Y Chromosome Genes May Play Roles in Directed Differentiation of Human Embryonic Stem Cells.

Farzaneh Khani
National Institute of Genetic Engineering & Biotechnology (NIGEB)
Simin Nafian Dehkordi
National Institute of Genetic Engineering & Biotechnology (NIGEB)
Sepideh Mollamohammadi
ACECR
Shiva Nemati
ACECR
Bahare Shokoohian
ACECR
Mehdi Alikhani
ACECR
Seyedeh Nafiseh Hassani
ACECR
Hossein Baharvand
ACECR
Hamid Reza Soleimanpour-lichaei (hrs149@yahoo.com)
National Institute of Genetic Engineering & Biotechnology (NIGEB)
Ghasem Hosseini Salekdeh
ACECR

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Abstract

The human Y chromosome harbors genes that are mainly involved in the growth, development, sexual dimorphism, and spermatogenesis process. Despite many studies, the function of the male-specific region of the Y chromosome (MSY) awaits further clarification, and a cell-based approach can help in this regard. In this study, we have developed four stable transgenic male embryonic stem cell (ESCs) lines that can overexpress male-specific genes *HSFY1, RBMY1A1, RPS4Y1, and SRY.* As a proof of principle, we differentiated one of these cell lines (*RPS4Y1* over-expressing ESCs) to the neural stem cell (rosette structure) and characterized them based on the expression level of lineage markers. RPS4Y1 expression in the Doxycycline-treated group was significantly higher than control groups in transcription and protein levels. Furthermore, we found Doxycycline-treated group had a higher differentiation efficiency than the untreated control groups. Our results suggest that the *RPS4Y1* gene may play a critical role in neurogenesis. Also, the generated transgenic ESC lines can be widely employed in basic and preclinical studies, such as sexual dimorphism of neural and cardiac functions, the development of cancerous and non-cancerous disease models, and drug screening.

1. Introduction

The human Y chromosome is a male sex-determination chromosome and escapes meiotic recombination. Despite its small size and limited gene content, the Y chromosome has crucial roles in development, differentiation, as well as gender-based diseases, which are often confined to the hypothesis (Bellott et al., 2014).

The Y chromosome harbors 47 protein-coding genes, where 35 of them have protein evidence (PE) at protein level (PE1), 5 proteins at the transcript level (PE2), and 7 genes encoding uncertain proteins (PE5) (https://www.nextprot.org/about/protein-existence). Therefore, Chromosome-centric Human Proteome Project (C-HPP) was launched in 2012, to identify human missing proteins and investigate their functions. (Meyfour et al., 2019). The human Y Chromosome Proteome Project (Y-HPP) is trying to recognize the functions of the Y chromosome's proteins (Jangravi et al., 2013).

In pursuit of this goal, several studies have been undertaken, including a tumor suppressor role for *KDM5D* in the prostate cancer cell line (Jangravi et al., 2015) and new roles for *DDX3Y* in neural differentiation of NTERA-2 human embryonal carcinoma cells (NT2) (Vakilian et al., 2015), as well as *TBL1Y* and also *KDM5D* in cardiac differentiation of hESC (Meyfour et al., 2019, Meyfour et al., 2017a).

Since many genes are silent in adult cells, investigating the functions of their proteins is challenging. To meet this challenge (pluripotent stem cells) PSCs might be used as a valuable tool to achieve proteins and missing proteins function, which is also a target of the Y chromosome project (Alikhani et al., 2020).

These studies highlighted the remarkable role of some genes that help to candidate the target genes for more investigations. Among genes of interest, theexpression of *RPS4Y1* showed sexual dimorphism of

the kidney, and *SRY* mediated kidney development and played a role in the blood pressure control in males (Meyfour et al., 2017b).

The expression of *RBMY1*, *HSFY1*, *RPS4Y1*, and *SRY* increased during the neural cell differentiation of the NT2 cell line (Vakilian et al., 2015). Furthermore, The overexpression of *RBMY1*, *HSFY1*, *RPS4Y1,* and several other MSY genes, was observed at the transcriptional level during the cardiac differentiation of hESCs (Meyfour et al., 2017a).

Genes located in the azoospermia factor (AZF) regions have a critical role in spermatogenesis and fertility, and AZF microdeletion can impair their functions. Our target genes, *RBMY1A1* and *HSFY1*, are in the AZF region. The *RBMY1* family consists of approximately 30 genes and pseudogenes in six subgroups on the Y chromosome. *RBMY1A1* contains a conserved domain called the SRGY motif (serine, arginine, glycine, and tyrosine) that modulated its function. *RBMY1* plays a significant role in cardiac development, spermatogenesis, infertility, prostate cancer, and hepatocellular carcinoma (HCC) (Meyfour et al., 2017b, Chua et al., 2015, Kido et al., 2020, Jangravi et al., 2013, Dasari et al., 2001).

The Heat Shock Factors (HSFs) are a family of transcription factors that encode chaperones (Duchateau et al., 2020). Their critical role is stress responses in abnormal conditions such as oxidative stress, thermal stress, hypoxia, and protein degradation (Chatterjee and Burns, 2017). They also participate in gametogenesis, embryonic development, and the integrity of the organ. Deregulation of HSFs could be a risk factor for reproductive failure, cancer, neurogenesis, and neurodegenerative disorders (Ma, 2000). *HSFY* (heat shock transcription factor, Y chromosome) is a member of HSFs and has been located in the AZFb region of the Y chromosome (Rosenfeld, 2017). This gene is associated with spermatogenic failure, infertility (Peng, 2009; Zenteno-Ruiz, 2001), varicoceles (Meyfour, 2017), maturation arrest (MA) (McCann-Crosby, 2014), sertolicell-onlysyndrome (Meyfour, 2017) and plays a crucial role in the brain and cardiac development (Meyfour et al., 2017b).

