

**REGULATION OF NEURAL DIFFERENTIATION IN HUMAN
PLURIPOTENT EMBRYONAL CARCINOMA STEM CELLS BY
RNA SIGNALING**

RUJAPOPE SUTIWISESAK

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ABSTRACT

Pluripotent stem cells are important sources for cell-based therapy, including treatment for neurological deficits. Understanding the process of neural differentiation is important for the development of methods needed to differentiate specific neural cells for treatment. It can also be a platform for studying pathogenesis of neurodevelopmental disorder. In the present study, human pluripotent embryonal carcinoma stem cells, NTERA2, were used as a model to study the neural differentiation process via induction by retinoic acid. The regulation by Paired-box 6 (PAX6), a transcription factor which has been shown as a neural fate determinant of human embryonic stem cells, was tested. An RNA-protein complex comprised of RNA signaling molecules; including CTCF, DDX5 and PUS1, with a long non-coding RNA, namely steroid receptor RNA co-activator (SRA), has been shown to regulate transcriptional activity at different targets. However, CTCF is the only component in the complex shown to regulate PAX6, the information of other components is still missing. Chromatin immunoprecipitation experiment was employed to identify the association of those proteins to the regulatory elements of PAX6 gene. Furthermore, the involvement of the RNA-protein complex in differentiation of NTERA2 was evaluated by loss of function and gain of function experiments, coupled with differentiation induced with retinoic acid treatment. The results revealed that both PAX6A and PAX6B isoforms are able to specify neural differentiation in NTERA2 cells. The association of PUS1 to P1 promoter provides an indication of the role of PUS1 on initiation of PAX6 transcription, while CTCF and DDX5 associations suggest a stabilizing mechanism for the transcription. Loss of SRA induced neural differentiation, while loss of DDX5 and PUS1 favored differentiation into other lineages and suppressed PAX6 expression. Over-expression of DDX5 and DDX17 in NTERA2 cells increased neural differentiation for which DDX17 was important for maturation of neuronal cells. Over-expression of PUS1 resulted in faster advancing the process of neural differentiation and expression of PAX6, whereas results from over-expression of catalytic inactive mutant form of PUS1 were not different compared to that of control. These results suggest a relationship between RNA pseudo-uridylation and transcription of PAX6 gene, which further suggests an engagement of RNA structure to the transcriptional machinery. In conclusion, this study demonstrates the collaboration between RNA and protein structure on regulation of gene expression as well as the process of neural differentiation from pluripotent stem cells.

KEY WORDS: HUMAN PLURIPOTENT EMBRYONAL CARCINOMA
STEM CELLS / NEURAL DIFFERENTIATION / PAX6 /
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REGULATION OF NEURAL DIFFERENTIATION IN HUMAN PLURIPOTENT EMBRYONAL CARCINOMA STEM CELLS BY RNA SIGNALING

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บทคัดย่อ

เซลล์ต้นกำเนิดแบบ pluripotent เป็นแหล่งสำคัญสำหรับการทำเซลล์บำบัด ซึ่งสามารถใช้ในการรักษาโรคทางระบบประสาทได้ การทำความเข้าใจกระบวนการเปลี่ยนแปลงเป็นเซลล์ในระบบประสาทจากเซลล์ต้นกำเนิดจึงมีความสำคัญต่อการผลิตเซลล์เพื่อนำไปใช้ในการรักษา และความเข้าใจดังกล่าวยังสามารถช่วยอดการศึกษากระบวนการก่อโรคของโรคพัฒนาการทางระบบประสาทได้อีกด้วย ในงานวิจัยนี้ผู้วิจัยได้ทำการศึกษากลไกการเปลี่ยนแปลงเป็นเซลล์ในระบบประสาทโดยใช้เซลล์มะเร็งต้นกำเนิด NTERA2 ผ่านการเหนี่ยวนำด้วยสาร retinoic acid โดยศึกษาผลของ Paired-box 6 (PAX6) ซึ่งมีบทบาทสำคัญต่อการเปลี่ยนแปลงของเซลล์ต้นกำเนิดตัวอ่อนของมนุษย์ให้เป็นเซลล์ในระบบประสาท และศึกษาการควบคุมการแสดงออกของยีน PAX6 ภายใต้การควบคุมของ RNA-protein complex จากการรวมตัวของ CTCF, DDX5 และ PUS1 ที่สร้างพันธะร่วมกับ RNA ชื่อ steroid receptor RNA co-activator (SRA) ซึ่งมีเพียง CTCF ที่มีการยืนยันความสัมพันธ์กับการควบคุมการแสดงออกของยีน PAX6 ผู้วิจัยยังได้ศึกษาความเชื่อมโยงระหว่าง RNA-protein complex ดังกล่าวกับการเปลี่ยนแปลงสภาพของเซลล์ NTERA2 ผลการศึกษาแสดงว่า ทั้งไอโซฟอร์ม PAX6A และ PAX6B สามารถกำหนดให้เซลล์ NTERA2 เปลี่ยนสภาพเป็นเซลล์ในระบบประสาท PUS1 มีความเกี่ยวข้องกับการเริ่มต้นถอดรหัสยีน PAX6 ผ่าน P1 promoter ในขณะที่บทบาทของ CTCF และ DDX5 คือการควบคุมความเสถียร เมื่อลดปริมาณ SRA พบว่าเซลล์ NTERA2 เปลี่ยนสภาพเป็นเซลล์ของระบบประสาท ในขณะที่เมื่อลด DDX5 และ PUS1 ให้ผลตรงกันข้าม เมื่อเพิ่มปริมาณ DDX5 และ DDX17 สามารถเหนี่ยวนำการเปลี่ยนแปลงเป็นเซลล์ระบบประสาทเพิ่มขึ้น โดย DDX17 สำคัญต่อการเปลี่ยนแปลงให้เป็นเซลล์ประสาทที่สมบูรณ์ เมื่อเพิ่มปริมาณของ PUS1 ส่งผลให้กระบวนการเปลี่ยนแปลงเป็นเซลล์ในระบบประสาท และการแสดงออกของ PAX6 เกิดเร็วขึ้น ในขณะที่ผลดังกล่าวไม่เกิดกับการเพิ่มปริมาณของ PUS1 ในรูปแบบที่ทำปฏิกิริยากับ RNA ไม่ได้ ผลการวิจัยนี้สรุปได้ว่า ในกระบวนการเปลี่ยนแปลงเป็นเซลล์ในระบบประสาทจากเซลล์มะเร็งต้นกำเนิดนั้น การทำงานร่วมกันระหว่างโครงสร้างอาร์เอ็นเอและโปรตีนมีบทบาทในการควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับระบบประสาท และมีความสำคัญในกระบวนการเปลี่ยนแปลงเป็นเซลล์ในระบบประสาทอีกด้วย

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LIST OF ABBREVIATION

5aCON	PAX6-5a consensus
ALP	Alkaline phosphatase
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CTCF	CCCTC-binding factor
DAPI	4',6-diamidino-2-phenylindole
DDX	DEAD-box RNA helicase
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxy-ribonucleic acid
DNMT1	DNA methyl-transferase 1
DNMT3B	DNA methyl-transferase 3B
EB	Embryoid body
EC cells	Embryonal carcinoma stem cells
ECL	Enhanced chemiluminescence
ENCODE	The Encyclopedia of DNA Elements
ES cells	Embryonic stem cells
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

LIST OF ABBREVIATION (cont.)

H3K27me3	Tri-methylation of lysine residue 27 of Histone H3
H3K4me3	Tri-methylation of lysine residue 4 of Histone H3
H3K9me3	Tri-methylation of lysine residue 9 of Histone H3
HD	Homeodomain
HDAC1	Histone de-acetylase 1
HMBA	Hexamethylene bisacetamide
HRP	Horse radish peroxidase
iPS cells	Induced pluripotent stem cells
LB broth/agar	Luria-Bertani broth/agar
LIF	Leukemic inhibitory factor
lnc RNA	Long non-coding RNA
LSD1	Lysine-specific de-methylase 1
MLASA	Mitochondrial myopathy and sideroblastic anemia
mRNA	Messenger RNA
N2102Ep CAG-PAX6A	PAX6A over-expressing N2102Ep cell line
N2102Ep CAG-PAX6B	PAX6B over-expressing N2102Ep cell line
N2102Ep CAG-Tomato	Control over-expression N2102Ep cell line
NT2 CAG-D146A	PUS1 mutant over-expressing NTERA2 cell line
NT2 CAG-DDX17	DDX17 over-expressing NTERA2 cell line
NT2 CAG-DDX5	DDX5 over-expressing NTERA2 cell line
NT2 CAG-PAX6A	PAX6A over-expressing NTERA2 cell line
NT2 CAG-PAX6B	PAX6B over-expressing NTERA2 cell line
NT2 CAG-PUS1	PUS1 wild type over-expressing NTERA2 cell line
NT2 CAG-Tomato	Control over-expression NTERA2 cell line
NT2 Ctrl KD	Control knockdown NTERA2 cell line
NT2 DDX5 KD	DDX5 knockdown NTERA2 cell line
NT2 PUS1 KD	PUS1 knockdown NTERA2 cell line

LIST OF ABBREVIATION (cont.)

NT2 SRA KD	SRA knockdown NTERA2 cell line
P6CON	PAX6 consensus
PAX6	Paired box 6
PAX6A	PAX6 isoform A
PAX6B	PAX6 isoform B
PBS	Phosphate buffer solution
pCAG	Chicken β -actin promoter
PD	Paired domain
PIC	Protein inhibitor cocktail
PMSF	Phenylmethanesulfonyl fluoride
Prg	Pargyline
PUS1	Pseudouridylate synthase 1
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RA	Retinoic acid
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOC	Super Optimal Broth with Catabolite repression
SRA	Steroid receptor RNA co-activator
SSEA1	Stage-specific embryonic antigen 1
SSEA3	Stage-specific embryonic antigen 3
SSEA4	Stage-specific embryonic antigen 4
TCP	Tranlycypromine
tRNA	Transfer RNA

CHAPTER I

INTRODUCTION

Several types of stem cells have been studied worldwide and proposed as a potential source for regenerative medicine and cell-based therapy, including the neurological diseases, gene therapy and for cancer studies (Binello & Germano, 2012; Borlongan & Sanberg, 2002; Colleoni & Torrente, 2008; Lederer & Santama, 2008; Lavdas *et al.*, 2011). Many sources of stem cells can be used for these purposes such as pluripotent stem cells (embryonic stem cells – ES cells - and induced pluripotent stem cells – iPS cells) and other somatic stem cells. However, to date, limited numbers of studies on cell-based therapy using stem cells have been clinically approved or proven successful in clinical trials. In order for them to achieve the clinical level transplantable cells, a sophisticated and defined method to differentiate them into target cells with fair ability to survive and without tumorigenicity has to be derived (Zhang & Zhang, 2010). The most promising strategy is to develop differentiation protocol according to the developmental cues of the required particular cell type.

Pluripotent stem cells become one of the most suitable cells to study differentiation, not only that they can be transferable to clinical usage, if success, but they also closely resemble to the beginning of cell differentiation at the embryonic stage. Several pluripotent cell lines have been used in the field of stem cell research (Yu & Thomson, 2008). Embryonic stem cells were first isolated from mouse blastocyst, cultured on a feeder cell layer and used to study developmental process *in vitro*. In 1998, first human ES cell lines were derived from discarded in vitro fertilization (IVF) blastocysts (Thomson *et al.*, 1998). Studies have been done in these embryonic cell lines to elucidate the mechanism of differentiation from pluripotent state to multipotent stem cells of each cell lineage, including differentiation of neural lineage. These mechanisms have been confirmed in the system of iPS cells later (Srikanth & Young-Pearse, 2014; Mohamad *et al.*, 2014).

Besides ES cells and iPS cells, there are other pluripotent stem cell lines, which have been long used to study ability to differentiate, called embryonal carcinoma stem cells (EC cells) (Andrews *et al.*, 1998). Unlike the first two, EC cells were derived from germ cell tumor. However, they also exhibit proliferating and differentiating abilities similar to ES and iPS cells. In fact, EC cells were discovered before ES and iPS cells and were the beginning of studies of pluripotent stem cells (Martin & Evans, 1975). Similar to derivation of ES cells, EC cells were derived from mouse teratocarcinoma before that of human. NTERA2 has become a widely used pluripotent EC cell lines. They were derived from an ancestor EC cell line, TERA2, and shown to predominantly differentiate into the ectodermal lineage (Andrews, 2002). The advantage of EC cells over ES cells and iPS cells is that they are easier to handle and have not much different in terms of differentiation potential. In turn, pluripotent EC cells are suitable for studying mechanism of cell differentiation; as such NTERA2 cells are widely used to demonstrate mechanisms of neural differentiation.

There are many regulatory mechanisms underlying neural differentiation from pluripotent stem cells (Christie *et al.*, 2013). Expression and function of intracellular signaling molecules as well as transcriptional factors have been studied for their involvement in neural specification from embryonic stem cells. In addition, following the increased understanding about epigenetic, epigenetic mechanisms and their regulator networks have been linked with differentiation of pluripotent stem cell as well, especially differentiation into neural lineage (Yeo *et al.*, 2008). Extracellular signaling molecules such as bone morphogenetic proteins (BMPs) and other growth factors have been shown to modulate activation of intracellular signaling cascades, which contribute to neural differentiation (Du & Zhang, 2004; Gerrard *et al.*, 2005; Willerth *et al.*, 2007).

Paired-box 6 (PAX6) is one several important transcriptional factors for regulating neurogenesis (Osumi *et al.*, 2008). Expression of PAX6 has been used to indicate neural differentiation in many studies both embryonic development and differentiation from pluripotent stem cells (Bibel *et al.*, 2004; Gerard *et al.*, 1995). It has been used as access point to study a network of transcriptional factors involving in cellular process of neural stem/progenitor cells (Sansom *et al.*, 2009). In the present

study, we attempt to determine importance of PAX6 in neural differentiation and used it as a reference transcriptional factor to study involvement of RNA signaling codes in neural differentiation process of human pluripotent stem cells.

Long non-coding RNA (Lnc RNA) is a class of RNA structures, which are excluded from or have limited protein encoding property. Recently, roles of lnc RNAs in transcriptional regulation as well as their involvements in developmental process have been widely studied (Kung *et al.*, 2013). It has been estimated that, of all transcripts in each cell, around one fifth is protein-coding RNA, leaving four fifths as non-coding RNA (Kapranov *et al.*, 2007). With the high abundance of non-coding species of RNA, only small examples of lnc RNA have been studied in context of their mechanism of transcriptional controlling and involvement in cell differentiation. Steroid receptor RNA activator (SRA) is an example of lnc RNA that have been studied for its transcriptional regulation on nuclear receptor (Leygue, 2007). SRA has been shown to regulate expression of transcriptional factor important for muscle differentiation as well as its involvement in muscle differentiation (Hubé *et al.*, 2011). However, its function in pluripotent stem cell and differentiation in other lineage have not been studied.

DEAD-box RNA helicases, DDX5 (p68) and DDX17 (p72), and Pseudouridylate Synthase 1 (PUS1) have been shown to be interacting partners with SRA, which function as transcriptional co-activator (Leygue, 2007). Besides being RNA-binding protein, all 3 of them can also function as RNA modification proteins. DEAD-box RNA helicases have an ability to unwind RNA structures as well as participate in RNA metabolism processes (Linder & Jankowsky, 2011). Pseudouridylation is an RNA modification process by which uridine bases are isomerized to pseudouridine with an action of, but not restricted to, Pseudouridylate Synthase family (Ge & Yu, 2013). Similar to their RNA partner, their roles in differentiation of pluripotent stem cell have never been unveiled.

CCCTC-binding factor (CTCF) is a zinc-finger DNA binding protein that have been shown to function as a chromatin organizing factor as well as insulator protein that prevent expression of certain genomic areas (Ghirlando *et al.*, 2012). Genome wide, there are a lot of CTCF-binding sites, which allow CTCF to be able to organize chromatin structure by bringing several CTCF-binding sites in close

proximity. Insulator function of CTCF has been considered another important genomic regulation during developmental processes, not only at embryonic stage but also development of certain lineage differentiation (Herold *et al.*, 2012). CTCF is another protein that has been shown to form a complex with SRA and DDX5. It has been shown to regulate expression level of PAX6 in mouse ES cells. Still, a role of CTCF in human ES cells and well as other pluripotent models has to be established.

In the present study, we hypothesize that over-expression of exogenous PAX6 in NTERA2 would induce neural differentiation and increase neuronal formation after treatment with retinoic acid. In addition, a protein-RNA complex comprised of CTCF, DDX5, SRA, and PUS1 could play a role as a transcriptional control over the regulatory element(s) of *PAX6* gene in pluripotent NTERA2 cells during differentiation process. Therefore, genetic manipulation, both gain and loss of function, of these factors in NTERA2 cells would change expression of *PAX6* mRNA and, in turn, induce differentiation into neural lineage.

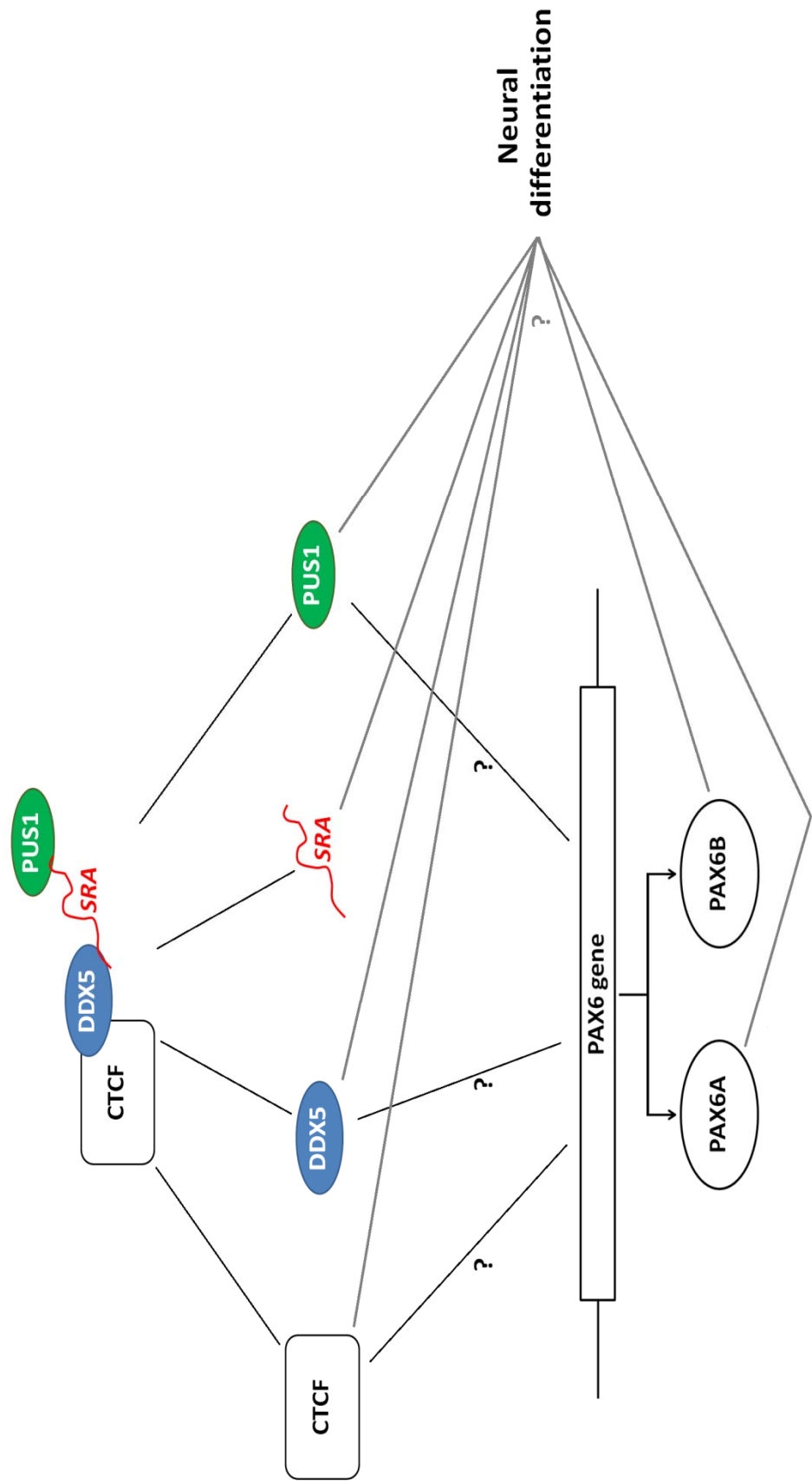


Figure 1.1: Diagram illustration of question asked in this study. The influences of PAX6; both PAX6A and PAX6B isoforms, CTCF, DDX5, SRA, and PUS1 on neural differentiation of NTERA2 cells are determined (grey line). In addition, regulatory roles of CTCF, DDX5, and PUS1 at transcriptional level on PAX6 gene are also studied (black line).

CHAPTER II

OBJECTIVES

PAX6 has long been considered as an important transcription factor for central nervous system (CNS) development (Götz *et al.*, 1998). Recently, it has been shown that PAX6A isoform has an ability to induce spontaneous neural differentiation when over-expressed in human embryonic stem cells (Zhang, *et al.*, 2010). With different human pluripotent stem cell used, experiments were done based on a hypothesis that PAX6 over-expression, similar to hES cells, could induce neural differentiation in pluripotent embryonal carcinoma NTERA2 cell line. In addition, over-expression of PAX6A isoform would favor neuronal lineage differentiation upon induction with all-trans retinoic acid. The ability to induce differentiation of PAX6 is also tested in N2102Ep, a nullipotent counter part of NTERA2 which resist to differentiation upon induction with retinoic acid (Andrews *et al.*, 1994).

Since PAX6 expression is important for neural differentiation from human pluripotent stem cells, understanding transcriptional control of *PAX6* gene during differentiation of pluripotent stem cells would offer insight to early stage of CNS development as well as potential strategy for induce neural differentiation from pluripotent stem cells. *SRA*, a long non-coding RNA, has been shown to play a role in differentiation of other but neural lineage. However, one partner of *SRA*, which is CTCF, has been shown to regulate *PAX6* expression in mouse embryonic stem cells (Gao *et al.*, 2011). We hypothesized that a complex of *SRA* and its binding partners would also participate in regulating *PAX6* gene expression. Furthermore, component(s) of this complex might influence neural differentiation of human EC cells NTERA2.

According to the hypothesis stated earlier, series of experiments were done with the following specific objectives;

1. To demonstrate role of PAX6 on differentiation potential of NTERA2 and N2102Ep cells after over-expression

2. To investigate effect of Lysine-specific histone de-methylase 1 (LSD1) inhibition on differentiation ability of N2102Ep cells upon induction with retinoic acid
3. To evaluate protein-DNA interaction of RNA signaling machineries with regulatory regions of PAX6 genes along the process of differentiation of NTERA2 cells by retinoic acid induction
4. To determine potential role of long non-coding RNA - SRA- and RNA signaling proteins on neural differentiation from NTERA2 cells by knockdown experiment
5. To examine effect of DDX5 over-expression versus DDX17 over-expression on cell differentiation from NTERA2 cells, particularly to neuronal lineage
6. To examine effect of PUS1 over-expression, wild type versus catalytic inactive mutant, on cell differentiation from NTERA2 cells, particularly to neural lineage

CHAPTER III

LITERATURE REVIEW

3.1 Human Pluripotent Stem Cells

Pluripotent stem cells refer to stem cells that are capable of differentiation into cells from all 3 germ layers. The term pluripotent was first used to describe the ability to differentiate and form teratoma of cells isolated from mouse teratocarcinoma, called embryonal carcinoma (EC) cells, which develop into another teratocarcinoma after transplanted into another host (Damjanov, 1993). They were called “embryonal” because they show similarities to cells in the normal mouse embryonic tissue, but are malignant in nature as well, hence the term “embryonal carcinoma” cells (Martin & Evans, 1975). Researches in EC cells, then, led to the derivation of first embryonic stem (ES) cells from primary mouse embryo (Evans & Kaufman, 1981; Martin, 1981). Notably, studies in the field of pluripotent stem cells began from cells derived from mouse tissue before translated to human tissue, both for human EC and ES cells. In 2006, another type of pluripotent stem cells were introduced by conversion of adult cells back to pluripotent state, called induced pluripotent stem (iPS) cells (Takahashi & Yamanaka, 2006).

Since pluripotent stem cells harbor an ability to differentiate into all cell types from 3 embryonic germ layers, they have been used as a model to study cellular and molecular mechanisms underlying differentiation process. After they have been proved that their differentiation process resemble that of embryonic development, information regarding cell differentiation and developmental process has been transferred back and forth between studies in embryo and pluripotent stem cells. Moreover, several differentiation protocols have been established based on information from developmental process of that particular cell type.

3.1.1 Discovery of human pluripotent embryonal carcinoma stem cells, NTERA2

At the very early phase of research in human development, directly investigation of human embryo is very difficult from ethical and technical standpoints. Using mouse EC cells in parallel with embryo was proved to be another way to study developmental process *in vitro* (Mintz & Illmensee, 1975), suggesting that using human counterpart would probably able to study human development as well. In 1980s, several human EC cell lines were derived (Andrews *et al.*, 1980; Bronson *et al.*, 1980; Hogan *et al.*, 1977). Few years later, the first pluripotent EC cell line was derived from another human teratocarcinoma cell line (Andrews *et al.*, 1984), and named NTERA2. NTERA2 has been shown to extensively differentiate into cells from all 3 germ layers under induction of several agents such as retinoic acid, hexamethylene bisacetamide (HMBA), and BMPs (Andrews *et al.*, 1994; Andrews, *et al.*, 1984; Mavilio *et al.*, 1988).

3.1.2 Derivation of human embryonic stem cells and induced pluripotent stem cells

Following advancement of human EC cells and mouse ES cells research, the first human ES cells were derived from human embryo at blastocyst stage (Thomson *et al.*, 1998). Mouse embryonic fibroblasts were originally used as feeder cells for human ES cells. Many other signaling molecules were employed into cell culture process such as leukemic inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), in order to achieve stable culture environment. Incorporation of human ES cells into mouse embryo showed that they can differentiate into various cell lineages. Another important characteristic of these derived human ES cells is that they can proliferate indefinitely in the culture, suggested from their high telomerase activity. Expression of cell surface markers of these human ES cells is similar to that of pluripotent human EC cells as well as ES cells derived from other species. Stem cell-related surface markers shown include stage-specific embryonic antigen 3 (SSEA3), stage-specific embryonic antigen 4 (SSEA4), TRA-1-60 and TRA-1-81, were shown. Stage-specific embryonic antigen 1 (SSEA1), which is associated with differentiation of human EC cells, cannot be detected from human ES cells as well.

Pluripotent state was able to induce from terminally differentiated cells by directly reprogramming with defined transcriptional factors (Takahashi & Yamanaka, 2006). These reprogrammed cells exhibited cell characteristics similar to those of ES cells. This work was first shown in mouse cells. A year later 2 separate studies in human cells showed that pluripotent stem cells can be induced from adult human cells as well (Takahashi *et al.*, 2007; Yu *et al.*, 2007). Even though these studies were successfully reprogrammed cells back to pluripotent state, they used different transcriptional factors. The overlapping factors are OCT4 and SOX2, which are later proved to be important factors for maintaining pluripotent state (Lam *et al.*, 2012). Several reports have shown that employing only these 2 factors is sufficient to establish iPS cells from certain cell types (Huangfu *et al.*, 2008; Meng *et al.*, 2012; Montserrat *et al.*, 2012).

3.2 NTERA2 clone D1 as a model to study neural differentiation

As mentioned earlier, NTERA2 cells are EC cells, which are pluripotent in nature (Andrews, 1998). Profiles of genes expression as well as surface markers expression in undifferentiated NTERA2 are similar to that of human ES cells (Sperger *et al.*, 2003). In addition, NTERA2 has been shown to predominantly differentiate into cells in ectodermal lineages, including neural lineage (Miyazono *et al.*, 1995; Pleasure & Lee, 1993). As such, many studies have employed this neural differentiation potential of NTERA2 as a tool to study mechanism of neural differentiation and develop a strategy for differentiating cells into certain neuronal subtypes. In addition to neuronal phenotype, differentiation of other glia cells, including astrocytes and oligodendrocytes, have been studied in the system of NTERA2 as well (Bani-Yaghoub *et al.*, 1999).

Several methods have been used to differentiate NTERA2 cells into mature cells of the central nervous system, both neurons and astrocytes. The most extensively used one is treatment with all-trans retinoic acid (Langlois & Duval, 1997). After treatment for 1-2 months, mature neurons and astrocytes can be detected in the culture, in terms of both cell morphology and physiology. Another differentiation method is to grow cells in suspension culture and let them form into an

aggregation, called embryoid body (EB) formation. EB formation mimics developmental process at blastocyst stage, which is the inner cell mass. Cell-cell interaction and paracrine effect allow cells to differentiate, not only into neuroectodermal lineage but also into other lineages (Pal & Ravindran, 2006).

NTERA2 cells have been used as a model to study differentiation of several neuronal specific subtypes as well as developmental process of the central nervous system. There are several neuronal subtypes differentiated from NTERA2 cells reported so far, for example, glutamatergic, GABAergic, catecholaminergic, and cholinergic neurons (Guillemain *et al.*, 2000; Podrygajlo *et al.*, 2009). NTERA2 cells have been used for studying differentiation process of dopaminergic neurons in place of human ES cells (Schwartz *et al.*, 2005). Metabolism of progesterone during neuronal differentiation process was studied in this system as well, comparing between undifferentiated and NTERA2-differentiated neurons (Pistritto *et al.*, 2009). Moreover, NTERA2 have been used as a tool to screen molecules that may have developmental potential and/or developmental toxicity (Barbaric *et al.*, 2011; Stern *et al.*, 2014).

Neurons differentiated from NTERA2 have been used to represent mature neurons in human central nervous system as well (Pleasure *et al.*, 1992). Milagre and colleagues utilize NTERA2-differentiated neurons as a model to study human neuronal cholesterol metabolism, both during development and in mature neurons (Milagre *et al.*, 2012a; Milagre *et al.*, 2012b). The role of neurotrophins on neuronal survival has been studied using neurons differentiated from NTERA2 (Piontek *et al.*, 1999). Using gene expression and protein markers as surrogates, regional specificity of differentiated cells from NTERA2 with retinoic acid treatment was identified (Coyle *et al.*, 2011). Following an experiment that NTERA2 was transplanted into rodent brain and showed potential in neuronal differentiation and incorporation into host brain (Trojanowski *et al.*, 1993), an attempt to use neurons differentiated from NTERA2 as a transplantation source for several neurodegenerative diseases and brain injury was tested in many animal models. Even though preclinical testing was promising and usage of NTERA2-derived neurons in stroke proceeded into clinical trials, malignant properties of NTERA2 is severe and proved that this cell source is not appropriate for clinical use (Hara *et al.*, 2008).

Moreover, differentiated NTERA2 cells were also used as a model for mature human astrocytes. Functional astrocytes have been differentiated from NTERA2 with defined method (Sandhu *et al.*, 2002) and their biochemical were studied (Lu *et al.*, 2005; Ozdener, 2007). In addition, extracellular signaling molecules involved in astrocytic maturation and function have been shown during differentiation process of NTERA2 towards astrocytes (Lim *et al.*, 2007).

Differentiation of NTERA2 with retinoic acid treatment results in heterogeneous population of neurons and astrocytes, in case that cell purification is not done. Researchers have taken advantages of this property to study *in vitro* interaction between 2 major cell types of the central nervous system (Hill *et al.*, 2012).

3.3 Paired-box 6 (PAX6)

PAX6 is a member of paired-box (PAX) transcription factor family. Almost every member of the family has been shown to play a role in differentiation and development of several cell/tissue lineages, while defects in PAX genes can cause developmental abnormalities in several organs (Chi & Epstein, 2002). The overall structure of PAX proteins is shown in figure 3.1. The main structure of PAX proteins is DNA binding domain, called paired domain (PD). DNA binding capacity of PD depends on interaction of 2 subdomains; PAI and RED, with DNA sequences, therefore changing in amino acid sequence in one of these subdomains also change PAX binding sites on genomic region (Czerny *et al.*, 1993; Xu *et al.*, 1995). Most of PAX genes encode another DNA binding domain called homeodomain (HD), leaving only PAX1 and PAX9 which contain no HD. The last motif of PAX protein is on the C-terminal of the protein containing transcriptional activation activity, called transactivation (PST) domain (Glaser *et al.*, 1994). This domain can function as both positive and negative transcriptional regulator depending on the protein complex it is forming with.

PAX proteins can be categorized into 4 groups according to domains composition (Table 3.1). Paired domain is an essential part for all PAX proteins, as well as transactivation domain for transcriptional activity. Thus, they are divided based on the extent of homeodomain transcribed into protein. PAX1 and PAX9 are

categorized in group I, which contains no HD. PAX2, PAX5 and PAX8 belong to group II, which have only one part of HD. The rest of PAX proteins contain full length HD, so they are divided with the presents of octapeptide linker region. This linker motif has been shown to have an inhibitory effect on transcriptional activity (Eberhard *et al.*, 2000). PAX4 and PAX6 belong to group III, which have no linker protein. Their roles in developmental control of each organ are also list in Table 3.1.

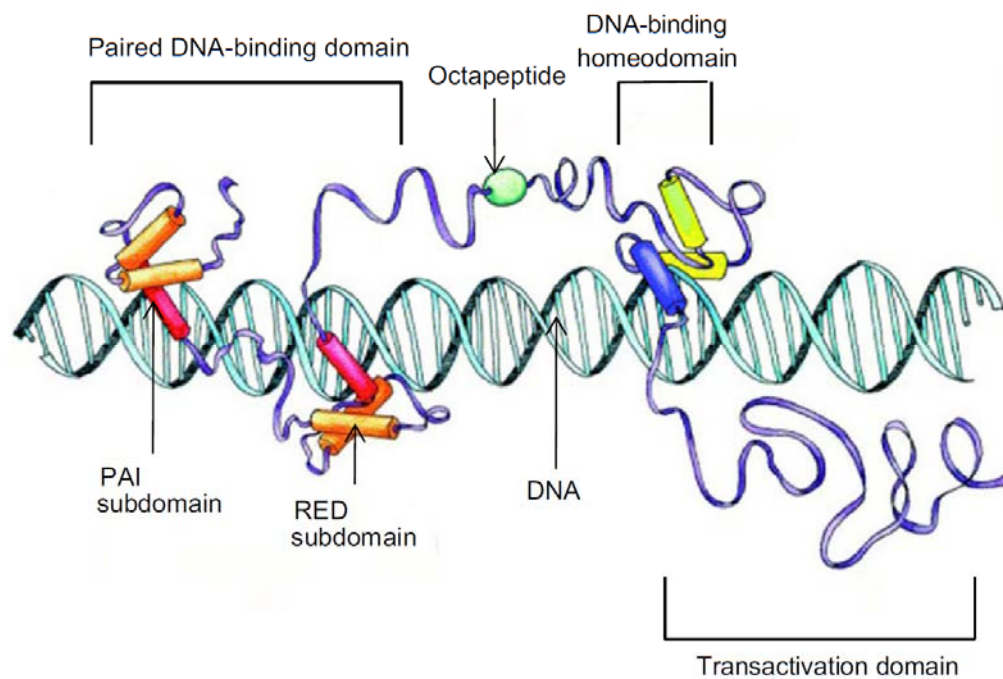


Figure 3.1: Structure of PAX proteins in DNA-protein complex (Modified from Blake & Ziman, 2014).

Gene	Structural domains ^a				Target organs/tissues	Chromosome ^b
	PD	OP	HD1	HD2/3		
<i>PAX1</i>	X	X			Skeleton, thymus	20p11
<i>PAX9</i>	X	X			Skeleton, cranio-facial, tooth	14q12–q13
<i>PAX2</i>	X	X	X		CNS, kidney	10q25
<i>PAX5</i>	X	X	X		CNS, B cells	9p13
<i>PAX8</i>	X	X	X		CNS, kidney, thyroid	2q12–q14
<i>PAX4</i>	X		X	X	Pancreas	7q
<i>PAX6</i>	X		X	X	CNS, eye, pancreas	11p13
<i>PAX3</i>	X	X	X	X	CNS, neural crest, skeletal muscle	2q35
<i>PAX7</i>	X	X	X	X	CNS, cranio-facial, skeletal muscle	1p36.2

^aStructural domains include the paired domain (PD), the octapeptide motif (OP), the first helix of the homeodomain (H) (HD2/3). X denotes presence of the domain.

^bHuman chromosome map position.

Table 3.1: Members of PAX family categorized according to their structural motifs (Chi & Epstein, 2002).

PAX6 has long been linked to development of several organs including central nervous system (CNS), eye, pancreas, pituitary and nasal structure (St-Onge *et al.*, 1997; Walther & Gruss, 1991). Mutation in *PAX6* gene and/or its regulatory elements can cause developmental defects in eye, such as aniridia, and CNS, such as polymicrogyria (Bhatia *et al.*, 2013; Hingorani *et al.*, 2012; Spalice *et al.*, 2009). As it is very important for CNS development, both upstream and downstream regulation of PAX6 have been extensively studied and used as reference for other members of PAX family.

PAX6 gene contains 16 exons, which can generate 2 protein isoforms, including PAX6A and PAX6B, by alternative splicing (Epstein *et al.*, 1994). PAX6A is the native form of PAX6, while PAX6B is the isoform that has exon 5a inserted into PAI domain region resulting in 14 amino acid added. Insertion of exon 5a in PAX6B results in restricted DNA binding capability of PD compared to native PAX6 (Kozmik *et al.*, 1997). However, exon 5a insertion only reduces DNA binding capability of PAI subdomain, leaving normal binding from RED subdomain. This results in different DNA consensus bound by PAX6A and PAX6B, named P6CON and 5aCON,

respectively (Epstein *et al.*, 1994). Figure 3.2 demonstrates the protein structure of PAX6, both isoforms, as well as their different DNA binding consensus.

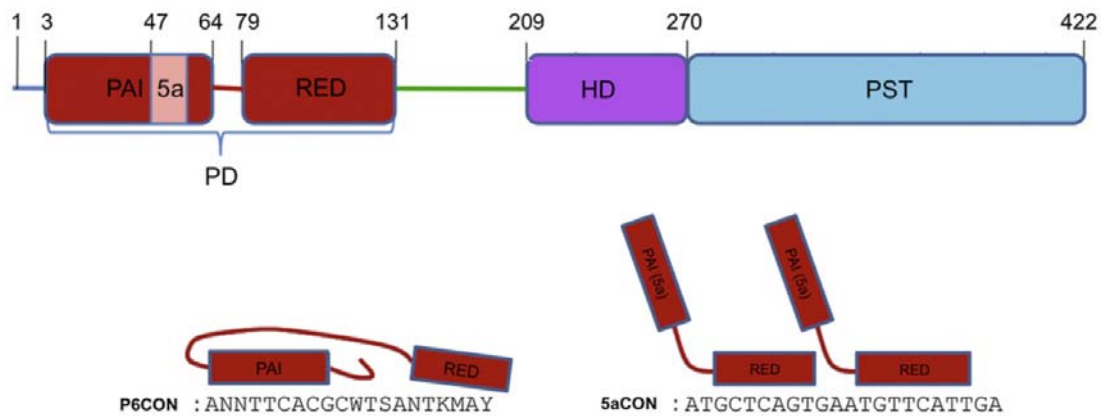


Figure 3.2: The top panel shows protein structure of PAX6. The numbers above the protein indicate amino acid residue at each point. The bottom panel demonstrates DNA-protein interaction between domains of PAX6 and different PAX6 targets (Shaham *et al.*, 2012).

3.3.1 PAX6 in neural fate determination

As discussed in the previous section, treatment of both mouse and human ES cells with external stimuli during the process of neural induction results in differentiation into neural lineages (Chambers *et al.*, 2009; Gaspard *et al.*, 2008; Watanabe *et al.*, 2005). Notably, PAX6 has been extensively used as a marker for neural cells differentiated from ES cells (Noisa *et al.*, 2012; Salehi *et al.*, 2011). Early strategies for directing differentiation of human ES cells towards neural lineage have shown that neuroepithelial cells, immediately differentiated cells, express SOX1 and PAX6 (Li *et al.*, 2005; Nat *et al.*, 2007). Differentiated human ES cells expressing SOX2, without PAX6, was labeled as “pre-neuroepithelial stage” and suspected to be a model for studying floor plate determination using human ES cells (Denham *et al.*, 2012). An important question is whether PAX6 itself has an ability to induce neuroectodermal differentiation directly from ES cells or not.

In 2010, Zhang and colleagues have shown that manipulation of PAX6 in human ES cells, but not mouse ES cells, has an effect on the extent of neural differentiation (Zhang *et al.*, 2010). Knocking down *PAX6* resulted in restricted neural

differentiation, while over-expressing PAX6, particularly PAX6A isoform, induced spontaneous neuroectodermal differentiation observed by morphology. Loss of ability to differentiation into neural lineage after PAX6 expression interfered is confirmed by heterozygous Pax6 knockout in mouse ES cells (Quinn *et al.*, 2010). The mechanism underlying neural induction by PAX6 was shown by ChIP experiment that it binds directly to promoter of stem cell-related genes, *OCT4* and *NANOG*, to suppress their expression. However, only PAX6A isoform was able to induce expression of neural-related genes, such as *MAP2*. They summarized that both isoforms of PAX6 inhibit stem cell state of ES cells, while only PAX6A leads cells to differentiate into neural lineage (Figure 3.3). Another report in 2009 has shown that over-expressing of PAX6B isoform facilitates differentiation of mouse ES cells into mature neurons (Shimizu *et al.*, 2009). These reports demonstrate neurogenic potential of both PAX6A and PAX6B, however their different results might be due to either cells used in the experiment or differentiation protocol employed or both.

