DEFINING ROLES FOR O-GLCNACYLATION IN HUMAN PLURIPOTENT STEM CELLS

by

XIANGRAN ZHAO

(Under the Direction of Stephen Dalton)

ABSTRACT

O-GlcNAcylation is one of the major forms of protein glycosylation and also an important post-translational modification discovered in recent years. To study the roles of O-GlcNAcylation in human pluripotent stem cells we carried out O-GlcNAc transferase gene knock-down and O-GlcNAcase inhibition assays to manipulate global O-GlcNAcylation level in human pluripotent stem cells, splanchnic mesoderm cells, definitive endoderm cells and neural progenitor cells. These experiments indicated that loss of high global O-GlcNAcylation in human pluripotent stem cells 1) elevates the expression of early mesoderm and early endoderm markers such as *T, GSC* and *EOMES,* 2) increases their doubling time, elongates G1 phase and shortens S and G2/M phases, however, 3) it doesn't affect differentiation to neural progenitor cells. 4) *OGT* knockdown during differentiation towards splanchnic mesoderm and definitive endoderm cells elevated their marker gene expression. 5) Enhanced O-GlcNAcylation impairs transcriptional expression of early mesoderm marker genes. The above findings serve to facilitate our understanding about the role of O-GlcNAcylation in human pluripotent stem cells.

INDEX WORDS: O-GlcNAcylation, human embryonic stem cells, pluripotency, differentiation, OGT

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XIANGRAN ZHAO

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XIANGRAN ZHAO

Major Professor: Stephen Dalton
Committee: Christopher West
Michael Pierce

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 ***Overview***

Being one of the major forms of protein glycosylation and post-translational modification, O-GlcNAcylation (O-GlcNAc) is reported to be involved in various biological processes, such as cellular survival, cell cycle, stress response and development[1-4]. Recent work has defined crucial roles of O-GlcNAcylation in mouse embryonic stem cells (mESCs) by showing that down-regulation of O-GlcNAc transferase (OGT) results in decreased number of alkaline phosphatase (a cell surface marker for pluripotent stem cells) positive cells as well as decreased reprogramming efficiency[5, 9-11]. Surprisingly, limited research has been done on their counterparts-- human embryonic stem cells (hESCs), which are heavily O-GlcNAcylated and potentially depend on O-GlcNAcylation to maintain the key features of ESCs including pluripotency and self-renewal[5]. On the other hand, though reported that fluctuation on O-GlcNAc level is able to impair spontaneous differentiation of both human and mouse embryonic stem cells[5-8], little is known about how O-GlcNAc affects differentiation of hESCs into specific cell types.

1.2 ***Characteristics and process of O-GlcNAcylation***

As a major and unique type of protein glycosylation, O-GlcNAcylation was first discovered as an *O*-beta-glycosidic attachment of beta-*N*-acetylglucosamine monosaccharide on the serine and/or threonine resides of polypeptide in the early 1980s[12]. Unlike other forms of protein glycosylation that attach complex oligosaccharides to intraluminal, membrane and/or secreted

proteins, O-GlcNAcylation only adds one single beta-*N*-acetylglucosamine monosaccharide to the serine and/or threonine and it only occurs on nuclear and cytoplasmic proteins. Among the O-GlcNAc modified proteins many are transcription factors, which offers great opportunity for cells to modify regulatory factors in a post-translational manner. Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the donor of monosaccharide for O-GlcNAcylation, is the ultimate product of hexosamine biosynthetic pathway (HBP) which is a branch-out from the glycolytic pathway (Figure 1). Instead of transferring into pyruvate ultimately, glucose uptaken by cells is catalyzed by hexokinase and phosphoglucose isomerase to generate fructose-6-phosphate, which then accesses the hexosamine biosynthetic pathway via the catalysis of glutamine fructose-6-phosphate aminotransferase along together with glutamine. In the next step, UDP-GlcNAc is formed via the attachment of acetyl from acetyl-CoA and UDP from UTP to glucosamine. As can be observed from the metabolism pathways, synthesis of UDP-GlcNAc is largely affected by both glucose and glutamine levels. In other words, O-GlcNAcylation can be regulated by metabolic changes. It has been shown that O-GlcNAcylation is sensitive to insulin, nutrients and cellular stress, which enlightens the possibility of its role in signaling and transcription regulation[13-16].

1.3 ***OGA and OGT***

O-linked-beta-*N*-acetylglucosamine transferase (OGT), the enzyme that is responsible for the attachment of O-linked-*N*-acetylglucosamine to proteins, was first discovered as a UDP-*N*-acetylglucosamine: peptide complex[17]. OGT was reported to use the synthetic peptide YSDSPSTST as the acceptor substrate and showed linearly activity according to substrate concentration as well as reaction time[17]. Switching proline in the peptide to a glycine results in ineffectiveness of OGT while replacement of aspartic acid to a glycine doesn't

have such effect. It is also found that activity of OGT can be severely inhibited by overdose of UDP yet GlcNAc doesn't seem to change its activity[17]. There are generally three domains of OGT: the N-terminal tetratricopeptide region (TPR) that is also the multimerization domain, the linker part and the C-terminus that has catalytic function. The TPR domain of OGT is highly conserved and is indicated to play essential role in protein-protein interactions[18, 19]. Substrate recognition of OGT enzyme is believed to be subject to the variance of the number of TPR domains[19, 20]. The linker domain contains a bipartite nuclear localization sequence, which is potentially responsible for its nuclear localization[19, 21, 22]. The gene encoding OGT locates on X chromosome and was initially discovered as identical to an open frame in the *C.elegans* gene, K04G7.3 on chromosome III[22, 23]. The researchers also found that forced overexpression of this gene after cloning the full-length cDNA results in effective increased level of global O-GlcNAcylation in human cells[22]. Until now, the gene encoding OGT has been isolated not only from *C.elegans*, but also from rat and human genomes[21, 22], and enzyme activity can be detected in nearly all metazoans examined[23]. There are two kinds of transcripts of OGT mRNA in mammalian cells, mitochondrial OGT (mOGT) and nucleocytoplasmic OGT (ncOGT), resulted from alternative splicing. Compared with mOGT, ncOGT is better studied and is used to represent O-GlcNAc transferase in most studies.

The names of O-GlcNAc hydrolase and O-GlcNAcase (OGA) are used interchangeably to represent the enzyme that removes O-GlcNAc from proteins. OGA was first discovered as hexosaminidase HexC[24-26] and then later distinguished by its neutral pH optimum and selectivity for GlcNAc over GalNAc[27]. There are at least two functional domains found in OGA: the N-terminal GH84 catalytic domain and the C-terminal HAT-like domain, with a stalk domain in between[28]. In human OGA, Asp-174 and Asp-175 were found to consist the

catalytic DD motif, which behaves as the active site for OGA activity[28, 29]. The C-terminal domain is reported to adopt a GCN5 acetyltransferase-like fold and therefore enable OGA to acetylate free-core and nucleosomal histones[30]. Co-presence of OGA and HAT domains in one enzyme convinced one research group that these two activities can work synergistically to regulate transcription. The general idea is that chromatin structures can be opened up via the acetylation of OGA and then transcription factors are activated upon removal of GlcNAc by the hydration activity of OGA[31]. However, those findings haven't been proved to be reproducible. MGEA5, the gene encoding OGA, has two isoforms in vertebrates and one isoform in lower eukaryotes[28, 32-34]. Among these isoforms the shorter form that lacks the C-terminal domain but pertains OGA catalytic activity resides in the nucleus and lipid droplets, while the full-length isoform is most abundant in cytoplasm and nucleus[31, 35-37].

To date up to hundreds of chemical molecules have been described for OGA activity inhibition, with the ultimate goal of studying O-GlcNAc in signaling, development and disease therapy. Among all the potential inhibitors of OGA, there are two types of highly selective chemicals that are most widely used, with Thiamet-G and GlcNAcstatin-G being their representatives respectively. The mechanism of how Thiamet-G inhibits OGA is rather straightforward. It mimicks the oxazoline reaction intermediate and therefore imparts high selectivity over other hexosaminidases[28]. Similarly, the rationale of GlcNAcstatin-G inhibition on OGA is to mimic the transition state of enzyme[28]. The NAGstatin family was originally discovered as hexosaminidase inhibitors. Due to the extension on *N*-acyl substituent that exploits the deep substrate-binding pocket in OGA, selectivity over other hexosaminidase is achieved[28, 38]. There are also other types of inhibitors, such as PUGNAc and streptozotocin, which have been applied in scientific researches and studies.

