UNIVERSITY OF CALIFORNIA, SAN DIEGO

Role of Matrix Metalloproteinase-9 in Neonatal Lung Development Under Hypoxic Stress

A thesis submitted in partial satisfaction of the requirements for the degree of

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in

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by

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This thesis of Mary Nguyen is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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To my parents and my mentor for all their support and confidence in me.

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ABSTRACT OF THE THESIS

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Professor Gabriel Haddad, Chair

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Hypoxia is a condition associated with many pulmonary diseases and can induce tissue and vascular remodeling, which can result in many pathological conditions. Since Matrix Metalloproteinases (MMP) family can alter the components of the matrix and thus is an important facilitator of the remodeling process; we chose to study the impact of MMP-9, a member of the MMP family that has been shown to be involved in many hypoxia-induced diseases, in hypoxia-induced lung remodeling in immature mice. MMP -9 knockout (KO) mice and FVB controls (background for MMP-9 KO) were both exposed to either 11%O_2 or room air from postnatal days 2 to 17. At P17, mice were sacri-

ficed and lungs were processed for protein and histology. In hypoxia, the survival rate of MMP-9 KO mice was significantly lower than MMP-9 WT mice. MMP-9 KO mice that survived hypoxia exposure were significantly smaller than MMP-9 WT mice. Histology revealed that MMP-9 KO mice that survived hypoxia had increased muscularized vessels and larger alveolar diameter than MMP-9 WT mice. Protein analysis demonstrated significant changes in ECM and overall increased in MMP expression in MMP-9 KO mice compared to MMP-9 WT exposed to chronic hypoxia. Our study indicates that MMP-9 may play a critical role in hypoxia-induced tissue and vascular remodeling. Deletion of this protein results in increased mortality that may be associated with increased smooth muscle proliferation and increased alveolar diameter.

I. Introduction

A. Lung development

Lung development in humans consists of 4 morphologically characterized phases called the pseudoglandular phase, the canalicular phase, the saccular phase, and the alveolar phase (Peter H. Burri). The pseudoglandular lungs (5-17 weeks) encompass prospective conductive airways and acinar outlines (Burri). During the canalicular phase (16-26 weeks), the lungs then undergo extensive angiogenesis, giving rise to a complex capillary network surrounding the developing airway structures (Ryu). The saccular phase (24-38 weeks) is characterized by alveoli formation. Lastly, the alveolar phase (36 weeks gestation to 1-2 years of age) allows for an increase in gas exchanging surface area. The alveolar phase continues even after birth into the first few year after birth. As the respiratory system develops, changes continue to occur in the lung extracellular matrix (ECM).

B. Extracellular matrix

The extracellular matrix is composed of proteins and polysaccharides that are assembled into organized meshwork. The extracellular matrix is the extracellular tissue substance that provides structural support and anchors cells. It also regulates intercellular communication and segregates tissues from one another. The composition of the ECM can indirectly affect the supply of oxygen in newly formed tissue during growth, wound healing, and fibrosis (Chen and Aplin; Gebb and Jones; Tokuda). In the normal healthy human lung, the ECM has a daily metabolic turnover rate of 10-15% and is controlled by 3 distinctive mechanisms: de novo synthesis (cellular synthesis), degradation of by matrix metalloproteinase (MMP), and inhibition of MMP by tissue inhibitors of

MMP (TIMP) (Leufgen). Therefore, degradation and restructuring of the ECM is crucial for maintaining or controlling cellular functions within a given tissue. For the restructuring of ECM to occur, cellular degradation must first occur to allow new tissues to develop to change the structure of the target tissue. Among the variety of enzymes that regulate cellular degradation and turnover of the ECM are the matrix metalloproteinases (MMPs) (Ryu).

C. Matrix Metalloproteinase

The MMP family of protein consists of 25 related endopeptidases that share common functional domains and activation mechanisms (Vu.). MMPs are Ca^{+2} and Zn^{+2} dependent enzymes that are active at neutral pH. These enzymes are usually secreted as proenzymes and can be further activated by the removal of their amino-terminal propeptide. When activated, MMP can degrade ECM molecules, including collagen, fibronectin, gelatin, and elastin, which can lead to cell migration or change the ECM microenvironment and can ultimately lead to alterations in cellular behavior (Cao). Therefore, changes in the ECM can modulate the activity of certain biological molecules through direct cleavage, release from bound molecule, or modulate their inhibitor's activities. One of the key enzymes that regulate the degradation of the ECM is the MMP family, which is regulated by the family of tissue inhibitors of metalloproteinases called TIMPs (Vu). The TIMP family consists of four members, which are capable of inhibiting MMP functions by interactions of their N-terminal domain with the active sites of MMPs (Ryu). Together, the TIMP family and MMP family work to maintain the homeostasis of the ECM.

D. Chronic Hypoxia

Hypoxia is a condition in which the body, or specific tissue, does not have an adequate amount of oxygen. Chronic hypoxia (CH) is a condition often associated with asthma, bronchopulmonary dysplasia (BPD), pulmonary hypertension, and congenital heart defects. CH can be seen in patients who have excessive degradation of collagen and deposition of elastin, some components of the extracellular matrix (ECM) (Ambalavanan). These changes to the ECM can interfere with cellular migration and repair mechanisms. Members of the MMP family are excellent candidate genes responsible for this thinning. ECM and certain growth factors no only help to maintain the balance of cell proliferation and differentiation but also help to direct organ development and tissue homeostasis (Leufgen) Since this family of proteins consists of about 25 types of proteins with various functions, this manuscript will focus on MMP-9 because it is an important member of the MMP family that has been associated with many of the lung disorders listed above. In addition, MMP-9 is a gelatinase that is mainly expressed in inflammatory and epithelial cells and is important in collagen degradation (Ambalavanan).

E. Matrix Metalloproteinase-9

MMP-9, also known as gelatinase B is a 92 kD proteinase that is produced by several cell types including inflammatory cells and stimulated connective tissue cells (Hussain). Gelatinases such as MMP-9 and MMP-2 are characterized by their ability to degrade gelatin. MMP-9 is also known for its role in pericellular basement membrane turnover by degrading collagen IV, which is main component of the basement membrane and the ECM (Hussain). However, MMP-9 is not constitutively expressed in nor-

mal healthy cells but is present in any repair or remodeling process and is also expressed by inflammatory cells in any diseased or inflamed tissue (Shapiro).

F. Significance

Many current studies show that an alteration of MMP/TIMP expression in both adult and neonatal with lung diseases, such as BPD, asthma, and emphysema, can cause changes to the ECM. Diseases where MMP/TIMP expression is altered are also influenced by the reduction in oxygen level available to the lungs. Chronic hypoxia is often present in newborn patients with pulmonary hypertension, BPD, and congenital heart disease (Ambalavanan). Bronchopulmonary dysplasia (BPD), also known as chronic lung disease (CLD), is a condition that affects premature infants who have diseased lungs, due to a number of factors including prematurity, arrested lung development and progressive lung injury due to high oxygen exposure and repeated infections. These repeated injuries cause changes in the structure and organization of the lungs, which adversely affects lung function and makes breathing difficult.