SRY (sex determining region Y) encodes a transcription factor (a member of the high mobility group [HMG]-box family) (Jangravi et al., 2013) and involved in spermatogenesis, male sex determination, brain sexual differentiation, epigenetic processes, brain, cardiac and kidney development (Kamachi and Kondoh, 2013, Rosenfeld, 2017, Peng et al., 2009, Meyfour et al., 2017b).

Moreover, *SRY* and some *SOX* genes participate in carcinogenesis. Some diseases associated with *SRY* include 46, Xx / 46, XY Sex Reversal 1 (Zenteno-Ruiz et al., 2001), campomelic dysplasia (CD) (Foster et al., 1994), gonadal dysgenesis (Swyer syndrome) (Hunter et al., 2016); testicular regression syndrome (TRS)(Hunter et al., 2016), Turner's syndrome (Oliveira et al., 2009), infertility and azoospermia (Hamada et al., 1807), testicular (Kido and Lau, 2015) and prostate cancers (Ely et al., 2010), and brain disorders (Wu et al., 2009).

SRY expression and function in the human brain have been examined in several studies (Rosenfeld, 2017, Wu et al., 2009, Kido et al., 2017). *SRY* suppression in the human neuroblastoma cell line led to a down-regulation in enzymes involved in the dopamine synthesis of males and may describe the cause of more

susceptibility of males in dopaminergic-based neurological disorders (Parkinson's disease and schizophrenia) (Loke et al., 2015).

Although the *SRY* may be needed for the normal function of the male brain, an aberrant expression of this gene could impair neurogenesis and other disorders in mice pups (Kido et al., 2017). The *SRY* expression level in primed hESCs was higher than naive and embryoid bodies derived from hESCs (Taleahmad et al., 2019, Dehkordi et al., 2021 Apr). The results of the *SRY* knockdown in the primed cells showed upregulation of pluripotency markers and *WNT* pathway target genes in si-SRY treated cells and also increased the expression of several Y chromosome-linked genes in the primed cells (Taleahmad et al., 2019).

The *RPS4Y1* encodes the S4 ribosomal protein, a part of the 40S subunit (Jangravi et al., 2013). This gene is associated with heart failure, turner syndrome, infertility, gonadoblastoma, prostate cancer, Parkinson's disease, GVHD, and also participated in the brain and cardiac development (Meyfour et al., 2017b, Heidecker et al., 2010, Jangravi et al., 2013, Dasari et al., 2001, Fisher et al., 1990, Nielsen et al., 2010, Miklos et al., 2005, Sun et al., 2014).

Each of the target genes has various molecular and biological functions in different signaling pathways and human diseases (Table 1). Although, the precise mechanisms of functions have not been entirely understood. Most of the information that researchers identified are speculations based on the similarity of homologous regions to other genes. Gene Ontology (GO) of target genes is listed in Table 1.

Based on the studies, we candidate *HSFY1*, *SRY*, *RBMY1A1,* and *RPS4Y1* genes for more investigations (Skuse, 2000, Serajee and Mahbubul Huq, 2009, Lau and Zhang, 2000). To this end, we described development of the transgenic cell lines over-expressing target genes in an inducible manner. Then, they were characterized in terms of karyotype, pluripotency, and integrated gene expression level. We also discussed the applications of them in clinic and research.

In this study, we used a cell-based approach, using hESCs to generate four inducible cell lines able to increase gene of interest expression. As a proof of principle, we then differentiated one of these cell lines (*RPS4Y1* over-expressing ESCs) to the neural stem cells.

RPS4Y1 is expressed during prenatal and infancy; indicating, it could play a critical role in early brain development (Meyfour et al., 2017b). Our results indicate a possible sex-dependent regulation of neural development that might underlie sexual dimorphism of human brain.

2. Results

2.1 Cell line generation and characterization

We cloned the genes of interest by gateway technology and verified them with DNA sequencing. After the production of the lentiviral particles, the transduced cells were titrated using puromycin treatment until all of the un-transduced cells (negative control) were died (Supplementary Fig 1. A

Before cell transduction, we required to know about the multiplicity of infection (MOI) of the target cells. We found out, at MOI of 40, almost 30% of cells were transduced. (Supplementary Fig 3. A-D). Furthermore, the cellular toxicity of polybrene was assessed. The optimal polybrene concentration for hESC-RH6 was 6 µg/ml (Supplementary Fig 3. E, F).

Following 10 to14 days, the stem cell colonies were formed and were transferred into the new plates (Fig 2. A). *Morphological characteristics of transgenic cell lines were observed* using light microscopy. Then their pluripotency was confirmed by alkaline phosphatase activity assay. The expression level of the pluripotency markers (*OCT4, NANOG*, and *SOX2*) were evaluated by qRT-PCR (Fig 1. A, B) and there were no significant differences between over-expressing cell lines and hESC-RH6 in pluripotency markers. The qRT-PCR results were statistically analyzed by *t*-test and SD's and showed a significant rise ($P < 0.05$). Giemsa banding pattern was shown a normal karyotype for new stable transgenic cell lines (Supplementary Fig 4. A).

The mock cell line (RH6-GFP) was characterized based on morphological characteristics. Doxycycline-induced expression of the mock promoter was observed by fluorescence microscopy. Purple dye detection in alkaline phosphatase staining confirmed the pluripotency state (Fig 1. C).

For the characterization of transgenic stable cell lines, PCR performed using puromycin primers and the accuracy of gene integration was verified. After that, the DNA band size of the puromycin resistance gene (383 bp) was confirmed. In the following *comparison between the* three cell groups, we have shown differences between our target genes expression in hESC-RH6 and untreated transgenic cell lines (as controls) with treated transgenic cell line (by Doxycycline) in transcript level (Fig 2. B, C).

