Characterization of Tumor-Suppressive Function of SOX6 in Human Esophageal Squamous Cell Carcinoma

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Abstract

**Purpose:** By using cDNA microarray analysis, we identified a transcriptional factor, SOX6, that was frequently downregulated in esophageal squamous cell carcinoma (ESCC). The aim of this study is to investigate the role of SOX6 in human esophageal cancer development, and to examine the prevalence and clinical significance of SOX6 downregulation in ESCC.

**Experimental Design:** Expressions of SOX6 mRNA in 50 ESCCs and SOX6 protein in 300 ESCCs were investigated by semiquantitative RT-PCR and immunohistochemistry, respectively. The tumor-suppressive function of SOX6 was characterized by cell growth, foci formation, wound-healing and cell invasive assays, and tumor xenograft experiment. Western blot analysis was applied to detect protein expression levels.

**Results:** SOX6 was frequently downregulated in primary ESCCs in both mRNA level (29/50, 58%) and protein level (149/219, 68.0%), which was significantly associated with the poor differentiation (P = 0.029), lymph node metastases (P = 0.014), advanced TNM stage (P = 0.000), and disease-specific survival (P < 0.001). Multivariate analysis indicated that the downregulation of SOX6 (P = 0.000) was a significant independent prognostic factor for ESCC. Functional studies showed that SOX6 was able to suppress both in vitro and in vivo tumorigenic ability of ESCC cells. The tumor-suppressive mechanism of SOX6 was associated with its role in G1/S cell-cycle arrest by upregulating expressions of p53 and p21WAF1/CIP1 and downregulating expressions of cyclin D1/CDK4, cyclin A, and β-catenin.

**Conclusions:** We provided the first evidence that SOX6 is a novel tumor-suppressor gene in ESCC development and is a potential prognostic marker in ESCC. Clin Cancer Res; 17(1); 46–55. ©2010 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common fatal malignancies of the digestive tract (1). Despite advances in diagnosis and multimodality therapies, ESCC continues to carry a grim prognosis (2). The major reason for this poor survival is the fact that ESCC frequently metastasizes to regional and distant lymph nodes, even at initial diagnosis. Recently, the treatment of cancer using molecular targets has brought promising results and attracted more and more attention. However, most of the molecular targets, which were commonly found in other epithelial neoplasias, have failed to show prognostic value for ESCC (3). Hence, characterization of genes involved in the development and progression of ESCC may lead to the identification of new prognostic markers and therapeutic targets (4, 5).

Systematic analysis of expression levels of thousands of genes by cDNA microarray is an effective approach to identify novel genes and pathways with importance in cancer development and progression, or as targets for new therapies. Recently, genome-wide expression profiles between pooled cDNA from 10 primary ESCCs and their corresponding nontumorous tissues were compared by cDNA microarray containing 47,000 genes. Using an arbitrary cutoff line at signal log ratio ≥2.0 or ≤−2.0, 185 upregulated genes and 225 downregulated genes were detected in ESCC tumors as compared with normal controls (unpublished data). One frequently downregulated gene in ESCC specimen, SOX6, was further characterized. SOX6, a member of the D subfamily of sex determining region y-related transcription factors, encodes a protein that binds to DNA through a high-mobility-group (HMG) domain and plays critical roles in cell fate determination, differentiation, and proliferation (6–9). Some Sox factors, such as, SOX2, SOX4, and SOX9, have been

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SOX6 in ESCC

Translational Interference

Increasing knowledge of genes and their biological pathways associated with cancer development and progression has provided opportunities to develop targeted therapeutics for cancer treatment with higher specificity, more efficiency, and safety. Here, we identify SOX6 as a tumor suppressor in esophageal squamous cell carcinoma (ESCC). Loss of SOX6 expression is significantly correlated with poor prognosis in ESCC patients. The tumor-suppressive mechanism of SOX6 is associated with its role in G1/S cell-cycle arrest via the upregulation of p53 and p21WAF1/CIP1, along with the downregulation of cyclin D1/CDK4, cyclin A, and β-catenin. These findings suggest that SOX6 could be used as a prognostic marker and a potential therapeutic target in ESCC.

identified as tumor suppressors that were frequently downregulated in various cancers (10–15). However, to our knowledge, the role of SOX6 in esophageal cancer development and progression has not been explored.

