

## VAMP2 Marks Quiescent Satellite Cells and Myotubes, but not Activated Myoblasts

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We examined the expression and intracellular localization of vesicle-associated membrane protein 2 (VAMP2) during the differentiation of skeletal muscle cells by immunofluorescence microscopy. In isolated single myofibers, VAMP2 was expressed in quiescent satellite cells, downregulated in proliferating myoblastic cells, and re-expressed with differentiation. In the myoblastic cell line C2C12, VAMP2 was expressed at a low level in the proliferating stage, and then increased after differentiation into myotubes. Based on these results, we propose that VAMP2 can be used as a molecular marker for both quiescent satellite cells and myotubes, but not for proliferating myoblasts. We also found the partial colocalization of VAMP2 with transferrin- or Rab11-labeled vesicles in myotubes, suggesting a role of VAMP2 in the trafficking of recycling endosomes.

**Key words:** satellite cell, myotube, C2C12 cells, VAMP, SNARE

### I. Introduction

Myofibers are highly specialized cells for force generation, and their organelles and plasma membrane are unique in structure and localization. Membrane traffic is expected to play roles in construction and maintenance of membrane structures in myofibers, as well as in other types of cells [18]. In addition, some muscular diseases result from mutations in genes involved in membrane traffic, *e.g.*, dysferlinopathy and caveolinopathy [4], indicating the significance of membrane traffic in myofibers. To gain insight into membrane traffic in myofibers, we examined the presence of SNARE proteins, which regulate docking and fusion of vesicles with their target membrane [9].

Vesicle-associated membrane protein-2 (VAMP2)/synaptobrevin-2 is a member of the SNARE protein family, which was first identified in synaptic vesicles [1]. VAMP2 is also found in non-neural tissue, and expressed in mature myofibers [14]. During the differentiation of L6 myoblasts, VAMP2 was detected in differentiated myotubes, but not in proliferating myoblasts [19]. Thus, it has been suggested that VAMP2 is expressed only after muscle differentiation.

However, we previously found that VAMP2 is expressed in satellite cells in the adult skeletal muscle [16]. To address this issue, we examined the expression of VAMP2 in isolated satellite cells by immunofluorescence microscopy. Isolated satellite cells allow analysis of the course of muscle differentiation, *i.e.*, the quiescent state, proliferation, and formation of myotubes. We also analyzed the intracellular localization of VAMP2 in C2C12 myotubes.

