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배아줄기세포에서 Sox2 오글루넥당화의 역할

Role of Sox2 *O*-GlcNAc modification in embryonic stem cells

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분자의학 및 바이오제약 전공
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A thesis of the Degree of Master of Philosophy

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ABSTRACT

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SRY (sex determining region Y) -box 2, also known as Sox2, is a transcription factor essential for the maintenance pluripotency of embryonic stem cells (ESCs) along with Oct4. *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification reflects cellular nutritional status and regulates pluripotency. Among transcription factors of core components of the pluripotency networks, Oct4 and Sox2 have been known to be modified by *O*-GlcNAc on threonine 228 (T228) for Oct4 and on Serine 248 (S248) and threonine 258 (T258) for Sox2. Although the role of *O*-GlcNAc modification on Oct4 has been studied extensively, that of Sox2 is not clear yet.

Here we found that *O*-GlcNAc modification of Sox2 on T258 is important for the maintenance of ESCs. When endogenous Sox2 was substituted with various *O*-GlcNAc-defective Sox2 mutants, a T258A point mutation reduces the capacity of Sox2 to maintain ESC self-renewal, whereas A S248A, T258A double mutation restores the capacity, suggesting that posttranslational

modifications on two sites play the opposite role. To confirm the role of T258A in more physiological condition, we mutated endogenous chromosome of Sox2 wild type (WT) to T258A using CRISPR-cas9 system. ESCs with one allele Sox2 T258A mutation were prone to differentiate, and ESCs with homologues Sox2 T258A mutation were not obtained.

Because *O*-GlcNAcylation usually controls cellular localization, protein stability, and protein-protein interactions, we investigated whether Sox2 T258A mutation affected those things. Both Sox2 WT and T258A were localized in nucleus and their protein stability is not significantly differ. We purified Sox2-interacting-proteins complex and identified them by liquid chromatography-mass spectrometry. We found about 800 Sox2 interacting proteins. Among them 164 proteins were not found in Sox2 T258A complex, suggesting that the interaction between these 164 proteins and Sox2 is dependent on T258 *O*-GlcNAcylation.

Combined analysis of RNA-sequencing, microarray, and chromatin immunoprecipitation- sequencing data showed that a Sox2 T258A point mutation increased the expression of genes in the lineage-developmental process, which suggests Sox2 suppressed developmental genes T258 *O*-GlcNAcylation dependently. Of those proteins whose interaction with Sox2 were dependent on T258 *O*-GlcNAcylation, we found 40 transcription factors, whose roles are negative regulation of gene expression and regulation of embryo development. Among them, we found Otx2, a transcription factor, which has been reported to regulate early stage embryogenesis and embryonic

stem cells, co-occupied lineage-specific genes promoters with Sox2 and nucleosome remodeling and deacetylation complex (NuRD) complex. In summary, *O*-GlcNAcylation of Sox2 T258 is important for the interaction between Sox2 and Otx2, which in turn is important for the suppression of genes in the developmental process.

Keywords: Nutritional status, ESC, *O*-GlcNAcylation, Pluripotency, Transcription factors, developmental biology, Sox2 (SRY (sex determining region Y) -box 2)

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LIST OF ABBREVIATIONS

Sox2	SRY (sex determining region Y)-box2
PTM	Posttranslational modification
O-GlcNAc	*O*-linked-N-acetylglucosamine
AP	Alkaline phosphatase
Dox	Doxycycline
CHX	Cycloheximide
TAD	Transcription activation domain
HMG	High mobility group DNA binding domain
ESC	Embryonic Stem cell
NuRD	Nucleosome Remodeling Deacetylase

I. INTRODUCTION

1-1. Role of Sox2 in ESCs

1-1-1. Sox2: Core regulator of embryonic stem cell pluripotency

In embryonic stem cells (ESCs), control of the gene expression that maintains ESC state is dependent on a remarkably small number of master transcription factor (Ng et al., 2011; Orkin et al., 2011; Young 2011). SRY (sex determining region Y) -box 2, also known as Sox2, is a transcription factor essential for the maintenance pluripotency of ESCs along with Oct4 and Nanog, and binds to the enhancer together with the mediator-assisted activator complex (Kagey et al., 2010). The mediator complex regulates the expression levels of the target genes by stimulating the ability of the enhancer binding transcription factor to induce RNA Pol II in the promoter of the target gene (Kornberg 2005; Malik et al., 2010; Borggrefe et al., 2011; Conaway et al., 2011). They activate the expression of pluripotent genes while repressing the expression of key genes that are causing the in vitro differentiation and in vivo developmental processes (Boyer et al., 2005; Chen et al., 2008).

1-1-2. Expression of Sox2 during development

Sox2 messenger RNA (mRNA) and protein can be detected in the oocyte and in the two-cell embryo (Avilion et al., 2003; Keramari et al., 2010). Subsequently, Sox2 mRNA is enriched in the inner cells of 16-cell morulae before becoming restricted to cell of the inner cell mass in early blastocysts

(Guo et al., 2010). By day 7.5, Sox2 expression becomes restricted to the neuroectoderm (Wood and Episkopou et al., 1999). At 15.5, Sox2 is expressed in the foregut endoderm derivatives, as well as in neuronal, inner ear, retina, and lens progenitors. Sox2 is not restricted to a role in early development. It is also important later during neurogenesis and lens placode formation (Miyagi et al., 2009). In adult mice, Sox2 is expressed in tissue stem cells of stratified and glandular epithelia of ectodermal and endodermal origin, including the glandular stomach, the esophagus, the tongue, the brain, the trachea, and the bronchiolar epithelium, as well as in sensory cells (Merkel and taste bud cells) and spermatogonial stem cells (Driessens et al., 2011).

1-1-3. Critical role of Sox2 in development

Sox2 is expressed in both the inner cell mass and trophectoderm of the blastocyst (Avilion et al. 2003). Sox2-null embryos die shortly after implantation as results of a failure to form a pluripotent epiblast, and blastocyst outgrowths form only trophectoderm-like cells (Avilion et al. 2003). Sox2-null embryonic stem (ES) cells down-regulate Oct4 and Nanog and differentiate into trophectoderm-like cells. (Masui et al. 2007). Also, Sox2 knockdown in two-cell embryos by RNAi, which depletes both maternal and embryonic Sox2, revealed a requirement for Sox2 in trophectoderm formation and development to the blastocyst stage (Keramari et al. 2010). Overexpression of Sox2 in ES cells induces differentiation toward the

neuroectodermal lineage, and Sox2 expression is maintained in the developing neuroectoderm (Avilion et al. 2003; Kopp et al. 2008; Thomson et al. 2011). These phenotypes lead to the conclusion that Sox2 may play an important role in ESC maintenance as well as development.

1-2. Regulation of Sox2

1-2-1. Sox2 protein structure

Sox (SRY-related HMG-box) family proteins are a conserved group of transcriptional regulators defined by the presence of a highly conserved high- mobility group (HMG) domain that mediates DNA binding. This domain was first identified in Sry, a crucial factor involved in mammalian male sex determination (Gubbay et al., 1990; Sinclair et al., 1990). Sox proteins are classified into groups A-H, depending on the amino acid sequence of the HMG domain (Fig. 1-2-1). Outside the HMG domain, strong homology, with regards to amino acid sequence and the overall organization of protein domains, is found only within a group (Bowles et al., 2000; Schepers et al., 2002) (Fig. 1-2-1). The SoxA group contains only Sry, which is encoded in mammalian Y chromosomes. Although the HMG box of Sry is highly conserved between species, sequences outside this domain are highly divergent among mammalian species (Sekido, 2010). The SoxB group is split into two sub-groups. SoxB1 comprises Sox1, Sox2 and Sox3. These family members have short N-terminal sequences followed by the HMG domain and long C-terminal sequences after the HMG domain. The C-terminal sequence includes a domain assigned as a transcriptional activation domain, based on an assay using fusion to heterologous DNA-binding domains (Kamachi et al., 1998).

