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COMPARISON OF HUMAN iPSC-DERIVED AND RODENT FOREBRAIN CULTURES REVEALS DISTINCTIVE MORPHOGENESIS PATTERNS OF HUMAN NEURONAL DEVELOPMENT

Marnelle Andersen, David Galitz, Susan Tousey, Charles Haitjema, Greg Herr, Jamie Van Etten, Sol Degese, Xi Lu, Jeff Cooper, Chris Heger, Sean Kevlahan, Scott Schachtele, and Kevin Flynn

Bio-Techne, 614 McKinley Place NE, Minneapolis, MN 55413

INTRODUCTION

While embryonic rodent pyramidal neurons have been extensively used for modeling neuronal development, interspecies differences, including alterations in developmental timings, mean that not all results are readily recapitulated in human models. There is a pressing need for *in vitro* models to fully capture the mechanisms and molecular pathways specific to human neural development and disorders including cortical gyrification, autism, and schizophrenia. To meet this objective, we describe a protocol for generating low density human iPSC-derived forebrain neurons and compare the development of various morphological features in human versus rodent-derived neurons. Using specific markers for developmental milestones, such as Tau-1 to mark axon formation, in conjunction with high content imaging, we observe that human derived neurons follow a similar pattern of development to rodent neurons, but with a prolonged time course. We use pharmacological treatments targeting the cytoskeleton to demonstrate that robust molecular mechanisms for neurite growth and polarization are preserved in human and rodent neurons. For example, myosin inhibition using Blebbistatin increased axon outgrowth and arborization in both rodent and in human neurons. Our findings indicate that while robust mechanisms for neuronal development are preserved, human iPSC-derived neurons follow a delayed growth pattern in vitro that can be used to explore nuanced mechanisms specific for human brain development.

