NEURAL REGENERATION RESEARCH
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Regenerative capacity of Müller cells and their modulation as a tool to treat retinal degenerations

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Vision is one of our most precious senses, and its impairment has a high socio-economic impact. In the industrialized world, degenerative diseases of the retina lead to vision loss, particularly among the elderly. These degenerations include, for instance, retinitis pigmentosa, age-related macular degeneration, and diabetic retinopathy. Although treatments are evolving to manage late-stage symptoms of retinal degenerations, no effective therapies to recover vision loss exist. Retinal degeneration often involves loss or damage to specialized neural cells, such as photoreceptors, and their death stimulates the activation and proliferation of Müller cells (Salman et al., 2021).

Müller cells are the predominant glial cell type in the retina, representing 90% of the retinal glia. They are radially oriented and span the entire depth of the neural retina. Initially, Müller cells were believed nothing more than an adhesive scaffold for retinal cells. However, numerous studies show that Müller cells have more functions, including homeostatic regulation, metabolic support to the retina, and light transmission under physiological conditions. They can also acquire the properties of a retinal stem cell. Furthermore, Müller cells are involved in response to injury, carrying out either a protective or detrimental function (Too and Simunovic, 2021). In zebrafish, they adopt certain progenitor/stem cell features, dislocate to the damaged retinal area, and produce new neurons. Transcriptome profiling of mammalian Müller cells demonstrated evidence for their neurogenic potential. However, it is still unclear why the regenerative response in mammals is minimal compared to the robust response in fish (Hoang et al., 2020). In mammals, Müller cells are also the glial cell type primarily involved in gliosis. Gliosis is a pathophysiological feature of retinal degenerations, and it is the activation and consequent proliferation of Müller cells in response to injury and/or disease. Müller cell reactivity differs between species and even among individual cells according to different pathological stimuli. The activated glial cells give a multifactorial signal, which potentially includes inflammatory features and triggers the development of a glial scar. This contributes to further degeneration and impedes tissue regeneration (Graca et al., 2018). Upon injury, Müller cells also generate neurotrophic factors to promote recovery. The complex molecular machinery that regulates retinal regeneration in fish and glial scar formation in mammals is undetermined to date, stimulating more research into the topic. Diverse molecular pathways drew the attention of researchers, such as tumor necrosis factor-alpha (TNFα), Wnt, and JAK-STAT pathways. In this perspective, we are going to focus mainly on the transforming growth factor-beta (TGFβ)-Smad3-Notch axis.

TGF-α belongs to a group of pleiotropic cytokines and regulates several cellular processes during embryogenesis and adulthood. TGFβ signaling is necessary for wound healing, including non-specific scar formation and tissue-specific regeneration. The TGFβ superfamily comprises 33 members: three multi-functional isoforms TGFβ1, TGFβ2, and TGFβ3, and downstream mediators of canonical and non-canonical signaling. This pathway affects the immune response, scar formation, and modulation of neurotrophic factors (Gilbert et al., 2016). The specific TGFβ isoforms and downstream mediators play different roles in each of these processes. For instance, in mammals, TGFβ1 and TGFβ2 promote collagen deposition and scar formation, while TGFβ3 is anti-fibrotic (Yang et al., 2020). In zebrafish, the TGFβ pathway is involved during heart, fin, and retina regeneration. In a light-induced model of retinal injury in zebrafish, TGFβ1 is primarily upregulated and then suppressed during the proliferative, neurogenic response of Müller cells (Lenkowski et al., 2013). Contrarily, TGFβ3 collaborates with Notch signaling to inhibit retina regeneration following retinal injury in zebrafish (Lee et al., 2020).