1-2-2. Post-translational modification of Sox2

The post-translational modifications (PTM) are considered as important events during ESCs early differentiation (Van Hoof et al., 2009). Sox2 activity can be regulated at multiple PTMs. PTMs of Sox2 are concentrated in two main areas. One within the DNA binding domain and one within the transactivation domain. Such a localization of modifications suggest that the main thrust of posttranslational regulation is to modulate both the binding of Sox2 to DNA and the ability of Sox2 to activate downstream effects after DNA binding. Most of these modifications affect the nuclear shuttling of Sox2 but possibly modulate interactions with partners and cofactors as well (Kamachi et al., 2013). PTMs have also been reported to modulate the activity, stability and intracellular localization of Sox2. The PTM of Sox2 was identifies by mass spectrometry, but there were few sites studied. These modifications are described in detail in Figure 1-2-2, Table 1 Acetylation of Sox2 on K75 in the nuclear localization sequence promotes Sox2 nuclear export, thus decreasing its function in the nucleus (Baltus et al., 2009). Sox2 monomethylation at T119 by Set7 inhibits its transcriptional activity and induces its ubiquitination and degradation, and Akt-mediated phosphorylation at Thr-1118 and Set-mediated Sox2 methylation at T119 mutually inhibit each other (Fang et al., 2014). CARM1 facilitates Sox2-mediated transactivation and methylates Sox2 at R113. Methylated Sox2 is more prone to self-associate than its unmethylated counterpart. In addition, the methylation level of Sox2 is restricted by the tight association of Sox2

with chromatin (Zhao et al., 2011). However, the study of methylation of Sox2 on R113 did not use normal stem cells, and the regulation of Sox2 activity was confirmed only as reporter gene assays. Mass spectrometry-based proteomics has identified four phosphorylation sites on Sox2, namely S39, K118, S85, P222, S248, S251, S252, and S253 (Swaney et al., 2009; Van Hoof et al., 2009; Jeong et al., 2010; Malak et al., 2015; Ouyang et al., 2015; Qi et al 2016). Among these phosphorylation, AKT-mediated phosphorylation of Sox2 S85 might influence its nuclear import and DNA-binding activity (Malak et al., 2015). But they did not use normal stem cells, and overexpressed Sox2 without removing endogenous Sox2. It has recently been shown that Sox2 phosphorylation at T118 by Akt promotes the establishment of the pluripotent state during reprogramming (Jeong et al., 2010). But they overexpressed Sox2 without removing endogenous Sox2. Phosphorylation of S251, S252, and S253 promotes SUMOylation of Sox2 on the site located upstream of the phosphorylation sites in human ESCs (Van Hoof et al., 2009). SUMOylation of Sox2 at Lys-247 inhibits the ability of Sox2 to bind to its transcriptional targets and its interaction with Oct4 (Tsuruzoe et al., 2006; Wu et al., 2012). In contrast, another group of studies reported that phosphorylation of Sox2 S39 and S253 by cdk2 is essential for maintaining the mESC and maintaining the pluripotent state during reprogramming (Ouyang et al., 2015). And aurora kinase A phosphorylates S222 and S253 of Sox2 in human ovarian cancer. A form or AURKA inhibition results in the loss of stem cell character and differentiation (Qi et al 2016). Thus Sox2

phosphorylation at S253 has controversy for its function in controlling ESC pluripotency.

In summary, various post-translational modifications have also been reported to modulate Sox2. However, it is not clear how each PTMs affects Sox2 activity, so it is necessary to study PTM more precisely to know the function of Sox2 in ESCs

1-3. *O*-GlcNAcylation of Sox2

External stimuli such as nutrient status must be controlled in order to maintain pluripotency in ESCs (Jang et al., 2012). *O*-GlcNAc (*O*-Linked β-N-acetylglucosamine) modification is PTM regulated by nutritional status and is essential for embryo viability. (Shafi et al., 2000; Yang et al., 2012). *O*-GlcNAcylation is the process, by which GlcNAc in UDP-GlcNAc is transferred to protein serine or threonine. These UDP-GlcNAc reflects various nutritional states of cells including glucose metabolism, amino acid metabolism, fatty acid metabolism, nucleotide metabolism (Slawson et al., 2010). *O*-GlcNAcylation controls diverse aspects of cellular physiology, such as nutrient, by regulating protein function, localization, protein stability, protein-protein interaction, transcriptional activity (Hart et al., 2007; Hanover et al., 2010; Hart and Cope- land, 2010; Love et al., 2010; Yang et al., 2014). Previous studies have shown that nutritional status can affect pluripotency and differentiation through *O*-GlcNAcylation of Oct4 (Jang et al., 2012). But *O*-GlcNAcylation of sox2 has not been studied well. Sox2 was modified by *O*-GlcNAcylation at two positions of serine 248 and threonine 258 (Myers et al., 2011). Serine 248 is also the site of phosphorylation (Swaney et al., 2009; Rigbolt et al., 2011). S248A point mutation that eliminates both *O*-GlcNAc modification and phosphorylation increase the capacity of Sox2 to maintain ESCs self-renewal (Myers et al., 2016), *O*-GlcNAcylation or phosphorylation of the S248 locus is expected to reduce Sox2 activity. And the *O*-GlcNAcylation of Threonine 258 and Threonine 259 has the potential to

enhance the function of Sox2 (Jang et al., 2012). However, it has not yet been elaborated on how the *O*-GlcNAcylation of Threonine 258 can increase the function of Sox2. In addition, it has not been studied how all *O*-GlcNAcylation occurring in Sox2 reflect the external nutritional status and act on the function of Sox2 as a whole.

1-4. Purpose

Nutritional status is important for embryonic stem cells (ESCs) to maintain self-renewal. Nutrient status may affect pluripotency and differentiation via *O*-linked-N-acetylglucosamine (*O*-GlcNAcylation). *O*-GlcNAc of OCT4 is well known to be important for regulating is pluripotency. However, the function of *O*-GlcNAc in Sox2 is poorly understand. So we elucidated the role of the *O*-GlcNAcylation of Sox2 in embryonic stem cells during development.

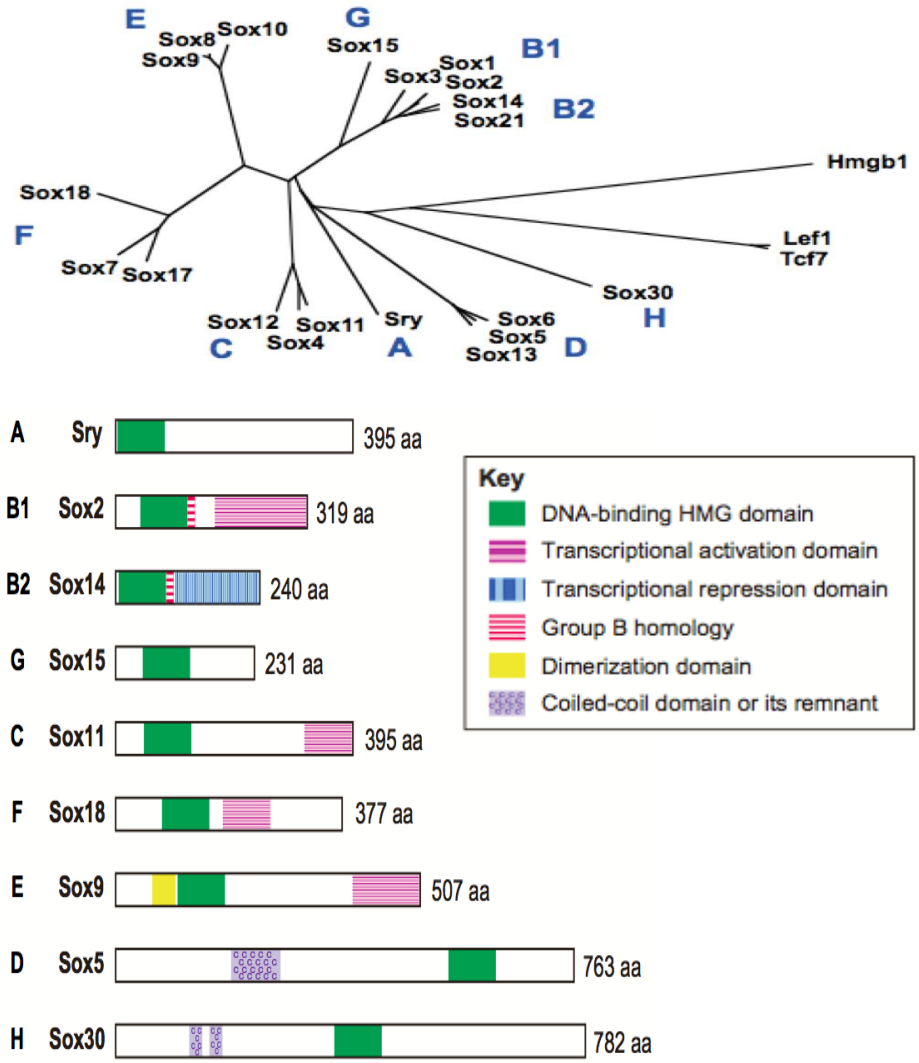


Figure 1-2-1. Diagram of Sox family and protein structure

Sox proteins are classified into groups A-H, depending on the amino acid sequence of the HMG domain

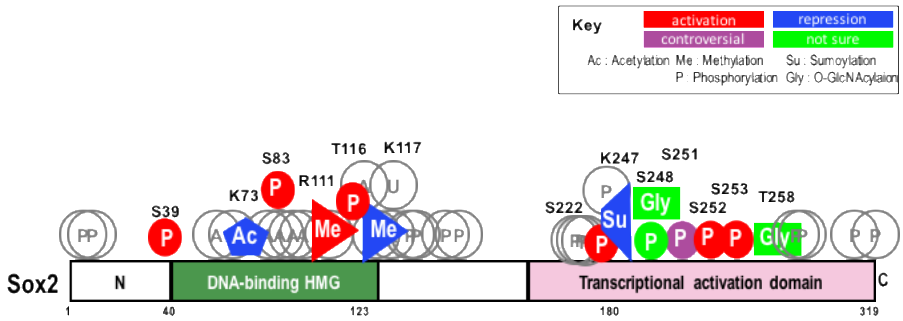


Figure 1-2-2. Diagram of Sox2 protein and covalent modifications

Types of posttranslational covalent modifications and their functional effects, based on studies using human and mouse Sox2, are indicated on the mouse Sox2 amino acid coordinate.

