Utilizing Fluorescent Fusion Proteins to Investigate SNAI2 Expression during Epithelial-Mesenchymal Transition in Chick Embryo Cranial Neural Crest Cells

Chi Lok WONG[1], Dennis PANG[2], Martin CHEUNG[2]
1. School of Biological Sciences, Faculty of Science, The University of Hong Kong
2. School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong

Name: Wong Chi Lok (Anson)
University No: 3035920962
Supervisor: Professor CHEUNG, Martin Chi Hang
Student's Major: Molecular Biology and Biotechnology (Intensive)
Poster No: E9
Poster Presentation for Summer Research Internship of Undergraduate Research Fellowship Programme (URFP) Programme 2024-25

Summary

❖ SNAI2 may act as a **pseudo-primed substrate** of GSK3

❖ The **mCherry-linker-SNAI2 fusion protein** was cloned into the pCIG plasmid to **examine the pseudo-priming site** on SNAI2 for GSK3 recognition

❖ Demonstrating the effect of **knocking down (KD)** *Serine/Threonine Kinase Receptor Associated Protein* ***(STRAP)*** on the **expression of neural crest markers Pax7 and Sox9**

Introduction

- EMT (Epithelial-Mesenchymal Transition) is critical in **neural crest migration**, **wound healing**, **proliferation** and **cancer progression**[1-3]
- SNAI2 is a **transcription factor (TF)** involved in **the initiation of EMT**[4]
  - SNAI2 represses cadherin6B to initiate EMT[5]
- SNAI2 can be **phosphorylated by GSK3** in the SLUG domain (Figure 1)[4]
  - This phosphorylation facilitates **ubiquitin-dependent proteasomal degradation**
- GSK3 has a **phosphate binding pocket (PBP)**
  - The PBP can **interact** with the **substrate with the pre-phosphorylation (priming) site**[6] to increase **the phosphorylation efficiency** by **at least 90%**[6].
- SNAI2 does not have priming site for GSK3 to recognize
  - Hence, the **detailed mechanism** by which GSK3 recognizes SNAI2 is **still unknown**
- STRAP has been found to **inhibit GSK3**, **maintaining SNAI2 expression** during neural crest EMT
  - However, the **effect of STRAP** on the **expression of neural crest marker** is **still unknown**

SNAI2:

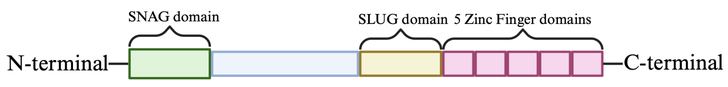
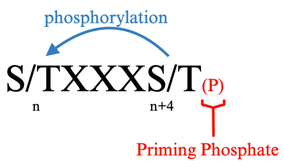


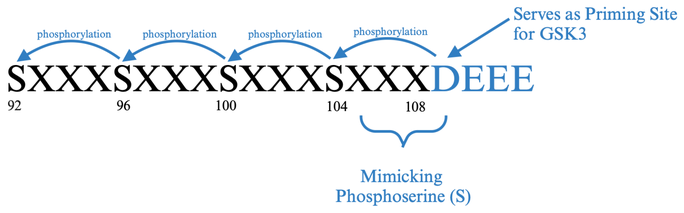
Figure 1. The main domains in SNAI2 protein.

Hypothesis

GSK3 consensus phosphorylation motif:



Proposed phosphorylation site on SNAI2:



SNAI2 consensus phosphodegron and negatively charged amino acids mimicking phosphoserine:

		Number of S/T:
Homo sapiens (Human):	SSSLGRVSPPPPSDTSSKDHSGSESPISDEEERLQSKLSDP	4
Gallus gallus (Chicken):	PSSLGRVSPPPPSDTSSKDHSGSESPISDEEERIQSKLSDP	4
Rattus rattus (mice):	SSSLGRVSPLPSSDTSSKDHSGSESPISDEEERLQPKLSDP	4
Oryctolagus cuniculus (rabbit):	PSSLGRVSPPPPSDTSSKDHSGSESPISDEEERLQSKLSDP	4
Danio rerio (Zebrafish):	LSPISGYPSSLSDTSSNKDHSGSESPRSDEDERIQSTKLSD	2

Figure 2. Proposed Mechanism for GSK3 Phosphorylation of SNAI2. The serine amino acids that can be phosphorylated by GSK3 and the pseudo-priming site on SNAI2 are highlighted in red. The number of serine or threonine residues that can be phosphorylated by GSK3 is also depicted in the figure.

