Construction and Characterization of EGFP Reporter Plasmid Harboring Putative Human RAX Promoter for in vitro Monitoring of Retinal Progenitor Cells Identity

Atefeh Atefi

Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran. Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran.

Pendar Shojaei kojouri

Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan,Iran.

Fereshteh Karamali

Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran.

Shiva Irani

Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Mohammad Hossein Nasr Esfahani (nasr.royan@gmail.com)

Royan Institute https://orcid.org/0000-0003-1983-3435

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Construction and characterization of EGFP reporter plasmid harboring putative human *RAX* promoter for *in vitro* monitoring of retinal progenitor cells identity

Atefeh. Atefi [1, 2], Pendar. Shojaei kojouri [2], Fereshteh. Karamali [2*], Shiva. Irani [1], Mohammad Hossein. Nasr-Esfahani [2*]

aatefi@royaninstitute.org

pendar_shojaei@yahoo.co.uk

s.irani@srbiau.ac.ir

*Corresponding Authors:

*Mh.nasr-esfahani@royaninstitute.org

*frkaramali@royaninstitute.org

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

[2] Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

Abstract

Background: In retinal degenerative disease, progressive and debilitating conditions result in deterioration of retinal cells and visual loss. In human, retina lacks the inherent capacity for regeneration. Therefore, regeneration of retinal layer from human retinal progenitor cells (hRPCs) is a challenging task and restricted *in vitro* maintenance of hRPCs remains as the main hurdle. Retina and anterior neural fold homeobox gene (*RAX*) play critical roles in developing retina and

maintenance of hRPCs. In this study, for the first time regulatory regions of human *RAX* gene with potential promoter activity were experimentally investigated.

Results: For this purpose, after *in silico* analysis of regulatory regions of human *RAX* gene, the expression of EGFP reporter derived by putative promoter sequences was first evaluated in 293T cells and then in hRPCS derived from human embryonic stem cells. The candidate region (*RAX*-3258bp) showed the highest EGFP expression in hRPCs. This reporter construct can be used for *in vitro* monitoring of hRPC identity and verification of an efficient culture medium for maintenance of these cells.

Conclusions: Furthermore, our findings provide a platform for better insight into regulatory regions of human *RAX* gene and molecular mechanisms underlying its vital functions in retina development.

Key words: Human retinal progenitor cells; Retinal regeneration; proliferation capacity; *RAX* Promoter

Background

Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are the most common types of retinal degeneration disease (RDD) [1]. In RDD, retinal cells are damaged and thereby visual ability is impaired [2]. The intrinsic regenerative capacity of the human retina is extremely restricted and there is a growing focus on using human retinal progenitor cells (hRPCs) as a potential therapeutic approach for restoring retinal function and visual ability [3]. In this regard, RPCs can reduce disease progression rate by secretion of growth factors and upon integration and differentiate into new rod and cone photoreceptors in retinal layer, can facilitate the process of visual rehabilitation [4-6]. In addition, transplanted RPCs exhibit low tumorigenic potential and

have tendency to differentiate into retinal cell layers [7]. However, their restricted *in vitro* maintenance and proliferation capacity have hampered their application in the field of regenerative medicine. It has been shown that they lost their proliferative ability after maximum of seven passages, with reduced capacity for retinal cells formation [3]. One indirect approach to assess hRPCs preservation while being maintained *in vitro*, is to target their cell morphology and molecular based tracking such as immunostaining. However, a more direct alternative is to use the advantage of a fluorescent reporter under control of a specific retinal promoter [8]. Retina and anterior neural fold homeobox gene (*RAX*) is one of the initial genes expressed in prospective retina derived from the anterior neural plate [9]. RAX transcription factor consists of two conserved domains of homeodomain proteins. The first one is an octapeptide motif in the N-terminus and the second one is a C-terminal OAR (otp, aristaless, and rax) domain, which plays prominent roles in the eye development [10]. RAX is essential for the eye field specification and normal development of retina. It is generally down-regulated during differentiation towards retinal cells [11]. Previous researches showed positive correlation between *RAX* expression and RPCs proliferation in mouse and xenapous [12, 13]. Human *RAX* promoter region has not been extensively examined yet [14]. In this study, after prediction of putative human *RAX* promoter regions, the expression of EGFP reporter derived by these regulatory regions was first evaluated in 293T cells and then in human embryonic stem cell (hESC) derived hRPCs. Distal region of human *RAX* gene containing −3097 to +161 resulted in the highest EGFP expression in hRPCs. Altogether, our findings provide a better understanding of regulatory regions of human *RAX* gene, and can be extended to study the mechanism behind RAX function in multipotent retinal progenitors. The identification of human *RAX* promoter sequence might be a valuable

supplementary tool for assessment of molecular pathways involved in retinal proliferation and differentiation.

Results

Verification of hESCs differentiation into hRPCs

After differentiation of hESCs to hRPCs by RDM, the identity of hRPCs was confirmed by immunocytochemical analysis of eye filed transcription factors (EFTF) panel including LHX2, RAX, PAX6, SIX3 and also stemness markers like OCT4 and NANOG (Fig. 1F, G). According to Figure 1, differentiation of hESCs towards retinal progenitors was verified by significant reduction of OCT4 and NANOG, whereas EFTFs had successfully higher protein expression in derived hRPCs. For further confirmation of proper retinal development, the relative expression of EFTFs were analyzed at mRNA level by quantitative RT-PCR. Based on our results, the expression of anterior neural and eye field genes including *SIX3*, *RAX* and *PAX6* were increased when cells cultured in RDM compared to hESCs. Moreover, the expression of stemness markers including *OCT4* and *NANOG*, were significantly decreased after differentiation of ESCs into hRPCs (Fig. 1G). These results successfully confirmed the potential of selected medium for differentiation of human stem cells towards retinal progenitor cells.

In silico analysis of *RAX* Promoter

In this study UCSC genome browser was applied for analysis of chromatin structure (DNase hypersensitivity), and chromatin state of human *RAX* gene upstream region.

DNase I hypersensitivity marks different classes of cis regulatory elements within genome, such as promoters and enhancers [15]. UCSC analysis showed several subset of DNase I clusters within ~ 3 kb region upstream of human *RAX* gene (chr18: 59273233-59276490). Also, Figure. 2A

demonstrated the distribution of H3K4me1, H3K4me3 and H3K27ac in 5' upstream region of *RAX* gene, which are marks of active promoter and enhancer regions.

Based on ChIP-seq analysis in UCSC database, there are three fragments upstream of transcription start site of human *RAX* gene enriched of RNA polymerase II which can be indicator of promoter activity of this region [16].

