# Phosphorylation state of tau in the hippocampus of apolipoprotein E4 and E3 knock-in mice

Mariko Kobayashi, Koichi Ishiguro, Yuko Katoh-Fukui,[1] Minesuke Yokoyama and Shinobu C. Fujita[CA]

Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida, Tokyo, 194-8511 Japan
[1]Present address: Department of Developmental Biology, National Institute for Basic Biology, Myodaiji-cho, Okazaki, Aichi, 444-8585 Japan

[CA]Corresponding Author: fujita@libra.ls.m-kagaku.ac.jp

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The apolipoprotein E (apoE) ε4 allele is associated with an increased risk of sporadic as well as late-onset familial Alzheimer's disease (AD). To accurately determine the isoform-specific effects of human apoE on AD-like phosphorylation of tau, hippocampi from human apoE knock-in (KI) mice were studied by quantitative immunoblotting. There was no significant difference in phosphorylation levels of tau at nine of the 13 epitopes, for six of eight tau kinases, or in protein levels of three tau phosphatases, between apoE3-KI and apoE4-KI mouse hippocampi. However, in apoE4-KI mice, phosphorylation of tau at Ser235 was increased to ~150%, that at Ser413 to ~140%, while that at Ser202/Thr205 and Thr205 were decreased to ~70%, and the protein level of tau protein kinase I/glycogen synthase kinase 3β (TPKI/GSK3β) was increased to ~120%, that of extracellular signal-regulated kinase 2 (ERK2) was increased to ~130%, compared with apoE3-KI mice. *NeuroReport* 14:699–702 © 2003 Lippincott Williams & Wilkins.

**Key words:** Alzheimer's disease; Apolipoprotein E; Hippocampus; Knock-in mouse; Phosphorylated tau; Quantitative immunoblotting; Tau kinase; Tau phosphatase

## INTRODUCTION

Human apolipoprotein E (apoE), a 34 kDa protein of 299 amino acids abundant in plasma and cerebrospinal fluid, is encoded by a single gene on chromosome 19 and plays an important role in the regulation of lipid transport and cholesterol homeostasis (reviewed in [1]). ApoE is primarily synthesized in the liver, followed by the brain. Three common variants of apoE protein result from three alleles ε2, ε3, ε4 coding for protein isoforms with single amino acid substitutions [1]. ApoE3 has cysteine at residue 112 and arginine at residue 158, whereas both residues are cysteines in apoE2 and arginines in apoE4. The apoE4 is an established and dose-dependent major genetic risk factor for the late-onset familial [2] and sporadic [3,4] Alzheimer's disease (AD).

The pathological hallmark of AD is the presence in the brain of extracellular senile plaques mainly composed of the amyloid β peptide, and intracellular neurofibrillary tangles composed of the abnormal hyperphosphorylated tau proteins aggregated into paired helical filaments (PHF).

It was reported that expression of human apoE4 in mouse neurons caused hyperphosphorylation of tau [5]. However, apoE3 transgenic mice were not available in that study. To study the apoE isoform-specific effect *in vivo* in the context of more physiological astrocytic expression of apoE, we chose human apoE knock-in (KI) mice, in which human apoE cDNAs were placed under the endogeneous apoE regulatory elements [6]. We focused on the hippocampal tissue, which is the brain region most susceptible to PHF pathology in AD.

## MATERIALS AND METHODS

*Animals:* Eight-week old homozygous ApoE3-KI or ApoE4-KI mice were analyzed. In these knock-in lines, a portion of the mouse apoE gene has been replaced by human apoE3 or apoE4 cDNAs through homologous recombination such that human apoE proteins are expressed under the endogenous apoE control region [6]. These mice lack mouse apoE protein entirely. Expression levels in brain homogenates and neuronal and astrocytic expression of human apoE were comparable in apoE3-KI and apoE4-KI mice [7]. Details of the generation of the ApoE3-KI line will be described elsewhere.

*Antibodies:* Purified rabbit polyclonal antibodies PS199, PS202, PT205, PT231/PS235, PT231, PS235, PS262, PS396, PS404, PS413 and PS422 are specific to tau phosphorylated at the respective residues indicated by the number referring to the longest human tau [8,9]. Antibody Tau-C specifically recognizes the C-terminus (422–438) of tau irrespective of phosphorylation status [8]. Mouse monoclonal antibody AT8 (Innogenetics) is specific to tau dually phosphorylated at Ser202 and Thr205, and Tau-1 (Boehringer) to tau dephosphorylated over residues 189–207. Anti-TPKI mouse monoclonal antibody T1.7 was produced against a C-terminal peptide of rat tau [10]. Rabbit antibody PS9 recognizes TPKI/GSK3β phosphorylated at Ser9, while antibody PY216 recognizes TPKI/GSK3β phosphorylated

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# FULL RECONSTITUTION OF HEMATOPOIETIC SYSTEM BY MURINE UMBILICAL CORD BLOOD

FUJIO MIGISHIMA,[1,3] ATSUHIKO OIKAWA,[2] SHUNZO KONDO,[3] HIDEO EMA,[4] YOHEI MORITA,[4] HIROMITSU NAKAUCHI,[4] MINESUKE YOKOYAMA,[3] SI-YOUNG SONG,[3] MASAHIRO NISHIJIMA,[1] MASARU OKABE,[5] AND NOBUKATA SHINOHARA[2,6]

***Background.*** **Murine umbilical cord blood cells (UCBCs) were studied for their ability to reconstitute the hematopoietic system.**

***Methods.*** **On average, 150 μL of cord blood per fetus containing 1.2 to $2\times10^4$ nucleated cells were collected from day 18.5 fetal umbilical cord, and 3 to $6\times10^3$ cells per fetus were obtained after separation by gradient centrifugation.**

***Results.*** **Although lineage marker$^-$, c-Kit$^+$, and Sca-1$^+$ cells were detectable among UCBCs, cells designated to be in the side population (SP) by Hoechst 33342 staining were hardly detectable within this population; the frequency of cells of this phenotype was less than 1 of $10^5$. Instead, the lineage marker$^-$, c-Kit$^+$, and Sca-1$^-$ population contained a considerable number of SP cells. Nevertheless, UCBCs obtained from fetuses of green fluorescent protein-transgenic mice successfully reconstituted the blood cells of lethally irradiated recipients. Fluorescent cells could be readily detected in every blood cell lineage and among immature cell populations. Furthermore, fluorescent SP cells sorted from the recipient bone marrow cells could also reconstitute the blood cells in the secondary recipients, indicating that UCBCs also replenished bone marrow stem cells.**

***Conclusion.*** **Murine UCBC could fully reconstitute the hematopoietic system of lethally irradiated recipients including hematopoietic stem cells in bone marrow.**

Since the first successful clinical application of human umbilical cord blood transplantation (*1*), a number of clinical trials have proven the usefulness of umbilical cord blood cells (UCBCs) for reconstitution of the hematopoietic system (*2*, *3*). Although the relatively slow engraftment is a drawback, UCBC transplantation has several advantages over bone marrow transplantation: the larger size of the available donor pool, enriched hematopoietic progenitor cells (*4*), low content of mature T cells causing graft-versus-host reaction (*5*, *6*), and absence of cytomegalovirus infection (*7*). Furthermore, recent investigations have indicated that UCBCs contain progenitor cells of a variety of cell lineages in addition to hematopoietic stem cells (*8*, *9*). Thus, the potential of UCBCs in clinical applications seems unpredictably broad. To thoroughly investigate the possible differentiation capacities of UCBCs, syngeneic transplantation models allowing long-term in vivo observations are essential. A murine experimental model fulfills such a requirement; however, there have been a limited number of reports describing murine experimental models of cord blood transplantation. Scaradavou et al. and de la Selle, Gluckman, and Bruley-Rosset reported successful long-term engraftment of peripheral blood cells in near-term fetal and newborn mice (*10*, *11*). Although successful engraftment of UCBCs in an irradiated host has been reported, the differentiation capacity of the repopulating cells has not been analyzed in detail. There have also been reports on hematopoietic stem cells in murine fetal liver (*12*). Nevertheless, the cell population taken from a hematopoietic organ may well be different from that in the circulating blood.

We have successfully established a murine model of UCBC transplantation that allows extensive analyses. Murine UCBCs virtually lacked cells with the typical staining profile of adult bone marrow stem cells, that is, lineage marker$^-$ (lin$^-$), c-Kit$^+$, and Sca-1$^+$ (LKS$^+$) cells designated to be in the side population (SP) by Hoechst 33342 staining. Nevertheless, UCBCs were capable of fully reconstituting the hematopoietic and lymphoid systems of lethally irradiated mice.

## MATERIALS AND METHODS

### *Animals*

Female C57BL/6(B6: H2$^b$) and ICR mice (randomly bred closed colony albino mice) were purchased from CLEA Japan Inc. (Tokyo, Japan). C57BL/6TgN(act-EGFP)OsbY01 mice (green mice: H2$^b$) (*13*) were bred in our animal facility. Mice were maintained in a specific pathogen-free barrier building.

### *Umbilical Cord Blood Cells*

Cryopreserved embryos (0.5 days old) were transferred into the uteri of pseudopregnant ICR female mice. Nontransgenic ICR mice were used as recipient mothers for fertilized eggs because of their high fecundity and to exclude the possible contribution of contaminating cells from the mother. Eighteen days after the transfer of embryo, the uteri were removed from the pregnant females under anesthesia. After washing, they were cut into individual fetal swellings, the muscle layer of each uterus was removed, and fetuses with membrane-covered placenta were obtained. They were then cooled down in ice-cold phosphate-buffered saline (PBS), and the visceral yolk sac and amnion were removed. The cleaned fetuses with umbil-

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[1] Department of Obstetrics and Gynecology, Kitasato University School of Medicine, Sagamihara, Japan.

[2] Department of Immunology, Kitasato University School of Medicine, Sagamihara, Japan.

[3] Mitsubishi Kagaku Institute of Life Sciences, Machida, Japan.

[4] Department of Immunology, Tsukuba University School of Medicine, Tsukuba, Japan.

[5] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

[6] Address correspondence to: Nobukata Shinohara, M.D., D.M.Sc., Department of Immunology, Kitasato University School of Medicine, 1–15–1 Kitasato, Sagamihara, Kanagawa 228–8555 Japan. E-mail: nobu@med.kitasato-u.ac.jp.

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# *jumonji* Downregulates Cardiac Cell Proliferation by Repressing *cyclin D1* Expression

Masashi Toyoda,[1,3,4] Haruki Shirato,[1,3]
Kuniko Nakajima,[1] Mizuyo Kojima,[1]
Miho Takahashi,[1,2] Misae Kubota,[1]
Rika Suzuki-Migishima,[1] Yoshiko Motegi,[1]
Minesuke Yokoyama,[1] and Takashi Takeuchi[1,*]
[1]Mitsubishi Kagaku Institute of Life Sciences (MITILS)
11 Minamiooya, Machida
Tokyo, 194-8511
[2]Graduate School of Environment and Information Sciences
Yokohama National University
79-1 Tokiwadai, Hodogaya, Yokohama
Kanagawa, 240-8501
Japan

## Summary

Spatiotemporal regulation of cell proliferation is necessary for normal tissue development. The molecular mechanisms, especially the signaling pathways controlling the cell cycle machinery, remain largely unknown. Here, we demonstrate a negative relationship between the spatiotemporal patterns of *jumonji* (*jmj*) expression and cardiac myocyte proliferation. *cyclin D1* expression and cell proliferation are enhanced in the cardiac myocytes of *jmj*-deficient mutant embryos. In contrast, *jmj* overexpression represses *cyclin D1* expression in cardiac cells, and Jmj protein binds to *cyclin D1* promoter in vivo and represses its transcriptional activity. *cyclin D1* overexpression causes hyperproliferation in the cardiac myocytes, but the absence of *cyclin D1* in *jmj* mutant embryos rescues the hyperproliferation. Therefore, Jmj might control cardiac myocyte proliferation and consequently cardiac morphogenesis by repressing *cyclin D1* expression.

