UNIVERSITAT DE BARCELONA

Setting up stem cell therapy for human retina: from organotypic cultures to cell fusion understanding

Sergi Àngel Bonilla Pons

Doctoral Thesis UB 2020

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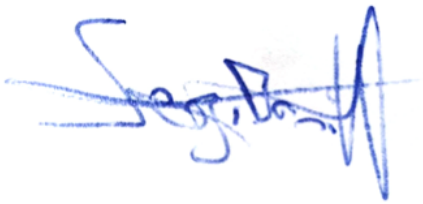


ABSTRACT

Visual impairments and different retinopathies have been silently increasing in the modern society and they become a medical hurdle in need to be addressed. Müller glial cells (MGCs), in lower vertebrates, show regenerative potential for the retina, however in the mammals this capacity is lost. However, it has been demonstrated in mice that fusion between MGCs and adult stem cells result in the formation of hybrids which detain some potential to regenerate the lost retinal neurons.

In the present work we hypothesize that also into the human retina cell fusion between adult stem cells and retinae resident MGCs can enhance a process resulting into retina rescue and regeneration. In this thesis we first demonstrated that cell fusion can occur between human MGCs (hMGCs) and adult stem cells in the human retinal organotypic cultures. We then isolated the hMGCs and we fused them with either hematopoietic stem cells or with human mesenchymal stem cells adipose-derived (hMSC AD) *in culture*. We found that the activation of the Wnt/β-catenin pathway can induce reprogramming of the hybrids, which, in turn, can then undergo differentiation and acquire some electrophysiological response. Finally, we set up a microinjection system to transplant the hybrids into human retinal organoids to finally study the hybrid differentiation *in vivo*.

With the strategy here exposed, we believe that it might represent the first step towards a potential regenerative therapy for human retinae via cell fusion and reprogramming. These observations might be the basis to develop an innovative approach to address in the long run the unmet medical need of the visual impairments.

PREFACE

By statistic, one out of two readers will reposition its spectacles before starting this read and, unluckily, 1 in 30 Europeans will not even have the possibility to read it because of blindness. With it, is quite obvious that visual impairments had become an unmet medical need hoping to be handled. The mentioned miss conditions most times are due to a retinal damage or degeneration resulting in an aberrant sense of sight.

Interestingly, retina regeneration is known to hold some regenerative potential. For instance, after different insults, zebrafish or xenopus are able to regenerate its retina through the regenerative potential of a special cell type of the retinae tissue, the MGCs. Unfortunately, this regenerative skill is lost through evolution or, at least, remain dormant. It is in this scenario were the scientific community started to work for a human treatment of the visual impairments, pointing the stem cell therapy as a putative therapy for retinal regeneration. Interestingly, once injected in the retina, stem cells can have different ways of action, rescuing the resident cells or replacing them. In addition, one might think that the stem cell treatment after injury could awake the retinae regenerative dormancy. Therefore, we hypothesize that, in addition, adult stem cells can awake the Müller glia dormant regenerative skills through a process of cell fusion, reprogramming its nucleus towards a more pluripotent state, the reprogrammed MGC can finally differentiate for instance, in neural ganglion cells like, in turn able to repopulate the damage cells of the tissue and acquire their characteristics.

Hopefully, the topic addressed in the present thesis, will shed some light in the retinae regeneration field and will open a new therapy approach for the

use of the adult stem cells, with the final aim of giving hope to those people suffering from visual impairments.

CONTENT

ABBREVIATIONS

AMD	Age-related macular degeneration
APC	Adenomatosis polyposis Coli
Ascl1	Achaete-Scute Family BHLH Transcription Factor 1
BMDC	Bone marrow derived cell
BMC	Bone marrow cell
BM-MSC	Bone marrow-derived mesenchymal stem cell
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
BUTC	Banc d'ulls I tractaments de ceguesa
FBS	Fetal Bovine Serum
BIO	6-bromoindirubin-30-oxime
CKIα	Casein kinase I α
CMZ	Ciliary margin zone
CNS	Central nervous system
Dkk1	Dickkopf-related protein 1
ESC	Embryonic Stem Cell
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPI	Glucose-6-phosphate isomerase
GSK3	Glycogen synthase kinase 3
hESC	Human embryonic stem cell
hMGC	Human Müller Glial cell
hMSC AD	Human Mesenchymal stem cell adipose derived
hPSC	Human pluripotent stem cell
HSPC	Hematopoietic stem progenitor cell
hHSPC CD34+	Human hematopoietic stem cells $CD34^+$
IGF-1	Insulin grow factor 1
INL	Inner nuclear layer
iPSC	Induce pluripotent stem cell
MGC	Müller Glial Cell
MHC	Major histocompatibility complex
NMDA	N-methyl-D-aspartic acid
MNU	N-methyl-*N*-Nitroso Urea

NOD	Non-obese diabetic
MSC	Mesenchymal stem cell
MSVI	Moderate and severe visual impairments
ONL	Outer nuclear layer
Rd10	Retinal degeneration 10
RP	Retinitis pigmentosa
RPC	Retinal progenitor cell
RPE	Retinal pigmented epithelium
RFP	Red fluorescent protein
qRT-PCR	Quantitative real time PCR
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell-derived factor 1
SPACA6	Sperm acrosome associated 6
TGF-β	Tumor growth factor β
Wnt1	Wnt family member 1
Wnt3a	Wnt family member 3a
YFP	Yellow fluorescence protein

INTRODUCTION

Section 1:

Retina architecture and related unmet medical needs; a deep lock

1.1.- Retina architecture

The retina is the organ in charge of allow sight. Physically located in the back part of the eye globe, has the ability to respond to light stimuli triggering the initial steps in the process of vision that will be finally decoded and interpreted in specific centres of the brain.

From the initial thoughts of Charles Darwin, who famously doubted about his postulated random selection when thinking about the eye complexity, extensive studies have been done to comprehend it, both its architecture and physiology. Retina complexity starts from its development process, known as retinogenesis. At pre-natal level, the anterior neural plate forms the eye field, containing the progenitors that will give rise to all the neural-derived cells and structures, developing the multilayered neural retina among them. Worth to be mentioned, the retina happens to be the more accessible part of the central nervous system (CNS) (Heavner and Pevny 2012; Quinn and Wijnholds 2019). Strikingly, a single common original cell type of retinal progenitor cells (RPC) give rise to all the different retinal cell types, thanks to an initial fasten cell division that leads to a posterior differentiation. (Cepko et al. 1996; Hoon et al. 2014; Jin and Xiang 2017; Marquardt and Gruss 2002).

By the end of the retinogenesis, the adult retina is architecturally structured in three different main layers: the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL). Within the same

layer, cells are connected building the so-called mosaic structure. Synaptic connections are established among layers (Hoon et al. 2014) (Figure 1a and b).

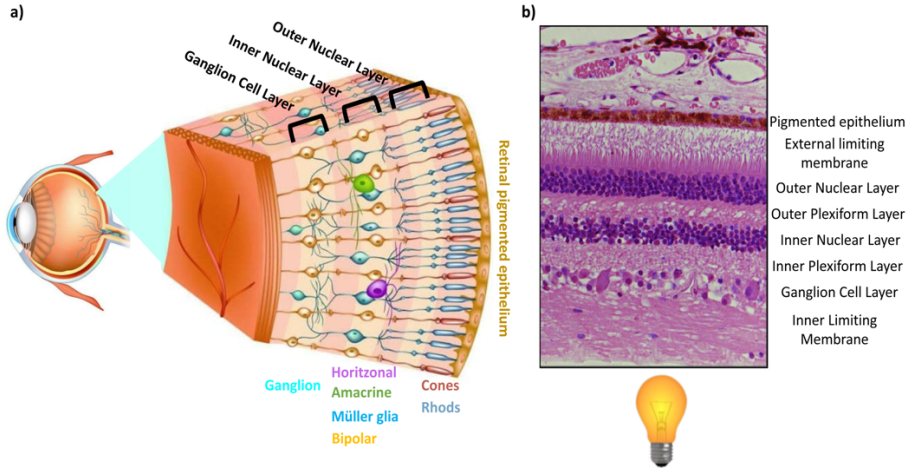


Figure 1. Architecture of the mammalian retina. a) Illustration of the retina placed in the back part of the eye globe, and a zoom in of a longitudinal section of the retina, showing the three-main layers and the predominant cells. Each cell type is written down with the cell representative colour (modified from *Bril Pharma*). **b)** Hematoxylin-Eosin staining of a human retina, exposing the real appearance of the retina and the mosaic architecture of the main layers (modified from: Li L. et al., 2012_ Photoreceptor sensory cilium and associated disorders).

Following the light stimuli, GCL is the first layer faced. As its name suggests, retinal ganglion cells populate this first mosaic (Figure 1a). Even do they are the first one to appear in the initial wave of neurodifferentiation, these cells are the less abundant population (Lukowski et al. 2019). A second differentiation wave of synaptogenesis give rise to the interneurons: horizontal, bipolar and amacrine cells are physically located in the INL, sharing it with the cell body of the MGCs. Due to their special characteristics, MGCs will be further deeply discussed. Briefly, MGCs are a special cell type that, being the last one to differentiate, are the principal glial type in the retina. As glial cells, their primary role is the retinal homeostasis maintenance. With their soma spread across all the three

different layers, they also play a dominant role in maintaining the tissue architecture and providing trophic support for the neurons (Hoon et al. 2014; Heavner and Pevny 2012; Hoshino et al. 2017). The last layer is the ONL, hosting the photoreceptors: rods and cones. While rods have exquisite sensitivity to light, cones compensate their relative low sensitivity with a faster phototransduction. Due to their specific properties, cones respond for the dim-light vision and rods are bright-light responders, being in charge of the high acuity colour vision. Their specific physiology accounting for their functionality, results in a specific distribution of the ONL mosaic. While cones are more scattered across the retina, rods are more concentrated in the central part, the macula, being highly packed in a rode-less slight retina depression known as fovea, which is the responsible for the high acuity and colour vision (Hoon et al. 2014).

Altogether, they participate in initial steps of the fragile yet captivating process of sight sense. Briefly summarized, light passes through all the three main layers and hit the rod and cone photoreceptors. Those cells depolarize and release neuro-transmitters to the bipolar and horizontal cells connected with ganglion cells at the inner retina. This signal received into ganglion cells is then refined by amacrine cells before generate an output signal that is transmitted by the ganglion axons through the optic nerve that will ultimately connect with the brain centres of vision (Lee, Martin, and Grunert 2010).

1.2.- Retina Organoids: Retina in a dish

Neurogenesis has never been easy to study. However, in the last years, new bio-molecular and sequencing techniques allowed these studies to be done in deep, deciphering the differentiation cascade of cells populating the

retina and their intrinsic genetic mechanism (Hoshino et al. 2017; Mao et al. 2019; Sridhar et al. 2020). Despite these encouraging findings, the full process was far from being understood since its morphogenesis had been a prohibit dare to face. Nevertheless, revealing work, published in 2011 by *Eiraku M.* et al., was the initial shot recapitulating the retinogenesis in a plate, being able to grow a *retina in a plate* starting from mouse embryonic stem cells (ESC). ESCs grow until they form a three-dimensional and stratified culture (neurosphere), able to first evaginate to later invaginate, resembling the optic cup structure, showing an apical-basal polarity. This mature structure is known as retina organoid (Eiraku et al. 2011).

These retina organoids were able to reproduce the mammalian optic-cup morphogenesis program and cell stratification in layers following an intrinsic self-organization and a stepped differentiation protocol (Eiraku and Sasai 2011).

After mouse retina organoids, great efforts were done to translate this procedure onto human samples. No more than one year was needed when *Nakano T.* et al. were able to reproduce the work from mouse ESC using human ESC (hESC) achieving larger organoids that grew multi-layered, showing rods and cones (Nakano et al. 2012). Not only hESC but also human induce pluripotent stem cells (iPSC) could give rise to fully development of properly layered human retina organoids. Even more interestingly, retina organoids derived from human iPSC, show the first hints of photosensitivity (Hallam et al. 2018; Zhong et al. 2014). Interestingly, not only the organoids could mimic the retinogenesis at morphological level. Posterior studies comparing human foetal retina, human pluripotent stem cells (hPSC) retinal organoids and long-term retinal cultures at single cell RNA sequencing level demonstrated that hPSC, besides slight differences in particular cell types, are able to recapitulate the cellular composition at different levels of differentiation of

foetal retina, being only differences at inner retinal lamination attributable to culture hPSC organoid conditions (Sridhar et al. 2020).

1.3.- Retina Failure: retinopathies and retina malfunction

As every single organ in our body, retina could be damage and/or degenerate. Retinopathies are known as a heterogeneous group of retina malfunctions that inevitably lead to a development of visual disabilities and, in the worst of the cases, blindness. To properly face this health problem and target it, should be first properly understood. A study published in 2017 estimates that in 2020, 237'1 million people will suffer from Moderate and Severe Visual impairments (MSVI) and among them, 38'5 million will be blind (Flaxman et al. 2017). Interestingly, the cases are not worldwide homogenously distributed, being in Asia the major number of cases of MSVI. There is also an age and gender stratification, being more prevalent in elderly and female population, respectively (Figure 2.a). Even more alarming is the fact that the majority of visual impairments could have been prevented or early addressed, pointing it as a huge unmet medical need (Figure 2.b) (Bourne et al. 2017).

Age-related macular degeneration (AMD) emerges as the first cause of visual impairment in the developed countries, followed by glaucoma and a large and heterogeneous group of hereditary disorders (Quartilho et al. 2016). AMD is a multifactorial disorder. From the genetic side, there are more than 50 genes described that could increase the susceptibility to suffer it. Concretely, the complement pathway, the *age–related maculopathy susceptibility 2* and the *TIMP metallopeptidase inhibitor 3* genes are the main

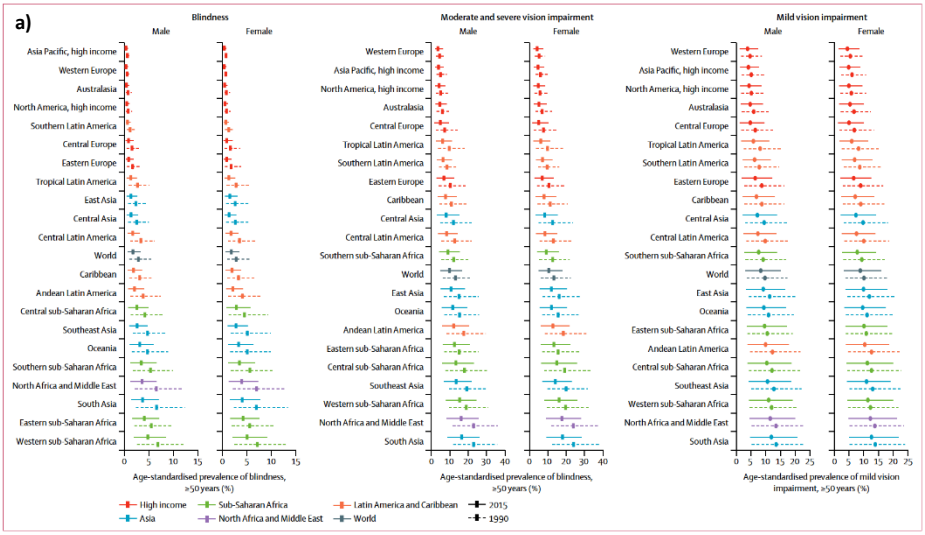
genetic alterations implied, ending in a macular degeneration, that cause a progressive loss of central vision. In addition, there are also non-genetic factors associated to AMD could increase the risk, such as smoking and low dietary intake of antioxidants (Bellezza 2018; Mitchell et al. 2018).

Glaucoma mainly affects ganglion cells, that suffer from a progressive degeneration that could result in changes on the optic nerve head. Like AMD, many genes are related to glaucoma susceptibility and had been identify through genome-wide scans. Besides its difficult to study, it is believed that an interaction is established between the affected genes and the transforming growth factor β, that regulates cell growth and survival. Thus, ganglion cells could have its surveillance compromised, degenerating and resulting in cell death, that promotes an intraocular pressure rise, ending in glaucoma disease (Weinreb, Aung, and Medeiros 2014).

In this devastating ranking, the heterogeneous group of hereditary disorders appears in the third position. The retinitis pigmentosa (RP) is, among all this group, the more prevalent accounting with 1 in 4,000 for a total of 1 million affected people. RP is developed by over 100 genes and its inheritance can be autosomal-dominant, autosomal-recessive or X-linked (Daiger, Bowne, and Sullivan 2007; Hartong, Berson, and Dryja 2006; Sohocki et al. 2001). Affected individuals suffer a progressive symptomatology, starting from a night vision lose in adolescence that progress into a side vision loss in the adulthood ending in a central vision lose later due to the progressive rods and cones photoreceptor cells death. Biochemically, all the cascade involved in light transduction and photoreceptor-cell polarization is affected. Generally speaking, the mutated genes affect the rods, leading to an impaired physiology (in example: an excessive proportion of cGMP-gated cation channels in the plasma

membrane) that will end with the rod's death. Interestingly, the subsequent cones death seems to be more associated with a neighbouring crosstalk yet to be properly elucidated (Hartong, Berson, and Dryja 2006).

In addition, it is estimated that by 2050 the blind people will rise up to 114'6 million people while more than 580 million will suffer from MSVI, representing also a public health concern (Figure 2.c) (Bastawrous and Suni 2020). Taken altogether, this astonishing estimation and the actual knowledge of the retinopathies point them as a clear unmet medical to be tackled in order to find an effective treatment to delay the vision loss or, ideally, a cure. With this, huge efforts are being driven to gain insight knowledge on the mechanisms behind the retinopathies. Furthermore, more interventional approach that can potentially treat or cure them are being optimized. So far, different approach had been tested as gene therapy, drug cocktails, stem-cell or grown tissues transplant or more technological approach as implanted neuro-devices (Bennett et al. 1996; Biswal et al. 2015; Lund et al. 2006; MacLaren and Pearson 2007; Mitrousis et al. 2020; Rolling 2004).



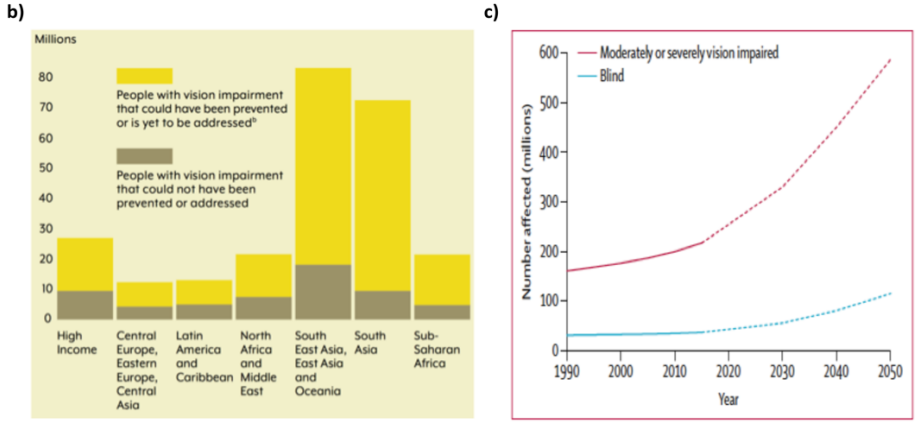


Figure 2. Visual impairments worldwide impact. a) Global trends and projection of the visual impairment and blindness in millions of people, from 1990-2050 (from Bourne et al., 2017). **b)** Millions of people visually impaired distributed by region and, among the total number per region, cases that could be prevented and could not (modified from world report on vision executive summary (who)). **c)** Future perspective on case rising of blindness and visual impairment until 2050 (from (Bastawrous and Suni 2020)).

Section 2

Regenerative medicine: awakening the regenerative potential of mammalian retina

2.1.- Regeneration

The idea of regeneration has been always as fascinating as controversial. A full healing and self-repair after injury, both functional and structural, has been always looked like mid-term between a tremendous loss and a dreamed goal from the species that cannot regenerate. Many questions can arise from the topic: what really regeneration is? why are some organisms able to regenerate and others do not? is it an adaptive gain or an evolutionary loss?

Regeneration can be understood as an evolutionary gain. This will imply large mechanisms involved, maybe new genes and a continued generational inheritance. Even possible, the most believed trend points regeneration as an intrinsic property of all the organisms that had been evolutionary lost or selectively silenced on those that are not able to regenerate (any more). Interestingly, this understanding of the regeneration also points it as a possible mechanism that can be awaken for those organisms non-regenerative, in example, the humans (Maden 2018; Slack 2017).

To arrive this far, first steps were done trying to understand the regeneration itself, being proposed in 1901 an initial classification by two models: (1) The reconstruction of a lost organ as a result of a regenerative

growth or (2) the growth of the remaining tissue without proliferation, passing through a process of dedifferentiation. These models had been classically named as (1) epimorphic and (2) morphallactic, respectively (Agata, Saito, and Nakajima 2007; Morgan 1901). Planarias had been a trustworthy model for epimorphic regeneration due to its adult stem cell population is capable to proliferate and further differentiate into an adult pluripotent stem cell known as neoblast. Additionally, its regeneration had been described as a multifunctional process involving a plethora of genes regulating not only regeneration but also tissue homeostasis (Reddien et al. 2005). On the other side, the Hydra polyp has been a good model for morphollactic regeneration with cells paused in G2 phase able to differentiate into head or foot cells (Vogg, Galliot, and Tsiairis 2019).

Additionally, further research in models with higher levels of complexity gave new insight in the regeneration process. Accounting not only for the organ structure but also for the cell level a new classification has been made. Five different types of regeneration models were defined according to its complexity: (1) cellular regeneration, (2) tissue regeneration, (3) organ regeneration, (4) structural regeneration and (5) whole body regeneration (Slack 2017).

Among these complex models, cold-blood vertebrates show impressive regenerative skills. Newts are a classical regeneration model, being able to regenerate entire limbs from the so called blastema, a regenerative structure able to recruit *in tissue* surrounding adult stem cells expressing pluripotency factors that will proliferate and later differentiate (Maki et al. 2009). Also, zebrafish possess astonishing regenerative capacities. It is known that they can regenerate their heart after 20% of ventricle amputation and not only this, since they can do it without leaving collagen-rich scars and repopulate

the heart with an early proliferative response of the cardiomyocytes in the surrounding non-damaged tissue (Schnabel et al. 2011).

As above discussed, in mammals this regenerative capacity seems to be progressively being lost or, at least, blocked. Also, the new understanding of the regeneration process opens room for awakening the mammalian regenerative potential. So, where does evolution place mammalian regeneration?

2.1.1.- Endogenous regeneration in mammals, where are we?

Large debates had been conducted to understand which is the mammalian regenerative potential, chasing the ambitious dream of human regeneration. Again, this topic is far from having a simple answer, but we might find different responses for different organs, pointing to the possibility of a selective retention of the mentioned regenerative capacity (Muneoka et al. 2008).

Liver regeneration could be the first example to emerge when thinking about mammal regeneration. Interestingly, the most famous example of mammalian regeneration could not totally merge the parameters of regeneration, even though the tissue is able to recover its functionality and structure. In mouse models, after partial hepatectomy, the liver recovery is mostly performed through a compensatory hyperplasia of the surrounding quiescent hepatocytes that go through a few rounds of replication (Fausto, Campbell, and Riehle 2006). Interestingly, after acute liver failure, was also early demonstrated a regenerative process involving the repopulation of the

tissue by the differentiation of progenitor cells (Dabeva and Shafritz 1993; Lemire, Shiojiri, and Fausto 1991).

Other organ that has shown their regenerative characteristics is the heart. It is known that cardiomyocytes hold some degree of renewal during both aging and when damaged. Pharmacologically, cardiomyocyte cell cycle activity could be enhanced, leading to a cardiomyocyte proliferation (in a "regeneration" approach similar to the liver). Additionally, this proliferation is accompanied by scar shrinkage and mechanical function improvement after infarct (Bersell et al. 2009; Laflamme and Murry 2011). Compellingly, a brilliant cell mapping study showed the cardiomyocyte progenitors contribution in heart regeneration. A transgenic mouse expressing green fluorescent protein (GFP) in the adult cardiomyocyte upon tamoxifen induction was the gold standard to differentiate between renewal from exiting cardiomyocytes or regeneration from resident cardiomyocyte progenitors. With this system, they found an overall GFP decrease after infarction, not only in the infarct area but also, in a lower proportion, in the surrounding tissue; pointing to the participation of progenitor cells in the regenerative process and discarding the adult cardiomyocyte contribution. However, not significant changes in GFP were observed through aging; highlighting that the mention progenitors participate only from the regenerative heart process (Hsieh et al. 2007; Laflamme and Murry 2011).

Among all mammal regeneration, the finger-tip regeneration found in mice and humans could be the more impressive and "classical" yet the "rarest" one, since is the one following the blastema paradigm mentioned in 2.1.- Regeneration chapter. Interestingly, it has been shown that is not only dependent in one cell-fate progenitor, but there are different populations of pluripotent cells following different dynamics to regenerate the finger-

tip in their own linage (Johnson, Masias, and Lehoczky 2020; Lehoczky, Robert, and Tabin 2011).

These few cases exposed exemplify the existence of adult stem cell population that can sustain different regeneration extents in their host organ. Interestingly, regeneration is present in diverse organs, independently of their complexity or function. In truth, even in the highly complex CNS, and specifically in the retina, regenerative features had been shown.

2.1.2.- Endogenous retina regeneration, a path to follow for mammals

As a general trend, retina regeneration in mammals is seen as dormant ability but yet possible to awake. On the other side, non-mammal species had shown extraordinary retina regenerative skills to learn from (Figure 3a). Amphibians, fish and birds possess a specific stem cell niche in the anterior margin of the retina, the ciliary margin zone (CMZ), able to repopulate the retina through the differentiation of RPC. Even though this area also exists in mammals and, more specifically, in humans, it does not play a significant role. Alternatively, cold blood vertebrates can dedifferentiate resident retinal cells to generate RPC that would further differentiate in specific cell types of the retina (Karl and Reh 2010).

Urodelian amphibians are the only ones able to regenerate the full retina, even in the adulthood by transdifferentiating retinal pigmented epithelium cells (RPE) and differentiating into RPC, being slightly supported by the resident progenitor cells in the CMZ (Araki 2007). Zebrafish can also regenerate to a large extend their retinae but starting from the MGCs that are able to re-enter the cell cycle (Nagashima, Barthel, and Raymond 2013).

Lately, also in chicks some extend of retina regeneration have been proved by MGCs (Karl and Reh 2010).

Mammals have also a regenerative potential in the retina, but poorer. It is known the existence of a retinal adult stem cells niche, aroused from the retinogenesis, in rodents but also in humans (Fischer, Bosse, and El-Hodiri 2013; Kuwahara et al. 2015; Tropepe et al. 2000). Due to its regenerative potential, in human those cells could be isolated from cadaveric donations, from where the neural stem progenitor marker Nestin^{+} cells had been isolated and shown capabilities to grown neuro-spheres (Mayer et al. 2005). Also, foetal retinal cells are able to be isolated from deceased foetus and kept in culture for regenerative purposes (Yang et al. 2002). Besides it, as it happens in zebrafish and chicks, the dormant regenerative potential of the mammalian retina relies in the MGCs. Their study and understanding are highlighted as the key to the mammalian retina regenerative potential (Figure 3b).

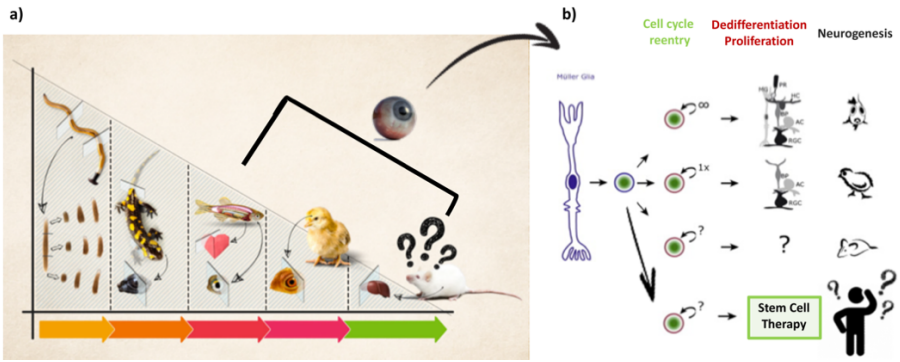


Figure 3. Regenerative potential dormancy across the evolution. The disadvantaged situation of humans in retina regeneration. a) From the left side part with Platyhelminthes, able to regenerate its entire body, to the right side one of the spectrum with mammals, with light regenerative capacity as the liver recovery after hepatectomy, it has been shown a regenerative loss or dormancy, enclose into the evolutionary process (modified from (Pesaresi, Bonilla-Pons, and Cosma 2018)). **b)** In the specific case of the retina, MGC are the ones leading the regenerative process. In zebrafish, they are able to re-enter the cell cycle an unlimited number of cycles and drive the neurogenesis. Its regeneration potential its loss, and able to happen just once in chicken and some initial hints are being shown for mouse. However, the regenerative human potential of retina is starting to be explored through a stem cell therapy enhancing (modified from (Karl and Reh 2010)).

2.1.2.1.-Müller glial cells: key player in mammalian retina regeneration

Interesting cell tracing studies have shown that Pax6+ is a neural precursor marker in MGCs. These studies pointed MGS regenerative potential and clear participation on photoreceptor regeneration after injury (Bernardos et al. 2007; Joly et al. 2011; Thummel et al. 2008) . Cell tracing experiments in transgenic zebrafish GFP labelled under Glial fibrillary acidic protein (GFAP) promoter, have shown colocalization of GFP and zpr1 cone marker 5 days after light photoreceptor damage, accompanied by a GFP decrease in the INL, highlighting the Müller glia involvement in photoreceptor regeneration (Bernardos et al. 2007; Goldman et al. 2001). Also, in chickens, after injury, bromodeoxyuridine (BrdU) labelled cells re-enter the cell cycle, express retinal progenitor markers, such as Pax6 and Chx10, and migrate from the INL. These cells are able to differentiate in different adult cell types within the following 10 days after injury (Fischer and Reh 2001) In regenerative retina, MGC appear to be a key player through evolution already in the regenerative models. Interestingly, even with the regenerative loss of the mammal retina, MGCs can still play a relevant role on the retina regeneration awakening.

As previously discussed, (in 1.1.- Retina architecture chapter) MGCs are the perfect example of multitasking. Being the retinal homeostasis and tissue architecture scaffold the main functions, this cell type is also known to support the neurons by releasing trophic factors, recycle neurotransmitters and keep the ionic balance of the extracellular space. Additionally, it has been recently found that actively participate in the visual cycle in a dual fashion: from one side they help to recycle the retinal chromophore for photo-detection by acting as cone phagocytes and, from the other side, they

act as optical fibbers guiding light to photoreceptors (Goldman 2014). On top of it, as seen, MGCs are the retinal cell type holding the regenerative potential through evolution. Indeed, it has been demonstrated in mice the high similarity by transcriptional overlap between MGCs and mitotic RPC. As example, in the specific genes expressed in MGCs, early genomic analysis already shown at one-week post-natal retinas a 68% of mitotic enriched genes and only 14% of photoreceptor-specific genes. Additionally, two-thirds of MGC enrich genes showed enriched expression in RPC (Blackshaw et al. 2004). First experiments conducted with rats could identify the BrdU+ MGCs in the INL cell cycle re-entry after N-methyl-D-aspartic acid (NMDA) induced damage and more interestingly, how those cells migrate to the ONL and suggest a further differentiation into bipolar cells and photoreceptors (Ooto et al. 2004). These results were lately confirmed in mice models. After NMDA injury, MGCs cell cycle re-entrance was proved by BrdU colocalization with the specific MGCs nuclear markers Sox2 or Sox9. Also, was noticed that after injury, MGCs go through a process of dedifferentiation, over-expressing a set of genes found in developing retina as *Notch1*, *FoxN4* and *Pea3*. This, together with the Pax6 expression in BrdU+ cells highlighted the dedifferentiation process of the MGCs and its re-entry in the cell cycle as a response to damage. Even more interestingly, they could identify the differentiation of the MGCs into amacrine cells by the expression of amacrine specific-cell markers such as Calretinin, NeuN, Prox1, and GAD67-GFP in a genetic mouse model (Karl et al. 2008). It is important to highlight the role of injury to skip the cell cycle arrest and awake the regenerative capacities, implying a tight regulation of the MGCs to keep their normal functions and not become potential malignant cells. In addition, it is also important the extent of damage. After an insult, the retina can undergo in a process known as gliosis to preserve the tissue from further damage and keep it structure. In

brief, the gliosis is a scarification process in which MGCs undergo morphological, biochemical and physiological changes; accompanied by a release of a plethora of neurotrophic factors. An exaggerate loss of the MGCs functions could then contribute to the tissue malfunction while trying to rescue it, disabling the MGC potential in the regenerative process (Bringmann et al. 2009; Goldman 2014). It is also notorious that to trigger the regeneration after injury, MGCs should be able to be first reprogrammed to a more naïve state to be able to further differentiate. With this, a second check point should be overcome for the MGCs to act as regenerating cells. In fact, in vitro experiments have demonstrated the potential of the MGCs to differentiate in different adult retinal cells linage (Jayaram et al. 2014; Ramachandran, Zhao, and Goldman 2011; Singhal et al. 2012)

To sum up, the MGCs regenerative potential awakening happen to be firmly regulated and only triggered after damage. Despite it, the inner MGCs could be aroused by targeting them. Great efforts had been done in understanding the biochemical process driving the sense of injury, reprogramming and posterior differentiation and how can them be targeted. However, it is still challenging and a deeper understanding and *in vivo* studies should be done. Additionally, different approaches should be considered, in order to reprogram the MGCs for regeneration purposes. Is in this lateral thinking approach where the stem cells field and the translational medicine could help in the fight for retinal regeneration, envisioning a potential stem cell-based treatment for ameliorating visual impairments. Focusing the research aim in a potential benefit for the patients, we can take advantages of the stem cells for a better MGC awakening, assisting in, for example, cell fusion mediated reprogramming and posterior hybrid cell differentiation.

