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Neural Stem Cells and Their Plasticity

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INTRODUCTION

Stem cells are functional units in both development and tissue homeostasis and can be found in a variety of embryonic and adult mammalian tissues. These cells are thought to arise from totipotent embryonic stem (ES) cells of the inner cell mass of the blastocyst from which distinct groups of precursors segregate into the three main germ layers (ectoderm, mesoderm, and endoderm) at around the time of gastrulation. Gradually, these cells will mature into fate-restricted organ- and tissue-specific somatic stem cells (SCs) *(1)*, which are responsible for the growth of tissues during development. The number of SCs declines when the tissues approach maturity and remains rather constant throughout life.

Throughout adulthood, SCs are responsible for tissue maintenance and repair, although the latter function may be carried out at very different rates in various organs. For instance, although many tissues like the hemopoietic system, the epidermis and the intestinal epithelium are known to undergo continuous, extensive cell replacement, the mature mammalian central nervous system (CNS) has long been considered incapable of significant cell turnover. This view has begun to change in the last few decades and, lately, the existence of *de novo* neurogenesis in the adult brain and the presence of stem cells in the mammalian CNS have emerged. Currently, a restricted area of the adult forebrain, the subventricular zone lining the forebrain ventricles (a remnant of the embryonic subventricular zone) is thought to be the largest stem cell compartment of the adult brain. Adult neural stem cells (ANSCs) have been isolated from this region and have been propagated in vitro (reviewed in refs. *2–5*). Although it was expected that ANSCs may display a certain degree of plasticity both in terms of growth and expansion rate and differentiation ability, it was also generally held that ANSC fate was restricted to generating exclusively the three major brain cell types, namely neurons, astrocytes, and oligodendrocytes.

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In this chapter, we focus on a series of recent findings that document an unexpected degree of plasticity of SCs. Emphasis is put on discussing the capacity of ANSCs to generate non-neural cell lineages. A preliminary examination of some basic notions on the functional properties and on the emerging role of the microenvironment in regulating ANSC behavior is preparatory to the discussion that follows on the extreme plasticity of these cells.

GENERAL CONCEPTS ON ADULT STEM CELLS

During development, stem cells proliferate and their progeny undergo a process of progressive lineage restriction and, eventually, generate the terminally differentiated cells that form the mature tissues. Although diversification of distinct mature cell types is complete at or soon after birth, many tissues in the adult organism undergo continuous physiological cell turnover and repair and must therefore embody a population of rather pliable SCs. These are often relatively quiescent or slow proliferating cells, but they retain a significant ability to increase their activity to replace dead and/or injured cells, and very often this occurs through the generation of an intermediate, fast-proliferating transit-amplifying cell population *(6,7)*.

Despite the significant effort that has gone into defining the specific molecular and/or antigenic markers for the various types of adult stem cell, their identification in many tissues is still carried out on an operational basis and, essentially, relies on the retrospective assessment of critical functional characteristics. A notable exception is represented by the hemopoietic stem cells, for which a wide array of such markers is, indeed, available.

According to the most widely accepted operational definition *(6,7)*, stem cells are undifferentiated cells (i.e., lacking antigenic markers typical of mature cells), displaying an extensive proliferation potential that is inextricably linked to their extensive (possibly throughout life) self-renewal capacity. SCs are also believed to be multipotent, in the sense that they can give rise to a wide array of mature progeny of the tissue in which they reside and should possess the ability to regenerate their tissue of origin, even following significant damage.

Self-renewal is defined as the capacity of a cell to perpetuate itself and, at least in invertebrates, it can be achieved at the single-cell level by a deterministic type of division in which one cell is identical to its mother and another more differentiated cell is invariably generated at each cell cycle. In vertebrates, self-renewal is rather viewed as the property of a cell population as a whole and is, thus, interpreted as the capacity to maintain the number of stem cells in a given cell compartment at a steady level. Yet, under particular

circumstances, the stem cell population can be expanded or reduced in size, if necessary. This is made possible by the fact that a fixed stem cell population is physiologically maintained by an even balance between the number of symmetric divisions that generate two stem cells or, alternatively, two differentiated cells at each cycle. Shifting this equilibrium in favor of the first or second type of division will either determine an increase or, alternatively, a decrease in the number of stem cells within the population. This mechanism is likely to provide a system by which the size of the stem cell population and the number of differentiated progeny generated can be varied in response to changes in the extracellular environment or in intercellular communication, brought about by various injuries or pathological situations.

Multipotency is the ability of a single cell to generate many different types of mature progeny. In principle, multipotent stem cells should be able to give rise to all the cell types that constitute their tissue. In vivo, this criterion may be difficult to assess, and stem cells may appear to generate only a subset of the differentiated cell types of a given tissue. However, the global fate potential of SCs may be unraveled by in vitro assays *(8–13)*, in which a candidate cell is challenged under environmental conditions that may not be as readily available in vivo.

A notable feature of SCs is that they are generally located in specific restricted tissue regions, within which a cytoarchitectural and/or biochemical confinement may create a specific niche. Within the niche, conditions are maintained so that SCs can retain their peculiar attributes and, particularly, their life-long ability to self-renew and generate mature progeny. Although in some tissues like the liver and the hemopoietic system the anatomical location of the stem cell niche and its relationship to the tissue-specific stem cells are not perfectly clear, in other systems like the epidermis in nonhaired skin, the hair follicle and the small intestine, the niche is neatly, spatially defined and the stem cells residing therein can be identified by their morphology and relative position within the niche itself *(14,15)*.

