

Screening the Brain: Molecular Fingerprints of Neural Stem Cells

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Abstract: With the development of high-throughput technologies like microarrays for genomic and transcriptomic analysis, and two-dimensional gel electrophoresis, mass spectrometry, and protein arrays for proteomic analysis, it is possible to monitor the changes in gene or protein expression of several hundreds, or even thousands of molecules simultaneously. Within the last years, these technologies have been applied successfully to stem cell research. One of the aims of stem cell expression profiling is to find specific marker genes or proteins which may determine the "stemness" of these cells. In the current review, we will evaluate the results of genomic, transcriptomic and proteomic approaches to find stem cell markers.

We compare the criteria of "stemness" to recent results in adult neural stem cell research. Neural stem cells have been isolated from various regions of the adult brain. They self-renew and give rise to progeny capable to generate neurons, astrocytes, and oligodendrocytes. Besides morphological differentiation, these cells can integrate into functional neuronal circuits, making them suitable targets for cell replacement strategies. General properties seem to be the responsiveness to growth factors, and the activation of developmental signaling pathways. In conclusion, we suggest that stem cell properties can be specified by global gene or proteomic expression patterns rather than by the analysis of individual genes or proteins.

Keywords: Adult neural stem cell, genomics, proteomics, stemness marker, microarray, two-dimensional gel electrophoresis.

INTRODUCTION

Molecular Fingerprints

In forensics, fingerprints are used to identify individuals unambiguously; they are unique characteristics of a person and remain unchanged during the whole lifetime. In molecular biology, "fingerprinting" means the use of high-throughput technologies which allow the simultaneous screening of hundreds or even thousands of "items", may it be genes, gene transcripts, or proteins. In theory, a molecular fingerprint is characteristic for the cell, or tissue of its origin. Its most precise definition is that of an expression pattern consisting of a large number of items, which, seen alone, are not necessarily characteristic of the object studied.

An example for the problem of defining a cell by one distinctive feature is given by the definition of an astrocyte. Originally, astrocytes were defined by their morphology visible in the microscope [1]. Only after the development of immunohistochemical methods, astrocytes were defined as cells expressing the Glial Acidic Fibrillary Protein (GFAP). However, nowadays it is well accepted that not all astrocytes are GFAP-positive and that, in addition, non-astrocytic cells can express GFAP [2, 3]. More generally, if a cell expresses a transcription factor, a metabolic enzyme, or a cell surface receptor which once has been regarded to be unique to this cell, it can happen that this molecule is found to be expressed in other types of cells. In this situation, molecular fingerprinting can help to define cells not only by a single "marker" but by a characteristic pattern of multiple entries.

To detect such a pattern, ways have to be found how to compare complex expression patterns represented by whole genomes or proteomes. The current methodology consists of a sequential application of different bioinformatic tools with the aim to find a common overlap [4].

In the following review we will discuss the use of molecular screening technologies in genomics, transcriptomics, and proteomics which are used to define expression patterns in neural stem cells. These cells give rise to new neurons, astrocytes, and oligodendrocytes. Although the physiological consequences of adult neurogenesis have not been elucidated in total [5], experiments nurture the hope for a therapeutic application of neural stem cells in a wide variety of brain diseases [6, 7]. The developmental potential of neural stem cells has opened a discussion about their use in neurodegenerative diseases like Alzheimer's and Parkinson's disease, cerebrovascular events, and developmental disorders [8, 9], since stem cell properties as defined by morphological and functional characteristics seem to be maintained over the entire lifetime of the organism. To be more definitive with the classification of a cell as a neural stem cell, it is of interest to detect common morphological features such as congruent gene or protein expression patterns. Are there common aspects of all brain-derived stem cells, subsumed under the term "stemness"? Currently, ethical and practical consequences arise from the potency of the zygote and embryonic stem cells [10-12]. In case stem cells isolated from the adult organism show the same gene or protein pattern for stemness, does this mean the same ethical and practical considerations have to be applied to these cells as to stem cells derived from the embryo? Perhaps it is not too visionary to imagine that genes, which normally are only active in the embryo and endow these cells with stem cell properties, can be turned on by biotechnological means in adult stem cells. Then, in

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consequence, must one attribute the same requirements by law to the genetically modified cells?

Stem Cell Definitions

Stem cells have been found in nearly all tissues investigated, but sometimes in very low densities. They can be defined by functional aspects common to all stem cells. These include (i) the ability to proliferate and self-renew, and (ii) the developmental competence ("potential") to give rise to cellular progeny which is able to differentiate into functional cells constituting the respective organ [13-15].

The potential of stem cells has been classified hierarchically: The zygote is the only totipotent cell, meaning that a whole and intact organism can develop from the totipotent cell. During the next developmental steps, pluripotent embryonic stem cells are generated, which can differentiate into all cells of the organism, but cannot give rise to the complete organism any more. In the next steps, multipotent stem cells emerge from the embryonic stem cells. These cells are not only present in the embryo, but also persist in the adult organism during its lifetime. Multipotent stem cells are able to generate at least all cell types of the same germinal layer. They give rise to progenitor cells of the respective tissue, which have only limited ability to self-renew, and seem to be restricted to specific lineages. Moreover, they already show some typical functional and morphological properties of the differentiated cell, but without full functional competence. It is only the terminally differentiated cell which is completely capable to fulfill the functional requirements of the respective tissue [16-20].

Often the terms "stem cell", "progenitor", and "precursor" are used synonymously. However, both terms are also differently used. In this sense, progenitor cells are cells with a limited capacity for self-renewal and only a limited

differentiation potential, whereas precursor cells are thought to be already committed cells with only a limited number of divisions before their terminal differentiation [21].

Stem Cells in the Brain

The careful reader has noted that we provided a general definition of stem cells that also applies for neural stem cells. What makes the difference between neural stem cells and other stem cells? In this review, we refer to "neural stem cells" as (i) proliferating and self-renewing cells isolated from the brain without regard of the developmental stage, may it be embryonic, fetal or adult, or (ii) cells with the ability to develop the phenotype of brain cells, which comprehend in this definition neurons, astrocytes, and oligodendrocytes (trilineage potential) (Fig. 1). Of note, some authors separate terminology between neural stem cells (giving rise to all three lineages) and neuronal stem cells (giving rise only to neurons), which we do not follow here.

Concerning the brain, neural stem cells have been isolated from various regions of the adult mammalian brain. At least two regions exist which show spontaneous neurogenesis persisting until late adulthood, i. e. the dentate gyrus of the hippocampus, and the subventricular zone [12, 17, 22, 23], which are shown in Fig. 1. Neural stem cells from the subventricular zone migrate along the rostral migratory stream towards the olfactory bulb, where they differentiate, mostly into interneurons [24]. *In vivo*, differentiated stem cells seem to integrate into functional neuronal circuits at their destination and are electrophysiologically active [25, 26].