Moreover, Western blot analysis confirmed the overexpression of our target proteins in the treated transgenic cell line (DOX positive) versus untreated (DOX negative) (Fig 2. D).

2.2 Neural differentiation

2.2.1 Characterization based on qRT-PCR, immunocytochemistry assay and western blotting

After the development of stable transgenic cell lines, "*RPS4Y1* over-expressing ESCs" were differentiated to neural stem cells. Following the *RPS4Y1* overexpression, the Rosette structures in the test group (treated by DOX) were more elegant and more than control groups (wild-type and untreated transgenic cell lines), especially on the last day (Fig 3. A, B).

The results of the immunofluorescence test showed an increase in RPS4Y1 protein expression in the treated group compared to untreated group in the process of neural differentiation (Fig 3, C). Moreover, the rosette markers (SOX1 and NESTIN) were increased in the treated group versus untreated group using immunostaining (Fig 3, D).

Based on Western blot data, RPS4Y1 upregulation in ESC-RH6 at pluripotent state (Day 0) was observed, and then RPS4Y1 downregulation during neural differentiation (Day 3) was seen. In the end, RPS4Y1

expression was re-increased (Day 8) (Fig 3.E). The transgenic-untreated group (DOX negative) was shown the same trend, but doxycycline treatment changed that in the treated group in comparison with the control groups (Fig 3.E).

To prove the neural differentiation, we showed overexpression of *SOX1*, *PAX6*, and *NESTIN* in all three groups (test and controls) by qRT-PCR. An increasing trend in all groups was observed, but in the treated group (DOX positive), neural lineage markers (*SOX1*, *PAX6*, and *NESTIN*) upregulated significantly compare to the control groups (Fig 3, F-H).

In the pluripotent state (ESC-RH6 on day 0), we observed *RPS4Y1* upregulation, and after inducing neural differentiation, *RPS4Y1* expression decreased (day 3) and on the last day (Day 8) re-increased. The same trend was in the transgenic cell lines; however, the difference in *RPS4Y1* expression of the treated group (DOX positive) was significantly more than the control groups (Fig 3. I).

3. Discussion

Although much of our current knowledge of the primary cell lines are derived from mouse models, the mice have weak similarities to the human in the developmental process (Petropoulos et al., 2016, Hughes and Page, 2016). These discrepancies are not only in autosomal chromosomes but also in the sexual and could make hESCs modeling a desirable alternative (Meyfour et al., 2017a, Hassani et al., 2019).

Manipulation of the MSY genes expression can provide clues about the functional roles of these proteins. The Y chromosome harbors genes that are mainly involved in the growth, development, spermatogenesis of the male genus, and deletion or defect in chromosomal regions can lead to developmental anomalies and fertility problems (Ma et al., 2000, Ahmadi Rastegar et al., 2015). In addition to the fertility and sexual development of male embryos, MSY genes are involved in a diverse range of biological functions (Jangravi et al., 2015, Vakilian et al., 2015, Meyfour et al., 2017b, Taleahmad et al., 2019).

The importance of balance in the expression of genes is obvious, and any alterations in copy number or expression level of a wild-type gene can lead to mutated phenotypes (Kido and Lau, 2015).

In this regard, the researchers investigated the functions of some MSY genes by knocking down gene expression. Knockdown of *RPS4Y1*, *KDM5D DDX3Y TBL1Y,* and *SRY expression* studied in human or mice models, and thereby the novel roles for these genes discovered in the brain, cardiac, carcinogenesis, pluripotency, etc. (Kido and Lau, 2015, Qin et al., 2019).

Overexpression experiments may be a powerful tool for linking genes to biological pathways. Generally, overexpression of individual genes performs for two purposes: desired amounts of gene products are obtained that can be used in drug screening and other studies. On the other hand, possible biological functions of target gene products can be determined (Prelich, 2012).

One of the most validated ways toward gain-of-function studies is to generate stable cell lines, and the best technique for gene integration in hESCs is transduction by lentiviruses (Magrin et al., 2019, Milone

and O'Doherty, 2018). After the generation of over-expressing stable cell lines, monitoring of the gene integration efficiency and the induced target gene overexpression during the differentiation is essential. Moreover, maintenance of pluripotency, viability, and normal morphology of the cells should be considered.

In addition to many roles, we have already mentioned for the Y chromosome, genes on this chromosome play crucial roles in brain function. Gender differences in brain development and prevalence of neuropsychiatric disorders such as depression, autism, parkinson, schizophrenia, and attention-deficit/hyperactivity disorder (ADHD) were reported. Expression of sex chromosome genes may contribute to gender differences in brain functions, and act independently of gonadal hormones (Liu et al., 2017, Kopsida et al., 2009).

One study investigated the effects of increasing in the copy number of genes. Data showed that increased doses of Y chromosome genes are associated with the risk of autism-related behaviors and ADHD symptoms in male with XYY syndrome. In this study, *NLGN4Y* expression level, through the effects on the synaptic function, is related to more severe degrees of the autism (Ross et al., 2015). Another study reported a set of preeclampsia molecular risk factors, which may lead brain development toward an inclusive risk of autism. One of the these stimuli, is the gene encoded by the Y chromosome; *RPS4Y1,* a STAT3 signaling inhibitor, that acts as a sex-based differential factor in male dominance in Autism (Xie et al., 2020).

In the current study, stable transgenic cell lines, were generated using lentivectors. First, we cloned target genes, produced lentiviral particles, and transduced ESC-RH6 cells *via* lentivectors. Ultimately, we generated four stable transgenic cell lines contain genes of interest (*HSFY1*, *SRY*, *RBMY1A1*, and *RPS4Y1*), and one as the mock cell line containing the same vector but expressing GFP reporter instead of the target gene. These transgenic cell lines, in addition to displaying the characteristics of the hESCs could overexpress the target genes by doxycycline treatment. The accuracy of the gene of interest integration was confirmed at the DNA level. The karyotype of the transgenic cell lines was normal, and also we showed the pluripotency potential in the test and control groups. Furthermore, we reported differences in mRNA expression level between test and control populations (DOX positive and negative) and proved that the mRNA expression level in the test group (DOX positive) was significantly higher than controls. Additionally, we showed these significant changes at the protein expression level between the test and control groups.