THE ADULT NEURAL STEM CELL AND ITS *INTRA-GERM-LAYER* PLASTICITY

The mammalian brain with its complex network of connections develops from a much simpler embryonic, neuroepithelial structure called the neural tube, contains uncommitted proliferating neural precursors that, initially, reside in the luminal cell layer or ventricular zone (VZ). As development proceeds, a new germinal layer appears beneath the original VZ, which is called the subventricular zone (SVZ). The thickness of the VZ then gradually

decreases and the latter is eventually reduced to a continuous cell monolayer (ependyma) lining the ventricular cavities of the adult brain. A remnant of the primitive SVZ persists in the forebrain throughout adulthood as an actively mitotic layer. A large body of evidence has been accumulating that suggests the persistence of intense neurogeneic activity within this region. This has raised questions as to the existence of neural stem cells in the adult brain and of specific niches within which the appropriate set of neurogeneic signals is preserved throughout life to continuously support neurogenesis (reviewed in refs. *16–18*). Recently, *bona fide* neural stem cells have been isolated from the adult SVZ *(8,19)* and numerous reports from different groups have suggested that a multipotent stem cell compartment resides within the periventricular region of the adult mammalian forebrain *(9,20)*. Furthermore, putative or "potential" stem cells have subsequently been isolated from non-neurogeneic regions of the adult brain *(10,21,22)*. This reinforces the idea that cells endowed with different degrees of stemness may reside throughout the adult CNS and that an appropriate neurogeneic microenvironment, which is likely found only within specific locations in the mature brain, is necessary for these stem cells to express their neurogeneic capacity in vivo.

The current view identifies the periventricular region of the forebrain as the main stem cell compartment of the adult mammalian CNS. From here, large numbers of cells are born in the neonatal *(23)* and adult *(11)* rodent brain that migrate along a restricted pathway to the olfactory bulb (OB), where they differentiate into interneurons. These stem cells can be isolated from the ependymal layer and/or the SVZ and, when propagated in long-term cultures, they retain extensive self-renewal, multipotency and stable functional features over time (Fig. 1). Culturing of these stem cells takes place in the presence of epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF2) *(8,9,19,20)* in serum-free medium, so that a steady expansion of the stem cell population is obtained. Notably, the actual rate of ANSCs expansion strictly depends on the pattern of mitogenic signals to which they are exposed. An intensive expansion of the stem cell number takes place in the concomitant presence of EGF and FGF2 *(12)*, indicating that symmetric divisions in which a stem cell gives rise to two daughter stem cells must occur with a high frequency under these conditions *(24)*. Yet, when only one of the two factors is used as a mitogen the very same ANSCs revert to a significantly slower rate of expansion, showing that symmetric divisions yielding two differentiated cells at each cycle are significantly increased in the presence of a single growth factor *(12)*. These findings describe an important aspect of the ANSCs' physiology, that is, their

