LR8 gene expression in normal and fibrotic human tissues

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Abstract

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LR8 gene was first isolated from a subpopulation of human lung fibroblasts expressing the C1q receptor. LR8 expression was found to be upregulated in human lungs with IPF and bleomycin-induced fibrotic mouse lungs. Previous studies on LR8 expression in gingival fibroblasts have shown that LR8 gene is expressed in fibroblasts cultured from only some healthy patients, whereas it was expressed in fibroblasts from all patients with phenytoin induced gingival overgrowth. The purpose of this study was to determine if fibroblasts expressing LR8 also express SMA and if these cells are selected and expanded in human fibrosis. Real time quantitative PCR was performed with normal and fibrotic liver and gingiva to quantitate expression of LR8, SMA and COL1A1 genes. Immunohistochemistry was performed in normal and fibrotic human tissues using LR8 and SMA antibodies. RT-PCR was performed to examine the presence of variants of LR8 gene in liver and gingiva. There was considerable variability in LR8 gene expression among the individual human patients in the results from PCR. LR8

expression was higher in fibrotic liver tissues as compared to normal liver tissues and there was lack of correlation between LR8 and SMA expression in tissues. Number of LR8 expressing cells increased in fibrotic tissues and number of cells coexpressing both LR8 and SMA increased in fibrosis. RT-PCR using three sets of primers from different LR8 domains showed tissue specific differences in LR8 expression between human liver and gingiva.

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I would like to thank my family for the support they provided me throughout my entire life. To my parents, Venkat Reddy and Suvarna, and my sister, Varsha, thank you for always loving me and believing in me. To my better half, Sirish, thank you for all the love and encouragement you have given me as a friend and life partner.

DEDICATION

This thesis is dedicated to my mentor and advisor, A Sampath Narayanan.

I would also like to dedicate this thesis to my husband, Sirish and my family.

CHAPTER I: Introduction and Literature review

Fibrosis

Fibrosis is a pathological phenomenon in which excessive deposition of collagen and other extracellular matrix (ECM) components leads to loss of normal tissue architecture and function. It is a disease that occurs in nearly every tissue and organ, and fibrotic lesions from various organs like the lung, liver, kidney, heart and gingiva have many common features. The major collagen species in fibrotic scars is type I collagen, but type III, IV and V collagens are also seen depending on the stage of the disease (Milani et al., 1990, Raghu et al., 1985, Wynn 2008).

Fibrosis is believed to be due to dysregulated wound healing response to chronic and progressive tissue injury. The injury activates inflammation, which results in the release of cytokines and growth factors like transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α) and interleukins-1β (IL-1β) by macrophages, platelets, neutrophils, lymphocytes and dendritic cells (Henry and Garner, 2003, Werner and Grose, 2003). Increased concentrations of TGF-β, connective tissue growth factor (CTGF) and other fibrogenic cytokines in the local environment have been shown to expand and activate mesenchymal cells to secrete excessive ECM components (Verrecchia & Mauviel, 2007, Wynn & Ramalingam, 2012). In most cases ongoing chronic inflammation is the main cause for the progression of fibrosis. Sometimes intrinsic defects in the wound healing can also lead to chronic fibrosis (Thannickal et al., 2004).

Fibroblasts are the most predominant cell type in connective tissues and these are the cells responsible for the synthesis of ECM components. In fibrosis TGF-β and other profibrotic cytokines activate the synthesis of ECM components by the fibroblasts. Fibroblasts from different anatomical sites as well as fibroblasts from within a tissue may vary in their properties, including morphology, surface marker expression, proliferative potential, growth rate and response to growth factors (Baglole et al., 2005, Koumas et al., 2001, McCulloch and Bordin 1991, Phan 2003). It has been demonstrated that fibroblast subpopulations derived from human lung, gingiva, skin, and other tissues vary significantly in the amount and pattern of collagen synthesis (Goldring et al., 1990, Lekic et al., 1997, Narayanan and Page, 1983), and selection of certain subpopulations characterized by activated ECM synthesis has been proposed as one mechanism contributing to fibrosis (Bordin et al., 1984; Fries et al., 1994, Phan 2003). Fibroblast subsets have been separated based on differences in expression of membrane proteins; examples of such cell surface protein markers, which distinguish subpopulations with differences in proliferation rate, collagen deposition and morphology, are the thymocyte 1 antigen (Thy 1) and C1q complement component (C1q) receptor (Akamine et al., 1992; Bordin et al., 1984; Phipps et al., 1989). However, differences in functional properties are not good markers to distinguish subpopulations. Lurton et al. (1999) identified a gene product, LR8, which is expressed by fibroblasts expressing the receptor for C1q-globular domain. This gene was expressed either poorly or not at all in other fibroblasts, smooth muscle cells, endothelial cells and alveolar macrophages, and the expression level was higher in human lungs with idiopathic pulmonary fibrosis (IPF) and in mouse lungs with bleomycin-induced fibrosis. Existence of fibroblast subpopulations with different phenotypic characteristics could provide for selection by

favoring expansion of clonal cell populations with phenotypic characteristics that promote disease expression.

Fibrotic tissues and granulation tissues contain myofibroblasts. The myofibroblasts are differentiated or modulated fibroblasts that exhibit some functional and structural characteristics similar to smooth muscle cells. These cells are identified by expression of smooth muscle cell protein, α-smooth muscle actin (SMA). Myofibroblast microfilaments are composed of SMA, which forms the major constituent of the contractile apparatus. The myofibroblasts play an important role in wound closure and they disappear by apoptosis after healing is complete (Gabbiani, 2003). Their presence contributes to the deposition and stabilization of excessive ECM components and is more permanent in fibrotic diseases (Tomasek et al., 2002). They produce interstitial collagens in significantly greater amounts than fibroblasts (Adler et al., 1989, Zhang et al., 1994). It is believed that fibroblasts are induced to differentiate to myofibroblasts by a change in the local microenvironment in the presence of growth factors like TGF-β, PDGF, and CTGF (Hinz, 2009, Hinz et al., 2012). In addition, they can also arise by transdifferentiation of epithelial cells through epithelial mesenchymal transition (EMT), and growth factors promoting EMT include TGF-β, epidermal growth factor (EGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF) (Kalluri & Neilson, 2003, Willis et al., 2006). In the process of EMT, cells acquire myofibroblast markers such as the SMA, fibroblast markers (eg, fibroblast specific protein-1) (Strutz et al., 1995) and loose epithelial markers (eg, E-cadherin, Zonula occludens-1) (Grunert et al., 2003).

LR8 gene

LR8 gene was first isolated from a subpopulation of human lung fibroblasts expressing the receptor for the globular domain of the complement component C1q (Lurton et al, 1999). It was observed that the LR8 gene was expressed by fibroblasts from lung, gingiva and skin, but its expression was not detectable in smooth muscle cells, endothelial cells, epithelial cells, gingival epithelial cells or alveolar macrophages (Lurton et al., 1999, Wisecup & Narayanan, 2009). LR8 expression was found to be upregulated in human lungs with IPF and bleomycin-induced fibrotic mouse lungs.

The LR8 gene is mapped on to chromosome 7q32 in humans (Nakajima et al., 2002) and it is located on chromosome 6 in the mouse genome. The approved gene symbol for LR8 gene is TMEM176B. The full length human LR8 gene is about 12kbp in size and contains a 772 base pair long open reading frame region encoding 270 amino acids. The molecular weight of LR8 protein is 29.1 KDa. LR8 protein has four transmembrane regions with amino and carboxy termini protruding into the cytoplasm. LR8 protein belongs to the CD-20 superfamily (NCBI Conserved Domain Database) which includes the CD-20 protein (expressed on the surface of B-lymphocytes) and the beta subunit of high affinity receptor for IgE Fc. LR8 gene is conserved in mouse, rat, chimpanzee, cow, dog and Rhesus monkey (NCBI Homologene).

The data from Human Protein Atlas indicates that some of the cells such as epithelial cells, glandular cells from gastrointestinal tract, smooth muscle cells, keratinocytes and Langerhans cells in skin, kidney tubule cells, and hepatocytes show LR8 expression. Certain cell

types from reproductive organs, endocrine glands, lymphoid organs also express LR8. However pneumocytes, bile duct cells, glomerular cells, hematopoietic cells, adipocytes, chondrocytes and fibroblasts in soft tissues do not show LR8 expression. These data were generated using anti-LR8 antibody HPA047509 and was submitted by the manufacturer, Atlas antibodies.

Of late there has been increasing interest in the LR8/TMEM176B gene. According to a recent study, TMEM176B induces the differentiation of myoblasts into an osteoblast lineage (Yano et al, 2013). Gehrau et al (2011) observed that TMEM176B transcript levels are elevated in transplanted human livers that showed recurrence of HCV infection. In another study, it was reported that the human TMEM176B transcripts are differentially expressed in various normal and cancer tissues with its expression being highest in liver, kidney, lungs and brain tissues (Cuajungco et al., 2012). TMEM176B is expressed at relatively low levels in peripheral blood B-cells (Zuccolo et al., 2013). TMEM176B protein levels were found to be elevated in lymphoma tissues, indicating that the LR8/TMEM 176B could be useful as a potential marker for certain types of cancers (Cuajungco et al., 2012).

LR8 gene was also found to be involved in the control of dendritic cell maturation (Condamine et al., 2010) and thus may play a role in the induction or maintenance of allograft tolerance. Louvet et al. (2005) reported that TORID/ LR8 mRNA is preferentially expressed in lymphoid tissues, immature macrophages and dendritic cells in rats, and its expression strongly declined following stimulation of macrophages or dendritic cells (Louvet et al., 2005). The above results indicate that the LR8 gene is involved in regulation of immune cells.

In a study by Spanakis & Boye (1997), it was observed that the pattern of gene expression varied among the clinical/anatomical subtypes of fibroblasts derived from human breast tissues. It was reported that specific clustering of gene expression is observed in subsets of fibroblasts studied using statistical multivariate analysis and that different types of fibroblasts give rise to different types of myofibroblasts. Therefore, identifying the gene expression patterns that determine the cell phenotype will provide valuable insights into the heterogeneity of fibroblast populations and their role in the disease. I will use LR8 and SMA gene expression to study if a certain subpopulation of fibroblasts (expressing LR8) are selectively expanded in fibrosis.

Previous studies on LR8 expression in gingival fibroblasts have shown that there is variability in LR8 expression among different patients. The LR8 gene was expressed in fibroblasts cultured from some, but not all healthy patients, whereas it was expressed in fibroblasts from all patients with phenytoin induced gingival overgrowth (Wisecup & Narayanan, 2009; unpublished data). The differences in LR8 gene expression and its possible role as a modulator of immune responses may be one reason for variability in human fibrotic responses to drugs like phenytoin. These findings lead me to believe that the LR8 gene may play an important role in fibroblast heterogeneity and mechanisms of fibrosis in various tissues.

CHAPTER II: Hypothesis and Study Objectives

Findings from previous studies conducted on fibroblast cultures from human lungs and gingiva have shown that:

- The LR8 gene is expressed in some cultured fibroblasts while it was poorly expressed in other fibroblasts.
- LR8 gene expression is upregulated in fibrotic human and mouse lungs as compared to their normal counterparts.
- In gingival fibroblasts cultured from human patients, cells from only some patients expressed LR8, whereas expression was not detectable in cells from other patients.
- Gingival fibroblasts obtained from overgrown gingiva of all patients expressed LR8.

In human gingival fibroblasts LR8 expression positively correlated with SMA expression. From these observations, I hypothesize that -

- LR8 expression will be higher in fibrotic tissues as compared to normal tissues.
- LR8 will be expressed by fibroblasts and the number of LR8 expressing cells will be higher in fibrotic tissues.
- LR8 expression in fibroblasts will correlate with SMA expression in tissues, and that
- Cells expressing both LR8 and SMA will increase in fibrosis.

My goal is to examine the general hypothesis that fibroblasts expressing LR8 also express SMA and determine if these cells are selected and expanded in human fibrosis. I will examine this hypothesis in normal and fibrotic human liver, gingiva, lung and kidney tissues. I will study the expression of LR8 and SMA by real time quantitative Polymerase Chain Reaction (qPCR).