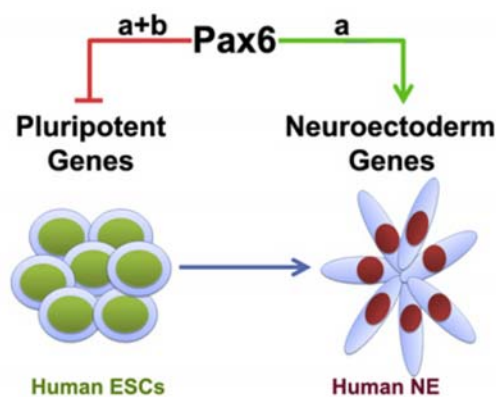


Figure 3.3: Roles of PAX6, both PAX6A and PAX6B isoforms, in differentiation of human ES cells are mediated through their transcriptional activities on set of genes important for maintaining pluripotent state or neuroectodermal lineage-related. (Zhang, *et al.*, 2010)

Differentiation potential of PAX6 in ES cells is not limited to neural structures in the CNS, but neuro-retina structures have also been shown as well. PAX6 is found to be very important for development of the eye, including fate determination of neuronal population in the retina (Oron-Karni *et al.*, 2008). Similar to other neural lineages, an attempt to determine differentiation strategies for retinal progenitor cells

from human ES cells is also focus on expression of PAX6 (Amirpour *et al.*, 2012; Ikeda *et al.*, 2005). Mouse ES cells with over-expression of PAX6 have been shown to differentiate into neural progenitor cells for retina (Kayama *et al.*, 2010).

3.3.2 Transcriptional control on PAX6 gene

The genomic structure of gene encoding PAX6 is shown in Figure 3.4. *PAX6* gene is localized on the short arm of chromosome 11 (11p13). This locus consists of 16 exons, 4 promoter regions, and variety of transcriptional regulatory elements (Glaser *et al.*, 1992; Kammandel *et al.*, 1999; Kim & Lauderdale, 2006). Two major transcriptional start sites are P0 and P1 (Plaza *et al.*, 1995; Xu & Saunders, 1997), which are relatively active in different tissues during development (Anderson *et al.*, 2002; Elso *et al.*, 2013). Along this *PAX6* locus, there are numerous regulatory elements, both enhancers and repressors, identified with different experiments and may be contributed to different cell types used to identify (Kleinjan *et al.*, 2004; Plaza *et al.*, 1995; Xu *et al.*, 1999). A collection of enhancers important for tissue development is presented in Table 3.2. Regulatory regions important for development of the CNS include enhancers in front of the P1 promoter and those located up to two hundreds kilobase downstream to *PAX6* gene (Griffin *et al.*, 2002; Kammandel, *et al.*, 1999; Kleinjan, *et al.*, 2004; Kleinjan *et al.*, 2006; Kleinjan *et al.*, 2001). Regulatory elements downstream to *PAX6* locus are found to be conserved between human and mice (Tyas *et al.*, 2006), introduction of these regulatory regions across species is able to restore *PAX6* expression.

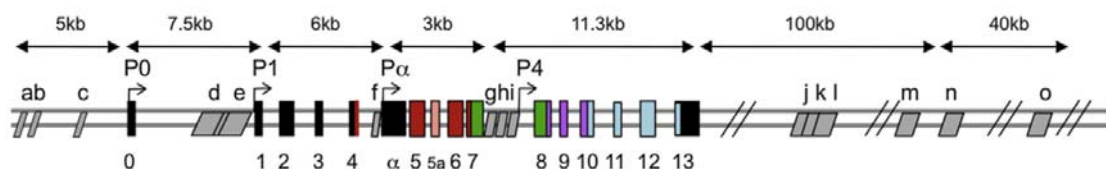


Figure 3.4: Genomic organization of *PAX6* gene illustrates all 16 exons (boxes with number labeled below), 4 promoter sites (marked by arrow), and other transcriptional regulators both enhancer and repressor regions (grey trapezoid with alphabet labeled above) (Shaham, *et al.*, 2012).

Reporter expression pattern	Approximate pair coordinates relative to (mouse) P0	Code
Pancreatic islets	−4.6k	a
Pax6 ectodermal enhancer (EE) surface ectoderm derivatives: cornea, lens, conjunctiva, lacrimal gland	−3.9k	b
Pancreatic islets, retinal progenitor cells	−2.3k	c
dorsal telencephalon, hindbrain, spinal cord	1.5–6.5k	d
Photoreceptor progenitors	3.5k	e
Distal retina α , amacrine cells, ciliary body, iris	14k	f
Late eye development	17.5k	g
Diencephalon	19k	h
Rhombencephalon	21k	i
Pretektum, neural retina and olfactory region	~ 105k, 106k and 107k	j,k,l
Lens, diencephalon, hindbrain, proximal retina, cerebellum	~ 110k	m
Telencephalon, neuroretina, retinal pigmented epithelium	~ 128k	n
Forebrain, diencephalon, pineal gland	~ 165k	o

Table 3.2: Enhancer regions within *PAX6* locus is presented according to their position from 5' most (code a) to 3' most. Their activities in different developing tissues are determined using reporter assay (Shaham, *et al.*, 2012).

The exact mechanism governing transcriptional regulation on *PAX6* gene is still unclear. However, one important mechanism is autoregulation of *PAX6* on transcriptional activity of its own promoters (Okladnova *et al.*, 1998), which is an important mechanism during development of the forebrain structures (Grindley *et al.*, 1997). Activation of TGF β signaling pathway has been shown to down-regulate of *PAX6* mRNA expression during development of the CNS and the eye (Pituello *et al.*, 1995; Timmer *et al.*, 2002; Wawersik *et al.*, 1999). Smad3 has been shown to underlie this interference with *PAX6* autoregulation by interacting with RED subdomain and resulting in reduction of DNA binding capacity of *PAX6* (Grocott *et al.*, 2007). In addition to TGF β pathways, subtle information has also pointed that FGFs, Wnt and Notch signaling pathways also have some mediating roles on transcription of *PAX6* (Faber *et al.*, 2001; Onuma *et al.*, 2002; Rasmussen *et al.*, 2001).

Regulatory regions of *PAX6* gene have been identified as target binding sites for other transcriptional factors. In mouse lens cell line, close relationship between Pax6 and homeoprotein Meis1 and Meis2 on each other regulatory elements have been shown (Zhang *et al.*, 2002). LHX2 facilitates neural differentiation in human ES cells by promoting *PAX6* expression after binds with active enhancer

region (Hou *et al.*, 2013). Transcriptional factors in basic helix-loop-helix (bHLH) family have been speculated to be regulators on PAX6 gene since a part of P1 promoter contains E-box, a target sequence for bHLH proteins such as NEUROD1, NGN2, HES1 and MASH1 (Cai *et al.*, 2000; Kageyama *et al.*, 1997; Ross *et al.*, 2003). NEUROD1 and NGN2 are factors that have been shown to interact directly with PAX6 genomic region (Marsich *et al.*, 2003; Scardigli *et al.*, 2001), while BHLHB5 has been reported as negative transcriptional modulator on human PAX6 promoter (Xu *et al.*, 2002).

3.4 RNA signaling

3.4.1 Long non-coding RNA: Steroid receptor RNA co-activator (SRA)

In 2012, the Encyclopedia of DNA Elements (ENCODE) project has reported that, of all human transcriptome, only 2.94% are protein-coding RNA (The ENCODE Project Consortium, 2012). The rest of transcribed RNAs are considered as “non-coding RNAs”, which those longer than 200 base pair are “long non-coding RNAs (lncRNAs) (Nagano & Fraser, 2011). Even though some of lncRNAs annotated in the literature have an ability to translate into protein (Washietl *et al.*, 2011), they are still considered as lncRNAs as biological role of the RNA structure can be displayed (Poliseno *et al.*, 2010).

There are many functions of lncRNAs demonstrated from experiments as shown in Figure 3.5. They are able to directly engage in transcriptional process via binding with their protein partners, to affect biogenesis of other types of both coding and non-coding RNAs, to modulate activity of their protein partners, as well as to form subcellular structure (Kugel & Goodrich, 2012; Wilusz *et al.*, 2009). Recent evidences also demonstrate interaction of lncRNAs with several chromatin modeling proteins and other epigenetic factors, suggesting a role of lncRNAs on regulating genomic formation as well (Hung & Chang, 2010). For the CNS, studies have elaborated the roles of several lncRNAs from the processes of neural development, neuronal

function, to the processes of aging and neurodegeneration (Lipovich *et al.*, 2012; Ng *et al.*, 2013; Qureshi *et al.*, 2010; Wu *et al.*, 2013).

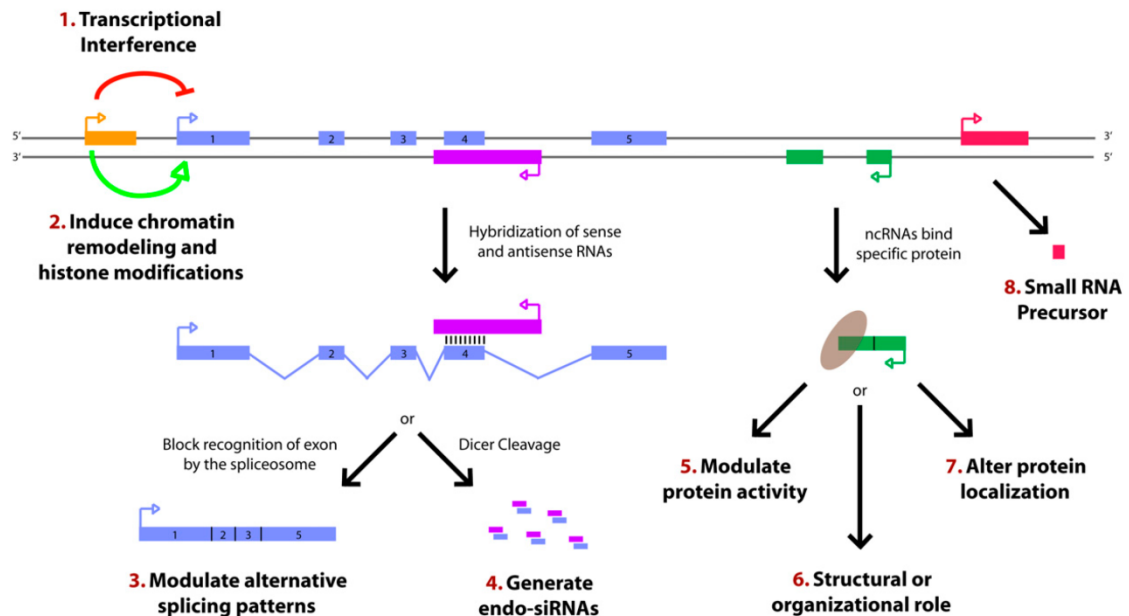


Figure 3.5.: Biological function of lncRNAs has been shown in different subcellular processes (Wilusz, *et al.*, 2009).

Certain lncRNAs have been shown to play an important role during neural differentiation step via different signaling pathways. One lncRNA transcribed from *Gli3* gene has found to modulate SHH signaling pathway contributing to fate determination in developing spinal cord in mouse (Meyer & Roelink, 2003). Some lncRNAs are detected after induction of neural differentiation from human ES cells (Ng *et al.*, 2012). Recently, another lncRNA named “Paupar” has been shown to regulate transcription of *PAX6* gene and induce differentiation from human neuroblastoma cell line (Vance *et al.*, 2014).

One of the most studied lncRNAs in the process of transcriptional regulation is SRA, steroid receptor RNA co-activator. The early studies have shown that SRA has an ability to modulate transcriptional activity of steroid hormone nuclear receptor, hence its name “steroid receptor RNA co-activator”. The first evidence showing direct transcriptional function of SRA, as an RNA transcript, was linked SRA with progesterone receptor (Lanz *et al.*, 1999). Later, other nuclear receptors were

shown to be bound by SRA as well (Colley & Leedman, 2009). In 2012, a secondary structure of SRA was shown in crystallized form (Novikova *et al.*, 2012; Figure 3.6), suggesting that transcriptional co-regulator of SRA lies within its secondary structure interacting nuclear receptors.

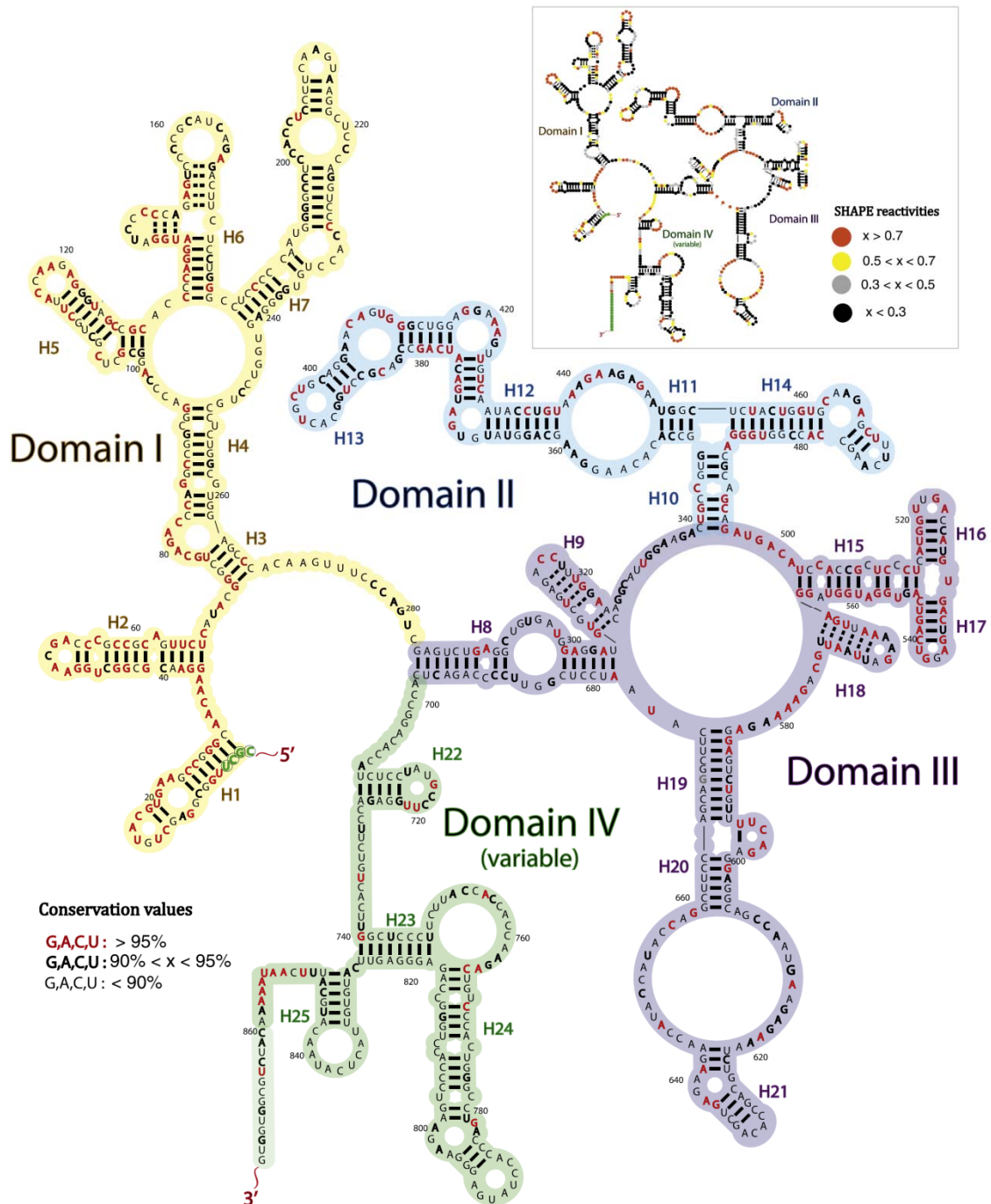


Figure 3.6: Secondary structure of lncRNA SRA (Novikova *et al.*, 2013)

Function of SRA has been reported to be exerted after forming into a complex with other proteins. Figure 3.7 summarizes functions, as both positive and negative regulators, of SRA-containing complexes. A number of reports points to function of SRA-mediated complex in cancer pathogenesis (Chooniedass-Kothari et al., 2006; Emberley et al., 2003; Kurisu et al., 2006; Lanz et al., 2003). However, it also involves in differentiation of several cell lineages. It has been reported that SRA plays a role in myocytes and adipocytes differentiation (Carette et al., 2006; Hubé et al., 2011; Kitamura et al., 2001; Xu et al., 2010). SRA is also suggested to participate in development of adrenal gland (Xu et al., 2009). However, the role of SRA-mediated cell differentiation from human pluripotent stem cells, into any cell lineages, is still to be presented.

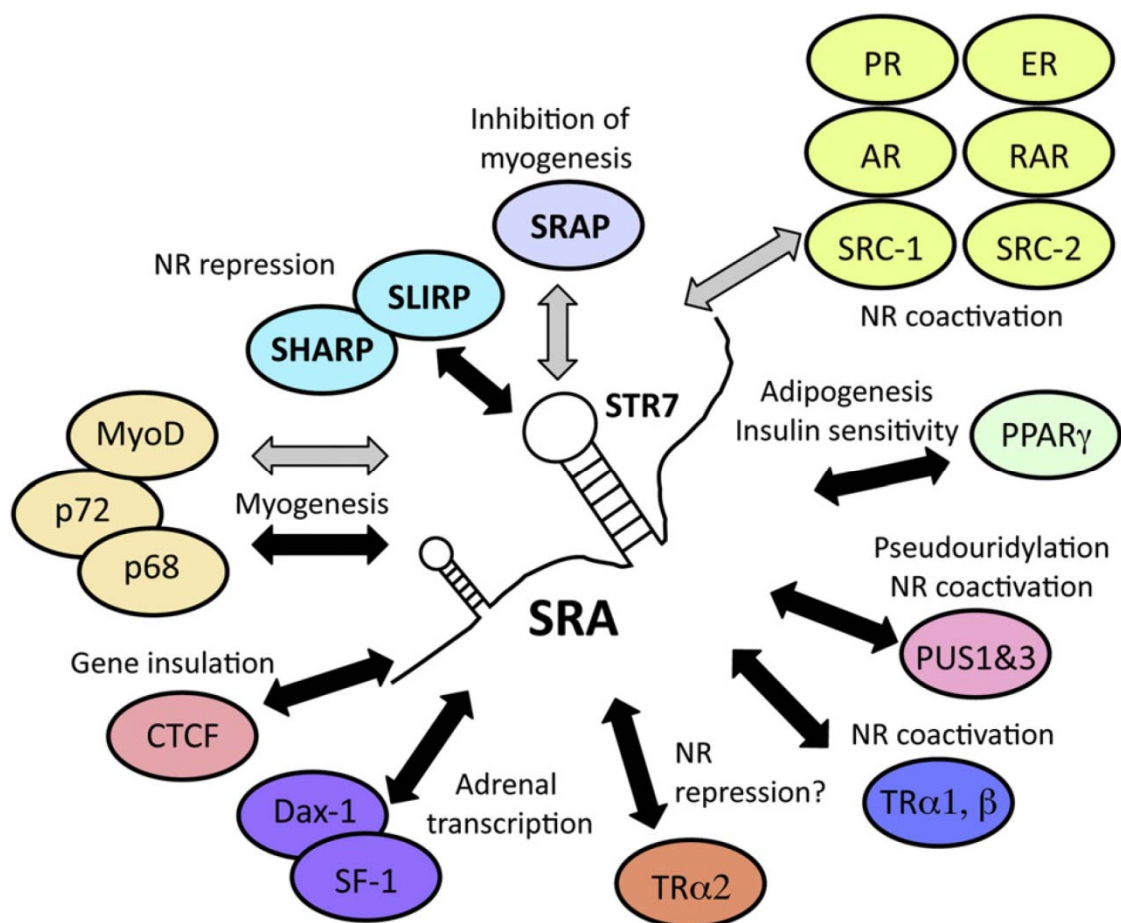


Figure3.7: Roles of different complexes forming from SRA with its partners (Colley & Leedman, 2011).

3.4.2 DEAD-box RNA helicase; DDX5 (p68) and DDX17 (p72)

The members of DEAD-box RNA helicase family were given the name from their conserved region of amino acid sequences (Asp-Glu-Ala-Asp) (Linder *et al.*, 1989). There are more than 500 different proteins in this family, which all of them share this similar amino acid consensus (Rocak & Linder, 2004). Their primary function is to unwind and separate double stranded RNA into single stranded one by ATP-dependent hydrolysis mechanism (Tanner *et al.*, 2003). In addition to unwinding RNA, DEAD-box RNA helicases have been shown to participate in almost every step of RNA metabolism (Cordin *et al.*, 2006). The most detailed studies of their function have been mainly shown from DEAD-box RNA helicases in bacteria, *S. cerevisiae* (Figure 3.8).

Another interesting function of DEAD-box RNA helicases is that they also function as transcriptional co-regulators as a complex with other proteins (Fuller-Pace, 2013). Figure 3.9 summarizes involvement of 2 most studied DEAD-box RNA helicases; DDX5 (p68) and DDX17 (p72), as either positive or negative transcriptional co-regulators. The decision on whether they would function as positive or negative co-regulator depends mostly on the components in the protein complex they are involved. DDX5 and DDX17 have been shown to participate in transcriptional control at certain genomic regions (Fuller-Pace & Ali, 2008). However, they contain no DNA binding motif structurally, indicating that they possess no direct transcriptional control. An early result regarding function of DDX5 indicated that it is involved in transcriptional activity of nuclear estrogen receptor (Endoh *et al.*, 1999). Subsequent identification of DDX17 as a homolog to and heterodimer partner of DDX5 led to a hypothesis that they might share some function (Jalal *et al.*, 2007; Lamm *et al.*, 1996; Ogilvie *et al.*, 2003), however distinct function of each one has been indicated from series of experiments (Bates *et al.*, 2005; Wortham *et al.*, 2009).

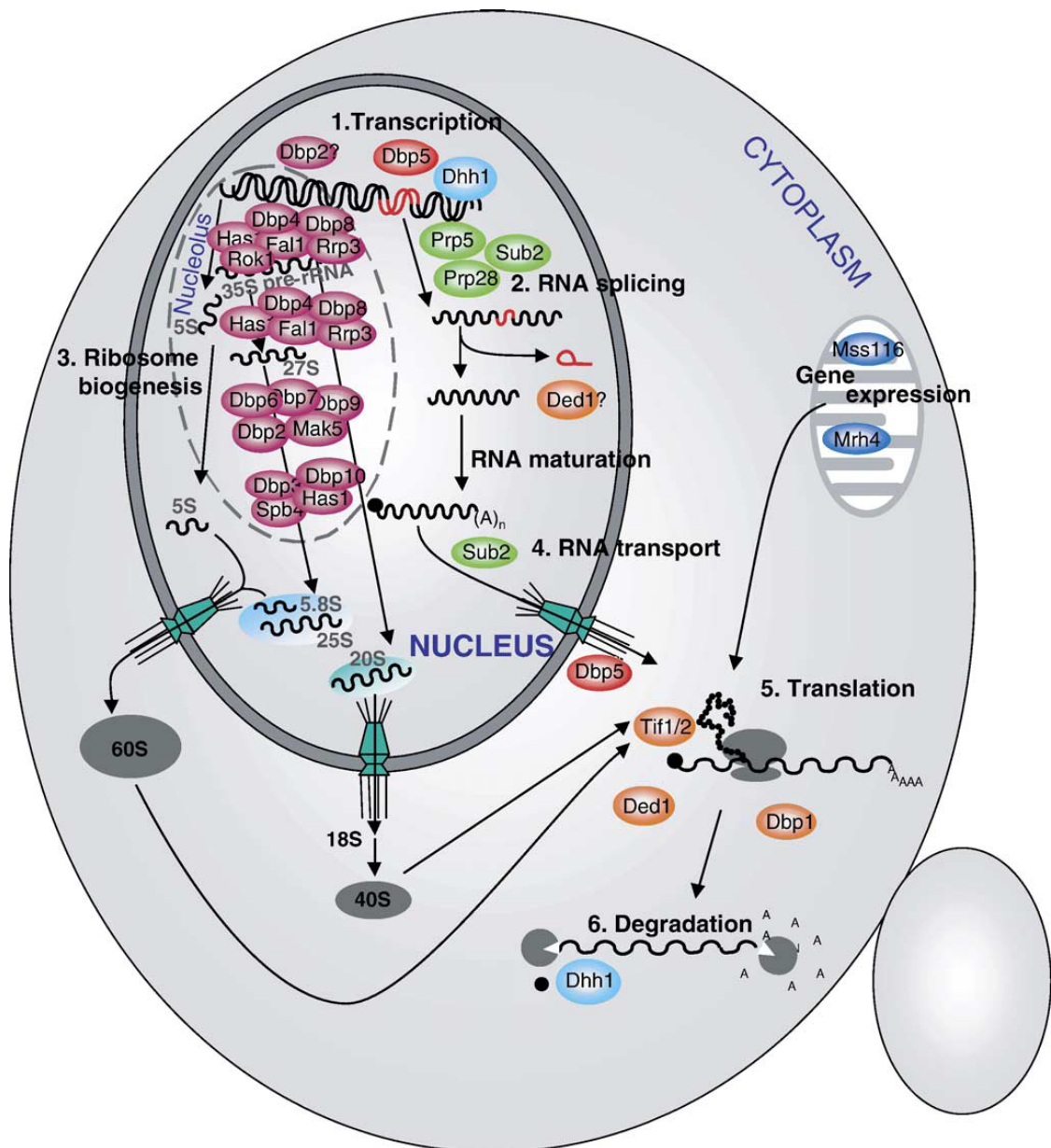
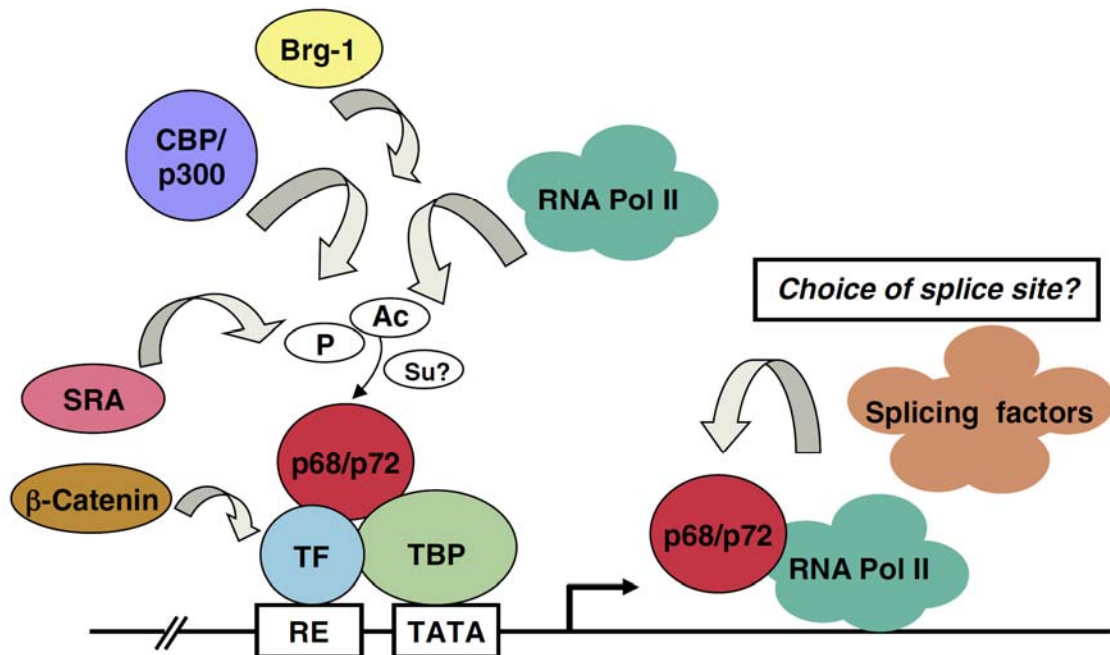


Figure 3.8: Illustration of DEAD-box RNA helicases involvement in metabolism of RNA, starting from generation until degradation (Cordin, *et al.*, 2006).

A *Transcriptional coactivation / regulation of splicing*



B *Transcriptional corepression / insulation*

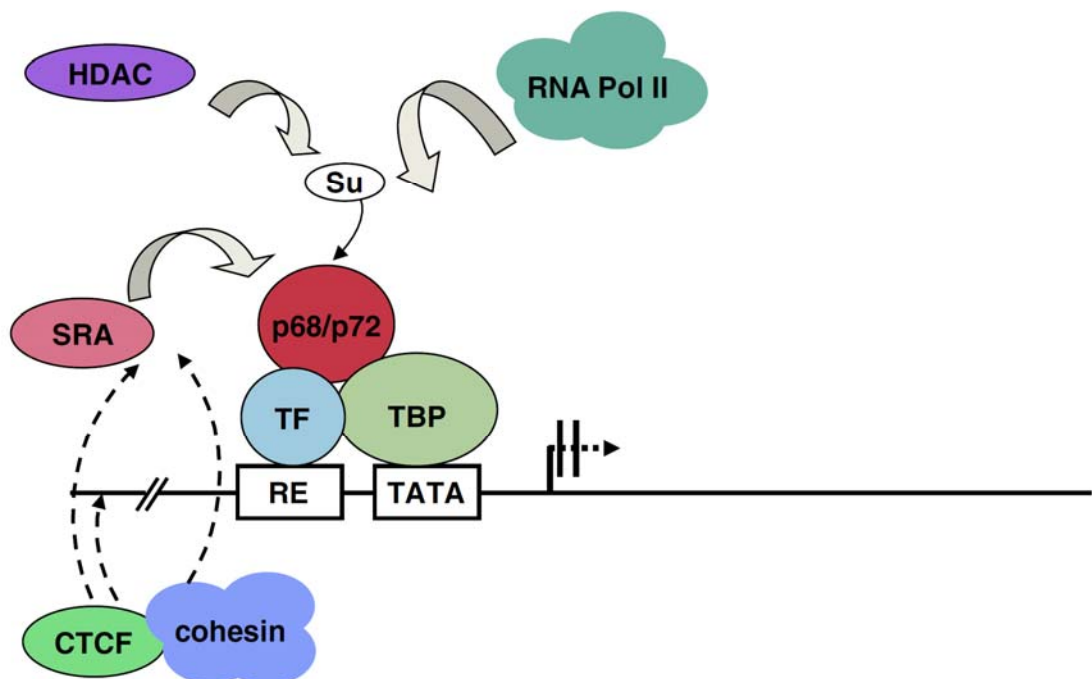


Figure 3.9: Positive and negative transcriptional co-regulators of DDX5 (p68) and DDX17 (p72) on generalized promoter regions (Fuller-Pace, 2013).

The role of DDX5 and DDX17 during development is still unknown since knockout either one of them results in death of the mouse embryo (Fukuda *et al.*, 2007). Moreover, knockout both of them simultaneously even fastens the embryonic death. However, this shows that both DDX5 and DDX17 are important for survival of mouse embryo during development. DDX5 expression at mRNA level has been shown to correlate with development of heart, kidney, liver and eye in mouse embryo (Stevenson *et al.*, 1998). DDX5 and DDX17 transcripts can be found in the developing CNS as shown from different species as well (Ip *et al.*, 2000; Seufert *et al.*, 2000). DDX17 might be tightly related to development of CNS as reduced expression of DDX17 was shown from fetal brain of Down's syndrome compared to controls (Kircher *et al.*, 2002).

Roles of many members of RNA helicase family in cell differentiation have been displayed including DDX5 and DDX17 (Abdelhaleem, 2005). Together with SRA as a complex, they have been shown to regulate transcription of MyoD and differentiation into muscle cells from C2C12 cell line (Caretti, *et al.*, 2006). DDX5 and RUNX2, a transcription factor important for osteocyte differentiation, show reciprocal interaction on both protein and genomic region suggesting complex regulation of DDX5 on differentiation of bone cells (Jensen *et al.*, 2008). Adipocyte differentiation may be involved by DDX5 as well (Kitamura, *et al.*, 2001). Unfortunately, role of these RNA helicases in cell differentiation has been demonstrated only in some lineages, especially for neural lineage even though they are expressed in the developing CNS. The role of these factors on inducing neural differentiation has to be elucidated.

3.4.3 Pseudouridylate Synthase 1 (PUS1)

Pseudouridylate synthase is an enzyme important for post-transcriptional modification of RNA. It isomerizes uridine base into pseudouridine, shown as Ψ in Figure 3.10. This modification allows RNA structure, at Ψ residue, to form one more hydrogen bond with other RNA resulting in stabilization of the complex RNA structure. Early studies of Ψ synthase were done mostly in micro-organism and categorize Ψ synthases into 5 subgroups according to the enzymes found in *E.coli* (Hamma & Ferré-D'Amaré, 2006; Kaya & Ofengand, 2003; Koonin, 1996). Human

PUS1 (hPUS1) belongs to TruA family and is shown to pseudouridylate only some specific RNA such as tRNAs and SRA (Sibert *et al.*, 2008). Even though some specific RNAs and specific sites within those RNAs were identified as target for pseudouridinylation, a specific RNA sequence for PUS1 recognition is still undetermined (Czudnochowski *et al.*, 2013; Sibert & Patton, 2012). A role of pseudouridinylation in the nervous system is indicated from under-development of cerebellum in a genetic disease, called Hoyeraal-Hreidarsson syndrome, which is caused by mutation in one pseudouridinylation synthase gene, *DKC1* (Knight *et al.*, 1999). However, mutations in *PUS1* gene have been linked to another rare genetic disease called mitochondrial myopathy and sideroblastic anemia (MLASA), which shows no neurological involvement (Bykhovskaya *et al.*, 2004; Fernandez-Vizarra *et al.*, 2007; Patton *et al.*, 2005).

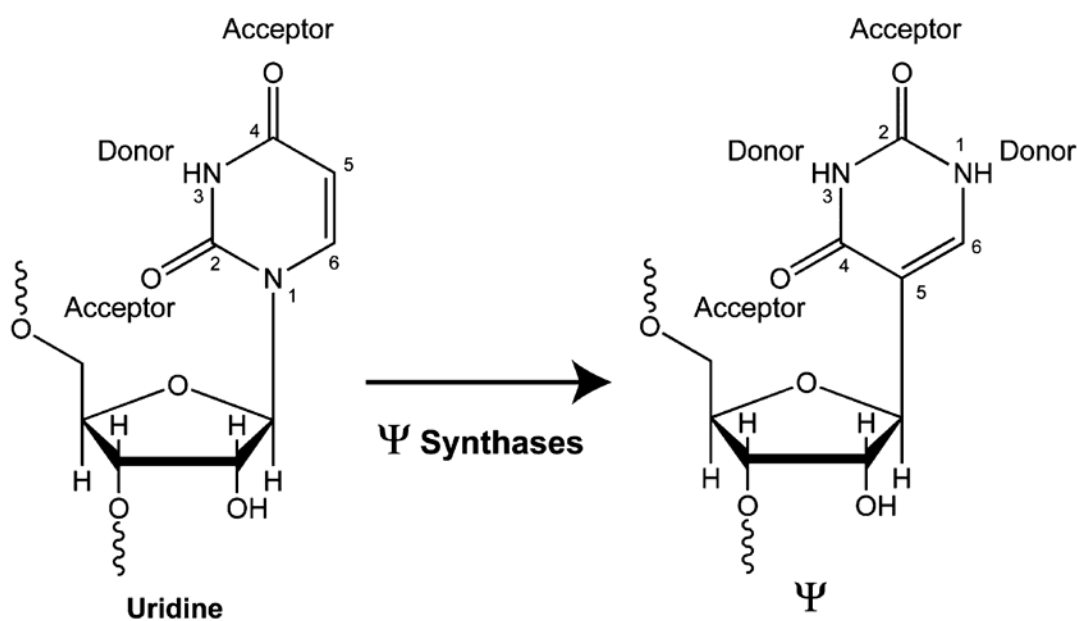


Figure 3.10: Structural difference between base uridine and pseudouridine (Hamma & Ferré-D'Amaré, 2006).

Since several RNA structures have been demonstrated as transcriptional modulator, pseudouridinylation might be another factor affecting their activity. The evidences linking pseudouridinylation and transcriptional modulation activity have been shown for pseudouridinylated SRA (Ghosh *et al.*, 2012; Zhao *et al.*, 2004; Zhao

et al., 2007). These reports show that PUS1-mediated SRA pseudouridylation can modulate a function as transcriptional co-regulator of SRA on steroid receptors. Even though modulation of transcriptional process might affect expression level of differentiation related genes, involvement of PUS1 in this process has not been published so far.

3.4.4. CCCTC-binding factor (CTCF)

CTCF is an 11-zinc finger protein, which has been shown to possess many regulatory functions on genomic organization. It is first shown as repressor transcription factor for *c-myc* oncogene in chicken (Filippova *et al.*, 1996; Klenova *et al.*, 1993; Lobanenko *et al.*, 1990). Different combinations of CTCF zinc fingers have been used to form interaction between related genomic areas. Genomic interaction is not limited to only nearby sequence, but recent studies have shown that CTCF also mediates long range interaction within the chromatin organization (Filippova, 2007; Kim *et al.*, 2007). In addition to transcriptional modulation and chromatin interaction modifying activity, CTCF can exert some roles in other cellular and molecular processes such as insulator function (Ghirlando *et al.*, 2012; Wallace & Felsenfeld, 2007), barrier function (Cuddapah *et al.*, 2009; Kim, *et al.*, 2007), genomic imprinting (Fedoriw *et al.*, 2004; Singh *et al.*, 2012; Zhang *et al.*, 2011) and X inactivation (Filippova *et al.*, 2005; Spencer *et al.*, 2011). The overall functions of CTCF are summarized in Figure 3.11.

A role of CTCF during development has been shown in mouse embryos. Knocking out of CTCF leads to death of embryo, which occurs before implantation into maternal womb (Heath *et al.*, 2008; Moore *et al.*, 2012; Splinter *et al.*, 2006). Activity of CTCF in neural development process has been suggested from studies in mouse and rat embryos. CTCF binding sites are identified at several regulatory elements of genes important for developing brain such as protocadherin- α gene (Hirayama *et al.*, 2012; Kehayova *et al.*, 2011) and amyloid precursor protein gene (Yang *et al.*, 1999). Specific depletion of CTCF in mouse telencephalon results in extensive cell death and loss of telencephalic regions (Watson *et al.*, 2014). Moreover, co-operation between CTCF and Pax6 has been shown to be another important process for development of neural part of mouse retina (Canto-Soler *et al.*, 2008).

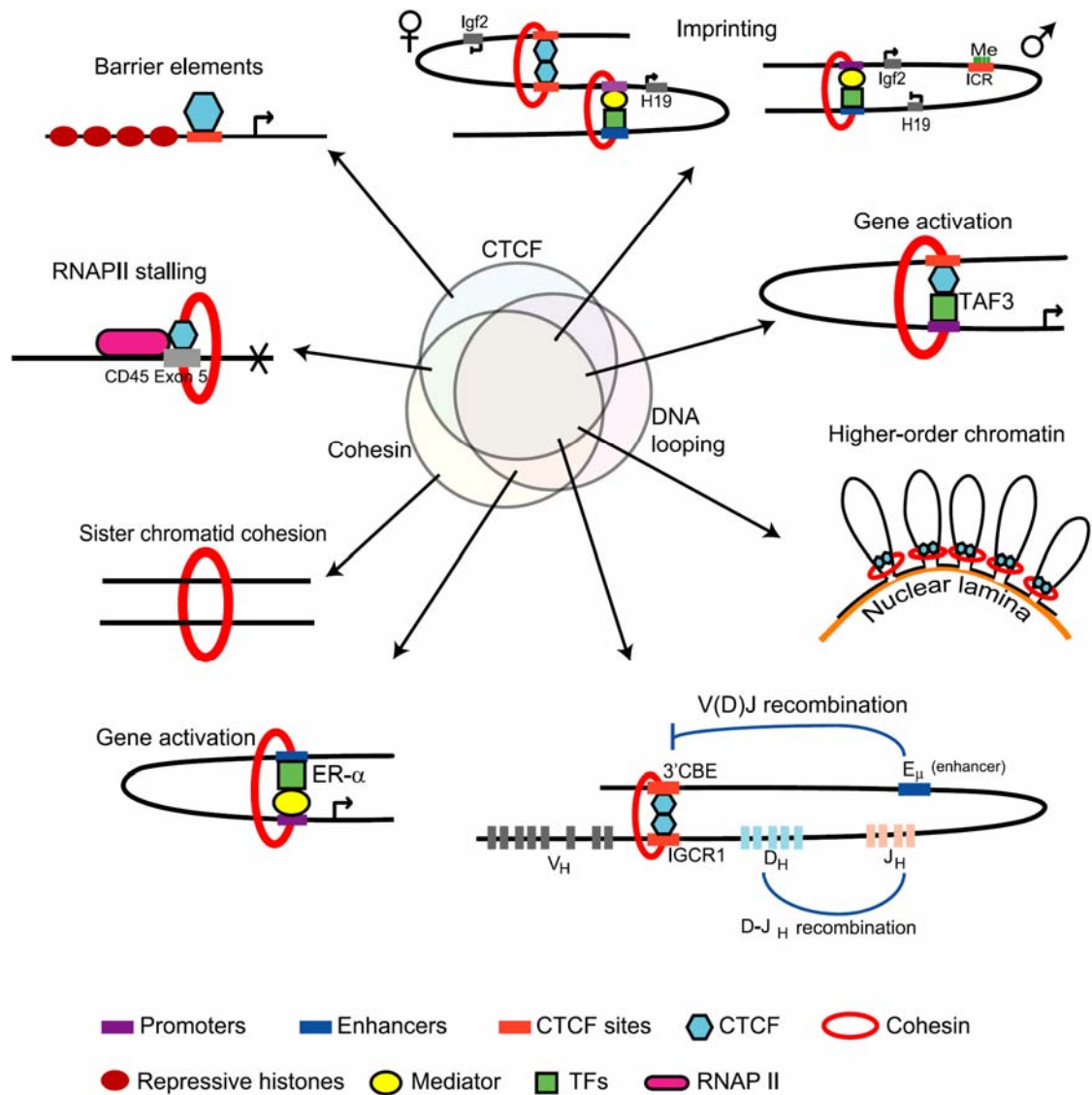


Figure 3.11: Function of CTCF in genomic organization and gene transcription (Lee & Iyer, 2012).

