

## Roles of carbonic anhydrase 8 in neuronal cells and zebrafish



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### ARTICLE INFO

#### Article history:

Received 13 December 2013

Received in revised form 7 April 2014

Accepted 23 April 2014

Available online 1 May 2014

#### Keywords:

Carbonic anhydrase 8

SK-N-SH

Neuro-2a

Zebrafish

Cerebellar granule neuron

Cell migration and invasion

### ABSTRACT

**Background:** Carbonic anhydrase 8 (CA8) is an isozyme of  $\alpha$ -carbonic anhydrases (CAs). Previous studies showed that CA8 can be detected in human adult brain, with more intense expression in the cerebellum. Single mutations in CA8 were reported to cause novel syndromes like ataxia, mild mental retardation or the predisposition to quadrupedal gait.

**Methods:** In the present study, we examine the functions of CA8 in neuronal cell lines, mouse cerebellar granule neurons and zebrafish.

**Results and conclusions:** We demonstrated that overexpression of CA8 in neuronal cells significantly decreased cell death under staurosporine treatment. Moreover, CA8 overexpression significantly increased cell migration and invasion ability in neuronal cells and in mouse cerebellar granule neurons, implicating that CA8 may be involved in neuron motility and oncogenesis. By using zebrafish as an animal model, motor reflection of 3 dpf zebrafish embryos was significantly affected after the down-regulation of CA8 through *ca8* morpholino.

**Conclusions:** We concluded that CA8 overexpression desensitizes neuronal cells to STS induced apoptotic stress and increases cell migration and invasion ability in neuronal cells. In addition, down-regulated CA8 decreases neuron mobility in neuronal cells and leads to abnormal calcium release in cerebellar granule neurons. Knock-down of the *ca8* gene results in an abnormal movement pattern in zebrafish.

**General significance:** Our findings provide evidence to support that the impaired protective function of CA8 contributes to human neuropathology, and to suggest that zebrafish can be used as an animal model to study the biological functions of human CA8 in vivo.

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### 1. Introduction

Carbonic anhydrase 8 (CA8) is one of the 3 catalytically inactive carbonic anhydrase isozymes (CA8, 10 and 11), also known as carbonic anhydrase-related proteins (CA-RPs), which lack the function to catalyze the reversible hydration of CO<sub>2</sub> due to the lack of zinc binding histidine residues [1,2]. Previous study showed that the sequence of CA8 has 97.9% identity at the amino acid level between human and mouse [3]. Phylogenetic study also showed that the protein sequence identities of CA8 ranged from 67% to 100% among different vertebrates like *Bos taurus*, *Canis familiaris*, *Danio rerio*, etc. [4], suggesting that CA8 may play biologically important roles in higher organisms. In human adult brain, CA8 was distinctly expressed in the neural cell body spreading to most parts of the central nervous system (CNS) like the cerebrum, diencephalon, cerebellum, pons and medulla [5]. In addition, it was found that CA8 can be detected in different types of mouse tissues, with more

intense expression in Purkinje cells [6]. Up to date, the only known biochemical function of CA8 is that it influences inositol 1,4,5-triphosphate (IP<sub>3</sub>) binding to its receptor IP<sub>3</sub>R1 on the endoplasmic reticulum and therefore modulates calcium signaling [6].

In 2005, a 19-bp deletion in exon 8 of the *CA8* gene (*ca8*) was found to cause ataxia and a lifelong gait disorder in waddles mice [7]. In addition, a homozygous mutation, S100P, in human *ca8* gene has been described in a consanguineous Iraqi family in which affected siblings had mild mental retardation and congenital ataxia characterized by quadrupedal gait [8]. Recently, the other homozygous mutation in CA8, G162R, was also identified in patients with variable cerebellar ataxia and mild cognitive impairment without quadrupedal gait [9]. These studies indicated that CA8 plays an important role in motor coordination and can be involved in human neurodegenerative disorders. On the other hand, significant expression of CA8 was observed in invasive lung adenocarcinoma but not in noninvasive adenocarcinoma. Interestingly, CA8 was strongly expressed in signet-ring cell cancer and invasive mucinous adenocarcinoma components [10]. Increased expression of CA8 has also been shown in colorectal carcinoma [11]. In addition, cell proliferation, colony formation, and cell invasion assays all demonstrated that colon cancer cell line (LoVo) bearing CA8 expression, LoVo-CA8, has

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significantly higher cell proliferative and invasive abilities than those found in parental LoVo and control LoVo-pCIneo cells *in vitro*. Furthermore, *in vivo* xenograft assay showed a higher tumor growth rate in LoVo-CA8 cells than that in parental LoVo cells [11]. Taken together, down-regulated CA8 and CA8 mutation may cause deficiencies in motor control but overexpression of CA8 may promote cell oncogenesis. However, the mechanisms that CA8 may be involved in the neural function and the exact biological functions of CA8 are still not clear.

In this study, we examined the functions of CA8 in neuronal cells because CA8 is distinctly expressed in the neural cells. First, the endogenous expression of CA8 was assessed in three easily accessible neuronal cell lines, including IMR32, SK-N-SH and Neuro-2a cells, as well as in mouse cerebellar granule neurons. To our surprise, no detectable expression of CA8 proteins was observed in neuroblastoma cell lines. Therefore, CA8 overexpression stable clones were established in SK-N-SH and Neuro-2a cells for analyzing the effects of CA8 overexpression. On the other hand, to examine the results of abolishing endogenous CA8 expression in normal neural cells, CA8 shRNA was used to eliminate the endogenous CA8 expression in cerebellar granule neurons. Together by using cell proliferation, cell migration/invasion, and staurosporine (STS)-induced cell apoptosis assays, we presented the results to define the functions of CA8 in neuron cells. Furthermore, to evaluate whether zebrafish may be used as an animal model to study the functions of CA8, we used *ca8* morpholino (*ca8* MO) to down-regulate CA8 expression for the observation of morphological and behavior changes in zebrafish, as well as the expression patterns of CA8 during embryo development.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Human neuroblastoma cell line SK-N-SH (HTB-11; ATCC) was provided by Dr. Shin-Lan Hsu (Taichung Veterans General Hospital, Taiwan); human neuroblastoma cell line IMR32 (CCL-127; ATCC) and mouse neuroblastoma cell line Neuro-2a (CCL-131; ATCC) were purchased from Bioresource Collection and Research Center (Hsin-Chu, Taiwan). All materials for cell culture were purchased from Gibco Life Technologies (Gaithersburg, MD). Reagents for Western blot were obtained from Pierce (Rockford, USA). Rabbit polyclonal antibody specific for CA8 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal antibody specific for Parvalbumin7 was provided by Dr. Masahiko Hibi (Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan); mouse monoclonal anti- $\alpha$ -tubulin was purchased from Sigma (St. Louis, MO, USA); rabbit polyclonal anti-IP<sub>3</sub>R1 was purchased from Millipore (Billerica, MA, USA). To generate plasmid pLKOAS3w-CA8.neo, a lentiviral transfer vector containing the full length CA8, CA8 DNA fragment was amplified by PCR and inserted between *NheI* and *PmeI* of pLKOAS3w.neo; plasmid pLKOAS3w-CA8-myc.puro, a lentiviral transfer vector containing the full length CA8 with myc tagged, was generated by PCR amplifying CA8-myc DNA fragment and inserted between *NheI* and *EcoRI* of pLKOAS3w.puro. The recombinant lentiviral production was generated by the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Meanwhile, control virus and virus carrying CA8 RNA interference (RNAi) vector, pLKO.1-shCA8 (shCA8-1, TRCN000153276; shCA8-2, TRCN000155916), were obtained from the National RNAi Core Facility.

### 2.2. Cell cultures

SK-N-SH, IMR-32 and Neuro-2a cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100  $\mu$ g/mL pyruvate, 1% PS, and 1% non-essential amino acid, and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. SK-N-SH cells were sub-cultivated every three days at a ratio of 1:2; IMR-32 cells were sub-cultivated every three days at a ratio of 1:3

and Neuro-2a cells were sub-cultivated every three days at a ratio of 1:6.

### 2.3. Plasmid construction

Full-length CA8 cDNA was PCR amplified from pcDNA3.1-CA8-myc-His (Chang W.H., unpublished work) using synthesized primers, CA8-*NheI* (5'TCCTGATGCTAATGGGGAATACCAG 3') and CA8-*PmeI* (5'CTAAGAGGCTGAGTGGGCCGAAAG 3'). CA8 cDNA was then subcloned into pLKOAS3w.neo lentiviral vector and their DNA sequences were confirmed.

### 2.4. Selection of stable cells and Western blot analysis

The recombinant pLKOAS3w-CA8.neo lentiviral production was generated by the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Infection of this modified virus for stable expression of CA8 was performed according to the instructions provided by the National RNAi Core Facility. SK-N-SH and Neuro-2a cells were infected with recombinant lentivirus and lines were then selected using 500  $\mu$ g/ml of G418 (GIBCO/BRL). Expression of CA8 proteins was examined by Western blot analysis using anti-CA8. After seven days of selection, the survival cells were diluted to a concentration of 10 cells/ml and keep growing in the medium containing 500  $\mu$ g/ml of G418. Stable cells were collected after 14–20 days. Western blot analysis was performed as previously described [12].

### 2.5. Cell toxicity studies and cell survival analysis

SK-N-SH, SK-N-SH-CA8, Neuro-2a and Neuro-2a-CA8 cells were maintained in DMEM containing 10% fetal bovine serum and then  $2 \times 10^5$  (Neuro-2a and Neuro-2a-CA8) and  $4 \times 10^5$  (SK-N-SH and SK-N-SH-CA8) cells were seeded in 3 cm dishes. One day after seeding, cultured medium was replaced with a medium containing 500 nM (Neuro-2a) and 1  $\mu$ M (SK-N-SH) STS and cells were incubated in this medium for different periods at 37 °C.

### 2.6. Characterization of apoptotic cells

Control or STS-treated cells were harvested by trypsin incubation, washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS buffer for 30 min at room temperature (RT), washed three times with PBST (0.1% of Tween 20 in PBS) and then exposed to 20  $\mu$ g/ml of propidium iodide (PI) in PBS for 15 min at RT, and washed three times with PBST. Apoptotic cells exhibited highly fluorescent condensed or fragmented DNA by fluorescence microscopy.

### 2.7. Transwell cell migration and invasion assays

Cell migration was studied using inserts incorporating 8  $\mu$ m polyethylene terephthalate (PET) track-etched membranes transwell coated with 10  $\mu$ g/ml collagen IV as chemo-attractant. Trypsinized cells were suspended in serum free medium and  $1 \times 10^5$  cells were seeded to the upper chamber of transwell inserts. 3% fetal bovine serum medium was added to the lower chamber. After 12 h of incubation for SK-N-SH and primary granule neurons, non-migrated cells on the upper surface of the membrane were scrapped off, cells on the lower surface were stained with 10  $\mu$ g/ml of 4',6-diamidino-2-phenylindole (DAPI) in PBS and counted from 7 to 8 randomly selected fields. Cell invasion assay was performed using matrigel coated (50  $\mu$ l of 5% matrigel) transwell inserts as described previously [13]. Invasion assay was performed similar to migration assay, only the cells were incubated for 36 h before observation. All the data were obtained from three separate experiments.