Recent studies indicate that MMP-9 and MMP family members play a major role in lung development (Ryu); therefore, while MMP-9 is associated with inflammation and some forms of lung injury, the absence of MMP-9 in premature infants under stress may cause even greater challenges in their lung development that can ultimately lead to further morbidity and mortality. In this study, we will investigate the impact of MMP-9 in neonatal lungs under hypoxic conditions by using MMP-9 KO mice. This study will help elucidate the role of MMP-9 expression during lung development.

II. Materials and Methods

A. Animals used

Two different strains of mice (Jackson Lab, ME) of were studied to analyze the role of MMP-9 in exposure to chronic hypoxia during postnatal lung development. MMP-9 knockout mice (KO) were used along with FVB (background mice of MMP-9 knockout mice, here on out will be referred to as MMP-9 WT) to study the role of MMP -9 in postnatal lung development under hypoxic stress. Newborn pups of both strains at postnatal day two were exposed to either 11%O2 (experimental) or room air of 21%O2 (control) for two weeks. After two weeks, at postnatal day 17, both groups were analyzed for histology and molecular analysis.

B. Overview

Four groups of newborn mouse pups were studied to analyze the role of MMP-9 in postnatal lung development under hypoxia. We exposed two groups (MMP-9 WT and MMP-9 KO) to 11% O2 (experimental) and two groups (MMP-9 WT(FVB background) and MMP-9 KO) to room air (control group) from postnatal day 2 through 17. Separate litters for each condition were analyzed for protein analysis and histology.

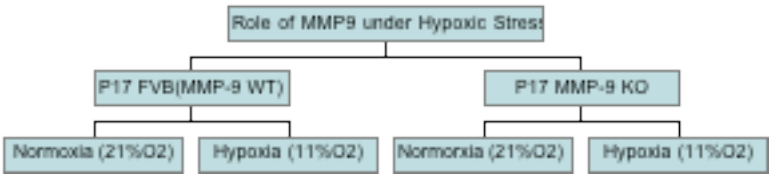

Figure 1: Overview of study. Flowchart of the design of study and animal groups.

C. Hypoxia exposure

A computer-controlled system (OxyCycler, Reming Bioinstruments, Redfield, NY) was utilized to introduce and maintain constant levels of 11%O2 and carbon dioxide levels of 5%. MMP-9 WT and MMP-9 KO were utilized for all experiments. All litters were culled to eight pups each. At postnatal day 2, litters and their dams were placed in Plexiglas chambers with regular 12:12-h light-dark cycles. Control litters were housed in the same room and exposed to room air. All studies were done in pathogen-free conditions. All experimental protocols were approved by the University of California, San Diego (UCSD) institutional Animal Care and Use Committee, which are accredited by the American Association of Laboratory Animal Care; animals were treated according to guidelines approved by UCSD.

D. Processing of lung tissues

For tissue collections and histological analysis, animals were euthanized using a lethal dose of inhales isofluorane. For histological purposes, lungs were perfused with 4% paraformaldehyde for 5 minutes at 25 cm pressure through tracheotomies. Lungs were then excised and submersed in 4% paraformaldehyde overnight and processed for paraffin embedding and sectioned into 5 m thick sections by the core histology facility at UCSD. For Western blot analysis, animals were euthanized as described above, and lungs were excised, frozen in liquid nitrogen, and stored in -80 C for further processing.

E. Western blot analysis

Frozen samples of mouse lung tissue from each group were homogenized in 650 μ L cell lysis buffer. Homogenized tissues were then quantified using Gen5 program, the bicinchoninic acid kit (Sigma-Aldrich, St. Louis, MO), and BSA standards to obtain

a standard curve. After protein quantification, cells were diluted in cell lysis buffer to achieve an equal amount of protein (5 μ g/μ L). An equal amount of samples of 35 μ g were then separated on 4-12% precast NuPAGE bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA) under both reduced and non-reduced conditions, and then were transferred to polyvinylidene difluoride membranes (Immobilin-P; Millipore, Bedford, MA). Membranes were then treated with a variety of antibodies and membranes were exposed to film. Films were then scanned and the volumes of protein expression were then quantified using HSC-70 (Stressgen/Assay Designs, Ann Arbor, Michigan).

Western blots were performed with antibodies against the constitutively expressed HSC-70 (1:50,000) as a loading control; Collagen types III, IV, VI, MMP-1/8, and elastin 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA; Fibronectin 1:5000 (B&D Bioscience, San Jose, CA); α-smooth muscle actin 1:5000; TIMP-I (R&D Systems, Minneapolis, MN); MMP-2; MMP-14; PECAM; VEGF; EMMPRIN; Specific bands were visualized after incubation with the respective secondary antibodies using Enhanced Chemiluminescense (GE Healthcare/Amersham Biosciences, Buckinghamshire, UK).

F. Densitometry measurements

Densitometry measurements of Western blots from all 4 experimental groups were obtained ($n > 6$ for each group) and absolute values were equalized by comparing the target signal against the constitutive HSC-70 signal. Results were reported in arbitrary units, comparing each value with that obtained from each respective HSC-70 measurement on each blot.

G. Immunohistochemistry

Left lung tissues from each group of animals were sent to UCSD Histology core for processing. After processing, slides are stained for specific proteins using specific antibody. Slides were deparaffinized in Xylene and rehydrated in decreasing ethanol concentration of 100%-70% and washed in water. Next, antigens were unmasked by cooking slides in 10 mM sodium citrate pH6.0 for 15 minutes and were then cooled to room temperature. Slides were then stained with 3% hydrogen peroxide for 10 minutes, with 1X PBS washes in between. Each section was then blocked with 200-300 μ L of blocking solution for 30 minutes at room temperature. Blocking solution was removed and 200-300 μ L of primary antibody, diluted in recommended antibody diluents, was added to each section. Slides were incubated over night at 4C. Antibody solution was removed and sections were washed in wash buffer. 200-300 μ L of biotinylated secondary antibody, diluted in PBS per manufacturer's recommendation, was added to each section. Slides were incubated at room temperature for 30 minutes. Secondary antibody solution were removed and washed with wash buffer. 200-300 μ L of ABC reagent were added to each section and incubated for 30 minutes at room temperature. ABC reagent was removed and sections were washed in wash buffer. 200-300 μ L of Nova Red were added to each section and monitored staining closely for sections to develop color. Slides were then immersed in water and then sections were counterstained with methyl green for 2 minutes. Slides were rinsed in water followed by dehydration steps with increasing concentration of ethanol of 70%-100% and Xylene. Slides were remounted with Permount.