### II. Materials and Methods

#### *Animals and cells*

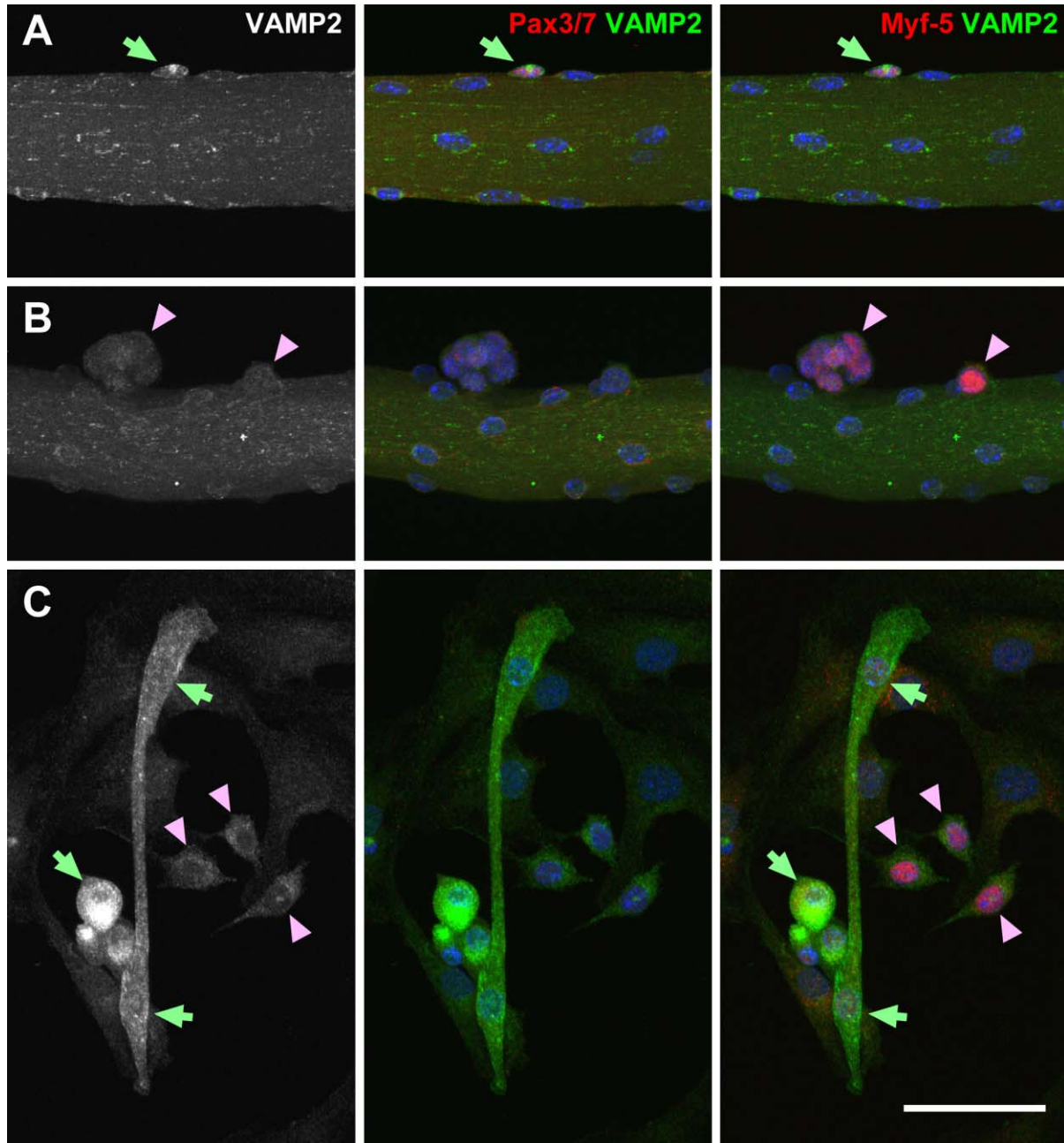
C57 BL/10-SCN mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). The protocol used in this study was approved by the Animal Care and Experimentation Committee, Gunma University (#07-094). For isolation of myofibers, extensor digitorum longus and flexor digitorum brevis muscles were carefully dissected, and digested with 0.2% collagenase type I (Worthington, Lakewood, NJ) in Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, MO) [6]. Isolated myofibers were cultured in suspension in DMEM containing 10% horse serum (Invitrogen, Carlsbad, CA), 5% chick embryo extract (Sera Laboratories International, West Sussex, UK), 1% penicillin/streptomycin solution (Invitrogen). C2C12 myoblasts were obtained from RIKEN Cell Bank (Tsukuba, Japan). For induction of differentia-

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tion, aliquots of  $4 \times 10^5$  cells in 3.5-cm dishes, or  $7 \times 10^4$  cells/well in 24-well plates were grown in 10% fetal bovine serum in DMEM for 24 hr, and the medium was then changed to 2% horse serum in DMEM which was replaced approximately every 24 hr.

