

Enriched Motor Neuron Populations Derived From Bacterial Artificial Chromosome–Transgenic Human Embryonic Stem Cells

Dimitris G. Placantonakis, M.D., Ph.D., Mark J. Tomishima, Ph.D., Fabien Lafaille, M.S., Sabrina C. Desbordes, Ph.D., Fan Jia, Ph.D., Nicholas D. Socci, Ph.D., Agnes Viale, Ph.D., Hyojin Lee, Ph.D., Neil Harrison, Ph.D., Lorenz Studer, M.D., and Viviane S. Tabar, M.D.

Human embryonic stem cells (hESCs)²¹ have received enormous attention because of their potential to produce somatic cells of all three germ layers, a property known as pluripotency. Recent advances in hESC biology have enabled the directed differentiation of hESCs into several types of neurons, including motor neurons,^{11,12,18} which are specifically lost in amyotrophic lateral sclerosis (ALS). However, in vitro differentiation protocols are flawed in that motor neuron populations are contaminated by hyperproliferative neural stem cells that can cause overgrowths in transplanted animals. The ability to obtain enriched populations of hESC-derived motor neurons is essential to their use in cell replacement therapy and to understanding the pathophysiology of ALS.

One approach to identifying and enriching hESC-derived motor neuron populations is the restricted expression of markers, such as green fluorescent protein (GFP), by motor neuron-specific promoters. Hb9 is a homeodomain transcription factor, which, within the central nervous system, is specifically expressed in spinal motor neurons.^{1,11,12,25} The *Hb9* promoter is, therefore, a good candidate for motor neuron-specific transgene expression. Here we describe a new technology that allows the transgenesis of hESCs via nucleofection^{9,17} with a bacterial artificial chromosome (BAC) expressing GFP under the control of the mouse *Hb9* promoter (*Hb9::GFP* BAC). BACs consist of large fragments of mammalian genomic DNA (150–250 kb), which contain genes along with their surrounding promoter and enhancer elements.⁸ A large library of engineered mouse BACs has been developed at Rockefeller University (GENSAT library). Such BACs contain the promoters and enhancers of specific genes whose coding sequences have been replaced by the GFP reporter gene (*Fig. 21.1A*). Thus, the GFP-expressing BACs may be used as transcriptional reporters. Because BACs typically contain many more promoter and enhancer

elements critical to the transcriptional regulation of a gene than conventional short promoter constructs, they confer a much higher fidelity of transgene expression. Moreover, BAC transgenesis does not require the precise mapping of promoter/enhancer sequences that is needed for the engineering of short promoter constructs. Thus, the use of GFP-expressing BACs as transcriptional reporters may be critical for accurate gene expression studies, disease model generation, and transplantation experiments.

METHODS

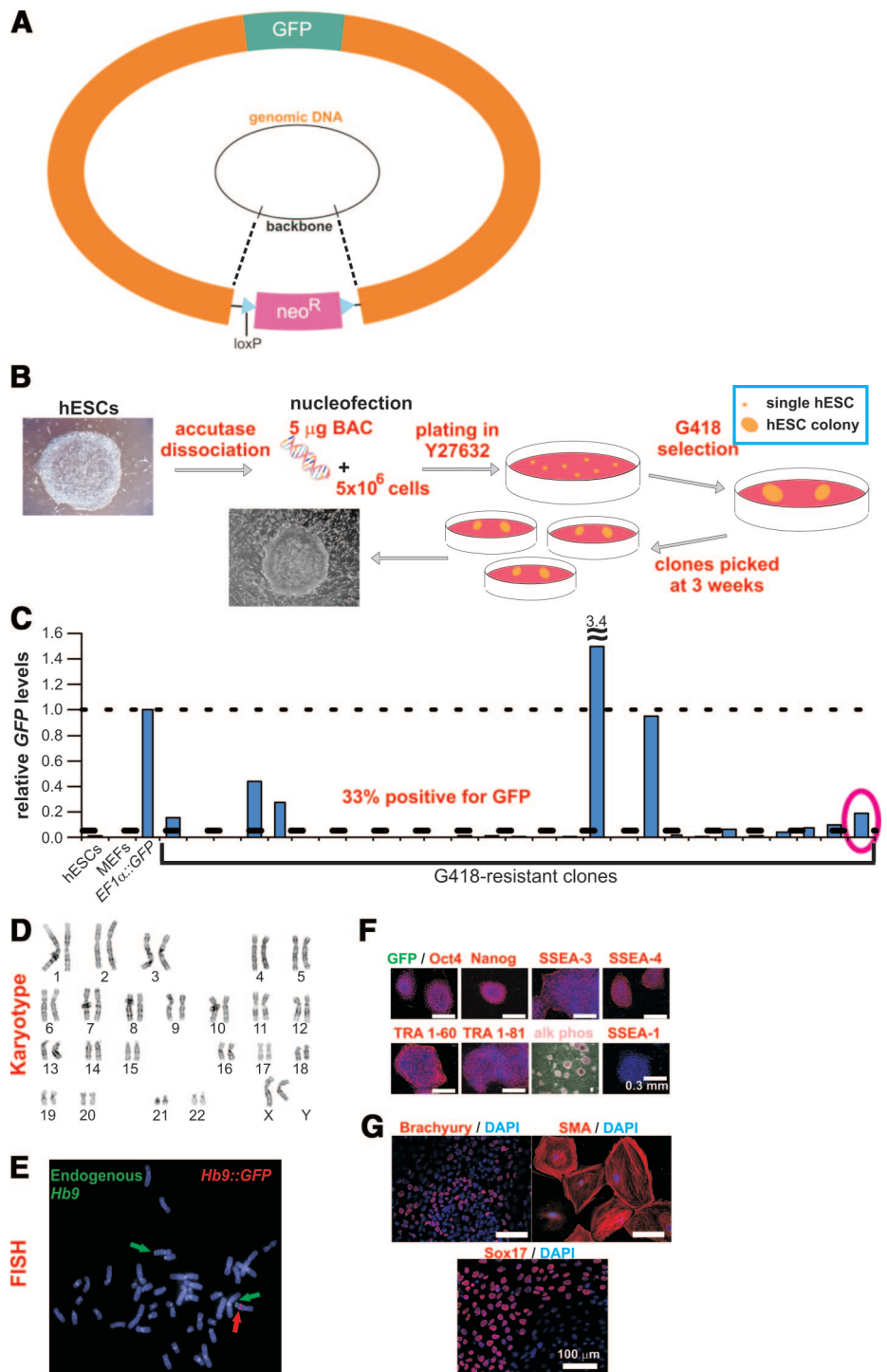
Human ESC Culture, Nucleofection, and BAC Engineering

