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Hyperpolarized ^3He Diffusion MRI and Histology of Secreted Frizzled Related Protein-1 (SFRP1) Deficient Lungs in a Murine Model

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Abstract

Secreted Frizzled Related Protein-1 (SFRP1) plays a key role in many diverse processes, including embryogenesis, tissue repair, bone formation, and tumor genesis. Previous studies have shown the effects of the SFRP1 gene on lung development using the SFRP1 knockout mouse model via histological and physiological studies. In this study, the feasibility of ADC (acquired via HP ^3He) to detect altered lung structure in the SFRP1 knockout (SFRP1^{-/-}) mice was investigated, and compared to analysis by histology. This study consisted of two groups, the wild type (WT) mice and the knockout (KO) mice with $n = 6$ mice for each group. ^3He ADC MRI and histology were performed on all of the animals. The global L_m of WT and KO mice were $35.0 \pm 0.8 \mu\text{m}$ and $38.4 \pm 3.8 \mu\text{m}$, respectively, which translated to an increase of 9.58% in the L_m of KO mice. The mean global ADC for the WT and KO mice were $0.12 \pm 0.01 \text{ cm}^2/\text{s}$ and $0.13 \pm 0.01 \text{ cm}^2/\text{s}$, respectively, which equated to a relative increase of 8.0% in the KO mice compared to the WT mice. In the sub-analysis of the anterior, medial and posterior lung regions, L_m increased by 10.50%, 6.66% and 11.84% in the KO mice, respectively, whereas the differences in ADC between the two groups in the anterior, medial, and posterior regions were 7.3%, 8.3%, and 4.6%, respectively. These results suggest that HP MRI measurements can be used as a suitable substitute for histology to obtain valuable information about lung geometry noninvasively. This technique is also advantageous as regional measurements can be performed, which can identify lung destruction more precisely. Most importantly, this approach extends far beyond the specific pathology analyzed in this study, as it can be applied to many other pathological conditions in the lung tissue, as well to many other embryonic studies.

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INTRODUCTION

Secreted Frizzled Related Protein-1 (SFRP1) is a member of a family of secreted proteins that contain a cysteine rich domain (CRD) analogous to the one found in the WNT binding region of the Frizzled family of transmembrane receptors. It has been shown that SFRP1 acts as an antagonist to the WNT pathway by competing with the Frizzled receptor for binding sites on WNT proteins. The WNT signaling pathway and its associated proteins play a key role in many diverse processes, including embryogenesis, tissue repair, bone formation, and tumor genesis.

Elucidating the mechanism of the SFRP family on the WNT pathway is an ongoing process. Previous work has suggested that SFRP1 acts as a tumor suppressor protein in various types of cancers, including breast cancer, colorectal tumors and pleural mesothelioma [1, 2, 3]. More recently, SFRP1 was demonstrated to play an important role in lung morphogenesis during embryogenesis. In the normal lung, SFRP1 is tightly expressed during early stages of development concomitant with the expression of WNT5A and WNT10B, resulting in healthy lung parenchyma and normal alveoli size. Knockout mouse studies demonstrate that blocking the expression of SFRP1 results in abnormally dilated alveoli with structural changes similar to what is seen in emphysematous tissue of adult lung parenchyma [4].

Although SFRP1^{-/-} knockout mice exhibit increased alveoli size similar to that observed in emphysematous tissue, there are several important differences between the two conditions. Firstly, although both conditions exhibit abnormally large alveoli, in emphysema SFRP1 expression is increased, contrary to what is observed in the knockout. Secondly, while both emphysema and loss of SFRP1 affect the lung fairly heterogeneously, the change in alveoli size due to SFRP1 deficiency is more heterogeneous and is greater in the periphery of the lung. Lastly, unlike emphysema, loss of SFRP1 does not result in an ongoing destructive process, since the alveoli do not continue to enlarge over time. Consequently, the compliance of the lungs is not altered in the lungs of SFRP1^{-/-} mice after development [4].