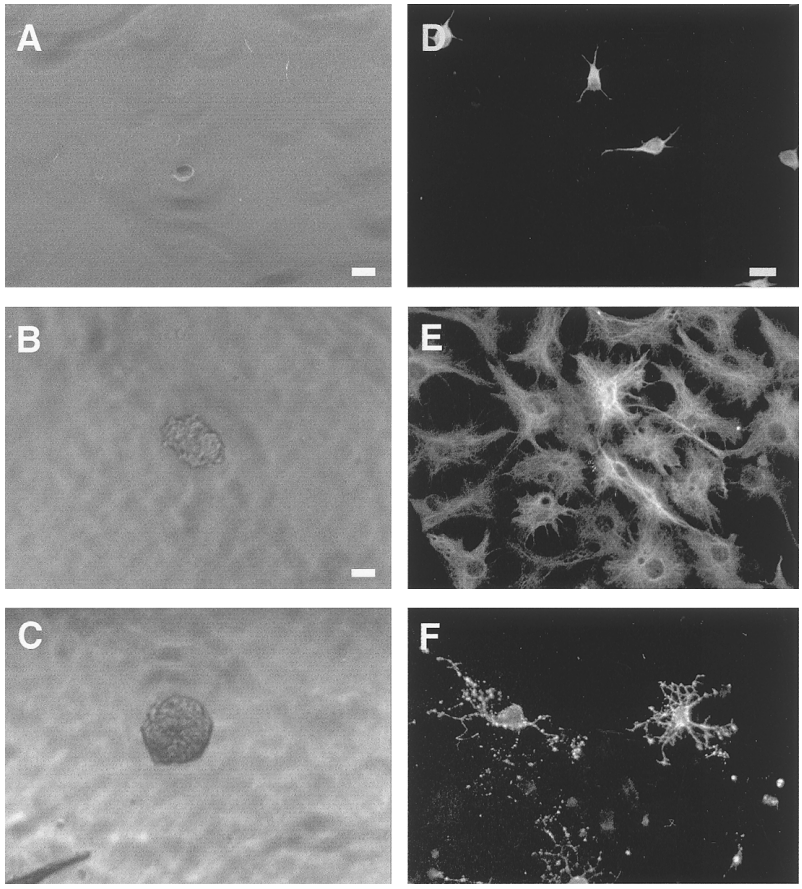

Fig. 1. Cloning of adult neural stem cells. An example of ANSC cloning is shown. **(A–C)** A single adult neural stem cell is shown after plating in isolation in a single well. After 7 d **(B)** and 14 d **(C)**, this cell formed a cluster, which was serially subcultured every 6 d to establish a continuous culture. Growing cells expressed the neuroepithelial antigen, nestin (data not shown). **(D–F)** To induce differentiation, a fraction of these cells were plated in 1% fetal calf serum (FCS), in the absence of growth factors. The detection of neurons (**D**; MAP2; 16.3 ± 0.55% of total cell number [TCN]; n = 5, ± SEM), astroglia (**E**; GFAP; 72.6 ± 5.9% TCN; n = 5) and oligodendroglia (**F**; GalC; 2.5 ± 0.9% TCN; n = 5) among the progeny of the cell in (A) indicate its multipotentiality *(3,4)*. Secondary clones of the cell displayed in (A) produced an average of 38 ± 5.8 (n = 6) cells capable of producing tertiary, multipotential clones, thereby demonstrating self-maintenance. Scale bars: A, 15 μm; B and C, 40 μm; D and E, 15 μm.

capacity to "interpret" the exposure to various combinations of epigenetic signals in order to vary their self-renewal activity in relation to changes in the extracellular environment.

That the fate of ANSCs is under tight environmental control is also shown by the observation that, upon removal of growth factors (GFs) from the culture medium, spontaneous differentiation of the ANSCs progeny into a mixed neuronal/glial cell population rapidly ensues (Fig. 1). Remarkably, the timing of appearance of the mature CNS lineages is similar to that observed within most embryonic, neurogeneic regions in vivo: neurons are generated first, followed by astroglial cells and, eventually, by the onset of oligodendrocyte production. Furthermore, survival of the various differentiated stem cell progeny, as well as their maturation (indicated by the expression of defined antigenic properties), is dependent on extracellular signals. For example, FGF2 prevents the differentiation/maturation of glial and neuronal/glial precursors in serum-free medium, and the addition of low concentration of serum is a necessary step to improve neuronal maturation and the expression of glial antigens *(25)*. Also, neurotrophins play a distinctive role in affecting both the survival and differentiation of neurons in culture *(26,27)*, whereas several other molecules (bone morphogenetic proteins [BMPs], platelet-derived growth factor [PDGF], FGFs, leukemia inhibitory factor [LIF], ciliary neurotrophic factor [CNTF]) regulate the proliferation, the lineage selection, and the differentiation/maturation of ANSC-derived astrodendroglial and oligodendroglial progenitors *(13,28–31)*.

An intriguing aspect of ANSCs' plasticity is the ability to alter their developmental fate in response to extrinsic cues that may act in an instructive fashion. In fact, it has been shown that once differentiation has begun, the ratio of neuronal versus glial differentiation can be altered by exposing the stem cell progeny to various combinations of epigenetic signals. For example, the final outcome of the differentiation process in murine ANSCs can be biased by PDGF in favor of the acquisition of a neuronal fate, so that almost half of the differentiated progeny turns into neurons *(32)*. A similar phenomenon is observed in fetal human NSC cultures in the presence of LIF *(33)*. In striking contrast, CNTF appears to act instructively on murine ANSCs to generate a "larger than normal" proportion of glial cells *(32)*.

It is worth noting that the neuronal progeny of the neural stem cells is truly functionally active, as demonstrated by the ability of neurons derived from long-term passaged stem cells to elicit action potentials *(9)*. With respect to the neurotransmitter phenotypes that this progeny appears to acquire in vitro, it is clear that the large majority of the cells display a GABA-ergic or glutamatergic phenotype when differentiation is induced by

the simple removal of GFs in serum-free culture medium. However, a certain degree of plasticity has been bestowed upon ANSCs also with respect to the choice of the neurotransmitter phenotype. In fact, there is evidence that specific neurotransmitter phenotypes such as the catecholaminergic one can be induced by exposing the ANSC progeny to various differentiation conditions *(34–36)*.

It should be emphasized that the epigenetic modulation of ANSC activity described above for ex vivo situations can also occur in vivo. This is shown by the dramatic increase in the number of newborn cells that is observed in the adult brain following intraventricular administration of EGF, FGF-2 *(36,37)*, or brain-derived neurotrophic factor (BDNF) *(38,39)*. Interestingly, whereas FGF-2 and BDNF induce an increase in the number of neurons, EGF enhances differentiation into the glial lineage *(36,37)*. Moreover, EGF has an inhibitory effect on the progression of SVZ-derived cells through their usual migratory routes and induces their displacement from the SVZ into the adjacent brain parenchyma *(36)*. This strongly suggests that different cell functions, including cell proliferation, survival, commitment, and migration, can be brought about under different environmental conditions in a rather complex fashion, in vivo. A recent confirmation of these phenomena has come from the recent work of Fallon et al., showing that the infusion of transforming growth factor (TGF)-α in the striatum of 6-hydroxy-dopamine-lesioned rats results in the proliferation of SVZ cells and in their migration toward the injection site, followed by differentiation of the newly born cells into intrastriatal, TH-immunoreactive neurons *(40)*.

