Physiopathology of osteoclasts

UE Cell Fate and Plasticity
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Cytoskeleton dynamics and osteoclast biology
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Osteoclasts and bone resorption

1- Bone dynamics and osteoclasts
2- Osteoclast differentiation
3- Bone degradation by osteoclasts
4- Osteoclasts and other bone cells
5- Genetic diseases caused by non functional osteoclast
6- Pathological activation of osteoclasts.
7- Osteoclasts as therapeutic targets in osteolytic diseases
8- A novel strategy against osteoporosis: example of an experimental design
Bone dynamics and osteoclasts

Osteoclast

Osteoblasts

Bone development and growth
Skeleton renewal and repair (turn over 5-10 years)
Calcium and phosphorus homeostasis
Hematopoiesis
Organ protection

Minerals (70%): % of total body content
- 99% Calcium
- 85% Phosphorus
- 50% Sodium
- 50% Magnesium

Hydroxyapatite

Proteins (22%):
- Type I Collagen (90% bone proteins)
- Osteocalcin and osteopontin (mineral binding)
- Growth factors

Water (8%)

Collagen organization in lamellar bone

Trabecular or cancellous bone

Cortical bone

Trabecular Bone
Cortical Bone

von Kossa staining (mineralized tissue)
1- Bone dynamics and osteoclasts: Bone Formation units

![Trabecular bone remodeling](image1)

![Cortical bone remodeling](image2)

Parra-Torres et al., 2013

Bone formation Unit
Lining the trabecular bone, osteoblasts synthesize the bone.

Osteocytes are osteoblasts that get embedded in the bone matrix during bone formation. They are connected by a canalicular network.
1- Bone cell orchestration during bone remodeling

Osteoclasts are multinucleated cells that come from the differentiation and fusion of precursor cells of the monocyte/macrophage lineage. Osteoclasts belong to the same hematopoietic lineage as dendritic cells and macrophages.
2- Osteoclast differentiation

Hematopoietic precursor cell

Polykarion

Osteoclast

Cytokines: MCSF (CSF1) and RANKL (TNFSF11)

Sealing zone:
Ring of podosomes allowing osteoclast adhesion on the bone.

Formation of a resorption pit

Ruffled border:
- secretion of H+ and Cl-
- secretion of proteases
  → solubilize bone minerals
  → degrade bone proteins

Actine/ADN
2- Osteoclast differentiation: Key transcription factors and receptors during osteoclastogenesis

Hematopoietic precursor cell

- CSF1R
- PU.1

→

RANK/TNFRSF11a

→

RANK

→

CSF1/MCSF

→

RANK/TNFRSF11a

→

RANKL

→

CSF1/MCSF

→

RANK/TNFRSF11a

→

RANKL

→

CSF1/MCSF

→

RANK/TNFRSF11a

→

RANKL

→

CSF1/MCSF

→

RANK/TNFRSF11q

→

RANKL

→

CSF1/MCSF

→

RANK/TNFRSF11q

→

RANKL

→

CSF1/MCSF

→

RANK/TNFRSF11q

→

RANKL

→

CSF1/MCSF
2- Osteoclast differentiation: Key transcription factors and receptors during osteoclastogenesis

With TREM2/ DAP12 and OSCAR/FcRγ co-stimulatory receptors, RANK induces oscillatory changes in intracellular Ca^{++}, resulting in activation of nuclear factor of activated T cells c1 (NFATc1) by dephosphorylation by the phosphatase calcineurin and of CREB by phosphorylation by CaM kinase IV. NFATc1 and CREB translocate to the nucleus and induce osteoclast-specific gene transcription.
2- Osteoclast differentiation: Expression of genes involved in osteoclast biology

- Osteoclast precursor fusion: DC-STAMP, OC-STAMP, Tks5
- Bone degradation:
  - acidification: CLCN7, V-ATPase
  - proteases: Cathepsine K, MMP9
  - phosphatase: TRAP
  - adhesion: Integrin αvβ3, Src...