CpG islands are typically located near TSS and might be associated with promoter regions [17]. In silico analysis of upstream region of *RAX* gene by UCSC genome browser confirmed the presence of a CpG island of around 1 kb length. Moreover, these results showed high evolutionarily conservation of this sequence in primates which reflects the significance of elements contained in this genomic regulatory region. Collectively based on these results 3258 bp upstream of human *RAX* gene was selected as a putative region with promoter activity.

In order to have a better view of potential regulation of *RAX* gene at transcriptional level, distal region was also analyzed for putative binding sites of transcription factors involved in retinal progenitor cell proliferation or development. For this purpose, GTRD which is a collection of ChiP-seq database for identification of TFBS in human and mouse, and also JASPAR data base were analyzed.

This sequence analysis revealed the presence of several presumptive binding sites for SOX2 and OTX2 in distal region of human *RAX* gene in accordance with previous studies in Xenopus which was introduced as a conserved noncoding sequence (CNS1) by Danno et.al (Fig. 3A) [18]. UCSC analysis revealed that CNS1 is highly conserved in primates and rodents (Fig. 2A). Also a number of putative binding sites were predicted for SMAD2/3 within human *RAX* gene. SMAD2/3 has been introduced as a key mediator for *in vitro* differentiation of mouse ESCs into retinal cells by direct binding to the regulatory elements of critical genes like *Rax*, *Pax6 and Otx2* [19].

Deletion analysis of human *RAX* promoter

To experimentally investigate the promoter activity of 5' flanking region of human *RAX* gene, we performed EGFP reporter assay using deletion constructs of regulatory regions. For this purpose, different candidate regulatory fragments were cloned upstream of EGFP reporter (Fig .3A) and transiently transfected in 293T cells (Fig. 3B, D) and hRPCs (Fig .3C, E). Based on protein atlas database 293T cells exhibited a low level of *RAX* mRNA expression. RT-PCR results also confirmed the amplification of *RAX* gene (100 bp) from CDNA synthesized from 293T total RNA (data not shown). So this cell line with high transfection efficiency was selected for analysis of *RAX* promoter activity. Before transfection of the putative promoter region into target cells, the integrity of expression constructs was examined by restriction digestions (Fig. 2C) and sequencing analysis (data not shown). Based on flow cytometry analysis 48 h post transfection, minimal upstream sequences of *RAX* gene with 267 and 525 bp length resulted in low rate of EGFP expression in 293T cells. After elimination of these two minimal regions, 3007 and 2761 bp fragments indicated lower EGFP expression compared to the minimal regions. On the other hand, 1665 and 3258 bp regions could highly derive the EGFP reporter and with a similar expression rate (Fig. 3B). Surprisingly, 3258 bp distal region indicated the highest *RAX* promoter activity in retinal progenitor cell and contributed to 2-fold EGFP enhancement in compared to 1665 proximal promoter sequence (Fig. 3C). These results suggest that there might be enhancer elements in distal region of human *RAX* gene which might cooperate with minimal regions and thereby modulating transcription of the associated *RAX* gene.

Discussion

In retina, proliferation, differentiation and cell fate decision are among cellular events being controlled by complex extrinsic and intrinsic signals [20, 21]. Transcription factors such as PAX6,

SIX3, OTX2, SOX2 and RAX are intrinsic regulators of maintenance and development of RPCs [22, 23].

In vertebrates, RAX transcription factor plays critical roles in early development of retina and has been implicated in RPCs maintenance [12]. Deletion studies of *Rax* in mice led to loss of optic vesicle development [24]. Moreover, studies have been showed that conditional knockout of *Rax* resulted in failure of laminar structure formation in retina, reduction of retinal progenitor cells, and retinal cell fate changes in conditional knockout mouse model [25]. However, there are still ambiguous aspects of RAX transcription factor roles in mammalian eye formation.

Most of retinal diseases are resulted from destruction of different retinal cell types [26]. To provide a sufficient pool of RPCs as a therapeutic approach for retinal degenerative disease, *in vitro* culture of retinal progenitor cells with high capability of proliferation and multi-potency is very critical. *In vitro* culture of ESC derived RPCs are usually led to down regulation of critical EFTFs in RPC and loss of their identity over passages [27-29]. These conditions are caused to failure of providing adequate pool of RPCs for downstream studies. In this study, to monitor *in vitro* maintenance of ESCs-derived hRPCs, an expression vector driving EGFP reporter by human *RAX* promoter was designed. For this purpose, for the first time we experimentally investigated the regulatory regions upstream of human *RAX* gene with potential promoter activity.

First we characterized hRPCs derived from hESCs by expression analysis of eye field markers [30]. In our previous studies, Noggin, IWR and IGF1 were included to the culture medium to differentiation of hESCs into hRPCs [31]. In this protocol, several sequential induction steps are needed to achieve retinal progenitor cells. For development of forebrain derivatives, BMP and Wnt pathways should be antagonized [32, 33]. Therefore, in order to direct ESCs to the anterior neural fate, EBs were treated with combination of noggin (a potent inhibitor of BMP pathway), IWR (an

antagonist of Wnt/β-catenin signaling pathway) and IGF-1as an inducer of retinal progenitors from ESCs under 3D culture conditions [34].

The prediction of putative promoter region was performed using different bioinformatics tools. Human *RAX* promoter region has not been thoroughly studied so far. For this purpose, upstream flanking region of human *RAX* gene was analyzed by UCSC in terms of chromatin state (H3K4me1, H3K4me3 and H3K27ac), DNase hypersensitive sites, CpG islands, POL II enrichment and sequence conservation [35]. DNase clusters indicated that transcriptional machinery might be enriched at these particular sequences with open chromatin structure [36]. Furthermore, our in silico analysis demonstrated the distribution of H3K4me1, H3K4me3 and H3K27ac in 5' upstream region of human *RAX* gene. H3K4me1 and H3K4me3 epigenetic modifications are normally signature of active promoter and enhancer regions. Enrichment of H3K27ac is also an active chromatin mark [37, 38]. Basically, RNA polymerase II binds to promoter region of genes for transcription initiation with aid of transcription factors. Most mammalian RNA polymerase II initiate transcription at CpG islands, which are devoid of DNA methylation [16]. UCSC analysis confirmed the presence of sites enriched by RNAP II and a CpG island in regulatory region of human *RAX* gene. Collectively, ~3.2 kb upstream of human *RAX* gene was considered as the putative promoter region. The promoter activity of this region was experimentally investigated using deletion constructs deriving EGFP reporter. Deletion analysis of ~ 3.2 kb human *RAX* promoter regions (−3097 to the +167) was analyzed in 293T and ESCs-derived hRPCs. The results of deletion construct analysis showed that region from −3097 to −336 (*RAX*-2761) and -3097 to -90 (*RAX*-3007) of human *RAX* gene could not independently drive downstream EGFP expression after elimination of minimal regions which basically include general regulatory binding sites required to trigger transcription. These findings indicated that the

minimal *RAX* regulatory promoter regions, from −106 to +161 (*RAX*-267) and -364 to +161 (*RAX*-525), consist of critical elements to derive *RAX* gene expression. Candidate distal region −3097/+161 (*RAX*-3258) and proximal region 1504/+161 (*RAX*-1665) showed a significantly higher EGFP expression in both 293T cells and hRPCs. Interestingly, distal region (*RAX*-3258bp) demonstrated a remarkable more EGFP expression than *RAX*-1665bp in hRPCs. Based on our results, we speculated that potential elements which are located in distal region, mediate the transcriptional stimulation and contributes to the higher promoter activity of *RAX*-3258 compared to *RAX*-1665.

