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Effects of fission neutrons on human thyroid tissues maintained in SCID mice

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ABSTRACT

Morphology and function (secretion of thyroid hormone) of human thyroid tissues from Graves' disease patients are well maintained in C57BL/6J-*scid* mice. Serum level of thyroid hormone was reduced by fission neutrons from the nuclear reactor UTR-KINKI, and changes in thyroid hormone by fission neutrons were bigger than those by low LET radiations, X-rays and ^{137}Cs γ-rays, suggesting high relative biological effectiveness (RBE; 6.5) of fission neutrons. Microarray analyses revealed that about 3% of genes showed more than 4-fold change in gene expression in the unexposed thyroid tissues against surgically resected thyroid tissues from the same patient, probably due to the difficult oxygen and nutrient supply shortly after transplantation. Dose-dependent changes in gene expression against unexposed concurrent controls were observed with increasing doses of fission neutrons (0.2–0.6 Gy) and ^{137}Cs γ-rays (1.0–3.0 Gy) and showed high RBE (4.2). Furthermore, there were some specific genes which showed more than 4-fold change in gene expression in all the thyroid tissues exposed to higher doses of radiation, especially neutrons (0.4 and 0.6 Gy), but none at lower doses (0.2 Gy of neutrons and 1.0 and 2.0 Gy of γ-rays). These genes related to degeneration, regeneration, apoptosis, and transcription, respond specifically and very sensitively to neutron injury in human thyroid tissues. This is the first experimental report that fission neutrons can induce some morphological and functional disorders in human tissues, showing high RBE against γ-ray exposure. These results are useful to evaluate the risks of fission neutrons and cosmic rays to humans.

1. Introduction

Radiation induces various types of damage in human and animals. Among various radiation sources, neutrons are several times more effective than X- and γ-radiation in inducing neoplastic cell transformation, mutation *in vitro*, germ-cell mutation *in vivo*, chromosomal aberrations *in vivo* and *in vitro* and cancer in experimental animals [1]. In spite of the evidence in experimental animals, there is a scarcity of evidence in humans; epidemiological study of A-bomb survivors on the difference between Hiroshima and Nagasaki and a few accidents at nuclear sites [1]. In humans, exposure to neutrons can occur from the nuclear fission reactions usually associated with the production of nuclear energy and from cosmic radiation (in the flying body) in the natural environment [2–4]. Consequently, it is of utmost importance to study the direct effects of fission neutrons on human organs and tissues for investigating the precise risk.

In the improved severe combined immunodeficient (super-SCID) mice, normal human organs and tissues are well maintained in morphology and function for a long period (~3 years) by the consecutive transplantation of these tissues [5–10]. For example, no substantial histological changes were observed in the human thyroid tissues maintained in SCID mice for 18 months, and rapid and high uptake of radioiodine into the transplanted human thyroid tissue was observed [10]. Furthermore, transplanted human thyroid tissues secreted thyroid hormone (T3), and T3 secretion was stimulated by the injection of human thyroid stimulating hormone (TSH)

Abbreviations: SCID, severe combined immunodeficiency; SPF, specific pathogen free; SSCP, single strand conformational polymorphism; RBE, relative biological effectiveness.

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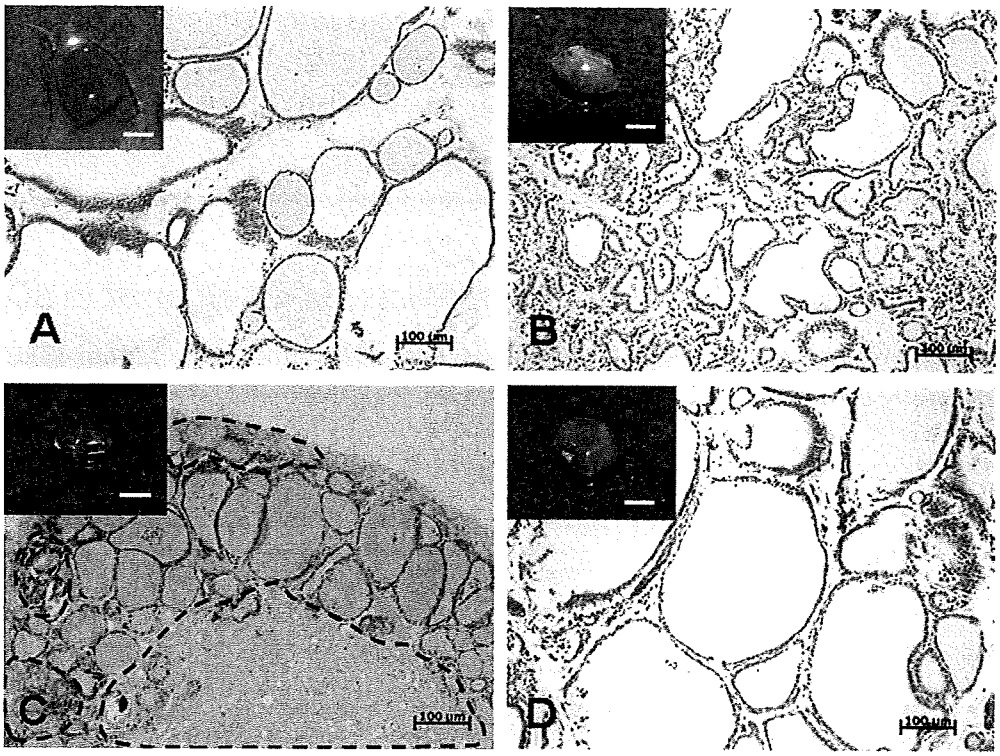

Fig. 1. Macroscopic and microscopic views of transplanted human thyroid tissues with or without radiation exposures. (A) Surgically resected human thyroid tissue from Graves' disease patient (20 years, female). (B) Human thyroid tissues from a Graves' disease patient (20 years, female) exposed to neutrons (0.2 Gy × 4 times at 1 week interval) and exposed tissue was removed 5 weeks after transplantation, (C and D) transplanted thyroid tissues exposed to neutrons (0.2 Gy × 6 times) and removed 7 months after transplantation (C) and concurrent unexposed controls (D), respectively. Histologically, follicles became small, some disappeared and were replaced to connective tissues (C) (marked by broken line). Scale bars in gross features: 3 mm. Microscopic views; haematoxylin and eosin staining. Scale bars: 100 μm.

[10]. Expression analysis by microarray indicated that gene expression was also well maintained in the transplanted human thyroid tissues [11]. The expression of only 3% of genes showed more than 4-fold change in gene expression during the first week after transplantation of surgically resected original tissues. However, further changes were not observed 2–4 weeks after transplantation, but instead recovered slightly [11].

The thyroid gland is one of the most important endocrine organs for the development and growth, and one of the most sensitive organs to radiation. Radiation exposure, therefore, causes general disorder in human beings [12]. In fact, consecutive irradiation with X-rays or ^{137}Cs γ-rays for approximately 2 years resulted in the disappearance of follicles and significant decrease of thyroid hormone secretion [11]. Mutations in *p53* and *c-kit* genes were induced significantly by high dose and high dose rate of X-rays and γ-rays in human thyroid tissues from old head and neck cancer patients and a Graves' disease patient, while mutations were not detected by low dose rate exposure [11]. Furthermore, lower doses (1–3 Gy) of ^{137}Cs γ-rays can induce changes in gene expression in the transplanted human thyroid tissues.

In the present study, human thyroid tissues from Graves' disease patients were transplanted into the improved SCID mice, and exposed to fission neutrons or ^{137}Cs γ-rays to examine the induced changes in morphology, function, cancer-related genes, and changes in gene expression in the transplanted human thyroid tissues to confirm the direct link between radiation sources.

2. Materials and methods

2.1. Human thyroid tissues

Thyroid tissues resected from two Graves' disease patients (20 and 23 years, females) were used for heterotransplantation to the SCID mice. Goiter was resected because of cosmetic problem, and blood level of thyroid hormone of the patients before the surgical resection was within normal range. Only the human tissues free of mycoplasma, human hepatitis B and C antigens/antibodies, adult T cell leukemia, human immunodeficiency virus, and Wassermann reaction were accepted into the SPF room of the barrier section of the Institute of Experimental Animal Sciences, Osaka University. Use of human tissues were permitted by the ethics committees of Osaka University, Graduate School of Medicine, Kuma Hospital and National Institute of Biomedical Innovation, and all experiments were performed following the guidelines of the Ministry of Education, Science and Culture and the Ministry of Health and Labor for the use of human tissues.

2.2. SCID mice

C57BL/6J-*scid*/*scid* mice ($N_{12}F_{22-24}$) were used for the experiment. C.B17-*scid*/+ male and female mice were provided by Dr. M.J. Bosma [13], Institute of Cancer Research, Philadelphia, in 1986, and then C.B17-*scid*/*scid* mice were maintained by selective sister-brother inbreeding of C.B17-*scid*/*scid* homozygote showing undetectable serum IgG and IgM (<1 μg/ml) by T. Nomura to diminish the leaky and leukemic mice [5,6,14]. C.B17*scid*/*scid* male (N_1F_3) was mated with C57BL/6J female (F_{153}) (provided by E.S. Russell, Jackson Laboratory at F_{129} in 1976 and inbred by sister-brother mating for further generations). Progeny was crossed and *scid* homozygote mouse was repeatedly back-crossed to C57BL/6J mouse to make congenic strain of C57BL/6J-*scid*/*scid* ($N_{12}F_{20}$) by T. Nomura [15]. Mice were maintained in the complete barrier condition, lit from 4:00 to 18:00, at 23 ± 1 °C and 50–70% humidity with autoclaved mouse diet CRF-1 (Charles River Japan, Kanagawa, Japan) and acidified, chlorinated, and filtrated (by MILLIPORE) water. Serum IgG and IgM were examined at 4–6 weeks after birth by enzyme-linked immunosorbent assay [5,6], and 2 months old C57BL/6J-*scid*/*scid* mice showing undetectable serum IgG and IgM (<1 μg/ml) were used for the heterotransplantation of human thyroid tissues. Animal experiments were carried out in the barrier section of the Institute of Experimental Animal Sciences following the Osaka University Guidelines for Animal Experimentation.

