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INTERVIEW

The people behind the papers – Megha Agarwal, Akashi Sharma, Pankaj Kumar, Amit Kumar and Sam Mathew

Myosin is a major component of the sarcomeres of muscle, but its roles during muscle development are still relatively poorly understood. A new paper in Development investigates the function of a developmentally expressed myosin heavy chain isoform during mice myogenesis. We caught up with the paper's four co-first authors, Megha Agarwal, Akashi Sharma, Pankaj Kumar and Amit Kumar, and their supervisor Sam Mathew (Associate Professor in the Regional Centre for Biotechnology in Faridabad, India) to find out more about the project.

Sam, Megha, Pankaj, Akashi and Amit (L to R).

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

I did my Bachelor's and Master's degree in Zoology in Kerala, India, and then got my first experience in research working as a research assistant for a year in Bangalore, in a lab that used *Drosophila* to study circadian rhythms. Soon after, in 2001, I joined the graduate program in Genetics and Functional Genomics at the University of Cologne, Germany. I joined Maria Leptin's lab for my graduate work, trying to characterize a genomic region involved in gastrulation in *Drosophila*. Maria's mentorship style of giving students a free hand helped me develop ideas and grow as a scientist. I got interested in understanding developmental processes using genetic tools and, although it took a while, this work led to the identification of the role of the TNF-Receptor Associated Factor 4 (TRAF4) in *Drosophila* gastrulation. I continued with Maria for 2 years as a postdoctoral fellow to complete this work and then wanted to switch to a vertebrate model. Thus, I joined Gabrielle Kardon at the University of Utah, USA, where she was using mouse models to understand the role of connective tissue fibroblasts in skeletal muscle development and regeneration. Using mouse genetics, we showed that Tcf4 is a marker of connective tissue fibroblasts and that Tcf4^{+} connective tissue fibroblasts are important regulators of muscle development and differentiation, especially regulating maturation of muscle fibre type. This is where I came across myosin heavy chain-embryonic (MyHC-emb), which we used as a marker for developing myofibres. Delving into the literature, I found that not much was known about MyHC-emb function even though it was used routinely as a marker for terminally differentiated myofibres during development and for regenerating fibres in adults. This led to discussions with Gabrielle, who was generous enough to allow me to start generating a targeted mouse model, which is an important tool used in the current work.

We are pursuing three main research directions in my lab: first, we are trying to understand the specific functions of developmental myosin heavy chains (MyHCs) and how mutations in these MyHCs lead to congenital diseases. Second, we are interested in how skeletal muscle differentiation occurs during development and stem cell-mediated regeneration, and are trying to identify genes and regulatory sequences involved in this. Third, we are working on signalling pathways that are misregulated and lead to the formation of a cancer type called rhabdomyosarcoma, in which the tumour cells exhibit muscle-cell characteristics. We use *Drosophila*, mouse and cell culture models, and employ imaging, biochemical methods and genetic tools to address all of these questions.

How did the four of you come to work in Sam's lab, and what questions drive your research?

MA I was very clear that I wanted to work on stem cells for my PhD. I had already worked on mesenchymal stem cells during my Master's degree project and was fascinated by how smart these cells are. How stem cells serve as a maintenance and repair resource for the entire body is something that has interested me all along. After checking out a few labs, I decided to join Sam's since it provided me with a great opportunity to study stem cells and their behaviour during animal development and adult tissue regeneration. Since this research topic is something close to my heart, it has been easy to keep myself motivated.

AS I joined as a project fellow in Sam's lab in 2014 and within 4 months I started my PhD with him! From my college days, I was interested in the processes of embryonic development that make an entire organism from a single cell. While working with Sam as a project fellow, I got an opportunity to develop a deeper understanding of the developmental processes that lead to muscle formation. Different muscles in our body have distinct muscle fibre type composition; however, all fibres express MyHC-emb during embryonic development. I was intrigued and wanted to explore more about this MyHC isoform and decipher the functional importance of MyHC-emb.

PK Before joining Sam's lab, I briefly worked on *Leishmania*, a parasitic protozoan that causes kala-azar (visceral leishmaniasis) in humans. I discovered how intriguing animal development is while preparing for my PhD fellowship exams. Therefore, I scouted around for labs working on animal development and joined Sam when he was in the early phase of setting up his lab at RCB. My research is driven by a quest to understand the complexities underlying gene regulation during animal development and tissue regeneration.

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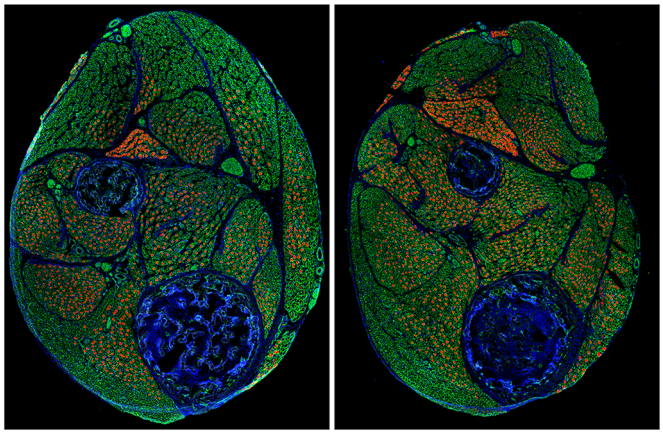

Cross-sections through the shank of postnatal day 0 wild-type (right) and *Myh3* knockout (left) mice, showing MyHC-slow (red), laminin (green) and DAPI (blue).

