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Research Paper

Curcumin suppresses proliferation and invasion in human gastric cancer cells by downregulation of PAK1 activity and cyclin D1 expression

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Curcumin (diferuloylmethane), is a natural chemopreventive agent known to inhibit the proliferation of several cancer cell lines. It has been previously demonstrated that curcumin is a potent inhibitor of EGF-receptor (EGFR) tyrosine kinase, but its inhibitive effect on p21-activated kinase 1 (PAK1), a downstream protein of EGFR, has not been defined. In this paper we found that curcumin repressed the expression of HER2 and inhibited the kinase activity of PAK1 without affecting its expression. Silencing HER2 in gastric cancer cells showed that even if PAK1 activity was transiently strengthened by EGF, curcumin still had a strong inhibitive effect. It should be emphasized that kinase assay in vitro showed that curcumin could act as an ATP-competitive inhibitor, which was supported by computer-aided molecular modeling. Curcumin also downregulated the mRNA and the protein expression of cyclin D1 and suppressed transition of the cells from G_1 to S phase. Therefore, curcumin inhibited the proliferation and invasion of gastric cancer cells. Overall, these results provided novel insights into the mechanisms of curcumin inhibition of gastric cancer cell growth and potential therapeutic strategies for gastric cancer.

Introduction

Cancer chemoprevention involves the use of natural or synthetic chemicals to prevent the tumorigenesis of cancer.[1] Curcumin has been widely studied for its anti-inflammatory, anti-angiogenic, antioxidant, wound healing and anti-cancer effects because of its medicinal properties in Indian and Chinese systems of medicine (Fig. 1A). As a natural anti-cancer agent, curcumin has been paid more attention because of its inhibition effect on tumor. Recently curcumin has been shown to be beneficial in all three stages of carcinogenesis,[2] due to its inhibition of protein kinase C (PKC), EGFR tyrosine kinase and IkappaB kinase. Subsequently, curcumin inhibits the activation of the transcription factor nuclear factor κB (NFκB) and the expression of oncogenes including MAPKs, ERK, PI3K, Akt.[3] Furthermore, the expression of cyclin D1, a proto-oncogene that is overexpressed in many cancers, is also downregulated by curcumin.[4] It is proposed that curcumin might suppress tumor promotion through blocking signal transduction pathways in the target cells.

The p21-activated kinases (PAKs) are important mediators of Rho GTPase signaling, and are implicated in biological processes ranging from cytoskeletal dynamics and motility to tumorigenesis.[5,6] Abnormal activation of PAK1 and thereafter high expression of cyclin D1 play an important role in tumorigenesis.[7] In addition, PAK1 regulates Cyclin D1 transcription via an NFκB-dependent pathway.[7] Therefore, PAKs which belong to the protein kinases are important therapeutic targets of tumor and are considered highly able to be drugged owing to their conserved ATP-bingding pocket.[14] Nowadays many small molecule inhibitors were reported targeting PAKs, especially PAK1.[8,9] However, most of these ATP-competitive inhibitors are not selective.[10,11] Recently, it was demonstrated that ATP-competitive inhibitors such as imatinib (Gleevec) achieved unusually high kinase selectivity by binding a less conserved region of kinase (inactive conformation).[12,13] It was also reported that IPA-3, a highly specific and potent non-ATP-competitive inhibitor, targeted the autoregulatory domain of PAK1.[14]

In a word, increasing data implicated PAK1 in tumor proliferation and metastasis,[15] thus inhibitors of PAK1 have been suggested as a novel oncologic therapy.[16] Here we report that curcumin targets PAK1 in gastric cancer cells, providing a novel mechanism for curcumin inhibitive effect on proliferation and invasion in human gastric cancer.