Altogether, these observations lead to the conclusion that, in ANSCs, epigenetic signals work in concert with the cell autonomous genetic program to regulate a series of critical neurogenetic steps during which growth, fate determination, differentiation, and maturation are finely and timely regulated. The above-discussed findings underline how the rate and extent by which the enormous number of different cell types that make up the mammalian CNS are generated is subjected to flexible regulation. These phenomena can be regarded as an example of intratissue or intra-germ-layer plasticity, because they concern the flexible generation of mature neural cell lineages, so that all the progeny that are produced by ANSCs are derivatives of the same embryonic germ layer (i.e., the ectoderm).

ADULT NEURAL STEM CELLS AND THEIR *TRUE* IDENTITY

In the last 3 yr, a plethora of studies have contributed to unravel the functional characteristic of the ANSCs. However, the true identity of these cells is still the object of heated debate. The cellular composition of the

SVZ region has been previously described, and four cell types have been characterized therein: ependymal cells, type A cells (neuroblasts), type B cells (astrocytes), and type C cells (immature precursors) *(41)*. Separate studies have shown that the ependymal cells that line the luminal surface of the adult ventricular wall *(42)* and the astrocytes that resides in the adjacent SVZ (type B cells) *(11)* are the source of the multipotent ANSCs. To provide direct evidence that type B cells can give rise to neurons, Doetsch et al. *(11)* used a transgenic mice expressing the avian leukosis viral (ALV) receptor driven by the promoter/enhancer of the gene encoding the glial-specific protein GFAP (glial fibrillary acidic protein). When these mice were infected with the ALV encoding alkaline phosphatase (AP), they generated AP-expressing SVZ astrocytes that gave rise to a population of rapid dividing cells (type C cells) that, in turn, gave rise to neuronal cells (type A cells) migrating to the OB. By using a similar experimental approach, Seri et al. *(43)* recently suggested that the granule neurons of the hippocampus, the other major neurogenetic region of the adult brain, are generated by astrocyte-like stem cells through a transit-amplifying cell population (type D cells).

A recent report has proposed that adult SVZ-derived astrocytes grown as a monolayer can resume neural stem cells features when replated in suspension cultures in the presence of FGF2 and EGF *(44)*. Moreover, SVZ-derived multipotent progenitors have been demonstrated to express radial glial markers *(45,46)*, indicating that monolayer astrocytes retain a sort of immature "radial glia" phenotype that could be functionally related to the type B astrocytes found in the SVZ. These results strongly suggest that multipotent stem cells of the adult brain may, indeed, represent a small subset of astrocyte-like V cells in the SVZ. However, the possibility that, in certain conditions, ependymal cells themselves may act as stem cells, directly or through the generation of SVZ, remains open.

NEURAL STEM CELL, THE NEUROGENETIC PROCESS, AND THE BRAIN MICROENVIRONMENT

The molecular signals that are required for the maintenance of a neurogenetic or, better, neuronogenetic capacity in the adult brain are poorly understood. Nevertheless, it appears that one can borrow the notion of stem cell niches found in many mature tissues and infer the existence of neurogenetic domains within the adult brain. For instance, adult rat hippocampus-derived progenitor cells grafted into the neurogenic (the SVZ) or non-neurogenic (the cerebellum) region of adult hosts specifically give rise to neurons only in the former *(47)*. Furthermore, SVZ cells transplanted

in the SVZ of a recipient animal generate large numbers of new neurons *(11,48)*, whereas the same cells transplanted to the non-neurogenetic brain region (cortex and striatum) produce astrocytes almost exclusively *(49)*. Similarly, transplantation of in vitro expanded spinal-cord-derived ANSCs *(22)* into the spinal cord of adult rats resulted in the production of glial cells only. However, after their heterotopic transplantation into the dentate gyrus of the hippocampus, a known neurogenetic region in the adult brain *(50)*, the same cells integrated in the granular layer and differentiated into neurons, whereas engraftment into other hippocampal regions resulted in the production of glial cells only *(51)*.

Many efforts have gone into unraveling the molecular signals that drive neurogenesis and in trying to determine their underlying mechanism of action. The scenario that has emerged so far proposes that many different types of molecule (secreted factors, membrane proteins, extracellular matrix components) as well as different cell types act and interact to contribute to the specification of particular domains in the neural stem cell's microenvironment.

Studies on the SVZ in vitro indicate that EGF and FGF2 may be essential components of the stem cell niche. As discussed previously, these mitogens can maintain the proliferation and self-renewal of ANSCs isolated from the SVZ. Infusion of EGF or FGF2 into the forebrain ventricles causes the expansion of the SVZ cell population *(52,53)*, and the importance of EGF signaling for adult neurogenesis in vivo is suggested by the reduced dorsolateral SVZ cell proliferation found in the TGF-α knockout mice *(54)*. Other factors have recently been implicated as potential regulators of stem cell activity in the SVZ. Among them are ephrins *(55)* and noggin, a polypeptide that binds bone morphogenetic proteins (BMPs), preventing their activation of BMP receptors *(56)*. Lim et al. *(57)* proposed that noggin produced by ependymal cells antagonizes BMP autocrine signaling of type B cells (which normally blocks the neurogenic pathway), creating a neurogenetic environment in the adjacent SVZ and driving SVZ cells toward the acquisition of a neuronal identity.