H. Immunofluorescence Staining

Slides were deparaffinized using the preceding protocols. After, antigen were unmasked using 10mM sodium citrate like previously done, slides were then incubated in 10% goat serum and 3% Bovine Serum Albumin in 1X PBS blocking solution for 1 hour follow by 2 hours of incubation in primary antibody in room temperature; 0.1% Triton-X 100 in PBS solution was used for washes in between. After primary antibody incubation, slides were then incubated with fluorescence secondary antibody for 30 minutes in the dark at room temperature, followed by 0.1% triton-X washes and a PBS rinse. Next, slides were then remounted with 4'6'diamidino-2-phenylindole anti-fade mounting media.

I. Statistical analysis

Student's *t*-test was used for comparison of effects of MMP9 KO on protein (Western blot) and histology; differences between sample means were considered significant if the p-values < 0.05.

III. Results

Body weight ratios. MMP-9 WT and MMP-9 pups exposed to 14 days of hypoxia had decreased weight gain as compared to those exposed to normoxia. However, in hypoxia, MMP-9 KO had lower weights than MMP-9 WT.

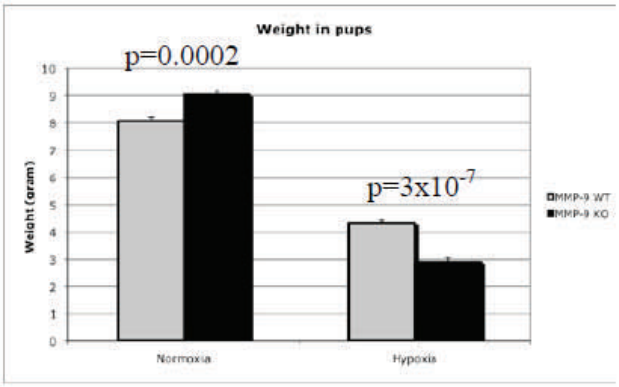

Figure 2: Weight differences in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. Hypoxia reduced weights in both MMP-9 WT and KO mice ($n > 6$ for each group of animal; in normoxia, $p=0.0002$ and in hypoxia, $p=3x10^{-7}$).

Lung structure. Pups exposed to hypoxia exhibited significantly larger alveolar diameters as determined by mean linear intercept measurements (MLI). However, under hypoxia, MMP-9 KO exhibited larger alveolar diameters than MMP-9 WT. Preliminary results show that animals exposed to hypoxia have increased MLI, indicating a decreased in surface area. However, MMP-9 KO animal exhibited higher MLI values than WT under these conditions, indicating significant decrease in gas-exchange surface area.

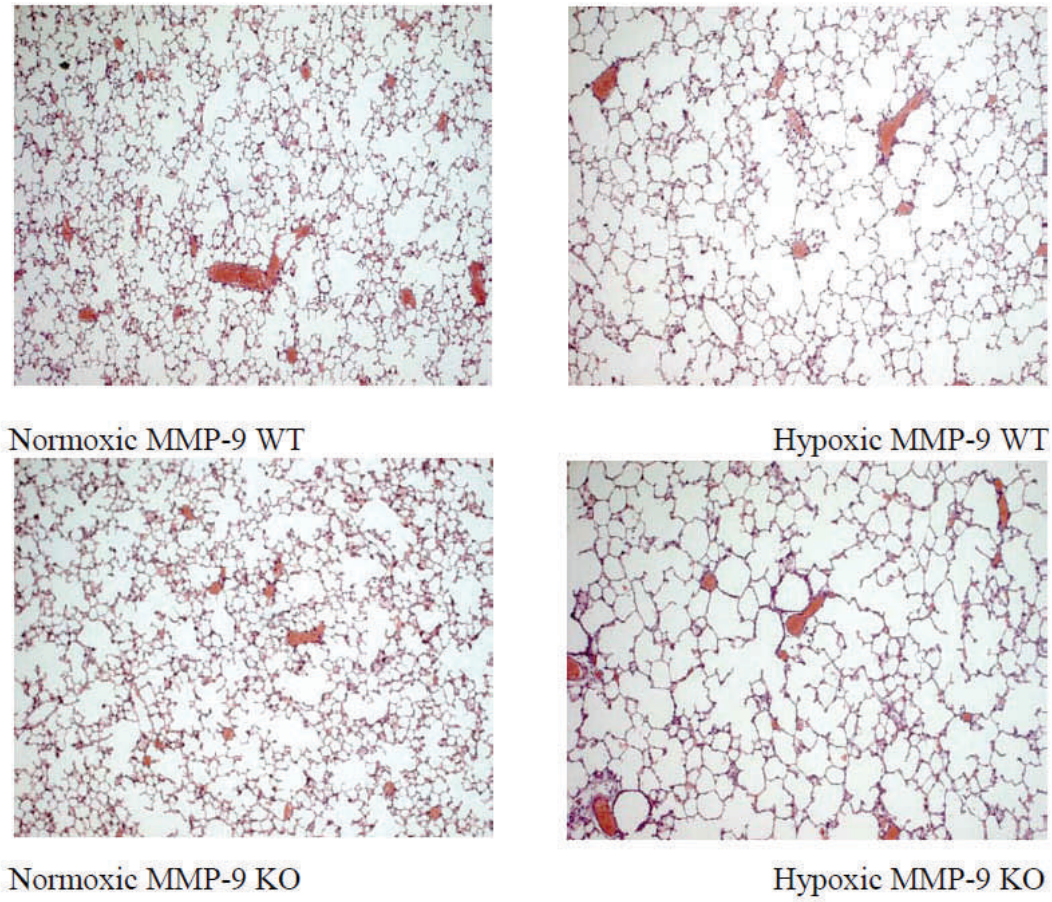

Figure 3: Haematoxylin and eosin (H&E) staining of MMP-9 WT and MMP-9 KO in normoxia and hypoxia. H&E staining of MMP-9 WT at 10X indicates an overall increase in alveolar diameter in MMP-9 WT and MMP-9 KO exposed to hypoxia. However, in hypoxia, MMP-9 KO shows larger alveolar diameter as compared to MMP-9 WT.

