Cell

Yoshiki Sasai 1962–2014

Yoshiki Sasai was one of the pre-eminent stem cell biologists. Known as the "Brain-maker," he developed methods to guide human embryonic stem cells (hESCs) into forming brain cortex, eyes, and other organs in tissue culture. Yoshiki was a man of rectitude and a scientist of high personal integrity, following in the line of his samurai ancestors. He always assumed responsibility. Since his post-doctoral studies, he was called Sensei, which means teacher, by those that knew him well. It was an affectionate sign of the high respect in which this scholar was held.

In addition to being a prodigiously original scientist, Yoshiki was one of the founding leaders of the RIKEN Center for Developmental Biology (CDB) in Kobe, a premier research institute for biology in Japan. As its Deputy Director, he embodied the dual nature of the institute, having both a solid grounding in the embryo and a deep and creative understanding of stem cell differentiation. Yoshiki played a key role in the initial design of CDB's beautiful new laboratories and in building the smaller adjacent hospital with the specific purpose of translating regenerative medicine to the clinic. With his boundless energy and keen eye for even obscure regulations, he became an indispensable person in helping CDB deal with issues that most other scientists would have been reluctant to approach. He was an attentive mentor and one of the guiding lights of the institute; the students who he trained will enrich Japanese stem cell biology for generations.

A man of strong physique, Yoshiki was recruited to play the sport he loved, American football, for Kyoto University when he studied medicine there. Yoshiki was then a resident at Kobe Municipal General Hospital. When Kobe was devastated by an earthquake during his postdoctoral training in the United States, Yoshiki was deeply affected by the destruction of this city he knew so well and would later greatly contribute to the Japanese government's efforts to rebuild it. A fully trained physician, his vocation from the beginning was focused on understanding the brain.

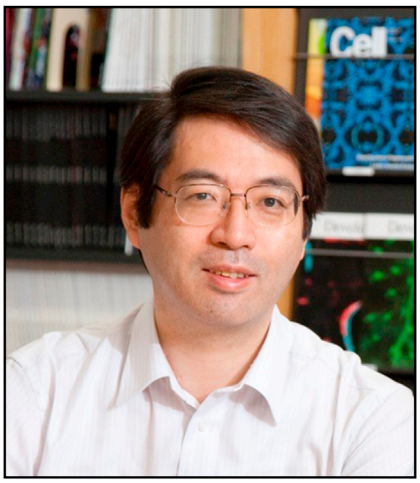
HES-1

For his Ph.D. training, Yoshiki joined the laboratory of molecular biologist Shigetada Nakanishi. His Ph.D. thesis work resulted in the cloning of a mammalian helix-loop-helix (HLH) transcription factor in the brain, *HES-1*. This discovery was a major contribution to cell signaling because HES-1 eventually proved to be the main transcription factor regulated by the Notch-signaling pathway.

His interest in brain formation prompted Yoshiki, still as a graduate student, to write to me in regards to a postdoctoral research position. We had just isolated a homeobox gene, *goosecoid*, expressed in Spemann's organizer, the region that induces the neural plate in *Xenopus*. During a brief visit to Japan, I interviewed him in the office of Professor Masatoshi Takeichi in Kyoto and recruited one of the best postdoctoral fellows one could ever hope for. He promptly secured a Human Frontiers Science Program fellowship and came to California in 1993.

Chordin

Yoshiki was a master molecular biologist. I proposed a differential screen between dorsal and ventral embryo mRNAs. He suggested that it would be much easier to screen for genes increased by lithium chloride, which expands the Spemann organizer by inhibiting GSK3, versus embryos ventralized by ultraviolet light in which the organizer does not form. Within a month of arrival, he had cloned a new cDNA, expressed in the gastrula organizer and later in the notochord, that he named *chordin*.



Yoshiki Sasai

This prompt achievement was performed in spite of a trying introduction to the USA. In the San Francisco airport, as Yoshiki was retrieving his luggage, a person approached his wife asking for directions. While holding her baby, she pointed to the directions while an envelope containing their passports and a few thousand dollars was being stolen from her baggage cart. Thus, while immigrating into a new country, starting a new job, finding an apartment, and cloning an important gene, Yoshiki was shuttling back and forth to the Japanese consulate to obtain new passports. When I asked him some time later how the United States compared to Japan, he responded that it was like *Through the Looking Glass*, the Lewis Carroll sequel to Alice in Wonderland. By this, he meant that everything seemed like the mirror image of what one might expect. While deeply rooted in Japanese culture, he did enjoy California and was a gregarious sushi and barbecue cook. Celebrations featuring these meals usually ended up in a hot tub. After his return, he would introduce many visitors to the marvelous traditional Japanese hot spring baths.