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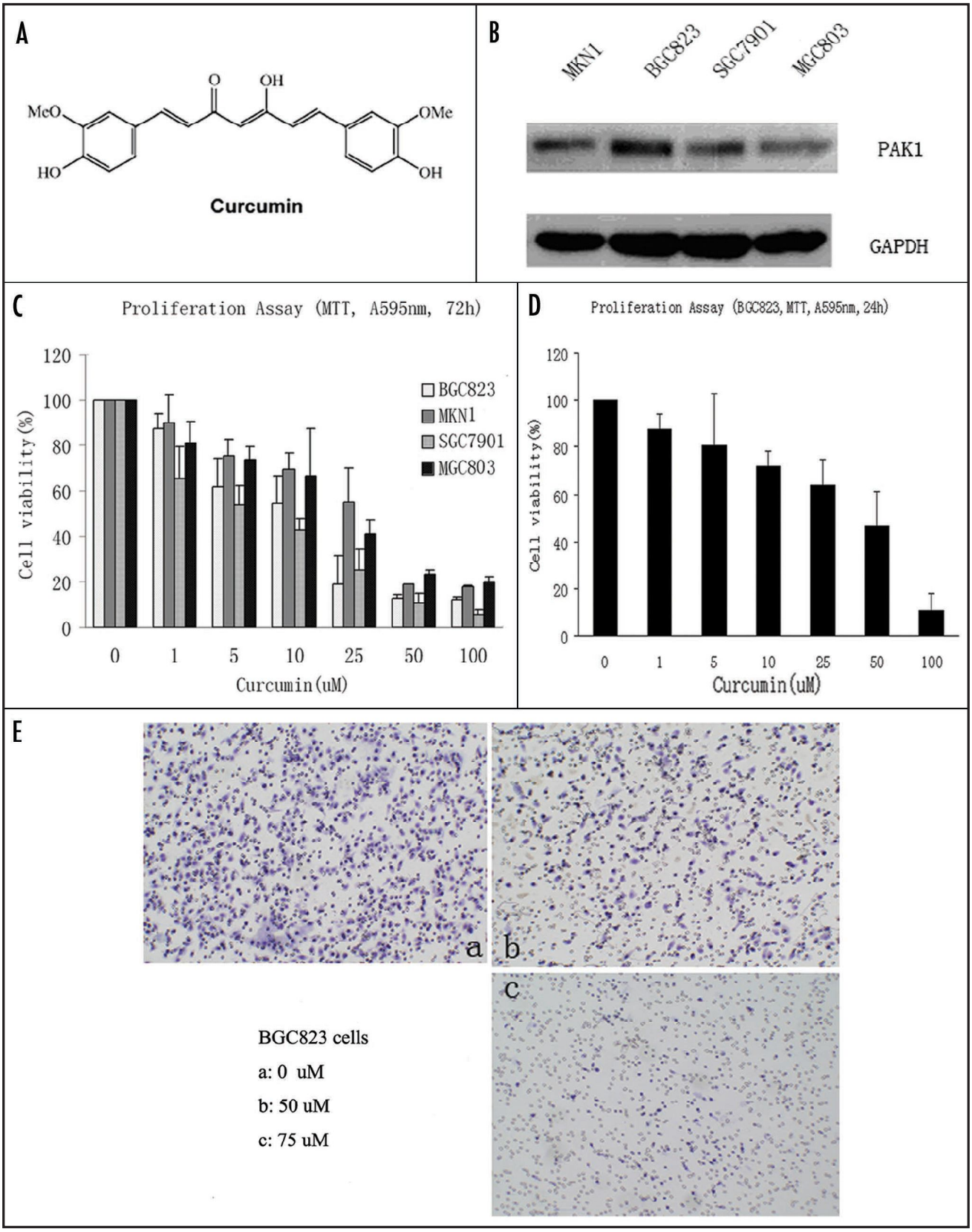

Figure 1. For figure legend, see page 1362.

Figure 1. (A) Chemical structure of curcumin. (B) To define the protein expression of PAK1 in BGC823, SGC7901, MKN28 and MKN45 cells, western blot analysis for the four cell lines was employed. (C) BGC823, SGC7901, MKN28 and MKN45 cells were plated in 0.1 ml of the medium containing 10% FBS in 96-well Corning plates; after 72 h medium was removed and replaced with 0.2 ml medium containing the indicated concentrations of curcumin for different times. At the end of the incubation, proliferation was measured by the modified tetrazolium salt-3-(4-5 dimethylthiozol-2yl) 2-5 diphenyl-tetrazolium bromide (MTT) assay. The experiment was repeated for three independent times. (D) BGC823 cells were plated in 96-well Corning plates, after 24 h MTT was performed. The experiment was repeated for three independent times. (E) BGC823 cells were pre-incubated with different concentrations of curcumin (0, 50 and 75 μM) for 24 h and equal amount of the cells (1 x 10^5 cells) were seeded into the upper part of transwell chamber with 100 μl serum-free DMEM supplemented with 0.1% bovine serum albumin. And the lower compartments were filled with 600 μl of the normal DMEM containing 10% serum. After 18 h at 37°C, non-invaded cells on the upper surface of the filter were wiped out with a cotton swab, and the invaded cells on the lower surface of the filter were fixed and stained with trypan blue.

Results

Curcumin inhibits the proliferation and invasion of gastric cancer cell lines. We chose four gastric cancer cell lines—BGC823, SGC7901, MKN1 and MGC803 to perform our research. We first detected the protein expression of PAK1 in these four cell lines and found that all of them had high levels of PAK1 protein expression, especially BGC823 with the highest protein level of PAK1 (Fig. 1B).

Exposure of cells to different concentrations of curcumin for 72 h inhibited the proliferation of BGC823, SGC7901, MKN1 and MGC803 cell lines when assayed by the MTT method. The effect of curcumin was dose-dependent (Fig. 1C). Before cell invasion assay, MTT assay was performed with BGC823 cells incubated together with curcumin for 24 h and the IC_{50} of curcumin was determined (Fig. 1D). Then BGC823 cells were pre-incubated with different concentrations of curcumin for 24 h and equal amount of the cells were seeded into the upper part of transwell chamber. After 18 h at 37°C, the invaded cells were fixed and stained with typan blue (Fig. 1E). These results clearly suggest that curcumin exhibits anti-proliferation and anti-invasion effects against gastric tumor cells.

Curcumin suppresses transition of gastric cancer cells from G_1 to S phase and downregulates the mRNA and protein expression of cyclin D1. To detect how curcumin inhibits poliferation of gastric carcinoma cells, cells were analyzed by flow cytometry (FCM). We performed FCM with BGC823 cells pre-incubated with different concentrations of curcumin for different times (Fig. 2A). We found that when cells were pre-incubated with 50 μM curcumin for 24 h, the percent of G_1 cells increased almost by 10% compared with the control group, and in contrast the percent of S-cells decreased almost by 15% (Fig. 2B), indicating that curcumin suppressed transition of gastric carcinoma cells from G_1 to S phase.

