Neurosci Bull June 1, 2015, 31(3): 371–378. http://www.neurosci.cn
DOI: 10.1007/s12264-014-1522-4

·Report·

Tunneling nanotubes between rat primary astrocytes and C6 glioma cells alter proliferation potential of glioma cells

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ABSTRACT

The tunneling nanotube (TNT) is a newly discovered, long and thin tubular structure between cells. In this study, we established a co-culture system for rat primary astrocytes and C6 glioma cells and found that TNTs formed between them. Most of the TNTs initiated from astrocytes towards C6 glioma cells. The formation of TNTs depended on p53. In addition, hydrogen peroxide increased the number of TNTs in the co-culture system. Established TNTs reduced the proliferation of C6 glioma cells. Our data suggest that TNTs between astrocytes and glioma cells facilitate substance transfer and therefore alter the properties, including the proliferation potential, of glioma cells.

Keywords: tunneling nanotube; astrocyte; glioma; proliferation; p53

INTRODUCTION

The tunneling nanotube (TNT) was first described as a freely-hovering tubular structure between two cells in 2004[1]. Similar structures had been described in *Drosophila* imaginal discs in 1999[2]. TNTs have been found in various types of cells, such as THP1 monocytes[3], rat astrocytes and neurons[4], PC12 cells[1, 5], DU 145 prostate cancer cells[6], HEK293 cells[1, 7], hematopoietic stem cells and progenitor cells[8], mouse macrophage J774 cells[9], TRVb-1 cells[10], human endothelial progenitor cells[11], T-cells[12], natural killer cells[13], human monocyte-derived macrophages[9, 14], and human mesothelioma cells[15, 16]. Studies have shown that TNTs can transfer a wide range of substances, such as cellular organelles[5, 17-19], polyglutamine aggregates[20], H-Ras[21], MHC class I molecules[22], Ca^{2+} [3, 23, 24], and even human immunodeficiency virus and PrP^{Sc}[12, 25-27]. Several studies have suggested that TNT-mediated cargo transport is part of the communication between cells[18, 27, 28].

In vivo evidence has shown that TNT-like structures exist in *Drosophila* imaginal discs[2] and MHC class II cells in mouse cornea[29]. Recent studies have shown that TNTs also exist in solid tumors[30]. Nevertheless, due to the lack of a specific TNT marker[31], it is still challenging to detect the TNTs between astrocytes and glioma cells *in vivo*.

Glioma is a central nervous system tumor, comprising about 80% of malignant tumors that develop in the brain[32]. More than 50% of gliomas are glioblastomas[33], which are the most aggressive form. The median survival time of glioblastoma patients is only 15 months[34]. In spite of limited progress, the treatment and prognosis of gliomas are still not satisfactory[35, 36]. Astrocytes are the most abundant surrounding cells in the glioma microenvironment *in vivo*[37]. TNTs, if they exist between glioma cells and astrocytes, could provide a communication pathway between them and thus affect their properties. This study was carried out to clarify this question.

MATERIALS AND METHODS

Cell Cultures and Cell Lines

Rat primary astrocytes were cultured from newborn Sprague-Dawley rat brain. The experiment protocol was approved by the Peking University Animal Care and Use

Committee and followed the NIH Guidelines regarding the care and use of animals for experimental procedures. Briefly, fresh rat brains were dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA). The mixture was filtered through 70-μm sterilized filters, then the cells were plated at 5×10^4 cells/mL. C6 glioma cells were obtained from the Cell Resource Center of Peking Union Medical College, Beijing, China. All cells were incubated at 37°C in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) with 10% FBS and 5% circulating CO_2. The culture medium was changed every 48 h.

Confocal Imaging and Movies of TNT Formation

Cells were imaged on a Zeiss LSM-510 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany). Image data were captured in the line-scanning mode with excitation at 488 nm for enhanced green fluorescent protein (EGFP) and 563 nm for red fluorescent protein (RFP). X–Z sections were taken at 1 μm per scanning step. Movies of TNT formation were shot and processed on a workstation for live cell imaging (API–Delta Vision-ELITE).