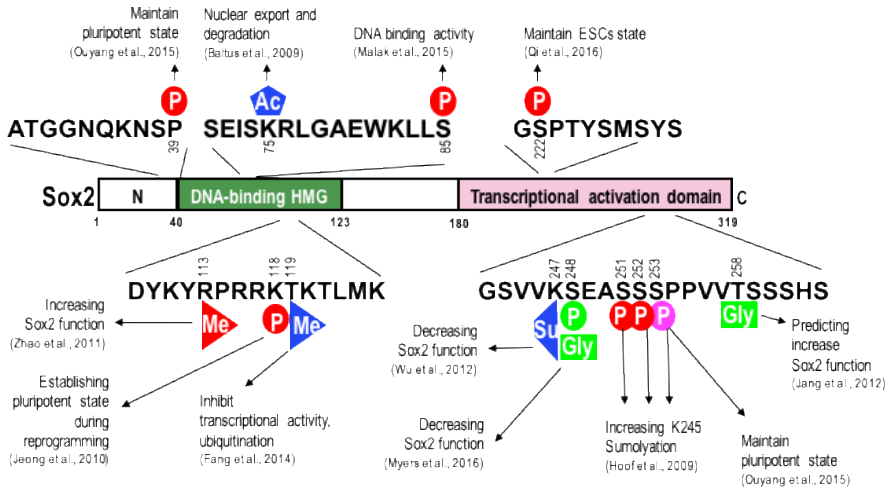


Figure 1-2-3. Diagram of Sox2 PTMs

The function of the Sox2 PTMs studied in Figure 1-2-2

	Published	Description	Sox2 function
S39, S253	Ouyang et al. JBC 2015	The phosphorylation of Sox2 S39 and S253 by cdk2 is essential for maintaining the mESC and maintaining the pluripotent state during reprogramming.	↑
K75	Baltus et al. Stem Cells 2009	mESC (J1) p300 acts as a nuclear export and degradation by acetylation to K75.	↓
S85	Malak et al. CELL CYCLE 2015	AKT-mediated phosphorylation of Sox2 might influence its nuclear import and DNA-binding activity.	↑
R113	Zhoa et al. Plos One 2011	mESC (p19) CARM1 methylation to R113. Increased Sox2 function.	↑
T118	Jeong et al. Cell Stem Cell 2010	Increased activation of AKT1 by phosphorylation at E14. Increase in establishing pluripotent state during reprogramming	↑
K119	Fang et al. Molecular Cell 2014	The monomethylation of Sox2 K119 by set7 plays a role in antagonizing the phosphorylation of T118 by AKT1, inhibiting Sox2 transcriptional activity, and acting as ubiquitination and degradation.	↓
S222, S253	Qi et al. CELL CYCLE 2016	Aurora kinase A phosphorylates S222 and S253 of Sox2 (human ovarian cancer). A form or AURKA inhibition results in the loss of stem cell character and differentiation.	↑
K247	Wu et al. Plos One 2012, Tsuruzoe et al. Biochem. Biophys. Res. Commun 2006	SUMOylation of K247 of Sox2 interferes with OCT4 and reduces the function of Sox2 binding to a transcriptional target.	↓
S248	Jang et al. Cell stem cell 2012	The phosphorylation or *O*-GlcNAcylation of S248 of Sox2 reduces Sox2 activity.	↑
	Myers et al. elife 2016.	In S248 of Sox2, *O*-GlcNAcylation mainly reduces Sox2 activity rather than phosphorylation.	

S251, S252, S253	Hoof et al. cell stem cell 2009	S251, S252 and S253 phosphorylation by Unknown kinase increased K247 SUMOylation	↓
T258	Jang et al. Cell stem cell 2012	*O*-GlcNAcylation of T258 in Sox2 is thought to be associated with increased Sox2 activity.	↑

Table 1. Description of Sox2 PTMs

Explanations of Sox2 PTMs studied in Figure 1-2-2 and their effect on Sox2 function

II. MATERIALS AND METHODS

2-1. Cell culture

E14 (mouse embryonic stem cells) was purchased from the Mutant Mouse Regional Resource Centers (MMRRC) (Jang et al., 2009). 2TS22C (mouse embryonic stem cells) (accession number AES0125), the parental cell line for derivation of tagged Sox2 lines (Masui et al., 2007), were obtained through Riken BioResource Center. E14 and 2TS22C cell lines were cultured in DMEM (Hyclone) supplemented with 15% FBS (Gibco), 2 mM l-glutamine, 55 μM β-mercaptoethanol, 1% (v/v) non-essential amino acid, 100 U/ml penicillin and 100 μg/ml streptomycin, and 1000 U/ml ESGRO (Millipore). Identity of 2TS22C cell line, which contains a Sox2 transgene under control of a tet-repressible promoter, was authenticated by culturing cells with and without doxycycline and examining Sox2 expression by Western blotting. To create the cell lines, 1 ug of the plasmid was transfected with Lipofectamine 2000 (Invitrogen) into a 6cm dish containing 2TS22C cells. Twenty-four hours after transfection 1 ug/mL doxycycline was added to silence expression of endogenous Sox2. After about two weeks, colonies exhibiting the typical ESC morphology were expanded and tested via western blot, morphology and colony forming assay. The same strategy was used to generate the Flag-tagged wild-type Sox2 (WT) and *O*-GlcNAc-deficient mutants (S248A and T258A), double mutant (S248A, T258A) ESC lines.

2-2. DNA construct, chemicals and antibodies

DNA construct of pMSCV-Flag puro Sox2 and pCAG-IP-Flag Sox2 were described previously (Jang et al., 2012). Various point mutants of pMSCV-Flag puro Sox2 and pCAG-IP-Flag Sox2 generated by site-directed mutagenesis. Sox2 (#MAB2018 (western blot), #MAB4343 (immunostaining)), Normal Mouse IgG (12-371) antibodies were purchased EMD Millipore; Actb (A2228), Flag (F3165) Sigma-Aldrich; CTD 110.6 (MMS-248R) BioLegend; Otx2 (ab21990) Abcam; Alexa Flour 488-conjugated anti-mouse (A11029) Invitrogen. DAPI (268298) was from Calbiochem. Doxycycline (D9891-1G), cycloheximide (01810-1G) was from Sigma-Aldrich

2-3. Colony forming assay

Colony forming assay using alkaline phosphatase (AP) staining was done as described (Jang et al., 2012).

2-4. Immunoprecipitation

Immunoprecipitation from 2TS22C Sox2 WT and T258A were done with AccuNanoBead Protein G Magnetic Bead™ (#TA-1021-1, Bioneer) as per the manufacturer's instruction.

2-5. Immunofluorescence

Immunofluorescence was done as described (Jang et al., 2009). Images were obtained an X20 at the Image Core (National Cancer Center) on a LSM510 META (Carl Zeiss, Germany)

2-6. Western blot analysis

Western blot were done as described (Jang et al., 2009). Briefly, cells were lysed using lysis buffer containing 20 mM Tris-Cl (pH7.4), 150 mM NaCl, 1mM EDTA, 1%(v/v) Triton X-100 and protease inhibitors (#p3100-010, GenDEPOT).

2-7. RNA, DNA precipitation

Total RNA from 2TS22C Sox2 WT and T258A cells were extracted with Trizol (#15596018, Life Technologies) as per the manufacturer's instruction. Genomic DNA from E14 mock and E14 T258A cells were preparation with MagListo™ 5M Genomic DNA Extraction Kit (#K-3603, Bioneer) as per the manufacturer's instruction.

2-8. RNA sequencing

Preparation of RNA library and sequencing were performed by Macrogen (Seoul, Korea). RNA sequencing was done using Next sequencing 500 system (Illumina).

2-9. Real-time PCR

Reverse-transcription PCR, real-time PCR was performed as described (Kim, Jang et al. 2015) with some modifications. Briefly, total RNAs from 2TS22C Sox2 WT and T258A cells were reverse transcribed according to the manufacturer's protocol (#2620A, Takara). Real-time PCR was performed by FastStart Essential DNA Green Master Kit (#06402712001, Roche) using Real-time PCR LightCycler96 (Roche) and normalized to Actb, Gapdh. The primers used for real-time PCR are listed in Table 2.