As the transition of cells from G_1 to S phase is in part regulated by cyclin D1, we next examined the effect of curcumin on the expression of cyclin D1. BGC823 cells were treated with various concentrations of curcumin for different hours and then examined for expression of cyclin D1 by western blot. Results shown in Figure 2C demonstrate that curcumin can downregulate the expression of cyclin D1 in a dose-dependent manner, achieving maximum effect at 100 μM. Figure 2D shows that the inhibitive effect is more obvious when an 8 h exposure was performed. However, when cells were treated for 24 h, no difference of cyclin D1 protein expression was found (Fig. 2E). Taken together, the results above manifest that curcumin inhibits proliferation of gastric cancer cell and blocks it in G_1 phase through suppressing the expression of cyclin D1.

To determine whether curcumin also downregulates mRNA expression of cyclin D1, BGC823 cells were treated with 50 μM curcumin for indicated times and then the mRNA was isolated for RT-PCR. As shown in Figure 2F, curcumin also significantly downregulated mRNA expression of cyclin D1 when cells were incubated for 4 and 8 h, respectively. Therefore, curcumin could affect the expression of cyclin D1.

Curcumin downregulates the expression of HER2 (Erb2) and the kinase activity of PAK1. Increased expression of kinase-active PAK1 in cells was also accompanied by elevated steady-state levels of cyclin D1 protein and it is implicated in biological process of cytoskeletal dynamics and motility.[6,7] We further detected the effects of curcumin on the activity of PAK1 by kinase assay. Before that, we first validated the reliability of the kinase assay system. ZD1839 (Iressa), the EGFR tyrosine kinase inhibitor, has been reported to suppress the activity of PAK1 indirectly.[17] So we employed ZD1839 and some other kinase inhibitors we discvoered before (unpublished data) to validate the kinase assay system. BGC823 cells were incubated for 24 h in DMEM with 10% FCS and then starved for 24 h in DMEM with 0.2% FCS. Cells were then pre-incubated with ZD1839 or the chemical compound for 90 min before further incubation in the absence or presence of EGF. Then immunoprecipitation and kinase assay were performed. The result showed that ZD1839 successfully suppressed the kinase activity of PAK1 compared to the other compound (Fig. 3A), which was consistent with the previous result that ZD1839 was an inhibitor of EGFR tyrosine kinase.[17] Then we applied the same method to check whether curcumin had the ability to inhibit the kinase activity of PAK1. Similarly, in the presence of curcumin the kinase activity of PAK1 mediated by EGF was strongly inhibited (Fig. 3B). However, curcumin had no effect on the protein expression of PAK1.

We have revealed curcumin inhibited the kinase activity of PAK1 in gastric cancer cells, and then we hypothesized curcumin could target the upstream protein of PAK1. The HER2 (erbB2/neu) gene-encoded p185 tyrosine kinase is a potent oncoprotein, which belongs to HER family. We investigated the effect of curcumin on protein expression of HER2, which decreased after curcumin treatment (Fig. 3C and D), proving our hypothesis. Meanwhile, the protein expression of cyclin D1 was also decreased correspondingly with it. However, the protein expression of cyclin D1 turned to increase when cells were incubated with curcumin for 16 h, which was consistent with the previous result that

