Direct Conversion of Pluripotent Human Embryonic Stem Cells into Functional Human Neuronal or Cardiomyocyte Cell Therapy Derivatives for Regenerative Medicine

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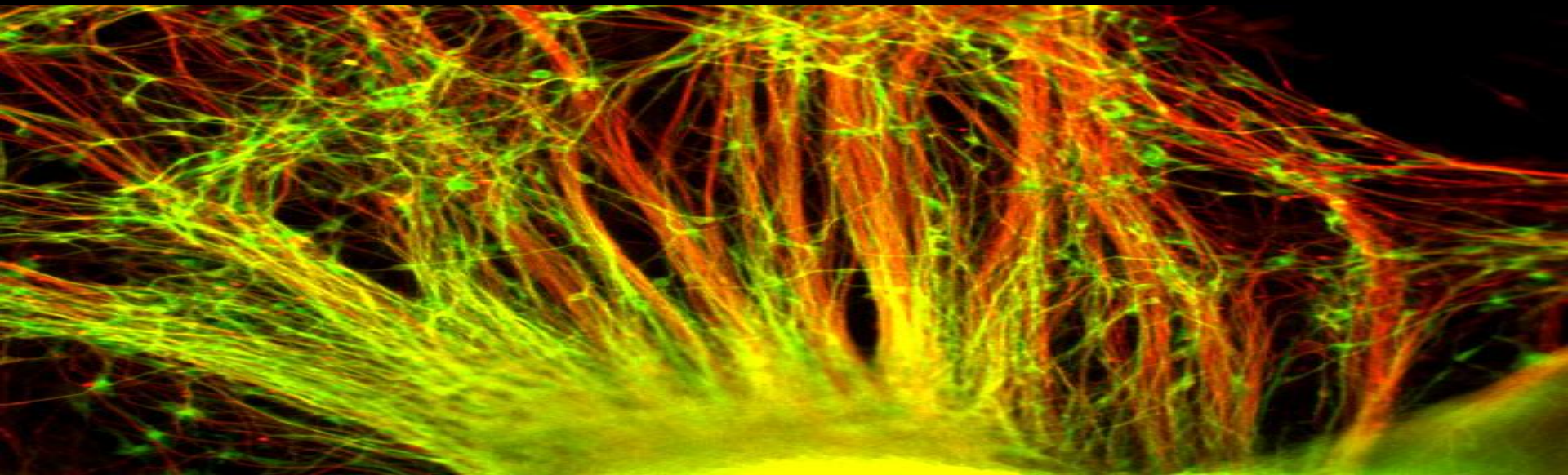


Unmet Medical Needs Cost Billions in Health Care

- **Heart disease: $190 billions**
- **Neurological disorders, including Parkinson's disease, ALS, Alzheimer disease, stroke, brain and spinal cord injuries: $1.5 Trillions**
- **No treatment option**
- **Given the limited capacity of the CNS and the heart for self-repair or renewal, cell-based therapy represents a promising therapeutic approach closest to provide a cure to restore normal tissue and function for neurological and cardiovascular disorders**

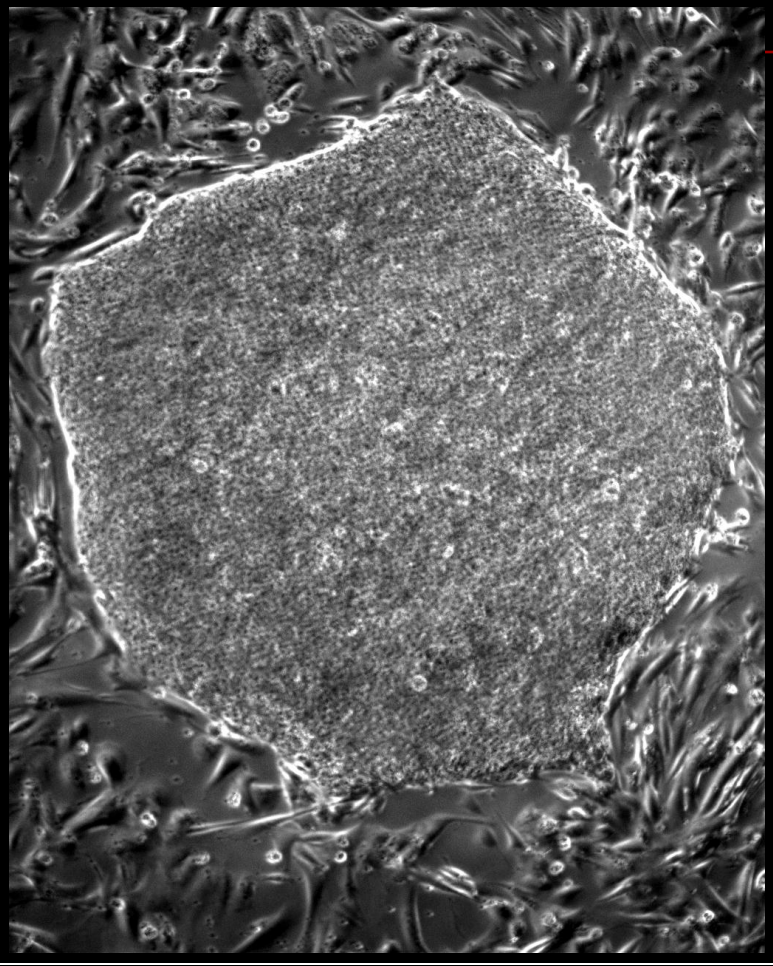
Limitations of Existing Approaches and Markets

Adult stem cells isolated from tissues or artificially reprogrammed from adult cells

- **Shortcomings: limited capacity for renewal and repair, accelerated aging, and immune-rejection following transplantation**
- **Major drawbacks of artificially reprogrammed adult cells: extremely low efficiencies and genetic defects associated with high risks of cancers**

Severely limited their utility as viable therapeutic approaches.

The Therapeutic Potential of Pluripotent Human Embryonic Stem Cells

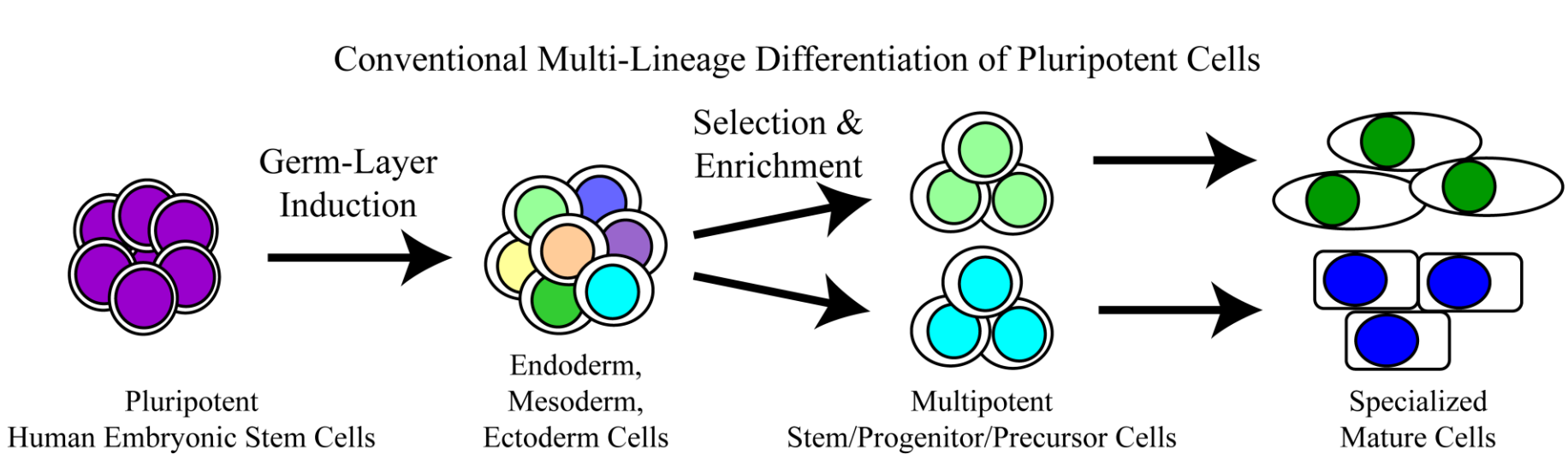


- **Pluripotent human embryonic stem cell (human ES cells) have the unlimited ability for both long-term stable undifferentiated growth in culture and differentiation into all functional somatic cell types in the human body, holding tremendous potential for restoring human tissue and organ function.**

Conventional Human Embryonic Stem Cell Differentiation Methods Require Multi-Lineage Differentiation of Pluripotent Cells

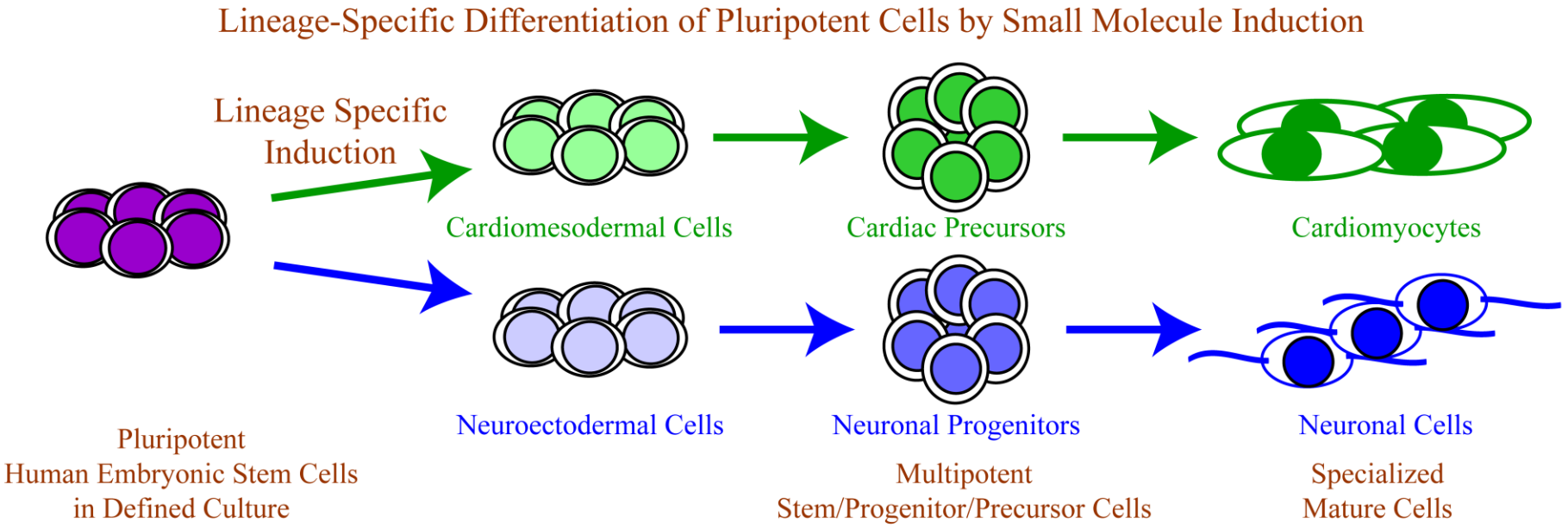
Shortcomings:

- **Unpredictable & uncontrollable**
- **Low-efficiency**
- **Phenotypic heterogeneity and instability**
- **High risk of tumor and/or inappropriate cell type formation following transplantation**
- **Require laborious, costly, and time-consuming purification or isolation procedures to generate only a small quantity of desired cells**
- **Impractical for commercial and clinical applications**

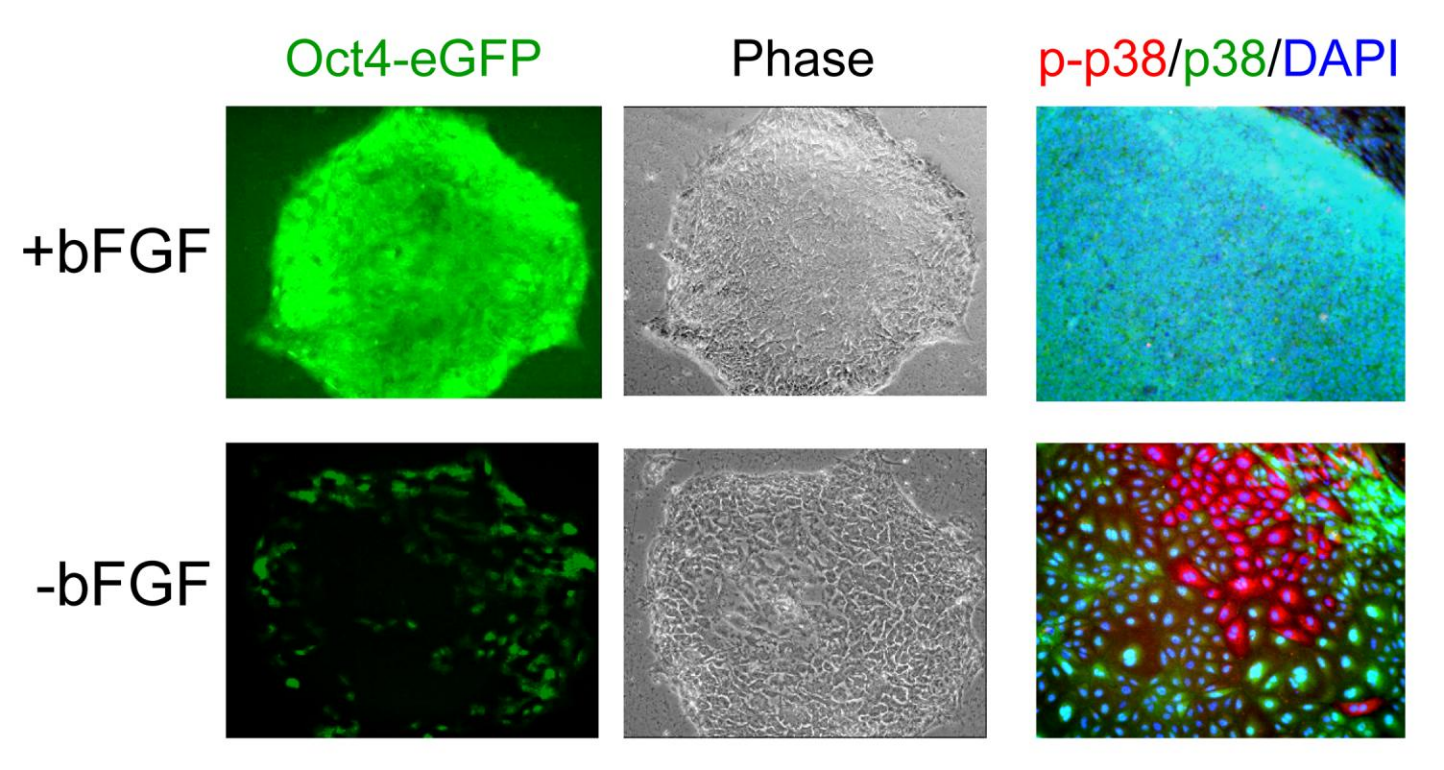


Major Challenges for Clinical and Developmental Studies

How to Channel the Wide Differentiation Potential of Pluripotent Human Embryonic Stem Cells Efficiently and Predictably to Desired Phenotypes ?

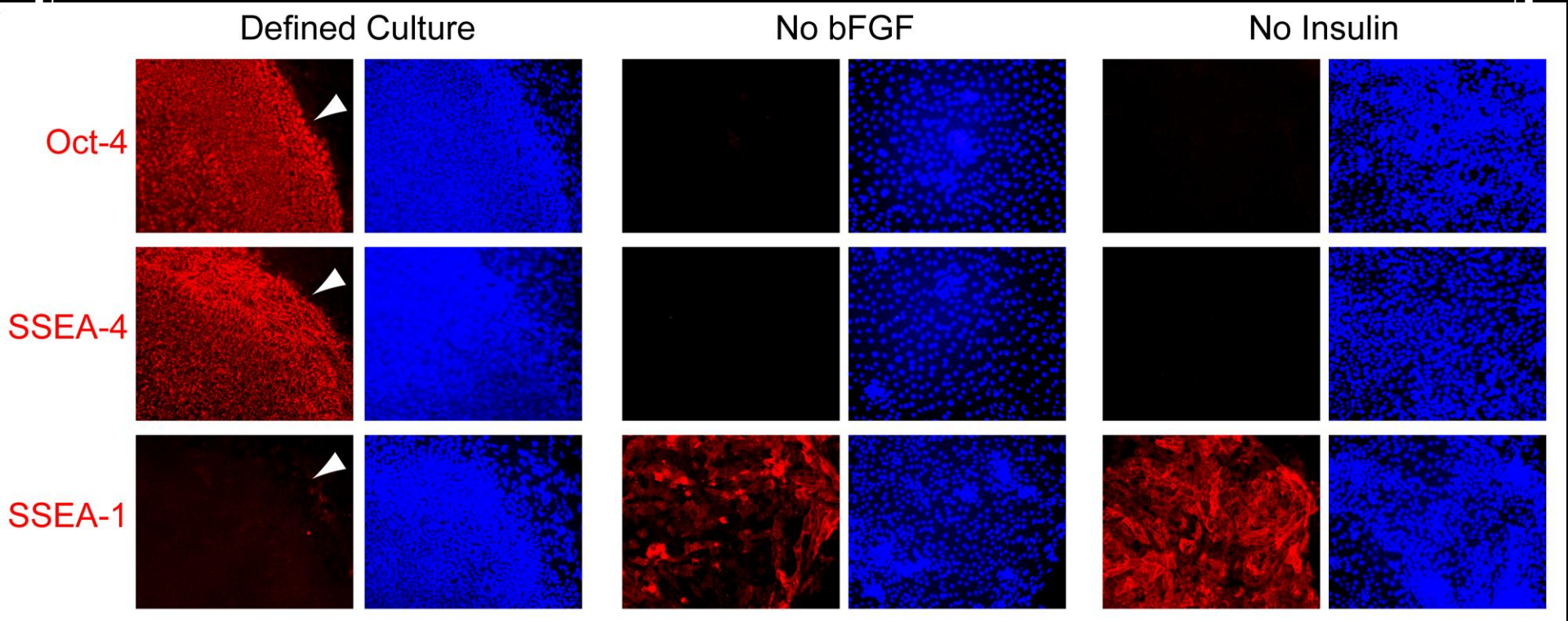


Developing Controlled Differentiation Strategies for Direct Induction of Pluripotent Human ES Cells into Clinically-Relevant Lineages First Requires Understanding the Minimal Essential Defined Elements for Sustaining the Self-Renewal and Pluripotence of Human ES Cells

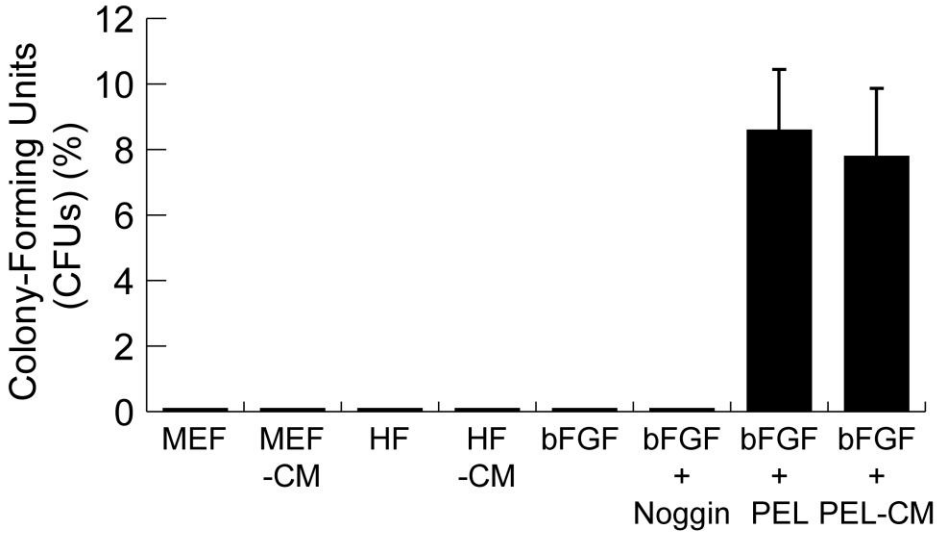


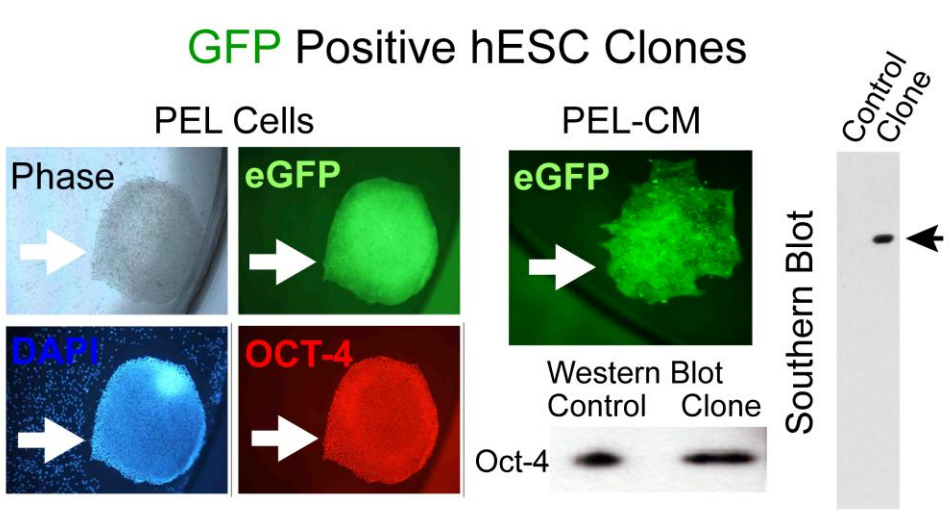
- **bFGF is one of the minimal essential defined elements for the maintenance of undifferentiated human ES cells**

Establishing a Defined Platform for Well-Controlled Derivation, Maintenance, and Differentiation of Clinical-Suitable Pluripotent Human ES Cells



Resolving Defined Elements for Sustaining Epiblast Pluripotence of Human ES Cells