Previous studies confirmed the necessity of conserved noncoding sequence 1 (CNS1) which is located ~2 kb upstream of *Rax* promoter as a regulatory region for expression of *Rax* in mice. CNS1 contains a highly conserved binding sites for Sox2 and Otx2 transcription factors across vertebrates, which are required for *Rax* transcription in mice [18]. These studies identified that Sox2 and Otx2 are potent modulators of *Rax* expression by direct binding to the promoter region and synergistically activate its transcription. Interactions between Sox2 and Otx2 proteins, regulate the expression of *Rax* during eye development [39].

Our bioinformatic analysis using JASPAR, GTRD and UCSC was also predicted binding sites for SOX2 and OTX2 in distal regulatory region of human *RAX* gene. This key conserved region was included in RAX-3258 bp distal promoter which exhibited the most promoter activity in hRPCs with high expression of SOX2 and OTX2. Interestingly, this candidate region was resulted in substantial reduced expression of EGFP in 293T cells which do not express SOX2 or OTX2 endogenously[18]. Albeit, the roles of other transcription factors in the regulation of human *RAX* expression should not been ignored.

Our in silico analysis also revealed several binding sites for SMAD2/3 in distal region of human *RAX* gene. Previous studies of *in vitro* differentiation of mouse ESCs into retinal cells, identified SMAD2/3 as a key regulator of several retinal genes like *Rax* [40, 41]. Moreover, this study showed that SMAD2/3 was able to directly bind to regulatory elements of retinal and photoreceptor precursor genes. In fact, SMAD2/3 binds to Smad binding elements (SBEs) which are located in distal promoter regions of target genes such as *RAX* and activates their expression (Fig. 4).

For further studies-regarding these molecular mechanisms in human, the effect of corresponding transcription factor overexpression on human *RAX* promoter activity with mutated TFBSs can be investigated.

Conclusion

In summary, the present study introduced the regulatory region of human *RAX* gene with high promoter activity. When, this region is included in an expression vector expressing EGFP, it may provide a molecular tool for monitoring retinal progenitor cell maintenance during *in vitro* culture. Furthermore, our study can be extended towards future investigations regarding the molecular mechanisms by which RAX play key roles in proliferation and development of retinal progenitor cells and reveal more aspects of hRPCs regulation.

Methods

Bioinformatics analysis

The sequence of human *RAX* gene was obtained from National Center for Biotechnology Information (NCBI). Different bioinformatic tools were used to predict the potential promoter regions of human *RAX* gene. These software are as follows; UCSC (http://genome.ucsc.edu/), and

Genomatix (https://www.genomatix.de/). Also, the putative transcription factor binding sites (TFBS) within the human *RAX* promoter region were analyzed using the gene transcription regulation database GTRD (http://gtrd.biouml.org/) and JASPAR (http:// http://jaspar.genereg.net/).

Primers design for amplification of putative human *RAX* promoter regions

Primers used to amplify the potential promoter regions of human *RAX* gene were designed from National Centre for Biotechnology Information (NCBI) database and confirmed by Primer-BLAST and Oligo 7. The primers were reconstituted in nuclease-free water to a concentration of 10 pM/µl. Then, 25 µL PCR reactions containing 2 µL of human genomic DNA (50 ng) as template, 1µl of each forward and reverse primers (10 pM/µl), 1X Multiplex PCR Master Mix (Yekta Tajhiz Azma Co., Tehran, Iran)., and 6 µL of nuclease-free water were prepared, Amplification was carried out in thermocycler (Thermoscientific) with amplification conditions shown in Table 1.

Cloning of putative human *RAX* promoter regions into pEGFP-C1 vector

Using the primers listed in Table 1, the candidate promoter region -3097/+161 of the human *RAX* gene, and a series of control fragments (-106/+161, -364/+161, -1504/+161, -3097/-336, -3097/-90) were amplified by PCR from human genomic DNA and inserted into *Sal*I/*Bgl*II site of the pEGFP-C1 vector upstream of EGFP reporter. The final expression vector was transformed into E. coli DH5-alpha cells. The integrity of all target sequences was verified by sequencing before evaluation of promoter activity in target cells.

Table 1. List of primers for amplification and characterization.

Putative Promoter regions /Genes	Primers	Sequences (5'>3')
RAX-267	Sense	ATTAATAGAGAAGGGGCTGGGT
RAX-525	Sense	ATTAATTAGTCTGAAGTGAGAGG
RAX-1665	Sense Anti-sense	GTCGACGAATTAATCAGAGGTGG AGATCTCTTTGGAGACGGAGAGG
RAX-2761	Anti-sense	AGATCTCTCCCCTTGCGTTTGT
RAX-3007	Anti-sense	AGATCTACCCAGCCCCTTCTC
RAX-3258	Sense Anti-sense	GTCGACGAATTAATCAGAGGTG AGATCTCTTTGGAGACGGAGAGG
OCT4	Sense Anti-sense	TCTATTTGGGAAGGTATTCAGC ATTGTTGTCAGCTTCCTCCA
NANOG	Sense Anti-sense	CAGCTACAAACAGGTGAAGAC TGGTGGTAGGAAGAGTAAAGG
NESTIN	Sense Anti-sense	TCCAGGAACGGAAAATCAAG TTCTCTTGTCCCGCAGACTT
SIX3	Sense Anti-sense	TCCTCCTCTTCCTTCTCC GTTGTTGATAGTTTGCGGTT
RAX	Sense Anti-sense	CAACTGGCTACTGTCTGTC CTTATTCCATCTTTCCCACCT
PAX6	Sense Anti-sense	CAGCTCGGTGGTGTCTTTG AGTCGCTACTCTCGGTTTA

In vitro cell culture of human embryonic stem cells

RH6 human embryonic stem cells (hESC, Royan institute) were seeded on 0.3 mg/ml Matrigel (Sigma-Aldrich, St. Louis, MO)-coated tissue culture dishes containing DMEM/F12 medium supplemented with 20% knockout serum replacement (KSR), 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1% ITS and 100 ng/ml bFGF with daily medium exchange (Fig. 1B).

