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14. ABSTRACT
In this grant, we sought to investigate the mechanisms that regulate the earliest events in cone photoreceptor development and to exploit this knowledge to program human stem cells directly into cones. Using RNA-seq, we identified several genes that are upregulated in advance of the earliest photoreceptor gene, Otx2. Two of these genes appeared to promote Otx2 expression in the developing mouse retina. These results provided a key link between what is occurring in retinal stem cells and the gene network expressed in nascent cone photoreceptors. We also found that transiently delivering the mRNA for three transcription factors promoted cone photoreceptor formation in retinal stem cells derived from human embryonic stem cells. These results are an important first step towards generating synchronized cone photoreceptor populations in the dish that could be used therapeutically to reverse vision loss.

15. SUBJECT TERMS
Cone photoreceptor, retina, retinal stem cell, Otx2, Onecut1, Blimp1, RNA-seq., transcription factors, and development.

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Introduction:

The cone photoreceptors of the retina are required for high acuity and color vision. Traumatic injuries can ultimately cause the death of cone cells. This results in permanent vision loss because cones cannot regenerate. Our goal is to restore vision using a regenerative medicine approach. We have shown that recapitulating early eye developmental events can efficiently program human embryonic stem (hES) cells into retinal stem cells. We hypothesized that recapitulating cone development *in vitro* can efficiently program retinal stem cells into transplantable cone photoreceptors. However, the mechanisms that regulate cone development are largely unknown. Several transcription factors play important roles. *Otx2* is required for rod and cone photoreceptor genesis as well as bipolar cell interneuron formation. Activating Otx2 is a key step in priming retinal stem cells for photoreceptor development. How retinal stem cells decide to activate *Otx2* is unknown and is a significant barrier to efficiently programming cone identity in stem cell cultures. Therefore, our first objective was to identify the gene regulatory network that controls *Otx2* expression and early photoreceptor fate commitment. Downstream of *Otx2* is *Blimp1* (*Prdm1*), which is transiently expressed by most Otx2+ cells and acts to maintain photoreceptor identity by blocking bipolar cell development within these cells. A smaller subset of Otx2+ cells transiently expresses *Onecut1* during development, which when overexpressed can promote cone marker expression. These data suggested that co-activation of Otx2, Blimp1, and Onecut1 is *sufficient* to promote cone development. Our second objective was to test whether transient and/or sequential activation of *OTX2*, *BLIMP1*, and *ONECUT1* are sufficient to program hES cell-derived retinal stem cells into transplant-competent cones.

Keywords:
1. Cone photoreceptor
2. Retina
3. Retinal stem cell
4. Otx2
5. Onecut1
6. Blimp1
7. RNA-seq.
8. Transcription factors
9. Development

Accomplishments:
The accomplishments under this one year award are described by specific aims and major tasks, as previously outlined in the approved Statement of Work. Administrative objectives (*e.g.*, animal approvals) were all achieved and are not discussed further in this report.

Specific Aim 1: To determine how retinal stem cells acquire cone photoreceptor potential (Brzezinski).
GOALS AND ACCOMPLISHMENTS:

A subset of retinal stem cells activates the transcription factor Otx2, which appears to give cells the potential to form photoreceptors and bipolar cells. Downstream of Otx2 is Blimp1, which acts to restrict bipolar cell potential in otherwise multipotent Otx2+ cells. Several cone-specific genes downstream of fate choice (*e.g. Rxrg*, *Thrb2*, and *S-opsin*) have been characterized, but how Otx2+/Blimp1+ cells become restricted to cone fate is unclear. Identifying the factors that control cone development is difficult because cones make up only ~2% of the mature retina and are formed over a relatively protracted time during mouse development (embryonic day (E)12.5 to E18.5). To bypass this problem, we treated intact E14.5 retinal explant cultures with DAPT, a γ-secretase inhibitor that effectively blocks Notch signaling. Inhibiting Notch causes retinal stem cells to exit the cell cycle and differentiate as cones in a synchronous fashion. We used RNA-seq to identify gene expression changes as retinal stem cells activated Otx2 and became cone photoreceptors (Major Task 1). Then we tested how upstream candidate factors influenced Otx2 expression (Major Task 2). We made significant progress on both tasks, paving the way for future studies of cone photoreceptor development.

Major Task 1: RNA-seq to identify genes upstream of Otx2.

To identify the genes that precede or coincide with Otx2 activation during cone genesis, we treated E14.5 C57BL/6 (wild-type) explants with DMSO (carrier control) or 10μM DAPT for 6, 7, 8, 9, and 10 hours. RNA from each time-point and condition was collected in triplicate and directionally labeled (Illumina TruSeq Stranded mRNA kit) for RNA-seq by the CU Denver Genomics and Microarray facility. From this, we obtained roughly 1.2 billion sequencing reads for an average sequencing depth of 41 million reads per sample. Dr. Ken Jones conducted ANOVA on this large dataset. From this, we identified 236 genes that were differentially expressed ($P < 0.01$, false discovery rate < 0.25) between DMSO and DAPT conditions at times that preceded *Otx2* upregulation (6-9 hours) (Appendix, Tables 1 & 2). Focusing on transcription factors, we identified genes such as *Hes1, Hes5,* and *Sox9,* which are likely negative regulators of *Otx2* expression (Fig. 1). Other transcription factors, such as *Onecut1* and *Neurod1* are likely to work in parallel to *Otx2* (see aim 2, below). Finally, a few genes like *Ascl1*, *Olig2*, and *Neurog2* peaked around the onset of Otx2 expression and are likely to activate *Otx2* expression (Fig. 2). These three factors are described in more detail in task 2 below.

