INTRODUCTION

Gastric cancer is the second most common cancer in the world. In China, there is a high incidence and mortality associated with this cancer (Yang, 2006). Helicobacter pylori is one of the contributing factors to the aetiology of gastric cancer. The International Agency for Research on Cancer has classified H. pylori as a group I carcinogen that causes gastric cancer in humans (Humans, 1994). H. pylori infects approximately 50% of the human population chronically, but only a subpopulation of those infected by H. pylori develop serious clinical consequences (Suerbaum & Michetti, 2002). Therefore, the identification of relevant bacterial factors will provide mechanistic insight into gastric carcinogenesis and identify populations at a high risk for H. pylori infection; these populations can then be targeted for therapeutic intervention.

H. pylori is a Gram-negative organism with a spiral shape, which allows colonization of gastric surface mucous cells, that resides in the mucus layer without invasion (Steer, 1985). Recent reports of H. pylori virulence factors have suggested that cytotoxin-associated protein A (cagA) and vacuolating cytotoxin A (vacA) are critical biomarkers related to H. pylori virulence (González et al., 2011; Montecucco & Rappuoli, 2001). However, cagA and vacA expression might not correlate with gastric diseases in China (Fock et al., 2008; Zhang et al., 2001), which implies that perhaps some other H. pylori virulence factors contributed to the characteristics of H. pylori infection in China.

Thioredoxin-1 (Trx1), encoded by HP0824 in strain 26695, is an antioxidant of H. pylori. Trx1 is a multifunctional protein with a low molecular mass; it contains a redox-active site, Cys-Gly-Pro-Cys, and is a ubiquitous enzyme that catalyses the reduction of disulfide bonds. Because reactive oxygen species and reactive nitrogen species are generated from inflammatory cells when H. pylori adheres to gastric epithelial cells, there must be an immediately available system to overcome these toxic redox compounds (Nardone et al., 2004). Trx1 is considered to be an arginase chaperone that protects against oxidative and nitrosative stresses, and it is responsible for the ability of H. pylori to persist for decades in hostile gastric environments (McGee et al., 2006; Wang et al., 2006; Windle et al., 2000). The involvement of Helicobacter pylori thioredoxin-1 in gastric carcinogenesis

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Abbreviations: cagA, cytotoxin-associated protein A; CI, confidence interval; CT, cycle threshold; PI, propidium iodide; Trx1, thioredoxin-1; vacA, vacuolating cytotoxin A.

A supplementary figure and table are available with the online version of this paper.
pathogenic mechanisms of *H. pylori* Trx1 are still under investigation.

In previous studies, we used two-dimensional gel electrophoresis to determine that Trx1 expression was higher in *H. pylori* isolated from gastric cancer tissues than in *H. pylori* isolated from tissues exhibiting gastritis and peptic ulcer (Zhang et al., 2006). In brief, the *H. pylori* protein isolated from three tissues displaying gastritis and three gastric cancer tissues was extracted by lysis buffer and sonication. One hundred micrograms of protein was applied to linear pH 3–10 immobilized pH gradient (IPG) strips (17 cm), and with 12.5 % linear vertical SDS-PAGE as the second dimension. Digitized images from the silver-stained gels were analysed using ImageMaster (Amersham Biosciences). The differentially expressed proteins were analysed using matrix-assisted laser desorption/ionization-time of flight MS and electrospray ionization quadrupole time-of-flight MS analysis, followed by Mascot (Matrix Science) search. The results showed that Trx1 intensity ratio of gastric cancer to gastritis was 5.69, with a Mowse score of 94, and sequence coverage showed that Trx1 intensity ratio of gastric cancer to gastritis tissues followed by Mascot (Matrix Science) search. The results were stored at −80 °C and RNA isolation and reverse transcription were performed within 2 weeks. Real-time PCR was carried out using SYBR Green on a Lightcycler 480II Real-time PCR Detection System (Roche). Each real-time PCR was performed in technical triplicates. All results were normalized to 16S rRNA amplification. The real-time PCR primers were the following: 16S rRNA: 5′-CCGCCCTACGGCCTCTTAC-3′ (forward primer) and 5′-GTAACGATAGCAGCCGCC-3′ (reverse primer); *H. pylori* Trx1: 5′-GGGTTCGTTAGTGGATTGTTTG-3′ (forward primer) and 5′-GACGAGCTTCCGCACTTTTGTA-3′ (reverse primer). The relative expression of the target gene *H. pylori* Trx1 was calculated based on the cycle threshold (CT) measurements. The ΔCT-values of each sample were calculated as ΔCT

