Contrasting Behavior of the p18INK4c and p16INK4a Tumor Supressors in Both Replicative and Oncogene-Induced Senescence

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Abstract

The cyclin-dependent kinase (CDK) inhibitors, p18INK4c and p16INK4a, both have the credentials of tumor suppressors in human cancers and mouse models. For p16INK4a, the underlying rationale is its role in senescence, but the selective force for inactivation of p18INK4c in incipient cancer cells is less clear. Here, we show that in human fibroblasts undergoing replicative or oncogene-induced senescence, there is a marked decline in the levels of p18INK4c protein and RNA, which mirrors the accumulation of p16INK4a. Downregulation of INK4c is not dependent on p16INK4a, and RAS can promote the loss of INK4c without cell-cycle arrest. Downregulation of p18INK4c correlates with reduced expression of menin and E2F1 but is unaffected by acute cell-cycle arrest or inactivation of the retinoblastoma protein (pRb). Collectively, our data question the idea that p18INK4c acts as a backup for loss of p16INK4a and suggest that the apparent activation of p18INK4c in some settings represents delayed senescence rather than increased expression. We propose that the contrasting behavior of the two very similar INK4 proteins could reflect their respective roles in senescence versus differentiation. Cancer Res; 72(1); 165–75. © 2011 AACR.

Introduction

The INK4 family of cyclin-dependent kinase (CDK) inhibitors, p16INK4a, p15INK4b, p18INK4c and p19INK4d, are structurally and functionally similar (1). They comprise between 3.5 and 5 ankyrin-type repeats and bind directly to CDK4 and CDK6, thereby preventing or destabilizing the interaction of these kinases with regulatory D-type cyclins. Although the INK4 proteins show equivalent affinity for CDK4 and CDK6 in vitro, and when overexpressed (2), the prevailing impression is that they associate preferentially with CDK6 in physiologic settings (3–6).

The prototypic member of the family, p16INK4a, is an accredited tumor suppressor that sustains inactivating germline deletions in familial melanoma and is incapacitated by mutated tumor suppressor that sustains inactivating germ line proteins show equivalent affinity for CDK4 and CDK6, thereby preventing or destabilizing the interaction of these kinases with regulatory D-type cyclins. Although the INK4 proteins show equivalent affinity for CDK4 and CDK6 in vitro, and when overexpressed (2), the prevailing impression is that they associate preferentially with CDK6 in physiologic settings (3–6).

The same applies to p18INK4c and p19INK4d, but there has recently been renewed interest in the potential role of INK4c in tumor suppression. The most compelling evidence comes from mice carrying null alleles of Ink4c. These animals develop spontaneous pituitary adenomas late in life (8), but when Ink4c deficiency is combined with other gene knockouts, the range and frequency of tumors become more extensive. For example, mice lacking both p16INK4a and p27KIP1 develop a tumor spectrum reminiscent of human multiple endocrine neoplasia type 1 (MEN1; ref. 9). Critically, menin, the product of the MEN1 gene, has been shown to activate p27KIP1 and p18INK4c by enhancing the binding of trithorax group (TrxG) complexes to the respective promoters (10, 11). Crossing of the knockout mice implicates Ink4c as the critical target of menin in this system (12). Another important example is the combination of Ink4c and p53 deficiency that results in early onset hemangiosarcoma and medulloblastoma and nicely models the genetic background of the human disease (13, 14).

In addition to these mouse models, loss or silencing of human p18INK4c has been recorded in a number of human malignancies (14–23). Glioblastoma multiforme (GBM) is an intriguing example because homozygous deletions and inactivating mutations of INK4c are observed in a tumor that is frequently associated with defects in the p16INK4a-CDK4-pRb pathway (20–23). As a number of the samples showed...
concordant loss of both INK4a and INK4c, implying that the selection against these components is not mutually exclusive, some have proposed that p18INK4c acts as a backup for p16INK4a (21, 23).

In considering what drives the inactivation of p18INK4c in human cancers, we investigated whether it contributes to replicative or oncogene-induced senescence. Remarkably, the accumulation of p16INK4a in senescent human fibroblasts is accompanied by the virtual elimination of p18INK4c. Loss of p18INK4c correlates with a reduction in the total levels of menin and E2F1, which positively regulate INK4c, but we saw no inverse correlation between INK4c and pRb status in human cancer cells or between p16INK4a and p18INK4c levels as previously suggested. Rather, depletion of p16INK4a delays the onset of senescence and concomitant downregulation of p18INK4c.