AK I joined Sam's lab with a deep interest in studying development, as it has always fascinated me. I worked extensively on fetal tissues during my doctoral training. Sam explained some of the observations he made on the myosins and how human muscle defects are recapitulated in myosin heavy chain knockout mouse models. This was exciting and I was keen to understand the role of myosins during development and regeneration.

How much was known about the role of the developmental MyHCs before your work?

MA, AS, PK, AK & SM Developmental MyHCs were discovered in the 1980s and although they were found to be expressed during development and regeneration, not much has been known about their function since then. A few studies on the regulation of their expression were carried out during the 1990s and early 2000s. Then in 2006, Michael Bamshad's lab identified that mutations in the *MYH3* gene, which codes for MyHC-emb, lead to Freeman–Sheldon and Sheldon–Hall congenital contracture syndromes, indicating that developmental MyHCs – and MyHC-emb specifically – have important functions. Surprisingly, animal models for studying and understanding the mechanisms underlying these syndromes were not pursued or were unsuccessful. This was in contrast to adult MyHCs, for which knockout mice for two isoforms were generated and characterized successfully in the 1990s by Leslie Leinwand's lab. This provided us an opportunity to explore the roles played by developmental MHCs.

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

MA, AS, PK, AK & SM In this paper, we describe the role of MyHC-emb in skeletal muscle development. By generating and making use of conditional targeted and null mouse alleles for *Myh3*, we characterize the role of MyHC-emb during embryonic, fetal and neonatal myogenesis. There are four key findings that we describe in this paper. First, we find that MyHC-emb has dual cell-autonomous and non-cell-autonomous roles during muscle development. MyHC-emb is expressed in myofibres, and in a cell-autonomous manner regulates muscle fibre type, fibre number and fibre size. In a non-cell-autonomous manner it regulates the rate of differentiation of myogenic progenitors and myoblasts during embryonic and fetal myogenesis (cells in which it is not expressed). Second, we identify fibroblast growthfactor (FGF) as the secreted signal from myofibres that mediates the non-cell-autonomous effects of MyHC-emb on muscle progenitors and myoblasts. Third, we find that, although MyHC-emb is expressed in all myofibres during development, different muscles respond differently to MyHC-emb loss. Fourth, adult mice null for *Myh3* exhibit scoliosis, a phenotype seen in individuals with Freeman–Sheldon Syndrome, a congenital muscle contracture syndrome in which *MYH3* is mutated. Thus, this work highlights the role of developmental MyHCs during development and how their loss of function leads to abnormalities.

Why do you think MyHC-emb has distinct effects in different muscle types?

MA, AS, PK, AK & SM This was a surprising finding since, to our knowledge, MyHC-emb is expressed by all myofibres during development. We believe that the distinct effects loss of MyHC-emb has on different muscles is down to unique fibre-type composition and metabolic characteristics, which in turn are determined by the anatomical location and functional needs of the specific muscle. We think that these differences between muscles are reflected in the distinct effects we observe upon loss of MyHC-emb.

How do you think an intracellular component of the sarcomere could act non-autonomously?

MA, AS, PK, AK & SM This was a puzzle for us until we came across some publications showing that the FGF pathway mediates differentiation and maintenance of the stem cell pool. FGFR4, a receptor in the FGF pathway, is important in regulating the rate of differentiation of myogenic progenitors and myoblasts during development, which are the cell populations that were affected upon loss of MyHC-emb. This led to additional experiments to test whether FGF signalling mediates the non-cell-autonomous effects of MyHC-emb on myogenic progenitors and myoblasts, which was indeed found to be the case. How MyHC-emb within myofibres controls the levels of FGF secreted by myofibres is a question we have been trying to find answers for, but have not been successful. This could hint at some novel function of MyHC-emb, which might be independent of its role in the sarcomere. Interestingly, Leslie Leinwand's lab reported in 2003 that MyHC-emb is one of three MyHCs that are expressed in non-myogenic cell types such as pulmonary myofibroblasts, indicating that MyHCs may have functions that are not restricted to skeletal muscle cells.

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

MA While deciphering the non-cell-autonomous effect of MyHC-emb on muscle progenitors, we hypothesized that it is mediated by FGFs. My eureka moment was when I successfully figured out, by mass spectrometric analysis, that secreted FGF levels are altered upon knockdown of MyHC-emb. This led us to the mechanism of how MyHC-emb regulates muscle differentiation non-cell-autonomously, confirming and validating our hypothesis.

AS One of our first *in vivo* experiments was to investigate the effect of loss of MyHC-emb on other MyHC isoforms. I was full of curiosity while performing the immunostaining for other MyHCs on *Myh3* knockout samples. I think finding more MyHC-slow^{+} fibres in *Myh3* knockout muscles was a result I can never forget.