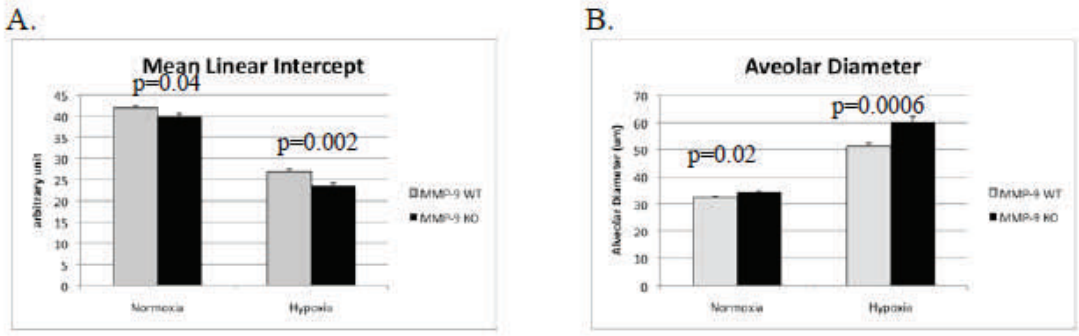

Figure 4: **Mean linear intercept and alveolar diameter of MMP-9 WT and MMP-9 KO in normoxia and hypoxia.** Analysis of 10X H&E staining indicates in hypoxia, WT animals have significantly lower MLI (n>4 for each group, in normoxia p=0.04, in hypoxia p=0.002) and larger alveolar diameter as compared to normoxic condition.(n>4 for each group, in normoxia p=0.02, in hypoxia p=0.0006). Similary,

a-Smooth muscle actin was increased in MMP-9 KO vessels in hypoxic conditions. Despite many significant changes in ta-Smooth muscle actin staining in MMP-9 KO pups, western blot analysis of whole lung tissues indicates no significant changes in a-Smooth muscle actin level.

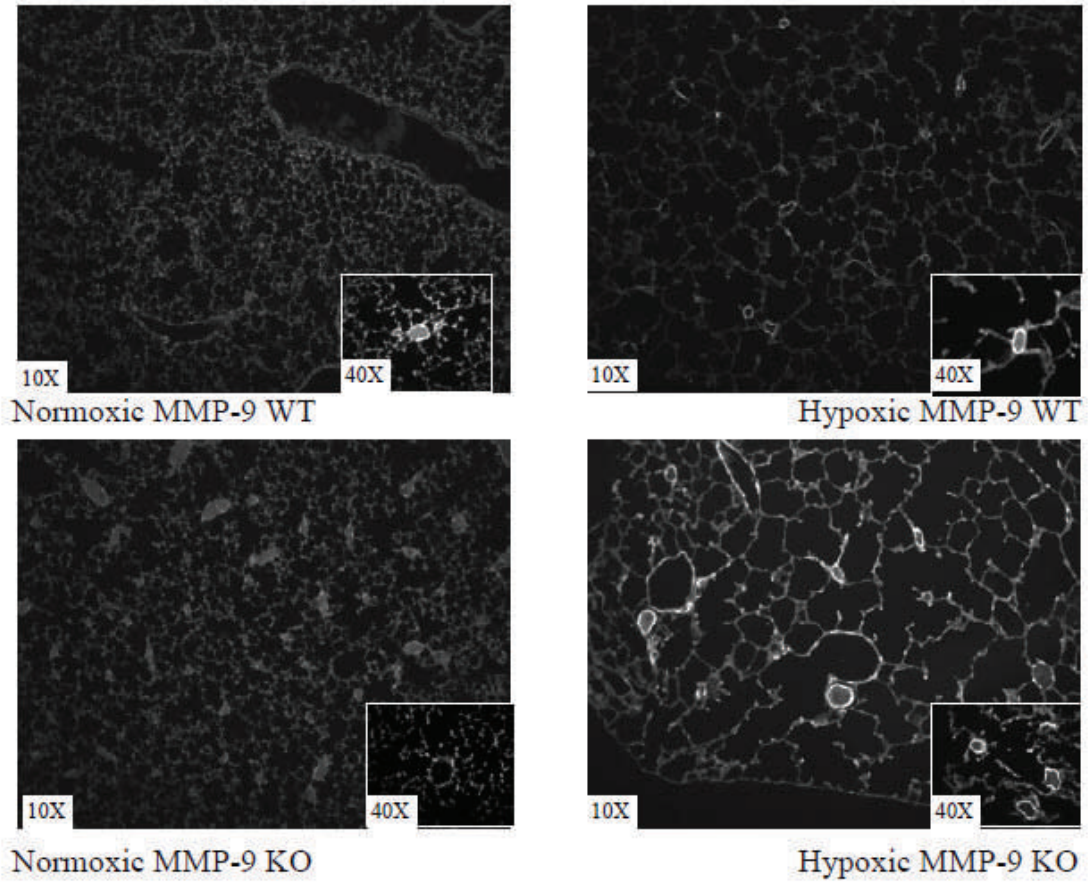

Figure 5: Immunofluorescence staining for α-smooth muscle actin in MMP-9 WT and MMP-9 KO in normoxia and hypoxia. Immunofluorescence staining for α-smooth muscle actin shows increased luminescence in MMP-9 KO and MMP-9 WT exposed to hypoxia. However, MMP-9 KO in hypoxia exhibited more α-smooth muscle actin than MMP-9 WT. (n>4 for each group)

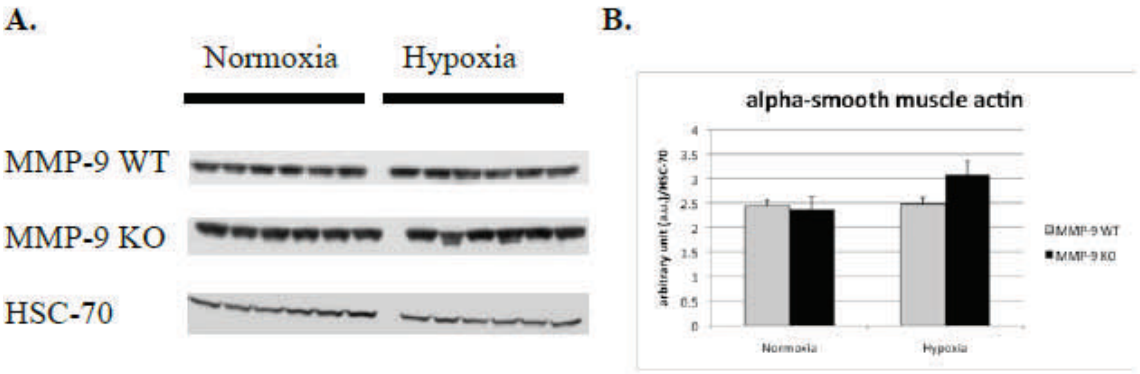

Figure 6: α-Smooth muscle actin(α-sma) protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from 6 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibodies for α-sma or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of α-sma protein expression in MMP-9 WT (grey bars) and MMP-9 KO(black bars) under both normoxia and hypoxia. MMP-9 KO had no significantly changes in α-smooth muscle actin expression in normoxia and hypoxia. (n > 6 for each group of animal)

Collagen levels were decreased in MMP-9 KO. Total lung proteins were processed and used for Western blotting analysis of different collagen levels of all four groups of animal since collagen is a major component of the ECM. In hypoxia, MMP-9 KO had a decreased in collagen IV (In normoxia $p=2x10^{-7}$, in hypoxia $p=0.002$; $n>6$ for each group of animal) and no significant changes in collagen VI level as compared to MMP-9 WT (In normoxia $p=0.002$; $n>6$ for each group of animal).