In this study, the expression pattern of SOX6 in clinical ESCC samples and ESCC cell lines was studied by RT-PCR. Both in vitro and in vivo assays were used to study the tumor-suppressive function of SOX6. In addition, the tumor-suppressive mechanism of SOX6 and its clinical significance in ESCC was also investigated.

Materials and Methods

Cell lines and primary tumor specimens

Chinese ESCC cell line HKESC1 was kindly provided by Professor Srivastava (Department of Pathology, The University of Hong Kong), and 2 Chinese ESCC cell lines (EC18 and EC109) were kindly provided by Professor Tsao (Department of Anatomy, The University of Hong Kong). Six Japanese ESCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410, KYSE510, and KYSE520; ref. 16) were obtained at Linzhou Cancer Hospital. A total of 300 formalin-fixed adjacent nontumorous tissues from the proximal resection margins were collected immediately after surgical resection at Linzhou Cancer Hospital. A total of 300 formalin-fixed and paraffin-embedded ESCCs and their corresponding nontumorous tissues were also kindly provided by Linzhou Cancer Hospital. Clinical data of patients included in this study are detailed in Supplementary Table S1 and S2. No patients recruited in this study have received any preoperative treatment. Samples used in this study were approved by the individual institutional Committees for Ethical Review of Research involving Human Subjects.

Semi-quantitative reverse transcription-PCR

Total RNA was extracted from cell lines and frozen ESCC tissues by the TRIzol reagent (Invitrogen). Reverse transcription of total RNA (2 μg) was done using an Advantage RT for PCR kit (Clontech), and cDNA was subjected to PCR for 28 cycles of amplification with the following pair of primers: SOX6Fw, 5'-GGCGTCCCCCTACCCGCTATCC-3' and SOX6Rv, 5'-TCGTCGACACGGCTCCCTCA-3'. GAPDH gene was used as a control.

Tissue microarray and immunohistochemistry

Tissue microarrays (TMA) containing 300 pairs of primary ESCC tissue samples and their corresponding nontumorous tissues were constructed as described previously (17). Standard streptavidin–biotin–peroxidase complex method was used for immunohistochemical staining (17). Briefly, TMA section was deparaffinized, blocked with 10% normal rabbit serum for 10 minutes, and incubated with anti-SOX6 polyclonal antibody (Novus; 1:75 dilution) overnight at 4°C. The TMA section was then incubated with biotinylated goat anti-rabbit immunoglobulin at a concentration of 1:75 at 37°C for 30 minutes. The status of nuclear expression of SOX6 was assessed by 3 independent investigators without prior knowledge of clinicopathologic data. Positive expression of SOX6 in normal and malignant ESCC tissues was primarily a nuclear pattern. Because the intensity of staining within each tumor tissue core was mostly homogeneous, the intensity of SOX6 staining was semiquantitatively evaluated on the basis of criteria used by our previous publication (18): strong positive (scored as 2+), dark brown staining in greater than 50% of normal or malignant esophageal squamous cells completely obscuring nucleus; weak positive (scored as 1+), any lesser degree of brown staining appreciable in cell nucleus; absent (scored as 0), no appreciable staining in normal or malignant esophageal squamous cells.

Tumor-suppressive function of SOX6

To test tumor-suppressive function of SOX6 gene, it was PCR amplified, sequence-verified, cloned into pcDNA3.1 (+) vector (Invitrogen), and transfected into ESCC cell line KYSE30 and KYSE510 cells. Stable SOX6-expressing clones (SOX6-30/SOX6-510) were pooled and the SOX6 cDNA was rescued. Blank vector-transfected KYSE30 and KYSE510 cells (Vec-30/Vec-510) were used as control. For foci formation assay, 1 × 104 SOX6-30/SOX6510 cells or Vec-30/Vec-510 cells were plated in wells of a 6-well plate. After 7 days culture, surviving colonies (>50 cells per colony) were counted with Giemsa staining. Triplicate independent experiments were carried out.