- GSK3 may utilize the **negatively charged amino acids** on SNAI2 as a **mimic of phosphoserine** to serve as a **priming site for recognition**.

Material and Methods

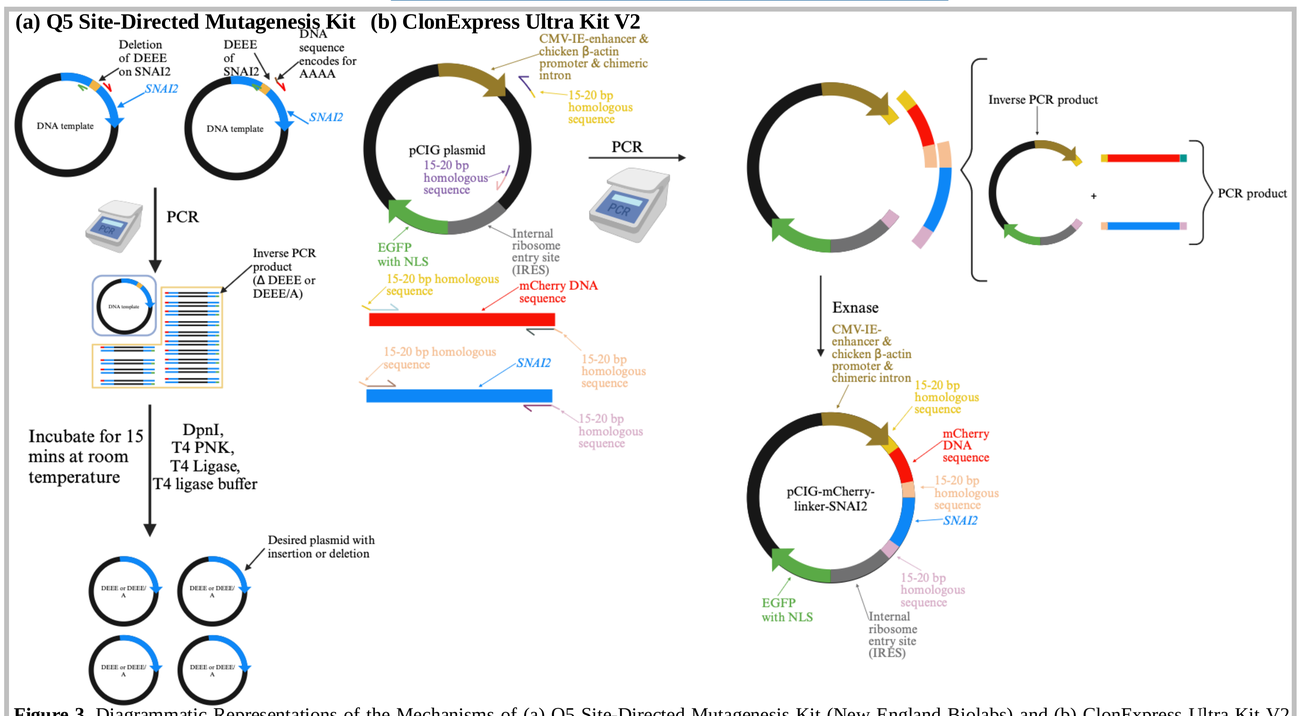


Figure 3. Diagrammatic Representations of the Mechanisms of (a) Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and (b) ClonExpress Ultra Kit V2 (Vazyme, Cat. No. C116). *SNAI2* refers to the DNA sequence encoding SNAI2 WT or one of the SNAI2 mutants. NLS stands for Nuclear Localization Signal.

