Transcription factors with a backbone:
regulation of skeletal homeostasis by NFATs

8-20-15

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BWH Rheumatology Division
Musculoskeletal disease...diverse presentations

Osteoarthritis

Back pain/arthritis

Rheumatoid Arthritis

Tendon tears

Osteoporosis

Ligament tears

Bone Tumors

Insight into each of these by studying one transcription factor (from the immune system)
What you will learn about today…

1. What are NFAT transcription factors?
   • Why are NFATs relevant to the musculoskeletal system?

2. Major bone and joint structures
   • Where is NFATc1 expressed?

3. Role of NFATc1 in osteoclasts, cartilage and ligaments
The NFAT Transcription Factor Family

- Nuclear Factor of Activated T-cells
  Originally identified in the early 1990s as a regulator of IL-2 in T-cells

  Redundancy...sometimes

- Family of 5 inducible transcription factors
  - NFATc1
  - NFATc2
  - NFATc3
  - NFATc4
  - NFAT5

  Ca^{++} influx

  Only in vertebrates!

  Osmotic stress

The NFAT Transcription Factor Family

Mechanical Stimuli

NFATc1-c4
Vertebrate transcription factors for vertebrate physiology

Physiology

• Axon patterning
• Heart valve formation
• Adaptive Immunity
• Hair growth
• Surfactant production

<table>
<thead>
<tr>
<th>Physiology</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon patterning</td>
<td>Down’s Syndrome</td>
</tr>
<tr>
<td>Heart valve formation</td>
<td>Cardiac hypertrophy</td>
</tr>
<tr>
<td>Adaptive Immunity</td>
<td>Autoimmunity</td>
</tr>
<tr>
<td>Hair growth</td>
<td>Cancer</td>
</tr>
<tr>
<td>Surfactant production</td>
<td>Pain sensation</td>
</tr>
</tbody>
</table>

NFATc1-c4
Vertebrate transcription factors for vertebrate physiology

Normal bones and joints
Key structures

- Cortical bone
- Trabecular bone
- Growth plate
- Articular surface
- Meniscus
- Ligaments and tendons

Is NFATc1 expressed at one or more of these sites?

$mT/mG; \text{NFATc1-Cre}$ reporter mouse – gift of Bin Zhou - Einstein
$mT/mG$ reporter mouse

**Before Cre**

Cells that never expressed NFATc1 will be **Red**

**NFATc1-Cre**

**After Cre**

Cells that express NFATc1 (and their progeny) will be **Green**

*genesis 45:593–605 (2007)*
Normal bones and joints

Key structures

Articular surface
Meniscus
Expression of NFATc1 in articular cartilage and meniscus

$mT/mG; NFATc1-Cre$

P0

P14

Nuclei = blue
Normal bones and joints

Key structures

Cortical bone

Trabecular bone

Ligaments and tendons
Expression of NFATc1 in ligaments

$mT/mG$

NFATc1-Cre

Nuclei = blue

200X
Normal bones and joints
Key structures

Trabecular bone
Growth plate
Expression of NFATc1 in osteoclasts

$mT/mG; NFATc1-Cre$

Nuclei = blue
Multinucleated cells at the growth plate = osteoclasts
NFATc1 expression within bone and joints

- **Cortical bone**
- **Trabecular bone**
  - Osteoclasts
- **Growth plate**
  - Osteoclasts
- **Articular surface**
- **Meniscus**
- **Ligaments and tendons**

Green text = sites of expression!
NFATc1 in skeletal biology

1. Osteoclasts

2. Cartilage

3. Ligaments
Why study the Osteoclast?

• Sole bone resorbing cell

• Physiologic bone remodeling
  – Growth
  – Fracture healing
  – Skeletal integrity

• Pathologic bone destruction
  – Inflammatory arthritis
  – Osteoporosis

Osteoporosis
Osteoclastogenesis and Bone Resorption

Myeloid Precursors

- c-fms
- RANK

Preosteoclasts

- M-CSF + RANKL

Osteoclast

- RANKL
- HCl
- Cathepsin K
- MMP9

Bone

Controlled by transcription factors

- NFATc1
Mutations in osteoclast pathways result in osteopetrosis

- **Two flavors**
  - Osteoclast poor - mutation affecting differentiation
  - Osteoclast rich - mutations affecting function

**Shared pathways in mice and humans!**

NFATc1 and the Osteoclast

- RANK signaling induces NFATc1 expression
- Co-stimulatory signals promote Ca\(^{++}\) influx and NFATc1 activation
- NFATc1 implicated in the control of various OC specific genes
  - TRAP, Cathepsin K, \(\beta_3\)-integrin and Calcitonin receptor

A tractable system to explore NFATc1 in adult mice was lacking....
NFATc1 KO mice die of cardiac malformations at E13.5

NFATc1 and the Osteoclast

Is NFATc1 required for physiologic bone remodeling?