The correlation between the expression of LR8 and SMA genes will be further studied using anti-LR8 and anti-SMA antibodies by immunohistochemistry (IHC) of normal and fibrotic human tissue sections. I will also examine by IHC whether specific cell types are expressing both LR8 and SMA in tissue sections.

I will examine these possibilities in the following specific aims:

Specific Aim 1

To quantitate LR8 and SMA gene expression in normal and fibrotic human tissues, and to correlate their expression to COL1A1 expression. The hypothesis is: LR8 and SMA gene expression (as well as COL1A1 expression) will be upregulated in fibrotic tissues, and a positive correlation between LR8 and SMA gene expression will be observed in both normal and fibrotic human tissues.

Specific Aim 2

To study the distribution of LR8 and SMA proteins in normal and fibrotic human tissue sections using immunohistochemistry. The hypothesis is: fibroblasts expressing and not expressing LR8 are present in normal tissues and cells expressing LR8 and SMA will increase in fibrosis.

Specific Aim 3

To analyze available information on the LR8 gene and its splice variants in normal and fibrotic tissues, using bioinformatics tools and databases. I will examine the presence of splice variants of LR8 gene in normal and fibrotic tissues by RT-PCR and correlate the results with the results from quantitative PCR.

CHAPTER III: Materials and Methods

Specific aim 1: Quantitate the expression of LR8, SMA and COL1A1 genes

Normal and fibrotic gingiva and liver tissues were studied. Normal human gingival cDNA s and fibrotic gingival tissues were provided by Dr. Manoj Muthukuru, Department of Periodontics, UWSOD. Fibrotic gingival tissues were stored at -80°C until RNA extraction was performed. Normal and fibrotic human liver tissues were obtained from the Department of Pathology, UWMC. Liver tissues were transferred to RNA*later*® solution (Invitrogen) immediately after tissue harvest, stored at 4°C and processed within 72 hours. The study was approved by University of Washington Human Subjects Division.

Total RNA was extracted using Qiagen RNeasy® miniprep and stored at -80^{0}C. RNA was quantitated spectrophotometrically prior to cDNA synthesis. RNA was stored at -80°C until further processing. First strand cDNA was synthesized by reverse transcription using RevertAid® First Strand cDNA Synthesis Kit (Fermentas), following the protocol recommended by manufacturer. Briefly, oligo (dT) 18 primer was added to about 5 µg of extracted RNA and the mixture was incubated at 65°C for 5 minutes, then enzyme mixture (5X reaction buffer, Ribolock® RNase inhibitor, 10mM dNTP mix, RevertAid M-MuLV reverse transcriptase) was added and incubated at 42°C for 2 hours. The reaction was terminated by incubation at 70°C for 15 minutes. RNase H (Fermentas) was added to remove any remaining RNA, and the incubation continued at 37°C for 20 minutes. The synthesized cDNA was used in RT-PCR analysis under the following conditions: Initiation at 95°C for 2 minutes followed by amplification protocol-denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 2

minutes. This cycle was repeated 40 times, followed by final elongation at 72°C for 5 minutes and cool down at 15°C for 10 minutes. Fermentas PCR master mix (2X) was used for the RT-PCR reaction. The primers used are listed in Table 3.1. The products were analyzed by 1.5% Agarose gel electrophoresis.

TABLE 3.1: List of primers used in PCR.

Gene name	Primer	Primer Sequence	Product length
LR8	A forward	CCTCTAGGCATGCCCAGCCCACC	155
	A reverse	GCCAGGATTGGTTATGAGCAGCTG	
	B forward	AAACTGAACCCTTTTTATACAT	162
	B reverse	CTGAGGAAGTTGTTCACAGCAATCC	
	C forward	CGAAACTTGTGTGGCCAGAGCTCC	126
	C reverse	CTCCACTGCCATTGTCCTGTG	
SMA	forward	CCGACCGAATGCAGAAGG	87
	reverse	ACAGAGTATTTGCGCTCCGGA	
COL1A1	forward	TGACGAGACCAAGAACTG	599
	reverse	CCATCCAAACCACTGAAACC	
GAPDH	forward	CCACCCATGGCAAATTCCATGGCA	597
	reverse	TCTAGACGGCAGGTCAGGTCCACC	

Real-time quantitative PCR was performed to measure the expression of LR8, SMA, COL1A1 and GAPDH genes. The DNA samples with known concentration of LR8, SMA, COL1A1 and GAPDH genes were used as positive controls for respective genes. Standard curve was generated using serial dilutions of β-actin cDNA. The qPCR reaction was carried out in a real-time thermal cycler (MyiQ™ Single color Real-Time PCR Detection System, Bio-Rad) using 2X iQ™ SYBR® Green Supermix (Bio-Rad). The protocol for Real-Time PCR was: Step 1 (initiation) - 95°C for 2 minutes, Step 2 (amplification and quantification) - denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 2 minutes with a single fluorescence measurement. Step 2 was repeated 40 times, followed by final elongation at 72°C for 5 minutes. A melt curve was generated at the end of the reaction. The fluorescence was measured using the IQ™5 optical system software (Bio-Rad). Each experiment was carried out in triplicate. The gene expression values of LR8, SMA, COL1A1 and GAPDH were generated using the standard curve. The gene expression for LR8, SMA and COL1A1 genes was normalized against GAPDH expression.

Statistical analysis:

The gene expression values for the normal and fibrotic tissues were expressed as mean ± standard deviation. These values were subjected to Students T-test, and p values ≤ 0.05 were considered statistically significant ($\alpha=0.05$). To correlate the expression of LR8 and SMA genes, ratios of their expression values in all the normal and fibrotic tissues were derived and were subjected to T-test.

Specific Aim 2: Immunohistochemistry of normal and fibrotic tissues

LR8 antibody used in this study was obtained as a generous gift from Dr. Math Cuajungco, California State University Fullerton. This is a rabbit polyclonal antibody produced against polypeptide sequence **EGSEKRLLGENSVPPSPSRE** in carboxyl terminus of predicted LR8 protein sequence (Cuajungco et al., 2012).

Paraffinized normal and fibrotic human lung, liver and kidney tissue sections were obtained from the Department of Pathology, UWMC. The tissue sections were deparaffinised in three washes of xylene (5 minutes each) followed by rehydration in ethanol (3 changes of 100% ethanol for total 5 minutes, 2 changes of 95% ethanol for 4 minutes and one minute in 75%). Endogenous peroxide was blocked using 3% hydrogen peroxide, followed by a wash in PBS, pH 7.4. Antigen retrieval was carried out using 1 mM EDTA, pH 8.0 in a pressure cooker. The tissue sections were then blocked in normal horse serum (2.5%) for 60 to 90 minutes and one of the following primary antibodies was applied: LR8, rabbit polyclonal antibody to polypeptide in the carboxyl terminus of LR8 protein (Cuajungco et al., 2012), SMA, mouse monoclonal antibody to SMA (Clone 1A4, DAKO, CA) or COL1A1, rat monoclonal antibody to collagen 1 proteins (Narayanan et al., 1985). After application of the primary antibody, diluted with PBS containing 1% bovine serum albumin (Sigma, St. Louis, MO), tissue sections were incubated overnight at 4°C or for 1 hour at room temperature. Tissue sections were then washed in two changes of PBS and incubated in respective secondary antibodies for 30 minutes, followed by two washes in PBS. Diaminobenzidine (DAB) reagent was then applied to the tissue sections for color development for 15 seconds to 1 minute. After washing in dH2O, the sections were counter

stained in hematoxylin and dehydrated using ethanol followed by placing cover slip using Xylene substitute mountant. Controls were non- and preimmune serum for LR8 antibody and were applied following the similar procedure described for LR8 antibody.

Double staining using LR8 and SMA antibodies was performed to study their co-localization in different cell types. The double staining consisted of horseradish peroxidase (HRP) activity in red and blue/grey using Vector® NovaRed™ stain and Vector® SG substrate kit for peroxidase stains. The tissue sections were first stained for SMA in red followed by staining for LR8 in blue/grey. Dako® N- Universal Negative control for Rabbit/Mouse primary antibodies was used as negative control for the IHC reactions.

The histology slides were analyzed using Nikon Eclipse E400 microscope and the digital images were taken using a SPOT camera mounted on the microscope. MetaVue™ Imaging System software was used to count the cells manually.

Specific Aim 3: Bioinformatics and splice variants of LR8 gene

The presence or absence of splice variants of LR8 gene in normal gingiva and normal and fibrotic liver was studied using RT-PCR analysis. The protocol followed was the same as described under Aim 1 and the primers used were LR8 A↑-A↓, LR8 B↑-B↓, LR8 C↑-C↓ and LR8 A↑-C↓ (Table 3.1, Fig. 3.1).

```
   1 ccccatctct ctctctctaa aaaaagagaa ctggccgtga gctattgtgc ccagctggga
  61 tcttgacaaa gacactattt ctctcctttc acctgtgctg tgtatttttc cctcgcctag
 121 ttcccagacc tcactgctat atgtcttctc cctggcaggc aggatgacgc aaaacacggt
 181 gattgtgaat ggagttgcta tggcctctag gcatgcccag cccacccacg tcaacgtcca
 241 catccaccag gagtcagctt tgacacaact gctgaaagct ggaggttctc tgaagaagtt
 301 tctttttcac cctggggaca ctgtgtcttc cacagccagg attggttatg agcagctggc
 361 tctaggggtg actcagatat tgctgggggt tgtgagttgt gttcttggag tgtgtctcag
 421 cttggggccc tggactgtgc tgcgtgcctc aggctgtgcc ttctgggcgg ggtctgtggt
 481 gatcgcagca ggagctgggg ccattgtcca tgagaagcac ccgggcaaac ttgctggcta
 541 tatatccagc ctgctcaccc tggcaggctt tgctacagct atggctgctg ttgtcctctg
 601 cgtgaatagc ttcatctggc aaactgaacc ctttttatac atcgacactg tgtgtgatcg
 661 ctcagaccct gtcttcccta ccactgggta cagatggatg cggcgaagtc aagagaacca
 721 atggcagaag gaggagtgta gagcttacat gcagatgctg aggaagttgt tcacagcaat
 781 ccgtgccctg ttcctggctg tctgtgtctt gaaggtcatt gtgtccttgg tttccttggg
 841 agtaggtctt cgaaacttgt gtggccagag ctcccagccc ctgaatgagg aaggatcaga
 901 gaagaggcta ctgggggaga attcagtgcc cccttcgccc tctagggagc agacctccac
 961 tgccattgtc ctgtgagccg ccaaagaccc cacggggtgc ccgcatgtcc ctgtctaggg
1021 cagcccaggg cccccactcc tggctcctca cacttgcctc ccctatggcc gctctccaga
1081 ccctcctcct ttcttctccc cacatccgca cctgctgttc ccactctggg gttctcaagt
1141 ccatgaacag atattgttgc attttccaca atgactgatt aaacataata aacaatccag
1201 aaaagcaa
```

Figure 3.1: LR8 mRNA sequence (accession no. AF115384) with primer sequences for LR8 A↑-A↓ indicated in red, LR8 B↑-B↓ in blue and LR8 C↑-C↓ in green.

A search of the databases- Nation Center for Biotechnology (NCBI) gene, NCBI protein and non-redundant database of expressed sequence tag (EST) was performed using the 1208 base pair long LR8 gene sequence and 270 amino acid long predicted LR8 protein sequence as the query (Lurton et al. 1999). Ensembl, Genecards, ENTREZ gene databases and Online Mendelian Inheritance in Man (OMIM) databases were searched using the keywords ‘LR8’ and ‘TMEM 176B’. The protein expression data from Human Protein Atlas with the same keywords were also reviewed. The results from these bioinformatics analysis of LR8 gene was correlated with results from the RT-PCR analysis and immunostaining.

