LIVER FIBROSIS; NOVEL FINDINGS ABOUT THE MECHANISM OF EXCESSIVE COLLAGEN SYNTHESIS

BRANKO STEFANOVIC
COM FSU GRAND ROUNDS 2009
PREVALENCE OF LIVER FIBROSIS IN USA

1 IN 679 PEOPLE; 400,000 PEOPLE IN USA

26,050 DEATHS PER YEAR

LEADING CAUSES OF DEATH IN USA IN 2005

1. HEART DISEASES
2. CANCER
3. STROKE
4. RESPIRATORY DISEASES
5. DIABETES
6. ALZHEIMER
7. INFLUENZA AND PNEUMONIA
8. KIDNEY DISEASES
9. LIVER FIBROSIS
MAIN CAUSES OF LIVER FIBROSIS IN USA

NASH SYNDROME: 1 IN 42 PEOPLE; 6.4 MILLION PEOPLE IN USA
HEPATITIS C: 1 IN 68 PEOPLE; 4 MILLION PEOPLE IN USA
ALCOHOLIC LIVER DISEASE: 1 IN 136 PEOPLE; 2 MILLION PEOPLE IN USA
CHEMOCHROMATOSIS: 1 IN 272 PEOPLE; 1 MILLION PEOPLE IN USA
Liver manifestation of the metabolic syndrome
Obesity, diabetes, hypertension, fatty liver
30% of obese people have fatty liver
80% of morbidly obese patients (BMI > 35) have NASH
9 year follow up study: 27% progressed to fibrosis
19% progressed to cirrhosis

NASH = NONALCOHOLIC STEATOHEPATITIS

Nonalcoholic fatty liver disease

- Deposits of fat cause liver enlargement.
- Scar tissue forms. More liver cell injury occurs.
- Scar tissue makes liver hard and unable to work properly.

Fatty liver
Liver fibrosis
Cirrhosis

obesity inflammation

NASH
FIBROSIS IS NOT LIMITED TO THE LIVER

SCLERODERMA: 1 IN 906 PEOPLE; 300,000 PEOPLE IN USA

PULMONARY FIBROSIS: 1 IN 2123; 128,000 PEOPLE IN USA

CARDIOMYOPATHIES: 1 IN 5439 PEOPLE; 50,000 PEOPLE IN USA

KIDNEY FIBROSIS: N/A

SCLERODERMA RESEARCH ORGANIZATION GRANT TO B.S.

AMERICAN HEART ASSOCIATION GRANT TO LE CAI
Hepatic Stellate Cells
UV Retinal
TWO MAJOR PROBLEMS WITH LIVER FIBROSIS

1. EARLY DIAGNOSIS CAN BE DIFFICULT

2. THERE IS NO CURE
EARLY DIAGNOSIS DIFFICULT: SERUM MARKERS OF LIVER FIBROSIS
“Now that the idea that hepatic fibrosis is reversible is taking root, many clinicians are beginning to ask why, if fibrosis is reversible, is there so little progress in the clinical setting, and will patients ever really benefit from antifibrotic therapies? Underlying such questions is a subtle cynicism that the reversibility of fibrosis and cirrhosis has been overhyped.”
WHAT CAN WE DO?

REMOVE THE CAUSE OF FIBROSIS

REVERSE ACTIVATION OF HEPATIC STELLATE CELLS

NEUTRALIZE PROFIBROTIC CYTOKINES (TGFβ)