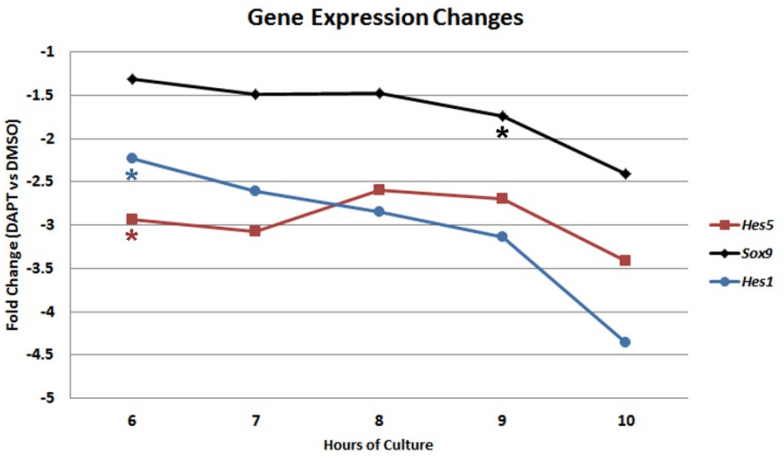


Fig. 1: RNA-seq analysis of E14.5 mouse retinas treated with DAPT or DMSO and cultured from 6 to 10 hours. Gene expression changes between DAPT and control are shown as fold changes. Asterisks mark the first point a gene expression change is statistically significant. The Notch signaling targets *Hes5* and *Hes1* are strongly downregulated by 6 hours of culture, showing that the DAPT is inhibiting the Notch pathway as intended. Blocking Notch signaling forces progenitors to exit the cell cycle and differentiate and the gene *Sox9*, which marks progenitors is downregulated at 9 hours. This correlates with the upregulation of Otx2.

Our milestone for Task 1 was to identify 5 candidate regulators of *Otx2* by 6 months of time. We identified more than a dozen candidate transcription factors (as positive or negative regulators) by about 8-9 months into this project. We met our goals, though we were a bit behind schedule (see changes and problems, below). This huge dataset has provided us with numerous hypotheses to test about the mechanisms of *Otx2* regulation and both cone photoreceptor fate potential and fate choice.

The earliest cone-specific markers are unknown, making understanding early steps in their development difficult. As a secondary measure, we also searched for genes that could mark cone

photoreceptors at the earliest stages of development. One gene that was activated around the time of *Otx2* upregulation was *Chrnb4*, a subunit of acetylcholine receptors. Preliminary *in situ* hybridization studies show this gene to be expressed in the area where developing cones reside during embryogenesis. A transgenic animal that makes GFP under the control of *Chrnb4* enhancer sequences has previously been made (*Chrnb4-eGFP*). We ordered this animal, though the re-derivation process has not yet been completed, to test whether Chrnb4-eGFP expression marks early developing cones. This mouse model may prove to be a key tool for studying cone development in the future.

Major Task 2: Functional analysis of candidate Otx2 regulators in cultured explants.

After identifying several candidate positive and negative regulators of Otx2 expression, we next wanted to test how these factors control *Otx2* expression. As the RNA-seq analysis was delayed a few months, we were limited as to how many candidates we could test. We started by looking at transcription factors whose expression was activated in advance of *Otx2*. Of these, three factors (*Ascl1*, *Olig2*, and *Neurog2*) were previously shown to be made by progenitors shortly before or during cell cycle exit; good candidate activators of Otx2. To test how they worked, we conducted loss-of-function studies in cultured explants. We started by electroporating plasmid vectors that stably express shRNAs that target these genes. However, these tools worked poorly in our hands (see changes and problems, below). We then switched to directly transfected siRNAs against each gene. These were much more robust, strongly (but not completely) repressing each factor in cultured explants. With validated tools, we sought to knock-down these factors singly and in combination to bypass any possible redundancy. As predicted from genetic mutants, knock-down of any factor singly had no appreciable effect on Otx2 expression when transfected into explants and cultured for one day. When we knocked-down multiple genes, it was evident that the combinations that contained siRNA against both *Ascl1* and *Neurog2* were able to inhibit Otx2 expression (Fig. 3). These