In contrast to other types of stem cells, no specific markers exist to identify neural stem cells unambiguously. Several candidate markers have been proposed, including nestin [27], Musashi-1 [28], Mcm2 [29], and the glial fibrillary acidic protein GFAP [30], but none of these

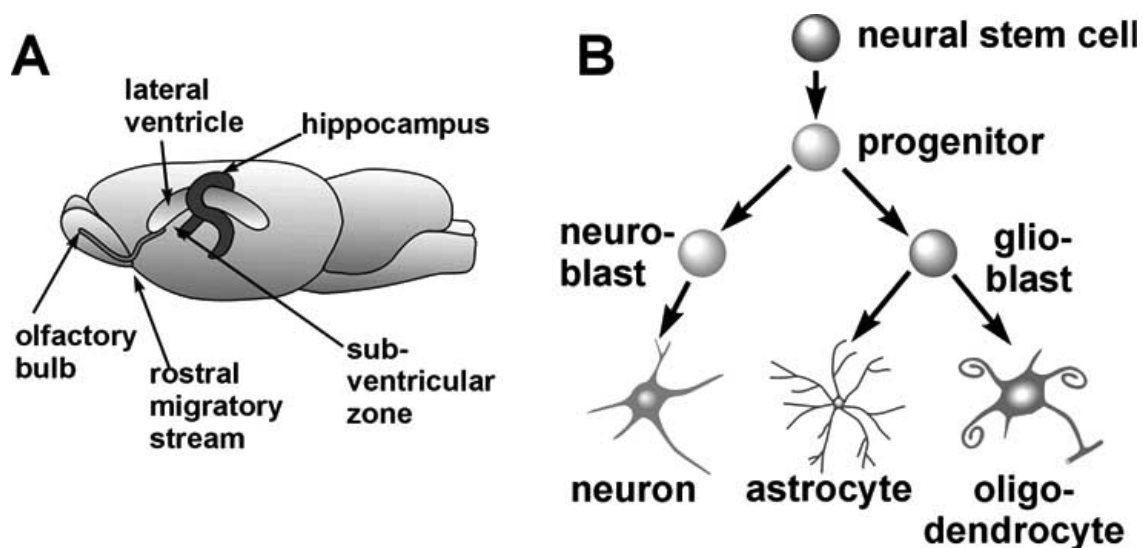


Fig. (1). (A) Anatomical origin of neural stem cells. Neural stem cells can be isolated from brain regions showing spontaneous neurogenesis throughout the whole life. These brain areas include the hippocampus and the subventricular zone. From the latter, neural stem cells migrate along the Rostral Migratory Stream (RMS) towards the olfactory bulb. (B) Stem cell hierarchy. This figure depicts the differentiation process of a neural stem cell to a terminally differentiated cell. It shows the trilineage potential of neural stem cells to give rise to neurons, astrocytes, and oligodendrocytes.

markers could comprehend all neural stem cells at any given time in development, or region. In all cases, only a respective subfraction is expressing the presumptive marker.

Additionally, the tissue of origin does not provide a basis for the neural stem cell definition. Several reports had reported the ability of stem cells isolated from e. g., bone marrow [31, 32], the umbilical cord blood [32], and several other organs (reviewed in [12]) have been shown to express "neuron-specific" proteins. There has been a recent debate about methodological artifacts in which this "transdifferentiation potential" has been questioned due to the occurrence of cell fusion mechanisms in transplantation studies [33-35]. Currently, the earlier enthusiastic view of stem cell potentiality with the ability of "transdifferentiation", or even "dedifferentiation" of already differentiated tissue, is seen very sceptically. This is best studied for bone-marrow derived stem cells. Although still partially controversial, the majority of evidence now speaks against true transdifferentiation [36-39].

Neural stem cells isolated from the brain can be maintained in culture for prolonged periods of time (several passages) without losing their ability to proliferate, self-renew, and differentiate [40, 41]. After harvesting from specific brain regions, these cells grow *in vitro* as neurospheres, each consisting of some hundreds, or even thousands of cells, whereas *in vivo* neural stem cells are rare, even in the neurogenic regions. When neurospheres are passaged for a longer time periods (>10 passages), they seem

to change their properties [39]. Then they become independent of growth factor stimulation, change their gene expression profile, and show altered proliferative kinetics. In consequence, only short-term neurosphere cultures (<10 passages) should be used for gene expression studies. On the other hand, stem cells have to be expanded in culture in order to exclude contamination by other types of neural cells and to yield sufficient amounts of DNA, RNA, or protein, which are necessary to specify gene and protein expression [42].

GENOMIC AND TRANSCRIPTOMIC SCREENING

Methods in Global Gene Expression Studies

The introduction of high-throughput methods to synchronously monitor the expression of myriad of genes or transcripts has revolutionized biological approaches and concepts: these types of large-scale experiments have shifted the concept of a "hypothesis-driven" approach towards that of a "hypothesis-generating" experimental setup [43]. In consequence, even new principles of publishing have occurred which allow the fast publication of large data sets, whenever necessary (see arXiv at <http://xxx.lanl.gov> and open access publishing at <http://www.pubmedcentral.nih.gov/about/openaccess.html>).

To this end, a multitude of methods has been developed for the analysis of a large number of genes/transcripts. Such

