Resilience to Transformation and Inherent Genetic and Functional Stability of Adult Neural Stem Cells Ex vivo

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Abstract
Recent observations have suggested that extensive culturing of adult neural stem cells (ANSCs) by exploiting the Neurosphere assay might select for aggressive cell clones, endowed with neoplastic potential, that overgrow the rest of the native stem cells. However, a detailed study of the propensity of ANSCs to transform has never been thoroughly undertaken. Here, we report the first demonstration that ANSCs can be propagated in vitro for over a year, maintaining a strikingly stable profile with regard to self-renewal, differentiation, growth factor dependence, karyotype, and molecular profiling. Most importantly, the long-term culturing of ANSCs did not result in the formation of tumors in vivo, even when ANSCs were transduced with Myc and Ras oncogenes. The cancer resistance could depend on specific mechanisms aimed at protecting ANSCs and preserved by optimal nonstressful culture conditions. In conclusion, besides a plentiful and safe source of cells for therapeutic applications, ANSCs provide an ideal model to study aging and cancer in the context of stemness. [Cancer Res 2007;67(8):3725–33]

Introduction
Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate the mature cells of their specific tissue through differentiation, persisting for long periods of time in highly proliferative areas of the body. The therapeutic efficacy of stem cells relies largely on their ability to replicate. Most adult stem cells do not have an unlimited self-renewal capacity, but reach a replicative senescent state, thus exhibiting a definite lifespan in vitro. On the other hand, other adult stem cells, including those from the central nervous system (CNS), are believed to divide indefinitely by self-renewal and this feature can be exploited for cell expansion in vitro. Indeed, the long life and large replicative potential of stem cells might link neoplastic growth to stem-cell biology, making stem cells good candidates for acquiring a tumorigenic phenotype (1).

Interestingly, subventricular zone–derived adult neural stem cells (ANSCs), which are of broad scientific interest for therapeutic cell replacement and gene transfer, may be at risk for malignant transformation, and, in fact, cancer stem cells have been identified and characterized in several CNS tumors, including glioblastoma (2–4).

Recent studies support the observation that spontaneous transformation of adult stem cells can take place after long-term culture in vitro (5, 6). In light of this, it has been suggested that extensive culture in vitro might generate malignant cells able to grow indefinitely (7, 8). To circumvent this presumptive limitation, it has become commonplace to avoid using ANSCs from neurospheres beyond the 10th passage (9, 10), thus hampering one of the most crucial advantages of neurospheres; that is, their significant cell expandability ex vivo (11).

Here, we report that ANSCs were propagated in culture for up to 70 passages without any transformation sign. ANSCs displayed a strikingly stable profile with regard to self-renewal, expansion, differentiation, growth factor dependence, and karyotype and molecular profiling. When forced to grow in vitro for over 100 passages, ANSCs did not undergo transformation and never gave rise to tumors in vivo, even when immortalized through delivery of Myc and Ras oncogenes.

This work shows that, under suitable culture conditions, ANSCs satisfy the most stringent requirements to qualify as a plentiful and safe source of neural cells.

Materials and Methods
Primary culture, culture propagation, population analysis, and cloning. Adult neural stem cell line (NSCL) cultures NSCL1, NSCL2, NSCL3, and NSCL4 were established from the subventricular zone of 2-month-old CD-1 albino mice (Charles River Laboratories, Wilmington, MA) as described previously (12–14). Tissues derived from 20 mice were pooled and processed to generate each culture. Cells were plated at a density of 8,000/cm² in DMEM/F-12 medium containing 20 ng/mL of both epidermal growth factor (EGF) and fibroblast growth factor (FGF-2; Peprotech, Rocky Hill, NY). For growth curves, 200,000 cells were replated in a 25-cm² flask after each subculturing passage. Population and serial subclonogenic analyses were done as described previously (15).

Differentiation of stem-cell progeny and immunocytochemistry. To assess multipotency, cells were plated at a density of 5 × 10³/cm² onto Matrigel-coated glass coverslips (12-mm diameter) in the presence of 20 ng/mL FGF-2. After 48 h, cultures were shifted to mitogen-free medium containing 2% fetal bovine serum (Invitrogen, Carlsbad, CA) for 5 days. Multiple immunofluorescence for neural antigens were done as previously described (12, 15).