Differentiation of hESCs into hRPCs

The process of retinal differentiation was briefly demonstrated in Figure 1A. For neural retinal differentiation, the over confluent feeder-free hESCs were dissociated and then a mechanical approach was used to initiate embryoid body formation (EBs) (Fig. 1C). So hESCs were transferred to low adherent dishes in order to form EBs in neural induction medium (NIM) containing 1 ng/ml noggin (R&D, 1976-NG), 3 μM IWR (R&D, 5439-DK/CF), and 5 ng/mL human recombinant insulin-like growth factor-1 (IGF-1) (R&D, 291-GI) in DMEM/F12 medium supplemented with 10% KSR, 0.1 mM nonessential amino acids, 2 mM L-glutamine, and 1% B27 (Gibco, 17504- 044) for 3 days. In the next step, the EBs were dissociated by Accutase (Millipore, SCR005) and were replated on 1 mg/ml laminin and 15 mg/ml Poly-L-ornithine (both from Sigma-Aldrich) coated 6-well tissue culture plates. The culture medium was replaced with retinal determination medium (RDM) containing DMEM/F12 supplemented with 1% B27, 2% N2 (Gibco, 17502-048), 10 ng/ml noggin, 3μM IWR, 10 ng/mL IGF-1, and 10 ng/ml bFGF as previously explained. This medium was exchanged every two days up to 21 days (Fig. 1D, E).

Transient transfection

For investigation the promoter activity of expression vectors harboring the potential promoter regions of human *RAX* gene by transfection into 293T cell line, these cells were maintained in DMEM supplemented with 1% L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/ml streptomycin and incubated at 37°C in 5% CO2. Next equal moles of expression vectors were transfected into 293T cells [42] by Lipofectamine LTX Transfection Reagent according to the manufacturer's instructions (Invitrogen, Germany) in a 24 well cell culture plate. Also control groups were transfected by pEGFP-C1 and promoter-less vector (pEGFP-np) for analysis the efficiency of transfection and confirmation of target promoter's activity respectively. The expression of EGFP reporter resulting from promoter activity of *RAX* regulatory regions were

assayed 48 hours post transfection. To explore the potential promoter activity of these regions in retinal progenitors, they were also transfected into hESCs- derived hRPCs, as described above.

Immunocytochemical analysis

To evaluate the expression of stem cell and retinal progenitor markers, immunocytochemistry staining was carried out using the primary antibodies: PAX6 (SC-11357, 1/50), RAX (LS-C53650, 1/200), NESTIN (ab-22035, 1/100), LHX2 (SC-81311, 1/100) and secondary antibodies Anti-Mouse IgG-FITC (Sigma, AP124F) and Anti-Rabbit IgG-TRITC (Sigma, T6778). Briefly, 5×10^4 cell/ well coverslips coated with Matrigel were plated in 24-well plates. One day later, samples were fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, these cells were permeabilized by 0.4% Triton X-100 for 30 min and were stained with blocking solution-diluted primary antibodies (BSA, 10 mg/ml) and kept at 4°C overnight. Then, they were treated with secondary antibodies at 37°C for 1hr. Furthermore, cell nuclei were stained with DAPI (3 ng/ml, Invitrogen). The images were taken by fluorescent microscope (Olympus, Center Valley, PA, USA) equipped with an Olympus DP70 camera.

Quantitative PCR (qPCR)

The total RNA of hRPCs and RH6 cells (as negative control) was extracted using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Then, cDNA was synthesized using Takara cDNA Synthesis kit using random hexamer primers. All qRT-PCR reactions were performed in triplicate, and data were normalized to human GAPDH mRNA. Relative fold changes in target gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. Table 1, represented primer sequences used in quantitative PCR. Moreover, to investigate the stemness state and multipotency capacity of hESCs-derived hRPCs, the relative expression of several markers including *OCT4*, *NANOG* and eye field markers like

294 *NESTIN*, *SIX3*, *PAX6* and *RAX* were evaluated[11]. Each experiment, had a negative template
295 control (NTC) for primer specificity analysis and lack of DNA contamination. SYBR Green I
296 Master reaction mix (Thermo Fisher Scientific) was used for qPCR analysis of gene expression,
297 and amplification was detected with Light Cycler 480 ABi System.

298 **Flow cytometry analysis**

299 Flow cytometry assessment was performed to evaluate the quantification of EGFP reporter derived
300 by promoter regions of human *RAX* gene. For this purpose, two days after transfection of target
301 cells with expression vectors, cells were detached and re-suspended in cold PBS^- and analyzed by
302 FACS Vantage flow cytometry (Becton Dickinson). Data were analyzed using BD Cell Quest Pro
303 and WinMDI 2.9 software.

304 **Statistical analysis**

305 All data were analyzed using one-way ANOVA, Tukey's post-hoc analysis and Student's t-test
306 and are shown as the mean±SD. In each experiment, at least three biological replicates were
307 examined. In this study, $P \leq 0.05$ was considered as statistically significant.

308 **List of abbreviations**

309 **hRPCs:** Human retinal progenitor cells

310 **RAX:** Retina and anterior neural fold homeobox

311 **RP:** Retinitis pigmentosa

312 **AMD:** Age-related macular degeneration

313 **RDD:** Retinal degeneration disease

EFTF: Eye filed transcription factor

CNS: Conserved noncoding sequence

hESC: Human embryonic stem cell

TFBS: Transcription factor binding site

Ethical approval and consent to participate

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Consent for publication

Not applicable.

Competing interest

There is no competing interests to report

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Authour Contributions

Conceived and designed the analysis were done by MHNE & FK, Collected the data was done by AA, PSH, Performed the analysis was done by AA, PSH & FK, Wrote the manuscript was done by PSH, AA, Interpretation of the obtained information was done by MHNE & SHI. All authors read and approved the final manuscripts.