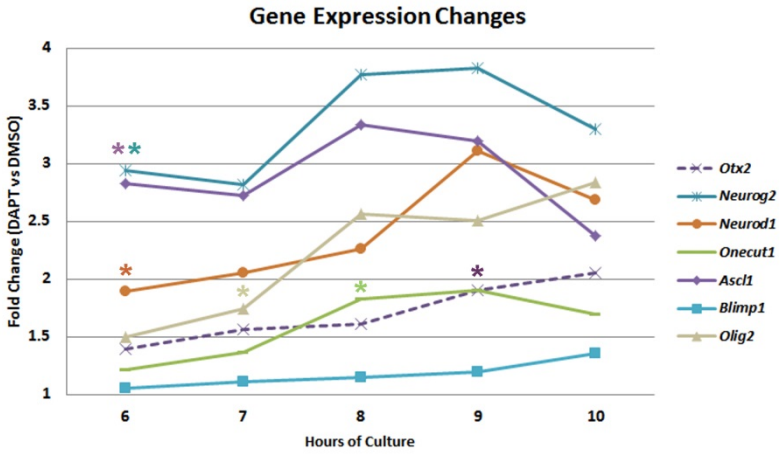


Fig. 2: RNA-seq analysis of E14.5 mouse retinas treated with DAPT or DMSO and cultured from 6 to 10 hours. Gene expression changes between DAPT and control are shown as fold changes. Asterisks mark the first point a gene expression change is statistically significant. At 9 hours of culture, the key photoreceptor gene *Otx2* is upregulated. Several genes are activated before this, including *Ascl1*, *Neurog2*, *Onecut1*, and Neurod1. The downstream target of Otx2, *Blimp1*, is not activated in this time-span as expected.

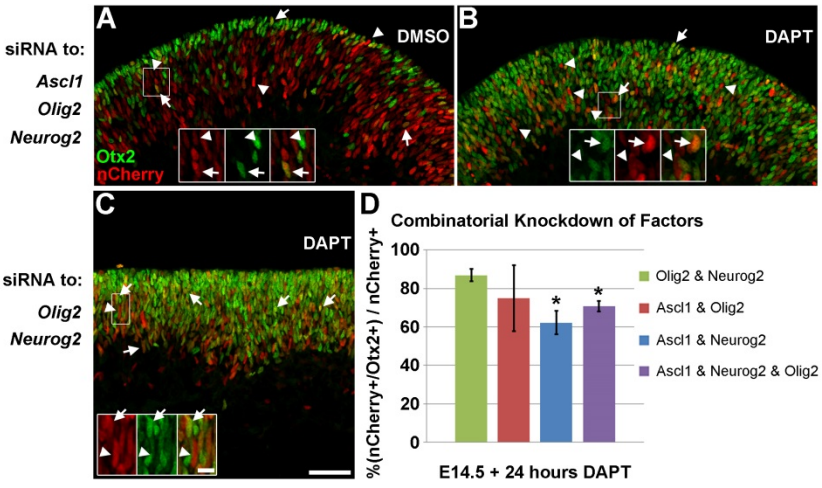


Fig. 3: Knock-down of *Ascl1*, *Neurog2*, and *Olig2* in E14.5 mouse retinas. Retinas were cultured with or without DAPT for 24 hours. The presence of DAPT strongly upregulates *Otx2*, allowing the testing of how *Ascl1*, *Olig2*, and *Neurog2* siRNAs affect Otx2 expression (green). Retinas were co-transfected with nuclear Cherry (red) to mark cells that take up siRNA. **(A)** An E14.5 retina treated with DMSO and siRNA against *Ascl1*, *Neurog2*, and *Olig2*. Arrows mark transfected cells that make Otx2 and arrowheads mark those that do not co-express Otx2. **(B)** An E14.5 retina treated with the same three siRNAs and cultured in DAPT. Note the strong increase in Otx2 expression. **(C)** An E14.5 retina treated with DAPT and transfected with siRNA to *Olig2* and *Neurog2*. This condition showed the lowest percentage of transfected cells that co-express Otx2. Scale bars are 50μm for panels and 10μm for insets. **(D)** Quantification of transfected cells in the multiple siRNA treatment experiments. Only knock-down of *Ascl1* and *Neurog2* (blue, purple) inhibits Otx2 expression. The asterisk denotes $P < 0.01$ by Student's T-test, $N = 3$ per condition. Error bars represent the SD.

experiments, while still preliminary, suggest that *Ascl1* and *Neurog2* combine in a dosage sensitive fashion during the final cell cycle to activate Otx2 and drive photoreceptor formation.

Our milestone for Task 2 was to identify the function of candidate regulators of *Otx2* expression and cone genesis by the end of the funding period. We were able to test how three of these candidates impact *Otx2* expression. With the delay in RNA-seq data genesis and the stable shRNA constructs failing to work, we were unable to do much functional testing during the funding period. Thus, we were partially able to meet our goals during the funding period. Nonetheless, we have identified Ascl1 and Neurog2 as regulators of Otx2 expression in the developing mouse retina. The intersection of these two factors may ultimately control the probability of a retinal stem cell activating Otx2 and becoming a photoreceptor.

TRAINING AND PROFESSIONAL DEVELOPMENT:
Nothing to report.

DISSEMINATION OF RESULTS:
Nothing to report. However, we are testing our candidates further and plan to publish a manuscript in about 6 months (see below).

NEXT REPORTING PERIOD:
Nothing to report. This is the final report for this award. However, using funds from philanthropic sources we have recently secured (11/2015), we are continuing to test candidate *Otx2* regulators (singly and in combination) and to evaluate *Chrnb4-GFP* mice for early labeling of cones.