The National Institutes of Health–approved WA-09 (H9) hESC line was used. hESCs were maintained on mitotically inactivated mouse embryonic fibroblasts (MEFs), as previously described.¹⁵ Accutase (Innovative Cell Technologies, San Diego, CA)-dissociated hESCs (5×10^6) were treated with 10 μ M Y27632 (Sigma-Aldrich, St. Louis, MO) to increase their clonogenic potential²⁴ and then were nucleofected^{9,17} (Amaxa) with 5 μ g of the mouse *Hb9::GFP* BAC. To select for stably transfected clones, G418 (Sigma-Aldrich) was started on day 4 at 25 μ g/mL followed by 50 μ g/mL on day 14. The mouse *Hb9::GFP* BAC was obtained from the GENSAT library at Rockefeller University and modified to express the neomycin resistance gene.²² Differentiation to mesoderm and endoderm were as previously described.²⁴

Human Motor Neuron Differentiation, Flow Cytometry, and Postsort Culture

Motor neurons were derived from hESCs as previously described.¹¹ Briefly, we obtained neural stem cells from hESCs through a process termed neural induction.^{6,11} Differentiation of such neural stem cells to the motor neuron lineage was achieved with C25II sonic hedgehog (Shh, 125 ng/mL; R&D Systems, Minneapolis, MN) and retinoic acid (1 μ M; Sigma-Aldrich), which act to ventralize and caudalize, respectively, neural stem

FIGURE 21.1. Basic characterization of bacterial artificial chromosome (BAC)–transgenic human embryonic stem cell (hESC) line. **A**, Schematic showing the basic architecture of engineered BACs. The genomic DNA (orange) contains the promoter/enhancer elements for a given gene, and the coding sequence has been replaced by the green fluorescent protein (GFP) transgene (green). These sequences are attached to a much smaller backbone that is engineered to express the neomycin resistance gene (*neo^R*) flanked by the *loxP* recombination sequences. **B**, Outline of the protocol for the stable transgenesis of hESCs with BACs. **C**, Summary of the quantitative polymerase chain reaction data for *GFP* using genomic DNA extracted from hESC clones transfected with the *Hb9::GFP* BAC. The data were normalized to the *GFP* content of an hESC line stably transfected with the *EF1 α ::GFP* plasmid. The clone that produced GFP-positive motor neurons is circled. Negative controls included untransfected hESCs and mouse embryonic fibroblasts (MEFs). **D** and **E**, Karyotype and fluorescent in situ hybridization (FISH) analysis of the BAC-transgenic clone. **E**, The BAC transgene is indicated by a red arrow, whereas the endogenous *Hb9* gene is indicated by green arrows. **F**, Immunofluorescence analysis of hESC marker expression in the undifferentiated transgenic hESCs. **G**, The transgenic hESCs maintain their pluripotency. When directed to mesodermal fates, the transgenic hESCs generated Brachyury and smooth muscle actin (SMA)–positive cells. Moreover, differentiation to endodermal precursors produced Sox17-positive cells. None of these cell types expressed GFP.



cells, thereby biasing them to the motor neuron fate. FACS analysis was performed on a FACSCalibur device (BD Biosciences, San Jose, CA), while either a MoFlo (Dako, Carpinteria, CA) or a FACSaria (BD Biosciences) device was used for cell sorting. After sorting, motor neurons were cocultured with

hESC-derived mesenchymal feeders² in medium containing glial cell line–derived neurotrophic factor (20 ng/mL), ciliary neurotrophic factor (50 ng/mL), brain-derived neurotrophic factor (20 ng/mL) (all obtained from R&D Systems), and ascorbic acid (0.2 mM; Sigma-Aldrich).

