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INTERVIEW

The people behind the papers – Maurício Rocha-Martins and Mariana Silveira

Retinal ganglion cells connect the retina to the brain, and their degeneration underlies glaucoma, which is the leading cause of irreversible blindness in humans and currently untreatable. Replacement of lost cells could be achieved by *in vivo* reprogramming of endogenous cells in the retina, a strategy explored by authors of a new paper in Development who focus on a transcription factor renowned for its reprogramming ability in other contexts. We caught up with first author Maurício Rocha-Martins and his former supervisor Mariana Silveira, Professor at the Federal University of Rio de Janeiro in Brazil, to find out more about the story.

Mariana, can you give us your scientific biography and the questions your lab is trying to answer?

MS My research started off in microbiology, first as a technician in biotechnology and then as an undergraduate at the Federal University of Rio de Janeiro (UFRJ). Although my degree was on Microbiology and Immunology and I carried out research in drug resistance in bacteria and in *Plasmodium falciparum*, this shifted to neuroscience when I joined the lab of Rafael Linden for my doctoral studies at the Institute of Biophysics Carlos Chagas Filho, a renowned biomedical institute in Brazil. There, co-mentored by Marcelo T. Bozza and Fernando G. Mello, my longstanding interest in retinal neurochemistry and molecular neurobiology started with studies on neuropeptide regulation of cell death and differentiation in the developing and mature retina. As a postdoc visiting Victor May at the University of Vermont, I was drawn to the Klf family of transcription factors even before *Klf4* became famous as one of the Yamanaka factors. In my own research group, my interest in developmental biology led me to start working on retinal cell reprogramming with talented students. We are interested in understanding the molecular mechanisms of cell fate acquisition as building blocks for the design of new regenerative strategies. Particularly, in this first study we wanted to elucidate *Klf4*-dependent mechanisms of retinal cell fate acquisition. The findings suggest that these mechanisms could be applied to design novel regenerative strategies to replace and reconnect retinal ganglion cells (RGCs) lost in the adult retina as a result of neurodegeneration in glaucoma.

And Maurício, how did you come to work in the Silveira lab?

MR-M My interest in science began at a young age. I used to do experiments around the house at the age of ten, and I even kept a little science notebook. My family was always supportive, even when, for example, I needed to use the freezer to test how long insects can sustain cold temperatures. It was no surprise then that I started a bachelor's degree in biology at the UFRJ, and soon enough I got a real internship in a real lab.

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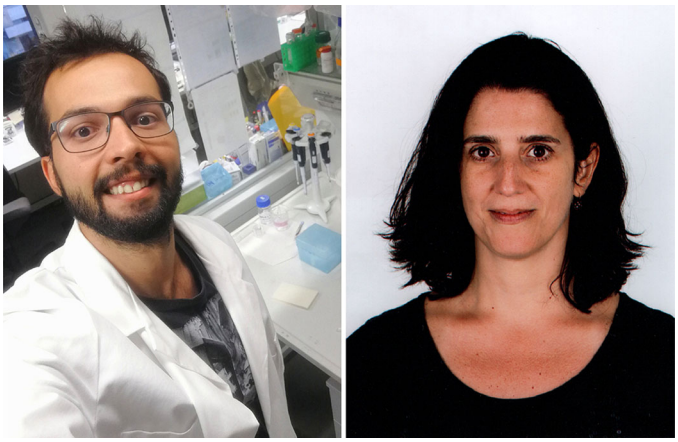

Maurício (L) and Mariana (R)

I was sure I wanted to be an entomologist, but slowly I became more and more interested in developmental biology, in particular how the different steps of embryo and organ formation are coordinated in time. The idea of an internal clock that determines when different types of cells are generated really fascinated me. Towards the end of my graduation I was fortunate enough to meet Mariana, who introduced me to a powerful system to study temporal control of development: the vertebrate retina. The neurons of the retina come in six 'flavours' and are generated in a stereotypic birth order. The predefined order and windows of cell type specification make the retina an excellent system for studying the mechanisms underlying temporal progression. I then dedicated my PhD in the Silveira lab to studying how neurogenesis is controlled in time and how to manipulate the birth order of RGCs, a disease-relevant cell type.

