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## **Identification of novel targets in prostate cancer progression**

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## CHAPTER 6

### MST1R SUPPORTS PROSTATE CANCER INVASION, DISSEMINATION AND FORMATION OF BONE METASTASES

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## ABSTRACT

The receptor tyrosine kinase, MST1R has been implicated in prostate cancer growth and angiogenesis and, in this context; an important role has been attributed to MST1R expressed on tumor-associated macrophages. Here, we observed that MST1R expression in human prostate cancer cell lines correlates with androgen-independency and metastatic capacity. We expressed MST1R shRNAs in androgen-independent, metastatic PC3 cells. Bulk selected stable populations showed ~50% reduction in MST1R mRNA and protein surface expression. As a consequence, cell scattering in 2D and invasive outgrowth in 3D collagen matrices was attenuated, which could be restored by HGF stimulation of the MST1R-related MET receptor. PC3shMST1R cells were also blocked in their ability to disseminate in a zebrafish embryo xenotransplantation model. Furthermore, RNAi targeting MST1R prevented the formation of bone metastases following intracardiac inoculation in mice. Together, these findings demonstrate that down regulation of MST1R can inhibit prostate cancer invasion, dissemination and metastatic colonization.

## INTRODUCTION

The macrophage stimulating 1 receptor (MST1R; RON; Met-related tyrosine kinase) is a receptor tyrosine kinase that has been implicated in various epithelial malignancies including breast, colon, ovarian, liver, and head and neck cancers (1-11). MST1R is also overexpressed in primary prostate cancer and at metastatic prostate cancer sites (12). Experiments using MST1R kinase-deficient mice have established a role for MST1R in prostate tumor growth and angiogenesis (13). Interestingly, this can be explained at least in part by a critical role for MST1R expression on macrophages in the tumor microenvironment (14). MST1R transgene expression in prostate epithelium and findings from in vitro experiments indicate that MST1R expressed on prostate cancer cells could contribute to tumor cell survival, production of angiogenic chemokines, and tumor growth (12, 15). Importantly, while localized prostate cancer has an almost 100% survival rate, the occurrence of metastases in bone and other distant sites lowers 5-year survival to ~30%(16). MST1R has not been implicated in prostate cancer progression. The MST1R-related MET receptor stimulates tumor growth and lymph node metastasis in a xenograft model using human PC3 prostate cancer cells (17) and HGF-MET signaling is an important target in prostate cancer progression (18). In this study, we took an RNAi-based approach to investigate the role of MST1R in several aspects of the prostate cancer metastatic cascade including cell migration, invasion, dissemination, and formation of bone metastases.

## MATERIALS AND METHODS

Prostate cancer cell lines were obtained from ATCC and cultured according to the standard protocol. PC3-M-Pro4luc cells have been described previously (19). Lentiviral shRNA constructs targeting MST1R (TRCN000012148; TRCN000012150) were obtained from the MISSION library (Sigma-Aldrich). Stable shRNA expressing PC3 and PC3-M-Pro4luc cells were bulk selected by puromycin. Generation of extracellular matrix (ECM)-embedded tumor cell spheroids was performed as previously described (20). Spheroid outgrowth and collagen invasion was quantified using an automated Image pro 7-based plugin to calculate surface area of the spheroid, number of cells migrating into the collagen, and mean cumulative distance (MCD) travelled by these cells. Recombinant hepatocyte growth factor (HGF; Sigma) was used at 5 ng/ml. For zebrafish xenograft assays, CMDiI-labeled PC3 cells were injected in the yolk of 2-day-old fli-EGFP-casper em-

bryos and fixed 6 days post-implantation as described previously (21). The automated process for collection of confocal image stacks, generation of in-focus composite images, alignment and orientation of the images, and subsequent quantification of tumor cell dissemination was done as described previously (21). Dissemination is described as mean cumulative distance (MCD) reflecting cumulative distance from the primary injection site of all tumor cells in each embryo, averaged over all embryos. Experimental bone metastasis in mice was analyzed by ~weekly whole body bioluminescent imaging (BLI) of nude mice following intracardiac injection with PC3-M-Pro4luc cells as described (19). Metastatic lesions in bone were identified by immunohistochemistry. FACS and Western blot experiments were performed as described previously (22) using MET phospho-Tyr1349 (Cell Signaling) and tubulin (Sigma) antibodies. Data for all experiments are presented as mean  $\pm$  SEM of at least 2 independent biological replicates. Student's t test (two-tailed) was used to compare groups.