Cell growth rates of SOX6-30/SOX6-510 and Vec-30/Vec-510 cells were detected by MTT assay. Cells were seeded at 103 per well. The cell growth rate was detected using cell proliferation MTT kit (Sigma) according to the manufacturer’s instruction. Triplicate independent experiments were carried out.

Tumor formation in nude mice

The in vivo tumor-suppressive ability of SOX6 was investigated by tumor xenograft experiment. About 2 × 106 SOX6-30 cells or Vec-30 cells were injected subcutaneously into the right and left hind legs of 4-week-old nude mice.
mice (10 mice per group). Tumor formation in nude mice
was monitored over a 4-week period. The tumor volume
was calculated by the formula $V = 0.5 \times L \times W^2$ (19).

**Migration and invasion assays**

For cell migration assay, SOX6-30/SOX6-510 or Vec-
30/Vec-510 cells were cultured in a 6-well plate until
confluent. The cell layer was wounded using a sterile
tip. After incubation for 24 or 36 hours, the cells were
photographed under a phase-contrast microscope. The
experiment was carried out in triplicate. For invasion
assay, SOX6-30 cells or Vec-30 cells were starved with
serum-free medium for 24 hours before the assay. Cells (5
× 10^4) were suspended in 0.5-mL serum-free medium
and loaded on the upper compartment of invasion cham-
ber coated with Matrigel (BD Biosciences). The lower
compartment was filled with complete medium as che-
moattractant. After 24 hours, invasive cells were fixed,
stained, and counted under a microscope. Triplicate
independent experiments were carried out.

**Cell-cycle analysis**

SOX6-30/SOX6-510 or Vec-30/Vec-510 cells (1 × 10^6 to
2 × 10^6) were cultured in RPMI medium containing 10%
fetal bovine serum (FBS). Serum was withdrawn from
culture medium when cells were 70% confluent. After 72
hours, 10% FBS was added in the medium for an additional
12 hours. Cells were fixed in 70% ethanol, stained with
propidium iodide, and DNA content was analyzed by
Cytomics FC (Beckman Coulter).

**Western blot analysis**

Western blotting was done according to the standard
protocol with antibodies for SOX6 (Novous), p53,
p21^WF1/CIP1, cyclin A, cyclin D1, CDK4 (Cell Signaling
Technology), and b-catenin (Santa Cruz Biotechnology).

**Statistical analysis**

Statistical analysis was done with the SPSS standard
version 13.0 (SPSS Inc). The correlation between SOX6
expression and clinicopathologic characteristics was ana-
yzed using the chi-square test. Disease-specific survival
(DSS) was calculated from the date of diagnosis to the
date of cancer-related death or last follow-up. Survival
curves were assessed by the Kaplan–Meier method and
compared by the log-rank test. Relative risks of cancer-
related death associated with SOX6 expression status and
other predictor variables were estimated by univariate
analyses. Multivariate survival analysis was done on all parameters that were found to be significant on univariate level using the Cox regression model. Differences were considered significant when $P$ value was less than 0.05.

### Results

#### Downregulation of SOX6 is frequently detected in ESCCs

The mRNA expression of SOX6 in 9 ESCC cell lines and 50 primary ESCC tumors and their paired nontumorous tissues were studied by semiquantitative RT-PCR. Expression of SOX6 was observed in all 50 tested nontumorous tissues. However, downregulation of SOX6 was detected in 29 of 50 (58%) of primary ESCCs (Fig. 1A). Similarly, downregulation of SOX6 was detected in 5 of 9 (EC109, HKESC1, KYSE30, 510, and 520) ESCC cell lines (Fig. 1B).