METHODS

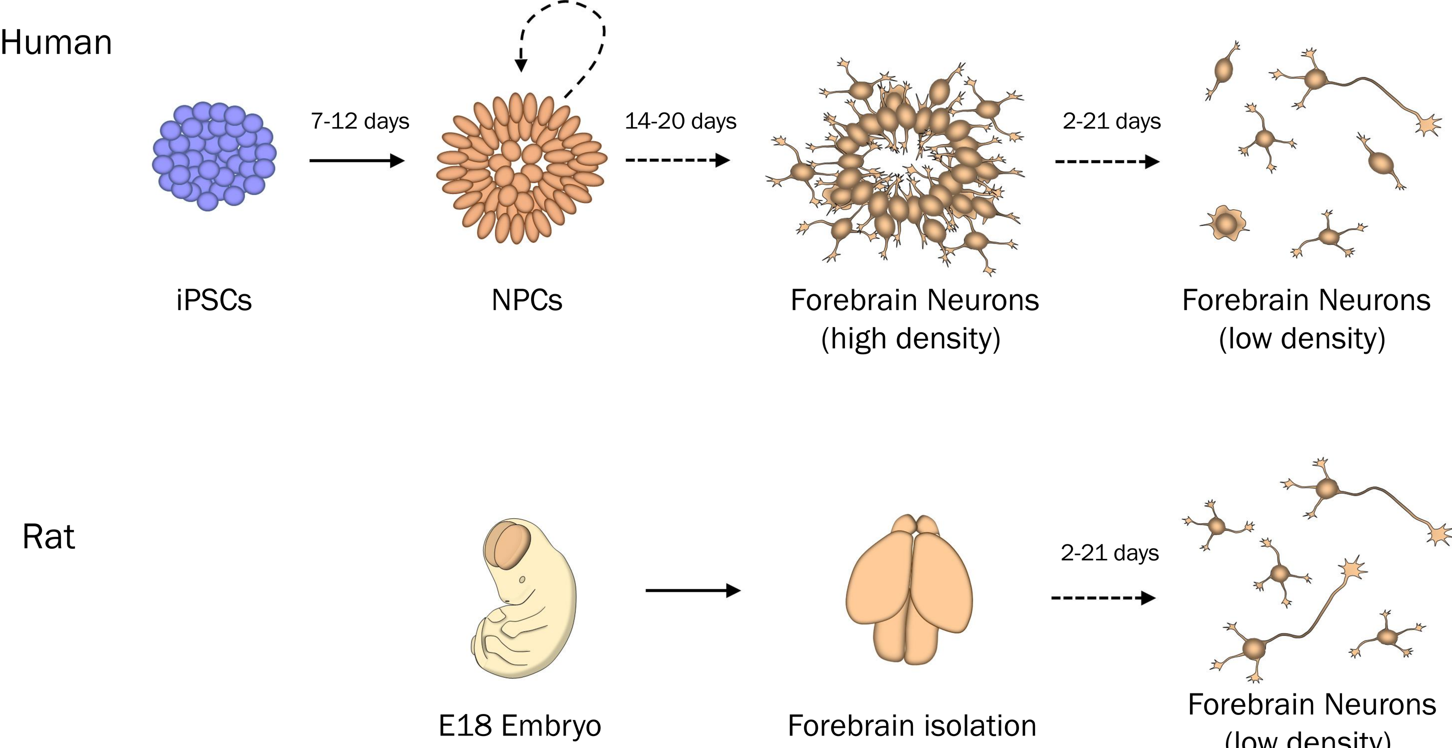

Figure 1. Analysis of neuronal morphogenesis workflow. Human induced pluripotent stem cells (iPSCs) were cultured under defined conditions with FGF2-GMP and TGFβ-GMP. Differentiation into neural progenitor cells (NPCs) was performed using the dual SMAD protocol (Chambers, et al., 2009) with Noggin-GMP and SB-43152-GMP and maintained in N2-MAX GMP and FGF2-GMP. Downstream differentiation into forebrain neurons proceeded by growth factor withdrawal and transitioning into neuronal media with animal-free N21-MAX GMP and BDNF-GMP. Upon differentiation into neurons (21-28 days) the cells were re-plated at low density for 2-21 days. Concurrently forebrain neurons were isolated from the cortices of E18 rat embryos. Rodent pyramidal neuronal cultures are a well-established paradigm for studying neuronal morphogenesis (Dotti and Banker, 1988, Flynn and Bradke, 2019). Neuronal cell cultures were performed side-by-side under identical conditions to compare the development of rat and human forebrain neurons.