Materials and Methods

Cell culture and retroviral infection

Conditions for the serial propagation and retroviral infection of human diploid fibroblasts (HDF; Hs68, TIG3, BF, IMR90, ESC, FDF, Leiden, and Q34) and mouse embryonic fibroblasts (MEF) were as previously described (24–27). As these are primary cells with a finite life span, they are considered to be genetically stable and have not been authenticated. The panel of human tumor cell lines, described previously (28), were authenticated by DNA fingerprinting by the LRI Cell Services facility. Retroviral vectors encoding HA-tagged p16INK4a, p18INK4c, p21CIP1, and SV40 large T-antigen were as described (24, 29), and the E2F1:ER, ER:RAS, and DGC1 (DCS118) mAbs were from Novus; the cyclin D1 and p18INK4c (DCS120) mAbs were from Santa Cruz; MEK1/2 (#9122) and p16INK4a (sc-1207) were from Cell Signalling; menin (AB2605), HRP-conjugated antimouse IgG, and rabbit antisera raised against bacterially expressed p18INK4c (sc-1208), mouse p16INK4a (sc-1207), β-tubulin (sc-9104), p53 (DO-1, sc-126), cyclin E (sc-247), cyclin A (sc-601), and p21(CIP1) (sc-397) were from Santa Cruz; MEK1/2 (#9122) was from Cell Signalling; menin (AB2605); HRP-conjugated anti-GAPDH (A9482) and a monoclonal antibody (mAb) against CDK6 (AB77674) were from Abeam; CDK4 (DCS31) and p14(INK4c) (DCS118) mAbs were from Novus; the cyclin D1 mAb (DCS6) was from BD Pharmingen; a pan-Ras mAb (OP41) was from Oncogene Research; and the mAb against p16INK4a (IC8) was provided by LRI Cell Services using cells originally obtained from J. Koh and E. Harlow. We also generated two rabbit antisera raised against bacterially expressed p18INK4c (FST1 and FST4) and have previously described in-house reagents against cyclin D1 (287.3), CDK6 (LBO-1) and p16INK4a (DPAR12; ref. 28).

Quantitative reverse transcription and PCR

Total RNA was extracted using the Ultra Pure RNA Extraction Kit (Roche). Reverse transcription was carried out with 0.5 to 1 μg of RNA using MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems). One fifteenth of the cDNA was used as a template for quantitative real-time PCR (qRT-PCR) on ABI 7900HT (Applied Biosystems) using Power SYBR Green Master Mix (Applied Biosystems) or TaqMan Universal PCR Master Mix (Applied Biosystems). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For detection of the INK4a transcript, annealing and extension was done for 1 minute at 66°C, whereas for other transcripts, this step was conducted at 60°C. Sequences of the primers can be found in Supplementary Table S1.

RNA interference

The validated short hairpin RNA (shRNA) against p16INK4a in the pRetroSuper vector was as described (30). Three different shRNAs targeting human p18INK4c were generated in pRetroSuper using the following 19-nucleotide sequences: A, 5'-CTGTTTGGCTGTGATTTCA-3', B, 5'-GGGAACTGGCCCTTGACACT-3', and C, 5'-GGGGGACACCGCCTGTGAT-3'. The C sequence was the most effective.

Chromatin immunoprecipitation

Cells were harvested and processed for chromatin immunoprecipitation as described (31). After sonication, the chromatin was centrifuged to pellet the debris and pre-cleared at 4°C for 2 hours with protein A-agarose and protein G-agarose beads. The samples were immunoprecipitated with 4 μg of relevant antibody or a control IgG at 4°C overnight, followed by a 3-hour incubation with blocked protein A- or protein G-agarose. The antibody-chromatin complexes were extensively washed with ice-cold immunoprecipitation buffer (150 mmol/L NaCl, 50 mol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 0.5% NP-40, and 1% Triton X-100) and eluted with 1% SDS, 100 mmol/L NaHCO3, 10 mmol/L DTT at 65°C for 5 minutes and at room temperature for 15 minutes. Cross-linking was reversed by incubation at 65°C overnight following the addition of NaCl to 150 mmol/L. The samples were treated with 100 μg/ml RNase (Roche) for 30 minutes at 37°C and DNA was purified using a Qiagen PCR cleanup kit. Antibodies against H3K4me3 (clone 07-473 or MC315) and H3K27me3 (clone 07-499) were from Millipore and Menin (AB2605) from Abcam.