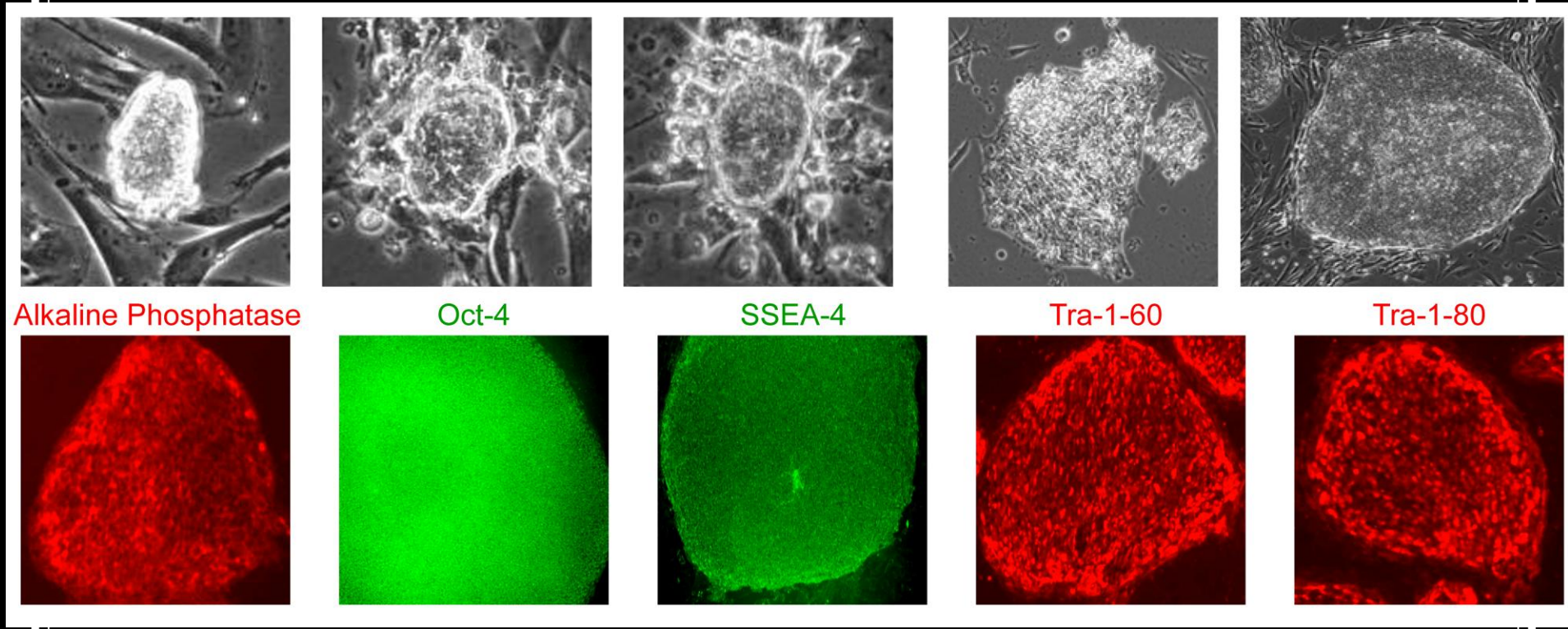




Clonogenicity Analysis by Clone Formation Units

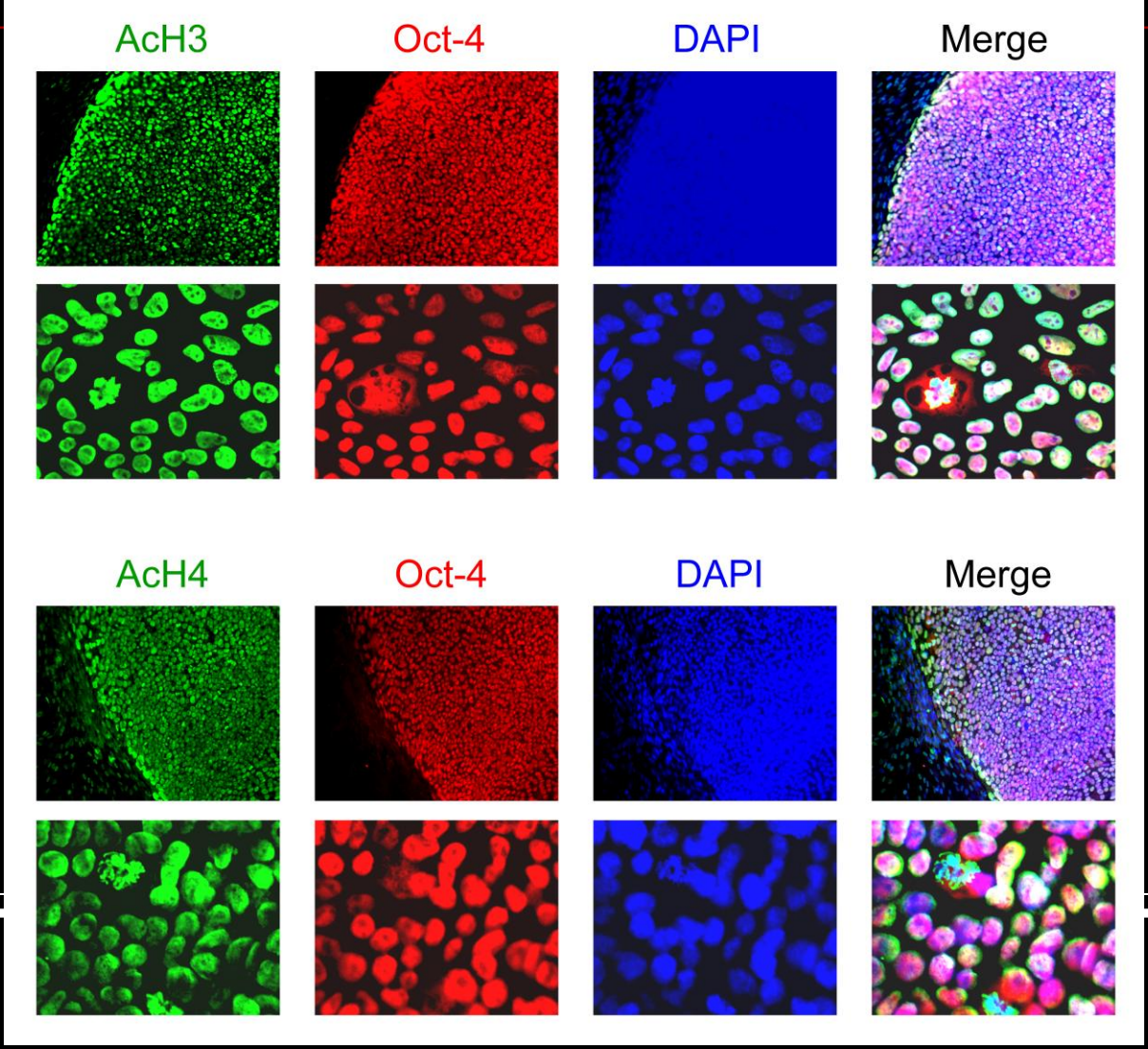
The human ES cell colonies maintained under the defined culture conditions are associated with a monolayer of human ES cell-derived fibroblastic cells that resembled the primitive endoderm (PE) cells surrounding the emerging epiblast in the developing pluripotent inner cell mass (ICM) of the blastocyst *in vivo*, suggesting that these PE-like cells may spontaneously act as auto feeder layers, preventing them from differentiating

The Defined Culture Platform Enables *de novo* Derivation of Clinically-Grade Human ES Cells

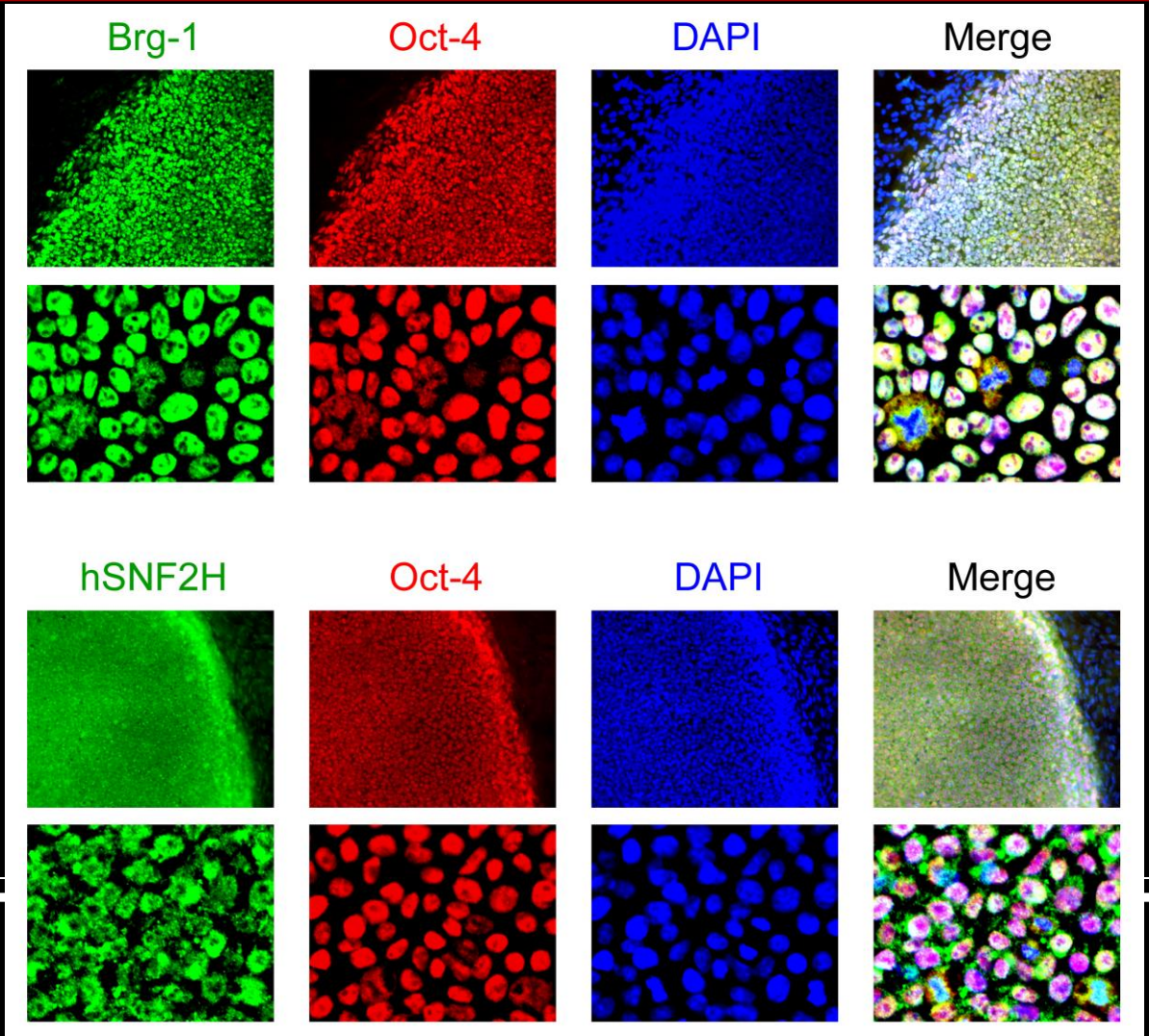


Animal-free, exogenous feeder-, serum-, and conditioned-medium-free human ES cells, suitable for therapeutic development and clinical application

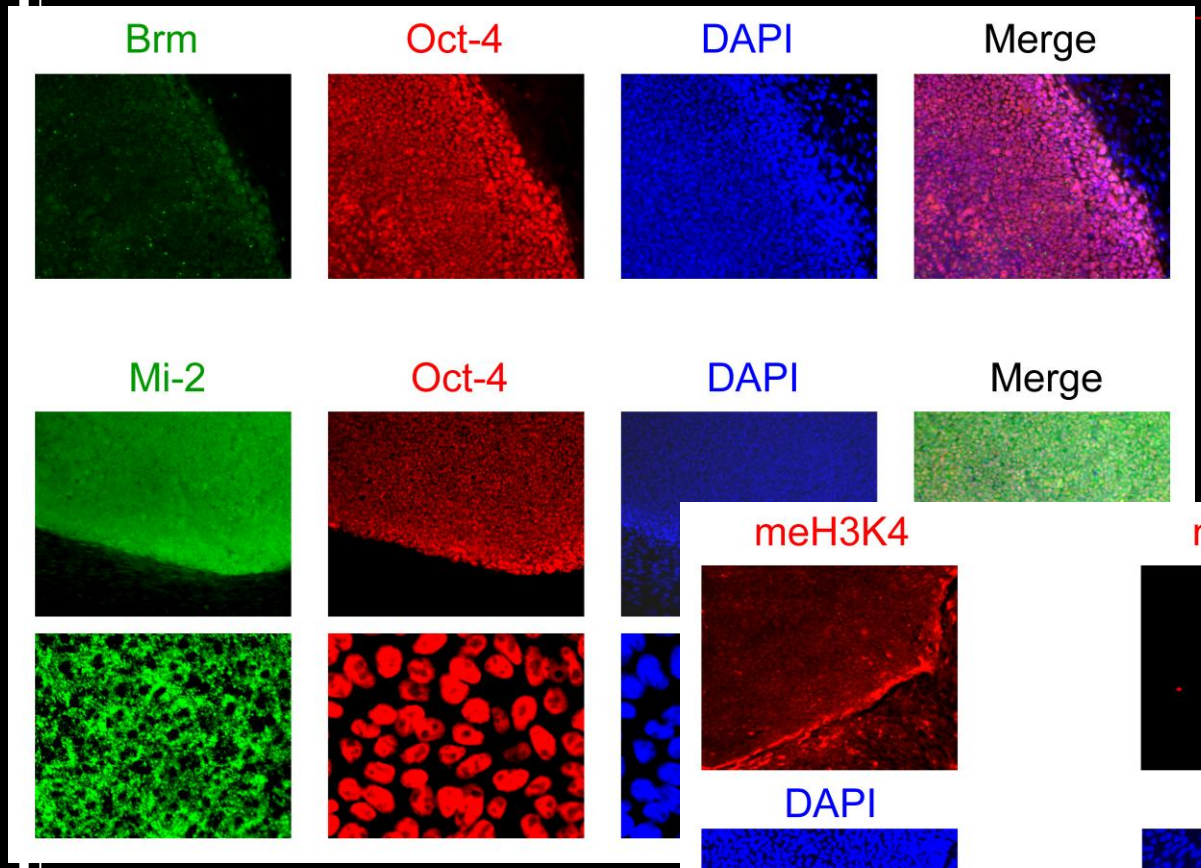
The Genomic Plasticity of Pluripotent Human ES Cells under the Defined Culture is Enabled by an Acetylated Globally Active Chromatin maintained by Oct-4

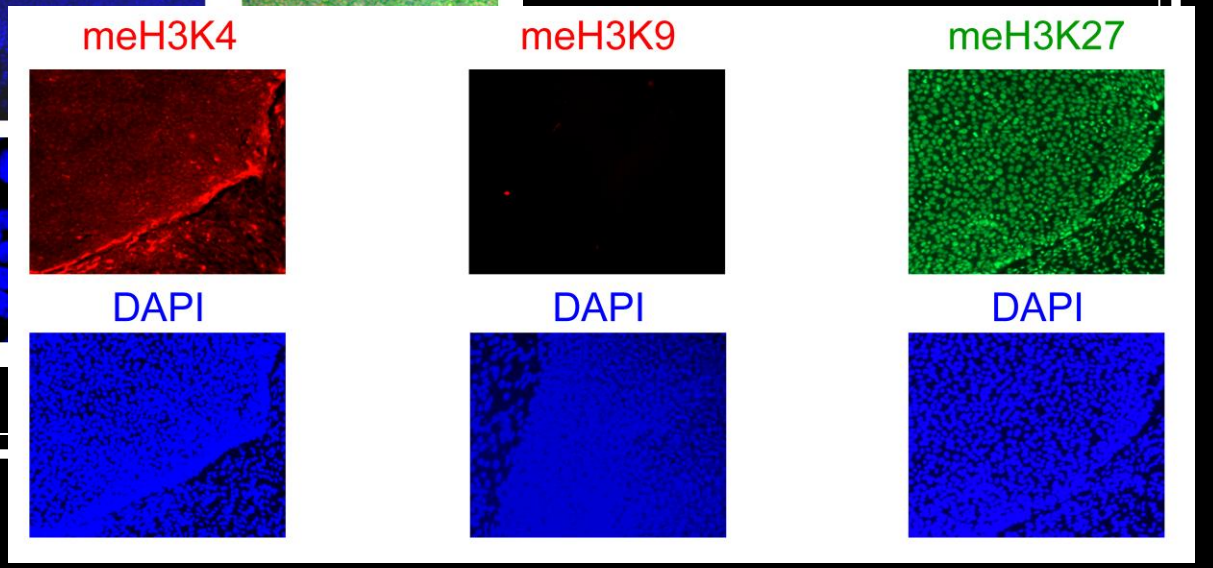


The Pluripotency of Human ES Cells that Display Normal Stable Expansion is Associated with High Levels of Expression and Nuclear Localization of Active Chromatin Remodeling Factors that Include Brg-1, hSNF2H, HAT p300, and HDAC1

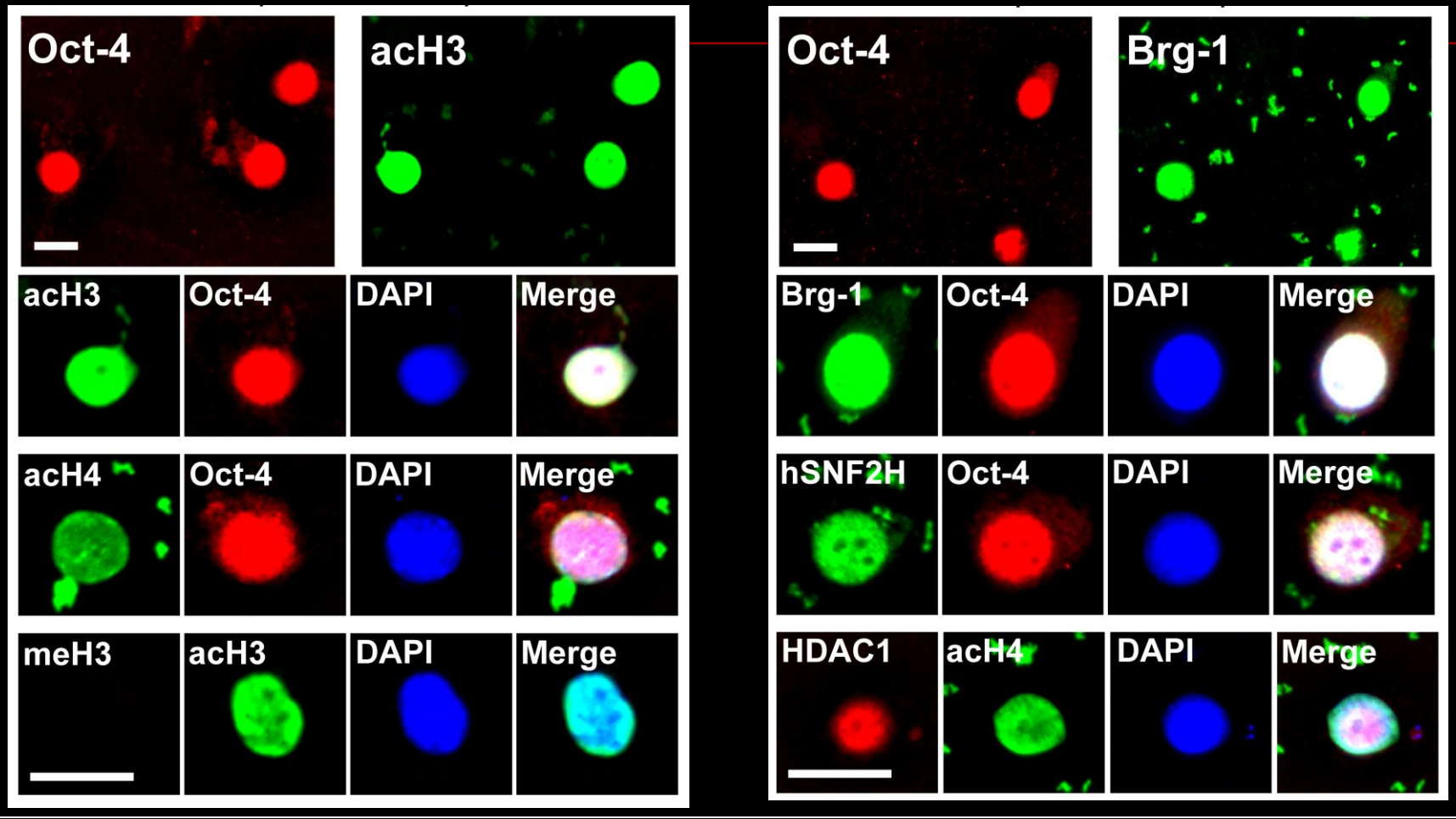


The Pluripotency of Human ES Cells that Display Normal Stable Expansion is Associated with Weak Expression or Cytoplasmic Localization of Repressive Chromatin Remodeling Factors that are Implicated in Transcriptional Silencing; and Residual H3 K9 Methylation





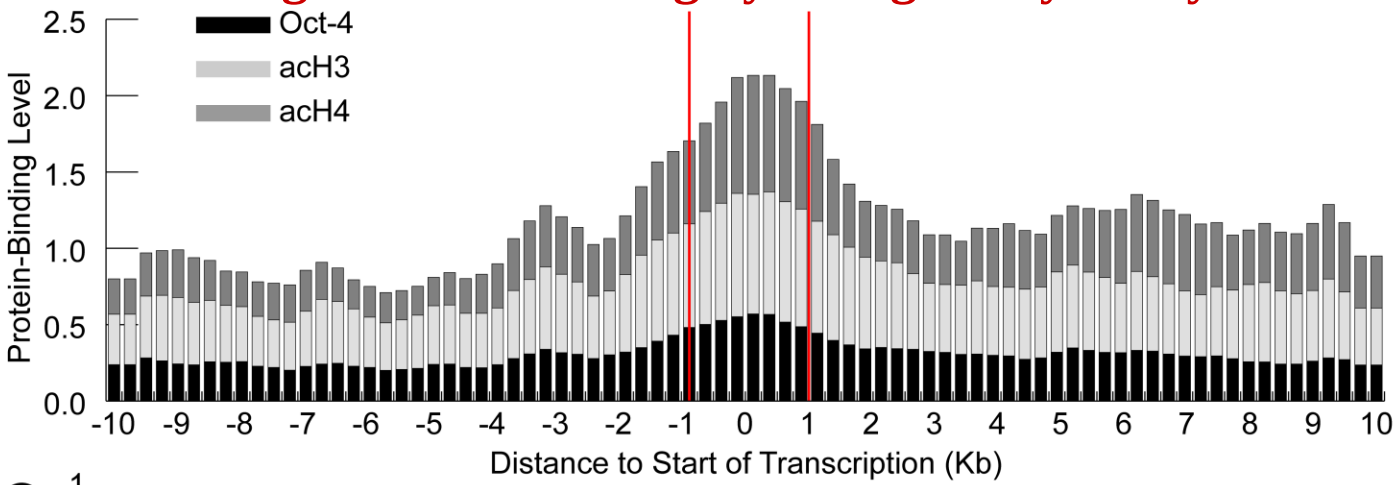
Oct-4 is Associated with Active Chromatin Modifiers Globally

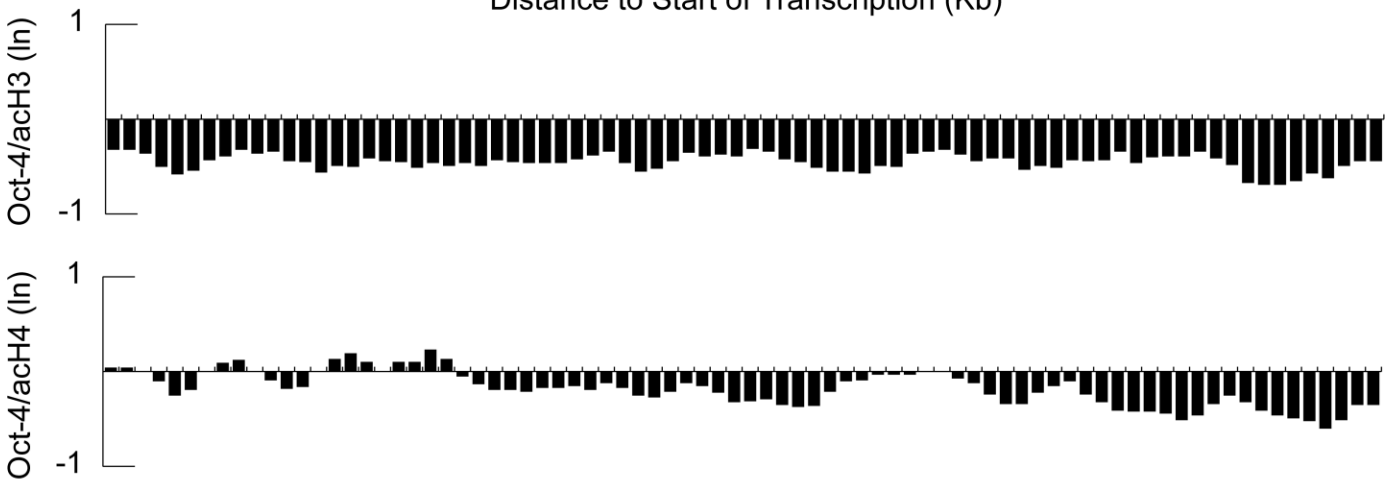


Chromatin Complexes of Pluripotent Human ES Cells

The broad potential of pluripotent Human ES Cells is defined by an epigenome constituted of open conformation of chromatin mediated by a pattern of Oct-4 global distribution that corresponds genome-wide closely with those of active chromatin modifications, as marked by either acetylated histone H3 or H4

