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Regulation of WNT Signaling by VSX2 During Optic Vesicle Patterning in Human Induced Pluripotent Stem Cells

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ABSTRACT

Few gene targets of Visual System Homeobox 2 (VSX2) have been identified despite its broad and critical role in the maintenance of neural retina (NR) fate during early retinogenesis. We performed VSX2 ChIP-seq and ChIP-PCR assays on early stage optic vesicle-like structures (OVs) derived from human iPS cells (hiPSCs), which highlighted WNT pathway genes as direct regulatory targets of VSX2. Examination of early NR patterning in hiPSC-OVs from a patient with a functional null mutation in VSX2 revealed mis-expression and upregulation of WNT pathway components and retinal pigmented epithelium (RPE) markers in comparison to control hiPSC-OVs. Furthermore, pharmacological inhibition of WNT signaling rescued the early mutant phenotype, whereas augmentation of WNT signaling in control hiPSC-OVs phenocopied the mutant. These findings reveal an important role for VSX2 as a regulator of WNT signaling and suggest that VSX2 may act to maintain NR identity at the expense of RPE in part by direct repression of WNT pathway constituents. STEM CELLS *2016;34:2625–2634*

SIGNIFICANCE STATEMENT

This study utilizes an hiPSC model of a retinal developmental disorder to gain insight into a novel mechanism underlying neural retinal (NR) vs. RPE fate choice during human retinogenesis. Using pharmacological manipulation and chromatin immunoprecipitation followed by massively parallel DNA sequencing, we show that the NRPC transcription factor VSX2 is a direct regulator of numerous WNT-related genes and that VSX2-mediated antagonism of WNT signaling plays an important role in the maintenance of NR identity at the expense of RPE. Our findings also underscore the utility of hiPSCs in investigating the earliest stages of human ocular development, which are otherwise inaccessible to study.

INTRODUCTION

The vertebrate eye develops from the anterior neural plate during late gastrulation as a result of intrinsic programming and signals from surrounding tissues, which in turn leads to the expression of a group of transcription factors that specify the eye field [1, 2]. Following eye field specification, the bilateral optic pits evaginate to form optic vesicles (OVs). OVs then undergo invagination, resulting in the creation of the bilayered optic cup, the inner and outer aspects of which become the neural retina (NR) and retinal pigmented epithelium (RPE), respectively.

OV patterning, optic cup formation, and subsequent retinal maturation are influenced by inductive signaling molecules, which are thought to include Fgfs [3–5], Tgfβ superfamily members, Hedgehog, [6, 7] and/or Wnts [8–12]. In addition to extrinsic instructional cues, there are multiple transcription factors that play crucial roles in early RPE and NR development. Two such factors are Microphthalmia-associated transcription factor (Mitf) and Visual system homeobox 2 (Vsx2). Mitf is initially expressed uniformly throughout the early mammalian OV, while at later stages its expression is restricted to the RPE, where it is involved in differentiation, proliferation, and pigmentation [13, 14]. The initiation of this restriction coincides with the onset of Vsx2 expression in the distal OV, which also serves to demarcate the future NR. Vsx2 is involved in maintenance and proliferation of the neural retinal progenitor cell (NRPC) pool, timing of photoreceptor production, and differentiation

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of one type of retinal interneuron, the bipolar cell [15–18]. In addition, Vsx2 has been shown to inhibit Mitf expression directly by binding and repressing Mitf promoter sites [19, 20], as well as through protein-protein interactions [21]. Disruption of *Vsx2* in animal models causes retinal defects, microphthalmia, and RPE layer duplication [15, 18, 21]; similarly, in humans *VSX2* mutations result in very small, nonfunctional eyes with malformed retinas [22–24]. However, the mechanism by which VSX2 influences these diverse processes remains the subject of investigation.

VSX2 expression has also been used in vitro to identify multipotent NRPCs derived from human pluripotent stem cells (hPSCs) [25–29]. In order to study the role of VSX2 in human retinogenesis and hPSC differentiation, we previously generated human induced PSCs (hiPSCs) from a microphthalmic individual bearing a homozygous R200Q mutation in VSX2 ($VSX2^{R200Q}$) and an unaffected sibling. The R200Q mutation eliminates the ability of VSX2 to bind DNA [21, 24, 30], thus rendering it unable to directly regulate gene expression. Populations of 3-dimensional OV-like structures were derived from the hiPSC lines (hiPSC-OVs) and differentiated into retinal cell types in a manner analogous to human retinogenesis in vivo [28, 31]. $VSX2^{R200Q}$ hiPSC-OVs demonstrated proliferation defects, enhanced RPE differentiation at the expense of NR, and absence of bipolar cells, which closely approximates the vertebrate disease phenotype [31]. Interestingly, RNA-seq performed on early $VSX2^{R200Q}$ hiPSC-OVs revealed upregulation of a striking number of genes belonging to the WNT pathway, which led us to further examine the association between VSX2 and WNT signaling during early retinal differentiation.

In the present study, we show that major components of the WNT pathway are gene targets for VSX2 binding during retinal differentiation in hiPSCs, and that pharmocological manipulation of the WNT pathway alters the expected phenotypes of both wild-type control ($VSX2^{WT}$) and $VSX2^{R200Q}$ hiPSC-OVs. Our findings indicate an important role for VSX2 as a direct regulator of WNT signaling and suggest that VSX2 may maintain NR identity in early hiPSC-OVs in part by antagonizing expression of WNT pathway constituents.

Materials and Methods

Cell Culture

hiPSCs derived from a microphthalmic patient[24] with homozygous R200Q mutations in VSX2 and an unaffected sibling were maintained on irradiated mouse embryonic fibroblasts and differentiated toward retina as previously described [31]. Briefly, on day 0 (d0), embryoid bodies (EBs) are lifted with 2 mg/ml dispase in the absence of FGF2 to initiate retinal differentiation. On d7, EBs are plated on laminin-coated wells, and by d10, 90% of cells express markers of anterior neuroectoderm [26]. By d12, MITF expression is detected in a subset of the cells, followed on d14 by expression of VSX2, which marks the production of NRPCs [20]. By d18-20, NRPC and RPE progenitors are established and OV structures consisting predominantly of NRPCs are manually separated and cultured en masse. These hiPSC-OVs subsequently generate all retinal neuron types in a sequence and time frame approximating normal human retinogenesis [29, 31].