**METHODS**

**Patient samples.** Gastric biopsy tissues were obtained from 43 patients with a clinical diagnosis of gastric cancer and gastritis who had undergone gastrectomy at Peking University Third Hospital from 2006 to 2008. All collected tissues had positive results from a rapid urease test that were confirmed by Warthin-Starry staining. These samples were divided into two groups: one group of tissues, which contained 16 samples that included 8 tissues displaying gastritis and 8 gastric cancer tissues, were used to directly test for *H. pylori* Trx1; the other group of tissues, which contained 27 samples that included 14 tissues displaying gastritis and 13 gastric cancer tissues, were used for the isolation and culturing of *H. pylori*. Written informed consent with a signature was obtained from each patient. Diagnoses of all the samples were confirmed histologically by two independent pathologists, and all tissues were assessed by haematoxylin-eosin staining.

**Culture of bacterial strains.** Tissues used for the isolation and culturing of *H. pylori* were collected in tubes containing brucella broth (Oxoid). Immediately after homogenization, the samples were plated on blood agar plates containing 39 g l−1 Columbia solid culture medium (Oxoid), 10 % (v/v) horse serum (Fisher Scientific) and the antibiotics amphotericin B (4 μg ml−1) (Life Tech), trimethoprim (4 μg ml−1) and vancomycin (4 μg ml−1). The plates were incubated at 37 °C for 5 or 7 days in a microaerobic environment [5 % (v/v) O2, 10 % (v/v) CO2 and 85 % (v/v) N2]. Before harvesting, the *H. pylori* cultures were examined using urease tests and Gram staining. Oxidase tests and catalase tests were also used to ensure that the strains were not contaminated.

**RNA isolation, reverse transcription and real-time PCR.** RNA isolation, reverse transcription and real-time PCR procedures were performed as described previously (Ma et al., 2008). For RNA extraction from *H. pylori* strains, *H. pylori* were resuscitated on blood agar plates and harvested into tubes containing precooled sterile PBS. After two washes with PBS, the total RNA was purified using Trizol (Life Tech). RNAs were isolated from bacterial strains and tissues, respectively, treated with RNase-free DNase I, and reverse transcribed using the RevertAid First strand cDNA Synthesis kit (Thermo). The samples were stored at −80 °C and RNA isolation and reverse transcription were performed within 2 weeks. Real-time PCR was performed in technical triplicates. All results were normalized to 16S rRNA amplification. The real-time PCR primers were the following: 16S rRNA: 5′-CCGCCCTACGGCCTCTTAC-3′ (forward primer) and 5′-GTAACGATAGCAGCCGCC-3′ (reverse primer); *H. pylori* Trx1: 5′-GGGTTCGTTAGTGGATTGTTTG-3′ (forward primer) and 5′-GACGAGCTTCCGCACTTTTGTA-3′ (reverse primer). The relative expression of the target gene *H. pylori* Trx1 was calculated based on the cycle threshold (CT) measurements. The ΔCT-values of each sample were calculated as ΔCT

**Construction of Trx1-knockout mutant *H. pylori* strain.** *H. pylori* strain 26695 was used as the parental strain in this study. To generate a *Trx1*-knockout mutant strain by allelic replacement mutagenesis, gene splicing by overlap extension for recombinating DNA molecules (Horton et al., 1989) was used. A kanamycin resistance (kanR) cassette was amplified from pET-30a (Novagen) using primers: 5′-CAAAATATATATTAGTAGAATATATAGCATTACAGG-3′ (forward primer) and 5′-CACATTCGCAATCTATCATATGATATATATATATTTT-3′ (reverse primer); *H. pylori* strain 26695 (Tomb et al., 1997), extracted according to the manufacturer’s instructions (Promega), was used to amplify the upstream and downstream fragments close to *Trx1* (UpTrx, DownTrx). The primers were as follows: UpTrx: 5′-CCGAAATTCCAGAATTTTTGATATAGCATTACACG-3′ (forward primer) and 5′-AGTTTTCCCGTGATATATGCTATATGATATGCGATG-3′ (reverse primer); DownTrx: 5′-TCATTGAGTGCCTGATGTTTCTGCATGATGATGCGG-3′ (forward primer) and 5′-GGCCGAATCCAGCAATAGGCGCGATGATGACCG-3′ (reverse primer). The UpTrx-F and DownTrx-R primers introduced EcoRI and BamHI restriction sites, respectively (shown in bold italics), for the cloning of recombed DNA fragment into pBluescript II SK (+) plasmid vector (Stratagene) digested with the same restriction enzymes. Electrottransformation and incubation on selective blood agar plates containing 20 mg kanamycin 1−1 (Life Tech) were carried out as described previously (Ferrero et al., 1992). Genomic DNA was extracted from putative recombinants, and the allelic replacement was evaluated by PCR and sequencing using the above primers.