Before your work, what was known about the role of *Klf4* in RGC development and regeneration?

MS Other research groups and our own had shown that RGCs express *Klf4*, and the influence of *Klf4* and other members of the family in axon growth have been studied in depth. Moore et al. (2009) showed that *Klf4* inhibits axonal growth, as its specific deletion in RGCs increased axon growth and regeneration, and the same group recently suggested that *Klf4* regulates axon density during retinal development, but not RGC specification and survival. However, a direct role of *Klf4* in RGC generation remained unexplored, which we addressed by combining a conditional knockout mouse in which the *Klf4* gene is deleted very early in retinal development and CRISPR/Cas9-based approaches in zebrafish. Interestingly, Todd and Fischer (2015) had also showed that *Klf4* was upregulated during endogenous reprogramming of Müller glial cells in chickens. Müller glia in fish are an endogenous source of regeneration, and upon damage can re-enter the cell cycle and generate all retinal neurons. However, this capacity is reduced in

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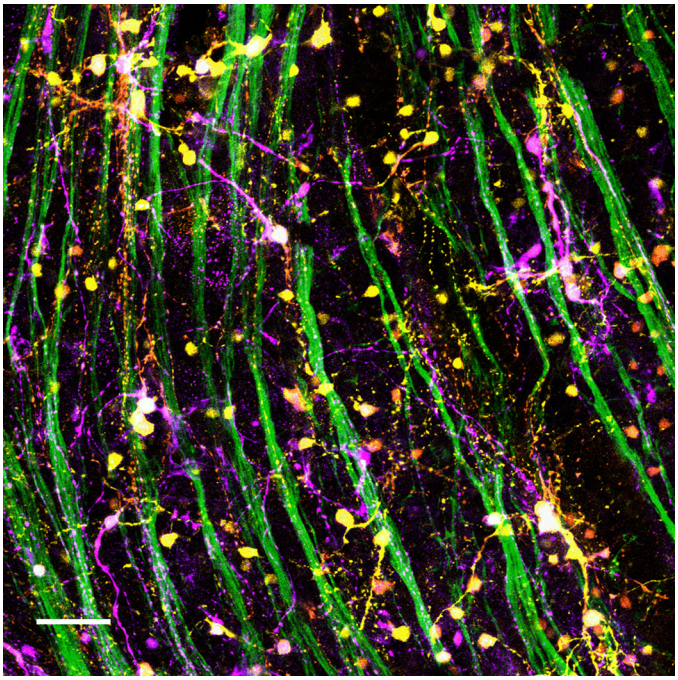

Induced ganglion cells (iRGC) generated *in vivo* upon Klf4 overexpression for 30 days. iRGC (GFP-positive, depth colour code) project TUBB3-positive axons which are aligned with endogenous fascicles (green).

chicken and almost lost in mammals. These lines of evidence encouraged us to pursue the study of *Klf4* in retinal development and cell reprogramming.

Can you give us the key results of the paper in a paragraph?

MS We wanted to know whether *Klf4* played a role in the development of specific cell types in the retina, in particular RGCs. To answer this, we used loss-of-function approaches in both mouse and zebrafish. The data showed that *Klf4* might not be essential for RGC generation, although other family members could compensate for its absence, as was shown in embryonic stem cells (Jiang et al., 2008). What was really striking was that when we overexpressed *Klf4* in late retinal progenitors, which have a restricted neurogenic potential, these progenitors re-acquired the potency to generate RGCs. Next, we asked what changed in the expression pattern of these late progenitors shortly after *Klf4* overexpression. We detected the upregulation of some characteristic elements of the molecular programme of early retinal development, such as *Atoh7*, which is essential for RGC generation. Although these induced RGCs (iRGCs) lacked some markers of mature RGCs (such as Brn transcription factors), they projected axons toward the head of the optic nerve and survived up to 30 days after the beginning of *Klf4* overexpression, the longest time analysed. In conclusion, this single transcription factor can reprogramme the fate of late retinal progenitors that normally generate other types of retinal neurons. This opens the door to investigating *Klf4* as a promising candidate for new strategies to regenerate RGCs at late disease stages of glaucoma.

