Direct Detection of Reward-Induced Dopamine Release in the Monkey Striatum: High-Speed Voltammetric Measurement with Carbon-Fibers

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Background: The temporal pattern of dopamine release is not necessarily the same as that of firing of midbrain dopamine neurons. Several mechanisms modifying the release at the synaptic terminals have been reported, such as nicotinic receptors (Rice and Cragg, 2004).

Methods: To elucidate the question, we tried a recording of dopamine release in the monkey caudate and putamen, during a typical Pavlovian reward task. We have previously reported the real-time detection of dopamine release using diamond microelectrodes (Yoshimi 2011), but the electrode sensitivity was easily lost in the brain. As we have succeeded in the simultaneous detection of electrically evoked dopamine release in caudate and putamen using fast-scan cyclic voltammetry on carbon-fibers, we next tried to detect reward-induced dopamine release in the striatum of an awake Japanese monkey.

Results: Significant event-related temporal changes were detected on 8 carbon fibers. Positive responses to unexpected free reward, trial initiation signal, differential responses to the positive and negative cues, and changes to a sudden reversal of positive and negative cues were observed. Interestingly, some examples showed marked enhancement of responses immediately after switching the behavioral task.

Conclusion: Real-time detection of dopamine release was successfully detected at multiple locations in the striatum of behaving monkey. This method could be useful to elucidate the modification of dopaminergic neurotransmission at the synaptic terminals, in both behaving and anesthetized primates.

Analysis of the Mechanisms of Recruitment by Inflammatory Cells in the Brain Ischemia-Reperfusion Injury

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Purpose: Ischemia-reperfusion (IR) injury is characterized by an initial restriction of blood supply to an organ followed by the subsequent restoration of perfusion and concomitant reoxygenation. An occlusion of the arterial blood supply is caused by an embolus and results in a severe imbalance of metabolic supply and demand, causing tissue hypoxia. A restoration of blood flow and reoxygenation is frequently associated with an exacerbation of tissue injury and a profound inflammatory response. But, the mechanisms of recruitment of the inflammatory cells into the brain after IR injury remain unknown. In our previous studies, we have demonstrated the physiological and pathological roles of leukotriene B4 receptor 1 (BLT1) in several inflammatory cells including neutrophils, eosinophils, dendritic cells, macrophages and mast cells. In this study, we analyzed the involvement of BLT1-expressing leukocytes in brain IR injury using the BLT1-deficient mice. Then, we identified BLT1-expressing cells by a home-made anti-mouse BLT1 monoclonal antibody, 7A8. Currently, we are analyzing the BLT1-expressing cells using 7A8 mAb in brain IR injury.

Methods: Briefly, mice (C57BL/6, male) were anesthetized with halothane in a mixture of 70% nitrous oxide and 30% oxygen. Head temperature was kept at 36°C with a warming lamp. After ligation of the right common carotid artery (CCA), the right middle cerebral artery (MCA) was occluded with a 7-0
nylon monofilament (11-mm-long). The distance from the suture tip to the right CCA bifurcation was ~9 mm. Sixty minutes after brain ischemia, the filament was withdrawn to allow reperfusion of the right MCA territory. On day 1 or 3 after reperfusion, 1-mm-thick serial coronal slices from the brain were stained with 2% tetrazolium chloride (TTC) in order to measure the infarct volume.

Results: We found that the size of ischemia regions (TTC-negative area) in BLT1−/− mice were slightly smaller than in BLT1+/+ mice on day 3 after reperfusion, but it was not significantly different. On day 1, there were no differences between BLT1+/+ and BLT1−/− mice.

Conclusion: We have not obtained the involvement of BLT1-expressing cells in the mouse model of brain IR injury on day 1 and 3. However, it is expected that inflammatory cells (i.e. neutrophils and monocytes/macrophages) express BLT1 and rapidly migrate to the lesion sites in brain. Thus, we will analyze the ischemia resins in BLT1+/+ and BLT1−/− mice for the early phase (a few hours) of IR injury.

Transient Increase in Proteinuria, Ubiquitinated Proteins and ER Stress Markers in Podocyte-Specific Autophagy-Deficient Mice After Unilateral Nephrectomy
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Objective: To determine the role of autophagy in podocytes during nephron reduction and adaptation to hypertrophy.
Method: We used the unilateral nephrectomy technique (UNX) and analyzed the physiological, anatomical and molecular changes on the remaining kidney. To measure the autophagy flux we used GFP–LC3 transgenic mice and analyzed autophagic activity for 3 days after UNX. We also analyzed lysosomal activity using immunofluorescence staining with the lysosomal marker LAMP-1. To confirm our observations we used the UNX model on newly generated podocyte-specific autophagy-deficient mice.
Results: Our results using GFP–LC3 transgenic mice showed a temporary downregulation of autophagic activity, suggesting that autophagy is dispensable in the recovery process from UNX. However, when using the UNX technique on podocyte-specific autophagy-deficient mice, we discovered an increase in proteinuria levels, and pathological ultrastructural changes in podocytes. We also observed a very dramatic increase in ubiquitinated proteins in podocytes, and an increase in ER stress markers after UNX on podocyte-specific autophagy-deficient mice. These observations show that the lack of autophagy delays the recovery process after UNX.
Conclusion: These results show the importance of autophagy in podocytes for maintaining a normal filtration function during the adaptation to compensatory kidney hypertrophy following UNX.

Heterologous Expression of Nestin Is Involved in Cell–Cell Contact Inhibition
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Background: Nestin is an intermediate filament protein and binds to vimentin and desmin. This molecule is also known as a marker protein of neuronal stem cell. In kidney, nestin is specifically expressed in podocytes. Like a vimentin intermediate filament, nestin is located in the cell body and primary processes, but not in foot processes, in which only actin filament cytoskeleton is involved (Kurihara et al. Am J Physiol 1998). However, the function of nestin in podocytes has not been elucidated.
Methods: We analyzed the function of nestin in podocyte by using unique rat podocyte cell line, which expresses most of cytoskeletal proteins seen in vivo podocytes.
Results: Cytochalasin D treatment induces the rearrangement of vimentin and nestin intermediate filaments and the cell process formation through the membrane retraction. Nestin silencing using siRNA, completely inhibited the cell process formation and the rearrangement of intermediate filament network, suggesting the involvement of nestin in these events. After nestin-siRNA treatment, fibroblast-like cells were found in the cell mass showing nestin-negative feature. Fibroblast-like cells still expressed nestin and higher amounts of actin. Live cell imaging analysis showed that fibroblast-like cells acquired higher motility. Immunostaining of ZO-1, a marker of tight junction demonstrated decreased cell–cell contact between the nestin-positive fibroblastic cell and the nestin-negative cell compared with each nestin-positive or nestin-negative cells, suggesting that heterologous expression of nestin between neighboring cells is involved in the downregulation of cell–cell contact.
Conclusion: Recently, epithelial–mesenchymal transition (EMT) of podocytes has been reported on the
pathogenesis of glomerulosclerosis. Our results suggest that the events shown in this study are closely associated with EMT of podocytes.

Deficiency of Transcription Factor Brn4 Disrupts Cochlear Gap Junction Plaques in a Model of DFN3 Non-Syndromic Deafness

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Objective: Brn4, which encodes a POU transcription factor, is the gene responsible for DFN3, an X chromosomelinked nonsyndromic hearing loss. Brn4 deficient (KO) mice show low endocochlear potential (EP), hearing loss and severe ultrastructural alterations in spiral ligament fibrocytes (Minowa O et al., Science, 1999). Mutations in the connexin26 (Cx26) and connexin 30 (Cx30) genes, which encode gap junction proteins and are expressed in cochlear fibrocytes and nonsensory epithelial cells (cochlear supporting cells) to maintain proper EP, are thought to be responsible for hereditary sensorineural deafness. The molecular pathologi of Brn4 deficiency causing low EP is still unclear.

Methods: It has been hypothesized that gap junction in the cochlea provide an intercellular passage by which K⁺ are transported to maintain high levels of the endocochlear potential essential for sensory hair cell excitation. In this study, we analyzed the formation of gap junction plaques in cochlear supporting cells of Brn4 KO mice in different stages by confocal microscopy and three dimensional graphic constructions.

Results: Gap junction composed of mainly of Cx26 and Cx30 in wild type mice formed large planar gap junction plaques (GJP). In contrast, Cx26R75W+ and Cx26f/fP0Cre showed fragmented small round GJPs around the cell border. In Cx26f/fP0Cre, some of the cells with Cx26 expression due to their cellular mosaicism showed normal large GJP with Cx26 and Cx30 only at the cell junction site between two Cx26 positive cells. These indicate that bilateral Cx26 expressions from both adjacent cells are essential for the formation of the cochlear linear GJP, and it is not compensated by other cochlear Connexins such as Connexin30.

Conclusions: In the present study, we demonstrated a new molecular pathology in most common hereditary deafness with different types of Connexin26 mutations, and this machinery can be a new target for drug design for hereditary deafness.

The Expression of TRPM4 in the Mouse Cochlea and its Putative Roles in the Inner Hair Cell Repolarization and the Endolymph Formation

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Role of Perlecan in Proliferation and Differentiation of Synovial Mesenchymal Cells

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**Purposes:** We changed the original plan that was related to the expression and functions of the inositol tris-phosphate receptor type 3 (IP3R3) in the mammalian inner ear. The present study was conducted to elucidate the spatio-temporal expression of the melastatin–related subfamily of transient receptor potential channels member 4, TRPM4, as a first step to elucidate its critical roles in the inner ear. We surmise that IP3R3 and TRPM4 may cooperate in the inner hair cells (IHCs), especially during the course of cell repolarization.