#### Downregulation of SOX6 correlates with poor survival outcome in ESCC

To investigate the clinical significance of downregulation of SOX6 in esophageal carcinogenesis, SOX6 expression in protein level was also studied using ESCC TMA containing 300 primary ESCC cases. The clinicopathologic features of these cases were summarized in Table 1. Positive staining of SOX6 was mainly observed at the nucleus (Fig. 1C) and the expression of SOX6 was classified into absent (scored as 0), weak-positive (scored as 1+) and strong-positive (scored as 2+) staining. Informative expression of SOX6 was detected in 219 ESCC cases. Noninformative samples included lost

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<td></td>
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<td>Sclerotic type</td>
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<td>Mushroom type</td>
<td>25</td>
<td>14 (56.0)</td>
<td>11 (44.0)</td>
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$^a$P < 0.05.

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$$\text{Figure 2. Kaplan–Meier analysis of survival in patients with ESCC. Black, patients with normal SOX6 expression (n = 70, median survival 18 months); gray, patients with downregulation of SOX6 (n = 149, median survival 37 months; P < 0.001, log-rank test).}$$

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**Table 1. Association between SOX6 expression and clinicopathologic characteristics of patients with ESCC (n = 219)**
samples, unrepresentative samples, samples with too few tumor cells, and samples with inappropriate staining; such were not used in data complication. Normal expression of SOX6 (strong-positive staining) was observed in 205 of 219 (93.6%) of normal esophageal epithelial cells. Downregulation of SOX6 (absent- and weak-positive staining) was detected in 149 of 219 (68.0%) of informative ESCC cases (Table 1).

The correlation of SOX6 expression with various clinicopathologic features was investigated and the result showed that downregulation of SOX6 was significant associated with poor differentiation \((P = 0.029)\), lymph node metastasis \((P = 0.014)\), and advanced stage \((P = 0.000, \text{Table 1})\). Furthermore, log-rank test showed that the cell growth rates in SOX6-transfectants were significantly inhibited \((P < 0.01)\) compared with control cells (Fig. 3B). Cell growth assay also revealed that the cell growth rates in SOX6-30 cells were significantly inhibited by SOX6 \((P < 0.01)\) compared with Vec-30 cells (Fig. 3D).

SOX6 inhibits cell migration and invasion

As the TMA result showed that downregulation of SOX6 was closely associated with ESCC metastasis, the effects of SOX6 on cell migration and invasion were studied by wound-healing and cell invasion assays, respectively. Wound-healing assay showed that the ectopic expression of SOX6 could dramatically inhibit cell migration ability in SOX6-transfectants compared with control cells (Fig. 4A). Matrigel invasion assay also found that SOX6 could inhibit the invasiveness of ESCC cells, as showed by a significant decrease in the number of invaded cells in SOX6-30 cells compared with Vec-30 control \((P < 0.001, \text{Fig. 4B})\).

SOX6 has strong tumor-suppressive ability

To determine if SOX6 has tumor-suppressive ability, SOX6 gene was stably transfected into KYSE30 and KYSE510 cells (Vec-30/Vec-510) were used as control. Expression of SOX6 in SOX6-30 and SOX6-510 was confirmed by RT-PCR or Western blot analysis, respectively (Fig. 3A). Tumor-suppressive function of SOX6 was studied by foci formation assay, cell growth assay, and tumor xenograft experiment. Foci formation assay showed that the efficiency of foci formation was significantly inhibited \((P < 0.001)\) in SOX6-transfectants compared with control cells (Fig. 3B). Cell growth assay also revealed that ESCC patients with SOX6 downregulation (median survival time, 18 months) experienced a shorter DSS than patients with normal SOX6 expression (median survival time, 37 months; \(P < 0.001\); Fig. 2). By univariable analysis, downregulation of SOX6 \((P < 0.0001)\), poor differentiation \((P = 0.012)\), presence of lymph node metastasis \((P < 0.0001)\), and advanced stage \((P < 0.0001)\) were significant negative prognostic factors for DSS in ESCC patients (Table 2). Nevertheless, multivariate analysis showed that downregulation of SOX6 and presence of lymph node metastasis were 2 independent prognostic predictors for ESCC patients enrolled in this study \((P < 0.001, \text{Table 3})\).

