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The role of DOCK10 in the regulation of the transcriptome and aging

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Abstract

DOCK10, a guanine-nucleotide exchange factor (GEF) for Rac1 and Cdc42 Rho GTPases whose expression is induced by interleukin-4 (IL-4) in B cells, is involved in B cell development and function according to recent studies performed in Dock10-knockout (KO) mice. To investigate whether DOCK10 is involved in regulation of the transcriptome, changes in the gene expression profiles (GEPs) were studied by microarray in three cellular models: DOCK10 expression induced by doxycycline (dox) withdrawal in a stable inducible HeLa clone, DOCK10 expression induced by transient transfection of 293T cells, and wild type (WT) *versus* KO mouse spleen B cells (SBC). In all three systems, DOCK10 expression determined moderate differences in the GEPs, which were functionally interpreted by gene set enrichment analysis (GSEA). Common signatures significantly associated to expression of DOCK10 were found in all three systems, including the upregulated targets of HOXA5 and the SWI/SNF complex, and EGF signaling. In SBC, Dock10 expression was associated to enrichment of gene sets of Cmyb, integrin, IL-4, Wnt, Rac1, and Cdc42 pathways, and of cellular components such as the immunological synapse and the cell leading edge. Transcription of genes involved in these pathways likely acts as a feedforward mechanism downstream of activation of Rac1 and Cdc42 mediated by DOCK10. Interestingly, a senescence gene set was found significantly associated to WT SBC. To test whether DOCK10 is related to

aging, we set out to analyse the survival of the mouse colony, which led to the finding that Dock10-KO mice lived longer than WT mice. Moreover, Dock10-KO mice showed slower loss of their coat during aging. These results indicate a role for Dock10 in senescence. These novel roles of DOCK10 in the regulation of the transcriptome and aging deserve further exploration.

Keywords: Bioinformatics, Cell biology, Immunology, Molecular biology, Physiology

1. Introduction

The Dedicator-of-cytokinesis (DOCK) protein family comprises 11 guanosine nucleotide exchange factors (GEFs) for Rho GTPases characterized by bearing a GEF domain called CZH2 [1,2]. Rho GTPases are small proteins that cycle between two states, GDP- or GTP-bound. GEFs promote the exchange of GDP by GTP that gives rise to the active GTP-bound form. Rho GTPases play roles in actin dynamics, vesicular trafficking, gene transcription, cell-cycle progression, and cell adhesion [3, 4, 5, 6, 7, 8]. Among them, Rac proteins are involved in formation of lamellipodia and membrane ruffles and Cdc42-related proteins in formation of filopodia [9, 10]. DOCK10 acts as a GEF for Rac1 and Cdc42 [10,11]. Accordingly, DOCK10 induces increased ruffling and filopodial activities in HeLa cells [10]. Consistent with its cell remodelling function, DOCK10 has been related to amoeboid-type movement in melanoma cells [12], breast cancer invasion [13], and epithelial to mesenchymal transition of squamous carcinoma cells [14]. DOCK10 expression is also upregulated in aggressive cases of papillary thyroid carcinomas [15].

DOCK10 is expressed at its highest levels in circulating leukocytes, principally in T and B cells, and is upregulated by interleukin-4 (IL-4) in B cells [16, 17]. Mouse models targeting expression of Dock10 in B cells or in the whole organism suggest that Dock10 is involved in B cell development and function [18, 19, 20, 21].

In this paper, we have investigated whether overexpression/deletion of DOCK10 affects the transcriptome, using two human cell line models, one with stable inducible and other with transient expression of DOCK10, and a mouse model targeting global expression of Dock10. In all three models, changes in the transcriptome were identified that suggest activation of certain transcription factors, pathways and cellular components, particularly those involved in formation of the cell leading edge and the immunological synapse. Interestingly, a senescence gene set was associated WT mice compared to Dock10-KO mice, which led us to perform a survival analysis, whose results showed that Dock10-KO mice had prolonged survival and slower loss of their coat during aging, suggesting a novel role for Dock10 in senescence.

Taken together, our data suggest novel roles for DOCK10 in the regulation of the transcriptome and aging.

