ was examined at 24 and 48 hours post-*siP* therapy.

As demonstrated in Fig. 3, the *siP* that was unable to dissociate NOX, *sipNox-1* showed a pattern of expression for phosphorylated MLC₂₀ and unphosphorylated MLC₂₀ (P- MLC₂₀) at 24 and 48 hours post-*siP* that differed significantly from that observed with the NOX-specific *siPs* (Fig 3B; (Cohen and Larson, 2006)). Although there was variation in the response, by 48 hours post-therapy, all NOX-specific *siPs* showed a decrease in P-MLC₂₀, the active form necessary for muscle contractions and stretch-induced differentiation. Actin levels were consistent between samples, however, more importantly; the levels of hrGFP expressed by the individual *siP* constructs via the IRES sequence were similar in each tissue. Thus, the infectivity and expression levels of the *siPs* were similar and concentration differences do not account for differences in MLC₂₀ expression. Protein stability may account for the variation seen in the response to the NOX-specific *siPs*. However, in concordance with the previous work showing an affect of NOX on muscle contraction (Lyle and Griendling, 2006), *siPs* that were found to disrupt NOX assembly and membrane association also indirectly affected the synthesis and phosphorylation of the regulatory subunit required for muscle contraction.

Use of *siP* technology to dissect the CFTR CK2 NOX sequential pathway interactions

Phosphorylation of NOX is known to be mediated by casein kinase 2 (CK2) (Park et al., 2001). Recently, it was demonstrated that CK2 is bound to a pathophysiologically important domain of the cystic fibrosis transmembrane conductance regulator (CFTR) by a specific sequence that can be allosterically regulated by the KENIIF peptide (Table 1; (Pagano et al., 2008)). This KENIIF peptide corresponds to the wild type sequence found in CFTR whereas the KENII mimics the common F508del mutant. Furthermore, interference with this interaction between CK2 and CFTR alters the function of CFTR (Treharne et al submitted) and CK2. Because both CFTR and NOX are known to affect muscle contraction in the developing lung (Fig. 3; (Cohen and Larson, 2006)), *sipKENIIF* and control *sipKENII* were examined for their ability to disrupt NOX in fetal lungs.

To confirm that our methods were sufficiently robust to detect inhibition of CK2-dependent phosphorylation and assembly of the NOX complex on the cell surface, fetuses were treated with either the CK2 specific inhibitor, DRB, or a G-protein coupled receptor inhibitor, Suramin. As can be seen in Fig. 4, the Suramin had no effect yielding results (Fig. 4) similar to *lacZ* and the non-specific, *sipNOX-1* (Fig 1 & 2). The CK2 specific inhibitor gave similar effects to that

seen with both the phosphorylation-specific, *sipNOX-3* (Fig. 1) in which p47 aggregates and does not full associate with gp91 in the normal diffuse membrane associated pattern (Fig. 4). Thus, the participation of CK2 in NOX assembly and association with the plasma membrane was confirmed using this method. This finding is consistent with a regulatory effect of CK2.

Comparison of the control *sipKENII* and reporter gene (*lacZ*; Figs. 1 & 2) treated cells showed that the *sipKENII* had no intrinsic affect on NOX assembly (Fig. 4). The *siP* that modifies the CFTR CK2 interaction (*sipKENIIF*), however, altered the interaction between p47 and gp91 in a manner similar to that seen with both the phosphorylation-specific, *sipNOX-3* (Fig. 1) and importantly the CK2-specific inhibitor DRB. Cytoplasmic aggregates (arrows) are present in *sipKENIIF* and in both DRB and *sipNOX-3* (Fig. 1) treated fetuses. It is clearly evident that CK2-CFTR specific *sipKENIIF* had less affect then either DRB or *sipNOX-3*, suggesting that the CFTR-CK2 interaction was only partially responsible for CK2-dependent activation of NOX.

