January 2014

Surfactant Enhanced Delivery Of C/ebp Homologous Protein And Angiopoietin 2 Silencing Rna To Prevent Bronchopulmonary Dysplasia In Mouse Lung

John Gregory Ramirez
Yale School of Medicine, jkat311@gmail.com

Surfactant enhanced delivery of C/EBP homologous protein and Angiopoietin 2 silencing RNA to prevent bronchopulmonary dysplasia in mouse lung

A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

John G. Ramirez

2014

Table of Contents

Abstract

SURFACTANT ENHANCED DELIVERY OF C/EBP HOMOLOGOUS PROTEIN AND ANGIOPOIETIN 2 SILENCING RNA TO PREVENT BRONCHOPULMONARY DYSPLASIA IN MOUSE LUNG. John G. Ramirez, Mansoor A. Syed, Vineet Bhandari. Section of Neonatal-Perinatal Medicine, Department of Pediatrics, Yale University, School of Medicine, New Haven, CT.

Objective. To determine if poractant alfa (Curosurf®) could serve as a viable vehicle for silencing RNA (siRNA) delivery. Specifically, we hypothesize that use of Curosurf® as an enhanced delivery vehicle for C/EBP homologous protein (CHOP) and Angiopoietin 2 (Ang2) siRNA would augment gene silencing, preventing cell death and bronchopulmonary dysplasia (BPD) in the mouse lung.

Design. Transfection efficiency of Curosurf® was compared to naked siRNA and Lipofectamine 2000®. MLE-12 cells were transfected with CHOP and Ang2 siRNA using Curosurf®, then exposed to hyperoxic conditions. Cultures were evaluated for respective protein expression and markers of cell death. Curosurf® enhanced siRNA therapy was tested in a hyperoxia-induced mouse model of BPD. Protein expression and lung morphology were assessed and compared to naked siRNA.

Results. Curosurf® significantly improved cell transfection rates when compared to naked siRNA alone. Cell cultures treated with Curosurf® enhanced CHOP and Ang2 siRNA delivery had significantly lower levels of expression of their respective protein products. Cleaved caspase 3, a marker of cell death, was also decreased in cells transfected using Curosurf®. Curosurf® enhanced delivery of Ang2 siRNA had a modest, but significant, improvement in mouse lung morphology in a hyperoxia-induced model of BPD.

Conclusions. Curosurf® enhances silencing RNA delivery of C/EBP homologous protein and Angiopoietin 2, resulting in an improvement in mouse lung phenotype in a hyperoxia-induced model of BPD.

Acknowledgements

I wish to acknowledge Dr. Michael O'Brien and Dr. Nancy Angoff – for their mentorship and support over the years, Dr. John Forrest and Dr. Jeffrey Gruen – for their assistance in finding an interesting and feasible research project, and Dr. Vineet Bhandari – for his support and guidance in the lab. Thanks to my fellow labmates, Mansoor Syed and Suresh Angara, who took me under their wings and taught me everything I needed to know to get my project off the ground.

I would also like to thank my fellow student Nataly Sumarriva for her friendship through these difficult times – it has been quite an adventure but we are finally almost done! A special thanks to my pediatrician Dr. Richard Levy, my role model and friend – thank you for everything you have done for my family and me. And finally, my old PI and very good friend Dr. Steven Nusinowitz – thank you for your support in nurturing my scientific interests and helping me reach my full potential.

Dedicated to my mother and father. Estoy agradecido por tener unos padres tan amorosos. Gracias por su apoyo incondicional.

Introduction

Background

Prior to the introduction of the mechanical ventilator to the neonatal intensive care unit in the 1960s, premature infants with respiratory distress syndrome (RDS) either died within the first week of life or survived with respiratory sequelae. In 1967 Northway et al described a new syndrome that affected premature infants with RDS who received prolonged treatment of high concentrations of supplemental oxygen via mechanical ventilation with high peak inspiratory pressures.[1] The surviving infants of this cohort had a mean gestational age of 34 weeks and a mean birth weight of 2.3kg. This form of chronic lung disease was characterized by an oxygen requirement at 28 days of life and progressive radiographic changes.

The syndrome was coined as bronchopulmonary dysplasia (BPD) in order to emphasize the fact that the disease process effected all tissues of the lung. Hyperinflation and atelectasis, along with a cystic and scarred lung appearance were characteristic findings on chest x-ray. Pathologic examination was characterized by smooth muscle hypertrophy, inflammatory infiltrates, interstitial fibrosis, and emphysema. These patients frequently remained oxygen and ventilator dependent beyond the initial few weeks of life and developed chronic lung changes and abnormal pulmonary function. Infants with severe lung damage had a high mortality secondary to progressive respiratory failure, usually within the first weeks of life.

To study the long-term sequelae Northway et al conducted a follow up study of their original cohort.[2] Most of these adolescents and young adults had some degree of pulmonary dysfunction characterized by airway obstruction, airway hyperreactivity, and hyperinflation. Approximately a quarter of the patients in this cohort had severe pulmonary dysfunction, including persistent symptoms of respiratory difficulty.

The "New" BPD

Preterm delivery affects 12.5% of pregnancies in the United States. These premature infants are at a higher risk for complications and unique medical problems. BPD is the most common chronic lung disease of infancy; it persists even with advancements having been made in the medical management of premature infants. During the era of "classical" or "old" BPD, few patients survived with birth weights less than 1 kg and a gestational age less than 28 weeks.[3] An increasing number of patients born at a younger gestational age are now surviving past infancy. Despite advancements in premature neonatal care the incidence of BPD in all preterm birth survivors has not decreased over time.[4] However, the demographics of the patient population has changed owing to the increased survival of the highly premature infants, specifically very low birth weight (<1500 g) and extremely low birth weight infants (<1000 g), and those <30 weeks' gestation.[5]

The "new" BPD, henceforth referred to simply as BPD, is a milder form of lung disease compared to "classic" BPD. The diagnosis and severity of BPD are defined by specific diagnostic criteria.[6] A clinical diagnosis of BPD for infants <32 weeks gestational age requires that the patient have a supplemental oxygen requirement at 28 days of life. The

severity of disease is defined by the supplemental oxygen need and/or respiratory support at 36 weeks corrected gestational age, or at the time of discharge. This can range from needing no supplemental oxygen to requiring positive pressure support.

The pathology of BPD is characterized by an impairment of alveolar development, resulting in alveolar hypoplasia.[7] Pathologic examination of infants who died with BPD reveals lungs with fewer number of alveoli and increased alveolar diameters.[8] Inflammation is generally milder with less fibrosis and absence of smooth muscle hypertrophy when compared to "old" BPD. Notably, the pulmonary microvasculature is also disrupted and features an abnormal vessel network with dysregulated vessel proliferation, dysmorphic vessels, and thickened alveolar septa.[9]

The possible sequelae of BPD is still under investigation, however, the available data would appear to suggest significant long-term morbidity. Studies show that BPD survivors are likely to suffer from persistent respiratory symptoms requiring medication, although no correlation was found between gestational age or duration of ventilatory support.[10] Investigations of lung function in this population have revealed the development of substantial airway obstruction, as well as persistence of impaired alveolar development.[3,10] These children were also more likely to suffer from chest deformities and asthma. Recent research has also identified pulmonary artery hypertension as a sequela of BPD, with one study finding a median age of 4.8 months at the time of diagnosis.[11]

In order to discuss the proposed therapeutic intervention explored in this paper, it is important to review pertinent fetal lung development. Gaining insight into human lung development will assist in understanding the etiology of BPD and potential avenues for intervention.

Fetal Lung Development

Human lung development is a complex and highly regulated process involving the coordination between surface epithelium, supporting interstitial tissue, blood vessels, and numerous regulatory factors.[12,13] During the first half of fetal life (embryonic phase) the major airways of the lung develop from the foregut. By 16 weeks' gestation (pseudoglandular phase), blood vessels that supply the lung and the major conducting airways have formed. Through 25 weeks' gestation (canalicular phase) the airways widen and lengthen, which is followed by the formation of saccules at the terminal bronchioles (saccular phase). The alveolar-capillary barrier undergoes reorganization, bringing the epithelial layer in close approximation with the capillary endothelium. By 35 weeks' gestation (alveolar phase) the lung is in its final phase of development – alveolarization of the distal lung saccules. The result is a 4-fold increase in lung volume from 29 weeks' gestation to term and a concomitant increase in available surface area for gas exchange. This maturation also coincides with a reduction of interstitial tissue, which serves to facilitate effective diffusion of gases. Lung development continues in the months and years following birth with continued alveolarization and remodeling of the interalveolar septa.[14]

