Stem Cell-derived Neural Stem/Progenitor Cell Supporting Factor Is an Autocrine/Paracrine Survival Factor for Adult Neural Stem/Progenitor Cells*

Hiroki Toda‡‡§, Masayuki Tsuji‡, Ichiro Nakano§, Kazuhiro Kobuke‡, Takeshi Hayashi‡, Hironori Kasahara‡, Jun Takahashi§, Akira Mizoguchi, Takeshi Houtani**, Tetsuo Sugimoto**, Nobuo Hashimoto§, Theo D. Palmer¶, Tasuku Honjo, and Kei Tashiro‡‡§§

From the ‡Department of Medical Chemistry, Kyoto University Graduate School of Medicine, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan, the §Department of Neurosurgery, Kyoto University Graduate School of Medicine, Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan, the ¶Department of Anatomy and Brain Science, Kansai Medical University, Osaka 570-8506, Japan, the ‡‡Department of Neurosurgery, Stanford University, Stanford, California 94305, and the §§Center for Molecular Biology and Genetics, Kyoto University Graduate School of Medicine, Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Recent evidence suggests that adult neural stem/progenitor cells (ANSCs) secrete autocrine/paracrine factors and that these intrinsic factors are involved in the maintenance of adult neurogenesis. We identified a novel secretory molecule, stem cell-derived neural stem/progenitor cell supporting factor (SDNSF), from adult hippocampal neural stem/progenitor cells by using the signal sequence trap method. The expression of SDNSF in adult central nervous system was localized to hippocampus including dentate gyrus, where the neurogenesis persists throughout life. In induced neurogenesis status seen in ischemically treated hippocampus, the expression of SDNSF was up-regulated. As functional aspects, SDNSF protein provided a dose-dependent survival effect for ANSC following basic fibroblast growth factor 2 (FGF-2) withdrawal. ANSCs treated by SDNSF also retain self-renewal potential and multipotency in the absence of FGF-2. However, SDNSF did not have mitogenic activity, nor was it a cofactor that promoted the mitogenic effects of FGF-2. These data suggested an important role of SDNSF as an autocrine/paracrine factor in maintaining stem cell potential and lifelong neurogenesis in adult central nervous system.

Studies carried out in the last few decades have revealed the potential for lifelong neurogenesis in the adult mammalian central nervous system (CNS). At present, discrete regions of the central nervous system, i.e., the adult brain, the subventricular zone of the forebrain and the subgranular layer of the hippocampal dentate gyrus, are known to mediate adult neurogenesis (1–4), but little is known about signaling that maintains the pool of self-renewing stem cells within these regions. Primary culture of adult neural stem/progenitor cells (ANSCs) is a potent tool to investigate signals controlling adult neurogenesis. ANSCs can be isolated and expanded by means of epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF-2). Cycling cells can maintain the properties of self-renewal and multipotency (5–8). The fate of ANSCs is under tight environmental control, and various extrinsic factors to promote lineage commitment have been described (9–11). ANSCs grafted into the neurogenic or non-neurogenic regions give rise to neurons in a site-specific manner, i.e., only within the neurogenic regions (7, 12–14).

Therefore, we anticipate that cell fate is tightly regulated by specific signaling molecules within neurogenic region or “stem cell niche.” However, the maintenance of the stem cell phenotype may also be an important attribute of the stem cell niche, and in vitro culture has provided evidence that stem cells themselves may produce autocrine/paracrine factors that facilitate proliferative self-renewal (7). Glycosylated cystatin C (15) and insulin-like growth factor-1 (16) are two such essential autocrine/paracrine molecules that have been identified as cofactors of FGF-2 and EGF, respectively. To further explore autocrine/paracrine signaling within the stem cell niche, therefore, we used the signal sequence trap method (17, 18) to isolate additional novel secretory molecules from ANSCs. Using this cDNA screening method, we have efficiently isolated many secreted molecules with a wide variety of functions (19–25).

In this report, we describe the cloning and characterization of a novel molecule, stem cell-derived neural stem/progenitor cell survival factor (SDNSF), from a cDNA library from rat adult hippocampal NSCs (9). SDNSF is secreted via the classical vesicular export pathway and provides trophic support for ANSC in the absence of mitogenic growth factors. In addition, SDNSF maintains the self-renewal potential and multipotency of ANSC in vitro. SDNSF maintains the self-renewal potential and multipotency of ANSC in vitro.

