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.
Backgrounds: 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
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)
Fibrosis affects a wide range of tissues, including:

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

Cardiac 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.

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
- Cause is unknown, but skin fibrosis can be treated

Liver cirrhosis

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

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

Used RT² Profiler PCR Array for Human Extracellular Matrix and Adhesion Molecules

SOCS3 and myocardial infarction

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

Used RT² Profiler PCR Array for Mouse Common Cytokines

Matrix metalloproteinases and LV remodeling


Used a Custom RT2 Profiler PCR Array.
RT² Profiler PCR Arrays

Pathway-focused gene expression profiling

- 84 (or 370) real-time PCR assays for genes related to specific pathways
- Gene lists chosen by our experts – bioinformatics and text mining
- Each assay is wet-lab tested for specificity and sensitivity
- More than 140 pathways available, including many for fibrosis-related processes
- Integrated controls for normalization, reverse transcription, genomic DNA contamination, and the PCR process, plus free data analysis tools

PCR Array Overview, Nov 15, 9:30am
https://www2.gotomeeting.com/register/263258378
RT² Profiler PCR Arrays for fibrosis and wound healing

Fibrosis (for Human, Rat, Mouse, Rabbit, and more)

Pro-Fibrotic: ACTA2 (a-SMA), AGT, CCL11 (Eotaxin), CCL2 (MCP-1), CCL3 (MIP-1a), CTGF, GREM1, IL13, IL13RA2, IL4, IL5, SNAI1 (Snail).

Anti-Fibrotic: BMP7, HGF, IFNG, IL10, IL13RA2.

Extracellular Matrix & Cell Adhesion:
ECM Components: COL1A2, COL3A1.
Remodeling Enzymes: LOX, MMP1 (Collagenase 1), MMP13, MMP14, MMP2 (Gelatinase A), MMP3, MMP8, MMP9 (Gelatinase B), PLAT (TPA), PLAU (uPA), PLG, SERPINA1 (a1-antitrypsin), SERPINE1 (PAI-1), SERPINH1, TIMP1, TIMP2, TIMP3, TIMP4.
Cellular Adhesion: ITGA1, ITGA2, ITGA3, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8.

Inflammatory Cytokines & Chemokines: CCL11 (Eotaxin), CCL2 (MCP-1), CCL3 (MIP-1a), CCR2, CXCR4, IFNG, IL10, IL13, IL13RA2, IL1A, IL1B, IL4, IL5, ILK, TNF.

Growth Factors: AGT, CTGF, EDN1, EGF, HGF, PDGFA, PDGFB, VEGFA.

Signal Transduction:
TGFB Superfamily: BMP7, CAV1, DCN, ENG (EVI-1), GREM1, INHBE, LTBP1, SMAD2, SMAD3, SMAD4, SMAD6, SMAD7, TGFβ1, TGFβ2, TGFβ3, TGFβ1 (ALK5), TGFβ2, TGFβ3, THBS1, THBS2
Transcription Factors: CEBPB, JUN, MYC, NFKB1, SP1, STAT1, STAT6

Epithelial-to-Mesenchymal Transition: AKT1, BMP7, COL1A2, COL3A1, ILK, ITGAV, ITGB1, MMP2 (Gelatinase A), MMP3, MMP9, SERPINE1 (PAI-1), SMAD2, SNAI1 (Snail), TGFβ1, TGFβ2, TGFβ3, TIMP1.

Others: BCL2, FASLG (TNFSF6).
RT² Profiler PCR Arrays for fibrosis and wound healing

Wound healing (for Human, Mouse, Rat, Pig, Rabbit, and more)
http://sabiosciences.com/rt_pcr_product/HTML/PAHS-121Z.html

Extracellular Matrix & Cell Adhesion:
ECM Components: COL1A1, COL1A2, COL2A1, COL3A1, COL4A1, COL4A3, COL5A1, COL5A2, COL5A3, VTN.
Remodeling Enzymes: CTSG, CTSS, CTSL2, F13A1, F3 (Tissue Factor), FGA (Fibrinogen), MMP1, MMP2, MMP7,
MMP9, PLAT (tPA), PLAU (uPA), PLAUR (uPAR), PLG, SERPINE1 (PAI-1), TIMP1.
Cellular Adhesion: CDH1 (E-cadherin), ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGAV, ITGB1, ITGB3,
ITGB5, ITGB6.
Cytoskeleton: ACTA2 (a-SMA), ACTC1, RAC1, RHOA, TAGLN.

