# A novel monoclonal antibody targeting TM4SF4 enhances antitumor activity through regulation of cellular levels of immune checkpoint ligands and antibody-dependent cellular cytotoxicity

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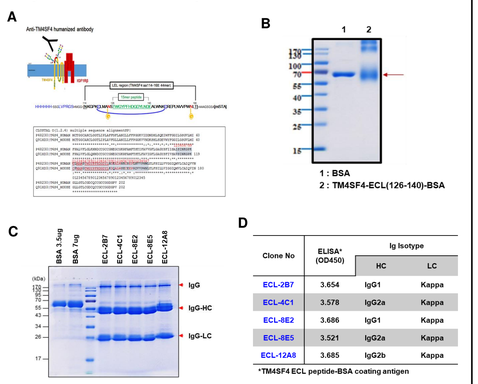
## Abstract

Transmembrane 4 super family member 4(TM4SF4) protein has been shown to be involved in EMT-associated stemness through autocrine of insulin-like growth factor 1(IGF1) and osteopontin(OPN) in non-small cell lung cancer(NSCLC) cells. However, its potential as a therapeutic target has not been evaluated. In this study, anti-human TM4SF4 monoclonal antibodies(anti-hTM4SF4 mAbs) were prepared in mouse as part of a strategy for developing anticancer drug using humanized antibody. A synthetic peptide(15 mer, 126-140th AA) derived from the extracellular loop(ECL) 2 of human TM4SF4 exposed on outer cell surface was used as an immunization antigen. Among the selected anti-hTM4SF4 mAbs, ECL-2B7 and ECL-12A8 showed the best affinity for the antigen (Kd), approximately 2.66 nM and 8.34 nM, as determined by surface plasmon resonance analysis. Using mouse xenograft of NSCLC cell line, it was confirmed that ECL-2B7 was superior to ECL-12A8 in producing humanized antibody. ECL-2B7 inhibited EMT-associated stemness of TM4SF4 positive cancer cells by blocking cellular signaling events such as IGF1R and CD44 and their downstream signals, PI3K/AKT/GSK3 /NF-κB and JAK2/STAT3 pathways. ECL-2B7 also enhanced antitumor activity by downregulating cellular and exosomal level of PD-L1 and B7-H4, immune-checkpoint ligands that elicit immunosuppression of T cell, and by mediating antibody-dependent cellular cytotoxicity. Treatment with OPN- and IGF1-neutralizing antibodies suppressed intracellular PD-L1 and B7-H4 level. It means that the regulation of PD-L1 and B7-H4 levels by TM4SF4 is closely related to the autocrine action of OPN and IGF1 induced by TM4SF4. Using patient-derived xenograft model, it was confirmed that the ECL-2B7 has excellent antitumor activity that effectively inhibits tumor progress. These results strongly suggest that it is necessary to construct humanized antibody based on ECL-2B7 to confirm its potential as a novel anticancer drug.

## Results

### ■ Preparation of peptide antigen, acquisition of B cell hybridoma clones and production of anti-hTM4SF4 mAbs

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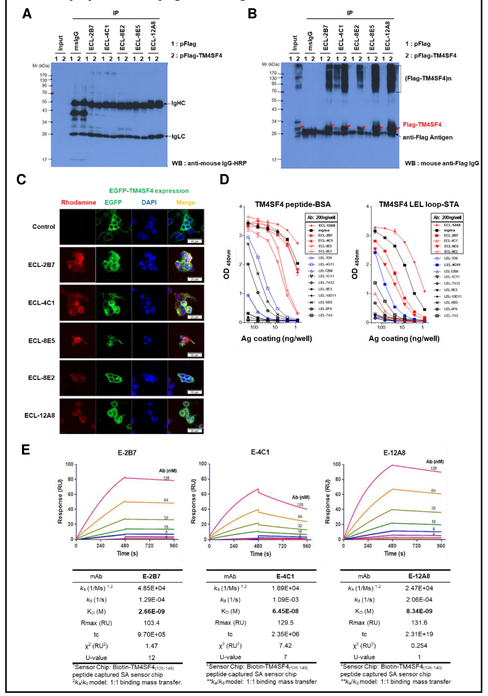
| Clone No | ELISA* (OD450) | Ig Isotype | |
|---|---|---|---|
| | | HC | LC |
| ECL-2B7 | 3.654 | IgG1 | Kappa |
| ECL-4C1 | 3.578 | IgG2a | Kappa |
| ECL-8E2 | 3.686 | IgG1 | Kappa |
| ECL-8E5 | 3.521 | IgG2a | Kappa |
| ECL-12A8 | 3.685 | IgG2b | Kappa |