Cell Stage	Proteins and Small Molecules	Supplements	Antibodies
Pluripotent Stem Cells	FGF2-GMP (233-GMP) TGFβ-GMP(240-GMP)	N/A	Oct-3/4 (AF1759) Nanog (AF1997) SSEA-1 (MAB2155)
Neural Progenitor Cells	Noggin-GMP (6057-GMP) SB-432152 (TB1614-GMP) FGF2-GMP (233-GMP)	GMP N2-MAX, AF (AR016) Ascorbic Acid	Pax6 (AF8150) SOX1 (AF3369)
Forebrain Neurons	BDNF-GMP (248-GMP)	GMP N21-MAX, AF (coming soon)	β-III-tubulin (MAB1195) Synaptotagmin (MAB4364) GluR2

Table of Bio-Techne reagents used in this work: R&D Systems and Tocris Bioscience

References

- Dotti, C. G., Sullivan, C. A. & Banker, G. A. (1988) The establishment of polarity by hippocampal neurons in culture. *Journal of Neuroscience*, 8(4), 1454-68.
- Flynn K.C. & Bradke F. (2019) Cytoskeletal and trafficking mechanisms underlying the development of neuronal polarity. In *Comprehensive Developmental Neuroscience*, 2nd Edition (Elsevier) (*in press*)
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RESULTS