During the late canalicular/early saccular phase (25-35 weeks' gestation) the cuboidal epithelium is replaced by simple squamous epithelium. Ninety-five percent of the alveolar surface is composed of Type I epithelial cells with Type II epithelial cells making up the remainder (although Type II pneumocytes dominate in numbers overall – 60%).[15] Type II pneumocytes serve as progenitor cells; they retain the capacity to divide and differentiate when Type I cells become damaged.[16] Type II epithelial cells are also responsible for producing and elaborating surfactant, a lipoprotein complex that lowers surface tension at the air-water interface, thereby preventing alveolar collapse during expiration. Lamellar bodies appear within Type II cells at 24 weeks' gestation, concomitant with the initiation of surfactant production, and are responsible for storing the lipoprotein complex. However, adequate amounts of surfactant are not produced until 35 weeks' gestation.[17] Type I epithelial cells are specialized to facilitate the diffusion of gases across the alveolar-capillary barrier. Organelles within these cells are clustered around the nucleus, resulting in a highly attenuated endothelial lining. Pinocytotic vesicles can be found within the cytoplasm of this thin portion, which are thought to play an important role in the turnover of surfactant.[18] However, surfactant reuptake is thought to be chiefly mediated by Type II cells via surfactant protein (SP) mediated endocytosis.[19-22]

Pathogenesis of BPD

Early investigations into the etiology of the "old" BPD hypothesized that the pathophysiology was due to oxygen toxicity and baro/volutrauma from mechanical ventilation.[23,24] Later studies in newborn mice demonstrated that exposure to oxygen alone could account for many of the changes seen in patients with BPD.[25,26] When these

mice were exposed to continuous atmospheric conditions of hyperoxia they developed an impairment of lung development, specifically a decrease in alveolarization and mild inflammatory response. It is postulated that hyperoxic acute lung injury (HALI) is mediated by reactive oxygen species, both from high oxygen ventilation and inflammatory cells, which activate extrinsic and mitochondrial cell death pathways, resulting in cell death with features of necrosis.[27]

BPD is a disease that effects newborn infants and involves the impairment of normal lung growth and development. The hallmark of the disease is impaired alveolarization. As stated above, premature infants comprise the patient population of BPD as it exists today. These premature infants are born with underdeveloped lung anatomy that is deficient in proper alveolar numbers and biochemical activity. The architecture of the lung is unoptimized for efficient gas exchange at the alveolar-capillary barrier, while surfactant production is inadequate to prevent alveolar collapse. As mentioned previously, lung development is a complex and highly coordinated process – thus BPD is likely the result of interference with the formation of terminal respiratory airways, alveoli, and microvasculature that supplies these areas.[12]

The etiology of BPD as we know it today is multifaceted, with multiple major inciting factors acting synergistically to develop the disease state. Preterm neonates are more susceptible to developing chronic lung disease as they are born with a surfactant deficiency secondary to immature Type II alveolar cells. The use of invasive, prolonged mechanical ventilation causes direct damage to the nascent alveoli which have limited repair capacity. Because the pulmonary antioxidant system develops alongside the

surfactant system, high levels of supplemental oxygen can cause significant oxidative stress injury.[17] Additionally, perinatal sepsis (specifically chorioamnionitis) has been shown to be a risk factor for BPD after premature birth.[28]

The common thread among these stressors is injury to the developing alveolar epithelium with subsequent release of proinflammatory cytokines, chemotactic factors, and an increase in microvascular permeability. The result is initiation of an inflammatory response, a process that is known to interfere with formation of the microvasculature and alveolarization in the developing lung.[29,30] An imbalance of inflammatory mediators causes the activation of cell death pathways followed by resolution and repair. It is this process which disrupts normal lung architecture and results in the characteristic features of impaired alveolarization and dysregulated angiogenesis.[6]

Various biomarkers are associated with BPD including regulatory factors, angiogenic factors, inflammatory mediators, and markers of oxidative injury.[31] Although not yet routinely used in clinical practice, these biomarkers offer potential avenues of intervention if they are indeed found to be in a causal relationship with BPD. Therefore they are of particular research interest. For the purposes of this paper we will focus on C/EBP homologous protein (CHOP) and Angiopoietin 2 (Ang2), two biomarkers thought to be involved in the cascade of events leading to dysregulated alveolar development.

Importance of CHOP and Ang2

In addition to the aforementioned extrinsic and mitochondrial cell death pathways, a third pathway exists which involves the endoplasmic reticulum (ER). Known as the ER

stress-dependent pathway, this pathway reacts to accumulated unfolded proteins within the ER by facilitating either improvement in protein folding and processing or inducing apoptosis.[32] This unfolded protein response (UPR) is dependent on the nature of the inciting stressor. Moreover, the UPR appears to upregulate the expression of genes that are protective against oxidative injury.[33] This implies that reactive oxygen species play a significant role in the ER stress-dependent cell death pathway. CHOP acts as an important late-phase mediator in this pathway, with increased levels of CHOP protein favoring cell death.[32]

Angiogenesis plays a fundamental role in embryonic and postnatal organ development. Vascular formation is regulated by growth factors and cytokines, including a family of vascular growth factors called angiopoietins. Angiopoietins are ligands of the tyrosine receptor kinase receptor with immunoglobulin and epidermal growth factor homology domain 2 (Tie2) located on endothelial cells.[34] Through this interaction they modulate the remodeling of blood vessels. Furthermore, angiopoietins can also induce the formation of new blood vessels when working in concert with vascular endothelial growth factor (VEGF).

Angiopoietin 1 (Ang1) is a Tie2 receptor agonist. It enhances angiogenesis, induces vascular maturation, and stabilizes blood vessels thereby decreasing vascular leak. Ang2, on the other hand, is a Tie2 receptor antagonist. It destabilizes blood vessels, induces vascular regression, and induces endothelial cell apoptosis.[27] Hence spatial and temporal regulation of Ang1 and Ang2 expression plays a key role in pulmonary vascular network development.[35] Indeed, tracheal aspirates from very preterm infants who go on to

develop BPD have been found to contain relatively high levels of Ang2, suggesting an association with abnormal fetal pulmonary angiogenesis.[9,36] Furthermore, hyperoxia has been shown to be a potent inducer of Ang2 mRNA and protein with resultant HALI (oxidative injury, inflammation, and cell death) and epithelial necrosis.[27]

Advancements in Treatment

Neonatal care has changed over the last decade following the recognition of the special needs of premature infants. Disease prevention starts during the antenatal course as the potential for developing BPD begins *in utero*. Tocolytics and antenatal corticosteroids can protect against respiratory distress by delaying delivery and allowing for lung maturation to occur.[17,37] Surfactant replacement therapy has also played a pivotal role in increasing patient survival by bridging the gap between birth and adequate endogenous surfactant production.[38] Exogenous surfactant therapy and gentle ventilation techniques have served to reduce the incidence of stretch injury. The use of lower oxygen concentrations for resuscitation, as well as administration of antioxidants such as vitamin A, has been shown to reduce oxidative stress in the newborn.[5,39] Aggressive treatment and prevention of infection is also important, as perinatal infection has been associated with an increased risk of developing BPD.[40] Postnatal corticosteroids have been shown to improve outcomes in some studies, presumably by stimulating surfactant production, enhancing antioxidant enzyme synthesis, and suppressing the inflammatory response to injury.[41]

The common theme in treatment is the prevention of inflammation, thus protecting from cellular death and promoting normal lung development.[5] In keeping with this theme, one

option that has been explored is the use of short interfering RNA, also known as silencing RNA (siRNA), to inhibit cell death pathways. Antisense agents, such as siRNA, are used by eukaryotic organisms to regulate gene expression via the prevention of translation. The cornerstone of this regulatory pathway involves the conjugation of a strand of siRNA to proteins of the Argonaute family, thereby forming an RNA-induced silencing complex (RISC).[42] RISCs are effector complexes that are responsible for silencing complementary single stranded RNA sequences through activation of ribonuclease (RNase).[43]

Recent studies have demonstrated that administration of CHOP and Ang2 siRNA have beneficial effects in cell and animal models. CHOP siRNA has been shown to prevent cell death in alveolar epithelial cells and hyperoxia-induced murine models of BPD.[44] Additionally, *in vivo* BPD models treated with CHOP siRNA benefited by preserved alveolarization.[44] Bhandari et al demonstrated that administration of Ang2 siRNA *in vivo* reduced hyperoxia-induced inflammation and cell death.[45] Both these studies were carried out with the use of a transfection reagent *in vitro* and naked siRNA alone *in vivo*.