CHAPTER IV: Quantitating LR8 and SMA gene expression in normal and fibrotic tissues

Specific Aim 1

Introduction

LR8 gene expression was studied previously in human lung and gingival fibroblasts. It was observed that LR8 expression was upregulated in human lungs with IPF, and in fibrotic mouse lungs (Lurton et al. 1999). In humans, LR8 expression correlated with SMA expression in gingival fibroblasts from different patients (Wisecup and Narayanan 2009). In Specific aim 1, I wanted to extend these findings to other tissues and test the following hypotheses: 1. LR8 gene expression will be higher in fibrotic human tissues relative to normal tissues, and 2. There will be a positive correlation between LR8 and SMA gene expression. The results of these experiments will provide evidence for the presence and clonal expansion of subpopulation of cells expressing LR8 and that these cells are a population of fibroblasts which are destined to differentiate into myofibroblasts. In this Aim, I will use SMA gene expression to identify the putative myofibroblast phenotype and COL1A1 gene expression as an indicator of fibrosis.

Methods

Normal and fibrotic human tissues were obtained from the UW Pathology Department. Total RNA was extracted from normal and fibrotic human tissues, and RT-PCR and quantitative real time PCR was performed as described in the Chapter III. Statistical analysis were performed as described in Chapter III.

Results

RT-PCR: RT-PCR products for LR8, SMA, COL1A1 and GAPDH were analyzed. All of the normal and fibrotic liver and gingiva tissues examined showed LR8 expression. They were also positive for SMA and GAPDH expression. All the gingival and liver samples, except one liver tissue (Normal Liver 2) expressed COL1A1.

Quantitative-PCR results: The gene expression values obtained for LR8, SMA and COL1A1 were normalized for GAPDH. There was variability in the expression of LR8 and SMA in the tissues obtained from different patients (Fig. 4.1). There was about 10^6 fold variation in the LR8 expression in normal liver tissues and a 100 fold difference in fibrotic liver tissues (Fig. 4.1 A, B). LR8 expression varied by about 1000 fold in normal gingival tissues and by 10^5 fold in fibrotic gingiva (Fig. 4.1 C, D). LR8 expression was higher in fibrotic liver as compared to normal liver (LR8 expression in normal liver vs. LR8 expression in fibrotic liver, $t=1.637$, $p=0.243$); (Fig. 4.1 A, B). Differences in LR8 expression between normal and fibrotic gingiva were not statistically significant (LR8 expression in normal gingiva vs. LR8 expression in fibrotic gingiva, $t=0.071$, $p=0.946$); (Fig. 4.1 C, D). LR8 expression was almost undetectable in some of the normal liver and gingival tissue samples.

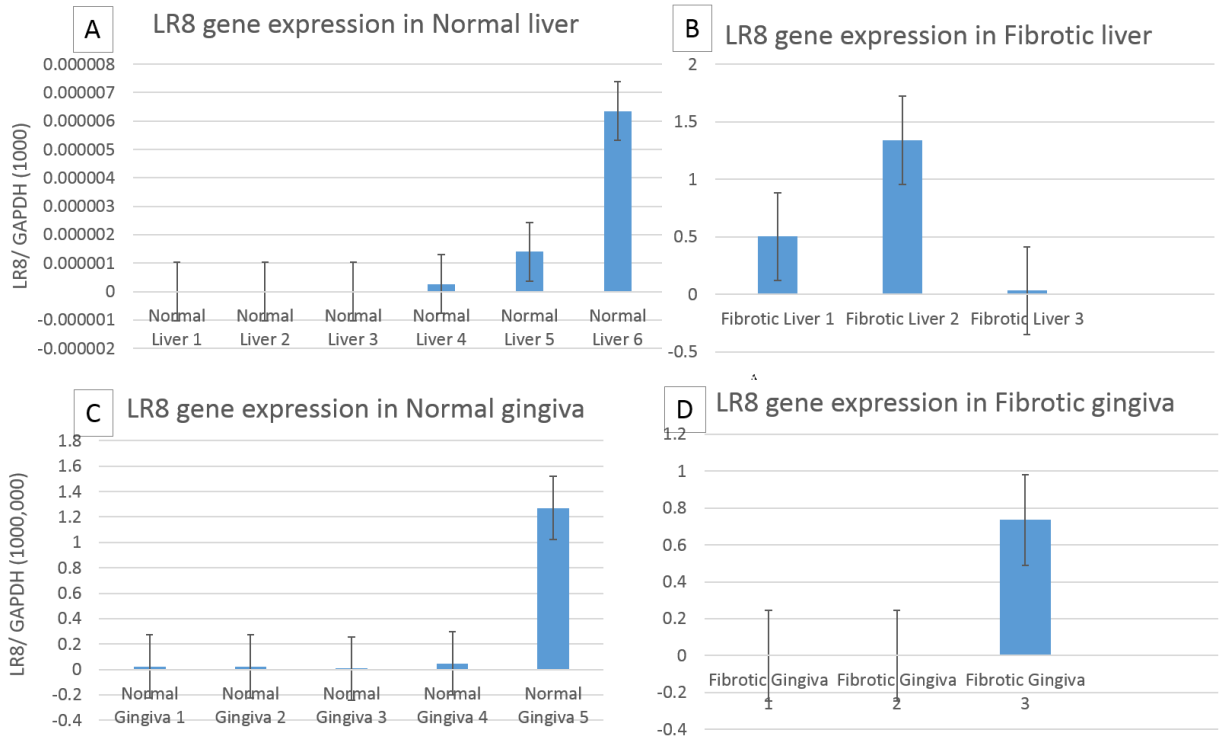

Figure 4.2: LR8 gene expression in Normal and Fibrotic human tissues. A. Normal liver. B. Fibrotic liver. C. Normal gingiva. D. Overgrown gingiva. Values were normalized for GAPDH. Values represent mean ± SD of 3 experiments.

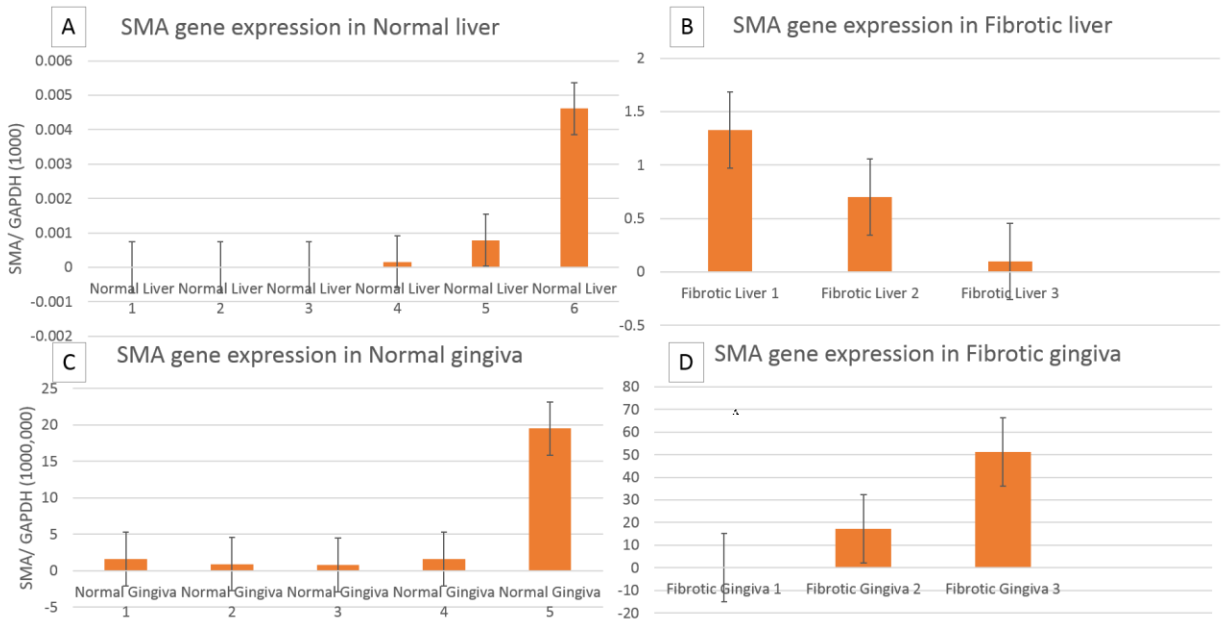

Figure 4.3: SMA expression in Normal and Fibrotic human tissues. A. Normal liver. B. Fibrotic liver. C. Normal gingiva. D. Overgrown gingiva. Values were normalized for GAPDH. Values represent mean ± SD of 3 experiments.

There was variability in SMA expression among individual patients in liver and gingiva. SMA expression was higher in fibrotic liver as compared to normal liver (Fig. 4.2 A, B), and in fibrotic gingiva compared to normal gingiva (Fig. 4.2 C, D), but differences were not statistically significant at $\alpha=0.05$.

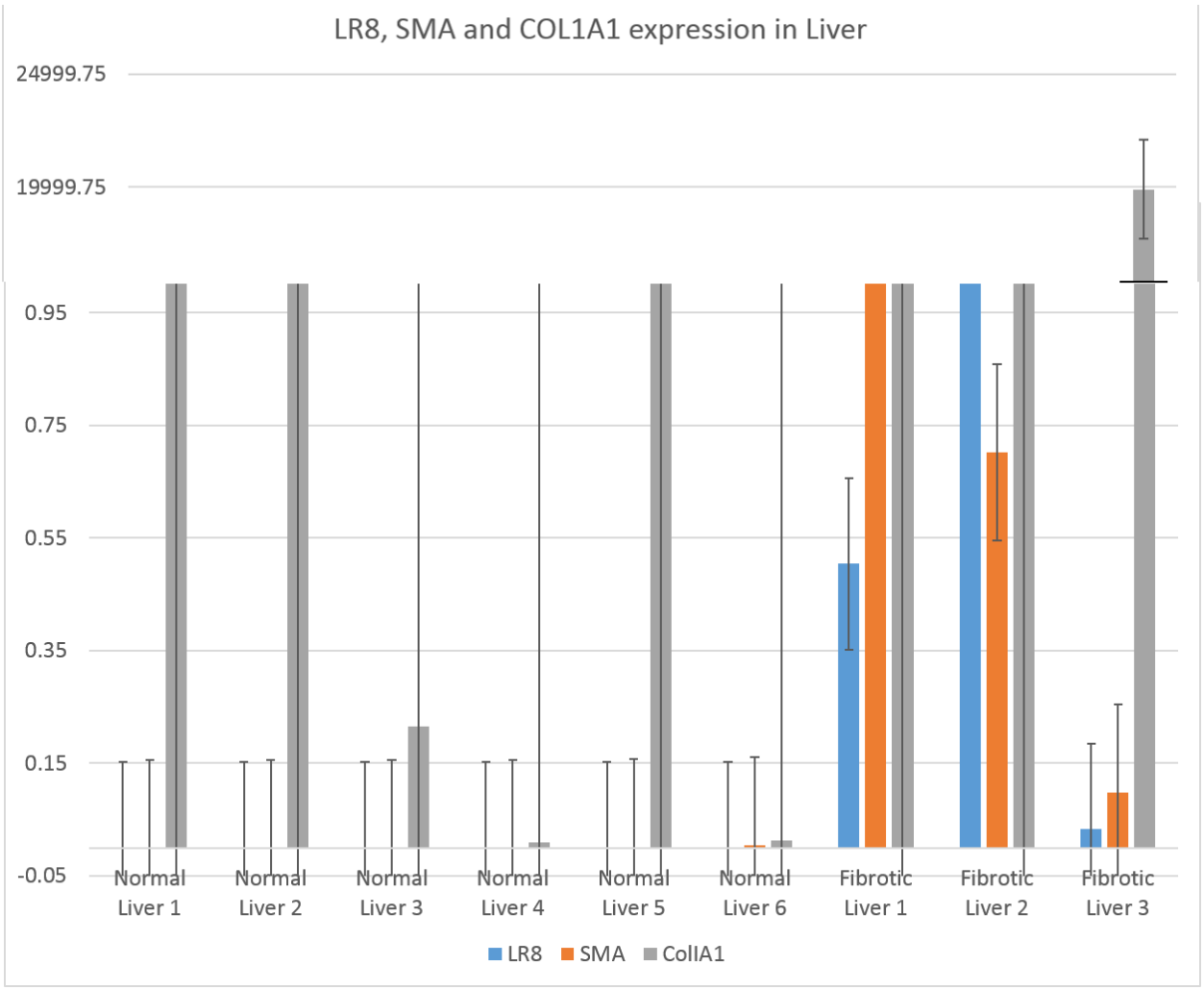

Figure 4.4: LR8, SMA and COL1A1 gene expression in Normal and Fibrotic liver tissues. All the values were normalized for GAPDH. Values represent mean ± SD of 3 experiments.