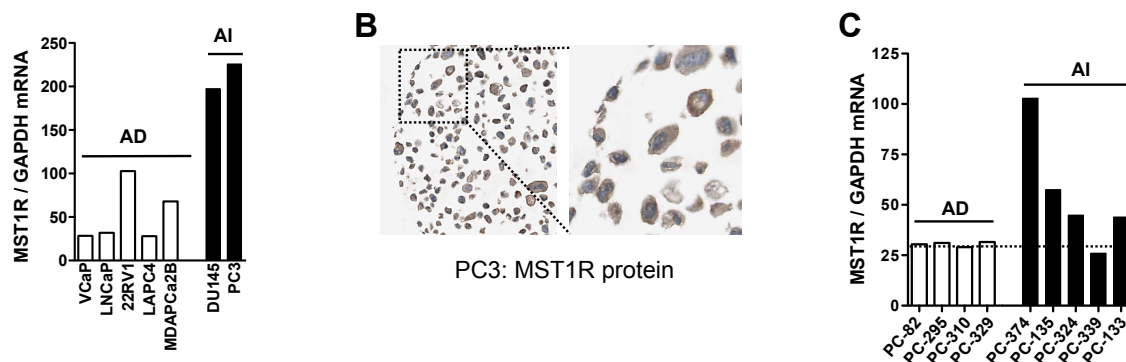


FIGURE 1. *MST1R* expression on human prostate cancer cells. A: *MST1R* mRNA expression determined by qPCR in panel of human prostate cancer cell lines. AD, androgen-dependent; AI, androgen independent. B: Immuno-histochemical staining showing *MST1R* expression in PC3 cells. Right panel shows zoom-in of area marked by dotted line in left panel. Adapted from [www.proteinatlas.org](http://www.proteinatlas.org). C: *MST1R* RNA expression determined by qPCR in series of human prostate cancer xenograft models.

## RESULTS AND DISCUSSION

We evaluated *MST1R* mRNA expression in a panel of prostate cancer cell lines. *MST1R* levels in cells reported to be androgen-independent and metastatic in mice, including PC3 cells were 2-8 fold higher than levels found in androgen-dependent non-metastatic cells (Fig 1A). *MST1R* protein expression was also detected in PC3 cells (Fig 1B). Likewise, in a series of human prostate cancer xenografts representing various aspects of human prostate cancer progression (23), *MST1R* mRNA levels were higher in most androgen-independent xenografts as compared to androgen-dependent xenografts (Fig 1C).

Based on these expression data, we asked if *MST1R* plays a role in aspects of prostate cancer progression. To address this question, *MST1R* expression in PC3 cells was silenced using lentiviral shRNAs. In bulk puromycin-selected stable PC3 shRNA populations, two distinct shRNAs caused ~50% silencing *MST1R* expression (Fig 2A).