2.3. Maintenance of human thyroid tissues in SCID mice

Procedures for the heterotransplantation of human organs and tissues into the SCID mice were reported previously [5–11]. Briefly, resected human thyroid gland was cut into 5–6 mm cubic masses in a 0.9% NaCl solution contain-

Table 1
Human thyroid hormone (T3) in peripheral blood of SCID mice with human thyroid tissues after neutron exposure.

Weeks after transplantation (first exposure)	Exposed			Unexposed[a]		
	Dose (Gy)	No.	Thyroid hormone (pg/ml)	No.	Thyroid hormone (pg/ml)	p^b
1	0.2 × 1	2	481, 409	1	445	1.000
2	0.2 × 2	2	509, 468	1	494	0.902
3	0.2 × 3	2	458, 385	1	461	0.644
4	0.2 × 4	2	385, 388[c]	2	443, 438	0.007
20	0.2 × 6	3	331, 275, 375[c]	2	391, 394	0.152
24	0.2 × 6	3	295, 334, 236[c]	2	328, 354	0.202

Thyroid tissues from Graves' disease patient (20 years, female) were used.
[a] Unexposed concurrent controls to the exposed groups.
[b] *t*-Test was applied after testing quality of variance by SPSS Statistics System.
[c] $p < 0.01$ vs. the mean of unexposed groups (5 samples from 1 to 4 weeks after transplantation).

ing high concentrations of antibiotics (penicillin G, 50,000 units/ml; panipenem, 25 mg/ml; streptomycin sulfate, 50 mg/ml). Mice were anesthetized with 0.77% tribromoethanol (Aldrich Chemical Co. Ltd., Milwaukee, WI, USA), and human thyroid tissues were implanted s.c. into the back of SCID mice by the surgical operation. Thyroid stimulating hormone (TSH) was not given to the SCID mice with thyroid tissues of Graves' disease which is characterized by stimulating autoantibody [16].

2.4. Neutron and γ-ray exposure of human thyroid tissues

SCID mice with human thyroid tissues were exposed to fission neutrons at about 0.2 Gy/h and γ-rays at 0.2 Gy/h by the nuclear reactor, UTR-KINKI in the Kinki University Atomic Energy Research Institute. UTR-KINKI has an ample space for the irradiation of biological materials in the central portion of its core, where neutrons and γ-rays of about 0.2 Gy/h is available during operation at a nominal output of 1 W. Neutron and γ-ray doses at the irradiation port in the presence of C57BL/6J mice were measured using the paired chamber method. Neutron dose was reduced with increasing numbers of mice; *i.e.* total weight (g) of mice [17]. Actual dose rate, when four C57BL/6J mice (25 g each) were placed, was 0.197 Gy/h of neutrons and 0.198 Gy/h of γ-rays. Human thyroid tissues from Graves' disease patients were also exposed to reference doses of ^{137}Cs γ- rays by Gammacell 40 Exactor (Nordion International Inc., Canada) at a dose rate of 1.19 Gy/min. Dosimetry of ^{137}Cs γ-rays was made each time by Condenser R-meter 500 Radcon with 550-3 probe (Victoreen Instr. Div. Cleveland, OH) adjusted to the standard ^{60}Co γ-ray source.

Human thyroid tissues from Graves' disease patient (20 years, female) were transplanted s.c. and then a half of SCID mice with human thyroid tissues were exposed to 0.2 Gy of fission neutrons by UTR-KINKI 1–4 times (2 samples in each group) at 1, 8, 15 and 22 days after transplantation. The other half were not exposed to neutrons and used for concurrent unexposed controls. Six days after exposure, blood was taken from each SCID mouse with thyroid tissue transplantation for the measurement of human thyroid hormone in the blood of SCID mice [18]. Blood was also taken from unexposed SCID mice with thyroid tissues 7, 14, 21 and 28 days after transplantation. Eleven thyroid tissues were also exposed to 0.2 Gy of fission neutrons 6 times at 1, 8, 15, 22, 29 and 36 days after transplantation, and then thyroid tissues were removed 5–13 months after transplantation (3–11 months after neutron exposure) for the examination of mutations. Peripheral blood was also taken from these exposed and unexposed SCID mice with thyroid tissue transplantation for the examination of human thyroid hormone secretion.

2.5. Measurement of thyroid hormone (T3)

Peripheral blood was taken from the SCID mice to which human thyroid tissue had been transplanted. Human thyroid hormone T3 was measured by the radioimmunoassay (T-3 RIABEAD, Dinabot, Tokyo, Japan) as reported previously [10,11,18].

2.6. Analysis of gene expression in the transplanted human thyroid tissue

Twenty two human thyroid tissues from the other Graves disease patient (23 years, female) were exposed to 0.2 Gy (4 thyroid tissues), 0.4 Gy (4 tissues) and 0.6 Gy (3 tissues) of fission neutrons and 1.0 Gy (4), 2.0 Gy (4) and 3.0 Gy (3) of ^{137}Cs γ-rays 1 day after transplantation to SCID mice. Eight thyroid tissues were not exposed to radiations and used for concurrent controls. Two weeks after radiation exposures, thyroid tissues were removed from SCID mice for the analysis of gene expression by microarray [11]. Human thyroid tissues removed from the SCID mice were kept in the liquid nitrogen.

Level of gene expression in transplanted thyroid tissue was compared with original (surgically resected) thyroid tissue to observe spontaneous changes after transplantation [11]. Radiation exposed thyroid tissues were also compared with concurrent unexposed controls by GeneChip (HG-Focus Array; 8500 genes, Affymetrix, Inc., Santa Clara, CA, USA) to examine the changes in gene expression by radiation exposure.

To extract RNA in high efficiency, thyroid tissues were crushed by pressing in frozen metal blocks (Cryopress, Microtec Co., Ltd., Funabashi, Japan) and then the powdered specimens were homogenized by microtube homogenizer (Homogenizer PT-101, PA Cosmo Bio., Tokyo, Japan) in Trizol reagent solution as described by manufacturer's protocol (Invitrogen, Japan K.K., Tokyo, Japan). The extracted RNA was purified by RNeasy Mini Kit (Qiagen, Tokyo, Japan). Quantity and quality of the RNA was characterized by spectrophotometer and gel electrophoresis. Total RNA (1.18–2 μg) was used to synthesize cDNA and biotinylated cRNA, and fragmentation of the cRNA. Those procedures were performed according to the manufacture's protocol (Affymetrix, http://www.affymetrix.com). The procedures for hybridization, staining and washing, and scanning were carried out as described by Affymetrix protocol. Expression analysis was made by the GeneChip operating software GCOS using the Microarray Suite 5 (MAS5) algorithm. The data of gene description were derived from NetAffx™ Analysis Center (Affmetrix Inc.) on July 4, 2009, and Entrez Gene (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, U.S.A.) in September, 2009.

Statistical analyses were carried out by SPSS Statistics System (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Morphological changes in human thyroid tissues by neutrons

There were no substantial differences in gross and histological features in the unexposed thyroid tissues after transplantation, as it was in previous reports [10,11]. However, macroscopic and his-

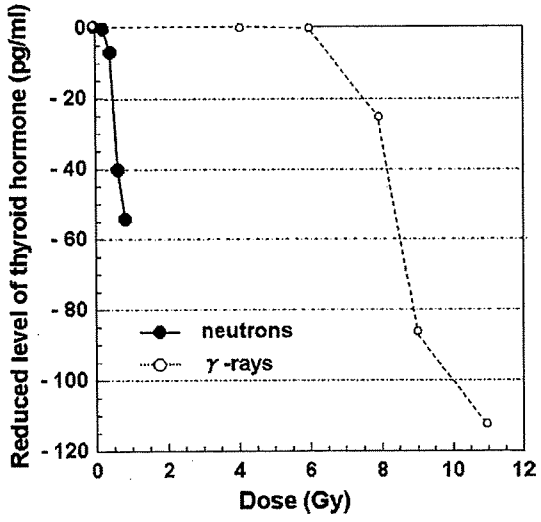

Fig. 2. Reduced concentration of human thyroid hormone (T3) in the peripheral blood of SCID mice with human thyroid tissues after neutron and γ-ray exposures. Thyroid tissues from a Graves' disease patient (20 years, female) were transplanted s.c. to the SCID mice and exposed to fission neutrons. Thyroid hormone (T3) in the blood of SCID mice with human thyroid tissue transplantation was measured by radioimmunoassay. Reduced level of serum thyroid hormone (T3) in exposed SCID mice from those of concurrent unexposed controls (Table 1) were plotted at each dose (closed circles, solid line). Similarly, reduced level of T3 after ^{137}Cs γ-ray exposure (open circles, broken line) were also plotted (from Table 2 of Ref. [11]) in the same figure in comparison with neutron exposed groups.

tological changes were observed in the thyroid tissues exposed to 0.8 Gy (0.2 Gy, 4 times) (Fig. 1B) and 1.2 Gy (0.2 Gy, 6 times) of fission neutrons and removed 7 months after transplantation (Fig. 1C), while such damage was not observed in concurrent unexposed controls (Fig. 1D). Histologically, follicles became small (Fig. 1B and C), some disappeared and were replaced by connective tissues (Fig. 1C, marked by broken line).

3.2. Functional changes in human thyroid tissues by neutrons

Table 1 shows the serum level of human thyroid hormone (T3) detected in the blood of the SCID mice to which human thyroid tissues had been transplanted. Serum level of T3 in unexposed control groups showed no decreases up to 4 weeks after transplantation, but slight decreases were observed at 20 weeks after transplantation. There were no differences in the serum level of T3 between exposed and concurrent unexposed groups at 0.2 Gy of neutrons. However, serum level of T3 decreased slightly and dose-dependently at doses from 0.4 to 0.8 Gy in comparison with unexposed concurrent controls (Table 1 and Fig. 2). Decrease of T3 was also observed 20 and 24 weeks after 1.2 Gy (0.2 Gy, 6 times) of neutron exposure. Results showed large differences from those by γ-rays which showed apparent threshold dose (~6 Gy) [11] (Fig. 2). Reduced serum levels of thyroid hormone by 0.4, 0.6 and 0.8 Gy of fission neutron exposure were nearly equivalent to those by approximately 6.5, 8.2, and 8.5 Gy of ^{137}Cs γ-rays (Fig. 2). This suggests high relative biological effectiveness (RBE) of fission neutrons from UTR-KINKI, the values estimated from the slopes from 0.2 to 0.8 Gy of neutrons and from 6 to 11 Gy of γ-rays (Fig. 2) being approximately 6.5 after the reduction of γ-ray component of the nuclear reactor UTR-KINKI.