Previous studies have primarily relied on histology to identify structural changes in animal models, both during development and adulthood. Histology is widely used to obtain anatomical measurements of both large-scale changes, such as alveoli size, as well as smaller scale changes, such as those that occur in the lung epithelium and parenchyma. However, using histology to obtain these data requires the animal to be sacrificed in the process. This approach becomes especially problematic if one is studying a developmental animal model longitudinally, or developmental abnormalities clinically. Hyperpolarized (HP) ³He MRI provides an alternative to histology; it does not require animal sacrifice, and can provide both structural and functional data on the lungs.

Hyperpolarized gases have been used previously to study structural and functional changes in lung tissue in human and animal species. Human pathologies studied include several disorders, such as, emphysema, asthma and COPD [5, 6, 7]. Animal models studied include rat and mouse models of elastase-induced emphysema [8, 9]. The simplest method to probe lung structure utilizes the apparent diffusion coefficient (ADC) of inhaled HP ³He, acquired by using diffusion-weighted magnetic resonance imaging (DW-MRI). An increase in

alveolar size correlates with elevated ^3He ADC values. This increase is caused by the diminished restriction of gas diffusion in larger airspaces due to the disease process [10]. Similarly, HP gas MRI can also provide functional information such as regional ventilation and regional alveolar partial pressure maps of lungs [11, 12].

Other alternatives to histological measurements for studying structural and functional changes in the lung include CT and PET. However, both of these methods suffer from their own shortcomings: the former requires ionizing radiation, and the latter requires a radionuclide. In contrast, HP ^3He MRI is a safe, non-invasive, non-ionizing technique that only requires the inhalation of an inert gas. As such, HP ^3He MRI is potentially a very useful tool for longitudinal studies as well.

Previous studies have shown the effects of the SFRP1 gene on lung development using the SFRP1 knockout mouse model via histological and physiological studies [4]. In the present study, the feasibility of ADC (acquired via HP ^3He) to detect altered lung structure in the SFRP1 knockout (SFRP1^{-/-}) mice was investigated, and compared to analysis by histology.

METHODS

Mouse Model

This study was performed in accordance with protocols approved by the respective Institutional Animal Care and Use Committees of the University of Pennsylvania and Columbia University. The mice were divided into a control (n=6) and a knockout (SFRP1^{-/-}) group (n=5). The SFRP1^{-/-} mouse model was generated using the protocol by Bodine et al. [13]. In brief, exon 1 of the SFRP1 gene was deleted because it encodes the majority of the functional protein. This exon was replaced with a LacZ/MC1-Neo gene to ensure that the SFRP1 promoter activity could be monitored with β -galactosidase expression in SFRP1^{-/-} mice. The control mice (SFRP1^{+/+}) carried the wild type (+/+) allele, the knockout (SFRP1^{-/-}) mice lacked this allele and contained the LacZ gene.

Mechanical ventilation

The mice were sedated with 100mg/kg intraperitoneal ketamine and 10 mg/kg xylazine. This dosage was repeated every 60-90 minutes or if any movement was detected. They were then tracheotomized with a 1.5mm endotracheal tube for intubation with a custom-designed, MRI-compatible, small animal ventilator equipped with real time monitoring of peak inspiration pressure (PIP) with an accuracy of 100 μL . A gas mixture of HP ^3He and oxygen in a 4:1 ratio was used for imaging. The heart rate and oxygen saturation were constantly monitored using a veterinary pulse-oximeter (Vet/Ox, Heska, Loveland, CO) with an optical probe attached to the hind foot. The temperature was monitored using a rectal probe and was maintained at 37°C by a flow of warm air through the bore of the magnet.

Helium Hyperpolarization

The helium gas used for imaging was hyperpolarized using spin exchange collisions with optically pumped rubidium (Rb) atoms, as described [14] using a commercial prototype polarizer (IGI.9600.He, GE Healthcare, Durham, NC). The gas was polarized to

approximately 30% over a period of 14 hours prior to use. During the imaging session, the HP ^3He was stored in a 400mL Tedlar bag (Jensen Inert Products, Coral Springs, FA) that was placed inside the magnet's bore. The T_1 relaxation time constant of this gas was in the order of 30-40min inside the bag. The ^3He was mixed with O_2 in a 4:1 ratio and delivered to the subjects at 100 BMP with I:E= 1:2 and V_T = 1.2mL/100g body mass.