Specific Aim 2: To program retinal stem cells into transplant-competent cone photoreceptors (Lamba).
GOALS AND ACCOMPLISHMENTS:

We and others have shown that hES cells can be efficiently programmed towards retinal fate by recapitulating early eye developmental events *in vitro*. After three weeks of culture, we found that >80% of cells adopted retinal stem cell fate. Upon further culture of 6-8 weeks, these cells spontaneously differentiated into all major retinal neuronal cell types. While rod photoreceptors were relatively abundant in these cultures, cones were sparse. Most studies have focused on rod photoreceptor integration; however, when a mixed population of retinal cells were transplanted into adult host mice, only a small subset of the integrated photoreceptors (<1%) were cones. While this shows that cone cell replacement is possible, it remains too inefficient to be clinically viable. To overcome this barrier, we must drastically increase the number of transplant-competent cones generated from stem cell cultures. We reasoned that recapitulating developmental events during cone genesis would improve hES cell differentiation into transplantable cone photoreceptors. To test this, we introduced mRNA for three genes (*OTX2*, *BLIMP1*, and *ONECUT1*) involved in cone development in multiple combinations to hES cell cultures and screened them for cone formation (Major Task 3). Then, we tested whether these cone-biased cultures could be successfully transplanted into adult wild-type host eyes (Major Task 4). We were able to complete both tasks and show that, while our initial attempts to program cone fate showed promise, these cultures were only poorly transplant competent.

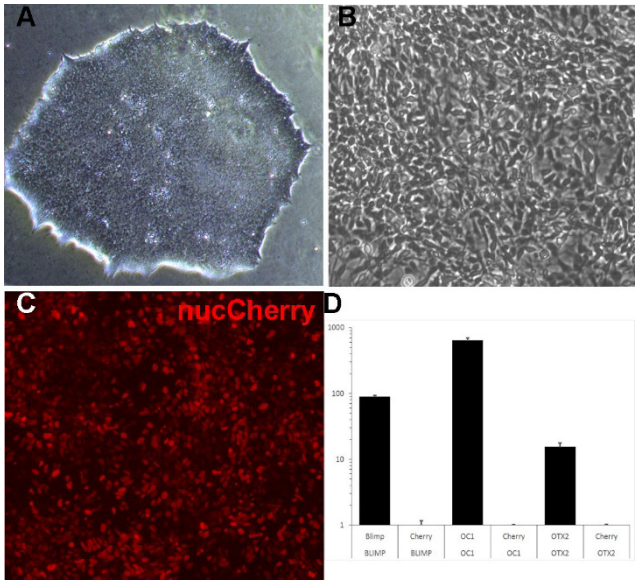


Fig. 4: Transfection of hESC-derived retinal cells. Undifferentiated hESCs **(A)** were differentiated for 2 months to generate retinal stem cells **(B)**. **(C)** Nuclear Cherry expression one day after mRNA transfection in over 70% of cells. **(D)** qRT-PCR analysis confirms strong expression (fold change) of transfected mRNAs 3 days later when compared to Cherry controls. Top line, transfected mRNA. Bottom line, RT-PCR product.

Major Task 3: Enhance cone differentiation from hESC-derived retinal progenitors using transcription factors.

A. Develop transfection parameters and protocols for mRNA transfection of hESC-derived retinal progenitors.

For generation of hESC-derived retinal cells, we differentiated them using our previously published protocol. The undifferentiated hESC cells (Fig. 4A) were differentiated over 2 months to generate retinal stem cells (Fig. 4B). These cells were then re-plated in 24-well format plates to identify optimal transfection conditions with short-lived synthetic mRNAs (nuclear *Cherry*). Using a number of different transfection agents and mRNA-transfection ratios, we identified Stemfect (at a ratio of 0.5μg mRNA / μl reagent) as the optimum reagent for efficient transfection of hESC-derived retinal cells (Fig. 4C). At this ratio we can transfect over 70% of all cells in a well. We then confirmed that this protocol works well for the mRNAs of interest involved in cone specification, *i.e. ONECUT1*, *BLIMP1*, and *OTX2*. As expected we observed strong expression (protein and mRNA) for each of these factors compared to *mCherry* control transfected cells (Fig. 4D and data not shown). With the successful optimization of mRNA transfection, we were then able to test how individual and combinations of factors influenced cone photoreceptor formation in hES cell cultures.

B. Assess efficiency of cone differentiation following mono/combination transfection of OTX2, BLIMP1, and ONECUT1.

We next tested how mRNA transfection affected photoreceptor gene expression in the hESC-derived retinal cells. Upon further optimization of our protocol in the final quarter of the funding period, we achieved good triple co-transfections of *OTX2*, *ONECUT1*, and *BLIMP1*. Figure 5 depicts the gene expression changes of various photoreceptor genes by qRT-PCR at three and five days post-transfection. *OTX2* had only modest effects on pan-photoreceptor genes (*CRX*, *NEUROD1*, *RECOVERIN*), rod- (*AIPL1*), and cone-specific genes (*THRB2*) compared to *mCherry* only transfections (Data not shown and Fig. 5). In contrast, *BLIMP1* and *ONECUT1* transfections were able to modestly increase photoreceptor gene expression, including cone-specific genes (Fig. 5). The combination of all three mRNAs had the maximal effect, driving the expression of the pan-photoreceptor gene *CRX* to high levels and increasing the cone-specific gene *THRB2* (*aka*, *TRB2*). Of note, these gene expression changes were seen 5 days after transfection, considerably longer than the lifetime of the transfected mRNAs (~24 hours). These results imply that combinations of mRNAs are able to stably shift retinal stem cells to photoreceptor fate. Future work is needed to determine what fraction of these cells are rods versus cones. Our milestone for this task was to achieve high efficiency programming of hES cells into cone photoreceptors. Our work does not quite reach this milestone, but represents a strong and promising first step towards reaching this goal.