Abbreviations used: CNS, central nervous system; NSC, neural stem/progenitor cell; ANSC, adult neural stem/progenitor cell; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor 2; IGF-1, insulin-like growth factor-1; SDNSF, stem cell-derived neural stem/progenitor cell supporting factor; DDEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ES, embryonic stem; dpc, days postcoitus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BrdU, 5-bromo-2-deoxyuridine; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; RT, reverse transcription; NTA, nickel-nitrotriacetic acid; DIVn, nth day in vitro; ANOVA, analysis of variance; LSD, least squares difference; En, embryonic day n; Pn, postnatal day n; GFAP, glial fibrillary acidic protein.
of ANSCs in the absence of FGF-2. SDNSF-treated ANSCs can proliferate in response to FGF-2 and produce neuronal and glial cell population after differentiation treatment.

### EXPERIMENTAL PROCEDURES

**Animals and Transient Forebrain Global Ischemia Model**—Fisher 344 rats, Sprague-Dawley rats, and C57BL/6 mice were maintained and used for experimentation according to the guidelines of the Kyoto University Animal Research Committee and Stanford University Animal Facility. The animals were deeply anesthetized, sacrificed with sodium pentobarbital, and then dissected immediately or fixed by intracardiac perfusion with 4% paraformaldehyde. For cell cultures, the brains were dissected with a 10× stereomicroscope to the cell culture settings as described previously. Then, the tissue extraction, the tissues were immediately frozen in liquid nitrogen. For in situ hybridization, 50-μm-thick coronal sections were prepared from adult female Fisher 344 rat brains after perfusion with 4% paraformaldehyde.

Transient global ischemia was induced on male Sprague-Dawley rats (300–350 g; Charles River Laboratories, Wilmington, MA) by bilateral common carotid artery occlusion and induced hypotension using the modified two-vessel occlusion method (26). In brief, the animals were anesthetized with 1.5% isoflurane, 65.5% nitrous oxide, and 30% oxygen and monitored from the femoral artery with PE-50 catheter (247410; Becton Dickinson, San Diego, CA). After exposure of the right jugular vein and both common carotid arteries, 150 IU/kg heparin was injected intravenously, and blood was withdrawn into the jugular vein. When the mean arterial blood pressure became 30 mm Hg, both common carotid arteries were clamped with surgical clips. Mean arterial blood pressure was maintained at 30–35 mm Hg for 5 min. After ischemic treatment, the clips were removed, and the blood was reinfused. Body temperature was monitored with a rectal probe and controlled at 37 °C. Sham-operated animals underwent exposure of vessels without blood withdrawal or clamping of carotid arteries. All animals were treated in accordance with the guidelines of the Kyoto University Animal Research Committee guidelines, Stanford University guidelines, and the animal protocol approved by Stanford University’s Administrative Panel on Laboratory Animal Care.

**Cell Culture**—ANSCs were isolated from adult rat hippocampi and cultured as described previously (6, 27). Briefly, hippocampi from adult female Fisher 344 rats were enzymatically dissociated with a papain (2.5 units/ml; Worthington, Freehold, NJ), dispase II (1 unit/ml; Roche Applied Science), and DNase I (250 units/ml; Worthington) solution. A cell suspension containing fractionated cells was washed free of Percoll and plated onto poly-l-ornithine/laminin-coated dishes in DMEM/Ham’s F-12 medium (1:1) containing 10% FCS medium for 24 h, and then the medium was replaced with serum-free growth medium consisting of DMEM/Ham’s F-12 medium (1:1) supplemented with N2 supplement (Invitrogen) and 20 ng/ml recombinant human FGF-2 (Genzyme, Cambridge, MA). To follow the proliferating single NSC, NSCs were infected by replication-deficient recombinant human FGF-2 (Genzyme). To follow the presence of FGF-2 at a concentration of 20 ng/ml. COS-7 cells, rat whole embryo at 8.5 days postcoitus (dpc) and 10.5; embryonic and postnatal rat brain from 12.5, 14.5, 16.5, and 18.5 dpc and P2, P4, and P7. Equal amounts of samples were subjected to RT-PCR as previously described (25). Each reaction was standardized against a GAPDH control to permit comparison between samples in each PCR. cDNA was generated using an image analyzer (BAS 2000, Fuji Film, Tokyo, Japan).

