Endogenous mobilization of bone-marrow cells into murine retina induces fusion-mediated reprogramming of Müller glia cells

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THESIS ABSTRACT

Müller glial (MG) cells are considered the most plastic cell type in the retina, especially in lower vertebrates. Upon damage in teleost fish and chicken, MG cells can efficiently proliferate by re-entering the cell cycle and generating different retinal cell types; however, this regenerative potential is very poor in mammals.

Some reports importantly highlighted that MG cells can act like adult stem cells of the retina with neurogenic potential also in mammals, but the mechanisms behind this ability are still not fully understood. We hypothesized that MG can de-differentiate after damage and generate new neurons in the mouse retina through a cell fusion process.

Moreover several studies emphasized the role of bone-marrow cells (BMCs) in participating to the repair of damaged tissue, thanks to their plasticity, and through a cell fusion mechanism.

We found that endogenous BMCs can be mobilized from peripheral blood and efficiently recruited into N-methyl-D-aspartate (NMDA)-damaged mouse retinas. The BMCs mobilized into the damaged retina fuse with MG cells, and the hybrids undergo dedifferentiation, to finally express markers of ganglion and amacrine cells over the long term.

Moreover, upon modulation of one of the major signaling pathway involved in bone-marrow (BM) mobilization, the stromal cell-derived factor 1 (SDF-1)/ C-X-C motif chemokine receptor type 4 (CXCR4) pathway we were able to enhance this process.

Overall, we show that an important mechanism by which MG cells can de-differentiate and reprogram after damage in mammals is due to this BMC migration and their fusion with MG cells.

In fact, MG cells neurogenic ability is severely impaired when BMC migration into damaged retina is blocked after interfering with the SDF-1/CXCR4 pathway.

Ultimately, our findings might open the path toward a new strategy to boost endogenous mechanism of repair in retinal degeneration.

RESUMEN DE TESIS

Las células gliales Müller (MG) son consideradas la mas plásticas entre todas las células de la retina, sobre todo en los vertebrados inferiores. Después de un daño determinado en las células retinianas en los peces teleósteos y en el pollo, las células MG pueden proliferar a través de la entrada en el ciclo celular y generar diferentes tipologías de células de la retina; de todas formas este potencial regenerativo es muy limitado en los mamíferos.

Algunos estudios ponen en evidencia que las células MG pueden actuar como células madre adultas de la retina con una capacidad neurogénica también en los mamíferos, pero los mecanismos que están detrás de esta habilidad no están todavía entendidos a fondo. Nosotros hemos hipotetizado que las células MG puedan desdiferenciarse y generar nuevas neuronas después de un daño a las células ganglionares de la retina del ratón, a través de un proceso de fusión celular.

Además varios estudios han enfatizado el papel de las células de la médula (BMCs) en la participación en el reparo de tejidos dañados, gracias a su plasticidad, y a través de un mecanismo de fusión celular.

Hemos descubierto que las células endógenas de la médula pueden ser movilizadas desde la sangre periférica, y eficazmente reclutadas en las retinas dañadas de ratón con N-methyl-D-aspartate (NMDA).

Las BMCs migradas en la retina dañada se fusionan con las células MG, y los híbridos formados empiezan un proceso de

desdiferenciación y reprogramación para finalmente expresar marcadores de células ganglionares y amacrinas a largo plazo.

Además, después de la modulación de una de las mejores vías de señalización (SDF-1)/ C-X-C motif chemokine receptor type 4 (CXCR4) involucrada en la movilización de las BMCs, hemos sido capaces de aumentar el referido proceso.

En general mostramos que un importante mecanismo a través del cual las células MG pueden desdiferenciarse y reprogramarse, después de dañar las retinas de los mamíferos, es debido a la migración de las BMCs y su fusión con dichas células MG.

De hecho, la capacidad neurogénica de las células MG queda severamente impedida cuando la migración de las BMCs en la retina dañada esta bloqueada, después de interferir con la vía de señalización SDF-1/CXCR4.

Finalmente, nuestro descubrimiento podrá abrir un nuevo camino hacia una nueva estrategia para estimular un mecanismo endógeno de reparación en la degeneración retiniana.

PREFACE

Adult stem cells were thought to produce only cell lineages characteristic of the tissue in which they reside. However, in the last years, recent findings suggest that bone marrow (BM) derived cells harbor a great plasticity and can differentiate into multiple lineages, including neurons. In pathological human retina, migration of endogenous BM cells toward the site of degeneration has been observed. This suggests a possible role of BM stem cells in the endogenous mechanism of tissue regeneration, opening new fascinating approaches to restore injured neural tissue.

Müller glia (MG) cells in the retina act as adult stem cells in lower vertebrates. Recently, also in mammal, MG cells were shown to have some limited potential to be de-differentiated de-differentiated in vivo. Endogenous regeneration through activation of resident MG cells is therefore an appealing alternative to cell therapy, because it might limit the hurdles associated with engraftment efficiency and integration in the existing neural circuit.

We identify a novel mechanism according to which, after mobilization of BM cells into the eye, the mammalian MG cells can be reprogrammed in vivo and their neurogenic potential can be increased.

This might give a new alternative for boosting endogenous repair as a possible therapeutic approach.

CONTENTS

THESIS ABSTRACT vii

RESUMEN DE TESIS ix

PREFACE xi

PART I INTRODUCTION

1. DEDIFFERENTIATION, REPROGRAMMING AND TRANSDIFFERENTIATION: CHANGES IN CELL IDENTITY 17

1.1 REPROGRAMMING TO PLURIPOTENCY 20

1.1.1 Reprogramming by Somatic Cell Nuclear Transfer 22

1.1.2 Direct Reprogramming: Induced Pluripotent Stem Cells (iPSCs) generation 23

1.1.3 Cell Fusion mediated Reprogramming 27

1.2 IN VIVO CELL FUSION 30

1.3 CELLULAR REPROGRAMMING: TRANSDIFFERENTIATION 33

1.4 WNT/B-CATENIN PATHWAY IN REPROGRAMMING 36

1.5 IN VIVO DEDIFFERENTIATION AND REPROGRAMMING 38

1.6 IN VIVO REPROGRAMMING IN THE CENTRAL NERVOUS SYSTEM 42

2. BONE MARROW DERIVED HEMATOPOIETIC STEM CELLS 44

2.1 HEMATOPOIETIC STEM CELLS IN HOMEOSTASIS 44

2.2 BONE MARROW CELL PLASTICITY AND THEIR ROLE IN TISSUE REPAIR 49

2.3 BONE MARROW CELL MOBILIZATION 54

2.4 SDF-1/CXCR4 SIGNALING PATHWAY 56

3. THE VERTEBRATES RETINA: A MODEL FOR CENTRAL NERVOUS SYSTEM (CNS) STUDIES 60

3.1 RETINAL STRUCTURE AND DEVELOPMENT 61

3.2 RETINAL REGENERATION 65

3.3 MÜLLER GLIA (MG) CELL FUNCTION IN HOMEOSTASIS AND TISSUE REPAIR 69

3.4 MG REPROGRAMMING AND MG-MEDIATED RETINAL REGENERATION IN LOWER VERTEBRATES 72

3.5 MG REPROGRAMMING AND MG-MEDIATED RETINAL REGENERATION IN MAMMALS 77

3.6 STEM CELL THERAPY FOR RETINAL DISEASES 79

PART II AIMS

AIMS 87

PART III **RESULTS**
RESULTS 91

PART IV **DISCUSSION AND CONCLUSIONS**
DISCUSSION 151
CONCLUSIONS 161

MATHERIALS AND METHODS **165**
REFERENCES **175**

ANNEX 1 **203**
ANNEX 2 **205**

PART I
INTRODUCTION

1. Dedifferentiation, Reprogramming and Transdifferentiation: changes in cell identity

Stem cells, which are characterized by the ability to both self-renew and to generate differentiated functional cell types, are classified according to their pluripotency grade. Totipotent cells like the zygote are formed after the fertilization process and have the highest differentiation potential. They can give rise to all kinds of tissues including extra-embryonic tissues such as the placenta, and can ultimately lead to the formation of an entire organism.

In the early embryo, totipotent cells divide and mature into more specialized cells that will form the placenta layer and inner cell mass (ICM). Embryonic stem cells (ESCs) can be derived from the ICM of the blastocyst, an early-stage in the pre-implantation embryo.

ESCs are pluripotent because they can generate all the cell types in the body but not the extra-embryonic tissue. As pluripotent stem cells divide, they specialize into the three germ layers: endoderm, mesoderm and ectoderm. Here, more specialized, cells are already partially committed to give rise to a limited range of somatically differentiated cells that in turn will form different tissues such as the brain, intestine, or muscle.

In the mid-20th century, Conrad Waddington developed a model that depicted normal embryonic development as a ball rolling downhill to its final differentiated state. Waddington's epigenetic

landscape is the most used hierarchical model to describe the progressive and irreversible restriction of cell differentiation potential during the normal development process (Figure 1) (Waddington, 1957).

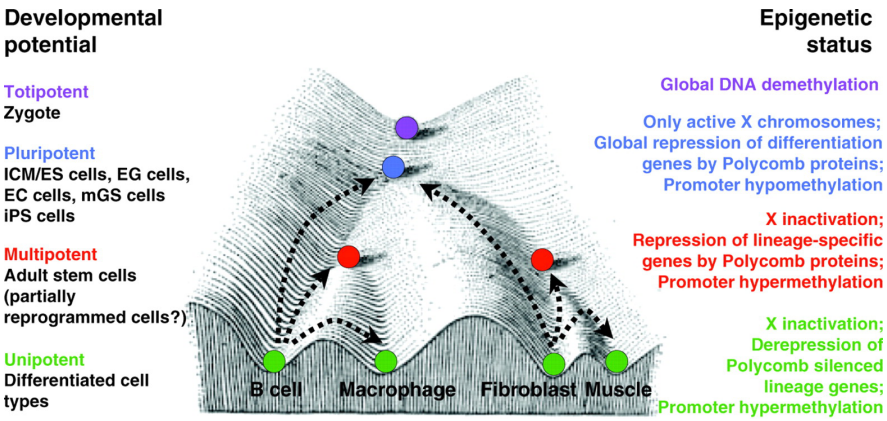


Figure 1. Progressive and irreversible restriction in cell differentiation potential and epigenetic states of cells at different stages of development Cell populations with different developmental potentials (left) and their respective epigenetic states (right) are shown in a modification of C. H. Waddington's epigenetic landscape model. Cells undergoing restriction in differentiation potential are illustrated as marbles rolling down a landscape. Several valleys indicate different cell fates. Colored marbles correspond to different pluripotency state (purple, totipotent; blue, pluripotent; red, multipotent; green, unipotent).

This process is regulated by the epigenetic machinery that mediates the changes in the gene expression profile during differentiation, and by the maintenance of the cellular identity by blocking a specific gene expression pattern.

In the last few decades, many attempts have been made to understand whether terminally differentiated cells, although very

specialized, keep a sort of cellular "memory" of their less specialized state. Attempts have also made to see if it is possible to unlock this cellular identity. The development of new strategies aimed at perturbing the epigenetic machinery, demonstrates that a somatic nucleus can acquire a new developmental potential. They also demonstrate that the gene expression profile of somatic nuclei can be totally resettled and rearranged to a less specialized state or to an early embryonic state, without any change in DNA sequence. These advances completely challenge the concept that cellular identity is locked in an irreversible state. Further, they demonstrate that it is possible to change the cell fate of somatic cells from different lineages or force them back to a pluripotent state. This process is called reprogramming.

The term "reprogramming" originally referred to the erasure and remodeling of epigenetic marks, such as the DNA methylation, during mammalian development. As an example, upon fertilization, the paternal and maternal genomes are de-methylated and re-methylated through a process of "epigenetic reprogramming", which is required for the totipotency of the newly formed embryo (Reik et al., 2001). However, the term "reprogramming" can also refer to the process by which a somatically differentiated cell can return to a less differentiated stage (i.e. dedifferentiation) or ultimately regress to a pluripotent state (i. e. reprogramming to pluripotency) (Jaenisch and Young, 2008) .

During dedifferentiation, terminally differentiated cells are capable of reverting to a less differentiated state within their

lineage, proliferating then differentiating once again to replace the correct amount of lost cells. Re-entry into the cell cycle, and thus the formation of a pool of proliferating less specialized cells, is a peculiar characteristic of a dedifferentiation process. However, the regression to a less specialized phenotype does not implicate the acquisition of a pluripotent state.

The process by which a differentiated cell of one type can be converted into another cell type is called cellular reprogramming. Cellular reprogramming includes transdifferentiation, which is the process that occurs when a specific specialized cell type is converted into another cell type of the same lineage or of a different lineage. In most cases this occurs without a dedifferentiation step into an intermediate state (Graf and Enver, 2009) (see Chapter 1.2).

1.1 Reprogramming to pluripotency

During reprogramming to pluripotency a somatic nucleus can acquire new developmental potential through a process, which requires epigenetic mechanisms. In fact, when a cell embarks on reprogramming, its gene expression is totally reset and rearranged to an early embryonic state without any change in DNA sequence.

Nuclear reprogramming has been demonstrated through three different experimental approaches: nuclear transfer, cell fusion and direct reprogramming through transcription-factor transduction (Figure 2) (Yamanaka and Blau, 2010). Using these three

approaches many scientists have demonstrated that "terminally differentiated" somatic cells, although very specialized, maintain the ability and all the genetic information to express genes that are typical of ESCs. Moreover, the three approaches have shown that although somatic differentiated cells remain in a stable state, they keep a sort of cellular "memory" which, under specific perturbations and gene regulation changes, can be dynamically controlled.

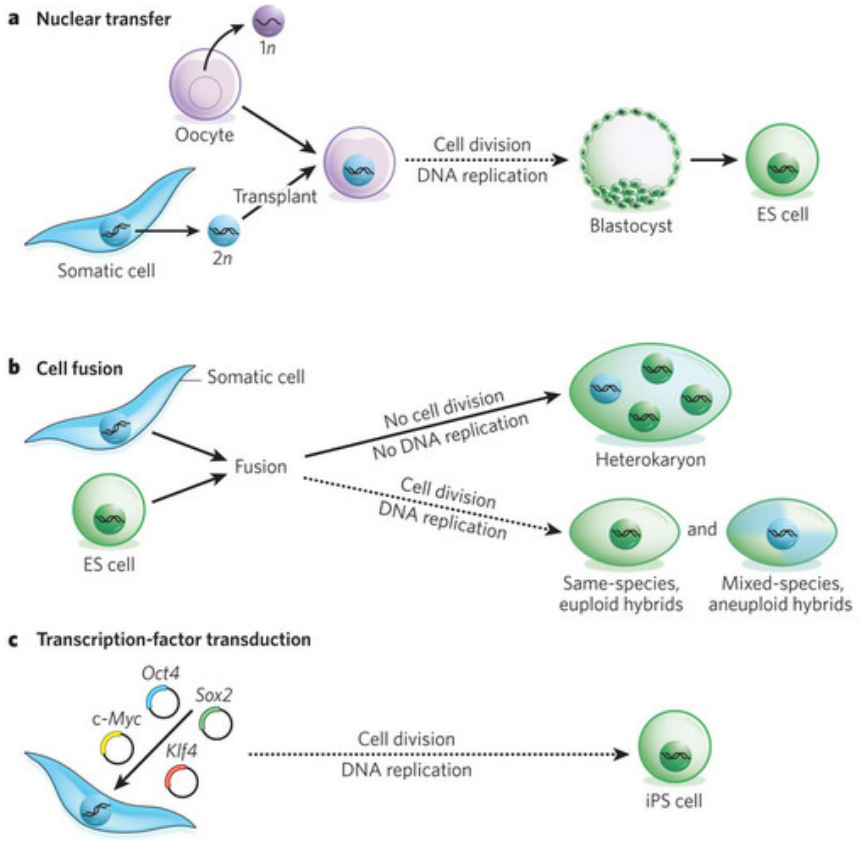


Figure 2. Three approaches for nuclear reprogramming to pluripotency (a) Nuclear Transfer. In this approach the nucleus of a somatic differentiated cell (diploid, 2n) is transferred into an enucleated

oocyte. Upon oocyte activation, maternal factors rapidly and globally remodel the somatic genome allowing the reprogramming of the somatic nucleus. ESCs tissue cultures are derived from the generated blastocyst. (b) Cell fusion. In this approach, two distinct cell types are combined together to form a single new entity. The result of cell fusion is the formation of a new cell type, which displays hybrid characteristics different from both the original cells. The hybrid, which becomes 4n, can be a synkaryon, if the two nuclei fuse together, or a heterokaryon, which is multinucleate and where the two nuclei from the original cells remain intact and distinct. Solid arrows indicate no division, like in the case of heterokaryons while dashed arrows show multiple rounds of cell division typical of a synkaryon hybrid. (c) Direct reprogramming-Transcription factor transduction. This approach consists in introducing four genes (Oct4 , Sox2 , Klf4 and c-Myc) into almost any kind of cell type by using retroviruses to generate induced pluripotent stem cells (iPSCs) with similar properties to ESCs.

1.1.1 Reprogramming by Somatic Cell Nuclear Transfer

In 1962, Dr. John Gurdon was the first who successfully demonstrated nuclear reprogramming and was awarded the Nobel Prize in Medicine in 2012 for this achievement. Gurdon managed to obtain swimming tadpoles by transferring nuclei from highly specialized tadpole intestinal epithelial cells into enucleated frog eggs (Gurdon et al., 1958). Gurdon's Somatic cell nuclear transfer (SCNT) experiments, also known as "cloning" experiments, demonstrated that differentiated cells maintained the extraordinary capability to create all the cells of the body after being converted back into an embryonic state (Gurdon, 1962). In addition, these first experiments showed that all the information necessary for the generation of an entire organisms is kept in the nucleus of specialized cells and can be "reactivated" following the contact with "reprogramming factors" present in the oocyte cytoplasm. This

outcome indicates that cell specialization processes can be totally reverted and involve changes in gene expression, not in gene content.

The first attempt to clone mammals was conducted by Bromhall in 1975. Bromhall attempted to fuse morula cells with unfertilized rabbit eggs, using both microinjection and virus induced fusion (Bromhall, 1975). The first murine nuclear transfer was achieved few years later, in 1981 by Illmensee and Hoppe. The researchers demonstrated that only the ICM and not trophectoderm (TE) cells could generate cloned mice once transplanted into enucleated zygotes (Illmensee and Hoppe, 1981).

Evidence, which indicated that a cell nucleus in an adult animal could possess the ability to generate a complete mature adult organism after nuclear transfer was finally found in sheep. In 1996, Campbell et al. obtained adult sheep from the nuclei of a cultured line of cells grown from a sheep embryo (Campbell et al., 1996). Finally, in 1997, Wilmut successfully cloned Dolly the sheep from nuclei of an adult sheep cell line (Wilmut et al., 1997).

1.1.2 Direct Reprogramming: Induced Pluripotent Stem Cells (iPSCs) generation

Scientific interest in the reprogramming field exploded after 2006, when Shinya Yamanaka's lab in Kyoto showed that the forced expression of just four genes into adult mouse differentiated

cells was sufficient to reprogram them back to the pluripotency, forming so-called induced pluripotent stem cells (iPSC). The researchers initially screened twenty-four genes, which were previously shown to be very important for the maintenance of ESC states. They delivered them into embryonic and adult mouse fibroblasts using retroviral infection. The recipient fibroblasts were engineered to express Fbx15, an ESC specific gene, fused to an antibiotic resistance. In this way the cells undergoing reprogramming could be identified and isolated using antibiotic selection. Finally, by removing one factor at a time from the pool of twenty-four, the researchers identified four factors: Oct4, Sox2, cMyc, and Klf4, which were necessary and sufficient to generate ESC-like colonies after selection for reactivation of Fbx15 (Takahashi and Yamanaka, 2006).

The authors identified that similar to ESCs, iPSCs were pluripotent and had unlimited self-renewal ability. Moreover, when forced to differentiate under specific culture conditions, iPSCs formed embryo bodies and were able to contribute to the formation of all three germ layer lineages. Finally, when transplanted into mice, these cells could form teratomas and could even give rise to fetal chimeras. However, from a molecular point of view, iPSCs showed different gene expression and epigenetic marks compared with ESCs.

In 2007 other groups improved the reprogramming protocol to generate iPSCs that were functionally identical to ESCs. In this case, iPSCs were again obtained by infecting mouse fibroblasts with a retrovirus that expressed the same four transcription factors (Oct4,

Sox2, cMyc, Klf4). This time, the reprogrammed clones were selected using the Nanog pluripotency gene, instead of Fbx15 (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). However, the authors realized that 20% of the chimeric mice developed cancer because two of the transcription factors (c-Myc and Klf4) were oncogenic. Because of this, in successive work, Yamanaka demonstrated that iPSCs could be created without the use of c-Myc. While their new method did not cause any cancer formation in the chimeras, the generation of iPSCs was less efficient and took longer (Nakagawa et al., 2008).

iPSCs formation has been obtained both in vitro after gene expression delivery by retroviral infection and, more recently, has also been shown to occur in vivo in the context of an entire organism. In a recent work, Manuel Serrano's group showed that full reprogramming could occur in vivo. They also showed that transitory induction of the four factors *Oct4, Sox2, Klf4* and *c-Myc* in a "reprogrammable mouse" resulted in iPSCs that were closer to ESCs compared with standard in vitro generated iPSCs. In fact such in vivo obtained iPSCs efficiently contributed to the trophectoderm lineage. This outcome suggests that they displayed totipotency features and achieved a more plastic and primitive state than ESCs (Abad et al., 2013).

The generation of human embryonic stem cells requires manipulation and destruction of the pre-implantation stage embryo; the blastocyst. This led to several ethical problems, which have limited the use of these cells for potential therapeutic applications.

Furthermore, the fact that ESCs can be derived from embryos, does not allow the generation of patient derived embryonic stem cell lines.

Similar to ESCs, induced pluripotent stem cells have unlimited self-renewal ability. They can proliferate indefinitely and can be induced to differentiate into almost any kind of cells in the body. Thus, induced pluripotent stem cells represent a great source of cells for regenerative medicine. Indeed, iPSCs can be used to replace lost or damaged cells in several diseases. Moreover, the fact that iPSCs can be derived directly from adult tissues, not only solves the ethical dilemmas related to the manipulation of embryos, but also allows the generation of pluripotent stem cell lines (Takahashi et al., 2007).

However, several problems need to be solved before these new technologies can be translated to the clinic. First, further studies are required to ensure the establishment of efficient reprogramming strategies that do not result in genetically modified cells. Moreover, the starting population of somatic cells often contains a mix of cells in a slightly different molecular state. This causes iPSCs generation to be inefficient and variable. Another limiting factor is that there is still no well-defined protocol for differentiating ESCs or iPSCs into lineage-committed cells for clinical use (Saha and Jaenisch, 2009).

1.1.3 Cell Fusion mediated Reprogramming

Artificial cell-cell fusion is also a well-known method used , to change the cellular identity. In general, fusion of uninuclear cells into one final multinuclear cell, defined as synchizium, can be achieved in vitro by treating cells with polyethylene glycol (PEG) (Ahkong et al., 1975). The result of a cell-cell fusion event is the formation of a new cell type, which displays hybrid characteristics, which are different from both original cells.

The fusion event can result in uninucleated cells "synkaryons", when the two nuclei are fused together, or "heterokaryon", when only the cytoplasmatic membranes fuse but the two nuclei remain separated (Chen et al., 2007). In general sare mostly formed as a result of homotypic cell fusion**,** where the same cell-types fuse. In contrast, heterokaryon are mostly obtained upon heterotypic cell fusion. An example of this is the case of hybridoma cells that are obtained as the result of the fusion of murine myeloma cells with B cells (Kohler and Milstein, 1975). However, synkaryons can also be formed by heterotypic cell fusion.

In vitro cell fusion was first used in the 1960s to assess whether the cytoplasm content of one differentiated cell could affect the gene expression of a different somatic nucleus. Through these investigations, in the late 1960s the existence of trans-activating repressors and tumor-suppressor proteins were discovered.

In the 1980s scientists started to use cell-cell fusion approaches to change cell identity and cell fate. Several studies

reported that proliferating hybrids (synkaryons) and post-mitotic hybrids (heterokaryons) could differentiate into specific cellular fates (Baron and Maniatis, 1986; Blau et al., 1983; Wright, 1984). One example is the fusion of murine hepatoma cells, which secrete mouse serum albumin, with human leukocytes. This allows the formation of hybrids that secrete both mouse and human serum albumin, suggesting a re-activation of the albumin gene in the human leukocytes. In addition, another study demonstrated that normally inactive globin genes can be activated in "transient" heterokaryons. These were obtained by fusing adult mouse erythroleukemia (MEL) cells and human embryonic/fetal erythroid cells with each other, or with a variety of mouse and human nonerythroid cell types (Baron and Maniatis, 1986). These results demonstrate that both pluripotent stem cells and differentiated cells can maintain the capacity to change the epigenetic state and gene expression of other nuclei.

Following these initial studies, which aimed to change cell identity through the cell fusion process, several groups have used cell fusion to induce differentiated cells to undergo reprogramming to a pluripotent state. Many studies have shown that somatic cells can acquire a pluripotent state after induced-fusion with pluripotent stem cells like ESCs, embryonic germ cells (EGCs), and embryonal carcinoma cells (ECCs) (Do and Scholer, 2004; Tada et al., 1997; Tada et al., 2001). Following cell fusion of somatic cells with these pluripotent stem cells, the resultant hybrids acquire pluripotency features. These include: the typical morphology of the respective pluripotent fusion partner, a specific gene profile and epigenetic

state, reactivation of pluripotent-related genes, inactivation of tissue-specific genes expressed in the somatic fusion partner, and the developmental and differentiation potential of the three germ layers (Do et al., 2006; Do and Scholer, 2006).

These results demonstrate that pluripotent cells possess an intrinsic capacity for inducing nuclear reprogramming following fusion with somatic cells. In particular, in heterokaryons, one of the two fusion partners imposes its own pattern of gene expression on the other partner, behaving like a "dominant" (Gurdon and Melton, 2008).

Cowan and colleagues have extended work on human cells by showing that nuclear reprogramming of human somatic cells can also be achieved by fusion with human ESCs (Cowan et al., 2005). Moreover, further results from cell-cell fusion experiments suggest that "reprogramming factors" can cross-act through species. This is reflected by mouse pluripotent stem cells that can reprogram nuclei of human somatic cells back to a pluripotent state (Flasza et al., 2003).

After these first discoveries, several experiments were performed to show that cell fusion could also occur spontaneously, and to provide a mechanism to study reprogramming in vitro. Hybrid cells were generated by growing mouse bone marrow cells (BMCs) on mitotically-inactive fibroblast feeder cells. These hybrids contained genetic markers from both cell types and showed endothelial potential (Que et al., 2004). In another study,

neurosphere cells derived from the embryonic day 14.5 mouse forebrain, were co-cultured with ESCs and spontaneously formed hybrids. These neurosphere/ESC hybrids had markers from both fusion partners, grew with ESC-like morphology, contained a tetraploid complement of chromosomes, and contributed to chimeras (Ying et al., 2002). Spontaneous cell fusion was also observed when hygromycin-sensitive mouse ESCs were co-cultured with hygromycin-resistant primary murine brain cells for five days (Pells et al., 2002).

In a similar approach, in 2002 Terada et al. co-cultured BMCs that were resistant to puromycin, with mouse ESCs. After three weeks, following the removal of growth factors, the clones that were morphologically similar to ESCs expressed ESC specific proteins and differentiated into heart muscle cells. These reprogrammed cells were synkaryon hybrids, as demonstrated by karyotype and polymorphism analysis (Terada et al., 2002). Yet, although the fusion-derived hybrids showed pluripotent characteristics, they were not identical to the pluripotent fusion partner cells. In fact, hybrid cells could form chimeras but not contribute to germline formation.

1.2 In vivo cell fusion

Cell fusion is a spontaneous process that naturally occurs in vivo during embryogenesis and morphogenesis. Indeed, cell fusion

events reportedly occur during several developmental processes including fertilization, placenta syncytiotrophoblast generation, myotube and osteoclast formation, and also during virus infection and immune response (Chen et al., 2007; Duelli and Lazebnik, 2007; Horsley and Pavlath, 2004; Ogle et al., 2005; Vignery, 2005) (Figure 3).

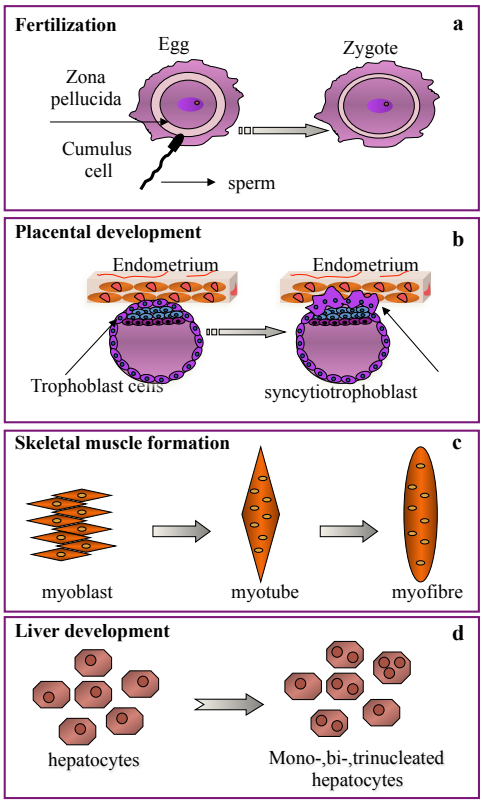


Figure 3. Physiological importance of cell fusion Cell fusion is one of the main mechanisms required during development to ensure the formation of several adult tissues and the maintenance of homeostasis. (a) During fertilization the fusion of the oocyte and sperm membranes is necessary to allow the formation of the zygote. (b) Placenta development requires the formation of the syncytiotrophoblast after cell fusion of endometrium cells with cells from the new forming embryo. This process allows the exchange of the nutrients between developing embryo and the mother´s blood (c) Fusion of many uninucleated myoblast cells leads to

the formation of a multinucleated cell called myotube, which is the functional unit of the skeletal muscle tissue. (d) The liver is the major example of an organ made by multinucleated cells formed after cell fusion of uninucleated hepatocytes among each other.

Over the last decade, many studies have applied cell fusion to stem cell biology. Several in vivo reports have suggested that adult stem cells may have the ability to differentiate into cell types, which are different from those of the tissues in which they reside, thus displaying an intrinsic "plasticity". One of the mechanism by which stem cells can change their cellular fate is through the cell fusion process.

Weismann and colleagues made the surprising discovery of Y-chromosomes in some cerebellar Purkinje neurons of women who had received bone marrow transplants from male donors. Moreover, they performed bone marrow transplants from transgenic mice, which ubiquitously expressed green fluorescent protein (GFP), using male mice as donors and female mice as recipients. Consistent with their earlier results, the researchers found that the transplanted cells contributed to some Purkinje neurons in the cerebellum. These GFP-positive Purkinje cells always contained two nuclei and, in many cases, the two nuclei were not identical. One nucleus resembled a typical Purkinje cell nucleus with dispersed chromatin. In contrast, the other was smaller and had compact, condensed chromatin, which was reminiscent of a bone marrow cell nucleus. The presence of two nuclei in every GFP-positive Purkinje cell, together with the confirmation that one of these nuclei originated from donor bone marrow, demonstrated that

cell fusion, rather than transdifferentiation or neurogenesis, had occurred (Weimann et al., 2003). After this study, several groups demonstrated that bone-marrow-derived cells (BMDCs) fuse spontaneously in vivo with hepatocytes in the liver, Purkinje neurons in the brain, and cardiac muscle cells in the heart. This was accomplished using a simple method based on Cre/lox recombination to detect cell fusion events (de Jong et al., 2012; Doyonnas et al., 2004; Johansson et al., 2008; Nygren et al., 2004; Vassilopoulos et al., 2003; Wang et al., 2003). These experiments provide the first in vivo evidence for cell fusion of BMDCs with neurons and cardiomyocytes. The findings introduce the fascinating possibility that cell fusion may contribute to the development or replacement of these key cell types (see Chapter 2.2).

1.3 Cellular reprogramming: transdifferentiation

The term “reprogramming” is also used to describe transdifferentiation processes that occur when a specific specialized cell type is converted into another cell type of the same or different lineage. This phenomenon was observed for the first time in vivo more than a hundred years ago. It was described as a spontaneous, naturally occurring mechanism during lens regeneration in the newt (Wolff, G.et al. 1895). After removal of lens, pigmented epithelial cells (PECs) underwent dedifferentiation, then following a proliferation step, PECs were re-differentiated to create a new lens vesicle (Tsonis et al., 2004).