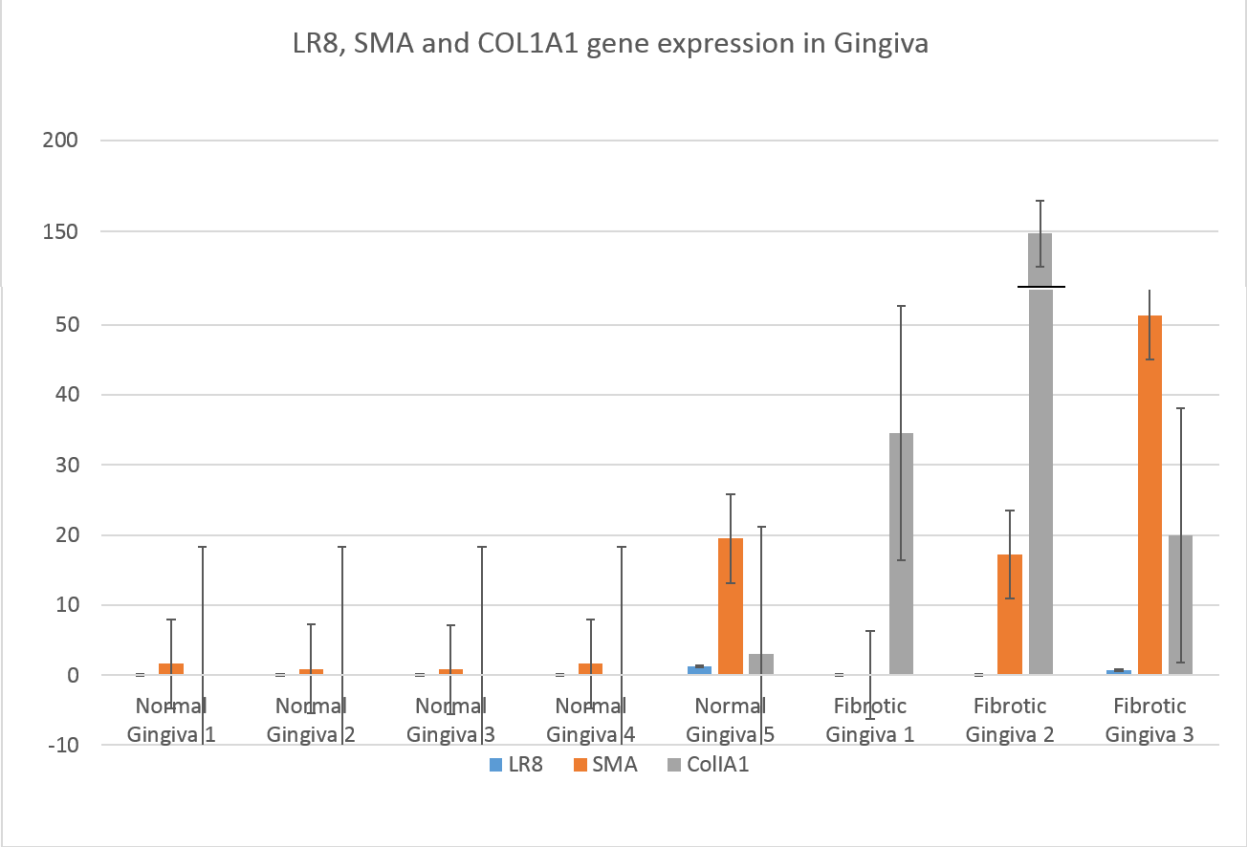

Figure 4.4: LR8, SMA and COL1A1 gene expression in Normal and Fibrotic gingiva tissues. Values were normalized for GAPDH. Values represent mean ± SD of 3 experiments.

COL1A1 expression was higher in fibrotic tissues as compared to its expression in normal tissues in both liver and gingiva. Its expression was much higher as compared to LR8 and SMA genes in all the fibrotic tissues (Fig. 4.3, Fig. 4.4). In normal tissues, COL1A1 expression was higher than LR8 and SMA expression in liver tissues (Fig. 4.3), but its expression was lower than SMA expression in normal gingival tissues (Fig. 4.4).

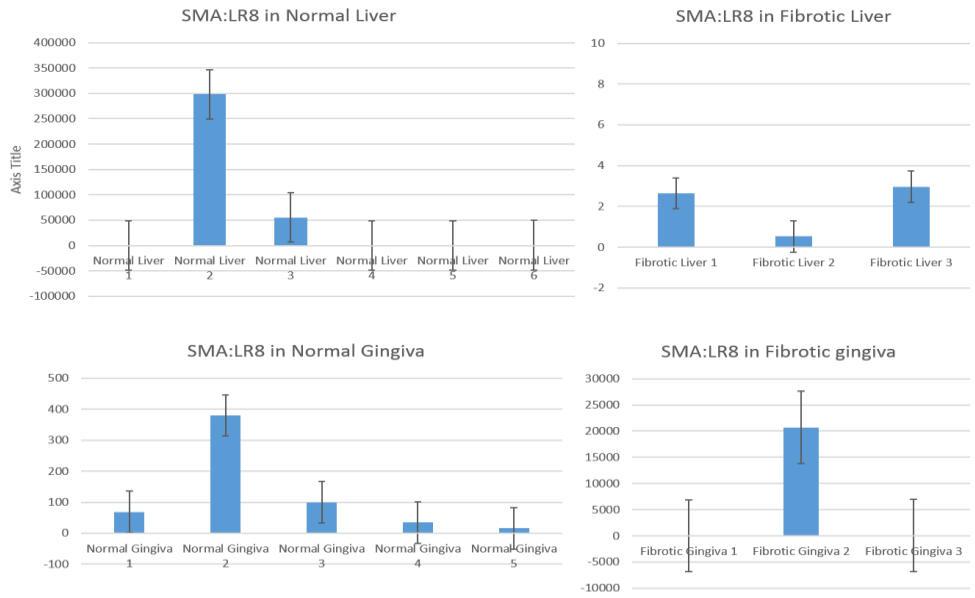

Figure 4.5: Graphs representing ratios of SMA and LR8 gene expression on Normal and Fibrotic liver and gingiva.

I did not find a positive correlation between LR8 and SMA gene expression amongst all normal liver tissues as compared to fibrotic liver tissues (SMA:LR8 in normal liver vs. SMA:LR8 in fibrotic liver, t= 0.8322, p= 0.4322). There was lack of correlation even in normal and fibrotic gingival tissues (SMA: LR8 in normal gingiva vs. SMA: LR8 in fibrotic gingiva, t= 1.3537, p= 0.2246) (Fig. 4.5).

Discussion

There was an increase in the LR8 gene expression in fibrotic liver as compared to normal liver; this is consistent with the previous results that LR8 gene expression is up regulated in fibroblasts derived from human IPF lungs and bleomycin-induced fibrotic lungs (Lurton et al., 1999) and in overgrown gingival fibroblasts (unpublished data from Dr. Narayanan's laboratory). However, normal and fibrotic gingival tissues did not show significant differences in

the level of LR8 expression. This may be due to various reasons. One reason could be that my hypothesis that LR8 expression will be higher in fibrotic tissues as compared to normal tissues does not hold true in gingiva. Alternatively, there may be cell types other than fibroblasts expressing LR8 and it is possible that LR8 may not be associated with only the subpopulation of fibroblasts that expand in fibrosis; I will examine this possibility by immunohistochemistry in Aim 2, Chapter V. The third reason could be decreased cellularity of the fibrotic tissue. The fourth reason could be the existence of more than one splice variant of LR8 RNA which are differentially expressed and affected in fibrotic tissues. This possibility will be examined by studying the splice variants of LR8 in different human tissues in Specific Aim 3 (Chapter VI).

As expected, COL1A1 was up regulated in liver and gingival fibrosis. The expression of LR8 and SMA was often low in most of the normal liver and gingival tissue samples. Elevated LR8 and SMA expression was seen in two out of three fibrotic liver, and one of the three gingival tissue samples. Comparing the averages of SMA expression among the normal and fibrotic tissue samples, there was up regulation of SMA expression in both liver and gingival fibrosis. The increased SMA expression in fibrosis indicates the presence of a myofibroblast phenotype in fibrotic tissues. In normal tissues the main source of SMA are endothelial cells and smooth muscle cells; therefore, the increased expression in fibrotic tissues may also be because of increased angiogenesis seen in early stages of fibrosis as a result of inflammation. However, the fibrotic liver tissues obtained for this study were mostly from cirrhotic livers, which is usually end stage fibrosis and may lack vascularity. Therefore, the increased SMA gene expression in liver is likely to be due to the myofibroblast phenotype; I will examine this possibility in Aim 2 (Chapter V).

It was observed earlier in gingival fibroblasts that there was positive correlation between LR8 and SMA expression and that fibroblasts from different patients showed variability in LR8 expression (Wisecup and Narayanan, 2009). In this study, I also observed that there was variability in individual patient LR8 gene expression in both normal and fibrotic liver and gingiva. But positive correlation between LR8 and SMA gene expression was not observed. To examine the possibility that this could be due to the expression of LR8 by cell types other than fibroblasts. I performed immunohistochemistry using LR8 and SMA antibodies in Specific Aim 2 (Chapter V).

CHAPTER V: Immunohistochemistry of normal and fibrotic tissue sections

Specific Aim 2

Introduction

Previous studies described under Specific Aim 1 (Chapter IV) demonstrated there is limited or no correlation between LR8 and SMA expression in tissues. One reason for this may be there is expression of these genes by other cells in addition to fibroblasts. To examine this possibility and to study the distribution of cells producing LR8 and SMA proteins in normal and fibrotic human tissues I performed immunohistochemistry using LR8 and SMA antibodies. The hypothesis is: fibroblasts expressing and not expressing LR8 are present in normal tissues and LR8 producing cells are present in increased numbers in fibrotic tissues. The immunostaining will reveal if cells other than fibroblasts express LR8. Presence of fibroblasts showing positive staining for both LR8 and SMA and increase in the number of these cells in fibrosis will support the hypothesis that subpopulations of fibroblasts expressing LR8 and SMA may represent the putative myofibroblast phenotype. For this experiment I examined tissue sections of healthy and fibrotic human liver, lung and kidneys.

Methods

Tissue sections of normal and fibrotic liver, lung and kidney were obtained from UW Department of Pathology. The sections were deparaffinized and immunostained as described under “Materials and Methods” (Chapter III). To study the co-expression of LR8 and SMA

genes in the same cells I performed double staining using LR8 and SMA antibodies. Staining was visualized by using either diaminobenzidine (DAB) reagent in single antibody staining, or Novared for SMA and Vector SG for LR8 in double immunostaining experiments (Chapter III).

Results

Histology:

Liver: Liver is made up of lobules. Each lobule contains portal triads, central vein and portal sinuses. The portal triad is composed of bile ducts, hepatic artery and portal vein. Plates of hepatocytes radiate from the central vein. Hepatic sinusoids are the micro vascular units of liver and these sinusoids contain endothelial cells, Kupffer cells and hepatic stellate cells (HSC) (Fig. 5.1A). In hepatic fibrosis, activation and proliferation of HSCs is mainly responsible for the increased ECM synthesis. Portal fibroblasts are also activated to differentiate into myofibroblasts. In chronic viral hepatitis there is portal-central fibrotic septa and nodule formation whereas portal- portal fibrotic septa formation and bile duct proliferation is seen in biliary cirrhosis. Alcoholic hepatitis is characterized by ballooning degeneration of the hepatocytes (Fig. 5.1B). Cirrhosis is the end consequence of progressive fibrosis, which ultimately leads to portal hypertension and liver failure.

