Sustained β-catenin activation in dermal fibroblasts promotes fibrosis by regulating cell proliferation and extracellular matrix protein-coding genes

by

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Role of Wnt/β-catenin signaling in fibrosis.

- Well established link Wnt signaling and desmoid tumors.
- What is the actual role of Wnt signaling in the etiology of fibrosis?
- Who are the causative cell types?
Fibrotic skin has elevated fibroblast β-catenin activity

Patient skin samples generously provided by Monique Hinchcliff & John Varga (Northwestern) and Kord Honda (UH Dermatology)
Wnt activation in other fibrosing diseases

- Dupuytren disease/superficial fibromatosis
- Idiopathic pulmonary fibrosis
- Chronic obstructive pulmonary disease (COPD)
- Liver cirrhosis
- Myelofibrosis
- Morphea
- Keloid & hypertrophic scars
- Post-MI scar
- Diabetic nephropathy
- Nephrogenic systemic fibrosis
- Systemic sclerosis
- Chronic GVHD

Adapted from *Fibrosis Research* (Varga et al, 2005)

+ Tumor stroma
Hypothesis

Activation of Wnt/β-catenin signaling in resident dermal fibroblasts can cause dermal fibrosis.
Advantages of genetic mouse models

- Manipulating a single causative cell type at a time: resident dermal fibroblasts
- Modulating a single signaling/factor at a time: Wnt signaling.
- Analyze whole dermis/obtain dermal fibroblasts at various time points after manipulating Wnt signaling.
- Identifying context-specific Wnt signaling targets in dermal fibroblasts.
- Wnt signaling sensitive biomarkers from specific cell types
- Testing therapeutic drug targets *in vivo*.

Our three genetic models

**In vivo** dermal fibrosis model: Genetically stabilize β-catenin in dermal fibroblasts.

Advantages: in vivo, cell-type restricted manipulation, analyze effect on the whole skin
Disadvantages: Difficult to isolate dermal fibroblasts from adult skin for molecular analysis.

**In vitro** dermal fibroblasts model from perinatal skin: Genetically stabilize β-catenin in dermal fibroblasts.

Advantages: dermal fibroblasts for analysis, molecular/genomics
Disadvantages: in vitro, culture conditions.

**Inducible/Deinducible over expression of stabilized β-catenin in dermal fibroblasts**

Advantages: Identify which aspects of the fibrotic phenotype requires sustained expression of β-catenin.
Disadvantages: Duration of deinduction is not known, difficult to isolate dermal fibroblasts from adult/fibrotic skin for molecular analysis.
Model 1: Genetically stabilize β-catenin activity in resident dermal fibroblasts

**control**

HoxB6CreER/++; R26R-YFP/+

**mutant**

HoxB6CreER/++; R26R-YFP/++; β-catenin$^{stab}$/+

![Diagram showing the genetic mechanism and expression analysis](image)
Sustained stabilized β-catenin activity in resident dermal fibroblasts leads to dermal fibrosis
Sustained stabilized β-catenin activity in dermal fibroblasts leads to increase in collagen deposition with thickened collagen fibrils.

- **Dermis**
  - Control: OH-pro (μg) per 5-mm punch biopsy
  - Mutant: OH-pro (μg) per 5-mm punch biopsy
    - P53: Control: 125, *P=0.004*, Mutant: 500
    - P100: Control: 125, *P=0.002*, Mutant: 500

- **Hypodermis**
  - β-catenin:
    - ↓ fibril diameter in fibrotic dermis
    - ↑ fibril diameter in fibrotic hypodermis

- Col1a1
  - *p=0.03*

- Fiber = bundle of fibrils
Sustained stabilized β-catenin activity in dermal fibroblasts leads thickened skin and increase in

**Increased skin thickness:**

![Graph showing increased skin thickness](image)

**Increased fibroblast proliferation:**

![Graph showing increased fibroblast proliferation](image)
What is the mechanism of pro-fibrotic β-catenin in dermal fibroblasts?