3.3. Changes of gene expression in human thyroid tissues by fission neutrons

Levels of gene expression in transplanted thyroid tissues were compared with those of the original tissues; *i.e.* surgically resected thyroid tissues. In the previous report [11], about 3% genes showed more than 4-fold change in gene expression in the first week after transplantation, probably due to the difficulty of oxygen and nutrient supply within 7 days after surgical extirpation and transplantation, but further changes were not observed 2–4 weeks after transplantation. In the present study, consequently, levels of gene expression were compared 2 weeks after transplantation and radiation exposure, and similar results were observed in the thyroid tissues from Graves' disease patient (23 years, female). About 3% of genes showed more than 4-fold change (both increase and decrease) in gene expression in 8 unexposed thyroid tissues [increases (>4); 201.8 ± 12.5 (mean ± SE, n = 8), decreases (<1/4); 69.8 ± 13.5 against surgically resected original thyroid tissue from the same patient]. Neutron exposed thyroid tissues from the Graves' disease patient (23 years, female) were also compared with unexposed concurrent control thyroid tissues from the same patient. Numbers of genes which showed more than 4-fold change in gene expression against concurrent unexposed thyroid tissues increased dose-dependently at dose ranges from 0.2 to 0.6 Gy of fission neutrons and from 1.0 to 3.0 Gy of ^{137}Cs γ-rays (Fig. 3). RBE was estimated as 4.2 after the reduction of γ-ray component of UTR-KINKI.

There were many genes which showed more than 4 folds change in gene expression among all the unexposed transplanted thyroid tissues against surgically resected original thyroid tissue (Fig. 4, Table 2), suggesting that these genes are sensitive to the lack of oxygen and nutrients just after surgical extirpation and trans-

Table 2
Genes showing more than 4-fold change in all the transplanted unexposed thyroid tissues.

	Gene name	Gene symbol
(1) Increase	Matrix metallopeptidase 12 (macrophage elastase)	MMP12
	Secreted phosphoprotein 1	SPP1
	Chemokine ligand 10	CXCL10
	Collagen, type I	COL1A1
	Perilipin 2	PLIN2
	Matrix metallopeptidase 9 (gelatinase B)	MMP9
	Chemokine ligand 9	CXCL9
	Guanylate binding protein 2	GBP2
	Tenascin C	TNC
	TIMP metallopeptidase inhibitor 1	TIMP1
	Rho family GTPase 3	RND3
	Versican	VCAN
	Collagen, type VI	COL6A3
	Collagen, type I	COL1A2
	Complement component 1, r subcomponent	C1R
	Collagen, type III	COL3A1
	Lysyl oxidase-like 2	LOXL2
	Cathepsin K	CTSK
	Transforming growth factor, beta-induced	TGFBI
	Chemokine ligand 11[a]	CXCL11
	Surfactant protein B[a]	SFTPB
	Plexin domain containing 1[a]	PLXDC1
(2) Decrease	Murine osteosarcoma viral oncogene homolog	FOS
	Murine osteosarcoma viral oncogene homolog B	FOSB
	Carbonic anhydrase IV	CA4
	Thyroid peroxidase	TPO
	Nebulin	NEB
	Solute carrier organic anion transporter family	SLCO2A1
	Deiodinase, iodothyronine, type I[a]	DIO1
	Chloride intracellular channel 3[a]	CLIC3
	Fc fragment of IgG binding protein[a]	FCGBP
	Solute carrier family 25[a]	SLC25A15
	Dual specificity phosphatase 1[a]	DUSP1
	Latent transforming growth factor beta binding protein 4[a]	LTBP4

Eight thyroid tissues from Grave's disease patient (23 years, female) were transplanted to SCID mice and removed 2 weeks after transplantation for gene expression analysis. Levels of gene expression were compared with surgically resected original thyroid tissue from the same patient.

[a] Seven of 8 thyroid tissues showed more than 4-fold change in gene expression.

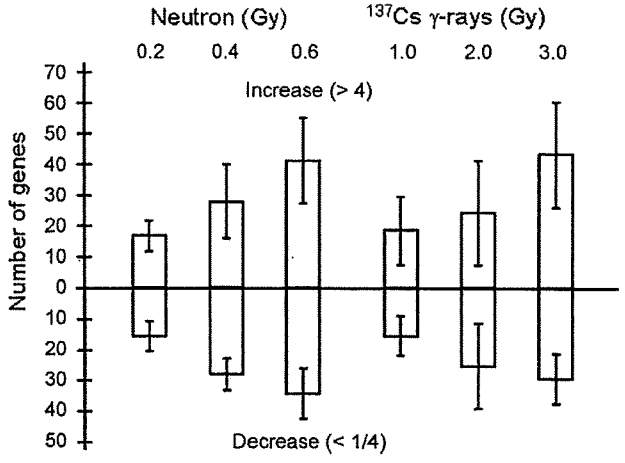

Fig. 3. Dose-dependent changes in gene expression in the transplanted human thyroid tissues after neutron and γ-ray exposures. Human thyroid tissues from Graves' disease patient (23 years, female) in SCID mice were exposed to 0.2, 0.4 and 0.6 Gy of fission neutrons and 1.0, 2.0 and 3.0 Gy of ^{137}Cs γ-rays, and compared with concurrent unexposed thyroid tissue from the same patient. Numbers of genes which showed more than 4-fold change in gene expression against unexposed concurrent controls were plotted on the ordinate. Histograms and horizontal bars show the mean and standard error of the mean. p values are 0.077 for neutrons and 0.157 for γ-rays by Kruskal Wallis test (SPSS statistics).

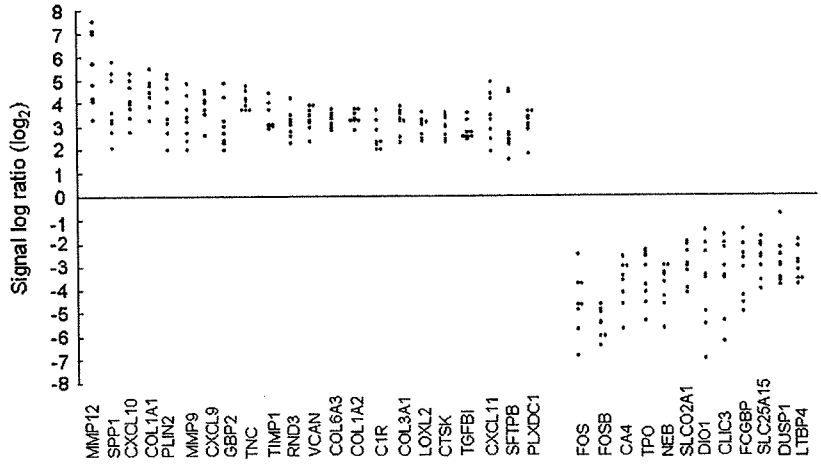

Fig. 4. Individual variation in gene expression of each unexposed thyroid tissue. Level of gene expression in 8 thyroid tissues from Graves' disease patient (23 years, female) was compared with that of surgically resected original thyroid tissue from the same patient. Genes showing signal log ratio (log_2) more than 2 (4-fold change) in gene expression in all the thyroid tissues were given on the abscissa and individual value was plotted on the ordinate.

plantation. Such genes are shown in Table 2. These spontaneous changes are related to degeneration and regeneration processes of transplanted thyroid tissues. In contrast, these findings were not observed in radiation exposed thyroid tissues at low doses of radiation. There were no common genes which showed more than 4-fold changes in gene expression in thyroid tissues exposed to 0.2 Gy of neutrons and 1.0 and 2.0 Gy of ^{137}Cs γ-rays, against unexposed concurrent controls, suggesting that radiation injury may be random phenomenon, while 4 and 14 genes for 0.4 and 0.6 Gy of neutron exposure and 5 genes for 3.0 Gy of γ-ray exposure showed more than 4-fold change (Table 3). Among 13 of 14 genes which showed more than 4-fold change in all the thyroid tissues exposed to higher doses of fission neutrons and ^{137}Cs γ-rays (Fig. 5, Table 3), eight were related to genes responding to injury or stress (C7orf68, ADM, SERPINEI, ENO2, ANGPTL4, PLIN2, COL9A3, and DIO1), 3 were related to apoptosis (BNIP3, HK2, and TFF3), and 2 were related to transcription (CEBRD and BHLHE40). This indicates that some genes are specifically sensitive to higher doses of radiation, especially to neutrons. Two genes, perilipin 2 and deiodinase showed more than 4-fold change in both unexposed and neutron exposed thyroid tissues. There were no changes in gene expression of *RET*, *BRAF* and Thyroxine (T3) related genes in all the thyroid tissues which were exposed to fission neutrons (0.2 Gy, 1–4 times at 1 week interval or single exposure of 0.2–0.6 Gy) and ^{137}Cs γ-rays (1.0–3.0 Gy).

4. Discussion

Human thyroid tissues were well maintained for a long period in C.B17-*scid* mice, showing no substantial differences in the histology, secretion of human thyroid hormone, and response to thyroid stimulating hormone [10,11]. Level of gene expression was also well maintained in the transplanted thyroid tissues for several weeks [11]. Large doses of X-rays and γ-rays at high dose rate significantly induced degeneration of the human thyroid tissue, reduced thyroid hormone secretion, and induced mutations in cancer related genes. However, all of these adverse effects of radiation on human thyroid tissues were not observed by the low dose rate exposure to the same dose of ^{137}Cs γ-rays, indicating significant dose rate effects in human thyroid tissues.