The permissive environment provided by extracellular matrix, cell surface molecules, and special supporting cells (i.e., radial glia) allows the displacement of neuronal precursors from the site of genesis to the site of their full differentiation during development. This is somehow reproduced within those regions of the adult brain that are endowed with structural plasticity and/or the capacity for active neurogenesis. For example, the polysialylated "embryonic" isoform of the neural cell adhesion molecule N-CAM (PSA-NCAM), which plays an important role in cell migration and cell shaping

(58–60), is selectively expressed on the membrane of newly generated cells in the SVZ *(61)* and in the hippocampus *(62)*.

The role of integrins in maintaining stem cells and progenitors in the proper position within a stem cell niche, as well as in activating signal transduction pathways essential for stem cell proliferation and survival, has been documented for extra-CNS stem cells (epidermis and intestinal stem cells) (reviewed in ref. *14*). The contribution of these molecules to the stem cell microenvironment in the adult CNS is currently unknown, but the interactions between integrins and their various ligands has been implicated in neuronal migration *(63)*, in the regulation of neurite outgrowth *(64)*, and in the enhancement of myelin membrane formation by oligodendrocytes *(65)*. Moreover, these molecules have been suggested to play both signaling and structural functions in adult synapses during plasticity *(66)*.

The formation of a specific microenvironment in the niche may also entail the presence of specific cell types that may provide mechanical and/or trophic sustenance to the stem cell. In a way somewhat similar to that observed with stromal and hemopoietic stem cells in the bone marrow, in the developing CNS, radial glial cells provide support for neuronal migration and supply instructive and neurotrophic signals that are required for the survival, proliferation, and differentiation (reviewed in ref. *67*). Similarly, in the adult brain, short radial glialike cells are present in the hippocampal dentate gyrus *(68)*, whereas SVZ astrocytes (type B cells) that surround the cells migrating toward the OB express embryonic cytoskeletal proteins such as vimentin and nestin *(17,69)*.

Once the appropriate set(s) of epigenetic cues is established/maintained within the niche, self-renewal of the neural stem cell compartment ultimately depends on the modulation imposed by these signals upon cell intrinsic regulatory mechanisms. The identity of these intrinsic molecules and the signaling cascades activated upon interaction with epigenetic factors is complex and still poorly understood. Nuclear factors controlling gene expression in stem and progenitor cells, molecules involved in the control of asymmetric divisions of stem cells, and clock mechanisms that set the number of division rounds within the population may function as cell intrinsic regulator factors (reviewed in ref. *70*). Interestingly, some of the transcription factors and their downstream effectors involved in the regulation of cell cycle and proliferation in the stem cell niches of the epidermis and intestinal epithelium (reviewed in ref. *70*) have also been found in the developing and adult CNS as well as in neural-derived primary cultures and cell lines *(71–74)*. This suggests that an evolutionarily conserved set of intrinsic mechanisms may be acting in different types of somatic

stem cell with the ultimate role of maintaining the appropriate size of stem cell compartment.

DEVELOPMENTAL REPERTOIRES OF ADULT SOMATIC STEM CELLS: *EXTRA-GERM-LAYER* PLASTICITY OF NEURAL STEM CELLS

As discussed, SCs from many different adult mammalian tissues, including the bone marrow, muscle, skin, gut, and central nervous system, have been isolated and characterized. In each tissue, these cells are responsible for the cellular turnover elicited by physiological cell depletion or by pathological situations or injury. Therefore, it is almost tautological to view the differentiation potential of SCs as being restricted to the sole production of mature cells that belong to the same tissue in which the SCs reside. However, several lines of evidence have recently challenged this dogmatic notion.

The initial finding of the intragerm layer transdifferentiation capacity of an adult stem cell—that is, the production of a mature progeny normally found in a tissue that is different from that in which the stem cell resides, but sharing a common embryonic germ layer origin—was reported in 1998, when Ferrari et al. *(75)* showed that mesodermal bone marrow precursors can differentiate into skeletal muscle cells following chemically induced damage of the adult tibialis anterioris in the mouse. This was followed by the report from Bjornson et al. demonstrating that, when injected into sublethally irradiated adult mice, ANSCs—that are ectodermal derivatives—could give rise to hematopoietic cells, which are mesodermal in origin *(76)*. This provided the first demonstration that somatic stem cells that were derived from a given germ layer could undergo *trans-germ-layer* differentiation and could generate cells of a distinct embryonic origin.

Later, this striking developmental flexibility began to emerge as a more general feature of other types of adult somatic stem cell. Thus, bone marrow precursors were soon proved to contribute to the regeneration of extramesodermal organs such as the liver and to be capable of converting into neural cell lineages *(77–80)*. Similarly, mesodermal derivatives such as mesenchymal stem cells were shown to give rise to astrocytes and, possibly, neurons, both in vivo *(81)* and in vitro *(82,83)*.