Immunofluorescence and Microscopy

Immunofluorescent analysis was performed either with a conventional epifluorescence microscope or with a confocal microscope (Leica Microsystems, Inc., Allendale, NJ). Cells were fixed with paraformaldehyde, blocked, and permeabilized with 1% bovine serum albumin/0.3% Triton X-100 and treated with antibodies to the following antigens: GFP (Abcam, Cambridge, MA), Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA), Nanog (R&D Systems), SSEA-3 (DSHB, Iowa City, IA), SSEA-4 (DSHB), SSEA-1 (DSHB), TRA 1-60 (Millipore, Bedford, MA), TRA 1-81 (Millipore), Pax6 (Covance, Princeton, NJ), Brachyury (R&D Systems), Sox17 (R&D Systems), smooth muscle actin (Sigma-Aldrich), Hb9 (DSHB), Isl1 (DSHB), Lim3 (DSHB), Nkx6.1 (DSHB), Nkx2.2 (DSHB), Tuj1 (Covance), nestin (Neuromics, Edina, MN), ChAT (Millipore), and Rap1A (Santa Cruz Biotechnology). Appropriate secondary antibodies were conjugated to the fluorophore Alexa 488, 546, 555, 568, or 633 (Invitrogen, Carlsbad, CA). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). In experiments aimed at identifying dividing cells, cultures were exposed to 5 $\mu\text{g}/\text{mL}$ BrdU (Sigma-Aldrich) for 16 hours, and on fixation, BrdU was detected with anti-BrdU antibody (Abcam). Alkaline phosphatase staining was performed using a commercially available kit (Sigma-Aldrich). Evaluation of the extent of colocalization of GFP with relevant markers was done on 0.15-mm² microscopic fields. A total of 1020 hESC-derived cells were counted.

Quantitative Polymerase Chain Reaction and Transcriptome Analysis

For quantitation of the *GFP* transgene in genomic DNA, purified DNA (Qiagen, Studio City, CA) was subjected to quantitative polymerase chain reaction using the primers 5'AA-GATCCGCCACAACATCGAG3' and 5'CGTTGGGGTCTT-TGCTCAGG3' and a SYBR green (Quanta Biosciences, Gaithersburg, MD)-based assay. A relative expression level of the *GFP* transgene at 5% of the levels observed in the *EF1 α ::GFP* line (a control hESC line stably transfected with a plasmid expressing GFP under the control of the *EF1 α* promoter) was defined as positive for the transgene. For quantitative reverse transcriptase polymerase chain reaction of the *Hb9*, *Isl1*, *Lim3*, and *ChAT* transcripts, we used the corresponding Taqman probes (Applied Biosystems, Norwalk, CT). Transcriptome analysis was performed on Affymetrix U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA) with duplicate GFP-positive and GFP-negative RNA samples. The transcriptomes of the two populations were compared with the transcriptome of hESC-derived neural stem cells⁶ using the LIMMA package.

Electrophysiological Analysis

Cells were recorded in current-clamp mode using patch pipettes at room temperature (20–22°C) using the Multi-

clamp 700B amplifier (Axon Instruments, Inc., Foster City, CA). Neurons were identified under Nomarski (differential interference contrast) optics. The membrane potential was manually adjusted to -60 mV. The extracellular solution contained 145 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES. The intracellular solution contained 135 mM potassium gluconate, 2 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM ethyleneglycol-bis-(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid, 2 mM Mg-adenosine triphosphate, and 0.4 mM Na-guanosine triphosphate. In some experiments, the fluorescent marker Alexa 594 (Invitrogen) was included in the intracellular solution to facilitate visualization of the recorded neuron. Current steps were applied for 500 ms to evoke action potentials. Tetrodotoxin (Sigma-Aldrich) was applied at a final concentration of 0.5 μM to block action potentials in some recordings. Data were analyzed with Clampfit (Axon Instruments).

Statistical Analysis

Student's *t* test was used for statistical comparisons. The cutoff for statistical significance was set at $P < 0.05$.

RESULTS

BAC Transfection Protocol

We previously showed that the GENSAT library of BACs⁸ can be used toward the stable transgenesis of mouse ESCs (mESCs) after retrofitting with a neomycin resistance cassette.^{22,23} The translation of BAC transgenesis from mESCs to hESCs has been hampered by the difficulty in transfecting the latter, especially given the large size of BACs (150–250 kb). Thus, when we attempted to transfect hESCs with BACs via electroporation²⁷ or lipofection,⁵ we did not obtain stably transfected clones.

We then tested a new strategy with a number of modifications for the stable transfection of hESCs with the *Hb9::GFP* BAC. Using nucleofection^{9,17} of Accutase-dissociated single hESCs treated with the rho-associated kinase inhibitor Y-27632 (10 μM)²⁴ to increase their clonogenic potential, we obtained BAC-transgenic hESC lines for the first time. An outline of our protocol is presented in *Figure 21.1B*.

A total of 28 clones were obtained after nucleofection with the *Hb9::GFP* BAC. After differentiation to the motor neuron fate and screening for GFP expression, only one clone (3.6%) produced GFP-labeled motor neurons. A secondary screen based on quantitative polymerase chain reaction of genomic DNA identified nine of 27 clones (33.3%) as positive for *GFP* (*Fig. 21.1C*), suggesting that only a fraction of the clones were transfected with the intact BAC.