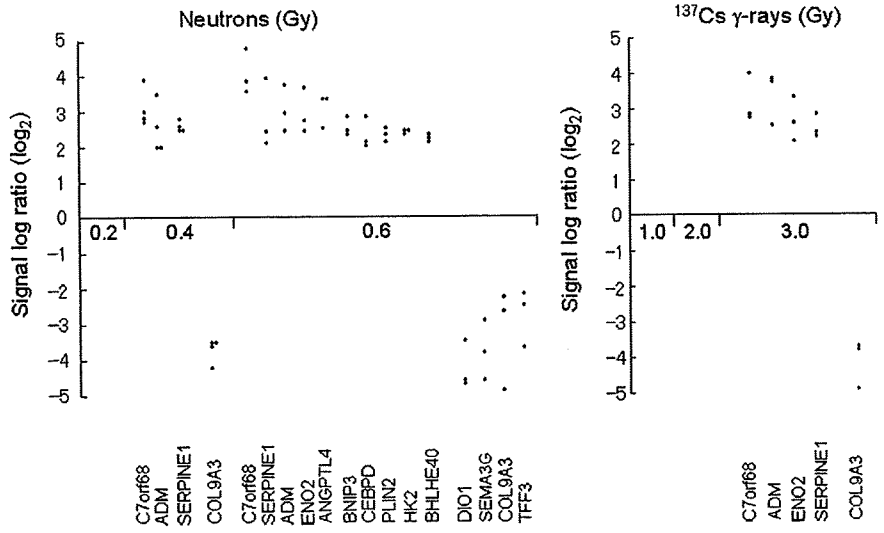

Fig. 5. Individual variation in gene expression of neutron and ^{137}Cs γ-ray exposed thyroid tissues. Level of gene expression in each thyroid tissue from Graves' disease patient (23 years, female) was compared with that of unexposed concurrent control thyroid tissue of the same patient. Genes showing signal log ratio (log_2) more than 2 (4-fold change) in gene expression in all the thyroid tissues were given on the abscissa and individual value was plotted on the ordinate.

Table 3
Genes showing more than 4-fold change in gene expression in all the thyroid tissues exposed to fission neutrons and ^{137}Cs γ-rays in comparison with unexposed concurrent controls.

Radiations	Dose (Gy)	Gene name	Gene Symbol
(A) Neutrons			
(1) Increase	0.2	None	
	0.4	Chromosome 7 open reading frame 68	C7orf68
	0.4	Adrenomedullin	ADM
	0.4	Serpin peptidase inhibitor, clade E (nexin)	SERPINE1
	0.6	Chromosome 7 open reading frame 68	C7orf68
	0.6	Serpin peptidase inhibitor, clade E (nexin)	SERPINE1
	0.6	Adrenomedullin	ADM
	0.6	Enolase 2	ENO2
	0.6	Angiopoietin-like 4	ANGPTL4
	0.6	BCL2/adenovirus interacting protein	BNIP3
	0.6	CCAAT/enhancer binding protein	CEBPD
	0.6	Perilipin 2	PLIN2
	0.6	Hexokinase 2	HK2
	0.6	Basic helix-loop-helix family	BHLHE40
(2) Decrease	0.2	None	
	0.4	Collagen, type IX	COL9A3
	0.6	Deiodinase, iodothyronine	DIO1
	0.6	Sema domain, immunoglobulin domain	SEMA3G
	0.6	Collagen, type IX	COL9A3
	0.6	Trefoil factor 3	TFF3
(B) ^{137}Cs γ-rays			
(1) Increase	1.0	None	
	2.0	None	
	3.0	Chromosome 7 open reading frame 68	C7orf68
	3.0	Adrenomedullin	ADM
	3.0	Enolase 2	ENO2
	3.0	Serpin peptidase inhibitor, clade E (nexin)	SERPINE1
(2) Decrease	1.0	None	
	2.0	None	
	3.0	Collagen, type IX, alpha 3	COL9A3

Twenty-two thyroid tissues from the Graves' disease patient (23 years, female) were transplanted to SCID mice and exposed to 0.2 Gy (4 tissues), 0.4 Gy (4) and 0.6 Gy (3) of fission neutrons or 1.0 Gy (4), 2.0 Gy (4) and 3.0 Gy (3) of ^{137}Cs γ-rays. Radiation exposed thyroid tissues were removed 2 weeks after transplantation for gene expression analysis. Levels of gene expression were compared with unexposed concurrent control thyroid tissue from the same patient.

In the present study, morphology and function (secretion of thyroid hormone) of human thyroid tissues are well maintained in congenic C57BL/6J-*scid* mice. Neutron exposure reduced thyroid hormone secretion and changes in serum level of thyroid hormone by fission neutrons were larger than those of low LET radiations, X-rays and γ-rays (Fig. 2) [11], suggesting high relative biological effectiveness (RBE; 6.5) of fission neutrons.

In the previous study, large doses of γ-ray exposure (11–33 Gy) at high dose rate for long period (~3 years) could induce mutations in *c-kit* and *p53* genes in the transplanted human thyroid tissues [11,19–21]. In the present study, however, no mutations of cancer-related genes, *p53*, *K-ras*, *c-kit*, *β-catenin*, *RET*, *BAK* and *BRAF* nor tumors have been detected in these eleven thyroid tissues exposed to 1.2 Gy of fission neutrons (0.2 Gy, 6 times at 7-day intervals) and in the unexposed concurrent control tissues which were observed for 5–13 months after transplantation (data not shown). Longer term exposure of the thyroid tissue to larger doses of fission neutrons may be necessary to induce specific mutations and cancer in human thyroid tissues. We succeeded to induce mutation and human skin cancer in normal human skin maintained on SCID mice only after the continuous (~2 years) ultraviolet light B (UVB) irradiation [9]. Topical exposures, in contrast to whole body exposure to neutrons, may be less hazardous to critical internal organs of SCID mice and can produce more changes to the transplanted human tissues, resulting in the induction of mutation and cancer in human tissues.

In contrast to gene mutation, changes in gene expression were detectable in the human thyroid tissues shortly after transplantation to SCID mice [11]. Spontaneous changes in gene expression were observed after surgical extirpation and transplantation of thyroid tissues to SCID mice, and many genes showed more than 4-fold changes in gene expression in all the unexposed thyroid tissues, suggesting these genes related to degeneration and regeneration processes are very sensitive to the lack of oxygen and nutrients (Table 2, Fig. 4). In the radiation exposed thyroid tissues, numbers of genes which showed more than 4-fold change in gene expression increased with increasing doses of γ-rays and neutrons (Fig. 3) and showed high RBE (4.2). Furthermore, there were several genes which showed more than 4-fold change in gene expression in all the thyroid tissues exposed to higher doses of radiation, especially neutrons (Fig. 5 and Table 3). Some genes related to degeneration, regeneration, apoptosis, and transcription, responded specifically and very sensitively to radiation injury. Mechanism on radiation effects will be elucidated.

This is the first experimental report that fission neutrons can induce morphological and functional disorders to human tissues, showing high RBE against γ-ray exposure. These results are essentially useful to evaluate the risks of fission neutrons and various environmental factors to humans, and also useful for preclinical studies to examine efficacy and safety of new drugs.

Conflicts of interest

None.

Acknowledgements

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宇宙環境の人体影響評価（2009年度ワーキンググループ活動報告）

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Evaluation of Human Risk in Space Environment; 2009 Working Group Report

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Abstract: To study the human risk of cosmic environment (including neutrons) in the flying body and space base, three projects are in progress: (1) Morphological and, functional effects (including changes in gene expression) of fission neutrons on human thyroid tissues maintained in super-SCID (severe combined immunodeficient) mice, (2) Microsatellite mutations and leukemia in the offspring of mice in the space environment, and (3) Effects of space environment (micro-gravity) on human diseases by using specific mouse models. The above experiments are ready to be carried out in the space environment. However, the first two projects were cancelled in Japan (and also in USA and EU). As for Project 3, micro-gravity is most important factor for development and protection of chronic diseases. We are testing the effects of micro-gravity on behavior mouse models by parabolic flight.

Key words; Space Environment, Neutrons, Human Thyroid Tissues, Super-SCID Mice, Microsatellite Mutation, Leukemia, F_1 Progeny, Animal Models for Human Diseases, Parabolic Flight

宇宙での人類の生活は、将来必要に迫られることが考えられ、最先端かつ安全な宇宙飛行技術の開発と宇宙環境を利用した新技術の開発等が大きく期待される。従って、人類が宇宙生活を行うにあたり不可欠なのが、宇宙環境および宇宙放射線（宇宙基地、飛翔体内のヒト被曝の主たる放射線である中性子線）による人体影響、即ち、忘れた頃に頭をもたげてくるがんや生活習慣病の防御である。また、当の本人だけでなく、子孫への影響も無視することはできない。この克服は、宇宙医学、宇宙創薬に直結する。

1．宇宙医学研究の3本柱と宇宙実験システム開発

「宇宙環境の人体影響評価」研究ワーキンググループでは、（1）ヒト組織に対する拒絶反応をなくしヒト臓器・組織機能を数年にわたる継代維持を可能にした超重度複合免疫不全マウス（super-SCID マウス）を用いたヒト組織の形態、機能、遺伝子変異、遺伝子発現への影響研究、（2）宇宙放射線等宇宙環境の子孫におよぼす影響、特に、マイクロサテライト遺伝子突然変異と白血病の検出、（3）がん他各種生活習慣病、情動行動異常等自然発症モデルマウスや安全性高感度検出モデルマウスを用いた研究など、宇宙生活や宇宙よりの帰還後を想定した基盤研究を20年間にわたり行ってきた。これらは、我が国独自発見、開発によるものであり、人類が宇宙環境利用、あるいは、宇宙環境で生活するためには避けて通れない研究課題である。また、その進展を計るためには、米、欧、日で中断している哺乳動物個体の打ち上げ実験を可能にする策が必要である。21年度も、JAXA, NASA いずれも哺乳動物を用いた宇宙実験は実施予定がないとの回答を得たので、三菱重工にワーキングに参加してもらい共同研究を開始した。三菱重工は、H2 ロケットにはじまり、小型回収カプセル型生物（哺乳動物）搭載実験システム（副衛星、ピギー衛星）開発のトップを切っているからである。これに、宇宙創薬を加え、最先端研究開発