## Introduction

Cell proliferation is an important factor in various developmental processes in tissue morphogenesis and is strictly regulated spatiotemporally so the tissue attains the necessary size, structure, and functions. Defects of the regulation result in disorganization of organogenesis or tumor formation. There are complex signaling pathways under the regulation of cell proliferation, of which the cell cycle machinery would hold a central position. It is still largely unknown, however, what molecules work in these pathways and how these pathways regulate the cell cycle during development.

The control of cell proliferation by extracellular signals occurs largely during the G1 phase of the cell cycle. During this phase, growth signals are transduced from the extracellular environment, ultimately reaching the cell cycle machinery operating in the cell nucleus, which is mainly regulated by molecules such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs). Complexes of G1 cyclins and their associated CDKs trigger a cell cycle progression by phosphorylating critical proteins such as the retinoblastoma tumor suppressor gene product (pRB) (for review, see Sherr, 1994). Thus, growth signals may influence the functions of G1 cyclin-CDK complexes.

Five mammalian G1 cyclins have been enumerated to date: cyclins D1, D2, D3 (D-type cyclins), and cyclins E1 and E2. Because the expression of D-type cyclins is controlled by extracellular signals, D-type cyclins are regarded as functional links between the extracellular environment and the cell cycle machinery (for reviews, see Sherr, 1993, 1994). Although there are many reports about the regulation of D-type cyclins, little is known about regulation for cell proliferation in vivo during development.

The *jumonji* (*jmj*) gene and *jmj* mutant mice were originally obtained by a mouse gene trap strategy (Takeuchi et al., 1995). The trap vector was introduced into the *jmj* gene and disrupted the gene. *jmj* heterozygous mice ($jmj^{+/trap}$) show no apparent abnormalities, whereas *jmj* homozygous mice ($jmj^{trap/trap}$), which lack *jmj* functions, die in utero. These embryos have various abnormalities such as neural tube, cardiac, and hematopoietic defects (Takeuchi et al., 1995, 1999; Motoyama et al., 1997; Kitajima et al., 1999, 2001; Lee et al., 2000). The *jmj* gene encodes a protein that is a member of the jumonji family (for review, see Balciunas and Ronne, 2000) and the AT-rich interaction domain (ARID) family (for review, see Kortschak et al., 2000). The ARID is a DNA binding domain, and several members of this family are known to be transcriptional factors and are involved in a variety of biological processes (Kortschak et al., 2000).

We previously reported that the number of megakaryocyte lineage cells increased and the regulation of growth arrest in these cells was affected in $jmj^{trap/trap}$ embryos (Motoyama et al., 1997; Kitajima et al., 2001) and that *jmj* overexpression in culture cells caused the inhibition of cell proliferation (Toyoda et al., 2000). To determine the distinct roles played by *jmj* in cell proliferation during development, we focused on the hyperproliferation of cardiac myocytes (Takeuchi et al., 1999) among various phenotypes of $jmj^{trap/trap}$ mice. In the present study, we show that the *jmj* gene downregulates cell proliferation of cardiac myocytes by repressing *cyclin D1* expression.

## Results

### Negative Relationship between Cardiac Myocyte Proliferation and *jmj* Expression during Cardiac Development

Previously we reported that cardiac myocytes in ventricles exhibited abnormal morphology that resulted from

*Correspondence: take@libra.ls.m-kagaku.co.jp
[3]These authors contributed equally to this work.
[4]Present address: Department of Genetics, National Research Institute for Child Health and Development, 3-35-31 Taishido Setagaya, Tokyo, 154-8567.

Cellular/Molecular

# Reversible Suppression of Glutamatergic Neurotransmission of Cerebellar Granule Cells *In Vivo* by Genetically Manipulated Expression of Tetanus Neurotoxin Light Chain

**Mutsuya Yamamoto,[1,2,3] Norio Wada,[2] Yasuji Kitabatake,[3] Dai Watanabe,[3] Masayuki Anzai,[4] Minesuke Yokoyama,[5] Yutaka Teranishi,[5] and Shigetada Nakanishi[2,3]**

[1]Mitsubishi Pharma Corporation, Discovery Technology Laboratory, Yokohama, 227-0033, Japan, [2]Department of Molecular and System Biology, Kyoto University Graduate School of Biostudies, Kyoto, 606-8501, Japan, [3]Department of Biological Sciences, Kyoto University Faculty of Medicine, Kyoto, 606-8501, Japan, [4]GenCom Corporation, Machida, 194-8511, Japan, and [5]Mitsubishi Kagaku Institute of Life Sciences, Machida, 194-8511, Japan

We developed a novel technique that allowed reversible suppression of glutamatergic neurotransmission in the cerebellar network. We generated two lines of transgenic mice termed Tet and TeNT mice and crossed the two transgenic lines to produce the Tet/TeNT double transgenic mice. In the Tet mice, the tetracycline-controlled reverse activator (rtTA) was expressed selectively in cerebellar granule cells by the promoter function of the $GABA_A$ receptor $\alpha 6$ subunit gene. In the TeNT mice, the fusion gene of tetanus neurotoxin light chain (TeNT) and enhanced green fluorescent protein (EGFP) was designed to be induced by the interaction of doxycycline (DOX)-activated rtTA with the tetracycline-responsive promoter. The Tet/TeNT mice grew normally even after DOX treatment and exhibited a restricted DOX-dependent expression of TeNT in cerebellar granule cells. Along with this expression, TeNT proteolytically cleaved the synaptic vesicle protein VAMP2 (also termed synaptobrevin2) and reduced glutamate release from granule cells. Both cleavage of VAMP2/synaptobrevin2 and reduction of glutamate release were reversed by removal of DOX. Among the four genotypes generated by heterozygous crossing of Tet and TeNT mice, only Tet/TeNT mice showed DOX-dependent reversible motor impairments as analyzed with fixed bar and rota-rod tests. Reversible suppression of glutamatergic neurotransmission thus can be manipulated with spatiotemporal accuracy by DOX treatment and removal. These transgenic mice will serve as an animal model to study the cerebellar function in motor coordination and learning.

*Key words:* transgenic mouse; $GABA_A$ receptor; tetanus neurotoxin; tetracycline-inducible system; cerebellum; granule cell; VAMP2; glutamatergic transmission

## Introduction

One useful approach to understanding mechanisms underlying information processing and integration in the neural circuit involves inactivating specific neurons in the neural network. This approach has been used effectively in *Drosophila* and *Caenorhabditis elegans* (*C. elegans*) in some cases by reversibly inactivating a subset of neurons (Sweeney et al., 1995; Dubnau et al., 2001; McGuire et al., 2001; White et al., 2001). In mammals, the cell targeting that allows selective ablation of a particular cell type within the network has been developed by genetically manipulating techniques (Nirenberg and Cepko, 1993; Kobayashi et al., 1995; Watanabe et al., 1998; Gogos et al., 2000). This technology has clarified mechanisms underlying development, information processing and integration, and behaviors but also often has led to adaptive and compensatory changes in the neural function (Watanabe et al., 1998; Kaneko et al., 2000). The technology that allows reversible suppression of a specific neuronal activity in mammals is desired but still is limited (Steele et al., 1998).

Neurotransmitter is stored in and released from synaptic vesicles by $Ca^{2+}$-regulated exocytosis (Südhof, 1995). VAMP2 (also known as synaptobrevin) is a core protein of synaptic vesicles (Baumert et al., 1989; Elferink et al., 1989; Archer et al., 1990; Söllner et al., 1993a,b) and is required for synaptic vesicle exocytosis (Südhof, 1995; Schoch et al., 2001). This vesicle protein is a target of tetanus neurotoxin. The light chain of tetanus neurotoxin (TeNT) proteolytically cleaves VAMP2 between Gln-76 and Phe-77 and impairs synaptic vesicle exocytosis (Link et al., 1992; Schiavo et al., 1992). The proteolytic inactivation of VAMP2 by genetic manipulation with TeNT thus would be approached to suppress synaptic transmission in the neural network. So that a temporally regulated expression of a transgene *in vivo* could be achieved, the tetracycline-controlled reverse transactivator (rtTA) system was developed (Gossen et al., 1995). In this system, rtTA, when bound to tetracycline or its derivative

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Correspondence should be addressed to Mutsuya Yamamoto, Mitsubishi Pharma Corporation, Discovery Technology Laboratory, 1000, Kamoshida-cho, Aoba-ku, Yokohama, 227-0033, Japan. E-mail: Yamamoto.Mutsuya@mf.m-pharma.co.jp.

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Original Article

# Ataxia and male sterility (AMS) mouse. A new genetic variant exhibiting degeneration and loss of cerebellar Purkinje cells and spermatic cells

**Takayuki Harada,[1] Liliam L. Pineda,[1] Akinobu Nakano,[1] Koji Omura,[1] Li Zhou,[1] Masaaki Iijima,[1] Yoji Yamasaki[1,2] and Minesuke Yokoyama[3]**

[1]*The Second Department of Pathology and* [2]*Department of Urology, Shimane Medical University, Izumo and* [3]*The Mitsubishi Kagaku Institute of Life Sciences, Machida, Tokyo, Japan*

**We describe a novel genetic variant mouse that exhibited ataxia and male sterility , named the AMS mouse. It arose in autoimmune-prone MRL/*lpr* strain and putative *ams* mutation showed an autosomal recessive inheritance pattern. Clinical symptoms were first discernible at approximately 21 days of age and consisting of subtle sway of the trunk followed by failure to maintain still posture and appearance of abnormal walk, but no further worsening was noted with advancement of age. The abnormal motor coordination was ascribed to almost complete loss of Purkinje cells of the cerebellum. The cell loss in the Purkinje cell layer began before onset of ataxia and rapidly progressed towards near-complete loss by 6 weeks of age. Another symptom was male sterility due to severe oligozoospermia associated with cellular degeneration during spermatic differentiation in the seminiferous tubules. Thus, the effects of the genetic variation were apparent in two different organs after the development of their basic histological structures, and degeneration and loss of particular cell types in these two tissues produced overt clinical symptoms. Genetic pleiotropism, provided that the nature of genetic variation is of a single gene mutation, is discussed.**

**Key words:** abnormal spermatogenesis, ataxia, cerebellum, genetic variant, male sterility, MRL/*lpr* mouse, Purkinje cell loss

Many mouse and rat genetic variants have been described and many of them are used as animal models of human diseases.[1] This depends upon the presence of various inbred strains with enormous knowledge of their genetic characteristics. The MRL/*lpr* strain, of which a new genetic variant is to be described in this report, is one of the frequently used mouse models for autoimmune diseases. Analysis of various types of autoimmune diseases such as glomerulonephritis, arteritis, arthritis or sialoadenitis have allowed the identification of autoimmune mechanisms of tissue damage. Moreover, cloning of the *lpr* gene revealed that it encodes a key molecule, Fas antigen or CD95 molecule, involved in the signal transduction system of apoptosis that transmits extracellular signals for apoptosis as a receptor of Fas ligand.[2] This has led to a unifying concept that a defect in programmed cell death system in lymphocyte causes immune dysregulation, consequently resulting in production of autoantibody and autoreactive T cells.[3] Therefore, studies of animal models provide important insights of the pathogenesis of human diseases, although the whole process of the disease is not necessarily similar to that of humans. In addition, cloning of the responsible gene for the disease might open a new avenue of research for important biological processes in the cells.