Basic Characterization of BAC Transgenic hESC Line

The GFP-expressing clone had a normal karyotype (46, XX; Fig. 21.1D). Fluorescent in situ hybridization analysis indicated a single BAC integration site at 7q31, a site distinct from the endogenous *Hb9* locus at 7q36 (Fig. 21.1E), indicating that BAC integration into the genome did not occur via homologous recombination. Undifferentiated transgenic hESCs expressed appropriate nuclear and surface markers and were GFP negative (Fig. 21.1F). In particular, undifferentiated hESCs were positive for the transcription factors Oct4 and Nanog; the cell surface markers SSEA-3, SSEA-4, Tra 1-60, and Tra 1-81; and the cytoplasmic marker alkaline phosphatase, but negative for the cell surface marker SSEA-1. Moreover, the transgenic hESCs could be differentiated to cells belonging to mesodermal (Brachyury and smooth muscle actin-positive cells) and endodermal (Sox17-positive cells) lineages in vitro (Fig. 21.1G), suggesting that they remained pluripotent.

Differentiation of BAC Transgenic hESCs to the Motor Neuron Lineage

Using our published differentiation protocol,¹¹ we patterned GFP and Hb9-negative neural stem cells⁶ derived from transgenic hESCs with retinoic acid and Shh for 11 to 15 days (Fig. 21.2A). We first observed GFP-expressing cells after 8 days of patterning (Fig. 21.2B, top). After 14 days of patterning, the number of GFP-positive cells and the fluorescence intensity increased (Fig. 21.2B, bottom). No GFP or Hb9 expression was seen when cells were directed to a dopaminergic fate,¹⁵ suggesting that the expression of GFP was specific to motor neurons (data not shown). Fluorescence-activated cell sorter analysis indicated that the induction of GFP expression was dependent on Shh (Fig. 21.2C and D) ($0.2 \pm 0.2\%$ GFP-positive cells with no Shh versus $5.8 \pm 1.1\%$ with Shh; *t* test, $P < 0.01$), a finding consistent with the hypothesis that GFP was expressed in motor neurons, whose ventral identity is established by Shh. Confocal microscopic analysis of the GFP-positive cells showed $66.1 \pm 3.0\%$ colocalization with endogenous Hb9 immunoreactivity (Fig. 21.2E). The transcription factor profile of the GFP-positive motor neurons was developmentally appropriate, as $85.5 \pm 5.7\%$ colocalized with *Isl1*, $80.8 \pm 2.4\%$ with *Lim3*, and $46.1 \pm 13.2\%$ with *Nkx6.1* (Fig. 21.2F), all transcription factors involved in the specification of motor neurons. Moreover, GFP showed little colocalization with *Nkx2.2* ($1.7 \pm 1.0\%$), a marker of a ventral spinal interneuron domain¹⁶ (Fig. 21.2F). The GFP-positive motor neurons expressed neuronal β -tubulin (Tuj1), a neuronal marker, but not Oct4, a marker of hESCs, or nestin, a neural stem cell marker (Fig. 21.2F). Likewise, GFP-positive cells did not stain with BrdU ($2.8 \pm 1.1\%$), indicating that they were postmitotic (Fig. 21.2F).

Enrichment for Motor Neurons via Cell Sorting

The GFP-positive motor neuron pool could be separated from GFP-negative cells by flow cytometry with yields averaging $2.6 \pm 0.7\%$ ($n = 6$; Fig. 21.3A). The enriched motor neuron pool was compared with the GFP-negative pool by quantitative reverse transcriptase polymerase chain reaction for the *Hb9*, *Isl1*, *Lim3* and *ChAT* (choline acetyltransferase, the enzyme responsible for acetylcholine biosynthesis in motor neurons) transcripts. We found robust enrichment (31.9 ± 19.5 -fold, $n = 3$) for *Hb9* in the GFP-positive pool compared with its GFP-negative counterpart, suggesting coordinate transcriptional regulation of the endogenous *Hb9* gene and the GFP transgene (Fig. 21.3B). Transcripts for *Isl1*, *Lim3* and *ChAT* were also enriched in the motor neuron pool by 4.3-, 20.8-, and 10.7-fold ($n = 2$ for each), respectively (Fig. 21.3B).

Fluorescence microscopy of living cells immediately after sorting showed an excellent separation of GFP-positive and -negative cells (Fig. 21.3C). The sorted motor neurons could be cultured long term on a layer of hESC-derived mesenchymal feeders.² Immunofluorescence analysis of such cocultures demonstrated *ChAT* expression in motor neurons (Fig. 21.3D) and the elaboration of dendritic and axonal processes, including putative growth cones. In contrast, the GFP-negative pool contained hyperproliferative nestin-positive neural stem cells, but few *ChAT*-expressing neurons.

Electrophysiological Analysis of Human Motor Neurons

Fluorescence-activated cell sorter-purified motor neuron populations cocultured on human mesenchymal feeders for 1 week were subjected to electrophysiological analysis using whole-cell patch recordings. Motor neurons were targeted using Nomarski (differential interference contrast) optics and filled with Alexa 594 through the recording electrode for easy visualization (Fig. 21.3E). When the membrane potential was held at -60 mV, neurons responded to 500-ms depolarizing current pulses with action potential (AP) firing (Fig. 21.3F). In 10 of 16 recorded neurons, trains of APs were evoked whose frequency increased with the amplitude of the current pulse (Fig. 21.3F). The AP trains were characterized by tonic firing without any obvious accommodation and were blocked by $0.5 \mu\text{M}$ tetrodotoxin ($n = 4$; Fig. 21.3G), indicating that they were sodium current dependent. There were no rebound calcium spikes elicited after hyperpolarization (data not shown). In summary, these recordings indicated that the transgenic human motor neurons are capable of tonic AP firing in vitro.

