MECHANISM STUDY OF SUMOYLATION IN REPRESSING RETROVIRUSES IN EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) have been found to have the potential to silence retroviral elements, including endogenous retroviruses (ERVs) through an established retroviral silencing pathway (ERSP). This ability is correlated to their pluripotency level, where ESCs with a high level of pluripotency are associated with an alleviated transcription of ERVs.

Genes coding for proteins involved in the SUMOylation pathway have been identified as important for retrovirus silencing. SUMOylation is a reversible process of adding small ubiquitin-related modifiers (SUMOs) to cellular proteins.

This study investigates whether the SUMOylation pathway is important for ESC pluripotency and whether it also contributes to silencing ERVs.

Relative expression level of ERVs generally increase after effective knockdown of SUMO and ERSP genes, with highest upregulation when sumo2 is knocked down.

The data collected on pluripotency and differentiation of the ESCs after effective knockdown of SUMO and ERSP genes was rather mixed and both generally increased, which is unusual. However, RNA-sequencing revealed Dub3, a gene responsible for maintaining pluripotency and decreasing differentiation, as a potential downstream target of SUMOylation of sumo2. When sumo2 is knock downed, expression of Dub3 and pluripotency level increase. Previous research has established that ESCs with high level of pluripotency are caused by alleviated transcription of ERVs. However, these results suggest a two-way association where decrease in pluripotency may also result in increased silencing of ERVs. This may further explain sumo2's ability to silence ERVs.

Embryonic stem cell; SUMOylation; pluripotency; endogenous retrovirus

I. INTRODUCTION

Embryonic stem cells (ESCs) are cells derived from the inner cell mass of blastocyst stage embryos. ESCs possess two unique properties. First, they have an infinite self-renewal capacity to go through numerous cycles of cell division while maintaining the undifferentiated state. Second, they are pluripotent and are able to differentiate to cells of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), and ectoderm (epidermal tissues and nervous system)[1].

In earlier studies, ESCs have been found to have the potential to silence retroviral elements[2] and that this ability is correlated to their pluripotency level, where ESCs with a high level of pluripotency are associated with an alleviated transcription of ERVs[3]. Retroviral elements include infectious exogenous retroviruses and endogenous retroviruses (ERVs). ERVs are normal genetic elements found in chromosomal DNA in the genome and they closely resemble retroviruses. In mice, ERVs display residual retrotransposition activity, leading to polymorphic integrations and differential gene regulation between mouse strains. Around 10% of spontaneous mutations in inbred mice are linked to ERVs.

The established mechanism of ESCs silencing retroviral infection is based on canonical machinery, as shown in Figure 1. KRAB-zinc-finger proteins (ZFPs) have specific binding capacity to different retroviral sequences. This family of proteins will then recruit KRAB-associated protein-1 (KAP1, also called TRIM28) which binds to the DNA of the retrovirus to prevent transcription, and SETDB1 (also called ESET) which causes the histone located in the retrovirus to undergo methylation, thus silencing the retrovirus[4].

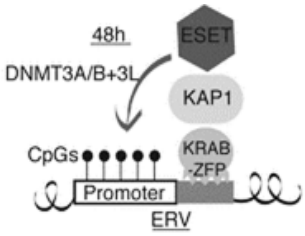

Figure 1: Diagram of established mechanism of ESCs silencing retroviral infection

In a previous unpublished study, our research mentor's laboratory identified groups of genes important for retrovirus silencing through a genome wide screening of the murine ESCs, among which were genes coding for proteins involved in the SUMOylation pathway.

SUMOylation is the process of adding small ubiquitin-related modifiers (SUMOs) to cellular proteins. A SUMO is a protein moiety that is ligated to lysine residues in a variety of target cellular proteins in a chain reaction[5]. This alters the way proteins interact with each other, modifies localisation patterns within the cell and controls protein stability for different cellular processes[6].

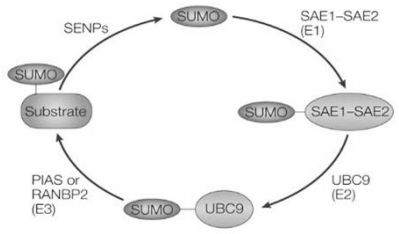

Figure 2: Diagram of SUMOylation pathway

SUMOylation involves three discrete steps: activation, conjugation and ligation. Each step is mediated by a specific enzyme (E1, E2 and E3, respectively), as shown in Figure 2. SUMO is activated by the E1, a heterodimer formed by sae1 (SUMO-activating enzyme subunit 1), and sae2 (also known as uba2). E1 uses ATP to form a thioester bond between the SUMO residue and a cysteine residue in uba2. In conjugation, SUMO is transferred from E1 to the cysteine residue of the SUMO E2 conjugating enzyme (E2) – ubiquitin-conjugating enzyme 9 (ubc9, also known as ube2i). The SUMO E3 ligase (E3) then promotes the transfer of SUMO from E2 to the target substrate, forming an isopeptide bond between SUMO and a lysine residue in the target protein. SUMO E3 ligases include PIAS (protein inhibitor of activated signal transducer and activator of transcription protein). In DeSUMOylation, senps remove SUMO from proteins, making the modification reversible[7].

