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Isolation of a novel gene down-regulated by v-*src***

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Abstract We have isolated a novel gene which was expressed in normal rat cells, but completely suppressed in cells transformed by v-*src*. The molecularly cloned cDNA was about 1.8 kb in size, containing an open reading frame composed of 464 amino acid residues. DNA sequence analysis showed that there was no corresponding gene in the data bases. Besides the suppression of gene expression in the v-*src* transformed cells, its expression was also strongly suppressed in cells transformed by other oncogenes such as v-*abl*, v-*fps*, v-*mos*, v-*sis*, v-K-*ras*, and polyomavirus middle T, but not affected in cells transformed by human papillomavirus type 16 E6E7 and polyomavirus large T. We named the gene *drs* for a gene down-regulated by v-*src*.

Key words: *drs* gene; *src* gene; Transformation; Down-regulation

1. Introduction

The v-*src* oncogene, the transforming gene of Rous sarcoma virus, encodes a protein kinase that phosphorylates tyrosine residues of specific target proteins. Its normal counterpart, c-*src*, is thought to play an important role in intracellular signal transduction which is related to cell proliferation [1] and differentiation [2,3], and the c-*src* kinase is tightly controled under normal conditions. However, the uncontrolled and activated protein-tyrosine kinase of v-*src* leads to cellular transformation [4]. Although v-*src* easily transforms many rodent cell lines that have been established from fibroblasts, such as NIH3T3, 3Y1 and F2408, its transforming ability is suppressed in primary rodent cells. In fact, it was shown that primary rat embryo fibroblasts (REF) had intracellular functions capable of suppressing v-*src*-mediated transformation [5].

Concerning alterations in gene expression upon transformation by v-*src*, several cellular genes are induced, such as 9E3/CEF4, c-*jun*, c-*myc*, c-*fos* and *Egr*-1, and some other genes are suppressed, such as fibronectin, MARCKS (a protein kinase-C substrate), DAN and 322 [6–16]. Although the relationship between v-*src*-induced transcriptional changes of these genes and the phenotypic alterations of transformed cells is unclear, down-regulated genes should be more important as candidates for transformation suppressor genes. Indeed, DAN and 322 genes have been shown to be tumor-suppressor genes [14–16].

In this study, we report a gene whose expression is down-regulated by v-*src*.

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**The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number D78359.

2. Materials and methods

2.1. Cells

No.7 cell line [17] is a hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) deficient strain of F2408 rat established cell line. SRD1 [18] is a No.7 cell line transformed by the SR-D strain of Rous sarcoma virus. No.7/HPVE6E7, No.7/PyMT and No.7/K-ras cells are No.7 cell lines transformed by human papillomavirus type 16 (HPV16) E6E7, polyomavirus middle T (PyMT), and v-K-*ras* genes, respectively, as described previously [19]. No.7/fps, No.7/mos, No.7/sis and No.7/PyLT cells are No.7 cell lines transformed by v-*fps*, v-*mos*, v-*sis*, and polyomavirus large T (PyLT) genes, respectively. OS7-1 cell line [20] is a No.7 cell line containing a temperature-sensitive viral mutant of the v-*src* gene (OS122) [21,22]. The cell line shows transformed phenotypes at 35°C (permissive temperature) and changes to normal phenotypes at 39°C (non-permissive temperature). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum.

2.2. Northern blot analysis

Cellular RNA was isolated by a guanidium thiocyanate-CsCl method [23]. Total RNA (20 μg) was separated on a 1% agarose gel containing 2.2 M formamide and transferred to a nitrocellulose filter. The filter was hybridized with a probe labeled by a multiprime DNA labelling system (Amersham) at 41°C overnight in a solution of 50% formamide, 0.6 M NaCl, 60 mM sodium citrate, 0.2% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 50 μg/ml of herring sperm DNA. The filter was washed with 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS at 50°C and autoradiographed.

2.3. Southern blot analysis

Isolation of cellular high-molecular-weight DNA and DNA blot hybridization were performed as described previously [24,25]. DNA from each sample was digested with restriction enzymes, separated by 1% agarose gel electrophoresis and transferred to a nylon filter. Hybridization and washing were performed as described for Northern blot analysis.

