Lentiviral vector-mediated gene transfer studies in animal models of arthritis: an application of RNAi in pre-clinical study

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Department of Internal Medicine
National Cheng Kung University Medical College
Multiple pathogenic mechanisms in rheumatoid arthritis (RA)

Gary S. Firestein, M.D.
Professor of Medicine
Dean and Associate Vice Chancellor of Translational Medicine
Director, Clinical and Translational Research Institute, UCSD
School of Medicine

Figure 3 A proposed model implicating multiple pathogenic mechanisms in RA. According to this model, a step-wise progression can begin with the activation of innate immunity by stimulating dendritic cells, macrophages, fibroblasts, and mast cells. After immune cells migrate into the synovium, an opportunity for adaptive immune responses arises in individuals with the appropriate genetic background. While antigen presentation can occur in the synovium, extra-articular sites can also participate if dendritic cells migrate to lymph nodes and bias T cells to a Th1 phenotype. In the destructive phase of disease, osteoclast activation mediates abundant bone resorption under the influence of RANKL, while synoviocytes can invade cartilage. These processes are not necessarily mutually exclusive. Activation of innate and adaptive immunity can also occur in a parallel fashion (denoted by bidirectional arrows), perhaps contributing to the patterns of disease flares and remissions. DC, dendritic cell; CCP, cyclic citrullinated peptide; FLS, fibroblast-like synoviocyte; Mo, macrophage.

Firestein 2007 *Science*; 315, 952-953

Firestein 2003; *Nature* 423, 356-361
Pathogenesis of RA, Harrison’s on line, 18th eds

Current Biological Agents Approved by FDA

TNF-alpha inhibitors: Infliximab, Etanercept, Adalimumab, Golimumab, Certolizumab
IL-1 antagonist: Anakinra
IL-6 antagonist: Tocilizumab
T cells blockade: Abatacept
B cells depletion: Rituximab

Current Targeted Agents Approved by FDA

Janus kinase inhibitor: Tofacitinib

Signaling Molecules and Transcription Factors in RA

<table>
<thead>
<tr>
<th>JAK</th>
<th>Tyrosine kinase that regulates cytokine-mediated leukocyte maturation and activation, cytokine production, and immunoglobulin production</th>
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</thead>
<tbody>
<tr>
<td>Syk</td>
<td>Tyrosine kinase that regulates immune-complex-mediated and antigen-mediated activation of B and T cells and other Fc receptor-bearing leukocytes</td>
</tr>
<tr>
<td>PI3K</td>
<td>Mediates signals that drive proliferation and cell survival</td>
</tr>
<tr>
<td>BTK</td>
<td>Plays important role in the activation of B cells, macrophages, mast cells, and neutrophils, through regulation of B-cell receptor and Fc receptor signaling as appropriate</td>
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<tr>
<td>NFκB</td>
<td>Helps integrate inflammatory signaling and is important for cell survival</td>
</tr>
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</table>

Evolving concepts of rheumatoid arthritis

RA as a locally invasive tumor

Nature 2003;423:356-61

Gary S, Firestein
Division of Rheumatology
UCSD School of Medicine
Origins of RA

• The earliest documentation of RA can be found in the paintings of the Flemish artist Peter Paul Rubens.

_The Holy Family with St. Anne (1630)_
Should we recognize a new type of gout to be called primary asthenic gout

- The sex distribution was predominantly female;
- Those affected were indigent and asthenic, rather than wealthy and robust;
- Several joints rather than a single joint were involved;
- Though the pains were less severe than gout, the attacks lasted much longer.

Augustin Jacob Landré-Beauvais (1772-1840)
He named it ‘rheumatoid’ arthritis to distinguish it from the two well-known forms arthritis, rheumatic fever and gout.