Adhesion structure organization

Fusion

Differentiation: 5 days

Bone resorption

acid and protease secretion

Life span: 2-3 weeks
3- Bone degradation by osteoclasts

Bone = 70% minerals (Hydroxyapatite) + 20% proteins (Type 1 collagen)

- Functional secretion domain
- Basolateral domain
- Sealing zone
- Exocytosis
- Transcytosis
- Endocytosis
- Ruffled border
- Resorption Lacuna
- Proton pump
- Chloride channel
- TRAP
- CtsK, MMP9
- Secretion vesicles
- Bone degradation products
- Nucleus
- Podosome

Actine/ADN

Bone degradation products include:
- TRAP
- CtsK, MMP9
- Secretion vesicles
- Podosome
3- Bone degradation by osteoclasts: the podosome

Luxenburg et al., 2007

Podosomes seal the osteoclast onto the bone matrix.

Podosomes are needed to concentrate ions, for extracellular medium acidification.

Adapted from Linder, 2003 and Luxenburg, 2007.
3- Bone degradation by osteoclasts: actin dynamics

Real duration: 14 heures

Slatel et al., 2004
3- Bone degradation by osteoclasts: actin dynamics

Real duration: 14 heures

Slatel et al., 2004
3- Bone degradation by osteoclasts: actin dynamics

Podosome dynamics and bone resorption cycles

(1) Resorption
(2) Spreading
(3) Actin organization
(4) Polarization
(5) Resorption

Until (6) apoptosis

Touaitahuata et al., Small GTPases, 2014
3- Bone degradation by osteoclasts: actin dynamics

Microtubules are intimately associated with podosomes
3- Bone degradation by osteoclasts: actin dynamics

Microtubules are required to maintain podosome organization

Effect of nocodazole treatment and wash-out on actin structures in osteoclasts.

- stack of 262 images.
- 1 image taken every 30 sec.
- accelerated 450 times.
- field approx. 325 X 325 μm.

Destaing, 2003
3- Bone degradation by osteoclasts: acidification

Extra cellular medium acidification is necessary to solubilize hydroxyapatite and make collagen fibers amenable to protease-mediated degradation.
3- Bone degradation by osteoclasts: collagen fiber degradation

Cathepsin K attacks collagen fibers


Triple helices of collagen (2 α1 et 1 α2) bound by proteoglycans et des glucosaminoglycans


(a) macroscopic bone, (b) osteons (~100 um in diameter) with circular arrangements of differently oriented collagen fibers. (c) A collagen fiber (~5 um in diameter) consisting of bundles of collagen fibrils (each with a diameter of ~500 nm). (d) A striped collagen fibril (each period is ~68 nm in length) consisting of a staggered arrangement of collagen molecules (each having a diameter of ~1.5 nm) with embedded mineral crystals (with diameters from ~2 to 20 nm and lengths of 30 nm). (e) A collagen molecule triple helix.
4- Osteoclasts and other bone cells: osteocyte produce RANKL

Osteocytes produce RANKL

Apoptosis induces osteocyte to produce RANKL

*Kennedy et al., 2012 *

RANKL production

Voide et al., 2011

Osteoclast differentiation and bone repair

Caspase 3 / RANKL

Kennedy et al., 2012

± 4 months

Seeman et al., 2006
4- Osteoclasts and other bone cells: dialogue between osteoclasts and osteoblasts

Semaphorine 4D prevents osteoblast differentiation

Sema4D
Plexin B1

Osteoclast

Osteoblast

No bone formation during resorption

EphrinB2 and OPG prevents osteoclast differentiation

EphB4
EphrinB2

OPG (TNFRSF11B gene)
RANKL

No resorption during bone formation
4- Osteoclasts and other bone cells: dialogue between osteoclasts and osteoblasts

**Stimulation of bone formation by osteoclasts**

Osteoclast activity frees growth factors from the bone matrix that stimulate osteoblasts formation: TGFβ, IGF1...

Osteoclasts secrete factors called clastokines that stimulate osteoblast formation: Complement component 3a, Sphingosine-1-phosphate, Collagen triple repeat containing1, Tartrate-resistant acid phosphatase...