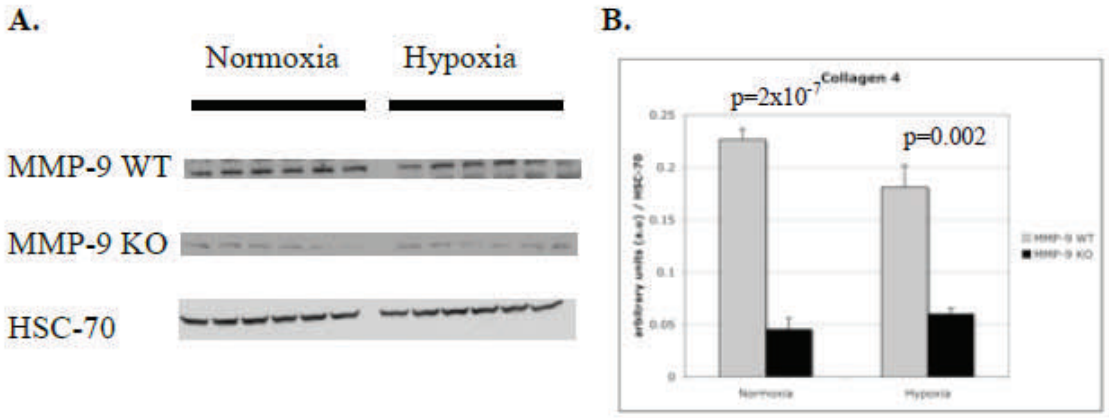

Figure 7: Collagen IV protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Representative Western blot analysis using specific antibody for collagen IV or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of collagen IV in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO had lower collagen IV in normoxia and hypoxia. ($n > 6$ for each group of animal; in normoxia, $p=2x10^{-7}$ and in hypoxia, $p=0.002$).

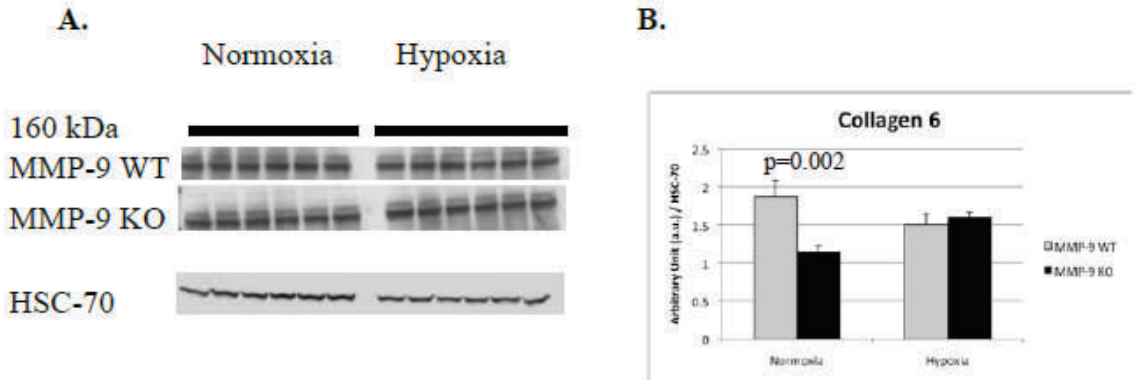

Figure 8: Collagen VI protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for collagen VI or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of collagen VI in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO had lowered collagen VI in normoxia. ($n > 6$ for each group of animal; in normoxia, $p=0.002$).

Elastin was significantly higher altered in MMP-9 KO. In hypoxia, MMP-9 KO demonstrated a four-fold increase in elastin level as compared to MMP-9 WT (In normoxia $p=1x10^{-4}$, in hypoxia p=0.0004, n>6 for each group of animal).

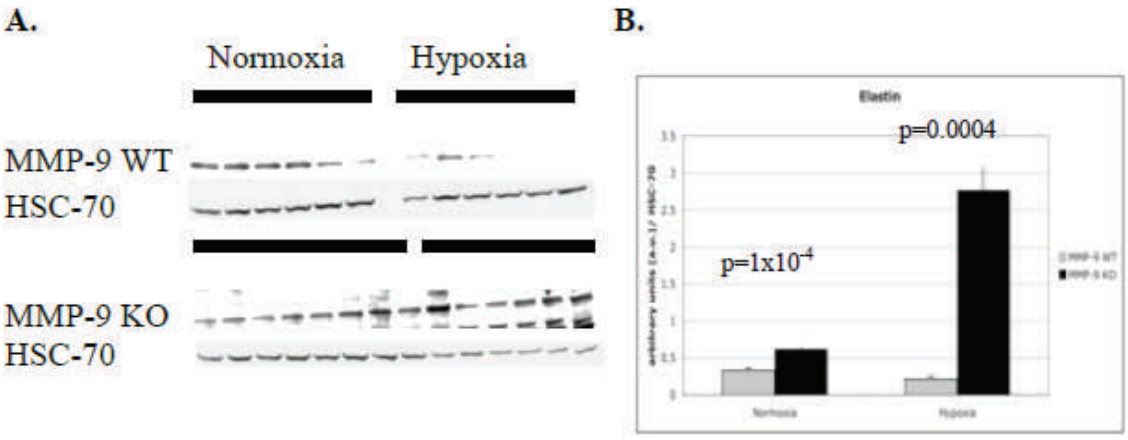

Figure 9: Elastin protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for elastin or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of elastin in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO had increased elastin expression in normoxia and hypoxia. (n > 6 for each group of animal; in normoxia, $p=1x10^{-4}$and in hypoxia, p=0.0004).

Fibronectin levels were increased in MMP-9 KO. In hypoxia, MMP-9 KO had a six-fold increase in fibronectin level as compared to MMP-9 WT (In normoxia p=0.05, in hypoxia p=0.02; n>6 for each group of animals).

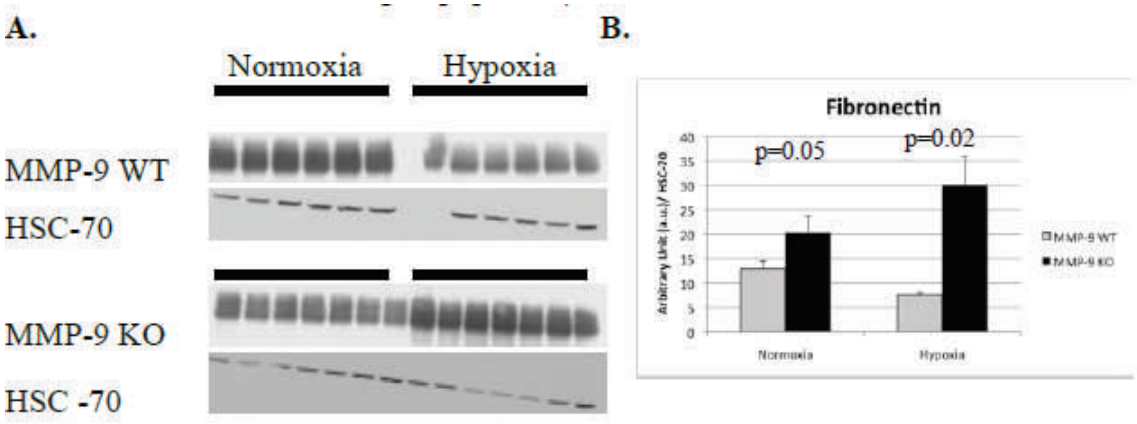

Figure 10: Fibronectin protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35µg) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for fibronectin or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of fibronectin in MMP-9 WT (grey bars) and MMP-9 KO(black bars) under normoxia and hypoxia. MMP-9 KO had higher levels of fibronectin expression in normoxia and hypoxia. (n > 6 for each group of animal; in normoxia, p=0.05 in hypoxia, p=0.02).