MR imaging and parameters

Imaging was performed in a 50-cm horizontal bore, 4.7T MRI scanner (Varian, Palo Alto, CA) equipped with 3.8 cm, 25 G/cm gradients and an 8-leg bird-cage chest coil (Stark Contrast, Erlangen, Germany). The coil was tuned to the ^3He resonance frequency of 152.95 MHz. The animal was placed in the supine position in the coil and inserted into the bore of the magnet. Diffusion imaging was performed using a double acquisition diffusion-weighted gradient echo pulse sequence [15]. In the double acquisition method a single pair of gradients with inverted amplitudes is used to obtain two sets of images. The following acquisition parameters were used: b-values = 0.00 and 2.18 s/cm², field of view = 3×3 cm², slice thickness = 4 mm, flip angle (α) = ~8°, matrix size = 64×64 pixels, TR = 5.6 ms, and TE = 3.2 ms. Three coronal slices were acquired: anterior, middle (plane through which the trachea passes), and the posterior lung. The RF pulse width was calibrated to estimate the flip angle by acquiring a series of consecutive images during a two-second single breath hold. Subsequently, series of 10 pre-wash breaths were administered before the 11th breath hold of 3 seconds, during which the diffusion-weighted images were acquired.

Histology

Each animal was euthanized following the image acquisition using intravenous, high concentration potassium cyanide. The drug was administered through the tail vein instead of the more commonly used intracardiac route to avoid damaging the lung with the needle. After euthanasia, the trachea and lungs were removed and filled with a 10% formalin solution at a pressure of 25 cmH₂O for 24 hours. The lungs were then paraffin embedded before being sectioned and stained with hematoxylin and eosin (H&E) [16]. A total of 10 5µm-thick axial tissue slices (5µm gap between slices) were cut for histology.

Data Analysis

Histology analysis

The histology tissue slices were used to generate morphometric measurements, particularly the mean linear-intercepts (L_m) for each subject. L_m is a measurement corresponding to the volume-to-surface-area ratio of alveoli, and has been used as a metric for alveolar changes in emphysema as well as in the SFRP1^{-/-} mice [4, 16]. The L_m measurements were generated using the protocol by Ishii et al. [9]. Slides were scanned using a ×20 slide scanner (Aperio-Technologies, Vista, CA), and processed using MATLAB (The MathWorks, Natick, MA). The slides were divided into anterior, medial, and posterior regions that corresponded to the respective MRI slices. Three slides were used to sample the anterior and posterior regions, whereas four slides were used to sample the medial region. Airways and vasculature were masked and then the images were thresholded. The thresholding removed the background noise, leaving the alveoli walls visible for mean linear-intercept calculations (L_m). A grid of

parallel lines spaced 75 μm apart was superimposed on each image, and L_x , which is defined by the intercept of the lines with the airspace walls in one orientation, was measured. Because mean linear intercept values depend on their orientation relative to the lung tissue slice, measurements were also made in orthogonal orientation, and labeled as L_y [18]. Global L_m and an L_m corresponding to the anterior, medial, and posterior MRI slices were calculated.

Imaging analysis

The acquired diffusion weighted images were processed offline using MATLAB (The MathWorks, Natick, MA). A custom script was used to fit to the following equation to yield the ADC value for each pixel in the image:

$$S_1 = S_0 * \exp \{ \ln [\cos(\alpha)] - b * ADC \}$$

The proximal conductive pathways (trachea, bronchioles) were masked prior to analysis. However, small airways were included in the ADC analysis. As such, the ADC values do not exclude airways completely, but are a combined measurement of the acinar airway and alveoli size [19].

A two-tailed, unequal variance, Student T-test was used to compare the ADC and histology measurements between the WT and KO mice. A significance level of 0.05 was applied for all the statistical analyses.