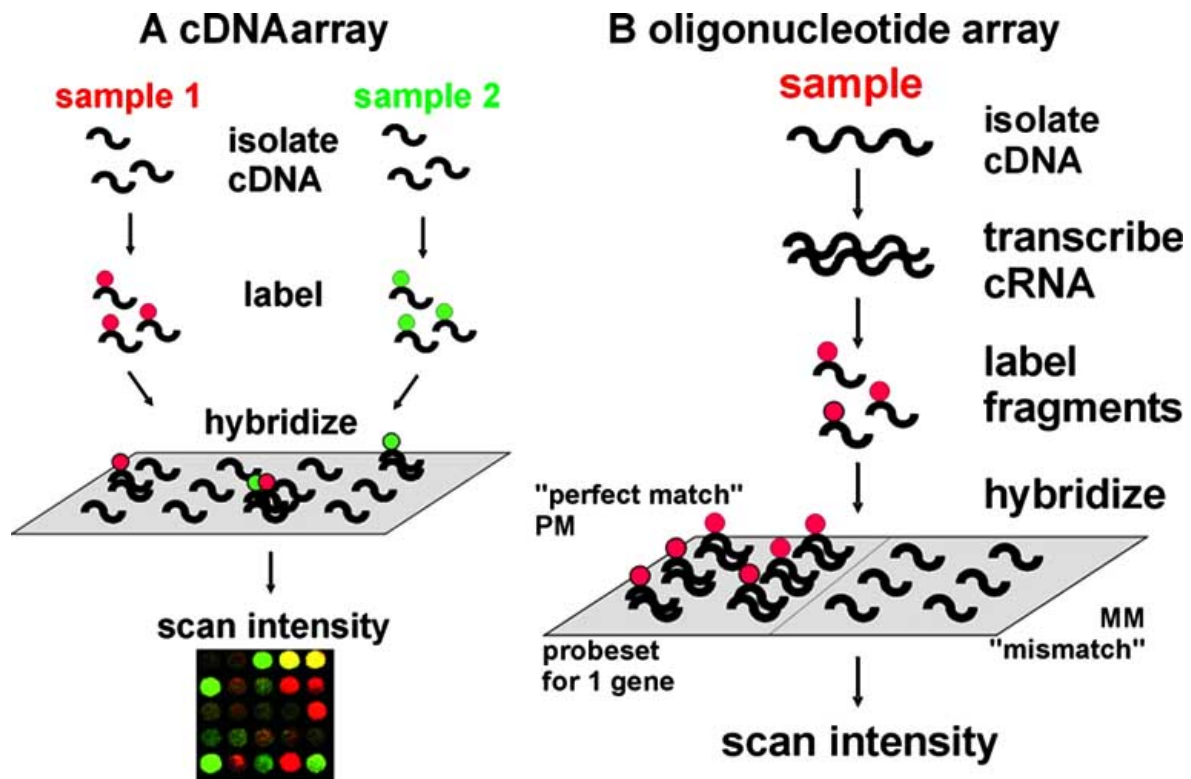


Fig. (2). Principle of microarray analysis. (A) cDNA array: RNA from two samples is extracted, reverse transcribed and labeled with two different fluorophores. Both samples are simultaneously hybridized to the probes immobilized on the microarray. Using a microarray scanner, fluorescent intensities are measured and compared to quantify the results. (B) oligonucleotide array: RNA from one sample is extracted, reverse transcribed, labeled, and transcribed *in vitro*. cRNA fragments are hybridized on glass arrays on which probesets for individual genes are synthesized. Hybridization efficacy to perfectly matching (PM) and mismatching (MM) oligonucleotides is measured.

methods can be categorized into those which allow detecting either known, or, in addition, unknown genes/transcripts. In closed systems, a preselected set of nucleic acids (or in proteomic experiments: antibodies, see below) is immobilized on a glass or membrane surface and then a sample is hybridized to these sequences. In open systems, novel sequences can be identified [44]. Whereas DNA arrays are the method of choice for the closed systems, open systems are not based on the knowledge of the exact gene sequence. These methods include fragment display (e.g. Differential Display, DD; Restriction-Mediated Differential Display, RMDD; Amplified Fragment Length Polymorphism, AFLP), tag sequencing (e.g. Massively Parallel Signature Sequencing, MPSS; Serial Analysis of Gene Expression, SAGE), and subtractive/competitive hybridization techniques (e.g. Suppression Subtractive Hybridization, SHH) (reviewed in [44]).

In this paragraph on genomic screening, we concentrate on gene expression studies using microarrays, as most genomic/transcriptomic studies in stem cell research have been based on the application of microarrays of a standardized format, making the experiments comparable. Mainly two approaches in microarray technology have been developed, cDNA and oligonucleotide microarrays (Fig. 2).

In the cDNA microarrays, nucleotide sequences are immobilized on a glass surface. Then RNA is extracted from a biological sample, reversely transcribed and labeled with two different fluorescent dyes (normally giving "red" and "green" signals). Consecutively, the cDNA is hybridized to its complementary sequence on the microarray [45]. In this context, "probes" are defined as the immobilized sequences of nucleotides on the array, whereas "samples" mean nucleotide sequences which are to be analyzed in the biological material. Practically, in most studies differential expression is investigated by the direct comparison of two samples. Therefore, the two cDNA samples are labeled with two different fluorophores. The samples are co-hybridized to the immobilized probes on the microarray slide. The competition in binding the immobilized target sequence as represented by the ratio of emission wavelengths can be used to quantify the relative expression in the respective sample [42, 46, 47]. The process is depicted in Fig. 2A. Besides fluorescent labeling which allows the direct comparison of the two samples, radiolabeled samples can be hybridized on filter-based arrays. In this case, no competitive binding assays can be performed, and the spotting density of filter-based microarrays is much lower. Filter-based arrays comprise only several hundred sequences, whereas slide-based microarrays reach densities of several thousand sequences per cm² on the slide. Using bioinformatic tools, quantitative data can be compared to identify differentially expressed sequences and to analyze gene expression pathways [48-50].

In the oligonucleotide microarray approach [51-53], small oligonucleotide sequences of 10-25 nucleotides in length are synthesized directly on the activated surface (see also the Affymetrix GeneChip homepage <http://www.affymetrix.com/technology/index.affx>). A target sequence ("probe") is represented by about 20 oligonucleotides, called probeset (Fig. 2B). This probeset consists of perfectly matching sequences (PM = perfect match) and sequences with a non-corresponding nucleotide (MM = mismatch). Hybridization

of cDNA samples results in a ration of hybridization signals for the perfectly matching and the mismatching sequences. This approach allows quantitative results in a single sample and the detection of single nucleotide polymorphisms (SNP) by including suitable single nucleotide mismatch sequences.

In addition to their use in the fields of biomedicine such as "pharmacogenomics" and "toxicogenomics" [47], microarrays have been used in developmental biology to find genetic differences which can be correlated to functional aspects, most importantly, the phenotype of the developing organism [54]. In stem cell research, microarrays are used to define the properties of stem cells, may it be the common properties of all stem cells, or the unique properties of a certain kind of stem cells. All studies have in common the aspect of comparing large-scale datasets, which emphasizes the need (i) to properly plan and design the experiment, and (ii) to have bioinformatic tools provided which allow statistical or categorical comparison of the samples.

Genomic and Transcriptomic Studies of Neural Stem Cells

During differentiation, neural progenitor cells have to undergo complex changes in their phenotype to accomplish diverse functional changes required for the mature brain cell. One of the most central questions refers to their intrinsic ability to proliferate and self-renew. It is currently unknown which mechanisms direct neural progenitor cells to leave the cell cycle and stop mitosis, although the molecular mechanisms of cell cycle control are available. The balance between symmetric and asymmetric division is one of the phenomena which enable neural progenitors both to self-maintain and to give rise to committed progeny [55, 56]. In the concept of symmetric cell division, the stem cell gives rise to two equal stem cells, whereas in asymmetric division, the stem cell gives rise to at least two daughter cells, of which one is again endowed with stem cell properties, whereas the other has lost these stem cell characteristics and is differentiating further [57-59]. Neural stem cells can divide either symmetrically, which happens during their proliferative phase in early development, or asymmetrically, yielding a high number of differentiated progeny which occurs in the later, differentiating phase [55, 60]. Some of the molecules involved in asymmetric cell division encompass members of the cytoskeleton like actin and tubulin, but also N-cadherin, beta-catenin, paxillin, and pp120 [61]. It seems that the developmental Wnt signaling pathway is a regulator for switching from symmetric to asymmetric division in neurogenesis [62], with other signaling pathways like Sonic Hedgehog (Shh), Bone Morphogenetic Proteins (BMP), or Notch balancing this system. Thus, activation of developmental pathways in stem cells is a key feature for the definition of these cells. Of note, asymmetric division can also mean that a stem cell does not only produce 2^N daughter cells, but a number of progeny not following simple power rules. Moreover, the proportion of stem cell progeny and differentiating progeny may be distorted. For example, a stem cell can produce two single stem cells for self-renewal, or one stem cell and one committed progenitor cell, or two progenitor cells. The pathways regulating the decision for either way are unknown. This is important to keep in mind, when interpreting the results of neurosphere assays [63].