Sir Alfred Baring Garrod (1819–1907)
## Gene therapy in animal models of RA

<table>
<thead>
<tr>
<th>Adenovirus</th>
<th>AAV</th>
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<tbody>
<tr>
<td>Genes demonstrated to successfully treat arthritis in various animal models</td>
<td>IL-1ra, sTNFR-Ig, sTNFRI, sTNFR:Fc</td>
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<tr>
<td>IL-1ra, sTNFRI-Ig fusion protein, sIL-1RacP IL-18-binding protein, IL-13, IL-4, vIL-10, CTLA4-Ig, TRAIL, Csk, IFN-β, p16INK4A, P21CIP1, prothymosin-α, VEGF receptor I, Tie2 soluble receptor, FADD, FasL, SOCS3, urokinase plasminogen inhibitor, TIMP-1, TIMP-3, Thrombospondin-1, dominant negative NFκB inhibitor</td>
<td>sTNFRI, sTNFR:Fc fusion protein, IL-4, IL-10, angiotatin, IKKβ</td>
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<table>
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<th>Retrovirus</th>
<th>Lentivirus</th>
<th>Plasmid DNA</th>
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<td>IL-1ra, sTNFRI variants, IL-4, TGF-β, angiotatin, soluble complement receptor I, superoxide dismutase, catalase</td>
<td>Angiostatin, endostatin</td>
<td>IL-1ra, sTNFRI receptor variants, sTNFR:Fc fusion protein, sTNFRII, TGF-β, IL-4, IL-10, vIL-10, soluble complement receptor I, TIMP-4, fibronectin peptide, sIL-1RacP</td>
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ICT: Modified by RA

*Mod. Rheumatol.* 2008; 18, 2-14
Animal models of RA
(Collagen-induced arthritis, CIA)

Sprague-Dawley Rat intradermal injection with bovine type II collagen in adjuvant

Trentham DE, et al.

Prophylactic Protocol
Treatment
Control

Therapeutic Protocol
Treatment
Control

Day 0
7
10~12
18~23
Sacrifice

First immunization
Second boost

Intra-articular injection

Ankle Circumference

0 = slight swelling and / or erythema
1 = low to moderate edema
2 = pronounced edema with limited joint usage
3 = excess edema with joint rigidity
4 = excess edema with joint rigidity

Articular Index

2\pi(\sqrt{a^2+b^2/2})

a: latero-lateral diameter
b: antero-posterior diameter

Trentham DE, et al.
CIA Induction in C57BL/6 Mice

Day 0

Day 14

Immunization with type II collagen
2 mg/ml chicken collagen + CFA 100 µl/mouse in tail base

Boost
2 mg/ml collagen + IFA100 µl/mouse

Scoring

Day 0

Day 21

Immunization with type II collagen
2 mg/ml bovine collagen + CFA 100 µl/mouse in tail base

Boost
2 mg/ml collagen + IFA100 µl/mouse

0 No evidence of erythema and swelling
1 Slight swelling and/or erythema of fingers
2 Pronounced edematous swelling
3 Joint rigidity with edematous swelling or ankylosis

# Characteristics of various gene delivery viral vectors

<table>
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<tr>
<th>Vector</th>
<th>Genetic material</th>
<th>Packaging capacity</th>
<th>Tropism</th>
<th>Inflammatory potential</th>
<th>Vector genome forms</th>
<th>Main limitations</th>
<th>Main advantages</th>
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<tr>
<td>Retrovirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Dividing cells only</td>
<td>Low</td>
<td>Integrated</td>
<td>Only transduces dividing cells; integration might induce oncogenesis in some applications</td>
<td>Persistent gene transfer in dividing cells</td>
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<tr>
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<td>RNA</td>
<td>8 kb</td>
<td>Broad</td>
<td>Low</td>
<td>Integrated</td>
<td>Integration might induce oncogenesis in some applications</td>
<td>Persistent gene transfer in most tissues</td>
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<td>HSV-1</td>
<td>dsDNA</td>
<td>40 kb(^+) 150 kb(^\ddagger)</td>
<td>Strong for neurons</td>
<td>High</td>
<td>Episomal</td>
<td>Inflammatory; transient transgene expression in cells other than neurons</td>
<td>Large packaging capacity; strong tropism for neurons</td>
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<tr>
<td><strong>Non-enveloped</strong></td>
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<tr>
<td>AAV</td>
<td>ssDNA</td>
<td>&lt;5 kb</td>
<td>Broad, with the possible exception of haematopoietic cells</td>
<td>Low</td>
<td>Episomal (&gt;90%) Integrated (&lt;10%)</td>
<td>Small packaging capacity</td>
<td>Non-inflammatory; non-pathogenic</td>
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<tr>
<td>Adenovirus</td>
<td>dsDNA</td>
<td>8 kb(^\star) 30 kb(^$)</td>
<td>Broad</td>
<td>High</td>
<td>Episomal</td>
<td>Capsid mediates a potent inflammatory response</td>
<td>Extremely efficient transduction of most tissues</td>
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Intra-articular gene delivery of lentiviral vectors into the knee joints of rats