The first evidence to indicate that cellular identity could be switched, came from the studies of Gehring and colleagues in 1987. The researchers showed that the forced expression of a single tissue-specific transcription factor could lead to changes in cellular phenotypes (Gehring, 1996). They showed that in D. melanogaster larvae, ectopic overexpression of the specific homeotic gene Antennapedia, under the control of a heat-shock gene promoter, caused a change in body plan and determined the development of an additional set of legs instead of antennae (Schneuwly et al., 1987). In 1989, Weintraub et al. provided one of the first examples of transdifferentiation within the same germ layer. The researchers were able to convert fibroblasts into muscle cells by overexpressing MyoD (Weintraub et al., 1989).

After these pioneering studies, several groups started to induce transdifferentiation of fibroblasts into almost any kind of cells, such as cardiomyocytes-like cells (Ieda et al., 2010). The conversion of astrocytes into neurons is another example of transdifferentiation within the same germ layer. In fact, astroglial cells can be converted into neurons (Heinrich et al., 2010; Torper et al., 2013) or reprogrammed into neuroblast cells (Niu et al., 2013).

In a more recent study, the forced expression of the three neuronal transcription factors (TFs): Ascl1, Brn2, and Myt1l, allowed rapid conversion of mouse fibroblasts into induced neuron-like cells (iN) that exhibit the biochemical and electrophysiological properties of neurons. iN cells share essential features with functional neurons, including morphological characteristics,

expression of cortical markers, the generation of action potentials, and the formation of synapses. However, it has not been resolved whether iN cells silence fibroblast-specific genes and maintained their newly acquired state independently of the expression of the transgenes (Vierbuchen et al., 2010). The same group later showed that, when combined with the bHLH transcription factor NeuroD1, the three TFs could also convert human fibroblasts into iN cells (Pang et al., 2011). Interestingly, iN cells could also be derived from defined endodermal cells such as primary hepatocytes, suggesting the existence of a more general mechanism of reprogramming (Marro and Yang, 2014).

Another group of researchers demonstrated that co-expression of the previously described neural TFs, together with Bcl-xL, induced fibroblast conversion into neural progenitor cells (NPCs). These proliferating NPCs predominantly differentiated into astrocytes but could also be induced to specifically differentiate into dopaminergic neurons by forced expression of Nurr1 and Foxa2 (Lim et al., 2015). All of the described studies have revealed that lineage conversions are not restricted to within the same lineage or germ layer but can also be achieved within different lineages.

In the majority of the cases, conversion of a cell into another of a different lineage occurs by two different steps. A first dedifferentiation step is often required to allow a cell type to regress to a less differentiated state and to proliferate. This is followed by a re-differentiation step to acquire a different cell fate. Contrary to this, other work has demonstrated that the forced expression of key

TFs can simultaneously mediate the down-regulation of a specific genetic program and the up-regulation of the new one. This work has also shown that this occurs in the absence of cell proliferation and without the need for a dedifferentiation step (Graf and Enver, 2009).

The induced expression of TFs is evidence of the above process. Such induced expression predominantly drives the conversion between cells states that are within the same lineage and are closely related developmentally, as is seen in the conversion of exocrine into endocrine pancreatic cells (Zhou et al., 2008). Thus, it appears that transdifferentiation in the absence of DNA replication, can occur when only a limited number of chromatin modifications are required.

1.4 Wnt/β-catenin pathway in reprogramming

As we have previously mentioned, reprogramming efficiency is quite low. For this reason, over the last decade several groups have tried to identify and manipulate the molecular components of signaling pathways that can enhance the efficiency of nuclear reprogramming.

Among others, research has shown that the Wnt/β-catenin pathway is intimately connected to the circuitry of pluripotency in ESCs (Raggioli et al., 2014; Sokol, 2011). The canonical Wnt signaling pathway centers on its downstream major player: β-

catenin. In the absence of a Wnt signal, the GSK-3β kinase, one of the major components of the "destruction complex", phosphorylates β-catenin and targets it for ubiquitin-mediated destruction. Activation of the pathway, by a Wnt ligand or by drugs such as chiron or BIO, inhibits glycogen synthase kinase-3 (GSK-3β) activity and results in the accumulation and stabilization of β-catenin (Hoppler and Kavanagh, 2007). Stable β-catenin accumulates into the nucleus where it can interact with DNA binding Tcf-Lef family factors and lead to the transcriptional activation of the target genes (Li et al., 2012; Willert and Jones, 2006).

The Wnt signaling pathway is involved in several biological processes during the development of vertebrates and invertebrates. These include cell proliferation and differentiation, cell fate decision, and organogenesis. It contributes to the self-renewal ability of undifferentiated adult stem cells in several tissues (Nusse, 2008; Reya and Clevers, 2005), and is involved in the maintenance of pluripotency, which has been demonstrated both in mouse and human ESCs (Cai et al., 2007; Ogawa et al., 2006; Sato et al., 2004; Singla et al., 2006).

Initial studies on iPSC generation suggest that constitutively active β-catenin may promote the reprogramming of fibroblasts to pluripotency (Merrill, 2008; Takahashi and Yamanaka, 2006). Jaenish´s group reported that the addition of Wnt3a to the media allows more efficient reprogramming of fibroblasts without the c-myc factor (OSK) (Marson et al., 2008; Merrill, 2008). Moreover,

in 2008 Lluis et al. demonstrated that treatment with Wnt3a or with an inhibitor of GSK-3β activity, enhances reprogramming frequencies up to 80-fold upon fusion between ESCs and different somatic cells, such as neural stem cells (NSCs), thymocytes or fibroblasts. In the same work, the authors also described that a specific threshold of β-catenin, in terms of dose and time of treatment, is needed to obtain higher efficiency in reprogramming. Indeed, the reprogramming failed when fusion was performed with ESCs harboring a genetic knockout for GSK-3β activity, or with ESCs that expressed high levels of β-catenin (Lluis et al., 2008).

1.5 In vivo dedifferentiation and reprogramming

Recent studies have highlighted that cellular plasticity, and thus the possibility that terminally differentiated cells can change their identity, can also occur under physiological conditions as part of an organ's normal injury response. Dedifferentiation into a cell with greater developmental potential (i.e., stem cells or progenitor cells) as well as the direct conversion of one differentiated cell type into another (transdifferentiation), have been shown to happen under "natural" conditions. This can occur in response to either intrinsic changes to the cell or following changes to the surrounding environment and signals.

Several examples exist of in vivo "spontaneous" dedifferentiation in both invertebrates, such as in Drosophila melanogaster testis (Brawley and Matunis, 2004), and in lower

vertebrates, such as in blastema formation during Amphibian limb regeneration (Tanaka and Reddien, 2011), or during heart regeneration in the Zebrafish (Kikuchi, 2014; Kikuchi and Poss, 2012; Poss et al., 2002). In these species tissue dedifferentiation was observed for the first time to be one of the main mechanisms associated with natural regeneration.

In mammals, recent studies have demonstrated that progenitor cells in different tissues de-differentiate into functional stem cells following injury, irradiation, or ablation of adult stem cells. These stem cells are able to give rise to all differentiated and specialized cells of the tissue, thus participating in a regenerative response. This has been shown in the hair follicle, (Fullgrabe et al., 2015) intestine (van Es et al., 2012), stomach (Stange et al., 2013), and lung (Tata et al., 2013).

However, vital organs such as the pancreas and the brain lack the capacity for effective regeneration. To overcome this limitation, several groups in the reprogramming field have explored the possibility of using cell type-specific TFs, which have been used for lineage reprogramming in vitro, to achieve reprogramming in vivo. In vivo lineage reprogramming or dedifferentiation consists of inducing the proliferation of residual cells or converting resident tissue-specific cells into the cell types that are lost due to disease (Heinrich et al., 2015).

The first achievement of in vivo reprogramming is the work of Murry et al. They demonstrated that adenovirus delivery

of *MyoD* to cryoinjured myocardium in rats could cause cardiac fibroblasts to covert to skeletal muscle cells (Murry et al., 1996). This work formed the foundation for the current line of investigation of direct, in vivo cell reprogramming.

The pancreas, the central nervous system (CNS), and also the heart in the adult, are tissues with very limited capacity for regeneration. These are therefore ideal targets for assessing the potential of lineage reprogramming *in situ.* Herrera and colleagues have reported that fate interconversion between endocrine cells can occur spontaneously following extreme toxin-induced β-cell damage in adult mice (Herrera P. et al 2001). In 2008, Melton and colleagues tested different combinations of TFs associated with β-cell development. They found that ectopic expression of Pdx1, Neurog3 and MafA allowed direct in vivo reprogramming of acinar cells into β-like cells (Zhou et al., 2008). In other cases, the in vivo conversion of acinar pancreatic cells into β-like cells has been observed after systemic exposure to cytokines and growth factors such as epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF) (Baeyens et al., 2014).

With regards to the heart, in vitro studies have elucidated the core transcriptional network during heart development and created the basis for in vivo reprogramming. In 2009 Takeuchi and Bruneau showed that overexpression of the transcription factors Gata4, Tbx5, and the chromatin remodeling protein, Baf60c, in the non-cardiogenic mesoderm, was sufficient to induce cardiomyocytes to

spontaneously contract in 50% of the transfected mouse embryos (Shirahata et al., 1997).

In most cases, this new in vivo reprogramming field is based on the full knowledge of developmental biology and thus of the key role that TF networks play in specific cell fate commitment and in the maintenance of cell identity during embryogenesis. On the other hand, another important factor for successfully achieving in vivo reprogramming is the choice of the source cell type most suited to undergo conversion into the desired cell type. The last important factor is the identification of the optimal reprogramming route and the safest method (Heinrich et al., 2015).

The route of lineage conversion may occur via direct switching of the source cell into the desired cell type. Alternatively, it may involve an intermediate fate-restricted stage, which allows for amplification of the desired cell type. However, most studies converge on one way to induce such direct conversion, the ectopic adenovirus-mediated expression of various specific TFs. Finally, other groups have investigated alternative experimental methods or the use of TF cocktails in combination with several growth factors or microRNAs, with the aim of improving reprogramming efficiency (Mathison et al., 2012; Qian et al., 2012).

1.6 In vivo reprogramming in the Central Nervous System

Among the different organs, the CNS seems to have very a limited ability to regenerate and replace lost neurons upon damage. However, several studies have shown that certain plasticity also exists at the level of post-mitotic neurons in the nervous tissue. After a decade of in vitro studies, over the past few years different groups have started to investigate the possibility of specifically manipulating gene expression in vivo via the ectopic expression of specific TFs in specific areas of the brain. In particular, the CNS harbors distinct glial cell populations, which are very abundant in nervous tissue and show the potential to divide. Because of this, these cells have been proposed as an ideal candidate cell type to generate new neurons.

Targeted expression of the TFs: Ascl1, Brn2, and Myt1l, in parenchymal astrocytes in the adult striatum in vivo could convert them into induced NeuN$^-$ neurons (Torper et al., 2013). In addition, Sox2 overexpression or genetic deletion of Notch signaling components could convert striatal astrocytes into NeuN$^+$ neurons, which do or do not pass through an intermediate proliferative neuroblast state (Niu et al., 2013). Chen and colleagues have also achieved the highly efficient conversion of reactive astrocytes into NeuN$^-$ and Tbr1$^-$ neurons, which showed functional activity with forced NeuroD1 expression. In addition, research has shown that NeuroD1 improves the conversion of active astroglia into NeuN$^+$ neurons in a mouse model of Alzheimer's disease (Lu et al., 2014).

After these first attempts, in 2014 Heinrich, C. et al. also showed that Sox2 mediated conversion of NG2 glia into induced neurons in the injured adult cerebral cortex (Heinrich et al., 2014). Thu, not only have glial cells been used as a source of in situ reprogramming, they have also been used for the conversion of neuronal progenitors or post-mitotic neurons of one subclass into neurons of another. This represents a promising strategy.

The Arlotta lab have conducted initial studies which have shown that the TF: Fezf2, could re-direct early post-mitotic callosal projection neurons, including corticospinal motor neurons, towards a corticofugal neuron fate (Rouaux and Arlotta, 2010). Moreover, in 2013 De la Rossa et al., developed an electrochemical in vivo gene delivery method to rapidly manipulate gene expression, specifically in post-mitotic neurons. They found that the molecular identity, morphology, physiology, and the functional input-output connectivity of layer 4 mouse spiny neurons could be specifically reprogrammed during the first postnatal week (De la Rossa et al., 2013).

Over the past few years, using this same scenario, our group has hypothesized that in vivo cell fate changes and the reprogramming process could also occur as a consequence of tissue damage and a cell fusion mechanism. We have demonstrated that in vivo reprogramming occurs after the fusion of transplanted bone marrow (BM)-derived hematopoietic stem and progenitor cells (HSPCs) with neurons and glia. We have shown this in both the damaged mouse retina and in a mouse model of Parkinson's disease

in the adult brain (Altarche-Xifro et al., 2016; Sanges et al., 2013; Sanges et al., 2016) (see Chapter 2.4).

2. Bone marrow derived Hematopoietic Stem Cells

The story of BM-derived hematopoietic stem cells (HSCs) began in the 20th century. Scientists were interested in understanding how ionizing radiation damages normal tissue and in finding a medically applicable intervention that could abolish the effects of a lethal dose. Several experiments have shown that intravenously injected BM cells can rescue irradiated mice from lethality by reestablishing blood cell production. This led to the discovery that, in the BM of adult mice, cells exist that have long-term hematopoietic repopulating activity (Ford et al., 1956; Jacobson et al., 1951). Because mature blood cells are predominantly short lived, stem cells able to replace multilineage progenitors and more committed precursors to form individual hematopoietic lineages that are continuously required throughout the lifespan

2.1 Hematopoietic Stem Cells in homeostasis

Hematopoietic Stem Cells (HSCs) are the adult stem cells, which give rise to all other blood cells during a process called hematopoiesis. The process of hematopoiesis is generally conserved throughout vertebrate evolution. In a healthy human adult,

approximately 10^{11}–10^{12} new blood cells are produced daily in order to maintain steady state levels in peripheral circulation. HSCs are found in the BM of adults, in particular in the pelvis, femur, and sternum. Although, HSCs have also been found in the umbilical cord blood and in fewer numbers in peripheral blood (Birbrair and Frenette, 2016).

HSCs are located in the red bone marrow in adult mammals. They are situated at the top of a complex hierarchy of progenitors that progressively undergo restriction to their multipotency to originate single or multiple lineages. These progenitors will give rise to more specialized and committed multipotent, oligopotent, and unipotent blood precursors. These in turn differentiate, producing both the myeloid and lymphoid lineages of blood cells, including red blood cells, megakaryocytes, myeloid cells (monocyte, macrophage and neutrophil), and lymphocytes (Orkin, 2000) (Figure 4).

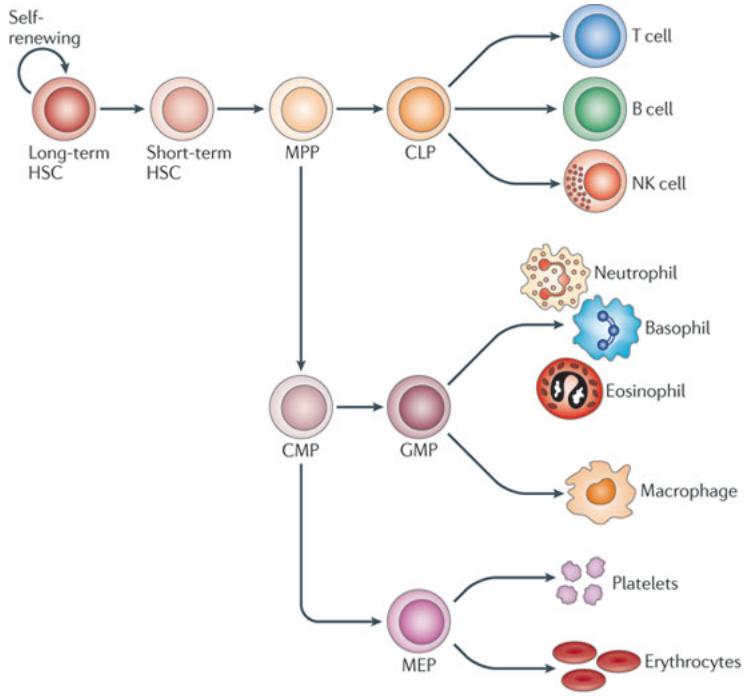


Figure 4. Hematopoietic stem cells (HSCs) hierarchy HSCs can be classified, according to their self-renewal and differentiation potential, into long-term hematopoietic stem cells (LT-HSCs) and short-term hematopoietic stem cells (ST-HSCs). These multipotent stem cells are located on the top of the hematopoietic tree and during hematopoiesis they gradually loose their self-renewal capability differentiating into more committed progenitors, in turn able to give rise to all differentiated cells of the blood. (MPP = multipotent progenitors; CLP = common lymphoid progenitors; CMP = common myeloid progenitors; GMP = granulocytes-megakaryocytes progenitors; MEP = monocytes-erythrocytes progenitors).

Several unique features that define their status characterize HSCs. Their principal property is the ability to choose between self-renewal and differentiation. Indeed HSCs are self-renewing cells, which means that they proliferate and asymmetrically divide (Brummendorf et al., 1998; Punzel et al., 2003; Wu et al., 2007). At

least some of their daughter cells remain as HSCs while the other daughter cells undergo differentiation. In this way, the pool of stem cells is not depleted.

In vertebrates, adult HSCs are derived from the ventral mesoderm. They are generated during embryonic development from distinct embryonic cells, which undergo specification in a variety of sites that change during development (Galloway and Zon, 2003). In particular, in mammals the yolk sac (YS) is the initial site of hematopoietic production. Subsequently, as development proceeds, the aorta-gonad mesonephros (AGM), the fetal liver and finally the bone marrow are the sequential sites of hematopoiesis (Boisset and Robin, 2012). Importantly, although the embryonic microenvironments of the different niches are still poorly described, it is clear that in each site HSCs acquire new intrinsic properties. These reflect the different niches and signaling environmental factors that support their proliferation and influence the balance between self-renewal and differentiation.

In contrast, HSC niches have been very well studied in adult bone marrow (Birbrair and Frenette, 2016). Three types of niches have been described so far: the endosteal (osteoblastic), the reticular, and the vascular (endothelial) niche. In the endosteal niche, the supporting cells are the osteoprogenitors. These maintain HSCs in a quiescent and slow cycling state (Calvi et al., 2003; Lo Celso et al., 2009; Zhang et al., 2003). The reticular niche consists of specialized reticuloendothelial cells that are in close contact with immune cells (B-lymphocytes, plasma cells, plasmacytoid dendritic

cells, and NK-lymphocytes), as well as sinusoidal endothelial cells and mesenchymal stem cells (MSCs). Finally, the third niche is represented by a specific microenvironment where sinusoidal endothelial cells are located. This microenvironment is also known to be rich in oxygen and low in calcium (Kiel et al., 2005). The stem cell niche is essential for the quiescence of HSCs. This is based on research, which has shown that more than 70% of these cells are in the G_0 phase of the cell cycle, while only 10% of their progenies are quiescent.

Adult HSCs can be highly purified and separated from more-committed progenitors and other BM cells by fluorescence-activated cell sorting (FACS). This is conducted using monoclonal antibodies directed to their surface specific markers.

In 1986, Müller-Sieburg et al. designed the lineage antibody panel (Lin), which has been routinely used to deplete mature cells from the erythroid, lymphoid, and myeloid lineages (Muller-Sieburg et al., 1986). However, Irvin Weissmann's group was the first to depict HSCs as a pool of highly heterogenous progenitors. In 1988, Weissmann's group both isolated mouse HSCs and was also the first to describe the markers currently used to distinguish between the two subpopulations of HSCs. These two populations include: mouse long-term (LT-HSC) and short-term (ST-HSC) hematopoietic stem cells which are capable of self-renewal, and multipotent progenitors (MPP) which have very low self-renewal ability (Spangrude et al., 1988).

The quiescence status of HSCs ensures their long-term properties. For this reason, it is difficult to maintain HSCs in culture, despite their self-renewal ability. This strictly restricts the full application and use of HSCs for transplantation in humans. Nevertheless, several attempts have been made in the field to expand in vitro HSCs. These include using various growth factors such as stem cell factor/steel factor (KitL), thrombopoietin (TPO), interleukins 1, 3, 6, 11, plus or minus the myeloerythroid cytokines GM-CSF, G-CSF, M-CSF, and erythropoietin (Domen and Weissman, 1999).

2.2 Bone marrow cell plasticity and their role in tissue repair

BM is a very heterogenous tissue comprised of several cell types. In addition to HSCs, at least one more adult stem cell type resides in BM, these are the mesenchymal stem cells (MSCs). Such MSCs not only provide a scaffold for the developing stem and progenitor cells, but also participate in the production of soluble proteins and extracellular matrix components. In fact, MSCs are capable of self-renewal. They are also capable of differentiating into many 'mesenchymal-derived' tissues such as osteoblasts, chondroblasts, and adipocytes, when exposed to the appropriate stimuli in vivo and in vitro.

MSCs also possess high differentiation capability in vitro toward cell types coming from germ layers other than the

mesodermal/mesenchymal germline where they originate (e.g, neurons) (Pereira et al., 1995; Pereira et al., 1998; Pittenger et al., 1999; Prockop, 1997). Kopen et al. were the first to demonstrate that MSCs injected into the CNS of newborn mice migrated throughout the brain and were able to acquire both morphological and phenotypic features of astrocytes and neurons (Kopen et al., 1999).

HSCs and MSCs retain the ability to differentiate into a wide range of tissues, more than other adult stem cells in the body. Recent studies strongly suggest that HSCs can be reprogrammed to differentiate into multiple lineage phenotypes that are different from the tissue where they reside (Grove et al., 2004). Researchers have asserted that once BM derived stem cells are in an environment which differs from hematopoietic niches, they can change their fate. As a result, they can also give rise to muscle cells, (skeletal myocytes and also cardiomyocytes) (Gussoni et al., 1999; Jackson et al., 2001; Orlic et al., 2001a; Orlic et al., 2001b), brain cells (Brazelton et al., 2000; Mezey and Chandross, 2000), liver cells (Lagasse et al., 2000; Petersen et al., 1999), skin cells, lung cells, kidney cells, intestinal cells (Krause et al., 2001), and pancreatic cells (Ianus et al., 2003) (Figure 5).

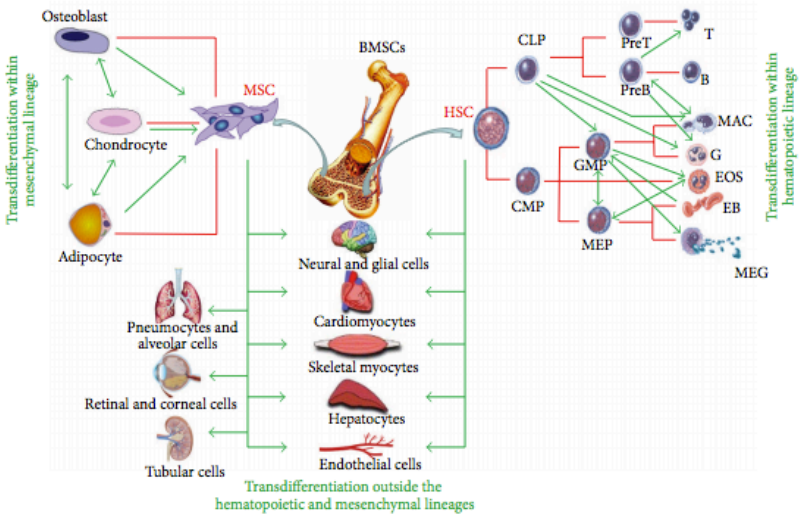


Figure 5. Bone marrow (BM) cell plasticity HSCs and MSCs possess the intrinsic capability to differentiate into a wide range of tissues different from the mesodermal germ layer from which they originally come from. Transdifferentiation outside the hematopoietic and mesenchymal lineages has been observed. Both HSCs and MSCs can be reprogrammed to differentiate into multiple lineage phenotypes including muscle cells, liver cells, neural and glial cells, skin and endothelial cells etc. Also transdifferentiation within the same hematopoietic cell lineage and within the mesenchymal lineage has been described as a physiologic mechanism to repair lost cells and to maintain tissue homeostasis.

Research has shown that after the transplantation of BM cells into the adult mouse brain, such cells are able to give rise to both microglia and macroglia cells (Eglitis and Mezey, 1997). This discovery challenges the original idea that HSCs were irreversibly committed to differentiate into a blood cell line. In 1998 an Italian group also demonstrated that BM cells could migrate into injured mouse muscle following transplantation, by participating in the muscle regeneration, although this occurred with low efficiency (Ferrari et al., 1998). The Gussoni E. lab has provided the clearest

demonstration of a potential clinical application of the BM cell capability to differentiate into a wide range of cell types. In a mouse model of muscular dystrophy, the researchers demonstrated that BM transplanted cells were able to be engrafted and expressed normal dystrophin in up to 10% of muscle fibrils after 12 weeks (Gussoni et al., 1999).

Following this pioneering research, other groups have demonstrated BMs ability to promote the repair of myocardial tissue in mouse and rat models of myocardial infarction, as well as in other damaged organs. These experiments have shown for the first time that the fate of BM adult stem cells is very "plastic". In addition, they have shown that under specific circumstances, BM cells can differentiate into the required cell type within the tissue and thus participate in the regeneration of non-blood tissues. This unexpected plasticity occurs not only under experimental conditions but also in humans following BM transplantation. As a result, BM transplantation has emerged as a novel fascinating approach to enhance neural regeneration and restore injured brain tissue.

Nevertheless, the molecular mechanism by which BM cells acquire a different phenotype is still unknown. One hypothesis is that HSCs, which are already committed to hematopoiesis, may undergo a reprogramming process to then differentiate into a different cell type. Another possibility is that more immature cells may be present within the BM compartment of a subpopulation of HSCs. These may be not yet be "committed" to adopt a blood phenotype and thus may be "prone" to differentiate into multiple

lineages. Researchers have suggested that the acquisition of a different identity by grafted BM derived stem cells could be ascribed to two different mechanisms (Vieyra et al., 2005). These mechanisms include the direct transdifferentiation of adult stem cells into a different phenotype, and the fusion of adult stem cells with somatic host cells from which they acquire all the information to change their identity.

The combination of the entire genomes and cytoplasms of two cells with different functions, and from different developmental states, may result in a rapid switch of gene expression profile from one genome to that of the fusion partner. Thus, upon cell fusion, it is possible in vitro to switch directly through different cell fates. Many of the earlier described studies have demonstrated that transplanted BM cells can fuse with several cell types in the body, including myocytes (Ferrari et al., 1998), hepatocytes (Wang et al., 2003), neurons, and many others (Alvarez-Dolado et al., 2003).

Transdifferentiation of BM HSCs can also occur within the hematopoietic system when progenitors that are committed to a specific myeloid lineage switch their differentiation into lymphoid cell types or vice versa. There are many described instances of lymphoid-to-myeloid and myeloid-to-erythroid changes after induction of TF expression, cytokine or drug treatment, and changes in environmental conditions (Wolff and Humeniuk, 2013).

2.3 Bone marrow cell mobilization

As we mentioned earlier, BM derived HSCs asymmetrically divide to originate two daughter cells. One daughter cell maintains self-renewal ability, and the other daughter cell migrates to the main BM compartments to differentiate into more committed progenitors. The fine balance between self-renewal and the differentiation of HSCs is strictly dependent on signaling factors released in an integrated specialized microenvironment where HSCs reside. This is called a "stem cell niche" and was defined for the first time by Ray Schofield in 1978.

Inside the niche, HSCs are maintained in a stable number under steady state conditions. The common belief is that, within the stem cell niche, a large variety of adhesion molecules keep HSCs in close contact with osteoblasts, other stromal cells, and the extracellular matrix. The most studied HSC niche interaction is between the CXC4 chemokine receptor (CXCR4) and its ligand, the stromal cell-derived factor 1α (SDF-1α). In addition to SDF-1, specialized spindle-shaped N-cadherin-expressing osteoblasts (SNOs), located in the "endosteal niche" (see chapter 2.1), express several signaling molecules that regulate HSCs function and retention in the BM niche. These include vascular cell adhesion molecule 1 (VCAM-1), osteopontin and others.

In addition, sinusoidal endothelial cells, which constitute the "endothelial niche" (see chapter 2.1), constitutively express cytokines such as CXC-chemokine ligand 12 (CXCL12), and

adhesion molecules such as endothelial-cell (E)-selectin and vascular cell adhesion molecule 1 (VCAM1). These are important for HSC mobilization, homing and engraftment (Avecilla et al., 2004).

The disruption of these tight interactions after any kind of "stress", can lead to a phenomenon called "peripheral blood stem cell mobilization". This is the release of HSCs from the niche and their translocation from bone marrow to the peripheral blood. Several factors such as chemotherapy, chemokines and small-molecule chemokine receptor inhibitors, and hematopoietic growth factors can trigger mobilization of BM cells (Papayannopoulou, 2004; Seggewiss et al., 2003). Disruption of the pre-existing connections and interactions, which maintain the HSC anchored to the stromal cells inside the niche, is mediated by proteolytic enzymes and methalloproteases. These are needed to cleave crucial microenvironmental retention factors. Neutrophil cells are also required for HSC mobilization because their proteolytic enzymes are essential. This is supported by research, which has shown that inhibition of these enzymes in protease deficient mice, remarkably reduces BM cells mobilization (Pelus et al., 2004).

Osteoblasts also have an essential function inside the HSC niche and are necessary for BM retention. Research has shown that deletion of these stromal cells using a transgenic model in which diphtheria toxin was applied, led to a massive mobilization of HSCs (Panaroni et al., 2014). In addition, evidence also indicates that osteoclasts contribute actively to the degradation of micro

environmental components by providing several enzymes (Kollet et al., 2006).

2.4 SDF-1/CXCR4 signaling pathway

The interaction between CXC4 chemokine receptor (CXCR4) and its ligand, the chemokine Stromal Cell Derived Factor 1 (SDF-1), also known as CXC chemokine ligand 12 (CXCL12), is one of the most investigated inside the niche. Osteoblasts are the major producers of SDF-1 (Peled et al., 2000) together with reticular specialized cells in endosteal and vascular niches, endothelial cells, and in the bone itself (Sugiyama et al., 2006). Recently, high levels of SDF-1 have also been found in a subpopulation of MSCs expressing Nestin, located in the stroma (Mendez-Ferrer et al., 2010). The CXCR4 receptor is expressed by HSCs, which are attracted in the BM (Aiuti et al., 1997; Kucia et al., 2004).

This interaction plays a central role both in the retention of BM cells inside the niche, as well as in their mobilization and homing. During the homing process, as well as during mobilization, the expression of several molecules including chemoattractants, selectins, and integrins, is critically required to enable BM cells to enter the niche.

Among the different chemoattractants, several studies have identified the chemokine SDF-1, as one of the major players

regulating HSC trans-endothelial migration (Aiuti et al., 1997; Tashiro et al., 1993). In addition to regulating migration to the BM, SDF-1 also plays an important role in the retention of HSCs in the BM. Indeed, several studies have shown that increase in SDF-1 levels in plasma, but not in BM, results in a strong mobilization of HSPCs expressing CXCR4, into the peripheral blood (Hattori et al., 2001).

Moreover, mice or human volunteers treated with AMD3100, a specific CXCR4 antagonist, also show an increased amount of HSCs in the peripheral blood. This finding indicates the role of SDF-1-CXCR4 interaction in the maintenance of HSC in the BM niche (Broxmeyer et al., 2005). Genetic knockout of either CXCR4 (Zou et al., 1998) or SDF-1α (Nagasawa et al., 1996) in mice is embryonically lethal because HSPCs cannot be mobilized from peripheral blood to bone marrow during development. On the contrary, conditional knockout mice show an increased trafficking of BM cells from the BM niche to peripheral blood (Nie et al., 2008). Moreover, HSPCs missing CXCR4 receptors, are not retained inside the BM niche after transplantation (Foudi et al., 2006).