Immunocytochemistry

EBs were plated on laminin-coated coverslips on d7 of retinal differentiation and fixed with 4% paraformaldehyde after an additional 7-11 days of differentiation. D35 and d50 hiPSC-OVs were fixed for 1 hour at RT in 4% paraformaldehyde, cryopreserved in 15-30% sucrose, and cut into 11 μm cryosections. Samples were blocked in 10% normal donkey serum, 0.5% Triton X100, 1% fish gelatin, and 5% bovine serum albumin for 45 min at room temperature and incubated with primary antibody overnight at 4°C in a humidified chamber (primary antibody sources and dilutions are listed in Table S1). AF546-, AF488-, and AF633-conjugated secondary antibodies (Thermo Fisher) were diluted 1:500 in blocking buffer and incubated at room temperature for 30 min. Samples were mounted in Prolong Gold antifade + DAPI (Thermo Fisher) and images were taken on a Nikon A1R-Si laser scanning confocal microscope (Nikon). Cell counts were performed with Nikon Elements module D and plotted with Graph Pad Prism 6.

WNT Agonist and Antagonist Treatments

EBs were plated at d7 on laminin-coated plastic wells or laminin-coated glass coverslips and treated with the WNT antagonist IWP2 (5 μM) (Tocris) from d12 to d20 or with the WNT agonist CHIR99021 (3 μM) (Tocris) from d14 to d20. DMSO served as the vehicle control for all experiments. Treated coverslips were fixed at d18 and immunostained as described above. Treated wells were collected after 30 days of differentiation and total RNA was extracted with RNAeasy mini spin columns (Qiagen) and reverse transcribed with iScript cDNA kit (BioRad) according to manufacturers' instructions. Quantitative PCR was performed with SSO Advanced SybrGreen master mix (BioRad) on a Step One Plus Real Time PCR system (Thermo Fisher). Relative expression was normalized to the geometric mean of two reference genes and the average $2^{-\Delta\Delta Cq} \pm$ SEM of three replicates was plotted using Graph Pad Prism 6. Statistical significance ($p < 0.05$) was calculated with an unpaired two-tailed Student's *t*-test. Primer sequences are listed in Table S2.

Chromatin Immunoprecipitation

Human iPSC-OVs were manually selected and differentiated to d30, fixed in 1% formaldehyde for 10 min at room temperature, washed, and lysed in Pierce IP lysis buffer (Thermo Fisher) supplemented with 40 μl/ml protease inhibitor cocktail P8340 (Sigma Aldrich). Cleared lysate was sheared in a Q700 ultrasonic processor (Qsonica) equipped with a cup horn, and shearing was monitored using 1% agarose gel electrophoresis. 10% volume was reserved for input and the remainder was incubated with 2 μg sheep anti-VSX2 antibody (Exalpha) overnight at 4°C with rocking. Immunoprecipitates were collected on protein-G conjugated Dynabeads (Thermo Fisher), washed 5x with sterile PBS, and eluted in 10 mM Tris/1 mM EDTA pH 8 + 1% SDS. Three volumes of 1% SDS, 0.1M $NaHCO_3$, and 200 mM NaCl were added to input and IP samples and cross-links were reversed by incubation at 65°C for 4 hours. DNA was extracted with phenol:chloroform:isoamyl alcohol, ethanol precipitated, and quantified with the Qubit high sensitivity double stranded DNA kit (Thermo Fisher). 8-10 ng of DNA were prepared for deep sequencing with either the Illumina

ChIP-Seq DNA or the TruSeq ChIP Sample Preparation Kit (Illumina) and quantified with a Qubit fluorometer. All samples were loaded at a final concentration of 8 pM and sequenced on the Illumina HiSeq 2500.

ChIP-Seq Analysis

ChIP-seq reads were aligned to the hg19 *Homo sapiens* assembly using Bowtie [32]. Duplicate reads were removed within each replicate. Transcription factor binding sites were first called after combining reads from both replicates using the R software package SPP [33] with the following settings: detection window halfsize = 300, False Discovery Rate (FDR) = 0.05. After subtracting the normalized input read counts [34], the number of ChIP binding reads were computed for every binding site within each replicate, and only sites having at least 10 ChIP binding reads within both replicates were considered high confidence sites. Transcription factor binding motifs were analyzed in an unbiased fashion using HOMER version 4.6 [35].

ChIP-PCR

Chromatin from both VSX2^{WT} and VSX2^{R200Q} hiPSC-OVs were immunoprecipitated as described above and purified DNA was diluted 1:10 for PCR. Genomic coordinates for selected WNT pathway peaks were used to generate primers, and sites in the *MITF-H* promoter known either to be bound or not to be bound by VSX2 [20] were used as positive and negative controls for these experiments, respectively. Primer sequences are listed in Table S2 and PCR analysis was performed with 2X PCR master mix (Promega) (35 cycles and Tm = 58°C) followed by visualization on a 2% agarose gel.