The first chordin cDNA was a partial clone lacking the carboxy-terminal half, but we had not realized this. We performed hundreds of mRNA microinjections into *Xenopus* embryos, but no phenotype was obtained. One day, Yoshiki came and said he had rescreened the library and had obtained a full-length mRNA and asked if I would like to microinject it. He was always thoughtful and respectful in the unique Japanese way. He gave his mentor the opportunity to perform the first experiment showing that *chordin* induced twinning in *Xenopus* embryos.

Chordin encoded a secreted protein with motifs later found to encode BMP-binding modules. The sequence,

expression pattern, and twinning phenotype allowed us to write a paper that Ben Lewin, the founding editor of *Cell*, promptly accepted.

Yoshiki next turned to his initial love of neural induction using ectodermal explants of *Xenopus* embryos. Two other secreted molecules, Noggin and Follistatin, discovered by Richard Harland and Douglas Melton, had been previously shown to induce neural tissue. Like them, chordin induced anterior brain differentiation. Herbert Steinbeisser in our lab was working on BMP4, a TGF-β growth factor expressed in the opposite side (ventral) of the embryo. Brain induction by *chordin* and *noggin* mRNA was antagonized by *BMP4* mRNA. As we were writing the paper, Yoshiki mentioned in passing that he had also prepared by PCR a *follistatin* clone. He gave me some mRNA to inject, and sure enough it too was antagonized by *BMP4*, though at the time Follistatin was thought to be exclusively an activin inhibitor. So we added an extra panel to our manuscript, showing this last-minute result. Yoshiki was a very meticulous scientist and thought that something might be wrong with his *follistatin* clone. So without telling me, he grew up plasmid from three other cloned colonies over the weekend, synthesized mRNA, injected it, and obtained the same result. Only then was he satisfied, and we submitted the paper to *Nature*. This exemplifies how careful he was to crosscheck others' experiments, including my own.

This 1995 *Nature* letter was the start of the realization that Spemann's organizer secretes a cocktail of BMP antagonists that are responsible for the formation of the neural plate in the embryo. There is another anecdote associated with this work. After we had written the paper together, Yoshiki accidentally erased the entire manuscript file while I was out of town. He asked my secretary not to tell and rewrote the entire manuscript from memory. I only realized this when I found one or two words that had changed from the original. He had an amazing photographic memory.

The purification of chordin protein from baculovirus constructed by Yoshiki was left in the hands of Stefano Piccolo. I found an old note of Yoshiki saying that "Stefano is a hardworking guy and is getting accustomed to the project slowly but steadily. As he is nervous about pressure, please give him only mild one if necessary." A kind-hearted man, Yoshiki felt it was his duty to look after everyone in the laboratory. This collaboration proved most productive and led to the finding that chordin was a BMP-binding protein.

Early Neural Target Genes in *Xenopus*

In 1996, Yoshiki returned to Japan to become an associate professor at the Kyoto University School of Medicine and was promoted to full professor in 1998. Together with other developmental biologists, he convinced the Japanese government to build a new institute for developmental and regenerative biology and persuaded Masatoshi Takeichi to become Director of this new initiative.

Now established in his own lab, Yoshiki trained a group of very talented students and began investigating the genes acting downstream of chordin to mediate the formation of the central nervous system. They discovered three early neural genes induced by *chordin* and suppressed by BMP4—*Zic1*, *Sox2*, and *SoxD*—which were required for neural differentiation. From his work in *Xenopus*, Yoshiki derived a unique feel for neural differentiation that led to his spectacular contributions to the mammalian stem cell field.

PA6 Cells Induce Dopaminergic Neurons

Mouse ESCs can differentiate into many cell types, and traditionally this was analyzed by preventing their attachment to substrate in the presence of high serum, which leads to the formation of embryoid bodies consisting of ectoderm, mesoderm, and endoderm. Yoshiki realized that it was critical to be able to differentiate ESCs in the complete absence of serum. He approached this problem by plating dissociated mESCs on feeder layers of a large number of cultured cell lines.

The breakthrough came with the discovery that the mouse calvaria PA6 bone marrow stromal cell line induced efficient neuronal differentiation. The resulting neurons were mostly dopaminergic and were able to improve symptoms of Parkisonism when transplanted into mouse brain basal ganglia. This stromal-cell-derived inducing activity was located on the cell surface of PA6 cells and was active even in formaldehyde-fixed cells. The gene responsible for this inducing activity has not yet been cloned. In addition to dopaminergic cells, some cultures developed large patches of confluent melanin-containing retinal pigmented epithelium (RPE). This observation would lead to Yoshiki's revolutionary investigations into eye development in vitro.