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Author information

Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

Atefeh Atefi & Shiva Irani

Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

Atefeh Atefi, Pendar Shojaei Kojouri, Fereshteh Karamali & Mohammad Hossein Nasr-Esfahani

Correspondence to Mohammad Hossein Nasr-Esfahani and Fereshteh Karamali

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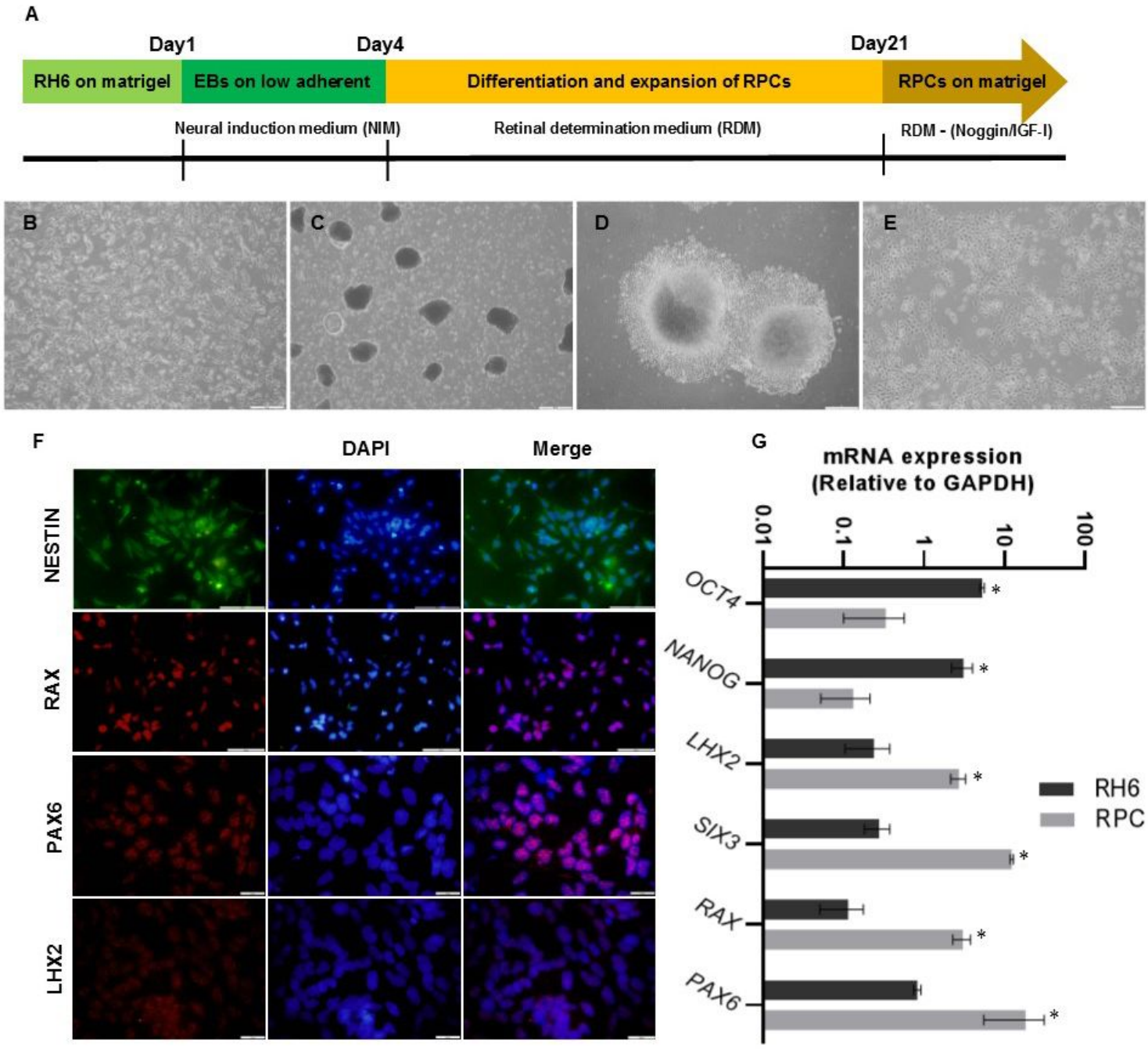
Fig. 2 Analysis of putative regulatory regions of human *RAX* gene to derive EGFP reporter plasmids. (A) Screenshot from UCSC genome browser indicating human *RAX* gene upstream region, H3K4me1, H3K4me3, H3K27ac and Pol2 patterns, CpG islands and DNase clusters. (B) Schematic representation of pEGFP-C1 plasmid in which CMV promoter was substituted with putative promoter regions of human *RAX* gene. (C) The final expression plasmids were confirmed by restriction digestion analysis.

Fig. 3 Deletion analysis of human *RAX* promoter regions. (A) A series of 5′ deletions from -3258 to +161 bp of human *RAX* gene. (B, C) Intensity of EGFP reporter in promoter deletion constructs was determined by flow cytometry in 293T cells (B) and RPCs (C). Data are expressed as mean ± SD (*$p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$, n=3). (D, E) Comparison of EGFP expression derived by different *RAX* regulatory regions in 293T cells (D) and RPCs (E) using flow cytometry and fluorescence microscopic analysis. The scale bar is 100µm.

Fig. 4 TFBS analysis of human *RAX* distal promoter region (−1665 to -3078) (A) Putative binding sites of SOX2, OTX2 and SMAD2/3 within this region were analyzed using the EPD database. (B) Profile summery of transcription factor binding sites using JASPAR data base.