Results

VSX2 Binds a Subset of WNT Pathway Genes

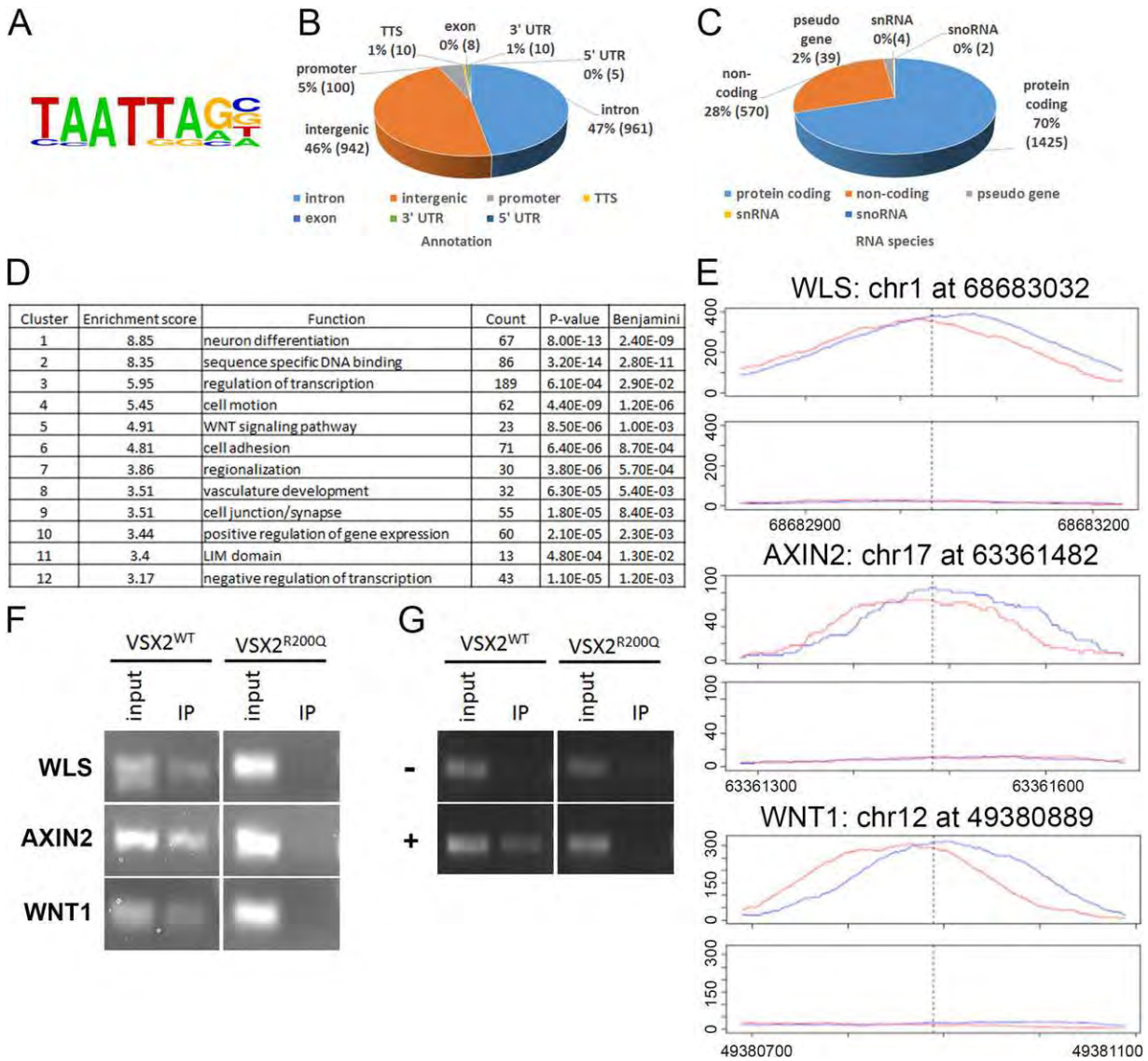
In a previous study, transcriptome comparison of VSX2^{WT} and VSX2^{R200Q} hiPSC-OVs suggested a regulatory role for VSX2 in WNT signaling, which in turn might contribute to the overproduction of RPE at the expense of NR seen in mutant OVs [31]. To further investigate this possibility, we performed unbiased searches for VSX2 DNA binding sites in two independent samples of d30 VSX2^{WT} hiPSC-OVs using chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-Seq). Western blot analysis of cell lysates and immunoprecipitates demonstrated VSX2 antibody specificity (Fig. S1A), although differences in binding affinities between lots of VSX2 polyclonal antibodies resulted in the second ChIP-seq displaying weaker signal strength. We then compared merged ChIP and input samples for peak calling and further stipulated that peaks be present in both replicates, which resulted in a list of 2038 high confidence VSX2 binding sites (Table S3). Examination of the DNA regions occupied by VSX2 in these analyses revealed a consensus binding motif identical to that previously found for VSX2 in various mammalian systems (Fig. 1A) [21, 24, 30]. Furthermore, 236 of these sites were located within 2.5 kb of a transcription start site. The majority of peaks were split between intronic and intergenic sequences (Fig. 1B), characteristic of enhancer targets, and more than half of the identified loci (1425 peaks) were associated with protein-coding genes (Fig. 1C), indicative of VSX2's role as a transcriptional regulator. Notably, DAVID annotation clustering [36, 37] not only identified numerous functions consistent with the known roles of VSX2 in early OV development, but also highlighted the WNT signaling pathway as a functional target of VSX2 (Fig. 1D). Target sites were identified in multiple WNT pathway genes, including *WLS*, *AXIN2*, and *WNT1* (Table 1).

To verify the potential for the canonical WNT pathway to serve as a direct target for VSX2 binding and regulation during early hiPSC-OV differentiation, we performed confirmatory ChIP-PCR analysis focusing on the three highest scoring WNT pathway genes: *WLS, WNT1,* and *AXIN2* (Fig. 1F; peak coverage for each is shown in Fig. 1E). Consensus binding sites from the *MITF-H* promoter that were previously shown to be either bound or unbound by VSX2 were also included as positive and negative controls, respectively (Fig. 1G) [20]. Two additional WNT pathway genes, *SMAD3* and *LEF1*, with strong peaks in the first ChIP-seq analysis (peak coverage shown in Fig. S1B) but not in the second (and thus not included in the high confidence list) were also subjected to ChIP-PCR (Fig. S1C). All five WNT targets were amplified from immunoprecipitates of d30 VSX2^{WT} hiPSC-OVs, but not VSX2^{R200Q} hiPSC-OVs (Fig. 1F and Fig. S1C). Thus, our findings confirm that these genes are direct targets for VSX2 binding during retinal differentiation.

