Radiation-Induced Response of Micro RNA Expression in Murine Embryonic Stem Cells

Hideshi Ishii[1,*] and Toshiyuki Saito[2,*]

[1]*Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan;* [2]*Transcriptome Profiling Group, National Institute for Radiological Science, Chiba 263-8555, Japan*

Abstract: Despite the importance of embryonic stem cells in embryology and regenerative medicine, their response to DNA damage is not fully understood. Here we studied the expression profiles of small RNAs approximately 22 nucleotides long called micro RNAs, which play an important role in developmental regulation in plants and animals. We identified a group of micro RNAs with increased expression after exposure to radiation (compared with murine fibroblasts) and characterized putative target genes. These micro RNA sequences are conserved in mice, chickens and humans with similar genomic organizations, suggesting that a common regulatory network is involved in stem cell regulation in response to DNA damage. Micro RNAs might be useful as therapeutic tools to control DNA damage response in pluripotent stem cells.

INTRODUCTION

Small RNA molecules play multiple roles in regulating gene expression, including transcriptional gene silencing [1, 2], targeted degradation of mRNA by small interfering RNAs (siRNAs) (posttranscriptional gene silencing) [3], and developmentally regulated, sequence-specific translational repression of mRNA [4]. Recent studies have revealed that some micro RNAs (miRNAs), which are an abundant class of noncoding RNAs of about 21-25 nucleotides in length, are part of a phylogenetically extensive family of small RNAs with potential roles in gene regulation [5]. These miRNAs regulate gene expression at the posttranscriptional or translational level [6,7]. They work either directly by cleaving the target mRNA using the RNA interference machinery, or indirectly by inhibiting protein synthesis [6,7].

Computational work has identified 200-255 human miRNA genes, but more recent research has predicted that the number is closer to 1000 [8] and that miRNAs repress more than one-third of human genes [9]. It has been suggested that miRNA genes are one of the most abundant classes of regulatory genes in mammals [8,9]. Since the discovery of the microRNA lin-4 in the nematode *Caenorhabditis elegans*, which regulates lin-14 translation *via* an antisense RNA-RNA interaction [10], a lot of these short regulatory RNA genes have been identified in flowering plants, worms, flies, fish, frogs and mammals [11]. Currently, about 2% of the known human genes encode microRNAs, which are essential for development [11], although their roles in cell growth, differentiation and development are only now beginning to be determined.

Embryonic stem (ES) cells were first derived from mice, and ES cells derived from various mammalian cells are now available [12,13]. They are characterized by nearly limitless self-renewal in an undifferentiated, pluri-potential state [14]. Because of their ability to differentiate into various somatic cell types, ES cells are useful in clinical and biotechnological applications [14]. Several molecules participate in the regulation of self-renewal and pluri-potency of mouse ES cells, including POU family transcription factor Oct4 [15], leukemia inhibitory factor (LIF), Stat3 [16], Sox2 [17], FoxD3 [18], and Nanog [19,20]. Considering that ES cells give rise to the diverse tissues of the mature organism, it is vital that ES cells maintain genetic stability. It is proposed that ES cells differ from their more differentiated counterparts by high levels of antioxidant defense and good DNA strand break repair capacity; reactive oxygen species are a major source of DNA damage, suggesting that stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells [21]. Recently, it was shown that the activity of ataxia-telangiectasia mutated- and Rad3-related kinase, Atr, is essential for normal cell cycle progression of exponentially proliferating mouse ES cells even in the absence of exogenously-introduced DNA damage, and Atr deregulation triggers p38alpha-dependent cell-cycle checkpoint and apoptotic responses, supporting the ability of DNA damage-induced response [22]. The alterations of miRNA expression after DNA damage in ES cells as well as in other types of cells, however, are poorly understood. In this study we assessed the involvement of miRNA expression in stem cells by comparing the responses to radiation-induced strand breaks of murine ES cells and embryonic fibroblasts, and provide data showing that miRNA may be involved in a DNA damage response network.

RESULTS AND DISCUSSION

ES Culture and Exposure to Radiation

The miRNAs are processed sequentially from primary miRNA (pri-miRNA) precursor transcripts, which were transcribed presumably by RNA polymerase II [23]. As suggested by the fact that some miRNA genes have been found

*Address correspondence to these authors at the Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan;
E-mail: hishii@ms.jichi.ac.jp

Transcriptome Profiling Group, National Institute for Radiological Science, Chiba 263-8555, Japan; E-mail: t_saito@nirs.go.jp

