RAP-536 (Murine ACE-536/Luspatercept) Inhibits Smad2/3 Signaling and Promotes Erythroid Differentiation By Restoring GATA1 Function in Murine β-thalassemia

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ACE-536 is a Modified ActRIIB Receptor Fusion Protein

- ACE-536 is a fusion protein that consists of a modified activin receptor (ActRIIB) - a member of the TGFβ superfamily - and the Fc of human IgG1

- Inhibits Smad2/3 signaling and acts as a ligand trap – GDF8, GDF11, ActB

- Robust stimulation of RBC production in mice, rats, cynomolgus monkeys and humans

- Co-developed with Celgene

RAP-536 is murine ortholog of ACE-536
Effect of ACE-536 on Erythropoiesis

- ACE-536 treatment increases RBCs – however activity is distinct from EPO
- Effect is focused on the differentiation of erythroid precursors while EPO affects proliferation of BFU-E and CFU-E
- ACE-536 promotes differentiation of Baso, Poly, and Ortho Erythroblasts
- ACE-536 does not affect other cell lineages
- ACE-536 has corrected anemia in various murine preclinical models of ineffective erythropoiesis such as MDS and β-thalassemia

Suragani et al., Nature Medicine 2014
RAP-536 Decreases Elevated pSmad2/3, Corrects co-morbidities and Attenuates Anemia in a Murine Model of β-thalassemia (Hbb\(^{-/-}\))

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### RBC

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>βthal</th>
<th>βthal RAP-536</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^6 cells/μL)</td>
<td>9</td>
<td><strong>8</strong></td>
<td><strong>7</strong></td>
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</tbody>
</table>

### EPO

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>βthal</th>
<th>βthal RAP-536</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum erythropoietin (pg/mL)</td>
<td>2000</td>
<td><strong>2500</strong></td>
<td><em>1500</em></td>
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### Reticulocytes

<table>
<thead>
<tr>
<th></th>
<th>βthal</th>
<th>βthal RAP-536</th>
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</thead>
<tbody>
<tr>
<td>Retic (%)</td>
<td>60</td>
<td><strong>50</strong></td>
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***p < 0.001 vs wt; **p < 0.01, *p < 0.05 vs th1/th1

Suragani et al., Blood 2014
β-thalassemic mice were treated with VEH or RAP-536, splenic basophilic erythroblasts were sorted post 16hrs, and RNA was isolated for RNA-seq

Unbiased enrichment analysis - **GATA1**, NEF2, and HSF transcription factors are activated while NfkB, etc., are repressed
158 GATA1 Downstream Target Genes are Differentially Regulated by RAP-536 Treatment in Basophilic Erythroblasts

These data indicate that pSmad2/3 negatively regulates erythropoiesis as RAP-536 binds strongly to GDF11 – preventing downstream phosphorylation of Smad2/3

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Expression</th>
<th>Genes</th>
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<tbody>
<tr>
<td>Erythroid differentiation</td>
<td>Up</td>
<td>Gata1, Fog1, p21, Klf1, Hri, Atf4, Bcl-xl</td>
</tr>
<tr>
<td>Heme biosynthetic pathway</td>
<td>Up</td>
<td>Alas2, Abcb6, Ppox, Mthfr, Bach1, Fech</td>
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<tr>
<td>Protein quality control</td>
<td>Up</td>
<td>Nqo1, Prdx2, Sod2, Hsp’s, Psmc3</td>
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<tr>
<td>Proliferation and cell death</td>
<td>Down</td>
<td>Fos, Jun, Igf1r</td>
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<table>
<thead>
<tr>
<th>Gene Symbols</th>
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<tbody>
<tr>
<td>Gata1</td>
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<tr>
<td>Fog1</td>
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<td>p21</td>
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<td>Klf1</td>
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<td>Hri</td>
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<td>Prdx2</td>
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<td>Psmc3</td>
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<td>Fos</td>
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<td>Jun</td>
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<tr>
<td>Igf1r</td>
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RAP-536 Administration to β-thal Mice Increased GATA1 and Erythroid Specific Genes in *late* Basophilic Erythroblasts
Differentiating erythroid cells (MEL cells treated with DMSO) or Fetal liver cells