Lung: In the lung, the alveoli are lined by two types of epithelial cells. The first type or type I pneumocytes are large flat cells which cover majority of the surface of alveoli. The second cell type is type II pneumocytes which are more cuboidal and are the progenitor cells of

alveolar epithelium. The bronchi are lined by respiratory mucosa and show the presence of smooth muscle in their walls. The interstitial space in the lung consists of fibroblasts, collagen, elastin, proteoglycans and also possibly myofibroblasts (Fig. 5.1C). In Idiopathic Pulmonary Fibrosis, hypertrophy of pneumocytes is seen with an increase in number of type II pneumocytes. Microscopic foci of injury, remodeling and repair is seen along with dense fibrosis and honey combing pattern. Bundles of collagen and myofibroblasts can be observed in the interstitium (Fig. 5.1D)

Kidney: The kidney is composed of an outer cortex and inner medulla and is lined by a capsule. Nephrons, the basic functional units of the kidney, are composed of glomerulus and tubules. The glomerulus consists of epithelial cells called podocytes and mesangial cells, which are modified smooth muscle cells. The tubule can be divided into proximal tubule, loop of Henley and distal tubule. The renal interstitium is composed of cells including fibroblasts and a collagenous matrix (Fig. 5.1E). In fibrotic kidney, areas of destruction foci are seen with lymphocytic infiltration. There is loss of normal tubule architecture and collapsed glomeruli are seen. The podocytes show leaking and some blood vessels show serous exudate. Calcifications are also seen in some areas (Fig. 5.1F).

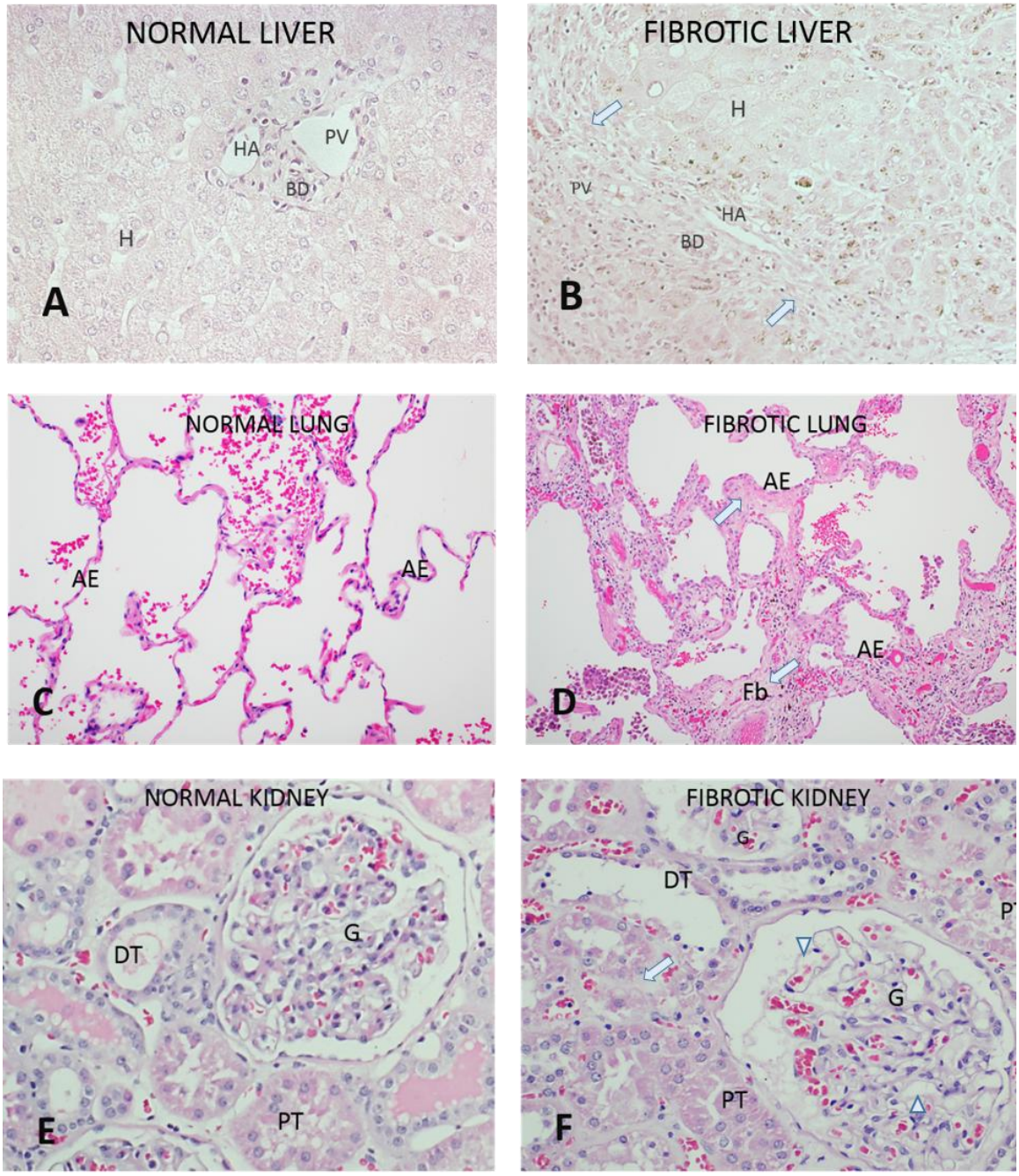

Figure 5.1: (A) Histology of normal liver showing Hepatocytes (H), portal triad consisting of Bile duct (BD), Hepatic artery (HA) and Portal vein (PV). (B) Fibrotic liver showing proliferation of fibroblasts and excessive extracellular matrix synthesis. (C) Normal lung showing alveoli lined by Alveolar Epithelial (AE) cells. (D) Fibrotic lung showing hypertrophy of pneumocytes and dense fibrosis with excessive collagen synthesis. (E) Normal kidney showing Glomeruli (G), proximal tubules (PT) and distal tubules (DT). (F) Early fibrosis in kidney showing leaking podocytes in glomeruli (arrowheads) and degenerating distal tubules. White arrows indicate fibrotic areas.

Immunostaining results

The results for immunohistochemistry are presented in Figures 5.2-5.6 and Tables 5.1-5.3. Fig. 5.2-5.4 and Fig. 5.6 show the double staining with anti-LR8 and anti-SMA antibody and Fig. 5.5 show pictures of tissue sections stained with anti- LR8 antibody only. The negative control reactions using Dako® N- Universal Negative control did not show measurable signals in lung, liver and kidney tissue sections.

In normal liver, all fibroblasts were positive for LR8, but not for SMA (Fig. 5.2). In fibrotic liver, fibroblasts manifesting positive and negative staining for LR8 antibody were present (Fig. 5.5). Most of the fibroblasts positive for LR8 were also positive for SMA (Fig. 5.2 B).

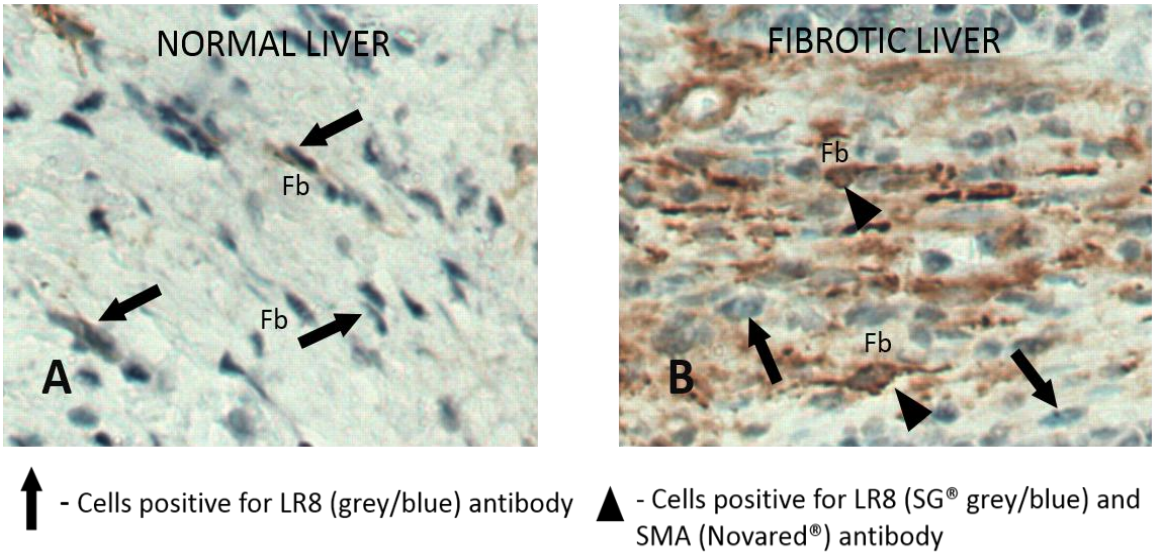

Figure 5.2: (A) Photomicrographs showing fibroblasts staining positive for only LR8 (grey) in normal liver, (B) fibroblasts staining positive for only LR8 and both LR8 and SMA (red) in fibrotic liver.

Normal lung fibroblasts were mostly positive for LR8 only (Fig. 5.3 A). Some areas in normal lung contained fibroblasts staining positive for both LR8 and SMA (Fig. 5.3 A). In fibrotic lung, the fibroblasts exhibiting differentiation into myofibroblasts were positive for both LR8 and SMA antibodies, but only undifferentiated fibroblasts were positive for LR8 (Fig. 5.3 B).

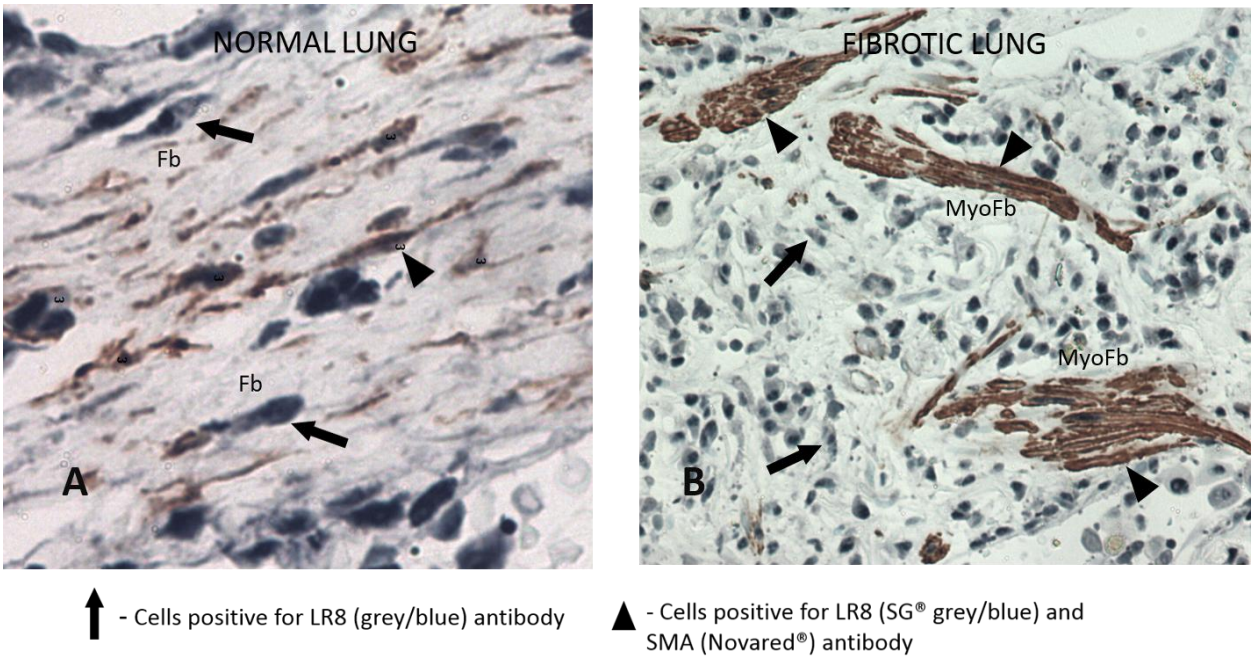

Figure 5.3: (A) Photomicrographs showing fibroblasts staining positive for only LR8 (grey) or both LR8 and SMA (red) in normal lung, (B) Fibroblasts staining positive for only LR8 and myofibroblasts staining positive for both LR8 and SMA in fibrotic lung,

In normal and fibrotic kidney, fibroblasts in the capsule displayed heterogeneity in LR8 expression; some were positive with variable staining intensities while others were negative (Fig. 5.4 A, Fig. 5.5). Fibroblasts in normal kidney cortex were positive for staining with LR8 antibody, but negative for SMA antibody (Fig. 5.4 A). In fibrosis, the fibroblasts were

heterogeneous in LR8 and SMA expression, with some of them being positive for LR8 and some fibroblasts not showing LR8 expression (Fig. 5.5). Some of the fibroblasts staining positive for LR8 were also positive for SMA (Fig. 5.4B).