We performed a cross-species analysis comparing animal models with fully regenerative capacity (zebrafish) and models with minimal/absent regeneration (mouse). Our study showed that TGFβ isoforms play a different role in retinal tissue repair, underscoring the pleiotropic nature of TGFβ action (Conedera et al., 2021b). Furthermore, canonical and non-canonical signaling pathways were activated differently among vertebrates, depending on TGFβ isoforms. In zebrafish, only TGFβ3 was expressed by Müller cells, and the expression of all activin receptors/ligands increased during injury response, promoting the canonical mothers against decapentaplegic homolog (Smad) signaling. Earlier studies showed that jun genes are highly expressed during regenerative processes. Thus, the simultaneous upregulation of *junb* and *mycb* suggested the activation of the canonical signaling via TGFβ3 in zebrafish during regenerative response. Instead, TGFβ1 and TGFβ2 were activated in murine Müller cells in our model of laser-induced retinal degeneration. Müller cells also expressed bone morphogenetic proteins (BMPs), such as BMP2 and BMP7, which are known to induce changes of markers usually associated with gliosis (e.g., γ-secretase, ciliary neurotrophic factor). Though BMPs can signal via both canonical and non-canonical TGFβ pathways, Smad was not significantly upregulated in response to injury. This suggested the activation of the non-canonical signaling during gliosis in murine Müller cells in our model. Non-canonical Smad-independent signaling has been identified for BMPs, and BMP2/7 upregulation was associated with p38 MAPK activation. Indeed, TGFβ1 supported reactive oxygen species production by impairing mitochondrial function and mediates the p38 MAPK pathway. We also detected upregulation of latent TGFβ-binding proteins (Ltbp1, Ltbp2, and Ltbp3), and reactive oxygen species production can directly mediate their modulation. Thus, we evaluated the activation of p38 MAPK signaling in the murine retina after laser-induced retinal degeneration. We found evidence of activation of the non-canonical p38 MAPK pathway - likely mediated by TGFβ1 and TGFβ2 - during gliosis in mice. We also detected increased leucine zipper transcription factors (Tsc22), such as Tsc22d1, in Müller cells. In agreement with our findings, Tsc22 has been shown to sequester Smad7 from binding to activated Tgfbr1 and hinder Smad7/Smurf-induced ubiquitination and degradation of the receptor (Xu, 2011). Tsc22 also promotes the expression of fibrotic genes, including *PAI1*. Such as Tsc22, PAI1 signal was upregulated during the experiment only in murine Müller cells. Many pro-fibrotic genes were overexpressed after injury in murine Müller cells in our model, suggesting that gliosis can be considered a fibrotic-like process. Overall, these results show that TGFβ isoforms have different effects on tissue regeneration and degeneration, and the role of TGFβ may be context-dependent. TGFβ3 is related to retinal regeneration via canonical signaling upon the regulation of *junb* and *mycb* genes in zebrafish Müller cells, while TGFβ1 and TGFβ2 are linked to the p38 MAPK pathway in mice. Therefore, retinal repair responses need to be discussed in relation to the species- and injury-specific effect on TGFβ to stimulate the de-differentiation of proliferating Müller cells.

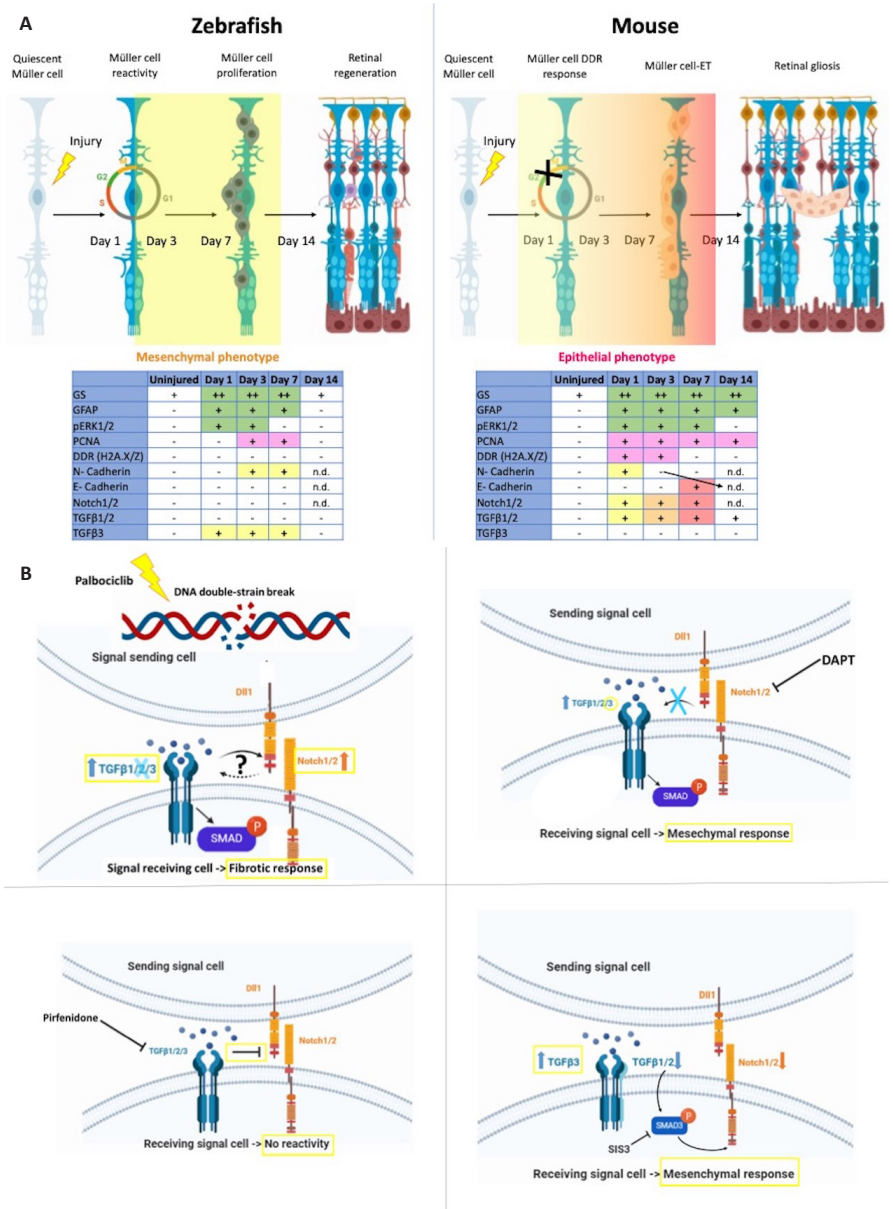
Recent evidence demonstrated that the regulation of TGFβ depends on its interaction with other pathways. Essential for tissue repair mechanisms in diverse organs (e.g., kidney, liver, and heart) is also the Notch pathway. Several physiological and pathological processes are regulated by Notch concomitant with TGFβ expression, thus setting the stage for a possible cross-talk between the two pathways. We determined the importance of TGFβ/Notch during laser-induced injury response of Müller cells by cross-species comparison. We showed that TGFβ/Notch interplay in a Smad3-dependent manner and triggers cycle arrest of Müller cells. This results in an unsuccessful reprogramming during reactive gliosis in mice, and Smad3 inhibition boosts the limited regenerative potential of murine Müller cells. Moreover, our findings suggest Müller cells shift towards an epithelial lineage [Müller cell - epithelial transition (MC-ET)] during reactive gliosis in mammals providing novel insights into the remodeling mechanism of retinal degeneration. In our model, we also detected a transient expression of gliotic markers in zebrafish, which suggests transient gliosis and its regression precedes the photoreceptors' regeneration. However, reactive gliosis persists in murine Müller cells throughout the injury response.