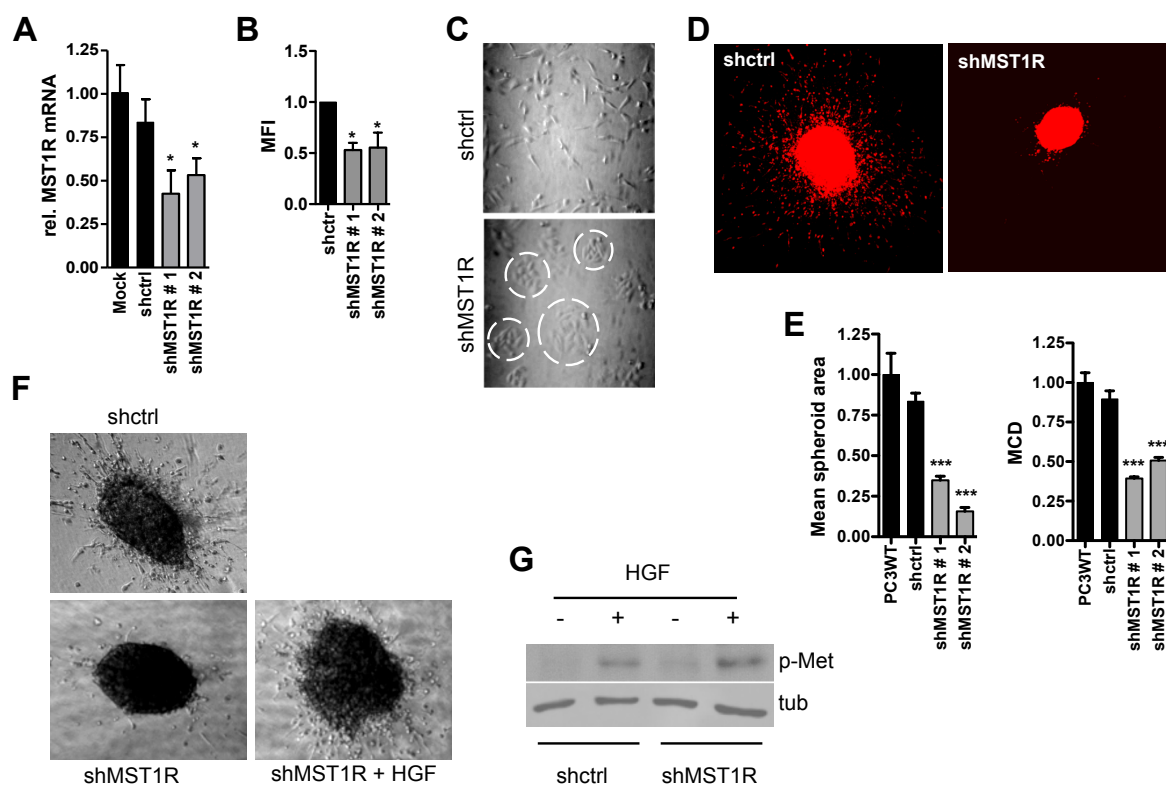


FIGURE 2. *MST1R* expression supports invasive growth of human prostate cancer cells. A: Relative *MST1R* mRNA expression determined by qPCR in PC3 cells bulk puromycin selected for expression of indicated lentiviral shRNAs. B: FACS analysis of *MST1R* surface expression on PC3 cells expressing indicated lentiviral shRNAs. MFI, mean fluorescence intensity. C: Microphotographs of 2D cultures of PC3 cells expressing indicated lentiviral shRNAs. Dotted circles indicate cell islands observed in PC3shMST1R cells. D,E: Representative images (D) and quantification (E) of spheroid outgrowth and ECM invasion (mean cumulative distance from spheroid center of migrating cells, MCD) for Phalloidin-stained PC3 cells expressing indicated shRNAs 6 days post-injection in collagen gels. F: Microphotographs of 3D collagen-embedded spheroids 6 days post-injection for PC3shctrl (upper) and PC3shMST1R cells in absence (lower, left) or presence of HGF (lower, right). G: Western blot showing MET phospho-Tyr1349 levels and tubulin (tub) loading control in PC3 cells expressing indicated shRNAs in absence or presence of HGF. Data are presented as mean  $\pm$  s.e.m.; \* $p < 0.05$ , \*\*\* $p < 0.001$ .

FACS analysis confirmed downregulation at the level of *MST1R* cell surface expression (Fig 2B). Interestingly, reduced expression of *MST1R* caused a conversion from a completely scattered phenotype to more cohesive growth in 2D culture with formation of multicellular islands (Fig 2C). Moreover, when these cells were grown as spheroids in 3D collagen matrices, wild type- and PC3shctrl cells were invasive whereas the number of cells migrating into the collagen was strongly reduced in PC3shMST1R cells (Fig 2D). Quantification of spheroid area and cell migration in Phalloidin-stained 3D cultures demonstrated that *MST1R* silencing significantly inhibited expansion of spheroids and invasion of cells into the collagen (Fig 2E).

