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Mammalian Neural Stem-Cell Renewal

Nature versus Nurture

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Abstract

Recent data show that the final events of mammalian brain organogenesis may depend in part on the direct control of neural stem cell (NSC) proliferation and survival. Environmental and intrinsic factors play a role throughout development and during adulthood to regulate NSC proliferation. The NSCs acquire new competences throughout development, including adulthood, and this change in competence is region-specific. The factors controlling NSC survival, undifferentiated state, proliferation, and cell-cycle number are beginning to be identified, but the links between them remain unclear. However, current knowledge should help to formulate an understanding of how a stem cell can generate a new stem cell.

Index Entries: Stem cell; neural precursors; neurogenesis; retina; spinal cord; telencephalon; neurons; glia.

Introduction

During brain development, neuroepithelial cells give rise successively to neurons, astrocytes, and oligodendrocytes. At the beginning of the century, while studying the presence of dividing cells in the embryonic brain, Ramón y Cajal found that neuroepithelial cells surrounding the ventricles divide, and should thus be at the origin of the future cells that build this organ. The origin of the neural cells that appear during the neural-tube formation was debated to identify whether multipotent cells able to generate the three cell phenotypes exist, or whether three restricted progenitors cohabit to produce either neurons, astrocytes, or oligodendrocytes. In the study of neural retina development, it appeared that a unique pool of cells gives rise to the different neuron phenotypes and then to astrocytes in a chronological mode. Clonal analysis at a

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different developmental time of the proliferating zone by infection with retrovirus carrying a reporter gene revealed that the retina contains multipotent cells capable of producing all cell phenotypes *(1).* Similar observations were made with the developing cortex *(2).* These data suggest that the brain is built by multipotent cells that are able to renew for an extended period of time. Multipotentiality and long-term renewal were defined as stem-cell characteristics *(3).* Renewal means that the stem cells can proliferate for an extended period of time and conserve their characteristics (multipotentiality and renewal). [1]Neural stem cells were isolated in vitro in 1992 *(4)* from various regions of the developing mouse brain, including the striatum, cortex, mesencephalon, and spinal cord. Thanks to the ability to isolate neural stem cells (NSC) in serum-free medium and to study them, NSCs provide(d) numerous new insights on neurogenesis, as reviewed in refs. *5–12.*

During the last two years, many studies describing the control of intrinsic and extrinsic factors on NSC proliferation have been published. Consequently, these findings have opened new perspectives for NSCs. This review focuses on the molecular control of mammalian neural stem-cell renewal of different central nervous system (CNS) locations, including the spinal cord, the telencephalon, and the retina. The similarities and differences between these stem cells may reveal important mechanisms that are specific to neural stem cells. In this article, only the factors that were shown to act directly on neural stem cells and, in certain cases, on neural progenitors*, are discussed. We first reveal the factors that are essential for NSC survival and renewal, and then the epigenetic and intrinsic factors that modulate NSC proliferation. Finally, because cell renewal depends on the regulation of the mitotic cycle, the tumor suppressors known to control stem-cell renewal are presented. In this description, data showing how the NSCs acquire new characteristics during development and adulthood are discussed.

* The term "neural progenitor" regroups all non-differentiated cells able to proliferate for a long period of time. Stem cells, bi-potent, and unipotent highly proliferative cells composed this group.

Multipotent Neural Stem Cells in the Developing and the Adult CNS

As described previously, NSCs can be isolated from various regions of the CNS *(4).* Isolation can be achieved in serum-free medium containing epidermal growth factor (EGF). NSCs proliferate and form large clusters known as spheres or neurospheres. Clonal analysis revealed that each sphere originates from one cell *(13),* providing an easy model to test stem-cell renewal and expansion. Indeed, a dissociated sphere is able to generate several other spheres, with the majority remaining multipotent (Fig. 1). This procedure can be repeated several times, thus revealing the stem-cell characteristic of renewal. It was also shown that clones of human telencephalic fetal stem cells can renew for at least 2 yr; the renewal capacity was demonstrated by subcloning procedures *(14).* As we will see, NSCs can be isolated early during development with fibroblast growth factor-2 (FGF-2) *(15–17),* and then later, with EGF. It appears that NSCs change their competence along development to respond to environmental cues. Also, certain adult neural stem cells present new characteristics in comparison to fetal NSCs, showing that NSCs change their biological behavior throughout life. Neurosphere isolation is not the unique in vitro technique to study NSCs. Infections with replication-incompetent viruses as well as monolayer cultures are also effective methods to investigate the biology of NSCs. It should be noted that, in a comparison of monolayer and neurosphere methods, there is no evidence that these two techniques allow the isolation of the same pool of NSCs.

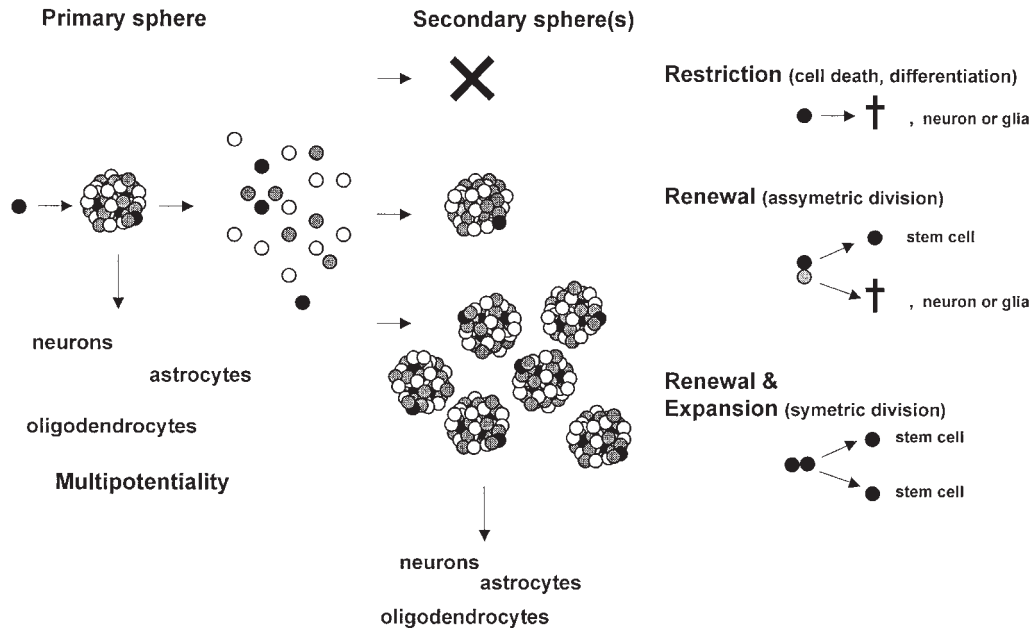

Fig. 1. A single stem cell is able to generate a cluster of neural progenitors termed "sphere." Under conditions that induce cell differentiation, this sphere generates neurons, astrocytes, and oligodendrocytes, revealing the multipotentiality of the stem cell. The renewal capacity can be tested by dissociating the sphere and incubating the single cells with medium known to induce the division of stem cells. If one or more new multipotent spheres appear, renewal has occurred. The generation of more than one multipotent sphere attests to the expansion of the stem cell through symmetric division (one mother stem cell gives rise to two daughter stem cells), whereas the presence of one sphere reveals stem-cell renewal by asymmetric division. If no spheres are generated, the initial cell at the origin of the primary sphere is restricted in its potential to renew (black cross), and is not considered as a stem cell. It remains unclear to what extent renewal testing is needed to ascertain the identity of a stem cell. In fact, the more renewal capacity is tested, the more confident we are.