Figures



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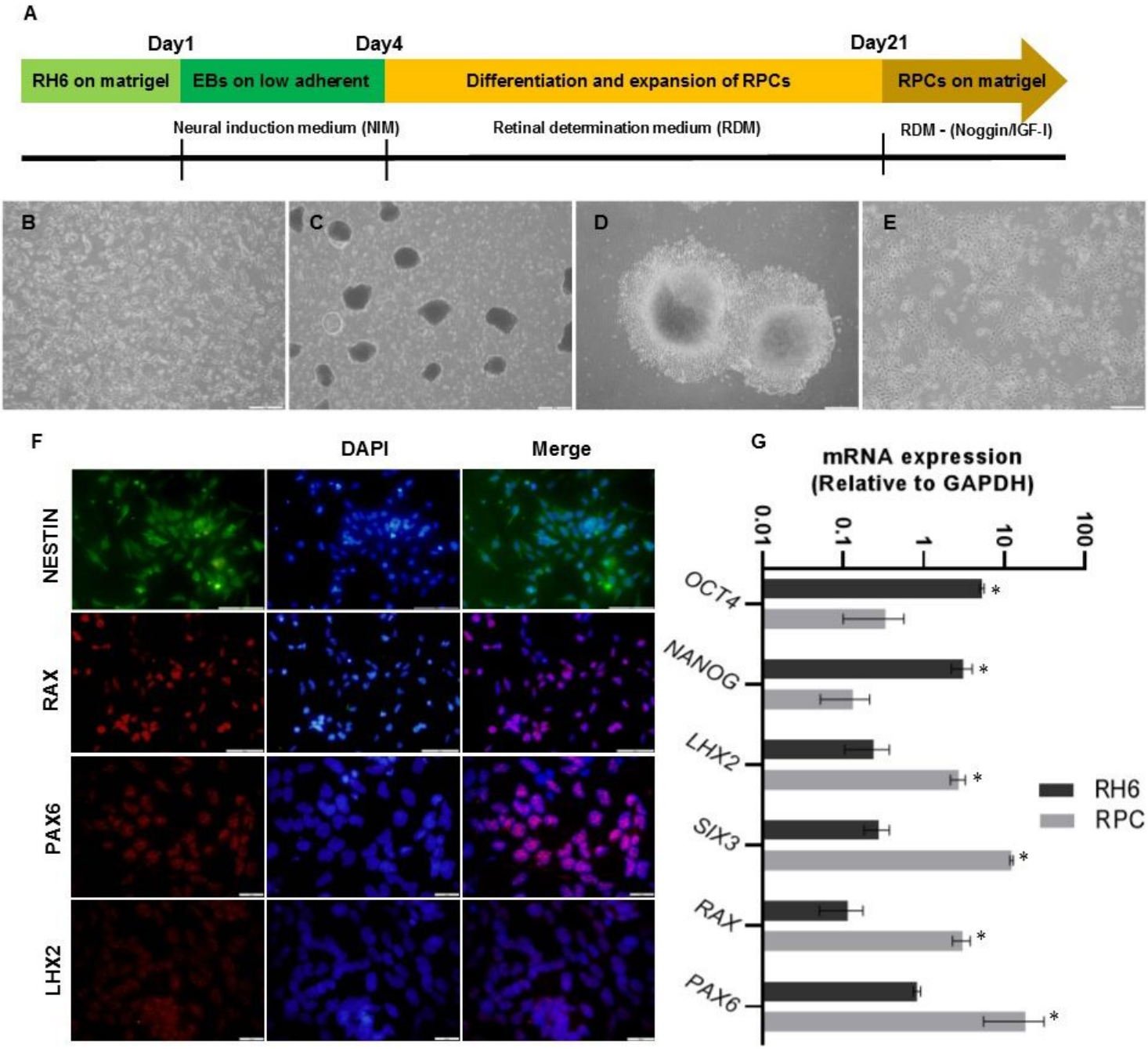

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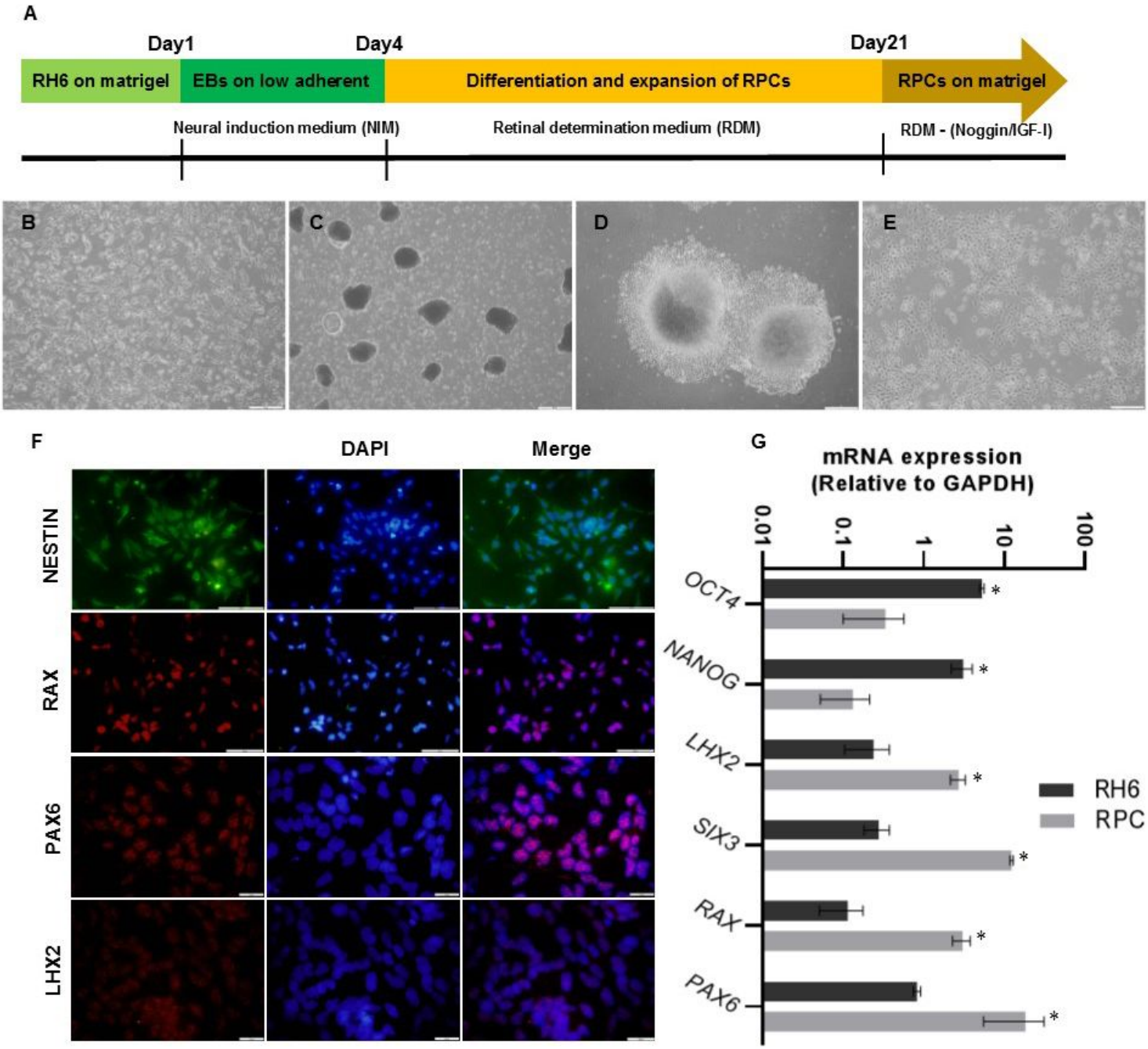

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Analysis of putative regulatory regions of human RAX gene to derive EGFP reporter plasmids. (A) Screenshot from UCSC genome browser indicating human RAX gene upstream region, H3K4me1, H3K4me3, H3K27ac and Pol2 patterns, CpG islands and DNase clusters. (B) Schematic representation of pEGFP-C1 plasmid in which CMV promoter was substituted with putative promoter regions of human RAX gene. (C) The final expression plasmids were confirmed by restriction digestion analysis.