VSV-G pseudotyped, HIV-based LVs

**TABLE 1:** Luciferase activity after intraarticular injection of a lentiviral vector encoding the firefly luciferase

<table>
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<th>Days post-injection</th>
<th>Knee</th>
<th>Liver</th>
<th>Lung</th>
<th>Heart</th>
<th>Spleen</th>
<th>Gonad</th>
<th>Plasma</th>
<th>WBC a</th>
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<td>2</td>
<td>$9.2 \times 10^5 \pm 11.2 \times 10^5$</td>
<td>$310 \pm 327$</td>
<td>$127 \pm 92$</td>
<td>$4 \pm 2$</td>
<td>$19 \pm 5$</td>
<td>$95 \pm 65$</td>
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<tr>
<td>5</td>
<td>$6.6 \times 10^5 \pm 1.7 \times 10^5$</td>
<td>$14 \pm 13$</td>
<td>$3 \pm 8$</td>
<td>$&lt; 1$</td>
<td>$144 \pm 133$</td>
<td>$93 \pm 73$</td>
<td>$&lt; 1$</td>
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<tr>
<td>10</td>
<td>$0.3 \times 10^5 \pm 0.2 \times 10^5$</td>
<td>$35 \pm 16$</td>
<td>$3 \pm 5$</td>
<td>$13 \pm 7$</td>
<td>$67 \pm 60$</td>
<td>$1 \pm 5$</td>
<td>$&lt; 1$</td>
<td>$&lt; 1$</td>
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</table>

Data are expressed as mean values ± SD.

aWBC, white blood cells.
Persistent Expression of Lentivirus Carrying Transgene in the Knee Joints of Nude Rats

LV.GFP injection into knee with long expression in fibroblasts of ligaments, tendon and capsules

Mol Ther 2007; 15: 1114-1120
Intra-articular lentivirus-mediated delivery of galectin-3 shRNA and galectin-1 gene ameliorates collagen-induced arthritis


Gal1 and Gal3 in CIA Synovium

Therapeutic Effect of Lt.shGal3 Rat Model of CIA

In vivo T cell Numbers and in Vitro T Cell Death by Type II Collagen

Ann Rheum Dis 2005;64:96-103
Construction of Lentiviral Vector-mediated Gal3 ShRNA Gene

Gal-3 gene: NM031832

sh44  sh597  sh654

Gal-3 mRNA: NM031832

ORF

Stop codon mutation (TAA→CAA)

RT-PCR

BamHI

Clai

EcoRI

sh537

EcoRI

Clai

Clai

H1 promoter

pLVTHM

11085 bp

pLVTHM/shGalentin3 537

11137 bp

pGEM-T Easy Vector

3015 bp

pGEM-T Easy Vector/rGal-3 delete Stop codon

3822 bp

pEGFP-N1

4722 bp

pEGFP-N1/rGal-3 fusion EGFP

5522 bp

M13 primer binding site

T7 primer binding site

EcoRI

ClaI

sh537

H1 promoter
Screening of Gal3 ShRNA Gene

Co-transfection

Flow cytometry analysis
Production of Lentiviral Vector-mediated Gal3 ShRNA Gene

Transfer plasmid

Co-transfection

Packaging plasmid (psPAX2)