Vascular markers PECAM, were increased in MMP-9 KO. In hypoxia, MMP-9 KO had significantly higher expression of PECAM than MMP-9 WT (In hypoxia p=0.04, n>6 for each group of animal).

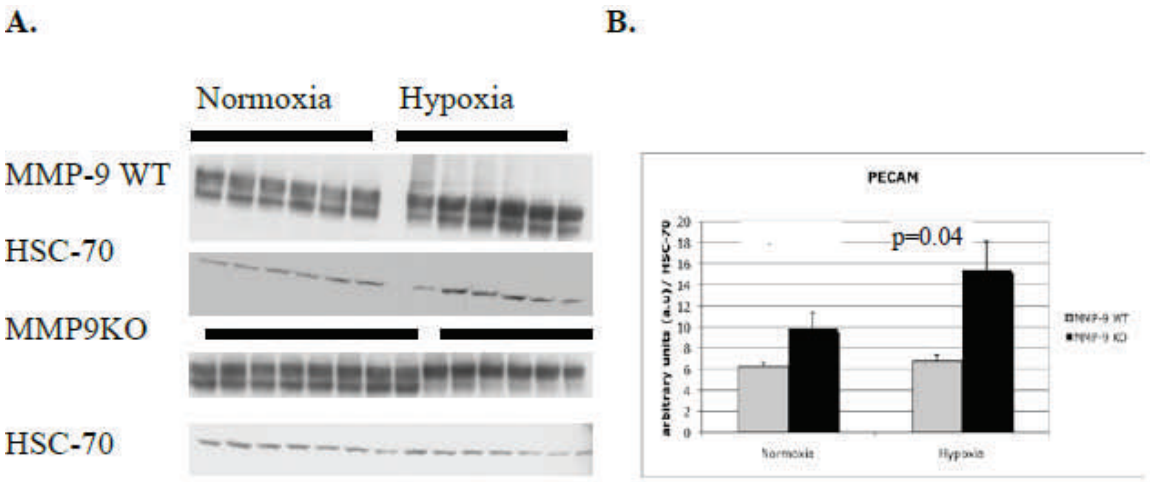

Figure 11: PECAM protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35µg) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for PECAM or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of PECAM in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO had increased PECAM expression in hypoxia. (n > 6 for each group of animal;in hypoxia, p<0.04).

Collagenase activity. Western blot analysis of whole lung tissues demonstrated that, in hypoxia, MMP-1/8 level in MMP-9 KO had no significant changes as compared to MMP-9 WT (Active MMP-8, in normoxia p=0.001; Latent MMP-8, in normoxia p=0.002, in hypoxia p=0.0004, n>6 for each group of animal). MMP-14 was also analyzed because it is another potent collagenase. In hypoxia, MMP-9 KO had a three-fold increase as compared to MMP-9 WT (In hypoxia p=0.02, n>6 for each group of animal).

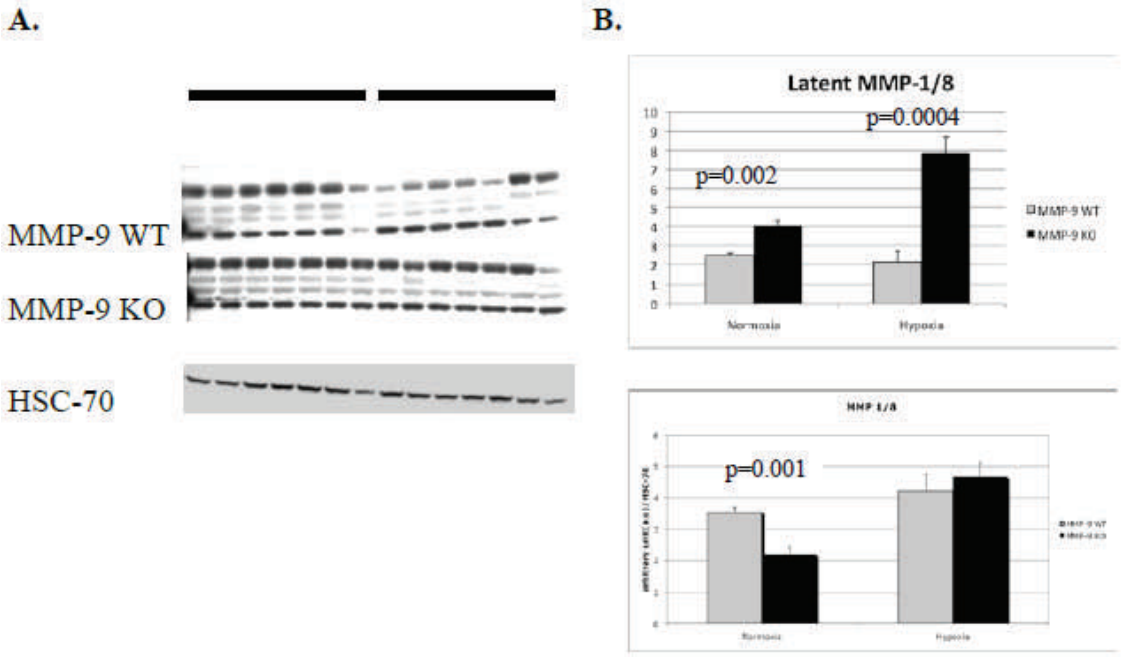

Figure 12: MMP-1/8 protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for MMP-1/8 or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of MMP-1/8 in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO expressed higher levels of latent MMP-1/8 in normoxia and hypoxia. (n > 6 for each group of animal; in normoxia, p=0.002 in hypoxia, p=0.0004). MMP-9 KO had lowered level of active MMP-1/8 in normoxia. (n > 6 for each group of animal; in normoxia, p=0.001).