Is the role of NFATc1 in vivo intrinsic to the osteoclast?

Identify novel regulators of osteoclast biology downstream of NFATc1
Understanding NFATc1 in bone
Generating NFATc1Δ/Δ mice with Mx1-cre

- **NFATc1**_${{\text{fl/fl}}}$ and **NFATc1**_${{\text{fl/fl}}}$, Mx1-Cre
- NFATc1 expressed normally until mice are treated with **poly I:C**
- Exon 3 excised in **NFATc1**_${{\text{fl/fl}}}$, Mx1-Cre = **NFATc1**ΔΔ mice

Birth

| ![](https://example.com/image1) |

poly I:C

Sacrifice and analyze

| ![](https://example.com/image2) | ![](https://example.com/image3) |

Exon 3

Floxed

Delta
NFATc1Δ/Δ mice develop osteoclast-poor osteopetrosis

**Plain film**
- NFATc1^{fl/fl}
- NFATc1^{Δ/Δ}

**Femoral growth plate**
- NFATc1^{fl/fl}
- NFATc1^{Δ/Δ}

*In Vitro*
- TRAP stain
  - NFATc1^{fl/fl}
  - NFATc1^{Δ/Δ}

But this is Mx1-Cre….
Mx1-Cre is non-specific

Inducible Gene Targeting in Mice
Ralf Kühn,* Frieder Schwenk, Michel Aguet,† Klaus Rajewsky

SCIENCE • VOL. 269 • 8 SEPTEMBER 1995

Is the NFATc1Δ/Δ phenotype is OC-intrinsic?

- Deletion in Osteoblasts
- Bone Marrow Chimeras
- Adoptive transfer of WT OC-precursors
Specific NFATc1 deficiency in osteoblasts does not increase bone mass.

Osteoblast-specific deletion

We didn’t see expression in osteoblasts anyway.
Bone marrow transplant transplant rescues the NFATc1Δ/Δ phenotype

**Remember!**

Osteoclasts are hematopoietic derived cells.

A bone marrow transplant should rescue the phenotype.

Micro-CT

Radiation
Adoptive transfer of osteoclast precursors into NFATc1Δ/Δ mice rescues osteoclastogenesis

Osteoclast Precursors are CD11b^{low}Ly6C^{hi}

Bone Marrow

What happens if these OCPs are injected into NFATc1Δ/Δ mice?
Adoptive transfer of osteoclast precursors into NFATc1Δ/Δ mice rescues osteoclastogenesis

CD11b\text{\textsuperscript{low}}Ly6C\text{\textsuperscript{high}} mT/mG; Ctsk-Cre

Osteoclast differentiation
(Express Cathepsin K)

Td Tomato

GFP
Adoptive transfer of osteoclast precursors into NFATc1Δ/Δ mice rescues osteoclastogenesis

CD11b\textsuperscript{low}Ly6C\textsuperscript{high}

\textit{mT/mG; Ctsk-Cre}

\textit{Intratibial Injection} \rightarrow \textit{NFATc1Δ/Δ mice}

\downarrow

\textit{7 days later}

\textit{IHC GFP}
Conclusions – Part 1

NFATc1 in osteoclasts

- Conditional deletion in the osteoclast precursor
  - Results in an osteoclast-poor osteopetrosis
  - Reduces osteoclast differentiation in vitro
  - Phenotype intrinsic to the osteoclast
    - Deletion in osteoblast – no phenotype
    - Bone phenotype rescued by bone marrow transplant
    - Lack of osteoclasts complimented by WT precursors

- Ongoing project – NFATc1 deletion in the mature osteoclast
Identification of NFATc1-dependent transcripts in the osteoclast