A highly complicated strategy is necessary to generate the enormous number of neurons and glia at the correct point of time in development, and to direct them correctly to their final destination. In this context, several molecular signaling pathways have been proposed for neural pattern formation, including Sonic Hedgehog (Shh) and Notch signaling pathways as well as transcription factors from the Paired Box (Pax) and Homeobox (Hox) families [64]. Some of these have been detected in the microarray experiments performed on developing neural tissue, although not consistently and not completely, indicating that they are important, but not exclusive, elements of neural fate decision. Whereas microarray technology has been used in "functional genomics" approaches for describing invertebrate development, microarray studies of mammalian development are rare [54]. This situation will presumably change during the next years, when results from cell lines, whole embryo, and organ studies will form a more unified picture. For more details, differences and perspectives of using microarray technology in developmental biology, and the limitations and prejudices related to mass screening approaches are discussed elsewhere [54].

Svendsen and coworkers have used oligonucleotide microarrays to screen gene expression in human embryonic neural stem cells isolated from human embryos in week 8-13 after conception [65]. One of their major findings was that cortical progenitor cells can be cultured over an extended series of cultures (up to 70 weeks) in the presence of Endothelial Growth Factor (EGF), Fibroblast Growth Factor-2 (bFGF), and Leukemia Inhibiting Factor (LIF) as mitogens without losing their stem cell properties. They found that LIF is an essential growth factor for the maintenance of stem cell properties in embryonic EGF-responsive neurospheres. Of note, gene expression did not change during this long time of cell passages, whereas gene expression showed major changes upon the withdrawal of LIF, for example, the expression of Glial Fibrillary Acidic Protein (GFAP) was decreased as well as the Major Histocompatibility Complexes (MHC) I and II, and CD44, a gene involved in cell shape, movement and interaction with the extracellular matrix. This decrease could be reversed by re-addition of LIF. The list of genes provided gives insights on the basal gene expression, as well as regulatory pathways that are activated during long-term self-renewal of human neurospheres.

Similar results have been demonstrated for the long-term culture of EGF-responsive neural progenitor cells isolated from the striatum of the rat brain of embryonic day E14 [66]. The authors cultured neurospheres for up to 6 years. The cells did not lose their self-renewal capacity and showed no major differences in gene expression over time. When differentiated for 24 h by the addition of serum and culturing on laminin and poly-L-lysine-coated slides, cellular processes spread rapidly, cells attached to the surface of the culture well, and more than 120 genes showed altered expression, among them genes associated with cell growth and proliferation, axon guidance, synaptogenesis, apoptosis and neurotransmitter synthesis.

Murine E14 neural stem cells were maintained in the presence of LIF for more than 30 passages before inducing neural phenotypes by retinoic acid [67]. Regulated gene sequences could be attributed to neuronal growth and

synaptogenesis, cell cycle control, transcriptional regulation and signal transduction.

In a further study, the changes in gene expression of embryonic neural progenitors were described. The authors compared murine ES cells to neurons differentiated from these *in vitro*, expressing a midbrain/hindbrain phenotype [68]. The authors maintained the neural stem cells in a LIF-containing medium and differentiated the cells *in vitro* by a 5-step induction protocol, including the formation of embryoid bodies, selection of nestin-positive cells, cell expansion in the presence of Fibroblast Growth Factor-2 (FGF-2), and final differentiation by withdrawal of the mitogen. They compared both cell types by cDNA microarrays containing more than 10,000 sequences. They found that neurogenesis was mainly regulated by Pax and Hox transcription factors, as well as Wnt, TGF-beta, and Shh signaling.

Dramatic changes occur in the phenotype of neurospheres when differentiating into neurons. The underlying processes are regulated by the activation and repression of gene and protein expression. In a large-scale gene expression study based on PCR methods, four steps of gene expression patterns have been identified [69]. Spinal cord tissue was dissected from the developing central nervous system at 9 different stages from E11 to P14 and from the adult rat brain. The expression of 120 genes was monitored by PCR and four patterns in temporal fluctuations of gene expression were found. The first expression pattern can be attributed to immature proliferative cells. The next two steps are characterized by the expression of genes for neurogenesis and synaptogenesis as well as neurotransmitters, but they differ in their proliferation properties. In the fourth step, gliogenesis and final maturation of the tissue can be seen. Although this study did not explicitly focus on stem cells, its results can be used to characterize stem cell differentiation.

In a microarray study, the changes in gene expression have been investigated during differentiation of neural stem cells. Gurok *et al.* [70] have cultured neurospheres from the subventricular zone of 7-day-old mice and compared gene expression profiles by cDNA microarrays containing about 14,000 sequences. In their time-course analysis of 1, 2, and 4 days after differentiation, they found alterations in gene expression which allowed them to separate five steps of *in vitro* differentiation. In the first step, cell cycle-related genes were down-regulated, including cyclins, replicating enzymes such as DNA polymerase, and transcription factors. This indicates that cells stop to proliferate and exit mitosis. In the next step, the neurosphere attaches to a surface. Therefore, cell adhesion molecules, extracellular matrix proteins, and cytoskeletal proteins are down-regulated. Third, individual cells detach from their connection within the neurosphere and migrate in the fourth step away from the sphere, which requires special metabolic components and cytoskeletal proteins. Only in the fifth and final step, final differentiation takes place including the expression of neural proteins.

Summarizing these studies, the differentiation of neural stem cells is controlled by a set of developmental signaling pathways [71], most prominently Pax, Hox, Wnt, TGF-beta, and Shh signaling. Therefore, the expression of appropriate cell surface receptors as well as of molecules linking the

signal from the receptor with intracellular effectors and with nuclear targets underlies the control of these genes. In most of the studies discussed in this review, the expression of members of the mentioned pathways was reported, indicating that they are intrinsic constituents of neural stem cells.

One of the most inherent problems of cell cultures using differentiation protocols is that not all cells are differentiating at once and completely. A number of cells always remains unresponsive to the differentiation stimuli. On the other hand, spontaneous differentiation may occur without a change of the medium in the cell culture well, for example when neurospheres attach to the surface. For investigating "pure" cultures of undifferentiated versus differentiated cells, a genetic subtraction method called Representational Difference Analysis (RDA) was applied to examine neurosphere cultures isolated from neonatal mouse cortex [72]. In principle, for RDA the RNAs from the different samples are ligated with specific adapters for binding of tester and driver primers. The samples are amplified by polymerase chain reaction (PCR) and tester and driver amplicons are mixed and differentially amplified by two additional rounds of PCR, resulting in representational amplification of the PCR products [73, 74]. Neurospheres were differentiated by removal of the mitogen FGF-2 for 24 h before RNA extraction, and microarray analysis was performed using RNAs from the RDA process. The authors identified 232 differentially expressed sequences out of a total of 3,360 sequences gridded on the array. The differentially expressed RNAs comprehend genes from signaling pathways, most prominently GTPases, cytoskeletal elements responsible for the outgrowth of cellular processes and transcriptional and cell cycle regulation. Of note, genes involved in developmental pathways seem to be underrepresented in this study, but this study yielded a number of genes characteristic to neural stem cells.