Section 3

Stem cells: a perfect trigger for regeneration

3.1.- Stem cell healing properties; the famed stem cell therapy

For a while now, stem cell therapy had emerged as a brilliant promise for tissue regeneration. Unequivocally, what it is aimed with the stem cell therapy is the repair of a damage tissue through a stem cell intervention.

Interestingly, the idea of the stem cell therapy mimics the own body regenerative skills but, trying to burst its inner potential. For instance, a basic classification could be done based on the activity of the stem cells on solid organs. From one side, the vast majority of organs are formed by long-lived cells with a low turn-over but, upon damage, those cells are able to react and restore the tissue after a short and fast cycle of proliferation. This is the well-known case of the liver (Michalopoulos 2017; Raven and Forbes 2018).

On the other hand, there are organs with a high turn-over, as the epidermis, able to drive a faster regeneration. It is also worthy to notice that are the more exposed organs the ones that possess a higher turnover and cell replacement also, because from a physiological point of view the elimination of discarded cells appear to be much easier (Post and Clevers 2019).

Under those specific circumstances of reaction against different insults, stem cells take a new role, not just as a precursor cells but as a healing unit.

3.1.1.- Stem cells as a healing unit

Starting from scratch, a stem cell is defined by two main characteristics: being able to self-renew and possess the ability to recreate all the differentiated cells of a healthy tissue (Weissman 2000); NHI stem cell information., 2020). Once they divide, the daughter cells could keep as a stem cell or give rise to different progenitors with a restricted linage in the tissue that will end up in the cellular rescue of the damaged organ (Clayton et al. 2007; Post and Clevers 2019). Under the premise of bursting the regeneration of a damaged area, stem cell therapy could act in two ways: via cell replacement or via cell rescue and paracrine effect. And, on the top of it, these activities can be complementary.

Must be also highlighted the possibility of *in vitro* stem cell culture to be further used as a healing unit. In fact, in 1975 was firstly described the possibility of culture human epidermal cells on mouse cell feeder layers (Rheinwald and Green 1975). These cultured cells were rapidly implemented for the treatment of third-degree burns, deriving a culture of stem cells from the unaffected skin areas that were lately transplanted in the affected skin surface ('Grafting of burns with cultured epithelium prepared from autologous epidermal cells' 1981).

Additionally, the possibility of culture the stem cells also opens the window for a previous differentiation into the desired cell phenotype to rescue into the tissue before transplantation. For instance, in the retinal context, challenging advances are still being done. For instance, the combination of notch signaling inhibitor, DAPT and retinoic acid differentiated

photoreceptors from mouse ESC-derived organoids. These organoids were isolated and cultured as a replacement cell source to be subretinically injected into an adult mice. After 3 weeks, ESC-derived organoids expressed both photoreceptor markers S OPSIN and M OPSIN pointing the retina differentiation potential. Additionally, these cells also showed an appropriate cell polarization in the host retina when injected in the end-stage retinal degeneration *Aipl*$^{-/-}$ mouse model; indicating a clear potential of these cells as a suitable replacement for the lost photoreceptors (Kruczek et al. 2017). Similar results had been shown from hESC, differentiating them under Bone morphogenic protein (BMP) inhibitor and insulin grow factor 1 (IGF-1). The mentioned differentiated cells were lately injected in immuno-depressed mice showing a proper integration on the retinal layers and even a functional rescue on *CRX*$^{-/-}$ mice, lacking the photoreceptors, showing light stimuli response (Lamba, Gust, and Reh 2009).

This just highlight the tremendous potential of the stem cells as a healing unit, since these cells could be easily accessible (as would be discussed in following lines), they are easy to culture and, if necessary, they can differentiate *in vitro*. In addition, having the cells of the own patient as a stem cell source avoid potential immune rejection (de Kleer et al. 2006; Michallet et al. 2000). Unluckily, this hopeful news also come with a dark side since different concerns must be taken when using stem cells. The administration path towards the damage tissue, after a proper encounter, the integration within it and the final polarization of the cells are already challenges in on-going research (Calonge et al. 2019; Freitag et al. 2019; Harris et al. 2018; Jin and Xiang 2017; Mead et al. 2015) . The pluripotent potential of the stem cells also entails an uncontrolled stem cell frown, leading to a chromosomal reorganization and a possible risk of teratoma formation (Atsumi et al. 1982; Ogle, Cascalho, and Platt 2005).

Taken all together, further steps must be taken to standardize the stem cell use, as well as protocols and procedures for a safety management (Tucker et al. 2011).

3.2.- Stem cell flavors: different stem cell sources for retinal repair and pathway perturbation for an enhanced reprogramming

Recapitulating, different sources of stem cells are available to tackle the tissue regeneration goal, starting from ESCs that could be further differentiate till adult stem cells more prone to a specific cell fate.

3.2.1.- Pluripotent stem cells

As mentioned above, the beauty of using pluripotent stem cells (PSC) is its unleashed differentiation potential. Interestingly, we can distinguish between two different sources of PSC, depending on their origin, embryonic or adult stem cells and induced pluripotent stem cells, being both of them suitable for tissue regeneration (Figure 4).

3.2.1.1.-Embryonic stem cells and Induced pluripotent stem cells (iPSC)

First hints of ESC were noticed as early as 1954, when LeRoy Stevens did the first definition of ESC without even knowing about it, since he was studying the teratoma formation (Evans 2011; Stevens and Little 1954).

Later in the 1981 Gail R. Martin established the first ESC line by isolating it from the inner cell mass of a mouse early blastocyst (Martin 1981). With this discovery, without even know it, Gail R. Martin opened a hopeful gate for the stem cell field in regenerative medicine.

Was not until 2006 when the next breakthrough milestone was achieved on the stem cell field. At that time, was already known that the cell reprogramming could be achieved through different process: somatic cell nuclear transfer, exposition to pluripotent cell extracts, iPSC generation and, the topic of the present thesis, cell-cell fusion. By then, Shinya Yamanaka hypothesized that some factors pivotal for ESC maintenance should be the responsible for pluripotency induction. After a screening of 24 driving factors candidates he ended up identifying the, since then known, four core Yamanaka factors (Oct3/4, Sox2, c-Myc and KLf4) that are able to reprogram mouse adult fibroblast cells into iPSC. Furthermore, the new iPSC showed the ability to produce teratomas after a subcutaneous injection into nude mice and also contributed to embryo development when iPSCs were injected into blastocyst (Takahashi and Yamanaka 2006). First results with human samples did not have to wait for long, since just one year later, in 2007, some researchers generate the human iPSC (Takahashi et al. 2007; Yu et al. 2007). The possibility of generating iPSCs from the own patient cells resolve one of the most important hiccups on stem cell regeneration field, the immune response.

Must be highlighted in both cases, (ESC and iPSC) their tremendous differentiation potential and their high proliferative activity. These features make these cells almost the gold candidate for tissue regeneration. In fact, a large plethora of protocols had been developed achieving different cell types such as insulin secreting pancreatic-island like structure or towards a

neural fate (Keller 2005; Lumelsky et al. 2001; Zhang et al. 2001). More interestingly, these differentiated cells can be later used for further transplants aiming the tissue recovery.

In fact, focusing on our interest, the retinopathies and its treatment, both mouse and humans differentiate cells had been used as a first brick towards the retina regeneration (Bibel et al. 2004; Hirami et al. 2009). Concretely, when a mouse retina is damaged by the injection of N-methyl-D-aspartic acid (NMDA) affecting the ganglion cells, ESC are capable to differentiated into eye-like structures, rescuing anatomically and functionally the mouse retina, and posteriorly, differentiating into ganglion-like cells (Aoki et al. 2008)). Similar results had been obtained with human ESC, driving a RPE differentiation through nicotinamide cultures. This RPE differentiated cells exhibiting typical markers and morphology of RPE cells, had been proved to show RPE65 markers of adult RPE cells as well as tight junctions and participate to the functional rescue in a rat model of RPE dysfunction (Idelson et al. 2009).

A part from the unlimited sources of healing unit cells that ESC and iPSC could represent, it is easy to imagine how far their differentiation skills can reach when unleashed and pushed, being able to reach even proto-organs or organoids (as previously discussed in former chapters). It is with the organoids when even more possibilities emerge, since it is not only that we can extract stem cells and adult stem cells from it but, we can use them as a model for study the development or even as a first model for proof of concept demonstration with human samples.

Unhappily, this PSC also hide a dark side. They are expensive to isolate and maintain in their pluripotent state but also the differentiation protocols are complex and costly. Even more important and as previously seen, their own

pluripotency makes them risky, since there is an inescapable risk of teratoma formation.

3.2.2.- Adult stem cells and stem cell niche

Being the stem cell a well renowned surname, the given name of all the stem cell family members seem to be easily forgettable. In fact, it is easy to conceive a stem cell as a PSC, in other words, as a cell able to give rise to all three different cell types of the body: endoderm, ectoderm and mesoderm. Interestingly, there is a higher and a lower potency step. Totipotent cells are the ones able to become all the embryonic cell types of the body plus the extra-embryonic ones. In the third potency position, are the multipotent stem cells. The multipotent stem cells had decided their fate and moved one differentiation step forward. In this case, the cells are still flexible enough to differentiate into different cell types, but restricted to the certain tissue that they will end up building (Evans and Kaufman 1981; Jiang et al. 2002).

Due to its late or advance stage of potency, these cells could also be identified as adult stem cells from the different tissues. In other words, we can have access to a reservoir of adult stem cells on their stem cell niche of a determined tissue, to potentially heal it or be used as a more specific healing unit, having leashed the uncontrollable differentiation potential of the pluripotent stem cells.

3.2.2.1.- Retinal progenitor cells

Being in the retinal niche, retinal progenitor cells (RPC) stand out as the first adult stem cells sources. In fact, the retina of cold blood vertebrate can undergo unlimited neurogenesis by incorporating neurons derived from the progenitors located in the CMZ (Miyake and Araki 2014; Wan and Goldman 2016). Similarly, it is in the CMZ where we can found a pool of RPC in mammal retina although its regenerative potential remains dormant after the embryo neurogenesis (Belanger, Robert, and Cayouette 2017). Besides it, there are rising evidences of the awaking of the mentioned dormant regenerative potential. Actually, GFP engineered cells had shown a lateral movement from the CMZ towards the central retina during the embryo retinogenesis, showing these cells a polarization in differentiation markers: they express Cyclin D2 in the CMZ and posteriorly, cells lose it in favor of the Brn3a neural expression. In addition, a lack of mitosis in CMZ cells was found in Cyclin D2 deficient mice (Marcucci et al. 2016). Moreover, RPC population is more complex, since the progenitor population $Msx1^{+}$ able to generate neural and non-neural cell fates, was also identified within the RPC (Belanger, Robert, and Cayouette 2017). In addition, when RPC were transplanted in human and mouse retina, they showed a slight amelioration of the visual skills (Humayun et al. 2000; Pearson et al. 2012).

Besides its indisputable skills and their retinal fate, RPC collection represents a huge obstacle. As seen, they are more active in foetal states, and it is only possible to collect a low number of cells, making them an interesting source for retina regeneration but not worthy yet.

3.2.2.2.- Hematopoietic stem progenitor cells

In the regenerative context, hematopoietic stem progenitor cells (HSPC) appeared as an unexpected player that rapidly took a huge prominence. First milestones achieved from the HSPC was their hematopoietic repopulating capability. This was shown by the protective and hematopoietic repopulation effect of a normal HSPC of normal mice bone marrow cells injected into lethally irradiated mouse (Ford et al. 1956; Eaves 2015; Jacobson et al. 1951). This regenerative potential of the hematopoietic system makes possible their use as a replacement therapy for multiple hematopoietic malignancies such leukemia like and anemia illnesses (Grewal et al. 2004; Hsu et al. 2005). Additionally, bone marrow derived cells had been shown to have an impressive plasticity, being able to differentiate into different cell fates organ specific (Helbling et al. 2019; Lee et al. 2015; Meyer et al. 2016; Shi et al. 2019). This, together with the mobility and access of the hematopoietic system to the entire body organs, opened a new promising gate into organ regeneration. Starting from the easiest example, bone marrow-derived cells had been shown to contribute to skin regeneration. The differentiated cells adopt a collagen fate expressing collagen type I and III that contributes to the collagen matrix. Functionally then, those differentiated cells are able to support the local cutaneous cells (Fathke et al. 2004). Moreover, HSPC mobilize into the liver differentiating into hepatocytes that reduce fibrosis and help the hepatocyte repopulation after a partial hepatectomy (Pedone et al. 2017; Tsolaki et al. 2014). They are also capable to migrate into the kidney and participate of the kidney repair after acute injury (Benigni, Morigi, and Remuzzi 2010; De Broe 2005) or even contribute on heart regeneration by cell fusion or by stimulating endogenous c-kit^{+} resident cells helping to cardiomyocyte

repopulation and heart regeneration (Loffredo et al. 2011; Nygren et al. 2004).

In this regenerative scenario, we can also find examples in the neural field and, more specifically, aiming for a retinal rescue by two different approaches, either by trans-differentiation or cell fusion. It is such the interest that it raised after some promising results in animal models that initial steps are being done in human samples (Alvarez-Dolado et al. 2003; Lu et al. 2010; Mathivanan et al. 2015; Quintana-Bustamante et al. 2006; Sanges et al. 2013). Indeed, from the trans-differentiation approach first trials are being run with human volunteers (Mehat et al. 2018; Oner et al. 2016; Schwartz et al. 2015).

Even the huge opportunities that they offer, some drawbacks should be taken in consideration as its collection, the heterogeneity of the working population and the culture conditions for preserving their pluripotency.

3.2.2.3.- Mesenchymal stem cells

Mesenchymal stem cells (MSC) are an extensive and heterogeneous stem cell population that could be found in almost every adult tissue. Despite it, initially they were classified according to their main differentiation potential into adipocytes, chondrocytes and osteocytes. Subsequent works demonstrate their hided potential since, under the appropriate environment, MSCs are able to adopt different fates, even neural and retinal fate (George, Hamblin, and Abrahamse 2019; Mahmoudian-Sani et al. 2019; Nitobe et al. 2019; Shiota et al. 2007). Due to its heterogeneity and variety of niches, even today it is difficult to find a clear definition on what a MSC really is. Looking for a consensus, basic features were defined. To

know, besides its osteogenic, adipogenic and chondrogenic capability, these cells must respond to fibroblast-like shape and show a spontaneous adherent capacity into plastic substrates. In addition, a minimum set of markers must be present, to know, CD73, CD90 and 105. In the contrary, CD34, CD45, CD14, CD11b, CD19, CD79α or HLA-DR must be absent. Furthermore, MSC must also self-renew (Dominici et al. 2006; Mushahary et al. 2018).

The beauty of this stem cell source also relies in their supplementary skills; non-existent in other stem cell sources, mainly related with its paracrine effect. These paracrine effects could be classified in six main groups: immunomodulation, chemoattraction, anti-apoptosis, support of growth and differentiation of stem and progenitor cells, antiscarring and angiogenesis (Samsonraj et al. 2017). Within them, the huge range of available trophic factors incorporate growth factors, morphogens, chemokines, cytokines, extracellular vesicles (as exosomes) and glycosaminoglycans (Harrell et al. 2019; Samsonraj et al. 2017).

Focalizing our attention in the field of retinopathies, a rising number of reports are being published delving on the neuroprotection and tissue regeneration effects of different sources of MSC (Mead et al. 2020; Usategui-Martin et al. 2020). Additionally, it has been showed that not only MSC have a neuroprotective effect but also that their enriched medium is able to stimulate the proliferation and neural differentiation towards ganglion and photoreceptor cells of the RPC (Jin et al. 2019; Zhang, Zhang, et al. 2017).

Furthermore, it is known the immune evasive condition (first named as immune privileged) of the MSC. In fact, MSC are able to suppress T lymphocyte activation and proliferation as well as interfere in the antigen presentation through impairing the dendritic cell differentiation; playing its

immune evasive skills independently of the major histocompatibility complex (MHC), expressing low levels of MHC-I and a complete lack of expression of MHC-II (Ankrum, Ong, and Karp 2014; Jacobs et al. 2013; Le Blanc et al. 2003; Nauta and Fibbe 2007).

This far, MSCs are postulated as a powerful stem cell source to drive tissue regeneration.

3.2.2.3.1.- Mesenchymal stem cell sources and their role in regenerative medicine

Happily, as mentioned in the beginning, MSC could be found in virtually all organs, being firstly discovered in bone marrow where they act as hematopoietic–supporting stromal cell (Dexter, Allen, and Lajtha 1977; Mendez-Ferrer et al. 2010). Their value in regeneration had been proved by its colony formation capacity, their differentiation potential into the four main linages (osteogenic, adipogenic, chondrogenic and myogenic) and also for the regenerative outcome seen in animal models and, even more specifically, in the neural tissue (Carr et al. 2019; Chen et al. 2019; Kfoury and Scadden 2015; Lin, Lin, et al. 2018). Therefore, MSC were the first alternative to use as a stem cell source. Unfortunately, important drawbacks were found. The donation is a very invasive procedure and, even if the isolation is very efficient, they show a decrease in proliferation and differentiation potential upon age and culture (Bara et al. 2014; Dahl et al. 2008; Zaim et al. 2012). Additionally, it has been proved that their efficiency is highly dependent on the extraction method. Despite it, and the need of new research and optimization of its collection, isolation and culture, bone

marrow-derived mesenchymal stem cells (BM-MSC) are still an interesting source for tissue regeneration to be considered.

The rising interest on the MSC in the regenerative field rapidly found other alternatives to the BM-MSC, being the MSC adipose derived (MSC AD) the best alternatives. But, which are the reasons that placed the MCS AD in the top preference? Compared with other mesenchymal sources, since they are easily accessible and have an extraordinary extraction efficiency (the initial material could be fat extracted from liposuctions and they have an isolation rate of 100%). Once extracted, they have a high colony frequency and are easy to culture and expand (Kern et al. 2006; Taghi et al. 2012). Additionally, as mentioned before, it is well-known the heterogeneity of its population but, interestingly, MSC AD are well conserved and do not show abrupt differences between different donations, showing more than 96% similarity measured by the expression markers CD105, CD90 and CD73 (Liu et al. 2019). Indeed, looking at their secretome, MSC AD showed higher secretion of neurotrophic factors that can contribute to tissue regeneration (Kern et al. 2006; Mitchell et al. 2019). To end with, MSC AD showed neuroprotection against different insults to the CNS as it could be in diabetic neuropathy or in Parkinson disease (Berg et al. 2015; Oses et al. 2017). This neuroprotective effect has been also seen in the retinal context. Concretely, it has been observed in models of ocular glaucoma and in hMSC culture protecting retinal ganglion cells (Mead et al. 2020; Mead et al. 2016).

Taken together, and with the first hints being explored currently, BM-MSC and, on top of it, MSC AD appear as an extraordinary opportunity to drive stem cell therapies for retina regeneration.

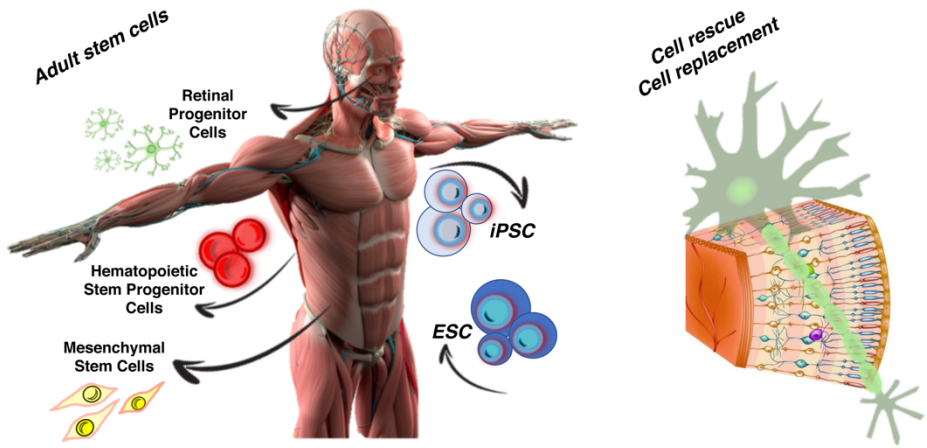


Figure 4. Stem cell sources for stem cell therapy towards retinal regeneration. Different sources of stem cells at different stages, from the ESC derived from the epiblast, to different sources of adult stem cells from different niches (as retinal progenitor cells, hematopoietic stem progenitor cells and MSC) and the iPSC derived from adult somatic cells from the same patient. Each source carries advantages and disadvantages but, all of them can lead a process of cell rescue or cell replacement within the retinal environment (retina image modified from *Bril Pharma)*.

3.3.- Stem cells contacting the human retina: the eye immune-privilege

Adult stem cells could be an interesting source to address different visual medical needs. Additionally, MGCs are placed within the human retinae, which have an evolutionary background lost across evolution as an "adult stem cell like" able to drive neurogenesis in cold blood vertebrates (Bernardos et al. 2007; Langhe et al. 2017; Nagashima, Barthel, and Raymond 2013). Besides it, in more evolved species, as humans, the MGCs had conserved some neural progenitor attributes. Taken this together, one could hypothesize that this regenerative potential hold by MGCs remain dormant waiting for some appropriate awakening stimuli as could be different sources of stem cells.

However, there is still the problem of physically putting in contact the adult stem cells with the human retina and how the retina itself could react against a transplant. To start with, it is important to highlight the eye globe exposure, being the easiest part to access of the CNS. Of course, it is also important the administration route and which is the retina layer target. For instance, one could think as an intravitreal injection if GCL is the target or in a sub-retinic injection if, by contrary, the idea is to contact with the photoreceptors (Barber et al. 2013; Hambright et al. 2012; Stout and Francis 2011).

However, we cannot exclude the fact that the retina could recruit their own inner adult stem cells to drive a process of retina regeneration. In fact, in our lab we recently published, in a mouse model that after NMDA damage, the retina is able to recruit bone marrow stem cells by stromal cell-derived factor chemokine (SDF-1) mediation through the chemokine CXCR4 pathway and drive a process of reprogramming and cell-fusion mediated regeneration (Pesaresi et al. 2018). Even this is possible, with the possibility of culture adult stem cells, and with the different routes of administration and easy access to the retinal tissue, it seems feasible to put in contact the adult stem cells and the retina, existing the only doubt on how the retina will react. Interestingly, the eye globe exhibits once more one its astonishing hided skills: the eye immune privilege, potentially giving an unlimited surveillance time for allogenic grafts. The way it works is believed to be not by the immune cell exclusion but by an active mechanism of antigen response suppression. These mechanisms go from developing blood-ocular barriers (blood-aqueous barrier and blood-retinal barrier) to an absence of MHC class II presenting cells and lack of expression of MHC-I on tissue resident cells, T cell suppression activity and the presence of immune modulators (tumor growth factor β, TGF-β as example) (Benhar, London, and Schwartz 2012; Forrester and Xu 2012).

The immune privilege of the eye, together with the regenerative potential of the MGCs and the adult stem cells as an awakening healing unit, make of them a worthy and exciting alternative to address the medical need of the visual impairments.

Section 4:

Cell fusion and reprograming: what follows after fusion between stem cells and retinal Müller glial cells

4.1.- Cell fusion as starting and natural process

As initial though, one may picture cell fusion as an ambiguous, rare and minority event when, actually, it is the starting of life. As profound this affirmation might sound, a quick thought would make the reader realize that the zygote starts between the fusion of a sperm and an ovule. Once realized, first question to irrupt might be: "How this cell fusion occurs?".

4.1.1.- How cell fusion occurs? A complex answer to an "easy" question

Despite it seems a straightforward answer, the different biological process implies different mechanisms and players, making the cell fusion a process that had been largely forgotten and deserve to be extensively studied.

Fortunately, there is some common features between all the cell fusion process. Briefly, two main events must happen in case of cell fusion: (1) the hydrostatic repulsion between the two cell membranes must be broken and (2) cell membranes must fuse and generate a fuse pore. First of all, the

hydrostatic, electrostatic and steric forces must be broken in a really energetic demanding process. Once overcome, the fusion itself can start by contacting the membranes, reaching the intermediate state of hemifusion where the two membranes bend and form a bilayer (diaphragm) that later, will lead to the formation of the fusion pore that will expand culminating in the cytoplasm merging in the syncytium formation. All this process is driven by a set of driver proteins commonly known as fusogens. They bring the membranes together, to later destruct the lipid bilayer reducing the energy barrier and opening the fusion pore (Martens and McMahon 2008; Ogle, Cascalho, and Platt 2005; Petrany and Millay 2019).

Cell-to-cell fusion give rise to a hybrid cell named either heterokaryon, a cell with an expanded cytoplasm and two nuclei, or a synkaryon, a cell with a unique tetraploid nucleus in the expanded cytoplasm (Figure 5). Therefore, in the heterokaryon model one could study the influence and interaction between the two nuclei while not in the synkaryon, where the nuclei contribution cannot be distinguished. Initial hints on the hybrids transition are being done. In fact, former work in our laboratory demonstrate the heterokaryon-to-synkaryon transition as a process depending on cell division. However, the gap information in these transition and its influence in the reprogramming and cell identity changes remain to be finally resolved (Dornen et al. 2020). What was already demonstrated in the late 1970s was the possibility to revert back a differentiated cell to a more pluripotent state or transdifferentiate it into a different cell fate by nuclear reprogramming (Miller and Ruddle 1977). This discovery, was further confirmed by different studies, showing nuclear reprogramming derived from the heterokaryon to synkaryon transition (Duncan et al. 2009; Matsumoto et al. 2020; Palermo et al. 2009).

With these compiling evidences and the implication of the merged cytoplasm, cell fusion-derived hybrids open a new field in tissue regeneration, challenging the mechanisms leading to cell plasticity and cell fate identity changes.

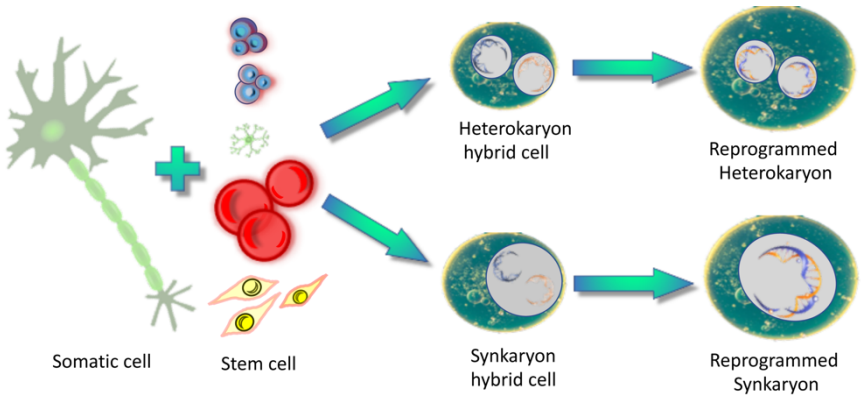


Figure 5. Cell fusion process between a somatic and stem cell. Fusion process between a somatic cell (fully differentiated cell) and stem cells. The fusion product can be a Heterokaryon hybrid cell (two nucleus, from the two fusion partners, sharing a fused cytoplasm generated by membrane merging) or a Synkaryon (one fused nuclei in a fused cytoplasm generated by membrane merging). Both types of new-born hybrids are able to be reprogrammed into a pluripotent state with a further differentiation potential.

4.1.2.- Starting from the beginning, the ovule fertilization

Starting from the very beginning, as mentioned in the initial lines, the zygote formation arises from the fusion between the sperm and the ovule, initiating the embryo development. Due to its indubitable interest, fertilization is a fusion process under constant study, with few bona fide fusogenic candidates. Among them, it is well stablished the formation of the adhesive protein complex between Juno (also known as Izumo1R) in the egg and Izumo1 in the sperm as the initiation point of the heterogenic cell fusion (Inoue et al. 2005). *IZUMO1*$^{-/-}$ mouse strand generate sterile male

mouse. Interestingly not any further deficiency in other fertilization proteins (as follow: ADAM2, CD147 and sp56) was observed, highlighting the essentiality of *IZUMO1* protein in the fertilization. To make sure that the deficiency was due to the fusion interruption, sperm from *IZUMO*$^{-/-}$ male mouse was injected into egg female mouse, developing functional embryos, pointing the fusion previous to fertilization as the disrupted mechanism (Inoue et al. 2005). From the egg side, *JUNO* was also shown to be essential for fertilization. Blocking it with monoclonal antibodies shows no fertilization *in vitro* while a female mouse *Folr4*$^{-/-}$ (folate receptor4 or *JUNO*$^{-/-}$) was unable to deliver newborn litters even if it showed a normal mating behavior, directly pointing that *JUNO* was essential for for the mice fertility (Bianchi et al. 2014; Hernandez and Podbilewicz 2017). Together with the Izumo complex, CD9 has been also pointed of capital relevance. CD9 was found exposed in the egg surface playing a role in the sperm-egg fusion. In fact, two independent laboratories found out that *CD9*$^{-/-}$ knock out female mice have a severe fertility mismatch, since the sperm is able to penetrate the zona pellucida but not properly fuse with the egg membrane, obstacle that could be overcome by a direct sperm intracytoplasmic injection pointing, again, on deficiencies in the cell fusion process (Le Naour et al. 2000; Miyado et al. 2000).

Further studies have been done to identify other fusogen proteins involved in the sperm-egg fusion as for example studies regarding the sperm acrosome associated 6 (SPACA6), that it is being hypothesized to cooperatively work with *IZUMO1* (Barbaux et al. 2020; Noda et al. 2020).

There are other developmental processes involving cell fusion and specific fusogenic protein in a tight regulated process. Among them, there is the syncytiotropholast establishment (that follows the fertilization ending in the placentation), the macrophages-derived giant cells formation in the immune

system and, structurally, the skeletal muscle and bone development (Black et al. 2004; Helming, Winter, and Gordon 2009; Oren-Suissa and Podbilewicz 2007; Sens et al. 2010).

4.2.- Cell fusion as a re-start. Regeneration of a damaged tissue

As seen in previous chapters (see section 2: "Regenerative medicine: awakening the regenerative potential of mammalian retina") the regeneration process in the human being is a process reserved for essential organs (as it could be the liver or the heart, showing a very discreet regenerative response) or to highly exposed organs to external insults (as it could be the skin). Therefore, we believe in the regeneration process not as something lost across evolution but a dormant mechanism that could be awaken after injury.

