PROGENITORS FROM THE POSTNATAL AND ADULT MAMMALIAN RETINA - NEUROGENIC COMPETENCE AND PLASTICITY

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-The eye to this day gives me a cold shudder-

Charles Darwin

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List of Abbreviations

bp	Base pair
BSA	Bovine Serum Albumin
BrdU	Bromodesoxyuridine
CB	Ciliary body
cDNA	complementary DNA
CNS	Central nervous system
°C	centigrade
Da	Dalton
DAPI	4',6'-diamidino-2-phenylindol-dihydrochlodire-hydrate
DCX	Doublecortin
DPBS	Dulbeccor's phosphate buffered saline
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleotide acid
dNTP	Desoxyribonucleotide-triphosphate
E	Embryonal day
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FGF-2	Basic fibroblast growth factor
Flk-1	Fetal liver kinase-1
g	Gram (10^{-3} g)
GAPDH	Glycerinaldehyde-3-Phosphate-Dehydrogenase
h	Hour(s)
HBSS	Hanks Balanced Salt Solution
HC	Hippocampus
HEPES	N-(2-Hydroxyethyl)piperazin-N'-(2-Ethansulfonacid)
H_2O_{dest}	Distilled water
l	liter
MEM	Modified Eagles Medium
mg	milligram
ml	milliliter
mRNA	messenger RNA
μ	micro (10^{-6})
NMDA	N-methyl-D-aspartate
NSC	Neural stem cell
PBS	Phosphate buffered NaCl-solution
PCR	Polymerase chain reaction
pH	Negative Logarithm of the H^+-Ion concentration
PFA	Paraform aldehyde
P	Postnatal day

rcf	Relative centrifugal force
RPE	Retinal pigment epithelium
RNA	Ribonuclein acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Spinal cord
SD	Standard deviation
SR	Sensory retina
SVZ	Subventricular zone
TBE	Tris-Borate-EDTA-Elektrophoresis Buffer
TBS	Tris-Borate buffered saline
TE	Tris –EDTA-buffer
TH	Tyrosine hydroxylase
Tris	Tris-(hydroxymethyl)-aminomethane
V	Volt
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

I. Introduction

I.1. The eye

The eye, throughout all species, is one of the most complex and fascinating organs of the body. The fact that it is designed so differently among various species and yet apparently underlies the same conserved genetic program makes the developmental process even more intriguing. In humans, the eye is one of the most important connections to the surrounding environment. The eye basically functions like a camera, but in comparison to its mechanical counterpart, links the visual input to a complex and sophisticated wired network in the brain, which leads to all kinds of reactions, both of physical and emotional nature.

A common hall mark of retinal diseases such as macular degeneration or retinitis pigmentosa is the selective loss of retinal neurons, mostly photoreceptor cells. The continuous loss of retinal cells points towards a therapeutic strategy to replace cellular elements, either by applying exogenous cells or by stimulating endogenous progenitor or stem cell populations. A key aspect is the characterization of retinal stem or progenitor cells for this purpose.

This thesis focuses on the cues of mammalian eye development and investigates the possibilities of finding retinal progenitor or even stem cells in the postnatal and adult retina. Comprehension of the nature of retinal progenitor cells and their biology might eventually lead to progress in the field of generating new neurons for a diseased retina.

I.1.1. Anatomical features of the mammalian eye

The eye is an organ that is specialized in detecting, localizing and analyzing sources of light. As shown in Fig.I.1., the pupil is the opening that enables light to enter the eye. The pupil is an adjustable part and its size is controlled by the iris, a circular muscle. Both the pupil and the iris are covered by the cornea, the external surface of the eye. The cornea does not contain blood vessels and is nourished by the fluid right behind it, the aqueous humor. The cornea is continuous with the sclera, which forms the resistant

wall of the eyeball. The sclera itself is continuous with the dura. Three pairs of extraocular muscles are inserted into the sclera and move the eye within the sockets of the skull. The lens is located right behind the iris. It is suspended by ligaments, the zonule fibers that are attached to the ciliary muscles. These muscles in turn attach to the sclera and thus form a ring within the eye. The changes in the shape of the lens help the eye to adjust the focus to different viewing distances.

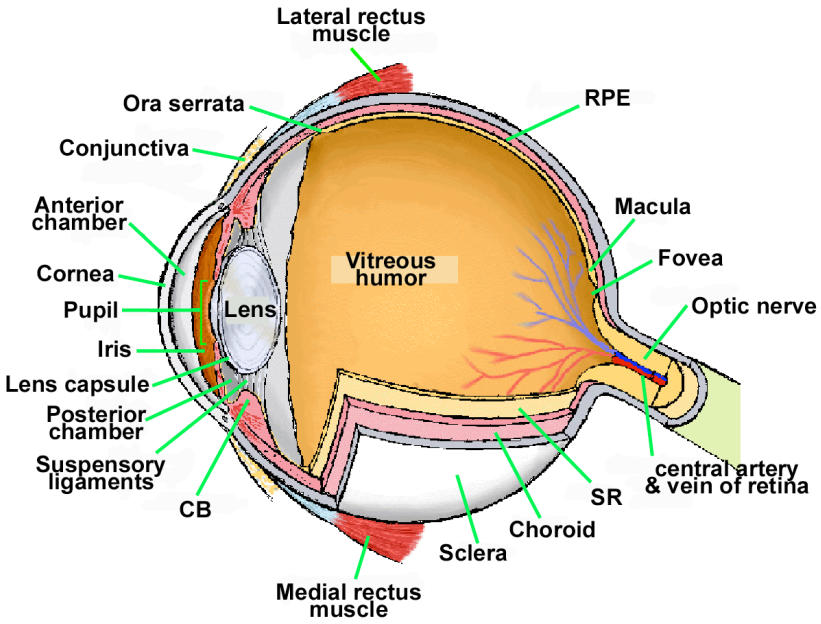

Fig.I.1. Gross anatomy of the eye. Saggital section through the adult human eye. RPE=retinal pigment epithelium; SR=sensory retina; CB=ciliary body. Adapted from W. Barry VanWinkle (http://medic.med.uth.tmc.edu/Lecture/Main/eye.htm)

The lens also divides the interior of the eye into two distinct compartments containing slightly different fluids. The aqueous humor is the watery fluid between the cornea and the lens. The more viscous vitreous humor lies between the lens and the retina, its pressure serves to keep the globe of the eye spherical. The retina serves as a sensor for incoming light and can turn this information into impulses transmitted via neurons, which makes it the major link to the visual system of the brain.

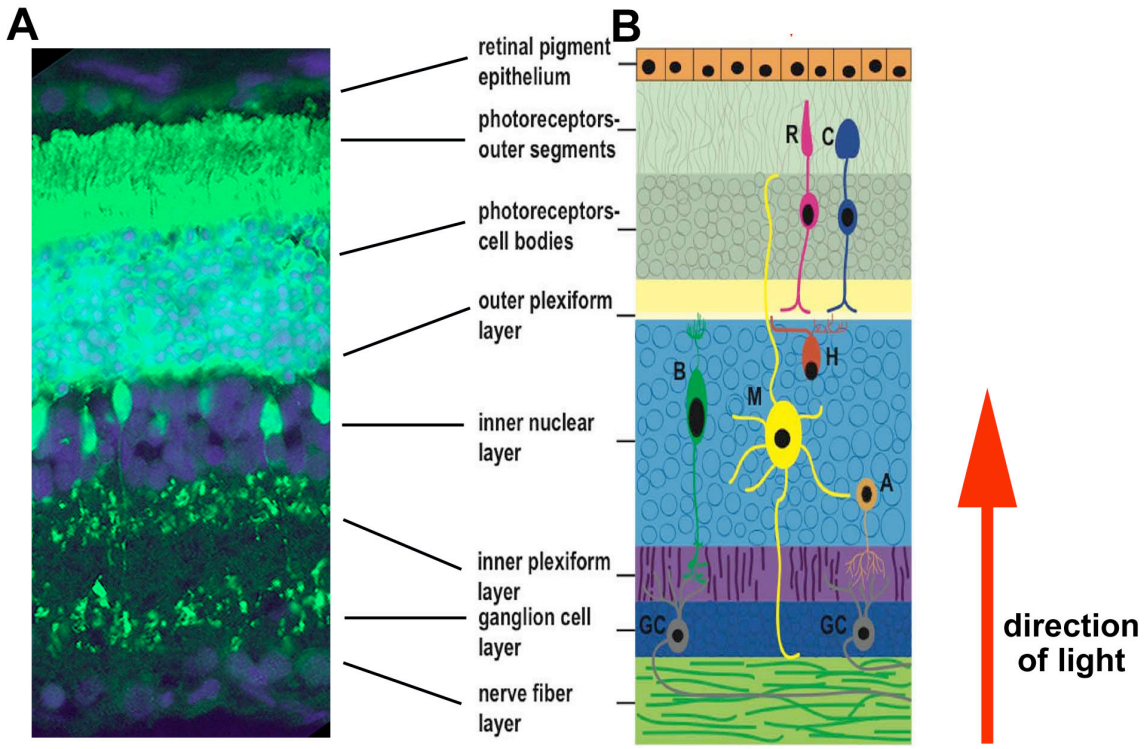

Fig.I.2. Laminar organization of the retina. A) Immunohistochemical staining of a postnatal day 3 derived rat retina with the photoreceptor marker recoverin [1] in green and the nuclear counterstain DAPI in blue. B) Distribution of retinal cell types in various layers in the adult. R=rod photoreceptor; C=cone photoreceptor; H=horizontal cell; B=bipolar cell; M=Müller glia; A=amacrine cell; GC=ganglion cell.

The cells of the retina are organized in layers (Fig.I.2). This laminar organization has an inside-out orientation. Light must pass from the vitreous humor through the ganglion cells and bipolar cells before it reaches the photoreceptors. Image distortion is kept at a minimum, as these cells are almost completely transparent. The layer of the photoreceptor outer segments contains the light sensitive elements of the retina and is embedded in the retinal pigment epithelium (RPE) that is specialized to absorb any light that passes entirely through the retina. Photoreceptors are not distributed evenly throughout the retina. The fovea defines the center of the retina, and is the region of highest visual acuity. In the fovea there are almost exclusively cones, and they are at their highest density. The ratio of ganglion cells to photoreceptors is about two to one here, the highest in the eye. In addition, at the fovea all of the other cell types are located out of the way of light to allow the most light to hit the cones. This makes the fovea visible microscopically. The blood vessels also leave a wide margin around the fovea. The area

in and around the fovea has a pale yellow pigmentation and is called the macula. Peripheral vision is dominated by rods. Overall, rods greatly outnumber cones. Rods are relevant for black and white vision and are very light sensitive, while cones allow for color vision and are not as sensitive.

I.1.2. Embryogenesis of the mammalian retina

The following chapter summarizes the embryonic development of the inner eye and, more specifically, the retina. During eye development, evaginations of the diencephalic neuroepithelium form optic vesicles around the third to fourth week *post conceptionem* in humans. The sensory retina (SR), posterior iris, ciliary body (CB), intraocular smooth muscle and retinal pigment epithelium (RPE) originate from the neural ectoderm, which is the medial portion of the ectodermal layer of the embryonic disc stage during development. The lateral portion of the ectoderm is the surface ectoderm. Derivates from this structure include the lens and the corneal epithelium. Furthermore, mesenchymal derivates of the neural crest generate the corneal and choroid stroma, corneal endothelium, anterior iris, smooth muscle of the CB and the meninges of the optic nerve (reviewed in [2]).

Simultaneously with the formation of the lens, the distal part of the optic vesicle invaginates (Fig.I.3.), resulting in the formation of the eye cup. Dividing cells in the invaginating cell layer start to differentiate, thus forming the future SR. The outer retinal layer of the optic cup differentiates and becomes the RPE, manifested by tyrosinase expression and melanin production. The cells bridging the invaginating inner and outer retinal layer, the retinal marginal zone or ciliary margin develop into 1) the future CB with the anterior portion extending processes to the lens and the posterior portion known as the ciliary ring or *pars plana*, and 2) the iris with its pigmented cells.

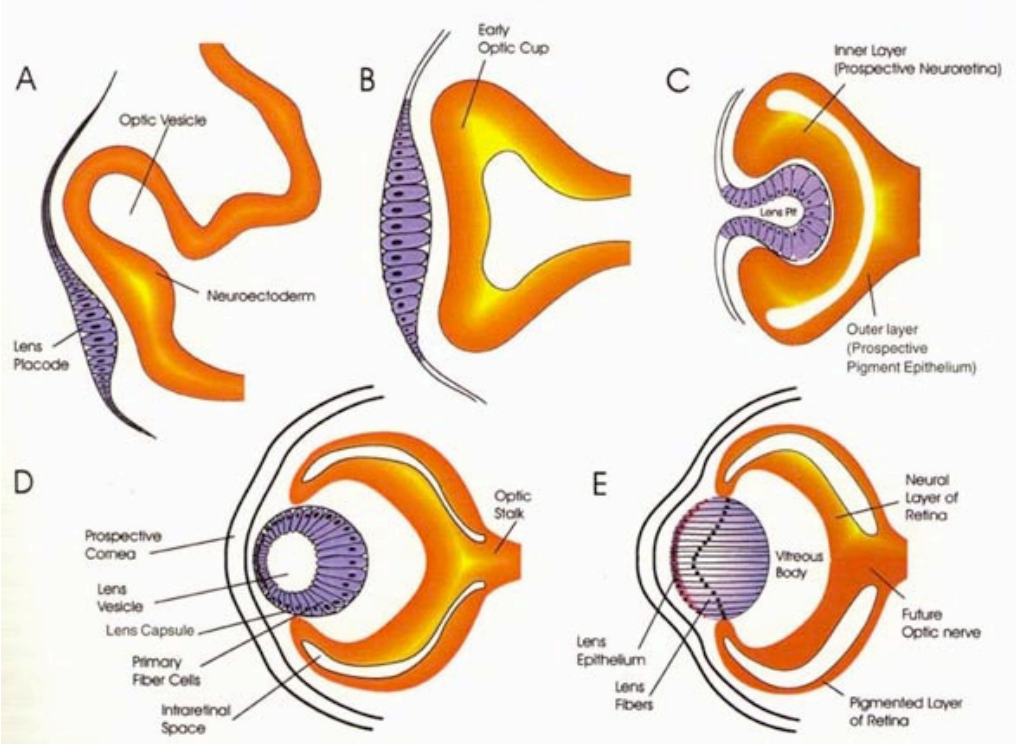

Fig.I.3. Embryogenesis of the mammalian eye (here: mouse). A)-C) Formation of the eyecup via evagination of the distal part of the optic vesicle. D) Dividing cells in the invaginating cell layer differentiate and become the future sensory retina while in E) the formation of lens, iris and ciliary body is driven by cells bridging the invaginating inner and outer retinal layer.
From Cvekl and Piatigorsky, 1995 (http://www.img.cas.cz/resrep/zk/eye-development.jpeg)

I.2. Stem cells

Stem cells are undifferentiated cells that proliferate, self-renew and produce daughter cells that are able to differentiate into various cell types. Per definition, three major types of stem cells are described: totipotent, pluripotent, and multipotent stem cells. A single totipotent stem cell can generate an entire organism. Pluripotent stem cells cannot give rise to an organism, but they can differentiate into any type of cell in the organism. Multipotent stem cells are organ-specific and differentiate into the appropriate organ-specific cell types (Fig.I.4.). The following chapters give an overview of the current knowledge in the field of stem cell biology. The role of stem cells during development and in the adult as well as a special focus on retinal stem cell biology will be introduced.

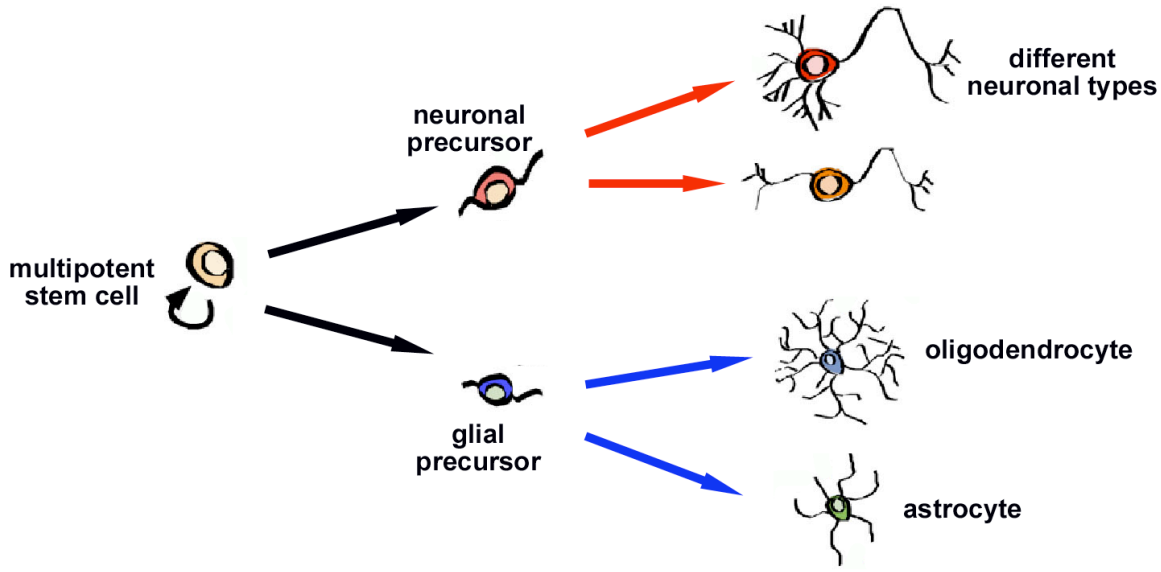

Fig.I.4. Neural stem cells and their progeny. Neural stem cells can renew and are multipotent. They can generate lineage restricted precursor cells, which further differentiate into the three major cell classes of the CNS: neurons, astroglia and oligodendroglia.

I.2.1 Somatic stem cells during development

During development, the first stem cell to appear is the zygote, forming with the unification of spermatozoon and oozyte. Later, stem cells are of embryonic nature and are derived from the inner cell mass of the blastula [3, 4]. Organ specific stem cells like neural stem cells (NSCs) are generated later during development. They can be identified first in the pseudostratified ventricular epithelium that lines the embryonic central ventricle wall [5, 6]. Cells that originate in this epithelium divide, resulting in two identical daughter cells, prior to the onset of neurogenesis, which is approximately at day 11 in mice and around the 5th week of gestation in humans [7, 8]. Some of the mitosis takes place in an asymmetrical mode, resulting in one cell maintaining "mother cell" characteristics (the so called stem cell). The majority of the cells in the brain derive from precursor cells that have proliferative potential and derive from stem cells. The bulk of neurogenesis during embryogenesis ends around embryonic day 17 in mice and the 5th month of gestation in humans [7, 9, 10].

Apart from multipotent neural stem cells, more fate-restricted progenitor cells have successfully been isolated from the CNS as well. Svendsen et al. were able to grow neural precursor cells from the developing fetal human cortex in culture and to

differentiate them into neurons and astroglia, both *in vitro* and *in vivo* after transplantation into an animal model of Parkinson's disease [11, 12]. These cells, however, appear to be lineage restricted as they do not generate oligodendrocytes [11, 13]. A similar finding is reported in the present work, with the isolation and analysis of postnatal retinal progenitor cells.

I.2.2. Adult neurogenesis - Somatic stem cells in the adult

A most prominent dogma in neuroscience was the statement that fully developed central nervous system (CNS) regions are devoid of mitotic activity and thus cannot generate new cells. However, as early as 1912, a group around Allen described mitotic activity in cells of the lateral ventricle of the adult rat [14]. Several decades later, in the mid 1960s, Altman and colleagues proposed a model of persistent neurogenesis in the adult rodent brain [15]. But only when Kaplan and Hinds showed convincing data on the fate of newly generated neurons in regions of the hippocampus in 1977, the dogma of the "static brain" became outdated.

Today, it is widely accepted that neurogenesis is a common phenomenon in the adult brain, with the two most active regions being the dentate gyrus of the hippocampus (HC) and the system of the subventricular zone (SVZ)/rostral migratory stream/olfactory bulb (Fig.I.5.). Other regions have been identified as well.

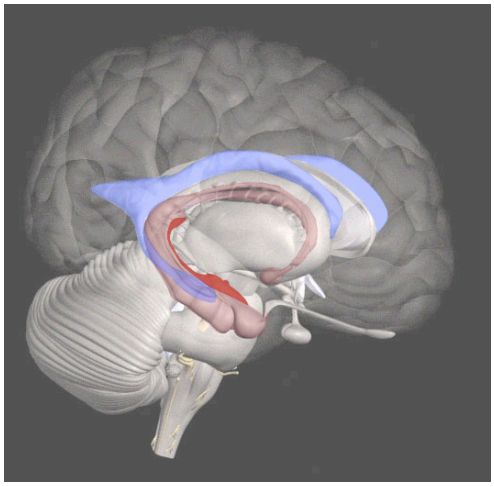

Fig.I.5. The prototypic neurogenic regions. The adult human brain with the dentate gyrus of the hippocampus (red) and the lateral ventricle system (blue). Neurons originate in these two systems in the adult. Modified from interBRAIN (Springer Publishing Group).

Neurogenesis in the adult brain seems to be a process of continuation of early postnatal neuronal production, as there is no significant onset of neurogenesis. It appears

that the process is a persisting neurogenesis that was started during the embryonic phase of development. The process of neurogenesis is composed of several distinct compounds. Stem cell and progenitor proliferation is only one aspect, fate determination (in the case of multipotent cells) is just as important, while migration and differentiation are key aspects of neurogenesis. Finally, cell survival and also cell death are major players for a balanced system and play a crucial role for the overall outcome of neurogenesis.

The typical characteristics of stem cells include proliferation, self-renewal, maintenance and multipotency. A number of different organ-specific cells types can be generated by stem and progenitor cells during development and in the process of regeneration in the adult. Based on its potential, there is a significant difference between adult and embryonic stem cells. The latter are pluripotent, meaning they can generate cell types required for the complete development of an organism and do not underlie germ layer restrictions. In contrast to pluripotent embryonic stem cells, several systems, including the CNS or the hematopoetic system, harbor multipotent somatic stem cells during development and in the adult. These cells are able to generate all organ-specific cell types. For CNS derived stem cells, this would include the three major cell classes of the CNS: neurons, astroglia and oligodendroglia.

Neural stem cells of the CNS have been shown to proliferate both *in vivo* and *in vitro* and generate all major cell classes of the CNS [16]. They typically appear during development and reside in the CNS during adulthood. They have been defined as slow dividing and uncommitted progenitor cells, generating fast dividing lineage restricted progenitor cells (precursor cells) that are able to undergo a limited number of cell cycles [17, 18]. Several aspects influence the final fate, of both intrinsic and extrinsic nature. Transcription factors and growth factors can function as intrinsic determinants, linking the extracellular signals via second messenger pathways and other intracellular responses to cell-intrinsic reactions. Important extrinsic determinants are growth factors and neurotrophins.

Neurogenesis occurs throughout life in the rodent and also in the human system. Kuhn and colleagues were able to show that the source of newly born neurons are stem or progenitor cells residing in the two major neurogenic areas of the brain, the HC and the wall of the lateral ventricle, including the ependymal and subventricular zone (SVZ) [19,

[20]. The continued production of new neurons throughout life has also been shown in humans [21].

Hippocampal neurogenesis is occurring in progenitor cells dividing along the border of the hilus and the granule cell layer. The daughter cells differentiate into granule cell neurons. Cells born in the SVZ leave the ventricle wall, migrate along the rostral migratory stream and, completing their last divisions, are a source of new neurons for the olfactory bulb, with 99% differentiating into GABAergic granule cells (reviewed in [22]). A clearly defined barrier between pluripotency and multipotency is hard to draw due to plasticity of cells as indicated by de-differentiation, reprogramming and trans-differentiation processes. Trans-differentiation describes the phenomenon of a differentiated organ or germ layer specific cell to acquire a different phenotype within the same organ or a differentiation phenotype of a cell from another organ or germ layer (Fig.I.6; red arrows). It also includes a somatic stem cell that acquires pluripotency and differentiates into a cell specific for another organ. De-differentiation describes the process of a differentiated, organ specific cell that looses its differentiation markers and acquires characteristics of a somatic stem cell from the same organ (Fig.I.6; green arrow).

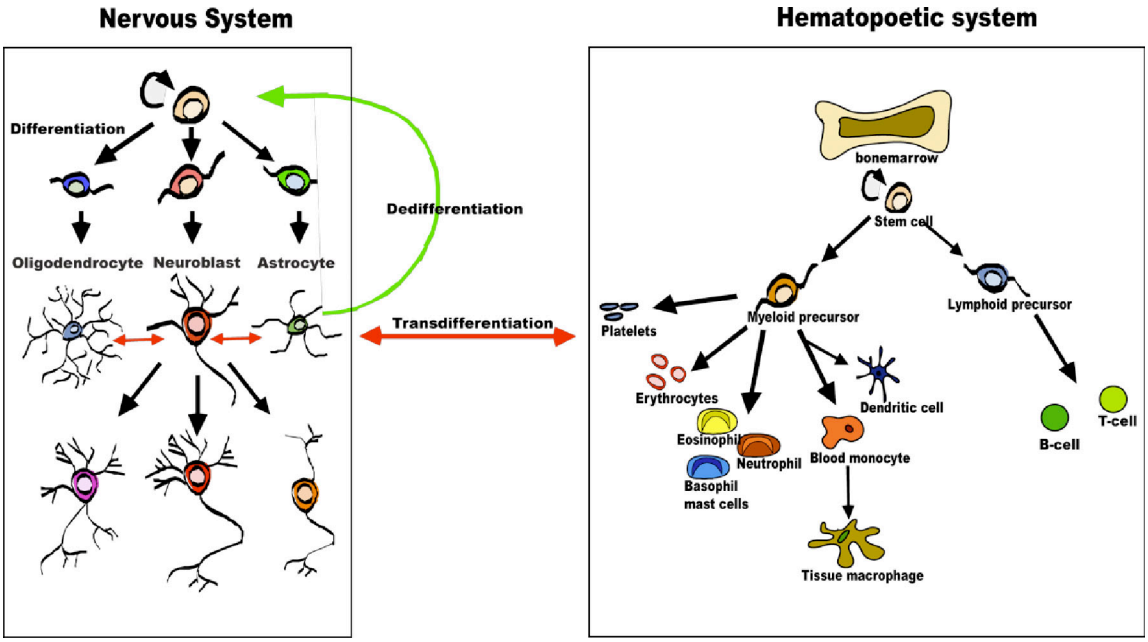

Fig.I.6. Differentiation, de-, and trans-differentiation profiles of somatic cells and somatic stem cells. The nervous system and the hematopoetic system as examples. Differentiation: black; trans-differentiation: red; de-differentiation: green.

The anatomical and topographical position of adult somatic neural stem cells is still controversial. However, it is agreed that this type of cell is probably an uncommitted progenitor cell that produces lineage-restricted precursors capable of a limited number of mitotic divisions. Evidence has been provided that a specialized glial fibrillary acidic protein (GFAP)-positive glia cell in the SVZ and the granule cell layer of the HC is a somatic stem cell [23, 24]. This GFAP positive neural stem cell in the SVZ is thought to derive from radial glia during development [25]. More recently, a discussion evolved around the role of radial glia during neurogenesis. These cells have been shown to constitute the neural stem cell population during the period of corticogenesis [25-29].

Another hypothesis states that ependymal cells, a developmental left over of the neuroepithelium, are neural stem cells [30]. One piece of evidence is the expression of Notch1, a most important developmental regulatory protein that can be used to enrich for neural stem cells *in vitro* [30]. More recent data suggest a close interaction between the vascular system and neural stem cell activity [31]. Palmer and colleagues were able to show that a vast majority of dividing progenitor cells in the hippocampal hilus were also immunoreactive for endothelial markers and that most of the newborn endothelial cells disappeared over several weeks. The authors concluded that neurogenesis is intimately associated with the process of active vascular recruitment and remodeling, suggesting that adult neurogenesis occurs within an angiogenic niche. Several possible explanations come to mind. First, the surrounding endothelium might provide inducing factors for residual stem cells. Secondly, the endothelium itself contains neural stem cells or thirdly, circulating neural stem cells enter the brain parenchyma through the endothelium.

To summarize the current situation, several models have been suggested that all try to explain a possible origin for somatic stem cells either as i) a residing stem cell in the adult brain or ii) an already differentiated glial or ependymal or maybe even unknown cell that de-differentiates into a somatic stem cell or iii) a differentiated somatic stem cell directly trans-differentiates into a neuron or finally iv) a circulating stem cell transforms into a somatic organ-specific stem cell.

It has been shown previously that somatic neural stem cells not only differentiate into neurons, astro- and oligodendroglia [16], but also into cells typical for the hematopoetic system, passing the germ layer restrictions [32]. Also, the opposite has been shown: hematopoetic stem cells can generate neurons in the CNS [33, 34].

However, a controversial discussion regarding the mechanisms of trans-differentiation was started by Terada and Ying in 2002. Both authors described cell fusion as the underlying mechanism of what other scientists thought to be trans-differentiation [35, 36]. The dispute is not settled and is object of further analysis. Prove for trans-differentiation could be the finding of the induction of a Drosophila leg to a wing by ectopic expression of *vestigial* [37]. In the controversy regarding the mechanisms behind trans-differentiation processes, it appears that, while the fate of cells is normally restricted to a limited number of differentiation pathways, these restrictions may be altered under certain circumstances (reviewed in [38, 39]).

I.2.3. Adult somatic neural stem cells in vitro

Adult somatic NSCs can be isolated from several regions of the CNS, including the already mentioned neurogenic regions of SVZ and HC [40, 41] as well as the non-neurogenic regions of spinal cord (SC), striatum and neocortex [42, 43]. These cells can be isolated and propagated *in vitro*. Palmer et al. showed that cells of glial origin de-differentiate and produce NSC cultures [42, 44, 45]. Recent data revealed that somatic NSCs from human surgical biopsy and from postmortem brain material can be propagated *in vitro* [30, 46-49]. The growth factor basic fibroblast growth factor (bFGF) has proven to be essential for proliferation in culture. It has been reported to be critical to propagate adult somatic neural stem cells [46-52]. Furthermore, the differentiation potential of these cells is of great interest. Adult NSCs can be differentiated *in vivo* and *in vitro*. Removal of growth factors prevents further divisions of NSCs and acts as initiation for neuronal and glial differentiation [46-49, 52]. Predominantly, neuronal differentiation can be triggered by stimulation with retinoic acid and neurotrophic factors like BDNF or NT-4. In addition, an activation of the cAMP signaling cascade induces neuronal differentiation [53].

In vivo, a wide spectrum of differentiation has been observed after transplantations. The microenvironment, in which the transplanted cells are placed, seems to be a determining factor for cell fate specifications. As an example, somatic NSCs derived from the HC differentiate into neurons when transplanted into the dentate gyrus of the HC or into glia, when placed in the surrounding non-neurogenic regions [51]. On the other hand, the same HC derived stem cells developed into dopaminergic neurons in the olfactory bulb, a heterotypic, neurogenic region [54]. The same authors were able to show

that grafts in non-neurogenic regions like the cerebellum did not induce neuronal differentiation. Similar results have been reported for the retina. After grafting adult rat HC derived progenitor cells into the vitreous cavity of the adult rat eye, the cells were well integrated into the retina after 4 weeks, adopting the morphologies and spatial positions of subsets of Müller glia cells, amacrine, bipolar, horizontal and photoreceptor neurons as well as general astrocytes [55]. However, although the cells expressed neuronal and glial markers, none expressed end stage markers unique to retinal neurons or glia.

I.2.4. Retinal stem cells during development

The neuroblast layer of the optic vesicle contains uncommitted stem cells as well as lineage restricted progenitor cells, much like the SVZ of the developing CNS. These cells are able to undergo a limited number of cell cycles. However, they show a tendency to produce only a limited range of cell fates [17, 56]. The role of extrinsic and intrinsic regulators and factors in controlling cell fate has been addressed in numerous cell fate determination studies. This led to a hypothetical model proposing that progenitor cells pass through intrinsically determined competence states, during which they have the capacity to generate limited subsets of different cell types, all under the influence and control of extrinsic signaling factors (reviewed in [57]). The different cell types of the retina are generated from proliferating stem cells in a certain temporal sequence. The mammalian retina is populated by two distinct groups of retinal neurons. One group is generated early during retinogenesis and consists of ganglion cells, cone photoreceptors, horizontal cells and a subpopulation of amacrine cells [58]. The other group is born later during retinogenesis and is made up of rod photoreceptors, bipolar cells and another subpopulation of amacrine cells [58]. Evidence has been presented that these two groups are generated by two different types of progenitor cells that may result from an asymmetrical final division of the original retinal stem cell [58].

I.2.5. Retinal stem cells in the adult

Several studies have shown that in the adult mammalian retina, a number of different cell types might be sources for somatic neural stem cells. These cells can be derived from the margin of the ciliary body (CB), the pigment epithelium layer (RPE) and the sensory retina (SR).

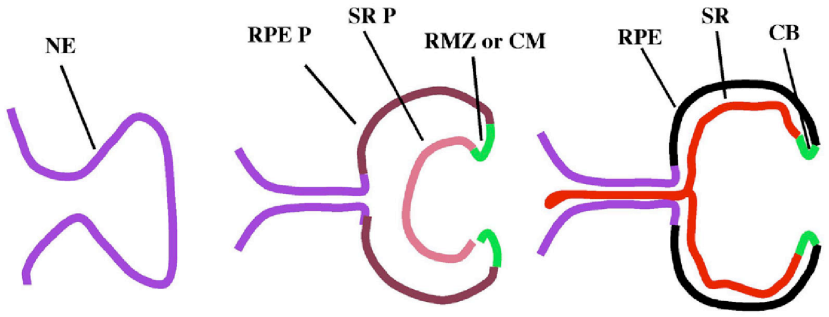

Fig.I.7. Development of neuroectodermal parts of the eyes: sources of stem/progenitor cells. NE: neuroepithelium, RPE P: retinal pigment epithelium restricted precursors, SR P: sensory retina restricted precursors, RMZ: retinal marginal zone, CM: ciliary margin, RPE: retinal pigment epithelium, SR: sensory retina, CB: ciliary body.

I.2.5.1. Pigmented cells in the ciliary body: multipotent somatic neural stem cells

It was thought that the adult mammalian retina - unlike its poikilothermic vertebrate analogues in fish, amphibians or reptiles – is devoid of proliferative or regenerative capacity. However, recent data suggest that there is indeed such proliferative capacity, leading to the identification of a stem or progenitor cell in the adult mammalian eye.

In fish, amphibia and reptiles, retinal stem cells in the periphery of the eye (ciliary marginal zone (CMZ) or CB) proliferate throughout life, continuously producing new neurons that are integrated into the already functioning retinal circuitry [59-62]. In these species, the retina grows constantly to keep up with the enlarging body. Proliferating cells in the marginal zone generate two clones containing cells of the SR and of the RPE, which might explain the coordinated growth of these two tissues [63, 64]. More recent data suggests that the neurogenic potential of the adult CMZ is not restricted to poikilothermic vertebrates, but that neurogenesis can be found in the postnatal and adolescent retina of the chicken in the proliferating marginal zone [65]. Furthermore, the adult primate can develop cysts in the peripheral retina and *pars plana* that contain cells expressing markers for retinal neurons [66].

Moreover, *in vitro* experiments suggest the presence of multipotent neural progenitor cells in the CB of the adult mammalian eye [67, 68]. Under the culture conditions used in these studies, pigmented cells from the CB, but not the SR, RPE or other retinal structures, formed neurospheres consisting of pigmented and non-pigmented cells [67].

Several cells in these neurospheres expressed nestin and Chx10, both markers for somatic neural precursor cells and retinal progenitors. Some CB derived cells differentiated into retinal neurons and glia [67]. Despite the lack of convincing clonal analysis, pigmented cells in the CB were proposed in this study to be multipotent neural stem cells [67].

Apart from the ciliary margin, other areas in the retina of poikilothermic vertebrates as well as embryonic homeothermic vertebrates like chicken and mammals have been identified to contain cells with proliferative capacity, especially triggered after injury [69]: i) the retinal pigment epithelium (RPE) in amphibians, birds and mammals, ii) a specialized rod progenitor cell in fish [70] and iii) the Müller glia cells, the only glial cell type of the retina.

I.2.5.2. A possible role as neural stem cell for Müller glia in the sensory retina

As Müller glia cells are among the latest cells in the retina to develop [58, 71], it is unlikely they are the source for generating retinal progenitor cells during development. However, after injury, Müller glia cells undergo reactive gliosis just like other glial cells types in the CNS. This process is associated with cell proliferation and upregulation of glial fibrillary acidic protein (GFAP) [72].

After an N-methyl-D-aspartate (NMDA) lesion in postnatal chicken retina, cell proliferation is induced and triggers the expression of the retinal progenitor markers CASH-1, Pax6 and Chx10 in Müller glia [73]. Newly born cells differentiate into retinal neurons, into Müller glia or remained undifferentiated [73], suggesting that Müller glia cells might be a potential source for de-differentiating cells that acquire a somatic neural stem cell phenotype. An interesting hypothesis was proposed by Willbold and Layer, which suggested the “gradual maturation of Müller cells” [74]. Müller cells have a remarkable plasticity, a fact that is reflected by their morphological shape, their biochemical equipment and their functions that all change continuously. The transition of early radial neuroepithelial precursors to adult Müller glia cells seems to be a graded process and does not run strictly unidirectional [74].

I.2.5.3. Retinal pigment epithelium cells: somatic neural stem cells in lower vertebrates and their possible role in mammals

The RPE is of neuroectodermal origin as it derives from the neural plate [75] and although it differentiates into a polarized epithelial monolayer sheet, it descends from precursors that later generate neural retina. The mature RPE is a single cell layer consisting of a mosaic of polygonal cells between the choroid and the neural retina. RPE cells are fully differentiated, polarized cells expressing genes required for melanin production, including tyrosinase [76]. It is a highly specialized epithelium that serves many functions, for example, the transport of metabolites to and from the choroid and photoreceptors. The RPE is of utmost importance in processes essential to vision such as the metabolism of intermediates of the visual cycle and the phagocytosis of photoreceptor outer segment (reviewed in [77]).