Misexpression of an Essential Canonical WNT Pathway Component in Neural Retinal Progenitors in VSX2^{R200Q} hiPSC-OVs

To further investigate effects on the WNT pathway stemming from the loss of VSX2 DNA binding ability, VSX2^{WT} and VSX2^{R200Q} hiPSCs underwent retinal differentiation for 18 days, a time point at which the expression of MITF and VSX2 (indicative of RPE progenitors and NRPCs respectively) becomes mutually exclusive [20]. D18 VSX2^{WT} and VSX2^{R200Q} hiPSC-OVs were then immunostained for VSX2, MITF, and the critical canonical WNT pathway protein WLS (also known as GPR177), which is necessary for WNT secretion and activation (Fig. 2A-F, Fig. S2) [38]. Expression of MITF was excluded from VSX2+ cells in VSX2^{WT} hiPSC-OVs at d18, consistent with VSX2's known role as a repressor of *MITF* (Fig. 2A) [16, 18]. Similarly, WLS expression was observed in MITF+ (Fig 2B), but not VSX2+ (Fig 2C), cells at d18. In stark contrast, VSX2+ cells in d18 VSX2^{R200Q} hiPSC-OVs co-labeled with both MITF and WLS (Fig. 2D-F).

We next examined hiPSC-OV cultures differentiated to d35, a time point at which the RPE and NR domains are well-established, and to d50, when RPE maturation and retinal neurogenesis are fully underway [28, 29]. An abundant population of VSX2+ NRPCs was present in d35 VSX2^{WT} hiPSC-OVs, which did not co-label with MITF or WLS (Fig. 2G and H; also see Fig. S2G and H). By d50, VSX2^{WT} hiPSC-OVs consisted primarily of VSX2+ NRPCs with rare MITF+ RPE cells (Fig. 2I). However, in d35 VSX2^{R200Q} hiPSC-OVs, a subpopulation of VSX2+ cells continued to co-express MITF, although co-labeling between VSX2 and WLS was no longer observed (Fig. 2J and K; also see Fig. S2J and K). By d50, VSX2^{R200Q} hiPSC-OVs contained MITF+ RPE with no detectable VSX2+ cells (Fig. 2L). Together, these data demonstrate that, in the absence of functional VSX2 DNA binding activity, both MITF and WLS exhibit abnormal and prolonged expression in VSX2^{+}



Cluster	Enrichment score	Function	Count	P-value	Benjamini
1	8.85	neuron differentiation	67	8.00E-13	2.40E-09
2	8.35	sequence specific DNA binding	86	3.20E-14	2.80E-11
3	5.95	regulation of transcription	189	6.10E-04	2.90E-02
4	5.45	cell motion	62	4.40E-09	1.20E-06
5	4.91	WNT signaling pathway	23	8.50E-06	1.00E-03
6	4.81	cell adhesion	71	6.40E-06	8.70E-04
7	3.86	regionalization	30	3.80E-06	5.70E-04
8	3.51	vasculature development	32	6.30E-05	5.40E-03
9	3.51	cell junction/synapse	55	1.80E-05	8.40E-03
10	3.44	positive regulation of gene expression	60	2.10E-05	2.30E-03
11	3.4	LIM domain	13	4.80E-04	1.30E-02
12	3.17	negative regulation of transcription	43	1.10E-05	1.20E-03

Figure 1. VSX2 ChIP-seq and ChIP-PCR analyses demonstrating binding of VSX2 to WNT pathway genes in d30 $VSX2^{WT}$ but not $VSX2^{R200Q}$ hiPSC-OVs. Confirmation of the VSX2 consensus binding motif in $VSX2^{WT}$ hiPSC-OV ChIP-seq target sequences (**A**). Distribution of high confidence VSX2 DNA binding targets in $VSX2^{WT}$ hiPSC-OVs as categorized by genomic location (**B**) or RNA species (**C**). List of GO terms with greater than 3-fold enrichment generated via DAVID functional analysis of high confidence ChIP-seq peaks (**D**). Peak localization and genomic coverage maps (±200 bp toward the 5′ or 3′ end) for *WLS*, *AXIN2*, and *WNT1* (red and blue lines represent forward and reverse DNA strand reads, respectively). The top panel designates ChIP coverage and the lower panel shows the input control for each gene (**E**). VSX2 ChIP-PCR from $VSX2^{WT}$ or $VSX2^{R200Q}$ d30 hiPSC-OVs confirming direct binding of $VSX2^{WT}$, but not $VSX2^{R200Q}$, to targets identified proximal to the WNT signaling pathway genes *WLS*, *AXIN2*, and *WNT1* (**F**). Regions in the *MITF-H* promoter previously shown by ChIP-PCR to be bound (+) or not bound (−) by VSX2[20] served as positive and negative controls, respectively (amplified from the same chromatin preparation presented in panel F) (G). See also Fig. S1.

cells. However, other factors must be involved in the regulation of *WLS* expression in hiPSC-OVs, since it is eventually turned off even in mutant NRPCs.

The Canonical WNT Pathway is Active in Early $VSX2^{R200Q}$ hiPSC-OVs

We next asked whether there were differences in WNT signaling activity between $VSX2^{WT}$ and $VSX2^{R200Q}$ hiPSC-OVs by examining βCATENIN nuclear localization (NL), a hallmark of canonical WNT pathway activation. For these experiments, we focused on d14 cultures as they contain a transient mixed population of MITF+/VSX2-, MITF+/VSX2+, and MITF-/VSX2+ cells as uncommitted hiPSC-OV cells transition to either NRPCs or early RPE precursors [20]. In $VSX2^{WT}$ hiPSC-OVs, βCATENIN NL was detected in MITF+/VSX2- cells and rare MITF+/VSX2+ cells but not in MITF-/VSX2+ cells (Fig. 3A-H; also see Fig. S3). In d14 $VSX2^{R200Q}$ hiPSC-OVs, only MITF+/VSX2- and MITF+/VSX2+ populations were present, and many cells demonstrated βCATENIN NL (Fig. 3I-P; also see Fig. S3). The percentage of VSX2+/βCATENIN NL+ co-labeled cells within the total VSX2+ cell population was quantified, revealing a mean of 0.37 ± 0.90% double-positive cells in $VSX2^{WT}$ cultures and 32.1 ± 19.3% double-positive cells in $VSX2^{R200Q}$ cultures ($p = .01$) (Fig. 3Q). These results show that