Reviewing the importance of cell fusion in the life development itself, regeneration appears as a biological process that could be enhanced through cell fusion with stem cells (see section 3: "Stem cells: a perfect trigger for regeneration"). It was in 1995 the first time that Gibson *et al.* pointed cell fusion as a putative mechanism for tissue regeneration when they investigate a putative therapy for Duchenne muscular dystrophy. Concretely, they transplanted dermal or muscle fibroblast into irradiated muscle of mdx mice (deficit on dystrophin) showning a mix phenotype of degenerative foci and regenerative fibers. More interestingly, they found both glucose-6-phosphate isomerase (GPI) isoenzyme types (GPI-1a from host and GPI-1b from donors or vice-versa) in the same cell, indicating the cell fusion (Gibson et al. 1995).

Despite the importance of these finding, was not until 2002 when two groups independently demonstrated heterotypic cell fusion *in vitro*. From one side, Terada *et al.* mixed a mouse bone marrow-derived cells (BMDC) population (GFP^+ and puromycin resistance) with mouse ESCs obtaining after 3 weeks GFP^+ puromycin-resistance ES like-cells. Additionally, when these cells were pushed toward differentiation, they were able to form beating cardiomyocytes and neural like morphology as well as were able to form teratomas when injected into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Terada et al. 2002).

Independently, Ying Q-L *et al.*, showed pretty similar results. In this case they demonstrated spontaneous fusion between ESC (hygromycin resistance) and neural precursor cells (GFP labelled) having resultant hybrids with enlarged nucleus and GFP-hygromycin resistance. Also, they demonstrate diverse differentiation potential forming embryo bodies and neural-like cells (Ying et al. 2002). It must be also mentioned that in both cases, the generated hybrids exhibit the expression of pluripotent genes, as *Oct-4* and *UTF1*, that were lost in favor of the differentiation phenotype. Therefore, these results from these two studies highlighted the *in vitro* cell fusion events as well as the potency and plasticity of the newly generated hybrids.

These results encourage an in deep research on cell fusion towards regeneration, creating a potential therapy through stem cells to address tissue regeneration. In fact, in the following years new methods were developed to identify fusion events, as the Cre-loxP system which consist in a reporter flanked by LoxP cassettes that can only be removed upon a cell counterpart fusion expressing Cre recombinase. With this system, Purkinje neurons, cardiomyocytes and hepatocytes were identified as cell fusion partners for BMDC (Alvarez-Dolado et al. 2003). Next step was to

prove it in *in vivo* mouse models. Due to their tissue architecture and tissue development, liver, muscle and heart were the first to be targeted, showing all of them *in vivo* cell fusion events (Alvarez-Dolado et al. 2003). For instance, the bone marrow cells fused with host hepatocytes could regenerate the liver and correct fumarylacetoacetate hydrolase deficiency (Wang et al. 2003); in the case of skeletal muscle, within the same tissue it is know the importance of the satellite cells and its fusion with injured myofibers, an *"in situ"* process that had been characterized highlighting the importance of the partnership between membrane micropeptide Myomixer in the satellite cells and the Myomaker transmembrane protein in the myoblast (Bi et al. 2018). This fusion process has also been seen with regenerative benefits between BMDC and myoblast (Wang et al. 2018); (Kasprzycka et al. 2019). Similar fusion events between BMDC and cardiomyocytes had been identified in the heart context that, even at low level, are able to drive regeneration in an infarcted myocardium (Nygren et al. 2004). What it is also important in all the regeneration cell fusion mediated events, is the pivotal role of the damage/inflammatory environment to favor cell fusion, pointing it as a putative *in vivo* regenerative mechanism, awaking an inner dormant potential of the damaged tissues.

Compelling evidences are being collected to spot the cell fusion as a regenerative process in different tissues and, luckily, retina is not an exception and will be addressed in following lines.

4.2.1.- Cell fusion-mediated reprogramming. One step backward, two forward

As seen, cell fusion-derived hybrids are able to participate in tissue regeneration but, how these newly born hybrids behave and how they

acquire the potential to differentiate again into adult tissue linage cells is something that remains unclear. The principle of cell fusion stem cell-mediated relies in the fact that an adult stem cell (either tissue resident, chemoattracted or exogenously delivered) will fused with a host tissue somatic cell. The resultant heterokaryon will be then reprogrammed to a more pluripotent state, to further differentiate into a cell linage of a specific tissue (Do and Scholer 2010; Fang et al. 2020; Lluis and Cosma 2010). Therefore, it is difficult to isolate the reprogramming itself (as it could be from the classical OSKM Yamanaka factors, previously discussed) but, we should understand the reprogramming as a cell fusion-mediated process in the *in vivo* regenerative context (Pesaresi, Bonilla-Pons, and Cosma 2018).

4.2.1.1.- Wnt/β-catenin pathway mediation on reprogramming, the needed burst for the tissue regeneration after damage

As concluded, cell fusion-mediated reprogramming is a whole process itself that emerges as a promising regenerative strategy through stem cell, within the pro-inflammatory environment where it occurs. Is then, in the pro-inflammatory environment, where we found a plethora of molecules and pathways involved in it (Figure 6). For instance, and focusing on the neural environment, it is known the chemotactic role of the glial cells and, more interestingly, how this effect is enhanced in the damage context (Zhang et al. 2005; Zhang, Zhang, et al. 2017). SDF-1 chemokine emerged as a main character in the adult stem cell recruitment towards many tissues after injury, being especially important in the neuronal context (Cheng and Qin 2012; Cheng et al. 2017; Dubovy et al. 2010). Putting an eye on the retinal tissue, in our lab we demonstrated how SDF-1 can enhance the BMDC recruitment and its further implication in the mouse retina regeneration

(Pesaresi et al. 2018). In this reprogramming assortment, Wnt/β-catenin pathway also claims its importance. Wnt/β-catenin pathway is an evolutionary conserved pathway implied in many aspects: stem cell self-renewal, cell proliferation and cell differentiation (Steinhart and Angers 2018). Additionally, it is a pathway remarkably implied in development (cell fate decision, cell migration and cell organization within the tissue) and homeostasis (Niehrs 2010).

The evolutionary Wnt/β-catenin pathway implication is also preserved in vertebrates. In fact, first experiments were conducted in *Xenopus laevis* embryos highlighting the essential role of the pathway on forming primary body axes (Wiese, Nusse, and van Amerongen 2018). In fact, it has been also proved the Wnt/β-catenin pathway in the pre-implantation mouse embryo. As early as pre-implantation blastocyst, Wnt ligands were detected, highlighting the presence and importance of the pathway in the embryo development. Additionally, some of the ligands, as Wnt family member 1 (Wnt1), were identified mostly in the blastocyst inner cell mass (Kemp et al. 2005). Following the blastocyst implantation, further studies demonstrated that Wnt/β-catenin pathway must be switched off to favor the following steps of embryo development (ten Berge et al. 2011). But, how this complex pathway works? In stationary conditions, β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK3) and degraded by a destruction complex form by casein kinase Iα (CKIα), Axin and adenomatosis polyposis coli (APC). Thus, the β-catenin degradation avoids the translocation into the nucleus. However, when Wnt ligands appear into scene and its recognized by the Frizzled transmembrane receptors, the destruction complex is recruited by the Wnt-receptor complex and is deactivated, allowing the translocation of β-catenin into the nucleus and promoting the transcription of target genes (Niehrs 2012). This referred

Wnt fluctuation in embryo development was also seen to be fundamental in the hybrid reprogramming. Lluis F. *et al.,* in our laboratory, firstly showed how the reprogramming could be enhanced after fusion by the activation of Wnt/β-catenin pathway, either by the Wnt family member 3a (Wnt3a) ligand or the small molecule 6-bromoindirubin-30-oxime (BIO) (GSK3 inhibitor); selecting the hybrids under a puromycin resistance that will only develop if the hybrids had pass through a proper reprogramming. Additionally, after activation, the hybrid differentiation potential was demonstrated both *in vitro*, by embryo body formation, and *in vivo* exhibiting their teratoma formation skills when injected in a severe combined immunodeficient mouse (SCID) (Lluis et al. 2008). Additionally, in our lab, Marucci *et al*., could demonstrate how the homeobox protein Nanog binds to a key negative regulator of the Wnt Pathway, the Dickkopf-related protein 1 (Dkk1). This binding represses Dkk1 transcription and, in turn, indirectly activates β-catenin, leading to a Wnt pathway activation leading to a better reprogramming. Therefore, this pulse on the activation of the pathway through Nanog will favor or repress the reprogramming of the newborn hybrids (Marucci et al. 2014).

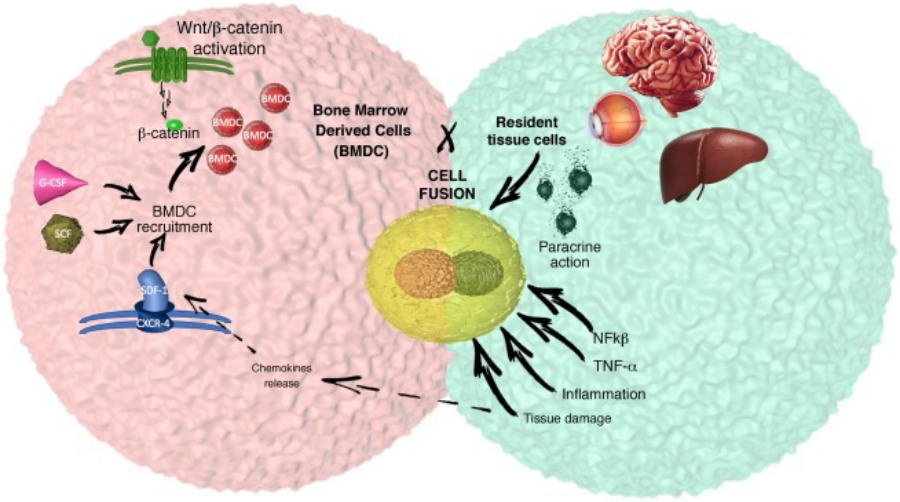


Figure 6. Cell fusion process between a somatic and stem cell. New-born hybrids cell fusion-derived go under a process of reprogramming. This process could be influenced by altering the plasticity of both cell fusion partners and by the environment within the *in vivo* cell fusion happens. Environment wise, inflammation has been shown to have a pivotal role on cell fusion. Additionally, the homing and recruitment of the stem cell partner can efficiently potentiate the cell fusion and reprogramming process, having the SDF1-CXCR4 as a main player on this. From the other side, activating the Wnt/β-catenin pathway of the stem cells also enhances the reprogramming capacity (Image from Pesaresi et al., 2018).

A comprehensive and inviting read might lead the reader to the idea that; the dormant regenerative potential of the hMGCs could be awaken through a process of cell fusion-mediated reprogramming (Wnt/β-catenin stimulated) with adult stem cells with, the ultimate aim of understand it as a putative stem cell therapy to address different visual impairments affecting the human retina. If so, it leads to the same process might have happen with well-studied animal models as mice and we could be one step closer to the ultimate referred goal.

4.2.1.2.- Wnt pathway activation to favor the cell fusion-mediated reprogramming; a bright path towards retina regeneration

With all the pieces on the table, was a matter of time to put the puzzle together and address the possibility of enhancing regeneration through a cell fusion-mediated reprogramming by stimulating Wnt/β-catenin pathway. As usual, first hints were discovered in zebra fish model. Concretely, Wnt/β-catenin pathway was demonstrated as instrumental, and within it, GSK3β inhibition as sufficient to lead towards retina regeneration. Through RNA analyses, they found a different set of genes expressed in injured retina when compared against uninjured retina, pointing an inverse expression correlation between *Dkk1* and *Achaete-Scute Family BHLH Transcription Factor 1(Ascl1)*, proving it with pharmacological and genetical models. More interestingly, it was also shown how the Wnt/β-catenin pathway stabilization through GSK3β inhibition was sufficient to dedifferentiate the MGCs and bring them into a pluripotent state able to regenerate the retina (Ramachandran, Zhao, and Goldman 2011).

Further studies show similar results, highlighting the importance of the Wnt pathway in both retina development and regeneration after injury, being able not only to dedifferentiate the MGCs but also to maintain the proliferation of retinal progenitors in the CMZ. In particular, they showed by light damaged or chemical damage how MGCs nucleus migrate from the INL towards the ONL, and how those cells re-enter the cell cycle by the expression of the nuclear protein named proliferating cell nuclear antigen (PCNA). Interestingly, this process was in co-expression with β-catenin accumulation in the cytoplasm and, when the Wnt-pathway was inhibited,

this regeneration was aberrant and unsuccessful (Meyers et al. 2012). With the aim of regeneration, our group challenged this idea in the CNS, in both retina and brain. Starting in the brain, the idea was to offer an alternative stem cell-based therapy targeting the dopaminergic neuron loss in the substantia nigra pars compacta. The hybrid generation upon fusion between transplanted Cre-HSPC and glial cells or neural cells was proved in two different mouse model of Parkinson-induced disease.

Yellow fluorescence protein (YFP) expressing cells could reprogram *in vivo* within one week and acquire astroglia features to survive up to four weeks and help in the physiological dopaminergic rescue. Additionally, this newly generated astroglia was able to secret Wnt1 which have a protective effect in neural cell survival and homeostasis (Altarche-Xifro et al. 2016). Driving our attention back to retina, promising results had been found in our laboratory in different mice model. A pioneer study confirmed the working hypothesis of cell fusion-mediated reprogramming as a regenerative mechanism in the mouse retina after damage. The cell fusion events in the mouse retina were proved through a Cre-Lox system. Specifically, $HSPC^{CRE/RFP}$ were intravitreally injected after NMDA damage (affecting ganglion and amacrine cells) in a host retina LoxP-STOP-LoxP-YFP; therefore, Sanges D. *et al.,* could identify RFP^+/YFP^+ hybrids, that also co-express by immunofluorescence with Glial (GS), Amacrine (Bielski et al.) and Ganglion markers (SMI-32). These fusion events were further proved with different stem cell sources as ESC and RPCs. Additionally, by Wnt pathway activation through BIO (GSK3 inhibitor) new generated hybrids showed a higher reprogramming potential since they expressed pluripotency markers as Oct4, Nanog and Nestin (that were not present in not BIO activated hybrids). Moreover, the reprogrammed hybrids showed a long term contribution on the retina regeneration as demonstrated via histological analysis of the GCL, which exhibited a higher density, and also

as demonstrated by the hybrids contribution with axon projections in optic nerve regeneration after one month (Sanges et al. 2013).

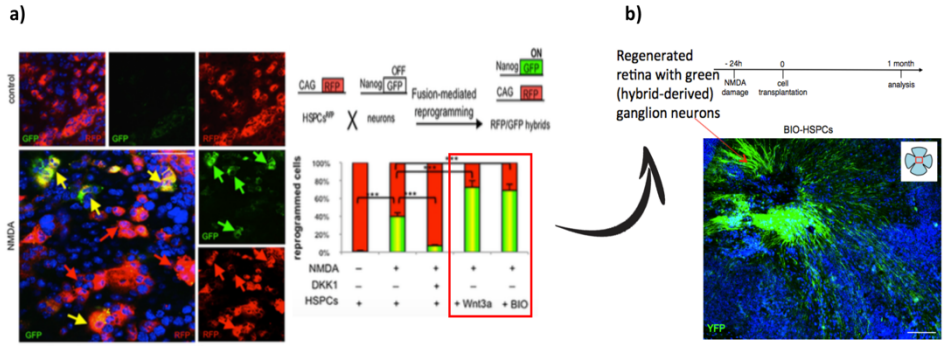


Figure 7. Evidences of mouse retina cell fusion-mediated regeneration and hybrid reprogramming enhancing by Wnt/β-catenin pathway in Ganglion cell regeneration in mouse retina. **a)** Immunostaining of mouse retina, showing the presence of hybrids between hematopoietic stem progenitor cells (HSPC) carrying red fluorescence protein (RFP) and reprogrammed retinal cells, expressing green fluorescence protein (GFP) under Nanog promoter in NMDA damage conditions. These hybrids events where not found in control conditions. Additionally, new-born hybrids are more reprogrammed under NMDA and Wnt/β-catenin pathway of the HSPC. **b)** Hybrid contribution (GFP+) into ganglion cells close to the optic nerve in a long-term analysis (1 month) after NMDA damage (Sanges et al. 2013).

Similar results were proved in different mouse models affecting the photoreceptors (recapitulating the RP disease): (1) the chemically induced *N*-methyl-*N*-Nitroso Urea (MNU) mouse model and, (2) the retinal degeneration 10 (rd10) genetic mouse model. As seen with the ganglion recovery, HSPCs were able to fuse with MGCs. The hybrids underwent reprogramming when Wnt/β-catenin pathway was activated in the HSPCs, to finally differentiate into photoreceptors (Figure 8). These newly generated photoreceptors, expressing the photoreceptor marker Recoverin, are able to rescue the photoreceptor layer of the mouse retina leading to a physical recovery of the ONL and physiological response to light measured by electroretinogram (Sanges et al. 2016).

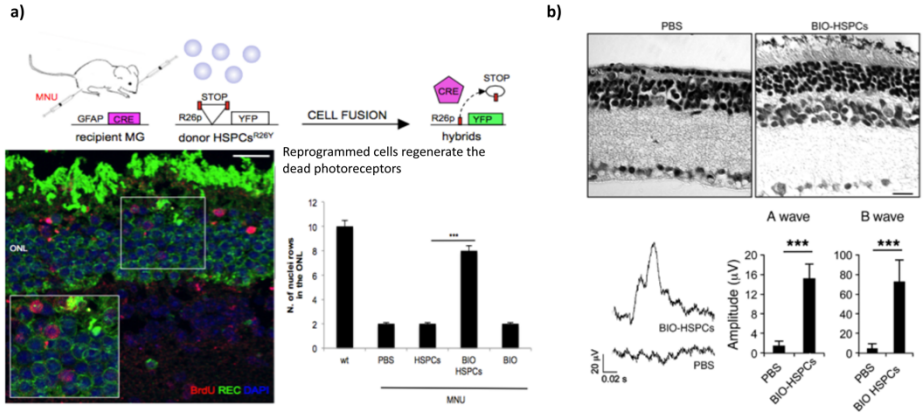


Figure 8. Evidences of mouse retina cell fusion-mediated regeneration and hybrid reprogramming enhancing by Wnt/β-catenin pathway. Photoreceptor cell regeneration in mouse retina. a) Mice model to detect cell fusion hybrids after N-methyl-N-Nitroso Urea (MNU) damage (photoreceptor in the outer nuclear layer depletion). Injection of hematopoietic stem progenitor cells (HSPC) carrying yellow fluorescence protein (YFP) expression under STOP codon is transplanted into recipient mice with Cre expression under GFAP promoter. If the HSPC fuse with GFAP expressing cells, STOP codon is chopped out and YFP shine identifying cell fusion. Fused hybrids are able to re-enter the cell cycle (BrdU) and repopulate the ONL. This repopulation is increased when hybrids had been previously Wnt/β-catenin pathway activated by BIO. **b)** Photoreceptors recovery of the ONL layer showing a major recovery when injected HSPC Bio activated. The recovered retina show difference both in A and B wave when Bio-HSPC treated in comparison with PBS control (Sanges et al. 2016).

It was also proved the implication of the SDF1/CXCR4 chemokine axis on the bone marrow cells (BMC) endogenously recruited in NMDA-damaged mouse retina of irradiated mice. Once recruited in the host retina, the $BMC^{Cre/RFP+}$ were able to fuse with MGC^{YFP+} (Figure 9a). The new born hybrids expressed progenitor markers as Pax6, Nestin or Chx10 as well as showed an increase of Cyclin D1, reentered in the cell cycle and finally expressed marker of differentiation, such as amacrine cell genes three weeks after the damage (Figure 9b) (Pesaresi et al. 2018).

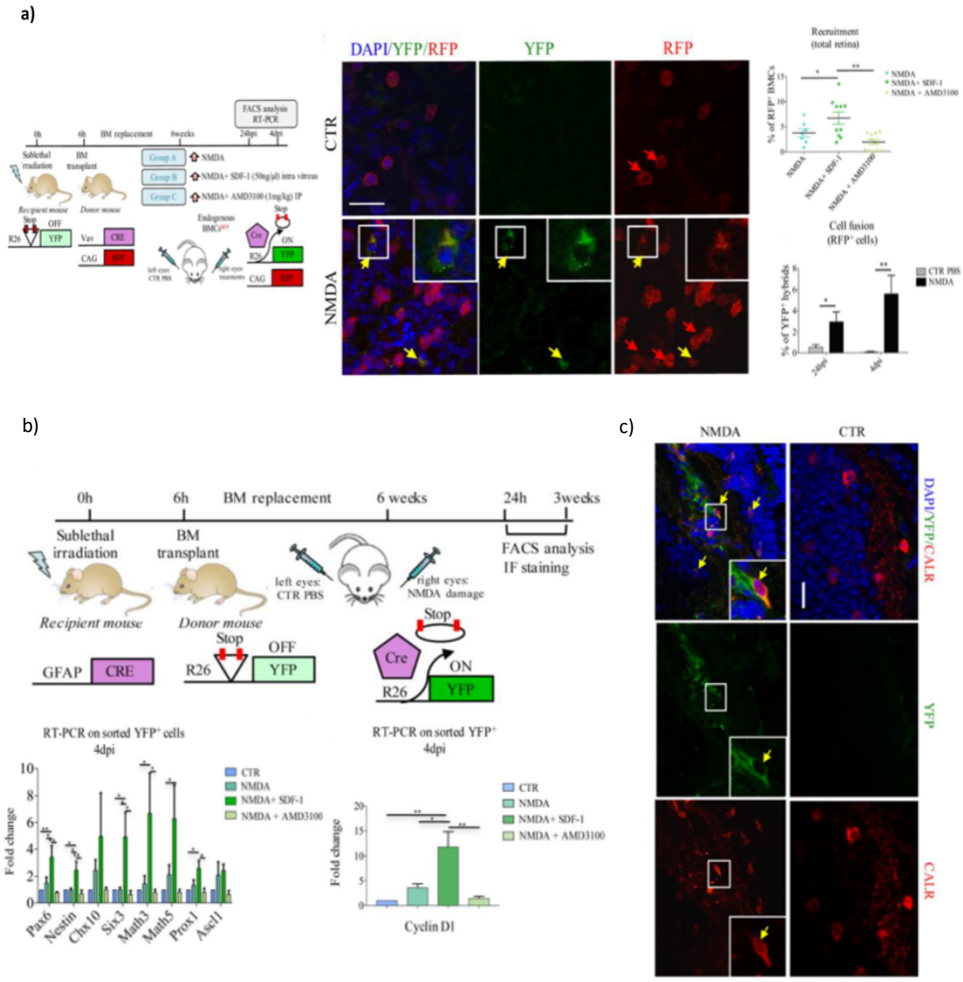


Figure 9. Evidences of mouse retina cell fusion-mediated regeneration and hybrid reprogramming enhancing by Wnt/β-catenin pathway. In vivo fusion after HSPC recruitment SDF1/CXCR4 mediated. a) Mice model Cre-lox based and RFP+ based to identify cell fusion. If the donor cells fuse with host cells, stop codon will be chopped out and YFP+/RFP+ events will be identified. In the mouse retina immunostaining, hybrids are present in NMDA conditions but not in control conditions. A major BMC recruitment is shown under damage and SDF1 activation. 4 days' post transplantation, in NMDA conditions is shown an increase on fusion percentage (Pesaresi et al. 2018). **b)** Another mice model identifying fusion through cre expression under GFAP expression when fused with BM transplanted cells YFP flanked by stop codon. Fused hybrids shown major progenitor marker expression and Cyclin D1 when resultant from BM chemotaxis was promoted by NMDA+SDF1. **c)** Resultant hybrids expressing CALR astrocyte marker in NMDA damage conditions but not in control conditions.

Even with these promising results pointing on the putative implication of the cell fusion-mediated reprogramming in the regenerative retina, further

steps should be done to consolidate the principals of the cell fusion-mediated reprogramming as a potential stem cell therapy. With this, the most natural step will be to challenge the laboratory ideas on human samples.

Closing, with the exposed in this introduction, the present thesis will present an initial translational work focused on the reprogrammed cell fusion stem cell in human samples as first steps towards a stem cell treatment for human retinal rescue and regeneration.

HYPOTHESIS, AIMS AND OBJECTIVES

HYPOTHESIS

At present, regenerative medicine is a well stablished field of medicine and an innovative approach to tackle unmet medical needs as visual impairments. Within the regenerative medicine field, one type of stem cell therapy, the cell fusion, has emerged as a potential mechanism for tissue regeneration. Interestingly, tissue regeneration has been reported to have different applications in a tissue-dependent way. Specifically, in the retinal tissue, where the regenerative ability is hold by the MGC, regeneration can potentially be achieved not only in cold blood vertebrate animal models but also in rodent models (Kara et al. 2019; Pesaresi et al. 2018; Sanges et al. 2016). Further studies should be taken in larger animals and human samples to eventually point cell fusion as a putative efficient stem cell therapy. Therefore, here we hypothesized the following:

- The fusion between human adult stem cells and human differentiated cells is capable to rescue/regenerate the human injured retinal tissue through a process of somatic cell reprogramming and differentiation

AIMS AND OBJECTIVE

All premises considered, lead to the overall aim of the present thesis:

Propose the cell fusion and reprogramming between adult stem cells, specifically, hMSC AD or hHSPC CD34^{+}, with hMGCs within human retinal organotypic cultures or human retinal organoids, or fusion of the above mentioned stem cells with isolated hMGCs as a potential regenerative therapeutic approach for human retinal regeneration.

To achieve this main goal the following objectives have been tackled:

1. Identify cell fusion events between hMSC AD or hHSPC CD34^{+} with hMGCs within the *ex vivo* human retinal organotypical explants;
2. Assess cell fusion efficiency of hMGCs and hMSC AD in different conditions (Control *vs* Damage) and characterize the reprogramming mechanisms of the resultant cell fusion hybrids;
3. Assess the differentiation potential of the cell fusion hybrids and their integration within a developing human retina organoid.

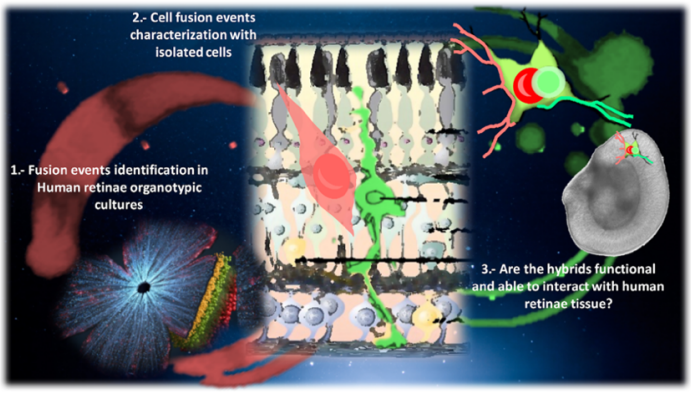


Figure 10. Brief summary of the project outline: cell fusion events between hMGCs and human adult stem cells could lead to a human retina regenerative process. With this project, we aim to: (1) Identify fusion events of MGCs within the human retinae organotypic culture with human adult stem cells (hMSC AD and hHSPC CD34+); (2) hMGC isolation and cell fusion characterization with the mentioned human adult stem cells; (3) assessment of the functionality of the newly generated hybrids with the aim to establish if cell fusion could be a putative regenerative strategy.

MATERIAL & METHODS

Cell lines and culture

ARPE19 cells were purchased from ATCC (ATCC®CRL-2302™) and maintained in DMEM/F-12-GlutaMax (GIBCO) supplemented with 10% fetal bovine serum (FBS), penicillin (100U ml^{-1}) and streptomycin (100μg ml^{-1}).

hHSPC $CD34^+$ were isolated from human cord blood donations of maximum of 26 hours. CD34+ cells were isolated by aspirating the dense interphase of the LymphoPrep (Stem Cell Technologies) solution and with a posterior CD34+ MicroBead Kit UltraPure human (MACS Miltenyi Biotec) column selection following manufacturer's instructions. Cells were counted and cryo-preserved at one million cells per milliliter in StemSpan™ SFEM (Stem Cell Technologies) supplemented with hIL3 0.02μg/μl at 1 μl/ml (PeproTech), hIL6 0.02μg/μl at 1 μl/ml (PeproTech), hSCF 0.1 μg/μl at 1 μl/ml (PeproTech), hTPO 0.1 μg/μl at 1 μl/ml (PeproTech), hFlt3 ligand 0.1 μg/μl at 1 μl/ml (PeproTech) in 10% DMSO. For CD34+ cells infection previous to cell fusion, cells were cultured in suspension at concentration of one million cells per milliliter in StemSpan™ SFEM (Stem Cell Technologies) supplemented with hIL3 0.02 μg/μl at 3 μl/ml (PeproTech), hSCF 0.1 μg/μl at 3 μl/ml (PeproTech), hTPO 0.1 μg/μl at 3 μl/ml (PeproTech), hFlt3 ligand 0.1 μg/μl at 3 μl/ml (PeproTech).

hMSC AD was a kind gift from Dr. Hoogduijn M.J., from Rotterdam Transplant Group, Department of Internal Medicine. The mentioned hMSC were isolated from the subcutaneous adipose tissue from kidney donation. Tissues were collected after explicit written informed consent and approval of the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam (protocol no. MEC-2006-190). hMSC AD were maintained in culture in α-MEM without ribonucleases (GIBCO)

supplemented with 15% FBS, 100 microliters of 50 ng/ml of human fibroblast growth factor hFGF2 (PeproTech), penicillin (100U ml^{-1}) and streptomycin (100µg ml^{-1}).
HEK 293 cells for lentiviral production were maintained with DMEM supplemented with 10% FBS, penicillin (100U ml^{-1}) and streptomycin (100µg ml^{-1}).
Characterized hMGCs were maintained in DMEM/F-12-GlutaMax (GIBCO) supplemented with 10% FBS, penicillin (100U ml^{-1}) and streptomycin (100µg ml^{-1}).

Human retinae organotypic cultures

Eye globes were donated through a collaboration with "Banc d'Ulls per a Tractaments de Ceguesa" (BUTC). Eye globes were from two different origins: from donors classified as non-suitable for cornea transplantation or from eye cups whose cornea was dissected for transplantation and the eye cup was stored for less than 24 hours. Explicit, written informed consent for the removal and use of the eye globes for diagnostic and research purposes was obtained from patients and/or relatives. The samples received were from donors between 40 and 98 years old.

Before retina extraction, the eye globe was decontaminated by submerging it in BSS (sterile irrigation solution, Alcon) during 2 min. Afterwards the globe is submerged in 5% Povidone-Iodine for 2 min and its followed a submersion in sterile sodium thiosulphate 0,1% for 1 min. Lastly, the globe was cleaned by submersion in PBS for 1 min.
Retina dissection was performed according to the set-up procedure optimized in our laboratory in collaboration with "Centre d'Oftalmologia Barraquer". Specifically, the eye globe was placed in *ad hoc* build holder

that generates vacuum, providing sufficient internal pressure for an appropriate dissection. Frontal part of the eye globe: cornea, iris and crystalline were removed by making an incision 6 mm away from the Iris. This opens a window on the eye globe, exposing its internal part and making the retina accessible attached to the retinal pigmented epithelium (RPE). The vitreal excess was removed by sponge absorption and the retina was separated mechanically from the RPE with the help of two forceps. The eye globe was then removed from the holder and placed up-side-down in a sterile petri dish. In this position, the retina junction with the optic nerve was exposed, and could be cut, releasing the retina from the optic cup. Vitreal leftovers and retina periphery were removed, and the central part of the retina was divided in four pieces and every piece was cultured with Neurobasal-A medium supplemented with 2% B-27 supplement (50X), 1% of N2 supplement and 1% GlutaMax and 1% penicillin (100U ml^{-1}) and streptomycin (100µg ml^{-1}).

Human retinae organoids differentiation

Organoids were generated and delivered to our group by our collaborators of the "Pluripotency for organ regeneration" group from IBEC. Human retina organoids were raised in monolayer and, from it, 3D optic-cup structures were grown. Organoids were delivered to our laboratory around day 30, and here were kept in culture with basic Differentiation Medium: DMEM/F-12-GlutaMax (GIBCO) supplemented with 1% of N-2 supplement (100X) (ThermoFisher), 1% B-27 supplement (50X) (ThermoFisher), 1% MEM NEAA (100X) (GIBCO), penicillin (100U ml^{-1}) and streptomycin (100µg ml^{-1}) and cultured for around 15 days, until the beginning of the human retina organoid lamination.

Within this time point, the experiment where dissected from the plate to its posterior immuno-staining or micro-injection.