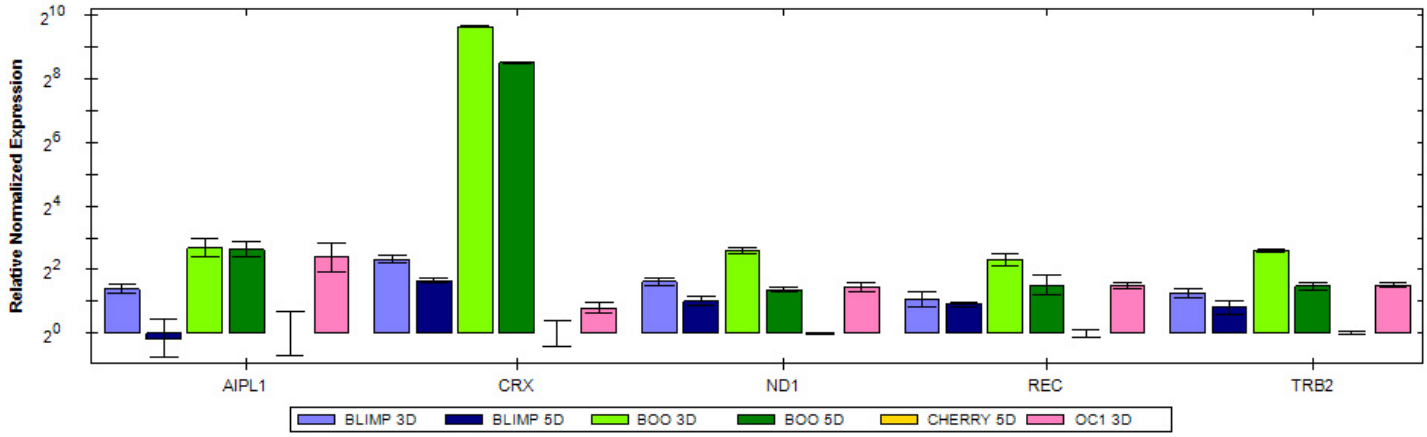


Fig. 5: Effects of single and combinatorial mRNA overexpression in hESC-derived retinal stem cells 3 days (3D) and 5 days (5D) post-transfection. A number of photoreceptor genes are up-regulated by *BLIMP1* (B), *ONECUT1* (O, OC1), and *OTX2* (O). The highest-cone specific gene expression (*TRB2*) was achieved using all three mRNAs simultaneously (BOO).

Major Task 4: Transplantation of stem cell-derived cones into adult host mice.

Finally, we wanted to test if the transfected retinal stem cells expressing cone-specific markers would survive transplantation and integrate into the host circuitry. For this we decided to try two conditions based on the *in vitro* data. We transplanted cells which had been transfected either with (a) *BLIMP1* alone, (b) *ONECUT1* alone, (c) the combination of *ONECUT1* and *BLIMP1*, (d) the triple combnation of *BLIMP1*, *ONECUT1* and *OTX2*, and (e) *mCherry* (control). Cells were first infected with a GFP-expressing lentivirus to permanently track the cells post-transplantation. Cells were transfected with the different mRNAs and transplanted 3 days later into WT host mice. For all the conditions, we saw good survival of transplanted cells,

confirming that transfected reprogrammed cells can survive dissociation and transplantation (Fig. 6). Cone integration into the retina was very low 2 weeks post-transplantation, and we did not observe any significant difference in the integration capacity between the different mRNA transfections (Fig. 6). Our milestone for this task was to characterize hES-derived cone integration. We feel that we mostly accomplished this milestone, showing that these cells can survive well upon transplantation. This is an important step in the path to implementing cone cell replacement therapy. Nonetheless, additional work is needed to test whether different times post-transfection or post-transplantation will improve the integration of cone photoreceptors into host retinas.