## 2.8. Differentiation of neuroblastoma cells by serum deprivation

$2 \times 10^5$  (SK-N-SH, SK-N-SH-CA8) and  $1 \times 10^5$  (Neuro-2a, Neuro-2a-CA8) cells grown in normal conditions were maintained in 3-cm culture dishes for 16–18 h under normal culture conditions. Next day, the culture medium was replaced by 0.5% fetal bovine serum medium. Morphological changes were assessed during different time points.

## 2.9. Mouse cerebellar granule neuron isolation and treatment

ICR mice (postnatal day 4–6) were anesthetized on ice for a few minutes before decapitation, then the crumbed meninges and cerebellum tissue were carefully removed using fine tweezers (#55, Dumont, Switzerland). The cerebellum tissue was then incubated for 10 min in 1 ml of dissociation solution containing 0.25% trypsin and 1 mM EDTA in Hank's Salts at 37 °C, pipetted with P1000 pipet every 5 min, centrifuged at 1000 rpm for 5 min, and then the supernatant was removed. 10  $\mu$ l of 10 mg/ml DNase I was added to the pellet, after briefly tipping the tube, 1 ml of DMEM containing 20% FBS was added into the tube. The mixture was then incubated for 10 min at 37 °C, pipetted every 5 min, centrifuged at 1000 rpm for 5 min, and then the supernatant was removed. 2 ml of 20% FBS DMEM was added in to the pellet (cerebellar granule neurons). After cell counting, we seeded the appropriate number of cerebellar granule neurons into 3 cm dishes for the following experiments. For lentivirus infection,  $2 \times 10^5$  of cerebellar granule neurons was seeded in 3 cm dishes with 20% FBS DMEM for 24 h. One day after, culture medium was replaced by 20% FBS DMEM containing 8  $\mu$ g/ml polybrene. According to the virus titer, we added the appropriate amounts of lentivirus carrying recombinant pLKOAS3w-CA8.neo, pLKOAS3w-CA8-myc-puro, CA8 shRNA (pLKO.1-shCA8, shCA8-1, TRCN000153276; shCA8-2, TRCN000155916), or control vector (M.O.I = 3) into cerebellar granule neuron for 12 h infection. After lentivirus infection, the infection medium was replaced by 20% FBS DMEM, selected by appropriate antibiotics for 3 days and then incubated until cells reached 80% confluence before the experiments.

## 2.10. Measurement of intracellular $Ca^{2+}$

Cells were seeded at the density of  $2 \times 10^4$  cells/well in a 96-well tissue culture plate and were incubated for 24 h in a 37 °C, 5%  $CO_2$  incubator. After 24 h, cells were washed by HEPES buffer with  $Ca^{2+}$ , and loaded with 2.5  $\mu$ M Fura-4AM in the HEPES buffer with  $Ca^{2+}$ . After 45 min, cells were washed by the HEPES buffer without  $Ca^{2+}$ , and incubated for 30 min. Finally, cytosolic  $Ca^{2+}$  signal in cells was assessed in response to 0.1 mM ATP. Fura-4AM fluorescence was recorded continuously at 37 °C in a spectrofluorometer at the excitation wavelength of 494 nm and an emission wavelength of 516 nm.

## 2.11. Animals

Zebrafish (*D. rerio*) were raised according to standard protocols [14]. Embryos were obtained from natural crosses of wild-type or transgenic fish, and staged by hours or days post-fertilization (hpf, dpf) [15]. The following lines were used: Oregon AB line (from Taiwan Zebrafish Core Facility at ZeTH), *Tg(HuC:EGFP)* (from Taiwan Zebrafish Core Facility at ZCAS), and *Tg(CM-is11:GFP)rwo* (a gift from Drs. Shinichi Higashijima and Hitoshi Okamoto) [16]. ICR mice were purchased from LASC Charles River Technology (Taipei, Taiwan). 4–6 days post-natal mice were used to perform cerebellum isolation experiment. All of the zebrafish- and mouse-use protocols in this research were reviewed and approved by the Institutional Animal Care and Use Committee of Tunghai University (IACUC number 100-17).

## 2.12. Morpholino oligonucleotides

The antisense morpholino oligonucleotides (MOs) were generated by Gene Tools (LLC, Corvallis, OR, USA). The sequences of *ca8* MO and standard control MO (STD-MO) were 5'-CATGGCAACTCAACCTTCTCTCAC-3' and 5'-CCTCTTACCTCAGTTACAATT TATA-3', respectively. The *ca8* and *p53* MOs were dissolved in and diluted into desired concentrations with Danieau solution, and 1 nl was injected into the yolk of each one- to two-cell-stage embryo.

## 2.13. Whole-mount immunofluorescence

Zebrafish larvae were fixed at 4 °C in 4% PFA in PBST (PBS, 0.1% Triton X-100) overnight. The fixed larvae were washed with PBST, and incubated in acetone at –20 °C for 30 min. Larvae were washed three times with PBST, and incubated in 10% FBS in PBST at RT for 1 h. The samples were incubated with the primary antibody solution at 4 °C overnight (1:1000 anti-Parvalbumin7; 1:200 anti-CA8). After washing with PBST for 6 times, the samples were incubated with secondary antibodies (1:200 dilution, anti-mouse IgG FITC and/anti-rabbit-IgG DyLight 594). The fluorescence images were obtained with a LSM5 laser scanning microscope (Zeiss). The fluorescence images were constructed from Z-stack sections by a 3D projection program associated with the microscope, and fluorescent intensities were quantified by the LSM 510 version 3.5 software.

## 2.14. Motility recording

Larvae at 3 dpf were placed in the 6 cm Petri dishes and videotaped using Nikon COOLPIX P500 camera. The motor reflection was initiated by tactile stimulation to the region near the tip of the tail. Five tactile stimuli with 30 s intervals were applied. A long distance reflection was defined as a distance longer than two folds of the body length, and assigned as score 3. On the other hand, a short distance reflection was defined as a distance shorter than the body length, and assigned as score 2. No response was defined as that zebrafish showed no response after needle stimulation, and assigned as score 1. Thirty-four larvae at 3 dpf of each group were counted in this experiment.

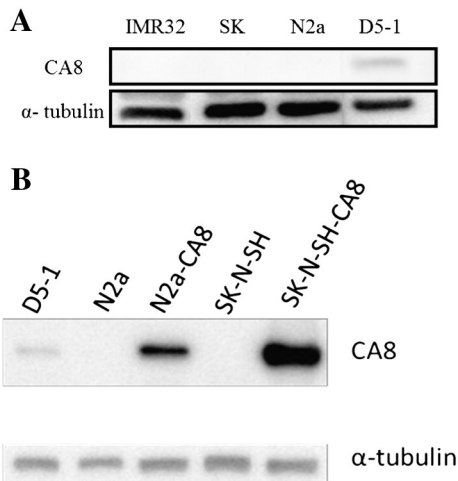
## 2.15. Statistical analysis

All cell numbers and values were expressed as means  $\pm$  SEM. Analysis of variance with subsequent Student's *t* test was employed to determine the significance of differences in comparisons. Values of  $p < 0.05$  were considered statistically significant.

## 3. Results

### 3.1. Ectopic expression of CA8 in neuronal cells

To understand the protein expression of CA8 in different neuronal cell lines; including human neuroblastoma cells (IMR32, SK-N-SH) and mouse neuroblastoma cells (Neuro-2a); Western blot analysis was performed first. To our surprise, no detectable signals around 38 kDa were observed in IMR32, SK-N-SH and Neuro-2a (Fig. 1A). The expected size for CA8 is about 38 kDa as indicated by a positive signal of CA8 from a cytoplasmic hybrid (cybrid) clone D5-1 [17], which is a cytoplasmic hybridization cell line between osteosarcoma and fibroblast cells. Given the fact that the expression of CA8 transcripts was observed in IMR-32 and SK-N-SH cells [5,18], our results suggested that the endogenous protein level of CA8 may be too low to be detected in these three neuronal cell lines. Therefore, ectopic expression of CA8 was used to examine the functions of CA8 in neuronal cells. Due to the low efficiency of transfection in neuronal cells, we used the CA8 expression lentivirus (pLKOAS3w.neo-CA8) to infect SK-N-SH and Neuro-2a cells; then stable clones were selected by G418. After selection, two



**Fig. 1.** Western blot analysis of CA8 expression in different neuronal cell lines. (A) No detectable endogenous CA8 expressed in a panel of neuronal cell lines. No detectable endogenous CA8 protein expression in human neuroblastoma cells (IMR32, SK-N-SH) and mouse neuroblastoma cells (Neuro-2a). Expression of CA8 was examined by immunoblotting whole cell lysates from different neuronal cells and MERRF cybrids (D5-1) were used as a positive control. Cell lysates were electrophoresed, blotted, and probed with anti-CA8 and antibody against  $\alpha$ -tubulin was used as an internal control. (B) Ectopic expression of CA8 in SK-N-SH-CA8 and Neuro-2a-CA8 cells. Expression of exogenous CA8 in SK-N-SH-CA8 and Neuro-2a-CA8 cells was detected by Western blot analysis using specific antibodies for CA8. The recombinant pLKOAS3w.neo-CA8 lentivirus was used to infect SK-N-SH and Neuro-2a cells and stable clones were selected by G418. Antibody against  $\alpha$ -tubulin was added as an internal control.

stable cell lines were obtained and designated as SK-N-SH-CA8 and Neuro-2a-CA8. It is noted that control cell lines stably expressing the lentiviral control vector showed no effects on cell morphology and proliferation under the same growth condition, as compared with the parental cell lines (data not shown). Therefore, all the following experiments were performed by comparing CA8 stably expressing cell line to the parental cell lines. We then examined CA8 protein expression by Western blot analysis. As shown in Fig. 1B, CA8 was significantly expressed in SK-N-SH-CA8 and Neuro-2a-CA8 cells compared with that in the parental cells. These two stable cell lines, SK-N-SH-CA8 and Neuro-2a-CA8, were further characterized in the following analysis.