Currently, it is unclear how the reactivity of Müller cells exacerbates chronically the injury response, which ultimately leads to glial scar formation. The reactivity of Müller cells is tightly connected with their exit from quiescence in response to injury. In both zebrafish and mice, Müller cells re-entered into the cell cycle in response to injury. In zebrafish, the signal returned to baseline in the restored retina, which supports the hypothesis of a transient reactive gliosis. In mice, Müller cells abnormally proliferated and that resulted in an arrested re-entry into mitosis. This development activated DNA damage response (DDR) and DNA repair mechanisms showing that murine Müller cells can protect the integrity of their genome from double-strand breaks. Once the repair is over, Müller cells should exit the checkpoints and restore the retina. Instead, Müller cells form a gliotic scar impeding retinal regeneration in mammals. We identify the initial expression of progenitor markers in both animals in our injury model, suggesting the possibility of Müller cells behave as progenitor/stem cells (Conedera et al., 2021a). In mice only, Müller cells undergo epithelial-like changes. The unsuccessful reprogramming during chronic gliosis illustrates that murine Müller cells cannot ensure proper segregation of the duplicated genome during injury response leading to arrested re-entry into mitosis. That induced double-strand breaks with the subsequent acquisition of epithelial features by Müller cells during glial scar formation. During epithelial transformation, characterized by DDR, Notch1/2 was upregulated in response to injury in mice only. The simultaneous activation of TGFβ and Notch in murine Müller cells indicates their combined action during chronic gliosis after laser-induced retinal degeneration. Interestingly, a gliotic reaction occurred with the acquisition of an epithelial phenotype in human Müller cells in pathological conditions. Furthermore, we detected both TGFβ1 and Notch2 expressions in human Müller cells during gliotic response. Altogether, these data link MC-ET to TGFβ/Notch during chronic reactive gliosis in humans comparable to retinal injury response in mice (**Figure 1A**).

The impact of arrested re-entry into mitosis on retinal regeneration in zebrafish and either TGFβ or Notch inhibition on reactive gliosis in mice was investigated upon laser-induced retinal degeneration. Palbociclib was used to induce cell-cycle arrest to examine whether it is necessary to stimulate MC-ET upon injury in zebrafish. We showed that the induced cell-cycle arrest in zebrafish could trigger DDR, in line with the arrested re-entry into mitosis in mice.