In the 1960s, Altman and Das *(18)* stated that the adult rodent brain can generate new neurons. However, these observations were considered as rare events, and thus received little interest. In 1982, Bayer et al. *(19)* revealed that adult neurogenesis is a robust phenomenon in the olfactory bulb and the dentate gyrus, rendering the study of adult neurogenesis attractive. If new neurons are generated throughout life, stem cells should still be present in adult rodent brains. The groups of Alvarez-Buylla and Luskin revealed through transplantation studies that the anterior part of the subventricular zone (SVZ) is at the origin of the neurons reaching the olfactory bulb *(20–22)*. In vitro isolation of adult NSC was achieved by Reynolds and Weiss *(23)*, showing that a population of striatal cells surrounding the mouse ventricle is able to proliferate in response to EGF in serum-free medium and these cells have all the characteristics of a stem cell. NSCs were also isolated in other brain areas, including the retina *(24,25)*. The nature of stem cells (astrocytes vs ependymal cells) is still under debate *(26–28)*. Adult NSCs have certain particularities that are distinct from fetal ones. In the following sections, the necessary events controlling NSC survival and renewal during development and adulthood are discussed.

Epigenetic Factors Controlling Stem-Cell Renewal and Expansion

The Factors Essential for NSC Survival and Renewal: The Triad EGF-FGF-2-IGF-I

In 1992, Reynolds and Weiss revealed that stem cells can be isolated from all the regions investigated at E 14 of the developing mouse CNS in the presence of EGF. Similar results also seem to be intrinsic to the developing retina *(29),* but long-term studies and subcloning are necessary to reveal the characteristics of these cells. Interestingly, mouse CNS stem cells, including those of the spinal cord, can also be isolated in the presence of FGF-2 *(17,30–32).* The existence of EGF- and FGF-2-responsive stem cells raises the question of their lineage relationships. Several studies have addressed whether two different NSCs exist or whether the NSC population responds to the two different growth factors *(17,30,33).* The work of Tropepe et al. (1999) showed that at low cell-culture density, EGF and FGF-2 have additive actions on E 14.5 NSCs, but such a phenomenon did not occur at high cell-culture density. Interestingly, FGF-2- but not EGF-responsive stem cells can be isolated at E 8.5. These results suggest that two populations of NSCs exist, and that yet another factor controls NSC proliferation or survival. Contradictory results nonetheless indicate that one stem cell exists *(30).* In fact, early NSCs acquire a new competence to respond to EGF *(15).* Some NSCs may lose their ability to react to FGF-2 alone, which would explain that at low cell density, two populations of NSCs are observed *(17).* Ultimately, a thorough understanding of the factors that regulate survival and/or proliferation of NSCs will be necessary to determine whether there are single vs multiple NSC populations in the forebrain.

CNS development is dramatically altered in the absence of FGF-2 or EGF receptor (EGF-R) *(34–36).* The lack of FGF-2 expression leads to marked brain growth failure starting at E 8.5, at the time when FGF-2-responsive stem cells can be isolated *(17),* whereas the EGF-R actions during CNS development appear to be required later (at E 13.5), when EGF-responsive stem cells appear to be active. As a result, a strong correlation exists between the time of brain defect resulting from the absence of FGF-2 or EGF-R and the time of stem-cell activations by FGF-2 and EGF. In an in vitro model using organotypic cultures, EGF-R was observed to be temporally expressed, with an increasing expression occurring during development *(37),* predominantly in the SVZ vs the VZ. Misexpression of EGF-R by replication incompetent retroviral vectors accelerates the appearance of EGF-responsive stem cells, and is involved in a switch from neuron to glia generation, probably acting directly on the stem cell. In support of these data, addition of FGF-2 to E12 cortical explant cultures accelerated the appearance of EGF-responsive stem cells *(38).* Confirming these results, FGF-responsive stem cells are isolated predominantly at early developmental stages, and mainly generate neurons in comparison to EGF-responsive stem cells, which are predominantly present at later stages and generate astrocytes and more oligodendrocytes *(39).* During spinal cord development (E 10.5), the neuroepithelial cells express receptors for the FGF family but not for EGF *(16).* Later (at E 14) EGF-responsive stem cells can be derived from this region. It appears that EGF and FGF-2 are essential for stem-cell proliferation throughout brain development, and participate in the cell fate decision of progenitors at a stem-cell level. Nonetheless, these factors require the presence of another factor in order to be active: insulin-like growth factor-I (IGF-I).

During early and midgestation brain development, both IGF-I ligand and receptors are present in rodent neuroepithelial cells at a time when FGF-2 and EGF are present, suggesting that these factors may interact to control NSC activity. Indeed, IGF-I was recently shown to be an essential factor in stem-cell proliferation: in the absence of IGF-I, EGF and FGF-2 are unable to induce stem-cell proliferation. In

vitro NSC generation is dose-dependent. If the number of proliferating NSCs is coupled to the IGF-I level, we can hypothesize that the final neural-cell number should depend on the concentration of IGF family peptides present during brain organogenesis. Deficits in brain development in IGF-I and IGF-I-R-deficient mice support this hypothesis *(40–42).* The temporal actions of IGF-I and EGF during development are well-correlated, suggesting a close interaction between these factors to sustain NSC proliferation, whereas FGF-2-responsive NSCs are probably controlled by another factor in addition to IGF-I. Insulin is one possible candidate, and is present throughout brain development. Supporting this view, in absence of IGF-I, physiological concentrations of insulin can induce stem-cell proliferation in the presence of FGF-2 or EGF (personal data). One of the actions of insulin was reported to control the early events of chick eye development *(43).* Moreover, insulin injection into the newborn chick induces neural-progenitor proliferation in the retinal margin *(44),* an area where stem cells are located *(45).*

Mouse null-mutation studies have revealed that IGF-I, FGF-2, and EGF are necessary for brain growth, but they have not demonstrated specific functions for each of these growth factors. The use of a neurosphere system is a powerful tool to understand the function and the mechanism of factors that support stem-cell proliferation (*see* Fig. 1). Studies testing continuous vs delayed administration of IGF-I, FGF-2, and EGF on mouse NSCs showed that IGF-I and EGF had no effect on NSC survival, and FGF-2 promoted NSC survival or maintenance. Also, short-term exposure to IGF-I induced the proliferation of NSCs in the presence of EGF, but not of FGF-2, through an autocrine secretion of IGF-I *(31).* These experiments show that EGF and FGF-2 have different functions and actions on NSCs. These differences may explain how NSCs can be quiescent or active. For instance, FGF-2 may maintain NSC survival, whereas the level of IGF-I can stimulate proliferation and determine the number of precursors generated.

In human fetal stem cells, the presence of both EGF and FGF-2 is required to allow stem-cell survival and proliferation in vitro, contrary to what is observed with murine fetal stem cells that require only one of these factors *(14).* The different actions observed for EGF and FGF-2 in murine cells may have been reinforced during evolution, with FGF-2 acting predominantly on stem-cell survival and EGF on proliferation. This hypothesis has not yet been tested. Another study using human fetal neural stem cells revealed that the Leukemia Inhibitory Factor (LIF) is required to allow stem-cell renewal *(46).* In fact, it is possible that LIF exerts a survival action on stem cells, as demonstrated on other neural cells *(47–49).* Human NSCs are delicate, and the presence of LIF may compensate for side effects such as cell dissociation. LIF may protect stem cells from entering into a senescent state. Interestingly, the LIF-R were also shown to have an effect on NSCs of adult mice. Indeed, the number of spheres generated from the SVZ of LIF-R–/– mice is lower in comparison to wild-type animals, as well as the number of interneurons in the olfactory bulb *(50).* In vivo and in vitro ciliary neurotrophic factor (CNTF) stimulation of SVZ cells increases the number of spheres. It is known that CNTF acts through the LIF-R. In fact, CNTF acts either at low concentration by binding the complex CNTF-Rα, gp130, and LIF-R, or at high concentration on the dimer gp130/LIF-R *(51).* These results suggest that the depletion of NSCs in the SVZ is responsible for the loss of neurons in the olfactory bulb. Single-sphere analyses have revealed that CNTF maintains multipotentiality and increases stem-cell renewal. Moreover, CNTF seems to counteract the EGF action that leads cell fate toward a glial phenotype *(50).* CNTF appears to play a role in maintaining stem-cell characteristics.

