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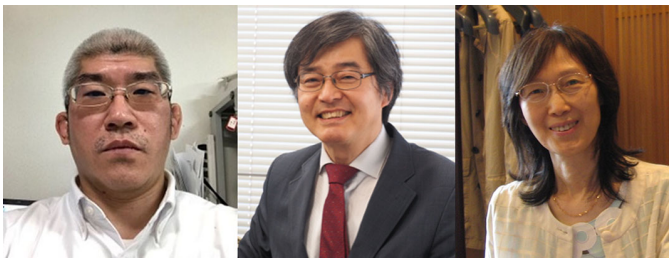
INTERVIEW

The people behind the papers – Masahito Irie, Fumitoshi Ishino and Tomoko Kaneko-Ishino

Viral-derived genes have had a huge impact during mammalian evolution, with many of the exapted genes being expressed in the placenta. Now, new research published in Development describes the importance of two genes with retroviral origins in microglia, the innate immune cells of the brain, which are derived from another extra-embryonic tissue, the yolk sac. We caught up with the first author, Masahito Irie, and the corresponding authors, Fumitoshi Ishino, Professor at Tokyo Medical and Dental University, and Tomoko Kaneko-Ishino, Professor at Tokai University, to hear about more about their research.



Masahito Irie, Fumitoshi Ishino and Tomoko Kaneko-Ishino (L-R).

Fumitoshi and Tomoko, can you give us your scientific biographies and the questions your labs are trying to answer?

FI: I completed both my undergraduate degree and my PhD at the University of Tokyo. After finishing my PhD in 1983, I was appointed Assistant Professor at the Institute of Applied Microbiology, University of Tokyo. Then, in 1991, I moved to the Gene Research Center at the Tokyo Institute of Technology to become an Associate Professor. During this time, I was a PREST (JST) researcher of inheritance and variation and a CREST (JST) research director in structure and function of genomes. I moved to my current position at the Medical Research Institute, Tokyo Medical and Dental University (TMDU) in 2003 and served as the Institute Director from 2014 to 2020.

TK-I: I studied at the University of Tokyo for both my undergraduate degree and my PhD, graduating in 1988. After completing my PhD, I moved to the Industrial Research Institute (now the National Institute of Advanced Industrial Science and Technology in Japan), followed by a year working as a British Counsel Fellow in Cambridge, UK. I then return to Tokyo, working as a JSPS fellow at the university, before moving on to a research assistant position at the Tokyo Institute of Technology. In 1995, I was appointed as an Associate Professor at the School of Health Sciences, Tokai University, and became a full Professor in 1999. From 2010 to 2014, I was a research representative of the JSPS Next Generation World-Leading Researchers (NEXT) program. I moved to the School of Medicine at Tokai University in 2019.

FI & TK-I: We started collaborating back in 1990, working on the isolation of imprinted genes. From 2000, we focused on *Peg10* (also known as *Rtl2* or *Sirh1*) and *Rtl1* (also known as *Peg11* or *Sirh2*), two retrovirus-derived genes in mammals. Then, from 2007, we expanded our research to look at all 11 eutherian-specific RTL/Sushi-ichi retrotransposon homologue (SIRH) genes, which include *Peg10* and *Rtl1*.

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Masahito, how did you come to work on this project and what drives your research today?

MI: I am interested in the genes that characterize mammals, and because of that I joined the Ishino lab, which studies mammalian-specific genomic imprinting. We work on the 11 RTL/SIRH genes that are eutherian specific, and we predicted that the functions of these genes would be important for present-day eutherian mammals. In the SIRH gene project, I worked mainly on analysing *Rtl6* (*Sirh3*), *Rtl5* (*Sirh8*) and *Rtl4* (*Sirh11*).

What was known about the role of the eutherian-specific retrotransposon Gag-like genes in early development prior to this research?