Profiling of Oct-4 Binding by Tiling Array Analysis





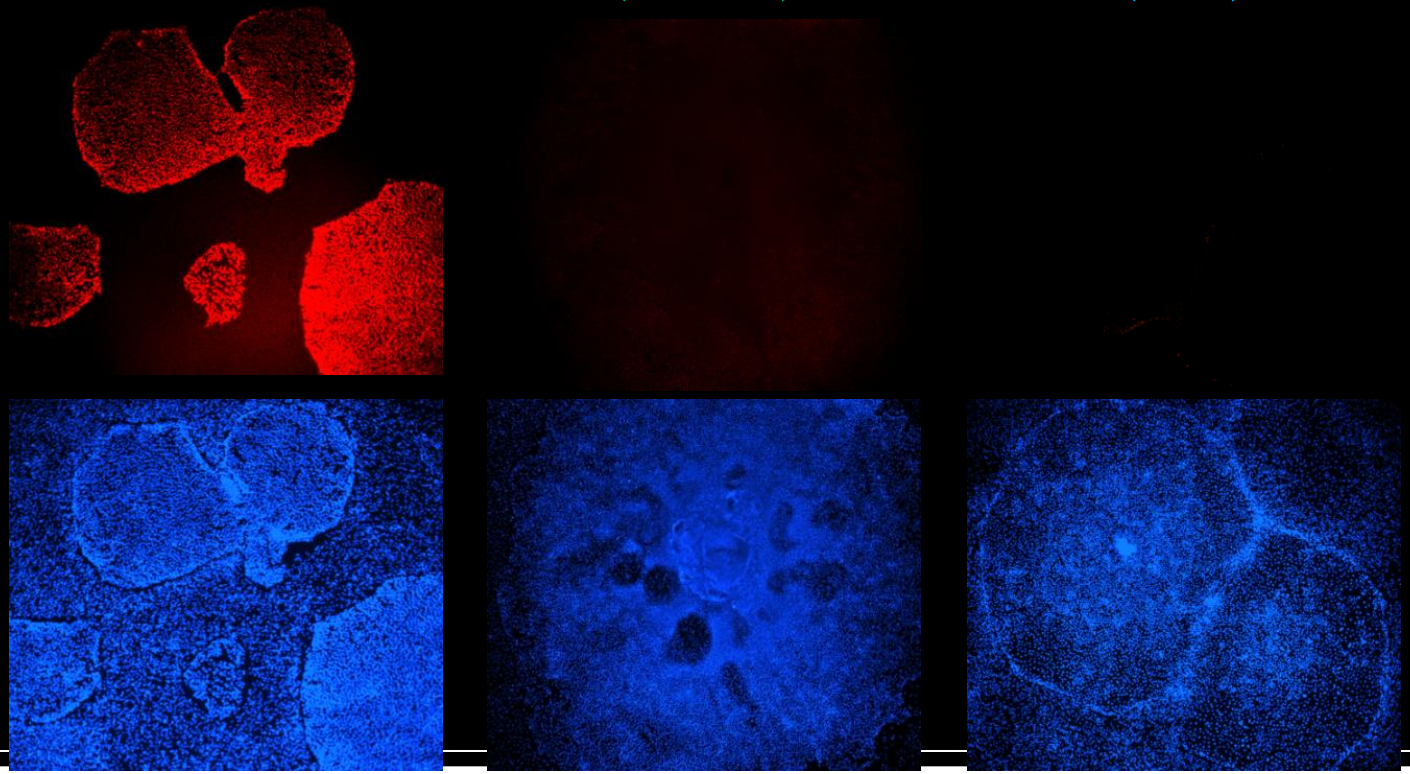
Chromatin/Nucleosome Immunoprecipitation and DNA Microarray Analysis (NuIP-on-chip)

A Defined Culture Platform Enables *de novo* Derivation of Clinically-Suitable Human ES Cells and Well-Controlled Efficient Specification of Clinically-Suitable Human ES Cells Exclusively towards a Cardiac or a Neural Lineage Direct from the Pluripotent Stage by Small Molecule Induction

Control

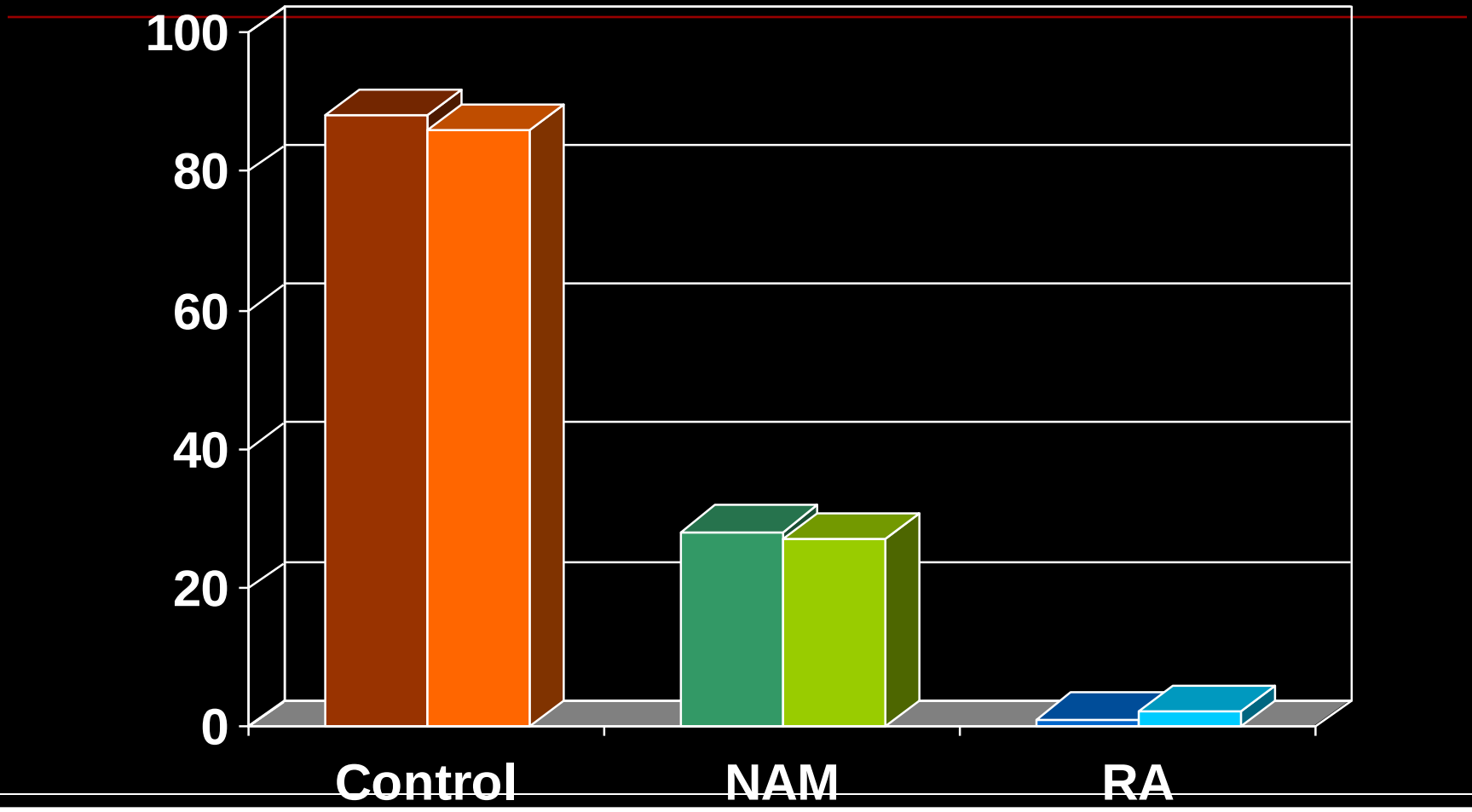
Nicotinamide (NAM)

Retinoic Acid (RA)



Oct-4 – Undifferentiated human ES cells

Upon Exposure of Undifferentiated Human ES Cells to Induced Molecules under the Defined Culture, All the Cells within the Colony Underwent Morphology Changes to Large Differentiated Cells that Cease or Down-Regulate Expression of Pluripotence-Associated Markers

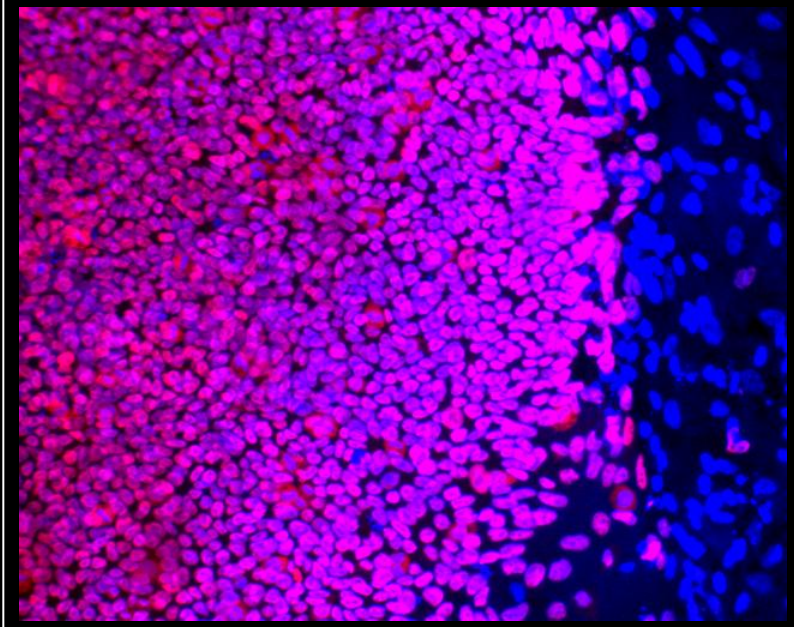


Oct-4 – Undifferentiated hESCs

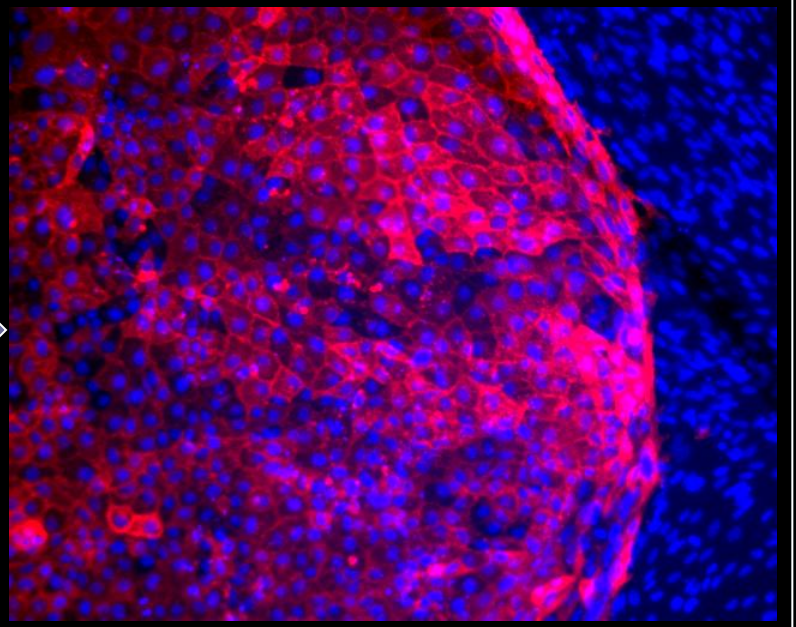
Retinoic Acid (RA) Induces Human ES Cells to Exit the Pluripotent Stage and the Appearance of Neuroectoderm Phenotype

Undifferentiated Human ES Cells

Neuroectodermal Cells



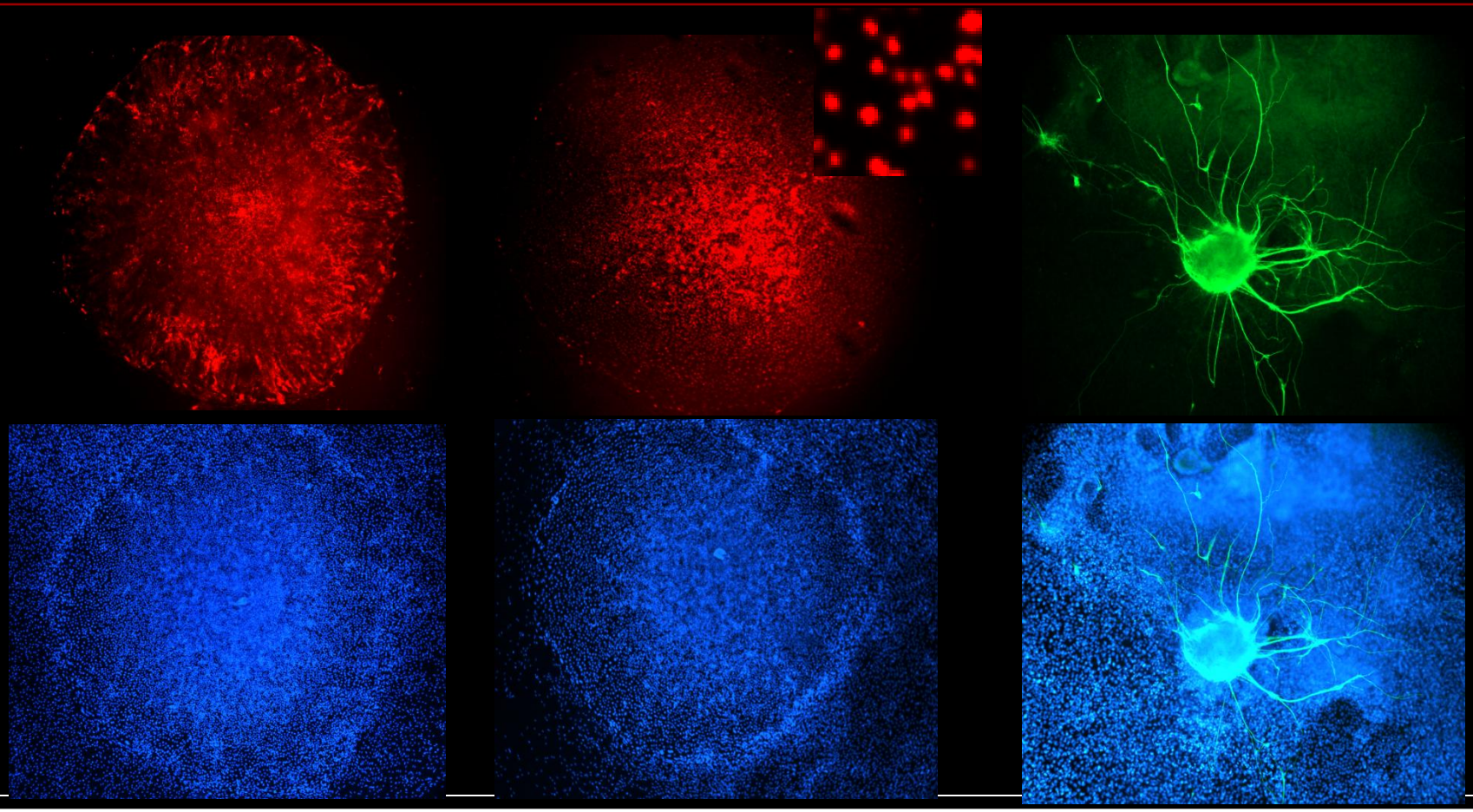
RA



Oct-4

SSEA-1

Retinoic Acid (RA) Induces Human ES Cells to Exit the Pluripotent Stage and the Appearance of Neuroectoderm Phenotype

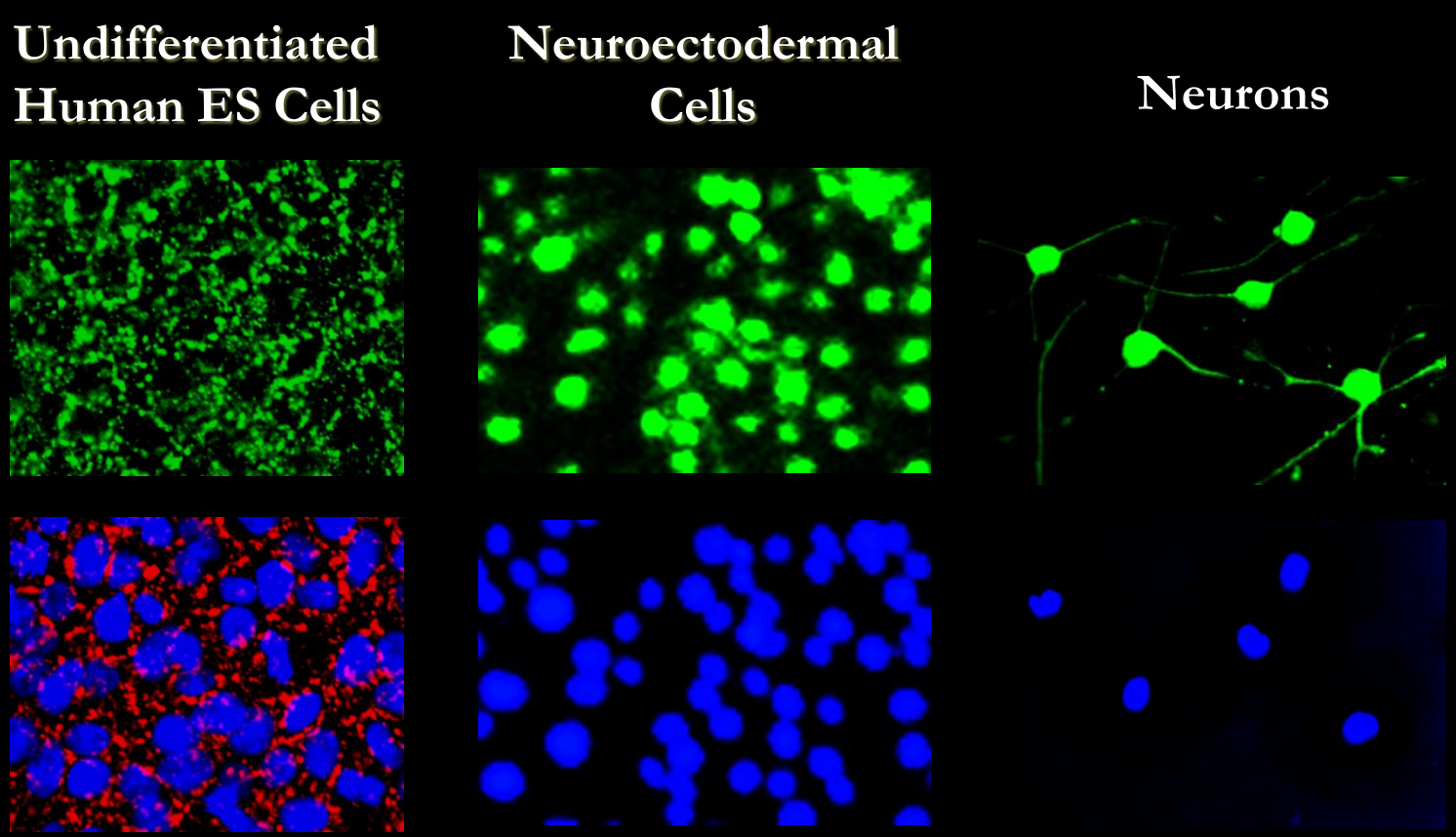


HNK1

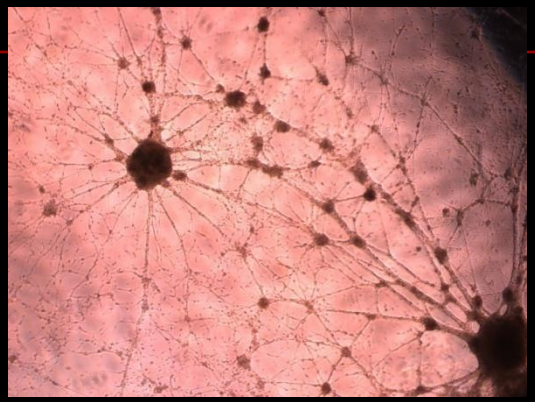
AP2

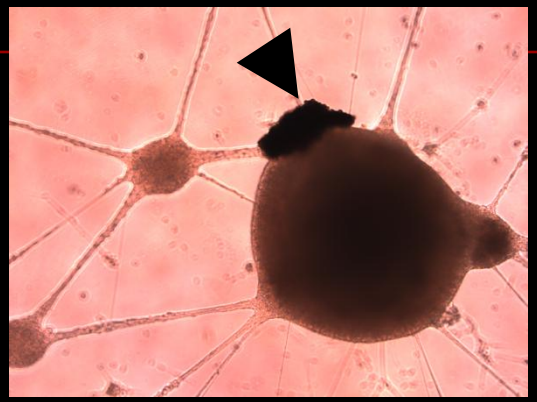
Map-2

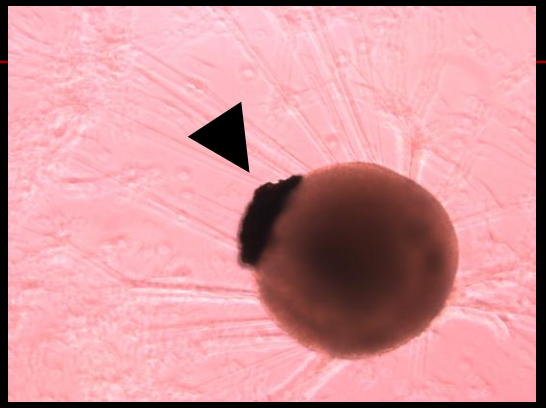
Retinoic Acid (RA) Induces Nuclear Translocation of the Neuronal Specific Transcription Factor Nurr-1