1.4 *O-GlcNAc and phosphorylation interplay in cellular signaling*

O-GlcNAcylation has long been believed to have cross-talk with phosphorylation, based on the similarities as well as various forms of competition existing between these two modifications. The serine and threonine residues are known to be popular sites for both O-GlcNAc and phosphorylation modifications, indicating potential competition between those two for occupancy at the same or adjacent sites. There are four major forms of cross-talk between O-GlcNAcylation and phosphorylation: competitive occupancy at the same site, reciprocal occupancy at different sites, simultaneous occupancy at different sites and site-dependent reciprocal or simultaneous occupancy. MYC and RNA polymerase II are two classical examples for which are reciprocally modified at the same site by either O-GlcNAc or phosphate. As for proteins like p53 and FOXO1, they can be modified either by O-GlcNAcylation or phosphorylation at proximal sites. One reasonable explanation for that is the large size of O-GlcNAc residue rebels charge between O-GlcNAc and phosphate. The conformational changes on proteins by either modification can be another reason for the resulted exclusivity between O-GlcNAcylation and phosphorylation[13, 39, 40]. The activity of some phosphor-enzymes is subject to O-GlcNAcylation modification. For example, CAMKIV, a kinase regulating transcription factors through phosphorylation, is inactivated with O-GlcNAcylation. This keeps itself from being phosphorylated at the key regulatory proximal site and prohibits it from transiting to the active form[41]. Interestingly, both OGT and OGA can be phosphorylated, with the consequences of OGT being activated and unclear influence on OGA[42-44]. However, it is also reported that virtually every O-GlcNAcylated proteins can also be phosphorylated[39, 45]. Some study even pointed out that inhibition of OGA or overexpression of OGT results in enhanced phosphorylation[46]. This supports the notion that there must be a complex interplay

between O-GlcNAcylation and phosphorylation. Another intriguing similarity between O-GlcNAc and phosphorylation is that they both cycle on and off according to upstream signaling on their catalytic enzymes. Nevertheless, not as much nor well has been studied on O-GlcNAc as on phosphorylation.

In recent years, the role of O-GlcNAcylation in cellular signaling regulation has been gradually uncovered by establishing relationship with already known key regulators involved in transcriptional machinery[47]. The C-terminal domain (CTD) of RNA polymerase II is shown to be exclusive for either phosphorylation or O-GlcNAcylation. In other words, O-GlcNAcylated RNA pol II is no longer available as substrate for CTD kinase phosphorylation[48]. The fact that both phosphorylation and O-GlcNAcylation modified RNA pol II exists in cells leads to the hypothesis that the O-GlcNAcylated RNA pol II inactivates transcription by anchoring at the promoter region in a poised state. Such inactivation can only be rescued by removal of O-GlcNAc[47, 49]. MYC, another example for reciprocal O-GlcNAcylation and phosphorylation modification, has threonine 58 as a target for both OGT and GSK3beta[47, 50]. Threonine 58 resides in the transactivation domain, and the phosphorylation of serine 62 is prerequisite for its phosphorylation[47]. O-GlcNAcylation on threonine 58 will disable its response to the GSK3 signaling. Changes of the binding properties of MYC's transactivation domain by the dynamic interplay between O-GlcNAcylation and phosphorylation will potentially result in altered downstream signaling[51].

Another important way that O-GlcNAcylation affects transcription is through altering the distribution and localization of transcription factors[52]. β-catenin has O-GlcNAcylation site and will change localization with this modification. Enhanced global O-GlcNAc level elevates O-GlcNAcylation on β-catenin, which results in increased translocation to cytoplasm. The direct

consequence of O-GlcNAcylaiton of β-catenin is the decreased transcriptional activity of β-catenin, which emphasizes the possibility that O-GlcNAcylation behaves like a novel transcriptional activity regulator[53-55]. As another example for how O-GlcNAcylation affects transcription factor behaviors, the activity of nuclear factor-κB (NFκB) is affected by O-GlcNAcylation indirectly. NFκB kinase-β (IKKβ), the upstream enzyme of NFκB, is able to phosphorylate the inhibitor of NFκB (IκB) and therefore make it exposed to ubiquitin-proteasome degradation[47]. As a consequence of elevated O-GlcNAcylation on IKKβ, increased amount of IκB are degraded by and NFκB gets to accumulate and translocate to the nucleus. Since NFκB is able to bind to DNA and is the hallmark of many cancers, O-GlcNAcylation of IKKβ may account for a possible reason for transcription of cancer genes[47].

1.5 ***O-GlcNAc and cancer***

Elevated O-GlcNAcylation and aberrant expression of OGA and OGT have been found in various kinds of cancer so far[56, 57]. Multiple groups have reported elevated levels of OGT and O-GlcNAc in prostate[58, 59], colon[60, 61], bladder[62], leukemia[63, 64], lung[60], breast[65, 66], and pancreatic[67] cancers compared to their normal counterparts. It is also found that oncogenic RAS could elevate O-GlcNAcylation via enhanced glycolysis and hexosamine biosynthetic pathways[68]. However, the exact roles of OGT or O-GlcNAcylation in cancer is not fully understood yet.

Till now two signaling pathways have been proposed to be responsible for the elevation of OGT and/or O-GlcNAcylation. In human breast cancer cells inhibitors of PI3K and mTOR results in reduced OGT expression as well as global O-GlcNAc level, which can be rescued by AKT and mTOR activation. It is also found that MYC, downstream of mTOR signaling, is able to elevate OGT expression level[69]. While in lung and prostate cancer cells positive correlation

is found between MAPK/ERK pathway and hyper-O-GlcNAcylation[70]. Taken together, previous studies indicate pathways regulating OGT and O-GlcNAcylation can be cancer type specific[71], which makes it harder to unmask the molecular correlation between signaling pathway and O-GlcNAcylation.

Studies on how OGT and O-GlcNAc regulate cancer phenotypes have been carried out in the past few years, and exciting findings were made regarding to how they behave as 'Hallmarks of Cancer', for example, proliferation and survival, invasion and metastasis, and metabolism[72, 73]. FoxM1 positively regulates transcription of cell cycle genes are is involved in various cancers. Reduced hyper-O-GlcNAcylation is found to yield decreased expression of FoxM1 and its target genes[65]. Emerging evidence shows that inhibition of OGT or O-GlcNAcylation can induce selective apoptosis in human breast cancer cells[74]. Possible mechanism behind this is reduced O-GlcNAcylation induces activation of RNA-like ER kinase and its downstream factors[74]. Hyper-O-GlcNAcylation is suggested to enhance invasion and metastasis in cancer cells by regulating epithelial-to-mesenchymal transition (EMT)[75, 76]. For examples, O-GlcNAcylation on Snail stabilizes itself and thus enhances its repression on E-cadherin directly, promoting invasion and metastasis[77]. Recently, O-GlcNAcylation is revealed to induce cancer glycolysis via regulation on HIF-1 pathway. High level of HIF-1α coexisting with elevated OGT and lower OGA expression in human breast cancer leads to the hypothesis that O-GlcNAcylation regulates cancer cell metabolism. Interestingly, in this research it's hard to ignore the facts that reduced O-GlcNAcylation results in increased HIF-1 hydroxylation and degradation, and overexpression of HIF-1 or GLUT1 can rescue the metabolic defects caused by OGT depletion.[71, 78] This emphasizes the notion that OGT/O-GlcNAc functions as an upstream

regulator of the HIF-1 pathway. All of the aspects discussed above connect O-GlcNAcylation tightly with tumorigenesis.

1.6 *O-GlcNAc and embryonic development*

Embryogenesis has been reported being influenced dramatically by nutrient availability, especially glucose[79, 80]. As a branch of the glycolytic pathway, HBP flux also depends on glucose uptake. Several studies have looked into the relationship between O-GlcNAc, downstream glycosylation resulted from HBP, and developmental progression[81]. Evidence on O-GlcNAc impacting embryonic stem cells (ESCs) viability and self-renewal have accumulated in recent years. Complete knockout of OGT in mouse ESCs generates poor embryogenesis and notably increased cell death was observed at the same time.[3] Mouse blastocyst formation is disordered by hyperglycemia, and such developmental disturbance is proved to be mediated via HBP[82]. Interestingly, the hyperglycemia-induced disorders can be terminated by OGT inhibition effectively, which demonstrates the role of O-GlcNAc in regulating embryonic development, though indirectly[82]. Besides manipulation of OGT, it has been shown that genetic disruption on OGA causes elevated O-GlcNAcylation and neonatal lethality in mouse embryo[4]. Loss of OGA is also shown to lead to mitotic defects, such as cytokinesis failure, increased lagging chromosome and micronuclei formation[4]. Generally, altering O-GlcNAcylation in developing zebrafish gives rise to reduced body size and formation defects in ectoderm, mesoderm and endoderm germ layer differentiation[83]. In another report, increased OGT activity and O-GlcNAcylation caused by GlcNAc supplement is demonstrated to accelerate mESCs migration and proliferation[84]. Proposed mechanism behind this is that O-GlcNAc elevates the intracellular calcium influx and the protein kinase phosphorylation on integrin β4 serine. Phosphorylated integrin β4 is less likely to form complex with plectin, and thus increase

cell migration[84]. Moreover, enhanced OGT expression can impact signaling pathway via Akt glycosylation, glycogen synthase kinase-3β phosphorylation and Snail1 glycosylation[84]. Several self-renewal regulators in ESCs have been reported to have O-GlcNAcylation modified forms, such as OCT4, SOX2, MYC, Zpf281 and mSin3a[5, 85, 86]. Notably, O-GlcNAcylation on OCT4 is reported as required for mESCs self-renewal and pluripotency. O-GlcNAcylated OCT4 is able to promote reprogramming through inducing other pluripotency gene expression, such as *Klf2*, *Klf5*, *Tbx3*, *Tcl1* and *Nr5a2*[5]. Taken these findings together, it is rather clear that a necessary correlation exists between precise regulation on OGA, OGT and O-GlcNAcylation and embryonic development[81].

