して同一年齢の野生型 Littermate を用いた。

(2) 組織解析：動物を深麻酔後、大動脈から4%PFA／カコジル酸溶液で還流固定後、同液で後固定し、パラフィン切片もしくは凍結切片を作成した。組織片は、HE 染色、および免疫染色として HGF および phospho-c-Met$^{1230,\ 1234,\ 1235}$（c-Met の 1230, 1234, 1235 番目のチロシン残基のリン酸化を特異的に認識し c-Met の活性化を反映する）を施行した。

(3) 定量的 Real-time (RT)-PCR 法を用いて HGF mRNA 量を定量した。

(4) HGF タンパク質量を ELISA 法にて定量した。

(5) ALT(GPT)の定量：WAKO トランスアミナーゼ CII Kit を用いて定量した。

(6) 抗 HGF 抗体投与による肝臓の組織変化による解析を行った。

C．研究結果

(1) 脊髄における c-Met のリン酸化（活性化）は動物個体が死亡するまで持続する：4%PFA 還流固定後の組織解析の結果、ヒト変異 SOD1(G93A)を高発現する ALS モデルトランスジェニックマウス（G1H）の脊髄運動ニューロンは、経時的に死細胞数が増え、回復する事はなかった。それにともなって、運動ニューロンの phospho-c-Met の免疫染色性が強くなり、その状態が基本的には個体死亡まで持続した。

(2) 肝臓の一過性組織変化に伴う肝臓における一過性の c-Met の活性化： ALS モデルトランスジェニックマウス（G1H）は、HE 染色で一過性の肝臓組織変化を示し、120 日齢までにその変化から回復した。すなわち脊髄では進行性に運動ニューロンの変性脱落が進行するのに対して肝臓での組織変化は一過性であった。肝臓における一過性組織変化の特徴は、vacuolation の亢進と HE 染色上の肝細胞の dark eosinophilic hepatocyte への変化であった。この組織変化時には肝細胞における phospho-c-Met の免疫染色性が一過性に強くなった。一方で肝臓の回復にともない phospho-c-Met の免疫染色性が低下した。

(3) ELISA 法による HGF タンパク質の定量：全経過の日齢（日齢 60, 70, 80, 90, 100, 110, 120 齢）において、血中 ALT(GPT)値の上昇を認めなかった。

(4) 腎臓および心臓における一過性組織変化と回復およびそれに伴う一過性の c-Met のリン酸化（活性化）：HE 染色において、腎臓や心臓においても肝臓と同様の一過性組織変化を認めた。これに伴って、phospho-c-Met の免疫染色性が一過性に強くなり、組織変化からの回復に伴って phospho-c-Met の免疫染色性は弱くなった。

(5) 組織および血漿中の HGF mRNA もしくは HGF タンパク質量の定量：脳神経系を除く一部のサンプルで、HGF の一過性の誘導が認められ、その time course は phospho-c-Met の免疫染色性の一過性の変化と一致した。

(6) 抗 HGF 抗体投与：Alzet mini pump を用いて抗 HGF 機能阻害抗体を肝臓の組織変化が顕著になる時期から持続的に 2 週間皮下投与し、HGF-c-Met system の一過性組織変化からの回復過程での contribution を評価したところ、preliminary には抗 HGF 機能阻害抗体の投与により、肝臓の一過性組織変化からの回復過程が修飾された。

D．考察

脊髄や脳幹部運動ニューロンが進行性、持続的に変性・脱落していくのに対して、神経以外の組織である肝臓については組織変化が一過性であり、その際 ALT の変動を伴わない、すなわち顕著な肝細胞死を伴わないで組織が回復することが明らかとなった。同様に腎臓や心臓においても組織変化が一過性で

あった。なぜ神経系では変化が進行性であり、神経以外の組織が一過性の変化の後、組織が回復していくかの分子機構は、現時点では不明であるが、この分子機構の解明は、神経変性の進行抑制だけでなく、変性神経細胞の回復機構を駆動できる可能性をもつ重要課題である。HGF は ALS の運動神経細胞変性に対して神経細胞保護作用を示す事に加えて、肝臓、腎臓、心臓の細胞保護、再生因子として機能することが明らかとなっている。本研究から、HGF の受容体である c-Met のリン酸化（すなわち活性化）が組織変化と time course がまさに一致していることは興味深い。今後例数を増やして検討する必要があるが、preliminary には抗 HGF 機能阻害抗体の投与により、肝臓の一過性組織変化からの回復過程が修飾された。したがって、HGF-c-Met system が組織回復過程に単純に平行して機能している可能性も否定できないものの、HGF が神経外の組織回復の中心分子として機能している可能性が示唆された。

E. 結論

肝臓、腎臓および心臓いずれの組織も ALS の進行過程で一過性に組織変化を示し、そこから回復したが、それに伴って c-Met の一過性の活性化を認めた。これらの回復には HGF-c-Met system の寄与が示唆された。

F. 健康危険情報

特記なし。

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得：なし。
2. 実用新案登録：なし。
3. その他：なし。

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

ヒトリコンビナントHGF蛋白の髄腔内投与によるALS治療法の開発

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研究要旨　肝細胞増殖因子（HGF）が筋萎縮性側索硬化症（ALS）のモデルマウス・ラットの両者で運動ニューロン保護、生存延長効果をもつことは既に報告されている。多くの神経栄養因子のなかでもこの様に変異Cu/Zn SODトランスジェニック動物によるALSモデルに対して明確な治療効果を示したものは少なく、この有効性をALS患者に臨床応用する意義と必要性がある。しかも、臨床応用の最も可能性の高いルートとしての髄腔内投与での効果がALSラットで確認されたので、霊長類（マーモセット）に対する髄腔内投与での安全試験を開始した。マーモセットによるALSモデルは確立されていないので、HGFの臨床用量決定には慶応大学の岡野らが確立したマーモセットによる脊髄損傷モデルを用いる。ヒトリコンビナントHGF蛋白によるALS治療は医薬品機構との安全性相談が終了し、現在の安全性試験計画をクリアできればフェーズ1の治験に進めることを確認している。

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A. 研究目的

進行性の運動ニューロンの選択的細胞死を惹起する筋萎縮性側索硬化症(ALS)に対してわが国で発見された神経栄養因子である肝細胞増殖因子（Hepatocyte Growth Factor、以下HGF）を用いた治療法の臨床応用を目的にしている。すでにALSラットに対してヒトリコンビナントHGF蛋白（rhHGF）の髄腔内持続投与で有効性を示したので、霊長類を用いてHGFの髄腔内投与による安全性を検証すると共に臨床用量の設定を行う。その結果を元に、ALS患者に対する治験フェーズ1に進む。同時にHGFの治療効果の機序を明らかにする。

B. 研究方法

本研究グループによるこれまでの研究により私たちが開発したALSラットに対するリコンビナントHGF蛋白髄腔内投与にて臨床的にも病理学的にも有効性が明らかになった。多くの神経栄養因子のなかでもこの様に変異Cu/Zn SODトランスジェニック動物によるALSモデルに対して明確な治療効果を示したものは少なく、この有効性をALS患者に臨床応用する意義と必要性がある。しかも、臨床応用の最も可

能性の高いルートとしての髄腔内投与での効果がALSラットで確認されたので、霊長類（マーモセット）に対する髄腔内投与での安全試験を開始した。その後にヒトへの臨床試験を計画している。マーモセットによるALSモデルは確立されていないので、HGFの臨床用量決定には慶応大学の岡野らが確立したマーモセットによる脊髄損傷モデルを用いる。

なお、すべての遺伝子操作は本学DNA組換え実験指針に従い、また動物実験は同動物実験指針に従った上で動物愛護面に配慮しかつ利用動物数を極力減らすように努めた。

C. 研究結果　および　D. 考察

1) 霊長類（マーモセット）に対する髄腔内投与での安全試験

マーモセットによる脊髄損傷モデルに対してrhHGFの髄腔内持続投与を行った。400μgのrhHGFを髄腔内に4週間持続投与したところ（実薬群；n=6, 対照群；n=5），rhHGF投与群で上肢筋力の有意な回復を認め、MRIでも病巣面積の縮小が確認された。12週の観察期間では安全性にも問題はない。損傷後12週までの間、異常行動ならびにMRI像における腫瘍形成は一切認められなかった。したがって今のところ安全性での問題点は認めていないと考えている。

（参考）ヒトリコンビナントHGF蛋白製剤
開発スケジュール

治験に必要なGMP基準を満たしたヒト型リコンビナントHGF蛋白は確保できているが、治験を開始するためにはGLP基準を満たしたカニクイザルに対する髄腔内投与による安全性試験をおこなっている。

2) 治験に関するプロトコールの作成

rhHGFによるALS治療は医薬品機構との安全性相談が終了し、現在の安全性試験計画をクリアできればフェーズ1の治験に進めることを確認した。東北大学トランスレーショナルリサーチセンターと共にプロトコールの検討している。

E. 結論

rhHGFによるALS治療は平成21年度中の治験届けの提出を目指している。わが国発のALS治療薬候補としてスーパー特区（代表　岡野栄之）に選定された。

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5) 青木正志, 割田 仁, 石垣あや, 船越 洋, 糸山泰人 内因性肝細胞増殖因子(HGF)によるALSラット脊髄再生阻害因子発現の抑制 同上

6) 水野秀紀, 割田 仁, 青木正志, 糸山泰人 ALSモデルラットにおけるプロテオグリカン・コア蛋白分子種による発現の差異 同上

H. 知的所有権の取得状況

1. 特許登録

ラットを用いたALSモデル(出願済)