2.4. Transfection and isolation of cDNA

A cDNA expression library prepared with REF mRNA and the expression vector pAP3-neo was a gift from H. Nojima. SRD1 cells (5×10^5) were transfected with 20 mg of the REF cDNA expression library by a calcium phosphate method modified by Chen and Okayama [26]. After transfection, the cells were selected with a neomycin analogue G418 (400 mg/ml) for 2 weeks and morphologically flat clones were isolated from surviving colonies with cloning cylinders. From one of the flat clones, we rescued a plasmid containing an about 1.0-kb cDNA insert by the Hirt method through cell fusion with COS7 cells as described by Okayama and Berg [27]. The insert was used as a probe to isolate a full length of cDNA. Thus, a plasmid which had a cDNA insert about 1.8 kb was obtained. DNA was sequenced by a Li-Cor sequencer, model 4000 series, using a SequiTherm Cycle Sequencing Kit (Epicenter Technology).

2.5. In vitro transcription-translation analysis

Plasmid DNA (1.0 μg) was incubated with [^{35}S]methionine (translation grade, Amersham) in a coupled transcription-translation assay system (TNT Kit, Promega). Products were analyzed by electrophoresis in SDS/5–20% polyacrylamide gradient gels. Then the gels were fixed in methanol-acetic acid, immersed in Amplify (Amersham), dried and subjected to autoradiography.

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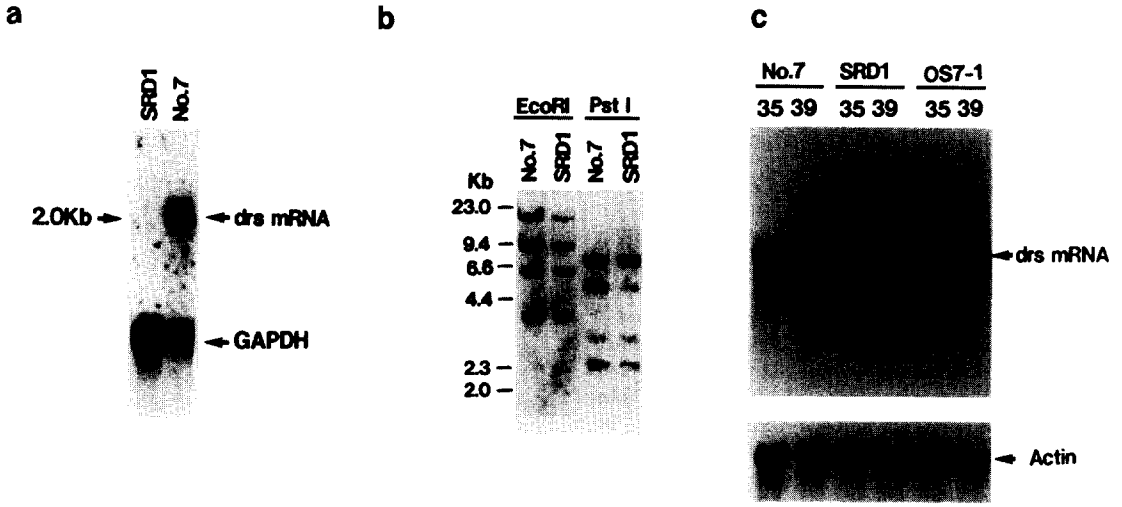

Fig. 1. Expression and genomic structure of *drs* gene in No.7, SRD1 and OS7-1 cells. (a,c) Northern blot analysis. Total RNA (20 μg) was subjected to Northern blot analysis with a multiprime labeled *drs* cDNA (between −5 to 1300 nt, Fig. 2), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), or β-actin DNA. (b) Southern blot analysis. *Eco*RI-or *Pst*I-digested genomic DNA (20 μg) prepared from No.7 and SRD1 were hybridized with the *drs* cDNA probe used for the above Northern blot analysis.