Beyond the pluripotent stage, O-GlcNAc also plays essential roles in cell differentiation. Researches done on 3T3-L1-adipocyte cell line indicate there's a direct relationship between O-GlcNAcylation and adipocyte differentiation[87, 88]. C/EBPα and C/EBPβ, belonging to the CCAAT/enhancer-binding protein family (C/EBP), are important leucine zipper transcription factors in adipocyte differentiation. Recently, two residues on C/EBPβ, Serine 180 and Serine 181, are identified as O-GlcNAcylation sites[89]. Occupancy of O-GlcNAc on these sites predominantly prevents phosphorylation on adjacent sites, which decreases its binding activity to DNA and delays adipocyte differentiation[89]. On the other side, enhanced O-GlcNAc increases expression of C/EBPα and PPARg, marker for adipocyte[8]. Reduced HBP flux resulting in low O-GlcNAc level correlates with decreased expression of C/EBPα and PPARg, together with poor lipid droplet formation[87, 88]. However, there isn't deeper insight into the molecular connections between adipocyte differentiation and O-GlcNAc. O-GlcNAc level is also indicated to be crucial in myoblast differentiation. In normal differentiation O-GlcNAc level drops in the first two days of myotubule formation, along with elevated OGA and OGT expression

levels[90]. Significant loss of myogenin- and MHC-positive cells were observed upon augmentation of O-GlcNAc via OGA knockdown or inhibition[90]. To conclude, proper level and timing of O-GlcNAcylation is crucial for proper cell differentiation.

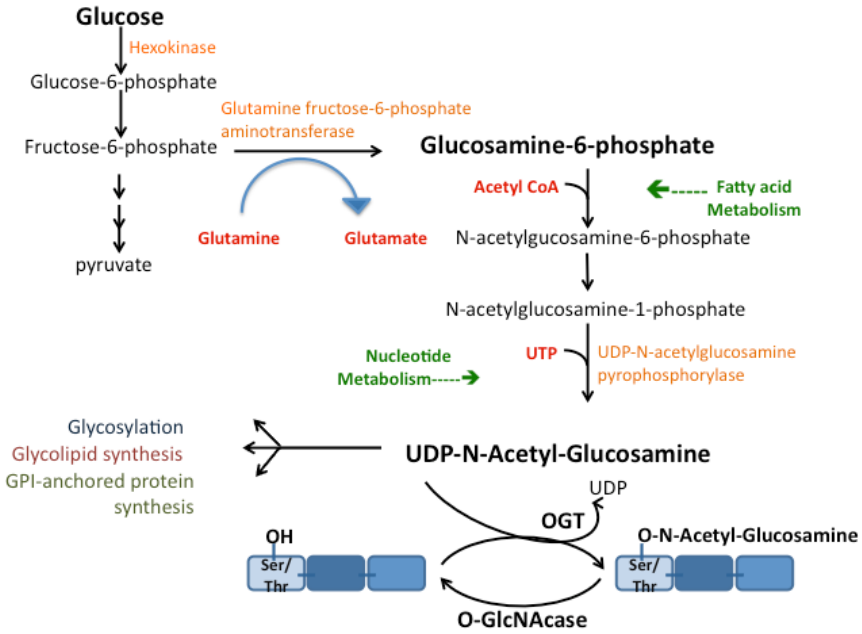

Figure 1. Synthesis of UDP-GlcNAc through hexosamine synthetic pathway (HBP) and process of O-GlcNAcylation on proteins. (Adapted from <Organic Chemistry>, 'Glycosylation', 'Chapter 13' by Paula S R) Synthesis of UDP-GlcNAc is affected by the availability of glucose, glutamine, Acetyl-CoA and UTP. In other words, HBP interacts with glycolysis, amino acid metabolism, fatty acid metabolism and nucleotide metabolism regulating the synthesis of UDP-GlcNAc. O-GlcNAcyaltion of protein is a dynamic process regulated by O-GlcNAc transferase and O-GlcNAcase.

CHAPTER 2

MATERIAL AND METHODS

2.1 *Human embryonic stem cells culture*

To culture hESCs, H9 (WA09) cells in our case, a chemically defined media (DM-) supplemented with four different signaling factors are used. Recipe for making the DM- base media is shown as following: DMEM-F12 w/o glutamine (Cellgro), 2% BSA (Millipore), 1x Penicillin/Streptomycin (Gibco), 1x non-essential amino acids (Cellgro), 1x Trace Elements A, B, and C (Cellgro), 50 µg/mL Ascorbic Acid (Corning), 10 µg/mL Transferrin (Athens Research Technology), 0.1 mM β-mercaptoethanol (Invitrogen), and 1x L-alanyl-L-glutamine. To make the media suitable for maintaining pluripotent stem cells, 8 ng/mL bFGF (Sigma), 200 ng/mL LR-IGF (JRH Biosciences), 10 ng/mL Activin A (R&D Systems), and 10 ng/mL Heregulin B (Peprotech) are supplied into them. This type of media is called 'HAIF media'. For regular maintenance, H9 cells are seeded at 50,000 cells/cm^2 onto 1:200 Geltrex (Invitrogen) coated plates with HAIF media. Media are changed every day and cells are passed every three to four days with Accutase (Invitrogen) when they reach 90-100% confluent.

2.2 *Differentiation of hESCs to neural progenitor cells, splanchnic mesoderm cells and definitive endoderm cells*

Differentiation of H9 cells to neural progenitor cells (NPCs), splanchnic mesoderm cells (ISL1$^+$ cells) and definitive endoderm cells (DE cells) also adopts the application of DM- base media described in the hESCs culture method part. For NPC differentiation, H9 cells are disassociated into single cells are and seeded at 90,000 cell/cm^2 onto 1:200 Geltrex coated plates with NPC

media. Media are changed every day till Day 6 when NPCs can be collected for experimental purposes. 10ng/ml Heregulin B, 200 ng/ml IGF, 500 nM LDN193189 (Sigma) and 8.33 µg/ml SB431542 (R&D) is supplemented into DM- for NPC differentiation. On Day 6, markers of NPCs, such as SOX1 and PAX6, are examined via qPCR and immunofluorescence staining. Differentiation towards splanchnic mesoderm (Isl1+) and definitive endoderm (DE) cells are carried out using the following protocol: H9 cells are seeded at 50,000 cells/cm^2 on 1:200 Geltrex coated plates and/or slides in DM- media described previously, plus 1ug/ml puromycin. For Isl1+ differentiation, 10 ng/ml Heregulin B, 10 ng/ml Activin A, 200 ng/ml IGF, 8 ng/ml FGF2, 25 ng/ml Wnt3a and 100ng/ml BMP4 are supplemented into DM- media. For DE differentiation, 100 ng/ml Activin A and 8 ng/ml FGF2 are used, noting that 25 ng/ml Wnt3a is only supplemented on day1. The differentiation periods of both Isl1+ and DE cells are hour days.

2.3 ***OGT knockdown***

To genetically manipulate OGT level, the gene responsible for adding O-GlcNAc to proteins, we use shRNA knockdown technique instead of knockout because complete knockout of OGT has been reported to be lethal to mESCs.[3] shRNA plasmids for human OGT gene are commercially available from Sigma, which have target shRNAs expressed in the pLKO-puro construct. After amplification using NEB 10 beta competent *E. coli* cells (BioLabs), shRNA plasmids together with packaging and enveloping plasmids, psPAX2 and pMD2.G are mixed and added to HEK 293ft cells (Thermo) using Lipofectamine3000 transfection reagents (Thermo). Lentiviruses are harvested from HEK 293ft cell supernatant at 24hr and 52hr post-transfection and concentrated using Lenti-X concentrator (Clontech). Virus titer is then measured using KAPA SYBR FAST qPCR Kit (KAPA Biosystems). Viruses are stored in -80 ^{0}C freezer as small aliquots. Upon transduction, ~70% confluent H9 cells are infected by lentivirus at MOI= 0.5, 1, 2, 5, 10, mixed

with 8 µg/ul polybrene. At 24hr post-transduction, 1ug/ml puromycin in fresh media is supplemented for drug selection. In another 48hr, drug resistant colonies are counted to find out the optimal MOI with highest transduction efficiency.

2.4 ***Western Blot Assay***

Cell pellet is lysed in RIPA buffer (Sigma) and supernatant is collected after centrifugation. Protein concentration is measured by doing Bradford assay (BioRrad) and stabilized in Laemmli sample buffer (Sigma). Gel running is performed using Bolt Bis-Tris Plus gels (Thermo) and Bolt running buffer (Thermo). Detection of OGT and global O-GlcNAc is performed using OGT antibody (Santa Cruz), anti-O-GlcNAc antibody (Thermo) and HRP immunoglobulins (Dako). Stable OGT-knockdown H9 cell lines are then frozen down in liquid nitrogen as stocks.

2.5 ***C^{13}-Glucose labeling and Nuclear Magnetic Resonance Spectroscopy***

Cells are cultured in regular conditions mentioned in above. Before labeling, cells are fed with fresh regular media four hours ahead of labeling when they are ready for experiment. At labeling the old C^{12}-glucose media are removed and the fresh C^{13}-glucose media are added to cell culture dishes. The C^{13}-glucose media are similar to regular media except for that DMEM (4.5g/L glucose, Invitrogen) is switched to DMEM (no glucose, Sigma), DMEM-F12 w/o glutamine (Cellgro) is switched to DMEM/F12 (no glucose, US Biological), Neurobasal Media (Life Tech) is switched to Neurobasal Media (no glucose, Life Tech), L-alanyl-L-gluatamine is not supplemented and uniform C13-glucose (Cambridge Isotopes) is added according to different media formulation requirement. After incubating the cells in C^{13}-glucose media for four hours, both media and cells are collected separately and shot-frozen to terminate all metabolic activities. Then cells are lysed by water and vortex. Aqueous and organic phases are extracted by water:

chloroform (J.T. Baker): methanol (CRS), and are lyophilized after collecting separately and frozen in liquid nitrogen. For NMR spectroscopy, aqueous lyophilized samples are resuspended in D2O (Cambridge Isotopes) with 10% of DSS (Cambridge Isotopes) in 3mm NMR tubes (Wilmad), while organic lyophilized samples are resuspended in DSS (Cambridge Isotopes) in 5mm NMR tubes (Wilmad). Samples are analyzed on the 800 MHz spectrometer (Oxford Instruments) with PROTON experiment for 1D (H1) spectrum and gHSQC for 2D (H1, C13) spectrum. Parameters used for gHSQC experiment are nt=104, ss=32, ni=120, and spectrum is folded to 10~120 ppm. Spectroscopy data collected from the NMR machine are analyzed on MNova software, using chemical shift standards from HMDB database and concentration standards made by Timothy Cliff. UDP-GlcNAc amount is normalized to nmole/cell its amount can be compared in different cell types.