Sick mice are not rare while maintaining mice strains. When a disease is caused by an external agent or mice are too ill to die of the disease, the disease trait will not be inherited over the generation. If they survive a disease of genetic origin and are able to give offspring, mice manifesting diseases could be segregated as a genetic variant. Many neurological mutants exhibiting ataxia have been separated from their mother strains particularly because abnormal behavior can readily be recognized. In this report, we describe a novel genetic variation that spontaneously occurred in a MRL/*lpr* mouse. The new variant manifests ataxia caused by very rapid and almost complete loss of Purkinje cells at young age. In addition, male mice are sterile because of severe oligozoospermia caused by defective spermatic differentiation. Based on these clinical features, we named this genetic variant the ataxia and male sterility (AMS) mouse.

Correspondence: Takayuki Harada, MD, Department of Pathology, Shimane Medical University, Izumo 693-8501, Japan.
Email: tharada@shimane-med.ac.jp

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T.H. and L.L.P. contributed equally to the work.

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# Impaired arterial pressure regulation during exercise due to enhanced muscular vasodilatation in calponin knockout mice

Shizue Masuki*, Michiko Takeoka†, Shun'ichiro Taniguchi†, Minesuke Yokoyama‡, and Hiroshi Nose*

*Departments of *Sports Medical Sciences, and †Molecular Oncology, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Matsumoto 390–8621 and ‡Mouse Genome Technology Center, Mitsubishi Kagaku Institute of Life Sciences, Tokyo 194-8511, Japan*

Calponin is known to be an actin binding protein in smooth muscle, inhibiting actomyosin ATPase activity *in vitro*. We previously reported that α-adrenergic vasoconstriction in calponin knockout (KO) mice was reduced compared with that in wild-type C57BL/6J (WT) mice and, as a compensation, arterial baroreflex sensitivity in KO mice was enhanced at rest. In the present study, we assessed arterial pressure regulation in WT and KO mice during graded treadmill exercise at 5, 10, and 15 m $min^{-1}$. Mean arterial pressure (MAP) in KO mice fluctuated more than that in WT mice at every speed of exercise with two-fold higher variances ($P < 0.001$). The baroreflex sensitivity (ΔHR/ΔMAP) in WT mice ($n = 6$), determined from the heart rate response (ΔHR) to spontaneous change in MAP (ΔMAP), was $-5.1 \pm 0.6$ beats $min^{-1}$ $mmHg^{-1}$ (mean ± S.E.M.) at rest and remained unchanged at $-5.0 \pm 0.9$ beats $min^{-1}$ $mmHg^{-1}$ during exercise ($P < 0.01$), while that in KO mice ($n = 6$) was $-9.9 \pm 1.7$ beats $min^{-1}$ $mmHg^{-1}$ at rest, significantly higher than that in WT mice ($P < 0.001$), and was reduced to $-4.7 \pm 0.4$ beats $min^{-1}$ $mmHg^{-1}$ during exercise ($P < 0.01$), not significantly different from that in WT mice. In another experiment, we measured muscle blood flow (MBF) in the thigh by laser-Doppler flowmetry, electromyogram (EMG), and MAP during voluntary locomotion in KO ($n = 7$) and WT ($n = 7$) mice. Muscle vascular conductance, MBF/MAP, started to increase immediately after locomotion, judged from EMG, and reached 50 % of the maximum after the time of $2.3 \pm 0.2$ s in KO mice, shorter than $5.8 \pm 0.6$ s in WT mice ($P < 0.001$). Prior administration of α-adrenergic blockade (phentolamine) shortened the time in WT mice to that in KO mice ($P < 0.001$), but did not shorten the time in KO mice. Thus, impaired MAP regulation in KO mice during exercise was caused by a blunted muscle vascular α-adrenergic contractile response and by the attenuated HR response to spontaneous change in MAP due to reduced baroreflex sensitivity.

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**Corresponding author** H. Nose: Department of Sports Medical Sciences, Shinshu University Graduate School of Medicine, 3-1-1 Asahi Matsumoto 390–8621, Japan. Email: nosehir@sch.md.shinshu-u.ac.jp

Calponin, referred herein to basic calponin or calponin h1 has been reported to reduce unloaded isometric forces and to shorten velocity (Jaworowski *et al.* 1995; Obara *et al.* 1996; Matthew *et al.* 2000; Takahashi *et al.* 2000) by the inhibition of actomyosin ATPase activity in a reconstituted isolated filament system (Winder & Walsh, 1990). On the other hand, there have been several studies suggesting that calponin increases the contractile response to noradrenaline (norepinephrine (NE)) (Nigam *et al.* 1998) or phenylephrine (PE) (Parker *et al.* 1994; Menice *et al.* 1997; Je *et al.* 2001) by facilitating agonist-induced signal transduction in isolated vascular smooth muscle.

Masuki *et al.* (2003) recently reported in genetically calponin-deficient mice (knockout (KO) mice) that the increase in mean arterial pressure (MAP) after a bolus intra-arterial injection of PE was reduced to half that in the wild-type (WT) mice during rest. Despite this, they found that the level of MAP in the KO mice remained the same as that in the WT mice while that of heart rate (HR) was significantly lower. On the other hand, the variability of HR was two-fold higher in the KO mice than that in the WT mice while that of MAP was controlled within the same range as in the WT mice, suggesting that the baroreflex control of HR compensates well for the impaired peripheral α-adrenergic vasoconstriction. Indeed, the arterial baroreflex sensitivity in the KO mice was two-fold higher than that in the WT mice as a compensatory adaptation (Masuki *et al.* 2003). These results suggest that in the KO mice the fast or dynamic vascular response to sympathetic nerve activity was impaired whereas the slow or static response remained intact. Thus, calponin may play an important role in enhancing the dynamic vasoconstrictive response to sympathetic nerve activity during rest.

However, there has been no study to show the role of calponin in arterial pressure regulation during exercise.

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原著

# ガラス化法による卵巣凍結保存の検討

右島富士男[*1] 横山峯介[*2] 西島正博[*3]

1960年parrot DMによる卵巣凍結保存後の自家移植による産仔獲得の成功[1]以来，さまざまな卵巣凍結保存の成功例が報告[2)~7)]された。しかしこれらの技術は緩慢凍結法によるものでその操作は煩雑で膨大な労力と時間を必要とした。しかしガラス化法による胚凍結保存技術は生殖医療に革命的な進歩をもたらした。また近年実際的な卵巣凍結保存法としてガラス化法による卵巣凍結保存技術が求められていた。われわれは，ガラス化法による卵巣凍結を行い融解後同系統のマウスに移植し産仔の獲得に成功した。その実際について述べる。

## はじめに

卵巣凍結保存は放射線治療や化学療法などによる医原性卵巣機能不全や早発閉経を危惧する患者に，妊孕性を温存する技術として注目されている。いままでの多くの報告が組織凍結保存時に細胞外マトリックスに氷晶を形成させ可能な限り組織損傷を抑えかつ細胞内の氷晶形成を最大限抑制することを目的とした“緩慢凍結法”によるもので，膨大な労力と時間を必要とし，また高価なプログラムフリーザーも必要となることから普及にいたっていない。

一方，ガラス化法は氷晶形成を行わず，組織構造を維持できるため組織の凍結保存法としては緩慢法よりむしろ良好な方法であるとも考えられる。さらにその凍結保存法は簡便かつ有効な卵子，胚の凍結保存技術として不妊治療技術に革命をもたらしたことは周知の通りであり，現在では未受精卵および胚の凍結保存法としては一般的なものとなっている。しかし凍結保護剤が高濃度であるためその毒性は致命的となる場合があり，必然的に凍結保護剤の暴露時間が長くなる組織ではガラス化法による凍結保存は不可能であろうと考えられていた。さらに凍結-融解時に形成される氷晶形成は組織破壊および細胞損傷の原因となるため，ガラス化法による組織凍結保存は凍結-融解時に必要とされる熱量に限りがあることから凍結-融解可能な組織体積に限界があった。これらの理由によりガラス化法での卵巣凍結保存法の報告例は数えるほどしかなくgraafian follicleまでの発育の確認をもって成功と判断されていた。しかし卵巣の凍結保存が成功したかどうかを確認する唯一の方法は凍結卵巣に由来した産仔を獲得することであり，生殖工学的技術の進歩によりGreen fluorescence protein(GFP)-transgenic mouse[8)]が作出されrecipient由来の産仔かdonor由来の産仔か明確に判別可能となったことは卵巣移植実験を容易なものとした。

今回われわれは，凍結保護剤としてDAP213[9)]を使用し，10日例のGFP-transgenic mouseの卵巣を液体窒素中に凍結保存し

*1Fujio MIGISHIMA
北里大学医学部産婦人科
*2Minesuke YOKOYAMA
三菱化学生命科学研究所マウスゲノム工学センター
*3Masahiro NISHIJIMA
北里大学医学部産婦人科
〒228-8555 相模原市北里1-15-1

Development/Plasticity/Repair

# Generation of Reelin-Positive Marginal Zone Cells from the Caudomedial Wall of Telencephalic Vesicles

**Keiko Takiguchi-Hayashi,[1] Mariko Sekiguchi,[1] Shizuko Ashigaki,[1,3] Masako Takamatsu,[3] Hiroshi Hasegawa,[3] Rika Suzuki-Migishima,[2] Minesuke Yokoyama,[2] Shigetada Nakanishi,[4,5] and Yasuto Tanabe[1,3]**

[1]Translational Research Department, Molecular Bio-Medicine Unit, [2]Mouse Genome Technology Center, and Precursory Research for Embryonic Science and Technology, Japan Science and Technology, Mitsubishi Kagaku Institute of Life Sciences, Machida, Tokyo, 194-8511, [4]Department of Biological Sciences, Faculty of Medicine, and [5]Department of Molecular and System Biology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan

An early and fundamental step of the laminar organization of developing neocortex is controlled by the developmental programs that critically depend on the activities of reelin-positive cells in the marginal zone. However, the ontogeny of reelin-positive cells remained elusive. To gain insights into the spatial and temporal regulation of reelin-positive marginal zone cell development, we used a transgenic mouse line in which we defined the green fluorescent protein (GFP) transgene as a novel reliable molecular marker of reelin-positive marginal zone cells from the early stages of their development. We further used *exo utero* electroporation-mediated gene transfer that allows us to mark progenitor cells and monitor the descendants in the telencephalon *in vivo*. We show here the generation of reelin-positive marginal zone cells from the caudomedial wall of telencephalic vesicles, including the cortical hem, where the prominent expression of GFP is initially detected. These neurons tangentially migrate at the cortical marginal zone and are distributed throughout the entire neocortex in a caudomedial-high to rostrolateral-low gradient during the dynamic developmental period of corticogenesis. Therefore, our findings on reelin-positive marginal zone cells, in addition to the cortical interneurons, add to the emerging view that the neocortex consists of neuronal subtypes that originate from a focal source extrinsic to the neocortex, migrate tangentially into the neocortex, and thereby underlie neural organization of the neocortex.

*Key words:* Cajal–Retzius cells; neocortex; cerebral cortex; laminar organization; tangential cell migration; caudomedial–rostrolateral axis

## Introduction

In the developing neocortex, the generation of distinct classes of cortical neurons is controlled by the hierarchical series of cellular interactions that culminate in the areal and laminar organization of distinct cortical areas. After the final cell mitosis, postmitotic cortical neurons migrate in a radial direction from the ventricular zone (VZ), form a cortical plate (CP), and subdivide the preexisting preplate into the superficial marginal zone (MZ) and the deeper subplate (Gupta et al., 2002; Nadarajah and Parnavelas, 2002). reelin-positive cells (Meyer et al., 1999) are one of the major neuronal subtypes located in the MZ and are distributed evenly throughout the entire cerebral cortex in a wide variety of mammalian species (Marín-Padilla, 1998; Supèr et al., 1998). The analyses of *reeler* mutant mice (Ogawa et al., 1995; Meyer et al., 1999; Rice and Curran, 2001; Tissir and Goffinet, 2003), together with studies in human congenital lissencephaly (Gleeson and Walsh, 2000), led to the suggestion that the neocortical neural organization depends on the activities of reelin-positive cells that govern the laminar arrangements of CP cells from the early stages of development. Studies on how reelin-positive cell generation is spatially and temporally controlled in the telencephalon might, therefore, provide a solid cellular framework for understanding neural organization during development of the neocortex.

