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Presence of Neuroglobin in Cultured Astrocytes

XIAO QIAN CHEN,[1,2] LU YE QIN,[1] CHEN GANG ZHANG,[3] LI TAO YANG,[1] ZHEN GAO,[1] SHUANG LIU,[1,2] LOK TING LAU,[1,2] YIN-WAN WENDY FUNG,[1,2] DAVID A. GREENBERG,[4] AND ALBERT CHEUNG-HOI YU[1,2]

[1]*Neuroscience Research Institute, Peking University, Key Laboratory of Neuroscience (PKU), Ministry of Education, Department of Neurobiology, Peking University Health Science Center, Beijing, People's Republic of China*

[2]*Hong Kong DNA Chips Limited, 1/F, Cosmos Centre, MongKok, Kowloon, Hong Kong SAR, China*

[3]*Beijing Institute of Radiation Medicine, Beijing, China*

[4]*Buck Institute for Age Research, Novato, California*

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ABSTRACT Neuroglobin (Ngb), a recently discovered intracellular respiratory globin in neurons, may play a crucial role in oxygen homeostasis in the brain. We report preliminary findings indicating the presence of functional neuroglobin in primary cultures of cerebral cortical astrocytes. Reverse transcription real-time polymerase chain reaction (RRT–PCR) and immunostaining confirmed such presence in cultured astrocytes isolated from newborn mouse brain. Ngb antisense treatment increased apoptosis in ischemic astrocytes. The discovery of Ngb in astrocytes may provide some insight into how oxygen homeostasis is regulated in the brain.

Astrocytes are the most abundant glial cells in the brain. In humans, they exceed neurons by a ratio of 10–50 astrocytes to every neuron. In recent years, astrocytes have been shown to play important roles in the central nervous system, including signal transduction (e.g., by calcium wave) and cellular communication (e.g., via gap junction), and in controlling synapse numbers (Kast, 2001; Hansson and Ronnback, 2003; Parri and Crunelli, 2003). In the present report, we outline the presence of neuroglobin transcript and protein in cultured astrocytes, suggesting further involvement of astrocytes in regulating brain function.

Neuroglobin (Ngb), named for its initial discovery in neuronal cells in the brain, is a small protein of 151 amino acids found to be expressed in the brains of a variety of species (Reuss et al., 2002; Zhang et al., 2002). The protein is highly conserved among vertebrates and has been classified as a tissue hemoglobin, a group of proteins that bind oxygen and mediate oxygen transport that also includes myoglobin (Mb) and hemoglobin (Hb). Despite the level of interest generated by the discovery of Ngb, the focus of research has been primarily on its roles in neurons. In fact, whether functional Ngb is expressed in glial cells has been questioned previously (Reuss et al., 2002). To address such question, we provide some preliminary data in this report to indicate the presence of functional Ngb in cultured astrocytes, the predominant glial cells in the brain. Ngb in astrocytes may be involved in the regulation of oxygen homeostasis in the brain.

The presence of the *Ngb* transcript in cultured astrocytes was examined by reverse transcription real-time PCR (RRT-PCR) with *Ngb*-specific primers and probe. More than 95% of the cultured astrocytes prepared from newborn ICR mice were positive for glial fibrillary acidic protein (GFAP) and 95% purity of the culture was consistently reproducible (Yu et al., 2003). Positive reporter signals for Ngb were observed for neurons and astrocytes (Fig. 1A), but not for Cos-7 cells (Fig. 1A, Ngb). The reporter signals for 18S rRNA for all tested cells were positive,

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*Correspondence to: Albert C.H. Yu, Neuroscience Research Institute, Peking University, 38 Xue Yuan Road, Beijing 100083, People's Republic of China. E-mail: achy@dnachip.com.hk; achy@bjmu.edu.cn