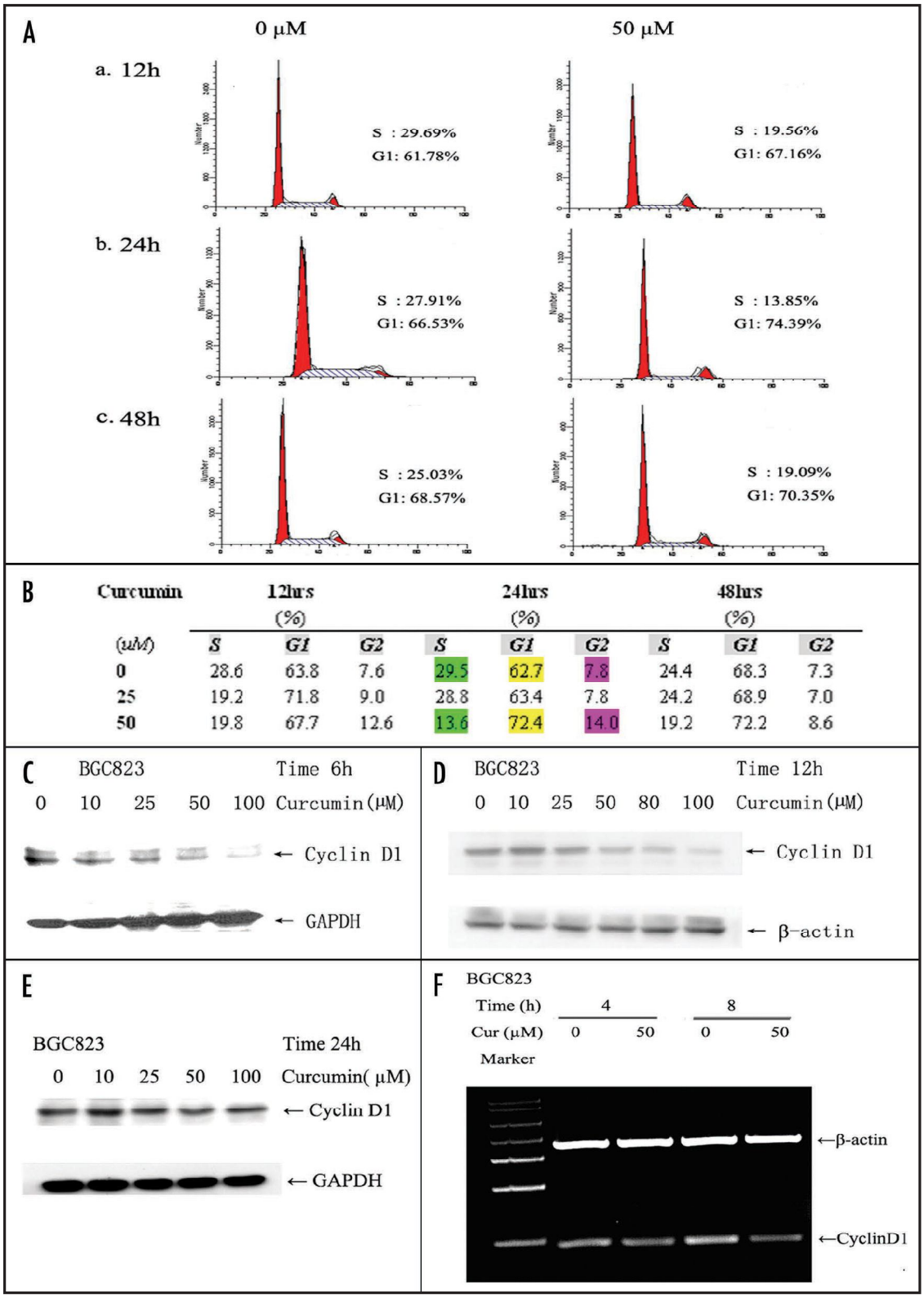

Figure 2. For figure legend, see page 1364.

Figure 2. (A and B) BGC823 cells were pre-incubated with different concentrations (0, 25 and 50 μM) of curcumin for 12, 24 and 48 h, washed and fixed with 70% ethanol. After incubation overnight at -20°C, cells were washed with PBS and then suspended in a staining buffer (10 μg/mL propidium iodide, 0.5% Tween 20, 0.1% RNase in PBS). The cells were analyzed using a FACSVantage flow cytometer with the CellQuest acquisition and analysis software program (Becton Dickinson and Co., San Jose, CA). The experiment was repeated twice. (C–E) BGC823 cells were treated with various concentrations of curcumin (0~100 μM) for different hours (6, 8, 24 h) and then examined for expression of cyclin D1 by western blot analysis. (F) 5 x 10^5 cells were plated in 1 ml medium overnight in six-well plates and treated with 50 μM curcumin for indicated times. Thereafter, mRNA was isolated and transcribed to cDNA. The cDNA was then used for PCR reactions. The PCR product was resolved on 3% agarose gel. β-actin was used as an internal control for equal amount of template.