**Cell lines, culture conditions and co-culture assays.** Two cell lines, GES-1 and BGC823 cells were cultured in RPMI1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS) (HyClone). Cell lines were cultured at 37 °C in a humidified incubator in 5 % (v/v) CO2. After the cultures had been resuscitated on blood agar plates, *H. pylori* expressing low and high levels of *Trx1*, *Trx1*-knockout mutant strain and its parental strain were harvested and washed with PBS three times, prepared in cell growth medium and diluted to a final concentration of 1 × 105 c.f.u. ml−1. GES-1 and BGC823 cells were plated 1 day before *H. pylori* treatment. For co-culturing of cells and strains, cells were rinsed once with PBS before fresh growth medium was added. The bacterial strains were then added to the cell medium at an m.o.i. of 200:1 and maintained under normal cell growth conditions. Uninfected GES-1 and BGC823 cells were used as controls.

**MTT assay.** After co-culturing, cell viability was quantified using an MTT assay. In brief, 1 × 105 cells per well were seeded into 96-well
culture plates and incubated for 24 h at 37 °C in 5 % (v/v) CO₂; the cells were then treated with the two H. pylori strains at an m.o.i. of 50 : 1, 100 : 1 or 200 : 1 as previously described. After co-culture for 24 h and 48 h, 50 μl MTT stock (final concentration 5 mg ml⁻¹) was added to the cell culture medium, and the cells were incubated for 4 h. The culture supernatant was removed, and 150 μl DMSO (Life Tech) was added to each culture to dissolve the crystals. After shaking the plate at room temperature for 10 min, cell viability was assessed by reading the absorbance at 495 nm using a spectrophotometer (Multiskan MK3; Thermo).

Cell cycle analysis. BGC823 cells were seeded in six-well plates (at a density of 1 x 10⁵ cells). After co-culturing the cells with H. pylori at an m.o.i. of 200 : 1 for 24 and 48 h, respectively, the cells were harvested and washed twice with cold PBS. Untreated BGC823 cells were used as negative controls. The cells were suspended in 0.5 ml 70 % (v/v) ethanol and chilled at −20 °C for 30 min. After extensive washing with PBS, the cells were resuspended in PBS containing 50 μg ml⁻¹ propidium iodide (PI) and 0.1 mg ml⁻¹ RNase A and incubated at 37 °C for 30 min. Cells were subsequently resuspended in PBS and assayed by flow cytometry (Becton Dickinson FACS Calibur). The results were analysed using ModFit LT Software (Verity Software House).

Apoptosis assay.GES-1 cells were seeded in six-well plates (at a density of 1.2 x 10⁵ cells). After co-culturing the cells with H. pylori at an m.o.i. of 200 : 1 for 24 and 48 h, an Annexin V-FITC/PI double-staining Apoptosis Detection kit (Becton Dickinson) was used to label the cells according to the manufacturer’s instructions. Untreated GES-1 cells were used as negative controls. Cells were washed with cold PBS, and 200 μl of the Annexin V-Binding Buffer was added. After the cells were stained with 10 μl FITC-labelled Annexin V and 5 μl PI, the samples were immediately analysed by flow cytometry.