SiRNAs

SiRNAs targeting p53 and MDM2, and scramble controls (Qiagen, Hilden, Germany) were diluted to 5 nmol/L before transfection by Hiperfect (Qiagen) following the manufacture's instructions. The off-target effects and silencing efficiency of siRNAs were verified by Qiagen.

Immunostaining

Cells were permeabilized in PBS-Triton and incubated with anti-Ki67 antibody (Abcam, Cambridge, UK, 1:200) at 4°C for 24 h. Cy3-conjugated anti-rabbit antibody was applied as the secondary antibody. The nuclei were stained with Hoechst (10 μg/mL) in the dark for 10 min. Then the cells were observed under a fluorescence microscope (Olympus BH2-RFCA).

Apoptosis Assay

Cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized by 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 10 min on ice. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the *in situ* cell death detection kit I (Roche, Basel, Switzerland) as described by the manufacturer. TUNEL-positive cells were counted among 500 cells in each group.

MTS Assay

The MTS assay was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI). Cells were plated on 96-well plates (1 000 cells per well) for 24 h, followed by treatment with Latrunculin A. Then 20 μL CellTiter 96® AQueous One Solution reagent was added to each well in 100 μL medium, and incubated for 2 h at 37°C humidified with 5% CO_2. The OD value at 490 nm was measured with a Varioskan Flash reader (Thermo, Waltham, MA). Relative OD value = $[(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})] \times 100\%$.

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was assessed by one-way analysis of variance (ANOVA). Sheffé's test was applied as a *post hoc* test. P <0.05 was considered to be statistically significant.

RESULTS

Formation of TNTs in the Astrocyte-C6 Glioma Cell Co-culture System

To establish the co-culture system, rat astrocytes were transfected with RFP, and C6 glioma cells with EGFP. Then the astrocytes and glioma cells were mixed together at 1:1 (Fig. 1A). In our co-culture system, most of the TNTs initiated from the astrocytes rather than the glioma cells (Fig. 1B, B1 and B2) since the number of red TNTs far exceeded that of green TNTs (Fig. 1C). The TNTs initiating from the astrocytes targeted either another astrocyte or a glioma cell (Fig. 1D and E). Furthermore, Z-axis scanning showed that the TNTs between cells did not attach to the bottom of the culture dish, rather, they hovered freely in the medium (Fig. 1D1 and E1). In addition, cargoes were transported through TNTs (Fig. 1D1 and F6), indicating communication between astrocytes and glioma cells *via* TNTs. Movies of TNT formation (Fig. 1F1–6, Supplementary Movie S1) clearly showed a TNT initiating from an astrocyte, then elongating and targeting a C6 glioma cell. At the same time, mitochondrial transport through TNTs from astrocytes to C6 glioma cells was also seen (Fig. 1G). The astrocytes were transfected with RFP, which included a mitochondrial-targeting sequence (Fig. 1G).