**Materials and Methods:** We performed immunohistochemistry using the customized anti-TRPM4 antibody (Teruyama et al., 2011) on frozen sections of the mouse inner ear of different developmental stages. We also evaluated the developmental change of TRPM4 transcripts by semi-quantitative RT–PCR in the lateral wall and the organ of Corti.

**Results:** TRPM4-IR was found in: (a) the cell body of IHCs, (b) the apical side of marginal cells of the stria vascularis, (c) the apical portion of the dark cells of the vestibule, and (d) a subset of the type II neurons in the spiral ganglion. Subsequently, the onset of TRPM4 expression in the stria vascularis coincides with the onset of endolymph formation. In contrast, the onset of TRPM4 expression in IHCs coincides with the onset of hearing.

**Conclusion:** Because TRPM4 is a Ca²⁺-activated monovalent selective cation channel, our results might be consistent with roles for TRPM4 in potassium ion transport: TRPM4 in the cell body of IHCs might contribute to the repolarization of IHCs just like KCNQ4 in OHCs. Meanwhile, in the marginal cells, TRPM4 potentially would play a role in the formation and maintenance of high K⁺ concentration that uniquely exists in endolymph in accordance with KCNQ1/KCNE1.
During mouse embryogenesis, hematopoietic stem cell (HSC) activity is first detected in the aorta–gonad–mesonephros (AGM) region. Hematopoietic clusters associated with the aorta play a pivotal role in the formation of the adult blood system. Both genetic and live-imaging data indicate that definitive hematopoietic progenitor/stem cells (visualized as hematopoietic clusters) are generated from specialized endothelial cells, termed hemogenic endothelium, which are localized in the middle segment of the aorta.

Recent transplantation and tracing studies showed that HSC activity is also detected in the mouse embryonic head and suggested that HSCs are generated from head vasculature (Cell Stem Cell 11, 663–675, 2012). However, hematopoietic clusters and hemogenic endothelium in the head region have not been well characterized. Therefore, it is still unclear whether head HSCs are generated in situ from vasculature or external HSCs colonize in the head.

In this study, by using whole-mount immunostaining and 3D confocal reconstruction techniques, we analyzed both c-Kit+ hematopoietic clusters and Runx1+ hemogenic endothelium in the whole head vasculature. The number of c-Kit+ hematopoietic cells was 20-fold less in the head arteries compared to the dorsal aorta. In addition, apparent nascent hematopoietic cells, characterized by "budding"–like structure, have not been observed in the head. These results suggest that HSC generation in the head is, if any, quite rare event.

**Advanced, Multimodal MRI Techniques in Parkinson Disease**

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The main histological changes in Parkinson disease (PD) are neuronal loss in the substantia nigra and the neuronal deposition of Lewy bodies (immunoreactive inclusions of alpha-synuclein). Visualizing pathological changes in PD by using conventional MRI is difficult, but advanced MRI techniques are now being used successfully.

Atrophy of the substantia nigra is not detected when voxel–based morphometry (VBM) and manual measurements are used to assess atrophy of the substantia nigra and basal ganglia. However, research has shown that high-resolution diffusion tensor imaging (DTI) can completely differentiate between early–stage PD patients and healthy individuals on the basis of reduced fractional anisotropy in the caudal part of the substantia nigra pars compacta. With neuromelanin imaging, high-intensity signals become obscured because of a reduction in neuromelanin in the substantia nigra pars compacta and locus ceruleus. With diffusional kurtosis imaging (DKI), which extends DTI by quantifying the non-Gaussianity of the water diffusion probability distribution function, highly sensitive and specific evaluation is possible because the mean kurtosis increases in the substantia nigra and basal ganglia.

The literature includes many studies on cerebral atrophy and cortical thinning, particularly in the prefrontal cortex and anteriomedial part of the temporal lobe where pathological changes occur relatively early. There are also reports on DTI showing white matter changes, including in the prefrontal cortex and supplementary motor area. In PD without dementia, significant cerebral atrophy or white matter changes may not be detected; however, in PD with dementia, atrophy and white matter changes become more widespread. Atrophy tends to occur in the medial temporal lobe and is similar in pattern to Alzheimer disease.

This study provides a general overview of MRI findings based on our own case studies and other reports, in relation to the progression of pathological changes and with a particular focus on the latest research that uses multimodal MRI in PD.

**Phenotypic Change from Rheumatoid Arthritis to Murine Lupus by Introducing Yaa or NZW Derived Gene in FcγRIIB-Deficient B6 Mice.**

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**Background:** FcγRIIB negatively regulates BCR-mediated activation signals. FcγRIIB-deficient C57BL/6 (B6)–congenic mouse strain (KO1) spontaneously developed pathology of rheumatoid arthritis (RA).

**Methods:** To analyze the phenotypic change of KO1 mice by crossing with lupus prone B6 Yaa mice and...
New Zealand White (NZW) mice, we established the KO1. Yaa, (KO1xNZW) F1 and (KO1xNZW) F2 hybrid mice. We evaluated the phenotype of these mice.

**Results:** The KO1. Yaa and F1 hybrid of the mice developed lupus nephritis including autoantibody production but they did not develop the phenotype of RA. F2 hybrid of the mice developed lupus (33.7%), sialoadenitis (27.6%) and RA (6.1%).

**Conclusion:** A phenotypic change was observed from rheumatoid arthritis to murine lupus by introducing Yaa or NZW derived gene in FcγRIIIB-deficient B6 mice.

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**Fish Oil Inhibit the Development of Abdominal Aortic Aneurysm in ApoE-Deficient Mice**

Takuma Yoshihara, Kazunori Shimada, Kosuke Fukao, Eiryu Sai, Hamad Alshahi, Tetsuro Miyazaki, Hiroyuki Daida

**Department of Cardiovascular Medicine**

**Background:** Dietary intake of fish oil, including ω3 polyunsaturated fatty acids (ω3-PUFAs), reduces progression of atherosclerosis and prevents future cardiovascular events. Macrophages have heterogeneous sub-populations such as classically pro-inflammatory macrophages (M1) and alternatively anti-inflammatory macrophages (M2). The effects of ω3-PUFAs on abdominal aortic aneurysm (AAA) formation and macrophage phenotype are unclear.

**Methods:** The AAA model was developed by continuous angiotensin II infusion for 28 days in apoE-deficient mice. Mice were supplemented with high purity eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). The development of AAA lesions and macrophage infiltration in the aorta were analyzed. Gene expressions of inflammatory markers in aortic tissues and peritoneal macrophages were measured by q-PCR.

**Results:** EPA administration and DHA administration significantly suppressed AAA formation and macrophage infiltration. Both ω3-PUFAs significantly suppressed the expressions of inflammatory cytokines, matrix metalloproteinase, and endothelial adhesion molecule in the aortas. The analysis of expressions of arginase 2, which is specific to M1, and Ym1, which is specific to M2, in the aortas was suggested skewing macrophage polarization toward M2 phenotype after EPA and DHA administration. Moreover, the analysis of peritoneal macrophages supported the shifting of M1/M2 balance.

**Conclusion:** Dietary intake of ω3-PUFAs prevented AAA development through the inhibition of aortic inflammation and skewing macrophage polarization toward M2 phenotype.

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**Circulating DPP-IV Levels Are Associated with Patients with Acute Decompensated Heart Failure**

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**Department of Cardiovascular Medicine**

**Objective:** DPP-IV, a modulator of blood glucose, is also known as an exopeptidase, which cleaves BNP, and DPP-IV correlates with heart failure. However, clinical significance of DPP-IV in the pathogenesis of acute decompensated heart failure (ADHF) remains obscure.

**Method:** We enrolled consecutive 200 patients admitted to coronary care unit in Juntendo University Hospital. Serum DPP-IV levels on admission were measured by ELISA.

**Results:** In 200 patients, 83 patients with ADHF (NYHA classification III or IV) showed significantly lower DPP-IV levels than patients without ADHF (340 ± 107 vs. 379 ± 87 ng/ml, p = 0.007). There were no significant associations of DPP-IV levels with age, BMI, lipid profiles, glucose levels on admission, and HbA1c levels, while serum DPP-IV levels were negatively associated with IL-6 ($r = -0.16$, $p = 0.02$) and CRP levels ($r = -0.26$, $p = 0.0002$). In addition, serum DPP-IV levels were negatively associated with BNP levels ($r = -0.16$, $p = 0.02$), but not with ejection fraction. DPP-IV levels positively correlated with E/A ($r = 0.28$, $p = 0.02$), which indicates diastolic dysfunction. Multivariate analysis demonstrated DPP-IV levels were an independent predictor for the presence of ADHF.

**Conclusion:** Decreased DPP-IV levels in patients with ADHF correlated with inflammation, increased BNP levels, and diastolic function, suggesting an important role of DPP-IV in the pathogenesis of ADHF.

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**Role of Oct4 in the Generation of Gefitinib-Resistant Lung Cancer Stem Cells**

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Background: Accumulating evidences indicate that a small population of cancer stem cells (CSCs) is involved in intrinsic resistance to cancer treatment. Oct4 is known to play an important role in reprogramming mouse or human somatic cells to undifferentiated, pluripotent stem cells.

Aim of Study: Our aim here was to elucidate the role of Oct4 in the resistance to gefitinib in non–small cell lung cancer (NSCLC) with activating epidermal growth factor receptor (EGFR) mutation.

Methods: NSCLC cell line, PC9, which expresses the EGFR exon 19 deletion mutation, was exposed to high concentration of gefitinib. Seven days after gefitinib exposure, a small fraction of viable cells were detected, and these cells were referred to as “gefitinib-resistant persisters” (GRPs).