RESULTS

Population of mice in each group

This study consisted of two groups, the wild type (WT) mice and the knockout (KO) mice with $n = 6$ mice for each group. ^3He ADC MRI and histology were performed on all the animals. However, the ADC measurements of one of the KO mice were excluded from the study due to incorrect insertion of the endotracheal ventilation tube used for intubation during the MRI sequence. Although the ADC measurements from this mouse were not used, the respective histology measurements were still included for analysis.

Histology Analysis

Representative histological slices for lungs of WT and KO mice are shown in Figure 1. The global L_m of WT and KO mice were $35.0 \pm 0.8 \mu\text{m}$ and $38.4 \pm 3.8 \mu\text{m}$, respectively. This translated to an increase of 9.58% ($p = 0.08$) in the L_m of KO mice. This result is consistent with previous results that show an increase in the alveolar size in the $\text{SFRP1}^{-/-}$ mice [4]. A statistically significant difference was not found, probably due to the study's lack of power. Figure 2(a) shows the box plots comparing the mean, median and range of the global L_m for each group. The greater spread of the transgenic mice as compared to the wild type mice is reflected in their respective standard deviations. In the sub-analysis of the anterior, medial and posterior lung regions, L_m increases of 10.50%, 6.66% and 11.84% were found in the KO mice, respectively (Table 1). L_m increases in the anterior and posterior regions were significantly different.

ADC Analysis

Representative ADC maps for WT and KO mice are shown in Figure 3. Global and regional ADC analyses were performed on the acquired diffusion MRI images. The mean global ADC for the WT and KO mice were $0.12 \pm 0.01 \text{ cm}^2/\text{s}$ and $0.13 \pm 0.01 \text{ cm}^2/\text{s}$, respectively. This equates to a relative increase of 8.0% in the KO mice compared to the WT mice. The result was not statistically significant, but again, was likely a limitation of the population size. Figure 2(b) shows the mean, median and range of ADC in the two mice populations. The histological findings confirm the increased alveolar diameter that results in higher ADC of the confined gas.

Table 2 shows the mean ADC of each region in both groups of mice. The differences in ADC between the WT and KO groups in the anterior, medial, and posterior regions were 7.3%, 8.3%, and 4.6%, respectively.

Comparison of Histology to ADC

The L_m measurements were compared to the corresponding ADC measurements in order to assess the value of ADC as an alternative technique for acquiring lung structure measurements in this particular model of pulmonary development deficiency. The comparison of the two sets of measurements is presented as a plot of global ADC values versus histology in figure 4(a). The two measurements cannot be quantitatively correlated due to fact that the ADC measurements were made at constant volume whereas the histological analysis was performed at fixed constant pressure. However, despite the differing conditions under which these techniques were performed, conclusions can still be made about the differences observed between the two groups of mice. Specifically, it is apparent that the WT mice clustered in the lower left region of the graph, corresponding to lower ADC and L_m values, whereas the KO mice clustered around the upper right region of the graph, corresponding to higher ADC and L_m values.

Both measurements were also plotted for the anterior, middle, and posterior lung slices, as shown in Figure 4(b). The general distribution was similar to that seen for the global ADC distribution, particularly for the anterior and middle slices. The posterior slice, however, shows a distribution different to that of the global ADC.

DISCUSSION

SFRP1 is a developmentally expressed gene that plays a role in alveoli formation and is a known inhibitor of the WNT signaling pathway. Previous studies have shown that mice with the SFRP1 gene knocked out present with abnormal WNT signaling and mesenchymal proliferation. SFRP1^{-/-} mice also exhibit down regulation of MMPs, which impairs the protease-dependent alveolar remodeling processes, resulting in alveolar duct dilation during embryogenesis. The conventional method for evaluating this type of abnormal alveolar dilation is through histology-derived morphometry. This process is inherently invasive as the mice have to be sacrificed prior to any measurements. As a result, any such study using histology is limited to a cross-sectional analysis of the data, since a longitudinal analysis of

pulmonary embryogenesis or other physiology would require multiple subsets of mice to be sacrificed in order to obtain sufficient data for each time point.

