

研究例 4

細胞の癌化・転移機構の解析

Sustained expression of LIP, a short repressive isoform of C/EBPβ, leads to epithelial-mesenchymal transition (EMT) in the mammary epithelial cells

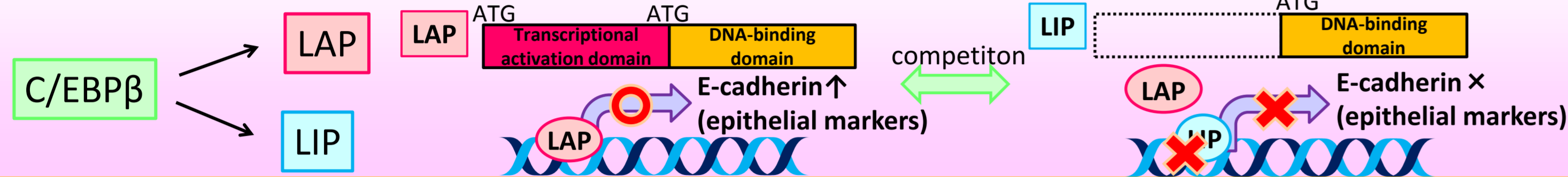
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1. Background

Upon oncogenic transformation, epithelial cells temporarily display a mesenchymal phenotype (EMT), in which a transcription factor Snail1 plays a causal role for various epithelia. Recently, another transcription factor CCAAT/enhancer binding protein C/EBPβ has been implicated to elicit the similar response in mammary epithelial cells. C/EBPβ mRNA has two translation initiation sites in its open reading frame, thereby is translated into two isoforms, LAP (liver-enriched activating protein) that is composed of the transcription and DNA binding domains, and LIP (liver-enriched inhibitory protein) that has solely DNA-binding domain. LIP is known as a competitor of LAP, and it cannot activate transcription of LAP targets. However, no difference between the amount of LAP protein and LIP protein was observed in epithelial and mesenchymal cells. Therefore, we focused on the temporal off-balance of expression level of LAP / LIP.