### 3.2. CA8 overexpression increases cell proliferation in Neuro-2a

Next, we were interested in knowing whether the overexpression of CA8 impacts cell proliferation in neuronal cells. In our experiment, Neuro-2a, Neuro-2a-CA8, SK-N-SH, and SK-N-SH-CA8 cells were seeded in a 24-well plate and cultured for 24, 48 and 72 h, respectively. The numbers of cells were counted in different time intervals under the normal growth conditions. As shown in Fig. 2A, Neuro-2a-CA8 cells showed increased cell numbers after 24 h and become significantly increased after 72 h of culture, as compared with parental Neuro-2a cells. In contrast, proliferative ability of SK-N-SH-CA8 showed no significant difference as compared with that of SK-N-SH cells during different time intervals (Fig. 2B). To understand whether the protein levels of CA8 influence cell proliferation, two single stable clones of Neuro-2a-CA8, designated Neuro-2a-CA8-2 and Neuro-2a-CA8-3, were further selected for comparison. Western blotting showed that CA8 expression was significantly lower in Neuro-2a-CA8-3 cells, as compared with that of Neuro-2a-CA8-2 cells (Fig. 2C). Consistently, a significant increased proliferative ability was observed in Neuro-2a-CA8-2 cells as compared with Neuro-2a-CA8-3 and parental Neuro-2a cells (Fig. 2D). Our results clearly demonstrated a positive effect of CA8 overexpression on cell proliferative ability in Neuro-2a cells, but not in SK-N-SH cells. We next investigated whether the transgenic expression of CA8 leads to any morphologic change in neuronal cells. Under normal culture conditions, the dendrite length, synapse number, and cell morphology of Neuro-2a-

CA8 cells showed no significant difference as compared with those of Neuro-2a cells (Supplementary Fig. 1A). In addition, similar results were obtained in the comparison of SK-N-SH and SK-N-SH-CA8 cells (Supplementary Fig. 1B). SK-N-SH-CA8 cells revealed no significant morphologic difference in dendrite length, synapse number, and cell morphology as compared with those of SK-N-SH cells.

### 3.3. Overexpression of CA8 significantly promotes neurite outgrowth in Neuro-2a cells

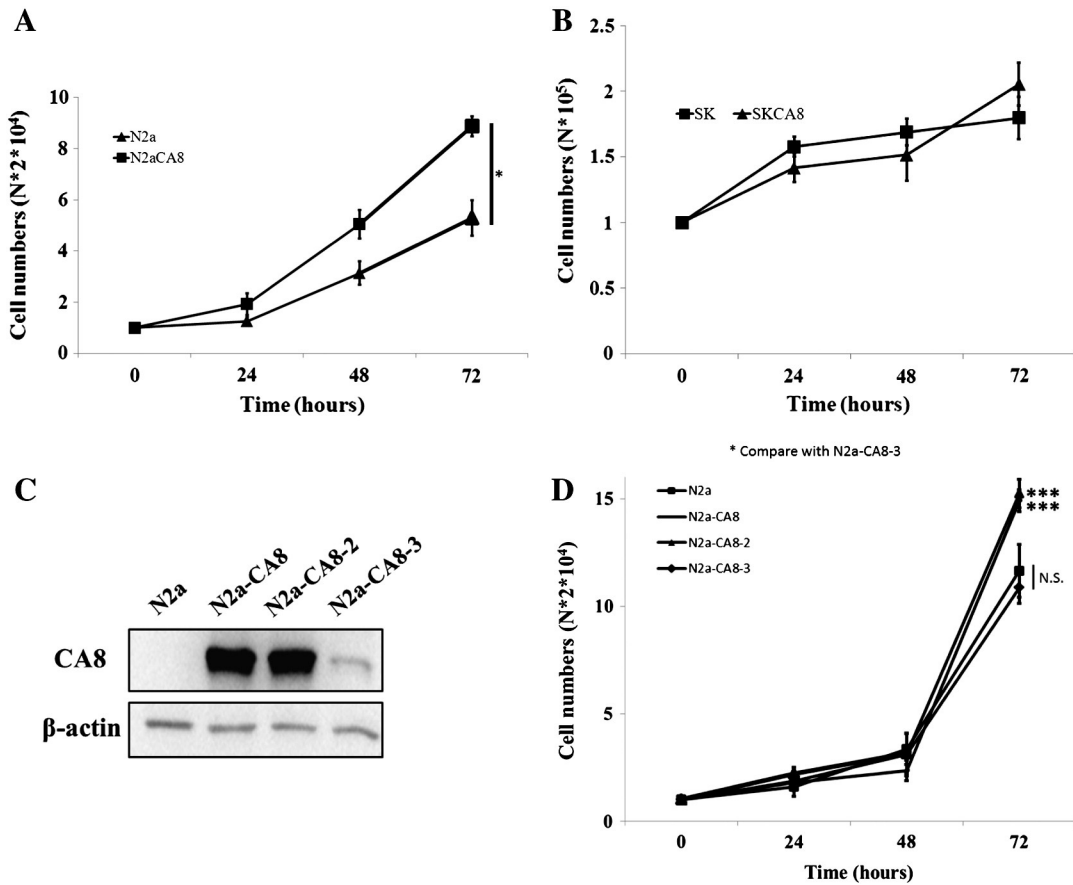
On the other hand, to understand whether CA8 expression induces morphological alteration in differentiated neuronal cells, a low serum medium was used to induce neuronal cell differentiation according to a previous report [19]. Low-serum-induced neurite outgrowth at various time points was observed with a phase contrast microscopy. To simplify the analysis, cells bearing neurites that were at least 2-fold longer than cell bodies were counted as differentiated cells. Cells bearing neurite outgrowth increased with time in Neuro-2a and Neuro-2a-CA8. At 24 and 36 h, CA8 significantly promoted neurite outgrowth in Neuro-2a-CA8 (Fig. 3B) as compared with Neuro-2a cells (Fig. 3A). The statistical assessment of the neurite outgrowth was shown in Fig. 3C. On the other hand, neurite outgrowth was also assessed in SK-N-SH and SK-N-SH-CA8 cells at every 24 h intervals until 72 h under low-serum conditions. However, no significant difference in neurite outgrowth was detected in these two cell lines (data not shown).

### 3.4. CA8 overexpression desensitizes neuronal cells to STS induced apoptotic stress

It is known that STS induces cell cycle G2/M arrest, cytotoxicity, and DNA fragmentation in Neuro-2a cells [20]. To investigate whether CA8 overexpression increases neuronal cell survival in response to STS treatment, Neuro-2a and Neuro-2a-CA8 cells were treated with STS and the apoptotic cells were assessed by PI staining. As shown in Fig. 4A, a significant decrease of apoptotic cell death was observed in Neuro-2a-CA8 cells as compared with parental Neuro-2a cells harboring no exogenous CA8. Meanwhile, STS treatment was also performed in SK-N-SH and SK-N-SH-CA8 cells. Based on a previous study in our laboratory, 1  $\mu$ M STS treatment for 4 h is the optimal condition for inducing cell apoptosis process in SK-N-SH cells [21]. Therefore, in this study, the same condition was applied to SK-N-SH cells with and without CA8 overexpression. After STS treatment, we examined apoptotic cells by DNA fragmentation with PI staining. The statistical assessment of the apoptotic cells was summarized in Fig. 4B. Consistently, our results demonstrated a significant decrease of cell death in SK-N-SH-CA8 cells as compared with that of SK-N-SH cells harboring no exogenous CA8 expression. Taken together, CA8 overexpression desensitizes both Neuro-2a and SK-N-SH to STS induced cell apoptosis.

### 3.5. Overexpression of CA8 increases cell migration and invasion ability in SK-N-SH cells

To evaluate whether CA8 overexpression increases cell mobility in neuronal cells, transwell assay was performed to study cell migration and invasion ability. After 12 h of incubation in cell migration assay, DAPI staining was performed to observe migrated cell numbers, as shown in Fig. 5A. Statistical assessment showed that CA8 overexpression significantly increased cell migration numbers to about 160% in SK-N-SH-CA8 cells as compared with those in parental SK-N-SH cells (Fig. 5B). In addition, cell invasion experiment demonstrated that overexpression of CA8 significantly increased the invaded cell numbers as compared with parental SK-N-SH cells (Fig. 5C). Statistical analysis of the invasion ability of SK-N-SH-CA8 cells showed an increase of about 5 folds as compared with that of parental SK-N-SH cells after 36 h of incubation (Fig. 5D). All the evidence suggests that CA8 overexpression significantly increases cell mobility in SK-N-SH cells.



**Fig. 2.** Effects of CA8 on neuronal cell proliferation. (A) Overexpression of CA8 increased cell proliferative ability in Neuro-2a-CA8. Neuro-2a and Neuro-2a-CA8 cells were seeded at  $1 \times 10^5$  cells/well in a 24-well plate and cultured with DMEM complete medium. We counted cell numbers every 24 h till 72 h. Cell number was assessed by trypan blue dye exclusion assay. The cell proliferative ability was significantly increased at 72 h in Neuro-2a-CA8, compared with that in Neuro-2a cells after 72 h of culture.  $*p < 0.05$ . (B) Overexpression of CA8 showed no effects on cell proliferative ability in SK-N-SH-CA8 cells. SK-N-SH and SK-N-SH-CA8 cells were seeded at  $1 \times 10^5$  cells/well in 24-well plates and cultured with DMEM complete medium. We counted cell numbers every 24 h till 72 h. Cell number was assessed by trypan blue dye exclusion assay. CA8 overexpression showed no significant changes in cell proliferative ability between SK-N-SH and SK-N-SH-CA8 cells. (C) Protein expression levels of CA8 in Neuro-2a stable clones. Expression of exogenous CA8 in Neuro-2a, Neuro-2a-CA8, Neuro-2a-CA8-2 and Neuro-2a-CA8-3 cells was detected by Western blot analysis using specific antibodies for CA8. Antibody against  $\alpha$ -tubulin was added as an internal control. (D) The effect of CA8 expression levels on cell proliferative ability in Neuro-2a cells. Neuro-2a, Neuro-2a-CA8, Neuro-2a-CA8-2 and Neuro-2a-CA8-3 cells were seeded at  $1 \times 10^5$  cells/well in a 24-well plate and cultured with DMEM complete medium. We counted cell numbers every 24 h till 72 h. Cell number was assessed by trypan blue dye exclusion assay. The cell proliferative ability was significantly increased at 72 h in Neuro-2a-CA8 and Neuro-2a-CA8-2, compared with that in Neuro-2a and Neuro-2a-CA8-3 cells after 72 h of culture.  $***p < 0.01$ .