Whereas the differentiation process of neural stem cells has been described in several studies, less is known about the differences and similarities of different stem cell types, such as embryonic, hematopoietic, and neural stem cells. Similar properties of all stem cells, termed "stemness", have been defined in two studies in which different microarray systems had been used [75, 76]. In the first of these studies [75], murine hematopoietic stem cells isolated from E14-E14.5 liver, neural stem cells from the lateral ventricle of E14 mice, and murine embryonic stem cells from the CCE cell line were compared by microarray analysis containing about 36,000 sequences. The authors found an overlap of 283 sequences which were expressed in all three samples. Similarly, in the second study, murine embryonic stem cells, neural stem cells isolated from the lateral ventricle of 8-week-old mice, and bone-marrow hematopoietic stem cells have been compared by microarray analysis containing about 12,000 genes. In this study, the authors found an overlap of 230 genes expressed in all three samples. Comparing both studies, only 15 sequences were common to the overlapping set in both approaches, mainly due to methodological differences with regard to isolation, purification, and maintenance, as well as the microarray screening [77].

In conclusion, several attributes of stemness can be identified (summarized in [78] from [76]): (i) the expression

of members of developmental signaling pathways, such as Notch, JAK/STAT, TGF- β , Wnt, or Shh, (ii) proliferation in the presence of growth factors, (iii) interaction with specific molecules of the extracellular matrix, such as integrins, (iv) expression of cell cycle regulating genes, (v) expression of transcriptional and translational regulating molecules, and (vi) molecules communicating the response to "cell stress" like DNA repair, molecular chaperones, detoxification systems and protein degradation.

Although the datasets of the microarrays differ significantly in size and composition, depending on the year of the study (with earlier studies comprising less sequences on the array) and the manufacturer, there are some genes, or gene families, which can be identified consistently in most of the microarray gene expression studies. This overlap seems to consist of genes of the transcription factor families involved in pattern formation (Pax, Hox), and developmental signaling pathways, most prominently Shh and Wnt signaling.

Whereas most microarray studies aimed at defining genes responsible for "stemness", studies identifying genes which are unique to different kinds of stem cells and allow to separate types of stem cells are rare. On the one hand, stem cells obviously have certain properties in common, mostly defined by their functional similarity: They are multipotent and self-renewing [17, 79]. So far, this definition comprehends stem cells from all tissues and all organisms investigated. On the other hand, no common type of stem cell has been defined, but rather heterogeneity in stem cell populations. This also holds for the brain, in which many regionally specified subpopulations of neural stem cells have been identified [80, 81]. Therefore care must be taken when comparing brain-derived stem cells isolated from different locations (spatial dimension), or different points in development (temporal dimension).

D'Amour and Gage isolated neural stem/progenitor cells from embryonic and fetal chimeric mice [82]. The transgenic mice expressed the promoter of the Sox2 transcription factor coupled with GFP. Cell sorting allowed the separation of pluripotent and multipotent stem cells. Then the authors used microarrays containing some 12,000 oligonucleotide sequences to find genes which are unique to each of the two populations. Overall, they found 269 genes with differential expression at stringent conditions with at least 2.0-fold change, and 666 genes with at least 1.4-fold change. Grouping these genes into functional categories, they were able to attribute different sets of regulated genes to the two classes of stem cells, each endowed with specific sets of unique genes for transcriptional regulation, reaction to cellular growth, cell cycle regulation, oxidative metabolism, signaling and many others. In contrast to other studies, in this study the term "stemness" is used as an inductive concept that is defined by unique properties of individual stem cell populations, rather than the deductive approach for finding stemness genes.

Transplantation of embryonic stem cells into adult individuals from the same species often results in the formation of embryonal tumors (see, for example, [83]). Distinct from the teratocarcinomas developing after transplantation, these tumors also mimic the growth and differentiation properties of stem cells, for example they

express neural proteins. In a study investigating the embryonal carcinoma cell line NTERA2, the authors induced neural differentiation by retinoic acid and compared gene expression profiles of carcinoma cells to *in vitro* differentiated cells by oligonucleotide microarrays [84]. They used custom-made arrays containing about 600 sequences to identify about 40 sequences which are differentially regulated during *in vitro* differentiation, including nestin, neuroD, and synaptophysin as markers for neurons, or neural progenitors. They also found differential expression of Hox and Pax genes and of cytoskeletal elements. Their findings indicate a close relationship between the teratocarcinoma cells and the neural stem cells, but one has to keep in mind that embryonal carcinoma cell lines may only imitate the neuronal phenotype by activating common growth and proliferation pathways.

Differential display (see section above: *Methods in global gene expression studies*) has been used for expression screening of PC12 cells differentiating into neurons [85]. Although PC12 cells are derived from a pheochromocytoma tumor and therefore can express neuronal phenotypes, these cells are distinct from neural stem/progenitor cells derived from the brain. The authors induced the neuronal phenotype by incubation with Nerve Growth Factor (NGF). They compared these cells to untreated PC12 cells, and found that 19 out of 466 transcripts were differentially expressed. These transcripts could be annotated as early response genes, neuron-specific genes, redox defense, and transcriptional control. These findings match the gene groups for stemness identified by microarray experiments, supporting the idea of neuronal differentiation in PC12 cells. Again, care must be taken in the interpretation of "neurogenesis" in immortalized non-neural cells imitating the neuronal phenotype.

PROTEOMIC SCREENING

Development of the Proteomic Technology

In analogy to the term "genome" describing the complete set of genes and "transcriptome" the complete set of RNA species, the term "proteome" is complementary denoting the complete set of proteins in a cell, an organ, or a whole organism, respectively [86].

The technology used to investigate the proteome, called proteomics, is more comprehensive than the mere listing of proteins contained in a specified compartment. It also includes the study of protein isoforms and post-translational modifications as well as information about their structure and interactions [87]. Whereas the genome seems to be stable throughout the lifetime of an individual, it is a characteristic of the proteome to be highly mobile. Thus a proteome study only represents a dynamic equilibrium between protein synthesis and protein degradation. Most common factors influencing the proteome are post-translational modifications such as phosphorylation, glycosylation, addition of carbohydrate sidechains (methylation and acetylation), proteolytic cleavage, sulfation and other biochemical reactions [88]. Therefore, a proteomic analysis only refers to a definite time and state of protein expression. The major advantage of proteomics is its ability to separate and characterize a multitude of proteins simultaneously, projecting the changes in concentration and

composition of individual proteins to biological phenomena in a cell.