2. Material and methods

2.1. Stable inducible cell clones

Three stable clones of the human cervix carcinoma epithelial HeLa cell line, designated as C33, C23, and the parental clone HeLa-tTA, generated using the plasmids of the tet-off system and previously reported [10], were used. C33 has regulatable expression of the HA-tagged DOCK10.1 isoform, and C23 and HeLa-tTA are negative controls. The clones were cultured in plastic flasks containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS; Biowhittaker, Cambrex, East Rutherford, NJ), 50 U/ml penicillin, 50 U/ml streptomycin, 2.5 µg/ml amphotericin B, and 2 mM L-glutamine (DMEM-C, for "complete") at 37 °C in a humid atmosphere of 5% CO₂. C33 and C23 were supplemented also with 1 µg/ml puromycin, 0.5 mg/ml G418, and 2 ng/ml doxycycline (dox), and HeLa-tTA with 1 µg/ml puromycin and 2 ng/ml dox but without G418. All the three HeLa cell clones grow as monolayers with fibroblast-like morphology, and were maintained subconfluent by detachment with trypsin 0.05%-EDTA 0.02% in PBS (EuroClone, Milano, Italy) and routine subculture. Replicate aliquots of the three clones were washed free of dox and reseeded in DMEM-C containing 2 ng/ml dox or lacking dox, then cultured during 24 h.

2.2. Transient transfections

Human embryonic kidney 293T cells were cultured in DMEM-C, transfected at subconfluency with pSG5-HA-DOCK10.1 or empty pSG5 plasmid [10] using lipofectamine, and cultured for 24 h.

2.3. Primary cells and mice

Spleen B cells (SBC) from 12-week-old C57BL/6N mice, Dock10-KO and WT, were obtained as previously described [20]. Briefly, Dock10 KO and WT mice cohorts were established from two heterozygous breeding pairs. The Dock10^{tm1a(EU COMM)Hmgu/leg} mutant allele (GenBank accession no. JN946611.1) bears the L1L2_Bact_P cassette just upstream of exon 4, and loxP sequences flanking exon 4, resulting in lack of Dock10 expression due to early protein truncation. Mice were killed by carbon dioxide asphyxiation and exsanguination. Spleens were excised, crushed through 100 µm cell strainers, and rinsed with PBS containing 2 % FCS, 0.06 % citrate, and 0.2 I.U./ml heparin. Splenocyte suspensions were treated with ACK Lysing Buffer (Lonza, Basel, Switzerland). Negative selection was

performed using the Pan B Cell Isolation Kit, mouse (Miltenyi Biotec, Bergisch Gladbach, Germany).

Survival analysis was performed in two cohorts of Dock10-KO (N = 26) and WT (N = 28) mice using GraphPad Prism software (San Diego, CA).

Mice were bred at the animal facility of the University of Murcia and at the specific-pathogen-free (SPF) animal facility of the Biohealth Research Institute of Murcia (IMIB-Arrixaca). All the procedures were performed according to the Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the Ethics Committee for Animal Experimentation of the Virgen de la Arrixaca University Clinic Hospital.

2.4. RNA isolation and microarray analysis

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples were quantitated on a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). RNA quality was examined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano Kit. RNA integrity numbers (RINs) ranged between 9.9 and 10 for HeLa samples, between 9.4 and 9.7 for 293T samples, and between 9 and 9.8 for SBC samples.

RNA samples from HeLa clones, transfected 293T cells, and SBC were labeled using Agilent Two Color Quick Amp Labeling and RNA Spike-In kits, according to the manufacturer's protocol. Reference and test samples were labeled with cyanine-3-CTP (Cy3) and cyanine-5-CTP (Cy5), respectively.

HeLa-C33-dox+/HeLa-C33-dox−, performed twice from biological replicates, were used as reference/test pairs, respectively, and these microarray experiments were designated as *HeLa-DOCK10* (N = 2). HeLa-tTA-dox+/HeLa-tTA-dox− and HeLa-C23-dox+/HeLa-C23-dox− pairs (N = 1 each) were used as controls for dox withdrawal, and these microarray experiments were designated as *HeLa-CTRL* (total N = 2). *HeLa-DOCK10* and *HeLa-CTRL* experiments were performed using Agilent SurePrint G3 Human Gene Expression 8 × 60K v2 Microarrays targeting 50,599 biological features.