In summary, it appears that EGF, FGF-2 and IGF-I are essential for mouse NSC regulation throughout development, and their interactions remain, unsolved. LIF could play an essential role in the maintenance of human NSCs, an action also identified in adult mice. It

is also possible that LIF exerts an effect on NSCs during early CNS development. Indeed, as we have seen, NSCs can only be isolated at E 8.5, and not before. These results suggest that other factors are necessary to isolate the most primitive NSCs. One possible candidate is LIF. In addition to the actions previously described, LIF allows the in vitro isolation of a pluripotent NSC able to generate various cell phenotypes and restricted NSCs from the mouse embryonic stem (ES) cells *(52).* Indeed, when ES cells are stimulated with LIF and FGF-2, they generate "spheres" that are capable of generating cells of the endo-, meso-, and the neuroectoderm. When these pluripotent spheres were dissociated and the single cells plated in the sole presence of FGF-2, neurospheres appeared. They are able to renew, but only generate neurons, astrocytes, and oligodendrocytes. It appears that between the ES cell and the NSC, an intermediate state is required, and that the intermediate state necessitates LIF. These results suggest that LIF could be an important regulator of NSCs during early induction of the CNS. The level of the NSC pool size during adulthood could already be determined during CNS development.

The Notch Pathway Is Essential for NSC Renewal

In order for a NSC to conserve its status, survival and proliferation are not sufficient. Epigenetic and intrinsic factors that prevent cell differentiation are also required. If we consider neurogenesis from invertebrates to mammals, Notch is certainly one of the most conserved pathways for the maintenance of the progenitor status at certain periods of development. Notch is a receptor that is present in neural progenitors, and its activation was first observed to prevent cell differentiation *(53).* When activated by ligands such as Delta and Jagged (in mammals), the intracellular domain of Notch (Notch-IC) is cleaved by presinilin (Fig. 2). Formation of Notch-IC can also occur independently of presinilin. Notch-IC activates gene expression through the binding of RBP-jk *(54);.* For Notch1 –/–, or RBP-jk –/– knockout mice, almost no neural stem cells were identified in E 10.5 and E 8.5 forebrain embryos, and a reduction of 93% of the stem-cell number in presenilin –/– mice was observed *(55).* These results suggest that the Notch pathway is essential for the formation of neural stem cells during this period. Regarding stem-cell renewal, when presenilin-1 is reduced or absent, a drastic reduction of the stem-cell number is observed when primary neurospheres were subcloned (*see* Fig. 1). Similar results were reported when neurospheres were derived from ES cells that were deficient for the RBP-jk protein *(52,55).* The target gene of Notch-IC/RBP-jk transactivation, Hes-1, also appears to play an important role in NSC renewal. In Hes1–/– mice, a 2.5-fold decrease in the stem-cell number was observed at E 10.5. The Hes1–/– spheres have a normal size, indicating that the proliferation of the progenitors derived from the stem cell is not affected by a loss of Hes-1 expression. However, the renewal ability is reduced by approx 50% *(56).* These results show that Hes-1 mediates Notch signaling, but that other genes may also be involved in such transduction—considering that the loss of RBP-jk results in a complete abolition of Notch signaling, whereas Hes-1 loss leads to partial disruption. Confirming this hypothesis, Hes-1 is present at normal level in RBP-jk–/– mice, whereas Hes-5 is downregulated in SVZ cells *(55).* Moreover, the generation of neurospheres is dramatically reduced in double knockout mice Hes1–/–/Hes5–/–, in comparison to single deficient mice *(57).* It appears that at least both Hes-1 and Hes-5 mediate Notch signaling in neural stem cells. When progenitors derived from stem cells of presinilin1–/– mice were induced to differentiate, a higher percentage of neurons and astrocytes was observed in comparison to wild-type animals, suggesting that a decreased activity of the Notch pathway induces a loss of the non-differentiated state. Forced Notch-1 expression in periventricular regions of the developing mouse brain (E 14.5) results in the reduction of neuron formation,

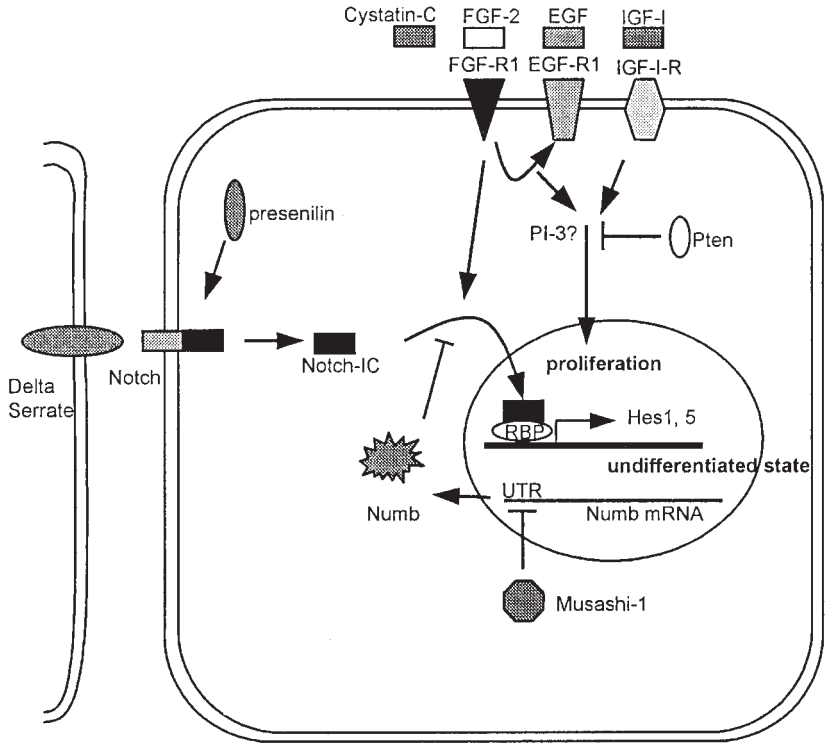

Fig. 2. This schema proposes several mechanisms necessary for a NSC to maintain the characteristics of multipotentiality and renewal. A non-differentiated state is probably conserved when a subtle level of Notch activation occurs between the stimulation by Delta/Serrate and the inhibition by Numb. First Delta binds to Notch, which is then cleaved in the cell by the presinilin protease, releasing the Notch-IC domain that complexes RBP in the nucleus. The result is the activation of Hes1 and Hes5 genes. Both Hes1 and Hes5 are necessary to maintain the stem-cell state. The downstream effectors preserving stem-cell identity are unknown. Musashi-1 might control the exit out of the stem-cell state by inhibiting Numb synthesis. Groups of receptors are involved in the control of stem-cell proliferation. Three receptors were identified to be necessary to maintain stem-cell renewal: FGF-R1, EGF-R, and IGF-I-R. The corresponding ligands are indicated here. In regard to the adult hippocampal NSC, cystatin-C controls renewal, and the receptor has not yet been identified.

supporting the role of Notch in preventing cell differentiation *(58).* Such action was also reported for cerebellar granule neuron precursors through the activation of the Notch2 receptor *(59).* These results contrast with those showing that overexpression of Notch1-IC or Notch3-IC in adult hippocampal stem-cell cultures (following the culture procedure of *[24]*) leads to the formation of astrocytes under proliferating conditions *(60).* It is known that hippocampal stem-cell cultures contain a variety of precursors and progenitors (neuroblasts, glioblasts, stem cells, or bi-potent precursors). thus from this study, it is not clear which cells are at the origin of astrocytes following overexpression of the Notch1-3 pathway. The experiments did not test whether Notch misexpression acts directly on stem cells or their derived progenitors. However, these results indicate that Notch can also intervene in favoring cell differentiation, as it was reported in vivo for the formation of cortical astrocytes *(58),* Müller cells in the retina *(61),* or Schwann cells in the peripheral nervous system (PNS)

(62), but not for olfactory bulb glia *(58).* Variations in mouse age, cell populations transduced to express Notch, and brain areas studied may explain the differences between these studies. However, these results are not necessarily contradictory, considering that radial glia *(63),* periventricular astrocytes *(26),* and Müller cells in the chick retina *(64)* may have a population that has retained some NSC characteristics. Nonetheless, the long-term renewal of these cells has still not been described. In fact, it is possible that, rather than driving stem cells to a glial fate, Notch increases the population by symmetric division—which later would express glial markers—because stem cells seem to be already committed to generate more glia than neurons at this stage.