Results: Oct4 and putative lung CSC marker CD133 were highly expressed in GRPs in PC9 cells, and PC9 GRPs exhibited sphere–forming ability in vitro and high tumorigenic potential in NOG mice, suggesting that GRPs have characteristic features of the CSCs phenotype. To investigate the role of Oct4 in the persistence of gefitinib–resistant lung CSCs, we introduced Oct4 gene into PC9 cells by lentiviral infection. Transfection of Oct4 gene significantly increased the number of sphere formation, reflecting the self–renewal activity, of PC9 cells under the high concentration of gefitinib. Furthermore, Oct4–overexpressing PC9 cells significantly formed tumors at 1 x 10 cells/injection in NOG mice as compared to the control cells.

Conclusion: These findings suggest that Oct4 plays a key role in the generation of lung CSCs resistant to gefitinib in EGFR mutation–positive NSCLC. Targeting Oct4 gene may be a promising strategy for overcoming gefitinib resistance in EGFR mutation–positive NSCLC induced by lung CSCs.

Matrix Coil Embolization Induce Favorable Tissue Proliferation and Maturation in Swine Experimental Aneurysm

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Background and Purpose: Recanalization of cerebral aneurysms postembolization remains a serious problem that influences treatment outcomes. Matrix2™ is a bioactive, bioabsorbable detachable coil that was developed for the purpose of reducing recanalization. The purpose of this study was to analyze the short–term efficacy of the Matrix coil system and evaluate the temporal profile of the tissue in a swine experimental aneurysm model.

Materials and Methods: Thirty–six experimental aneurysms were created in 18 swine. All of the aneurysms were tightly packed with Matrix or Guglielmi detachable coils (GDC). Comparative histologic and morphologic analyses were analyzed at 1, 2, and 4 weeks postembolization.

Results: Both of the GDC and Matrix coil group, histological analysis showed endothelial–like cells lining the aneurysm opening partially 1 week postembolization. At 2 and 4 weeks postembolization, the aneurysms treated with Matrix coils exhibited a more extensive area of organized thrombus when compared with the aneurysms treated with GDC. Neck tissue thickness was higher in the Matrix coil group at 2 and 4 weeks. In immunohistochemical analysis, these cells were suggested more functional proliferating endothelial cells. Moreover morphological analysis suggested that these cells were more matured endothelial cells in Matrix coil group compared with the GDC coil group.

Conclusion: Our results indicated that the aneurysm embolized with Matrix coils builds stable scaffold for tissue engineering, and occurs easily and with early tissue proliferation, therefore induced early maturation compared with GDC coils. These findings suggest that the Matrix coils might prevent aneurysmal recanalization after endovascular treatment of cerebral aneurysms.

Adrenomedullin Deficiency Exacerbates Ischemic White Matter Injury After Prolonged Hypoperfusion in Young/Aged Mice

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Background and Purpose: Adrenomedullin (AM), a potent vasodilator peptide, was originally isolated from pheochromocytoma cells and reduces insulin resistance by decreasing oxidative stress. White matter lesions induced by aging and hyperglycemia play a crucial role in cognitive impairment in post-stroke patients. Here, we examine whether adrenomedullin deficiency and aging exacerbate ischemic white matter injury after prolonged hypoperfusion.

Materials and Methods: AM heterozygous (AM$^{+/-}$) and wild-type mice were subjected to prolonged hypoperfusion. Immunohistochemical analysis was used to evaluate WM injury at several time points after prolonged hypoperfusion. We also analyzed aged mice to investigate WM injury in aging.

Results: After prolonged hypoperfusion, WM damage progressed in a time-dependent manner in the AM knock out (AM$^{+/-}$) group compared with the wild-type group. The number of oligodendrocyte progenitor cells gradually increased after prolonged hypoperfusion, whereas oligodendrocytes decreased following a transient increase, but the ratio of increase was mild in the AM$^{+/-}$ group compared with the wild-type group ($p < 0.05$). Oxidative stress was detected in oligodendrocytes after prolonged hypoperfusion, with a larger increase in the AM$^{+/-}$ group ($p < 0.05$). Aged mice showed the same tendency, but WM damage was worse compared with young mice, and was increased in the aged AM$^{+/-}$ group.

Conclusion: Our results demonstrated that WM injury was increased in AM deficiency, which induced oxidative stress in young groups. WM injury was exacerbated because of hyperglycemia in aged groups. AM may be an important target in the control of ischemic WM injury.

Inflammatory Mediators’ Role in Epileptogenesis Caused by Cavernous Angioma

Objective: We performed tissue immunostaining for iron, oxidative stress and inflammatory mediators using tissues around the cavernous angioma removed during epileptogenic focal excision to further elucidate the mechanism of epilepsy pathogenesis in the tissues around the cavernous angioma.

Methods: We examined the cavernous angioma and the surrounding brain tissues of six patients who underwent epilepsy surgery at the Neurosurgery Department of Juntendo University.

After identifying the epileptogenic focus based on the preoperative EEG findings, the focus of the angioma was resected including the brain cortex in which the focal epileptic discharge was observed. The specimen was fixed in formalin, and iron staining were performed using MAP-2, GFAP and CD68 as markers for neurons, astrocytes and microglia, respectively. Immunostaining was performed using TLR4, HMGB1 and IL-1$\beta$ as the inflammatory mediators, and 4-HNE as an oxidation stress marker.

Results: In all patients who underwent focal resection, no postoperative symptomatic epilepsy was noted. When iron staining was performed for the obtained tissues around the cavernous angioma, three patients showed less iron deposition in the tissue around the angioma, while the other three patients showed iron diffusing throughout the tissue. The immunoreaction for oxidant stress was confirmed in many sites surrounded by iron, as well as in sites with no iron. In the samples in which many iron depositions were confirmed in the tissues, the amount of reactive astrocytes (gemistocytic astrocytes) and microglia, which phagocytize iron, were increased, as indicated by the expression of HMGB1 and TLR4. Furthermore, there were neurons showing HMGB1 and TLR4 expression in the cells of the brain cortex located far from the angioma. Throughout the brain cortex, IL-1$\beta$ expression was confirmed. However, even in the tissues with unclear iron diffusion, HMGB1 and TLR4-positive astrocytes, microglia, and neurons were confirmed.

Conclusion: In the sites around the cavernous angioma, in which spikes were identified based on ECoG, oxidant stress and inflammatory mediators were confirmed throughout the brain tissue samples, suggesting a relationship with epilepsy pathogenesis. In addition, the appropriate range of focal resection by sufficient electrophysiological evaluation should be determined for surgical treatment of the cavernous angioma in patients with epilepsy.

Electrophysiological Analysis of Transgenic Mice Overexpressing LRG1 in Glia Cells

Objective: We performed tissue immunostaining for iron, oxidative stress and inflammatory mediators using tissues around the cavernous angioma removed during epileptogenic focal excision to further elucidate the mechanism of epilepsy pathogenesis in the tissues around the cavernous angioma.
Diseases. For various diseases, been implicated in TSC and other neurological autism. Aberrant activation of mTORC1 pathway has

TSC1 mutation and is characterized by tumorous TSC2 autosomal dominant disorder caused by

Purpose

Cells

Diseases Using Tsc2-Deficient Rat Embryonic Stem

sis Complex and Other mTOR Signal-Related Neural

Elucidation of the Pathogenesis of Tuberous Sclerosis Complex and Other mTOR Signal-Related Neural Diseases Using Tsc2-Deficient Rat Embryonic Stem Cells

Yoshitaka Ito1, Haruna Kawano2, Fumio Kanai3, Eri Nakamura3, Norihiro Tada1, Hajime Arai1, Toshiyuki Kobayashi2, Okio Hino5

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Expression of leucine-rich alpha-2 glycoprotein (LRG1) increases with aging in the brain, and its association with cognitive disorders is assumed. Transgenic mice (Tg) overexpressing this glycoprotein in the brain were created, and immunohistological staining of the brain, observation of changes in cognitive behavior, and a patch clamp experiment using hippocampal slices were performed at 8 and 24 weeks old experimental animals. The brain morphology was markedly different, and LRG1 overexpression in the hippocampus was confirmed. Expression of cAMP response element-binding protein was inhibited in the brain of 24-week-old Tg mice, but no significant difference was noted in the cognitive behavior or electrophysiology. However, regarding the learning time and frequency of stimulation-induced neuronal firing, the response to stimulation was smaller and the field excitatory postsynaptic potential (fEPSP) was slightly lower in LRG-Tg mice than in the wild type (Wt) group.

Elucidation of the Pathogenesis of Tuberous Sclerosis Complex and Other mTOR Signal-Related Neural Diseases Using Tsc2-Deficient Rat Embryonic Stem Cells

Methods: We stained tissue sections of teratomas for various neural markers by immunohistochemistry. To induce neural differentiation of Eker ESCs, we employed a three-dimensional aggregation culture (SFEBq culture) established for mouse and human ESCs.

Results: In the teratomas from Eker ESCs, including homozygous Tsc2-/- cells, neural epithelia-like tissues (NETs) were observed. They were stained with neural differentiation markers. Cell division in the lumen side was observed. However, Tsc2-/- NETs were negative for gamma-tubulin, an apical marker of neural tube. In neural induction method, we observed positive staining of neural markers, and neural rosette-like structures in the part of the colonies, but the efficiency was significantly lower compared with mouse ESCs.

Conclusion: Tsc2-/- ESCs could be committed into neural cells, but unknown defects associated with gamma-tubulin function or polarity during their differentiation was expected. For rat ESCs, conditions of SFEBq culture should be optimized further.