For in situ hybridization, we used riboprobes for the in situ detection of mRNAs derived from rat SDNSF as described previously (36). One-kb cDNA fragments including coding region and 3′-noncoding region of the rat SDNSF were generated and subcloned into pGem-T vector (Promega, Madison, WI). Digoxigenin-labeled sense and antisense rat SDNSF probes were generated with SP6 and T7 polymerases, respectively, using the digoxigenin RNA labeling kit (Roche Applied Science).

RT-PCR, total RNA was extracted using Trizol reagent from undifferentiated ANSCs, differentiated ANSCs, embryo NSCs, C6, N18, U251 cells; rat whole embryo at 8.5 days postcoitus (dpc) and 10.5; embryonic and postnatal rat brain from 12.5, 14.5, 16.5, and 18.5 dpc and P2, P4, and P7. Equal amounts of samples were subjected to RT-PCR using a set of primer pairs: 5′-GCACCACCAACT-3′ and 5′-GTCGAGCGCGAAGCGCTT-3′. The Northern analysis was performed essentially as described (35). Rat SDNSF and rat GAPDH (459–1001 of GenBank accession number M17701) cDNAs were labeled with [α-32P]dCTP by random priming. The filtering for Northern analysis was prepared from 4 μg of poly(A) RNA prepared as above from all cells and tissues: ANSCs, adult rat brain, heart, lung, liver, spleen, muscle, kidney, testis, and skeletal muscle. The filter was washed with Quick-Hyb solution (Stratagene, La Jolla, CA). Blotting was analyzed using an image analyzer (BAS 2000, Fuji Film, Tokyo, Japan).

**Protein Analysis and Immunological Methods**—Rat SDNSF was cloned into pEF-6V5-His (Invitrogen) with swapping the original V5 epitope to the FLAG epitope tag at the C terminus (pEF-His.FLAG) to generate FLAG fusion protein, SDNSF-C.FLAG/His. We also inserted
FLAG epitope tag at the predicted signal sequence cleavage site and constructed rat SDNSF-N.FLAG fusion protein. The primer sequences for rat FLAG-SDNSF construction were 5'-GACTAGTCTAGGATCC-CTCGAGCTGCTAGAGGCTTTGCCCTTGTGCTGCTGCTGAGCG-3' and 5'-CTACTGCTAGGCAAGTGGGTGTCCTGAC-3'. HEK293T cells and COST cells were transfected with these expression vectors pEF-SDNSF/C.FLAG/His, pEF-SDNSF-N.FLAG, or pEF-HisFLAG in serum-free DMEM/Ham's F-12 medium (1:1) with N2 supplement using CellPhect (Amersham Biosciences) and were treated with lysis buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris-HCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). The conditioned medium from these transfected cells was filtered through a 0.22 μm filter and stored at −20 °C. The conditioned medium was dialyzed with Slide-A-Lyzer (Pierce) overnight at 4 °C. The concentration of the purified SDNSF protein was determined with Coomassie protein assay reagent (Pierce). We used antibodies against phosphate-buffered saline at 4°C. The protein concentrations were determined with Coomassie protein assay reagent (Pierce). We measured endotoxin/lipopolysaccharide activity by Toxocolor LS-6 (Shionogi, Osaka, Japan) and 450 positive signals in the Toxocolor protocol; also by following clonal expansion of single GFP-labeled ANSC, SDNSF effects on proliferational activity were estimated. To test the self-renewal and differentiation potential of SDNSF-treated ANSCs, after ANSCs were cultured in the SDNSF / FGF-2 medium (100 ng/ml of SDNSF without FGF-2) for 6 days, those ANSCs were replated on noncoated Nunc 48-well plates at the density of 2,000 cells/cm² and grown for 6 days in growth medium containing 20 ng/ml of FGF-2, and the number of neurospheres were counted. To assess the differentiation potentials, the newly formed neurospheres were replanted on poly-L-ornithine/laminin-coated Labtek 4-well chamber slides (Nunc) at the density of 10–30 spheres/well and cultured in differentiation medium for further 6 days. Under confocal laser microscope or fluorescent microscope, positively stained cells were quantified at least 100 fields systematically across the coverslips from three to four independent experiments of parallel cultures.