#### RNA interference

siRNAs, AUGAUGAUGAGGAUGAUGGCGCAGA and UAAACGAUGAUGAUGAUGAGGAUGA targeting nt 403–427 and 412–436 of VAMP2 sequence (NM\_009497), were purchased from Invitrogen (Stealth RNA). Scrambled



**Fig. 1.** Expression of VAMP2 in isolated myofibers. (A) 2 hr after sacrifice, (B) 48 hr after isolation, and (C) 6 days after isolation. Myofibers and migrated cells were immunostained for VAMP2, Pax3/7, and Myf-5. The left column shows VAMP2 staining. The center column shows merged images of Pax3/7 (red) and VAMP2 (green), and the right column shows merged images of Myf-5 (red) and VAMP2 (green). Nuclear DNA stained with DAPI is shown in blue. VAMP2 is expressed in Pax3/7-positive satellite cells (arrow in A), and is absent from Myf-5-positive proliferating cells (B). On day 6, VAMP2 was found in myotubes and mononuclear cells (arrows in C), but not in Myf-5-positive proliferating cells (arrowheads in C). Multiple confocal images with intervals of 2  $\mu$ m were projected onto single images. Images were acquired and processed according to the same settings. Bar=50  $\mu$ m.

siRNAs, AUGCGAGUUAAGGGUAAGGUCGAGA and UAAAGAUAGCGUAGAGUAGAGUUGA were used as controls. These siRNAs were indicated as 1, 2, 1s, and 2s, respectively, in experimental data. Transfection of siRNAs was performed on day 0 and 3 of differentiation, using CombiMag, or SilenceMag (OZ Biosciences, Marseille, France). Cells from 2 wells of 24-well plates were homogenized and used for Western blot analysis, and cells on 3.5 cm dish were processed for immunofluorescence.

### **Immunofluorescence**

Myofibers and C2C12 cells were fixed with 3% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min at 37°C, or methanol for 10 min at -20°C. Specimens from the bottom of each 3.5 cm cultured dish were cut into small pieces, and processed for immunostaining. PFA-fixed specimens were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After blocking with 5% normal donkey serum, specimens were incubated with primary antibodies and subsequently with secondary antibodies. Antibodies and reagents used were as follows: mouse anti-VAMP2 antibody (1:500, clone 69.1; Synaptic Systems, Göttingen, Germany) [16, 17], rabbit anti-VAMP2 antibody (1:200; Wako Pure Chemical Industries, Osaka, Japan) [7], mouse anti-MyHC (1:200, clone MF20; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-myf-5 antibody (1:200, sc-302; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-MyoD1 (1:200; clone 5.8A; Dako, Glostrup, Denmark), mouse anti-Golgi 58K antibody (1:200, clone 58K-9; Sigma), rabbit anti-VAMP4 antibody (1:200; Affinity BioReagents, Rockford, IL), mouse anti-Rab11 antibody (1:100, clone 47; BD Bioscience, Franklin Lakes, NJ), goat anti-EEA1 antibody (1:200, sc-6415; Santa Cruz Biotechnology), Rhodamine RedX or Cy5-conjugated donkey antibodies against mouse, rabbit, or goat IgG (1:500; Jackson ImmunoResearch, West Grove, PA), AlexaFluor488-conjugated goat anti-mouse IgG antibody (1:500; Invitrogen), AlexaFluor488-conjugated donkey anti-mouse IgG antibody (1:200; Invitrogen and Jackson ImmunoResearch), AlexaFluor555-conjugated phalloidin (1:50; Invitrogen), ER-Tracker Blue-White DPX (Invitrogen), LysoTracker Red DND-99 (Invitrogen), Alexa488-conjugated transferrin (Invitrogen), DAPI or Sytox Green (Invitrogen) for nuclear staining. Confocal images were obtained with an Olympus IX81 fluorescence microscope equipped with the Fluoview FV1000 confocal system (Olympus, Tokyo, Japan). Phase contrast images were obtained with Nikon Eclipse TS100 (Tokyo, Japan) and Olympus E-330 digital camera. Images were processed using FV10-ASW1.6 (Olympus) or Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