The RPE is the source of newly generated retinal neurons during retinal regeneration in amphibians. Devascularization-induced retinal degeneration in the frog is followed by the generation of new retina by RPE cells and by increased proliferation of the marginal zone [78]. Removal of the retina induces proliferation of the RPE cells in the frog creating a second layer of RPE cells that lose pigmentation and rebuild a new retina in a sequence similar to the one observed during developmental retinal histogenesis [62]. *In vitro*, neuronal trans-differentiation of frog RPE cells was found to be influenced by the substrate on which the cells are cultured. In particular, RPE cells plated on laminin-containing substrates frequently trans-differentiated into neurons [79]. Additionally, FGF-2 promotes the genesis of neurons and glia by RPE cells during development [80-82]. With respect to homeothermic vertebrates, first evidence of RPE based retinal regeneration arose from the work of Coulombre, demonstrating that after surgical removal of the retina in chick embryos, the remaining RPE formed a new retina *in vivo*, although in an inverted orientation [83]. In the late embryonic chicken, RPE based retinal regeneration does not occur spontaneously, but can be induced by FGF [84, 85] and requires cell-cell interaction [86]. However, no cases of RPE regeneration in higher mammals have been reported so far. Substantial evidence exists for de- or trans-differentiation of RPE cells. *In vivo*, experimentally induced retinal detachment causes focal proliferation of RPE cells at the site of detachment in a number of mammalian species [87-91]. RPE cells round up, loose their apical processes and divide to form a three to four cells thick layer. In humans, RPE

proliferation is described as a consequence of retinal detachment surgery in which the subretinal space, the junction between SR and RPE, has been disturbed [88]. In addition, epiretinal membranes that are neoplastic structures derived from proliferating RPE cells contain cells expressing β III tubulin, a marker for neuronal differentiation [92].

In vitro, de- and trans-differentiation of mammalian RPE cells is well documented. Primary cultures of RPE cells loose differentiation markers such as pigmentation and their specific markers retinal pigment epithelium protein 10 (RPE10) and RPE65 [93, 94]. Neuronal trans-differentiation was found in human RPE cultures by expression of the early neuronal marker β III tubulin [92] and in a minor population of cultures RPE cells that express the neuron-specific enolase (NSE) and the adult neuronal marker neurofilament 200 (NF200kDa) [92]. Additionally, the mRNA for the neuronal specific microtubule-associated protein MAP 1B is induced in human cultured RPE cells [95]. Cultured RPE cells from neonatal rats, fetal and adult humans express voltage-gated Na^+ channels and can produce action potentials, properties normally associated with neurons [96, 97].

In conclusion, RPE cells from poikilothermic vertebrates and birds are able to i) either trans-differentiate into retinal neurons and glia or ii) de-differentiate into multipotential retinal stem or progenitor cells. FGF induces or promotes this process. In contrast, homeothermic vertebrates apparently lost this capacity (Fig.I.8.). RPE cells partially trans-differentiate and acquire normal features, including expression of β III tubulin and voltage-gated Na^+ channels. However, they do not de-differentiate into a multipotent somatic neural stem cell nor do they trans-differentiate completely to acquire the full phenotypic pattern of a nerve cell or regenerate a retina.

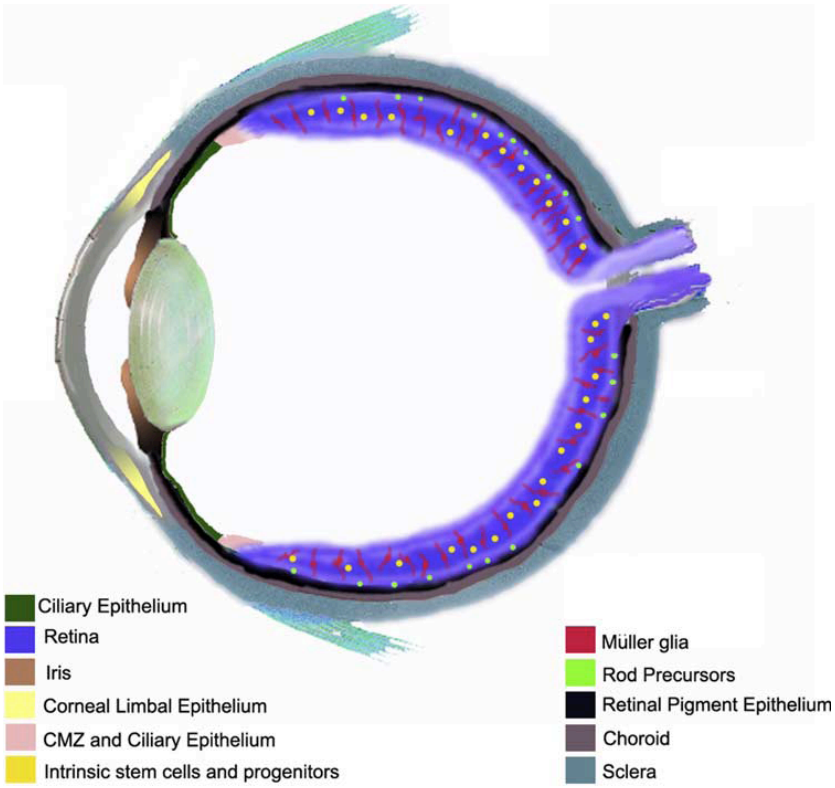

Fig.I.8. Possible sources for retinal regeneration compiled from different animal models. In amphibians: RPE=black, ciliary marginal zone (CMZ)=pink. In birds: RPE=Black, CMZ=pink, CB=dark green (embryonic), Müller glia=red (posthatch). In fish: circumferential germinal zone (CGZ)=pink (embryonic/larval), rod precursors=light green (embryonic/larval and adult), intrinsic stem and progenitor cells=dark yellow (embryonic/larval and adult), Müller glia=red. In mammals: RPE (embryonic), pigmented cell of the CB=dark green, iris=light brown, corneal limbus area=light yellow, choroids=dark brown, sclera=aqua. Adopted from [98]

I.2.6. Fate determination of retinal stem cells: the molecular basis

The pathway from a multipotent neural retinal stem cell to a differentiated retinal neuron or glia cell involves different regulatory steps, including maintenance of multipotency and self-renewal of retinal stem cells, cell lineage determination, and the specification and differentiation of mature cell types (reviewed in [99]).

I.2.6.1. The role of the Notch/Delta system during retinal development

Notch is among the best characterized genes in *Drosophila melanogaster*. It plays an important role during early neurogenesis and provides a feedback signal from postmitotic neuroblasts towards undifferentiated stem cells, inhibiting their neuronal differentiation (reviewed in [100]). The signaling pathway includes close cell-cell contacts and interaction of Notch1 with its ligand Delta, in a model called lateral inhibition

(summary in Fig.I.9.), supporting the idea of Notch1 as a stem cell regulator. Both Notch1 and Delta are expressed in the neural retina during the period of cell fate determination and differentiation in the rat [101].

The historic view of Notch/Delta function has been that its signaling is used to maintain a pool of uncommitted precursors, while a subset of cells is selected to leave this pool and differentiate [102]. Recently, however, several studies in vertebrates have shown that rather than simply inhibiting neuronal differentiation, Notch1 may promote the acquisition of glial identity (reviewed in [103]). Transient activation of Notch1 is sufficient to cause an irreversible loss of neurogenic potential associated with accelerated glial differentiation [104]. Interestingly, in some cases in vertebrates, Notch1 signaling promotes glial cell types that may retain progenitor character (e.g. radial glia, astrocytes, Müller glia). Furukawa and colleagues showed that in the retina, Notch1 is expressed in retinal progenitor cells and downregulated in differentiating, mature neurons. However, it is expressed in Müller glia cells, consistent with the proposed idea of a possible role in Müller glia development [105]. Notch1 might be arresting NSCs in a resting position that is associated with the expression of glial differentiation markers, since radial glia have been shown to serve as a NSC during corticogenesis [27, 28] and since astroglial cells are stem cells in the SVZ [23].

A downstream mediator of Notch1 signaling is Hes1 (Hes1 in rat and Hes5 in mouse) [106, 107]. Hes genes are basic helix-loop-helix (bHLH) transcriptional repressors that may inhibit proneuronal transcription factors such as Mash1 or neurogenin 2, which have been shown to promote neurogenesis and block gliogenesis [108]. Misexpression studies of Hes1 have revealed that Müller glia fate is promoted, while loss-of-function studies led to reduced numbers of glial cells [109]. In general, the fate promoted by Notch signaling might represent a so far undescribed population of persisting adult progenitors, whose morphology and gene expression overlaps considerably with those of Müller glia cells [105].

I.2.6.2. Pax6, a homeobox gene regulating cell fate in the retina

Pax6 is a master regulatory gene of the paired-domain homeobox gene family, which induces eye development in a broad range of animal species. It is highly conserved among vertebrates and invertebrates. The Pax6 protein is expressed in a large area of the

rostral neural plate within which all of the cells that contribute to the optic vesicle are likely to originate. It is unlikely to be required for the initiation of optic vesicle formation, as this process occurs well in homozygous Pax6$^{-/-}$ *Small eye* (*Sey*) mice. However, although optic vesicle evagination does occur in *Sey* mice, the resultant morphogenesis and growth are abnormal and the optic vesicle fails to form a recognizable optic cup. Reduction of Pax6 activity causes the small eye phenotype in rodents [110] and induces ectopic eyes in invertebrates and in amphibians [111, 112]. This suggests that Pax6 may have a more fundamental role in maintaining the proliferation of cells within the optic vesicle. Further support for a role of Pax6 in proliferation comes from the observation that Pax6 expression is maintained in cells at the proliferative margins of the retina during retinal regeneration in goldfish and lens regeneration in urodeles. It is also widely expressed in other dividing cells in the CNS.

Pax6 expression is downregulated in the optic stalk and in the RPE, while its expression in the developing SR persists in retinal progenitor cells. In the mature retina, expression is retained in amacrine and ganglion cells (reviewed in [99]). After conditional incativation of Pax6 in the developing distal SR, the pool of retinal progenitors is not affected, but an impairment of the differentiation into various retinal cell types has been detected [113]. The same authors were able to show that retinal progenitors deficient of Pax-6 exclusively produce amacrine phenotypes [113].

While amacrine cell differentiation is driven by the bHLH transcription factor neuroD, a protein not regulated by Pax6, other transcription factors mediating neuronal or glial differentiation in the retina such as Brn3b, Math5, Mash1, Ngn2, Crx1, Rx1 or HES1 seem to depend on Pax6 activity [113]. Since in the developing CNS, Pax6 is expressed in radial glia and is required for radial glia to exhibit their full differentiation program [27], it might be a proneuronal regulating factor.

I.2.6.3. Basic helix-loop-helix transcription factors during retinal development

When cells are released from the inhibition mediated by the Notch/Delta pathway, bHLH factors act as intrinsic mediators that bias neuroblasts towards specific fates. The progenitors are maintained in the proliferative state by Pax6 expression and Hes1 and Id bHLH proteins and the transition from proliferation to neurogenesis involves a

coordinated increase in other bHLH factors such as Brn3b, Math5, Mash1, Ngn2, Crx1, Rx1 or NeuroD [114, 115].

Mutational analysis has implicated Math5 in promoting ganglion cell fate while restricting differentiation into amacrine cell fate [116, 117]. Mash1 regulates bipolar cell differentiation [118] and NeuroD promotes amacrine and rod, but restricts bipolar cell development [119].

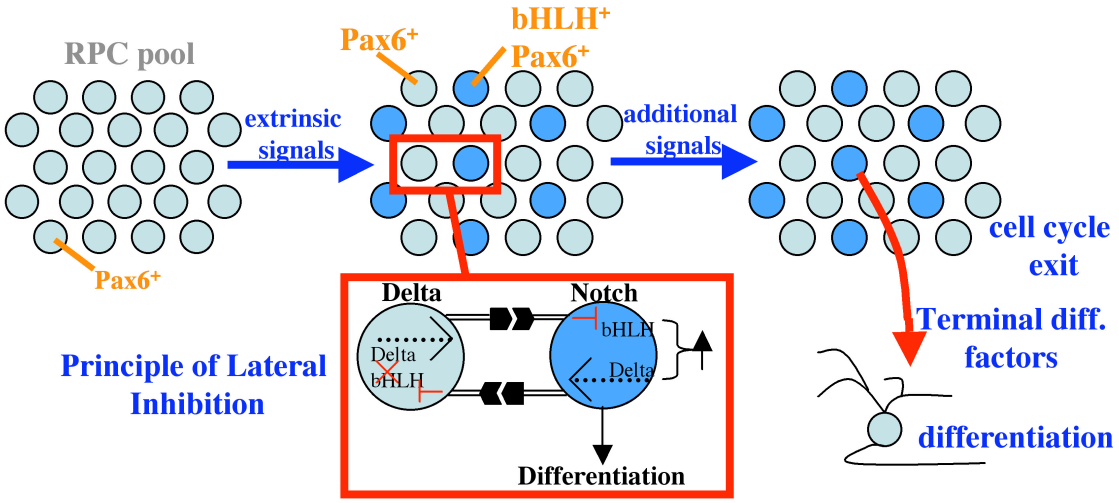

Fig.I.9. Interaction of molecular factors during retinogenesis. The activity of Pax6 in all retinal progenitor cells (RPCs) is a prerequisite for the activation of retinogenic bHLH transcription factors (such as Math5, Ngn2 and Mash1) [113], possibly triggered by extrinsic signals, such as Sonic hedgehog (Shh) or epidermal growth factor (EGF) [120] [121]. Presumably, their activation, underlies the transition from uncommitted to lineage-restricted RPCs. Insert: activation of Notch receptor by high levels of Delta ligand present on the surface of the adjacent RPC leads to the suppression of Delta and bHLH factor expression. The resulting lateral inhibition consequently assures that the activation of bHLH factors occurs only in a subset of RPCs. The action of bHLH factors is thought to accelerate cell cycle exit [122], possibly assisted by additional signals. In addition, bHLH factors, such as Math5, are implicated in activating terminal differentiation factors, such as Brn3b, in postmitotic precursor cells [117]. Adapted from [123].

In summary, retinal stem and precursor cell fate is regulated by the Notch/Delta system, the homeobox transcription factors Pax6 and Chx10 and a variety of bHLH factors. Notch inhibits retinal stem or precursor cells from neuronal differentiation by activating the proneural gene repressors Hes1 and triggers gial differentiation, while the proneuronal gene Pax6 activates other proneuronal and neuronal differentiation genes to bias retinal stem cells towards a neuronal cell fate.

II. Aim of study

Several aspects of retinal development and histogenesis point towards the existence of retinal progenitor or stem cells in the adult mammalian eye. However, the sources of progenitor and stem cells derived from the adult eye are still not sufficiently analyzed and it was therefore a goal of this study to broaden the current knowledge especially by focusing on the existence of other locations of retinal stem or progenitor cell in the postnatal and adult eye. A major focus is also the plasticity possibly displayed by adult derived retinal cells to de-differentiate and acquire a more progenitor or even stem cell like phenotype.

This dissertation focuses on three major characteristics of retinal progenitor and stem cells:

1. Existence
2. Potential
3. Plasticity

A first step was to optimize cell culture protocols in order to have sufficient amounts of cells available for the studies. Adult derived NSCs from the neurogenic regions of the brain served as model system and were also analyzed with respect to their differentiation properties both *in vitro* as well as after retroviral infection.

The isolation and expansion of postnatal retinal progenitors *in vitro* was addressed to bridge the gap in knowledge between the existence of early embryonic and adult derived retinal stem and progenitor cells. Furthermore, the differentiation potential of these postnatal derived progenitor cells was investigated.

While the existence of stem cells from the adult CB has been described in detail, possible progenitor or stem cell characteristics for RPE derived cells have not yet been

reported. Another aim of this study was therefore, to analyze this particular compartment of the eye and its potential to harbor cells displaying some characteristics of retinal progenitor cells. RPE derived progenitor cells were closely compared to CB derived progenitor cells and the known differentiation profile of the two other major CNS derived stem cell classes: SVZ and HC derived stem cells.

Finally, the controversial discussion about cell plasticity and possible trans- or de-differentiation processes in adult derived retinal stem cells is addressed by a comprehensive expression study in CB and RPE derived progenitor cells.

III. Material and Methods

III.1. Material

III.1.1. Chemicals

III.1.1.1. Cell culture

Chemical	Source
Accutase®	Innovative Cell Technologies, USA
B27 supplement	Gibco BRL, Germany
BIT9500	StemCell Technologies Inc., Canada
Bromodesoxyuridine (BrdU)	Sigma, Germany
cyclic AMP	Sigma, Germany
Dispase II	Boehringer, Germany
DMEM/F12	Gibco BRL, Germany
Dnase I	Worthington Biochemicals, England
Dulbecco's phosphate buffered saline	Sigma, Germany
Epidermal growth factor (EGF)	R&D Systems
Fetal calf serum (FCS)	PAN, Germany
Fibroblast growth factor (FGF)	R&D Systems
Forskolin	Sigma, Germany
Glucose	Merck, Germany
Hank's Balanced Salt Solution	PAN, Germany
Heparin	Sigma, Germany
IMEM	Gibco BRL, Germany
Laminin	Sigma, Germany
L-glutamine	PAN, Germany
Neurobasal Medium (NB)	Gibco BRL, Germany
N2	Gibco BRL, Germany
Papain	Worthington Biochemicals, England
Penicillin/streptomycin	PAN, Germany
Poly-L-ornithine	Sigma, Germany
Trypan Blue	Sigma, Germany
Trypsin	PAN, Germany

III.1.1.2. Immunodetection

Chemical	Source
4'6-diamidino-2-phenylindole, dihydrochloride (DAPI)	Molecular Probles, USA
Donkey serum	PAN, Germany
Fluoromount G	Southern Biotechnology Ass. Inc., USA
TOPRO-3iodide	Molecular Probles, USA
Triton X-100	Sigma, Germany

III.1.1.3. Antibodies

Primary antibody[(a)]	Experimental application	Dil.	Source
	Neuronal marker		
ms α βIII tubulin	early postmitotic neuron [124]	1:250	Promega, USA
rb α βIII tubulin	early postmitotic neuron [124]	1:250	BabCO, USA
rb α neuron specific enolase (NSE)	mature neuron (not specific) [125]	1:100	Chemicon, USA
ms α microtuble assoc. protein (MAP2)	mature neuron [126]	1:200	Sigma, Germany
rb α neurofilament 200kD	mature neuron [127]	1:250	Chemicon, USA
rb α Calbindin	Ca^{2+}-binding protein [128]	1:300	Swant, Swizerland
rb α Calretinin	Ca^{2+}-binding protein [128]	1:300	Chemicon, USA
	Glial marker		
ms α A2B5	glial progenitor [129]	1:100	Chemicon, USA
rb α S100 β	glial progenitor [129]	1:1000	Swant, Swizerland
rb α NG2 chondroitin sulfate proteoglycan	glial/oligodendroglial progenitor [130]	1:200	Chemicon, USA
rb α glial acidic fibrilary protein (GFAP)	mature glia [131]	1:500	Dako, Denmark
rb α basic lipid binding protein (BLBP)	radial glia [28]	1:3000	gift N. Heintz, Rockefeller Univ., USA
	Oligodendrocyte marker		
rb α NG2	oligodendroglial progenitor [130]	1:200	Chemicon, USA
rb α Galactocerebroside (GalC)	mature oligodendrocyte [132]	1:250	Chemicon, USA
	Intermediate filament marker		
ms α Nestin	progenitor [133]	1:150	PharMingen Intl, USA
ms α Vimentin	ubiquitous filament [134, 135]	1:300	Chemicon, USA
	Proliferation marker		
rb α Ki-67 nuclear antigen	cell cycle protein [136]	1:500	NOVO Castra, UK
rt α Bromodesoxyuridine (BrdU)	base analog [137]	1:500	ImmunologicalsDirect, USA
	Stem cell and progenitor markers		
ms α Pax6	paired homeobox transcription factor [113]	1:100	DSHB, USA
gt α Doublecortin (DCX)	neuronal progenitor [138]	1:350	Santa Cruz, USA
sh α Chx10	homeobox transcription factor [139]	1:200	Exalpha Biol., USA
rb α Notch1	transmembrane receptor [100]	1:200	Upstate Biotech., USA
rb α NeuroD1	bHLH transcription factor [140]	1:200	Chemicon, USA
	Retina-specific marker		
rb α recoverin	photoreceptors, subsets of bipolar and amacrine cells [1]	1:150	gift Dr. Koch, Jülich, Germany
ms α Glutamine Synthetase	bipolar cells [141]	1:200	Infection Labs, USA
rb α Flk-1	retinal progenitor [142]	1:250	Santa Cruz, USA
ms α Rhodopsin	cone and rod photoreceptors [143]	1:150	Leinco Technol., USA
rb α retinoschisin	photoreceptors and bipolar cells [144]	1:500	gift B. Weber, Würzburg, Germany
	RPE markers		
rb α tyrosine hydroxylase	secreted in RPE cells [145]	1:200	Chemicon, USA
ms α keratin	intracellular marker in RPE [146]	1:150	NeoMarkers, USA

(a) ms=mouse; rb=rabbit; rt=rat; gt=goat; sh=sheep

Western Blot antibody	Experimental application	Dil.	Source
ms myc tag	myc tag associated with Notch1	1:500	Invitrogen, USA
dk ms-IgG-Peroxidase		1:10000	Jackson Immuno, USA

Secondary antibody	Dilution	Source
gt ms Fluorescein (FITC)	1:1000	Molecular Probes, The Netherlands
gt ms Rhodamine Red (RHOX)	1:1000	Molecular Probes, The Netherlands
gt rb Rhodamine Red (RHOX)	1:1000	Molecular Probes, The Netherlands
gt sh Fluorescein (FITC)	1:1000	Molecular Probes, The Netherlands
dk gt Fluorescein (FITC)	1:1000	Jackson Immuno Research, USA
dk rt 7-Amino-4-methylcoumarin-3-acetic acid (AMCA)	1:1000	Jackson Immuno Research, USA

III.1.1.4. Viral infections

Reagent	Application	Source
2x BES buffered saline	- 50mM BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid	Sigma, Germany
	- 280mM NaCl	Merck, Germany
	- 1.5mM Na_2HPO_4 * $2H_2O$	Merck, Germany
	- dissolve in 90ml dH_2O, adjust pH to 6.96 and add dH_2O ad 100ml.	
2.5M $CaCl_2$		Merck, Germany

III.1.1.5. HPLC

Reagent	Source
Ethylenglycole-bis-aminoethylether-N,N,N,N-tetraacide (EGTA)	Sigma, Germany
Glutathion	Merck, Germany
NaOH	Sigma, Germany

III.1.2. Solutions

Reagent	Application	Source
Borate Buffer (0.1M), pH 8.5	- 3.08g boric acid	Sigma, Germany
	- 450 ml H_2O	
	- 5N NaOH to pH 8.5	Sigma, Germany
	QS to 500ml	

Reagent	Application	Source
Coomassie stain	- 2.0g Coomassie Brilliant Blue R250	Sigma, Germany
	- 0.5g Coomassie Brilliant Blue G250	Sigma, Germany
	- 425ml EtOH	J.T. Baker, Holland
	- 50ml MetOH	J.T. Baker, Holland
	- 100ml HCOOH	
	- 425ml Bidest H_2O	
Coomassie differentiation sol	- 45% EtOH	see above
	- 10% HCOOH	
	- 45% Bidest H_2O	
Donkey serum blocking buffer	- 0.1 M Tris-Hcl, pH 7.5	Merck, Germany
	- 0.15 M NaCl	Sigma, Germany
	- 3% donkey serum	PAN, Germany
	- 0.1% Triton X-100	Sigma, Germany
Fish skin gelatine blocking buffer	- 497.5ml TBS	
	- 1% BSA	Sigma, Germany
	- 0.2% fish skin gelatine	Sigma, Germany
	- 0.1% Triton-X 100	Sigma, Germany
	- 0.02% sodium azide	Merck, Germany
Loading/Lysis buffer for Western Blot (WB)	- 312.5μl 1M Tris-HCl, pH 6,8	
	- 1000μl 10% SDS	Merck, Germany
	- 250μl 2-mercapto-ethanol	Sigma, Germany
	- 580μl glycerin	Sigma, Germany
	- some crystals bromophenol-Blue	Sigma, Germany
	- 2857.5μl H_2O	
Phosphate Buffer (0.2M)	- 1L ddH2O	
	- 5.52g NaH_2PO_4, water free	
	- 21.9g Na_2HPO_4, water free	
PFA (4%, 100ml)	- 4g paraformaldehyde in 80ml H_2O, heat to 60°C	Aldrich, Germany
	- dissolve while stirring, add 10N NaOH until solution is clear	
	- 10ml 10xPBS and H_2O until 100ml is reached	
	filter solution and keep at 4°C	
PBS (0.1M)	- 500ml 0.2M Phosphate Buffer	
	- 500ml dH_2O	
	- 9g Natriumchloride	
Ponceau Solution		Sigma, Germany
PPD (100ml)	- 0.01% Papain	Worthington, USA
	- 0.1% Dispase II	Boehringer Mannheim
	- 0.01% Dnase I	Worthington, USA
	- 149 mg $MgSo_4*7H_2O$	
	in Hank's Balanced Salt Solution w/o Ca^{2+}/Mg^{2+}	PAN, Germany
Running buffer for WB	- 3.02g Tris-Base	Sigma, Germany
	- 14.4g glycerin	Sigma, Germany
	- 1.0g SDS	Sigma, Germany
	- dissolve in 0.8l H_2O, set at pH 8,3 with 2M HCl and fill up to 1l with H_2O	
30% Succrose	- 400ml 0.1M PO_4	
	- 150g Succrose	Sigma, Germany

Reagent	Application	Source
Stabilization solution for HPLC	- 900mg EGTA - 700mg glutathion - in 0.5M NaOH (pH 7.0-7.5) ad 10ml with H_2O	Sigma, Germany Merck, Germany
SSC	- 3.0M NaCl - 0.3M NaCl x $2H_2O$ QS 500ml H_2O, pH 7.0	
TBST	TBS+0.25% TWEEN	Sigma, Germany
Transfer buffer	- 3.03g Tris-Base (25mM) - 11.26g glycin (150mM) - 100ml Ethanol (100%)	Sigma, Germany Merck, Germany
Western Fish buffer	- 20ml 1M Tris HCl (20mM, pH 7,3) - 9g NaCl (0.9%) - 20ml 50% Fish gelatin (1%) - 1ml TWEEN (0.1%)	 Sigma, Germany Sigma, Germany

III.1.3. RT-PCR primers

III.1.3.1. Rat primers

cDNA		Sequence 5' 3'	Temp	Size
GAPDH	sense	GGT CGG TGT GAA CGG ATT TG	58°C	500bp
	antisense	GTG AGC CCC AGC CTT CTC CAT		
III tubulin	sense	ATG GAC AGC GTT CGG TCT G	58°C	140bp
	antisense	ATA GTG CCC TTT GGC CCA GT		
DCX	sense	TCG TAG TTT TGA TGC GTT GC	56°C	142bp
	antisense	GCT TTC CCC TTC TTC CAG TT		
Flk-1	sense	GCC AAT GAA GGG GAA CTG AAG AC	59°C	537bp
	antisense	TCT GAC TGC TGG TGA TGC TGT C		
HES1	sense	TAC CCC AGC CAG TGT CAA CA	56°C	140bp
	antisense	TTC ATT TAT TCT TGC CCG GC		
musashi	sense	GAG TCA TGC CCT ACG GGA TG	61°C	140bp
	antisense	CAA GGC CTG TGT AAC TCC GG		
nestin	sense	TCC CAG TTG CTC AGT CAA TGC	59°C	140bp
	antisense	CTG GCA GCC TCT AAC CCA AG		
neuroD1	sense	CAT GAA GCG CTG CGT TTA AC	58°C	140bp
	antisense	CTT CAG CTC CCT CTC CCT CA		
Notch1	sense	TGT GCC AGT ATG ACG TGG AT	58°C	140bp
	antisense	CAC ACT CGT CAA TGT CCA CC		
Pax6	sense	CTC CGT ACA TGC AAA CAC AC	58°C	140bp
	antisense	GTC AGG TTC ACT TCC GGG AA		
RPE 65	sense	CCG GAT TCT TAC CCA TCT GA	57°C	200bp
	antisense	AGT CCA TGG AAG TGC ACA GG		

III.1.3.2. Human primers

cDNA		Sequence 5'□ 3'	Temp	Size
GAPDH	sense	GGT CGG TGT GAA CGG ATT TG	58°C	500bp
	antisense	GTG AGC CCC AGC CTT CTC CAT		
DCX	sense	GGA AGG GGA AAG CTA TGT CTG	59°C	138bp
	antisense	TTG CTG CTA GCC AAG GAC TG		
Flk-1	sense	ATG CAC GGC ATC TGG GAA TC	59°C	537bp
	antisense	GCT ACT GTC CTG CAA GTT GCT GTC		
nestin	sense	GGC AGC GTT GGA ACA GAG GTT GGA	64°C	300bp
	antisense	CTC TAA ACT GGA GTG GTC AGG GCT		
neuroD1	sense	GCC CCA GGG TTA TGA GAC TAT CAC T	64°C	522bp
	antisense	CCG ACA GAG CCC AGA TGT AGT TCT T		
Notch1	sense	ACT GTG AGG ACC TGG TGG AC	57°C	200bp
	antisense	AGA ACT TGT AGG TGT TGG GG		
Pax6	sense	CCA GCC AGA GCC AGC ATG CAG AAC A	64°C	907bp
	antisense	GGT TGG TAG ACA CTG GTG CTG AAA CT		

III.1.4. Equipment and Instruments

Appartus	Source
Confocal laser scanning microscope Leica DMRX with confocal software	Leica, Germany
Fluorescence microscope Leica DMR with SPOT software	Leica, Germany Diagnostics Instruments, USA
Hera Cell incubator	Heraeus, Germany
Hera Safe cell culture hood	Heraeus, Germany
Inverse fluorescence microscope for cell culture:	Olympus, Germany
Olympus IX70 with Color View software	Soft Imaging Systems, Germany
Photodocumentation Ethidium bromide gels	MWG Biotech, Germany
Thermocycler mastercycler gradient	Eppendorf, Germany

III.2. Methods

III.2.1. Preparation and cell culture

III.2.1.1. Postnatal and adult derived cells from the sensory retina

All animal experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC). All animals were obtained from Charles River, Germany. Male and female Long Evans rats of age postnatal day 1 (P1), P3, P8, P14 and adult (4 weeks) were sedated and sacrificed by decapitation. Eyes were dissected

by first opening the eye at the periphery of the sclera and the rest of the eye (see Fig.III.1.).

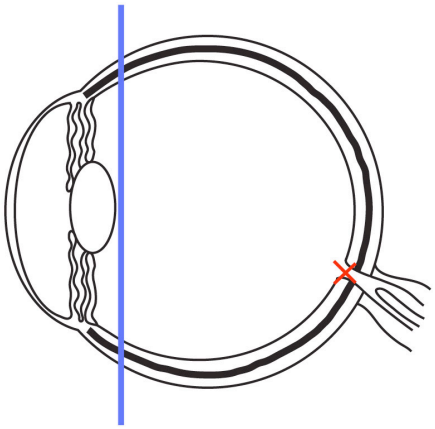

Fig.III.1. Preparation of the sensory retina. Image of a mammalian eye with the blue bar indicating where the eye was opened for isolation of retinal progenitor cells. The red cross marks the section of the retina not included in the isolation.

Next, the posterior part of the eye cup was placed with the retina facing upward. Finally, the retina was peeled off and special care was taken not to contaminate the sample with retinal pigment epithelium cells, glial cells form the optic nerve or with cells from the ciliary margin. Typically, retinae from 10 eyes were digested in 10 ml PPD solution for 30 min at room temperature. The cell suspension was triturated every 10 min. Dissociated cells were collected by centrifugation (188 rcf) and resuspended in 5 ml cold DMEM/F12. Cells were washed three times with thorough trituration. Finally, cells were plated at 10^5 cells/2 ml in Neurobasal medium supplemented with B27 (NB/B27), 0.1 g/L penicillin/streptomycin, 2 mM L-Glutamine, 20 ng/ml EGF, 20 ng/ml FGF and 2μg/ml Heparin. This medium/supplement composition was chosen since it provides optimal growth conditions for adult NSCs [13]. Cells were seeded in 6 well plates (each well at 35 mm) and cultures were maintained at 37°C in an incubator with 95% air and 5% CO_2. Single cells began to form neurospheres within 2 to 3 days in culture and continued to grow in mass and number over the next weeks. Half of the medium was changed every 4 days.

III.2.1.2. Adult derived cells from the ciliary body

Ciliary body (CB) derived cells were isolated from adult (6 weeks old) female Long Evans rats. The eye was opened as described above, only this time, the frontal part of the eye was dissected clean of the surrounding tissues, including the vitreus, retinal pigment epithelial cells, iris and neural retina (see Fig.III.2.). Pieces of CB were placed in

a petridish and mechanically disrupted with a scapel. Cells and remaining tissue was harvested by washing the dish with colt Dulbecco's phosphate buffered saline (DPBS). The solution was centrifuged at 188rcf for 5 min and supernatant was discarded. The pellet was resuspended and digested in PPD solution. Cells were further treated as described in section III.2.1.1.

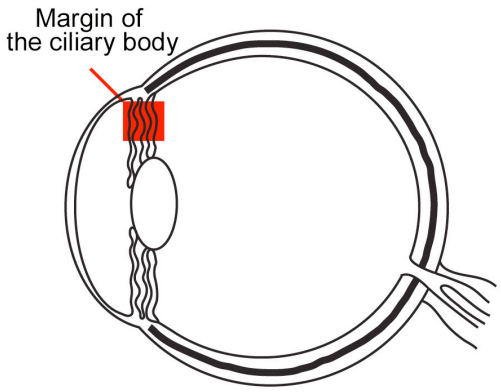

Fig.III.2. Preparation of the ciliary body. The image indicates which region was processed for the preparation of adult ciliary body derived stem cells (red square).

III.2.1.3. Adult derived cells from the retinal pigment epithelium

Cells of the retinal pigment epithelium (RPE) were isolated by first opening the eye and removing all posterior parts (lens, CB). The neural retina was peeled off the remaining eye cup. To exclude quiescent NSCs present in the optic nerve [42] from the preparation, a circular incision around the optic nerve head was made and the tissue was discarded (Fig.III.3.). The eye cups were collected in a 96 well plate (one cup per well) and 2% Dispase II solution was incubated at 37°C for 30 min. After that the RPE cells were gently washed from the underlying choroid tissue and harvested in ice-cold PBS. After a 15 min incubation step in PPD solution, the cells were processed as described in section III.2.1.1.

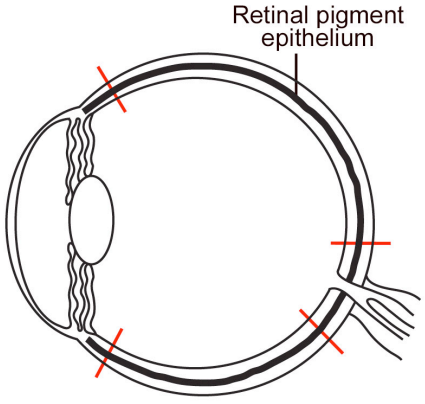

Fig.III.3. Preparation of the retinal pigment epithelium. Image of a rat eye, indicating the region of interest, the retinal pigment epithelium. The dissection of that region was focused on those areas that were distant enough from CB or optic nerve structures. The bars in the graph indicate the region where cells were harvested.

III.2.1.4. Adult derived stem cells from the subventricular zone and hippocampus

For subventricular (SVZ) and hippocampus (HC) cell preparations, adult female Fischer-344 rats (3–4 months old) were sedated, decapitated and brains were removed and put in 4°C cold DPBS with 4.5 gm/L glucose (DPBS/glu). Overlying meninges and blood vessels were removed. The subventricular zones from the lateral wall of the lateral ventricle and the entire hippocampi were aseptically removed (Fig. III.4.).

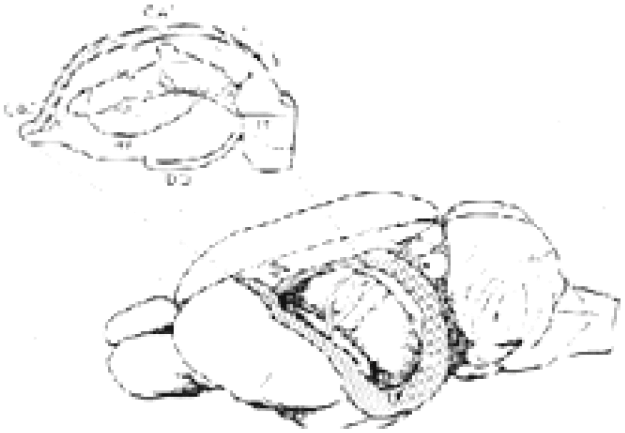

Fig.III.4. Preparation of the HC and SVZ. Image of a rat brain. The hippocampus is pictured as taken out. This is the fragment that is further processed in the preparation of hippocampal stem cells. After the hippocampus has been removed, access is granted for carefully scraping off the first few layers of the subventricular zone. The material is then processed as described in section III.2.1.1.

The dissected tissue was transferred to fresh DPBS/glucose, washed once, transferred to Petri dishes, and dissociated mechanically. The cell suspension was washed in DPBS/glu to rinse off excess blood and resuspended in PPD solution and processed as described above. Rat NSCs used in this study were derived from cultures passaged between 3 and 20 times.