Analysis of the Human Motor Neuron Transcriptome

We then analyzed the motor neuron transcriptome using microarray technology (Affymetrix U133 Plus 2.0). We

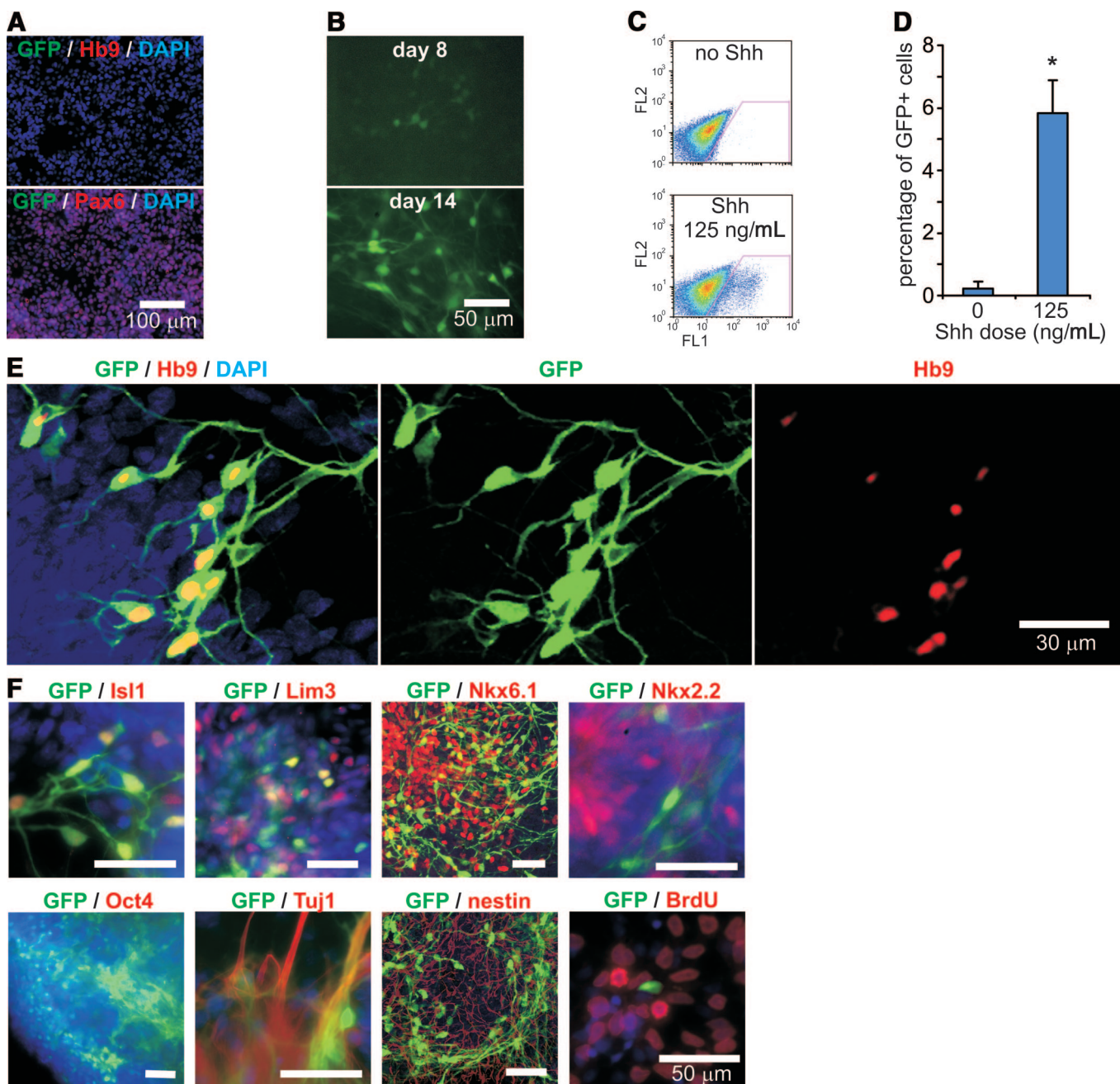


FIGURE 21.2. Motor-neuron specific expression of green fluorescent protein (GFP) in the transgenic human embryonic stem cell (hESC) line. **A**, Neural stem cells derived from transgenic hESCs were used for generating motor neurons after treatment with retinoic acid and Shh. Such neural stem cells were negative for GFP and Hb9, but positive for Pax6, a transcription factor expressed in neural precursors. **B**, After 8 days of treatment with retinoic acid and Shh, we observed GFP-positive neurons in the cultures. By day 14, the number of GFP-positive cells had increased substantially. **C** and **D**, Fluorescence-activated cell sorter analysis indicated that the production of GFP-positive cells required Shh. **E**, Confocal microscopic images showed extensive colocalization between the GFP signal and the endogenous Hb9 immunoreactivity, suggesting that the bacterial artificial chromosome BAC transgene functioned appropriately. **F**, Immunofluorescence analysis of the GFP-positive motor neurons demonstrated colocalization of GFP with the transcription factors Isl1, Lim3 and Nkx6.1, but not Nkx2.2 or Oct4. Moreover, the GFP-positive cells were positive for neuronal β -tubulin (Tuj1), but not nestin or BrdU.