The numerous phosphate groups in RNA are charged at physiologic pH, giving the molecule an overall negative charge. This would make entry of siRNA into cells difficult as ionic substances generally cannot cross the lipophilic plasma membrane. Thus, transfection reagents are commonly used in cell cultures as internalization of naked oligonucleotides is usually inefficient.[43] Typical transfection reagents are lipid-derived, cationic substances capable of forming liposomes that are specifically designed to deliver exogenic nucleic acids into eukaryotic cells.[43] However, these products are only used in

cell culture experiments. They are not cleared for use in the clinical setting and can have considerable cytotoxic effects.[46]

Surfactants are lipoprotein substances that readily form liposomes in aqueous solution.[47,48] Curosurf® (Cornerstone Therapeutics Inc, Cary, NC) is an extract of natural porcine lung surfactant consisting of 99% polar lipids and 1% hydrophobic low molecular weight proteins including SP-B and SP-C, but not SP-A.[49] It is routinely used in the treatment of RDS in premature infants.[38,50] Of the commercially available therapeutic surfactants, Curosurf® has physiologic phosphatidylcholine composition most similar to that of human surfactant.[49] Furthermore, the ultrastructural composition of Curosurf® consists of roughly 80% lamellar/vesicular structures.[49]

Given the capacity of surfactants like Curosurf® to form liposomes, the potential for uptake by alveolar epithelial cells (surfactant turnover), and the obvious therapeutic benefits for very premature infants, we postulate that Curosurf® could serve as a viable vehicle for siRNA delivery. Specifically, we hypothesize that use of Curosurf® as an enhanced delivery vehicle for CHOP/Ang2 siRNA would augment gene silencing, preventing cell death and BPD in the mouse lung. Our goals in this project were to deliver CHOP/Ang2 siRNA using Curosurf® as the delivery vehicle in *in vitro* and *in vivo* systems to evaluate the impact on CHOP/Ang2 gene silencing, cleaved caspase 3 protein expression, and lung morphology in a hyperoxia-induced BPD mouse model.

Materials and Methods

Cell Culture Work

Cells. Mouse transformed lung epithelial cells (MLE-12) (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured in a humidified incubator under normal growth conditions of 5% CO_2 at 37°C. All cell culture work was carried out by the author.

Transfection fluorescence. One microliter of 20 µM labeled, scrambled siRNA (Thermo Fisher Scientific, Waltham, MA) was diluted in 500 µL of DMEM. Transfection reagent – 20 µL of Lipofectamine 2000® (Life Technologies, Carlsbad, CA) or 5/10/20 µL of Curosurf® (Cornerstone Therapeutics Inc, Cary, NC) – was diluted in a separate 500 µL of DMEM. After 5 min, both solutions were combined, briefly vortexed, and allowed to sit for 15 min at room temperature to permit transfection complexes to form. MLE-12 cells (2.5×10^5) were seeded per well in 24-well plates, in 500 µL DMEM enriched with 2% FBS. Three hundred microliters of transfection complex solution was then added drop-wise to cultures with gentle swirling to ensure uniform distribution. Cells were then incubated under normal growth conditions for 24 hours. Cells were then stained with DAPI (Thermo Fisher Scientific, Waltham, MA) and visualized using an Olympus IX70 fluorescence microscope with the appropriate fluorescence filters. Photomicroscopy was performed using cellSens imaging software (Olympus Corp), with a minimum of 3 fields per group. Cell counts for each image were manually performed.

siRNA transfection. One microliter of 20 µM siRNA (CHOP or Ang2) (Life Technologies, Carlsbad, CA) was diluted in 500 µL of DMEM. Transfection reagent – 20 µL of Lipofectamine 2000® or 10 µL of Curosurf® – was diluted in a separate 500 µL of DMEM. After 5 min both solutions were combined, briefly vortexed, and allowed to sit for 15 min at room temperature to permit transfection complexes to form. MLE-12 cells (1×10^6) were seeded per 60 mm plate in 3000 µL DMEM enriched with 2% FBS. One thousand microliters of transfection complex solution was then added drop-wise to cultures with gentle swirling to ensure uniform distribution. Cells were then incubated under normal growth conditions for 24 hours prior to hyperoxia exposure.

Oxygen exposure. MLE-12 cell cultures were placed in an airtight metallic chamber ($30 \times 20 \times 20$ cm^3) containing a plate of autoclaved water to promote proper humidification. The chamber was flushed with 95% O_2 for 5 minutes to purge the room air from the container. The chamber was then placed in a humidified incubator at 37°C for 16 hours.

Protein isolation and detection. Cell cultures were washed with cold PBS and lysed using radioimmunoprecipitation assay (RIPA) buffer. Following incubation, lysates were centrifuged and supernatant containing whole protein was collected. Western analysis was used to evaluate lysates. Whole protein samples were electrophoresed using 4-20% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. CHOP and Ang2 protein was detected, using β-actin as control. Cleaved caspase 3 was detected as a surrogate marker for cell death. CHOP antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ang2 antibody was obtained from R&D Systems (Minneapolis, MN). β-actin and cleaved caspase 3 antibodies were purchased from Cell

Signaling Technology (Danvers, MA). Detection of protein-bound antibody was achieved using enhanced chemiluminescence. Protein levels were evaluated by densitometry using ImageJ (National Institutes of Health).

Animal Work

Animals. Wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were kept at atmospheric pressure and room temperature, 12-hour light-dark cycle, and allowed to feed ad lib. Two lactating dams were used, which were alternated between hyperoxia and room air every 24 hours. A murine model of hyperoxia-induced BPD was used as described in the literature.[44,51] Briefly, newborn mice were exposed to hyperoxia (100% O_2) from postnatal day (PN) 1-4, during the saccular stage of murine lung development. A subgroup of animals were sacrificed on PN5 for lung protein analysis. Mice were returned to room air and allowed to recover until the BPD phenotype developed (circa PN14).[51] Mice were then sacrificed on PN14 to assess lung morphology. All animal work was approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine (New Haven, CT). The author received help with animal care, siRNA administration, and oxygen exposure. All other animal work was carried out by the author.

siRNA administration. Ang2 siRNA (20 μM) was prepared in either RNase-free water or Curosurf®. One to two drops of these solutions was introduced intranasally into the pulmonary tree of newborn mice on PN1 and PN3.

Oxygen exposure. Newborn mice were placed in cages in an airtight Plexiglas chamber (55 × 40 × 50 cm^3) ventilated with 100% O_2. Oxygen levels were monitored throughout via a sensor connected to a relay switch integrated into the oxygen supply circuit. Hyperoxia exposure was initiated on PN1 and continued through PN4.

Protein isolation and detection. Mice were sacrificed using standard CO_2 protocol. Median sternotomy was performed and right heart perfusion with PBS was done to clear the pulmonary intravascular space. The lungs were rapidly removed en bloc and frozen in liquid nitrogen. Tissue samples were added to RIPA buffer solution and homogenized with an electric homogenizer. Following incubation, lysates were centrifuged and supernatant containing whole protein was collected. Lung lysates were analyzed for protein content as described above.

Histology. Lung tissues obtained from newborn mice at PN14 were subjected to a standard protocol for lung inflation (25 cm) and fixed overnight in 10% buffered formalin. The fixed tissues were then embedded in paraffin, sectioned (5 µm), and hematoxylin & eosin stained by the Research Histology Laboratory of the Department of Pathology at Yale University School of Medicine.

Lung morphometry. Alveolar size was estimated from the mean chord length of the airspace, as the chord length increases proportionally with alveolar enlargement.[52] Images were obtained using an Olympus IX70 fluorescence microscope at 10x magnification and cellSens imaging software (Olympus Corp). A minimum of 3 fields per mouse were obtained. Each field was analyzed on a Macintosh computer using a modified

custom macro in NIH Image (National Institutes of Health).[53] Briefly, images were run through an automated algorithm which processed the images via threshold, smoothing, and inversion. The processed images were then subjected to logical image match 'and' operations with horizontal and vertical grids. At least 200 measurements per field were made for each animal. The length of the lines overlying air space was averaged as the mean chord length.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). A minimum of 3 samples per group was used. As appropriate, groups were compared with an unpaired two-tailed Student's *t*-test with GraphPad Prism 6.03 (GraphPad Software Inc, San Diego, CA). In all analyses, $P < 0.05$ was considered statistically significant.

Results

Effect of Curosurf® on siRNA transfection in MLE-12 cells

The phenomena of gene silencing expression via RNA interference is dependent on effective transfection of siRNA. Hence, we first wanted to assess the efficacy of Curosurf® as a transfection reagent with fluorescent-labeled scrambled siRNA. Lipofectamine 2000® was used as a positive control as it is known to have very good transfection efficiency. MLE-12 cells, a pulmonary adenocarcinoma cell line with alveolar ell characteristics, were selected as a surrogate for alveolar epithelial cells.

As expected, immunofluorescent analysis revealed that detection of scrambled siRNA within cells was dependent on the use of a transfection reagent (**Fig. 1A**). Lipofectamine 2000® had a transfection rate of 53%, over a 30-fold increase in efficiency when compared to siRNA alone ($P \leq 0.0001$; **Fig. 1B**). Among the Curosurf® groups, cells treated with 10 µL had the highest transfection rate at 4.5% ($P \leq 0.0001$; **Fig. 1B**). Cells treated with 20 µL Curosurf® actually had a slight decrease in transfection efficiency when compared to siRNA alone ($P < 0.05$; **Fig. 1B**). Taken together, these studies show that at proper concentrations Curosurf® is a modest enhancer of siRNA transfection in alveolar epithelial cells.

