July-December 2014 Progress Report to the AHCF
Kevin Ess and Al George, P.I.'s

Summary

We have made overall excellent progress in 2014 with progress on all proposed Specific Aims. These completed and ongoing experiments allowed us to establish a human stem cell model of AHC and explore disease mechanisms that lead to severe neurologic manifestations in patients with AHC. We have generated stem cell lines using AHC patient cells that have the most common mutations in the *ATP1A3* gene (encodes the Na/K ATPase alpha-3 subunit) known to cause AHC. In addition, we have generated stem cells from paired control lines obtained from healthy volunteers. These stem cells lines were verified to be bona fide stem cells (based on gene expression profiles and ability to differentiate into many different cells types). We then used specialized protocols to turn these stem cell lines into neurons. As the alpha-3 subunit is mainly expressed in human neurons, these are key approaches to generate relevant cell models to test new ideas about disease mechanisms in AHC.

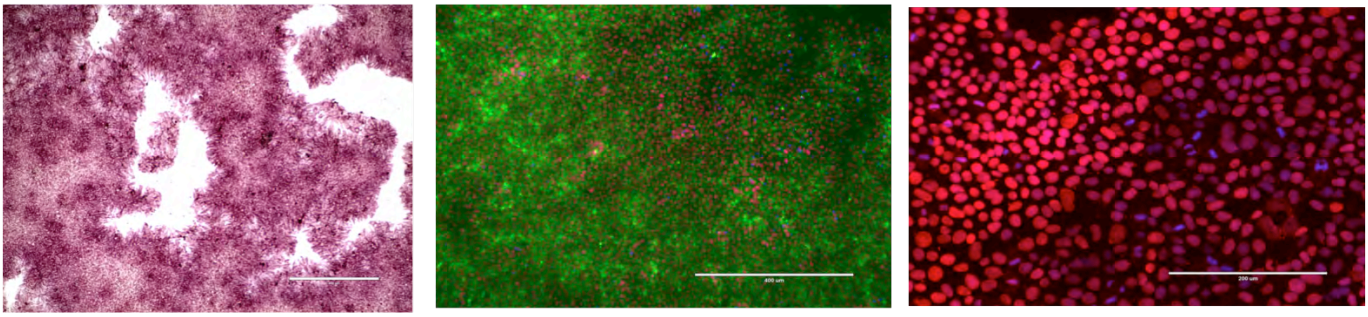
In addition to our progress on Specific Aims 1, 2 and 3 as detailed below, we have also continued refining previous work and have now completed our first manuscript and recently submitted it for scientific peer review and publication.

Specific Aim 1 – To generate iPSC-derived neurons from AHC patients.

We focused on the generation of induced pluripotent stem cells (iPSCs) that harbor *ATP1A3* mutations associated with most cases of AHC. As detailed in Table 1 (below), D801N, E815K and G947R mutant human iPSCs have been generated and in most cases, also differentiated to neurons. Each patient derived fibroblast line is reprogrammed to several independent lines of stem cells to account for any possible variations that may arise during their creation This redundancy is a theoretical concern and we have not to date found any variability in lines made from individual patients. Control lines (CA, CC, CD) from healthy volunteers were also used. Examples of derivative iPSC lines and neurons are shown in Figures 1-3.

Patient ID	***ATP1A3* Mutation**	**iPSC**	**Neuron**
AHC-4 (AHC 35189)	D801N	yes	yes
AHC-8 (AHC 91759)	E815K	yes	yes
AHC-12 (AHC 41935)	G947R		
AHC-14 (AHC 46337)	G947R	yes	yes
AHC- 20 (AHC-42101)	G947R	yes	
AHC 95454	Splice c.2542+2A→G	yes	
CA	None (control line)	yes	yes
CC	None (control line)	yes	yes
CD	None (control line)	yes	yes

iPSC Pluripotency Validation of AHC 46337 (G947R)



AHC 46337/Alkaline Phosphatase

AHC 46337/Oct4/TRA-1-60

AHC 46337/Nanog

Figure 1. Using a non-integrating episomal method, iPSCs were generated from patient fibroblasts harboring a G947R mutation in the *ATP1A3* gene. Validation experiments included expression of alkaline phosphatase (left image, red stain, size bar 400 microns), Oct4 (red) and TRA-1-60 (green) (middle image, size bar equals 400 microns), and Nanog (right image, red stain, size bar 200 microns).