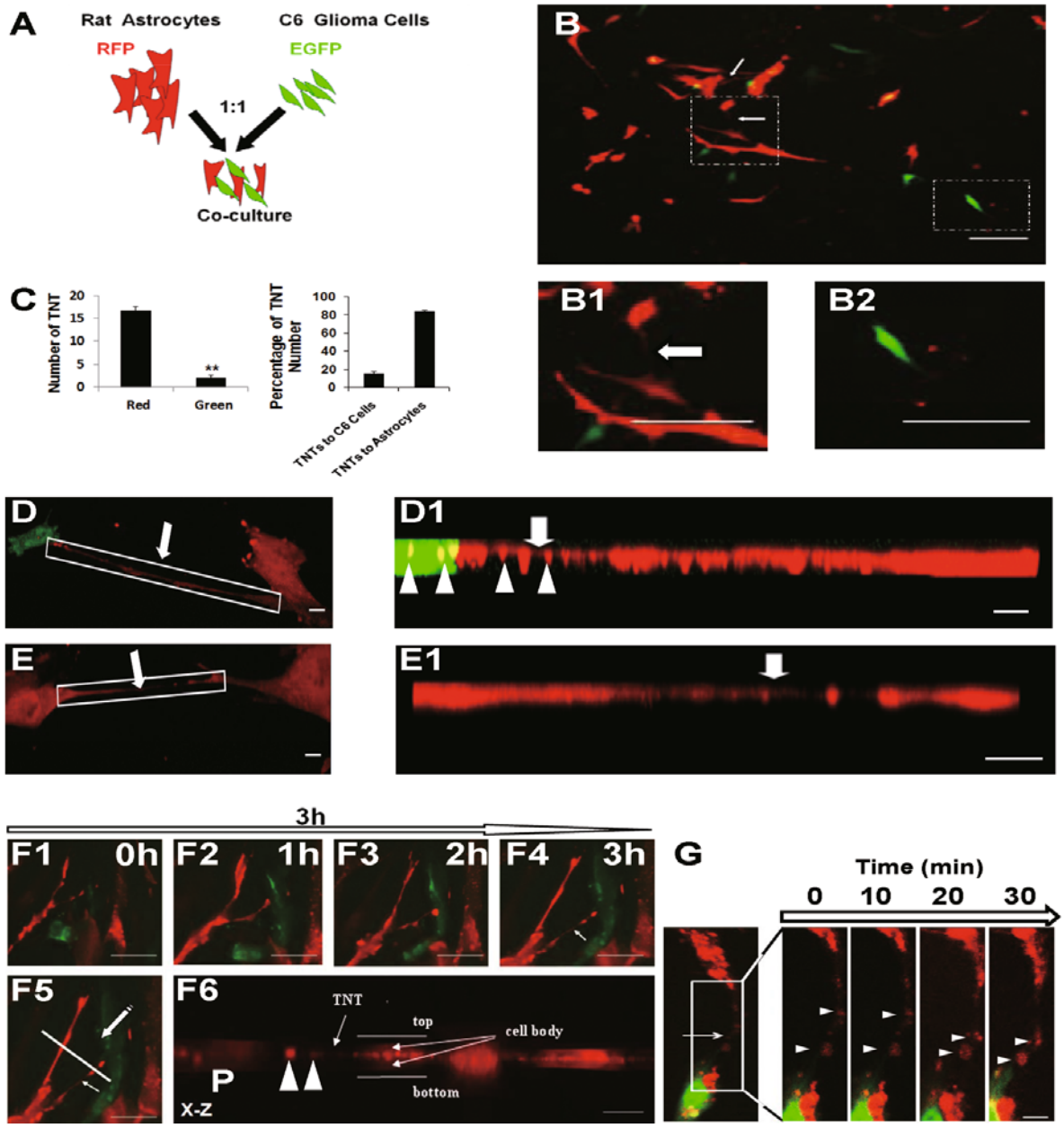

Fig. 1. Establishment of the astrocyte-C6 glioma cell co-culture system and the formation of TNTs in the system. (A) Scheme for establishing the astrocyte-C6 glioma cell (1:1) co-culture system. Astrocytes expressed RFP while C6 glioma cells expressed EGFP. (B) Cells in the co-culture system. Arrows indicate TNTs. (B1) High-magnification image of an astrocyte in the co-culture system (arrow, TNT). (B2) High-magnification image of a C6 glioma cell in the co-culture system. Scale bars, 200 μm. (C) Quantification of TNTs initiating from astrocytes (red) and C6 glioma cells (green) and percentage of TNTs initiating from astrocytes to astrocytes and C6 glioma cells. TNTs were counted among 100 cells in three independent preparations (mean ± SEM, **P <0.01 compared with red TNTs). (D) TNT between an astrocyte and a C6 glioma cell in the co-culture system. (E) TNT between two astrocytes in the co-culture system. (D1 and E1) Images of the z-axis view of the TNTs in D and E, showing that they did not attach to the bottom (arrowheads, cargoes transported through TNTs; arrows, TNTs; scale bars, 10 μm). (F1–F5) TNT initiated from one astrocyte and targeted at a C6 glioma cell (arrows, TNT). (F6) Image of the z-axis view of line P in F5 (arrow with dotted tail in F5 indicates direction of view). The TNT in F4 and F5 is hovering freely. Arrowheads in F6 indicate cargoes transported through the TNT. Scale bars, 30 μm in F1–F5; 15 μm in F6. (G) Mitochondrial transport from an astrocyte to a C6 glioma cell through a TNT (arrow, TNT; arrowheads, mitochondria transported through the TNTs; images captured every 10 min; scale bar, 10 μm).