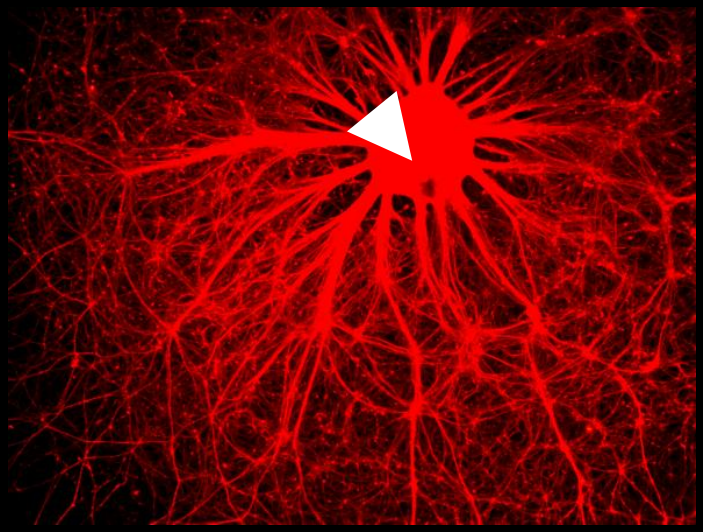


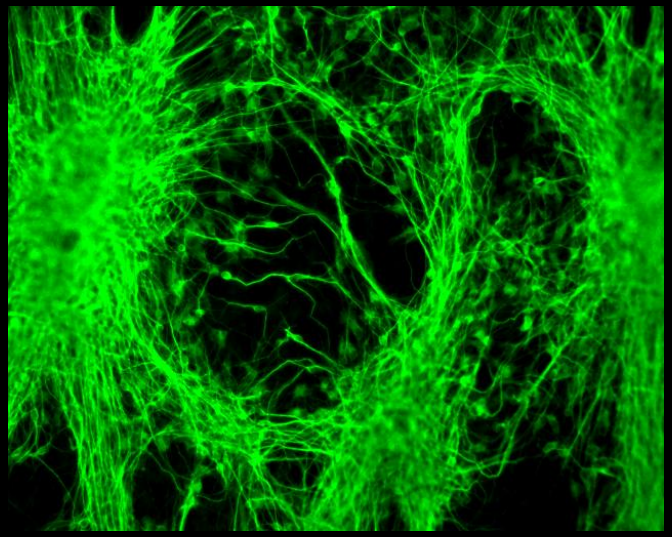
RA-Induced Human ES Cells are Capable of Progression to a Neuronal Phenotype with High Efficiency







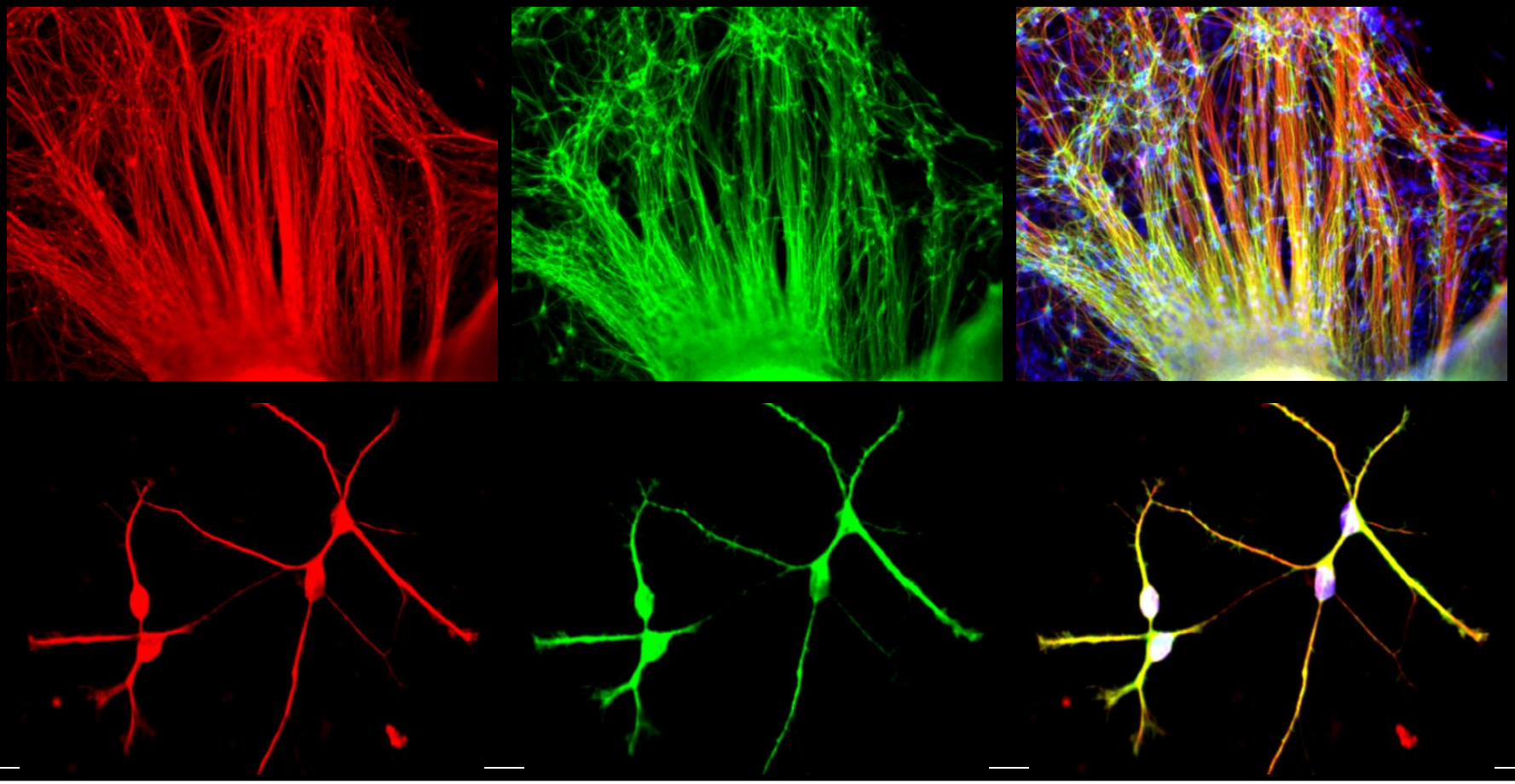




β-III-Tubulin

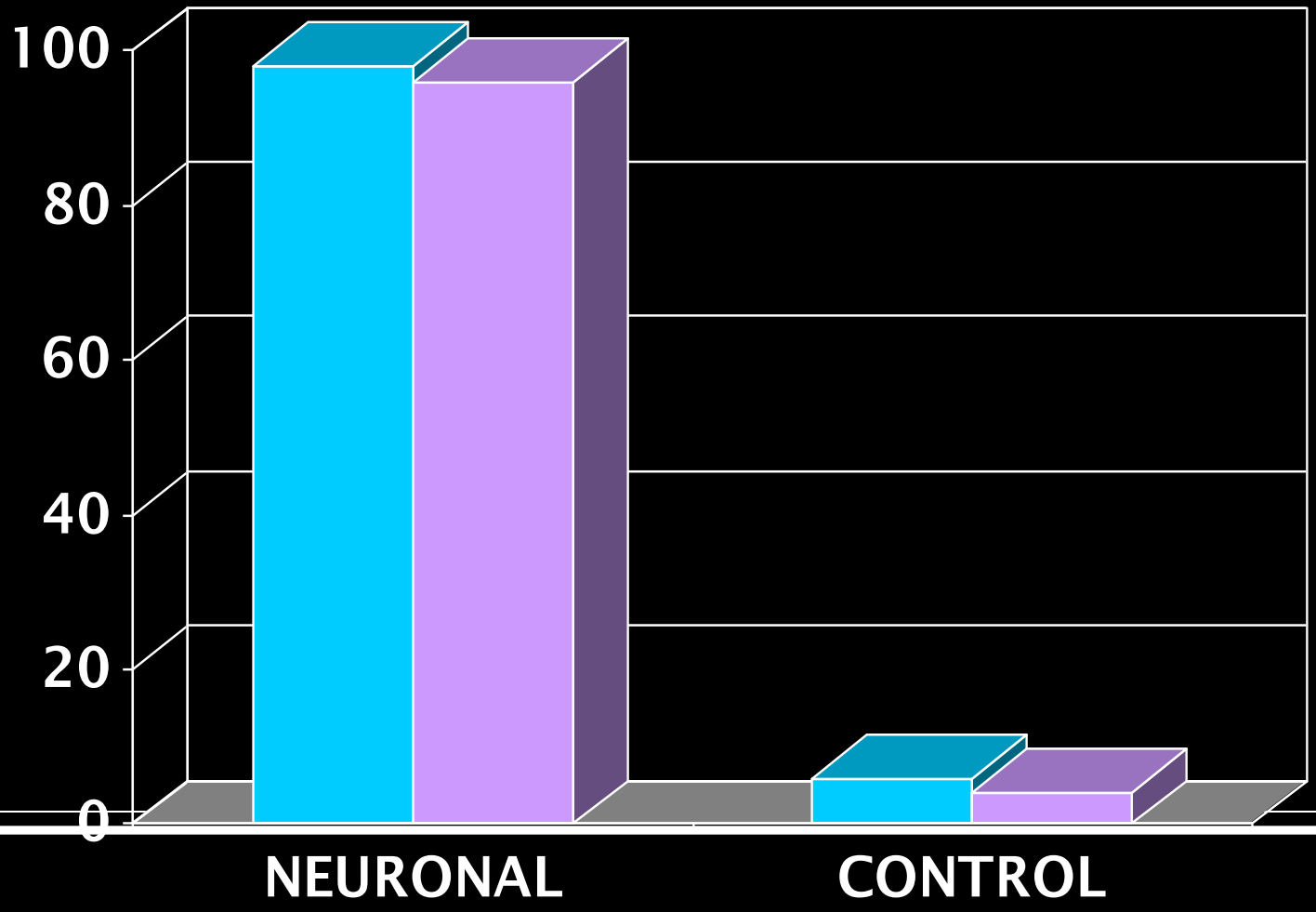
Map-2

RA-Induced Human ES Cells are Capable of Progression to a Neuronal Phenotype with High Efficiency



β-III-Tubulin Map-2 Merge

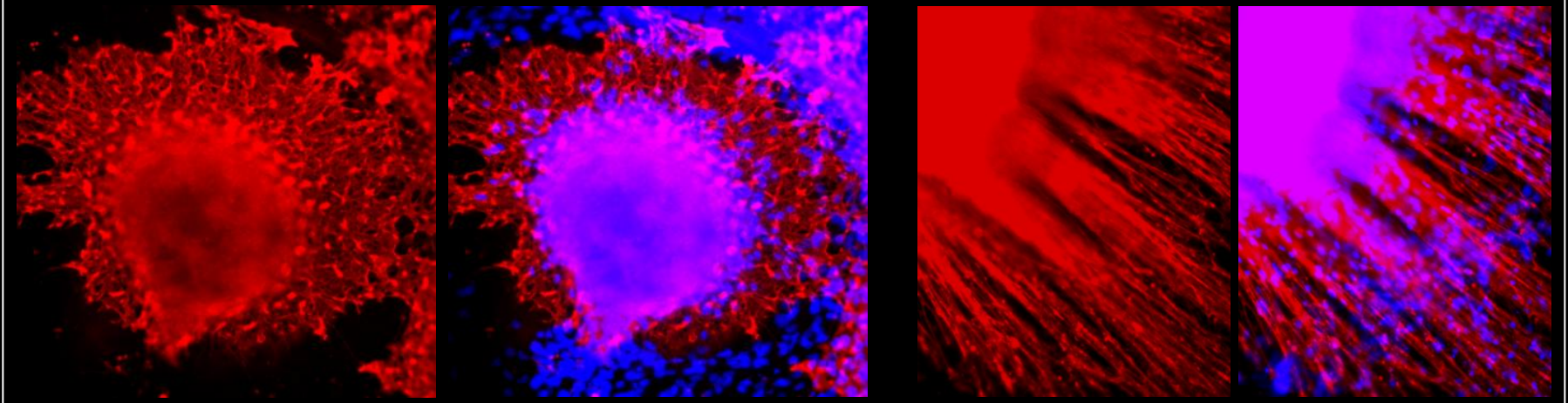
Comparison of Neuronal Differentiation Efficiencies of Pluripotent Human ES Cells by Small Molecule Induction (NEURONAL) with Conventional Multi-Lineage Neural Differentiation Approaches (CONTROL)



Under Neuronal Subtype Specification Conditions, these Human ES Cell-Derived Neuronal Cells Further Proceed to Express Subtype Neuronal Markers Associated with Ventrally-Located Neuronal Populations, such as Tyrosine Hydroxylase (TH) (Dopaminergic Neurons) and Hb9/Lim3/Isl1 (Motor Neurons)

+ Sonic Hedgehog

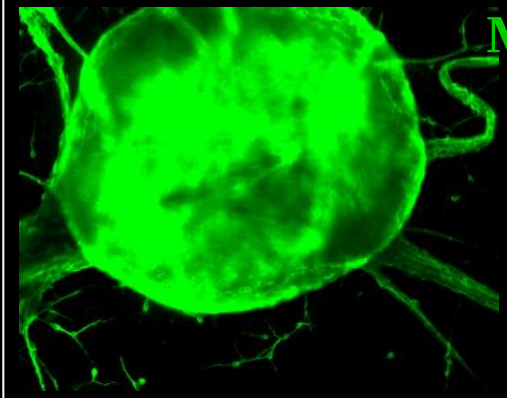
- Sonic Hedgehog

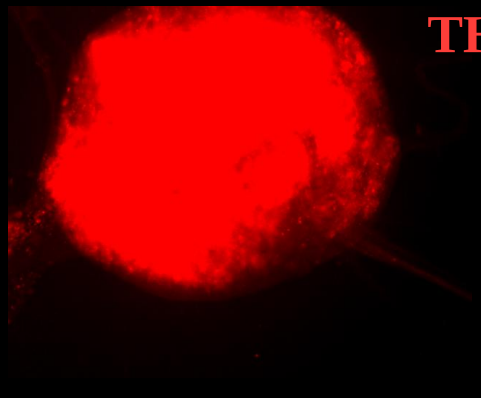


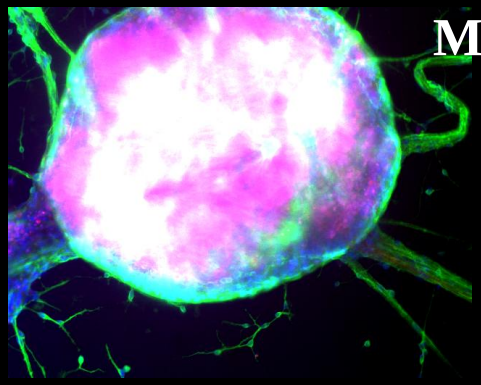
Tyrosine Hydroxylase (TH)

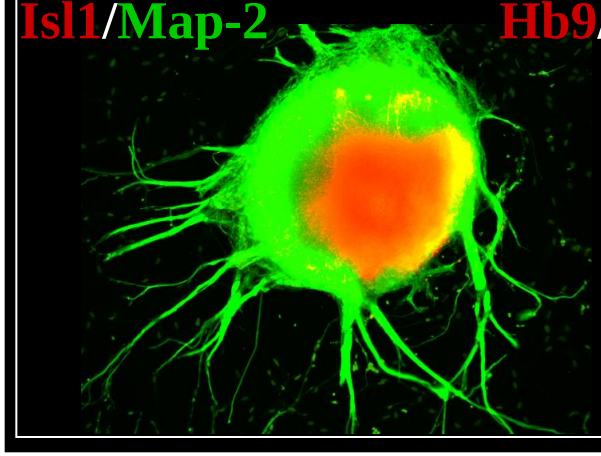
2D Culture

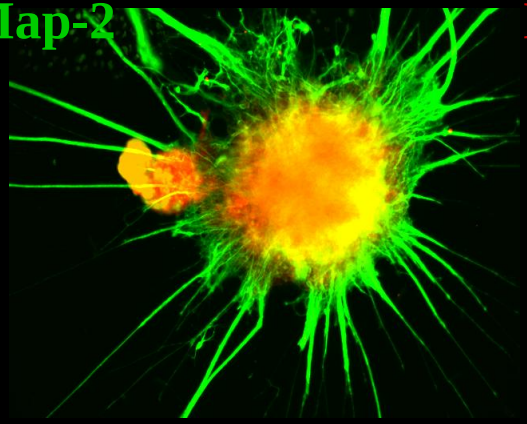
Under Neuronal Subtype Specification Conditions, these Human ES Cell-Derived Neuronal Cells Further Proceed to Express Subtype Neuronal Markers Associated with Ventrally-Located Neuronal Populations, such as Tyrosine Hydroxylase (TH) (Dopaminergic Neurons) and Hb9/Lim3/Isl1 (Motor Neurons)