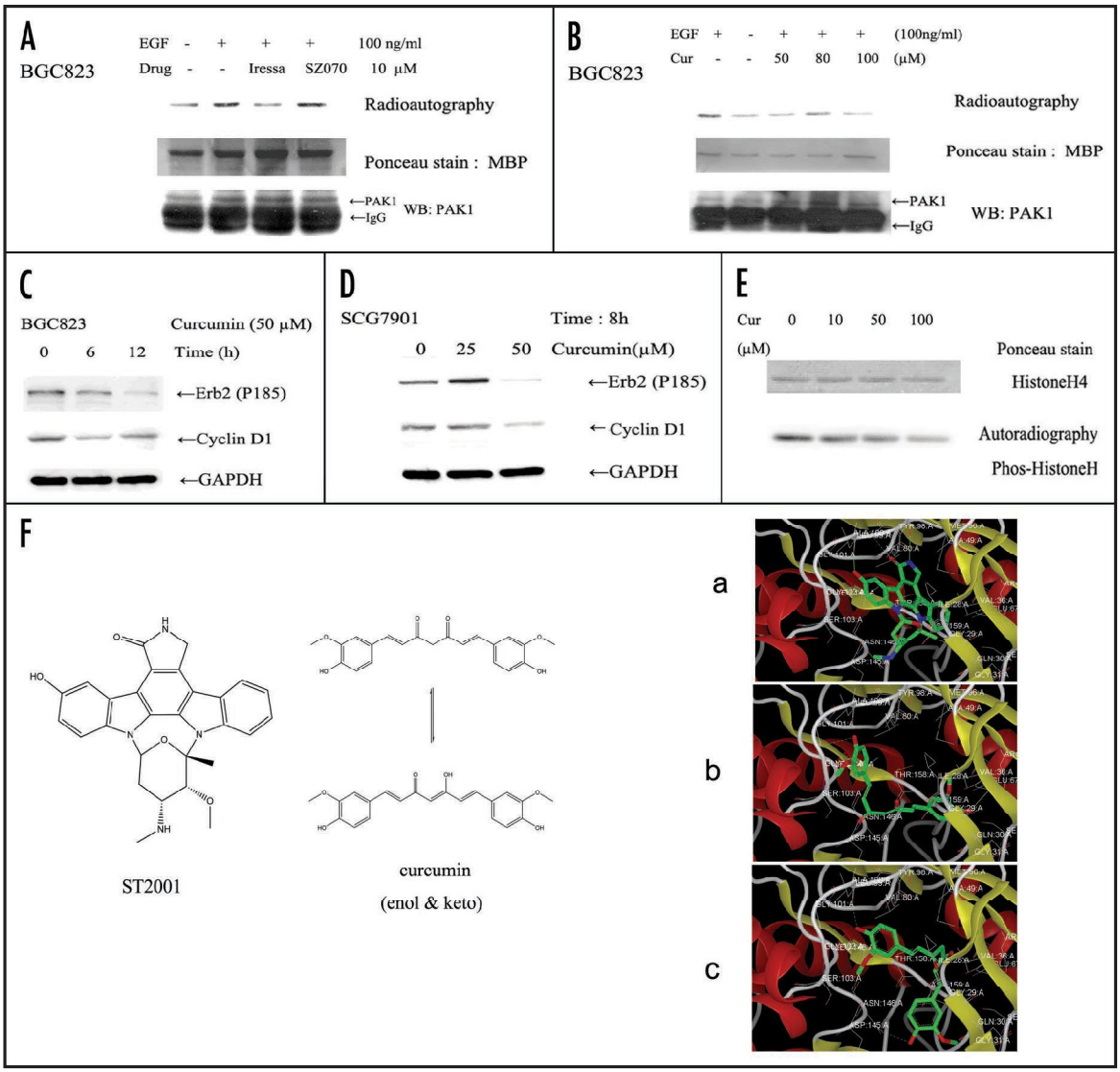

Figure 3. (A) BGC823 cells were incubated for 24 h in DMEM with 10% FCS and then starved for 24 h in DMEM with 0.2% FCS. Cells were then pre-incubated with ZD1839 or the chemical compound for 90 min before further incubation in the absence or presence of EGF. Then immunoprecipitation and kinase assay were performed. (B) BGC823 cells were incubated for 24 h in DMEM with 10% FCS and then starved for 24 h in DMEM with 0.2% FCS. Cells were then pre-incubated with different concentrations of curcumin for 90 min before further incubation in the absence or presence of EGF. Then immunoprecipitation and kinase assay were performed. (C) BGC823 cells were treated with curcumin (50 μM) for different hours (0, 6, 12 h) and then examined for expression of cyclin D1 and Erb2 by western blot analysis. (D) SGC7901 cells were treated with different concentrations of curcumin for 8 h and then examined for expression of cyclin D1 and Erb2 by western blot analysis. (E) For curcumin direct inhibition of PAK1 kinase activity in vitro, PAK1 was preincubated with the indicated concentrations of curcumin. Kinase reactions were started by addition of histone H4 and a mixture of 1 mM ATP and [γ-^{32}P] ATP. (F) Molecular modeling of potential curcumin interactions with PAK1, the crystal structure of PAK1 (PDB code: 2HY8) was used as receptor, the predicted binding modes with PAK1 binding pocket is demonstrated for: (a) co-complex ligand ST2001, (b) curcumin (enol form) and (c) curcumin (keto form). The PAK1 structure is shown in ribbon form, and ST2001 (a), curcumin (b) and curcumin (c) are presented as stick, colored by atom types. The important amino acid residues are labeled with name and number. Hydrogen bonds are indicated in green. The chemical structures of ST2001 and curcumin are shown above.

curcumin had no inhibitive effect on protein expression of cyclin D1 when cells were incubated for 24 h.

It was proved that curcumin could inhibit the activity of PAK1 mediated by EGF, therefore we hypothesized that it might directly inhibit PAK1 kinase activity through competitive inhibition of ATP binding. Actually, curcumin did act as an ATP-competitive inhibitor, which directly inhibited the phosphorylation of substrate when incubated in vitro together with PAK1 kinase, substrate and ATP (Fig. 3E). The hypothesis was also supported by the binding modes of curcumin in the ATP binding region of PAK1 through molecular docking studies (Fig. 3F). Both keto- and enol-curcumin formed hydrogen bonds with residues in the ATP binding pocket of PAK1.

Curcumin can still inhibit the activity of PAK1 induced by EGF after silencing HER2 (erbB2/neu) transiently in gastric cancer cells. To detect whether the activity of PAK1 induced by EGF and the inhibitive effect of curcumin was related to HER2, we silenced HER2 transiently and then carried on the kinase assay. Before that, we first chose BGC823 as HER2-high expression cells amoung the four cell lines (Fig. 4A) and employed the 48-h time point as the suitable silencing time (Fig. 4B). Kinase assay showed that even if the activity of PAK1 induced by EGF was strengthened after silencing HER2 in gastric cancer cells, curcumin still markedly inhibited PAK1 activity (Fig. 4C). This indicated that PAK1 activity induced by EGF was related to HER2, but considering EGFR (HER1)—the direct receptor of EGF, the inhibitive effect of curcumin was some degree of dependence on EGFR.