Western blot analysis. Proteins related to the cell cycle and apoptosis were detected by Western blot analysis. GES-1 and BGC823 cells cultured with or without H. pylori were individually harvested. For protein extraction, cells were suspended in cell lysis buffer containing a protease inhibitor mixture and shaken on ice for 30 min. The cell lysate was centrifuged at 15 000 g at 4 °C for 10 min, and the supernatant was collected. The total protein concentration was measured by the Bradford method using a BCA Protein Assay kit. Proteins (40 μg) were separated on 12 % (w/v) SDS-PAGE gels and electrophoretically transferred onto PVDF membranes (Millipore). The membranes were blocked in 5 % (w/v) fat-free milk in Tris-buffered saline–0.5 % (v/v) Tween-20 at room temperature for 1 h and incubated overnight at 4 °C with antibodies against p21 (MBL; 1 : 500), cyclin D1 (MBL; 1 : 500) or α-tubulin (MBL; 1 : 2000). After three washes in PBS supplemented with 0.1 % (v/v) Tween-20 for 15 min, the membranes were incubated with a secondary antibody, goat anti-Rabbit IRDye 680 or goat anti-Mouse IRDye 800CW (LI-COR; 1 : 5000), for 1 h at room temperature. Proteins were identified by scanning the membranes using the Odyssey Imager (LI-COR Biosciences).

Statistical analysis. The significant differences between two groups were analysed using Student’s t-test. Comparisons between multiple sample sets and the control were analysed using a one-way ANOVA test followed by a Student–Newman–Keuls test. Data are presented as the mean ± SD of three independent experiments. The 90 % confidence interval (CI) of ΔCT-values was estimated to distinguish the high or low expression of H. pylori Trx1. All statistical analyses were performed using SPSS 13.0 computer software (SPSS). P-values that were less than 0.05 were considered statistically significant.

RESULTS

Trx1 expression of H. pylori in gastric cancer tissues is higher than in tissues with gastritis

We initially evaluated the Trx1 expression of H. pylori in eight gastric cancer tissues and eight tissues displaying gastritis using real-time PCR three times. As shown in Fig. 1a, the mRNA expression level of H. pylori Trx1 in gastric cancer tissues was significantly higher than in tissues exhibiting gastritis (P<0.05). For the selection of strains expressing high and low levels of Trx1 for use in co-culture experiments, H. pylori were isolated from 13 gastric cancer samples and 14 samples displaying gastritis, and the Trx1 expression was evaluated. Consistent with our previous results, the mRNA expression level of Trx1 in H. pylori isolated from the gastric cancer tissues was significantly higher than the level observed for tissues exhibiting gastritis (P<0.05) (Fig. 1b). The 90 % CI of ΔCT-values was 8.86–28.05. Two strains of H. pylori, which expressed high and low levels of Trx1, respectively, were chosen for co-culture with cell lines based on ΔCT-values. The ΔCT-values of these two strains were 8.103 and 31.029. The ΔCT-value of the strain 26695, which was used as the parental strain to construct the Trx1 mutant, was 10.037.

H. pylori expressing high levels of Trx1 decreased cell viability and induced apoptosis in GES-1

MTT assay was performed three times to examine the effects of H. pylori expressing high or low Trx1 levels on cell growth in GES-1. After samples were treated with H. pylori at an m.o.i. of 50 : 1, 100 : 1 or 200 : 1 for 24 h or 48 h, the cell number was found to be decreased in all cases. When the m.o.i. increased, this effect was more obvious. Compared with H. pylori expressing low Trx1 levels, H. pylori expressing high levels of Trx1 had a more significant impact on the reduction in cell survival based on MTT assays (Fig. 2a).

An apoptosis assay was used to detect cell apoptosis induced by H. pylori in GES-1 cells three times. To evaluate the cell changes induced by H. pylori, an m.o.i. of 200 : 1 was used. The results (Fig. 2b) indicated that H. pylori expressing either high or low Trx1 levels induced apoptosis in GES-1 cells at 24 h and 48 h post-infection. Compared with the negative control, H. pylori expressing low or high Trx1 levels caused significant cell apoptosis; the cell apoptotic effect was more pronounced in the high Trx1 H. pylori-infected cells (P<0.05).