Relationship between muscle contraction and the CFTR CK2 NOX interactonome

Previously, CFTR was shown to affect muscle contractions necessary for mechanosensory regulation of lung development through altered MLC₂₀ and Wnt/ β -catenin pathways (Cohen and Larson, 2006; Cohen et al., 2008). To examine the role of the CFTR-CK2-NOX interaction in this process MLC₂₀ physiology was examined following *sipKENII* and *sipKENIIF* challenges. All *sipNOX* constructions showed an increased unphosphorylated MLC₂₀ at 24 hours post-treatment of the fetal lung (Fig. 3B), likewise, a similar effect was obtained with *sipKENIIF* when compared to the inactive *sipKENII* (Fig. 5). Elevated LC₂₀ at 48 hours following *sipKENIIF*, however, was less severe than that of the phosphorylation specific NOX, *sipNOX-3*, which exhibited decreased unphosphorylated LC₂₀. Increased phosphorylation of LC₂₀ following *sipKENIIF* occurred at both 24 and 48 hours post-transfer a result distinct from that of the NOX-specific *siPs*. This result is not surprising given that disruption of the CK2-CFTR interaction was recently shown to block the chloride channel function (Pagano et al., 2008) necessary for production of MLC₂₀ (Cohen and Larson, 2006). Thus, these results are consistent with a duality of CFTR function in which the CK2 binding function influences both NOX for expression of unphosphorylated MLC₂₀ and the previously demonstrated chloride channel function required for P- MLC₂₀ levels.

To confirm the increased expression of MLC₂₀ following *sipNOX* or *sipKENIIF*, fluorescent immunohistochemistry was performed on lung tissues collected at 24 hours post-*siP* treatment (Fig. 6). Control *lacZ* and *sipKENII* were compared to *sipKENIIF* and *sipNOX-4*, the most effective *sipNOX* in altering MLC₂₀ (Fig 3). Low levels of MLC₂₀ were detected in both *lacZ* and *sipKENII* treated lungs (Fig. 6A&B). In direct contrast both the *sipKENIIF* and *sipNOX-4* treated lungs showed increased expression (Fig. 6C&D). The *sipNOX-4* which caused aggregation of NADPH oxidase has a much greater effect.

The stoichiometry of the in utero gene transfer system is such that the total number of infected cells is relatively small ($<10^6$), because only 10^7 infectious particles total are injected into the amniotic fluid and this inoculum is distributed between the amnion, skin, lung, and intestines (Sekhon and Larson, 1995; Larson et al., 2000; Garrett et al., 2003). Thus, only a small fraction of the total epithelial cell layer of the developing lung is targeted. Consistent with previous results on in utero gene transfer, only a small proportion of cells are hrGFP positive (arrows). That the observed physiologic effects were due to disruption of NADPH oxidase and not non-specific *siP* interference is shown by the *hrGFP* positive cells distinct from the MLC₂₀ positive cells. Thus, reactive oxygen produced by NADPH oxidase expressing cells is involved in regulation of MLC₂₀ expression (Fig. 6C & D) and phosphorylation (Figs. 3 & 5).

Wnt/ β -catenin signaling activity following disruption of the CFTR CK2 NOX interactome

Previously we demonstrated that disruption of stretch induced differentiation by antisense and sense CFTR resulted in altered Wnt/ β -catenin signaling (Cohen et al., 2008). In these studies the Bat-gal mice which has β -galactosidase with a canonical Wnt promoter was used. To confirm that the CFTR-CK2-NOX interaction was involved in stretch induction, the effect of the various *siP* constructs on Wnt signaling in Bat-gal mice was examined. As shown in Figure 7 the non-functional *siP*, *siPNOX-1*, shows little or no evidence of Wnt signaling activation at E16 following gene transfer at E15. However, all the NOX function-specific *siPs*, *siPNOX-2*, 3, & 4, showed statistically significant increases in Wnt signaling consistent with altered stretch. In addition, the *siP* specific for CFTR CK2 interaction, *siPKENIIF*, also showed significant increases in Wnt signaling. Furthermore, those interfering peptides with the most direct activity on NOX assembly, *siPNOX-2*, and phosphorylation, *siPNOX-3*, had greater effects than the partial homology, *siPNOX-4* or the CFTR-CK2 interfering peptide, *siPKENIIF*.

Discussion

At the time of these experiments, the role of NOX in muscle contraction was not known. Recently, Ristic and co-workers (Ristic et al., 2007) showed in a *RNAi* inducible system that inactivation of NOX had a negative effect on muscle contraction. Thus, our data that inhibition of NOX association and/or phosphorylation altered production of the phosphorylated regulation MLC₂₀ necessary for muscle contraction (Figs. 3, 5 & 6) are consistent with the result obtained with their inducible *RNAi* system. In addition, the effect of the short interfering peptides on Wnt/ β -catenin signaling (Fig. 7) was consistent with previously published data on altered stretch in lung development by our (Cohen and Larson, 2006; Cohen et al., 2008) and that of Torday and Rehan (Sanchez-Esteban et al., 1998; Torday et al., 1998; Torday and Rehan, 2007b).