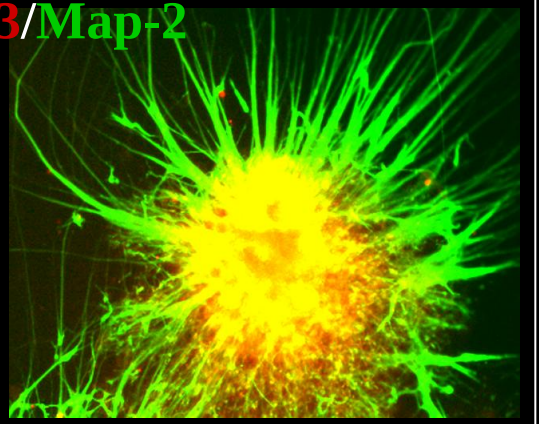






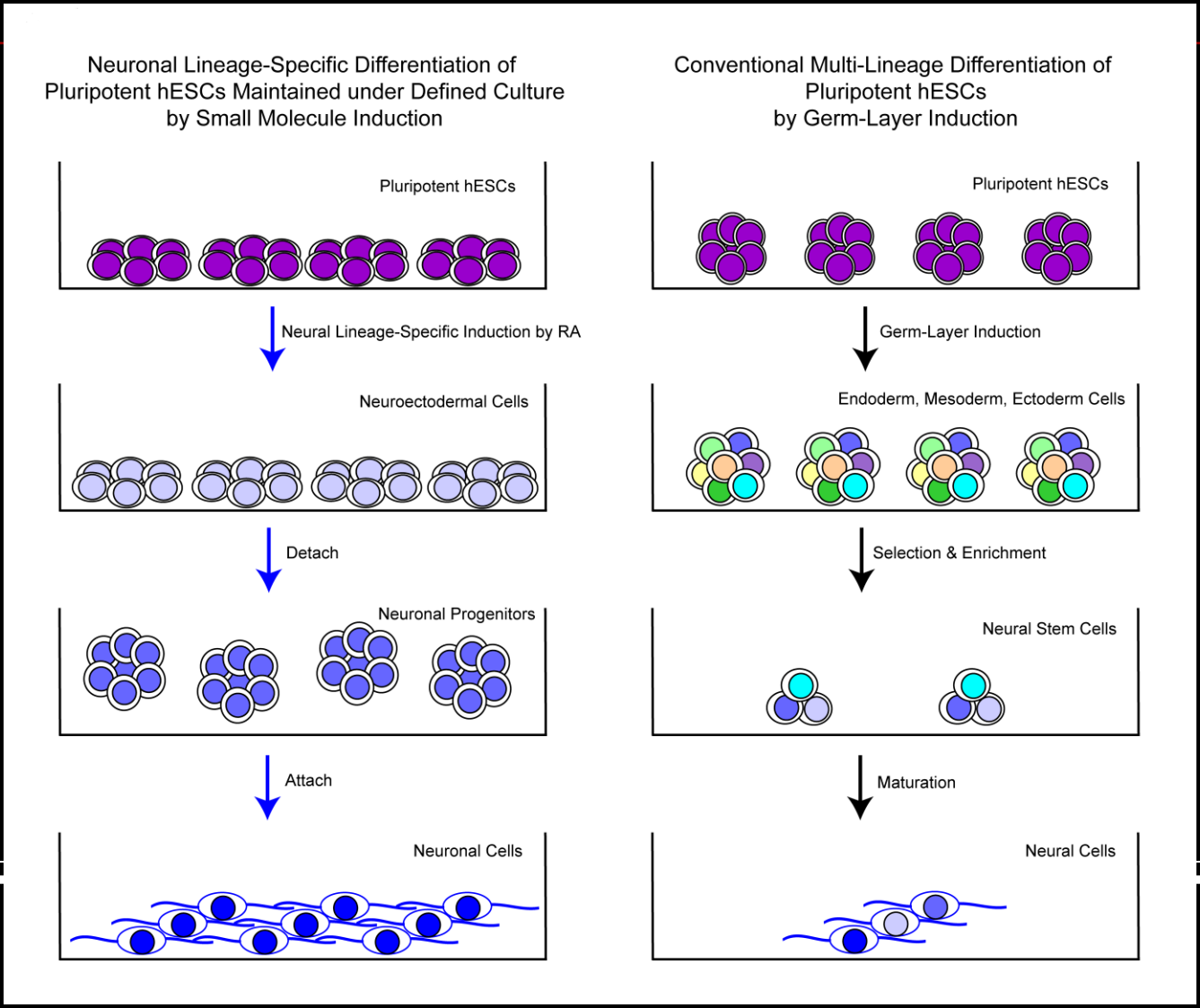




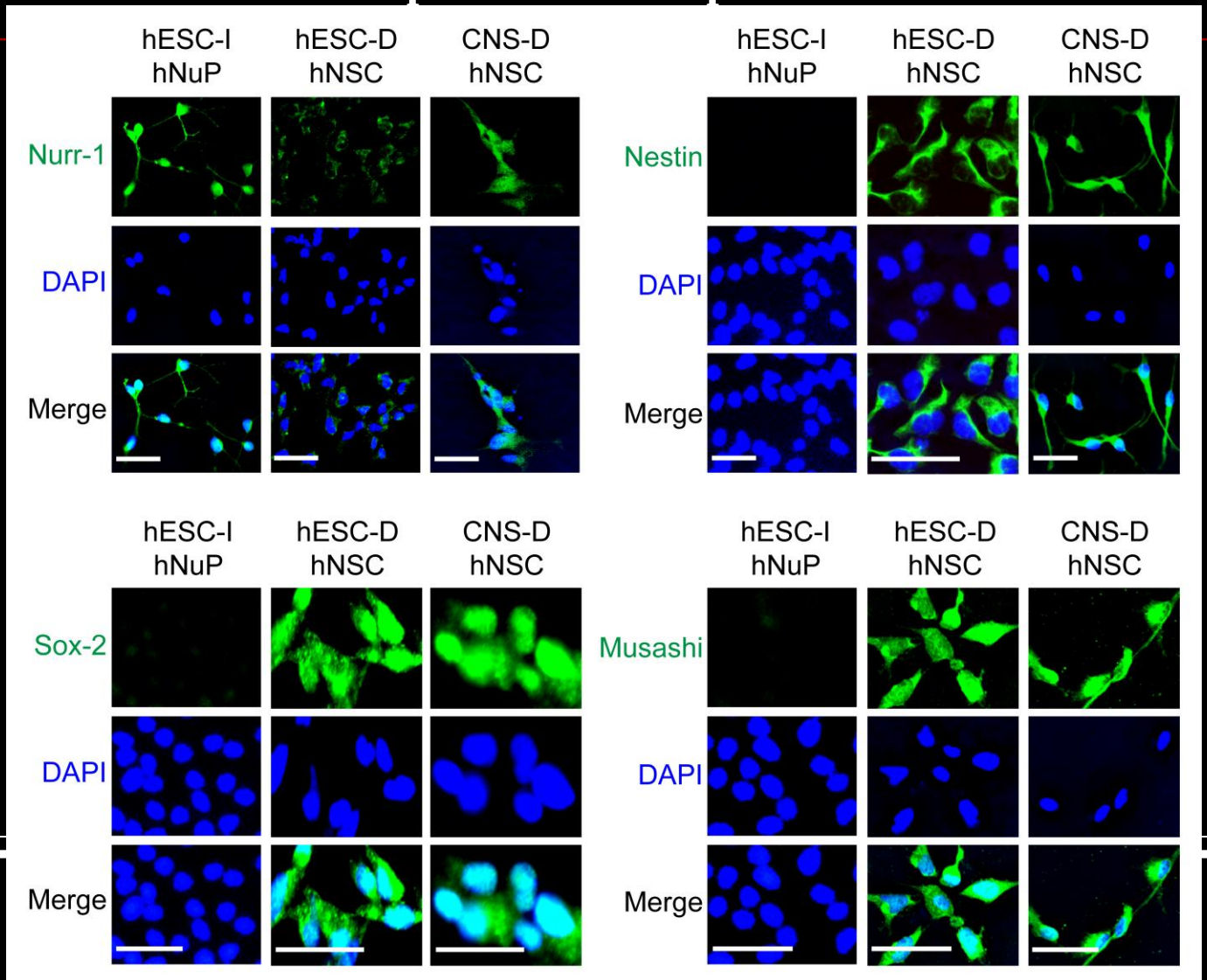


3D Culture

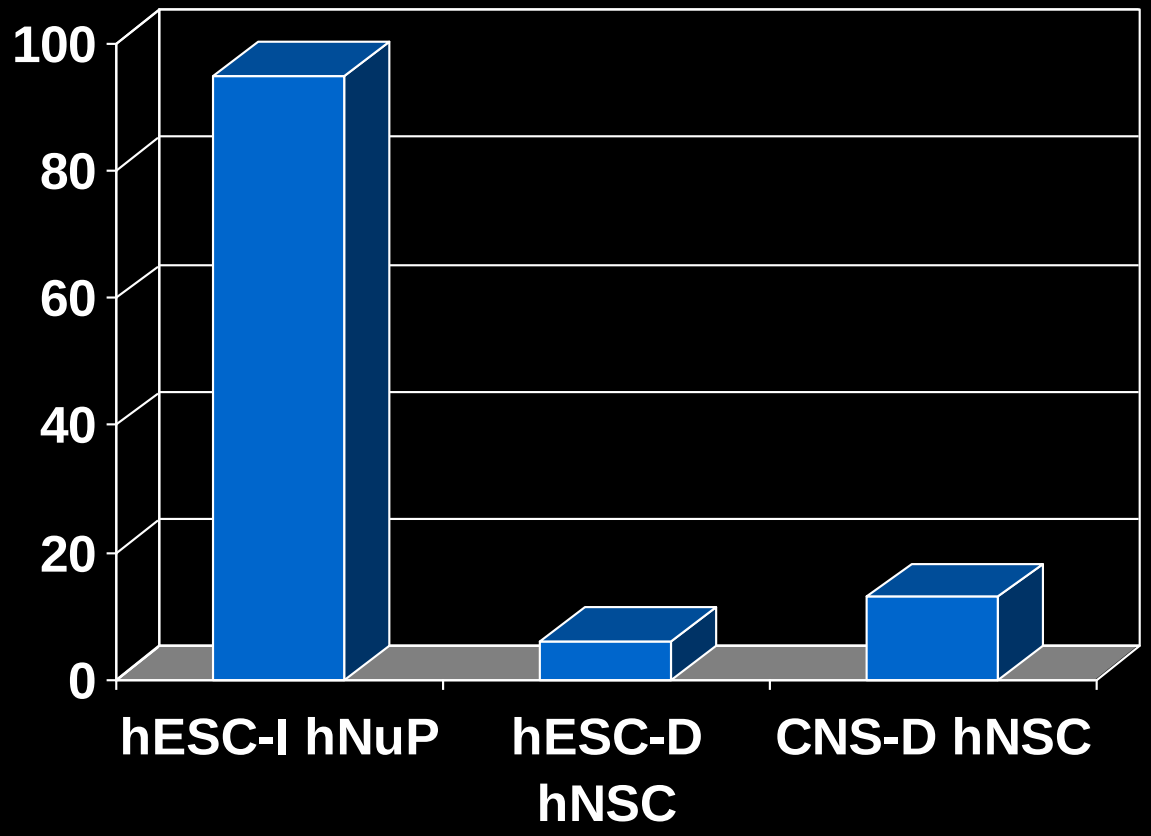
Comparison of Neuronal Lineage-Specific Differentiation of Pluripotent Human ES Cells Exclusively to a Neuronal Fate by Small Molecule Induction without Going through a Multi-Lineage Embryoid Body (EB) Stage vs. Conventional Multi-Lineage Differentiation Approaches through Germ Layer Induction



Unlike the two prototypical human neural stem cells (hNSCs) derived either from human ES cell *in vitro* or isolated directly from CNS *in vivo*, human ES cell neuronal progenitors (hESC-I hNuP) do not express the canonical hNSC markers, but assumed uniformly strong expression and nuclear localization of the neuronal specific transcriptional factor Nurr-1

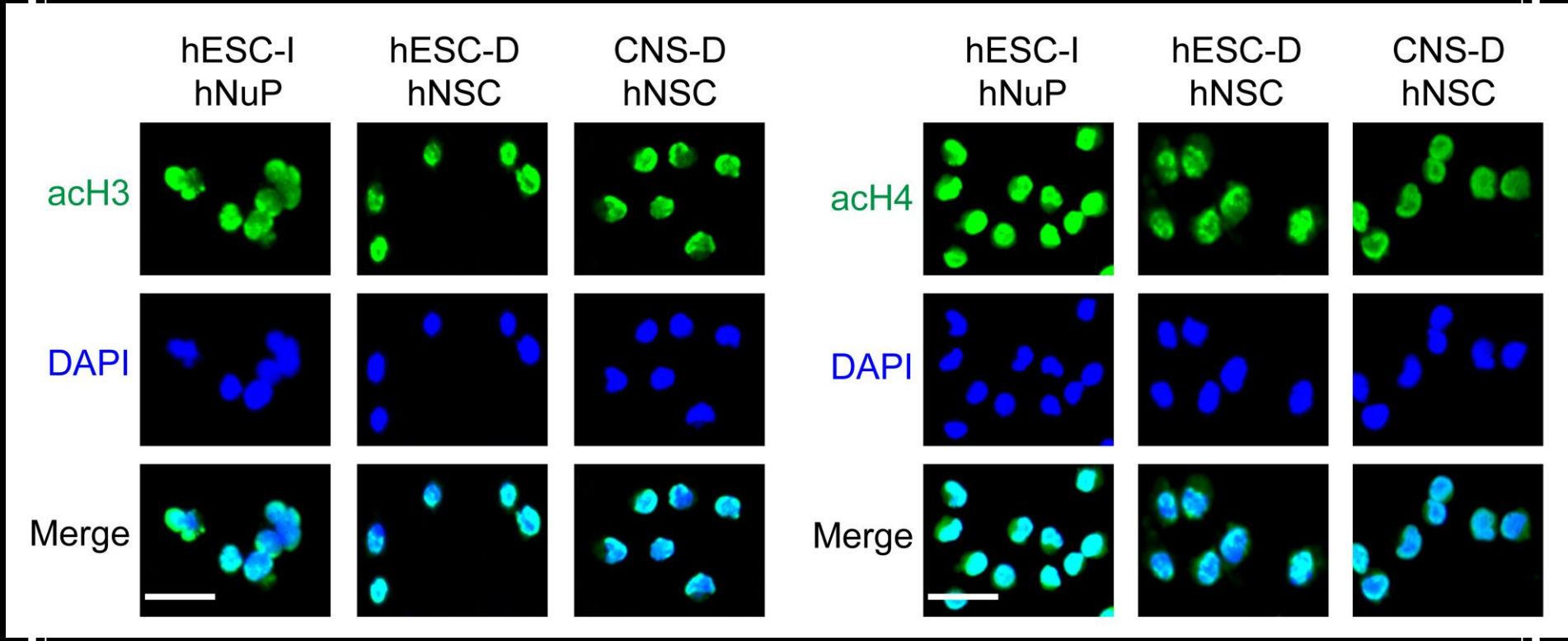


Neuroectoderm Specification Transforms Pluripotent Human ES Cells Uniformly into a More Neuronal Lineage-Specific Embryonic Neuronal Progenitor than the Prototypical Neuroepithelial-Like Nestin-Positive hNSCs Derived either from CNS or hESCs



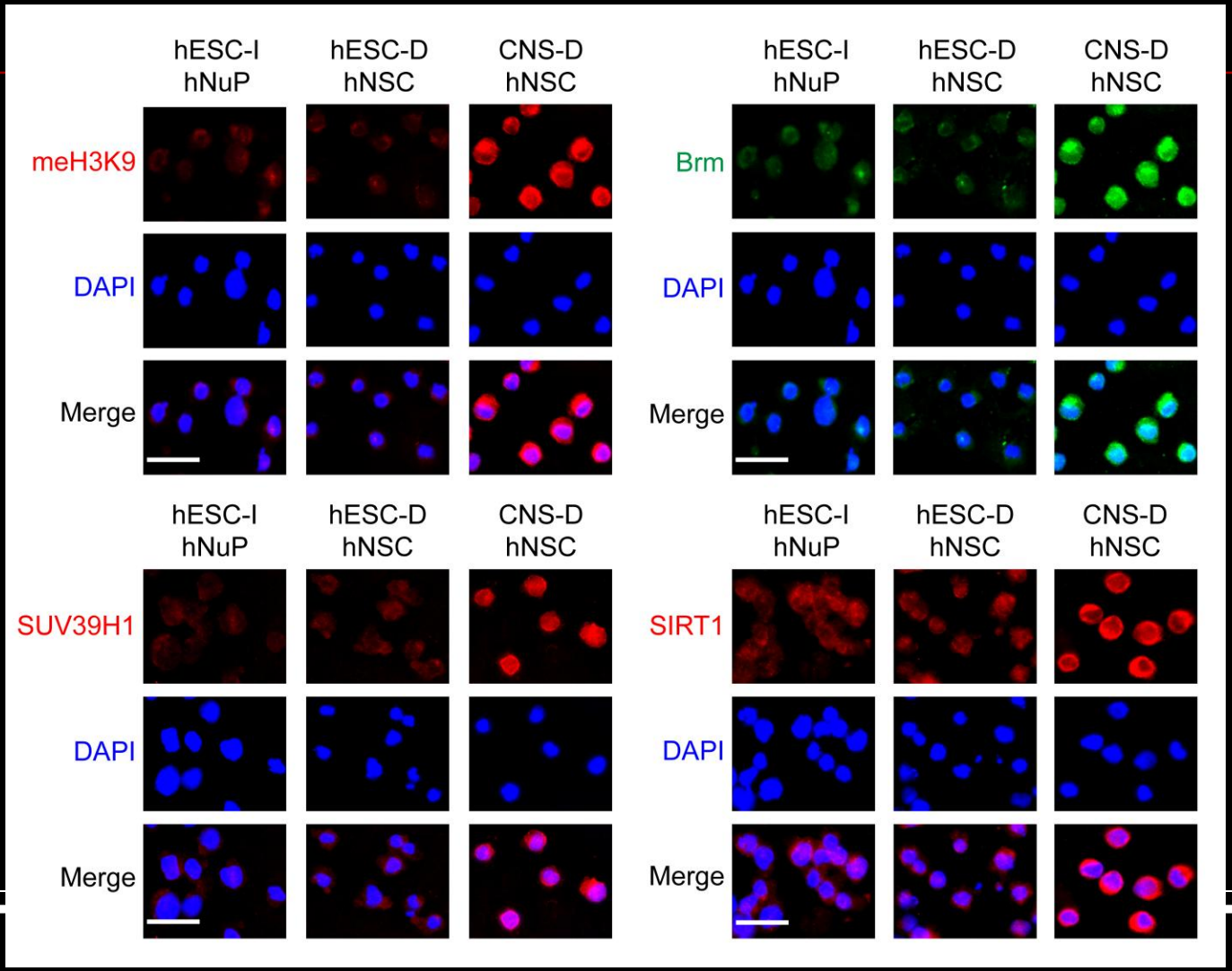
Beta-III-Tubulin-Positive Neurons

These *in vitro* Neuroectoderm-Derived Nurr1-Positive hESC-I hNuP Retain an Rmbryonic Acetylated Globally Active Chromatin State, Which Suggests that They are a More Plastic Human Embryonic Neuronal Progenitor



Chromatin Complexes

Repressive Chromatin Remodeling Factors Regulating Histone H3K9 Methylation, including SIRT1, SUV39H1, and Brm, were Inactive in hESC-I hNuP

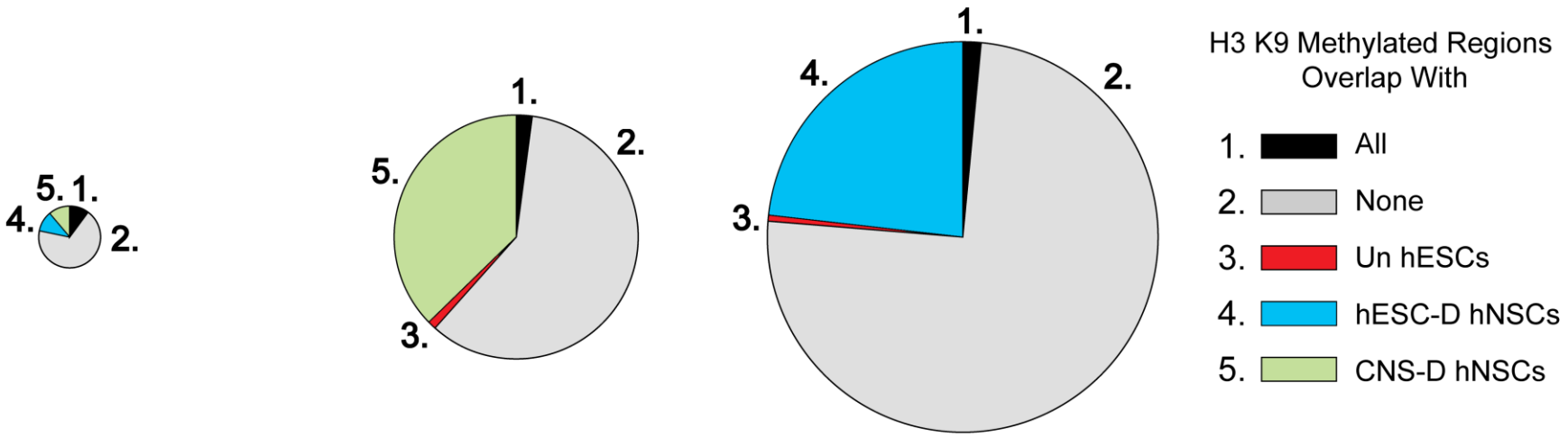


Chromatin Complexes

The Global Increases in Repressive Chromatin-Remodeling Mediated by H3 K9 Methylation Suggest that the Tissue-Resident CNS-derived hNSCs have Acquired a More Silenced Chromatin

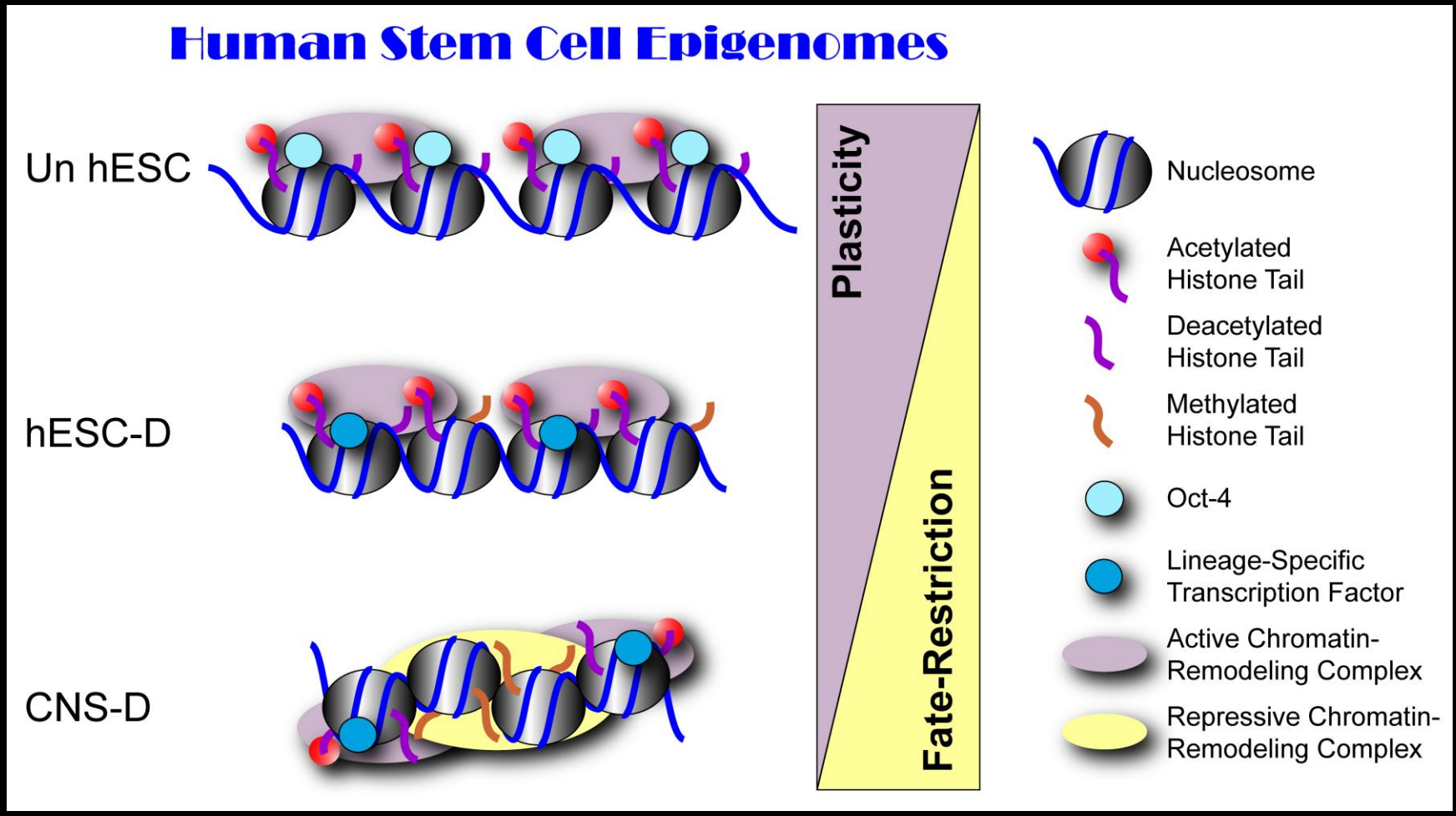
Although the two human stem cell derivatives shared ~ 1/4 of the methylated regions, tissue-resident CNS-derived hNSCs proceeded to acquire a substantial number of additional silenced regions (increased meH3K9), such that ~ 75% of its H3 K9 methylated regions were unique and not present in embryonic derivatives

Un hESCs **hESC-I hNuP** **CNS-D hNSC**



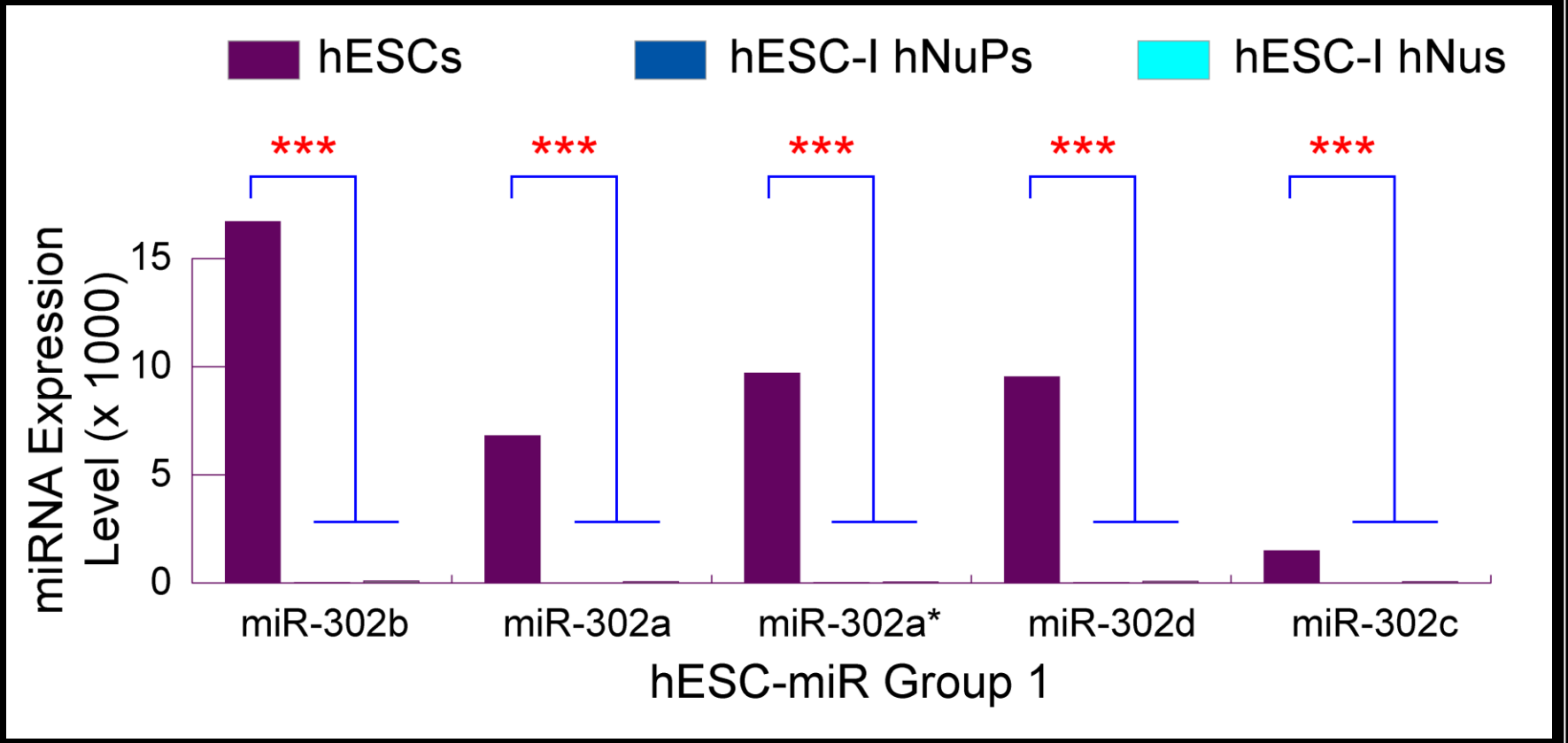
Profiling of H3 K9 Methylation by Tiling Array Analysis

Epigenomic Progression from Pluripotency to Lineage Restriction is Associated with Global Increases in Chromatin-Silencing



The Expression of Pluripotence-Associated hsa-miR-302 Family was Silenced in Human ES Cell Neuronal Derivatives

