Uncovering the mechanisms of wound healing and fibrosis
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**Background: process of wound healing**

Wound healing — a 4-step process

**Step 1: Hemostasis (clotting)**
- Vasoconstriction
- Coagulation cascade is initiated by interaction of coagulation factor FVII with TF
- Von Willebrand factor helps platelets bind at wound sites, where they activate and degranulate

**Step 2: Inflammation**
- Enhanced blood vessel permeability due to release of histamine and other factors
- Inflammatory cells infiltrate (PMN, macrophages) and kill any microbes accompanying the injury
- Leukocytes produce cytokines, chemokines, and growth factors, some of which (IL-1beta, TGF-beta, TNF) recruit fibroblasts

**Step 3: Proliferative phase**
- Fibroblasts are recruited and activated, and secrete ECM components (type III collagen, fibronectin)
- Formation of granulation tissue (fibroblasts, inflammatory cells, new blood vessels, fibronectin, hyaluronan, collagen, endothelial cells)
- Epithelialization

**Step 4: Wound closing/tissue remodeling**
- Wound contraction via myofibroblasts at the edges
- Type III collagen is replaced by Type I, and fibers are rearranged and crosslinked.
- Remodeling will continue for weeks to months.
Background: Key components of wound healing/fibrosis

**Cells/cell fragments:**
- Epithelial and endothelial cells
- Platelets
- Fibroblasts / Myofibroblasts
- Inflammatory cells (macrophages, neutrophils)

**Proteins:**
- ECM components (collagens, fibronectin, etc.)
- Proteases (MMPs, collagenase)
- Growth factors (TGF-beta, PDGF)
- Inflammatory mediators (histamine)
- Cytokines (TNF-alpha, IL-1-beta)
- Intracellular signaling pathways (NFkappaB, JAK/STAT, more)
Background: wound healing and fibrosis

Uncontrolled wound healing response results in fibrosis

Fibrosis develops if any stage in the tissue repair program is dysregulated.

- Chronic inflammation
- The tissue-damaging agent is not removed
- The repair process is not regulated properly

*Review, Integrating mechanisms of pulmonary fibrosis. JEM Vol. 208, No. 7*
Fibrosis affects a wide range of tissues, including:

- Lungs (idiopathic pulmonary fibrosis, cystic fibrosis)
- Heart (post-myocardial infarction, endomyocardial fibrosis / hypereosinophilic syndrome)
- Kidney (renal fibrosis)
- Liver (cirrhosis)
- Skin (Scleroderma, nephrogenic systemic fibrosis)
- Joints (arthrofibrosis)
- Bone marrow (myelofibrosis)
Background: Fibrosis and diseases

Heart fibrosis
- Can occur in response to left ventricular pressure-overload, as “reactive interstitial fibrosis”, which leads to cardiac hypertrophy and necrosis
- Alternatively, “replacement fibrosis” could occur in response to myocardial infarction, inflammation, and myocyte death.
- Excess serotonin or serotonin receptor agonist drugs

Systemic scleroderma - skin
- Autoimmune disease of the skin and, in some cases, the internal organs
- Arteriole endothelial and smooth muscle cell apoptosis, followed by inflammation and fibrosis

Liver cirrhosis
- Develops as a result of chronic liver disease (alcoholic, fatty liver disease, autoimmune disease, or hepatitis virus, etc.), or idiopathic

Renal fibrosis
- End result of most kinds of CKD, leading to end-stage kidney failure
- Myofibroblast accumulation, increased matrix deposition, tubular cell death, loss of peritubular and glomerular capillaries
Background: Fibrosis and diseases

**Pulmonary fibrosis**

- Idiopathic (possibly linked to surfactant protein C mutation), or as a result of injury or disease, including:
  - Inhalation of particulates and gases
  - Smoking
  - Infections
  - Drugs (bleomycin, amiodarone, etc.)
- Involvement by inflammatory mediators such as TNF, IL-beta, and IL-17, has been implicated, as well as Th2 cytokines such as IL-13 and IL-4

*Review, Integrating mechanisms of pulmonary fibrosis. JEM Vol. 208, No. 7*
Pulmonary fibrosis: role of T helper cells and macrophages

Review, Integrating mechanisms of pulmonary fibrosis. JEM Vol. 208, No. 7
A few key open questions

- To what extent are immune cells and factors involved in driving fibrosis? Can they be effective therapeutic targets?

- What are the cellular origins of myofibroblasts in various fibrotic settings? Is **epithelial to mesenchymal transition** a significant source of myofibroblasts?

- What are the root causes of idiopathic fibroses? Can we identify further genetic or environmental causes, in addition to those already identified?

- What role might the **microbiome** play in either disrupting a normal wound healing response and causing fibrosis, or in promoting normal wound healing?