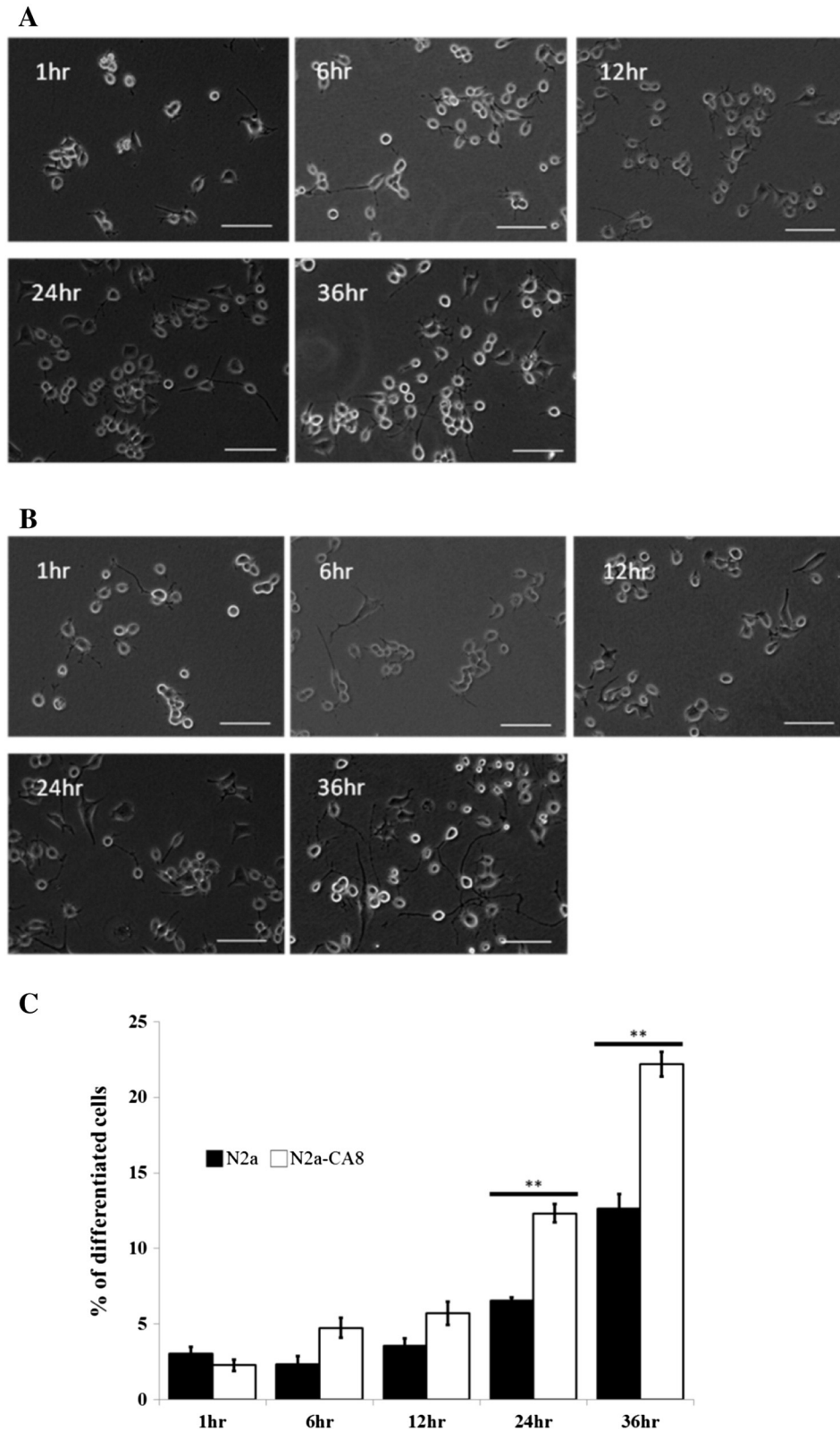
### 3.6. Down-regulation of CA8 significantly decreases cell migration and invasion ability in cerebellar granule neurons

In mouse, CA8 expression was not only observed in Purkinje cells but also found, even though less intensely, in cerebellar granule neurons by immunohistochemistry [7]. Therefore, we used mouse cerebellar granule neurons as a model system to examine the functions of endogenous CA8 in neurons. It has been previously determined that CA8 knockdown can be efficiently achieved by using CA8-shRNA vector, pLKO.1-shCA8-1 and shCA8-2 (Cheng C.K., unpublished work). Therefore, the cerebellar granule neurons were isolated and infected with CA8 shRNA and overexpressing CA8 lentivirus to down-regulate and overexpress CA8, respectively. As shown in Fig. 6A, granule neurons with CA8 overexpression showed the most intense expression of CA8 as compared with those in granule neurons and granule neurons with CA8 down-regulation. It was also noted that normal granule neurons showed more intense CA8 expression as compared with that in CA8 shRNA down-regulated granule neurons. To evaluate whether CA8 knockdown significantly decreases cell mobility in neural cells, mouse cerebellar granule neurons with down-regulated and overexpressed CA8 were used to perform transwell cell migration and invasion assays. As shown in Fig. 6B, CA8 down-regulation significantly decreased migrated cell numbers in CA8 knockdown granule neurons as compared with those in normal granule neurons and CA8 overexpressed granule neurons. Statistical analysis

demonstrated that the migration ability of CA8 down-regulated granule neurons significantly decreased to about 85% as compared with that of normal granule neurons. Consistently, granule neurons with CA8 overexpression significantly increased to about 134% of that of normal granule neurons, as shown in Fig. 6C. Furthermore, similar results were obtained in cell invasion experiment. Down-regulation of CA8 significantly decreased cell invasion ability in CA8 knockdown granule neurons when compared with those of CA8 overexpressed granule neurons and normal granule neurons (Fig. 6D). As compared with that of normal granule neurons, CA8 knockdown granule neurons showed a significant decrease of 58% in cell invasion ability. In contrast, CA8 overexpressed granule neurons significantly increased invaded cell numbers to about 161% as compared with that of normal granule neurons, as demonstrated in Fig. 6E. Taken these results together, CA8 knockdown significantly decreases cell mobility in mouse cerebellar granule neurons.

### 3.7. Increased ER-dependent $Ca^{2+}$ release in cerebellar granule neurons with down-regulated CA8 expression

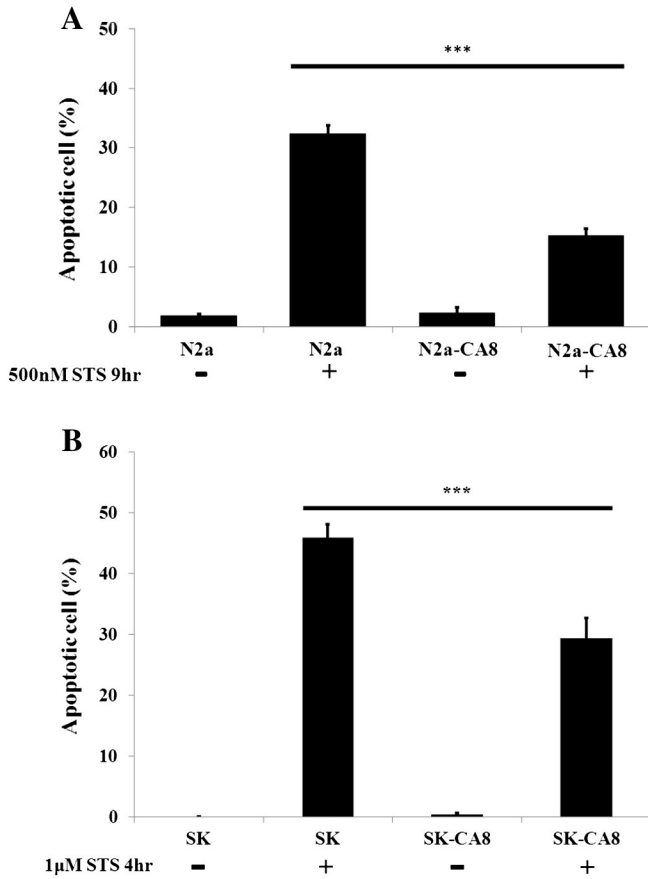
Because CA8 is known as a novel binding protein to inositol 1,4,5-trisphosphate receptor type 1 (IP<sub>3</sub>R1) [5,6], we were interested in the effects of CA8 loss-of-function on the expression of IP<sub>3</sub>R1 and cytosolic calcium concentration in granule neurons. Western blot analysis was performed for the assessment of the IP<sub>3</sub>R1 expression. As shown in



**Fig. 3.** CA8 overexpression significantly promotes neurite outgrowth in Neuro-2a cells.  $1 \times 10^5$  Neuro-2a (A) and Neuro-2a-CA8 (B) cells were seeded in 3 cm dishes and differentiated with 0.5% FBS DMEM medium. (C) To assess the percentage of cells bearing neurite, at least 5 fields at different time points were analyzed under a phase contrast microscope. Cells bearing neurites at least 2-fold longer than cell bodies were counted as differentiated cells. Data were expressed as means  $\pm$  SEM from three experiments. Scale bar = 100  $\mu$ m. \*\* $p < 0.01$ .

**Fig. 7A**, no detectable difference was observed among neuron cells with different expression levels of CA8, indicating no effects of CA8 loss-of-function on the IP<sub>3</sub>R1 expression in cerebellar granule neurons. Meanwhile, to investigate whether the knockdown of CA8 influences Ca<sup>2+</sup>

signaling in primary neurons, we measured the increase in Ca<sup>2+</sup> in cerebellar granule neurons with and without down-regulated CA8 under a 0.1 mM ATP stimulus. To block Ca<sup>2+</sup> influx due to the activity of the store-operated channel, the experiments were performed in the



**Fig. 4.** Overexpression of CA8 desensitizes neuronal cells to STS induced cell apoptosis. (A) Overexpression of CA8 significantly increases cell survival in Neuro-2a-CA8 cells. Neuro-2a and Neuro-2a-CA8 were seeded at a density of  $2 \times 10^5$  cells/ml in 3 cm dishes and treated with 500 nM STS for 9 h. After STS treatment, PI staining was performed to observe DNA fragmentation. The apoptotic cell percentage was obtained by referring these values to untreated control cells. Data were expressed as means  $\pm$  SEM from three separate experiments. More than 200 cells were counted for each group.  $***p < 0.001$ . (B) Overexpression of CA8 significantly increases cell survival in SK-N-SH-CA8 cells.  $4 \times 10^5$  SK-N-SH and SK-N-SH-CA8 cells were seeded in 3 cm dishes and treated with 1  $\mu$ M STS for 4 h. PI staining was performed after STS treatment to observe DNA fragmentation. The apoptotic cell percentage was obtained by referring these values to untreated control cells. Data were expressed as means  $\pm$  SEM from three separate experiments. More than 500 cells were counted for each group.  $***p < 0.001$ .

absence of extracellular  $\text{Ca}^{2+}$  ions (see the [Materials and methods](#) section for details). Under the experimental conditions, the increase in cytosolic  $\text{Ca}^{2+}$  after the addition of ATP was largely due to release from the ER pool. Our results demonstrated that ATP induced a transient elevation of  $\text{Ca}^{2+}$  for all neurons. As shown in [Fig. 7B](#), cerebellar granule neurons with down-regulated CA8 showed significantly increased cytosolic  $\text{Ca}^{2+}$  signaling in response to 0.1 mM ATP, as compared with the lentiviral-CA8 infected and lentiviral-control infected neurons. This finding demonstrates that the knockdown of CA8 in neurons leads to abnormal effects on cytosolic  $\text{Ca}^{2+}$  release.