III.2.1.5. Passaging of NSCs and retinal progenitor cells

For passaging of cells, the culture medium containing the floating neurospheres was collected in a 15 ml centrifuge tube and centrifuged at 188 rcf for 5 min. The pellet was resuspended in 200 μl of Accutase® and triturated 5 times. Additionally, the cell suspension was placed at 37°C for 10 min. After dissociation, the cells were again triturated, resuspended in 800μl NB/B27 medium and centrifuged at 188 rcf. After that, cells were resuspended in NB/B27 medium in final concentration (10^4 cells/ml medium). For cell counts, an aliquot was counted by trypan blue exclusion assay in a hemocytometer to determine the amount of viable cells. Cells obtained after the enzymatic treatment of primary neurospheres proliferated and produced secondary neurospheres. These were passaged 7 days after plating primary neurosphere cells.

Similar to primary cultures and neurospheres, single cells obtained after Accutase®-treatment of secondary neurospheres again proliferated and produced tertiary neurospheres. Rat retinal progenitor cells used in this study were derived from cultures passaged between 3 and 4 times. To visualize proliferation in immunocytochemical studies, the thymidin-analogue Bromodesoxyuridin (BrdU) was used. Retinal progenitor cells after passage #3 were supplemented with 10 μM BrdU in NB/B27 for 24 hrs prior to seeding on poly-L-ornithine/laminin coated glass coverslips. During the time of differentiation (7 days), medium was changed every 3 days, but no new BrdU was added.

III.2.1.6. Clonal assays

Single freshly isolated cells (P1, P3, CB, RPE and SVZ) or cells from already established cultures were transferred to 96-well plates either by limited dilution assay or FACS sorting. For limited dilution experiments, single cells were plated at a density of 0.5 cells/well of 96-well plates and for FACS sorting experiments, individual cells were sorted (1 cell/well) in 96-well plates in 200 μl of growth medium (FACStarPlus, Becton Dickinson, Dept. of Pathology, University of Regensburg). After the first two weeks, half of the medium was changed, then, the medium change was performed weekly. After 6 weeks of culture, each well was manually screened for colonies using phase contrast microscopy and only cells in wells that originally contained one single cell were referred to as clones. Individual clones were used to establish clonal NSC cell lines by dissociating the clonal neurosphere and replating the single cells under the same culture conditions. NSCs were grown for an additional 14 passages and then analyzed for their differentiation potential. CB and RPE cells only grew clonally under adherent conditions. Cells were transferred to 50μg/ml collagen I coated 96-well plates under limited dilution conditions (0.5cells/well) in 200μl NB/B27 medium supplemented with 1% FCS. After 7 days of culture, each well was manually screened for colonies using phase contrast microscopy. Clones were picked and expanded in collagen I coated 12-well plates in NB/B27 medium.

III.2.1.7. Differentiation of cells

In order to study the differentiation capacities of retinal progenitors, CB, RPE, SVZ and HC derived stem cells, spheroid cultures after passage #3, dissociated with

Accutase®, were grown as adherent single cells at a density of 10^5 cells/well on glass coverslips coated with 250 μg/ml poly-L-ornithine and 5 μg/ml laminin for additional 7 days. Differentiation-promoting conditions included withdrawal of growth factors and addition of 1% or 5% FCS or addition of a cocktail consisting of 2μM Forskolin, 100nM cAMP and 25μM KCl.

III.2.2. Immunocytochemistry

Immunocytochemistry was performed on fixed adherent cultures following fixation with phosphate-buffered, pre-warmed (37°C) 4% paraformaldehyde for 30 min at room temperature. After 3x10min wash steps in TBS, cultures were blocked in donkey serum blocking buffer of Fish skin gelatine buffer. If BrdU immunocytochemistry was performed, an HCl-incubation step prior to blocking the cultures was performed (2N HCl, 30 min at 37°C, followed by 3x10min wash steps in TBS). All primary antibodies used are displayed in Table II.1.1.3. Primary antibodies were diluted in blocking buffer and samples were incubated at room temperature for 2 hrs prior to 4°C incubation over night. In the case of A2B5, GalC ad NG2, where the antigen is sensitive to detergents, Triton X-100 was not applied.

After over night incubation, the cultures were washed in TBS (4x5min) and fluorochrome-conjugated secondary antibodies were diluted 1:1000 in blocking buffer and incubated at room temperature in the dark for 2 hrs. After the incubation of the secondary antibodies, cultures were washed 5x5 min in TBS in the dark. Nuclear stains for fluorescence microscopy were performed by using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) at a 1:5000 dilution in TBS. Nuclear stain for confocal microscopy was TO-PRO-3-iodide (TOPRO) at a 1:5000 dilution in TBS. After the final wash, samples were mounted on slides using Fluoromount-G.

Negative controls for antibodies were performed by applying only secondary antibodies to fixed cells, which yielded no signals. To exclude unspecific non-neuronal labeling in the case of β III tubulin antibody, it was tested for immune-reactivity in COS-7 cells, which are of non-neuronal origin, with a negative result (data not shown).

III.2.3. Quantitative analysis

For the quantification of stainings, numbers of BrdU+ or DAPI+ cells respectively, and other immunoreactive cells were determined by counting specifically stained cells in at least 10 random fields on the coverslip, using a fluorescent microscope (Leica DMR) with a 40x objective and CCD camera. Photomicrographs of subcellular structures like mitotic spindles were taken with a 100x objective. Analysis of co-labling and sections of neurospheres was furthermore conducted by using a confocal microscope (Leica DMRXE) with a 40x objective. The Mann-Whitney test for non-parametric comparison and Student's T-test were used for statistical analysis. Data is expressed as mean value +/- standard error of the mean (S.E.M.). Significant differences are expressed as p-values.

III.2.4. RT-PCR

To further analyze the progenitor state as well as the de-differentiation and trans-differentiation potential of the retinal neurospheres and adherent progenitor cell cultures, RT-PCR as performed for various precursor, stem cell, proneural and differentiation factors (Table III.1.3.). All primer sets were designed in a manner that sense and antisense primers recognized different exons. Total RNA of P1, 3, 8 and 14 retinal cells as well as adult SR, RPE and CB derived cells grown under proliferation and differentiation conditions described above and after passage #3 or 21 days in culture, respectively, was extracted using the RNeasy® Kit (Qiagen, Germany). cDNA was generated by RT-reaction using the RETROScript® kit (Ambion, USA). PCR conditions were 94°C, 2 min, follow by 94°C, 45 s; annealing time according to Table I, 1 min; then 72°C, 2 min, for a total of 35 cycles. All controls were carried out using embryonic-derived rat RNA and cDNA, respectively. All experiments were conducted in triplicate on three different cell preparations. Negative controls included RNA, but no reverse transcriptase, to ensure that the PCR product was not amplified from genomic DNA. PCR products were visualized on a 2% agarose gel against Roche marker VIII (range 19-1114bp, Roche, USA)

III.2.5. HPLC

HPLC was performed to detect potential dopamine secretion by RPE cells into the cell culture supernatant. Prior to the chromatographic separation, the catecholamines adrenalin, noradrenalin and dopamine are selectively adsorbed to aluminiumoxyde and therefore isolated from the solution. Here, the Agilent 1090 LCD detector (ESA, Coulochem II) with a 5μm column (Phonomenex, Luna C182) of 250mm length and with a diameter of 2mm was used. The compounds were detected in the eluant with an Agilent 1090 LCD detector (ESA, Coulochem II) using two Au/Hg electrodes in series with potentials of 400mV and 900mV for the upstream and downstream electrodes, respectively. A PM-80 solvent delivery system was used to recirculate the following mobile phase at 0.3 ml/min: 8% MeOH, 92% of (0.1M $NaH_2PO_4*H_2O$, 2.6mM 1-octane-sulfonate sodium, 0.1mM EDTA, $2.5*10^{-4}$ triethylamine; pH 3.35). All chemicals were of analytical quality.

III.2.6. Retroviral vector design and production

The retrovirus used here is a gift from Dr. Norbert Weinder and Maurice Vroemen, Department of Neurology, University of Regensburg, and is based on a cytomegalovirus/murine leukemia virus construct with a modified protein capsule. The pCLE vector containing a hybrid CMV/MLV 5'LTR is modified by the introduction of an EF1 enhancer/promotor as an internal transcriptional regulatory sequence. The coding sequence of the extracellular Notch1 domain is cloned into the multiple cloning site (total size 7.9 kb). As control vector served a pCLE construct with enhanced GFP (eGFP; size 6.5 kb), amplified from PCR and cloned into the multiple cloning site.

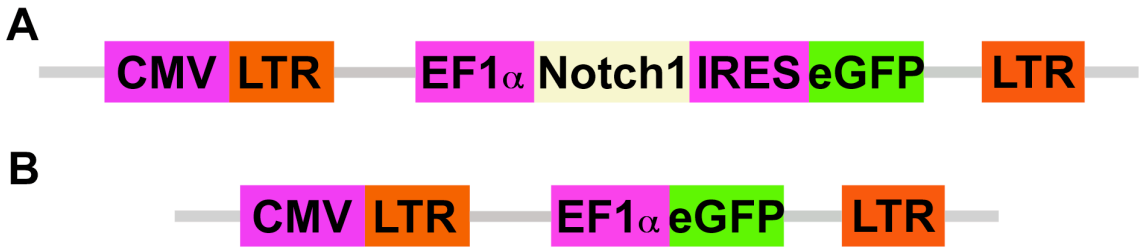

Fig.III.5. Vector maps of Notch1 and control construct. A) pCLE-Notch1. B) pCLE-GFP. CMV=cytomegalovirus; LTR=long terminal repeat; EF1=promoter for the contructs; followed by multiple cloning site with either Notch1 extracellular domain (A) or enhanced GFP (B); IRES=internal ribosome binding site.

Retrovirus containing cell culture supernatant was produced by sodium acetat/ethanol precipitation of plasmid DNA, utilizing the 293T producer cell line.
Ampho cells were plated at $3x10^6$ cells per T75 flask (volume=10ml) in IMEM medium supplemented with Pen/Strep, L-Glutamine and 10% FCS. 3 hrs prior to infection, the medium was replaced. For the sodium acetat/ethanol precipitation, a total of 30μg plasmid DNA (pDNA) was mixed with 0.1 volume of 3M NaCOOH and 2.5 volume 100% ethanol. The cup was gently turned several times and the DNA was pelleted for 10 min at 14,000 rpm. The pellet was washed once with 1 volume of 70% EtOH and dried at room temperature under a sterile cell culture hood. The DNA was then resolved in 675μl sterile water and mixed with 75μl 2.5M $CaCl_2$. Finally, 2x BES-buffered saline was added drop-wise and under constant agitation and the mix was incubated at room temperature for 20 min. 1.5ml of the solution was added per T75 flask. After an incubation period of 24 hrs, the medium was replaced by fresh NB/B27 medium supplemeted with Pen/Strep, L-Glutamine, FGF, EGF and Heparin. The retrovirus containing supernatant was harvested after another 24 hrs by filtering it through a 0.45μm syringe filter. The supernatants were used immediately or stored at –80°C.

III.2.7. Retroviral infection of SVZ and HC cells in vitro

For effective infections with retroviral supernatant, the cells were grown in NB/B27 medium supplemented Pen/Strep, L-Glut, FGF, EGF and Heparin and 1/3 of the total medium volume was exchanged with retrovirus containing supernatant. Cells were cultured, passaged and grown as described in section III.2.1.5. After 1 week in culture, the infected cells were sorted for GFP positive signals with a desktop FACS sorter (FACStarPlus, Becton Dickison, two argon laser at 488nmand 635nm; multiline-UV at 351-363nm, Dept. of Pathology, University of Regensburg).

The GFP positive cells were further cultured in NB/B27 medium for additional 2 weeks. For immunocytochemical stainings, cells were plated and grown as described in section III.2.1.7. All further analysis of immunodetection was conducted in the way described in section III.2.2.

III.2.8. Detection of the myc tag in retrovirally infected cells by Western Blot

To determine, if the gene construct containing the Notch1 fragment was successfully introduced and correctly translated in the infected stem cells, a Western Blot analysis for the myc tag associated with the Notch1 construct was performed.

III.2.8.1. Gel preparation

The SDS-PAGE was conducted with a gel containing 12.5% acrylamide (see Table II.1.2. for details). The glass plates for the gels were rinsed with 100% EtOH and assembled using silicone seals and 3 clips. The running gel was prepared freshly and poured between the glass plates. After the running gel had polymerized completely, the stocking gel was prepared and immediately poured on top of the other gel. The combs were inserted. After polymerization, the silicone seals were removed and the unit was mounted into the gel apparatus. The apparatus was then filled with 500ml running buffer (see II.1.2. for details) and special care was taken to remove all air bubbles at the bottom of the running gel. The combs were removed and the slots were washed with running buffer before the samples were applied.

III.2.8.2. Sample preparation

The cells were harvested and resuspended in lysis buffer (see table II.1.2. for details). The samples were then mixed with loading buffer and boiled for 5 min. Finally, the samples were loaded on top of the gel (15μl protein total). The standard used was the Sigma Caleidoscope standard at 10μl. The running conditions included a 10 min incubation at 10mA (Volt and Watt at maximum) and then a step at 25mA for 60-90 min.

III.2.8.3. Blotting procedure

Six pieces of Whatman paper and one piece of nitrocellulose membrane were cut per gel (for a minigel a size of 75mm x 100mm was sufficient). Three pieces of Whatman paper were soaked in transfer buffer and placed on the blotting surface. The gels were disassembled right after the run and the stacking gel was cut out with a scapel. The gel and the nitrocellulose membrane were placed carefully on the blotting surface, ensuring that no air bubbles were trapped underneath. The remaining three pieces of Whatman paper were soaked in transfer buffer as well and placed on top. All remaining air bubbles

were rolled out of the sandwich using a 10ml pipette. The surface around the blotting sandwich was dried with filter paper and the lid of the apparatus was connected. The power supply blotted at 10W and 250V (250mA per gel) for 30 min. After blotting, the gel was stained for 30-60 min in Coomassie staining solution (see table II.1.2. for details) and briefly de-stained in Coomassie differentiation solution (see table II.1.2. for details) before it was mounted in a drying apparatus.

The blot was stained for 2 min in 0.1% Ponceau solution. To differentiate, the blot was washed with distilled water and the bands of the standard were marked with a pencil. For final antibody reaction, the blot was washed in Fish skin gelatine buffer twice for 30 min. The incubation of the primary antibody (1:5000) lasted 1 hr at room temperature. After the incubation, the blot was washed in Fish skin gelatine buffer (1x15 min, 2x5 min). In the following step, the peroxidase conjugated secondary antibody was incubated (1:10,000) for 1 hr at room temperature. The final washing step lasted 3x15 min in TBST and finally, the blot was processed for chemoluminescent detection.

IV. Results

In this study, the different aspects of isolating, expanding and characterizing postnatal and adult derived stem and progenitor cells from the mammalian eye were analyzed. The results of several abalysis are presented in the following sections.

IV.1. Adult derived neural stem cells from the subventricular zone and hippocampus of the rat

Parts of this work are published in Wachs et al., 2003 [13]

With the discovery of adult neural stem cells from the CNS, a new chapter was opened for the strategic use of these cells in cell replacement strategies focusing on autologous transplantations. A key role is played by high expansion rates of these cells in culture in order to generate sufficient amounts of graftable cells. Therefore, a major focus is placed on tools and strategies to reach efficient growth *in vitro* and to further characterize the differentiation potential these cells harbor.

Furthermore, a retroviral infection study indicated that the phenotype of NSCs could be altered when the cells were infected to express the determination factor Notch1. Notch1 is a transmembrane receptor that is a key player during several aspects of development, including patterning and lateral inhibition. Together with its ligand Delta, the protein regulates cell fate choices and maintains cells in an undifferentiated state, while in late progenitors, signaling through Notch/Delta often induces the differentiation of cells along the glial lineage (reviewed in [103]).

IV.1.1. Optimization and standardization of cell culture conditions for NSCs from adult rats

NSCs from the adult CNS are currently investigated for their potential use in autologous cell replacement strategies. High expansion rates of NSCs in culture are crucial for the generation of a sufficient amount of cells needed for transplantation. In the present study, the efficient growth of adult NSCs in Neurobasal medium containing B27 supplement (NB/B27) under clonal and low-density conditions in absence of serum or conditioned medium is described. An expansion of up to 15 fold within one week was achieved in low-density NSC cultures derived from the lateral ventricle wall (SVZ), the

hippocampal formation (HC), and the spinal cord (SC) of adult rats. A 27% single cell cloning efficiency in NB/B27 medium further demonstrates its growth promoting ability. Multipotency and non-tumorgenicity of NSCs was retained despite the high rate of culture expansion. In addition, increased cell survival was obtained when Accutase™, instead of trypsin, was used for enzymatic dissociation of NSC cultures.

IV.1.1.1. Comparison of Accutase™ and trypsin treatment for neurosphere passaging

NSCs from the adult rat SVZ, HC and SC were used. The cells were maintained in suspension cultures in which they grew as neurospheres. Following a growth phase of typically 1 to 2 weeks for SVZ, HC and SC cells, neurospheres became too large to allow for proper nutrient supply in the central part of the cell mass and needed to be dissociated. Hence, the first parameter investigated in this study was the passaging process. Two different dissociation methods are commonly used, i) enzymatic treatment using trypsin or ii) mechanical sectioning of neurospheres, also referred to as "chopping". The latter one maintains cell-cell interactions and results in faster recovery and higher expansion rates [12]. However, this method is not appropriate when single cells are required. [12, 147-149]. Since dissociation of neurospheres using trypsin provokes massive cell death, Accutase™ was tested, a different enzyme preparation that is proposed to be less aggressive than trypsin (see www.innovativecelltech.com/accutase.html).

To compare the effects of the two different enzyme preparations, HC, SVZ and SC derived NSCs from passage number 10 were plated at low density (10 cells/μl) and allowed to grow for seven days (Fig.IV.1A). The resulting neurospheres were then dissociated into single cells using trypsin or Accutase™ and re-seeded. The single cell suspension was grown for additional four days. At the end of the experiment, the total number of neurospheres and cells was determined. One day after enzymatic treatment, a reduced cell number was observed in rodent cultures dissociated with trypsin as compared to Accutase™ treated or untreated cultures (Fig.IV.1B). As opposed to trypsinized cells, Accutase™ treated cultures readily formed neurospheres within 24 hrs (Fig.IV.1B). Compared to untreated control cultures, Accutase™ dissociation increased the number of neurospheres by three-fold by the end of the experiment (Fig.IV.1E). Starting with equal cell numbers (10^4 cells per 2ml), the initial cell survival after Accutase™ treatment was significantly higher (90 to 95%) as compared to trypsin (70 to

80%) (data not shown). This enhanced survival was observed in NSC cultures derived from the different CNS regions used. Within four days post-dissociation, a large proportion of the remaining cells in trypsinized rodent NSC cultures died (Fig.IV.1C). In contrast, dissociation using Accutase™ not only increased the initial number of viable cells, but also significantly improved the survival of cells. As a consequence, an increase in total cell and neurosphere number was measured in Accutase™ treated cultures (Fig.IV.1D). To exclude the possibility that the observed effects are not specific to the different enzyme compositions, a series of dissociation and passaging experiments was performed, using different enzyme concentrations and incubations times. The results consistently demonstrated that at lower concentrations, trypsin is insufficient to generate single cell suspensions, and at higher concentrations it results in low survival of cells and is insufficient re-growth of neurospheres (data not shown). In addition, other enzymes such as thermolysin, collagenase II or the enzyme composition PPD were insufficient to yield good cell dissociation and survival at the same time (data not shown).

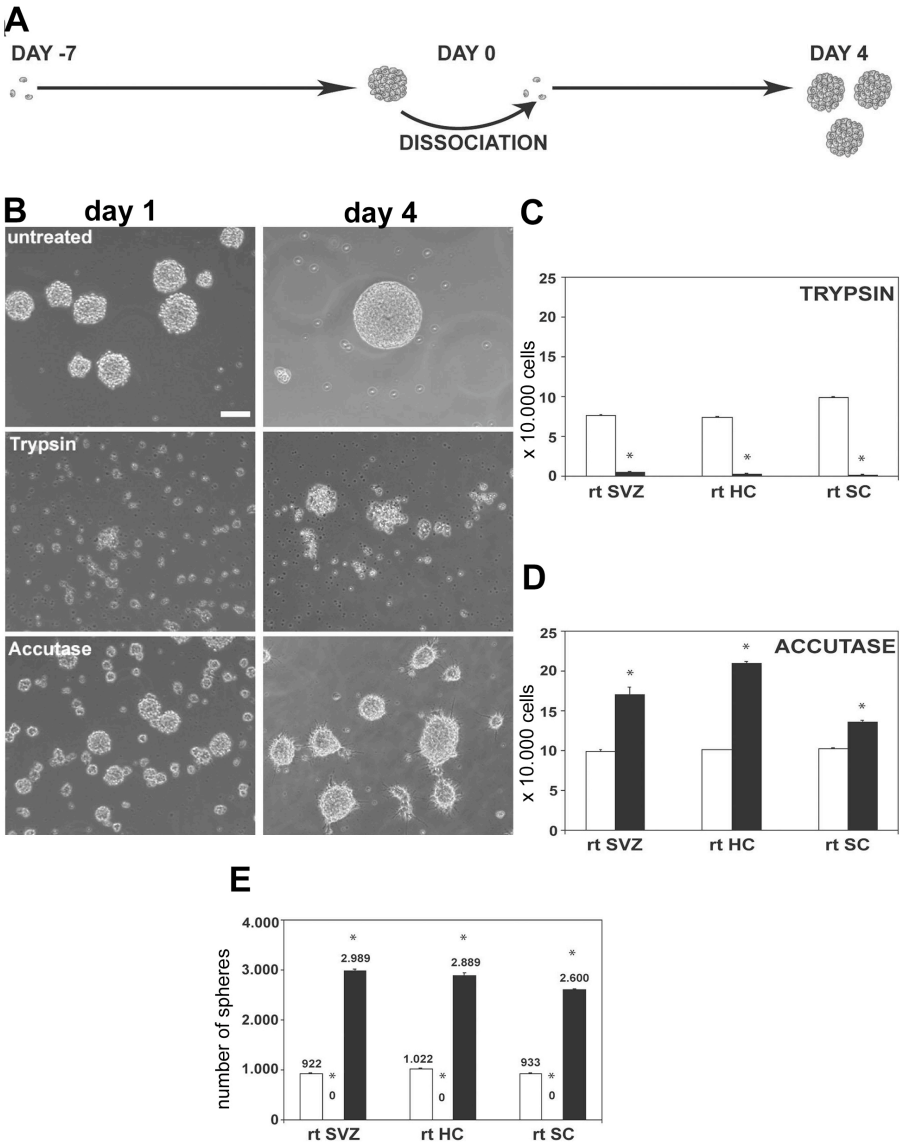

Fig.IV.1. Comparison of trypsin and Accutase® dissociation of NSC neurospheres. A) Schematic representation of the dissociation paradigm. 10^4 cells from adult rat NSC of passage 3-20 were grown for 7 days in 1ml medium. At day 7, the number of neurospheres was determined. Neurospheres were then dissociated as described in section III.2.1.5. and replated. After additional 4 days *in vitro*, the number of neurospheres was counted again. B) Phase contrast micrographs of untreated (top), trypsinized (middle) and Accutase® treated (bottom) rat SVZ neurosphere cultures on day 1 (left) and day 4 (right) after dissociation (scale bar=100µm). C) Quantification of the total number of viable cells of adult rat SVZ (rt SVZ), rt HC and spinal cord (rt SC) immediately after trypsinization (white bars) and 4 days after dissociation (black bars). D) Quantification of the total number of viable cells from SVZ, HC and SC immediately after Accutase® treatment (white bars) and 4 days after dissociation (black bars). E) Number of newly formed neurospheres on post-dissociation day 4 in untreated cultures (white bars), trypsinized cultures (n=0; gray bars) and Accutase®-treated cultures (black bars). Viable cells were determined by trypan blue exclusion assay. Data was generated using adult rat NSCs from passage number 10. Data expressed as average ± SD from three experiments performed in triplicate. Significance values according to Student's t test are * $p<0.003$.

IV.1.1.2. Effects of different culture media and supplements on NSC expansion and survival

Neurospheres from passage number 3 to 20 were dissociated and seeded as single cells in six different media/supplement combinations at low density (10 cells/μl) to assess the influence of culture medium compositions on adult rat NSC proliferation. After a growth cycle of seven days in the presence of FGF/EGF, the total cell number in the culture was quantified. Cell proliferation rates, obtained in Dulbecco's modified Eagles medium, HAMS F12 (DMEM/F12), the most prevalent NSC culture medium, versus rates obtained in Neurobasal (NB) medium, a medium previously described for the maintenance of differentiated neurons [150], were compared. These media were completed with three different supplements: B27, BIT9500 and N2 (see Table I). N2 was traditionally used for NSC cultures, whereas B27 was described originally for the maintenance of primary neurons in culture [43, 149]. BIT9500 was initially used for hematopoietic stem cell cultures [151] and recently applied on adult human NSCs [49].

Table I. Composition of B27-, N2-, and BIT9500 supplements

	B27 [150]	N2**	BIT9500***
BSA	+	+	+
Transferin	+	+	+
Insulin	+	+	+
Progesterone	+	+	-
Putrescine	+	+	-
Sodium selenite	+	+	-
Biotin	+	-	-
L-carnitine	+	-	-
Corticosterone	+	-	-
Ethanolamine	+	-	-
D(+)-galactose	+	-	-
Glutathione (reduced)	+	-	-
Lionelic acid	+	-	-
Linoleic acid	+	-	-
Retinyl acetate	+	-	-
Selenium	+	-	-
T3 (triodo-1-thyonine)	+	-	-
DL-α-tocopherol (vitamin E)	+	-	-
DL-α-tocopherol acetate	+	-	-
Catalase	+	-	-
Superoxide dismutase	+	-	-

** provided by manufacturer (GibcoBRL, Germany)
*** provided by manufacturer (StemCell Technologies Inc., Canada)

The different media/supplement combinations significantly influenced cell expansion rates. NSCs readily grew as neurospheres in NB/B27 medium (Fig.IV.2A, C) resulting in a high cell expansion rate of 10 to 15 fold within seven days (Fig.IV.2B). In media supplemented with BIT9500, cells were able to reform neurospheres, however, the total number of cells did not increase within seven days, whereas in the other media, the total number of cells decreased within the same period of time (Fig.IV.2A, B, C). As shown in figures 2B and C, the number of neurospheres corresponded to the total number of cells counted.

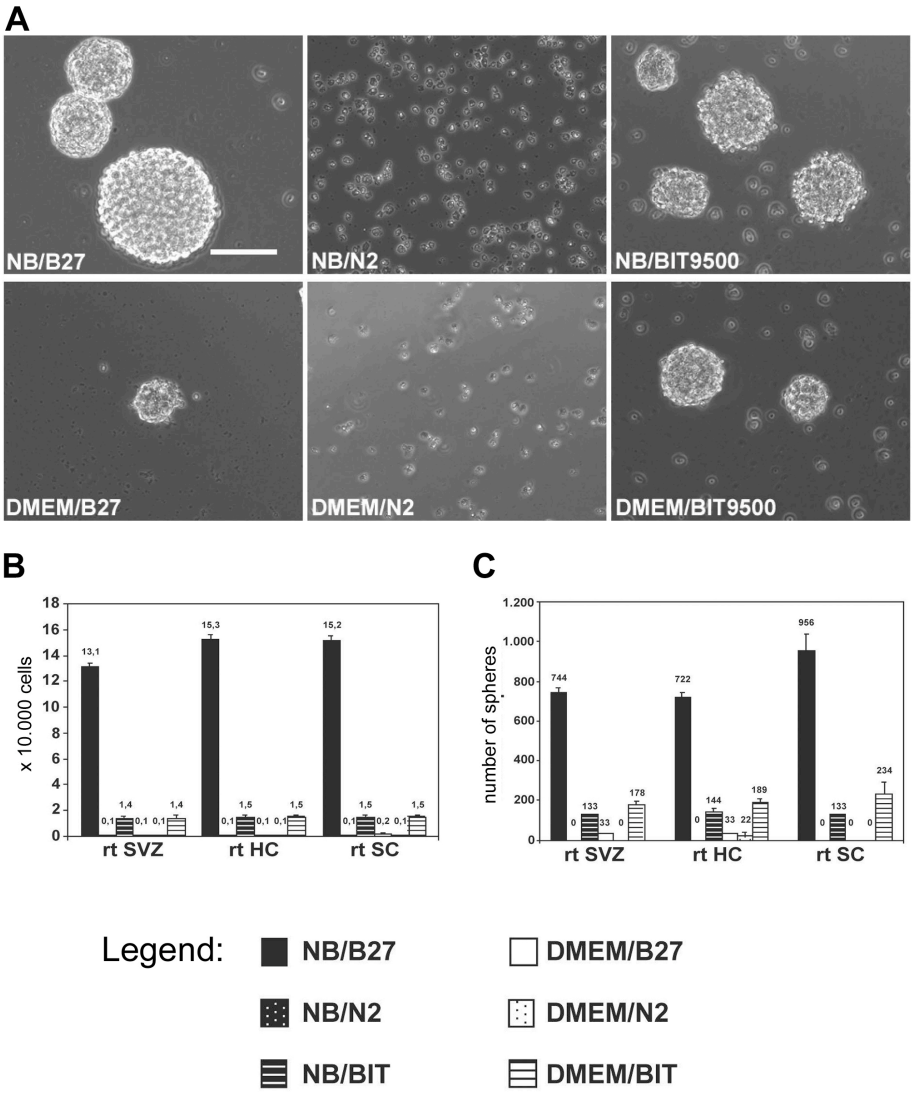

Fig.IV.2. Expansion of NSC cultures in different media/supplement combinations. Single cells were plated at a density of 10 cells/µl in 12 well plates and allowed to grow for 7 days in six different media/supplement combinations. A) Phase contrast micrographs of rat HC NSCs after 7 days of growth (scale bar=100µm). B) Total number of viable cells after 7 days of growth in

Neurobasal (NB) medium supplemented with B27 (black bars), N2 (n=0.1; black bars with dots) or BIT9500 (white bars with stripes) and in DMEM/F12 medium supplemented with B27 (n=0.1; white bars), N2 (n=0.1; white bars with dots or BIT9500 (white bars with stripes). C) Number of neurospheres present in the culture described in B). Data expressed as average ± SD from three experiments performed in triplicate.

IV.1.1.3. Comparison of different media for establishing primary neurospheres

The next set of experiments tested, whether the growth promoting effect of NB/B27 on already established neurosphere cultures was also applicable on freshly isolated NSCs. Following dissociation of adult rat HC and SVZ, the number of viable cells was estimated and seeded in NB/B27 or DMEM/F12/N2 at three different densities (10 cells/μl, 50 cells/μl and 100 cells/μl). Single cells began to form small neurospheres within 5 to 7 days of culture and grew in mass and number over the next several weeks. Highest yields of primary neurospheres were obtained in NB/B27. The higher efficacy of NB/B27 compared to DMEM/F12/N2 was observed at the three cell densities investigated.

Primary neurospheres were passaged using Accutase™ and further expanded in NB/B27. Both SVZ and HC derived NSCs yielded a 6.000 fold expansion within 21 days (Fig.IV.3.). In addition, the proliferation rate for both cell types remained constant for at least three weeks of culture.

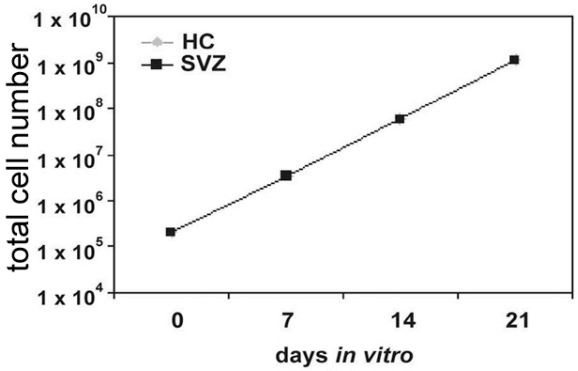

Fig.IV.3. Growth rates of different NCS populations. Growth rates of rat SVZ and HC cells in NB/B27 for 21 days in which the total number of cells at each passage has been calculated. Every 7 days, adult rat SVZ and HC neurospheres were dissociated and reseeded with a density of 20 cells/µl (200,000 cells per flask). Both cell types yielded similar results (data points overlaying).

IV.1.1.4. Comparison of different media and supplements for single cell cloning

The gold standard for the *in vitro* identification of NSCs is demonstration of multipotency of clonally derived cells [152]. Accutase™ dissociated rat SVZ NSCs derived from established passage number 4 cultures were placed in 96-well plates at a density of 0.5 cells/well by limited dilution. Comparison of the ability of six media/supplement-

combinations to promote neurosphere formation revealed significant differences. In agreement with the proliferation data on bulk cultures, B27-supplemented NB medium induced NSC proliferation under clonal conditions most effectively. After 6 weeks in culture, about 27% of the seeded cells produced new neurospheres (Fig.IV.4). Albeit to a lesser extent, media supplemented with BIT9500 were also able to support substantial formation of neurospheres from single cells (4-10%). FACS-sorting is a widely used method to isolate single cells. Therefore, the impact of FACS-sorting on rat SC and HC derived NSCs that were dissociated and sorted into 96-well plates at a density of 1 cell/well in NB/B27 was assessed. As with the limited dilution experiment, 25 to 30% of the plated single cells formed neurospheres (data not shown).

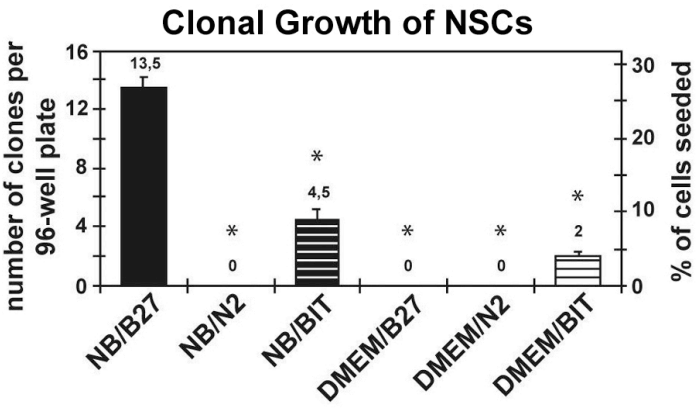

Fig.IV.4. Quantification of total number of clonally derived neurospheres of rat SVZ in different media 6 weeks after seeding. Adult SVZ neurospheres were dissociated and reseeded in the different media: NB/B27 (black bars), N2 (n=0; black dotted bars) or BIT9500 (black striped bars) and DMEM/F12 medium (DMEM) supplemented with B27 (n=0; white bars), N2 (n=0; white dotted bars) and BIT9500 (white striped bars) by limited dilution with a density of 0.5 cells/well. Data expressed as average ± SD from two experiments. Significant values according to Student's t test are * $p<0.0001$ related to cells grown in NB/B27.

IV.1.2. Immunocytochemical analysis of expression patterns for progenitor and adult markers in NSCs

The analysis of the expression of progenitor markers and markers for the three major cell classes of the CNS (neurons, astroglia and oligodendroglia) is a key procedure to determine the differentiation potential of stem cell cultures. All these aspects were part of the following evaluation.

IV.1.2.1. Detection of proliferation markers in NSC derived neurospheres

To determine the proliferation status of NSCs under proliferation conditions, neurospheres from SVZ suspension cultures were transferred onto glass coverslips coated with poly-L-ornithine and laminin and cultured in NB/B27 in the presence of EGF and FGF. Within a few hours of incubation on the poly-L-ornithine/laminin matrix,

neurospheres became adherent and cells started to migrate out and formed a surrounding monolayer (Fig.IV.5A). Two days post-plating, cells were fixed with 4% paraformaldehyde, and processed for Ki-67 immunocytochemistry. Immunodetection of the Ki-67 antigen, which is detected in the nucleus during the G1, S, G2 and M phases of the cell cycle, was used to assess proliferation in adherent cultures. As shown in Fig.IV.5B, Ki-67 could be detected, suggesting that cultures were still in a proliferative state. Fig.IV.5C shows a SVZ derived neurosphere on a coated glass cover slip after 24 h of growth. Prior to fixation, the cells were treated with 10μM BrdU (24 h) and then processed for immunocytochemistry. The outline of the spheroid body is already diffuse due to migration of the cells.

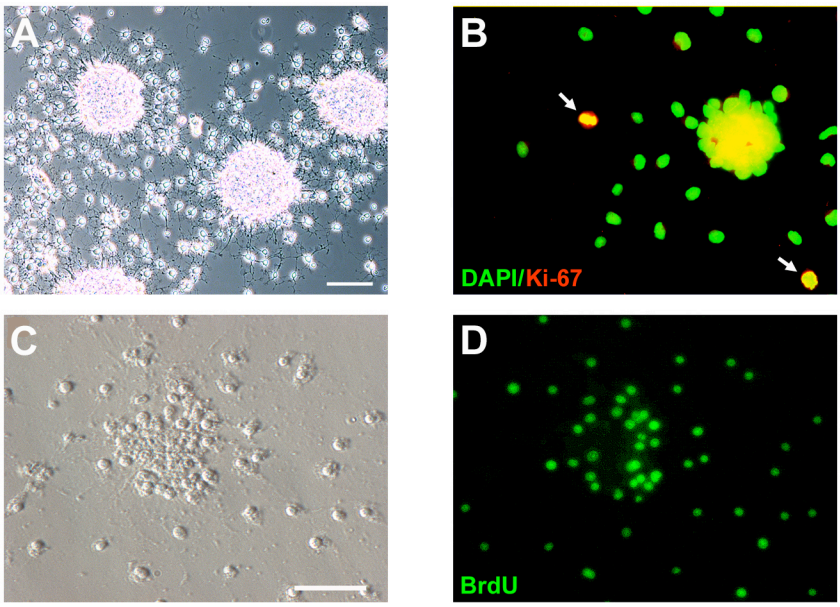

Fig.IV.5. Proliferation and growth in neurospheres *in vitro*. A) Phase contrast micrographs of rat SVZ neurospheres plated for 2 days. B) Immunodetection of the Ki-67 antigen (red, arrowheads) in rat SVZ cultures plated for 2 days on poly-L-ornithine/laminin coated glass coverslips. Nuclei were counterstained with DAPI. Doublelabeling for both is indicated by a yellow overlay. The neurosphere depicted in B is not from the same culture as neurospheres in A. C-D) Adult SVZ derived neurosphere from passage number 3 incubated with 10µM BrdU for 24 hrs and seeded overnight on poly-L-ornithine/laminin coated glass coverslips. BrdU+ in green. Scale bar=50µm

IV.1.2.2. Detection of the progenitor marker nestin in NSCs

SVZ and HC derived neurospheres were dissociated and plated on poly-L-ornithine/laminin coated glass coverslips for additional 7 days. Cells grew adherently and were processed for immunocytochemistry. A subpopulation of adult rat NSCs was

positive for nestin (Fig.IV.6), an intermediate filament found in uncommitted neural stem cells [133].