As a proof of principle, to investigate the overexpression effects in the differentiation process, the *RPS4Y1* over-expressing cell line, was differentiated to neural stem cells based on the literature. Then, we evaluated the expression of neural and pluripotency markers, as well as *RPS4Y1* on days 0, 3, and 8 in both test and control groups. The morphological and molecular changes of the test and controls were monitored during neural differentiation up to the formation of rosette structures. In general, following the *RPS4Y1* overexpression, differentiation efficiency increased and the number of Rosette structures in the treated group was more than control groups, especially on the last day. On the other hand, *the*

RPS4Y1 expression pattern at the transcription and protein levels indicates a decrease in the expression of this gene in all groups on day 3, followed by an increase in expression on day 8 of neural differentiation, compared to the undifferentiated state.

Therefore, it could be concluded that in the natural state, the *RPS4Y1* gene increases the efficiency of neural differentiation and may contribute to create Rosette structures. Accordingly, *RPS4Y1*, as a structural protein of the ribosome, may play a vital role in the development of the brain and nerve cells.

Moreover, these stable transgenic cell lines are valuable tools for the gain-of-function studies in Y chromosome-linked genes. Inducible overexpression of target genes during differentiation into the desired cell fate may lead to changes in the efficiency or morphology of the differentiated cells. These changes could indicate a possible modification in signaling pathways. Further analysis is required to identify a protein interaction network of target genes. The generated cell lines could be widely used in basic and preclinical studies, such as sexual dimorphism of neural and cardiac functions, the cancerous and non-cancerous modeling, and drug screening. These stable cell lines would provide a step forward in the identification of MSY genes functions and their network of protein interactions. Inducible overexpression of the MSY genes in female ESCs can use to further understanding of their roles and consider as promising progress in C-HPP goals.

4. Materials And Methods

4.1 Cell culture.

The male Royan H6 (RH6, RRID: CVCL_9387) line was obtained from Royan Stem Cell Bank (RSCB). The generation of hESC-RH6 has been previously described by Baharvand and colleagues [14]. hESCs were cultured under adherent condition in DMEM/F12 medium (Gibco Dulbecco's Modified Eagle Medium, Nutrient Mixture F-12) supplemented with 20% knock-out serum replacement (KSR, Gibco/BRL), 2mM L-glutamine (Gibco/BRL), 0.1 mM β–mercaptoethanol (Sigma-Aldrich, Germany), 1% nonessential amino acid (Gibco/BRL), 100 unit's/ml penicillin, 100 µg/ml streptomycin (Gibco/BRL), and 100 ng/mL basic fibroblast growth factor (bFGF). The cells were incubated at 37°C and 5% CO_2, and they were further passaged every 6-7 days.

4.2 Cloning.

The Y chromosome cDNA (*HSFY1, RPS4Y1,RBMY1A1,* and *SRY)* were prepared synthetically by Integrated DNA Technologies (IDT). YTA Plasmid DNA Extraction Mini Kit (Yekta Tajhiz Azma, Iran) was used for plasmid isolation. Templates were amplified by PCR at a T100™ thermal cycler (Bio-Rad, Hemel Hempstead, UK). The primers for the *target genes werelisted* in Supplementary *Table*1. We cloned target genes by the Gateway™ technology (Invitrogen, USA) according to the manufacturer's protocol. Amplification was performed by *using pfu DNA polymerase* (Thermo Fisher Scientific, USA).

attB1, and B2 fragments (attB1: GGGGACAAGTTTGTACAAAAAAGCAGGCT, attB2: GGGGACCACTTTGTACAAGAAAGCTGGGT) were *then added* to PCR products. Competent E. coli Stbl3 (Invitrogen, USA) was prepared and transformed. Gateway® destination vector of choice was pLIX-403 (was provided by Dr. David Root, Addgene 41395). All cloned fragments were confirmed by sequencing experiments at Macrogen Inc. (http://www.macrogen.com). All the plasmids were isolated using the Nucleobond-extra-maxi kit (MACHEREY-NAGEL, Germany) and prepared for lentivirus production.

4.3 Production of lentiviral particles.

We used a second-generation packaging vector including 5µg packaging vectors (psPAX2 and pMD2.G from Sigma-Aldrich, Germany) and 5µg inducible vector contains target genes (pLIX-403 vector -Tet-On) for lentiviral particles production. On the first day, the HEK293T cells were cultured at 5×10^5 cells/cm^2 in DMEM (Dulbecco's Modified Eagle's Medium) high glucose (Sigma-Aldrich, Germany) medium containing 10% FBS. As the cell confluency reached 70 -80 percent, the medium was refreshed. The cells were transfected with the cocktail of lentiviral vectors with polyethyleneimine (PEI 1µg / µL from Sigma-Aldrich, Germany) according to the manufacturer's protocol. The medium of cell culture was collected after 24 to 72 hours and filtered with 0.45 micrometers (Millipore, USA). Lentiviral particles were concentrated by centrifugation at 20,000 rpm for 2 hours at 4^{o}C. The biological titration of the lentiviral particles was performed using puromycin (2µg / mL) selection on HT1080 cells and crystal violet staining (Invitrogen protocol). The production and titration of lentiviral particles was shown in Supplementary Figure 1.A, B.