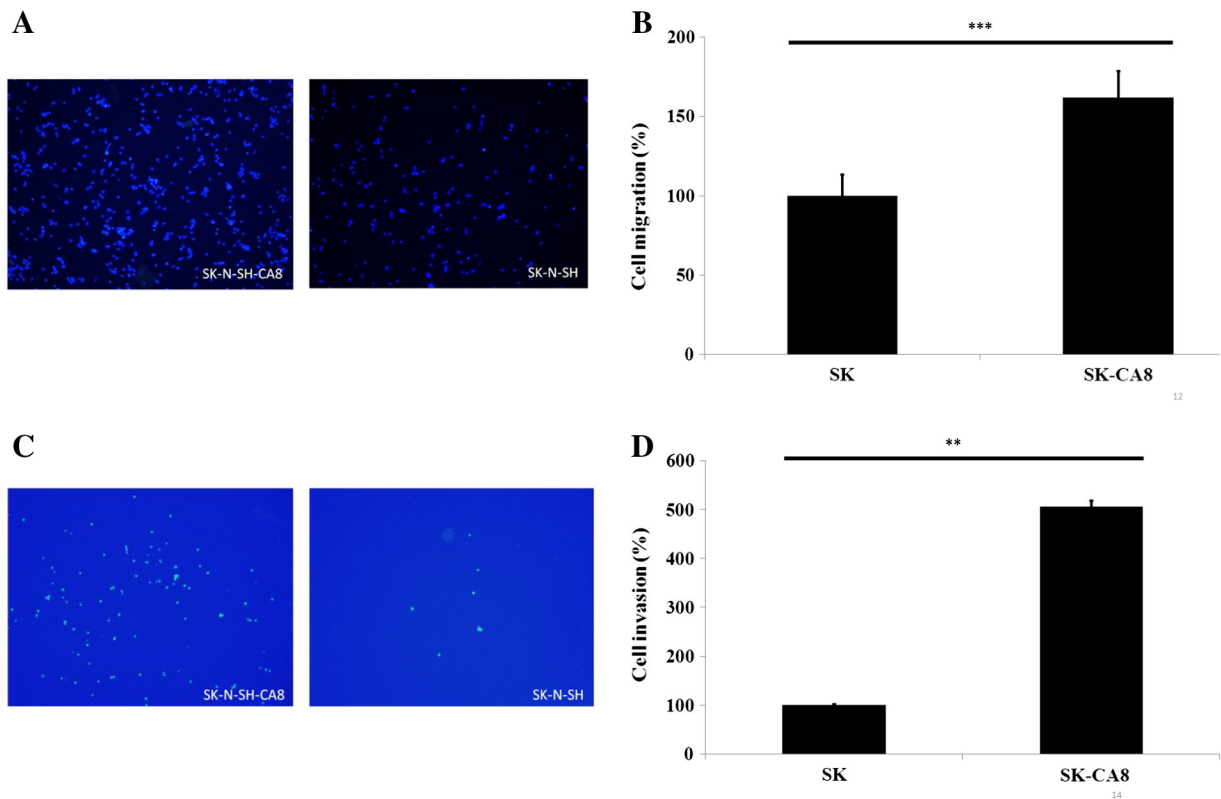
### 3.8. *ca8* MO successfully decreases CA8 protein expression in 3 dpf zebrafish

To evaluate the possibility to create a zebrafish CA8-knockout model for future study, we first investigated the CA8 protein expression pattern in zebrafish embryos. Zebrafish embryos at 24 hours post-fertilization (hpf), 48 hpf and 72 hpf were used to perform immunohistochemical analysis for detecting CA8 protein expression in the cerebellum. Cerebellum-specific protein, Parvalbumin7, was used as an indicative marker to localize the cerebellar tissue. As shown in [Fig. 8](#), CA8 started to appear in the cerebellum at the stage of 72 hpf. Furthermore, the expression of CA8 was co-localized with that of Parvalbumin7.

Our results are consistent with the previous report that the expression of CA8 protein is highly intense in the cerebellum [22]. To knockdown the expression of CA8, antisense MO targeting the translation initiation site of *ca8* gene was designed to evaluate the down-regulating efficiency of CA8 protein expression in 3 dpf zebrafish embryos. First of all, we injected 1.2 pmol of *ca8* MO into one- to two-cell stage embryos to inhibit CA8 expression. Our results showed that 1.2 pmol/embryo of *ca8* MO completely inhibited CA8 protein expression in 3 dpf zebrafish embryos (Supplementary Fig. 2). Meanwhile, severe morphological defects such as heart edema, shorter trunk and developmental delay were also observed after 1.2 pmol/embryo of *ca8* MO injection. These observations were accompanied with 90% of embryo lethality within three days. In order to avoid the serious morphological defects due to high MO dosage, we decreased the amount of injected *ca8* MO to 0.6 pmol/embryo. As indicated in Supplementary Fig. 2, CA8 protein expression was successfully inhibited in 3 dpf zebrafish embryos after 0.6 pmol/embryo of *ca8* MO injection, while mild morphological defects were shown. Although the *ca8* MO designed in our study was effective in inhibiting the expression of CA8, the general morphological defects in the heart and muscles implicated a possibility of p53-dependent off-target effects which has been reported to be associated with certain morpholinos and short-interfering RNAs [23]. Therefore, it is essential to lower the dosage of *ca8* MO, and to verify whether any specific effect caused by *ca8* MO is independent of a non-specific massive activation of p53.

### 3.9. Down-regulation of CA8 significantly affects zebrafish locomotion in 3 dpf zebrafish after 0.3 pmol/embryo of *ca8* MO injection

In order to reduce the morphological effect of *ca8* MO on the early embryo, so that we could evaluate the role of CA8 knockdown on the zebrafish motor ability at 3 dpf, the *ca8* MO dosage was next lowered to 0.3 pmol/embryo. The immunohistochemistry assay was performed to examine whether the CA8 protein expression could be specifically down-regulated by the dosage of *ca8* MO at 0.3 pmol/embryo. As demonstrated in [Fig. 9A](#), the *ca8* morphant at 3 dpf showed a significant down-regulation of CA8 expression, as compared with that in the control embryo. The fluorescent intensity of CA8 in the cerebellar region of *ca8* morphants ( $n = 8$ ) was measured to be 64% ( $p < 0.05$ ) of that in the control embryo ( $n = 6$ ). Meanwhile, fluorescent intensities of Parvalbumin in the cerebellar region were not significantly different between *ca8* morphants and control embryos. Therefore, *ca8* MO injection at 0.3 pmol/embryo was able to significantly knock down the CA8 protein without altering the cerebellar structure and the Parvalbumin expression. Next we checked whether the *ca8* MO injection would affect the zebrafish development. In order to evaluate the effect of *ca8* MO on general morphology and the nervous system, the *ca8* MO was injected into embryos of *Tg(HuC:EGFP)* line which recapitulates the expression of *HuC (elav)* and is an excellent fluorescent reporter for differentiating neurons [24]. Furthermore, p53 MO at the dosage of 0.45 pmol/embryo (1.5 fold of the *ca8* MO concentration) was co-injected with *ca8* MO to test whether the observed defects were caused by an off-target effect of *ca8* MO by p53 activation [23]. In terms of the gross morphology of the heart, somitic muscles and nervous structures, our results showed no noticeable phenotypic difference between all of the *ca8* morphants ( $n = 93$ ) and control embryos ( $n = 145$ ) ([Fig. 9B](#)). In addition, there was no general morphological difference among embryos injected with *ca8* MO alone, with p53 MO alone ( $n = 85$ ), and with a combination of *ca8* and p53 MOs ( $n = 53$ ). Therefore, 0.3 pmol/embryo of *ca8* MO injection was determined to be the effective dosage with minimal morphological effects on the early embryo, which then allowed us to perform tactile stimulate motor assay to assess the motor ability of *ca8* morphants. We used a needle to stimulate the tail of 3 dpf embryos to induce tactile stimuli, and the locomotion of zebrafish was recorded with Nikon COOLPIX P500. Five tactile stimuli with 30 s interval were applied to each zebrafish and the first tactile-stimuli-induced motor response was counted for behavior score (for details please see the



**Fig. 5.** Overexpression of CA8 in SK-N-SH cells significantly increases cell migration and invasion ability. (A)  $1 \times 10^5$  SK-N-SH and SK-N-SH-CA8 cells were seeded in the upper chambers of the trans-well with serum free DMEM medium and incubated for 12 h. After 12 h of cell migration, DAPI staining was performed to observe the migrated cells of SK-N-SH and SK-N-SH-CA8. (B) To assess cell migration ability, at least 7 different fields of each cell line were analyzed under a fluorescence microscope. Data were expressed as means  $\pm$  SEM from three separate experiments. \*\*\* $p < 0.001$ . (C)  $1 \times 10^5$  SK-N-SH and SK-N-SH-CA8 were seeded in the upper chambers of trans-well with serum free DMEM medium and incubated for 36 h. After 36 h of incubation, DAPI staining was performed to observe invaded SK-N-SH and SK-N-SH-CA8 cells. (D) To assess cell invasion ability, at least 7 different fields of each cell line were analyzed under a fluorescence microscope. Data were expressed as means  $\pm$  SEM from three separate experiments. \*\* $p < 0.01$ .