2-10. Purification of Sox2 complex

Sox2-binding proteins were immunoprecipitated using anti-Flag antibody-conjugated agarose beads (80 μl of 50% slurry) from about 90 mg of extracts that were washed with buffer containing 20 mM Tris-HCl (pH 7.9), 15% Glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM PMSF, 0.05% Nonidet P40, and 150 mM KCl to remove non-specific contaminants, and the

bound materials were eluted by competition with the Flag peptide (0.1 mg/ml). The bound proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2-11. Identification of Sox2 interacting Proteins

2-11-1. Protein Digestion and Peptide Cleanup

The protein samples were precipitated using cold acetone, reduced by 10mM DTT and alkylated by idoacetamide (IAA). The alkylated samples were digested with MS grade trypsin 25 mM NH4CO3 for 12 h at 37 ‘C. Digested peptides were cleaned-up using C18 spin columns (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer's instruction.

2-11-2. LC-MS/MS analysis

The digested peptides were analyzed by a Q ExactiveTM hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific). The peptides were loaded onto trap column (100µm x 2cm) packed with Acclaim PepMap100 C18 resin, separated by the analytical column (EASY-Spray column, 75µm x 50cm, Thermo Fisher Scientific), were sprayed into nano-ESI source. The Q Exactive Orbitrap mass analyzer was operated in a top 10 data-dependent method. Full MS scans were acquired over the range m/z 300–2000 with mass resolution of 70,000 (at m/z 200). The AGC target value was

1.00E+06. The ten most intense peaks with charge state ≥2 were fragmented in the higher-energy collisional dissociation (HCD) collision cell with normalized collision energy of 25 and 30, and tandem mass spectra were acquired in the Orbitrap mass analyzer with a mass resolution of 17,500 at m/z 200

2-11-3. Database search

Database searching of all raw data files was performed in Proteome Discoverer 2.1 software (Thermo Fisher Scientific). SEQUEST-HT was used for database searching against Swissprot – Mus musculus database. Database searching against the corresponding reversed database was also performed to evaluate the false discovery rate (FDR) of peptide identification. The database searching parameters included precursor ion mass tolerance 10 ppm, fragment ion mass tolerance 0.08 Da, fixed modification for carbamidomethyl cysteine and variable modifications for methionine oxidation. We obtained a FDR of less than 1% on the peptide level and filtered with the high peptide confidence.

2-12. Flow cytometry analysis and cell sorting

Flow cytometry analysis was done as reported previously (Kim, Jang et al. 2015) at Flow Cytometry Core (National Cancer Center). GFP positive cells were sorted by FACSort flow cytometry (BD Biosciences) 36 hr after transfection. Single cell clones were identified by DNA sequencing. The

primers used for Site mutation of threonine 258 of Sox2 in ESCs by CRISPR-Cas9 are listed in Table 2.

2-13. Site mutation of threonine 258 of Sox2 in ESCs by CRISPR-Cas9

Site mutation of threonine 258 of Sox2 in ESCs by CRISPR-Cas9 was done as described (Abulaiti et al. 2017) with some modifications. Briefly, Substitution of threonine 258 by alanine was made by CRISPR-Cas9 editing. The 20 bp guide RNA sequence containing the motif NGG nearby threonine 258 was inserted into PX458 (Addgene plasmid no. 48138) vector at the BbsI site, and then was transfected into ESCs together with SSDNA by lipo2000 (Invitrogen).

2-14. Statistical analysis

Numerical values were expressed as the mean ± standard error of the mean (SEM) of three independent experiments. The statistical differences between two groups were analyzed by the two-tailed, unpaired Student's t test. The significant differences between two groups were declared as * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$.

Genomic DNA precipitation		
Gene	Forward (5' - 3')	Reverse (5' - 3')
Sox2	CCTACATGAACGGCTCGCCC	CTCCTCTTTTTGCACCCCTCC
Real time PCR		
Gene	Forward (5' - 3')	Reverse (5' - 3')
Peg10	CCTGACCAACTACGACCTGG	GCCTGTTCCACACGAGGATT
Basp1	CAACTCGCTCCACGCTCC	CCCATCTTGGAGTTCGGCTT
Sfmbt2	CTCAGACCCCTGCTCCCTA	GTCTCCCCAGCTGAAGTCAT
Krt8	CGGCTACTCAGGAGGACTGA	CAGCTTCCCATCTCGGGTTT
Hand1	CGGAAAAGGGAGTTGCCTCAG	CGTCCTTTCCTCTCCTCTCCA
Zic2	CATACAGGGGAGAAACCTTTCCA	CCTTCATGTGCTTCCGCAAC
Gapdh	TCAAGATCATCAGCAATGCC	CAGGGATGATGTTCTGGAGAG
Actb	ATCACTATTGGCAACGAGCG	TCAGCAATGCCTGGGTACAT
Site mutation of threonine T258 of Sox2 in ESCs by CRISPR-Cas9		
	Forward (5' - 3')	Reverse (5' - 3')
SSDNA	CTCCATGGGCTCTGTGGTCAAGTCCGAGGCCAGCTCCAGCCCCCCCGTGGTTGCCTCTTCCTCCCACTCCAGAGC GCCCTGCCAGGCCGGGGACCTCCGGGACATGATCAGCATGTACCT	AGGTACATGCTGATCATGTCCCGGAGGTCCCCGGCCTGGCAGGGCGCTCTGGAGTGGGAGGAAGAGGCAACCACGGGGGGGCTGGAGCTGGCCTCGGACTTGACCACAGAGCCCATGGAG
PX458-gRNA	CACCGTTACCTCTTCCTCCCACTCC	AAACGGAGTGGGAGGAAGAGGTAA C

Table 2. Primer sequences used in the present study

Ⅲ. RESULTS

3-1. Sox2 T258A disrupts self-renewal ability in mESCs

We used a 2TS22C cell line to eliminate endogenous Sox2 by treating doxycycline. (Masui et al. 2007). Sox2 has been reported to be modified by *O*-GlcNAcylation at two positions of serine 248 and threonine 258 (Myers et al., 2011). Serine 248 is also the site of phosphorylation (Swaney et al., 2009; Rigbolt et al., 2011). First, *O*-GlcNAc-deficient mutants (S248A, T258A) and phospho-mimetic Sox2 mutant (S248D) that only blocking *O*-GlcNAcylation were overexpressed using retroviral system, and DOX (Doxycycline) was treated to eliminate endogenous Sox2. As a results, T258A exhibited reduced number of colonies compared to Sox2 WT in colony forming assays with the majority of colonies staining negative for alkaline phosphatase (AP), suggesting that T258A affects ESC self-renewal. S248A and S248D were not different from WT (Figure 3-1 A). These results suggest that the self-renewal ability of Sox2 is affected only by T258-*O*-GlcNAcylation. Difference in self-renewal is not caused by the different virus titers (Figure 3-1 B).

We adjusted the amount of Sox2 protein to the endogenous level and observed more precisely the effect of T258A on self-renewal ability. We generated mESC lines that expressing Flag-tagged wild-type Sox2 (WT) and *O*-GlcNAc-deficient mutants (S248A and T258A), double mutant (S248A, T258A) at the same level as endogens Sox2 (Figure 3-1 C). To confirm the *O*-GlcNAcylation level of T258A, whole-cell lysates from 2TS22C Sox2 WT and T258A cells overexpressing Flag-tagged Sox2 were immunoprecipitated with anti-Flag. Western blot analyses with anti-*O*-GlcNAc (clone CTD110.6)

show that reduction in the *O*-GlcNAcylation of the T258A (Figure 3-1 D). In this case, the T258A mutant reduces the capacity of Sox2 to maintain ESC self-renewal (Figure 3-1 E), whereas S248A, T258A double mutation restores the capacity (Figure 3-1 F), suggesting that posttranslational modifications on two sites play the opposite role. To study T258A self-renewal ability in more physiological condition, we introduced 258 threonine of Sox2 to alanine in endogenous chromosome using CRISPR-Cas9 technic. Despite of several attempts, we could get only heterozygous mutants, but not homozygous mutant (Figure 3-1 G). To determine the impact of heterozygous mutant on ESC pluripotency, we performed an experiment to compare ESCs differentiation tendency. Typically, the withdrawal of LIF sufficiently drives WT, T258A populations to differentiate completely. But readdition of LIF in a short time, these ESCs can restore the pluripotent state. Unlike WT, T258A lost pluripotency and differentiated with withdrawal of LIF for only 18 hours. The withdrawal of LIF for 24 hours was still insufficient to drive WT to differentiate, as readdition of LIF nearly completely restored ESC pluripotency. In contrast, 42 hours was sufficient to drive WT to differentiate, and readdition of LIF failed to restore any pluripotent cells (Figure 3-1 H). Overall, these data suggest that the *O*-GlcNAcylation of Sox2 T258 is very important for maintain of embryonic stem cells.