Even though your iRGCs matured and survived for a long time, they didn't express known regulators of RGC maturation and survival: why do you think that is?

MS Our working hypothesis is that the role of *Klf4* as a pioneer factor might affect epigenetic modifications crucial to the progression of retinogenesis, which normally prevents the neurogenic programming of RGCs at late stages of retinal development. Then, to reprogramme late retinal progenitors, *Klf4* might interfere in mechanisms that, in combination with *Atoh7*, succeed in the generation of RGCs. However, although iRGCs do survive for at least 30 days, the programme activated by *Klf4* may not follow the exact time course of gene expression of the key transcription factors responsible for terminal differentiation and survival, such as *Brn3b*. Alternatively, the sustained expression of *Klf4* could interfere with later steps in iRGC differentiation. In fact, *Klf4* has been described as an inhibitor of axonal outgrowth. So, we believe that although it activates a programme that is efficient in giving rise to RGCs, in the long run *Klf4* may impair the final stages of the process. Either there is a missing step and/or *Klf4* may actively inhibit maturation.

More longer term, what do you think are the advantages (and disadvantages) of cell reprogramming compared with cell therapy for the treatment of glaucoma?

MS There are different strategies involving cell therapy and cell reprogramming. One which has shown promising results uses stem cells as a source for the secretion of neurotrophic factors. This and other neuroprotective approaches, including gene therapy, are efficient to prevent the progression of RGC degeneration. However, in late stages of disease progression, RGC regeneration would be essential. The generation of RGCs *in vitro* either from embryonic stem cells or induced pluripotent stem cells to transplant into affected eyes is an alternative, but cost, safety and low efficiency are the major limitations of this approach. Finally, an appealing option is to coax endogenous cell sources to replace the lost RGCs. In this context, re-activating the intrinsic regenerative potential of Müller glial cells is an interesting option, and has potential advantages related to cell integration; however, it is very early to state that it may lead to a treatment for glaucoma. The first step would be to demonstrate that functional RGCs can be generated *in vivo* from Müller glia, and to attain visual function recovery in preclinical models of glaucoma.

When doing the research, did you have any particular result or eureka moment that has stuck with you?

MR-M As a junior group leader, Mariana was very hands-on, helping with bench work whenever possible, so it was no surprise that we shared the eureka moment of this project. We went together to the microscope to check what long-term overexpression of *Klf4* does to progenitor cells: could *Klf4*'s reprogramming abilities could be used to promote *de novo* genesis of RGCs? I had electroporated the retina of rat pups *in vivo*, a method that consists of delicate surgery and injection of DNA into the subretinal space. To be honest, because this injection is tricky, we would have been happy to see any sign of electroporation at this point. But, to our surprise, the retinas were nicely electroporated – 'beginner's luck' – and we observed a striking change in the position of the neurons within the retina. In the control, most neurons were located at the apical side, whereas in the *Klf4* group they were positioned at the basal side. This change in position indicated a possible change in fate. Looking through more sections we saw beautiful bundles of axons coming from the basal neurons all the way to the optic nerve, a unique feature of RGCs. We were literally jumping with excitement in the microscope room. This finding motivated us to characterize in detail the fate of these basal neurons, and we confirmed that *Klf4* overexpression is sufficient to induce *de novo* genesis of RGCs.

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We were literally jumping with excitement in the microscope room

And what about the flipside: any moments of frustration or despair?

MR-M The moments of frustration were usually associated with the lack of infrastructure that is common even in the best universities and institutes in Brazil. Although I was lucky to work in a well-equipped lab, many times I lost experiments due to power blackouts or could not start because there was no water, so no ice. Another recurrent issue is the time it takes to import lab reagents. It is not uncommon to wait 3 months or more to receive an antibody or a restriction enzyme. For these reasons, simple experiments that should take weeks were stretched for months. So, I really would like to thank the labs at the Institute of Biophysics (UFRJ) and neighbouring institutes for sharing reagents and equipment. This work would not have been possible without this collaborative spirit.

So what next for you after this paper – I understand you're now in Germany?