3. Results

3.1. Down-regulation of drs gene in v-src transformed cells

To isolate a gene capable of suppressing v-src transformation, we transfected a cDNA expression library prepared from REF mRNA into a v-*src* transformed cell line (SRD1), isolated several flat revertant clones and rescued plasmids carrying REF cDNAs from those revertant clones through cell fusion with COS7 cells. To examine whether expression of the genes corresponding to those cDNAs was affected by v-*src* transformation, we first performed Northern blot analyses of the corresponding genes in an untransformed cell line, No.7, and a v-*src* transformed cell line. For the analyses, each rescued cDNA was used as a probe. The results showed that expression of a gene hybridized with a 1.0-kb cDNA was almost completely suppressed in v-*src* transformed cells, as shown in Fig. 1a. An about 2.0-kb mRNA hybridizing with the cDNA was recognized in No.7 at a relatively high level, but almost completely disappeared in SRD1. REF and another rat cell line 3Y1 also showed a level of mRNA similar to No.7 cells (data not shown). Down-regulation of the mRNA was also observed in two other independently isolated No.7 cell lines transformed by v-*src* (data not shown). To verify that the disappearance of the mRNA was not due to rearrangement of the genomic DNA but was due to the regulation of mRNA expression, Southern blot analysis of the genomic DNA prepared from No.7 and SRD1 cells was carried out. As shown in Fig. 1b, the hybridization patterns of SRD1 DNA were identical to those of No.7 DNA. These results suggested that disappearance of the mRNA in SRD1 cells was not due to loss or alteration of the genomic DNA. We also examined expression levels of the mRNA in a No.7 cell line, OS7-1, containing a temperature-sensitive v-*src* mutant: the expression was similar to that in No.7 cells at 39°C (non-permissive temperature), whereas the expression was almost completely suppressed at 35°C (permissive temperature) (Fig. 1c). These results indicate that the down-regulation of the 2.0-kb mRNA is dependent on the expression of the v-*src* oncogene.

3.2. drs gene encoded a transmembrane protein which had three consensus repeats of the selectin family

To isolate a full length of cDNA, screening of the REF cDNA library was performed with a probe of the 1.0-kb cDNA insert. We obtained positive clones with about 1.8-kb cDNA inserts. The longest cDNA we obtained consisted of 1830 base pairs (Fig. 2a), supposedly covering almost the entire 2.0-kb mRNA because an average size of polyadenylation sequences is usually 2–300 bases. The cDNA had one long open reading frame (ORF) which extended from nucleotide position 66 to 1522, encoding 464 amino acids. Although no termination codon in frame was found in the 5′ region upstream of the presumed initiation codon, the sequence around the initiation codon (CTCGCCATGG) was consistent with Kozak's consensus sequence (GCCA/GATGG) [28]. The molecular weight of the protein which encoded 464 amino acids was estimated to be 51.6 kDa. This was consistent with the result obtained from an in vitro transcription-translation experiment, by which an about 52 kDa product was identified (Fig. 3). Although homology search was performed at the nucleotide level by both BLAST and FASTA programs through the Genbank data base, no corresponding gene was found, suggesting that this gene was a novel one. We named this gene *drs* for a gene *d*own-*r*egulated by v-*src*. Further homology search of *drs* protein revealed that the protein had one transmembrane domain (TM) (amino acid residues 376–413) and three consensus repeats (CR) (amino acid residues 57–115, 120–174 and 262–317) which were conserved as various numbers of CR in the extracellular domain of the selectin family, such as P-selectin, E-selectin and L-selectin. Based on these results, it is likely that the *drs* gene encodes a transmembrane protein with the three CR in the extracellular domain.

To examine the genetical conservation of the *drs* gene, we carried out Southern blot analyses of genomic DNA obtained from mouse, monkey and human. As shown in Fig. 4, hybridized bands were recognized in all samples, suggesting that the *drs* gene was conserved in the various mammalian cells.