The potential role of Notch on stem cells was also identified in living embryonic retinal slices *(65).* During CNS development, NSCs are known to divide asymmetrically to give rise to one stem cell and one neuroblast, with the plane of division parallel to the surface of the ventricle *(66),* whereas when one stem cell divides symmetrically to generate two stem cells, the plane is perpendicular. This rule is not absolute (*see* the percentage of mitotic cells that are neither in a horizontal or in a vertical plane in ref. *67*). Studies of stem cells during retinal and cortical neurogenesis have revealed that the apical cell that gives rise to a new stem cell expresses Numb, whereas the basal cell does not *(68).* Both cells express Notch, and Numb is believed to be implicated in the stem-cell-state maintenance of the apical cell *(69).* Indeed, in the developing rat retina or mouse cortex, when division occurs on a vertical plane, two stem cells are generated, both containing an equal amount of Numb protein in their apical pole *(65,69).* When division separates cells on a horizontal plane, only the apical cell conserves Numb, and only this cell maintains stem-cell characteristics. Moreover, Numb knockout mice have profound defects in the neural tube, characterized by an early appearance of neurons in the telencephalon, which suggests that the progenitor state was altered in these animals, provoking an advanced formation of neurons *(70).* All these experiments suggest that Numb may participate in retaining the stem-cell state. However, recent data revealed that Numb decreases the Notch activation pathway. When Numb is misexpressed in cells expressing Notch and activated by the ligand Delta, the transactivation of Hes-1 by Notch is repressed by Numb *(71),* as was previously observed in the *Drosophila (72).* Numb is believed to bind the PEST sequence of the Notch-IC preventing Notch-pathway activation (demonstrated in avian CNS, *[73]*), as well as by preventing Notch-1 transactivation of the HES1 promoter *(71).* Intriguingly, it seems that the maintenance of the stem-cell state by Numb is linked to an inhibition of the Notch activation. We previously described Notch as necessary for stem-cell renewal *(55).* How can we reconcile these findings? First, Numb and Notch are not unique to control the stem-cell state. Other factors, such as Emx2, can have redundant actions such as the different forms of Notch-1, -2, and -3; or the Numb-like protein. Or, different levels of Notch activation might result in different cell states. For instance, low activation of Notch caused by the presence of Numb may lead to a stem-cell state, whereas higher activation leads to the generation and the limited proliferation of committed neural precursors (neuroblasts early, and glioblasts late in development). Perhaps, during the absence of Numb, Notch alone cannot maintain the stem-cell state, but may allow the amplification of neuroblasts, resulting in an accelerated neurogenesis. Notch would only be necessary to amplify a non-differentiated cell, a stem cell, or a precursor, with other factors controlling their specific fate. Supporting this hypothesis, Notch overexpression in eye *Drosophila* provokes an enlarged eye and not a non-differentiated mass *(74),* and overexpression of Notch in hematopoietic stem cells allows in vitro proliferation of these stem cells without altering the differentiation capacity of the derived progenitors to differentiate *(75).* I believe that this hypothesis is compatible with apparent contra-

dictory observations in neurogenesis studies involving Numb and Notch misexpression.

Another modulator of this pathway was recently identified: musashi. Mouse Musashi-1 was first identified as a regulator of asymmetric division necessary for the formation of sensory neurons in Drosophila *(56).* In mammals, Musashi was shown to be an RNA-binding protein, and is believed to regulate posttranscriptional events that control cell differentiation *(76).* Isolation of fetal human neural cells that express Musashi-1 revealed that the majority of them are also positive for the marker of undifferentiated cells, nestin, and have the potential to proliferate *(77).* Moreover, cell isolation based on Musashi expression allows a 10-fold enrichment of stem cells from cell culture and a 100-fold enrichment from cells isolated directly from the parenchyma, showing that NSCs contain Musashi *(77).* A target of mouse Musashi was recently identified: Musashi binds the 3′-untranslated sequence (UTR) of the numb mRNA *(71).* Overexpression of Musashi results in the downregulation of the Numb protein level but not of its mRNA. In fact the promoter activity of numb mRNA is reduced by approx 75% in the presence of Musashi. Moreover, the presence of Musashi slightly activates (fivefold) the Hes1 promoter and enhances Notch activation on this Hes-1 promoter. The Hes1 promoter is also the target of Numb that inhibits the transactivation *(71).* This series of observations suggests that similar phenomena could occur in NSCs, and that Musashi could be an intrinsic factor controlling the non-differentiated state. These actions have not yet been demonstrated in neural stem cells and under physiological conditions, but nonetheless, they point out an important possible mode of stem-cell-state regulation. Because musashi is also expressed in neuroblasts *(78,79),* and blocks Numb protein synthesis, it is possible that musashi intervenes in the transition of the stem-cell state toward the neuronal fate. Loss-of-expression studies of musashi should reveal its role.

Does a Link Exist Between NSC Mitogens and the Notch Pathway?

What is the link between the Notch pathway and the mitogens that allow NSC renewal? Some studies show a possible cross-communication between these pathways. Interestingly, a link between the FGF family and the Notch pathway was recently observed in neuroepithelial cells *(80).* The stem cell's characteristics were not studied in these experiments, but FGF-1 or FGF-2 prevented neurogenesis of neuroepthelial cells, upregulated the expression of Notch-1 and Notch-3, and decreased Delta 1. Moreover, FGF action is blocked when Notch-1 expression is suppressed by anti-sense *(80).* These observations are in agreement with the results of Hitoshi et al. *(55)* and suggest that FGF-2 stimulates stem-cell expansion (symmetric division) through the activation of the Notch pathway. EGF probably acts in a similar fashion (although this has not been confirmed) because ectopic EGF stimulation on retinal explant expand eye neuroepithelial cells *(12,29,81),* and prevents cell differentiation (or prolong the non-differentiated state) *(29).* The maintenance of precursor proliferation by epigenetic stimulation through the activation of the Notch pathway is also documented for cerebellar granule neuron precursors stimulated by sonic hedgehog (Shh) *(59).* Shh was initially identified as a factor secreted by the notochord that ventralizes the CNS *(82–85)* and more recently, as a mitogen for neural precursors suggested by mutation of the Shh signaling pathway, leading to brain tumors *(86–88).* Overexpression of Shh at E 10 in the dorsal tube of transgenic mice, after the dorsoventral patterning of the CNS, provokes a blocking of SVZ neuroepithelial cells in a non-differentiated state *(89),* suggesting that Shh may prevent differentiation of neural stem cells, or derived progenitors. In support of these observations, Shh stimulates in vitro the proliferation of neural progenitors of the retina *(90)* or the telencephalon *(91).* However, whether stem cells are included in the pool of proliferating cells is unclear.