Many molecules and reagents have been discovered so far and tested for their efficiency in the disruption of the interaction between SDF-1 and its receptor. Among these, the CXCR4 antagonist AMD3100 seems to be the most efficient (Broxmeyer et al., 2005). In addition, research has shown that treatment with AMD3100, in combination with G-SCF, strongly enhances HSCs

mobilization (Broxmeyer et al., 2005), and results in stimulation of angiogenesis at ischemic sites (Capoccia et al., 2006). Furthermore AMD leads to the recruitment of endothelial progenitor cells (EPCs) into injured areas contributing to neovascularization after myocardial infarction (Jujo et al., 2010).

Likewise, by cleaving and degrading many components of the extracellular matrix, activity of proteolytic enzymes can negatively regulate the SDF-1/CXCR4 axis interaction and consequently actively participate in the regulation of BM progenitor cell migration. Specifically, evidence indicates that matrix metalloproteinases (MMP) 2/9 mediate the cleaving of SDF-1 at specific residues. This impairs the interaction between this chemokine and the CXCR4 receptor, thus finally promoting the mobilization of HSCs out from bone marrow niche (Yoder and Williams, 1995).

Together with this fundamental function in regulating homing and trafficking of BM derived HSPCs, SDF-1 has been found to be strongly up regulated in several tissues after damage including in the liver, brain, and retina. These findings indicate that chemokine is implicated in tissue repair (DeLeve et al., 2016; Lima e Silva et al., 2007; Mocco et al., 2014).

In fact, in basal conditions, the expression of SDF-1 at low levels in peripheral blood ensures that HSCs and MSCs are kept at a certain level, in both the peripheral blood and in distant tissues.

When tissue damage occurs, the level of this chemo attractant chemokine increases in peripheral organs, creating a change in the normal gradient of this factor. This process serves the purpose of driving efficient migration of $CD34^{+}$ stem cells to the injured sites (Figure 6).

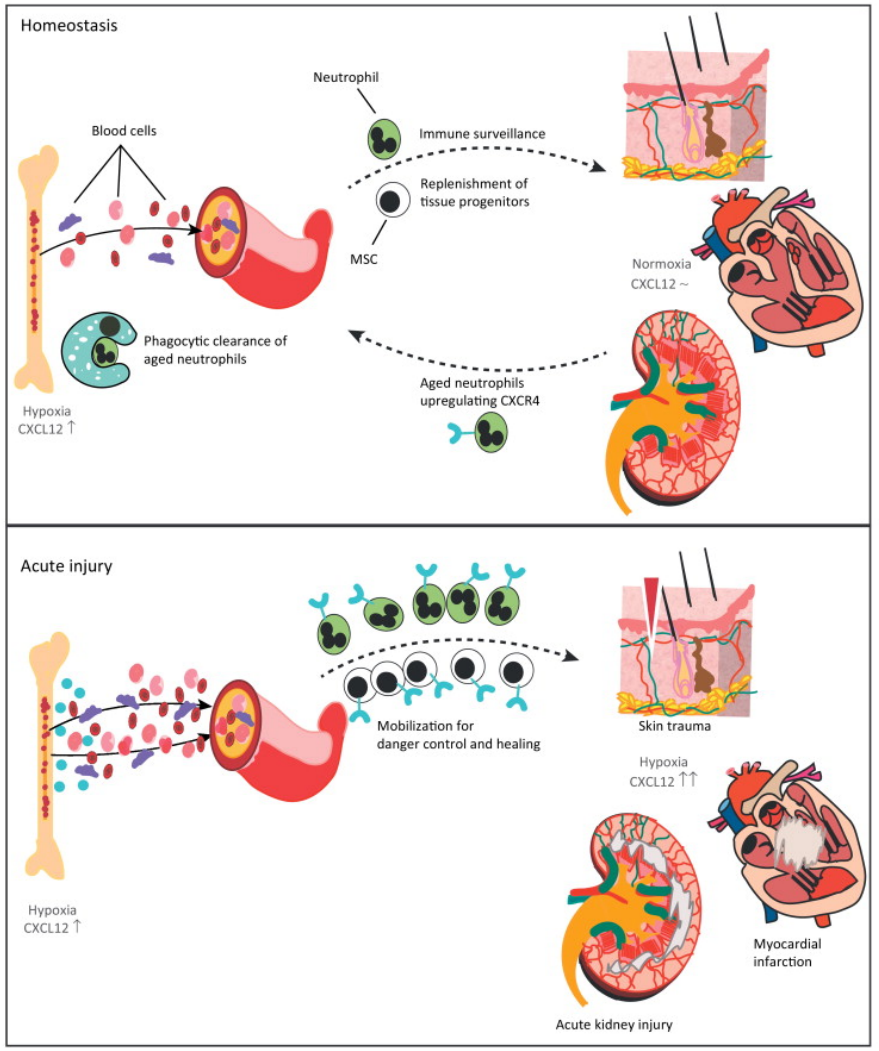


Figure 6. SDF-1/CXCR4 interaction in homeostasis and upon tissue damage SDF-1 is mainly produced by osteoblast cells in the niche and its interaction with the CXCR4 receptors on the membrane of HSCs and

MSCs ensures the retention of BM cells inside the niche. Upon tissue damage the increased level of SDF-1 chemokine in the peripheral blood and in distant organs causes a change in the gradient leading to the mobilization of BM cells from the niche to peripheral blood.

The importance of SDF-1 in the homing of stem cells to damaged sites is supported by the observations that SDF-1 is up regulated after several types of damage and under hypoxic conditions. These include during myocardial ischemia (Yu et al., 2010), cerebral ischemia (Shen et al., 2007), and renal failure (Togel et al., 2005). The increased level of expression correlates with adult stem cell recruitment and tissue regeneration (Askari et al., 2003; Ceradini et al., 2004; Kollet et al., 2003; Yamaguchi et al., 2003). Moreover SDF-1 is secreted from vascular endothelial cells and reactive astrocytes in injured regions of the brain (Ohab et al., 2006; Thored et al., 2006). It has also been shown to be up-regulated in a rat model of retinal ischemia-reperfusion injury (Lai et al., 2008), and after retinal pigmented epithelium damage (Li et al., 2006).

3. The vertebrates retina: a model for Central Nervous System (CNS) studies

In 1894, Santiago Ramón Cajal was the first to characterize retinal neurons in his work, *Retina der Wirbelthiere* (The Retina of Vertebrates)(Bergua, 1994). For the first time, Cajal described that the retina was composed of individual neurons, thus contradicting

the theory at that time that the nervous system was a synzythium. In 1967, George Wald, Haldan Keffer Hartline and Ragnar Granit were awarded the Nobel Prize in physiology for Medicine for their discoveries concerning the mechanisms by which light triggers reactions in the sensory cells of the eye and thus for identifying the primary physiological and chemical visual processes in retina. They discovered that visual pigments, the light-sensitive substances in the sensory cells, are comprised of two components: the chromophore and the opsin. When a light quantum is taken up by the visual pigment there is a molecular transformation induced by light. This is the isomerization from II-*cis* to all-*trans* that triggers the subsequent events in the visual system.

3.1 Retinal structure and development

The retina (from Latin *rēte*, meaning "net") is the light-sensitive nervous tissue of the eye and is considered a part of the Central Nervous System (CNS) and thus a “brain tissue”. This is because during embryonic development the retina forms from outgrowths of the developing brain in vertebrates. Specifically, during vertebrate embryo development, the retina extends from the diencephalon where it elongates in two branches, forming the optic vesicles and finally the optic cup. The optic cup is comprised of two layers that later differentiate in different directions. The cells of the outer layer give rise to the pigmented retina (PR), while the cells of the inner layer proliferate and ultimately become neural retina (NR)

(Heavner and Pevny, 2012). Because the retina can be thought as a `stripped down' version of the brain, and because it is accessible and can be easily visualized, it is often utilized as a model system to elucidate fundamental questions regarding the CNS at large.

The neural retina is a highly specialized tissue that consists of six major types of morphologically and functionally different retinal neurons, with cell body distributions and connectivity arranged in stereotypic patterns. All retinal cell types derive from a common retinal progenitor cell (RPC) population. Here, the specific, sequential, and coordinated expression of pro-neural TFs ensures the differentiation of the distinct subpopulations (Cepko, 2014; Wetts and Fraser, 1988).

The fundamental plan of the retina is conserved across vertebrates although there are some specializations in circuit design across species. The non-random ‘mosaic-like’ distribution of cells belonging to the same type is a common organizational principle of the vertebrate retina. Neighboring cells of the same layer are linked to one another through electrical synapses, also called gap junctions. These are small ionic channels allowing the bidirectional circulation of ions between the cells’ two cytoplasms. At the same time, neighboring cells from different layers are also linked one to another, this time through chemical synapses (Wassle and Riemann, 1978).

The retina is comprised of ten different layers that can be simplified into three major cell layers: the outer nuclear layer

(ONL), the inner nuclear layer (INL) and the ganglion cells layer (GCL) (Jeon et al., 1998).
Coding of visual information begins with conversion of light energy into membrane potential changes in the photoreceptors (PRs) that alter neurotransmitter release. PRs, the "sensitive" cell types of the retina, are localized in the onl and can be categorized into rods and cones. These represent 70 % of the total retinal population.

Rods are responsible for dim-light vision since because they have exquisite sensitivity to light and can detect even a single photon (Rieke, 2000). Cones are engaged for bright-light, high-acuity color vision. In fact, they are 100 times less sensitive than rods, but exhibit much faster response kinetics during phototransduction. Rod and cone photoreceptors make synapses with bipolar cells at the outer plexiform layer (OPL).
Together with horizontal and amacrine cells, bipolar cells represent the interneurons, which are located in the middle layer of the retina, the inl. Bipolar cells can be divided into two major classes: rod and cone bipolar cells. Rod bipolar cells primarily synapse with rod photoreceptors, whereas cone bipolar cells primarily synapse with cone photoreceptors. In addition, bipolar cells form two functional subclasses: those that depolarize (ON) and those that hyperpolarize (OFF) to increments in light intensity.

Cone bipolar cells contact retinal ganglion cells and amacrine cells within the inner plexiform layer (IPL). Horizontal cells mainly modulate the signaling between photoreceptors and bipolar cells. Amacrine cells are synaptically active in the IPL and

function to integrate, modulate and control the signals that ganglion cells receive from the outer retina. Ganglion cells are the first retinal cell type that differentiates during development. They represent around 2% of total retinal neurons in retina, and are the sole output neurons of the retina projecting their axons to higher visual centers. A part from these retinal neurons, Müller glia cells are the only glial cell type in the retina. They play an important role in the maintenance of retinal architecture and structure (Masland, 2012) (Figure 7).

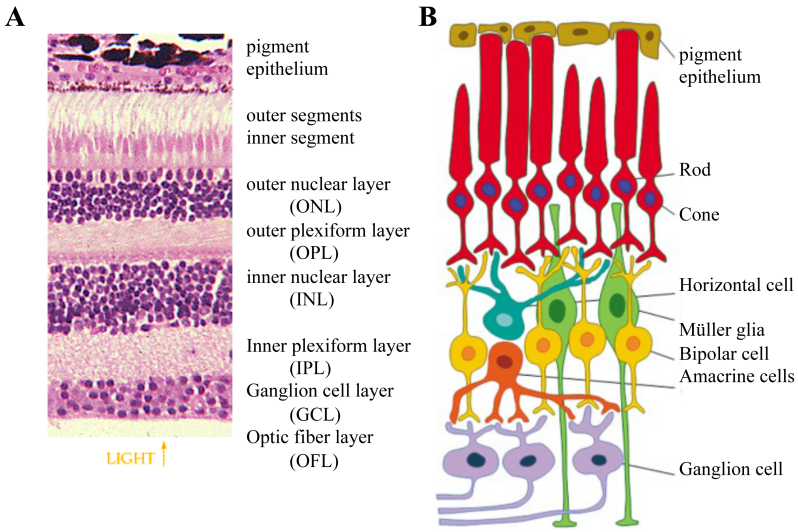


Figure 7. Mammalian retinal structure (A) Hematoxylin and eosin staining of retinal sections showing all the different retinal cell layers. (B) Light from outside passes all the retinal layers and is captured by rods and cones photoreceptors (red), the light sensitive cells, which are part of the outer nuclear layer (ONL). Bipolar cells (yellow), horizontal cells (dark green) and amacrine cells (orange) are the interneurons, which form the inner nuclear layer (INL). Also Müller glia cells (light green) bodies are located in the INL Ganglion cells (violet) make the ganglion cell layer (GCL) and with their axons form the optic nerve, which transmits the light

stimulus to the brain. Photoreceptors communicate with interneurons making synapses at the level of the outer plexiform layer (OPL), while interneurons contact the ganglion cells at the level of the inner plexiform layer (IPL).

3.2 Retinal regeneration

Studies conducted over the past 100 years using a wide variety of animals, have contributed to the current knowledge that amphibians, fish, birds and mammals all possess regenerative properties in the retina. Yet, even though retina regeneration is carried out at specific developmental stages, only a few animals have been shown to possess retinal regenerative potential as adults.
In vertebrates, the strategies used to regenerate retinal tissue seem to be evolutionarily conserved and can be summarized into two major groups. These are: regeneration through the activation of cells located in the CB (ciliary body)/ CMZ (ciliary marginal zone), and transdifferentiation of resident Müller glial cells or cells from the pigmented retinal epithelium. In the first case, the "activation" of stem/progenitor cells that reside in the CB or CMZ and their proliferation, are both needed before differentiation into retinal cells.

In the case of transdifferentiation, the regeneration process requires an additional dedifferentiation step whereby RPE or MG cells lose their original properties and proliferate before acquiring a different phenotype (Del Rio-Tsonis and Tsonis, 2003; Haynes and Del Rio-Tsonis, 2004).

In amphibians and fish retina growth continues until adulthood due to the contribution of progenitor cells present in the CMZ (Hollyfield, 1968; Johns, 1977). In birds, the CMZ can also provide retinal cells for retinal growth, but in this case regenerative properties of the CMZ are lost postnatally (Fischer and Reh, 2000).

Nevertheless, several groups have shown that potential regenerative activity can be stimulated by several exogenous factors. Such exogenous factors are required for the proliferation and survival of stem/progenitor cells in the CMZ during regeneration. These include fibroblast growth factor (FGF), endothelial growth factor (EGF), insulin-like growth factor-1 (IGF-1), insulin, and Sonic Hedgehog (Shh) (Fischer and Reh, 2000, 2003; Moshiri et al., 2005). In addition, bone morphogenetic protein (BMP) also promotes proliferation of stem/progenitor cells via its canonical pathway (through SMADs) during the early stages of retina regeneration (Haynes et al., 2007). Recently, Wnt signaling was also identified to be essential for maintaining the stem cell niche of the CB/CMZ after retina removal (Zhu et al., 2014).

Following retinal injury in fish and amphibians, progenitor cells from the CMZ can contribute to retina regeneration. They do so by proliferating and eventually differentiating to replace various types of lost retinal cells. However, the largest contributors to retinal regeneration are “regenerative cells” within the RPE in amphibians and in MG cells in fish. In both Xenopus and Rana

catesbienna, retinal regeneration is predominantly achieved via RPE transdifferentiation (Yoshii et al., 2007).

Several in vivo and in vitro studies have identified the association between RPE cells and the vascular membrane as a crucial step during the process of transdifferentiation. Such research has highlighted the importance of environmental factors, cell–cell contact, and cell–extracellular matrix (ECM) interactions to successfully achieve retina regeneration via transdifferentiation (Reh et al., 1987).

In the embryonic chicks, retinal regeneration through RPE transdifferentiation also occurs during a small window of its development, between stages 23 and 25. This transdifferentiation process appears to be dependent on the presence of exogenous FGF2. The molecular mechanism by which FGF2 induces cell fate conversion includes the activation of the mitogen-activated protein kinase (MAPK) pathway and the up-regulation of Pax6 in the transdifferentiating RPE cells (Pittack et al., 1991; Spence et al., 2004).

Unlike birds and mammals, teleost fish such as zebrafish can regenerate a damaged retina and functionally restore visual behavior (Sherpa et al., 2008). In fish, MG cells represent the main source of retinal regeneration. MG cells can give rise to different progenitor populations: rod precursors and retinal progenitors cells (RPCs). Rod precursors are only able to differentiate into rods and are

continuously produced in the adult to respond to the constant growth demands of the retina (Johns, 1977). In addition, following injury, MG can de-differentiate generating RPCs. RPCs are multipotent progenitors that are able to differentiate into all retinal cell types (Bernardos et al., 2007).

In birds, MG cells have been reported as the major source of retinal regeneration, although the retinal cell types that can be replenished postnatally are more limited than in fish. In mammals, although MG cells share many characteristics with retinal stem cells(Wilken and Reh, 2016).

In mammals MG cells, although they share many characteristics with retinal stem cells (Roesch et al., 2008) they do not behave like retinal progenitors in vivo. Their neurogenic capability is markedly reduced, and they have a demonstrable low rate of proliferation (Goldman, 2014) (Figure 8). In the next chapter, we will describe the studies made in mammals in greater detail (see Chapter 3.5).

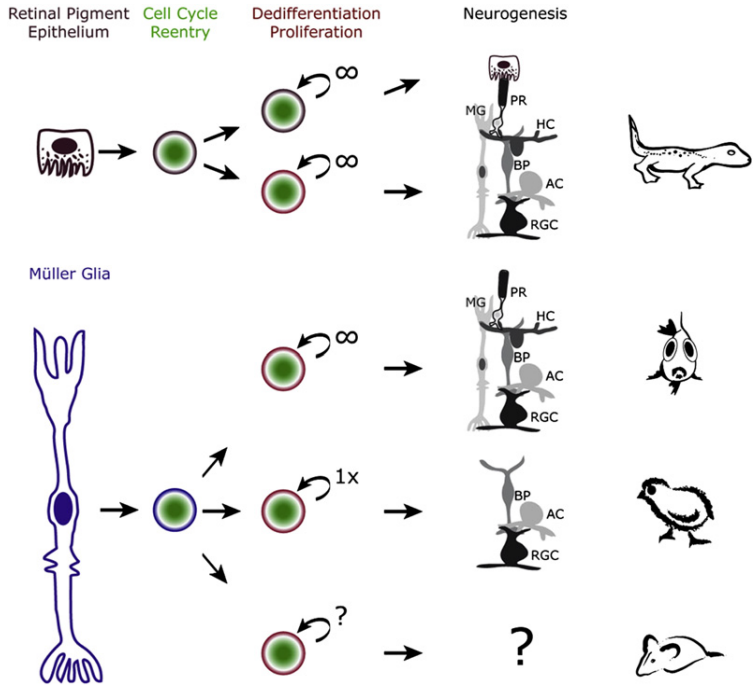


Figure 8. Retinal regeneration in lower vertebrates and mammals In amphibians retinal pigment epithelium (RPE) represents the main source of adult retinal stem cells able to reenter the cell cycle and to proliferate to replace any retinal cell type. In other species such as fishes, chickens and mammals Müller glia (MG) cells de-differentiate by acquiring proliferative potential and generating a pool of retinal progenitor cells (RPCs), which contributes to retinal regeneration. However the retinal cell types that can be replenished after retinal damage differ among the different species, with the greatest neurogenic capability in fishes.

3.3 Müller glia (MG) cell function in homeostasis and tissue repair

MG cells are the main glial cell type in the retina, and they are one of the final retinal cell type to be formed during development (Young, 1985). MG cells share a common embryonic

origin with retinal neurons and are generated from common multipotent progenitors although both intrinsic and extrinsic factors will finally determine the temporal order in which the different retinal cell types are born (Cepko, 2014; Turner and Cepko, 1987). During this developmental process several molecules influence the fate of RPCs like Notch, Janus kinase (JAK), ciliary neutrophic factor (CNTF) and homeobox protein Rx (RAX) (Bhattacharya et al., 2008; Furukawa et al., 2000; Jadhav et al., 2006). Importantly, a recent study in mouse suggests that a subset of Müller cells may be derived from the neural crest (Liu et al., 2014).

MG cells bodies are located in the INL but their cytoplasm extends toward all retinal layers. With their processes they contact neighbouring neurons and form part of the outer and inner limiting membranes. As a result of this favorable position, MG cells typically contribute to the maintenance of retinal structure and homeostasis (Bringmann et al., 2009; Reichenbach and Bringmann, 2013). They also function as a "physiologic" barrier by participating in the transfer and transport of several molecules, neurotransmitters, and ionic and trophic factors between the different retinal compartments and the extracellular space (Nagelhus et al., 1999; Pow and Crook, 1996). Moreover, they also have phagocytic capacity because they can contribute to the assembly of the cone outer segments. MG cells also play an active role in the recycling of the retinal chromophore for photo detection (Wang et al., 2004).

In addition to this homeostatic role, recent research has

demonstrated that MG actively contributes to vision by functioning as optic fibres that guide light to the PRs (Franze et al., 2007). Although they share a lot of features with radial glia in the cortex of the CNS including their radial morphology and shape, under normal conditions MG cells do not function as neural progenitors, nor as "scaffolds" for cell migration during development. Nonetheless, transcriptomic analysis has revealed a great similarity between the molecular profile of MG and RPCs (Blackshaw et al., 2004; Jadhav et al., 2009; Roesch et al., 2008). This could explain why MG cells act as adult stem cells, especially in lower vertebrates such the teleost fish (Lenkowski and Raymond, 2014; Nagashima et al., 2013) and chickens (Fischer and Reh, 2003). In these animals, MG cells respond to injury by expressing retinal stem cell genes. Like RPCs, they can proliferate, re-enter the cell cycle, and finally re-differentiate to replace damaged retinal neurons (Jadhav et al., 2009; Thummel et al., 2008).

In the adult mouse retina, MG cells undergo proliferation following pharmacological damage of ganglion cells and photoreceptors, although the number of proliferative MG cells in rodent models is relatively small (Karl and Reh, 2010). In addition, MG cells can also contribute to neuronal regeneration, though with very low frequency. However, the mechanism behind this limited regenerative potential in mammals remains to be fully explored (Ooto et al., 2004).

3.4 MG reprogramming and MG-mediated retinal regeneration in lower vertebrates

After retinal injury, as well as in many retinal diseases, MG cells respond by activating a "damage-response program", which consists of changes in their morphology, biochemistry, and physiology. However, during the response to damage, which is accompanied by a global change in gene and protein expression, MG also undergoes hypertrophy and subsequently causes "gliosis". Gliosis can be beneficial for retinal neurons because it leads to the release of factors that protect neurons from glutamate neurotoxicity and cell death. Yet, if prolonged, gliosis can have detrimental effects (Bringmann et al., 2009).

Of the different species studied, fish show a strong and efficient MG regenerative response to retinal damage. The majority of studies conducted in zebrafish have highlighted that the process of MG reprogramming and participation in retinal repair requires three different steps. These are: reprogramming of MG cells to acquire retinal stem cells properties, generation of MG-derived multipotent progenitors, and finally cell cycle exit to allow differentiation into a neuronal cell fate.

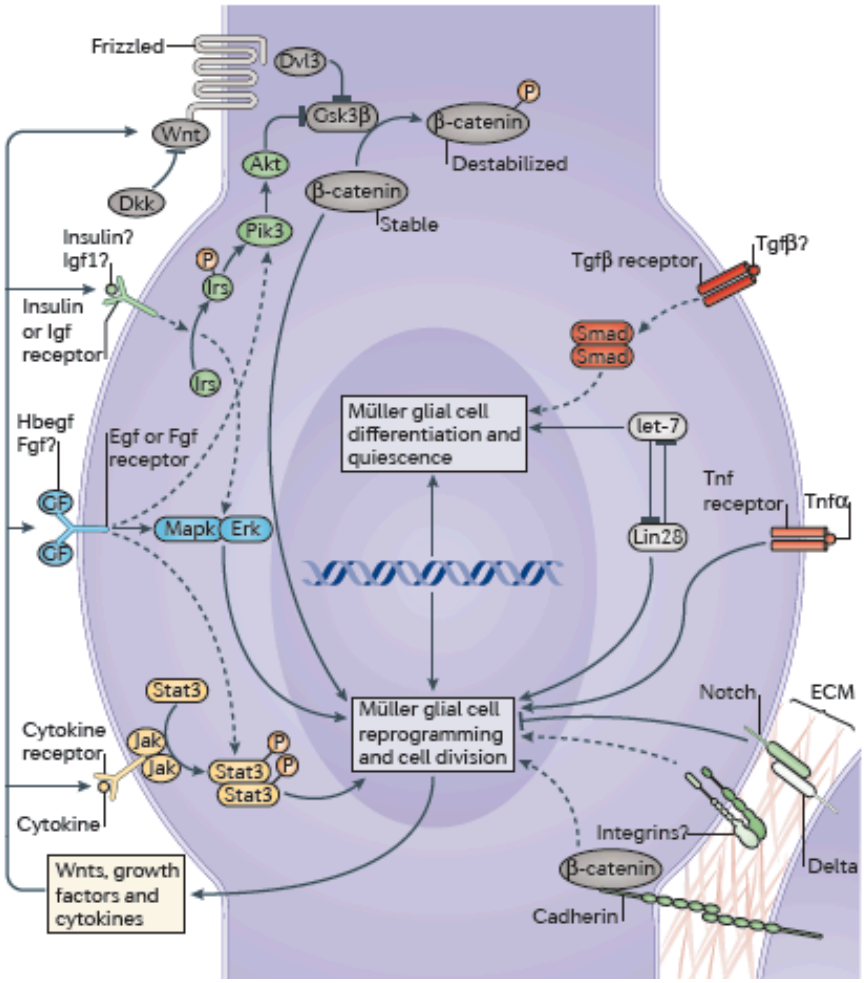
Transgenic zebrafish lines in which MG or MG-derived progenitors were specifically labelled with green fluorescent protein (GFP), can be used by researchers to track the progeny of MG proliferating cells. They can also be used to identify all the major retinal cell types produced after damage by taking advantage of

bromodeoxyuridine (BrdU) lineage tracing strategies and Cre–*loxP* technology (Fausett and Goldman, 2006; Fimbel et al., 2007; Kassen et al., 2007; Ramachandran et al., 2010). MG cells activate a "sensory response" after several kind of retinal damage, including mechanical damage (Fausett and Goldman, 2006), damage by intense light (Bernardos et al., 2007; Vihtelic and Hyde, 2000), chemical damage (Fimbel et al., 2007), and cell type-specific expression of toxic genes (Montgomery et al., 2010). However, independent of the kind of the damage sustained, several factors, which are produced both by dying cells and by MG cells, have been identified and shown to stimulate MG cells response to the injury in a "autocrine or paracrine" manner. These factors cross-communicate with each other and converge on the same common signaling pathways.

In particular, soluble factors such as tumour necrosis factor-α (Tnfα), heparin-binding EGF-like growth factor (Hbegf), Wnts, adenosine diphosphate (ADP), and ciliary neurotrophic factor (CNTF) promote progenitor formation after injury-induced Müller glial cell reprogramming in fish (Battista et al., 2009; Faillace et al., 2002; Kassen et al., 2009; Nelson et al., 2013; Wan et al., 2012). Further, research has shown that the formation and proliferation of MG-derived progenitors during MG regenerative response requires the induction of the transcription factor Ascl-1 expression. In turn, it requires the activation of transcription 3 (Stat3) (Nelson et al., 2013) after Tnfα has bound to its receptor (Fausett et al., 2008; Ramachandran et al., 2010). In fact, following retinal injury, Stat3

is induced in both quiescent MG cells and in MG-derived progenitors. Its knockdown prevents progenitor cell generation (Kassen et al., 2007; Nelson et al., 2012) (Figure 9).

A



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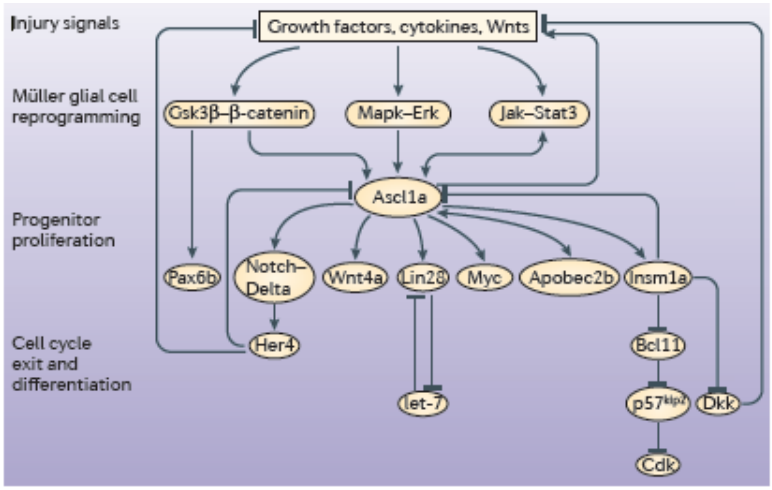


Figure 9. Signaling pathways that contribute to MG cell reprogramming and retinal progenitor proliferation in Zebrafish (A) The major signaling pathways described as crucial in regulating MG cell reprogramming and proliferation are shown and indicated by the continue and solid line arrows, whereas those indirectly involved or hypothesized to be involved are indicated by dashed lines. Growth factors and cytokines are shown outside the cell. (B) Regeneration associated genes are shown. Growth factors and cytokines acting in an autocrine or paracrine activate several signaling pathway in MG cells. The downstream transcriptional cascade converges on the transcription factor Ascl-1a, which promotes MG cells reprogramming after injury. Several TFs mediating cell cycle exit and finally differentiation of progenitors into the different retinal cell types are also shown in the bottom.

Finally, further studies have demonstrated that immune response and phagocytosis also play a role in MG cells reprogramming. Their proliferation capacity can be influenced by soluble factors released as consequence of immune cell and microglia cell migration in the damaged tissue (Craig et al., 2008; Fischer et al., 2014) and blocked when phagocytosis is inhibited (Bailey et al., 2010).

Amphibians such as the newt or salamander possess a high regenerative capability following ablation. In 2007, Yoshii et al. demonstrated that such amphibians also retain the ability to regenerate their retinas at post metamorphic stages following retinectomy (Yoshii et al., 2007). It remains unknown whether their MG cells participate in retinal repair. This is because the models used to study retinal regeneration included large ablation or even retinectomy; thus resident MG cell response could not be investigated. However, a few studies suggest that in amphibians there is also a subpopulation of MG cells that proliferate and re-enter the cell cycle following damage, such as after retinal detachment (Grigorian et al., 1996; Novikova Iu et al., 2008).

In birds, here are first reports of the regeneration of some types of retinal neurons. These were found in the chicken post hatching, following acute neurotoxic damage, and with a proliferative response observed in MG cells (Fischer and Reh, 2002). Here, MGs re-entered the cell cycle, de-differentiated into retinal progenitors, and generated new retinal neurons and MG cells. Also in birds, as in fish, the inflammatory response of Müller cells and the activation of microglia and macrophages, stimulated MG cell dedifferentiation, reprogramming, and cell cycle re-entry (Fischer et al., 2015). In addition, studies have found that exogenous growth factors such as insulin and FGF2 may influence MG dedifferentiation ability (Fischer and Reh, 2002; Todd et al., 2015). Finally the Notch signaling pathway, which is up regulated in MG cells after damage (Ghai et al., 2010), together with the

Hedgehog signaling (Todd and Fischer, 2015) and the Wnt/β-catenin pathway (Gallina et al., 2016) have been shown to be necessary for the dedifferentiation and proliferation of MG cells in chickens.

3.5 MG reprogramming and MG-mediated retinal regeneration in mammals

In mammals, the regenerative response of MG and their reprogramming and proliferation potential is very limited. This is the case even though important studies in rodent and human cell cultures have shown that MG cells can generate both neurons and glia (Das et al., 2006; Lawrence et al., 2007). Notably, primary human MG cell culture generates photoreceptors and retinal ganglion cells that exhibit some regeneration potential when transplanted into damaged mouse retinas. These MG-derived neurons integrate into the correct retinal layer and allow improvements in rod and ganglion cell function (Singhal et al., 2012). Several studies have tried to investigate mammalian MG behavior and to enhance their regenerative response in vivo but with limited success.

In 2004 for the first time Ooto et al. demonstrated that murine MG cells possess neurogenic ability. They undergo reprogramming and dedifferentiation by re-entering the cell cycle after N-Methyl-D-aspartate (NMDA)-induced retinal damage, finally generating new bipolar and photoreceptors (Ooto et al.,

2004). After this pioneer study, other work has shown that photoreceptors regeneration can also be achieved in retinal explants (Wan et al., 2008) and following N-methyl-N-nitrosourea (MNU) retinal damage (Osakada et al., 2007). Yet, the mechanism behind this limited regenerative potential in mammals has not been fully investigated. Some studies suggest that pharmacological perturbation of many signaling pathways, such as the Wnt/β-catenin, Shh, epidermal growth factor (EGF)–EGF receptor (EGFR), Notch signaling, and overexpression of the TF Ascl-1, could stimulate the proliferation and neural regenerative potential of MG cells in the mammalian retina (Del Debbio et al., 2016; Karl et al., 2008; Wan et al., 2007). Overall, these reports foresee the possible establishment of an appealing regenerative approach for retinal neurons in the treatment of retinal degeneration that is based on stimulation of MG cells.