RNA from 293T cells transfected with HA-DOCK10.1 or empty plasmid were used as tests, and untransfected 293T cells as reference. These microarray experiments were designated as *293T-DOCK10* (N = 1) and *293T-pSG5* (N = 1), respectively, and were performed using Agilent Whole Human Genome Microarrays (4 × 44K) targeting 19,596 Entrez Gene RNAs.

RNA samples from SBC isolated from Dock10-KO mice and WT mice were used as tests, and a pooled sample composed of equimolar amounts of RNA from 5 KO mice was used as reference [20]. These microarray experiments were designated as *KO* (N

= 10) or WT (N = 10), and were performed using Agilent Mouse GE 4 × 44K v2 Microarrays targeting 39,430 Entrez Gene RNAs.

The labeled cRNAs were mixed together and hybridized onto the microarray slides using the Agilent Gene Expression Hybridization kit. After hybridization, the microarray slides were washed and scanned in an Agilent G2565CA DNA Microarray Scanner. Datasets, expressed as $\log_{10}$ ratios for test *versus* (vs) reference, were computed from images by the Agilent Feature Extraction software, using normalization by linear and Lowess methods.

The three dataset series were deposited at the Gene Expression Omnibus (GEO) database under the superseries accession number GSE114243 [GSE114227 (HeLa), GSE114226 (293T), and GSE73760 (SBC, *KO* from GSM1911156 to GSM1911165, and *WT* from GSM1911136 to GSM1911145)].

Datasets were transformed into $\log_2$ ratios. Entities flagged as *Not Detected* in any of the HeLa and 293T experiments, and exceeding half the total SBC experiments, were excluded. Comparison between values from *CTRL* and *DOCK10* experiments (HeLa) and from *KO* and *WT* experiments (SBC) was performed using the Student *t* test, and *p* values were computed using the Benjamini-Hochberg false discovery rate (FDR) correction. Mean values from the different conditions were subtracted, and fold changes (FCs) calculated using the formulas $2^{(DOCK10 - CTRL)}$, $2^{(DOCK10 - pSG5)}$, and $2^{(WT - KO)}$ for positive changes, and $-2^{-(DOCK10 - CTRL)}$, $-2^{-(DOCK10 - pSG5)}$, and $-2^{-(WT - KO)}$ for negative changes, in HeLa, 293T, and SBC experiments, respectively.

2.5. Gene set enrichment analysis (GSEA)

GSEA was performed to determine whether both biological states (*CTRL* and *DOCK10*, for HeLa, *pSG5* and *DOCK10*, for 293T, or *KO* and *WT*, for SBC) present significant differences for *a priori* defined set of genes, included in the Molecular Signatures database [MSigDB (<http://software.broadinstitute.org/gsea/>)] [22, 23].

3. Results and discussion

3.1. Gene expression profiles (GEPs) of HeLa cells, 293T cells and SBC with differential expression of DOCK10

To explore whether DOCK10 affects gene expression, a microarray study in HeLa clones with inducible expression of DOCK10, in which we had been previously observed that DOCK10 induces activation of Rac1 and Cdc42, and membrane ruffling and filopodia [10], was performed. After filtering the data by expression, 27,155 features representing 17,823 genes were significantly expressed. None of the genes were differentially expressed between *DOCK10* and *CTRL* according to

the Student's *t* test at adjusted (*adj.*) $p < 0.05$. Transient transfection of 293T cells with DOCK10 is efficient [10], and a single microarray experiment using these cells transiently transfected with DOCK10 was performed. After filtering the data by expression, 20,999 features representing 12,446 genes were significantly expressed. Differential expression could not be statistically analysed though only 49 genes and 16 genes were > 2 -fold upregulated and downregulated, respectively, in *DOCK10* compared to *pSG5* (data not shown), indicating that changes were mild in 293T cells.