2.6 ***Real-time polymerase chain reaction Assay***

Cell pellets are collected and washed in DPBS (Thermo) and stored in -80^{0}C for qPCR and Western Blot. E.Z.N.A total RNA isolation kit (Omega) is used for RNA preparation. cDNAs are made from isolated RNA via RT-PCR using iSCript Reverse Transcription Supermix (Bio-Rad). Gene expression of OGT is detected by qPCR using commercial Taqman primer for OGT (Thermo) and Taqman Universal PCR Master Mix (Thermo).

2.7 ***Immunofluorescence staining***

Cells are cultured in 4-well chamber slides (Fisher) and fixed with 4% PFA when ready for analysis. OCT4 antibody (Santa Cruz) is used at 1:50, SOX2 (R & D) antibody (R & D) is used at 1:100, GOOSECOID antibody (R & D) is used at 1:100, BRACHYURY antibody (R & D) is used at 1:100, SOX1 antibody (Abcam) is used at 1:100, PAX6 antibody (Biolegend) is used at 1:200. Images are captured with Olympus FV10-ASW confocal microscope.

2.8 ***Doubling time calculation***

To calculate doubling time, seeding density and time point are recorded for each passage of cells. Cell numbers are counted using hemocytometer and Trypan blue solution (Corning). Doubling time can be obtained via the online Doubling time calculator http://www.doubling-time.com/compute.php. The theoretical formula used is shown as following:

$$DoublingTime = \frac{duration * \log(2)}{\log(FinalConcentration) - log(InitalConcentration)}.$$

2.9 ***Cell cycle analysis***

Cell cycle analysis is applied using Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry kit (Thermo). Cells are cultured to around 90% confluent before being incubated with fresh HAIF media with EdU supplemented at 1:1000. Incubation time need to be optimized for the first time. Usually labeling time for ESCs can vary between 15min to 1hr. After labeling for desired amount of time, EdU is washed away, and cells are digested into single cell solution using Accutase. One million cells are fixed and permeabilized using reagents supplied with the kit. Then Click-iT reaction is started with cocktail included in the kit. FxCycle Violet stain (Thermo) works as DNA dye and is used at 1:1000. To view the distribution of each phase in cell cycle, cells labeled with EdU and FxCycle Violet are run through flow cytometry machine, and then analyzed via FlowJo software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 *O-GlcNAcylation in hESCs and early differentiated cells*

To gain a general understanding of the global O-GlcNAcylation in human embryonic stem cells (hESCs) and other early differentiated cells, such as neural progenitor cells (NPCs), splanchnic mesoderm cells ($ISL1^+$ cells) and definitive endoderm cells (DE cells), we ran the Western Blot assay on the whole cell lysate and blot them against RL2, the pan-specific anti-O-GlcNAcylation antibody. Interestingly, unexpected similarity between hESCs and NPCs emerges, which distinguished themselves from the early mesoderm and early endoderm cells. More enhanced O-GlcNAcylation accumulates in both H9 cells and NPCs, while relatively less O-GlcNAcylation is obtained in $ISL1^+$ cells and DE cells (Figure 2A). This result echoes the metabolic patterns in hESCs, NPCs, $ISL1^+$ cells and DE cells, where C^{13}-labeled UDP-GlcNAc, substrate of OGT, is detected via NMR Spectroscopy (Figure 2B, by Timothy Cliff). After a four-hour C^{13}-glucose feeding period, hESCs and NPCs separate themselves nicely from $ISL1^+$ and DE cells with steady accumulation on the amount of intracellular C^{13}-labeled UDP-GlcNAc. This result points to the notion that relatively high level of O-GlcNAcylation in hESCs and NPCs results from abundant substrate accumulation and implies that different levels of O-GlcNAcylation in each cell type can be a substrate-dependent event. Also, the variance of global O-GlcNAcylation level in each cell type as well as its consistency with the amount of UDP-GlcNAc indicate a possible relationship between pluripotency maintenance, differentiation onset and O-GlcNAcylation.

3.2 ***Knockdown of OGT in hESCs elevates expression of early mesoderm and endoderm markers***

To view how O-GlcNAcylation regulates pluripotency in hESCs (H9 cells), we reduced the global O-GlcNAcylation by down-regulating expression of *OGT* using shRNA knockdown technique, and stable cell lines were established. *OGT* knockdown efficiency are monitored via qPCR assay (Figure 3A), and diminution of OGT protein and reduced global O-GlcNAcylation in *OGT* knockdown cell lines are validated via Western Blot assay (Figure 3B). hESCs are typically featured with high expression of pluripotency markers, such as *NANOG*, *OCT4* and *SOX2*, and minimal expression of differentiation markers, such as *T* (BRACHYURY), *GSC* (GOOSECOID) and *EOMES*. To examine whether *OGT* knockdown will affect this feature of hESCs, transcriptional expression of both two types of markers are checked via qPCR. Although expression of *NANOG* and *OCT4* don't change, *SOX2* expression at transcriptional level drops upon *OGT* knockdown (Figure 4). However, immunostaining results indicated that SOX2 expression at protein level isn't affected by *OGT* knockdown in hESCs (Figure 5). What surprised us is that even cultured in HAIF media which are supposed to maintain cells in undifferentiated state, hESCs with *OGT* knockdown show dramatic up-regulation of the expression of *T*, *GSC* and *EOMES*, which are markers for early mesoderm and early endoderm differentiation (Figure 4). Immunostaining results confirm the presence of BRACHYURY and GOOSECOID in *OGT* knockdown H9 cells (Figure 5). Interestingly, cells that are positive for BRACHYURY or GOOSECOID are still OCT4 and SOX2 positive (Figure 5), indicating though maintaining the expression of pluripotency markers, these cells are already primed to differentiating by expressing the differentiation factors such as BRACHYURY and GOOSECOID upon reduction of O-GlcNAcylation.

3.3 ***Knockdown of OGT alters the doubling time and cell cycle of hESCs***

Besides expression of pluripotency markers, we also want to obtain insight of how other features, such as doubling time and cell cycle, of human embryonic stem cells are affected by reduced O-GlcNAcylation. Doubling time of sh*OGT* and control H9 cells from passage 3 to passage 9 post transduction is measured and recorded. As shown in Figure 6, doubling time of hESCs with *OGT* knockdown ranges from 25 hours to 40 hours, and is notably longer than the control group, which has doubling time of around 23 hours at average. Cell cycle analysis shows increased population in G1 phase and decreased populations in S and G2/M phases in hESCs when they lose high level of O-GlcNAcylation via *OGT* knockdown (Figure 7). Larger population in G1 phase corresponds to elongated doubling time of sh*OGT* H9 cells and implies blockage at G1 to S phase transition due to loss of O-GlcNAcylation. This also suggests potential relationships between O-GlcNAcylation and cell cycle regulators, such as CDK2, Cyclin D, may exist.

3.4 ***Knockdown of OGT doesn't affect neural progenitor cell differentiation***

Since high level of O-GlcNAcylation are found in both hESCs and neural progenitor cells (NPCs), and loss of this feature results in changing of hESCs characteristics, we're interested to see whether heavy O-GlcNAcylation is also required for proper differentiation from hESCs to NPCs. To test our hypothesis, we differentiate sh*OGT* H9 cells to NPCs and evaluate the expression of NPC markers via qPCR and immunofluorescence staining. SOX1 and SOX2 are not affected by *OGT* knockdown at either transcriptional level or translational level (Figure 8 and Figure 9). Two groups of NPCs show decreased *PAX6* expression at transcriptional level by one-fold upon *OGT* knockdown, yet there is no change at protein level according to the

immunostaining results. These results indicate moderate reduction on global O-GlcNAcylation in human embryonic stem cells doesn't affect their differentiation to neural progenitor cells.

3.5 ***Knockdown of OGT elevates maker gene expression during early mesoderm and endoderm differentiation***

Besides examining its influence on differentiation from hESCs to neural progenitor cells, we also explore how reduced global O-GlcNAcylation affects differentiation to splanchnic mesoderm cells (Isl1+ cells) and definitive endoderm cells (DE cells). Upon *OGT* knockdown, at transcriptional level two groups of sh*OGT* ISL1+ cells (#1 and #2) show up-regulation of *ISL1* expression, and two groups of sh*OGT* DE cells (#1 and #3) show more than one-fold up-regulation of *SOX17* and *FOXA2* expression (Figure 10). These indicate that O-GlcNAcylation is capable of altering gene transcription. More interestingly, it is highly possible that reduced O-GlcNAcylation favors transcription of early mesoderm and endoderm marker genes, which may be blocked in the heavy O-GlcNAcylation environment.