MR-M Part of my PhD work was carried out in Caren Norden's lab at the Max Planck Institute of Molecular Cell Biology and Genetics. The goal of this collaboration was to develop zebrafish mutants to complement our mouse data on *Klf4* function. But during this time I took on a side project investigating neuronal migration in the retina. This was when I did my first live-imaging experiments of zebrafish embryos and realized how dynamic retinal morphogenesis is. I saw that the different cell types initially share the same space and later sort themselves into layers. It became clear to me that the formation of a functional retina crucially requires that each cell finds its way through the crowded tissue to its correct destination. But, although this neuronal layering is clearly essential for proper circuit assembly and vision, we are just beginning to understand how retinal neurons migrate and de-mix to form layers. So, fascinated by this question, I returned to the Norden lab as a postdoc. I am currently investigating the formation of the layer responsible for collecting light from the environment: the photoreceptor layer.

What is the situation like for developmental biology – and research in general – in Brazil at the moment?

MS These are hard times for research and education in Brazil. We are facing a drastic reduction in research investment from the federal government and the budget now is the lowest in the last 14 years. This affects the number of fellowships awarded to PhD students and postdocs and the budget for research projects. The impact is evident in the reduction of students seeking PhD programmes and in the increase of dropout rates. For this reason, many talented students go to other countries and are not attracted to return to Brazil. This state of affairs, which has been the subject of many articles in scientific journals and reports from scientific societies (see for example https://www.nature.com/articles/d41586-019-01079-9 and https://www.sciencemag.org/news/2019/05/brazil-useful-idiots-protest-cuts-research-and-education), is something that will threaten the future of Brazilian research. I believe one way to overcome difficulties and to survive through this hard period is to intensify international and local research collaborations. But the government must soon realize and embrace the slogan from the Brazilian Academy of Sciences: 'Science is not an expense. It's an investment'.

Where will this work take the Silveira lab?

MS Understanding the basic mechanisms associated with the acquisition of specific cell identities in neural development underpins my interests. This work opens crucial questions in the fields of neuronal development and *in vivo* reprogramming. For example, we are interested in understanding the mechanisms behind the *Klf4*-induced change in cell fate; in the design of complementary strategies to guarantee the generation of mature RGCs; and in the development of reprogramming strategies directed to Müller glia in disease models of glaucoma. Müller glial cells are good targets if we think about the replacement of RGCs from an endogenous source. To re-activate the regenerative potential that has been lost over evolutionary time is a promising alternative.

I hope that the visibility of this study helps us to build international collaborations to pursue these lines of investigation. Collaborative work with other colleagues and a group of very motivated and talented students were essential to achieve the results we have obtained up to now. However, the situation in Brazil is now very difficult for those who want to pursue a scientific career and it is hard to keep the students in the lab motivated in such scenario. This was also one of the reasons that made me decide to spend a sabbatical period at João Relvas' lab at the Institute for Investigation and Innovation in Health (i3S, University of Porto, Portugal) to diversify our research lines and build new collaborations. It is a survival strategy.

Finally, let's move outside the lab – what do you like to do in your spare time?

MR-M: Coming from Rio de Janeiro, I had low expectations about life in a 'small' city in Germany. But soon I was surprised by the cultural diversity the city of Dresden has to offer. Now, I occasionally enjoy jazz concerts and even jam sessions of Brazilian chorinho. Dresden also taught me to appreciate nature in a different way. I have replaced the beaches of Rio with hikes through the beautiful Sächsische Schweiz national park. These hikes are usually slow because the kid in me still wants to turn over every rock. Also, my love for insects stays strong. I currently keep a family of stick insects at home … but I don't do experiments on them anymore.

MS Besides spending time playing with my 4-year-old son, I really love the diversity of Brazilian music, the rhythm and beat of samba, maracatu, coco, congo, ciranda. I try to enjoy it by participating in groups that use the instruments from samba schools to play not only samba but this rhythmic diversity. I learned how to play with musicians in groups like *Monobloco* and *Bangalafumenga*, which in the early 2000s contributed to the rebirth of street carnival in Rio de Janeiro. It is really a fantastic experience. I do recommend it.

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