Inflammatory Cytokines & Chemokines: CCL2 (MCP-1), CCL7 (MCP-3), CD40LG (TNFSF5), CXCL1, CXCL11 (ITAC/IP-9), CXCL2, CXCL5 (ENA-78/LIX), IFNG, IL10, IL1B, IL2, IL4, IL6.

Growth Factors: ANGPT1, CSF2 (GM-CSF), CSF3 (GCSF), CTGF, EGF, FGF10, FGF2, FGF7, HBEGF (DTR),
HGF, IGF1, MIF, PDGFA, TGFA, TGFβ1, TGFβ3, VEGFA.

Signal Transduction:
TGFβ: TGFβ1, TGFβR3, STAT3.
WNT: CTNNB1, WISP1, WNT5A.
Phosphorylation: MAPK1 (ERK2), MAPK3 (ERK1), PTEN.
Receptors: EGFR, IL6ST (GP130).
Other: PTGS2.
RT² Profiler PCR Arrays for fibrosis and wound healing

ECM & Adhesion Molecules (for Human, Mouse, Rat)
RT² Profiler PCR Arrays for fibrosis and wound healing

Additional pathways available

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

For complete list, see http://www.sabiosciences.com/ArrayList.php

Other species and custom array

<table>
<thead>
<tr>
<th>Human (Homo sapiens)</th>
<th>Cow (Bos taurus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Mus musculus)</td>
<td>Chicken (Gallus gallus)</td>
</tr>
<tr>
<td>Rat (Rattus norvegicus)</td>
<td>Horse (Equus ferus caballus)</td>
</tr>
<tr>
<td>Fruitfly (Drosophila melanogaster)</td>
<td>Zebrafish (Danio rerio)</td>
</tr>
<tr>
<td>Dog (Canis lupus familiaris)</td>
<td>Chinese Hamster: CHO (Cricetulus griseus)</td>
</tr>
<tr>
<td>Pig (Sus scrofa)</td>
<td>Rabbit (Oryctolagus cuniculus)</td>
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<tr>
<td>Rhesus macaque (Macaca mulatta)*</td>
<td></td>
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<tr>
<td>*Compatible with: Crab-eating macaque (Macaca fascicularis) (Cynomolgus monkey)</td>
<td></td>
</tr>
</tbody>
</table>
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
## Researching the causes of fibrosis

### Scleroderma and cytokines

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

Used RT² Profiler PCR Array for Human Extracellular Matrix and Adhesion Molecules

### SOCS3 and myocardial infarction

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Used RT² Profiler PCR Array for Mouse Common Cytokines

### Matrix metalloproteinases and LV remodeling


Used a Custom RT2 Profiler PCR Array.
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 fibroblasts. This was rescued by siRNA for TGF-beta1 or Smad knockdown.

The RT²Profiler PCR Array showed that IL-17A treatment caused downregulation of pro-fibrotic CTGF. Alpha1(I) collagen expression remained the same, but was lower by immunoblotting.

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

Application 2: SOCS3 and myocardial infarction

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**Matrix metalloproteinases and LV remodeling**


Used a Custom RT2 Profiler PCR Array.
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: 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.

- RT² Profiler PCR Array showed expression of multiple JAK-STAT-activating cytokines following AMI, and many were diminished in SOCS3 knockouts (including G-CSF, IL-11, and IL-6).

- 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.

### Application 3: MT1-MMP, LV remodeling, and fibrosis

#### Fibrosis research with RT² Profiler PCR Arrays

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| **SOCS3 and myocardial infarction** | 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 |


#### Used RT² Profiler PCR Arrays
- **Scleroderma and cytokines**: Used RT² Profiler PCR Array for Human Extracellular Matrix and Adhesion Molecules
- **SOCS3 and myocardial infarction**: Used RT² Profiler PCR Array for Mouse Common Cytokines
- **Matrix metalloproteinases and LV remodeling**: Used a Custom RT2 Profiler PCR Array.
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 3: 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 profibrotic 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

Application 3: MT1-MMP, LV remodeling, and fibrosis

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.

- The RT² Profiler PCR Array showed increases in various profibrotic genes, including the TGF-beta receptor, collagens, serine protease inhibitors, LTBP, and CTGF, one week following PO.

- 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 NFkappaB 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

Interested in trying RT² Profiler PCR Arrays?

Visit us at www.sabiosciences.com/rt_pcr_product to learn more!

Call 1-888-503-3187 for more information

Email: support@SABiosciences.com (US & Canada)
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