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧

原著論文

著者	論文タイトル	掲載誌名	巻 頁	出版年
Okada Y, Matsumoto A, Shimazaki T, Enoki R, Koizumi A, Ishii S, Itoyama Y, Sobue G, Okano H.	Spatio-temporal recapitulation of central nervous system development by ES cell-derived neural stem/progenitor cells.	Stem Cells	26 3086-3098	2008
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Akita. H, Takagi. N, Ishihara. N, Takagi. K, Murotomi. K, Funakoshi. H, Matsumoto. K, Nakamura. T, Takeo. S	Hepatocyte growth factor improves synaptic localization of the NMDA receptor and intracellular signaling after excitotoxic injury in cultured hippocampal neurons.	Exp Neurol.	210(1) 83-94	2008
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加藤信介	ALS1の神経病理と発症機序	Clinical Neurosci	26(3) 319-322	2008
Mizuno H, Warita H, Aoki M, Itoyama Y.	Accumulation of chondroitin sulfate proteoglycans in the microenvironment of spinal motor neurons in amyotrophic lateral sclerosis transgenic rats.	J Neurosci Res	86(11) 2512-2523	2008
Tanaka K, Okada Y, Kanno T, Otomo A, Yanagisawa Y, Shouguchi-Miyata J, Suga E, Kohiki E, Onoe K, Osuga H, Aoki M, Hadano S, Itoyama Y, Ikeda JE.	A dopamine receptor antagonist L-745,870 suppresses microglia activation in spinal cord and mitigates the progression in ALS model mice.	Exp Neurol	211(2) 378-386	2008
Dagvajantsan B, Aoki M, Warita H, Suzuki N, Itoyama Y. astrocytes in the spinal cord of amyotrophic lateral sclerosis transgenic rats.	Up-regulation of insulin-like growth factor-II receptor in reactive astrocytes in the spinal cord of amyotrophic lateral sclerosis transgenic rats.	Tohoku J Exp Med	214(4) 303-310	2008

著者	論文タイトル	掲載誌名	巻 頁	出版年
Takahashi Y, Seki N, Ishiura H, Mitsui J, Matsukawa T, Kishino A, Onodera O, Aoki M, Shimozawa N, Murayama S, Itoyama Y, Suzuki Y, Sobue G, Nishizawa M, Goto J, Tsuji S.	Development of a high-throughput microarray-based resequencing system for neurological disorders and its application to molecular genetics of amyotrophic lateral sclerosis.	Arch Neurol	65(10) 1326-1332	2008

STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

Spatiotemporal Recapitulation of Central Nervous System Development by Murine Embryonic Stem Cell-Derived Neural Stem/Progenitor Cells

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Key Words. Neural stem/progenitor cells • Embryonic stem cells • Neurosphere • Temporal identity • Spatial identity • Regenerative medicine

ABSTRACT

Neural stem/progenitor cells (NS/PCs) can generate a wide variety of neural cells. However, their fates are generally restricted, depending on the time and location of NS/PC origin. Here we demonstrate that we can recapitulate the spatiotemporal regulation of central nervous system (CNS) development in vitro by using a neurosphere-based culture system of embryonic stem (ES) cell-derived NS/PCs. This ES cell-derived neurosphere system enables the efficient derivation of highly neurogenic fibroblast growth factor-responsive NS/PCs with early temporal identities and high cell-fate plasticity. Over repeated passages, these NS/PCs exhibit temporal progression, becoming epidermal growth factor-responsive gliogenic NS/PCs with late temporal identities; this change is accompanied by an alteration in the epigenetic status of the glial fibrillary acidic protein promoter, similar to that observed in the developing brain. Moreover, the rostrocaudal and dorsoventral spatial identities of the NS/PCs can be successfully regulated by sequential administration of several morphogens. These NS/PCs can differentiate into early-born projection neurons, including cholinergic, catecholaminergic, serotonergic, and motor neurons, that exhibit action potentials in vitro. Finally, these NS/PCs differentiate into neurons that form synaptic contacts with host neurons after their transplantation into wild-type and disease model animals. Thus, this culture system can be used to obtain specific neurons from ES cells, is a simple and powerful tool for investigating the underlying mechanisms of CNS development, and is applicable to regenerative treatment for neurological disorders. STEM CELLS *2008;26:3086–3098*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Neural stem/progenitor cells (NS/PCs) can proliferate to self-renew and are multipotent; that is, they can generate the neurons and glial cells constituting the central nervous systems (CNS). They are expected to be useful in the study of neural development and to provide a variety of neural cells for regenerative treatments of neurological disorders. However, because their fates are generally determined and restricted spatiotemporally, a given NS/PC cannot generate all of the cell types existing in the CNS. For example, early NS/PCs generate neurons but not glial cells, whereas later and adult NS/PCs generate both neurons and glial cells; these late NS/PCs, however, do not normally produce early-born neurons, such as forebrain cholinergic, midbrain dopaminergic, and spinal motor neurons. Moreover, they cannot respond to cues for regional specification [1, 2].

Neurospheres and other methods for culturing NS/PCs in vitro have been reported and are widely used [3–5]. In these culture systems, NS/PCs are usually derived from the brain at mid to late gestation or from the adult brain, both of which are easy to manipulate and yield NS/PCs in large quantities, but these NS/PCs have limited plasticity. On the other hand, the culture of early embryonic brain requires special dissection techniques, and only a limited number of NS/PCs can be obtained from each brain. Furthermore, it is impossible to expand these early NS/PCs maintaining their early identities. Thus, it would be valuable to establish a culture system for generating NS/PCs with early temporal identities and high cell-fate plasticity from ES cells and to be able to control the spatiotemporal identities of the NS/PCs in vitro.

Several methods have been reported for deriving neural cells from mouse embryonic stem (ES) cells [6–14]. However, each

Author contributions: Y.O.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript: A.M., R.E., and A.K.: collection and assembly of data, data analysis and interpretation: T.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing: S.I.: collection and assembly of data: Y.I.: provision of study materials: G.S.: financial support: H.O.: conception and design, financial support, manuscript writing, final approval of manuscript.

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method directs the induction of only certain types of neural cells with specific temporal and spatial identities, such as forebrain progenitors, dopaminergic neurons, motor neurons, cerebellar neurons, and neural crest cells. In addition, the differentiation protocols are quite varied, and therefore the methods used must be changed according to the desired cell type. Moreover, the requirement for different conditions means that neither the characteristics of NS/PCs with different spatial or temporal identities nor the possible associations between temporal and spatial identities can be evaluated within the same culture system. Furthermore, most of these culture protocols have a risk of contamination by undifferentiated cells, non-neural cells, and feeder cells or rely on a long and complicated protocol to obtain particular cells.

To solve these problems, we examined whether we could recapitulate in vivo CNS development in vitro and established a simple ES cell culture system in which purified early NS/PCs are derived as neurospheres and their spatial (rostrocaudal and dorsoventral) and temporal identities are regulated in a single culture system. This system provides a powerful in vitro model for investigating the mechanisms underlying early CNS development and the pathogenesis of neurological disorders and will be applicable to regenerative therapy for neurodegenerative disorders.

Materials and Methods

ES Cell Culture and Differentiation

Mouse ES cells (EB3) grown on gelatin-coated (0.1%) tissue culture dishes were maintained in standard ES cell medium and used for EB formation in the presence of noggin or retinoic acid (RA) as described previously with slight modifications [15, 16]. For primary neurospheres, the EBs were collected on day 4 (high-RA) or day 6 (noggin or low-RA), dissociated, washed twice, and cultured in suspension at 5×10^4–1×10^5 cells/ml in media hormone mix (MHM) medium with 20 ng/ml fibroblast growth factor (FGF) 2 (PeproTech Inc., Rocky Hill, NJ, http://www.peprotech.com) for 7 days. For secondary and tertiary neurospheres, the neurospheres were dissociated and cultured at 5×10^4 cells/ml in MHM with FGF2 and/or epidermal growth factor (EGF) (PeproTech). To assay differentiation, neurospheres were plated on poly-L-ornithine/fibronectin-coated cover glasses and allowed to differentiate without growth factors for 5–7 days. The culture protocol is detailed in the supplemental online Materials and Methods.

Lentivirus Transduction and Clonal Neurosphere Formation

For clonal neurosphere analysis, primary neurospheres were initiated from dissociated EBs transduced with lentivirus expressing either Venus or monomeric red fluorescent protein (mRFP) under the EF1α promoter (pCSII-EF-Venus or pCSII-EF-mRFP) [17]. Venus- and mRFP-labeled primary neurospheres were dissociated, plated at a 1:1 ratio at a cell density of 0.5–1×10^4 cells/ml, and cultured for 10–13 days in the MHM with 0.8% methylcellulose (22223-52; Nacalai Tesque, Kyoto, Japan, http://nacalai.co.jp) and 20 ng/ml FGF2 to form secondary neurospheres as described previously [18, 19].

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

RNA isolation and reverse transcription (RT)-polymerase chain reaction (PCR) were performed as described previously [16]. The amount of cDNA was normalized to *β-actin* mRNA. Total RNA from embryonic day (E) 11.5 whole embryos was used as a positive control. Real-time RT-PCR was performed using MX3000P (Stratagene, La Jolla, CA, http://www.stratagene.com), with SYBR Premix ExTaq (Takara, Otsu, Japan, http://www.takara.co.jp). Data are expressed as the amount of mRNA relative to that of neurospheres derived from E14.5 striatum. Primer sequences and PCR cycling conditions are listed in supplemental online Table 1.

Immunohistochemical Analysis

Immunohistochemical analyses for cultured cells (immunocytochemistry [ICC]) and embryonic tissues (immunohistochemistry [IHC]) were performed as described previously [16]. Detailed conditions for the ICC and IHC are in the supplemental online Materials and Methods. For statistical analysis of the ICC results, at least 60 colonies/cover glasses were examined, and the number of colonies that immunoreacted with each antibody was counted and expressed as the percentage of the total number of colonies.