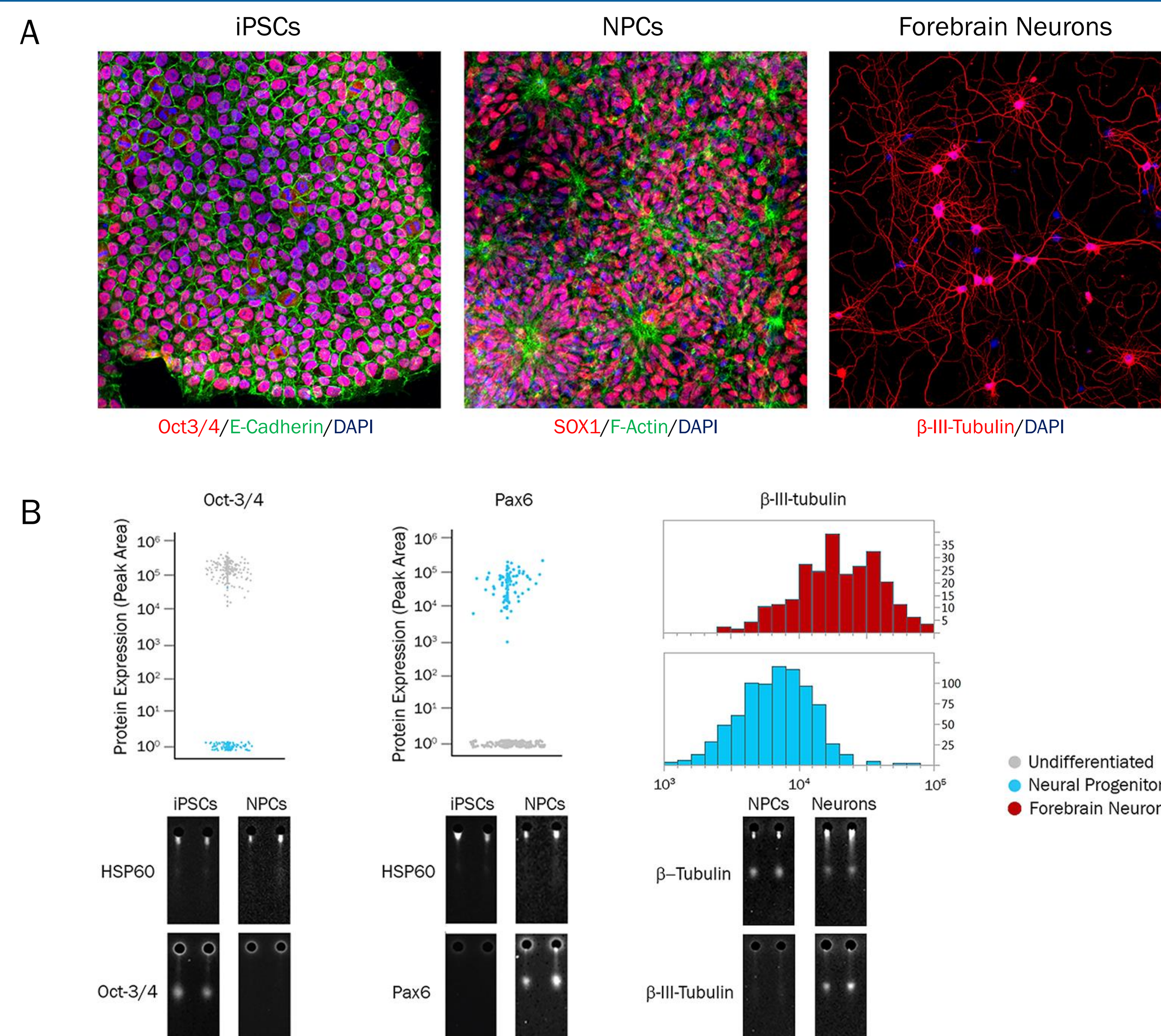

Figure 2. Differentiation of forebrain/cortical neurons and verification of neuronal identity. A. Under maintenance conditions, human induced pluripotent stem cells (iPSCs) stain for the pluripotency marker Oct-3/4 (red). Differentiation into neural progenitor cells (NPCs) results in neural rosettes which stain for the NPC marker SOX1 (red). Terminal downstream differentiation into neurons is confirmed with the pan-neuronal marker, β-III-tubulin. **B.** Detection of homogeneity of cell populations at different stages of neuronal differentiation. MILO™ Single-Cell Western technology (ProteinSimple) was used to analyze marker expression at a single cell level. In scatter plots, undifferentiated human induced pluripotent stem cells (iPSCs) show high expression for the pluripotency marker Oct-3/4 while neural progenitors have lost Oct-3/4 expression (left). Conversely, the NPC marker Pax6 is undetectable in undifferentiated iPSCs while NPCs have gained high expression of this marker (middle). Histogram analysis shows that following downstream differentiation into neurons there is an increase in the expression the pan-neuronal marker, β-III-tubulin as compared to NPCs (right).