Chromosome analysis and telomere length assessment. Q-banding staining was done as described previously (2). Forty metaphases were analyzed for each sample. The length of telomeres was determined using Telomere Assay (Roche, Gipf-Oberfrick, Switzerland) according to the manufacturer’s instructions. Two micrograms of genomic DNA of each sample were digested with HinI/BsuAI.

Macroarray analysis. Five micrograms of total RNA, extracted from NSCLs, were used to synthesize [32P]dATP-radiolabeled cDNA probes for hybridization to Atlas Mouse Cancer 1.2 Array (BD Biosciences Clontech, Palo Alto, CA). Probe labeling and hybridization were done as described in the Clontech Atlas cDNA Expression Arrays User’s Manual (PT3140-1). The
hybridization signals were scanned with a Phosphorlmager and subsequently analyzed using AtlasImage software.

**Retroviral vector and infection.** Replication-deficient retrovirus PK-VM-2 coding for the v-myc oncogene (described in ref. 16) was kindly provided by Dr. L. DeFilippis (Department of Biotechnology and Biosciences, University of Milan Bicocca, Milan, Italy). The Myc-tagged oncogenic form of H-Ras (H-RasG12V), a kind gift of Dr. R. Brambilla (DIBIT, H. San Raffaele, Milan, Italy), was cloned into the pLENITI6 vector and used to prepare lentiviral construct by the Viral Power Lentiviral Expression System (Invitrogen) according to the manufacturer's recommendations.

Cell transduction was carried out by dissociation of the neurospheres and plating in the presence of the viral supernatant containing 8 μg/mL polybrene for 24 h. Mouse embryonic fibroblasts (MEF) were infected as described previously (17). G418 at 500 μg/mL and blasticidin at 5 μg/mL were used for drug selection of resistant clones of ANSCs and MEFs.

**Reverse transcription-PCR.** Total RNA was isolated from ANSCs by Trizol (Invitrogen). Two micrograms of total RNA were reverse-transcribed by Superscript Rnase H-Reverse Transcriptase (Life Technologies, Gaithersburg, MD) and oligo(dt) (Amersham Biosciences, Piscataway, NJ), Products corresponding to the v-myc and the H-RasG12V integrated provirus were amplified by PCR using Taq DNA Polymerase (Qiagen, Valencia, CA) and the following primers: v-myc (gag-myc fusion region) forward, 5′-CCTTGTGTTGATTTCGCCAAT-3′; reverse, 5′-AGTGTCTCCTCCCTCCCGTG-3′; H-RasG12V (myc-tagged region) forward, 5′-AGGAGGACCTGATCAGCAG-3′; reverse, 5′-GGGAGGACCCACTGTGCCA-3′. cDNAs used as templates were previously normalized throughout glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse transcription-PCR (RT-PCR).

**In vitro tumorigenic assays.** Tumorigenicity was determined by injecting NSCLs either s.c. or orthotopically into 6-week-old Scid/bg mice. Cells (3 × 10^6) in 200 μL of DMEM were s.c. injected into the right flank of mice, whereas 2 μL of a 1 × 10^6 cells/mL cell suspension in DMEM were delivered into the right striatum (0.2 μL/min) by stereotactic injection (2). Immunohistochemistry was done on 60-μm-thick vibratome sections of mouse brains using the antiserum rabbit anti-Ki67 (1:1,000; NovoCastra, Newcastle, United Kingdom). Sections were processed as described previously (18).

**Results**

**Reproducible long-term–cultured ANSC lines maintained the functional properties expected from bona fide NSCs.** The Neurosphere assay (NSA) is the most widely used technique to culture ANSCs (11) and represents the bona fide methodology to identify, isolate, and propagate ANSCs (12–15). By exploiting this method, we have been able to generate and culture a remarkable number of ANSC lines through the years. Under our standard culture conditions, a common trend in the growth properties of ANSCs was observed. In fact, although each ANSC line could exhibit an intrinsic proliferative rate, all ANSC lines were endowed with long-term self-renewal capacity and expanded in an exponential manner for many subculturing passages, with little changes in their global proliferation or differentiation potential. Figure 1A shows a representative selection (n = 20) of the growth curves of individually established mouse ANSC lines.