1573-4064/06 $50.00+.00

in close conjunction, miRNAs are sometimes transcribed in clusters and frequently in introns (25% of human miRNAs [24]), which harbor several miRNAs in tandem. After transcription, miRNAs undergo nuclear cleavage by the nuclear RNA endonuclease III, Drosha, which produces the stem-loop pre-cursors of approximately 70-80 nucleotides (pre-miRNAs) with a 5' phosphate and a 2-nt 3' overhang stem-loop [25]. The pre-miRNAs are subsequently transported across the nuclear membrane through the mechanism that the protein exportin 5 is involved in [26]. Dicer cleaves the pre-miRNAs in the cytoplasm from the ends of the pre-miRNA molecules, producing double-stranded RNA. A helicase unwinds the cleaved double-stranded RNA in a strand-specific direction [27]. The processed miRNAs are subsequently incorporated into a ribonuclear particle (RNP) complex, RNA-induced silencing complex (RISC) [28]. In the RISC the PAZ domains play an important role in binding to single-stranded RNAs, which may have implications for prediction of miRNA targets [29-31]. Thus the miRNAs are endogenous molecules and play an important role in regulation of the cellular protein expression network. Biochemical experiments will be required to define roles of the miRNAs in cellular response to genotoxic stress, and the biological effect of altered expression of miRNAs in response to DNA damage in ES cells but not in MEFs.

To assess the alterations of miRNA expression in ES cells, ES cells were cultured in medium with LIF and ES-certified serum, with a feeder layer of mouse embryonic fibroblasts (MEFs). A culture of MEFs was used as a control. Cell viability and death were assessed by direct visualization of cell morphology, trypan blue exclusion, Hoescht 33342 vital staining, and flow-assisted cytometric analysis of cells with sub-G1 DNA content, which showed that more than 95% of cells were viable before and two hours after irradiation. Total RNA was extracted from the ES culture and MEFs before and two hours after exposure to 7.5 Gy radiation. RNA quality was assessed by electrophoresis. As shown by Fig. **1**, the ribosomal RNAs were observed as sharp peaks with no apparent degradations. Assessment by chromatography indicated that total RNA from the ES culture was very similar to that of MEFs, suggesting that there were no apparent alterations or degradations in the complexity of the RNA. The micro RNAs were extracted and subjected to hybridization using a micro RNA chip.