- GDF11
  +/− ACE-536

- Phosphorylation of Smad2/3
- GATA1
- Reactive Oxygen Species (ROS)

Normal: 5
Thalassemia patients: 19

pSmad2 – 60kDa
GAPDH – 37kDa

GDF11(100 ng/mL⁻¹), 30 mins

* p< 0.05
GATA1 levels were Decreased in the Nucleus with GDF11 Treatment – ACE-536 Reverses this Effect in MEL Cells
GATA1 levels were Decreased and Active Caspase 3 is Elevated Post GDF11 Treatment – ACE-536 Reverses this Effect in MEL Cells
GATA1 Levels are Restored in the Nucleus of β-thalassemic Murine BM Cells

**GATA1**  
**DAPI**  
**Merge**

**β-thalassemia**

**β-thalassemia + ACE-536**
Key Points

- GATA1 is decreased in the nucleus with GDF11 treatment
- Caspase 3/7 is elevated post GDF11 treatment – indicating the decrease of GATA1 is possibly due to cleavage by active Caspase 3
- Furthermore, an increase in GATA1 and erythroid genes was shown post ACE/RAP-536
- Because ROS is elevated in β-thal, we decided to investigate its role
Overview of NOX Enzymes

NOX Enzymes: NOX1, NOX2/Cybb, NOX3, NOX4, NOX5, DUOX1, DUOX2

Block et al., Nature Reviews 2012
George et al., Blood 2013
Effect of RAP-536 in a Murine Model of β-thalassemia ($Hbb^{th1/th1}$) on ROS

Suragani et al., Blood 2014
ACE-536 Decreases NOX4 (responsible for ROS production) in MEL Cells
RAP-536 Decreases NOX1 (responsible for ROS production) in β-thalassemic Mice to WT levels

![Normalized Expression of NOX1](chart.png)
RAP-536 also Decreases NOX2 and NOX4 (Responsible for ROS Production) in β-thalassemic Mice
GDF11 Treated Mice Show a Block in Differentiation and an Increase in NOX4
Model - β-thalassemia

- GDF11
- pSmad2/3
- ROS
- C-Cas-3

= Erythroid differentiation is inhibited

Nucleus = Erythroid differentiation is inhibited
Model - β-thalassemia + ACE-536

Block removed
Proper erythroid maturation

GDF11

ACE-536

ROS

pSmad2/3

C-Cas-3

Nucleus

GATA1
Conclusions

• Luspatercept (ACE-536) binds and inhibits signaling by certain Smad 2/3 signaling ligands

• RNA seq analysis revealed that ACE-536 upregulates genes involved in erythroid differentiation through GATA1

• GDF11 increases ROS and limits the availability of GATA1 in the nucleus

• ACE-536 inhibits ROS via a decrease in NOX enzymes and restores GATA1 availability

• Luspatercept is currently being tested in patients with β-thalassemia and MDS
Acknowledgements

• Dr. Suragani

• Dr. Pearsall

• Dr. Bhasin – Collaborator at Beth Israel Medical Center at Harvard

• Dr. Kumar

• Acceleron Team

• Celgene Team
GDF11 and other Smad2/3 Ligands Contribute to the Activity of Luspatercept

**Red Blood Cells**

- VEH
- RAP-536
- Anti-activin B
- Anti-GDF8/11
- Anti-actB + Anti-GDF8/11

**Hemoglobin**

- VEH
- RAP-536
- Anti-activin B
- Anti-GDF8/11
- Anti-actB + Anti-GDF8/11

Dosage: 10mg/kg, twice/week for 2 weeks, N= 5/group ** P<0.01, *** P< 0.001 vs VEH