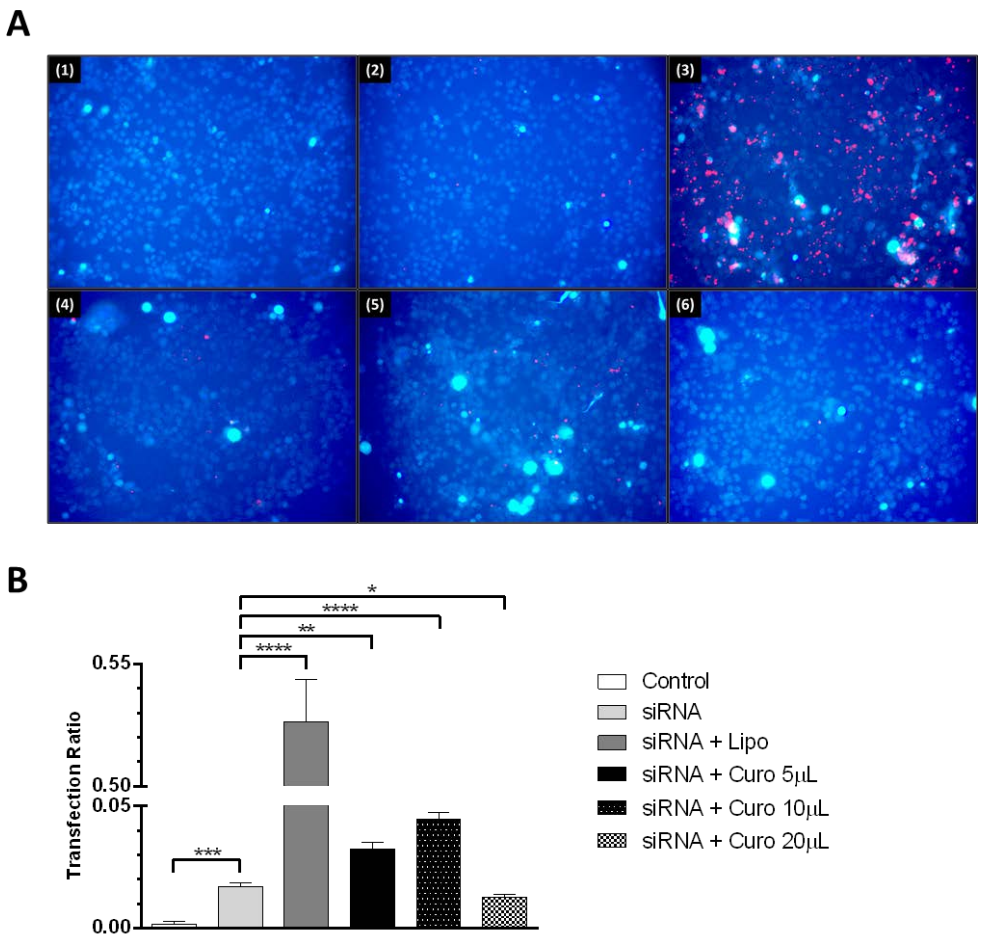

Figure 1. *In vitro* effect of Curosurf® on siRNA transfection. *(A)* Representative fluorescence microscopy images for (1) untreated, (2) scrambled siRNA, (3) siRNA and Lipofectamine 2000®, (4) siRNA and 5 µL Curosurf®, (5) siRNA and 10 µL Curosurf®, and (6) siRNA and 20 µL Curosurf®. Red areas indicate presence of siRNA. *(B)* Transfection ratios for each group. Each bar represents the mean ± the SEM for a minimum of 3 samples per group. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, unpaired *t*-test. (Curo = Curosurf®, Lipo = Lipofectamine 2000®)

Curosurf® as a transfection reagent on CHOP expression in MLE-12 cells

Elevated levels of CHOP are associated with cell death via the ER stress-dependent pathway in the hyperoxia-induced model of BPD.[44] Hence, we wanted to evaluate the use of Curosurf® to augment gene silencing using CHOP siRNA. CHOP protein expression was assessed by western blot analysis (**Fig. 2A**). Exposure to hyperoxia resulted in an increase

in CHOP when compared to room air. Both Lipofectamine 2000® and Curosurf® treated groups benefited from a decrease in CHOP protein expression when compared to untreated hyperoxia and naked siRNA ($P \leq 0.05$; **Fig. 2B**). There was no difference between the Curosurf® and Lipofectamine 2000® treated groups, suggesting comparable efficiency in gene silencing. Interestingly, there was no appreciable difference between hyperoxia and naked siRNA groups. Thus, when used as a transfection reagent, Curosurf® serves as a potent enhancer of CHOP gene silencing via RNA interference.

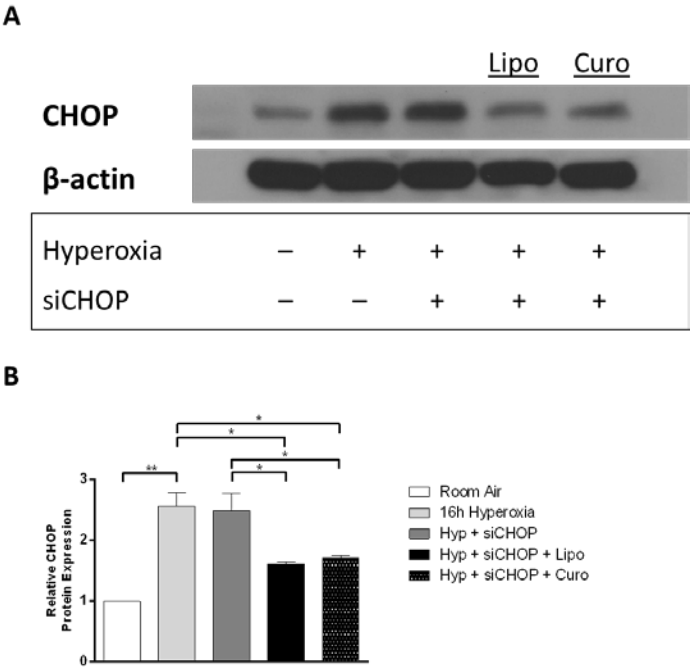

Figure 2. *In vitro* effect of Curosurf® as a transfection reagent on CHOP expression in hyperoxic exposure. *(A)* The protein expression of CHOP in MLE-12 cells was ascertained under the conditions described, with β-actin controls. *(B)* Corresponding relative CHOP protein expression in MLE-12 cells. Each bar represents the mean ± the SEM for a minimum of 3 samples per group. **P* ≤ 0.05, ***P* ≤ 0.01, unpaired *t*-test. (Curo = Curosurf®, Hyp = hyperoxia, Lipo = Lipofectamine 2000®, siCHOP = CHOP siRNA)

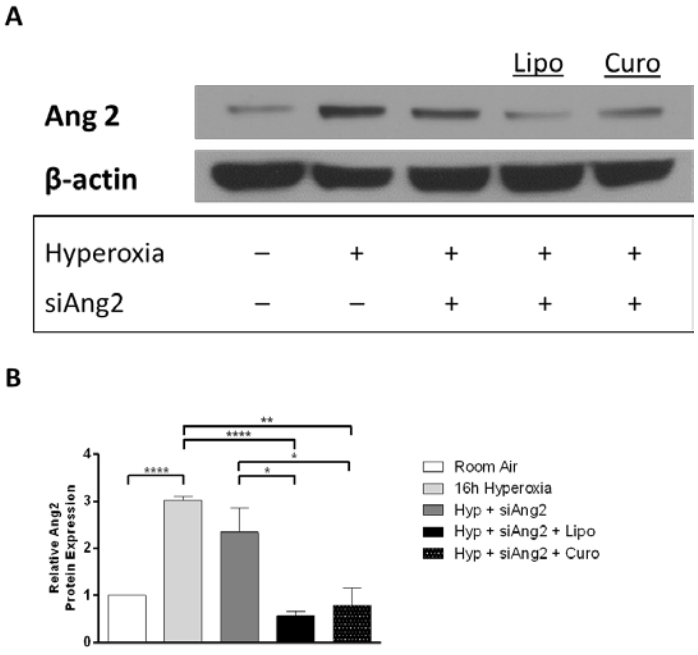

Figure 3. *In vitro* effect of Curosurf® as a transfection reagent on Ang2 expression in hyperoxic exposure. *(A)* The protein expression of Ang2 in MLE-12 cells was ascertained under the conditions described, with β-actin controls. *(B)* Corresponding relative Ang2 protein expression in MLE-12 cells. Each bar represents the mean ± the SEM for a minimum of 3 samples per group. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, unpaired *t*-test. (Curo = Curosurf®, Hyp = hyperoxia, Lipo = Lipofectamine 2000®, siAng2 = Ang2 siRNA)

Curosurf® as a transfection reagent on Ang2 expression in MLE-12 cells

Ang2 is an important mediator of epithelial cell death in the hyperoxia-induced model of BPD.[45] Therefore, we wanted to evaluate the potential use of Curosurf® to augment gene silencing using Ang2 siRNA. Ang2 protein expression was assessed by western blot analysis (**Fig. 3A**). Exposure to hyperoxia resulted in an increase in Ang2 when compared to room air. Both Lipofectamine 2000® and Curosurf® treated groups benefited from a decrease in Ang2 expression when compare to untreated hyperoxia (*P* ≤ 0.001 and *P* ≤ 0.01, respectively; **Fig. 3B**). There was no difference between the Curosurf® and

Lipofectamine 2000® treated groups, suggesting comparable efficiency in gene silencing. Interestingly, there was an appreciable difference between hyperoxia and naked siRNA groups but it did not reach statistical significance. There was a significant decrease in protein expression when comparing naked siRNA to Lipofectamine 2000® and Curosurf® ($P \leq 0.05$; **Fig. 3B**). Thus, when used as a transfection reagent, Curosurf® serves as a potent enhancer of Ang2 gene silencing via RNA interference.