Involvement of CTCF activity on its binding site has been linked with an ability to differentiate of mouse ES cells (Plasschaert *et al.*, 2014). Long range interaction between *OCT4* and *SOX2* enhancers is mediated by CTCF in human ES cells (Gao *et al.*, 2013), confirming that CTCF is important for co-operating between stem cell-related genes as well as stem cell biology of human ES cells (Balakrishnan *et al.*, 2012). A genome-wide study comparing between CTCF binding sites in mouse ES cells and mouse brain reveals that many unconventional CTCF binding sites are tissue-specific (Prickett *et al.*, 2013). CTCF is able to recruit a factor important for activation of promoter region as well as important for endodermal lineage commitment, namely TAF3 (Liu *et al.*, 2011). In differentiation of muscle lineage, CTCF is found to promote expression of myocytes-related genes as well (Delgado-Olguín *et al.*, 2011). Activation of other differentiation related genes is also under control of CTCF through DNA methylation in human ES cells (Yu *et al.*, 2013). CTCF has been shown to be specifically involved with neural differentiation of mouse ES cells by repressing expression of *Pax6* on its promoter (Gao *et al.*, 2011).

Transcription of *PAX6* has been shown to be under modulation of CTCF in mouse ES cells, developing retinas as well as in other human cell lines (Gao, *et al.*, 2011; Li *et al.*, 2006; Wu *et al.*, 2006). However, this regulatory role of CTCF on *PAX6* gene in human pluripotent stem cell has yet to be shown. CTCF is also found as a complex with other RNA signaling molecules, including SRA and DDX5 (Yao *et al.*, 2010). The complex was shown to modulate expression of *IGF2/H19* imprinting region via activity of CTCF. Another RNA signaling molecule, PUS1, has been shown to directly interact with SRA and DDX5 (Colley *et al.*, 2008). All together, it is interesting to study the involvement of a complex comprising of CTCF, long non-coding RNA, and other protein binding factors on transcriptional regulation of *PAX6* gene as well as their role in regulating neural differentiation of human pluripotent stem cells.

CHAPTER IV

MATERIALS AND METHODS

4.1 Cell culture

NTERA2/D1 and N2102Ep were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco/Invitrogen) supplemented with 10% Fetal bovine serum (FBS; Gibco/Invitrogen), L-Glutamine (Cellgro/Corning) and MEM non-essential amino acid (Cellgro/Corning). Cells were maintained at 37°C in 5% CO₂ incubator. When cells reached confluent, they were dissociated and replated 1:3 with 0.25% trypsin/EDTA (Gibco/Invitrogen).

All-trans retinoic acid (RA; Sigma) was used to induce differentiation. Cells were trypsinized and plated at density of 1x10⁶ cells per well in 6-well plate, 3x10⁶ cells in T25 culture flask or 9x10⁶ cells in T75 culture flask. Cells were allowed to attach on culture vessel for 1 day. On the next day, culture medium was replaced with culture medium containing 10 µM RA to treat culture. RA-containing culture medium was replaced every 3 days. The culture usually reached 80% confluent by 3-4 days after treatment, so trypsinization and 1:2 splitting were done on 8th day after treatment. After splitting, culture medium was changed every 7 days.

To determine effect of Lysine-specific histone de-methylase 1 (LSD1) inhibition on cell differentiation of N2102Ep, cells were plated at 1x10⁴ cells per cm² density, either in 6-well plate or T25 culture flask. Pretreatment with LSD1 inhibitor started on the next day after allowing cells to attach overnight. LSD1 inhibitors used in this experiment included tranilcypromine (TCP; Sigma) and pargyline (Prg; Sigma). Culture medium (DMEM/F12; Gibco/Invitrogen) containing 200 µM TCP or 750 µM Prg (final concentration) or vehicle (DMSO) was fed to the cells. On the 4th day of pretreatment, the pretreated culture was split 1:2. Pretreated cells were treated with LSD1 inhibitor with or without 10 µM RA for 7 and 14 days. Culture medium was changed every 2 days afterwards.

4.2 Reverse-transcription polymerase chain reaction

4.2.1 RNA extraction and quantification

RNA samples were extracted from either cell pellet or adherent culture. Pellet of adherent culture were homogenized with 1 mL of TRIzol reagent (Invitrogen). The reagent was pipetted up and down until cells were not visible, then the solution was transferred to 1.7 mL microcentrifuge tube. Vortexing microcentrifuge tube was done to ensure complete cell homogenization, before incubating on ice for 5-10 minutes. After incubation, 200 μ L of ice-cold chloroform was added into the homogenate. The solution was vortexed for 15-30 seconds and incubated on ice for 15 minutes. Then, the solution was centrifuged at 14,000 rpm at 4°C for 15 minutes. The result solution consisted of 3 layers, including upper clear aqueous phase, intermediated phase, and lower red phenol-chloroform phase. 450 μ L of aqueous phase was transferred carefully to another tube, without disturbing the intermediate phase. RNA was precipitated by adding 500 μ L of ice-cold isopropanol, vortexing to mix the solution, and incubation at -20°C overnight. On the following day, precipitated RNA was centrifuged at 14,000 rpm at 4°C for 30 minutes to pellet RNA. Supernatant was carefully discarded without disturbing the pellet. The pellet was washed with 500 μ L of ice-cold 75% ethanol in DEPC-treated sterile distilled water (sdH₂O) and centrifuged at 14,000 rpm at 4°C for 15 minutes. Supernatant was discarded and the pellet was left to air dry for 15 minutes. To dissolve RNA pellet, 50 μ L of DEPC-treated sdH₂O was added. It was let stand for 15 minutes and pipetted up-down to ensure completely dissolved.

RNA concentration was measured using NanoDrop ND1000 Spectrophotometer (Thermo Scientific). Absorbance (A) at 260 and 280 nm wavelength was read, RNA samples that had ratio of A₂₆₀/A₂₈₀ more than 1.50 were acceptable for further experiments. Absorbance at 260 nm was set to blank using distilled water; the absorbance at 260 nm of each sample was used to calculate RNA concentration. After the concentration was determined, RNA was diluted to make 100 ng/ μ L RNA samples with DEPC-treated sdH₂O for further complementary DNA synthesis and stored at -80°C.

4.2.2 Complementary DNA synthesis

Complementary DNA was reverse transcribed from 1 µg of total RNA using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). 2 µL of reverse transcriptase enzyme was used in 20 µL reaction. The reactions were then incubated at 25°C for 10 minutes, 50°C for 30 minutes, and 80°C for 10 minutes respectively. After finished incubation, each reaction was diluted with 20 µL sdH₂O. The synthesized cDNA was used immediately or kept at -20°C.

4.2.3 RT-PCR primers

Table 4.1: Primers used in qRT-PCR for amplification of each gene

Gene	Forward primer	Reverse primer
<i>ACTB</i>	AAACTGGAACGGTGAAGGTG	AGAGAAGTGGGGTGGCTTTT
<i>OCT4</i>	GTACTCCTCGGTCCCTTTCC	CAAAAACCCTGGCACAACCT
<i>NANOG</i>	GGATGGTCTCGATCTCCTGA	CCTCCCAATCCCCAAACAATA
<i>SOX2</i>	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT
<i>PAX6</i>	ACTGGGGAAGGAATGGACTT	AATTCGTGGCAAAGCTTGTT
<i>T</i>	GTCCACCTGCAAATCCTCAT	CACAGGCTGGGGTACTGACT
<i>GATA6</i>	CCACCTTATGGCGCAGAAAC	GAGCCCATCTTGACCCGAAT
<i>CDX2</i>	CCTCCGCTGGGCTTCATTCC	TGGGGGTTCTGCAGTCTTTGGTC
<i>NEUROD1</i>	AAGCCATGAACGCAGAGGAGGACT	AGCTGTCCATGGTACCGTAA
<i>NESTIN</i>	GGAATCTCTTGCTGCTCCA	CAAAAGCCAGCATGTCACCC
<i>DCX</i>	TTTGGTTTGCTGACCCCACT	AGCCCTCATTGAATTGCGGA
<i>MAP2</i>	TTGAGCACCCTCTTGTGGG	GCAAGTGACCCAGAGGTTGA
<i>S100β</i>	TGTAGACCCTAACCCGGAGG	TGCATGGATGAGGAACGCAT
<i>GLAST</i>	CTTTCAGCTGGAGTGGGGTT	TCCCACTCAGCGTCTTTGAC
<i>MBP</i>	CTTCCAAAGAGTCCCACGCT	TTTCCGTTTCCAGCCATCGTCA
<i>CTCF</i>	GTGCTGGCCCAGATGGCGTA	GGTTCTGCCAGGGGGTCTGTC
<i>SRA</i>	CGGTGGGACGCAGCAGATGA	TGAAGCCTGCTGGAAGCCTGGT
<i>DDX5</i>	GTCAACAAGAGCGTGACTGG	TGTGCCTGTTTTGGTACTGC
<i>PUS1</i>	CCTTTGTGCGGGAGGGCCTG	CCCAGTCCAGCGGCTCATGC

4.2.4 Quantitative RT-PCR

Quantitative RT-PCR was performed with SYBR Green-based ABI Prism 7900HT Sequence Detection System (Applied Biosystems/Invitrogen) using 96-well plate. Technical triplication was done with every cDNA template sample. Reaction in each well was prepared to make 20 μ L reaction mixtures as following;

1 μ L of cDNA template

2 μ L of 10 μ M forward and reverse primers mixture (MWG Eurofins)

10 μ L of 2X Power SYBR Green PCR Master Mix (ABi/Invitrogen)

7 μ L of sdH₂O

PCR condition used to amplify target fragment was;

95°C for 2 minutes

95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute

Repeat for 50 cycles

After amplification finished, dissociation curve was analyzed using the following condition;

95°C for 15 seconds

Dissociation curve analysis started from 60°C to 95°C for 15 seconds each, rate of temperature ramping was set at 2%, dissociation data was collected every 10 seconds while temperature ramping.

After finished PCR and dissociation curve processed, data was analyzed with SDS software (ABi/Invitrogen). Ct values for each sample calculated with the program were exported for further analysis. Expression of the gene of interest was normalized with expression of ACTB of the similar sample. Biological duplication of different experiments was done on different qRT-PCR analysis.

4.3 Western blot analysis

4.3.1 Protein extraction and quantification

Protein samples were extracted from cell pellet equivalent to a confluent culture in T25 culture flask. Protein extraction was done from either freshly prepared

or frozen pellet at -80°C . The pellet was washed once with phosphate buffer solution (PBS) and transferred to 1.7 mL microcentrifuge tube. PBS supernatant was discarded and the pellet was resuspended in 100 μL of RIPA buffer supplemented with 1X protein inhibitor cocktail (PIC). Cell suspension was homogenized with sonication at maximum level for 7.5 minutes per cycle with 30 seconds on and 30 seconds off interval, 2 cycles in total. The sonicated samples were centrifuged at 14,000 rpm at 4°C for 10 minutes. The resulting supernatant was transferred to fresh tube as the protein samples, which were kept at -80°C until use.

Protein concentration was measured with BCA assay kit (Thermo Scientific). Protein concentration standard curve was made with bovine serum albumin (BSA) at 0, 2, 4, 6, 8, and 10 $\mu\text{g/mL}$ final concentrations. 1 μL of each protein samples was diluted in 4 μL of sdH_2O , making 5 dilution factor. 1 μL and 3 μL of diluted samples were mixed with 24 μL and 22 μL respectively of sdH_2O , to make 25 μL total volumes for concentration measurement. 200 μL of BCA buffer was added to 25 μL of sample to make 225 μL final reactions. Every reaction was incubated at 37°C for 30 minutes. Protein concentration was determined by optical density at 562 nm wavelength using NanoDrop ND1000 spectrophotometer. OD_{562} of BSA was used to plot a standard protein concentration curve. Concentration of each sample was calculated from mean OD_{562} of the respective sample over the plotted standard protein concentration curve.

4.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were mixed with 4X NuPAGE LDS Sample Buffer (Novex/Invitrogen) supplemented with 5% β -mercaptoethanol, to make 2 $\mu\text{g}/\mu\text{L}$ solutions. The solutions were incubated at 95°C for 15 minutes. 4-12% precast gradient Bis-Tris polyacrylamide gel (Novex/Invitrogen) was inserted into a chamber containing 1X NuPAGE MES Running Buffer (Novex/Invitrogen). Forty micrograms of protein solutions were loaded, with 5 μL of prestained protein marker (Bionexus) loaded in the outer most well. Protein electrophoresis was performed with constant voltage set at 200 V on a power supply (Bio-rad) for 40 minutes.

4.3.3 Protein transfer and Immunoblotting

Protein electrophoresis was transferred onto polyvinylidene fluoride (PVDF) membrane (Novex/Invitrogen). Before assembling transfer cassette, both gel and methanol pre-soaked PVDF membrane were incubated in 1X NuPAGE Transfer Buffer (with 20% methanol and 0.01% SDS) for 15 minutes at room temperature. Transfer unit was assembled with sponges and filter papers on the outer most layers, gel and PVDF membrane were placed next to each other. When inserted into the transfer chamber, PVDF membrane was placed facing positive electrode of the chamber. Protein transfer was done with constant ionic current at 400 mA set on a power supply for 1 hour.

After protein transfer was finished, protein blot-containing PVDF membrane was taken out of the transfer cassette and incubated in TBST (1X TBS; Novex/Invitrogen and 0.1% (v/v) Tween-20) for 15 minutes at room temperature. Non-specific binding was blocked by blocking solution (5% non-fat milk in TBST) for 2 hours at room temperature. Blot was incubated with primary antibody immediately after blocking overnight at 4°C. Primary antibodies and their concentration used were listed in Table 4.2. On the next day, blot was washed with TBST, for 10 minutes, 3 times. Secondary antibody conjugated with horse radish peroxidase (HRP) corresponding with the primary antibody used was added to blot. List of secondary antibodies used was shown in Table 4.3. Protein blot was incubated at room temperature for 1 hour.

Table 4.2: Primary antibodies used in western blot analysis

Primary antibody	Company	Dilution
Anti-ACTB mouse monoclonal antibody	Sigma	1 in 3000
Anti-PAX6	Santa Cruz	1 in 500
Anti-FLAG	Sigma	1 in 500
Anti-CTCF	Millipore	1 in 500
Anti-DDX5	Millipore	1 in 500
Anti-PUS1	Sigma	1 in 500

Table 4.3: Secondary antibodies used in western blot analysis

Secondary antibody	Company	Dilution
HRP-conjugated Goat anti-Mouse antibody	Sigma	1 in 10000
HRP-conjugated Goat anti-Rabbit antibody	Sigma	1 in 10000

4.3.4 Enhanced chemiluminescence detection of proteins

After incubation with secondary antibody, membrane was washed with TBST for 10 minutes, 3 times. Illumination of protein blots was developed with enhanced chemiluminescence (ECL) reagents (Invitrogen) for 1 minute at room temperature. Membrane was placed between 2 sheets of transparent film inside of an X-ray film cassette (Kodak). X-ray film exposure was done in the dark room and developed with X-ray film processor machine (Kodak). Protein blots on processed X-ray films were scanned to digital images.

4.4 Cloning efficiency assay

Confluent culture of NTERA2 miCtrl, NTERA2 miSRA-2, NTERA2 miDDX5-2, and NTERA2 miPUS1-2 was washed with phosphate buffer solution (PBS; Gibco/Invitrogen) and dissociated with 0.25% trypsin. Cell numbers were counted. Eight hundreds cells were taken and diluted in 12 mL of DMEM/F12 (Gibco/Invitrogen) supplemented with 10% FBS. 3 mL of cell suspension (200 cells) was seeded into each well of 6-well plate, 3 wells per cell line. Cells were put into the incubator immediately after seeding and left undisturbed for 2 weeks. On the 14th day after seeding, cells were taken out for analysis with Alkaline Phosphatase Detection Kit (Millipore). Culture was washed gently with PBS and fixed with 4% paraformaldehyde in PBS for 2 minutes. After washed with TBST, culture was incubated in dark with staining solution, supplied with the kit, for 15 minutes at room temperature. The stained plate was washed with TBST once more and covered with PBS. Alkaline phosphatase-positive colonies with colony morphology representing that of pluripotent cells were counted.

4.5 Flow cytometry

Cell samples were stained with antibody against surface antigens to determine the percent of positive population in each culture. There are 6 antigens analyzed in total, including P3X, SSEA3, SSEA4, TRA-1-60, SSEA1 and A2B5. Flow cytometry was done from 3 different cultures for each cell line and treatment. Cultures were washed with PBS once before dissociation and harvesting. Cell numbers were estimated by counting with hemacytometer. 500,000 cells were used to stain with each antigen, 3×10^6 cells were taken and resuspended in 600 μL of 10% FBS in PBS. 100 μL of cell suspension was divided into each microcentrifuge tube. Primary antibody against the antigen was added into cell suspension at 1 in 10 dilution and incubated dark on ice for 30 minutes. Primary antibody was washed out by centrifugation and discarded the supernatant. Cell pellets were then incubated with 100 μL of secondary antibody-containing 10% FBS in PBS, at 1 in 200 dilution, dark on ice for 30 minutes. Secondary antibody used was FITC-conjugated Goat anti-Mouse antibody (Cayman Chemical). Then, secondary antibody was washed out. Cell pellets were resuspended in 700 μL of PBS and transferred to tubes used with flow cytometry machine. Flow cytometric analysis was performed on (Beckman Coulter).

4.6 Immunocytochemistry

Cells used for immunocytochemical staining were cultured with RA-containing culture medium for 30 days in T25 culture flask. On the 30th day of RA treatment, cells were dissociated and replated into 24-well plate. On the following day, cells were washed with PBS once and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Fixative was washed out with PBS and cells were kept at 4°C with 1 mL PBS covered, culture plate was sealed with parafilm.

On the staining day, cells were permeabilized with 0.5% Triton-X 100 (Sigma) in PBS for 10 minutes at room temperature. They were washed by washing buffer (0.1% Triton-X 100 in PBS) for 5 minutes, 3 times. Blocking buffer (20% FBS in washing buffer) was added to each well to block non-specific binding of the antibodies and incubated on shaker for 30 minutes at room temperature. Primary

antibody against β III-tubulin (Sigma) was diluted at 1 in 500 in blocking buffer and added into each well. Cells were incubated with primary antibody overnight at 4°C on the shaker. Primary antibody was washed on the following day with washing buffer for 5 minutes, 3 times. Alexa488-conjugated Goat anti-Mouse antibody (Sigma) was used as secondary antibody, diluted at 1 in 200 in blocking buffer. Cells were incubated with secondary antibody in dark for 1 hour at room temperature, on a shaker. From this step onwards, cells were processed with caution not to expose to light for a long period. After washed 3 times with washing buffer, cells were incubated with DAPI (???) -containing blocking buffer (diluted at 1 in 200) for 3 minutes at room temperature. Cells were washed last time with PBS and covered with 500 μ L PBS for image captures. Fluorescence images were captured with EVOS fl (Life Technologies) at 10X magnification, 3 areas in each sample were captured for statistical analysis.

4.7 Chromatin immunoprecipitation

Sample preparation and immunoprecipitation step were done using ChIP IT Express kit (Active Motif). Immunoprecipitated chromatin preparation for qPCR was done using QIAquick PCR Purification Kit (Qiagen). qPCR was done with SYBR Green-based system (Applied Biosystems/Invitrogen), similar to RT-PCR experiments. Primers used to quantify amount of precipitated chromatin was designed with NCBI-Primer BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), using target sequences acquired from genome database – UCSC Genome Browser (<http://genome.ucsc.edu/>)

4.7.1 Sheared chromatin preparation

Sheared chromatin was collected from undifferentiated NTERA2 culture and RA-treated for 3, 7, 14 and 21 days cultures. Undifferentiated sample was collected from 2 confluent culture of NTERA2 in T75 culture flasks, while RA-treated samples were collected from 3 T75 flasks of treated cells. On the day of sample collection, culture was washed with PBS once, trypsinized, harvested and pooled together. Cell number was estimated by counting with hemacytometer. Cells were centrifuged and washed once more with PBS. Cell pellet was resuspended in PBS and

2×10^7 cells were fixed with 1% formaldehyde (final concentration) in 20 mL volume on rotator at room temperature for 10 minutes. After that, 2 mL of Glycine Stop-Fix Solution (1 M Glycine) was added into the fixed sample and incubated on rotator for 5 more minutes at room temperature. Samples were centrifuged and cell pellets were washed with PBS. Pellets were resuspended with ice-cold Lysis Buffer, 3 fixed samples (equivalent to 6×10^7 cells) were pooled together and resuspended by 1 mL of lysis buffer. Resuspended samples were vortexed for 10-15 seconds and incubated on rotator at 4°C for 30 minutes. Following incubation, samples were centrifuged at 5,000 rpm at 4°C for 10 minutes to pellet nuclei. Supernatant was carefully discarded and nuclear pellets were resuspended in 350 μ L Shearing Buffer. They were sheared with sonication at maximum level for 7.5 minutes with 30 seconds on and 30 seconds off interval, for 5 cycles. PMSF and Protein Inhibitor Cocktail (PIC) were used throughout the lysis and sonication steps to preserve protein content bound with the chromatin. The sonicated samples were centrifuged at 14,000 rpm at 4°C for 10 minutes. Sheared chromatin samples in supernatant of sonicated samples were transferred to fresh 1.7 mL microcentrifuged tube and stored at -80°C until used.

4.7.2 Chromatin quantification and shearing efficiency determination

20 μ L of sheared chromatin sample was taken for quantification and shearing efficiency determination before storage. The sample was mixed with 150 μ sdH₂O and 10 μ L of 5 M NaCl. The solution was mixed together and incubated at 65°C overnight in order to de-crosslink formaldehyde fixation. On the following day, sample was treated with RNase A, incubated at 37°C for 15 minutes, and Proteinase K, incubated at 42°C for 1.5 hours. Phenol-chloroform (1:1, pH 8), 200 μ L, was added into the treated sample. The solution was vortexed for 10-15 seconds and centrifuged at 14,000 rpm at 4°C for 5 minutes. Chromatin was precipitated using all of the upper aqueous phase by mixing with 20 μ L of 3 M sodium acetate and 500 μ L of ice-cold absolute ethanol and incubation overnight at -20°C.

On the following day, precipitated chromatin was centrifuged at 14,000 at 4°C for 30 minutes to make chromatin pellet. Supernatant was carefully discarded; the pellet was washed with 70% ethanol in sdH₂O and left to air dry for 15 minutes. It was dissolved with 20 μ L of sdH₂O, pipetting up and down after 15 minutes incubation

with sdH₂O was done to ensure complete dissolved. Chromatin concentration was measured using NanoDrop ND1000 Spectrophotometer (Thermo Scientific). Similar to RNA samples, samples with A260/A280 over 1.5 were acceptable for further experiments. Chromatin concentration was calculated from A260. After concentration was measured, 10 µg of the sample was used to check shearing efficiency by running in 1.2% agarose gel (Life Technologies/Invitrogen) at 70 V for 30 minutes. Chromatin fragment between 200 base pair to 1 kilobase pair was acceptable for further immunoprecipitation experiments.

4.7.3 Immunoprecipitation

Chromatin immunoprecipitation was performed with 50 µg of sheared chromatin. The immunoprecipitation reactions were mixed into the 1.7 mL siliconized microcentrifuged tubes. The component of each reaction included

- 50 µg sheared chromatin sample
- 20 µL ChIP Buffer I
- 2 µL of protein inhibitor cocktail
- 2 µL of RNase inhibitor (NEB)
- 25 µL of Protein G Magnetic beads
- 3 µg of Antibody (1 µg of IgG for negative control)
- x µL of sdH₂O, which made the total reaction volume to 200 µL

Amount of chromatin used in the reaction was calculated from the concentration determined from the corresponding sample in the previous step. Magnetic beads were resuspended before added into the reaction, without vortexing as it can cause damage to the beads. The antibody was the last component that was added into the reaction. Caution was made not to cause bubbles inside the tubes since the electrostatic force from the bubbles might interfere with binding of antibody to magnetic beads. After finishing reaction assembly, tube caps were closed and sealed with parafilm. The reactions were incubated overnight on the rotator at 4°C.

On the following day, reaction tubes were briefly spun to collect solution inside the cap. Magnetic beads were washed with ChIP Buffer I once and ChIP Buffer II twice on the magnetic stand. After last washed with ChIP Buffer II,

immunoprecipitated chromatin was eluted out of the magnetic beads by incubation with 50 μ L of Elution Buffer AM2 on rotator for 15 minutes at room temperature. 50 μ L of Reverse Cross-linking Buffer was added into the eluted chromatin and mixed by pipetting up and down. The solution was placed on the magnetic stand to separate eluted chromatin with reverse cross-link buffer from the magnetic beads and transfer the supernatant to a fresh tube. DNA input sample was prepared from 0.5 μ g of sheared chromatin sample (1% of DNA input), the similar sample used for immunoprecipitated reaction, mixed with 2 μ L of 5 M NaCl and x μ L of sdH₂O to make a 100 μ L total volume. The samples were incubated at 95°C for 15 minutes to remove formaldehyde fixation. Samples were returned to room temperature and treated with 2 μ L of Proteinase K, incubated at 37°C for 1 hour. The Proteinase K Stop Solution was brought to room temperature about 30-60 minutes before stopping Proteinase K reaction. Samples were returned to room temperature and 2 μ L of stop solution was added to the reaction.

4.7.4 Immunoprecipitated chromatin preparation

It is recommended that the chromatin samples should be processed with DNA clean up step before used in qPCR quantification. Column purification with QIAquick PCR Purification Kit (Qiagen) was used. 500 μ L of Buffer PB was added into each sample and mixed completely. The solution was transferred into QIAquick column and allowed to bind to the column. It was centrifuged at 13,000 rpm at room temperature for 1 minute. The column, containing immunoprecipitate chromatin, was washed with 750 μ L of Buffer PE by centrifugation for 1 minute. After discard the flow-through, the column was centrifuged again for 1 minute to remove the residual buffer. The column was transferred to a new tube; and 50 μ L of Buffer EB was added into the membrane to elute chromatin sample. The column was centrifuge for 1 minute; the resulting solution inside the tube was ready to use for further analysis.

4.7.5 Chromatin Immunoprecipitation (ChIP) primers

Table 4.4: Primers used in qPCR for amplification of each target genomic region

Target region	Forward primer	Reverse primer
<i>5' Enhancer</i>	GGGCACACTGGAGGGATATAG	AGGTACATGAGAGGGATGGC
<i>P0 promoter</i>	CCGATGCCTCTGATTCCTCA	CACTCTCGCTGCACAAACTT
<i>P1 promoter</i>	CCACAGAGCCTCTTCGTCTT	GAGAAGTTTCCACGCAGGAG
<i>Intronic enhancer</i>	GGGACCCTCTTAGCACCAAA	CTTTAACGCGCCTAGTTGCT

4.7.6 Quantitative PCR

Quantitative PCR was performed with SYBR Green-based ABI Prism 7900HT Sequence Detection System (Applied Biosystems/Invitrogen) using 96-well plate. Technical triplication was done with every purified-immunoprecipitated chromatin sample. Reaction in each well was prepared to make 20 μ L reaction mixtures as following;

2 μ L of purified-immunoprecipitated chromatin sample

2 μ L of 10 μ M forward and reverse primers mixture (MWG Eurofins)

10 μ L of 2X Power SYBR Green PCR Master Mix (ABi/Invitrogen)

6 μ L of sdH₂O

PCR condition used to amplify target fragment was;

95°C for 2 minutes

95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute

Repeat for 50 cycles

After amplification finished, dissociation curve was analyzed using the following condition;

95°C for 15 seconds

Dissociation curve analysis started from 60°C to 95°C for 15 seconds each, rate of temperature ramping was set at 2%, dissociation data was collected every 10 seconds while temperature ramping.

After finished PCR and dissociation curve processed, data was analyzed with SDS software (ABi/Invitrogen). Ct values for each sample calculated with the program were exported for further analysis. Ct values of 1% DNA input were used to

estimate Ct values of 100% DNA input. After that %input from IgG and other antibodies was calculated according to their Ct values compared to that of 100% DNA input. Binding of IgG to each region was normalized to 1, while binding of other antibodies was normalized over that of IgG to determine fold enrichment of binding compared to negative control.

4.8 Complementary DNA cloning, plasmid preparation and cell transfection

4.8.1 cDNA cloning of *PAX6A* and *PAX6B* coding sequences

cDNA encoding human *PAX6A* and *PAX6B* were amplified using Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Scientific). Plasmids used for over-expression of both isoforms of *PAX6* gene were given from Prof. Su-Chun Zhang (University of Wisconsin, USA) and used as DNA template for amplification. Oligonucleotides were used as primers for amplification including XhoI-FLAG-*PAX6*-F, which contained *XhoI* restriction site and a FLAG-tag at 5', (CCCCTCGAGATGGATTACAAGGATGACGACGATAAGATGCAGAACAGTCACAGCGGAGTG) and NotI-*PAX6*-R, which contained *NotI* restriction site at 3', (CCCGCGGCCGCTTACTGTAATCTTGGCCAGTATTGAGACATATCAGGTTCACTTCCGGG) as forward and reverse primer respectively (MWG Eurofins). The PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen). The purified products were measured for concentration with spectrophotometer (NanoDrop ND1000/ Thermo Scientific) and confirmed in size by running with agarose gel (E gel/ Life Technologies/Invitrogen).

The purified PCR products were cloned using TOPO TA Cloning Kit (Life Technologies/Invitrogen). First, adenosine base-overhang was added to 3' end of the products, A-tailing, by incubation the following mixture at 72°C for 30 minutes;

5 µL of 10X buffer

1.5 µL of 50 mM MgCl₂

1 µL of dNTPs

42 μ L of gel-extracted PCR products

0.5 μ L of Platinum Taq DNA Polymerase

The A-tailed products were immediately cloned with TOPO TA Cloning Kit by incubating the following reaction mixture overnight at 22°C;

4 μ L of A-tailed products

1 μ L of pCR4-TOPO vector

1 μ L of salt solution

The ligation product was transformed into competent *E.Coli* (section 4.8.2) for further application and maintenance.

4.8.2 Plasmid transformation

Plasmids or ligation products were transformed into OneShot Top10F Chemically Competent *E.Coli* (Life Technologies/Invitrogen). They were mixed with 50 μ L of competent cells and incubated on ice for 30 minutes. After that, competent cells were challenged with heat shock at 42°C for 1 minute, before returning to ice for 2 minutes. Cells were supplemented with 500 μ L of SOC medium and incubated at 37°C on shaking heat block for 1 hour. Then, 100 μ L of transformed cells were spread on LB agar plate containing 100 μ g/mL ampicillin. The agar plate was incubated overnight at 37°C.

4.8.3 Plasmid extraction

Plasmids were extracted from bacterial culture using QIAGEN Plasmid Mini Kit (Qiagen). 10 mL of bacteria was cultured with LB broth containing 100 μ g/mL ampicillin in 50 mL conical tube on shaking incubator overnight at 37°C. Bacterial culture was pelleted by centrifugation at 3,500 rpm at 4°C for 15 minutes. Supernatant was discarded and bacterial pellet was resuspended in 300 μ L of Buffer P1 (with LyseBlue reagent) before transferred to microcentrifuge tube. The pellet was lysed by adding 300 μ L of Buffer P2 and vigorously inverting the tube until the lysate turned blue. Bacterial lysate was incubated at room temperature for 5 minutes. The reaction was neutralized with 300 μ L of Buffer P3. Vigorous inverting was done until blue solution vanished and solution was incubated on ice for 5 minutes. The lysate was

subjected to centrifugation at 14,000 rpm at 4°C for 10 minutes to pellet bacterial debris. During centrifugation, QIAGEN-tip 20 was equilibrated with 1 mL of Buffer QBT in 15 mL tube. Supernatant from lysate was applied to the tip and allowed to run through. After all supernatant was passed through the tip, the tip was washed twice with 2 mL of Buffer QC. To elute DNA from the tip, the tip was moved to another 15 mL tube and 800 µL of Buffer QF was added into the tip. The solution was mixed with 560 µL absolute ethanol in another microcentrifuge tube, in order to precipitate plasmid DNA. The mixture was incubated overnight at -20°C.

On the following day, precipitated plasmid was pelleted by centrifugation at 14,000 rpm at 4°C for 30 minutes. DNA pellet was washed with 70% ethanol in sdH_2O and left to air dry for 15 minutes. It was dissolved with 50 µL of 10 mM Tris-HCL, pH 8 by letting stand for 15 minutes and pipetting up-down to ensure completely dissolved. The extracted plasmid was confirmed in size by running in 0.8% agarose gel (E gel/Life Technologies/Invitrogen). The plasmid was stored at -20°C until used.

4.8.4 Enzymatic digestion of DNA

PAX6 PCR fragment cloned into pCR4-TOPO was digested with restriction enzymes, *XhoI* and *NotI*, for subcloning into pCAG vector. Digestion reaction was prepared as following mixture;

30 µg of pTOPO-PAX6 plasmid

9 µL of Buffer 3 (New England Biolabs)

6 µL of *XhoI* (New England Biolabs)

6 µL of *NotI* (New England Biolabs)

0.5 µL of BSA

x µL of sdH_2O , which made the total reaction volume to 90 µL

The reaction mixture was incubated at 37°C for 6 hours. After the incubation step, reaction was run in 0.8% agarose gel. DNA band at position corresponding to size of PAX6 PCR product was excised from agarose gel and subjected to QIAquick Gel Extraction Kit (Qiagen). Gel extracted DNA fragment concentration was measured with NanoDrop ND1000 spectrophotometer. After that, it

was verified by size after running in 0.8% agarose E-gel (Life Technologies/Invitrogen). The product that showed only 1 band in verification step was used for further subcloning process.

In addition, after pCAG-PAX6 plasmids were successfully constructed, the plasmids were also subjected to enzymatic digestion with *PvuI* in order to linearized plasmids before transfection. Digestion reaction was prepared as following mixture;

- 10 µg of pCAG plasmids
- 10 µL of Buffer 3 (New England Biolabs)
- 3 µL of *PvuI* (New England Biolabs)
- 1 µL of BSA
- x µL of sdH₂O, which made the total reaction volume to 100 µL

The reaction mixture was incubated at 37°C for 6 hours. In case that more than 10 µg of plasmid wanted to be digested, the reaction volume was scaled up at the ratio of 10 µg plasmid per 100 µL reaction volume (containing 3 µL of restriction enzyme). After the incubation period, digestion reaction was run in 0.8% agarose gel together with the original plasmid in order to ensure complete digestion of the plasmid to only linearized form. Reaction was kept incubated for longer period in case of incomplete digestion. In case of complete digestion, linearized plasmids were precipitated from phenol/chloroform extraction sample in order to use for cell transfection.

4.8.5 Subcloning of pCAG over-expressing plasmid

PAX6 fragment was digested with *XhoI* and *NotI* restriction enzymes (section 4.8.4) for subcloning into pCAG vector. Plasmid vector was also prepared from pCAG-Tomato by digested with similar set of restriction enzymes to take Tomato-encoding region out. Digested vector and PAX6 inserts were ligated together using T4 DNA ligase (??). The ligation reaction was prepared as following mixture;

- 1 µL of *XhoI-NotI* digested pCAG vector
- 7 µL of *XhoI-FLAG-PAX6-NotI* insert
- 1 µL of T4 ligation buffer
- 1 µL of T4 ligase

The reaction was incubated overnight at 16°C on temperature controlled block. On the following day, all of the ligation reaction was used to transform into OneShot Top10F competent cell (Life Technologies/Invitrogen) using similar method in section 4.8.2 with slightly modification that all of the transformed cells were plated onto LB agar plate. Bacterial colonies grew on LB agar plate were screened with conventional PCR and plasmid DNA was sequenced. Cells from positive screening were cultured and made into glycerol stock, stored at -80°C. After plasmid DNA sequence was confirmed, cells from the positive colony were propagated, extracted and linearized for transfection.

4.8.6 Stable transfection with nucleofection

Transfection in NTERA2 was performed with nucleofection system (Amaxa/Lonza). Transfection reagent used with NTERA2 was Amaxa Cell Line Nucleofector Kit L (Amaxa/Lonza), which was prepared into transfection solution immediately before used. On the day of transfection, confluent culture of native NTERA2 was washed with PBS once, trypsinized and harvested. T25 culture flask was filled with DMEM/F12 (Gibco/Invitrogen) supplemented with 10% FBS and put into 37°C CO₂ incubator until used. Cell number harvested from the culture was estimated by counting in hemacytometer. Cell suspension equivalent to cell number needed for transfection was centrifuged to pellet cells, 1x10⁶ cells were used for each transfection. Cell pellet was resuspended in transfection solution, using 100 µL per million cells. 3-5 µg of linearized plasmid was added into cell suspension in transfection solution. The solution was transferred into transfection cuvette, ensured that the solution reached the bottom of cuvette without having bubbles in between. The cuvette was placed in the Nucleofector machine (Amaxa/Lonza). High viability transfection program for NTERA2 cells were selected on the machine. After transfection was finished, 1 mL of DMEM/F12 was added into cuvette and cell suspension was transferred into pre-incubated culture flask. The transfected cells were immediately moved into the CO₂ incubator for recovery.

4.8.7 Over-expressing cell lines establishment and culture

Transfected cells were allowed to recover and attach to culture surface for 1 night. DMEM/F12 supplemented with 10% FBS (culture medium) was changed on the following day. Cells were allowed to grow for 3 days. Puromycin-containing DMEM/F12 (3 $\mu\text{g/mL}$ final concentration) was used as selection medium for the transfected cell line. On the 3rd day after transfection, normal culture medium was replaced by puromycin-containing selection medium in order to select for cells that contained transfected transgene only. Massive cell death was observed during the first week of selection, the selection medium was changed every day as well. After less cell death was observed, selection medium was changed every 5 days. Transfected cells were maintained with selection medium until they grew into colonies. As the transfected cells were maintained with selection medium all the time, the colonies appeared were considered as colonies of stable transfected cell line. When colonies were visible without using microscope, they were dissociated and seeded onto another culture vessel. They were maintained and expanded with selection medium; stock in liquid nitrogen was made after several passages of the transfected cell line. Stable transfected cells were continually maintained with selection medium throughout the experiments.

4.9 Data analysis

Data from every experiment was recorded using Microsoft Excel. Data used for statistical analysis included Ct value for quantitative PCR (both qRT-PCR and ChIP) and percentage of population stained with surface antigens for flow cytometry. Each experiment was repeated 3 times for statistical analysis with Student's t-test. Mean / Relative expression / Fold enrichment with standard deviation of each sample were plotted as the bar graphs.

In qRT-PCR experiments, Ct values from genes of interest were compared with Ct value of ACTB from the respective sample. Difference between Ct of gene of interest and Ct of ACTB (ΔCt) was used to calculate relative expression of gene of interest compared with the appropriate control. Relative expression of gene of interest is calculated from difference between ΔCt of the sample and that of the control

($\Delta\Delta Ct$). Assuming that cDNA synthesis by PCR occurred from every double stranded DNA template, relative expression is determined from the following equation;

$$\text{Relative expression} = 2^{(\Delta\Delta Ct)}$$

In ChIP experiment, Ct values from 1% input samples were adjusted with dilution factor to estimate Ct values of the genomic DNA input signal. After that, difference between Ct value of immunoprecipitation reaction and respective input signal was determined as ΔCt , which is further used to calculate percentage of genomic DNA precipitated with each antibody (%input) from the following equation;

$$\%input = 100 \times 2^{(\Delta Ct)}$$

Finally, enrichment of precipitated genomic DNA by each antibody was calculated by dividing %input of the interested antibody by that of negative control (IgG).

$$\text{Fold enrichment} = (\%input \text{ of IgG}) / (\%input \text{ of interested antibody})$$

CHAPTER V

RESULTS

5.1 NTERA2 differentiates in response to retinoic acid

NTERA2 is a pluripotent human EC cells line, which possesses an ability to differentiate in response to retinoic acid (Andrews *et al.*, 1984; Andrews, 1984). In order to confirm differentiation of NTERA2 induced by retinoic acid, qRT-PCR analysis was performed. RNA samples were collected from cells treated with 10 μ M retinoic acid at day 3, 7, 14, and 21 following the treatment. SYBRGreen-based qPCR was used to determine mRNA expression and the data was shown as relative expression compared to undifferentiated cells without retinoic acid treatment.

Expression level of stem cell-related genes, *OCT4* and *NANOG*, was significantly reduced as early as 3 days of treatment. Expression level of *SOX2*, another stem cell-related gene, was relatively maintained until day 7, at which point the level started to reduce. By day 14 and 21, expression level *OCT4* and *NANOG* was remarkably down-regulated as compared to undifferentiated state, whereas that of *SOX2* was approximately 10-fold decreased (Figure 5.1).

Next, expression level of germ layers-associated and an extraembryonic trophectodermal gene were evaluated (Figure 5.2). *PAX6*, *T*, *GATA6*, and *CDX2* were used to represent neuroectodermal, mesodermal, endodermal, and trophectodermal lineages respectively. NTERA2 showed comparable amount of *T* mRNA level throughout the experiment. An up-regulation of gene expression was observed for *PAX6*, *GATA6*, and *CDX2*, albeit with different patterns. An increased level of *PAX6* was shown to start since 3 days of treatment, reached highest level by 7 days, and maintained at high level on 14 and 21 days. As for *GATA6* up-regulation, the mRNA level gradually increased from 3 days and reach highest level on 21 days of treatment. *CDX2* up-regulation was shown to be only in a brief period that started 3 days and reduced by 7 days after retinoic acid treatment.