To extrapolate an unambiguous proliferative trend of long-term *in vitro* expanded ANSCs, we characterized in detail four ANSC lines, named NSCL1, NSCL2, NSCL3, and NSCL4, generated ad hoc by exploiting the NSA (13). This method allows the positive selection of undifferentiated ANSCs by taking advantage of their capacity to extensive proliferation and self-renewal while progressively eliminating lineage-restricted progenitors (11).

All four NSCLs were expanded for up to 70 passages, corresponding to 240 days *in vitro* and analyzed for the fulfillment of the cardinal requirements for "stemness." The concept of *in vitro* self-maintenance implies that stem cells should retain the same functional properties as detected in the early stages of culture, even when grown for extended periods of time. Thus, the defining criteria expected from bona fide ANSCs (i.e., self-renewal capacity, functional stability, and capacity to differentiate into neurons, astrocytes, and oligodendrocytes) must be steadily maintained throughout extensive culturing.

To investigate both self-renewal and the proliferation ability of ANSCs at different passages *in vitro*, we generated long-term growth curves from each of the four independent NSCLs. All the NSCLs maintained stable growth capacity over time, displaying a constant proliferation rate between passages, while consistently expanding in number (Fig. 1B). However, the overall kinetics of expansion as well as the average value of total cell amount yielded were characteristic of each established NSCL. Within 70 passages of culturing and starting from the initial 2 × 10^6 cells, the estimated final yield was ~10^9 cells.

As shown in Fig. 1C, we analyzed the effect of cell subculturing on the global growth rate by plotting the number of cells generated after each single passage against the time the cells spent *in vitro* (assessed as the number of passages). To have a better view of the underlying trend, while de-emphasizing the random noise superimposed on each observation, we smoothed the original time series using a simple five-point moving average (i.e., the observation at the generic passage j was substituted by the average value of the five adjacent observations measured at passages j ± 1, and j ± 2). This smoothing revealed a gentle upward trend in the number of cells per passage over the 70 passages covered by our data. The average number of cells, yielded per passage during 240 days of *in vitro* culture, ranged from 1.2 × 10^6 at the early passages (p1–p15) to 2.3 × 10^6 at later passages (p55–70), suggesting that long-term culturing resulted in a very modest proliferation rate increase considering the long time of total cell culturing. Most interestingly, NSCLs maintained this trend for over 400 days *in vitro* (see next sections), thus showing unlimited self-renewal ability.

Moreover, cell density does not seem to affect the stability of the growth profiles. The growth curves in which the plating density was increased (24,000 cells/cm^2 versus 8,000 cells/cm^2) displayed a growth rate trend consistent with low-density experiments (data not shown).

The relative frequency between symmetrical proliferative divisions and symmetrical differentiative divisions is one of the mechanism by which ANSC self-renewal is regulated (12, 13, 15). To evaluate the contribution of this variable, every 10 passages in culture, all four NSCLs were subjected to serial clonogenic assay. This assay allows the estimate of ANSC clonal efficiency by assessing the frequency of secondary neurospheres generated after dissociation of primary neurospheres into single cells and then replating under identical condition. The generation of secondary clones of comparable size could be observed in all tested passages. However, in accordance with the growth curve trend, the clonal frequency significantly increased throughout subculturing. This suggests that our culture conditions were likely to select a "true" stem-cell population by excluding step-by-step residual progenitors and progressively enriching the stem-cell component (Fig. 2A; ref. 11).

Multipotency is the other fundamental requirement expected from bona fide ANSCs. The withdrawal of mitogens from the culture conditions induces the differentiation of ANSCs into neurons, astrocytes, and oligodendrocytes, all the three lineages being generated within each individual clonal neurosphere (12, 13). Indirect immunofluorescence for neural-specific antigens showed that, at different subculturing steps, all NSCLs exhibited prompt...
differentiation upon growth factor withdrawal. Both multipotency and differentiation characteristics of ANSCs were preserved over time, and no appreciable quantitative and qualitative differences could be detected between early and late cultures (Fig. 2B and C). It is important to highlight that, unlike tumor stem cells (2), neuronal, astroglial, and oligodendroglial markers never colabeled in ANSCs, even in very high-passage cultures (Fig. 2D).

Long-term culturing of ANSCs does not result in the acquisition of transformed features. Although we never observed important modifications in the behavior of ANSCs following prolonged growth in culture, we set out to evaluate in NSCLs in vitro indicators of cellular transformation, including loss of growth factor dependence, karyotype changes, and altered patterns of gene expression.