Other Factors Known to Influence NSC Proliferation

In the previous section we saw that NSCs require the presence of specific factors to sustain cell survival and proliferation. Other factors modulate these cell states and participate in the homeostasis of NSCs throughout development. Indeed, analyses of the stem-cell population revealed that at high cell density some secreted factors favor the expansion of stem cells, suggesting that unidentified factors may control NSC renewal *(17).* A paracrine action on NSCs was also described for the developing mouse cortex *(38):* we previously mentioned that EGF-R expression increases in a temporal manner in cortical explant cultures, allowing the proliferation of EGF-responsive stem cells *(37).* Interestingly, a co-culture with explants derived from younger embryos prevents an EGF-R increase, suggesting that secreted factors may delay stem-cell activation. The secreting factor was identified as Bmp4, a bone morphogenic protein that intervenes in the dorsalization of the CNS *(92–94).* FGF-2 and Bmp4 have antagonistic action on the appearance of EGF-responsive stem cells *(38).* Knowing that FGF-2 is required for neuronal formation, it is reasonable to theorize that when FGF-2 is present in large amounts during later stages of neurogenesis *(95–97),* it helps to start the gliogenesis program by promoting the appearance of EGF-R on NSCs, and overcomes the inhibition of Bmp4. Bmp2 and Bmp4, as well as their receptors, are also present in the adult SVZ, their presence being associated to neurogenesis inhibition *(98).* Noggin, a Bmp inhibitor, is present in ependymal cells that are adjacent to the SVZ. Bmp7 acts on the same receptors as those recognized by Bmp2 and Bmp4, and although it has a lower affinity for Noggin, it decreases SVZ proliferation by 50%. These experiments show that Bmps regulate neurogenesis at the SVZ cell level throughout development until adulthood.

EGF is not the unique ligand for the EGF-R. TGFα is known to bind this receptor with high affinity. The in vivo stem-cell cycle in the SVZ was previously evaluated by H^3-thymidin or bromodeoxyuridine (BrdU) incorporation *(99).* BrdU incorporation in TGFα–/– mice revealed that NSC proliferation is reduced in the SVZ lateral corner, a region known to contain the NSCs at the origin of the neuroblasts populating the olfactory bulb. In TGFα knockout and wild-type mice, the same number of stem cells were isolated in vitro from the SVZ lateral corner *(100).* Studies of BrdU incorporation reveals that the absence of TGFα produces a lengthening of the NSC mitotic cycle. These and the previous experiments discussed here show that growth factors can independently regulate NSC survival, proliferation, and cell-cycle duration.

The ephrin family and their receptors was first described to be involved in axon targeting *(101),* cell migration *(102),* and brain boundary *(103),* and they may participate in NSC regulation. No mitogenic action on neural progenitors was reported until recently. The Eph family is formed of 8 ligands and 14 receptors. The adult SVZ contains receptors for ephrin as well as the Ephrins-B2/3, suggesting a role of the Ephrin family in the SVZ cell state. Infusion of the clustered ectodomain of Ephrin-B2 (an active form of Ephrin) leads to increased proliferation in the SVZ, as shown by BrdU incorporation and a decrease of migratory neuroblasts *(104).* An increase of astrocytes was simultaneously observed. Under these conditions, astrocytes have the type-B cell phenotype described as stem cells, showing that ephrin can stimulate the proliferation of stem cells in vivo. The ultimate demonstration that stem cells are the target of Eprin-B2 should be revealed by the in vitro stem-cell characterization after in vivo ephrin infusion.

Several other factors may modulate stem-cell proliferation; some of them enhance stem-cell proliferation, and others inhibit it. The FL ligand for the FLT3-receptor kinase was originally reported to stimulate hematopoietic stem-cell proliferation. In a recent study, it was observed that progenitors derived from neural stem cells isolated from postnatal rats express

the FLT3 receptor *(105).* When neurospheres were incubated in the presence of EGF and FGF-2 + FL and H^3-thymidine, a decreased proliferation was observed in comparison to the stimulation with EGF + FGF-2. Because the renewal capacity of the stem-cell population was not challenged (for example, in Fig. 1) it is difficult to conclude that stem-cell expansion was inhibited by FL. Moreover, FL synergizes with NGF to enhance dorsal-root ganglion neuron survival, indicating that FL might control neuronal differentiation. In this hypothesis, the decrease in proliferation of a subpopulation of progenitor cells may reflect the entry of neural precursors into G0 of the cell cycle. The direct potential FL action on stem cells has not yet been determined.

Other factors might control NSC—such as TNFα, which increases proliferation in the adult SVZ *(106),* or the ganglioside GM3, which decreases BrdU incorporation in the postnatal SVZ *(107).*

It appears that the control of the neural stem-cell number during development depends on the levels of various factors that control NSC survival, proliferation, and cell state as well as cell-fate determination. A picture of the temporal actions of these factors is beginning to emerge. The understanding of how these factors coordinate and influence the role of each other will be the next challenge. Nonetheless, certain links have already been identified.

Intrinsic Control of Stem-Cell Renewal

The previous sections mainly described the action of epigenetic factors as well as some potential intracellular regulatory mechanisms. Several intrinsic factors that may control NSC proliferation were also recently discovered, but no links with known extrinsic factors acting on stem cells have been identified so far. One of these intrinsic factors is the transcription factor Emx2. Homeodomain transcription factors are involved in the control of body segmentation, organogenesis, cell specification, and differentiation. During development and adulthood, some homeodomain transcription factors are expressed in the neuroepithelium, suggesting a possible NSC regulation by these factors. One of them—Emx2—is expressed in the germinal area *(108–110)* and in the SVZ of the adult mouse brain *(111).* These observations suggest that Emx2 could play a role in the regulation of neural progenitor proliferation or cell fate. In fact, Emx2 has region- and age-specific action on neural progenitors. Moreover, Emx2 can have opposite effects, depending on the brain area where it is expressed. Emx2 is expressed in neurospheres derived from the adult mouse SVZ, but down-regulated in differentiated cells, indicating that progenitors or stem cells express Emx2 *(111).* Neural progenitors derived from Emx2 knockout mice show a greater ability to proliferate in comparison to wild-type cells, and these mice generate 50% less neuroblasts that migrate through the rostral migrating stream, suggesting that the undifferentiated state is favored in the absence of Emx2. Using subcloning analysis as described in Fig. 1, it was shown that the presence of Emx2 reduced the frequency of secondary stem-cell generation (one stem cell generates less stem cells). This effect is not related to a control on cell survival or on cell-cycle time, but appears to be linked to the capacity of stem cells to divide symmetrically or asymmetrically. By reducing the generation of new NSC, Emx2 favors asymmetric division of adult SVZ stem cells *(111).* A completely opposite action of Emx2 is described for the regulation of cortical multipotent progenitor cells: when Emx2 is overexpressed in vitro in cortical E14 progenitors cultivated at low density, an increase of clone size was observed *(67),* whereas no effect was recorded when progenitor cells of the ganglionic eminence were transduced with Emx2. This increase in cell number is not related to an enhancement of survival, but to an increase in cell proliferation by favoring symmetric division, as attested by BrdU incorporations and BrdU dilution throughout cell division. This observation was confirmed by analyzing stem cells in the cortical neuroepithelium of knockout mice for Emx2. In these mice, the

frequency of symmetric division is reduced by 30% (mitotic cell observation), whereas asymmetric division is increased by 50% in comparison to wild-type animals showing a switch in cell-fate decision. This switch is confirmed by the analysis of clones generated in vitro. At E 14, the majority of clones are composed of pure neurons, and only 25% are multipotent. When Emx2 is overexpressed in vitro, 60% of the clones become multipotent *(67)*. It appears that Emx2 in the cortex enhances stem-cell symmetric division and instructs progenitor cells toward a multipotential fate, whereas Emx2 favors asymmetric division in the adult SVZ. These opposite effects of Emx2 in different regions revealed that other modulators interacting with Emx2 exist. We can speculate that Emx2 acts on different promoters. It would be of great interest to reveal whether the regulation of Emx2 action at the promoter level is region-specific and/or age-related.