The HP gas MRI technique used in this study holds numerous advantages over conventional histology. It has been previously used to characterize emphysematous lungs in animal models [20, 21], and human specimens [5, 22] and subjects [23]. Most of these studies have used HP ^3He MRI to detect alveolar changes using ADC, and compared the results to histology. As mentioned above, this technique can also be used to study different models of lung embryogenesis non-invasively as compared to invasive histology, which necessitates postmortem conditions. Thus, the HP gas MRI technique not only allows for other cross-sectional studies similar to this study, but allows us to carry out longitudinal analysis as well.

Compared to conventional methods, the HP gas MRI technique not only reduces the number of animals required, but also allows for physiological changes to be tracked on a per subject basis, contributing to a better understanding of pathological conditions. This is similar to other longitudinal studies performed in different tissues, in both animals and humans. For example, Kirby et al. studied chronic obstructive pulmonary disease (COPD) in lungs using HP ^3He , and Blumenkrantz, et al. studied osteoarthritis in knees using multiple MRI techniques [24, 25]. Both studies demonstrate the potential of non-invasive imaging techniques to study pathology longitudinally, a goal that can also be accomplished after making minor adjustments to the technique presented in this study.

Another shortcoming of using histology is that detection is limited by the sampling technique. An abnormal or pathological condition can be overlooked if that particular region was not biopsied or sampled. Changes in inflation or fixation can alter the quantitation of L_m as well. Conversely, global parameters are difficult to quantify accurately, especially in larger animals. HP MRI techniques circumvent these shortcomings by acquiring an image of the entire tissue that can be analyzed on both regional and global bases. As a result, HP MRI has the potential to probe early disease pathology that may only be visible in some particular regions of the tissue, but not globally. And as demonstrated in our study, HP MRI can also be used as an alternative for histology to measure changes in alveolar size in pathological conditions non-invasively. In contrast, the histological measurements made in previous studies showing that $\text{SFRP1}^{-/-}$ transgenic mice undergo alveolar dilation during embryogenesis required the mice to be sacrificed.

Previous studies have shown that, in general, the alveolar size in mice is not isotropic across the lung [18]. As such, the mean linear intercept was calculated along two orthogonal orientations, labeled L_{My} and L_{Mx} . The two values were slightly anisotropic, with a difference of $9 \pm 2\%$ between L_{Mx} and L_{My} for both groups. The data pertaining to the two measurements are not shown because the relative change between the WT and KO mice for both parameters was very similar. Given that the directional changes were not particularly anisotropic, the root mean squared (RMS) average of the two measurements was calculated, with the results presented as L_m .

As noted earlier, the transgenic mice exhibited a global L_m increase of 9.6%. This trend was also present in the anterior, medial and posterior lung slices. These results are consistent with the previously published $SFRP1^{-/-}$ model study, however, the increase in magnitude in our study was less than the 22% reported in the previous study at five weeks post-partum [4].

The histology of the anterior, medial, and posterior lungs showed increases in L_m as well. The anterior and posterior regions showed a similar magnitude of change, 10.5% and 11.9% respectively. The medial slice exhibited a much smaller increase of 6.7%. The smaller increase in the medial region may be the result of embryogenesis itself. Previous work on $SFRP1$ has demonstrated that the protein is primarily expressed during the pseudoglandular stage of lung development. In the early pseudoglandular stage $SFRP1$ can be detected in both the epithelium and mesenchyme. However, in the mid and late pseudoglandular stages, $SFRP1$ is only seen in the distal mesenchyme, which matures into the distal portions of the mature lung.

As a result of these differences during the varying stages of embryogenesis, a knockout mouse with the $SFRP1^{-/-}$ phenotype might experience the most significant changes in the distal regions of the lung, as this is where $SFRP1$ is detected for the longest duration during embryogenesis. Those regions will show the greatest change in magnitude in the mature mice. Such a change will translate to large changes in the distal, i.e. the posterior and anterior regions, as compared to the medial region of the mature mice.