*Charles and Aliprantis, Tends in Molecular Medicine, 2014*
<table>
<thead>
<tr>
<th></th>
<th>Gene</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>autosomal recessive osteopetrosis</strong></td>
<td></td>
<td></td>
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<tr>
<td>50%</td>
<td>TCIRG1</td>
<td>Proton pump V-ATPase, subunit a isoform 3</td>
</tr>
<tr>
<td></td>
<td>CA2</td>
<td>Carbonic anhydrase, proton production</td>
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<tr>
<td></td>
<td>OSTM1</td>
<td>CLCN7 cofactor</td>
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<tr>
<td></td>
<td>PLEKHM1</td>
<td>Vesicle transport and acidification</td>
</tr>
<tr>
<td></td>
<td>TNFRSF11A</td>
<td>RANK receptor</td>
</tr>
<tr>
<td></td>
<td>SNX10</td>
<td>Vesicular traffic, partner of the V-ATPase</td>
</tr>
<tr>
<td></td>
<td>KINDLIN3</td>
<td>Integrin activation, sealing zone formation</td>
</tr>
<tr>
<td></td>
<td>CTSK</td>
<td>Cathepsin K, collagenase (Pycnodysostosis)</td>
</tr>
<tr>
<td><strong>autosomal dominant osteopetrosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75%</td>
<td>CLCN7</td>
<td>CLC7 H+/Cl- membrane transporter</td>
</tr>
</tbody>
</table>

Osteopetrosis Osteoporosis Osteoclast Osteoblast

Balanced bone remodeling
6- Genetic diseases caused by hyperactive osteoclasts.

**Paget disease of bone**

Genetic bone disease caused by increased osteoclast activity inducing increased bone formation by osteoblasts. Bone remodeling is too fast to ensure the building of correct bone architecture: no formation of lamellar bone, which takes several months.

The genetic is complex: mutations identified in SQSTM1/p62, TNFRSF11A/RANK, TNFRSF11B/OPG, CSF1/M-CSF, OPTN/OPTINEURIN...
Several mechanisms could be involved, including viral causes (paramyxovirus=measles).

Disorganized collagen fibers in woven bone

Improper mineralization of bone
6- Pathological activation of osteoclasts

Many physio-pathological situations lead to increased osteoclast activity.

Absence of / reduced gravity (bed rest, space flights, paralysis, myopathies...)

Apoptose → RANKL
Many physio-pathological situations lead to increased osteoclast activity.

Sex hormone deficiencies (hypogonadism): menopause, andropause

- Estrogens
- OPG: decoy receptor
- RANKL
- Hif1α
- FasL
- Apoptosis
- Activity

Menopause

Increased fracture risk
Women have a 40% chance of experiencing an osteoporotic fracture during their lifetime.
Tumor cell growth

IL-6, -8, -11, TNF, MCSF, PTHrP...

TGF-β, IGF, FGF, PDGF, BMPs...

Breast, prostate cancers (70%), Lung, colon, stomach... (15-30%)
Multiple Myeloma

The vicious cycle of bone metastases

Metastatic cells

Osteoblast

Tumor cell growth

RANKL

Osteoclast

TGF-β, IGF, FGF, PDGF, BMPs...

Bone destruction

Severe pain, fractures
Inflammatory synovial tissue

Fibroblasts, Monocytes, Macrophages, Lymphocytes

Production of RANKL

RANKL

Production of inflammatory cytokines

TNFα, IL-1, IL-6, IL-17

Fibroblasts, Monocytes, Macrophages, Lymphocytes

Production of RANKL

Monocytes differentiation into osteoclasts

Monocytes (Mo)

RANK

TNFα, IL-1, IL-6, IL-17

Degradation of mineralized cartilage and bone

Pain, disabilities

Inflammatory synovial tissue

Balanced bone remodeling

Osteoporosis

Osteopetrosis

Osteoblast

OSTEOCLAST

RA Erosions
7- Osteoclasts as therapeutic targets in osteolytic diseases: Bisphosphonates