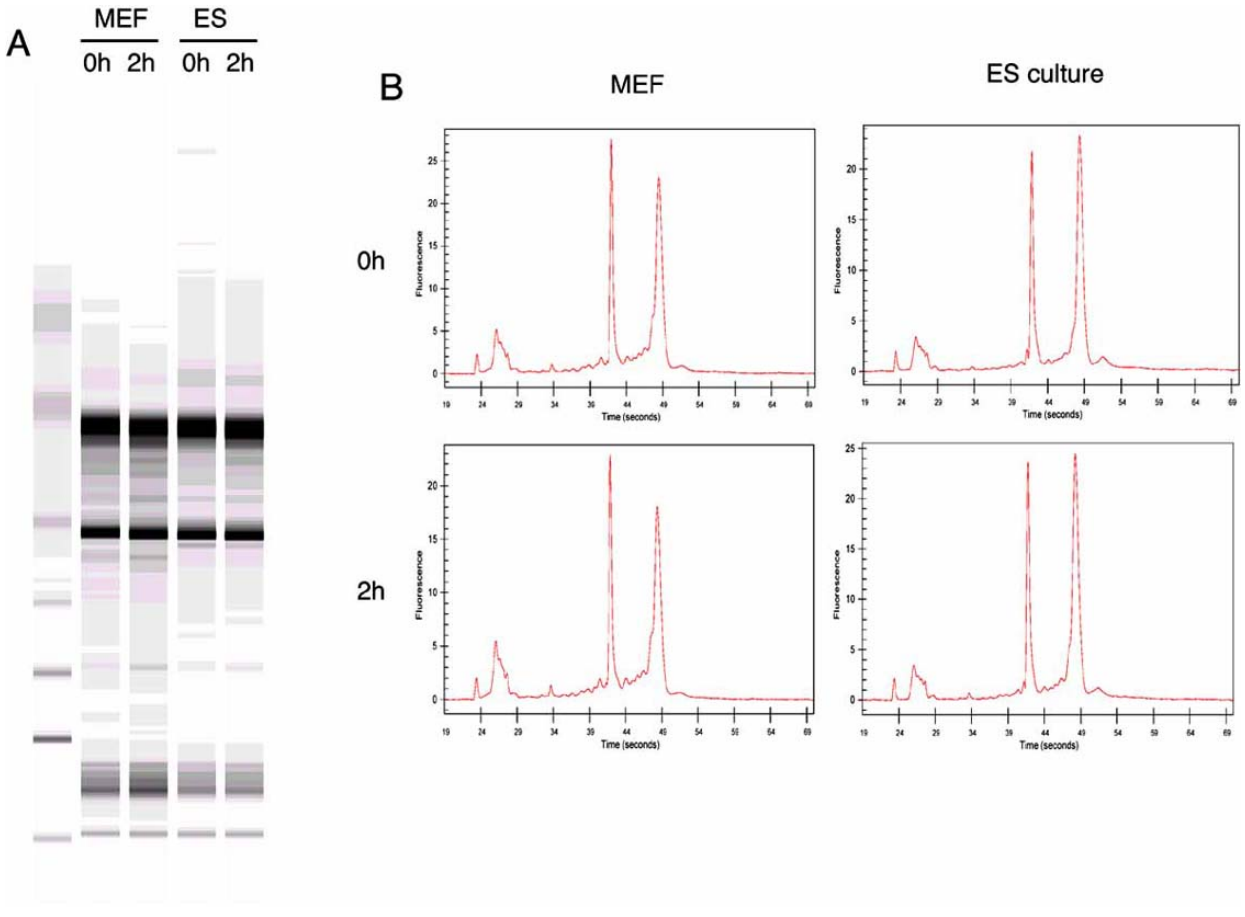

Fig. (1). Assessment of the quality of RNAs. RNA quality was assessed by electrophoresis. Gel images (A) and chromatogram (B) are shown. In panel A, the left lane indicates a ladder marker.

Radiation-Induced Alterations of miRNA Expression in ES

During differentiation, the expression level of miRNAs varies [32-36]. We recorded the intensity of the hybridized signal. Alteration was assessed by comparison with a hybridization control and classified as "absence", "margin" or "presence", which were defined as described in Materials and Methods. We studied miRNAs, which showed an increase (absence to margin; margin to presence; or absence to presence) or a decrease (presence to margin; margin to absence; or presence to absence) of the expression before and after exposure to radiation. As data was summarized in Table **1**, 16 of 181miRNAs (8.8%) from MEFs showed a decrease of expression, whereas no miRNAs from MEFs were detected showing an increase of expression. In a sharp contrast, 61 of 181 miRNAs (33.7%) from ES culture showed an increase of expression, whereas one miRNA (miR-412) from ES culture showed a decrease. Since our samples of ES culture are supposed to contain ~20 % of MEFs, the miRNA data showing decrease in ES and MEF (miR-412) might be affected by contaminated MEFs; however, the other data were unlikely overestimated because the altered expression were not coincident in ES culture and MEFs.

Prediction of Target Sequences of miRNAs, Showing Radiation-Induced Altered Expression in ES Culture

To assess the biological significance of altered expression of miRNAs, we studied miRNAs showing alterations of expression between ES culture and MEFs. By excluding data with marginal alterations, we focused on 17 miRNAs whose expression in ES culture increased from "absent" to "present". All of these cases showed the presence of expression in MEFs before and after irradiation, except for miR-19a with the absence of expression despite of radiation (Table **2**). We searched for the chromosome locations, strands, lengths of mature sequences and stem-loops at the web site (http://microrna.sanger.ac.uk/sequences/search.shtml), and the results are summarized in Table **3**. The putative target genes were predicted using computational software (http://www.microrna.org/mammalian/index.html). We analyzed the 15 miRNAs available at the web site (out of our 17) and identified 14 common candidate targets with more than five hits, including quaking homolog, KH domain RNA binding isoform HQK-7 (score: 11) and mesoderm induction early response 1 (MI-ER1; score: 12). These miRNAs and putative targets are summarized in Table **4**.

Our study unequivocally indicates that multiple target genes are involved in DNA damage response in ES cells, and that there are common putative targets. The quaking homolog KH domain RNA binding isoform HQK-7, which was identified in our search for putative targets of the miRNA group, is a member of the signal transduction and activation of RNA (STAR) family of RNA-binding proteins, whose members are evolutionarily conserved from yeast to humans and are important for a number of developmental decisions. For example, in the mouse, quaking proteins (QKI-5, QKI-6, and QKI-7) are essential for embryogenesis, whereas a closely related protein in *Caenorhabditis elegans*, germline defective-1 (GLD-1), is necessary for germ-line development [37]. On the other hand mesoderm induction early response 1 (MI-ER1), which we identified as a putative target by bioinformatics, associates with Sp1, which is involved in the negative regulation of Sp1 target promoters [38]. The repressor activity is due to interaction and recruitment of a trichostatin A-sensitive histone deacetylase 1 (HDAC1) [39]. It is suggested that the expression network of miRNAs might play a role in the regulation of differentiation and in the maintenance of genome integrity of undifferentiated stem cells.