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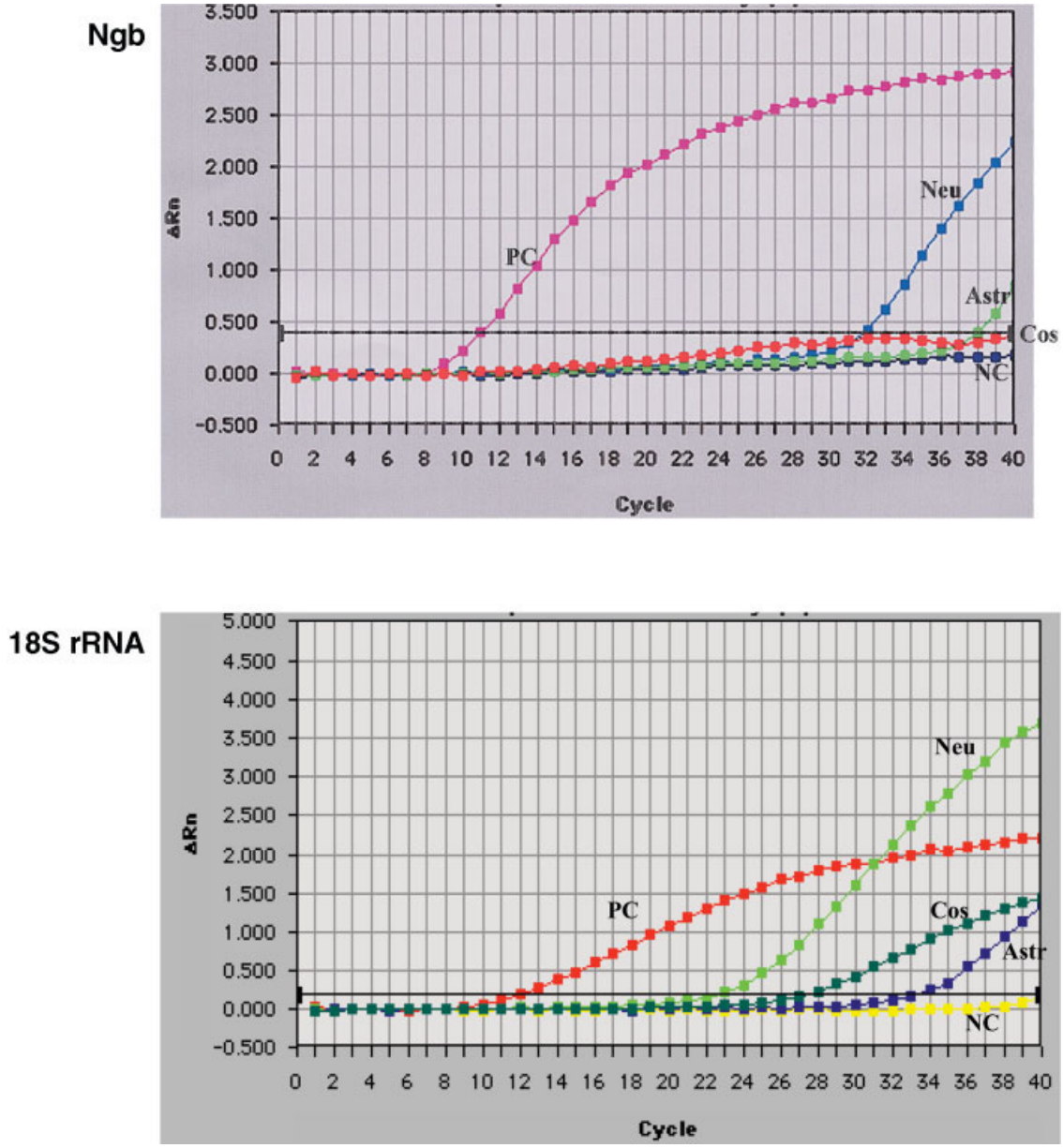