Elucidation of the Pathogenesis of Tuberous Sclerosis Complex and Other mTOR Signal-Related Neural Diseases Using Tsc2-Deficient Rat Embryonic Stem Cells

Aberrant Differentiation of Tsc2-Deficient Teratomas Associated with Activation of the mTORC1-TEF3 Pathway

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1 Department of Urology, 2 Department of Molecular Pathogenesis, 3 Department of Neurosurgery, 4 Laboratory of Genome Research, Research Institute for Diseases of Old Age

Purpose: Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by TSC1 or TSC2 mutation and is characterized by tumorous lesions and neural symptoms, such as seizure and autism. Aberrant activation of mTORC1 pathway has been implicated in TSC and other neurological diseases. For various diseases, in vivo differentiation experiments of mutant pluripotent stem cells have been utilized to elucidate mechanisms of pathogenesis and discover new therapeutic targets. We have established embryonic stem cells (ESCs) from blastocysts of Eker (Tsc2 mutant) rat, an animal model of TSC. Here, we analyzed the neural tissues in the teratomas from the ESCs transplanted into immunodeficient mice. To generate the experimental system to investigate neural differentiation abnormalities associated with Tsc2 deficiency, we tried to induce neural differentiation of Eker ESCs.

Methods: We stained tissue sections of teratomas for various neural markers by immunohistochemistry. To induce neural differentiation of Eker ESCs, we employed a three-dimensional aggregation culture (SFEBq culture) established for mouse and human ESCs.

Results: In the teratomas from Eker ESCs, including homozygous Tsc2-/-deficient (Tsc2-/-) cells, neural epithelia-like tissues (NETs) were observed. They were stained with neural differentiation markers. Cell division in the lumen side was observed. However, Tsc2-/- NETs were negative for gamma-tubulin, an apical marker of neural tube. In neural induction method, we observed positive staining of neural markers, and neural rosette-like structures in the part of the colonies, but the efficiency was significantly lower compared with mouse ESCs.

Conclusion: Tsc2-/- ESCs could be committed into neural cells, but unknown defects associated with gamma-tubulin function or polarity during their differentiation was expected. For rat ESCs, conditions of SFEBq culture should be optimized further.

Aberrant Differentiation of Tsc2-Deficient Teratomas Associated with Activation of the mTORC1-TEF3 Pathway

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The activation of mTORC1 is related to development of cancer, diabetes, and some neuropathies. The Eker rat is a germline mutant model of the tuberous sclerosis complex 2 gene (Tsc2), which is a negative regulator of mTORC1. Heterozygous mutants develop renal cell carcinomas (RCCs) by second hit in the wild-type Tsc2 allele, whereas homozygous mutants are embryonic lethal. To establish a novel cell differentiation model for exploring the mechanism of Tsc2 mutation-associated pathogenesis, we have generated Tsc2-deficient ES cells (ESCs) from the
Eker rat. Not only Tsc2\(^{+/+}\) and Tsc2\(^{-/-}\) but also Tsc2\(^{++/-}\) ESCs could differentiate to all three germ layers. However, interestingly, epithelial tumor–like abnormal ductal structures were reproducibly observed in Tsc2\(^{++/-}\) teratomas from different ESC lines. In this study, we immunohistochemically characterized those abnormal ductal structures. The activation of mTORC1 signaling was observed in Tsc2\(^{-/-}\) teratomas, especially in the abnormal ductal structures as judged by staining for p-S6 and p-4EBP1. We observed the expression of epithelial markers, megalin and cubulin, as well as the cytoplasmic localization of E-cadherin and \(\beta\)-catenin in abnormal ductal cells, which were similar to those in Eker rat RCC. Moreover, we found the nuclear accumulation of TFE3, a transcription factor regulated by mTORC1, in abnormal ductal structures as well as Eker rat RCC. As TFE3 was recently reported to be a negative regulator of ESC differentiation, it may cause tissue–specific differentiation defects, related to the tumorigenesis of Eker rat, in Tsc2\(^{-/-}\) teratomas. ESCs derived from the Eker rat will be novel experimental tool to analyze the differentiation defects and cell–type specific pathogenesis associated with the Tsc2\(^{-/-}\)-deficiency.

The Role of the Lipid Raft on the Erc–Regulated Signal Pathways in Mouse Renal Tumor Cells

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**Purpose:** Mouse Erc (Expressed in renal carcinoma) is a homologue of human mesothelin gene. Erc/ mesothelin (described hereafter as Erc), a GPI–anchored protein, is expressed in normal mesothelium and induced in several species of human malignant tumors, as well as in renal tumors of Tsc2 KO mice. We reported that the size and number of renal tumors of Tsc2 KO mice were reduced by Erc deficiency and Erc is involved in integrin–related pathway. To investigate further the role and position of Erc in signal pathway, we continue the studies.

**Methods:** (1) Erc–deficient Tsc2 KO mouse renal tumor cell line T1–9 was transfected with Erc expression vector or empty vector. (2) Cells were lysed in TNE buffer and fractionated on discontinuous sucrose gradients by centrifugation to isolate lipid raft.

**Results:** The phosphorylation of epidermal growth factor receptor (EGFR; Tyr 1068) was reduced but total EGFR was remarkably increased in Erc–deficient cells compared with Erc–restored cells. Multiple bands were observed in Western blots of EGFR on lipid raft, the lowest band was clearly observed in Erc–restored cells. The expression level of Egfr mRNA in Erc–deficient cells was higher than in Erc–restored cells as revealed by RT–PCR, real time PCR and GeneChip exon array. It is plausible that the expression of EGFR may be regulated by feedback from Erc–regulated signal pathways other than mTOR axis involved in the Tsc2 mutation–induced tumorigenesis.

**Conclusion:** These results suggest a novel function for Erc in the regulation of membrane protein dynamics and EGFR signaling on lipid raft. Further analysis of the Erc pathway may help to develop potential targets for novel anti-cancer therapies.

Analysis of Endosomal Proteins, Which Specifically Interact with APP–\(\beta\)CTF

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**Introduction:** Amyloid–\(\beta\) peptide (A\(\beta\)) is the major component of senile plaques deposited in the brains of patients with Alzheimer’s disease (AD). Although several lines of evidence suggest that the accumulation of A\(\beta\) is linked to the pathogenesis of AD, clinical trials of agents designed to reduce A\(\beta\) continue to fail. It may suggest that A\(\beta\) is not the only pathogenic molecule. A\(\beta\) is produced from sequential cleavage of APP by \(\beta\)-secretase and \(\gamma\)-secretase. Intriguingly, \(\beta\)-cleaved C–terminal fragment (\(\beta\)CTF), the product of \(\beta\)-secretase processing of APP and a direct precursor of A\(\beta\), is also accumulated in AD brains. It is suggested that the accumulation of \(\beta\)CTF in endosomes have a correlation with the increased number and morphological enlargement of endosomes in AD brains, which are observed before A\(\beta\) deposition.

**Methods:** To gain insight into the molecular mechanisms, we screened endosomal proteins that interact with \(\beta\)CTF.

**Results:** We found two candidates that are known to regulate lipid metabolisms and neural functions. One of the candidates interacts with \(\beta\)CTF in endosomes, and this interaction results in the endosomal enlargements.

**Conclusion:** Our results propose a novel mechanism of \(\beta\)CTF accumulation in endosomes to disturb
vesicular traffic systems, and suggest a novel therapeutic aspect of AD.

**Parkinson’s Disease–Related Protein for the Analysis of PINK1 Substrate**

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**Purpose:** Senescent and damaged mitochondria undergo selective mitophagic elimination through mechanisms requiring two Parkinson’s disease factors, the mitochondrial kinase PINK1, and the cytosolic ubiquitin ligase Parkin. In the last year, we have determined that Parkin and protein TA, are phosphorylated by PINK1. We tried to build the assay system using measurable multiplex bead ELISA at the same time the parkin phosphorylation and TA.

**Methods:** The antibody against Parkin phospho (Ser65) was generated by injection of the KLH-conjugated phospho-peptide CDLDQQpSIVHIVQR (where pS is phospho-serine) into rabbit and was affinity-purified by positive and negative selection against the phospho- and de-phospho-peptides, respectively. The antibody against Protein TA phospho (Ser 696) was generated by injection of the KLH-conjugated phospho-peptide CSPVVSGDTpSPR into rabbit and was affinity-purified by positive and negative selection against the phospho- and de-phospho-peptides, respectively. We confirmed the antibody specificity by Western blotting. We prepared anti-phosphorylated parkin antibody and anti-phosphorylated protein TA antibody each bound multiplex beads.

**Results:** Anti-phosphorylated protein TA antibody showed a strong reaction to the WT mice brain mitochondrial fraction. Furthermore it is necessary to investigate. Anti-phosphorylated parkin antibody was able to react in the phosphorylation parkin specifically by CCCP treatment in Hela cells. Also, Anti-phosphorylated parkin antibody did not react replace Ala with Ser of parkin.

**Conclusion:** Preparation of multiplex beads for anti-phosphorylated parkin antibody, resulted in good fluorescence intensity.

**The Relation Between Toll Like Receptor and Autophagy Induction in Neurons**

Takehiko Sunabori, Masato Koike

**Fate of Olfactory Placode-Derived Early Migratory Cells**

Momoko Miyakawa, Shizuko Murakami, Yasuo Uchiyama
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Inactivation of autophagy by Atg7 deletion and Toll like receptor (TLR) –2 null mice both shows tolerance against neonatal Hypoxic–Ischemic (H–I) brain injury. However, the relation between TLR2 and autophagy induction still remains unclear.