Envelope plasmid (pMD2.G)

Calcium phosphate precipitation

Transduction Unit
Virus titers determination by transfection of TE671 cells

Viral Particle
Viral particles detected by measurement of virus-associated p24 core protein

2,000 VP equaling to 1 TU
Suppression of collagen-induced arthritis by intra-articular lentiviral vector-mediated delivery of Toll-like receptor 7 short hairpin RNA gene

S-Y Chen¹,², A-L Shiu³, Y-T Li¹, Y-S Lin³, C-H Lee³, C-L Wu¹ and C-R Wang¹,²

Toll-like Receptor 7 Signaling

Endogenous Expression of TLR-7 on CIA Rats Synovium

TLR7 expression on synovium

Nat Rev Immunology 2008; 10: 1038-2358
Suppression of collagen-induced arthritis by intra-articular lentiviral vector-mediated delivery of Toll-like receptor 7 short hairpin RNA gene

S-Y Chen\textsuperscript{1,5}, A-L Shiau\textsuperscript{3,5}, Y-T Li\textsuperscript{1}, Y-S Lin\textsuperscript{3}, C-H Lee\textsuperscript{6}, C-L Wu\textsuperscript{1} and C-R Wang\textsuperscript{2,3}

**TLR7 expression on CIASF**

**Endogenous TLR7 ligand on IL-6 from CIASF**

NSF: Normal synovial fibroblast
CIASF: Collagen-induced arthritis synovial fibroblast
TLR7 in autoimmune arthritis

- TLR-3 and TLR-7 were highly expressed in RA synovium.
- TLR-3 and TLR-7/8 stimulation resulted in a skewed balance toward IL-12 (Th1).

Arthritis Rheum. 2005; 52: 2313-2322
Construction of Lentiviral Vector-mediated TLR7 ShRNA Gene

**TLR 7 gene: NM001097582**

sh1842

*EcoR I/Cla I*  
*EcoR I*  
*Cla I*  
*H1 promoter*  
*sh1842*

**pLVTHM**

- **Amp**
- **GFP**
- **WPRE**
- **LTR**
- **SIN**
- **tetO**
- **ORI**
- **gpt**
- **psi**
- **RRE**
- **CpPT**
- **EF1-alfa**
- **SV40**
- **SD**
- **SA**
- **LoxP**
- **Cla I (5681)**
- **EcoRI (5393)**

**pLVTHM-shTLR7 sh1842**

- **11137 bp**

**pSuper.basic/TLR7-sh1842**

- **3230 bp**

**An amazing gift from Dr. D. Trono, Ecole Polytechnique Federale de Lausanne (EPFL), Lausanne, Switzerland**
Production of Lentiviral Vector-mediated TLR7 ShRNA Gene

Transfer plasmid

Co-transfection

Ultracentrifugation

293T

Calcium phosphate precipitation

Transduction Unit
Virus titers determination by transfection of TE671 cells

Viral Particle
Viral particles detected by measurement of virus-associated p24 core protein

2,000 VP equaling to 1 TU
Efficacy of Lentiviral Vector-mediated TLR7 ShRNA Gene

48h post-infection
Infection (8 ug/ml polybrene)

RT-PCR (CIASF)

Western blot (PBMC)

mock  Vector only  Scramble  Lt. shTLR7

TLR7

β-actin
Suppression of collagen-induced arthritis by intra-articular lentiviral vector-mediated delivery of Toll-like receptor 7 short hairpin RNA gene