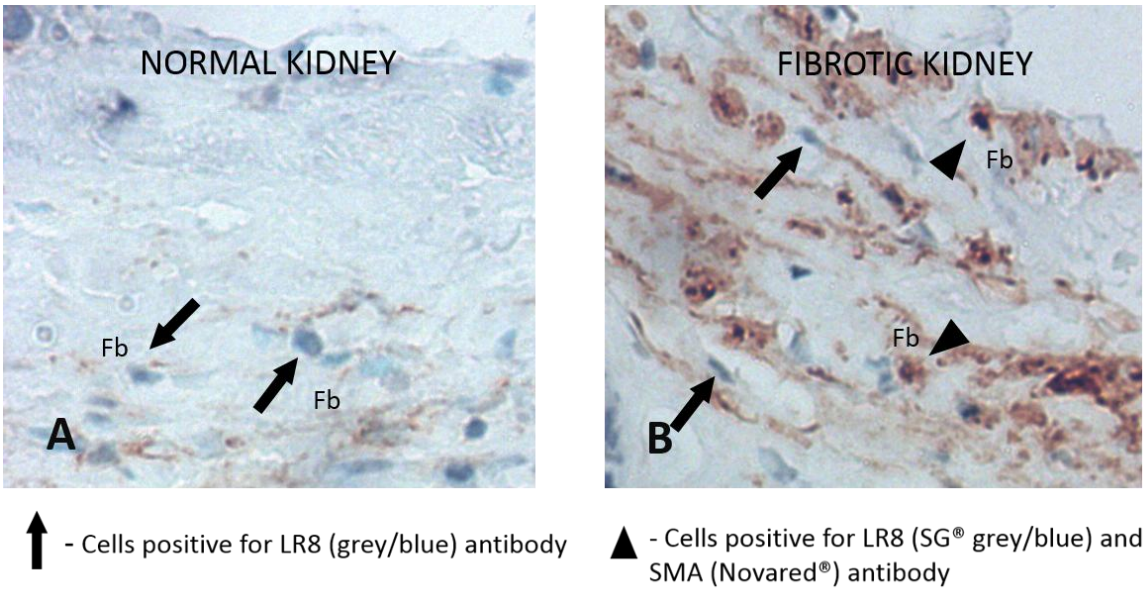

Figure 5.4: (A) Photomicrographs showing fibroblasts staining positive for only LR8 (grey) in normal kidney capsule, (B) fibroblasts staining positive for only LR8 and both LR8 and SMA (red) in fibrotic kidney capsule.

Table 5.1: Immunostaining in fibroblasts from normal and fibrotic liver, lung and kidney tissue sections double stained with LR8 and SMA antibodies

Organ	Cell type	Normal		Fibrotic	
		LR8	SMA	LR8	SMA
Liver	Fibroblasts				
Kidney	Fibroblasts				
Lung	Fibroblasts				

Strong
Moderate
Weak
No staining

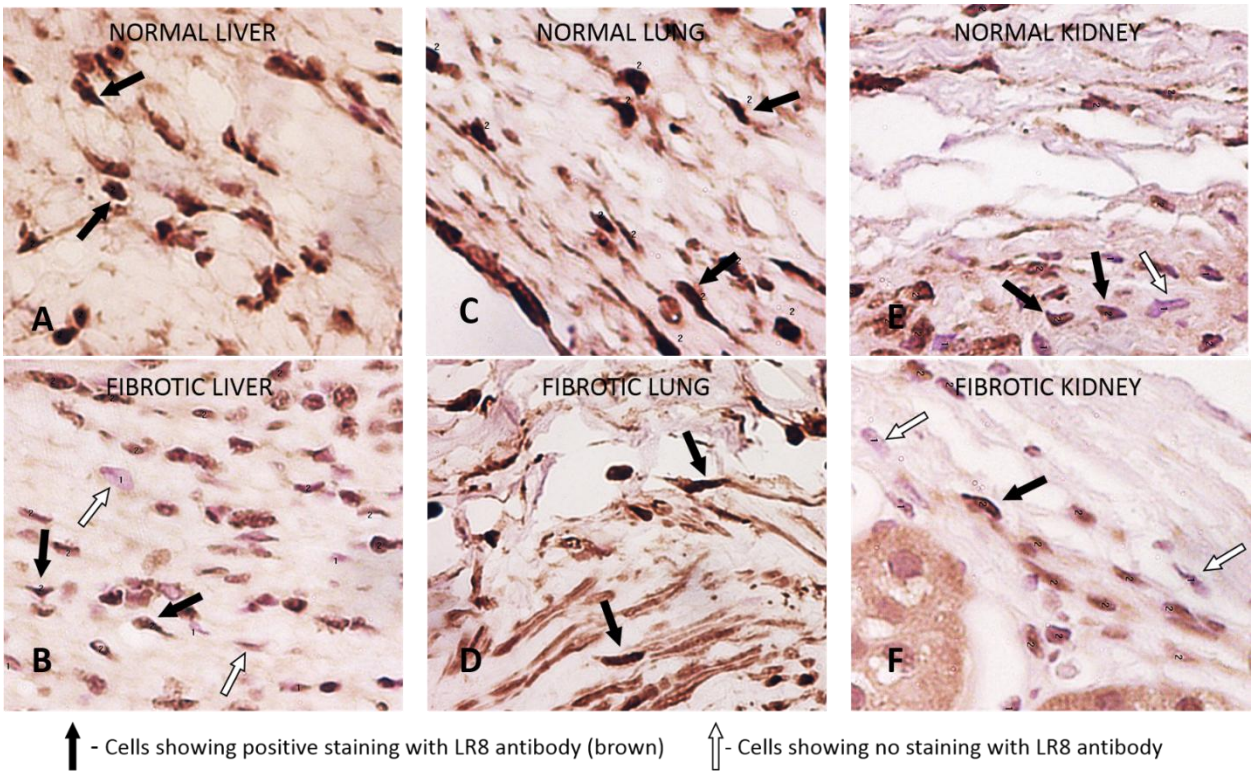

Figure 5.5: Immunostaining in fibroblasts with only anti-LR8 antibody. DAB reagent (brown) was used for color development.

Overall, fibroblasts without LR8 expression were seen in fibrotic liver and normal and fibrotic kidneys; fibroblasts expressing LR8 only were present in normal human liver, lung and kidney; cells expressing LR8 and SMA were present in normal lung and fibrotic tissues (Table 5.1) (Fig. 5.5). The number of these cells were quantified in 3 fields (double staining with LR8 and SMA antibodies and single staining with LR8 antibody) each of normal and fibrotic specimens (Table 5.2). I performed Students T-test with the values obtained and the results are: number of LR8 positive cells in normal tissues vs. fibrotic tissues: t=1.4826, p=0.2348, number of LR8 and SMA positive cells in normal tissues vs. fibrotic tissues: t=4.478, p=0.0208. The results showed that number of fibroblasts staining positive for both LR8 and SMA increased in fibrosis (α= 0.05).

Table 5.2: LR8 $^{-}$ & LR8+ cells counted in single stained tissue sections stained, LR8+ & SMA+ positive cells counted in double stained tissue sections. The fibroblasts were identified based on their appearance.

	LR8 $^{-}$		LR8^{+}		LR8^{+}, SMA^{+}	
	Normal	Fibrotic	Normal	Fibrotic	Normal	Fibrotic
LIVER	02 ± 1	20 ± 2	43 ± 10	60 ± 25	05 ± 2	36 ± 9
LUNG	0	0	30 ± 3	38 ± 2	08 ± 4	23 ± 9
KIDNEY	15 ± 5	11 ± 1	19 ± 7	17 ± 6	01 ± 1	09 ± 2

Production of LR8 by cells other than fibroblasts:

In liver, hepatocytes, bile duct cells, endothelial cells and smooth muscle cells showed weak to moderate staining with LR8 antibody (Fig. 5.6 A, B). In lung, alveolar cells, macrophages, endothelial cells in the blood vessels, smooth muscle cells were all positive for LR8 expression. But pneumocytes were heterogeneous in LR8 expression with varying staining intensities (Fig. 5.6 C, D). Kidney cells such as podocytes, interstitial cells and cells of Bowman's capsule are mostly negative for LR8 expression. Proximal tubule cells showed strong staining and distal tubule cells displayed moderate staining. But in kidney fibrosis, the collapsed ducts did not exhibit much staining with LR8 antibody (Fig. 5.6 E, F).

Endothelial cells and smooth muscle cells in all the tissue sections demonstrated strong staining with SMA antibody (Fig. 5.6). These results are summarized in Table 5.3.

Table 5.3: LR8 and SMA staining intensity by different cells in normal and fibrotic liver, lung and kidney tissue sections.

Organ	Cell type	Normal		Fibrotic	
		LR8	SMA	LR8	SMA
Liver	Hepatocytes				
	Bile duct cells				
	Endothelial cells, Smooth muscle cells				
Lung	Alveolar cells				
	Respiratory epithelium in bronchioles				
	Endothelial cells, Smooth muscle cells				
Kidney	Distal tubule cells				
	Proximal tubule cells				
	Cells in glomeruli				
	Endothelial cells, Smooth muscle cells				

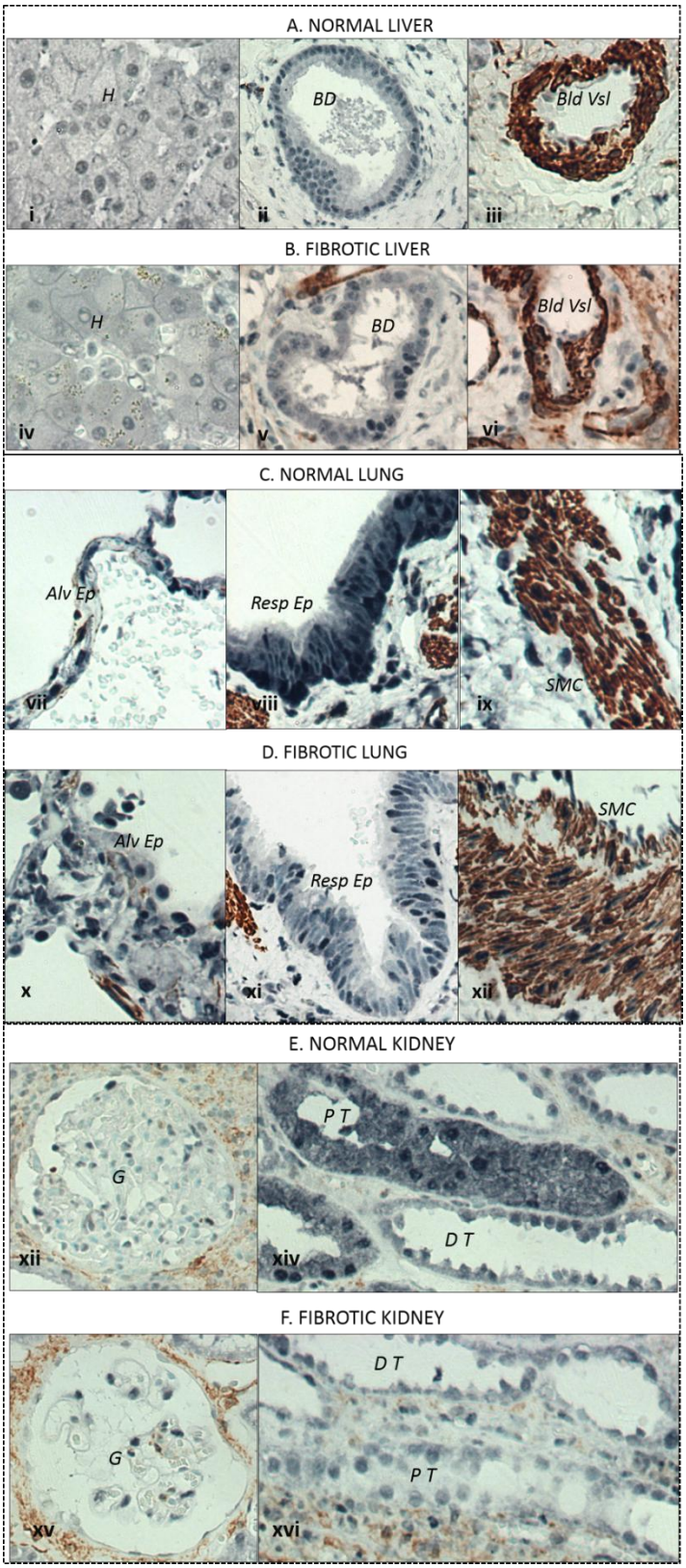

Figure 5.6: Photomicrographs showing staining with anti-LR8 and anti-SMA antibodies in normal and fibrotic liver, lung and kidney.