References

1 Zhou, W., Gross, K. M. & Kuperwasser, C. Molecular regulation of Snai2 in development and disease. *J Cell Sci* 132 (2019). https://doi.org/10.1242/jcs.235127

2 Thiery, J. P., Sheng, G., Shu, X. & Runyan, R. How studies in developmental epithelial-mesenchymal transition and mesenchymal-epithelial transition inspired new research paradigms in biomedicine. *Development* 151 (2024). https://doi.org/10.1242/dev.200128

3. Cao, Y. Lack of basic rationale in epithelial-mesenchymal transition and its related concepts. *Cell & Bioscience* 14, 104 (2024). https://doi.org/10.1186/s13578-024-01282-w

4 Nieto, M. A., Sargent, M. G., Wilkinson, D. G. & Cooke, J. Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* 264, 835-839 (1994). https://doi.org/10.1126/science.7513443

5 Taneyhill, L. A., Coles, E. G. & Bronner-Fraser, M. Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. *Development* 134, 1481-1490 (2007). https://doi.org/10.1242/dev.02834

6 Sutherland, C. What Are the bona fide GSK3 Substrates? *Int J Alzheimers Dis* 2011, 505607 (2011). https://doi.org/10.4061/2011/505607

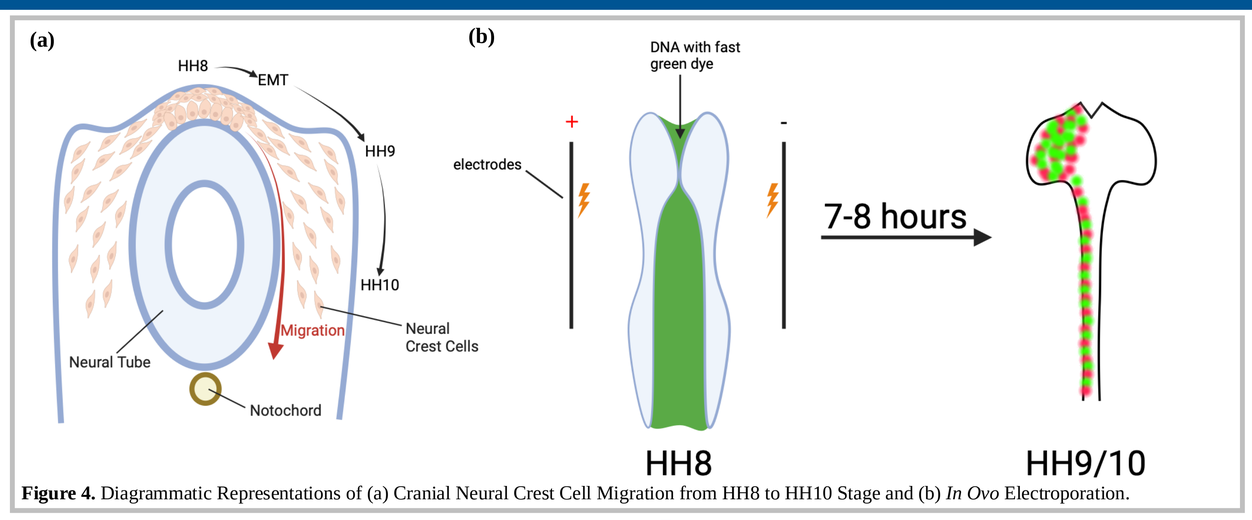


Figure 4. Diagrammatic Representations of (a) Cranial Neural Crest Cell Migration from HH8 to HH10 Stage and (b) *In Ovo* Electroporation.

Results

Generating Fusion Proteins with mCherry and SNAI2 Mutants (WT, 4A, 108A, 108E, 108N, ΔDEEE, DEEE/A) (Figure 5(a))

- Study whether the **DEEE protein sequence** on SNAI2 is acting as a **priming site** for GSK3 recognition
- The fusion protein **was cloned into pCIG plasmid** (Figure 5(b))
- The EGFP contains the **nuclear localization signal (NLS)** to enter the nucleus and act as an **internal control** for comparing the mCherry signal in the nucleus