Pyrophosphate PPi

O
OH

P
O
OH

OH

Bisphosphonate

O
OH

P
C
P
OH

R1

OH

R2

OH

Hydroxyapatite

Several year half-life of bisphosphonates in the skeleton.
7- Osteoclasts as therapeutic targets in osteolytic diseases: Bisphosphonate absorption by osteoclasts

Modifié de Rogers et al., Bone 49 (2011) 34–41
7- Osteoclasts as therapeutic targets in osteolytic diseases: Bisphosphonate, 2 modes of action

First generation of bisphosphonates
- Etidronate
- Clodronate
- Tiludronate
- Medronate (scintigraphy Technétium 99m)

Incorporation in ATP biosynthesis pathway → non-hydrolysable ATP analogue → Osteoclast apoptosis

Modifié de Sutherland KA et al., Arthritis Res Ther 2009;11:R58
### 7- Osteoclasts as therapeutic targets in osteolytic diseases: Bisphosphonate, 2 modes of action

**Next generations**: nitrogen-containing bisphosphonates

<table>
<thead>
<tr>
<th>Risedronate</th>
<th>Alendronate</th>
<th>Ibandronate</th>
<th>Zoledronate</th>
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<tr>
<td><img src="image" alt="Risedronate" /></td>
<td><img src="image" alt="Alendronate" /></td>
<td><img src="image" alt="Ibandronate" /></td>
<td><img src="image" alt="Zoledronate" /></td>
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</table>

**Inhibition of Farnesyl pyrophosphate synthase**

- No protein prenylation
- Perturbation of small GTPase signaling pathways (Ras, Rho, Rab)

- Accumulation of ATP analog Apppl
- Osteoclast apoptosis

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*Russell, Bone 49 (2011) 2–19*
Perturbation of RhoGTPase signaling pathways → perturbation of actin cytoskeleton

Perturbation of RabGTPase signaling pathways → perturbation of vesicular traffic


de Fraser P. Coxon et al. J. Biol. Chem. 2001; 276:48213-48222

Denosumab (Amgen), is a human monoclonal antibody that targets RANKL. (AMM in 2011)
Denosumab prevents osteoclast differentiation.

Another solution under development (Merk):

Odanacatib, an inhibitor of Cathpsin K.
It prevents collagen fiber destructuration and proteolysis.
Phase 3 clinical trials until 2016 showed efficiency to prevent pathological bone loss.
Discontinued september 2016 (strokes and cardiac fibrillation).
7- Osteoclasts as therapeutic targets in osteolytic diseases

Target osteoclasts to control bone loss

Another solution that we are investigating: prevent the formation of the podosome belt

Sealing zone= tight seal  
Lower pH in the lacuna, mineral dissolution

No sealing: no mineral dissolution  
No protein degradation
Osteoclast differentiation and function

Bone marrow precursor

MCSF  RANKL

Bone

Sealing zone

Polykaryon

Bone resorbing osteoclast

Functional secretory domain

Basolateral domain

HCO₃⁻

Cl⁻

H⁺

Resorption lacuna

CtsK

MMP9

Sealing zone

Actin/DNA

Actin

RhoGTPases
8- A novel strategy against osteoporosis: example of an experimental design.

Complexity of the actin regulatory network

18 RhoGTPases

± 80 Dbl & Dock/CZH-family exchange factors (RhoGEFs)

D R Cook, K L Rossman and C J Der, Oncogene 2013

Nishikimi et al., Exp Cell Res, 2013

GTPase

GDP

Inactive

GTP

GTPase

GEF

GDP

GTPase

GTP

Active

Actin Dynamics
About 40 GEFs out of ± 80 expressed in osteoclasts, 7 are induced by RANKL.
Dock5 associates with the podosome belt and activates Rac in osteoclasts

Vives, JBMR, 2011
Dock5 is necessary for sealing zone formation

shDock5

Disorganized podosomes

Dock5

Dock5+/+

Dock5-/

Mineralized matrix

Sealing zone

Vives, JBMR, 2011
Dock5 is essential for mineral dissolution.