The optimal concentration is the lowest amount that kills around 100% of un-transduced cells versus transduced cells in 3-4 day (Duchateau, 2020). Before titration and antibiotic selection, the optimal antibiotic concentration was estimated by GFP labeled lentiviral particles *tittering* with flow cytometry (Dasari, 2001). These viral particles (GFP labeled) were used to determine the Multiplicity of infection (MOI) and also to produce a mock cell line (Supplementary Method 1) (Ma et al., 2003).

4.4 Cell culture and transduction.

After some passages, approximately 1×10^5 single hESCs were seeded in two tests, and one control well of a six-well Matrigel-coated plate. hESC medium with 10 mM Rho-associated protein kinase inhibitor (ROCKi; Sigma-Aldrich, Germany) was used for that. 24 hours after seeding, the cells were transduced with lentiviral particles that contained 6 µg/ml polybrene and incubated in the condition mentioned above (Supplementary Method 2).

4.5 Antibiotic selection and colony pickup.

The medium was refreshed 24 hours after transduction with the hESC medium. For the cell line selection, transduced cells were treated by the optimal dosage of puromycin for around 4-5 days, and the medium was refreshed every day until the stable colonies were observed. However, due to the toxicity of puromycin, an appropriate dosage needed to be determined (Supplementary Method 3). Consequently,

the colonies were picked up, and after some sub-culturing, were treated with 5 µg/mL Doxycycline (Takara Bio USA) for 24-65 hours. The new colonies called *HSFY1, RBMY1A1, RPS4Y1,* and *SRY* over-expressing transgenic cell lines.

4.6 Neural differentiation

We induced feeder-free hESC-RH6 and *RPS4Y1* over-expressing cell line that was differentiated for eight days into the neural stem cell in the Dulbecco's modified Eagle's medium and Ham's F-12 (Invitrogen, USA), 5% knockout serum replacement (KOSR; Gibco BRL, Paisley, UK), 1% nonessential amino acids (NEAAs; Invitrogen, USA), l-glutamine (2 mM; Invitrogen, USA), penicillin (100 U/mL), Streptomycin (100 mg/mL; Invitrogen, USA), b-mercaptoethanol (0.1 mM), Retinoic acid (10 µM; Sigma-Aldrich, Germany), 1% N2 (Invitrogen, USA), 0.5% B-27 minus vitamin A (Gibco BRL, Paisley, UK), Dorsomorphin (10 µM; Stemgent), SB431542 (10 µM; (Cayman Chemical, Ann Arbor, MI, USA), CHIR99021 (3 µm; Stemgent) and LDN193189 (5 µM; Stemgent). Also, we have divided the *RPS4Y1* over-expressing cell line into two gropes during neural differentiation. The first group was treated with Doxycycline and the second was untreated.

4.7 Characterization.

The pluripotency, karyotype, and mycoplasma contamination of over-expressing cell lines were investigated by alkaline phosphatase activity assay (Sigma-Aldrich, 86R, USA), G- banding chromosome analysis, and PCR assay, respectively. These experiments were performed based on the manufacturer's protocols.

4.8 Molecular confirmation of genomic insertion.

The genomic insertion of target genes in hESCs-RH6 is required to confirm by the PCR. Therefore, genomic DNA from resistant colonies was isolated manually (Supplementary Method 4). We used primer sequences of the puromycin resistance gene as follows:

(Forward: 5'- GGTCACCGAGCTGCAAGAAC -3', Reverse: 5'- GCTCGTAGAAGGGGAGGTTG -3'). 150 ng of the DNA template was pooled in a 20 µl total reaction volume containing the primers and Taq master mix 2x (Ampliqon, Herlev, Denmark). The cycling program and subsequent processes were provided in Supplementary Methods.

4.9 Quantitative real-time PCR (qRT-PCR).

Total RNA was extracted from cultured cells by the TRIzol® Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, USA). RNA purity was assessed by absorbance *measurement* of 260/280 and 260/230 ratios. All the extracted RNAs were purified after *DNase treatment* by Recombinant DNase I kit (TaKaRa, Japan), according to the manufacturer's instructions. cDNA was synthesized from 2 mg total RNA using Revert AidTM H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. PCR was performed for cDNA samples by specific primer sequences. The PCR products were analyzed by gel electrophoresis the *same*as in the Supplementary Method 5. qRT-

PCR was performed with SYBR green (TaKaRa, Japan) according to the manufacturer's protocol in a thermal Cycler Rotor-Gene 6000 (Corbett, Australia). The PCR mixture for each reaction was contained in a final volume of 20 µL (10 µL Rotor-Gene SYBR Green PCR Master Mix (TaKaRa), 25 ng cDNA and 3 pmol of each primer). Gene expression analysis was done by the ΔΔCT method and the normalization of the relative expression level was carried out against a housekeeping gene (GAPDH) expression level. The detailed information about the primers has been presented in Supplementary Table 1.

4.10 Western blot analysis.

The proteins were extracted using a Qproteome Mammalian Protein Prep Kit (Qiagen, Germany) according to the manufacturer's instructions. The protein quantitation and subsequent processes were presented in Supplementary Method 6 and details of sources and concentrations of antibodies used for western blot were included Supplementary Table 2.

4.11 Immunocytochemistry

Cells plated on a four-well Matrigel-coated plate (Sigma-Aldrich, Germany) were washed in PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, Germany) for one hour. Then cells were washed two times with PBS-Tween, permeabilized, and blocked in blocking buffer consisting of 0.1% Triton, secondary host serum, and 5% bovine serum albumin (BSA; Sigma-Aldrich, Germany) in Tris buffer for 20 min. Primary antibodies were added in blocking buffer for 45 min at room temperature or overnight in 4ºC and washed three times in with PBS-Tween. Primary and secondary antibodies and dilutions used were included Supplementary Table 2. The cells were incubated with secondary antibody diluted in blocking buffer according to supplementary table 2 for 45 min at room temperature. After two washes in PBS, the cells' nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) based on the manufacturer's protocol (Sigma-Aldrich, Germany), and cells were observed under the fluorescence microscope.