The unexpected plasticity of adult CNS stem cells was quite astounding, particularly because the nervous system has always been depicted as the most "quiescent" of the adult tissues, at least with reference to cell turnover in adult life. Yet, documentation of such extraordinary capability has been extended by the work of Clarke et al. that demonstrated that, upon injection

into the mouse blastocyst, ANSCs can integrate into many different tissues derived from the three main germ layers *(84)*. In this work, the contribution of ANSCs to two major mesodermal lineages such as blood and the skeletal muscle was not observed, whereas our group demonstrated soon thereafter that ANSCs or human fetal neural stem cells do differentiate into skeletal muscle, in vivo and in vitro *(85)* (Fig. 2). This discrepancy is likely justified by the different cell systems and assays used. In the work of Clarke et al., ependymal-derived ANSCs were injected into an embryonic environment whereas, in our experiments, adult SVZ stem cells underwent trans-germ-layer differentiation in the context of an adult organism. Thus, whereas in a regenerating adult tissue the "inducing" cues direct transdifferentiation toward the generation of the local, tissue-specific lineages *(85)*, distinct signals are likely to act upon exogenous cells injected into a blastocyst. These signals will compete in trying to direct the fate of the transplanted cells toward many alternative developmental pathways. In the latter situation, the final fate acquired by the implanted cells will be the result of their exposure to numerous, yet unpredictable factors. To compound the problem, the overall pattern of composition of these factors will depend on the precise stage of blastula development and on a series of unpredictable positional cues that relate to the initial site of integration of the transplanted cells. This situation is likely to cause a significant degree of variability in the outcome of the blastula experiments, which will require the analysis of a significant number of grafts to allow for a categorical conclusion to be reached as to the actual overall potential of the donor cells. Recent data from Pipia et al. seem to confirm this view *(86)*. In contrast with the work of Clarke et al., when these authors injected neural stem cells into blastocysts, the very first lineage to be colonized by the neural cells in their assay was, indeed, the hemopoietic one *(86)*.

The many reports describing CNS stem cell plasticity underline the need for specific, rather peculiar, conditions in order for SCs to be able to express their latent, generalized developmental potential(s). Two main conditions have been identified to be necessary for CNS stem cell transdifferentiation to occur both in vivo and in vitro. First, the stem cells ought to be in a highly undifferentiated state. In fact, we reported that the proliferation state of ANSCs represents an important element that can influence the rate at which ANSCs convert into non-CNS cells. Thus, ANSCs need to be in a state of active proliferation in order to undergo efficient conversion into non-neural cells either in vivo or in vitro *(76,84,85)*. As described by Galli et al. *(85)*, when ANSC-derived terminally differentiated progeny (neurons and glia) were exposed to cues that induce the myogenic phenotype, almost no

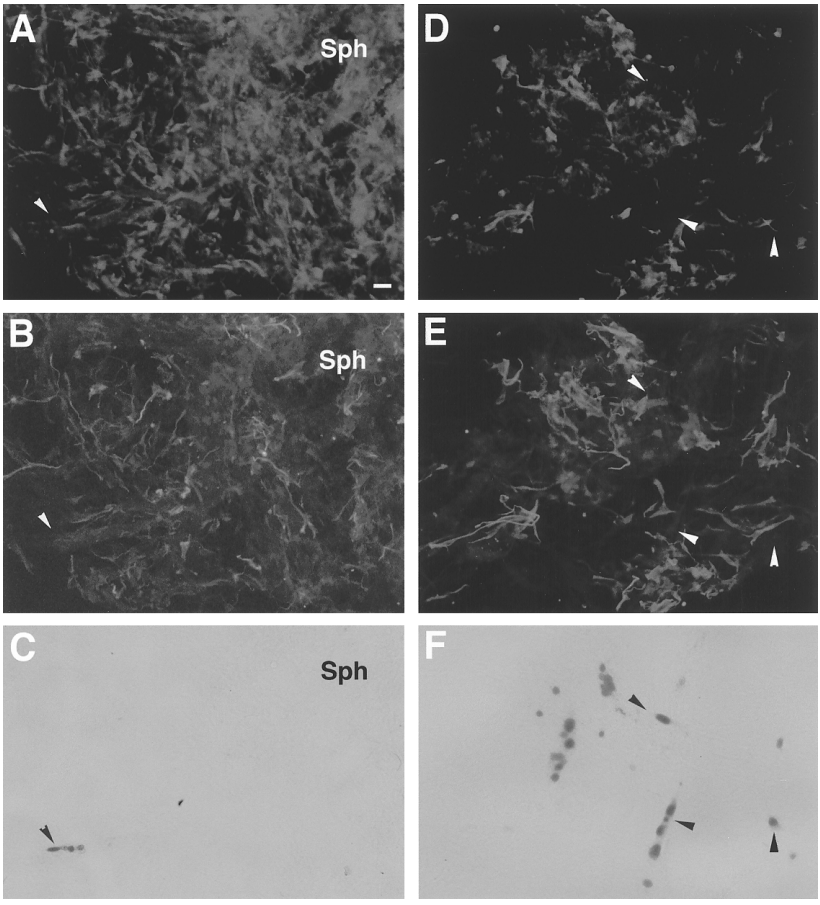

Fig. 2. Inhibition of myogenic conversion in ANSCs by neural cell contact. Equal numbers of ANSCs derived from MLC3F animals that carry the nuclear *lacZ* transgene under the control of the heavy-chain myosin promoter were plated as clustered cells (neurospheres; **A–C**) or following dissociation to single cells **(D–F)** onto C2C12 myogenic cells to induce conversion into muscle. **(A–C)** Myogenic conversion never took place within neurosphere cores (Sph), where densely packed neurons (**A**, microtubule-associated protein 2, MAP2) and astrocytes (**B**, glial fibrillary acidic protein, GFAP) were present. ANSC-derived myocytes were detected by X-gal reaction only at the outer margins of the same sphere (**C**, arrowhead). When dissociated cells from NSMLC-derived neurospheres were cocultured with C2C12 **(D–F)**, a significantly greater number of myocytes (arrowheads in **D–F**) developed: These were uniformly dispersed among neurons (**D**, MAP2) and astrocytes (**E**, GFAP). Expression of neural antigens in MLC3F-derived myogenic cells was never observed (arrowheads, **A–F**). Scale bar = 8 μm.

conversion to skeletal muscle was observed, thus emphasizing the need for ANSCs to be in a high immature "naive" state, in order to successfully deal with a cohort of new and "unfamiliar" signals.