Despite many advances in the characterization of the way CP neurons are generated and organized into distinct layers (Gupta et al., 2002; Nadarajah and Parnavelas, 2002), the ontogeny of reelin-positive cells has remained elusive. The concept of a dual origin of the cerebral cortex, together with the classical concept from the time of Ramón y Cajal, provided a prevailing view that both reelin-positive cells and subplate are generated as preplate from the underlying VZ before the formation of CP (Marín-Padilla, 1978, 1998). Consistent with these ideas, several studies suggest the local generation of reelin-positive cells within the neocortical area (Meyer et al., 2000; Hevner et al., 2003). In contrast, several lines of evidence suggest that neocortical reelin-positive cells may originate from locations extrinsic to the neocortex (Meyer et al., 1998, 2002; Lavdas et al., 1999; Shinozaki et al., 2002). Together, these studies raised the possibility that neo-

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Correspondence should be addressed to Dr. Yasuto Tanabe, 11 Minami-Ooya, Machida, Tokyo, 194-8511, Japan. E-mail: ytanabe@libra.ls.m-kagaku.co.jp.

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# Cardiac abnormalities cause early lethality of *jumonji* mutant mice

Miho Takahashi[a,b], Mizuyo Kojima[a], Kuniko Nakajima[a], Rika Suzuki-Migishima[a], Yoshiko Motegi[a], Minesuke Yokoyama[a,1], Takashi Takeuchi[a,b,*]

[a] *Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida, Tokyo 194-8511, Japan*
[b] *Graduate School of Environment and Information Sciences, Yokohama National University, 79-1 Tokiwadai, Hodogaya, Yokohama 240-8501, Japan*

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**Abstract**

*jumonji* (*jmj*) mutant mice, obtained by a gene trap strategy, showed several morphological abnormalities including neural tube and cardiac defects, and died in utero around embryonic day 11.5 (E11.5). It is unknown what causes the embryonic lethality. Here, we demonstrate that exogenous expression of *jmj* gene in the heart of *jmj* mutant mice rescued the morphological phenotypes in the heart, and these embryos survived until E13.5. These results suggest that there are at least two lethal periods in *jmj* mutant mice, and that cardiac abnormalities may cause the earlier lethality. In addition, the rescue of the cardiac abnormalities by the *jmj* transgene provided solid evidence that the cardiac abnormalities resulted from mutation of the *jmj* gene.


*Keywords:* *jumonji*; Transcriptional repressor; Cardiac morphogenesis; Cardiac myocyte; Embryonic lethality; Gene trap; Transgenic mouse; Mutant mouse

Embryonic lethality is one of major phenotypes in mutant animals and the embryos die of various defects, for example, hematopoietic or cardiac defects.

*jumonji* (*jmj*) homozygous mice, originally obtained by a mouse gene trap strategy, show embryonic lethality [1]. These mice also exhibit various abnormalities such as neural tube, cardiac and hematopoietic defects [1–6]. These phenotypes are dependent largely on the mouse genetic backgrounds. Mutant mice with a C3H/He background exhibit neural tube defects, abnormal morphology of the right ventricle, and hyperproliferation of trabecular cardiac myocytes, and die around E11.5 [3]. Our recent studies on hyperproliferation of trabecular cardiac myocytes showed that this abnormality resulted from enhanced expression of cyclin D1 [7], which is one of the key components of the cell cycle machinery. On the other hand, mutant mice with a BALB/c background exhibit hematopoietic defects but not neural tube defects or hyperproliferation of trabecular cardiac myocytes, and die around E15.5 [2,4,6].

Molecular analysis has indicated that trap vectors were introduced into one intron of the gene designated as *jmj* and disrupted the function of this gene [1,8]. The *jmj* gene encodes a protein that is a member of the jumonji family [9] and the AT-rich interaction domain (ARID) family [10]. The ARID is a DNA-binding domain and several members of this family are known to be transcriptional factors and are involved in a variety of biological processes [10]. Recently, we and Kim et al. showed that Jmj protein is a transcriptional repressor [7,11]. We also showed that Jmj downregulates cardiac myocyte proliferation by repressing *cyclin D1* transcription [7].

Despite these results, the reasons for the embryonic lethality are still unknown. In the case of mutant mice with a C3H/He background, embryos are likely to die

* Corresponding author. Fax: +81 42 724 6267.
*E-mail address:* take@libra.ls.m-kagaku.co.jp (T. Takeuchi).
[1] Present address. Department of Comparative and Experimental Medicine, Niigata Brain Research Institute, 1-757 Asahi-machi-dori Niigata, Niigata, 951-8585 Japan.

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# Naso-maxillary deformity due to frontonasal expression of human transthyretin gene in transgenic mice

Hiromitsu Noguchi[1,5], Tadashi Kaname[1], Tomohisa Sekimoto[1], Kei Senba[1], Yasushi Nagata[1], Masatake Araki[2], Makoto Abe[3], Naomi Nakagata[4], Tomomichi Ono[5], Ken-ichi Yamamura[1] and Kimi Araki[1,*]

[1]*Division of Developmental Genetics, Institute of Molecular Embryology and Genetics, Kumamoto University, 4-24-1 Kuhonji, Kumamoto 862-0976, Japan*

[2]*Gene Technology Center, Honjo 2-2-1, Kumamoto 860-0811, Japan*

[3]*Department of Oral Anatomy and Developmental Biology, Osaka University Faculty of Dentistry, 1-8, Yamadaoka, Suita, Osaka 565-0871, Japan*

[4]*Division of Reproductive Engineering, Center for Animal Resources and Development, Honjo 2-2-1, Kumamoto 860-0811, Japan*

[5]*Department of Dermatology, Kumamoto University School of Medicine, Honjyo 1-1-1, Kumamoto 860-8556, Japan*

## Abstract

*Background*: **Retinoic acid, a metabolic product of retinol, is essential for craniofacial morphogenesis. Transthyretin (TTR) is a plasma protein delivering retinol to tissues. We produced several transgenic mouse lines using the human mutant *TTR* ($hTTR^{Met30}$) gene to establish a mouse model of familial amyloidotic polyneuropathy. One of the lines showed an autosomal dominant inheritance of naso-maxillary deformity termed *Nax*.**

*Results*: **The *Nax* malformation was characterized by a hypoplastic developmental defect of the frontonasal region. Homozygous mice with higher transgene expressions showed more severe phenotypes, but a subline, in which the copy number and expression of the transgene was reduced, showed a normal phenotype, indicating that the $hTTR^{Met30}$ expression caused the malformation. *Nax* mice began to express the $hTTR^{Met30}$ gene in the nasal placode from embryonic day 10.5 (E10.5), which was 2 days earlier than in the other transgenic lines with a normal phenotype. Excessive cell death was observed in the nasal placode of the E10.5 *Nax* embryos. In addition, the forced expression of $hTTR^{Met30}$ in the nasal placode of transgenic mice resulted in similar phenotypes.**

*Conclusion*: **The expression of the $hTTR^{Met30}$ gene in the nasal placode at E10.5 induced apoptotic cell death, leading to hypoplastic deformity in the frontonasal region.**

## Introduction

Craniofacial morphogenesis is a complex process that requires the coordinated growth of small buds of tissue as facial prominences. The cephalic neural crest, which has been shown to be the major cell line populating the face (Noden 1991), and retinoid signalling are known to play important roles during the development of the frontonasal region. In humans, both excess ingestion and deprivation of vitamin A (retinol) during pregnancy induce an abnormal development of foetuses (Rothman *et al.* 1995; Shenai *et al.* 1985). In experimental models using rodents, exposure to retinoic acid (RA), the major bioactive metabolic product of vitamin A, resulted in craniofacial defects. In mice, RA exposure on embryonic days (E) 8.25 or 10 severely affected the frontonasal neural crest-derived tissues, but on E9, exposure completely spared the tissues (Grant *et al.* 1997). In rats, temporally regulated RA depletion between E11.5 and E13.5 resulted in specific malformations of the face, neural crest, eyes, heart and nervous system (Dickman *et al.* 1997). Recently, it has been demonstrated that local RA signalling regulates facial morphogenesis (Schneider *et al.* 2001), and the precise control of RA concentration is involved in specifying

*Communicated by*: Shinichi Aizawa

**Correspondence*: E-mail: arakimi@gpo.kumamoto-u.ac.jp

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# *Effect of the Intestinal Flora on Amyloid Deposition in a Transgenic Mouse Model of Familial Amyloidotic Polyneuropathy*

Hiromitsu NOGUCHI[1, 6], Mika OHTA[1], Shoji WAKASUGI[6], Kazuhiro NOGUCHI[2], Naoko NAKAMURA[2], Osamu NAKAMURA[5], Kazuhisa MIYAKAWA[5], Motohiro TAKEYA[5], Misao SUZUKI[3], Naomi NAKAGATA[4], Toru URANO[2],Tomomichi ONO[6], and Ken-ichi YAMAMURA[1]

*[1]Division of Developmental Genetics, Institute of Molecular Embryology and Genetics, Kumamoto University, 4–24–1 Kuhonji, Kumamoto 862-0976, [2]Division of Microbiology and Genetics, [3]Division of Transgenic Technology, [4]Division of Reproductive Engineering, Center for Animal Resources and Development, Honjo 2–2–1, Kumamoto 862-0811, [5]The Second Department of Pathology, and [6]Department of Dermatology, Kumamoto University School of Medicine, Honjo 1–1–1, Kumamoto 860-8556, Japan*

**Abstract:** *Familial amyloidotic polyneuropathy (FAP) is a hereditary disease characterized by the systemic accumulation of amyloid fibrils. A mutant transthyretin (TTR) gene is mainly responsible for the disease. However, the variable age of onset and low penetrance might be due to environmental factors, one of which is the intestinal flora. Three types of intestinal flora were introduced into a transgenic (Tg) mouse FAP model, 6.0-hMet30. The CV1 and CV2 group transgenic mice were transferred with the intestinal flora from two different mouse facilities housed under conventional conditions, and the SPF group transgenic mice were kept under specific pathogen free conditions in our facility. All the mice were maintained under controlled temperature, humidity and bacterial conditions. Over a period of 28 months, amyloid was not deposited in the SPF and CV1 groups. In contrast, amyloid was deposited in the esophagus and small intestine of two of the three CV2 mice at 18 months. Many neutrophils infiltrated the lesions. The numbers of tissue neutrophils were higher in the CV2 group than in the SPF and CV1 groups at 18 months. The CV2 flora included fewer gram-positive anaerobic cocci as well as higher proportions of yeasts, staphylococci and enterobacteriaceae compared with the SPF and CV1 flora. These findings suggest that the intestinal flora plays an important role in amyloid deposition.*

**Key words:** *Familial amyloidotic polyneuropathy, amyloid, intestinal flora*

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*Address corresponding: K. Yamamura, Division of Developmental Genetics, Institute of Molecular Embryology and Genetics, Kumamoto University, 4–24–1 Kuhonji, Kumamoto 862-0976, Japan*

# Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells

Yuichi Oike, Yasuhiro Ito, Koichi Hamada, Xiu-Qin Zhang, Keishi Miyata, Fumio Arai, Tomohisa Inada, Kimi Araki, Naomi Nakagata, Motohiro Takeya, Yaz Y. Kisanuki, Masashi Yanagisawa, Nicholas W. Gale, and Toshio Suda