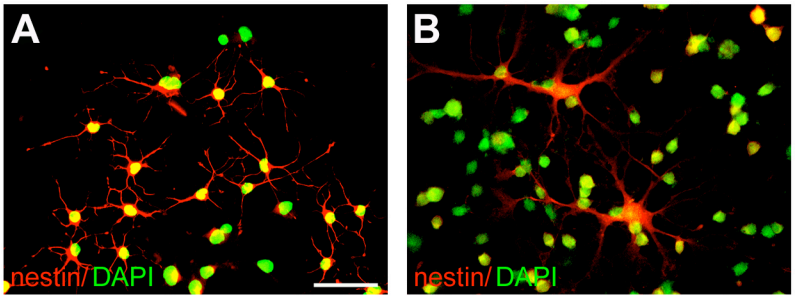

Fig.IV.6. Expression of the progenitor marker nestin. A) Immunodetection of nestin antigen in rat SVZ NSCs (red) after growth on poly-L-ornithine/laminin coated glass coverslips for 7 days after initial expansion in neurosphere cultures. Nuclei were counterstained using DAPI in green. B) Immunodetection of nestin antigen in rat HC NSCs (red). Scale bar=50µm

IV.1.2.3. Analysis of differentiation potential of SVZ and HC derived stem cells

To determine the differentiation status of NSCs under proliferation conditions, clonally derived rat neurospheres from passage 14 post-cloning were transferred onto glass-coverslips coated with poly-L-ornithine and laminin and cultured in NB/B27 in the presence of EGF and FGF. To detect neuronally committed cells, we used antibodies directed against III tubulin, an early neuron specific tubulin isotype [153]. In rodent NSC cultures, no III tubulin positive cells were detected under proliferative conditions. The detection of glial fibrillary acidic protein (GFAP), an intermediate filament found in astrocytes, was rare in rat NSCs. Finally, consistent with other reports [154], some rat cells under proliferative conditions were immunoreactive for galactocerebroside (GalC), a marker for mature oligodendroglial cells.

In the next set of experiments, clonally derived rat SVZ NSCs grown in NB/B27 were tested for their differentiation potential. NSCs were plated onto poly-L-ornithine/laminin coated glass-coverslips in NB/B27 without growth factors, but in the presence of 1% fetal calf serum (FCS), a standard paradigm used to induce differentiation in NSCs [40, 41, 47, 52]. Two different SVZ clones were analyzed by immunofluorescence staining for III tubulin, GFAP and GalC. All three markers were expressed in the SVZ cultures (Fig.IV.7A-C), indicating that the original clones were multipotent and cells grown in NB/B27 retained multipotency.

To test if the different media/supplement combinations used in the proliferation tests affected neuronal and glial differentiation, NSC bulk cultures derived from rat SVZ

and HC were differentiated as described above in the different media/supplement combinations. Cultures were analyzed by immunostaining for β III tubulin and GFAP. Quantitative analysis revealed that in SVZ cultures, the most potent medium for neuronal differentiation, as indicated by β III tubulin immunoreactivity, was NB/B27 (22.5±5%). In HC derived cultures, DMEM/B27 facilitated neuronal differentiation best (33±5%). Glial differentiation was best mediated by NB/BIT9500 media in SVZ cultures (15±5%), while in HC derived cultures, NB/N2 induced differentiation in 9±4% of the cells. In HC derived cultures grown in DMEM medium, a tendency towards neuronal versus glial differentiation was observed. In contrast, this effect was not observed in SVZ derived cultures. A similar amount of cells survived the differentiation condition in the different media/supplements (data not shown). Therefore, selective cell death of one or more cell types is not likely.

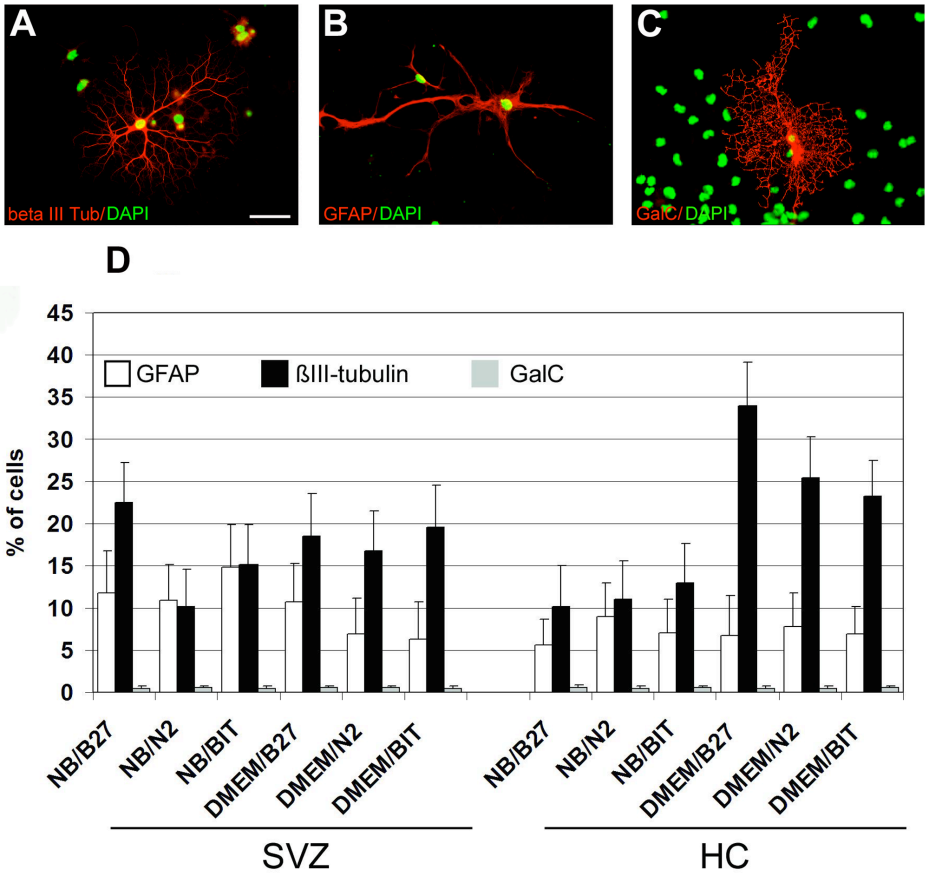

Fig.IV.7. Lineage potential of cloned rat NSC cultures. Individual clones derived from rat SVZ cultured on poly-L-ornithine/laminin matrix were differentiated in NB/B27 medium supplemented with 1% FCS for 7 days and immunostained for the presence of neurons with β III Tubulin (A), astrocytes with glial fibrillary acidic protein (GFAP) (B) or oligodendrocytes with galactocerebroside (GalC) (C). Cells were cloned at passage number 4 and passaged an

additional 14 times after cloning. D) Quantification of the number of GFAP-immunolabeled (*white bars*), β III tubulin-immunolabeled (*black bars*) and GalC-immunolabeled (*gray bars*) cells detected 7 days after plating in different media/supplement combinations onto poly-L-ornithine/laminin matrix in the absence of growth factors and the addition of 1% FCS. NSCs were able to generate neuronal and glial cells in all media/supplement combinations used. Data expressed as average ± SEM. Scale bar=40µm.

IV.1.3. Analysis of cell fate in modified NSC cultures constitutively expressing Notch1

The Notch1 and Delta proteins, functioning as receptor and ligand, play a key role in regulating cell fate choices by inhibiting the onset of differentiation of retinal progenitor cells (reviewed in [155]). Furthermore, it was shown that rather than simply inhibiting neuronal differentiation and maintaining a neuronal progenitor state, Notch1 signaling may promote the acquisition of a glial identity in various progenitor populations [104, 105, 156, 157]. Convincing data demonstrates that Notch1 signaling promotes glial cell types, which retain progenitor character. Notch1 can therefore maintain progenitor identity in cells that acquire glial characteristics. Cell types in this context are astroglial cells, radial glia and the retina-specific Müller glia (reviewed in [103, 156]).

In this study, a gene construct coding for a fusion protein including Notch1 and eGFP (see section III.2.6.) was introduced into NSCs to analyze, if the constitutive expression of Notch in adult NSCs can alter the expression pattern of these cells towards the glial lineage and if the neurogenic potential of these cells changes.

IV.1.3.1. Detection and sorting of NSCs constitutively expressing Notch1-eGFP

NSCs were isolated from adult rat SVZ and HC and expanded in culture as previously described. After passage number 3, the cells were infected with the pCLE-Notch1 or pCLE-eGFP control constructs (see section III.2.6.) and grown for an additional 1.5 weeks. After the green fluorescent signal could be detected (Fig.IV.8A-D for SVZ and E-H for HC), cells were subjected to FACS sorting.

The sorting of 1,5Mio cells (2x75ml flasks) yielded a total of 33.000 SVZ cells and 97.000 HC cells positive for Notch1 (Fig.IV.9A-D). Western Blot analysis showed the β-myc tag with Notch1 in both cell preparations (Fig.IV.9E). The sorted cells were kept in culture on poly-L-ornithine/laminin coated glass coverslips and under differentiation conditions (NB/B27 with 1% FCS and removal of growth factors). After differentiation for 14 days, the cultures were analyzed for expression of the early

neuronal marker β III tubulin, the mature neuronal markers neurofilament 200kDa (NF200kDa) and Map2ab, the glial progenitor marker NG2 and the mature glial marker GFAP.

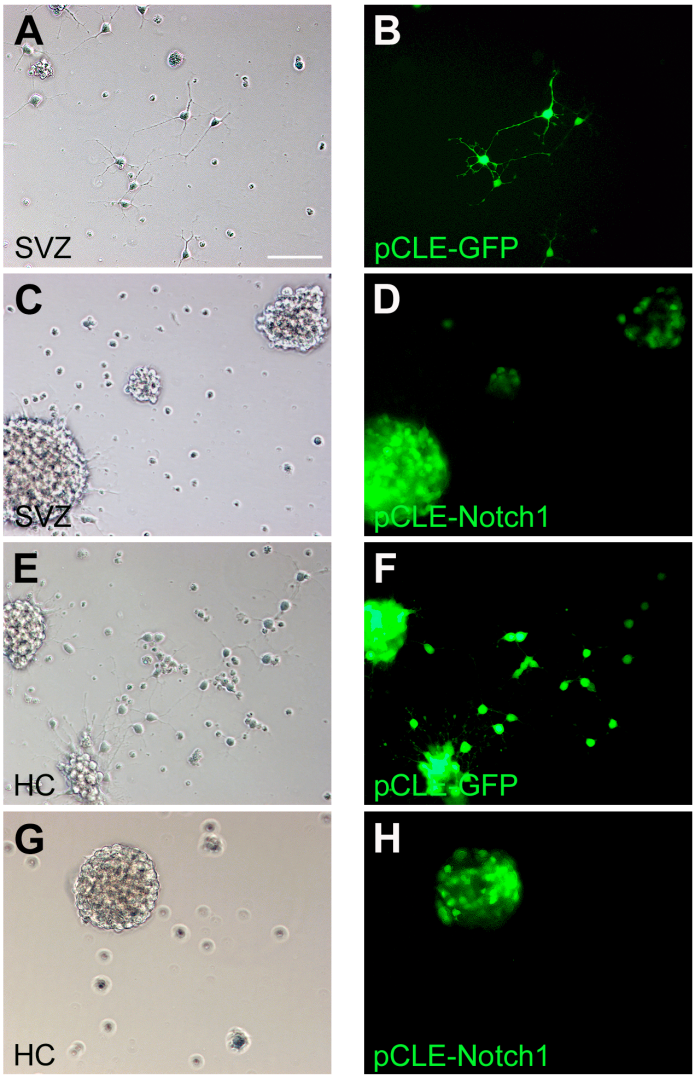

Fig.IV.8. Analysis of FACS sorted, infected NSCs. Cells were grown as neurosphere cultures for 14 days and then subjected to retroviral infection. A) Phase contrast micrograph of rat SVZ cells infected with the control vector pCLE-GFP and B) expressing GFP. C) Phase contrast micrograph of rat SVZ cells infected with pCLE-Notch1 and D) expressing GFP. E) Phase contrast micrograph of rat HC cells infected with the control vector pCLE-GFP and F) expressing GFP. G) Phase contrast micrograph of rat HC cells infected with pCLE-Notch1 and H) expressing GFP. Scale bar=100μm.

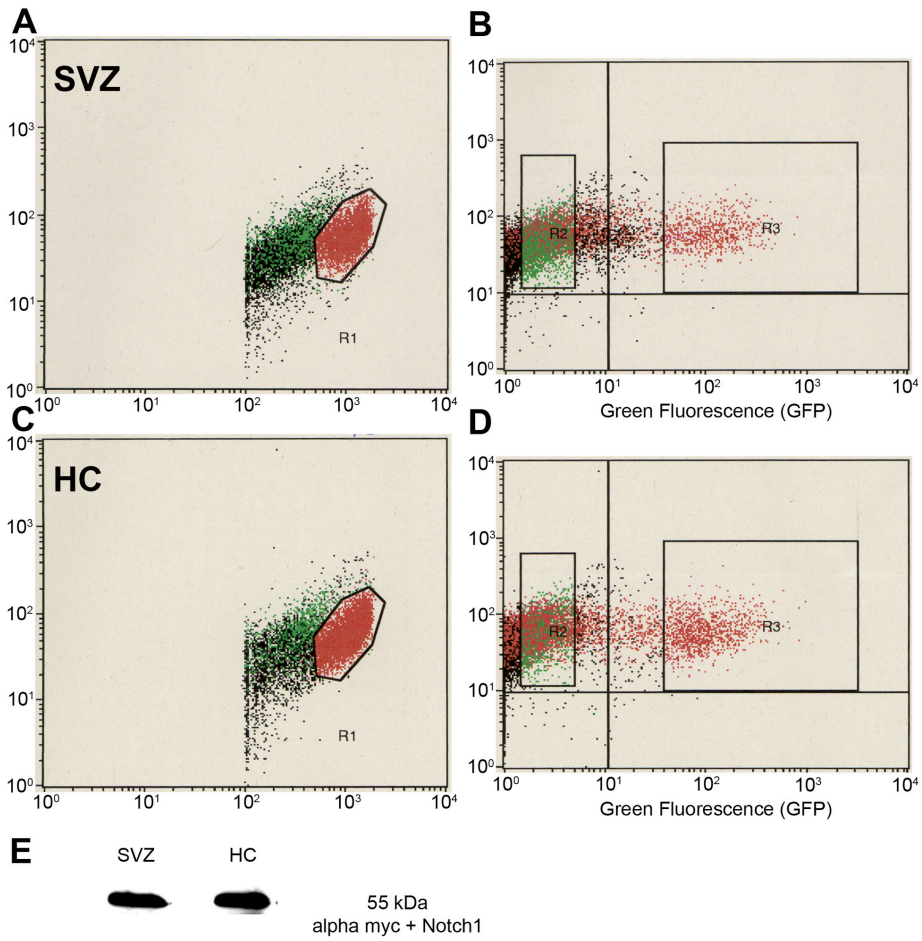

Fig.IV.9. FACS sorting. A-B) Spectrum of counted and sorted SVZ derived cells. C-D) Spectrum of counted and sorted HC derived cells. E) Western blot analysis revealed the -myc tag of the Notch1 construct in both SVZ and HC sorted cell cultures. Y-axes show total counts of events.

IV.1.3.2. Neuronal and glial differentiation of NSCs constitutively expressing Notch1

As illustrated in Fig.IV.10, the early neuronal marker III tubulin was detected in both control and infected cultures. Very defined neuronal morphology of the cells was apparent. The mature neuronal markers NF200kDa and Map2ab could not be detected in the Notch1-expressing cultures.

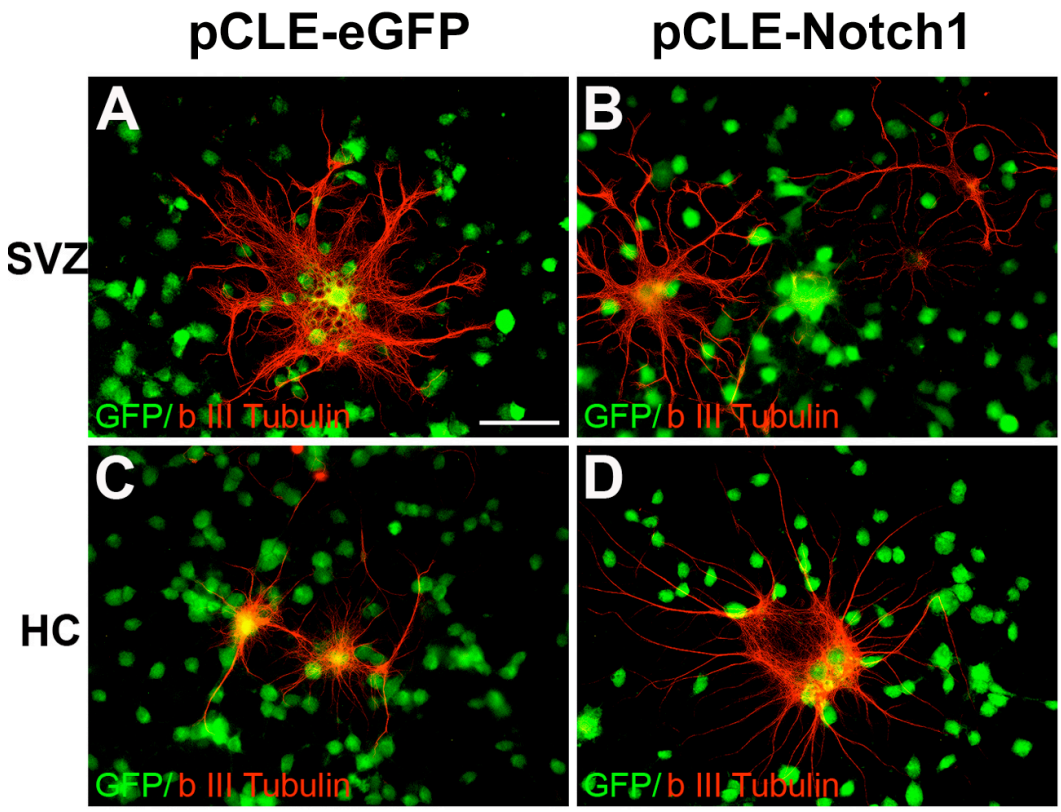

Fig.IV.10. Neuronal expression pattern in cells constitutively expressing Notch1 or control vector. A) adult rat SVZ NSCs immunostained for the early neuronal marker III tubulin (red). Cells have been cultured on poly-L-ornithine/laminin coated glass coverslips for 7 days after FACS sorting. The green signal results from the GFP expression of the control vector pCLE-GFP. B) adult rat SVZ NSCs immunostained for III tubulin (red) after pCLE-Notch1 infection. C) adult rat HC NSCs immunostained for III tubulin (red), also labeling for the control GFP signal after infection. D) adult rat HC NSCs immunostained for III tubulin (red) after pCLE-Notch1 infection. Neuronal differentiation is still observed after constitutive expression of Notch1, but as quantification revealed (see Fig.IV.12.), was downregulated. Scale bar=50µm.

Expression of NG2 could be observed as well (Fig.IV.11B,C,E,F). NG2 is an integral membrane chondroitin sulfate proteoglycan that can be found on the surface of early oligodendrocyte precursor cells and other, early glial cells [158]. Expression of GFAP did not show any other phenotype than the one observed in the regular NSC cultures (Fig.IV.11A+D).

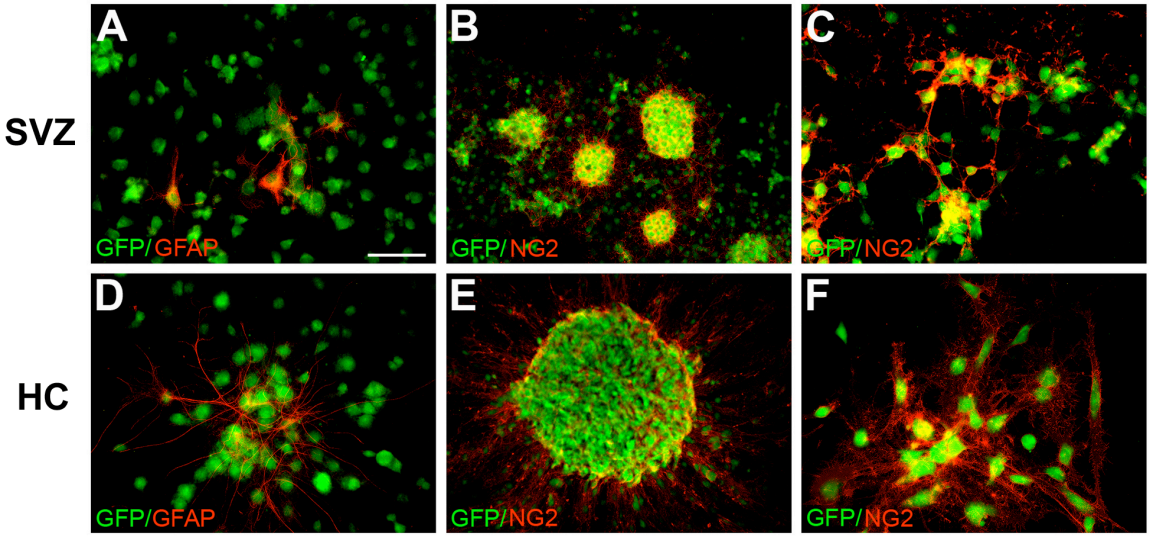

Fig.IV.11. Expression of glial markers in NSCs constitutively expressing Notch1. A) Immunodetection of the adult glial marker GFAP in rat SVZ NSCs (green GFP signal). B-C) Immunodetection of the glial progenitor marker NG2 in neurospheres (B) and dissociated rat SVZ NSCs (C) after 7 days on poly-L-ornithine/laminin coated glass coverslips. D) Immunodetection of GFAP in rat HC NSCs (green GFP signal). E-F) Immunodetection of NG2 in neurospheres (E) and dissociated rat HC NSCs (F). Scale bar=50μm.

Quantification revealed the expression profile of NSC cultures could be altered by the constitutive expression of Notch1 (Fig.IV.12.). The expression of Notch1 in SVZ cultures resulted in a significant decrease of both GFAP and □ III tubulin expression and only a slight trend towards more NG2 expression, which was not significant (Fig.IV.12A). The expression of GFAP and □ III tubulin in HC derived cultures was not affected by the introduction of Notch1 in these cultures. However, a significant upregulation of NG2 expression was observed after the constitutive expression of Notch1 (Fig.IV.12.B).

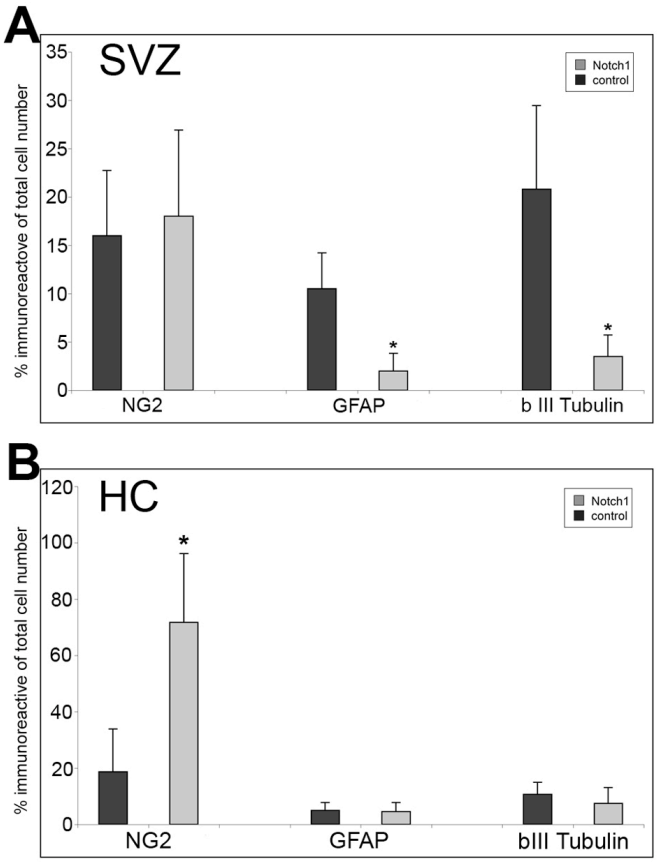

Fig.IV.12. Quantification of the effects of Notch1 expression in SVZ and HC derived adult stem cell cultures. A) The graph indicates the number of immunoreactive cells per total cell number, showing that the expression of GFAP and III tubulin was significantly reduced after the introduction of Notch1 expression. B) In HC derived cultures, the number of NG2 positive cells significantly increased with the introduction of Notch1, while the expression of the other markers was not effected. Data expressed as mean values ± SEM; Significance values according to Student's t test are * $p<0.005$ related to cells expressing the control construct.

IV.2. Postnatal derived progenitor cells from the sensory retina of the rat eye

Parts of this work are published in Engelhardt et al., 2004 [159]

The results and observations of the initial study on the optimization of cell culture conditions for the known NSC populations (HC, SVZ and SC) were applied to retina derived cultures. The study indicated that in terms of growth and clonal expansion, retinal derived progenitor cells have different attributes than the NSCs of the CNS and are somewhat limited in their potential to self-renew and proliferate. On the other hand, they share distinct properties with NSC populations, which are illustrated in this chapter.

In the case of retinal progenitor cells, it has long been known that they reside in the embryonic mammalian eye and recent results also suggested such a cell population in

the adult eye [67, 68]. The analysis of the postnatal stage during development was conducted to bridge the gap of knowledge in progenitor cell biology in this field.

IV.2.1. Isolation, proliferation and generation of retinal neurospheres

In retinal cultures derived from the early postnatal period (postnatal day 1 (P1) and P3), cell number initially increased during the first two weeks three-fold (Fig.IV.13). Then, cell proliferation ceased and the number of cells reached a plateau, suggesting that the generated cells survived in culture. The increase in cell number was delayed in P8 and P14 derived retinal cultures and reached a plateau phase at 1,5 fold of their initial cell-density after three weeks *in vitro*. This plateau remained stable in the culture for one more week, indicating cell survival.

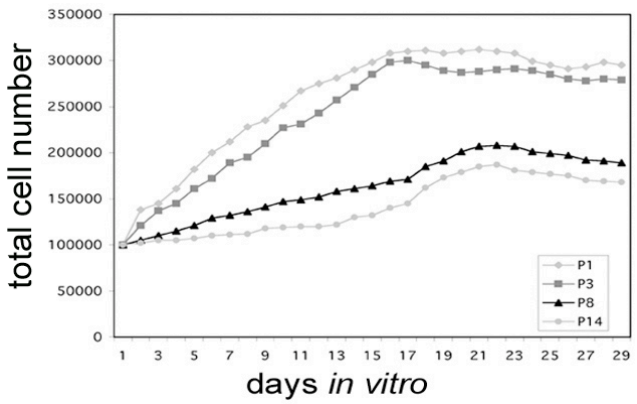

Fig.IV.13. Growth rates of postnatal derived sensory retinal progenitor cells over a period of 28 days. Cells were seeded at 10^5 cells per well in 12 well plates in NB/B27 medium and counted daily via trypan exclusion assay in a hematocytometer.

Postnatal derived retinal progenitors (P1, passage number 3) grew as neurospheres approximately 7-9 days after isolation. The neurospheres ceased to grow in diameter after another two days. Migrating cells extended long processes, which kept the neurospheres adherent to the poly-L-ornithine/laminin substrate (Fig.IV.14A). Cells within these neurospheres proliferated as indicated by BrdU staining (Fig.IV.14B). Dissociated and passaged postnatal cells readily reformed neurospheres with similar growth kinetics compared to primary neurospheres. When retinal tissue older than P14 or adult was used, no neurospheres formed, unless the region of the CB or the pars plana was included in the preparation. To further analyze the potential for clonal growth, NSCs (HC) and retinal progenitors (P1 and P3) were compared. Single cells derived from primary tissue or from already established cultures were seeded into 96 well plates by

limited dilution or by FACS sorting. In contrast to NSCs from the adult hippocampus that showed clonal growth in 27% of the cells (see section IV.1.1.4.), none of the postnatal retina derived cells grew as neurospheres under clonal conditions (data not shown). This suggests that initial cell-cell contact might be essential for cell proliferation.

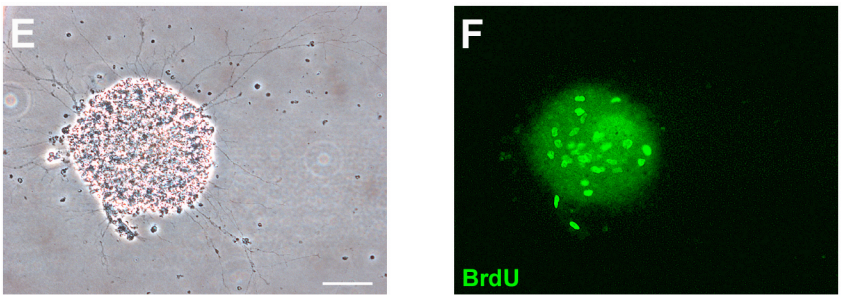

Fig.IV.14. Proliferation and growth in neurospheres *in vitro*. Postnatal day 1 (P1) derived retinal neurosphere from passage number 3 incubated with 10µM BrdU for 24 hrs and seeded overnight on poly-L-ornithine/laminin coated glass coverslips. BrdU incorporation is indicated by green labeling. Scale bar=100µm.

IV.2.2. Expression of progenitor markers in postnatal retinal cells

Retinal progenitors are characterized by the expression of nestin and additional specific markers, such as the tyrosine-kinase receptor Flk-1 and the homeobox proteins Chx10 and Pax6 [133, 139, 160, 161]. Retinal cultures derived from different postnatal stages were tested for Flk-1 and Chx10 expression. Since radial glia cells have been shown to function as NSCs during development, expression analysis for the radial glia marker basic lipid binding protein (BLBP) was included [28]. Postnatal retinal neurospheres were grown under proliferation conditions in the presence of BrdU for 24 hrs, transferred on poly-L-ornithine/laminin-coated glass coverslips and grown for additional 7 days. As shown by immunostainings, subpopulations of cells expressed the progenitor markers nestin, Flk-1, Chx10 and BLBP (Fig.IV.15).

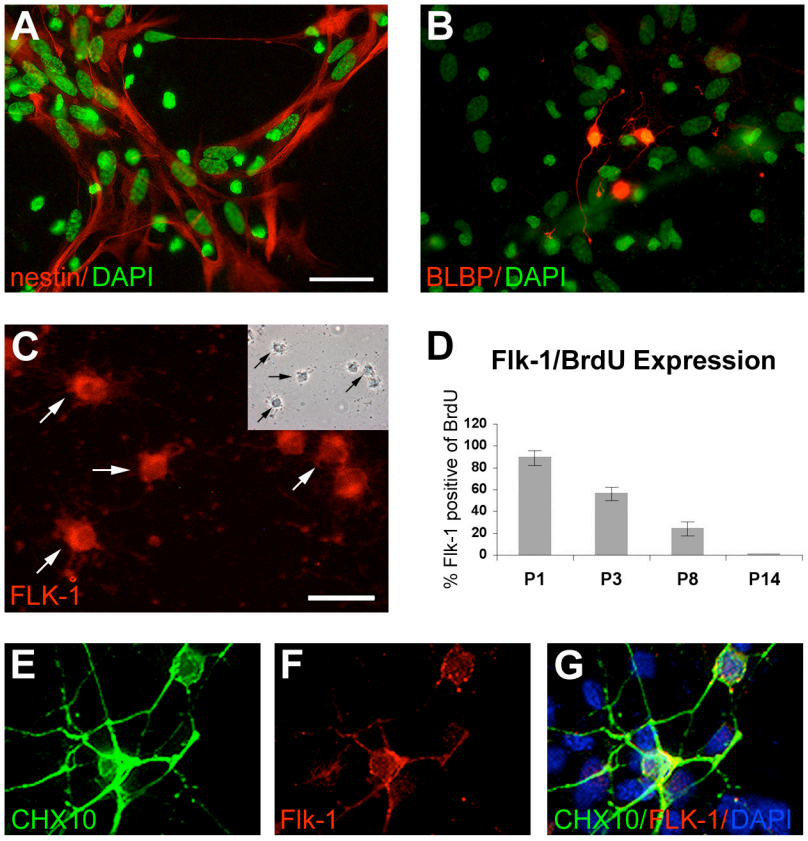

Fig.IV.15. Postnatal retinal cells express progenitor and stem cell markers *in vitro*. P1 derived retinal progenitor cultures of passage number 3, grown for additional 7 days on poly-L-ornithine/laminin coated glass coverslips in proliferation medium, are shown. A) Immunostaining for the nestin antigen (red). B) Immunostaining for the BLBP antigen (red). C) Immunostaining for the Flk-1 antigen (red). Insert shows phase contrast micrograph of the same cells. D) Quantification of BrdU and Flk-1 doublestaining at P1, P3, P8 and P14 shows a significant decrease in Flk-1 expression with dependence on age. E-G) Doublestaining for Chx10 (in green) and Flk-1 (red) and overlay with nuclear counterstain DAPI (blue). Scale bar=50µm.

In addition, an age-dependent decrease in Flk-1 expression was detected by immunofluorescence stainings and quantified (Fig.IV.15D). Flk-1 expression in cells that had incorporated BrdU *in vitro* was readily detected in P1 cultures (89±5% of cells co-labeled for Flk-1 and BrdU) and lower in P3 cultures (56±11%). P8 cultures showed yet another significant reduction in number of double labeled cells (24±8%), and in P14 cells, no Flk-1/BrdU doublelabeling was detected. Moreover, colabeling of Chx 10 and Flk-1 in P1 derived progenitor cells was detected under proliferation conditions (Fig.IV.15E-G). RT-PCR analysis showed a significant reduction in mRNA expression of the progenitor marker Flk-1, with the highest level of expression at P1 and no detectable expression at P8 and P14 (Fig.IV.16.). The proneuronal element Pax6 was detectable in RT-PCR at all stages, but with a slight decrease in older cultures.

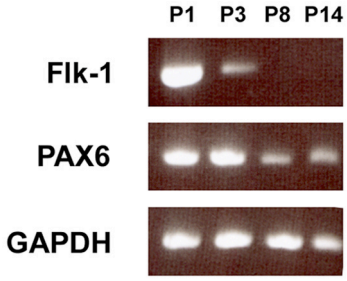

Fig.IV.16. RT-PCR for Flk-1 and Pax6 expression in sensory retina (SR) cells derived from P1, P3, P8 and P14 rat. Products are 537bp for Flk-1, 497bp for Pax6 and GAPDH was used as standard. The analysis revealed that the specific retinal progenitor marker Flk-1 was downregulated at P3 and not detectable at P8 and P14. Pax6 remained expressed at all stages, although a decreasing tendency in its expression was detected.

IV.2.3. Differentiation potential of postnatal retinal progenitors

Neural stem cells are characterized by their potential to proliferate, to self-renew and to differentiate into the three cell classes of the CNS: neurons, astroglia and oligodendroglia. Postnatal derived retinal progenitor cells (P1, P3, P8 and 14) were tested for these characteristics.

IV.2.3.1. Expression of early and late neuronal and glial markers in postnatal retinal cells

P1 derived retinal cultures were analyzed for the expression of neuronal and glial markers. The cells were derived from passage number 3 and grown on poly-L-ornithine/laminin coated glass coverslips under differentiation conditions (NB/B27, 1%FCS, no growth factors). Approximately 9±2% of cells were found to be immunoreactive for DCX, a marker migrating neuroblasts [162, 163] (Fig.IV.17A-C). In addition, β III tubulin was expressed in up to 11±2% of cells, showing a distinct, neuronal morphology indicated by long processes (Fig.IV.17D-F). A marker for adult neurons is neuron specific enolase (NSE) [164], which was expressed by 12±2% of the cells (Fig.IV.17G-I). A similar expression pattern was detectable for young glial markers such as A2B5 [165], which was expressed in about 6±1% of all cells (Fig.IV.17J-L) and NG2 [158], which was expressed in 7±1% of all cells (Fig.IV.17M-O). Adult glial phenotypes were detected by GFAP stainings in about 21±3% of all cells (Fig.IV.17P-R).