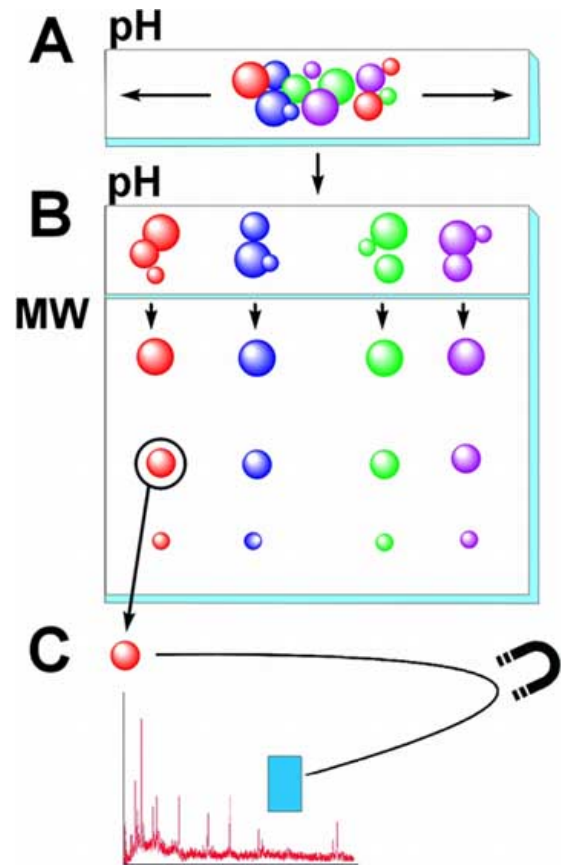


Fig. (3). Principle of two-dimensional gel electrophoresis. (A) In first dimensional isoelectric focusing, protein lysates are separated according to their isoelectric points. (B) In the second dimensional polyacrylamide gel electrophoresis, proteins are separated according to their molecular weight. Gel images can then be analyzed by specific gel software to map and compare spots. (C) To identify proteins, spots of interest are excised, digested enzymatically, and subjected to mass spectrometry.

Fig. (3) shows the principle of proteomic technology based on two-dimensional gel electrophoresis for separation of proteins and mass spectrometry for protein identification [87, 89, 90]. In this approach, proteins are separated in the first dimension by isoelectric focusing, where proteins migrate in a gel with an immobilized pH gradient to the position in the gel where their net charge is zero. This gel strip is superposed on a polyacrylamide gel for second dimension separation according to the proteins' molecular mass. Proteins are visualized in the gel by staining procedures, and the digitized gel images are analyzed using specialized software for detecting and matching gel spots in the different gels with one another [91]. The results are then compared statistically. Spots of interest are excised, enzymatically digested, and subjected to mass spectrometry. The mass spectra obtained are submitted to protein sequence databases, and proteins are identified by spectral similarities. Recent advances in proteomic technology encompass direct mass spectrometric strategies to identify components in complex protein mixtures [92, 93] as well as use of protein chips with immobilized protein reaction partners (e.g. antibodies) [94, 95].

In recent years, the technological strategies of proteomics have been broadened by the use of direct mass spectrometric analysis of complex protein mixtures, including the specific tagging of proteins [93, 96]. Also liquid chromatography has been coupled with mass spectrometry (LC-MS) to separate and identify proteins. Additional tools for large-scale protein analysis include yeast two hybrid (Y2H) screening [97] and protein tagging and pulldown [98]. Moreover, antibody arrays complete the current set of high-throughput protein analysis systems [99, 100].

In principle, two fields of proteomic analysis have emerged, called "protein profiling" on the one hand, and "functional proteomics" on the other hand [101]. Protein profiling describes the aim to identify all proteins in a biological sample in completeness, and to create a database containing the information of these proteins. Moreover, proteomic profiling also subsumes comparing the differences in two given samples, which have been treated under different experimental conditions (e. g. undifferentiated vs. differentiated stem cells). In contrast, functional proteomics is mostly understood as measuring protein activities, interactions, and post-translational modifications. No strict borderline can be drawn between both types of studies, and one can think of all kinds of overlap between them.

Proteomics of Neural Stem Cells

Focusing on stem cell biology, proteome analysis can be used to define specific properties of stem cells by identifying protein markers specific for the origin of cells from a certain tissue, or for their developmental stage. It is also possible to define cellular properties with regard to proliferation and differentiation by comparing protein expression patterns.

Whereas different types of stem cells have been compared in the several studies, only few proteomic studies exist describing the proteomic profile during brain development. These studies mainly concentrate on the differentiation process from the stem cell to the neuronal phenotype. The number of studies investigating neural stem cells by proteomics is low. Due to the novel technologies involved in both fields, the combination of expertise is rare.

Pearce and Svendsen [102] investigated stem cell differentiation from the human fetal cortex by two-dimensional gel electrophoresis. They compared neurospheres to cells differentiated *in vitro* by removal of the mitogens epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) in the presence of serum. Even after an extremely short differentiation time of one hour, they found differentially expressed proteins between the two groups.

Other studies investigated embryonic stem cells by proteomics. For example, Guo *et al.* [103] cultured PKU cells, an embryonic stem cell line from Beijing University, China. Leukemia inhibiting factor (LIF) was used to maintain stem cell properties and cells were differentiated by adding all-*trans* retinoic acid for 4 days. Comparing proteomes from undifferentiated and differentiated cells by two-dimensional gel electrophoresis, the authors found 24 differentially expressed protein spots, of which 12 spots could be identified. Several of the proteins were associated with neural differentiation and survival. Their findings

indicate major changes in the proteome of differentiating ES cells in the functional groups cytoskeleton and cell cycle.

Adult neural stem cells isolated from rat brain hippocampus have been compared to *in vitro* differentiated cells by two-dimensional gel electrophoresis [104, 105]. Cells were grown in neurosphere cultures in the presence of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) and differentiated *in vitro* by removal of the mitogens and addition of serum. A differential expression was reported for 367 protein spots, of which 128 could be identified by mass spectrometry and database searching. Regulated proteins included participants in transcription and DNA metabolism, signal transduction and Ca²⁺-signaling, MAP kinase pathways, cytoskeletal rearrangement, regulation of cell cycle, proliferation and survival as well as protein biosynthesis, folding, degradation, and glycine and glutamate metabolic pathways.

The rat cerebellum matures within the first 3 weeks after birth and is therefore well-suited for the analysis of postnatal neuronal differentiation. In a study based on two-dimensional gel electrophoresis [106], the authors isolated proteins from rat cerebella of early postnatal age (up to 3 weeks) and separated proteins by two-dimensional gel electrophoresis. They identified 68 proteins by mass spectrometry. Comparing the gels for P0, P3, P7, P14, P21, and P56, the authors found that 42 protein spots were transiently expressed in the immature cerebellum. Of these, the authors identified 29 protein spots, of which most were related to the cytoskeleton, or intermediary metabolism.

Although two-dimensional gel electrophoresis and mass spectrometry remained the basic techniques of proteome research over the past 10 years, chip-based strategies emerge to constitute a suitable alternative. Mainly two techniques have been established: Either antibodies or antigens are immobilized on a solid surface [94, 107], or the molecules of interest are fractionated on a surface and identified by mass spectrometry on the chip. The latter technique is known as surface-enhanced laser desorption/ionization (SELDI-TOF) [108].