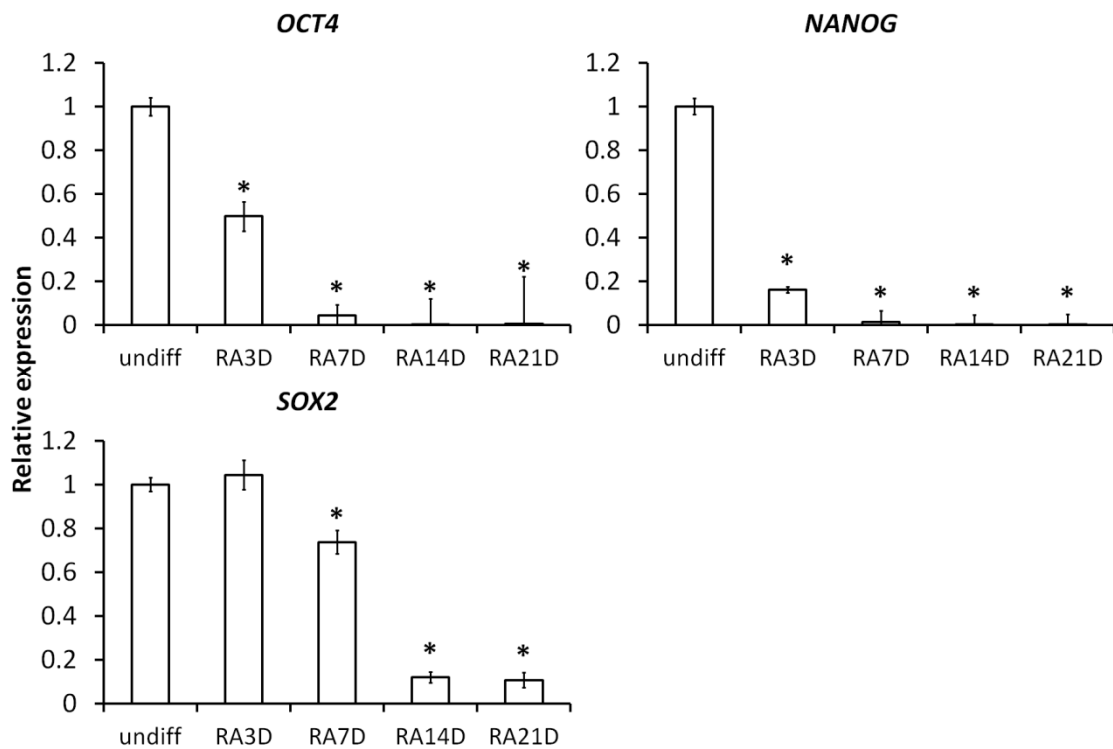


Figure 5.1: Gene expression profile of stem cell-related genes; *OCT4*, *NANOG*, and *SOX2*; in NTERA2 cells from undifferentiated culture or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as relative mRNA level compared to undifferentiated cells ($\pm$ SEM). Rapid reduction of *OCT4* and *NANOG* mRNA level can be seen as soon as 3 days after retinoic acid treatment, while *SOX2* mRNA level started to reduce by 7 days. (n=3, *p<0.05)

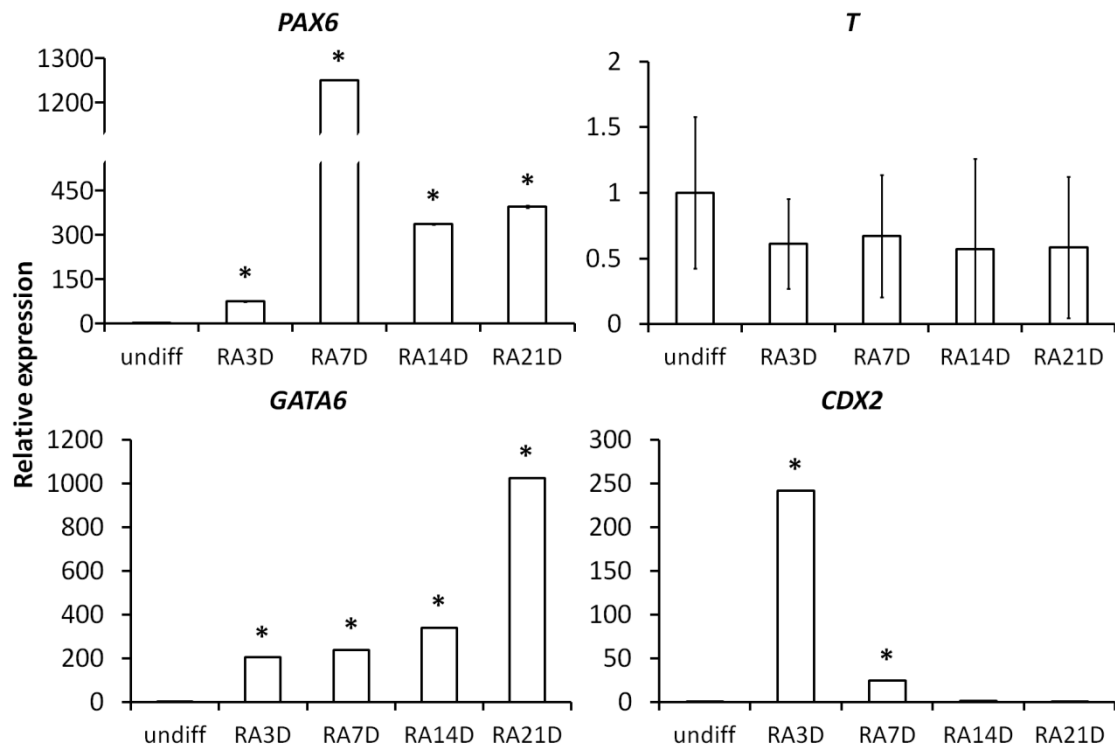


Figure 5.2: Gene expression profile of differentiation associated transcription factors; *PAX6*, *T*, *GATA6*, and *CDX2*; in NTERA2 cells from undifferentiated culture or following 10 μ M retinoic acid treatments for 3,7, 14 and 21 days. Results were shown as relative mRNA level compared to undifferentiated cells ($\pm$ SEM; n=3, *p<0.05).

Neural stem cells can give rise to 2 different lineages, which are neuronal and glial lineages. To test whether gene specific to both fates are induced during retinoic acid-induced differentiation, qRT-PCR was performed on 2 sets of genes, which define the two lineages (Figure 5.3-5.4). Expression of bHLH transcription factor *NEUROD1* was found to express highly at 7 days after treatment, but quickly disappeared afterwards. Expression level of neuronal marker at different states also suggested differentiation dynamic of NTERA2 into neuronal lineage. *NESTIN* mRNA level showed peak expression at 3 days after treatment, and down-regulated later. While mRNA level of *DCX* was highest at 7 days and gradually decreased as approaching day 21, level of *MAP2* was found to express at high level started 7 days and maintained until 21 days after treatment.

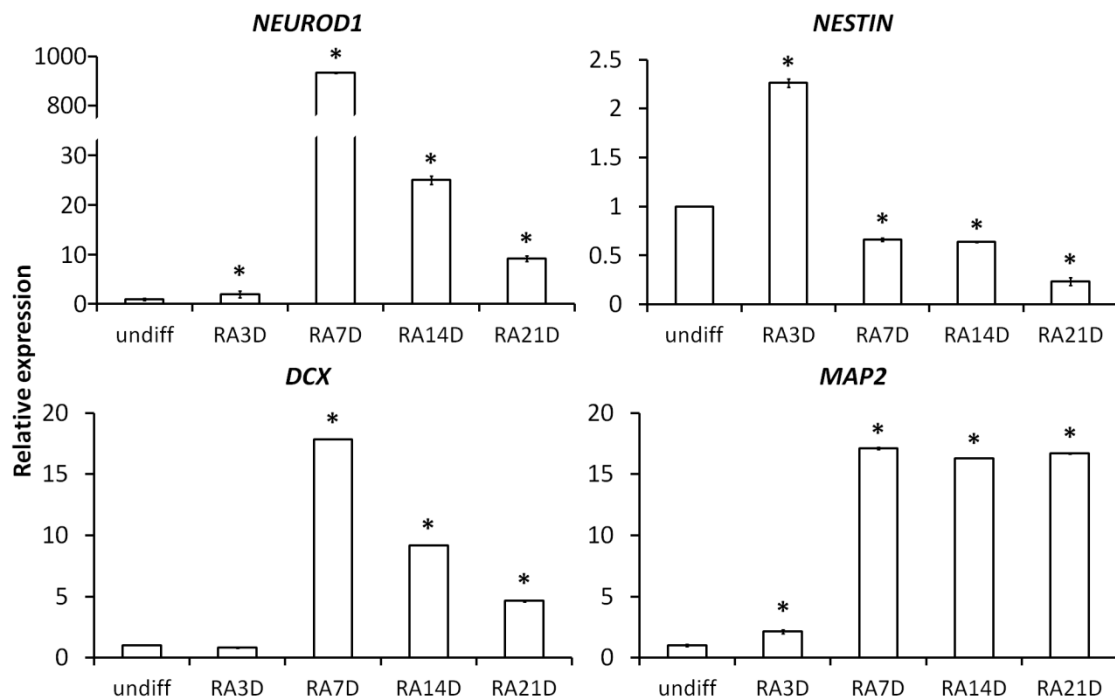


Figure 5.3: Gene expression profile of neuronal-related genes; *NEUROD1*, *NESTIN*, *DCX*, and *MAP2*; in NTERA2 cells from undifferentiated culture or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as relative mRNA level compared to undifferentiated cells ($\pm$ SEM; $n=3$, $*p<0.05$).

Finally, *S100 β* and *GLAST* were used as astroglial markers, while *MBP* was used to represent oligodendrocyte. Expression of both astrocytic markers mRNA were shown to gradually increase following RA treatment and reached highest level on day 21. Notably, even though the pattern of expression of *S100 β* and *GLAST* were similar, the relative level of mRNA changes compared to undifferentiated cell was more notable in *S100 β* than that of *GLAST*. On the other hand, oligodendrocyte lineage seems to be rarely differentiated by RA treatment in NTERA2 as expression of *MBP* showed only slightly changes, 2 fold increments only on day 21.

Altogether, these results confirm that the human pluripotent EC cell line, NTERA2, differentiates in response to retinoic acid and that both neuronal and glial lineages can be produced by this differentiation inducing agent.

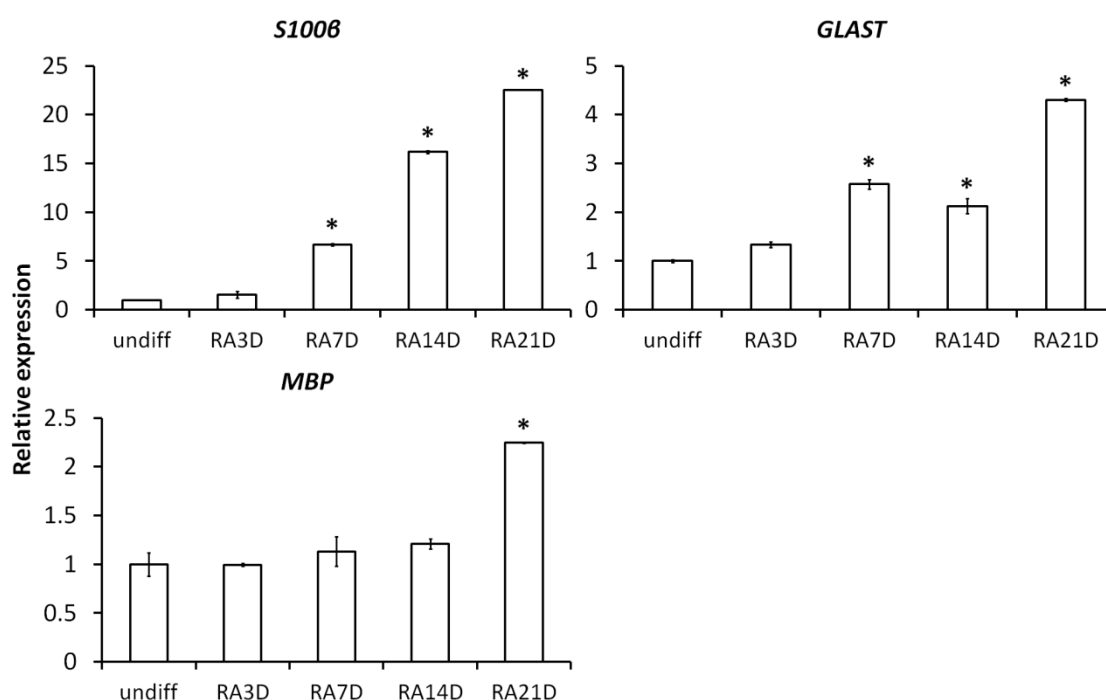


Figure 5.4: Gene expression profile of glial cell markers; *S100 β* , *GLAST*, and *MBP*; in NTERA2 cells from undifferentiated culture or following 10 μ M retinoic acid treatments for 3,7, 14 and 21 days. Results were shown as relative mRNA level compared to undifferentiated cells ($\pm$ SEM; n=3, *p<0.05).

5.2 Characterization of PAX6 over-expressing NTERA2 cell lines

Expression of PAX6, which is a master regulator of neuroectodermal differentiation in human ES cells (Zhang *et al.*, 2010), was found to be highly up-regulated upon differentiation induced by retinoic acid in NTERA2 cells (Figure 5.2). There are 2 isoforms of PAX6, namely PAX6A and PAX6B. While the former isoform has been reported to induce spontaneous neural differentiation from human embryonic stem cell, the latter isoform failed to do so (Zhang *et al.*, 2010). To evaluate the effect of PAX6A and PAX6B in neural differentiation of pluripotent stem cells NTERA2, both isoforms of PAX6 with FLAG tag version were over-expressed under CAG promoter. A stable cell line transfected with CAG parental plasmid was used as a control throughout the experiment, referred as NT2 CAG-Tomato.

5.2.1 Establishment of PAX6 over-expressing NTERA2 cell lines

Linearized over-expression plasmid was transfected into NTERA2 to establish stable cell lines. (Materials and Methods) Figure 5.5 shows the representative micrograph of PAX6A and PAX6B cell lines. Culture medium containing puromycin at 3 µg/mL final concentration was used as selection medium, starting on day 3 after transfection. During the first week of selection, most of the cells died as a result from toxicity of puromycin, leaving only those containing transgene survived. By 1-2 weeks after selection, survived cells proliferated and formed into colony, which continued to expand. After colonies can be clearly visible without seeing under microscope, cells in colonies were dissociated with trypsinization and replated into another culture vessel for further experiments.

To ascertain that the established cell lines expressed the ectopic PAX6, western blot analysis was done using antibody against PAX6 and FLAG (Figure 5.6). Expression level of PAX6 from both cell lines was higher than that of control transfected cell line. In addition, expression of FLAG-contain protein confirmed that the increased PAX6 expression was from the introduced transgene.

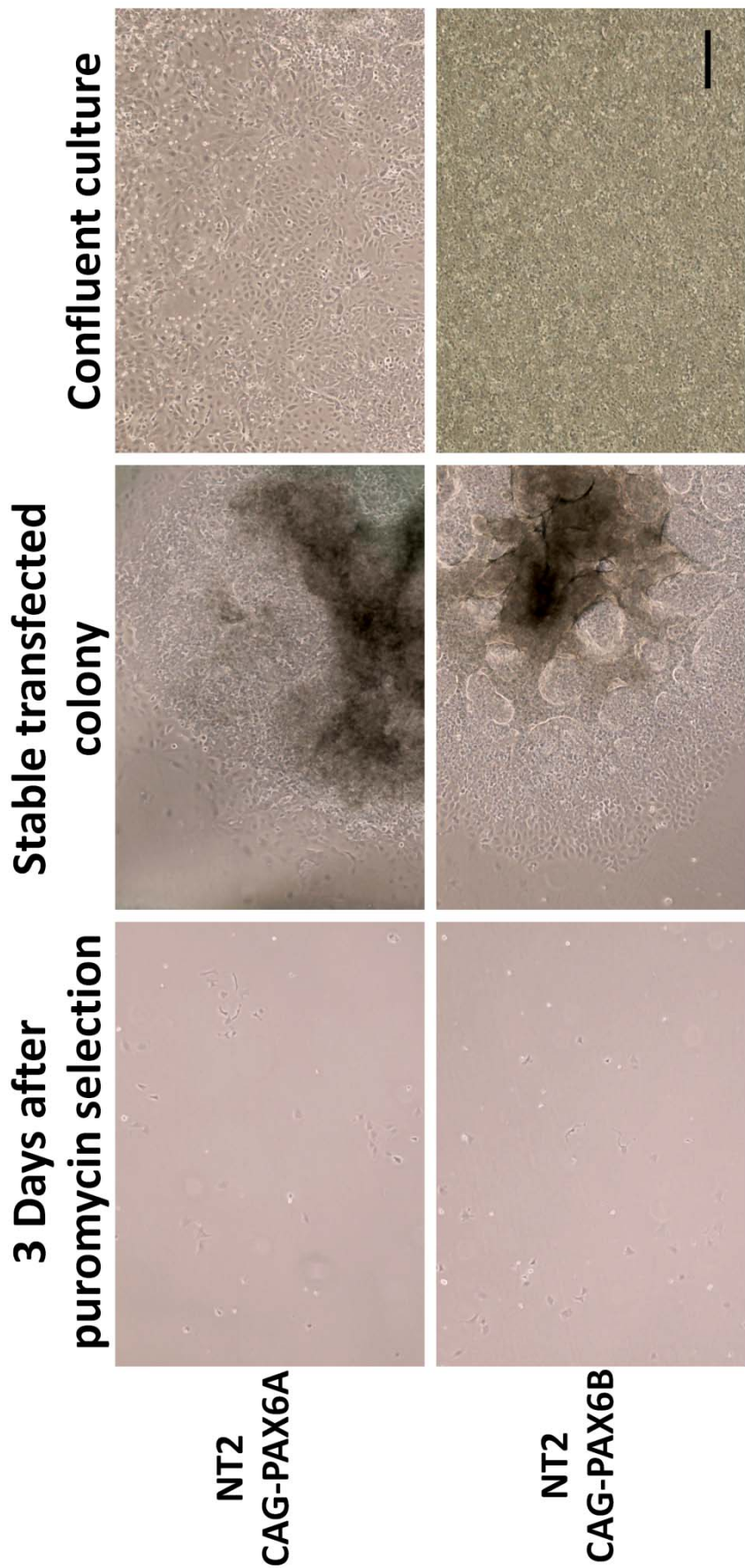


Figure 5.5: Phase contrast images of transfected cells were captured at 4X magnification. The upper row shows images of NT2 cells transfected with PAX6A over-expression plasmid, while the lower one shows that of PAX6B over-expression plasmid. The left column shows cells after puromycin selection for 3 days, the picture was taken after replacing culture medium in order to wash off dead cells. The middle column shows colony of stable transfected cells after 2 weeks of puromycin selection, a big clump of cells can be observed. The right column is the images of confluent culture after dissociation and replating of cells from colony. Notably, rosette formation can be seen in confluent culture of PAX6A over-expressing NT2 cell line. (Scale bar = 100 μ m)

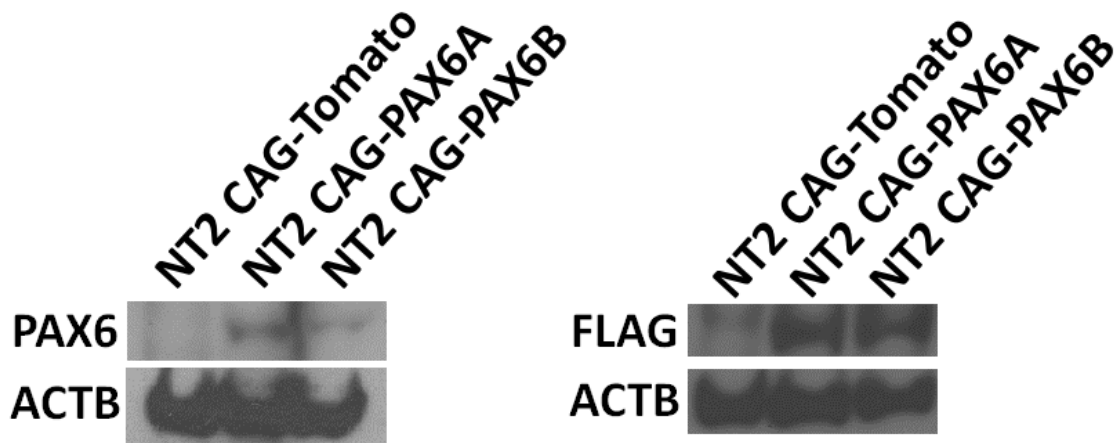


Figure 5.6: Western blot analysis showed increased expression of PAX6, both isoforms, in transfected cell lines compared to control transfection cell line. FLAG-containing proteins also visualized on a separate blot to ensure that the increased expression of PAX6 was from exogenous one produced by transfected transgene.

5.2.2 Stem cell property of PAX6 over-expressing cell lines

After stable PAX6 over-expressing cell lines were established and maintained in culture, stem cell characteristics were assessed using flow cytometric analysis of stem cell-related surface markers and mRNA expression level of stem cell-related genes.

PAX6A over-expression reduced number of stem cell-related surface markers-positive population compared to control transfected cells and PAX6B over-expressing cells (Figure 5.7a). SSEA3, SSEA4, and TRA-1-60 had been used together to determine numbers of human pluripotent stem cell population. Flow cytometric analysis shows that population of NT2 CAG-Tomato and NT2 CAG-PAX6B possess higher level of SSEA3-, SSEA4-, and TRA-1-60 positive cell that those of NT2 CAG-PAX6A. This result indicates that the ectopic expression of PAX6A leads to a reduction in stem cell property of NTERA2.

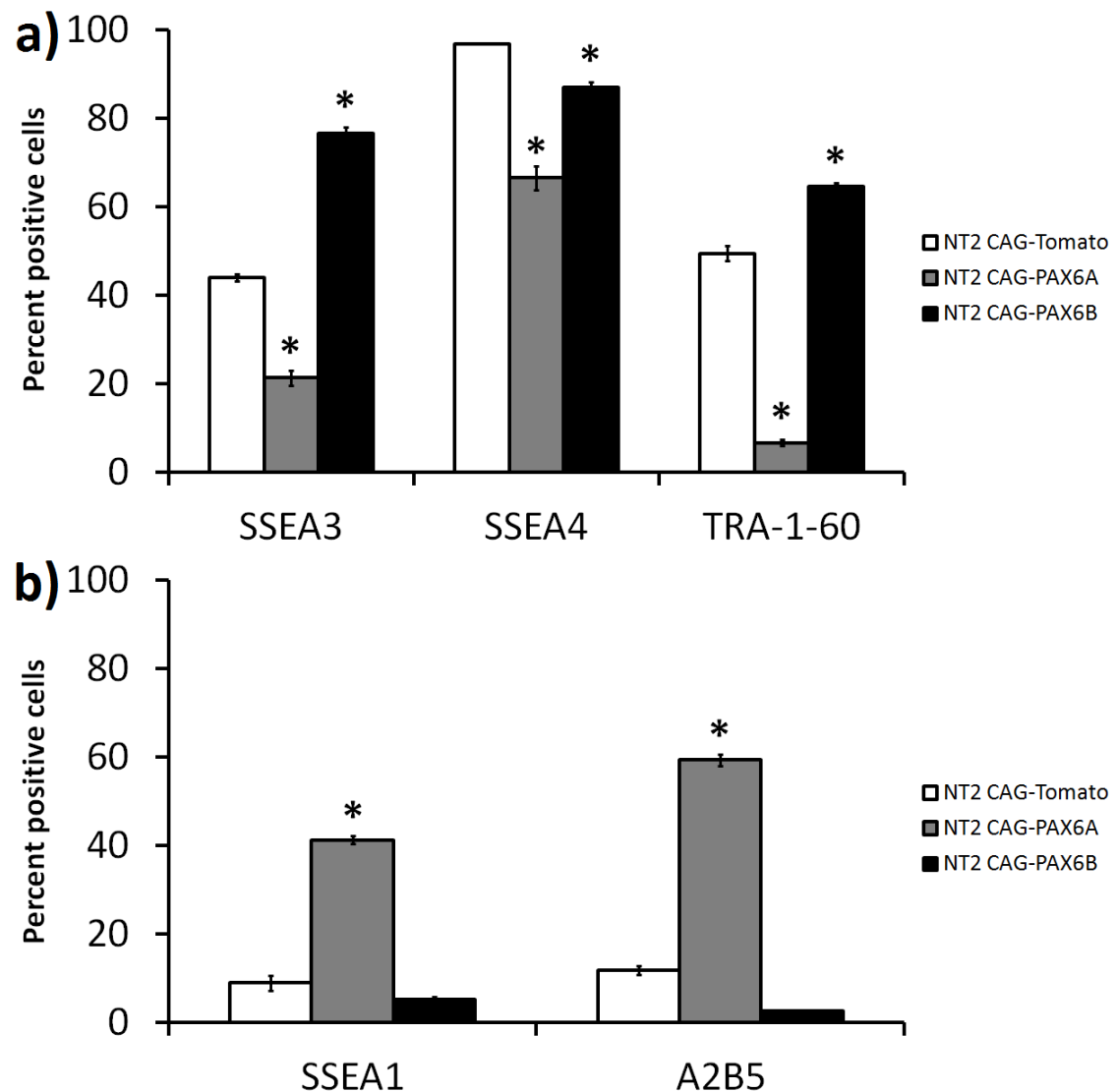


Figure 5.7: Flow cytometric analysis of surface markers from PAX6 over-expressing cells compared with control transfected cells. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n=3$, $*p<0.05$). **a)** population positive with stem cell-related surface markers; SSEA3, SSEA4, and TRA-1-60 **b)** population positive with differentiation related surface markers; SSEA1 and A2B5. PAX6A decreased population with pluripotent markers and, at the same time, increased population with differentiation markers.

On the other hand, PAX6A over-expression increased population that positive with differentiation related surface markers (Figure 5.7b). Even though SSEA1 had been used as pluripotent marker in mouse stem cells, human pluripotent stem cells lacked SSEA1 expression. SSEA1 can be detected when pluripotent stem cells differentiated into extraembryonic trophectodermal lineage. A2B5 was also used to detect differentiation of human pluripotent stem cells, mainly neural differentiation from pluripotent stem cells accompanied by increased in number of A2B5-positive population. In this experiment, number of SSEA1- and A2B5-positive population of PAX6A over-expressing NTERA2 cell line were higher than those of control and PAX6B over-expressing cells. This result indicates that ectopic PAX6A expression promotes cell differentiation of NTERA2.

Expression at mRNA level also changed as a result of over-expression of PAX6, both isoforms. Quantitative RT-PCR analysis showed that although only a marginal decreased of *OCT4* expression level was observed for PAX6B over-expression, PAX6A over-expression led to a 2-fold decrease in *OCT4* expression compared with control. However, expression level of NANOG was not dramatically reduced in both cell lines. While *OCT4* and *NANOG* expression level were decreased, that of *SOX2* was comparable with control (data not shown). Endogenous expression of *PAX6* mRNA was induced by PAX6A over-expression, but not by PAX6B. Furthermore, up-regulation of several genes related to neural lineage can be seen as well, including *NESTIN*, *MAP2*, and *SI00 β* (Figure 5.8). Together with the result from flow cytometry, these results suggest that PAX6 isoform a, but not isoform B, might induce spontaneous neural differentiation of NTERA2 cells.

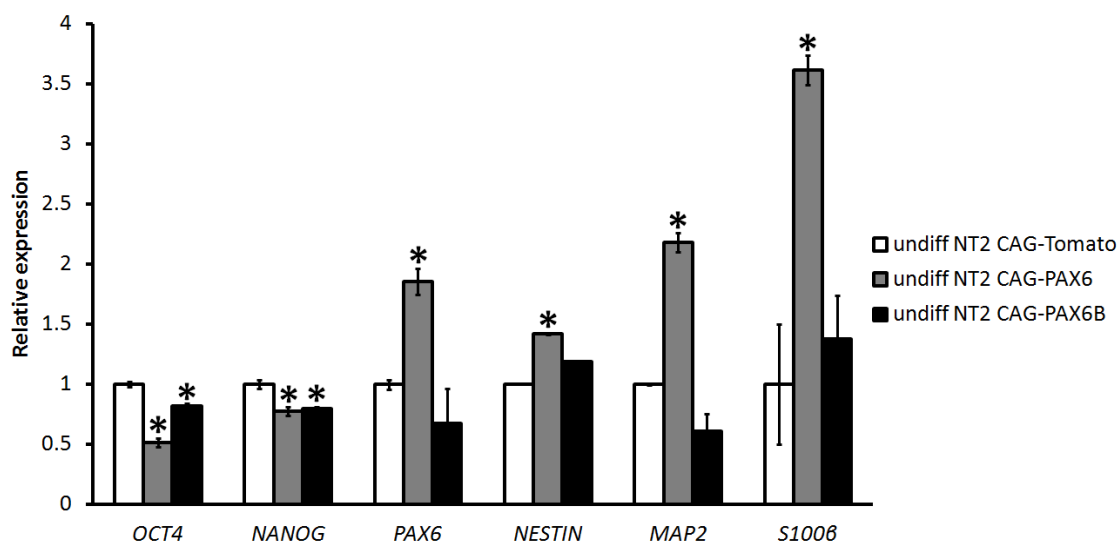


Figure 5.8: mRNA expression in undifferentiated PAX6, both isoforms, over-expressing cells compared to the of control transfected cells. Results were shown as relative expression level to that of undifferentiated NT2 CAG-Tomato ($\pm$ SEM; $n=3$, $*p<0.05$).

5.2.3 Differentiation of PAX6 over-expressing NTERA2 cell lines

Next, cell differentiation in response to retinoic acid treatment was tested in over-expressing cell lines of both PAX6 isoforms. Flow cytometric analysis and mRNA expression were evaluated during the course of differentiation. Finally, immunofluorescent staining with β III-tubulin antibody was done to assess the extent of neural differentiation after RA treatment for 30 days.

Figure 5.9 shows changes of stem cell-related surface markers; SSEA3, SSEA4, and TRA-1-60, expression during retinoic acid treatment in NT2 CAG-Tomato, NT2 CAG-PAX6A, and NT2 CAG-PAX6B. During differentiation, population expressed these markers was reduced in all 3 cell lines. In NTERA2 CAG-PAX6A, total loss of SSEA3 and TRA-1-60 population was shown on day 7 and 14 after treatment respectively. In contrast, both PAX6 over-expressing cell lines were able to maintain number of SSEA4 positive population on day 3 after treatment.

Expression of stem cell-related genes, including *OCT4*, *NANOG*, and *SOX2*, was also consistent with the expression of surface markers. Loss of expression level of these genes is shown in figure 5.10. Almost none of *OCT4* and *NANOG* transcript can be detected on day 14 of differentiation. On the other hand, even though

mRNA level of *SOX2* was decreased, pattern of mRNA expressions were different among 3 cell lines. Loss of *SOX2* transcript was greatest in NT2 CAG-PAX6A. In NT2 CAG-PAX6B, slight increased level of *SOX2* mRNA on day 3 of treatment, before reduced on day 7 can be observed. These results suggest different influence of PAX6 isoforms on *SOX2* mRNA expression.

To ascertain cell differentiation from PAX6 over-expressing cell lines, flow cytometric analysis of differentiation related surface markers was performed. Figure 5.11 shows changes in population expressing SSEA1 and A2B5 during retinoic acid-induced differentiation. SSEA1-positive population was gradually reduced in NT2 CAG-PAX6A, while that in NT2 CAG-Tomato and NT2 CAG-PAX6B were gradually increased before rapidly disappeared on day 14. On the other hand, A2B5-positive population was increased during the first 7 to 14 days of treatment before disappeared. The highest population of A2B5-positive cells from NT2 CAG-Tomato was found at day 14 after differentiation, while those of PAX6 over-expressing cell lines were found as early as 7 days. However, at the end of the experiment, the A2B5-positive population differentiated from NT2 CAG-Tomato was higher than those of NT2 CAG-PAX6A and NT2 CAG-PAX6B.

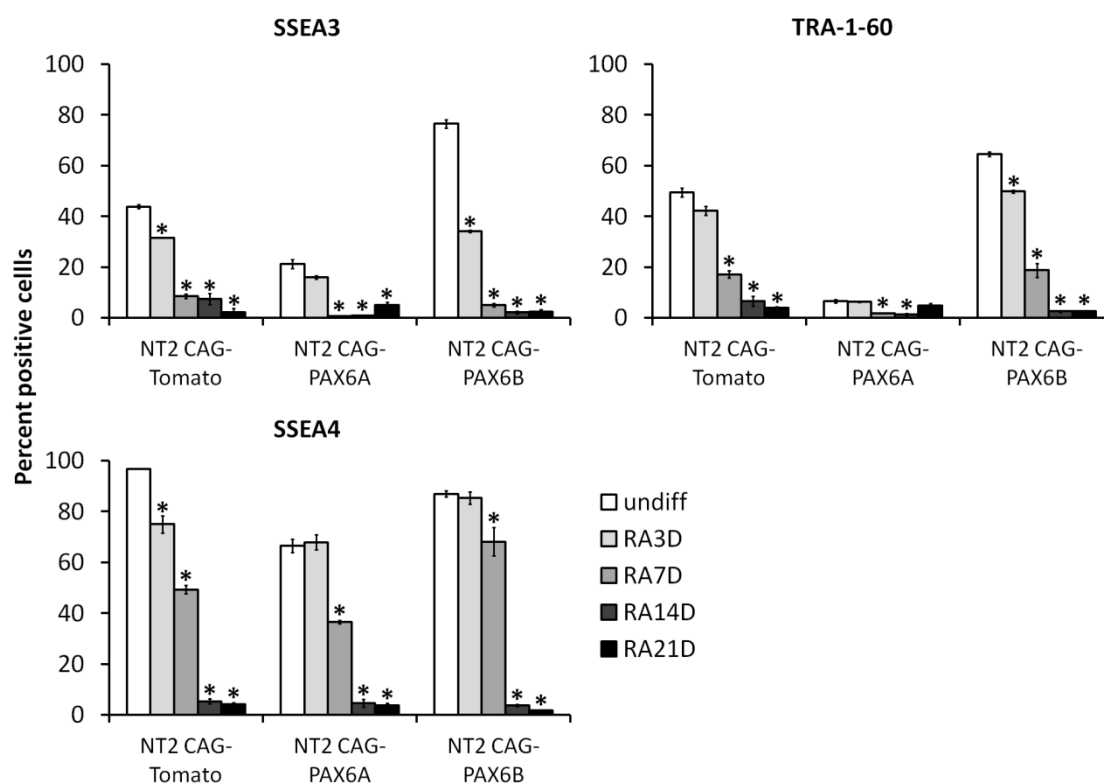


Figure 5.9: Flow cytometric analysis of stem cell-related surface markers from PAX6 over-expressing cell lines, compared with control transfected cells, at undifferentiated state and during differentiation with 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. The surface markers analyzed included SSEA3, SSEA4 and TRA-1-60. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n=3$, $*p<0.05$ compared with undifferentiated sample of its own cell line).

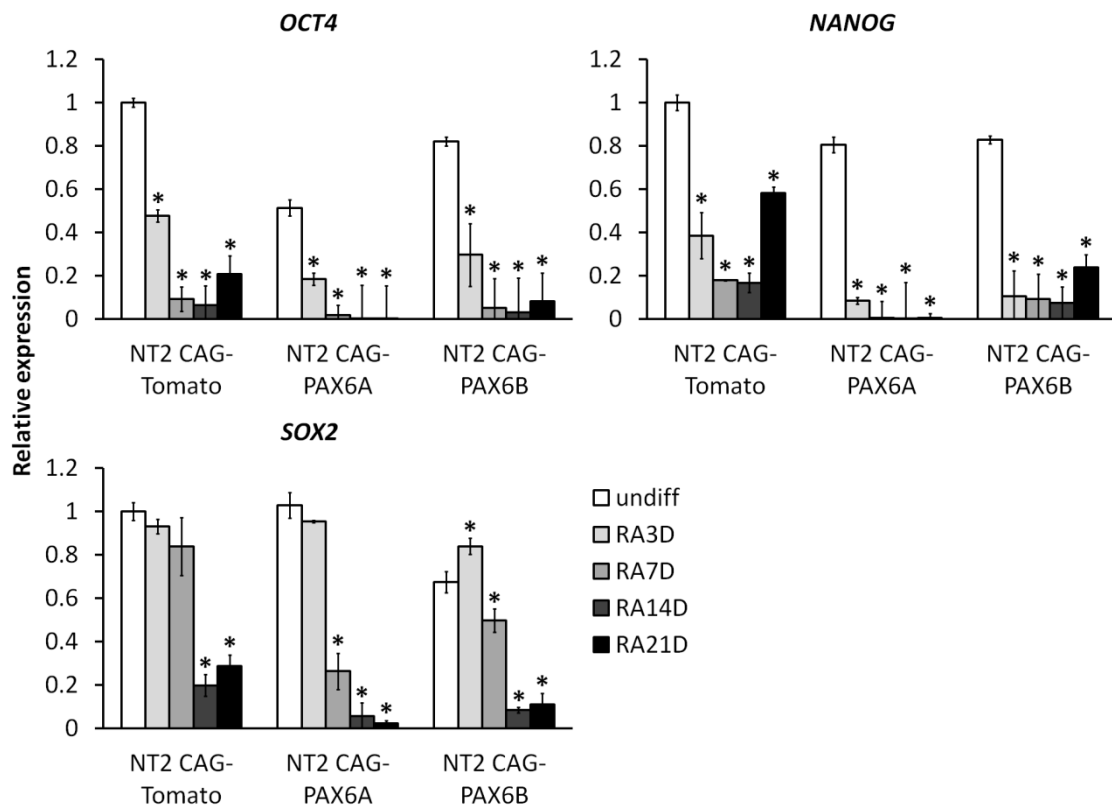


Figure 5.10: Gene expression profile of stem cell-related genes; *OCT4*, *NANOG*, and *SOX2*; in PAX6 over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n=3, *p<0.05 compared with undifferentiated sample of its own cell line).

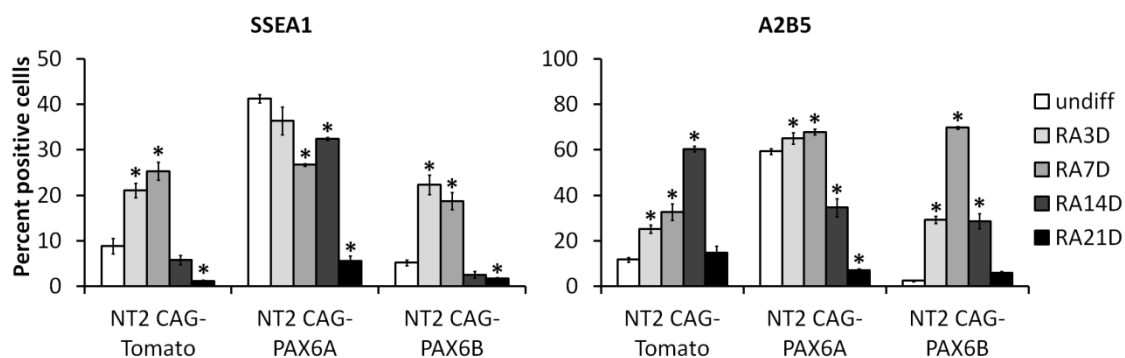


Figure 5.11: Flow cytometric analysis of differentiation related surface markers from PAX6 over-expressing cell lines, compared with control transfected cells, at undifferentiated state and during differentiation with 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. The surface markers analyzed included SSEA1 and A2B5. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n=3$, $*p<0.05$ compared with undifferentiated sample of its own cell line).

Figure 5.12 shows expression level of differentiation-related transcription factors, including *PAX6*, *T*, *GATA6*, and *CDX2*, during the period of RA induction. In order to avoid detection of PAX6 transgene introduced to the cell lines, primers used for *PAX6* mRNA amplification were designed to recognize at 3' UTR of the RNA. Generally, pattern of gene expression of these 4 genes were similar among 3 over-expressing cell lines, especially that of *PAX6* mRNA. However, mRNA transcript of *T*, *GATA6*, and *CDX2* was lower in NT2 CAG-PAX6A compared with NT2 CAG-Tomato and NT2 CAG-PAX6B. This result suggested that PAX6A over-expressing cells were refrained from undergoing differentiation into lineages other than neural lineage. Lastly, PAX6B over-expressing cells showed highest level of *GATA6* transcript at every time point, suggesting the involvement of this isoform of PAX6 in endodermal lineage differentiation.

PAX6A over-expression also favored neuronal lineage differentiation induced by retinoic acid. Even though expression level of bHLH transcription factor *NEUROD1* was lower in both PAX6A and PAX6B over-expressing cell lines,

expression level of neuronal related markers were significantly higher in NT2 CAG-PAX6A (Figure 5.13). *NESTIN* expression in NT2 CAG-PAX6A, unlike the other cell lines, cannot be induced by retinoic acid, but the transcripts were gradually reduced towards the lowest level on day 21. Both PAX6A and PAX6B over-expression resulted in constant level of *DCX* mRNA on day 14 and 21 after treatment, while that of NT2 CAG-Tomato was decreased. Lastly, expression level of *MAP2* induced in NT2 CAG-PAX6A was highest among the cell lines at every time point, except on day 21 when the level was comparable with that of control.

These results indicate that PAX6A over-expression do not only reduced stem cell population in the culture, but also facilitated neural differentiation process in NTERA2 cells after treatment with retinoic acid. Even though PAX6B over-expression does not affect stem cell state, over-expression still increase neural differentiation from NTERA2.

Finally, the extent of neuronal differentiation from these cell lines was assessed by immunocytochemical staining of neuronal specific marker. Cells were treated with retinoic acid for 30 days prior to the staining. Figure 5.14 shows percentage of β III-tubulin positive cells in the culture on the top panel and representative images of each cell line on the bottom panel. Even though overall β III-tubulin positive cells differentiated from NT2 CAG-PAX6A were lower, positive cells with neuronal morphology were higher than other two cell lines. β III-tubulin positive cells differentiated from NT2 CAG-PAX6B were slightly more abundant than control, however, number of cells with neuritis was comparable.