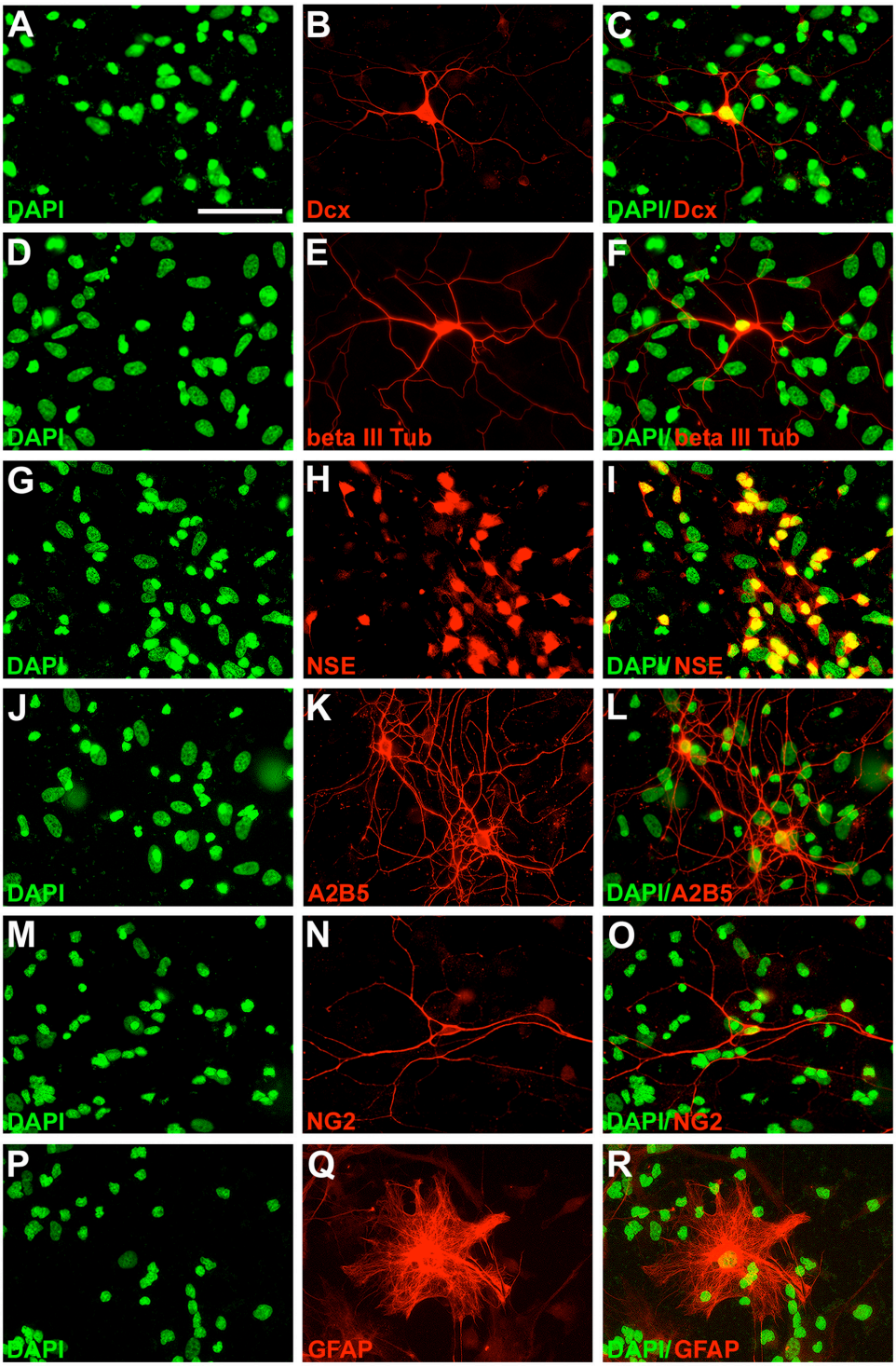

Fig.IV.17. Neuronal and glial phenotypes of postnatal retinal cell cultures under differentiation conditions. Cells from P1 derived retinal progenitor cultures of passage number 3, grown for 7 days on poly-L-ornithine/laminin coated glass coverslips, are shown. A-C) Immunodetection of the neuronal precursor marker protein DCX (red). D-F) Immunodetection of the III tubulin antigen (red). G-I) Immunodetection of the neuronal marker neuron specific enolase (NSE) antigen (red). J-L) Immunodetection of the glial progenitor marker A2B5 antigen (red). M-O) Immunodetection of the glial precursor marker NG2 antigen (red). P-R) Immunodetection of the GFAP antigen (red). Nuclear counterstain is DAPI (in green). Scale bar=50μm

Using β III tubulin as a marker for young post-mitotic neurons and GFAP as a glial marker, postnatal retinal cultures and adult SVZ derived NSCs have similar differentiation properties, even though postnatal retinal progenitor cultures show a higher percentage of a more glial defined phenotype (Fig.IV.18.).

Postnatal retina derived cells expressed high levels of DCX (8%; Fig.IV.18.), a transient marker for neuronal precursor cells. In contrast, only a small fraction of cells derived from the adult SVZ expressed DCX (less than 1%). Low expression of DCX in adult SVZ derived NSCs could either relate to the more transient expression of DCX in comparison with β III tubulin, or to neuronal subpopulations with different expression levels of the two markers. The analysis for expression of the oligodendrocyte specific marker GalC revealed that postnatal retinal progenitors lack the potential to differentiate into oligodendrocytes (Fig.IV.18.). In contrast, oligodendrocytic differentiation was observed in SVZ derived stem cells (1% of total cells number, Fig.IV.18.).

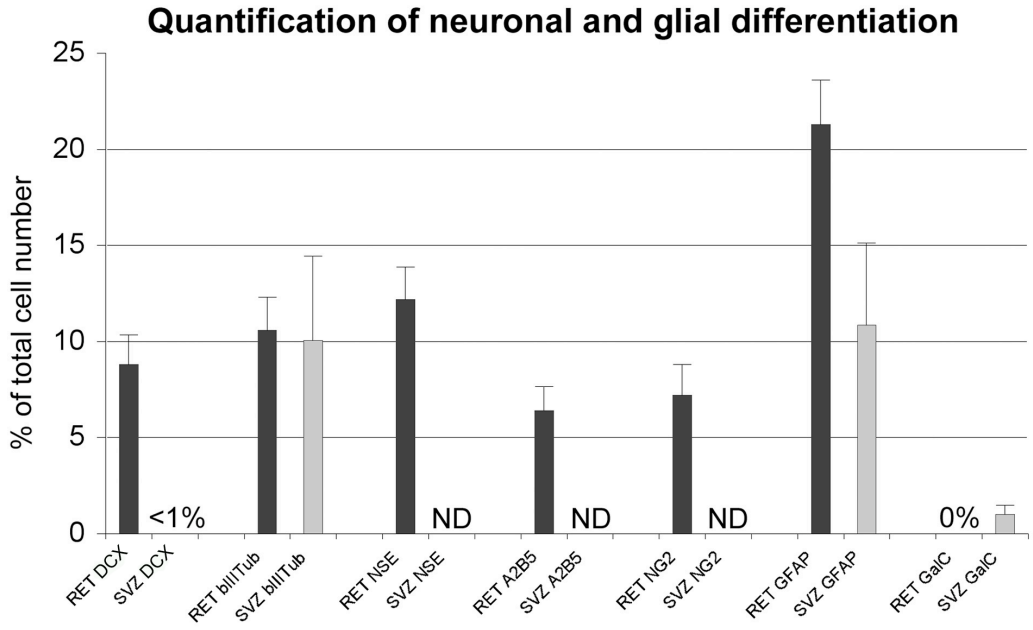

Fig.IV.18. Quantification of neuronal and glial differentiation of postnatal SR and adult SVZ cells. P1 derived retinal progenitor cultures of passage number 3 (RET) and adult SVZ derived neural stem cells (SVZ) were grown for 7 days on poly-L-ornithine/laminin coated glass coverslips under differentiation conditions, fixed, immunostained and quantified for the following markers: neuronal precursor marker doublecortin (DCX), young neuronal marker β III tubulin, neuronal marker neuron specific enolase (NSE), glial progenitor marker A2B5, glial precursor marker NG2, mature glial marker GFAP and oligodendrocyte marker GalC. ND=not determined. Experiments were performed in triplicate. Data expressed as mean value ± SEM.

IV.2.3.2. Generation of new neurons and glia from postnatal retina in vitro

To test the potential of postnatal retinal progenitors to generate new neurons and glia *in vitro*, retinal neurospheres were grown in the presence of BrdU for 24 hrs, plated on poly-L-ornithine/laminin coated glass coverslips and then grown for additional 7 days under proliferation and differentiation conditions. In cultures derived from P1 and P3 retinae and maintained under proliferation conditions (NB/B27 medium, growth factors), a small percentage of BrdU positive cells expressed the neuronal marker β III tubulin and the glial marker GFAP, indicating that these particular neurons and glial cells were newly generated (Fig.IV.19G, H). BrdU positive cells derived from P8 and P14 were not β III tubulin positive, but showed colabeling of BrdU and GFAP (Fig.IV.19G, H). Under differentiation conditions (NB/B27 medium, 1%FCS, no growth factors), the morphology of retinal neurospheres changed within two to three days. Cells began to migrate out of the spheroid bodies and flattened. Several cells elaborated processes contacting other cells, while retinal neurospheres grown under proliferation conditions maintained their spheroid morphology for a longer period of time. Some cells derived from P1 or P3 cultures that incorporated BrdU, expressed β III tubulin revealing that a population of precursors is proliferating *in vitro* to generate new neurons (Fig.IV.19D-F, H). Cells derived from P8 and P14 cultures were devoid of BrdU/β III tubulin double labeling (Fig.IV.19H).

Cells co-labeled for BrdU and GFAP could be observed in cultures derived from all ages (Fig.IV.19A-C, G). GFAP was mainly expressed by cells with epitheloid morphology and elongated processes. The proportion of BrdU positive cells expressing GFAP was larger than that of cells expressing BrdU and β III tubulin (Fig.IV.19G, H). In addition to the expression of β III tubulin and GFAP, P3 derived BrdU+ retinal progenitor cells also expressed markers for more mature neurons such as Map2 and NF200kD (data not shown). The oligodendrocyte specific marker GalC however remained undetectable in the postnatal cultures. The quantitative analysis revealed that the capacity to generate new neurons is significantly reduced with increasing age of the cultures (Fig.IV.19G, H).

While in P1 and P3 cultures 25±4% of the BrdU+ cells express GFAP under both proliferation and differentiation conditions, the number is significantly reduced to 4±3% at P8 and P14 (Fig.IV.19G, H). The ability to generate new neurons, described by

double-stainings for BrdU and β III tubulin, is even more reduced. While under both conditions in P1 and P3 cultures 7.5±5% of cells were newly generated neurons, no such cells were detectable in P8 and P14 cultures (Fig.IV.19G, H).

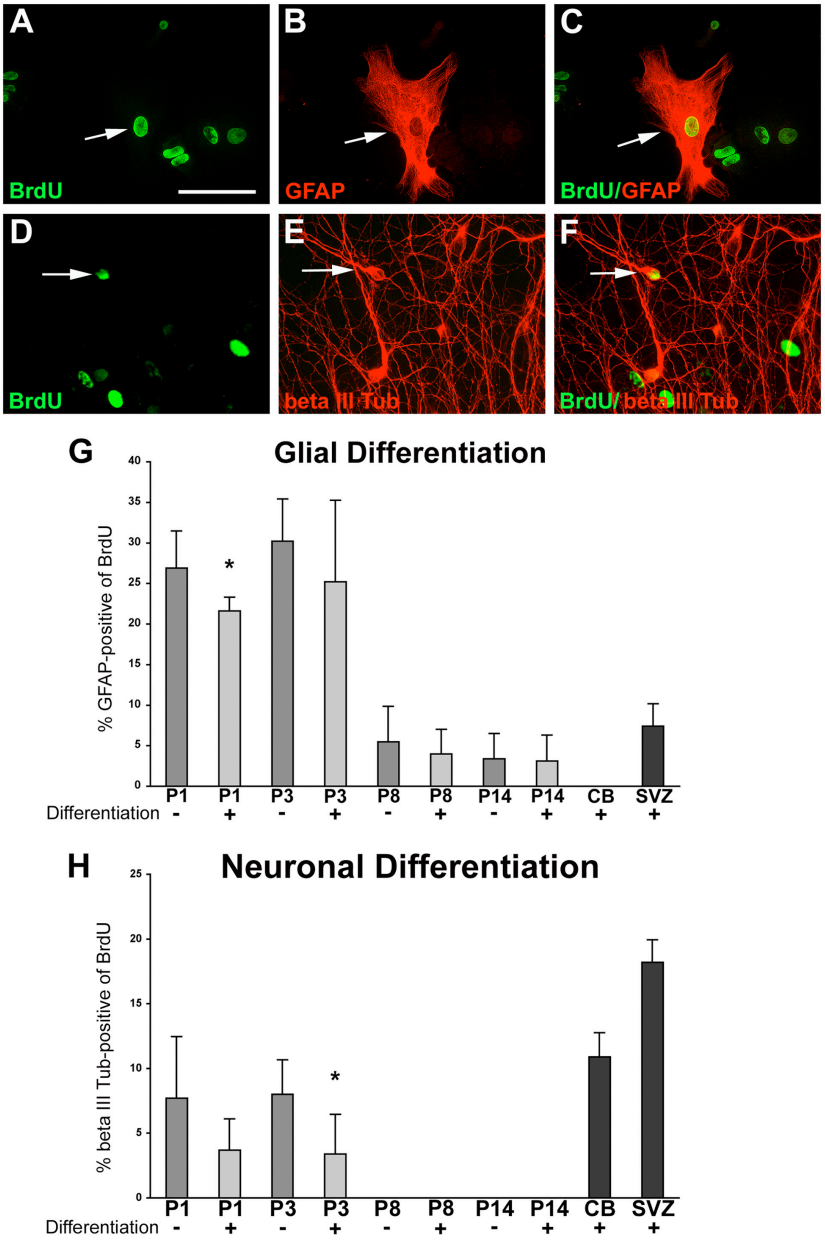

Fig.IV.19. Neuronal and glial phenotypes in retinal progenitor cells, CB derived stem cells and SVZ derived NSCs *in vitro* under proliferation and differentiation conditions. A-C) Doublelabeling for GFAP (red) and BrdU in green in P1 derived retinal progenitor cultures of passage number 3. Cells were labeled with BrdU under proliferation conditions for 24 hrs and subsequently plated for 7 days on poly-L-ornithine/laminin coated glass coverslips under differentiation conditions. Doublelabeled cell indicates newborn glia. Arrow points to cell body. D-F) Doublelabeling for β III tubulin (red) and BrdU in P1 derived retinal progenitor cultures. Doublelabeled cell indicates newborn neuron. Arrow points to cell body. G) Quantification of glial and H) neuronal differentiation of postnatal (P1-P14) derived retinal progenitor cells of passage number 3, in comparison to adult derived CB progenitor cells and SVZ derived NSCs,

proliferation: -; differentiation: +. Expressed are GFAP and β III tubulin positive cells as percentage of total BrdU-positive cells. Experiments were carried out in triplicates. Data are expressed as mean value ± SEM, * indicates significance (p<0,005) of difference between differentiation and proliferation. Scale bar=50µm.

In contrast to cells derived from P8 and P14 postnatal retinae, adult CB and SVZ derived cells, which incorporated BrdU, expressed β III tubulin upon differentiation (Fig.IV.19H). The highest number of BrdU/β III tubulin positive cells was observed in adult SVZ derived cultures (Fig.IV.19H). These cultures also contained cells that differentiated into GalC expressing oligodendrocytes, which was not observed in cells from the adult CB.

Similar results for the generation of new neurons and glia *in vitro* in postnatal retinal cultures were observed with the cell cycle marker Ki-67. P1 and P3 derived postnatal retinal progenitor cells co-labeled for Ki-67 and GFAP or Ki-67 and β III tubulin under proliferation and differentiation conditions. No co-staining for the cell cycle marker and β III tubulin in P8 or P14 cultures could be observed.

IV.2.3.3. Analysis of neurogenic competence and stem cell character in postnatal retinal cells

It has been shown previously, that specific culture conditions can influence not only the ability to differentiate, but also the lineage along a progenitor cell will differentiate. Therefore, three differentiation conditions were tested: i) NB/B27 Medium with 1% FCS and growth factor removal (control), ii) NB/B27 Medium with 5% FCS and growth factor removal (FCS) and iii) NB/B27 Medium with 1% FCS and a cocktail consisting of 2µM Forskolin, 100nM cAMP and 25µM KCl (Forskolin).

Postnatal retinal cells were tested for expression of the proneural gene Pax6, early neuronal markers (β III tubulin), and neural stem cell markers (DCX, nestin, musashi, neuroD, notch1, Flk-1, Hes1) by RT-PCR experiments under the three described differentiation conditions. Cells were analyzed after growth as neurospheres for three passages (total of three weeks) and additional plating as adherent cultures for 7 days on poly-L-ornithine/laminin coated glass coverslips. After 21 days *in vitro* under neurosphere conditions and additional growth for 7 days as monolayers, postnatal derived retinal cells (P1) expressed β III tubulin, nestin, DCX, Pax6, Flk-1, notch1 and musashi (Fig.IV.20A). GAPDH was used for standardization. The comparison of the growth

conditions in the P1 group revealed that both 5% FCS and the Forskolin cocktail had the greatest influence on the neurogenic and stem cell related program in the cells. In particular, the expression of the retinal progenitor specific VEGF receptor Flk-1 as well as the expression of the proneural gene Pax6 and the neural progenitor marker DCX was increased under differentiation conditions as compared to control cultures. In P3 preparations, the expression of DCX and Flk1 decreased compared to the overall levels at P1, while the cell fate-controlling genes Hes1 and neuroD were increased in their expression as compared to P1 derived cells (Fig.IV.20B). Significant differences among the two differentiation conditions could not be observed in P8 cultures, except for the reduction of DCX and Flk-1 expression under Forsolin stimulation (Fig.IV.20C). Notch1 and Hes1, which acts downstream of Notch1 as a bHLH repressor and inhibits proneuronal genes during neurogenesis, are still prominently expressed at this stage. P14 cultures showed no expression of any of the progenitor markers, only the early neuronal marker β III tubulin and nestin under high serum were expressed, indicating that at this point, the adult sensory retina is indeed fully developed and does not contain any stem or progenitor like cells (data not shown).

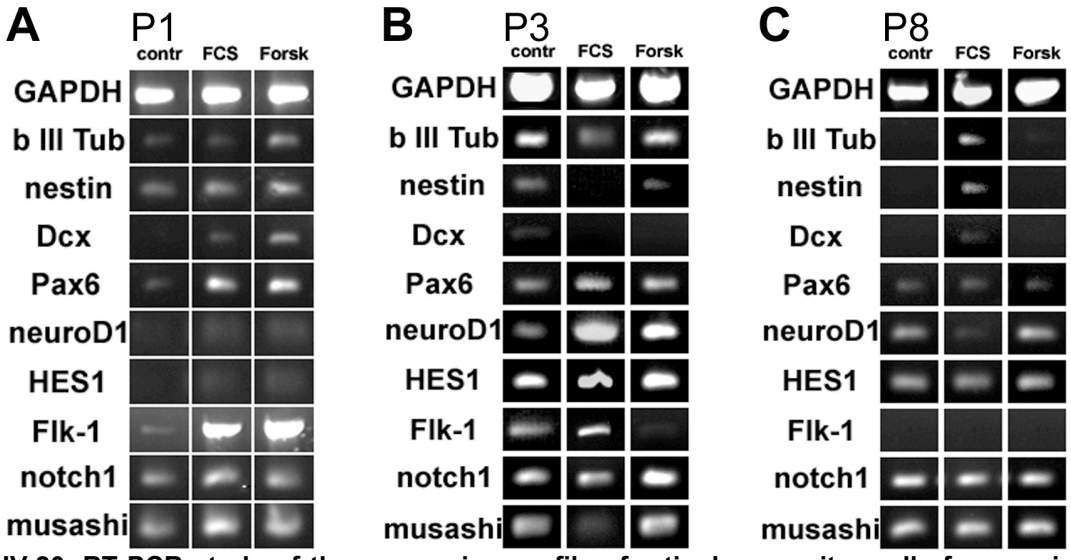

Fig.IV.20. RT-PCR study of the expression profile of retinal progenitor cells from various ages and under the influence of selective differentiation conditions. A) P1 derived retinal progenitor cells from passage number 3 and after growth under control, FCS and Forskolin conditions for 7 days on poly-L-ornithine/laminin coated glass coverslips. B) P3 derived retinal progenitor cells from passage number 3 and after growth under control, FCS and Forskolin conditions. C) P8 derived retinal progenitor cells from passage number 3 and after growth under control, FCS and Forskolin conditions. P14 cells did not express any of the selected markers except for GAPDH, β III tubulin and nestin.

IV.2.3.4. Expression of retina-specific markers in differentiated retinal cultures

To further assess the differentiation potential of postnatal retinal neurospheres, the expression of the retinal Ca^{2+}-binding protein recoverin was investigated. This protein is expressed in rods, cones, some bipolar cells and a rare population of ganglion cells in the rat retina [1]. While in P1 derived retinal neurospheres, the expression of recoverin was restricted to a small population of cells, the expression of recoverin in P3 cultures was significantly increased (Fig.IV.21E). The highest amount of recoverin-immunoreactive cells was found in cultures derived from P14 animals, indicating that at this point, retinal differentiation *in vivo* is finalized and the majority of cells have become photoreceptors.

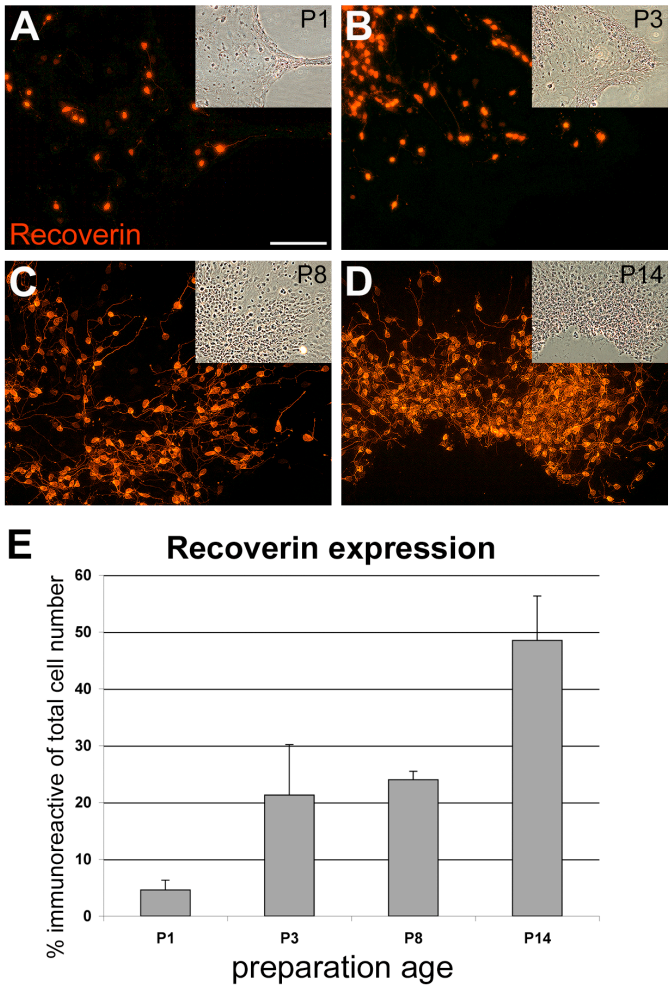

Fig.IV.21. Expression of the retina-specific Ca^{2+}-binding protein recoverin in postnatal derived cultures. A) Recoverin expression in P1 derived retinal cultures. Inserts show corresponding optic fields in phase contrast. B) Recoverin expression in P3, C) P8 and D) P14 derived retinal cells. E) Quantification of recoverin immunorecative cells in % of total cell number, showing a significant increase in immunoreactive cells with the progression of age at the date of preparation. Cells were derived from passage number 3 and plated for 7 days on poly-L-ornithine/laminin coated glass coverslips under differentiation conditions (1%FCS). Scale bar=50µm. Data expressed as average ± SD.

A very small subset of cells showed doublelabeling for BrdU and recoverin (Fig.IV.22.), indicating photoreceptors differentiated from mitotic progenitor cells. Other mature retina-specific markers that were immunoreactive in P8, P14 and adult derived SR cultures included rhodopsin, the primary light sensor of the visual system in vertebrates [166]. Additionally, retinoschisin was used to stain mature retinal cells. Retinoschisin is secreted by mature photoreceptors and is critical for cell adherence in the outer nuclear layer [144, 167]. Both markers, rhodopsin and retinoschisin, were observed in P8, P14 and adult derived retinal cultures, but never co-labeled with progenitor or stem cell markers (data not shown).

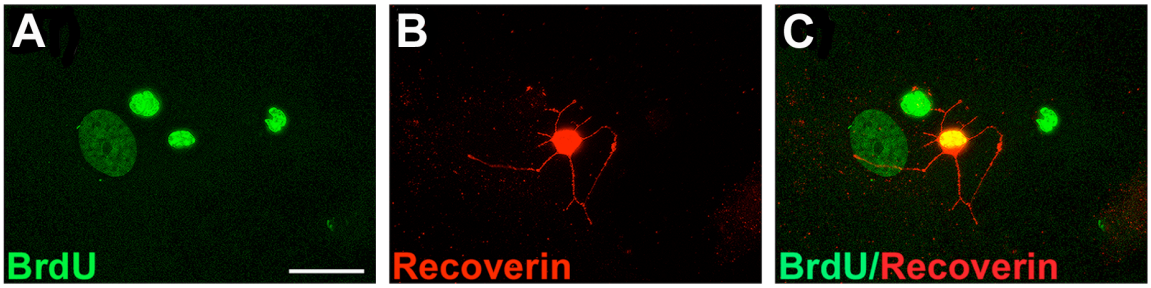

Fig.IV.22. Generation of new photoreceptors *in vitro*. A) BrdU incorporation indicating proliferation in P3 derived retinal progenitor cells after passage 3 and 7 days on poly-L-ornithine/laminin coated glass coverslips under differentiation conditions. B) Recoverin-immunoreaction (red). C) Expression of recoverin and BrdU, resulting in a yellow overlap at higher magnification (100x). Scale bar= 50µm. A small percentage of recoverin-positive cells were BrdU positive, indicating that a subset of progenitors differentiated into photoreceptors in culture.

IV.3. Adult derived progenitor cells from the ciliary body and retinal pigment epithelium of the rat eye

Submitted to Brain Research, Engelhardt et al., 2004

The existence of retinal stem cells from the adult mammalian eye has already been demonstrated for the margin of the ciliary body (CB) [67]. Other studies described that the retinal pigment epithelium (RPE), which in poikilothermic vertebrates is able to generate new retinal cells, has no such properties in adult mammals [67, 168]. Therefore, the next focus of this work was the evaluation of the potential of these cells to de-differentiate into a neurogenic cell type with certain properties of retinal stem cells. A direct comparison of RPE and CB cells in a differentiation study followed.

IV.3.1. Isolation, proliferation and generation of neurospheres

Adult derived rat RPE cells were tested for their proliferative potential under culture conditions that rpmote growth of NSCs. Since the CB was previously shown to contain neural stem cells [67, 68], this cell type served as control. Special care was taken during the dissection procedure to avoid contamination of the RPE preparation with cells from the CB. Therefore, a circular incision was made at least 1,5 mm posterior to the CB and the anterior part of the eye including CB and lens was lifted off and not used for the RPE preparation. To exclude quiescent NSCs present in the optic nerve [42] from the preparation, a circular incision around the optic nerve head was made and the optic nerve head was discarded. RT-PCR for the RPE specific marker RPE65 confirmed its presence in the RPE preparation (Fig.IV.23B).

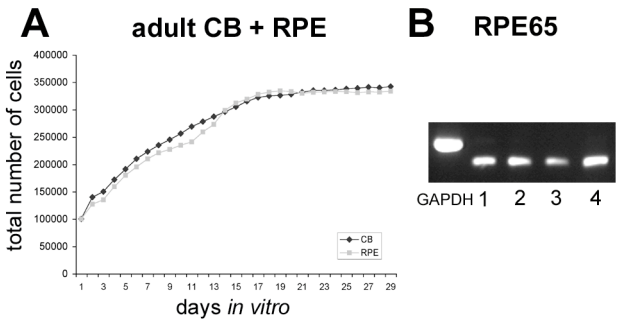

Fig.IV.23. Cell culture data. A) Growth rates of adult CB and RPE derived progenitor cells over a period of 28 days. Cells were seeded at 10^5 cells per well in 12 well plates in NB/B27 medium and counted via trypan exclusion assay. B) RT-PCR for the RPE-specific marker RPE65 on 4 different preparations of RPE cells indicates the presence of RPE cells in the preparation. GAPDH for standardization, product size of RPE65 200bp.

Cell number initially increased during the first two weeks three-fold with a cease in proliferation when the cell number reached a plateau phase (Fig.IV.23A). The increase in cell number was slightly delayed in RPE derived cells, but showed similar growth kinetics as the CB cells. Both CB and RPE derived cells could be kept in culture for up to 12 weeks (6 passages) before an arrest of proliferation was observed. Similar to postnatal retinal progenitors and in agreement with published data [67, 68], adult CB derived progenitors grew as neurospheres by cell proliferation as indicated by BrdU incorporation (Fig.IV.24A, B). The neurospheres consisted of both pigmented and non-pigmented cells. A similar finding was observed for RPE derived progenitor cells (Fig.IV.24C, D). During the time of proliferation, dissociated and passaged cells reformed neurospheres with similar growth kinetics compared to primary neurospheres.

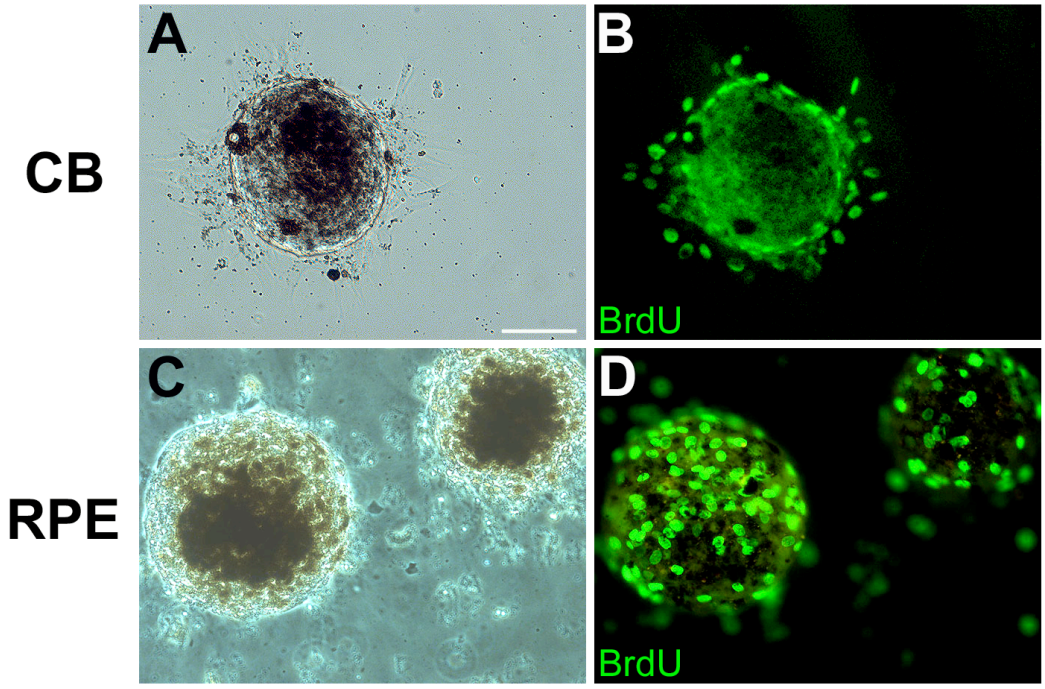

Fig.IV.24. Detection of proliferation of RPE and CB derived cells in neurospheres *in vitro*. A-B) Adult CB derived neurosphere, incubated with 10µM BrdU for 24 hrs and seeded overnight on poly-L-ornithine/laminin coated glass cover slip. BrdU incorporation is indicated by green labeling. C-D) Adult RPE derived neurosphere with BrdU incorporation. Scalebar =75µm

IV.3.2. Clonal expansion and growth as adherent monolayer cultures

CB and RPE derived cells, either as bulk cultures or under clonal conditions, adhered, proliferated, acquired a flat mesenchymal like morphology and lost their pigmentation within a few days under proliferation conditions (Fig.IV.25A, C). The same was observed with CB and RPE cells that were initially grown as neurospheres and then changed to adherent conditions. Cells proliferated rapidly and needed to be passaged every three days after a 1/3 split ratio due to confluency. An attempt to derive CB or RPE cultures under strict clonal and neurosphere conditions failed. However, when grown as adherent monolayers, primary RPE cells grew clonally with a cloning efficiency of 72% (81 of 113). Vimentin is the most ubiquitous intermediate filament in various cell types, which is replaced by other intermediate filaments during differentiation. It has been shown to be a marker expressed by the majority of proliferating RPE cells in the pathological condition of proliferative vitreoretinopathy and in RPE cells dissociated and placed in culture [169, 170]. Thus, vimentin was used as first indicator for proliferation of RPE cells exposed to proliferation medium under adherent conditions *in vitro*. As shown in Fig.IV.25B, most of the RPE cells expressed vimentin after the first passage. After five

to six passages, cell proliferation slowed down and ceased. An indication for the acquisition of a more de-differentiated state was the loss of pigmentation, which occurred in both CB and RPE derived cultures (Fig.IV.25C).

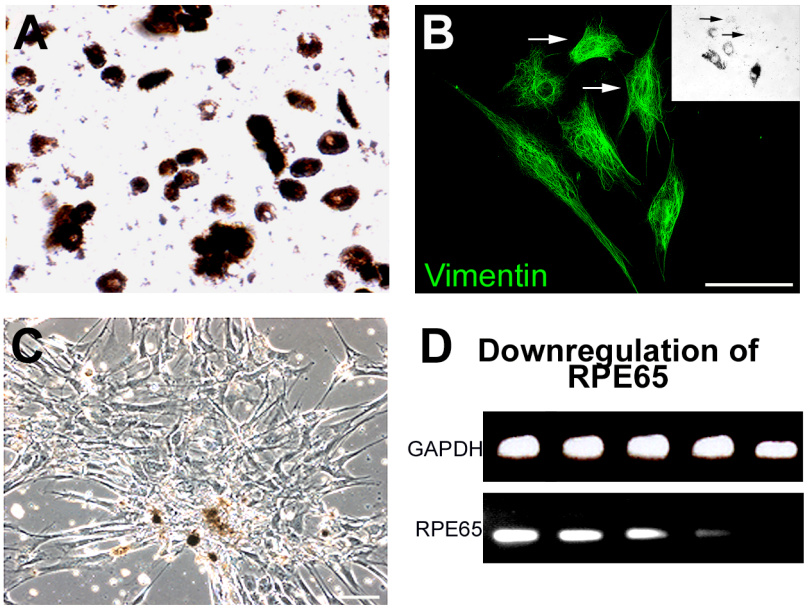

Fig.IV.25. RPE Monolayer cultures. A) Adult derived RPE cells one day after isolation. All cells were pigmented and a few appeared in clusters. B) RPE cells after 1 week in culture in NB/B27 with 1% FCS on collagen I coating. Up to 95% of cells are vimentin positive and still carry their pigmentation. Insert shows phase contrast micrograph of the same cells. C) RPE derived cells after 2 weeks in culture in NB/B27 with 1% FCS on collagen I coating. The cells adhered and many lost their pigmentation. D) RT-PCR for the RPE specific marker RPE65. 1=RPE cells 4 hrs after isolation, 2=pigmented neurospheres after 1 week in culture, 3=adherent RPE cells after 14 days *in vitro* (DIV), 4=adherent RPE cells after 21DIV and on poly-L-ornithine/laminin coated glass cover slips, 5=adherent RPE cells after 28DIV on poly-L-ornithine/laminin coated glass cover slips. GAPDH for standardization, product size of RPE65=200bp. Scale bar A-C=50µm

The loss of pigmentation in RPE cultures was coherent with the observed downregulation of the RPE specific marker RPE65. RT-PCR showed that in feshly isolated cells (4 hrs past isolation, cells in NB/B27 medium), RPE65 was strongly expressed (Fig.IV.25.D). The same culture grown as neurospheres after one week (NB/B27, FGF/EGF/Heparin, 1% FCS) showed a similar expression of the marker. After growth as adherent monolayer cultures for 14 days on collagen I, RPE cells began to downregulate RPE65 expression. Adherent RPE cultures plated on poly-L-ornithine/laminin coated glass cover slips for 21 days *in vitro* under differentiation conditions (NB/B27, no growth factors, 1%FCS) showed a distinct reduction of RPE65 expression, while RPE cells after 28 days *in vitro* on poly-L-ornithine/laminin coated

glass cover slips and under differentiation conditions were devoid of RPE65 signals as analyzed by RT-PCR.

IV.3.3. Expression of progenitor markers in adult derived retinal cells: RT-PCR

CB and RPE cells were tested for expression of the proneural gene Pax6, early neuronal markers (β III tubulin), neural stem cell markers (nestin, DCX, musashi, neuroD, notch1, Flk-1, Hes1) by RT-PCR experiments. Cells were analyzed directly after initial cell preparation and after growth in neurospheres for three passages (total three weeks) and additional plating as adherent cultures for 7 days on poly-L-ornithine/laminin coated glass coverslips under differentiation conditions (removal of growth factors, addition of 1% FCS). When tested directly after dissection and dissociation, only β III tubulin in CB and Hes1 in RPE cells could be amplified (Fig.IV.26.). However, after 21 days *in vitro* under neurosphere conditions and additional growth for 7 days as monolayers, CB and RPE derived cells expressed nestin, Flk-1, neuroD, Hes1, notch1, musashi, Pax6, β III tubulin, and DCX (Fig.IV.26.), indicating that a de-differentiation process has been turned on which leads towards a more progenitor-like genotype in these cells.