Curosurf® as a transfection reagent on cleaved caspase 3 expression in MLE-12 cells

Studies have shown that hyperoxia exposure causes cellular injury in alveolar epithelial cells and induces cell death.[25-27] Caspase 3 is an executioner protease zymogen that plays a central role in the activation of cellular death via the intrinsic, extrinsic, and UPR pathways.[32,54] Since cleaved caspase 3 correlates directly with cell death, we wanted to evaluate the expression of cleaved caspase 3 in cells transfected using Curosurf®.

Cleaved caspase 3 protein expression was assessed by western blot analysis. Cells treated with CHOP siRNA alone did not experience an appreciable decrease in cleaved caspase 3 expression (**Fig. 4A**). Qualitatively, Curosurf® appears to enhance attenuation of cleaved caspase 3 when compared to CHOP siRNA alone (**Fig. 4A**). Cells treated with Ang2 siRNA alone did not experience an appreciable reduction in cleaved caspase 3 expression (**Fig. 4B**). Qualitative analysis suggests that transfection with Curosurf® appears to offer a considerable reduction of cleaved caspase 3 expression when compared to cells treated with Ang2 siRNA alone (**Fig. 4B**). Thus, transfection with CHOP/Ang2 siRNA using

Curosurf® appears to enhance attenuation of cleaved caspase 3 expression in alveolar epithelial cells.

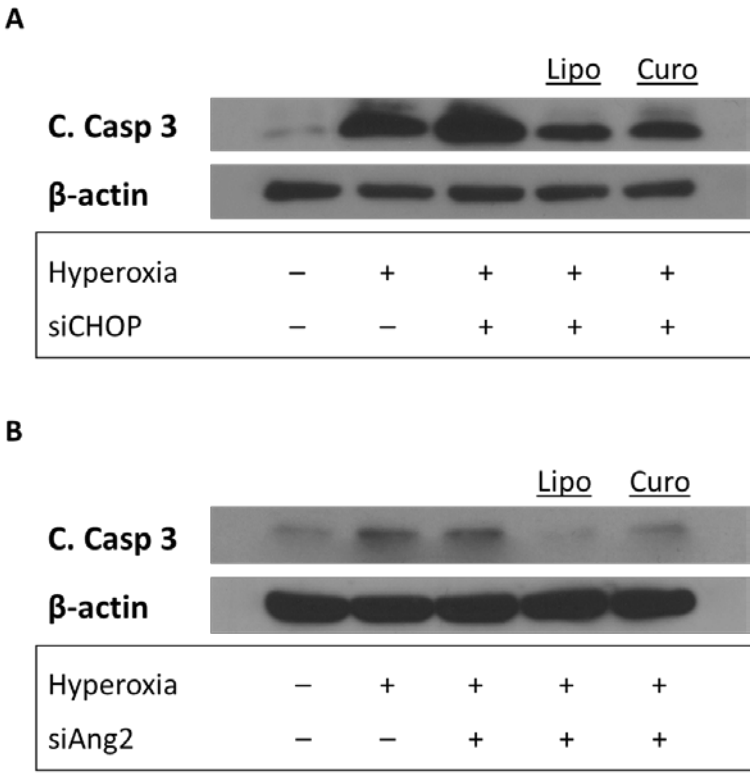

Figure 4. *In vitro* effect of Curosurf® as a transfection reagent on cleaved caspase 3 expression in hyperoxic exposure. *(A)* The protein expression of cleaved caspase 3 in CHOP siRNA transfected MLE-12 cells, with β-actin controls. *(B)* The protein expression of cleaved caspase 3 in Ang2 siRNA transfected MLE-12 cells, with β-actin controls. (C. Casp 3 = cleaved caspase 3, Curo = Curosurf®, Lipo = Lipofectamine 2000®, siAng2 = Ang2 siRNA, siCHOP = CHOP siRNA)

Curosurf® as a transfection reagent on Ang2 expression in mice

A murine hyperoxia model of BPD was chosen as previous research has shown that anatomical and functional changes found in this model mimic those found in human disease.[26,51,55] Ang2 expression was assessed by western blot analysis. Among mice

treated with Ang2 siRNA, protein expression appears to be attenuated when transfected with and without Curosurf® (**Fig. 5**). Qualitative analysis suggests that treatment with Ang2 siRNA alone appears to offer comparable Ang2 gene silencing when compared to cells transfected with Curosurf®.

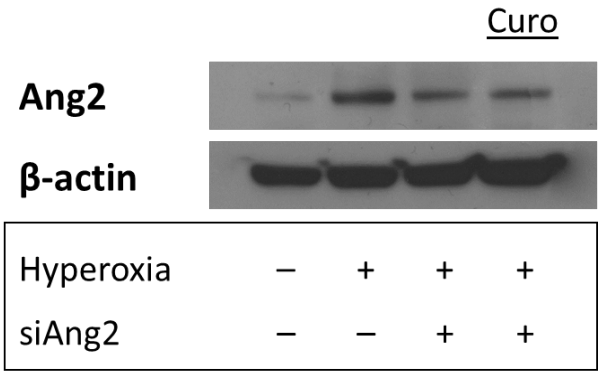

Figure 5. *In vivo* effect of Curosurf® as a transfection reagent on Ang2 expression in hyperoxic exposure. The protein expression of Ang2 in C57BL/6 mice was ascertained under the conditions described, with β-actin controls. (Curo = Curosurf®, siAng2 = Ang2 siRNA)

Effect of Curosurf® as a transfection reagent on mouse lung morphology

BPD lung morphology is characterized by impaired alveolarization secondary to cellular injury and inflammation.[7] Histologic sections of lung tissue from PN14 mice exposed to hyperoxia exhibit characteristic enlarged, simplified alveoli (**Fig. 6A, Panel 2**). The lung architecture is better preserved in mice treated with Ang2 siRNA with improved alveolarization (**Fig. 6A, Panel 3**). Additionally, mice treated with Ang2 siRNA and Curosurf® seemed to respond with further improvement in alveolarization when compared to siRNA alone (**Fig. 6A, Panel 4**).

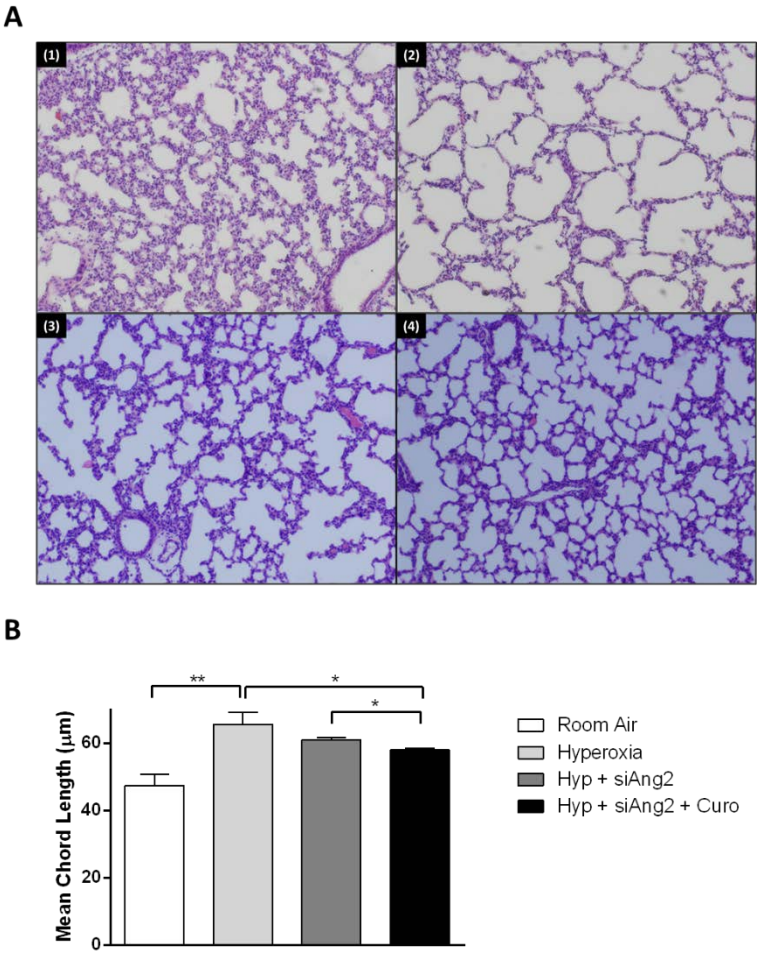

Figure 6. Effect of Curosurf® as a delivery vehicle for Ang2 siRNA on pulmonary phenotype. *(A)* Representative microscopy images of pulmonary structure in PN14 WT mice exposed to (1) room air, (2) hyperoxia, (3) hyperoxia treated with siAng2, and (4) hyperoxia treated with siAng2 and Curosurf®. *(B)* Mean chord length for each group. Each bar represents the mean ± the SEM for a minimum of 3 samples per group. **P* ≤ 0.05, ***P* ≤ 0.01, unpaired *t*-test. (Curo = Curosurf®, Hyp = hyperoxia, PN = postnatal, WT = wild-type)

