The Role of Enhancers in Genetic and Epigenetic Control of Gene Expression

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Why Study Enhancers?

• Enhancers govern cellular phenotype through selective control of gene expression

• Detailed enhancer studies provide relevant insight into basic gene regulatory mechanisms

• Understanding specific enhancer features may reveal roles for SNVs in genome evolution and SNPs in human disease

• Unique enhancer properties could facilitate the development of next generation therapeutics for personalized medicine

• Enhancer/promoter segments of genes can be utilized to create diverse basic as well as clinically relevant animal models
Characterization of the VDR Cistrome in Differentiating Osteoblasts

Pre-Osteoblasts

POB

VDR

Veh (947)

158

786

6,215

1,25D₃

(7,007)

POB

VDR

1,25D₃

(7,007)

Pre-Osteoblasts

POB

VDR

1,25D₃

(7,007)

POB

RXR

Veh (7,834)

2,565

5,244

2,334

1,25D₃

(7,591)

Osteoblasts

POB

VDR

Veh (277)

22

255

617

1,25D₃

(873)

POB

RXR

Veh (4,815)

561

4,254

3,853

1,25D₃

(8,128)


<table>
<thead>
<tr>
<th>Rank</th>
<th>TF match</th>
<th>Peak (bkgd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POB</td>
<td>1</td>
<td>VDR/RXR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>RUNX</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB</td>
<td>1</td>
<td>VDR/RXR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>RUNX</td>
</tr>
<tr>
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</table>
Contraction of the 1,25(OH)$_2$D$_3$ Transcriptome After Osteoblast Differentiation

Differential Target Gene Responsiveness to $1,25(OH)_2D_3$ Due to Differentiation

HUB Tracks:
- Yellow, Vehicle
- Blue, $1,25(OH)_2D_3$
- Green, Overlap
Enhancers are highlighted by signature histone modifications that are dynamic and include H3K4me1, H3K4me2, H3K9ac and H3K27ac (ENCODE)

Differentiation/trans-differentiation is characterized by significant changes in histone modification at selected gene loci (ENCODE)

Changes in histone marks and regulatory factors can contribute to responsivity to secondary regulators such as the vitamin D receptor

1,25(OH)$_2$D$_3$ and other hormones provoke changes in histone modification/acetylation and factor binding in a gene-selective manner

The Osteoblast Enhancer Complex (OEC): An Example of a Consolidated Enhancer

<table>
<thead>
<tr>
<th>Venn Part</th>
<th>Rank</th>
<th>TF match</th>
<th>Peak (bkgd)</th>
<th>Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR/RXR (802, 6,722)</td>
<td>1</td>
<td>VDR/RXR</td>
<td>36% (1%)</td>
<td>AGGTCAAGGTCAAGGTCA</td>
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<tr>
<td>RUNX2 (6,722)</td>
<td>1</td>
<td>RUNX2</td>
<td>45% (6%)</td>
<td>TGTTGGTTGGTTGGTTGGTT</td>
</tr>
<tr>
<td>C/EBPβ (13,335)</td>
<td>1</td>
<td>C/EBPβ</td>
<td>48% (4%)</td>
<td>GAGCCAGAGCCAGAGCCAG</td>
</tr>
<tr>
<td>VDR/RXR / RUNX2 / C/EBPβ (1,744 peaks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 RUNX2</td>
<td>50%</td>
<td>12%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 C/EBPβ</td>
<td>24%</td>
<td>8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 VDR/RXR</td>
<td>7%</td>
<td>2%</td>
<td></td>
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</tbody>
</table>

**Key Features of Enhancers Thus Far**

**Distal Binding Site Locations:** *Cis*-regulatory modules (CRMs or enhancers) are dispersed across the genome; located in a cell-type specific manner near promoters, but predominantly within introns and distal intergenic regions; frequently located in clusters of elements.

**Modular Features:** Enhancers contain binding sites for multiple transcription factors that facilitate both independent or synergistic interaction.

**Epigenetic Enhancer Signatures:** Defined by dynamically regulated post-translational histone H3 and H4 modifications.

**Transcription Factor Cistromes (VDR) are Highly Dynamic:** Cistromes change during cell differentiation, maturation, and disease activation and thus have broad consequential effects on gene expression.
Mmp13 is Regulated by 1,25(OH)_2D_3 and Differentiation

- Collagenase-3 (Mmp13) degrades extracellular collagens at skeletal sites in bone
- The gene is aberrantly expressed in nearly every cancer or disease with fibrotic complications (breast, prostate, pancreatic, and atherosclerosis)
- *Mmp13* is regulated by a variety of factors including FGF2, PTH, estrogens, 1,25(OH)_2D_3, and cytokines
- Previous work on regulation has focused almost exclusively on the promoter proximal region of *Mmp13*

ChIP-Seq Analysis Identifies Distal Upstream Enhancers in the *Mmp13* Locus
CRISPR/Cas9 Mediated Enhancer and TF Deletion in an Osteoblastic Cell Line

**Vdr**
- Exon 3
- chr15: 97,715,284 - 97,715,335

**Runx2**
- Exon 3
- chr17: 44,861,800 - 44,861,840
Genome Deletions have Dramatic Effects on Basal Mmp13 Expression and on 1,25(OH)$_2$D$_3$ Inducibility

- Deletion of the promoter proximal region of Mmp13 reduces Mmp13 RNA expression.