4.12 Statistical analysis.

Analysis by the qRT-PCR and western blot was performed with three biological and technical replicates (control and test). Significant differences between groups were statistically analyzed using an ordinary one-way, two-way ANOVA tests and a two-tailed unpaired student's *t*-test in Graphpad Prism 8 (Graphpad Software). Data are displayed as mean ± standard error of the mean (SEM) and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ indicated statistically significant in all the experiments.

Declarations

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Authors' Contributions

F.K., S.N.D.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. F.K.; Wrote the manuscript. F.K.; Provided scientific advice throughout the project and performed cell culture. HR.S. L, G.H.S; Supervised the project scientifically and contributed to establishing the main idea of the presented work and designing the experimental study. HR.S. L, G.H.S, H.B, and S.- N.H.; Contributed to financial support and final approval of the manuscript. All authors have read and approved the final version of this manuscript.

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Table

Table 1.

Gene ontology (GO) of target genes and related disorders. Abbreviations: *HSFY1*, Heat Shock Transcription Factor Y-Linked 1; *RBMY1A1,* RNA Binding Motif Protein, Y-Linked, Family 1, Member A1; *RPS4Y1,* Ribosomal Protein S4 Y-Linked 1; *SRY,* Sex Determining Region Y.

Gene list	Gene ontology			Disorders and diseases
	Biological process	Molecular function	Cellular localization	
HSFY1	Cellular response to heat Regulation of transcription by RNA polymerase II Regulation of transcription from RNA polymerase II promoter in response to stress Role in fertility	DNA binding transcription factor activity Protein binding RNA polymerase II transcription factor activity Sequence-specific DNA binding Transcription factor activity	Cytosol Nucleus	Azoospermia Cardiovascular system disease Embryonal carcinoma Oligospermia Sertoli cell-only syndrome Testicular cancer Varicoceles Y chromosome infertility
RMY1A1	Cell differentiation Gene editing regulation Immune system process Regulation of alternative mRNA splicing RNA processing and splicing System development	mRNA binding DNA binding Protein binding	Nucleus	Aortic stenosis Azoospermia Liver cancer Myotonic dystrophy Partial Deletion of Y Prostate cancer Sertoli cell-only syndrome Spermatogenic Failure, Y-Linked, 2
RPS4Y1	Activation of the mRNA upon binding of the cap-binding complex and eIFs, HIV Life Cycle Influenza Viral RNA Transcription and Replication Nuclear-transcribed mRNA catabolic	Protein binding RNA binding Structural constituent of ribosome	Nucleus Cytosol	Autism Azoospermia Duchenne muscular dystrophy Gonadoblastoma Graft-versus-host disease (GVHD) New-onset heart failure (HF)

	process rRNA processing in the nucleus and cytosol SRP-dependent cotranslational protein targeting to membrane Translational initiation Viral mRNA Translation			Parkinson Prostate cancer Turners syndrome
SRY	Androgen signaling pathway Cell differentiation Gene regulation GPCR signaling pathway Regulation of transcription Sex differentiation Wnt signaling pathway	A member of the HMG box family DNA binding and bending Protein binding TDF The maintenance of motor functions of (DA) neurons Transcription factor activity	Nucleus	Campomelic dysplasia (46,XY Sex Reversal) Glioma Gonadal dysgenesis (Swyer syndrome) Gonadoblastoma Infertility and azoospermia Possible role in brain disorders Prostate cancer Testicular regression syndrome Testis cancer Turner syndrome XX male syndrome (46,XX Sex Reversal)