Human retinae damage, collection of conditioned medium and cell co-culture experiments for fusion induction

Human retina was cultured up to seven days in Neurobasal-A medium supplemented with 2% B-27 supplement (50X), 1% of N2 supplement and 1% Glutamax. Human retinae were cultured in 2,5 milliliters and the medium was refreshed every two days. Conditioned medium enriched of secreted factors from the retina was collected at day 1 (as "natural" control condition) and at day 7 of culture (as "natural" damage condition).

Cell co-culture fusion experiments were done culturing adult stem cells, either human MSC AD H2B::mRFP or human CD34+ H2B::mRFP. hCD34+ cells were infected with pHIV-H2B::mRFP two consecutive days before the co-culture.

Human retinae were extracted from the eye globe, and after the dissection were placed one day in culture. Medium was refresh at day one and the retinae were infected with AAV2/ ShH10Y445- pRLBP_eGFP. The medium was changed every two days, until the day 5. With the new medium, the retinae were placed facing up the ganglion cell layer and, 500.000 adult stem cells expressing H2B::mRFP and resuspended in 50 microliters were cultured on top of the retinae. After one over-night co-culture, retinae were harvested for further experiment, such as FACS or immunohistochemistry.

Western Blot experiments to characterize hMGCs

Cells were cultured until 85% of confluence. Then harvested and washed once with PBS. The wash was done after pelleting down the cells at 300rcf for 5 min at room temperature. Cell lysis was performed on ice for 15 min, in RIPA buffer (SIGMA R0278) containing protease (SIGMA P8340) and phosphatase inhibitors (SIGMA P5726). By centrifugation, insoluble material was pelleted at 16.000 rcf for 30 mint at 4ºC. Protein concentration was determined by Bradford assay (BIO-RAD 500-0006). The protein extract was prepared by mixing it with 4x sample buffer (40% glycerol, 240 mM Tris/HCL, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol). Then, denatured at 99ºC for 10 minutes and spined down before gel running. Samples were separated by SDS-PAGE on 10% gel, and then transferred to poly vinylidene difluoride membrane (BIO-RAD 162-0177). Membranes were blocked with 5% non-fat dry milk (SIGMA 70166) in TBS-Tween 20 (0.1%) (SIGMA P1397) for 60 minutes and, just after, incubated overnight with primary antibodies in TBS-T with 5% non-fat dry milk at 4ºC. After, membranes were washed three times with TBST-T for 10 minutes each and incubated with conjugated secondary antibody (1:2000 Amersham Biosciences NA931V) in TBS-T with 5% non-fat dry milk for 60 minutes and finally washed three times, 10 minutes each, with TBS-T. Immunoreactive proteins were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific 32106) and final images were taken with Amersham Imager 600.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Cells were cultured until 85% of confluence. Then cells were harvested and washed once with PBS. The wash was done by pelleting down the cells at 300rcf for 5 min at room temperature. RNA was isolated with Mini kit (QIAGEN), according to the manufacturer protocol. RNA was then reverse transcribed to cDNA with SuperScript III CellsDirect cDNA Synthesis Kit (Invitrogen). qRT-PCR reactions were performed using Platinum SYBR green qPCix-UDG (Invitrogen) in a LyghtCycler 480 (Roche) machine, according to the manufacturer recommendations. The used oligos are listed in the following table (table 1). qRT-PCR obtained data was normalized to GAPDH expression. Each sample was performed in technical triplicates. A minimum of three independent experiments were averaged.

Gene	Primer FW	Primer RV
hNMDA R2	AAATGCTGCTGCTTTTCCTC	CTGCATGATCTTCAGCATG
hIL1-β	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTTGGGATCT
hSox9	GTACCCGCACTTGCACAAC	TCTCGCTCTCGTTCSGAAG
hC-Myc	GCTGCTTAGACGCTGGATTT	TAACGTTGAGGGGCATCG
hAxin 2	GATATCCAGTGATGCGCTGA	ACTGCCCACACGATAAGGAC
GAPDH	TCAAGAAGGTGGTGAAGCAGG	ACCAGGAAATGAGCTTGACAAA

Lentiviral constructs and MSCs and MGCs infection

The lentiviral construct pHIV-H2B::mRFP was an acquired plasmid from addgene (#18982). For the pRLBP_eGFP plasmid, pRLBP human gene promoter was amplified by reverse transcribing human ARPE19 RNA

SuperScript III CellsDirect cDNA Synthesis Kit (Invitrogen) and then amplified by PCR (using the Phusion hot start high fidelity polymerase, Thermofisher). An eGFP reporter was also extracted by PCR from pH2B-eGFP plasmid (addgene #11680). SgrAI/AgeI were added to the pRLBP sequence and SgraI/SalI were added to eGFP by PCR (using the Phusion hot start high fidelity polymerase, Thermofisher). Both sequences were subcloned into a lentiviral vector with a p1494 backbone giving the final plasmid. For the Tuj1_Crimson_Hygro similar procedure was followed. Due to the rich GC content of the Tuj1 promoter region, Tuj1 promoter was synthetized by GeneArt company (Thermofisher). pEF.myc.ER-E2-Crimson plasmid was purchased from addgene (#38770). An SV40_Hygro with its expression being driven by a constitutive SV40 promoter, was PCR extracted from a previous generated plasmid in the laboratory (EF1α_HA-Cxcr6-SV40_Hygro). The same restriction enzyme strategy was followed, adding by PCR AgeI/SgrAI, SgrAI/NheI and NheI/SalI to the enumerated fragments. The three plasmids were then sub-cloned into a lentiviral vector with a p1494 backbone. pCMV-ΔR8.2 dvpr (Addgene #8455) and pCMV-VSVG (Addgene #8454) were used as lentiviral packaging constructs. The oligonucleotides used for the mentioned plasmids are listed in the following table (table 2). Lentiviral production was performed following the RNA interference consortium (TRC) instruction for lentiviral particle production (http://www.broadinstitute.org/rnai/public/). HEK 293 cells were plated at a known density of 5*104 cell cm-2 in p150 plates at day 0. Following, at day 1, cells were co-transfected with: (A) 19.5 µg pCMV-DR8.2; (B) 10.5 µg pCMV-VSV-G; (C) 30 µg of the corresponding plasmid construct (H2B::mRFP, pRLBP1_eGFP or Tuj1_Crimson_SV40_Hygro) with calcium phosphate transfection kit (Clontech 631312). At day 2, medium was refreshed with new DMEM medium supplemented with 30% FBS and 1% PenStrep. Medium was harvested at day 3 and day 4 for the viral

collection. Virus were filtered and ultracentrifuged and aliquoted in batches of 20 microliters for a posterior infection when needed.

Infected cells with H2B::mRFP or either pRLBP_eGFP were then FACS sorted on the basis on fluorescent intensity. The generated hMGCs pRLBP_eGFP cells were re-infected with Tuj1_Crimson_SV40_Hygro and selected by hygromycin resistance (50 µg ml^{-1}) starting two days after infection.

Name	Target	Amplified region	Sequence
Hs_pCRALBP_1_Fw	hs_pRLBP promoter	chr15:89,221,447-89,222,025	CATCTTGGGACGTGGTCTCC
Hs_pCRALBP_1_Rv			GCAATAGGCCTGGATTTGCC
Hs_pCRALBP_1_SgrAI_Fw			AAAACACCGGTGCATCTTGGGACGTGGTCT
Hs_pCRALBP_1_AgeI_Rv			AAAAACCGGTGCAATAGGCCTGGATTTGCC

Adeno-associated constructs and human retina infection

Labelling of human retina was done through adeno-associated viral infection. Adeno-associated viruses were manufactured by Unitat de Vectors Virals. First tropism and Müller Glia specificity infection experiment were performed by AAV2/8-CMV-GFP. More specific Müller Glia infective virus were prepared by modifying the capsid and introducing the generated pRLBP_eGFP construct. The ShH10Y445 capsid plasmid was a kind donation from Wijnhold J. (Pellissier et al. 2014). Unitat de Vectors Virals (Piedra et al. 2015) generated the final Adeno-associated virus AAV2/ ShH10Y445- pRLBP_eGFP.

Human retinas were infected with a $5x10^{10}$ viral particles. Virus and medium were refreshed daily during 4 days before running the co-culture fusion experiments.

Immunofluorescence and antibodies used

Human retinae were washed in PBS for 5 minutes and fixed by immersion in 4% PFA over-night at 4ºC. For organoid and retinal sections, serial transversal sections of 5µm of thickness were prepared and processed for immunofluorescence. Briefly, sections were left at 60ºC over-night to melt down the paraffin, after that, sections were totally de-paraffinized by sequential de-hydration treatment, first with Xylene and then decreasing EtOH concentration to re-hydrate the samples. Slices were then placed in a plastic coplin jar with a permeabilization buffer 0.3%Triton X-100 and 0.1M NaCitrate in PBS (1h at RT). After this, antigen retrieval was carried out by boiling the slides for 4 minutes in a microwave. Three washes with PBS during 10 minutes per wash were then done and the sections were blocked for 1h (3% BSA, 300 µM glycine, 0.03% Triton X-100, 0.01M NaCitrate in PBS). Sections were then incubated over-night with primary antibodies diluted in 1:2 blocking buffer for two over-nights at 4ºC. On the following day, washing were done again and the sections were incubated with secondary antibodies and DAPI ($5mg/ml^{-1}$) during two hours at room temperature. Three more washes of 15 minutes each were done before mounting the samples with VectaShield with dapi (Vector Laboratories, 42 Burlingame, CA, USA).

For retinal-piece flat mount, after fixing retinas were then permeabilized (0.3% Triton X-100 in PBS, 1.5h at RT) and blocked for 1h at RT (3% BSA, 300 µM glycine, 0.03% Triton X-100, in PBS). For the following two over-

nights, retinae were incubated with primary antibodies at 4ºC. Retinae were posteriorly washed with PBS 3 times 15 minutes each and incubated with secondary antibodies over-night at 4ºC. This incubation was carried out together with DAPI (5mg/ml^{-1}) and were mounted with VectaShield with dapi (Vector Laboratories, 42 Burlingame, CA, USA).

Following flat mount imaging, retinae pieces were embedded in paraffin to proceed for the section re-staining, proceeding as described.

Spotted cells in 8 multi-well chamber (Lab-Tek Chambered #1.0 Borosilicate Coverglass System) or in cover glass ∅ 22 mm (VWR, Borosilicate glass 631 – 1089) were immuno-stained as follow. Cell samples were washed in PBS for 5 minutes and fixed by immersion in 4% PFA for 15 minutes. Just after, the cells were permeabilized with permeabilization buffer 0.3%Triton X-100 and 0.1M NaCitrate in PBS for 15 minutes and after three washes of 5 minutes each, blocked with blocking buffer for 45 minutes (3% BSA, 300 μM glycine, 0.03% Triton X-100, 0.01M NaCitrate in PBS). Samples were then incubated for 2 hours with primary antibodies diluted in 1:2 blocking buffer. Three large washes, of 15 minutes each, were done before incubation with secondary antibodies and DAPI (5mg/ml^{-1}) one hour at room temperature. Three more washes of 15 minutes each were done before mounting the samples with VectaShield with dapi (Vector Laboratories, 42 Burlingame, CA, USA).

The following antibodies were used: chicken anti-GFP (1:500; ab13970, Abcam); mouse anti-*βIII-tubulin (1:200; ab7751, Abcam); rabbit anti-RFP (1:250;* ab62341, Abcam); mouse anti-cralbp (1:200; ab15051, Abcam); rabbit anti-GS (1:200; G2781, Sigma); mouse anti-Tuj1 (1:200; MAB1637, Millipore); rabbit anti-Pax6 (1:200; ab5790, Abcam); mouse anti-GFAP

(1:200; MAB360, Millipore); mouse anti-Sox9 (1:200; MA5-17177; ThermoFisher Scientific); mouse anti-rhodopsin (1:200; MAB5356, Millipore); mouse anti-calretinin (1:200; MAB1568, Abcam); rabbit anti-IBA1 (1:200; 019-19741, Fujifilm). All secondary antibodies were from Molecular Probes (Invitrogen) and used 1:1.000 in PBS. DAPI (5mg ml^{-1}) was used to stain for cell nuclei.

For all the samples, images were taken using either Leica laser SP5 or SP8 confocal microscopy systems.

Immuno-TUNEL assay and frozen tissue sections

Human retinae chopped in pieces were washed in PBS for 5 minutes and fixed by immersion in 4% PFA over-night at 4ºC. Three washes with PBS of 20 minutes each were done immediately after, to later proceed to the cryo-protection by immersing the sample in rising concentrations of sucrose: 15% sucrose for 30 minutes, 20% sucrose for 1 hour and 30% sucrose over-night at 4 ºC. Samples were then embedded in OCT and cryo-sectioned in sections of 10-12 μm. For the immuno-TUNEL staining, immuno-staining was performed with primary antibodies as described previously, then after the primary incubation, the TUNEL staining was carried out. TUNEL staining was performed following manufacturer's instructions (In Situ Cell Death Detection Kit, TMR Red, Merck). Briefly, human retinae chopped in pieces, already permeabilized and blocked from the immuno-staining, were incubated with the TUNEL reaction mixture at 37ºC in a humidified chamber for 1hour and 30 minutes. Just after, we proceed with the secondary antibodies and DAPI (5mg/ml^{-1}) for the final

immuno-staining step. Lastly, samples were mounted with VectaShield with dapi (Vector Laboratories, 42 Burlingame, CA, USA).

Images were taken using either Leica laser SP8 confocal microscopy systems.

Flow cytometry analysis

For Flow cytometry of human retinae chopped in pieces and co-cultured with adult stem cells for hybrids identification, we used the protocol described in "Human retinae damage, enriched medium collection and cell co-culture fusion". Retinae chopped in pieces were then disaggregated mechanically and chemically with 0.05% Trypsin-EDTA (1X) (GIBCO) for 20 minutes, filtered through a 70 μm filter, pelleted and later resuspended with 1 millilitre of PBS supplemented with 2% FBS and DAPI ($5mg/ml^{-1}$) before analysis. Cell fusion events were detected by first excluding the dead DAPI+ cells and the double GFP and RFP cells, as detailed before. Flow cytometry analysis was performed using LSR Fortessa (Becton Dickinson) with FACSDiva software (Becton Disckinson) and FlowJo software.

FACS sorting analysis

Cell fusion was performed as described in "co-culture and cell fusion" section. In this case, a more refined selection was done, to have a cleaner population of the sorted hybrids for posterior experiments. The entire fused population of cells was incubated with Hoescht 33342 (ThermoFisher) at 37ºC for 45 minutes to determine the DNA content of

the cells. Cells were pellet down and resuspended in PBS with To-Pro™-3 Iodide (ThermoFisher) for cell viability. Before sorting, cells were filtered with a 70 μm filter. Double GFP+RFP+ cells, 4n cells and higher ploidy cells, were sorted for subsequent experiments. Cell sorting was performed using BD Influx sorting machine (Becton Dickinson) and analyzed with FlowJo software.

Time-lapse imaging

FACS-sorted hybrids were plated in Time-Lapse poly-ornitine/laminin coated Thermo Scientific Nunc Lab-Tek chambered coverglass. After 3 hours, cells were attached in the plate and cells were treated with differentiation medium: 20 ng/ml of human fibroblast growth factor hFGF2 (Peprotech) and 50 μM of Notch inhibitor DAPT (Sigma-Aldrich) (Singhal et al. 2012) or control conditions (DMSO and PBS). Images of the process were acquired on Andor Revolution XD inverted Olympus microscope. Images of the cells were taken up to 24 hours and in an incubator chamber at 37ºC and 5% CO_2.

Co-culture and cell fusion

Co-culture and cell fusion experiments were adapted from previous work from our laboratory (Sottile et al. 2016). To mimic damage conditions, monolayer of human AREP19 cells were grown. In parallel, hMGCs pRLBP_eGFP and hMSC H2B::mRFP were cultured until 75-80% of confluence. At day -1 of fusion, ARPE19 monolayer was exposed to either 1mM NMDA damage or PBS control. At day 0, cells were separately detached and plated in co-culture in suspension, in a volume of 200

microliters, in a ratio 1:1, with a maximum of 500.000 cells per cell fusion partner during 45 minutes. After 45 minutes, the cells were plated in a T75 cell culture flask (Falcon) and left in culture over-night. At day 1, the hybrids were detached and prepared for FACS cell sorting and for the following experiments.

In vitro patch clamp

Cells to patch, both hMGCs and GFP^+RFP^+ 4n hybrids, were placed in a recording chamber in a Olympus BX51WI microscope. Electrophysiological recordings were acquired at 20kHz with Multiclamp 700B (Axon Instruments) and were digitized at 16 bits (Axon 1550B Digidata, Molecular Devices). Patch pipettes of borosilicate glass were pulled (Sutter P-97) and filled with internal solution for whole-cell somatic current clamp recordings (135 mM KMeSO4, 10 mM KCl, 10 mM HEPES, 5 mM NaCl, 2.5 mM ATP-Mg, 0.3 mM GTP-Na). Intracellular solution was adjusted to pH 7.2 and displayed osmolarity values in the interval 285–295 mOsm. Pipette resistance ranged 5-7 MOhms, pipette capacitance ranged from 7-7.5pF and series resistance ranged 10-40 MOhms. Electrophysiological properties of the patched cells were obtained in current-clamp configuration by applying a sequence of hyperpolarizing and depolarizing current pulses and were analyzed with Clampfit 9.

Human retina organoids micro-injection and imaging

Human retina organoids were detached from the cell cultured dish by dissecting with a needle. Organoids were then transferred to a micro-

injection dish into M2 medium (Sigma-Aldrich) drops covered with NidOil™ (Nidacom). Organoids were micro-injected by holding them with a holding pippete and micro-injected with a manufactured sharpened injecting pippete of ∅ 70 μm. Organoids were micro-injected twice, injecting 20-25 cells per injection, in opposite sites.
Once injected, each single organoid was mounted in a bed of low melting agarose base and confined in a capillary for the mesoscopy imaging. Once set, the imaging chamber was load with DMEM/F-12-GlutaMax (GIBCO) without phenol red supplemented with 1% of N-2 supplement (100X) (ThermoFisher), 1% B-27 supplement (50X) (ThermoFisher), 1% MEM NEAA (100X) (GIBCO), penicillin (100U ml^{-1}) and streptomycin (100μg ml^{-1}) and imaged for 24 hours at 5%CO_2 and 37 ºC. Images were taken with MuViSPIM microscope (LuXendo).

The non-live imaged organoids were fixed, sectioned and immuno-stained as described.

Image processing and quantification

All the immunofluorescence images were processed with FiJi ImageJ software. Quantifications were based on analysis of at least three biological replicates. For each sample analyzed, we analyzed at least three random fields. Labelled positive cells quantification, both TUNEL+ and marker specific+, were manually acquired with the cell counter tool.

GFP decrease of the sorted 4n hybrids pRLBP_eGFP+/H2B::mRFP was calculated by a home-made code. GFP values decrease was expressed in percentage of decrease, normalized by the initial GFP value of every single cell analyzed, ± SEM.

Statistical analysis

As specified in the figure legends, data is presented as mean ± SEM or scattered points (with line at median). All statistical tests and graphs were generated using the Prism 6.0 software (GraphPad, San Diego, CA). FACS of the hybrids was defined by odd ratio, measuring that an event happens by a treatment (co-culture or not co-culture) and not by chance. Odd ratio follows the below formula. If OR ≥ 1 the identified events are due to the treatment conditions.

		Outcome	
		Yes	No
Treatment	Yes	A	B
	No	C	D

OR= (A*D)/(B*C)

Different statistical analysis was run according to the experimental setup. We used Mann-Whitney test, Two-tailed Student's T-test or One Way Anova. In all the mentioned cases, a p value < 0.05 was considered significant (*, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001; ns, not significant).

RESULTS

Part I: Hybrid identification formed after fusion between human Müller glial cells and human adult stem cells in human retina organotypic cultures

The human retina preserves its integrity for up to seven days in organotypic cultures

Organotypic human retina culture has become a procedure routinely used. Even more, slightly different protocols and culture mediums reported their capability to maintain the human retina for up to two weeks (Fernandez-Bueno et al. 2012; Niyadurupola et al. 2011; Osborne et al. 2016). Within this broad time window and, to conduct our further experiments on fusion events identification, we firstly analyzed how long a human retina could be greatly preserved. To address this question, we decided to evaluate two different methods of culture: The eye globe culture and the retina organotypic culture.

To perform the eye globe culture, the entire eye globes were disinfected following adapted methods from the clinic. One eye was cultured for 1 day and the other eye donated from the same patient was cultured for 4 days, until it started to undergo deterioration (Figure 11a). For the human retina organotypic cultures, the retinae were dissected from the eye globe following clinical advices and adapting the protocol from *Niyadurupola et al.,* (Niyadurupola et al. 2011) (see materials and methods). After that, each retina was split in four pieces and cultured at four different time points (day 1, day 3, day 5, day 7; Figure 11b). Finally, retina samples from eye globes and organotypic cultures were prepared and stained with hematoxylin and eosin (H&E).

As it could be observed in Figure 11c, the entire eye globe culture had almost an intact tissue architecture after one day of culture. In contrast, after four days of culture, we observed almost a total decomposition of the retinal tissue and even an initial sclera deterioration (Figure 11c). In contrast, the human retina organotypic culture showed a progressive architectural lost more severe in the periphery than in the central retina. Moreover, this architectural tissue collapse appeared to be more dramatic from day 5 and onward with a total loss of retinal architecture by day 7 (Figure 11d). With these results, we defined a time window of 5 days suitable to use the central part of the retina in organotypic cultures to perform further experiments.

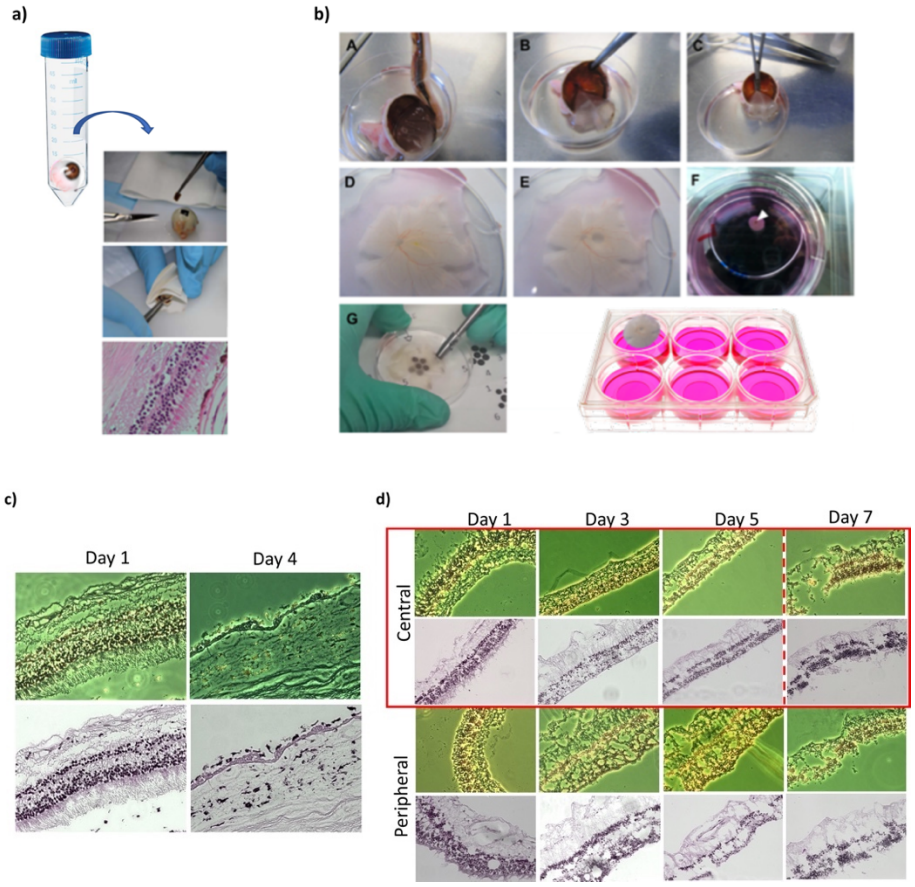


Figure 11. The two culturing methods used to study retina architecture and culture. a) Entire eye globe culture from a human eye globe donation and the posterior sectioning of the retina. **b)** Eye globe dissection and retina extraction to establish organotypic cultures. **c)** Hematoxylin and Eosin staining of the retina sections. Total tissue loss occurred after 4 days of culture. **d)** Hematoxylin and Eosin staining of human retina organotypic cultures. The retina showed a progressive de-cellularization and architectural lost. Drastic changes were visible from day 5 in advance, ending in total architectural loss in day 7. There were also differences between central and peripheral part of the retina, being the mentioned changes more severe in the periphery. **c)** and **d)** Representative images of H&E staining visualized through two different filters

Next, human retinal pieces from a single eye donation were cultured in order to define the cell death in the central part of the retinal organotypic cultures during seven days. To do that, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to mark death cells in red and, the cellular retinaldehyde–binding protein (CRALBP) immunostaining was also performed to mark in green the specific hMGCs.

Results showed that cell death increased progressively during seven days in agreement with the tissue architecture deterioration (Figure 12a). In addition, cell death percentage was calculated counting the $TUNEL^+$ cells and normalizing them against the total number of retinal cells. Results showed non-significant differences between the first three days in the cell death percentage. In addition, and correlating with the architectural loss, a significant increase in the cell death percentage was observed in days five and seven (Figure 12b).

Moreover, to quantify the hMGC death, we also calculated the cell death percentage by counting the double positive cells, $CRALBP^+$ $TUNEL^+$, and normalizing the count by the total number of hMGCs. As it could be observed in Figure 12b), hMGCs showed a significant progressive increase in cell death that also started to appear in day five.

According to these results, we decided to narrow down our experimental time window to the first five days of culture.

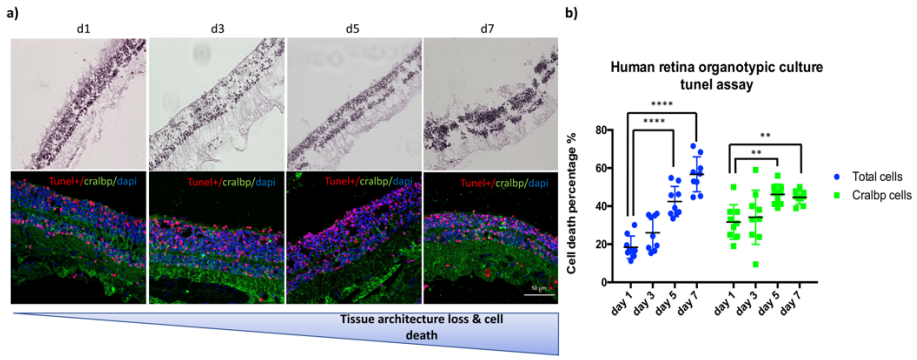


Figure 12. Architectural loss and cell death quantification of human retina organotypic culture by *in situ* cell death detection. **a)** TUNEL assay was combined with an immunostaining against CRALBP to specifically label the hMGC. TUNEL+ cells were labeled in red while CRALBP+ cells were labelled in green. In blue we can observe cell nuclei stained by DAPI. We could observe a general and specific (hMGCs) increase in cell death through the tissue culture. d1, day 1; d3, day 3; d5, day5; d7, day 7. **b)** Significant increase of cell death was observed from day 5 in advance. Data is presented as individual values, mean ± Standard error Mean (SEM) from n = 3 independent experiments (quantifying 3 random fields per time point and experiment). Unpaired T-test was used for statistical analysis. ** p_{value}< 0.01; **** p_{value}<0.0001.

Adeno-Associated viruses 2/8 can infect and label human Müller glia cells while adult stem cells can be labelled by H2B::mRFP lentiviral infection.

Once known the time and culture limitations of our model, the next step to tackle was the cell fusion partners labelling and identification.

Previous *in vivo* studies with rodent models proved that mouse MGCs can fuse with bone marrow derived stem cells in the retinal tissue (Pesaresi et al. 2018; Sanges et al. 2013; Sanges et al. 2016) . Live-cell staining or live-cell infection appeared as the only possibility to identify hMGCs in the human retinae tissue. In this context, previous studies pointed to the efficiency of adeno-associated viruses (AAV) to infect the human retina tissue, AAV are even used for gene-replacement therapies (Bennett et al. 2016; Tolmachova et al. 2013). Therefore, considering these previous publications, AAV2/8 virus carrying the cytomegalovirus (CMV) promoter expressing the enhanced green fluorescent protein (eGFP) was selected for human retinae tissue infection and labelling.

Experiments showed that AAV2/8-CMV-eGFP was able to infect a maximum of 1.84% of hMGCs when used at a $5x10^{10}$ IU/ml concentration. The data were evaluated with both EVO's live-cell imaging of the tissue (Figure 13a) and by fluorescence-activated cell sorting (FACS) analysis, after selecting the bigger cells by cell shape (FSC-A) to then perform GFP analysis (Figure 13b).

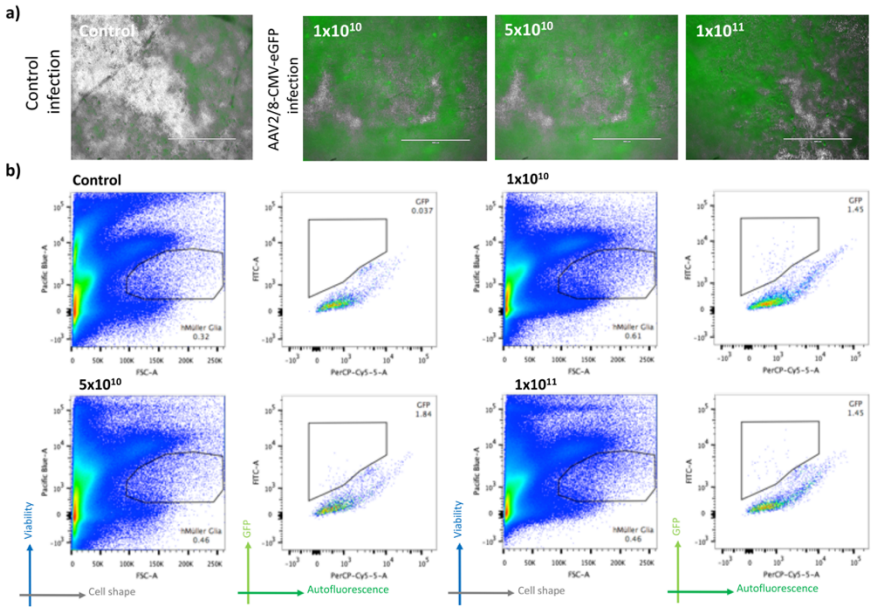
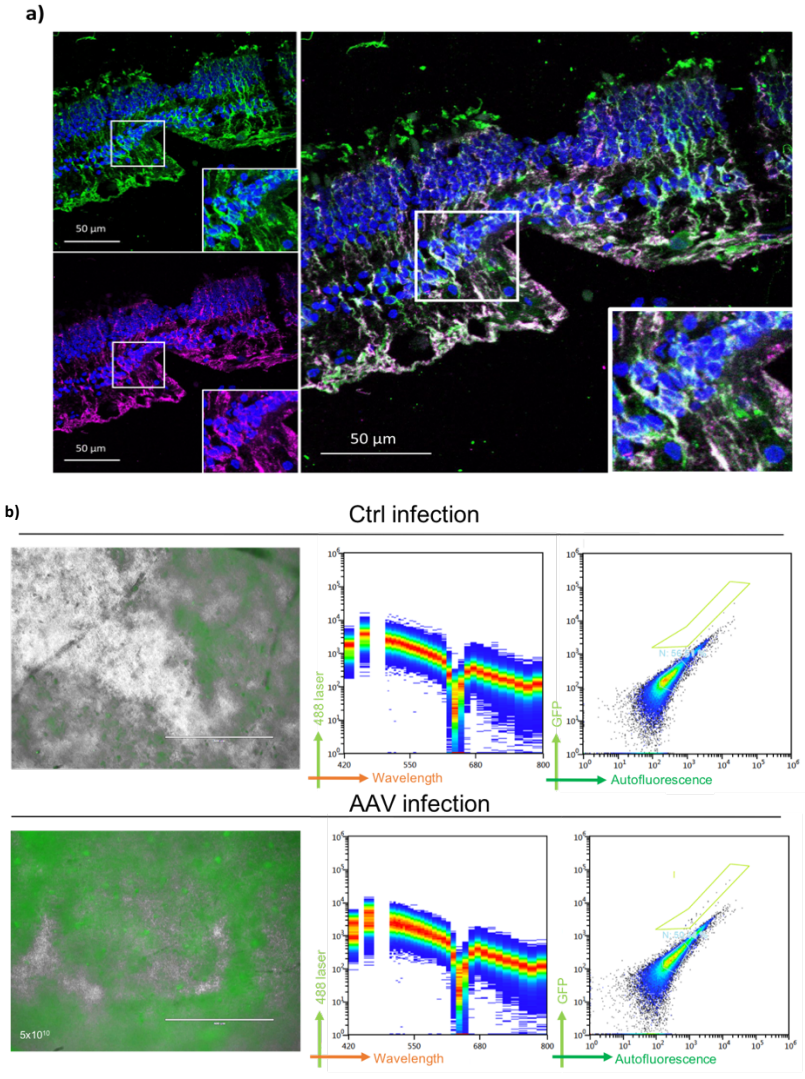


Figure 13. Assessment of the adeno-associated virus concentration to infect hMGCs in human retinal organotypic explants. a) Live-tissue imaging of the adeno-associated infection after testing three different viral concentrations ($1x10^{10}$, $5x10^{10}$; $1x10^{11}$ IU/ml) in comparison with the control retina. Cell infection was identified in all three concentrations compared with the control autofluorescence. **b)** The FACS analysis of the infection showed a higher percentage of infection at $5x10^{10}$ IU/ml concentration. n = 1, due to the shortness and value of the sample.