Combining the results from *siPNOX* studies with those obtained with *siPKENIIF/KENII* and previously published work by others allows the construction of a possible CFTR-CK2-NOX interactome for developmental regulation of muscle contraction necessary for stretch-induced differentiation of the lung (Figure 8). Because this ion channel is inactivated by disruption of the cytoskeleton (Cantiello, 2001; Chasan et al., 2002) CFTR would be ideally located to act as a stretch sensor. This role is supported by CFTR modulation of the Torday-Rehan stretch model involving PTHrP and Wnt (Torday and Rehan, 2006; Torday and Rehan, 2007b; Cohen et al., 2008). The chloride secretion function would regulated production of ATP and a wave of calcium production necessary for peristaltic muscle contractions in the developing airway (Cohen and Larson, 2006). The CK2 function associated with active CFTR (Pagano et al., 2008) would promote muscle contraction by removing H₂O₂ and producing ROS blocking the effects of H₂O₂ and NO on muscle relaxation. Upon smooth muscle contraction and induced mechanical stretch, disruption of the actin CFTR cytoskeleton interaction would inactivate the CFTR sensory function turning calcium and activated NOX production off to inhibit further contractions and allowing H₂O₂ and NO dependent muscle relaxation. At this point, the direct interaction between CFTR, CK2, and NOX affecting stretch induced differentiation is supported by multiple, independent lines of evidence, however, a direct link between them and specific stretch-dependent markers is needed. Tentative steps in this direction have been taken in this direction by D Litchfield and co workers who report the presence of a CK2 regulated actin capping protein called CKIP (Olsten et al., 2005; Canton et al., 2006).

The *siP* technology has the potential to be of significant use in discerning specific amino acids involved in protein-protein interactions and as an adjunct to more traditional methods for gene knockout/knockdown methods. Because the peptides used in this method were <20 amino acids in length, all the DNA sequences used in cloning were readily synthesized and cloned with minimal effort and expense. The Helper-Free rAAV system requires a simple transfection of

3 plasmids and results in high yields of virus particles. Thus, new *siP* variations were generated in weeks rather than the months needed for more traditional transgenic technologies. In addition, rAAV was shown to readily infect fetuses from mice, rats, and non-human primates with prolonged expression and physiologic effect for over a year (Garrett et al., 2003; Garrett et al., 2004) making them suited for application across many species and genetic backgrounds.

Like *RNAi*, *siP* targeting the same site can have variable efficiencies. Comparison of *sipNOX-2* and *sipNOX-4* showed that although the former had complete homology with the target sequence, the latter had different physiologic effects (Fig. 3 and 7). This may reflect variable translational efficiency, peptide stability, or destabilization of the target resulting in its degradation. Regardless, it does demonstrate that like *RNAi*, it is not possible to completely predict the efficiency of specific sequences in generating a physiologic effect.

In addition to their investigative role, *siPs* may be used therapeutically. Because they can be designed to interfere with either protein assembly or modifications necessary for activation, *siPs* may be useful as antiviral agents for example. Should the small size of the *siP* may permit its escaping from immune surveillance, properly designed *siPs* could be repeatedly used to “vaccinate” bone marrow cells to compete with proteins necessary for the assembly and replication of HIV.

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Figure 1. Interference of NOX assembly with *sip* rAAV constructs

Rat fetuses were infected at E16 with either rAAVCMV*lacZ* or one of three different (top to bottom rows) rAAV*sips* (Table 1) and fetuses harvested at 24 hours post-gene transfer. Cryosections of fetal lung were immunostained for NOX subunits p47 (green) and gp91 (red). DAPI (blue) was used to stain the nucleus and GFP was visualized (Pink). High resolution, 3 dimension, deconvoluting microscopy was performed. Vertical panels from left to right are specific for hrGFP, p47, gp91, and merge p47/gp91. A 3-D surface expression of each p47/gp91 merge is presented (rightmost). Scale bar is presented in the lower right Immunofluorescent panel and each grid mark in the 3-D reconstruction is 5 μ m.

Figure 2. Functionality of partial *sip* homology

Rat fetuses were infected at E16 with either rAAVCMV*lacZ* or rAAV*sipNOX-4* (Table 1) and fetuses harvested at 24 hours post-gene transfer. Cryosections of fetal lung were immunostained for NOX subunits p47 (green) and gp91 (red). DAPI (blue) was used to stain the nucleus. Arrows indicate the punctuate aggregation of p47. Scale bars are present in merge panels and 3-D reconstructions are on a 5 µm grid.