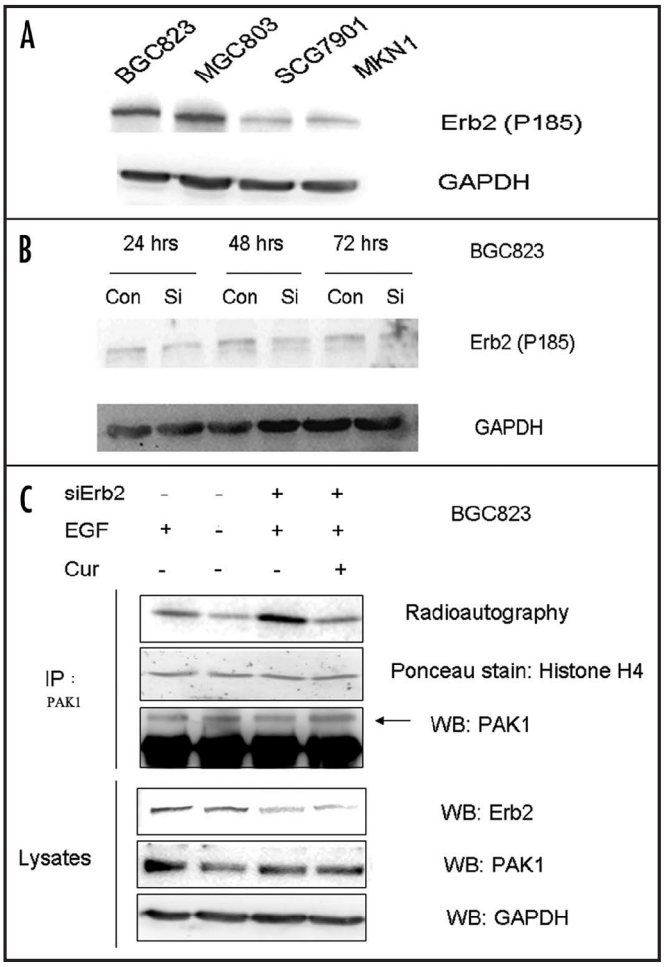

Figure 4. (A) To define the protein expression of HER2 in BGC823, SGC7901, MKN28 and MKN45 cells, western blot analysis for the four cell lines was employed. (B) For transfection of siRNA, BGC823 cells were transfected with siRNA using the Oligofectamine protocol according to Elbashir et al. and the manufacturer's instructions using 0.37% (vol/vol) of Oligofectamine. One day prior to transfection, 2 x 105 cells per six-well plate were seeded without antibiotics, corresponding to a density of 30–40% at the time of transfection. Control cells were incubated with OPTI-MEM I and Oligofectamine (mock-transfected cells). Cells were harvested 24, 48 and 72 h after transfection for protein analysis. (C) After 24 h transfection of siRNA, BGC823 cells were starved for another 24 h in DMEM with 0.2% FCS. Cells were then pre-incubated with different concentrations of curcumin for 90 min before further incubation in the absence or presence of EGF. Then immunoprecipitation and kinase assay were performed, and the lysates were reserved for western blot analysis.

Discussion

The present study demonstrated that curcumin inhibited proliferation, invasion and metastasis of different cancers through interaction with multiple cell signaling proteins.[18] It was found that curcumin could inhibit RON tyrosine kinase, Akt, NFκB and p38 MAPK to affect the biological function of cancer cells, such as apoptosis and invasion.[19-21]

The results presented here indicate that curcumin, a chemopreventive agent, inhibits the proliferation of gastric cancer cells and its inhibition correlates with the downregulation of the expression of cyclin D1. Cyclin D1 is required to mediate the G_1 to S transition, in turn leading to DNA synthesis and cell cycle progression.[22] Our results also reveal that curcumin makes G_1 cells increase but S-cells decrease, so it is reasonable that the antiproliferative effects of curcumin are related to the downregulation of cyclin D1 expression. However, there is a time lag between the inhibition of transition of the cells from G_1–S and the downregulation of Cyclin D1 expression.

Data from the literature suggest that cyclin D1 transcription could be upregulated through multiple signaling pathways, including NFκB.[23] Because PAK1 signaling has been shown to modulate NFκB activation,[24] and we have revealed that the activity of PAK1 is inhibited by curcumin, it is concluded that curcumin downregulates the expression of cyclin D1 through PAK1 signaling pathway. Considering that PAK1 is implicated in biological process ranging from cytoskeletal dynamics and motility to tumorigenesis and also the stimulation of PAK1 kinase activity contributes to the actin reorganization and cell migration,[25-27] thus the inhibition of PAK1 activity leads to the decrease of proliferation and invasion in gastric cancer cells.

HER2 (also known as c-*erb*B2 or c-*neu*), one hypotype of EGFR family, encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity that has been shown to be overexpressed, amplified or both, in a number of human malignant cancer.[28,29] Overexpression of the HER2 receptor is associated with increased progression and metastasis.[30,31] There is

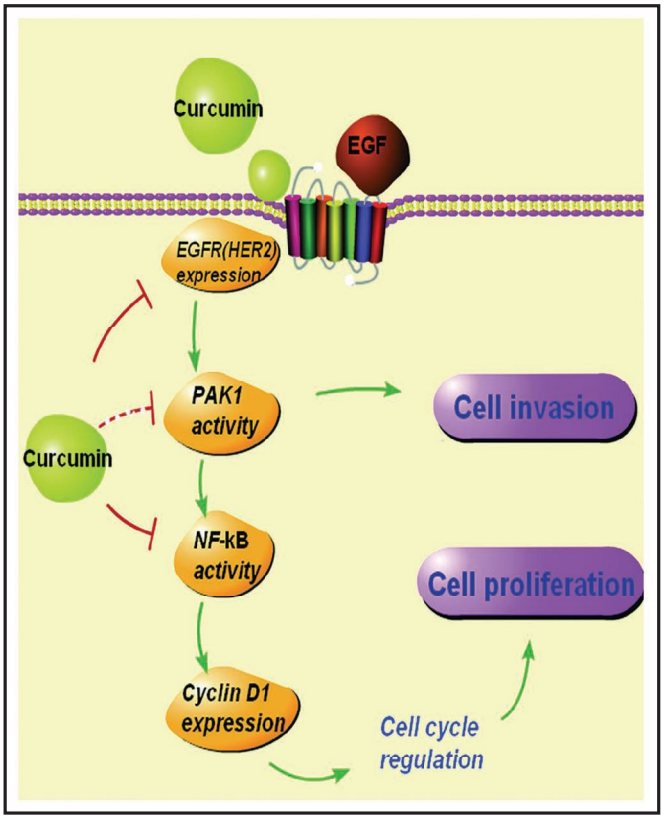

Figure 5. Schema of the putative mechanisms of curcumin suppressing proliferation and invasion in human gastric cancer cells.