Aberrant growth occurs when cell lines exhibit lower growth factor dependence, becoming self-sufficient in the production of growth factors (19). ANSCs are strongly dependent on the mitogenic stimulation provided by EGF and FGF-2 to proliferate and self-renew (13). When low and high passages of NSCLs were plated in the absence of mitogenic stimulation, ANSCs did not survive. On the rare occasion in which few neurospheres formed, they could be subcultured without mitogens for only one passage and never gave rise to tertiary neurospheres, in contrast with previous observations (7). Notably, the sporadic detection of growth factor–independent neurospheres was never correlated with the time spent by stem cells in culture (Fig. 3A).

Aneuploidy and chromosomal aberrations frequently occur in transformed cells (20); however, stem cells are considered to have special machinery to maintain intact their replicative potential without accumulating abnormalities (21). Accordingly, Q-banding karyotypic analysis of all four NSCLs at regular time points showed that the karyotype did not change significantly during long-term culture (Fig. 3B). Indeed, >85% of ANSCs were euploid (38 paired autosomes and two sex chromosomes) as were ANSCs tested at the second passage from isolation (Fig. 3B). Only after the 60th passage, we detected a single metacentric chromosome in 10% of cells of NSCL4 (Fig. 3B and C). The presence of a single metacentric chromosome (called marker 1) was probably the result of chromosomal end-to-end fusions of Robertsonian type, suggesting the appearance of a putative aberrant marker. These rearrangements are detected in transformed murine stem cells (5) but also in long-term cultured normal embryonic stem cells (22).

Figure 1. Proliferation analysis of NSCL lines. A, representative selection of the growth curves of 20 established mouse NSCLs, cultured by exploiting the NSA. B, long-term proliferation curves for the ad hoc established four independent cultures, NSCL1, NSCL2, NSCL3, and NSCL4. The total number of cells cultured for >240 days in vitro was calculated at each subculturing passage. Exponential expansion rate was maintained over time and the total amount of cells, yielded from a starting number of $2 \times 10^5$, was $\sim 10^{66}$ cells for each cell line. C, relative increase of NSCL growth rate was assessed by plotting the number of cells yielded at each single subculturing passage (>70 total number of passages). Curves reveal a modest but constant upward trend of proliferation rate throughout long-term culturing.
Notably, at the 70th passage, marker 1 was present in an increasing number of cells (40% of metaphases); in addition, 10% of total metaphases displayed the appearance of a new metacentric chromosome marker (called marker 2). Manually aligned karyotype analysis pointed out that both markers were linked with a gain of chromosomes (Fig. 3C). However, growth curve stability, together with regular capacity to differentiate and the maintenance of strict growth factor dependence, confirm the absence of any predisposition to transformation of NSCLs, regardless of the presence or absence of chromosomal anomalies.

The onset of a malignancy-associated phenotype implies genetic changes involving the regulation of many genes controlling the cell cycle, cell differentiation, and immortality (23). Atlas Mouse Cancer 1.2 Array, containing 1,176 murine cDNAs related to cancer, was used to obtain a comprehensive picture of possible genetic changes occurring in long-term cultured ANSCs (Fig. 3D). The array data of all NSCLs showed that the expression of specific cancer-related genes did not change significantly (selection criteria: 2-fold change in signal intensity between the two samples under analysis with \( P < 0.05 \); Fig. 3D). Thus, these results confirmed that a substantial transcriptional stability was maintained in ANSCs. Interestingly, comparison of the expression profiles among the four different NSCLs (\( n = 4 \)) underlined high degree of interline homogeneity in their molecular profiling, attesting to the reliability of the NSA to identify, isolate, and propagate ANSCs.

Long-term expanded ANSCs do not display malignant potential after in vivo transplantation. The cardinal trait of a malignant cell is the capacity to generate tumors in vivo. To ultimately rule out that the extensive in vitro culturing of ANSCs might result in the acquisition of a “latent” malignant phenotype that could be reactivated after in vivo implantation, we did orthotopic transplantation of green fluorescent protein (GFP)-labeled NSCLs at passages 20 and 70 into adult severe combined immunodeficient (SCID) mice (2). Histologic and immunofluorescence stainings were carried out on brains of mice sacrificed 14 and 60 days after the cell implant. The presence of several viable nonproliferating (i.e., Ki67-negative) GFP-positive cells, intermingled with autofluorescent cellular debris, could be observed.