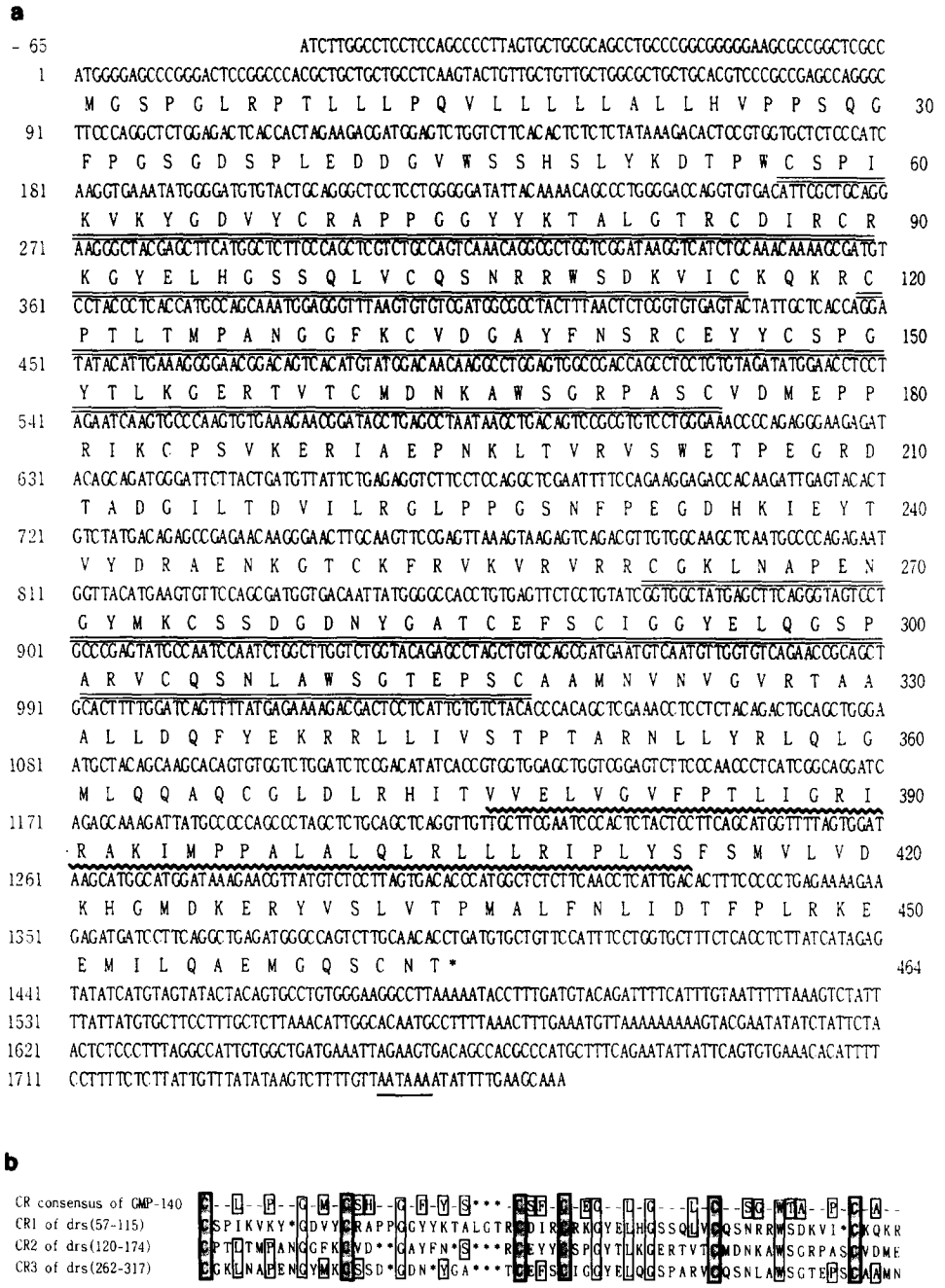

Fig. 2. (a) The nucleotide sequence of *drs* cDNA and its deduced amino acid sequence. The nucleotides and amino acids are numbered from the beginning of translation initiation. Double underlines indicate consensus repeat domains and a wavy line shows a transmembrane domain. A stop codon is shown by an asterisk. A polyadenylylation signal is underlined. (b) Comparison of the CR amino acid sequences of Drs protein with a consensus sequence of P-selectin CR (GMP-140). The top is the consensus sequence of CR in P-selectin (GMP-140) [29]. Below this, the three CR sequences of Drs are shown. The boxed residues show conserved residues in at least five out of the nine repeats in GMP-140. The shaded areas show the position of conserved cysteine residues. Asterisks show deletion of the corresponding amino acid residues.