The alveolar size was also measured using ADC. As seen in the results, the transgenic mice exhibited an ADC increase of 8.02%. This result is consistent with, although slightly lower, than the magnitude of change measured by L_m globally. This is most likely due to the fact that our ADC quantification technique includes the smaller airways in the measurement, which do not change in the $SFRP1^{-/-}$ mouse model. The ADC of the anterior, medial, and posterior lung slices showed an increase in transgenic mice as well. The increases were 7.31%, 8.35%, and 4.61%, respectively. The magnitude of these increases did not match their corresponding L_m measurements for each region. While a similar increase in ADC was seen in the anterior and medial regions, the posterior region has a much smaller increase.

The smaller increase in the posterior region can be most likely attributed to the MRI scanning procedure. A previous study has shown that the alveoli can collapse due to gravity mediated atelectasis [17]. The posterior region, which is closest to the surface, will experience the greatest gravitational effect. This will result in a greater number of collapsed alveoli, which will lower the average ADC value for the posterior lung region. A potential way to avoid this in future studies would be to apply positive end-expiratory pressure (PEEP) ventilation, or to increase the tidal volume (V_T) to reduce the number of collapsed alveoli.

The comparison of the two techniques, L_m and ADC, are presented in Figure 4(a). It is clear that for all cases, global as well as regional, the wild type mice cluster in the lower left region towards lower ADC and L_m values, whereas the KO mice cluster around the upper right towards greater ADC and L_m values. The consistency of this pattern suggests that both

measurements can be used to measure changes in alveoli sizes. Unfortunately a quantitative correlation between the two cannot be performed due to the different conditions under which the two measurements were made. The histology was performed using a constant pressure (25cm H₂O) fixing procedure that minimizes atelectatic changes, whereas the MRI measurements were done using ventilation proportional to the body mass. Ideally, a tidal volume (V_T) proportional to the total lung capacity (TLC) of each mouse would have been measured and used for ventilation during the MRI procedure, which would have allowed for a direct correlation with the morphometry data.

This study demonstrates the numerous advantages of hyperpolarized MRI as an alternative to conventional morphometric techniques used for studying the physiology of healthy and pathological tissue. Using a SFRP1 knockout mouse model, this study has established that HP MRI is a suitable substitute for histology to obtain valuable information about lung geometry non-invasively. This technique is also advantageous as regional measurements can be performed which can identify the lung destruction more precisely. Finally, it should be recognized that this approach extends far beyond the specific pathology analyzed in this study, as it can be applied to many other pathological conditions in the lung tissue, as well to many other embryonic studies.

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ABBREVIATIONS

SFRP1	Secreted Frizzled Receptor Protein-1
HP	HyperPolarized
L_m	Mean Linear Intercept
WT	WildType
KO	KnockOut

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Figure 1. Representative Histology of wildtype (WT) and knockout (KO) mice. It can be seen that the alveoli in the KO model are larger than that of the WT mice in the same region (medial lung slice).

Figure 2.
(a) Global <LM> of KO and WT mice. **(b).** Global <ADC> of KO and WT mice.

Figure 3. Representative ADC images of WT (above) and KO (below) mice in, from left to right, the anterior, medial, and posterior slices.

Figure 4.

(a) Mean global ADC vs mean global L_M . (b). Regional comparison of ADC vs L_M .

Table 1

Regional mean linear intercept (L_m) analysis of lung.

<Lm>	Anterior		Medial		Posterior	
	Mean	SD	Mean	SD	Mean	SD
Wild type	35.34	1.02	35.47	1.08	34.22	1.41
Transgenic	39.05	3.38	37.83	4.49	38.27	3.22
Difference (%)	10.50		6.66		11.85	

All measurements are in μm . The $SFRP1^{-/-}$ lungs show an increase in mean linear intercepts in all three regions.

Table 2

Regional apparent diffusion coefficient (ADC) measurements of the lung.

<ADC>	Anterior		Medial		Posterior	
	Mean	SD	Mean	SD	Mean	SD
Wild type	.125	.01	0.127	.01	0.112	0.01
Transgenic	0.134	0.01	0.138	0.01	0.117	0.01
Difference (%)	7.31		8.35		4.61	

All measurements are in cm^2/s . The gas imaging technique shows an increase in alveolar size in all three regions, similar to the trend seen with histology.