Other intrinsic factors that are not directly linked to the cell-cycle machinery have recently been identified in the SVZ. The transcription factor Sox2 is expressed in the inner cell mass (where ES cells reside) of blastocytes, and is ubiquitously present in the neural tube during early developmental stage *(112)*. Some regulatory regions of the Sox2 gene drive the expression to the ventricular zone, where progenitor cells are located. Neurosphere isolation from transgenic mice that expressed the β-galactosidase and the neomycine transgenes under the activation of the specific telencephalic regulatory element of the Sox2 gene have revealed that the Sox2 gene is expressed in stem cells and derived progenitors *(113)*. Indeed, the transgene construction contained a gene of resistance against neomycine, so in vitro proliferation of stem cells can be achieved in G418 only if the transgene is expressed, showing that Sox2 is specifically expressed in stem cells. Because of its constant presence in NSC and in nondifferentiated cells throughout development, Sox2 is likely to play an important role on the stem-cell state.

Particularities of Adult Neural Stem Cells

Adult striatal stem cells conserve their embryonic ability to respond to either EGF or FGF-2 *(23,33)*, whereas other regions respond differently to environmental factors. The hippocampus was also described as a region containing stem cells. The subependyma juxtaposing the hippocampus is supposed to be the location for stem cells *(114)*. The hippocampal stem-cell amplification necessitates the presence of FGF-2, serum, and high cell density *(24)*. In fact, conditioned medium (resulting from high cell density) can be substituted by the cystatin-C protein *(115)*. Cystatin-C is secreted in cell cultures, providing a neurotrophic factor necessary for stem-cell division. Cystatin-C is a protease, but the neurotrophic effect is determined by another domain than the one implicated in proteolysis. Moreover, the neurotrophic domain must be glycosylated in order to allow mitogen activity *(115)*. As a result, hippocampal NSCs can be isolated and propagated in the presence of FGF-2 and cystatin-C. The spinal cord also presents a dependence on two factors to induce its proliferation state. Indeed, the presence of both EGF and FGF-2 is required to induce spinal cord stem cell proliferation *(116)*. Similar observations were made for NSCs located around the third and fourth ventricles. It appears that all NSCs in the caudal region of the CNS require two factors for their expansion (in addition to all the unknown factors that are acting through an autocrine or a paracrine mode). Interestingly, the most rostral region of the brain—the eye—contains stem cells that can be isolated without EGF, or FGF-2 *(25)*, but by adding insulin and transferrin in the media. High concentrations of insulin are known to mimic the action of IGF-1 that is required for fetal stem-cell proliferation *(31)*. These observations suggest two possible mechanisms regulating NSCs during adulthood: the retinal stem cells may control their activity in an autocrine manner, and the environment may

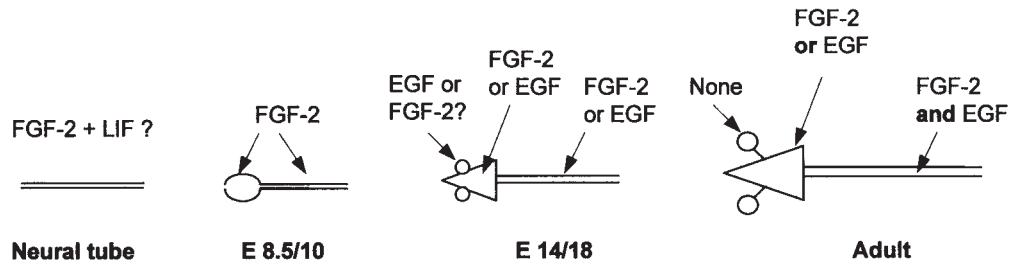

Fig. 3. Neural stem cells are regulated differently throughout age and depending on the brain area. No data concerning the isolation of NSCs at the time of neural tube formation are available, but from the studies of Tropepe et al. *(52)* on embryonic stem cells, we can suggest that FGF-2 and LIF could be required for the activation of NSCs. At E8.5, telencephalic NSCs as well as spinal cord NSCs (at E10) can only be isolated with FGF-2. Later, NSCs proliferate either after FGF-2 or EGF stimulations. In regard to the retina, only data concerning the role of EGF are available. During adulthood, a strong difference in NSC responsiveness to mitogens is established along the rostro-caudal patterning of the CNS. Indeed, in vitro isolation of retina NSCs does not necessitate the presence of factors in the medium, whereas telencephalic NSCs require the presence of FGF-2 or EGF, and for spinal cord NSCs, both.

provide inhibitory factors to prevent uncontrolled stem-cell proliferation. In fact, the study of Tropepe et al. *(25)* has shown that in the absence of EGF or FGF-2, retinal stem cells release FGF-2. Indeed, blocking FGF-2 with antibodies decreased the formation of neurospheres by one-half. The possible autocrine release of IGF-I must be investigated to understand the molecular control of stem-cell renewal. These studies indicate that stem cells are regulated in a different manner from the rostro-caudal axis of the CNS, with an increasing dependence on factors necessary for stem-cell proliferation: in vitro, the eye NSC requires no exogenous mitogen for proliferation, the telencephalic NSC needs EGF or FGF-2, and the spinal cord NSC EGF and FGF-2 (*see* Fig. 3). What is the origin of this difference? Until now, the lineage relationship between stem cells of the various brain areas was unknown. All NSCs could be derived from a homogenous pool of primitive stem cells, and are then specified during rostro-caudal induction. Another scenario could be that certain NSCs forming the neural tube are already committed to certain functions. The differences between NSCs could be linked to the evolution of the brain. During evolution, the eye appeared very early, and is already present in very primitive animals. Interestingly, some animals have eyes without a brain. For instance, the jellyfish *Cladonema* or the scallops have primitive eyes directly connected to a muscle *(117).* In one review, Gehring suggests that the brain was formed during evolution after the apparition of the eye *(117).* These observations point out that the origin of the eye is one of the most primitive regions of the CNS. As a result, it is possible that the retinal stem cells may have evolved differently from the other NSCs. The understanding of the origin of the differences between NSCs should reveal important intrinsic mechanisms that regulate NSCs.

Intriguingly, the difference in growth-factor responses of NSCs is present during adulthood, but not during embryogenesis. At mid-gestation, NSCs of the retina, the telencephalon, and the spinal cord can respond either to EGF or to FGF-2 (Fig. 3). These observations show that all along the lifespan, NSCs acquire new abilities to respond to environmental signals. The role of such changes from birth to adulthood is thus far unclear.

Concerning the adult human brain, EGF and FGF-2 are both required for the isolation of multipotent neural stem cells/progenitors *(118–121).* An approximate 100-fold increase of the cell number in 20 d was observed when neurospheres derived from the olfactory bulb were expanded with EGF and FGF-2 *(121).* However, a long-term renewal was only documented in the presence of cystatin-C *(122),* allowing a 10^8 fold increase in cell number. It remains unclear whether isolated neural progenitor cells have characteristics of stem cells related to long-term cell renewal and whether these progenitors contain NSCs (multipotent). Nonetheless, the use of cystatin-C make it possible to obtain a sufficient number of cells to study neurogenesis derived from adult human tissue. The fact that cystatin-C is only active in its glycosylated form necessitates a cell line expressing the protein under the correct isoform, rendering the access to this molecule still complicated. It appears that during evolution between rodents and humans, stem cells were controlled by a more complex environment.