Previous studies performed intensive cloning of or used bioinformatics approaches for miRNA genes using cultured mammalian cells, cancer cells, adult tissues and stem cells [32-36]. It would be interesting to look for uncharacterized

Table 1. Alterations of miRNA Expression in Mouse Embryonic Stem Cells and ES Cell Culture

	Increase in ES	**no changes in ES**	**decrease in ES**
Increase in MEF	Not assigned	Not assigned	Not assigned
no changes in MEF	miR-7, miR-10b,miR-96,miR-103,miR-126-3p,miR-132,miR-134, miR-136,miR-146,miR152,miR-154,miR-155,miR-182,miR188, miR-191,miR-193,miR-296,miR-297,miR-298,miR-301,miR-337, miR370,miR-376a,miR-376b,miR-379,let-7e,miR-15a,miR-15b, miR-195,miR-19a,miR19b,miR-106b,miR-25,miR-26b,miR27a, miR-29c,miR-30a-5p,miR-30b,miR30c,miR-30d,miR-30e, miR-34b,miR-99a,miR-99b,miR-101b,miR-125a,miR-130a, miR-130b,miR-181a,miR-300,miR140*,miR-424,miR-431		Not assigned
decrease in MEF	miR-1, miR-9, miR-17-3p, miR-124a, miR-128a,miR-129-5p,miR-92,miR-200b	miR-150,miR-190,miR-205,miR-213,miR-292-3p,miR-377,miR-410	miR-412

The alterations were assessed by the comparison of expression before and after irradiation. The miRNAs showing increased or decreased expression in either ES culture or MEFs are listed. See definition text.

Table 2. miRNAs with Differential Expression Between Mouse Embryonic Stem Cells and ES Cell Culture. A, Absent; P, Present Expression. See Definition in Text

miRNA ID	Sequence	MEF 0h_Detection	MEF 2h_Detection	ES+MEF 0h_Detection	ES+MEF 2h_Detection
mmu-miR-19a	MIMAT0000651	A	A	A	P
mmu-miR-19b	MIMAT0000513	P	P	A	P
mmu-miR-26b	MIMAT0000534	P	P	A	P
mmu-miR-27a	MIMAT0000537	P	P	A	P
mmu-miR-30b	MIMAT0000130	P	P	A	P
mmu-miR-30c	MIMAT0000514	P	P	A	P
mmu-miR-103	MIMAT0000546	P	P	A	P
mmu-miR-130b	MIMAT0000387	P	P	A	P
mmu-miR-152	MIMAT0000162	P	P	A	P
mmu-miR-181a	MIMAT0000210	P	P	A	P
mmu-miR-191	MIMAT0000221	P	P	A	P
mmu-miR-195	MIMAT0000225	P	P	A	P
mmu-miR-296	MIMAT0000374	P	P	A	P
mmu-miR-298	MIMAT0000376	P	P	A	P
mmu-miR-300	MIMAT0000378	P	P	A	P
mmu-miR-301	MIMAT0000379	P	P	A	P
mmu-miR-379	MIMAT0000743	P	P	A	P

miRNAs, which may play a role in DNA damage response in ES cells. For instance, the study of the regulation of undifferentiated cells in response to DNA damage would be highly informative for understanding how stem cells are protected against transformation, and how they maintain genomic integrity. We found that a group of miRNAs was expressed differentially in ES cells and MEFs after exposure to radiation. Although the exact roles of miRNA molecules in DNA damage response and a possible crosstalk with the characterized response [40], remain to be more fully defined, the present miRNAs with altered expression might be useful as monitoring markers of cellular response and therapeutic tools to modify DNA damage response in pluripotent stem cells.

At present our incomplete knowledge of bona fide miRNA gene targets, which play a role in the regulation of pluripotent stem cells and the maintenance of genome integrity, hampers a full understanding of "stemcellness", which is possibly regulated by miRNAs [32-36]. To partially overcome this limitation, we introduced radiation-induced damage into the genome whose integrity should be maintained in ES cells, surveyed miRNAs whose expression was affected, and performed computational analysis to predict gene targets. The present study indicates that the expression of 17 miRNAs increased in undifferentiated ES cells after exposure to radiation, while miRNA expression was not altered significantly in MEFs, indicating a role for miRNAs in the radiation-induced response of undifferentiated ES cells. miRNAs were conserved among human, mouse, rat and chicken. Our study suggests that a group of miRNAs play a role in regulating the radiation-induced damage response of ES cells.

MATERIALS AND METHODS

Cell Culture

Mouse embryonic stem (ES) cells (Incyte Genomics, Palo Alto, CA) were cultured on a feeder cell layer of mouse embryonic fibroblasts (MEFs) (Incyte Genomics), in DMEM supplemented with 10% ES-certified serum (Invitrogen-Gibco, Carlsbad, CA) and 1000 units/ml of leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA). ES cells were trypsinized every other day for passages on freshly prepared feeder cells; the feeders were pre-treated on 0.1% gelatin-coated dishes. Cell viability and death were assessed by direct visualization of cell morphology, trypan blue exclusion, Hoescht 33342 vital staining, and flow-assisted cytometric analysis of cells with sub-G1 DNA content. Cell cycle was assessed by flow cytometry after 1 x 10^5 cells were fixed with 70% ethanol for 10 min, incubated with RNase A, and stained with propidium iodide. Viable cells were defined as