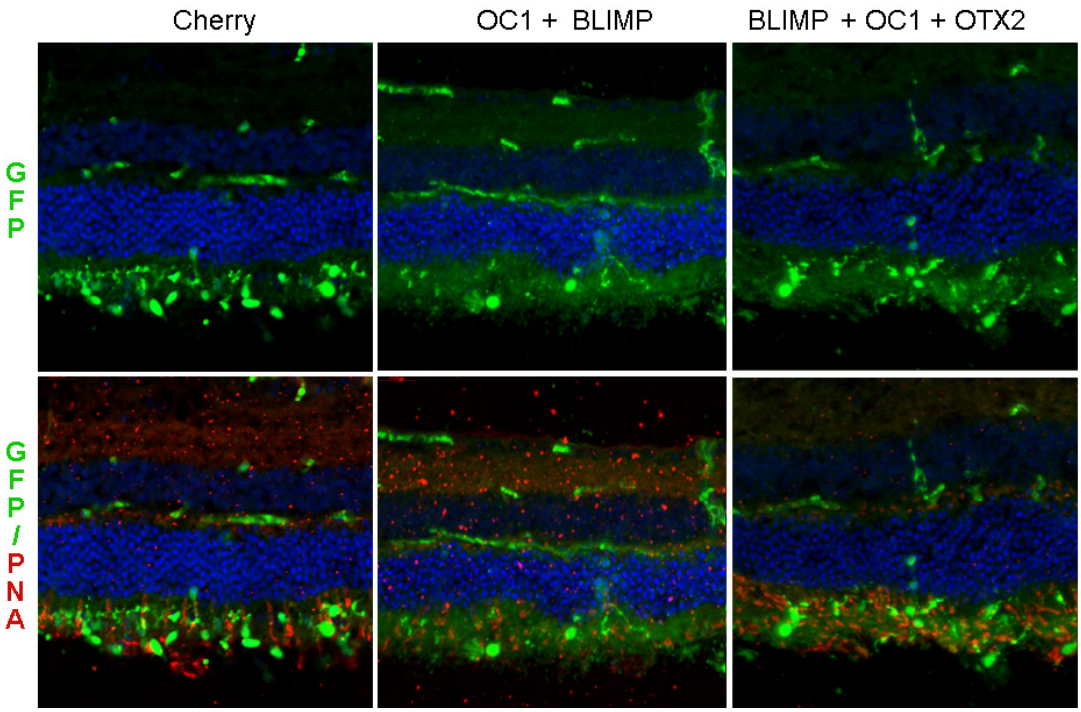


Fig. 6: Transplantation of programmed hESC-derived retinal cells. Survival and integration of hESC-derived retinal cells transfected with either cherry (control), or dual (*OC1* and *BLIMP1*) and triple (*OC1*, *BLIMP1*, and *OTX2*) combinations of transcription factors. Cells were permanently marked with GFP (green) and stained for the cone marker PNA lectin (red). Cell survival was seen as cells residing in the subretinal space (bottom of images), but few GFP+/PNA+ cones escaped this space and entered the retina in any transfection conditions.

TRAINING AND PROFESSIONAL DEVELOPMENT:
Nothing to report.

DISSEMINATION OF RESULTS:
Nothing to report. However, our ongoing experiments on this aim will allow us to publish a manuscript in the coming 12 months.

NEXT REPORTING PERIOD:
Nothing to report. This is the final report for this award. However, using funds from philanthropic sources we have recently secured (11/2015), we are continuing to explore the tasks in this aim. In particular, we are expanding the conditions and timing of mRNA administration to cultured hES cells and will attempt this experiment in 3D cultures, which may improve nascent cone photoreceptor survival and transplantability.

Impact:

ON THE FIELD:

The goal of our projects was to collaboratively generate data to form and test new hypotheses about the mechanisms of cone photoreceptor development, stem cell programming, and human-derived cone transplantation. Our findings, while preliminary in this brief funding period, have informed several new avenues of research. First, we have for the first time characterized gene expression in cone photoreceptors developing synchronously and identified several potential gene regulatory networks that promote cone formation. Indeed, two genes (*Ascl1*, *Neurog2*) we identified in this analysis appear to be required to co-activate *Otx2*, the key transcription factor required for rod and cone photoreceptor formation. These data will allow the field to test multiple factors, both singly and in combinations, to identify how retinal stem cells become cone photoreceptors. Second, we have looked downstream of Otx2 and attempted to directly program human ES cells into cone photoreceptors using transient ("scar-free") mRNA transfection. Our findings show that this technique can promote cone formation in the dish, though much optimization is still required to generate pure cone photoreceptors. This is an important early step for making cone photoreceptor transplantation a viable clinical strategy. It is highly likely that transient mRNA transfection strategies will be used broadly by researchers that want to reprogram stem cells for clinical applications. Lastly, we attempted to transplant cone photoreceptors derived from human retinal stem cells into host mice. We had success getting these cells to survive, but have yet to identify the conditions that allow them to best integrate into the host. Understanding how cones integrate upon transplantation remains a major barrier to therapy, but our approach going forward will allow us to test multiple hypotheses about what allows cones to integrate. In sum, our experiments have significantly advanced the fields of cone development, stem cell programming, and cell transplantation in the retina.

OTHER DISCIPLINES:

Nothing to report

TECH TRANSFER:

Nothing to report

SOCIETY:

Nothing to report

Changes and Problems:
We encountered some modest technical challenges during the course of our experiments. These challenges were overcome and none required a major change in the objectives or expenditures. These are briefly detailed below. IACUC approvals were renewed by both Dr. Brzezinski and Lamba without any significant changes during the funding period.

Specific Aim 1: To determine how retinal stem cells acquire cone photoreceptor potential (Brzezinski)

Major Task 1: RNA-seq to identify genes upstream of Otx2.

We were delayed in our RNA-seq experiments primarily by a backlog in our high throughput sequencing core to process the samples. This resulted in our experiments being delayed about 6-8 weeks. This had a ripple effect that modestly delayed the experiments of major task 2

Major Task 2: Functional analysis of candidate Otx2 regulators in cultured explants.

Our experiments to perturb candidate Otx2 regulators was modestly delayed by the RNA-seq (see above). We also experienced a technical problem when trying to knock-down candidate Otx2 regulators. The plasmid based shRNA approach was initially favored as it will result in stable, semi-permanent knock-down. However, none of the plasmids we acquired was efficient at knocking down our first three targets, Ascl1, Neurog2, or Olig2 in retinal explant cultures. To overcome this barrier, we acquired siRNAs to each gene. These have the advantage of working quicker (as they are just RNA), but do not last as long as plasmid systems. When we transfected these siRNA into retinal explants, we saw robust knock-down of our targeted genes. We decided to use this system going forward, but having to optimize a second system put us behind schedule and we were only able to screen three candidates during the funding period.