支援会議に応募したが、波及効果、経済効果が少ないとの意見があり採択されなかった。

3 本柱の内、ヒト組織への直接影響と次世代影響に関しては、宇宙放射線の影響が中心となり、長期滞在が必要となることから、昨秋カザフスタンを訪問し、関係者を集め、本年 4 月スタートを目処にして共同研究契約書を作成中である。3 番目の疾患自然発症モデルマウスを用いた宇宙医学（創薬）研究に関しては、重力等宇宙環境が重要なファクターである。従って、パラボリックフライトでも十分目的が達せられると考え、医薬基盤研究所の野村プロジェクト特有のモデルマウスを用い研究を開始した。

これら研究は予算化されておらず、現在、他の競争資金で研究を継続している。21 年度の成果を少し紹介する。

2．ヒト臓器・組織置換マウス等を用いた宇宙環境の人体影響

宇宙環境、宇宙放射線の人体影響を想定した地上実験を計画するにあたり用意したヒト甲状腺組織置換超重度複合免疫不全マウス（super-SCID マウス）は、少数例で環境因子等の影響を高感度に検出できる最新、独創的、革新的、究極の人体影響評価システムである。通常の宇宙実験はヒトが動物、細胞等を宇宙に運ぶが、本実験では、マウスがヒト組織をおんぶして運ぶ計画である。

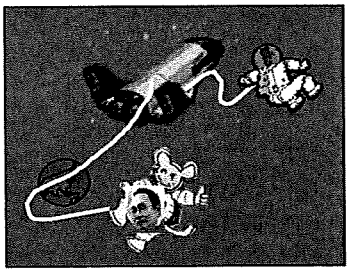

Fig. 1. SCID mice carry the human organs and tissues into the space.

1）ヒト甲状腺組織に対するγ線、中性子線の形態・機能への影響

近大原子炉 UTR-KINKI（毎時 0.2 Gy 中性子線＋0.2 Gy ガンマ線)、大阪大学ガンマセルで照射した。

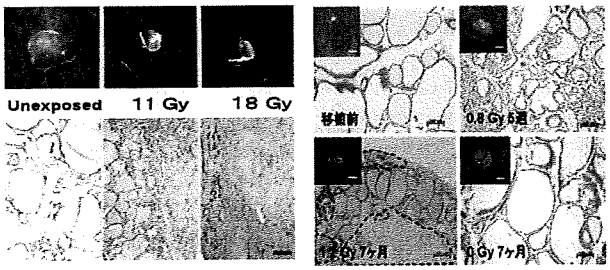

Fig. 2. Morphological changes by γ-rays and neutrons. (Mutation Research, 2009, 2010)

ガンマ線、中性子線照射で甲状腺組織の濾胞減少、壊死が起こっている(Fig. 2)。また、甲状腺ホルモンの分泌の減少が線量依存的に認められ、高い RBE 値を示した(Fig. 3)。

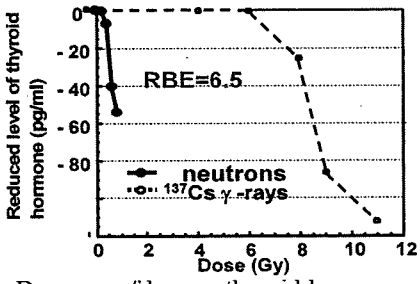

Fig. 3. Decrease of human thyroid hormone after γ-ray and neutron exposure. (Mutation Research, 2010).

2）ガンマ線、中性子線照射を受けた甲状腺組織において 4 倍以上の遺伝子発現の変化を示す遺伝子が線量依存的に増加した(RBE 値は 4. 2)。しかも、照射を受けた全てにおいて、4 倍以上の遺伝子発現の変化を示す特定の遺伝子 14 個が見つかった（ストレス、損傷、アポトーシス及び転写に関与する遺伝子。Mutation Research, 2010）。

3）今後の方針：何時でも宇宙実験を実施できる。民間企業あるいは国外機関との共同研究を計画。

3．宇宙環境の次世代に及ぼす影響

宇宙での長期滞在計画で考慮しなければならないのは宇宙環境の子孫に及ぼす影響である。遺伝影響（継世代影響実験）は、40 年間続けてきた野村のライフワークであり、Fig. 4 のごとく、放射線、化学物質が親マウスに作用すると、次世代に突然変異だけでなく、ヒトによくみられるがん、形態異常、生活習慣病も発生することを発見した。その要旨は国連レポート、大阪レポートとして紹介されている。

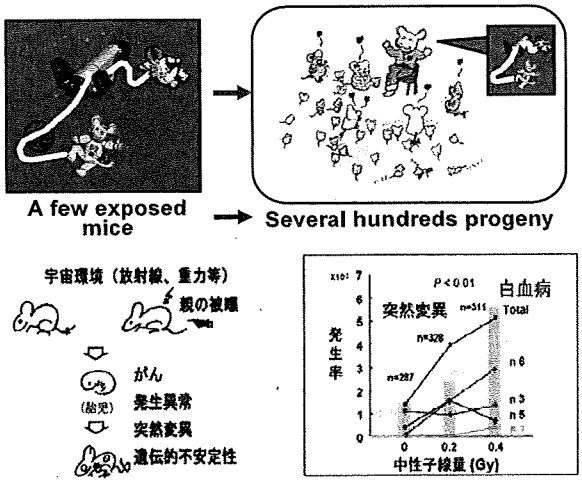

Fig. 4. Scheme of experimental procedures on transgenerational effects of radiations and results of neutron effects on microsatellite mutation and leukemia. (in preparation).

本計画は 20 年前に計画したものであり、少数（5～10 匹）の N5 マウス雄を宇宙に上げ、帰還後被曝雄マウスと正常雌マウスを交配し数千匹の F_1 マウスの発がん、突然変異等の検出を目指していたが、中断されている。現在、近大原子炉において、飛翔体内での宇宙放射線類似核分裂中性子線（中性子対ガンマ；1 対 1）を照射し、突然変異、白血病が線量依存的に誘発されていることを確認している。
今後の方針：何時でも宇宙実験を実施できる。民間企業あるいは国外機関との共同研究を計画。

4．自然発症疾患モデルマウスを用いたヒト疾病への影響研究（宇宙創薬）

宇宙環境での生活により発生すると思われる宇宙生活習慣病等の予防と治療法を考えておく必要がある。自然発症疾患モデルマウスは野村が 30 年以上前に世界中から収集した変異マウス中に見つけたものをもとにしており、医薬基盤研究所野村プロジェクトにおいて、予防、治療法の有効性、安全性を迅速、正確に判定するマウス系統を維持・開発している。

本課題に関しては、宇宙放射線よりも重力等宇宙環境の変化が重要であり、かなりの部分がパラボリックフライト等で代用できる。2009 年 12 月 17 日に、三菱重工との共同研究のひとつとして、モデルマウスの中から「パニックになりやすいマウス BH-4」を選び、パラボリックフライト（μG）実験を行った。時田偉子、行徳淳一郎が搭乗した。

Fig. 5. Parabolic flight experiment.

今回のパラボリックフライトでは、対照として最もおとなしいマウス BH-8 を選んだ。基礎実験では、BH-4 は尾を小さなクリップで挟むと、慌てふためいて噛み切るのに対し、BH-8 は平気である。両系統のマウスを 1 匹ずつケージに入れ、飛行中ビデオ撮影を行った。また、パラボリックフライト直前と直後にマウス脳等臓器を採取し、マイクロアレイを用い、遺伝子発現の 2 系統間の差、同一系統での微少重力による差を解析することにした。マイクロアレイ解析は進行中である。ビデオ解析は進んでいるので少し成果を報告する。

μG 開始から 3 回目の μG 終了までの間、不安レベル指標となる、壁際へのうずくまり（Fig. 6）の時間および回数を計測しグラフに示した（Fig. 7）。

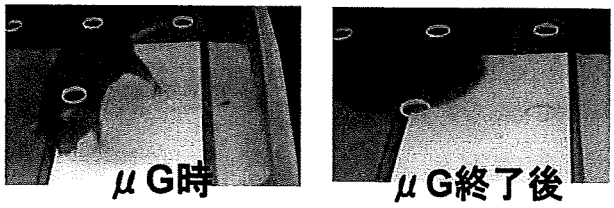

Fig. 6. Behavior before and after μ G.

うずくまる時間の割合もうずくまる回数も「パニックになりやすいマウス系統」の方が圧倒的に多く、大きな差がでた。遺伝子発現解析の結果が楽しみである。

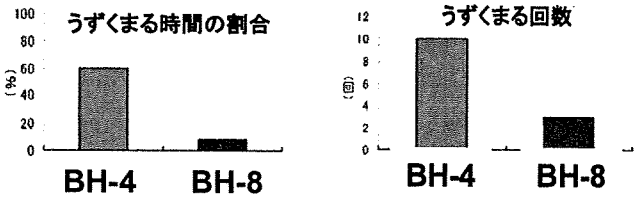

Fig. 7. Squatted position at the corner of the mouse cage.

謝辞： 課題 1 に関しては、未来開拓、文科省基盤研究 A、宇宙フォーラム、厚労省科研（HS 財団等）、武田科学財団、課題 2 に関しては、文科省基盤研究 A、宇宙フォーラム、平和中島財団、課題 3 に関しては、疾患モデル動物研究プロジェクト、武田科学財団、アミノアップ科学助成金の支援を受けた。

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UICC
global cancer control

Database of misidentified cell lines

R. Ian Freshney

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Dear Sir,

Much of current cancer and cell biology research depends on the use of cell lines cultured from normal and malignant tissue. However, ever since the time when continuous cell lines were first established, there has been a problem of the more vigorous lines contaminating and overgrowing more slowly growing cultures. This has been compounded by confusion of one cell line with another by mislabeling in routine culture or during and after cryopreservation. The result is that some 15–20% of cell lines in current use may not be what they are claimed to be. This has prompted a number of recent reports in the literature[1–7] and discussions at scientific meetings. One of the main conclusions is that there needs to be a way to alert scientists using established and frequently propagated cell lines that there is a significant risk that they may be using cell lines which are not what they need them to be. This issue of International Journal of Cancer will address this problem and wants to increase the awareness of authors submitting their work for publication and of reviewers considering the merit of the work. Restrictions and conditions will be imposed regarding proof of authentication of cell lines used and advice given on how to authenticate cell lines (see editorial and letter by W. Dirks). My purpose in this letter is to notify the scientific community of the existence and free availability of a list of cell lines which are known or suspected to be falsely identified or cross contaminated. This will allow scientists embarking on a project or reviewers considering the work for publication, to have access to a data source which will advise them on the respective cell line's authenticity. This list is available for download from: http://www.hpacultures.org.uk/services/celllineidentityverification/misidentifiedcelllines.jsp by following the link after my and Amanda Capes-Davis's names. It has been compiled from quality assurance carried out by a number of cell banks (ATCC, CellBank Australia, sDSMZ, ECACC, JCRB, and RIKEN) and published on their websites, from an entry in Wikipedia, and from reports in the scientific literature. It must be emphasized that while many of the cell lines listed are clearly and incontrovertibly not what they are supposed to be, original and authentic stocks of other lines may yet exist. Where this is believed to be the case the line is included in the second table. This list will be published (Capes-Davis *et al.*, ms in preparation).