Table 3. Characteristic miRNAs with Differential Expression Between Mouse Embryonic Stem Cells and ES Cell Culture

miRNA ID	Chromosome	Strand	Length Mature Seq	Stem-loop length
mmu-miR-19a	14	+	23	82
mmu-miR-19b	X or ND	- or ND	23	84 or 87
mmu-miR-26b	1	+	22	85
mmu-miR-27a	8	+	21	87
mmu-miR-30b	15	-	22	96
mmu-miR-30c	4 or 1	- or +	23	89 or 84
mmu-miR-103	11 or 2	+	23	86
mmu-miR-130b	16	-	22	82
mmu-miR-152	11	+	22	73
mmu-miR-181a	2	+	23	76
mmu-miR-191	9	+	22	74
mmu-miR-195	11	+	21	94
mmu-miR-296	2	-	21	79
mmu-miR-298	2	-	22	82
mmu-miR-300	12	+	22	79
mmu-miR-301	11	+	23	86
mmu-miR-379	12	+	21	66

those excluding trypan blue, with uncondensed chromatin or mitotic chromosomes visualized by vital staining with G1 or greater DNA content. These methods showed good general agreement. For radiation, 60-70% confluent ES culture and growing MEFs were washed with PBS and irradiated to ^{137}Cs, 661 keV at 7.5 Gy, and assessed as indicated. Control cells were taken into the radiation exposure source like irradiated cells but not irradiated.

RNA and Micro RNA Extraction

Total RNA was extracted from ES cells and mouse embryonic fibroblasts using the TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Assessment of total RNA amounts of ES cells and MEFs used for the culture indicated that about 85% of the total RNA of the co-culture originated from ES cells. RNA quality was assessed by electrophoresis using 2100 Bioanalyzer system (Caliper Technologies, Hopkinton, MA). RNA labeling and hybrizidation were performed using miRNA chips (Kurabo, Osaka, Japan). 12.5 μg of RNA from each sample was purified using the Qiagen kit. Briefly, RNA was dissolved in 40 μl nuclease-free water, mixed with 10 μl of the standard nucleotide (Mitsubishi Rayon, Tokyo, Japan) as an external spike control, incubated at 70° C for 2 min and cooled on ice for 2 min. Each sample (50 μl) was mixed with RLT buffer (250 μl) and with 240 μl of ethanol, which was loaded on an RNeasy MinElute spin column. After centrifugation at 9000 g for 1 min, the flow-through was mixed with 760 μl of ethanol, loaded on a new RNeasy MinElute spin column and centrifuged. It was washed by loading 500 μl of RPE buffer on the RNeasy MinElute spin column and centrifuging at 9000 g for 1 min. The column was then washed with 500 μl of 80% ethanol. After centrifugation of the column for dry-up, each sample was eluted by 20 μl of nuclease-free water.

The Platinum Bright nucleic acid labeling kit (Kreatech Biotechnology, Huntsville, AL) was used to label samples. Briefly, 16 μl of sample RNA was mixed with 2 μl of 10x labeling solution and 2 μl of ULS dye and incubated at 85° C for 30 min. Samples were purified by loading them onto a KREA pure column and centrifuging. Samples were suspended in a 130 μl reaction volume of 2xSSC/0.2% SDS solution (the final concentration) and analyzed with a micro RNA chip, Genopal MICM (Mitsubishi Rayon, Tokyo, Japan), which is a fibrous DNA chip concentrating on 181 specific miRNA gene sequences using sequence information obtained from Sanger Institute miRNA Data Base (http://microrna.sanger.ac.uk/). For hybridization, the chip was incu-

Table 4. Predicted Targets of 15 miRNAs with Differential Expression Between Mouse Embryonic Stem Cells and ES Cell Culture. Common Putative Targets Sequences of 15 miRNAs Identified in this Study are Shown as Conserved Human Sequences