SELDI-TOF-MS was used in a recent study to identify biomarkers in human pluripotent stem cells derived from human embryonic carcinoma stem cell lines SP12 and NTERA2.d1 [109]. Differentiation was induced by addition of 10 μ M trans-retinoic acid, or 3 mM hexamethyl-bis-acetamide, for 28 days. The authors reported the finding of 3 biomarkers in the differentiating cells, which could unfortunately not be identified. Their SELDI-TOF approach is particularly useful to identify smaller peptides or proteins with a molecular weight of less than 15 kDa. These peptides/proteins are often poorly resolved on 2D gels. Although the cells used in this study were not brain-derived but from teratocarcinoma, they share several common features of embryonic stem cells. Investigating their proteomic properties may help to understand the "stem-like" character of early developmental cells in general.

Protein arrays constitute a different approach. The major advantage of protein arrays is that they may overcome the inability of two-dimensional gel electrophoresis to resolve membrane proteins adequately. The hydrophobicity of membrane proteins prevents their proper isoelectric focusing in the first dimension. On the other hand, protein arrays are

still "low-throughput", as only few antibodies, or targets, can be screened simultaneously. In the future, the trend in protein chip application will go towards further miniaturization and automatization, thus allowing a more common use of protein chips.

Although some progress has been made in improving the solubility of hydrophobic proteins in the extraction procedure [110], a simple protocol still does not exist which allows assessing membrane proteins by proteomics. Protein microarrays may help to fill the gap. For example, Iwata and coworkers [111, 112] have developed antibody-based microarrays for the analysis of cell surface markers. In two studies, they showed the proof of principle to use surface-bound antibodies for the detection of markers located on the cell surface. For a whole cell assay, they isolated neurospheres from fetal (E16) striatum of Fischer 344 rats. These cells were incubated either on a cellulose membrane on which 8 antibodies against cell surface antigens had been immobilized [112], or on glass slides with 15 bound antibodies [111]. In the glass surface microarray, they found that most of the neurospheres bound fibronectin, laminin, peanut agglutinin (PNA) and CD15 (Lewis-X), whereas smaller amounts of neurospheres bound to CD44 (ECMR III; H-CAM; HUTCH-1; Hermes), CD54 (ICAM-1), CD56 (Leu-19; NKH1; NCAM), CD57 (HNK1; Leu-7), the polysialic acid-neural cell adhesion molecule (PSA-NCAM), and the endothelial growth factor receptor (EGFR). Only few cells bound to CD9 (DRAP-27; MRP-1), CD31 (PECAM-1), CD34 (L-selectin ligand), CD81 (TAPA-1), CD90 (Thy-1), the oligodendrocyte surface antigen O4, the fibroblast growth factor receptor (FGFR), and the nerve growth factor receptor (NGFR). In contrast, the cellulose membrane array showed highest immunoreactivity for fibronectin and laminin, and a moderate number of cells binding to CD56, CD57, and O4. Only a small number of cells was present on CD44, CD81, and CD90 antibody spots, whereas no reactivity was found for CD105 (endoglin), CD115 (M-CSFR), IgG, and IgM. Double-staining with nestin and vimentin antibodies revealed that about 90% of the CD57-positive cells were also nestin-positive, whereas about 60% of the CD57-positive cells were also vimentin-positive, making the CD57 antigen the most probable cell surface marker for neural stem cells. Although several interesting candidates have been identified, no unambiguous cell surface marker has been identified.

CONCLUSIONS

The Need for Standardization

When microarray screening was at its beginning, it soon became evident that reporting of genomic/transcriptomic experiments required a standardized format to enable the reader to recapitulate the experimental details, and to make studies comparable. This effort resulted in the adoption of the Minimum Information About a Microarray Experiment (MIAME) [113]. Although the same requirements should be fulfilled in proteomic experiments, the standards are less well defined [114]. Comparing the different types of studies reviewed in this article, we are confronted with an overwhelming variety of experimental procedures, instrumentation, individual technical "improvements", as well as a large set of databases in different data formats.

In addition to variations in technology, the definition of neurobiological features of stem cells is far from common standards. Differences in cell preparation, media formulation, addition of growth factors, culturing conditions, and in protocols to induce cell differentiation make it impossible to find a unifying description or even a general definition of neural stem cells.

The possibility exists that the difficulties to define a stem cell are not only caused by the various methodological approaches but arise also from heterogeneities in stem cell types. Several studies imply that different regional neuronal precursors exist in the brain [80, 81], and a serious discussion has evolved about the contribution of cell culture conditions to the trilineage potential of neural stem cells to differentiate into neurons, astrocytes, and oligodendrocytes [115]. Although it is too early to attribute the *in vitro* potential of stem cells to proliferate and to differentiate generally as artificially caused by cell culture conditions, the drawbacks of *in vitro* models are obvious. In this context, it is worth considering significant changes occurring in the cultured stem cells due to the composition of the media. For example, we cultured bone-marrow stem cells in two different commercially available media and found major differences in the cellular proteomes of the two cell cultures (Wagner, Feldmann *et al.*, manuscript in preparation). Taking into account that many protocols for embryonic stem cell differentiation are built up by 5 to 9 steps including frequent changes of the media, and addition or removal of growth factors to either adhesive and free floating cells, we suspect that substantial variations in cellular properties are likely to occur.

A principle question is how to compare different datasets obtained from different study designs. Besides the efforts to create standards for microarray [113] and two-dimensional gel electrophoresis experiments [114], the use of a unifying database seems to be a solution. In this database, the results of multiple studies are collected in a single format. This makes the experiments more comparable. For example, StemBase (<http://www.scgp.ca:8080/StemBase/>) collects information about microarray experiments obtained from over 200 different samples, including neural stem cells [116], mostly of mouse and human origin. Although some algorithms are available which allow to compare whole proteomes on the basis of protein sequences [4], satisfying bioinformatic tools do yet not exist to compare protein patterns [117]. This is mostly due to the fact that the term proteome does not define the kind of data it is describing. For example, protein sequences, strength of expression, or mass spectrometry data may be included, all of them in different data formats. Currently, there is no agreement which information are contained in a dataset describing a given "proteome". Thus it is difficult to compare protein patterns in total.

Strategies for Defining Molecular Fingerprints of Neural Stem Cells

Although several comprehensive studies have been performed in which either the differentiation of neural stem/progenitor cells or stem cells from different origin have been compared, no unambiguous markers have been found which enable us to describe the "stemness" of neural