I would request that anyone who uses this list and finds that some misidentified cell lines have been omitted or that some cell lines reported as misidentified do have authentic stocks available should contact me (i.freshney@ntlworld.com), and I will arrange to have the database updated.

The recommended procedure for anyone contemplating the use of cell lines is as follows:

- Check that the cell line that you intend to use is not listed in the above database.
- Ensure that the cell line is obtained from a properly authenticated source (and that may not be the originator), preferably from one of the recognized cell banks.
- Authenticate cell lines received from a nonauthenticated source on receipt (see letter of W. Dirks, this issue, and instruction for authors of IJC).
- Repeat authentication at intervals of 3–6 months for cell lines used for an extended study, before cryopreservation, and after thawing for further use.

It may not be possible to eliminate misidentification entirely, as new examples will continue to appear, but following these precautions should reduce the frequency and minimize the spread of the problem.

Yours sincerely,
R. Ian Freshney

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Cell line cross-contamination initiative: an interactive reference database of STR profiles covering common cancer cell lines

Wilhelm G. Dirks[1], Roderick A. F. MacLeod[1], Yukio Nakamura[2], Arihiro Kohara[3], Yvonne Reid[4], Herbert Milch[1], Hans G. Drexler[1], Hiroshi Mizusawa[3]

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[2] RIKEN—Cell Engineering Division, Tsukuba, Japan
[3] JCRB—Japanese Collection of Research Bioresources, Osaka, Japan
[4] ATCC—American Type Culture Collections, Manassas, VA

Dear Sir,

Recent reports[1–4] demonstrate the growing perception in the scientific community that cross contamination (CC) of mammalian cell lines represents a major risk for generating false scientific data. The level to which research has been compromised by the use of contaminated or misidentified cell lines has become a major concern for scientists, granting agencies, and, increasingly, scientific journals. In 2007, a group of cell biologists led by Roland M. Nardone petitioned the United States Secretary of Health and Human Services to develop an active program for cell line authentication.[5] They stressed that research and teaching tools in diverse fields of science and industry would be unimaginable without cell cultures. Despite the key importance of cell cultures, only little consensus exists regarding the technical means by which cell line identity can be controlled and how to follow through the results of any such testing.

The key problems of CC are known and chronic in nature: neglecting guidelines for quality control and disregarding adequate cell culture techniques are the main reasons why cell lines have been misidentified or cross contaminated. The incidence of CC in directly and indirectly provenanced cell lines alike[1,3] implies that the majority of false cell lines are perpetrated in originators' own laboratories, presumably by failures during the establishment of new cell lines. A plethora of reports unmasking bogus cancer cell lines, including members of the NCI-60 panel used to generate reference baseline transcriptional drug responses has triggered calls for remedial action.[5,6] Nevertheless, standard authentication procedures for testing cell line identity have yet to be defined.

Short tandem repeat (STR) microsatellite sequences are highly polymorphic in human populations, and their stability throughout the lifespan of individuals renders STR profiling (typing) ideal for forensic use. STR typing has served as a reference technique for identity control of human cell lines at Biological Resource Centers (BRCs) since the turn of the millennium.[7] Ideally, authentication involves coincident STR typing of paired donor and derived cell line samples. However, this ideal is met by a few recently established cell lines only. Most widely used cell lines are decades old and their identification is largely retrospective and multidisciplinary, combining diverse criteria such as uniqueness and the congruence of STR profiles across independent samples with the consistency of observed karyotypes with those reported by the originators.

The DSMZ as well as the ATCC, JCRB, and RIKEN repositories have generated large databases of STR cell line profiles. By using the same microsatellite loci at these BRCs, individual databases could be merged, thereby facilitating interactive searches. This work was piloted at the DSMZ to generate an international reference STR profile database for human cell lines. To render it user friendly, a simple search engine for interrogating STR cell line profiles has now been made available on the homepages of JCRB and DSMZ (http://cellbank.nibio.go.jp/cellbank_e.html, http://www.dsmz.de/STRanalysis). Registered users simply login at the search-site on the DSMZ homepage and will be guided. Aided by simple prompts, users can input their own cell line STR data to retrieve best matches with authenticated cell lines listed on the database.

Once the problem of false negatives due to discrepant representation of single STR alleles, *e.g.*, by losses of heterozygosity and bottlenecking selection—has been tackled and unambiguous search results are produced, human cell lines will need to be consistent with consensus STR reference data sets. STR profiles of all human cell lines distributed by DSMZ, JCRB, and RIKEN and one-third of the cell lines distributed by ATCC are now publicly accessible on interactive databases where match criteria have been arbitrarily set to 95%. Inevitably, reference profiles remain subject to revision until all commonly held cell lines have been STR typed across participating repositories. At present, about 2,342 such cell lines have been STR typed and are represented as reference sets on the database.

The authors of this article are currently participating in an international workgroup organized by the ATCC Standards Development Organization, (ATCC SDO) to develop a standardized methodology (protocols and procedures for STR analysis) for authenticating human cell lines. An additional

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goal of the workgroup is to establish a global database for STR profiles of human cell lines. The development of the consensus standard offers a new tool to the cell biology community that will foster reproducibility and comparability of cell lines used in different laboratories. Armed with these tools, online verification of cell line identity should prove a vital weapon to combat the havoc of cell line cross contamination which has dogged cancer research since inception.

Yours sincerely,
Wilhelm G. Dirks
Roderick A. F. MacLeod
Yukio Nakamura
Arihiro Kohara
Yvonne Reid
Herbert Milch
Hans G. Drexler
Hiroshi Mizusawa

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Aneuploidy in immortalized human mesenchymal stem cells with non-random loss of chromosome 13 in culture

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Abstract Aneuploidy (an abnormal number of chromosomes) is commonly observed in most human cancer cells, highlighting the need to examine chromosomal instability in tumorigenesis. Previously, the immortalized human mesenchymal stem cell line UE6E7T-3 was shown to undergo a preferential loss of one copy of chromosome 13 after prolonged culture. Here, the loss of chromosome 13 was found to be caused by chromosome missegregation during mitosis, which involved unequal segregation, exclusion of the misaligned chromosome 13 on the metaphase plate, and trapping of chromosome 13 in the midbody region, as observed by fluorescence in situ hybridization. Near-diploid aneuploidy, not tetraploidy, was the direct result. The loss of chromosome 13 was non-random, and was detected by analysis of microsatellites and single nucleotide polymorphism-based loss of heterozygosity (LOH). Of the five microsatellite loci on chromosome 13, four loci showed microsatellite instability at an early stage in culture, and LOH was apparent at a late stage in culture. These results suggest that the microsatellite mutations cause changes in centromere integrity provoking loss of this chromosome in the UE6E7T-3 cell line. Thus, these results support the use of this cell line as a useful model for understanding the mechanism of aneuploid formation in cell cultures.

Keywords Aneuploidy · Human mesenchymal stem cell · Loss of chromosome 13 · Loss of heterozygosity · Microsatellite instability

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Introduction

In human tumors, aneuploidy (an abnormal number of chromosomes) occurs frequently, and has been proposed to drive tumor progression by enhancing genomic instability, as originally proposed by Boveri nearly a century ago. But even now, how aneuploidy arises in cells and whether it causes tumors remains to be fully elucidated (Pellman 2007). Currently, two principal mechanisms have been proposed to generate aneuploidy through chromosome missegregation (a chromosome distribution error) during mitosis. One hypothesis states that nondisjunction yields a tetraploid intermediate, and aneuploid cells are subsequently formed through chromosomal loss from the tetraploidy (Shi and King 2005). Recently, Shi and King, using fluorescence in situ hybridization (FISH) in a human cell line, proposed that nondisjunction-induced failure of cytokinesis, coupled with tetraploidization and subsequent aberrant mitosis, underlies the aneuploidy that is frequently found in human cancer cells (Shi and King 2005). Their hypothesis is supported by evidence showing that tetraploidy is frequently present with aneuploidy in many human cancers (Galipeau et al. 1996; Nigg 2002; Olaharski et al. 2006). However, the gain of a single chromosome during meiosis of a human egg or sperm is well known, as seen with trisomies involving chromosomes 13, 18, or 21, or the sex chromosomes (resulting in XXX, XXY, or XYY genotypes). Such chromosome missegregation is also frequently observed during mitosis of human cancer cells (Lengauer et al. 1997; Weaver and Cleveland 2006) and with mosaic variegated aneuploidy syndrome (Hanks et al. 2004). These observations can be explained by a different mechanism; nondisjunction through gain or loss of a single chromosome results directly in near-diploid aneuploidy, and does not involve a tetraploid intermediate (Weaver et al. 2006).

Chromosomal instability has been studied in human mesenchymal stem cells immortalized with human papillomavirus type 16E6/E7 (HPV-16E6/E7) and human telomerase reverse transcriptase (hTERT) genes. Preferential loss of one copy of chromosome 13, yielding near-diploid aneuploidy, occurred in all three cell lines examined (Takeuchi et al. 2007). This phenomenon is not unique to these three cell lines, and is observed frequently in cultured human vascular endothelial cells (Zhang et al. 2000; Kimura et al. 2004; Wen et al. 2006; Anno et al. 2007) suggesting that it might be associated with telomere length. However, cells immortalized with hTERT gene also lost chromosome 13 during culture as shown in our studies (Takeuchi et al. 2007) and those of others (Wen et al. 2006; Anno et al. 2007). These observations suggest that near-diploid aneuploidy with loss of chromosome 13 occurs independently of telomere length in most cases.