Can we leverage the fact that NFATc1 is a transcription factor to find new genes of importance in osteoclasts?
Identification of NFATc1-dependent transcripts in the osteoclast

Osteoclast Precursors

NFATc1fl/fl

M-CSF + RANKL
3 days

mRNA

Microarray

NFATc1D/D

M-CSF + RANKL
3 days

mRNA

Microarray
Identification of NFATc1-dependent transcripts in the osteoclast

80 unique genes up or down 6-fold

Known NFAT-target genes identified

\textit{Itgb3, Calcr, Oscar, Tnfrsf11b (OPG)}
Six genes for follow-up

- **Ion channels** – *Nhedc2, Slc4a2*

- **Small GTPases** – *Rhoc, Rab38*

- **Signal Transducers** – *Adcy3*

- **Protease inhibitor** – *Serpind1*

The collection of NFATc1-dependent transcripts in the osteoclast includes numerous genes non-essential to physiologic bone resorption

Julia F. Charles *, Fabienne Coury h,c,f,1, Rosalyn Sulyanto b,g,l, Despina Sitara h,b,1, Jing Wu b,1, Nicholas Brady b,1, Kelly Tsang b, Kirsten Sigrist h,k, Douglas M. Tollefsen b, Li He d, Daniel Storm *, Antonios O. Aliprantis h,b,g

**Bone 51 (2012) 902–912**
What is SLC4A2/AE2?

- Plasma membrane bicarbonate/chloride exchanger
- Large, central, multi-pass transmembrane domain mediates transport
- Large N-terminal and small C-terminal cytoplasmic domains
pH homeostasis in Osteoclasts

Is this Slc4a2/Ae2?

The Osteoclast needs:

• A way to release excess base
• A source of Cl⁻
Osteoclast-specific deletion of SLC4A2

- Crossed to Ctsk-Cre (an osteoclast Cre)
- Deletion → Frameshift → Stop codon in exon 9
- Renders all isoforms non-functional

Thank you Gary Shull, U. Cinn !!
**Slc4a2/Ae2**−/− phenotype is OC-intrinsic

Crossing Slc4a2fl mice with Ctsk-Cre

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**MicroCT**

- Slc4a2fl/fl
- Slc4a2fl/+ Ctsk-Cre
- Slc4a2fl/fl Ctsk-Cre

**TRAP Stain**

- Slc4a2fl/fl

**MicroCT – BV/TV**

- SLC4A2-mediated Cl−/HCO3− exchange activity is essential for calpain-dependent regulation of the actin cytoskeleton in osteoclasts

*PNAS* | February 5, 2013 | vol. 110 | no. 6 | 2163–2168
A deletion mutation in bovine SLC4A2 is associated with osteopetrosis in Red Angus cattle

**Slc4a2/Ae2**-/- mice develop osteoclast-rich osteopetrosis

**Slc4a2/Ae2** is a key regulator of the osteoclast

- **In vivo**
  - Deficient mice develop osteopetrosis
  - Similar phenotype in cattle!
  - Phenotype is osteoclast intrinsic

- **In vitro (not shown)**
  - Functional defect – acidification
  - Function depends on anion exchange
NFATc1 in skeletal biology

1. Osteoclasts
2. Cartilage
3. Ligaments
What about NFATs and OA/cartilage?

The Nuclear Factor of Activated T Cells (NFAT) 
Transcription Factor NFATp (NFATc2) is a Repressor 
of Chondrogenesis 
By Ann M. Ranager, Louis C. Gerstenfeld, Jinxin Wang, Tamiyo Kon, Hyungan Bae, Ellen M. Gravallese, Melvin J. Glimcher, and Laurie H. Glimcher

J. Exp. Med. © The Rockefeller University Press 
Volume 191, Number 1, January 3, 2000 9–21

Old Nfatc2−/− mice develop limited joint mobility periarticular calcifications

Ectopic chondrocyte proliferation → endochondral ossification around joints

Chondrosarcomas in old mice

NFATc2 is a repressor of chondrogenesis

Plain radiographs
What about NFATs and OA/cartilage?