H_2O_2 Increased TNT Induction, which Depended on p53 Activation

H_2O_2 is known to induce TNTs *in vitro*[18, 38]. In the present study, we confirmed that H_2O_2 (100 μmol/L, 24 h) increased the number of TNTs in the co-culture system (Fig. 2A and B). Similarly, most of the increased TNTs initiated from astrocytes (Fig. 2B). We had previously shown that TNT development depends on p53 activation[18]. In this study, the induction of TNTs initiating from astrocytes also depended on p53 (Fig. 2C and D). When p53 was knocked down by siRNA, the H_2O_2 (100 μmol/L, 24 h) induction of TNTs initiating from astrocytes was greatly reduced (Fig. 2D). In contrast, when the negative regulator of p53, MDM2, was knocked down by siRNA, the number of TNTs increased significantly (Fig. 2D). However, p53 or MDM2 siRNAs did not alter the number of TNTs initiating from C6 glioma cells (Fig. 2D).

TNTs Reduced Proliferation Potential of C6 Glioma Cells

As TNTs were established between astrocytes and C6 glioma cells, we then investigated whether alteration of the TNT number affected the proliferation potential of C6 cells. It has been reported that Latrunculin A, an F-actin-disrupting agent, blocks the formation of TNTs[18]. Here, we confirmed that Latrunculin A (25 nmol/L) markedly reduced the number of TNTs in the co-culture system (Fig. 3A, B1, and B2). In addition, transwells (0.4 μm pore size, Corning, New York, NY) were used to separate astrocytes and C6 glioma cells (Fig. 3C), and this kind of co-culture served as a control group to exclude the effects of secreted molecules. After induction by H_2O_2 (100 μmol/L) and/or Latrunculin A (25 nmol/L) for 24 h, we sorted C6 glioma cells from the co-culture system by flow cytometry and plated the same number of glioma cells from each experimental group on a 96-well plate. Cells were counted at 4, 24, 72, 96 and 120 h of culture according to the method used previously[39]. We found that if there were more TNTs in the co-culture system, the proliferation of C6 glioma cells decreased significantly (Fig. 3D and E). The decrease in proliferation was also demonstrated by Ki67 immunostaining (Fig. 3F and G). Meanwhile, H_2O_2 (100 μmol/L, 24 h) did not induce significant apoptosis in C6 glioma cells (Fig. 3H and I) while Latrunculin A (25 nmol/L, 24 h) alone did not markedly affect their proliferation (Fig. 3J).

DISCUSSION

The interactions between normal astrocytes and glioma cells are very important *in vivo*[40], but very few studies have addressed TNTs between normal cells and cancer cells. Here, we showed that in our co-culture system most of the TNTs initiated from astrocytes rather the glioma cells. Few studies have reported TNTs initiating from glioma cells, and the reason for the rare TNT initiation from C6 glioma cells in this study is still unknown.

We also found that ~80% of the TNTs initiating from astrocytes targeted other astrocytes while ~20% targeted C6 glioma cells. This difference may be explained in three ways: (1) different levels of RFP/EGFP expression; (2) inhibited proliferation of glioma cells which then decreased the chance of TNTs encountering them; and (3) molecules secreted by the target cells determined the direction of TNT growth[4], i.e., different kinds or levels of molecules secreted by astrocytes and glioma cells could differentially attract TNTs. Here, we found that TNTs in the co-culture system reduced the proliferation potential of C6 glioma cells, which suggests a new way to inhibit glioma proliferation. Since intensive communications between glioma cells and normal cells make it difficult to control glioma cells separately, our idea was to modify this communication rather than just focusing on glioma cells. Although H_2O_2 did not induce significant apoptosis in C6 glioma cells (Fig. 3H and 3I), it decreased their number at 120 h (Fig 3E–3G). In addition, at 96 h or before, H_2O_2 did not decrease cell numbers. As H_2O_2 is associated with oxidative stress[41], this treatment might cause such stress and cause long-term damage. Nonetheless, the cell number in the "H_2O_2 + C6" group was far higher than in the groups with more TNTs between astrocytes and C6 glioma cells, indicating that TNTs play important roles in the decrease of glioma cell proliferation.