Fig. 1. Presence of neuroglobin (Ngb) in cultured astrocytes. Astrocyte cultures were prepared from newborn ICR mice as reported previously (Yu et al., 2003). Briefly, cerebral cortices free of meninges were isolated and cut into small cubes (<1 mm^3) in minimum essential media (MEM) (Invitrogen Inc., Ontario, Canada), and then mechanically dissociated by vortexing for 1.5 min. The resulting cell suspension was sieved through 70- and 10-μm sterile Spectra/Mesh® nylon filters (Spectrum Medical Industries), respectively. Sieved cell suspension equivalent to approximately one-tenth of the cerebrum volume was seeded in 35-mm Falcon tissue culture dishes (Becton Dickinson). Fresh MEM supplemented with 20% fetal calf serum (FCS; HyClone, Logan, Utah, USA) was added to yield a final volume of 2 ml per 35-mm dish. All cultures were incubated in a 95% humidified incubator at 37°C with 5% CO_2. Culture medium was changed 2 days after initial seeding, and subsequently, twice per week with Dulbecco's modified Eagle's medium (DMEM)/10% FCS for the first 2 weeks and DMEM/7% FCS thereafter. RRT–PCR was performed. **A:** Typical amplification plot for real-time PCR. Positive fluorescent reporter signals for *Ngb* were observed in cultured neurons and cultured astrocytes, but not Cos-7, by real-time TaqMan PCR (upper panel). (18S rRNA [lower panel] was included as internal control for the same real-time PCR run.) The change of normalized reporter signals (ΔRn) was calculated by normalizing the reporter signals with the fluorescent signals given by a passive reference. Ngb-specific real-time PCR primers were 5′-aggactgtctctcctctccagaatt and 5′-gctggtcaggtactcctccaat, and the TaqMan probe was 5′-accacattaggaaggtgatgctagtga. PC, positive control; Neu, neurons; Astr, astrocytes; Cos, Cos-7; NC, negative control. **B:** Cultured astroctyes were transfected with a Ngb-GFP construct and immunostaining was performed on the transfected cells as previously described (Chen et al., 2003). Here, one representative astrocyte is shown, indicating specificity of the anti-Ngb antibody to mouse Ngb with co-localization of GFP and Ngb signals. **C:** Regularly cultured astrocytes were immunostained with antibodies against Ngb (green) and GFAP (red). A representative culture is shown here to indicate the co-localization of Ngb and GFAP signals in the same cells.

indicating the general presence of transcripts in all tested samples (Fig. 1A, 18S RNA). Therefore the negative Ngb signal displayed by Cos-7 cells was not due to the absence of transcript in the Cos-7 sample. The real-time PCR results indicate the presence of Ngb transcript in astrocytes, but of a much less