### **Immunoblotting**

Protein concentrations of homogenates were determined by Bio-Rad protein assay (Hercules, CA). Homogenates of C2C12 cells (30 µg/lane), TA muscle (50 µg/lane) and brain (5 µg/lane) were run on 13% polyacrylamide gels,

and transferred onto PVDF membranes. Antibodies used were as follows: mouse anti-VAMP2 antibody (1:5000, clone 69.1; Synaptic Systems), mouse anti-desmin antibody (1:500, clone DE-R-11; Leica Microsystems, Newcastle upon Tyne, UK), rabbit anti-GAPDH antibody (1:2000, clone 14C10; Cell Signaling Technology, Danvers, MA), HRP-conjugated donkey F(ab')<sub>2</sub> fragments against mouse or rabbit IgG (1:100000; Jackson ImmunoResearch). Chemiluminescence (Immobilon Western HRP substrate; Millipore, Billerica, MA) was detected with cooled CCD camera (QICAM, Qimaging, Surrey, BC, Canada) or X-ray film.

## **III. Results**

### ***VAMP2 in isolated satellite cells***

Expression of VAMP2 in isolated satellite cells was analyzed by immunofluorescence microscopy (Fig. 1). Pax3/7 and Myf-5 were detected simultaneously to define quiescent satellite cells and activated satellite cells, respectively [20]. Isolated mouse myofibers were fixed within 2 hr of sacrifice to observe satellite cells in the near-quiescent state [6]. VAMP2 was expressed in Pax3/7-positive quiescent satellite cells and the perinuclear region of myofibers (Fig. 1A). Forty-eight hours after isolation, activated and proliferating satellite cells were observed as Myf-5-positive and Pax3/7-negative cells. VAMP2 was downregulated in these activated satellite cells (arrowheads in Fig. 1B). Six days after isolation, satellite cells migrated from myofibers and some fused to form myotubes. VAMP2 reappeared in myotubes and mononuclear cells nearby (arrows in Fig. 1C). Myf-5-positive proliferating cells were weakly positive for VAMP2 (arrowheads in Fig. 1C).