Discussion

The anti-LR8 antibodies that are commercially available were generated using a polypeptide fragment of the LR8 protein. As a part of this study, I performed experiments to generate a polyclonal antibody against recombinant LR8 protein at Dr. Timothy Rose/Greg Bruce's laboratory, Seattle Children's Research Institute. LR8 gene was sequenced and a construct was made by introducing LR8 gene into expression vector pQE30. The plasmid was then transfected into *E. coli* cells to produce his-tagged LR8 protein. Protein expression was induced using IPTG and Western Blot analysis was performed using protein extracts, which gave negative results. Another expression vector, pRSET, was also tried. But the results were also negative. The lack or poor LR8 expression appeared to be due to presence of codons which were not suitable for expression in *E. coli*. Therefore, we obtained LR8 antibody as a generous gift from Dr. Math Cuajungco, California State University Fullerton. This is a rabbit polyclonal antibody produced against polypeptide sequence **EGSEKRLLGENSVPPSPSRE** in carboxyl terminus of predicted LR8 protein sequence (Cuajungco et al., 2012).

Most of the cell types in all the tissue sections showed no- to moderate staining with LR8 antibody. Though this result does not agree with the previous cell culture studies performed on LR8 (Lurton et al., 1999), it is similar to the information available regarding LR8 expression in the Human Protein Atlas database (HPA047509 antibody, Atlas antibodies. ENSEMBL gene ID-ENSG00000106565). However, there was a very specific staining pattern for SMA. Only endothelial cells and smooth muscle cells showed positive staining in the normal tissue sections, and fibroblasts/myofibroblasts were additionally positive in fibrotic tissue sections (Fig. 5.2-4).

My results showed that cells other than fibroblasts were also positive for LR8 (Table 5.3; Fig. 5.6). In kidney there was strong positive staining seen with LR8 in the proximal tubule cells. Proximal tubule cells play an important role in renal damage in proteinuria. In proteinuria, the interstitial inflammation activates the fibroblasts to synthesize extracellular matrix proteins. In a study using gene expression profiling of renal proximal tubules in proteinuria, GS188, a gene closely related to LR8 was found to be up regulated. Since these genes are located very close to one another on human chromosome 7, Nakajima et al. (2002) concluded that they may be regulated similarly and LR8 is possibly involved in the regulation of proteinuria as well as renal fibrosis.

In fibrotic lungs, myofibroblasts were positive for both LR8 and SMA, but the fibroblasts which were not yet differentiated to become myofibroblasts showed positivity only for LR8. In fibrotic liver and kidney, fibroblasts showed staining for LR8 and SMA, but they did not appear to be clearly differentiated to become myofibroblasts. Pneumocytes in fibrotic lung showed heterogeneity in LR8 expression. Fibroblasts in the capsule of normal and fibrotic kidneys and fibrotic liver also showed heterogeneity in LR8 expression; this result supports my hypothesis.

The qPCR analysis showed SMA expression by fibroblasts expressing LR8 (Wisecup and Narayanan 2009); however, I found all fibroblasts producing LR8 were not positive for SMA. One reason for this may be that the SMA mRNA produced by these cells is not translated.

These results show that expression by cell types other than fibroblasts could be one explanation for the lack of correlation between LR8 and SMA gene expression measured by quantitative PCR in the tissues. In normal and fibrotic tissues SMA is strongly expressed by endothelial cells and smooth muscle cells; this is expected. However, it is expressed by fibroblasts usually in fibrotic tissues (one exception is normal lung, where some of the fibroblasts showed SMA expression).

The presence of cells expressing both LR8 and SMA in fibrotic tissues, and their presence in lower numbers (or absence) in normal tissues support the hypothesis that fibroblasts expressing LR8 are enriched in fibrosis and that fibroblasts co-expressing LR8 and SMA may be putative myofibroblasts.

CHAPTER VI: Bioinformatics of LR8

Specific Aim 3

Introduction

In 1999, Lurton et al. first identified LR8 as a 724 bp product in a subpopulation of human lung fibroblasts. A composite sequence was constructed using the 724bp LR8 cDNA and sequences from five different human EST clones corresponding to this LR8 cDNA and PCR product with predicted length and sequence was obtained (Fig. 6.1). The predicted LR8 protein is 270 a. a. long and contains four hydrophobic domains (Lurton et al., 1999). A significant amount of bioinformatics data on LR8 gene is now available in various bioinformatics databases and I have reviewed the current information regarding the LR8 gene available in the bioinformatics databases NCBI, Ensembl, Genecards and Human protein atlas databases.

In Specific Aim 1, I could not show upregulation of LR8 in fibrotic tissues, contrary to my proposed hypothesis. There was also a lack of correlation in LR8 and SMA expression in normal and fibrotic liver and gingival tissues. One of the reasons for this could be the existence of more than one variant of LR8 which might be differentially expressed in tissues. For this reason and because splice variants of LR8 might be implicated in regulating the function of fibroblasts in fibrosis, I performed RT-PCR using RNA extracted from normal and fibrotic liver tissues and normal gingiva using three primer pairs. The PCR product using primer pair A↑-C↓ will represent the majority of the coding region of LR8 gene (Fig. 6.1). These primer pairs were

selected from three different domains in the LR8 gene in order to identify variants from different regions of the gene. In order to examine if these variants are the splice variants of LR8 gene, I have used the data from different bioinformatics databases.

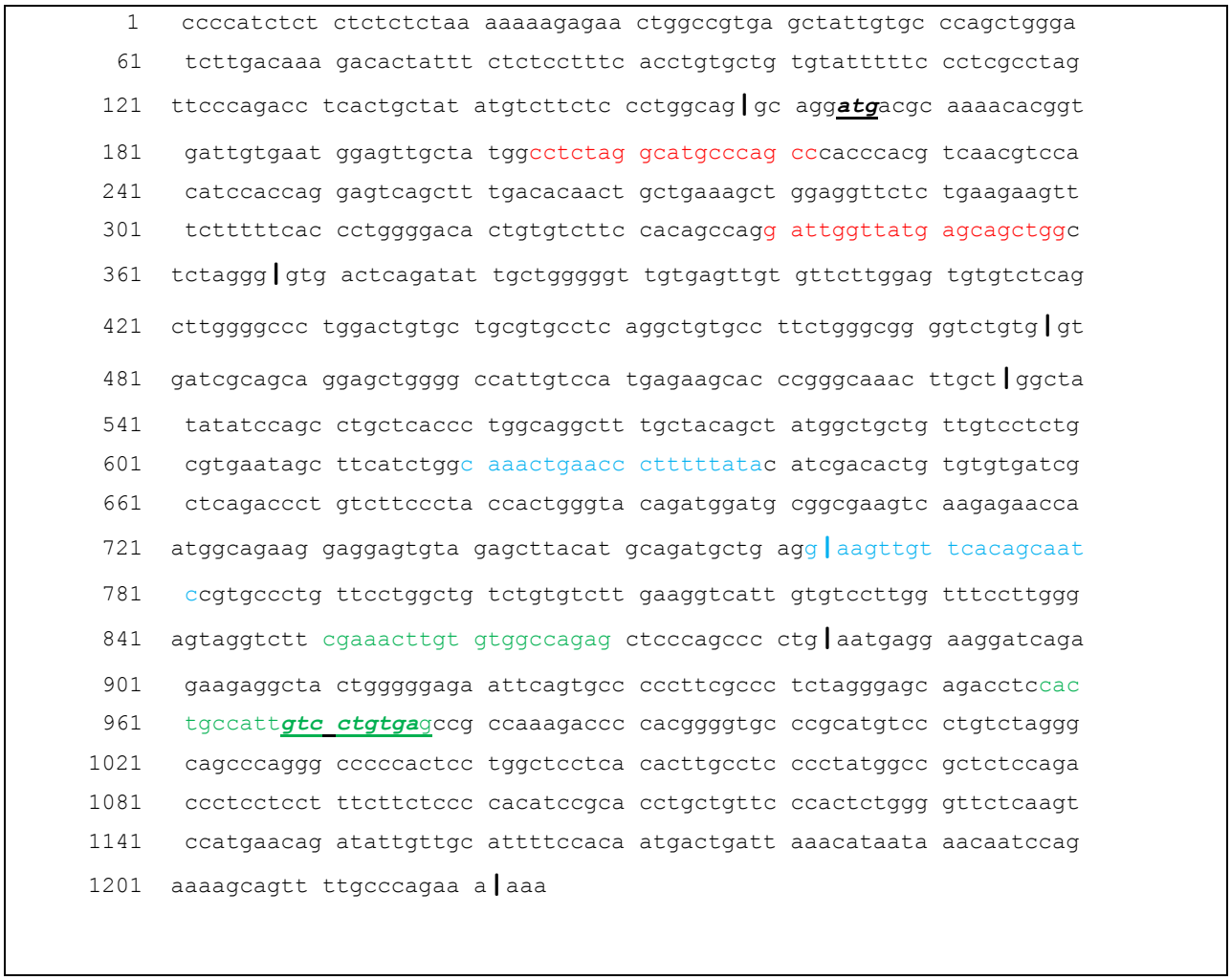

Figure 6.1: LR8 gene sequence with Exon junctions indicated with symbol '|' and primer sequences for LR8 A↑-A↓ indicated in red, LR8 B↑-B↓ in blue and LR8 C↑-C↓ in green. The start and end of the protein sequence is italicized and underlined.

Methods

Total RNA was extracted from normal and fibrotic liver tissues and gingiva and cDNA was synthesized. RT-PCR experiments were performed as described previously in Materials and Methods (Chapter III) using three primer pairs, LR8 A↑-A↓ , LR8 B↑-B↓ and LR8 C↑-C↓ (Table 3.1); these primers represent the N-terminal, middle and C-terminals regions of the predicted LR8 protein. The products were analyzed using Agarose gel electrophoresis.

Results

RT-PCR: PCR products of the expected size were detectable using primer pair LR8 A↑-A↓ (155 bp) in the gingival and liver tissue cDNAs (Figure 6.2). Primer pair LR8 B↑-B↓ (162 bp) also gave products in both tissues (Fig. 6.3), but the intensity of the DNA fluorescent band varied among normal gingival cDNAs. The most prominent display of products were seen in normal gingiva-1, and least prominent products in the normal gingiva-2 (Fig. 6.3). PCR products using primer pair LR8 C↑-C↓ (126 bp) was detectable in the normal and fibrotic liver tissues, but it was not detectable in all three of the gingival cDNAs (Fig. 6.4). When the PCR was performed using A↑-C↓ primers, expected product was obtained from liver. It was not detected in gingiva (Fig. 6.5).