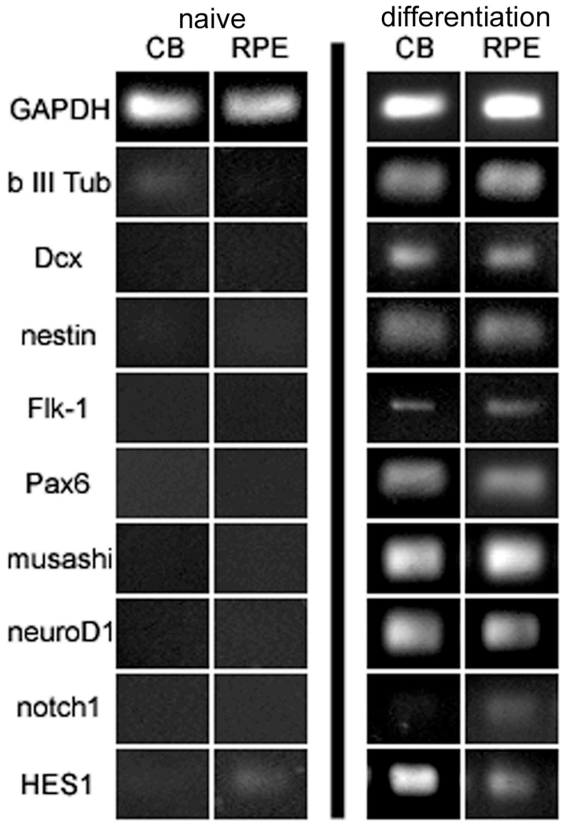

Fig.IV.26. CB and RPE derived cells up-regulate the expression of neural progenitor and neuronal differentiation markers *in vitro*. A) RT-PCR on RNA obtained from adult derived CB and RPE cells 4 hrs post preparation. Except for slight expression of β III tubulin in CB cells and Hes 1 in RPE cells, no other markers could be found. B) RT-PCR on RNA obtained from adult derived CB and RPE cells after passage number 3 and subsequent differentiation on poly-L-ornithine/laminin for additional 7 days. Expression of β III tubulin is now observed in both cell preparations, and other neural progenitor markers like nestin, DCX and Flk-1 have been up-regulated. Furthermore, the retinal determination factor Pax6 as well as the translational regulator of stem cells, musashi, the transcription factor neuroD and the determination factors Notch1 (only RPE) and Hes1 are expressed. GAPDH was used for standardization.

IV.3.4. Expression of progenitor and early neuronal markers: morphological alterations

Expression of NSC and neuronal precursor markers was further investigated by immunocytochemistry using antibodies specific for nestin, β III tubulin and DCX. Neurospheres derived from both cell types after 15 days *in vitro* in NB/B27 medium and 1% FCS were plated on collagen I coated glass coverslips and adhered immediately. Fixation only 24 hrs later and staining for nestin and the cell cycle marker Ki-67 revealed nestin-positive cells in pigmented neurospheres (Fig.IV.27.). RPE derived neurospheres adhered more rapidly than CB derived neurospheres and immediately flattened with cells beginning to migrate out, which caused the less spheroid morphology of the aggregate in Fig.IV.27.B.

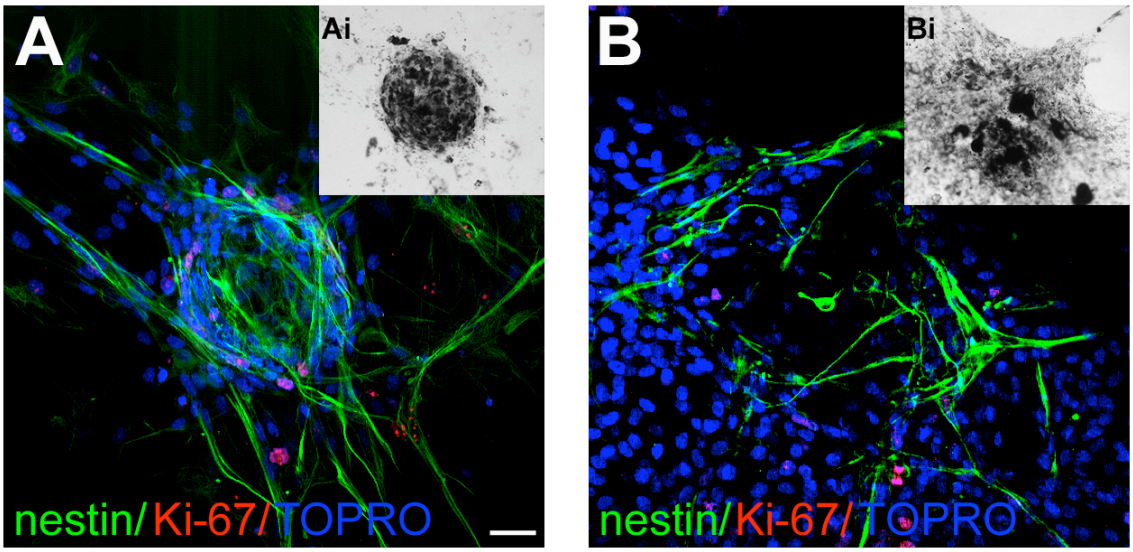

Fig.IV.27. Expression of the progenitor marker nestin and the cell cycle protein Ki-67 in adult CB and RPE cells. A) Immunodetection of nestin and Ki-67 antigen in an adult rat CB derived neurosphere. Insert shows brightfield micrograph of the same neurosphere. B) Immunodetection of nestin and Ki-67 antigen in an adult rat RPE derived neurosphere. Neurospheres were stained after growth in NB/B27 medium supplemented with 1% FCS for 1 week and attachment on collagen I coated glass coverslips for 24hrs. TOPRO iodide is used as nuclear stain. Visualization of one plane in the neurosphere by confocal microscopy. Scale=50µm

Quantification showed that 13±2% of the CB and 9±2% of the RPE derived cells in monolayer cultures were immunoreactive for nestin after 7 days of differentiation on poly-L-ornithine/laminin coated glass coverslips.

In addition, β III tubulin was expressed in up to 21±7% of CB and 13,2±2% of RPE cells (Fig.IV.28.). A morphological analysis of the β III tubulin expressing cells revealed two types of cells: i) cells with flat epithelial appearance (14±11% CB; 1,2±2% RPE; Fig.IV.28G-I&P-R) and ii) cells with neuronal morphology including bi- or pluripolar structures and elongating processes (7±5% CB; 12±7% RPE; Fig.IV.28.A-F+J-O). In an attempt to describe the β III tubulin positive RPE and CB fraction with neuronal morphology in more detail, the neuronal precursor marker DCX was used. Doublelabeling of CB and RPE derived cells for DCX and β III tubulin illustrated that DCX identifies the β-III tubulin subpopulation with neuronal morphology (Fig.IV.28D-F+M-O). In average, 6,3±2% of CB and 6±2% of RPE derived cells were found to be immunoreactive for DCX by immunostaining. All DCX positive cells were immune-

reactive for β III tubulin and displayed a neuronal morphology. They did not co-label with nestin, and not with the glial marker GFAP (data not shown).

In contrast to the postnatal sensory retina cultures, RPE derived cells were not able to generate retina-specific phenotypes such as photoreceptors under the applied conditions. None of the cells tested expressed the photoreceptor markers rhodopsin and recoverin, a CA^{2+}-binding protein predominantly found in cones and rods as well as a small subpopulation of bipolar neurons [1]. In addition, RPE derived cells expressed no signs of transmitter specialization such as expression of tyrosine hydroxylase (TH) or GAD67. Absence of a dopaminergic phenotype was further indicated by the lack of dopamine in the cell culture supernatant tested by HPLC (data not shown).

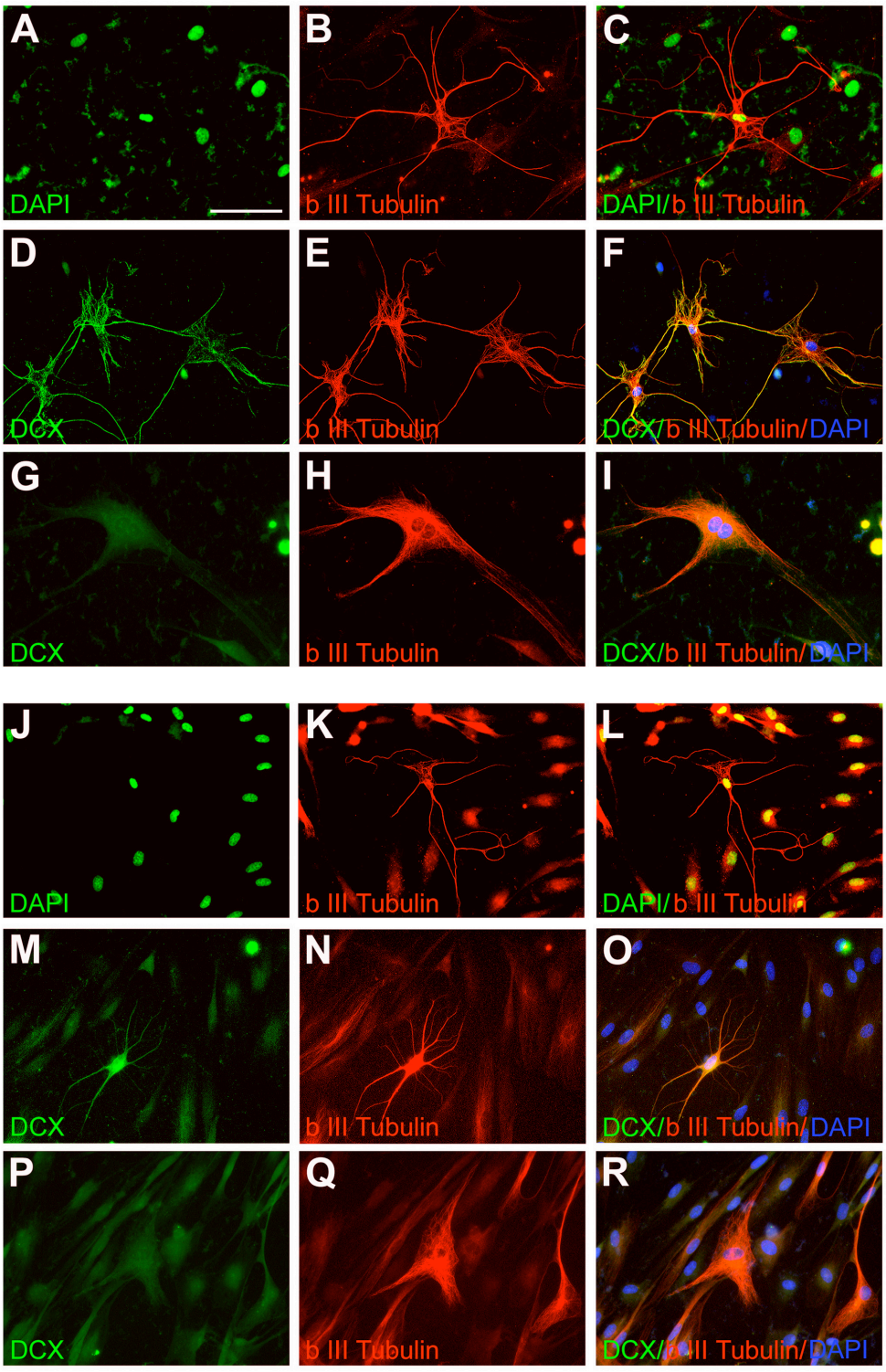

Fig.IV.28. Expression of neuronal markers in adult CB and RPE derived cells. Cells from adult derived CB and RPE cultures of passage number 3 after 7 days of differentiation on poly-L-ornithine/laminin coated glass coverslips were analyzed. A-C) Immunostaining for III tubulin (red) in RPE cultures. Stained cells exhibit distinct neuronal morphology. D-F) Doublelabeling for III tubulin (red) and DCX in green in RPE cultures. Stained cells exhibit distinct neuronal morphology. G-I) Doublelabeling for III tubulin (red) and DCX with nuclear counterstain DAPI (blue) in a RPE culture, showing a flat mesenchymal/epithelial morphology. Note that this cell

type is devoid of DCX expression. J-L) Immunostaining for β III tubulin (red) in a CB culture, staining a cell with a neuronal morphology. M-O) Doublelabeling for β III tubulin (red) and DCX in a CB culture, staining a cell with a distinct neuronal morphology. P-R) Doublelabeling for β III tubulin (red) and DCX with nuclear counterstain DAPI (blue) in a CB culture, showing a flat mesenchymal/epithelial morphology. This cell type is devoid of DCX immunoreactivity. Green nuclear counterstain is DAPI. Unspecific binding of antibody can be observed in background staining in G, M-N, P-Q. Scale bar=50μm.

IV.3.5. Differentiation along the glial lineage

Furthermore, NSC identity requires differentiation into the glial lineages. Adherent monolayer cultures derived from both CB and RPE cells differentiated into glial cells as indicated by immunoreactivity for GFAP and NG2 in CB and RPE derived cells (Fig.IV.29.). Remarkably, glial differentiation cannot be observed in the natural setting of CB or RPE cells, since these regions of the eye are devoid of any glial phenotypes. Differentiation into oligodendroglia was not observed under the chosen conditions, suggesting that CB and RPE derived cells are not multipotent.

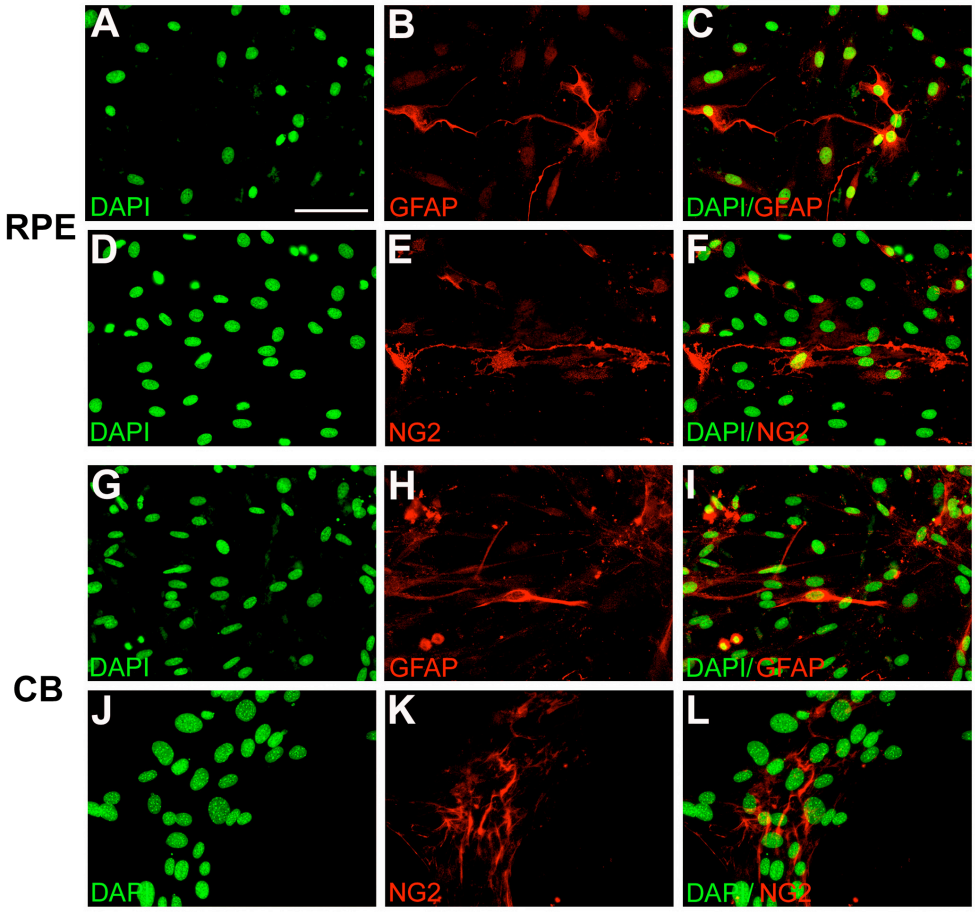

Fig.IV.29. Expression of glial markers in adult CB and RPE derived cells. Cells from adult CB and RPE were cultured under neurosphere conditions for 21 days and subjected to differentiation

(NB/B27, 1%FCS, no growth factors) for additional 7 days on poly-L-ornithine/laminin coated glass coverslips. A-C) Immunostaining for GFAP (red) in a RPE derived culture. D-F) Immunostaining for NG2 (red) in a RPE derived culture. G-I) Immunostaining for GFAP (red) in a CB derived culture. J-L) Immunostaining for NG2 (red) in a CB derived culture. Nuclear counterstain is DAPI. Scale bar=50µm.

IV.4. Adult derived progenitor cells from the human retinal pigment epithelium

In the final analysis of this study it was investigated, whether adult human derived RPE cell cultures harbor similar properties in terms of expansion rates, growth as neurospheres and differentiation potential as previously shown for rodent cultures. Human donor material was obtained from the Department of Ophthalmology, University of Regensburg. A recent study revealed that RPE cell lines can differentiate into neurons expressing early and late neuronal markers such as III tubulin, Map2 and Neurofilament 200kD (NF200kDa) [171]. That study, however, did not cover the precursor cell potential of these cell lines, nor did it reveal how similar these RPE cells are in comparison to the published rodent retinal stem cells. An evaluation of the proliferation potential as well as the differentiation capacities of adult human derived RPE cells is therefore included in this work.

IV.4.1. Cell culture conditions: growth as neurospheres and adherent cultures

Adult human retinae and RPE derived cells were isolated from donor eyes provided by the Department of Ophthalmology, University of Regensburg. The isolation procedure was the same as described for RPE cells from the rat eyes (see section III.2.1.3.). CB cells could not be obtained due to the fact that the entire lens/iris/CB region was taken out for transplantation purposes. RPE cells were placed in NB/B27 medium with FGF/EGF/Heparin and addition of 1% FCS and showed similar properties as the rodent RPE cells *in vitro* (Fig.IV.30A, B). Neurospheres were generated after approximately 2 weeks in culture. Neurospheres were dissociated by Accutase treatment and reformed new neurospheres under proliferation conditions (NB/B27, 1% FCS, FGF/EGF/Heparin). After plating on poly-L-ornithine/laminin coated glass coverslips, the cells readily adhered and migrated out of the spheroid bodies, losing their pigmentation during the course of this (Fig.IV.30C). Proliferation was observed by Ki-67

immunodetection in adherent monolayer cultures. Doublelabling with DAPI showed distinct mitotic activity as revealed by spindle apparatus staining (Fig.IV.30D-I).

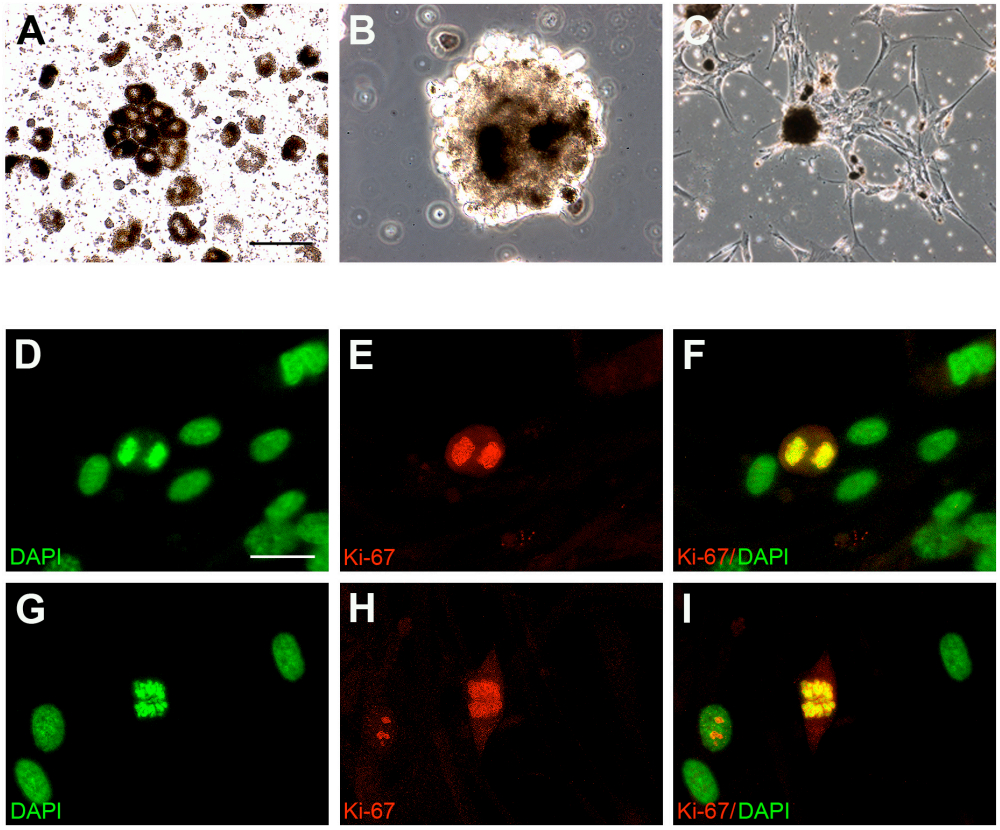

Fig.IV.30. Adult RPE derived cells from human donor material. A) RPE derived cells 2 days after isolation, seeded in NB/B27 medium with 1% FCS and on collagen I coated 6-well plates. All cells were pigmented and could be observed either as single cells or in small clusters. B) RPE derived neurosphere after 2 weeks *in vitro*. While still many cells remain pigmented, a few have lost their pigmentation. C) RPE derived cells grown as adherent monolayer cultures. Neurospheres were plated on poly-L-ornithine/laminin coated glass coverslips in the presence of 1% FCS. Soon, cells started to migrate out of the neurospheres and lost their pigmentation. D-I) Immunodetection of the Ki-67 antigen (red) in mitotically active cells. The nuclear counterstain DAPI clearly marks the spindle apparatus of cells that actively proliferate *in vitro*. Scale bar=25µm.

The expression profile of adult human derived RPE cells changed after growth as neurospheres and under proliferation conditions in NB/B27 medium supplemented with growth factors (FGF, EGF, Heparin). PCR analysis of naïve, untreated cells shortly after isolation showed no significant expression of progenitor markers. However, after 21 days *in vitro* under neurosphere conditions (NB/B27 medium, FGF/EGF/Heparin), cells that were dissociated from RPE neurospheres upregulated the proneural homeobox gene Pax6, the bHLH transcription factor NeuroD and the progenitor cell marker nestin

(Fig.IV.31.). The determination factor Notch1, the progenitor marker Flk-1 and the neuronal precursor marker DCX remained undetectable at this stage. The upregulation of especially Pax6 could be an indication for the de-differentiation potential of adult human RPE cells cultured under strict growth conditions. Pax6 is a known determination factor for RPE development during embryogenesis, but is downregulated in the mature mammalian RPE [172].

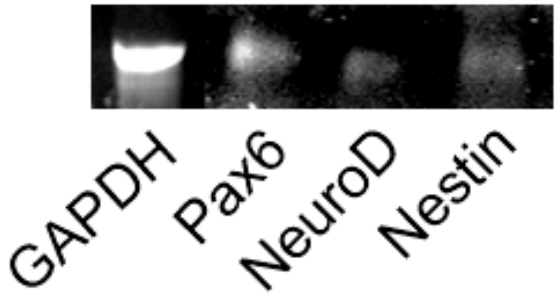

Fig.IV.31. PCR for progenitor markers expressed in adult human derived RPE cells. Expression of the proneural gene Pax6, the bHLH transcription factor NeuroD and the progenitor cell marker nestin was upregulated in human RPE cells after growth as neurospheres for 21 days in NB/B27 medium supplemented with FGF, EGF and Heparin. Freshly isolated RPE cells showed no expression of these markers. Expression of Notch1 and DCX was not detected at this stage.

IV.4.2. Differentiation potential of human RPE cells: neuronal lineage

After an initial growth phase of 21 days as neurospheres and additional cultivation under differentiation conditions (NB/B27, 1%FCS, no growth factors), human derived RPE cells started to express the early neuronal marker β III tubulin (Fig.IV.32A-C). The majority of the cells expressing β III tubulin exhibited an epithelial phenotype, while a small subset of cells also showed a distinct neuronal morphology (Fig.IV.32D-F), as it was previously described for rodent derived RPE cultures (see Fig.IV.28.)

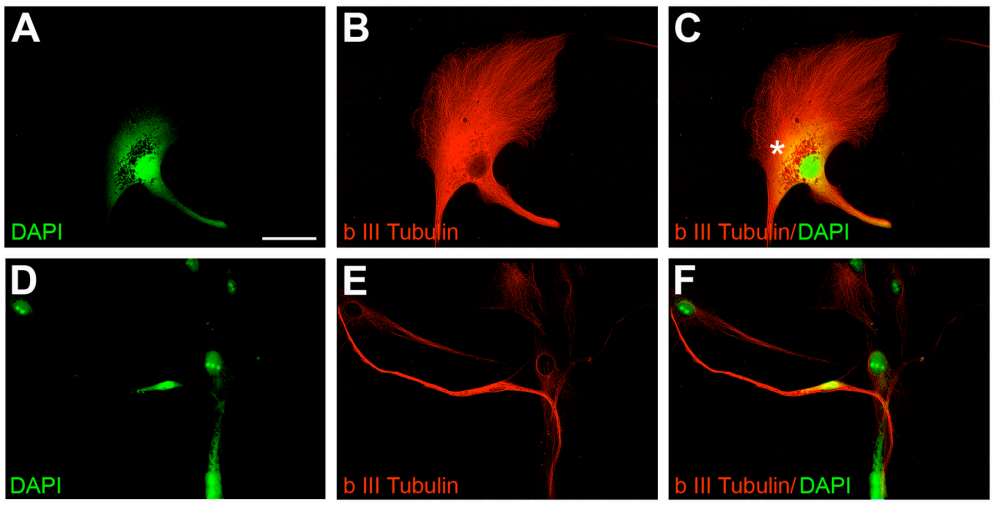

Fig.IV.32. Expression of the early neuronal marker β III tubulin in human derived RPE cells.

A-C) Immunodetection of β III tubulin (red). Cultures were grown for 7 days on poly-L-ornithine/laminin coated glass cover slips in NB/B27 medium with 1%FCS. The cell pictured shows a clear epithelial morphology and is representative for the majority of β III tubulin positive cells in these cultures. Most of the cells were pigmented (C, asterisk). D-F) Immunodetection of β III tubulin (red) in a cell that has altered the morphology towards a mostly neuronal phenotype. This example can be compared to the CB and RPE cells from adult rats shown in Fig.IV.28. Nucelar counterstain is DAPI in green. Scale bar=25µm.

Pigmentation was still prevalent in the epithelial phenotype (Fig.IV.32C, asterisk). Furthermore, a small subset of cells expressed the adult neuronal marker neurofilament 200kDa (Fig.IV.33.) and also co-labeled for the proliferation marker BrdU, indicating that a small subset of cells were generated *in vitro* and de-differentiated into cells expressing adult neuronal markers. However, the percentage of these cells was very low, and therefore quantification would not have been representative.

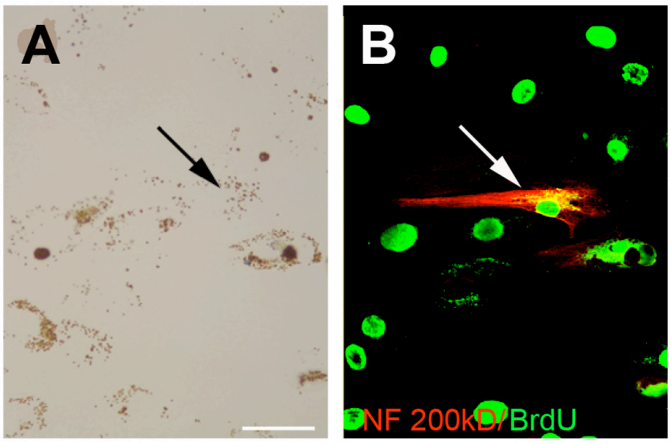

Fig.IV.33. Expression of adult neuronal markers in human derived RPE cultures. A) Phase contrast micrograph of pigmented human RPE cells after 21 days under neurosphere conditions and subsequent plating on poly-L-ornithine/laminin coated glass coverslips for another 7 days. Prior to staining, the cells were exposed to 10µM BrdU for 24 hrs. B) Immunodetection of the NF200kDa antigen (red), showing a cell that also incorporated BrdU in green. Scale bar=25µm.

IV.4.3. Differentiation potential of human RPE cells: glial lineage

Similar to reports from the rodent CB and RPE cultures, human derived RPE cells also differentiated along the glial lineage, as shown by S 100 β immunodetection (Fig. IV.34A-C) for immature [129] and GFAP (Fig. IV.34D-F) for mature glial cells. While a distinct astrocytic morphology is missing, the expression of GFAP is nevertheless increased and reflects a state of de-differentiation, since RPE cells do not harbor any glial cell types in their natural setting. However, multipotency can be excluded for these cells, as they were unable to generate oligodendrocytic markers like GalC (data not shown). As already demonstrated in rodent cells (see section IV.3.3.), human RPE cells have the potential to de-differentiate to a certain degree, but cannot be called multipotent neural

stem cells since they fail the requirements of differentiating into neurons, astroglia and oligodendroglia.

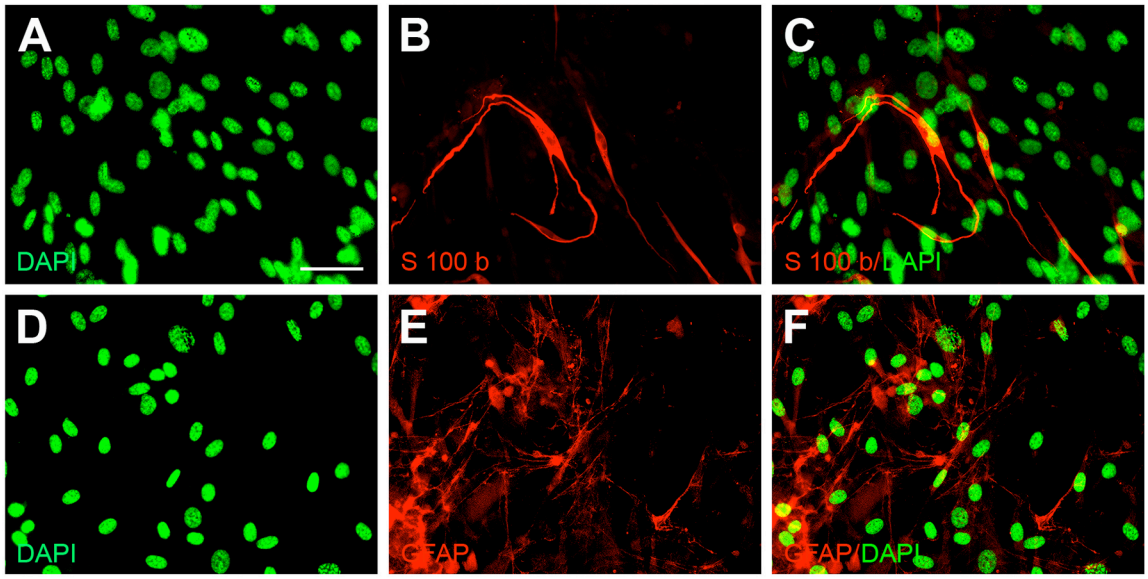

Fig.IV.34. Expression of glial markers in human derived RPE cultures. A-C) Immunodetection of the early glial marker S 100 □ (red) in human RPE cells derived from adult eyes after an initial growth phase of 21 days under neurosphere conditions and subsequent plating on poly-L-ornithine/laminin coated glass coverslips for another 7 days. D-F) Immunodetection of the adult glial marker GFAP (red). Nuclear counterstaining is DAPI in green. Scale bar =50µm

V. Discussion

The present work aimed at analyzing and understanding the characteristics of postnatal and adult derived retinal progenitor cells from distinct parts of the eye, while applying optimized culture conditions that were established using adult derived CNS stem cells.

V.1. Differentiation of NSCs and their phenotype plasticity after improvements of growth conditions

The first part of this thesis describes i) a protocol for increased cell survival and efficient growth of dissociated multipotent rodent derived neurospheres using Accutase™ instead of trypsin, ii) high efficiency of NSC growth under clonal and low density conditions independent of the presence of serum, conditioned medium or factors like cystatin C, iii) the differentiation potential under various media conditions, and iv) the phenotype plasticity of NSCs constitutively expressing Notch1.

V.1.1. Growth and proliferation of NSCs as neurospheres

NSCs are typically grown as monolayer cultures or in three-dimensional neurosphere aggregates [12, 30, 40, 43]. Neurospheres are free-floating structures of cell aggregates that are usually obtained by exposing dissociated NSCs to growth factors [40, 173, 174]. The advantages of neurosphere cultures are the easy isolation technique and the maintenance in larger numbers. Heterogeneous neurospheres are composed of a mix of neural stem cells, progenitors and fully differentiated cells that are arranged in a complex, three-dimensionally organized extracellular matrix. This 3D structure creates a unique microenvironment, which facilitates the discrimination of proliferation of committed progenitors versus stem cell maintenance and renewal [175]. Recent results addressing the composition of neurospheres generated from NSC cultures suggest a core of differentiating GFAP- and III tubulin-positive cells that are surrounded by nestin-

positive, undifferentiated cells that also express epidermal growth factor receptor (EGFR) and integrins [176].

In the present analysis, adult derived NSCs formed neurospheres after only a few days *in vitro* and continued to proliferate inside the neurospheres (see section IV.1.1.3.). Indication for proliferation was a positive immunocytochemical signal for the thymidine analog bromodeoxyuridine (BrdU), which is incorporated into DNA by polymerase activity during S phase of the cell cycle [177]. However, the use of BrdU to determine proliferation in a subset of cells is to some extend controversial. Several authors argue that labeling of cells with BrdU is necessary, but not sufficient, to demonstrate cell division. BrdU might rather be a marker for DNA synthesis or repair mechanisms, not for newly generated cells [178]. In addition, BrdU is known to be a mutagen under certain conditions, but this is dependent on administered concentrations [179]. On the other hand, studies combining BrdU with other histological methods to label proliferating cells and also combining BrdU detection with methods to detect apoptotic events, argue against the hypothesis of DNA repair being the major contribution to BrdU-positive cells [137, 180]. The usage of BrdU *in vitro* is a widely accepted method to monitor proliferation events, but despite that, the cell cycle marker Ki-67 was additionally used in the present study (see sections IV.1.2. and IV.4.1.).

V.1.1.1. Improved passaging of NSCs using Accutase ™

After reaching a critical cell-density or neurosphere size, or when clonal analysis of NSCs is required, single cell suspensions can be generated by enzymatic treatment. Enzyme-dependent dissociation of neurospheres at clonal densities is generally an impairment for growth of isolated cells, since besides matrix molecules, vital growth factor receptors are affected as well. An alternative and more gentle approach to trypsinization for passaging the neurosphere cultures is the “chopping”-method, described for human fetal neural progenitor neurospheres [12]. In contrast to the enzyme-dependent dissociation of neurospheres, the mechanical disruption causes no drastic growth arrest [181], but has the disadvantage that growth under clonal conditions (including limited dilution assays) is not possible. The type of enzyme used for dissociation of neurospheres is critical for cell growth, since more aggressive treatments with highly

reactive enzymes can lead to major cell damages. This could influence the outcome of stem cell assays, especially in terms of self-renewal and proliferation capacities of NSCs. In order to address this problem, a comparative analysis using two different enzymes, trypsin and Accutase™, was conducted.

The present results demonstrated high cell viability and fast recovery of adult NSC neurospheres following Accutase™ dissociation. Accutase™ has been specifically developed for the preparation of cell samples for fluorescent activated cell sorting (FACS) analysis (http://sciencepark.mdanderson.org/flow/files/Aggregates.html). It is a proprietary formulation containing proteolytic and collagenolytic activities and was developed for rapid dissociation with high viability of cells (see http://www.innovativecelltech.com/accutase.html). Since the precise formulation of Accutase™ is not available, it can only be speculated about the nature of the selective effect of Accutase™. Despite its efficient cell dissociation activity, Accutase™ is less aggressive to NSCs than trypsin, as suggested by the higher cell viability seen immediately after enzymatic treatment. The increase in the total number of cells and neurospheres observed four days after dissociation also reflects a higher survival of cells after Accutase™- versus trypsin-dissociation (see section IV.1.1.1.). Since the integrity of cell surface markers is crucial for antibody binding and FACS analysis, it is plausible that Accutase™ does not degrade membrane bound receptors, such as EGF and FGF receptors, as extensively as trypsin. Alternatively, Accutase™ might be less destructive to cell adhesion molecules required for neurosphere formation. The deleterious effect caused by trypsin was also reported after dissociation of primary olfactory receptor neurons and human tumor xenografts [182, 183].

V.1.1.2. Adult derived NSCs self-renew under clonal conditions

An additionally fundamental assay of neurosphere cultures is the demonstration of self-renewal of NSC cultures. One way is the utilization of clonal secondary neurosphere assays that test the hypothesis whether isolated stem cells can generate new neurospheres. Of even greater importance is the clonal analysis to identify multipotent NSCs. The successful application of a clonal analysis depends on the capacity of the initial cell to survive in culture once isolated from its complex surroundings, be it the neurosphere or

the *in vivo* situation. Cell survival seems to be the key aspect for clonal assays, as it has been shown previously that there is a direct link between the loss of proliferation potential in stem cells and cell survival of isolated single cells [184].

For the clonal analysis, the isolated cell has to retain the ability for cell division and to be responsive to growth factors or other environmental cues. In this respect, the aspect of cell-cell interaction and cell-density has come into focus. Recently, cell-density has been shown to be a major factor for influencing embryonic stem cell behavior and differentiation [185]. Interestingly, Tschumperlin and colleagues suggested that autocrine binding of EGF ligands to the respective receptor can be triggered by applying pressure on cells "packed together", physically decreasing the intercellular space between cells [186]. Adult SVZ derived NSCs have the capacity to grow clonally, either after FACS sorting or under limited dilution conditions (see section IV.1.1.4.), but their regenerative properties are strictly dependent on optimal cell culture conditions including specific media and supplements, emphasizing the necessity of the required optimization of cell culture conditions.

