RESUMEN

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"Rol de las células gliales de Müller en la regeneración neuronal de la retina"

Las enfermedades neurodegenerativas se caracterizan por la pérdida progresiva e irreversible de neuronas, lo cual perjudica seriamente las funciones neurológicas, por lo que es indispensable encontrar una solución efectiva a esta problemática.

El sistema nervioso es de difícil acceso y de una enorme complejidad. Utilizar modelos que permitan analizar la génesis y la muerte neuronal, resulta clave para comprender la evolución y establecer potenciales tratamientos para las enfermedades neurodegenerativas. La retina es un excelente sistema para indagar en estos procesos, dado que forma parte del sistema nervioso central y presenta una estructura laminar sencilla.

En la retina, las enfermedades neurodegenerativas que afectan a las neuronas fotorreceptoras - como la Retinitis Pigmentaria o la Degeneración Macular - culminan con la ceguera de los pacientes afectados. Actualmente se presentan dos posibles estrategias de tratamiento para estas enfermedades: la primera busca conocer los

mecanismos que conducen a la apoptosis neuronal, de manera de evitarla mediante el uso de factores que promuevan la supervivencia de los fotorreceptores. La segunda propone regenerar las neuronas perdidas durante la enfermedad mediante el uso de *stem cells* o células madre, las cuales se caracterizan por la auto-renovación y multipotencialidad. Las células gliales de Müller (CGM) serían *stem cells* en la retina. Numerosos trabajos indican que las CGM son capaces de de-diferenciarse y proliferar luego de un daño en la retina, para luego expresar marcadores de fotorreceptores. Este comportamiento es ampliamente reconocido en peces y anfibios, sin embargo resulta más restringido en aves y mamíferos. Para poder utilizarlas en el tratamiento de patologías degenerativas, es clave conocer varios aspectos de la biología de las CGM como *stem cells* en animales superiores. Por otra parte, la rápida muerte por apoptosis de las células regeneradas dificulta notablemente el éxito de las terapias de reemplazo. Por lo tanto, es importante dilucidar cómo evitar dicha muerte.

Estudios previos de nuestro laboratorio indicaron que las CGM expresaron marcadores de multipotencialidad por largos períodos *in vitro*, y que esta expresión era regulada por la interacción con neuronas de retina o el agregado de factores tróficos. El objetivo del presente trabajo de Tesis fue evaluar el potencial comportamiento como células madre de las CGM de roedores, determinando si eran capaces de generar neuronas fotorreceptoras *in vitro*. Para ello diseñamos tres sistemas de cultivos celulares de retina (neuronales puros, gliales puros y cultivos mixtos neuro-gliales) en los cuales analizamos la presencia, origen y evolución de progenitores multipotentes, determinando si retenían las características de multipotencialidad a través de los pasajes. A continuación

evaluamos la capacidad de diferenciación de dichos progenitores en fotorreceptores maduros y funcionales; y si el agregado de factores lipídicos podía prevenir la apoptosis de los fotorreceptores regenerados.

En el **Primer Capítulo** evaluamos si las CGM presentaban dos características de las células madre: la capacidad de expresar marcadores de multipotencialidad a través de los pasajes, y de generar progenitores neuronales. Determinamos que tras el repique de cultivos enriquecidos en CGM, las CGM retuvieron la expresión de Nestina, a la vez que co-existieron con una población de progenitores que bajo condiciones adecuadas se diferenciaron en neuronas. La existencia de progenitores en estos cultivos secundarios planteó cuatro preguntas: ¿De dónde provienen los progenitores? ¿Expresan otros marcadores de multipotencialidad? ¿Es posible diferenciarlos en fotorreceptores? ¿Existen factores que promuevan su supervivencia y/o diferenciación? Antes de avanzar con ellas evaluamos si las dos poblaciones mayoritarias en cultivos primarios de retina (CGM y neuronas), influían en el destino que los progenitores adoptaban *in vitro.* Determinamos que sólo la interacción de CGM con neuronas de retina permitía la generación y/o conservación de progenitores multipotentes por largos períodos en cultivo.