Bisulfite Sequencing

Sodium bisulfite treatment of genomic DNA was performed as described previously [20] with slight modifications, as described in supplemental online Materials and Methods. The DNA fragment containing the Stat3 recognition sequence was amplified by PCR, the products were cloned into the pGEM-T easy vector (Promega, Madison, WI, http://www.promega.com), and 10–14 clones randomly picked from each of four independent PCRs were sequenced.

Patch-Clamp Recording Procedure

ES cell-derived neurospheres were allowed to differentiate for 10–14 days on poly-L-ornithine/fibronectin-coated cover glasses, on an astrocyte feeder layer, and processed for patch-clamp analysis as described in the supplemental online Materials and Methods.

Transplantation of ES Cell-Derived Neurospheres and Immunohistochemistry

Low-RA neurospheres were transplanted into wild-type Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, http://www.clea-japan.com) or amyotrophic lateral sclerosis (ALS) model rats harboring a mutant human $SOD1^{G93A}$ gene [21, 22] at approximately 90 days of age. The procedure for the transplantation is described in the supplemental online Materials and Methods. All of the animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Keio University, School of Medicine.

Results

Neurosphere Culture System from Mouse ES Cells That Mimics In Vivo CNS Development

The easiest way to generate various types of specific neural cells in vitro is to establish a culture system in which the spatiotemporal identities of NS/PCs can be manipulated to mimic in vivo CNS development. With this goal in mind, we established an ES cell culture system that efficiently and easily generated NS/PCs (Fig. 1A).

First, ES cells were dissociated and cultured in suspension as EBs, which contain progenitor cells of the three germ layers. We dissociated the EBs and selectively expanded the NS/PCs in serum-free medium containing FGF2 as neurospheres [3]. To enrich for NS/PCs in the EBs, we added noggin, which inhibits bone morphogenetic protein (BMP) signals and is involved in forebrain formation [23–26], or RA, a neural inducer of ES cells, during EB formation. RA is also a caudalizing factor that is important in the formation of the hindbrain and rostral spinal cord [27, 28]. We previously found that noggin and a low concentration of RA (10^{-8} M: low-RA) induces more nestin$^+$ and Sox1$^+$ neural progenitors, whereas a high concentration of RA (10^{-6} M: high-RA) induces more βIII-tubulin$^+$ postmitotic neurons [16]. Therefore, we expected to obtain more neurospheres from EBs treated with noggin or low-RA. To confirm this idea, we first examined the efficiency of neurosphere for-

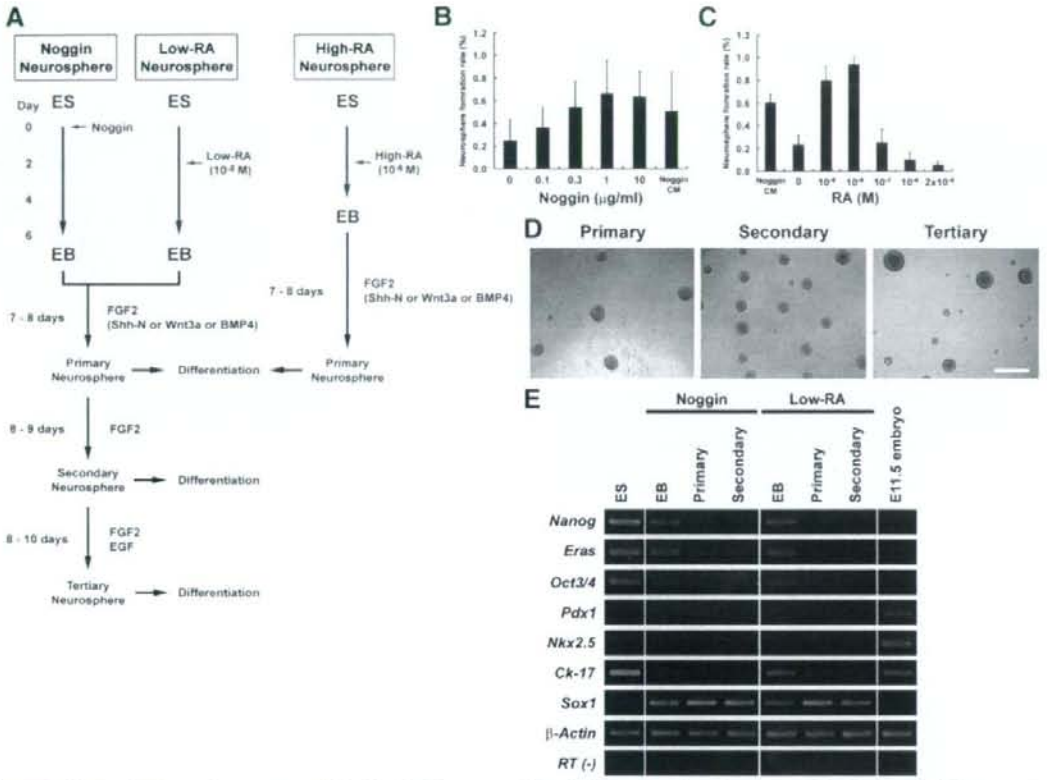

Figure 1. Neurosphere formation from mouse ES cells. **(A):** Three protocols for deriving neurospheres from mouse ES cells through EB formation. EBs were cultured in suspension in bacteriological dishes for 6 days in the presence of noggin or low RA (10^{-8} M). RA was added on day 2 of EB formation. The EBs were then dissociated and cultured in suspension for 7 days to form neurospheres in serum-free medium (media hormone mix) containing FGF2. Primary neurospheres were dissociated and cultured in suspension again with FGF2 and/or EGF to form secondary and tertiary neurospheres. EBs cultured for 4 days with high RA (10^{-6} M) also formed neurospheres but not secondary neurospheres. To regulate the dorsoventral identity, Shh-N, Wnt3a, or BMP4 was added during primary neurosphere formation in some experiments. **(B, C):** EBs treated with various doses of recombinant mouse noggin-Fc **(B)** or various concentrations of RA **(C)** were dissociated and cultured with 20 ng/ml FGF2 at a density of 1×10^4 cells/200 μl/well in an ultra-low cluster 96-well plate (Costar) for 1 week, and neurospheres larger than 50 μm in diameter were counted. Data are presented as the percentage of total cells plated that formed neurospheres ($n = 5$, mean ± SEM). **(D):** Representative morphologies of primary, secondary, and tertiary neurospheres. Scale bar = 200 μm. **(E):** Reverse transcriptase-polymerase chain reaction of undifferentiated and lineage-specific markers in ES cells, EBs treated with noggin or low RA, and primary and secondary neurospheres. Total RNA from embryonic day 11.5 whole embryos was used as a positive control. Abbreviations: BMP, bone morphogenic protein; CM, conditioned medium; EB, embryoid body; ES, embryonic stem; EGF, epidermal growth factor; FGF, fibroblast growth factor; RA, retinoic acid; Shh-N, sonic hedgehog N-terminal peptide; Wnt, wingless.

mation from EBs treated with various doses of noggin (0–10 μg/ml) or RA ($0–2 \times 10^{-6}$ M). As expected, EBs treated with more than 1 μg/ml noggin or with low-RA (10^{-8} M) generated, respectively, 2.6- and 4.0-fold more neurospheres than the control EBs, and those treated with high-RA (10^{-6} M) generated fewer neurospheres (70% of control) (Fig. 1B, 1C).

To confirm whether these neurospheres were derived from NS/PCs, we examined their self-renewal and multipotency. Most of the single primary noggin or low-RA neurospheres that were cultured at low density (2.5×10^4 cells/ml) and deposited into 96-well plates (single neurosphere/well) generated secondary neurospheres (103/133 [77%] and 115/122 [94%], respectively, from more than two independent experiments). Moreover, both types of ES cell-derived neurospheres could be passaged repeatedly to form tertiary neurospheres (Fig. 1D), indicating that they could self-renew.

We also examined the differentiation potentials of noggin and low-RA neurospheres grown at low density (<2.5×10^4 cells/ml at plating) by subjecting 5-day differentiated neurospheres to immunocytochemical analysis with markers for neurons (N) (βIII-tubulin), astrocytes (A) (glial fibrillary acidic protein [GFAP]), and oligodendrocytes (O) (O4). Most of the colonies from primary neurospheres contained neurons only (93.4% and 74.5% in noggin and low-RA neurospheres, respectively), whereas only a small number contained glial cells. When single primary neurosphere-derived secondary neurospheres and tertiary neurospheres cultured at low density ($2.5–5 \times 10^4$ cells/ml) were differentiated using the same method, they generated more colonies containing glial cells, and the proportions of multipotent colonies (NAO colonies) increased gradually in the secondary and tertiary neurospheres (Fig. 2A–2C; supplemental online Fig. S1A–S1C). This sequential generation of neurons and glial cells also corresponds well with in vivo CNS development, in which neurons are generated first and glial cells later [1, 29]. Notably, colonies that only generated neurons (N colonies) were observed in up to 44.9% of the secondary noggin neurospheres, whereas all of the colonies from secondary low-RA neurospheres contained glial cells, sug-