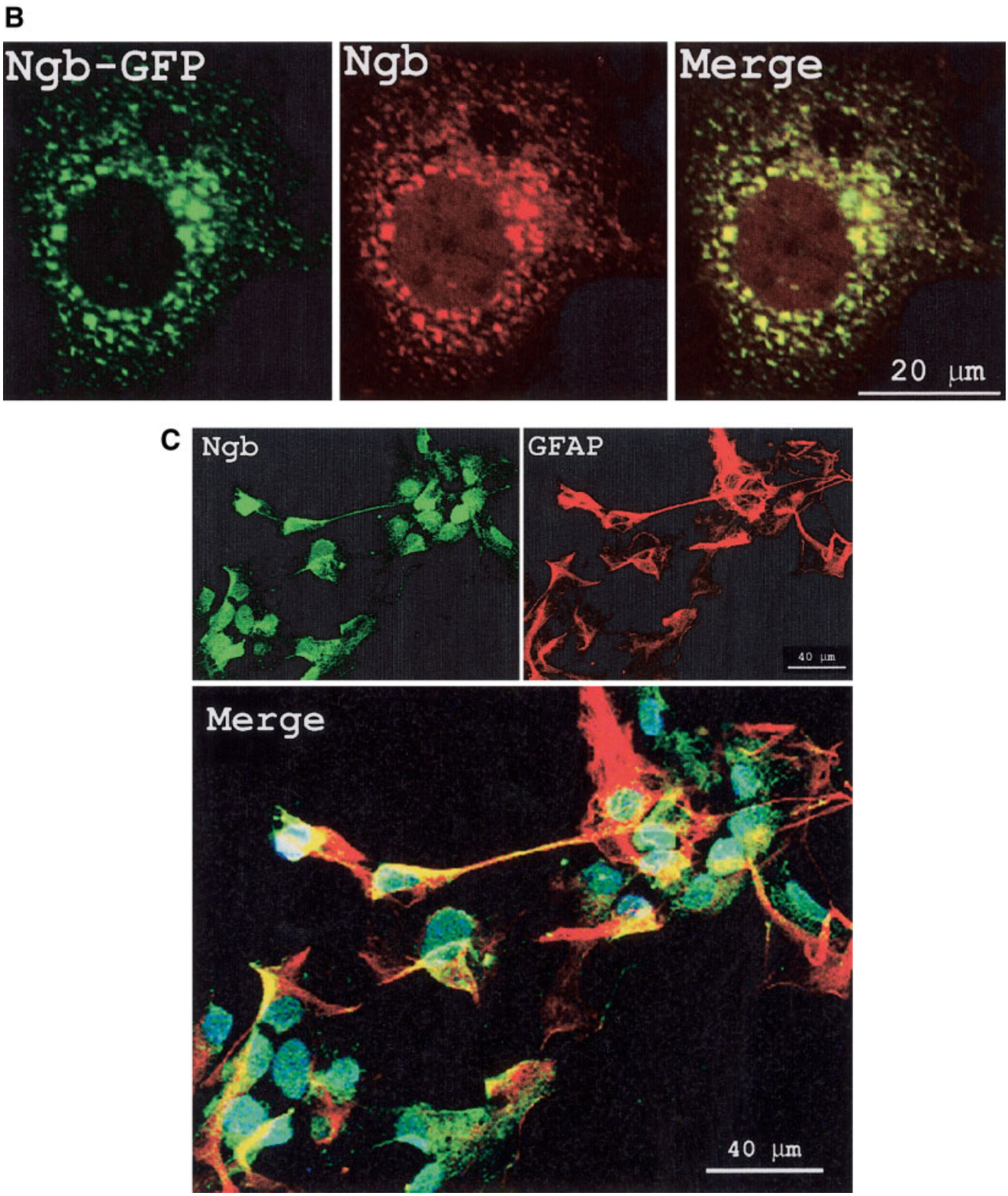

Fig. 1. (Continued)

amount when compared with neurons. DNA sequencing confirmed that the amplified product from *Ngb*-specific primers matched to *Ngb*.

After showing the presence of *Ngb* transcript in cultured astrocytes, immunostaining technique (according to conditions described in Chen et al., 2003) was used to