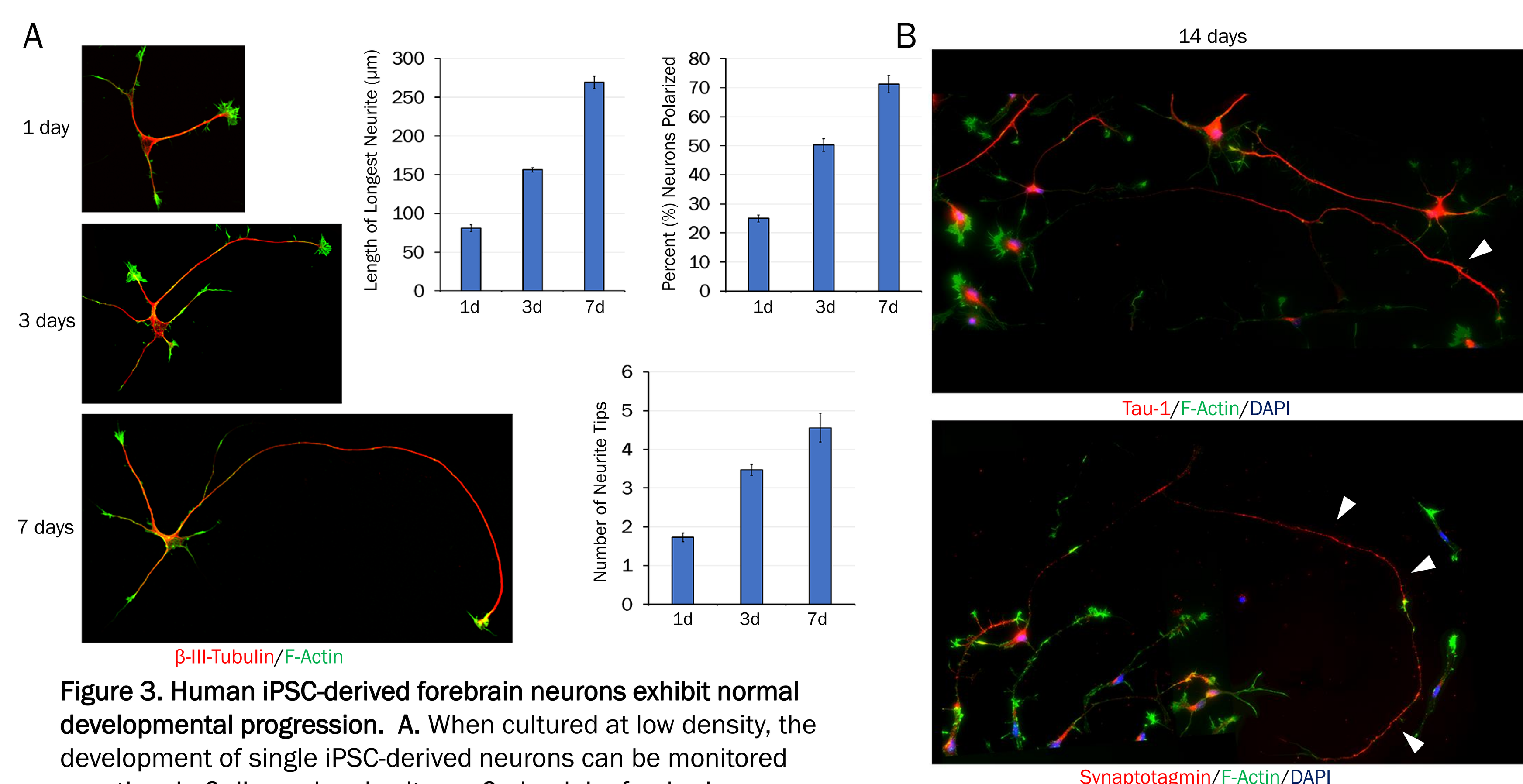

Figure 3. Human iPSC-derived forebrain neurons exhibit normal developmental progression. A. When cultured at low density, the development of single iPSC-derived neurons can be monitored over time in 2-dimensional culture. On laminin, forebrain neurons initially extend minor neurites after 1 day in culture, as indicated by β-III tubulin labeled neurites. The number of neurites, the length of the longest neurites and the percent (%) of neurons with a morphologically distinct axon increases over time over 7 days in culture. This is noticeably slower than rodent pyramidal neurons (see below). **B.** After 14 days of low-density culture, there is continued growth and maturation of forebrain neurons with long axons that express Tau-1 and the synaptic marker Synaptotagmin. **C.** Further characterization of the differentiation was performed with simple western (ProteinSimple). Using the forebrain differentiation protocol the culture tested positive for GluR2, a marker for excitatory cortical neurons. Under these conditions, neurons were not positive for the interneuron marker GAD67 or the motor neuron marker Islet-1.