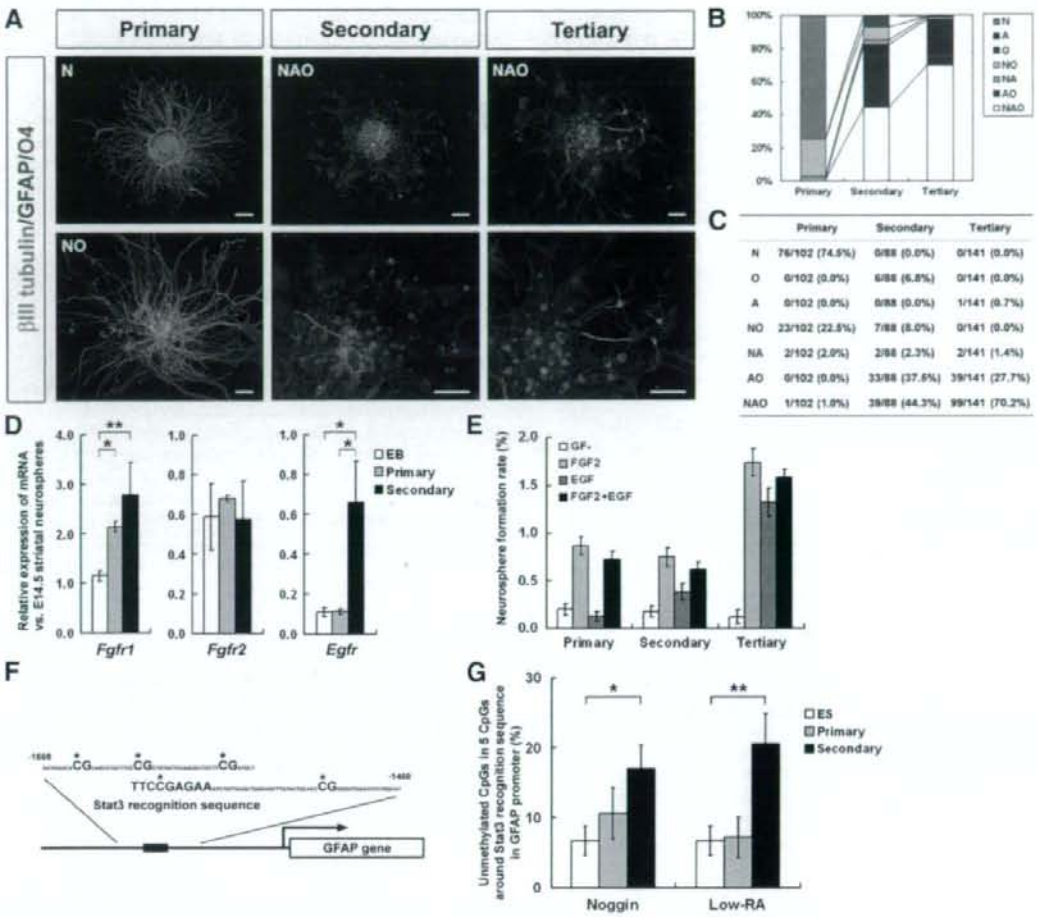

Figure 2. Primary and secondary/tertiary neurospheres corresponded to early and late neural stem/progenitor cells, respectively. **(A–C):** Primary, secondary, and tertiary neurospheres derived from low-RA-treated EBs cultured at low density ($< 2.5 \times 10^4$ cells/ml) were allowed to differentiate for 5 days followed by immunocytochemical analysis for βIII-tubulin (neurons), GFAP (astrocytes), and O4 (oligodendrocytes). The frequency of colonies consisting of neurons, oligodendrocytes, and astrocytes is presented as the percentage of total colonies **(B, C)**. More than 80 colonies from at least two independent experiments were examined. Scale bar = 50 μm. **(D):** Expression of mRNAs of growth factor receptors (*Fgfr* and *Egfr*) in EBs and in primary and secondary low-RA neurospheres was analyzed by real-time reverse transcription-polymerase chain reaction. Data are presented as the expression relative to that in neurospheres derived from E14.5 striatum ($n = 5$, mean ± SEM; *, $p < .01$; **, $p < .05$). **(E):** Neurosphere formation rate in the presence of FGF2 and/or EGF. Low-RA-treated EBs and primary and secondary neurospheres were dissociated and plated onto ultra-low cluster 96-well plates (coaster) at a density of 1×10^4 cells/200 μl/well to form primary, secondary, and tertiary neurospheres, respectively, in the presence of FGF2, EGF, both FGF2 and EGF, or no growth factors. Neurospheres larger than 50 μm in diameter were counted on day 7. Data are presented as the percentage of plated cells that formed neurospheres ($n = 3–5$, mean ± SEM). **(F, G):** The methylation status of CpGs around the Stat3 recognition sequence (TTCCGAGAA in **F**) of the GFAP promoter in ES cells and in primary and secondary neurospheres was examined by bisulfite sequencing. The proportion of unmethylated cytosines in the five CpGs (−1568 to −1460) around the Stat3 recognition sequence (−1518 to −1510) (asterisks in **F**) is shown in **(G)** ($n = 4$, mean ± SEM; *, $p < .05$; **, $p < .01$, one-way analysis of variance). Abbreviations: A, astrocytes; E, embryonic day; EB, embryoid body; ES, embryonic stem; EGF, epidermal growth factor; FGF, fibroblast growth factor; GF, growth factor; GFAP, glial fibrillary acidic protein; N, neutrons; O, oligodendrocytes; RA, retinoic acid.

gesting that the low-RA neurospheres preceded noggin neurospheres in their temporal differentiation properties (Fig. 2; supplemental online Fig. S1).

We also performed a clonal neurosphere analysis in which secondary neurospheres were formed for 10–13 days in medium containing 0.8% methylcellulose, which forms a semisolid. We previously reported that this method effectively prevents neurosphere aggregation, resulting in clonal neurosphere formation [18, 19]. In fact, more than 70% of the secondary neurospheres that formed from mixed cultures of wild-type cells along with cells expressing Venus or mRFP (both introduced via lentivirus) were homogeneous with regard to the expression of Venus, mRFP, or no fluorescence (wild type). However, some nonclonal chimeric neurospheres, composed of a mixture of Venus-positive, mRFP-positive, and fluorescence-negative cells, were also observed (24.1 ± 2.7 and 15.1 ± 6.2% of the secondary noggin and low-RA neurospheres, respectively, $n = 4$). Differentiation studies of these clonally derived neurospheres revealed that more than half of them exhibited a multipotent character (NAO) (supplemental online Fig. S2), indicating that the sec-

ondary neurospheres could be initiated from NS/PCs that were multipotent. These results also suggested that neurogenic NS/PCs derived from ES cells could generate gliogenic NS/PCs during repeated passages of the neurosphere culture.

We also examined the expression of cell type-specific markers in ES cell-derived neurospheres by RT-PCR. Both noggin-treated and low-RA-treated EBs expressed markers for neural progenitors (*Sox1*), along with markers for undifferentiated ES cells, such as *Nanog*, *Eras*, and *Oct3/4* (Fig. 1E). In contrast, primary and secondary neurospheres derived from EBs treated with either agent expressed much lower levels of markers for undifferentiated ES cells or other lineages, including markers for endoderm (*Pdx1*), mesoderm (*Nkx2.5*), and epiderm (*Ck-17*). These results indicate that the repeated formation of neurospheres from single-cell suspensions in serum-free medium facilitates the selection of NS/PCs and the elimination of unwanted undifferentiated and non-neural cells.

Primary Neurospheres and Secondary/Tertiary Neurospheres Have the Properties of Early and Late NS/PCs, Respectively

Because the long-term expansion of ES cell-derived neurospheres through repeated passages resulted in a sequential generation of neurons and glial cells similar to that seen in vivo (Fig. 2A–2C; supplemental online Fig. S1A–S1C), we next focused on the temporal specification of ES cell-derived NS/PCs, characterizing them by several other temporally restricted properties of NS/PCs seen in vivo.

In vivo, NS/PCs initially proliferate only in response to FGF, and then acquire responsiveness to EGF when the EGF receptor is expressed at mid gestation [30, 31]. We therefore first studied the expression of the involved receptors by NS/PCs and their responsiveness to these growth factors during neurosphere formation. Real-time RT-PCR analysis showed that *Fgfr1* and *Fgfr2* were constantly expressed by EBs, primary neurospheres, and secondary neurospheres. In contrast, *Egfr* expression was low in EBs and primary neurospheres but was dramatically upregulated in the secondary neurospheres (Fig. 2D; supplemental online Fig. S1D). Consistent with these results, EBs and primary neurospheres efficiently generated primary and secondary neurospheres only in the presence of FGF2 and not EGF, but tertiary neurospheres could be efficiently formed in the presence of either FGF2 or EGF (Fig. 2E; supplemental online Fig. S1E). These results suggest that this in vitro system also recapitulates the temporal change in growth factor responsiveness that occurs during NS/PC development in vivo.

Our results so far showed that the temporal changes in differentiation potentials and proliferation in ES cell-derived NS/PCs were similar to those of embryonic NS/PCs. These changes are thought to be regulated in vivo by epigenetic mechanisms such as DNA methylation and chromatin modification, at least in part. To examine these mechanisms in our system, we focused on the methylation status of CpGs around the Stat3 recognition sequence in the GFAP promoter. This region is methylated during neurogenesis, at around E11.5–12.5 in mice, and gradually demethylated during gliogenesis, after E14.5, to regulate the transcription of the GFAP gene and astrocyte differentiation [20, 32, 33]. By bisulfite sequencing, we found that the proportion of unmethylated CpGs around the Stat3 recognition sequence (−1568 to −1460) (Fig. 2F) gradually increased in the primary and secondary noggin and low-RA neurospheres (Fig. 2G). Thus, temporal epigenetic changes in the ES cell-derived neurospheres also recapitulated those seen in vivo.