3.3. drs gene expression was also down-regulated in cell lines transformed by other oncogenes

To examine whether the suppression of *drs* gene expression was specific to v-*src* or general to cell transformation, we analyzed the *drs* mRNA expression in various cell lines transformed by other oncogenes using Northern blot hybridization. As shown in Fig. 5, in the cell lines transformed by v-*abl*, v-*fps*, v-*mos*, v-*sis*, PyMT or v-K-*ras*, its expression was extremely down-regulated. In contrast, it did not change in cell lines transformed by HPV16 E6E7 or PyLT. These results indicated that down-regulation of *drs* gene was not a general event for cell transformation, and suggested that the down-regulation may have been related to some specific signal transduction pathways.

4. Discussion

We have isolated a novel gene, drs, whose mRNA expression was down-regulated by v-*src* or some other oncogenes. The cDNA consisted of 1830 nucleotides and encoded an open reading frame of 464-amino-acid protein. Although no highly homologous genes were found by homology search, the

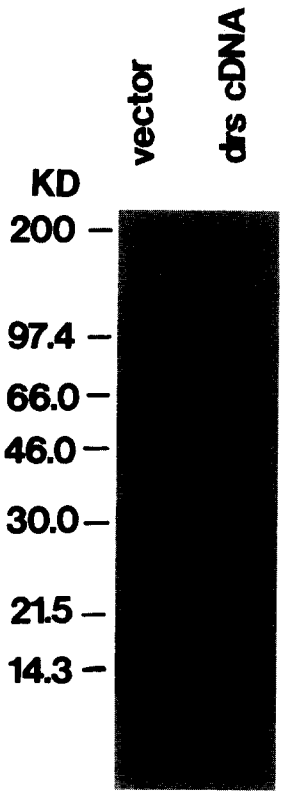

Fig. 3. In vitro transcription-translation of *drs* cDNA. Coupled transcription-translation assay was performed with 1 mg of *drs* cDNA or vector as described in section 2.

drs gene contained three CR sequences identified in the selectin family (Fig. 2b).

The selectins are carbohydrate-binding adhesion molecules that regulate leukocyte entry into lymphoid tissues or sites of inflammation [30,31]. This family includes E-, P-and L-selectins, and all of them consist of five structurally different domains, i.e. an amino-terminal lectin (Leu) domain, an epidermal growth factor (EGF)-like domain, 2 to 9 CR sequences, a transmembrane domain, and a short cytoplasmic carboxyl-terminal domain [29,32–35]. Although there is shared sequence homology among those three members of the selectin family, they differ in the number of CR repeats. L-, E-and P-selectins have two, six and nine CR repeats, respectively. Mutational analyses have demonstrated the crucial roles of the lectin and EGF-like domains in selectin-mediated adhesion [36]. Although the role of CR in selectin is not yet well defined, there are some reports that suggested involvement of CR repeats in adhesion function of both L-and E-selectins [37,38]. However, because *drs* protein has no Leu and EGF-like domains, it is unclear whether this protein acts as an adhesion molecule like selectin.