Many proteins are involved in the maintenance of correct chromosome number during cell division. A significant number have been identified, and include members of the kinetochore complex such as Mad1, Mad2, BubR1, Bub1, Bub3, and CEMP-E. The signaling pathways involving these proteins have been extensively investigated (Bharadwaj and Yu 2004; Weaver and Cleveland 2006). Recently, direct evidence has shown that the reduced levels of these components lead to chromosome instability: cells in which the mitotic checkpoint was completely inactivated by siRNA-mediated depletion of *Mad2* or *BubR1* or by knockout of *CENP-E* showed missegregation of large numbers of chromosomes during anaphase, and the cells died due to chromosome loss (Dobles et al. 2000; Putkey et al. 2002; Weaver et al. 2003; Kops et al. 2004; Meraldi et al. 2004; Michel et al. 2004), while partial loss or mislocation of these components led to weakened signal generation at an individual, unattached kinetochore; thus, the cells survived and exhibited chromosome instability (Michel et al. 2001; Weaver et al. 2003; Kops et al. 2004). Therefore, cells with reduced levels of these components became aneuploid due to the random missegregation of one or a few chromosomes, that is, random loss of any one of the chromosomes. In contrast, UE6E7T-3 cells lost only one copy of chromosome 13. This finding suggests that there was loss of heterozygosity (LOH) of chromosome 13, not loss of spindle checkpoint components.

This question could be elucidated by analyzing for alterations in microsatellite alleles (microsatellite instability: MSI) on chromosome 13. Based on the random occurrence of microsatellites on all chromosomes, such an analysis not only provide significant information about genomic instability, but also allows the detection of the corresponding allelic deletion (LOH) (Ionov et al. 1993; Powierska-Czarny et al. 2003). MSI and LOH have been detected in a wide variety of human neoplasmic tumors, both sporadic and hereditary cancers, and are therefore used as important indicators of genomic instability, during the progression of cancer (Miturski et al. 2002). Recently, analysis of single nucleotide polymorphism (SNPs) analysis has emerged as a promising method for detecting chromosome copy number changes and LOH in cancer (Lindblad-Toh et al. 2000; Zheng et al. 2002; Pfeifer et al. 2007). Using a high-resolution analysis, we explored an SNP array kit with 2×10^5 SNPs in order to detect LOH of chromosome 13 in UE6E7T-3 cells.

In the present study, aneuploidy with loss of chromosome 13 was examined in UE6E7T-3 cells after prolonged culture. Using FISH with a specific probe for chromosome 13, the loss of chromosome 13 was found to arise through chromosome missegregation, resulting directly in near-diploid aneuploidy. Moreover, analyses of the microsatellite DNA of chromosome 13 and the SNP-based LOH assay showed that one copy of chromosome 13 in UE6E7T-3 cells had MSI and the chromosome was lost after prolonged culture.

Materials and Methods

Cell culture. A human mesenchymal stem cell line, UE6E7T-3 (JCRB1136), was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan), which entailed no ethical problems. The UE6E7T-3 cells have been described previously (Takeuchi et al. 2007). The UE6E7T-3 cells were cultured in POWEREDBY10 medium (Med-Shirotori Co.; Tokyo, Japan). Cells were seeded at a concentration of 5×10^3 cells/ml and were cultured for 6–10 d. When culture plates were subconfluent, cells were treated with 0.25% trypsin and 0.5 mM EDTA (both from Invitrogen; Tokyo, Japan), before re-plating at a density of 5×10^3 cells/ml, as described previously (Takeuchi et al. 2007). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The population doubling level (PDL) was calculated according to the following formula:

$$PDL = \log(\textit{cell output}/\textit{input})/\log 2$$

At the start of cultivation, the PDL of the UE6E7T-3 cells was 60.

Measurement of chromosome number. Metaphase chromosome spreads used for measurement of chromosome number and FISH were prepared from exponentially growing cells at various PDLs. The cells were treated in a hypotonic solution (0.075 M KCl) after exposure to 0.06 μg/ml colcemid (Invitrogen; Carlsbad, CA) for 1.5–2 h. After removal of the hypotonic solution, the cells were fixed in a solution of methanol and acetic acid (3:1). This

procedure was repeated several times with fresh fixative. The cells were then mounted on microscope slides.

In order to count the number of chromosomes, cells were stained with DAPI (4'-6-diaminido-2-phenylindol; Vector Laboratories, Inc.; Burlingame, CA) and examined using an Axioplan II imaging microscope (Carl Zeiss Microimaging GmbH; Jena, Germany) equipped with Leica QFISH software (Leica Microsystems Holding; Buckinghamshire, UK). In each assay, 50–54 metaphase spreads were scored.

Cell staining. For the staining of mitotic cells, on day 2 of culturing, the culture medium was removed and loosely adhered mitotic cells were collected in PBS using a shake-off protocol described elsewhere (Piel et al. 2001). The harvested cell suspension was fixed with 2% paraformaldehyde in PBS at room temperature for 10 min and centrifuged using a Cytospin (Shandon Cytospin 4; Thermo; Leicestershire, UK). The cells adhered to the glass coverslip were stained using a painting probe that was specific for chromosome 13 (XCP13 kit; MetaSystems; Atlussheim, Germany), described previously (Takeuchi et al. 2007).

Slides were analyzed using a motorized epifluorescence microscope (Axio Imager Z1; Carl Zeiss Microimaging) equipped with the appropriate filter sets and a MetaSystems Isis/mFISH imaging system (MetaSystems).

Microsatellite DNA assay. For detection of MSI and LOH of chromosome 13, polymerase chain reactions (PCR) were carried out using primers for specific microsatellite markers (D13S1493, D13S153, D13S788, D13S800, and D13S154) for the chromosomes 13. All primers are shown in Table 1. Microsatellite PCR was performed in a reaction volume of 20 μl that contained 10 ng of cell DNA and 10 ng of each oligonucleotide primer. AmpliTaq Gold DNA Polymerase (Applied Biosystems; Foster City, CA) was used for polymerase reaction of five markers. Reaction mixtures containing sample DNAs were heated at 96°C for 12 min, and cycled for 25 s at 95°C, and maintained for 30 s at 53°C for annealing, and for 50 s at 72°C for polymerase reaction. PCR products were applied to Capillary Electrophoresis equipment (Model HAD-GT12; eGene, Inc.; Irvine, CA) for molecular weight analysis using a QIAxcel DNA analysis kit.

SNP analysis. UE6E7T-3 DNAs were isolated using an isolation kit (Amersham BioSciences; Little Chalfont, UK). Hybridization of UE6E7T-3 DNA was performed according to a protocol of the Affymetrix Gene Chip Human Mapping 250K Nsp Array Set (Affymetrix, Inc.; Santa Clara, CA). Signal intensity of the fluorescence was gained using Gene Chip Operation Software GOS1.4 (Affymetrix). The SNP types indicating heterozygote or homozygote was estimated with Gene Chip Genotyping Analysis Software (GTYPE) 4.0 (Affymetrix).

Results

Changes in chromosomal number in long-term UE6E7T-3 cell cultures. Previously, aneuploid formation, accompanied by the loss of chromosome 13, was demonstrated in three immortalized human mesenchymal stem cell lines cultured for prolonged periods of time (Takeuchi et al. 2007). Changes in chromosomal numbers were, in detail, re-examined in a human mesenchymal stem cell line, UE6E7T-3, in order to ascertain whether or not the origin of aneuploidy in the UE6E7T-3 cells was diploid or tetraploidy.

As shown in Fig. 1, nearly 90% of the cell population contained 46 chromosomes up to PDL62, but by PDL85 this proportion had decreased markedly to 35.3%, and a new population that contained 42–45 chromosomes appeared (31.3%). From PDL100 to PDL147, population patterns remained similar, and a population that contained 44 chromosomes became dominant (26–44% of the cell population). Near-tetraploidy appeared at PDL85 but was

Table 1. Sequence of primers used for PCR analysis

Marker	Location	Sequence of primer		PCR product (bp)
D13S1493	13q13.2	FW	ACCTGTTGTATGGCAGCAGT	223–248
		Rev	GGTTGACTCTTTCCCCAACT	
D13S153	13q14.2	FW	GACTCCTGTTTCTCCTCCCTG	155
		Rev	ATTTGTGGAAAGGAGCGTAT	
D13S788	13q14.3	FW	GATTGAGGTAGGGTCCCAAG	240–270
		Rev	GCTCCATAATTGTGTGAGCC	
D13S800	13q22.1	FW	AGGGATCTTCAGAGAAACAGG	295–319
		Rev	TGACACTATCAGCTCTCTGGC	
D13S154	13q32.1	FW	GTGCTATAAAGGCTTGCTGC	243–277
		Rev	CTCTTGCCCTGGTCTTGACT	