- Deletion of the -10k Mmp13 enhancer or VDR reduces basal expression of Mmp13 RNA and highlights secondary regulation by 1,25(OH)$_2$D$_3$.

- Deletion of the -30k Mmp13 enhancer or RUNX2 eliminates basal expression of Mmp13 RNA.
A dispersed osteoblast enhancer complex at the *Mmp13* locus coalesces at the promoter through chromatin reorganization.

The promoter proximal region is unable to mediate independent regulation.

The -10 kb enhancer mediates hormonal regulation by 1,25(OH)$_2$D$_3$ yet is dominated by the -30 kb enhancer.

The -30 kb region is central to the basal activity of *Mmp13* and exhibits hierarchical activity over the remaining enhancers.

Repression by 1,25(OH)$_2$D$_3$ in the absence of the -10 kb enhancer is likely due to independent RUNX2/OSX downregulation by the VDR.

The Diverse Biological Activities of RANKL
Regulatory Complexity at the *Tnfsf11* (Rankl) Gene Locus Involves Multiple Upstream Distal Enhancers

Genetic Deletion of *Tnfsf11* (Rankl) Enhancers in the Mouse

Phenotype

- **Δ RL-P1 (-500 b to -7 kb):** No effect on regulatory expression of Rankl
- **Δ RL-D2:** Reduces expression of Rankl in mesenchymal cells, limits regulation by PTH and induces age-related osteopetrosis
- **Δ RL-D5:** Reduces Rankl expression in mesenchymal and hematopoietic cells, limits regulation by PTH and 1,25(OH)_{2}D_{3} and induces age-related osteopetrosis
- **Δ RL-D6:** Limits mesenchymal response to inflammatory cytokines with no skeletal phenotype
- **Δ RL-T1:** Prevents Rankl expression in hematopoietic but not skeletal cells

High RANKL Expression in Atherosclerotic Plaques is Compromised in RL-D5 Enhancer Deleted ApoE-null Mice

Shamsuzzaman et al. 2016
Deletion of the RANKL RL-D5 Enhancer Induces Osteopetrosis in Mice

**Rankl (Tnfsf11)**

- **Tibia**
  - Relative RNA Levels (x10^4)
  - * vs ApoE+/+;D5+/+
  - *# vs ApoE−/−;D5+/+

**Opg (Tnfrsf11b)**

- Relative RNA Levels (x10^4)
- * vs ApoE+/+;D5+/+
- * vs ApoE−/−;D5+/+

**BMD (g/cm²)**

- **Total Body**
  - 12 Weeks: * vs ApoE+/+;D5+/+
  - 18 Weeks: *# vs ApoE−/−;D5−/

- **Spine**
  - 12 Weeks: * vs ApoE+/+;D5+/+
  - 18 Weeks: *# vs ApoE−/−;D5−/

- **Femur**
  - 12 Weeks: * vs ApoE+/+;D5+/+
  - 18 Weeks: # vs ApoE−/−;D5−/
Analysis of Atherosclerotic Plaques by μCT

- Perfuse with 4% PFA
- Fix in 10% Formalin
- Perform μCT
- Clean Aorta
- Histology

WT  ApoE−/−

von Kossa
Reduced RANKL Expression in the Atherosclerotic Plaques of RL-D5 Enhancer Deleted Mice Delays the Progression of Calcification

**CONCLUSION**

RANKL plays a significant role in atherosclerotic plaque calcification, perhaps by promoting bone formation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency of Calcification</th>
<th>Presence of Fatty Streak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 Weeks</td>
<td>18 Weeks</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;;D5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0% (0/10)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;;D5&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>75% (6/8)</td>
<td>100% (8/8)</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;;D5&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>12.5% (1/8)</td>
<td>100% (8/8)</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;;D5&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>0% (0/10)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;;D5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>100% (8/8)</td>
<td>100% (8/8)</td>
</tr>
</tbody>
</table>
So What Have We learned About Enhancers?

• Located distal to, yet interact collectively at promoters

• Integrate multiple incoming signals at genes through modular and often hierarchical mechanisms

• Are highly dynamic during differentiation and disease

• Retain temporal, tissue- and hormone-specific expression properties in vivo

• Are active in disease settings, often in a unexpected manner

• Provide the mechanistic environment for the selective activity of SNPs that cause gene mis-expression

• May represent highly selective approaches for therapeutic targets