One possible explanation for the limited regenerative ability of mammalian MG cells could be ascribed to the suppression, during postnatal development, of the EGFR and to a concomitant increase in transforming growth factor-β (TGFβ) signaling. In fact, TGFβ signaling, stimulates the expression of the cyclin-dependent kinase inhibitor p27kip1, and is necessary for the formation and the maintenance of the mitotic quiescence in rodent MG cells (Close et al., 2005).

In accordance with this hypothesis, EGF treatment following NMDA-induced retinal damage strongly enhances the proliferative

ability of MG cells (Karl et al., 2008). This occurs through the activation of MAPK, phosphoinositide 3-kinase (PI3K), and BMP signalling pathways (Ueki and Reh, 2013). Regarding the molecular mechanisms driving mammalian MG cell reprogramming, it seems that ASCL1 expression represents a key step. In fact, this transcription factor plays a key role in the conversion of fish MG cells into RPCs and a lack of expression of *Ascl1* has been observed in the mouse retina after NMDA-induced damage (Karl et al., 2008).

Interestingly, overexpression of *Ascl1* together with EGF treatment in postnatal mouse retinal explants triggers reprogramming and enhances proliferation of Müller glia and the consequent formation of bipolar neurons (Pollak et al., 2013).

3.6 Stem cell therapy for retinal diseases

Retinal neurons strictly communicate with each other via an intricate net of connections. Thus any loss or defect in these connections, or degeneration of cells located in the different layers, can lead to retinal disease. Degenerative retinal diseases are a leading cause of irreversible blindness and debilitating loss of visual function, some of which have no treatment to date. Thus, over the past decade much work has focused on the replacement of damaged cells, initially with an autologous cell source and more recently with stem cells. Stem cell therapies represent an attractive possibility to

treat retinal diseases and the retina is an ideal target for many reasons. The eye is a relatively small organ, easily accessible (Perez et al., 2013), with a very low immune response. Moreover, novel surgical approaches allow the transplantation of cells to specific locations (Tibbetts et al., 2012). The numerous tools available allow the continuous assessment of ocular structure and monitoring of stem cell function.

The most common strategy used for the therapeutic application of stem cells is cell replacement therapy. Through this therapy, stem cells are differentiated into the needed cell type then transplanted into the damaged tissue to be integrated and thus functionally restore retinal function. Another method is via a "paracrine effect". This is where the transplanted stem cells provide secreted growth or trophic factors, which in turn help resident cells to proliferate and to self-repair (Baglio et al., 2012).

The source of harvest for human stem cells is a very important issue in stem cell therapy. The first attempts used cells from fertilized embryos. For example, ESCs, which can be efficiently differentiated toward a particular lineage, have been largely used for stem cell therapy. Both neural stem cells (Lu et al., 2013) and retinal progenitor cells (Gonzalez-Cordero et al., 2013) have been derived from mouse and human ESCs and then transplanted into degenerating retinas, or differentiated into RPE cells under specific culture conditions (Diniz et al., 2013) and PRs (Decembrini et al., 2014). The transplantation of human ESCs, which have been previously differentiated in vitro into RPE cells, is

another approach currently undergoing clinical trials in myopic macular degeneration (MMD), Stargardt disease, and age-related macular degeneration (AMD).

iPSCs have also been used to obtain the desired cell type in vitro then transplanted in vivo to replace damaged cells (Maeda et al., 2013; Satarian et al., 2013; Zhou et al., 2011). However, in the majority of the cases, transplantation of ESC and iPSC-derived retinal cells has led to teratoma and malignant tumor formation (Cramer and MacLaren, 2013). Improvements in visual behavior have been achieved using retinal precursors, which have been isolated from fetal tissue then transplanted into the sub retinal space of both mice and human subjects with PR loss (Humayun et al., 2000; Pearson et al., 2012; Radtke et al., 2004). Nevertheless, research has demonstrated that only cells that are already committed to a PRs fate are able to be integrated into the mature retina and be able to correctly differentiate into functional rod PRs (MacLaren et al., 2006).

Adult stem cells have also been considered as potential sources for treating retinal diseases i.e., using undifferentiated cells from several specialized tissues such as bone marrow, tooth pulp, and corneal limbus. In particular, bone tissue represents a rich source of adult stem cells because it contains both hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

Researchers have used autologous bone marrow–derived stem cells as potential treatments for ganglion cell loss associated

with glaucoma, in both basic research and early clinical trials.

In addition, one group (Otani et al., 2004) has transplanted intravitreally derived bone marrow HSCs in a mouse model of Retinitis Pigmentosas (RP) and shown an improvement in visual parameters. Another group has attempted to apply the same therapy to subjects with AMD and vascular retinopathies such as retinal vein occlusion and diabetic retinopathy, both of which result in retinal ischaemia (Siqueira et al., 2011).

In 2013, our group investigated the intravitreal transplantation of Wnt-activated bone marrow derived hematopoietic stem and progenitor cells (HSPCs) into a mouse model with NMDA induced retinal degeneration. We observed regeneration of ganglion and amacrine cells with a partial functional rescue after a cell fusion-mediated reprogramming process (Sanges et al., 2013) (Figure 10).

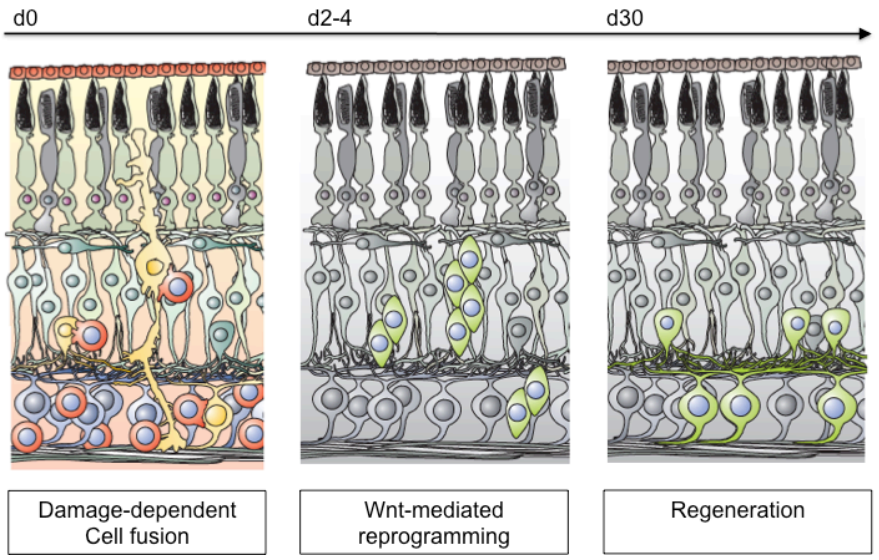


Figure 10. **Bone marrow derived hematopoietic stem and progenitor cells (HSPCs) fuse in vivo with damaged retinal neurons after transplantation into mouse damaged retina** HSPCs purified from donor Cre-RFP mice (red cells) and transplanted into N-Methyl-D-aspartate (NMDA) damaged retina of R26-LoxP-YFP recipient mice, fuse with retinal neurons. Ganglion cells, amacrine cells and Müller glia cells (yellow cells) are the main retinal cell types involved in cell fusion with BM cells. The resulting hybrids (green cells) proliferate and undergo reprogramming in vivo when the Wnt/β-catenin pathway is activated into HSPCs before transplantation. One month after the transplantation the survived hybrid cells differentiate into ganglion and amacrine cells replacing lost cells and participating in retinal regeneration.

Likewise, in a recent work we also investigated a mouse model with MNU induced photoreceptor damage and an inherited genetic mouse model of Retinitis Pigmentosa. In these models we demonstrated that subretinal transplantation of bone marrow derived HSPCs, induces new PRs and has beneficial effects on the survival of remaining PRs in vivo (Sanges et al., 2016). In these two studies

we have shown that the process that allows the integration, survival, and finally the differentiation of the resulting hybrids into functional ganglion and amacrine cells and photoreceptors, respectively, is dependent on the Wnt/β-catenin pathway. Moreover we have shown that such processes passes through a cell fusion mechanism. We found that fusion of transplanted cells with resident retinal neurons and MG cells allowed the formation of reprogrammed hybrid cells when the Wnt/β-catenin pathway was pre-activated in HSPCs before transplantation. The hybrids were able to finally differentiate into mature ganglion, amacrine cells and photoreceptors, thus participating in the repair of the retinal tissue.

PART II
AIMS

In adult mouse retina, MG cells were shown to undergo proliferation upon pharmacological damage of ganglion cells and of the photoreceptors, although the number of observed proliferative MG cells in rodent models is relatively small (Karl and Reh, 2010).

In addition, MG cells can contribute to neuronal regeneration, although with very low frequency (Karl et al., 2008). The mechanism of this limited regenerative potential in mammals remains to be explored fully (Ooto et al., 2004; Wan et al., 2008).

Previous studies have suggested that pharmacological perturbation of some signaling pathways, such as the Wnt/β-catenin, the Notch, and insulin pathways, as well as the overexpression of retinal developmental transcription factors, such as ASCL-1, can stimulate the proliferation and neural regenerative potential of MG cells in mammalian retina (Del Debbio et al., 2016; Karl et al., 2008; Osakada et al., 2007; Pollak et al., 2013; Wan et al., 2007). Overall, these reports foresee the possible establishment of an appealing regenerative approach for retinal neurons in the treatment of retinal degeneration that is based on stimulation of MG cells.

Bone-marrow cells (BMCs) are known to be very plastic and to participate in changes in cellular identity through cell-fusion-mediated mechanisms. BMCs have been shown to fuse with several cell types, such as neurons, hepatocytes, cardiomyocytes, and gut cells (de Jong et al., 2012; Doyonnas et al., 2004; Johansson et al., 2008; Nygren et al., 2004; Ogle et al., 2005; Sanges et al., 2011).

Moreover, several reports in recent years showed that BMCs could participate in the repair of damaged organs and tissues

(Sanges et al., 2013; Wagers et al., 2002; Wagers and Weissman, 2004; Wang et al., 2003). This ability of BMCs to contribute to the repair of damaged tissues might reside in their plasticity after recruitment into damaged organs, and the consequent exposure to different environmental factors (Wagers et al., 2002; Wagers and Weissman, 2004).

However, the factors and the signaling pathways, and the mechanisms involved, that drive BMCs from their niche to the peripheral blood and finally to the injured tissues are still not understood. Based on these observations the aims of my Project have been the followings:

- To study the neurogenic potential of Müller glia cells in mouse retina following N-Methyl-D-aspartate (NMDA) damage

- To explore the molecular mechanism of Müller glial (MG) cells dedifferentiation and reprogramming in vivo

- To investigate endogenous bone marrow cell (BMCs) recruitment into NMDA damaged mouse retina and their possible cell fusion with retinal neurons

- To investigate whether the SDF-1/CXCR4 signaling pathway can be perturbed to “boost” endogenous BM cell mobilization and BM-mediated retinal regeneration.

PART III
RESULTS

1. Müller glial cells are 'activated' and undergo dedifferentiation after NMDA damage

Upon tissue damage in lower vertebrates, MG cells can undergo dedifferentiation, and thereby re-enter the cell cycle. In contrast, several studies in mammalian retina have indicated that MG cells have very limited proliferative ability, although they can be stimulated to re-enter the cell cycle after injury (Goldman, 2014) (see Introduction Chapter 3.3-3.5).

We thus aimed to more deeply investigate MG cells behavior in adult mouse retina to determine how MG cells are re-activated by retinal damage and undergo dedifferentiation. In addition, we aimed to investigate whether MG cells can be reprogrammed, thereby contributing to the generation of new retinal neurons. For this purpose, we used adult mice that were double transgenic for the LoxP-STOP-LoxP-YFP [R26Y] and GFAP-Cre transgenes (GFAP-Cre/R26YFP). *Gfap* (glial fibrillary acidic protein) is expressed in MG cells, and upon NMDA damage, its expression is increased. Therefore MG cells can be tracked, as the Cre recombinase induces expression of yellow fluorescent protein (YFP) specifically in the MG cells (Karl et al., 2008). We injected NMDA into the right eyes of a group of GFAP-Cre/R26Y mice to induce ganglion and amacrine cell damage (Lucas and Newhouse, 1957; Siliprandi et al., 1992; Sucher et al., 1997), with the left eyes treated with phosphate-buffered saline (PBS) as controls (Figure 1 A). At 24 h (24 hpi) and 4 days (4 dpi) post-injection (i.e., after the NMDA damage) we investigated the localization and morphology

of MG cells in retinal sections. YFP was expressed in the MG cells and it co-localized with GLUTAMINE SYNTHETASE, a marker of MG cells that spans the entire thickness of the retinal tissue (Figure 1 B, yellow arrows).

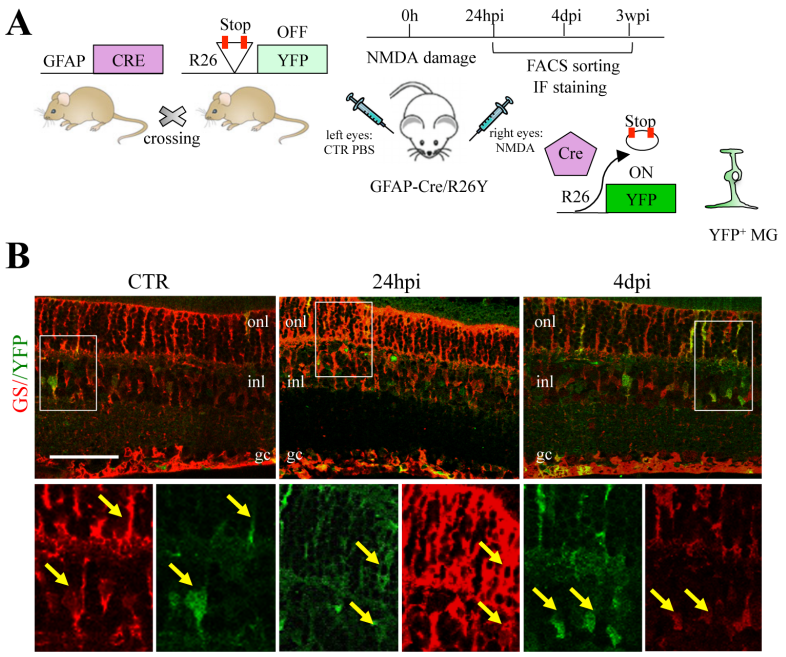


Figure 1. GFAP-Cre/R26Y lineage tracing mice allow to track endogenous Müller glia cells following N-Methyl-D-aspartate (NMDA) damage (A) Experimental scheme: Retinas of transgenic GFAP-Cre/R26Y lineage mice, where Cre recombinase allows the expression of YFP specifically in MG cells were damaged with NMDA to analyze YFP^+ MG cells at different times. Left eyes were injected with PBS, as controls. (B) Representative immunostaining of sections of retinas harvested from GFAP-Cre/R26Y mice sacrificed 24h (24 hpi) and 4 days (4 dpi) after NMDA and PBS (CTR) injections. Higher magnification images from the white boxes are shown. YFP^+ cells (green) (yellow arrows) are also positive to the marker of MG cells, GLUTAMINE SYNTHETASE (GS) (red) and span the entire thickness of the retina (onl, outer nuclear layer; inl, inner nuclear layer; gc, ganglion cells layer). Scale bar: 100 μm. At least three different sections for each mouse were stained (n = 3).

To determine whether the MG cells underwent dedifferentiation upon this retinal damage, we looked at the expression of neural and retinal progenitor markers using real-time PCR. YFP$^+$ MG cells were FACS-sorted from undamaged (i.e., PBS treated) and damaged retinas at different times after NMDA treatment (Figure 2 A), to analyze their gene expression and cell fate. Interestingly, *Nestin*, *Chx10, Pax6*, and *Sox2* progenitor markers were significantly up-regulated either at 24 hpi (*Nestin*, *Chx10*) or at 4 dpi (*Pax6*, *Sox2*) in YFP$^+$ MG cells isolated from the damaged retinas, as compared to the levels in cells purified from the undamaged retinas (Figures 2 B, C).

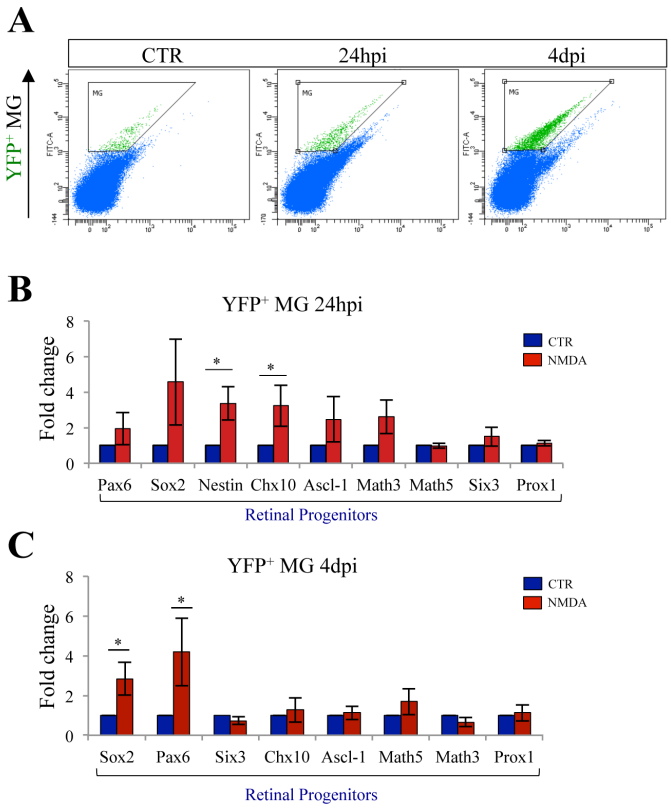


Figure 2. Müller glial cells activated following NMDA damage undergo dedifferentiation (A) Representative FACS profiles showing YFP^+ MG cells sorted from undamaged PBS-treated (CTR) and NMDA-damaged retinas of GFAP-Cre/R26Y lineage mice at 24 h (24 hpi) and 4 days (4 dpi) post-NMDA injection, with respect to the total retinal population. Dead cells were excluded from the analysis by gating on $DAPI^+$ cells. (C-D) qRT-PCR expression of retinal progenitors genes analyzed using total RNAs of FACS-sorted YFP^+ MG cells harvested from undamaged PBS-treated (CTR) and damaged (NMDA) retinas of GFAP-Cre/R26Y mice at 24 hpi and 4dpi. Data are means ±S.E.M. of five independent experiments (n = 4-5). The transcript levels are expressed as fold-changes relative to CTR YFP^+ MG cells sorted from undamaged PBS-injected retinas (CTR) after normalization to *Gapdh* levels. Statistical analyses are based on unpaired Student's T-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s, not significant.

The activation of *Pax6* suggests that MG cells acquire characteristics of retinal progenitors after NMDA damage. *Pax6* is expressed by retinal progenitors in the developing retina and in ganglion and amacrine cells in the inner retinal layer (Hitchcock et al., 1996). Moreover, *Pax6* has been well documented to be a gene that is activated during dedifferentiation of MG cells after damage in fish and chick retina in vivo (Fischer and Reh, 2001; Hitchcock et al., 1996; Thummel et al., 2010; Thummel et al., 2008), as well as in adult rat retina in vitro (Ooto et al., 2004; Osakada et al., 2007). *Nestin* is up-regulated after injury in higher vertebrates (Kohno et al., 2006; Wan et al., 2007), and it is expressed in retinal progenitor cells during development (Lee et al., 2012). *Chx10* is also indispensable for retinal progenitor cell proliferation during development (Livne-Bar et al., 2006). *Sox2* is a multipotent neural stem cell marker, and it is also expressed by retinal progenitors during development, furthermore it has been shown to be important

for both maintenance of progenitor cell state (Surzenko et al., 2013) and promotion of amacrine cell fate (Lin et al., 2009).

At 24 hpi, there was also a tendency, although not significant, to increased expression of the *Sox2*, *Ascl1*, *Math3*, and *Six3* retinal progenitor genes, which are also expressed during development. *Ascl-1* is the master regulator of MG-cell-mediated retinal regeneration in lower vertebrates (Pollak et al., 2013). *Math3* has been shown to be essential for amacrine cell generation during development (Inoue et al., 2002), and *Six3* is required for neuroretina formation during development (Loosli et al., 1999). However, many of these retinal progenitor genes showed very poor, and not significant, trends for increased expression at 4 dpi (Figure 1 C). This might be due to the progressive dedifferentiation of the YFP^+ MG cells from 24 hpi to 4 dpi, a period when *Sox2* and *Pax6* are significantly up-regulated. Interestingly, at 24 hpi, *Cyclin D1*, which is expressed in the G_1 phase of the cell cycle and in proliferative cells (Kohno et al., 2006), was strongly increased in MG cells isolated from damaged retinas, with respect to the controls, and remained very high at 4 dpi (Figure 3).

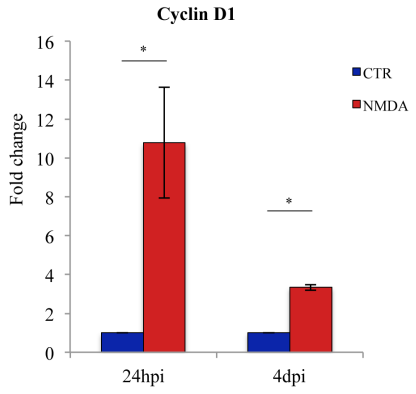


Figure 3. Müller glial cells activated following NMDA damage exit from a quiescence state qRT-PCR expression level of CyclinD1 cell cycle gene analyzed on total RNA harvested from FACS-sorted YFP^+ MG cells of undamaged PBS-treated (CTR) and damaged (NMDA) retinas at 24 hpi and 4dpi. Data are means ±S.E.M. of four independent experiments. The transcript levels are expressed as fold-changes relative to CTR YFP^+ MG cells after normalization to *Gapdh* levels. Statistical analyses are based on unpaired Student's T-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s, not significant.

This suggests that MG cells can re-enter the cell cycle upon NMDA damage. We therefore also investigated in sections the YFP^+ MG cells for the expression of PCNA and for phosphorylated Hystone H3 (phH3), which are well-known proliferation markers (Goto et al., 1999; Maga and Hubscher, 2003). We observed that already at 24 hpi, the YFP^+ MG cells expressed PCNA and phosphorylated H3, while in the control retinas there were no $PCNA^+$ or phosphorylated $H3^+$ MG cells (Figures 4 A, B). This expression of PCNA and phosphorylated H3 remained high also at 4 dpi.

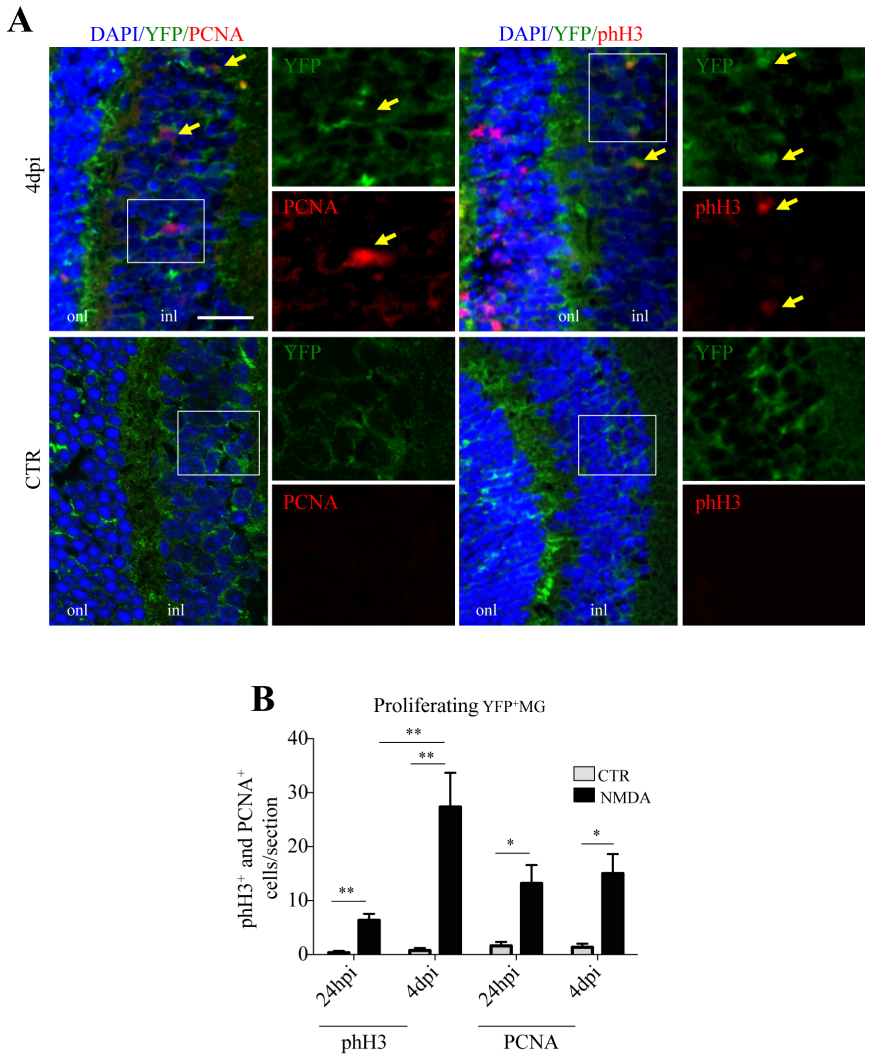


Figure 4. Müller glial cells proliferate upon NMDA damage (A) Confocal microscopy images of immunostained sections from damaged (4 dpi) and undamaged PBS-treated (CTR) retinas of GFAP-Cre/R26Y lineage mice sacrificed 4 dpi. Proliferative cells are immunopositive for PCNA (red, left panels) and phosphorylated H3 (phH3; red, right panels). Higher magnification areas included in the white boxes are shown. Yellow arrows show proliferative YFP^+ cells. Nuclei were counter stained with DAPI (blue). Scale bar: 20 μm. (n = 3) (inl = inner nuclear layer; onl = outer nuclear layer). (B) Number of proliferating $PCNA^+$/ YFP^+ and $phH3^+$/ YFP^+ MG cells per section, counted for damaged retinas (NMDA) as compared to healthy, PBS-treated, retinas (CTR) at 24 hpi and 4 dpi.

Data are means ± S.E.M. counted for three different sections for each mouse, from two independent experiments (n = 6).

These data show that upon NMDA damage, MG cells can de-differentiate and re-enter the cell cycle. Interestingly, this dedifferentiation of MG cells upon NMDA damage has been reported before, even if only after treatment with specific growth factors that stimulates MG cell proliferation (Goldman, 2014; Ooto et al., 2004).

We then asked whether de-differentiated YFP^+ MG cells could generate new inner retinal neurons. In particular, to determine whether YFP^+ MG cells can generate ganglion and amacrine cells (i.e., the damaged cell type in this experimental model), we followed the fate of the YFP^+ MG cells in the long term after damage in GFAP-Cre/R26Y mice. In retinal flat mounts we found YFP^+ MG cells expressing CALRETININ, which is a specific marker of ganglion and amacrine cells (Figures 5 A, B). Specifically three weeks after the retinal damage (3 wpi), 5% of the YFP^+ MG cells expressed CALRETININ (Figure 5 C), which suggested that the MG cells generated ganglion and amacrine cells by passing through a proliferative stage, which also confirmed previous observations (Karl et al., 2008).

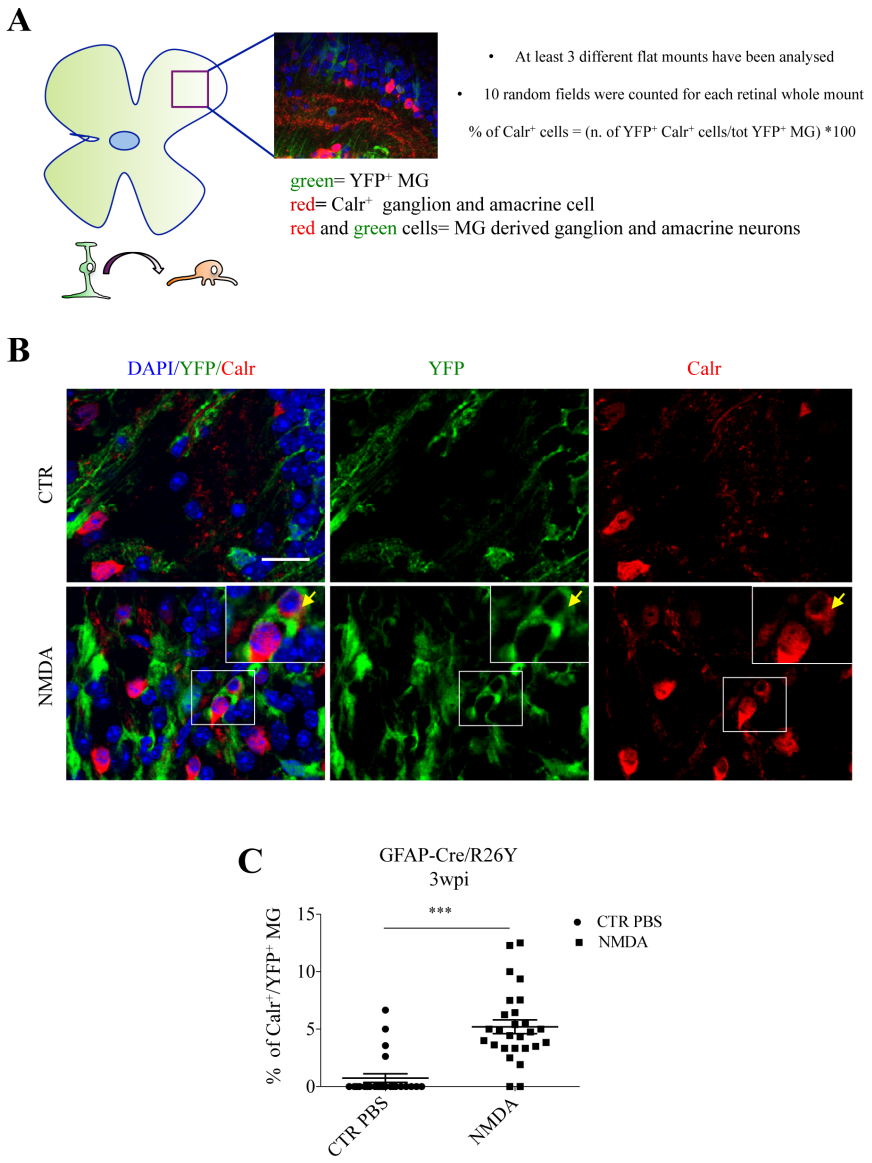


Figure 5. Müller glial cells activated following NMDA damage differentiate into ganglion and amacrine cells at long term (A) Schematic representation of the method for counting marker-positive cells in retinal flat mounts. The number of YFP^+ MG cells expressing CALRETININ, the ganglion and amacrine cell marker, 3 wpi is expressed as percentages of $Calr^+$ cells on the total YFP^+ MG. YFP^+/ $Calr^+$ cells were counted in 10 random fields from at least three different retinal flat mounts for each group of treatment. (B) Representative immunostaining of retinal flat mounts of GFAP-Cre/R26Y mice sacrificed 3 weeks after

NMDA damage (3 wpi), and of control retinas injected with PBS (CTR). YFP^+ MG cells (green) differentiated into $CALRETININ^+$ cells (Calr-red) are indicated by yellow arrows. Nuclei were counterstained with DAPI (blue). (n = 3) Scale bar: 20 μm. (C) Quantification of the percentage of YFP^+ MG cells expressing CALRETININ (Calr). Cells were counted in 10 random fields of retinal flat mounts harvested from undamaged PBS-injected (CTR) and damaged (NMDA) retinas 3 weeks after injection. Data are means ±S.E.M. of three independent experiments (CTR PBS, n = 19; NMDA, n = 22). Statistical analyses are based on unpaired T-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s, not significant.