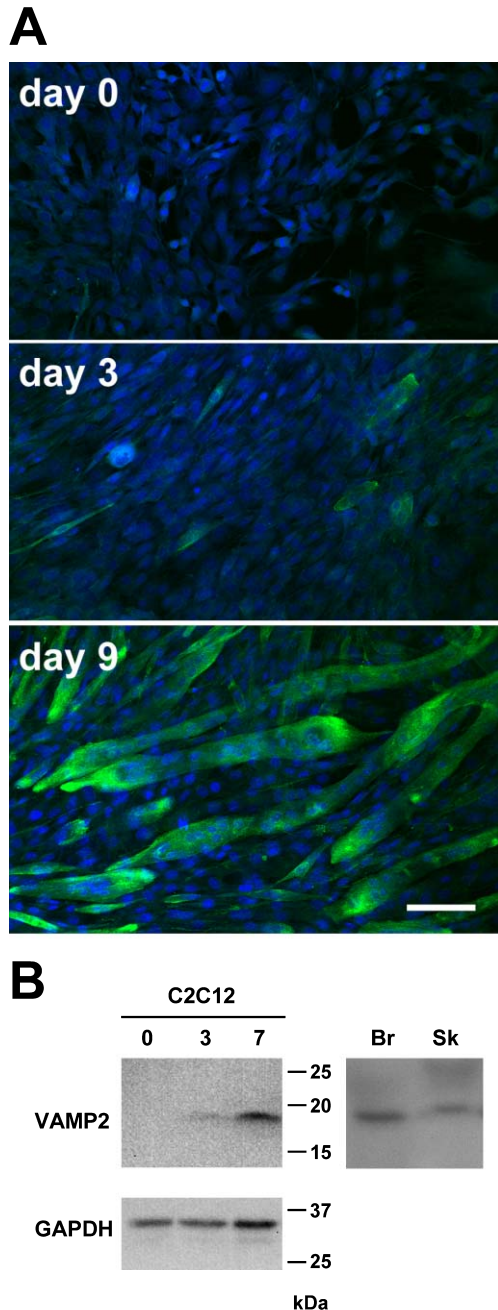
### ***VAMP2 in C2C12 cells***

For detailed analysis of the expression and intracellular localization of VAMP2, we used the myoblastic cell line C2C12. VAMP2 in C2C12 cells was detected by immunofluorescence microscopy (Fig. 2A). Before the differentiation (day 0), the levels of signals corresponding to VAMP2 were very low. On day 3 of differentiation, VAMP2 was found in a few cells. On day 9, VAMP2 was abundant in myotubes, and was localized mainly at the periphery of the cytoplasm. Increases in VAMP2 level after differentiation were also confirmed by immunoblotting (Fig. 2B).

### ***Onset of reappearance of VAMP2 during muscle differentiation***

To determine the stage of differentiation when VAMP2 expression appears, we compared the expression profiles of myogenic marker proteins and VAMP2 in C2C12 cells (Fig. 3). Double immunostainings were performed on day 2 and 3 of differentiation when VAMP2-positive cells were initially observed. Myf-5 and MyoD, myogenic transcription factors, mark specific phase of cell cycle with mutually exclusive expression pattern, *i.e.*, high MyoD and low Myf-5 levels indicate G1, where cells exit from cell cycle to enter

into differentiation [11, 12]. VAMP2-positive cells were negative or weakly positive for Myf-5 (arrows in Fig. 3A), and were positive for MyoD (arrows in Fig. 3B). MyHC is a marker for cells undergoing terminal differentiation [5].



**Fig. 2.** Expression of VAMP2 in C2C12 cells. (A) Immunostaining of C2C12 cells on day 0, 3, and 9 after differentiation. VAMP2 (green) was found in a few cells on day 3, and myotubes on day 9. Nuclear DNA stained with DAPI is shown in blue. Bar=100  $\mu$ m. (B) Immunoblotting showing the increase of VAMP2 during differentiation. VAMP2, bands of 18 kDa, were seen in the brain (Br, 5  $\mu$ g/lane), skeletal muscle (Sk, 50  $\mu$ g/lane), and C2C12 cells on days 3 and 7 after differentiation (30  $\mu$ g/lane). GAPDH was used as a loading control.

MyHC-positive cells initially appeared on day 2, and were negative for VAMP2 (arrowheads in Fig. 3C). On day 3, VAMP2 was detected in cells that were strongly positive for MyHC (arrow in Fig. 3C). These observations indicated that VAMP2 appeared in cells that exited from the cell cycle and entered into terminal differentiation.