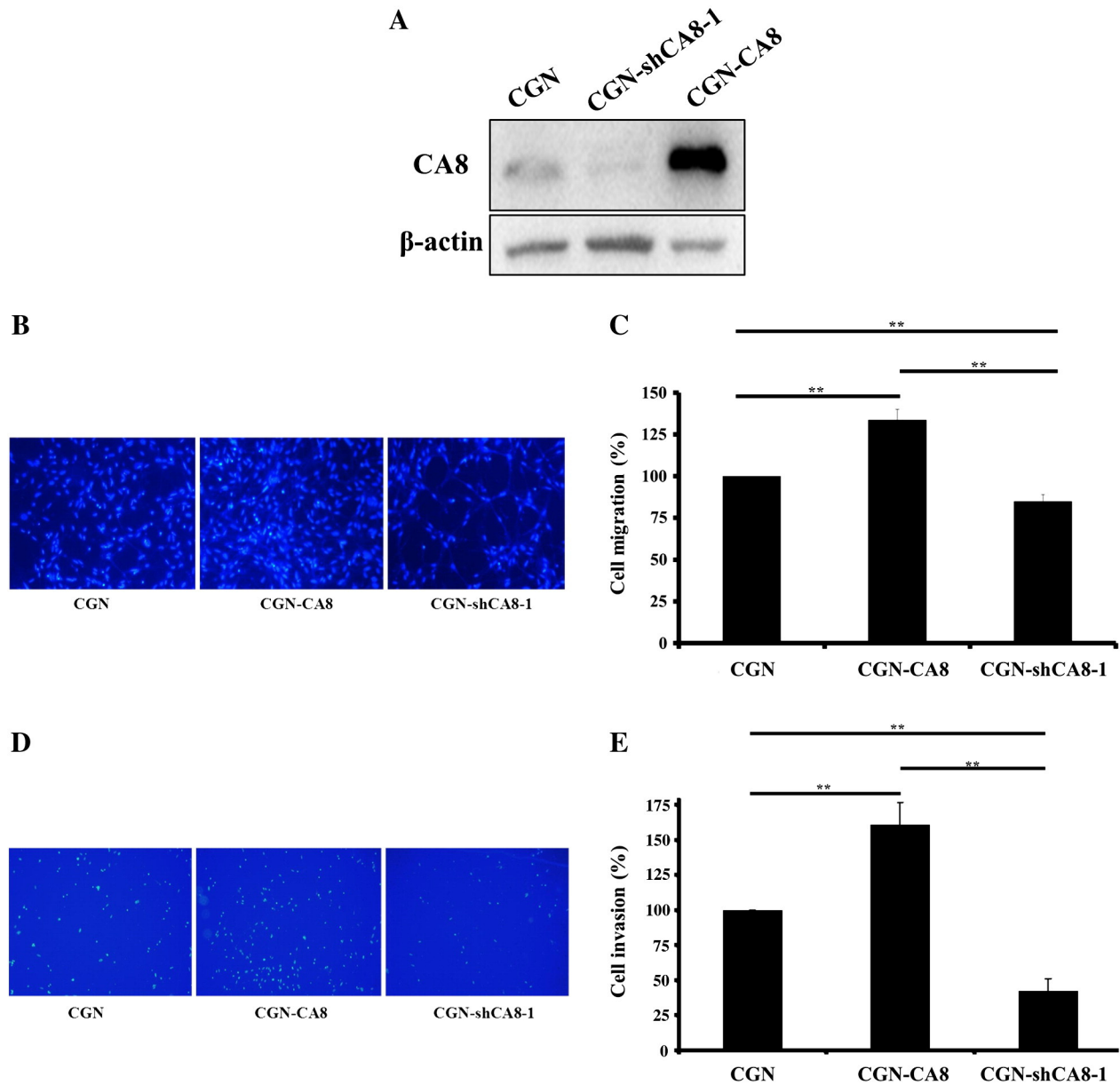
Materials and methods section). As shown in Fig. 9C, the motor reaction in *ca8* morphants was significantly decreased as compared with that in the controls. In order to verify whether the decreased motor reaction in the *ca8* morphant was due to defective development of sensory neurons and muscles, we also tested the effect of *ca8* MO, with or without co-injected *p53* MO, on embryos of the *Tg(CM-isl1:GFP)*rwo** which is a reporter line for motor and sensory neurons [16]. As shown in Fig. 9D, the ontogeny of cranial motor neurons such as trigeminal motor neurons (V), facial motor neurons (VII) and vagus motor neurons (X) at 3 dpf was not affected in embryos injected with *ca8* MO, *p53* MO, or a combination of *ca8* MO and *p53* MO (representative images of 21, 4, and 16 samples respectively). Moreover, the development of Rohon Beard sensory neurons and spinal motor neurons in the trunk region appeared normal in all the morphants. These results indicate that the reduced motor response of *ca8* morphants was specifically due to defective cerebellar function, but not to a general disruption of either motor or sensory neuron development in the early embryo. The morphology of somitic muscles was also examined (Supplementary Fig. 3). The formation and organization of myofibrils and somite boundaries were generally unaffected in all the morphants at 3 dpf, suggesting that the low locomotor activity in the *ca8* morphant was not a result from somitic muscle malformation. In summary, the studies of expression, phenotype and function performed on the zebrafish embryo support that the CA8 expression in the cerebellum during development is required for the neural function that coordinates motor control.

#### 4. Discussion

It is known that CA8 is highly expressed in neural cell body spreading to most parts of the central nervous system and is a novel binding

protein to inositol 1,4,5-trisphosphate receptor type 1 (IP<sub>3</sub>R1) [5,6]. Furthermore, it was demonstrated that CA8 deficiency is associated with a distinctive lifelong gait disorder in Waddles (*wdl*) mice [7]. Consistently, homozygous mutations in CA8, S100P and G162R, were reported to cause human ataxia and mental retardation [8,9]. All these lines of evidence indicate that CA8 may play important roles in motor control of neuron cells. In addition, expression of CA8 in invasive lung adenocarcinoma and colorectal carcinoma promotes cancer oncogenesis [10,11]. These findings prompted us to examine the expression and roles of CA8 in neuroblastoma cell lines and in primary neurons. Therefore, we first examined the protein expression in three different neuroblastoma cell lines. In contrast to a previous study showing that CA8 mRNA expression was distinctly expressed in human neuroblastoma cell lines (IMR32, TGW) and human schwannoma cell line (HS-Sch II) [5]; our study did not reveal detectable endogenous CA8 protein in human neuroblastoma cells (IMR32, SK-N-SH) and mouse neuroblastoma cells (Neuro-2a) (Fig. 1A). Therefore, to study the possible functions of overexpressed CA8 in neuronal cells, a lentiviral expression plasmid pLKOAS3W-CA8.neo, a lentiviral transfer vector containing the full-length CA8, was constructed in this study.

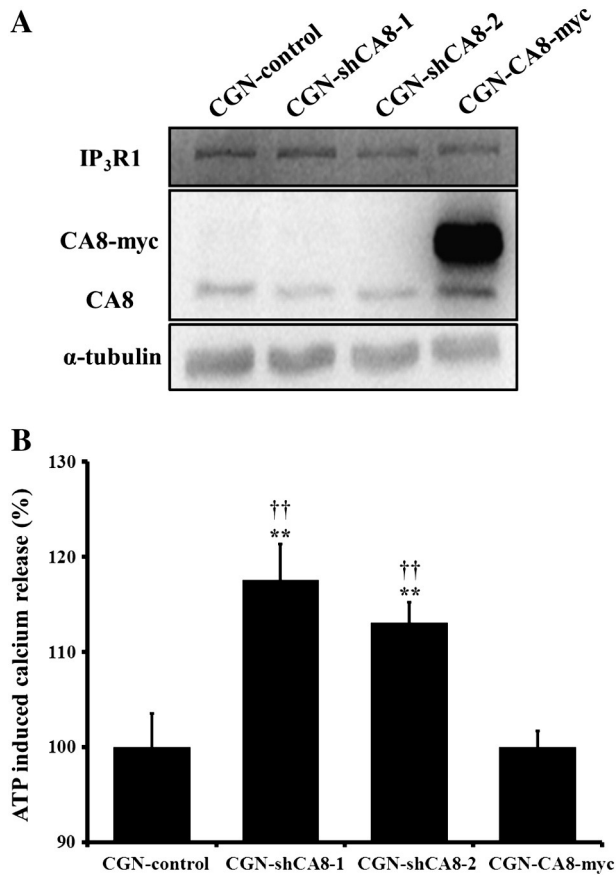
We then established CA8 overexpression neuronal cells by infecting SK-N-SH and Neuro-2a cells with CA8-containing lentivirus. Up to 90% of cells (SK-N-SH and Neuro-2a) were successfully infected after G418 selection under immunofluorescence analysis (data not shown). It is noted that control cell lines stably expressing the lentiviral control vector showed no effects on cell morphology and proliferation under the same growth condition, as compared with those of the parental cell lines (data not shown). Therefore, most of the experiments were performed by comparing stably-expressing CA8 cell line to the parental cell lines. The successful overexpression



**Fig. 6.** Knockdown of CA8 in mouse cerebellar granule neurons significantly decreases cell migration and invasion ability. (A) Western blot analysis of CA8 in mouse cerebellar granule neurons with down-regulation and overexpression of CA8. Mouse cerebellar granule neurons were seeded at  $1 \times 10^5$  cells/well in a 24-well plate with DMEM medium containing 20% FBS. After incubation for 24 h, CA8 overexpression lentivirus (pLKOAS3w.neo-CA8) and CA8 shRNA lentivirus were added for 12 h. Western blot analysis was then performed to study the expression of CA8 in cerebellar granule neurons that were treated with CA8 shRNA lentivirus (CGN-shCA8-1), cerebellar granule neurons that were treated with CA8 overexpression lentivirus (CGN-CA8), and cerebellar granule neurons (CGN). (B) Overexpression of CA8 in mouse cerebellar granule neurons significantly increased cell migration ability.  $1 \times 10^5$  neurons with and without lentivirus infection were seeded in the upper chambers of the trans-well with serum free DMEM medium; then incubated for 12 h. After 12 h of cell migration, DAPI staining was performed to observe migrated cells in CGN, CGN-CA8, and CGN-shCA8-1. (C) To assess cell migration ability, at least 7 different fields of each cell type were analyzed under a fluorescence microscope. Data were expressed as means  $\pm$  SEM from three separate experiments. \*\* $p < 0.01$ . (D)  $1 \times 10^5$  neurons with and without lentivirus infection were seeded in the upper chambers of the trans-well with serum free DMEM medium and incubated for 36 h. After 36 h of cell invasion, DAPI staining was performed to observe cell invasion in CGN, CGN-CA8, and CGN-shCA8-1. (E) To assess cell invasion ability, at least 7 different fields of each cell type were analyzed under a fluorescence microscope. Data were expressed as means  $\pm$  SEM from three separate experiments. \*\* $p < 0.01$ . CGN: cerebellar granule neurons.

of CA8 in the resulting cells, designated as SK-N-SH-CA8 and Neuro-2a-CA8 cells, was confirmed by Western blot analysis and these cell lines were used for further analysis. Our results demonstrated that Neuro-2a-CA8 cells showed increased proliferative ability as compared with parental Neuro-2a cells. To confirm the impact of CA8 protein levels on cell proliferation; CA8 stably expressed single clones, Neuro-2a-CA8-2 and Neuro-2a-CA8-3, were further selected for comparison. Our results clearly demonstrated a positive dosage effect of the expression levels of CA8 on cell proliferative ability in

Neuro-2a cells, as evidenced by the increased cell proliferation in selected Neuro-2a-CA8 single clone with higher expression level of CA8. However, we did not detect alterations in cell proliferation between SK-N-SH-CA8 and SK-N-SH cells, suggesting differential effects of CA8 on cell growth in different cell types/organisms. Furthermore, because the function of CA8 in neurogenesis is currently unclear, whether the transgenic expression of CA8 leads to any morphologic change in neuronal cells was examined under normal and differentiated conditions. Even though there was no



**Fig. 7.** Increased ER-dependent  $\text{Ca}^{2+}$  release in cerebellar granule neurons with down-regulated CA8 expression. (A) Western blot analysis of IP<sub>3</sub>R1 in mouse cerebellar granule neurons with down-regulation and overexpression of CA8. Mouse cerebellar granule neurons were infected with control virus, CA8 shRNA lentivirus (shCA8-1 and shCA8-2), and CA8 overexpression lentivirus (pLKOAS3w-CA8-myc-puro) according to the standard protocol. Western blot analysis was then performed to study the expression of IP<sub>3</sub>R1 in CGN-control, CGN-shCA8-1, CGN-shCA8-2, and CGN-CA8-myc. (B) Cytosolic calcium signals in cerebellar granule neurons in response to 0.1 mM ATP. Fura-4AM fluorescence was recorded continuously at 37 °C in a spectrofluorometer at the excitation wavelength of 494 nm and an emission wavelength of 516 nm. Peak  $\text{Ca}^{2+}$  increase in response to 0.1 mM ATP in CGN-control, CGN-shCA8-1, CGN-shCA8-2, and CGN-CA8-myc. The results were represented as the percentage compared with CGN-control. \*\*Significant difference with CGN-control; ††Significant difference with CGN-CA8-myc. CGN: cerebellar granule neurons.