The specificity of AAV2/8-CMV-eGFP for hMGCs was further confirmed by immunostaining in which we observed the colocalization of GFP from the AAV infection (Rheinwald and Green) with CRALBP (magenta). As expected, the nucleus of GFP^+ cells appear to be localized in the INL while the cell body is spread across the three main retinal layers, following the hMGC pattern (Figure 14a). Moreover, due to the high autofluorescence of

the sample, we further validated the GFP$^+$ hMGC signal by FACS. Retinas infected with AAV2/8-CMV-eGFP were analyzed by Spectra view FACS analysis, plotting the spectra of emission of the cells when illuminated by specific laser, in our case, by the GFP laser 488 nm. Results showed that in the control retinas there were not GFP cells while a clear GFP$^+$ signal appear in the infected human retina (Figure 14b).



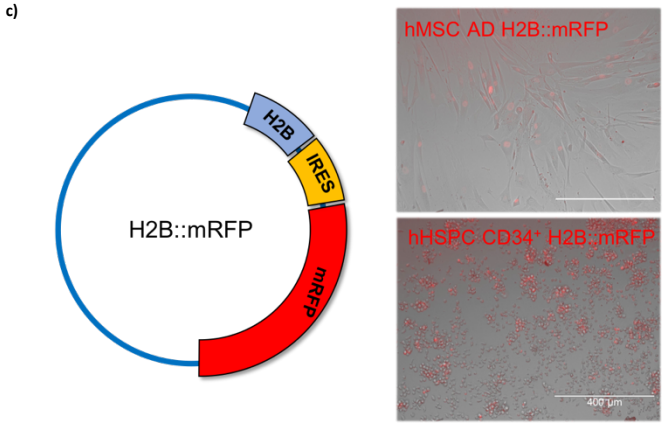


Figure 14. Infection of the MGCs by AAV2/8 virus and lentiviral infection of the stem cells. a) co-immunostaining of eGFP from the adeno-associated infection (Rheinwald and Green) and CRALBP hMGC marker (magenta) by Spectra view analysis. This colocalization confirmed the specificity of AAV2/8 virus infecting hMGCs. Scale: 50µm; **b)** FACS analysis showing the wavelength emission of every single cell when excited with the laser 488 nm. In the AAV infection plot we detected a small GFP population through FACS techniques. Scale: 400µm. **a)** and **b)** Sample analyzed, n = 2. **c)** Scheme of the H2B::mRFP lentiviral vector used for the infection of the selected adult stem cells, the hMSC AD and hHSPC CD34+. Representative images of hMSC AD and HSPC CD34+ H2B::mRFP infected. Scale: 400 µm.

We then proceed with the selection and labeling of the stem cells. hMSC AD and hHSPC CD34$^+$ were selected as adult stem cell types. To label them, we generated a vector carrying the histone H2B tagged with monomeric red fluorescence protein (mRFP) that was used for lentiviral infection (Figure 14c).

Cell fusion between human Müller glial cells and different adult stem cell types can be identified in human retinal tissues

Although we confirmed AAV2/8 viruses infective competence of the hMGCs, we aimed to increase the infection specificity by engineering AAV2/8-CMV-eGFP construct. Published studies showed that a ShH10Y modification in the capsid AAV could increase the human Müller specificity infection (Pellissier et al. 2014). With this information, we decided to change the capsid of our AAV2/8-CMV-eGFP into AAV2/ShH10Y. We

also changed the CMV promoter by the retinaldehyde binding protein 1 promoter (pRLBP), that it is specific of hMGCs. With the AAV2/ShH10Y-pRLBP_eGFP construct, we ensured that only those cells expressing pRLBP promoter would be GFP^+ when infected.

We then performed cell fusion experiments by co-culturing overnight the hMSC AD H2B::mRFP with the human retinae infected with AAV2/ShH10Y-pRLBP_eGFP (Figure 15a). After the overnight co-culture, samples were prepared for its disaggregation and subsequent FACS analysis. Through this analysis, cells that were positives to GFP and RFP (GFP^+RFP^+) were identified as hybrids. We also included as control experiment the retina (GFP^-) which was not co-cultured with hMSC AD H2B:mRFP (RFP^-).

Results showed that the control condition had a small GFP^+RFP^+ population, due to the autofluorescence and apoptotic cells left in the sample (Figure 15b, upper panel). However, AAV2/ShH10Y-pRLBP_eGFP infected retina (GFP^+) co-cultured with hMSC AD H2B:mRFP, showed a clearly defined GFP^+RFP^+ population (Figure 5b, lower panel). Therefore, to verify that the GFP^+RFP^+ hybrids were a product of cell fusion, we performed an Odd Ratio (OR) analysis (Bland and Altman 2000). In brief, OR is a measure of association between an exposure (co-culture AAV2/ShH10Y-pRLBP_eGFP infected retina with hMSC AD H2B:mRFP) with an outcome (to have GFP^+RFP^+ hybrids). Therefore, OR>1 indicates an increased occurrence of the outcome with a given exposure. Results showed that OR from both GFP^+ and GFP^+RFP^+ hybrids were higher than 1 with values of 3,06 and 3,78, respectively, indicating that these identified populations were obtained through the experimental conditions (exposures) and double confirmed the GFP^+RFP^+ hybrid population (Figure 15c).

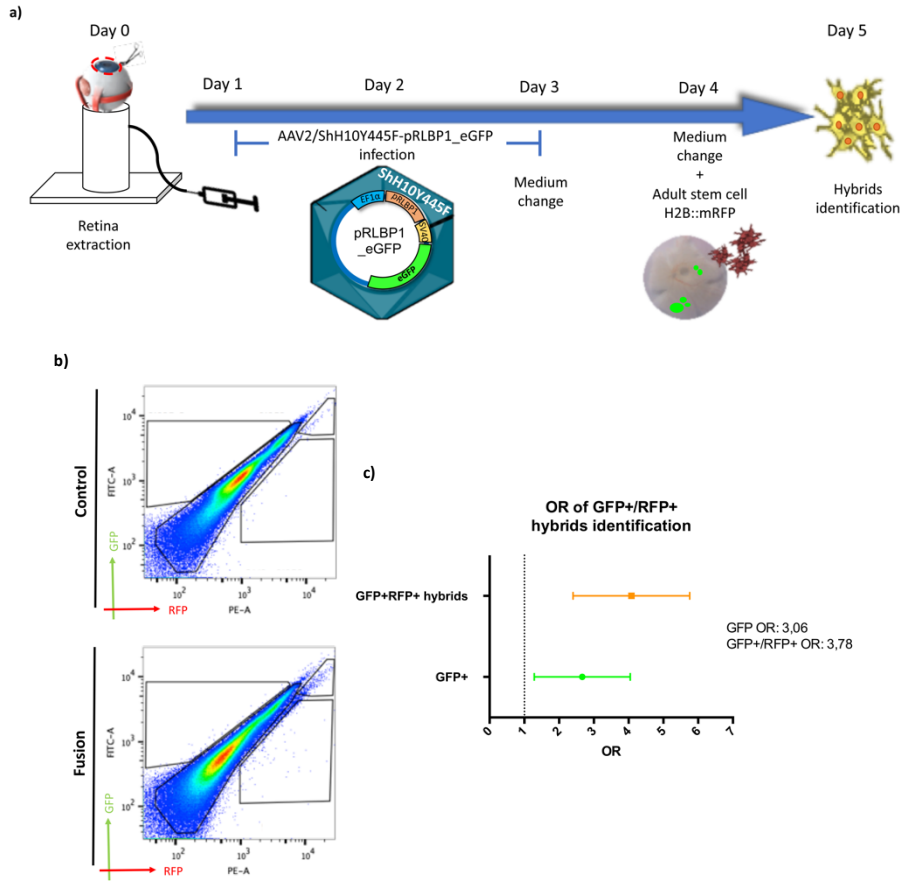


Figure 15. Fusion events identification in human retina organotypical tissue. a) Experimental scheme of cell fusion assessment co-culturing adult stem cells H2B::mRFP with AAV2/ShH10Y-pRLBP_eGFP infected retina **b)** FACS analysis of fusion events between human retina AAV2/ShH10Y-pRLBP_eGFP labelled and hMSC AD H2B::RFP (below) and the control autofluorescence retina (above). Fused population $GFP^{+}RFP^{+}$ preset a more defined population on the infected and co-cultured retina. **c)** Odd Ratio of GFP+ cells and $GFP^{+}RFP^{+}$ hybrids identification by FACS analysis. Data is presented as individual values, mean ± SEM from n = 3 independent experiments.

In addition to using FACS for identifying $GFP^{+}RFP^{+}$ hybrids, we aimed to confirm the presence of hybrids by triple marker co-localization in the human retina, both in whole mount retina and human retina sections (Figure 16). To perform the whole mount retina immunostaining, the AAV2/ShH10Y-pRLBP_eGFP infected retina (GFP^{+}) co-cultured with

hMSC AD H2B::mRFP were used. Thus, human retina samples upon AAV2/ShH10Y-pRLBP_eGFP infection were stained against GFP, hMSC-AD H2B::mRFP were stained against RFP and finally, hMGCs were stained against CRALBP Müller glial specific marker.

Moreover, to further examine the hybrid capabilities of interaction within the tissue, after co-staining the GFP (from AAV2/ShH10Y-pRLBP_eGFP infection), RFP (from hMSC AD H2B::mRFP) we also performed a the Tuj-1 (Ganglion cell specific marker) staining. We confirmed the presence of hybrids by triple marker ($GFP^+RFP^+CRALBP^+$) co-localization in the human retina, both in whole mount retina (Figure 16a) and human retina sections (Figure 16b). In addition, we found a faint Tuj-1 co-localization signal together with GFP^+RFP^+ hybrids, suggesting the possibility of hybrid differentiation into a neural phenotype in both human whole mount staining's (Figure 16c) and retinae sections (Figure 16d).

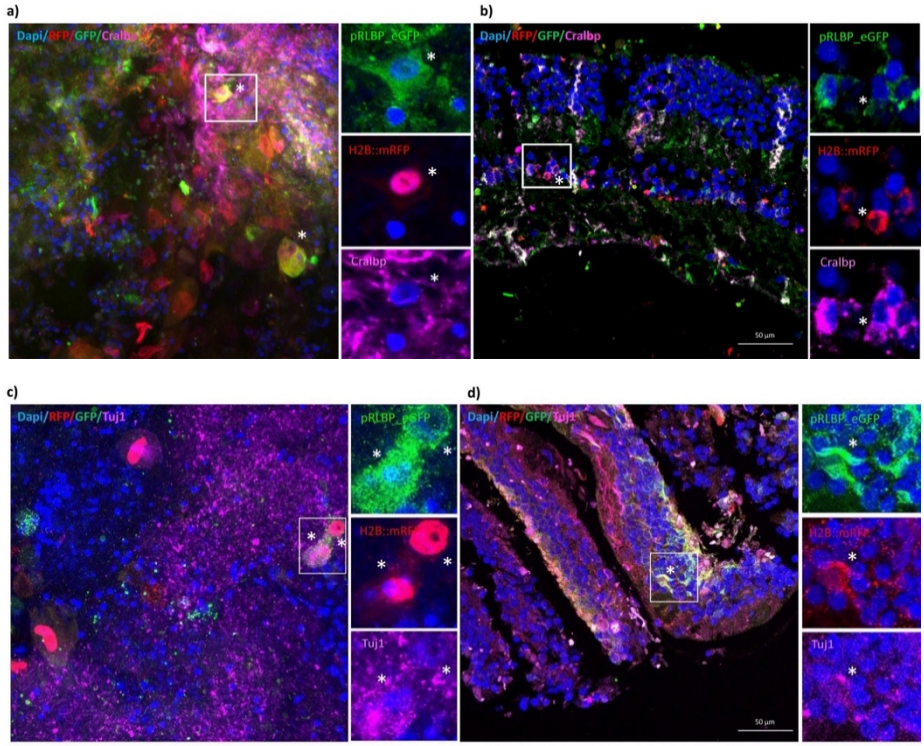


Figure 16. Fusion events detected in human retinae between human mesenchymal stem cells adipose derived with hMGC and faint ganglion marker expression. Colocalization in organotypic culture of GFP, RFP and Far Red staining against AAV2/ShH10Y-pRLBP_eGFP, H2B::mRFP and CRALBP, respectively, in whole mount retinae **a)** and sections **b)**. Asterisk are pointing to the hybrid cells. Colocalization in organotypic culture of GFP, RFP and Far Red staining against AAV2/ShH10Y-pRLBP_eGFP, H2B::mRFP and Tuj-1, respectively, in whole mount retinae **c)** and sections **d)**. Asterisk are pointing to the hybrid cells.

The same fusion phenotype reveled by triple co-localization with CRALBP has been identified in both whole mount staining's and human retinae sections after fusion with another adult stem cell type, the hHSPC CD34^{+} (Figure 17). Due to the scarce availability of this kind of samples, immunostaining experiments against Tuj-1 could be performed. Thus, we are still in the process of gathering enough data. However, considering the progenitor nature of hHSPC CD34^{+} and the results shown by hMSC AD hybrids, we speculate that the hybrids resulting from hHSCP CD34^{+} fusion might have a comparable potential to acquire neural features.

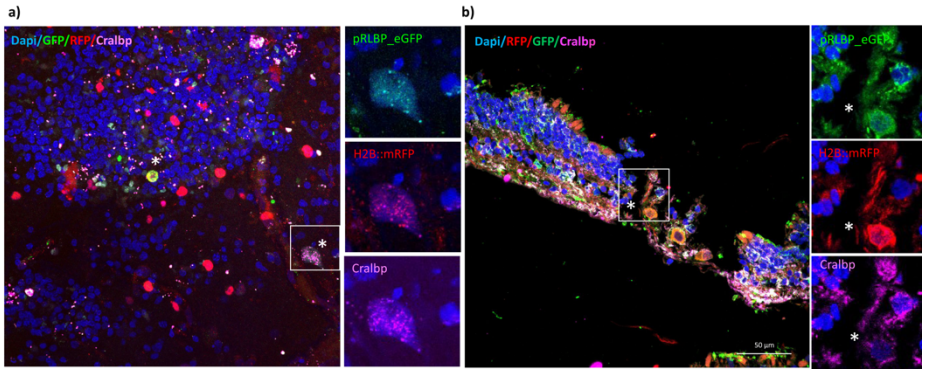


Figure 17. Fusion events with hHSPC CD34+ in human retinae with Müller glia partners. Colocalization in organotypic culture of GFP, RFP and Far Red staining against AAV2/ShH10Y-pRLBP_eGFP, H2B::RFP and CRALBP respectively, in whole mount retinae **a)** and sections **b)**. Asterisk are pointing to the hybrid cells.

Part II: Investigating whether damage can enhance *In vitro* cell fusion and the role of the Wnt/β-catenin pathway on the fate of the hybrids

Having identified fusion events between hMGCs infected with AAV2/ShH10Y-pRLBP_eGFP and hMSC AD H2B::mRFP, both by FACS and immunostaining in retinal explants, as well as fusions between hMGCs and hHSPC $CD34^+$, we aimed to a further understanding of the fusion process. To deeply interrogate the fusion complexity, we performed fusion *in vitro* studies, focusing on both fusion partners: hMGCs with hMSC AD or with hHSPC $CD34^+$.

Isolation, characterization and manipulation of human Müller glial cells

To study cell fusion *in vitro*, we first had to set up the isolation and the cell line establishment for the hMGC.

We first isolated the hMGCs from the human retinal organotypic explant. After that, we had to establish the primary cell line of hMGCs. To do that, we took advantage of the adherent capacity of the hMGCs. We carried out the isolation slightly modifying the protocol from *Limb. A et al* (Limb et al. 2002). Briefly, after disaggregating the retina we passed the pool of cells through a sieve and left the filtered cells in culture. After three passages, we obtained a cell line to characterize and work with (Figure 18).

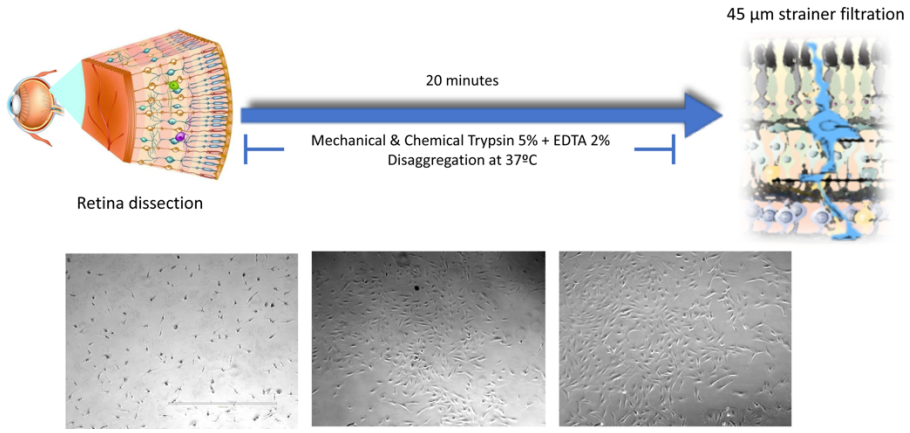


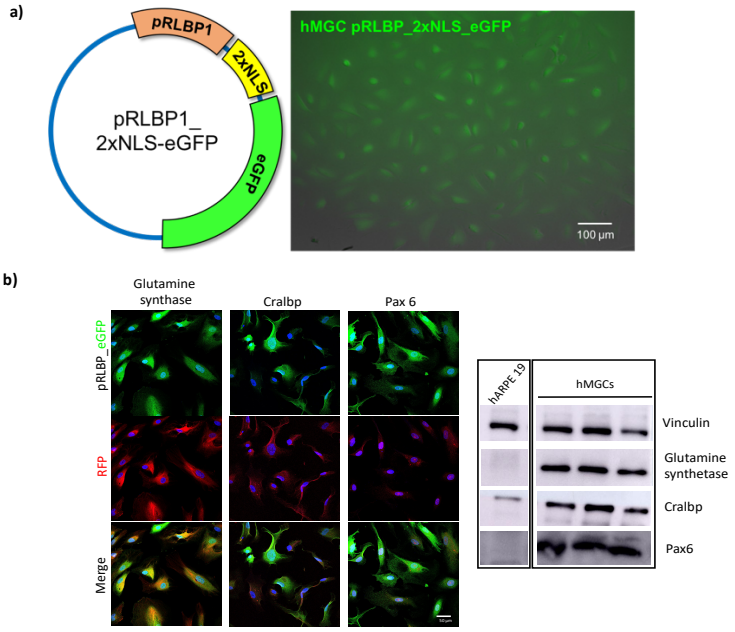
Figure 18. Human Müller Glia Cells isolation and culture. Representative scheme of hMGCs isolation process from human retinae. In the lower panel, cell line establishment of hMGCs: First picture after filtering the hMGCs at passage 1. At passage 3 the hMGCs were stable to culture.

After culturing the hMGCs *in vitro*, we aimed to confirm that the culture was enriched in hMGCs. For this we designed a plasmid expressing GFP under the specific Müller glial promoter pRLBP (so only the hMGC could be GFP^+). Furthermore, we included into the plasmid two nuclear localization signals (2xNLS) that produced a dimmer nuclear signal. Subsequently, we infected hMGCs with the obtained plasmid using lentiviruses. Finally, we sorted the GFP^+ cells (Figure 19a).

In the sorted human GFP^+ MGCs, we tested expression of Glutamine synthase (GS) glial marker, a specific MGC marker (CRALBP) and a neuro-precursor marker (Pax6) both by immunostaining and Western Blot (WB). The WB analysis was done taking samples of three different passages of the hMGCs and comparing them against the human adult retinal pigmented epithelium cell line (hARPE 19) as a negative control for GS and Pax6 and positive control for CRALBP (Figure 19b). In the case of the immuno-characterization, we used a secondary antibody linked to GFP for the pRLBP_eGFP and a secondary RFP for the specific markers. We performed immunostainings against two markers of the glial family: the glial fibrillary acidic protein (GFAP) from the astrocytes and the ionized calcium

binding adaptor molecule 1(IBA1) for the microglia. As it shown it Figure 19c, pRLBP_eGFP co-localized with dim RFP signal indicating the presence of glial family cells. Finally, expression of two markers not belonging to the glial family were also evaluated. We selected Calretinin as neuronal marker and Rhodopsin for photoreceptors. In this case, no signal was found discarding the presence of non-glial cell types in the generated cell line. (Figure 19c). Altogether, these results revealed that our generated primary cell line was hMGC.

Having characterized the hMGC cell line (hMGC_pRLBP_2xNLS_eGFP), to study the fusion, we used as cell fusion partners the hMSC AD H2B::mRFP and the hHPSC CD34^{+} H2B::mRFP (Figure 14c).



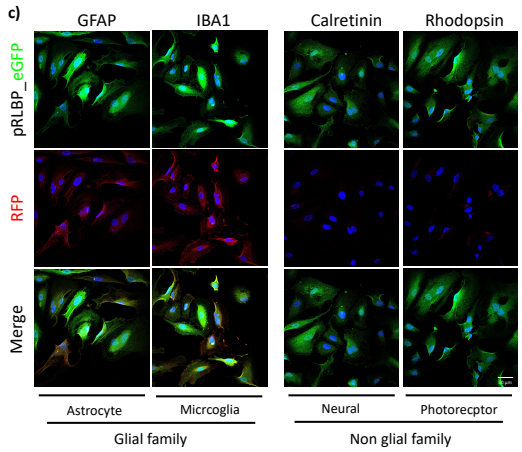


Figure 19. Human Müller Glia Cells characterization . a) Cloning performed to assess cell fusion *in vitro*. Specific eGFP labelling under human Müller glia promoter pRLBP. MGC characterization by **b)** immunostaining against glial marker glutamine sintase (GS), the specific Müller glia marker (CRALBP) and the neuro-precursor marker (Pax6) (left side panel) and **Western Blot** of the same markers in the human MGC line and using the human adult retinal pigmental epithelium cell line (hARPE19) as control (right side panel) **c)** Further immunocharacterization of the hMGC cell line (hMGCs_pRLBP_eGFP). Evaluation of glial markers such as glial fibrillary acidic protein (GFAP) expressed by astrocytes and ionized calcium binding adaptor molecule 1 (IBA1) expressed by microglia cells. Non-glial family markers were also invesrigated such as Calretinin and Rhodopsin expressed by neural and photoreceptor cells, respectively.

Wnt/β-catenin pathway can be activated in the human mesenchymal stem cells adipose-derived previous to fusion

Previous results from our laboratory in rodent models showed an increased reprogramming efficiency when Wnt/β-catenin pathway is activated (Sanges et al. 2013; Sanges et al. 2016) . In our experimental approach, we activated the Wnt/β-catenin pathway by Chiron, an agonist of Wnt signaling, triggering a downstream cascade of events resulting in the upregulation of transcription factors such as Axin2, Sox9 or c-Myc (Figure 20a). Therefore, we evaluated the expression level of these transcription factors by quantitative real time polymerase chain reaction (qRT-PCR) by treating hMSC AD with three different concentration of Chiron: 1μM, 3μM and 6μM. As it could be seen in Figure 20b, Axin2 and Sox9 were

significantly overexpressed when the cells were treated with 6μM Chiron in comparison with the DMSO control treatment. Moreover, , we could also observe a slightly increased expression of C-Myc not canonical Wnt factor. Additionally, phenotype changes of the treated cells were not observed at any of the different Chiron treatment (Figure 20c).

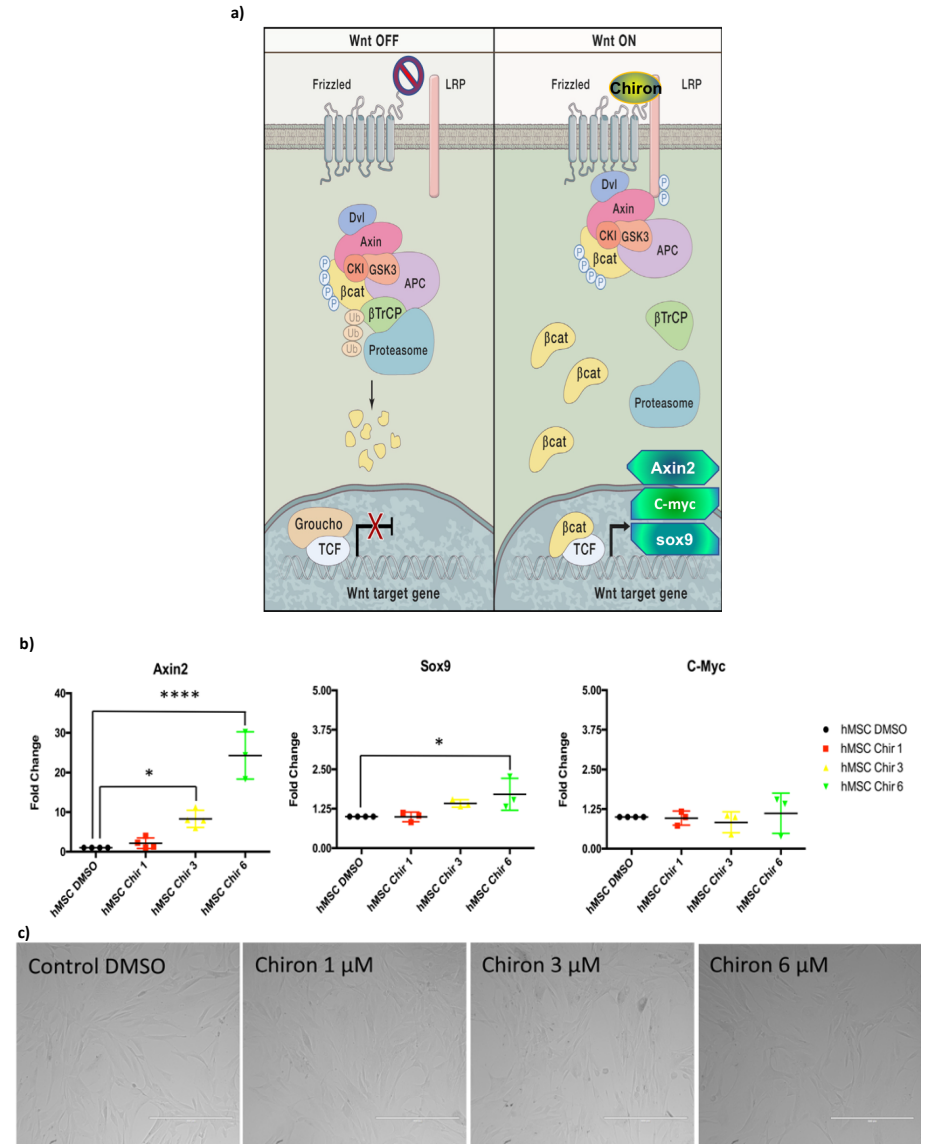


Figure 20. Wnt/β-catenin pathway activation in human Mesenchymal Stem Cells. a) Scheme modified from *Nuse R. et al.*, showing the activation of the Wnt pathway. Briefly, when the pathway is activated it results in the transcription of downstream factors. **b)** Scatter Plots showing the fold change expression of the Axin2, Sox9 and C-Myc for the Chiron β-catenin/Wnt pathway activation

of hMSCs at three different concentrations (1μM, 3μM and 6μM). DMSO treatment was used as control. **c)** Confocal microscopy images of hMSCs with the control treatment (DMSO) or at different Chiron concentration (1μM, 3μM and 6μM) showing that morphology was not affected by increasing Chiron concentration. Data is represented as individual values, mean ± SEM from n ≥ 3 independent experiments. One-way Anova Dunnett's multiple comparison was used for statistical analysis. * p_{value}< 0.05; **** p_{value}<0.0001.

In vitro cell fusion between hMGCs_pRLBP_2xNLS_eGFP and hMSC AD H2B::mRFP or hHSPC CD34$^+$ H2B::mRFP is enhanced by pro-inflammatory damage conditions while is not affected by the Wnt/β-catenin pathway activation of the adult stem cells.

We next performed *in vitro* cell fusion experiments. Briefly, first we growth a monolayer of hARPE 19 cells, which are cells resembling the epithelial retinal tissue. One day before starting the experiment (day -1), we treated them with NMDA to mimic a damage condition or with phosphate buffered saline (PBS) as control condition. At day 0, we collected the enriched medium from the NMDA damage or PBS control conditions. In parallel with the enriched medium collection, we had the cell fusion partners in culture. We detached the hMSC AD H2B::mRFP or hHSPC CD34$^+$ H2B::mRFP as well as the hMGCs_pRLBP_2xNLS_eGFP. We mixed them in a ratio 1:1, 500.000:500.000 cells of each partner fusion cell type (hMGCs_pRLBP_2xNLS_eGFP x hMSC-AD H2B::mRFP or hHSPC CD34$^+$ H2B::mRFP) in a total volume of 200 microliters (μL) of hARPE 19 NMDA-treated or PBS-treated conditioned media. Mix of cells was left in suspension during 45 min at 37ºC to promote cell fusion. After that, cells were plated in a flask overnight with the previously collected enriched media (NMDA or PBS). At day 1, plated cells were analyzed by detaching and incubating them during 1h with Hoechst 33342 (Figure 21a). After that, to purify the hybrid population and apply a high selection, we analyzed and sorted the hybrids using a two steps analysis. First, we selected

the hybrids as those cell population expressing both GFP from the hMGCs and RFP from the hMSC-AD or hHSPC CD34^{+}, thus our initial hybrid population was GFP^{+}RFP^{+}. The second layer of selection was applied considering the DNA content measured by Hoechst 33342. Since we expected the fusion of two cells, the hybrids should have at least a DNA content of 4n or higher, thus we selected a population of hybrids defined as GFP^{+}RFP^{+} 4n hybrids (Figure 21b).