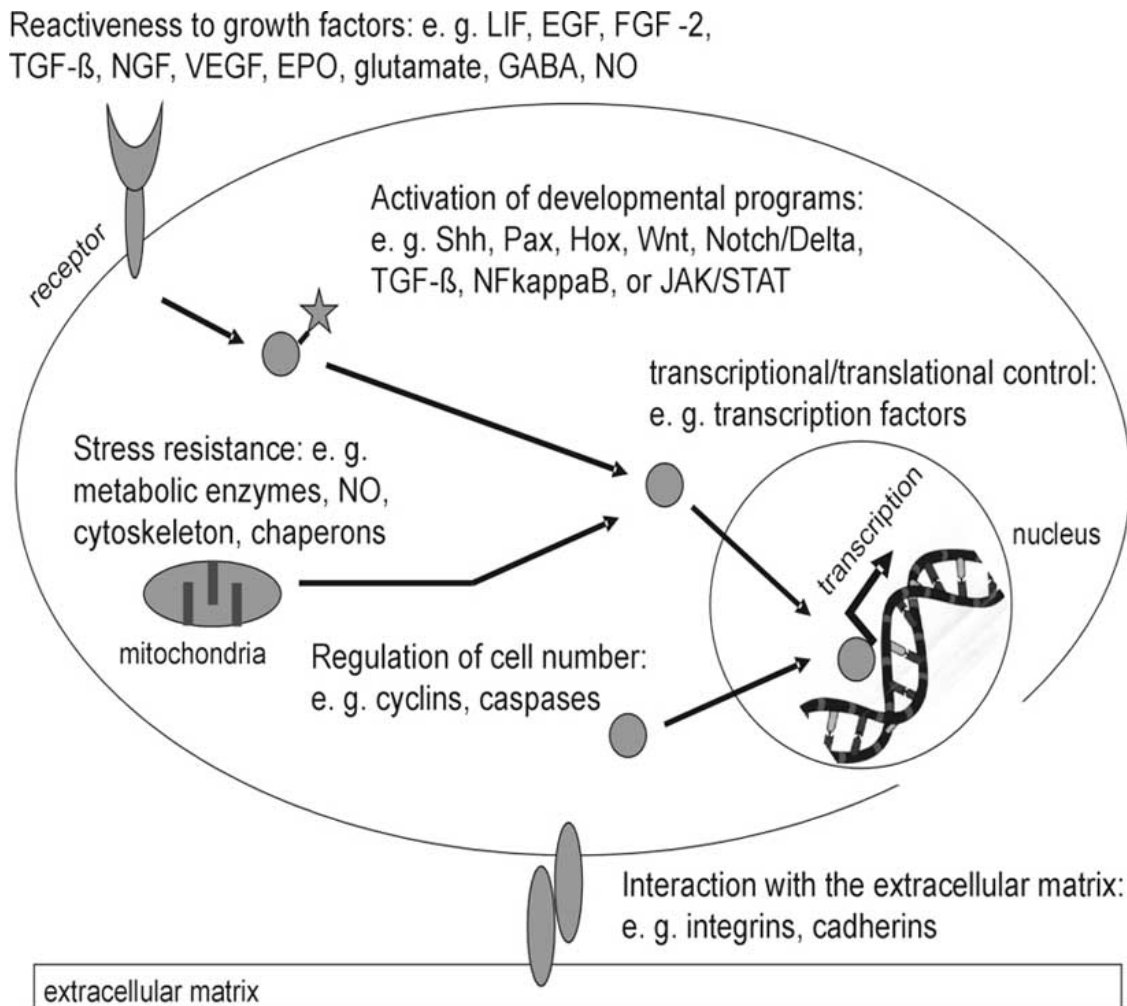


Fig. (4). Proposition for defining neural stem cells by their molecular fingerprints. Several common properties of neural stem cells with regard to signaling pathways and defining molecules are depicted (see text for details).

progenitors on a molecular basis. However, evaluating the analogies and similarities in the results of all these studies, molecular definitions of neural stem cells appear to be possible.

These common properties are not based on individual genes or proteins shared by all neural stem cells, but rather on the capacity of the cell to perform the following specific functional tasks (Fig. 4):

- (i) Reactiveness to growth factors. Neural stem cells react to a variety of growth factors and neurotransmitters in the local microenvironment [118], including LIF, EGF, FGF-2, TGF- β , NGF, VEGF, EPO, glutamate, GABA, NO. Of note, not all neural stem cells react to all of the growth factors, but, for example, the expression of VEGF receptors in the brain is highest in the zones of spontaneous neurogenesis [119, 120]. The signaling induced by growth factors is transduced by specific membrane-bound receptors which activate an intracellular signaling cascade. Therefore, one aim of studies investigating the reactiveness of neural stem cells to growth factors is to define the corresponding receptors and intracellular pathways in expression studies.
- (ii) Activation of developmental genetic programs. In early embryonic development, specific pathways control the fate of stem cells. At least 17 pathways have been identified [71]. Most of them have also been found in the expression analysis of neural stem cells, including Shh, Pax, Hox, Wnt, Notch/Delta, TGF- β , NFkappaB, or JAK/STAT [64, 71]. Of note, some of the developmental pathways seem still to be active in adult neural stem cells.
- (iii) Capacity of transcriptional/translational control. A specific machinery for gene transcription and protein translation is required to change the cellular phenotype from its undifferentiated form to the new functional requirements of a differentiated stem cell. Several families of transcription factors, DNA or RNA binding proteins, and chromatin remodeling enzymes have been identified in this context. Again, no specific molecules exist in all stem cells which mediate the transition to the differentiated stage, but the existence of such specific genes/proteins in the expression pattern contributes to the stem cell pattern.
- (iv) Interaction with the extracellular matrix. During differentiation and maturation, stem cells change their

spatial orientation. They extend processes, attach to the extracellular matrix, or adhere to nearby surfaces. They also migrate towards their final destination *in vivo*, requiring the expression of motor proteins, lytic enzymes, and enzymes for providing metabolic energy. Several extracellular matrix proteins have been identified in neural stem cells, like integrins and cadherins, which mediate cell-cell interaction [121]. It is not unlikely that further proteins of the extracellular matrix may be detected interacting with neural stem cells.

- (v) Regulation of cell number. The number of cells is controlled by three processes: Mitosis in the cell cycle, differentiation, and apoptosis. Proliferating and self-renewing neural stem cells have to stay in the cell cycle, whereas differentiating precursors leave it. Moreover, the balance between a constant proliferation and apoptosis which exists in the stem cell pool is disturbed during differentiation. Cyclins and caspases have a major influence on tissue maturation by negative selection, and asymmetric division is the essential underlying mechanism for both the maintenance of stem cell properties and the differentiation potential [55, 58, 60, 122, 123]. Therefore, genes and proteins which are essential for cell cycle regulation are expressed in neural stem cells, but in the respective set of functionally required proteins.
- (vi) Stress resistance. Neural stem cells are surprisingly resistant to a wide variety of noxious events, such as nutrient deprivation, NO toxicity, mechanical distortion, hyperthermia, or hypoxia (Maurer, Bürgers *et al.*, unpublished data). Some of these effects may be mediated by molecular chaperones, i. e. proteins responsible for protein folding. Molecular chaperones also play an important role in stem cell maturation and tissue regeneration [124, 125]. Moreover, detoxifying systems like NO scavengers, or peroxide destructing enzymes as well as proteins related to degradation in the proteasome are expressed in neural stem cells.

In summary, we think that molecular stem cell properties, especially those of neural stem cells, can be characterized by the expression patterns of specific genes and proteins, but not by the presence or absence of an individual gene or protein in the cell. Genes and proteins in this characteristic pattern are members in functional pathways directing proliferation, differentiation, and cell death as well as interaction with the local microenvironment. This definition results in a new understanding of the term "genome" or "proteome" expression profile in a sense that this profile is constituted by a group of genes and proteins in an ever-changing differing combination, but sharing a common functional background.

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