INHIBIT COLLAGEN SYNTHESIS?
CLASSIC VIEW OF BIOSYNTHESIS OF TYPE I COLLAGEN

PRO 

α1(I) mRNA

α2(I) mRNA

NUCLEUS

α1(I) gene

α2(I) gene

ENDOPLASMIC RETICULUM

proα1

proα2

GalGal

Triple helix

OH

OH

Proteolytic processing

Polymerization

Crosslinking
FSU COM VIEW OF BIOSYNTHESIS OF TYPE I COLLAGEN

NUCLEUS

α1(I) gene

LARP6

α1(I) mRNA

AGGREGATION OF COLLAGEN mRNAs

BINDING TO NONMUSCLE MYOSIN

ENDOPLASMIC RETICULUM

Triple helix

LARP6

α2(I) mRNA

α2(I) gene

LARP6

AGGREGATION OF COLLAGEN mRNAs

BINDING TO NONMUSCLE MYOSIN

DEGRADATION OF LARP6?
COORDINATED TRANSLATION

α1(I) mRNA

α2(I) mRNA

Proα1

Proα2

G1G1

OH

OH

G1G1
5’ STEM-LOOP IS UNIQUE FOR COLLAGEN mRNAs

RNA PROTEIN

PROBE: WT WT A A
+ + EXT EXT

ALPHA 1(I)
ALPHA 2(I)
ALPHA 1(III)

5’-STEM-LOOP IS UNIQUE FOR COLLAGEN mRNAs

WT + EXT

RNA PROBE:

G G     U
GC AUUACGUAGCUA
AU CG AU
U     U
C            C
U               A
G A       G
GCAUAUAUCGGUCGCG
A

5' 3'

ALPHA 1(I)
ALPHA 2(I)
ALPHA 1(III)

G
G
GC
AU
UA
CG
U
A
AU
CG
AU
AU
AU
AU
AU
AU
CG
GU
CG
CG

85 nt

7mG

RNA

*
EXPRESSION CLONING OF 5’ STEM-LOOP BINDING PROTEIN

cDNA library
Amplified in pools of 100 clones
Each pool transfected into mammalian cells
Cell lysates tested for 5’ SL binding by gel mobility shift
One pool identified (pool 110)
Single clone isolated from pool 110
LARP6
LARP6 IS 5' STEM-LOOP BINDING PROTEIN

RNA (LARP6)_2
RNA LARP6
RNA LARP6

EXTRACT: LARP6
CONTROL

COMP: SP 50X SP 250X NS 50X NS 250X
SP 50X SP 250X NS 50X NS 250X

MUTANT: WT A B2 S1 S2
WE HAVE TWO UNIQUE MOLECULES TO WORK WITH

5’ STEM-LOOP RNA
LARP6

MUTATION OF 5’ STEM-LOOP IN ENDOGENOUS COLLAGEN α1(Ⅰ) GENE

INACTIVATION OF LARP6 BY siRNA
Generation of Knock-out Mice

Transfect knock out construct into Embryonic Stem (ES) cells

Select ES cells which have integrated knock out construct into its DNA thru homologous recombination.

(positive and negative selection)

Inject selected ES cells into mouse blastocyst

Reimplant blastocyst into pseudopregnant females

COLLAGEN α1(I) GENE

E1

20 NT MUTATION

I1

SELECTION MARKER

E2

agcagacggg agtttctcct cggggtcgga gcaggaggca cgcggagtgt gaggccacgc
atgagcggac gctaaccccc tccccagcca caaagagtct acatgtctag ggtctagaca
tttcagctt tgtggacctc cggctcctgc tcctcttagc ggccaccgcc ctcctgacgcacggccaaga ggaaggccaa gtcgagggcc aagacgaaga catcccacca atcacctgcg
tacagaacgg cctcaggtac catgaccgag acgtgtggaa acccgagccc tgccggatct
gcgtctgcga caacggcaag gtgttgtgcg atgacgtgat ctgtgacgag accaagaact
cgccggcgc cgaagtcccc gagggcgagt gctgtcccgt ctgccccgac ggctcagagt
cacccaccga ccaagaaacc accggcgtcg agggacccaa gggagacact ggcccccgag
gcccaaggg acccgcaggg ccccctggcc gagatggcat ccctggacag cctggacttc
cggaccccc cggaccggg cggacctggcc gagatggcat cctggacag cctggacttc
agcagacggg agtttctcct cggggtcgga gcaggaggca cgcggagtgt gaggccacgc
atgagcggac gctaaccccc tccccagcca caaagagtct acatgtctag ggtctagaca
tgttcagctt tgtggacctc cggctcctgc tcctcttagc ggccaccgcc ctcctgacgcacggccaaga ggaaggccaa gtcgagggcc aagacgaaga catcccacca atcacctgcg
tacagaacgg cctcaggtac catgaccgag acgtgtggaa acccgagccc tgccggatct
gcgtctgcga caacggcaag gtgttgtgcg atgacgtgat ctgtgacgag accaagaact
cgccggcgc cgaagtcccc gagggcgagt gctgtcccgt ctgccccgac ggctcagagt
cacccaccga ccaagaaacc accggcgtcg agggacccaa gggagacact ggcccccgag
gcccaaggg acccgcaggg ccccctggcc gagatggcat ccctggacag cctggacttc
cggaccccc cggaccggg cggacctggcc gagatggcat cctggacag cctggacttc
KNOCK-IN MICE ARE DEFICIENT IN SYNTHESIS OF TYPE I COLLAGEN