This study will investigate whether the SUMOylation pathway is important for ESC pluripotency and whether this pathway also contributes to silencing endogenous retroviruses.

II. Methodology

A. Cell culture

For cell sample preparation, E14 mouse embryonic stem cells (mESCs) were cultured with mouse ESC medium (DMEM high glucose supplemented with 15% defined FBS (Hyclone), 2mM L-glutamine (Gibco), 1X PenStrep (Gibco) 100μM MEM non-essential amino acids (Gibco), 100μM β-mercaptoethanol (Gibco), and 1000 U/mL leukaemia inhibitory factor (LIF; ESGRO, Millipore)). ESC cell lines used in this study were cultured on gelatin-coated tissue culture plates and were cultured at 37°C with 5% CO2.

B. shRNA interference

E14 was seeded as single cells 24 hours before transfection of shRNA plasmids. Lipofectamine 2000 (Invitrogen) was used for transfection of shRNA plasmids according to the manufacturer's instructions. Puromycin was added to the culture medium 16 hours post transfection and cells were harvested for experiments after 3 days of knockdown. E14 was selected with 1 μg/ml puromycin. Knockdown efficiency was measured by real-time qPCR.

C. RNA extraction, reverse transcription and real-time PCR

Total RNA was then extracted using the Trizol reagent (Invitrogen). Contaminating DNA was removed by DNaseI (Ambion) treatment, and the RNA was further purified using QIAGEN RNeasy Kit. First strand cDNA was synthesised using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative Real-time PCR was performed on the CXF384 Real-time System (Bio-Rad), using a Kapa SYBR Fast qPCR kit (Bio-Rad).

D. RNA sequencing and clustering

Real K-means clustering algorithm was performed and a heat map in "R" was plotted.

III. Results and Discussion

A. SUMO genes knockdown efficiency

Knockdown efficiency of each gene (sae1, senp6, sumo2, uba2, ube2i, eset, trim28, zfp809) was measured by real-time qPCR. Two primers were used to target different areas of each gene to ensure that other genes would not be amplified by chance.

In general, expression levels for mRNA for each gene were similar as quantified by the two different primers. The relative gene expression levels of mRNA for both SUMOylation genes (sae1, senp6, sumo2, uba2, ube2i) and genes in the established retroviral silencing pathway (ERSP) (eset, trim28, zfp809) is less than 0.5 (Figs. A1 and A2).

As relative gene expression levels for mRNA experienced a substantial decrease, it can be concluded that knockdown of genes is effective and these cell lines can be used for further experiments.

B. SUMO genes knockdown on pluripotency genes expression in E14 mESCs

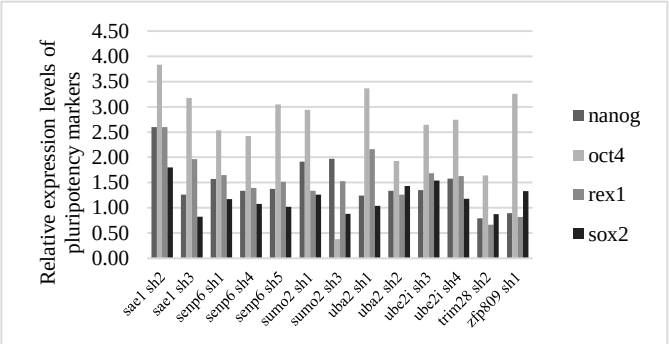

Figure 3: Graph of relative expression levels of pluripotency markers

The data for the expression level of both pluripotency markers and differentiation markers is rather mixed and unexpected. The expression level of both pluripotency markers and differentiation markers increased, though an increase in pluripotency usually means a decrease in differentiation. However, genes with relatively higher expression levels of

pluripotency markers (sae1, sumo2) still had relatively lower expression levels of differentiation markers.