Gel filtration

The procedures for gel filtration and associated data were as described in the work of Ruas and colleagues (32).

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Results

Downregulation of p18INK4c in replicative and oncogene-induced senescence

When primary HDFs reach the end of their proliferative lifespan in culture, there is a substantial accumulation of p16INK4a, preceded by a peak in p21CIP1 expression which subsequently declines (25). However, to our knowledge, there have been no reports on the expression of p18INK4c in this classical model of senescence. We found that whereas p18INK4c levels remained relatively constant with cumulative population doublings, they declined dramatically when the cells became senescent, essentially the mirror image of p16INK4a expression levels (Fig. 1A). This pattern of expression was recapitulated in different HDFs irrespective of their maximum life span and basal levels of p16INK4a and p18INK4c (data not shown).

As replicative senescence reflects both telomere-dependent and -independent events, we asked whether a similar phenomenon occurs during oncogene-induced senescence. Expression of H-RASG12V in HDFs caused a marked downregulation of p18INK4c, contrasting with the anticipated upregulation of p16INK4a (Fig. 1B). Analogous effects were observed in MEFs (Fig. 1C) and with other oncogenes (data not shown).

Downregulation of p18INK4c at senescence is independent of p16INK4a

One simple explanation for these observations would be that the accumulation of p16INK4a at senescence might displace p18INK4c from CDK complexes, resulting in its turnover. Indeed, ectopic expression of HA-tagged p16INK4a in early-passage HDFs caused a marked reduction of p18INK4c (Fig. 1D). However, there are several reasons why this explanation is untenable. First, both p18INK4c and p16INK4a are stable proteins with half-lives in excess of 15 hours as judged by blocking protein synthesis with cycloheximide or by pulse-chase analyses (Supplementary Fig. S1; refs. 5, 28, 33, 34). Second, the downregulation of p18INK4c was not reversed by treatment with the proteasome inhibitor MG132 (Fig. 1E). Third, extinction of p18INK4a at senescence was observed in the Leiden strain of human fibroblasts (24), which lack functional p16INK4a (Fig. 1F).

Downregulation of p18INK4c RNA at senescence

As these data imply that the loss of p18INK4c at senescence is not caused by protein turnover, we asked whether the downregulation of INK4c occurs at the RNA level. When different strains of HDF, including p16INK4a-deficient strains,
were passaged to senescence, there was a marked reduction in the levels of *INK4c* RNA relative to early-passage cells, as assessed by qRT-PCR (Fig. 2A and B). *INK4c* RNA levels also declined in cells infected with an H-RAS<sup>G12V</sup> retrovirus (Fig. 2C) or following addition of OHT to HDFs transduced with a RAS:ER fusion protein (Fig. 2D). As expected, *INK4a* RNA levels were substantially increased under the same conditions. Unlike other members of the *INK4* family, the *INK4c* gene specifies two distinct transcripts that originate from alternative promoters but encode the same protein (35). Using primers that discriminate between the two transcripts, we confirmed that both are downregulated by RAS (Supplementary Fig. S2).

HDFs arrested by overexpression of p16<sup>INK4a</sup> or other members of INK4 and CIP/KIP families acquire many features of senescence, although they do not display the senescence-associated secretory phenotype associated with chronic DNA damage (36, 37). We therefore investigated the fate of p18<sup>INK4c</sup> in these circumstances. Interestingly, ectopic expression of p21<sup>CIP1</sup>, p16<sup>INK4a</sup>, or p18<sup>INK4c</sup> itself resulted in downregulation of the endogenous *INK4c* gene (Fig. 2E). These findings suggest that transcriptional silencing of *INK4c* is a consistent feature of senescent cells but make it unlikely that the loss of p18<sup>INK4c</sup> has a causal role in the phenotype.