Figure 3. Myosin light chain regulatory subunit (MLC₂₀) expression and phosphorylation following *sip*- dependent NOX dissociation

Lungs from fetuses infected with different rAAVsips were harvest at 48 and 24 hours post-gene transfer. Panel A – Western blot with MLC₂₀, phosphorylated MLC₂₀, hrGFP, and actin specific antibodies; Pane B – Level of expression of MLC₂₀ and P- MLC₂₀ standardized to the level of hrGFP protein.

Figure 4. Effect of disruption of the CK2-CFTR interaction on NOX assembly

Fetuses were treated with rAAV carrying either *sipKENIIF*, *sipKENII* (Table 1), DRB, or Suramin and harvested at 24 hours post-treatment and immunohistochemistry performed for p47 (green) and gp91 (red) with DAPI (blue) nucleus staining. Scale bars are present in merge panels and 3-D reconstructions are on a 5 μ m grid.

Figure 5. Characterization of MLC₂₀ expression following treatment with *sipKENII* and *sipKENIIF*

Fetuses were treated with either *sipKENII* or *sipKENIIF* and lungs harvested at 24 and 48 hours post-gene transfer. Panel A -Non phosphorylated and phosphorylated (P-MLC₂₀) forms of MLC₂₀ were identified by Western blotting. Expression of hrGFP from the *siP* clones and actin levels for all samples were visualized with specific antibodies. Panel B – Level of expression of MLC₂₀ and P-MLC₂₀ standardized in each sample to the level of hrGFP protein.

Figure 6. Impact of NOX interfering peptides on myosin light chain contraction regulatory subunit MLC₂₀

Recombinant AAVs with either *lacZ* (A) *sipKENII* (B), *sipKENIIF* (C), or *sipNOX-4* (D) were infected into rat fetuses at E16. At 24 hours post-gene transfer frozen sections of lung were stained for MLC₂₀ (red) or nuclei (DAPI; blue) Arrows indicated hrGFP positive cells. Panel A construct has no GFP as negative control

Figure 7. Wnt/β-catenin signaling activity following *siP* challenge

Bat-gal mice were injected at E15 with rAAV containing either *sipNOX-1*, -2, -3, -4, or *sipKENIIF* and lungs harvested 24 hours later. Tissue was homogenized and β-galactosidase enzyme assays and total protein determinations performed. * $p < 0.05$.

Figure 8. Proposed CFTR-CK2-NADPH oxidase interactome

A. 1- CFTR is known to promote muscle contraction via chloride and ATP release (Cohen and Larson, 2006). 2 – CFTR interacts with CK2 to control CFTR activity (Pagano et al., 2008) 3 - CK2 phosphorylation of NADPH oxidase is inhibited by *sipNOX-3* and/or *sipKENIIF* (Figs. 4–6). 4 – Reactive oxygen species (ROS) are generated during lung development from H₂O₂ which is involved in muscle relaxation. ROS is also known to block NO dependent muscle relaxation (Marechal and Gailly, 1999). B. 5 – Stretch inhibits CFTR function (Cantiello, 2001; Chasan et al., 2002). 6 – NO and H₂O₂ promote muscle relaxation (Marechal and Gailly, 1999).

TABLE 1Short Interfering Peptides (*siP*)

<i>siP</i>	Gene (amino acids)	Sequence	Predicted domain function
<i>siPNOX-1</i>	p47 (61–75)	EAGAINPENRIIPHL	None (Morozov et al., 1998)
<i>siPNOX-2</i>	p47 (37–51)	EKVYRRFTEIYEFH	Binding site between p47 and gp91 (Morozov et al., 1998)
<i>siPNOX-3</i>	p47 (357–371)	QRSKPQPAVPPRPSA	Phosphorylation site for activation of NOX (Morozov et al., 1998)
<i>siPNOX-4</i>	P 47 61.5% Homology (40–52; Lower Sequence)	ERIGTVYRGFSDIYEIYKN VYRRFTEIYEFHK	Binding site between p47 and gp91 (Morozov et al., 1998)
<i>siPKENII</i>	CFTR	GTI KENII GVSYDEYR	F508del Mutant CFTR Casein Kinase 2 (CK2) Binding site (Pagano et al., 2008)
<i>siPKENIIF</i>	CFTR	GTI KENIIF GVSYDEYR	CFTR CK2 Binding site; (Pagano et al., 2008)