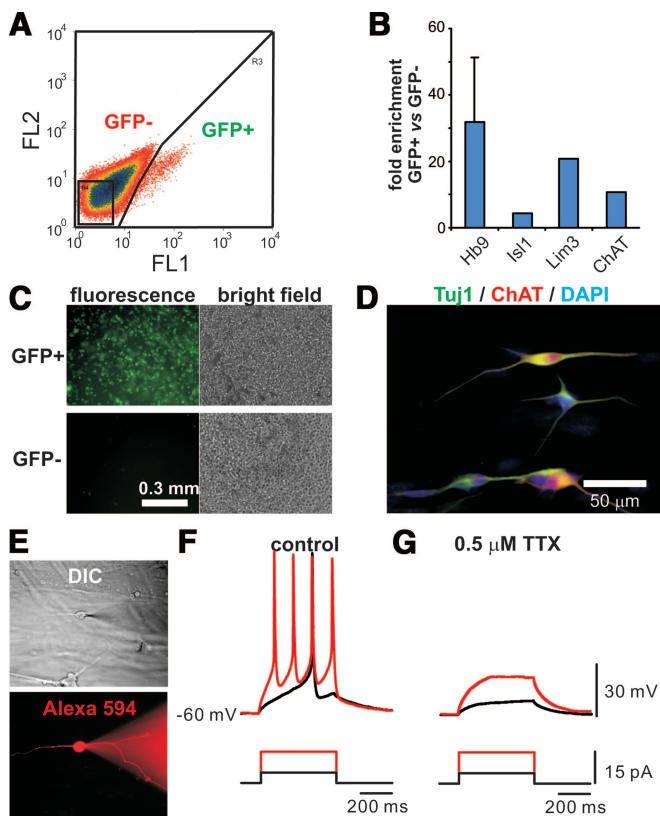


FIGURE 21.3. A, Cell sorting of green fluorescent protein (GFP)-positive and -negative cell populations. B, Quantitative reverse transcriptase polymerase chain reaction analysis of RNA extracted from the GFP-positive and negative pools indicated robust enrichment of the human *Hb9* transcript in the GFP-positive cells, again suggesting coordinate regulation of the *GFP* transgene and the endogenous *Hb9* gene. The motor neuron markers *Isl1*, *Lim3*, and *ChAT* were also enriched in the GFP-positive pool. C, Plating of cells immediately after cell sorting and fluorescence microscopy showed good separation of the GFP-positive and -negative pools. D, Motor neurons cultured on human embryonic stem cell-derived mesenchymal feeders for 1 week after cell sorting expressed ChAT and β -tubulin (Tuj1). E, Motor neurons were visualized with Nomarski optics (top) and filled through the patch electrode with Alexa 594 for confirmation of their neuronal character (bottom) before electrophysiological recordings. DIC, differential interference contrast. F and G, Motor neurons responded to current injection with tonic action potential firing that was blocked by tetrodotoxin (TTX).

identified 78 transcripts (three duplicates) specifically up-regulated in the GFP-positive population compared with neural stem cells,⁶ of which 49 (62.8%) were enriched more than twofold when compared with the GFP-negative cells (Fig. 21.4A and B). Importantly, the *Hb9* transcript (green in Fig. 21.4B) was 16.7-fold enriched compared with the GFP-negative pool. Other notable genes up-regulated in motor neurons whose function is consistent with motor neuron

biology were the transcription factors *Isl2*²⁰ and *Lim4*,¹³ *Grin2A* (*N*-methyl-D-aspartate receptor subunit 2A), *Ret* (glial-derived neurotrophic factor receptor),¹⁴ and *Slc5A7* (choline transporter).⁷ The majority of the 49 genes have not been implicated before in motor neuron biology. To validate the results of the transcriptome analysis, we performed immunofluorescence analysis of GFP-positive motor neurons with an antibody against Rap1A, a Ras-like guanosine triphosphatase, previously shown to modulate synaptic function.²⁶ The experiment showed enrichment of Rap1A immunoreactivity in motor neuron neurites (Fig. 21.4C), consistent with the finding that the *Rap1A* transcript is 60.4-fold enriched in motor neurons. This experiment allowed us to analyze the transcriptional signature of human motor neurons for the first time.

We are currently working on analyzing the electrophysiological behavior of human motor neurons and establishing an ALS disease model by overexpressing mutant *SOD1*, an established genetic cause of inherited ALS. Moreover, we are examining the engraftment potential of human motor neurons in healthy and mutant *SOD1*-transgenic rats.¹⁰

DISCUSSION

The field of regenerative medicine has witnessed an unprecedented renaissance over the past decade since the establishment of the first hESC lines.²¹ The excitement surrounding their use stems from two fundamental properties of hESCs. First, they can be cultured for prolonged periods of time while remaining in an undifferentiated state. Second, they possess a theoretically unlimited potential to differentiate into every cell type in the body under the right conditions, a property known as pluripotency. Harnessing the power of hESCs will enrich our understanding of human biology and pathophysiology and lead to novel treatments of human disease.