3.6 ***Enhanced global O-GlcNAcylation inhibits expression of early mesoderm marker genes***

Facts that global O-GlcNAcylation is kept at relatively low level in ISL1+ cells and DE cells, and that expression of genes such as *ISL1, SOX17* and *FOXA2*, are slightly up-regulated in *OGT* knockdown differentiated cells lead to a feasible hypothesis that enhanced global O-GlcNAcylation will possibly block differentiation towards those two types of cells. To test this, we forced elevation on the global O-GlcNAcylation level during differentiation to $ISL1^+$ cells, DE cells and NPCs by treating cells with GlcNAcstatin, an OGA enzyme inhibitor. GlcNAcstatin is supplemented during the whole differentiation period and global O-GlcNAcylation level is confirmed by Western Blot (Figure 11 and Figure 12). Dramatic down-regulation on *ISL1* expression at transcriptional level is found in GlcNAcstatin treated $ISL1^+$

cells (Figure 11A). Though global O-GlcNAcylation is also elevated to a comparative level to H9 cells during DE differentiation, no change in transcriptional expression of *SOX17* (marker gene for DE cells) are observed (Figure 11B). In contrast, when NPCs are treated with GlcNAcstatin, no obvious elevation on global O-GlcNAcylation is observed until high concentration drug is used. Even so, the expression of NPC marker genes are not affected by manipulation of the global O-GlcNAcylation in NPCs (Figure 12).

3.7 ***Summary***

With the hypothesis that heavy O-GlcNAcylation is required for the maintenance of pluripotency in hESCs as well as proper differentiation to neural progenitor cells, while reduced O-GlcNAcylation is crucial for the onset of differentiation to splanchnic mesoderm and definitive endoderm cells, we carried the above studies discussed in previous part of this chapter. We find that knockdown of *OGT* in hESCs doesn't change expression of pluripotency markers. But it is able elevates expression of the early differentiation markers at both transcriptional and protein level. Tough knockdown of *OGT* elongates the doubling time, as well as alter cell cycle of hESCs, it doesn't affect the differentiation from hESCs to NPCs. Knockdown of *OGT* either elevates or doesn't change transcriptional expression of marker genes during splanchnic mesoderm and definitve endoderm differentiation. Though enhanced O-GlcNAcylation during differentiation to splanchnic mesoderm cells inhibits transcriptional expression of marker gene such as *ISL1*, yet it doesn't affect differentiation to either definitive endoderm or neural progenitor cells.

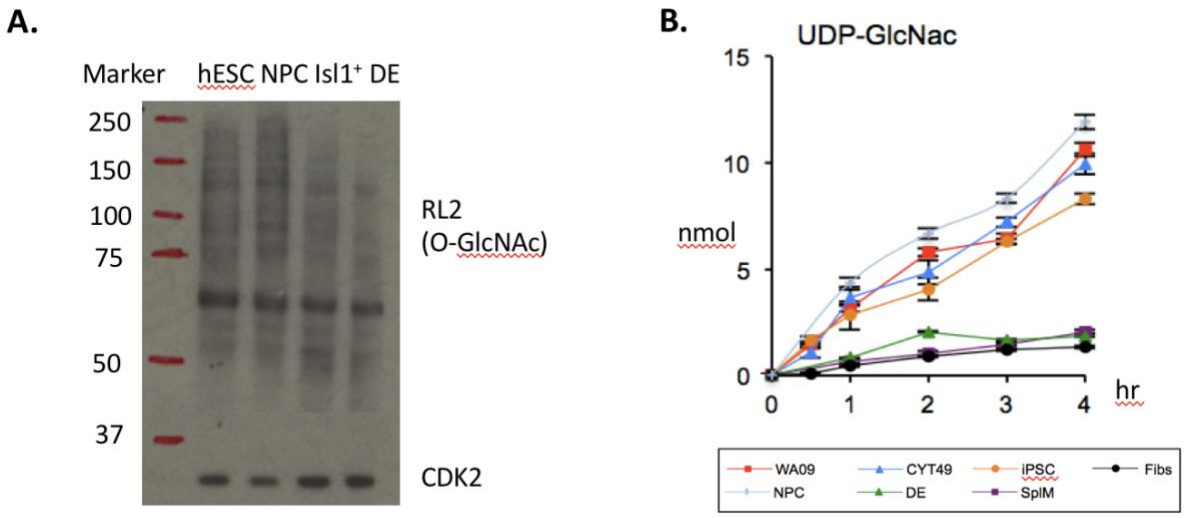

Figure 2. Global O-GlcNAcylation and accumulation of UDP-GlcNAc in hESCs, NPCs, ISL1^{+} cells and DE cells.

(A) Western Blot shows relatively heavier O-GlcNAcylation in hESCs and NPCs, and light O-GlcNAcylation in ISL1^{+} and DE cells. RL2, antibody used to detect global O-GlcNAcylation; CDK-2, internal loading control.

(B) Amount of accumulated C^{13} labeled UDP-GlcNAc in pluripotent and differentiated cells, detected by nuclear magnetic resonance spectroscopy (by Timothy S. Cliff). W09, NPC, DE and SplM cells in this panel correspond to the hESC, NPC, DE and Isl1^{+} cells in panel (A).

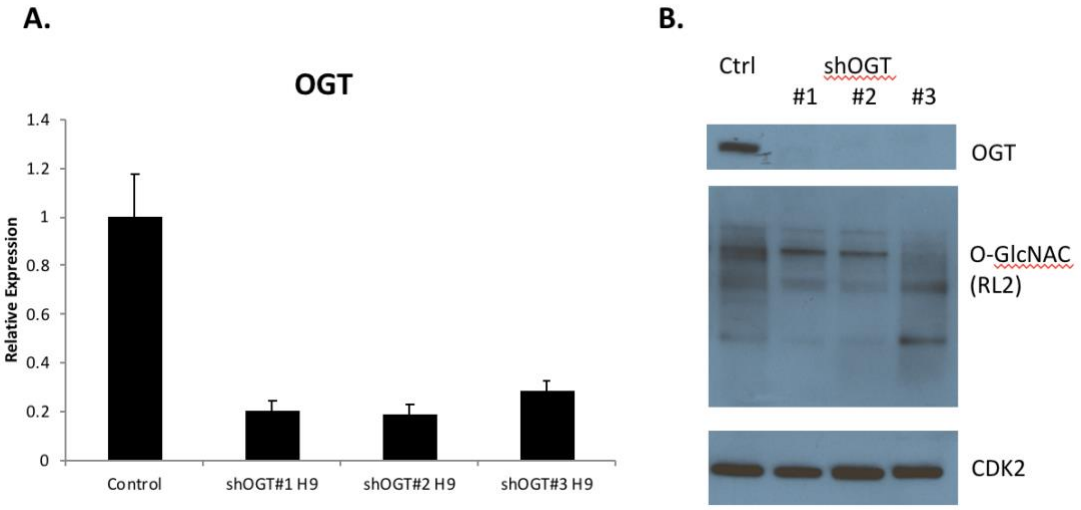

Figure 3. Knockdown of *OGT* in human embryonic stem cells.

(A) qPCR result of *OGT* expression at transcriptional level in H9 cells using shRNA knockdown technique. Three different shRNAs of *OGT* are used. sh*GFP* H9 cells are used as control and OGT expression in sh*GFP* H9 cells is normalized to 1. MOI = 5.

(B) Western Blot assay demonstrates the absence of OGT at protein level, and the reduction on global O-GlcNAcylation in each *OGT* knockdown cell line.

Cell line establishment and gene knockdown experiments were all carried out for one time.