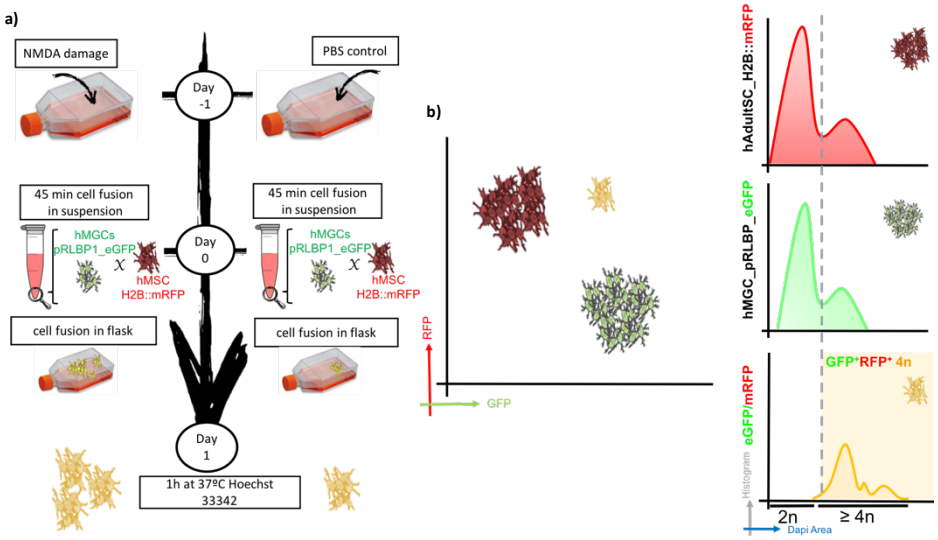


Figure 21. Experimental set up for *In vitro* fusion assessment. a) Scheme of the fusion protocol for *in vitro* fusion assessment under two different conditions: PBS control and NMDA damage conditioned media. **b)** Identification and cell-sorting of the GFP^{+}RFP^{+} 4n *in vitro* hybrids by FACS analysis

Importantly, we first assessed if NMDA treatment in hARPE 19 monolayer cells induced a pro-inflammatory phenotype through the NMDA receptors (Ola et al. 2001; Reigada, Lu, and Mitchell 2006) . We observed that after the NMDA or control treatment, hARPE 19 did not show morphological changes (Figure 22a). In addition, in hARPE 19 we analyzed by qRT-PCR the expression of the pro-inflammatory factor IL1β after 24h of treatment. Results showed that NMDA treatment induced a significant increase of IL1β in comparison with the PBS control condition. Moreover, this

increase was not followed by an increased expression of the NMDA receptor (Figure 22b).

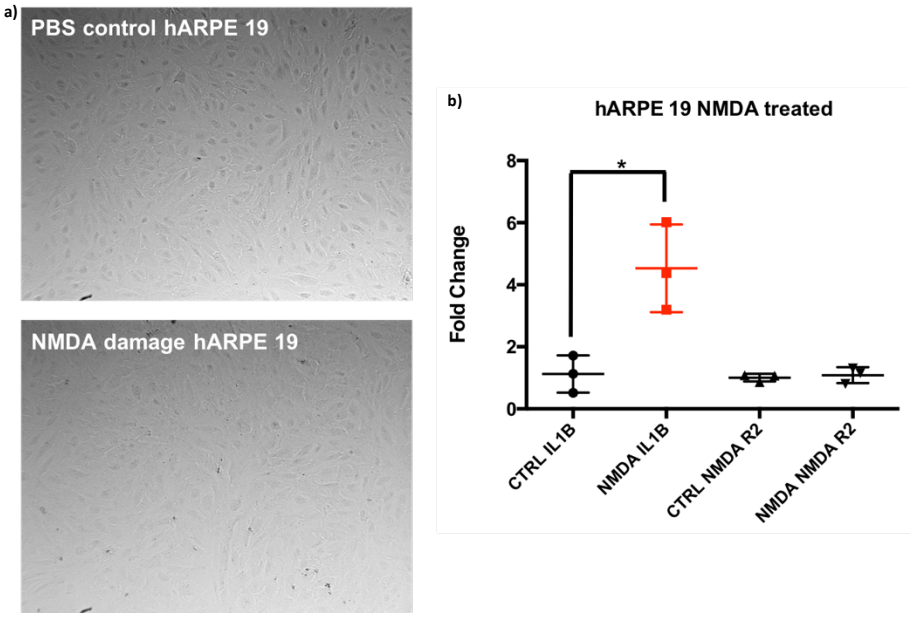


Figure 22. NMDA Chemical damage resembling a pro-inflammatory environment. a) hARPE 19 cells grown in monolayer under control or NMDA damage conditions morphology. **b)** Expression analysis of the pro-inflammatory chemokine IL1β and NMDA receptor 2 (NMDAR2) by qRT-PCR in treated hARPE 19 cells under control (CTRL) or NMDA damage conditions. Data is presented as individual values, mean ± SEM from n = 3 independent experiments. Unpaired T-test was used for statistical analysis. * p_{value}< 0.05.

In vitro cell fusion between hMGCs_pRLBP_2xNLS_eGFP and hMSC-AD H2B::mRFP is enhanced in pro-inflammatory damage conditions

It was previously showed in mouse models the importance of the Wnt/β-catenin pathway on the hybrids reprogramming (Sanges et al. 2013; Sanges et al. 2016). Then we tested if: (1) NMDA pro-inflammatory conditions promoted cell fusion efficiency, increasing the hybrids percentage and, (2) if the Chiron treatment of adult stem cell enhanced the fusion percentage or the reprogramming of the hybrids.

Obtained results by FACS analysis confirmed that PBS control conditions showed lower percentage of $GFP^{+}RFP^{+}$ 4n hybrids when compared with NMDA pro-inflammatory conditions indicating that NMDA-derived conditioned media enhanced the cell fusion efficiency (Figure 23a and b). Moreover, we asked whether Chiron-mediated Wnt/β-catenin pathway activation increased the cell fusion efficiency. We found that fusion of adult stem cells treated by Chiron was not increased, suggesting that fusion is enhanced by pro-inflammatory conditions and not by the Chiron activation of the adult stem cells (Figure 23c).

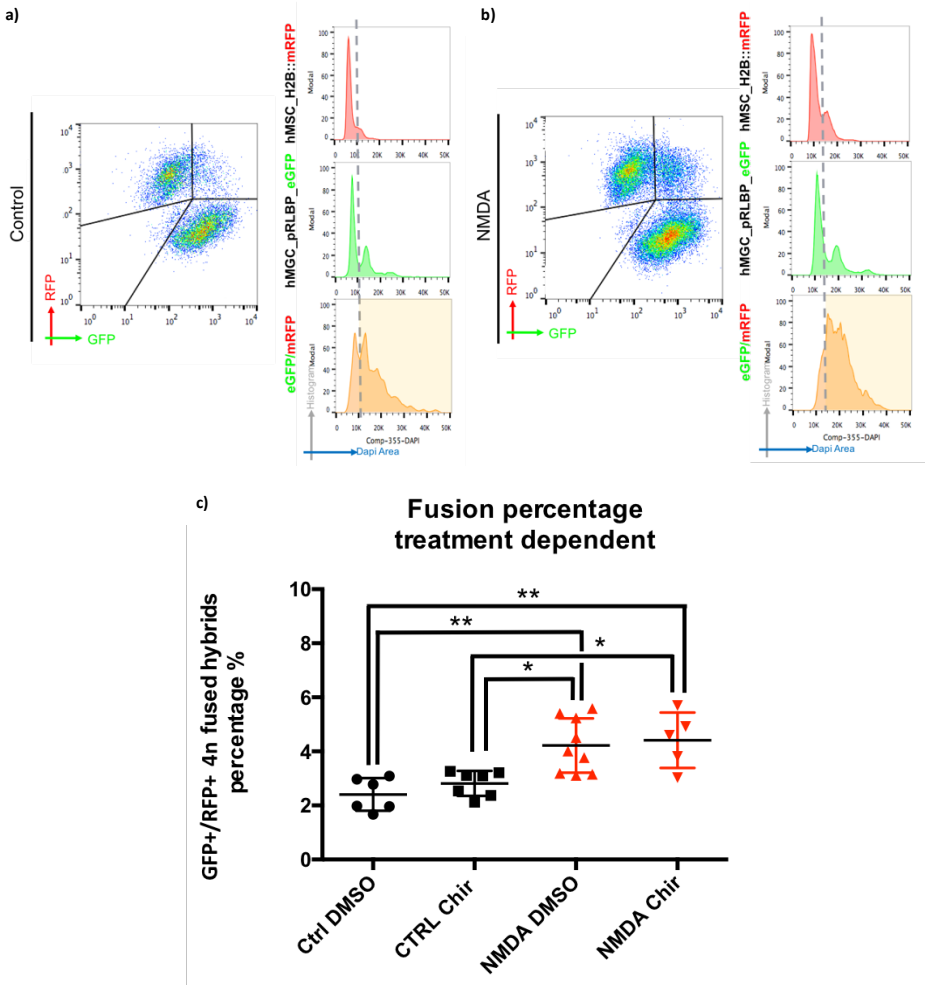
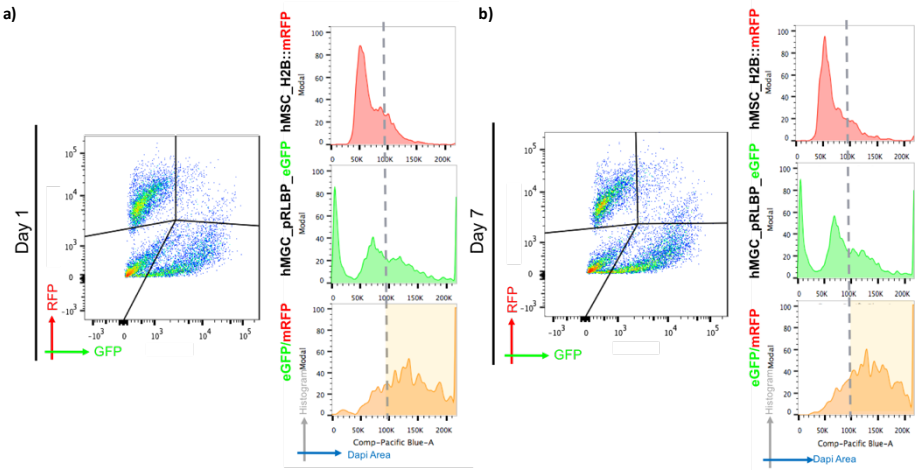


Figure 23. Increase of *in vitro* fusion percentage in pro-inflammatory damage conditions for hMGCs_pRLBP_2xNLS_eGFP x hMSC-AD H2B::mRFP fusion partners. FACS analysis of fusion percentages. FACS plots of **a)** PBS control condition and **b)** NMDA damage condition gating the GFP^+RFP^+ population and the subsequent gating of the hybrids with 4n or increased ploidy (orange squares). **c)** FACS data quantification of 4n GFP^+RFP^+ hybrids resulting from PBS control medium conditions (with and without Chiron-dependent β-catenin/Wnt pathway activation) and NMDA damage medium conditions (with and without Chiron-dependent β-catenin/Wnt pathway activation). Data is presented as individual values, mean ± SEM from n ≥ 5 independent experiments. One-way anova Dunnett's multiple comparison was used for statistical analysis. * $p_{value}< 0.05$; ** $p_{value}<0.01$.

Next, we asked if we could observe comparable results in cell fusion experiments when harvesting medium just after 1 day (D1) of human retinal organotypic culture as "natural control" (Figure 24a) and comparing it with medium collected after 7 days (D7) of culture as "natural damage" (Figure 24b). Additionally, we also tested Chiron-mediated Wnt/β-catenin pathway activation of the hMSC AD in the fusion efficiency. Also, in these experiments, cell fusion percentage was increased upon culturing in "natural damage" conditions (D7), as compared with "natural conditions" (D1). Additionally, the Chiron-mediated Wnt/β-catenin activation of the hMSC AD did not affect the cell fusion percentage (Figure 24c).



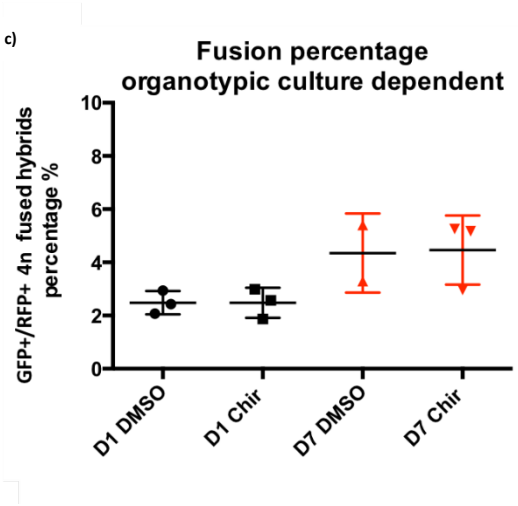


Figure 24. ***In vitro*** **fusion percentage in "natural control" condition (day 1, D1) and "natural damage" conditions (day 7, D7). a)** FACS analysis at D1 of the natural control condition. Gating of the GFP+RFP+ population and the subsequent gating of the hybrids with 4n or higher ploidy. **b)** FACS analysis at D7 of the natural damage condition. Gating of the GFP+RFP+ population and the subsequent gating of the hybrids with 4n or higher ploidy. **c)** FACS data quantification of 4n GFP+RFP+ hybrids resulting from natural control medium conditions (with and without Chiron-mediated β-catenin/Wnt pathway activation) and natural damage medium conditions (with and without Chiron-mediated β-catenin/Wnt pathway activation). Data is presented as individual values, mean ± SEM from n ≥ 2 independent experiments.

Next, we compared cell fusion efficiency when using conditioned media from the culturing of the retinae or conditioned media after NMDA or PBS treatment of the hARPE 19 cells. Interestingly, when comparing the effect of conditioned medium collected from retina at D1 and the medium from PBS-treated hARPE 19 cells we did not observe any difference in cell fusion percentage (Figure 25a). Similarly, we did not observe any difference on cell fusion when using the NMDA-conditioned medium and the D7 conditioned medium collected from retinae (Figure 25b). Additionally, Chiron activation of the Wnt pathway did not alter the cell fusion percentage in any of the treatments (Figure 25a & b). Taken together these results, we concluded that cell fusion efficiency is enhanced only by the pro-inflammatory conditions and the differences are consistent with the two models used.

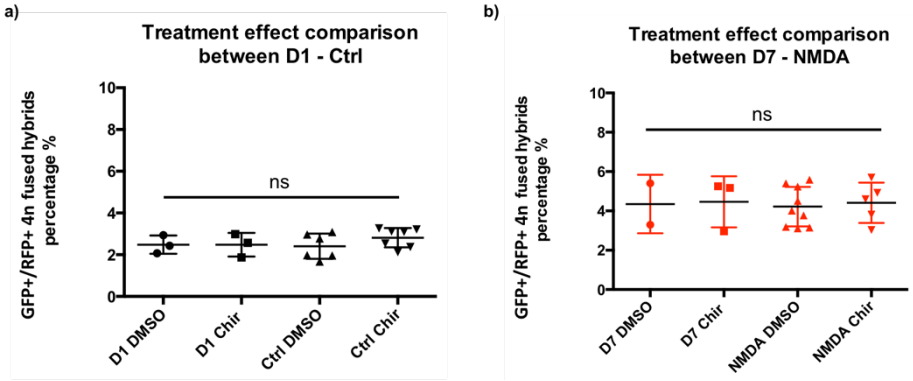


Figure 25. ***In vitro*** **fusion percentage was comparable between natural and chemical conditions and it was not affected by Chiron-mediated β-catenin/Wnt pathway activation of human MSC AD.** FACS analysis of fusion percentages. **a)** Comparison of 4n GFP^+RFP^+ hybrids percentage obtained with PBS control medium harvested from hARPE 19 cells and medium harvested from human retinae organotypic culture for 1 day (D1). **b)** Comparison of 4n GFP^+RFP^+ hybrids percentage obtained with medium harvested from NMDA-induced damage of hARPE 19 cells (NMDA) and with medium harvested from human retinae organotypic culture for 7 days (D7). **a & b)** Comparison of Chiron-mediated β-catenin/Wnt pathway activation between fusion conditions and the control (DMSO). Data is presented as individual values, mean ± SEM from $n \geq 2$ independent experiments. One-way annova was used for statistical analysis. ns = non-significant

In vitro cell fusion between hMGCs_pRLBP_2xNLS_eGFP and hHSPC $CD34^+$ H2B::mRFP is enhanced in pro-inflammatory damage conditions as observed for the fusion of hMSC AD H2B::mRFP.

As previously discussed we observed fusion in organotypic cultures in human retinae between hMGCs infected AAV2/ShH10Y-pRLBP_eGFP and hHSPC $CD34^+$ H2B::mRFP (Figure 17). First, we investigated whether fusion efficiency of hHSPC $CD34^+$ H2B::mRFP was also enhanced by the pro-inflammatory NMDA damage conditions. As mentioned at the beginning of this section, GFP^+RFP^+ 4n hybrids were sorted with our set up method (Figure 21). Similarly to what observed with hMSC AD, we found a significant increase of GFP^+RFP^+ 4n hybrids in the NMDA damage condition in comparison with the PBS control conditions (Figure 26a). Unfortunately, the scarce availability of hHSPC $CD34^+$ samples

precluded us to evaluate the effect of the Wnt/β-catenin pathway activation in this set of experiments. On the other hand, the results obtained using hMSC AD showed that Chiron treatment does not affect cell fusion, thus we speculated that Chiron had no effect in enhancing fusion of hHSPC $CD34^+$.

In conclusion, by comparing PBS control conditions (DMSO and Chiron) and NMDA damage conditions (DMSO and Chiron) we asked if the adult stem cell type could influence the efficiency of *in vitro* cell fusion with hMGCs. Remarkably, no differences were observed in fusion percentage when comparing hHSPCs $CD34^+$ or hMSC AD both in NMDA and in control conditions. Significant differences only appear when comparing NMDA damage against PBS control conditions, further confirming that only the damage enhances the fusion efficiency (Figure 26b).

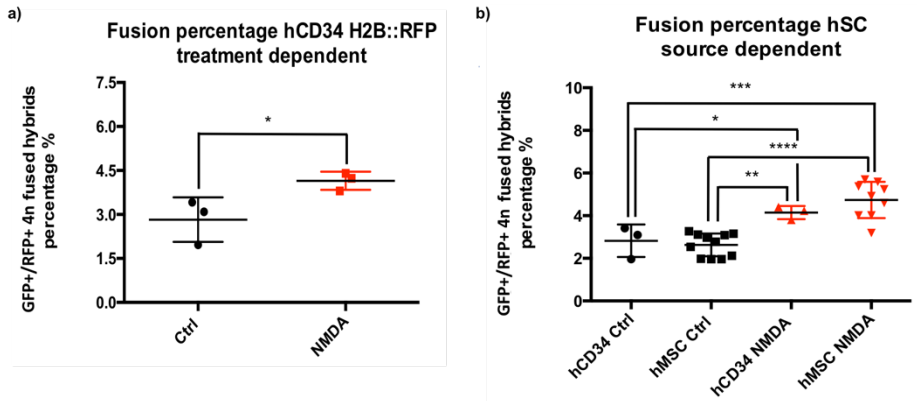


Figure 26. *In vitro* fusion percentage of hMGCs_pRLBP_2xNLS_eGFP and hHPSC $CD34^+$ H2B::mRFP fusion partners. Efficiency follows a comparable trend when compared to the fusion of hMSC AD H2B::mRFP a) Data quantification of 4n GFP^+RFP^+ hybrids resulting from PBS control medium and NMDA damage medium for hHPSC $CD34^+$ H2B::mRFP (named as hCD34). Data is presented as individual values, mean ± SEM from n≥3 independent experiments. Unpaired T-test was used for statistical analysis. **b)** Comparison of 4n GFP^+RFP^+ hybrids fusion percentage between hHPSC $CD34^+$ H2B::mRFP and hMSC-AD H2B::mRFP upon PBS control or NMDA damage conditions. Data is presented as individual values, mean ± SEM from n≥3 independent experiments. One-way annova Dunnett's multiple comparison was used for statistical analysis. * p_{value}< 0.05; ** p_{value}<0.01; *** p_{value}<0.001; **** p_{value}<0.0001.

In vitro sorted GFP$^+$RFP$^+$ 4n hybrids can undergo reprogramming and differentiation towards a neural-like phenotype

Our results show that cell fusion is enhanced by a pro-inflammatory signal but is independent of Wnt/β-catenin pathway activation. Notwithstanding, previous data in mice from the laboratory showed that the activation of Wnt/β-catenin pathway in the stem cells induced increased reprogramming of the hybrids (Sanges et al. 2013; Sanges et al. 2016). Then, we hypothesized that also in the human context the Chiron-dependent Wnt/β-catenin pathway activation of the hMSC AD H2B::mRFP could increase the reprogramming of the resulting hybrids. To quantify the reprogramming effect we used our engineered cell line hMGC_pRLBP_2xNLS_eGFP (Figure 19). We aimed to test whether the GFP$^+$RFP$^+$ 4n hybrids forced to differentiate, would silence hMGC markers, thus shutting off the GFP under the Müller glial specific promoter pRLBP. We then cultured the GFP$^+$RFP$^+$ 4n hybrids obtained after culturing with PBS control conditions or with NMDA medium towards ganglion cell differentiation using 20 ng/ml human fibroblast growth factor 2 (hFGF2) and 50 μM of *NOTCH* inhibitor called DAPT (Singhal et al. 2012). We then performed live cell imaging of the differentiating GFP$^+$RFP$^+$ 4n hybrids for 24 hours using the Andor XD Revolution Spinning disk confocal microscope. As control we imaged hybrids in control not differentiating medium.

In brief, we had 8 different conditions to image, with either activation or not of the Wnt pathway and either differentiation or not-differentiation culturing condition (Figure 27).

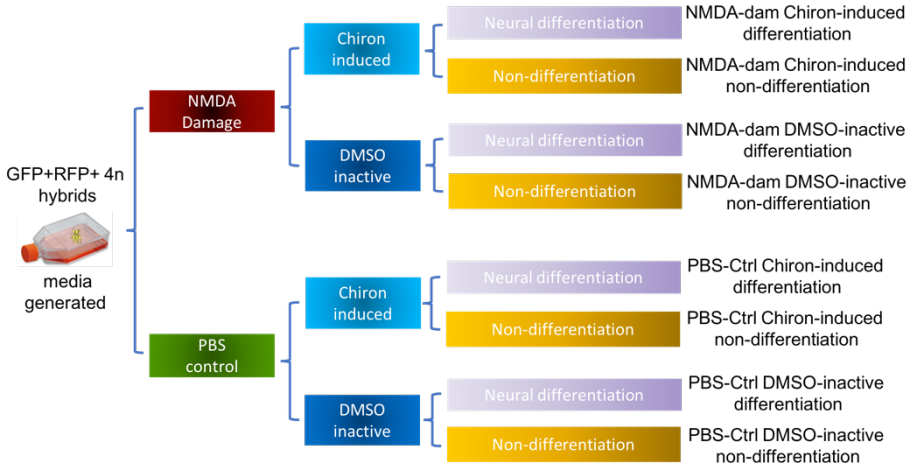


Figure 27. Decision tree of the GFP+RFP+ 4n hybrids treatment. Decision tree of the 8 different conditions to which the hybrids were subjected to interrogate their differentiation potential.

The hybrids generated in in PBS control condition cultured in non-differentiation media did not acquire appreciable morphological changes upon 24 hours. Even though, they showed slight changes in the soma, after Chiron treatment (Figure 28, upper rows). Interestingly, both the Chiron-induced and DMSO-inactive hybrids cultured in differentiation medium, showed a neural like phenotype, they displayed an elongated soma and emitted projections resembling podocyte-like structures (Figure 28, lower rows).

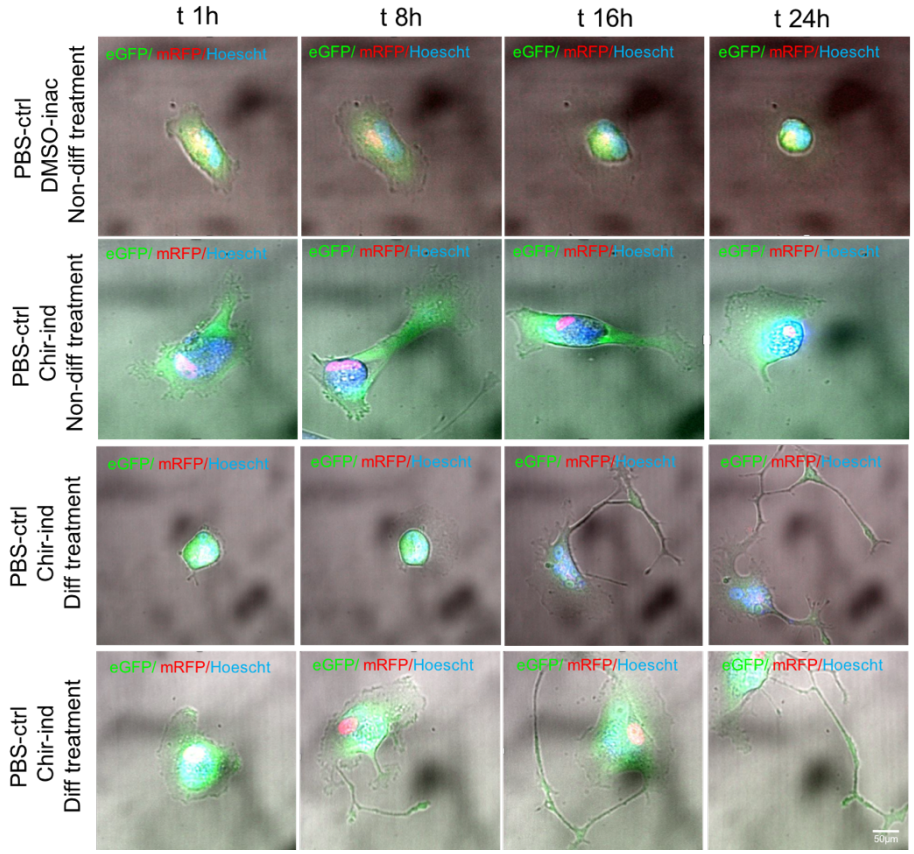


Figure 28. GFP⁺RFP⁺ 4n hybrids from PBS control medium conditions differentiate into neural like phenotype when cultured in the presence of human fibroblast growth factor 2 (hFGF2) and NOTCH inhibitor (DAPT) differentiation treatment for 24 hours. Both 4n DMSO-inactive and 4n Chiron-induced hybrids showed no differences in morphology without differentiation treatment as it is shown in the first and second row, respectively. 4n DMSO-inactive hybrids showed phenotype changes when treated with FGF2/DAPT differentiation treatment (third row) as well as 4n Chiron-induced hybrids which exhibit drastic changes in morphology showing podocyte-like structures (last row). Representative experimental images are shown. eGFP from the pRLBP of hMGC and RFP from the H2B of the hMSC AD.

Moreover, we observed a comparable differentiation capacity in the hybrids generated with NMDA-damage medium. In the case of GFP^+RFP^+ 4n hybrids exposed to non-differentiation conditions, we did not identify major changes but, in both Chiron-induced and DMSO-inactive, we observed soma expansion (Figure 29). Interestingly, in the case of the differentiation of DMSO-inactive GFP^+RFP^+ 4n hybrids, we identified the formation of aberrant and poor podocyte-like structures as well as an

important soma expansion. It appears that this type of hybrids generated in NMDA-damage conditioned medium but without Chiron-induction even if forced to differentiate, show an aberrant morphology (Figure 29, 3rd row). On the other hand, in the NMDA-damage Chiron-induced $GFP^{+}RFP^{+}$ 4n hybrids we noticed a morphological differentiation toward ganglion cells since the hybrids exhibited some polarized thin axon-like projections (Figure 29).

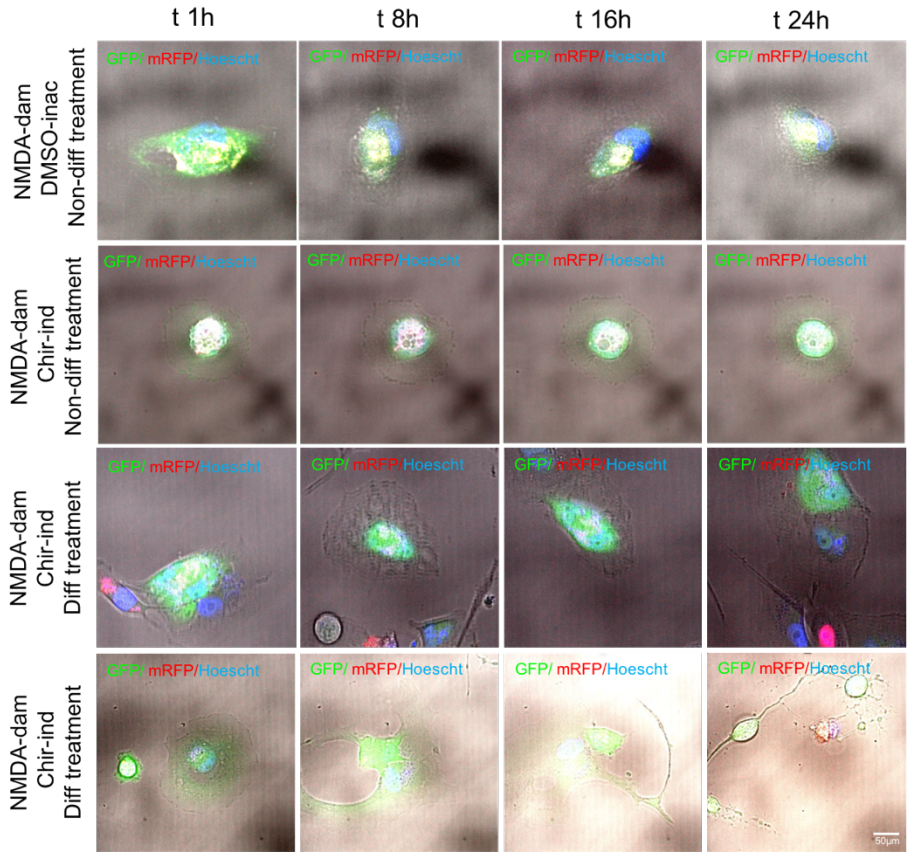


Figure 29. GFP+RFP+ 4n hybrids obtained with NMDA damage conditioned medium differentiate into neural like phenotype when cultured in the presence of human fibroblast growth factor 2 (hFGF2) and NOTCH inhibitor, (DAPT) differentiation treatment for 24 hours. Neither 4n DMSO-inactive nor 4n Chiron-induced hybrids (1st and 2nd row, respectively) showed morphological differences when cultured without differentiation treatment. 4n DMSO-inactive hybrids (3rd row) showed a slight phenotype elongation changes when treated with differentiation treatment (FGF2/DAPT) whereas 4n Chiron-induced hybrids exhibit drastic changes in morphology showing podocyte like structures at both sides of the nuclear body of the cell. Representative experimental images are shown. eGFP from the pRLBP of hMGC and RFP from the H2B of the hMSC AD.

Next, we quantified the GFP intensity decrease over time of the non-differentiation controls and the differentiation treatment of the GFP^+RFP^+ 4n hybrids generated under PBS-control or NMDA-damage conditioned media. As mentioned before, the GFP silencing is a proxy of hMGC loss of identity. To analyze the data, the GFP fluorescence intensity was normalized by the initial fluorescence of each cell imaged 1 hour after the start of the time course imaging experiment. This initial value was considered as 100% and we studied the decrease in signal in time for the different samples.

First, we investigated how the GFP expression changed in the hybrids cultured in differentiation medium (hFGF2/DAPT). Hybrids generated in PBS-control medium cultured under non-differentiation conditions showed a slight fluctuation in the expression of the GFP signal, moreover there was no differences among the Chiron-induced or the DMSO-inactive hybrids. In contrast, the hybrids cultured under differentiation conditions exhibited a significant decrease of GFP expression, which dropped to 40% of intensity at 24 hours. As in the case of the non-differentiation conditions, no differences were observed among the Chiron-induced or the DMSO-inactive hybrids (Figure 30a).

For the hybrids generated with NMDA-damage conditioned medium, the results obtained were slightly different. First, surprisingly the NMDA-damage DMSO-inactive hybrids showed a significant decrease of the GFP expression in non-differentiating medium. Considering the morphological analysis obtained, we hypothesized that the hybrids cultured with NMDA-damage conditioned medium, can acquire astrocyte-like features and down-regulate GFP expression to 50% level. On the other hand, the NMDA-damage Chiron-activated hybrids under control non-differentiating conditions did not show GFP decrease. Importantly, under differentiation

conditions, both Chiron-induced and DMSO-inactive hybrids responded to the differentiation stimuli since they showed GFP decrease (Figure 30b).