Specific Aim 2: To program retinal stem cells into transplant-competent cone photoreceptors (Lamba).

Major Task 3: Enhance cone differentiation from hESC-derived retinal progenitors using transcription factors.

We were delayed in testing triple transfections due to technical issues transfecting our cultures. Importantly, we were able to get these experiments working and our data confirmed our hypothesis that transfecting *OTX2*, *BLIMP1*, and *ONECUT1* promoted cone formation. Nonetheless, this delay caused the transplant experiments in Task 4 to be modestly delayed as well.

Major Task 4: Transplantation of stem cell-derived cones into adult host mice.

We had little difficulty executing these experiments, but due to delays described above for Task3, we only had time to conduct one major transplantation experiment during the funding period. As described above, we plan to do future experiments that test additional cell treatment paradigms and post-transplantation time-points.

Products:

Nothing to report.

Participants and Other Collaborating Organizations:
Note that this report includes the activities done at both research sites, as directed by Dr. Brzezinski (University of Colorado Denver) and Dr. Lamba (The Buck Institute for Research on Aging).

CU Denver:

Name: Dr. Joseph A. Brzezinski IV
Role: PI
Person Months: 1
Contribution to Project: Designed & supervised research at CU Denver, representing the experiments for Aim 1.
Changes: No significant changes to funding support during the award period.

Name: Dr. Kenneth L. Jones
Role: Collaborator
Person Months: 1
Contribution to Project: Analyzed RNA-seq datasets.

Name: Grace Randazzo
Role: Technician
Person Months: 9
Contribution to Project: Conducted the experiments for Aim 1.

Buck Institute:

Name: Dr. Deepak A. Lamba
Role: PI
Person Months: 1
Contribution to Project: Designed & supervised research at The Buck Institute, representing the experiments for Aim 2.
Changes: No significant changes to funding support during the award period.

Name: Dr. Jie Zhu
Role: Postdoc
Person Months: 6
Contribution to Project: Conducted the experiments for Aim 2.

Special Reporting Requirements:
See appendix for Quad Chart.

Appendix:

The appendix includes:

1. Data tables summarizing RNA seq results.
2. Quad chart for the award period.

Table 1: Genes upregulated after 9 hours culture in DAPT vs. DMSO controls.

Gene	DMSO[1]	DAPT[1]	Fold Change	P-value
Rn7sk	5.66	33.24	**5.87**	0.0141
Tfap2c	1.31	6.34	**4.84**	0.0001
Cck	1.26	5.67	**4.50**	0.0018
Yam1	207.11	905.29	**4.37**	0.0022
Gadd45g	8.62	36.62	**4.25**	0.0003
Hpse	1.58	6.30	**3.99**	0.0000
Snord70	14.04	54.87	**3.91**	0.0313
Dll1	34.52	133.63	**3.87**	0.0000
Neurog2	38.08	145.74	**3.83**	0.0003
Cerkl	2.12	6.95	**3.28**	0.0137
Lars2	15.63	50.88	**3.26**	0.0167
Bhlhe22	4.92	15.75	**3.20**	0.0001
Ascl1	23.80	75.87	**3.19**	0.0059
Neurod1	30.07	93.59	**3.11**	0.0003
Pkib	3.16	9.42	**2.98**	0.0219
Dll4	12.09	35.27	**2.92**	0.0003
Hes6	161.72	470.76	**2.91**	0.0002
Ccno	1.16	3.13	**2.70**	0.0102
Mybl1	48.46	129.65	**2.68**	0.0002
Insm1	15.87	42.21	**2.66**	0.0014
Itga4	1.38	3.55	**2.57**	0.0000
Olig2	5.03	12.61	**2.51**	0.0308
Dll3	10.50	25.11	**2.39**	0.0289
Cdc25b	28.83	67.75	**2.35**	0.0023
Penk	10.71	23.21	**2.17**	0.0268
Sstr2	5.05	10.78	**2.13**	0.0036
Btg2	61.18	130.25	**2.13**	0.0001
Frrs1l	2.69	5.61	**2.09**	0.0010
Mfng	34.59	70.10	**2.03**	0.0000
Otx2	57.88	109.97	**1.90**	0.0159
Chrnb4	11.05	17.25	**1.56**	0.0032

[1.] In reads per kilobase of exon sequence per million mapped reads.

Table 2: Genes downregulated after 9 hours culture in DAPT vs. DMSO controls.