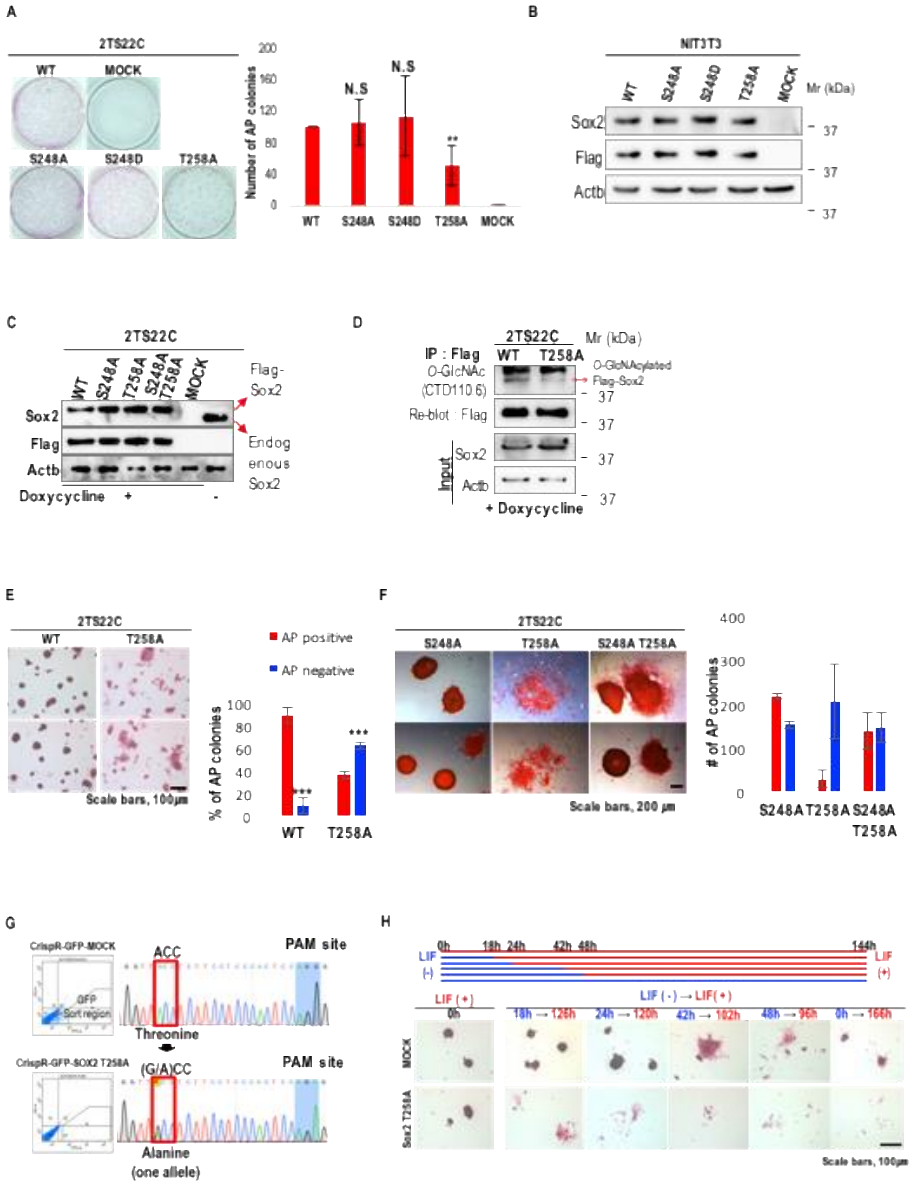


Figure 3-1. Sox2 T258A disrupts self-renewal ability in mESCs

(A-B) Equal numbers of 2TS22C cells were infected with same titer of retroviral Sox2 point mutants. After selecting infected cells in the presence of Dox to eliminate endogenous Sox2, these cells were re-plated for colony

forming assay. The undifferentiated state was assessed by AP staining. AP-positive colony numbers are expressed as relative percent mean ± standard deviation (n = 3). Western blot (B) of Sox2 showed no apparent difference virus titer between wild type and mutants. (C-D) Stably expressed Flag-Sox2 WT and T258A under the control of the constitutive CAG expression unit. After selecting infected cells in the presence of Dox to eliminate endogenous Sox2, these cells were re-plated for colony forming assay. Western blot (C) of Sox2 protein levels showed no apparent difference between wild type and mutants. (D) Western blot analyses with anti-*O*-*Glc*NAc (clone CTD110.6), which recognizes *O*-GlcNAcylated serine and threonine residues show that reduction in the *O*-GlcNAcylation of the T258A. (E) The T258A decreased self-renewal ability. (F) Self-renewal ability becomes rescue in double mutant. (G-H) Substitution of threonine 258 by alanine was made by CRISPR-Cas9 editing. The guide RNA sequence was inserted into PX458 (With a sequence that expresses GFP) vector, and then was transfected into ESCs together with ssDNA. GFP positive cells were sorted by flow cytometry (G). Single cell clones were identified by DNA sequencing. The *O*-GlcNAc-deficient mutant (T258A) interrupts ESC self-renewal capability. E14 cells cultured in serum plus LIF were plated for colony forming assay. After withdrawal and readdition of LIF at the indicated time points, the undifferentiated state was assessed by alkaline phosphatase staining (H). Abbreviations: WT, wild type; S248A, Sox2 248 serine to alanine; S248D, Sox2 248 serine to aspartate; T258A, Sox2 258 threonine to alanine; LIF, leukemia inhibitory factor.

3-2. WT and T258A Sox2 have no difference in cellular localization and protein stability

Next, we investigated the mechanism by which *O*-GlcNAcylation of Sox2 influences mESC self-renewal. Since *O*-GlcNAcylation usually controls cellular localization, protein stability, and protein-protein interaction (Hart et al., 2007; Hanover et al., 2010; Hart and Cope- land, 2010; Love et al., 2010; Yang et al., 2014), we investigated whether T258A affects these effects. WT and mutants exhibited no difference in cellular localization (Figures 3-2-A). In addition, T258A showed no apparent difference in protein stability compared to WT (Figure 3-2-B).

These results demonstrate that the Sox2 self-renewal ability is not dependent on cellular localization or protein stability. So we looked at the protein-protein interaction of Sox2 in WT and T258.