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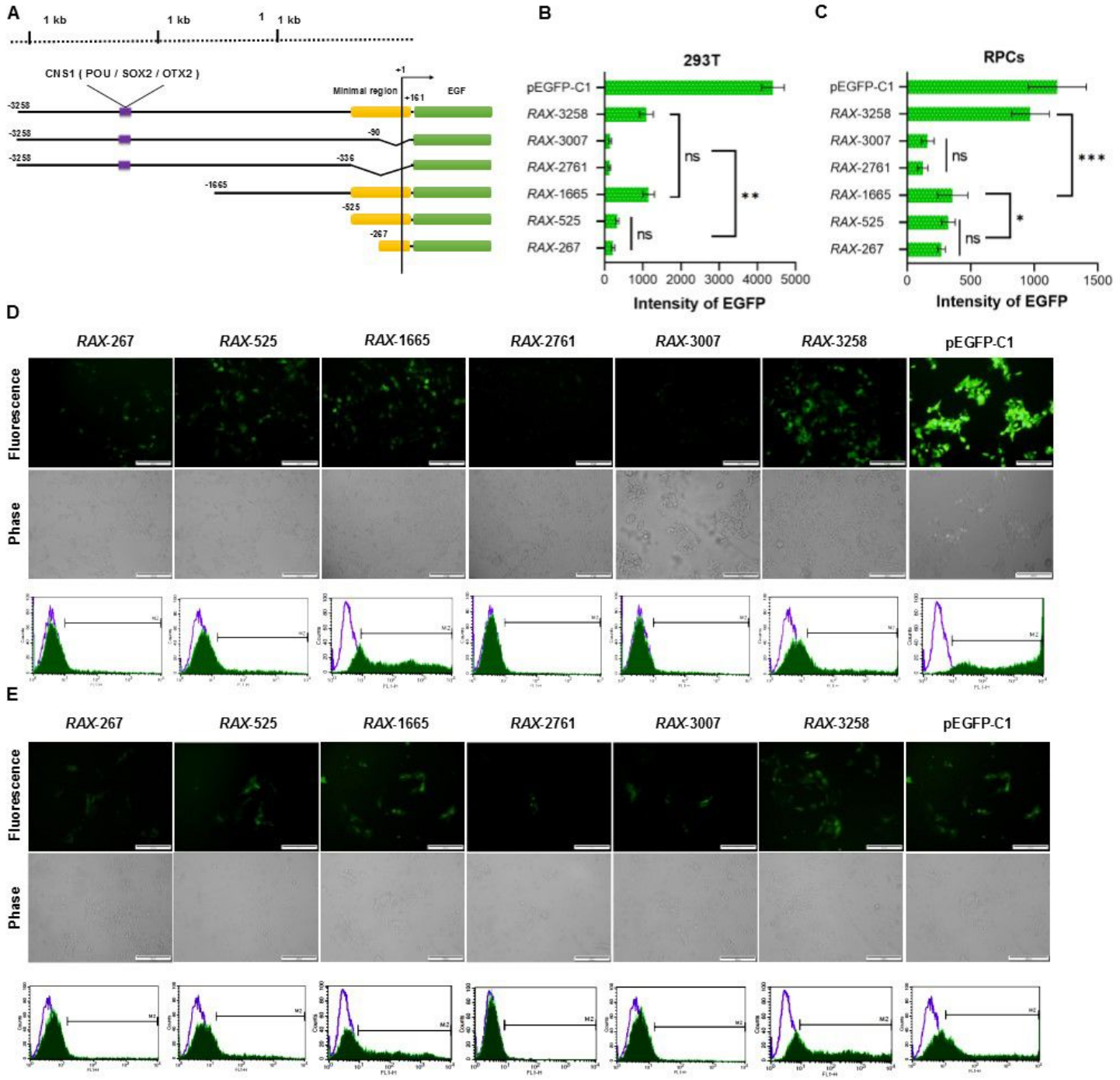

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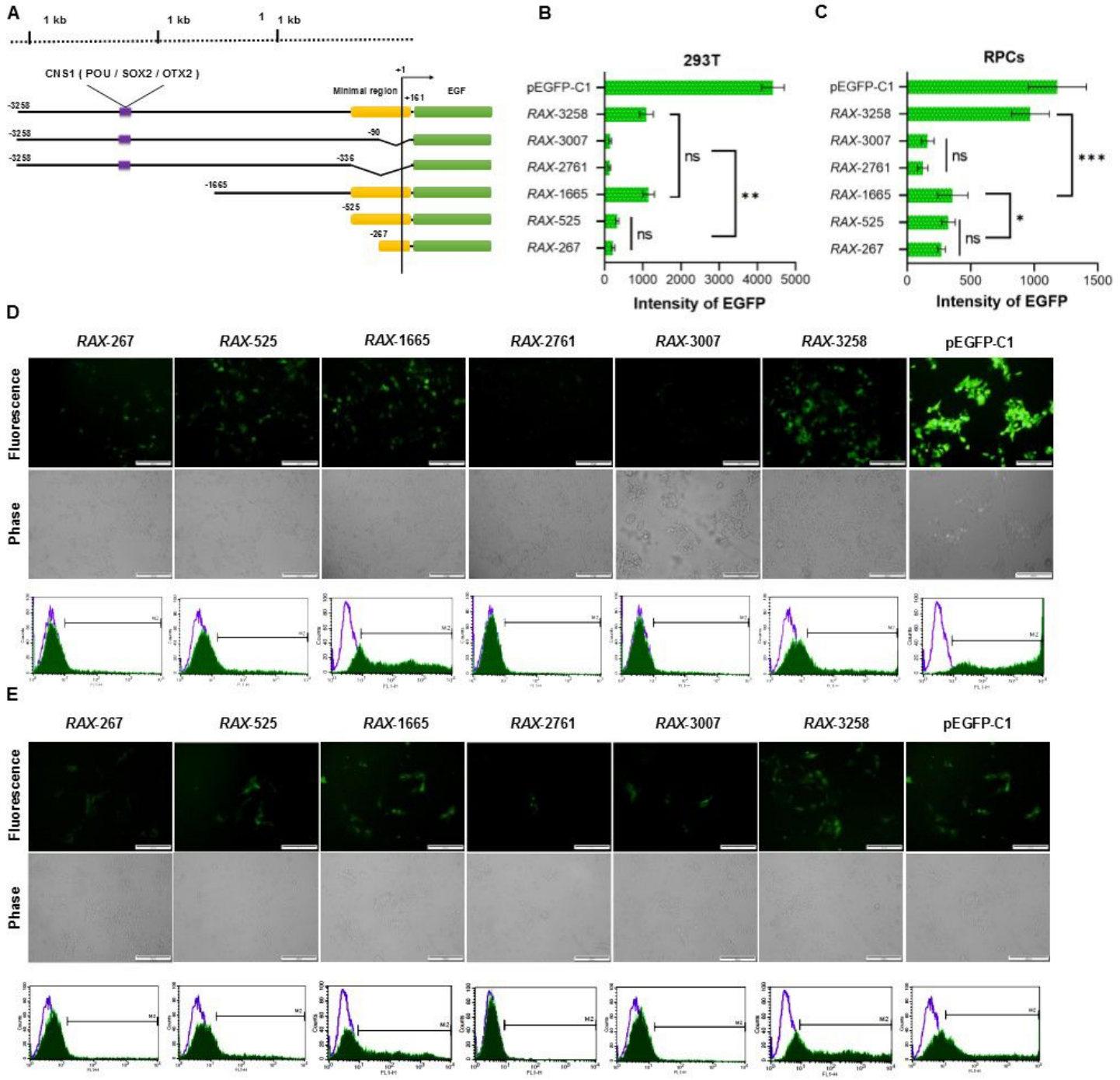