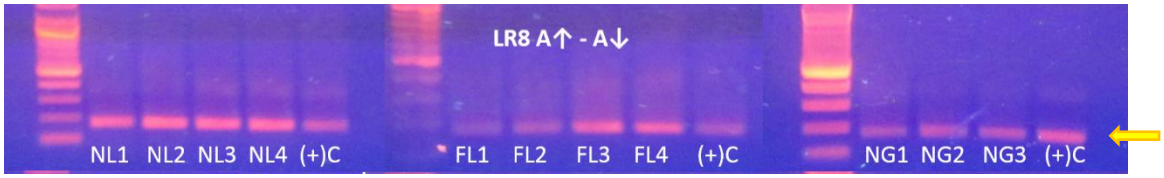

Figure 6.2: RT-PCR of liver and gingival cDNA using LR8 A↑-A↓ primers (155bp), Left lane is 100bp ladder. NL1-4 are Normal liver cDNAs, FL 1-4 are fibrotic liver cDNAs and NG1- NG3 are normal gingival cDNAs. (+) C is the positive control.

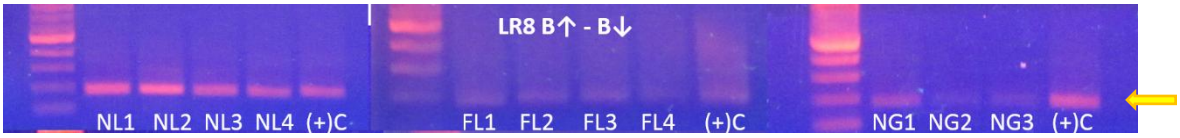

Figure 6.3: RT-PCR of liver and gingival cDNA using LR8 B↑-B↓ primers (162 bp). Left lane is 100bp ladder. NL1-4 are Normal liver cDNAs, FL 1-4 are fibrotic liver cDNAs and NG1- NG3 are normal gingival cDNAs. (+) C is the positive control.

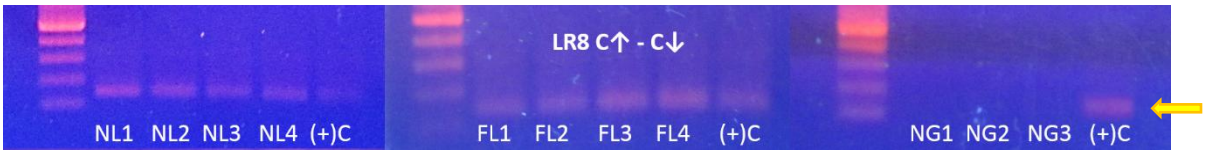

Figure 6.4: RT-PCR of liver and gingival cDNA using LR8 C↑-C↓ (126 bp). Left lane is 100bp ladder. NL1-4 are Normal liver cDNAs, FL 1-4 are fibrotic liver cDNAs and NG1- NG3 are normal gingival cDNAs. (+) C is the positive control.

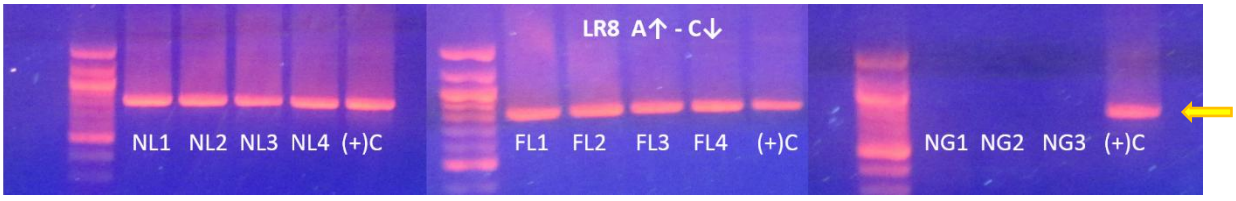

Figure 6.5: RT-PCR of liver and gingival cDNA using LR8 A↑-C↓ (774 bp). Left lane is 100bp ladder. NL1-4 are Normal liver cDNAs, FL 1-4 are fibrotic liver cDNAs and NG1- NG3 are normal gingival cDNAs. (+) C is the positive control.

Bioinformatics of LR8

Human LR8 gene is about 12kb long (accession no. AF115384) and contains a 772 base pair long open reading frame. According to the genome browser Ensembl, LR8 gene has 7 RNA splice variants and 2 protein isoforms (Ensembl Gene ID for LR8/TMEM176B - ENSG00000106565). Figure 6.6 shows the exon structure of the splice variants of LR8. In order to mark the splice variants in LR8 sequence, I performed a multiple alignment of the splice variant sequences with 1208 bp LR8 sequence. There is no splicing occurring in the region with PCR products and the only significant splice variant reported within the LR8 mRNA sequence was TMEM176B-003. This sequence is the smallest among all splice variants and a 67 bp fragment was missing in this sequence as compared to the 1208 bp LR8 sequence (between LR8 A↑-A↓ and LR8 B↑-B↓) (Table 6.1). All other splice sites are located upstream to the 1208 bp main sequence. This is the only splice variant encoding the LR8 protein isoform 2 which is 233 a.a. long. LR8 protein isoform 1 is 270 a.a. long.

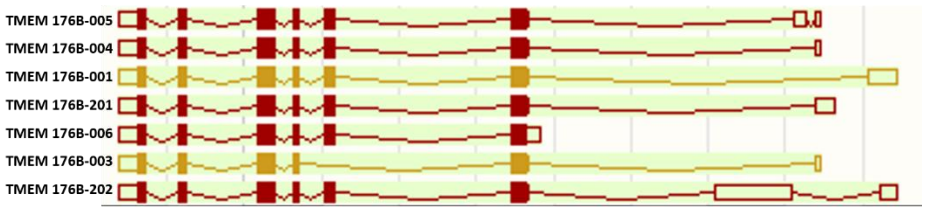

Figure 6.6: Exon structure of the splice variants of TMEM 176B/LR8 gene.

Table 6.5: splice variants of LR8

Splice variant	Length in bp	Size of protein in a.a
TMEM176B-001	1430	270
TMEM176B-003	1006	233
TMEM176B-004	1129	270
TMEM176B-005	1265	270
TMEM176B-006	1206	270
TMEM176B-201	1303	270
TMEM176B-202	2252	270

A search of the NCBI Expressed Sequence Tag database (dbEST) revealed that there were 53 different Expressed Sequence tags (EST) that are matching LR8 cDNA. Of these 19 ESTs were obtained from human tissues and 19 ESTs were from mouse. The human ESTs were cloned from various organs including brain, pancreas, lung carcinomas and fibrothecoma of ovary. The mouse ESTs were from small intestine, pancreas, spleen, salivary gland, thymus and carcinomas of mammary glands. According to Ensembl genome database, there are 34 orthologs of LR8 gene and TMEM 176A gene is considered to be a paralog.

Based on the data available from bioinformatics databases, it was inferred that LR8 gene can be involved in cell differentiation, negative regulation of dendritic cell differentiation (Condamine et al., 2010) and organ morphogenesis. The LR8 protein could be integral to a membrane and may be a component of nuclear membrane.

Discussion

The results from RT-PCR show that there is variability in LR8 expression. LR8 expression varied from tissue to tissue. The evidence for this is the presence of PCR product LR8 C↑-C↓ and A↑-C↓ in liver and its absence in gingiva, indicating that the variant of LR8 present in gingiva is different than liver. The 774 bp product using A↑-C↓ primers was also not detected in gingiva and this was expected because of the absence of PCR product LR8 C↑-C↓. It is also noteworthy that the product of C↑-C↓ has its nucleotide sequence spread across two exons.

The results also show that there is variability between individual patients. These findings were also supported by the findings from qPCR in Specific Aim 1. Variability in LR8 expression from tissue to tissue and among patients could therefore be one reason for the lack of correlation between LR8 and SMA expression. These findings can be useful to design the appropriate primers for analyzing LR8 expression in different tissues. The presence of different variants for LR8 needs to be considered in designing the qPCR assays in future.

The IHC results reported in Chapter V are consistent with the data from Human Protein Atlas generated using anti- LR8 antibody HPA047509.

Variation in LR8 expression among gingival fibroblasts obtained from different patients was also observed in previous studies (unpublished data from Dr. Narayanan's laboratory). This result indicates that presence of variants may be one reason for differences in LR8 expression

among healthy gingiva of different patients. These results also support the notion that LR8 can be a potential marker for distinguishing fibroblasts.

Conclusions

There was considerable variability in LR8 gene expression among the individual human patients in the results from qPCR. Although overexpression of LR8 gene was observed in some of the fibrosis patients, it was not consistent and the results were not statistically significant for normal-fibrosis comparisons. There can be several reasons for this and I examined these possibilities.

One of the reasons for the variability could be the expression of LR8 by fibroblasts as well as by other cells. Immunostaining with anti-LR8 antibody revealed that LR8 is expressed by fibroblasts, but also by smooth muscle cells, endothelial cells, bile duct cells, hepatocytes, alveolar cells and proximal and distal tubule cells within nephrons. Nevertheless, my hypothesis that LR8 producing cells are present in increased numbers in fibrotic tissues and that fibroblasts coexpressing LR8 and SMA may be putative myofibroblasts holds true, if only the fibroblasts are considered. Immunohistochemistry also revealed that there is co-expression of LR8 and SMA in some of the fibroblasts in healthy tissues, but their numbers were higher in fibrotic tissues. This result indicates that the population of fibroblasts expressing both LR8 and SMA expand in fibrosis, and that these cells may differentiate into myofibroblasts.

Another reason for variability in qPCR results could be the differences in LR8 expression pattern. RT-PCR using three sets of primers from different LR8 domains showed tissue specific differences in LR8 expression between human liver and gingiva. I conclude that presence of

different variants, possibly splice variants, could also contribute to the variability in patient to patient and tissue to tissue differences in LR8 expression.

Future studies

Immunostaining results revealed that fibroblasts staining positively for anti-LR8 antibodies and fibroblasts staining positively for both anti-LR8 and anti–SMA antibodies are present in increased numbers in fibrotic human tissues. These results indicate that LR8 positive cells expand in fibrosis and that they may contribute to myofibroblasts in fibrosis. This possibility needs to be further investigated more systematically by examining other fibroses. Further experiments will address the mechanisms by which the fibroblasts are transformed to myofibroblasts; these could be studied using cultured LR8 expressing gingival and lung fibroblasts, which are already available. Another important question is if the "myofibroblasts" in fibrosis are the same as those responsible for granulation tissue formation during would healing; this could be examined by determining LR8 and SMA expression by fibroblasts and myofibroblasts in normal and healing human tissues by methodology described in this thesis.

To further pursue LR8 gene expression in fibroblasts in fibrotic tissues, it would be beneficial to select certain cells using Laser microdissection and then studying LR8 expression in the selected cells. This approach will distinguish fibroblasts from other LR8 expressing cell types. Studying the expression patterns in various cell types might also provide new insights into the regulation of LR8 expression.

In Aim 3, RT-PCR showed that there is presence of a variant of LR8 in gingival tissues. Presence of this variant may explain why LR8 is not expressed in gingival fibroblasts of some patients. This possibility can be verified by examining gingival tissues from a spectrum of

patients with healthy gingiva and with overgrown gingival tissue. The results from these experiments may help to identify patients who respond to drugs by gingival overgrowth. Performing quantitative PCR with different primer pairs might also provide useful information about patient variability in LR8 expression in gingiva. .

So far there is not much information available regarding the function of LR8 gene. Knocking down LR8 gene expression using RNA interference techniques in cultured cells and studying the effects would be interesting. LR8 knock out animal models manifest *ataxia*; these animals should be further studied to determine the effects of LR8 knock out on different organs and cell types in health and in fibrosis.

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