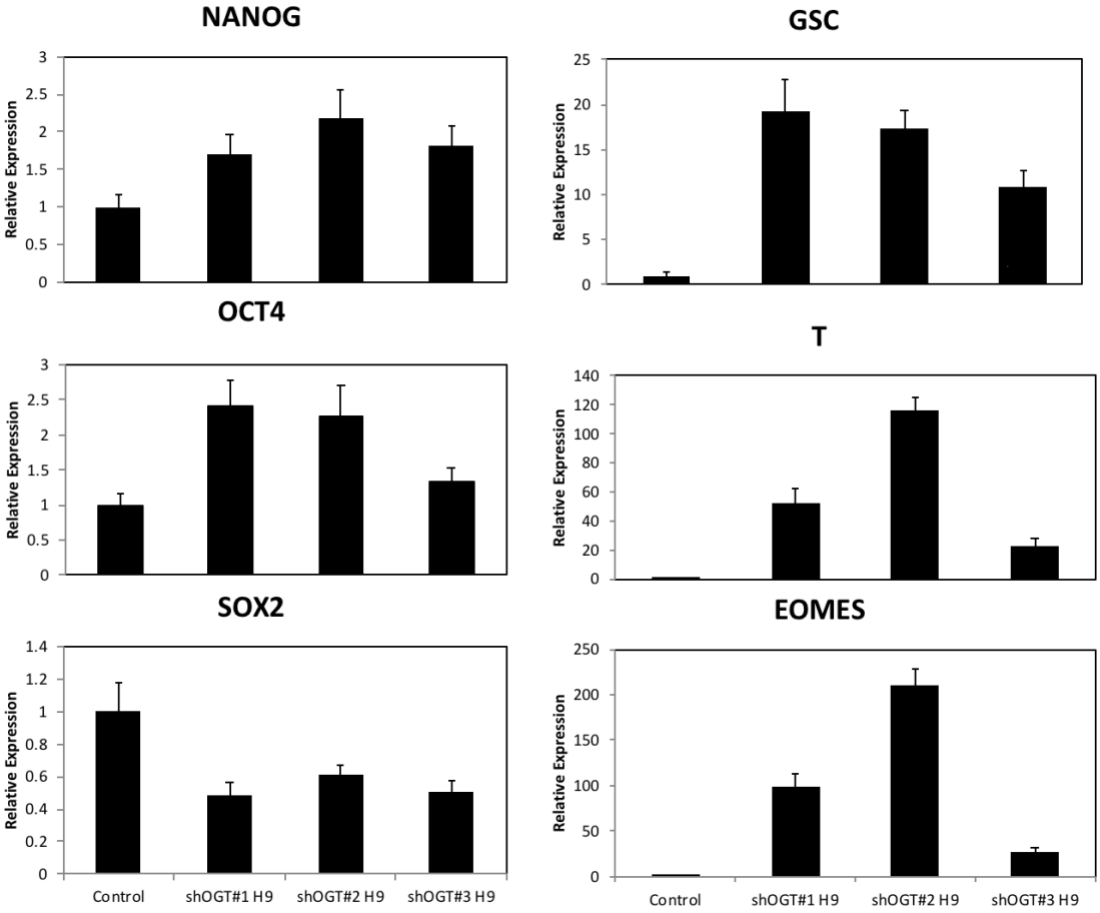

Figure 4. qPCR result of gene expression of pluripotency and early differentiation markers. Up-regulation of early mesoderm and early endoderm markers, such as *T*, *GSC* and *EOMES*, are found in sh*OGT* H9 cells together with stable expression of pluripotency markers such as *NANOG* and *OCT4.*

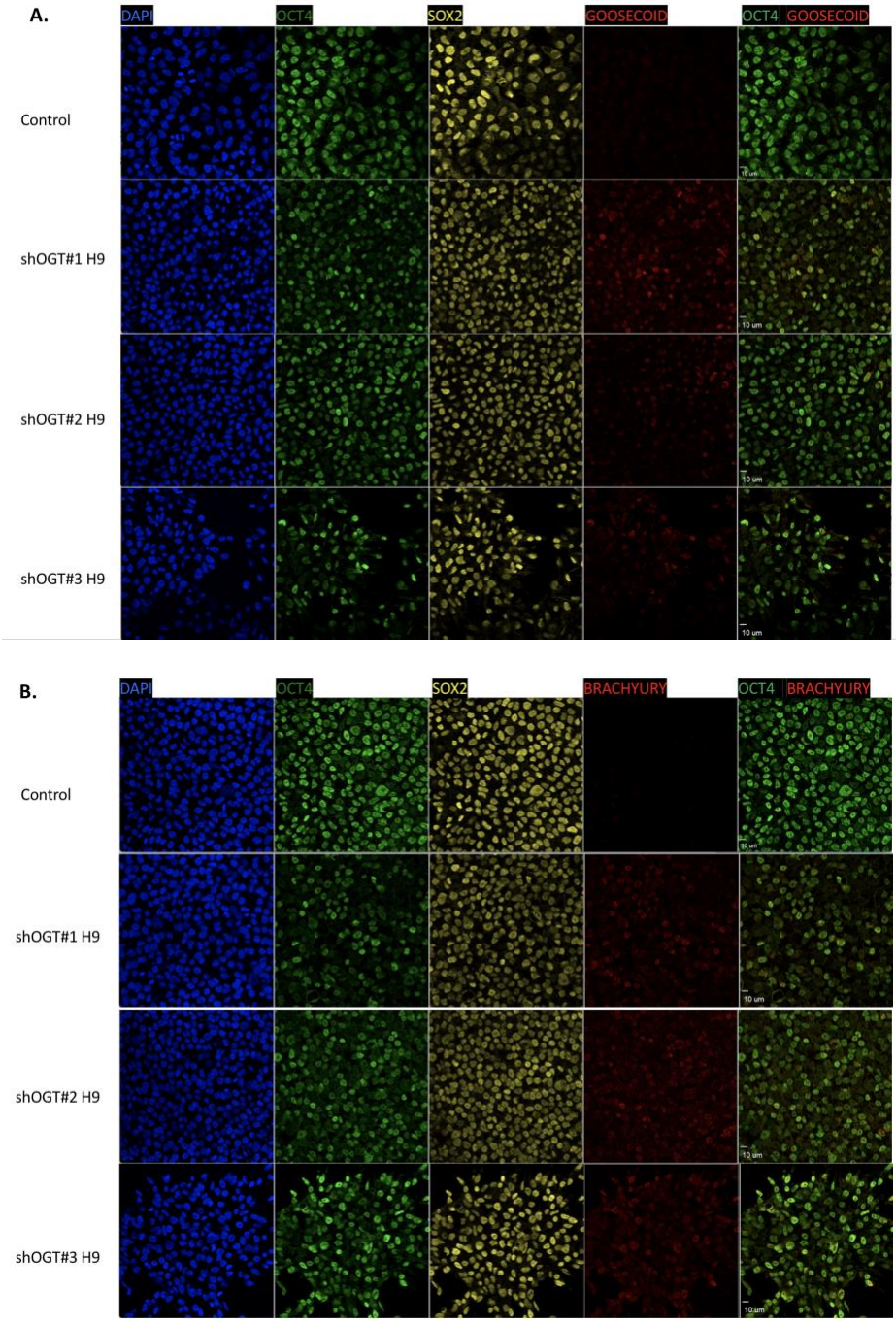

Figure 5. Immunofluorescence staining images on OCT4, SOX2, GOOSECOID and BRACHYURY (*T*) in control and sh*OGT* H9 cells.

(A, B) Blue: DAPI, green: OCT4, yellow: SOX2, red in (A): GOOSECOID, red in (B): BRACHYURY. Objective scale: 40x.

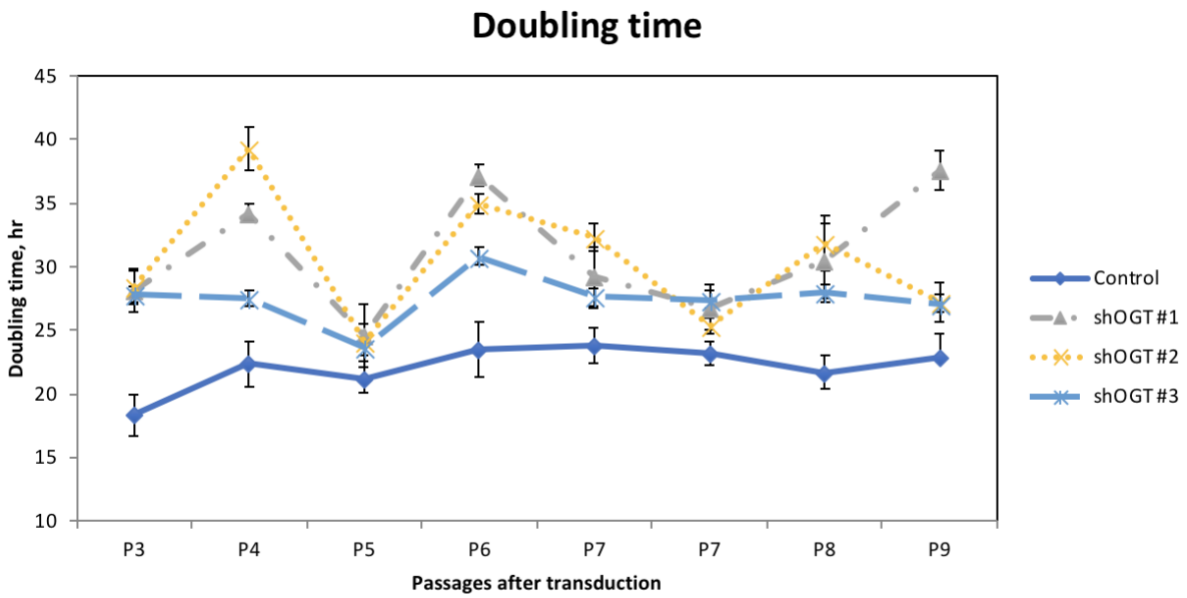

Figure 6. Doubling time of control and sh*OGT* H9 cells from passage 3 to passage 9 after transduction. Knockdown of OGT alters the doubling time of hESCs.