Regulation of Rostrocaudal Identities by Noggin and Various Concentrations of RA in ES Cell-Derived NS/PCs

Because noggin and RA are involved in the formation of the rostral and caudal neural tube, respectively, we next examined whether exposure to noggin or various concentrations of RA during EB formation could alter the rostrocaudal identities of the neurospheres that were subsequently derived. We found that noggin neurospheres expressed forebrain to midbrain markers (*Foxg1*, *Otx1*, *Otx2*, and *En1*), whereas low-RA neurospheres were caudalized to some extent, expressing hindbrain markers (*Pax2*, *Gbx2*, *Hoxb1*, *Hoxa2*, and *Hoxb4*) as well as forebrain to midbrain markers (Fig. 3A). However, neither type of neurosphere expressed spinal cord markers such as *Hoxc4* and *Hoxc6*.

Although high-RA-treated EBs cultured for 6 days acquire the caudal spatial identities of the hindbrain to spinal cord region [16] but do not form neurospheres efficiently because of the high proportion of postmitotic neurons in the EBs (Fig. 1C), we expected to obtain neurospheres with more strongly caudalized characteristics from EBs treated with high-RA but cultured for shorter periods (4 days) (Fig. 1A). This protocol resulted in the formation of some neurospheres (high-RA neurospheres) from the high-RA-treated EBs, but they formed at a lower frequency than under the other conditions. These neurospheres mainly expressed markers for the hindbrain to the rostral spinal cord (*Pax2*, *Gbx2*, *Hoxb1*, *Hoxa2*, *Hoxb4*, and *Hoxc4*) and did not express forebrain markers. Unfortunately, these high-RA neurospheres rarely formed secondary neurospheres, suggesting that they did not contain many NS/PCs, which can self-renew, but rather consisted largely of committed progenitors and postmitotic neurons (progenitor spheres).

This regulation of marker expression correlating with the rostrocaudal axis was confirmed by subjecting 5-day differentiated neurospheres to immunocytochemical analysis for Otx1 (forebrain to midbrain), Pax2 (midbrain to spinal cord), and Hoxb4 (hindbrain and spinal cord) (supplemental online Fig. S5A). Approximately 70% of the colonies from noggin neurospheres contained $Otx1^+$ cells, whereas few or none contained $Pax2^+$ or $Hoxb4^+$ cells (Fig. 3B, 3C), suggesting a forebrain-to-midbrain identity. In contrast, many colonies from low-RA neurospheres contained $Pax2^+$, $Hoxb4^+$, and $Otx1^+$ cells (forebrain-to-hindbrain identity), and high-RA neurospheres generated $Pax2^+$ and $Hoxb4^+$ but not $Otx1^+$ colonies (hindbrain-to-spinal cord identity). These data indicate that the caudalization of NS/PCs in neurospheres could be driven by RA added during EB formation in a concentration-dependent manner. These rostrocaudal marker-positive cells were also positive for neural progenitor markers, such as Sox1/2/3 and hSox (supplemental online Fig. S3) ([34, 35]), or the neuronal marker NeuN, confirming that they were neural cells (Fig. 3B). Moreover, noggin and low-RA neurospheres differentiated into many neurons that were glutamic acid decarboxylase (GAD67)-positive (GABAergic) or choline acetyltransferase (ChAT)-positive (cholinergic), and small numbers of neurons that were tyrosine hydroxylase (TH)-positive (catecholaminergic) or serotonin (5-HT)-positive (serotonergic). In contrast, the high-RA neurospheres differentiated into $GAD67^+$ and $ChAT^+$ neurons but not into TH^+ or $5\text{-}HT^+$ neurons (Fig. 3D, 3E). There was no significant difference in the frequency of differentiation into $GAD67^+$ or $ChAT^+$ neurons between the noggin and low-RA neurospheres. The generation of these neuronal subtypes shows overall consistency with the identity of neurons generated in the corresponding rostrocaudal region in vivo. Thus, the rostrocaudal identity of ES cell-derived NS/PCs was controlled by administering noggin or various concentrations of RA during EB formation.

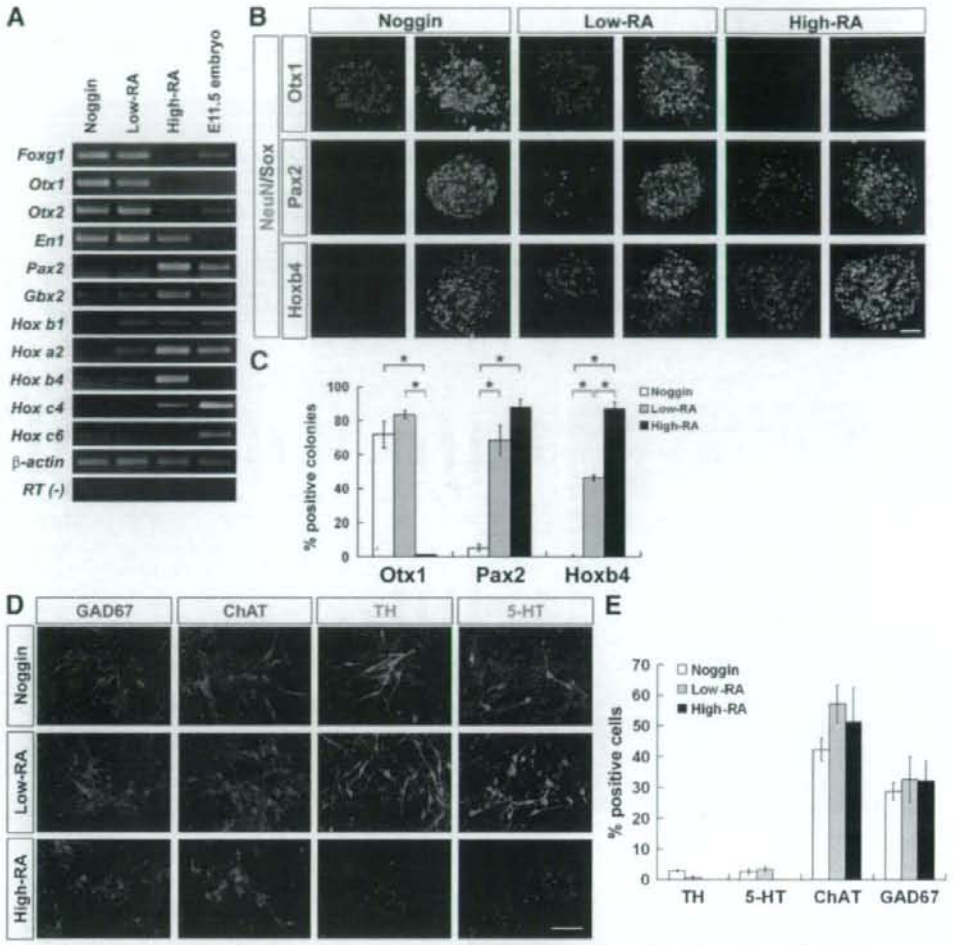

Figure 3. Regulation of rostrocaudal regional identity in embryonic stem (ES) cell-derived neural stem/progenitor cells. **(A):** Reverse transcription-polymerase chain reaction analysis of the expression of rostrocaudal marker expression in ES cell-derived neurospheres derived from noggin-, low-RA-, and high-RA-treated embryoid bodies (EBs). Total RNA from E11.5 whole embryos was used as a positive control. **(B, C):** ES cell-derived neurospheres were differentiated en bloc for 5 days and immunostained with rostrocaudal markers Otx1, Pax2, and Hoxb4. The frequency of colonies containing immunopositive cells is shown as the percentage of total colonies **(D)** ($n = 5$, mean ± SEM; *, $p < .01$). Scale bar = 50 μm. **(D):** Neurotransmitter subtypes of neurons that differentiated from neurospheres derived from EBs treated with noggin, low-RA, and high-RA. Representative photographs showing relatively high amounts of marker-positive cells in a particular field are shown. Scale bar = 50 μm. **(E):** Quantification of neuronal subtypes in primary neurosphere-derived neurons ($n = 3$). Abbreviations: ChAT, choline acetyltransferase; E, embryonic day; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; 5-HT, serotonin; RA, retinoic acid; TH, tyrosine hydroxylase.

Dorsoventral Regulation of ES Cell-Derived NS/PCs

In addition to the rostrocaudal axis, we next attempted to control the dorsoventral identities of ES cell-derived NS/PCs by adding several secreted factors during neurosphere formation, including Sonic hedgehog (Shh), wingless (Wnt) 3a, and BMP4, which act as morphogens in dorsoventral specification in the developing neural tube [2, 36].

Because the neuronal subtypes and the dorsoventral axis are determined during the neural fold and neural tube stages in vivo by Shh from the ventral notochord and floor plate and by Wnts and BMPs from the dorsal roof plate [2, 36] and because this follows the determination of the rostrocaudal axis (during gastrulation in vivo or EB formation in vitro) [2, 27, 37], we added Shh, Wnt3a, and BMP4 during neurosphere formation.

As with BMP4, only small-cell clusters (ragged "spheres") were formed, and very few spheres were formed from noggin-treated EBs (supplemental online Fig. S4). Therefore, we examined the effects of sonic hedgehog N-terminal peptide (Shh-N) and Wnt3a on the expression of dorsoventral markers of the forebrain in noggin neurospheres, and the effects of Shh-N, Wnt3a, and BMP4 on the expression of caudal dorsoventral markers in low- and high-RA neurospheres.