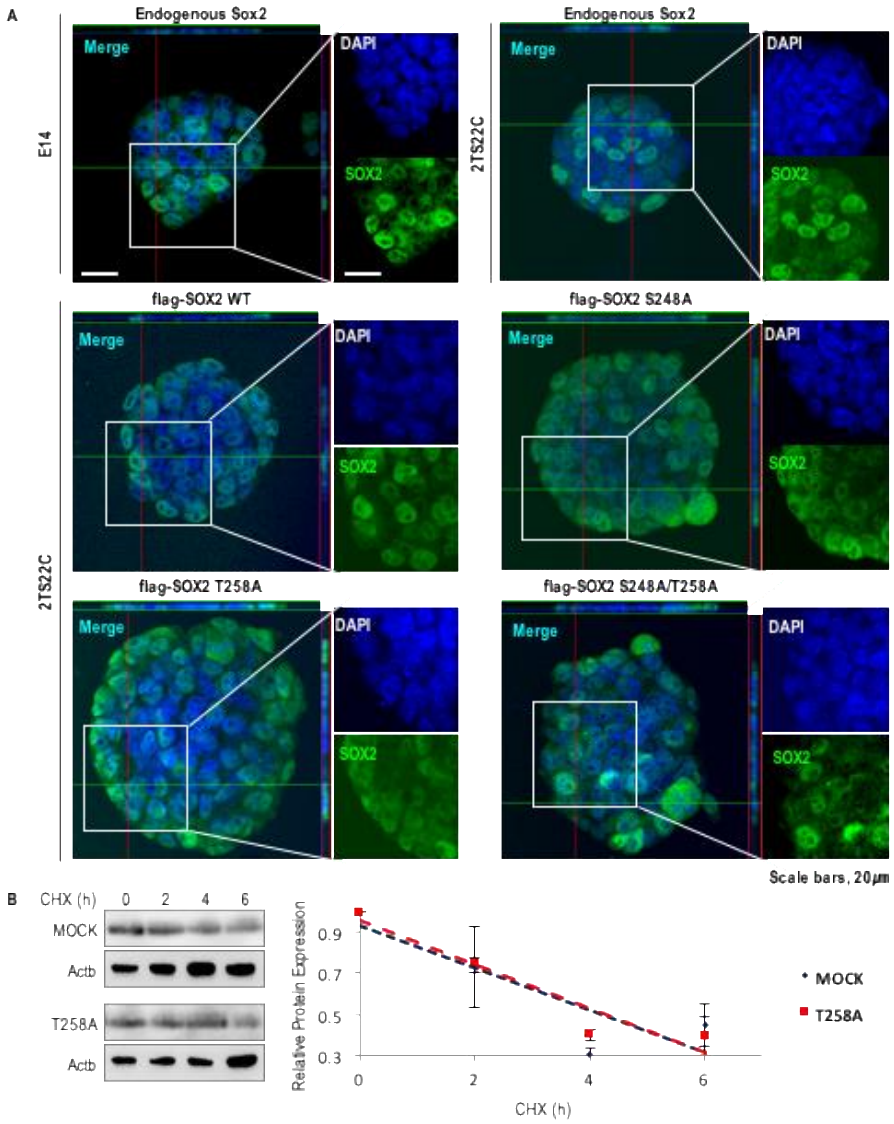


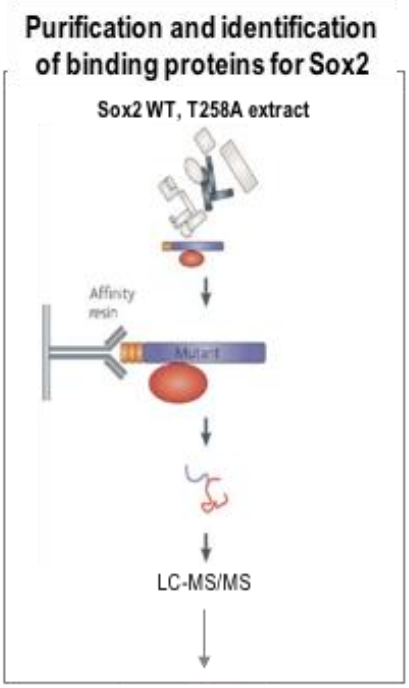
Figure 3-2. T258A and WT have no difference in cellular localization or protein stability

(A) No difference in the cellular localization of wild type and Sox2 mutants. The localization of Sox2 in 2TS22C, E14 cells was analyzed by immunofluorescent staining and 3D-confocal microscopy. (B) No apparent

difference in protein stability between Sox2 WT and T258A. Endogenous Sox2 was replaced with T258A in E14 cells. These cells were treated with CHX (20 ug/ml) for indicated time periods and the Sox2 protein levels were determined by Western blot. Abbreviations: CHX, cycloheximide

3-3. Sox2 *O*-GlcNAcylation affects interaction of Sox2 with various proteins

T258 lies in the TAD of Sox2, a region responsible for interactions with transcriptional regulatory machinery (Kamachi et al., 1998; Myers et al., 2011). Therefore, we tested whether the T258A altered Sox2 centered protein-protein interactions. We performed affinity purifications of Sox2 complex from extracts of Mock, WT and T258A. Co-purified proteins were identified by LC-MS/MS. We identified 800 proteins enriched in Sox2 WT, but not in Mock. Several Sox2 interactors previously described (Mallanna et al., 2010;Engelen et al., 2011;Gao et al., 2012;Cox et al., 2013;Myers et al., 2016) were included in our Sox2 complex. Among them 164 proteins did not found in T258A complexes. These 164 proteins might be the proteins that bind Sox2 T258 *O*-GlcNAcylation dependently. Of these, there are 40 transcription factors, whose roles are negative regulation of gene expression, regulation of embryo development (Figure 3-3). These results suggest that Sox2 *O*-GlcNAcylation directly alters its interaction with a transcriptional regulatory protein involved in maintaining the balance of self-renewal and differentiation (Figure 3-3).



Sox2 binding protein
(800)

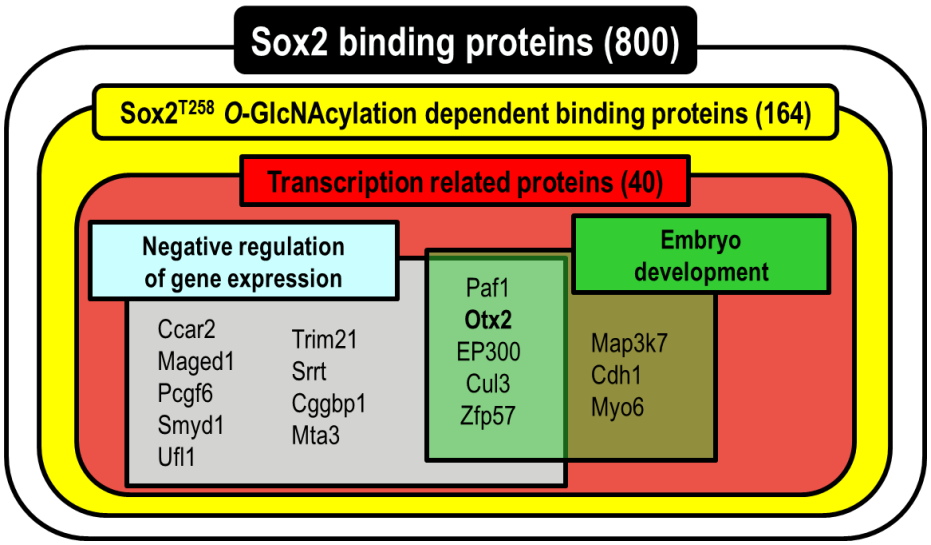


Figure 3-3. *O*-GlcNAcylation of Sox2 at T258 alters protein-protein interactions

Interaction diagram of a subset of Sox2 interactors that exhibit differential association with WT relative to T258A. 800 proteins enriched in both WT and T258A, but not Mock. Among them 164 proteins did not found in mutant complexes. Among these, there are 40 transcription factors, whose roles are negative regulation of gene expression, regulation of embryo development.

3-4. Sox2 T258A is upregulated in developmental signature

To understand why Sox2 mutant could not maintain self-renewal, we screened differentially expressed genes between the cells expressing WT and T258A Sox2 using RNA sequencing. Significant changes in mRNA levels were observed, with 186 genes up regulated and 118 genes down regulated in T258A (Figure 3-4 A). Cells expressing T258A are enriched in ectoderm and mesendoderm development related genes by Gene Set enrichment analysis (GSEA), whereas pluripotency related genes were not altered significantly (Figure 3-4 B-D). This data suggested that activation of developmental genes is the main mechanism why T258A easily lose self-renewal ability.

By combining previous ChIP-sequencing, microarray and our RNA-sequencing results, we identified direct targets of Sox2. The 4255 tentative target genes of Sox2 were found in a microarray paper (Masui et al., 2007, Sharov et al., 2008) and the 5865 Sox2 target genes were found in a paper with Chip-seq (Marson et al., 2008, Lodato et al., 2013. We have identified 1378 putative Sox2 target genes that are common among these genes. When comparing the putative target genes of Sox2 with the RNA sequencing results of WT and T258A, 31 common genes were found. Of the 31 genes, 27 genes were expressed higher in T258A than WT and only 4 genes were higher. Seven of the 27 genes were found to play an important role in development and differentiation (Figure 3-4 E-F). Real-time qPCR confirmed the differential expression of development related genes including (Figure 3-4 G).

These data suggest the T258A alters the development gene expression in mESCs.

