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Paternal irradiation perturbs the expression of circadian genes in offspring

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The circadian system represents a complex network which influences the timing of many biological processes. Recent studies have established that circadian alterations play an important role in the susceptibility to many human diseases, including cancer. Here we report that paternal irradiation in mice significantly affects the expression of genes involved in rhythmic processes in their first-generation offspring. Using microarrays, the patterns of gene expression were established for brain, kidney, liver and spleen samples from the non-exposed offspring of irradiated CBA/Ca and BALB/c male mice. The most over-represented categories among the genes differentially expressed in the offspring of control and irradiated males were those involved in rhythmic process, circadian rhythm and DNA-dependent regulation of transcription. The results of our study therefore provide a plausible explanation for the transgenerational effects of paternal irradiation, including increased transgenerational carcinogenesis described in other studies.

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1. Introduction

Recent animal studies have provided strong evidence that exposure to mutagens not only results in mutation induction in the germline of treated parents, but can also destabilise the genomes of their non-exposed offspring [1]. According to the results of numerous studies, mutation rates in the germline and somatic tissues of non-exposed offspring of irradiated male mice are significantly elevated [2–7]. It has also been reported that in the first-generation (F_1) offspring of irradiated males transgenerational instability affects the frequency of chromosome aberrations [8], as well as mutations at protein-coding genes [6,9] and tandem repeat DNA loci [2–7]. Taken together, these data imply that the transgenerational effects in the offspring of irradiated male mice may be explained by a genome-wide destabilisation which manifests in many all tissues. The phenomenon of transgenerational instability was also observed in the offspring of male mice exposed to chemical mutagens and anticancer drugs, thus implying that exposure to a variety of mutagens can destabilise the F_1 genomes [10,11]. Curiously enough, maternal exposure to ionising radiation does not affect stability of their F_1 offspring [4,5].

As destabilisation of the offspring's genomes results in accumulation of mutations, a number of health-related traits can be affected by this process. Given that tumour progression is attributed to accumulation of oncogenic mutations, these results imply that elevated mutation rates would contribute towards an elevation in the number of tumour initiation events and could enhance tumour progression in the destabilise offspring of exposed parents. Indeed, according to the results of some studies the incidence of cancer among carcinogen-challenged offspring of irradiated males is elevated [12,13]. Taken together, these observations indicate that transgenerational effects can be regarded as a substantial component of the genetic risk factors for humans.

It should be stressed that further analysis of the clinical implications of transgenerational effects is currently limited as the mechanisms underlying this phenomenon remain unknown. Evidence accrued to date, such as the non-Mendelian segregation of transgenerational effects, suggests that this phenomenon can be attributed to epigenetic mechanisms [2–7,11]. It has also been proposed that parental exposure to ionising radiation or other mutagens may alter the epigenetic landscape of germ cells and that such epigenetic marks, following the transmission to subsequent generations, can destabilise their genomes [1]. To evaluate the transgenerational effects of paternal irradiation, the amount of endogenous DNA damage in the first-generation offspring of exposed male mice was measured [6]. This study revealed abnormally high levels of both single- and double-strand DNA breaks in the somatic tissues of F_1 offspring. Further analysis revealed that the elevated levels of DNA damage cannot be attributed either to (i) compromised DNA repair, as normal repair profiles were

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observed or (ii) oxidative stress, as the level of oxidatively damaged nucleotides in the offspring did not significantly differ from that in controls [6]. Considering these data, it would appear that destabilisation of the F_1 genome could be related to the deregulation of other systems responsible for the maintenance of genome stability. Here we present the results of a systematic mouse study aimed to establish the effects of paternal irradiation on gene expression in their non-exposed F_1 offspring.

2. Materials and methods

2.1. Animals

BALB/c and CBA/Ca mice were obtained from Harlan (Bicester, UK) and housed at the Division of Biomedical Services, University of Leicester. Seven-week-old male mice were given whole-body acute irradiation of 1 Gy of X-rays delivered at 0.5 Gy min^{-1} (250 kV constant potential, HLV 1.5 mm Cu, Pantak industrial X-ray machine, Connecticut, USA) and were then mated 8 weeks after to non-exposed females from the same strain. Tissue samples were taken from 8-week-old male offspring of control and irradiated males, sacrificed between 9 and 11:30 am. For each strain, RNA samples were analysed from 6 offspring of both control and irradiated males. All animal procedures were carried out under the UK Home Office project licence No. PPL 80/2267.

2.2. Microarray analysis

RNA samples were extracted from whole brain, kidney, liver and spleen using TRI reagent (Sigma–Aldrich, Poole, UK). RNA quality was determined by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Wokingham, UK).cDNA synthesis was performed using the SuperScript^{TM} Double-Stranded cDNA synthesis kit (Invirogen, Paisley, UK). cDNA was then labelled using the NimbleGen One-Colour DNA Labelling Kit (Roche Diagnostics, Burgess Hill, UK) and hybridised to NimbleGen 12x135K Mouse Expression Arrays (080925_mm8_60mer_expr_HX12 and 090901_mm8_60mer_expr_HX12, Roche Diagnostics, Burgess Hill, UK) using the NimbleGen Hybridisation System 4 according to the manufacturer's protocol. The slides were scanned using a GenePix 4200 AL scanner and the GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, USA).