Protease secretion is not affected

<table>
<thead>
<tr>
<th></th>
<th>Dock5^{+/+}</th>
<th>Dock5^{+-}</th>
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<tbody>
<tr>
<td>MMP9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CtsK</td>
<td></td>
<td></td>
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<tr>
<td>Actin</td>
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</table>

Calcium Phosphate mineral substrate

Von Kossa mineral staining

Dissolution area (mm^2)

Dock5^{+/+} and Dock5^{+-} show differences in mineral dissolution.

MMP9, CtsK, and Actin levels are compared.

Dock5-KO

Touaitahuata, Dev Biol, 2014
Dock5 is necessary for bone resorption

Dock5 is required for bone resorption by facilitating the process of mineralized matrix sealing. In Dock5 knockout (Dock5−/−) models, bone collagen degradation is impaired compared to wild-type (Dock5+/+) controls.

Acidification and proteases play a role in bone collagen degradation. The dosage of bone collagen degradation can be measured using CTx as a biomarker. Osteoclasts are involved in bone resorption, and bone slice staining is used to assess the extent of bone collagen degradation.

Vives, JBMR, 2011
Dock5-/- mice have increased bone mass with normal osteoclast numbers.

Dock5 is mainly expressed in osteoclasts, KO mice are normal and fertile.

**Use Dock5 as a therapeutic target in osteolytic diseases**

Principle of the Yeast Exchange Assay to identify Dock5 inhibitors

**Yeast 2-hybrid system**

- **Inactive Rac**
  - Rac-GDP
  - LexA
  - βgal
  - β-gal - HIS-

- **Active Rac**
  - Dock5
  - Rac-GDP
  - LexA
  - βgal
  - β-gal + HIS+

**Yeast Exchange Assay**

- Dock5 expression
  - Dock5
  - Rac-GDP
  - LexA
  - βgal

**Exchange factor**

<table>
<thead>
<tr>
<th>Exchange factor</th>
<th>-</th>
<th>Dock5</th>
<th>+</th>
</tr>
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<tbody>
<tr>
<td>Rac1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cdc42</td>
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**Graph**

- Yeast growth (OD$_{600}$)
- Time (hours)
- DOCK5 +His
- no GEF +His

- DOCK5 -His
- no GEF -His

- 50%
Screening for Dock5 inhibitors with the Yeast Exchange Assay

Identification of Dock5 inhibitors

Inactive Rac

Dock5 expression

Active Rac

Dock5

Dock5 Inhibitor

Screening for Dock5 inhibitors:

Identify inhibitors of Rac activation by Dock5:
Molecules that prevent yeast growth in the absence of histidine but not in complete medium.

Yeast growth (OD\textsubscript{600})

0.2
0.4
0.6
0.8
1
1.2
0
8
16
24

Time (hours)

- Inhibitor +His
- Inhibitor -His
+ Inhibitor +His
+ Inhibitor -His
The inhibitor C21 blocks Rac activation specifically by Dock5

**Yeast 2-hybride-derived assay**

C21

N-(3,5-dichlorophenyl) benzenesulfonamide

**Pull down assay**

Dock5 or TrioN

Rac - GDP
Inactive

Rac - GTP
Active

TrioN or Dock5 : - +

Rac-GTP

Rac Total

**In vitro exchange assay**

C21 slows down the exchange reaction

Spontaneous + C21

Spontaneous

Dock5

Dock5 + C21

**C21 blocks Rac activation by Dock5 but not by TrioN**

<table>
<thead>
<tr>
<th>C21 (µM)</th>
<th>0</th>
<th>0</th>
<th>100</th>
<th>50</th>
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<tbody>
<tr>
<td>Rac-GTP</td>
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<tr>
<td>Total Rac</td>
<td></td>
<td></td>
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<tr>
<td>Anti-GFP</td>
<td></td>
<td></td>
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<tr>
<td>Total Rac</td>
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</tr>
</tbody>
</table>

Vives, JBMR, 2011
Dock5 inhibitor C21 blocks bone degradation by osteoclasts

Vives, JBMR, 2011
Vives, Cres, Nat Comms, 2015
8- A novel strategy against osteoporosis: example of an experimental design.