The degree of alveolarization can be characterized quantitatively because alveolar size is directly proportional to chord length.[52] Mean chord length of PN14 mice treated with Ang2 siRNA alone was decreased when compared to the hyperoxia model of BPD; however, this difference was not statistically significant (*P* = 0.095; **Fig. 6B**). Mean chord

length of mice treated with Ang2 siRNA and Curosurf® was decreased when compared to both hyperoxia and siRNA alone ($P \leq 0.05$; **Fig. 6B**). Thus, Curosurf® enhances gene silencing via RNA interference and augments alveolar preservation *in vivo*.

Discussion

The use of siRNA therapy in the treatment of BPD offers many advantages and can act in concert with other treatment modalities. The central premise of siRNA therapy is interruption of key inflammatory cascade components that lead to impaired vascularization and alveolarization. RNA interference has the advantage of gene silencing without altering host DNA. Exogenous nucleic acids introduced into the host are temporary – they act transiently and are degraded by host RNases. An added benefit of using RNA interference is the ability to directly targeting the lung when administering siRNA intranasally. Thus, siRNA therapy is suited for a disease of the premature neonate as it protects the developing lung until the patient is capable of being weaned from respiratory support.

Although the use commercially available transfection reagents to enhance transfection in siRNA therapy is appealing, these reagents are not cleared for clinical use in the United States. Additionally, studies have shown that these transfection reagents can have significant cytotoxicity.[56,57] Moreover, cationic liposomes cause a release of reactive oxygen species and induce a marked inflammatory response in pneumocytes.[46] This necessitates the need to explore other transfection enhancing candidates such as Curosurf®.

Properties of Curosurf®

Curosurf® is unique among the exogenous surfactants currently approved by the FDA for clinical use in the United States. It is the only natural surfactant extracted from porcine

lung. Studies have suggested that porcine derived surfactants are most similar in composition to human surfactant with regards to phosphatidylcholine content.[49] However, the relevance of this similarity to clinical practice remains to be investigated. Furthermore, due to mincing of whole lung in the extraction process, Curosurf® has a significantly higher proportion of sphingomyelin.[49] Orborina et al have shown that sphingomyelin is resistant to oxidation, particularly when compared to phosphatidylcholines like dipalmitoylphosphatidylcholine (DPPC).[58] DPPC is a common additive in many commercial surfactant preparations; however it is not added to Curosurf®.[49] Thus, in addition to its role as a transfection reagent, Curosurf® itself may offer direct protection against oxidative damage.

Like the other commercially available surfactants, preparations of Curosurf® lack surfactant protein SP-A since it is removed in the lipid extraction process.[49] SP-A is found in abundance in natural surfactant and plays an important role in surfactant recycling via receptor-mediated endocytosis at Type II alveolar cells.[19-21] The relatively low transfection efficiency of Curosurf® in our experiments may be partially attributed to this lack of SP-A. The use of native SP-A is restricted secondary to limitations imposed by its size and solubility in isolation and recovery. Animal experiments using surfactant enriched with a synthetic SP-A analogue have shown superiority in improved lung compliance over surfactant deficient in SP-A.[59] However, the effect on surfactant reuptake was not investigated. The use of Curosurf® preparations enriched with synthetic SP-A in siRNA therapy is something that could be investigated in future studies.

Hyperoxic injury during the late canalicular/early saccular phase of lung development has been implicated in the pathogenesis of BPD.[27] Hence, we decided to investigate the use of Curosurf® in RNA interference using two mediators of cell death in BPD: CHOP and Ang2. These two proteins were chosen because they act upon differing pathways that ultimately lead to inflammation and hyperoxia-induced cell death.

Curosurf® as a transfection reagent *in vitro*

Internalization of naked RNA is inefficient in cell cultures because negatively charged oligonucleotides have to cross the hydrophobic cell membrane. Transfection regents such as Lipofectamine 2000® can enhance internalization by an order of magnitude or more. In our study, Lipofectamine 2000® improved transfection by 30 fold, whereas Curosurf® had a transfection rate of 4.5% – a modest 2.5 fold improvement over naked siRNA (**Fig. 1**). Despite this, transfection with Curosurf® proved to have a potent effect on gene silencing (**Fig. 2** & **Fig. 3**). Moreover, Lipofectamine 2000® has the potential for considerable cytotoxicity.[46,56] Curosurf® on the other hand is relatively innocuous – animal studies and clinical trials have shown that it has a low cytotoxic profile.[60-62] Taken together, this makes Curosurf® an appealing candidate for use in siRNA therapy.

Cleaved caspase 3 expression, a marker of cell death, was also attenuated with Curosurf® enhanced siRNA therapy *in vitro* (**Fig. 4**). Hence, although the transfection efficiency of Curosurf® is relatively low when compared to Lipofectamine 2000®, it had an appreciable effect on suppressing cell death. This suggests that RNA interference is sensitive to even

modest increases in siRNA internalization – small improvements in transfection efficiency can markedly prevent cell death.

Choo-Wing et al have previously investigated the relationship between CHOP expression and cell death in alveolar epithelial cells exposed to hyperoxia.[44] They found that CHOP siRNA reduced CHOP protein expression and cell death in MLE-12 cells exposed to 24 h hyperoxia. Our studies did not show a decrease in CHOP protein expression with naked siRNA alone. However, we did find that administration of CHOP siRNA with Curosurf® significantly reduced CHOP protein expression (**Fig. 2**). This difference is likely due to our higher seeded cell count, resulting in a higher cell density and relatively low dose of siRNA in our experiments. Although not measured directly, our work suggests that cell death would also be ameliorated as levels of cleaved caspase 3 are reduced when Curosurf® is employed as a transfection reagent (**Fig. 4A**). Hence, our results suggest that Curosurf® has a marked effect on enhancing CHOP gene silencing, decreasing cell death.

The use of Ang2 siRNA has not been investigated in MLE-12 cells. Our results suggest that internalization of Ang2 siRNA is inefficient without the use of a transfection reagent. Although the protein expression of Ang2 was decreased relative to hyperoxia, it was not found to be statistically significant (P = 0.26, **Fig. 3**). However, Curosurf® enhanced the transfection efficacy of Ang2 siRNA as evidenced by the decrease in protein expression (**Fig. 3**). Cleaved caspase 3 was also attenuated with Curosurf®, suggesting a reduction in cell death (**Fig. 4B**). Transfection with naked siRNA alone did not reduce cleaved caspase 3 expression. The findings suggest that *in vitro* transfection of naked siRNA is ineffective, whereas Curosurf® significantly enhances transfection efficiency.

Curosurf® as a transfection reagent *in vivo*

Numerous *in vivo* studies have shown modest internalization of naked oligonucleotides without the use of a transfection reagent.[63,64] Furthermore, RNA interference therapy using naked siRNA in animal models of BPD has been reported in the literature.[44,45,55] Prior studies investigating Ang2 siRNA therapy have used young adult mice (4-6 weeks of age) in their BPD models.[45,55] The temporal regulation of various angiopoietins plays a key role in pulmonary vascular network development.[35] Hence, the use of appropriately aged specimens is crucial when investigating animal models of BPD. To our knowledge, the data we present herein marks the first time that newborn mice with a hyperoxia-induced model of BPD were used to evaluate the efficacy of Ang2 siRNA therapy.