One of the major endeavors in the hESC field has been the development of protocols that allow their directed differentiation to specific cell types, such as neurons. However, such protocols are prone to contamination by undesirable cell types, including hyperproliferative stem cells, which may cause overgrowths when transplanted into experimental animals.^{3,19} Our study aims at eliminating this contamination problem by providing a means for enriching differentiation protocols for specific cell types using developmentally regulated cell type-specific promoters in BACs to drive GFP expression. Future cell replacement therapies will require purified cell populations devoid of contaminants that may cause adverse effects in vivo.

This study is the first to demonstrate the use of BAC transgenesis in hESCs as a tool to molecularly and functionally define a human neural lineage. BAC transgenesis has been routinely used for the generation of transgenic mice. An extensive library of BAC-transgenic mice with reporters relevant to neural development has been established at Rock-

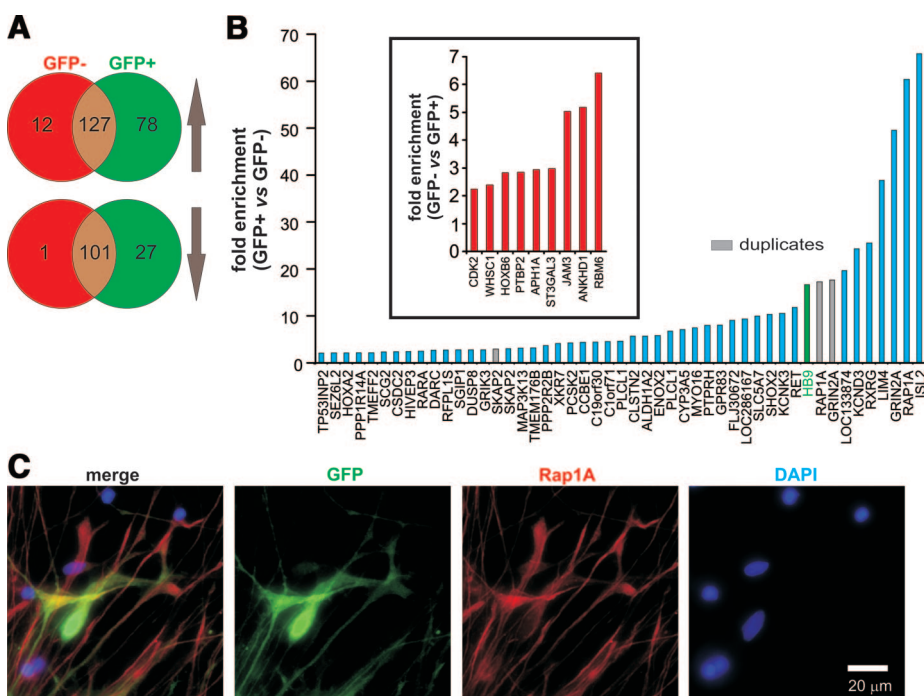


FIGURE 21.4. Microarray analysis of the human motor neuron transcriptome. **A**, The transcriptome of the green fluorescent protein (GFP)-positive and -negative pools was compared with the transcriptome of neural stem cells,¹² to help uncover genes that were developmentally regulated. The number of genes that were differentially expressed is shown, with the arrows indicating whether they were up-regulated or down-regulated in the GFP-positive and -negative pools. **B**, When the GFP-positive and -negative cell populations were compared, 49 transcripts were specifically up-regulated in motor neurons, whereas 9 were down-regulated (inset). **C**, Rap1A immunoreactivity was enriched in the neurites of GFP-positive motor neurons.

efeller University (<http://www.gensat.org>). The technology presented here should enable the establishment of large-scale hESC-based BAC transgenic reporter libraries as a novel genetic model system of human development.

Spinal motor neurons are lost in several neurodegenerative diseases, including ALS and spinal muscular atrophy. The *Hb9::GFP* hESC reporter line may facilitate studies on disease-related genes in purified human motor neurons. One of our ongoing efforts is the introduction of mutant *SOD1* transgenes into the *Hb9::GFP* BAC-transgenic hESC line in an effort to establish a human disease model for ALS. Such a model would allow us to characterize transcriptional, cellular, and biochemical changes in diseased human motor neurons and to test novel treatments. In particular, our recent implementation of high-throughput screening of drug libraries in hESCs⁴ represents an attractive technology for the testing of the effects of novel compounds on human motor neuron biology.

Our experiments demonstrated that enriched motor neuron populations can survive in vitro for prolonged periods of time. We are currently testing the engraftment potential of enriched human motor neuron populations in the rodent spinal cord. Importantly, we are pursuing such experiments with both normal rats, as well as rats transgenic for mutant *SOD1*,¹⁰ which represent an animal model for ALS. As mentioned above, our ability to obtain enriched human motor neuron populations is critical in performing such experiments.

CONCLUSIONS

We have developed a novel technology that allows the stable transfection of hESCs with the *Hb9::GFP* BAC, which drives GFP expression specifically in motor neurons. The use of BACs theoretically confers a much higher fidelity of transgene expression compared with short promoter constructs. This technology represents the first step toward the easy identification of motor neurons and their enrichment by flow cytometry. Our strategy allowed us to examine the electrophysiological profile and transcriptome of human motor neurons for the first time. In addition, the enrichment of motor neuron populations is a prerequisite for transplantation studies because of the elimination of contaminant hyperproliferative neural stem cells that may cause overgrowths in vivo.