- Can we identify noninvasive biomarkers for liver fibrosis that might help catch the disease early and stratify patients for improved treatment?

- Is reversal of fibrosis feasible?
Researching the causes of fibrosis

4 recent case studies

<table>
<thead>
<tr>
<th>Scleroderma research</th>
<th>Cardiac fibrosis</th>
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RT² Profiler PCR Arrays

Pathway-focused gene expression profiling

- 84 or 370 real-time PCR assays for pathway-related genes
- Gene lists chosen by our experts via bioinformatics and text mining
- Wet-lab tested of each assay ensures specificity and sensitivity
- 170+ pathways available, many for fibrosis-related processes
- Integrated controls for normalization, reverse transcription, genomic DNA contamination, and the PCR process, plus free data analysis tools

Pathways for fibrosis / wound healing:

- **Fibrosis** (human, mouse, rat, rabbit)
- **Wound Healing** (human, mouse, rat, pig, rabbit)
- **ECM & Adhesion Molecules** (human, mouse, rat, pig)

Additional related pathways:

- Common Cytokines
- Inflammatory Cytokines & Receptors
- TGF-beta Signaling Pathway
- Endothelial Cell Biology
- Epithelial-to-Mesenchymal Transition

Get the full list at [www.qiagen.com/search/rt2-profiler-pcr-arrays](http://www.qiagen.com/search/rt2-profiler-pcr-arrays)
An application example using RT² Profiler PCR Arrays

Cytokine expression changes in human PBMC on PMA/ionomycin treatment.

Plot and Chart Format:
- Heat Map
- Scatter Plot
- Volcano Plot
- Clustergram
- Multigroup Plot
### 4 recent case studies

#### Scleroderma research

- Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts. Nakashima, T. et al. (2012) *Journal of Immunology*

#### Cardiac fibrosis

- Cardiac-specific deletion of SOCS-3 prevents development of left ventricular remodeling after acute myocardial infarction. Oba, T. et al. (2012) *Journal of the American College of Cardiology*

#### Idiopathic pulmonary fibrosis

- Severe lung fibrosis requires an invasive fibroblast phenotype regulated by hyaluronan and CD44. Li, Y. et al. (2012) *Journal of Experimental Medicine*

#### Cardiac fibrosis (2)

Application 1: Fibroblast collagen production and IL-17

Background and research question

- Fibroblasts from SSc patients show intrinsic TGF-beta1 activation, and other cytokines are also implicated in disease progression.

- Previous studies yielded conflicting reports on the association of IL-17 with SSc, and the authors sought to clarify its involvement.

- Are IL-17A&F and IL-17RA expressed differently in SSc vs. healthy subjects?

- Is IL-17 involved in regulating ECM during SSc?

Application 1: Fibroblast collagen production and IL-17

Approach

- Cytokines and receptors were measured in serum, fibroblast cultures, and tissue samples by ELISA, immunoblotting, and immunohistochemistry.

- An RT² Profiler PCR Array for Human Extracellular Matrix and Adhesion Molecules was used to profile ECM gene expression.

- An RT² miRNA PCR Array was used to profile miRNA expression.

- siRNA against TGF-beta1, Smad3, and IL-17RA were used to assess the effects of TGF-beta signaling on IL-17 receptor expression and IL-17 signaling on miR-129-5p expression, respectively.
IL-17A levels were higher in sera and involved skin of SSc patients, and IL-17RA was lower at both the protein and mRNA level in cultured SSc fibroblasts. This was rescued by siRNA for TGF-beta1 or Smad knockdown.

IL-17A treatment caused fibroblast downregulation of pro-fibrotic CTGF (RT² Profiler PCR Array data). Alpha1(I) collagen gene expression wasn’t significantly different, but protein was decreased (immunoblot).

miR-129-5p (among others) was downregulated in SSc fibroblasts (RT² miRNA PCR Array data).

In conclusion:
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Application 2: SOCS3 and myocardial infarction

Rationale and research question

- Left ventricular remodeling after acute myocardial infarction (AMI), including fibrosis, contributes to heart failure.

- Previous work had shown that cytokines activating the JAK/STAT pathways could prevent LV remodeling in animal models after AMI.

SOCS3 acts in a negative feedback loop induced by JAK/STAT-activating cytokines – could inhibition of SOCS3 prevent LV remodeling?