Ensembl Gene ID	Gene Names	Hits	Hit by	Hit by	Descriptions
ENSG00000112531	QKI	11	30c, 195, 152, 301, 27a, 30b, 130b, 19b	hs, mmu, rno, gga	quaking homolog, KH domain RNA binding isoform HQK-7
ENSG00000125266	EFNB2	8	103, 152, 130b, 301, 19b, 27a, 19a	hs, mmu, rno, gga	Ephrin-B2 precursor (EPH-related receptor tyrosine kinase ligand 5)
ENSG00000008083	JARID2	9	103, 195, 152, 130b, 301, 19b	hs, mmu, rno, gga	Homolog of Homo sapiens "Jumonji protein"
ENSG00000196914		7	103, 152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Rho guanine nucleotide exchange factor 12 (Leukemiaassociated RhoGEF)
ENSG00000146776	ATXN7L 1	7	103, 152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Ataxin 7-like protein 1 (Fragment)
ENSG00000124487	DDX3X	7	103, 195, 181a, 301, 27a, 19a	hs, mmu, rno, gga	DEAD-box protein 3, X-chromosomal (Helicase-like protein 2) (HLP2) (DEAD-box, X isoform)
ENSG00000198160		12	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	mesoderm induction early response 1
ENSG00000155111	CDK11	7	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Cell division cycle 2-like protein kinase 6 (CDC2- related protein kinase 6) (Death-preventing kinase) (Cyclin-dependent kinase 11)
ENSG00000134698	EIF2C4	9	103, 195, 152, 130b, 301	hs, mmu, rno, gga	Eukaryotic translation initiation factor 2C 4 (eIF2C 4) (eIF-2C 4) (Argonaute-4)
ENSG00000090905	TNRC6A	7	30c, 152, 301, 26b, 30b	hs, mmu, rno, gga	Trinucleotide repeat-containing gene 6A protein (CAG repeat protein 26) (Glycin-tryptophan protein of 182 kDa) (GW182 autoantigen) (GW1 protein) (EMSY interactor protein)
ENSG00000165891	E2F7	6	195, 152, 130b, 301, 27a	hs, mmu, rno, gga	E2F transcription factor 7
ENSG00000134852	CLOCK	6	195, 152, 301, 19b, 19a	hs, mmu, rno, gga	Circadian locomoter output cycles kaput protein (hCLOCK)
ENSG00000155545		7	30c, 130b, 301, 27a, 30b	hs, mmu, rno, gga	No description
ENSG00000177200	CHD9	7	30c, 152, 181a, 301, 30b	hs, mmu, rno, gga	chromodomain helicase DNA binding protein 9
ENSG00000099250	NRP1	7	30c, 152, 130b, 301, 30b	hs, mmu, rno, gga	Neuropilin-1 precursor (Vascular endothelial cell growth factor 165 receptor)
ENSG00000170989	EDG1	8	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Sphingosine 1-phosphate receptor Edg-1 (Sphingosine 1phosphate receptor 1) (S1P1)
ENSG00000112182	BACH2	6	103, 195, 152, 130b, 30	hs, mmu, rno, gga	Transcription regulator protein BACH2 (BTB and CNC homolog 2)

(Table 4. Contd….)

Ensembl Gene ID	Gene Names	Hits	Hit by	Hit by	Descriptions
ENSG00000137075	RNF38	6	152, 130b, 301, 19b, 27a	hs, mmu, rno, gga	RING finger protein 38
ENSG00000113300	KIAA1194	8	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Carbon catabolite repressor protein 4 homolog (Cytoplasmic deadenylase) (CCR4 carbon catabolite repression 4-like) (Nocturnin homolog)
ENSG00000130699	TAF4	6	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Transcription initiation factor TFIID subunit 4 (Transcription initiation factor TFIID 135 kDa subunit) (TAFII-135) (TAFII-130)
ENSG00000165029	ABCA1	6	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	ATP-binding cassette sub-family A member 1 (ATP-binding cassette transporter 1) (ATP-binding cassette 1) (ABC-1) (Cholesterol efflux regulatory protein)
ENSG00000108924	HLF	6	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Hepatic leukemia factor
ENSG00000140873	ADAMTS 18	6	195, 152, 130b, 181a, 301	hs, mmu, rno, gga	ADAMTS-18 precursor (A disintegrin and metalloproteinase with thrombospondin motifs 18) (ADAM-TS18)
ENSG00000165699	TSC1	6	195, 301, 19b, 27a, 19a	hs, mmu, rno, gga	Hamartin (Tuberous sclerosis 1 protein)
ENSG00000109184		6	103, 152, 130b, 301, 27a	hs, mmu, rno, gga	No description
ENSG00000006576	PHTF2	6	30c, 19b, 26b, 30b, 19a	hs, mmu, rno, gga	putative homeodomain transcription factor 2
ENSG00000110958		6	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Telomerase-binding protein p23 (Hsp90 co-chaperone) (Progesterone receptor complex p23)
ENSG00000054267	ARID4B	6	130b, 301, 19b, 27a, 19a	hs, mmu, rno, gga	AT rich interactive domain 4B (RBP1- like) isoform 1; RBP1-like protein; breast carcinoma-associated antigen BCAA; breast cancer-associated antigen BRCAA1; Rb-binding protein homolog; retinoblastoma-binding protein 1-like 1; SIN3Aassociated protein 180; retinoblastoma binding protein 1-like 1
ENSG00000125149	UPF0183 protein	6	130b, 301, 19b, 26b, 19a	hs, mmu, rno, gga	No description
ENSG00000173744	HRB	6	103, 152, 130b, 301, 19a	hs, mmu, rno, gga	Nucleoporin-like protein RIP (HIV-1 Rev-binding protein) (Rev interacting protein) (Rev/Rex activation domain-binding protein)
ENSG00000056586	MNAB	6	152, 130b, 301, 19b, 19a	hs, mmu, rno, gga	Membrane-associated nucleic acid binding protein (RING finger protein 164)

(Table 4. Contd….)