**Methods:** Primary hippocampal neurons were stimulated with an autophagy inducible factor (Rapamycin; 1mM) and TLR2 agonist (Pam3CSK4: 0.1mg/ml) for 3 hours. The induction of autophagy in vitro was evaluated by Western blot analysis with anti-LC3 antibodies and the puncta–formation of transfected GFP–LC3. The induction of autophagy in vivo after H–I brain injury was evaluated by Western blot analysis with anti–LC3 antibodies and phosphorylated–p70–S6K.

**Results:** The conversion of LC3–I to LC3–II, an index of autophagy induction, was observed by stimulating TLR2 as Rapamycin in primary hippocampal neurons and increase of GFP–LC3 puncta was observed in Rapamycin and Pam3CSK4 stimulated primary hippocampal neurons. The conversion of LC3–I to LC3–II was attenuated 6 hours after H–I brain injury in TLR2 null mice compared to the control mice. Moreover, the reduction of phosphorylated–p70–S6K after H–I brain injury in the ipsilateral–hippocampus against the contralateral–hippocampus in the control mice was cancelled by TLR2 deletion.

**Conclusion:** These results suggest that the induction of autophagy is closely related to the stimulation of TLR2.

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**Introduction:** Olfactory placode is known to produce various kinds of cells other than olfactory receptor neurons. In chick embryos when the first olfactory nerve axons grow and GnRH neurons start to migrate on embryonic day (E) 3 (Hamberger and Hamilton stage 21), there are cellular cord between the olfactory epithelium (OE) and the rostral forebrain (anlage of the olfactory bulb). The cells which
form the cellular cord are immunoreactive for polysialylated NCAM (PSA–NCAM) and HuC/D (neuronal markers). In the present study, development of the cellular cord and origin of the early migratory cells were examined in whole-mount specimens by immunohistochemistry and labeling with GFP vector.

**Material and Methods:** GFP vector was introduced to epithelial cells of the olfactory placode by an electroporator. About 40 hours later, electroporated embryos were fixed and GFP-labeled cells were detected after immunohistochemical staining for PSA–NCAM and HuC/D by a confocal laser scanning microscope (Carl Zeiss LSM 510 META).

**Results:** GFP-labeled cells were found in the OE and PSA–NCAM-immunoreactive cellular cord between the olfactory epithelium and the rostral forebrain, and also in the dorsally or caudally-oriented branches of the cellular cord during earlier stages than formation of the single rostrally oriented cellular cord. In addition, a few cells expressing GFP, PSA–NCAM and HuC/D were detected in the neuronal network which seemed to locate in the marginal zone of the telencephalon.

**Conclusion:** These results indicate the possible involvement of the olfactory placode-derived neurons to development of the telencephalon.

**Involvement of RCAN–Calcineurin–Signaling in the Brain of LAMP–2–Deficient Mice**

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**Purpose:** Lysosome-associated membrane protein–2 (LAMP–2) is a highly glycosylated protein localized to the lysosomal membranes. Recently, it has been reported that each subtype of LAMP–2 has a specific function such as chaperone-mediated autophagy as well as nucleic acid degradation; however, the role under the neuropathological conditions has not been elucidated. To study the role of LAMP–2 in the CNS, we examined expression of affected proteins in the LAMP–2–deficient mice.

**Methods:** Two-dimensional gel electrophoresis and mass spectrometry were performed in the brain of LAMP–2–deficient mice. We also examined the brain lesions from the neonatal hypoxia ischemia and middle cerebral artery (MCA) occlusion in the LAMP–2–deficient mice.

**Results:** Two-dimensional gel electrophoresis revealed altered expression of proteins in relation to the ubiquitin–proteasome system, heat shock proteins, synaptic proteins, cytoskeletons, membrane trafficking, and calcium modulators. Among these proteins, we have focused on calcineurin because it is involved in important pathways of the CNS. We found an increased protein expression of regulator of calcineurin 1 (RCAN1), which negatively regulates calcineurin expression. Histopathological examination revealed extensive tissue damage with glial reaction in both models of LAMP–2–deficient mice.

**Conclusion:** RCAN1–calcineurin–signaling was affected in the CNS of LAMP–2–deficient mice. Suppression of the calcineurin–induced NFAT pathway by RCAN1 accumulation may enhance the brain damage such as neonatal hypoxia ischemia and MCA occlusion.

**Enrichment of GABARAP Relative to LC3 in the Axonal Initial Segments of Neurons**

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**Background:** GABAA receptor–associated protein (GABARAP) was initially identified as a protein that interacts with GABAA receptor. Although LC3 (microtubule–associated protein 1 light chain 3), a GABARAP homolog, has been localized in the dendrites and cell bodies of neurons under normal conditions, the subcellular distribution of GABARAP in neurons remains unclear.

**Aims:** Using GFP–GABARAP transgenic mice, we investigated the intracellular distribution of GFP–GABARAP in hippocampal pyramidal neurons and cerebellar Purkinje cells by comparing its distribution with that of endogenous LC3.

**Methods:** Using brain tissues and cultured cortical neurons from GFP–GABARAP transgenic mice, we performed confocal immunofluorescence microscopy was performed for multiple immunostaining.

**Results:** Immunohistochemistry in GFP–GABARAP transgenic mice showed that positive signals for GFP–GABARAP were widely distributed in neurons in various brain regions, including the hippocampus and cerebellum. Interestingly, intense diffuse and/or
Fibrillary expression of GFP-GABARAP was detected along the axonal initial segments (AIS) of hippocampal pyramidal neurons and cerebellar Purkinje cells, in addition to the cell bodies and dendrites of these neurons. In contrast, only slight amounts of LC3 were detected along the AIS of these neurons, while diffuse and/or fibrillary staining for LC3 was mainly detected in their cell bodies and dendrites.

**Conclusions:** Compared with LC3, GABARAP is enriched in the AIS, in addition to the cell bodies and dendrites, of these hippocampal pyramidal neurons and cerebellar Purkinje cells.

**Selective Autophagy of Lysosomes with Ceroid-Lipofuscin in Neurons Deficient in Cathepsin D**

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Department of Cell Biology and Neuroscience

**Introduction:** p62 and NBR1, adaptor proteins for selective autophagy, have binding regions with ubiquitin and LC3 on the isolation membrane of autophagosomes (AP). We have previously shown that mice deficient in lysosomal cathepsin D (CD) exhibit a new form of lysosomal accumulation disease with a phenotype resembling neuronal ceroid lipofuscinosis (NCL). Electron microscopic observations revealed accumulation of granular osmiophilic deposits (GRODs) and AP, morphological hallmarks of NCL, in the perikarya of neurons deficient in CD. Since GRODs were frequently found within AP that were localized in the perikarya of CD-deficient neurons, we speculated that enwrapment of GRODs into AP is due to selective autophagy. In CD-deficient neurons, GRODs, p62 and NBR1 were found only in somatodendritic portions but not in axons and their terminals.

**Method:** Primary cerebral cortical neurons were obtained from mouse brains at embryonic day 16–17, while they were transfected with gene constructs of autophagy/lysosome-related proteins on 5 days in vitro (DIV) using Lipofectamine 2000. They were further incubated for 2 days after transfection. Fluorescence images of neurons were obtained using a confocal laser scanning microscope.

0.5% triton-100 was used for brain tissue fractionation of the WT, cathepsinD (CD) deficient, and CD/Atg7 double deficient mouse. Marker molecules of selective autophagy were detected by Western blotting.

**Result:** In primary cultured neurons obtained from mouse embryonic cerebral cortex at E16, Lysotracker red-positive acidic compartments were largely localized in cell bodies and dendrites at 10 days after the start of cultures (DIV). Although such positive vesicles were abundantly present in axons and filopodia at 3 DIV. Moreover, not only endogenous p62 and NBR1 but also GFP-tagged p62 and NBR1 were detected only in cell bodies and dendrites but not in the distal part of axons beyond the ankyrin G-positive initial segment. These results indicate that AP is non-selectively formed in the axons and axon terminals, while it is retrogradely sent back to the cell bodies of neurons where it receives lysosomal enzymes and become autolysosomes.

**Facilitatory Effects of Somatostatin on GnRH Neuron Migration and Olfactory Axon Fasciculation**

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**Purpose:** Transient expression of somatostatin (SS) has been found in the olfactory-forebrain region of chick embryos. The times of appearance and distribution of SS immunoreactive (-ir) neural elements are similar to those of GnRH neurons which originate in the olfactory placode and migrate into the forebrain. Based on these results, we hypothesize that SS play a role in development of GnRH neurons and olfactory axons. To examine the effect of SS on the migration of GnRH neurons, olfactory placode explant cultures were as formed.

**Materials and Methods:** Olfactory placode explants from embryonic day 3.5 chick embryos were treated with SS (1 nM), octreotide, an SS analog (100 nM), and Neurobasal medium as a control once at 2–3 hours after the start of cultures, and twice per day on 1–3 days in vitro (div). Explants were fixed on 4 div and processed for triple immunofluorescence labeling for GnRH, the highly polysialylated form of NCAM (PSA) and SS.

**Results:** The migration distance of GnRH neurons into the gel from the peripheral site of the explant was significantly increased in explants treated with SS and octreotide than in those without stimulants. Moreover, the diameter of PSA-ir fibers was measured at 100 μm away from the peripheral site of the explants and found that the rate of PSA-ir fibers above 2 μm in diameter was significantly increased in...
the treated groups than in the control ones. **Conclusion:** These results indicate that SS exerts facilitatory influences on GnRH neuron migration and olfactory axon development.