2 Perturbation of the SDF-1/CXCR4 pathway affects the ability of Müller glial cells to undergo dedifferentiation

Previous studies have reported that stromal-derived factor 1 (SDF-1; also known as C-X-C motif chemokine 12 [CXCL12]) and its interaction with C-X-C motif chemokine receptor type 4 (CXCR4) is not only involved in invasion and metastasis of malignant tumors (McIver et al., 2013; Mukherjee and Zhao, 2013), but also that it plays a key role in the migration of stem cells (Du et al., 2012; Theiss et al., 2011).

The observations that SDF-1 is up regulated after several types of damage such as in liver, brain, and retina suggests that SDF-1 also plays an important role in the homing of stem cells to damaged sites (DeLeve et al., 2016; Lima e Silva et al., 2007; Mocco et al., 2014). Moreover it has been shown that SDF-1 is up regulated under hypoxic conditions, including during myocardial ischemia (Yu et al., 2010), cerebral ischemia (Shen et al., 2007), and renal failure (Togel et al., 2005), and that this correlates with adult stem cell recruitment and tissue regeneration (Askari et al.,

2003; Ceradini et al., 2004; Kollet et al., 2003; Yamaguchi et al., 2003).

Furthermore SDF-1 is secreted from vascular endothelial cells and reactive astrocytes in injured regions of the brain (Ohab et al., 2006; Thored et al., 2006). In addiction SDF-1 has been shown also to be up regulated in a rat model of retinal ischemia-reperfusion injury (Lai et al., 2008), and after retinal-pigmented epithelium damage (Li et al., 2006).

We found that at 24 hpi, there was up-regulation of SDF-1 expression also in the NMDA damaged retinas as compared to the control PBS-treated retinas as well as in the YFP^+ MG cells sorted from damaged retinas as compared to the YFP^+ MG sorted from control retinas (Figures 6 A, B).

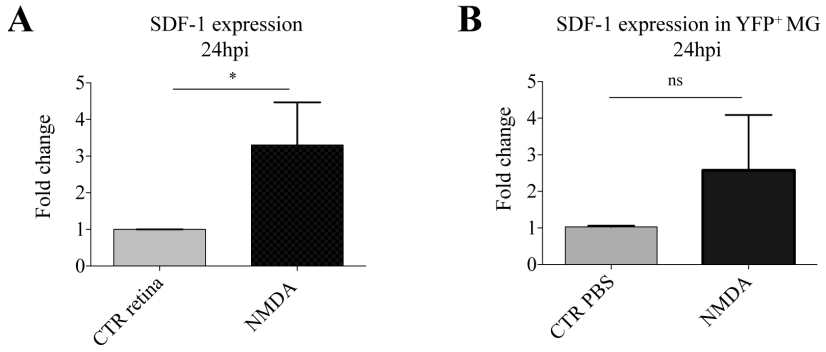


Figure 6. SDF-1 is up-regulated into NMDA damaged retinas and in YFP^+ Müller glial cells sorted from damaged retinas (A-B) qRT-PCR quantification of SDF-1 chemokine expression levels using total RNAs extracted from damaged (NMDA) and undamaged PBS-treated (CTR retina) total retinal samples (A) or from YFP^+ MG cells FACS-sorted from NMDA-damaged retinas (NMDA) of GFAP-Cre/R26Y lineage mice, as compared to YFP^+ MG cells sorted from undamaged PBS-treated (CTR PBS) retinas (B), 24 hpi. Data are means ±S.E.M. of three independent experiments (n = 3). The transcript levels are expressed as relative to CTR retina (A) or to CTR YFP^+ MG cells (B) after

normalization to *Gapdh* levels. Statistical analyses based on unpaired Student's T-tests. *P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

Thus we hypothesized that activation of the SDF-1/CXCR4 pathway is involved in the dedifferentiation process of MG cells. Therefore, we aimed to determine whether this signaling pathway and the mobilization of BMCs from peripheral blood affects the reprogramming potential of the MG cells.

For this, we first investigated the effect of SDF-1 and its antagonist AMD3100 treatment on BM cell mobilization by taking advantage from mice double transgenic for LoxP-STOP-LoxP-YFP [R26Y] and Vav-Cre transgenes (Vav-Cre/R26Y) (Figure 7 A). In Vav-CRE mice, Cre recombinase expression is controlled by the hematopoietic specific promoter Vav (Stadtfeld and Graf, 2005) thus in Vav-Cre/R26Y all the cells from hematopoietic compartment are labeled in green (BMCs YFP^{+}) (Figure 7 B).

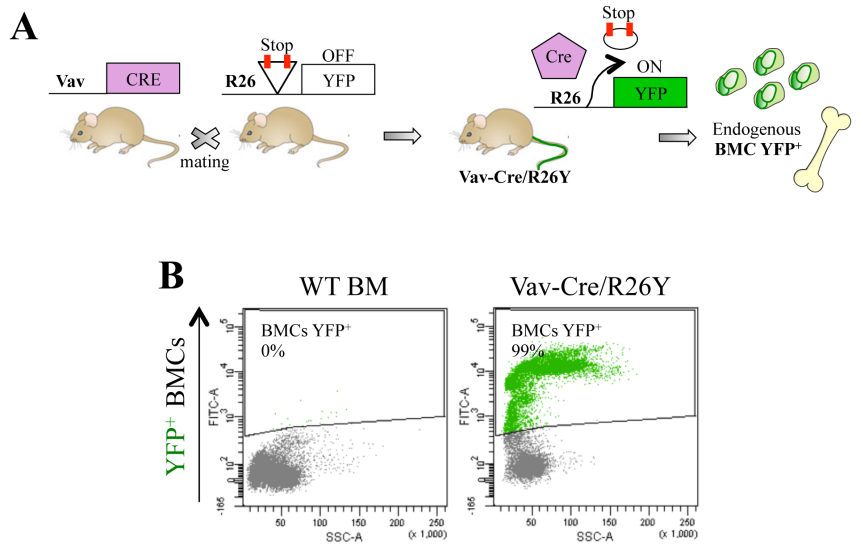


Figure 7. Vav-Cre/R26Y mice allow to track endogenous BMCs and to follow their mobilization (A) The Vav-Cre/R26Y mice were generated by crossing together the Vav-Cre mice expressing the Cre recombinase under a BM cells promoter together with R26-LoxP-YFP mice. The Vav promoter drives the expression of the Cre recombinase specifically in BM cell compartment. (B) FACS plots showing the YFP^+ BMCs in the total bone marrow isolated from Vav-Cre/R26Y double transgenic mice as compared to bone marrow harvested from wild type C57 mice.

We injected a group of Vav-Cre/R26YFP mice with NMDA into the right eyes to induce the damage; another group of mice were injected intravitreous with the chemokine SDF-1 together with NMDA, while a group of mice received the injection of SDF-1 antagonist AMD3100 intraperitoneally (IP) soon after NMDA (figure 8 A). In all the groups of treatment left eyes were injected with PBS (CTR PBS) and used as control.

In a first analysis we investigated the effect of NMDA retinal damage on endogenous YFP^+ BMC mobilization, at different time points. We analyzed the percentage of YFP^+ BMCs recruited into damaged retinas and we found that the NMDA damage caused a mobilization of YFP^+ BMCs from peripheral blood into damaged eyes as compared to the control PBS injected eyes, already at 24hpi (figure 8 B).

We also studied the consequences of the manipulation of the SDF-1/CXCR4 signaling on BMC potential recruitment into damaged retinas (figure 8 B). We analyzed the percentage of YFP^+ BMCs recruited into damaged retinas after the modulation of the SDF-1/CXCR4 pathway, at 24hpi by FACS analysis (Figure 8 C).
The treatment with the chemokine SDF-1 enhanced BMC mobilization into damaged eyes while, on the contrary, AMD3100 treatment strongly reduced the percentage of YFP^+ BMCs recruited into NMDA damaged eyes at 24hpi (figure 8 B, C, D). However, this effect was stronger in the first 48 hours after damage and then decreased during time (figure 8 B).

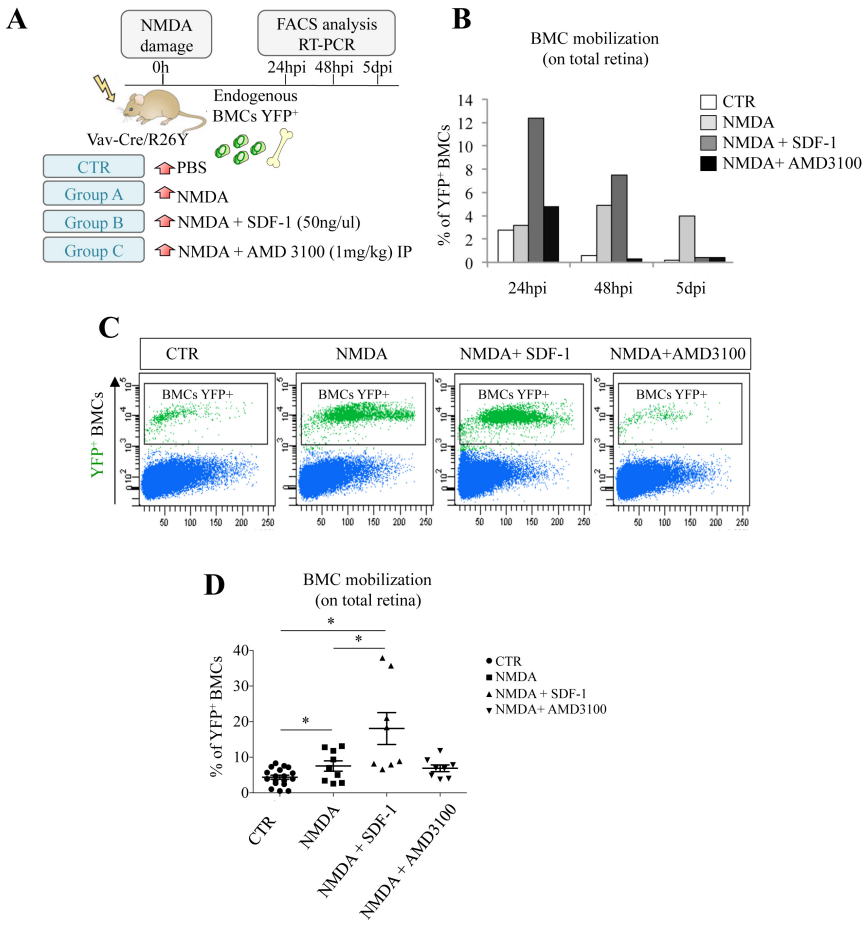


Figure 8. YFP$^+$ BMCs are mobilized into NMDA damaged retina and modulation of the SDF-1/CXCR4 pathway affects their recruitment (A) Experimental scheme: Vav-Cre/R26Y lineage tracing mice mice were divided into 4 different groups of treatment: a group of mice was injected with SDF-1 after NMDA damage (group B; NMDA+ SDF-1), another group of mice was injected intraperitoneally (IP) with AMD3100 after NMDA damage (group C; NMDA+ AMD3100); a group of mice injected with NMDA (group A; NMDA) and a group of mice injected with PBS has been used as control (CTR). (B) Quantification of the percentage of YFP$^+$ BMCs detected at different time points into the total retina of transgenic Vav-Cre/R26Y mice after the different treatments evaluated by FACS analysis on the total retinal population. (C) Representative FACS plots showing YFP$^+$ BMCs recruited into undamaged PBS treated (CTR PBS) and damaged (NMDA) retinas from transgenic Vav-Cre/R26Y mice at 24hpi after the different treatments gated on the total live retinal population. (D) Percentages of YFP$^+$ BMCs

mobilized into damaged (NMDA) retinas of Vav-Cre/R26Y mice from the different groups of treatment (NMDA SDF-1, NMDA AMD3100) as compared to the percentage of YFP^+ BM cells migrated into undamaged PBS injected control retinas (CTR PBS) evaluated by FACS analysis at 24hpi and gated on the total retinal population. DAPI positive dead cells were excluded from the analysis. Statistical analyses based on unpaired Student's T-tests. *P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

We also attempted to identify and characterize the subpopulations of BM-derived cells migrated into damaged retinas. To this aim we used the Vav-Cre/R26Y mice where endogenous BM cells were labeled with YFP (Figure 7 A) and we performed FACS analysis for several markers specifically enriched into the distinct HSC subpopulations. We induced the damage by injecting NMDA into the right eyes of a group of Vav-Cre/R26Y mice sacrificed 24hpi (NMDA) and we used the contralateral eyes injected with PBS as controls (CTR).

Retinal samples were harvested 24hpi and analyzed by FACS to assess the amount of YFP^+ BM cells migrated into retinas upon damage and to determine if these mobilized cells expressed HSC markers (Figure 9 A). We performed staining for c-kit, sca-1, CD150, CD34, which are well documented membrane markers used to identify HSC and to distinguish the different subpopulations of MPP, LT-HSC, ST-HSC, according to Weissman BM hierarchy classification (see Introduction Chapter 2.1). We found that inside the population of YFP^+ BMCs migrated into retina following NMDA damage (which represents a mean of 18 % of the total retinal cell population) (Figure 9 B) the 0,5% of the cells expressed c-kit and sca-1 markers, suggesting recruitment of BM-derived

progenitor cells, lineage negative and c-kit$^+$/sca-1$^+$ (LSK) into damaged retinas (Figure 9 C). However we should note that the majority of YFP$^+$ BMCs mobilized into retinas upon NMDA damage were most likely more differentiated progenitor cells as suggested by the low percentage of c-kit/sca-1 double positive cells inside the YFP$^+$ BMC population (Figure 9 C). Further and detailed analysis would be needed to clarify this point.

We then analyzed the expression of CD150 and CD34 inside the c-kit$^+$/sca-1$^+$ BMCs. Both these markers are used to distinguish between MPP (CD150$^-$/CD34$^+$) and LT-HSC (CD150$^+$/CD34$^-$) or ST-HSC (CD150$^+$/CD34$^+$) distinct populations in the total BM. Interestingly, we found that in damaged retinas (NMDA) there was a high percentage of MPP cells inside the c-kit$^+$/sca1$^+$ YFP$^+$ population that did not change after NMDA damage as compared to PBS treated control retinas (Figure 9 D first panel). On the contrary, when we analyzed the number of YFP$^+$/c-kit$^+$/sca-1$^+$ cells expressing HSC markers we found a significant increase in the number of ST-HSCs and LT-HSCs in damaged retinas as compared to control retinas (Figure 9 D second and third panels).

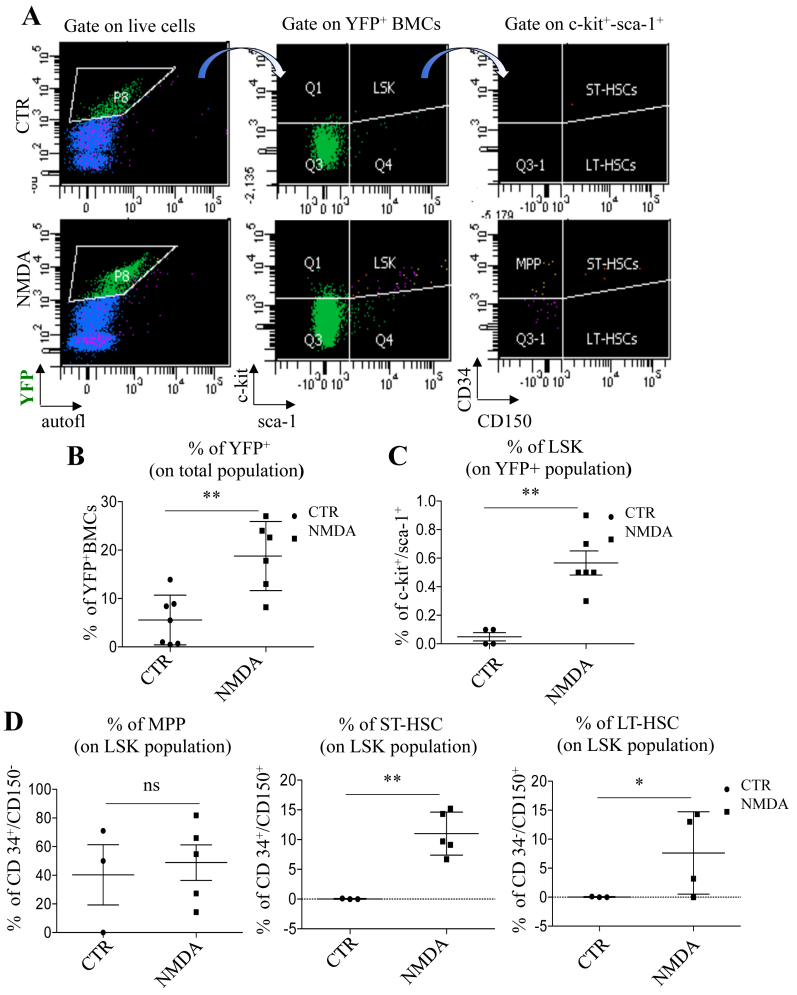


Figure 9. YFP$^+$ BMCs mobilized into NMDA damaged retina express HSC markers (A) FACS plots showing the gate strategy used to identify and characterize YFP$^+$ BMCs mobilized into damaged (NMDA) retinas of Vav-Cre/R26Y lineage tracing mice at 24hpi as compared to PBS injected control (CTR) retinas. (B) Quantification of the YFP$^+$ BMCs detected by FACS analysis into damaged (NMDA) retinas as compared to undamaged PBS injected control retinas (CTR) expressed as percentage with respect to the total retinal population. (C) Quantification of c-kit$^+$ /sca-1$^+$ (LSK) BMCs migrated into damaged (NMDA) retinas as compared to undamaged PBS injected control retinas (CTR) expressed as percentage on YFP$^+$ live BMCs. (D) Quantification of CD34$^+$/ CD150$^-$ multipotent progenitors (MPP), CD34$^+$/ CD150$^+$ short term hematopoietic stem cells (ST-HSCs) and CD34$^-$/ CD150$^+$ long term hematopoietic stem cells (LT-

HSCs) into damaged (NMDA) and undamaged (CTR) retinas of Vav-Cre/R26Y mice, gated on the c-kit$^+$/sca-1$^+$ (LSK) population. Died cells DAPI$^+$ cells were excluded from the analysis. Data showed are means (+/- S.E.M.) of 2 different experiments (n = 3-6); each dot represents a single retinal sample. Statistical analyses based on unpaired Student's T-tests. *P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

After having analyzed the functionality of the drugs in the Vav-Cre/R26Y mice we decided to modulate the SDF-1/CXCR4 signaling pathway in the mouse model where we can track endogenous Müller glial cells (GFAP-Cre/R26Y) to assess if the mobilization of BMCs could affect MG cells ability to undergo reprogramming.

We injected a group of GFAP-Cre/R26Y mice with the chemokine SDF-1 intravitreally, and at the same time we induced NMDA damage to the right eyes, while the left eyes were injected with PBS as the controls (Figure 10; Group B). Another group of mice was damaged by NMDA injection into the right eyes and was injected intraperitoneally with the SDF-1 antagonist AMD3100 (Figure 10; Group C). Finally a group of mice that received NMDA injection into the right eyes was used as control group (Figure 10; Group A).

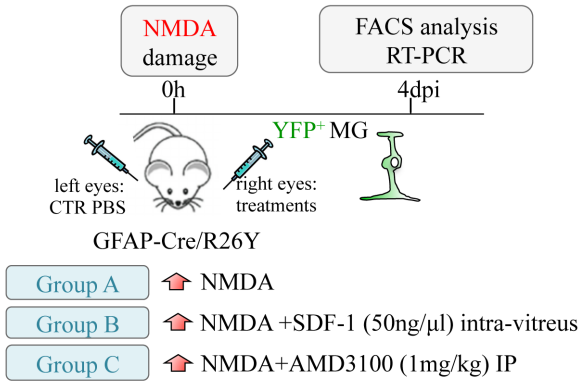


Figure 10. SDF-1/CXCR4 signaling pathway modulation in GFAP-Cre/R26Y mice to evaluate Müller glial cell reprogramming and dedifferentiation (A) Experimental scheme: GFAP-Cre/R26Y lineage mice, where Cre recombinase allows the expression of YFP specifically in MG cells, were divided into three different groups: retinas that were NMDA-damaged (group A); retinas that were damaged and treated with SDF-1 chemokine (group B); retinas that were damaged and mice which received IP injection of AMD3100 (group C). The mice were sacrificed 4 dpi to investigate changes in the number of YFP^+ MG cells and in the dedifferentiation and reprogramming state, using FACS and RT-PCR analysis.

To determine whether the dedifferentiation of the MG cells, and thus their ability to become retinal progenitors after NMDA damage, was dependent on mobilization and migration of BMCs through the SDF-1/CXCR4 pathway, we FACS-sorted the YFP^+ MG cells 4 dpi (Figure 11 A), and we used qRT-PCR to investigate the expression of neural and retinal progenitors genes (Figure 11 B). Interestingly, the YFP^+ MG cells sorted from the retinas treated with the chemokine SDF-1 showed a more de-differentiated retinal-progenitor-like phenotype (Figure 11 B). Many of the neural multipotent (e.g., *Pax6, Nestin*) and retinal progenitor genes that are normally expressed during retinal development (e.g., *Chx10, Six3,*

Math3, Math5, Prox1), together with the cell-cycle gene *Cyclin D1*, were strongly up-regulated in the SDF-1–treated YFP$^+$ MG cells, which suggested that this chemokine increases the dedifferentiation and proliferation of MG cells (Figure 11 B, C, NMDA+SDF-1). In contrast, the YFP$^+$ MG cells sorted from the retinas of mice that received the antagonist AMD3100 did not undergo dedifferentiation, as indicated by the decrease in the expression of these genes (Figure 11 B, C, NMDA+AMD3100).

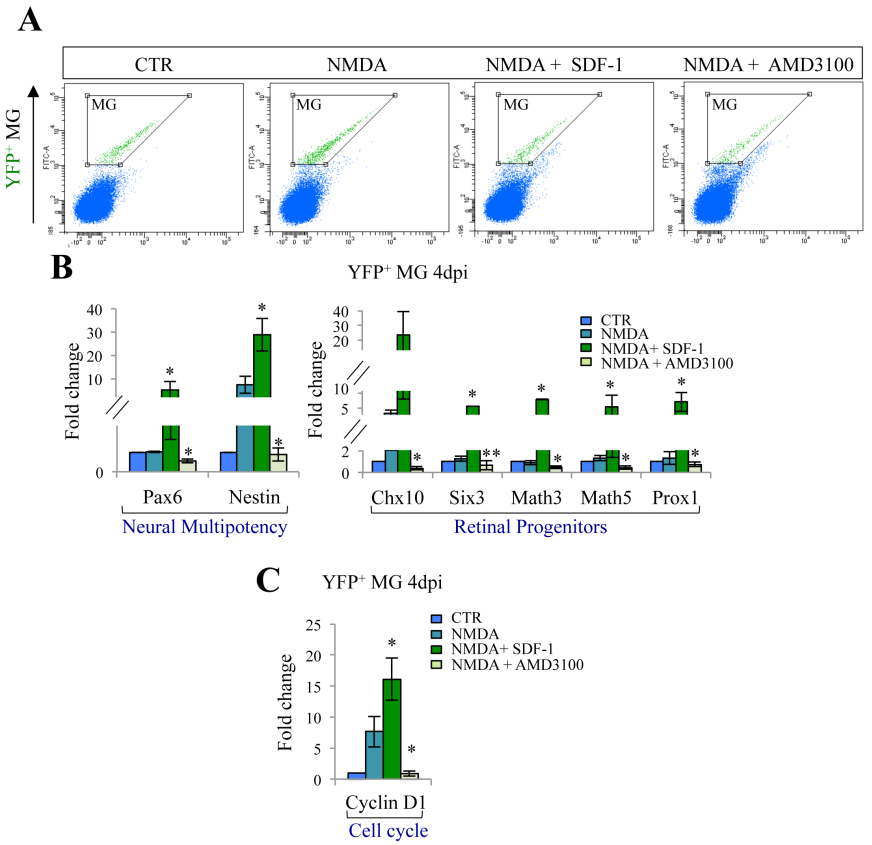


Figure 11. SDF-1/CXCR4 signaling pathway modulation affects Müller glial cell reprogramming (A) FACS analyses showing YFP$^+$ MG cells sorted from undamaged PBS-treated retinas (CTR) and NMDA-damaged retinas of GFAP-Cre/R26Y lineage tracing mice after the

different treatments (NMDA+SDF-1, NMDA+IP injection of AMD3100), 4 dpi. YFP^+ cells were gated on total retinal population, and dead cells were excluded from the analysis by gating on $DAPI^+$ cells. (B, C) Expression levels of neural progenitors, retinal progenitors (B), and cell-cycle genes (C), are evaluated by RT-PCR in total RNA from FACS-sorted YFP^+ MG cells at 4 dpi following different treatments (NMDA, NMDA+ SDF-1, NMDA+AMD3100). Data are means ±S.E.M. from five independent experiments (n = 5), and transcript levels are expressed as fold-changes relative to YFP^+ MG cells sorted from PBS-injected control retinas (CTR), after normalization to *Gapdh* levels. Statistical analyses based on unpaired Student's T-tests. *P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

Interestingly, there was also an increase in the number of YFP^+ MG cells analyzed by FACS analysis after the SDF-1 treatment (Figure 12 A, NMDA+ SDF-1) maybe due to MG cells proliferation after SDF-1 injection. On the contrary when we treated the mice with the AMD3100 we noticed a tendency to a decrease in the percentage of YFP^+ MG cells as compared to the YFP^+ MG isolated from NMDA damaged retina evaluated by FACS (Figure 12 A, NMDA+ AMD3100).

Moreover, when we analyzed the expression of the neural progenitor marker NESTIN in YFP^+ MG cells on retinal flat mounts 4 dpi, there was an increase of $YFP^+/NESTIN^+$ cells in SDF-1–treated retina, while these cells were reduced after AMD3100 treatment (Figure 12 B, C).

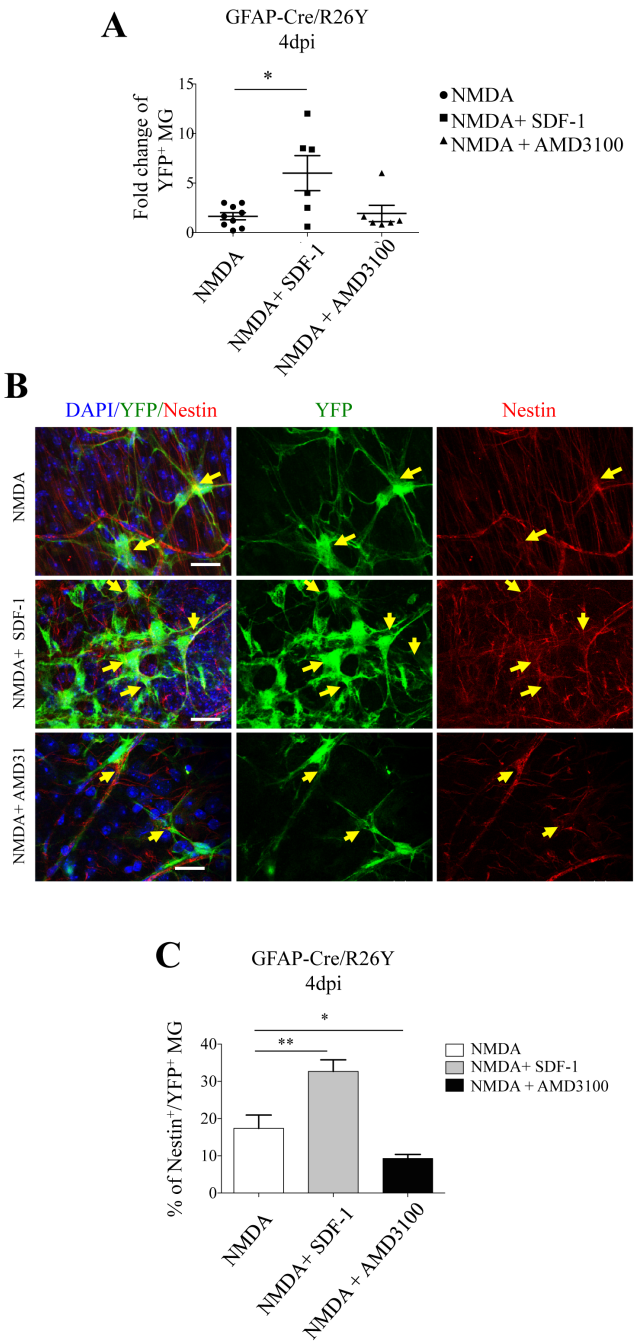


Figure 12. Modulation of the SDF-1/CXCR4 pathway influences the number of YFP$^+$ Müller glial cells and their reprogrammed state (A) Percentage of YFP$^+$ MG cells in GFAP-Cre/R26Y lineage mice at 4 dpi after the different treatments (NMDA, n = 9; NMDA+SDF-1, n = 6;

NMDA+AMD3100, n = 6), analyzed by FACS analysis. Data are expressed as fold-changes between the percentages of YFP^+ MG cells in treated retinas with respect to control retinas injected with PBS. The percentage of YFP^+ MG is calculated by FACS with respect to the total retinal cells. (B) Representative immunostaining images of retinal flat mounts from GFAP-Cre/R26Y lineage mice sacrificed 4 dpi after different treatments (NMDA, NMDA+ SDF-1, NMDA+ AMD3100). Co-localization between YFP^+ MG cells (green) and neural stem cell marker NESTIN (red) is shown (yellow arrows). Images were chosen from random fields of stained flat mounts from two different mice for each treatment (n = 2) and the cell nuclei were counterstained with DAPI. Scale bar: 20 μm. (C) Percentages of $NESTIN^+$ cells calculated on the total YFP^+ MG cells. Double-positive cells were counted in random fields of retinal flat mounts harvested from GFAP-Cre/R26Y mice 4 dpi after different treatments (NMDA, n = 7; NMDA+SDF-1, n = 4; NMDA+AMD3100, n = 7). Statistical analyses based on unpaired Student's T-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ is applied for statistical analysis.

Importantly, the treatment with the SDF-1 chemokine not only caused a more de-differentiated phenotype of YFP^+ MG cells but 3 wpi we also found a significant increase in the number of YFP^+/$CALRETININ^+$ cells in retinal flat mounts harvested from mice treated with SDF-1, while a strong reduction in mice treated with the antagonist AMD3100 (Figure 13 A, B). These data suggest that the number of ganglion and amacrine cells derived from YFP^+ MG cells was increased upon SDF-1 injection, while it was decreased when CXCR4 was blocked. Furthermore, the increase or block of BMC mobilization through the SDF-1/CXCR4 pathway modulated the ability of MG cells to proliferate (Figure 11 C). All in all, our data suggest that after NMDA damage in the mouse retina, one of the mechanisms by which MG cells undergo reprogramming to change their cell fate is dependent on the activity

of the SDF-1/CXCR4 signaling pathway.