V.1.1.3. Media-supplements differentially affect growth rates

Current techniques used for the growth of NSCs in neurospheres or as monolayers are based on serum-free DMEM/F12 medium supplemented with N2, B27, or BIT9500 [41, 49, 149, 187, 188]. DMEM/F12 promotes the growth of NSCs under high cell-density conditions ranging from 20 cells/μl to 4.5 x 10^7 cells/ml [52, 188, 189]. However, it is insufficient for the growth of NSCs under low density or clonal conditions and requires conditioned medium [42] or additional compounds such as cystatin C [190].

In the present work, media/supplement combinations that allow for high efficiency of NSC growth under clonal and low-density (10 cells/μl) conditions, independent of the presence of serum or conditioned medium, are described. Rat NSCs derived from SVZ, HC or SC showed highest proliferation rates when grown in NB/B27 medium. Similar results were obtained with NSCs derived from adult mouse neurogenic regions SVZ and HC. The elevation in rat NSC number in NB/B27 (6.000 fold within 21 days) represents a higher expansion rate as compared to previously established NSC culture protocols (e.g. 10^5 to 10^{10} in 100 days *in vitro*) [49, 191]. In addition, the data

demonstrated clonal growth of single NSCs in a serum-free medium (NB/B27) for the first time. This represents a major advantage for future cell therapy and transplantation experiments in a clinical context. Despite the high expansion rate, *in vitro* propagated NSCs did not form tumors two months after transplantation into the cortex of adult Fisher-344 rats, indicating that these cells did not transform.

NB medium has been originally described as a supportive medium for primary neuronal cultures [192, 193], but was so far not used for adult NSC cultures. In combination with the supplement B27, it has been described to be supportive for the maintenance of neurons derived from various brain regions, including cortex, cerebellum, dentate gyrus, striatum, and substantia nigra [194]. B27 was first reported for the maintenance of embryonic hippocampal neurons and neuronal cell lines [192]. Compared to the Bottenstein and Sato N2 formulation [195] with transferrin, insulin, putrescine, progesterone, and sodium selenate, it includes a range of additional substances (see Table I and [192]). As a supplement to DMEM/F12, B27 was described to promote growth and expansion of freshly isolated rat embryonic precursor cells and of adult rodent NSCs [149, 188]. The application of NB/B27 for cultures of adult derived NSCs, however, represents a new and highly efficient approach. The BIT9500 supplement, originally used for hematopoietic stem cell cultures [151], has previously been reported to induce the growth of adult human progenitor cells isolated from post-mortem tissue [49]. It is an efficient supplement for the growth of adult human NSCs, however, B27 in combination with NB was the most potent growth medium for adult rat NSCs.

In conclusion, the present results suggest that a cell-density-dependent response to growth factors, either via intrinsic or extrinsic signals, could be due to the activation of extracellular matrix molecules or growth factor receptors whose interaction is promoted by cell proximity (reviewed in [196]). The choice of medium, supplements and growth factors in addition to a gentle enzymatic dissociation have great impact on the proliferation and self-renewal properties of adult derived NSCs.

V.1.2. NSC derived neurospheres express markers for all three major cell classes of the CNS

The expansion potential of a given cell culture is an important factor of NSC-based transplantation strategies. However, it is also crucial to determine the differentiation potential of the expanded cultures. The differentiation of NSC cultures was induced by removal of growth factors and addition of 1% FCS, which was previously shown to be sufficient for the differentiation of rodent NSC cultures for young neuronal markers (see section IV.1.2.2.). Stronger differentiation stimuli, such as retinoic acid exposition or prolonged differentiation period, may be required to further analyze the differentiation into more mature neuronal markers.

The observations using different media/supplement combinations during differentiation of adult rat NSCs suggested that all, including NB/B27, facilitated neuronal and glial differentiation. Furthermore, the percentages of generated neurons and glia per analyzed culture were not significantly influenced by the different combinations. Despite the high expansion rate in NB/B27, the results demonstrate that adult rodent-derived cultures maintained multipotency and could differentiate into the three main neural cell classes: neurons, astroglia and oligodendroglia. The designation neuron in this context is based on the detection of β III tubulin, but it should be kept in mind that the expression of β III tubulin implies only a certain degree of cell maturation towards the neuronal phenotype. Other neuronal markers, such as neurofilament proteins and neurotransmitters, as well as the electrophysiological property of a cell, indicate a mature neuronal phenotype. Further discussion of the usage of β III tubulin will follow in section V.2.3.

In conclusion, it was demonstrated that media and supplements currently used for *in vitro* expansion of NSCs promote significantly different rates of proliferation. Optimization of the growth conditions allowed for the expansion of cells under clonal conditions without the use of serum or conditioned medium. It is noteworthy that the different medium compositions did not alter the differentiation potential of adult NSCs.

V.1.3. Constitutive expression of Notch1 has different effects on the SVZ and HC derived neural stem cell populations

The Notch/Delta signaling pathway, first described in *Drosophila melanogaster*, is a key pathway regulating the maintenance of a pool of uncommitted precursor cells and inhibiting cells from differentiating along the neuronal lineage during neurogenesis (reviewed in [100, 102]). A recent study by Gaiano revealed that the signal via Notch1, in addition to inhibiting neuronal differentiation of progenitor cells, might actually promote the acquisition of a glial phenotype [103]. Interestingly, several studies showed that Notch signaling effectively promoted glial cell types that at the same moment, are discussed to retain properties of progenitor or stem cells. This includes radial glia [156, 197], Bergmann glia, a radial glia type in the developing vertebrate cerebellum [198, 199], GFAP-positive astrocytes of the SVZ [200, 201] and Müller glia cells of the vertebrate retina throughout several species [101, 105, 157, 202]. In this context, an important finding was made by Tanigaki and colleagues. The promotion of astrocyte identity by activation of Notch1 ultimately leads to a decrease of neuronal and oligodendroglial fate [203]. In fact, the differentiation along the oligodendroglial lineage is actively inhibited [204].

In order to assess the plasticity and differentiation potential of NSCs, cells constitutively expressing Notch1 were generated. This was performed by the introduction of a Notch1 gene construct via retroviral infection. For indirect assessment of Notch1 expression, a reportergene was added downstream of Notch1. For this, the green fluorescent protein (GFP) was used, which can be directly quantified in intact cells due to its autofluorescence. Preferred vehicles for infection of NSCs are viral vectors, since they provide the most efficient and stable introduction of desired gene constructs. In particular, retroviral infection of NSCs is considered to be a reliable tool to stably integrate gene constructs into the host genome [205]. Moreover, retroviral vectors efficiently infect proliferating NSCs both *ex vivo* and *in vivo* [156, 203], but face one major obstacle with respect to introducing a gene construct: the integrated viral construct faces significant, time-dependend down-regulation by several silencing mechanisms [206-209]. Therefore, suitable viral vectors need to be carefully selected and optimized to overcome gene silencing in NSCs [210]. The retroviral construct used in the present study proved to be stably expressed in adult derived NSC populations (SVZ and HC from rat brain) for

the duration of the differentiation study (14 days *in vitro* after infection plus 7 days on poly-L-ornithine/laminin coated glass coverslips).

In the differentiation study, diverse results were obtained for the two neural precursor cell populations isolated from the adult rat SVZ and HC. While in the SVZ, the constitutive expression of Notch1 resulted in a significant decrease of neuronally committed cells as indicated by a reduction in β III tubulin-positive young neurons, the number and morphology of cells expressing β III tubulin in the HC derived cultures was not affected. Interestingly, the activation of Notch1 in SVZ cultures also led to a significant reduction of GFAP-positive glia cells *in vitro*. At the same time, expression of the early glial marker NG2, which is also expressed in a population of oligodendrocyte precursor cells [211], was upregulated slightly in SVZ derived NSCs. This suggests that a population of NG2-positive cells remains uncommitted, resulting in a lower number of GFAP-positive mature glial cells. This conclusion is in accordance with previous publications that suggest a role of glia-specified, yet uncommitted precursor cells to arise from Notch 1 positive cells (reviewed in [103]).

Furthermore, in HC derived cultures, the expression of NG2 was dramatically increased in the Notch1 expressing cells, accompanied by no effect on the GFAP- and β III tubulin-positive population. The observation suggests an increase in an early glial or oligodendroglial phenotype in HC derived stem cells. The population of cells not detectable by either β III tubulin, GFAP or NG2 immunocytochemistry probably has no stem or progenitor-like properties. Their identity was not detected with the antibodies used in this study. Moreover, detailed studies addressing the self-renewal and clonal capacities of these cells need to be conducted in order to comprehend the nature of these genetically modified NSC populations.

An interesting hypothesis, resulting from a study of Walz [212] and in accordance with a recent review [213], outlines that not all astrocytes are necessarily GFAP-positive. In fact, a population of smooth protoplasmic astrocytes that express mRNA for GFAP in 75% of the cells, but never mature to express the protein, are more general precursor cells. They reside in the adult brain grey and white matter and are typically S100β positive [214]. This specific cell type is an oligodendrocyte precursor cell (OPC), as it shares NG2 immunoreaction with cells that have been shown to give rise to

oligodendrocytes [130, 211]. The authors suggested that these cells are bi-potent in terms of performing both physiological as well as progenitor functions. A most interesting observation in that respect is the function of these OPCs as neuronal precursors [215] and the fact that NG2+/GFAP- cells persist in the SVZ where they most likely represent a new class of postnatal multipotent progenitor cells [216]. The same authors suggest that these cells represent a cellular reservoir for renewal of postnatal and adult inhibitory interneurons in the hippocampus [216]. In conclusion, the population of GFAP-negative NSCs in SVZ cultures indicate a population of yet undifferentiated, GFAP-negative but NG2-positive astrocytic cells, which retain their progenitor character during constitutive Notch1 expression. The results of the immunocytochemical study in the HC derived cultures suggests that Notch1 expression leads to an increase in cells that will most likely differentiate along the glial lineage (as indicated by the NG2 immunoreaction), but that retain some of their progenitor character.

V.2. Postnatal derived progenitor cells from the sensory retina share typical characteristics with NSCs

Neurogenesis in the developing mammalian retina ceases during the postnatal period and is absent in the adult [71, 217]. This correlates well with the presence of neural progenitors during the embryonic period [168] and the absence of active neural stem cells in the adult sensory retina [218, 219]. To determine, if the postnatal mammalian retina harbors progenitor cells and to complete the analysis of progenitor populations in the eye, a major focus of this study was the identification and characterization of retinal progenitor cells during the postnatal period [159].

Retinal progenitors described in the present work were isolated at postnatal days 1 (P1), P3, P8 and P14. They were characterized by i) their potential to proliferate, as analyzed by total cell number in growth curves over 28 days *in vitro*, incorporation of BrdU and their capacity to self-renew in clonal assays, ii) by expression of the progenitor markers nestin, Flk-1, Pax6, Chx10, the radial glia marker BLBP, and iii) by their potential to differentiate into neuronal and glial cell types, including retina-specific types (expression of recoverin).

V.2.1. Proliferation and growth as neurospheres

The isolation of retinal progenitor cells was performed in accordance with previously described methods [168, 220-223]. Special care was taken in preparations to avoid contamination of the cultures with parts from the optic nerve or the central retina, because in the early postnatal period, these regions mostly consist of astrocytes and endothelial cells [224]. Of optimal culture conditions in terms of plating, cell-density and medium composition were developed. Traditionally, the growth of retinal progenitor cells has been shown to be maintained at high levels when the cells were cultured as explants or aggregate cultures [225-227]. However, in order to assess the similarities of retinal progenitors and CNS stem cells, equal conditions should be utilized. This was accomplished by strictly transferring the experimental approach of the cell culture optimization study for adult derived CNS stem cells (see section IV.1.1). Therefore, retinal progenitor cells were grown in single cell cultures after their initial growth in neurospheres. Highest growth rates for CNS derived adult stem cells were supported by NB/B27 medium. Therefore, it was also chosen for the postnatal retinal progenitor cultures. The expansion of cells under serum-free conditions was performed according to published data [228]. The addition of bFGF and EGF to the culture medium is essential for growth rates and proliferation. Extensive documentation in the literature convincingly shows that both factors are important to generate sufficient amounts of neurospheres and to maintain a pool of surviving progenitors in retinal cultures, which was reported for CNS stem cells as well [152, 168, 229].

Similar to adult derived CNS stem cells, postnatal retinal progenitor cells grew after isolation as neurospheres, which were dissociated for passaging using Accutase®. Once dissociated, cells were capable of generating secondary neurospheres.

In other respects, retinal progenitors are not similar to NSCs. Regarding the growth curves *in vitro*, postnatal retinal progenitors display a linear growth and, when monitored over 28 days (see section IV.2.1.), reach a plateau, indicating a growth arrest. However, cell survival does not seem to be affected, as the total cell number is stable. In contrast to this, adult derived NSCs both from the SVZ and HC show an exponential growth (see section IV.1.1.3), yielding much higher total cell numbers. Furthermore, the proliferation rate in NSC cultures is much higher than in retinal progenitors and can be

monitored over a longer period of time. Eventually, this results in an increasing number of passages in comparison to the retinal progenitor cells. In particular, retinal cells derived from P14 animals were very restricted in their proliferation capacities, indicating that at this age, a population of proliferating progenitors is diminished in the sensory retina. Development-dependent changes in the ability to self-renew have been described for progenitors derived from mouse embryonic day 10 (E10) to E17 brain [230]. In that study, E17 derived progenitors were much more restricted than E10 derived cells. Further studies with E17 derived retinal progenitor cells reported poor survival of secondary neurospheres and failure to generate tertiary neurospheres [231]. In addition, studies on BrdU incorporation as mitotic indicator in P13-15 mice revealed that no proliferation can be observed at this age *in vivo* [232].

All studies, including the present one, coherently demonstrate that unlike NSCs, postnatal retinal progenitors only have a limited potential to proliferate, which decreases with increasing age of the animals. This might be due to intracellular mechanisms such as control of cell cycle exit of progenitors [233, 234] or due to external factors, such as the lack of growth promoting cell culture conditions (reviewed in [235]). One precise example for a group of external factors are the vertebrate hedgehog (Hh) proteins, which are secreted molecules that are involved in various developmental processes, including cell proliferation [236]. During eye morphogenesis and retinogenesis, Hh proteins are secreted by the anterior ventral midline tissues, RPE cells and some types of differentiated retinal neurons. RPE cells have been excluded from isolations of postnatal retinal progenitors and differentiated retinal neurons are generally not supported by the chosen media conditions. Therefore, the Hh signal, which promotes proliferation in the cultures of progenitors, might simply be missing.

The clonal analysis further revealed differences between postnatal retinal cultures NSCs. While adult derived NSCs from the HC are capable of self-renewal under clonal conditions (see section IV.1.1.4.), postnatal retinal progenitors completely failed to grow in limited dilution assays as well as after FACS sorting as single cells. In contrast, E17 and P0 derived mouse progenitor cells isolated from the SR exhibited clonal growth under limited dilution conditions [237]. The lack of clonal growth in the present study can be explained by the higher age of the cells (first isolation at P1) and the apparent loss of

self-renewal properties. Cell-cell contact seems to be of great importance for these cells too (see section V.1.1.) and the lack of either direct contact and the associated signaling from neighboring cells or diffusible factors from other cells in culture might be the explanation for the complete inability of these cells to self-renew under clonal conditions.

V.2.2. Postnatal derived retinal progenitors express markers specific for precursors and stem cells

A commonly accepted procedure to determine the progenitor or stem cell character of a cell population is the expression analysis of markers such as the intermediate filament nestin, which is specifically expressed in uncommitted precursor cells and is downregulated once the cells leave the cell-cycle at the commencement of a fate choice and differentiation [133]. Nestin expression was observed in all postnatal progenitor cultures from P1 to P14, but a slight, age-dependent decrease in the total number of nestin-positive cells was observed, indicating a more differentiated state of the retinal cells during later developmental stages. This result is in accordance with previous data, showing that the down-regulation of nestin is followed by the upregulation of alternative intermediate filaments during neuronal [238] as well as glial differentiation [40], when cells acquire a more mature phenotype.

The tyrosine kinase receptor Flk-1 binds vascular endothelial growth factor (VEGF) and is specifically expressed in retinal progenitor cells [142]. The Flk-1 immunocytochemistry revealed that while almost all P1 cells were positive, the expression decreased in P3 and in P8 and P14 cultures, no Flk-1 immunoreaction was present. The age-dependent decline was confirmed at mRNA level as shown by RT-PCR (see section IV.2.2.). The down-regulation of Flk-1 protein and mRNA levels was also shown for mouse retina *in vivo* during neurogenesis and in the adult [142].

Furthermore, the paired-type homeobox gene Chx10 was used as a progenitor marker in this study. The expression of Chx10 is restricted to undifferentiated retinal neuroepithelial cells early during development [139], and only a subset of bipolar interneurons maintains expression of this putative transcription factor. Chx10 expression was observed in cells at all ages, but at P8 and P14, the total number of positive cells was

distinctly reduced. In addition, P1 derived retinal progenitors co-labeled for Flk-1 and Chx10.

Another important progenitor marker for retinal cells is Pax6, which is a paired-domain homeobox gene and belongs to a big family of highly conserved genes that regulate crucial developmental processes in various species [239]. Besides being a proneuronal gene, Pax6 is essential for proliferation and multipotency in a pool of undifferentiated progenitor cells within the optic vesicle [113, 240]. The expression of Pax6 was observed at all developmental stages, but showed a slight, age-dependent decrease over time, reflecting the *in vivo* situation, in which cells increasingly differentiate and downregulate Pax6 expression.

The radial glia marker basic lipid binding protein (BLBP) in retinal cultures was expressed in a subset of cells, suggesting the presence of radial glia type stem-or progenitor cells. This indicates a similarity between retinal progenitor cells, radial glia and neural stem cells, which was shown previously for the postnatal chicken retina [73, 241]. The authors showed that Müller glia cells are a source of proliferating progenitors that can generate neurons.

In conclusion, progenitors from the postnatal rat retina share common characteristics with adult rat derived NSCs with regards to growth as neurospheres and expression patterns of specific precursor and stem cell markers.

V.2.3. Postnatal retinal progenitors differentiate along the neuronal and glial lineage

Multipotency is characteristic for NSCs and describes the potential to differentiate into neurons, astroglia and oligodendroglia [16]. The expression of various early and late neuronal and glial markers was evident. In contrast, markers for oligodendroglia were not detected in postnatal retina derived progenitors under permissive differentiation conditions.

The expression of β III tubulin as an indicator for early postmitotic neurons, was analyzed by immunostaining and RT-PCR (see sections IV.2.3.1. and IV.2.3.3.). Both RT-PCR and immunocytochemistry were positive in postnatal retinal progenitor cells at all ages. The morphology of cells expressing β III tubulin was distinctively neuronal, with multiple processes protruding from the cell body. The use of β III tubulin as a

specific neuronal marker needs careful evaluation. For one, β III tubulin is transiently expressed in the telencephalic subventricular zones, where putative neuronal and glial precursor cells reside [242]. In addition, the protein is expressed in several tumors of non-neuronal origin, indicating that the expression of β III tubulin alone cannot be sufficient to localize postmitotic neurons [242, 243].

In order to analyze the neural progenitor state of the cells, doublecortin (DCX) was used as marker. DCX encodes for a microtubule-associated protein that is essential for proper brain development as it is expressed in migrating neuronal precursors during development (reviewed in [10]). Furthermore, DCX is transiently expressed during neurogenesis and can be detected in postmitotic and proliferating neuronal precursors in the areas of continuous neurogenesis [138]. Since DCX is downregulated in cells that start to express more mature neuronal markers, it is an ideal marker to detect ongoing neurogenesis [138]. The expression of DCX in postnatal retinal cultures showed that neuronally committed, but yet undifferentiated progenitor cells persist in these cultures and strong colabeling with β III tubulin indicated their neuronal fate determination. Co-expression of DCX with either nestin or GFAP was not detected.

Neuron specific enolase (NSE) and microtubuli-associated protein (Map2ab) are markers that are associated with more mature neurons. NSE is expressed in most neuronal retinal cell types from later developmental stages to adult life [164]. Map2ab is a major component of the neuronal cytoskeleton with a function in assembly and stabilization of microtubules during neuronal differentiation [126]. Both markers were frequently detected in older postnatal retinal cultures, corresponding to the *in vivo* situation, in which the retina is increasingly differentiated and therefore, expresses more mature neuronal markers. In contrast to the neuron specific marker Map2ab, some reports indicate that the NSE antibody reacts with astrocytes and/or oligodendrocytes [244, 245].

Differentiation along the glial lineage was monitored by the expression of two markers for immature glia cells: A2B5 and NG2. A2B5 is a marker for glial progenitors expressed in various tissues [129]. It is downregulated with the acquisition of a more mature glial phenotype and previous work showed that a subset of A2B5 positive precursor cells from the embryonic cortex can actually generate neurons later during neurogenesis [246]. Therefore, the pool of A2B5 positive progenitors is heterogeneous.

A2B5 expression was observed in all postnatal retinal cultures, and the number of positive cells corresponded with those for another early glial marker, NG2, suggesting that not all of these cells are committed to mature to glia, but rather differentiate along the neuronal lineage.

NG2 is a chondroitin sulfate proteoglycan and originally was thought to be expressed exclusively in oligodendrocyte progenitors, but more recent studies demonstrated that NG2-positive cells are capable of generating neurons after they de-differentiated and built neurospheres [215]. The postnatal retinal progenitor cells showed distinct staining patterns for NG2, but failed to up-regulate more adult oligodendrocytic markers like GalC, indicating that most of all cells acquired a bi-potent phenotype. These results are in agreement with a previous study, in which the authors tested mouse retinal progenitor cells in clonal-density cultures for their multipotency [237]. Only neuronal and glial cells types were generated. Developmental changes in the intrinsic competence for multipotency might contribute to the bi-potent phenotype, since progenitors derived from E17 retina are able to express oligodendrocytic markers such as O4 and GalC [168].

The expression of the adult glial marker GFAP was detectable in approximately 25% of postnatal retinal progenitor cells. A direct comparison with SVZ derived stem cells (see section IV.2.3.1.) revealed that while the differentiation along the neuronal lineage (as indicated by III tubulin expression) occurred in equal percentages in both cell types, postnatal retinal progenitors showed significantly higher percentages of GFAP-positive glial cells. Considering the fact that there is only one glial cell type in the mature retina (the Müller glia cell), while the neuronal differentiation leads to six distinct retinal cell types (rods, cones, ganglion-, bipolar-, amacrine-, and horizontal cells), the high amount of GFAP-positive cells *in vitro* is surprising. A possible explanation could be that the applied differentiation conditions (NB/B27, 1% FCS, no growth factors) promote the maturation along the glial lineage. Similar results were observed for other cell types [154].

V.2.4. New neurons and glia can be generated from postnatal retina in vitro

The crucial question whether neurons and glia are newborn or only co-stain for BrdU incorporation due to DNA repair mechanisms in postmitotic cells, demands critical

interpretation (see section V.1.1. and [178, 247]). Therefore, the observed colabeling of β III tubulin or GFAP with BrdU in a small subset of postnatal retinal progenitor cells at a younger stage (see section IV.2.3.2.) was confirmed by immunocytochemichal analysis of the cell cycle specific marker Ki-67. An interesting result in this respect is the decrease of doublelabeled cells with an increase of the age of the animals. While a small subset of P1 and P3 derived postnatal progenitors generated new neuronal cells, P8 and P14 derived cells did not have that capacity. This result is coherent with the observation of reduced expression of precursor markers in P8 and P14 cultures (see section IV.2.2.). The generation of new glia, indicated by colabeling for GFAP and BrdU, was more frequently observed than generation of neurons at all isolation ages. These results are supported by the observation that generally in all cultures of different age groups, postnatal retinal progenitors predominantly differentiate along the glial lineage (see section IV.2.3.1.).

V.2.5. The neurogenic competence of postnatal retinal progenitors is age-dependent

After the immunocytochemical analysis of postnatal retinal progenitor cells under the influence of one differentiation conditions, a more detailed expression study was conducted under three differentiation conditions by RT-PCR to evaluate their neurogenic competence. The applied differentiation conditions were NB/B27 supplemented with i) low serum concentration (1% FCS, no growth factors), ii) high serum concentration (5%FCS, no growth factors) and iii) a cocktail comprised of forskolin, cAMP and KCl. Forskolin is a diterpene, which increases the intracellular level of cyclic adenosine monophosphate (cAMP) [248]. The forskolin mediated increase of intracellular cAMP is most likely induced by direct interaction with adenylate cyclase, the enzyme that catalyzes the formation of cAMP [249]. Elevated levels of cAMP have been shown to be important for GFAP gene expression [131].

Forskolin is a widely used agent in a variety of aspects (reviewed in [250]), since it not only increases the cAMP levels, but because it also interacts with other proteins like glucose transporters and ion channels [249, 251]. Thus, forskolin mimics spontaneous neuronal activity, which is, for instance, essential for the development of the CNS. Spontaneous neuronal activity has been observed between neighboring ganglion and amacrine cells during development, long before photoreceptors can respond to light

(reviewed in [252]), and is required for rod photoreceptor development *in vitro* [253]. Furthermore, it induces the expression of cortical neurotrophins. *In vivo*, neurotrophins are the master regulatory elements of cortical and subcortical cell growth (reviewed in [254]) and are essential determination signals during retinogenesis [255-257]. *In vitro*, they act as critical factors in stem cell biology, both as ligands [258-260] as well as secreted factors in NSC cultures [261]. Furthermore, the application of forskolin in different culture systems caused decreased expression of neuronal markers and increased expression of glial markers [262]. Functional expression of voltage-gated Na^+ and Ca^{2+} channels during neuronal differentiation [263] and promotion of astroglial differentiation in human neurocytoma cells [264] has also been ascribed to forskolin.

The addition of cAMP and KCl to the cocktail enhances the effects of forskolin and mimics electrical activity in the cultures. The differentiation of hippocampal neurons [265] as well as the induction of neuronal differentiation in rat pheochromocytoma PC12 cells [266] are both dependent on the combined effects of forskolin, cAMP and KCl. Considering all aspects, the forskolin condition was chosen to mimic electrical activity and because of its influence on developmental processes known, from *in vivo* and *in vitro* studies. cAMP and KCl were added to enhance the effect of forskolin.

Analysis of the expression of the proneural gene Pax6 and the retinal progenitor marker Flk-1 revealed downregulation in both cases with increasing age of the rats (see section IV.2.2.). The detailed expression study by RT-PCR showed no alteration of the expression of Flk-1, nestin, DCX and Pax6 under the influence of the three applied differentiation conditions. A noteworthy change was the upregulation of the bHLH transcription factor neuroD in P3 derived retinal progenitor cells both under 5% FCS and forskolin conditions in comparison to 1% FCS. NeuroD is a essential factor for the development of amacrine and photoreceptor cells during retinogenesis [119, 267]. The peak of amacrine cell generation occurs at E14, with most of the cells completely matured at P3. The upregulation of neuroD in P3 derived retinal progenitors suggests that the maturation process underlies plasticity and that the applied culture conditions are capable of extending this phase of maturation. The model of retinal cell fate determination, as it was proposed by Cepko and colleagues, seems to be the theoretical basis for this plasticity [71]. The model is based on the principle that retinal progenitors, both embryonic

and postnatal, undergo a series of cellular states. The state of a cell is defined by its ability to respond to signals from the environment and to generate one or more cell types. The capacity to respond to signals is strictly correlated to the expression of certain transcription factors, such as neuroD. This state of competence is modulated in a transient manner. Previous data suggests that once a cell has changed its state, the step is irreversible [268, 269]. Results from the expression study by RT-PCR yet suggests a partial reversibility. On one hand, retinal cells could acquire an immature, stem cell like type and upregulate determination factors. On the other hand, a pool of already lineage-restricted progenitors could exist during the postnatal period that has upregulated determination factors, but has not yet reached a mature state. The observed upregulation of the downstream element of the Notch/Delta signaling pathway, Hes1, in P3 and P8 derived cultures under all conditions suggests a similar de-differentiation process.

In conclusion, markers that *in vivo* have been downregulated due to maturation of retinal progenitor cells are re-expressed in postnatal retinal cultures *in vitro* and indicate that retinal progenitors of the postnatal period undergo plastic changes.

V.2.6. A subset of early postnatal retinal progenitors is mitotic and expresses the retina-specific photoreceptor marker recoverin

The retina-specific differentiation of retinal progenitor cells grown in NB/B27 media with 1%FCS and without growth factors was analyzed by immunocytochemical stainings of the Ca^{2+}-binding protein recoverin. Recoverin can be found in adult rods and cones, a subpopulation of bipolar and retinal ganglion cells [1, 270], but not in all photoreceptors [271]. It has been reported that some recoverin-positive cells resemble proliferating neuroepithelial cells because of their bipolar-shaped morphology [272]. These immunoreactive cells are not postmitotic photoreceptors, but possibly precursor cells that started the expression of markers of their eventual progeny [252].

In the present study, recoverin immunoreactivity was observed in retinal progenitor cells of all age groups, but a distinct increase in recoverin-positive cells was found (see section IV.2.3.4.). This increase is coherent with the increase of mature photoreceptor cells in older postnatal cultures. The expression of recoverin in P1 and P3 cultures might indicate cells that are not yet completely differentiated photoreceptors, but

are bound to differentiate into mature ones. A very small amount of recoverin-positive cells at P1 and P3 also colabeled with BrdU, indicating cell division of these cells after preparation. Previous reports were not able to demonstrate Ki-67 or BrdU labeling in recoverin-positive cells *in vivo* [273]. Therefore, the authors concluded that recoverin-positive cells must be postmitotic. The results of the present study suggest that the application of differentiation conditions *in vitro* induces plasticity of retinal progenitor cells and allow these cells to remain mitotic for a longer period of time.

V.3. Adult derived progenitor cells from the ciliary body and retinal pigment epithelium share characteristics with NSCs

This study provides comparative analyses of NSC properties and the neurogenic potential of adult CB and RPE derived cells *in vitro*. Both CB and RPE derived cells characteristics of NSCs and were tested in reference to i) proliferation, ii) self-renewal, based on primary and secondary neurosphere formation and clonal analysis, iii) NSC identity, based on the expression of markers used to identify neural stem and progenitor cells, such as nestin, Flk-1, Hes1 and notch1 and iv) differentiation into neuronal and glial cells, based on the expression of differentiation markers, such as DCX, β III tubulin and GFAP.

V.3.1. Adult CB and RPE derived progenitor cells proliferate and self-renew in vitro

Multipotential retinal stem cells reside in adult stages of several vertebrate species such as poikilothermic vertebrates (fish and amphibians) as well as reptiles, where only a small portion of the retina is generated during the embryonic phase and most of the retina is produced in the larval period and grows throughout the entire life of the animal (reviewed in [62]). Extensive regenerative capacities have been shown for the retina of mature uroldele amphibians, fish and embryonic homeothermic vertebrates such as birds (chicken) and mammals [274]. The source of these newly generated cells is the peripheral margin of the eye. There, cells are produced and subsequently inserted into the existing and functional retinal circuits as long as the animal is alive. Interestingly, clones generated by the most peripheral cells in the ciliary margin contain cells of the neural

retina and the RPE [63]. However, retinal regeneration in amphibians is mostly driven by the RPE [79]. Since RPE cells share common ancestors with neural retinal and CB cells, their potential to contribute to regeneration in the retina is likely. Homeothermic animals have the ability to regenerate as well, although the capacities are restricted to the embryonic stage in chicken [83] and depend on the effects of basic fibroblast growth factor (FGF) [82]. Only few results have been published on the regeneration potential of the RPE in embryonic mammals [81]. Despite that, a pool of multipotent stem cells has been identified in the ciliary body of adult mammals [67, 68].

In order to assess the proliferative potential of RPE cells, the already known population of CB derived stem cells served as controls. The culture conditions used in the present study were adapted from a recent publication that compared different culture media and supplements for adult NSCs [152]. This study concluded that the combination of NB/B27, which is the medium used in the present work, provides improved growth and proliferation conditions for adult SVZ, HC or SC derived neural stem cells than the widely used DMEM/F12/N2 combination. Moreover, the NB/B27 combination has been demonstrated to allow growth of postnatal retinal progenitor cultures as well [159]. DMEM/F12/N2 was the standard medium used by Tropepe et al. [67] and Ahmad et al. [68], the two studies that revealed retinal stem cells in the adult mammalian CB. Both studies indicated that compared to cells from the CB, RPE cells are devoid of stem or progenitor cell properties in adult rodents. The addition of FGF proved to be essential for NSC populations and has previously been shown to influence the regenerative capacities of retinal cells [84, 86, 275]. Growth curves for CB and RPE derived cells showed an initial increase in cell number after plating (see section IV.3.1.), but similar to postnatal retinal progenitors, the growth ceased after approximately two weeks in culture, when a plateau phase was reached. There was no significant difference in the growth kinetics of CB and RPE derived cells. Both grew as neurospheres and proliferated as demonstrated by BrdU incorporation and expression of the cell cycle marker Ki-67 (see sections IV.3.1. and IV.3.3.).

The results present for the first time that RPE cells have progenitor properties. In the Tropepe study, RPE cells showed no clonal expansion and lacked the capacity to generate spheres [67], but different culture conditions were used. The authors concluded

that RPE cells are in no way similar to CB cells and excluded them from their further analysis on differentiation potential of adult retinal derived stem cells. The present study demonstrates that RPE cells share characteristics with CB derived stem cells. The gold standard for NSC identification *in vitro* is proof of proliferation, self-renewal and neural differentiation of a clonally derived cell. Clonal growth of adult RPE cells has been demonstrated under adherent conditions on collagen matrix [171]. In the present study, clonal growth of RPE cells under neurosphere forming and adherent conditions was compared. Clonal growth of RPE cells was only successful under adherent conditions, but not under neurosphere conditions, suggesting that cell–matrix interactions are required for RPE proliferation. This is reminiscent of RPE based retinal regeneration in the embryonic chicken, which besides FGF requires cell-cell interaction [86]. Self-renewal of RPE derived cells is indicated by the fact that secondary neurospheres were obtained *in vitro*.

V.3.2. Loss of pigmentation in adult derived progenitors expanded under adherent conditions

When plated on collagen I and cultured in the presence of 1% FCS, both CB and RPE derived retinal progenitor cells started to adhere within hours after plating. After 2-3 days, cells began to migrate and grow on the provided substrate. With the process of migration, a loss of pigmentation was observed (see section IV.3.2.). The loss of pigmentation is typically a sign of a continuing de-differentiation process, since the cells start to downregulate genes that are generally expressed in their mature state [79, 80]. Furthermore, the loss of pigmentation is characteristic for the RPE cells with regenerative potential in poikiolothermic vertebrates, since the cells lose their melanin pigment granules prior to the initiation of cell division [276]. The choice of substrate is critical, since besides collagen I, also for laminin an inductive effect on de-or trans-differentiation processes in RPE cells was reported [79]. Whether RPE and CB cells indeed undergo trans-differentiation or if the process has a different background, will be discussed in section V.3.5.

V.3.3. Specific progenitor markers are expressed in adult derived CB and RPE cells

A direct comparison of naive, untreated and freshly isolated RPE and CB cells with cells grown for several weeks under differentiation conditions (NB/B27, 1% FCS, no growth factors) revealed that a set of specific progenitor markers is upregulated in long term cultures (see section IV.3.3.). The progenitor markers nestin and Flk-1 as well as the determination factors neuroD, Hes1, notch1, musashi and Pax6, the young neuronal marker β III tubulin and the neurogenesis marker DCX were expressed in both CB and RPE derived cells. The expression of the VEGF-receptor Flk-1, a specific marker for retinal progenitor cells [142], indicates that some characteristics of retinal progenitors have been acquired by the adult CB and RPE cells. The most interesting observation is the upregulation of the proneural homeobox transcription factor Pax6, which is transiently expressed by the prospective RPE *in vivo*, but is downregulated when the RPE finally differentiates [277]. More recent studies showed that FGF is efficient to induce trans-differentiation processes in postnatal chicken RPE and promotes the upregulation of transcription factors such as Chx10 and Pax6 in the pigmented cells of the pars plana[278].

Yan and Wang used of the bHLH gene neuroD as a trigger to induce photoreceptor markers in chicken RPE [279]. Interestingly, the application of FGF again proved to be essential in that case [280]. Therefore, the upregulation of neuroD in CB and RPE derived cells in the present study could indicate de-differentiation of the mature cells towards an undifferentiated phenotype that has the capacity to differentiate into neurons or photoreceptors. The upregulation of the young neuronal marker β III tubulin and the neural stem cell marker DCX was in parallel observed in immunocytochemical analyses of the cells. Furthermore, β III tubulin/DCX expression exhibited alterations in the morphology, which is discussed in section V.3.4.

In conclusion, it was evident that the cultivation as neurospheres in NB/B27 medium with FGF/EGF and the differentiation as adherent monolayer cultures on poly-L-ornithine/laminin had an effect on the plasticity and therefore, expression pattern as well as morphology of CB and RPE derived cells. Several key molecules of retinogenesis and cell fate determination, which are specific for retinal stem and progenitor cells, were upregulated in both cell types. This demonstrates that the adult derived cells i) have the

potential to extend their phase of plasticity and ii) can de-differentiate to become immature and similar to stem or progenitor cells.

V.3.4. CB and RPE derived cells expressing young neuronal and progenitor markers change their morphology

There is substantial evidence for neuronal markers in adult mammalian RPE derived cells like neuronal Na^+ currents [96, 97], β III tubulin [92], microtuble-associated protein 1b (Map1b) [95] and NF200kDa [171] (reviewed in [62]). RPE cells start to express the young neuronal marker β III tubulin once they are removed from their *in vivo* environment and placed in culture [281]. These observations suggest that RPE derived cells are in the early stages of de-differentiation [76], or that an immediate onset of neuronal trans-differentiation occurs. However, most of the RPE cells in culture maintain an epithelial morphology and only a minority of cells display neuronal morphology. The latter cells express mature neuronal markers like neuron-specific enolase (NSE) or NF200kDa [171, 281].