To explore whether DOCK10 affects gene expression *in vivo*, a microarray study in SBC from Dock10-KO mice was performed. These mice show decreased numbers of SBC [20]. After filtering the GEPs of SBC by expression, 30,140 features representing 19,074 genes were compared between *WT* and *KO*. None of the genes were differentially expressed according to the Student's *t* test at *adj.* $p < 0.05$.

3.2. Gene set enrichment analysis (GSEA)

GSEA is appropriate to identify small but consistent gene expression changes in a particular direction. A search for gene sets which were significantly enriched at FDR $q < 0.25$ for HeLa (*DOCK10* vs *CTRL*), 293T (*DOCK10* vs *pSG5*), and SBC (*WT* vs *KO*) was conducted. Several recurrent significant signatures were found within the Chemical and Genetic Perturbations collection of the MSigDB. First, a gene set comprising the upregulated targets of homeobox A5 (HOXA5), was associated to *DOCK10* with high significance in HeLa cells, and also significantly to *DOCK10* in 293T cells and to *WT* SBC (Fig. 1A). HOXA5 is a helicase that regulates morphogenesis and differentiation [24]. Second, an epidermal growth factor (EGF) signaling gene set was associated with high significance to *WT* SBC, and that same gene set or a related neuregulin 1 (NRG1) signaling gene set were significantly associated to *DOCK10* in HeLa cells and 293T cells, respectively (Fig. 1B). Last, a gene set comprising the upregulated targets of switch/sucrose non fermentable (SWI/SNF) related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 (SMARCA2), a helicase which regulates chromatin condensation [25], was associated with high significance to *DOCK10* in HeLa cells. Expression of components of the SWI/SNF complex was also significantly associated to *DOCK10* in 293T cells and to *WT* SBC (Fig. 1C). Considering that common pathways identified in the different models could not be selected by chance, recurrence of these signatures suggests that DOCK10 induces cellular changes that drive induction of HOXA5, EGF or SWI/SNF activity.

Within the Canonical Pathways collection, signatures for the c-Myb pathway and integrin pathway were among the most significantly associated to *WT* SBC, as well as those for diverse cytokine (IL-2, IL-4, IL-6, IL-12) signaling pathways, the Wnt, Rac1, and Cdc42 signaling pathways (Fig. 2A). Finally, signatures for the immunological synapse and the cell leading edge, comprised in the Cellular Components

