Therapy resistant recurrence in breast cancer: ING4 tumor suppressor and immune modulation

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City of Hope 4th Annual Gayle Brinkenhoff Breast Cancer Symposium
November 14, 2017
DISCLOSURES

I do not have anything to disclose.
ING4 tumor suppressor in breast cancer

**Myc** suppression by ING4 – discovery and mouse study

**NF-κB** inhibition by ING4 – **inflammation and therapy resistance**

**ER** inhibition by ING4 – tamoxifen vs fulvestrant resistant recurrence

Molecular Mechanism of ING4?
“Conditional transcription regulation”
Screen for genes that suppress MYC induced loss of contact inhibition


Rat1A N-mycER/tTA

→ cDNA expression library (TRE)

→ Confluent monolayer

AZT/5-FU

→ Surviving cells

4-OHT

→ Surviving cells

21 days

Molecular identification of cDNAs by PCR/Sequencing

→ ING4

14 days

2 days
Genomic deletion of ING4 in T47D human breast cancer cell line

Southern blot

<table>
<thead>
<tr>
<th>Probe</th>
<th>H23</th>
<th>H82</th>
<th>HeLa</th>
<th>T47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>ING4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H23  lung adenocarcinoma
H82  small cell lung carcinoma
HeLa  cervical cancer
T47D  breast ductal carcinoma
CGH (Comparative Genomic Hybridization) analysis of deletion in the ING4 locus (12p13.3)

55 breast cancer cell line data set (UCSF Cancer Center - K. Chin and J.W. Gray): 11-24% have ING4 locus deletion
10-20% deletion in the ING4 locus in 146 primary human breast tumors

CGH data set (UCSF Cancer Center - Chin K, Gray JW, and Waldman FM)

In the literature:
Chromosome 12p13 LOH
5-26% childhood ALL
12-43% prostate cancer
26% ovarian cancer
Suppression of MYC-induced mouse mammary hyperplasia/tumors by ING4
ING4 attenuates MYC-initiated mouse mammary tumors

MMTVrtTA/TRE-MYC/TRE-ING4-IRES-GFP

Decreased tumor penetrance by 20% - suppression of tumors
Increased average tumor latency by 2 months - delayed onset

“In vivo evidence” for ING4 tumor suppressor function in breast cancer

Patient data?
Fluorescent In situ Hybridization (FISH) to detect ING4 deletion

Coya Tapia, MD

BAC clone
Bacterial Artificial chromosome

Ch12 centromere
ING4 BAC

Normal lymphocyte metaphase

T47D breast cancer cells metaphase

Normal breast interphase
Breast Tumor Tissue Microarray (TMA) survey of ING4 deletion using FISH
### ING4 Deletion in 16.5% of all breast cancers

FISH on TMAs (Institute for Pathology, University of Basel, Switzerland)

<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>ING4 (n=1,033)</th>
<th></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deletion (n=73)</td>
<td>No deletion (n=392)</td>
<td></td>
</tr>
<tr>
<td>Luminal A (ER+PR+HER2-)</td>
<td>25 (14.5%)</td>
<td>148 (85.5%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Luminal B (ER+PR+HER2+)</td>
<td>1 (20%)</td>
<td>4 (80%)</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>HER2</strong> (ER-PR-HER2+)</td>
<td><strong>23 (28.4%)</strong></td>
<td><strong>58 (71.6%)</strong></td>
<td>0.002</td>
</tr>
<tr>
<td>Basal-like (ER-PR-HER2-)</td>
<td>24 (11.7%)</td>
<td>182 (88.3%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

### ING4 deletion is prevalent in HER2+ breast cancer

Low ING4 expression correlate with poor disease-free survival in breast cancer

NKI295 data set: DFS in all tumors

![Graph showing disease-free survival comparison between high and low ING4 expression](graph.png)

- **p = 0.0355**
- **Hazard Ratio: 1.549**
- **95% CI of ratio: 1.028-2.208**
Low ING4 expression = faster breast cancer recurrence