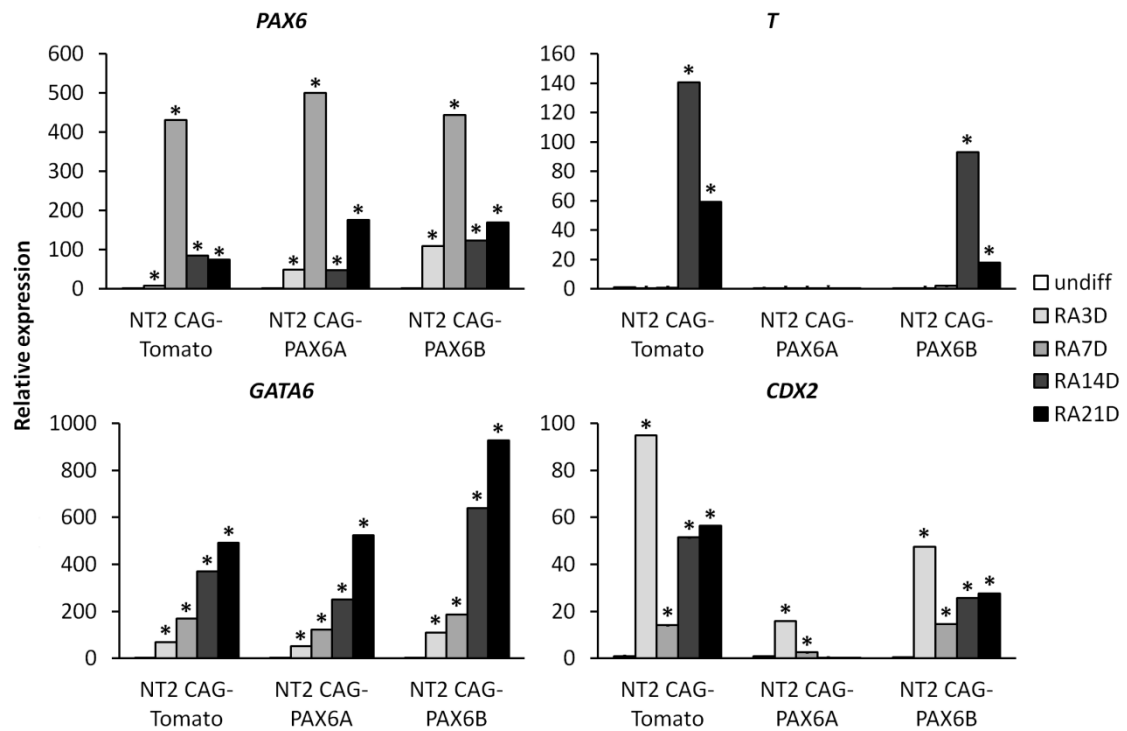


Figure 5.12: Gene expression profile of differentiation associated transcription factors; *PAX6*, *T*, *GATA6*, and *CDX2*; in *PAX6* over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n=3, *p<0.05 compared with undifferentiated sample of its own cell line).

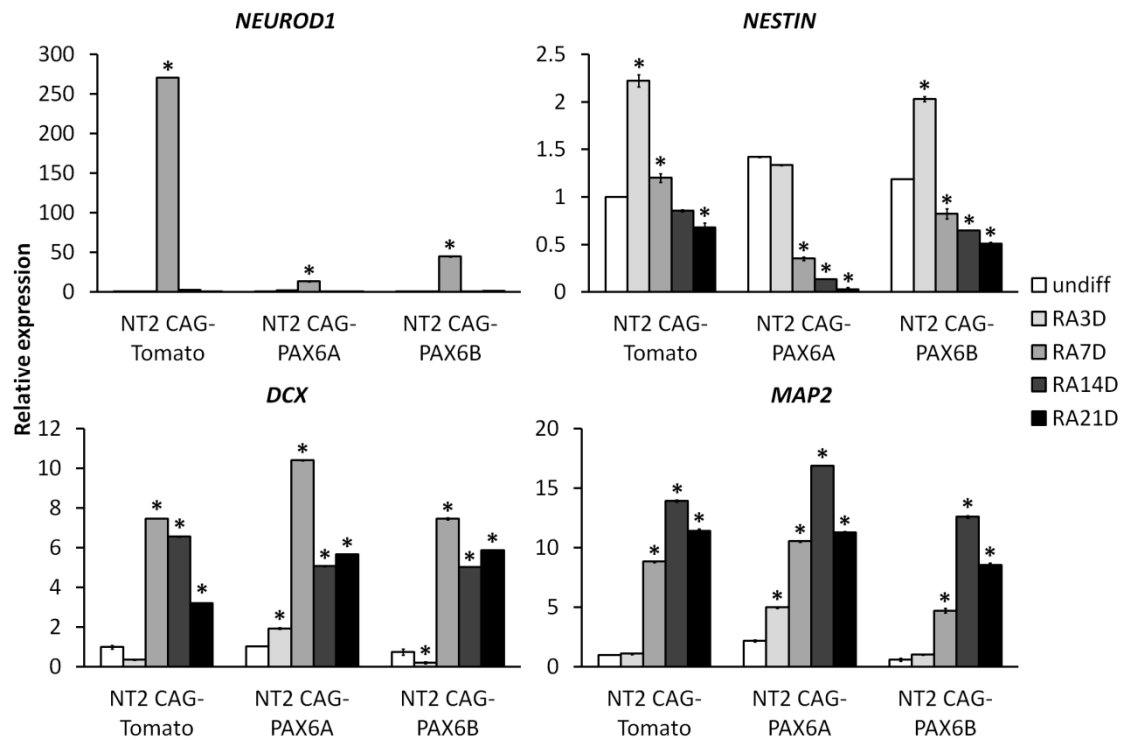


Figure 5.13: Gene expression profile of neuronal-related genes; *NEUROD1*, *NESTIN*, *DCX*, and *MAP2*; in PAX6 over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n=3, *p<0.05 compared with undifferentiated sample of its own cell line).

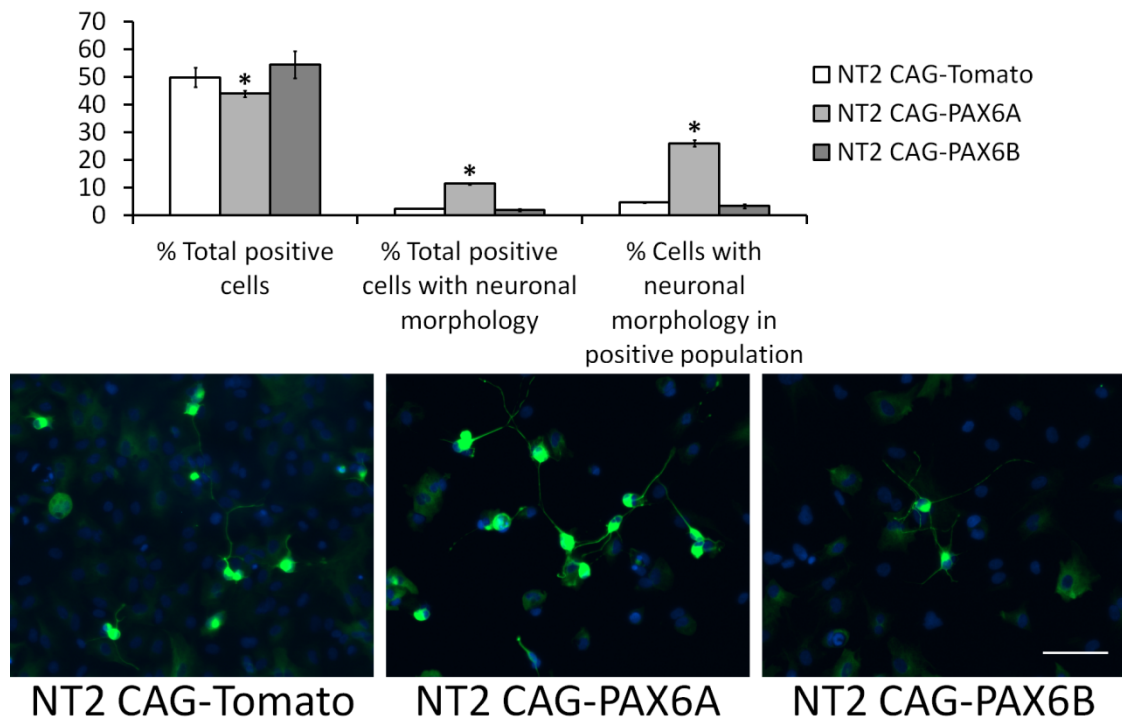


Figure 5.14: Fluorescent images taken from cells differentiated from PAX6 over-expressing cell lines comparing with control. Cells were differentiated with 10 μ M retinoic acid treatments for 30 days. On the last day of treatment, cells were dissociated and replated on 24-well plate for 24 hours. They were stained with β III-tubulin (green) and DAPI (blue). Cell numbers were count manually and present as percentage of immunoreactive cells ($\pm$ SEM; n=3, *p<0.05 compared to control). Scale bar = 100 μ m.

5.3 Establishment of PAX6 over-expressing N2102Ep cell lines

Next, we asked whether PAX6 over-expression in N2102Ep, a nullipotent counterpart of NTERA2, could result in neural differentiation as shown in NTERA2. As mentioned earlier, nullipotent N2102Ep cells cannot be induced to differentiate even though their mRNA and surface marker expression were similar to those of NTERA2. Since PAX6 over-expression in NTERA2 changed status of cell differentiation as well as mRNA expression, PAX6 over-expression in N2102Ep would also result in alteration at mRNA transcription that might unlock the inability to differentiate after treated with RA.

To address the hypothesis, PAX6 over-expressing N2102Ep cell lines were constructed with the similar method with that of NTERA2. However, massive cell death was observed in both N2102Ep CAG-PAX6A and N2102Ep CAG-PAX6B after puromycin selection. However, control transfected cells with the parental plasmid were able to maintain and formed into colony by 1-2 weeks after selection (Figure 5.15). By 14 days after selection, almost all of PAX6 over-expressed N2102Ep underwent cell death and could be rarely seen under the microscope.

To ensure that PAX6 could be over-expressed in N2102Ep cells, western blot analysis using protein samples from transient transfection was performed (Figure 5.16). Cells were let to grow in growth medium for 5 days after transfection without challenging with antibiotic before harvesting for protein samples. Western blot analysis confirmed that both PAX6 isoforms can be ectopic expressed in N2102Ep, but the long-term over-expression was toxic to the cells.

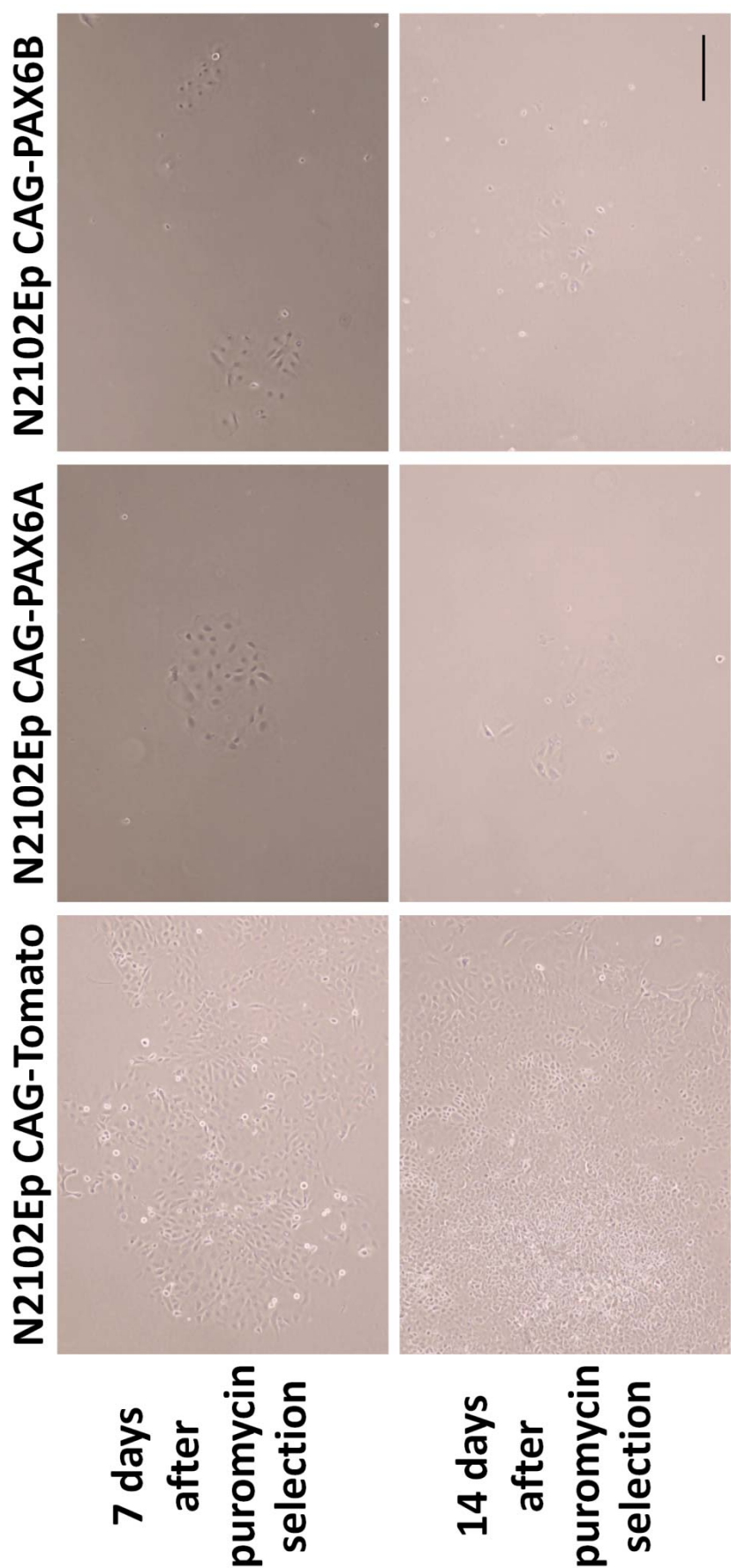


Figure 5.15: Phase contrast images of transfected cells were captured at 4X magnification. The left column showed images of N2102Ep cells transfected with control empty plasmid, the middle and right column showed cells transfected with PAX6A and PAX6B over-expressing plasmids respectively. The upper row images were taken at 7 days after selection with puromycin-containing culture medium, while the lower row was taken on day 14. Images were taken after replacing culture medium in order to wash off dead cells as much as possible. N2102Ep transfected with control plasmid formed into colony as soon as 7 days after puromycin selection, while those transfected with both isoforms of PAX6 failed to grow and continually underwent cell death. (Scale bar = 100 μ m)

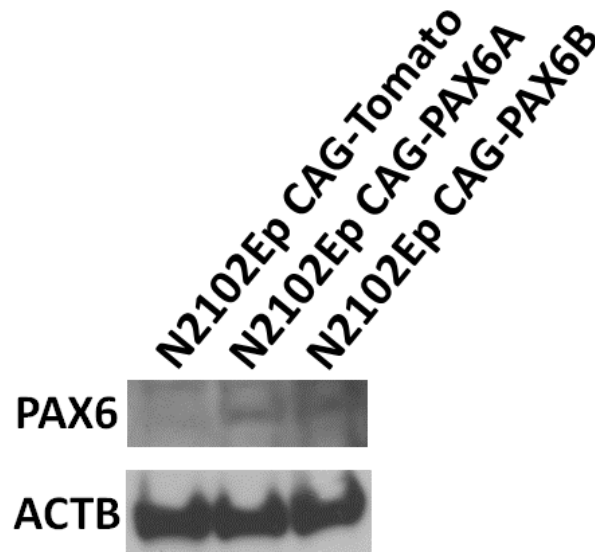


Figure 5.16: Western blot analysis showed increased expression of PAX6, both isoforms, in N2102Ep transfected cell lines compared to control transfection.

5.4 Effect of Lysine-specific demethylase 1A (LSD1) inhibition on differentiation of N2102Ep

The failure to establish PAX6 over-expressing N2102Ep cells led to the hypothesis that what prevents N2102Ep from differentiate upon retinoic acid induction would be at epigenetic level, rather than at the mRNA expression level. Previous experiment had shown that neither inhibition of Histone De-acetylase (HDAC) nor DNA Methyl-transferase 3B (DNMT3B) were able to induce differentiation of N2102Ep with retinoic acid. In fact, these treatments resulted in cell death. (Dr.Patompon Wongtrakoongate, unpublished data) To further test this hypothesis, N2102Ep cells were treated with Lysine-specific demethylase 1A (LSD1) inhibitor with or without combination with retinoic acid in order to determine the effect on cell differentiation. The inhibitors used in this experiment included tranylcypromine (TCP) and pargyline (Prg). FACS analysis of surface markers and mRNA expression were performed after treatment for 7 and 14 days.

Flow cytometric analysis of stem cell-related surface markers showed only slight differences in population number of combination treatment at day 14, compared to untreated control (Figure 5.17). At 14 days of treatment, percent population of all 3

markers; SSEA3, SSEA4 and TRA-1-60, in every group were reduced from native N2102Ep population, but showed no difference between groups. Population at day 14 was also comparable to that of day 7, except combination treatment of both LSD1 inhibitors, which was reduced from 7 days.

Expression of stem cell-related genes; *OCT4*, *NANOG* and *SOX2*, was not different between the 2 time point (Figure 5.18). Even though TCP-treated group showed reduction of stem cell-related genes expression, the level was not different between TCP alone and combination with retinoic acid groups.

Even though TCP treatments or combination of Prg and retinoic acid resulted in increased of SSEA1-positive population on day 7, the population quickly disappeared on day 14. On the other hand, A2B5-positive population cannot be induced by any LSD1 inhibitor treatment (Figure 5.19). On day 7, A2B5-positive cells were found at very low percentage (not more than 2%). The highest percentage of A2B5-positive population was found by retinoic acid treatment alone for 14 days (6.3%). Pargyline treatment alone induced about 4% of A2B5-positive population, while TCP failed to induce more A2B5-positive population.

Combination treatment of LSD1 inhibitor and retinoic acid induced mRNA expression of transcription factor specific for endodermal, *GATA6*, and trophectodermal lineage, *CDX2*. However, the mRNA induction cannot be seen for *PAX6* and *T* expression (Figure 5.20).

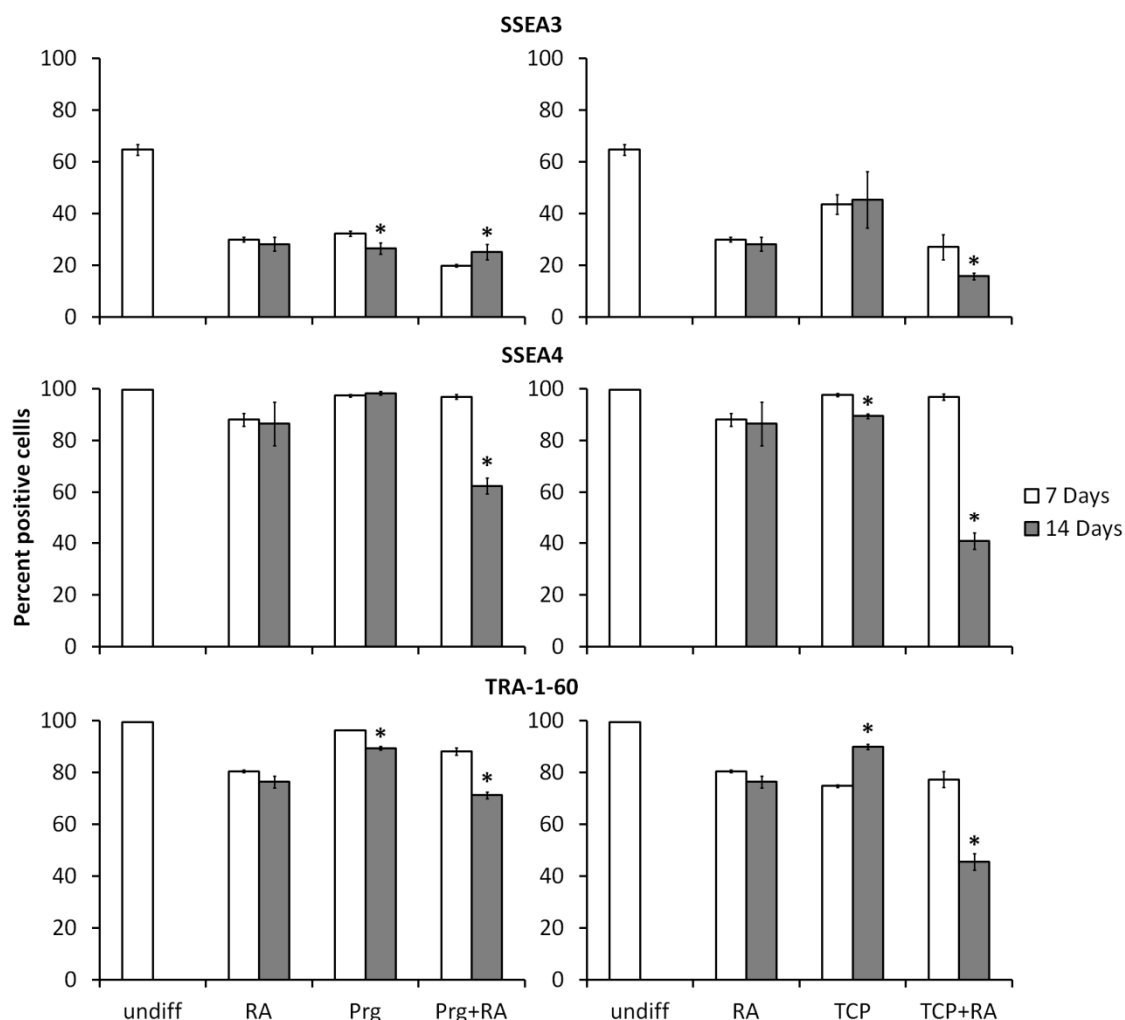


Figure 5.17: Flow cytometric analysis of stem cell-related surface markers from LSD1 inhibitor-treated N2102Ep cells. The surface markers analyzed included SSEA3, SSEA4 and TRA-1-60. Untreated culture was used to determine basal proportion of cells positive for each surface marker, designated as “undiff”. N2102Ep cells were pre-treated with vehicle or pargyline (Prg) or TCP for 4 days. Changes of population expressed each marker were tested on day 7 and 14 after retinoic acid treatment. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n=3$, $*p<0.05$ compared with 7 days treated sample).

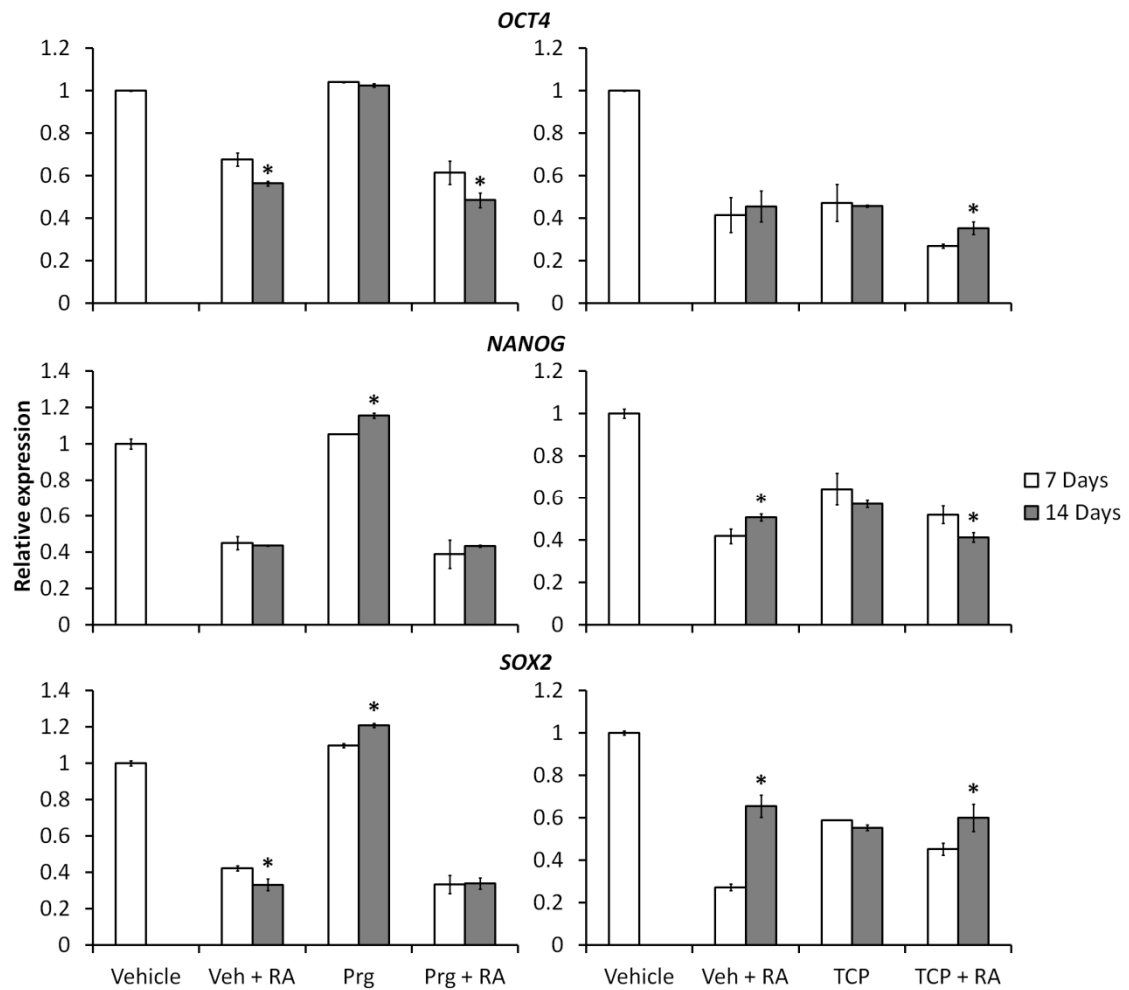


Figure 5.18: mRNA expression level of stem cell-related genes – *OCT4*, *NANOG* and *SOX2* – in LSD1 inhibitor-treated N2102Ep. Cells were pretreated with either vehicle or pargyline (Prg) or TCP for 4 days before starting retinoic acid treatment. RNA samples were collected for the experiment on day 7 and 14 after treatment. Results were shown as mRNA level relative to expression in 4 days vehicle-pretreated N2102Ep sample ($\pm$ SEM; $n=3$, $*p<0.05$ compared with 7 days treated sample).

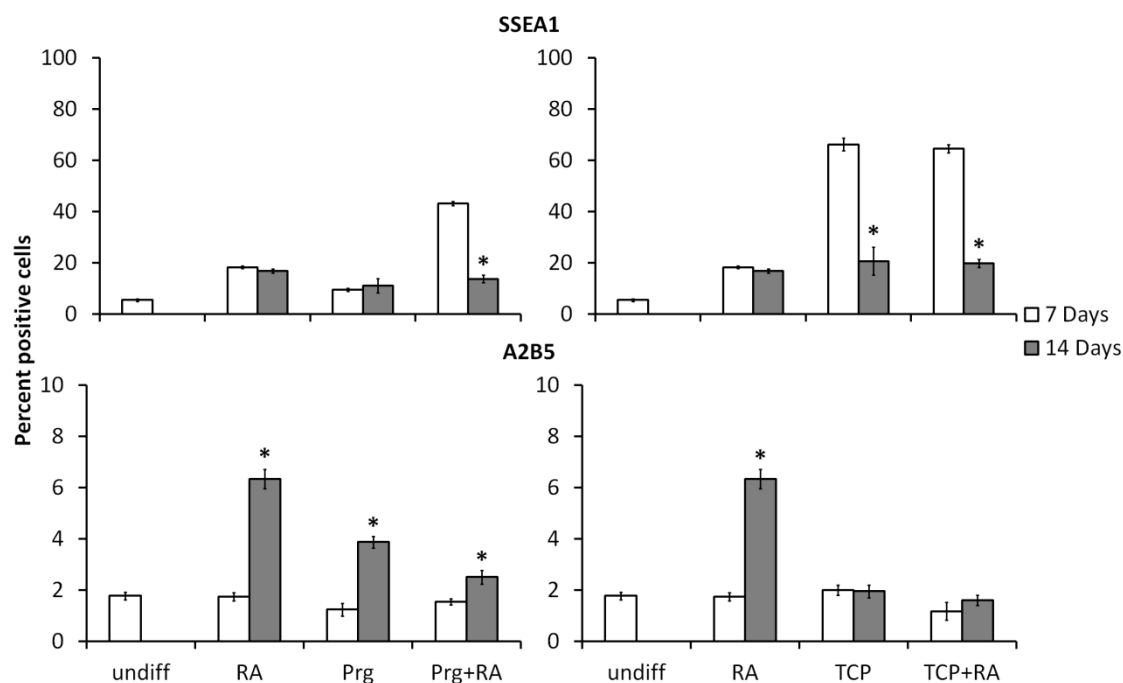


Figure 5.19: Flow cytometric analysis of differentiation related surface markers from LSD1 inhibitor-treated N2102Ep cells. The surface markers analyzed included SSEA1 and A2B5. Untreated culture was used to determine basal proportion of cells positive for each surface marker, designated as “undiff”. N2102Ep cells were pre-treated with vehicle or pargyline (Prg) or TCP for 4 days. Changes of population expressed each marker were tested on day 7 and 14 after retinoic acid treatment. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n=3$, $*p<0.05$ compared with 7 days treated sample).

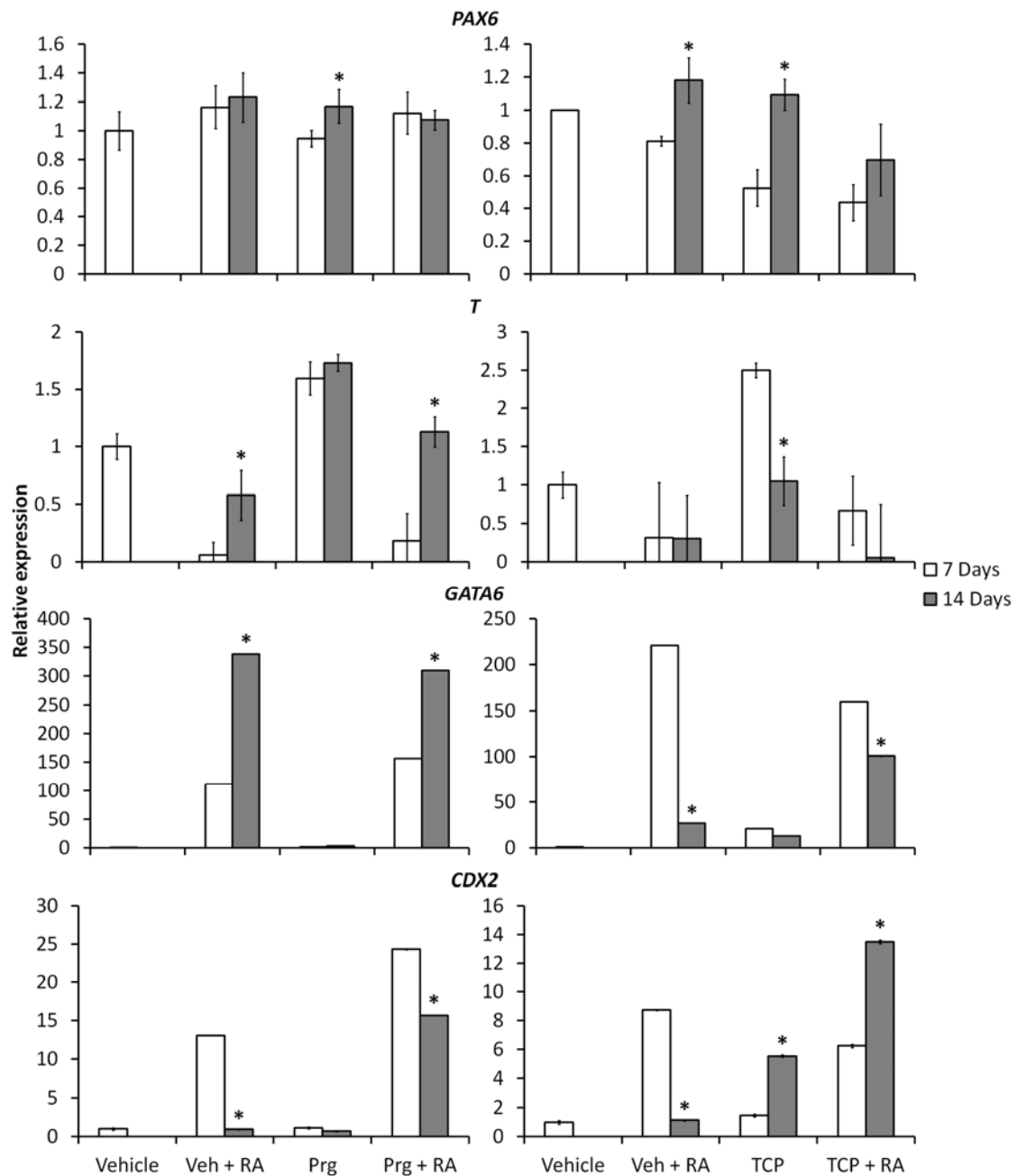


Figure 5.20: mRNA expression level of differentiation related genes – *PAX6*, *T*, *GATA6* and *CDX2* – in LSD1 inhibitor-treated N2102Ep. Cells were pretreated with either vehicle or pargyline (Prg) or TCP for 4 days before starting retinoic acid treatment. RNA samples were collected for the experiment on day 7 and 14 after treatment. Results were shown as mRNA level relative to expression in 4 days vehicle-pretreated N2102Ep sample ($\pm$ SEM; n=3, *p<0.05 compared with 7 days treated sample).

5.5 Expression of RNA signaling machineries in response to RA treatment in NTERA2

In this part, we would like to determine whether retinoic acid treatment in NTERA2 could change expression level of chromatin organizing protein, CTCF, RNA signaling molecules, DDX5 and PUS1, as well as long-non-coding RNA, *SRA*, or not. This experiment would give information on how RNA modification molecules were regulated during differentiation of NTERA2.

Expression at mRNA was determined by qRT-PCR (Figure 5.21). Fluctuation of mRNA level of all 4 genes was shown during NTERA2 differentiation with retinoic acid induction. *CTCF* mRNA was down-regulated to about 70% of that of undifferentiated NTERA2 on day 7, before gradually restored back to the same level by day 21. *PUS1* mRNA was the only one that showed down-regulation to about half of that of undifferentiated cells at the end of differentiation. *DDX5* and *SRA* were slightly up-regulated on day 7 onwards.

To confirm the expression level of these molecules at protein level, western blot analysis was performed (Figure 5.22). Protein bands of CTCF, DDX5 and PUS1 during retinoic acid treatment were not different from that of undifferentiated cells.

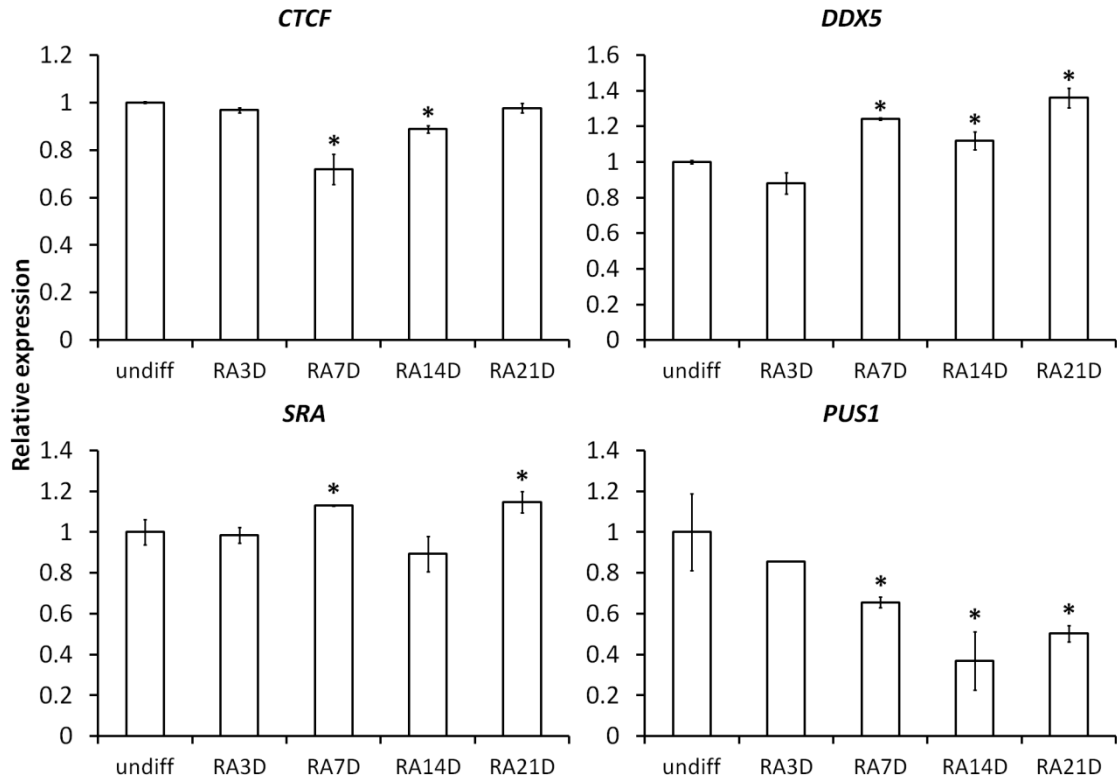


Figure 5.21: Gene expression profile of RNA signaling genes; *CTCF*, *DDX5*, *SRA* and *PUS1*; in NTERA2 cells from undifferentiated culture or following 10 μ M retinoic acid treatments for 3,7, 14 and 21 days. Results were shown as relative mRNA level compared to undifferentiated cells ($\pm$ SEM; n=3, *p<0.05).

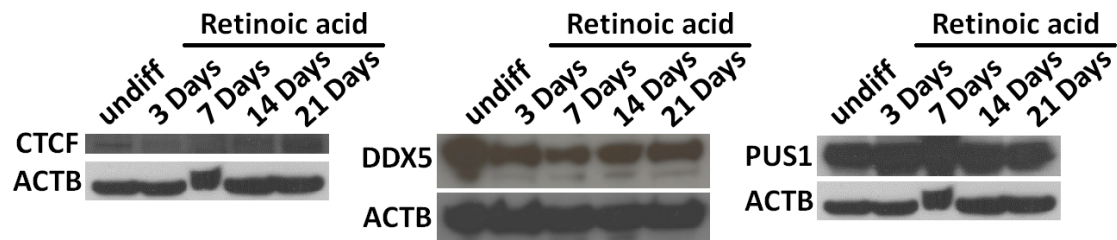


Figure 5.22: Western blot representation of expression at protein level of RNA signaling molecules; *CTCF*, *DDX5* and *PUS1*. Protein samples were collect from undifferentiated NTERA2 culture or following 10 μ M retinoic acid treatments for 3,7, 14 and 21 days.

5.6 Occupancy of CTCF, DDX5, and PUS1 on *PAX6* regulatory loci

Next, we asked whether *PAX6* expression is regulated by a complex comprised of a chromatin organizing protein, CTCF, and RNA modification machineries, DDX5 and PUS1, or not. Chromatin immunoprecipitation was performed to determine association of these proteins on *PAX6* regulatory regions, including *P0* and *P1* promoters, *5' enhancer* and *intronic enhancer* regions (Figure 5.23-24). Chromatin samples were prepared from NTERA2 cells, both untreated (undifferentiated) and retinoic acid treated (differentiated), similar to previous experiments. Primers used to quantify amount of precipitated chromatin were designed based on sequence from online database, “The UCSC Genome Browser” (<http://genome.ucsc.edu>, Figure 5.25). Immunoprecipitation with rabbit IgG was performed in parallel as a negative control. Results were normalized with IgG binding and presented as fold enrichment from non-specific binding by IgG. Fold enrichment higher than 2 fold after IgG normalization was considered as actual binding.

In native NTERA2 cells, binding of all 3 proteins were concentrated at *intronic enhancer* region (Figure 5.26). CTCF binding on other regions were comparable to each other, suggesting that CTCF bound along regulatory regions of *PAX6*. RNA helicase DDX5, besides *intronic enhancer*, showed moderate binding at *5' enhancer* and *P0* promoter (about 5-6 fold enrichment). On the contrary, PUS1 binding were shown to present at these regions except *P0* promoter.

After RA induction for 3 days, localization of CTCF and DDX5 on *PAX6* regulatory elements was lost, while PUS1 was recruited to *P1* promoter (Figure 5.27). CTCF binding on these elements was still present, but lack of concentrated localization. Binding on *P1* and *intronic enhancer* of DDX5 was completely lost, leaving only weak binding at *5' enhancer* and *P0* promoter (about 2-3 fold enrichment). On the other hand, binding of PUS1, which was found on all regulatory elements, exhibited with highest enrichment at *P1* promoter.

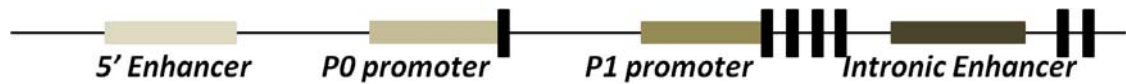


Figure 5.23: A representative of PAX6 gene structure. Regulatory regions of PAX6 including 2 promoters and 2 enhancer regions are represent with horizontal box in different color. A small vertical boxes represent each exon of PAX6, starting with Exon 0 next to P0 promoter (the left most exon) to Exon 5a (the right most exon). Note that this diagram includes only regions used in the experiment.

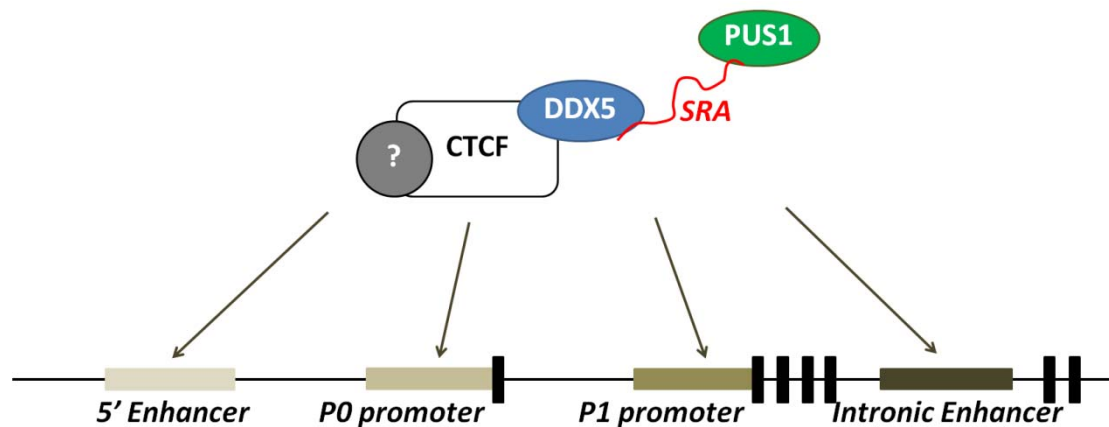


Figure 5.24: A diagram representing a hypothetical interaction between protein-RNA complex and regulatory elements of *PAX6* gene. The protein-RNA complex comprise of CTCF (a chromatin remodeling factor), DDX5 (DEAD-box RNA helicase, p68), long non-coding RNA (Steroid receptor RNA co-activator, *SRA*), and PUS1 (Pseudouridylate Synthase 1).