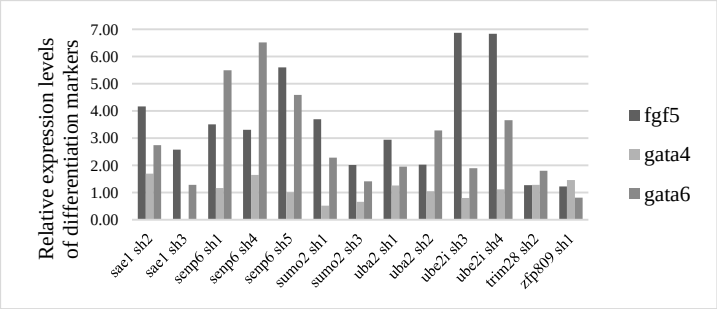

Figure 4: Graph of relative expression levels of differentiation markers

The unexpected results may be due to the period of knocking down being insufficient to observe the decrease of pluripotency genes. Oct4 is also known to be upregulated at the early stage of differentiation, which may account for the relatively higher expression levels. It may also be because the SUMOylation pathway does not have direct effect on pluripotency and the expression level change is actually indirect fluctuation.

C. Outreach of SUMOylation genes on pluripotency network regulation of E14 mESCs

As shown in Figure 5, formation of cell colonies was less distinct after knockdown of each of the two SUMO genes, with senp6 having the least distinct of colonies. This is because the ESCs now have less capacity for self-renewal.

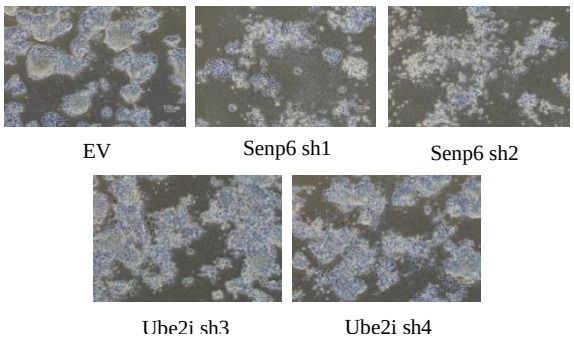

Figure 5: E14 mESCs after knockdown of genes

The gene ube2i is involved in SUMOylation while senp6 is involved in DeSUMOylation. Hence, both SUMOylation and DeSUMOylation are shown to be important for ESCs self-renewal.

D. Dub3 as potential downstream target of SUMOylation

The RNA-sequence shows that the knockdown of each of the five genes (senp6, sumo2, uba2, sae1, ube2i) all cause expression of genes in Cluster 3 to be upregulated. The gene ontology of this cluster was found to code for deubiquitinating proteins (DUBs). A gene of particular interest is Dub3, which has been shown to be important for maintaining pluripotency and knockdown of Dub3 increases spontaneous differentiation levels.

Knockdown of each of the SUMO genes all resulted in increased fold of Dub3 expression, except sumo1 and sumo3. Knockdown of sumo2 resulted in highest Dub3 expression while knockdown of sae1 and uba2 (responsible for initiation of SUMOylation) resulted in next highest Dub3 expression. This shows that the SUMOylation pathway involving sumo2 is critical in maintaining pluripotency levels through regulation of downstream target Dub3.

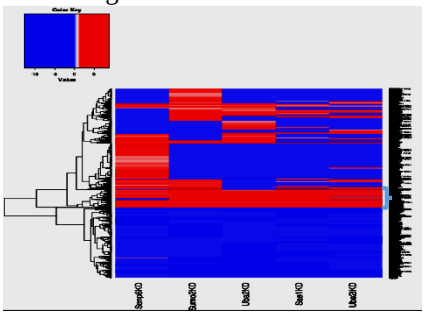

Figure 6: RNA-seq of E14 mESCs after knockdown of genes

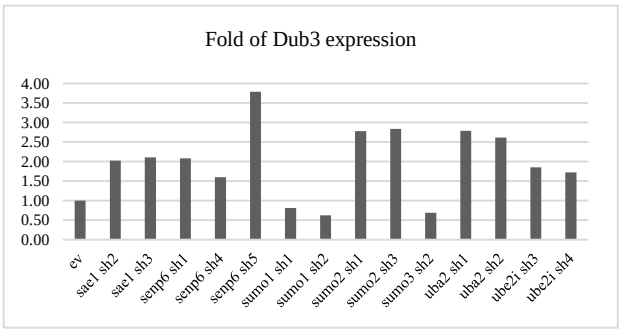

Figure 7: Graph of relative gene expression levels of mRNA of genes in established retroviral silencing pathway (ERSP)