increasing evidence of the role of HER2 overexpression in patients with gastric cancer.[32] Both homo- and hetero-dimeric receptor complexes are formed upon cell stimulation by growth factor, but the signal generated by activated Erb1/Erb2 heterodimers leads to greater tumorigenic cell growth than homodimer-induced signal.[33,34] The physical and functional interaction of HER2 and EGFR leads to the formation of a highly active, heterodimeric tyrosine kinase complex which synergistically activates cellular transformation.[35] Moreover, it is known that the EGFR-initiated signaling pathway is a potent inducer of c-Src and PAK1 pathways, as well as of reorganization of the cytoskeleton, which allows increased tumor invasiveness.[17] Therefore, inhibitive expression of the upstream protein HER2 by curcumin is accompanied by suppression of PAK1 activation and invasiveness of cancer cells. However, Bowers G, et al. found that inhibiting HER2 caused the enhanced Tyr phosphorylation of EGFR, which was the opposite to what one would predict.[36] This is consistent with our results that PAK1 activity was enhanced after silencing HER2 transiently. The reason for the finding may be that the studies examined immediate responses to the extrinsically added growth factor, which was not intrinsic growth regulatory event.[36] Still, curcumin strongly inhibited the activity of PAK1 when HER2 was silenced. Together with the previous study that curcumin could inhibit the expression and the tyrosine phosphorylation of EGFR,[37] these indicate that curcumin plays its role of an inhibitor dependent on EGFR—a direct and potent receptor of EGF.

As indicated above, PAK1 plays a central role in motility, invasion and cell survival in human cancer (Fig. 5), and the results show that curcumin does effectively inhibit PAK1 activity induced by EGF. Interestingly, curcumin inhibits the kinase activity of PAK1 not only when it is added in cells, but also when incubated diredtly together with PAK1, ATP, ^{32}P and substrates. The predicted binding modes of curcumin in the PAK ATP binding domain also indicates that curcumin has the ability of acting as an ATP-competitive inhibitor. Since curcumin exists in two tautomeric forms: ketone and enol, both were used in molecular docking studies. Enol and ketone curcumin bind in same position of ATP binding site, however, their binding orientation and affinity were different from each other, as ketone curcumin showed higher putative affinity towards PAK1 than enol curcumin, with fitness value of 44.43 and 36.20 according to GOLD Score function, respectively. Therefore, the role of the ATP-competitive inhibitor of curcumin and its inhibitive effect on HER1/2 both contribute its inhibition of PAK1 activity.

The inhibitor belongs to many current kinase inhibitors targeting enzyme active sites.[38] However, this kind of inhibitor is not selective. More and more inhibitors of PAK1 kinase activity that target the PAK1 autoregulatory strategy have been described (such as IPA-3 and Merlin), suggesting that modulation of the autoregulatory domain may be a normal physiological strategy for modulating PAK catalytic activity.[14,38] This indicates that the derivates of curcumin should be studied for finding a potent inhibitor.

Materials and Methods

Cell culture and reagents. Curcumin (diferuloylmethane) was purchased from Sigma-Aldrich Corporation and was prepared with dimethyl sulfoxide (DMSO) at the concentration of 10 mM, stored as small aliquots at -20°C, and thawed and diluted as needed in cell culture medium. Four different human gastric cancer cell lines (BGC823, SGC7901, MKN1, MGC803) were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) at 37°C in incubator with humidified atmosphere of 5% CO_2 and 95% air.

Transfection of siRNA. Cells were transfected with siRNA using the Oligofectamine protocol according to Elbashir et al. and the manufacturer's instructions using 0.37% (vol/vol) of Oligofectamine. One day prior to transfection, 2 x 10^5 cells per six-well plate were seeded without antibiotics, corresponding to a density of 30–40% at the time of transfection. Control cells were incubated with OPTI-MEM I and Oligofectamine (mock-transfected cells). The siRNA of HER2 sequences: sense-5' GGA GCU GGC CUU GUG CCG 3'; antisense-5' GCA CAA GGC CGC CAG CUC CAU 3'. Cells were harvested 24, 48 and 72 h after transfection for protein analysis.

Antiproliferative assays. Briefly, cells (10^4/well) were plated in 0.1 ml of the medium containing 10% FBS in 96-well Corning plates; after 24 or 72 h medium was removed and replaced with 0.2 ml medium containing the indicated concentrations of curcumin for different times. At the end of the incubation, proliferation was measured by the modified tetrazolium salt-3-(4-5 dimethylthiozol-2yl) 2-5 diphenyl-tetrazolium bromide (MTT) assay. For this,

0.02 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 4 h incubation at 37°C, medium was replaced by 0.15 ml DMSO. After a 15 min incubation at 37°C, the optical densities at 595 nm were measured using a Microplate Reader (BIO-RAD).