MI, FI & TK-I: During the course of our genomic-imprinting research, we demonstrated that two paternally expressed genes, *Peg10* and *Rtl1*, play an essential role in placenta formation (Ono et al., 2006) and maintenance of placental function (Sekita et al., 2008) as therian- and eutherian-specific genes, respectively. These results indicate the important role of mammalian-specific genes acquired from retroviruses and prompted us to start a systematic analysis on eutherian-specific RTL/SIRH genes. We found that *Ldoc1* (also known as *Sirh7* or *Rtl7*) is another essential placental gene involved in endocrine regulation that determines the timing of parturition (Naruse et al., 2014) and *Sirh11* (also known as *Zcchc16*) is an important gene in the brain, regulating cognitive ability and space memory via noradrenaline regulation (Irie et al., 2015). *Sirh11* was later implicated in autism spectrum disorder (Lim et al., 2013). We also reported that *Rtl1* plays important roles in muscle development during fetal and neonatal stages and in the central nervous system. All these results indicated that *Rtl1* is the major gene responsible for two genomic imprinting disorders: Kagami–Ogata and Temple syndromes (Kagami et al., 2008; Kitazawa et al., 2020, 2021). Recently, we reported that the *Peg10* protease domain is essential for the maintenance of fetal capillaries during late gestation (Shiura et al., 2021).

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

MI, FI & TK-I: Two phylogenetically related retrovirus-derived genes, *Rtl5* and *Rtl6*, play important roles in the front line of the innate brain immune response. Both RTL5 and RTL6 are expressed in microglia and are involved in removing pathogens, which we mimic using LPS, dsRNA and non-methylated DNA. It is interesting that they function against bacteria and viruses although

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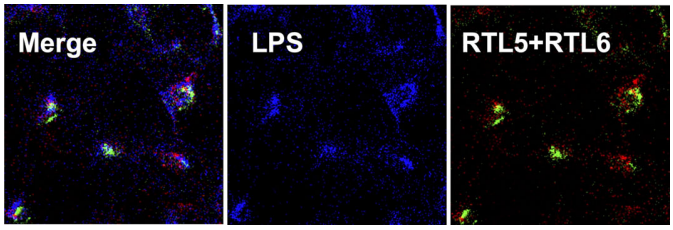

Microglia expressing RTL6 (green) react to and surround LPS (blue) that has been injected into mouse brains.

they themselves are derived from a retrovirus. It suggests that the eutherian innate immune system recruited additional members via exaptation to complement the universal Toll-like receptor system found throughout the animal kingdom. As microglia are derived from the yolk sac, we have proposed that the extra-embryonic tissues, such as placenta and yolk sac, may be the birthplace of exapted genes during mammalian evolution.

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Does the amount of extracellular RTL5/6 increase upon damage to the brain and, if so, could this be via regulated exocytosis from the microglia?

MI, FI & TK-I: In our preliminary data on isolated microglia, *Rtl6* mRNA does not increase after LPS injection. Extracellular RTL5 and RTL6 proteins are found in small dots and granules in the brain at steady state, but we think it probable that single RTL5-mCherry and RLT6-Venus are not detectable in our system, and we can only detect them when they aggregate as small dots and granules. Similarly, when they form big complexes with pathogens, we can detect them more easily. We think it is likely that they are secreted from microglia because the dots and granules inside of microglia have a structure that is similar to secretory vesicles.

Do RTL5 and RTL6 interact directly with the Toll receptors already implicated in pathogen recognition in the brain?

MI, FI & TK-I: No, we don't think so. It takes about 30 min to increase *Tnfa* and *Il6* mRNAs in microglia, but the RTL5 and RTL6 reaction to the pathogens seem to take place more quickly. Our unpublished preliminary *in vitro* experiments suggested that the reaction takes place just after LPS administration. Therefore, we think that RTL5 and RTL6 are constantly present throughout the brain and immediately aggregate around pathogens in an emergency reaction that prevents spreading before the major Toll-like receptor system starts work. They presumably act as the first barrier against the pathogens.

Why do virus-derived genes play such important roles specifically in extra-embryonic tissues?

MI, FI & TK-I: It is known that endogenous retroviruses (ERVs) and retrotransposons are completely repressed by DNA methylation in the fetus while being constantly transcribed in the extra-embryonic tissues owing to the lower DNA methylation level. Therefore, we think that the extra-embryonic tissues play a role in testing the function of mutated ERV-derived genes. In other words, the ERV-derived genes might have been functionally selected based on whether they are advantageous for these tissues or not. Thus, it is likely that ERV-derived genes were first domesticated as genes functioning in the placental or yolk sac, including microglia.

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

TK-I: When I started screening for imprinted genes in Azim Surani's lab in 1990 in Cambridge, UK, I assumed that there would be some mammalian-specific genes that were derived from retroviruses because I hypothesised that genomic imprinting arose as a defence mechanism against the introduction of exogenous DNAs. Therefore, I was very excited to find that my idea and research direction were correct when we identified *Peg10* and also *Rtl1* as novel imprinted genes in 2000, although we did not publish the result of *Rtl1*. Using *Peg10*, *Rtl1* and *Ldoc1* knockout mice, we identified clear placental phenotypes, which lead to lethality in embryos, fetuses and pups, respectively, allowing this project to proceed relatively easily.