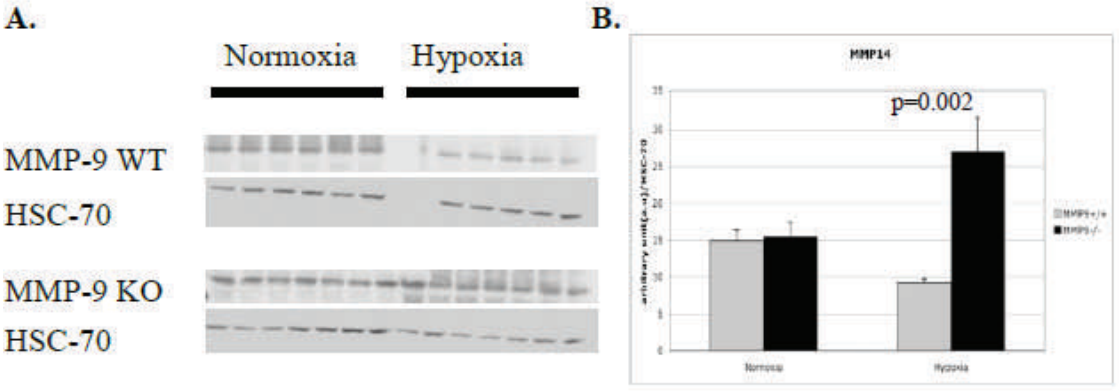

Figure 13: MMP-14 protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for MMP-14 or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of MMP-14 in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO had higher level of MMP-14 expression in hypoxia. (n > 6 for each group of animal; in hypoxia, p=0.002).

MMP-2 was lower in MMP-9 KO. Since MMP-2 and MMP-9 are both gelatinases, Western blotting analysis was performed to see if MMP-9 down-regulation would change the level of both latent and active form of MMP-2. In hypoxia, MMP-9 KO had significantly lower level of active MMP-2 no changes in latent MMP-2 level. (in normoxia $p<0.005$; in hypoxia $p<0.02$; $n>6$ for all groups).

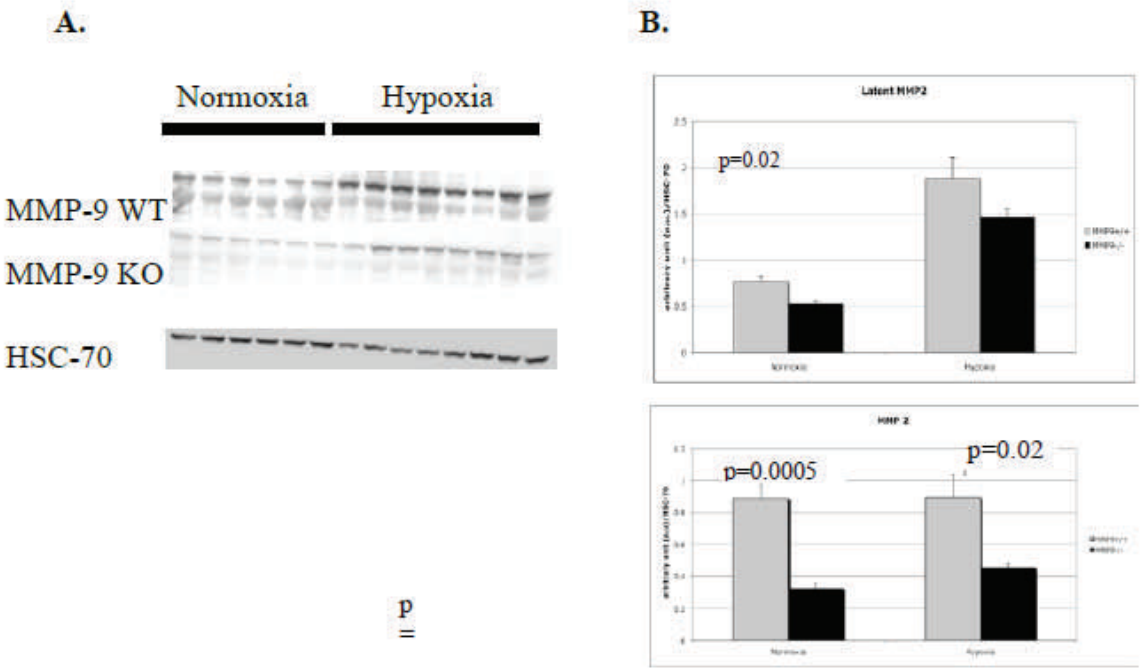

Figure 14: MMP-2 protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for MMP-2 or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of MMP-2 in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 KO had lower level of latent MMP-2 expression in normoxia. ($n > 6$ for each group of animal; in normoxia, $p=0.02$). MMP-9 KO had lower MMP-2 expression level in normoxia and hypoxia. ($n > 6$ for each group of animal; in normoxia, $p=0.0005$ in hypoxia, $p=0.02$).

EMMPRIN expression. Western blot analysis of whole lung tissues demonstrated that, in hypoxia, EMMPRIN level in MMP-9 KO was significantly higher than MMP-9 WT (In normoxia p=0.004, in hypoxia p=0.01, p>6 for each group of animal).

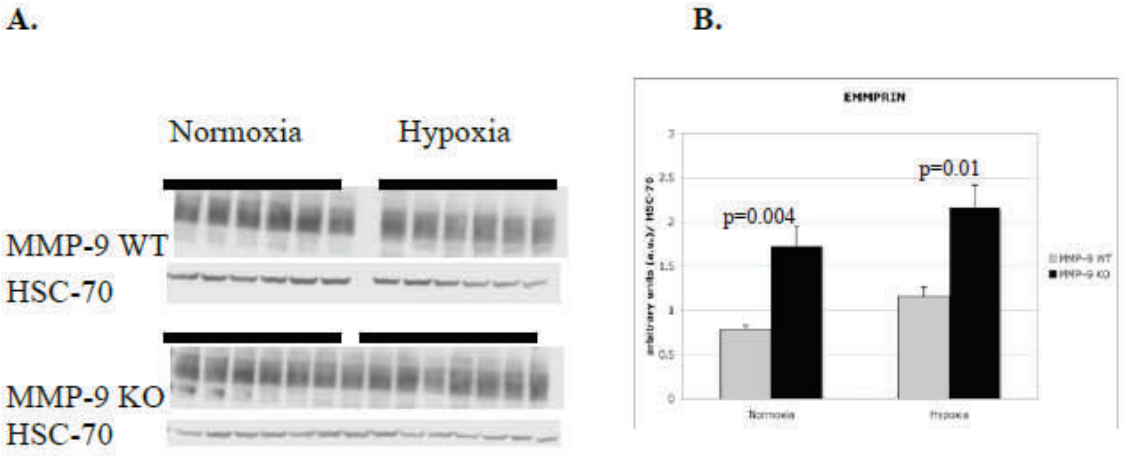

Figure 15: EMMPRIN protein expression analysis in MMP-9 WT and MMP-9 KO mice after 14 days of normoxia or hypoxia. (A) Total lung proteins (35 μ g) from at least 5 different MMP-9 WT and MMP-9 KO neonatal mice lungs exposed to either normoxia or hypoxia were separated by SDS-PAGE, blotted to nitrocellulose membranes, and analyzed by Western blot analysis using specific antibody for EMMPRIN or heat shock protein 70 (HSC-70) as indicated. (B) Blots were scanned and densitometry was performed. Bar graphs show the densitometric analysis of EMMPRIN in MMP-9 WT (grey bars) and MMP-9 KO (black bars) under normoxia and hypoxia. MMP-9 had higher levels of EMMPRIN expression in both normoxia and hypoxia. (n > 6 for each group of animal; in normoxia, p=0.004 in hypoxia, p=0.01).