Transcription factor NFAT1 deficiency causes osteoarthritis through dysfunction of adult articular chondrocytes

Jinxi Wang,1,2* Brian M. Gardner,1 Qinghua Lu,1 Marianna Rodova,1 Brent G. Woodbury,1 John G. Yost,1 Katherine F. Roby,1 David M. Pinson,4 Ossama Tawil4 and Harrison C. Anderson1

Journal of Pathology
J Pathol (2009)
Published online in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/path.2578

2 months
Loss of proteoglycans

Safranin O stains of hips or shoulders

2 months
4 months
6 months
12 months

Loss of proteoglycans
Chondrocyte clustering
Fibrillation
Articular surface effacement
Thick subchondral bone

• Increased catabolic (e.g. MMP13) and inflammatory (e.g. IL1) genes

• Decreased anabolic gene expression (e.g. COL2A)
What about other NFATs and OA/cartilage?

What is the function of NFATc1 in chondrocytes?

NFATc1\(^{fl/fl}\), Col2Cre mice = Nfatc1\(^{col2}\)
NFATc1 in articular chondrocytes

- *Nfatc1*<sup>col2</sup> mice
  - Grossly normal with no baseline phenotype
  - Destabilization of the medial meniscus OA model

**Wild-type**

**Nfatc1**<sup>col2</sup>

Safranin O stain

8 weeks after DMM

Glasson Scoring System
NFATc1 in articular chondrocytes

• Why is there no phenotype?
  – Is there redundancy with other NFAT family members?

• *Nfatc2*^−/−^ mice develop an OA-like phenotype

• Does loss of NFATc1 in chondrocytes alter the *Nfatc2*^−/−^ OA phenotype?

• Generated *Nfatc1*^col2^ *Nfatc2*^−/−^ mice
  – X-ray, histopathology, skeletal preparations, micro-CT and qPCR
*Nfatc*¹⁺*Nfatc²⁻⁻* mice

Post-natal elbow and tarsal subluxations

3 week old mice
**Nfatc1\textsuperscript{col2} Nfatc2\textsuperscript{-/-} mice**
Post-natal elbow dislocation

3 week old mice

H&E

\textit{Nfatc1}\textsuperscript{fl/+} \textit{Nfatc2}\textsuperscript{+/+} \quad \textit{Nfatc1}\textsuperscript{col2} \textit{Nfatc2}\textsuperscript{-/-}

Phenotype never observed in \textit{Nfatc2}\textsuperscript{-/-} littermates
Nfatc1<sup>col2</sup> Nfatc2<sup>-/-</sup> mice
Destructive elbow arthropathy

6 month old mice

H&E

Nfatc1<sup>fl/+ Nfatc2<sup>+</sup>-/.</sup> Nfatc1<sup>col2 Nfatc2<sup>-/-</sup></sup>
**Nfatc1**<sup>col2</sup> **Nfatc2**<sup>-/-</sup> mice

Characterization of cartilage matrix degradation

1 week old mice

Safranin O

*Nfatc1<sup>fl/+</sup>Nfatc2<sup> +/-</sup>**<sup>**Nfatc1<sup>col2</sup>Nfatc2<sup>-/-</sup>**

Loss of proteoglycans
**Nfatc1^{col2} Nfatc2^{-/-} mice**

Characterization of cartilage matrix degradation

3 week old mice

9A4 neoepitope antibody

- **Nfatc1^{fl/+} Nfatc2^{+-}**
- **Nfatc1^{col2} Nfatc2^{-/-}**

9A4 – Collagenase generated type I/II collagen neoepitope

No Increase in staining at one week

Nfatc1<sup>col2</sup> Nfatc2<sup>−/−</sup> mice
Characterization of cartilage matrix degradation

3 week old mice

Aggrecan neoepitope

**Aggrecanase generated NITEGE neoepitope**
\textbf{Nfatc}^{\text{col2}} \textbf{Nfatc}^{2/-} \textbf{mice}

Gene expression

qPCR on elbow joints at 3 weeks

\textbf{Mmp13} \quad \textbf{Adamts5}

p<0.001
Summary

• Loss of NFATc1 alone in cartilage does not
  – Cause OA
  – Exacerbate post-traumatic OA

• Loss of NFATc1 in chondrocytes in NFATc2 KO mice causes
  – Joint subluxations
  – Aggressive degenerative osteoarthritis (?secondary)
    • Increased cartilage catabolism

Joint subluxations suggest
there may a function for NFATs in ligaments....