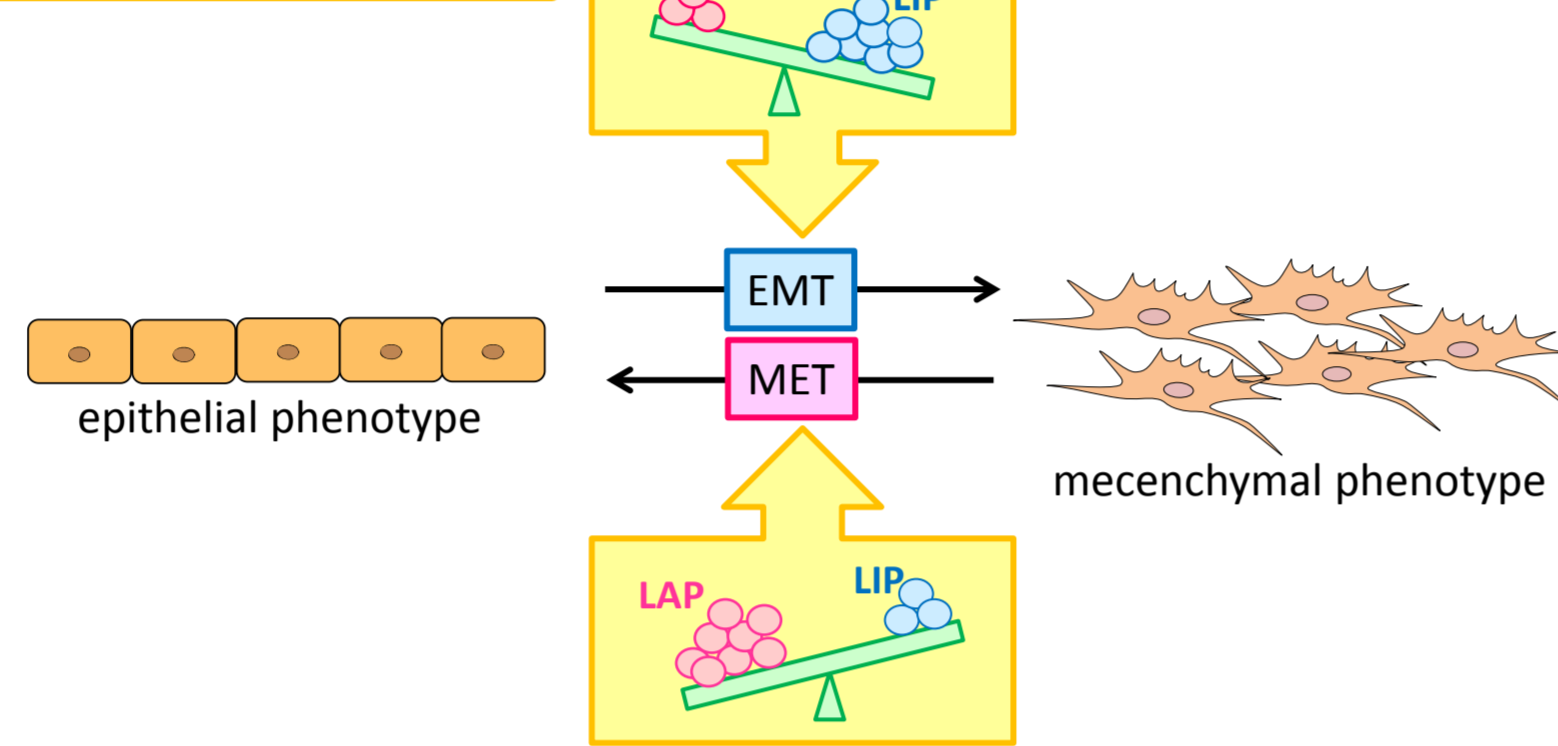


2a. Aim

Elucidation of the possible involvement of LAP / LIP in epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET).

- Forced expression of LIP in epithelial cells leads to EMT?
- Forced expression of LAP in mesenchymal cells leads to MET?

2b. Hypothesis



3. Model cells and markers for EMT / MET

COMMA1-D (mammary epithelial cells)

- P2 : pure epithelial population
- G6 : transformed population (fibroblastic)

| | P2 | CID9 | G6 |
|------------|------------|--------------------------|-------------|
| cell | epithelial | epithelial / mesenchymal | mesenchymal |
| keratin | ⊙ | ○ | × |
| E-cadherin | ⊙ | ○ | × |
| vimentin | × | ○ | ⊙ |

• P2 cells express epithelial markers, **keratin** and **E-cadherin**.
 • G6 cells express a mesenchymal marker, **vimentin**.
 • CID9 cells express both epithelial and mesenchymal markers.
 • EMT-transitioning cells are resemble to CID9 cells.
 • P-cadherin, MMP2, MMP3 are other EMT markers.

4. Generation of inducible cells (tet-off system)

(expression) off on

HA-LIP (off) HA-LIP (on)

(cell) P2tetLIP G6tetLIP

(expression) off on L-on off on L-on

• expression (on) : 3days
 • expression (L-on) : 7days

Exogenous expression of the transgene product (LAP or LIP) became suppressed in a week both in P2 and G6 cells.

5. Effects of exogenous LIP on P2 cells

epithelial phenotype → EMT??? → mesenchymal phenotype?

W.B. (expression) off on L-on

vimentin P-cadherin β-actin

RT-PCR (expression) off on L-on

P-cadherin vimentin snail1 mmp2 gapdh

immunostaining vimentin / keratin / DAPI keratin ↓

ChIP assay

Signal intensity (relative)

LIP (off) LIP (on)

primer : E-cadherin promoter

zymography (expression) off on L-on

MMP2 MMP3

• Forced expression of LIP protein temporally induced EMT-like cell behaviors.
 • The expression profile of the EMT-markers was consistent with that of exogenous LIP protein.

6. Direct introduction of LIP protein into P2 cells

Arg-LIP

add to medium

Recombinant LIP / GFP protein added to medium was successfully delivered to nuclei.

Again, LIP protein transported to cell nuclei elicited EMT-like changes in P2 cells.