Figures

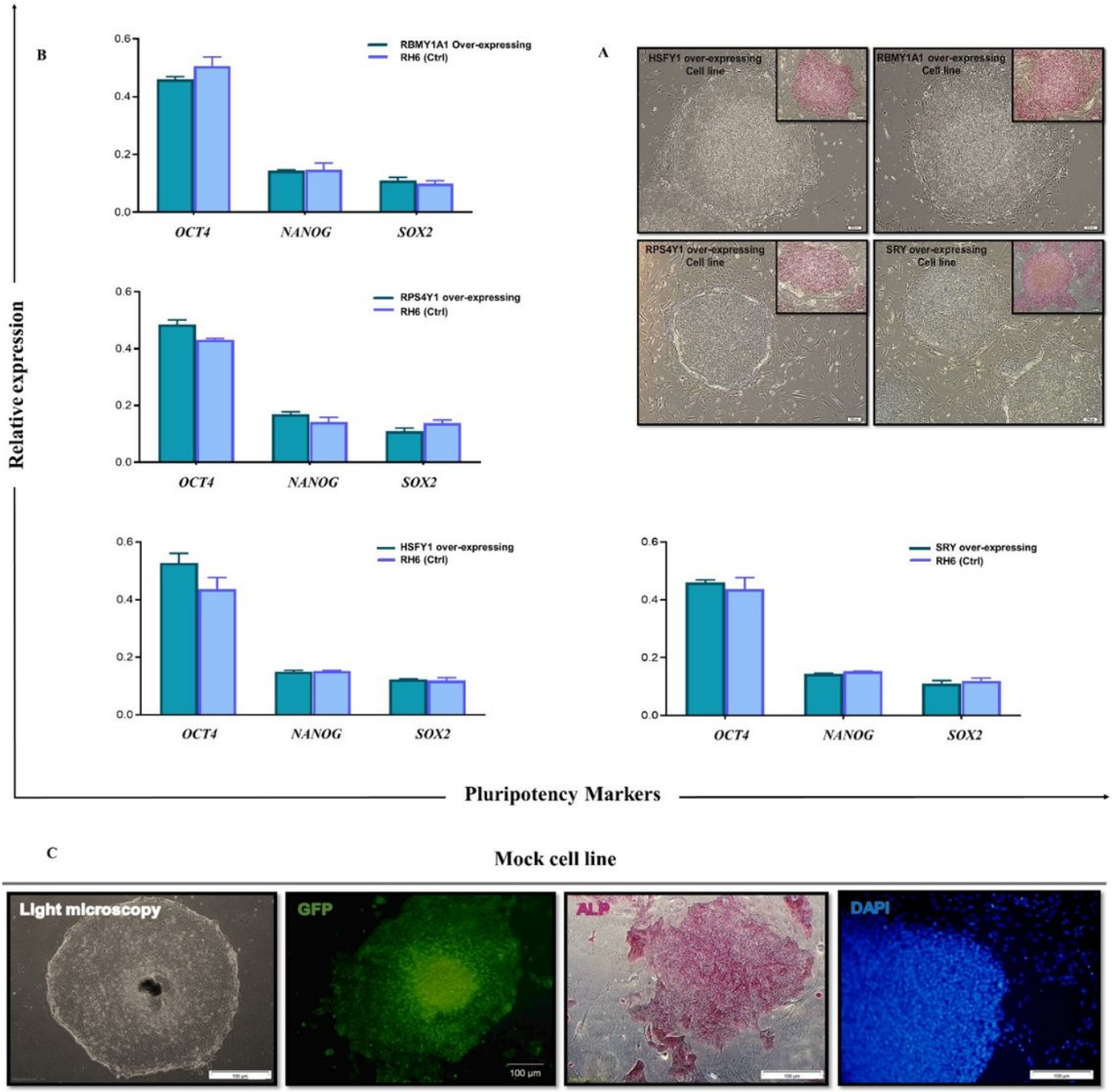

Figure 1

Morphology and pluripotency analysis. (A) Microscopic images and alkaline phosphatase activity of over-expressing cell lines shown. Scale bar: 100μm. (B) Pluripotency markers (OCT4, NANOG, and SOX2) analyzed and there were no significant differences between over-expressing cell lines vs. hESC-RH6 as control by qRT-PCR. The data statistically analyzed by the t-test and expressed as mean ± SEM ($P > 0.05$). (C) Mock cell line characterization. From left to right: light microscopy, Fluorescence microscopy, Alkaline phosphatase staining and DAPI staining. Scale bar: 100μm.