NCBI GEO data set GDS806 (Gene expression profile data)
Primary ER+ tumors from patients who did not recur (n=32) and who recurred (n=28) with adjuvant tamoxifen therapy

\[
\text{ING4} \quad p=0.0181
\]

\[
\begin{align*}
\text{Disease Free Survival} \\
0 & 25 & 50 & 75 & 100 & 125 & 150 & 175 \\
0 & 25 & 50 & 75 & 100 & 125 & 150 & 175 \\
0 & 25 & 50 & 75 & 100 & 125 & 150 & 175 \\
0 & 25 & 50 & 75 & 100 & 125 & 150 & 175 \\
\end{align*}
\]

Hazard Ratio \quad 3.22
95% CI of ratio \quad 1.241-5.632
No correlation between other ING family member expression and breast cancer recurrence.
34% of breast tumors express low ING4 protein levels correlative with aggressive breast tumor features
breast tumor tissue microarray (n=249)

<table>
<thead>
<tr>
<th>Pathologic feature</th>
<th>ING4 &lt;1.5</th>
<th>ING4 ≥1.5</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77 (33.9%)</td>
<td>150 (66.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological subtype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCIS only</td>
<td>0</td>
<td>6 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>51 (32.1%)</td>
<td>108 (67.9%)</td>
<td>1</td>
</tr>
<tr>
<td>Lobular carcinoma</td>
<td>6 (30.0%)</td>
<td>14 (70.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2.0cm</td>
<td>8 (27.6%)</td>
<td>21 (72.4%)</td>
<td>0.207</td>
</tr>
<tr>
<td>≥ 2.0cm</td>
<td>47 (40.9%)</td>
<td>68 (59.1%)</td>
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</tr>
<tr>
<td><strong>BRE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>8 (28.6%)</td>
<td>20 (71.4%)</td>
<td>0.284</td>
</tr>
<tr>
<td>Grade 2</td>
<td>29 (40.3%)</td>
<td>43 (59.7%)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>17 (40.5%)</td>
<td>25 (59.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>23 (34.8%)</td>
<td>43 (65.2%)</td>
<td>0.143</td>
</tr>
<tr>
<td>&gt; N0</td>
<td>19 (51.4%)</td>
<td>18 (48.6%)</td>
<td></td>
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</table>
Inverse association **ING4 vs NF-κB**

Breast tumor microarray survey

<table>
<thead>
<tr>
<th>Normal Breast tissue</th>
<th>ING4</th>
<th>Activated NF-kB p-p65 (ser276)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>![Image A]</td>
<td>![Image B]</td>
</tr>
<tr>
<td>Invasive Papillary carcinoma</td>
<td>![Image C]</td>
<td>![Image D]</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>![Image E]</td>
<td>![Image F]</td>
</tr>
</tbody>
</table>
Low ING4 prevalent in breast tumors with NF-κB activation (phospho-p65 subunit)

ING4 $\downarrow$ NF-κB $\uparrow$

In Low ING4 tumors, NF-κB activation is prevalent.

% tumors per group

- Total
- ER+
- ER-
- HER2-
- HER2+
- p-p65-low
- p-p65-high

p-values:
- p=0.429
- p=1.000
- p=0.018

ING4-low
ING4-high
Canonical NF-κB activation

Stress
UV, metal, heat shock

Virus, bacteria
Toll-like receptor (TLR)

Oxidative stress

Growth signal
EGF, HER2

TNFα, RANK

BCR/TCR

Inflammatory signal

IKKα
IKKβ

IKKγ

NF-κB
p65
p50

Ub

Nucleus

Transcription activation of genes
Cytokines, chemokines, etc
600-800 genes

Cell invasion
**ING4 inhibits NF-κB in breast cancer cells**


---

**Inflammatory signal**

- **pMIG**
  - ING4
  - p65
  - p-p65(ser536)
  - Histone H3

- **p65**
  - p-p65(ser536)
  - Tubulin

**Nucleus**

- **p65**
  - p-p65(ser536)