**RESULTS**

**Isolation of Human, Rat, and Mouse SDNSF cDNA Clones**— Yeast transformants (3 × 10⁶) from ANSC cDNA library were plated onto the concentric circular plates obtained by the signal sequence trap method as described previously (18). Nucleotide sequences of all positive clones were determined. Of 29 independent clones, 27 clones were identical or homologous to sequences reported in rat or other mammals and two clones were novel. Examples of isolated known secretory or surface molecules, which bear secretory signal sequences include CD44, CD164 (MGC-24), chemokine CX3C, neural adhesion molecule F3, seizure-related gene (SEZ-6), CNS myelin membrane glycoprotein M6, growth/differentiation factor-15/macrophase-inhibiting cytokine-1, transforming growth factor-β2, serpin receptor Cyt28, neurotrimin, podocalyxin, protocadherin 7, and cystatin C. The full-length cDNA for rat SDNSF was isolated from rat ANSC and rat brain cDNA library. The sequence of the full-length rat SDNSF encodes a 145-amino acid protein (Fig. 1A). The translation start site methionine was assigned at nucleotide position 80 because of the presence of an N-terminal signal sequence and the compatibility of sequences immediately upstream with the Kozak consensus initiation sequence (44).

Homology searches on DNA data bases revealed partial cDNA fragments of mouse and human SDNSF, whose full-length sequences were integrated and confirmed on the product of RT-PCR from mouse brain and human heart poly(A)⁺ RNA. The deduced amino acid sequences of the mouse and human SDNSF were compared with the rat SDNSF (Fig. 1A). The mouse and human SDNSF have 91.0% (132 of 45) and 85.5% (124 of 145) identity with rat SDNSF in their amino acid sequences, respectively. Data base searches also revealed that Caenorhabditis elegans gene NP 505967, which had been reported from the genetic analysis of developmental arrest and longevity in C. elegans (45), had 44.8% (65 of 145) identity, and the CG17271 gene product of Drosophila melanogaster had 39.3% (57 of 145) identity with rat SDNSF (Fig. 1A). These data base searches suggest that SDNSF genes are highly conserved.
SDNSF Is Secreted via the Classical Secretory Pathway—
The deduced amino acid sequence has an N-terminal hydrophobic domain, which is presumed to be the signal peptide, and has no other hydrophobic regions that can serve as a transmembrane domain. Among the known motifs, EF hand motifs were recognized (Fig. 1); however, there were no known Golgi or endoplasmic reticulum retention signals at its C terminus such as KDEL, HDEL, or HDEF (20, 21). These data base search results suggested that SDNSF was a secreted protein. To determine the validity of this signal sequence, HEK293T cells and COS7 were transfected with SDNSF plasmids, which were tagged with inserted FLAG at the predicted cleavage site (pEF-SDNSF-N/H11032 FLAG) or with FLAG/His at C terminus (pEF-SDNSF-C/H11032 FLAG/His). The expression of these tagged SDNSF proteins was examined with Western blot analysis, and their subcellular localization was analyzed with immunofluorescent staining and immunoelectron microscopy. In both of the cell lysates and their concentrated conditioned mediums, tagged SDNSF proteins were detected at 19 kDa on the blotted membrane with anti-FLAG antibody (Fig. 2A). The slight size difference on blotted membrane between SDNSF-N/H11032 FLAG or SDNSF-C/H11032 FLAG/His in electrophoresis might come from the different tags inserted at the different positions in synthetic SDNSF protein.

To visualize the subcellular localization of SDNSF, the transfected COS7 cells with the SDNSF-C/H11032 FLAG/His construct were stained with anti-FLAG antibody. Under a confocal laser microscope, the staining profile exhibited a perinuclear distribution, which is a typical pattern of endoplasmic reticulum staining (Fig. 2B). Furthermore, the immunoelectron microscope using anti-FLAG antibody revealed that the expressed fusion proteins were densely packed in the nuclear envelope, endoplasmic reticulum, Golgi apparatus, and secretory vesicles (Fig. 2C). The subcellular localization pattern of sorted SDNSF was in accordance with classic vesicular pathway. According to the results shown above, we conclude that the predicted signal peptide of SDNSF functioned normally in the intracellular sorting process and that SDNSF is a secreted protein via classic vesicular pathway.