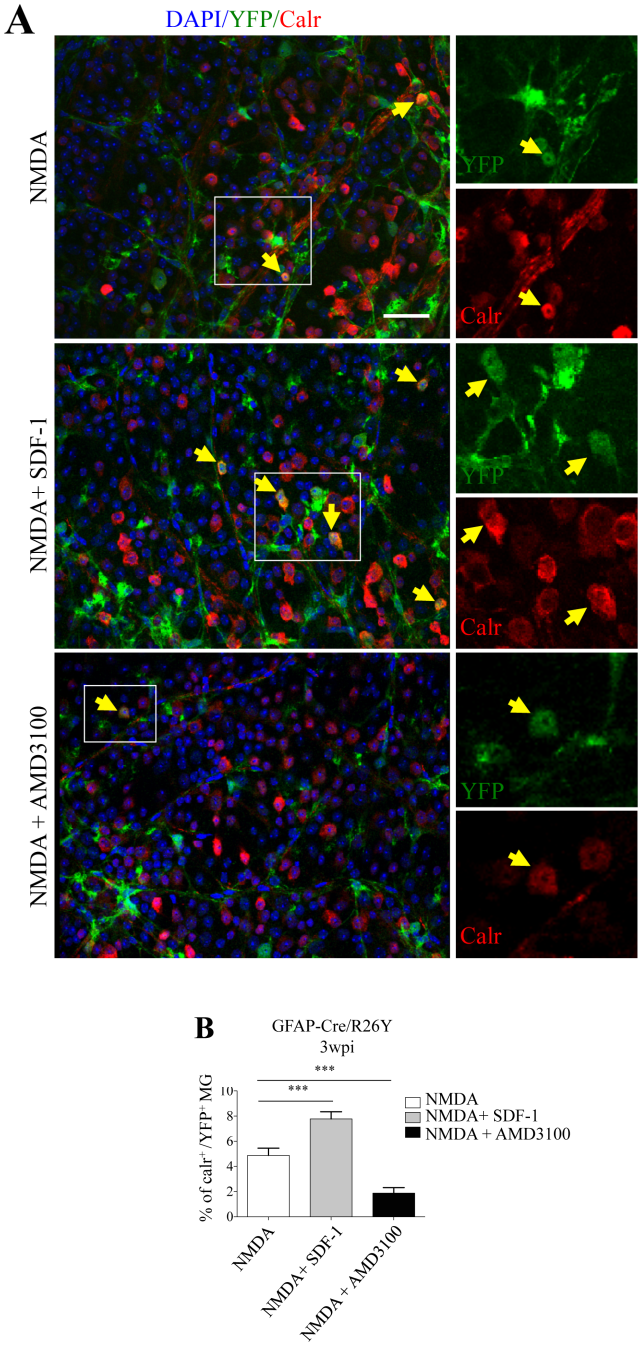


Figure 13. SDF-1/CXCR4 signaling pathway modulation controls Müller glial cell reprogramming and their neurogenic capability (A) Representative immunostaining images from retinal flat mounts harvested at 3 wpi from GFAP-Cre/R26Y lineage mice from three different treatments groups (NMDA, NMDA+ SDF-1, NMDA+ AMD3100). YFP^+ MG cells (green) are also positive for the CALRETININ (Calr-red) marker. Yellow arrows indicate co-localization of YFP with CALRETININ. Representative higher magnification images from the white boxes are shown. Scale bar: 40 μm. Images were taken from 10 random fields for each retinal flat mount from at least three different mice for each group (n = 3). (B) Percentages of $YFP^+/Calr^+$ cells with respect to total YFP^+ MG cells in retinal flat mounts in groups of mice treated as indicated and sacrificed 3 wpi. YFP and Calr immunopositive cells were counted in 10 random fields (magnification, 20×) from each retinal flat mount from at least three different mice for each group (NMDA, n = 30; NMDA+SDF-1, n = 43; NMDA+AMD3100, n = 21). The statistical analysis is based on unpaired Student's T-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s, not significant.

3. SDF-1/CXCR4 pathway controls BMC migration into mouse retina after NMDA damage, and mobilized BMCs fuse with retinal neurons

Bone-marrow cells have been shown to participate in the repair of several tissues after damage (de Jong et al., 2012; Johansson et al., 2008; Wang et al., 2003). We previously showed that transplantation of Wnt-activated HSPCs into damaged mouse retina leads retinal cells to undergo cell-fusion-mediated reprogramming, and that these hybrids, in turn, enhance retinal regeneration (Sanges et al., 2013; Sanges D., 2016). Moreover, cell fusion between transplanted HSPCs and neurons occurs in vivo also in the brain of two Parkinson's disease mouse models (Altarche-Xifro et al., 2016). In addition, we previously showed that cell fusion can occur between endogenous migrated BMCs and retinal

cells after NMDA damage (Sanges et al., 2013). Here, we want to study the regenerative potential of these in vivo-formed hybrids upon endogenous BMC migration.

As the dedifferentiation potential of MG cells is strongly affected by modulation of the SDF-1/CXCR4 pathway, we hypothesized that this signaling pathway could controls BMC migration into damaged retinal tissue, and that these cells can in turn fuse with retinal neurons. To assess better this hypothesis, we generated R26Y/BM$^{CRE-RFP}$ chimeric mice by replacing the BM of sub-lethally irradiated R26Y recipient mice carrying the Rosa26-LoxP-stop-LoxP-YFP transgene with the BM from donor CAG-RFP/Vav-CRE double transgenic mice. In Vav-CRE mice, as we mentioned before, Cre recombinase expression is controlled by the hematopoietic specific promoter Vav (Stadtfeld and Graf, 2005). Six weeks after the replacement of the BM$^{CRE-RFP}$, we injected a group of chimeric mice with NMDA in the right eyes to induce damage, and with PBS in the controlateral eyes as controls (Figure 14, Group A).

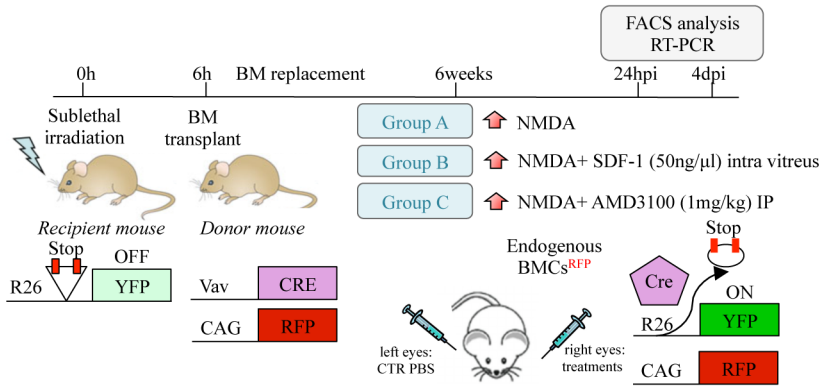


Figure 14. Chimeric mice to follow endogenous BMC mobilization and cell fusion with retinal neurons Schematic representation of the

experimental plan: Chimeric R26Y/BM$^{CRE-RFP}$ mice were damaged in the right eye with NMDA injection 6 weeks after sub-lethal irradiation and transplantation. Cell fusion between BMCs (Cre$^+$/RFP$^+$) and retinal neurons (R26-LoxP-YFP) leads to excision of the floxed stop codon, and in turn, to expression of YFP in hybrid cells. The mice were divided in three groups: NMDA damaged (group A), NMDA damaged and treated with SDF-1 (group B), NMDA damaged and IP injected with AMD3100 (group C). Mice were sacrificed 24 hpi and 4dpi to investigate BMC recruitment (RFP$^+$) and cell fusion with retinal neurons (RFP$^+$/YFP$^+$). Left eyes injected with PBS (CTR) were used as controls.

To evaluate the endogenous BMC mobilization in the retinas, we analyzed the percentage of RFP$^+$ BM cells, calculated with respect to the total retinal cell number, using FACS analysis. There was up to 3% RFP$^+$ BMCs in the damaged retinas, which indicated efficient damage-dependent mobilization of these cells within 24 h (Figure 15 A, B).

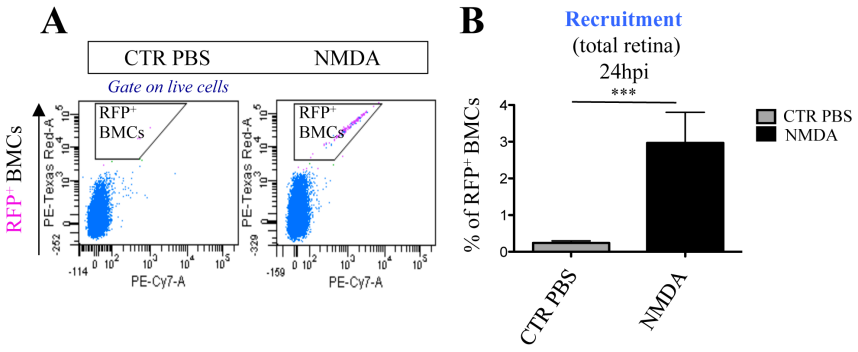


Figure 15. BMCs are mobilized into NMDA damaged retina in R26Y/BM$^{CRE-RFP}$ chimeric mice (A) Representative FACS plots showing recruitment of BMCs (RFP$^+$) into NMDA-damaged and undamaged (CTR PBS) retinas of chimeric R26Y/BM$^{CRE-RFP}$ mice, at 24 hpi (NMDA, 24 hpi) calculated on total living retinal cells. (B) Percentages of RFP$^+$ BMCs recruited into NMDA-damaged retinas as compared with healthy PBS-injected control retinas (CTR) 24 hpi, plotted as ratios with respect to total retinal cell population. Data are means ±S.E.M. of 10 different

experiments (CTR, n = 13; NMDA, n = 8). The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

To investigate the possibility that modulation of the SDF-1/CXCR4 pathway affects mobilization of BMCs into NMDA-damaged retinas, we injected a group of R26Y/BM$^{CRE-RFP}$ mice with the chemokine SDF-1 intravitreally, and at the same time we induced NMDA damage in the right eyes, with the left eyes injected with PBS as controls (Figure 14 A; Group B). Another group of mice was damaged with NMDA in the right eyes and intraperitoneally injected with the SDF-1 antagonist AMD3100 (Figure 14 A; Group C).

Retinal samples were analyzed by FACS 24 hpi, and the migration of RFP$^+$ BMCs into the damaged right eyes in the different groups was compared with the control group, which was only damaged with NMDA (Figure 14 A; Group A). Surprisingly, the percentage of RFP$^+$ cells was significantly increased in the group of mice that received the injection of SDF-1 in the damaged eyes, as compared to the group that received only NMDA (Figure 16, A, B NMDA+SDF-1). In contrast, the group of mice treated with AMD3100 showed partial block of the recruitment of RFP$^+$ cells, as indicated by the decrease in the percentage of RFP$^+$ cells in the total retinal tissue after the damage, as compared to the NMDA-damaged eyes (Figure 16 A, B NMDA+AMD3100).

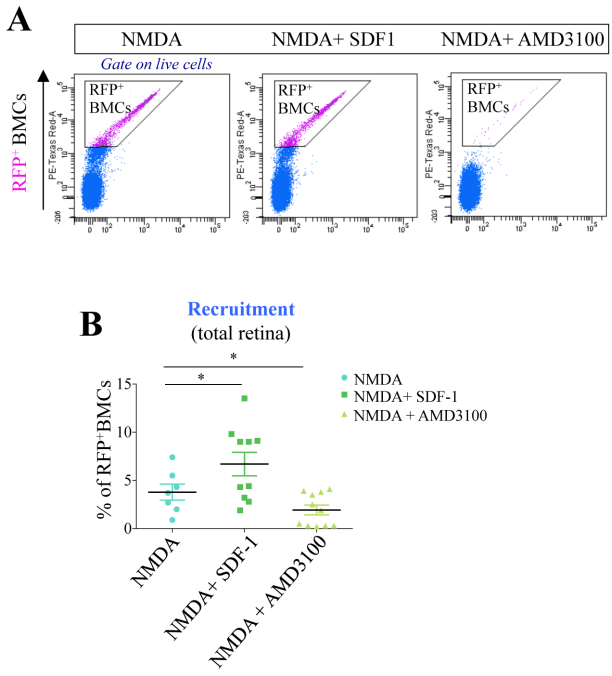


Figure 16. SDF-1/CXCR4 pathway modulation increases BMC recruitment into NMDA damaged retina in R26Y/BM$^{CRE-RFP}$ chimeric mice (A) Representative FACS profiles of RFP$^+$ BMCs migrated into damaged retinas of chimeric R26Y/BM$^{CRE-RFP}$ mice at 24 hpi after different treatments (NMDA, NMDA+SDF-1, NMDA+AMD3100). RFP$^+$ cells are plotted with respect to the total retinal population. DAPI was added to all retinal samples to exclude dead cells from the analysis. (B) Percentages of RFP$^+$ BMCs recruited into the damaged right eyes of R26Y/BM$^{CRE-RFP}$ chimeric mice from different treatment groups were evaluated by FACS analysis respect to the total retinal cell population. (NMDA+SDF-1, n = 10; NMDA, n = 8; NMDA+AMD3100, n = 10). The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

We then wanted to investigated whether BMCs migrated into the damaged retinas also fuse with retinal neurons. Damaged retinas of R26Y/BM$^{CRE-RFP}$ chimeric mice were analyzed by FACS 24 hpi. FACS analysis showed that approximately 3% of the RFP$^+$ BMCs recruited into the damaged eyes expressed YFP, which

suggested the occurrence of cell fusion events, and therefore the presence of hybrids (Figure 17 A, B). However, the YFP^+/RFP^+ hybrids were very low with respect to the total retinal cell population, which suggested that cell fusion with endogenous BMCs occurs with low efficiency (Figure 17 C, D).

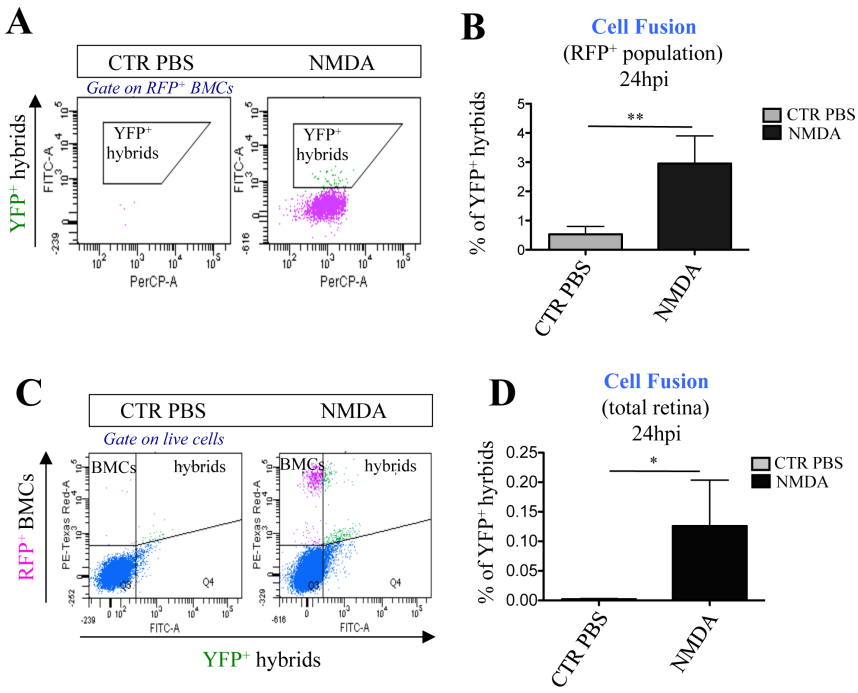


Figure 17. BMCs mobilized into NMDA damaged retina fuse with retinal neurons (A, C) FACS profile of YFP^+ hybrid cells formed in damaged (NMDA) and undamaged (CTR PBS) retinas of chimeric $R26Y/BM^{CRE\text{-}RFP}$ mice analyzed at 24 hpi with respect to the RFP^+ BMC population (A), and to the total retinal population (C). (B, D) Percentages of YFP^+ hybrids formed after BMC recruitment in damaged (NMDA) retinas as compared to healthy control retinas (CTR PBS) at 24 hpi, plotted as ratios with respect to recruited RFP^+ BMCs (B), and to the total retinal population (D). Data are means ±S.E.M. of 10 different experiments (CTR, n = 13; NMDA, n = 8). The statistical analysis is

based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

On the other hand, 6 weeks after BM replacement into sub-lethally irradiated recipient mice, the percentage of RFP^+ cells in the BM (i.e., chimerism) was around 45%, which suggested a possible underestimation of the number of hybrids formed in vivo (Figure 18 A, B).

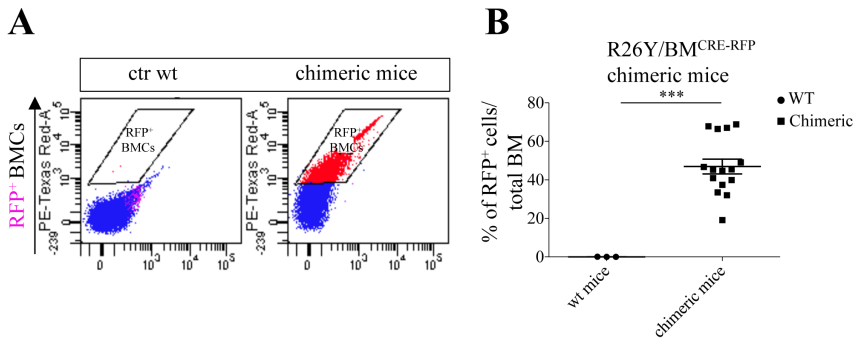


Figure 18. Chimerism analysis of R26Y/BM$^{CRE-RFP}$ chimeric mice after irradiation and transplantation (A) Representative FACS plots showing percentage of RFP^+ cells in the reconstituted total bone marrow harvested from chimeric R26Y/BM$^{CRE-RFP}$ mice 6 weeks after sub-lethal irradiation, and BM transplantation calculated with respect to total BM population. Chimerism was calculated taking into account BM analysis from a wild-type control mouse (CTR). (B) Chimerism was calculated as percentages of RFP^+ cells in the total BM of chimeric R26Y/BM$^{CRE-RFP}$ mice analyzed by FACS 6 weeks after irradiation and BM transplantation (n = 15). Cells of total BM from wild-type mice were used as the negative control. The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

The amount of RFP^+ cells detected into damaged retinas decreased over time (Figure 19 A, B, 4 dpi). On the contrary the number of

YFP$^+$ hybrids increased over time, to reach approximately 5% of the RFP$^+$ BM cell population in retinas analyzed by FACS at 4 dpi (Figure 19 C D, 4 dpi). This indicated the survival of the hybrids integrated into the retinal tissue.

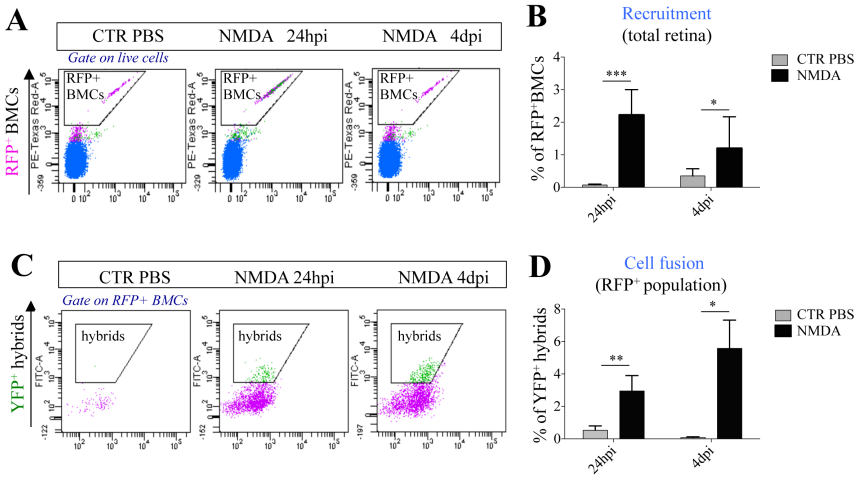


Figure 19. BMCs fuse with retinal neurons and YFP$^+$ hybrids survive into the damaged retinas (A) Representative FACS plots showing BMCs (RFP$^+$) recruited into damaged (NMDA) and undamaged (CTR PBS) retinas of chimeric R26Y/BM$^{CRE\text{-}RFP}$ mice calculated on total living retinal cells at 24hpi and 4dpi. (B) Percentages of RFP$^+$ BMCs recruited into NMDA-damaged retinas compared with healthy PBS-injected control retinas calculated on total retinal population. (C) FACS plots showing hybrid cells (YFP$^+$) formed in damaged (NMDA) retinas as compared to undamaged (CTR PBS) retinas of chimeric R26Y/BM$^{CRE\text{-}RFP}$ mice analyzed at 24 hpi (NMDA, 24 hpi) and at 4 dpi (NMDA, 4dpi) with respect to the RFP$^+$ BMC population. (D) Quantification of the percentage of YFP$^+$ hybrids formed into damaged retinas (NMDA) as compared to healthy control retinas (CTR PBS) 24 hpi and 4 dpi plotted as ratios with respect to recruited RFP$^+$ BMCs. Data are means ±S.E.M. of 10 different experiments (24 hpi: CTR, n = 13; NMDA, n = 8; 4 dpi: CTR, n = 4; NMDA, n = 4). The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

Since SDF-1 treatment enhances the percentage of RFP^+ BMCs into NMDA damaged retinas we wanted to investigate whether the presence of this chemokine also increases the efficiency of cell fusion. Thus we analyzed the number of YFP^+ hybrid cells detected into damaged retinas of chimeric $R26Y/BM^{CRE\text{-}RFP}$ mice at 24hpi after the different treatments (Figure 14). We found that, although the SDF-1/CXCR4 pathway modulation influenced the amount of RFP^+ BMCs recruited into damaged retinas (Figure 16 A, B), neither the treatment with SDF-1 chemokine nor with the antagonist AMD3100 did not change the percentage of YFP^+ hybrids formed into NMDA damaged retinas at 24hpi (Figure 20 A, B). This result suggested that the increase in recruitment of BMCs, due to the SDF-1 chemokine, did not correspond to an increased number of YFP^+ hybrids inside the RFP^+ population thus indicating that the efficiency of cell fusion events was not affected by the modulation of the SDF-1/CXCR4 pathway.

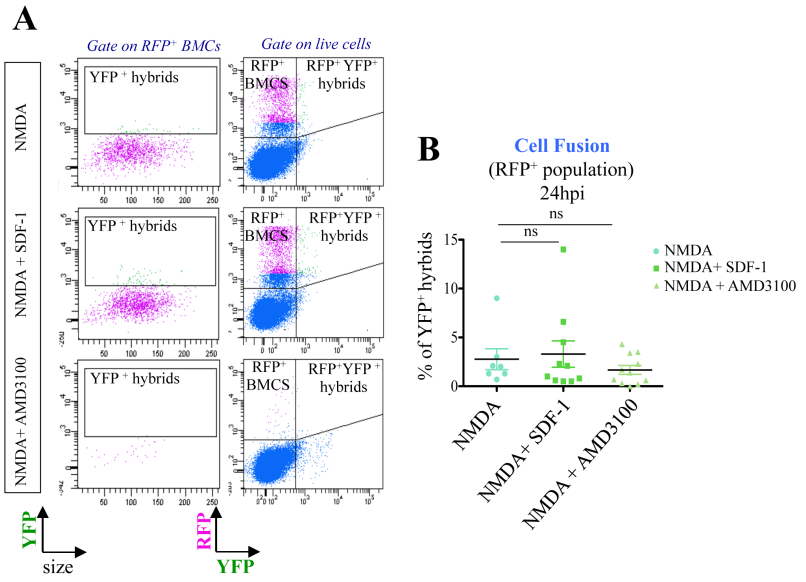
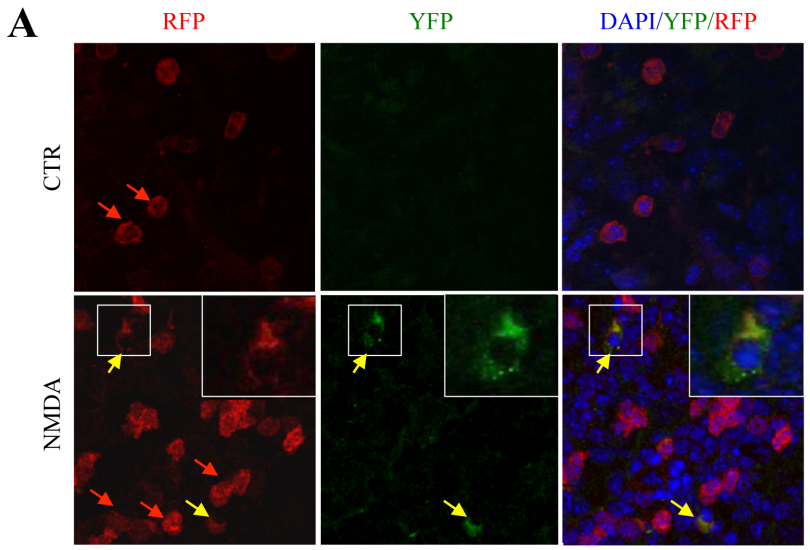


Figure 20. SDF-1/CXCR4 pathway modulation does not affect cell fusion efficiency (A) Representative FACS profiles of YFP^+ hybrids detected into damaged retinas of chimeric $R26Y/BM^{CRE\text{-}RFP}$ mice at 24 hpi after different treatments (NMDA, NMDA+SDF-1, NMDA+AMD3100). YFP^+ cells are plotted with respect to RFP^+ BMC population (left panel) or to total retinal population (right panel). DAPI was added to all retinal samples to exclude dead cells from the analysis. (B) Percentages of YFP^+ hybrids formed after BMC recruitment into the damaged right eyes of $R26Y/BM^{CRE\text{-}RFP}$ chimeric mice from different treatment groups were evaluated by FACS analysis with respect to the RFP^+ BMC population. (NMDA+SDF-1, n = 10; NMDA, n = 8; NMDA+AMD3100, n = 10). The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

We confirmed the presence of YFP^+/RFP^+ hybrids at 24 hpi also in retinal flat mounts and in vertical sections upon NMDA damage, there was a significant increase of cells recruited into damaged retinas that also expressed YFP (Figure 21 A, B, C).

All in all, our data indicate that modulation of the SDF-1/CXCR4 pathway enhances endogenous recruitment of BMCs into NMDA-damaged retinas, which in turn fuse with retinal neurons.



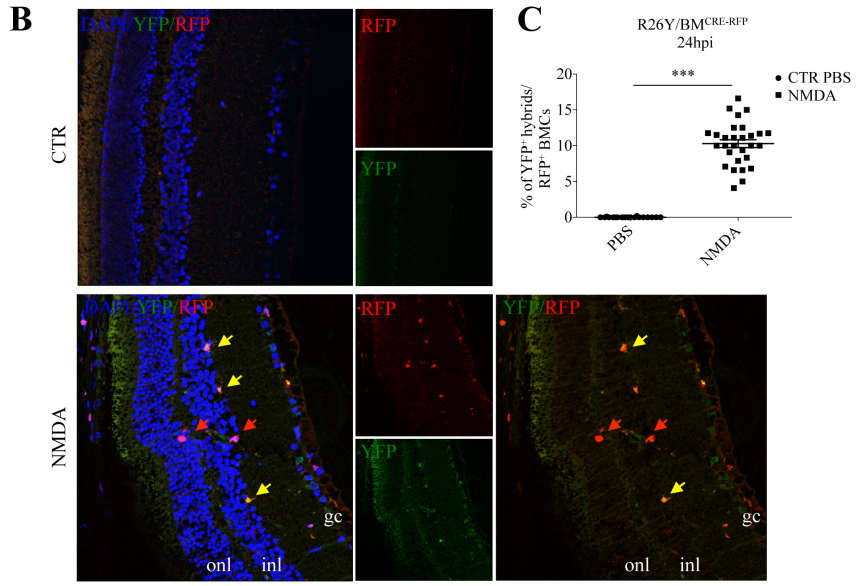


Figure 21. Endogenous BMCs mobilized into NMDA damaged retinas fuse with retinal neurons (A, B) Representative immunostaining of NMDA-damaged and undamaged PBS-injected (CTR) retinal flat mounts (A) and retinal sections (B) of chimeric R26Y/BM$^{CRE-RFP}$ mice harvested 24 hpi. Recruited RFP^{+} BMCs (red arrows) and RFP^{+}/YFP^{+} hybrids (yellow arrows) are detected in damaged retinas. Cell nuclei were counterstained with DAPI (blue). (onl= outer nuclear layer; inl= inner nuclear layer; gc= ganglion cells layer). Scale bar: 40 μm (n = 3). Representative, higher magnification images included in the white boxes are shown. (C) Quantification of YFP^{+} hybrids counted in damaged (NMDA) or undamaged PBS injected (CTR PBS) retinal flat mounts of chimeric R26Y/BM$^{CRE-RFP}$ mice sacrificed at 24hpi. The number of the hybrids is represented as percentage of YFP^{+} fused cells on the total of migrated RFP^{+} BMCs counted in 7-10 random fields from each mouse retina. The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

4. BMCs fuse with Müller glia cells after mobilization into damaged retina

We then asked which retinal cell types underwent cell fusion. Thus, we performed immunofluorescence stainings for

several retinal cell markers at 24 hpi both in retinal flat mounts and in retinal sections of chimeric R26Y/BM$^{CRE-RFP}$ mice. We found YFP^{+} hybrids that expressed GLUTAMINE SYNTHETASE, which suggested that the endogenous BMCs migrated into the damaged retina fused with MG cells (Figure 22 A, B).

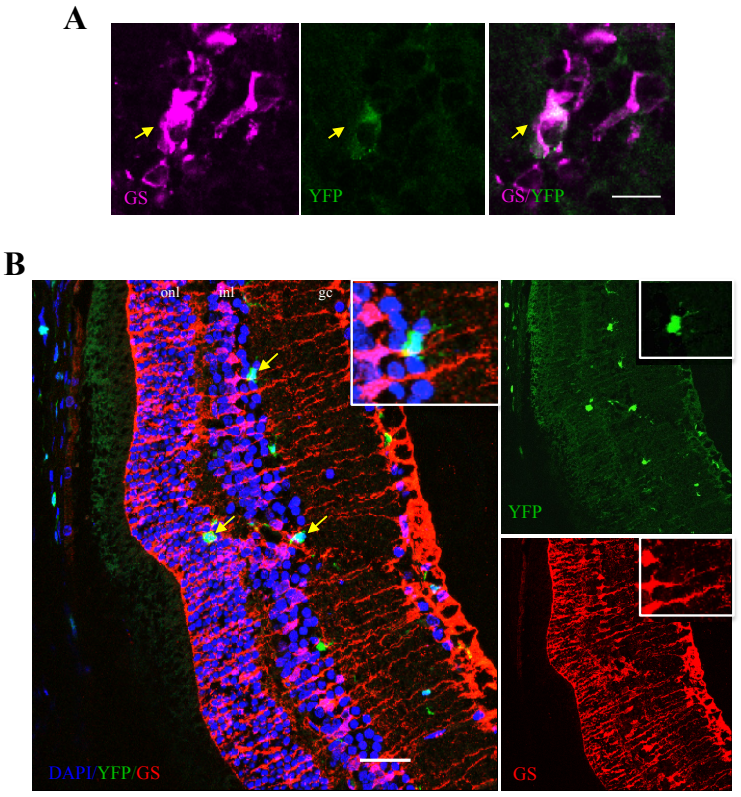


Figure 22. Endogenous BMCs migrated into NMDA damaged retinas fuse with Müller glial cells (A, B) Immunostaining of retinal flat mounts (A) and retinal sections (B) from NMDA-damaged retinas of R26Y/BM$^{CRE-RFP}$ chimeric mice sacrificed at 24hpi, showing co-localization of YFP^{+} hybrids (green) with MG cell marker GLUTAMINE SYNTHETASE (red). Higher magnification images included in the white boxes are also shown. Cell nuclei were counterstained with DAPI (blue). Scale bar: A, 10 μm; B, 50 μm

Indeed, we analyzed the expression of bipolar (PKC), horizontal (CALBINDIN), and photoreceptor (RECOVERIN) markers in YFP^+ hybrids, and we did not find any co-localization of YFP with these retinal neuron markers. On the other hand, some YFP^+ hybrids expressed CALRETININ, which suggested that there was also cell fusion with ganglion and amacrine cells (Figure 23).

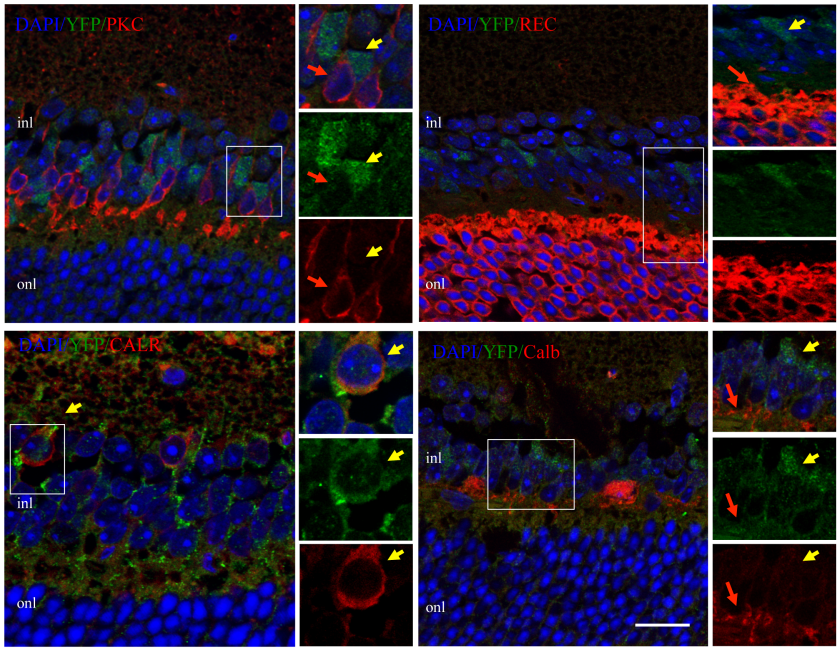


Figure 23. Bone-marrow cells migrated into NMDA damaged retinas fuse with Müller glial cells and amacrine and ganglion cells Representative immunostainings of YFP^+ hybrids (green) in sections of damaged retinas of $R26Y/BM^{CRE\text{-}RFP}$ chimeric mice at 24 hpi. Co-staining of YFP^+ hybrids and retinal cell markers is shown: photoreceptors (RECOVERIN, red), bipolar (PKC, red), ganglion and amacrine (CALRETININ, red), horizontal (CALBINDIN, red) (onl, outer nuclear layer; inl, inner nuclear layer; gc, ganglion cells layer). Red arrows indicate retinal neurons positive to specific markers, yellow arrows indicate YFP^+ hybrids. Higher magnification areas included in the white

boxes are shown. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

To then determine the frequency of BMC fusion with MG cells and with ganglion and amacrine cells, we created two different types of chimeric mice.