(day) 1 2 3

Arg-LIP P2

β-actin

W.B. E-cadherin ↓

(day) 1 2 3

Arg-GFP Arg-LIP G6

E-cadherin vimentin

number of cells

WST-1 cell growth ↑

Absorbance (450nm)

day1 day2 day3

7. Effects of exogenous LIP on P2 cells on matrigel

DH5 + 2% matrigel

alveoli

matrigel

LIP (off) LIP (on)

Overexpression of LIP perturbed alveolar-like morphogenesis in P2 cells cultured on Matrigel.

W.B. (expression) off on long-on

LIP β-actin

In contrast to on plastic, P2 cells cultured in matrigel maintain LIP-overexpression.

immunostaining integrin α1 / ZO-1 / DAPI

8. Effects of exogenous LIP on P2 cells in vivo

injection P2 cells into nude mice

bred 2months

groupA : feed tetracyclin (+) water = expression (off)

groupB : feed normal water = expression (on)

W.B. P2 tumor

LAP P-cadherin E-cadherin vimentin β-actin

In vivo, P2 cells with overexpression of LIP formed metastatic tumors. The expression of LIP was sustained for a long period, which was accompanied with a dramatic alternation in the expression of EMT-markers in the tumors.

| | A1 | A2 | A3 | A4 | B1 | B2 | B3 |
|------------------|------|------|------|------|------|------|------|
| LIP expression | off | off | off | off | on | on | on |
| palpable cancers | × | × | × | × | ⊙ | ⊙ | ⊙ |
| weight (g) | 19.5 | 20.7 | 21.0 | 19.0 | 23.6 | 24.9 | 24.0 |
| tumors (g) | 0 | 0 | 0 | 0 | 0.8 | 0.5 | 2.0 |
| spleen (g) | 0.1 | 0.1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.7 |

9. Proteasome-dependent elimination of excess amount of LIP

(expression) off on on L-on

(MG132) - + + +

Proteasome inhibitor (MG132) was added to the medium. Incubate 24h at 37°C.

LAP LIP

E-cadherin P-cadherin β-actin

MG132 (+) ⇒ LIP ↑, E-cad ↓ ⇒ EMT

Matrigel was added to the medium (final 1%) after cells attached to dish.

LIP (long-on)

(gel) - + - +

LAP E-cadherin P-cadherin β-actin

1% matrigel (+) ⇒ LIP ↑, E-cad ↓, P-cad ↑ ⇒ EMT

Long-term expression of LIP led to clearer EMT in P2 cells.

10. Relationship between C/EBPβ and TGFβ (popular EMT inducer)

(rTGFβ) - +

LAP LIP

E-cadherin P-cadherin β-actin

rTGFβ (+) ⇒ EMT ⇒ LAP ↓, LIP ↓

(expression) off on L-on

(RTAce) + - + - + -

tgfb gapdh

LIP(on) ⇒ no changes in mRNA level of TGFβ

C/EBPβ is a downstream of TGFβ signaling pathway.

11. Effects of exogenous LAP on G6 cells

epithelial-like morphology

LAP construct

ATG

LAP LIP

epithelial phenotype? → MET??? → mesenchymal phenotype

W.B. vimentin ↓

(expression) off on L-on

vimentin β-actin

LAP binds to E-cadherin promoter

immunostaining keratin ↑

WST-1 cell growth ↓

Absorbance (450nm)

day 1 2 3 4 5

ChIP assay

Signal intensity (relative)

LAP (off) LAP (on)

Primer : E-cadherin promoter

RT-PCR vimentin ↓

(expression) off on off L-on

(RTAce) + - + - + -

vimentin gapdh

Overexpression of LAP resulted in the cell shape change and growth arrest in G6 cells. LAP also led to the down-regulation of a mesenchymal marker and the up-regulation of an epithelial marker. LAP elicited cellular response opposite to LIP. ⇒ LAP might induce MET.

12. Conclusion

- LIP induced EMT-like changes and abrogated morphogenic potential in mammary epithelial cells.
- LIP overexpression in the epithelial cells resulted in the formation of metastatic tumors.
- LAP induced MET-like changes in mesenchymal cells.
- The expression profile of LIP protein was affected by cellular microenvironment.
 - ⇒ Excess amount of LIP protein was actively eliminated.
 - ⇒ Under the influence of basement membrane, the LIP-elimination was suppressed.
- Possibility : LIP also acts as a transcriptional activator in mammary.
- C/EBPβ is a downstream of TGFβ signaling pathway.