



A Cell Culture Model for SMA Using Embryonic Stem Cell-Derived Motoneurons¹

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We have developed a unique cell culture system in which mouse embryonic stem (ES) cells are differentiated into mature motoneurons in co-culture with skeletal muscle. ES cell-derived motoneurons extend long axons toward skeletal muscle, synthesize synaptic vesicle machinery, develop neuromuscular junctions and initiate skeletal muscle contraction. Currently, we will establish a cell culture model for SMA using ES cells that are deficient in mouse SMN and express human SMN from the SMN2 genomic sequence. We have derived SMA ES cells from blastocysts of the genotype *Smn*^{-/-}, hSMN2⁺ and by stably knocking down mouse *Smn* expression in ES cells with the *Smn*^{+/+}, hSMN2 genotype. Both types of SMA ES cells are phenotypically normal, yet when they are differentiated into motoneurons, they undergo axonal degeneration followed by cellular death.

In the present project, we will define critical features of this system so that it can be used by the scientific community to understand the pathophysiology of SMA. We will rigorously quantitate the dysfunction of SMA ES cell-derived motoneurons to serve as a baseline in screening agents that modulate this phenotype. We will define the minimal requirements of this system in order to understand how scalable the system is. Once we have defined the critical cellular participants, have defined the spatial requirements for these participants and have defined a quantifiable phenotype, we will initiate transfer of the protocols and reagents to a centralized facility. While this transfer is occurring, we will define the rescuability/reversibility of this phenotype in order to inform the development of screening assays. We will define the ability of transfected SMN derivatives and drugs that modulate splicing of SMN2 to rescue this phenotype. These studies will allow us to understand when during differentiation screening studies must be initiated and will serve as a proof of concept of the utility of this system.

Task 1. Determine the minimum culture requirements to recreate the SMA phenotype, 8 months to complete (Months 1-8). Spatially segregated chamber systems such as Campenot chambers are not amenable to high-throughput assays, and will be used only as comparison control experiments in initial experiments. A layered spatial segregation system (i.e., motoneurons on top of agar on top of skeletal muscle) may be amenable to a medium-throughput assay. Since a high-throughput assay is desired, the simplest variation of this system that yields consistent, accurate results will be determined. Milestone 1 (Month 6) is definitive evidence of whether simple co-cultures (without agar layering) can be used for these assays. If simple co-cultures are not sufficient, Milestone 2 (Month 6) is definitive evidence of whether layered spatial segregation (i.e., motoneurons on top of agar on top of skeletal muscle) can be used for these assays. Experiments to achieve Milestone 2 will take place concurrently with experiments to achieve Milestone 1. If neither of the previous two co-culture systems is sufficient to recreate the phenotype, the project will not continue. Milestone 3 (Month 8) is validation of GFP ELISA, axonal length, and NMJ immunohistochemistry as methods to quantify the phenotype of SMA ES cell-derived motoneurons in simple or layered co-cultures (depending on the results at Milestones 1 and 2).

Task 2. Determination that the phenotype can be rescued by overexpression of SMN from an inducible transgene, 3 months to complete (Months 9-11). In order to consider this culture system a viable one for large-scale screening, the phenotype must be reversible. The simplest proof-of-principle experiment is to overexpress SMN from a transgene within differentiating SMA ES cells. Milestone 4 (Month 11) is definition of when SMN overexpression rescues the phenotype. The milestone will be unachievable if the phenotype cannot be reversed even by overexpression of SMN at an early stage in development.

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Task 3. Transfer assay to the centralized facility, 3 months for transfer and replication of assay (Months 12-14). Transfer to and replication of this assay to a centralized facility are critical to the completion of this project. Beginning at Month 12, the investigators will initiate transfer of the reagents and protocols to the centralized facility. They will work with the centralized facility to ensure that personnel at this facility are able to carry out the protocols. This task will not involve scale-up of the assay; the centralized facility can automate the assay if and when necessary. *Milestone 5 (Month 14)* is successful replication by the centralized facility of the SMA phenotype in the system developed in Task 1.

Task 4. Test a drug known to increase SMN2 expression for its ability to act as a positive control, (Month 12). It is important to demonstrate that enhanced expression of SMN from an endogenous genomic construct (SMN2) reverses the phenotype. Therefore, the investigators will perform a simple time-course experiment with a drug known to increase SMN2 expression (a positive control) to define whether and when the SMA phenotype can be rescued. The PI will consult with SAIC and NINDS beforehand to determine the best drug to use. Testing of experimental drugs will be done by the centralized facility, and is not part of this project. *Milestone 6 (Month 10)* is the validation of an agreed-upon drug as a positive control for the assay.

Description of Approach: We will demonstrate a stable, reproducible and quantifiable phenotype in response to reduced SMN expression in SMA ES cell-derived motoneurons. We will define and quantify abnormalities in axonal extension, formation of neuromuscular junctions and induction of skeletal muscle contraction. Further, since motoneurons in this system express GFP, we will determine whether release of GFP into culture supernatant can be used as a quantifiable marker for cellular dysfunction/injury. In parallel, we will examine whether skeletal muscle is required and needs to be in a spatially distinct chamber to create this phenotype. These studies will define how effectively this system can be scaled up to medium-throughput or even high-throughput screening. By transfecting SMN derivatives or by including drugs that modulate SMN2 splicing in the medium, we will define the ‘point of no return’ during differentiation for rescuing the phenotype.