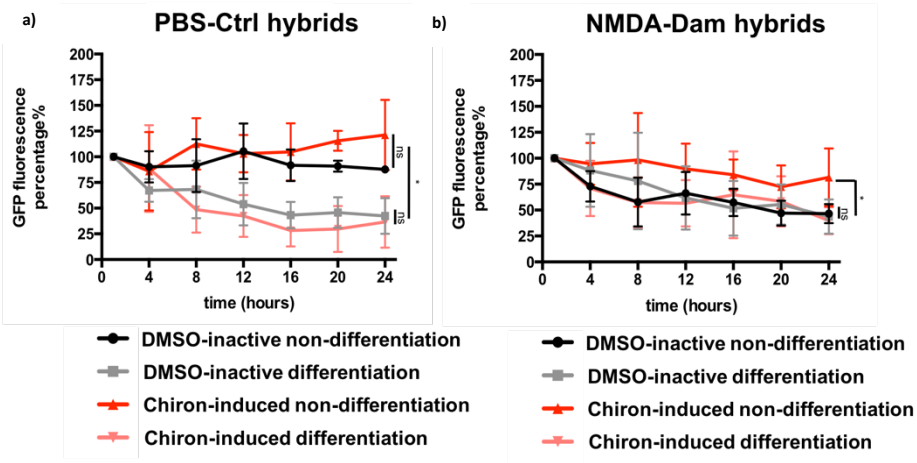


Figure 30. GFP fluorescence percentage in hybrids generated in control and NMDA damage conditions. a) The GFP fluorescent percentage measured in hybrids generated in DMSO-inactive or Chiron-induced conditions and under non-differentiating or differentiating media. **b)** GFP fluorescent percentage measured in NMDA-damage conditioned medium hybrids generated in DMSO or Chiron under non-differentiating control conditions or differentiating conditions. Data is presented as grouped values, mean ± SEM from n ≥ 3 independent experiments. One-way anova Dunnett's multiple comparison was used for statistical analysis. ns = non-significant; * p_{value}<0.05.

Finally, for an in-depth analysis, we compared the 4n hybrids cultured in differentiating medium. Under non-differentiating control condition, we could observe that only the NMDA-induced/DMSO-inactive hybrids showed a significant GFP decrease to 60% of intensity as early as 8 hours. These results were consistent with the previous observations, suggesting that the NMDA-damage condition, in where the hybrids were generated, promotes their differentiation. Interestingly, the GFP^+RFP^+ 4n hybrids generated in PBS-control condition, both Chiron-induced/DMSO-inactive, and exposed to non-differentiating control medium, were fluctuating around values of 100% GFP intensity but did not show significant changes (Figure 31a).

In conclusion in the differentiation medium the four types of hybrids showed a significant GFP decrease to more than 50%. Interestingly, when

hybrids were generated in the presence of Chiron we could identify a slightly higher GFP decrease. Furthermore, the GFP decrease showed a faster kinetics, decreasing already at the 8 hour time point, in the case of Chiron-induced and for both NMDA damage or PBS control hybrids, while DMSO-inactive hybrids show significant decrease of GFP signal only at the 12 hour time point (Figure 31b).

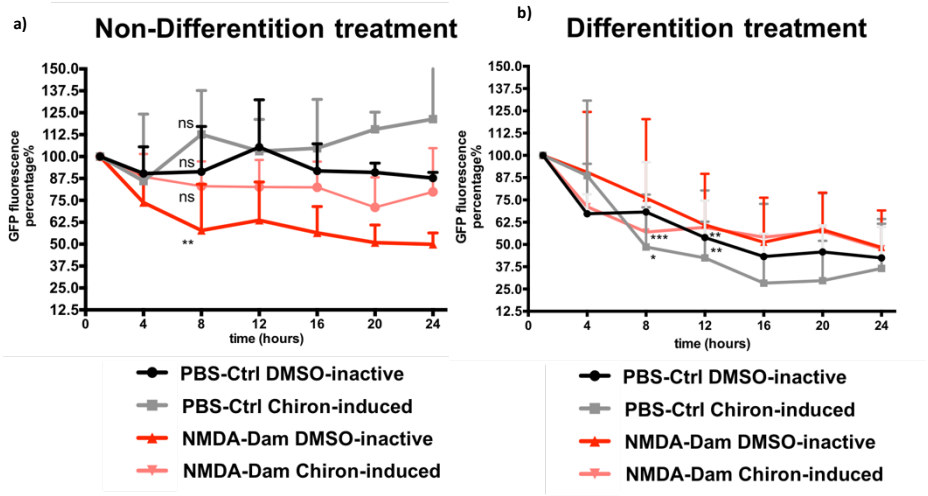


Figure 31. GFP fluorescence percentage of the hybrids under non-differentiation control or DAPT/FGF2 differentiation treatment. a) GFP fluorescent percentage measured under non-differentiation control treatment in PBS-control or NMDA-damage generated hybrids, although not significant. **b)** GFP fluorescent percentage decrease under differentiation treatment in PBS-control or NMDA-damage generated hybrids. Chiron-induced hybrids were the earliest responders of the differentiation treatment. Data is presented as grouped values, mean ± SEM from n ≥ 3 independent experiments. Two-way annova Tukey's multiple comparison was used for statistical analysis ns = non-significant; * p_{value}<0.05; ** p_{value}<0.01; *** p_{value}<0.001.

Overall, we identified the pro-inflammatory medium as a trigger to enhance fusion efficiency. Moreover, we also found that Chiron activation of the hMSC AD adult stem cells did not affect fusion percentage. In addition, we sorted out the hybrids with a new experimental method and cultured them under differentiation conditions while imaging them. From these experiments, we could identify the hybrids differentiation potential according to morphological changes towards a neural-like phenotype. Interestingly, when measuring the GFP decrease expressed from the

specific hMGC promoter pRLBP, we could identify a proper response to the treatment and also see that Chiron was able to promote an early response towards differentiation.

Therefore, we concluded that the GFP^+RFP^+ 4n hybrids have the potential to become neural-like cells able to rescue the degenerating retinal tissue.

Part III: *In vitro* generated GFP$^+$RFP$^+$ 4n hybrids show functional electrophysiological response and their differentiation can be tested *in vivo* after organoid injection

Summarizing the results obtained so far, we first identified cell fusion events in human retinal organotypic explants between hMGCs and both hMSC AD and hHSPC CD34$^+$. Interestingly, in the case of fusion with hMSC AD, the hybrids showed faint expression of the ganglion marker Tuj1, suggesting a first hint about the hybrid differentiation potential. Then, we characterized the fusion events between hMGCs and hMSC AD. We observed that cell fusion is promoted by a pro-inflammatory environment. Also, we could observe morphological phenotypical changes towards a neural phenotype when the hybrids were cultured under differentiation conditions. Moreover, we excluded the role of Chiron-mediated Wnt/β-catenin pathway activation in cell fusion efficiency however, we showed that the pathway activation can induce early reprogramming of the hybrids.

Ultimately, we aimed to study the functionality of the hybrids and their capacity to differentiate within the human retinal tissue, to suggest that cell fusion-mediated reprogramming of hMGCs with human adult stem cells can become a retinal regeneration or rescue strategy.

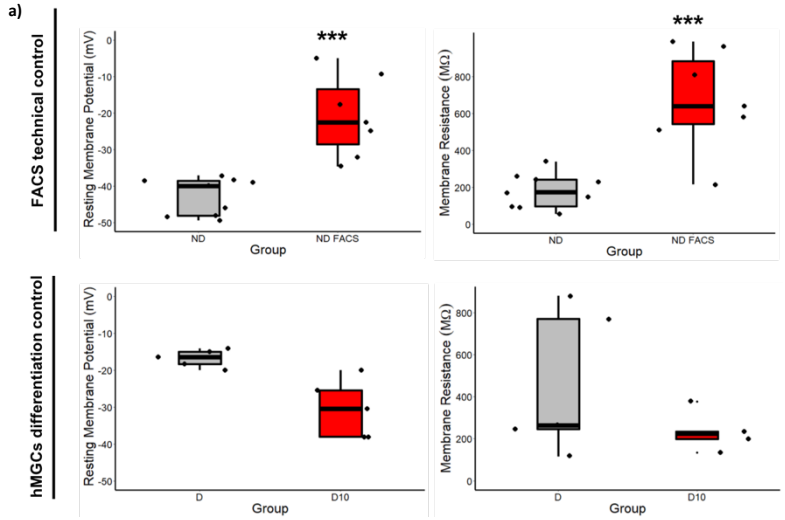
Patch clamp of *in vitro* ganglion cell-like differentiated hybrids showed cell membrane depolarization as a response to electrical stimulus

As it has been mentioned in part II results, GFP^+RFP^+ 4n hybrids showed ganglion cell-like phenotype when they were cultured in differentiation conditions. Thus, we wondered if this morphological phenotype was accompanied with a neuronal functionality. To investigate that, we used patch clamp techniques in order to characterize the electrophysiological response of the differentiated hybrids. Briefly, patch clamp experiments can give information about the membrane excitability, its functional properties and the densities of the ion channels by directly applying a current to the neural soma (additionally, also the dendrites can be targeted) (Bekkers 2000); (Davie et al. 2006); (Boinagrov et al. 2012). We analyzed the resting membrane potential, defined as the electrical potential differences across the membrane in a non-excited cell and the membrane resistance, that is a function of the number of open ion channels in the membrane. In a normal retinal ganglion cell, the resting membrane potential is around -60mV and the membrane resistance of 150 mΩ.

We are gathering results of GFP+RFP+ 4n hybrids that were put under differentiation (D) or non-differentiation (ND) treatment during two days and patch clamped. Non-sorted hMGC differentiated during two (D) or ten days (D10) were used as treatment control, and non-sorted or sorted hMGCs under non-differentiation conditions were used as the patch clamp technical control (Figure 32a). Interestingly, we could observe how the FACS procedure itself sensitizes the cells, both hybrids and hMGC controls. Additionally, in our system we could identify a better ganglion-like phenotype when culturing the hMGCs up to ten days, these exhibited an electrophysiological response that resemble a ganglion cells, showing a

resting membrane potential of -30 mV and a membrane resistance of 250 mΩ (Figure 32a, lower panel). Looking into our 8 conditions (figure 27) in the PBS-control generated hybrids we could not identify electrophysiological changes even though, both Chiron-induced and DMSO-inactive showed a lower membrane potential when compared with the non-differentiation condition (ND) (Figure 32b). NMDA-damage Chiron-induced hybrids under differentiation conditions exhibit a resting membrane potential even lower than hMGCs D10. In addition, they showed a membrane resistance of 400 mΩ (Figure 32c lower panel). On the other hand, the NMDA-damage DMSO-inactive showed a membrane resistance potential around -15 mV, pointing to an aberrant cell behavior.

To sum up, NMDA-dam Chiron-induced hybrids were the ones exhibiting better electrophysiological characteristics after two days of differentiation treatment. Enthrallingly this goes in line with our hypothesis, since the Chiron-activated hybrids generated in a pro-inflammatory (damage) condition should be the ones to replace damaged ganglion cells in the tissue.



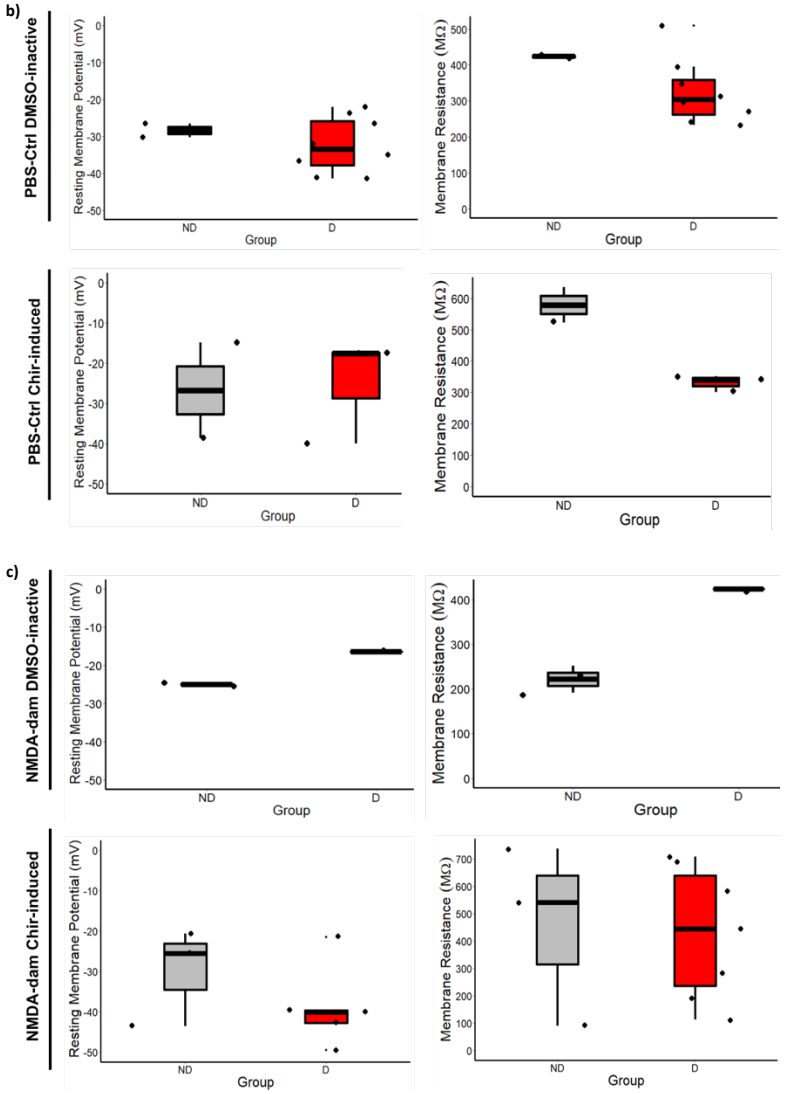


Figure 32. GFP⁺RFP⁺ 4n hybrids differentiated into neural-like phenotype showed membrane depolarization responding to increase electrical stimulation. Electro characterization through resting membrane potential and membrane resistance of **a)** FACS technical control and differentiation control on hMGCs **b)** GFP⁺RFP⁺ 4n hybrids generated under PBS-Control conditions and **c)** GFP⁺RFP⁺ 4n hybrids generated under NMDA-damage conditions. Data is presented as columns with single values, mean ± SEM from n ≥ 1 independent cells per condition. One-way anova Dunnett's multiple comparison was used for statistical analysis. ns = non-significant; *** p_{value}<0.001.

Testing the integration and differentiation of the hybrids in human retinal organoids.

Having characterized the hybrid differentiation potential towards ganglion-like phenotype *in vitro*, we wanted to investigate the differentiation *in vivo* within the retinal tissue, thus we used the human retina organoids. First, we had to engineer the stem cells to follow the hybrids differentiation. To do so, we engineered hMGC_pRLBP_2xNLS_eGFP cells and introduced a second fluorescence protein named Crimson, on Far-Red, under the expression of ganglion cell specific promoter, Tuj1. Hygromycin resistance cassette was also added to select the infected cells (Figure 33a). Therefore, we expected that the engineered cells (hMGCs-pRLBP_eGFP_Tuj1_Crimson) undergoing differentiation could decrease GFP intensity while increase the Far-Red Crimson signal (Figure 33b).

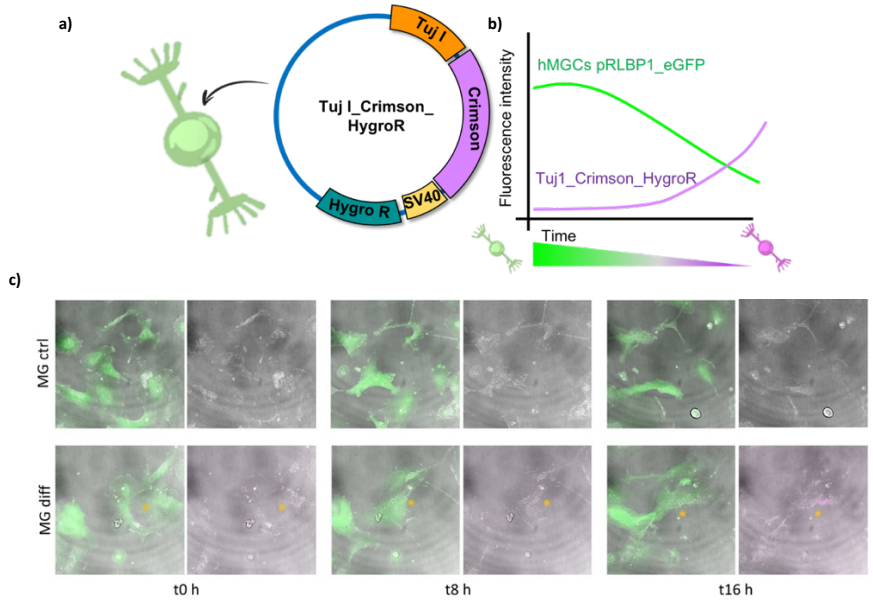


Figure 33. Engineering hMGC_pRLBP_2xNLS_eGFP cells to follow *live* cell differentiation of the GFP⁺RFP⁺ 4n hybrids. a) Plasmid for the introduction of the specific fluorescent protein Crimson (Far-Red) under the human ganglion promoter Tuj1 and Ampicillin resistance. **b)** Representative scheme of the differentiation strategy output. **c)** Representative images of hMGCs-pRLBP_eGFP_Tuj1_Crimson cells under non-differentiating conditions (upper row) or differentiating conditions (lower row) for 16 hours. Faint Tuj1_Crimson expression is marked with an asterisk.

We then aimed to inject the hybrids in the human retina organoids while ganglion cells are developing. This will allow us to track hybrid differentiation along with ganglion cell development.

Organoids were generated using a specific differentiation protocol. In brief, hESCs were plated in semi-confluent conditions, and cells were growing in islet-like structures from which the organoids started to develop (Figure 34, day 0). In the following day (Figure 34, day 6) the hESCs kept growing, built a monolayer and raised the first doom shape proto-organoids, composed by small spheres of neural precursor cells. Was from day 15 in advance when clear 3D structures could be identified growing on the top of hESC monolayer (Figure 34, day 15 & day 22) and from day 30 in advance was when the first lamination of the cell started to be identified (Figure 34, day 30) and build up and optic-cup like structure with small invaginations (Figure 34, day 42).. It has been reported that the initiation of differentiation in optic cups starts at day 30 of culturing and around day 35 at the time of the retinal lamination (Zhong et al. 2014). Thus, to identify the optimal micro-injection time point, we performed immune-staining against the ganglion specific marker β-III-tubulin in whole organoids, from day 35 to day 50, at four different time points: day 35, 40, 45 and 50. We identified the retina lamination in all time points analyzed although an intense immunostaining signal was detected at day 40 (Figure 25a). Additionally, organoids were sectioned and immuno-stained against β-III-tubulin (red) and against the glial family marker GS (Rheinwald and Green). GS^+ and β-III-tubulin$^+$ cells were quantified and we observed comparable numbers of GS^+ cells at all time points analyzed, while the ganglion cells, β-III-tubulin$^+$, formation increased significantly by day 40 (Figure 35b and c).

We also studied the β-III-tubulin^{+} cells location. As it could be observed in the images, all cells were found in the inner part of the organoid (yellow asterisk) at day 40, whereas this pattern was less defined at day 35, 45 and 50 in which cells were located in the inner (yellow asterisk) and outer (orange asterisk) part of the organoid (Figure 35b). Taken together, we concluded that day 40 was the best time point to perform the micro-injection.

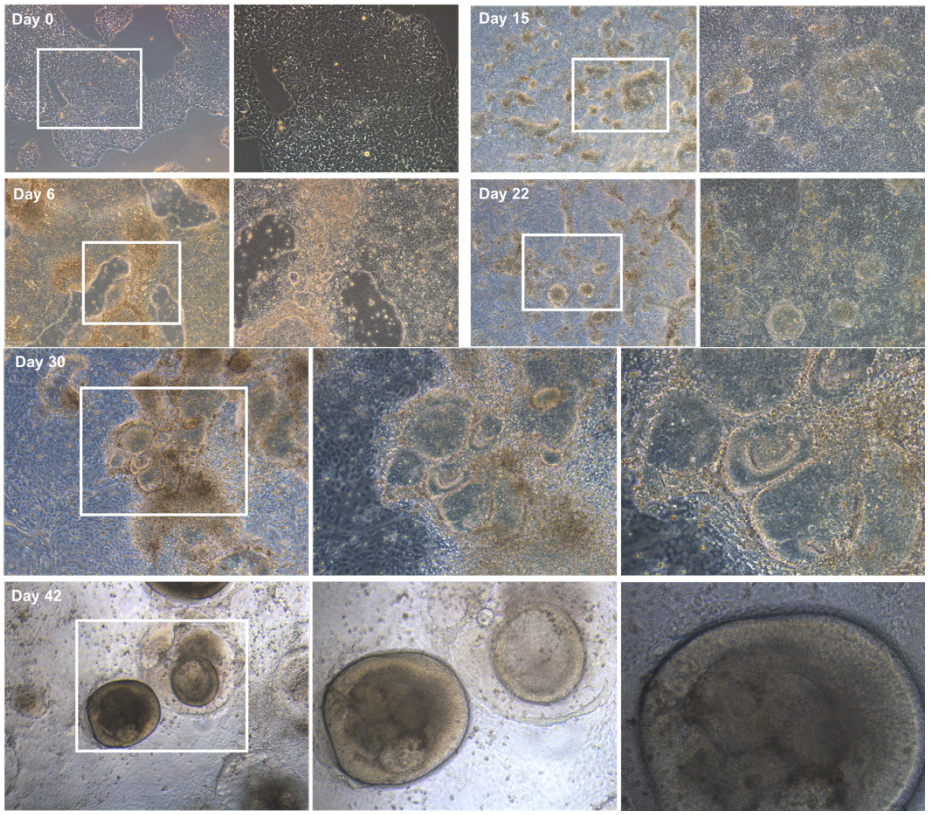


Figure 34. Organoid development from human ESC islets to optic-cup like structures. Representative image of developing organoids starting from human ESC. First 3D structures growing on top of human ESC monolayer are recognize from day 15, while spheroids appear at day 22 and human retinal tissue proto-laminated from day 30.

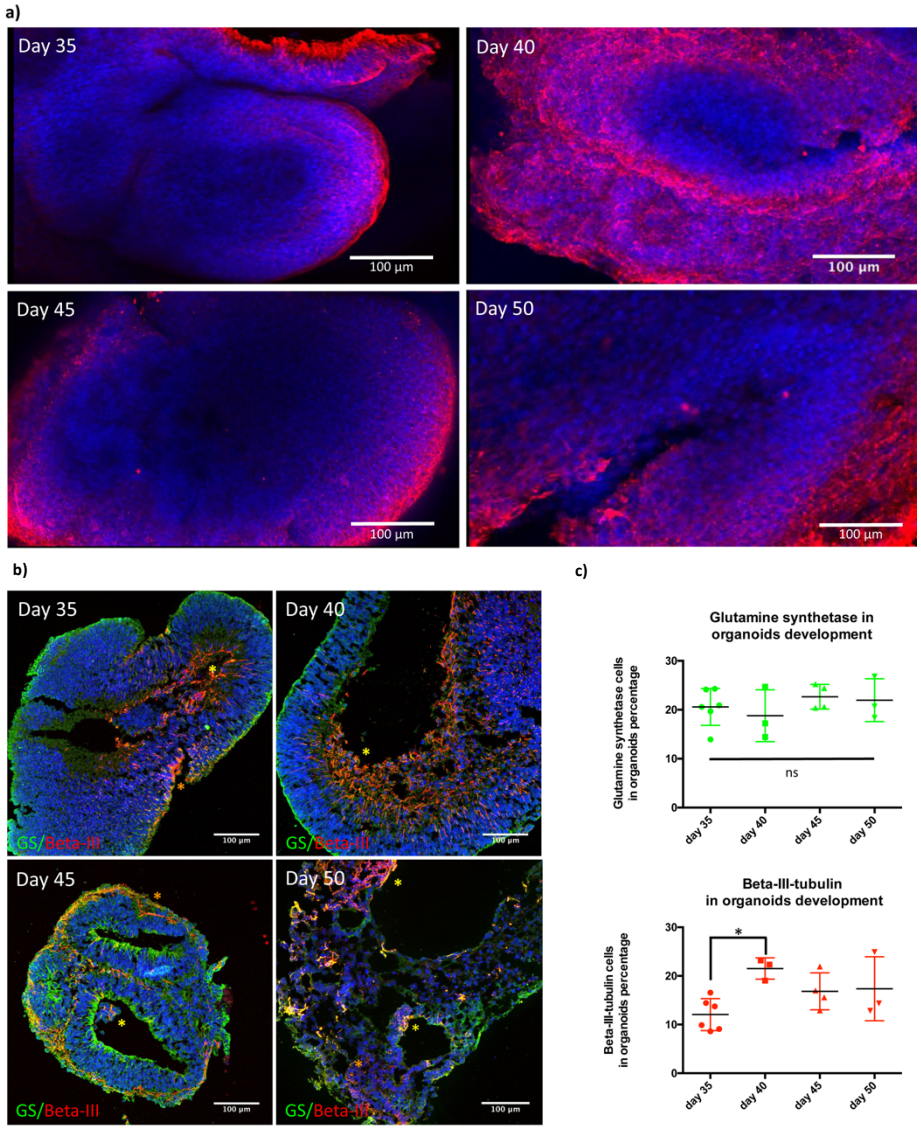


Figure 35. Ganglion cells development in developing human retina organoids. a) *In toto* RFP staining against β-III-tubulin marker tracing the ganglion development in growing organoids from day 35 to day 50. Representative experimental images are shown. **b)** Section immunostaining of β-III-tubulin (RFP staining) and GS (GFP staining) marker in growing organoids tracing the ganglion development and the glial cell development, respectively, from day 35 to day 50. **c)** Cell type quantification in growing organoids from day 35 to day 50. Data is presented as individual values, mean ± SEM from n ≥ 2 independent experiments (quantifying at least 2 random fields per time point and experiment). One-way anova Dunnett's multiple comparison was used for statistical analysis. ns = non-significant; * p_{value}<0.05.

Mesoscopic imaging after microinjection experiments of GFP^+RFP^+ 4n hybrid into a human retina growing organoids.

To put in contact the GFP^+RFP^+ 4n hybrids we adapted the embryo microinjection technique for the organoids. In this case, the goal was to micro-inject cells in the cellularized part of a growing organoid. In brief, GFP^+RFP^+ 4n hybrids to be micro-injected were resuspended in 10 µL of medium. Then, they were placed in suspension into a 35 mm petri dish and about 20-25 cells were aspirated by the injection pipette to perform the microinjection (Figure 36a). The injection was done in the bright retina laminated part of the organoid, fixed by a holding pipette. Cells were released and the exact number of cells released were quantified as well as the quality and reliability of the micro-injection (Figure 35b). After injection, the organoids were mounted in a low-melting agarose base and confined by a plastic capillary. Then, the organoids were imaged in medium without phenol red in the MuVi SPIM microscope for 24 hours mesoscopic imaging.

Based on the previous results and, in our hypothesis that hybrids generated under proinflammatory conditions will be the ones able to repopulate the cells of the damage tissue, we injected two different types of hybrids: NMDA-damage Chiron-induced and NMDA-damage DMSO-inactive. While we are still gathering results, first experiments showed that within the first 16 hours of imaging, the human retinae organoid exhibited a normal phenotype and were growing normally. Interestingly, the injected hybrids could be identified by the RFP signal expressed by the hMSC-AD H2B::mRFP and the GFP signal expressed by the hMGCS_pRLBP_2xNLS_eGFP (Figure 36c & d). For a close analysis of the hybrids, we focused on the 100µm volume where the GFP^+RFP^+ 4n hybrids were injected. Micro-injected NMDA-damage DMSO-inactive

hybrids and NMDA-damage Chiron-induced hybrids showed similar phenotype within the organoid. In this initial experiment, we could see a loss of GFP and RFP signals in time for both types of hybrids. Interestingly however, at 12 hours in the NMDA-damage Chiron-induced hybrids we could see some minor expression of FarRed signal (from the Tuj1 promoter). Further confirmation will be obtained with additional imaging experiments as well with section immunostainings of the micro-injected organoids.

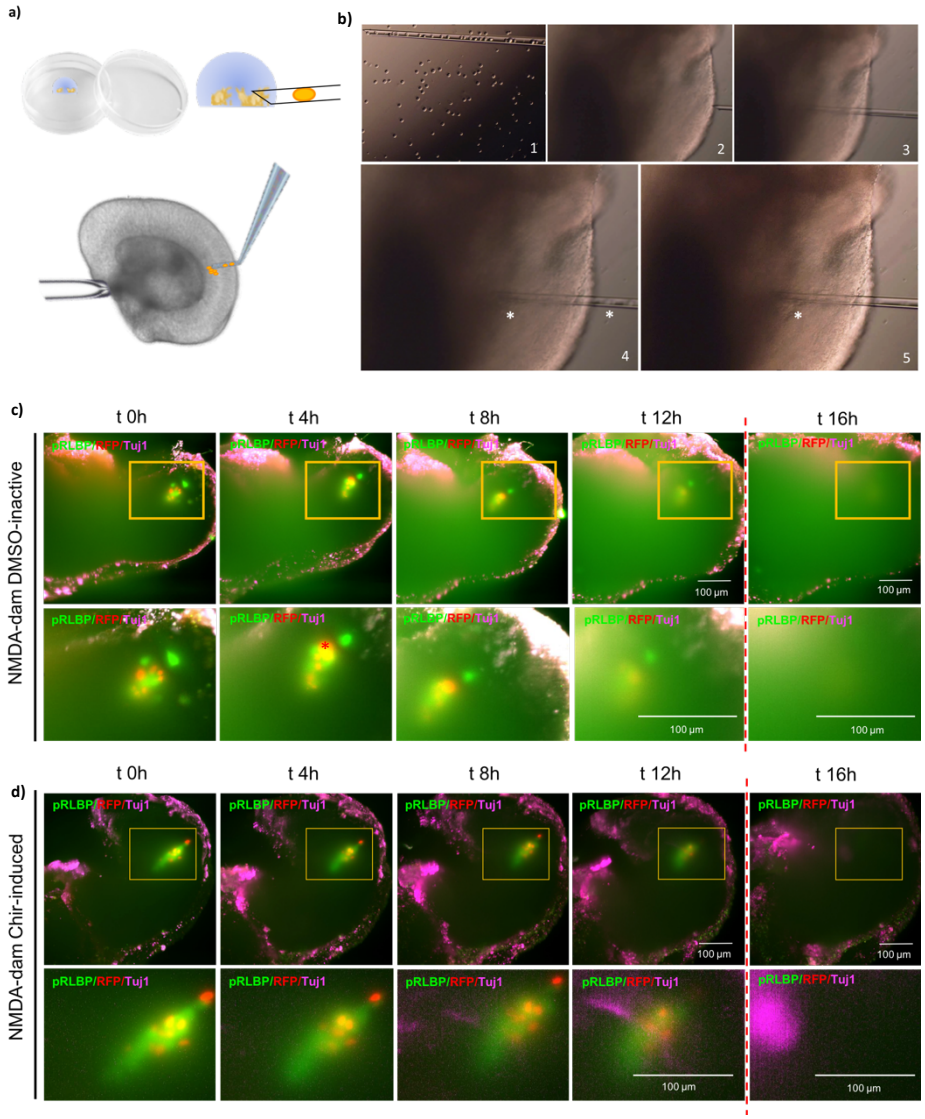


Figure 36. Micro-manipulation and micro-injection set up of the human retina organoids model. a) Scheme of sample micro-manipulation of the 4n GFP^+RFP^+ hybrids and the *in vivo* organoids. Cell aspirated one by one were kept in the micro-injection pipette (Black et al.). Holding pipette holds the organoid allowing the cell micro-injection by the micro-injection pipette from the opposite side (lower). **b)** Live images of the micro-injection procedure. (1) Cell loading into the micro-injection pipette. (2) Organoid micro-injection penetrating the surface. (3) Reaching the center with the microinjecting pipette (4) release of the microinjecting pipette in the cellularized part. Asterisk indicate the cells passing through the microinjection pipette. (5) Cells were released into the organoid and placed in the tissue. Asterisks indicate the cell placement. **c** & **d)** Representative image of the max-projection of growing organoids micro-injected with NMDA-dam DMSO-inactive hybrids and NMDA-dam Chiron-induced hybrids. Lower panels show a zoom-in of the injected hybrids. Cells on the organoid surface were labelled by VivoTrack 680.

With the established microinjection method, future experiments will be performed to investigate the hybrids differentiation within the tissue.

Overall, the experiments presented in this thesis point to the possibility of using cell-fusion mediated reprogramming of MGCs as a novel cell therapy approach to fight visual impairments.