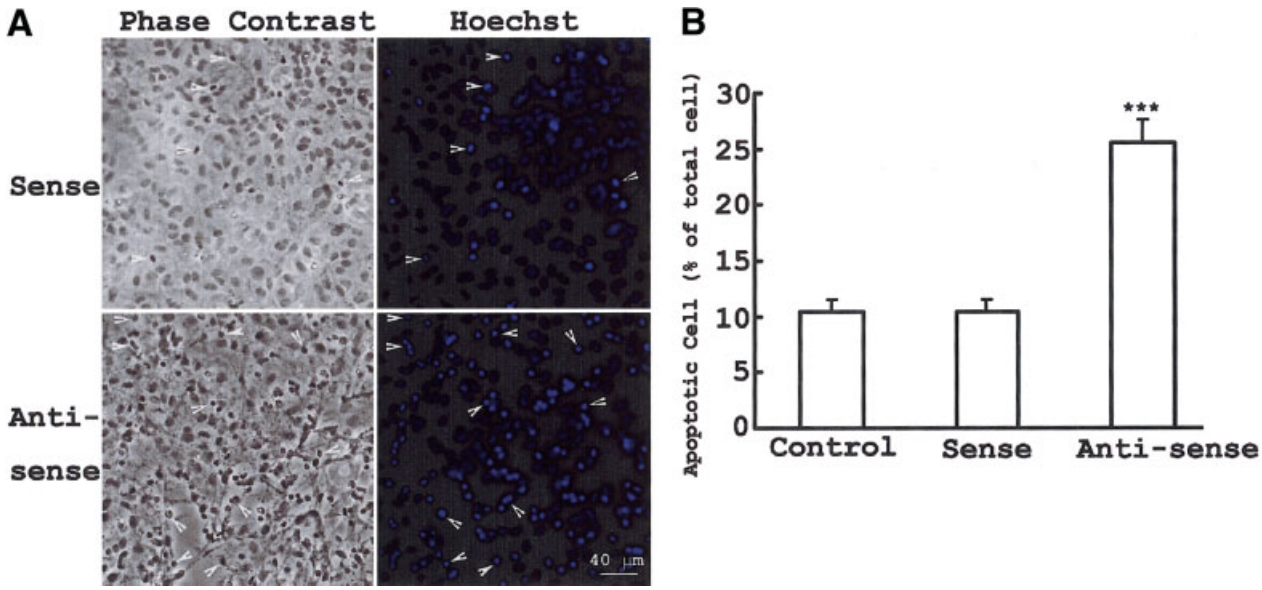

Fig. 2. Effect of Ngb anti-sense treatment on ischemia-induced apoptosis in astrocytes. **A:** Primary cultures of astrocytes were transfected with anti-sense (or sense) Ngb ODN, and then subjected to 5 h of ischemic treatment. Transfection was accomplished using Lipofectamine™ 2000 (LF) reagent (Invitrogen) according to the manufacturer's instructions. The cells were fixed immediately after ischemia and stained with Hoechst 33342 (Jiang et al., 2002) to identify highly condensed apoptotic nuclei (indicated by concave arrowhead). **B:** Statistical analysis was accomplished by counting the number of cells in nine random fields, using Student's unpaired *t*-test at a confidence interval of ≥95% (i.e., $P \leq 0.05$). The graph shows that apoptotic cells significantly increased in cultures transfected with anti-sense Ngb ODN compared with the untransfected and sense-transfected controls. Data represent mean ± SEM from three independent results. ***$P < 0.01$.

determine if this transcribed product gave rise to the Ngb protein. Ngb immunostaining experiments were accomplished using a rabbit polyclonal antibody raised against a rat Ngb fusion protein (Zhang et al., 2002). The specificity of this rabbit polyclonal antibody was examined by transfecting cultured astrocytes with a Ngb-GFP construct (Fig. 1B). The overlapping patterns of GFP fluorescence (green) and Ngb staining (red) on one representative transfected astrocyte indicate the specificity of this anti-rat Ngb antibody to mouse Ngb. Cross-reactivity of the antibody to rat and mouse Ngb is anticipated, as rat and mouse Ngb share 96% amino acid similarity (Zhang et al., 2002). With confirmed specificity of the Ngb antibody, endogenous Ngb protein in regularly cultured astrocytes was detected by immunostaining (procedures as previously described in Chen et al., 2003), followed by confocal microscopy analysis. All astrocytes in culture were stained positive for Ngb and GFAP (green and red respectively; Fig. 1C), both of which were co-localized to the same cells in the merged image (Fig. 1C).

Since Ngb has been demonstrated to be upregulated in neurons by hypoxia-ischemia injury (Sun et al., 2001), the functional role of Ngb in astrocytes during ischemia was examined using the anti-sense technique. An anti-sense oligonucleotide (ODN) (5′-tgactccgggcgctccatgctc) acting against the initial coding region of *Ngb* was designed based on the GenBank mouse *Ngb* sequence, NM 022414. This *Ngb* anti-sense ODN is similar to the ODN used previously (Sun et al., 2001), with demonstrated efficiency in blocking Ngb protein production (Sun et al., 2001). A corresponding *Ngb* sense ODN was used as control to examine the specific effect of the anti-sense ODN. Cultured astrocytes were transfected with the anti-sense (or sense) Ngb ODN, after which cells were subjected to 5 h of ischemic treatment. Untransfected cultured astrocytes were used as controls. Apoptosis in astrocytes was assessed by Hoechst 33342 nuclear staining as previously described (Jiang et al., 2002). In cultured astrocytes transfected with the sense Ngb ODN, only a few apoptotic astrocytes were visible after 5 h of ischemia (Fig. 2A, top, arrowheads). However, anti-sense Ngb ODN transfection resulted in a higher number of apoptotic cells (Fig. 2A, bottom, arrowheads). The percentage of apoptotic cells to total cells in the untransfected controls and sense-transfected culture was approximately 10% after 5 h of ischemic incubation, while transfection of anti-sense Ngb ODN led to a 2.5-fold increase in apoptotic cells (~25%, Fig. 2B) after 5 h of ischemic treatment. These results suggest a possible protective role for Ngb in astrocytes against ischemic insult.

Although the human brain consists of only 2% of the total body weight, it utilizes 20% of in-taken oxygen. Therefore, it is reasonable to assume that some intracellular respiratory proteins exist to regulate oxygen balance in the brain, much like Mb for muscle (Merx et al., 2001). The recently discovered Ngb is expressed mainly in the brain (Burmester et al., 2000; Zhang et al., 2002; Geuens et al., 2003) and binds oxygen reversibly with a higher oxygen-binding affinity than Hb.

Thus, it is likely that Ngb plays a critical role in regulating oxygen homeostasis in the brain (Burmester et al., 2000; Trent et al., 2001; Kriegl et al., 2002). However, how Ngb might facilitate oxygen transport and diffusion into the brain cells remains largely unknown. The discovery of Ngb in astrocytes may provide some insight into how oxygen homeostasis is regulated in the brain through regulated expression of Ngb in neurons and astrocytes.

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