Table 1. List of WNT-related genes identified by VSX2 ChIP-SEQ

ID	Gene name
CXXC4	CXXC finger 4
DIXDC1	DIX domain containing 1
FBXW4	F-box and WD repeat domain containing 4
RSPO2	R-spondin 2 homolog (Xenopus laevis)
WWOX	WW domain containing oxidoreductase
AXIN2	axin 2
CSNK1A1	casein kinase 1, alpha 1
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
DKK2	dickkopf homolog 2 (Xenopus laevis)
FRAT2	frequently rearranged in advanced T-cell lymphomas 2
FZD1	frizzled homolog 1 (Drosophila)
FZD5	frizzled homolog 5 (Drosophila)
FRZB	frizzled-related protein
NXN	nucleoredoxin
SFRP1	secreted frizzled-related protein 1
SFRP2	secreted frizzled-related protein 2
TLE1	similar to transducin-like enhancer of split 1
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)
TLE4	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)
WLS	WNT ligand secretion mediator
WNT1	wingless-type MMTV integration site family, member 1
WNT4	wingless-type MMTV integration site family, member 4
WNT7B	wingless-type MMTV integration site family, member 7B

inactivation of canonical WNT signaling coincides with the onset of VSX2 expression in d14 $VSX2^{WT}$ hiPSC-OVs, whereas WNT signaling remains active in cells expressing mutant $VSX2^{R200Q}$, which lacks DNA binding ability [21, 24, 30].

Pharmacological Manipulation of WNT Signaling Interconverts Early $VSX2^{WT}$ and $VSX2^{R200Q}$ hiPSC-OV Phenotypes

The observation that canonical WNT activity is inversely associated with the presence of functional VSX2 suggested that stimulation or inhibition of WNT signaling might alter the phenotypes of $VSX2^{WT}$ and $VSX2^{R200Q}$ hiPSC-OVs, respectively. Since d12 through d20 represents the critical period for hiPSC-OV formation, we applied pharmacological treatments at various intervals within that time frame, followed by continued growth of cultures without exogenous WNT modulation until d30. When $VSX2^{WT}$ hiPSC-OVs were treated with the WNT agonist CHIR99021 beginning at d12, cultures were pushed toward a nonretinal fate (data not shown). However, when CHIR99021 treatment of $VSX2^{WT}$ hiPSC-OVs was initiated on d14, aberrant MITF and VSX2 co-labeling was detected on d18, analogous to what was observed in $VSX2^{R200Q}$ hiPSC-OVs (Fig. 4A-C; compare Fig. 4C to Fig. 2D). Control, vehicle-treated $VSX2^{WT}$ hiPSC-OVs showed the typical segregation of VSX2 and MITF expression at d18 (Fig. 4D-F). Upon removal of CHIR99021 at d20 and further culture to d30, expression of WNT pathway (*WLS* and *AXIN2*) and RPE (*MITF* and *OTX2*) genes were increased, whereas NR-related genes (*VSX2, SIX6, RX, FGF9*) were down-regulated (Fig. 4G). By contrast, CHIR99021 treatment of $VSX2^{R200Q}$ hiPSC-OVs over the same time periods had no significant effect (data not shown).

Next, $VSX2^{R200Q}$ hiPSC-OVs were treated with the WNT inhibitor IWP2. Unlike WNT agonist treatment of $VSX2^{WT}$ hiPSC-OVs, IWP2 treatment of $VSX2^{R200Q}$ hiPSC-OVs beginning at d12 did not lead to the production of nonretinal lineages. Thus, we applied IWP2 from d12 to d20 for all experiments. When sampled at d18 (Fig. 4H-M), abundant VSX2+ cells were present in IWP2-treated hiPSC-OVs (Fig. 4H), similar to vehicle-treated hiPSC-OVs (Fig. 4K). However, unlike vehicle-treated cultures, MITF expression was no longer detected in mutant VSX2+ cells at d18 in the presence of IWP2 (compare Fig. 4I to Fig. 4L). Likewise, when IWP2 treatment of $VSX2^{R200Q}$ hiPSC-OVs was discontinued at d20, followed by an additional 10 days of culture to promote further differentiation, WNT and RPE gene expression decreased and NR-related gene expression increased relative to d30 vehicle-treated $VSX2^{R200Q}$ hiPSC-OVs (Fig. 4N). Thus, pharmacological blockade of WNT signaling during early NR:RPE patterning partially rescues the $VSX2^{R200Q}$ hiPSC-OV phenotype at d30, while early augmentation of WNT signaling in $VSX2^{WT}$ hiPSC-OVs mimics the effects of the $VSX2^{R200Q}$ mutation.

Discussion

The role of VSX2 as a transcriptional repressor is well-documented, although the number of known gene targets for VSX2 is small considering its pleiotropic effects throughout retinogenesis [19, 30, 39–41]. One of the earliest and most important functions of VSX2 is in the maintenance of a proliferating pool of NRPCs in the developing OV, which it accomplishes at least in part by repressing *MITF* expression, as demonstrated in both animal and hPSC model systems [16, 18, 20]. In this report we provide evidence that VSX2 can regulate expression of WNT pathway genes in early hiPSC-OVs, and that repression of WNT signaling by VSX2 may contribute to the maintenance of NR identity at the expense of RPE. Furthermore, pharmacological manipulation of WNT signaling can partially mimic or rescue the effects of a functional null VSX2 mutation in early hiPSC-OVs.

The Wnt pathway is necessary for axis formation and early embryonic patterning, and also serves as an essential regulator of neural and ocular development in part through the canonical βCatenin/TCF/Lef transcriptional pathway [8, 9, 42, 43]. With specific regard to ocular development, Wnt signaling is required for proper dorsal-ventral patterning, RPE production, and optic cup morphogenesis in vivo [10, 11, 44–47]. Conditional knockout (CKO) of βCatenin at an early OV stage in mouse resulted in transdifferentiation of RPE to VSX2+ NRPCs, leading to partial duplication of the NR [10, 46]. Conversely, ectopic βCatenin activation at later OV stages caused a thickened RPE layer with aberrant Mitf expression in the NR [46]. Interestingly, *Mitf*, which acts as a regulator of RPE differentiation and pigmentation, is a direct gene target for the Wnt/βCatenin/Lef transcriptional complex, and *Mitf* null mutations exhibit a similar RPE to NR phenotype as the βCatenin CKO [4, 7].