The Special Case of the Telomerase

To be considered as NSC, a cell must be maintained in a non-differentiated multipotential state and proliferate. Proliferation potential is one of the most essential characteristics for a NSC to conserve its rank. This state depends on an internal clock that regulates the lifespan of a cell. It is obvious that cell renewal is tightly linked to this potential, but the control of neural-cell lifespan is poorly understood. At each division, cells lose 8–12 bp on each chromosome *(123).* To prevent alteration of the genes located at the extremities of the chromosomes, a repetitive sequence, CCTTAG, is added at each end by an enzyme known as telomerase *(124–126).* The repeat of CCTTAG is termed telomeric sequence. As cells proliferate in vitro, telomeres become progressively shorter *(127,128).* When telomeres disappear, ends of chromosomes can fuse, provoking recombinations of genes located at the extremities, and leading in some cases to cell transformation *(129,130).* To prevent this event, the cell induces the senescence state and cell death *(131)* through the activation of *p19* and *p16,* and then *p53* (reviewed in ref. *132*). The real demonstration of the role of telomerase on cell lifespan was shown in senescent fibroblasts by transferring the gene coding for the catalytic subunit of the telomerase, Tert, and thus, inducing immortalization *(133,134).* Tert is still present in the adult brain (cortex, eye, but not the brainstem), although telomerase activity decreases throughout development *(135,136),* in accordance with the presence of neural progeny. Telomerase activity is present in rodent neural progenitors at early and late passages *(137),* showing that these cells maintain the capacity to renew, and suggesting that telomerase activity might be mainly located in the stem-cell population. Interestingly, FGF-2 increases telomerase expression in mouse cortical neural precursors *(138),* leading to the theory that environmental factors may also control telomerase activity in NSCs. Such observation was not reported for human fetal neurospheres, where a decrease in the telomerase activity was recorded from early culture to passage 20 *(137).* But it is not known whether the decrease is a result of the cell culture used, or if it is inherent to the population studied. Because the loss of telomeres can induce cell senescence, it would be interesting to restore telomerase activity in fetal and adult human neural progenitors in order to maintain cell expansion and survival, and to thus have material to study human neurogenesis.

Cell-Cycle Machinery and Stem-Cell Renewal

Cell-Cycle Arrest and Replicative Senescence

Cell renewal implicates the control of the cell-cycle entry. The cell cycle is activated when the transcription factor of the E2F family is separated from the tumor suppressor Rb,

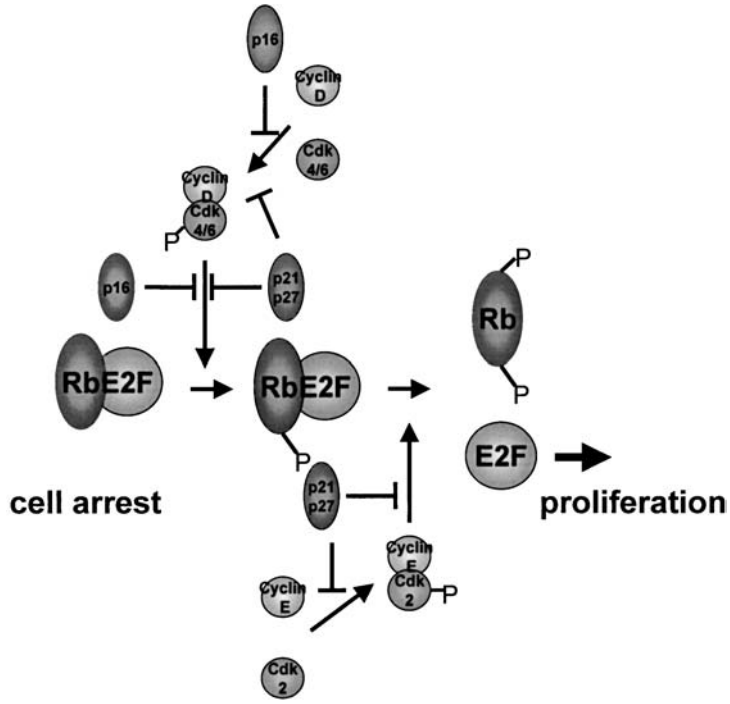

Fig. 4. This simplified schema shows the key controls of cell proliferation: the transcription factors of the E2F family induce cell proliferation only when it is separated from the tumor suppressor Rb. Indeed, the unphosphorylated form of Rb inhibits E2F action, whereas phosphorylation of Rb releases Rb from E2F, allowing E2F proliferation induction. Entry into the cell cycle depends on the cyclin D/cdk4/6 complex (upper part of the figure) that phosphorylates Rb on one site, and progression of the cell cycle can be achieved only when a second site of Rb is phosphorylated by the cyclin E/cdk2 complex. The tumor suppressor *p21/p27* prevents the activation of both cyclin/cdk complexes, whereas *p16* blocks the action of cyclin D/cdk4/6 only.

allowing E2F to transactivate genes necessary for cell division (*see* Fig. 4). These two families of molecules are the key regulators of cell proliferation, and numerous other proteins participate in their activation and inhibition. Indeed, the unphosphorylated form of Rb inhibits E2F action, whereas phosphorylation of Rb releases E2F from Rb, allowing E2F action. Cell-cycle entry depends on the cyclin D/cdk4/6 complex that phosphorylates Rb on one site, and progression of the cell cycle can be achieved only when a second site of Rb is phosphorylated by the cyclin E/cdk2 complex. The tumor suppressors *p21/p27* prevent the activations of both cyclin/cdk complexes, whereas *p16* blocks the action of cyclin D/cdk4/6 only (for review see ref. *139*). Numerous other factors are involved in cell-cycle control, but it is important to note that three sets of tumor suppressors can block the cell cycle transiently or definitively. Irreversible growth arrest is termed replicative senescence. A stop in cell-cycle progression can be imposed by the *p21* and *p27* tumor suppressors, whereas replicative senescence is caused by the expression of *p16* and *p14* (*p19* in mouse) and finally *p53* to induce cell death *(140–142)* (*see* Fig. 1, 2). *p21* and *p27* are more involved during the growth of an organ, when cell proliferation is controlled by the presence of mitogens or inhibited by cell-cell contacts. Different signals can lead to senescence-like trophic-factor removal, telomere shortening *(127,131,143,144),* oxidative damage *(128,145,146),* and oncogene acti-

vation *(141,147,148).* These alterations can potentially lead to cell transformation and oncogenicity. As a result, an irreversible growth arrest (and often cell death) is a protection against tumorigenicity. It also appears that if the cell-culture medium is not optimal, cells can enter senescence because of free radical attacks or the absence of neurotrophic factors. The following sections outline approaches that attempt to identify which tumor suppressors are involved in the control of stem cell/progenitor renewal. Contrary to common beliefs, the cell cycle is not regulated in a homogeneous manner in all cells. Numerous tumor suppressors control the cell cycle, and recent data in developmental biology have revealed that specific tumor suppressors only act in specific cells. Here, I present data from studies that reveal that different functions of neural stem cells in the brain and the retina are controlled by different tumor suppressors.

Pten and Brain Growth

The Pten tumor suppressor is a phosphatase of phosphatidylinositol-triphosphate that can antagonize the mitogenic effect of certain mitogens that act through the PI-3 kinase-signaling pathway (for review, see ref. *149*). Pten is ubiquitously expressed during CNS development, suggesting a potential role for this protein in brain-growth control *(150,151).* Confirming this hypothesis, the Pten gene is often mutated in glioblastoma *(152).* The excision of the Pten gene in Pten-lox+ transgenic mice by the Cre-recombinase gene controlled by the nestin promoter induces the loss of Pten expression in neural progenitors at mid-gestation. The loss of Pten inhibition results in a larger brain growth because of increased cell proliferation in the ventricular zone and decreased cell death *(153).* Isolation of stem cells by the neurosphere approach has revealed that mutant mice have approx 70% more stem cells in comparison to controls and that knockout neurospheres contain 6.5 times more stem cells, as shown by a stem-cell renewal assay *(153).* These data clearly show that Pten controls stem-cell renewal by inhibiting proliferation.