SOX6 inhibits tumor formation in vivo

To further explore the in vivo tumor-suppressive ability of SOX6, tumor formation in nude mouse was carried out by injection of SOX6-30 cells \((n = 10)\), whereas Vec-30

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**Table 2.** Univariate Cox regression analysis of factors possibly influencing disease-specific survival in patients with ESCC

<table>
<thead>
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<th>Variables</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>(P)</th>
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<td>SOX6</td>
<td>0.338</td>
<td>0.242-0.471</td>
<td>0.000a</td>
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<td>Age, y</td>
<td>1.206</td>
<td>0.912-1.595</td>
<td>0.188</td>
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<tr>
<td>Gender</td>
<td>1.052</td>
<td>0.793-1.395</td>
<td>0.726</td>
</tr>
<tr>
<td>Differentiation</td>
<td>1.349</td>
<td>1.067-1.706</td>
<td>0.012a</td>
</tr>
<tr>
<td>pN factor</td>
<td>1.859</td>
<td>1.401-2.467</td>
<td>0.000a</td>
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<tr>
<td>TNM stage</td>
<td>2.148</td>
<td>1.622-2.843</td>
<td>0.000a</td>
</tr>
</tbody>
</table>

\(aP < 0.05.\)

**Table 3.** Multivariate Cox regression analysis for factors possibly influencing disease-specific survival in patients with ESCC

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>(P)</th>
</tr>
</thead>
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<td>SOX6</td>
<td>0.360</td>
<td>0.257-0.506</td>
<td>0.000a</td>
</tr>
<tr>
<td>Differentiation</td>
<td>1.220</td>
<td>0.963-1.548</td>
<td>0.100</td>
</tr>
<tr>
<td>pN factor</td>
<td>1.733</td>
<td>1.301-2.309</td>
<td>0.000a</td>
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\(aP < 0.05.\)
cells were used as controls. Within 4 weeks, solid tumors were readily visible in left hind legs of all 10 mice (injected with Vec-30 cells), but only observed in 4 of 10 of mice injected with SOX6-30 cells. In addition, the size of tumors caused by SOX6-30 cells (tumor volume: $385 \pm 50 \text{ mm}^3$) was significantly smaller than tumors ($750 \pm 37 \text{ mm}^3$) induced by Vec-30 cells ($P < 0.001$; Fig. 4C). These results suggested that SOX6 had a strong tumor-suppressive ability in vivo.

**SOX6 arrests cell cycle at G1/S transition**

To elucidate the mechanism underlying growth inhibition by SOX6, flow-cytometry was used to compare cell distributions in cell cycle between SOX6-transfectants and
control cells. The percentage of SOX6-transfectants in G0/G1 phases and in S-phase were significantly increased and decreased, respectively, compared with that in control cells, suggesting that SOX6 was able to inhibit DNA synthesis and G1/S phase transition (Fig. 5A and B). To further reveal the potential molecular mechanism of SOX6 in cell-cycle arrest, the effects of SOX6 on several key cell-cycle regulators, including p53, p21^{WAF1/CIP1}, cyclin A, cyclin D1, CDK4, and β-catenin were investigated by Western blot analysis. Increased expression of p53 and p21^{WAF1/CIP1}, downregulation of cyclin D1/CDK4 complex, cyclin A, and β-catenin were detected in SOX6-30 and SOX6-510 cells, compared with those in control cells (Fig. 5C).