Similar results were observed in the present work. While almost all CB and RPE derived cells expressed β III tubulin with a distinct epithelial morphology, only a small subset of cells changed their morphology to express the same marker with a neuronal phenotype, showing longer processes protruding from the cell body (see section IV.3.4.). Further neuronal characterization of the subpopulation was performed by immmunocytochemical doublelabeling of DCX and β III tubulin. DCX is a specific marker for neuronal precursors and young neuronal cells during development and in the adult [138, 282-284]. It is transiently expressed in neuronally determined cells. Expression starts during the last cell cycles and ceases with the onset of mature neuronal markers such as NeuN [138]. In the eye, DCX expression is found during the developmental period with high expression between E18 and E20 [285]. The data presented here demonstrate that DCX can identify the RPE and CB subpopulation with neuronal morphology via colabeling with β III tubulin.

Retina-specific differentiation, as it has previously been described for CB derived stem cells [67, 68], was not observed under the conditions applied in the present work. RPE cells also lacked the expression of tyrosine hydroxylase (TH) as well as dopamine

receptor markers and did not secrete dopamine into the culture supernatant as tested by HPLC (see section IV.3.4.), although it is known that the RPE of several vertebrate species can react to dopamine treatment and express dopamine receptors [286]. Dopamine is stored and released by neurons of the retina and it has been shown that RPE cells can act as dopaminergic support cells in the neural retina and produce L-dopa [287]. Recently, clinical trials commenced, which utilized human derived RPE cells for transplantation into patients suffering from Parkinson's Disease. Functional improvement was observed, suggesting an increase in dopamine release by the transplanted cells [288-292]. The complete lack of TH immunoreactivity or secreted catecholamines in the RPE cultures could be another indication for the de-differentiation process within these cells.

V.3.5. Expression of neuronal and glial markers in adult derived RPE cells: de-differentiation or trans-differentiation?

The differentiation potential of CB and RPE derived cells is not restricted to neuronal fate. The present work revealed that cells of both areas express the young glial markers NG2 as well as the adult glial marker GFAP (see section IV.3.5.). The expression of NG2 indicates an oligodendrocyte lineage potential of these cells, since it is also expressed in oligodendrocyte precursor cells in the CNS [130]. On the other hand, expression of the mature oligodendrocyte marker GalC was not detected, suggesting that the adult derived retinal cells are not multipotent, but rather bi-potent.

Previous studies indicated that CB derived cells differentiate into cells expressing markers for photoreceptor and bipolar cells [67, 68]. The expression of retina-specific markers, especially photoreceptor markers, was not observed in CB and RPE derived cells in the present study. This might be due to missing extracellular stimuli or intrinsic competence to respond to the stimuli. Furthermore, restricting determinants might be present, either in the extracellular environment or intracellular by transcriptional repressors or epigenetic determinants. Molecular determinants, which might promote NSC properties or retina-specific phenotypes in RPE cells, have recently been revealed by developmental studies (reviewed in [172, 293, 294]). These studies conclude that by the inductive activities of lens placode derived FGFs and extraocular mesenchymal derived transforming growth factors (TGFs), initially indistinguishable neurepithelial progenitors

in the optic vesicle are induced to form neural retina or RPE, respectively. Differences in cell fate are induced by the transcription factors Otx, Wnt, Pax6 and Mitf, which are expressed in the prospective RPE and Pax6, Rx1, Six3 and Chx10, which are expressed in the prospective neural retina. Some of these molecules might be interesting targets for the future clinical application of RPE cells in cell replacement strategies, either by stimulation of endogenous RPE cells to proliferate and to differentiate into photoreceptor cells or by transplanting RPE derived cells with the potential to integrate into the diseased retina.

The fundamental question resulting from the present observations and the recently published results is, whether RPE cells de-differentiate to neural stem cells or directly trans-differentiate to neurons and glial cells. The process of trans-differentiation has been defined by Okada as "an alteration of the state of differentiation of cells that have already been, at least partially, specialized or programmed in a given direction under normal conditions *in situ*" [295]. In contrast, de-differentiation describes the process of a gradual loss of differentiation markers of a mature cell, which enables the cell to become a progenitor or stem cell and subsequently differentiate into a distinct lineage. Trans-differentiation of the RPE into neural retina has been observed in a number of vertebrate species (reviewed in [62, 76]). Other reports show trans-differentiation of newt, chicken and human RPE cells into lens [296-298]. Furthermore, FGF was found to be an essential factor for cell proliferation and trans-differentiation of the epithelial cell types into neuronal cell types [299].

An early notable change during trans-differentiation is the loss of pigmentation and the downregulation of RPE specific markers, including RPE10 and RPE65 [93, 94]. A change in morphology from epithelial to columnar type, resembling proliferating neuroepithelium, has been observed as well [79, 80]. Furthermore, an extensive proteome comparison of differentiated and de-differentiated human RPE cells showed that the expression of proteins related to cytoskeleton organization, cell shape, cell migration and mediation of proliferative signals, was induced [300].

In contrast to the extensive data on trans-differentiation in lower vertebrates, there is only limited data about this process in mammals available. Trans-differentiation is

typically analyzed by the cultivation of embryonic rat retina *in vitro* under the influence of various growth factors. Under these conditions, the differentiation pathway of rat RPE cells could be altered by the addition of FGF, which induced expression of both neuronal and retina-specific markers [81]. However, this event is dependent on a narrow time frame during the early phase of eye development. A study by Zhao et al. showed that at about E14 in rat, the commitment of developing cells to the RPE phenotype became irreversible. The latest stage during which neuronal markers like β III tubulin or NF200kDa, and retina-specific marker like HPC-1 (amacrine cells) were detectable, was at E12 and E13 in FGF treated rat RPE cells [81].

The results obtained in the present study suggest a de-differentiation process and the acquisition of an intermediate or late phenotype typical for NSCs for RPE derived cells. These results are coherent with a recent analysis using adult human RPE cell lines in combination with culture conditions that promote growth of NSCs [171]. Under these circumstances, RPE derived cells altered their morphology from epithelial to spherical or spindle-like morphology, which is reminiscent of NSCs [171]. Moreover, these cells did not express epithelial or mature neuronal markers, but upregulated neuronal markers after stimulation. The differentiation potential of RPE derived cells is not restricted to neuronal fate. In the present study, RPE cells expressed the glial markers GFAP and NG2, but no GalC. This observation suggests a de-differentiation into a bi-potent neural progenitor or stem cell like phenotype, rather than direct neuronal trans-differentiation.

The analysis of environmental signals or genetic triggers, which induce de-differentiation or trans-differentiation *in vitro*, is necessary for therapeutic applications of RPE cells. A key role may be assumed by FGF and its synergistic effect with fate controlling proteins. Several studies indicated that FGF possibly has an influence on the upregulation of crucial determination factors like neuroD [280] and Mitf [301]. In addition, FGF is a major activator of the mitogen-activated protein kinase (MAPK) pathway, which targets Mitf [301-303], cath5 [304] and Pax6 [305]. The observed upregulation of Pax6 and neuroD in RPE cells in the present study (see section IV.3.3.) once more suggests a de-differentiation process in RPE cells.

Epidermal growth factor (EGF), which is present in the culture medium used in this study, is important for amphibian RPE cell survival and like FGF, can signal along

the MAPK/ERK pathway [306]. Further factors that can induce de- or trans-differentiation events in RPE cells include light [307], the substrate [79, 308], as well as the extracellular matrix [309] and cell-cell contact [310]. In the present study, the substrate was changed from collagen I in the initial growth phase to laminin for the differentiation experiments, because a previous study revealed that laminin can induce trans-differentiation of RPE cells into neurons [78].

Future studies need to identify the detailed molecular mechanisms, which are associated with distinct steps of de-differentiation processes to provide more insight in changes of cell state and in NSC potential of RPE cells. The insertion of genes like the H-ras proto-oncogene in adult derived human RPE cells [311], or the overexpression of neuroD in chicken RPE can trigger trans-differentiation in RPE cells to express photoreceptor spcific markers [280]. Analyzing the molecular basis of RPE plasticity harbors tremendous potential for the application of de-differentiated RPE cells in the field of retinal degenerative diseases. The discussed results are summarized in Fig.V.1. to provide an overview for RPE plasticity.

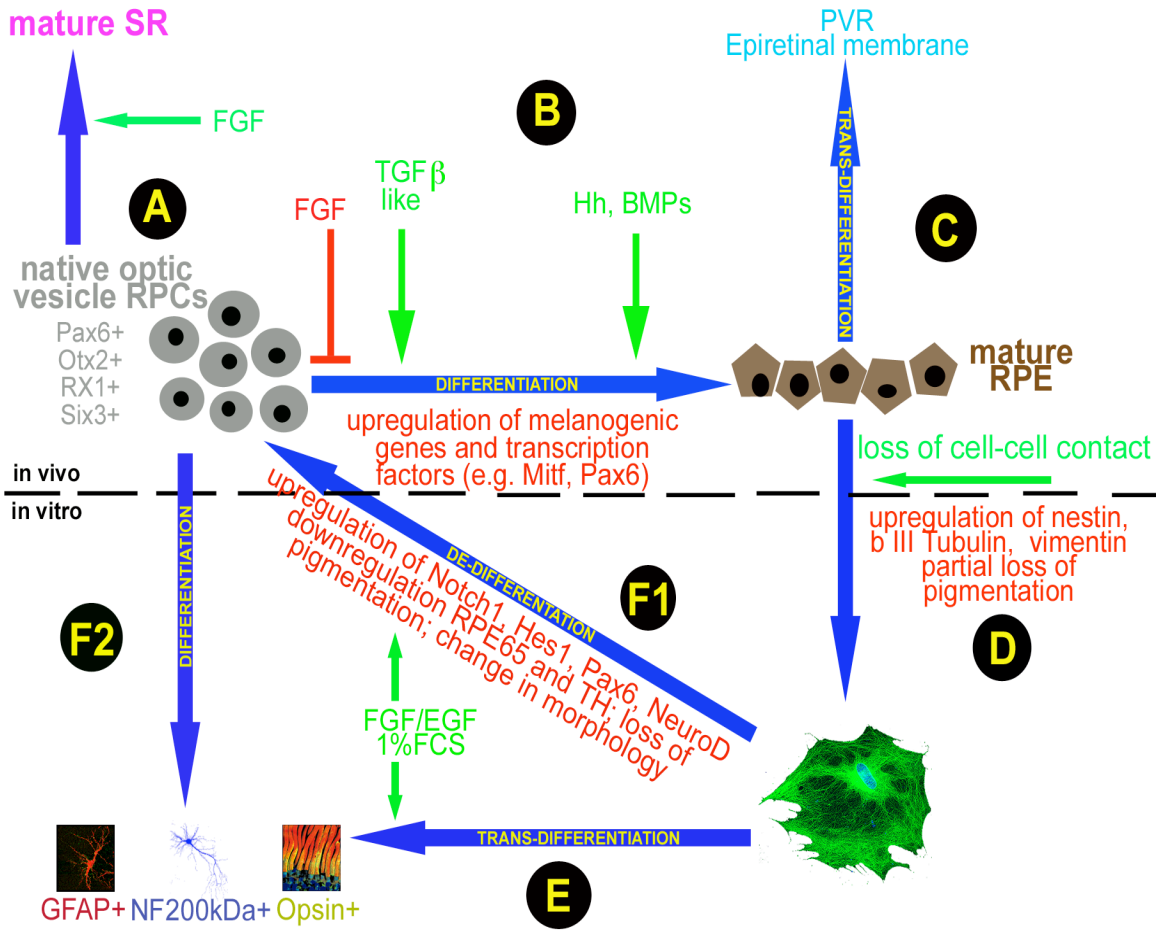

Fig.V.1. Proposed model of RPE plasticity. ***In vivo*** **A)** retinal progenitor cells (RPCs) of the optic vesicle are indistinguishable during early retinogenesis and develop either into sensory retina (SR) via FGF signaling or into RPE via TGF□-related signaling. **B)** Upregulation of transcription factors such as Mitf or Pax6, and signaling via Hedghog (Hh) and bone morphogenic proteins (BMPs) further induce formation of mature RPE. **C)** Loss of cell-cell contact causes proliferative vitreoretinopathy (PVR) or epiretinal membranes. ***In vitro*** **D)** Cultivation of RPE cells also causes loss of cell-cell contacts. **E)** Previous studies indicate that RPE cells can directly trans-differentiate into cells expressing mature neuronal markers (NF200kDa) and neuron specific enolase [171] accompanied by a loss of pigmentation. **F1)** The present study suggests the gradual de-differentiation of RPE cells towards their original status as RPCs, indicated by upregulation of the progenitor markers Hes1, Notch1, Pax6 and NeuroD, downregulation of the RPE specific marker RPE65 and tyrosine hydroxylase (TH), loss of pigmentation, and change towards neuronal morphology. **F2)** Finally, the acquisition of neuronal and glial phenotypes as differentiating, intermediate progenitor cells was observed. Both events under **E)** and **F)** are most likely triggered by growth factors and/or differentiation factors in the culture medium. The signaling mechanisms remain unknown.

V.4. Adult human derived RPE cells proliferate *in vitro* and express early and late neuronal and glial markers

Spontaneous proliferation of RPE cells in mammals was demonstrated in the adult primate, where with increasing age, cysts containing pigmented cells formed in the peripheral retina and pars plana [66]. The authors described that these cysts contained cells expressing the Ca^{2+}-binding proteins calbindin and calretinin as well as the homeodomain transcription factor Prox1. These antigens are generally found in Müller glia cells and in subsets of retinal neurons. However, the primary cause for the formation of adult primate cysts remains unknown. The pathological condition of epiretinal membranes in humans is another example of phenotypically altered RPE cells, which trans-differentiate after detachment from the retina and express markers of neuronal lineage [92].

Trans-differentiation of the RPE in humans occurs sporadically and is the cause of proliferative vitreoretinopathy. This condition often results from surgery treating retinal detachment. Due to the detachment, RPE cells extend into the subretinal fluid, enter the vitreous cavity, and proliferate on both the anterior and posterior surfaces of the detached retina [312, 313]. The trans-differentiation process is marked by an epithelial-mesenchymal transition. The cells undergo a differentiation program, which is marked by loss of their epithelial morphology and expression of specific markers (e.g. E-cadherin). They acquire a mesenchymal phenotype accompanied by the expression of markers such as □-smooth muscle actin [314, 315]. Chen and colleagues were able to show that retinamide, a synthetic derivate of retinoic acid, can induce the expression of mature neuronal markers in a human derived RPE cell line [316]. Interestingly, retinoic acid also induces the downregulation of the mature RPE specific marker RPE65 [317], which represents a critical development for the initiation of de-differentiation in RPE cells. A very recent study showed that the de-differentiation of adult human RPE cells lines, triggered by addition of FGF, EGF and retinoic acid, induces the expression of adult neuronal, but not retina-specific markers [171]. Furthermore, the authors described that glial differentiation, even under high serum conditions (8% FCS), could not be observed in human RPE cells.

Similar results were obtained in the present study. Adult human derived RPE cells proliferated *in vitro* as shown by the expression of the cell cycle protein Ki-67 in mitotic spindles of dividing RPE cells (see section IV.4.1.). The cultures expanded as

neurospheres and, like the adult derived CB and RPE cells from rats, could only be grown under clonal conditions if grown adherently. This result indicates a limited potential to self-renew. Almost all RPE cells were immunoreactive for the young neuronal marker β III tubulin, but showed a distinct epithelial phenotpe as previously reported [92]. In addition, β III tubulin-positive cells partially lost their pigmentation, suggesting that the process of de-differentiation had started. A small subset of cells showed a change in morphology towards a bipolar, neuronal-like phenotype without any pigmentation. In addition, the expression of the mature neuronal marker NF200kDa could be observed in these cells (see section IV.4.2.). Remarkably, BrdU was incorporated in a rare subset of NF200kDa-positive cells, indicating that new neurons could fully mature after isolation and proliferation *in vitro*. This observation suggests that BrdU/NF200kDa-positive cells first acquired a proliferative state resembling progenitor or stem cells and then differentiated into mature neurons. Furthermore, it might be an indication for the potential of adult RPE cells to de-differentiate instead of direct trans-differentiation.

Expression of glial markers in de-differentiated adult human RPE cells has not been reported so far. Amemiya and colleagues demonstrated that despite the presence of high serum concentration (8% FCS) in the culture media, which normally triggers astrocyte differentiation [154, 318, 319], no immunoreaction for GFAP was detectable [171]. The present results showed expression of both young (S100β) and mature (GFAP) glial markers in adult human RPE cells in NB/B27 with 1% FCS (see section IV.4.3.). This result strongly suggests that the de-differentiation of adult human RPE cells is bi-directional. In comparison to NSC characteristics, RPE cells lack the potential to self-renew under clonal conditions and to express markers of the three major cell classes of the CNS (neurons, astroglia, oligodendroglia). On the other hand, the de-differentiation process results in a cell state that can be induced to differentiate along two lineages and also harbors properties of progenitor and stem cells.

V.5. Potential therapeutic application of retinal progenitor and stem cells

Currently, there is no satisfying treatment for blinding disorders that result from degenerative processes of the retina and mainly affect photoreceptors. The main focus of

present cell therapy development is the replacement of degenerated and thus lost photoreceptors by transplantation of suitable cells into the subretinal space between the outer retinal layers and the RPE. Several strategies have been developed and include transplantation of retinal sheets (reviewed in [320]), RPE cells (reviewed in [321]), iris pigment epithelium [322, 323], Schwann cells [324], retinal progenitor cells (reviewed in [325]), neural stem cells (reviewed in [221]) or bone marrow mesenchymal cells [326-328]. The application of trophic factors (reviewed in [329]) or dietary supplements [330] as well as retinal prosthesis [331-333] were considered as alternatives. More recently, gene therapy has become an interesting modality [334, 335].

V.5.1. Replacement strategies for degenerating photoreceptor and RPE cells

Retinal degenerative diseases are classified three major groups: those affecting primarily photoreceptors (retinitis pigmentosa (RP) and related diseases), those involving the RPE (e.g. age-related macular degeneration (AMD)), and those affecting retinal ganglion cells (glaucoma). Most of the RP group are caused by single gene mutations, which contribute to photoreceptor death. Over 100 single gene mutations for RP have been identified [336]. The AMD group is mostly comprised of diseases caused by polygenic incidents with a strong environmental influence. Glaucoma results in optic nerve degeneration due to high intraocular pressure.

Numerous studies regarding replacement strategies have been conducted in the past, which address fundamental questions, e.g. if the grafted cells will survive, and function normally in their ectopic environment and if they will support visual functions. The results of these studies revealed that embryonic and fetal derived neural retinal tissues survive and differentiate after transplantation to the subretinal space [337-342]. Furthermore, several modalities have been tested, which focus on the distinct diseased parts of the retina. RPE transplantation has been conducted to replace degenerated RPE in AMD [343-345]. In animals models, transplantations of rodent RPE cells have been shown to promote photoreceptor cell survival and regeneration of outer segments in the Royal College of Surgeon's (RCS) rat retina, a model for AMD [346]. Similar results were obtained with human fetal RPE cells transplanted into the same animal model [347]. However, RPE cells cause immune-mediated rejection of RPE grafts, since they express

class I and II major histocompatibilty antigens [348] and thus, are a limitation to allogenic grafts. The major disadvantage of RPE cell grafts is their inability to attach as a monolayer in the host, mimicking RPE cells *in vivo*. Even when transplanted as freshly isolated sheets, RPE cells do not maintain their monolayer structure with junctional complexes, but migrate into the host retina and build cellular aggregates, which further damage the retina [349]. Similar results were observed in clinical trials with AMD patients [350].

The transplantation of retinal sheets was first conducted by Silverman and colleagues [340, 351]. Optimization of this procedure in past years has resulted in a method to transplant a cograft of fetal retinal neuroblastic progenitor cells as a sheet attached to the RPE [352-354]. This new method is supposed to compensate many of the addressed problems related to pure RPE transplantations. A beneficial reciprocal relationship between the two donor tissues (retina and RPE) can be maintained, which includes the nourishment of photoreceptors from the choroids. Moreover, the RPE will produce growth factors and signal molecules, which interact with the retinal transplant. Seiler and colleagues demonstrated in detail that these transplantation procedures harbor tremendous potential for the rescue of visual functions in degenerative models and have taken this approach into clinical trials (reviewed in [320]).

With respect to the present study, transplantations of neural and retinal stem and progenitor cells are another major focus in the field of degenerative diseases. Neural stem cells, which can be derived from the adult brain and propagated *in vitro* under the presence of FGF and EGF [41], have also been transplanted into retinal degeneration models. The grafted cells integrated into the laminar structures of the retina and extended processes into the optic nerve head [55, 355], but no expression of retina-specific markers was observed. When transplanted into the immature retina, NSCs adopted expression profiles similar to retinal neurons [356].

Retinal progenitor cells from the adult mammalian CB [67], postnatal retina [159] and fetal retina [228, 231] have been characterized in detail. Transplantation studies with these cells indicated that the degree of integration and migration into the host retina depends on the age or stage of the diseased or injured recipient retina. Grafted retinal progenitor cells

express the retina-specific marker opsin [357], but their differentiation potential seems to be limited to the glial lineage after transplantation to an adult host with retinal degeneration [231]. It would be of interest to introduce regulated expression of factors like Notch1 in these cells, which mediate not only glial differentiation but also retain progenitor potential, including Müller glia characteristics such as regenerative capacities (see section IV.1.3.2.).

The essential role of optimized culture conditions for expanding progenitor cell cultures for transplantation approaches was not only demonstrated in the present work, but also by recent studies from Qui et al. [228] and Miu et al. [358]. Grafts derived from retinal progenitors, which were maintained in optimized culture conditions, mostly contained opsin expressing cells [228]. A transplantation study revealed that these cells integrated well into young recipients of slow and fast retinal degeneration models [358].

V.5.2. Neuroprotection and stimulation of and endogenous regeneration potential in the diseased or injured retina

One of the major limitations in successful transplantation strategies is probably the fact that cells are transplanted into a pathological, hostile environment. This environment is unlikely to provide the necessary stimuli for differentiation and integration of grafted cells. *Ex vivo* gene transfer has been shown to harbor potential to overcome this barrier. The advantage of this strategy is the inclusion of survival-promoting factors into the grafts. These factors could also act in an autocrine manner to simulate a physiological environment for differentiation after transplantation and integration into the retina. *Ex vivo* gene transfer could facilitate neuroprotection to prevent retinal cell loss in RP, AMD or glaucoma. Several studies have been conducted, which address optic nerve degeneration and gene transfer via viral vectors. Results indicated that the integration of neurotrophins such as nerve growth factor (NGF) [359], glial cell line derived neurotrophic factor (GDNF) [360], ciliary neurotrophic factor (CNTF) [361, 362] or brain derived neurotrophic factor (BDNF) [363] by viral vectors can rescue photoreceptors and retinal ganglion cells of the optic nerve in degeneration models. Genetically modified human derived RPE cells, which overexpress BDNF, have been shown to promote cell survival [364] and to inhibit aberrant retinal neovascularization

[365]. The application of FGF, the major growth-promoting factor in the cell culture conditions used in the present study, has also been implemented in endogenous stimulation of regeneration in degenerative retinal models. Sapieha et al. demonstrated that FGF gene delivery stimulates axon growth of adult retinal ganglion cells after optic nerve injury [366]. They concluded that factors essential for axon outgrowth during neural development might just as well promote regeneration in the adult retina. The survival promoting effects of FGF have also been demonstrated by direct intravitreal or subretinal application of the growth factor into eyes of an inherited degeneration model [367, 368]. Finally, an approach using small interfering RNA (siRNA), which targeted vascular endothelial growth factor (VEGF), effectively inhibited ocular neovascularization in a mouse model [369, 370]. This suggests that siRNA techniques harbor the potential to address retinal degeneration and neural protection in disease models by targeting factors that drive disease mechanisms.

A most promising idea for replacement strategies in the retina is the stimulation of endogenously persisting stem or progenitor cell populations. Even though it has been reported that the mammalian retina is devoid of regenerative capacities, numerous studies have indicated that with appropriate stimuli, regeneration can be induced, especially in chicken and neonatal mammalians (reviewed in [219, 371, 372]). With the observation that glial cells of the CNS provide a source of neural regeneration, a focus has been placed on the glial cell type of the retina, the Müller glia. Fischer and Reh showed that Müller glia cells can respond to injury or exogenous growth factors by de-differentiation, proliferation and expression of neuronal and glial markers [241]. Following these reports, the evaluation of Müller glia cells as a source of endogenous regeneration in the mammalian retina revealed the potential this cell type harbors with respect to therapeutic strategies. First indications for the successful endogenous stimulation of Müller glia regeneration in the adult mammalian retina were published by Ooto et al. [373]. The authors applied NMDA lesions to the adult rodent retina and demonstrated that Müller glia cells were stimulated to proliferate in response to the toxic injury. Furthermore, the cells produced bipolar cells and rod photoreceptors and their numbers could be promoted by application of retinoic acid. In addition, misexpression of bHLH factors and homeobox genes induced the formation of other retinal cell types such as amacrine or horizontal cells and rod

photoreceptors. The authors convincingly showed that they could partially control the fate of the newly generated neurons with extrinsic factors and intrinsic genes. The analysis of the integration of newly generated neurons and their functionality remains to be conducted. Müller glia cells might be an endogenous source of retinal progenitor cells and may become a target for both, drug delivery and gene therapies to effectively treat retinal degenerative diseases.

In conclusion, the present study provides an analysis and characterization of progenitor cells from the postnatal and adult mammalian retina under the influence of optimized culture conditions of CNS derived NSCs. Adult retinal progenitor cells display changes of plasticity and share characteristics with NSCs, suggesting an ideal role for therapeutic strategies. Their potential to acquire stem cell characteristics after they differentiated into their mature state is documented and reveals that the adult mammalian retina might be induced to regenerate from endogenous sources during disease or injury.

VI. Summary

The mammalian retina develops from stem cells that are of neuroectodermal origin and derive from bilateral evaginations of the neuroepithelium, the optic vesicles. In rats, the differentiation of the six neuronal and the one glial cell types is terminated around postnatal day 12. The retina of adult mammals is a non-neurogenic region and the diseased retina is devoid of any spontaneous regeneration, while in poikilothermic vertebrates, cells at the ciliary margin of the eye proliferate throughout the life of the animals and generate new retinal cells that are integrated into functional retinal circuits. This knowledge initialized the search for such pools of stem and progenitor cells in the mammalian ciliary body (CB) from which neural stem cells (NSCs) have been isolated and characterized. These cells are capable to differentiate into glia and neurons, including retina-specific cell types like photoreceptors.

In course of the present study, cell culture protocols for NSCs from the neurogenic regions subventricular zone (SVZ) and hippocampus (HC) of the adult CNS were optimized. Efficient growth of the cells was accomplished by using Neurobasal medium (NB), Accutase® in enzyme treatments and B27 as supplement, which additionally promoted cell survival and clonal growth. Furthermore, optimal conditions for differentiation of SVZ and HC cells into the three major cell classes of the CNS, namely neurons, astroglia and oligodendroglia, were established by supplementing NB/B27 with 1% FCS, but without growth factors. Artificial constitutive expression of Notch1 in adult SVZ and HC derived stem cells resulted in upregulation of glial differentiation and downregulation of neuronal differentiation, suggesting that adult CNS derived stem cells are subject to plasticity in terms of their fate determination. Therefore, these cells could provide a promising source for cellular replacement strategies in neurodegenerative models. The results of the NSC culture optimization were the basis for analyses of retinal progenitor cells.

The presence and neurogenic potential of mammalian progenitors of the postnatal sensory retina were analyzed by immunocytochemistry and RT-PCR. The results demonstrate that postnatal rodent retina derived cells proliferate *in vitro* and display some

characteristics of NSCs, such as the expression of specific progenitor markers or the ability to incorporate BrdU. On the other hand, self-renewal as determined by clonal assays was not observed, indicating that postnatal cells are restricted in their stem cell potential. Furthermore, postnatal retinal cells grown under the optimized differentiation condition only differentiated along two neural lineages. Neuronal and glial markers were detected in immunocytochemical studies of the cells, but they were devoid of markers for oligodendroglial differentiation. Expression of the retina-specific marker recoverin indicated differentiation of postnatal progenitors into mature retinal cell types. Doublestaining for the proliferation marker BrdU and recoverin revealed a subpopulation of mitotic cells *in vitro*.

Stem cell capacities have been demonstrated for pigmented cells of the adult mammalian CB. Under *in vitro* conditions, these cells form neurospheres, consisting of pigmented and non-pigmented cells, and differentiate into glial and neuronal cells. Other cell types such as Müller glia and RPE cells have been shown, for instance, to activate a neurogenic program after a lesion or to regenerate the retina in poikilothermic vertebrates. RPE based neuronal regeneration in adult mammals has not been reported so far. This is surprising, since RPE cells are of neuroectodermal origin. They derive from the neural plate, and although they differentiate into a polarized epithelial monolayer sheet, they descend from precursors that later generate neural retina.

A comparative study of adult rodent CB and RPE cells revealed that both cell types share characteristics, which are reminiscent of NSCs. Growth as neurospheres, proliferation and self-renewal capacities were all comparable to NSCs. Furthermore, both cell types expressed a set of specific retinal stem and progenitor cell markers, such as the proneural homeobox transcription factor Pax6 or the basic helix-loop-helix transcription factor neuroD. Differentiation in the optimized media on poly-L-ornithine/laminin coating indicated that CB and RPE cells can differentiate along neuronal and glial lineages, but are devoid of oligodendroglial differentiation. Trans- or de-differentiation processes induce changes in phenotypes and expression profiles of CB and RPE cells. While substantial evidence exists for trans-differentiation of embryonic RPE cells, only limited results on trans- or de-differentiation of adult RPE cells have been published. Therefore, one further focus of this study was the analysis of adult mammalian RPE cells

with respect to de- and trans-differentiation under the optimized conditions. Differentiated RPE cells acquired neuronal and glial phenotypes *in vitro*, although the RPE is devoid of such phenotypes *in vivo*. Furthermore, detection of doublecortin, a transient marker for neuronal precursors, identified de-differentiating RPE cells that not only expressed β III tubulin, but also acquired a neuronal morphology. Similar results were also obtained for human derived RPE cells.

The presented results eventually aim at the comprehension of the basic biological mechanisms, which regulate the restrictions that stem or progenitor cells experience during cessation of development in the mammalian retina. An intriguing idea is to provide stimuli that alter the gene expression from a quiescent somatic cell in the adult retina towards a retinal stem cell and activate its developmental program to generate new neurons in a diseased or injured retina.

VII. Zusammenfassung

Die Säugerretina entwickelt sich aus Stammzellen neuroektodermalen Ursprungs und entsteht aus bilateralen Einstülpungen des Neuroepithels, den optischen Vesikeln. In der Ratte ist die Differenzierung der sechs neuronalen und der einen glialen Zellklasse der Retina nach dem Postnataltag 12 abgeschlossen. Die Retina adulter Säuger ist ein nicht-neurogenes Gewebe und die erkrankte Retina ist nicht in der Lage, spontan zu regenerieren. Im Gegensatz dazu proliferieren in poikilothermen Vertebraten Zellen des Ziliarkörpers über die gesamte Lebensdauer des Tieres. Retinale Zellen werden generiert und in das bereits bestehende Netzwerk integriert. Dieses Wissen hat dazu veranlasst, ähnliche Vorkommen proliferierender Stamm-oder Vorläuferzellen im Ziliarkörper von Säugern zu suchen. So wurden Stammzellen im Ziliarkörper der adulten Säugerretina gefunden, isoliert und charakterisiert. Dabei wurde deutlich, dass diese Zellen zu Neuronen und Gliazellen differenzieren können und auch retina-spezifische Zelltypen, wie z.B. Photorezeptoren, generieren können.

Im Verlauf dieser Studie wurden Zellkulturbedingungen für neurale Stammzellen aus dem adulten ZNS (subventrikuläre Zone (SVZ) und Hippocampus (HC)) optimiert. Effizientes Wachstum der Zellen wurde unter der Verwendung von Neurobasal Medium (NB) mit dem Zusatz B27 und enzymatischem Verdau mittles Accutase® erreicht. Zusätzlich wurden optimale Differenzierungsbedingunen für SVZ und HC Stammzellen entwickelt, die eine Differenzierung der Zellen entlang der drei Hauptzellklassen des ZNS (Neurone, Astroglia und Oligodendroglia) ermöglichen. Hierzu wurde dem NB/B27 Mendium 1% fötales Kälberserum zugesetzt und alle Wachstumsfakoren aus dem Medium entfernt. Die konstitutive Expression von Notch1 in SVZ und HC Zellen, vermittelt durch retrovirale Infektion des Zellen, führte zu einer vermehrten glialen Differenzierung bei gleichzeitigem Rückgang der neuronalen Differenzierung. Dies lässt darauf schliessen, dass adulte Stammzellen des ZNS plastischen Veränderung im Bezug auf ihre Phänotypspezifizierung unterliegen. Daher könnten diese modifizierten Zellen eine interessante Möglichkeit zur Behandlung neurodegenerativer Erkrankungen sein.

Die Ergebnisse der Zellkulturstudie waren der Ausgangspunkt für die Analyse der retinalen Vorläuferzellen.

Das Vorkommen und neurogene Potenzial postnataler Vorläuferzellen aus der Säugerretina wurde mittels immunzytochemischer Färbungen und RT-PCR analysiert. Die Ergebnisse zeigen, dass postnatale retinale Zellen aus dem Nager *in vitro* proliferieren und einige Gemeinsamkeiten mit neuralen Stammzellen des ZNS haben, wie z.B. die Expression spezifischer Vorläufermarker oder der Fähigkeit, BrdU zu inkorporieren. Auf der anderen Seite sind postnatale Vorläuferzellen aber nicht in der Lage, klonal zu expandieren, was ein Anzeichen für limitiertes Stammzellpotenzial ist. Weiterhin können postnatale Vorläuferzellen unter Differenzierungsbedingungen nur in zwei der drei neuralen Zellklassen differenzieren (Neurone und Astroglia). Marker für Oligodendrozyten wurden nicht nachgewiesen. Expression des retina-spezifischen Photorezeptorproteins Recoverin zeigte an, dass die Zellen auch retina-spezifische Phänotypen annehmen können. Eine Subpopulation dieser Zellen war doppeltmarkiert mit BrdU, was darauf schliessen lässt, dass einige postnatale Vorläufer *in vitro* Teilungen durchlaufen und neue Neurone generieren können.

Stammzell-Eigenschaften wurden für adulte Ziliarkörperzellen (CB) aus der Säugerretina nachgewiesen. Unter *in vitro* Bedingungen können diese Zellen Neurosphären bilden, die aus pigmentierten und nicht-pigmentierten Zellen bestehen und sowohl zu Neuronen als auch Gliazellen differenzieren können. Andere Zelltypen der adulten Retina wie beispielsweise Müllerglia oder retinales Pigmentepithel (RPE) können ebenfalls ein neurogenes Programm aktivieren und in poikilothermen Vertebraten zur Regeneration der Retina nach Verletzung beitragen. RPE-basierte Regeneration in adulten Säugern ist bis heute noch nicht nachgewiesen worden. Dies Überrascht vor dem Hintergrund, daß RPE Zellen neuroektodermalen Ursprungs sind und obwohl sie zu einem epithelialen Phänotyp differenzieren, stammen sie doch von Zellen ab, die auch neurale, sensorische Retina produzieren können.

Eine vergleichende Studie von adulten CB Zellen und RPE Zellen zeigte, dass beide Zelltypen Charakteristika mit neuralen Stammzellen teilen. So wurde für CB und RPE Wachstum in Neurospären, klonale Expansion sowie die Expression Vorläufer-spezifischer Marker wie dem Homeobox Gen Pax6 oder dem Helix-Loop-Helix

Transkriptionsfaktor NeuroD nachgewiesen. Unter Differenzierungsbedingunen konnten sowohl neuronale Marker als auch gliale Marker in CB und RPE Kulturen gefunden werden.

Trans- bzw. De-differenzierungsprozesse verursachen phänotypische Veränderungen und alternieren das Expressionsprofil der Zellen. Bis heute sind aber nur wenige Studien zum De-differenzierungspotenzial adulter retinaler Vorläuferzellen des RPE publiziert worden. Aus diesem Grund war die Analyse von RPE Zellen vor diesem Hintergrund ein wichtiger Bestandteil dieser Arbeit. Differenzierte RPE Zellen exprimierten neuronale und gliale Marker *in vitro* obwohl das RPE *in vivo* keine dieser Marker exprimieren kann. Zudem konnte auch eine morphologische Veränderung in den RPE Zellen, die neuronal differenzierten, nachgewiesen werden. Ähnliche Resultate wurden ebenfalls für humane RPE Zellen gefunden.

Ein Kernstück der vorliegenden Arbeit war demnach das Verständnis der biologischen Grundlagen retinaler Stamm- oder Vorläuferzellen der postnatalen Periode und des adulten Säugers. Bereits vorhandenes Wissen zur Embryonalentwicklung der Säugerretina und der Plastizität von Stamzellen in die Hauptklassen neuraler Zellen im ZNS zu differenzieren, wurden mit den postnatalen und adulten Phasen der retinalen Differenzierung verglichen. Die Analyse der molekularen Mechanismen, die während der Entwicklung die Stammzellkapazitäten von retinalen Zellen einschränken, ist von größtem Interesse. Eine interessante Idee ist die Bereitstellung entsprechender Stimuli, die eine ruhende Stammzelle der adulten Retina so beeinflusst, dass es zu einer Neuauflage des Entwicklungsprogramms dieser Zelle kommt und somit die Generierung neuer Neurone in einer erkrankten oder verletzten Retina möglich würde.

VIII. Bibliography

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