*TM4SF4 ECL peptide-BSA coating antigen

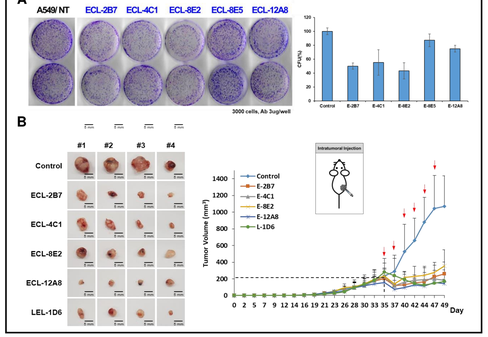
### ■ Characterization of anti-hTM4SF4 mAbs specific for BSA-peptide conjugate antigen



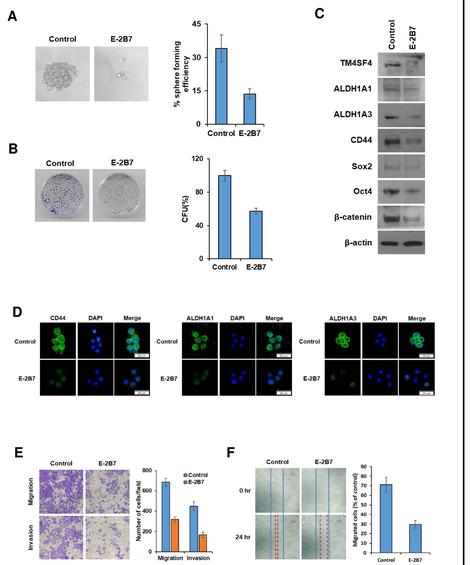

| mAb | E-2B7 | E-4C1 | E-12A8 |
|---|---|---|---|
| $k_a$ (1/Ms) [1,2] | 4.85E+04 | 1.68E+04 | 2.47E+04 |
| $k_d$ (1/s) | 1.29E-04 | 1.09E-03 | 2.06E-04 |
| $K_D$ (M) | **2.66E-09** | **6.45E-08** | **8.34E-09** |
| Rmax (RU) | 103.4 | 129.5 | 131.6 |
| tc | 9.70E+05 | 2.35E+06 | 2.31E+19 |
| $\chi^2$ ($RU^2$) | 1.47 | 7.42 | 0.254 |
| U-value | 12 | 7 | 1 |

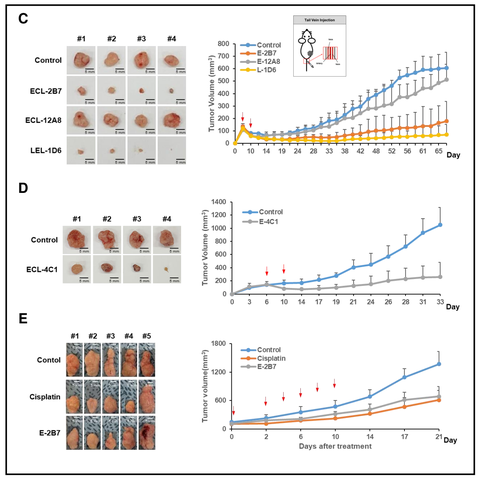
*Sensor Chip: Biotin-TM4SF4$_{(126-140)}$ peptide captured SA sensor chip
[1]$k_a$/$k_d$ model: 1:1 binding mass transfer.