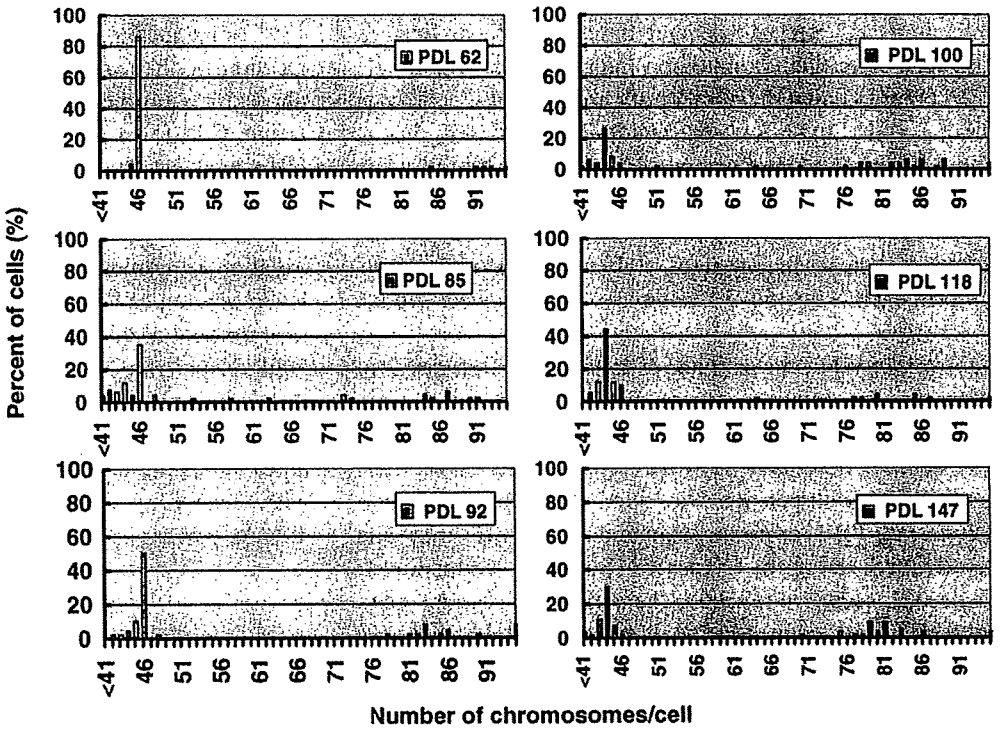

Figure 1. Changes in chromosomal numbers during prolonged cultivation of UE6E7T-3 cells. The chromosomal numbers at various culture stages (PDL62-147) were counted, using DAPI staining of 50–54 metaphase spreads for each PDL. Note the changes in chromosomal numbers from 46 per cell to 44–45 per cell in prolonged cultures. *PDL* population doubling level.

relatively infrequent. These results suggest that the loss of one or two chromosomes in UE6E7T-3 cells occurred in diploid cells at an early stage of culture.

Aneuploid cells are generated from diploids. In order to examine whether or not one of the lost chromosomes was in fact chromosome 13 and was generated directly from a diploid, whole chromosome painting–FISH analysis was applied using a DNA probe specific for chromosome 13. More than 80% of the UE6E7T-3 cells that contained 46 chromosomes had two copies of chromosome 13 at both interphase and metaphase at PDL 62, whereas the cells with 43–45 chromosomes at PDL118 contained only one copy of chromosome 13 (Fig. 2*B*). The near-tetraploidy increased gradually but remained infrequent even after a prolonged culture time, as shown in Fig. 1. If the tetraploid was generated after the loss of chromosome 13, the cells should have contained two copies of chromosome 13, and not four copies (Fig. 2*A*). In fact, the near-tetraploid cells, which constituted approximately 20% of the population at PDL118, contained two copies of chromosome 13 (Fig. 2*C*). These results indicate that near-diploid aneuploidy arises through the loss of one or two chromosomes (one of which is chromosome 13) from a diploid and that some diploid cells then spontaneously become tetraploid via cleavage failure during cell culture passage.

Chromosome missegregation during UE6E7T-3 mitosis. In order to investigate how near-diploid aneuploidy arises in UE6E7T-3 cells, segregation of chromosome 13 during anaphase and telophase of mitosis was followed by FISH analysis with a probe specific for chromosome 13. During mitosis, cells in culture detached from the glass coverslip. The loosely adhered cells were collected, fixed, and plated onto a glass coverslip using a Cytospin centrifuge (Shandon Cytospin 4).

During anaphase, the two sister chromatids separated and moved to opposite poles of the cell. Following normal segregation, two copies of chromosome 13 were observed in each daughter nucleus (Fig. 3*A*), whereas only one copy of chromosome 13 was detected in the two daughter nuclei derived from the parental cell that contained only one copy of chromosome 13 (Fig. 3*B*). When improper segregation of chromosome 13 occurred, the pattern of segregation fell into three categories. The first pattern, as shown in Fig. 3*C* and *D*, involved unequal segregation and nondisjunction whereby one daughter nucleus contained one copy and the other daughter nucleus contained two or three copies of chromosome 13. Notably, however, very few cells that contained three copies of chromosome 13 were observed at late passages. The second pattern of improper segregation, as shown in Fig. 3*E*, involved lagging of the chromosome during metaphase. The lagging chromosome did not align with the duplicated chromatid pairs on the metaphase plate, most likely resulting in loss of chromosome 13 through exclusion. The third pattern of segregation (Fig. 3*F*) involved a lagging anaphase bridge chromosome. Here, chromosome 13 was trapped in the midbody region between the dividing daughter cells. It is possible that the lagging chromosome 13 was excluded from the reforming

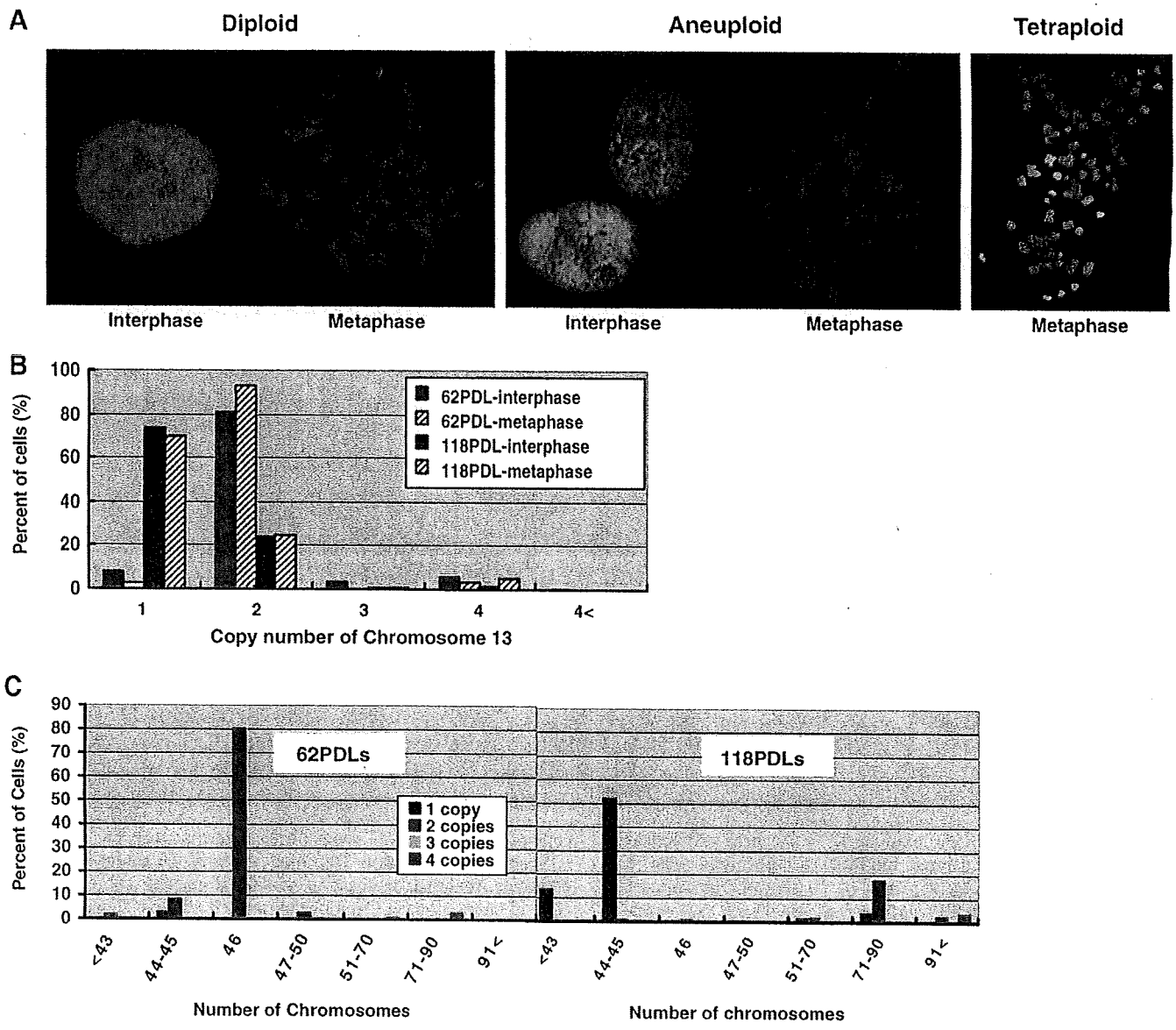

Figure 2. FISH analysis of chromosome 13 in the UE6E7T-3 cell line at early and late passages. (*A*) FISH images using a DNA probe specific for chromosome 13 (*red*) at interphase and metaphase in diploid, aneuploid, or tetraploid cells. *Blue fluorescence* shows other chromosomes. (*B*) Chromosome 13 copy numbers obtained by direct counting in metaphase spreads or interphase nuclei using FISH at PDL62 (*red*) and PDL118 (*blue*). (*C*) Analysis of chromosome 13 copy numbers in each metaphase chromosome spread. UE6E7T-3 cells contained two copies of chromosome 13 within the set of 46 chromosomes, showing a normal karyotype at PDL62, but contained one copy of chromosome 13 within the set of 44–45 chromosomes at PDL118. *PDL* population doubling level.

daughter nucleus, resulting in its loss. Together, these results suggest that near-diploid aneuploidy in UE6E7T-3 cells can arise through three different mechanisms, each of which confers errors in segregation of chromosome 13 during mitosis.

Assessment of LOH and MSI. MSI often causes chromosome instability and is a common phenomenon observed in many kinds of tumor cells (Miturski et al. 2002). MSI for DNA samples was assessed as positive in cases in which there were additional bands in the test sample that were not observed in the corresponding reference sample (Powierska-Czarny et al. 2003). By PCR analysis employing five microsatellite markers of chromosome 13 (D13S1493, D13S153, D13S788, D13S800, and D13S154), the instability of chromosome 13 was evaluated (Fig. 4*A*). In general, microsatellite markers of heterozygotes generate two bands during PCR analysis. However, as shown in Fig. 4*B*, more than two bands were detected for four of the microsatellite marker products obtained from the UE6E7T-3 cells at PDL78. It has been reported that mismatch repair during the process of DNA replication results in MSI, which produces more than two bands (Miturski et al. 2002). MSI was detected for all four microsatellite markers of