Application 2: SOCS pathway
Application 2: SOCS3 and myocardial infarction

Approach

- Made cardiac-specific SOCS3 knockout mice, induced AMI, and observed LV remodeling in knockouts vs wild-types
- Performed western blot analysis, TUNEL staining for apoptosis, echocardiograph, and real-time PCR
- Used Mouse Common Cytokines RT2 Profiler PCR Array to profile cytokines in the system

Application 2: SOCS3 and LV remodeling

Major findings

- Survival was enhanced in SOCS3 knockouts after AMI induced by coronary ligation – 100% survived to 14 days, compared to 55% of controls

- LV remodeling was diminished in knockouts, as was apoptosis

- Multiple JAK-STAT-activating cytokines were expressed following AMI, and many were diminished in SOCS3 knockouts (including G-CSF, IL-11, and IL-6) (RT² Profiler PCR Array data)

- Western blot showed greater activation of STAT3, AKT, and ERK pathways in knockouts

- Mallory-AZAN staining showed smaller fibrotic areas in knockout hearts, and MMPs, TGF-beta2, and collagen showed lower expression as well

Conclusions

Cardiomyocyte SOCS3 may drive fibrosis development/LV remodeling following AMI, and may be a useful therapeutic target.

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HA accumulates in the lung after bleomycin injury, and CD44 has been shown to play a role in fibroblast recruitment after injury.

HA and CD44 are implicated in tumor metastasis, and invasiveness of metastatic cells could be compared to the ECM-invasive ability of fibroblasts and myofibroblasts in IPF.

Are HA and CD44 involved in the invasiveness of fibrotic fibroblasts in IPF?
Application 3: Hyaluronan, CD44, and IPF

Approach

- Cells/mice:
  - murine myofibroblasts (alpha-smooth-muscle-actin-expressing) expressing human HAS2 (a hyaluronan synthase)
  - HAS2 knockouts (fibroblasts)
  - CD44 null mice (or mice treated with anti-CD44)
  - Cells from patients with IPF (+/- HAS2 siRNA or anti-CD44)
- Measured HA, collagen, and/or fibroblast accumulation after bleomycin challenge.
- Used a Matrigel assay with fibroblasts to assess invasiveness.
- Compared gene expression with the RT² Profiler PCR Array for Extracellular Matrix & Adhesion Molecules
hHAS2 expression was linked to greater fibrosis, and Has2 knockout in mesenchymal cells or fibroblasts, to less.

HAS2 expression also drove invasiveness in Matrigel experiments and vice versa.

CD44 is upregulated after bleomycin treatment, particularly in ASMA-HAS2 mice, and anti-CD44 antibodies block collagen accumulation. Fibroblasts from CD44-null mice also showed diminished invasive capacity.

Fibroblasts from IPF patients were more invasive in Matrigel and showed higher HAS2 mRNA. siRNA knockdown of HAS2 inhibited invasiveness, as did anti-CD44 antibody treatment.

Murine HAS2-expressing invasive fibroblasts showed upregulation of HAS2, CD44, and MMPs, and downregulation of TIMP1 and TIMP3. IPF patient invasive fibroblasts showed MMP9 upregulation, which was diminished by siRNA knockdown of HAS2 (along with CD44).

Conclusions: HAS2 drives invasiveness of fibroblasts in fibrosis, possibly through control of MMP and CD44 expression.

Major findings
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Application 4: MT1-MMP, LV remodeling, and fibrosis

Research question

- Myocardial fibrosis develops during chronic pressure-overload (PO), which causes LV hypertrophy

- Membrane type I MMP (MT1-MMP) is implicated in fibrosis development, and its transcription is enhanced by mechanical forces

Could mechanical forces from chronic PO increase MT1-MMP expression and fibrosis?

Application 4: MT1-MMP, LV remodeling, and fibrosis

Approach

- Developed an MT1-MMP promoter reporter mouse and used transverse aortic constriction to model PO
- Used a Custom RT² Profiler PCR Array for MT1-MMP, procollagen type I, CTGF, TGF-betaR1, and other fibrosis-related genes in myocardial samples
- Measured LV by echocardiography and collagen by light microscopy
- Isolated papillary muscles from reporter mice and subjected to stimulation, then observed expression of MT1-MMP as well as transcription factors
Major findings

- PO led to LV hypertrophy and collagen volume fraction increase.

- MT1-MMP protein abundance increased over the course of 4 weeks after PO, and MT1-MMP promoter activity increased at 1 and 4 weeks, with a dip at week 2.

- Various profibrotic genes showed increases one week after PO, including the TGF-beta receptor, collagens, serine protease inhibitors, LTBP, and CTGF (RT² Profiler PCR Array data)

- Increases in mechanical load led to strong increases in MT1-MMP expression in isolated papillary muscles, as well as expression of transcription factors including NFKappB, RELA, and c-Fos.

Conclusions

Mechanical forces during PO may activate MT1-MMP transcription via NFKappB or c-Fos, exacerbating fibrosis through TGF-beta signaling. The temporal associations in this study suggest that further research into MT1-MMP as a driver of LV remodeling is warranted.
RT² Profiler PCR Arrays for fibrosis research

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PCR arrays of any pathway
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• Required Reagents
  • RT² First-Strand cDNA Synthesis Kit
  • RT² SYBR Green Mastermix (2-Pack)

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