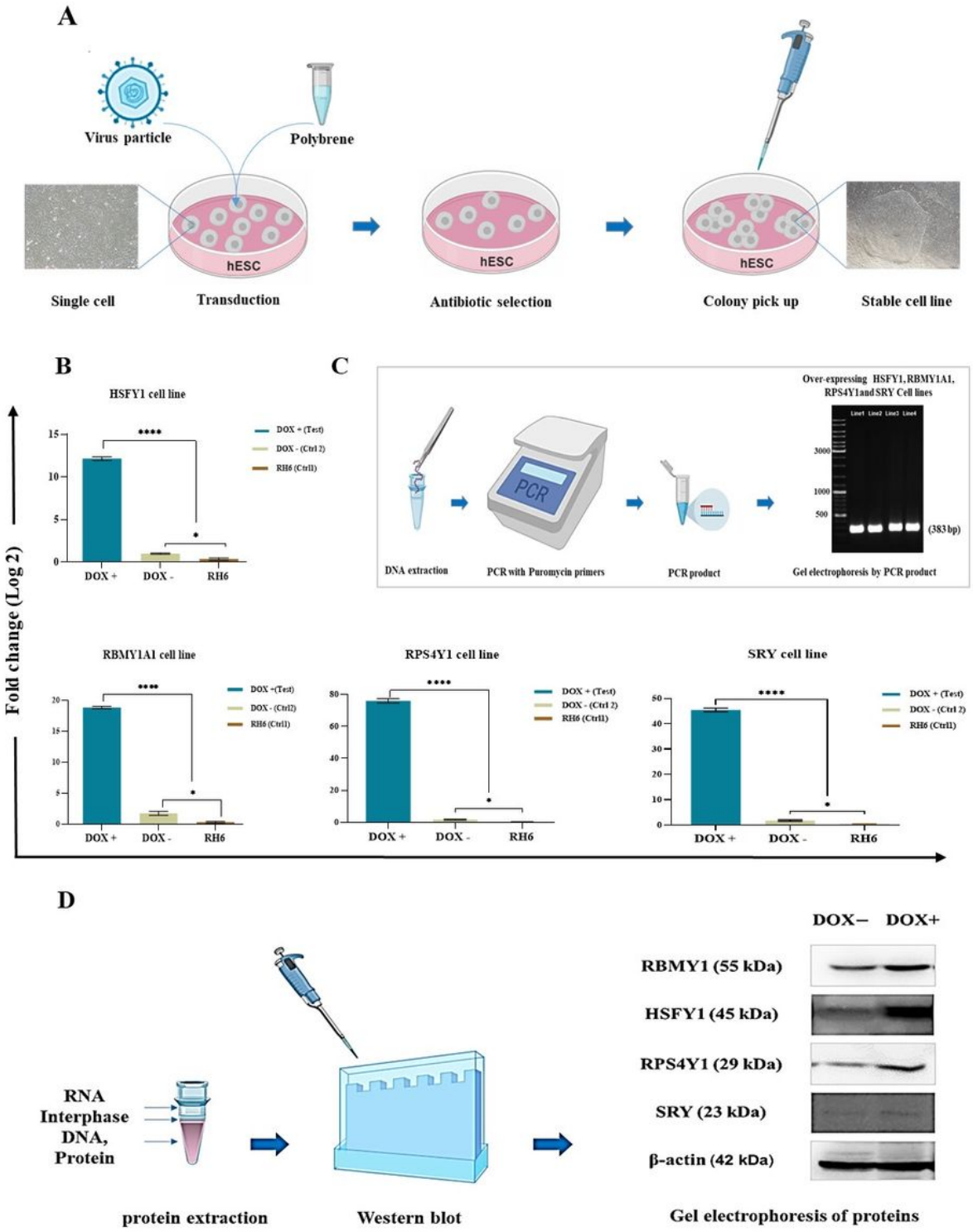

Figure 2

(A) The workflow of cell line development. First step; cell culture, transduction with virus particles, and polybrene, second step; antibiotic selection and colony pick up. (B, C) PCR-based assay for confirmation of the genomic insertion of lentiviral plasmids carrying SRY, HSFY1, RPS4Y, and RBMY1A1 genes in hESCs. An insert checking PCR for a fragment of puromycin resistance DNA was carried out. The mRNA expression levels of target genes. The differences between the expression level of genes of interest in

hESC-RH6 (wild-type group) and DOX negative (untreated group) with DOX positive (treated group) lines shown by qRT-PCR. Data expressed as mean ± SEM. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001 and ∗∗∗∗p < 0.0001 (ordinary one-way ANOVA test). (D) The protein expression levels of target genes. The differences between the expression level of target proteins in DOX negative compare with DOX positive groups was shown by western blot assay. β-actin is used as loading control.

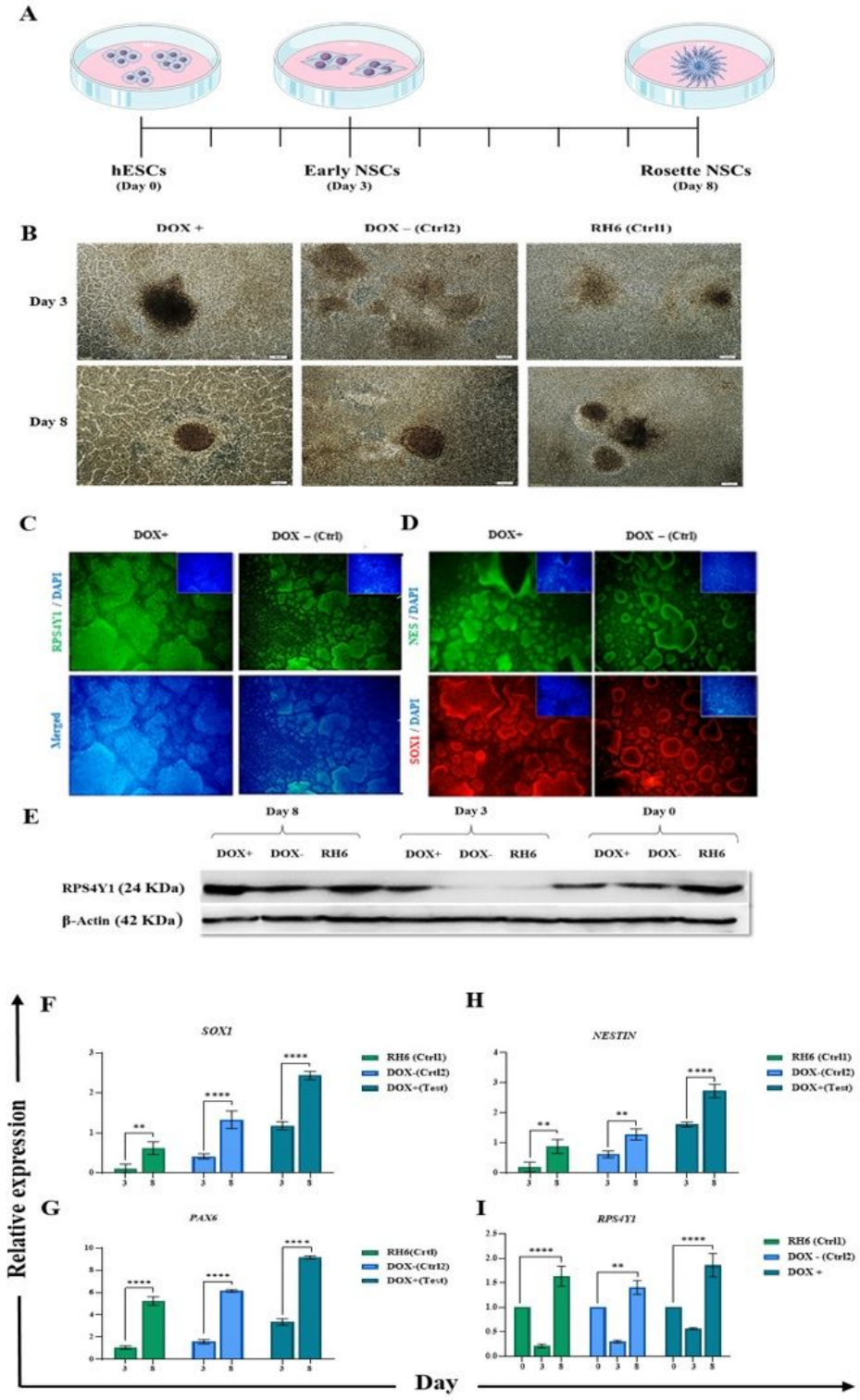

Figure 3

(A) Schematic image of neural differentiation. (B) Morphology of treated, untreated and wild-type groups during neural differentiation. Scale bar: 200µm. (C, D) Immunofluorescence of RPS4Y1, SOX1 and NESTIN in treated and untreated groups. (E) Western blot of RPS4Y1 in test and control groups. (F-H) Transcript expression of SOX1, NESTIN and PAX6 in treated, untreated and wild-type groups on day 3 and 8. (I) Transcript expression of RPS4Y1 in treated, untreated and wild-type groups on day 3 and 8. Data expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ (two-way ANOVA test).

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