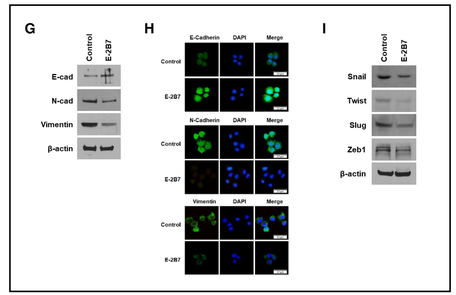
### ■ Anti-hTM4SF4 mAbs inhibit NSCLC growth in vitro cell culture and in vivo mouse xenograft model



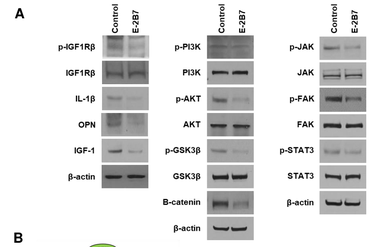

### ■ Effect of anti-hTM4SF4 mAbs, ECL-2B7, on self-renewal and EMT in NSCLC cells



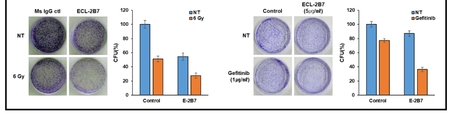



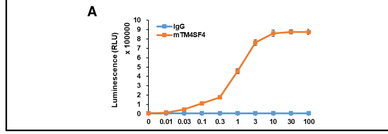

### ■ ECL-2B7 inhibits the autocrine secretion of IGF1 and OPN

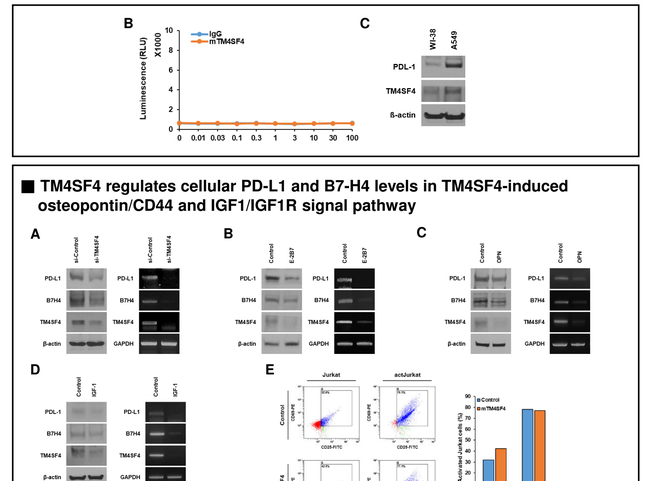



### ■ ECL-2B7 treatment increases γ-radiation sensitivity of NSCLC cells

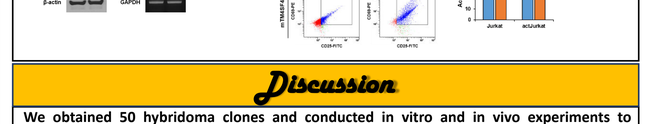



### ■ ECL-2B7 mediates cellular cytotoxicity against TM4SF4-expressing cancer cells




### ■ TM4SF4 regulates cellular PD-L1 and B7-H4 levels in TM4SF4-induced osteopontin/CD44 and IGF1/IGF1R signal pathway




## Discussion

We obtained 50 hybridoma clones and conducted in vitro and in vivo experiments to evaluate and select mouse monoclonal antibodies for early-stage candidates in developing potential humanized antibody therapeutics. Among the initially chosen anti-hTM4SF4 mAbs (ECL-2B7, ECL-4C1, ECL-8E2, and ECL-12A8), ECL-2B7 was selected as the most promising performance for humanized antibody preparation. ECL-2B7 mAb correctly recognized TM4SF4 on cell surfaces, inhibiting TM4SF4-related signaling events associated with cancer malignancy. It also suppressed invasion and proliferation of NSCLC cells, reduced tumor size in mice with NSCLC cell lines and patient-derived xenografts, and mitigated cancer treatment resistance. ECL-2B7, seemed suitable for chimeric and humanized antibody manufacturing, demonstrated antitumor activity in various cancer cells besides NSCLC, including HCC. Additionally, it possessed ADCC and immune checkpoint inhibitory functions, suggesting its potential as a cancer therapeutic antibody candidate, especially in enhancing radiotherapy efficacy. Further studies will focus on producing optimal humanized antibodies based on ECL-2B7.

## References

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