Taken together, we provide evidence that TNTs exist between astrocytes and C6 glioma cells in a co-culture system. Most of the TNTs initiate from the astrocytes and target the glioma cells. In addition, H_2O_2 increases the number of TNTs. The TNTs alter the proliferation potential of glioma cells. Our findings disclose a novel function of TNTs and we hope this will result in innovative ways to study the treatment of glioma.

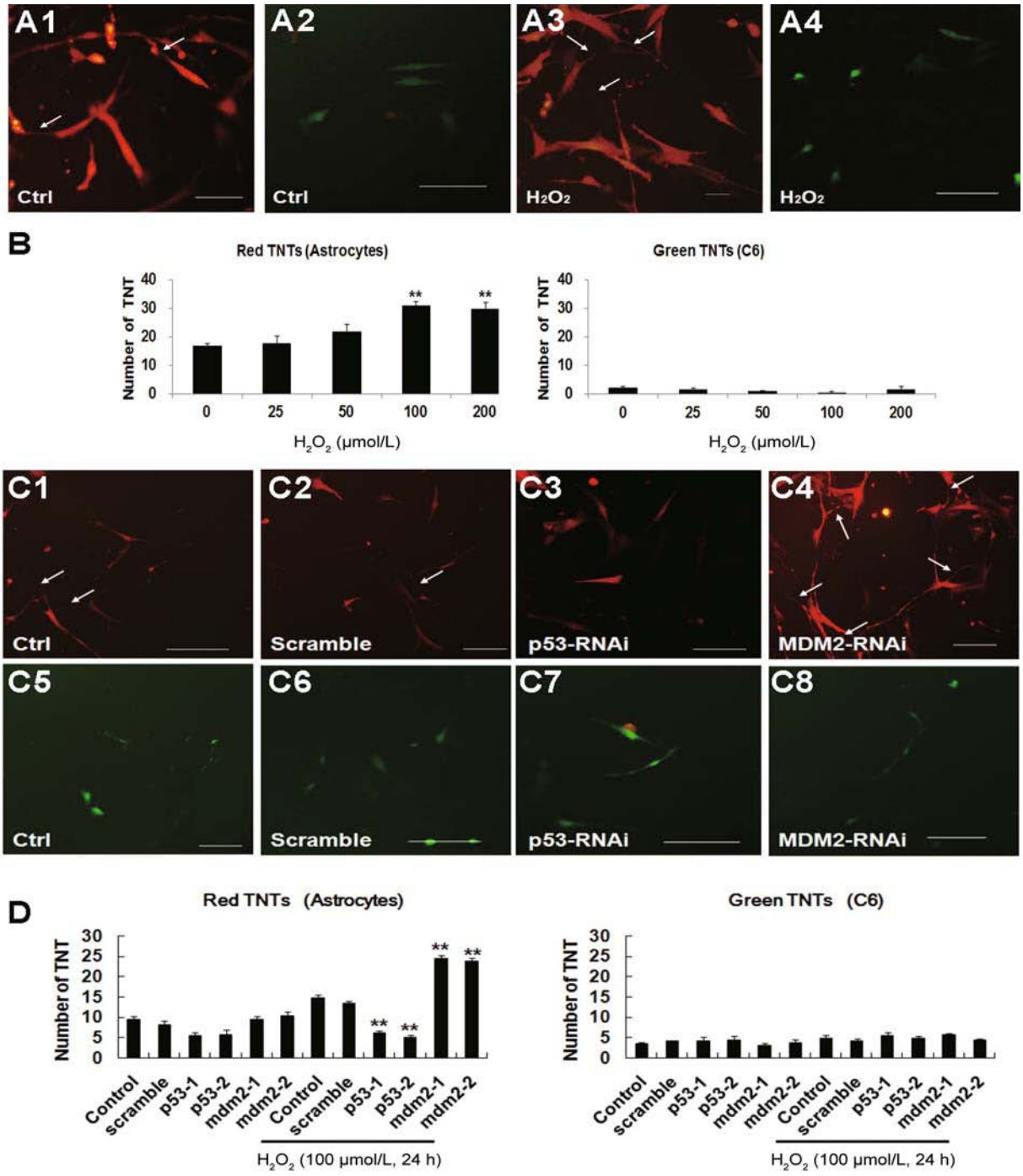

Fig. 2. H_2O_2 induced more TNTs in the co-culture system and this depended on p53. (A1–4) Astrocytes (red) and C6 glioma cells (green) in the co-culture system without (A1 and A2) or with (A3 and A4) H_2O_2 (100 μmol/L, 24 h) (scale bars, 200 μm). (B) Quantification of TNTs initiating from astrocytes (red) and C6 glioma cells (green). Treatment of cells with different doses of H_2O_2 lasted 24 h in each group. TNTs were counted among 100 cells in three independent preparations (mean ± SEM, $^{**}P$ <0.01 compared with control groups). (C1–8) Astrocytes (red) and C6 glioma cells (green) in the co-culture system treated with H_2O_2 (100 μmol/L, 24 h) and transfected with scrambled siRNA (C2 and C6), siRNA to p53 (C3 and C7), and siRNA to MDM2 (C4 and C8) (scale bars, 200 μm). (D) Quantification of TNTs initiating from astrocytes (red) and C6 glioma cells (green) (TNTs counted among 100 cells in three independent preparations; mean ± SEM, $^{**}P$ <0.01 compared with control groups).