Disclosure

The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

Acknowledgments

This work was supported by Project ALS (LS, VT), Starr Foundation (LS, VT), ALS Association of America (LS, VT), and an NREF grant from the American Association of Neurological Surgeons (DGP). We thank the GENSAT library at Rockefeller University for providing the *Hb9::GFP* BAC. We are grateful to Jan Hendriks, Patrick Anderson, and Diane Domingo of the Flow Cytometry facility at Memorial

Sloan-Kettering Cancer Center (MSKCC) for performing cell sorting and fluorescence-activated cell sorter analysis, Margaret Leversha of the Molecular Cytogenetics facility at MSKCC for performing karyotyping and fluorescent in situ hybridization analysis of the transgenic hESCs, Katia Manova and Tao Tong of the Molecular Cytology facility at MSKCC for confocal microscopic analysis, and Jeffrey Zhao of the Genomics Core Laboratory at MSKCC for technical help in microarray data analysis. We also thank George Al Shamy, Chris Fasano, Inna Lipchina, Yechiel Elkabetz, Gab-sang Lee, Yosif Ganat, and Asif Maroof for technical assistance and helpful discussions.

REFERENCES

- Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S: Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* 23:659–674, 1999.
- Barberi T, Bradbury M, Dincer Z, Panagiotakos G, Socci ND, Studer L: Derivation of engraftable skeletal myoblasts from human embryonic stem cells. *Nat Med* 13:642–648, 2007.
- Bjorklund LM, Sánchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O: Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 99:2344–2349, 2002.
- Desbordes SC, Placantonakis DG, Ciro A, Socci ND, Lee G, Djaballah H, Studer L: High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. *Cell Stem Cell* 2:602–612, 2008.
- Eiges R, Schuldiner M, Drukker M, Yanuka O, Itskovitz-Eldor J, Benvenisty N: Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. *Curr Biol* 11:514–518, 2001.
- Elkabetz Y, Panagiotakos G, Al Shamy G, Socci ND, Tabar V, Studer L: Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes Dev* 22:152–165, 2008.
- Ferguson SM, Bazalakova M, Savchenko V, Tapia JC, Wright J, Blakely RD: Lethal impairment of cholinergic neurotransmission in hemicholinium-3-sensitive choline transporter knockout mice. *Proc Natl Acad Sci U S A* 101:8762–8767, 2004.
- Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N: A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425:917–925, 2003.
- Hohenstein KA, Pyle AD, Chern JY, Lock LF, Donovan PJ: Nucleofection mediates high-efficiency stable gene knockdown and transgene expression in human embryonic stem cells. *Stem Cells* 26:1436–1443, 2008.
- Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD: Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci U S A* 99:1604–1609, 2002.
- Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrision NL, Panagiotakos G, Socci ND, Tabar V, Studer L: Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. *Stem Cells* 25:1931–1939, 2007.
- Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC: Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23:215–221, 2005.
- Liu Y, Fan M, Yu S, Zhou Y, Wang J, Yuan J, Qiang B: cDNA cloning, chromosomal localization and expression pattern analysis of human LIM-homeobox gene LHX4. *Brain Res* 928:147–155, 2002.
- Oppenheim RW, Houenou LJ, Parsadian AS, Prevet D, Snider WD, Shen L: Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: Regulation of programmed cell death among motoneuron subtypes. *J Neurosci* 20:5001–5011, 2000.
- Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L: Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* 101:12543–12548, 2004.
- Shirasaki R, Pfaff SL: Transcriptional codes and the control of neuronal identity. *Annu Rev Neurosci* 25:251–281, 2002.
- Siemen H, Nix M, Endl E, Koch P, Itskovitz-Eldor J, Brüstle O: Nucleofection of human embryonic stem cells. *Stem Cells Dev* 14:378–383, 2005.
- Singh Roy N, Nakano T, Xuing L, Kang J, Nedergaard M, Goldman SA: Enhancer-specified GFP-based FACS purification of human spinal motor neurons from embryonic stem cells. *Exp Neurol* 196:224–234, 2005.
- Tabar V, Tomishima M, Panagiotakos G, Wakayama S, Menon J, Chan B, Mizutani E, Al-Shamy G, Ohta H, Wakayama T, Studer L: Therapeutic cloning in individual parkinsonian mice. *Nat Med* 14:379–381, 2008.
- Thaler JP, Koo SJ, Kania A, Lettieri K, Andrews S, Cox C, Jessell TM, Pfaff SL: A postmitotic role for Isl-class LIM homeodomain proteins in the assignment of visceral spinal motor neuron identity. *Neuron* 41:337–350, 2004.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147, 1998.
- Tomishima MJ, Hadjantonakis AK, Gong S, Studer L: Production of GFP transgenic embryonic stem cells using the GENSAT bacterial artificial chromosome library. *Stem Cells* 25:39–45, 2007.
- Wang Z, Engler P, Longacre A, Storb U: An efficient method for high-fidelity BAC/PAC retrofitting with a selectable marker for mammalian cell transfection. *Genome Res* 11:137–142, 2001.
- Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K, Sasai Y: A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* 25:681–686, 2007.
- Wichterle H, Lieberam I, Porter JA, Jessell TM: Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385–397, 2002.
- Zhu JJ, Qin Y, Zhao M, Van Aelst L, Malinow R: Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* 110:443–455, 2002.
- Zwaka TP, Thomson JA: Homologous recombination in human embryonic stem cells. *Nat Biotechnol* 21:319–321, 2003.