- How does dermal gene expression change in response to expression of stabilized β-catenin?
- Which of this β-catenin-responsive genes are also dysregulated in human fibrotic tissues?

**Hypothesis:**

Elevated nuclear β-catenin activity in dermal fibroblasts

Δ amount of matrix proteins?
Δ post-translational modifications to matrix proteins?
Δ turnover of matrix proteins?
Measuring differentially expressed genes in stabilized β-catenin
After 3 weeks in whole dermis in vivo

1. Isolate whole dermis RNA (all transcribed genes)
2. RNA-sequencing & alignment of transcribed genes
3. Determine differentially expressed genes
Stabilized β-catenin results in increased expression of 175 genes
β-catenin-responsive matrisome genes are putative targets of TCF binding

16 matrisome genes have ↑ expression & Tcf/Lef binding sites:
Model #2: Early responders of Wnt/β-catenin signaling in dermal fibroblasts after 4 days in vitro

(1) Isolate dermal fibroblasts, culture 3 days, AdCre infect, verify recombination
(2) Mouse dermal fibroblasts RNA (all transcribed genes, IncRNAs)
(2) RNA-sequencing & alignment of transcribed genes
(3) Determine differentially expressed genes

Nathaniel Mullin ‘16
Early target genes of Wnt/β-catenin signaling in dermal fibroblasts are enriched for matrisome genes

- **Input/output considered**: Upregulated genes (FC > 1.5, p< 0.05) from B-cat. Stab P4 ventral dermal fibroblasts (in vitro)

Functional annotation clustering of Genes FC>1.5 GOF P4 Ventral Dermal Fibroblasts

<table>
<thead>
<tr>
<th>Representative annotation terms</th>
<th>Enrichment Score</th>
<th>% of total genes present in cluster</th>
</tr>
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<tbody>
<tr>
<td>1 Glycoprotein</td>
<td>10.10</td>
<td></td>
</tr>
<tr>
<td>2 Glycoprotein/Membrane</td>
<td>8.01</td>
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<tr>
<td>3 Vessel development</td>
<td>6.52</td>
<td></td>
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<tr>
<td>4 Pleckstrin Homology</td>
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<tr>
<td>5 Lipoprotein</td>
<td>4.70</td>
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</table>
Early responders to Wnt/β-catenin signaling in dermal fibroblasts are targets of Epigenetic repression and poised for regulation by Wnt signaling.

- **Input data:** All DE genes (p<0.05) from B-cat. Stab P4 ventral dermal fibroblasts (in vitro).
- **MSigDB Gene Set selected:** Ben-Porath PRC2 targets (ranked #2 of all C2 gene sets; NES = 2.640442, p<.001, FDR = 2.79E-05).

**Representative annotation terms:**

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Enrichment Score</th>
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<tr>
<td>Signal/ disulfide bond</td>
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<td>Wnt Signaling</td>
<td>2.42</td>
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<tr>
<td>Growth Factor/ TGFβ</td>
<td>2.39</td>
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<tr>
<td>Regulation of signal transduction</td>
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</table>

Gene sets most enriched from *c2 Curated Gene Sets*. Selected by GSEA, FDR < 0.05, FWER p Value < 0.05, Nom. p Value < 0.001.
### Genes Upregulated in desmoid tumor & In β-catenin stabilized dermal fibroblasts

<table>
<thead>
<tr>
<th>Genes</th>
<th>EARLY B-cat Stab. (P4) (1535)</th>
<th>LATE B-cat Stab. (P21) (171)</th>
<th>Both EARLY and LATE</th>
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<tbody>
<tr>
<td>Desmoid Tumor (1556) (Bacac et al.)</td>
<td>162</td>
<td>51</td>
<td>8</td>
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<tr>
<td>Desmoid Tumor and β-Catenin Chip-seq (Watanabe et al. 2014)</td>
<td>MYO1F</td>
<td>AXIN2 WNT11 HYAL1 SYK SMAD7</td>
<td>--</td>
</tr>
</tbody>
</table>

**Upreg in P21, P4 and Desmoid Tumor**

- Cd53
- Cadps
- Ahr
- Qpct
- Twist1
- Rarres1
- Col7a1
- Nfatc4
On our way to targeting the Wnt pathway

• Test whether Wnt/β-catenin signaling activation needs to be maintained to continue the fibrotic state.