Ensembl Gene ID	Gene Names	Hits	Hit by	Hit by	Descriptions
ENSG00000118257	NRP2	6	103, 152, 130b, 301, 19b, 27a, 19a	hs, mmu, rno, gga	Neuropilin-2 precursor (Vascular endothelial cell growth factor 165 receptor 2)
ENSG00000055070		9	152, 130b, 301, 27a	hs, mmu, rno, gga	putative MAPK activating protein PM20,PM21
ENSG00000132640	BTBD3	7	152, 130b, 181a, 301	hs, mmu, rno, gga	BTB/POZ domain containing protein 3
ENSG00000156642	SDFR1	6	103, 152, 130b, 301	hs, mmu, rno, gga	stromal cell derived factor receptor 1 isoform a
ENSG00000168502	KIAA0802	6	152, 130b, 181a, 301	hs, mmu, rno, gga	No description
ENSG00000182621	PLCB1	7	130b, 301, 26b	hs, mmu, rno, gga	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 1 (Phosphoinositide phospholipase C) (PLC-beta-1) (Phospholipase C-beta-1) (PLC-I) (PLC-154)
ENSG00000085832	EPS15	6	152, 130b, 301	hs, mmu, rno, gga	Epidermal growth factor receptor substrate 15 (Protein Eps15) (AF-1p protein)
ENSG00000106511	MEOX2	8	152, 130b, 301	hs, mmu, rno, gga	Homeobox protein MOX-2 (Mesenchyme homeobox 2) (Growth arrest-specific homeobox)
ENSG00000152601	MBNL1	7	103, 181a, 301	hs, mmu, rno, gga	Muscleblind-like protein (Triplet-expansion RNA-binding protein)
ENSG00000164742	ADCY1	7	130b, 301, 19a	hs, mmu, rno, gga	Adenylate cyclase type I (ATP pyrophosphate-lyase 1) (Ca(2+)/calmodulin activated adenylyl cyclase)
ENSG00000015171	ZMYND11	6	19b, 19a	hs, mmu, rno, gga	Zinc finger MYND domain containing protein 11 (Adenovirus 5 E1A- binding protein) (BS69 protein)

bated at 50° C for 16 hours. The chip was washed in 1 ml of 2xSSC/0.2% SDS solution at 50° C for 1 min, and this was repeated 40 times. The chip was then immersed in 1 ml of 2xSSC solution at 50° C, and this was repeated 10 times. Hybridization signals were detected and analyzed using Image-Pro (Media Cybernetics, Silver Spring, MD). Data was normalized so that the external controls would be equal in each group. Signals were classified into three categories:

- "absent" if SI - BG < Bkg + 2SD;
- else "marginal" if SI - BG < Bkg + 3SD;
- else "present"

where SI is the value of the sample signal, BG is the background signal of blank spots on an array, Bkg is the average of the control signals and SD is the standard deviation of the control signals. The normalized data in ES culture and MEFs were compared before and after exposure to radiation.

ACKNOWLEDGEMENTS

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from Japan Science and Technology Agency.

REFERENCES

[1] Pal-Bhadra, M.; Bhadra, U.; Birchler, J.A. *Mol. Cell*, **2002**, *9*, 315.
[2] Birchler, J.A.; Pal-Bhadra, M.; Bhadra, U. (E. G. J. Hannon, Editor) *RNAi, A Guide to Gene Silencing,* Cold Spring Harbor Laboratory Press: New York, **2003**, pp. 23–42.
[3] Hammond, S.M.; Caudy, A.A.; Hannon, G.J. *Nat. Rev. Genet.,* **2001**, *2,* 110.
[4] Carrington, J.C.; Ambros, V. *Science*, **2003**, *301*, 336.
[5] Lim, L.P.; Glasner, M.E.; Yekta, S.; Burge, C.B.; Bartel, D.P. *Science*, **2003**, *299*, 1540.
[6] Bartel, D.P. *Cell*, **2004**, *116*, 281.
[7] Ambros, V. *Nature*, **2004**, *431*, 350.