S-Y Chen, A-L Shiu, Y-T Li, Y-S Lin, C-H Lee, C-L Wu and C-R Wang

Clinical, Radiological and Histological Evaluation on Lt.shTLR7 gene transfer

TLR7 Expression on CIA Synovium by Lt.shTLR7

<table>
<thead>
<tr>
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<th>Lt.scramble</th>
<th>Lt.shTLR7</th>
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</thead>
<tbody>
<tr>
<td>TLR7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
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</tr>
<tr>
<td>TLR7/β-actin</td>
<td>0.25</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Clinical results:

- **Ankle circumference**: Lt.shTLR7 Shows a significant decrease compared to Lt.scramble.
- **Articular index**: Lt.shTLR7 also shows a significant decrease.
- **Radiologic score**: Lt.shTLR7 has a lower score compared to Lt.scramble.
- **Histologic score**: Lt.shTLR7 has a lower score compared to Lt.scramble.
Suppression of collagen-induced arthritis by intra-articular lentiviral vector-mediated delivery of Toll-like receptor 7 short hairpin RNA gene

S-Y Chen, A-L Shiu, Y-T Li, Y-S Lin, C-H Lee, C-L Wu and C-R Wang

Imiquimod TLR7 ligand-Induced VEGF and IL-6 production from CIASF by Lt.shTLR7

Microvessel density and VEGF levels by Lt.shTLR7 treatment
**RA as a locally invasive tumor**

- Synovial fibroblasts (SFs), which are responsible for the progressive destruction of articular cartilage and bone, play an important role in the pathogenesis of RA.

- RASFs have been shown to grow *in vitro* in an anchorage-independent manner, a property that correlates closely with *in vivo* tumorigenicity.

- In a severe combined immunodeficiency mouse co-implantation model, RASFs maintain their invasive and destructive behavior, and these effects are independent of T cells.
Characteristics of RASF

• Include somatic gene mutations, oncogene activation, alterations in tumor suppressor gene expression, and enhanced telomerase activity.


• Accumulation of p53 mutations have been noted in RA synovial tissue and synovial fibroblasts.

  Proc Natl Acad Sci U S A 1997;94:10895–10900

• High telomerase activity is also detected in RASFs.

  Rheumatol Int 2000;19:123–128
Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors

Nunzio Bottini and Gary S. Firestein

Amelioration of Experimental Arthritis by a Telomerase-Dependent Conditionally Replicating Adenovirus That Targets Synovial Fibroblasts

Shih-Yao Chen, Ai-Li Shiau, Gia-Shing Shieh, Chih-Hau Su, Che-Hsin Lee, Hwei-Ling Lee, Chrong-Reen Wang, and Chao-Liang Wu

Oncolytic Adenoviral Vector (Ad.GS1)

Mutant p53

Telomerase

CIA

Adenovirus Alleviates Arthritis

Arthritic symptoms can be ameliorated by targeting synovial fibroblasts—responsible for progressive destruction of cartilage and bone—with a novel genetically engineered adenovirus (Ad.GS1) designed by Chen et al. “The growth of pannus in arthritis is similar to tumor growth and so we thought antitumor strategies could be applied to treat rheumatoid arthritis,” explains Chao-Liang Wu, lead investigator of the study.

The researchers designed this adenovirus to be telomerase-dependent and to target cells with TP53 mutations because enhanced telomerase activity and accumulation of TP53 mutations are observed in synovial fibroblasts from individuals with rheumatoid arthritis (RA). Ad.GS1 induced cytolysis of synovial fibroblasts from patients with RA and from rats with collagen-induced arthritis (CIA), but had no effect on control cells. The investigators observed replication of Ad.GS1 in synovial fibroblasts by inserting and quantitating the expression of an Ad.GS1 derivative containing a luciferase gene, and in synovial tissue from the ankles of arthritic rats by measuring levels of adenovirus late proteins. By contrast, they did not detect replication in rat lymph node cells or ankle joints from normal rats.