**STEADY-STATE**

- Wt
- Wt
- M
- M

Procollagen type I

A-tubulin

**RATE OF SECRETION**

- Wt
- M

Procollagen

Time (h):

1 2 3 1 2 3
KNOCK DOWN OF LARP6 DECREASES COLLAGEN SYNTHESIS

CELLULAR

FIB

LARP6 siRNA      CON siRNA

MEDIUM

COL

FIB

LARP6 siRNA      CON siRNA

RNA/PROTEIN

RNA

LARP6
siRNA      CON siRNA
TWO KEY STEPS IN COLLAGEN SYNTHESIS ARE MEDIATED BY LARP6

1. AGGREGATION OF COLLAGEN mRNAs

2. ASSOCIATION WITH NONMUSCLE MYOSIN
LARP6 INTERACTS WITH ITSELF

5’ STEM-LOOP BINDING
+
+
-

PROTEIN:

IP: HA-LARP6
- RNase

IP: HA-LARP6
+ RNase
EXTRACT STREPTAVIDIN
PULL DOWN

COL1A2
LARP6

COL1A1
LARP6

COL1A2
LARP6

LARP6

1             2            3
1             2            3

COL1A1
COL1A2

ACTIN

PROTEIN: - LARP6 CON

PROTEIN: - ΔC LARP6 CON
FORMATION OF DISCRETE GRANULES CONTAINING COLLAGEN mRNAs

SUCROSE DENSITY

CONTROL

LARP6

DENSITY: 1.146 \( \text{G/cm}^3 \)  1.117 \( \text{G/cm}^3 \)
LARP6 INTERACTS WITH NONMUSCLE MYOSIN

FS

1 85 183 296 491
N-TERM LA RBD C-TERM

XCM

1 300

5’ STEM-LOOP BINDING

LARP6 INTERACTS WITH NONMUSCLE MYOSIN

MYO IIB

1 2 3

HA

IP: LARP6 ΔC-LARP6 CON

MYO IIA

1 2

IP: LARP6 ΔC-LARP6
NONMUSCLE MYOSIN IS UPREGULATED IN ACTIVATED HSCs

DAYS IN CULTURE: 4 5 6 7 8

- NONMUSCLE MYOSIN IIB
- COLLAGEN
- TUBULIN
MYOSIN FILAMENTS ARE NECESSARY FOR COLLAGEN EXPRESSION
QUIESCENT VS ACTIVATED HSCS

ACTIVATED HSCS

QUIESCENT HSCS

ML7

SHORT HALF-LIFE OF mRNAs

Triplet helix

GalGal

OH

proα1

proα1

proα2

OH
FOCAL COLLAGEN SYNTHESIS
CONCLUSIONS

TWO KEY ASPECTS OF COLLAGEN SYNTHESIS ARE REGULATED BY LARP6

INHIBITION OF LARP6 BINDING TO 5’ STEM-LOOP = LESS COLLAGEN

LARP6 AS TARGET FOR ANTIFIBROTIC THERAPY

DEVELOPMENT OF SCREENING SYSTEM FOR SMALL COMPOUNDS TARGETING LARP6
Figure 1. Truth survival in original articles and meta-analyses on hepatitis and cirrhosis.
ACKNOWLEDGMENTS

LE CAI
LELA STEFANOVIC
DILLON FRITZ
AZARIYAS CHALLA
BRANKO STEFANOVIC

RUTH DIDIER: CONFOCAL IMAGING FACILITY
DORIS TERRY: PROTEOMICS FACILITY