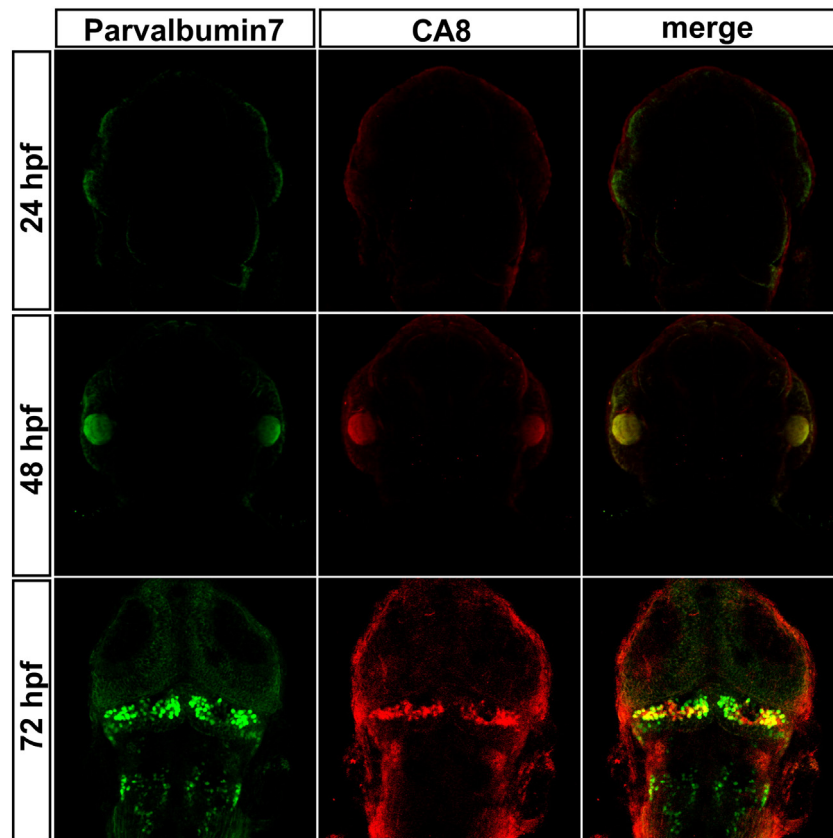
significant morphologic change in neuronal cells overexpressing CA8 under normal growth conditions, our results demonstrated that CA8 overexpression significantly promoted neurite outgrowth in Neuro-2a-CA8, as compared with parental Neuro-2a cells at low-serum treatment for 24 and 36 h. On the other hand, compared with that of SK-N-SH cells, there was no significant difference in neurite extension in SK-N-SH-CA8 cells under low-serum conditions. So far, we do not know the reasons for the discrepancies between these two cell lines in cell proliferation and neurite outgrowth. Our results suggest that there may be differential effects of CA8 on various cell types because of CA8's respective role/function in controlling cell growth and morphology in specific cell types.

It is well-documented that CA8 overexpression significantly increases cell migration and invasive ability in colorectal carcinoma and non-small cell lung carcinoma [10,11,25]. To understand whether overexpression of CA8 in neuronal cells has similar functions, neuronal cells with stable CA8-expression were examined in this study. Our results showed that CA8 overexpression significantly increased cell migration and invasive abilities in SK-N-SH-CA8 as compared with those of SK-

N-SH cells. However, we failed to examine the migration and invasive abilities in Neuro-2a and Neuro-2a-CA8 cells by using either wound-healing or transwell assay (data not shown), which may be due to slow motility of Neuro-2a cells under the experimental conditions. To further confirm the results obtained from ectopically expressed CA8 in neuronal cell lines; primary cerebellar granule neurons, with endogenous CA8 expressed, were isolated for comparison. Again, CA8 overexpression lentivirus was used to infect primary granule neurons for overexpression study. Meanwhile, to test the effects of down-regulated CA8 contributes to primary neurons, lentivirus-carrying CA8 RNA interference (RNAi) vectors were used to knock down CA8 expression in cerebellar granule neurons. Consistent with what was observed in SK-N-SH cell line, CA8 down-regulated granule neurons significantly decreased cell migration and invasive abilities as compared with those in normal granule neurons and CA8 overexpressed granule neurons, indicating a specific role of CA8 in neuron motility. Even though our results gained in the neuroblastoma cells are similar to the previous findings that overexpression of CA8 affects the proliferation and invasive ability of colon cancer cells [11], our study further provided evidence that down-regulation of CA8 significantly decreased cell migration and invasive abilities, not only in neuroblastoma cells but also in primary cerebellar granule neurons. Our results support the notion that down-regulated CA8 in neurons may cause deficiencies in controlling neuron mobility but overexpression of CA8 in neuronal cells may be involved in promoting oncogenesis.

Meanwhile, it is noteworthy that the deduced amino acid sequence of human CA8 is 98% identical to the mouse homologue [3]. The high degree of conservation of the CA8 protein and intensely-expressed CA8 in Purkinje cells of human and mouse brain imply that CA8 may play some important roles in neuron cells. Recently, we have reported a protective function of CA8 in cells harboring the A8344G mutation of mitochondrial DNA [17]. Therefore, we speculated that the proper expression of CA8 protein in neurons may also play an important protective role. To investigate the putative role in neuron cells, STS treatment was applied to both neuronal cell lines with and without CA8 overexpression. Interestingly, overexpression of CA8 in both Neuro-2a and SK-N-SH cells significantly increased cell survival as compared with that of parental cells after STS treatment (Fig. 4). Our results evidently demonstrated a positive effect of CA8 overexpression on protective abilities in both human and mouse neuroblastoma cell lines. Furthermore, to assess the effects of CA8 loss-of-function on the cytosolic calcium concentration in cerebellar granule neurons, our results demonstrated that down-regulated CA8 in primary neurons leads to abnormal ATP-induced  $\text{Ca}^{2+}$  release, which may be a contributing factor in the decreased protective ability observed in cells with down-regulated CA8. Taken together, our observations support and extend the notion that the expression of CA8 is critical in the modulation of intracellular  $\text{Ca}^{2+}$  signaling, which may be responsible, at least in part, for the protective functions in human neuronal cells.

To further understand the biological roles of CA8 in vivo, an animal model is indispensable. Bio-information analysis showed that the protein sequence of CA8 is up to 84% identical between *D. rerio* and *Homo sapiens*, *D. rerio* and *Mus musculus*, and *D. rerio* and *Rattus norvegicus*; and up to 78% identical between *D. rerio* and *Xenopus* [4,26]. It is known that CA8 is significantly expressed in Purkinje cells of human, mouse and zebrafish [5,22,27–29]. CA8 may have an important function in the development and maturation of Purkinje cells because of the high degree of conservation of the protein and DNA sequence between human, mouse and zebrafish [3,4]. In addition, the high intracellular CA8 levels in Purkinje cells were found throughout the development of mouse cerebellum [30] and zebrafish [22]. These studies suggest that zebrafish is a good animal model to study the functions of CA8. Therefore, we evaluated whether zebrafish can be used to create an animal model for CA8-deficient neuromuscular diseases. From our observations, CA8 protein started to appear in embryos at 3 dpf, as evidenced by immunofluorescence staining using specific cerebellum



**Fig. 8.** Significant effects of *ca8* morpholino on CA8 protein expression in 72 hpf zebrafish embryos. Expression of CA8 during the cerebellar development in early zebrafish embryos. Whole mount immunofluorescence of 24, 48 and 72 hpf zebrafish embryos was performed with anti-Parvalbumin7 (representing cerebellum signals) and anti-CA8. The images were presented in dorsal view, with head pointed to the top. Double staining indicated colocalization (yellow) of Parvalbumin7 (green) and CA8 (red) in the cerebellum. The images were presented in dorsal view, with head pointed to the top.

marker, Parvalbumin7. In addition, we demonstrated that the 5'UTR *ca8* MO can efficiently down-regulate CA8 protein expression in embryos at 3 dpf. However, we found that higher dosages of *ca8* MO injection also resulted in general morphologic defects not only in the cerebellum but also in different organs of zebrafish larvae. This developmental abnormality accompanied with CA8 knockdown is not expected because no developmental alteration was observed in the *wdl* mice that do not have detectable CA8 expression [7]. To make sure that our *ca8* MO can specifically target *ca8* gene with minimal off-target effects, an optimized BLAST search was performed to reduce the risk. Additionally, STD-MO and p53-MO were used as control-MO for comparison. After applying different dosages of *ca8* MO to zebrafish embryos, 0.3 pmol of *ca8* MO was the optimal amount, which did not lead to severe developmental defects, to be used for further experiments. The results from immunofluorescence showed that 0.3 pmol *ca8* MO injection partially decreased the expression of CA8 protein in the cerebellum of 3 dpf zebrafish embryos. Furthermore, we performed tactile stimulate motor assay to assess whether CA8 down-regulation leads to defects in zebrafish motor reaction. As we expected, down-regulation of CA8 in zebrafish significantly decreased the motor response after tactile stimuli. This observation is similar to defects in CA8, which lead to ataxia, mental retardation and quadrupedal gait in human patients and ataxia [8,9] and lifelong gait disorder in *wdl* mice [7].