### Hippocampal Pyramidal Neurons Lacking LC3A and LC3B Are Resistant to Hypoxic–Ischemic Brain Injury in Neonatal Mice

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Although LC3, a mouse homologue of yeast Atg8, has mainly been used as a marker protein of autophagy, little is known about the role of subtypes of LC3 or whether these proteins compensate their roles for each other during autophagic processes. For this, we produced LC3A/B-double deficient mice, which were born normally and were able to reproduce. Using these KO mice, both mRNA and protein levels of LC3 homologous proteins, a GABARAP family of proteins, were examined in brain and other tissues and found that they did not significantly alter between wild-type and LC3A/B-double deficient mice. These results indicate that basic autophagy may work normally in neurons of LC3A/B-deficient brains. Since we have previously shown that Atg7-deficiency rescues mouse neonatal hippocampal pyramidal neurons from hypoxic–ischemic injury (H/I), LC3A/B-double deficient mice were applied to this H/I model. As previously reported, hippocampal pyramidal neurons in wild-type mice were vulnerable to H/I injury, while the protein amount of LC3-II was markedly elevated from 9 to 24 hours after H/I injury. In contrast, hippocampal pyramidal neurons deficient in LC3A/B were resistant to the H/I injury. These results suggest that LC3A/B, but not GABARAP family of proteins, are involved in H/I injury-mediated pyramidal neuron death that occurs in the hippocampal pyramidal layers of neonatal mouse brains. As shown in our previous study, excess autophagic stress may induce neuron death. Our present data using LC3A/B-deficient mice strongly support the presence of H/I injury-mediated autophagic neuron death in neonatal brains. Further study is required to elucidate the molecular mecha-nism of why H/I injury-mediated neuron death is suppressed by deficiency in Atg7 or LC3A/B.

### Bortezomib Induces Schwann Cell Injury Due to Activation of the Autophagy/Lysosomal System

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Bortezomib (BTZ) is a proteasome inhibitor and it is used as an anticancer agent for multiple myeloma. The most significant side-effect of BTZ is peripheral sensory neuropathy. The main theme of our study is to reveal the mechanism of BTZ-induced neuropathy. **Methods:** Rats were administered with BTZ intraperitoneally (0.15–0.30 mg/kg/three times/week for 4 weeks). The sciatic nerve, dorsal root ganglion (DRG), and spinal cord were processed for morphological as well as immunohistochemical analyses. **Results:** In immunohistochemistry showed that, staining for ubiquitin was more intense especially in Schwann cells that attached to axons of the sciatic nerve, and in the DRG neurons than that in saline-treated control cells. Moreover, relating to the sciatic nerve immunoreactivity, LC3 and p62 were also highly detected in the cytoplasm of Schwann cells. Electron microscopic observations showed an increase in cisternal dilatation of endoplasmic reticulum (ER) in the Schwann cells of the myelinated axons of BTZ treated rats. On the other hand, unmyelinated axons of the BTZ treated rats showed an increase in lamellarily arranged bodies (LB) and collapsed structures (CS) in the axons and the cytoplasm of Schwann cells. Some unmyelinated axons of BTZ treated rats were almost completely disintegrated. **Conclusion:** Our present data suggest that BTZ-induced neuropathy in a rat model is characterized by Schwann cell toxicity and axonal degeneration, both of which were observed dominantly in unmyelinated axons. Moreover, activation of the autophagy/lysosomal system is involved in BTZ treatment, resulting in peripheral neuropathy.

### Progress Report on Services for the Assisted Reproductive Technologies in Laboratory of Genome Research (5)

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The Laboratory of Genome Research (LGR) was established in 2002 to provide services for the assisted reproductive technologies (ART) including pronuclear or sperm microinjection (1), cleaning by in vitro fertilization (IVF) and embryo transfer (ET) (2), cryopreservation of embryos and spermatozoa (3), rederivation of vitrified embryos and frozen spermatozoa from transgenic/knockout (Tg/Ko) mice (4). These services have contributed for the effective and proper animal experimentation in our animal facility. Therefore, we report a record of achievement for the assisted reproductive technologies in our animal facility in 2013.

Method: (1) mouse spermatozoa with foreign DNA were microinjected into the cytoplasm of unfertilized eggs via intracytoplasmic sperm injection (ICSI). After incubation, the resulted embryos were transferred into the oviducts of recipient mice to generate transgenic mice. The genotype of these mice obtained by ICSI/ET was analyzed by PCR with the use of genomic DNA from their tails. (2) cleaning was performed by IVF/ET using spermatozoa and eggs collected from Tg/Ko mice. Recipients or foster mothers were microbiologically examined after weaning of their offspring developed from embryos via ET to confirm specific pathogen free status. (3) 2-cell embryos obtained from Tg/Ko mice via IVF were vitrified with vitrification solution (DPS) containing 2.75M DMSO, 2.75M propylene glycol and 1M sucrose and then stored in liquid nitrogen tank. A part of these embryos cryopreserved were thawed by rapidly warming at 40℃ with 0.3M sucrose in PBI to confirm their survivability and viability. The genotype of E13.5 fetuses from vitrified–thawed embryos was analyzed by PCR after transferring them into recipient mice. Spermatozoa collected from Tg/Ko mice were also rapidly frozen with stock solution containing 18% raffinose and 1.75M glycerol in −80℃ deep freezer and then stored in liquid nitrogen tank. To test fertilizing and developmental abilities, a part of frozen spermatozoa was thawed and subsequent injected into eggs via ICSI. The resulted 2-cell embryos were transferred into the recipient mice, and the presence of transgene in fetuses was analyzed by PCR at E13.5 after ET. (4) Tg/Ko-derived embryos or spermatozoa cryopreserved and transported from outside institutes were rederivered via ET or ICSI/ET after thawing.

Result: (1) Generation of Tg mice via ICSI has not done in 2013. (2) 24 lines from Tg/Ko mice were successfully cleaned and appeared to be all negative for specific pathogen. (3) 5,253 embryos from 19 lines were vitrified and stored in liquid nitrogen tank. A part of them were thawed and transferred into recipient mice. Twenty–four % of embryos survived developed to normal E13.5 fetuses after transferring into recipient mice while survival rates of embryos just after thawing was 77%. (4) rederivations of cryopreserved embryos and spermatozoa from 5 Tg/Ko lines, transported from 3 outside institutes, were performed via ET or ICSI/ET just after thawing. All lines could be successfully rederivered via ART.

Conclusion: Our services for the assisted reproductive technologies facilitate effective management of animal experiment in animal facilities, and may support more rapid and efficient recovery of Tg/Ko lines. This service also contributes to maintenance of valuable Tg/Ko lines via ART.
biological and histological analysis. For cell biological study, C2C12 cells, a murine myoblast cell line, were used.

**Results:** PMM deficiency in denervated slow-twitch soleus muscles delayed skeletal muscle atrophy, reduced mitochondrial activity, induced oxidative stress and increased the accumulation of polyubiquitinated proteins. In addition, denervation induced proteasomal activation via transcription factor Nrf1 nuclear translocation in wild-type mice, whereas it had little effect in autophagy-deficient and Parkin-deficient mice. Nrf1 nuclear translocation was inhibited by reactive oxygen species produced by damaged mitochondria.

**Conclusion:** The accumulation of damaged mitochondria by denervation suppresses proteasomal activation via Nrf1 nuclear translocation, and causes the delay of atrophy in PMM deficient soleus muscle.

**Functional Analysis of PINK1–Parkin Pathway in Drosophila Neural Model System**

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**Objective:** Our purpose is to reveal molecular mechanisms underlying the mitochondrial maintenance and the neuronal activities regulated by PINK1–Parkin pathway using *Drosophila* models.

**Methods:** To examine the consequence of phospho-modification of Parkin by PINK1 in neuronal activities, wild-type (WT–Parkin), a phosphor–inactive form (SA–Parkin), or phospho–mimetic form (SE–Parkin) of Parkin were expressed in dopaminergic (DA) neurons. VMAT–pHluorin, which is a pH-sensitive GFP conjugated with vesicle monoamine transporter, was co-expressed with three kinds (WT, SA or SE) of Parkin in DA neurons to record presynaptic activity by using a live-imaging technique.

**Results:** Aggregation of mitochondria and age-dependent loss of DA neurons were observed in WT– or SE–Parkin–expressing flies. SA–Parkin overexpression induced age-dependent loss of DA neurons while mitochondrial morphology was largely unaffected. Live imaging of the whole mount brain tissues revealed that presynaptic activity–dependent fluorescent recovery after photobleaching was normal in WT–Parkin flies compared with a control flies whereas it was reduced in SA– or SE–Parkin–expressing flies.

**Conclusion:** Our results suggest that 1) mitochondrial morphology in DA neurons is regulated by Parkin phosphorylation by PINK1; 2) expression of both SA– and SE–Parkin impairs presynaptic activity and leads to age-dependent loss of DA neurons.

**Molecular Mechanism of Early Onset Parkinson’s Disease**

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**Objective:** Parkinson’s disease (PD) is the most common neurodegenerative movement disorder, affecting 1% of the population above the age of 60. The classical form of the disease is characterized clinically by rigidity, resting tremor, bradykinesia and postural instability. The cellular abnormalities in Parkinson’s disease (PD) include mitochondrial dysfunction and oxidative damage, which are probably induced by both genetic predisposition and environmental factors. Mitochondrial dysfunction has long been implicated in the pathogenesis of PD. The recent discovery of genes associated with the etiology of familial PD has emphasized the role of mitochondrial dysfunction in PD. The discovery and increasing knowledge of the function of PINK1 and parkin, which are associated with the mitochondria, have also enhanced the understanding of cellular functions. Another autosomal recessive early-onset disorder characterized by levodopa–responder Parkinsonism, supranuclear gaze palsy, pyramidal sign, and dementia; it is also called as Kufor–Rakeb syndrome (KRS). Recently, ATP13A2 was identified as the causative gene for PARK9.