• Wnt is a well-appreciated therapeutic target. . .
  – Cross talk with other pathways
  – There are many mesenchymal derivatives that are implicated in various human and animal models
  – How to measure the therapeutic efficacy of systemic Wnt inhibitors on fibroblast-specific effects \textit{in vivo}?
  – Wnt responsive biomarkers in specific cell types.
  – Develop guides for our expectation to evaluate the effect(s) of Wnt/β-catenin inhibitors (antiproliferative or ECM modulator).
Model#3: New genetic model of Inducible/deinducible β-catenin in mouse dermal fibroblasts

A

Engrailed1
Cre recombinase

R26-rtTA
neopApApA
rtTA-IRES-EGFP-pA

β-catenin
Tet-O
ΔN89β-catenin

Cre recombinase

B

**Induction**

1 Week Induction

+/+; R26rtTA/+
En1cre/+; R26rtTA/+

3 Week Induction 5 Week Deinduction

+/+; R26rtTA/+
En1cre/+; R26rtTA/+; TetOβ-cat/+
Deinducing β-catenin in dermal fibroblasts does not amelioration of matrix in 5 weeks.
Deinducing β-catenin leads to recovery of cell number and may be adipose tissue.
Summary

- Increased dermal fibroblasts in fibrotic human skin express nuclear β-catenin.
- Expression of stabilized β-catenin in mouse dermal fibroblasts is sufficient for skin fibrosis.
- Stabilized β-catenin in dermal fibroblasts produces fibrotic extracellular matrix by altering expression of matrisome genes that may be targets of epigenetic regulation.
- Sustained expression of stabilized β-catenin is not required for matrix turnover, but for fibroproliferative response.
Inspired by my father,
Oct 24, 1941-2014
Thanks so much

Atit lab Present/Past members: Thu Tran, Ph.D., Peggy Myung M.D./Ph.D, Henry Goodnough, M.D./Ph.D., Demeng, Ph.D., James Ferguson, Jennifer Ohtola, Adrie Welsh, Ozimba Anyangawe, Nikhil Mallipeddi, Mahima Devarajan

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March of Dimes, Scleroderma Research Foundation, Global Fibrosis Foundation, Skin Disease Research Center (CWRU).
Active Wnt/β-catenin signaling might be due to increased expression of Wnt ligands

↑ WNT4, WNT10A, and WNT10B in SSc skin

↑ Wnt1 protein in SSc skin

↑ AXIN2 in SSc skin

AXIN2 = Wnt/β-catenin target gene

Beyer et al. (2012)
β-catenin-responsive genes are also up-regulated in human fibrotic tissues

Publicly available microarrays (10 total)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>SSc skin</th>
<th>Tumor stroma</th>
<th>IPF lung</th>
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Scleroderma biopsy & fibroblasts
Tumor stroma
IPF lung biopsy & fibroblasts
Dupuytren contracture fibroblasts
Keloid fibroblasts

Nathaniel Mullin ‘16

↑ in a private scleroderma dataset
Are the differentially upregulated matrisome genes potential transcriptional targets of Wnt/beta-catenin signaling?

* Which of these genes encode matrix proteins?

Differentially expressed genes

Genes poised for beta-catenin regulation

*
β-catenin-responsive matrisome genes are putative targets of TCF binding

oPOSSUM: 2 statistical measures of over-represented transcription factor binding sites (TFBS)

- Fisher score ~ proportion of the gene set that includes a TFBS / proportion of background genes that include that TFBS
- Z-score ~ rate of occurrence of a TFBS in gene set / rate of occurrence of that TFBS in background genes