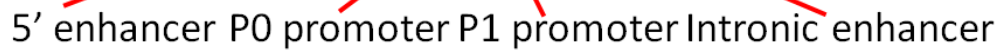


Figure 5.25: Captured screen from <http://genome.ucsc.edu> used for designing primers for ChIP experiments. The locations were searched mainly based on location of CTCF binding sites analyzed signals from ChIP-Seq experiments submitted into the database. The red boxes indicate the regions of interest. Genomic sequences at each region were taken from the database and primers designing were done using with NCBI primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>).

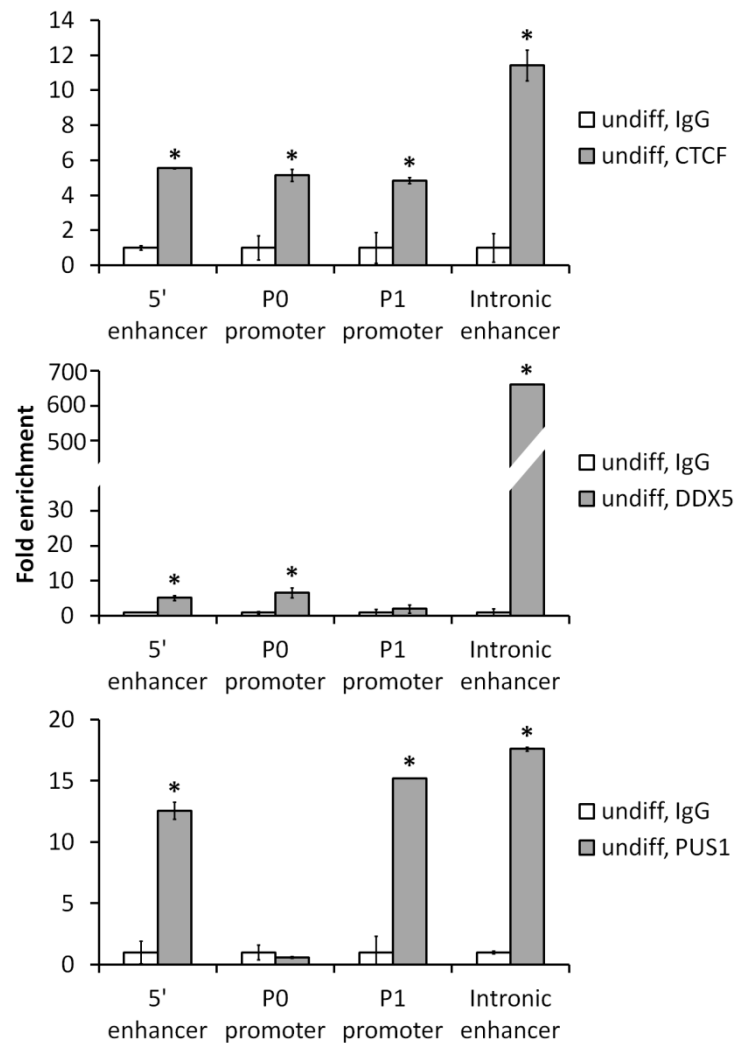


Figure 5.26: Association of CTCF, DDX5 and PUS1 on regulatory regions of *PAX6* gene was determined with chromatin immunoprecipitation. Protein-crosslinked chromatin collected from undifferentiated NTERA2 culture was precipitated with 3 μ g of antibody specific to CTCF, DDX5 and PUS1. Immunoprecipitation with IgG was done to determine non-specific binding of the antibody. Amount of precipitated chromatin from each antibody was determined with qPCR. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3, *p<0.05).

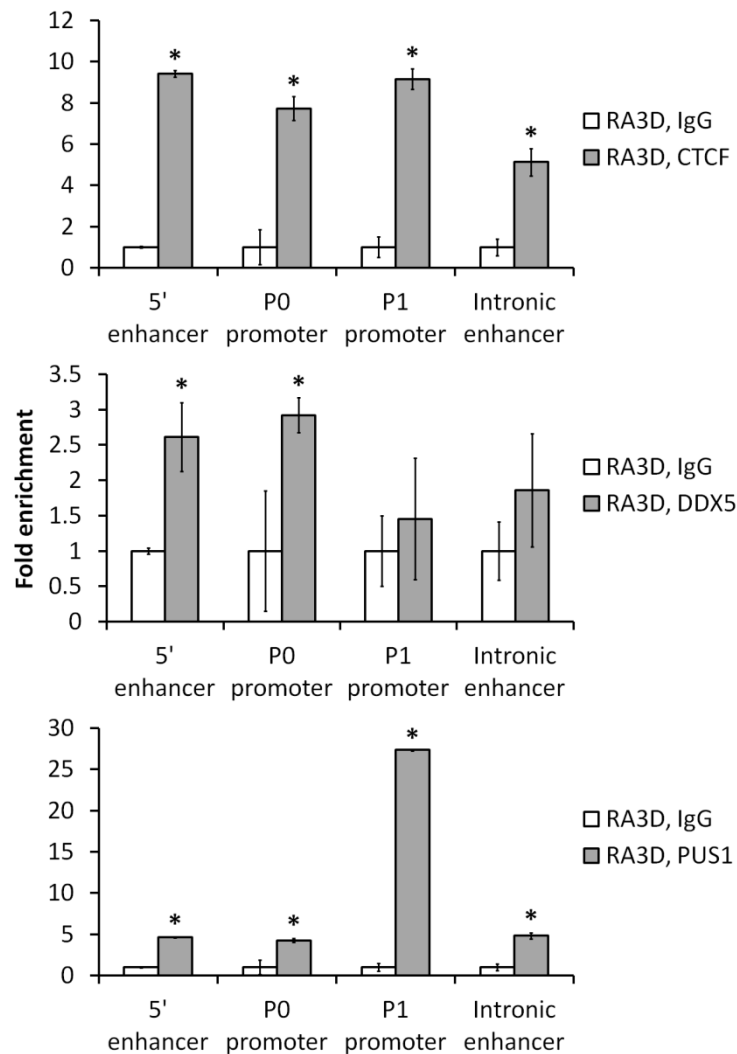


Figure 5.27: Association of CTCF, DDX5 and PUS1 on regulatory regions of *PAX6* gene was determined with chromatin immunoprecipitation. Protein-crosslinked chromatin collected from 3 days retinoic acid-treated NTERA2 culture was precipitated with 3 μ g of antibody specific to CTCF, DDX5 and PUS1. Immunoprecipitation with IgG was done to determine non-specific binding of the antibody. Amount of precipitated chromatin from each antibody was determined with qPCR. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3, *p<0.05).

CTCF, DDX5 and PUS1 were recruited to *P1* promoter at day 7 of differentiation (Figure 5.28). CTCF and DDX5 bindings were highest at *P1* promoter and distributed equally at other regions. PUS1 maintained high binding at *P1* promoter similar to that on day 3. However, it dissociated from *P0* promoter and bound weakly at 5' *enhancer*.

Localization of CTCF, DDX5 and PUS1 occupancy was lost on day 14 and 21, especially recruitment at *P1* promoter (Figure 5.29-30). CTCF bound highly at *intronic enhancer* and *P0* promoter on day 14 and 21 respectively. Binding of DDX5 on *PAX6* regulatory elements was moderately high (6-8 fold) on day 14, but decreased to weak or no binding on day 21. PUS1 binding at *P1* promoter was completely loss on day 14, then restored to weak binding on day 21.

In order to understand dynamics of binding of these proteins on *PAX6* regulatory regions, results were rearranged to represent binding of each protein at specific location over time course of retinoic acid induction. Binding of CTCF, DDX5 and PUS1 at 2 enhancer regions of *PAX6* gene during differentiation of NTERA2 by retinoic acid treatment were shown in figure 5.31. CTCF bound to the enhancers at the highest level on day 7, when the level of *PAX6* mRNA was at peak. PUS1 binding on both enhancers also showed only slight difference, highest binding at undifferentiated state and reduced during differentiation until completely loss at day 21. Unlike those two, DDX5 occupancy on both enhancers showed different dynamic during NTERA2 differentiation. DDX5 binding at 5' *enhancer* showed slight changing during differentiation, but loss of binding at *intronic enhancer* during differentiation was shown despite extremely high binding at the undifferentiated state.

Figure 5.32 shows dynamic of CTCF, DDX5 and PUS1 binding at the promoters of *PAX6* genes. Protein occupancy at *P0* promoter changed randomly with no consistency with *PAX6* mRNA expression at all. At *P1* promoter, PUS1 occupancy was relatively high in native NTERA2 cells and was increased as soon as 3 days of treatment, while occupancy of CTCF and DDX5 followed a similar pattern of *PAX6* mRNA expression in response to retinoic acid treatment.

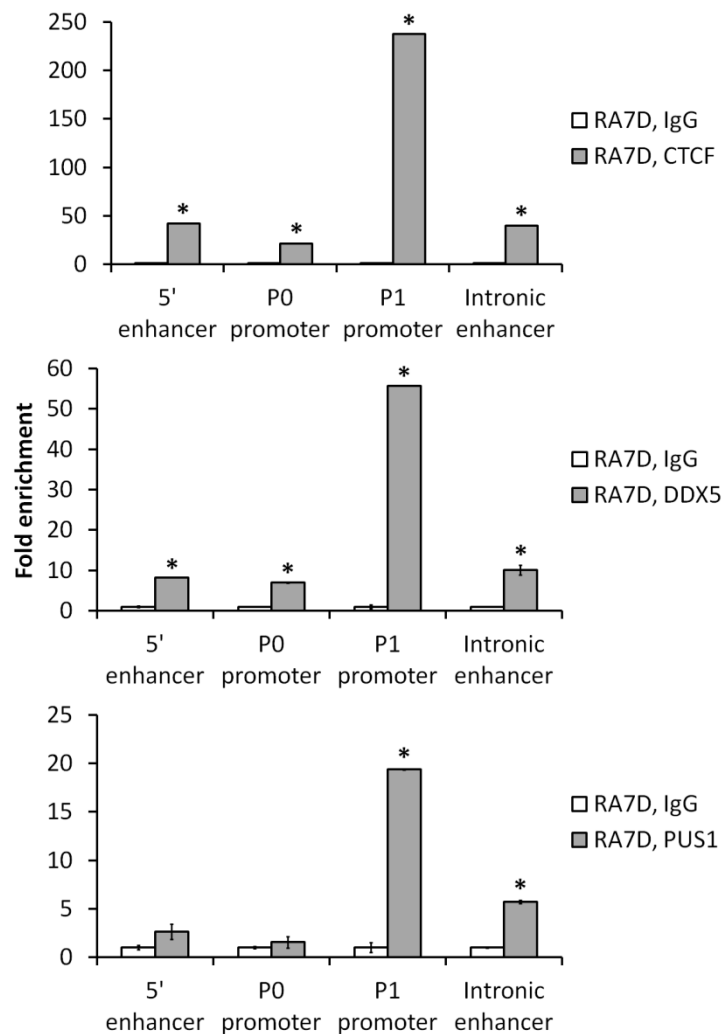


Figure 5.28: Association of CTCF, DDX5 and PUS1 on regulatory regions of *PAX6* gene was determined with chromatin immunoprecipitation. Protein-crosslinked chromatin collected from 7 days retinoic acid-treated NTERA2 culture was precipitated with 3 μ g of antibody specific to CTCF, DDX5 and PUS1. Immunoprecipitation with IgG was done to determine non-specific binding of the antibody. Amount of precipitated chromatin from each antibody was determined with qPCR. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3, *p<0.05).

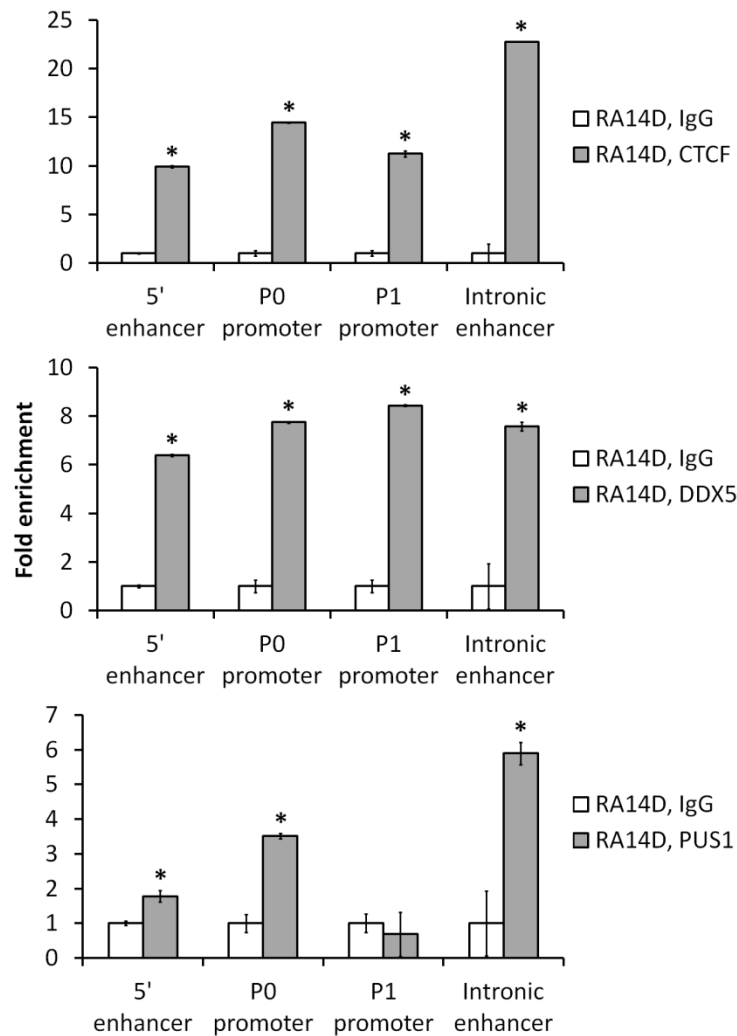


Figure 5.29: Association of CTCF, DDX5 and PUS1 on regulatory regions of *PAX6* gene was determined with chromatin immunoprecipitation. Protein-crosslinked chromatin collected from 14 days retinoic acid-treated NTERA2 culture was precipitated with 3 μ g of antibody specific to CTCF, DDX5 and PUS1. Immunoprecipitation with IgG was done to determine non-specific binding of the antibody. Amount of precipitated chromatin from each antibody was determined with qPCR. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3, *p<0.05).

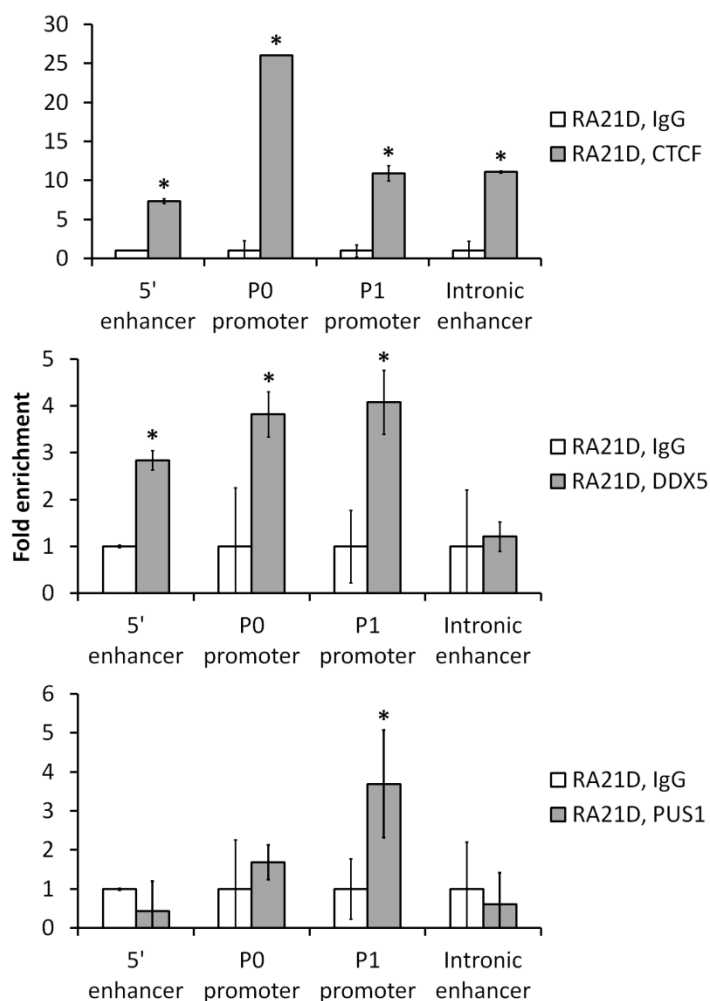


Figure 5.30: Association of CTCF, DDX5 and PUS1 on regulatory regions of *PAX6* gene was determined with chromatin immunoprecipitation. Protein-crosslinked chromatin collected from 21 days retinoic acid-treated NTERA2 culture was precipitated with 3 μ g of antibody specific to CTCF, DDX5 and PUS1. Immunoprecipitation with IgG was done to determine non-specific binding of the antibody. Amount of precipitated chromatin from each antibody was determined with qPCR. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3, *p<0.05).

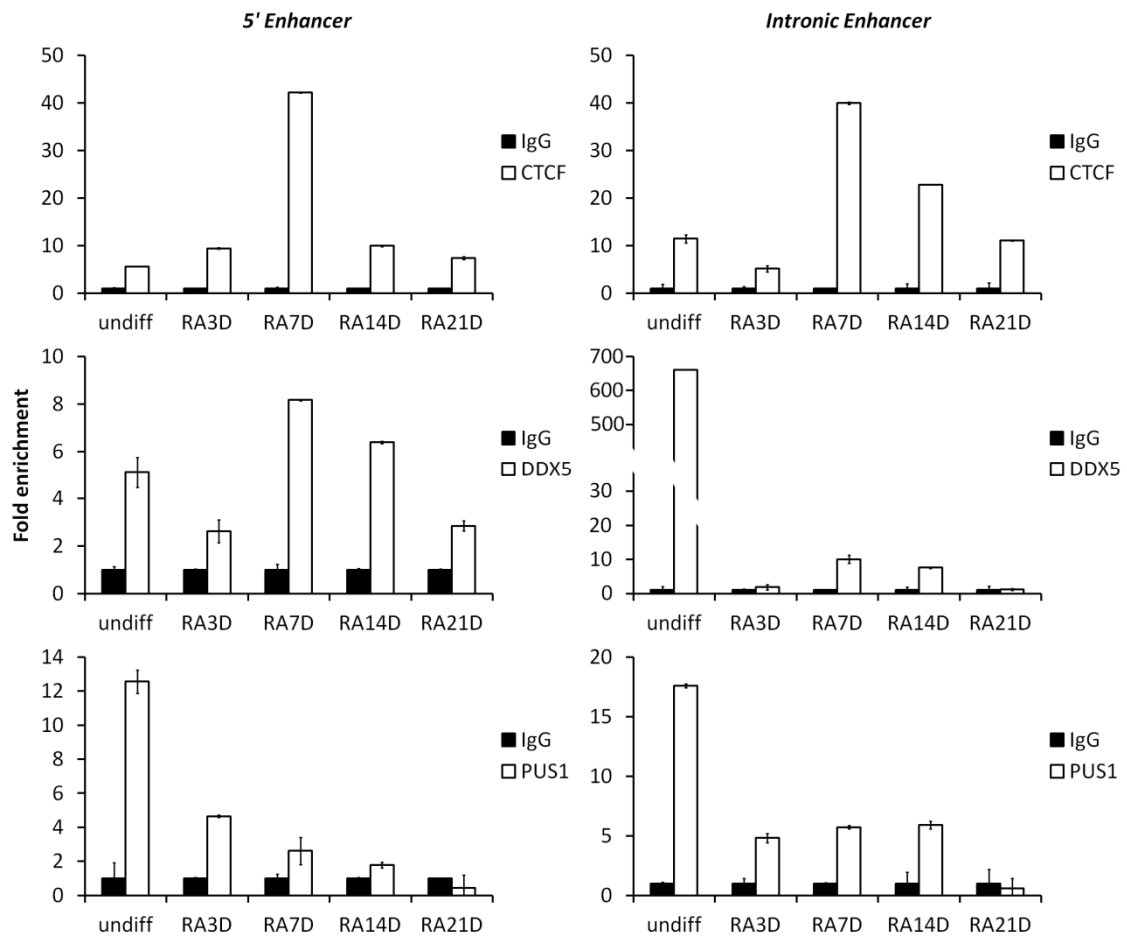


Figure 5.31: Rearranged chromatin immunoprecipitation results represented dynamic of protein-DNA association at enhancer regions of *PAX6* gene during differentiation of NTERA2 cells by retinoic acid induction. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3).

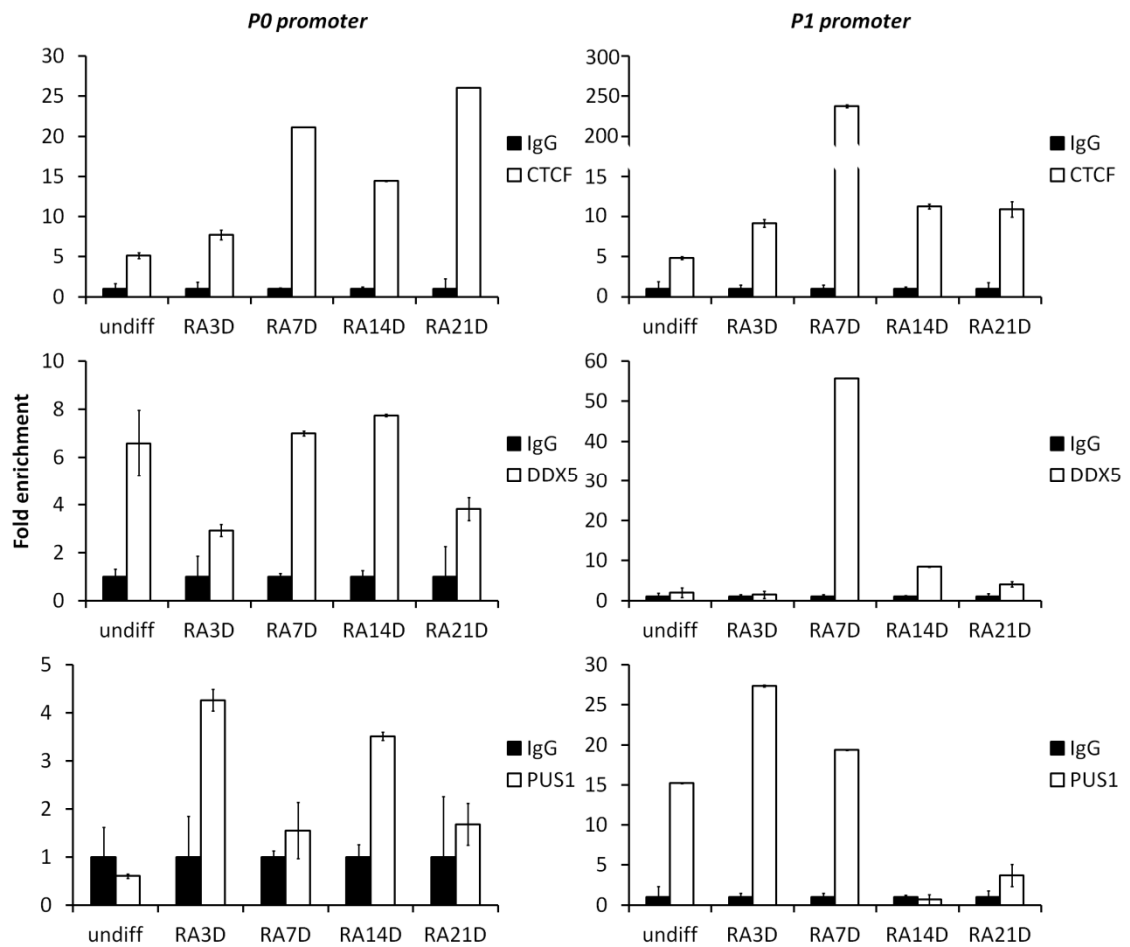


Figure 5.32: Rearranged chromatin immunoprecipitation results represented dynamic of protein-DNA association at promoter regions of *PAX6* gene during differentiation of NTERA2 cells by retinoic acid induction. Results were shown as fold enrichment of chromatin pulled down by each antibody over that of negative control IgG ($\pm$ SEM; n=3).

5.7 Characterization of stable *SRA*, *DDX5* and *PUS1* knockdown NTERA2 cell lines

To elucidate the role of RNA signaling molecules on differentiation of NTERA2 cells, stable knockdown cell lines of those molecules were established. Stable knockdown cell lines were constructed by transfecting short hairpin RNA (shRNA) recognizing mRNA of target gene, which in turn activate silencing mechanism that results in degradation of target mRNA. *SRA*, *DDX5* and *PUS1* knockdown was confirmed at both mRNA and protein level (Figure 5.33). An attempt to establish CTCF knockdown cell line was made. However, massive cell death occurred after challenging with selection culture medium (data not shown).

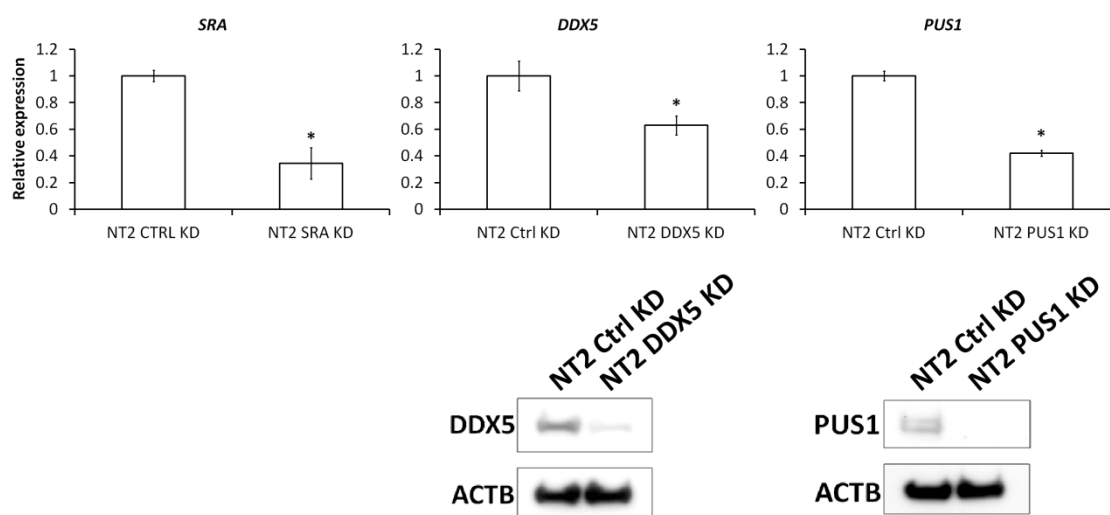


Figure 5.33: qRT-PCR and western blot analysis confirming knockdown of *SRA*, *DDX5* and *PUS1* in NTERA2 cells after establishment of stable knockdown cell lines. RNA and protein samples were collected from confluent culture of each knockdown cell line, including knockdown control. Expression at mRNA level was shown as relative to that of knockdown control cell line ($\pm$ SEM; $n = 3$, $*p < 0.05$). Reduction of *SRA* and *PUS1* mRNA was around 60%, while *DDX5* mRNA was reduced about 40%. Protein bands from western blot analysis of *DDX5* and *PUS1* expression were also reduced after knockdown.

5.7.1 Stem cell property of knockdown NTERA2 cell lines

In order to evaluate pluripotent property of knockdown cell lines, cloning efficiency assay was done with all knockdown cell lines (Figure 5.34). Two hundred cells were trypsinized from confluent culture and plated in 6-well plate. They were left undisturbed in the incubator for 2 weeks before taken out for alkaline phosphatase (ALP) staining to determine number of pluripotent colonies. Colonies grown on culture plate were count manually. Their morphologies were confirmed under microscope. Only colonies stained strongly with ALP with the morphology shown in Figure 5.34a were counted, those with weaker staining or with other morphology (such as the one shown in Figure 5.34b) were excluded. The numbers of pluripotent colonies from all knockdown cell lines were less than that of control knockdown cell line.

The percentage of SSEA3-, SSEA4- and TRA-1-60-positive population was determined from confluent culture of the knockdown cell lines by flow cytometry (Figure 5.35). Compared to control knockdown cell line, NT2 SRA KD showed drastic loss of SSEA3- and TRA-1-60-positive population, while maintained relatively high SSEA4-positive population. NT2 DDX5 KD cells were similar to that of NT2 Ctrl KD, high percentage of SSEA3-, SSEA4- and TRA-1-60-positive population. NT2 PUS1 KD loss some of TRA-1-60-positive population, but SSEA3-positive cells were about half of that from knockdown control.

Figure 5.36 presents the mRNA expression of stem cell-related genes; *OCT4*, *NANOG* and *SOX2*. Consistent with flow cytometric results, expression of stem cell-related genes in NT2 DDX5 KD was retained at high level compared to control. While PUS1 knockdown resulted in loss of only *SOX2* expression, *SRA* knockdown resulted in loss of expression from all of 3 genes.

Taken together, knockdown of *SRA*, DDX5 and PUS1 resulted in loss of cell pluripotency as shown by cloning efficiency assay, even though FACS analysis and mRNA expression of NT2 DDX5 KD and NT2 PUS1 KD suggested the other way.

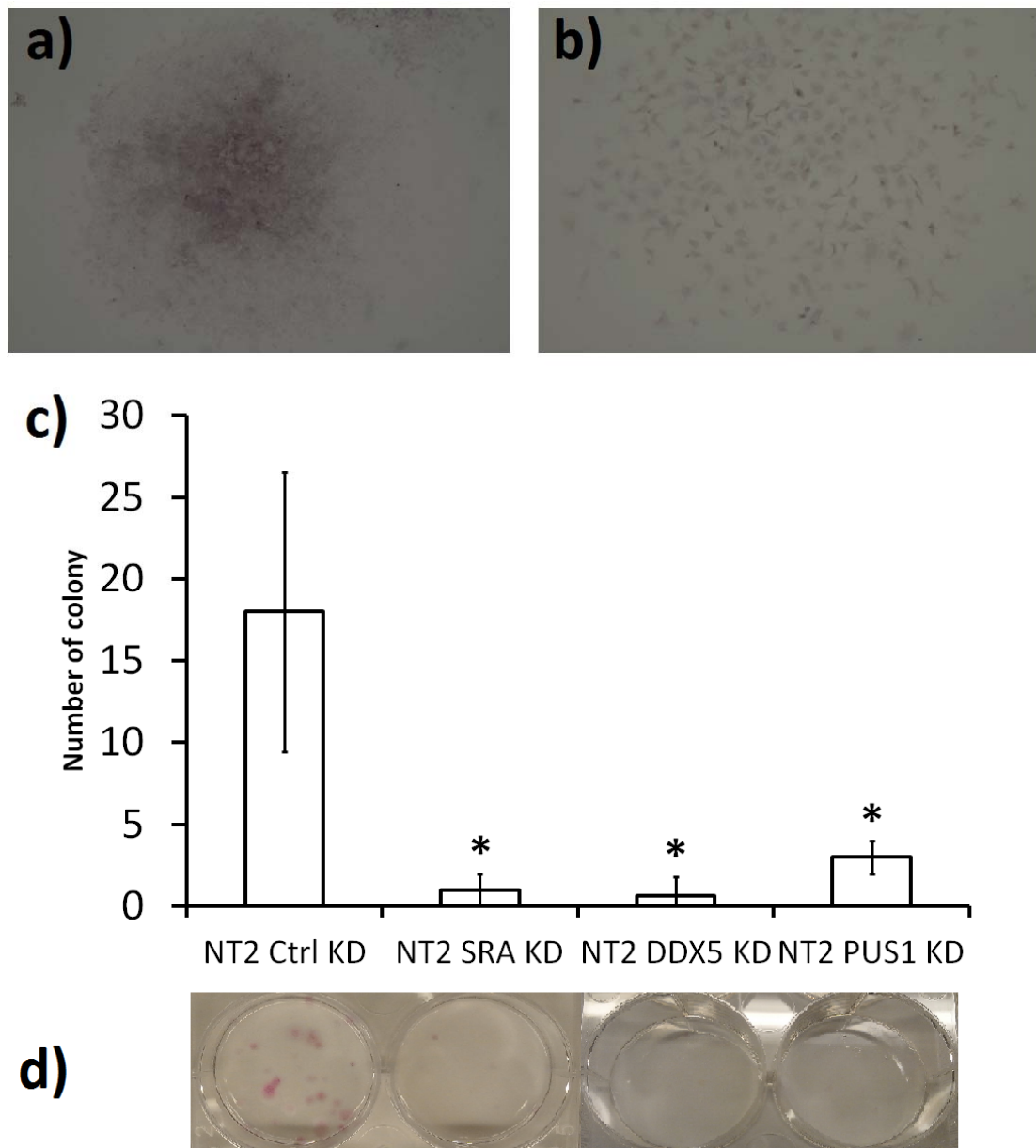


Figure 5.34: Cloning efficiency assay was done to demonstrate pluripotency of all knockdown cell lines as functional assay. 200 cells from confluent culture of each cell line were seeded in a well of 6-well plate, 3 wells per cell line. Alkaline phosphatase (ALP) staining was done 14 days after seeding. Colonies positive for ALP were counted in each well, confirming by observation under microscope. Results were shown as mean number of ALP-positive colonies per well ($\pm$ SEM; $n = 3$, $*p < 0.05$). a) representative of ALP-positive colony b) representative of ALP-negative colony c) bar graph representing number of colony in each cell line d) representative colonies grew from each cell line.

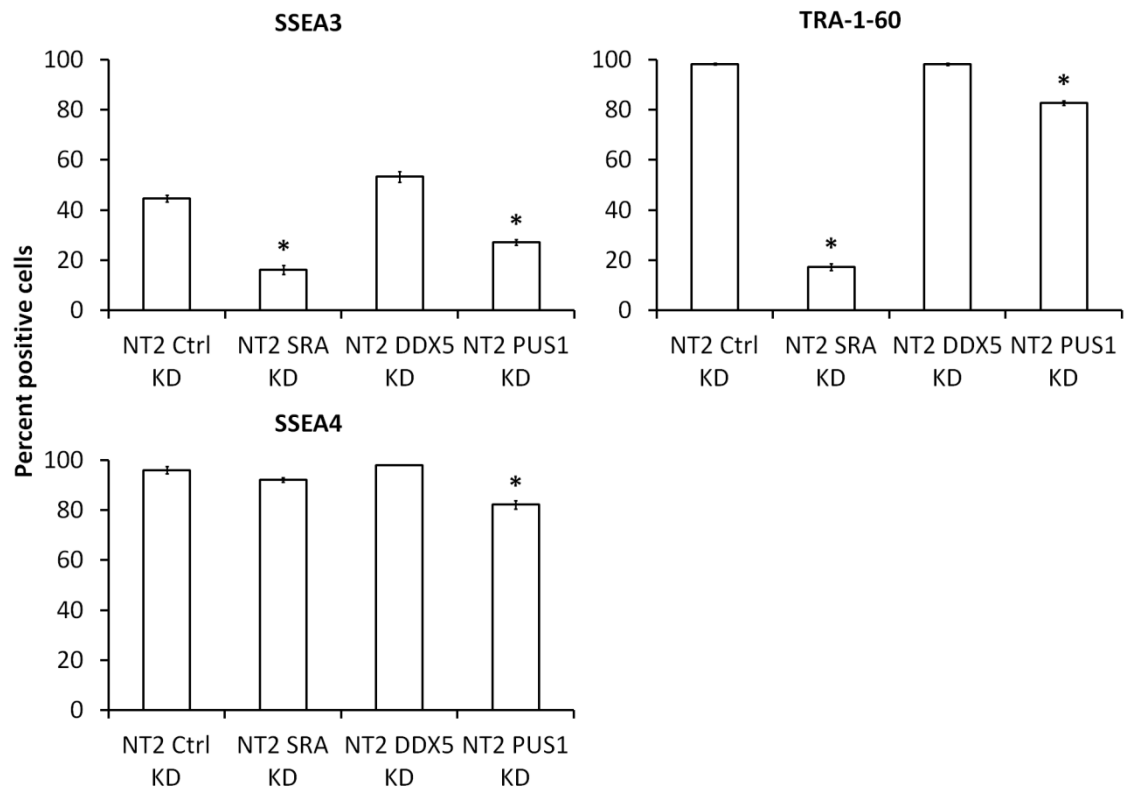


Figure 5.35: Flow cytometric analysis of stem cell-related surface markers from *SRA*, *DDX5* and *PUS1* knockdown cell lines compared with knockdown control cell line. Cell samples were collected from confluent culture of each cell line, stained with each marker individually. The surface markers included SSEA3, SSEA4 and TRA-1-60. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n = 3$, $*p < 0.05$).

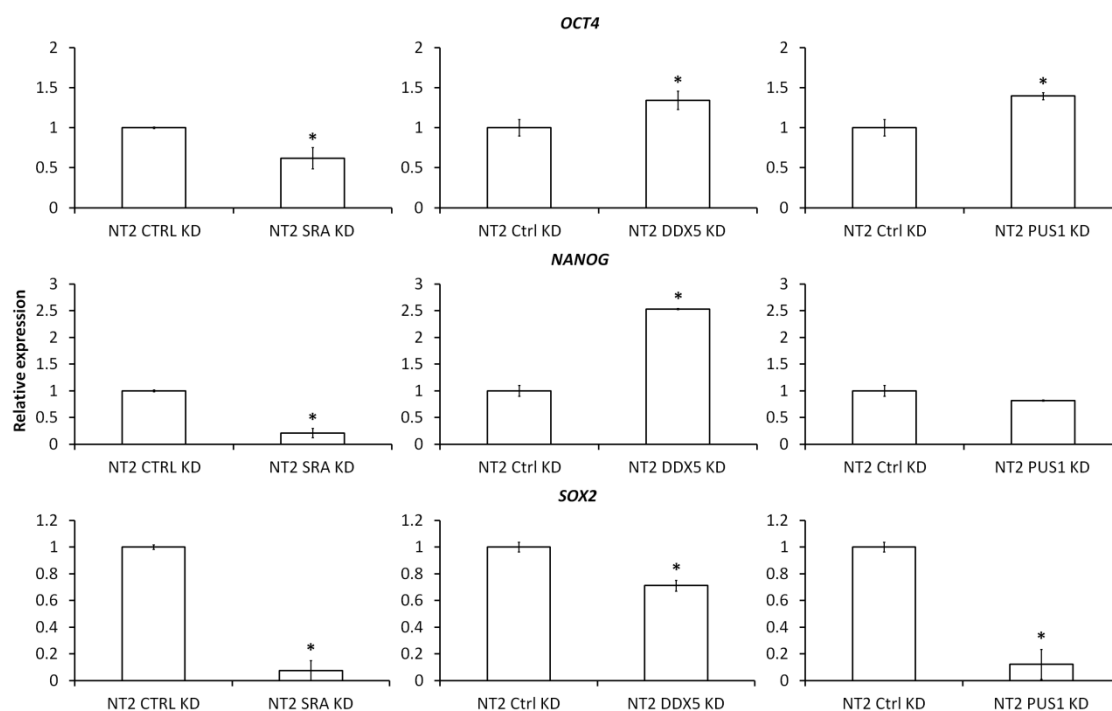


Figure 5.36: Gene expression profile of stem cell-related genes; *OCT4*, *NANOG*, and *SOX2*; in *SRA*, *DDX5* and *PUS1* knockdown NTERA2 cell lines. RNA samples were collected from confluent culture of each cell line, including knockdown control cell line. Expression at mRNA level was shown as relative to that of knockdown control cell line ($\pm$ SEM; $n = 3$, $*p < 0.05$).

5.7.2 Differentiation potential of knockdown NTERA2 cell lines

First, flow cytometric analysis of differentiation related surface markers was done from confluent culture of knockdown cells (Figure 5.37). *SR4* knockdown reduced SSEA1-positive population, at the same time increased A2B5-positive population compared to NT2 Ctrl KD. SSEA1-positive cells from PUS1 knockdown cell line were less than 2% of total population. A2B5-positive population from both NT2 DDX KD (12.7%) and NT2 PUS1 KD (9.8%) was not different from that of NT2 Ctrl KD (8.8%)

Expression of differentiation associated transcription factors; *PAX6*, *T*, *GATA6* and *CDX2*, was determined (Figure 5.38). Knockdown of DDX5 and PUS1 reduced mRNA expression of *PAX6*, while *SR4* knockdown did not change the expression level. *T* and *GATA6* transcripts were up-regulated as a result of DDX5 and PUS1 knockdown. On the other hand, they were down-regulated in *SR4* knockdown cells.

Taken together, knockdown of *SR4*, DDX5 and PUS1 changed differentiation potential of NTERA2 cell. FACS and mRNA expression results suggested that *SR4* knockdown favor neural lineage differentiation as A2B5-positive population was induced and down-regulation of transcription factors for differentiation into other lineages. DDX5 and PUS1 knockdown suppress neural differentiation as *PAX6* was down-regulated, *T* and *GATA6* were up-regulated, and A2B5-positive cells were not induced, indicating that DDX5 and PUS1 played some roles in neural differentiation from NTERA2 cells.