**Methods:** The function of ATP13A2 protein remains largely unknown. We examined localization and molecular mechanism by use of ATP13A2 stable knockdown cells.

**Results:** Normal ATP13A2 localizes in the lysosome, whereas disease–associated variants remain in the endoplasmic reticulum (ER). Stable ATP13A2–knockdown cells displayed lysosome–like bodies characterized by granular deposits and fingerprint–like structures.

**Conclusion:** We supposed that ATP13A2 might associate with protein degradation via autophagy and lysosomal pathway. Further investigation will be needed to elucidate how ATP13A2 influences the
pathomechanisms of neuronal degeneration.

Phosphorylation Enhances Recombinant HSP27 Neuroprotection Against Focal Cerebral Ischemic in Mice
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Background: Heat shock protein 27 (HSP27) exerts cytoprotection against many cellular insults, including cerebral ischemia. We previously showed that intravenous injection of HSP27 purified from human lymphocytes (hHSP27) significantly reduced infarct volume against cerebral ischemia-reperfusion injury. While recombinant HSP27 (rHSP27) purified from Escheria coli was less effective against ischemic injury. hHSP27 contained more larger amount of phosphorylated HSP27 compared to recombinant HSP27. Phosphorylation is important for HSP27 function. This led us to hypothesize that phosphorylated recombinant HSP27 (prHSP27) might gain brain protection.

Methods: rHSP27 was phosphorylated by MAPKAP kinase 2 in vitro. Mice were subjected to transient 1-h MCAO, and then randomly divided into 4 groups. (1) hHSP27 group as positive control, (2) rHSP27 group, (3) prHSP27 group, (4) a control group: bovine serum albumin (BSA) group, that received tail-vein injections of each materials 1h after reperfusion. Mice were evaluated for infarct volume, neurological score, cell-deaths, oxidative stress, and inflammatory response.

Results: Infarct volume was reduced by 69% treated with hHSP27 (10.5 ± 4.6 mm³, p < 0.001, n = 5), 19% treated with rHSP27 (27.8 ± 4.2 mm³, p < 0.05, n = 5), by 49% treated with prHSP27 (17.5 ± 4.5 mm³, p < 0.001, n = 9) vs. BSA–treated controls (34.4 ± 2.8 mm³ n = 5). prHSP27 treatment considerably reduced infarct volume and improvement functional deficit compared to rHSP27 and BSA–treated control. It also significantly suppressed apoptosis, oxidative stress and inflammatory response after reperfusion.

Conclusion: prHSP27 was phosphorylated at Ser15, Ser78 and Ser82, which phosphorylation were important for brain protection against focal cerebral ischemia in mice. prHSP27 may be a useful therapeutic agent to protect against acute cerebral ischemic stroke.

Exploration of the Function of a Novel Candidate Causative Gene for Parkinson's Disease Using D. Melanogaster
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Purpose: Through linkage analysis of one of the families registered in our institutional gene bank, an orphan gene ₇₉ has just been screened as a novel candidate causative gene for autosomal dominant Parkinson's disease (PD). Previous reports and our preliminary experiment suggest that ₇₉ mainly localizes in mitochondria, which all the more makes us associate ₇₉ with PD, considering that mitochondrial stressors such as complexI inhibitors bring about PD-like pathology and that mitochondrial quality control (MCQ) failure has been believed to cause PD. The purpose of this study is to examine whether ₇₉ has some role in mitochondrial stress response and MCQ.

Method: We chose flies (D. melanogaster) as model animal, which have a homologue for ₇₉ (₇₉). We planned to examine the effect of ₇₉ modulation. First we prepared strains of flies with modulated ₇₉ alleles (overexpression, knock-down and knock-out.) and examined their phenotypes. Second we crossed them with PD-related flies (PINK1-knockout and parkin-knockout flies.), and observed the phenotypes of the offsprings.

Result: In comparison to controls, ATP production tended to be increased in flies with either knocked-out or knocked-down ₇₉, whereas it tended to be reduced in flies with overexpressed ₇₉. Over-expressed ₇₉ significantly worsened the normal flies' vulnerability to paraquat. Observation on 15 day after eclosion revealed that abnormal wing posture, muscle degeneration, and ATP production of PINK1-knockout flies were worsened by knocked-down ₇₉ whereas it was rescued by knocked-out ₇₉. The crossing of ₇₉ modulated flies with parkin-knockout flies is underway.

Conclusion: So far, ₇₉ modulation appears to affect mitochondrial function and the pathway in which PINK1 is involved, possibly MCQ though it has yet to
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be determined whether the effect is positive or negative.

Mutation Analysis of EIF4G1 in Japanese Patients with Parkinson’s Disease.
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Objective: Recently EIF4G1 was identified as a novel causal gene for familial Parkinson’s disease. In this study, to examine the mutation frequency of EIF4G1 in Japanese patients with Parkinson’s disease, we screened EIF4G1 mutations.
Methods: We sequenced coding exons of EIF4G1 in 95 patients with autosomal dominant Parkinson’s disease by PCR-direct sequencing. All detected variants in 95 patients were validated in the case-control series (224 patients and 374 controls).
Results: We detected novel mutations of EIF4G1 in a patient with autosomal dominant Parkinson’s disease. This patient harbored three point mutations (c.1388AG>GA and c.1394A>C) on the same allele, however these three point mutations were found in two healthy siblings of the patient. In addition, we found the same three point mutations in two patients with sporadic Parkinson’s disease.
Conclusions: No pathogenic mutations of EIF4G1 were found in this study, suggesting that EIF4G1 mutations are rare in Japanese patients with Parkinson’s disease.

Clinicogenetic Study of GBA Mutations in Patients with Familial Parkinson Disease
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Objective: The glucocerebrosidase gene (GBA) is the causative gene of Gaucher disease (GD), and recently rare variants of GBA have been found as risk factor of sporadic Parkinson disease (SPD). However there are few large studies of GBA mutations familial PD (FPD), so that we aimed to clarify the role of GBA mutations in FPD.
Methods: We sequenced entire coding exons and exon/intron boundaries of GBA in 147 Japanese FPD patients from 144 families and 100 unrelated controls.
We compared 19 items concerning prominent PD symptoms between GBA mutation-positive and GBA mutation-negative groups.
Results: Eight heterozygous mutations were detected in 31 index patients (31/144=21.5%), suggesting the GBA heterozygous mutations are strongly associated with FPD (OR=21.5, 95% confidence interval=3.6–202.7). The frequency was significantly higher in autosomal dominant PD (ADPD) compared to autosomal recessive PD (ARPD). PD patients with GBA mutations exhibited typical manifestations of PD or dementia with Lewy bodies (DLB), such as L-dopa responsive parkinsonism with psychiatric problems and/or cognitive decline. Nine patients with GBA mutations were underwent I-metaiodobenzylguanidine (MIBG) scintigraphy, and all of them showed marked reduction of myocardial MIBG uptake.
Conclusion: Heterozygous GBA mutations play a greater role in FPD, especially in ADPD, and are likely to facilitate the development of PD and DLB via different genetic and pathogenic mechanism. We speculated that GBA dysfunction may promote Lewy body formation. Our findings suggest that further functional analyses for GBA should elucidate the pathogenesis of PD and Lewy body diseases.

Identification of Fibulin-7 Peptides Active for Endothelial Cell Adhesion and Tube Formation
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Introduction: Fibulin-7 (Fbln7) is an extracellular matrix glycoprotein that promotes cell adhesion for dental mesenchyme cells and endothelial cells. We recently found that endothelial cells adhere to the recombinant C-terminal Fbln7 protein Fbln7-C, which inhibits endothelial tube formation in culture and endothelial sprouting in the mouse aortic ring assay. In this study, we screened 12 synthetic peptides from the C terminal part of the Fbln7 protein to identify the potential active sites for endothelial cell adhesion and in vitro angiogenesis inhibition.
Material and Methods: Endothelial cells–Adhesion Assay–Western Blot–Angiogenesis Assays.
Results and Discussion: Fbln7-C bound to human umbilical vein endothelial cells (HUVECs), but it did not promote cell spreading and actin stress fiber formation. Fbln7-C binding to HUVECs induced integrin clustering at cell adhesion sites with other
focal adhesion molecules, and sustained activation of FAK, p130Cas, and Rac1. Additionally, RhoA activation was inhibited, thereby preventing HUVEC spreading. We showed that Fbn7-C inhibited the HUVEC tube formation and the vessel sprouting from aortic rings. These findings suggest that the Fbn7 C-terminal region contains the inhibitory site for angiogenesis. Peptides have emerged as effective therapeutic agents for angiogenesis-dependent diseases due to their low toxicity and high specificity. Therefore, we screened a series of peptides spanning the C-terminal Fbn7 region using a synthetic peptide approach to identify a site(s) for the anti-angiogenesis activity. We have found that three peptides bound to endothelial cells and reduced the tube formation of HUVEC, suggesting that these peptides could represent a new therapeutic agent to inhibit angiogenesis.

Laminin α1 Regulates Age-Related Mesangial Cell Proliferation and Mesangial Matrix Accumulation Through the TGFβ Pathway

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Object: Laminin α1 (LAMA1), a subunit of the laminin-111 basement membrane component, has been implicated in various biological functions in vivo and in vitro. Although LAMA1 is present in kidney, its roles in the kidney are unknown because of early embryonic lethality. Here, we used a viable conditional knockout mouse model with a deletion of Lama1 in the epiblast lineage (Lama1CKO) to study the role of LAMA1 in kidney development and function.