Second, it can be speculated that, to undergo *trans-germ-layer* differentiation, ANSCs ought to become exposed to specific microenvironments rich in instructive cues. These cues, apparently, become available during the regenerative phase that follows an injury in an adult target tissue or during embryonic development, as CNS stem cells have been shown to repopulate both the early developing gastrula *(84)* as well as the regenerating hemopoietic system *(76)* and muscle *(85)*.

The fact that only ANSCs but not their differentiated neuronal or glial progeny are susceptible to *trans-germ-layer* differentiation *(85)* highlights an important difference between this phenomenon and transdifferentiation as conceived in its classical form. In fact, transdifferentiation implies the ability of differentiated cells to acquire a new identity by turning off one set of lineage-specific genes and activating genes found in another differentiated cell type, whereas *trans-germ-layer* differentiation seems to reflect the *de novo* expression of a broader developmental potential of ANSCs that may become evident only under peculiar conditions. In the context of this observation it is worth noting that no molecular and biochemical overlapping of specific neural and non-neural markers could ever be observed between ANSCs and their extra-CNS progeny. In fact, markers of hematopoietic precursors were never detected in ANSCs before injection into damaged animals *(76)* and expression of muscle determination factors or muscle differentiation markers (MyoD, Myf5, myogenin and myosin) was never observed in ANSCs prior to their exposure to "myogenic" environments *(85)*. In addition, the total absence of expression of mesodermal- and endodermal-specific antigens (i.e., TROMA-1) was demonstrated in ANSCs before injection into the developing blastula *(84)*, yet these cells can give rise to derivatives from both layers. In all these examples, ANSCs expressed appropriate neural stem cell antigens such as nestin in their undifferentiated state and neuronal and glial markers upon differentiation.

An important issue ensues from the unexpected *trans-germ-layer* plasticity of somatic stem cells and concerns the mechanisms that govern this striking phenomenon and the identification of the signals released by the "inductive" environments. In spite of the relative abundance of examples of *trans-germ-layer* differentiation, very little is known as to the identity of the factors responsible for this transition. Nevertheless, based on our recent work showing the generation of skeletal muscle from ANSCs, it is still possible to propose some hypothesis. We have established the initial in

vitro model by which neuro-myogenic conversion can be elicited and studied under controlled conditions. Using this system, it appeared immediately clear that neuro-myogenic conversion could be observed only when ANSCs were cocultured with C2C12 myogenic cells or with primary myoblasts, and never with nonmyogenic cells. Interestingly, conversion required direct cell–cell contact and did not take place when neural and myogenic cells were physically separated by a porous membrane, nor was it observed when ANSCs were exposed to myogenic cell-derived extracellular matrix or to medium in which muscle cells had been grown. Furthermore, when ANSC underwent myogenic induction as undissociated clonal colonies, the proportion of cells that underwent conversion to skeletal muscle dropped by almost 80% when compared to the same number of ANSCs that were dissociated before coculturing. This happened despite the fact that ANSCs clusters rapidly spread onto myoblasts—so that the cells in the sphere were in contact with the C2C12 cells. The occasional myogenic conversion in these cocultures occurred exclusively at the exterior of the cluster of neural cells and never within its core, for the latter contained only neurons and glia. Thus, when neural cells are clustered together, neural-to-neural signals override the myoblast-derived myogenic cues, blocking neuro-myogenic conversion of ANSCs. Therefore, in analogy to what is observed during the dedifferentiation of retinal pigmented epithelial into lentoids *(87)*, loss of cell-to-cell communication among ANSCs seems to emerge as one of the major determinants influencing the onset of neural-to-muscle conversion.

Based on these findings, we can infer that, at least in the specific case of neuroectodermal–mesodermal conversion, cell-to-cell contact between the inducing (muscle) and the induced (neural) cells must occur. Because cell fusion was not a prerequisite for neuro-myogenic conversion in our system, this kind of direct interaction underlines the necessity of a direct exchange of information between the inducer and the induced cells. This may imply the interaction of cell surface receptors and ligands, the formation of gap junction structures, as well as the involvement of short-range-acting molecules. However, it should be emphasized that, based on these observations, it cannot be ruled out that also secreted factors may be implicated in neuro-myogenic conversion. Yet, it can be argued that diffusible molecules alone cannot enforce such an extreme change of cellular identity. This finds confirmation in the observation that culturing ANSCs in the sole presence of hematopoietic growth factors and cytokines—that, alone, would allow for the growth and maturation of hemopoietic precursors—fails in eliciting conversion of neural cells into blood. In fact, the latter can only be achieved through transplantation into irradiated animals, following their integration

into the bone marrow *(76)*. Finally, it emerges that a fine interplay between antagonistic cues takes place in the neuro-myogenic conversion phenomenon. In this system, the induction of the muscle fate elicited upon ANSCs is simultaneously counteracted by a "neuralizing" kind of signaling that takes place between the neural cells when they are in direct contact with each other. This phenomenon may be viewed as a classical "community effect." Hence, a cohort of instructive signals rather than a single effector is likely to direct the change from a brain-specific fate to a mesodermal one, as observed in ANSCs. In vivo, these signals can be found either in the extracellular microenvironment that has been perturbed by a lesion or are elicited through a direct cell-to-cell interaction between the host and donor cells (Fig. 3).