Noggin neurospheres expressed *Nkx2.1*, a marker for the basal forebrain (supplemental online Fig. S5B), in an Shh-N concentration-dependent manner, generating 69.2 ± 8.2% $Nkx2.1^+$ ventral colonies with 300 nM Shh-N (Fig. 4A, 4D, 4E). On the other hand, these neurospheres expressed *Pax6* and *Emx1*, markers for dorsal telencephalon (supplemental online Fig. S5B), in a Wnt3a concentration-dependent manner, gener-

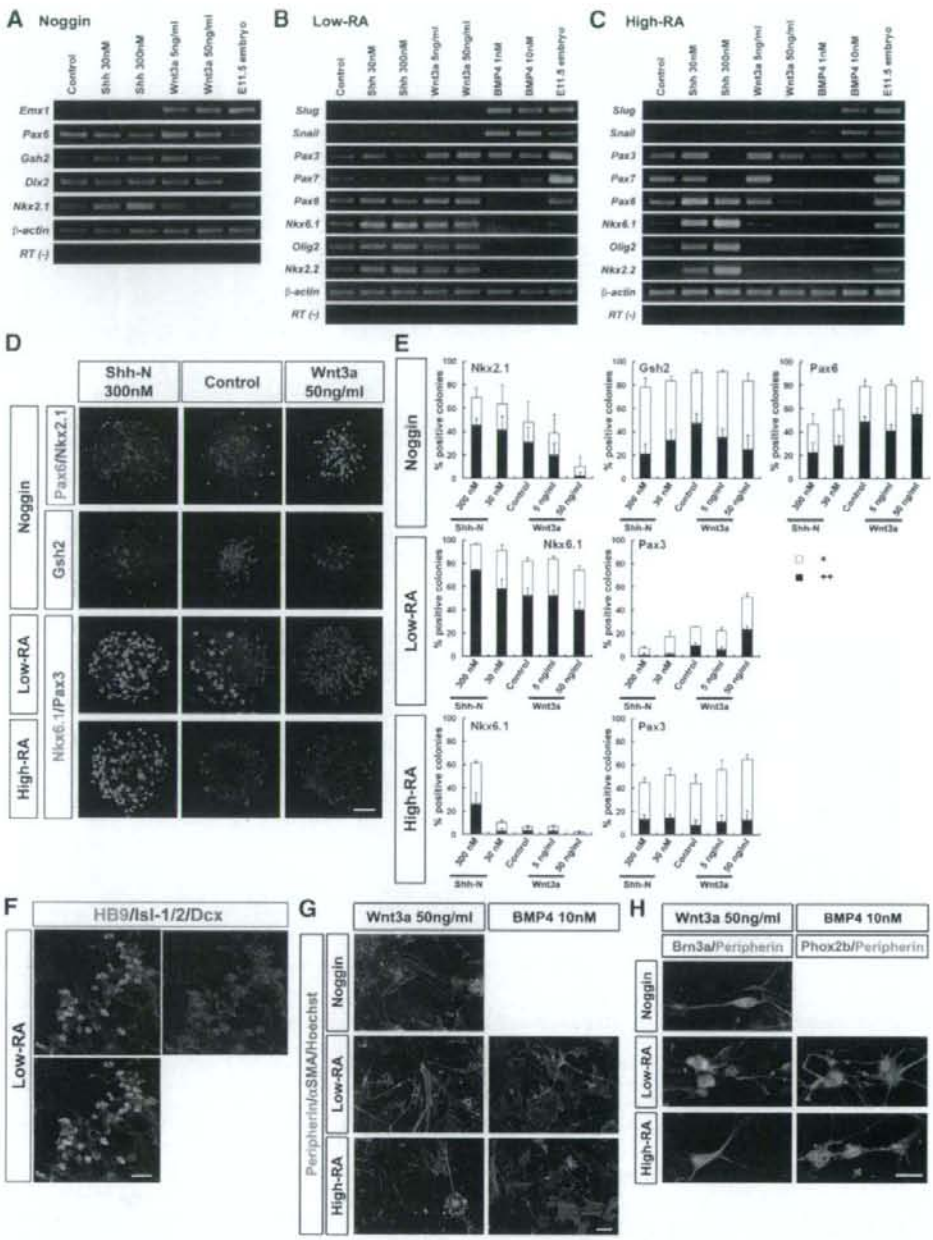

Figure 4. Dorsoventral regulation by treatment with Shh, Wnt3a, and BMP4 during neurosphere formation. **(A–C):** Reverse transcription-polymerase chain reaction analysis of dorsoventral markers in embryonic stem (ES) cell-derived neurospheres. Total RNA from E11.5 whole embryos was used as a positive control. **(D, E):** ES cell-derived neurospheres were differentiated en bloc for 5 days and immunostained with dorsoventral markers. The frequency of colonies containing immunopositive cells is shown as the percentage of total colonies **(E)** (mean ± SEM). Colonies containing more than 10 positive cells/colony are indicated as strongly positive (++, ■), whereas those containing fewer than 10 are indicated as weakly positive (+, □). Scale bar = 50 μm. **(F):** Low-RA neurospheres were differentiated for 5 days and immunostained for Isl-1/2 and HB9, markers for ventral somatic motor neurons and with the neuronal marker Dcx. Scale bar = 20 μm. **(G):** Immunocytochemical analysis of differentiated neurospheres treated with dorsalizing factor, Wnt3a or BMP4. Differentiated neurospheres were immunostained for αSMA and peripherin, markers for smooth muscle cells and peripheral nerves, respectively, both of which are derived from neural crest. Scale bar = 50 μm. **(H):** ES cell-derived neurospheres cultured in the presence of Wnt3a or BMP4 were allowed to differentiate and then were immunostained for Brn3a, Phox2b, and peripherin. Wnt3a-treated neurospheres differentiated into Brn3a$^+$ sensory neurons, whereas more than 85% of the peripherin$^+$ neurons from the BMP4-treated neurospheres were Phox2b$^+$ autonomic neurons. Scale bar = 20 μm. Abbreviations: BMP4, bone morphogenic protein 4; Dcx, doublecortin; RA, retinoic acid; Shh, sonic hedgehog; Shh-N, sonic hedgehog N-terminal peptide; Wnt, wingless.

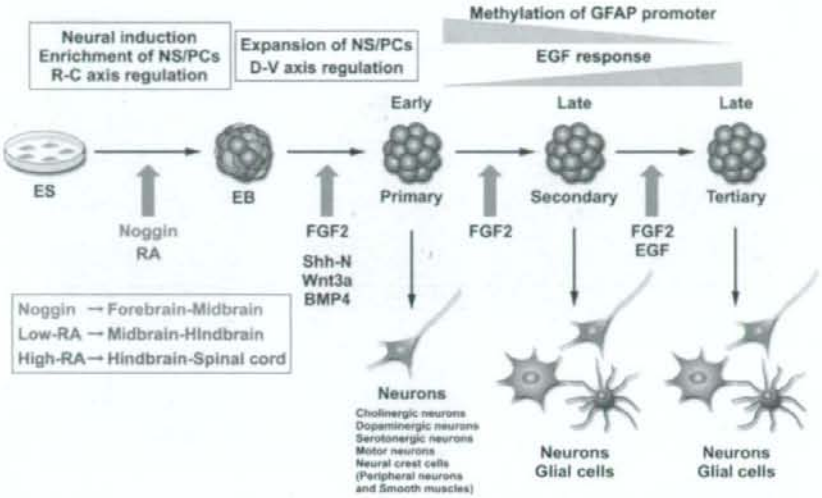

Figure 5. Schematic presentation of the spatiotemporal recapitulation of central nervous system development in ES cell-derived neurospheres in vitro. Abbreviations: BMP4, bone morphogenic protein 4; D-V, dorsoventral; EB, embryoid body; ES, embryonic stem; EGF, epidermal growth factor; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; NS/PCs, neural stem/progenitor cells; RA, retinoic acid; R-C, rostrocaudal; Shh-N, sonic hedgehog N-terminal peptide; Wnt, wingless.

ating 84.0 ± 3.1% Pax6$^+$ dorsal colonies with 50 ng/ml Wnt3a (Fig. 4A, 4D, 4E). The expression of *Gsh2* and *Dlx2*, which are found in the mid forebrain (supplemental online Fig. S5B), was not significantly altered by any of the treatments, and Gsh2$^+$ colonies were generated in the presence or absence of Shh-N or Wnt3a (Fig. 4A, 4D, 4E).

Similarly, in low- and high-RA neurospheres, 30–300 nM Shh-N induced ventral markers such as *Nkx6.1*, *Olig2*, and *Nkx2.2*, and 5–50 μg/ml Wnt3a upregulated the expression levels of dorsal markers, including *Pax3* and *Pax7* (Fig. 4B, 4C). Although the control low-RA neurospheres normally exhibited mainly ventral identities (Fig. 4B) and gave rise to mainly Nkx6.1$^+$ ventral colonies (82.4 ± 5.9%) and fewer with the Pax3$^+$ dorsal identity (25.7 ± 2.9%), 300 nM Shh-N increased the proportion of Nkx6.1$^+$ ventral colonies to 96.7 ± 1.5%, and 50 ng/ml Wnt3a increased the Pax3$^+$ dorsal colonies to 51.4 ± 5.8% (Fig. 4D, 4E; supplemental online Fig. S5C). Note that some colonies from low-RA neurospheres contained HB9$^+$ and Isl-1/2$^+$ ventral somatic motor neurons, regardless of the culture conditions (Fig. 4F; data not shown). In contrast, high-RA neurospheres exhibited more dorsalized characteristics (Fig. 4C). Whereas high-RA neurospheres gave rise to 44.4 ± 11.7% Pax3$^+$ dorsal colonies and a small number of Nkx6.1$^+$ ventral colonies in controls, 300 nM Shh-N induced a significant number of Nkx6.1$^+$ ventral colonies (61.2 ± 10.9%), and 50 ng/ml Wnt3a increased the Pax3$^+$ dorsal colonies (65.5 ± 9.3%) (Fig. 4D, 4E). Although Shh-N and Wnt3a could regulate the dorsoventral identities of ES cell-derived NS/PCs in all three types of neurospheres, the effects of these factors on dorsoventral identities in low-RA neurospheres was relatively small compared with those seen in noggin- or high-RA neurospheres (Fig. 4A–4E).