Gene	DMSO[1]	DAPT[1]	Fold Change	P-value
Heyl	2.96	0.17	**-17.41**	0.0107
Ano1	11.02	1.37	**-8.04**	0.0008
Hhipl2	1.16	0.20	**-5.80**	0.0013
Rnf144b	3.47	0.88	**-3.94**	0.0113
Fgf3	12.41	3.25	**-3.82**	0.0095
Fam69c	11.11	3.42	**-3.25**	0.0066
Fst	1.66	0.51	**-3.25**	0.0172
Hes1	96.14	30.75	**-3.13**	0.0001
Ttyh1	18.69	6.05	**-3.09**	0.0090
Sfrp2	421.32	142.28	**-2.96**	0.0355
Cntnap2	25.95	9.20	**-2.82**	0.0004
Cyp26a1	18.69	6.76	**-2.76**	0.0000
Hes5	116.63	43.24	**-2.70**	0.0006
Egfr	1.21	0.47	**-2.57**	0.0255
Pmepa1	28.51	11.51	**-2.48**	0.0152
Cyp26c1	2.30	0.94	**-2.45**	0.0006
Kirrel2	4.46	1.83	**-2.44**	0.0142
Etv1	3.98	1.70	**-2.34**	0.0002
Erich2	1.25	0.55	**-2.27**	0.0222
Nr2e1	51.53	22.95	**-2.25**	0.0012
Ptprz1	10.02	4.49	**-2.23**	0.0005
Tanc1	14.30	6.54	**-2.19**	0.0231
Id4	10.87	5.00	**-2.17**	0.0096
Hey1	18.44	8.52	**-2.16**	0.0002
Oas2	1.23	0.58	**-2.12**	0.0170
Cdo1	11.90	5.66	**-2.10**	0.0001
Orc1	16.80	8.06	**-2.08**	0.0209
Hhipl1	2.81	1.36	**-2.07**	0.0025
P2rx1	1.73	0.84	**-2.06**	0.0033
Tgm2	2.42	1.18	**-2.05**	0.0095
Trpc3	4.84	2.40	**-2.02**	0.0362
Dio3	29.51	14.73	**-2.00**	0.0009
Smad6	2.05	1.03	**-1.99**	0.0165
Notch3	8.35	4.26	**-1.96**	0.0093

[1.] In reads per kilobase of exon sequence per million mapped reads.

Programming Retinal Stem Cells Into Cone Photoreceptors

PI: Joseph A. Brzezinski and Deepak A. Lamba **Org:** University of Colorado Denver and The Buck Institute **Award Amount:** $249,979

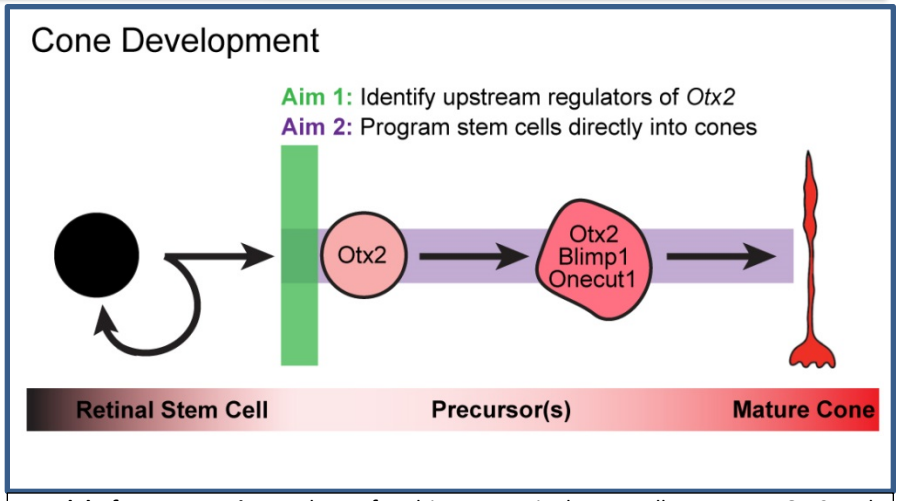
Study/Product Aim(s)

- **Aim 1:** To determine how retinal stem cells acquire cone photoreceptor potential.
- **Aim 2:** To program retinal stem cells into transplant-competent cone photoreceptors.

Approach

Aim 1: We will culture retinal explants forced to adopt cone fate and identify genes upstream of *Otx2* by RNA-seq. Candidate regulators will be tested in explants by gain- and loss-of function approaches.

Aim 2: We will transiently administer key transcription factors (*Otx2*, *Blimp1*, and *Onecut1*) (together or sequentially) in human retinal stem cell cultures to determine whether they promote cone fate. Stem cell derived cones will be transplanted into wild-type mouse eyes to evaluate their therapeutic potential.



Model of cone genesis: A subset of multipotent retinal stem cells expresses Otx2 and can adopt cone photoreceptor and a few other cell fates. Other factors (*e.g.* Blimp1 & Onecut1) may specifically promote cone fate from uncommitted Otx2+ precursor cells.

Timeline and Cost

Activities CY	9/2014	9/2015
Aim 1: RNA-seq of explants (Denver)		
Aim 1: Gain- and loss-of-function (Denver)		
Aim 2: Stem cell programming (Buck)		
Aim 2: Cone transplants (Buck)		
Estimated Budget		**$249,979**

Updated: 12/8/15

Goals/Milestones:

CY14-15 Goals (Dr. Brzezinski at CU Denver)

- Collect retinal explants and conduct RNA-seq.
- Statistical analysis of RNA-seq data.
- Gain- and loss-of-function analysis of top 5 candidate *Otx2* regulatory factors (only 3 candidates screened).

CY14-15 Goals (Dr. Lamba at The Buck Institute)

- Program retinal stem cells with combinations of *OTX2*, *BLIMP1*, and *ONECUT1* and count cones formed.
- Program retinal stem cells with sequential treatment of factors and with new candidates identified in Aim 1 (no new candidates screened).
- Transplant stem cell-derived cones into wild-type mouse eyes.

Budget Expenditure to Date
Projected Total Expenditure: $249,979
Total expenditure in Q1-Q4: $249,979