Expression of the *drs* gene is strongly suppressed in the cell lines transformed by not only v-*src* gene but also other oncogenes such as v-*abl*, v-*fps*, v-*mos*, v-*sis*, v-K-*ras* and PyMT. However, its expression was not altered in the cell lines transformed by HPV16 E6E7 or PyLT. As well as the v-*src* gene product, the products of v-*abl* and v-*fps* genes have protein-tyrosine kinase activity, and PyMT is a constitutive activator of $pp60^{c-src}$ tyrosine kinase activity [39–41]. v-*sis* encodes B chain of PDGF [42], binds to PDGF receptor, and activates tyrosine kinase of the receptor. The *ras* product is known to mediate the signals from those tyrosine kinases to serine-threonine kinases like *raf* kinase. The *mos* gene product is a member of the serine-threonine kinases [43] and suspected to have a similar function to *raf* kinase. Further, the products of v-*abl*, v-*src*, v-*fps*, v-*ras* and v-*mos*, and PyMT are known to activate MAP kinase through *raf*-1 [44,45]. Some of these oncogenes can also activate the PI3 kinase or the protein kinase C dependent pathways [46,47]. Therefore, all of these oncogenes may promote cell growth by activating mitogenic signal transduction pathways. On the other hand, the mechanism of growth promotion by HPV16 E6E7 and PyLT may be quite different. These oncogenes are thought to promote cell cycle by binding to and inactivating p53 or the pRB tumor suppressor gene product, either of which negatively regulates cell cycle [48,49]. These suggest that down-regulation may be the event that is directly or indirectly related to specific signal transduction pathways rather than a general phenomenon of transformation. Some transcriptional factor downstream of the signal transduction pathway(s) may regulate the expression of the *drs* gene.

As *drs* gene expression is down-regulated in cell lines transformed by various oncogenes, the gene may function inhibitingly to cell transformation. Therefore, it is interesting to examine whether expression of *drs* gene could result in suppression of cell transformation. In a preliminary experiment, we transfected SRD1 cells with *drs* expression vector, but could not detect obvious transformation suppressor activ-

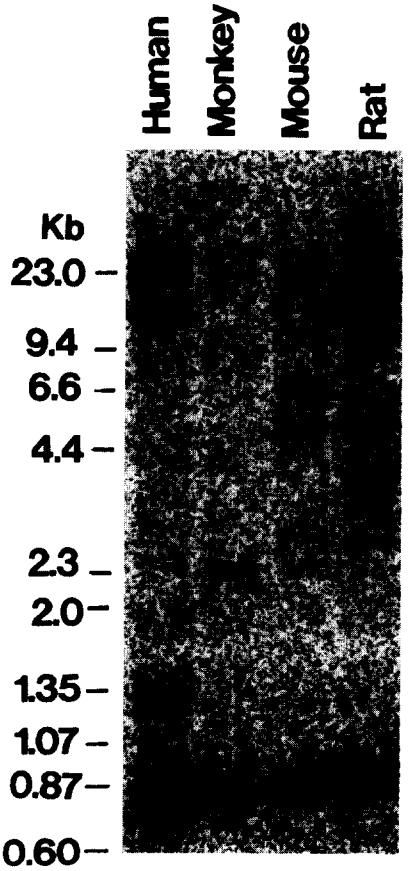

Fig. 4. Detection of *drs* gene in various mammalian species. *Pst*I-digested genomic DNAs (20 μg) derived from the human, monkey, mouse and rat were hybridized with the same *drs* cDNA probe used in Fig. 1a.

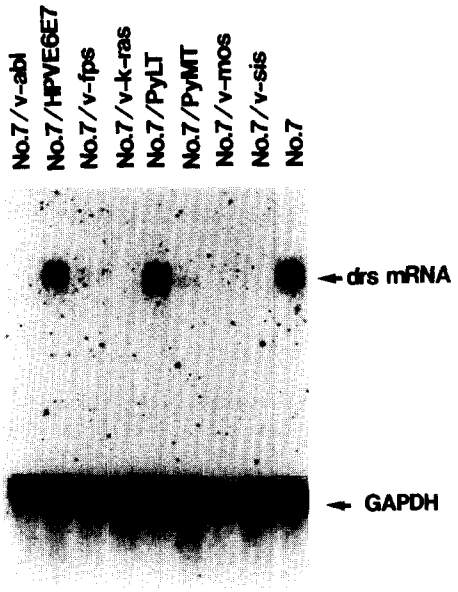

Fig. 5. Expression of *drs* mRNA in cell lines transformed by various oncogenes. Total RNA (20 μg) from the cell lines transformed by each oncogene was subjected to agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with labeled *drs* probes used in Fig. 1a.

itv thus far. Further studies are necessary to reach a final conclusion on this matter.

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