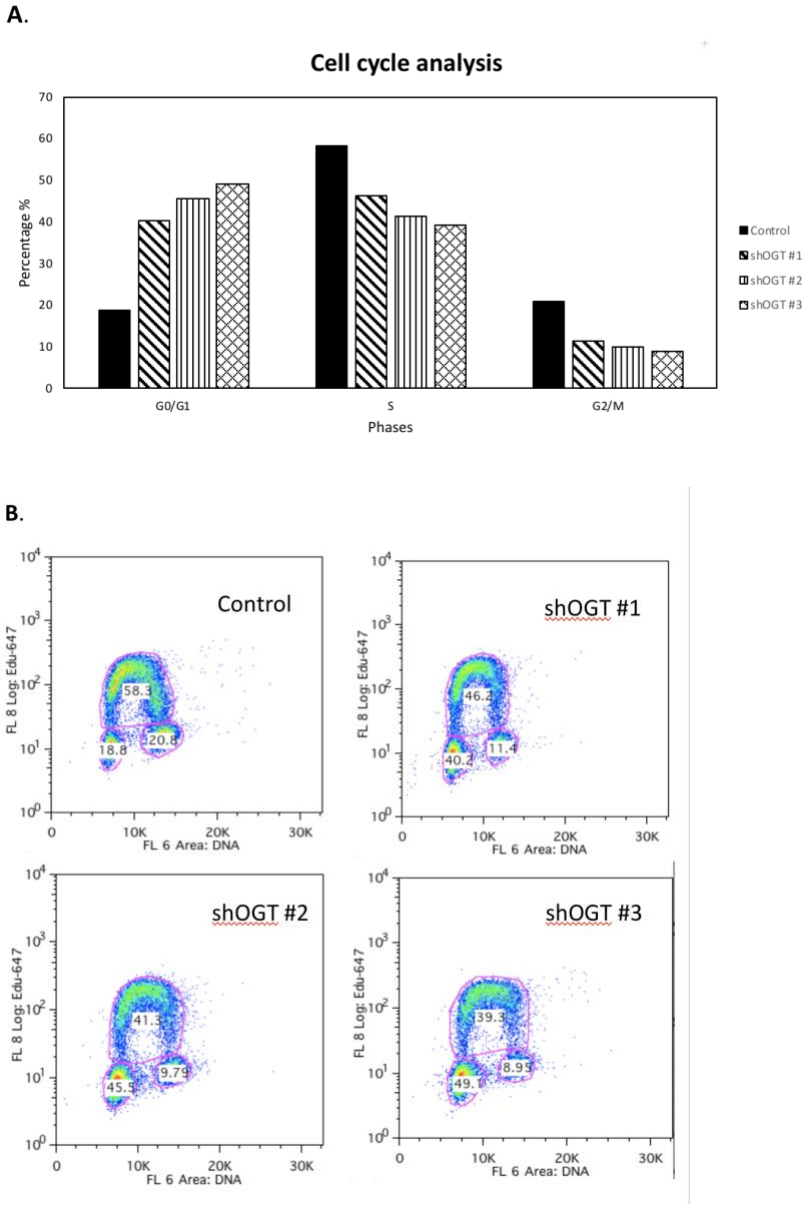

Figure 7. Knockdown of *OGT* alters the cell cycle of hESCs.

A. Histogram of cell cycle analysis in control and sh*OGT* H9 cells in each phase. Increase population in G1/0 phase and decreased populations in S and G2/M phases in are found in sh*OGT* H9 cells.

B. Flow cytometry of cell cycle analysis of control and sh*OGT* H9 cells. X axis- DNA, y axis—EdU reacted azide fluor dye.

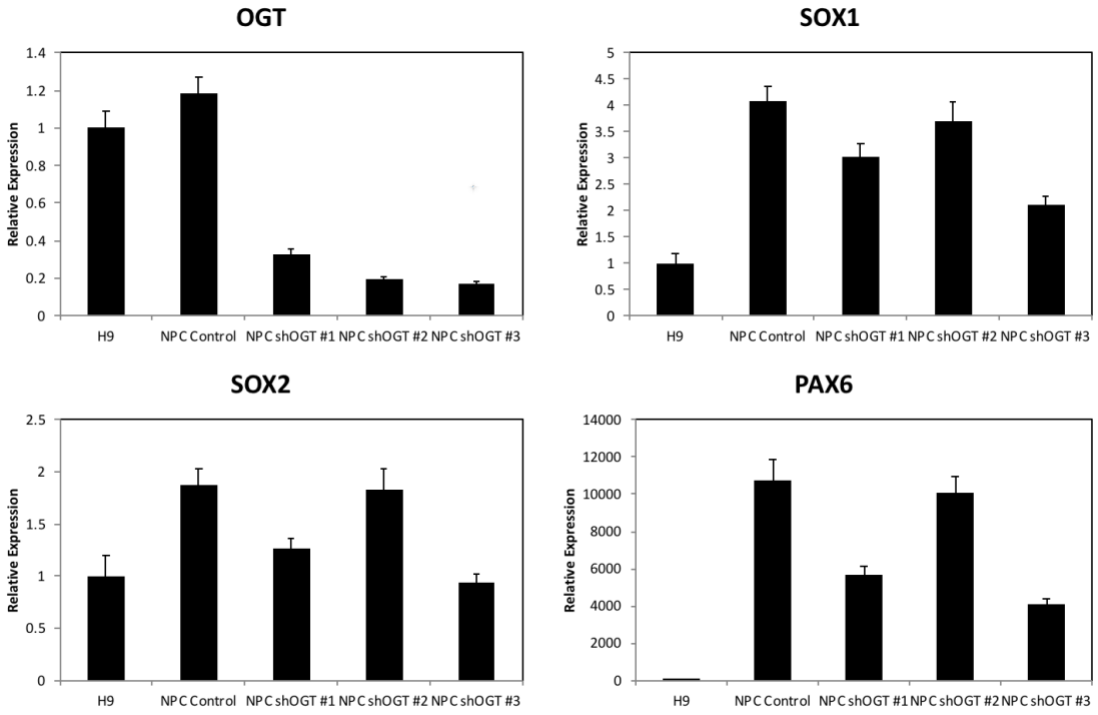

Figure 8. qPCR results of marker genes in control and sh*OGT* neural progenitor cells. No change in *SOX2* and *SOX1* expression upon *OGT* knockdown.

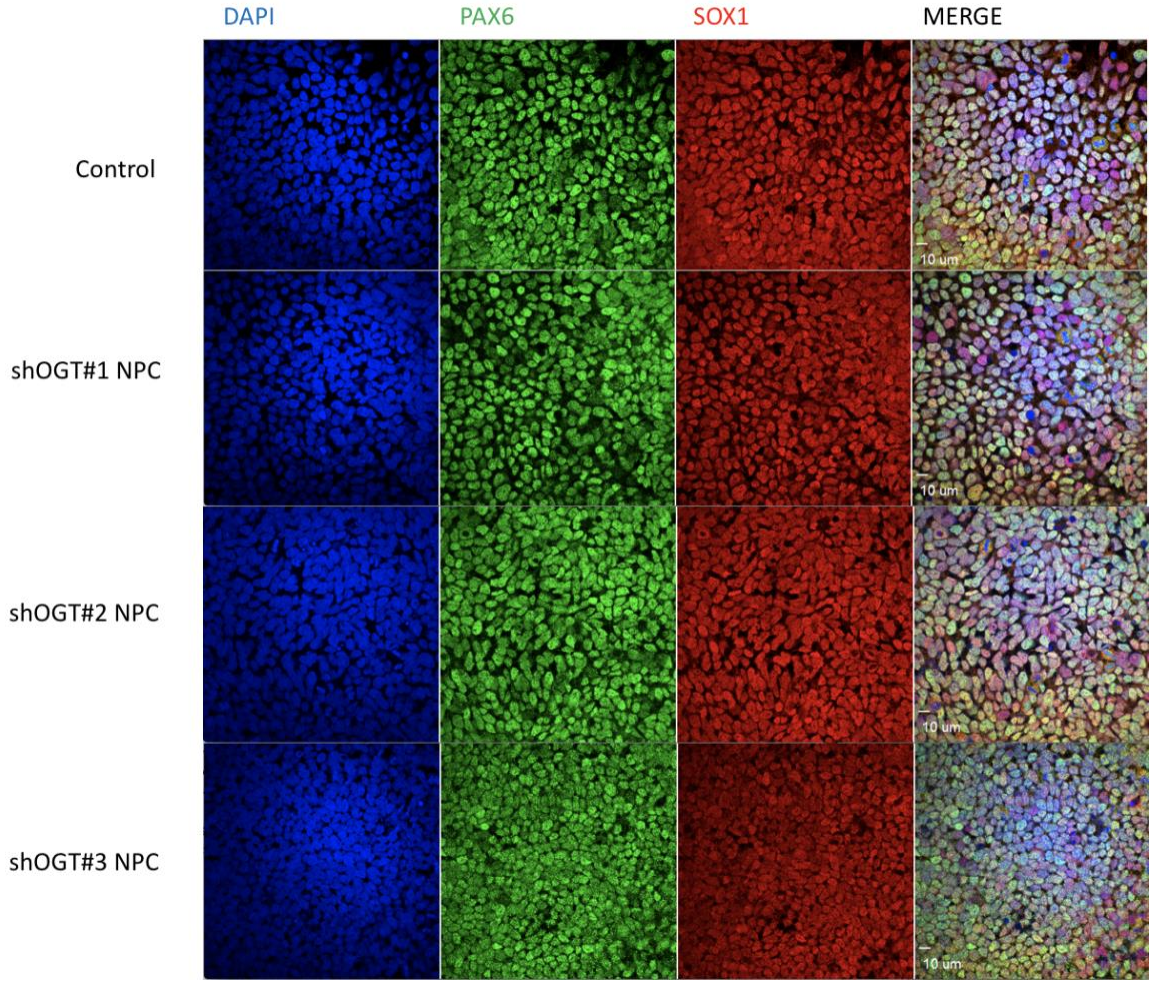

Figure 9. Immunofluorescence staining images of control and sh*OGT* neural progenitor cells. No change in expression of SOX1 or PAX6 is observed upon *OGT* knockdown. Blue: DAPI, green: PAX6, red: SOX1. Objective scale: 60x.