Moreover, Ad.GS1-treated rats with CIA had considerably smaller ankle circumferences, attenuated bone erosion, and lower radiographic and histologic scores in comparison to rats receiving control adenovirus or no treatment. The researchers also noted reduced production of disease-related proteins—interleukin-1β, matrix metalloproteinase 9 and prolyl 4-hydroxylase—in Ad.GS1-treated rats with CIA compared with control rats.

“Our study provides proof of the principle that synovial fibroblasts can be targeted to treat RA,” concludes Wu.
Mutant p53 might be responsible for the invasiveness of SF via snail stabilization

• Invasiveness of synovial fibroblasts is regulated by p53 in the SCID mouse in vivo model of cartilage invasion.  

  Arthritis Rheum 2001;44:676–681

• P53 inhibits tumor cell invasion via the degradation of snail protein in hepatocellular carcinoma.  

  FEBS Lett 2010;584:2231-2236
Gene expression in the synovium of CIA
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**RNAi資料庫**
*(Knockdown Information)*

**clone ID**: TRCN0000218784

**TRC / RNAiCore knockdown**

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</table>

**說明：**
*Good*: qPCR data quality is good
*Low qPCR Replicates*: Data from less than 3 qPCR replicate wells was used for at least one qPCR assay in the analysis (standard deviation cannot be calculated)
*High Error*: qPCR replicate wells had high standard deviation (>2 fold, ie dCt stdev > 1)
*High ENC*: Detection of endogenous control gene failed (no knockdown calculated)
*Enoutry*: Detection of endogenous control gene was at least 2 cycles different than other samples in the same experiment. Assumptions of ΔΔCt analysis might not be valid.

**User Feedback**

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此為使用者回報之shRNA Knockdown efficiency (可能與TRC資料庫所提供之 knockdown資訊不同)，僅供其他使用者作為參考之用。
Production of **Lentiviral Vector-mediated Snail ShRNA Gene**

**Transfer plasmid**

**Packaging plasmid (psPAX2)**

**Envelope plasmid** (pMD2.G)

**Co-transfection**

**Calcium phosphate precipitation**

**Ultracentrifugation**

**Lt.shSnail**

Relative infection Unit (R.I.U.)

Virus titers determination by transfection of A549 cells
Snail knockdown in CIA SF

Primarily cultured SF from synovium

48h post-infection

<table>
<thead>
<tr>
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<tbody>
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<td>β-actin</td>
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</table>
Reductions in IL-6 production, cad-11 expression, and invasive ability in CIA SF by knockdown of snail
Changes in arthritis signs in CIA rats by modulation of snail

10^6 RIU
Conclusion

• Our present studies demonstrate lentiviral vector-based shRNA gene transfer strategies to downregulate putative molecules in animal models of RA. Molecular mechanisms responsible for arthritis amelioration include immunomodulation, anti-cell invasion and - inflammation.
Future perspectives

• Detail mechanisms
  → Epithelial mesenchymal transition

Chen SY, Shiau AL, Li YT, Lin CC, Liu MF, Wang CR, Wu CL.
Snail as a regulator of cadherin-11 expression and a potential therapeutic target for rheumatoid arthritis.

→ Epigenetic regulation
Histone deacetylase, DNA methyltransferase, microRNAs

• Novel therapeutics
  → Established microRNA microarray database in CIA mice

Li YT, Chen SY, Wang CR, Liu MF, Lin CC, Jou IM, Shiau AL, Wu CL.
Amelioration of collagen-induced arthritis in mice by lentivirus-mediated silencing of microRNA-223.

Acknowledgements

• Chao-Liang Wu, Ph.D.  
  Department of Biochemistry and Molecular Biology

• Ai-Li Shiau, Ph.D.  
  Department of Microbiology and Immunology

• Chrong-Reen Wang, M.D. Ph.D.  
  Section of Rheumatology and Immunology, Department of Internal Medicine

National Cheng Kung University Medical College
Who suffered from RA?

The Phillips Collection, Washington, D.C.

Pierre-Auguste Renoir-1841~1919

Luncheon of the Boating Party
1880-1881