The WNT pathway has also been exploited in order to enrich for desired cell types in vitro [48, 49], including production of NRPCs or RPE cells from hESCs and hiPSCs [50–53]. Our prior finding that the expression of multiple WNT-related genes was dramatically increased in $VSX2^{R200Q}$ hiPSC-OVs led us to further examine the relationship between VSX2 and the WNT pathway using ChIP and massively parallel DNA sequencing. These analyses showed that VSX2 directly targets a host

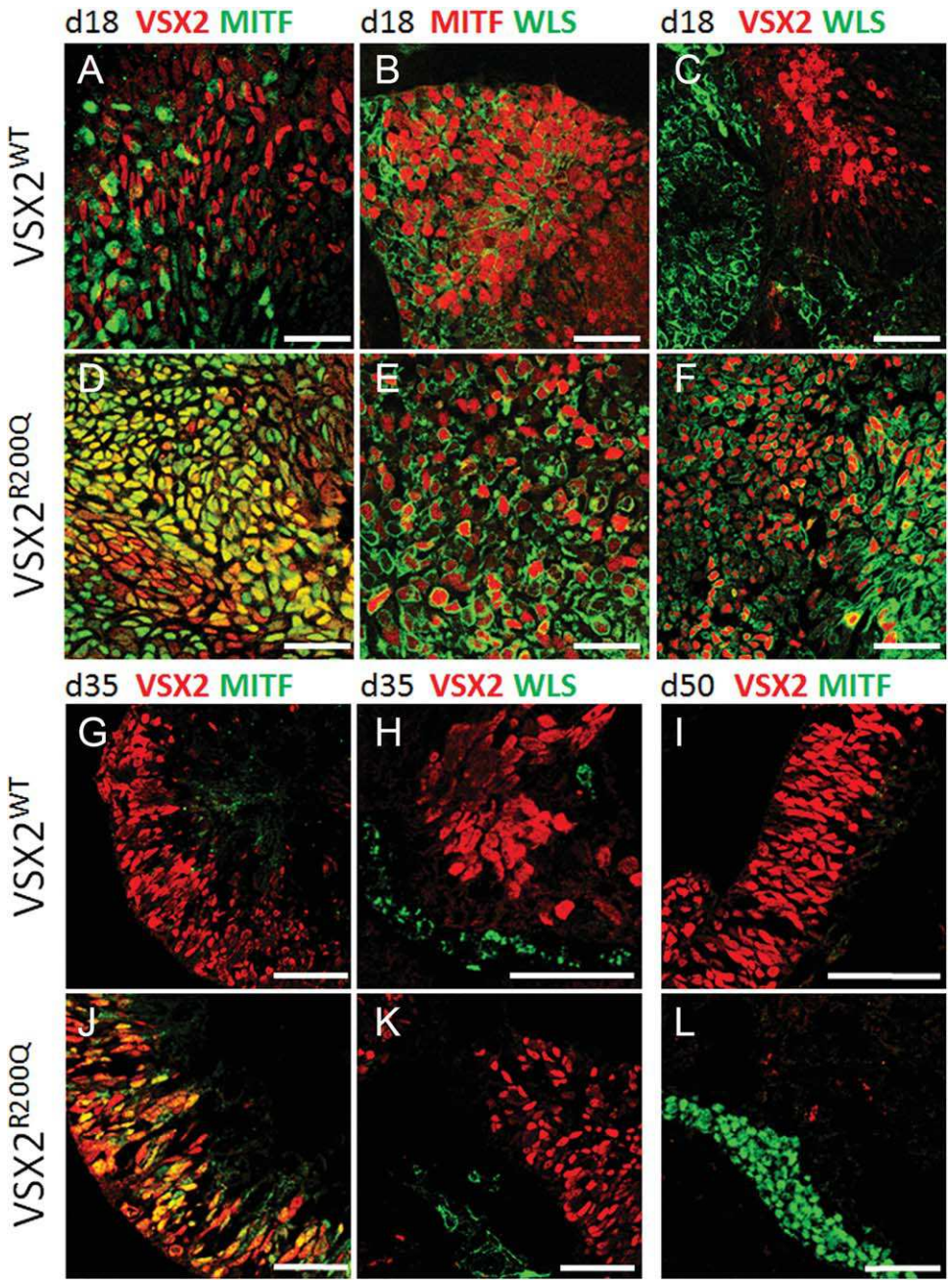


Figure 2. Comparison of VSX2, MITF, and WLS localization in $VSX2^{WT}$ and $VSX2^{R200Q}$ hiPSC-OVs. D18 OVs from $VSX2^{WT}$ (**A–C**) or $VSX2^{R200Q}$ (**D–F**) hiPSCs were immunostained for VSX2 (red) and MITF (green) (A, D), MITF (red) and WLS (green) (B, E), or VSX2 (red) and WLS (green) (C, F). D35 and d50 $VSX2^{WT}$ hiPSC-OVs (**G–I**) were examined for VSX2 (red) and MITF (green) (G, I) or VSX2 (red) and WLS (green) (H) expression and compared to d35 (**J, K**) and d50 (**L**) $VSX2^{R200Q}$ hiPSC-OVs. Identical images with DAPI-labeled nuclei are shown in Fig. S2. Scale bars = 50 μm.

of genes encoding critical WNT pathway constituents in early hiPSC-OVs. Therefore, results from the present study, combined with prior reports in hiPSCs [20] and animal models [19, 21] showing direct regulation of *MITF* expression by VSX2, suggest that VSX2 antagonizes RPE development on multiple levels (Fig. 5). In addition to repressing *MITF*, VSX2 is capable of downregulating expression of key WNT pathway genes, including *WLS*, which is required for WNT trafficking and secretion [38, 54], and *AXIN2*, which has a complex role in both βCatenin destabilization and recruitment of key Wnt receptor components to the cell membrane [55]. These examples, along with others (Tables 1 and S3), point to WNT pathway components as important targets for VSX2-mediated gene repression during early NR development in hiPSCs. However, the observation that *WLS* is eventually downregulated even in $VSX2^{R200Q}$ mutant NRPCs indicates that gene regulatory mechanisms independent of VSX2 are also in place to control expression of WNT pathway components in hiPSCs.