**Figure 2.** Self-renewal capacity, multipotency, and mitogen dependence of NSCLs. A, clonal efficiency of ANSCs at different passages. Cells (1 \( \times 10^5 \)/cm²) were cultured for 7 d in the presence of EGF and FGF-2. The observed increase in clonal efficiency fits well with the proliferation trend plotted in long-term growth curves. B, quantitative analysis of the frequency of CNS lineages in the differentiated progeny of NSCLs at different passages. Neuronal (Tuj1), glial (GFAP), and oligodendroglial (GalC) markers were detected by immunofluorescence. Values are expressed as percentage of immunoreactive (IR) cells over the total cell number. C, analysis of ANSC multipotency at passages 5, 30, and 70 by immunofluorescence for neuronal (Tuj1; red), astroglial (GFAP; green), and oligodendroglial (GalC; red) markers (representative of all NSCL lines). Bar, 30 \( \mu \)m. D, simultaneous triple-labeling immunofluorescence of differentiated ANSCs never revealed the presence of cells colabeled for neuronal (Tuj1; green), astroglial (GFAP; blue), or oligodendroglial (O4; red) markers. Bar, 30 \( \mu \)m.
at the site of transplantation at both time points (Fig. 4A). Of note, some GFP-positive NSCLs were found to be integrated in the striatum and morphologically resembled neuron-like cells (ref. 24; Fig. 4A, white arrows). Most importantly, generation of tumors was never observed in the brains of any of the injected mice (n = 40; Fig. 4B), thus demonstrating the in vivo non-tumorigenic behavior of ANSCs cultured for long periods of time in vitro.

Protracting long-term culturing of ANSCs affects their growth rate but does not result in the acquisition of any transformed or tumorigenic features. Because the process of cell transformation requires an initial step of cell immortalization and, subsequently, the gradual acquisition of the full tumorigenic phenotype, we assessed whether passage 70 ANSCs, cultured for additional 50 passages in vitro, had accumulated part of the genetic alterations that could make them not yet fully malignant but more susceptible to transformation than ANSCs grown in vitro for fewer passages (<70).

We chose to extend our long-term analysis up to passage 120 for selected NSCLs; that is, NSCL4, the only cell line exhibiting structural chromosomal aberrations, and NSCL3, a cell line representative of those that had a normal karyotype.

Strikingly, both NSCLs continuously proliferated for over 400 days of culture in vitro, which represents a very long lapse of time when compared with the overall mouse lifetime, within this time frame, the total cell yield was ~ 9 X 10^{11} cells.

At the end of analysis, NSCL3 line still exhibited a normal karyotype, whereas markers 1 and 2, originally found in NSCL4,
we present in a growing number of metaphases. In addition, NSCL4 showed the appearance of a new marker (marker 3; Fig. 5A). In spite of this, the growth curves of two NSCLs maintained the same slight upward trend displayed throughout the first 70 passages (Fig. 5B).

Consistently with the growth trend, clonogenic assays confirmed a statistically significant increase in the self-renewal ability of NSCLs at the 120th passage with respect to passages between the 1st and the 70th (clonal efficiency at the 120th passage: 28.6 ± 1.7%, mean ± SE, n = 3 for each NSCL3 and NSCL4 lines, ANOVA F = 16.894 P < 0.001). Moreover, NSCLs maintained strict growth factor dependence.

Importantly, NSCL3 and NSCL4 lines underwent terminal differentiation upon removal of mitogens even at passage 120, without significant differences compared with earlier passages [12.6 ± 1.8% Tuj1-positive neurons, 66.8 ± 4.5% glial fibrillary acidic protein (GFAP)–positive astrocytes, 5.9 ± 0.8% GalC-positive oligodendrocytes; mean ± SE, n = 3 for each line, ANOVA, F < 1.78563, P > 0.1097; Fig. 5C]. Coexpression of neuronal and glial markers was never observed (Fig. 5C).

The gene expression profiling done by the same macroarrays on NSCLs at the 120th passage did not exhibit substantial differences when compared with the same lines at the 6th and 70th passages (data not shown).