As mentioned earlier, Ang2 destabilizes blood vessels, induces vascular regression, and induces endothelial cell apoptosis.[27] Bhandari et al previously investigated the relationship between Ang2 expression and acute lung injury.[45,55] They found that administration of naked Ang2 siRNA reduced protein expression, BAL cellularity, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cell death in a murine model of hyperoxia-induced BPD.[45] Our animal studies also suggest that Ang2 expression is attenuated with Ang2 siRNA therapy. Surprisingly, this reduction is comparable between naked siRNA and Curosurf® enhanced transfection (**Fig. 5**). However, analysis of lung morphology reveals that only mice transfected using Curosurf® had a further significant preservation of lung architecture compared to naked siRNA alone (**Fig. 6**). One explanation for this may be the inherent differences between the *in vivo* and *in vitro* transfection processes. Furthermore, Curosurf® may play a more significant role

in facilitating effective distribution of siRNA to the alveoli than enhancing siRNA transfection into Type II pneumocytes.

In sum, our research demonstrates that Curosurf® has a modest effect on the internalization of CHOP/Ang2 siRNA in transfection experiments. We subsequently appreciated a significant decrease in CHOP/Ang2 protein expression, cleaved caspase 3 expression, and improved lung alveolarization. A proposed sequence of events summarizing these results is illustrated below (**Fig. 7**).

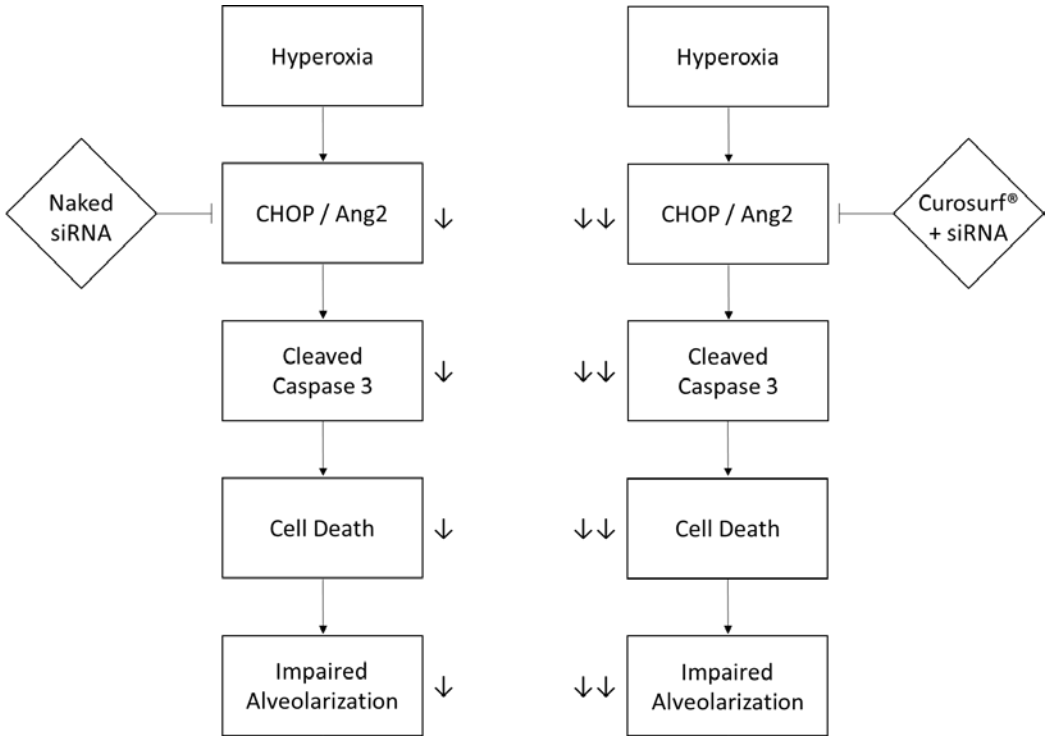

Figure 7. Proposed pathway for the effects of siRNA *in vivo* and *in vitro*. Hyperoxia leads to increased expression of CHOP/Ang2, which is decreased with naked siRNA against those two specific genes. This leads to decreased cleaved caspase 3, resulting in decreased cell death and improved alveolarization. In the right panel, use of Curosurf® as a delivery vehicle for CHOP/Ang2 siRNA augments the decreased expression of their respective gene products. This leads to further decrease in cell death and improves alveolarization.

Limitations and Future Work

The animal work presented here is limited due to low numbers of mice in each group (n≥3). Repeating the studies with larger cohorts should provide enough power to resolve whether or not there is a significant difference in Ang2 protein expression in mice transfected with and without Curosurf®. Given the improved lung morphology in mice treated with Curosurf®, we expect that there should indeed be a difference. Although not covered in our study due to time constraints, investigation is warranted into the effect of Curosurf® enhanced delivery of CHOP siRNA in newborn mice – specifically, the effect on hyperoxia-induced pulmonary damage. Furthermore, future studies expanding on the concepts presented here should include a Curosurf®-only control in order to assess whether the beneficial effect of Curosurf® enhanced siRNA therapy is in part due to Curosurf® itself.

We expect that our protocol for Curosurf® enhanced Ang2 siRNA delivery *in vivo* could be generalized for any oligonucleotide sequence. This opens up the possibility of testing Curosurf® enhanced siRNA delivery of many other causal biomarkers and proteins of interest. It would also be interesting to compare transfection rates of other commercially available surfactants to Curosurf®.

There are a number of studies that can add to our understanding of how Curosurf® enhanced siRNA therapy effects the development of BPD. Bronchoalveolar lavage (BAL) can be performed prior to lung harvest to assess the degree of inflammation. Neutrophil counts of the BAL fluid give an indication of the degree of inflammation and is thus

appropriate in ascertaining if Ang2 siRNA ameliorates inflammation. Additionally, the effect on angiogenesis of the microvasculature should be investigated. This can be performed *in vivo* via the use of antibodies against endothelial markers such as platelet endothelial cell adhesion molecule (PECAM). Finally, plasticity and degree of dysplasia can be assessed by measuring the Type II epithelial cell count via flow cytometry. Type II pneumocytes serve as progenitor cells in the lung and play an essential role during normal lung development.

Bourbon et al have shown some success with the reuptake of surfactant components using Curosurf®.[22] In their studies, liposomes were prepared by sonication of the suspension for 30 s·min^{-1} over a 15 min period. Future RNA interference studies using Curosurf® may benefit from the use of sonication to facilitate siRNA-laden liposome formation. Furthermore, as stated earlier, SP-A is thought to be an important mediator in receptor-mediated endocytosis at Type II alveolar epithelial cells. Enrichment of Curosurf® with synthetic SP-A may also yield an improvement in transfection ratio in future studies.[59]

Conclusion

In summary, our data shows a modest but significant improvement in mouse lung morphology when Curosurf® is used to enhance Ang2 siRNA delivery in a hyperoxia-induced model of BPD. Our *in vitro* work with CHOP also suggests that cell death is better mitigated with Curosurf® enhanced CHOP siRNA delivery. Possible mechanisms for the observed improvement includes better alveolar distribution of therapeutic siRNA,

improved surfactant/oligonucleotide uptake by Type II pneumocytes, and intrinsic antioxidative protection offered by Curosurf®. Further studies are needed to optimize and validate the observed positive effect of Curosurf® enhanced siRNA delivery in BPD.

References

1. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. The New England journal of medicine 1967;276:357-68.

2. Northway WH, Jr., Moss RB, Carlisle KB, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. The New England journal of medicine 1990;323:1793-9.

3. Jobe AH. The new bronchopulmonary dysplasia. Current opinion in pediatrics 2011;23:167-72.

4. Fanaroff AA, Stoll BJ, Wright LL, et al. Trends in neonatal morbidity and mortality for very low birthweight infants. American journal of obstetrics and gynecology 2007;196:147 e1-8.

5. Wright CJ, Kirpalani H. Targeting inflammation to prevent bronchopulmonary dysplasia: can new insights be translated into therapies? Pediatrics 2011;128:111-26.

6. Bhandari A, Bhandari V. Pitfalls, problems, and progress in bronchopulmonary dysplasia. Pediatrics 2009;123:1562-73.

7. Coalson JJ. Pathology of bronchopulmonary dysplasia. Seminars in perinatology 2006;30:179-84.

8. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Human pathology 1998;29:710-7.

9. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. American journal of respiratory and critical care medicine 2001;164:1971-80.

10. Bhandari A, Bhandari V. “New” Bronchopulmonary Dysplasia: A Clinical Review. Clinical Pulmonary Medicine 2011;18:137-43.

11. Khemani E, McElhinney DB, Rhein L, et al. Pulmonary artery hypertension in formerly premature infants with bronchopulmonary dysplasia: clinical features and outcomes in the surfactant era. Pediatrics 2007;120:1260-9.

12. Bourbon J, Boucherat O, Chailley-Heu B, Delacourt C. Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. Pediatric research 2005;57:38R-46R.

13. Fishman AP, Elias JA. Fishman's pulmonary diseases and disorders. 4th ed. New York: McGraw-Hill Medical; 2008.

14. Burri PH. Structural aspects of postnatal lung development - alveolar formation and growth. Biology of the neonate 2006;89:313-22.

15. Young B. Wheater's functional histology : a text and colour atlas. 5th ed. Edinburgh: Churchill Livingstone/Elsevier; 2006.