PK Two moments actually, both related to when I was quantifying data: first when I found that the muscle progenitor numbers reduced significantly upon *Myh3* knockout, and second when FGF supplementation led to a rescue of the progenitor numbers *in vitro*.

AK To me, corroborating the *in vivo* results from the *Myh3* knockout mice using C2C12 myogenic cells *in vitro* was highly

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satisfying. The *in vitro* system proved really handy for the demonstration of the non-cell-autonomous effect of MyHC-emb.

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

MA I started working on this project about 5 years ago and there were several instances when I got frustrated. For a while, we had difficulty in explaining the MyHC-emb loss-of-function phenotype, especially the mechanism of how MyHC-emb regulates muscle stem cells. Although we followed several directions, most were unsuccessful until we came upon FGF signalling. I think many of these failed experiments formed the basis of this manuscript and I kept going because, as Steve Jobs said, I considered what I do to be great work!

AS Not really a specific moment, but in the early days of the project, we did not have an animal facility on campus and were reliant on the samples brought by Sam from the laboratory of our collaborator, Gabrielle Kardon, at the University of Utah. This meant that every sample was precious and there were times when I had to wait for several months to get samples for new experiments, which was frustrating.

PK I faced some frustrating moments with the muscle-fibre size quantification. This was mainly related to finding a reliable software application that would make these measurements accurately. These problems went away after we came across a software application called SMASH, which was developed precisely for muscle fibre measurements.

AK I remember that one of the challenging moments early on was to draw an understanding of the sequence of events affecting normal myogenesis in the absence of MyHC-emb, and to identify the mechanisms underlying them. This led to testing a lot of possibilities without success, which for a while was frustrating.

So what next for the four of you after this paper?

MA This PhD training with all its ups and downs has helped me decide that research is what I want to do. Although there might be difficulties in research, with constant effort one can definitely achieve good results. I am keen to switch fields and am on the lookout for a postdoctoral position in computational modelling and bioinformatics.

AS I am currently working on other projects and plan to complete my PhD soon. In addition to skeletal muscle, I am also interested in the cardiac and smooth muscle. I would like to pursue postdoctoral research in the development of any or all of these three muscles.

PK I am also working on another project in the lab, related to the regulation of myogenesis. I am trying to complete this work and will be looking for postdoctoral opportunities in developmental biology, with translational relevance.

AK I left the lab in 2016, when this paper had started to take shape. I am now a postdoctoral fellow at the University of California Los Angeles, working on haematopoietic stem cells and cancer from a gene regulation perspective.

Where will this work take the Mathew lab?

SM This work originated when I was working as a postdoctoral fellow and I remember starting work on the gene-targeting construct almost 10 years ago! There have been a few milestones along the way, such as successfully identifying the gene-targeted mice, initial characterization of the knockout mice, moving to India and becoming an independent investigator, getting the mice shipped to India and now getting the work published. Although it took a while to get this paper out, we should now be able to come out with more interesting results, especially with respect to MyHC-emb in muscle regeneration. We are also keen to understand the precise mechanisms that underlie the phenotypes seen in individuals with Freeman–Sheldon Syndrome. I think skeletal muscle development, regeneration and homeostasis are all research areas with immense translational significance and hope to continue making new discoveries in these fields.

Am I right in thinking there is an increasing amount of developmental biology going on in India at the moment? As someone who left India for your PhD and postdoc but then returned to set up your own lab, what has your experience been like?

SM Yes, I think it is true. Actually, there are a lot of relatively young investigators, working in diverse areas, who set up their labs in the past 10-15 years. In my opinion, this resulted from several new research institutes being set up and an increase in funding over this period. Coming back to developmental biology in India, we have a set of diverse researchers working on different model systems, who are doing well. We also have an Indian Society for Developmental Biology with an ever-increasing number of members.

Returning to India was a decision I made after being abroad for more than 12 years. Although it took some getting used to, it is a decision I am quite happy with. Since I joined a relatively new research institute, some amount of time was spent initially on ordering equipment and getting facilities up and running. Some of the flexible funding I received, especially from the Wellcome Trust DBT India Alliance, really helped run the project during the initial days, when I had to travel to Gabrielle's lab to carry out the mouse work. I have been lucky to work with some really smart, talented and dedicated graduate students and postdoctoral fellows, and mentoring them has been a lot of fun.

There are many labs working on developmental biology today in India

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

MA When I have free time, I like to work out in the gym. I also like to go out shopping with friends.

AS Our campus in Faridabad is a really beautiful place, and I like to spend my leisure time enjoying the beauty of nature around me, while reading books.

PK I like playing badminton, going out with friends and especially visiting my cousins to enjoy home-cooked food!

AK I remember how we used to go out together with the lab for lunch, and also remember a trip to the Himalayan mountains for hiking and fun. Currently, I am in Los Angeles, where I like to try out different cuisines and go on long drives over weekends.

SM I like spending time with family and friends in my spare time. I also like to travel; Faridabad is close to a lot of places with historical significance, which I try to visit.

Reference

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