Finally, NSCL3 and NSCL4 again did not display any significant differences in telomeric length between early (7th and 30th) and late (70th and 120th) passages (Fig. 5D), suggesting that telomere length in ANSCs is probably not correlated with susceptibility to transformation (25).

Oncogenic challenge through Myc and Ras overexpression is not sufficient to induce tumorigenic transformation of ANSCs. Recent evidence suggests that primary rodent cells can be efficiently converted into tumorigenic cells by the delivery of two oncogenes, whereas immortalized rodent cell lines could be transformed by even a single oncogene (17, 26, 27). To challenge the ANSC resistance against transformation events, we assessed the contribution of individual or combined genetic alterations on the acquisition of transformed features.

The proto-oncogene c-Myc, deregulated in a wide variety of tumors, has been used to induce neoplastic transformation in cultured cells or transgenic animals (5, 6). We delivered the v-Myc oncogene (the p110 gag-Myc fusion protein derived from the avian retroviral genome; ref. 16) by retroviral transduction into the karyotype-normal NSCL3 and the karyotype-aberrant NSCL4 both at passage 100 in vitro. The v-Myc–expressing NSCLs were subsequently infected with a lentiviral vector coding for the oncogenic H-Ras (mutated H-RasQ61L). From here on, we will refer to single transduced NSCLs as NSCL3-M and NSCL4-M and to double-transduced NSCLs as NSCL3-MR and NSCL4-MR. Primary MEFs, chosen as a well-characterized model for testing the efficacy of the viral constructs used to transduce ANSCs, were subjected to the same serial infection (17). The expression of Myc- and Ras-coding integrated provirus in all transduced NSCLs was confirmed by RT-PCR (Fig. 6A).

According to prior observations (28), normal untransduced MEFs underwent a progressive decline in the growth rate and, at passage 6, showed a widespread senescence-like phenotype. Only when double transduced with Myc and Ras, MEFs (called MEF-MR) bypassed senescence and started to proliferate extensively. Conversely, both NSCL-MRs displayed unchanged clone morphology (Fig. 6B) and still maintained strict growth factor dependence.

Finally, NSCL3, NSCL3-M, NSCL3-MR, NSCL4, NSCL4-M, and NSCL4-MR cells were orthotopically injected into adult SCID mice. MEF-GFP and MEF-MR-GFP were used as controls (Fig. 6C). Ten days after intracranial transplantation, rapidly growing tumors were observed in mice transplanted with MEF-MRs (Fig. 6D, bottom). Conversely, although some Ki67-negative GFP-positive neuron-like cells, well integrated in the striatum (24), could be detected, no sign of tumorigenesis was ever observed in the brains of mice transplanted with NSCLs, either naïve or genetically modified, even 2 months after intracranial transplantation (Fig. 6D, top).

**Discussion**

The proliferative features of ANSCs, characterized as having a long lifespan and continuous self-renewal, make their behavior similar to that of tumor cells. Accordingly, ANSCs have been recently proposed as precursors to cancer in the brain (4, 8), and, as a consequence, a major criticism raised against their therapeutic applications is their potential propensity to undergo rapid and spontaneous transformation. In fact, a single previous report has proposed that the extensive culturing of ANSCs by the NSA (11) might select for aggressive cell clones. These cells were apparently able to overgrow the rest of the native stem cells generating a mutated population, which grew indefinitely in culture and was endowed with neoplastic potential (7).
To bypass this drawback, several groups have proposed using ANSC lines grown for very few subculturing passages in vitro, thus limiting greatly their potential applications (9, 10). However, rather than the long-term culturing per se, it might be improper execution of the NSA that inevitably results in subtle, yet significant, modifications to the original procedure, which leads to critical inconsistencies in the behavior and properties of cultured ANSCs. In fact, when correctly done, the NSA enables the maintenance of ANSCs as functionally and genetically stable cells that self-renew for an extended period of time (in theory, indefinitely) with no change in their differentiation potential (11).