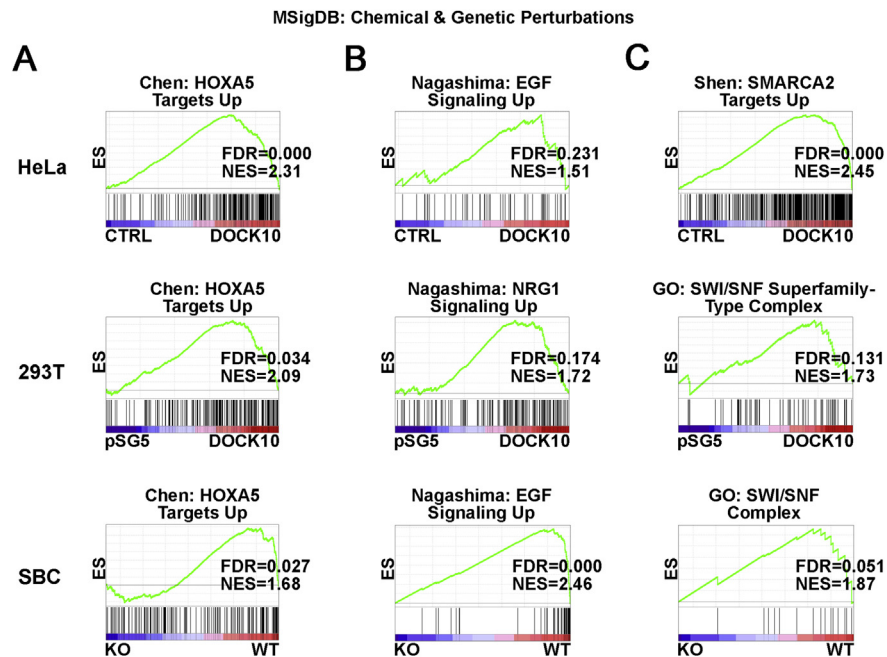


Fig. 1. Common and related signatures of the Chemical & Genetic Perturbations collection of the MSigDB associated to expression of *DOCK10* in HeLa cells, 293T cells and SBC, according to GSEA. (A) *Chen: HOXA5 Targets Up* gene set. (B) *Nagashima: EGF Signaling Up* or closely related *Nagashima: NRG1 Signaling Up* gene sets. (C) *Shen: SMARCA2 Targets Up* gene set or the related *GO: SWI/SNF Superfamily-Type Complex* or *GO: SWI/SNF Complex* gene sets. In HeLa and 293T, CTRL and pSG5 were the reference conditions for *DOCK10*, respectively. In SBC, KO was the reference condition for WT. ES, enrichment score; FDR, false discovery rate; GO, gene ontology; NES, normalized enrichment score.

collection, were among the most significantly associated to WT SBC (Fig. 2B). In the 293T model, the cell leading edge signature was also significantly associated to *DOCK10*, and those related to the mitotic spindle, such as the microtubule organizing center and kinesins, were significantly associated to *DOCK10* in 293T (Fig. 2C) and HeLa cells (Fig. 2D). Therefore, transcriptome changes in SBC fit well with what was previously known about *DOCK10*, i.e., its induction by IL-4 and its role as a GEF for Rac1 and Cdc42. Moreover, it is known that the EGF signaling pathway activates Rac1 and Cdc42 [26], and the IL-4 signaling pathway induces activation of the Rac1, Cdc42, and Wnt signaling pathways [27]. These findings suggest that *DOCK10*-mediated transcription of genes involved in the integrin, IL-4, Wnt, Rac1, and Cdc42 signaling pathways likely operates as a feedforward mechanism that acts downstream of *DOCK10*-mediated activation of Rac1 and Cdc42 and enhances the effects of *DOCK10* on the cytoskeletal dynamics.

3.3. *DOCK10* and senescence

A senescence gene set of the Chemical and Genetic Perturbations collection of the MSigDB was found significantly associated to WT SBC (Fig. 3A) [28]. Bearing

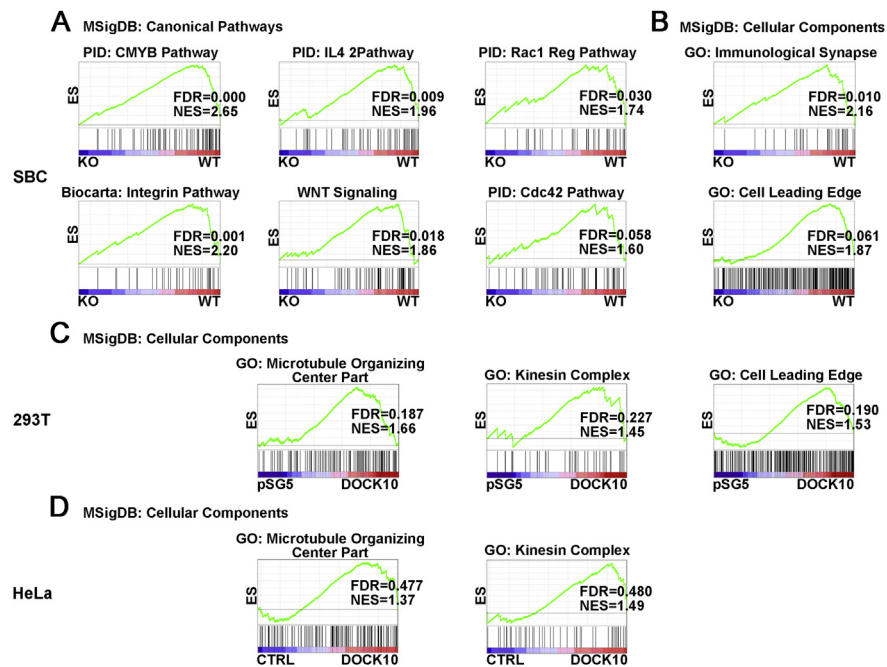


Fig. 2. Canonical pathways and cellular components of the MSigDB regulated by DOCK10 in SBC, 293T and HeLa cells, according to GSEA. (A) Canonical pathways in SBC. (B) Cellular components in SBC. (C) Cellular components in 293T cells. (D) Cellular components in HeLa cells. In SBC, *KO* was the reference condition for *WT*. In 293T and HeLa, *pSG5* and *CTRL* were the reference conditions for *DOCK10*, respectively. ES, enrichment score; FDR, false discovery rate; GO, gene ontology; NES, normalized enrichment score; PID, Protein Interaction database.