Embryoid Body Differentiation of AHC 46337 (G947R)

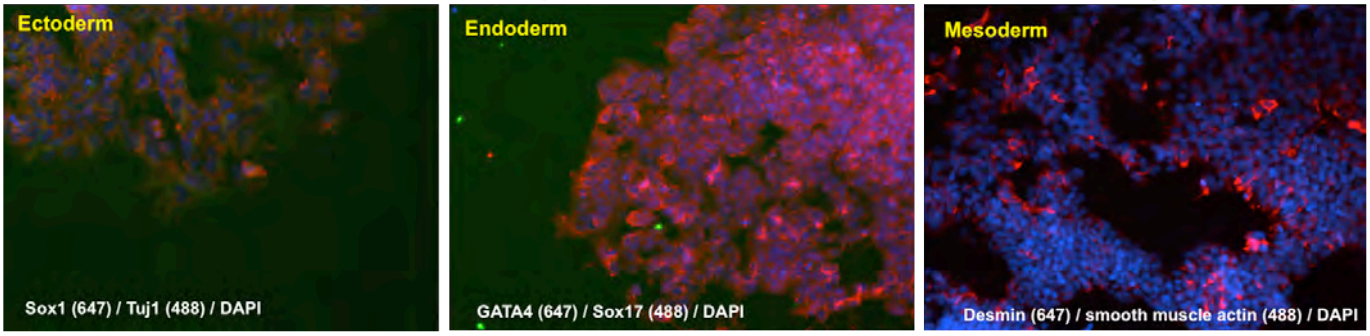


Figure 2. An additional assay of pluripotency was performed to test the ability of iPSCs to generate all germ lines (ectoderm, endoderm, and mesoderm type cells) through embryoid body formation(left image, Sox1 red, Tuj1 (neuronal) green stain); (middle image, GATA4 red stain, Sox17 green stain); (right image, desmin red stain, smooth muscle actin green stain).

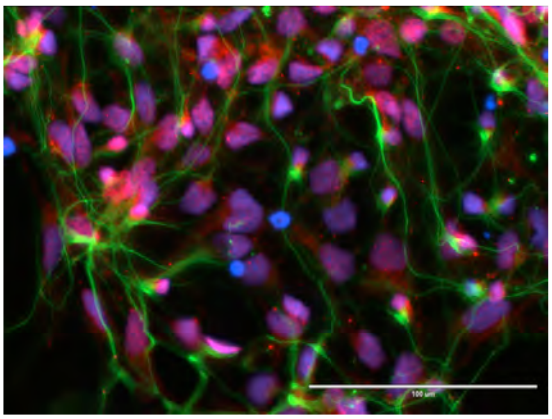


Figure 3. AHC iPSC with G947R mutation were differentiated to neural progenitors (not shown) and then neurons. Cells were fixed after 98 days in culture. Neurons are positive for Tuj1 (green, neuronal marker), TTF-1 (red, specific neuronal lineage marker), nuclei by Hoechst stain (blue). Size bar equals 100 microns.

Specific Aim 2 – To elucidate physiological effects of *ATP1A3* mutations using iPS cell-derived neurons from AHC patients.

These experiments are ongoing and will follow validation of pluripotentcy status as well as verification of these cells to generate specific neuronal lineages. Experiments as previously proposed will be similar to drug screening experiments done using HEK cells expressing either wild-type or AHC patient related *ATP1A3* mutations. In this Aim however, we will be testing the ability of identified compounds to rescue ATP1A3 function in human neurons. As detailed in the previous progress report, the three compounds we have identified (chlormadinone acetate, aklavine hydrochloride, and 3-hyroxyflavone) will be added to cultures of neurons derived from AHC patient iPSCs. Cells will then be screened for rescue of surface expression of the alpha3 subunit as well as functional rescue. Results will be examined for neurons derived from *ATP1A3* mutations including D801N, E815K and G947R as well as the splice site mutation c.2542+2A→G.

Derivative neurons from control and AHC patient iPSCs are being shipped to the George lab at Northwestern where they are used in electrophysiologic experiments to define the impact of the various *ATP1A3* gene mutations on neuronal function. We have established robust methods where neurons are created at Vanderbilt and then frozen in vials, each containing 100s to 1000s of neurons. These cells are very stable and can be shipped overnight on dry ice to Northwestern. They are then thawed as necessary, so live neurons can be recovered, plated onto dished and subsequently used for electrophysiologic experiments.

Specific Aim 3 – To continue efforts to identify drug or drug-like compounds capable of restoring ATP1A3 function in cells expressing AHC-associated mutations.

We are in the process of performing a larger scale screen for compounds that can rescue mutant ATP1A3. We are screening a library of ~6,000 compounds selected due to chemical properties known to modulate the function of ion transporters. Results from this expanded screen will demonstrate the feasibility of restoring *ATP1A3* function using a chemical approach and could inform us about “scaffolds” on which we can base the design of new drug-like compounds using a medicinal chemistry approach. All techniques are already operational in our laboratory and ongoing.

References

***ATP1A3* Mutations Associated with Alternating Hemiplegia of Childhood Have Divergent Loss-of-function Mechanisms.** Christine Q. Simmons, Emily Days, Paige N. Vinson, Bryan Cawthon, Kathleen Swoboda, C. David Weaver, Kevin C. Ess, and Alfred L George, Jr. (Submitted).