**Although the cellular and molecular mechanisms governing angiogenesis are only beginning to be understood, signaling through endothelial-restricted receptors, particularly receptor tyrosine kinases, has been shown to play a pivotal role in these events. Recent reports show that EphB receptor tyrosine kinases and their transmembrane-type ephrin-B2 ligands play essential roles in the embryonic vasculature. These studies suggest that cell-to-cell repellent effects due to bidirectional EphB/ephrin-B2 signaling may be crucial for vascular development, similar to the mechanism described for neuronal development. To test this hypothesis, we disrupted the precise expression pattern of EphB/ephrin-B2 in vivo by generating transgenic (CAGp-ephrin-B2 Tg) mice that express ephrin-B2 under the control of a ubiquitous and constitutive promoter, CMV enhancer-β-actin promoter-β-globin splicing acceptor (CAG). These mice displayed an abnormal segmental arrangement of intersomitic vessels, while such anomalies were not observed in Tie-2p-ephrin-B2 Tg mice in which ephrin-B2 was overexpressed in only vascular endothelial cells (ECs). This finding suggests that non-ECs expressing ephrin-B2 alter the migration of ECs expressing EphB receptors into the intersomitic region where ephrin-B2 expression is normally absent. CAGp-ephrin-B2 Tg mice show sudden death at neonatal stages from aortic dissecting aneurysms due to defective recruitment of vascular smooth muscle cells to the ascending aorta. EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells plays an essential role in vasculogenesis, angiogenesis, and vessel maturation. (Blood. 2002;100:1326-1333)**



## Introduction

Angiogenesis, the formation of new vessels from the pre-existing primary plexus, occurs through several processes: vascular sprouting, branching and pruning, and the differential growth of blood vessels to form more mature vascular networks.[1] These vascular events are required for embryogenesis and are also involved in several pathophysiologic conditions such as neovascularization in tumorigenesis, inflammatory disease, and ischemic disease in adulthood. Although the cellular and molecular mechanisms governing angiogenesis are only beginning to be understood, signaling through endothelial-restricted receptors, particularly receptor tyrosine kinases (RTKs),[2,3] plays a pivotal role in these events. Three subfamilies out of at least 14 RTK subfamilies, vascular endothelial growth factor (VEGF) receptors (VEGFRs), Tie receptors, and Eph receptors are primarily expressed in the endothelial lining of blood vessels from embryonic stages to adulthood.[3] VEGFs,[4-7] ligands for VEGFRs, angiopoietins (Angs),[8] and ligands for Tie-2 receptor[9-11] play an essential role in vasculogenesis and angiogenesis. In contrast to VEGFs and Angs, ephrins, which were initially characterized by their roles in axon guidance and neuronal patterning,[12,13] cannot act as soluble mediators but rather must be membrane-bound to activate their receptors. This observation indicates that Eph/ephrin signaling is mediated by cell-to-cell interaction, resulting in repulsive or attractive signaling.

The recent observation of vascular defects in ephrin-B2 and EphB4 knockout mice strongly suggests that the interaction between the ephrin-B2 ligand and its cognate EphB4 receptor defines the boundaries of arterial-venous domains.[14,15] Subsequent work demonstrating expression of ephrin-B2 and its cognate EphB receptors in mesenchymal cells adjacent to vascular endothelial cells (ECs) suggests an EphB/ephrin-B2 interaction at endothelial-mesenchymal contact zones.[16] Eph receptors and ephrin ligands are divided into 2 broad subclasses, A and B, based on structural homologies and binding specificities. High redundancy exists within each subclass in terms of receptor/ligand binding. Ephrin-B ligands are transmembrane proteins that preferentially bind to receptors of the EphB subclass.[3,13,17] It is notable that ephrin-B ligands not only activate their respective receptors but also are in turn activated upon engaging their receptors, as judged by tyrosine phosphorylation of the ephrin-B cytoplasmic domain.[18,19] These findings and more recent reports[20] indicate that ephrins may provide key bidirectional cues in an obligate cell-to-cell, contact-dependent fashion in the vascular system. The mechanism of

From the Department of Cell Differentiation, the Department of Developmental Genetics, the Institute of Molecular Embryology and Genetics, and the Animal Resource Center, Kumamoto University, Kumamoto, Japan; the Second Department of Pathology, Kumamoto University School of Medicine, Kumamoto, Japan; the Howard Hughes Medical Institute and Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, TX; and Regeneron Pharmaceuticals, Tarrytown, NY.

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**Reprints:** Toshio Suda, The Sakaguchi Laboratory, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan; e-mail: sudato@sc.itc.keio.ac.jp.

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# SH2-B Is Required for Both Male and Female Reproduction

Satoshi Ohtsuka,[1] Satoshi Takaki,[2] Masanori Iseki,[2] Kanta Miyoshi,[3] Naomi Nakagata,[4] Yuki Kataoka,[5] Nobuaki Yoshida,[5] Kiyoshi Takatsu,[2] and Akihiko Yoshimura[1,3]*

*Division of Molecular Genetics, Institute of Life Science, Kurume University, Kurume 839-0861,[1] Division of Immunology, Department of Microbiology and Immunology,[2] and Laboratory of Gene Expression and Regulation, Center for Experimental Medicine,[5] Institute of Medical Science, The University of Tokyo, Minato-ku 108-8639, Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka 812-8582,[3] and Division of Reproductive Engineering, Center for Animal Resources & Development, Kumamoto University, Kumamoto 860-0811,[4] Japan*

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**Many growth factors and hormones modulate the reproductive status in mammals. Among these, insulin and insulin-like growth factor I (IGF-I) regulate the development of gonadal tissues. SH2-B has been shown to interact with insulin and IGF-I receptors, although the role of SH2-B in these signals has not been clarified. To investigate the role of SH2-B, we generated mice with a targeted disruption of the SH2-B gene. Both male and female SH2-B$^{-/-}$ mice showed slight retardation in growth and impaired fertility. Female knockout mice possess small, anovulatory ovaries with reduced numbers of follicles and male SH2-B$^{-/-}$ mice have small testes with a reduced number of sperm. SH2-B$^{-/-}$ cumulus cells do not respond to either follicle-stimulating hormone or IGF-I. These data suggest that SH2-B plays a critical role in the IGF-I-mediated reproductive pathway in mice.**

Cytokine and growth factor receptors trigger multiple signaling cascades that regulate cell growth and differentiation. Many growth factor receptors have a protein tyrosine kinase domain in their cytoplasmic region (receptor tyrosine kinase [RTK]). In contrast, cytokine receptors, such as those for interleukins, interferons, and colony-stimulating factors, do not have an intrinsic kinase domain but instead constitutively associate with Janus tyrosine kinases (JAKs). Binding of growth factors and cytokines to their cognate receptors induces the homo- and heterodimerization of the receptors, an event which positions the kinase domains close to each other. This leads to transphosphorylation and thereby activation of RTKs and receptor-associated JAKs. The activated kinases further phosphorylate other tyrosine residues in the cytoplasmic region, where various signaling molecules containing Src homology 2 (SH2) or phosphotyrosine binding domains are recruited. As a consequence, these recruited adaptor molecules contribute to specification and amplification of signaling downstream of the receptors. Lnk family proteins, including Lnk, APS, and SH2-B, are some of these adaptor molecules (39).

SH2-B was originally identified by using a yeast trihybrid system as a protein associated with an immunoreceptor tyrosine-based activation motif in the high-affinity immunoglobulin E (IgE) receptor Fcε-RI (29). SH2-B contains a proline-rich domain, a Pleckstrin homology (PH) domain, and an SH2 domain. APS was initially cloned from a B-cell cDNA library using a yeast two-hybrid screening with the c-Kit RTK as bait, and it was shown to associate with a B-cell receptor (44). Lnk was cloned from a rat lymph node cDNA library and was shown to participate in T-cell signaling (20, 38, 39). In Lnk$^{-/-}$ mice, T-cell development was unaffected, but pre-B and immature B cells accumulated in the spleen and in the bone marrow, thereby indicating that the Lnk protein negatively regulates the production of pro-B cells and c-Kit (39, 40).

Recently, SH2-B was reported to mediate signaling through many cytokine and growth factor receptors, including growth hormone (GH), insulin, insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF), and nerve growth factor (NGF) receptors (23, 31–33, 36, 37, 47). SH2-B has been shown to mediate mitogenic signals as well as ERK activation through these receptors (44, 45). A variant form of SH2-B, SH2-Bβ, was reported to be a substrate of the tyrosine kinase JAK2 and to potentiate JAK2 kinase activity (34, 35). However, these studies were performed using an in vitro cultured cell system, and the conclusions were obtained from the overexpression of wild-type or domain negative forms. To clarify the physiological role of SH2-B adaptor molecules, we used gene targeting to acquire mice lacking the SH2-B gene. SH2-B$^{-/-}$ mice displayed normal development of lymphoid organs but decreased body weight and developmental defects in gonadal organs similar to the phenotype seen in mice with IGF-I or follicle-stimulating hormone receptor (FSH-R) deficiencies (24, 26). We propose that while SH2-B is dispensable for JAK2 activation, it does play an important role in the IGF-I pathway that up-regulates FSH-R levels in vivo.

## MATERIALS AND METHODS

**Generation of SH2-B$^{-/-}$ mice.** Genomic clones of the SH2-B locus, including all exons, were isolated from a 129sv mouse strain genomic library (Stratagene). The targeting vector was constructed by replacing the second through the eighth exons of the SH2-B gene with a PGK-NEO cassette, preserving 8.0-kb (left arm) and 3.8-kb (right arm) flanks of homologous sequences (see Fig. 1). The diphtheria toxin A gene was inserted for negative selection. Homologous recombination in murine embryonic stem cells was performed as described previously (19) and was confirmed by Southern blot analysis (probes are shown below in Fig.

* Corresponding author. Mailing address: Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-6822. Fax: 81-92-642-6825. E-mail: yakihiko@bioreg.kyushu-u.ac.jp.

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—Note—

# *Experimental Evaluation of Cross-contamination between Cryotubes Containing Mouse 2-cell Embryos and Murine Pathogens in Liquid Nitrogen Tanks*

Shigeru KYUWA[1], Takaki NISHIKAWA[2, 3], Takehito KANEKO[2], Tatsuyuki NAKASHIMA[2, 4], Kayo KAWANO[2, 5], Naoko NAKAMURA[2], Kazuhiro NOGUCHI[2], Toru URANO[2], Toshio ITOH[6], and Naomi NAKAGATA[2]

*[1]Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113-8657, [2]Center for Animal Resources and Development, Kumamoto University, Kumamoto, Kumamoto 860-0811, [3]Chemicals Evaluation and Research Institute, Japan, Hita Laboratory, 3–822 Ishiimachi Hita, Ooita 877-0061, [4]Ark Resource, 383–2 Nakaharacho Kumamoto, Kumamoto 861-5271, [5]Panapharm Laboratories, 1285 Kurisakimachi Uto, Kumamoto 869-0425 and [6]Central Institute for Experimental Animals, 1430 Nogawa, Miyamae-ku Kawasaki, Kanagawa 216-0001, Japan*

**Abstract:** *It has been suspected that embryos stored in liquid nitrogen tanks may become contaminated with murine pathogens, if some pathogens had been introduced to the tanks accidentally. To examine this, we stored tubes containing embryos with tubes containing mouse hepatitis virus (MHV) or Pasteurella pneumotropica in liquid nitrogen tanks and examined whether progeny mice derived from the embryos were contaminated with the pathogens or not. After storing for 6 months or 1 year the frozen embryos were thawed and implanted into the oviducts of pseudopregnant female mice, and the mice were bred in vinyl isolators. We could not detect serum antibodies to MHV and isolate Pasteurella pneumotropica in the progeny mice, suggesting that cross-contamination between tubes in a liquid nitrogen tank scarcely occurs.*

**Key words:** *contamination, embryo, liquid nitrogen tank*

The number of transgenic and gene-knockout mice has been increasing, because these genetically modified animals are believed to be not only an effectual tool to figure out the role of a gene of interest *in vivo*, but also beneficial in research on the cause and treatment of human diseases [2]. Breeding of mice is costly and laborious, and needs a lot of space, and in order to save money, labor and space, it is practical to freeze mouse embryos in liquid nitrogen. Actually, approximately two hundred thousand mouse embryos have been stored in liquid nitrogen tanks in the Center for Animal Resources and Development (CARD), Kumamoto University, a mouse embryo bank in Japan. In addition to CARD, several mouse embryo banks have recently been established and the number of frozen embryos in the world has been increasing.