Along with the influence of the external milieu in controlling stem cell flexibility, cell-autonomous modifications may be involved in the transdifferentiation process. Therefore, it appears of fundamental importance to identify the genetic determinants that can activate the cascade of events that lead to cell lineage interconversion. Initial data are now becoming available in this area of investigation. For instance, the gene called *Pax7* has been involved in controlling the capacity of adult muscle satellite cells to transdifferentiate into hemopoietic cells *(88)*. In fact, gene deletion by homologous recombination provided adult muscle stem cells with a markedly increased potential for hematopoietic differentiation. Furthermore, similarities between the gene expression pattern observed during embryonic development and during transdifferentiation have been documented for the cornea–lens transdetermination phenomenon *(89)*. This implies that the different processes of embryogenesis, regeneration and transdifferentiation are likely highly interrelated at the molecular level. In a similar fashion, PTF-1 and PDX-1 expression in pancreatic acinar cells has been associated to the neogeneration of endocrine islet cells, and exocrine cells can transdifferentiate and acquire characteristics typical of precursors active during β-cell neogenesis *(90)*.

CONCLUSIONS

The different studies demonstrating the *trans-germ-layer* developmental potential of adult stem cells lead to a quite clear conclusion; that is, that many SCs possess the capacity to reactivate an apparently dormant set of developmental programs when challenged under peculiar environmental conditions. The reactivation of these programs does not require genetic manipulation or nuclear transplantation and is driven, at least in part, by a cohort of extracellular cues that exist in the developing organism as well as within regenerating adult tissues. This observation seems to reinforce the

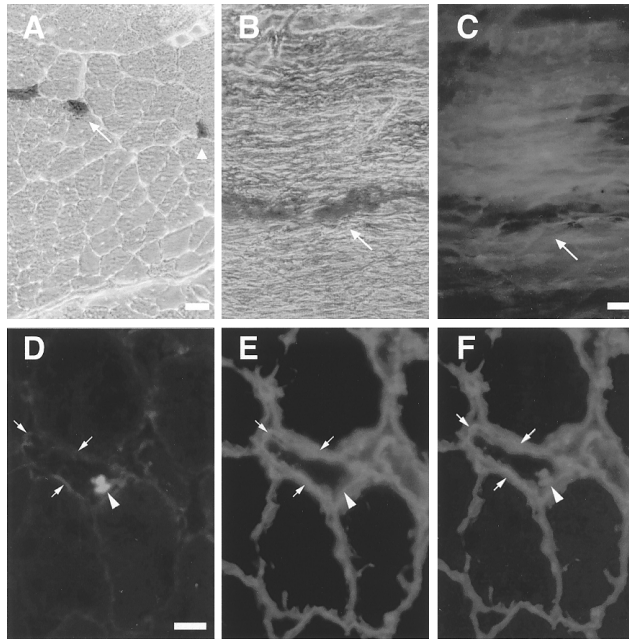

Fig. 3. Clonally derived mouse adult ROSA-26 and human embryonic neural stem cells differentiate in vivo to muscle fiber. 5×10^5 ANSCs that were clonally derived from ROSA26 animals **(A–C)** that constitutively express the *lacZ* transgene or human fetal neural stem cells **(D–F)** were injected into regenerating tibialis anterior (TA) of *scid/bg* mice. **(A)** After 3 wk, both ROSA26-derived single cells (arrowhead) and small regenerating fibers (arrow) expressing cytoplasmic β-gal were detected at the injection site. **(B–C)** Longitudinal 20-μm-thick sections show β-gal-positive fibers (arrow in **B**) within a group of fibers expressing a sarcomeric myosin heavy chain (arrow in **C**). The fluorescence of the β-gal-positive fibers in **(C)** is quenched by the X-gal reaction product. Scale bar in **A**: 100 μm; **B–C**: 50 μm. **(D–F)** In **D**, two human nuclei are detected deep inside a dystrophin-positive fiber (**E**, dystrophin) (**F**, merged), as expected in regenerating muscle fibers. Scale bar: 25 μm. (Courtesy of U. Borello; see also ref. *65*.)

idea that the cellular and molecular mechanisms that are responsible for the appropriate development of the mammalian body become partly reactivated during tissue regeneration. This underlines the importance and the impact that studies on mammalian development and on basic stem cells physiology bear on the establishment of novel therapeutic strategies for many human diseases that are untreatable with conventional therapies.

The existence of the *trans-germ-layer* differentiation phenomenon opens new therapeutic vistas that were unimaginable until a few years ago.

One may envision a scenario in which cells from a healthy tissue can be "transengineered" and used for the therapy of an ill part of the body in the same individual. The development of such a refined kind of autologous intervention hinges on the understanding of the basic cellular and molecular mechanisms that underline the conversion of cells derived from different germ layers into another. It is clear that this area of investigation will likely become a most explosive one in the years to come.

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