To quantify the fusion events with MG cells, we generated GFAP-Cre/BM^{R26Y} mice by transplanting sub-lethally irradiated GFAP-Cre mice with BM from R26Y mice (Figure 24).

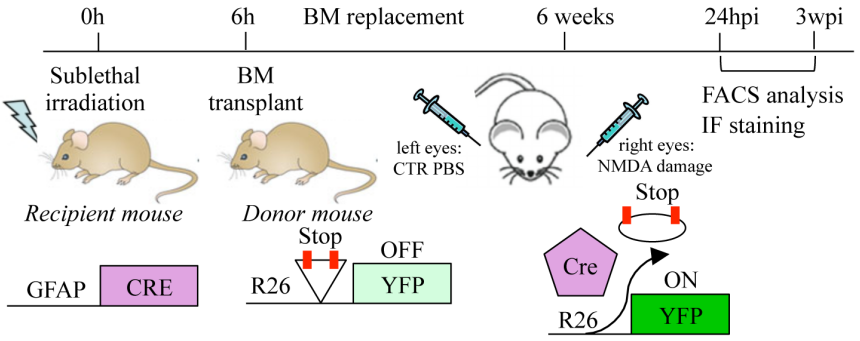


Figure 24. GFAP-Cre/R26Y chimeric mice to study cell fusion of endogenous BMCs with retinal Müller glial cells. Experimental scheme: GFAP-Cre/BM^{R26Y} chimeric mice were generated to analyze fusion of endogenous BMCs (R26-LoxP-YFP) with MG cells (GFAP-Cre). YFP^{+} hybrids were formed only if endogenous BMCs that migrated into NMDA-damaged retinas specifically fused with MG cells, which express Cre recombinase. Left eyes injected with PBS were used as controls. Mice were sacrificed 24 h and 3 weeks after NMDA damage, to investigate cell fusion using FACS analysis and immunofluorescence staining.

Six weeks after BM replacement, a group of mice received the injections of NMDA into the right eyes and PBS into the contralateral eyes, as the controls. We analyzed the percentage of YFP^+ hybrids at 24hpi by FACS analysis and we found that 0.6% of the total retinal cell population expressed YFP, which indicated fusion events between BMCs that had migrated into the damaged retinas and MG cells (Figure 25 A). We confirmed the presence of MG-BMCs hybrids in NMDA-damaged retinal sections of chimeric GFAP-Cre/BM^{R26Y} mice, where the YFP^+ hybrids expressed SOX2 and GLUTAMINE SYNTHETASE, both markers of MG cells (Figure 25 B, C).

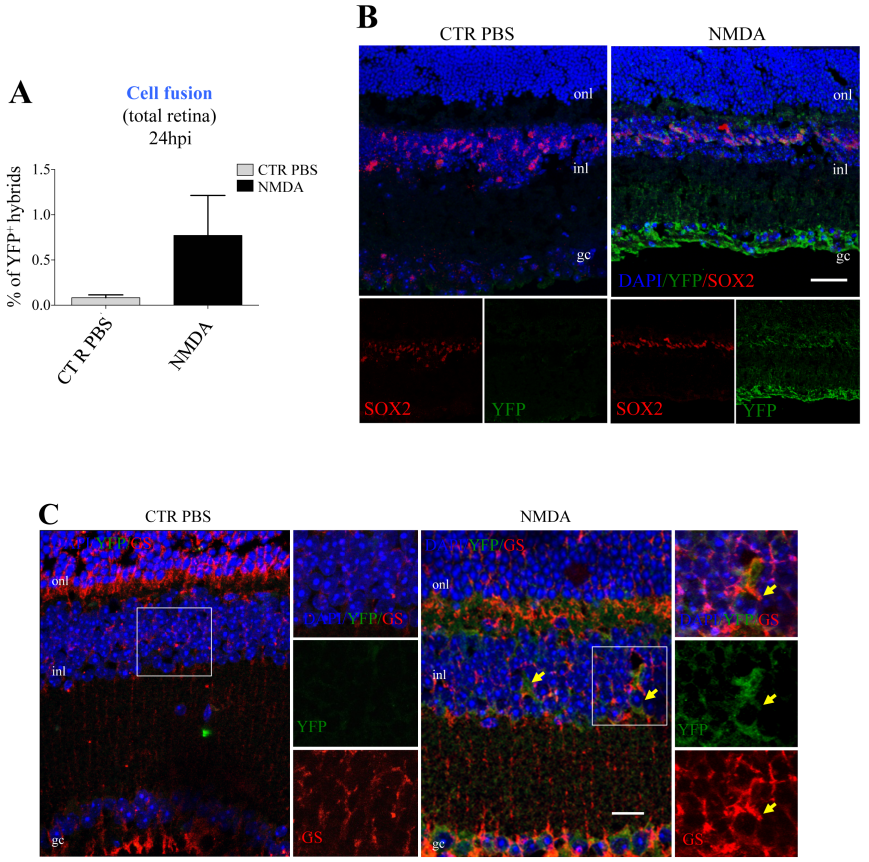


Figure 25. BMCs recruited into damaged retinas fuse with retinal Müller glial cells (A) Percentage of YFP^+ hybrids detected into FACS-sorted retinas from GFAP-Cre/BM^{R26Y} chimeric mice after BMC mobilization in damaged retinas (NMDA) and in undamaged PBS-injected control retinas (CTR PBS), 24 hpi. Data are means ±S.E.M. of five independent experiments (CTR PBS, n = 5; NMDA, n = 7). (B, C) Representative immunostaining images of retinal sections from damaged (NMDA) and undamaged PBS-treated (CTR) retinas of chimeric GFAP-Cre/BM^{R26Y} mice sacrificed 24hpi. YFP^+ hybrids (green) formed after cell fusion between endogenous BMCs (R26-LoxP-YFP) and Müller cells (GFAP-Cre) are shown. Co-localization of YFP^+ hybrids and SOX2 (B) or GLUTAMINE SYNTHETASE (C) showing cell fusion with MG cells are indicated by yellow arrows. Representative higher magnification images from the white boxes are also shown. Sections from at least three different mice were analysed (n = 3). Nuclei were counterstained with DAPI (blue). (onl= outer nuclear layer; inl= inner nuclear layer; gc ganglion cell layer). Scale bar: B= 45 μm, C= 20 μm.

To evaluate cell fusion of endogenous BMCs with ganglion and amacrine cells, we generated Calr-Cre/R26Y/BM^{RFP} mice by transplanting sub-lethally irradiated double transgenic mice for LoxP-STOP-LoxP-YFP [R26Y] and Calr-Cre transgenes (Calr-Cre/R26Y) with BM from donor CAG-RFP mice (Figure 26 A). Here, only 0.7% of the RFP^+ BMCs recruited into the damaged retina fused with ganglion and amacrine cells, which corresponded to 0.03% of the total retinal cells (Figure 26 B, C, D).

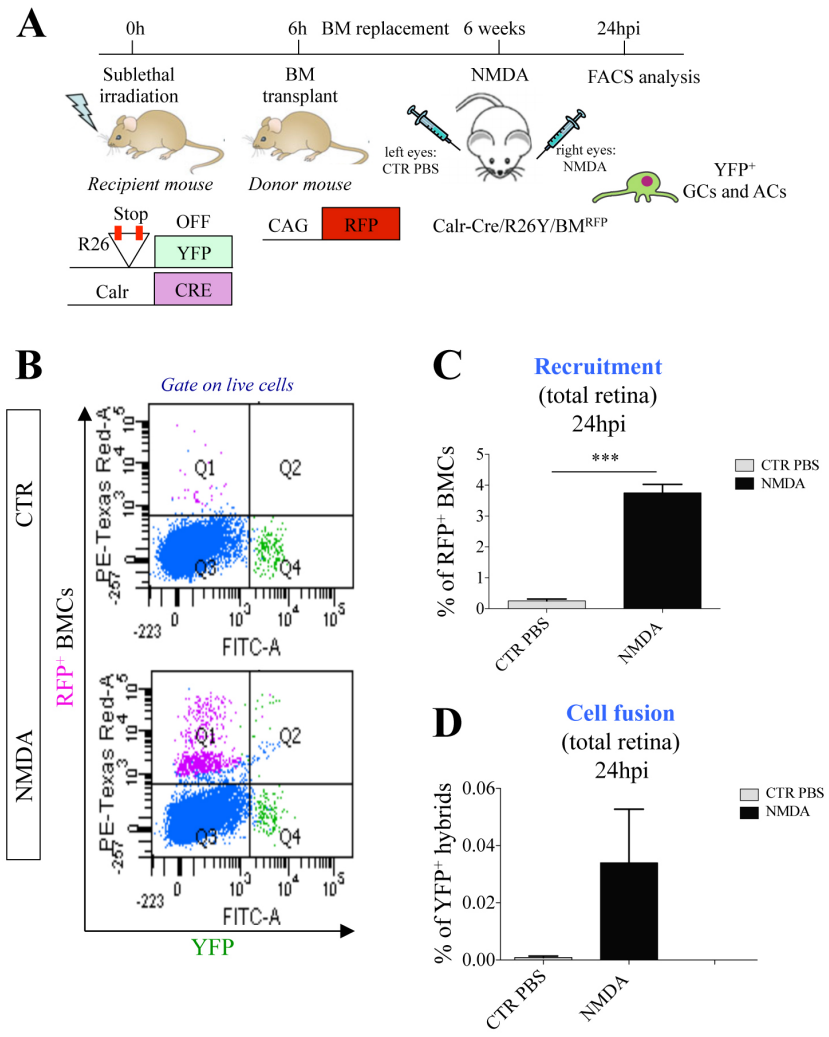


Figure 26. Calr-Cre/R26Y/BM^{RFP} chimeric mice to study cell fusion of endogenous BMCs with ganglion and amacrine cells

(A) Experimental scheme: Calr-Cre/R26Y/BM^{RFP} chimeric mice were generated to investigate fusion with ganglion and amacrine cells. YFP^{+}/ RFP^{+} hybrids derived from fusion between endogenous RFP^{+} BMCs and YFP^{+} ganglion cells (GCs) and amacrine cells (ACs), where Cre recombinase expression is driven by the Calretinin promoter.(B) Representative FACS plots showing fusion events (RFP^{+}/YFP^{+} hybrids) in chimeric Calr-Cre/R26Y/BM^{RFP} mice in damaged (NMDA) and undamaged PBS-injected (CTR) retinas 24 hpi (n = 3). (C) Percentages of RFP^{+} BMCs recruited into NMDA-damaged or control PBS-treated (CTR) retinas of Calr-Cre/R26Y/BM^{RFP} chimeric mice, 24 hpi, relative to

total retinal cells. (D) Percentages of YFP^+ hybrids formed after BMC recruitment into NMDA-damaged and PBS-injected (CTR PBS) retinas of Calr-Cre/R26Y/BM^{RFP} chimeric mice, 24 hpi, relative to total retinal population. Data are means ±S.E.M. of three different biological samples (n = 3). The statistical analysis is based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

5. BMC–Müller glial cell hybrids undergo reprogramming, and in turn, differentiate into ganglion and amacrine cells

As the majority of fusion events occurred with MG cells, we followed the hybrids derived from cell fusion with MG cells in the chimeric GFAP-Cre/BM^{R26Y} mice (Figure 24).

We investigated whether the hybrids undergo proliferation through immunofluorescence staining for proliferative markers in retinal sections. Here, already at 24 hpi, many YFP^+ hybrids also expressed phosphorylated H3 (Figure 27 A).

Moreover we analyzed the presence of YFP^+ hybrids at longer time points by FACS analysis and the hybrids derived from cell fusion between endogenous BMCs and Müller glial cells survived longer till 3wpi into damaged retinas (Figure 27 B, C).

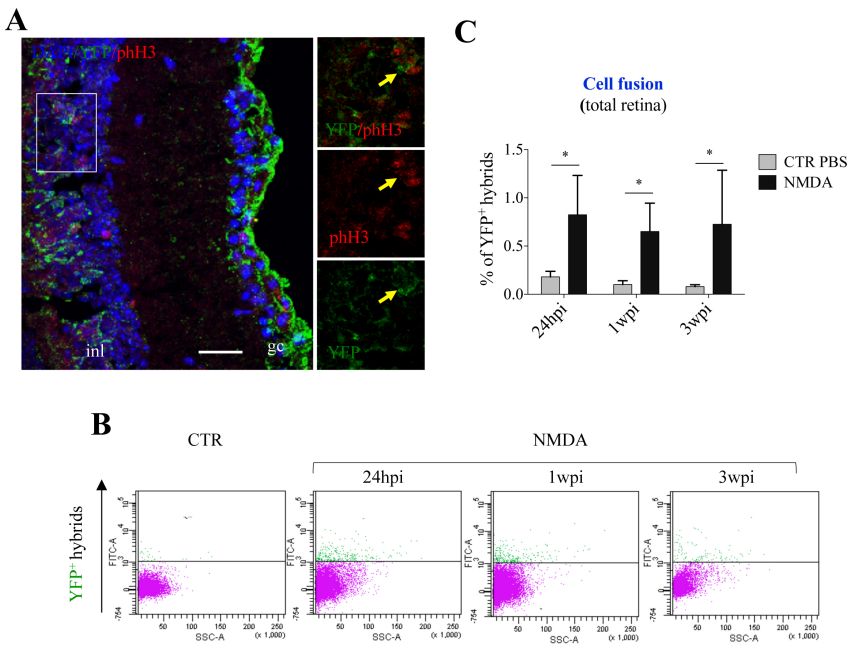


Figure 27. MG-derived hybrids proliferate and survive at long term into damaged retina (A) Immunostaining of retinal sections from NMDA-damaged retinas of chimeric GFAP-Cre/BM^{R26Y} mice, 24hpi, showing YFP^{+} hybrids expressing phosphorylated H3 (phH3; red; yellow arrows). Higher magnification areas included in the white boxes are shown. Scale bar: 20 µm. (B) FACS profile of the YFP^{+} hybrids identified into damaged retinas (NMDA) and undamaged PBS injected control retinas (CTR PBS) of GFAP-Cre/BM^{R26Y} chimeric mice at different time points after damage (24pi, 1wpi, 3wpi). (C) Quantification of the percentages of fused YFP^{+} hybrids derived from cell fusion between endogenous BMCs^{R26Y} and MG cells (GFAP-Cre) at different time points after damage (NMDA), relative to the total retinal population. Data are means ±S.E.M. of five different biological samples (n = 4-6). The statistical analysis is based on ANOVA unpaired T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

To determine whether the hybrids can undergo dedifferentiation, we analyzed the expression of some pluripotency and retinal progenitor markers in the FACS-sorted YFP^{+} hybrids at

24 hpi by q-RT-PCR (Figure 28 A, B). The expression of the pluripotency genes Oct4 and SSEA1 showed only a tendency to increase in the hybrids, as compared to the control retinas (Figure 28 B-*pluripotency genes*). In contrast, the hybrids showed some significantly up-regulated neural progenitor genes, such as *Pax6* and *Nestin*, as well as some retinal progenitor genes, such as *Ascl-1*, which has been shown to be essential for MG-cell-mediated retinal regeneration in fish (Pollak et al., 2013). Furthermore also *Six3*, *Prox1*, *Chx10*, and *Math3*, which have been shown to be important for retinal development and differentiation of retinal progenitors (Dyer et al., 2003; Inoue et al., 2002; Livne-Bar et al., 2006; Oliver et al., 1995) showed an increased expression in the hybrids as compared to the control retinas (Figure 28 B- *retinal progenitors*). The hybrids also up-regulated the cell-cycle gene *cyclin D1* (Figure 28 B- *cell cycle*), which provides further confirmation of their proliferative potential.

To determine whether the MG-cell-derived hybrids can ultimately differentiate into mature retinal neurons and specifically into the cell types that are damaged in our model, we followed their fate at later times. We analyzed the molecular profile of the MG-derived hybrids FACS sorted from damaged retinas 3 weeks after NMDA damage. We evaluated the level of expression of several retinal progenitors genes and mature retinal genes by q-RT-PCR to study the differentiation potential of the hybrids at later time points (Figure 28 C).

We found that the hybrids sorted from damaged retinas 3wpi started to up regulate ganglion and amacrine cells genes such as

Brn3b and *Calr*. Whereas, on the contrary *GS*, a Müller glia cells gene was down regulated into MG-derived hybrid cells. These results further suggest a differentiation of the hybrids into amacrine cells and ganglion cells. However this differentiation was not complete as indicated by the fact that progenitors gene such as *Prox1* and *Math5* were still expressed at 3wpi (Figure, 28 C).

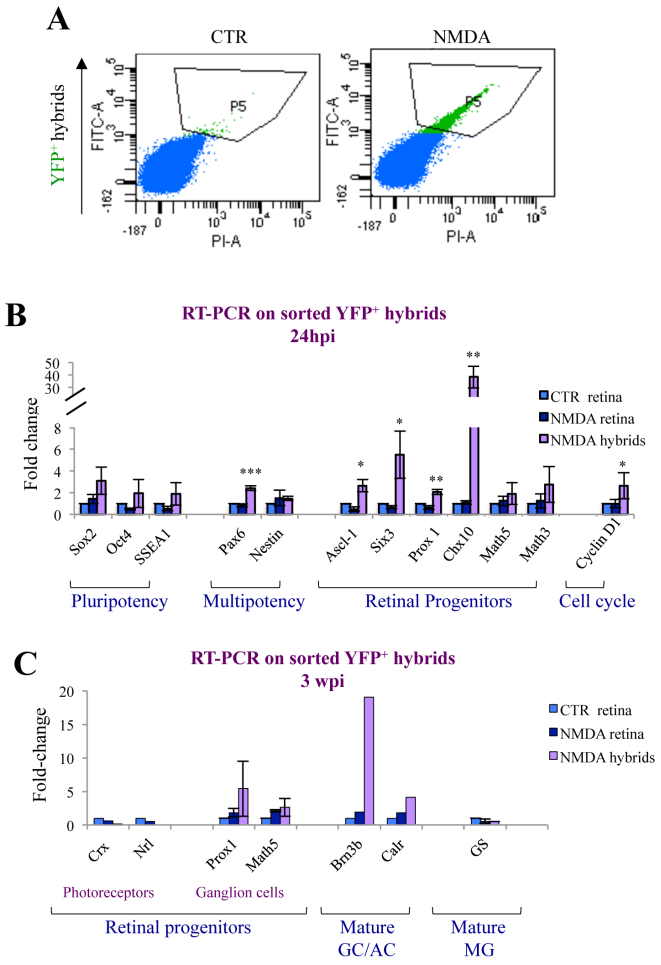


Figure 28. MG-derived hybrids undergo reprogramming showing a retinal progenitor phenotype (A) Representative FACS plot showing YFP^{+} hybrids sorted from damaged retinas (NMDA) of GFAP-Cre/BM^{R26Y} chimeric mice at 24hpi as compared to undamaged PBS

injected control retinas (CTR). YFP^- retinal cells were sorted as well from damaged retinas (NMDA retina) and used as control in the molecular profile analysis. (B) RT-PCR analysis on total RNAs harvested from YFP^+ hybrids FACS-sorted from NMDA-damaged retinas (NMDA hybrids) of GFAP-Cre/BM^{R26Y} chimeric mice, 24 hpi (B) and 3wpi (C). Expression levels of pluripotency, neural stem cells, retinal progenitor, cell-cycle and mature neurons genes are expressed relative to levels in undamaged PBS injected control retinas (CTR retina), after normalization to *Gapdh* levels. Damaged retinas depleted of sorted hybrids (YFP^-, NMDA retina), were also included in the analysis as further control. Data are means ±S.E.M. of three independent experiments (24hpi; n = 3-4) (3wpi; n = 2-3). The statistical analysis is based on unpaired Student's T-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s, not significant.

We checked also in retinal flat mounts at 3wpi the expression of GCs and ACs markers into MG-derived hybrid cells. Surprisingly, already at 3 wpi, some of the YFP^+ hybrids expressed CALRETININ also in retinal flat mounts (Figure 29 A, yellow arrows), which suggested differentiation toward ganglion and amacrine cell fate upon damage after passing through a proliferative and dedifferentiation step. However, this process occurred with low frequency, as the majority of the YFP^+ hybrids detected 3 wpi in retinal flat mounts still co-expressed GLUTAMINE SYNTHETASE (Figure 29 C, magenta arrows). This suggested that most of the hybrids remained as MG cells, or were still not fully differentiated. Importantly, 15% of these cells differentiated toward a ganglion and amacrine cell fate (Figures 29 A, B, C, yellow arrow). In addition, there were some YFP^+ hybrids that were in an intermediate differentiation stage, as these expressed both GLUTAMINE SYNTHETASE and CALRETININ (Figure 29 C, red arrows). It was, however, difficult to calculate the number of each type of hybrid.

All in all, these data suggest that one of the mechanisms by which MG cells de-differentiate and participate in ganglion and amacrine cell replacement after NMDA damage is through a cell fusion process with endogenous BMCs recruited into the damaged retinas.

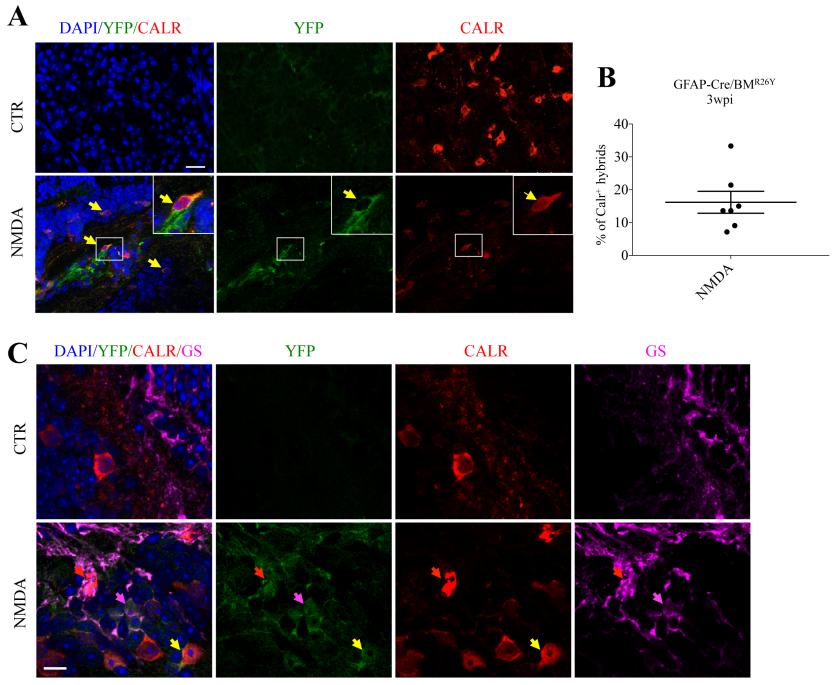


Figure 29. Müller glial-derived hybrids differentiate into ganglion and amacrine cells over the long term (A) Representative immunostaining images of retinal flat mounts from damage (NMDA) or undamaged PBS-injected (CTR PBS) eyes of GFAP-Cre/BM^{R26Y} chimeric mice 3 wpi. Yellow arrows show co-localization of YFP^{+} hybrids (green) with CALRETININ (Calr^{+}, red). Images were taken from at least three independent experiments (n = 3). Scale bar: 20 μm. (B) Percentages of YFP^{+} hybrids (green) expressing CALRETININ (Calr^{+} red) in retinal flat mounts 3 wpi. YFP^{+}/Calr^{+} hybrids were counted in three different mouse retinal flat mounts from three different experiments (n = 3), and each plot represents the percentage of Calr^{+} /YFP^{+} cells with respect to the total of YFP^{+} hybrids counted in each field. (C) Immunostaining of retinal flat mounts from damaged retinas (NMDA) and undamaged PBS-treated

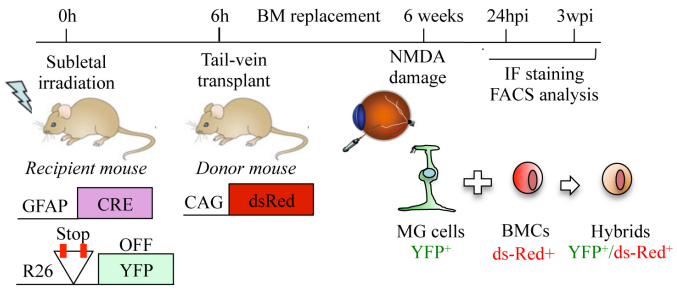
retinas (CTR) of chimeric GFAP-Cre/BM^{R26Y} mice, 3 wpi, showing YFP$^+$ hybrids cells (green) expressing GLUTAMINE SYNTHETASE (purple) and CALRETININ (CALR; red). Higher magnification areas included in the white boxes are shown. Nuclei were counterstained with DAPI (blue). Scale bar: 10 μm. Statistical analyses are based on unpaired Student's T-tests. *, P <0.05; **, P <0.01; ***, P <0.001; n.s, not significant.

We finally aimed to understand if after a cellular damage cell fusion is the main mechanism through which MG cells can undergo dedifferentiation and reprogramming. To do this we generated chimeric mice by using as recipient GFAP-Cre/R26Y lineage tracing mice. We replaced their BM with the whole BM from donor CAG-ds-RED transgenic mice after sub-lethal irradiation generating the GFAP-Cre/R26Y/BM$^{ds\text{-}RED}$ chimeric mice (Figure 30 A). By using this model we could distinguish between YFP$^+$ MG cells, activated by NMDA damage and undergoing reprogramming and MG-derived YFP$^+$/ds-RED$^+$ hybrids. Thus we first checked in retinal flat mounts the presence of hybrid cells and found many YFP$^+$/ds-RED$^+$ cells at 24hpi suggesting the occurrence of cell fusion between endogenous ds-RED$^+$ BMCs mobilized into damaged retinas (NMDA) and YFP$^+$ MG cells (Figure 30 B; yellow arrows). We then FACS sorted separately the population of YFP$^+$ MG cells both in NMDA damaged retinas (NMDA MG) and in PBS injected control retinas (CTR MG) and finally also the YFP$^+$/ds-RED$^+$ MG-derived hybrids formed after cell fusion into damaged retinas 24hpi (Figure 30 C). In these sorted cells we investigated reprogramming by real time PCR. Only YFP$^+$/ds-RED$^+$ hybrids, sorted from damaged retinas (NMDA hybrids) expressed progenitor genes indicating a partial reprogramming of these hybrid cells (Figure 30 D). While, on the contrary YFP$^+$ MG cells,

activated upon damage (NMDA MG) did not show any up regulation of retinal progenitor genes as compared to control MG cells. These results suggest that cell fusion between endogenous BMCs migrated into damaged retina and MG cells might represent an important mechanism by which MG undergo reprogramming acquiring neurogenic potential. However, further experiments are needed to confirm these results.

In addition we found few fused cells at long term and very few of them differentiating into CALRETININ^{+} amacrine/ganglion neurons when we analyzed retinal flat mounts of these chimeric mice at 3wpi (Figure 30 E; magenta arrows). On the contrary the majority of ds-RED^{+} BM cells were retained into damaged retinas and did not express YFP indicating that they did not derived from fusion with MG cells. These ds-RED^{+} /YFP^{-} cells changed their shape and morphology most of them resembling microglia (Figure 30 E; red arrows). This could suggest a possible transdifferentiation mechanism through which BMCs, migrated into damaged retinas, directly differentiate into glia or neurons, once in the retinal environment. However, we cannot exclude the possibility that these ds-RED^{+}/YFP^{-} cells were, at least in part, the product of cell fusion with other retinal cell types not tracked in this model.

A



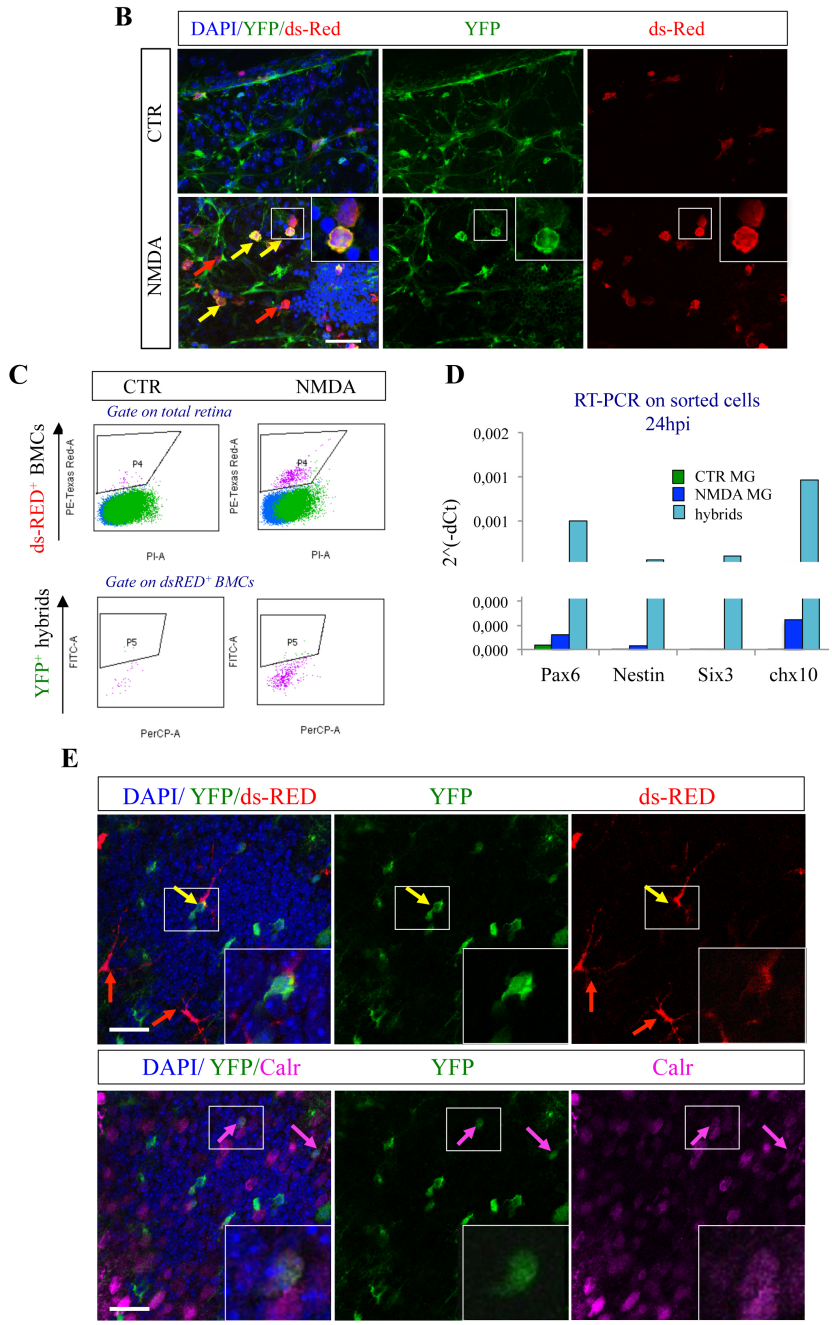


Figure 30. MG derived hybrids undergo reprogramming into damaged retinas of GFAP-Cre/R26Y/BM$^{ds\text{-}RED}$ chimeric mice (A) Experimental scheme: GFAP-Cre/R26Y/BM$^{ds\text{-}RED}$ chimeric mice were generated to study cell fusion of endogenous BMCs (ds-RED^{+}) with MG

cells (YFP^+). The mice were damaged into the right eyes (NMDA) while left eyes were injected with PBS and used as control (CTR). The mice were sacrificed at 24hpi and 3wpi to assess cell fusion and reprogramming by FACS analysis and RT-PCR. (B) Representative pictures of immunostained retinal flat mounts from GFAP-Cre/R26Y/$BM^{ds\text{-}RED}$sacrificed at 24hpi. Colocalization of YFP with ds-RED into hybrid cells is indicated by the yellow arrows. Red arrows indicate unfused ds-RED^+ BMCs. Zoomed pictures are included in the white boxes. Scale bar: 20 μm**.** (C) FACS plots showing few cell fusion events between endogenous BMCs (ds-RED^+) and Müller glia cells (YFP^+) into damaged retinas (NMDA) as compared to undamaged PBS injected control retinas (CTR). ds-RED^+ cells were gated on the total retinal population while YFP^+ cells were gated on the ds-RED^+ BM population. Dead cells were excluded from the analysis. (D) Expression levels of retinal progenitors genes were analyzed by RT-PCR on total RNAs harvested from GFAP-Cre/R26Y/$BM^{ds\text{-}RED}$ mice sacrificed at 24hpi and represented as absolute 2^(-dCt) value after normalization on GAPDH levels. (E) Representative pictures of retinal flat mounts from GFAP-Cre/R26Y/$BM^{ds\text{-}RED}$ mice sacrificed at 3wpi stained for YFP (MG cells, green), ds-RED (BMCs, red) and CALRETININ ($Calr^+$, magenta). Scale bar: 30 μm. Yellow arrows indicate fused hybrid cells. Magenta arrows indicate MG-derived hybrids expressing CALRETININ.