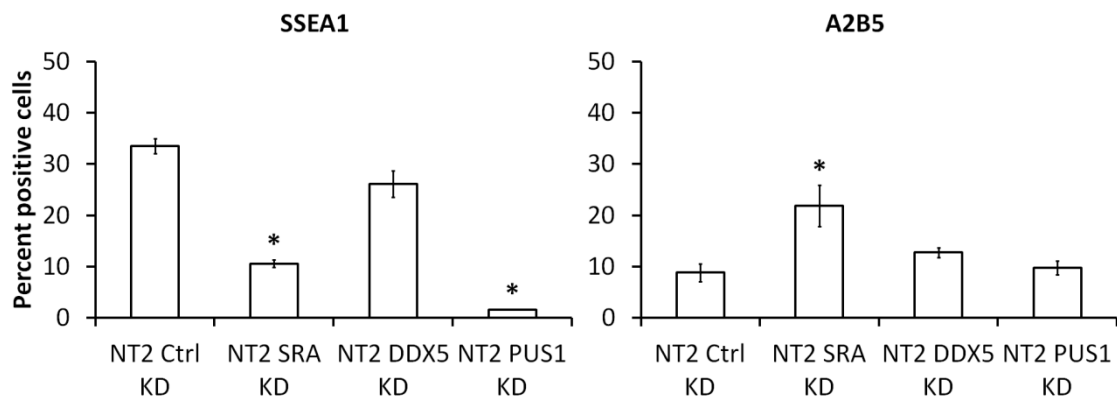


Figure 5.37: Flow cytometric analysis of differentiation related surface markers from *SRA*, *DDX5* and *PUS1* knockdown cell lines compared with knockdown control cell line. Cell samples were collected from confluent culture of each cell line, stained with each marker individually. The surface markers included SSEA1 and A2B5. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n = 3$, $*p < 0.05$).

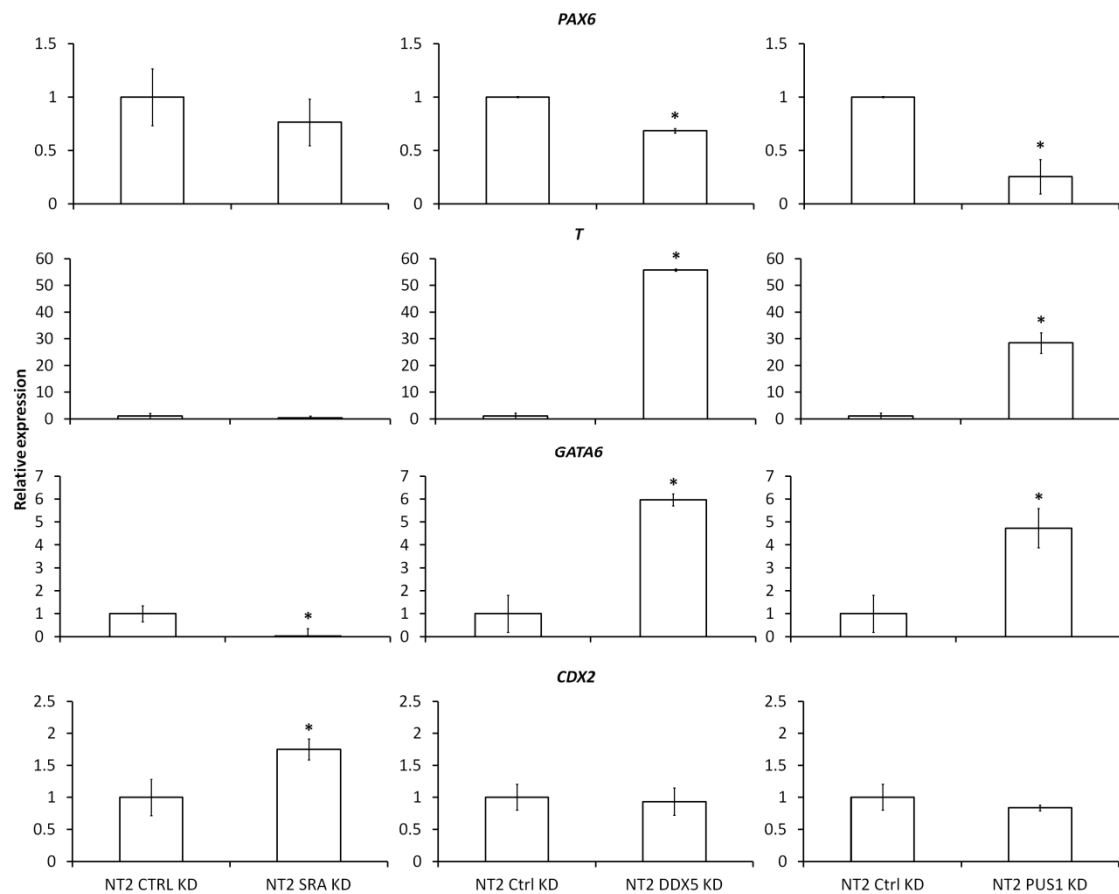


Figure 5.38: Gene expression profile of differentiation associated transcription factors; *PAX6*, *T*, *GATA6*, and *CDX2*; in *SRA*, *DDX5* and *PUS1* knockdown NTERA2 cell lines. RNA samples were collected from confluent culture of each cell line, including knockdown control cell line. Expression at mRNA level was shown as relative to that of knockdown control cell line ($\pm$ SEM; $n = 3$, $*p < 0.05$).

5.8 Characterization of DDX5 and DDX17 over-expressing NTERA2 cell lines

To demonstrate the role of DDX5 on neural differentiation of NTERA2 cells, DDX5 over-expressing NTERA2 cell line was established. Besides DDX5, there are other members of RNA helicase family. One of them that had been shown to have homology with DDX5 is DDX17. Knockout of either DDX5 or DDX17 or double knockout resulted in embryonic lethality, suggesting that they possessed a role during development (Fukuda *et al.*, 2007). So, we chose to study effects of DDX17 over-expression in NTERA2 along side with those of DDX5. Transfected NTERA2 with DDX5 or DDX17 over-expressing plasmid were selected with puromycin, maintained, expanded in culture, and designated as NT2 CAG-DDX5 and NT2 CAG-DDX17 respectively. Figure 5.39 shows western blot analysis for FLAG-tagged protein, confirming that the established cell lines express exogenous DDX5 or DDX17.

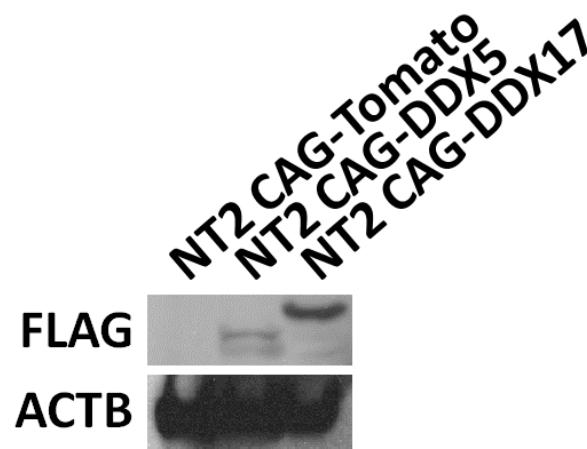


Figure 5.39: Western blot analysis showed increased expression of FLAG-containing proteins indicating that the increased expression of both DDX5 and DDX17 was from exogenous one produced by transfected transgene.

5.8.1 Stem cell property of DDX5 and DDX17 over-expressing NTERA2 cell lines

Flow cytometric analysis and mRNA expression of stem cell-related markers were studied in those over-expressing cell lines, at undifferentiated state and after differentiated with retinoic acid induction. Both over-expressing cell lines rapidly lost cell population positive for SSEA3, SSEA4 and TRA-1-60 upon retinoic acid treatment (Figure 5.40). From undifferentiated culture, SSEA3- and TRA-1-60-positive population was lower in NT2 CAG-DDX17 compared to NT2 CAG-Tomato and NT2 CAG-DDX5. However, complete loss of SSEA3-, SSEA4- and TRA-1-60-positive population was shown as soon as 7 days (for SSEA3) or 14 days.

Loss of *OCT4* and *NANOG* mRNA expression was also faster in over-expressing cell lines compared to control (Figure 5.41). In consistent with FACS results, expression level of *OCT4* and *NANOG* mRNA was lower in NT2 CAG-DDX17 compared to NT2 CAG-Tomato. After retinoic acid treatment, down-regulation of *OCT4* and *NANOG* was faster and with greater magnitude in both DDX5 and DDX17 over-expressing cell lines. Surprisingly, we did not see down-regulation of *SOX2* after retinoic acid treatment as shown in native NTERA2 (Figure 5.1), instead, mRNA level was up-regulated on day 3 and 7 after treatment before reduced on day 14 and 21. In addition, higher *SOX2* up-regulation can be observed in NT2 CAG-DDX17.

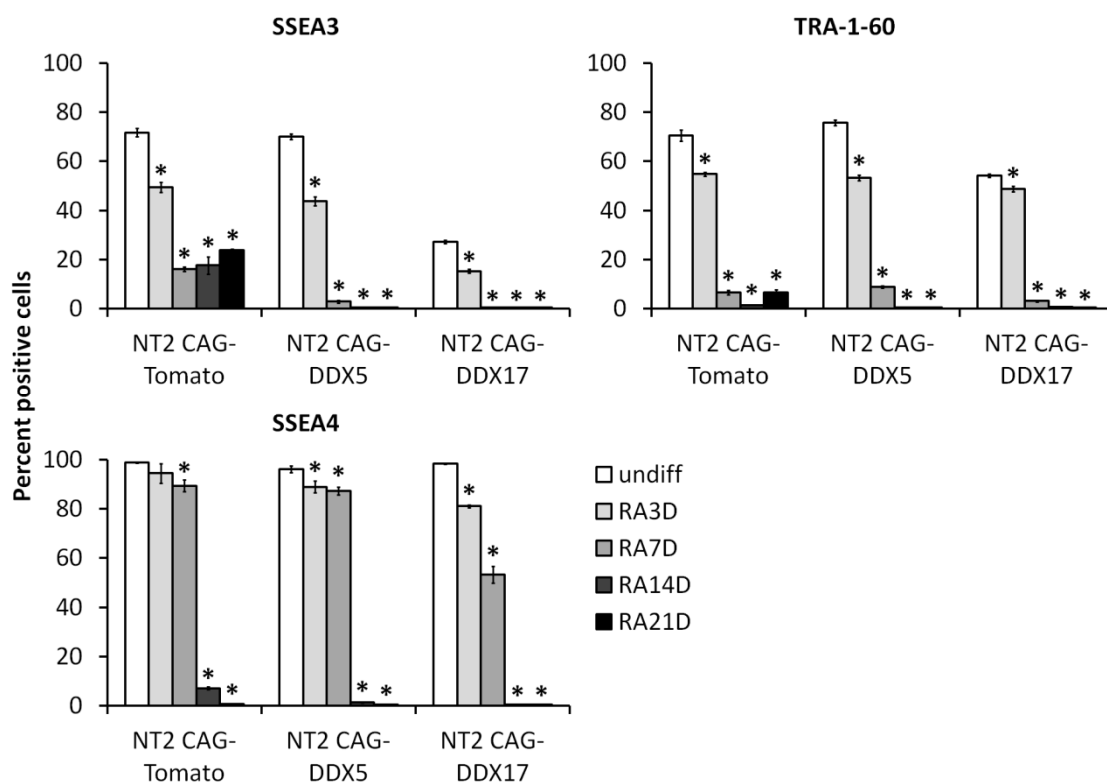


Figure 5.40: Flow cytometric analysis of stem cell-related surface markers from DDX5 and DDX17 over-expressing cell lines, compared with control transfected cells, at undifferentiated state and during differentiation with 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. The surface markers analyzed included SSEA3, SSEA4 and TRA-1-60. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n = 3$, $*p < 0.05$ compared with undifferentiated sample of its own cell line).

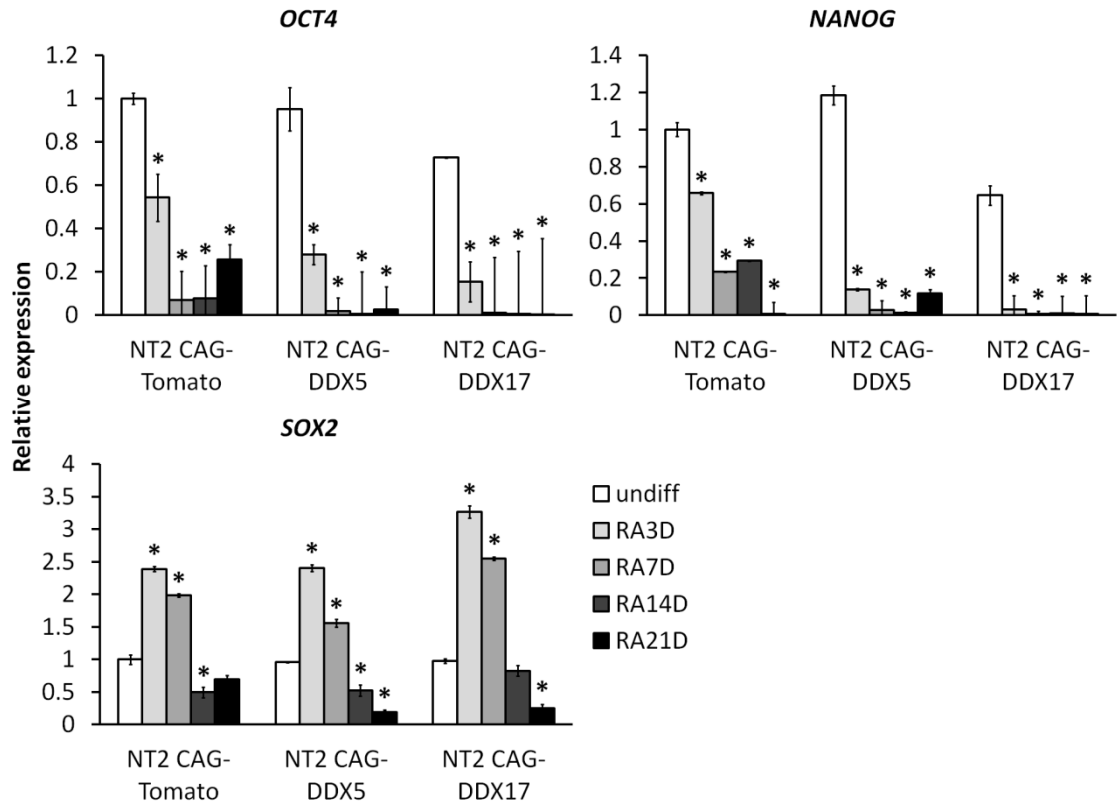


Figure 5.41: Gene expression profile of stem cell-related genes; *OCT4*, *NANOG*, and *SOX2*; in DDX5 and DDX17 over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; $n = 3$, * $p < 0.05$ compared with undifferentiated sample of its own cell line).

5.8.2 Differentiation of DDX5 and DDX17 over-expressing NTERA2 cell lines

To clarify the role of DDX5 and DDX17 over-expression on differentiation of NTERA2 cells, we began with flow cytometric analysis of differentiation related surface markers – SSEA1 and A2B5 (Figure 5.42). SSEA1-positive population increased after treatment with retinoic acid, reaching highest proportion on day 7 for NT2 CAG-Tomato. Even though, SSEA1-positive cells can be induced from NT2 CAG-DDX5 and NT2 CAG-DDX17, they disappeared faster than that of NT2 CAG-Tomato (to about 10% on day 14). On the other hand, A2B5-positive population was induced easier from DDX5 and DDX17 over-expressing cell lines. Highest proportion of A2B5-positive cells was around 50% on day 14 after retinoic acid induction for NT2 CAG-Tomato, while almost 80% of A2B5-positive cells were induced on day 7 in both DDX5 and DDX17 over-expressing cell lines.

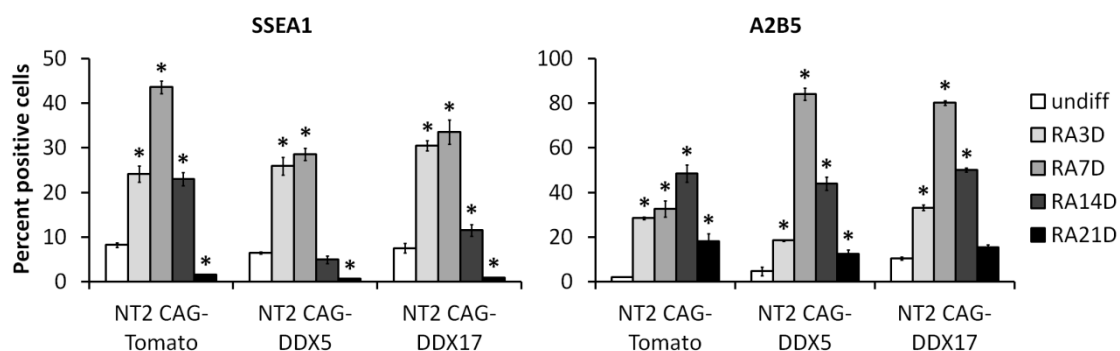


Figure 5.42: Flow cytometric analysis of differentiation related surface markers from DDX5 and DDX17 over-expressing cell lines, compared with control transfected cells, at undifferentiated state and during differentiation with 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. The surface markers analyzed included SSEA1 and A2B5. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n = 3$, * $p < 0.05$ compared with undifferentiated sample of its own cell line).

In accordance with high A2B5-positive population, higher level of *PAX6* mRNA was detected in NT2 CAG-DDX5 and NT2 CAG-DDX17 treated with retinoic acid compared to NT2 CAG-Tomato (Figure 5.43). *T* and *GATA6* were also highly increased on day 14 and 21 of treatment in NT2 CAG-DDX5. On the other hand, NT2 CAG-DDX17 showed only subtle changes compared with NT2 CAG-Tomato.

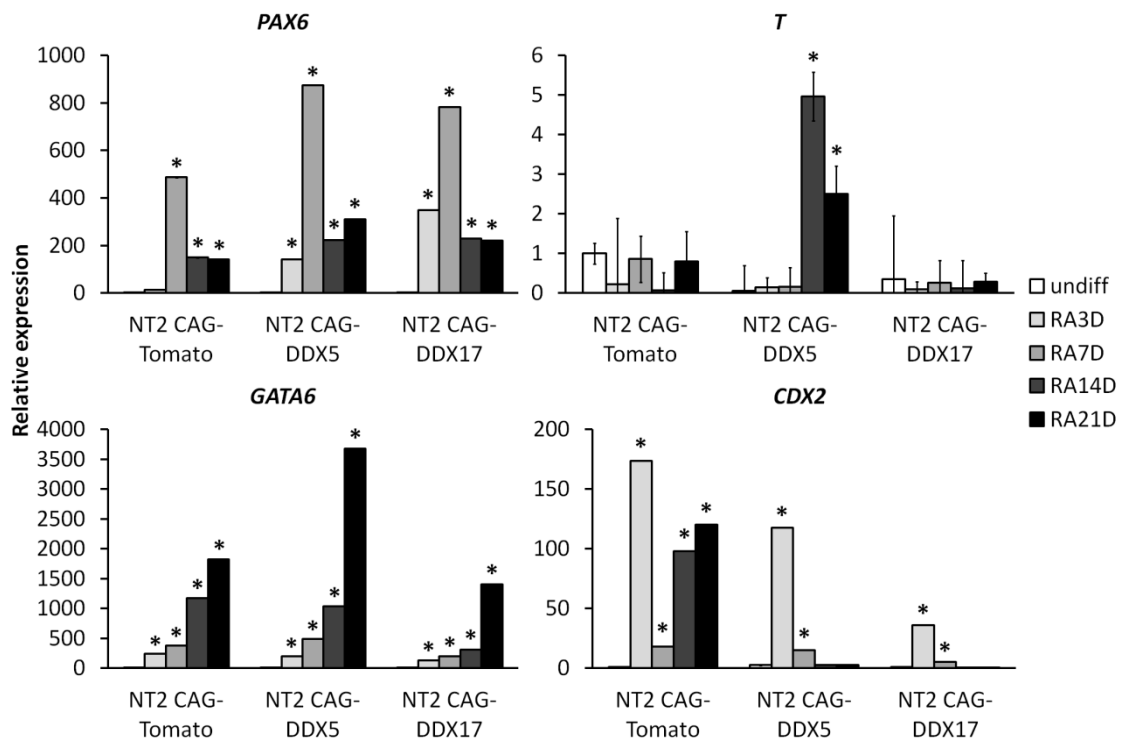


Figure 5.43: Gene expression profile of lineage-specific transcription factors; *PAX6*, *T*, *GATA6*, and *CDX2*; in DDX5 and DDX17 over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n = 3, *p<0.05 compared with undifferentiated sample of its own cell line).

To confirm neuronal differentiation potential of over-expressing cell lines, mRNA expression of neuronal related genes was also determined. NT2 CAG-DDX17 showed highest expression level of *NEUROD1*, *NESTIN*, *DCX* and *MAP2* after induced with retinoic acid. (Figure 5.44) Increased expression level of these genes was found in NT2 CAG-DDX5 as well, but with a lower magnitude.

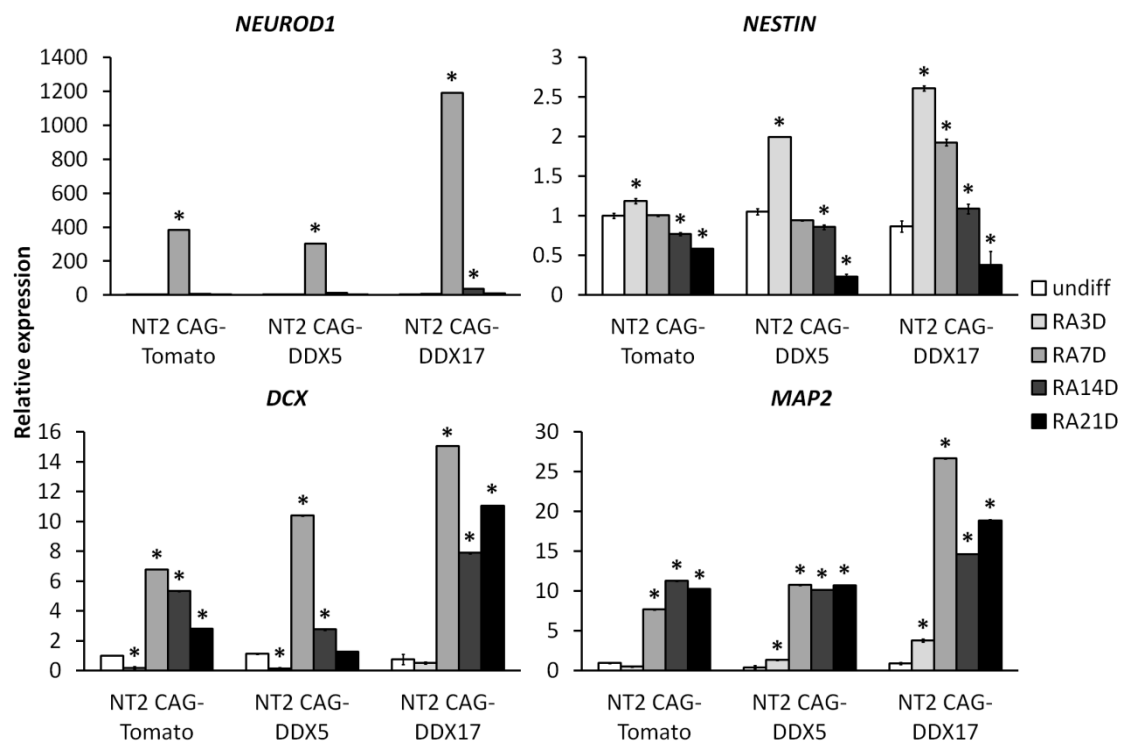


Figure 5.44: Gene expression profile of neuronal-related genes; *NEUROD1*, *NESTIN*, *DCX*, and *MAP2*; in DDX5 and DDX17 over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n = 3, *p<0.05 compared with undifferentiated sample of its own cell line).

Finally, neuronal differentiation was confirmed by immunocytochemical staining with β III-tubulin antibody in cells that were treated with retinoic acid for 30 days (Figure 5.45). The top panel demonstrated percentage of cells with β III-tubulin immunoreactivity, while the bottom panel showed the representative images from each cell line. Total positive cells were dramatically lower in both over-expressing cell lines compared with control. However, when taken into account about mature neuronal morphology, neurons differentiated from NT2 CAG-DDX17 were significantly higher than the other two cell lines. Even though DDX5 over-expressing cells differentiated into mature neurons, the percentage to all cell population was very small.

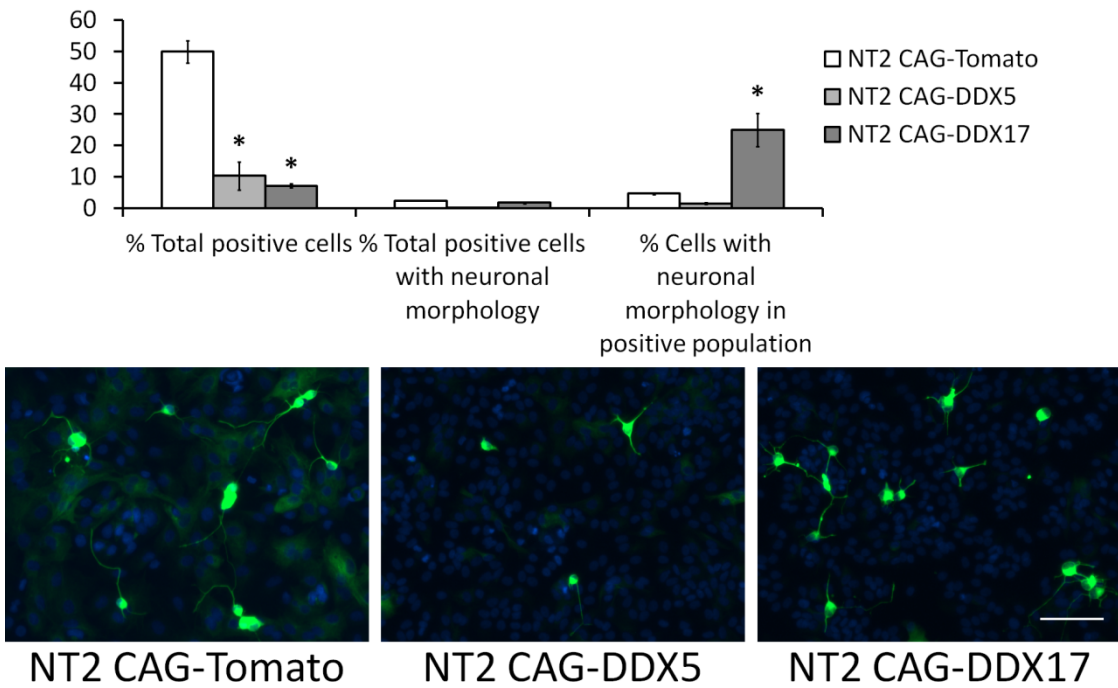


Figure 5.45: Fluorescent images taken from cells differentiated from DDX5 and DDX17 over-expressing cell lines comparing with control. Cells were differentiated with 10 μ M retinoic acid treatments for 30 days. On the last day of treatment, cells were dissociated and replated on 24-well plate for 24 hours. They were stained with β III-tubulin (green) and DAPI (blue). Cell numbers were count manually and present as percentage of immunoreactive cells ($\pm$ SEM; n = 3, *p<0.05). Scale bar = 100 μ m.

5.9 Characterization of PUS1 over-expressing NTERA2 cell lines

Next, effect of PUS1 over-expression on NTERA2 differentiation was tested, compared with control transfected NTERA2 cell line (NT2 CAG-Tomato) and over-expression of catalytic inactive PUS1 mutant (NT2 CAG-D146A). After cell line establishment, protein samples were collected from confluent culture of all 3 cell lines. Western blot analysis was done to confirm over-expression of PUS1, both wild type and mutant (Figure 5.46). Neural differentiation potential was assessed after treatment with 10 μ M retinoic acid for 3, 7, 14 and 21 days compared with undifferentiated culture. Flow cytometric analysis and measurement of mRNA expression were done at each time point of treatment in all 3 cell lines.

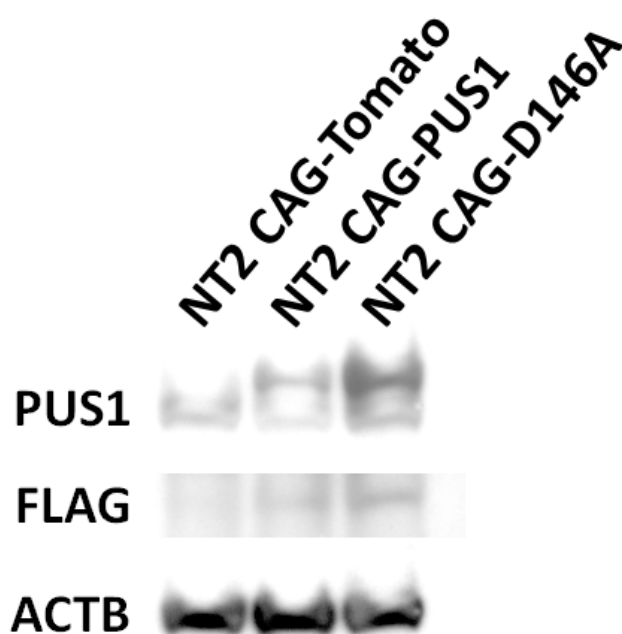


Figure 5.46: Western blot analysis showed increased expression of PUS1, both wild type and catalytic inactive mutant, in transfected cell lines compared to control transfection cell line. FLAG-containing proteins also visualized on a separate blot to ensure that the increased expression of PAX6 was from exogenous one produced by transfected transgene.

5.9.1 Stem cell property of PUS1 over-expressing cell lines

FACS analysis of stem cell-related surface markers showed that loss of SSEA4- and TRA-1-60-positive population was faster in NT2 CAG-PUS1 compared to NT2 CAG-Tomato and NT2 CAG-D146A (Figure 5.47), even though the populations were not different among the cell lines at undifferentiated state.

PUS1 wild type over-expression reduced mRNA level of *OCT4* and *NANOG*, while increased *SOX2* mRNA in undifferentiated cells compared to control transfection and PUS1 mutant over-expression (Figure 5.48). After treatment with retinoic acid, down-regulation of stem cell-related genes in NT2 CAG-PUS1 was faster than those of NT2 CAG-Tomato and NT2 CAG-D146A. Even though *SOX2* mRNA level was induced in differentiated NTERA2 CAG-PUS1, it was reduced to similar level of *SOX2* in other 2 cell lines after treatment with retinoic acid.

These results indicated that over-expression of PUS1 alone was able to reduce expression level of stem cell-related genes, *OCT4* and *NANOG*, which in turn reduced SSEA3-positive population in the culture and let the over-expressing cells differentiated faster when induced with retinoic acid.

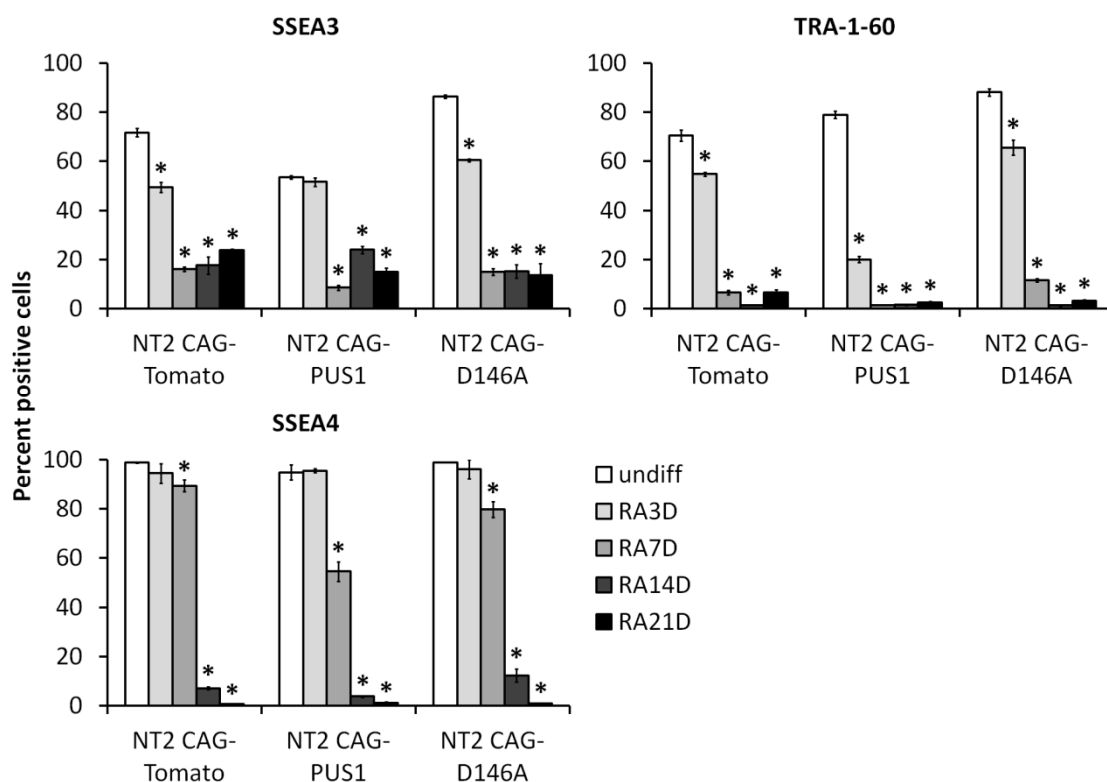


Figure 5.47: Flow cytometric analysis of stem cell-related surface markers from PUS1 and catalytic inactive PUS1 mutant over-expressing cell lines, compared with control transfected cells, at undifferentiated state and during differentiation with 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. The surface markers analyzed included SSEA3, SSEA4 and TRA-1-60. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n = 3$, * $p < 0.05$ compared with undifferentiated sample of its own cell line).

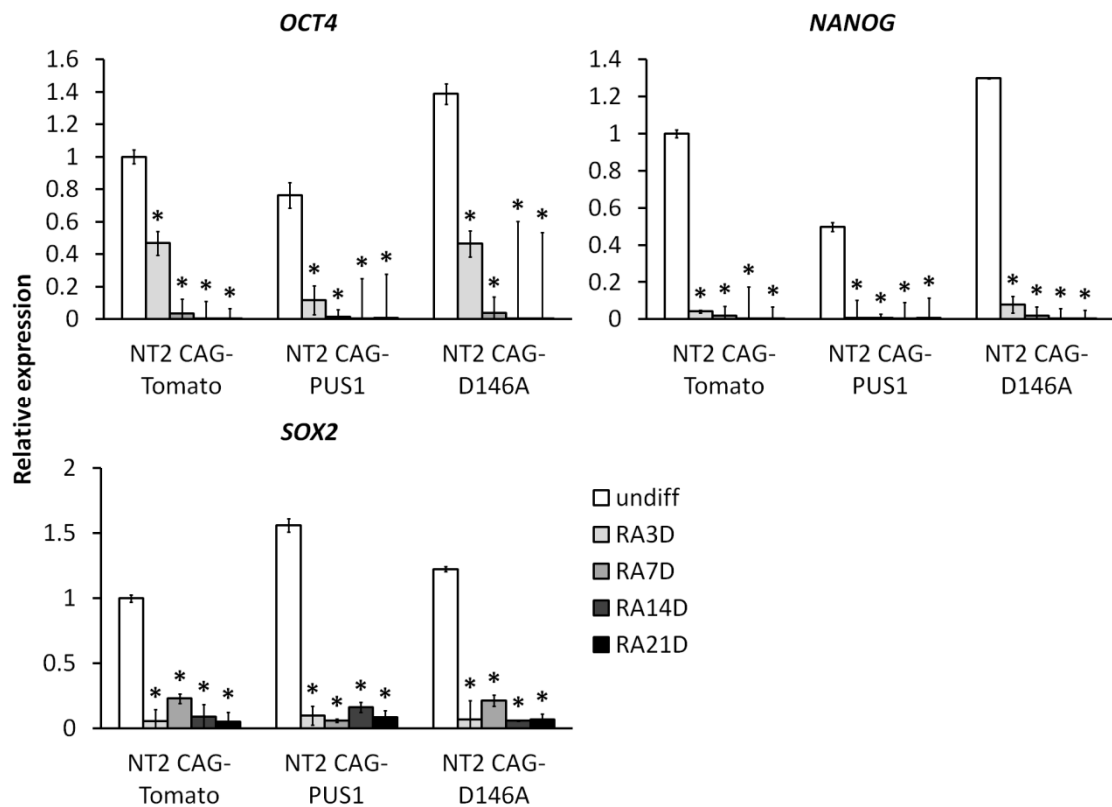


Figure 5.48: Gene expression profile of stem cell-related genes; *OCT4*, *NANOG*, and *SOX2*; in PUS1 and catalytic inactive PUS1 mutant over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; $n = 3$, * $p < 0.05$ compared with undifferentiated sample of its own cell line).

5.9.2 Differentiation potential of PUS1 over-expressing NTERA2 cell lines

To confirm differentiation potential of NT2 CAG-PUS1, flow cytometric analysis of differentiation related surface markers – SSEA1 and A2B5 – was done. Higher A2B5-positive population can be induced from NT2 CAG-PUS1 compared to NT2 CAG-Tomato and NT2 CAG-D146A (Figure 5.49). Population positive with A2B5 from undifferentiated culture from NT2 CAG-PUS1 was also highest (28.6%), compared to NT2 CAG-Tomato (2.1%) and NT2 CAG-D146A (10.7%). A2B5-positive population was induced to the highest level on day 14 of treatment in control and PUS1 mutant over-expressing cells, while those of NT2 CAG-PUS1 reached highest level on day 7 of treatment. However, PUS1 over-expression seemed to have no effect on SSEA1-positive population throughout the experiment.

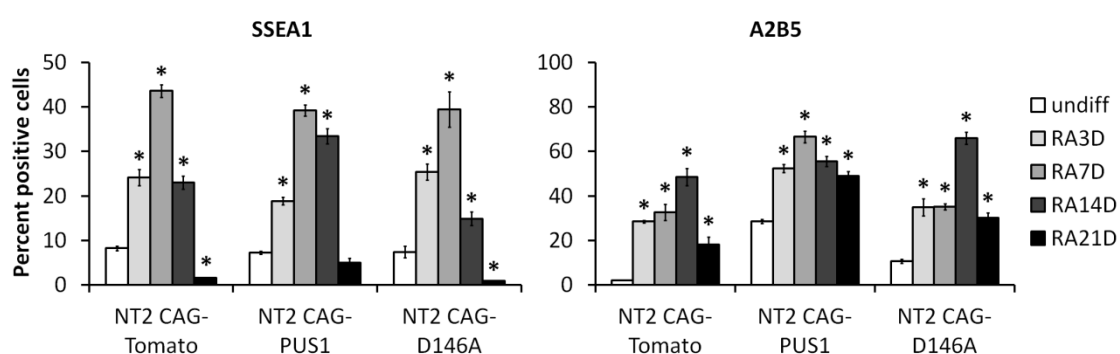


Figure 5.49: Flow cytometric analysis of differentiation related surface markers from PUS1 and catalytic inactive PUS1 mutant over-expressing cell lines, compared with control transfected cells, at undifferentiated state and during differentiation with 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. The surface markers analyzed included SSEA1 and A2B5. Population positive with each surface marker was calculated from biological triplication samples, analyzed in parallel. Results were shown as mean of percent positive population ($\pm$ SEM; $n = 3$, * $p < 0.05$ compared with undifferentiated sample of its own cell line).

PUS1 over-expression suppressed mRNA expression of transcription factors important for differentiation, except for neural lineage differentiation (Figure 5.50). *T* and *CDX2* were almost completely suppressed. Even though *GATA6* expression was induced in NT2 CAG-PUS1, the expression level was still lower than those of NT2 CAG-Tomato and NT2 CAG-D146A. Up-regulation of *PAX6* in NT2 CAG-PUS1 showed different pattern from NTERA2 cells, lacking spike of *PAX6* expression and remained at certain level at every time point tested.

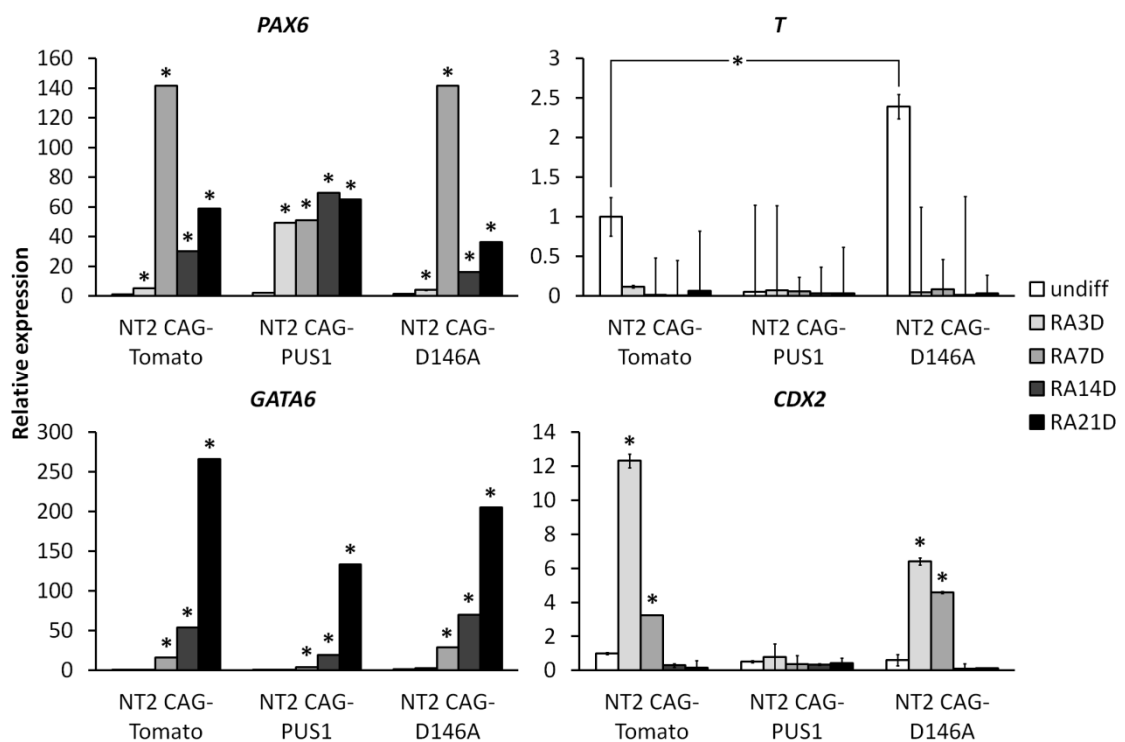


Figure 5.50: Gene expression profile of differentiation associated transcription factors; *PAX6*, *T*, *GATA6*, and *CDX2*; in PUS1 and catalytic inactive PUS1 mutant over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n = 3, *p<0.05 compared with undifferentiated sample of its own cell line).