IV. Discussion

In this study, the impact of MMP-9 during lung development in hypoxia was investigated by using MMP-9 KO mice. We demonstrated that MMP-9 down-regulation in hypoxia causes a) increased morbidity and mortality, b) increased alveolar diameter, c) increased blood vessel muscularization, and d) significant changes to the ECM.

In our study, MMP-9 WT and MMP-9 KO were both exposed to either normoxia (room air) or hypoxia (11% O_2) at postnatal day 2 for 14 days; at postnatal day 17 (P17), animals were sacrificed and processed for tissue analysis. We noticed MMP-9 KO mice had a decreased survival rate as compared to MMP-9 WT, nearly a quarter of MMP-9 KO litters did not survive in hypoxia. From the previous literature, we know that hypoxia can decrease growth rate by decreasing appetite, food ingestion, and affecting lactation in the mother (Alippi; Moore). Our results indicate that our hypoxic groups of animal possessed decreased growth rate as reported by previous literature. Interestingly, MMP-9 KO animal in hypoxia exhibited a significantly lower weight than did MMP-9 WT. Since inadequate lactation may significantly limit growth, we rotated dams between hypoxic and normoxic groups to reduce the impact of hypoxia on the dams. We found that changing dams did not improve MMP-9 KO weight gain in hypoxia. Thus, our results demonstrate that lactation/nutrition is not the only factor in hypoxia-induced growth retardation. In fact, we believe that MMP-9 is involved in essential adaptive mechanisms to hypoxia.

To investigate the potential causes for increased morbidity and mortality, we examined lung tissue to study the structure of the MMP-9 WT and KO lungs in hypoxia. We chose to start with lung tissue because it is an organ that can be directly impacted by

hypoxia and is immature at this experimental period. Previous studies have shown that low O_2 tension can increase alveolar sizes and possibly number (Mortola). As expected, our histological analysis indicates that both MMP-9 WT and KO animals showed evidence of larger alveolar diameter in hypoxia. More significantly, in hypoxia, MMP-9 KO exhibited noticeably larger alveoli sizes than MMP-9 WT. Investigators have suggested that alveolar changes in neonatal lung may be a hyperplastic response to hypoxia aiming to protect and expand the gas-exchange surface area (Mortola). We believe this further increase in alveolar diameter in MMP-9 KO may contributed to the increased morbidity and mortality. Therefore, we hypothesize that MMP-9 may play a role in the repair mechanisms that are required to respond to hypoxic stress.

Since the ECM is a major component of the lung, we chose to studied the components of the ECM to further evaluate the changes in the lung parenchyma and vessel composition. Since collagens are major structural components of the lung matrix, we hypothesized that it would be altered by MMP-9 deletion. Studies have shown that chronic hypoxia results in increased collagen deposition, which can promote the progression of pulmonary hypertension (Estrada). However, our results indicate that in hypoxia, MMP-9 KO had over lower collagen 4 level and no changes in collagen 6 level as compared to MMP-9 WT. Our results indicate that the significant changes in collagen level expression in MMP-9 KO may play an important role in their decreased survival and growth rate, and may have contributed to the changes seen in alveolar sizes that is seen in the KO animals. Due to the changes seen in collagen level, analysis of other matrix components like fibronectin and elastin were done. Analysis demonstrates that in

hypoxia, MMP-9 KO had overall higher elastin and fibronectin level as compared to MMP-9 WT.

Since the MMP family consists of many members that can regulate the ECM, we investigated changes in levels of other MMP members. To further understand the changes in the ECM, we looked at other collagenases such as MMP-1/8 to determine if MMP-1/8 expressions were altered. Our study indicates that in hypoxia MMP-9 KO mice had an overall increased in expression of MMP-2 and MMP-14, and no significant changes in MMP-8 level as compared to MMP-9 WT. To further investigate the changes in MMP level, we analyzed the expression of MMP regulating factors, one of which is EMMPRIN. Western blot analysis indicates that in hypoxia, MMP-9 KO had significantly higher level of EMMPRIN as compared to MMP-9 WT. Due to the increased in MMP and EMMPRIN level that are seen in MMP-9 KO animals, we speculate that the overall increase in MMP production in KO mice may play a significant role in changes that are occurring to lung's structure.

In addition to the changes in alveolar diameter, at 40X magnification, we also found significant changes around pulmonary blood vessels. We found that in hypoxia, MMP-9 KO mice had thicker blood vessels than MMP-9 WT. Structural remodeling of pulmonary vessels, including proliferation of vascular smooth muscle cells, are characteristic of pulmonary hypertension which is a well described response to chronic hypoxia (Eddahibi). It is also known that pulmonary hypertension is accompanied by increased muscularization of normally muscularized arteries and extension of muscularization to smaller arteries (Eddahibi). Our mice exhibited increased muscularized small and large blood vessels when exposed to hypoxia. We also found that α-smooth muscle

actin immunofluorescence staining was greatest in KO mice. Surprisingly, MMP-9 KO mice had significantly more α-smooth muscle actin staining than WT even in control condition. Recent studies suggest that MMP-9 plays a role in development of chronic pulmonary hypertension (Uzuelli). However, our study shows that MMP-9 KO resulted in muscularization phenotypes similar to those seen in pulmonary hypertension models. Thus, we hypothesize that although MMP-9 may play both a role in the development and repair mechanisms in hypoxia.

Since blood vessel muscularization was an apparent phenotype in KO mice, vascular markers PECAM was analyzed. Our results demonstrate that in hypoxia, KO animals had higher PECAM expression than WT animals. Previous studies have shown that in chronic hypoxia, PECAM level is increased. Interestingly, our results indicate that in hypoxia, MMP-9 KO mice had significantly higher expression of PECAM as compared to MMP-9 WT. We hypothesize that such significant changes in vascular markers may directly or indirectly affect pulmonary vasculatures, however, further studies still needs to be done with other vasculature marker.

In conclusion, although MMP-9 has been associated to many pulmonary disorders, our study has provided evidence that MMP-9 KO may result in worsened conditions. Our data suggests that MMP-9 KO in neonatal mice results in overall increases in MMP levels, ECM components, and vascular markers. Since so many changes were seen in KO animals in hypoxia, further studies with regards to these changes should be investigated. Our study has provided more information on the role of MMP-9 in hypoxic stress and we hope that this research will provide further insights on the mechanisms that are involved in hypoxic repair.

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