In the present study, we report the first demonstration that ANSCs can be propagated for over a year in vitro (corresponding to >120 passages), maintaining all of the functional properties expected from bona fide ANSCs with no sign of neoplastic degeneration. Our results suggest that the unlimited proliferative capacity of ANSCs is an inherent cell feature that can be preserved under suitable culture conditions. Indeed, all of the established cell lines analyzed in this study maintained a steady growth profile over extensive culturing. Consistently, the analysis of their growth rate did not detect any abrupt changes, as opposed to what has been documented for other adult stem cells such as the mesenchymal, which undergo immortalization upon long-term in culture (5, 6, 29). The very modest increase in global cell proliferation correlated with the increased clonal efficiency observed throughout culturing and suggested that the growth rate increase could be related to the progressive enrichment in the stem-cell component. Importantly, unlike cancer cells (19), ANSCs maintained strict growth factor dependence, indicating that they are normal cells that require mitogenic stimulation to proliferate (13). Also, multipotency persisted throughout in vitro culturing and it is worth noting that ANSCs never showed promiscuous expression of lineage markers, as opposed to the population of stem cells with malignant properties recently isolated from human glioblastomas (2).

The further characterization of ANSCs with respect to genetic and epigenetic variables typically linked to the transformation process confirmed their stability over time. The expression profile of a panel of genes, known to be involved in the malignant phenotype, did not show any significant alteration in ANSCs analyzed at different passages. To the same extent, karyotype analysis did not reveal aberrations as those reported in previous studies concerning in vitro spontaneous cell transformation (5, 6). However, the only end-to-end chromosomal fusions evidenced in NSCL4 did not confer any proliferative advantage or differentiation anomaly to this cell line that, cultured for an additional 50 passages, kept on displaying the same behavior as the lines with normal karyotype (5). Indeed, it has been reported that in long-term cultured embryonic stem cells, the presence of chromosomal end-to-end fusion has the role of maintaining or protecting eroded telomeres, favoring cell viability during prolonged cell growth with no sign of cell transformation (22). At early and late passages, ANSCs maintained similar telomere length, and their expression of telomerase activity did not significantly increase from its moderate basal level (data not shown). The persistence of long telomeres not associated with altered phenotype correlates well with recent reports, suggesting that the presence of long telomeres in mouse

Figure 5. Characterization of NSCLs cultured for >120 passages in vitro. A, metaphase spread and manually aligned karyotype of abnormal cell line NSCL4 at passage 120. Marker 1 was found in 100% of the metaphases, whereas marker 2 was found in 30% of the metaphases. A new aberrant metacentric chromosome (marker 3, white arrow and box) was found in 10% of the metaphases. B, NSCL growth curves, prolonged up to 120 passages in vitro, continued to exhibit the same modest upward trend observed in the previous 70 passages (gray box). The total number of cells cultured was calculated at each subculturing passage. As a whole, long-term culturing did not have radical effects on cell growth, although the average number of cells yielded per passage ranged from 1.2 x 10^6 cells at the early passages (p1–p15) to 4.5 x 10^6 cells at later passages (p105–p120), with an increase in the proliferation rate of 3.7-fold during the 400 d of continuous culture. C, at passage 120, the differentiation potential of NSCLs was stably maintained compared with lower passages and without significant differences between NSCL3 (normal karyotype) and NSCL4 (anomalous karyotype) line. Neuronal (TuJ1; red), astroglial (GFAP; green), and oligodendroglial (GalC; red) markers were detected by immunofluorescence in the NSCL4 at passage 120. Bar, 30 μm. D, telomere length for NSCL3 and NSCL4 cells was analyzed after 7, 30, 70, and 120 passages in culture. As expected for mouse cells, ANSCs showed heterogeneous and very long telomeres of over 21 kb in length. Telomere length did not consistently change during continuous culturing. The size marker (kb) is indicated at the left. LW, low-weight standard; HW, high-weight standard.
stem cells is not necessarily an indicator of reduced aging or enhanced cell transformation (30). However, other studies describing long-term cultured mouse germ cell lines as well as murine mesenchymal stem cells have suggested that these cells undergo malignant transformation when displaying considerable telomere erosion (31) or when maintaining long telomeres by gradually increasing their telomerase activity (5).

Interestingly, the maintenance of long telomeres correlates with the observation that these cells can be kept in culture for a long time without entering into replicative senescence. We hypothesize that the presence of long telomeres is a physiologic property of self-renewing ANSCs that can be preserved in vitro in appropriate culture conditions. A recent study has shown that rodent oligodendrocyte precursors do not undergo replicative senescence and have unlimited proliferative capacity, with no evidence of malignant transformation, if cultured in the correct manner (32, 33).