**Cytosolic**

- **p65**
  - p-p65(ser536)
  - Tubulin

**Western blot**

- **ING4**
- **qRT-PCR**

**Boyden chamber assay**

**Cell invasion**

**Relative expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vector</th>
<th>ING4</th>
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<tbody>
<tr>
<td>IL8</td>
<td><img src="chart1.png" alt="Bar chart" /></td>
<td><img src="chart2.png" alt="Bar chart" /></td>
</tr>
<tr>
<td>IL6</td>
<td><img src="chart3.png" alt="Bar chart" /></td>
<td><img src="chart4.png" alt="Bar chart" /></td>
</tr>
<tr>
<td>PTGS2 (COX2)</td>
<td><img src="chart5.png" alt="Bar chart" /></td>
<td><img src="chart6.png" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

**Cell number per field**

- **pMIG**
- **ING4**

**Byron et al (2012)** PLoS ONE

**ING4 inhibits NF-κB in breast cancer cells**

**NF-κB**

**IKKα**

**IKKβ**

**IKKγ**

**NF-κB**

**p65**

**p-p65(ser536)**

**Histone H3**

**Tubulin**

**INHIBITION**

**Boyden chamber assay**

**qRT-PCR**

**Western blot**

**Cell invasion**
ING4 inhibits NF-κB in breast cancer cells

MCF7

<table>
<thead>
<tr>
<th>pMIG</th>
<th>ING4</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ING4
p65
p-p65(ser536)
Histone H3

Nuclear

Cytosolic

p65
p-p65(ser536)
Tubulin

Western blot

NF-κB

p65
p50
Ub

qRTPCR

Boyden chamber assay

Cell number per field

Cell invasion

Relative expression

IL8

IL6

PTGS2 (COX2)

PMA

pMIG

ING4

ING4 inhibits NF-κB in breast cancer cells

ING4 represses NF-κB-target genes in breast cancer

Sara Byron, PhD

PMA induces 35/84 NF-kB target genes
ING4 suppresses 27/35 genes 2-fold
27 and 14 ING4-repressed NF-κB-target gene signature

Fold Increase with PMA Treatment

- 4x repression
- 2x repression

14 gene signature
27 gene signature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR4A2</td>
<td>250</td>
</tr>
<tr>
<td>PTGS2</td>
<td>500</td>
</tr>
<tr>
<td>CXCL1</td>
<td>2x repression</td>
</tr>
<tr>
<td>CXCL10</td>
<td>4x repression</td>
</tr>
<tr>
<td>CCL5</td>
<td>2x repression</td>
</tr>
<tr>
<td>CCL2</td>
<td>4x repression</td>
</tr>
<tr>
<td>CCL2</td>
<td>2x repression</td>
</tr>
<tr>
<td>PLAU</td>
<td>4x repression</td>
</tr>
<tr>
<td>LTA</td>
<td>2x repression</td>
</tr>
<tr>
<td>IL8</td>
<td>2x repression</td>
</tr>
<tr>
<td>CBF</td>
<td>4x repression</td>
</tr>
<tr>
<td>NF-κB</td>
<td>2x repression</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>4x repression</td>
</tr>
<tr>
<td>EGR2</td>
<td>2x repression</td>
</tr>
<tr>
<td>NFKB1</td>
<td>4x repression</td>
</tr>
<tr>
<td>BIRC3</td>
<td>2x repression</td>
</tr>
<tr>
<td>IFNB1</td>
<td>4x repression</td>
</tr>
<tr>
<td>GADD45B</td>
<td>2x repression</td>
</tr>
<tr>
<td>SELE</td>
<td>4x repression</td>
</tr>
<tr>
<td>MYC</td>
<td>2x repression</td>
</tr>
<tr>
<td>RELB</td>
<td>4x repression</td>
</tr>
<tr>
<td>IFN1</td>
<td>2x repression</td>
</tr>
</tbody>
</table>
14 inflammatory gene signature associates with poor disease free survival in breast cancer

25 NF-κB- target gene signature

14 NF-κB- target gene signature

Disease Free Survival

Time (months)

Low NFκB Score
High NFκB Score

p=0.5421
Hazard Ratio 1.123
95% CI of ratio 0.6499-1.596

p=0.0413
Hazard Ratio 2.23
95% CI of ratio 1.03-4.57
“ING4-regulated” inflammatory gene signature associates with poor disease free survival in breast cancer.