**Regulation of p18<sup>INK4c</sup> by menin**

The *INK4a* locus is subject to regulation by the Polycomb group (PcG) of transcriptional repressors (7) via trimethylation of histone H3 on lysine 27 (H3K27me3). Although published data sets indicate that *INK4c* is also occupied by PcG complexes in embryonic stem cells (38), we found no evidence for the H3K27me3 mark at *INK4c* in human fibroblasts under conditions in which it is readily detectable at *INK4a* (Supplementary Fig. S3). As previously shown (26), H-RAS<sup>G12V</sup> caused a loss of H3K27me3 at *INK4a*.

The repressive effects of PcG complexes can be countered by the TrxG proteins and a number of studies have shown that *INK4c* is positively regulated by the tumor suppressor menin as part of a TrxG complex (10, 11). In line with these reports, we found that in human fibroblasts undergoing H-RAS<sup>G12V</sup>–induced senescence, there was a measurable decrease in the levels of menin and H3K4me3 at the *INK4c* locus (Fig. 3A and B). In essentially all of the situations in which we documented a senescence-associated decline in *INK4c* expression, there was a concomitant but less pronounced decline in the levels of menin RNA (Fig. 3C–G).

**Regulation of p18<sup>INK4c</sup> by E2F**

As it seemed unlikely that the changes in menin levels could account for the dramatic downregulation of *INK4c*, we considered the possibility that as an E2F-regulated gene (21, 39, 40), *INK4c* was simply reacting to cell-cycle arrest. However, agents that cause acute arrest in different cell-cycle phases had no effect on the levels of p18<sup>INK4c</sup> RNA or protein, and we saw no substantive changes in p18<sup>INK4c</sup> levels following serum stimulation of quiescent cells (Supplementary Fig. S4A and S4B and data not shown). Moreover, p18<sup>INK4c</sup> levels were relatively unaffected by introduction of SV40 large T-antigen, whereas p16<sup>INK4a</sup> expression was clearly elevated in this setting (Fig. 4A). We also surveyed p18<sup>INK4c</sup> expression in a panel of human tumor cell lines that we had previously used to show the inverse correlation between pRb status and p16<sup>INK4a</sup> expression (28). There was no consistent relationship between p16<sup>INK4a</sup> and p18<sup>INK4c</sup> expression or between p18<sup>INK4c</sup> and pRb status (Fig. 4B), in line with an earlier report (41).

Taken together, our data imply that p18<sup>INK4c</sup> expression is largely unaffected by the fluctuations of E2F activity that occur during the cell cycle or as a consequence of pRb inactivation. However, it was quite clearly induced by over-expression of E2F1, as confirmed using HDFs expressing a tamoxifen-regulated ER–E2F1 fusion protein (Supplementary Fig. S6).
We therefore asked whether the downregulation of INK4c at senescence correlated with reduced expression of E2F1. As shown in Fig. 4C–F, the levels of E2F1 RNA and protein are consistently and dramatically reduced in fibroblasts undergoing either replicative, oncogene-, or p16-induced senescence.

**Distinction between activation and delayed repression of INK4c**

It was previously reported that shRNA-mediated knockdown of p16INK4a in normal astrocytes caused an increase in the levels of p18INK4c (21). As this appeared to be at odds with our findings, we carried out analogous experiments in human astrocytes.
fibroblasts. In general, we found that shRNA-mediated knock-down of p16INK4a had no discernible impact on the levels of p18INK4c despite robust depletion of p16INK4a (Fig. 5A). This negative result was confirmed in multiple experiments with several different strains of logarithmically dividing cells (not shown). However, we occasionally noted higher levels of p18INK4c in p16INK4a-knockdown cells that had been in culture for some time. We reasoned that agents that suppress or disable the pRb-p16INK4a pathway can extend the replicative life span of cells and potentially delay the downregulation of p18INK4c that occurs at senescence. Depending on when the cells are analyzed, this could give the impression that p18INK4c levels are elevated relative to the control cells.