Inhibition of p53 *and Rb leads to Neural-Cell Immortalization: The SV40 large T-antigen*

When the conditions necessary for mouse embryonic cerebellar or hippocampal progenitor expansion were still unknown, blocking of the tumor suppressors *p53* and Rb was performed to allow cell proliferation. The inhibition of *p53* and Rb was achieved using the temperature-sensitive allele of SV40 large T-antigen *(154):* cells transduced with Sv40 large T proliferated at 33°C, and cell stimulation stopped at 39°C. The multipotentiality of clonogenic hippocampal cell lines was tested using transplantation procedures in neurogenic brain sites such as the neonatal hippocampus and cerebellum *(155).* After transplantation, grafted cells harbored either a glial (astrocyte and oligodendrocyte) or a neuronal morphology, showing that these cells are derived from a multipotent progenitor *(155,156).* It is probable that the primary neural cultures expressed *p53* and Rb because of inappropriate culture conditions capable of maintaining progenitor survival and proliferation. It is not clear whether Sv40 acts on the NSC population or on other progenitors. Interestingly, Sv40 large T failed to allow the establishment of a human neural-cell line from progenitors derived from neurospheres that originated from fetuses *(157).* These results suggest that human neural multipotent progenitor/stem cells are controlled by other tumor suppressors under these conditions.

p27 *and Neural Cell Immortalization*

Various tumor suppressors could be involved in the regulation of NSCs. Moreover, the expression of tumor suppressors appears to have a specific pattern. Interestingly, in the adult mouse, *p27* appears to regulate the cell cycle of the transit-amplifying progenitors of the SVZ but not of stem cells, whereas *p19* con-

trols the proliferation of neuroblasts *(158).* It appears that different populations of progenitors have their cell cycle controlled by different tumor suppressors. It is important to determine whether these proteins are expressed in a similar pattern (*p19* in neuroblasts) in other areas of the brain and in cultured stem cells and derived progenitors, because they could serve as specific markers to determine subpopulations in nestin-positive cells.

The retina also exhibits a different expression pattern of tumor suppressors, depending on the cell phenotype (for review, see ref. *159*). For instance, the tumor suppressor *p57* is expressed by a subpopulation of amacrine cells *(160),* whereas *p27* is found in the proliferative zone and persists in Müller cells *(161,162).* Misexpression of *p27* leads to premature differentiation, showing that *p27* regulates the retinal progenitor cell-cycle exit. These results suggest that the exit of stem cells from the mitotic cycle is regulated according to the neuronal phenotype they will generate. However, contrary to *Xenopus (163),* there is no evidence that cell fate depends on the identity of the tumor suppressor expressed,

It appears that by controlling *p27* expression, it is possible to increase or repress neural progenitor proliferation. Several studies reported the induction of neural-cell division by mimicking the action of c-Myc *(157,164,165).* c-Myc is known to activate the cell cycle through the inhibition of *p27* action by different mechanisms. First c-Myc can induce the expression of a *p27*-sequestering protein *(166).* Second, it may prevent the interaction of *p27* with the cyclinE-cdk2 complex (see ref. *167*). In addition, c-Myc can induce the expression of cyclin E, leading—after several steps—to the release of *p27* from the cyclinE-cdk2 complex and thus allowing cells to enter the mitotic cycle *(168).* Confirming these actions, the deregulated, enhanced expression of Myc genes is linked to tumor proliferation in various cell types *(169–171).* Some viruses contain homologs of c-Myc in their genome. The viral homolog of c-Myc, v-Myc, has first been identified as the transforming element in the MC29 avian-transforming retrovirus. The molecular action of v-Myc seems to be similar to those linked to c-Myc function (reviewed in ref. *172*).

The expression of v-Myc by the MC29 retrovirus in primary chicken fibroblasts or murine and avian hematopoietic cells provokes the transformation of these cells *(173–175).* In contrast, the transfer of v-Myc has virtually no action on adrenocortical cells *(176).* These results show that each cell type determines its cell-cycle progression with different levels of controls. Regarding the CNS, v-Myc has no effect on quiescent retinal cells *(177).* Interestingly, it was recently shown that c-Myc expression can stimulate telomerase expression *(178),* suggesting, that in certain cases, c-Myc can lead to immortalization through telomerase activity. Moreover, the c-Myc level is increased after prolonged hTert expression, provoking a positive feedback loop even after the removal of the hTert transgene *(134).* Such an effect has not yet been tested in neural progenitors. Nonetheless, the proliferation of cerebellar neural progenitors can be definitively established by v-Myc expression only *(165).* This series of data suggests that Myc stimulates the proliferation of neural progenitors, but does not reprogram proliferation in cells that are definitively post-mitotic. Similar results were obtained with human fetal neural progenitors that overexpress v-Myc, the cell-doubling rate of which was accelerated and maintained constant under stimulations of exogenous mitogens *(157,179,180).* No tumorigenic properties of this cell line were reported *(157).* Interestingly, the differentiation capacity seems to be unaltered by the expression of the oncogene *(181–183).* Murine and human fetal neural progenitors immortalized by v-Myc can differentiate into neurons and glia once injected into the developing mouse brain or the adult rat striatum, respectively *(165,181–183).* In fact, the effect of v-Myc on neural-cell fate determination is not a clear-cut action. Other studies revealed that v-Myc can interfere with differentiation. Hippocampal neural progenitors genetically engineered with v-Myc controlled by an inducible promoter differentiate only

when v-Myc is shut down *(164).* The level of v-Myc in various studies on neural cells may explain the differences observed for the action of v-Myc on differentiation.

This series of data shows that the level of stimuli needed to increase progenitor proliferation is a subtle balance between renewal and maintenance of cell differentiation. Such a critical equilibrium was recently observed for NSCs cultivated in the long term in the presence of EGF. After several passages, cells cultivated as neurospheres attach (approx 10 passages), lose their dependence on EGF to proliferate (approx 27 passages), and acquire characteristics of transformed cells. These observations have revealed that constant mitogenic stimulation of NSCs can lead to tumorigenicity *(184).*

An understanding of the dosage of NSC mitogens and inhibitors of tumor suppressors is thus crucial in order to maintain NSC long-term renewal without inducing cell transformation.

Perspectives

Interestingly, neural cells transduced with v-Myc necessitate the presence of mitogens to undergo sustained proliferation. This characteristic provides an opportunity to explore the mechanisms leading to self-renewal. One can study gene expression (induction and repression) after v-Myc induction, and then the stimulation by exogenous mitogens. Moreover, comparisons of neural stem-cell gene-expression profiles could identify the genes that are required for cell proliferation in neural progenitor and stem cells. This approach, combined with those of various groups looking for gene-expression homology in different stem cells (e.g., hematopoietic, embryonic, or neural) *(185),* may help to identify candidate genes. Then, these genes could be investigated by *in situ* hybridization to reveal their location and temporal pattern of expression in order to define whether this expression is compatible with the location of stem cells and their functions. Similar approaches have recently been described using hematopoietic and neural stem cells *(185).*

Another promising approach in the study of the mechanism endowed by stem cells is the use of nuclear transfer. The actual situation in this domain seems to show that efficacy in cloning after nuclear transfer is tightly linked to the developmental state (differentiated cells vs stem cells) of the donor nucleus *(186).* The most primitive cell state seems to need less genomic remodeling in comparison to adult differentiated cells. The dissection of the genes re-expressed after nuclear transfer in comparison to those already expressed (or even inactive) in different cell-donor populations should help to reveal important genes leading to self-renewal.

The understanding of how to amplify stem cells without inducing cell transformation represents a challenge to produce NSCs in order to adequately study neurogenesis in vitro and to produce safe NSCs for cell transplantation, as well as to identify key mechanisms at the origin of brain tumors.

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