En el **Segundo Capítulo** analizamos el origen y la expresión de marcadores de multipotencialidad en los progenitores observados tras el repique. Determinamos que los progenitores expresaron -además de Nestina- Sox-2 y Pax-6 a la vez que conservaron su capacidad proliferativa. Estas características se observaron sólo en los pasajes de cultivos mixtos neuro-gliales: los cultivos puros neuronales no sobrevivieron al repique, mientras

que los cultivos puros de CGM no generaron progenitores de retina. Los progenitores retuvieron la expresión de Pax-6 y Sox-2 y continuaron proliferando aún luego de cuatro pasajes sucesivos de cultivos mixtos. Algunos de ellos, inclusive, expresaron Crx, hecho que indicaba su capacidad de diferenciación en neuronas fotorreceptoras. Para establecer el origen de los progenitores de retina en los repiques, utilizamos una sonda fluorescente que nos permitió identificar la progenie tanto de los progenitores como de las CGM. Este análisis sugirió que los progenitores multipotentes observados en los sucesivos repiques se originarían en progenitores pre-existentes preservados por la interacción con las CGM y no en las CGM.

En el **tercer capítulo** investigamos la capacidad de diferenciación de los progenitores multipotentes en neuronas fotorreceptoras. Determinamos que en los cultivos mixtos neuro-gliales, bajo condiciones adecuadas de cultivo los progenitores se diferenciaron en fotorreceptores maduros y funcionales, que expresaron marcadores característicos de fotorreceptores (como Crx, Opsina, Periferina) a la vez que fueron capaces de responder a la luz y de capturar glutamato por mecanismos de alta afinidad. Por último, investigamos si era posible bloquear o retrasar el desarrollo de la apoptosis en las células regeneradas. Determinamos que la administración de DHA y S1P - dos moléculas lipídicas con efectos anti-apoptóticos en fotorreceptores-disminuyeron significativamente la apoptosis neuronal en los repiques.

En conclusión, en este trabajo de Tesis establecimos una nueva función para las células gliales de Müller: su capacidad de transformar a las células progenitoras de retina en células multipotentes, y de promover su diferenciación en neuronas fotorreceptoras.

Esta novedosa función podría ser relevante al momento de diseñar nuevas estrategias para el tratamiento de enfermedades neurodegenerativas de la retina.

SUMMARY

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"Role of Müller glial cells during neuronal regeneration in the retina"

Neurodegenerative diseases are characterized by progressive and irreparable neuronal death, which ends up in major neurological dysfunctions. This scenario has prompted researchers to find an effective cure for these diseases; however, few results have been achieved so far.

Studying the nervous system is very complicated, because of its difficult access and the vital functions that rely upon it. Thus, having an appropriate model to study neuronal genesis and degeneration is very important in order to find a valid treatment for neurodegenerative diseases. The retina is an excellent model for this type of studies: it is part of the central nervous system, has a very simple structure and is readily accessible.

Retinal neurodegenerative diseases, like Retinitis Pigmentosa (RP) or age- related macular degeneration (AMD), are characterized by extensive photoreceptor loss, which results in visual impairment and/or complete blindness. Two possible strategies are now being considered to find a cure for these pathologies: one strategy is to avoid neuronal

degeneration, by using trophic factors that promote neuronal survival. The second strategy proposes using stem cells to replace the neurons lost during neurodegenerative diseases. Müller glial cells (MGC) are possible candidate to behave as stem cells in the retina. Several studies indicate that, after retinal injury, MGC are capable of de-differentiating and proliferating, and later expressing photoreceptor markers. This regenerative capacity is very robust in lower vertebrates, but much more restricted in birds and mammals.