To explore this possibility, we monitored the changes in p16INK4a and p18INK4c expression in human fibroblasts infected with a retrovirus encoding the PcG protein Bmi1. As expected (25), Bmi1 enabled the TIG3 strain of fibroblasts to grow for about 10 additional population doublings relative to the cells infected with the empty vector (Fig. 5B). It also reduced the basal levels of p16INK4a but did not prevent the eventual accumulation of p16INK4a at senescence (Fig. 5C). Importantly, the p18INK4c levels declined much later in the Bmi1-transduced
cells than in the controls. To illustrate this point more clearly, Fig. 5D shows a direct comparison of cell lysates prepared on days 50 and 64 postinfection. At these time points, cells expressing Bmi1 appear to have substantially more p18INK4a than the control, giving the false impression that p18INK4c is being activated by Bmi1. Similar effects were observed in cells whose life span was extended by overexpression of CDK4 or p16-shRNA (data not shown).

Senescence-dependent and -independent effects on p18INK4c

One of the characteristic features of senescent fibroblasts is the appearance of senescence-associated heterochromatin foci (SAHF) which cause irreversible silencing of a subset of E2F target genes (42). We wondered whether the silencing of INK4c at senescence was related to the formation of SAHFs. To avoid altering the levels of key components, we treated HDFs with the CDK4/6 inhibitor PD0332991, which causes a pRb-dependent G1 arrest (43) and was recently shown to induce senescence in GBM cells that have codeletion of INK4a and INK4c (22, 23). The stable arrest caused by PD0332991 was accompanied by a decline in the expression of p18INK4c RNA, as well as other E2F target genes (Fig. 6A). Importantly, the cells developed characteristic features of senescence and stained positively for SA-β-galactosidase and SAHFs (Fig. 6B). Of note, the loss of p18INK4c expression was accompanied by downregulation of both menin and E2F1 (Fig. 6C). However, for technical reasons, we were unable to draw a direct correlation between the appearance of SAHFs and elimination of p18INK4c at the single-cell level.

Reversing the logic, we asked whether H-RASG12V would cause downregulation of INK4c in the absence of SAHFs. The Leiden strain of p16INK4a-deficient HDFs are resistant to oncogene-induced senescence under conditions that efficiently arrest other HDF strains (24). H-RASG12V caused downregulation of INK4c and upregulation of the mutant INK4a in Leiden cells, although the effects were less dramatic than in the control fibroblasts (Fig. 6D and E). As H-RASG12V caused downregulation of p18INK4c in MEFs (Fig. 1D) where SAHFs are not observed (44), our data suggest that SAHFs are not required for the downregulation of INK4c by H-RASG12V. Importantly, the correlation between p18INK4c, E2F1, and menin expression still applied in Leiden cells, despite the avoidance of senescence (Fig. 6F). Taken together, our data suggest that there could be two distinct pathways leading to downregulation of INK4c; one instigated by CDK inhibition and the other instigated by RAS that appears to be independent of CDK inhibition.
Consequences of p18INK4c downregulation in senescent cells

At face value, the behavior of p18INK4c at senescence is at odds with its role as a tumor suppressor. Loss or downregulation of p18INK4c would in principle make it more difficult for cells to mount a senescence-like arrest in response to oncogenic signals by depleting the total INK4 pool. To investigate this possibility, we used shRNA to knock down the expression of p18INK4c in primary HDFs. As exemplified in Fig. 7, cells transduced with p18INK4c shRNA had a proliferative advantage and extended life span relative to control cells. Similar effects were observed in p16INK4a-deficient strains of HDF (not shown). However, the cells eventually underwent replicative senescence. Moreover, cells expressing p18INK4c shRNA remained susceptible to H-RAS G12V-induced senescence. Interestingly, these cells had higher levels of p16INK4a at senescence than controls, suggesting an increased dependence on p16INK4a to enforce the arrest (Fig. 7C).