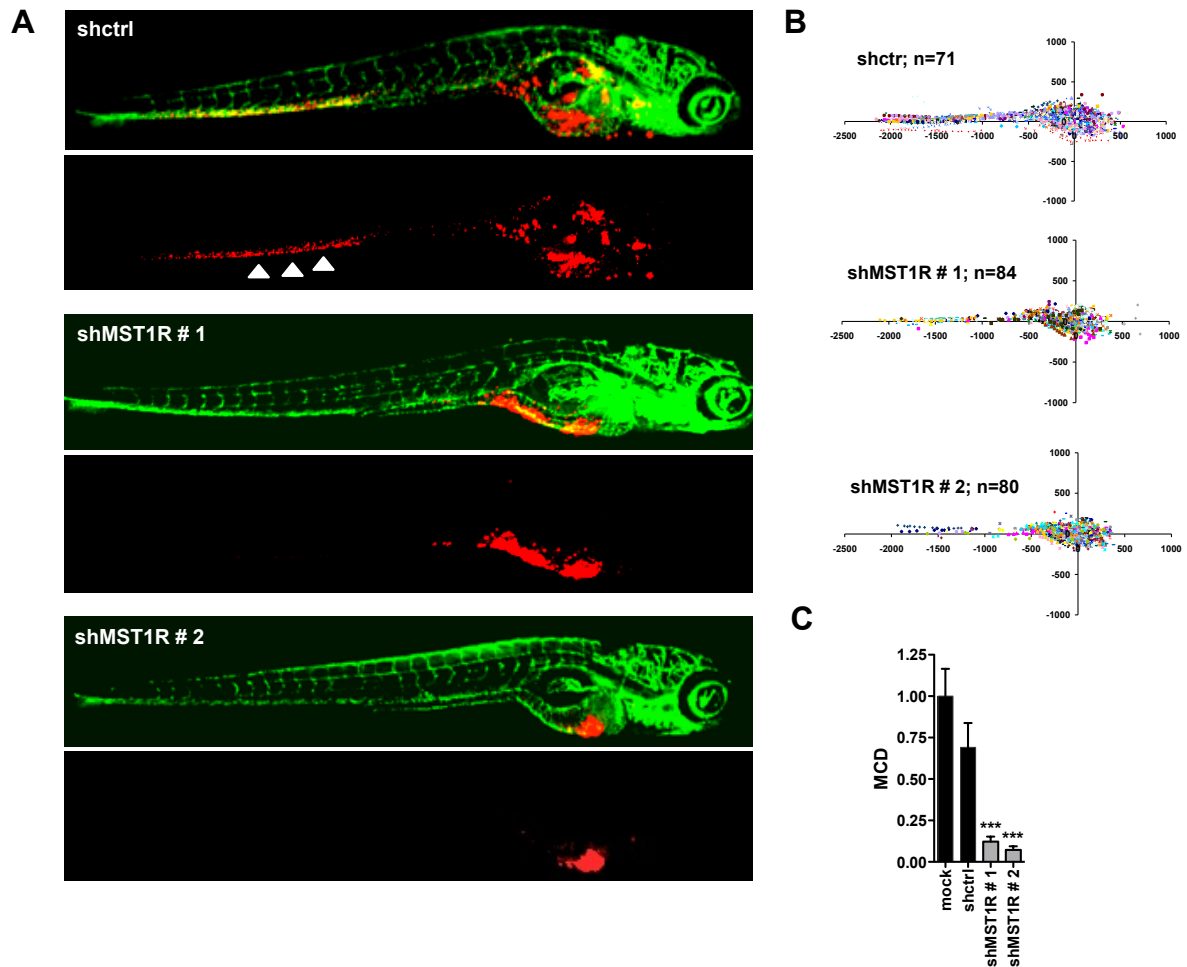


FIGURE 3. *MST1R* expression on human prostate cancer cells supports dissemination in zebrafish xenotransplantation model. A: Confocal microscopy images showing dissemination of PC3 cells expressing indicated *MST1R* shRNAs 6 days post implantation in yolk of Fli-GFP-Casper zebrafish embryos. Red, CM-Dil-labeled tumor cells; green, GFP-marked endothelial cells. Arrow heads indicate PC3 cells disseminated to tail region. B: Scatter plot representation of dissemination of PC3 cells expressing indicated *MST1R* shRNAs. Colors represent individual embryos. N=number of injected embryos from 2 biological replicates. C: Mean cumulative distance (MCD) determined from data represented in B. Data are presented as mean  $\pm$  s.e.m. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