RESULTS

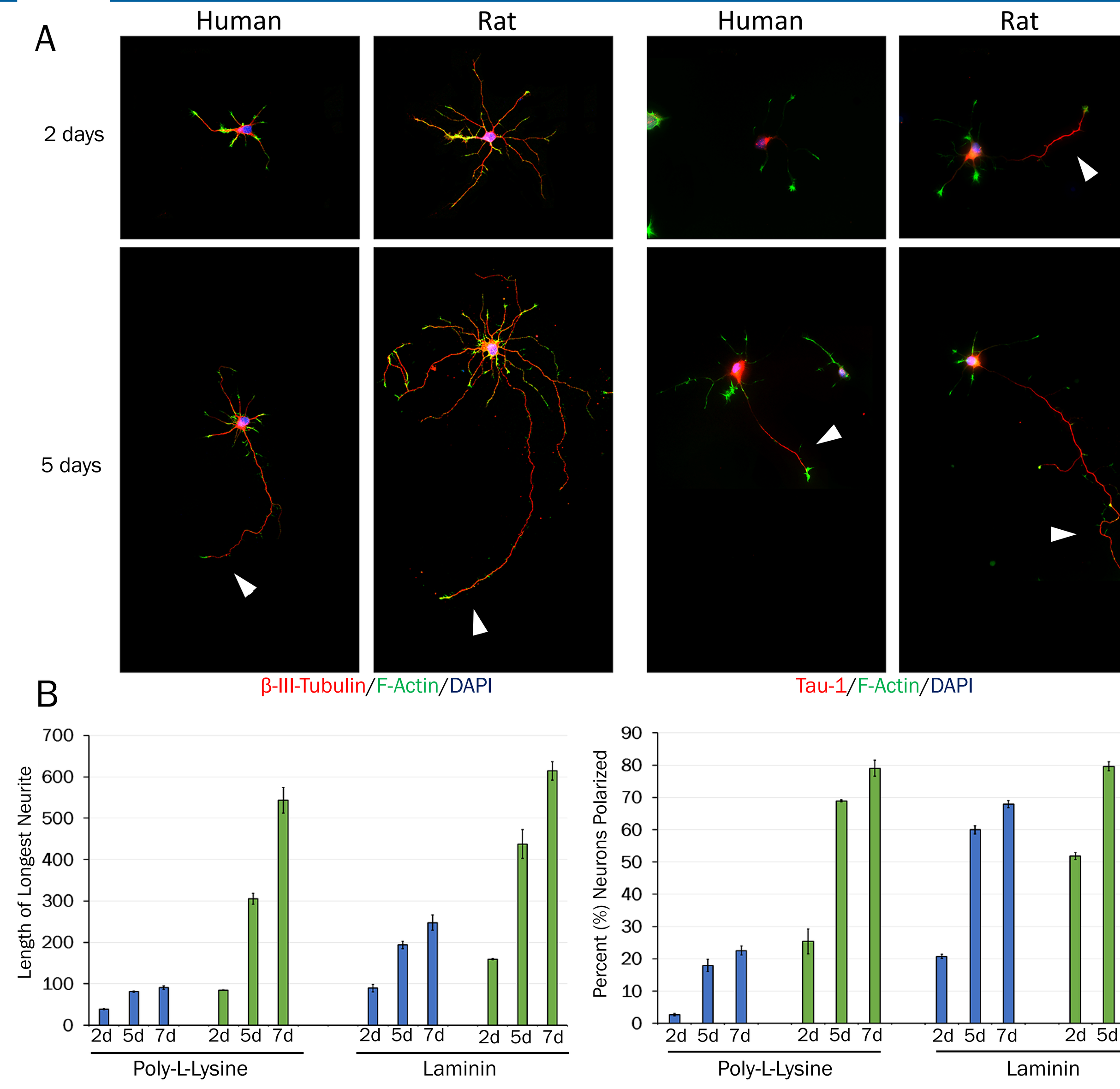

Figure 4. iPSC derived human forebrain neurons display reduced neurite growth and delayed polarization compared to rat forebrain neurons. A. iPSC-derived forebrain neurons and E18 rat forebrain neurons were cultured for 2-7 days (d) on Poly-L-lysine or Laminin and fixed and stained for β-III-tubulin (red, left), Tau-1 (red, right), F-Actin with Phalloidin-488 (green) and DAPI (blue). Note that rat neurons have longer neurites than human neurons at 2d and 5d in culture (white arrowheads). **B.** High content quantification of the length of the longest neurite in human (blue) and rat (green) forebrain neurons at 2d, 5d, and 7d in culture on Poly-L-lysine or Laminin-1 (left). Quantification of neuronal polarization in human (blue) and rat (green) forebrain neurons as indicated by percent (%) of neurons with an axon (right).