- The pCIG-mCherry-linker-SNAI2 plasmid (Figure 5(b)) can be used to determine the **half-life of SNAI2** by examining **the ratio of mCherry to EGFP in the nucleus**
  - The **expected result** should be that **the ratio of mCherry to EGFP for the WT** is **lower** compared to **that of the other mutants**

(a)

SNAI2 phosphodegron (80 … 108)

Wild Type (WT):	PSSLGRVSPPPPSDTSSKDHSGSESPISDEEERIQSKLSD
4A:	PSSLGRVSPPPPADTSAKDHAGSEAPISDEEERIQSKLSD
108A:	PSSLGRVSPPPPSDTSSKDHSGSESPISAEEERIQSKLSD
108E:	PSSLGRVSPPPPSDTSSKDHSGSESPISEEEERIQSKLSD
108N:	PSSLGRVSPPPPSDTSSKDHSGSESPISNEEERIQSKLSD
ΔDEEE:	PSSLGRVSPPPPSDTSSKDHSGSESPISRIQSKLSDPHAI
DEEE/A:	PSSLGRVSPPPPSDTSSKDHSGSESPISAAAARIQSKLSD

(b)

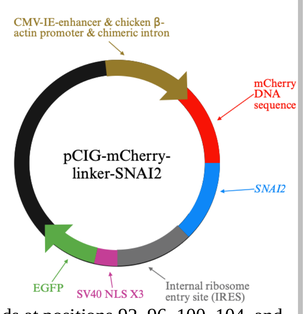


Figure 5. (a) The protein Sequences of the Phosphodegron in SNAI2 Mutants and the Wild Type (WT). The amino acids at positions 92, 96, 100, 104, and 108 to 110 are highlighted in red. (b)The Plasmid map of the pCIG-mCherry-linker-SNAI2. *SNAI2* refers to the DNA sequence encoding SNAI2 WT or one of the SNAI2 mutants. NLS refers to nuclear localization signal.

Verification of pCIG-mCherry-linker-SNAI2 Plasmid in HEK293T Cell Line

- The pCIG-mCherry-linker-SNAI2(WT) plasmid and GSK3 expressing plasmid (in 1:1 ratio) were **transfected into 293T Cell line** using PolyJet™ In Vitro DNA Transfection Reagent with 4 duplicates
- Proteins were collected using RIPA lysis buffer and centrifugation
- **Western blot analysis** was performed (Figure 6)

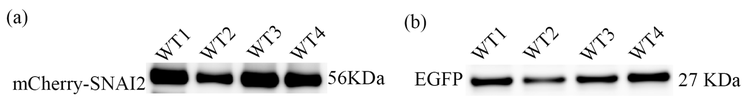


Figure 6. The Results of the Western blot analysis. (a) mCherry-SNAI2 fusion protein at 56 kDa, and (b) GFP protein at 27 kDa.

- The result shows **large batch-to-batch variations**
  - The variation may be attributed to the **limitations of using transfection**, which **largely depend on the researcher's technique**
- The amount of mCherry-SNAI2 protein is **unexpectedly higher** than that of the EGFP protein
  - This discrepancy may be due to the **limitations of using the IRES**

Pax7 and Sox9 Protein Expression in STRAP KD Chick Embryo

- Both **whole mount immunofluorescent (WMIF)** staining (Figure 7(a)) and **cryosectioning** (Figure 7(b)) were performed

Figure 7. (a) The whole mount immunofluorescent staining of *a STRAP* KD chick embryo at the HH9/10 stage. (b) The immunofluorescent staining of a *STRAP* KD chick embryo at the HH9/10 stage after cryosection.

Discussions

Future Directions:

1. Package the **mCherry-linker-SNAI2 DNA into lentivirus** for a more stable infection
   - Clone the mCherry-linker-SNAI2, GFP, and GSK3β into the lentivirus plasmid
   - These three protein are **linked with T2A peptide** as the final protein
2. Perform ***in ovo*** **electroporation** of the **pCIG-mCherry-linker-SNAI2 plasmid** into chick embryo **cranial neural crest cells**
3. Perform ***in ovo*** **electroporation** of ***STRAP*** **KD control gRNA plasmid** into chick embryo **cranial neural crest cells**