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General Summary

Our group is interested in the developmental and evolutionary aspects of the human body. By comparing organ development among vertebrates, we are attempting to reconstitute the evolutionary path that each of our organs has taken, at both the molecular and morphological levels, thus identifying fundamental molecular mechanisms that shape each organ.

Research Activities

Pathological and molecular biological investigation of congenital ataxia mouse bearing abnormal iron metabolism
Our congenital ataxia mouse had genetic alterations in the vicinity of rs13476689 in chromosome 2. Mutation analysis identified about 1,600 mutations, including single nucleotide polymorphisms, insertions, and deletions. In addition, a deletion of about 7,000 base pairs was found in the second intron of the Gm13912 gene. These genetic alterations made it possible to genotype individual animals to maintain the strain. However, the genetic alterations responsible for ataxia could not be determined. Vacuolar degenerations found in the spinal nerves and trigeminal nerves were the spheroid formation in the axons of NF200-positive neurons. The neurodegeneration had occurred before disease onset. Real-time polymerase chain reaction (PCR) analysis of gene expression suggested that iron deposition in the kidney was caused by decreased levels of divalent metal ion transporter.

The efficacy and the target specificity of genome editing with the CRISPR/Cas9 system
This year clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genome editing was developed for efficient genome editing in cells and organisms. We examined the efficacy and the target specificity of this method. We constructed CRISPR/Cas9 vectors targeting the human *HDAC8* and *NIPBL* genes, which are the responsible genes for Cornelia de Lange syndrome. We choose 2 highly specific target loci in each of *HDAC* and *NIPBL*. In addition, we predicted candidate off-target loci, including up to 3 mismatches against each target, using Bowtie, a short read mapping software program (available at http://bowtie-bio.sourceforge.net/index.shtml). We found a total of 64 potential off-target loci. We prepared multiplex primers for 4 target loci and 32 potential off-target loci for high-throughput deep sequencing.
We transfected the CRISPR/Cas9 vectors to HEK293 cells using FuGene HD transfection reagent (Promega Corp., Madison, WI, USA). Forty-eight hours after transfection, cells were harvested and the genomic DNAs were extracted. Then, multiplex PCR and

library construction were performed. High-throughput deep sequencing was performed with the Ion PGM system (Life Technologies, Carlsbad, CA, USA). We could obtain at least 10^4 reads with respect to each locus in each experiment. The efficacy of target-specific insertions/deletions ranged from 22.8% to 48.5%. On the other hand, off-target insertions/deletions were observed at a range of 0% to 3.1% (mean, 1.2%). Our results suggest that CRISPR/Cas9-mediated genome editing is highly active, even with imperfectly matched RNA-DNA interfaces in human cells. Therefore, when using CRISPR/Cas9 systems in research and therapeutic applications, careful evaluation of the off-target effects is required.

The vertebrate-specific structures—the neural crest and the placode—arise from the neural plate border: development of a new culture method for a possible precursor of exterior epithelium of the neural plate

Previous studies have shown that the neural crest is induced in the neural plate-embryonic ectoderm border by the action of bone morphogenetic protein (BMP) 4, which is derived from the embryonic ectoderm, and that the neural plate explant is likewise transformed to the neural crest cells by BMP4. We described a new culture method we developed and used it to find that the additive effects of BMP4 and fibroblast growth factor 2 on the neural plate explant results in morphological change to the simple squamous epithelium, which characteristically expresses Dlx5, which is a neural plate border specifier that positions the neural crest and future epidermis.

The induced epithelia were first tested by reverse transcription PCR (RT-PCR) for GATA3/keratin19 as epidermis specific markers, Sox1/Sox3/Neurogenin1/NCAM as neural plate markers, Slug/Snail1/Msx1/AP2/Zic1 as neural crest markers, and Dlx5/Six1/Six4/Eya2 as neural plate border markers using cells collected from neural plate explants and the corresponding control cells to evaluate the validity of RT-PCR testing. As a result, the expression levels of epidermis, the neural crest, and the neural plate border markers were all increased in the induced epithelia in telling contrast to those in the control cells.

We next examined the effect of Dlx5 downstream genes that are expressed in the neural plate and its border region on the induced epithelium by using real-time quantitative reverse transcription PCR (qPCR). The expression levels of epidermis specific markers GATA3/keratin19 and neural crest markers Slug/Msx1 in the induced epithelium were increased at the expense of the expression of neural plate marker Sox2. The pre-placodal ectoderm or ridge (PPE or PPR) arises in the anterior border of the neural plate in the form of an inverted-U shape and is regarded as a presumptive placode area at the late neurula-early pharyngula stage. The expression levels of the PPE-specific genes Six1/Eya2 known as the direct downstream genes of Dlx5, and of some placode-specific markers such as Pax3/Brn3a were also increased, albeit only slightly.