ING4

Hazard Ratio: 3.22
95% CI of ratio: 1.241-5.632

p = 0.012

14 NFkB-target gene signature

Hazard Ratio: 2.23
95% CI of ratio: 1.03-4.57

p = 0.0413
“The plot thickens”
Breast TMA survey for immune cells (n=185)

CD68
Macrophages

CD4
T helper cells

CD8
Cytotoxic T cells

CD20
B cells

<table>
<thead>
<tr>
<th></th>
<th>ING4 &lt;1.5</th>
<th>ING4 ≥1.5</th>
<th>p</th>
<th>NF-κB &lt;1.5</th>
<th>NF-κB ≥1.5</th>
<th>p</th>
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<tbody>
<tr>
<td><strong>CD68</strong></td>
<td>&lt;1.5</td>
<td>9%</td>
<td>9%</td>
<td>1</td>
<td>12%</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>≥1.5</td>
<td>90</td>
<td>90</td>
<td></td>
<td>87</td>
<td>91</td>
</tr>
<tr>
<td><strong>CD4</strong></td>
<td>&lt;1.5</td>
<td>79</td>
<td>72</td>
<td>0.36</td>
<td>74</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>≥1.5</td>
<td>20</td>
<td>27</td>
<td></td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td><strong>CD8</strong></td>
<td>&lt;1.5</td>
<td>56</td>
<td>62</td>
<td>0.50</td>
<td>63</td>
<td>49%</td>
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<tr>
<td></td>
<td>≥1.5</td>
<td>43</td>
<td>37</td>
<td></td>
<td>36</td>
<td>51%</td>
</tr>
<tr>
<td><strong>CD20</strong></td>
<td>&lt;1.5</td>
<td>96</td>
<td>90</td>
<td>0.24</td>
<td>90</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>≥1.5</td>
<td>3</td>
<td>9</td>
<td></td>
<td>9</td>
<td>7</td>
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</tbody>
</table>

NF-κB activation → CD8+ T cells
(CXCL10 chemokine = CD8+ T cell recruitment)
ING4 deficiency-correlated exhausted T cell immune environment

<table>
<thead>
<tr>
<th>ING4 positive</th>
<th>pp65</th>
<th>CD4</th>
<th>CD8</th>
<th>PD-1 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="ING4_positive" alt="Image" /></td>
<td><img src="pp65" alt="Image" /></td>
<td><img src="CD4" alt="Image" /></td>
<td><img src="CD8" alt="Image" /></td>
<td><img src="PD-1_negative" alt="Image" /></td>
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</tbody>
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<tr>
<th>ING4 negative</th>
<th>pp65</th>
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<th>CD8</th>
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<tr>
<td><img src="ING4_negative" alt="Image" /></td>
<td><img src="pp65" alt="Image" /></td>
<td><img src="CD4" alt="Image" /></td>
<td><img src="CD8" alt="Image" /></td>
<td><img src="PD-1_positive" alt="Image" /></td>
</tr>
</tbody>
</table>
Working hypothesis

ING4-low/NF-κB activation/immune microenvironment (CD8+)

→ Recurrence (therapy resistance)

Tumor-initiated chronic inflammation

Tumor cells

ING4

NF-κB

MØ

CD8+ T cells

(Exhaustion → immune suppressive)
Working hypothesis and therapeutic opportunity

ING4-low/NFκB activation/immune microenvironment (CD8+)
→ Recurrence (therapy resistance)

Tumor cells

- ING4
- NF-κB

EGFR/Jak2 activation

Tumor-initiated chronic inflammation

- CD8+ T cells
- MØ
- Anti-chemokine agents
- Immune checkpoint Inhibitors (anti-CTLA-4, anti-PD-1)

Jakt inhibitors
EGFR pathway inhibitors

(Exhaustion → immune suppressive)
Therapy resistant recurrence of ING4-deficient breast tumors

- Heterotrophic interactions – immune cells (for now)
- Intra tumor Heterogeneity – ING4 expression levels
Single Cell RNAseq Analysis – normal breast