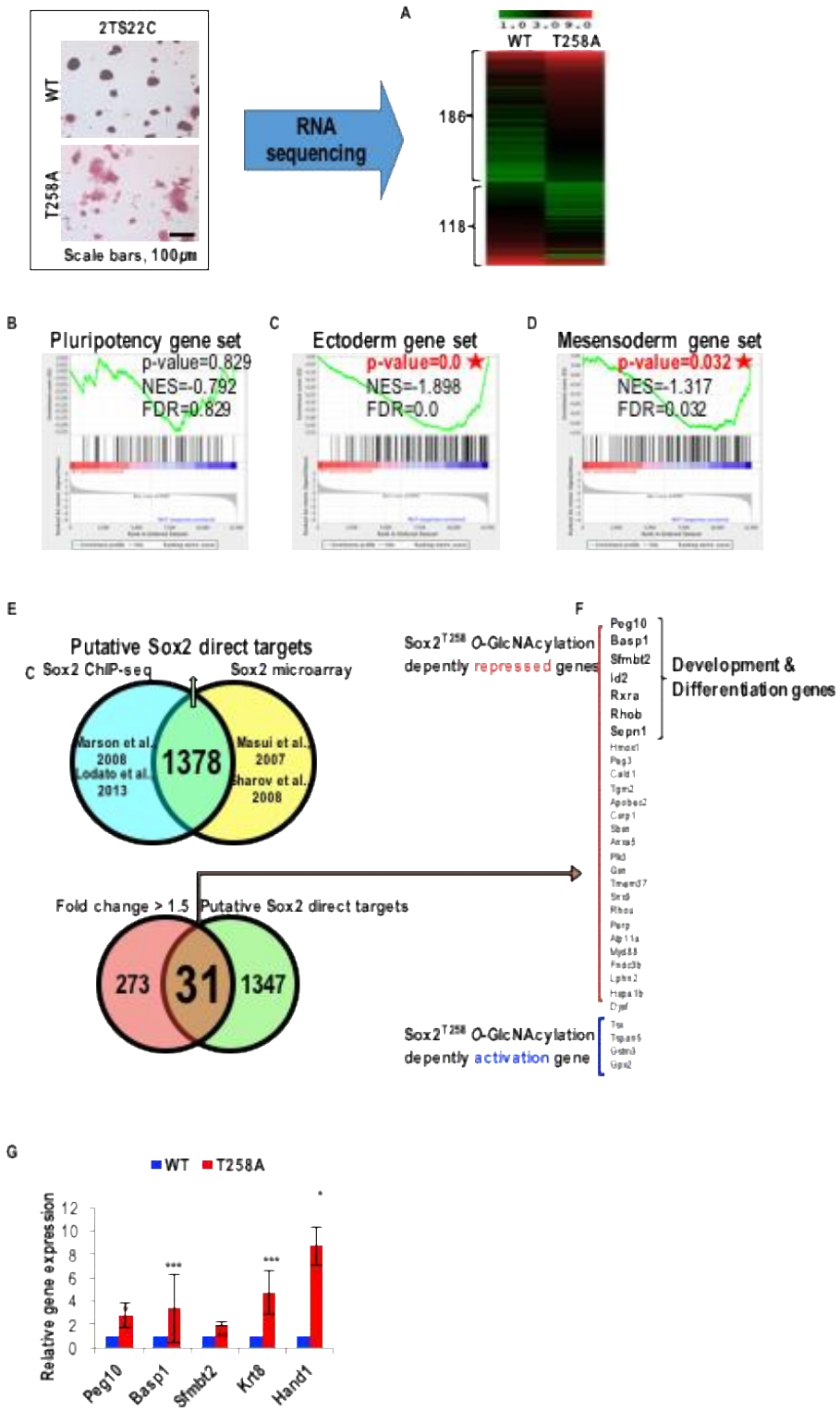

2TS22C
WT
T258A
Scale bars, 100μm
RNA sequencing
A
WT T258A
186
118
B
Pluripotency gene set
p-value=0.829
NES=-0.792
FDR=0.829
C
Ectoderm gene set
p-value=0.0
NES=-1.898
FDR=0.0
D
Mesensoderm gene set
p-value=0.032
NES=-1.317
FDR=0.032
E
Putative Sox2 direct targets
Sox2 ChIP-seq
Sox2 microarray
Marson et al., 2008
Lodato et al., 2013
1378
Masui et al., 2007
Kharov et al., 2008
Fold change > 1.5
Putative Sox2 direct targets
273
31
1347
F
Sox2T258 O-GlcNAcylation depently repressed genes
Peg10
Basp1
Sfmbt2
Id2
Rxra
Rhob
Sepn1
Development & Differentiation genes
Sox2T258 O-GlcNAcylation depently activation gene
G
WT T258A
Relative gene expression
12
10
8
6
4
2
0
***
***
*
Peg10
Basp1
Sfmbt2
Krt8
Hand1

Figure 3-4. T258A are enriched in ectoderm, mesendoderm development signature

(A) Differentially expressed genes (DEGs) between WT and T258A were analyzed by RNA sequencing. Genes whose expression changed more than 1.5-fold change, lower than 5 threshold are shown. (B-D) Gene Set enrichment analysis (GSEA) showed that T258A were enriched in ectoderm and mesendoderm development related gene sets. The gene sets were adapted from Jang et al (2017). (E) 1378 genes common among known genes in microarray and Chip-sequencing published papers. 31 common genes were found in the putative target genes and RNA sequencing results of WT and T258A. (F) Of the 31 genes, 27 are highly expressed in T258A, of which 7 genes (Peg10, Basp1, Sfmbt2, Id2, Rxra, Rhob, and Sepn1) play an important role in development and differentiation. (G) Changes in expression of development related genes were validated by real-time qPCR. Normalized to Actb, Gapdh. Abbreviations: NES, Normalized Enrichment Score; FDR, False Discovery Rate.

3-5. Otx2 and Sox2 suppresses developmental genes in dependent of *O*-GlcNAcylation

Combinatorial analysis of the RNA sequencing and analysis of protein-protein interaction using mass spectrometer. We looked at cofactors that repress the gene expression, influence the developmental stage, and bind to the Sox2 target gene promoter. As a results we found Otx2. We inspected the genomic distribution of the annotated ChIP-sequencing peaks over the mouse genome using Integrative Genome Viewer (IGV) (Robinson et al., 2011). And in published ChIP-sequencing data (Liu et al., 2017; Buecker et al., 2014), Sox2 and Otx2 are enriched the regions around development related genes such as Peg10, Id2, Basp1, Sfmbt2, Rxra (Figure 3-5). These results demonstrate that the Sox2 regulates the expression of target genes by binding with Otx2 in promoter/enhancers of development related genes.

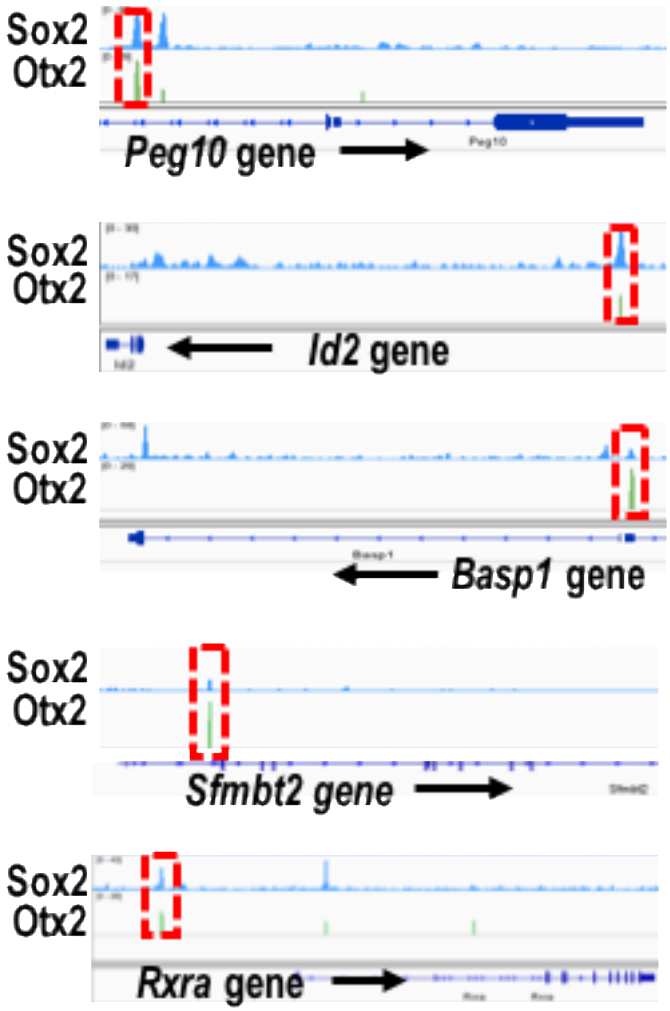


Figure 3-5. Otx2 is correlated with Sox2 and is a downstream target of Sox2 in ESC development

ChIP-seq peaks indicating binding of enhancers Sox2 and Otx2, as shown using IGV outputs for genomic. The direction of gene transcription is indicated by arrows. The Sox2 enhancer is 5' of the Peg10, Id2, Basp1, Sfmbt2, Rxra gene (Buecker et al. 2014; Liu et al. 2017), with ChIP-seq peaks formed at the same position (boxed), indicating the co-binding of Otx2.

IV. DISCUSSION

In this study, we demonstrate that Sox2 *O*-GlcNAcylation is an important regulator of ESCs maintain. Our previous data demonstrate that *O*-GlcNAcylation is critical for Oct4 transcriptional activity and that inhibition of Oct4 *O*-GlcNAcylation disrupts pluripotency (Jang et al., 2012). *O*-GlcNAc-defective Sox2 mutant (S248A) that eliminates both *O*-GlcNAc modification and phosphorylation increase the capacity of Sox2 (Myers et al., 2016), *O*-GlcNAcylation or phosphorylation of the S248 locus is expected to reduce Sox2 activity. Considering that *O*-GlcNAcylation of Oct4 acts to maintain the pluripotency of stem cells, it is not appropriate that *O*-GlcNAcylation of Sox2 inhibits the ability to pluripotency. Since Serine 248 is a site of both *O*-GlcNAcylation and phosphorylation, it is unreasonable to conclude that Sox2 *O*-GlcNAcylation inhibits self-renewal ability. To understand the role of *O*-GlcNAcylation on Sox2, it is necessary to test nutrient dependency of the modification.