E. Comparison of SUMO genes knockdown on ERV expression with ERSP genes

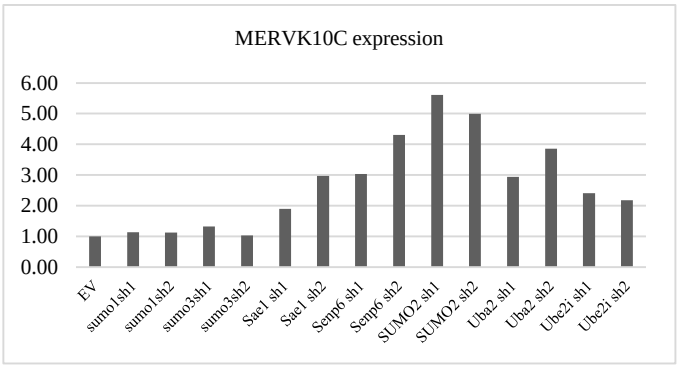

Figure 8: Graph of relative expression levels of ERV with knockdown of SUMO genes

The relative expression level of the 10 investigated ERVs generally increase after knockdown of SUMO and ERSP genes, showing that these genes do play a part in silencing these ten ERVs (Figs. A3 to A9). It is also clear that both the ERVs MERVL and MERVK constantly have the highest relative expression levels after the knockdown of sumo2 genes as well as relatively higher expression levels after knockdown of ERSP genes. Hence, SUMOylation involving sumo2 may play a potential role in the regulation of these two ERVs.

Genes of proteins involved in SUMOylation appear to play a part in silencing ERVs in ESCs, especially for the ERVs MERVL and MERVK. This may further help in the understanding of the SUMOylation mechanism and how some SUMO genes regulate ERVs.

The upregulation of expression levels of all ERVs (MERVL, MERVK, GLN, IAP, EtnmusD, MTA) after knockdown of sumo2 and senp6 genes increased the most. The sumo2 gene is involved in the initial step of SUMOylation because it produces the SUMO itself. Hence, it can be concluded that silencing of ERVs occurs more upstream in SUMOylation rather than during the process, especially for MERVL and MERVK.

The senp6 gene is involved in DeSUMOylation where SUMO is removed from proteins. An explanation for the results obtained is that knockdown of senp6 gene causes concentration of SUMO to decrease as SUMO is permanently bound to a substrate once SUMOylation occurs and cannot undergo SUMOylation again to modify other proteins. Hence, with knockdown of senp6, silencing of ERVs will be much less.

IV. Conclusion

SUMOylation involving sumo2 may play a potentially crucial role in the regulation of ERVs, especially MERVL and MERVK

The data collected on pluripotency and differentiation of the ESCs after knockdown of SUMO genes was a bit mixed and both generally increased. However, RNA-sequencing revealed Dub3, a gene responsible for maintaining pluripotency and decreasing differentiation, as a potential downstream target of SUMOylation of sumo2. When sumo2 is knock downed, expression of Dub3 and pluripotency level increase. Previous research has established that ESCs with a high level of pluripotency are caused by alleviated transcription of ERVs. However, these results suggest a two-way association where decrease in pluripotency may also result in increased silencing of ERVs. This may further explain sumo2's ability to silence ERVs.

Acknowledgment

We would like to thank our mentor, Mr Yu Tao of the A*STAR Institute of Molecular and Cell Biology, and our teacher-mentor, Mr Chong Han Guang, for their guidance throughout the course of this project. We would also like to thank Dr. Jonathan, Yuin Han Loh of the A*STAR Institute of Molecular and Cell Biology for funding this project. This project is a small part of an existing, ongoing project of Mr Yu Tao.

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Figures

[1] De novo DNA methylation of endogenous retroviruses is shaped by KRAB-ZFPs/KAP1 and ESET. Development (Cambridge, England), 140(3), pp. 519–29. http://doi:10.1242/dev.087585

