Luspatercept inhibits pSMAD2/3 signaling and promotes erythroid maturation through a GATA1 dependent mechanism.

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Luspatercept is a Modified ActRIIB Receptor Fusion Protein

- Luspatercept 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 traps GDF8, GDF11, ActB
- Stimulates RBC production in mice, rats, cynomolgus monkeys and humans
RAP-536 Enhances Erythroid Maturation in WT mice

Suragani et. al, 2014
Differentially Regulated Genes with RAP-536 treatment Are Involved in Erythroid Maturation – β-thalassemic mice

These data indicate that pSmad2/3 negatively regulates erythropoiesis as RAP-536 binds ligands that prevent downstream phosphorylation of Smad2/3.
Western blot analysis of extracts from MEL cells untreated or GDF-11 (100ng/mL), ActB (1ug/mL), and ACE-536 (1ug/mL) treated. treated (+/- SB431542 inhibitor) MEL cells using pSMAD2 from Millipore. Treatments were for 30 and 60 minutes respectively. Cells were in MEM serum free media for 2 hours prior to treatment.

Based on these results, GDF-11 induces strong phosphorylation of pSmad2/3, which is completely ablated when treated with ACE-536 as expected, in both time points. However, ActB which also induces pSmad2/3 phosphorylation has a different offrate with ACE-536 compared to GDF-11. At 30mins, ActB + ACE-536 shows ablation of pSmad2/3, however at 60 minutes the effect is clearly gone.
pSmad2/3 Levels are increased with GDF11 and decreased with ACE-536 treatment
Increased pSmad2/3 in GDF11 treated MEL cells
Cell size is normal in MEL cells treated with DMSO
Cell size is compromised in MEL cells treated with GDF11 – larger cells
Cell size is restored to a smaller diameter post treatment with ACE536
GDF11 prevents condensation of nucleus = larger cells

DAPI

pSmad2/3
Luspatercept Treatment Increases Nuclear Accumulation of GATA-1

Control

GDF11

ACE-536

MFI - GATA1

Control

GDF11

GDF11 + ACE-536

DAPI

GATA1
ACE-536 Treatment Prevents GATA-1 Degradation

Nuclear extracts

<table>
<thead>
<tr>
<th>Time</th>
<th>DMSO</th>
<th>GDF11</th>
<th>ACE-536</th>
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<td>48hr</td>
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GATA1

Caspase 3/7 Flow

- MFI - Caspase 3/7

DMSO | GDF11 | GDF11 + ACE-536

p < 0.05*
Transgenic Mice Overexpressing GDF11 with enhanced pSmad2/3 signaling have Reduced Erythroblasts

Control

WT - Tamoxifen

GDF11 Overexpression

n=5 per group. Treated for 3 weeks **** p<0.0001
GATA1 and TER119 double positive population in the BM decreases *in vivo* in GDF11 overexpression mice.
What is the molecular link between pSmad2/3 and GATA1?
TIF1γ competes with Smad4 to form a complex with Smad2/3 that promotes erythropoiesis.

He et al., 2006

Monteiro et al., 2011
MEL Control – TIF1γ localizes primarily in the nucleus

He et. al, Cell 2006
MEL GDF11 treatment – TIF1γ localizes to the cytoplasm to a greater extent

He et. al, Cell 2006
TIF1γ locates mostly to the cytoplasm of MEL cells upon GDF11 treatment
TIF1γ in the nucleus of MEL cells is decreased upon treatment with GDF11

Control | GDF11 Treated | ACE536 Treated

TIF1γ

White area – co-localization area of TIF1γ with the nucleus

DAPI
TIF1γ co-localization is significantly decreased in GDF11 treated MEL cells and restored upon ACE536 treatment.
TIF1γ is decreased \textit{in vivo} in GDF11 overexpression mice due to elevated pSmad2/3.
Key messages

• pSmad2/3 signaling pathway negatively regulates erythroid differentiation

• RAP-536/Luspatercept treatment inhibits pSmad2/3 signaling and promotes erythroid maturation in β-thalassemia

• Block of terminal erythroid maturation in GDF11 over-expression mice due to increased pSmad2/3 signaling

• Mechanistically, Luspatercept treatment increased nuclear accumulation of GATA-1

• Important to note that GDF11 is one amongst the ligands that are trapped by Luspatercept that cause elevated pSmad2/3, but not the only player in this signaling pathway
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