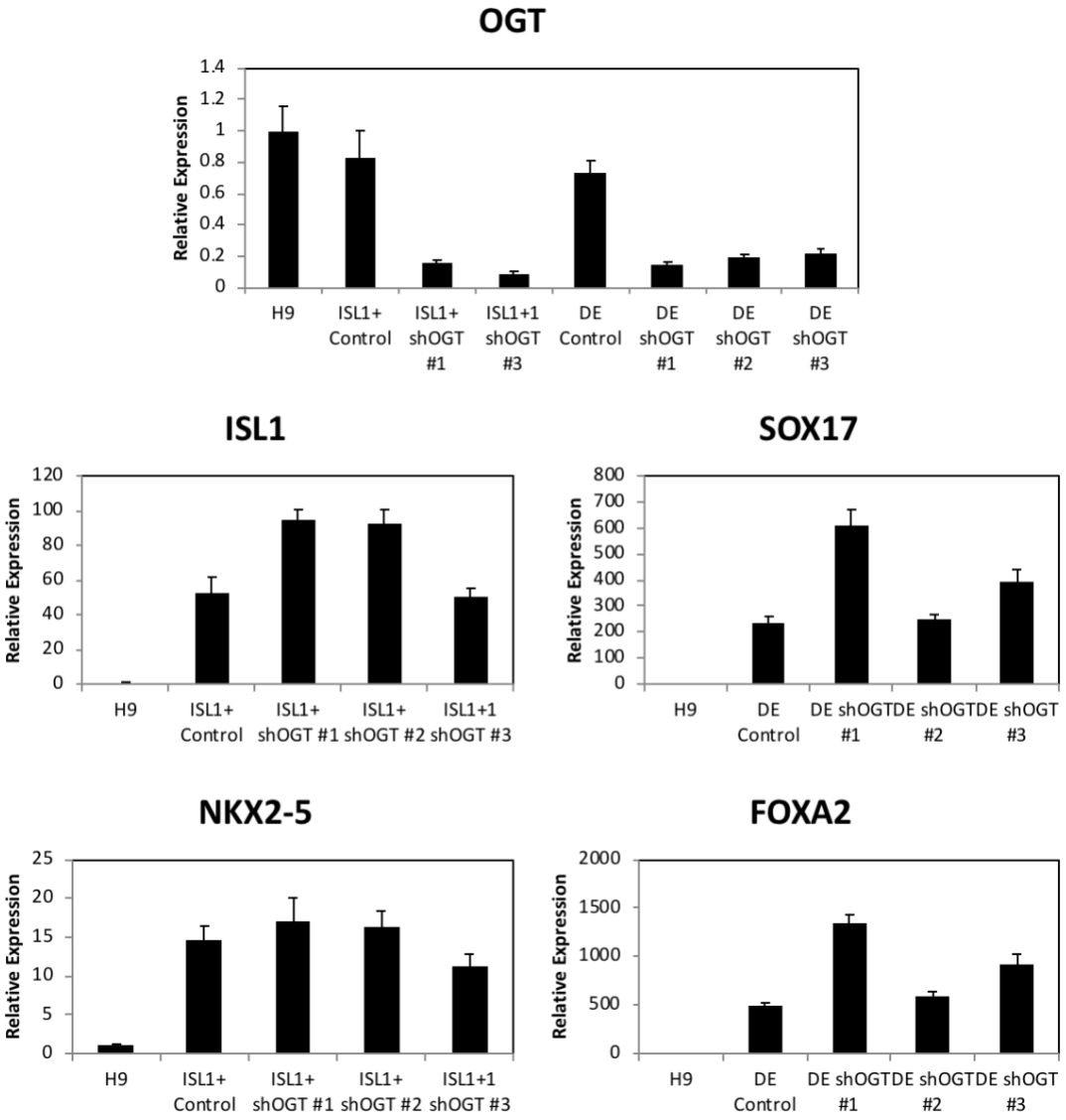

Figure 10. Knockdown of *OGT* elevates expression of marker genes for ISL1^{+} and DE cells. *OGT* knockdown slightly elevates expression of early mesoderm and endoderm marker genes, such as *IS1*, *SOX17* and FOXA2, at transcriptional level during differentiation from hESCSs to ISL1+ and DE cells.

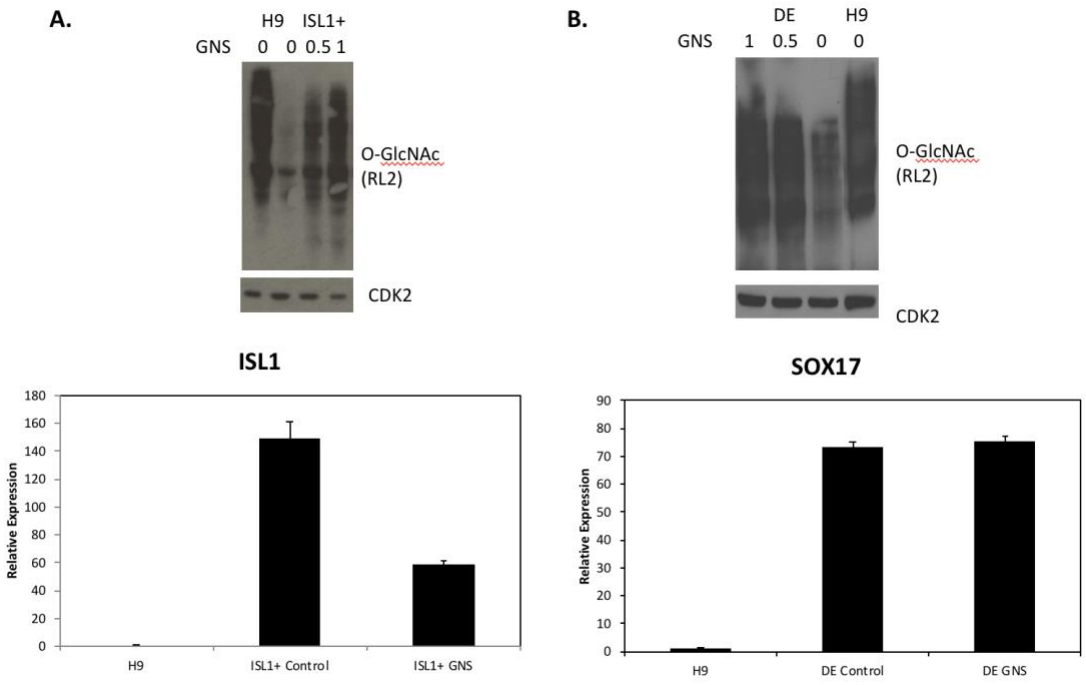

Figure 11. Enhanced global O-GlcNAcylation inhibits expression of *ISL1*.

(A) OGA inhibitor- GlcNAcstatin (GNS) during differentiation to IS1^{+} cells elevate the global O-GlcNAcylation level at 1μM, which results in decrement in *ISL1* expression at transcriptional level.

(B) Supplement of GlcNAcstatin (GNS) at both 0.5uM and 1uM is able to elevate the global O-GlcNAcylation level during differentiation towards DE cells. Expression of *SOX17* at transcriptional level is not affected.

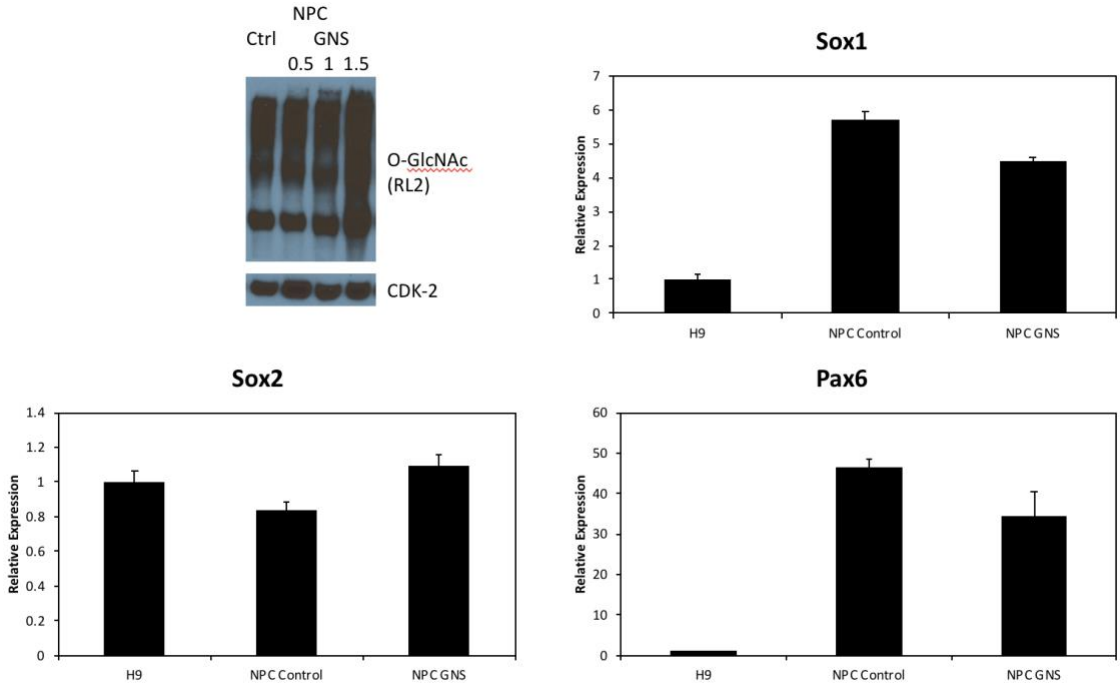

Figure 12. Enhanced global O-GlcNAcylation doesn't affect expression of markers for neural progenitor cells. Global O-GlcNAcylation in NPCs are not elevated until 1.5uM GlcNAcstatin is supplemented. Expression of marker genes for NPCs such as *SOX2*, *SOX1* and *PAX6* are not affected at transcriptional level.

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