Both serine 248 and threonine 258 has been identified as *O*-GlcNAcylated residues in Sox2 by ETD MS/MS in ESCs (Myers et al., 2011). Among them, Sox2 *O*-GlcNAc-deficient mutant (T258A) disrupts self-renewal ability in mESCs (Figure 3-1). *O*-*Glc*NAcylation controls diverse aspects of cellular physiology, such as nutrient, by regulating protein function, localization, protein stability, protein-protein interaction, transcriptional activity (Hart et al., 2007; Hanover et al., 2010; Hart and Cope- land, 2010; Love et al., 2010; Yang et al., 2014). Both Sox2 WT and T258A were localized in nucleus and their protein stability is almost same (Figure 3-2). Also, proteomic analyses indicated there are transcription factors play a role

in negative regulation of gene expression, regulation of embryo development (Figure 3-3). Of those proteins whose interaction with Sox2 were dependent on T258 *O*-GlcNAcylation. These results suggest that Sox2 *O*-GlcNAcylation directly alters its protein binding related to pluripotency and differentiation

Repression of Sox2 in mouse ESCs induces trophectoderm differentiation (Masui et al., 2007), whereas over expression of Sox2 in mouse ESCs induces non-specific lineage differentiation, neuronal differentiation or massive cell death (Mitsui et al., 2003; Zhao et al., 2004; Kopp et al., 2008). Furthermore, Sox2-deficient mice are defective in the maintenance of the ICM／epiblast and trophoblast development (Avilion et al., 2003), leading to the conclusion that Sox2 may have an important role in trophoblast development as well as in ESC maintenance. Reduction in Sox2 expression in hESCs resulted in the loss of the undifferentiated stem cell state accompanied by increased expression of trophectoderm markers (Fong et al., 2008). Depletion or over-expression of Sox2 in hESCs induced trophectoderm differentiation (Adachi et al., 2010). Like this, Sox2 expression level is greater than or lower than normal level, ESCs do not maintain pluripotency and differentiated. Many existing papers do not match the amount of Sox2 protein, so it is not clear whether the studied PTM results precisely reflect the function of Sox2 (Jeong et al., 2010; Zhao et al., 2011; Malak et al., 2015; Qi et al 2016). So we adjusted the amount of Sox2 protein and observed more precisely the effect of T258A on self-renewal ability (Figure 3-1-C).

To confirm the role of T258A in more physiological condition, we mutated endogenous chromosome of WT to T258A using CRISPR-cas9 in

E14 ESCs. Despite of several attempts, we could not get homozygous mutant (Figure 3-1-G). To determine the impact of alterations in metabolism on ESC pluripotency, we performed an experiment to compare tendencies with regard to ESC differentiation. Typically, the withdrawal of LIF sufficiently drives all E14 ESC populations to differentiate completely, but readdition of LIF cannot restore these ESCs to the pluripotent state. However, if we withdraw LIF for the short term, certain ESC populations fail to cross the threshold between differentiation and pluripotency, for which the readdition of LIF at this time point restores pluripotency. Heterozygous mutant alone reduced self-renewal ability by commitment assay (Kim, Jang et al. 2015). Therefore, we think ESCs with homozygous mutant cannot expanded from single cells. Only one allele mutation was sufficient to reduce Sox2 activity to maintain ESC self-renewal (Figure 3-1-H).

Embryonic stem cells (ESCs) are defined by two essential features - pluripotency and self-renewal - whose balance requires the concerted action of signal transduction pathways, transcription factor networks, and epigenetic regulators. Recent findings have implicated the NuRD chromatin remodeling complex in the sophisticated choreography of ESC regulatory pathways (Hu et al. 2012). Components of Otx2 was bind to WT, suggesting the possibility that these complexes may function in development regulation (Figure 3-5). Consistent with this hypothesis, Otx2 Transcription factor, is known that regulate early stage embryogenesis and embryonic stem cells. And Otx2 co-occupied lineage-specific genes with the nucleosome remodeling and deacetylation complex (NuRD) complex to regulate pluripotency in ESCs.

This study shows that new mechanism for the regulation of Sox2 through *O*-GlcNAcylation, and elaborates that role of *O*-GlcNAcylation in pluripotency and self-renewal more delicate than previously appreciated.

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Ⅵ. ABSTRACT IN KOREAN

국문 초록

Sox2 (SRY (sex determining region Y)-box 2)는 Oct4 와 함께 배아줄기세포의 전분화능을 유지하는 핵심인자이다. 오글루넥당화는 세포의 영양상태를 반영하여 전분화능을 조절한다. 전분화능 핵심 네트워크 전사인자들 중 Oct4 의 트레오닌 228 위치와 Sox2 세린 248, 트레오닌 258 위치는 오글루넥당화가 된다고 알려져 있다. 이 중 Oct4 의 오글루넥당화의 역할에 대해서는 잘 연구되어 있지만 Sox2 의 오글루넥당화의 역할은 아직 불분명하다.

이 연구에서 우리는 Sox2 트레오닌 258 (T258) 위치의 오글루넥당화가 배아줄기세포 유지에 중요하다는 것을 발견했다. 배아줄기세포에서 내재되어 있는 Sox2 를 다양한 오글루넥당화 불가 돌연변이체들로 치환했을 때, 248 번째 세린을 알라닌으로 치환한 돌연변이체 (S248A)는 자가재생능력이 유지되는 반면, 258 번째 트레오닌을 알라닌으로 치환한 돌연변이체 (T258A) 치환 세포는 자가재생능력이 현저히 감소하였다. 또한 248 번째 세린과 258 번째 트레오닌을 모두 알라닌으로 치환한 돌연변이체 (S248A, T258A)는 무너진 자가재생능력이 복구되는 것을 보아 두 위치의 수식화가 반대의 역할을 하고 있음을 알 수 있다. 보다 생리학적인 조건에서 T258A 의 역할을 확인하기 위해 CRISPR-

cas9 system 방법을 사용해 염색체상에서 정상 Sox2 를 T258A 로 돌연변이 시켰다. 두 개의 상동염색체가 모두 치환된 줄기세포는 얻을 수 없었고, 한 개의 상동염색체만 T258A 로 바뀐 경우에도 줄기세포 자가재생능력이 현저히 감소하였다.

오글루넥당화는 주로 단백질 안정화, 세포 내 위치, 단백질 결합 상호작용에 영향을 주는 것으로 보고되어 왔기 때문에, Sox2 T258A 돌연변이가 이런 현상에 미치는 영향을 살펴보았다. Sox2 T258A 는 정상과 비교했을 때 단백질 안정화 정도, 세포 내 위치에는 크게 차이가 없었다. T258A 가 Sox2 와 다른 단백질과의 결합에 영향을 주는 지 살펴보기 위해, Sox2 정상과 T258A 단백질 복합체를 각각 정제한 후, 질량분석법을 이용하여 결합이 달라지는 단백질들을 동정하였다. 그 결과 Sox2 정상 단백질에 결합하는 800 여개의 단백질을 동정하였고, 그 중 164 개의 단백질이 Sox2 정상 단백질 복합체에서는 발견되었으나 T258A 복합체에서는 발견되지 않았다. 이 164 개의 단백질들은 Sox2 T258 오글루넥당화 의존적으로 Sox2 에 결합할 가능성이 큰 후보군이다.

Sox2 정상과 T258A 의 RNA-시컨싱 결과 및 기존의 마이크로어레이 결과, 크로마틴 면역침전- 시컨싱(ChIP-seq) 결과를 종합해서 Sox2 T258A point mutation 이 계통 발달 과정의 유전자들의 발현을 직접적으로 증가시킨 다는 것을 확인하였다.

이는 Sox2 가 T258 오글루넥당화 의존적으로 발달 관련 유전자들의 발현을 억제함을 시사한다. 앞에서 찾은 164 개의 Sox2 T258 오글루넥당화 의존적 결합 단백질 후보군에서 40 여개의 전사관련 인자를 찾았고, 이중 유전자의 발현을 억제시키면서 배아 발달에 기능을 하는 단백질로 Otx2 를 동정하였다. Otx2 는 전사인자로 초기의 배아 발생 및 전분화능 조절에 관여하고, NuRD (nucleosome remodelling and deacetylation complex) 복합체와 함께 계통 특이성 유전자들 영역에 결합한다고 알려져 있다. ChIP-seq 결과 분석을 통해 실제로 Otx2 와 Sox2 가 T258A 에서 발현이 증가하는 유전자들 프로모터 또는 enhancer 영역에 같이 결합하고 있음을 확인하였다. 결론적으로, Sox2 의 T258 위치의 오글루넥당화는 Sox2 와 Otx2 의 상호작용에 중요하며, Sox2 와 Otx2 는 함께 발달 관련 유전자들의 발현을 억제하여 전분화능을 조절하는 것으로 생각된다.

주요어 : 영양상태, 배아줄기세포, 오글루넥당화, 전분화능, 핵심전사인자, 발달생물학, Sox2 (SRY (sex determining region Y)-box 2)

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