Tissue collection:
Lora Hebert, MD
Jenny Eschbacher, MD

Estimated Number of Cells
3,353

Mean Reads per Cell
50,858
Median Genes per Cell
1,290

Sequencing
- Number of Reads: 170,529,217
- Valid Barcodes: 98.1%
- Reads Mapped Confidently to Transcriptome: 53.3%
- Reads Mapped Confidently to Exonic Regions: 57.2%
- Reads Mapped Confidently to Intronic Regions: 18.8%
- Reads Mapped Confidently to Intergenic Regions: 7.8%
- Sequencing Saturation: 75.2%
- Q30 Bases in Barcode: 97.1%
- Q30 Bases in RNA Read: 73.2%
- Q30 Bases in Sample Index: 94.6%
- Q30 Bases in UMI: 96.9%
Epithelial cells:
- TFF1
- KRT7
- KRT8
- CLDN4
- MUCL1

Immune cells:
- CCL2
- IL1β
- HLAs
- IL6
- IL8
- CD36

Stroma fibroblasts:
- COL1A1
- COL1A2
- COL6A1
- COL6A2

Myoepithelial cells?
- KRT5

Endothelial cells?
- SOX7
- SOX17

Adipocytes:
- APOE
- TGFB1
- NR4A1

Epithelial cells:
- TFF1
- KRT7
- KRT8
- CLDN4
- MUCL1

Immune cells:
- CCL2
- IL1β
- HLAs
- IL6
- IL8
- CD36

3,353 Single Cells captured
50,853 Reads per Cell
1,290 Genes per Cell
20,345 Total Genes detected
**PROPOSAL** to identify therapy resistant clones during initial neoadjuvant therapy by single cell analysis

- Therapy response assessment
- Biomarker discovery
- Therapeutic targets
- Precision medicine

**Pre therapy biopsy**

**Post therapy resection**

Therapy resistant clone

de novo
Resistant clone

Immune cells?
Acknowledgement

Kim Lab
Jeremiah Stricklin
Paul Heaton
Emily Szeto
Ashley Unger
Madeline Keenen (UCSF PhD program)
Tanya Thal (Retired)
Sara Byron, PhD (TGen)
Elizabeth Min (Yale PhD program)
Timothy Whitsett, PhD (Norton Thoracic Institute/St. Joe’s)
Coya Tapia, MD (University of Bern, Switzerland)

Collaborators
Janine LoBello, DO (TGen/Ashion)
David Azorsa, PhD (Phoenix Children’s Hospital)
Daphne deMello, MD (Phoenix Children’s Hospital)
Galen Hostetter, MD (Van Andel Research Institute)
Cindy Miranti, PhD, Joyce Schroeder, PhD (University of Arizona)
Landon Inge, PhD (Norton Thoracic Institute/Dignity Health)
Michael Grant, MD; Jack Snipes, MD (Baylor Scott White)
Daruka Mahadevan, MD (UA Cancer Center), Will Hendricks (TGen)
Lora Hebert, MD (UACC/Dignity Health) Jenny Eschbacher, MD (Dignity Health)
Jonathan Keats, PhD (TGen), Venupresad Poojary (Baylor Immunology Institute)

Funding
NIH/NCI K01 CA115681
NIH/NCI T32 CA009213
DOD/CDMRP PC130616
AACR
Novartis Foundation
TGen-VARI Integration Grants
UACOM-PCH Seed Funding
Valley Research Partnership
Flinn Foundation
TGen-BSWRI Research Partnership
In order to visually represent the data, first each endpoint was normalized across all samples to have a mean of 0 and standard deviation of 1. Then the data are plotted in a matrix heatmap, high relative expression appears red and low relative expression is shown as green. For this data, the samples are ordered by increasing Her2 expression and endpoints are organized by molecular pathway affiliation.
Tumor-initiated immune modulation in BC therapy resistance

Multicolor immunofluorescent labeling on breast tumor

Jack Snipes, LuAnn Snipes, Brent Hart (Baylor University Medical Center)

Pink: cytokeratin
Yellow: CD68 (MØ)
Red: CD8 (Tc)
Green: CD4 (Th)
Blue: DAPI
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<td><img src="image4" alt="Image" /></td>
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<td><img src="image8" alt="Image" /></td>
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