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*Address corresponding: S. Kyuwa, Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

# Angiopoietin-related growth factor (AGF) promotes epidermal proliferation, remodeling, and regeneration

Yuichi Oike*†, Kunio Yasunaga‡, Yasuhiro Ito*, Shun-ichiro Matsumoto‡§, Hiromitsu Maekawa*, Tohru Morisada*, Fumio Arai*, Naomi Nakagata¶, Motohiro Takeya||, Yasuhiko Masuho§, and Toshio Suda*†

*Department of Cell Differentiation, The Sakaguchi Laboratory, Keio University School of Medicine, Tokyo 160-8582, Japan; ‡Molecular Medicine Laboratories, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba 305-8585, Japan; §Helix Research Institute, Inc., Kisarazu 292-0812, Japan; and ¶Center for Animal Resources and Development and ||Department of Pathology, Kumamoto University, Kumamoto 860-0811, Japan

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**We report here the identification of an angiopoietin-related growth factor (AGF). To examine the biological function of AGF *in vivo*, we created transgenic mice expressing AGF in epidermal keratinocytes (K14-AGF). K14-AGF mice exhibited swollen and reddish ears, nose and eyelids. Histological analyses of K14-AGF mice revealed significantly thickened epidermis and a marked increase in proliferating epidermal cells as well as vascular cells in the skin compared with nontransgenic controls. In addition, we found rapid wound closure in the healing process and an unusual closure of holes punched in the ears of K14-AGF mice. Furthermore, we observed that AGF is expressed in platelets and mast cells, and detected at wounded skin, whereas there was no expression of AGF detected in normal skin tissues, suggesting that AGF derived from these infiltrated cells affects epidermal proliferation and thereby plays a role in the wound healing process. These findings demonstrate that biological functions of AGF in epidermal keratinocytes could lead to novel therapeutic strategies for wound care and epidermal regenerative medicine.**

Skin tissues, especially epidermis, are a barrier against the environment, which becomes accessible in wounds and various infections. An important goal in wound management is to achieve rapid wound closure. Analysis of gene activation in skin tissues shows that transforming growth factor-α (TGF-α) (1) produced by keratinocytes and keratinocyte growth factor (KGF) (2, 3) made by dermal fibroblasts are both powerful growth factors for epidermal keratinocytes, indicating an important role for the interaction between dermis–epidermis in skin development. Although these two growth factors play critical roles in wound healing, gene inactivation studies show that these factors are not essential for epidermal growth or regeneration and suggest that regulation of epidermal growth is more complex than has been previously appreciated (4, 5).

Angiopoietin-1 (Ang-1) (6) is a ligand for the receptor tyrosine kinase TIE2 (7, 8), which contributes to signaling in angiogenesis (9). Ang-1 is characterized structurally by two domains, a coiled-coil domain and a fibrinogen-like domain (10). Recently, members of the angiopoietins (Angs) family have been identified by a domain homology-based molecular cloning strategy. One of these proteins, angiopoietin-related protein 2 (ARP2), was also reported as an angiogenic factor (11). Several recent reports demonstrate that angiopoietin-related proteins (ARPs) show pleiotropic effects not only on vascular cells but on cells of other lineages. For example, it has been shown that ANGPTL3 (12, 13) and FIAF/PGAR/ARP4 (14) may play central roles in lipid/adipocyte metabolism as well as in angiogenesis. Here we identify, by screening EST databases, a previously undescribed angiopoietin-related growth factor (AGF), which is abundantly expressed in hepatocytes. To determine whether AGF promotes *in vivo* angiogenesis as does Ang-1, we created transgenic (TG) mice overexpressing mouse AGF in epidermal basal keratinocytes of skin (K14-AGF) and investigated vascularization of their dermal layer. K14-AGF mice show not only increased numbers of microvessels in the skin but thickened epidermal layers, resulting in rapid wound closure in the healing process and unusually rapid closure of holes punched in the ears of K14-AGF mice. Here we focus on the role of AGF in skin and show that AGF promotes the proliferation of epidermal keratinocytes and its biological functions could lead to novel therapeutic strategies for wound care and epidermal regenerative medicine.

## Materials and Methods

**Generation of TG Mice.** The pK14-AGF-pA plasmid was generated by inserting the coding region of mouse AGF cDNA into the pK14-pA plasmid (15). We subsequently generated K14-AGF mice according to standard methods (16). We identified transgenic offspring by PCR of tail genomic DNA using forward (5′-GCTCCTGGGCAACGTGCTGG-3′) and reverse (5′-CTGCTGTCTCAAGCTCTGC-3′) primers. Three independent K14-AGF TG lines were backcrossed with wild-type BALB/c mice (purchased from SLC, Shizuoka, Japan). Mice were housed in environmentally controlled rooms of the Laboratory Animal Research Center under the guidelines of Keio University for animal and recombinant DNA experiments.

**Preparation of cDNA from Hematopoietic Cells and RT-PCR Analysis.** A cell suspension from femur bone marrow of C57BL/6 mice (SLC) was prepared. For preparation of bone marrow-derived mast cells (BMMCs), total bone marrow cells were cultured as described elsewhere (17). For purification of various hematopoietic cells, total bone marrow cells were analyzed and sorted by FACSVantage (BD Biosciences, Palo Alto, CA). The mAbs used in immunofluorescence staining and procedures for flow cytometry were as described (18). Procedures for RT-PCR analysis were as described (19). For AGF the forward primer was 5′-CATGGAGGGATTGTGCAGAG-3′ and the reverse was 5′-AGCCGGGTCAACATAACAGC-3′ For GAPDH the forward primer was 5′-AATCCCATCACCATCTTCCA-3′ and the reverse was 5′-CCAGGGGTCTTACTCCTTG-3′. Each PCR cycle consisted of a 1-min denaturation at 94°C, 1 min of annealing at 64°C, and 1 min of extension at 72°C.

**Immunohistochemical Analysis.** To detect AGF protein in sections and by Western blotting, we prepared anti-mouse AGF polyclonal antibodies that were produced by immunizing rabbits with a synthetic peptide corresponding to amino acids 202–217 of mouse AGF (NTSRRLDQTPEHQREQ). Fixed sections from liver, back skin, and ears of K14-AGF mice and controls were stained with 1:500 diluted anti-mouse AGF antibody, anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake

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Abbreviations: AGF, angiopoietin-related growth factor; ARP, angiopoietin-related protein; Ang, angiopoietin; TG, transgenic; KGF, keratinocyte growth factor; BMMC, bone marrow-derived mast cell.

†To whom correspondence may be addressed. E-mail: oike@sc.itc.keio.ac.jp or sudato@sc.itc.keio.ac.jp.



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# Study of Low-Temperature (4°C) Transport of Mouse Two-Cell Embryos Enclosed in Oviducts

Eikichi Kamimura,[1,*] Tatsuyuki Nakashima,[2] Mami Ogawa,[2] Kazuo Ohwada VMD, PhD,[1] and Naomi Nakagata, PhD[2]

***Purpose:*** **We examined usefulness of a mouse embryo transportation system for low-temperature transport of oviducts containing mouse two-cell embryos.**

***Methods:*** **Oviducts containing two-cell mouse embryos were stored at 4°C for 36 h. After that, embryos were collected and cultured for 96 h in Potassium Simplex Optimized Medium (KSOM) medium and evaluated for their rate of development to hatched blastocysts. Embryos were transferred to recipients, and the rate of survival to live young was investigated. The oviducts were then transported from Yamagata to Kumamoto (distance of approx. 1,000 km). At the destination, embryos were implanted in recipient dams and were studied to evaluate their survival to live young.**

***Results:*** **After preservation for 36 h at 4°C, 68.3% of two-cell embryos developed to hatched blastocysts. As a result of transplanting 546 embryos into 25 recipients, 109 normal live young mice were obtained; the rate of development was 20.0%. Results of oviduct transport from Yamagata to Kumamoto indicated that 30.2% of transplanted embryos developed to live young.**

***Conclusion:*** **Low-temperature transport of two-cell embryos in oviducts is useful as a method of shipping mouse embryos between institutes.**

Recently, many laboratories have produced genetically modified mice, such as transgenic and knockout mice. Cryopreservation of embryos and spermatozoa is widely used to maintain these mice (3, 4, 7, 12, 13, 15, 16, 21, 26). Transport of mice between institutions is increasing concomitantly with use of genetically modified mice.

When sending and receiving mice, shipping mouse embryos is more advantageous than is shipping postnatal live mice for several reasons. There are reports of embryo transport by use of freezing techniques (2, 10, 18, 25), but in the case of cryopreserved transport, a freezing and thawing technique is required at donor and recipient laboratories; an exclusive nonliquid-type liquid nitrogen container is needed (10). Recently, several researchers have studied nonfreezing preservation techniques of embryos from mated female mice (5, 6, 22, 24, 27, 28). However, in such instances, the embryos must be collected from oviducts by flushing. Therefore, transferring oviducts containing embryos is more convenient because every researcher, even a beginner with no experience manipulating embryos, can do it easily. Thus, the objective of the study reported here was to investigate transport of oviducts containing two-cell embryos and to evaluate subsequent embryo viability by use of in vitro culture.

## Materials and Methods

**Animals.** Female mice (C57BL/6J, nine weeks old; CLEA Japan, Inc., Tokyo, Japan) were used as donors to obtain eggs (15 female mice per group). Injections of 5 IU of pregnant mare serum gonadotropin (PMSG; Serotropin, Teikoku Hormone Manufacturing Co., Tokyo, Japan) and 5 IU of human chorionic gonadotropin (hCG; Gonatropin, Teikoku Hormone Manufacturing Co.) were given 48 h apart to donor mice. After hCG administration, females were mated with 15-week-old male mice of the same strain and presence of a vaginal plug was confirmed the next morning. Donor mice were euthanized by cervical dislocation between 8 and 9 a.m. of the day after vaginal plug observation; oviducts were then separated from the ovaries and uterus and were used for this experiment.

Female mice (CD-1(ICR), 12 weeks old; Charles River Japan, Inc., Tokyo, Japan) were used as recipient mothers. The mice were mated with 16-week-old vasectomized male mice. Then, mice with confirmed formation of a vaginal plug the next day were used as pseudopregnant recipient mothers.

Donor and recipient mice were maintained in barrier system. Every month, microbial monitoring for the following agents was carried out: mouse hepatitis virus, Sendai virus, *Citrobacter rodentium*, *Clostridium piliformis*, *Corynebacterium kutscheri*, *Helicobacter bilis*, *Helicobacter hepaticus*, *Mycoplasma* spp., *Pasteurella pneumotropica*, *Salmonella* spp., *Aspiculuris tetraptera*, *Giardia muris*, *Syphacia* spp., *Spironucleus muris*, trichomonads, and ectoparasites. Mice had negative test results for all the aforementioned infective agents.