The microarray data were extracted and normalised using the Robust Multichip Average (RMA) algorithm [14] from the NimbleScan v2.5 software (Roche Diagnostics, Burgess Hill, UK). The normalised data were then imported into the SYSTAT13 software (http://www.systat.com/). Lists of differentially expressed genes were generated at the false discovery rate (FRD) <0.05 using the Benjamini and Hochberg method. Gene ontology (GO) analysis was performed using the BioProfiling.de analytical web portal [15]. The Bonferroni-adjusted *P*-values were used to determine statistical over-representation of GO categories. Network analysis was performed using the Cytoscape 3.0.2 software (http://www.cytoscape.org/).

The microarray data were deposited on the Gene Expression Omnibus (GEO) database, submission number GSE42621.

2.3. Quantitative PCR

Single-stranded cDNA synthesis was performed using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA). qPCR was performed on a DNA Engine Opticon 2 PCR machine (Bio-Rad, Hemel Hempstead, UK) using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Loughborough, UK). The expression of the target genes *Arntl* and *Per2* was quantified against the housekeeping genes *Aprt* and *Actb*. Primer sequences used in qPCR are given in supplementary Table S1. The qPCR data were analysed using the Opticon Monitor v3.1 (Bio-Rad, Hemel Hempstead, UK) and REST 2009 (http://rest.gene-quantification.info/) software.

3. Results

3.1. Experimental design

To evaluate the effects of paternal irradiation on the pattern of gene expression, RNA samples extracted from the F_1 male offspring of irradiated CBA/Ca and BALB/c male mice were compared with those from age-matched controls (Fig. 1a). These strains have previously been used in our studies on radiation-induced transgenerational instability in mice, where male mice were exposed to the same dose of acute X-rays [3,4,6,7]. As according to the results of these studies the F_1 mutation rates were considerably elevated across multiple tissues, here we established the pattern of gene expression in four tissues – brain, kidney, liver and spleen.

3.2. Gene expression analysis

To delineate the effects of paternal irradiation we used a three-way ANOVA. This technique was used as it allowed the estimation of independent effects of three factors (tissue, strain and paternal treatment) on the pattern of gene expression. Fig. 1b summarises the results of the ANOVA analysis. Microarray analysis revealed highly significant effects of paternal irradiation on the pattern of expression of 56 transcripts (across 45 genes). The list of differentially expressed genes is given in supplementary Table S2. The expression of 34 transcripts (61%) was up-regulated in the offspring of irradiated males (Fig. 2). The changes in *Per2* and *Arntl* mRNA

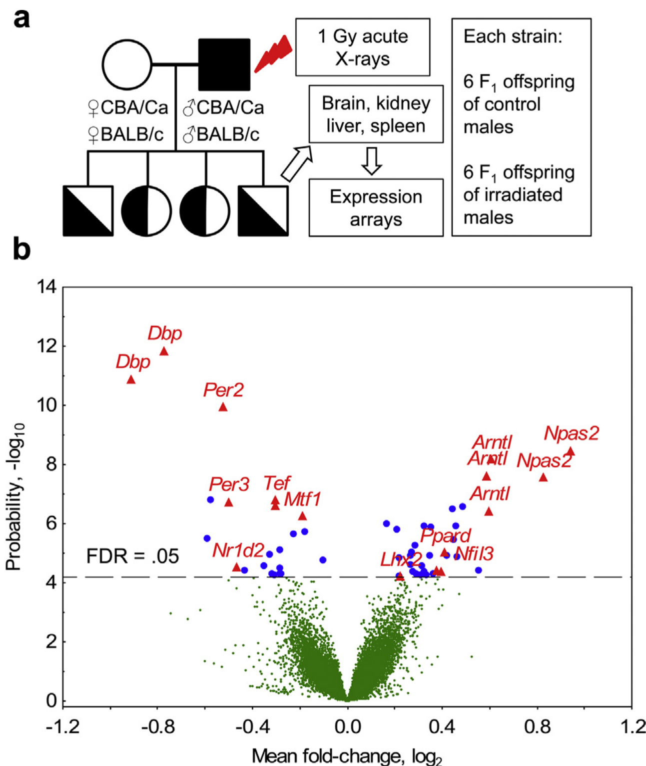

Fig. 1. (a) Design of microarray study. (b) Volcano plot showing the effects of paternal irradiation on gene expression in the offspring. Probabilities for the effects of paternal irradiation (ANOVA) and mean values of fold-changes in the F_1 offspring for each transcript are shown. Differentially expressed transcripts (FDR < 0.05) of the genes involved in rhythmic process and circadian rhythm are shown in red; other significantly deregulated transcripts are shown in blue.

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