Finally, we examined whether Wnt3a and BMP4 directed ES cell-derived neurospheres into the neural crest lineages. Although Wnt3a increased the expression of dorsal markers, including *Pax3* and *Pax7*, it only slightly upregulated markers for neural crest lineages, *Slug* and *Snail*, in contrast to BMP4, which strongly upregulated *Slug* and *Snail* (Fig. 4B, 4C). Consistent with these results, only a small number of cells in Wnt3a-treated neurospheres appeared to be differentiated into peripherin$^+$ peripheral neurons and SMA$^+$ smooth muscle progenitors, as in control cultures, whereas virtually all of the cells that differentiated from BMP4-treated spheres expressed peripherin or SMA (Fig. 4G). Moreover, Wnt3a-treated neurospheres generated Brn3a$^+$/peripherin$^+$ sensory neurons and a small number of Phox2b$^+$/peripherin$^+$ autonomic neurons, whereas most of the peripherin$^+$ neurons derived from BMP4-treated low- and high-RA neurospheres were Phox2b$^+$ autonomic neurons (85.8 ± 5.0 and 89.2 ± 0.6%, respectively) and none at all were Brn3a$^+$ sensory neurons (Fig. 4H). These results were consistent with the in vivo effects of Wnt3a and BMP4 on neural crest stem cells [38]. Taken together, these results indicate that our culture system provides a variety of neural progenitors with a wide range of temporal and spatial identities, including those of the neural crest lineages (summarized in Fig. 5).

ES Cell-Derived Neurospheres Differentiated into Electrophysiologically Functional Neurons That Formed Synaptic Contacts In Vitro

To test whether the neurons generated by our in vitro system are actually functional, neurospheres were allowed to differentiate for 10–14 days without growth factors on an astrocyte feeder layer, and then the differentiated neurons were subjected to electrophysiological analysis by the whole-cell patch-clamp technique (Fig. 6). Voltage-clamp recordings of individual neurons revealed transient inward and sustained outward currents (Fig. 6C) (data not shown). From their activation voltages and time courses, we identified the transient inward current as a Na^+ current and the sustained outward current as a delayed rectifier K^+ current (Fig. 6C–6F). The transient Na^+ current was tetrodotoxin-sensitive (data not shown). The injection of a sustained positive current induced the repetitive firing of action potentials in all of the neurons tested (n = 6) (Fig. 6D). Similar results were obtained in neurons derived from low-RA neurospheres, in which a transient Na^+ current was recorded under voltage clamp in 20 of 22 neurons tested, and depolarization (single or multiple action potentials) was induced by the positive

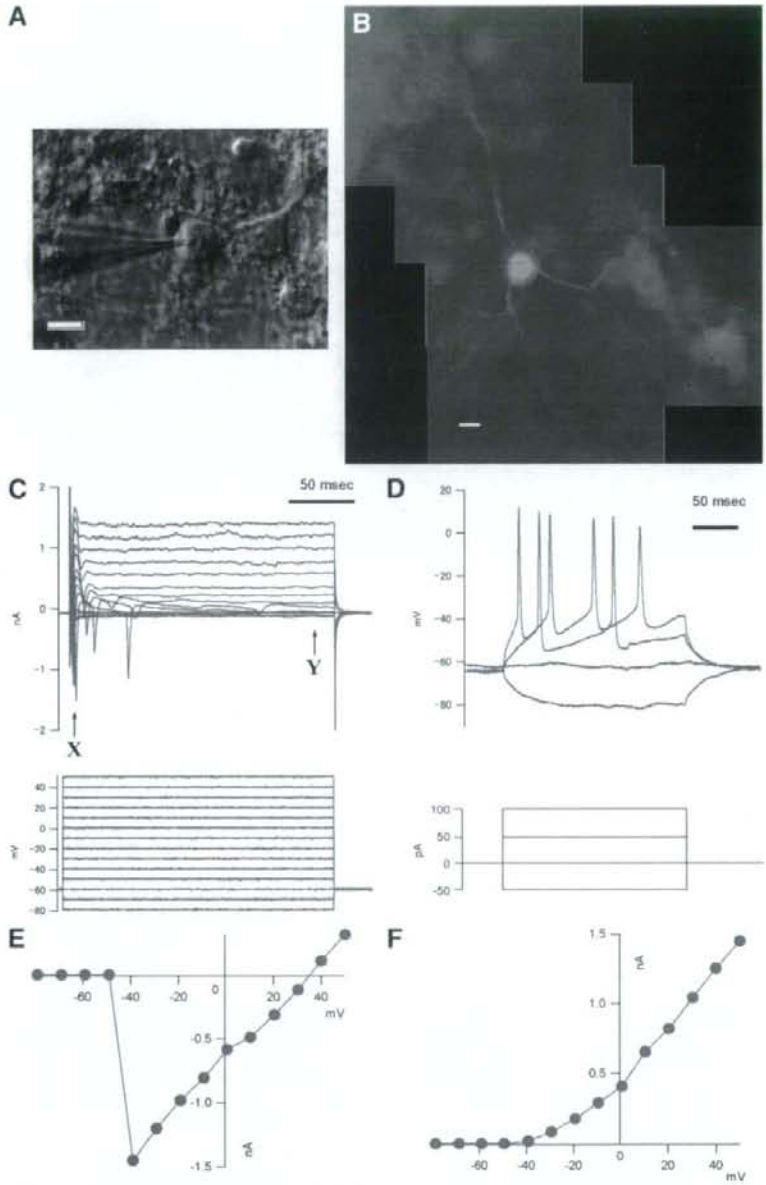

Figure 6. Electrophysiological properties of embryonic stem cell-derived neurons. Neurospheres derived from noggin-treated embryoid bodies were dissociated and differentiated on poly-L-ornithine/fibronectin-coated cover glasses, on an astrocyte feeder layer for 10–14 days, before electrophysiological analysis. **(A, B):** The cell used for the recorded data in (**C**) and (**D**) is shown in differential interference contrast and fluorescence (Lucifer yellow) micrographs. Scale bar = 10 μm. **(C):** A transient Na^+ and a sustained K^+ current were detected under voltage clamp (holding voltage, −60 mV; command voltage, from −80 to 50 mV; 10-mV step). I-V curves in panels (**E**) and (**F**) correspond to X and Y in (**C**). X represents the transient Na^+ current and Y the sustained K^+ current. **(D):** Repetitive firing of action potentials was detected when a depolarizing current was injected under the current clamp (−50, 0, 50, and 100 pA from the bottom).

current in 3 of 4 neurons tested (data not shown). These results indicate that the ES cell-derived NS/PCs generated electrophysiologically functional neurons.

We also asked whether these neurons could form synaptic contacts in vitro. We generated an ES cell line genetically tagged to express enhanced green fluorescent protein (EGFP) under a ubiquitously expressing promoter (CAG-EGFP ES cells) and derived low-RA neurospheres from it. Because a portion of low-RA neurospheres produced $HB9^+$ somatic motor neurons (Fig. 4F), we cocultured these neurons with C2C12-derived myotubes. $EGFP^+$ ES cell-derived neurons showed neuromuscular contacts labeled by rhodamine-conjugated

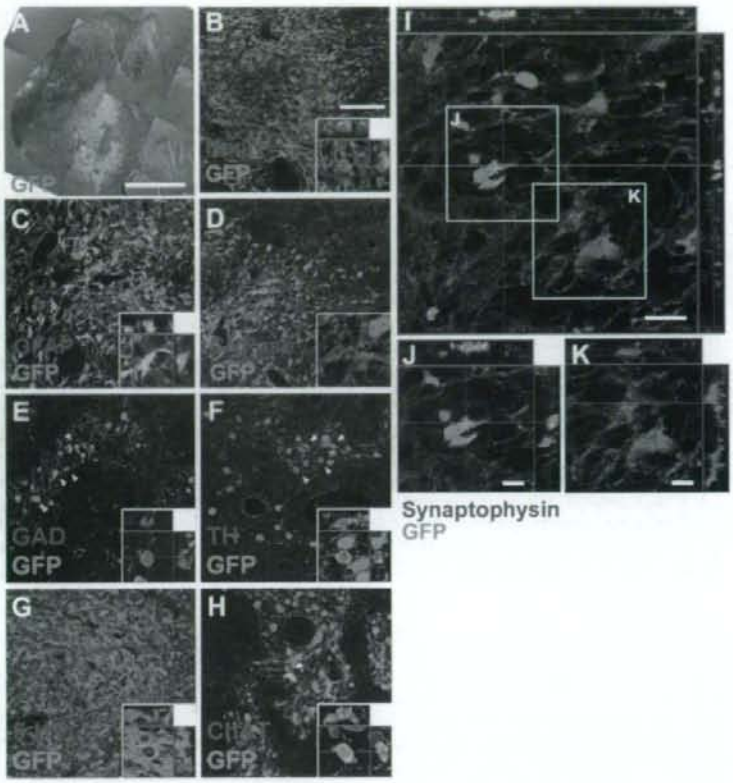

Figure 7. Low-RA neurospheres differentiated into NeuN$^+$ neurons, and when transplanted into rat lumbar spinal cord, formed synaptic contacts with the host neurons. **(A):** Low-RA neurospheres derived from CAG-enhanced GFP embryonic stem cells were transplanted stereotactically into the ventral lumbar spinal cord of Sprague-Dawley rats. Scale bar = 1 mm. **(B–H):** The rats were processed for immunohistochemical analysis 2 weeks after transplantation. Insets show higher magnifications of cells positive for both GFP and NeuN (**B**), GFAP (**C**), or subtype-specific markers (**E–H**). GFP-positive grafted cells did not differentiate into CNPase-positive oligodendrocytes (**D**). Scale bar = 100 μm for low magnification. **(I–K):** The synaptic connections of transplanted cells were studied by capturing a series of 0.5-μm optical sections with a confocal laser microscope from synaptophysin- and GFP-immunostained sections. Higher magnification images are shown in **J** and **K**. Scale bar = 20 μm (**I**), 10 μm (**J, K**). Abbreviations: ChAT, choline acetyltransferase; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; 5-HT, serotonin; NeuN, neuronal marker; TH, tyrosine hydroxylase.