DISCUSSION

Discussion

The unstoppable grow of the visual impairment cases highlights that loss of sight is an unmet medical need of the modern society that should be addressed. Among all the innovative treatments that have been studied, the stem cell therapy stands as an attractive option. It is well known the medical potential of the stem cells, since they are able to rescue the injured tissues, mostly by their paracrine effects, or also by replacing the dead cells through their differentiation into lineage specific cells of the damage tissue. Interestingly, it is also well known the regenerative potential of the retinal tissue, a potential that is gradually lost across evolution. Additionally, stem cell fusion has emerged over the last years as an exciting alternative for tissue regeneration, in a process were stem cells fuse with somatic cells and the resulting hybrids go back to a pluripotent state able to differentiate into lineage tissue specific cell types. Therefore, the retina regeneration potential could be stimulated through stem cell treatment.

In this work, we identified cell fusion events in human retina *ex vivo*. In addition, by studying the fusion process *in vitro*, we demonstrated an increase in cell fusion upon damage. Additionally, we proved the reprogramming of the new generated hybrids when the Wnt/β-catenin was activated in adult stem cells prior to fusion. Moreover, we observed that new-born hybrids had a potential to differentiate into a neural-like cells, exhibiting morphological changes resembling neurons and showing some electrophysiological characteristics. Finally, we set up a system to study the hybrid viability within a growing retinal tissue, as the human retina organoid, developing a micro-injection protocol to integrate the hybrid cells

into the growing tissue. This will allow us to assess the engraftment and differentiation potential of the hybrids in an *in vivo* system.

Cell fusion is emerging as a mechanism contributing to tissue regeneration. In fact, even if rare, cell fusion events have been detected in human tissues, often in post-mortem analysis. Concretely, fluorescence *in situ* hybridization experiments identified cell fusion events in different human tissues such as the liver, brain or gastrointestinal tract. (Silk et al. 2013; Theise et al. 2000; Weimann et al. 2003). Likewise, we could identify cell fusion events in human retinae. Our observations regarding the potential of the hybrids to undergo differentiation go in accordance with previous published data, highlighting the neural differentiation potential of the retinal progenitor cells when they were co-cultured with bone marrow-derived conditioned medium. This co-culture promoted either both neural differentiation of the adult stem cells into neural lineage and neural protection via paracrine effects (Goldenberg-Cohen et al. 2014; Mead et al. 2020; Okano et al. 2008; Tomita et al. 2006; Xia et al. 2013).

Despite the promising results obtained, adult stem cells also show some disadvantages. Thus, we must consider this issue when envisioning a putative treatment. Cell fusion process must be tightly controlled to enhance regeneration. On the other hand, we did not induce fusion, but the latter occurs spontaneously, as we also previously demonstrated in mice retina (Pesaresi et al. 2018; Sanges et al. 2013; Sanges et al. 2016). Indeed, our results demonstrate that fusion between MSCs and MGCs is enhanced in "natural damage", though the medium collection of human retinae organotypic cell cultures at day 7 or "chemical damage", by conditioned medium collected from hARPE19 cells damaged with NMDA. Moreover, we found that cell fusion is increased when we used both the hMSC AD

and hHSPC $CD34^+$. It appears that cell fusion occurs spontaneously for different cell types and upon damage in different tissues.

For instance, there are some studies reporting cell fusion, both in vitro and in vivo, in the multinucleated myofibers of the muscle or, at very low frequency, among hepatocytes (Zhang, Vashisht, et al. 2017; Hochreiter-Hufford et al. 2013; Willenbring et al. 2004; Yu et al. 2015). Besides it, it has been extensively proved that cell fusion occurs in different organs upon damage, as we reported in this work, such in the liver, where it was observed that BMDC can fuse with damage hepatocytes and increased cell fusion during partial hepatectomy (Pedone et al. 2017; Wang et al. 2003). Other studies reported acute inflammation as an enhancer of cell fusion; it was observed fusion increase between Purkinje neurons and stem cells, to mitigate cell injury in the brain or, between BMDC with intestinal progenitors in the intestinal tract (Altarche-Xifro et al. 2016; Davies et al. 2009; Kemp et al. 2018). It is clear then, the relevant paper of the inflammation context. On it, and specifically in the case of the retinal MGC, it happens a "reactivation" process known as gliosis. This process starts as a retinal rescue, releasing a plethora of cytokines and pro-inflammatory factors, therefore, making the MGC the first putative target to fuse. In addition, and since these cells are undergoing also phenotypical changes (Iandiev et al. 2006), their membranes might be more fluid, facilitating the putative fusion. Then, if this gliosis could be kept in the very early time points, could benefit the cell fusion with adult stem cells. On the other hand, longer reactive gliosis could go against of the regenerative goal. To avoid this gliosis in cell transplants, it has been already proved that the adult stem cell inhibition of STAT3 in MSC BMD co-cultured with retinal explants could reduce the reactive gliosis facilitating cell engraftment (Tassoni et al. 2015). Therefore, treated adult stem cells could buffer the

gliosis once they reach the tissue, helping on this retinal gliosis control and taking advantages of its early steps to promote the fusion.

After fusion, the new-born hybrids will go through a process of reprogramming, where the adult stem cell usually is dominant over the somatic cell and the genome of the latter go back to a pluripotent state. Finally, the hybrids can differentiate and induce regeneration (Sanges, Lluis, and Cosma 2011; Silva et al. 2006). Cell reprogramming is a complex process and many players can drive the process after cell fusion in the retina. Zebrafish has been the spearhead of this regenerative process. The helix-loop-helix transcription factor *Ascl1* was found over-expressed in MGC after retina injury and a key factor for reprogramming induction (Fausett, Gumerson, and Goldman 2008). In addition, *Ascl1* was also found to control mouse postnatal MGC reprogramming after damage (Pollak et al. 2013). We therefore can speculate that some mechanisms are evolutionary conserved between species, not only *Ascl1*, but also signaling pathways as Wnt/β-catenin or signals from the pro-inflammatory environment, that could control retinal regeneration (Osakada et al. 2007; Pesaresi et al. 2018).

In this thesis we evaluated the effect of the Wnt/β-catenin pathway in the hybrid reprogramming after fusion. As previously demonstrated in other studies, the Wnt/β-catenin pathway activation by Wnt3a ligand, leads to an enhanced reprogramming of the somatic cells through the accumulation of β-catenin. Previous studies were focused on studying the reprogramming of hybrids after fusion between ESC and the neural stem cells. The reprogrammed hybrids expressed pluripotent markers as *Oct4, Nanog* and *Rex1* while they silenced neural stem cell markers as *Blbp* and *Olig2* (Lluis et al. 2008). Similarly, we asked about the human hybrid reprogramming by measuring the decay on pRLBP expression of the generated hybrids under differentiation towards ganglion cell phenotype (Singhal et al. 2012).

Interestingly, the hybrids generated after the Wnt/β-catenin pathway activation showed an early reprogramming, suggesting the importance of the Wnt/β-catenin pathway in the reprogramming process. Further, together with the Wnt/β-catenin pathway, the Hippo pathway is also evolutionary conserved, driving two principal process: the transition from cell proliferation to quiescence and the proper cell differentiation. Even more, its action is context dependent (Pan 2010). For instance, in the zebrafish retina, Hippo pathway acts as a regulator between cell proliferation and photoreceptor differentiation. By knocking down the core Mst1/2 kinases, the YAP cofactor was not inhibited and the zebrafish shown unpaired retinal pigmentation and malformation. Same phenotypes were seen when injecting zebrafish embryos with a YAP-constitutive active form; pointing at YAP as a repressor of photoreceptor differentiation from retinal progenitor cells (Asaoka et al. 2014). Additionally, recent studies identified Hippo pathway as the stopper of mammalian retina regeneration through YAP repression, since non-responding YAP to Hippo pathway shown an accentuate increase of Cyclin-D1, increased proliferation and Müller glia fate loss (Rueda et al. 2019). Interestingly, the interaction between Wnt ligands and YAP has been proved, being Wnt ligands YAP activators through the alternative Wnt pathway. From another hand, in this same study they show how YAP antagonize Wnt/β-catenin pathway (Park et al. 2015). Altogether, and accounting for the Wnt/β-catenin role enhancing the reprogramming together with the YAP implication on the Müler glia proliferation, a time-refined crosstalk in a pro-inflammatory regenerative context, could enhance the dormant Müller glia regenerative capabilities to favor the retina regeneration.

Once reprogrammed, the 4n generated hybrids, as seen in mouse tissues, should be able to differentiate towards different cell types within the resident tissue and, participate or help into the functional rescue or tissue regeneration (Alvarez-Dolado et al. 2003). Specifically, in the retinae, the cells able to regenerate the tissue, must be able to differentiate into different retinae cell types. For instance, the MGCs in zebrafish can functionally regenerate the retina an unlimited number of times, generating all the retinal cell types (Lust and Wittbrodt 2018; Raymond et al. 2006).

In mice, previous studies demonstrated the potential of the hybrids generated *in vivo*, to differentiate towards retinal ganglion cells and being able to contribute to the optic nerve regeneration and to photoreceptor regeneration, leading a functional rescue measured by electrophysiology tests (Sanges et al. 2013; Sanges et al. 2016). Other study also demonstrated the differentiation of the *in vivo* generated hybrids which were formed after BMDC were chemoattracted in the retina via the SDF1/CXCR4 pathway (Pesaresi et al. 2018). Likewise, we observed that the human new-born hybrids could differentiate toward ganglion cell phenotype, and exhibit a neural-like phenotype as well as showing the polarization of the soma and the formation of podocyte-like structures. We also asked about the functionality of the differentiated hybrids into retinal ganglion cell-like. To prove that, we assessed the membrane polarity. To electro-characterize the cells we used patch clamp (Davie et al. 2006) that can be carried out with low number of cells as well as has better sensitivity. Our results shown an electro-phenotype towards neural cell under the differentiation treatment. This intermediate phenotype could be due to the fact that the cells are producing the sodium and potassium channels to react to the electrical stimuli but they are insufficient or immature yet. Similarly, we also could discuss about the N-Glycans influence on the membrane characteristics, since could be that the ion channels are being produce but not yet the glycan

shift towards a neural fate. Interestingly, it has been shown that the glycosylation pattern can change together with the fate potential in the neural stem cells. Also, favor the glycosylation could prime the astroglia fate in detriment of ganglion fate. Therefore, since the newborn hybrids are differentiating starting from the Müller glia, is reasonable to think that more accentuated electrophysiological changes should be accompanied by glycosylation modifications (Yale et al. 2018) and that changes are not happening yet in our differentiated hybrids.
Additionally, and due to the high surface stress to which the cells are subdued when FACS sorted, one could speculate that this initial electro physiological functionality is not completed due to the need of a general recovery of the cell.

Lastly, we studied how hybrids could differentiate within a living tissue. Due to the impossibility to study the hybrids in the retinae organotypic cultures, because of their degeneration already after one week of culture, we decided to use human retinae organoids (Eiraku and Sasai 2011; Eiraku et al. 2011; Zhong et al. 2014). Interestingly, growing organoids have been found to recapitulate human retinal development, structure and cellular organization (Sridhar et al. 2020). Additionally, retinal organoids show light response and neurons can build functional synapses (Cowan et al. 2020). When comparing the developmental organoid process with the natural human retinogenesis (between week 7 and 20) at single-cell transcriptome level, negligible differences were found. These results showed that the "mature" organoids resemble the human retinae and that neural development within the organoids goes in accordance with a natural neurogenesis (Cowan et al. 2020). Thus, organoids open a broad number of possibilities that could be combined with the newest technologies as the single-cell sequencing and single-cell multi-omics to, for example, do an in

depth study of the neural development and the origin of the visual disorders (Brancati, Treutlein, and Camp 2020). In this endless sea of possibilities, we thought about using growing organoids as host of the generated hybrids, to study their engraftment and interaction within a living tissue as well as their differentiation potential towards the ganglion cell type in an *in vivo* system. To achieve our goal, we adapted the protocols of embryo manipulation by designing in-house injection. Interestingly, we could count the number of injected cells and ensure that they were placed within the tissue to then perform further studies. Once injected, we could ensure using mesoscopic imaging that cells could interact within the tissue and did not interfere with the normal organoid development since, the organoid structure was well preserved and it kept growing during 16 hours of imaging. With this micro-injection set-up, we propose a new use for the human retina organoids. First, this system opens the gate for putative studies of cell engraftments in the tissue. Also, it will allow to study the proper cell positioning, for instance, after the adult stem cells are chemoattracted to the tissue. Additionally, in a functional organoid the differentiated cells could be electro-characterized in an *in vivo* human model.

This, together with the new technologies as specific mesoscopic microscopes for longer *in vivo* imaging, the CRISPR/CAS9 technology to generated visual impaired organoids or, lastly, the adaptation of cell tracer techniques as brain bow to study the human retina interconnections or the differentiation potential of micro-injected hybrids in a more primitive context of neural precursors pre-invaginated organoid, could be a step further on the study of the human retina regeneration.

Our approach to use stem cell-mediated regenerative therapy as a future perspective for treatment, goes hand in hand with other approaches focused on regenerating the human retina hold with the hMGCs as well as

using other stem cell. For instance, the are some studies reporting the possibility of developing *in vitro* differentiated retinal cell types, as the retinal ganglion cells or photoreceptors and their transplantation into the damage retina (Hambright et al. 2012; Lamba, Gust, and Reh 2009). Another approach that is being studied is the use of scaffolds to grow monolayers of differentiated cells, to transplant the full retinal sheet, ensuring the proper positioning of the cells in the eye environment (Lin, McLelland, et al. 2018; Mitrousis et al. 2020).

Additionally, it is important to highlight that although the stem cells are in contact with the retina, most of them do not fuse with resident cells. Therefore, they could act in an alternative way or go to apoptosis. Previous studies demonstrated that entosis is one of the resultant process from cell fusion, where the stem cell include the differentiated cells and there is not nuclear fusion (Sottile et al. 2016). Thus, we could hypothesize cell entosis as an additional mechanism to eliminate those cells that cannot be rescued. It is also important to mention that, even if the adult stem cells were transplanted into the subretinal or intravitreal space, this does not mean that they will be in close contact with the damage tissue. Hence, these stem cells must be attracted to the damage area in order to heal it. A recently published work show that chemokines were over-expressed during retinal damage and that could attract MSCs. The study demonstrated that the overexpression of chemokines receptors Ccr5 and Cxcr6 in mouse MSCs, improved cell migration and integration in a mouse damaged retina, the integrated cells could then trans-differentiate and enhance the retinal rescue (Pesaresi et al. 2020). Therefore, similar approach should be considered with the adult stem cells to enhance the regenerative process.

In this thesis we also found that upon damage cell fusion efficiency is increased at comparable level with both hMSC and hHSPCs. Interesting, it has been demonstrated that hMSC and hBMDC when co-transplanted

result into and increased regenerative effect and engraftment. Indeed, hematopoietic stem cells labeled with GFP when co-transplanted with bone marrow stromal cells showed an increased reconstitution and expansion and it was shown more than double increase of chimerism when compared with a single transplant (Abbuehl et al. 2017). MSCs have been shown to have a supportive effect on the HSPCs, via the release of the HIF-1α (Kiani et al. 2014). Lastly, some interesting results have been shown with human adult stem cells in a type 1 diabetic mice model, that show homing of hematopoietic stem cells in the pancreas when they are co-transplanted intravenously with MSCs (Arjmand et al. 2019). Therefore, we hypothesize that something similar could happen in the human retina. It will be important to perform future works and test if the co-transplant of different types of adult stem cells could enhance the therapeutic treatment for visual impairments.

Lastly, one of the most important points to investigate about the hybrids is the possibility of ploidy reduction. Hybrids after fusion are tetraploid, this might potentially lead to chromosomal instability and cancer development (Bielski et al. 2018; Delespaul et al. 2019). On the other side, as we here describe, cell fusion and tetraploid hybrids can undergo reprogramming and contribute to the tissue rescue and regeneration. Hence, we hypothesize that there might be tightly regulated ploidy reduction to avoid chromosome miss-segregation and consequent cancer development. Interestingly, previous work showed that tetraploid hybrids, after being injected into blastocysts can undergo ploidy reduction upon tripolar mitosis, with random and non-random chromosome segregation, finally the generated diploid cells can contribute to the adult tissues in chimeric mice (Frade et al. 2019). A similar mechanism can occur also for the 4n hybrids

contributing to tissue regeneration. Further studies are needed to test this possibility.

In conclusion, the need of treatment for visual impairment and our above discussed results indicate that cell fusion might be a promising stem cell-mediated therapy for human retinae rescue and, potentially, regeneration. However, only initial investigations have been carried out and much work remains to be done for developing an efficient therapy.

CONCLUSIONS

Conclusions

- Organotypic cultures of human retinae can be maintained for 7 days without a major collapse or cell death of the tissue.

- Human MGCs can be fused with the same efficiency with human MSC AD and HSPC $CD34^+$ within the human retinae organotypic cultures.

- Spontaneous *in vitro* cell fusion is enhanced by natural or chemical pro-inflammatory conditions.

- Chiron-mediated activation of the Wnt/β-catenin in human MSC AD prior to fusion does not affect the cell fusion efficiency but promotes an early reprogramming of the hybrids.

- *in vitro* cell fusion-derived hybrids show a neural-like phenotype and electrophysiological response under neural differentiation conditions.

- The microinjection approach we set up allow the study of the hybrid differentiation into ganglion neurons within the growing human retinal organoids

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SUMMARY IN CATALAN

Resum en català

Establiment d'una teràpia amb cèl·lules mare per la retina humana: des dels cultius organotípics a la comprensió de la fusió cel·lular

Introducció

És molt probable que el lector d'aquesta tesi, abans de començar a llegir-la, es re-col·loqui les ulleres, o es tiri unes gotes de llàgrima artificial per hidratar-se les lents de contacte. Aquest fet, no és més que un reflex del creixement silenciós de les deficiències visuals i les diferents retinopaties de la societat moderna. Així doncs, ens trobem davant d'una necessitat mèdica que mereix ser adreçada.

El primer que cal entendre és el propi òrgan afectat: la retina. Tal com el lector haurà deduït, la retina és l'òrgan al càrrec del sentit de la visió. Arquitectònicament, està composada per tres capes principals: la capa nuclear externa, on es troben els fotoreceptors, la capa nuclear interna, on es troben les cèl·lules horitzontals, amacrines, bipolars i Müller de la glia i, per últim, la capa ganglionar, on trobem les cèl·lules ganglionals.

Un altre aspecte força interessant de la retina es que del propi teixit se'n coneix un potencial regeneratiu que, malauradament, s'ha perdut al llarg de l'evolució. Per exemple, en *zebrafish* (*Danio rerio*), s'ha vist que de les principals cèl·lules residents de la retina, les cèl·lules Müller de la glia (MGC; Müller Glial Cells) són les responsables de re-entrar en el cicle cel·lular i

dirigir un nou cicle de retinogenesis un nombre indefinit de cops. D'igual manera, en el cas dels pollets, s'ha vist que després d'una lesió de la retina, les MGC són capaces de regenerar la retina un cop. És a partir d'aquí on es comença a perdre o silenciar de manera més accentuada el potencial regenerador. És per això que es va pensar en les cèl·lules mare com a intermediari per despertar el potencial regenerador de la retina.

D'entre les diferents fonts de les cèl·lules mare, dues sobresurten com les més interesants, les cèl·lules mare hematopoètiques $CD34^+$ (HSPC $CD34^+$; Hematopoietic Stem Progenitor Cells $CD34^+$) degut a la seva gran plasticitat o, les cèl·lules mare mesenquimals derivades del teixit adipós humà (hMSC AD; Human Mesenchymal Stem Cells Adipose Derived) degut a la seva fàcil extracció, els efectes paracrins derivats que aporten i així com la bona plasticitat que presenten. D'igual manera, es pot pensar en, augmentar el propi potencial de les cèl·lules mare per tal que, els híbrids resultants, siguin de més fàcil reprogramació. Aquesta fita es podria assolir, per exemple, pertorbant vies moleculars conservades en l'evolució per la seva importància com la via Wnt/β-catenin.

Hipòtesi i Objectius

Així doncs, sembla fàcil hipotetitzar amb la possibilitat que la fusió entre cèl·lules mare adultes, bé hHSPC $CD34^+$ o bé hMSC AD, amb la vía Wnt/β-catenin activada, i les cèl·lules MGC resident a la retina humana, doni com a resultat una població de cèl·lules híbrides, on el genoma de les cèl·lules mare sigui dominant sobre el de les MGC, reprogramant aquestes últimes per, posteriorment, diferenciar en altres tipus cel·lulars de la retina. D'aquesta manera, els híbrids resultants de la fusió cel·lular, després de

reprogramar-se a un estat més pluripotent, podrien diferenciar i repoblar les cèl·lules malmeses de la retina. Resultats preliminars s'han pogut provar al laboratori amb ratolins, demostrant que les cèl·lules híbrides resultants, amb la via metabòlica Wnt/β-catenin activada, són capaces de tenir una major eficiència de reprogramació i diferenciar, per exemple, en cèl·lules ganglionars que repoblen el nervi òptic o en fotoreceptors, participant del rescat funcional de la retina.

Així doncs, el nostre objectiu es identificar events de fusió cel·lular en la retina humana i caracteritzar aquesta fusió cel·lular per presentar-la com una potencial alternativa en la lluita contra les deficiències visuals.

Resultats

Per tal de testar la nostra hipòtesi, el primer pas consistia en identificar esdeveniments de fusió cel·lular en teixit retínic. Per poder-ho fer primer vàrem testar la viabilitat del cultiu de les retines, tant dins de l'ull com de forma organotípica *ex vivo.* Així, vàrem poder concloure que disposàvem d' una finestra de treball de 5 dies per provar la nostra hipòtesi de fusió cel·lular abans que el teixit es veies compromès al cultivar-lo *ex vivo*. Per tal d'identificar els events de fusió, es va infectar la retina amb un virus adeno-associat fent una infecció selectiva només de les MGCs amb una proteïna fluorescent en verd (GFP) expressada només sota el promotor pRLBP, específic de les MGCs. Un cop infectada, aquesta retina es va co-cultivar amb cèl·lules mare adultes marcades a les histones H2B amb la proteïna fluorescent en vermell (RFP). Així doncs, es van poder identificar cèl·lules fusionades en teixit retínic, resultants de la fusió de les MGC tant amb MSC AD com amb HSPC $CD34^+$.

Un cop confirmada la presencia de cèl·lules híbrides resultants de la fusió cel·lular, es volia entendre com aquesta fusió havia tingut lloc, és a dir: sota quines condicions es dona la fusió cel·lular. És possible augmentar-la? I, un cop tenim els híbrids: Són aquests híbrids reprogramables? Pertorbant la via metabòlica Wnt/β-catenin aconseguim influir en la reprogramació dels híbrids? Tenen els híbrids resultants potencial per diferenciar-se en altres tipus cel·lulars de la retina?...Pas per pas, vam anar adreçant les diferents preguntes, optimitzant primer un sistema per poder estudiar la fusió cel·lular *in vitro*, tenint els dos participants de la fusió aïllats. Es van extreure les MGCs de la retina i després es van caracteritzar tant per immunofluorescència com per Western Blot amb anticossos específics (CRALBP, específic de les MGC, Glutamine Synthase, específic de les cèl·lules de la Glia i PAX 6, que marcar neuroprecursors). Un cop aïllades, es va dissenyar un clonatge que expresses GFP sota el promotor específic de Müller pRLBP, seguint la mateixa estratègia que la dissenyada amb el teixit retínic. Les cèl·lules en aquest cas van ser infectades amb un lentivirus on es va inserir el constructe dissenyat i seleccionades per FACS. Així doncs, ja teníem els dos participants de la fusió disponibles per estudiar-la. Per tal d'entendre sota quines condicions era més favorable la fusió, vàrem dissenyar un nou model experimental. En aquest cas, havíem de mimetitzar les condicions de dany. Per fer-ho, vam fer créixer en monocapa les cèl·lules ARPE 19 de l'epiteli pigmentari i un cop crescudes, es va similar el dany afegint NMDA al medi o bé PBS, com a control. Després de 24h, aquest medi enriquit es recollia i s'utilitzava com a medi per la fusió de les MGCs i les MSC AD o HSPC $CD34^+$, amb la via Wnt/β-catenin activada o no, en un ratio 1:1 i es deixaven les dues poblacions juntes fins al dia següent. Després de la fusió, les cèl·lules eren incubades amb el marcador nuclear Hoescht 33342 i s'estudiava els percentatges de cèl·lules fusionades RFP^+GFP^+ i amb una dotació nuclear de 4n resultants. Es va poder veure

com els percentatges de fusió eren significativament més elevats quan la fusió es donava en condicions de dany i que, aquests percentatges, no variaven entre les diferents fonts de cèl·lules mare (MSC AD o HSPC $CD34^+$) i també que l'activació de la via Wnt/β-catenin no afectava els percentatges de fusió. Per altra banda, es va repetir la fusió però en condicions control o de dany naturals, és a dir, recollint el medi de retines humanes en cultiu a dia 1 (com a control) o a dia 7 (com a dany) i es va poder veure que les percentatges de fusió eren els mateixos que els assolits ens condicions químiques (de NMDA o PBS), confirmant també en condicions naturals que la fusió cel·lular es veu afavorida per condicions pro-inflamatòries.

Ara doncs, es tractava d'entendre si els híbrids resultants s'havien reprogramat i tenien potencial per diferenciar-se en altres tipus cel·lulars. Per entendre la reprogramació i diferenciació, vam sotmetre els híbrids, generats sota diferents condicions ((1) medi control, MSC AD no Wnt/β-catenin activades; (2) medi control, MSC AD Wnt/β-catenin activades; (3) medi NMDA pro-inflamatori, MSC AD no Wnt/β-catenin activades; (4) MSC AD Wnt/β-catenin activades) i, cadascuna d'aquestes condicions, es van posar sota condicions de diferenciació cap a fenotip neuronal, pel tractament de DAPT (inhibidor de la via Notch) i hbFGF (human basic-Fibroblast Growth Factor) o en condicions control de no diferenciació. Sota aquestes condicions, es va mesurar la pèrdua de senyal GFP, és a dir, es va estudiar com es reprogramaven les cèl·lules, al perdre la expressió del pRLBP (sota el que s'expressa la GFP) i es va quantificar aquesta pèrdua d'intensitat. El que es va veure, es que en tots els casos els híbrids responien a la diferenciació, experimentant pèrdues de més del 50% de la expressió de GFP. De igual manera, es van poder veure canvis fenotípics en direcció a

un fenotip proto-neuronal, ensenyant projeccions i estructures que recorden a les neurites. El que es interessant però, és que aquells híbrids generats sota condicions pro-inflamatòries NMDA, es veia que també perdien la expressió del GFP, però en aquest cas el fenotip era expandint el soma, recordant un procés que seria similar al de la gliosis, responent així a la inflamació.

Un cop vist el potencial dels híbrids de diferenciar en cèl·lules neuronals, es va voler testar la seva funcionalitat. Per fer això, s'està duent a terme una electró-caracterització *in vitro* de les cèl·lules híbrides. Tot i que aquests experiments estan encara en procés, ja s'ha pogut veure com les cèl·lules responen a les corrents aplicades. S'està, a dia d'avui, recopilant suficients dades per obtenir-ne resultats concloents. D'altra banda, també pretenem entendre com aquests híbrids es comportarien dins del teixit retínic. Donat que es impossible estudiar la integració dins de la retina en cultiu de forma organotípica, es va estudiar una altra aproximació. En aquest cas, vam decidir que el més similar serien els organoids de retina generats de cèl·lules humanes. Per tal de veure la onada de diferenciació cap a cèl·lules ganglionars, es va fer una quantificació per immunofluorescència del marcador β-III tubulin, específic de cèl·lules ganglionars, del dia 35 al 50 de desenvolupament. És va identificar el dia 40 de desenvolupament com el que presentava un pic d'expressió de cèl·lules ganglionars i que, a més, aquestes cèl·lules estaven posicionades a la part interna de l'organoid. En paral·lel, es va posar a punt un mètode per tal de microinjectar les cèl·lules híbrides resultants de la fusió dins de l'organoid de retina humà en desenvolupament. Així doncs, es va adaptar la tècnica de microinjecció embrionària, resultant en un sistema d'injecció que ens permetia injectar cèl·lules d'un diàmetre més gran (d'uns 60-70 μm) i contar el número de cèl·lules injectades per, poder extreure dades del percentatge de cèl·lules

que diferenciarien dins de l'organoid de retina en creixement. Així doncs, el que faltava per fer era trobar un marcador que expressés si els híbrids diferenciaven cap cèl·lules ganglionars. Així doncs, es va dissenyar un nou clonatge, on es va posar la proteïna fluorescent Crimson (que brilla al vermell llunya, Far Red) sota l'expressió del promotor del marcador Tuj1, específic de les cèl·lules ganglionars. Del plàsmid dissenyat se'n van fer uns lentivirus per infectar les cèl·lules MGC que ja prèviament expressaven el marcador GFP sota el promotor específic pRLBP. Així doncs, els híbrids generats a partir d'aquests cèl·lules, inicialment tindrien una expressió de GFP que s'aniria perdent en favor de l'expressió de Crimson. Per poder seguir aquesta evolució, es planeja prendre imatges amb el sistema de mesoscopia MuVi Spim (departament de mesoscopia de la EMBL Barcelona). De moment, hem pogut veure que som capaços d'injectar MGC a l'organoid en creixement i treure imatges in vivo durant 24 hores. Durant aquestes 24 hores, hem vist que questes cèl·lules no són expulsades pel teixit i que l'organoid continua creixent sense experimentar complicacions. Actualment, s'esta duent a terme el mateix experiment però injectant els híbrids dins de l'organoid per poder dilucidar el seu potencial de diferenciació dins del teixit retínic humà.

Discussió

Amb això doncs, tenim uns resultats en la línia amb diferents estudis publicats que subratllant la fusió cel·lular com un mecanisme partícip de la regeneració de teixits, on pot ser més clar al fetge, el múscul o el cor. Des del nostre laboratori s'han publicat diferents estudis centrats en el paper de les cèl·lules mare en la fusió cel·lular com a mecanisme regenerador, tant en fetge, com a nivell cerebral. Tot i així, és en la retina on més hem centrat

l'atenció, veient que les cèl·lules híbrides, tal com en els resultats aquí resumits, poden diferenciar en cèl·lules ganglionars i formar part tant de la retina com del nervi òptic, o bé diferenciar en fotoreceptors que participen del rescat funcional del teixit. No només això sinó que també hem demostrat la importància de l'ambient pro-inflamatori per reclutar cèl·lules mare, tant injectades directament a l'espai ocular, com per reclutar-les del propi cos. De igual manera, es pot discutir sobre el paper de les cèl·lules mare que un cop dins del teixit, no es fusionen però poden participar de la regeneració o, es fusionen, però no diferencien. Per una banda, es sap del paper paracrí de les cèl·lules mare i, per altra banda, s'ha vist que un altre possible resultat de la fusió cel·lular es la entosi. Així doncs, s'hipotetitza amb la possibilitat que aquelles cèl·lules que després de fusionades no poden ser rescatades, serien eliminades. D'igual manera, no es pot obviar el fet que les cèl·lules híbrides resultant són tetraploides i que tenen risc de malignizar si no experimenten una reducció de la ploidia. En aquest sentit, s'ha pogut veure que els híbrids poden reduir la seva ploidia al dividir-se o que, en el cas de la retina, al diferenciar en cèl·lules neurals adultes veurien arrestat el seu cicle cel·lular.

Conclusions

A mode de conclusió, podem dir que en teixit retinic humà, cultivat *ex vivo* de forma organotípica, hem estat capaços d'identificar events de fusió cel·lular entre cèl·lules MGC i MSC AD o HSPC DC34^{+}. D'igual manera, hem pogut estudiar aquest events de fusió *in vitro*, on hem vist que el percentatge de fusió es superior si aquest procés es dona en condicions pro-inflamatòries, bé siguin naturals o químiques. També, que aquest percentatge de fusió és manté independentment de la font de cèl·lules mare

que utilitzem. A més, hem vist que l'activació de la vía Wnt/β-catenin de les cèl·lules mare (MSC AD o HSPC DC34^{+}) no afecta els percentatges de fusió però promou una reprogramació primerenca. Dels híbrids resultants, n'hem pogut concloure el seu potencial de diferenciació cap a un fenotip proto-neuronal. En aquesta mateixa direcció, s'estan recollint les últimes dades per fer-ne la seva electró-caracterització *in vitro*. Addicionalment, s'ha posat a punt un sistema per microinjectar el híbrids en organoids de retina humans en creixement, per veure el seu potencial de diferenciació *in vivo* per mitja de la presa d'imatges durant 24 hores per mesoscopia.