16. Barkauskas CE, Cronce MJ, Rackley CR, et al. Type 2 alveolar cells are stem cells in adult lung. The Journal of clinical investigation 2013;123:3025-36.

17. Roberts D, Dalziel S. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. The Cochrane database of systematic reviews 2006:CD004454.

18. Corrin B. Phagocytic potential of pulmonary alveolar epithelium with particular reference to surfactant metabolism. Thorax 1970;25:110-5.

19. Chander A, Claypool WD, Jr., Strauss JF, 3rd, Fisher AB. Uptake of liposomal phosphatidylcholine by granular pneumocytes in primary culture. The American journal of physiology 1983;245:C397-404.

20. Ryan RM, Morris RE, Rice WR, Ciraolo G, Whitsett JA. Binding and uptake of pulmonary surfactant protein (SP-A) by pulmonary type II epithelial cells. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 1989;37:429-40.

21. Wissel H, Looman AC, Fritzsche I, Rustow B, Stevens PA. SP-A-binding protein BP55 is involved in surfactant endocytosis by type II pneumocytes. The American journal of physiology 1996;271:L432-40.

22. Bourbon JR, Chailley-Heu B, Gautier B. The exogenous surfactant Curosurf enhances phosphatidylcholine content in isolated type II cells. The European respiratory journal 1997;10:914-9.

23. Bancalari E, Abdenour GE, Feller R, Gannon J. Bronchopulmonary dysplasia: clinical presentation. The Journal of pediatrics 1979;95:819-23.

24. Philip AG. Oxygen plus pressure plus time: the etiology of bronchopulmonary dysplasia. Pediatrics 1975;55:44-50.

25. Bonikos DS, Bensch KG, Northway WH, Jr. Oxygen toxicity in the newborn. The effect of chronic continuous 100 percent oxygen exposure on the lungs of newborn mice. The American journal of pathology 1976;85:623-50.

26. Warner BB, Stuart LA, Papes RA, Wispe JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. The American journal of physiology 1998;275:L110-7.

27. Bhandari V. Hyperoxia-derived lung damage in preterm infants. Seminars in fetal & neonatal medicine 2010;15:223-9.

28. Young KC, Del Moral T, Claure N, Vanbuskirk S, Bancalari E. The association between early tracheal colonization and bronchopulmonary dysplasia. Journal of perinatology : official journal of the California Perinatal Association 2005;25:403-7.

29. De Paepe ME, Mao Q, Powell J, et al. Growth of pulmonary microvasculature in ventilated preterm infants. American journal of respiratory and critical care medicine 2006;173:204-11.

30. Pierce MR, Bancalari E. The role of inflammation in the pathogenesis of bronchopulmonary dysplasia. Pediatric pulmonology 1995;19:371-8.

31. Bhandari A, Bhandari V. Biomarkers in bronchopulmonary dysplasia. Paediatric respiratory reviews 2013;14:173-9.

32. Rutkowski DT, Kaufman RJ. That which does not kill me makes me stronger: adapting to chronic ER stress. Trends in biochemical sciences 2007;32:469-76.

33. Harding HP, Zhang Y, Zeng H, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Molecular cell 2003;11:619-33.

34. Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. The quarterly journal of nuclear medicine : official publication of the Italian Association of Nuclear Medicine 2003;47:149-61.

35. Hato T, Kimura Y, Morisada T, et al. Angiopoietins contribute to lung development by regulating pulmonary vascular network formation. Biochemical and biophysical research communications 2009;381:218-23.

36. Thomas W, Seidenspinner S, Kramer BW, et al. Airway Angiopoietin-2 in Ventilated Very Preterm Infants: Association With Prenatal Factors and Neonatal Outcome. Pediatric pulmonology 2011;46:777-84.

37. Haas DM, Caldwell DM, Kirkpatrick P, McIntosh JJ, Welton NJ. Tocolytic therapy for preterm delivery: systematic review and network meta-analysis. Bmj 2012;345:e6226.

38. Surfactant replacement therapy for severe neonatal respiratory distress syndrome: an international randomized clinical trial. Collaborative European Multicenter Study Group. Pediatrics 1988;82:683-91.

39. Vento M, Moro M, Escrig R, et al. Preterm resuscitation with low oxygen causes less oxidative stress, inflammation, and chronic lung disease. Pediatrics 2009;124:e439-49.

40. Bancalari E. Changes in the pathogenesis and prevention of chronic lung disease of prematurity. American journal of perinatology 2001;18:1-9.

41. Avery GB, Fletcher AB, Kaplan M, Brudno DS. Controlled trial of dexamethasone in respirator-dependent infants with bronchopulmonary dysplasia. Pediatrics 1985;75:106-11.

42. Wilson RC, Doudna JA. Molecular mechanisms of RNA interference. Annual review of biophysics 2013;42:217-39.

43. Kurreck J. Antisense technologies. Improvement through novel chemical modifications. European journal of biochemistry / FEBS 2003;270:1628-44.

44. Choo-Wing R, Syed MA, Harijith A, et al. Hyperoxia and interferon-gamma-induced injury in developing lungs occur via cyclooxygenase-2 and the endoplasmic reticulum stress-dependent pathway. American journal of respiratory cell and molecular biology 2013;48:749-57.

45. Bhandari V, Choo-Wing R, Lee CG, et al. Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. Nature medicine 2006;12:1286-93.

46. Dokka S, Toledo D, Shi X, Castranova V, Rojanasakul Y. Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes. Pharmaceutical research 2000;17:521-5.

47. Lasic DD. Liposomes. Science & Medicine. Philadelphia, PA: Science & Medicine; 1996:34-43.

48. Gregoriadis G. Liposome Technology: Interactions of Liposomes with the Biological Milieu. 3rd ed. New York: Informa Healthcare; 2007.

49. Bernhard W, Mottaghian J, Gebert A, Rau GA, von Der HH, Poets CF. Commercial versus native surfactants. Surface activity, molecular components, and the effect of calcium. American journal of respiratory and critical care medicine 2000;162:1524-33.

50. Speer CP, Sweet DG, Halliday HL. Surfactant therapy: past, present and future. Early human development 2013;89 Suppl 1:S22-4.

51. Yee M, Chess PR, McGrath-Morrow SA, et al. Neonatal oxygen adversely affects lung function in adult mice without altering surfactant composition or activity. American journal of physiology Lung cellular and molecular physiology 2009;297:L641-9.

52. Hsia CC, Hyde DM, Ochs M, Weibel ER, Structure AEJTFoQAoL. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. American journal of respiratory and critical care medicine 2010;181:394-418.

53. Ray P, Tang W, Wang P, et al. Regulated overexpression of interleukin 11 in the lung. Use to dissociate development-dependent and -independent phenotypes. The Journal of clinical investigation 1997;100:2501-11.

54. Salvesen GS. Caspases: opening the boxes and interpreting the arrows. Cell death and differentiation 2002;9:3-5.

55. Bhandari V, Choo-Wing R, Harijith A, et al. Increased hyperoxia-induced lung injury in nitric oxide synthase 2 null mice is mediated via angiopoietin 2. American journal of respiratory cell and molecular biology 2012;46:668-76.

56. Breunig M, Lungwitz U, Liebl R, Goepferich A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. Proceedings of the National Academy of Sciences of the United States of America 2007;104:14454-9.

57. Masotti A, Mossa G, Cametti C, et al. Comparison of different commercially available cationic liposome-DNA lipoplexes: Parameters influencing toxicity and transfection efficiency. Colloids and surfaces B, Biointerfaces 2009;68:136-44.

58. Oborina EM, Yappert MC. Effect of sphingomyelin versus dipalmitoylphosphatidylcholine on the extent of lipid oxidation. Chemistry and physics of lipids 2003;123:223-32.

59. Walther FJ, David-Cu R, Leung C, et al. A synthetic segment of surfactant protein A: structure, in vitro surface activity, and in vivo efficacy. Pediatric research 1996;39:938-46.

60. Fornasier M, Puccini P, Razzetti R, Acerbi D, Bongrani S, Ventura P. Evaluation of potentially injurious effects of exogenous surfactant lysophospholipids on the alveolar epithelium and pulmonary mechanics. Biology of the neonate 1997;71:337-44.

61. Ramanathan R, Rasmussen MR, Gerstmann DR, Finer N, Sekar K, North American Study G. A randomized, multicenter masked comparison trial of poractant alfa (Curosurf) versus beractant (Survanta) in the treatment of respiratory distress syndrome in preterm infants. American journal of perinatology 2004;21:109-19.

62. Halliday HL, Tarnow-Mordi WO, Corcoran JD, Patterson CC. Multicentre randomised trial comparing high and low dose surfactant regimens for the treatment of respiratory distress syndrome (the Curosurf 4 trial). Archives of disease in childhood 1993;69:276-80.

63. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science 1990;247:1465-8.

64. Lai J, Gold MS, Kim CS, et al. Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1.8. Pain 2002;95:143-52.