Keeping hESCs Alive

Human ESCs mimic the embryonic epiblast epithelium. When hESCs are dissociated, cadherins are released from the cytoskeleton, causing an activation of the Rho small GTPase that, in turn, activates the Rho-associated kinase (ROCK). The ROCK activation triggers myosin hyperactivation, blebbing, and apoptosis via mitochondria. Yoshiki and colleagues discovered that the simple addition of the ROCK inhibitor Y-27632 was sufficient to prevent death of dissociated hESCs. This opened the door for the clonal expansion and differentiation of hESCs, which they went on to exploit fully in order to differentiate the human brain in vitro.

Making Brains

Realizing that it was necessary to minimize signals from neighboring cells before triggering the intrinsic brain differentiation program, Yoshiki and colleagues developed a culture procedure in which human or mouse ESCs are dissociated into single cells, allowed to re-aggregate in the absence of serum, and then directed to specific differentiation pathways. The cultured floating ESC aggregates developed into neuroepithelial vesicles that were then cultured further to allow for the expression of their intrinsic self-differentiation programs. According to the initial treatment, the vesicles would generate various neural organs, such as telencephalic cortex. The neuron precursors could be transplanted into mouse brains, where they survived and established synaptic contacts corresponding, for example, to typical pyramidal neurons. In his most recent article, Yoshiki was able to grow highly sophisticated human brain cortex structures. The brain cortex formed multilayered neuronal structures in a similar arrangement as that seen in human fetal cortex at the beginning of the second trimester. The potential of

these self-organizing forebrain tissues for the study of human embryonic development is enormous.

Making Eyes

The result that stunned the stem cell world was obtained in 2011, when Yoshiki and colleagues discovered that their brain vesicles self-organized into retina and eyes. They used a mESC line with a retina homeobox (Rx) GFP knockin to find that strongly fluorescent patches developed within the neuroepithelial vesicles. When the Rx-GFP tissues were excised with forceps from larger aggregates, these fragments were able to recapitulate normal eye morphogenesis, invaginating to form an eye cup consisting of a retina including all of the correct neuronal cell types and layers covered on the outside by RPE. On occasion, eye lenses developed. Similar results were then obtained with hESCs. This galvanized the regenerative medicine world because the eye, like the brain, is an immune-privileged region and is very suitable for transplantation. Photoreceptor precursors and RPE cells have a great and immediate potential for use in stem cell therapy to cure major causes of blindness such as retinitis pigmentosa and macular degeneration.

Making Pituitary Glands

Another striking demonstration of the power of stem cells, if allowed to follow their biological destiny, was the generation of anterior pituitary. Yoshiki and colleagues were able to obtain fully differentiated pituitary glands secreting multiple hormones from ESCs in culture. When transplanted into the brain of mice, these ESC-derived organs were able to function and alleviate hypopituitarism. ESCs contain the regulatory gene networks that allow them to self-organize, and Yoshiki realized that all that was necessary was only to point them in the right direction in the right culture condition and then allow them to develop their genetic programs and cell-cell interactions on their own.

A Brilliant Scholar

This remarkable body of work, in which the differentiation of entire organs for transplantation therapy was achieved, was based on a profound knowledge of developmental biology. In the 1940s, Holtfreter and Barth had found that, in salamanders, animal cap ectoderm could in some conditions self-organize into brain vesicles, eyes, lens, and olfactory placodes. To achieve this in human stem cells, Yoshiki Sasai had to develop new methods to keep alive dissociated ESCs, to re-aggregate them in the absence of growth factors, and then to allow cells to interact with each other to elicit the genetic programs of organogenesis. HES-1, chordin, and self-differentiation of organs from stem cells were all remarkable achievements that opened entirely new fields of research. One of the leading lights of Japanese stem cell science is now extinguished. He is survived by his wife, daughter, and son. His teachings will be continued by his many students and the magnificent RIKEN CDB institute that he tirelessly built. His passing represents a great loss to science. He was only 52.

Requiescat in pace, Sasai-san.

Edward M. De Robertis[1,*]
[1]Howard Hughes Medical Institute and University of California, 675 C. Young Drive South, Los Angeles, CA 90095-1662, USA
*Correspondence: ederobertis@mednet.ucla.edu
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