Dock5 inhibitor C21 reversibly perturbs osteoclast podosome organization

- **Vehicle**
- **C21 1 hour**
- **C21 4 hours**
- **C21 4 hours + recovery**

8- A novel strategy against osteoporosis: example of an experimental design.

Can C21 prevent menopause-induced bone loss?

Menopause → Estrogens

Estrogens → Osteoblast

OPG → RANKL

RANK → Mo

Increased bone degradation

C21 → apoptotic pathway

Estrogen Receptor α → FasL

Can C21 prevent menopause-induced bone loss?
Ovariectomy (menopause)-induced bone loss

Protocol

C57Bl/6 mice, 12 week-old
8 ovariectomized (OVX),
8 OVX+C21,
15 SHAM

C21 (25 mg/kg/day, 5 days/week)

D0

D28

Ovariectomy or sham operation

Sacrifice
Micro-tomography
Uterus weight

Uterus

μCT

C57Bl/6 mice + ou - C21
8- A novel strategy against osteoporosis: example of an experimental design.

Dock5 inhibitor C21 prevents ovariectomy (menopause)-induced bone loss

** p< 0.01, Kruskal-Wallis test with Dunn’s post test

Inflammation (arthritis)-induced bone loss

Published Results

Male DBA1 mice
8 week-old

D0
Bovine collagen
100 µg

D20
C21 (25 mg/kg/day, 5 days/week)

D53
Sacrifice
Bovine collagen
100 µg

Micro-computed tomography

Bovine collagen
100 µg

Arthritis score

* p< 0.05, Kruskal-Wallis test with Dunn’s post test

8- A novel strategy against osteoporosis: example of an experimental design.

Dock5 inhibitor C21 prevents inflammation (arthritis)-induced bone loss

* p< 0.05, Kruskal-Wallis test with Dunn’s post test

What is the effect of Dock5 inhibitor on the development of bone metastases?

Inhibition of Dock5 in vivo

Breast, prostate cancers (70%), Lung, colon, stomach... (15-30%)

Multiple Myeloma

Metastatic cells

Tumor cell growth

IL-6, -8, -11, TNF, MCSF...

Osteoclast activity

Bone destruction

TGF-β, IGF, FGF, PDGF, BMPs...

C21
Metastasis-induced bone loss

C21 (25 mg/kg/day)

2 Weeks

Induction of bone metastases

Development of metastases

Effect on bone

Syngenic model for bone metastases

B16-F10 melanoma cells expressing luciferase

C21

C57BL/6

Balanced bone remodeling

Osteoporosis

Osteoblasts

Osteoclasts

Hormone deficiency
Bone metastasis
Rheumatoid arthritis
8- A novel strategy against osteoporosis: example of an experimental design.

**Inhibition of Dock5 prevents metastasis-induced bone loss**

![Graph showing BV/TV (%) for vehicle and C21 treatments.](image)

**Diagram illustrating balanced bone remodeling**

- Osteopetrosis
- Osteoporosis
- Osteoblast
- Osteoclast

**Genetic variant**

- Hormone deficiency
- Bone metastasis
- Rheumatoid arthritis

---

8- A novel strategy against osteoporosis: example of an experimental design.

Inhibition of Dock5 prevents metastasis-induced bone loss and bone tumor burden

Present treatments against osteoporosis inhibit bone formation

Therapeutic challenge in osteolytic diseases:
Block bone degradation **without** affecting bone formation
Dock5 inhibition prevents pathological bone loss

What about bone formation?
Dock5 inhibitor C21 does not affect bone formation in healthy mice

Dock5 inhibitor C21 does not affect bone formation in ovariectomized mice

Targeting podosome organization to protect against bone loss and preserve bone formation

Denosumab

Bisphosphonates

RANKL

Dock5

Osteoblasts

Bone degradation

Bone formation

→

Bone formation
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