Tumor transition states occurring during EMT

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Tumor heterogeneity

Inter-tumour heterogeneity

Intra-tumour heterogeneity

Marusyk A et al. Nat Rev Cancer 2012
Implication of EMT for tumor heterogeneity and metastasis

De Craene & Berx, Nat Rev Cancer 2013
Squamous cell carcinoma

- 2\textsuperscript{nd} most frequent cancer
- 500,000 patients /year in US
- Surgical excision
- 5% with metastasis leading to poor survival prognosis

Mutations of the \textit{Ras} pathway in mice and human SCC
\Rightarrow Kras (Spencer et al., 1995; Sutter et al., 1993; van der Schroeff et al., 1990, Nassar et al. Nature Medicine 2015)
\Rightarrow RRas2 (Nassar et al. Nature Medicine 2015)
Genetic lineage tracing in mouse model of skin SCC that undergo spontaneous EMT

Latil et al, Cell Stem Cell 2017
Loss of Epcam expression during EMT

![Image showing loss of Epcam expression during EMT](image-url)
Does EMT occur through distinct transitional states?

Plasticity through transitional states during EMT/MET

**EMT regulators**

**E/M score**

**Energy**

**EM1**
- Metastable
- Dissolution of junctions
- Loss of apico-basal polarity

**EM2**
- Stable
- Residual apical junctions

**EM3**
- Metastable
- Residual junction puncta
- Front-back polarity

**Epithelial**
- Stable
- Intact junctions
- Well developed polarity

**Mesenchymal**
- Stable
- No junctions
- Front-back polarity

**Residual junctions**

**Stromal interactions**
**ECM remodeling**

**Epithelial Promoters**
- GRHL2, OVOL1/2
- ESRP1/2
- miR200 family, miR34a

**Mesenchymal Promoters**
- SNAI1/2
- SRSF1, RBFOX2
- ZEB family, TWIST1

Nieto et al. Cell 2016
Identification of the tumor transition states occurring during EMT in vivo

Screening 176 cell surface markers

FACS analysis
YFP+ Ep+ and YFP+ Ep- tumor cells

Analysis of marker expression

Not expressed
Heterogeneously expressed
Homogeneously expressed
Identification of cell surface markers heterogenously expressed during EMT in vivo

Heterogeneously expressed markers

<table>
<thead>
<tr>
<th>CD24</th>
<th>CD51</th>
<th>CD61</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="CD24" alt="Histogram" /></td>
<td><img src="CD51" alt="Histogram" /></td>
<td><img src="CD61" alt="Histogram" /></td>
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</table>

<table>
<thead>
<tr>
<th>CD106</th>
<th>CD140a</th>
<th>CD147</th>
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<tbody>
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<td><img src="CD106" alt="Histogram" /></td>
<td><img src="CD140a" alt="Histogram" /></td>
<td><img src="CD147" alt="Histogram" /></td>
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Homogeneously expressed markers

<table>
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<tr>
<th>CD9</th>
<th>CD29</th>
<th>CD47</th>
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<table>
<thead>
<tr>
<th>CD49a</th>
<th>CD73</th>
<th>CD98</th>
</tr>
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<tbody>
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<td><img src="CD49a" alt="Histogram" /></td>
<td><img src="CD73" alt="Histogram" /></td>
<td><img src="CD98" alt="Histogram" /></td>
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</tbody>
</table>

Number of surface markers expressed:

- **Ep+**
  - Homogeneous: 24
  - Heterogeneous: 11

- **Ep-**
  - Homogeneous: 2
  - Heterogeneous: 17

**Figure 1**
Identification of the tumor transition states occurring during EMT in vivo
Uncovering the order of transition during EMT
EMT transition states revealed by tumor by single cell RNA-seq

![Graph showing EMT transition states](image)

- 

![Cumulative Density Plot](image)
Metaplastic mammary tumors progress through the same EMT transition states.
EMT transition states in MMTV-PyMT mammary tumors
EMT transition states in human cancer
EMT transition states present similar TPC capacity but exhibit different plasticity