To obtain the purified SDNSF protein, overexpressed SDNSF-C’/FLAG/His were treated with Ni-NTA-agarose, and eluted samples were analyzed after SDS-polyacrylamide gel electrophoresis and silver staining with using NIH Image 1.62. Intensity of the band for SDNSF was compared with the total bands visualized (Fig. 2D). After Ni-NTA-agarose treatment, the major fractions of bovine serum albumin were removed, and the ratio of SDNSF to the total proteins was 88.4% in elute 3 (Fig. 2D). From the amount of SDNSF protein in elution 1 through 4, the recovery rate were estimated as 51%. In the following experiments, 88.4% of the obtained SDNSF protein was treated as purified SDNSF protein.

Although the SDNSF amino acid sequence has two EF hand motifs, SDNSF could not show the calcium binding activity with 45Ca2+ blotting assay (40) and Ca2+-dependent mobility shift assay (41). SDNSF has no predicted N-glycosylation site and deglycosylation assay (42), and lectin blot assay (43) did not reveal any detectable glycosylation (data not shown).

SDNSF Is Localized at Dentate Gyrus, CA3, and Subiculum in Adult Rat Brain—To examine the systemic distribution of SDNSF, the poly(A)+ RNA from various rat tissues were tested.
with Northern blot analysis, and it was revealed that every tested organ expressed the major transcript of 1.8 kilobases (Fig. 3). To localize the distribution of SDNSF in the adult brain, especially for the relationships with neurogenesis, in situ hybridization was performed. The positive signals for SDNSF probe that includes the 3/7-untranslated region were detected in hippocampus (Fig. 4, A and C) and subiculum (Fig. 4, D). In hippocampus, some immunoreactive cells were localized at the stratum oriens and in dentate gyrus, they were distributed at the polymorphic cell layer also called hilus. Some immunoreactive cells are localized at the subgranular zone of granule cell layer, where the neurogenesis persists throughout the life (46). In another major region of adult neurogenesis, the subventricular zone of lateral ventricles, we could not detect positive signals for SDNSF expression (Fig. 4D). According to these in situ hybridization data, we can expect that the expression of SDNSF is related with ANSC and/or neurogenesis-related neurons in hippocampal dentate gyrus.

To observe the correlation of SDNSF with neurogenesis, the expression of SDNSF in several neural cell types and developing brain tissue was examined with RT-PCR. The SDNSF transcript was moderately or highly expressed in developing brains, especially late embryonic stages, primary hippocampal
neurons, embryonic NSCs, and neuroblastoma N18 cells, glioblastoma U251 cells, and ANSCs (Fig. 5A). The signal levels in primary astrocytes and C6 glioma cells were low (Fig. 5A).

Therefore, we assumed that SDNSF was expressed not specifically to nervous system, but their distribution in the CNS might be restricted to several cell types such as NSCs and neuronal cells and less astroglial cells.

Further, the expressions of SDNSF in the induced adult neurogenesis were also tested. It is known that ischemic stress to the brain induces neurogenesis in hippocampal dentate gyrus (47).
We performed forebrain ischemic treatment on adult rat and induced hypoxic stress on hippocampus (26). Semiquantitative RT-PCR results showed on postoperative days 1 and 7 that the SDNSF transcripts were highly up-regulated (Fig. 5, B and C).

**SDNSF Improves ANSCs Viability in the Absence of FGF-2**—To test the SDNSF biological effects on ANSC, SDNSF activity was assessed in the absence of FGF-2. The addition of recombinant SDNSF in growth medium without FGF-2...
FGF-2/H11002 growth medium had a significant effect on ANSC viability. The purified recombinant SDNSF protein was added to FGF-2/H11002 growth medium at concentrations of 0.1, 1, 10, 100, and 500 ng/ml when ANSCs were plated at a density of 1,000 cells/cm². At DIV6, premix WST-1 assay was performed to measure the number of viable cells or cell viability by detecting the cleavage of tetrazolium salts and mitochondrial enzyme activity. It was shown that SDNSF improved ANSCs viability in a dose-dependent manner (Fig. 6A). SDNSF at the concentration of 100 ng/ml has a statistically significant difference on viability of ANSCs (Fig. 6B). To see whether this difference of viability came from proliferation activity of SDNSF, mitogenic activity was examined by tracking single cell proliferation via BrdU incorporation. In addition, the progeny from ANSCs were tracked by marking single ANSCs with replication-deficient GFP-expressing recombinant retrovirus. ANSCs treated with 100 ng/ml of SDNSF did not proliferate as much as FGF-2-treated ANSC, and the sizes of the colonies in SDNSF group were similar to that of the control group (Fig. 6C). BrdU ELISA assay did not reveal an increased uptake of BrdU in the ANSCs treated with SDNSF (Fig. 6D). To test the possibility of SDNSF as a cofactor for FGF-2, ANSCs were cultured in FGF-2 growth medium with 100 ng/ml of SDNSF, however, mitogenesis was not enhanced by the addition of SDNSF (data not shown). Therefore, we conclude that SDNSF improves ANSC viability in the absence of FGF-2. To test similar effects on embryonic stem cells, the mouse ES cell line R1 was used; however, the addition of SDNSF did not show significant improvement of ES cell survival nor proliferation (Fig. 6E).