This result would be consistent with the idea that the total levels of INK4 proteins in the cell determine the availability of CDK4 and CDK6 to associate with the D-type cyclins. Human fibroblasts contain substantially more CDK4 than CDK6 RNA, as judged by deep sequencing (unpublished observations of H. Pemberton and the authors), and we and others have previously shown that only a small fraction of the total CDK6 is associated with D-type cyclins (5, 32). Excluding the high-molecular-weight complexes with heat-shock chaperone proteins, most of the CDK6 is bound by INK4 proteins in 1:1 complexes of around 50 to 60 kDa, whereas the bulk of the CDK4 is in 150 to 170 kDa complexes with D-cyclins and CIP/KIP proteins (Supplementary Fig. S5A). In proliferating cells, CDK6 is primarily associated with p18INK4c as judged by co-immunoprecipitation, but in senescent cells, virtually all of the CDK6 becomes bound to p16INK4a (Fig. 7D and Supplementary Fig. S5B). Although CDK4 can be found associated with p16INK4a and p18INK4c in early-passage cells, its association with cyclin D1 is preserved at senescence despite the excess of p16INK4a (Fig. 7D and ref. 32). In summary, the downregulation of p18INK4c at senescence results in reassortment of the D-type cyclins, CDKs and CDK inhibitors to retain CDK6 in an apparently inactive state.

Discussion

A motivation for this study was the evidence that p18INK4c acts as a tumor suppressor not only in mouse models but also...
in a number of human cancers. Remarkably, deletions of INK4c have been found in tumors that also have defects in INK4a (17, 21–23, 45) prompting questions about the selective rationale for concomitant inactivation of two members of the same family. As mutation or silencing of p16INK4a can enable incipient cancer cells to escape from oncogene-induced senescence, it was suggested that p18INK4c serves as a backup in these circumstances, causing pressure to escape from p18INK4c-mediated arrest (21, 23). The data we describe make this scenario unlikely.

In primary human fibroblasts undergoing senescence, whether in response to an activated oncogene, continuous passaging or CDK inhibitors, there is a marked decline in the total levels of E2F1. Again, our findings agree with published evidence that E2F1 is downregulated in replicative and H-RASG12V-induced senescence (46, 49). However, the interpretation is complicated by the fact that agents that modulate E2F1 activity rather than its total levels, such as cell-cycle arrest or inactivation of pRb, had little effect on p18INK4c. While it is clear that the response of E2F target genes can be context dependent, our data do not support the notion of a linear pathway through which loss of p16INK4a induces E2F-dependent expression of p18INK4c (21).

The concept of a linear pathway was based on evidence that shRNA-mediated knockdown of p16INK4a in primary astrocytes resulted in upregulation of p18INK4c (21). However, this inverse relationship was not apparent in the logarithmically growing human fibroblasts studied here and, whereas there could be differences between cell types, our data suggest that the passage history of the cells can have an important bearing on the interpretation of such experiments. Agents that neutralize p16INK4a, such as PcG proteins, INK4a shRNAs, or ectopic expression of CDK4, have the potential to extend the replicative life span of cells and thereby delay the downregulation of p18INK4c. Depending on how close the control cells are to...
senescence, this can give the false impression that p18INK4c is being upregulated. Although they are virtually indistinguishable biochemically, a substantial body of evidence implies that the INK4 family of proteins and their target kinases, CDK4 and CDK6, have distinct roles. Whereas p16INK4a is primarily involved in senescence in response to diverse forms of stress, most studies link p14ARF-INK4a with differentiation and suggest that p18INK4c and CDK6 are partners in crime (3, 4, 6, 50–53). Thus, the overexpression of CDK6 or loss of p18INK4c, as observed in a variety of human cancers, would be expected to impair differentiation.

Interestingly, in terminally differentiated myoblasts, virtually all of the CDK6 is bound to p18INK4c (3), whereas in senescent fibroblasts, almost all of the CDK6 is bound by p16INK4a. This suggests a fundamental difference between these states of cell-cycle arrest. But what purpose is served by expressing p18INK4c and CDK6 if the major fraction of both proteins is locked in a futile embrace and why would the substitution of p18INK4c by p16INK4a at senescence make a difference? One possibility would be that senescence requires an INK4 protein that is more able to inhibit CDK4. Another is that while differentiation is reversible, as evident from the reprogramming of pluripotency in somatic cells, senescence is generally considered to be irreversible. Interestingly, INK4c levels decline when human fibroblasts are transduced with the OSKM reprogramming factors, consistent with reports that senescence acts as a barrier to reprogramming (A. Banito and J. Gil, personal communication). It will be interesting to explore the dynamics of p16INK4a and p18INK4c expression and their regulation by PcG complexes during this process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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