We lastly confirmed SEM images of the cultured cells, in which many epithelium-specific desmosomes were observed near boundaries of the adjoining big- and flat cells, which were cultured with BMP4 and FGF2 as the experimental group, although in which many filopodia and lamellipodia were surrounding cells, which were cultured with BMP4 as the positive control group to form motile neural crest cells.

This study thus suggests that the neural plate cells have a latent ability to be transformed into exterior epithelium of the neural plate, such as the neural crest, the PPE, and the embryonic ectoderm through the action of BMP4 and FGF2. The induced epithelium might be a precursor of all exterior epithelium of the neural plate. We are now investigating molecular cascades and cell differentiation in both the epithelium induced by the explant culture and the neural plate border in the embryo.

Analysis of cell distribution in diaphragm development
The diaphragm consists of mesodermal tissues from several sources. Because of its complexity, the development of the diaphragm remains unclear. For this reason, we observed the distribution of cells that are involved in diaphragm development. We used the Wilms' tumor 1 (*Wt1*) gene, because it is the responsible gene for congenital diaphragmatic hernia. We observed *Wt1*-positive cells in the left side of posterolateral region, which is the most common site of congenital diaphragmatic hernia. We plan to analyze the mechanism of the distribution of *Wt1*-positive cells in this region.

Formation of vertebrate appendages (limbs and fins) during development and regeneration
Limbs and fins, which are paired appendages of gnathostomes used for locomotion, are formed by shared developmental mechanisms, although the final morphologies of limbs and fins differ from each other. While limb bones are formed by endochondral ossification, fins comprise both endochondral bones and intramembranous bones. To understand the mechanisms of fin bone development and fin-to-limb evolution in gnathostomes, we are investigating pectoral fin formation in zebrafish (*Danio rerio*). We found a mutant fish whose pectoral fin lacked intramembranous bones.
In adults, limbs (of amphibians) and fins can regenerate completely by about 2 weeks after amputation. Previous work indicates that regenerative fin growth is greater after proximal amputation than after distal amputation. Therefore, we investigated levels of gene expression during such position-dependent regeneration by using quantitative polymerase chain reaction analyses. We found that the position-dependent regeneration already occurred in the inflammatory period before the blastema proliferation period. We would like to find a candidate for factor during position-dependent regeneration and to understand similarities and differences of morphogenesis during development and regeneration.

Publications

Komoike N, Kato T, Saijo H, Arihiro S, Hashimoto H, Okabe M, Ito M, Koido S, Homma S, Tajiri H. Photodynamic diagnosis of colitis-associated dysplasia in a mouse model after oral administration of 5-aminolevulinic acid. *In Vivo.* 2013; **27:** 747-53.
Dobashi A, Imazu H, Tatsumi N, Okabe M, Ang TL, Tajiri H. Quantitative analysis of VEGF-C mRNA of extrahepatic cholangiocarcinoma with real-time PCR using samples obtained during endoscopic retrograde cholangiopancreatography. *Scand J Gastroenterol.* 2013; **48:** 848-55.
Nikaido M, Noguchi H, Nishihara H, Toyoda A, Suzuki Y, Kajitani R, Suzuki H, Okuno M, Aibara M, Ngatunga BP, Mzighani SI, Kalombo HW, Masengi KW, Tuda J, Nogami S, Maeda R, Iwata M, Abe Y, Fujimura K, Okabe M, Amano T, Maeno A, Shiroishi T, Itoh T,

Sugano S, Kohara Y, Fujiyama A, Okada N. Coelacanth genomes reveal signatures for evolutionary transition from water to land. *Genome Res.* 2013; **23:** 1740-8.
Mimoto R, Taira N, Takahashi H, Yamaguchi T, Okabe M, Uchida K, Miki Y, Yoshida K. DYRK2 controls the epithelial-mesenchymal transition in breast cancer by degrading Snail. *Cancer Lett.* 2013; **339:** 214-25.

Reviews and Books

Hayashi S, Yano T, Kawasumi A, Tamura K, Yokoyama H. Dedifferentiation, redifferentiation and memory in limb regeneration (in Japanese). *Jikken Igaku.* 2013; **31:** 2075-82.