In addition, for stem cells to be useful in the treatment of retinal neurodegenerative diseases, an important issue needs to be solved: the rapid and extensive apoptosis of newly generated neurons. In this regard, it is essential to find molecules able to promote neuronal survival.

Previous studies from our laboratory indicate that MGC express stem cell markers for several days *in vitro.* Moreover, this expression can be regulated by both interactions with retinal neurons and supplementation with trophic factors. The present Doctoral Thesis studied the behavior of MGC as stem cells in rodent retinas, evaluating the ability of MGC to originate photoreceptors *in vitro* through the generation of multipotent retinal progenitors. We used three different types of retinal cultures (pure neuronal, pure glial and mixed neuro-glial cultures), in which we analyzed the presence, origin and evolution of multipotent retinal progenitors. We evaluated their multipotentiality in passages of the diverse culture systems and their later ability to differentiate into functional photoreceptors. Finally, we investigated if supplementation with docosahexaenoico acid

and sphingosine-1-phospate (two lipid molecules with anti-apoptotic effects in photoreceptors *in vitro*) could promote the survival of newly regenerated neurons.

In the **First Chapter,** we evaluated if MGC presented at least two of the main stem cell features: multipotentiality preserved through successive re-seedings and the ability to give rise to neuronal progenitors. Our results indicated that MGC retained the expression of stem cell marker Nestin after being re-seeded; in addition, they co-existed with a population of progenitors that also expressed Nestin and, eventually, differentiated into neurons. The presence of neuronal progenitors in these secondary cultures raised four important questions: Which cells originate these progenitors? Do they express other stem cell markers, besides Nestin? Is it possible to induce their differentiation into photoreceptors? Is there any trophic factor that may promote their survival and/or induce their differentiation into neurons? Before looking for answers to these questions, we evaluated if the two most abundant cells in retinal cultures (MGC and neurons) could influence photoreceptor fate in culture. We determined that progenitors were only able to retain the expression of stem cell marker Nestin for several days *in vitro* in mixed neuron-glial cultures.

In the **Second Chapter** we analyzed the source and the expression of other stem cell markers in multipotent progenitors present in secondary cultures. Our results indicated that progenitors expressed Sox-2 and Pax-6, and preserved the ability to proliferate. These characteristics were only found in secondary mixed neuro-glial cultures; re-seeding of pure neuronal cultures led to generalized cell death, while re-seeding of pure glial cultures only generated glial cells, since no progenitors where found in this

condition. To our surprise, progenitors from mixed cultures could be consecutively re-seeded until the fourth passage still preserving the expression of stem cell features while some of them began to express Crx (an early transcription factor for photoreceptors). To address the question about the source of the progenitors in the re-seedings, we used a fluorescent probe that separately labeled the progeny of both MGC and progenitors. We determined that multipotent progenitors did not originate from MGC; instead, they derived from pre-existent progenitors present in the donor retina, which were preserved after passages due to their interactions with MGC.

In the **Third Chapter** we investigated the ability of multipotent progenitors to differentiate into photoreceptors. We determined that in secondary mixed cultures, progenitors differentiated into mature photoreceptors that expressed Opsin, Crx and peripherin. Moreover, they displayed functional features: they responded to light stimuli and showed high affinity-glutamate uptake, characteristics found in mature photoreceptors in the retina *in vivo*. Finally, we evaluated if docosahexaenoic acid (DHA) and sphingosine-1-phosphate (S1P) promoted the survival of newly generated neurons. Our results indicated that supplementation of secondary mixed cultures with both DHA and S1P significantly reduced the number of apoptotic neurons, suggesting they might be useful in preventing neuronal apoptosis after their regeneration is achieved.

We conclude that MGC may have an alternative function in retina regeneration: besides giving birth to neurons -as many others researchers suggest-, we believe that MGC may help to restore neuronal populations by preserving a pool of progenitors in a

multipotent state, and by later inducing their differentiation into mature and functional photoreceptors.

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