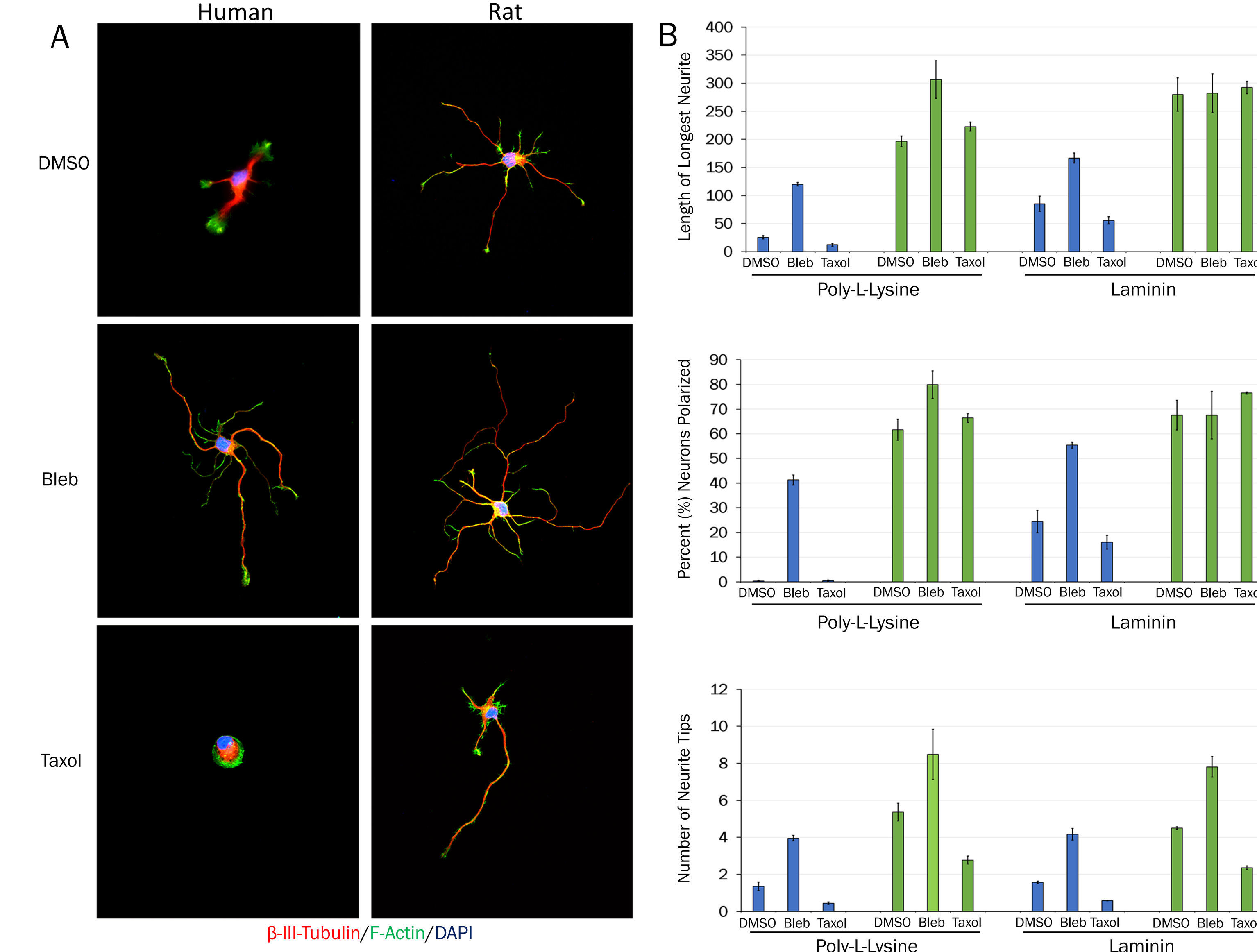

Figure 5. iPSC-derived human forebrain neurite outgrowth is enhanced by myosin inhibition. A. iPSC-derived forebrain neurons and rat forebrain neurons were cultured for 2 days (d) on Poly-L-lysine (PLL) and treated with vehicle (DMSO), 5µM Blebbistatin or 5nM Taxol for 2d. Neurons were fixed and stained for β-III-tubulin (red), F-Actin with Phalloidin-488 (green) and DAPI (blue). Note that on PLL both rat neurons and human neurons displayed drastically longer neurites in the presence of the myosin inhibitor, Blebbistatin. While low levels (5nM) of the microtubule stabilizer Taxol increases axon growth in rat neurons, it is inhibitory to neurite growth in human neurons. **B.** High content quantification of the length of the longest neurite (top), the percent of neurons with an axon (middle), and number of neurite tips (bottom) in human (blue) and rat (green) forebrain neurons at 2d.

CONCLUSIONS/FUTURE STUDIES

- The protocol for deriving low density cultures of human iPSC-derived neurons provides groundwork for examining temporal and morphological features specific for the development of human neurons.
- Human iPSC-derived neurons develop in a manner generally analogous to the prototypical model system, rodent pyramidal neurons, albeit on a delayed time-course.
- Future work will determine timing of other critical aspects of neuronal morphogenesis such as the formation of the axon initial segment, dendritic arborization, and dendritic spine formation.
- The techniques described here can be used to study mechanisms of human developmental disorders.