Furthermore, we associated DDR with fibrotic-like outcomes in Müller cells at the expense of their mesenchymal-neural potential (**Figure 1B**). These data propose MC-ET as a repair mechanism following cell-cycle arrest. Pirfenidone showed the potential to regress the TGFβ-induced MC-ET, and TGFβ inhibition trapped Müller cells into quiescence even after injury in mice (**Figure 1B**). However, targeting TGFβ may affect other physiological mechanisms, owing to its pleiotropic nature. Our data revealed that TGFβ might act upstream of Notch. The Notch pathway was inhibited by DAPT (inhibitor of γ-secretase) to preserve TGFβ and prevent MC-ET-associated fibrosis after laser-induced retinal degeneration. Transient exposure to DAPT confirmed the link between TGFβ and Notch. However, pro-fibrotic TGFβ response seems independent of Notch inhibition. Inhibitions of either TGFβ or Notch were unsuccessful in stimulating further improvements during injury response in mice (**Figure 1B**). Finally, we tested if the combined action of TGFβ/Notch mediates MC-ET. TGFβ cooperates with Notch in a Smad3-dependent manner, and both synergistic and antagonistic effects of TGFβ/Notch interplay have been reported (Wang et al., 2017). Smad3 was suppressed using a selective inhibitor of Smad3 (SIS3) during early injury response, MC cell-cycle arrest, and MC-ET in mice (24-hour treatment). TGFβ3 favored a mesenchymal response in Müller cells at the expense of their epithelial transformation via TGFβ1/2 after laser-induced retinal degeneration (**Figure 1B**). Based on this promising data, we extend SIS3 treatment throughout the experiment. SIS3 treatment showed that murine Müller cells are capable of exiting their quiescence state, a critical step toward regeneration. Thereby, SIS3 showed the potential to modulate MC-ET-associated fibrosis and reduce the glial scar *in vivo* by stimulating the de-differentiation of murine Müller cells.

In our opinion, reactive gliosis, Müller cell-epithelial transition, and ensuing fibrosis are the main characteristics of retinal degeneration. Thus, alleviating the detrimental effects of glial scar formation via modulation of these players might enable retinal regeneration in mammals.



	Uninjured	Day 1	Day 3	Day 7	Day 14
GS	+	++	++	++	+
GFAP	-	+	+	+	-
pERK1/2	-	+	+	-	-
PCNA	-	-	+	+	-
DDR (H2A.X/Z)	-	-	-	-	-
N- Cadherin	-	-	+	+	n.d.
E- Cadherin	-	-	-	-	n.d.
Notch1/2	-	-	-	-	n.d.
TGFβ1/2	-	-	-	-	-
TGFβ3	-	+	+	+	-

	Uninjured	Day 1	Day 3	Day 7	Day 14
GS	+	++	++	++	++
GFAP	-	+	+	+	+
pERK1/2	-	+	+	+	-
PCNA	-	+	+	+	+
DDR (H2A.X/Z)	-	+	+	-	-
N- Cadherin	-	+	-	-	n.d.
E- Cadherin	-	-	-	+	n.d.
Notch1/2	-	+	+	+	n.d.
TGFβ1/2	-	+	+	+	+
TGFβ3	-	-	-	-	-

Figure 1 | Graphical representation of Müller cell-dependent changes during retinal degeneration/regeneration. (A) Cellular and molecular outcomes during retinal repair in mice and zebrafish. (B) Modulation of Müller cell response. Schematic presentation of the effect of pharmacological treatments performed in zebrafish (Palbociclib) to induce development of a chronic scar and mouse (Pirferidone, DAPT and SIS3) on TGFβ, Notch and their interaction via Smad3 to shift Müller cell response towards regeneration. DAPT: 2,4-Diamino-5-phenylthiazole; DDR: DNA damage response; Dll1: delta-like protein 1; GFAP: glial fibrillary acidic protein; GS: glutamine synthetase; MC-ET: Müller cell-epithelial transition; PCNA: proliferating cell nuclear antigen; pERK 1/2: phosphorylated extracellular signal regulated kinase 1/2; Smad3: mothers against decapentaplegic homolog; SIS3: selective inhibitor of Smad3; TGF: transforming growth factor; TNF: tumor necrosis factor.

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Date of submission: November 23, 2021
Date of decision: December 20, 2021
Date of acceptance: January 13, 2022
Date of web publication: April 25, 2022

https://doi.org/10.4103/1673-5374.340408
How to cite this article: *Conedera FM, Enzmann V (2023) Regenerative capacity of Müller cells and their modulation as a tool to treat retinal degenerations. Neural Regen Res 18(1):139-140.*
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C-Editors: Zhao M, Liu WJ, Wang Lu; T-Editor: Jia Y