<table>
<thead>
<tr>
<th>Number of grafted cells</th>
<th>Ep+</th>
<th>Ep-</th>
<th>TN</th>
<th>CD106</th>
<th>CD51</th>
<th>CD106/51</th>
<th>CD51/61</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>7/9 (n=3)</td>
<td>10/12 (n=3)</td>
<td>9/12 (n=2)</td>
<td>15/17 (n=2)</td>
<td>12/15 (n=3)</td>
<td>6/6 (n=2)</td>
<td>14/18 (n=3)</td>
<td>10/12 (n=2)</td>
</tr>
<tr>
<td>100</td>
<td>1/9 (n=3)</td>
<td>15/18 (n=4)</td>
<td>23/24 (n=4)</td>
<td>17/24 (n=4)</td>
<td>3/3 (n=3)</td>
<td>13/18 (n=3)</td>
<td>19/24 (n=3)</td>
<td>13/17 (n=4)</td>
</tr>
<tr>
<td>10</td>
<td>1/9 (n=3)</td>
<td>18/24 (n=3)</td>
<td>7/30 (n=4)</td>
<td>21/46 (n=4)</td>
<td>14/19 (n=3)</td>
<td>6/18 (n=3)</td>
<td>14/30 (n=3)</td>
<td>12/21 (n=3)</td>
</tr>
<tr>
<td>TPC frequency</td>
<td>1/614 (1/1266-1/297)</td>
<td>1/93 (1/159-1/54)</td>
<td>1/146 (1/246-1/86)</td>
<td>1/99 (1/156-1/63)</td>
<td>1/130 (1/246-1/68)</td>
<td>1/59 (1/99-1/35)</td>
<td>1/168 (1/285-1/99)</td>
<td>1/124 (1/226-1/69)</td>
</tr>
</tbody>
</table>

\[ p = 0.004 - 3.35e-08 \quad p = 0.0049 \quad p = 0.001 \]
Different EMT transition states present different metastatic potential.
Transcriptional landscape of EMT transition states

mRNA expression (fold change over TP)

Epithelial differentiation markers

Epithelial adhesion molecules

Basal lamina

Epithelial transcription factors

Splicing reg

Adhesion

EMT signalling

Extracellular matrix

Secreted molecules

EMT transcription factors

Krt14, Krt5, Tgm1, Iv1, Evpl, Cdh1, Itga3, Dsg2, Cldn4, L1cam, Lama3, Lamb3, Trp63, Ghr11, Ghr13, Ovol1, Esrp1, Cdh2, Pdgfra, Pdgfrb, Fap, Fn1, Lox, Loxl1, Col3a1, Col24a1, Mmp19, Mmp23, Ctgf, Snai1, Prrx1, Zeb1, Zeb2
Uncovering transcription factors operating during EMT at each transition states

(A) Epithelial tumor cells
   - Cdh1

(B) Early hybrid EMT cells
   - Krt5

(C) Late hybrid EMT cells
   - Vim

(D) Mesenchymal tumor cells
   - Mmp19

Epithelial tumor cells
Early hybrid EMT cells
Late hybrid EMT cells
Mesenchymal tumor cells

- p63
- Lhx2
- Nfatc
- Sox2
- Ap2g
- Klf5

- Ap1
- Nfl
- Sp1
- Runx
- Tead
- Nfl

Smad2/3
Nfatk
Zeb1
Grhl2

- Ap1
- Ets
- Ap1
- Ets
- Ap1
- Ets
- Ap1
- Nfl
- Ap1
- Nfl
- Ap1
- Nfl

bHLH
RbpJ
Ctcf

- Ap1
- Runx
- Ap1
- Runx
- Ap1
- Runx
- Ap1
- Tead
- Ap1
- Tead
- Nfl
- Nfl
- Nfl

TGFβ/Smad2 inhibition accelerates tumorigenesis and blocks EMT progression

a.

Lgr5CreER;Kras;p53;RYFP

- Control - Anti-TGF

% of Tumor free mice

% of EpCAM+ TCs

% in EpCAM- TCs

0 1 2 3 4 5 6 7 8 9 10
0 10 20 30 40 50 60 70 80 90 100
p=0.0006
p=0.02

p=0.0001
p=0.0007
p=0.013
NS
NS
NS

Control Anti-TGF

Control Anti-TGF

p=0.0001

Anti-TGF

TN CD106 CD51 CD106/51 CD51/61 TP

p=0.0007

Analysis Subpopulations (FACS)
Histological analysis (d-f)
FACS isolation subpopulations qPCR (g)
p63 overexpression blocks late steps of EMT progression

![Diagram of genetic modifications and EMT progression](image)
Different EMT transition states are localized in different niches.
Different EMT niches are associated with different immune and vascular infiltration.
Macrophages regulate EMT transition states

a. Diagram showing the timeline and treatments:

- Lgr5CreER;Kras;p53;RYFP
- 10mg Tamoxifen IP
- Anti-Csf1r and Anti/Ccl2 inhibitors

Timeline:
- 0w
- 5w
- 16w

b. Bar graph showing the number of tumors per mouse:

- Control
- Anti-Csf1r
- Anti/Ccl2

Number of tumors per mouse:
- Control: 3
- Anti-Csf1r: 2
- Anti/Ccl2: 1

Significant difference (*)

NS: Not significant

Analysis macropahes blood (FACS)

Number of tumors

Analysis Subpopulations (FACS)

Histological analysis

c. Bar graph showing the percentage of EMT tumor cells:

- Control
- Anti-Csf1r
- Anti/Ccl2

Significant difference (*)

NS: Not significant

d. Bar graph showing the percentage of Macrophages in total YFP cells:

- Control
- Anti-Csf1r
- Anti/Ccl2

Significant difference (*)

NS: Not significant

e. Line graph showing the percentage of Macrophages in total YFP cells:

- Control
- Anti-Csf1r
- Anti/Ccl2

Significant difference (*)

NS: Not significant

Legend:
- Control
- Anti Csfr1 Ccl2

Markers:
- TN
- CD106
- CD51
- CD106/51
- CD51/61
- TP

Histological analysis

Anti-Csf1r and Anti/Ccl2 inhibitors

Lgr5CreER;Kras;p53;RYFP

Macrophages regulate EMT transition states
Transition through the different EMT states

- EMT occurs through distinct transition states
- Early hybrid EMT exhibit the highest metastatic capacity
- Different transition states are associated with different microenvironment
- Depletion of macrophages prevents progression towards complete EMT

Pastushenko et al. Nature 2018
Role of Netrin-1 in EMT

Netrin1

Count per 20 million

Hybrid EMT → Full EMT

Unc5b

Count per 20 million

Hybrid EMT → Full EMT

Epcam+  TN  CD106  CD51  CD106/51  CD51/61  TP

0  1000  2000  3000  4000

0  1000  2000  3000  4000  5000  6000  7000  8000

0  100  200  300  400  500  600  700  800  900  1000

Comparison of Netrin1 and Unc5b expression in different subpopulations of EpCAM+ cells during EMT. The graphs show counts per 20 million for each subpopulation, indicating the role of Netrin-1 in EMT.
Netrin-1 inhibition reverts EMT upon subcutaneous grafting of Epcam- tumor cells

![Diagram showing the process of Netrin-1 inhibition and its effects on EMT](image)

**p=0.01**

**% Epcam+ in total TCs**

- Control
- NP137

![Images showing the expression of Krt14, Vim, and DAPI in Control and NP137 conditions](image)
Netrin-1 inhibition reduces tumorigenesis and prevents EMT in primary skin SCC

Impact of Netrin1 inhibition in tumor initiation and EMT in mouse model of HF-derived skin SCC
Netrin-1 inhibition reduces macrophage infiltration in primary skin SCC
Netrin-1 inhibition prevents lung colonization upon intravenous injection of Epcam- TCs
Regulation of EMT and metastasis through modulation of the microenvironment

Pastushenko & Lengrand, unpublished data
Can genetic hints stabilize specific EMT state?
Fat1 mutations in human cancers

- Head and neck SCCs
- Lung SCCs
- Oral SCCs

Pan Cancer

Lung SCCs

- Significantly mutated genes
- Focal amplifications
- Focal deletions

**Significantly mutated genes**
- KRAS
- KEAP1
- EGFR
- STK11
- ARID1A
- NRAS
- PIK3CA
- TP53
- RB1

**Focal amplifications**
- MCL1
- MDM2
- TERT
- MYC
- ERBB2
- MET
- 19p13.1
- CDK6
- REL-BCL11A

**Focal deletions**
- CDKN2A
- PTEN
- FAT1
- FOXP1

**Pan Cancer**

- panCancer
- FAT1
- ARID2
- SMAD4
- SMAD5
- SMAD7
- WWOX
- B3M
- PD34D
- LRPIB

**Mutations in Fat1**

- Mutations in human cancers: 23%
- Mutations in Head and neck SCCs: 99%
- Mutations in Lung SCCs: 99%
- Mutations in Oral SCCs: 99%

**Significance**

- q value (combined pan-cancer cohort)
- q value (most significant tumour type)

**Analysis**

- Mutations in Fat1 are significantly higher in Head and neck SCCs compared to other cancer types.
- Gene expression analysis indicates potential targets for therapeutic intervention.
Fat1 deletion promotes hybrid EMT in SCC
Deletion of Fat1 promotes hybrid EMT state in human SCCs
Somatic mutations in Fat1 is associated with hybrid EMT state in human cancers
Fat1 deletion increases lymph node and lung metastasis

**Figure 4**

(a) % of mice with LN mets

(b) % of LN mets

(c) % of LN mets

(d) % of LN mets

(e) % of mice with Lung mets

(f) # of Lung metastasis

(g) # of Lung metastasis

(h) K14 Vim

Pastushenko et al, under review
• EMT occurs through distinct transition states
• Early hybrid EMT exhibit the highest metastatic capacity
• Different transition states are associated with different microenvironment
• Depletion of macrophages prevents progression towards complete EMT

• Role of microenvironment in the modulation of EMT can be explored as therapeutic opportunity
• Specific mutations can stabilize highly metastatic hybrid EMT state and should be be explored as predictive factor of poor outcome