Under normal conditions, FGF-2 withdrawal induces apoptosis or differentiation of ANSCs (6, 9, 10); however, the addition of SDNSF without FGF-2 improved the survival of ANSCs as we have shown above. To determine whether SDNSF support maintain the multipotency of ANSCs, we test the response of the SDNSF-treated ANSC to FGF-2 and check the formation of neurosphere (30) and phenotype of descendent cells. The formation of neurospheres was used as an indicator for the abundance of stem-like cells (30). ANSCs that had been treated with 100 ng/ml of SDNSF for 6 days could form neurospheres. The number of neurospheres/well was smaller than those from FGF-2-treated ANSCs but more than those from control ANSCs. The data are expressed as the means ± S.D. (n = 8). Statistical analysis was performed by one-way ANOVA followed by a post hoc Fisher LSD test. Significant differences between results for SDNSF, FGF-2, and control are indicated by an asterisk (p < 0.01). B, differentiation profiles of the ANSC that were treated with SDNSF in the absence of FGF-2. The neurospheres formed in FGF-2 growth medium after treatment with SDNSF, FGF-2, or control were differentiated in the differentiation medium. Percentage of Tuj1+ cells and GFAP+ cells from neurospheres were shown with their mean values ± S.D. (n = 10). Statistical analysis was performed by one-way ANOVA followed by a post hoc Fisher LSD test. Significant differences versus control are indicated by an asterisk (p < 0.01).

C–E, immunofluorescence images of the differentiated ANSCs cells from the differently treated ANSCs neurospheres. Tuj-1+ cells were labeled with Cy3. Similarly, NG-2 were with fluorescein isothiocyanate and GFAP with Cy5. ANSCs that had been treated with SDNSF (C), FGF-2 (D), or control (E) for 6 days were stimulated with FGF-2 for the neurosphere formation and then were differentiated in the presence of retinoic acid.
Neural Stem/Progenitor Cell Supporting Factor, SDNSF

6.2 ± 2.0%. Giall marker, GFAP-positive cells were 5.9 ± 2.1%, and oligodendrocyte marker, NG-2-positive cells were 1.2 ± 0.6% (Fig. 7B) from the SDNSF-treated neurospheres. From FGF-2 or control CM-treated neurospheres, Tuj-1-positive cells were 8.8 ± 3.3% or 1.3 ± 0.9%, GFAP-positive cells were 4.8 ± 2.1% or 7.2 ± 2.4%, and NG-2-positive cells were 2.3 ± 1.1% or 0.9 ± 0.5% (Fig. 7, B, D, and E). From these results, the surviving cells in SDNSF-treated ANSCs still retained self-renewal potentials and maintained multipotency to differentiate into neuronal and glial phenotypes as has been seen in FGF-2-treated ANSCs.

**DISCUSSION**

ANSC conditioned medium is known to provide both trophic and mitogenic support for ANSCs, and previous analysis of the ANSC conditioned medium led to the characterization of glycosylated cystatin C as a cofactor for FGF-2 (15). By analyzing roles of IGF-1 in EGF-dependent ANSCs, IGF-1 was also identified as a cofactor of EGF (20). Our attempt here was to identify additional autocrine/paracrine molecules from FGF-2-dependent ANSCs by selecting molecules bearing the secretory signal sequence. By using the signal sequence trap method on a cDNA library from ANSCs, we isolated a previously unknown secreted protein, SDNSF, in addition to several known molecules bearing signal sequences.