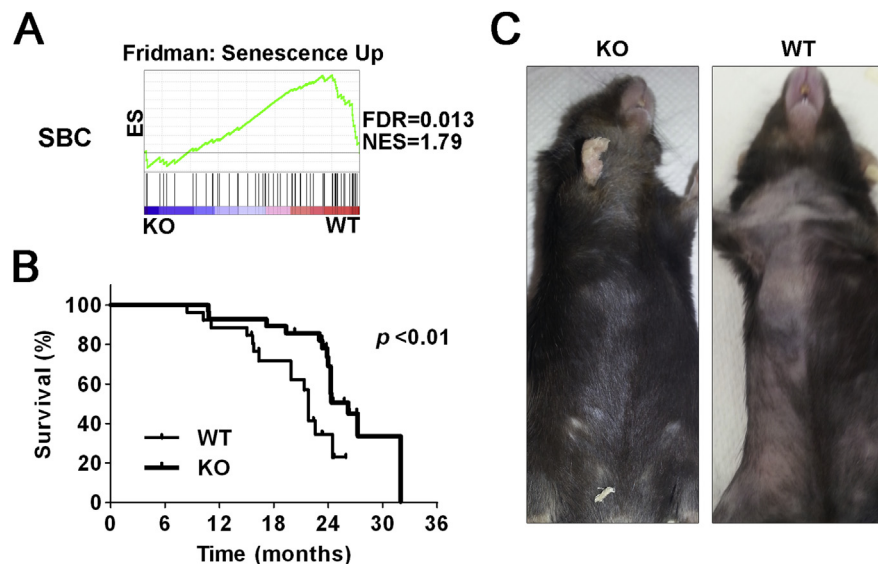


Fig. 3. Survival analysis of Dock10-KO mice and WT mice. (A) Enrichment of the *Fridman: Senescence Up* gene set in WT condition compared to the reference *KO* condition according to GSEA. ES, enrichment score; FDR, false discovery rate; NES, normalized enrichment score. (B) Survival analysis of two cohorts of Dock10-KO mice (N = 26) and WT mice (N = 28) using GraphPad Prism software. (C) Coat of 24-month-old Dock10-KO mice and WT mice, showing reduced hair loss in KO mice.

in mind that its homologous gene, DOCK11, is related to aging [21, 29], we set out to investigate whether DOCK10 affected aging. With this purpose, we conducted a survival analysis of the Dock10-KO mice and WT mice, using larger mice cohorts than in our previous study [20]. This analysis showed that Dock10-KO mice lived significantly longer than WT mice (Fig. 3B). Moreover, at older ages, Dock10-KO mice kept their fur longer than WT mice (Fig. 3C). These results indicate that Dock10 could play a role in senescence.

In summary, expression of DOCK10 affected the transcriptome in HeLa and 293T cell lines and in primary mouse B cells, leading to enrichment of gene sets comprising the targets of HOXA5, the SWI/SNF chromatin remodeling complex, the EGF signaling pathway and, in mouse B cells, the IL-4 signaling pathway. Transcription of genes involved in these pathways likely functions as a feedforward mechanism downstream of DOCK10-induced activation of Rac1 and Cdc42. Last, survival, phenotype, and transcriptome analyses of Dock10-KO and WT mice indicate that Dock10 could play a role in aging and that it could be driven through transcriptional regulation of senescence-related genes.

Declarations

Author contribution statement

Natalia Ruiz-Lafuente: Performed the experiments.

Alfredo Minguela, Manuel Muro: Contributed reagents, materials, analysis tools or data.

Antonio Parrado: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study has been deposited at the Gene Expression Omnibus (GEO) database, under the superseries accession number GSE114243.

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