H. pylori expressing high levels of Trx1 induced cell proliferation in BGC823

After co-culturing with H. pylori, an MTT assay of BGC823 showed that cell number had increased. Compared with H. pylori expressing low Trx1 levels, the increase in cell number was more significant after BGC823 was co-cultured with H. pylori expressing high Trx1 levels at all
To certify the roles of Trx1 separated from other virulence factors in H. pylori, a Trx1-knockout mutant strain and its parental strain were used. Cell apoptosis induced by H. pylori in GES-1 cells was assayed three times as described above. The results (Fig. 4a) indicated that compared with the negative control, Trx1-knockout mutant H. pylori and its parental strain induced apoptosis in GES-1 cells at 24 h and 48 h post-infection. The cell apoptotic effect was more pronounced in parental H. pylori-infected cells compared with the Trx1-knockout mutant H. pylori (P<0.05).

Cell cycle analysis was performed to further determine the effects of H. pylori in BGC823 cell proliferation three times as described above. As shown in Fig. 4(b), no obvious cell cycle changes were found after treatment for 24 h with Trx1-knockout mutant H. pylori and its parental strain. However, 48 h of parental strain infection induced a significant increase in the percentage of S phase cells compared with the control (P<0.05). Treatment with Trx1-knockout mutant H. pylori did not increase the percentage of cells in the S phase significantly (26.78 vs 29.88 %). In comparison with Trx1-knockout mutant strain, infection by its parental H. pylori showed an obvious regulative effect on the S phase (41.90 vs 29.88 %).

Cyclin D1/p21 are involved in the effects of H. pylori expressing high Trx1 levels on cell growth

In support of the above results, Western blot analysis was conducted to monitor changes in the level of previously described proteins implicated in cell cycle and apoptosis. In comparison with the negative control and treatment with H. pylori expressing low levels of Trx1, a marginal reduction in the level of cyclin D1 and a remarkable upregulation in p21 were demonstrated in GES-1 cells treated with H. pylori expressing high levels of Trx1. Strikingly, for BGC823 cells, compared with the negative control, the protein level of cyclin D1 was significantly increased after infection with H. pylori expressing low or high levels of Trx1. This upregulation of cyclin D1 expression by H. pylori was dose-dependent. The level of p21 in BGC823 cells remained unchanged after H. pylori treatment (Fig. 5).

DISCUSSION

Many virulence factors of H. pylori have been reported to be associated with gastric carcinogenesis. It is well known that H. pylori has marked genetic diversity. CagA is a critical biomarker of H. pylori virulence in Western countries (Saadat et al., 2007). However, in the Chinese
population with a 90% prevalence of CagA-positive *H. pylori* infection, only a minority (approx. 1% instead of 90%) of the infected population would ultimately develop gastric cancer. Thus, the assessment of additional virulence proteins is essential for the identification of individuals with a high risk of developing gastric cancer (Epplein et al., 2012). In our study, a virulence factor related to gastric cancer, called Trx1, was identified. Trx1 was shown to be an electron donor in vitro for alkyl-hydroperoxide reductase, a member of the antioxidant peroxidases in *H. pylori* (Comtois et al., 2003). However, investigations of the relationship between *H. pylori* Trx1 and clinical diseases are still lacking, especially studies of gastric cancer associated with *H. pylori*.

Our results suggested that *H. pylori* expressing high levels of Trx1 are associated with gastric cancer. Moreover, the investigation of Trx1-mutant and its parental strain separated the effects of Trx1 from other virulence factors. *H. pylori* Trx1 may contribute to the pathogenesis of gastric cancer, and *H. pylori* expressing high levels of Trx1 would be expected to be highly pathogenic in gastric diseases in China. We also explored Trx1 functions in GES-1 and BGC823 cells. To investigate the mechanism underlying Trx1 effects on cell growth, two key factors involved in controlling cell cycle progression, p21 and cyclin D1, were assayed by Western blot. Cyclin D1 was markedly downregulated, and p21 was upregulated in GES-1 cells infected by *H. pylori* expressing high levels of Trx1. Cyclin D1 can