6. Perturbation of SDF-1/CXCR4 pathway is not sufficient to induce retinal regeneration

We found that the modulation of SDF-1/CXCR4 signaling pathway affects MG cells ability to undergo reprogramming and to differentiate into new ganglion and amacrine cells by acting on endogenous BMC mobilization into damaged retinas.

We aimed to verify the hypothesis that, by boosting endogenous BMC recruitment into NMDA damaged retinas, we could induce endogenous retinal repair.

We therefore used mice double transgenic mice for LoxP-STOP-LoxP-YFP [R26Y] and Calr-Cre transgenes (Calr-Cre/R26Y). Here the Cre recombinase expression, driven by CALRETININ

promoter, allows the presence of YFP specifically into ganglion cells (GCs) and amacrine cells (ACs) (Figure 31 A).

We first confirmed that YFP was specifically expressed into ganglion and amacrine cells by performing immunostaining for the neurofilament SMI32 and for CALRETININ, both markers of GCs and ACs. We found that YFP signal colocalized with these two markers both in total retinal flat mounts and in retinal sections specifically labeling ganglion and amacrine cells (Figure 31 B).
Thus we took advantage from this lineage tracing mouse model to evaluate the amount of GCs and ACs in the total retinal tissue 3weeks after the different treatments. Specifically we injected a group of Calr-Cre/R26Y mice with the chemokine SDF-1 intravitreally, and at the same time we induced NMDA damage in the right eyes, with the left eyes injected with PBS as controls (Figure 31 A; Group B). Another group of mice was damaged with NMDA in the right eyes and received intraperitoneally injection of the SDF-1 antagonist AMD3100 (Figure 31 A; Group C).

Retinal samples were analyzed by FACS 3wpi (Figure 31 C), and to evaluate the effects of the different treatment on retinal regeneration we quantified the percentage of YFP^+ ganglion and amacrine cells into the damaged right eyes in the different groups as compared with the control group, which was only damaged with NMDA (Figure 31 A; Group A). We did not found any significative change in the percentage of YFP^+ GCs and ACs counted at long term 3wpi by FACS analysis in the total retinas upon the different treatments as compared to control damaged retinas (NMDA) (Figure 31 D). In fact we noticed that the perturbation of the SDF-

1/CXCR4 signaling pathway, although increased the reprogrammed MG cells and the number of MG cells differentiating into GCs and ACs (Figure 11, 12, 13), globally, did not have effect on retinal regeneration (Figure 31 D).

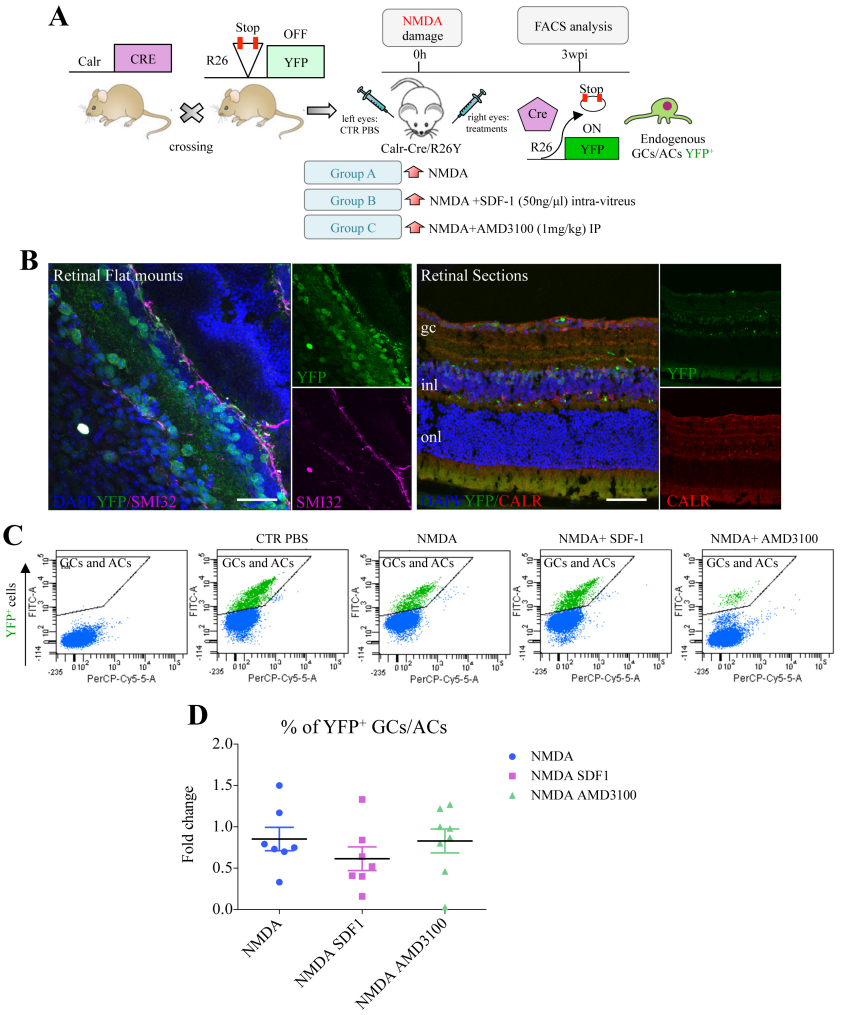


Figure 31. Modulation of the SDF-1/CXCR4 pathway into Calr-Cre/R26Y does not affect retinal regeneration (A) Experimental scheme of Calr-Cre/R26Y mice where Cre recombinase expression is driven by CALRETININ promoter allowing the YFP expression specifically into ganglion (GCs) and amacrine cells (ACs). The mice were divided into three different groups of treatment and sacrificed 3wpi:

damaged with NMDA into the right eyes, which is the control group (NMDA, group A), damaged with NMDA and intravitreally injected with the chemokine SDF-1 (NMDA+ SDF-1, group B), damaged with NMDA and intraperitoneally injected with the AMD3100 (NMDA+AMD3100, group C). Left contralateral eyes injected with PBS were used as controls. (B) Representative immunostaining on retinal flat mounts (left panel) and retinal sections (right panel) of Calr-Cre/R26Y mice showing colocalization of YFP signal (green) with ganglion-amacrine cell markers SMI32 (magenta) and CALR (red). Scale bar: 20 μm. (C) FACS profile of total retinas from Calr-Cre/R26Y mice showing the percentages of YFP^+ ganglion and amacrine cells evaluated on the total retinal population 3wpi. Retinas from Calr-Cre/R26Y mice differently treated (NMDA, NMDA+SDF-1, NMDA+AMD3100), untreated PBS injected control mice (CTR PBS) and from Calr-Cre (as negative control for the YFP) are shown in the plots. Dead cells DAPI positive were excluded from the analysis. (D) Quantification of the percentages of the YFP^+ ganglion and amacrine cells counted by FACS 3wpi represented as fold changes respects to the contralateral PBS injected eyes used as a control.

PART IV
DISCUSSION AND CONCLUSIONS

DISCUSSION

We have shown here that endogenous BMCs can be recruited into mouse retina in an NMDA-damage-dependent manner. After migration into the damaged retina, these BMCs can fuse with retinal cells, although with low efficiency, and mainly with MG cells. The hybrids generated can give rise to CALRETININ^{+} ganglion and amacrine cells 3 wpi upon NMDA damage.

Although neurons are still considered among the most immutable of cell types, in the last years, the possibility to reprogram these cells or to reprogram MG cells in vivo has become more promising, and this has promoted the idea that self-repair might be possible in nervous tissue (Niu et al., 2013; Torper et al., 2013).

Different reports have highlighted that MG cells are the more plastic cells in the retina, and they can undergo changes in gene expression and generate retinal progenitors. These, in turn, can proliferate and differentiate into functional retinal neurons, even in mammals.

Several in vitro studies have enhanced MG cell proliferation and promoted their transdifferentiation into other retinal cell types. Ascl1 has been reported to be the master transcription factor that is necessary for MG cell reprogramming and the generation of proliferating MG-derived multipotent retinal progenitors (Pollak et al., 2013; Ueki et al., 2015). Other transcription factors have been used to promote MG cell conversion into retinal neurons, such as Math5, which was shown to promote the differentiation of MG-derived retinal stem cells into retinal ganglion cells (Lust et al.,

2016; Song et al., 2015). In addition, mitogenic factors, such as insulin, EGF, Wnt3a, and also inhibition of TGFβ and Notch signaling, have been shown to increase the numbers of proliferating MG cells in vivo in the mammalian retina (Close et al., 2006; Ooto et al., 2004; Osakada et al., 2007; Takeda et al., 2008; Wan et al., 2008; Wan et al., 2007; Wohl and Reh, 2016).

Although the mechanisms that drive MG cell reprogramming and all the different steps have been extensively dissected out in zebrafish (Goldman, 2014; Powell et al., 2016; Wan et al., 2014; Wohl and Reh, 2016), it is still not known how MG cells change their identity and generate new retinal neurons in mammals.

It has already been reported that MG cells have neurogenic potential also in mammals, and specifically that MG-derived $BrdU^+$ progenitors can undergo proliferation, to thereby generate ganglion and amacrine cells in an NMDA damage mouse model, after stimulation of the EGF pathway (Karl et al., 2008). Moreover several reports have shown that MG-derived retinal progenitors but also mature differentiated neurons (both ganglion cells and photoreceptors) after transplantation into retinal disease mouse models may contribute to an improvement in retinal function (MacLaren et al., 2006; Pearson et al., 2012). However, one of the major problems after cell transplantation is the inefficiency of cell integration into the adult tissue due to inflammatory causes that makes stem cell therapy for retinal diseases a big challenge. Here, we have shown that even in the absence of signaling activation, $GFAP^+$ activated MG cells can undergo reprogramming by re-

expressing retinal stem cell markers, and can ultimately generate CALRETININ^{+} ganglion and amacrine cells passing through a proliferative step.

BMCs can be mobilized in peripheral blood and participate in the repair of several tissues and organs (Doyonnas et al., 2004; Johansson et al., 2008; Nygren et al., 2004). Many studies have shown that transplanted BM-derived cells can generate hybrids, which in turn differentiate into different lineages in vivo, such as myocytes (Ferrari et al., 1998), hepatocytes (Petersen et al., 1999), neurons (Mezey et al., 2000), and other cell types (Krause et al., 2001). Moreover, more recently, BM-derived cells were seen recruited in the retinas of diabetic mice and in murine hypoxic retinas (Boettcher et al., 2008; Chakravarthy et al., 2016; Lima e Silva et al., 2007). However, fusion of BM-recruited cells was not investigated, nor whether these cells contributed to regeneration of the retinal tissue.

We recently showed that HSPCs can fuse with retinal cells (Sanges et al., 2013). The pre-activation of the Wnt signaling pathway in the transplanted cells promoted cell-fusion-mediated reprogramming. During this process retinal cells were reprogrammed back to a pluripotent/ neural progenitor cell fate in vivo. The possibility that this process can also happen as a physiological response to damage in vivo after endogenous BMC mobilization might explain possible cell fate changes for BM-derived cells recruited into the retina. This process would also open new therapeutic strategies aimed at boosting and enhancing endogenous repair, by overcoming problems related to rejection of

transplanted cells, their engraftment, and their correct integration into the neural circuit.

We have shown here that endogenous hybrids formed between BMCs and MG cells can undergo partial reprogramming through the re-expression of progenitor genes. Surprisingly, we also found that Ascl-1 was up-regulated in the hybrids sorted 24 hpi, and that there was a tendency also to increase in the YFP^+ MG cells sorted from damaged retinas as compared to the control ones. Ascl-1 has been shown to have an important role in regenerative responses in lower vertebrates (Pollak et al., 2013). Ascl-1 was not seen to be up-regulated in the mouse retina upon damage previously. However, here we FACS sorted the $GFAP^+$ activated MG cells, where Ascl-1 expression was slightly increased in this specific population of cells, and highly up-regulated in YFP^+ MG-derived hybrids. It might well be that Ascl-1 activation, as well as that this general endogenous mobilization of BMCs occurs also after other kind of damage (i.e upon photoreceptor damage). This will be a matter for future investigation. Indeed, we recently showed that MG cell reprogramming occurs upon their fusion with transplanted HSPCs in photoreceptor degeneration mouse models (Sanges D., 2016).

Previous studies have reported that SDF-1/CXCR4 axis is not only involved in invasion and metastasis of malignant tumors (McIver et al., 2013; Mukherjee and Zhao, 2013), but also that it is critical in the migration of stem cells (Du et al., 2012; Theiss et al., 2011). The importance of SDF-1 in the homing of stem cells to damaged sites is suggested by the observations that SDF-1 is up-

regulated after several types of damage and under hypoxic conditions, including during myocardial ischemia (Yu et al., 2010), cerebral ischemia (Shen et al., 2007), and renal failure (Togel et al., 2005), and that this correlates with adult stem cell recruitment and tissue regeneration (Askari et al., 2003; Ceradini et al., 2004; Kollet et al., 2003; Yamaguchi et al., 2003). Moreover SDF-1 is secreted from vascular endothelial cells and reactive astrocytes in injured regions of the brain (Ohab et al., 2006; Thored et al., 2006) and it has been shown also to be up-regulated in a rat model of retinal ischemia-reperfusion injury (Lai et al., 2008), and after retinal pigmented epithelium damage (Li et al., 2006). Therefore, based on these observations, we hypothesized that also in the NMDA retinal damage model, SDF-1 could mediate recruitment of BMCs into the degenerated retina. Interestingly, there was increased recruitment of BMCs into the damaged retina when we injected the chemokine SDF-1 directly into the vitreous, and higher conversion of MG cells into ganglion and amacrine neurons. This was not due to an increase in cell fusion efficiency in the short term, as this was unchanged in our experimental setting. It is likely that as soon as the hybrids were formed, they were committed to be converted in newly generated ganglion and amacrine neurons.

On the contrary, block of CXCR4 by injection of AMD3100 led to decreased BMC recruitment and to fewer YFP^+ MG cells being converted into ganglion and amacrine neurons. Overall, through pharmacological modulation of the SDF-1/CXCR4 interaction, we showed that the ability of MG cells to undergo reprogramming and subsequent differentiation was strongly

dependent on the migration of endogenous BMCs into damaged retina. In fact the neurogenic ability of MG cells, activated following NMDA damage, to differentiate into new ganglion and amacrine cell types was severed impaired when BMC migration into damaged retina was blocked. However it would be useful to deeply compare the molecular profiles of de-differentiating MG cells, which undergo reprogramming with that of MG-derived hybrids to definitively prove that cell fusion is the only mechanism by which MG cells contribute to neurogenesis. In fact the increased recruitment of BM derived cells due to SDF-1 treatment could also promote other transdifferentiation mechanisms of BM cells into glia or neurons. Indeed we found many ds-RED^{+} BM cells (not expressing YFP and therefore unfused cells) when we analyzed damaged retinal flat mounts at long term in chimeric mice carrying ds-RED BM (GFAP-Cre/R26Y/BM$^{ds\text{-}RED}$ chimeric model Figure 30 E). These unfused BM cells once migrated in the retina changed their morphology due to the retinal microenvironment and showed a glial and neuronal–like shape suggesting a possible transdifferentiation mechanism.

It would be also interesting to investigate whether MG cells, undergoing reprogramming could give rise to other retinal cell types different from the ones affected by the damage in our model. In fact, recent findings suggest that MG cells acting as retinal stem cells are likely biased to move and finally to differentiate specifically into the cell types located in the retinal layer, which has been damaged (Powell et al., 2016).

In conclusion, we have identified here a new cell-fusion mediated mechanism according which MG cells can change their fate undergoing reprogramming and participating in retinal regeneration. Nevertheless when we checked weather the modulation of SDF-1/CXCR4 pathway could definitively help endogenous retinal regeneration or at least rescue the NMDA damage at the level of ganglion and amacrine neurons we did not find any increase in the total number of GCs and ACs (Figure 31). This could be due to the fact that the number of MG that proliferate, undergo reprogramming and differentiation into ganglion and amacrine cells is very little, thus this conversion is not sufficient to achieve a rescue of the retinal degeneration. Moreover it would be interesting also to investigate whether ganglion and amacrine cells generated from MG glia reprogrammed cells and from MG-derived hybrids are electrophysiologically functional and if the treatment with the chemokine can improve this functionality. Further studies will be also needed to fully identify the environmental signals released after NMDA damage that mediate BMC recruitment in order to boost this endogenous retinal regeneration potential and to set up an innovative therapeutic approach.

CONCLUSIONS

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- Müller glia cells undergo dedifferentiation and reprogramming following N-Methyl-D-aspartate damage into mouse retina

- Müller glia cells proliferate and are converted into amacrine and ganglion cells at long term after retinal damage

- SDF-1 is up-regulated into NMDA-damaged mouse retina and in Müller glia cells sorted from damaged retina

- SDF-1/CXCR4 signaling pathway controls endogenous bone-marrow cell migration into NMDA-damaged retina

- Bone-marrow cells recruited into damaged retina express hematopoietic stem cell markers

- SDF-1/CXCR4 pathway modulation affects Müller glia cell reprogramming and neurogenic ability

- SDF-1/CXCR4 signaling pathway modulation enhances/decreases bone-marrow cell mobilization into NMDA-damaged retina

- Bone-marrow cells recruited into damaged retina fuse with retinal neurons and mostly with Müller glia cell

- Müller glia-derived hybrids express neural and retinal progenitor markers, and differentiate into ganglion and amacrine cells at long term after damage

- Cell fusion is one of the major mechanisms by which Müller glia cells undergo reprogramming in mammals

- Perturbation of the SDF-1/CXCR4 pathway is not sufficient to induce endogenous retinal regeneration

MATHERIALS AND METHODS

MATHERIALS AND METHODS

Animal care and treatment

Animal Care and Treatments and all the procedures on the mice were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were maintained under a 12 hr light/ dark cycle, with access to food and water and according with the CEEA (Ethical Committee for Animal Experimentation) of the Government of Catalonia. We used the following transgenic mice: Vav-Cre (Stadtfeld and Graf, 2005) CAG-RFP (Long et al., 2005), CAG-ds-RED (Vintersten et al., 2004), GFAP-Cre (Zhuo et al., 2001), CALR-Cre (Taniguchi et al., 2011), R26Y (Srinivas et al., 2001). Male and female between 8-12 weeks were used for the whole study. We chose groups of minimum 3 mice that were randomly assigned to treatment groups. A general anaesthesia was induced when needed with an intraperitoneal (i.p.) injection of ketamine (70 mg/kg, Imagene®; Merial) and medetomidine (10 mg/kg, Domitor®; Pfizer Animal Health). To collect eye samples, mice were euthanized with CO2 at different time points.

Transgenic mice

GFAP-Cre/R26Y mice (Figure 1 A) are double transgenic mice used to trace Müller glia cells with the YFP reporter. They were generated by crossing the Rosa 26-LoxP-stop-LoxP-YFP strain (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J) with the GFAP-Cre strain where the Cre recombinase (Cre) expression is controlled by the Müller glia specific promoter GFAP.

Vav-Cre/R26Y mice (Figure 7 A) are double transgenic mice used to trace endogenous BM cells with the YFP reporter. They were generated by crossing the Rosa 26-LoxP-stop-LoxP-YFP strain (B6.129X1-Gt (ROSA) 26Sortm1(EYFP) Cos/J) with the Vav-Cre strain where the Cre recombinase (Cre) expression is controlled by the hematopoietic specific promoter Vav.

R26Y/BM$^{CRE-RFP}$ chimeric mice (Figure 14 A) were generated by replacing the BM of transgenic mice carrying the Rosa26-LoxP-stop-LoxP-YFP allele with the BM of donor mice expressing the Cre under the hematopoietic specific promoter VAV and expressing the RFP under CAG ubiquitous promoter.

GFAP-Cre/BM^{R26Y} chimeric mice (Figure 24) were generated by replacing the BM of GFAP-Cre mice, where the Cre expression is under GFAP promoter, with the BM of donor transgenic mice carrying the Rosa26-LoxP-stop-LoxP-YFP allele.

Calr-Cre/R26Y mice (Figure 31 A) are double transgenic mice used to trace ganglion and amacrine cells with the YFP reporter. They were generated by crossing the Rosa 26-LoxP-stop-LoxP-YFP strain (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J) with the CALR-Cre strain where the Cre expression is regulated by the ganglion-amacrine specific promoter CALRETININ (Calr).

Calr-Cre/R26Y/BM^{RFP} mice (Figure 26 A) were generated by replacing the BM of Calr-Cre/R26Y mice where the YFP expression is restricted to ganglion-amacrine cells with the BM of donor CAG-RFP mice where the RFP reporter gene is ubiquitously expressed.

GFAP-Cre/R26Y/BM$^{ds\text{-}RED}$ (Figure 30 A) were generated by replacing the BM of GFAP-Cre/R26Y mice where the YFP expression is restricted to Müller glia cells with the BM of donor CAG-ds-RED mice where the ds-RED reporter gene is ubiquitously expressed.

Sub-lethal irradiation and bone marrow (BM) transplantation

BM transplantation was carried out as previously reported (Fazel et al., 2006). Recipient mice were total body irradiated with 9 Gγ (double dose of 4,5 Gγ) six weeks before retinal damage and/or drugs treatment. The BM of 8-12 weeks old R26Y, GFAP-Cre or Calr-Cre/R26Y recipient mice was reconstituted with BM cells from the tibias and femurs of young Vav-CRE/RFP, R26Y or CAG-RFP transgenic mice, respectively. Total BM cells (obtained by gently flushing donor mice femurs with phosphate-buffered saline PBS) were counted and 0.1 ml of a cell suspension ($1x10^7$ cells) were intravenously injected into recipient mice 3-4 h after irradiation.

Retinal damage and drug treatment

Mice between 8-12 weeks of age were anaesthetized by an intraperitoneal injection of ketamine: medetomidine (70 mg/kg: 1.0 mg/kg). To induce retinal damage, mice were treated intravitreally with 2 l of NMDA (20 mmol, total 40 nmol; Sigma) (Timmers et al., 2001) and eye samples isolated 24 hours, 4 days or 3weeks after treatment. Control eyes were injected with PBS. Briefly, a 30-G

needle was used to carefully make a small incision at the upper temporal ora serrata. A 33-gauge needle coupled with Hamilton's 5 ml syringe was inserted into the incision to inject NMDA and any other drug or PBS into the vitreus, Upon injection, the needle was left in place for 10 seconds before being retracted to avoid reflux along the injection track.

To study the effect of the modulation of SDF-1-CXCR4 signaling pathway on BM recruitment, 1μl of SDF-1 (50ng/μl, Sigma) was intra-vitreally injected in recipient eyes soon after the NMDA treatment. To block BMCs migration, mice received daily intraperitoneal injections of the CXCR4 antagonist AMD3100 (1 mg/kg, Sigma A5602), starting the same day of the NMDA treatment.

FACS sorting of MG and hybrids for gene expression analysis

For FACS analysis, retinal samples were dissected from the eye balls, disaggregated in trypsin for 20-30 minutes at 37ºC. Retinal samples were then mechanical triturated, filtered, pelleted and resuspended in PBS with 2% FBS. A solution of 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) was also added to exclude dead cells from the analysis. The flow cytometry analysis was performed in a LSR For-tessa (Becton Dickinson) with FACSDiva (Becton Dickinson) software in order to assess the percentage of MG cells and RFP^+/ or hybrids. For gene expression analysis, a BD FACSAria II sorting machine (Becton Dickinson) was used to isolate the activated MG, RFP^+/YFP^+ hybrids and YFP^+ MG derived hybrids from the whole retinal samples. Depleted

retinas were also collected as control.

RNA extraction and real-time PCR

For the RNA extraction, retinal samples were dissociated from the eye balls and immersed into a Lysis Buffer (QIAGEN). Sorted samples were directly collected into Lysis Buffer. Total RNA was extracted using RNA Isolation Mini or Micro kits (QIAGEN), according to the manufacturer protocol. The eluted RNA was reverse-transcribed with SuperScript III (Invitrogen) and real-time qPCR reactions were performed using Platinum SYBR green qPCix-UDG (Invitrogen) in a LyghtCycler 480 (Roche) real-time PCR machine, according to the manufacturer recommendations. The oligos used are listed in Supplementary Table S1. The qPCR data were normalized to the expression of GAPDH. The results were the average of three-five independent experiments performed in triplicate. Relative mRNA levels were evaluated as the fold-changes with respect to the untreated or PBS injected control total retinas or undamaged YFP^+ MG sorted cells or to retinal samples depleted of sorted YFP^+ hybrid population.

Fixing, sectioning, immunohistochemistry

Eyes were enucleated and fixed by immersion in a 4% paraformaldehyde solution overnight at 4°C and the following day were embedded in paraffin. Serial transversal sections of 5µm of thickness were prepared and processed for immunofluorescence stainings. Briefly, eye sections were deparaffinated by treating with serial washing in Xilene 2', EtOH 100% 1', EtOH 95% 1', EtOH 90

1', EtOH 80% 1', EtOH 70% 1', EtOH 50% 1' and H_2O. Slices were placed in a plastic rack with an antigen retrieval buffer (NaCitrate 0.1M Triton X-100 0.1-0.2%) and boiled for 4' into a domestic microwave. After a wash with cold water, sections were blocked in NGS 10% for 30' and NGS 1% for 30'. Primary antibodies were incubated in PBS O/N at 4ºC.

The sections were then washed with PBS and incubated with secondary antibodies for 1h at RT. For retinal flat mount immunostaining the whole retinas were dissected and fixed with 4% PFA for 1 h. Retinas were then placed into a permeabilization solution (10% NGS, 0.2% Triton X-100 in PBS) and left 2-3 days into primary antibodies and 1 day into secondary antibodies after washing with PBS. The primary antibodies used were: rabbit anti-GLUTAMINE-SYNTHETASE GS (1:300, Sigma G-2781) mouse anti-PCNA (1:200, Sigma P8825), rabbit anti-PHOSPHO-HYSTONE H3 (Ser10) (1:200, Millipore 06-570), chicken anti-GFP (1:600, Abcam AB13970), rabbit anti-RECOVERIN (1:500, Millipore AB5585), rabbit anti-CALBINDIN D-28K (1:50, Sigma C7354), mouse anti-CALRETININ (1:200, Millipore MAB1568), mouse anti-SMI32 (1:200, Millipore), mouse anti-Nestin (1:200, Abcam AB6142), rabbit anti PKC (1:200, santa Cruz Biotechnology sc-208, Lot L2414). The secondary antibodies used were: anti-chicken Alexa Fluor 488 (1:1000; Molecular Probes, Invitrogen), anti-chicken Alexa Fluor 568 (1:1000; Molecular Probes, Invitrogen), anti-mouse Alexa Fluor 568 (1:1000; Molecular Probes A11031, Invitrogen), anti-rabbit Alexa Fluor 568 (1:1000; Molecular Probes A11036, Invitrogen), anti-mouse Alexa Fluor 633

(1:1000; Molecular Probes A21050, Invitrogen) and anti-rabbit Alexa Fluor 633 (1:1000; Molecular Probes A21070, Invitrogen). Nuclei were counterstained with DAPI (Vectashield, Vector Laboratories, 42 Burlingame, CA, USA).

Fluorescence microscope analysis and image acquisition were carried out with either an Axioplan microscope (Zeiss) or a Leica laser SP5 or SPE confocal microscopy systems.

Preparation of Flat-Mounted Retina and quantification of immunostainings

Confocal laser-scanning microscope (LEICA TCSSP5) was used to quantify immunopositive cells both in sections and whole retinal flat mounts. All quantifications of immunostainings were based on analysis of at least three sections per animal, from at least three animals. Serial transversal retinal sections were selected and used to quantify the stained area.

To quantify the number of YFP^+ MG differentiating into ganglion-amacrine cells on retinal flat mounts (prepared as previously described in Fixing, sectioning, immunohistochemistry paragraph), YFP^+ total MG cells and double $YFP^+/CALR^+$ cells were visualized in 10 random x20 fields of the whole retina and photographed with a confocal laser-scanning microscope (LEICA TCS SP5). Images were processed with the ImageJ software (US National Institutes of Health, Bethesda, Md., USA; http://rsb.info.nih. gov/ij/). The "transdifferentiation rate" was expressed as percentage of $YFP^+/CALR^+$ cells on the total YFP^+ MG cells counted in each

field. At least 3 different retinas were counted for each treatment group (Figure 1F,G Figure S1D, Figure 2E,F). To quantify the proliferating YFP^+ MG cells (Figure S1C), the number of either $phH3^+/YFP^+$ or $PCNA^+/YFP^+$ cells was evaluated by analyzing 3 serial retinal sections/eye from at least three different mice for each group and is expressed as number of proliferating cells/section. To quantify the number of YFP^+ /$Nestin^+$ cells after the different treatments (Figure S2D, E), double immunopositive cells were counted in at least 5 random fields from 2 different mouse retinas/each group of treatment. The percentage of $Nestin^+$ cells was expressed respect to total YFP^+ MG counted in each field.

Statistical Analysis

For statistical analysis, the data were expressed as means ± S.E.M., as pooled from at least three independent experiments. Statistical comparisons were examined using unpaired Student's t-test as indicated in the figure legends. All of the statistical tests and graphical presentations were performed using the Prism 5.0 software (GraphPad, San Diego, CA). A P value < 0.05 was considered significant.

Study approval

All of the procedures on mice were reviewed and approved by the Comité Ético de Experimentación Animal del Parc de Recerca Biomèdica de Barcelona, (Spain) and were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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ANNEX 1

SUPPLEMENTARY TABLE

List of the oligos used for RT-PCR analysis

OCT4	fw CGTGGAGACTTTGCAGCCTG	rv GCTTGGCAAACTGTTCTAGCTCCT
SOX2	fw GGCAGAGAAGAGAGTGTTTGC	rv TCTTCTTTCTCCCAGCCCTA
PAX6	fw CCACCCATGCCCAGCTT	rv AACTGACACTCCAGGTGAAATGAG
NESTIN	fw TGGAAGTGGCTACA	rv TCAGCTTGGGGTCAGG
SIX3	fw GTGGACGGCGACTCTGC	rv CAACTGGTTTAAGAACCGGC
CHX10	Fw ATCCGCAGAGCGTCCACT	rv CGGTCACTGGAGGAAACATC
MATH3	fw AGCTGACCCCGGGAAAGAGAATC	rv AGCCCGGTCTTCTCTCTTGCT
MATH5	fw TGGGGCCAGGACAAGAAGCTGT	rv ATGCGGGTGAGCGCGATGAT
PROX1	fw TGAATCCCCAAGGTTCTGAG	rv AAAGGCATCATGGCATCTTC
ASCL-1	fw GTTGGTCAACCTGGGTTTTG	rv CCTTGCTCATCTTCTTGTTGG
CYCLIND1	fw GAGATTGTGCCATCCATGC	rv CTCCTCTTCGCACTTCTGCT
GAPDH	fw GTATGACTCCACTCACGGCAAA	rv TTCCCATTCTCGGCCTTG

ANNEX 2

PUBLICATIONS

Endogenous mobilization of bone-marrow cells into murine retinas induces fusion-mediated reprogramming of Müller glia cells. Simonte G, Sanges D, Di Vicino U, and Maria Pia Cosma. J Clin Invest Insight, under revision

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