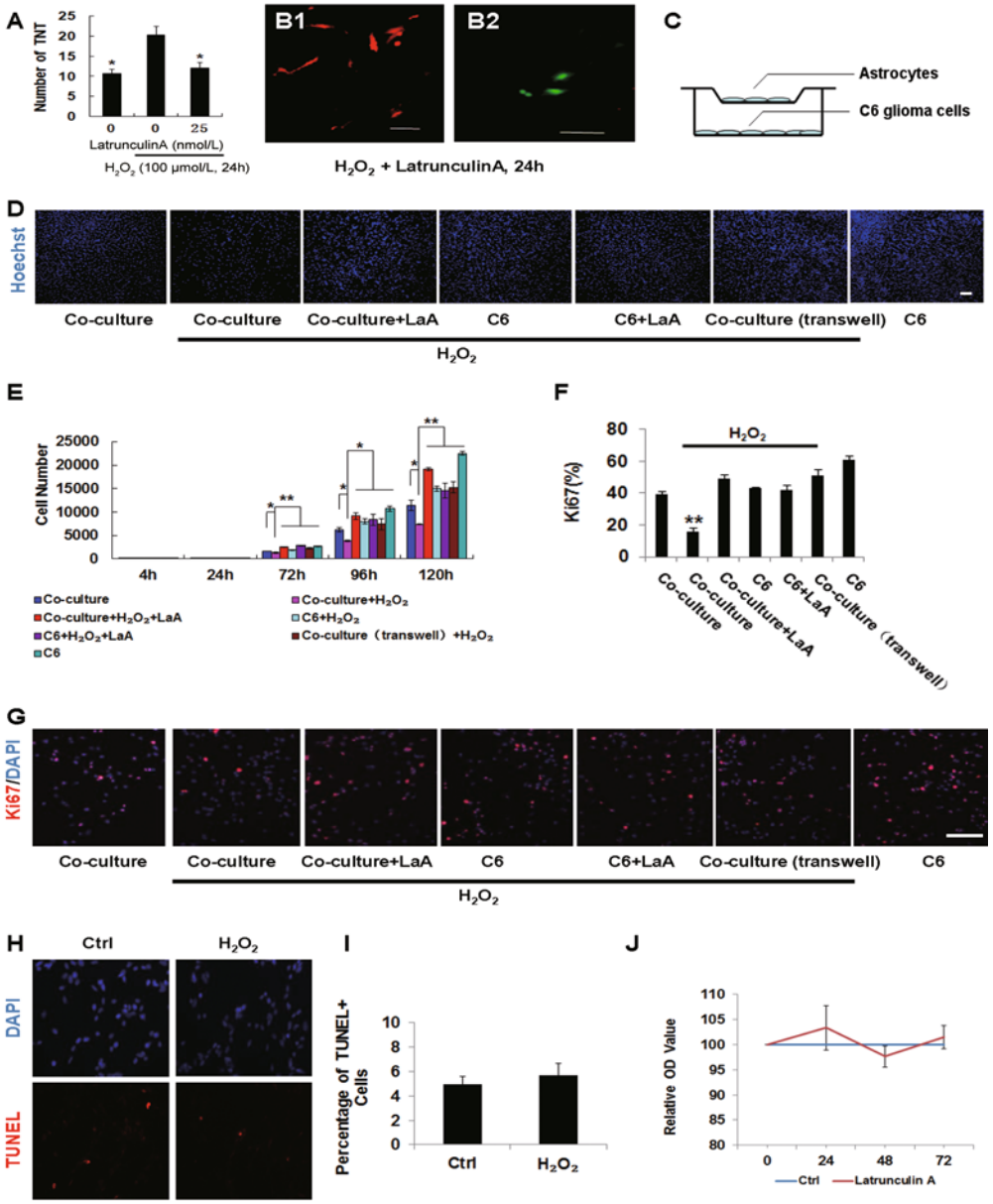

Fig. 3. Increase of TNTs in the co-culture system reduced the proliferation of C6 glioma cells. (A) Quantification of TNTs in the co-culture system. TNTs were counted among 200 cells (100 astrocytes and 100 C6 glioma cells) in three independent preparations [mean ± SEM, *P <0.05 compared with H_2O_2 (100 μmol/L, 24 h) treatment only]. (B1 and B2) Astrocytes (B1) and C6 glioma cells (B2) in the co-culture system 24 h after treatment with 100 μmol/L H_2O_2 and 25 nmol/L Latrunculin A (scale bars, 200 μm). (C) Schematic of transwell co-culture of separated astrocytes and C6 glioma cells. (D) C6 glioma cells stained with Hoechst 120 h after sorting from co-culture (scale bar, 200 μm). (E) Cell numbers at 4, 24, 72, 96, and 120 h after the C6 glioma cells had been sorted from co-culture (counts from three independent preparations; mean ± SEM, *P <0.05, **P <0.01). (F) Percentage of Ki67+ cells among 500 cells in three independent preparations (mean ± SEM; **P <0.01 compared with other groups; in D–H, 25 nmol/L Latrunculin A and 100 μmol/L H_2O_2 were added 24 h before the C6 glioma cells were sorted from co-culture). (G) C6 glioma cells stained for Ki67 (red) and counterstained with Hoechst 24 h after sorting from the co-culture (scale bar, 200 μm). (H) Apoptosis detected by TUNEL assay. C6 glioma cells were treated with 100 μmol/L H_2O_2 for 24 h. (I) TUNEL-positive cells among 500 cells in three independent preparations (mean ± SEM; no significant difference between control and H_2O_2 groups). (J) Latrunculin A (25 nmol/L, 24 h) alone did not markedly affect the proliferation of C6 glioma cells. Cell numbers were assessed by MTS assay. OD values were recorded at 24, 48, and 72 h in three independent preparations (mean ± SEM; no significant difference between control and Latrunculin A groups).

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-014-1522-4.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (91132718) and the Beijing Natural Science Foundation, China (7142085).

Received date: 2014-10-21; Accepted date: 2015-02-11

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