[8] Berezikov, E.; Guryev, V.; van de Belt, J.; Wienholds, E.; Plasterk, R.H.; Cuppen, E. *Cell,* **2005**, *120*, 21.
[9] Lewis, B.P.; Burge, C.B.; Bartel, D.P. *Cell*, **2005**, *120*, 15.
[10] Lee, R.C.; Feinbaum, R.L.; Ambros, V. *Cell,* **1993**, *75*, 843.
[11] Alvarez-Garcia, I.; Miska, E.A. *Development*, **2005**. *132*, 4653.
[12] Evans, M.J.; Kaufman, M.H. *Nature*, **1981**, *292*, 154.
[13] Martin, G.R. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 7634.
[14] Smith, A. *Embryonic stem cells*, Cold Spring Harbor Laboratory Press: New York, **2001**.
[15] Palmieri, S.L.; Peter, W.; Hess, H.; Scholer, H.R. *Dev. Biol.,* **1994,** *166*, 259.
[16] Matsuda, T.; Nakamura, T.; Nakao, K.; Arai, T.; Katsuki, M.; Heike, T.; Yokota, T. *EMBO J.,* **1999**, *18*, 4261.
[17] Avilion, A.A.; Nicolis, S.K.; Pevny, L.H.; Perez, L.; Vivian, N.; Lovell-Badge, R. *Genes Dev.,* **2003**, *17*, 126.
[18] Hanna, L.A.; Foreman, R.K.; Tarasenko, I.A.; Kessler, D.S.; Labosky, P.A. *Genes Dev.,* **2002**, *16*, 2650.
[19] Chambers, I.; Colby, D.; Robertson, M.; Nichols, J.; Lee, S.; Tweedie, S.; Smith, A. *Cell,* **2003**, *113*, 643.
[20] Mitsui, K.; Tokuzawa, Y.; Itoh, H.; Segawa, K.; Murakami, M.; Takahashi, K.; Maruyama, M.; Maeda, M.; Yamanaka, S. *Cell*, **2003**, *113*, 631.
[21] Saretzki, G.; Armstrong, L.; Leake, A.; Lako, M.; von Zglinicki, T. *Stem Cells*, **2004**, *22*, 962.
[22] Jirmanova, L.; Bulavin, D.V.; Fornace, A.J. Jr. *Cell Cycle*, **2005**, *4*, 1428.
[23] Lee, Y.; Jeon, K.; Lee, J.T.; Kim, S.; Kim, V.N. *EMBO J.,* **2002**, *21*, 4663.
[24] John, B.; Enright, A.J.; Aravin, A.; Tuschl, T.; Sander, C.; Marks, D.S. *PLoS Biol*., **2004**, *2*, e363.
[25] Lee, Y.; Ahn, C.; Han, J.; Choi, H.; Kim, J.; Yim, J.; Lee, J.; Provost, P.; Radmark, O.; Kim, S.; Kim, V.N. *Nature*, **2003**, *425*, 415.
[26] Yi, R.; Qin, Y.; Macara, I.G.; Cullen, B.R. *Genes Dev.,* **2003**, *17*, 3011.
[27] Khvorova, A.; Reynolds, A.; Jayasena, S.D. *Cell*, **2003**, *115*, 209.
[28] Hutvagner, G.; Zamore, P.D. *Science*, **2002**, *297*, 2056.
[29] Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. *Nature*, **2003,** *426*, 465.
[30] Song, J.J.; Liu, J.; Tolia, N.H.; Schneiderman, J.; Smith, S.K.; Martienssen, R.A.; Hannon, G.J.; Joshua-Tor, L. *Nat. Struct Biol.,* **2003**, *10*, 1026.
[31] Yan, K.S.; Yan, S.; Farooq, A.; Han, A.; Zeng, L.; Zhou, M.M. *Nature*, **2003,** *426*, 468.
[32] Murchison, E.P.; Partridge, J.F.; Tam, O.H.; Cheloufi, S.; Hannon, G.J. *Proc. Natl. Acad. Sci. USA*, **2005**, *102*, 12135.
[33] Houbaviy, H.B.; Dennis, L.; Jaenisch, R.; Sharp, P.A. *RNA*, **2005**, *11*, 1245.
[34] Rao, M. *Dev Biol*., **2004,** *275*, 269.
[35] Suh, M.R.; Lee, Y.; Kim, J.Y.; Kim, S.K.; Moon, S.H.; Lee, J.Y.; Cha, K.Y.; Chung, H.M.; Yoon, H.S.; Moon, S.Y.; Kim, V.N.; Kim, K.S. *Dev Biol*., **2004**, *270*, 488.
[36] Houbaviy, H.B.; Murray, M.F.; Sharp, P.A. *Dev. Cell.,* **2003**, *5*, 351.
[37] Saccomanno, L.; Loushin, C.; Jan, E.; Punkay, E.; Artzt, K.; Goodwin, E.B. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 12605.
[38] Ding, Z.; Gillespie, L.L.; Mercer, F.C.; Paterno, G.D. *J. Biol. Chem*., **2004**, *279*, 28009.
[39] Ding, Z.; Gillespie, L.L., Paterno, G.D. *Mol. Cell. Biol*., **2003**, *23*, 250.
[40] Sancar, A.; Lindsey-Boltz, L.A.; Unsal-Kacmaz, K.; Linn, S. *Annu. Rev. Biochem*., **2004**, *73*, 39.

Received: 05 January, 2006 **Revised: 31 May, 2006** **Accepted: 01 June, 2006**