In summary, our findings indicate a heretofore undescribed role for VSX2 as a regulator of WNT signaling during

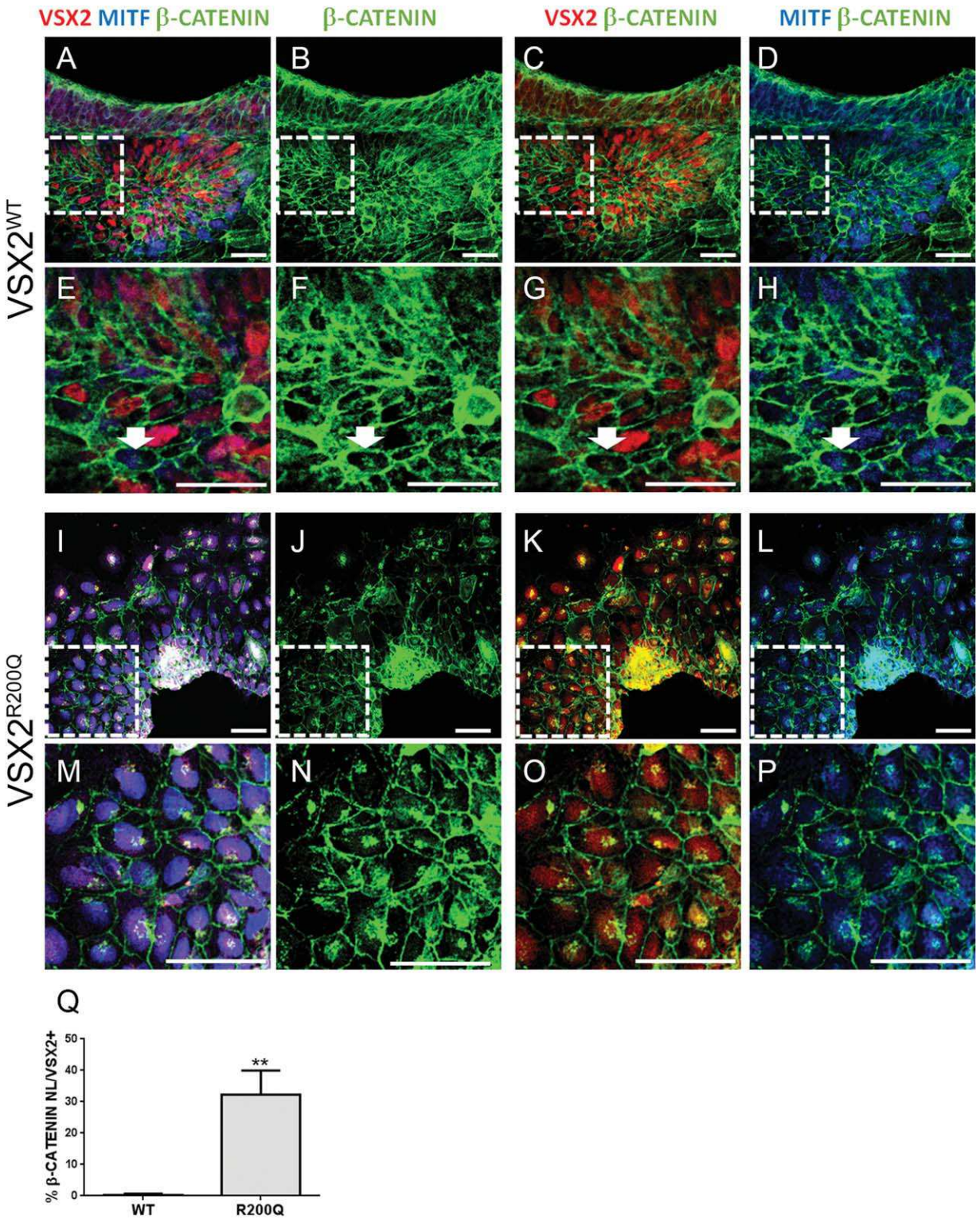


Figure 3. βCATENIN localization in d14 VSX2^{WT} and VSX2^{R200Q} hiPSC-OVs. D14 VSX2^{WT} (**A–H**) or VSX2^{R200Q} (**I–P**) hiPSC-OVs were immunolabeled with βCATENIN (green), VSX2 (red), and MITF (blue) primary antibodies. Examples of βCATENIN nuclear localization are designated by white arrows (E–H). Panels E–H and M–P are cropped magnifications of the outlined areas in panels A–D and I–L, respectively. Also see Fig. S3 for identical images with βCATENIN and DAPI-labeled nuclei. (**Q**) Graph of percent of βCATENIN nuclear localization in VSX2+ nuclei for WT and R200Q d14 hiPSC-OVs. $^{**}p = .01$. Scale bars = 50 μm.