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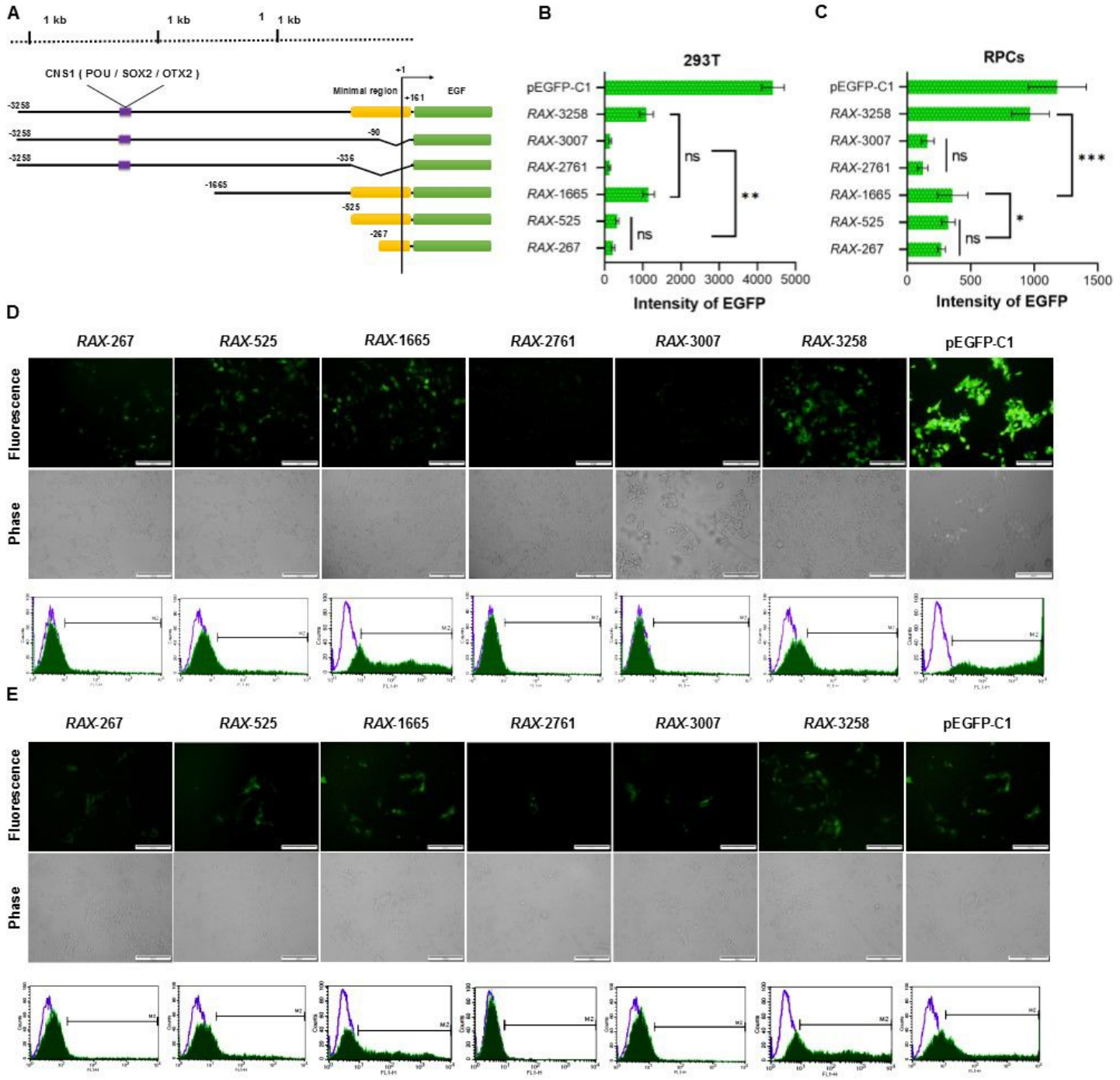

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A

-3078

GAATTAATCAGAGGTGGGGTTCAGAAGCCAGGCTCCTTACTGTTGCTGAAGCAGGGCTGA
TTATGAGCTAGCCTAACTGCTGGAAAAATGCAGCTTCATGCTAGAAGAATTAGGGACATG
GCCCAGTCCATTCATCTTCCCTAATCGAGTTCTTGGGAAATACACACGTAGATTAGCCCC
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TTTTTCTTAAAAGATAATTTCTGACTTGAATTAG

-1665

B

Transcription factor	Motif logo	Matrix ID
SMAD2/3		MA0513.1
SOX2		MA0143.4
OTX2		MA0712.1

Figure 4

TFBS analysis of human RAX distal promoter region (−1665 to -3078) (A) Putative binding sites of SOX2, OTX2 and SMAD2/3 within this region were analyzed using the EPD database. (B) Profile summery of transcription factor binding sites using JASPAR data base.

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GAATTAATCAGAGGTGGGGTTCAGAAGCCAGGCTCCTTACTGTTGCTGAAGCAGGGCTGA
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TTTTTCTTAAAAGATAATTTCTGACTTGAATTAG

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TTATGAGCTAGCCTAACTGCTGGAAAAATGCAGCTTCATGCTAGAAGAATTAGGGACATG
GCCCAGTCCATTCATCTTCCCTAATCGAGTTCTTGGGAAATACACACGTAGATTAGCCCC
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TTTTTCTTAAAAGATAATTTCTGACTTGAATTAG

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