An interesting characteristic of SDNSF is an autocrine/paracrine effect on ANSCs themselves. Unlike glycosylated cystatin C or IGF-1, the addition of SDNSF into FGF-2-treated cultures did not modify the proliferation activity of FGF-2, and SDNSF itself did not display mitogenic activity. However, our data indicate that a high concentration of SDNSF improves the viability of ANSCs in the absence of FGF-2. In addition, SDNSF-treated ANSCs were shown to retain stem-cell characteristics, i.e., neurosphere formation as an indirect indicator of self-renewal and multipotency and also retninoic acid-induced differentiation assay. Therefore, we conclude that SDNSF has two effects on ANSCs: (a) to prevent ANSC cell death and (b) to maintain stem cell characteristics. These two effects on ANSCs have been observed in mitogenic growth factors, FGF-2 and EGF. However, SDNSF is unlikely to possess mitogenic activity and also unlikely to be cofactor for other mitogenic growth factors. As for the effect on maintenance of stem cell characteristics, the ability of SDNSF was lower than that of FGF-2 (Fig. 7A). Although SDNSF and FGF-2 (14.41 kDa) have similar molecular weights, SDNSF needs a higher concentration to effect the survival of ANSCs. Considering these characteristics of SDNSF, we could hypothesize that SDNSF delayed cell death and the differentiation processes that usually proceed in the absence of mitogenic growth factors.

The expression analyses probe us another conjecture on the role of SDNSF. SDNSF was expressed in every organ tested in vivo (Fig. 3). However, the different expression and the results of NCSE viability assays, SDNSF could be a novel circulating tumor marker for neuroblastoma or glioblastoma. Because there are no available neural tumor markers secreted in circulating blood or cerebrospinal fluid, it is worth studying further to test SDNSF availability.

Another unique feature of SDNSF is its motif structure. Generally, it is rare that molecules within the EF hand superfamily have a signal peptide. Most of the EF hand molecules with secretory signal sequences usually also retain signals to the organelle, and so they are not secreted extracellularly. Calumenin (20) and FKB23 (21) are localized to the lumen of the endoplasmic reticulum, and Calb45 (22) is localized to the Golgi lumen. The only exception among those EF hand family members with secretory signal sequences is BM-40, also known as SPARC (50), which is known to be secreted. One of the essential roles of EF hands in BM-40 is suspected to be Ca²⁺ binding for folding and secretion of BM-40 (30). Although amino acid sequences around the two EF hand motifs of SDNSF are highly conserved from Drosophila to human, the importance of EF hands in SDNSF remains unknown, and the Ca²⁺ binding capacity remains unconfirmed. In addition, the function of EF hand proteins and involvement of Ca²⁺ in the regulation of ANSCs viability are still unknown.

The unique characteristic of SDNSF is its survival effect on ANSCs without proliferation activity, which was not reported previously for other growth factors or cofactors. This effect of SDNSF might be able to explain why the quiescent ANSCs could persist and maintain the multipotency in adult CNS, and utilizing SDNSF might help the therapeutic application of ANSC for diverse neurological entities by preventing further loss of adult neurogenesis.

**Acknowledgments**—We thank Dr. Tomoyuki Nakamura for modified pEFVS3-His plasmid, Dr. Ami Okada for LZRS-CAMmut4GFP retrovirus, Dr. Yuichi Hori for mouse ES cell R1, and Prof. Hiroshi Nakata (Kyoto Sangyo University) for helpful comments on glycosylation/modification. We also thank Naoko Tomikawa and Masami Tanaka for excellent technical assistance.

**REFERENCES**

1. Altman, J., and Das, G. D. (1965) J. Comp. Neurol. 124, 319–335
2. Bayer, S. A. (1982) Exp. Brain Res. 46, 315–323
3. Kaplan, M. S., and Bell, D. H. (1984) J. Neurosci. 4, 1429–1441
4. Lasliz, M., B. (1996) Neuroeut 11, 173–189
5. Reynolds, B. A., and Weiss, S. (1992) Science 255, 1707–1710
6. Palmer, T. D., Ray, J., and Gage, F. H. (1995) Mol. Cell Neurosci. 4, 744–486