Scattering and invasion are stimulated by HGF binding to the *MST1R*-related MET receptor and this signaling axis is a candidate drug target to halt prostate cancer progression (18). Cross talk between *MST1R* and MET has been shown to support the transforming potential of oncogenic MET mutants (24). To test if the attenuated invasion caused by *MST1R* downregulation was due to inactivation of MET signaling PC3sh*MST1R* were treated with HGF. HGF treatment could restore invasion of PC3sh*MST1R* cells (Fig 2F). In agreement, *MST1R* silencing did not prevent MET phosphorylation in response to HGF (Fig 2G). Together, this indicates that the level of downregulation of *MST1R* in PC3sh*MST1R* cells attenuates invasion without compromising HGF-MET signaling.

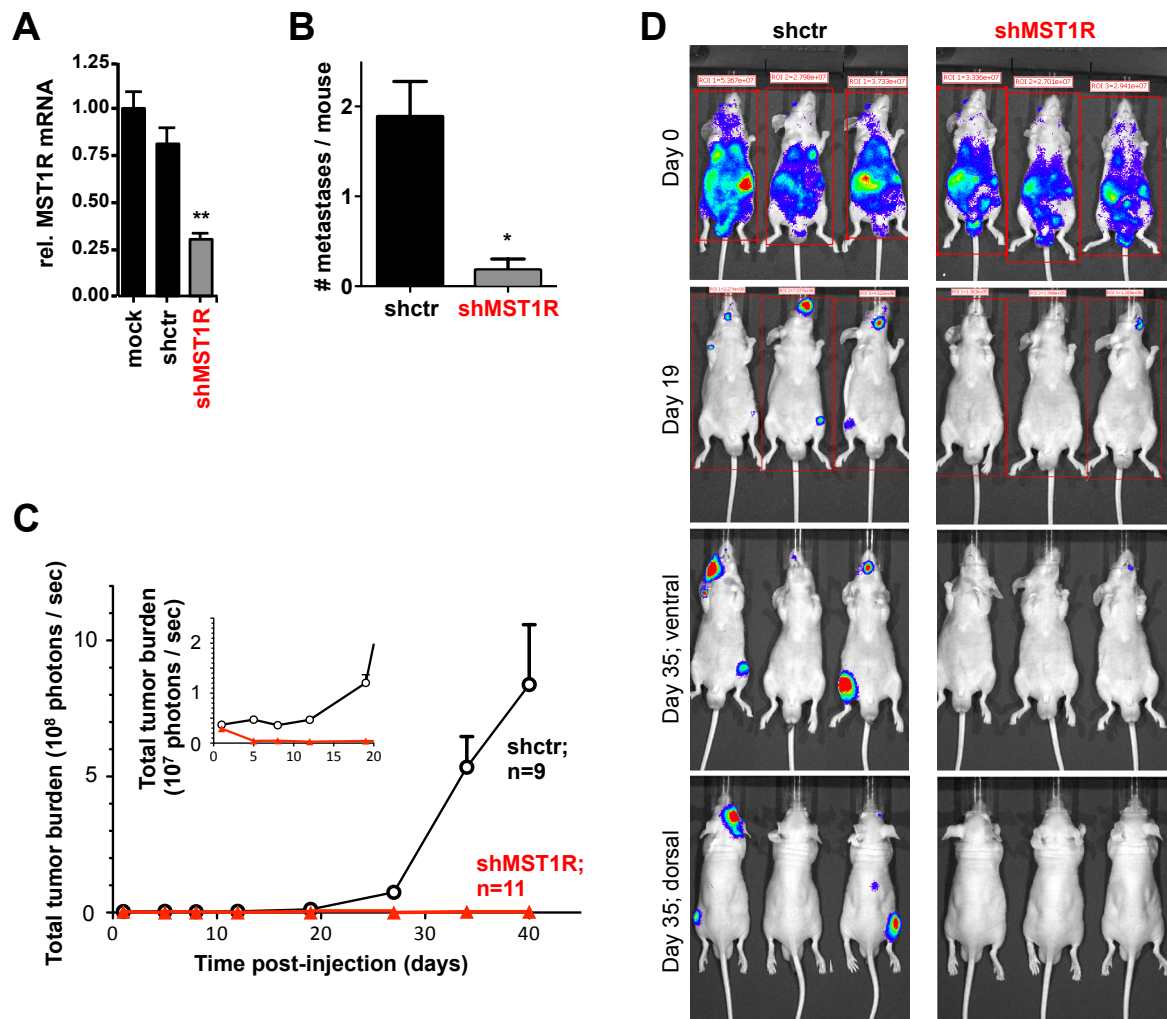


FIGURE 4. *MST1R* expression on human prostate cancer cells supports metastatic colonization in mouse experimental bone metastasis model. A: Relative *MST1R* RNA expression determined by qPCR in PC3-M-Pro4luc cells bulk puromycin selected for expression of indicated lentiviral shRNAs. B: Number of bone metastases per mouse determined by immunohistochemistry 40 days post intracardiac injection of 100,000 PC3-M-Pro4luc cells expressing indicated lentiviral shRNAs. C: Total tumor burden determined by whole body BLI at indicated time points post intracardiac injection of 100,000 PC3-M-Pro4luc cells expressing indicated lentiviral shRNAs. Inset shows tumor burden during the first 20 days. N=number of injected mice. Data are presented as mean  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.005$ .

These in vitro results indicated that enhanced levels of *MST1R* as observed in prostate cancer cells not only regulate tumor growth but also stimulate cell migration/invasion of prostate cancer cells in extracellular matrix. To study effects on cell migration in vivo, we made use of zebrafish embryo xenografts. Here, a primary tumor is formed at the injection site and subsequent dissemination throughout the embryo is assessed 6 days post-injection. The small size and optical transparency of zebrafish embryos and the use of transgenic strains with a fluorescently marked vasculature allows automated confocal imaging and image analysis to quantify dissemination in large numbers of embryos (21). Injected PC3 cells and PC3 cells expressing con-