α-bungarotoxin, suggesting that these neurons could form synaptic contacts in vitro (supplemental online Fig. S6).

ES Cell-Derived NS/PCs Survived and Differentiated into a Variety of Neuronal Subtypes That Formed Synaptic Connections in Rat Spinal Cord

Finally, we transplanted low-RA neurospheres derived from CAG-EGFP ES cells into the ventral lumbar spinal cord of approximately 90-day-old Sprague-Dawley rats to assess the in vivo differentiation potentials of the ES cell-derived NS/PCs. Two weeks later, the rats were sacrificed and processed for immunohistochemical analysis. The transplanted cells survived in the ventral spinal cord as cell clusters (Fig. 7A). In the treated rats, the ES cell-derived NS/PCs differentiated into NeuN$^+$ neurons and GFAP$^+$ astrocytes in some animals, but never into 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-positive oligodendrocytes (Fig. 7B; supplemental online Table 2). The neurons were mostly GAD$^+$ (GABAergic), and there were relatively few ChAT$^+$ (cholinergic), TH$^+$ (catecholaminergic), or 5-HT$^+$ (serotonergic) neurons (Fig. 7B–7F; supplemental online Table 2). Moreover, the EGFP$^+$ neurites of the transplanted neurons were extensively labeled for and surrounded by the presynaptic marker synaptophysin, indicating the formation of synaptic contacts with host neurons (Fig. 7I–7K). Similar results were obtained when low-RA neurospheres were transplanted into the lumbar spinal cord of ALS model rats harboring the mutant human *SOD1*^{G93A} gene [21, 22], although their differentiation properties in vivo varied somewhat from animal to animal (supplemental online Fig. S7; supplemental online Table 2). Thus, our ES cell-derived neurosphere culture system could be applicable to regenerative therapy for neurological disorders.

Discussion

In the present study, we successfully developed an in vitro system for the efficient derivation of NS/PCs from mouse ES cells, whose temporal and spatial identities can be controlled simultaneously. By applying the neurosphere method [3], we selectively and easily cultured NS/PCs with early temporal identities and high plasticity and achieved a precise wide-range recapitulation of in vivo CNS development in vitro. This is the first report of an ES cell differentiation system that broadly and

closely mimics in vivo CNS development using a single culture protocol to generate NS/PCs.

We used noggin and RA for neural induction, which also respectively determined the rostral and caudal identities of the NS/PCs. Several lines of evidence have suggested a model in which the default positional fate of the neural plate is the rostral brain, with factors such as RA, FGFs, Wnts, and growth differentiation factors (GDFs) inducing the caudalization of neural cells in early vertebrate development [39]. One report demonstrated the effective induction of rostral neural progenitors with a combination of Dickkopf-1 and LeftyA [10]; here, we chose to use noggin instead, because BMP antagonism is essential for mammalian forebrain development [25, 26]. In our present study, noggin treatment of EBs dose dependently increased the number of neurospheres by inhibiting BMP signals in the serum-containing EB medium. Moreover, consistent with the above-mentioned model, caudalizing signal-independent noggin neurospheres adopted rostral identities. On the other hand, RA promotes the neural differentiation of ES cells concurrently with the caudalization of neural progenitors in EBs in a concentration-dependent manner in vitro: low RA produces more neural progenitors with slightly caudalized identities around the midbrain to hindbrain, and high RA induces postmitotic neurons with caudal neural tube identities rather than proliferative neural progenitors [16]. RA also causes cell cycle arrest in neuroblastoma cells by increasing the level of cyclin-dependent kinase inhibitors, such as $p27^{kip1}$, through downregulation of the ubiquitin-proteasome-dependent degradation pathway [40]. Thus, the efficient generation of neurospheres from low-RA-treated rather than high-RA-treated EBs in the present study was consistent with this concentration-dependent effect of RA on neural induction from ES cells. Moreover, we obtained neurospheres with caudal identities in an RA concentration-dependent manner by using the low-RA neurosphere protocol (forebrain, midbrain, and hindbrain) and the high-RA/short-exposure protocol (hindbrain and spinal cord). These results indicated that the rostrocaudal identity in primary neurospheres could be preserved from that acquired in the EB stage, except that the low-RA neurospheres exhibited not only midbrain-to-hindbrain but also forebrain identity. Taking into consideration the effect of RA on cell cycle arrest, these findings suggest that the rostral NS/PCs, a relatively minor population in the low-RA-treated EBs, were selectively expanded in the neurosphere condition.

In the developing CNS, distinct NS/PCs with different temporal identities are generated, depending on the developmental stage. The earliest are leukemia inhibitory factor-dependent primitive NS/PCs (E5.5–E7.5) and FGF-responsive (but not EGF-responsive) NS/PCs (E8.5–E11.5), which have the potential to generate early-born neurons; the latest are EGF-responsive NS/PCs with gliogenic potentials that cannot generate early-born neurons [1, 29–31, 41]. In addition, although ES cell-derived NS/PCs that are identical to primitive NS/PCs have been reported, these cells do not show a transition into EGF-responsive NS/PCs unless stimulated by an exogenous Notch signal [9, 41]. In contrast, our neurosphere culture system successfully enables the sequential generation of NS/PCs with early and late temporal identities just as in vivo, and it could clearly recapitulate the temporal transition of neurogenic early NS/PCs into gliogenic late NS/PCs and the acquisition of EGF responsiveness.

The gradual increase in the number of unmethylated CpGs in the GFAP promoter region, which regulates the timing of GFAP expression [20, 32, 33], from undifferentiated ES cells to secondary neurospheres, suggests that in vivo developmental changes in the epigenetic status of this region are also recapitulated to some extent in our system. Despite the remarkable augmentation of differentiation into $GFAP^+$ astrocytes from secondary neurospheres, the increase in unmethylated CpGs was not as dramatic. It is possible that cells of the neuronal lineage within the neurospheres masked this change in stem cells.

It is also noteworthy that the acquisition of gliogenic potential in the noggin neurosphere cultures was delayed compared with that in the low-RA neurosphere cultures (Fig. 2A–2C; supplemental online Fig. S1A–S1C). This is consistent with in vivo development, in which the acquisition of later identities in the caudal neural tube precedes that in the forebrain [29–31, 42, 43], indicating another advantage of our culture system, the simultaneous recapitulation of temporal and spatial specification.

We also showed that dorsoventral identity can be controlled by the administration of Shh-N, Wnt3a, and BMP4 during the neurosphere formation. The dorsoventral identity in noggin neurospheres was sharply regulated by Shh-N and Wnt3a, but the neurosphere-initiating progenitors from low- and high-RA-treated EBs seemed relatively less competent to respond to these factors. We previously showed that EBs treated with low-RA express more of the active form of Shh-N and are ventralized and those treated with high-RA express less Shh-N and acquire a dorsal identity [16]. Thus, low- and high-RA neurospheres exhibited the default identities of ventral and dorsal neural tubes, respectively. On the other hand, BMP4 had a drastic effect. BMP4 induced neurospheres that largely adopted neural crest lineages, generating $peripherin^+$ peripheral neurons and α-SMA^+ smooth muscle cells. Given that BMP2 and BMP4 promote cell death and inhibit the proliferation of rat early cortical progenitors in vitro [44], this difference between BMP4 and other factors in the magnitude of their effects on NS/PCs, including negative effects on neurosphere formation and the potential to differentiate into neural crest lineage cells, may be explained by a mechanism of selective survival. Focusing on neural crest development, because BMP2/4 and Wnt1/3a play important, concerted roles in the formation of the dorsal neural tube, including the neural crest, and are respectively involved in the generation of $Phox2b^+$ autonomic and $Brn3a^+$ sensory neurons in vivo [36, 38], the differentiation of $Phox2b^+$ autonomic and $Brn3a^+$ sensory neurons from neurospheres treated with BMP4 and Wnt3a, respectively, was consistent with the in vivo development of the peripheral nervous system (PNS), demonstrating the possible application of our culture system to the generation of neural crest lineages, including the PNS.

Another important finding of this study is that low-RA neurospheres differentiated into GAD^+, $ChAT^+$, TH^+, and $5\text{-}HT^+$ neurons both in vitro and when transplanted into the lumbar spinal cord of adult rats (Figs. 3 and 7; supplemental online Fig. 7; supplemental Table 2), indicating that these ES cell-derived NS/PCs may have maintained their in vitro-acquired identities of mainly midbrain to hindbrain even after transplantation into the more caudal in vivo environment. Interestingly, the low-RA neurospheres generated some GAD^+ GABAergic neurons (20%–40%) but relatively few $ChAT^+$ cholinergic neurons (up to 5%) in vivo (Fig. 7E, 7H; supplemental online Fig. S7G, S7J, S7K, S7N; supplemental online Table 2), even though they differentiated into many GAD^+ GABAergic and $ChAT^+$ cholinergic neurons in vitro (Fig. 3D, 3E). It is well known that neurons that make contact with their target cells and form the appropriate synaptic circuitry selectively survive better than those that do not form such connections in the nervous system [45–47]. Thus, it is reasonable to speculate that GABAergic interneurons derived from the grafted low-RA neurospheres might have been able to form synaptic contact with their target cells, which are abundant within the host spinal cord [48] and thereby survived well even in vivo. On the other hand, grafted neurosphere-derived cholinergic neurons, including motor neurons, might not have been able to