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Appendix

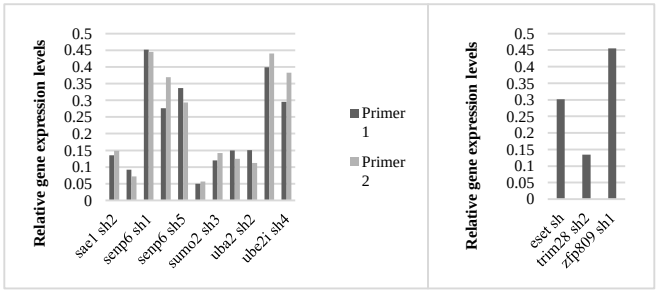

Figure A1: Graph of Relative Gene Expression Levels of mRNA of SUMOylation genes

Figure A2: Graph of relative gene expression levels of mRNA of genes in ERSP

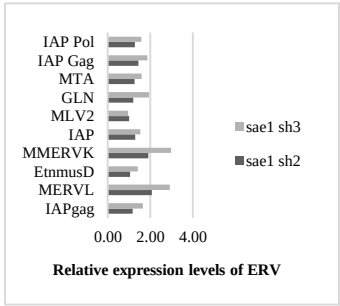

Figure A3: Graph of relative expression levels of ERV with knockdown of sae1

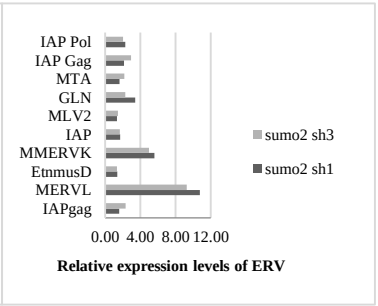

Figure A4: Graph of relative expression levels of ERV with knockdown of senp6

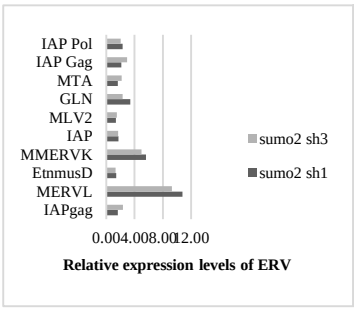

Figure A5: Graph of relative expression levels of ERV with knockdown of sumo2

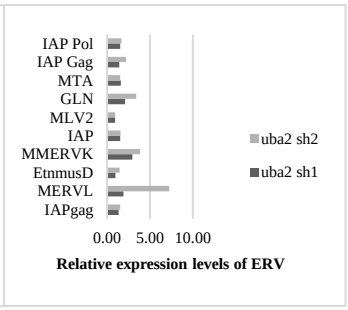

Figure A6: Graph of relative expression levels of ERV with knockdown of uba2

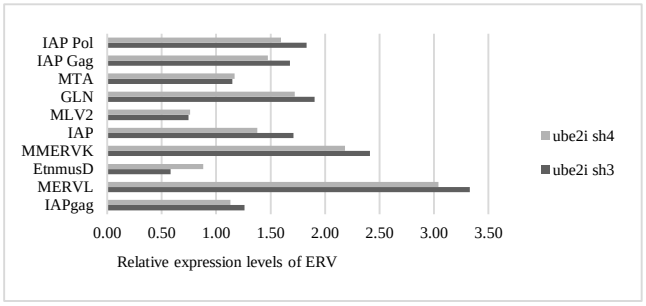

Figure A7: Graph of relative expression levels of ERV with knockdown of ube2i

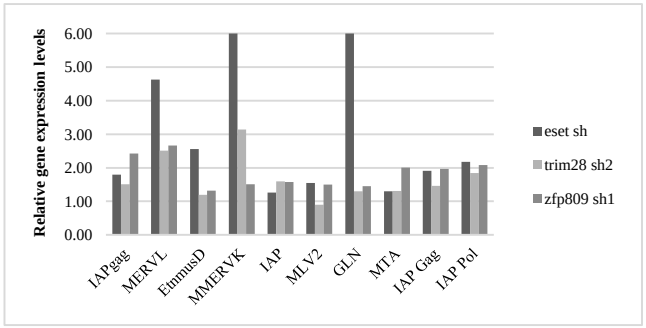

Figure A8: Graph of relative expression levels of ERV with knockdown of esset, trim28, zfp809

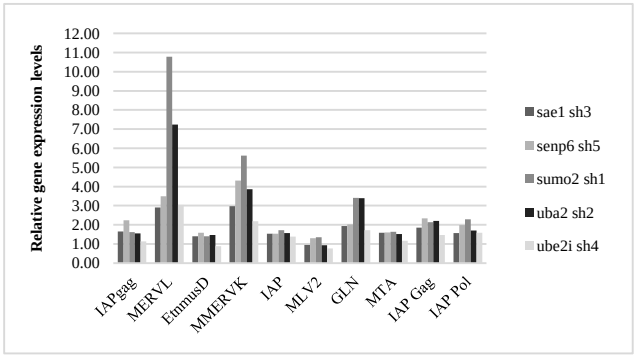

Figure A9: Graph of relative expression levels of ERV with knockdown of SUMO genes