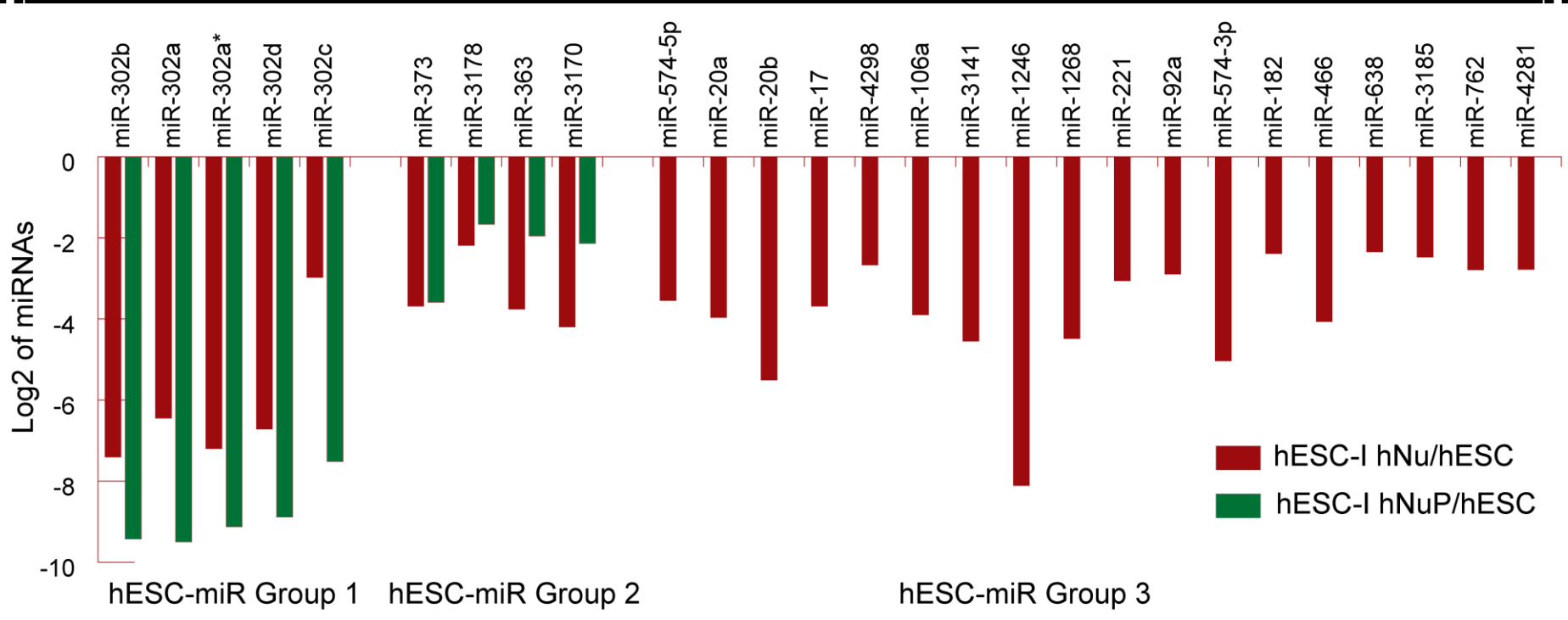
Genome-Scale Profiling of microRNA (miRNA) Differential Expression



The expression of the prominent cluster of human pluripotence-associated miRNA hsa-miR-302 family was silenced upon human ES cell neuronal lineage specification

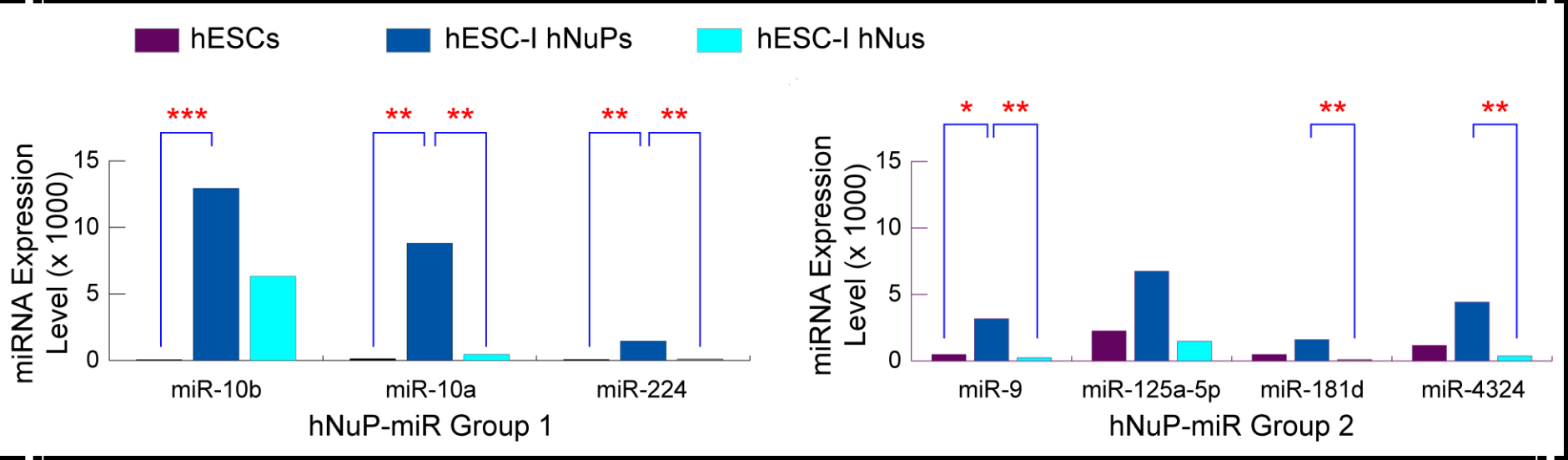
The Expression of Pluripotence-Associated hsa-miR-302 Family was Silenced in Human ES Cell Neuronal Derivatives

Genome-Scale Profiling of microRNA (miRNA) Differential Expression



The Expression of Hox miRNA hsa-miR-10 Family that Regulates Gene Expression Predominantly in Neuroectoderm was Induced to High Levels in hESC-Derived Neuronal Progenitors

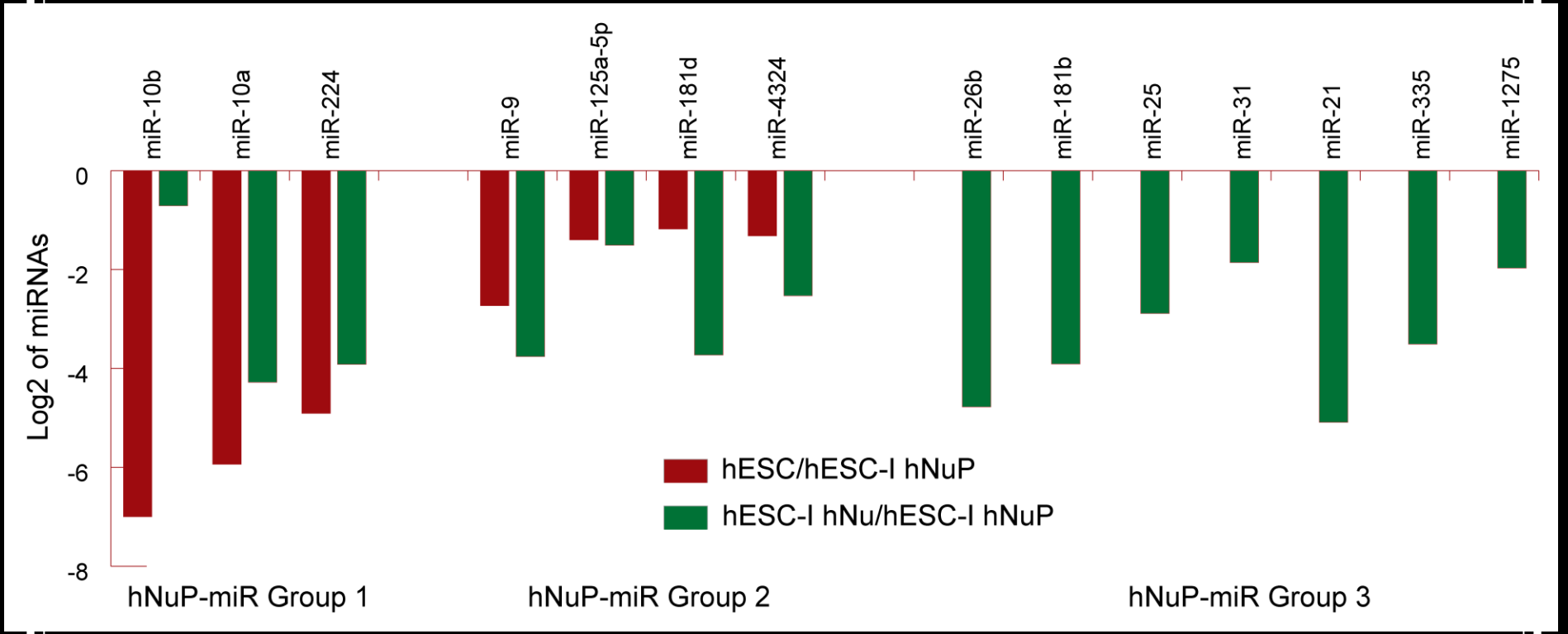
Genome-Scale Profiling of microRNA (miRNA) Differential Expression



- miR-10 genes locate within the Hox clusters of developmental regulators
- miR-10 is coexpressed with a set of Hox genes
- miR-10 repress the translation of Hox transcripts
- Polycomb (PcG) and trithorax (trxG) group proteins control the silence and activation of Hox genes, which play essential roles in epigenetic developmental processes

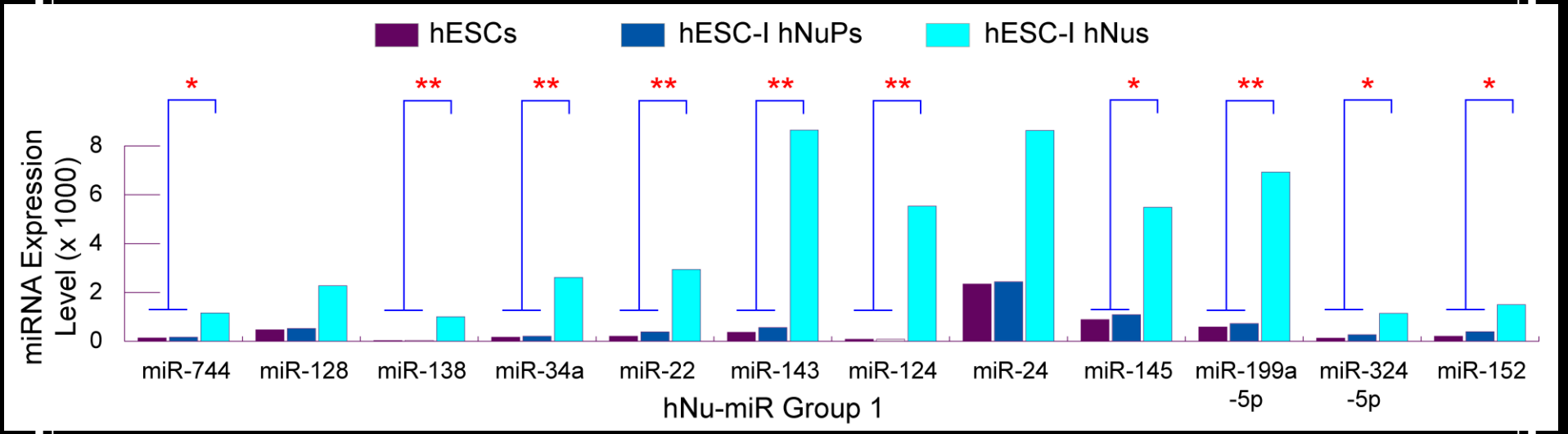
The Expression of Hox miRNA hsa-miR-10 Family that Regulates Gene Expression Predominantly in Neuroectoderm was Induced to High Levels in Human ES Cell-Derived Neuronal Progenitors

Genome-Scale Profiling of microRNA (miRNA) Differential Expression



Distinct Sets of Stage-Specific Human Embryonic Neurogenic miRNAs, Many of Which were not Previously Linked to Neuronal Development and Function, Contribute to the Neuronal Identity of the Developing CNS

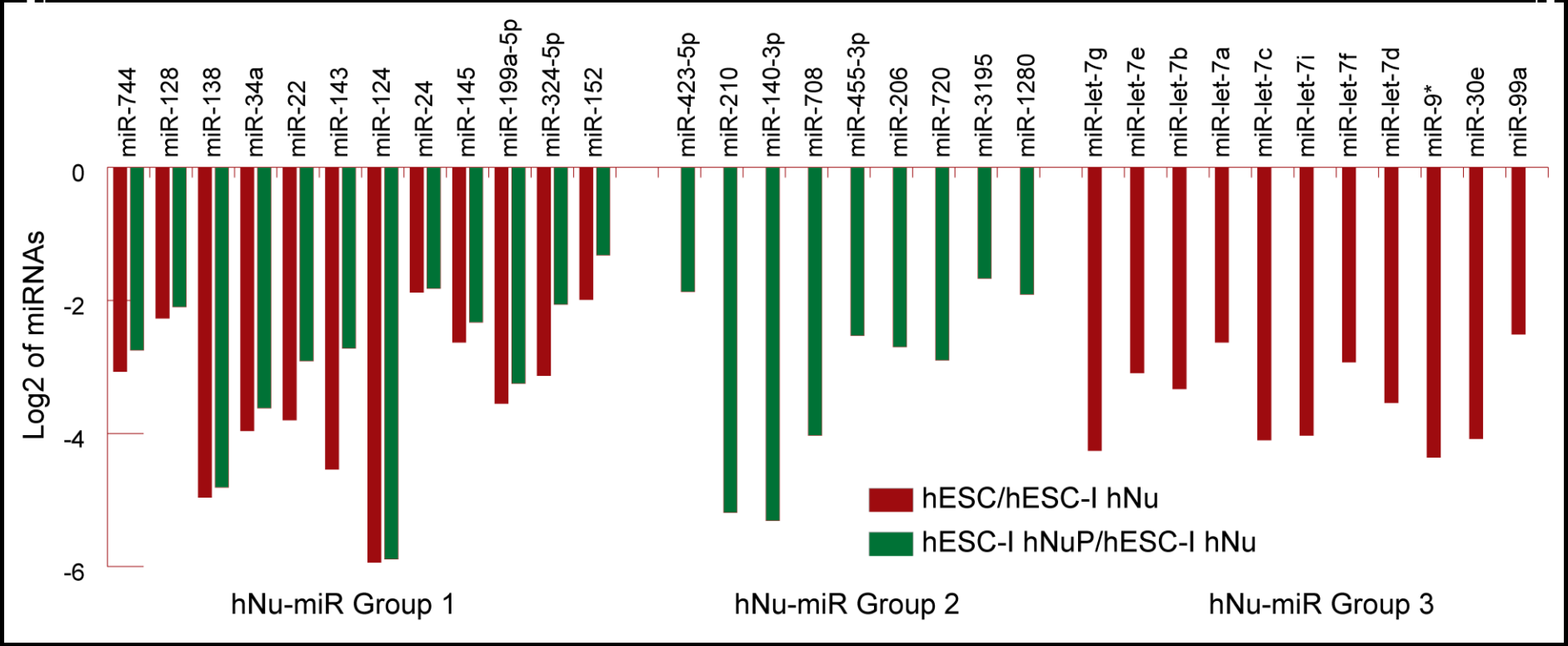
Genome-Scale Profiling of microRNA (miRNA) Differential Expression



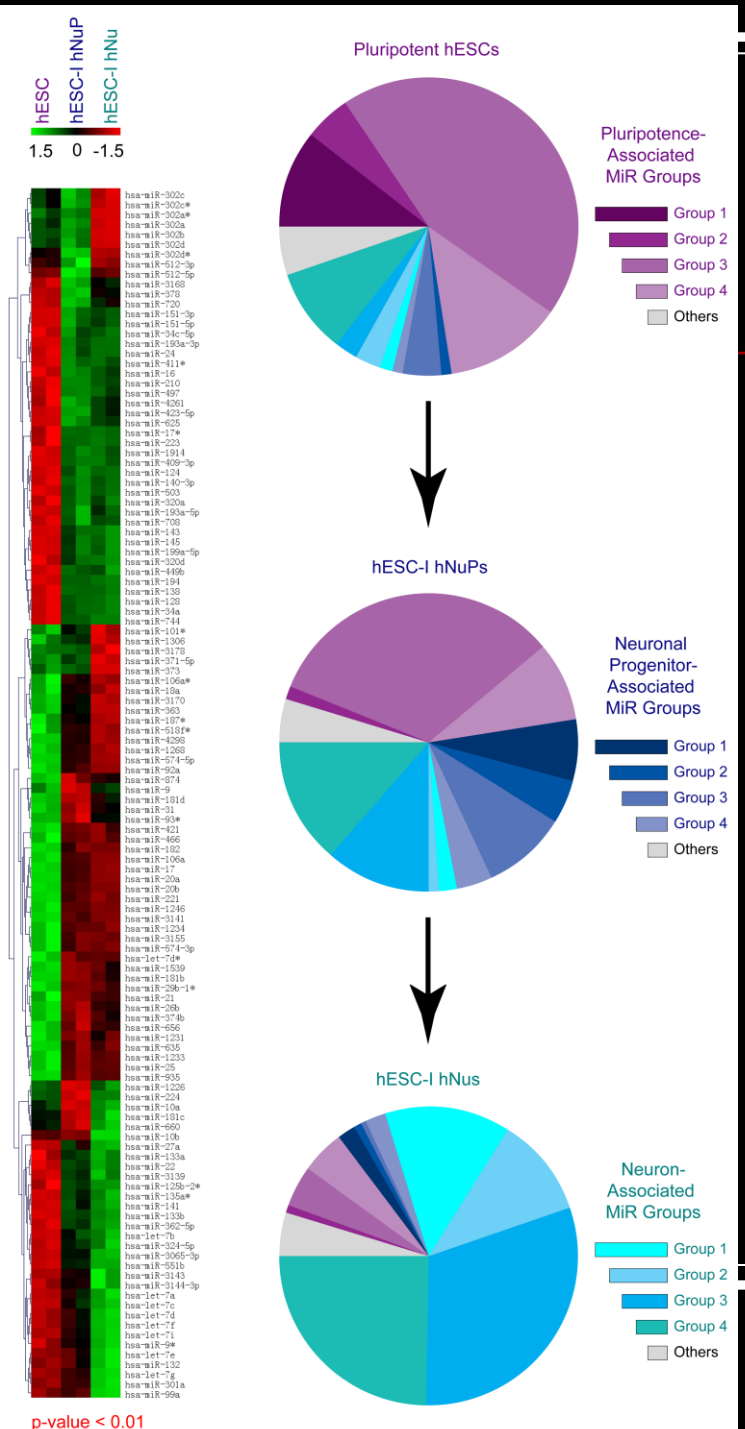
Distinct Sets of Stage-Specific Human Embryonic Neurogenic miRNAs, Many of Which were not Previously Linked to Neuronal Development and Function, Contribute to the Neuronal Identity of the Developing CNS

Genome-Scale Profiling of microRNA (miRNA) Differential Expression

let-7 family



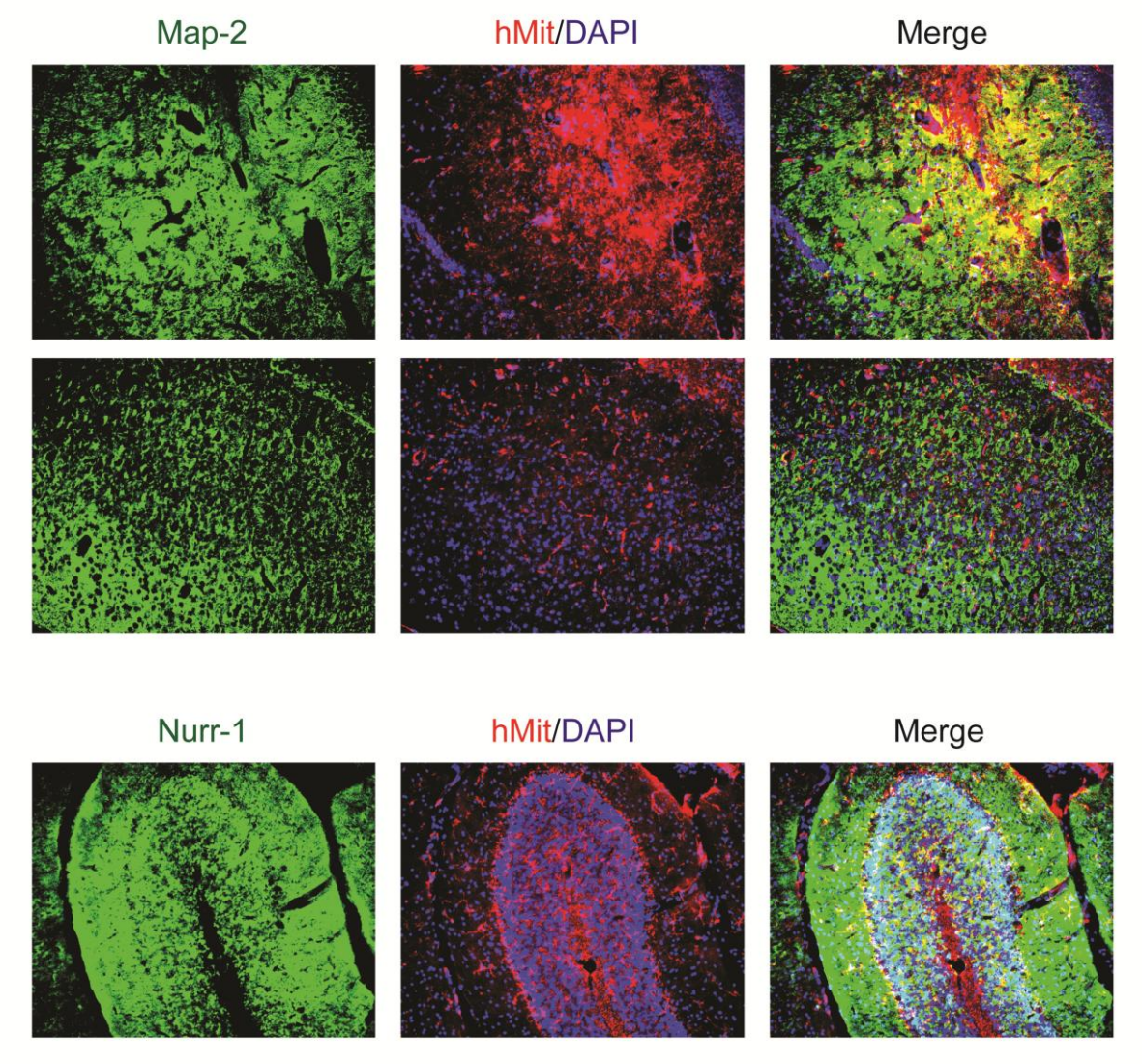
MicroRNA (MiR) Signatures of Human Neuronal Progenitor Cells (Xcel-hNuP or hESC-I hNuP) and Neuronal Cells (Xcel-hNu or hESC-I hNu) Derived from Human ES Cells by Small Molecule Induction



Pie charts showing decreased contribution of a set of **pluripotence-associated miRNAs (purple)** and increased contribution of distinct sets of **neuronal progenitor-associated miRNAs (blue)** and **neuron-associated miRNAs (cyan)** to the entire miRNA populations during human ES cell neuronal lineage-specific progression. Note that the expression of pluripotence-associated **hsa-miR-302 clusters (dark purple)** was silenced and the expression of **Hox miRNA hsa-miR-10 cluster (dark blue)** was induced to high levels in *in vitro* neuroectoderm-derived hESC-I hNuPs.