I feel that understanding the function of *Rtl6* was the toughest project. It is extremely well conserved among eutherians, suggesting it is a very important gene, but although knockout mice exhibited several interesting behaviours, we could not address its real function using these mice. Then, we generated *Rtl6*-Venus knock-in mice, but it still took more than 3 years for us to detect the fluorescent signal from the fusion protein efficiently and to get a hint of its function.

Then, on 16 February 2021, I saw Fumi carefully dissect a brain without damage using ice-cold buffer, but we found that the RTL6 signal was actually weaker than before. This result made me think that our experimental conditions were wrong, and we went the opposite direction and realised that we needed to damage the brain in order to activate the RTL6 reaction. It was a turning point that led to a great surprise: that RTL6 reacted to injected LPS in the brain. Then, we tried dsRNA and non-methylated DNA and found that RTL5 mainly reacts to these pathogens rather than RTL6.

FI: I started genomic imprinting research with Tomoko in 1989. My target imprinted genes were essential placental genes because genomic imprinting appears to be deeply related to viviparity. I knew that Tomoko was looking at the imprinted genes *Peg10* and *Rtl1* and I thought that it was highly possible that they may be essential placental genes, although they looked like LTR retrotransposon-derived genes. At that time, it seemed like a big project to knock out these two genes because nobody even knew whether such LTR retrotransposon-derived genes would have real functions. However, as my research aim was to identify essential placental genes, there was no reason to hesitate in making these transgenic mice. In early 2004, we got the results of both the *Peg10* and *Rtl1* knockout mice finding that they exhibit early embryonic and late embryonic/neonatal lethality, respectively. During this time, we generated a list of 11 candidate genes in humans and mice. So, we had established that mammalian-specific genes derived from retroviruses, the RTL/SIRH genes, are important in mammals, and it was exciting to find that this was not only in the placenta but also for the innate immune response in the brain via the microglia.

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

TK-I: Although we made the *Rtl6* knock-in mice 5 years ago, we weren't sure if the signal that we observed was the true signal or

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autofluorescence because we did not have laser scanning confocal microscopy (LSM880) with automatic component extraction (ACE) function at that time. We went to the ZEISS Show Room in Tokyo with Johbu Itoh to try out an LSM880 equipped with ACE function on 24 December 2015. We did not get the images we needed from the demo, and we returned home in the glare of neon lights on Christmas Eve without a word because we knew we'd have to wait until Tokai University introduced the ZEISS LSM880 to our campus. It was not until December 2017 that we could restart the analysis of *Rtl6* knock-in mice.

FI: It was essential to use the LSM880 in this experiment. Unfortunately, we, as well as many researchers doing basic research in universities in Japan, have not had a chance to improve our equipment for more than 15 years because of budget cuts. I think it has been a serious problem for development of basic science in Japan.

What is next for you after this paper?

MI: We have some interesting results from our analysis of the *Rtl6* and *Rtl5* knockout mice analysis. I would like to be able to explain these knockout mice phenotypes based on the results from this paper.

Where will this story take your labs next?

TK-I: Among RTL/SIRH genes, I would like to elucidate the functions of *Rtl3* (*Sirh10*) and *Rtl4*, and the molecular mechanisms behind them.

FI: I think that our series of work on RTL/SIRH genes provided evidence that mammalian-specific genes from retroviruses are very important in elucidating mammalian-specific functions in development. I think that this concept is a key to link ontogeny (development) and phylogeny (evolution) in current genome biology.

We assume that primate- and human-specific genes from retroviruses have important roles in the creation of several human features as well as the evolution of humans. It should be a neo-human genome project and we hope that many young researchers will want to be involved in this intriguing research.

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

TK-I: I am a travel lover, and also like having dinner with close friends, but I had to give up both for the past 3 years because of COVID-19. This summer, I have got involved with watching the video of the Viennese musical 'Elisabeth' and try to sing their songs in German. I learned German in my undergraduate days, so that helps!

FI: I like reading books, especially novels, science fiction, philosophy, and so on, which combines well with activities such as travelling with my family. We need a break after the COVID-19 restrictions.

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