NEUROD1, *NESTIN* and *MAP2* mRNA expression was also up-regulated in retinoic acid-treated NT2 CAG-PUS1 (Figure 5.51). Up-regulation of *NEUROD1* in NT2 CAG-PUS1 was higher than that of NT2 CAG-Tomato, while *NEUROD1* expression of NT2 CAG-D146A showed only subtle changes. *NESTIN* mRNA induction was dramatically higher in NT2 CAG-PUS1 at day3 of retinoic acid treatment. *MAP2* mRNA expression was at high level since the undifferentiated state, and can be induced to higher level after retinoic acid induction. On the other hand, *DCX* expression was the only gene that show reduction after induction in NT2 CAG-PUS1 compared to control.

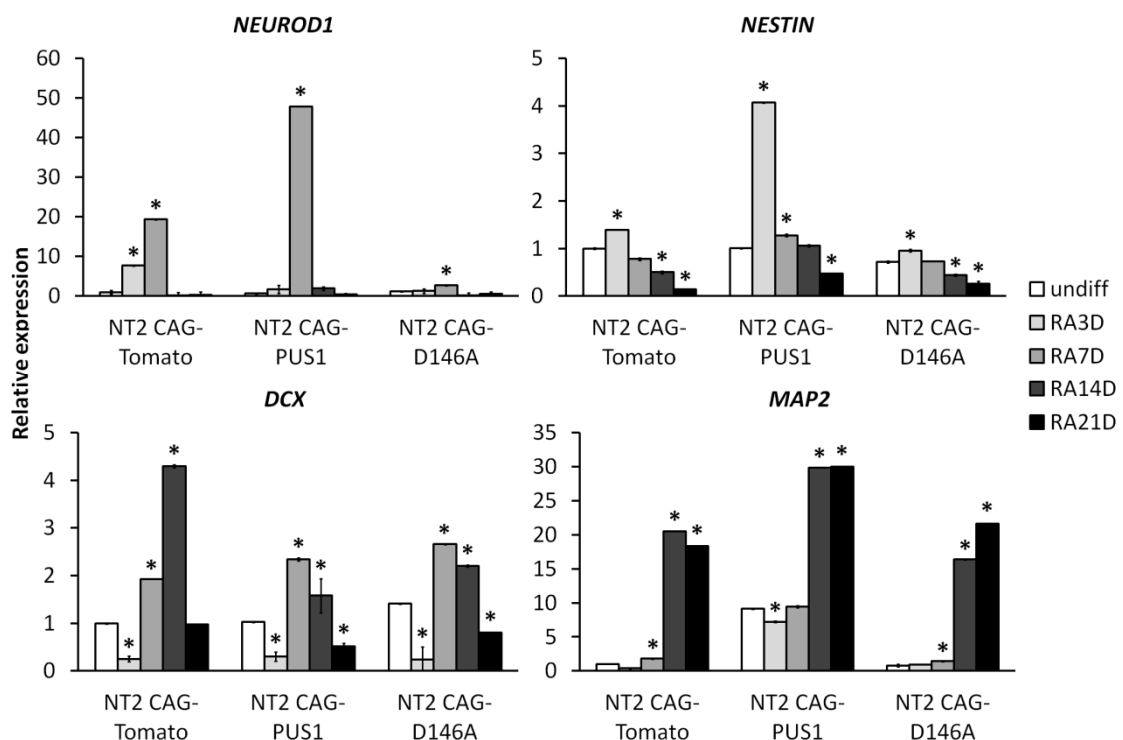


Figure 5.51: Gene expression profile of neuronal-related genes; *NEUROD1*, *NESTIN*, *DCX*, and *MAP2*; in PUS1 and catalytic inactive PUS1 mutant over-expressing cells in undifferentiated cells or following 10 μ M retinoic acid treatments for 3, 7, 14 and 21 days. Results were shown as mRNA level relative to undifferentiated-control transfected cells ($\pm$ SEM; n = 3, *p<0.05 compared with undifferentiated sample of its own cell line).

Neuronal differentiation was confirmed by immunofluorescent staining with antibody against neuronal specific marker, β III-tubulin, in cells treated with retinoic acid for 30 days (Figure 5.52). The top panel demonstrated percentage of β III-tubulin positive population, while the bottom one was the representative images taken from each cell line. NT2 CAG-PUS1 differentiated substantially lower than control, while almost every cell in NT2 CAG-D146A expressed β III-tubulin. However, when looking for immunoreactive cells with neuronal morphology, they can be rarely observed from both over-expressing cell lines.

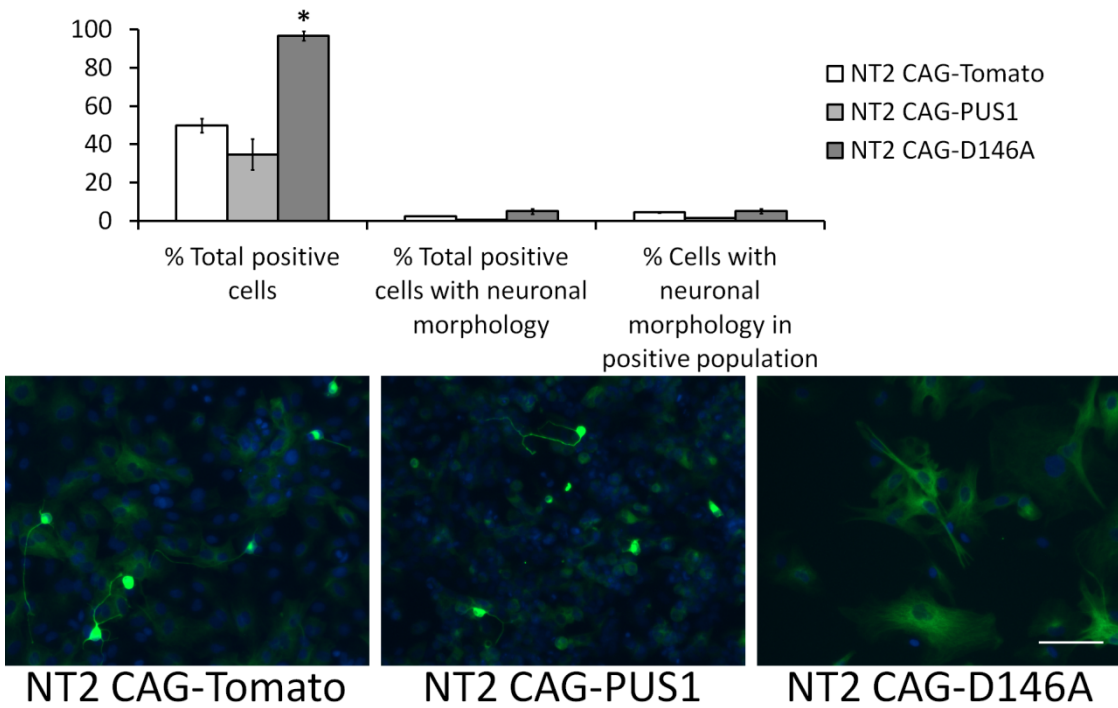


Figure 5.52: Fluorescent images taken from cells differentiated from wild type and mutant PUS1 over-expressing cell lines comparing with control. Cells were differentiated with 10 μ M retinoic acid treatments for 30 days. On the last day of treatment, cells were dissociated and replated on 24-well plate for 24 hours. They were stained with β III-tubulin (green) and DAPI (blue). Cell numbers were count manually and present as percentage of immunoreactive cells ($\pm$ SEM; n = 3, *p<0.05). Scale bar = 100 μ m.

CHAPTER VI

DISCUSSIONS

6.1 PAX6 over-expression induces neural differentiation of pluripotent embryonal carcinoma NTERA2 cells

The transcriptional factor PAX6 has been reported to be important for inducing differentiation at different stages in neural lineage. During development of cortical structure, PAX6 was found to induce radial glia cells differentiation and to be specifically expressed in radial glia population of the developing cortex, not elsewhere (Englund *et al.*, 2005; Götz *et al.*, 1998). In addition to radial glia commitment, PAX6 was also shown to maintain number of neural progenitor cells of the developing cortex as well (Quinn *et al.*, 2007). The role of PAX6 in radial glia differentiation has been demonstrated in the system of human embryonic stem cells as well (Suter *et al.*, 2009). Moreover, in 2010, PAX6A isoform has been shown to induce spontaneous differentiation from human ES cells into neuroectodermal lineage, the earliest stage of neuro-developmental process (Zhang *et al.*, 2010).

In this study, over-expression of PAX6A led to spontaneous differentiation of NTERA2, while this is not the case for PAX6B over-expression. We have shown by flow cytometric analysis of stem cell-related surface markers that PAX6A over-expression alone reduces stem cell properties of NTERA2 cells, while the effect cannot be seen in PAX6B over-expression. Number of cells positive with SSEA3, SSEA4, and TRA-1-60 were reduced by PAX6A over-expression. Expression at mRNA level of *OCT4* and *NANOG* was found to be reduced, while that of *SOX2* was not changed in both over-expressing cell lines. The result is consistent with other studies, which show that PAX6 has an ability to suppress expression of stem cell-related genes such as *OCT4* and *NANOG*, and at the same time, to activate expression of another stem cell-related gene *SOX2* (Wen *et al.*, 2008; Zhang, *et al.*, 2010). Even though PAX6B is also capable of inhibit expression of *OCT4* and *NANOG* mRNA,

higher level of expression of *OCT4* in PAX6B over-expressing cell line compared to those of PAX6A indicates that PAX6A is more potent in repressing *OCT4* expression.

PAX6A over-expression maintains high proportion of neural differentiated cells during retinoic acid treatment, while PAX6B allow majorly neural lineage differentiation after treatment start. This was shown by A2B5 positive population from PAX6A over-expressing cells, which were the majority of the population even without differentiation induced by retinoic acid. Nonetheless, A2B5 positive population can be highly induced in PAX6B over-expressing cells at day 7 compared with control NTERA2. These results suggest that not only PAX6 isoform A but also isoform B which are neurogenic in nature consistent with a previous study suggesting that PAX6B also plays a role in development of the nervous system (Carbe *et al.*, 2013). Since PAX6B over-expression did not reduce expression level of stem cell-related surface markers and genes as much as PAX6A did, PAX6B over-expressing cells are, somehow, able to withstand differentiation process. The neurogenic property of PAX6 is supported by homozygous PAX6 mutation study in mouse ES cells, which show reduced ability to differentiate into neural lineage (Quinn *et al.*, 2010). Neurogenic function of PAX6 is also confirmed by RNAi experiment in human ES cells, which also shows that PAX6 knockdown cells lose their neural differentiation propensity (Zhang, *et al.*, 2010). The study also suggested that PAX6 is a prime transcription factor that determines neuroectodermal fate decision from human ES cells.

In this study, the results have also shown that, even though the bHLH *NEUROD1* was up-regulated during differentiation, its expression level in PAX6 over-expressing cells was lower than the control. This result suggests that neuronal differentiation in PAX6 over-expressing cells might be achieved via pathway(s) regulated by pro-neural gene(s) other than *NEUROD1*. *NEUROD1* has been implicated in the process of terminal differentiation from progenitor cells to mature neuronal cells at different region of the brain (Boutin *et al.*, 2010; Cho & Tsai, 2004). However, it has been shown that PAX6 is associated with promoter region and substantially promote expression of *NEUROD1* gene in mouse embryonic cortical tissue as well as in rat pancreatic islet cells (Gosmain *et al.*, 2010; Sansom *et al.*, 2009). Another explanation for this result is that down-regulation of *NEUROD1* is an indirect effect from changes in overall phenotypic changes as a result PAX6 over-

expression. Two potential candidates are bHLH transcription factor *HES-1* and *HES-5*, which have been shown to be induced by PAX6 and to negatively regulate transcriptional process of *NEUROD1* gene (Akazawa *et al.*, 1992; Sansom, *et al.*, 2009).

6.2 PAX6 over-expression induces cell death in nullipotent embryonal carcinoma N2102Ep cells

N2102Ep cells are nullipotent counterpart of pluripotent NTERA2 cells that are incapable of response to differentiation induction treatment (Andrews *et al.*, 1980; Duran *et al.*, 2001). However, the profiles of surface markers expression as well as gene expression of N2102Ep are very similar to human ES cells (Skotheim *et al.*, 2005; Sperger *et al.*, 2003). Thus, nullipotent N2102Ep cell line has been suggested to be one of reference tool for studying ES cells as well as other pluripotent stem cells (Josephson *et al.*, 2007). Therefore, since PAX6 has been shown to induce spontaneous differentiation in NTERA2 cells, an attempt to study the ability to induce differentiation in N2102Ep by PAX6 was done by similar over-expression experiment. Unfortunately, N2102Ep stably expressing PAX6 was unable to be maintained in culture after several days of puromycin selection.

The toxicity of PAX6 over-expression in N2102Ep cells comes from the *PAX6* transgene, but not the plasmid itself. This notion is supported by the successful establishment of control over-expression N2102Ep cell line, N2102Ep CAG-Tomato, leaving the only possibility that *PAX6* transcripts might induce cell death in N2102Ep cells through apoptosis. Studies using over-expression of PAX6 have shown that apoptotic cell death is induced in several cell types including human cervical cancer cell line, rabbit corneal epithelial cell line, and human glioblastoma cell line (Ouyang *et al.*, 2006; Zhang *et al.*, 2010; Zhou *et al.*, 2005).

To summarize the effect of PAX6 over-expression in NTERA2 and N2102Ep cells, Figure 6.1 depict the result from the experiments as well as hypothetical standpoint for both cell lines. PAX6 over-expression in NTERA2 down-regulates expression of stem cell-related genes as well as up-regulates expression of neural-related genes, leading to differentiation into neural lineage. PAX6 over-

expression in N2102Ep results in extensive cell death with unknown cause. Possibilities include direct toxic effect of PAX6 over-expression on cell viability and/or other intracellular cues, which maintain nullipotent state of N2102Ep, leading to cell death mechanism rather than to undergo differentiation.

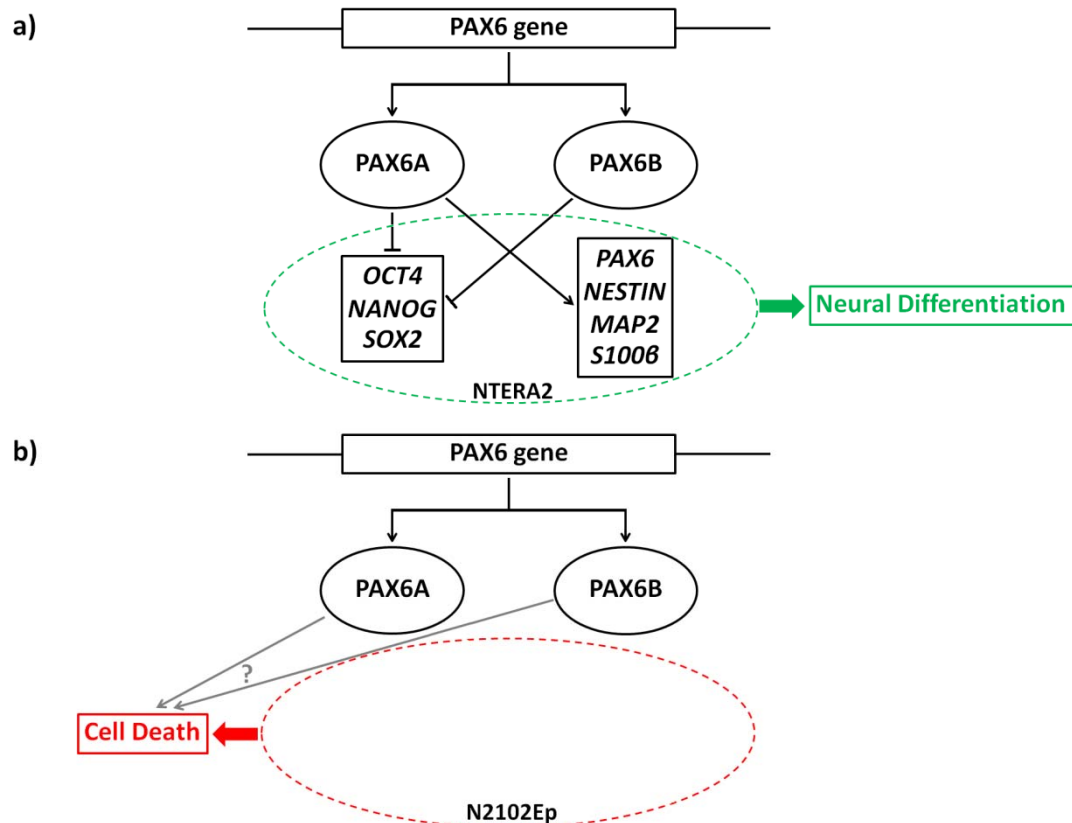


Figure 6.1: Effect of PAX6 over-expression in a) NTERA2 and b) N2102Ep cells.

6.3 Inhibition of histone lysine de-methylase (LSD1) activity is insufficient to induce differentiation of N2102Ep cells

Epigenomic status is unique in each cell type, which can be changed due to overall gene expression in cells such as induced pluripotent stem cells (Liang & Zhang, 2013). There are several epigenetic mechanisms discovered until now including DNA methylation and histone modifications, which involve in both activation and repression of the genomic region they are governed. Methylation at different lysine residues of histone protein has been used to determine state of gene

activity at the particular region. Tri-methylation at lysine 4 of histone H3 (H3K4me3) is a marker for active gene, while tri-methylation at lysine 9 and 27 of histone H3 (H3K9/27me3) are repressive markers (Black *et al.*, 2012). Lysine-specific de-methylase 1 (LSD1), which is a member of the histone de-methylase family, removes methyl group from di- or mono-methylated, but not tri-methylated, of lysine 4 and 9 residue of histone H3 (Shi *et al.*, 2004). Thus, de-methylation with LSD1 activity would prevent switching on and off several genes. On the other hand, inhibition of LSD1 would eventually let chromatin structure to achieve stable tri-methylated lysine residue of histone H3 protein, either active H3K4me3 or repressive H3K9me3. This activity of LSD1 in maintaining pluripotent state has already been shown in human ES cells (Adamo *et al.*, 2011).

TCP, a LSD1 inhibitor, has been reported to be another target for cancer therapy as it can induce differentiation from other cancer stem cells (Schenk *et al.*, 2012; Zhu *et al.*, 2014). The effect of LSD1 inhibitor on NTERA2 proliferation and cell growth has been demonstrated (Wang *et al.*, 2011). The mechanism of LSD1 inhibitor-induced cell differentiation has been shown in SOX2-positive cancer cells by reduction of stem cell-related gene *SOX2* expression, promoting cell cycle arrest as well as selectively change methylation status of genes involved in differentiation (Zhang *et al.*, 2013). Activity of LSD1, together with another histone lysine de-methylase KDM5B, has been shown recently to regulate methylation level at promoter of genes important for pluripotency retention in ES cells (Kidder *et al.*, 2014). In the present study, inhibition of LSD1 activity by TCP or pargyline together with retinoic acid treatment were employed to test whether they can induce differentiation of nullipotent EC cells N2102Ep. However neither TCP nor pargyline, could enable nullipotent N2102Ep to differentiate after induction with retinoic acid treatment. Changes in population expressing stem cell-related surface markers as well as expression level of several differentiation related genes did not differ between vehicle-treated group and LSD1 inhibitor-treated group. This result suggests that inhibition of LSD1 activity is not sufficient for N2102Ep to undergo cell differentiation.

Epigenetic barrier preventing N2102Ep from differentiation may comprise from a complex of epigenetic factors, since inhibition of either DNA methylation, histone de-acetylation or LSD1 cannot permit N2102Ep to differentiate. Recent

evidence has shown that LSD1 and HDAC1 present in a similar transcriptional repression protein complex, CoREST. LSD1 and HDAC1 modulate binding of each other to the complex and are “mutually dependent” in term of functional consideration (Yin *et al.*, 2014). LSD1 has been shown to interact with another DNA methyl-transferase, DNMT1, and be recruited to genomic region where DNMT1 is activated (Clements *et al.*, 2012). On the other hand, loss of LSD1 in ES cells results in instability and loss of function of DNMT1 as well (Wang *et al.*, 2009). Thus, an epigenetic manipulation using chemical inhibitors aimed at differentiation of the nullipotent stem cells N2102Ep might require a combined treatment of inhibitors which target different pathways of epigenetics.

6.4 Association of RNA signaling molecules at regulatory regions of *PAX6* gene during differentiation of NTERA2 cells

Expression of *PAX6* at transcriptional level is controlled by several regulatory elements including promoters and enhancers. In this experiment, we chose to demonstrate association of RNA signaling molecules; including CTCF, DDX5 and PUS1, on 4 regulatory elements on genomic region of *PAX6* gene, including 2 promoters and 2 enhancer regions, using chromatin immunoprecipitation (ChIP) technique. We have shown different patterns of association of each protein at particular site during differentiation of NTERA2 cells. Overall, the binding of these transcriptional regulators generally reflect expression status of *PAX6*.

In this experiment, association of CTCF on regulatory regions of *PAX6* gene is found to bind strongly at intronic enhancer region in undifferentiated NTERA2 cells. As the differentiation process continues, stronger association is found at *PAX6* P1 promoter with highest association on day 7 after retinoic acid treatment corresponding to highest level of *PAX6* transcripts. At the same time, association of CTCF at intronic enhancer region is also restored, to a higher level of binding than that in undifferentiated state, before gradually reduced as the differentiation proceed. CTCF has been reported to repress *PAX6* mRNA expression in mouse ES cells as well as human corneal epithelial cell line (Gao *et al.*, 2011; Wu *et al.*, 2006). However, repressive function of CTCF in these reports has been shown to mediate via

interrupting transcriptional activation at P0 promoter, while in this study, dynamic of CTCF at P0 promoter seems to be irrelevant to mRNA expression of *PAX6*. Unfortunately, action of CTCF at other regions of *PAX6* gene has yet to be shown. An important function of CTCF is to form an insulator on chromatin structure, which seems to be the most relevant explanation for inhibitory effect of CTCF on *PAX6* transcription (Bell *et al.*, 1999). This indicates that, at undifferentiated state, *PAX6* is kept silenced by CTCF governed over enhancer region. However, it still cannot explain how increasing association of CTCF to P1 promoter after differentiation is induced. One possibility is that CTCF is brought to P1 promoter via protein-protein interaction, and this protein should be a transcriptional factor that is promote mRNA expression of *PAX6*. As transcriptional process of *PAX6* is increased, binding of this factor to the promoter increases as well as CTCF binding. A promising target for this hypothesis is *PAX6* itself. *PAX6* has an autoregulation mechanism, by which it can bind to its own promoter to increase transcriptional level (Aota *et al.*, 2003). However, evidence showing direct protein-protein interaction between these two factors should be demonstrated in order to confirm this hypothesis.

This study is the first one showing that DEAD-box RNA helicase DDX5 has association to regulatory regions of *PAX6* gene. It shows extremely strong association with intronic enhancer element in undifferentiated NTERA2. As differentiation proceeds, major binding site of DDX5 shifts from intronic region to P1 promoter. Notably, at P1 promoter, the pattern of association of DDX5 is similar to CTCF, supporting our prior hypothesis that they form a protein complex together. DDX5 has been shown to directly interact with and be important for insulator function of CTCF (Yao *et al.*, 2010). This is in agreement with our results that DDX5 knockdown suppress level of *PAX6* mRNA, while DDX5 over-expression enable higher *PAX6* expression during differentiation with retinoic acid treatment (Figure 5.38 and 5.43). Since DDX5 might be in a complex with CTCF, in this context, The primary role of DDX5 might be to facilitate CTCF function on regulation of *PAX6* gene at CTCF binding sites.

This is also the first report showing that PUS1 might play a role in transcriptional control, apart from post-transcriptional RNA modification (Cantara *et al.*, 2011). Association of PUS1 to *PAX6* regulatory elements also shows interesting

pattern. It binds to both enhancer regions, while the only promoter bound by PUS1 at undifferentiated state is P1 promoter. We also show that manipulation of PUS1 affect expression of *PAX6*. Loss of PUS1 reduces *PAX6* expression, while gain of PUS1 function increase *PAX6* mRNA level (Figure 5.38 and 5.50). Differential activity of each promoter during development of the CNS has been shown in mouse embryo (Anderson *et al.*, 2002). P1 promoter has been utilized highly for both telencephalon and diencephalon development, while P0 promoter usage is much lower. Together with our result, specifically association of PUS1 to P1 promoter suggests a role of PUS1 for development of the CNS, which still has to be elucidated. Increased binding of PUS1 to P1 promoter, while those to enhancers reduce, after retinoic acid treatment indicates that positive regulatory role of PUS1 on *PAX6* gene is mediated mainly via activation at P1 promoter. However, since PUS1 is not a DNA binding protein, there might be a transcriptional factor tethering in PUS1 to *PAX6* regulatory regions.

Figure 6.2 summarizes association of these factors on transcriptional regulatory elements of *PAX6* gene. Four regulatory regions of *PAX6* gene are depicted, not in scale. Strength of binding signal of each protein to each regulatory region is represented by amount of protein bound on the gene locus. Since we cannot demonstrate association of *SRA* with ChIP experiment, we assume that it would be in the same complex with CTCF and DDX5 as reported in 2010 (Yao, *et al.*, 2010).

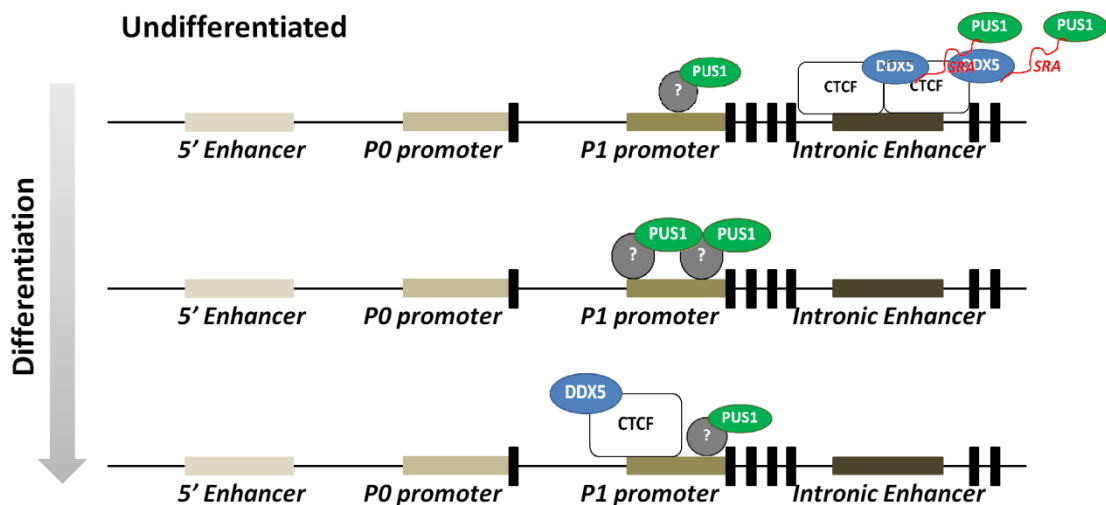


Figure 6.2: Association of RNA signaling proteins; CTCF, DDX5, and PUS1, on regulatory elements of PAX6 gene during differentiation of NTERA2 with retinoic acid treatment.

6.5 Differentiation inducing potential of long non-coding RNA, Steroid receptor RNA activator (SRA), in NTERA2 cells

To demonstrate a role of SRA RNA in differentiation of NTERA2, SRA knockdown and over-expression cell lines were established. SRA knockdown cell line establishment is success, however the over-expression cell line fails to increase SRA RNA level. The failure might be due to the choice of plasmid used in the experiment. SRA over-expression is driven under CMV promoter, which has been shown to be silent in pluripotent stem cell line such as human ES cells (Liew *et al.*, 2007; Norrman *et al.*, 2010). In this case, SRA transgene might not be transcribed due to silencing of the CMV promoter in NTERA2 cells.

This study is the first report a role of lnc SRA RNA in human pluripotent stem cell. Knocking-down SRA results in reduction of cell population positive for stem cell-related surface markers, especially SSEA3 and TRA-1-60. In consistent with flow cytometric results, mRNA level of stem cell-related genes are reduced as well. SRA-knockdown NTERA2 cells also fail to form pluripotent colony in cloning efficiency assay. These results suggest that SRA is involved in maintaining stem cell state in NTERA2 cells. Studies in human ES cells have identified several lnc RNAs that

potentially involved in pluripotency state of ES cells, and induce differentiation of ES cells after knockdown with siRNA (Ng *et al.*, 2012).

Using expression level of differentiation-related gene and population expressing differentiation-related surface markers as surrogates, results indicate that of *SRA*-knockdown NTERA2 cells undergo differentiation. A2B5-positive population in *SRA*-knockdown NTERA2 culture is increased by 2 fold compared to control knockdown cell line. Differentiation-related transcription factors are down-regulated, except *PAX6*. These results suggest that *SRA* might involve directly with control of pluripotent property of NTERA2, since knockdown cause cell differentiation. They also point out that *SRA* might as well involve in *PAX6* transcriptional regulation as a weak negative regulator or might not be involved at all, but the involvement is in regulation of transcription factor for other lineage commitment. However, evidence regarding influence of *SRA* on these transcription factors is still lacking.

6.6 Differentiation inducing potential of DEAD-box RNA helicases, DDX5 and DDX17, in NTERA2 cells

Many members of RNA helicase family have been shown to be involved in cell differentiation process, including DDX5 and DDX17, with unclear mechanisms (Abdelhaleem, 2005). In the present study, DDX5 knockdown NTERA2 cell line loses pluripotent state shown by reduced clonal ability, even though percentage of population expressing stem cell-related surface markers is on the same level with that of control knockdown cell line. In fact, DDX5 knockdown results in up-regulation of stem cell-related genes, *OCT4* and *NANOG*. DDX5 has been shown to be a member of micro RNA processing complex, which possesses a function to inhibit expression of self-renewal machineries in mouse ES cells (Fukuda *et al.*, 2007; Wang *et al.*, 2007).

Knockdown of DDX5 in NTERA2 also reduces mRNA expression of *PAX6*, while highly increase expression of *T* and *GATA6* at the same time. Unfortunately, there is still no report linking between regulatory roles of DDX5 on transcription of these genes. Even so, it has been shown that DDX5 can act as both co-activator and co-repressor for transcriptional regulation, depending on the complex it presents and regulatory elements where the complex binds (Wilson *et al.*, 2004).

Using this as a standpoint, DDX5 might function as co-activator for *PAX6* as well as co-repressor for *T* and *GATA6* transcriptional process during differentiation of NTERA2.

Another important consideration is that the results of SRA-knockdown and DDX5-knockdown cell lines are in opposite direction. We earlier hypothesized that they are in the same complex and may function together in mediating differentiation of NTERA2. However, our results indicate that SRA and DDX5 do not always work together in the similar pathway, at least in differentiation of NTERA2.

Over-expression of DDX5 in NTERA2 cells increase expression of transcriptional factors related to differentiation of all 3 germ layers, including *PAX6*, *T*, and *GATA6*, during cell differentiation with retinoic acid treatment. DDX5 has been shown to be expressed in neural and mesodermal parts of xenopus, chick and mouse embryos (Seufert *et al.*, 2000), raising a question that it might play a role in differentiation into these lineages during development. Rate of decline in number of cells expressing stem cell-related surface markers as well as stem cell-related gene, *OCT4* and *NANOG*, are greater in DDX5 over-expressing cell line compared to control. These results indicate that DDX5 promote differentiation of NTERA2 cells. Even though DDX5 and SRA have been reported to promote NOTCH signaling pathway, which is involved in neural differentiation, through mediating activity of NICD (Jung *et al.*, 2013), up-regulation of neural-related genes studied in this experiment is only slightly increased compared to that of control.

Over-expression of DDX17 in NTERA2 cells induce mainly neural differentiation as shown by up-regulation of neural-related genes (*PAX6*, *NEUROD1*, *NESTIN*, *DCX*, and *MAP2*) as well as reduced induction of genes for other lineages (*T* and *GATA6*). Even though overall β III-tubulin positive cells in DDX17 over-expressing group is lower than control, cells exhibited mature neuronal morphology is especially higher than control. These results suggest that DDX17 is more specific to neuronal differentiation and maturation than that of DDX5. It is supported by a report in 2000 showing that, expression level of DDX17 is gradually increased after differentiation of NTERA2 (Ip *et al.*, 2000).

6.7 Differentiation inducing potential of Pseudouridylate Synthase 1 (PUS1) in NTERA2 cells

This study is the first to demonstrate that manipulation of PUS1 level in pluripotent stem cells affect their self-renewal and differentiation. Knockdown of PUS1 expression results in loss of clonogenic ability, which might reflect loss of self-renewal. Stably increased level of PUS1 expression also reduces population expressed stem cell-related surface marker, SSEA3. When differentiation is induced with retinoic acid treatment, population expressing stem cell-related marker quickly reduces as well. Loss of stem cell characteristic is also supported by reduction in mRNA level of stem cell-related genes, *OCT4* and *NANOG*, even though *SOX2* level is found to be elevated.

Over-expression of wild type PUS1 highly increases percentage of A2B5-positive population and maintains high percentage after retinoic acid treatment, while moderately high percentage can be induced in mutant PUS1 over-expressing cells. This result indicates that PUS1 over-expression favors neural differentiation in NTERA2 cells. Wild type PUS1 also increases level of *PAX6* mRNA expression, even before retinoic acid treatment. This result is consistent with previous result in association of PUS1 with *PAX6* regulatory regions, where PUS1 is presented highly at P1 promoter of *PAX6* gene. After over-expressed, PUS1 might be gathered at the promoter and recruit another factor that activates transcription of *PAX6* gene. This is also the mechanism mediating increased level of *SOX2* expression as well. Since both *SOX2* and *PAX6* are important for development in neuroectodermal lineage, they might share similar regulatory mechanism with each other (Gómez-López *et al.*, 2011; Malchenko *et al.*, 2014). Another possible explanation is that PUS1 activity might change post-transcriptional modification, since expression profiles in mutant PUS1 over-expressing cells are not different from control. Unfortunately, these can still be only the hypothesis that has to be further tested.

Wild type PUS1 over-expressing cells also express high level of neuronal-related mRNA during differentiation with retinoic acid treatment. On the other hand, expression level of these genes in mutant PUS1 over-expressing cells is comparable with control. Even though over-expression changes level of mRNA expression, immunocytochemical analysis suggest otherwise. Only small numbers of cells are

β III-tubulin positive cells. In addition, almost none of mutant PUS1 over-expressing cells develop into cells with mature neuronal morphology. These results suggest that PUS1 might not involve in terminal differentiation and maturation into neuronal cells, even though PUS1 favor differentiation into neural lineage.

CHAPTER VII

CONCLUSION

In the present study, we have shown that

1) Over-expression of PAX6 induces neural differentiation from pluripotent EC cells, NTERA2, especially PAX6A isoform.

2) Over-expression of PAX6, both isoforms, induces cell death in nullipotent EC cells, N2102Ep, rather than induce differentiation.

3) Inhibition of histone lysine de-methylation, LSD1, activity in N2102Ep cannot overcome epigenetic mask preventing cells from differentiate in response to retinoic acid treatment.

4) RNA signaling molecules, including CTCF, DDX5 and PUS1, show dynamic association with regulatory elements of *PAX6* gene during differentiation process of NTERA2 cells.

5) DDX5 and PUS1 have a role to induce neural differentiation, while a role of long non-coding RNA – *SRA*- is to prevent neural differentiation in NTERA2 cells.

6) Over-expression of DDX5 and DDX17, another RNA helicase homolog to DDX5, favor neural differentiation from NTERA2 induced by retinoic acid treatment. DDX17 over-expressing NTERA2 give rise to high proportion of cells with neuronal processes, even though overall β III-tubulin positive population is markedly reduced in both DDX5 and DDX17 over-expressing cell lines.

7) Over-expression of wild type PUS1 hastens the process of neural differentiation. However, both wild type and mutant PUS1 over-expressing NTERA2 rarely differentiate into mature neurons.

In conclusion, we have demonstrated that PAX6, especially PAX6A isoform, is important for neuronal differentiation from pluripotent stem cell line. Transcriptional control of *PAX6* mRNA seems to be involved with RNA signaling

process. RNA signaling molecules have been shown to participate in transcriptional regulation on *PAX6* gene. PUS1 is recruited to *PAX6* promoter at the early stage of differentiation. DDX5 and CTCF are recruited to the promoter for maintaining high level of *PAX6* mRNA expression. DDX5 and PUS1 also exhibit a role in inducing neural differentiation from NTERA2 cells as well. Even though both DDX5 and PUS1 are not DNA binding proteins, modification of their expression can effect neural differentiation of NTERA2. These suggests an involvement of RNA signaling molecule as a complex with other DNA binding proteins on regulation of neural differentiation process from pluripotent stem cell line.

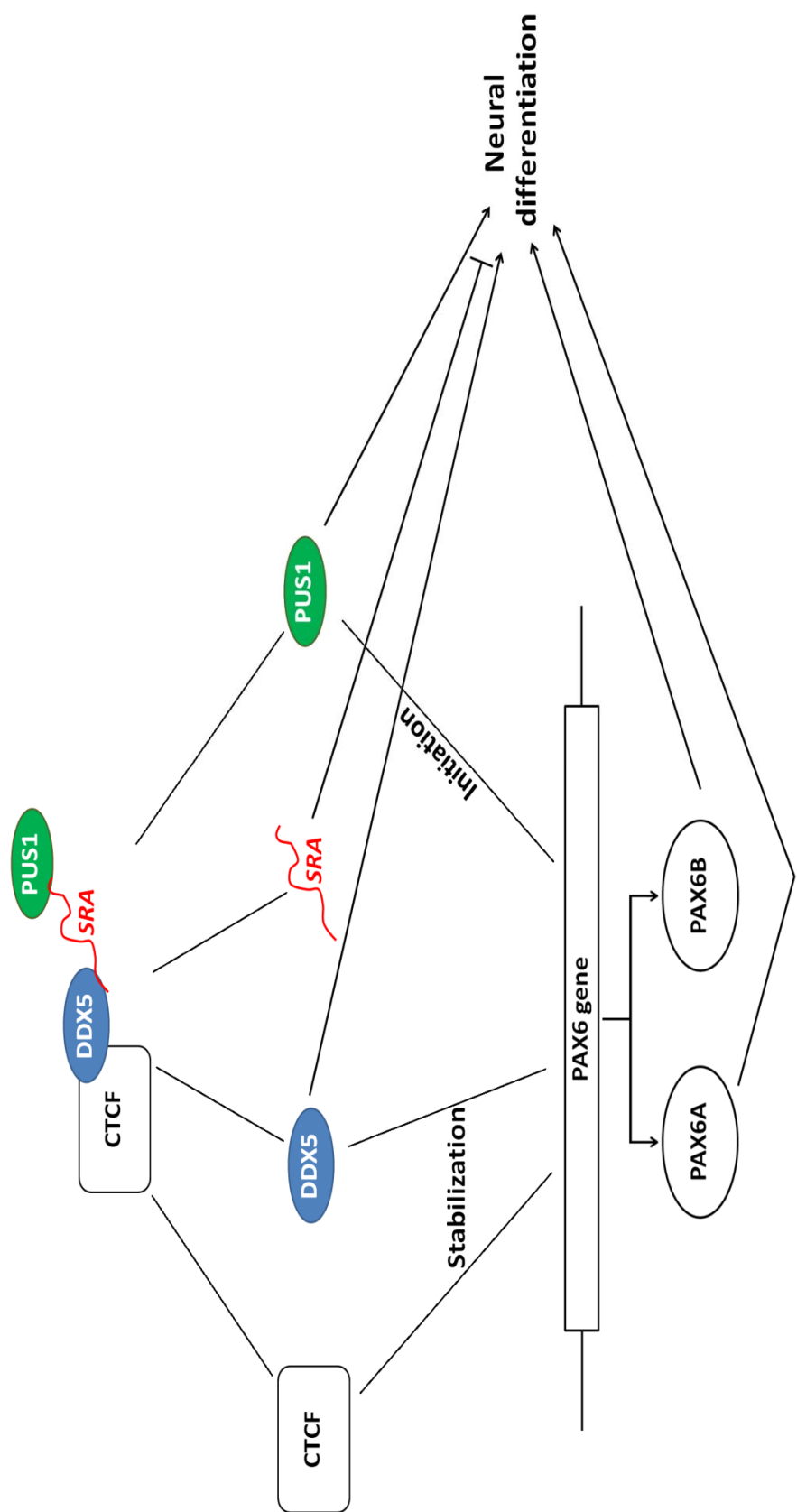


Figure 7.1: Diagram illustration of conclusions of this study. PAX6A and PAX6B are both neurogenic in NTERA2 cells, with higher potent exhibited from PAX6A. A role of SRA in differentiation of NTERA2 is to suppress neural differentiation. On the contrary, over-expressions of DDX5, DDX17, and PUS1 favor neural differentiation of NTERA2 upon induction with retinoic acid. At transcriptional level, a role of PUS1 is involved in initiating transcription of PAX6 gene, while those of CTCF and DDX5 are to stabilize transcriptional machineries.

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