At Kumamoto University, animal rooms were kept at 22 ± 2°C and 50 to 70% humidity under a light:dark regimen of 12:12 h (lights on from 7 a.m. to 7 p.m.). Mice were housed in polyolefin resin cages (120 × 323 × 145 mm [length by depth by width]) with sterilized wood chip bedding. Mice were fed a standard diet (CE-2; CLEA Japan, Inc.), and water was supplied automatically after UV-light sterilization.

At Yamagata University, mice were housed at 22 ± 1°C and 40 to 60% humidity; lights were on from 6 a.m. to 6 p.m.). Five mice

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*[1]Laboratory Animal Center, Yamagata University School of Medicine, 2-2-2 Iidanishi, Yamagata 990-9585, Japan, and [2]Division of Reproductive Engineering, Center for Animal Resources & Development (CARD), Kumamoto University, 2-2-1 Honjo, Kumamoto, 860-0811, Japan.*
*[*]Corresponding author.*

# Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells

Keisuke Ito[1,2*], Atsushi Hirao[1*], Fumio Arai[1], Sahoko Matsuoka[1], Keiyo Takubo[1], Isao Hamaguchi[1], Kana Nomiyama[1], Kentaro Hosokawa[1], Kazuhiro Sakurada[3], Naomi Nakagata[4], Yasuo Ikeda[2], Tak W. Mak[5] & Toshio Suda[1]

[1]*Department of Cell Differentiation, The Sakaguchi Laboratory of Developmental Biology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan*
[2]*Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan*
[3]*BioFrontier Laboratories, Kyowa Hakko Kogyo Co. Ltd, Machida-shi, Tokyo 194-8533, Japan*
[4]*Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, Honjo, Kumamoto 860-0811 Japan*
[5]*Advanced Medical Discovery Institute, Ontario Cancer Institute, Departments of Medical Biophysics and Immunology, University of Toronto, 620 University Avenue, Suite 706, Toronto, Ontario M5G 2C1, Canada*

*These authors contributed equally to this work

**The 'ataxia telangiectasia mutated' (*Atm*) gene maintains genomic stability by activating a key cell-cycle checkpoint in response to DNA damage, telomeric instability or oxidative stress[1,2]. Mutational inactivation of the gene causes an autosomal recessive disorder, ataxia–telangiectasia, characterized by immunodeficiency, progressive cerebellar ataxia, oculocutaneous telangiectasia, defective spermatogenesis, premature ageing and a high incidence of lymphoma[3,4]. Here we show that ATM has an essential function in the reconstitutive capacity of haematopoietic stem cells (HSCs) but is not as important for the proliferation or differentiation of progenitors, in a telomere-independent manner. $Atm^{-/-}$ mice older than 24 weeks showed progressive bone marrow failure resulting from a defect in HSC function that was associated with elevated reactive oxygen species. Treatment with anti-oxidative agents restored the reconstitutive capacity of $Atm^{-/-}$ HSCs, resulting in the prevention of bone marrow failure. Activation of the $p16^{INK4a}$-retinoblastoma (Rb) gene product pathway in response to elevated reactive oxygen species led to the failure of $Atm^{-/-}$ HSCs. These results show that the self-renewal capacity of HSCs depends on ATM-mediated inhibition of oxidative stress.**

It has been shown that many factors are crucial in determining the fate of stem cells, yet molecular mechanisms governing the maintenance of the self-renewal capacity have not been well studied. Wong *et al.*[5] recently reported an interesting linkage between ATM and stem cell function. This group demonstrated that *Atm* deficiency, in combination with telomerase RNA component (Terc) deficiency, led to neural stem cell failure characterized by increased telomere erosion. In contrast, *Atm* deficiency alone did not affect neural stem cells. To investigate roles of ATM on self-renewal of stem cells in detail, we evaluated the effects of *Atm* deficiency on the haematopoietic system in this study.

Other than the previously reported defect in T cell development[6–9], $Atm^{-/-}$ mice showed normal numbers and subsets of haematopoietic cells in peripheral blood at 8 weeks of age. The loss of ATM did not show any defect in the differentiation or proliferation potential of progenitors at this age (Supplementary Fig. 1). The percentages of S/G2/M phases of the cell cycle in c-Kit$^+$Sca-1$^+$ Lineage$^-$ (KSL) cells were equal in wild-type (WT; 12.15 ± 1.60%) and $Atm^{-/-}$ mice (13.36 ± 2.81%). Next we investigated whether more primitive haematopoietic cells, HSCs, are affected in $Atm^{-/-}$ bone marrow (BM). The frequencies of the stem cell subsets CD34$^{-/low}$KSL, Thy1$^{low}$KSL and Tie2$^+$KSL (as defined by cell surface markers[10–12]) were significantly reduced in $Atm^{-/-}$ BM (Fig. 1a). The number of colony-forming cells derived from $Atm^{-/-}$ KSL cells after 6 weeks of culture on stromal cells was significantly decreased compared with that of the WT, although a short-term culture (less than 2 weeks) did not show a significant difference (Fig. 1b). To assess the repopulating ability of $Atm^{-/-}$ HSCs directly *in vivo*, we performed a competitive reconstitution assay in which BM mononuclear cells (MNCs) from $Atm^{-/-}$ mice competed with BM MNCs from congenic (Ly5.1/Ly5.2) WT mice to reconstitute the haematopoietic compartment of an irradiated recipient mouse (Ly5.1). Flow cytometric analysis of peripheral blood of the transplanted recipients taken at 4 weeks after transplantation revealed that $Atm^{-/-}$ BM MNCs were just as able as the competitor cells to contribute to haematopoietic reconstitution (Fig. 1c, left panel). Thus, short-term repopulation was not affected. However, there were markedly fewer haematopoietic cells derived from $Atm^{-/-}$ BM MNCs than from competitor MNCs at 16 weeks after transplantation (Fig. 1c, right panel), indicating that $Atm^{-/-}$ HSCs lack long-term repopulating capacity. To examine this phenomenon more closely, we transplanted purified $Atm^{-/-}$ KSL cells into congenic recipients. We observed a marked impairment of long-term repopulation capacity (Fig. 1d), which affected the B, T and myeloid cell lineages (data not shown). These transplantation data did not result from altered homing or apoptosis of HSCs in the absence of ATM (Supplementary Fig. 2 and 3). Thus, ATM has an essential role in the self-renewal of adult HSCs but is less important for their proliferation or differentiation into haematopoietic progenitor cells.

The capacity for self-renewal under conditions of stress such as transplantation may or may not reflect the expansion of HSCs under normal homeostatic conditions. We therefore evaluated the effects of *Atm* deficiency on haematopoiesis in older mice. Although $Atm^{-/-}$ mice often die of lymphoma after 9 weeks of age[6–9], some lymphoma-free animals do survive. When we monitored haematopoiesis in these mice at 24 weeks of age, they all exhibited a progressive anaemia not seen in the WT mice (mean haemoglobin and haematocrit values at 24 weeks were 15.3 ± 0.94 g dl$^{-1}$ and 50.3 ± 0.47%, respectively, in the WT ($n = 5$), in comparison with 9.93 ± 0.37 g dl$^{-1}$ and 28.0 ± 1.41%, respectively, in $Atm^{-/-}$ mice ($n = 5$); Fig. 1e). The numbers of leukocytes and platelets in $Atm^{-/-}$ mice were also significantly lower than in the WT (mean white blood cell and platelet counts were 10,800 ± 210 μl$^{-1}$ and 126 ± 19 × 10$^4$, respectively, in the WT ($n = 5$), in comparison with 7,300 ± 940 μl$^{-1}$ and 76 ± 6 × 10$^4$, respectively, in $Atm^{-/-}$ mice ($n = 5$)). Analysis of BM from $Atm^{-/-}$ mice revealed a decrease in cellularity accompanied by a relative increase in adipose tissue (Fig. 1f; the mean ratio of number of BM MNCs from $Atm^{-/-}$ mice to that from WT mice was 0.37 ± 0.02). Neither cells with abnormal morphology nor lymphoma-like cells were observed in $Atm^{-/-}$ BM. Flow cytometric analysis of BM revealed that the absolute cell numbers of multiple lineages, including myeloid cells (Mac-1$^+$Gr-1$^+$), B cells (B220$^+$) and erythroid cells (Ter119$^+$ cells), were decreased in $Atm^{-/-}$ BM (Fig. 1g). Colony-forming assays confirmed that the numbers of myeloid and erythroid precursors among $Atm^{-/-}$ BM MNCs were markedly decreased (Supplementary Fig. 4a). Co-culture of BM MNCs on stromal cells showed that $Atm^{-/-}$ cells were no longer able to form any colonies after 2 weeks of culture (Supplementary Fig. 4b).

To analyse the HSC population in older $Atm^{-/-}$ mice in more detail, we examined the marker expression and colony-forming ability of the KSL fraction of $Atm^{-/-}$ BM cells. In contrast to the normal frequency of KSL cells observed in 8-week-old $Atm^{-/-}$ mice (Supplementary Fig. 1a), the c-Kit$^{high}$ fraction of Sca-1$^+$Lineage$^-$ cells had disappeared in 24-week-old $Atm^{-/-}$ mice, and most c-Kit$^+$Sca-1$^+$Lineage$^-$ cells expressed only low levels of c-Kit

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Regular Paper

# Spontaneous and Radiation-induced Leukemogenesis of the Mouse Small Eye Mutant, *Pax6$^{Sey3H}$*

Yumiko NITTA[1]*, Kazuko YOSHIDA[2], Kenichi SATOH[3], Kei SENBA[4], Naomi NAKAGATA[5], Jo PETERS[6] and Bruce M. CATTANACH[6]

**Chromosome deletion/Acute myeloid leukemia/$^{60}$Co gamma rays/Mouse small eye mutant/*Pax6$^{Sey3H}$*.**

Allelic loss on the chromosome 2 is associated with radiation-induced murine acute myeloid leukemia. However, the gene, which contributes mainly to the leukemogenesis has not yet been identified. Expecting any predisposition to acute myeloid leukemia, we performed a radiation leukemogenensis experiment with *Pax6$^{Sey3H}$*, one of the small eye mutants carrying a congenital hemizygosity of the chromosome 2 middle region. A deletion mapping of *Pax6$^{Sey3H}$* with 50 STS markers indicated that the deleted segment extended between the 106.00 and 111.47 Mb site from the centromere with a length of 5.47 Mb. In the deleted segment, 6 known and 17 novel genes were located. *Pax6$^{Sey3H}$* mutants that crossed back into C3H/He did not develop myeloid leukemia spontaneously, but they did when exposed to gamma-rays. The final incidence of myeloid leukemia in mutants (25.8%) was as high as that in normal sibs (21.4%). Survival curves of leukemia-bearing mutants shifted toward the left ($p = 0.043$ by the Log rank test). F1 hybrids of *Pax6$^{Sey3H}$* with JF1 were less susceptible to radiation than *Pax6$^{Sey3H}$* onto C3H/He in regard to survival ($p = 0.003$ and $p < 0.00001$ for mutants and normal sibs, respectively, by a test of the difference between two proportions). Congenital deletion of the 5.47 Mb segment at the middle region on chromosome 2 alone did not trigger myeloid stem cells to expand clonally *in vivo*; however, the deletion shortcut the latency of radiation-induced myeloid leukemia.