trol shRNA disseminated throughout the embryo with multiple tumor cell foci in the tail region (Fig 3A,B). By contrast, in agreement with the observed effect in PC3 migration/invasion in vitro, PC3shMST1R mostly remained close to the area of injection and very few tumor cell foci were observed in the tail. In two independent shMST1R lines, reduced expression of MST1R significantly impaired dissemination in this model (Fig 3A-C). Subsequently, we assessed if MST1R plays a role in later stages of prostate cancer metastasis that involve extravasation and homing and expanding in bone lesions. For this purpose, we silenced MST1R in the PC3-derived PC3-Pro4-luc cells (19) and analyzed stable bulk-selected shMST1R populations that had ~70% reduction in MST1R expression (Fig 4A) in a preclinical mouse model for bone metastasis (19). Following intracardiac injection of control or shMST1R cells whole animal BLI was used to measure outgrowth of metastatic lesions over time and immunohistochemistry of bone sections at the end of the experiment was used to determine the number of metastases. Mice injected with PC3 cells expressing a control shRNA showed on average ~ 2 bone metastases whereas very few metastatic lesions were detected in the bone of mice injected with PC3shMST1R cells (Fig 4B). In agreement, the total metastatic tumor burden determined by BLI was strongly suppressed as a consequence of the reduction in MST1R expression (Fig 4C,D).

In summary, our findings in 3D in vitro and preclinical in vivo models show that MST1R expression in human prostate cancer cells can support local invasion, dissemination, and formation of bone metastases. These results significantly extend earlier findings on the role of MST1R in prostate cancer formation. It has been previously established that MST1R supports prostate tumor growth and angiogenesis and this can involve MST1R expressed on prostate cancer cells as well as MST1R expressed on cells in the tumor microenvironment (13-15). Our current report also implicates MST1R expressed on prostate cancer cells in aspects of prostate cancer metastasis. Altogether, the data from several studies indicate that MST1R represents an attractive potential drug target for molecular targeted therapy.

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## AUTHOR CONTRIBUTIONS

VPSG, SH, SN, GvdH, and SB designed and performed experiments. GvdP, GJ, BvdW, BES-J, and EHJD analyzed and interpreted data. VPSG and EHJD wrote the manuscript.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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