Cell invasion assay. Matrigel invasion assay were done using modified boyden chambers with polycarbonate Nucleopore membrane. Precoated filters (6.5 mm in diameter, 8-μm pore size, Matrigel 100 μg/cm^2) were rehydrated with 100 μl medium. Then, 1 x 10^5 cells in 100 μl serum-free DMEM supplemented with 0.1% bovine serum albumin were seeded into the upper part of each chamber, whereas the lower compartments were filled with 600 μl of the normal DMEM containing 10% serum. Following incubation for 18 h at 37°C, non-invaded cells on the upper surface of the filter were wiped out with a cotton swab, and the invaded cells on the lower surface of the filter were fixed and stained with trypan blue.

RNA isolation and RT-PCR. To determine the effect of curcumin on cyclin D1 mRNA levels, cells were treated with curcumin, the mRNA was extracted, and equal amounts were transcribed to cDNA from a kit (Takara). The cDNA was taken from samples at various times and used as the DNA templates for PCR. Primers used for cyclin D1 were 5'-CCG TCC ATG CGG AAG ATC-3' and 5'-ATG GCC AGC GGG AAG AC-3'. For a control, β-actin was used in the reaction. Primers for β-actin were 5'-ACA CTG TGC CCA TCT ACG-3' and 5'-CTC GTC ATA CTC CTG CTT G-3'. For each cDNA, 2 μl was used as the template and 1 μl of each primer was used. The samples went through 30 rounds of PCR. Samples were separated on a 3% agrose gel and visualized by ethidium bromide.

Western blot analyses. To determine the expression of protein, whole cell extracts were prepared from 1 x 10^6 cells in lysis buffer (20 mM Tris pH 7.4, 250 mM sodium chloride, 0.1% TritonX-100, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate and 1 mM DTT), and 60 μg of the protein was resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were eletrotransferred to nitrocellulose filters, the membrane (Amersham) was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 3 h at room temperature, and the proteins were probed with specific antibodies—cyclin D1 (NeoMarkers), PAK1 (Cell Signaling Technology), HER2 (Santa) and detected by chemiluminescence (ECL, Amersham). To assaure equal loading, gels were stripped and reprobed with antibodies against GAPDH (Kangchen Bio-tech Inc., Shanghai, China).

Immunoprecipitation and kinase assays. Cells were preincubated with the chemical compound before further incubation in the absence or presence of EGF (Peprotech, America). Cells were washed three times with ice-cold PBS and suspended in cold lysis buffer containing 1% Nonidet P-40, 50 mM Tris, pH 7.5 and 150 mM NaCl supplemented with protease and phosphatase inhibitors as described.[39,40] Cleared lysates were preadsorbed with protein A-Sepharose 4B beads (GE Healthcare Bio-Science Inc., Sweden) for 1 h at 4°C before incubation with PAK1 antibody (Santa), and centrifuged, and the supernatants (equal amounts of protein) were subjected to immunoprecipitation using the indicated antibodies and the protein A-Sepharose 4B beads. PAK1 kinase assays were performed as described using the exogenous substrate myelin basic protein (Sigma) to assess activity.[30] Protein A-Sepharose 4B beads containing immunoprecipitated with PAK1 were washed twice with lysis buffer and three times with kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$ and 0.2 mM DTT). Kinase activity was measured in 60 μl of kinase buffer containing 10 μCi of [γ-^{32}P] ATP (5,000 Ci/mmol) for 20 min at 30°C. Reactions were stopped by addition of SDS sample buffer and loading on a 10% SDS-PAGE. Proteins were transferered onto nitro-cellulose membranes and ^{32}P-labeled proteins were visualized by autoradiography with Molecular Imager RX (BIO-RAD). To assure equal loading, PAK1 were detected by immunoblotting analysis and MBP was detected by ponceau staining. For curcumin direct inhibition of PAK1 kinase activity in vitro, commercialized PAK1 kinase (Cell Signaling Technology Inc.,) was preincubated with the indicated concentrations of curcumin. Kinase reactions were started by addition of Histone H4 (Sigma) and a mixture of 1 mM ATP and [γ-^{32}P] ATP.

Cell cycle analysis. To determine the effect of curcumin on the cell cycle, cells were treated with various concentrations of curcumin for 12 h, 24 h and 48 h, washed, and fixed with 70% ethanol. After incubation overnight at -20°C, cells were washed with PBS and then suspended in a staining buffer (10 μg/mL propidium iodide, 0.5% Tween 20, 0.1% RNase in PBS). The cells were analyzed using a FACSVantage flow cytometer with the CellQuest acquisition and analysis software program (Becton Dickinson and Co., San Jose, CA). Gating was set to exclude cell debris, doublets and clumps.

Computer modeling. The three-dimensional structures of curcumin (both keto and enol form) were generated and minimized, using Tripos force field, by SYBYL 6.91 software package.[41] The crystal structure of PAK1 kinase (entry code 2HY8)[42] was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB).[43] The heteroatom entries were deleted. The protein structure was then subjected to the addition of all hydrogens, Amber charges. Docking was performed with GOLD4.0,[44] on a personal computer Pentium 4 (1.8 GHz) with 1 Gb RAM under Linux OS. Default parameters were used. The co-complexed ligand was extracted from the crystal structure and was re-docked into the ATP-binding pocket to reproduce the binding modes observed in the crystal structure, afterwards, the curcumins were docked into the PAK1 active site with GOLD Score to measure the binding affinity. The binding modes were displayed with Vida2 software.[45]

Acknowledgements

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