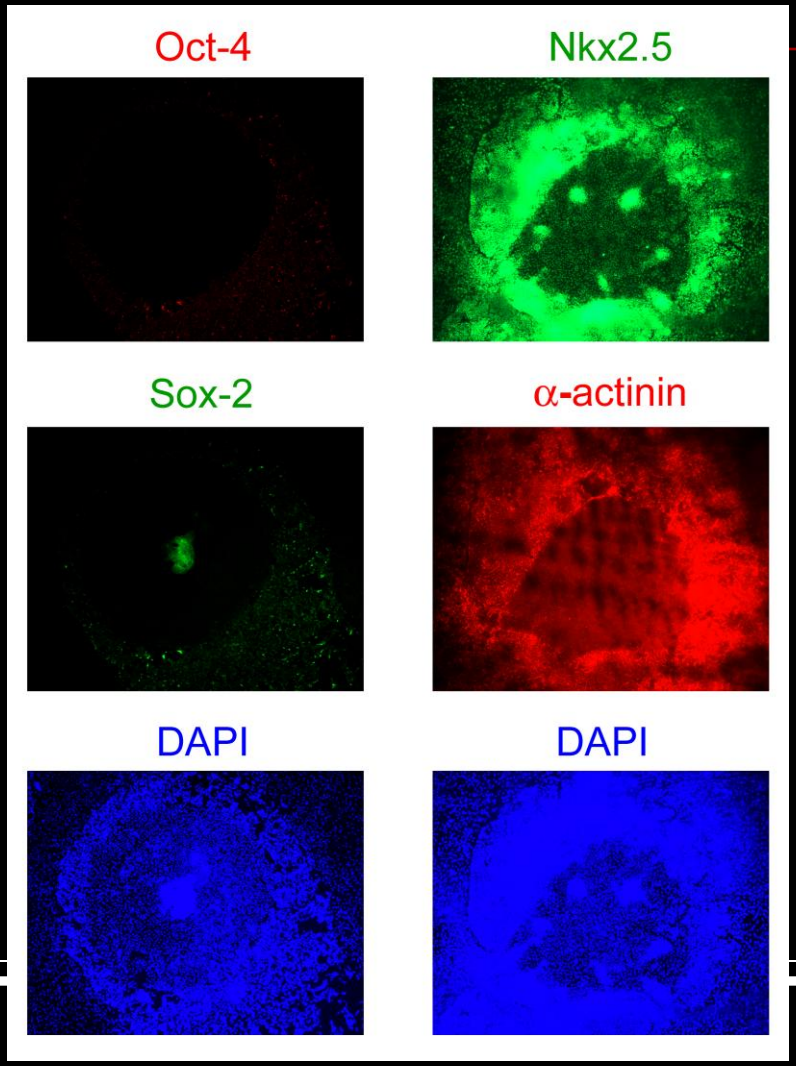
A unique set of human embryonic neurogenic miRNAs contributes to the neuronal identity of the developing CNS

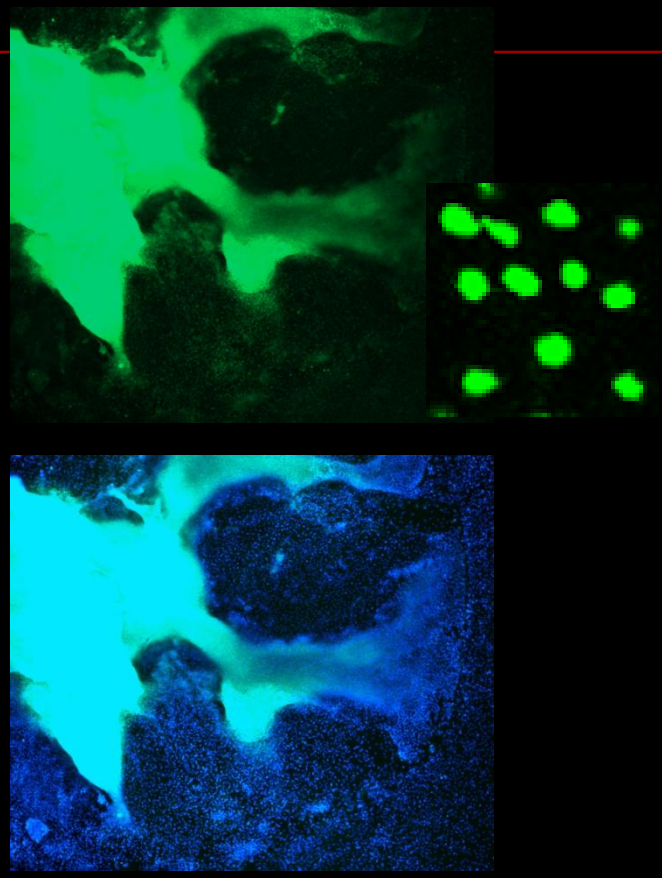
The Neuroectoderm-Derived Human Neuronal Progenitors Specified from Human ES Cells by Small Molecule Induction are Highly Neurogenic Following Transplantation into the Brain



Transplanted Mice Developed Hyper-Active Behavior, such as Fast Movement and Fast Spin

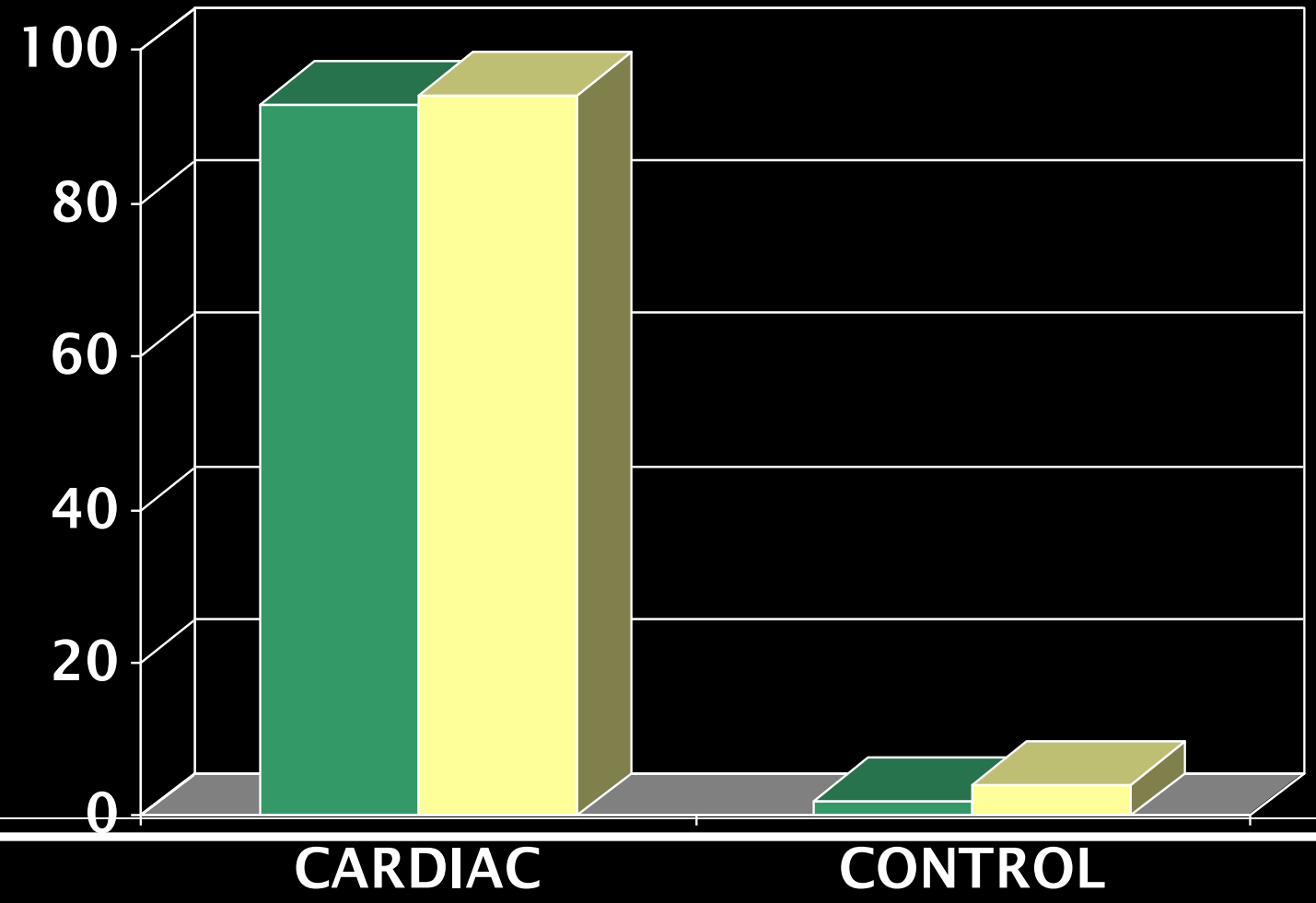
Nicotinamide (NAM) Induces Human ES Cells to Exit the Pluripotent Stage and the Appearance of Cardio-Mesoderm Phenotype by Promoting the Expression of Cardiac-Specific Transcription Factor Nkx2.5



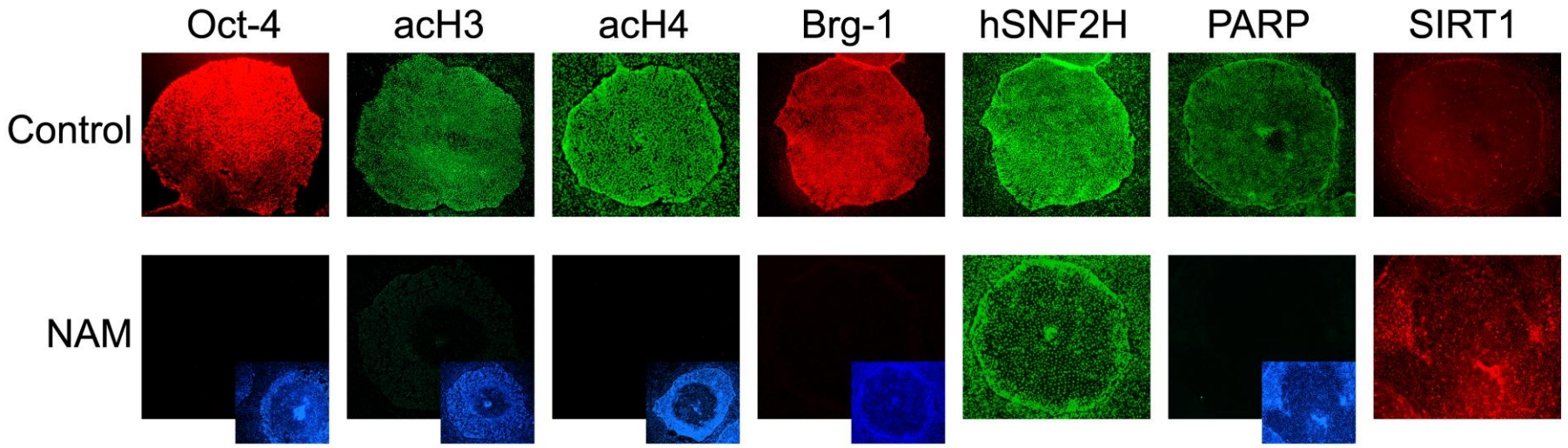


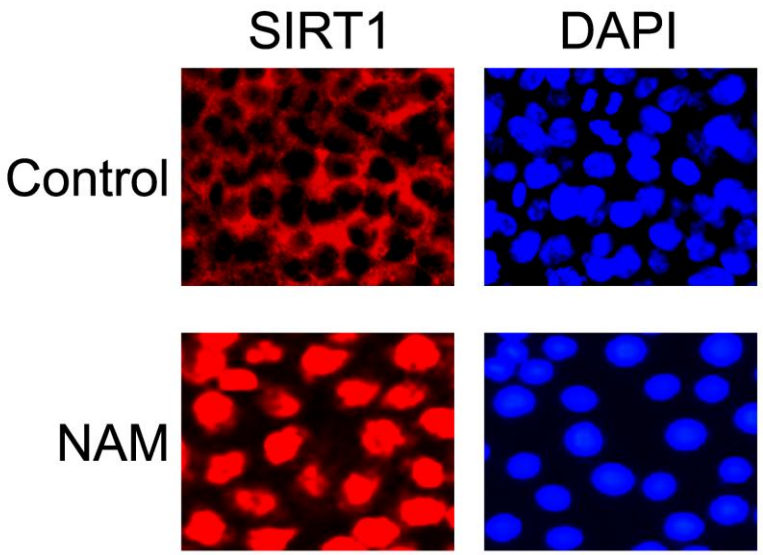
Nkx2.5: The earliest marker for heart precursor cells and indispensable for normal cardiac development

Comparison of Cardiac Differentiation Efficiencies of Pluripotent Human ES Cells by Small Molecule Induction (CARDIAC) with Conventional Multi-Lineage Cardiac Differentiation Approaches (CONTROL)

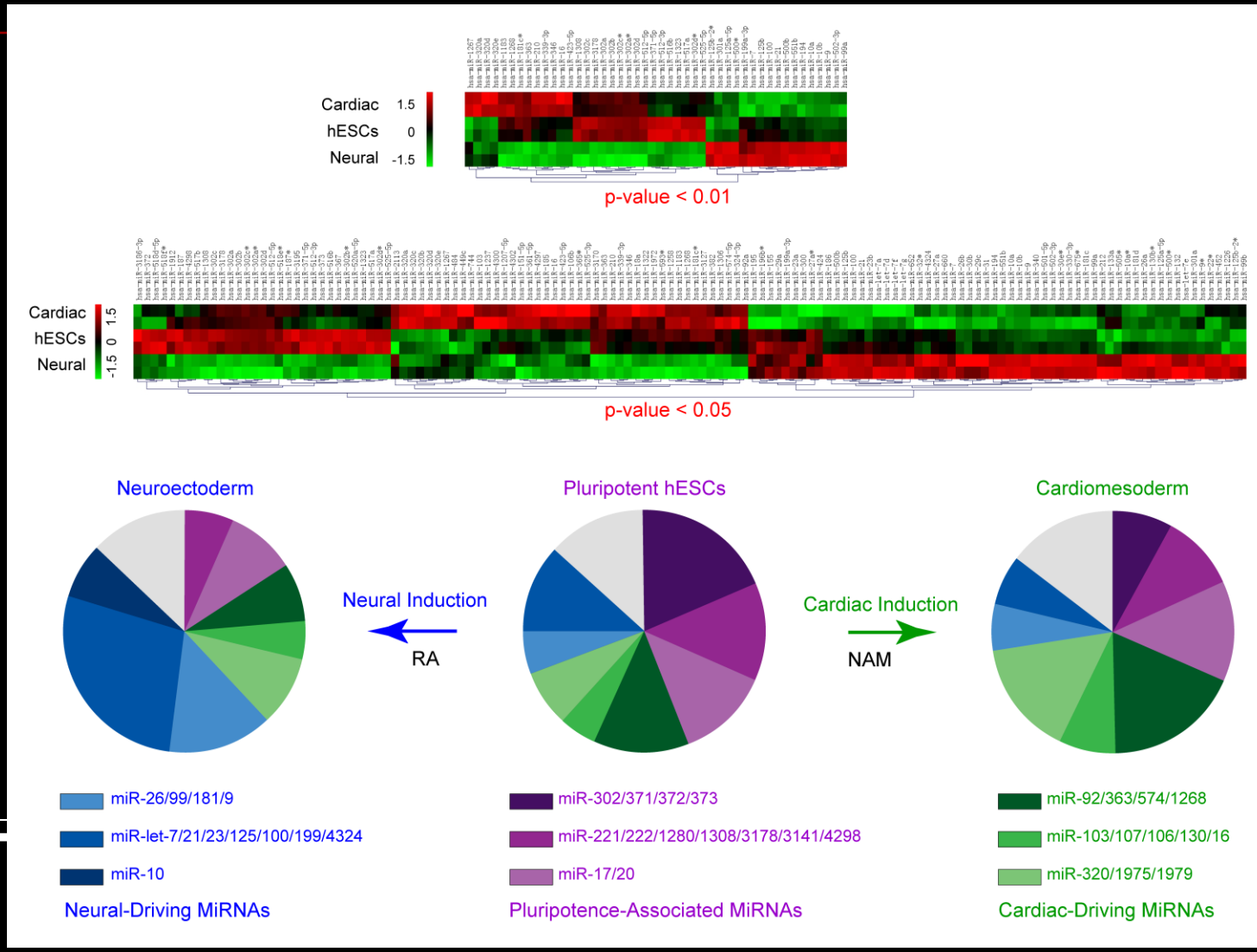


NAM Induces Nuclear Translocation of NAD-Dependent Histone Deacetylase SIRT1 and Global Chromatin Silencing

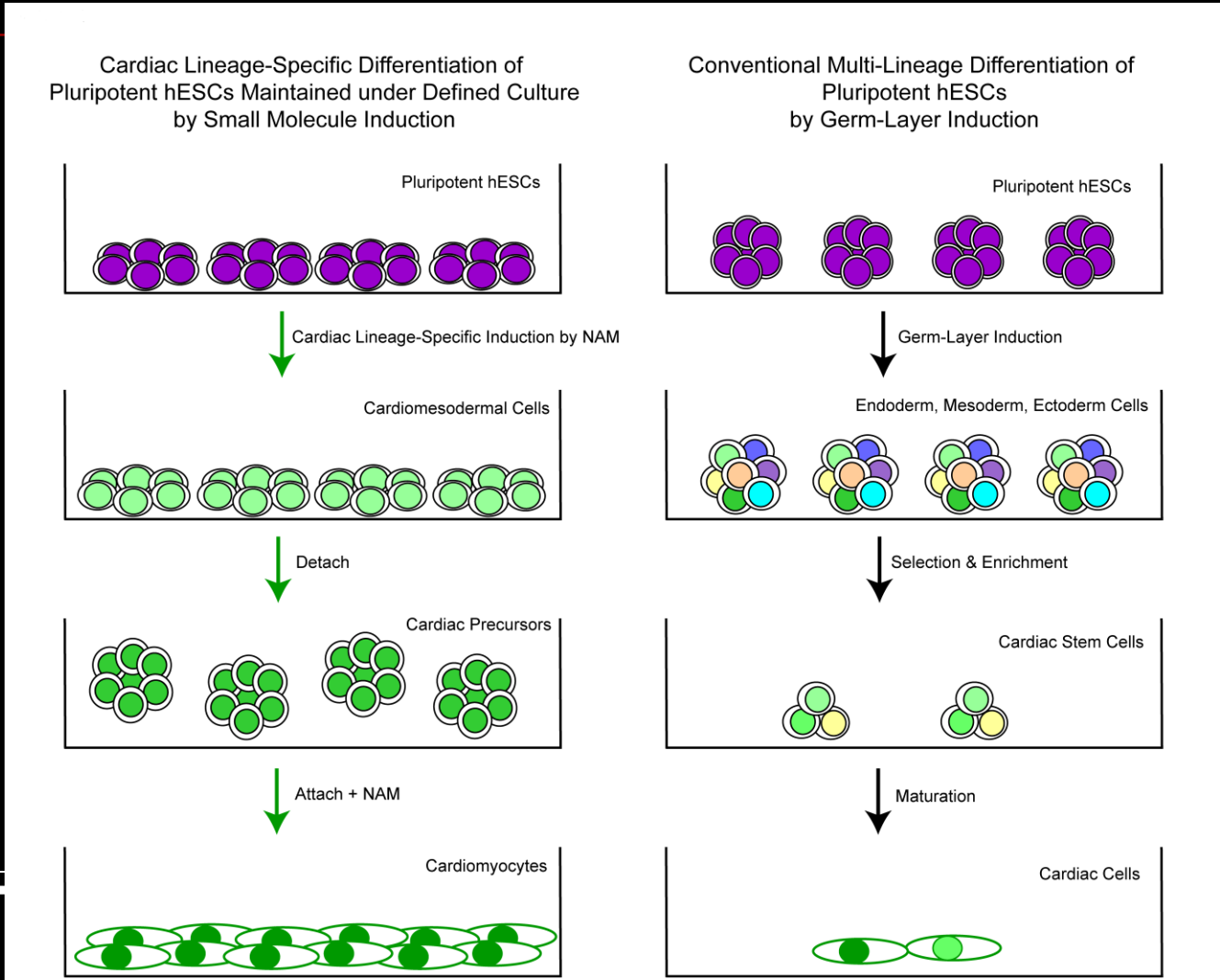




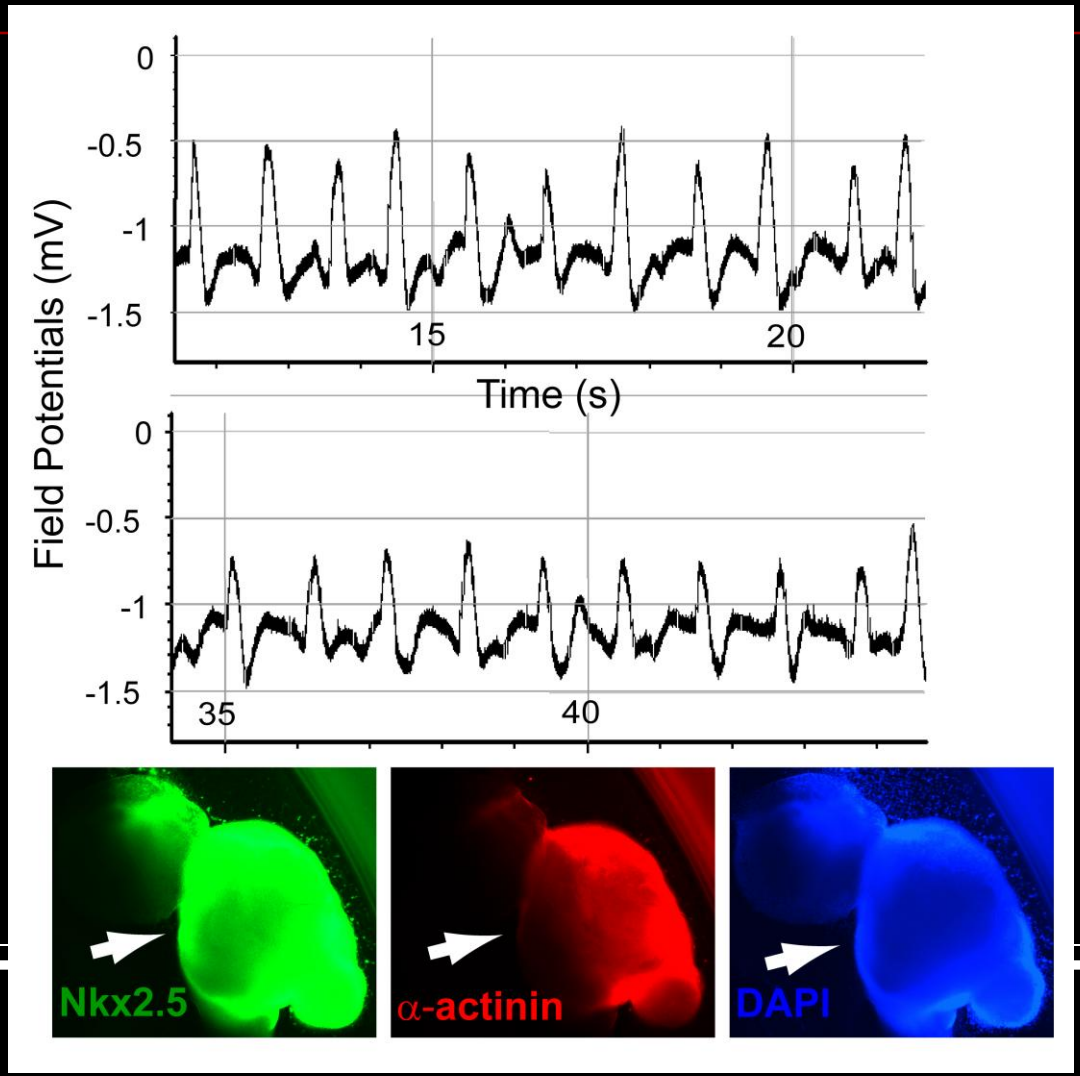
A Unique Set of Pluripotence-Associated miRNAs was Down-Regulated, While Novel Sets of Distinct Cardiac- and Neural-Driving miRNAs were Up-Regulated Upon the Induction of Lineage-Specification Direct from the Pluripotent State of Human ES Cells