Result: Adult Lama1CKO mice developed focal glomerulosclerosis and proteinuria with age. In addition, mesangial cell proliferation was increased, and the mesangial matrix, which normally contains laminin-111, was greatly expanded. In vitro, mesangial cells from Lama1CKO mice exhibited significantly increased proliferation compared to those from controls. This increased proliferation was inhibited by the addition of exogenous LAMA1-containing laminin-111, but not by laminin-211 or laminin-511, suggesting a specific role for LAMA1 in regulating mesangial cell behavior. Moreover, the absence of LAMA1 increased TGF-β1-induced Smad2 phosphorylation, and inhibitors of TGF-β1 receptor I kinase blocked Smad2 phosphorylation in both control and Lama1CKO mesangial cells, indicating that the increased Smad2 phosphorylation occurred in the absence of LAMA1 via the TGF-β1 receptor.

Conclusion: These findings suggest that LAMA1 plays a critical role in kidney function and kidney aging by regulating the mesangial cell population and mesangial matrix deposition through TGF-β/Smad signaling.

Reduced Expression of Endothelial Nitric Oxide Synthase in Perlecan Deficiency Aorta

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Aim: Perlecan (HSPG2; Perl) is expressed in all basement membranes and cartilage, and has multiple biological activities. Perl is essential for cartilage development, and the perl null (HSPG2-/-) mice die perinatally. We previously created a lethality rescued HSPG2-/-Tg mouse model by expressing the HSPG2 transgene, specifically in cartilage of HSPG2-/- mice. In this study, we focus on the role of perl in aortic endothelial cell activity in the regulation of vascular tone.

Methods: Aorta tissue samples were prepared from the thoracic aorta of HSPG2-/-Tg and control (HSPG2+/+Tg) mice. We measured endothelial cell activity in response to aortic tension using Sodium Nitroprusside or Acetylcholine. We evaluated the RNA expression or protein expression by qRT-RCR or western blot assay, respectively Human aortic endothelial cells (HAEC) were transfected with Perl siRNA, and RNA expression level of eNOS was analyzed.

Results and Conclusion: We found that a deficiency of Perl results in the impairment of endothelium-dependent vascular relaxation in mice aorta, whereas endothelium-independent relaxation remained well preserved. We found that eNOS expression levels were decreased at both RNA and protein levels in the Perl-null aorta tissues. We further examined the relationship between perl deficiency and the decreased eNOS expression by treating HAECs with Perl siRNA and found that a reduction in the Perl gene expression induced a decrease in eNOS gene expression. In conclusion, we showed that deficiency of perl led to endothelial dysfunction. This dysfunction was due, at least in part, to a reduction in eNOS expression, indicating that Perlecan plays a role in the activation of the eNOS gene expression during the
normal growth process.

Clarity-Cleared Whole Brain Imaging: Application in a Mouse Model of Autism
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Background: Autism spectrum disorder (ASD) is a cognitive syndrome affecting one in 100–150 children. It is characterized by a lack of social interaction, impaired communication, and repetitive behavior. BTBR mice are an inbred mouse strain that recapitulate key features of ASD. Interestingly, BTBR mice also present a total lack of corpus callosum, a structure often smaller and disorganized in autism patient. Recently, new approaches to image the whole brain in order to reconstruct neuronal circuits have arisen. "Scale" makes use of high concentration of urea to render the brain transparent (Hama et al., Nat Neurosci 2011) and “SeeDB” uses high fructose concentration to achieve the same purpose (Ke et al., Nat Neurosci 2013). While both techniques successfully render adult mouse brain transparent, they do not permit the diffusion of antibodies throughout the brain. Here, using a technique developed recently called CLARITY (Chung et al., Nat Neurosci 2013), we investigate the neuroanatomical defect of BTBR mice.

Methods: We clarity cleared the brain of newborn B6 (control) and BTBR mice. Using a Zeiss LSM 780 2-photon microscope we recorded images following whole brain staining with antibodies against myelin, neurofilament or vasculature (laminin). We used Imaris software to visualize and analyze 3D data set.

Results: We observed that myelin fibers accumulated medially instead of crossing to the contralateral hemisphere to form the corpus callosum. This was correlated with impaired vascularization. Together this technique appear very promising to decipher complex anatomical defect since we can have both an overview of the whole brain while keeping a single cell resolution.

Age-Related Alteration of Sonic Hedgehog Signaling in the Subventricular Zone of the Lateral Ventricle
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Background: In the subventricular zone (SVZ) of the adult brain, neural stem cells (NSCs) are intimately associated with fractones and capillaries basement membrane. Fractones are specialized extracellular matrix (ECM) composed of laminin, collagen IV, nidogen, and heparan sulfate proteoglycan such as agrin or perlecan. We previously reported that fractones capture growth factors such as FGF-2 from the extracellular milieu and thus promote proliferation in the neurogenic niche (Kerever et al., 2007). During aging, the NSC pool decrease and neurogenesis declines. However, the reasons behind these events are not yet understood.

Methods: In this study we investigated if the reported age-related decline of neurogenesis in the SVZ was correlated with changes in the ECM niche and Sonic Hedgehog signaling. Using 10 weeks (young) and 100 weeks (old) mice brain, we performed immunostaining for different ECM components, in situ growth factor binding assay and real time quantitative PCR.

Results: We observed that in the aged neurogenic niche, fractone size dramatically increases. Also, the composition of heparan chains contained in fractones was modified. However, FGF-2 was still capable to bind fractones. Furthermore, RNA level of smoothen, Gli1, Gli2 and Cyclin D2 were decreased with aging, suggesting that sonic hedgehog signaling was impaired. Theses results suggest a critical role of ECM in aging. How the modification in heparan sulfate chain impacts on sonic hedgehog pathway and the crosstalk between FGF-2 and sonic hedgehog need to be further investigated.

Attempt of Stemness-Potentiation of Adipose-Derived Stem Cells by the Culture Supernatant of Human iPSC Cells
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The potential of cultured adipose-derived mesenchymal stem cells (ASCs) in regenerative medicine and new cell therapeutic concepts has been shown recently by many investigations. However, although ASCs can differentiate into multi-lineage cells, their stemness has not been well investigated. Recent reports demonstrated that older ASCs, compared with younger donor cells, secrete less cytokines. Furthermore, the potential of ASCs differs depending on the patient’s conditions such as diabetes. Meanwhile, induced pluripotent stem (iPS) cells can be
reprogrammed from adult somatic cells by the transduction of genes or chemical agents. iPS cells share the features of embryonic stem cells and are capable of self-renewal and tri-dermal differentiation. Further, some researchers demonstrated that the administration of iPS cells-conditioned medium (iPS-CM) also contributes the tissue repair such as lung injury. Therefore, secreted cytokines from iPS cells have a potential for the improvement of tissues and cells. The purpose of this study is to investigate whether iPS-CM can improve the stemness of ASCs.

This study was designed as follows. The human iPS cell line (201B7, Riken Bio Resource Center, Tsukuba, Japan) is cultured and expanded to collect the culture supernatant of iPS cells. Samples of human subcutaneous adipose tissue are obtained as waste material after resection and donated after informed consent from donors. ASCs are expanded in culture medium (DMEM + 10%FBS + 1% antibiotic-antimycotic) into the 3-passage. After ASCs are cultured with 10% iPS-CM, proliferation assay, FACS (CD29, CD73, CD90 and CD105) and RT-PCR (Oct-4, Nanog, SOX2 and SSEA1) are performed. Secreted cytokines from iPS cells are analysed with ELISA. The goal of this study is to reveal the mechanism and related factors for stemness-potentiation of ASCs.

Lysosomal Proteases in Podocytes May Play an Important Role in the Pathogenesis of Proteinuria and Chronic Kidney Disease
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Objective: We aim to investigate the role of lysosomal proteases, cathepsin L (CL) and D (CD) in podocytes, and the mechanism by which they cause proteinuria and chronic kidney disease.

Methods: ① Previous studies showed that up-regulated CL in podocytes is required for the development of proteinuria. We examined whether CL-mediated proteolysis plays a role in the development of glomerulosclerosis and proteinuria using the doxorubicin (DOX) induced nephrosis and glomerulosclerosis model on CL knockout mice.

② The role of CD in podocytes has not been explored previously. We generated podocyte-specific CD knockout mice (CD$^{\Delta podocyte}$), and evaluated the natural course.

Results: ① The levels of proteinuria in CL knockout mice were significantly lower than those in wild-type (WT) mice from day 24 to day 56 after DOX injection. The number of sclerotic glomeruli in the CL knockout mice was also significantly less than that in WT mice at 56 day after DOX injection. These data provides genetic evidence that CL is essential for the progression of glomerulosclerosis.

② CD$^{\Delta podocyte}$ mice had a significantly lower survival ratio than WT mice. By 5 months of age, CD$^{\Delta podocyte}$ mice developed significantly higher levels of proteinuria compared with WT mice. Electron microscopy analysis revealed characteristic cytoplasmic inclusions in podocytes from CD$^{\Delta podocyte}$ from 5 months of age.

Also, immunofluorescence revealed altered expression patterns for slit diaphragm protein podocin. In addition, lysosomal-associated membrane protein 1 was co-localized with podocin. These results suggest that the podocytes in CD$^{\Delta podocyte}$ mice develop chronic kidney disease because of the presence of lysosomal storage disease.