Fig. 2. *H. pylori* decreased cell viability and induced apoptosis in GES-1 cells. (a) The optical density of GES-1 cells was measured using an MTT assay at 495 nm after cells were co-cultured with *H. pylori* expressing high or low Trx1 at m.o.i. of 50:1, 100:1 or 200:1. (b) A representative experiment of Annexin V-FITC/PI double-staining of GES-1 cells at 24 and 48 h of treatment is shown with proportions of Annexin V positive cells (AV+, apoptotic cells). The cells were treated with *H. pylori* at m.o.i. of 200:1. Data are presented as the mean ± SD of three independent experiments. *When compared with the control, a *P*<0.05 was considered statistically significant. †When compared with *H. pylori* expressing low levels of Trx1, a *P*<0.05 was considered statistically significant.
accelerate cell cycle progression and promote cells to enter S phase (Coqueret, 2002); thus it is considered to be a marker of cell proliferation (Motohashi et al., 2011). P21 is a potent inhibitor of cyclin-dependent kinases capable of arresting cell cycle progression (Radhakrishnan et al., 2004). The expression of p21 is positively correlated with cell apoptosis (Zoli et al., 2005). The changes in cyclin D1 and p21 levels can explain the apoptosis of infected GES-1 cells. Interestingly, for BGC823, the results of the MTT proliferation assay and the cell cycle analysis showed that H. pylori expressing high levels of Trx1 increased cell proliferation. The upregulation of cyclin D1 in BGC823 cells treated with H. pylori also confirmed the oncogenic effects of Trx1 in H. pylori. The regulation of cyclin D1 likely contributes to the increased cell growth caused by H. pylori infection.

Invasive gastric carcinoma is preceded by a cascade of precancerous lesions (Correa & Piazuelo, 2012). According to this model, intestinal gastric cancer is a multistep process from chronic gastritis, progressing through chronic atrophic gastritis, intestinal metaplasia and dysplasia (Correa, 1995). H. pylori infection has been reported to trigger increased apoptosis in the glandular epithelium and atrophy of the mucosa (Correa & Piazuelo, 2008). Further, exposure of gastric epithelial cells to H. pylori results in an inflammatory reaction (Nardone et al., 2004). In our studies, H. pylori infection induced apoptosis in a gastric epithelial cell line. This result might be related to the first recognizable histological change proposed in the Correa model, active chronic inflammation. Sustained stimulation of apoptosis might result in excessive cell loss and ulcer development. In this process, because H. pylori infection can result in the deamination of DNA that causes mutations, the surviving cells might undergo genomic events leading to malignant transformation and driving the development and progression of gastric tumours (David & Meltzer, 2011). Persistent survival of abnormal cells can

Fig. 3. H. pylori induced proliferation in BGC823 cells. (a) The optical density of BGC823 cells was measured using an MTT assay at 495 nm after cells were co-cultured with H. pylori expressing high or low levels of Trx1 at m.o.i. of 50 : 1, 100 : 1 or 200 : 1. (b) BGC823 cells infected by H. pylori with different Trx1 expression at m.o.i. of 200 : 1 and control cells were harvested, stained with PI and analysed by flow cytometry. Data are presented as the mean ± SD of three independent experiments. *When compared with the control, a P<0.05 was considered statistically significant. †When compared with H. pylori expressing low levels of Trx1, a P<0.05 was considered statistically significant.
lead to the accumulation of sequential genetic mutations and the initial transformation process, which would result in tumorigenesis (Moss, 1998). Our results imply that the effects of *H. pylori* were different in the developing stages of gastric cancer. After normal gastric epithelial cells transform into malignant cells, *H. pylori* infection might not cause cell apoptosis and tissue damage, but this infection might promote cell proliferation. Increased cell proliferation is a common event in tissue hyperplasia, which is a neoplastic change in gastric glands (Shinohara et al., 1996).

Our study shows that *H. pylori* Trx1 is associated with the clinical cascade stages of gastric cancer.

Collectively, our findings demonstrate that the expression of *H. pylori* Trx1 is higher in gastric cancer tissues than in tissues displaying gastritis. This finding suggests that *H. pylori* expressing high levels of Trx1 play an important role in gastric carcinogenesis. Our study is a pilot study heralding the clinicopathological significance of *H. pylori* Trx1. Future studies will be performed to confirm the
relationship between <i>H. pylori</i> Trx1 and gastric cancer. It is also important to consider oxidative stress levels of host, which would be closely related with <i>H. pylori</i> Trx1 and might result in worsening of symptoms. We plan to create animal models of gastric cancer using Mongolian gerbils infected by <i>H. pylori</i> to determine the exact mechanisms of <i>H. pylori</i> with high Trx1 expression during gastric carcinogenesis.

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