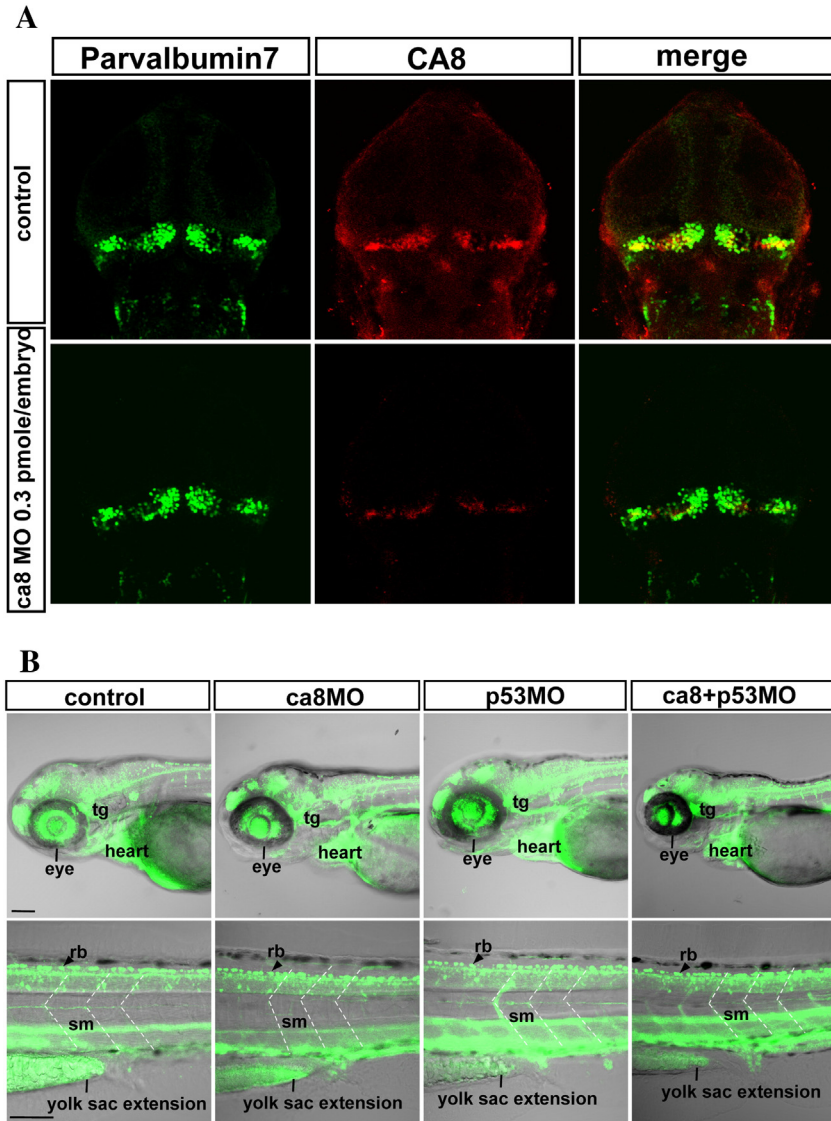
It is noted that a recent publication by Aspatwar et al. also used antisense morpholino oligonucleotides against the *ca8* gene to study the effects of CA8 knockdown in zebrafish [31]. Consistent with our study, the *ca8* morpholino designed by Aspatwar et al. is able to suppress CA8 expression up to 5 dpf as evaluated by Western analysis using

whole-embryo lysates, and also leads to dose-dependent effects on the general morphology. However, embryos injected with a dosage at 0.125 pmol/embryo manifested general developmental defects as early as 1 dpf, which include pericardial dilation, abnormal head shape and disrupted muscle formation, and displayed apoptotic neuronal cells at the cerebellum at 5 dpf. In addition, Aspatwar et al. demonstrated that suppression of CA8 expression leads to defects in motor and coordination functions, as evidenced by the trajectories of the 5 dpf larvae injected with different concentrations of *ca8* MO when compared with uninjected 5 dpf larvae [31]. By contrast, the *ca8* MO designed in this study and injected at a dosage of 0.3 pmol/embryo led to a 36% reduction of the cerebellum-specific CA8 expression at 3 dpf, as quantified by fluorescent intensities of CA8 immunostaining. Although this is not a complete knockdown, it allowed us to perform a motor activity assay for CA8-deficient embryos without significant general disruption of early neuronal or muscular structures. Furthermore, the embryos injected with *ca8* MO at 0.3 pmol in this study did not display a change of either general cerebellar morphology or Parvalbumin expression at 3 dpf. As our results showed that a highly restrictive expression of CA8 protein at the cerebellum started to be evident at 3 dpf, the design and dosage of the *ca8* MO in this study enabled us to evaluate the specific function of CA8 without the confounding factors of early defects. Our results further demonstrated that the effect of CA8 knockdown on the zebrafish motility was evident as early as 3 dpf, the onset of embryonic to larval transition [32], implicating an essential role of CA8 for the early function of the cerebellum. Despite the different designs and dosages of morpholinos, our results are consistent with their work that CA8 plays an important role in zebrafish motor control and further provide

evidence of a useful animal model for neuromuscular disease analysis in the future.

In summary, our results demonstrated for the first time that CA8 overexpression significantly increased cell migration and invasion ability in neuronal cell lines, consistent with what was previously reported in adenocarcinoma, colorectal carcinoma and lung carcinoma cells [10,11,25]. The decreased cell mobility and abnormal calcium release in mouse cerebellar granule neurons with down-regulated CA8 strongly support that a proper expression level of CA8 in neurons is critical in motor coordination. Moreover,

overexpression of CA8 in Neuro-2a and SK-N-SH cells significantly increased cell survival in response to the apoptotic stresses, indicating an important protective function of CA8 in neuronal cells. In addition, motor reflexion of 3 dpf zebrafish embryos was significantly affected after the down-regulation of CA8 through *ca8* MO, mimicking the movement disorder in humans and mice. From the results showing that the knockdown of the *ca8* gene resulted in an abnormal movement pattern in zebrafish, we expect that zebrafish can be a useful animal model to study the biological functions of CA8 in human diseases.



**Fig. 9.** Low dosage of *ca8* morpholino inhibits CA8 protein expression and zebrafish locomotion in 3 dpf zebrafish embryos. (A) *ca8* morpholino inhibited CA8 protein expression in 3 dpf zebrafish embryos. Whole mount immunofluorescence of *ca8* morphants was performed with anti-Parvalbumin7 (green) and anti-CA8 (red). CA8 protein expression was down-regulated in CA8 morphants after low dosage (0.3 pmol/embryo) of *ca8* MO injection as compared with control. (B) No significant general morphological defects were observed after injection of 0.3 pmol/embryo *ca8* MO with or without *p53* MO. *Tg(HuC:EGFP)* embryos injected with control dye, *ca8* MO alone, *ca8* MO plus *p53* MO, or *p53* MO alone during one- to two-cell stage and harvested for phenotypic analysis after three days of development. The upper panels are lateral views of the anterior part of embryos where the EGFP was detected in nervous cells including those located in the eye and trigeminal ganglia (tg). The lower panels are lateral views of tail trunks where the EGFP was detected in Rohon Beard (rb) sensory neurons. The boundaries of somites (sm) are marked by white dashed lines. Scale bar = 100  $\mu$ m. (C) Down-regulation of CA8 by *ca8* MO significantly reduced mobility response of 3 dpf zebrafish embryos after tactile stimulation. 3 dpf morphants (or control embryos) were placed in the middle of a 6 cm Petri dish. Five tactile stimuli with 30 s intervals were applied to the region near the tip of the tail. First tactile stimuli were counted for the behavioral score analysis. The motor response of *ca8* morphants significantly decreased as compared with that of the control. The results represent the mean scores of 34 larvae in each set of experiment. \*\*\* $p < 0.01$ . (D) No significant defects of motor and sensory neurons were observed at 3 dpf after injection of 0.3 pmol/embryo *ca8* MO with or without *p53* MO into *Tg(isl1:GFP)rwo* embryos. The upper panels are dorsal views of the anterior part of *Tg(isl1:GFP)rwo* embryos where the EGFP was detected in cranial motor neurons including trigeminal motor neurons (V), facial motor neurons (VII) and vagus motor neurons (X). The lower panels are lateral views of tail trunks where the EGFP was detected in Rohon Beard (rb) sensory neurons and spinal motor neuron (smn). Scale bar = 100  $\mu$ m.

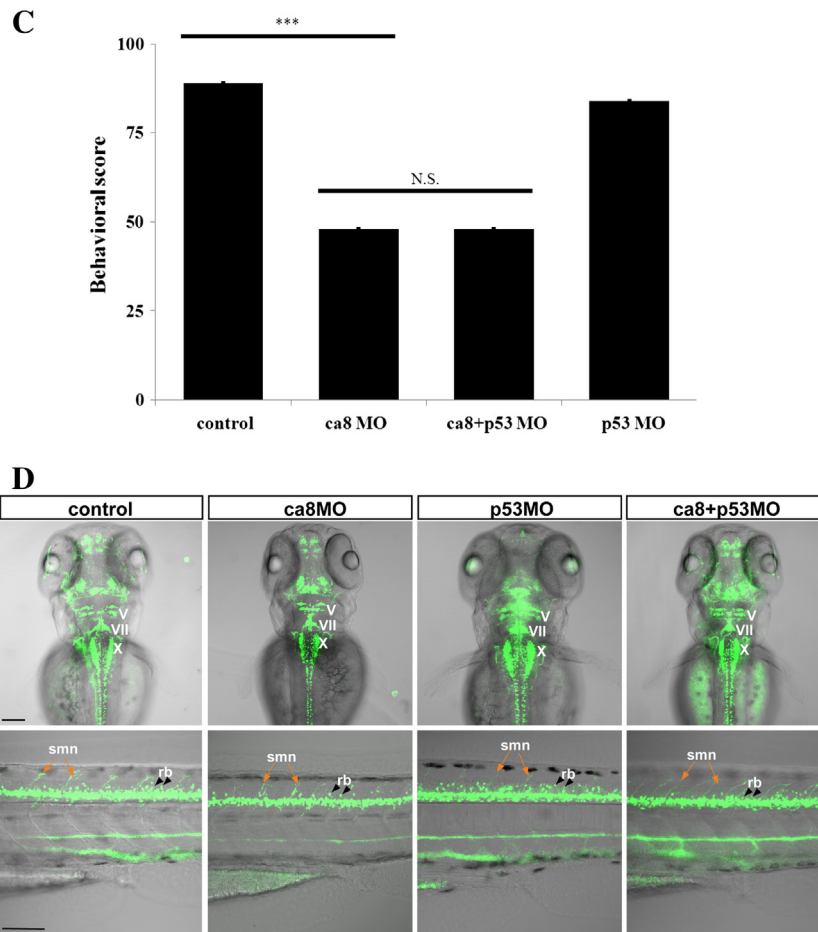


Fig. 9 (continued).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2014.04.017>.

### Acknowledgements

We thank Dr. Masahiko Hibi for Parvalbumin7 antibody; Drs. Shinichi Higashijima and Hitoshi Okamoto and the support of the National Bioresource Project of Japan for *Tg(CM-isl1:GFP)row*; Dr. Yi-Chuan Cheng for providing materials and helpful discussions; Dr. Jiann-Jou Yang for technical assistance in zebrafish handling; Dr. Hong-Lin Su for teaching us to isolate mouse cerebellar granule neurons; Dr. Wei-Ting Chao for advice on cell migration and invasion assays; Che-Min Lo for technical assistance with cell culture and Benjamin Y.T. Hsieh for editing the manuscript. We thank the National RNAi Core Facility for providing the RNAi reagents; Taiwan Zebrafish Core Facility (TZCF) at ZeTH and TZCF at Academia Sinica (TZCAS) for providing fish and trainings. This work was supported by grants from the Ministry of Science and Technology of the Republic of China (NSC99-2632-B-029-001-MY3; NSC100-2311-B-029-003; NSC101-2311-B-029-001; NSC102-2628-B-029-002-MY3).

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