Comparison of Cardiac Lineage-Specific Differentiation of Pluripotent Human ES Cells Exclusively to a Cardiomyocyte Fate by Small Molecule Induction without Going through a Multi-Lineage Embryoid Body (EB) Stage vs. Conventional Multi-Lineage Differentiation Approaches through Germ Layer Induction

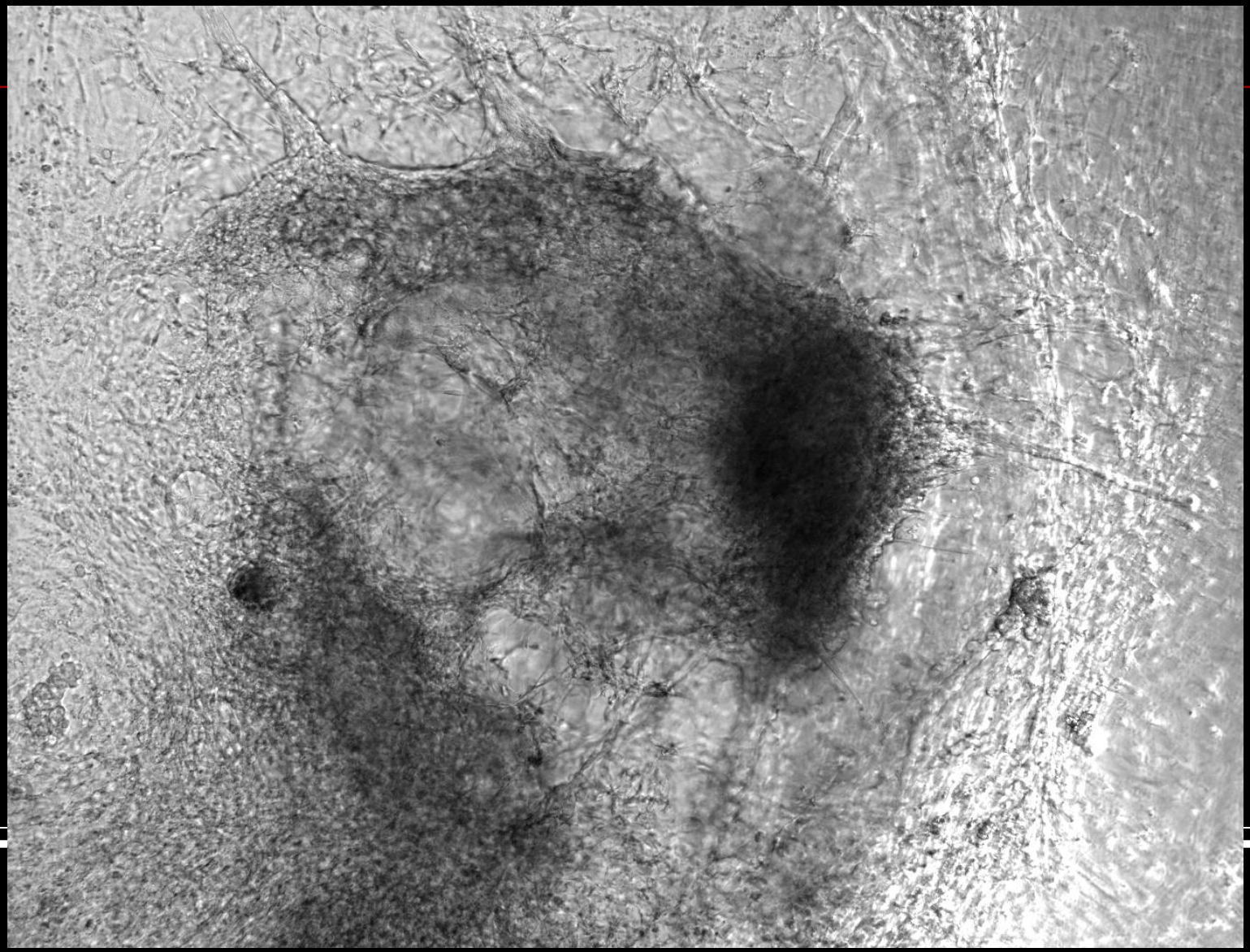


NAM-Induced Human ES Cells are Capable of Progression to Beating Cardiomyocytes with High Efficiency

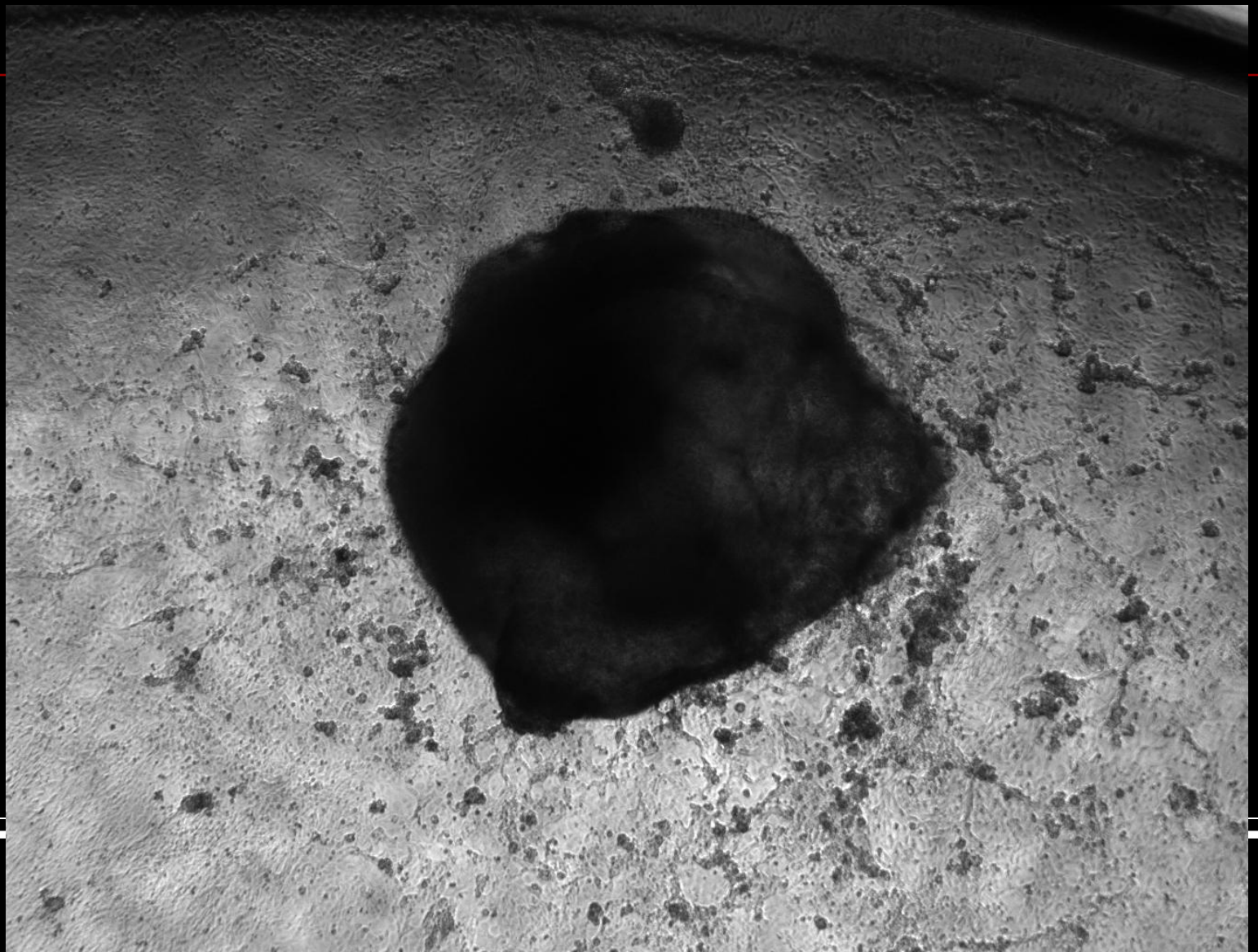


Beating Cardiomyocytes Show Rhythmic Impulses Reminiscent of the p-QRS-T-complexes Seen in Clinical Electrocardiograms and express markers characteristic of cardiomyocytes

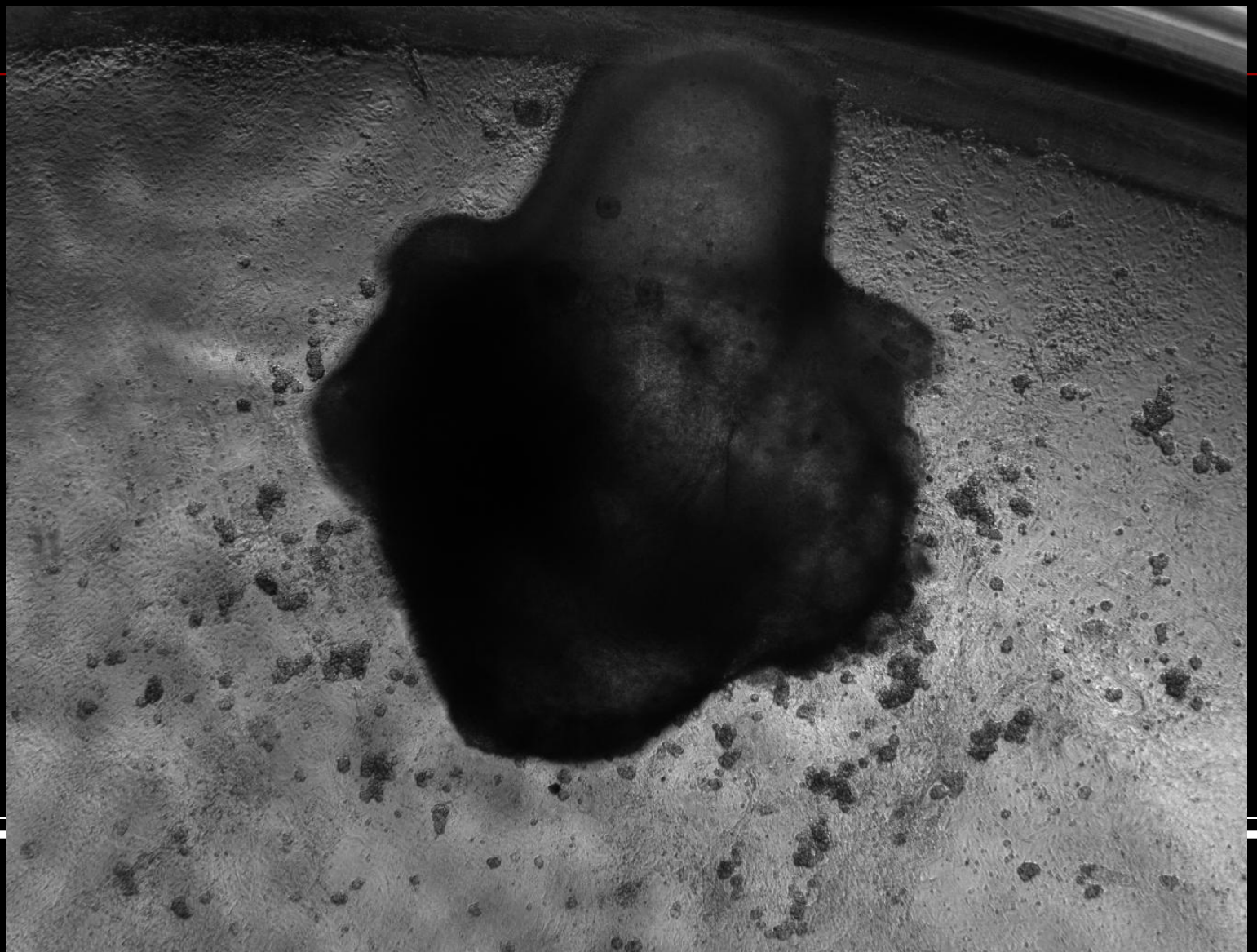
Beating Cardiomyocytes Show Rhythmic Impulses Reminiscent of the p-QRS-T-complexes Seen in Clinical Electrocardiograms



Beating Cardiomyocytes Show Rhythmic Impulses Reminiscent of the p-QRS-T-complexes Seen in Clinical Electrocardiograms



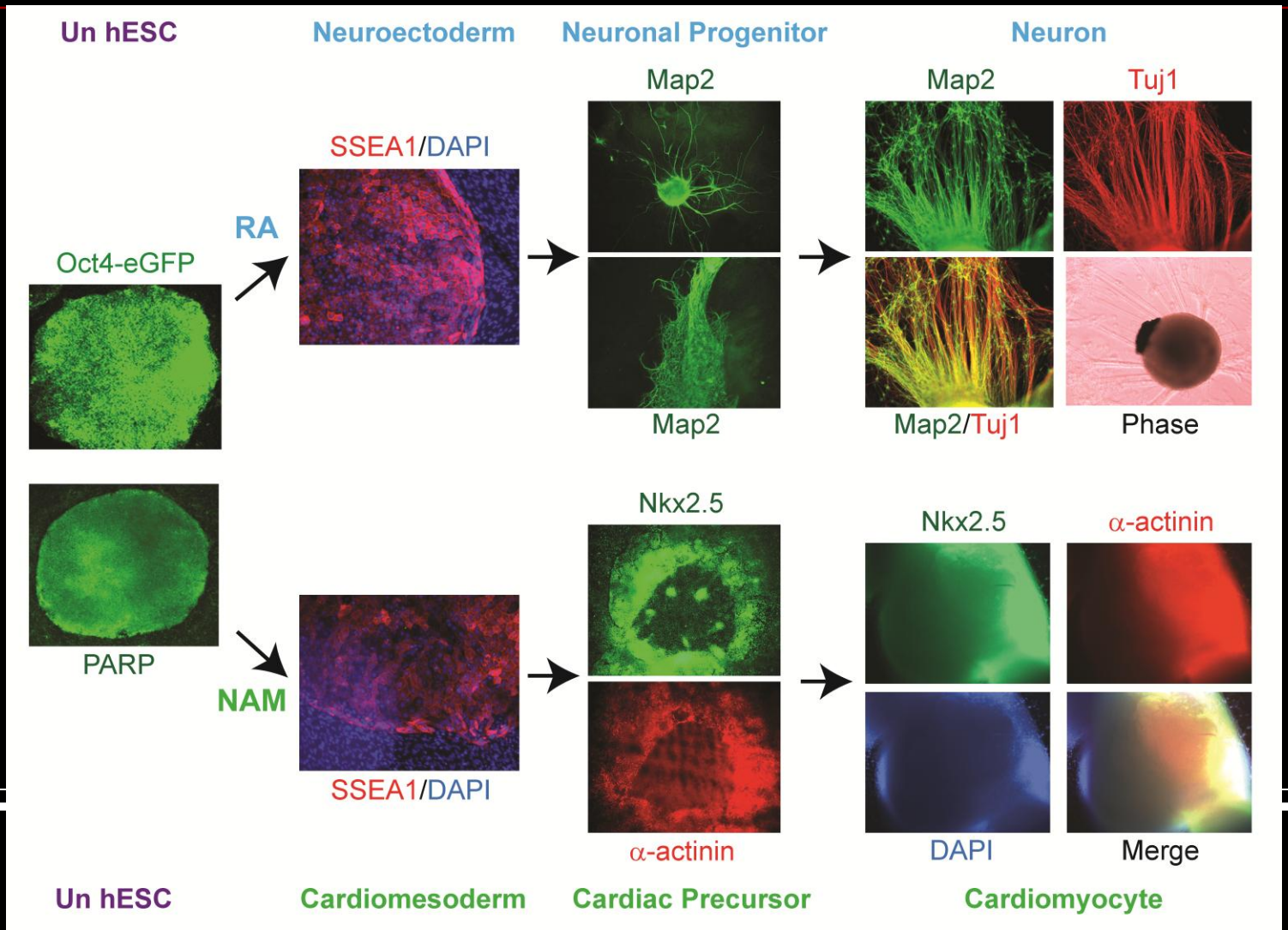
Beating Cardiomyocytes Show Rhythmic Impulses Reminiscent of the p-QRS-T-complexes Seen in Clinical Electrocardiograms



PluriXcel Novel Human Stem Cell Technology Platforms Enable Large Scale Production or Manufacture of High Quality Clinical-Grade Human Neuronal and Heart Muscle Cell Therapy Products as Cellular Medicines that can Offer Pharmacologic Utility and Capacity adequate for CNS and Heart Regeneration

- **PluriXcel-DCS: Defined culture systems for derivation and maintenance of clinical-grade high quality human ES cell lines. PluriXcel-DSC allows all poorly-characterized and unspecified biological components and substrates in the culture system, including those derived from animals, to be removed, substituted, and optimized with defined human alternatives for *de novo* derivation and long-term maintenance of cGMP-quality xeno-free stable human ES cell lines and their human cell therapy derivatives, which have never been contaminated with animal cells and proteins, thus, suitable for therapeutic development and clinical applications.**

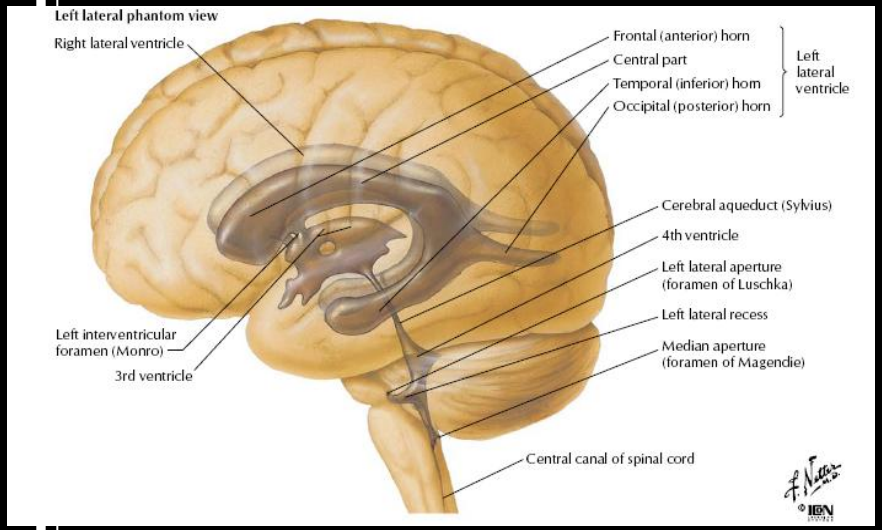
PluriXcel Human Stem Cell Technology -- a Major Milestone towards Clinical Application of Human ES Cell Therapy Derivatives, Offering the Benefits in Efficiency, Stability, Safety, Efficacy, and Large-Scale Production of High Quality Clinical-Grade Human Stem Cell Therapy Products for Commercial and Therapeutic Uses



The Designation of Human Stem Cell therapy Products for Human Trials or First-in-Human Studies

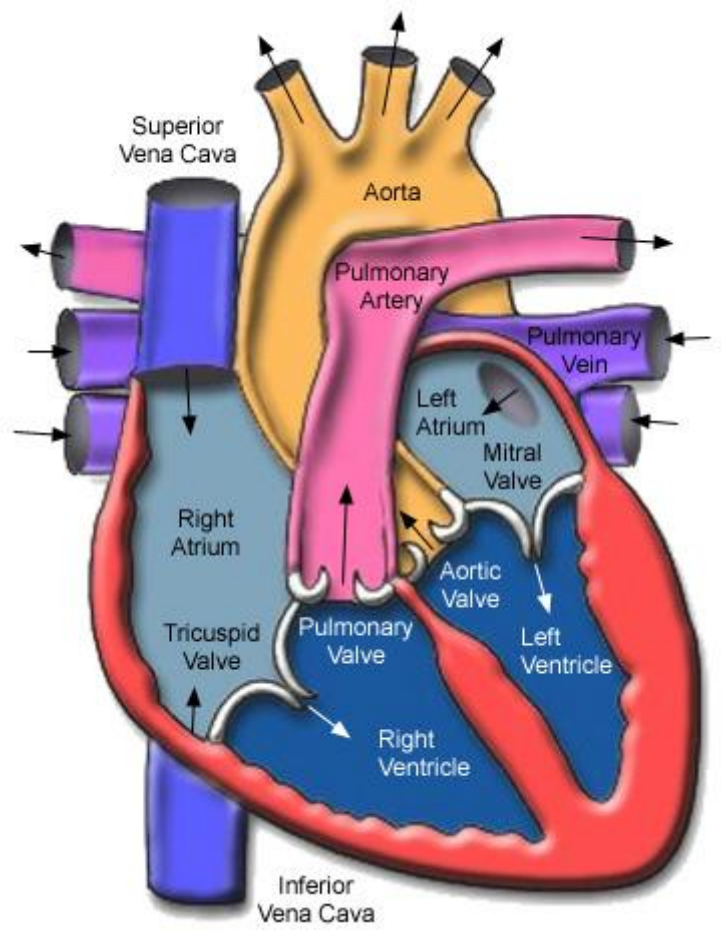
- Such human stem cells or their progenies or derivatives must be able to be manufactured in a commercial scale
- Such human stem cells and their progenies or derivatives must be able to retain their normality or stability for a long term
- Such human stem cells must be able to differentiate or generate a sufficient number of the specific cell type or types in need of repair or regeneration

Xcel Prototypes, Generated from Human ES Cells using our Novel PluriXcel Technology, Represent the Next Generation of Human Cell Therapy Products, Offering Purity, Large-Scale Production, High Quality, Safety, and Effectiveness for Commercial and Therapeutic Uses over all other Existing Cell Sources



- **Xcel-hNuP & Xcel-hNu: Clinical-grade high purity human neuronal progenitor cells and neuronal cells for CNS neuron regeneration. *Therapeutic Sector:* cellular medicine or cell therapy product for a wide range of neurological disorders, including Parkinsons disease, ALS, Spinal muscular atrophy, Alzheimer disease, motor neuron diseases, neurodegenerative diseases, stroke, brain and spinal cord injuries.**

Current State of Regenerative Medicine: Moving Stem Cell Research from Current Studies in Animals towards Human Trials



- **Xcel-hCardP & Xcel-hCM: Clinical-grade high purity human heart precursor cells and cardiomyocytes (heart muscle cells) for contractile heart muscle regeneration. *Therapeutic Sector:* cellular medicine or cell therapy product for cardiovascular disease, including heart disease and failure, Myocardial infarction, Cardiomyopathy, Ischemic heart disease, Congestive heart failure.**

Acknowledgement

- **Xcelthera, INC.**
- **San Diego Regenerative Medicine Institute**

- **NIH/NIA K01AG024496, PI**
- **NIH/NICHD R21HD056530, PI**