Thus, the proper application of the in vitro conditions required for the NSA not only implement the self-renewing properties of ANSCs (advantageous for eventual therapeutic purposes), but may also recreate a permissive environment, allowing ANSCs to actively maintain all of the sophisticated mechanisms that preserve them from tumorigenic transformation.

![Figure 6](image_url)

Figure 6. Tumorigenicity of nontransduced and Myc- and Ras-transduced NSCL3 and NSCL4 lines cultured over passage 110. **A,** expression of the integrated Myc and Ras provirus was confirmed by RT-PCR using primers specific for the two transgenes. GAPDH expression was used for sample normalization. **B,** phase-contrast microphotographs showing examples of neurospheres from NSCLs at passage 110, either untransduced or transduced with Myc and Ras oncogenes. No appreciable morphologic differences are observed. Bar, 80 μm. **C,** summary of transplantation in SCID mice. Transduced NSCLs at passage 110 were tested for tumorigenicity by orthotopic injection of 2 × 10^6 cells. Cells injected into SCID mice were GFP tagged before transplantation. MEFs expressing Myc and Ras (positive control) formed tumors 7 to 10 d after transplantation. **D,** confocal analysis of brain sections of mice transplanted with genetically modified NSCLs shows integrated GFP-positive cells (green), morphologically resembling neurons (arrows), and autofluorescent cellular debris (red; top panels). Brain sections from mice transplanted with MEFs transfected with Myc and Ras oncogenes display GFP-positive tumors with cells invading the whole parenchyma (bottom right panels). Sections from brain implanted with nontransduced MEFs show only cellular debris at the inoculation site (red; left bottom panels). Bar, 48 μm.
Although *in vivo* tumor formation by short-term expanded ANSCs with otherwise intact *in vitro* differentiation potential has been suggested (7), here we show that ANSCs, cultured for over 100 passages, were never able to generate tumors. Most importantly, ANSCs never displayed tumorigenic potential even when challenged by exploiting a well-established paradigm of induced transition from normal to fully transformed cells (17). This experimental protocol is based on a normal cell being (a) first immortalized by the Myc oncogene, which confers a limitless replicative potential to the cells, and (b) subsequently transformed by the Ras oncogene. The introduction of these two oncogenes readily induces transformation of primary rodent fibroblasts, whereas only one oncogene is sufficient to transform MEFs spontaneously immortalized by prolonged culture *in vitro* (27). Even when ANSC lines, previously cultured for over 100 passages, were transduced with both *Myc* and *Ras* oncogenes, they never generated tumors *in vivo*, although similar levels of transgene expression were led to induced transformation and *in vivo* tumorigenesis of primary MEFs. Different requirements have been shown necessary to drive different cell types to full malignant transformation (the number and/or type of mutational events, oncogene expression levels, etc.), both *in vitro* and *in vivo* (34). Such context-dependent differences in susceptibility to acquire neoplastic phenotype are attributed to specific transcriptional programs active in diverse tissues or at various differentiation points (35). We cannot rule out the possibility that intrinsic biological differences in *Myc-* and *Ras*-dependent pathways in ANSCs might render the oncogenic challenge less effective than in MEFs. However, the extreme resilience of ANSCs to transformation might also depend on mechanisms specifically active in stem cells and aimed at protecting them from the accumulation of genetic alterations (21, 36). *In vitro* culture under optimal, nonstressful conditions can preserve ANSCs from environmental insults that might induce mutagenic events at the origin of neoplastic transformation and play an important role in preventing immortalization and tumor progression.

Recent studies provide evidence that undifferentiated mitotically active precursors within the adult brain might be probable targets for tumorigenic transformation, although neoplastic degeneration might occur at low frequency (4). The extreme genetic and epigenetic stability of ANSCs upon extensive culturing emerging from our study might justify the low incidence of brain tumors with respect to other types of cancer derived from different actively proliferating stem-cell compartments.

In summary, we have described conditions, variables, and time frame(s) under which ANSC cultures satisfy the most stringent criteria required to provide a plentiful and safe source of neural cells, fulfilling the expectations raised by their discovery over a decade ago. In addition, the NSA, as a method for successful isolation and propagation of stem cells, makes ANSCs an ideal model to study the molecular mechanisms that might link stem-cell properties to critical physiologic and pathologic processes, such as senescence and cancer.

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**References**

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