## INTRODUCTION

It is well known that leukemia occurs more frequently among atomic bomb survivors than in the general population.[1–3] Clinical, cytogenetic, and molecular-genetic examinations indicated that complex chromosome abnormalities without a specific type of translocation and a high incidence of genetic instability of leukemic cells were characteristic to radiation-related acute myeloid leukemia in humans.[4]

To clarify the mechanism of leukemogenesis, the experimental model is useful. Hayata *et al.* reported the high susceptibility of C3H/He mice to myeloid leukemia and the consistent occurrence of the deleted chromosome 2 in mouse myeloid leukemias.[5,6] Since this publication, there have been three independent articles that demonstrated the occurrence of the deleted chromosome 2 in mouse radiation-induced acute myeloid leukemia).[7–9] Alexander *et al.* reported the commonly deleted region of 6.5 cM from markers *D2Mit214* to *D2Mit395*, where *Wt1* and *Pax6* genes located.[7] Silver *et al.* reported the minimal deleted region for radiation-induced acute myeloid leukemia on chromosome 2.[8] The interval was 1 cM between markers *D2Mit126* and *D2Mit185*, in which *Wt1* and *Pax6* genes were involved. The genes flanking the SFFV proviral integration 1 (*Sfpi1*) were suggested to work tumor suppressive in leukemogenesis in accordance with the observation of homozygous deletion. Rithidech *et al.* reported another commonly deleted region on chromosome 2.[9] The interval was 4.6 cM between *D2Mit272* and *D2Mit394*, in which *Wt1* and *Pax6* genes existed.

WAGR (*Wilms'* tumor, aniridia, genitourinary anomalies, mental retardation) syndrome is one of the well-known congenital disorders, patients of which have carried the simultaneous deletion of *PAX6* and *WT1* (OMIM#194072). Mouse small eye mutants, *Pax6$^{Sey1H}$*, *Pax6$^{Sey2H}$*, and *Pax6$^{Sey3H}$* are animal models of the WAGR syndrome, characterized by the genomic deletion at the two genes' locus and by the small eye

*Corresponding author: Phone/Fax: +81-82-257-5877,
E-mail: yumiko@hiroshima-u.ac.jp
[1]International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi, Minami-ku, Hiroshima 734-8553, Japan; [2]Radiation Hazards Research Group, National Institute for Radiological Science, Anagawa, Inage-ku, Chiba 263-8555, Japan; [3]Division of Bio-Medical Informatics, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi, Minami-ku, Hiroshima 734-8553, Japan; [4]Division of Developmental Genetics, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan; [5]Divigion of Reproductive Engineering, Institute of Resource Development and Analysis, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan; and [6]Mammalian Genetics Unit, Medical Research Council, Harwell, Didcot, OXON OX11 0RD, UK.

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# Decrease of Fertilizing Ability of Mouse Spermatozoa after Freezing and Thawing Is Related to Cellular Injury[1]

**Hirofumi Nishizono,[3,4] Masaki Shioda,[5] Toru Takeo,[6] Tetsumi Irie,[6] and Naomi Nakagata[2,4]**

*Kyudo Company Limited,[3] Kumamoto 861-0104, Japan*
*Center for Animal Resources and Development,[4] Kumamoto University, Kumamoto 860-0811, Japan*
*Department of Materials and Life Science,[5] Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan*
*Department of Clinical Chemistry and Informatics,[6] Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 860-8555, Japan*

## ABSTRACT

**In general, the fertilizing ability of cryopreserved mouse spermatozoa is less than that of fresh spermatozoa. This ability is especially low in C57BL/6, the main strain used for the production of transgenic mice. To solve this problem, the relationship between cell damage and fertilizing ability in cryopreserved mouse spermatozoa was examined in this study. Sperm motility analysis revealed no significant difference among the motilities of cryopreserved C57BL/6J, BALB/cA, and DBA/2N sperm (67.6%, 43.4%, and 60.0%, respectively) after thawing. However, the results of in vitro fertilization (IVF), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) showed a strong correlation between the frequency of aberrant spermatozoa (FAS) and fertilization rates (FR; C57BL/6J: FAS, 83.7%; FR, 17.0%; BALB/cA: FAS, 67.2%; FR, 24.2%; and DBA/2N: FAS, 10.2%; FR, 93.6%), and damage to spermatozoa was localized particularly in the acrosome of the head and mitochondria.**

*fertilization, in vitro fertilization, male reproductive tract, sperm, sperm motility and transport*

## INTRODUCTION

Over the past 15 years, a large number of transgenic and targeted mutant mice have been produced worldwide [1, 2]. In addition, N-ethyl-N-nitrosourea mutagenesis projects have been progressing, leading to an enormous increase in the number of strains of mutant mice that will be produced over the next few years [3, 4]. As a result, across the world animal facilities have an excess of mutant mice [5]. To solve this problem, sperm freezing may provide a much simpler and more economical alternative to embryo freezing [6–8].

In 1990, Yokoyama et al. [9] and Tada et al. [10] reported the successful freezing of mouse sperm using a solution containing glycerol and raffinose. Okuyama et al. [11] then found that mouse sperm can be frozen in a solution containing raffinose and skim milk without glycerol. We were also subsequently successful in the cryopreservation of mouse spermatozoa, including transgenic strains (luciferase transgenic mouse), using an improved method [12, 13]. These results indicated that slow dilution after thawing prevents the sharp change in osmolarity and viscosity between the cryopreservation solution and diluent. Moreover, Thornton et al. [14] have demonstrated that it is possible to establish efficient, comprehensive, and extensive archives, and that potentially large numbers of offspring (>7000) can be derived from the frozen spermatozoa of a single mutant male mouse.

However, in general, high fertilization rates are not always obtained for the frozen spermatozoa of all mouse strains [15]. Notably, the fertilization rate of frozen C57BL/6 spermatozoa remains very low, although the rate can be increased by in vitro fertilization with partial zona pellucida dissection or the intracytoplasmic sperm injection technique [16, 17]. C57BL/6 is a major inbred strain, and its genetic background is well known. Furthermore, this strain is used not only for the production of transgenic mice [18], but also as a backcross for targeted mutant mice. Therefore, it is necessary to establish a cryopreservation method for C57BL/6 mouse spermatozoa that can maintain high fertilizing ability after thawing. In this study, C57BL/6 frozen-thawed mouse spermatozoa were examined ultrastructurally for any damage that could account for their low fertilizing ability.

## MATERIALS AND METHODS

### Animals

Inbred male (12- to 20-week-old) and female (8- to 12-week-old) C57BL/6J, BALB/cA, and DBA/2N mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Homozygous transgenic male (12- to 20-week-old) mice expressing the enhanced green fluorescent protein (EGFP) gene under the acrosin promoter on C57BL/6J background, acr3-EGFP [19, 20], were provided from the mouse embryo bank of Mitsubishi Kagaku Institute of Life Sciences (Machida-shi, Tokyo, Japan). All mice were kept according to the Guidelines for Animal Experiments of Kumamoto University and the Guide for the Care and Use of Laboratory Animals. They were maintained on a constant 12D:12L cycle with standard mouse chow and water available ad libitum.

### Sperm Freezing and Thawing

Spermatozoa were obtained from C57BL/6J, BALB/cA, DBA/2N, and acr3-EGFP male mice (5 males/strain). After the male mice were killed humanely, one caudae epididymis was removed and placed into an 18% raffinose/3% skim milk solution. Spermatozoa from other caudae epididymides were used as a noncryopreserved control (fresh). Sperm cryopreservation and thawing were performed as described previously [15]. Briefly, 0.25-ml plastic straws (IMV, Paris, France) with 10-μl sperm aliquots collected at room temperature were frozen by exposure to liquid nitrogen

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[2]Correspondence: Naomi Nakagata, 2-2-1 Honjo, Kumamoto 860-0811, Japan. FAX: +81 96 373 6560; e-mail: nakagata@gpo.kumamoto-u.ac.jp

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**Original**

# Effects of a Hemizygous Deletion of Mouse Chromosome 2 on the Hematopoietic and Intestinal Tumorigenesis

Yumiko Nitta[1], Kazuko Yoshida[2], Naomi Nakagata[3], Toshihide Harada[4], Fumiko Ishizaki[5], Kohsaku Nitta[6], and Mikinori Torii[7]

[1]International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi, Minami-Ku, Hiroshima 734–8553, Japan
[2]Environmental and Toxicological Sciences Research Group, National Institute for Radiological Science, Anagawa, Inage-Ku, Chiba 263–8555, Japan
[3]Division of Reproductive Engineering, Institute of Resource Development and Analysis, Kumamoto University, 2–2–1 Honjo, Kumamoto 860–0811, Japan
[4]Third department of Internal Medicine, School of Medicine, Hiroshima University, 1–2–3 Kasumi, Minamiku, Hiroshima 734–8551, Japan
[5]Hiroshima Prefecture College of Health Science, 1–1 Gakuennmachi, Mihara, Hiroshima 723–0053, Japan
[6]Ootani Rehabilitation Hospital, 1970 Kakiura, Ohgakicho, Saiki, Hiroshima 737–2211, Japan
[7]Developmental of Research Laboratories, Shionogi Co. Ltd., 3–1–1 Futaba, Toyonaka, Osaka 561–0825, Japan

**Abstract:** Allelic loss of chromosome 2 is associated with radiation-induced murine acute myeloid leukemia. However, the gene, which contributes mainly to the leukemogenesis in a tumor suppression manner, has not been identified, yet. Expecting predisposition to acute myeloid leukemia, a radiation leukemogenensis experiment was performed with *Pax6*$^{Sey3H}$, one of the small eye mutants. Deletion mapping of *Pax6*$^{Sey3H}$ indicated that the deleted segment extended from 106.00 to 111.47 Mb from the centromere with a length of 5.47 Mb on chromosome 2. Six known and seventeen novel genes were located in the segment. *Pax6*$^{Sey3H}$ mutants crossed back into C3H/He did not develop hematopoietic tumors spontaneously, but they did after exposure to γ-rays. The final incidence of hematopoietic tumor in mutants (45.2%) was higher than that in normal sibs (26.2%), and the survival curve of mutants shifted toward the left ($p<0.05$ by the Cox-Mantel test). Mutants developed intestinal tumors spontaneously with long latency as well as showing abnormality in the Wirsung's duct from young ages. Congenital deletion of the 5.47 Mb segment at the middle region on chromosome 2 alone did not trigger hematopoietic tumors, however, the deletion promoted the development of hematopoietic tumors initiated by radiation. The deletion developed intestinal tumors spontaneously. Radiation exposure at 10 weeks of age did not contribute to the intestinal tumorigenesis. (J Toxicol Pathol 2004; **17**: 105–112)

**Key words:** chromosome deletion, *Pax6*$^{Sey3H}$, Wilsung's duct, intestinal tumor, radiation

## Introduction

It is well known that leukemia occurs more frequently among atomic bomb survivors than in the general population[1–3]. Clinical, cytogenetic and molecular-genetic examinations indicate that complex chromosome abnormalities without specific types of translocation and high incidence of genetic instability of leukemic cells are characteristic of radiation-related acute myeloid leukemia in humans[4].

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Mailing address: Yumiko Nitta, International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi, Minami-Ku, Hiroshima 734–8553, Japan
TEL & FAX: 81-82-257-5877
E-mail: yumiko@hiroshima-u.ac.jp

To clarify the mechanism of leukemogenesis, an experimental model is useful. Hayata et al. reported the high susceptibility of C3H/He mice to myeloid leukemia and the consistent occurrence of chromosome 2 deletions in mouse myeloid leukemias[5,6]. Since this publication, three independent articles have demonstrated the deletion of chromosome 2 in mouse radiation-induced acute myeloid leukemias[7–9]. Interestingly, the length of these three deleted regions was different, 6.5 cM, 1.0 cM and 4.6 cM for each reference, respectively, but they involved *Wt1* and *Pax6* genes commonly.

The WAGR (*Wilms'* tumor, aniridia, genitourinary anomalies, mental retardation) syndrome is one of the well known congenital disorders, the patients of which have simultaneous deletion of *PAX6* and *WT1* (OMIM#194072). The mouse small eye mutant, *Pax6*$^{Sey1H}$ is the animal model of the WAGR syndrome, and is characterized by the