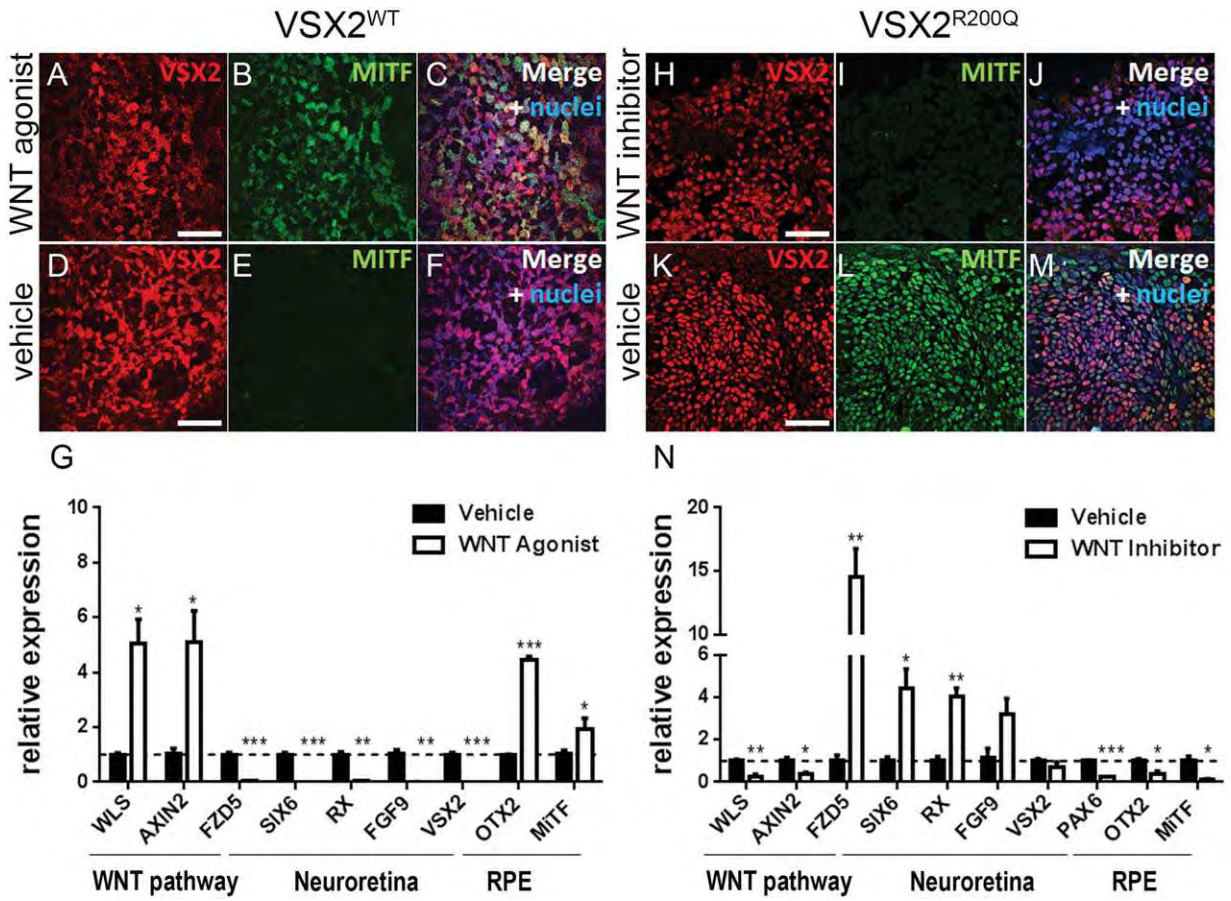


Figure 4. Interconversion of early VSX2^{WT} and VSX2^{R200Q} hiPSC-OV phenotypes by pharmacological manipulation of WNT signaling. Immunocytochemistry analysis on d18 VSX2^{WT} hiPSC-OVs treated with the WNT agonist CHIR99201 (**A-C**) or vehicle (**D-F**) showing VSX2 (red) and MITF (green) coexpression (A–C) or lack thereof (D–F). RT-qPCR analysis of d30 VSX2^{WT} hiPSC-OVs treated from d14-d20 with vehicle or CHIR99201 (**G**). Immunocytochemistry analysis on d18 VSX2^{R200Q} hiPSC-OVs treated with the WNT inhibitor IWP2 (**H-J**) or vehicle (**K-M**) showing lack of coexpression (H–J) or coexpression (K–M) of VSX2 (red) and MITF (green). (**N**) RT-qPCR analysis of d30 VSX2^{R200Q} hiPSC-OVs treated from d12-d20 with vehicle or inhibitor. Nuclei are shown in blue. *$p < .01$; **$p < .001$;***$p < .0001$. Scale bars = 50 μm.

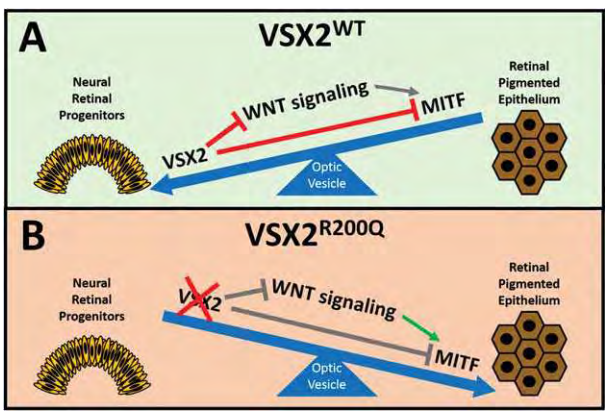


Figure 5. Schematic model depicting the impact of VSX2-mediated regulation of WNT signaling on early retinal patterning in wild-type hiPSC-OVs, and the consequence of the (R200Q)VSX2 mutation. VSX2^{WT} acts in multiple ways to maintain NR identity. On a gene expression level, VSX2 antagonizes expression of WNT pathway genes and *MITF*, resulting in the promotion of NR at the expense of RPE in hiPSC-OVs (**A**). In the absence of functional VSX2 (i.e., VSX2^{R200Q} hiPSC-OVs), expression of WNT pathway genes and *MITF* are unchecked, leading to RPE production over NR (**B**).

retinal differentiation and suggest a mechanism whereby VSX2 maintains NR identity in part by regulation of WNT pathway genes. This study also offers the first unbiased search for VSX2 binding sites in a mammalian developmental model system, which may lead to the discovery of other genes and mechanisms involved in the production and maintenance of early NR.

Conclusion

This study utilized an hiPSC model of a retinal developmental disorder, combined with pharmacological manipulations, chromatin immunoprecipitation, and massively parallel DNA sequencing to gain insight into a novel mechanism underlying NR vs. RPE production during human retinogenesis. We show that the NRPC transcriptional factor VSX2 is a direct regulator of numerous WNT-related genes and that VSX2-mediated antagonism of WNT signaling plays an important role in the maintenance of NR identity at the expense RPE. Our findings also underscore the utility of hiPSCs in investigating the earliest stages of human ocular development, which are otherwise inaccessible to study.

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Author Contributions

E.E.C.: conception and design, collection of data, data analysis and interpretation, manuscript writing; L.S.W.: conception and design, collection of data, data analysis and interpretation, manuscript writing; K.L.: Data anlysis and interpretation; M.J.P.: conception and design, data analysis and interpretation; K.W.: collection of data; A.P.: collection of data; A.H.: collection of data; I.P.: collection of data; K.B.: collection of data; J.L.: collection of data; J.H.M.: collection of data; S.K.: financial support, data analysis and interpretation; J.A.T.: financial support, data analysis and interpretation; D.A.G.: financial support, conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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