**Discussion**

The known functions of the SOX protein in development and disease are both diverse and complex (20–22). In this study, we found that SOX6 was frequently downregulated in ESCC cell lines and primary ESCC tumors. TMA study showed that the downregulation of SOX6 was detected in 68% primary ESCCs, which was significantly associated with advanced stage, increased lymph node metastasis, and poor survival of patients with ESCC. Multivariate analysis showed that downregulation of SOX6 could be used as an independent prognostic predictors for ESCC patients. In contrast, it has been reported that high SOX6 expression may be a potential diagnostic marker for some types of brain tumors (23, 24). However, the biological role of high SOX6 expression in brain tumor is still unknown. The contradictory results indicated that SOX6 expression might thus be tissue specific. In this study, the mechanisms underlying silencing of SOX6 in ESCC cells have also been investigated. However, neither methylation nor mutation of SOX6 was detected (data not shown), suggesting that other mechanisms such as, micro-RNA regulation or histone modification might be involved in the inactivation of SOX6.

The tumor-suppressive function of SOX6 showed by both in vitro and in vivo assays further supports its role as a TSG in the development and progression of ESCC. The results showed that SOX6 could suppress cell growth, decrease foci formation and cell motility, and inhibit tumor formation in nude mice. Further study revealed that SOX6 was able to inhibit G1/S-phase transition through the upregulation of p53 and p21^{WAF1/CIP1}, and downregulation of cyclin D1/CDK4 complex and cyclin A in ESCC cancer cells. G1/S phase transition is known to be a major checkpoint for cell-cycle progression. The cyclin-dependent kinase inhibitor (CKI), p21^{WAF1/CIP1}, serves as a critical negative mediator during this transition through CDK inhibition and regulate the activity of cyclin D-CDK complex, which are essential for S phase entry (25–27). Because an inhibition of CKI activity is one of the factors causing uncontrolled tumor cell growth (27), one possible strategy to control cancer cell proliferation is to induce CKI expression, which would lead to G1 arrest and inhibit tumor growth. Although the tumor suppressor p53 itself is not a...
CKI, it has been implicated in various cellular processes (28, 29), including induction of G1 arrest by transactivating its downstream target gene, p21WAF1/CIP1 (30). Taken together, it is reasonable to speculate that SOX6 is able to induce p53 expression, in turn, activate the expression of p21, p53, β-catenin, cyclin A, cyclin D1, and CDK4 were determined by Western blot analysis. GAPDH was used as an internal control.

Recent study by Iguchi et al. has shown that SOX6 suppresses cyclin D1 promoter activity by interacting with β-catenin, and its downregulation induces pancreatic β-cell proliferation (9). Because the Wnt/β-catenin signaling pathway regulates a variety of cellular processes, including cell proliferation, differentiation, development, and apoptosis (34, 35), the suppression of β-catenin signaling by SOX6 may be of biological significance. Previous studies have also shown that some other Sox proteins, including SOX1, SOX3, SOX9, and SOX17, could bind β-catenin and inhibit β-catenin signaling (36–39). In this study, we did found that SOX6 could downregulate β-catenin expression in ESCC cells, suggesting that the tumor-suppressive function of SOX6 might also be associated with the inhibition of β-catenin signal transduction pathway. Interestingly, the oncogenic role of β-catenin in enhancing proliferation could also be inhibited by p53 in response to growth arrest.
Thus, it will be important to know whether SOX6 could exert the tumor suppressor effect in esophageal cancer through interaction with p53 directly.

In summary, we provided evidences that SOX6 is a tumor suppressor in ESCC, based on the following facts: (i) SOX6 is frequently downregulated in ESCC tissues; (ii) down-regulation of SOX6 was significantly associated with poor prognosis; (iii) the introduction of SOX6 into ESCC cells could inhibit cell proliferation and tumor formation in nude mice; (iv) the ectopic expression of SOX6 is able to arrest cell cycle at G1/S checkpoint by upregulating p53 and p21, downregulating cyclin D1/CDK4, cyclin A and β-catenin. A better understanding of the tumor-suppressive role of SOX6 will significantly improve our knowledge in the development of ESCC, and may lead to a more effective management of ESCC with the inactivation of SOX6.

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Disclosure of Potential Conflicts of Interest

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