#### *Intracellular localization of VAMP2 in myotubes*

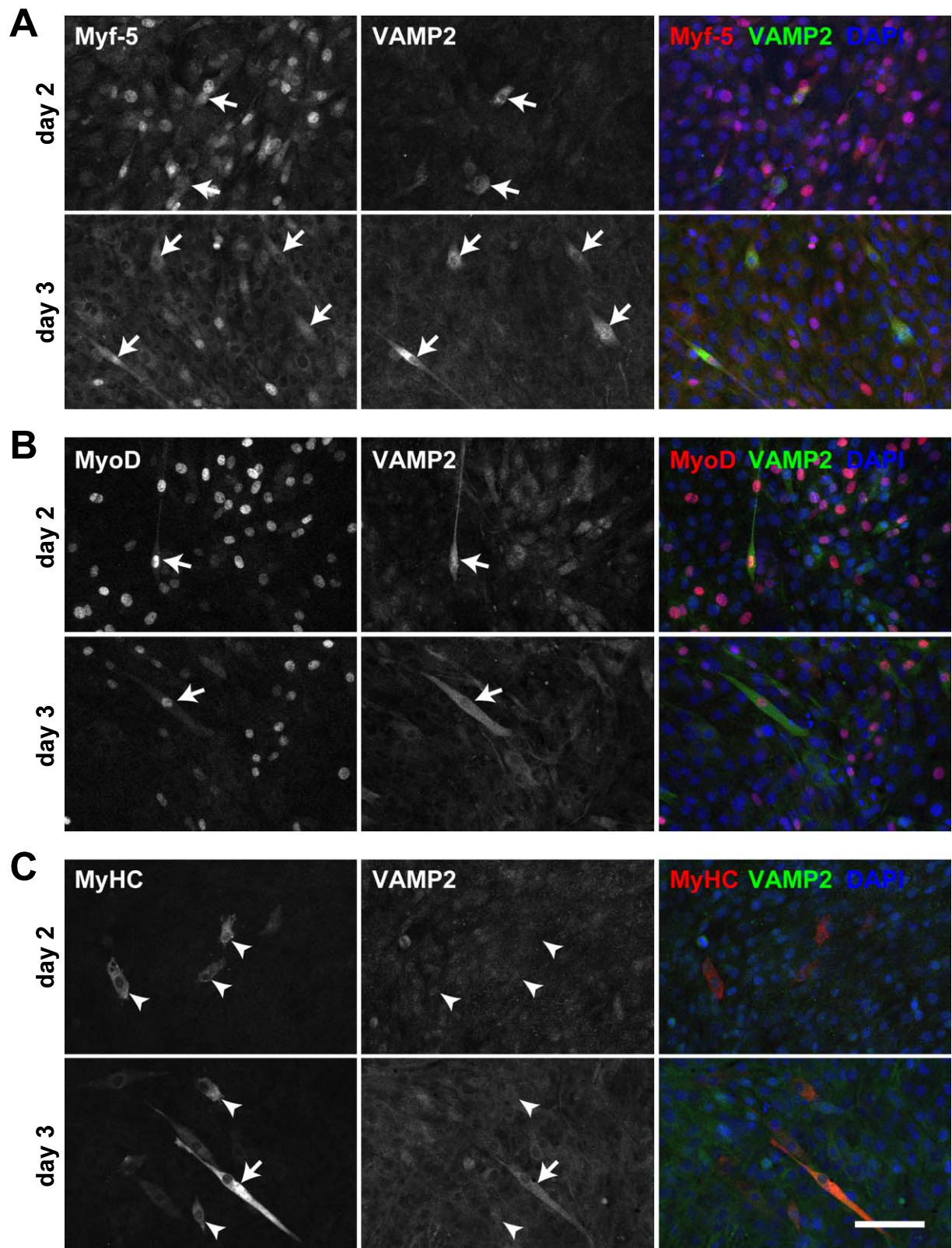
To characterize the intracellular vesicles bearing VAMP2, we performed double labeling using antibodies and reagents recognizing various cytoplasmic organelles (Fig. 4). Endoplasmic reticulum (ER, Fig. 4A) and lysosomes (Fig. 4B) were detected widely throughout the cytoplasm except in the peripheral region, and were not colocalized with VAMP2. The trans-Golgi network (TGN, Fig. 4C) was seen as tubular and dotted staining around the nuclei, and the Golgi apparatus (Fig. 4D) showed a similar staining pattern to the TGN, but was closer to the nuclei. These observations indicated that the TGN and Golgi apparatus were not colocalized with VAMP2. Recycling endosomes were labeled by uptake of transferrin (Fig. 4E) or immunostaining for Rab11 (Fig. 4F). VAMP2 was partially colocalized with these recycling endosomes (arrows in Fig. 4E, F). Early endosomes labeled by EEA1 were relatively larger vesicles, and were localized close to the VAMP2-positive vesicles (Fig. 4G). These observations suggested that VAMP2 may play roles in the trafficking of recycling endosomes in myotubes.

#### *siRNA-mediated knockdown of VAMP2 does not affect differentiation of C2C12 cells*

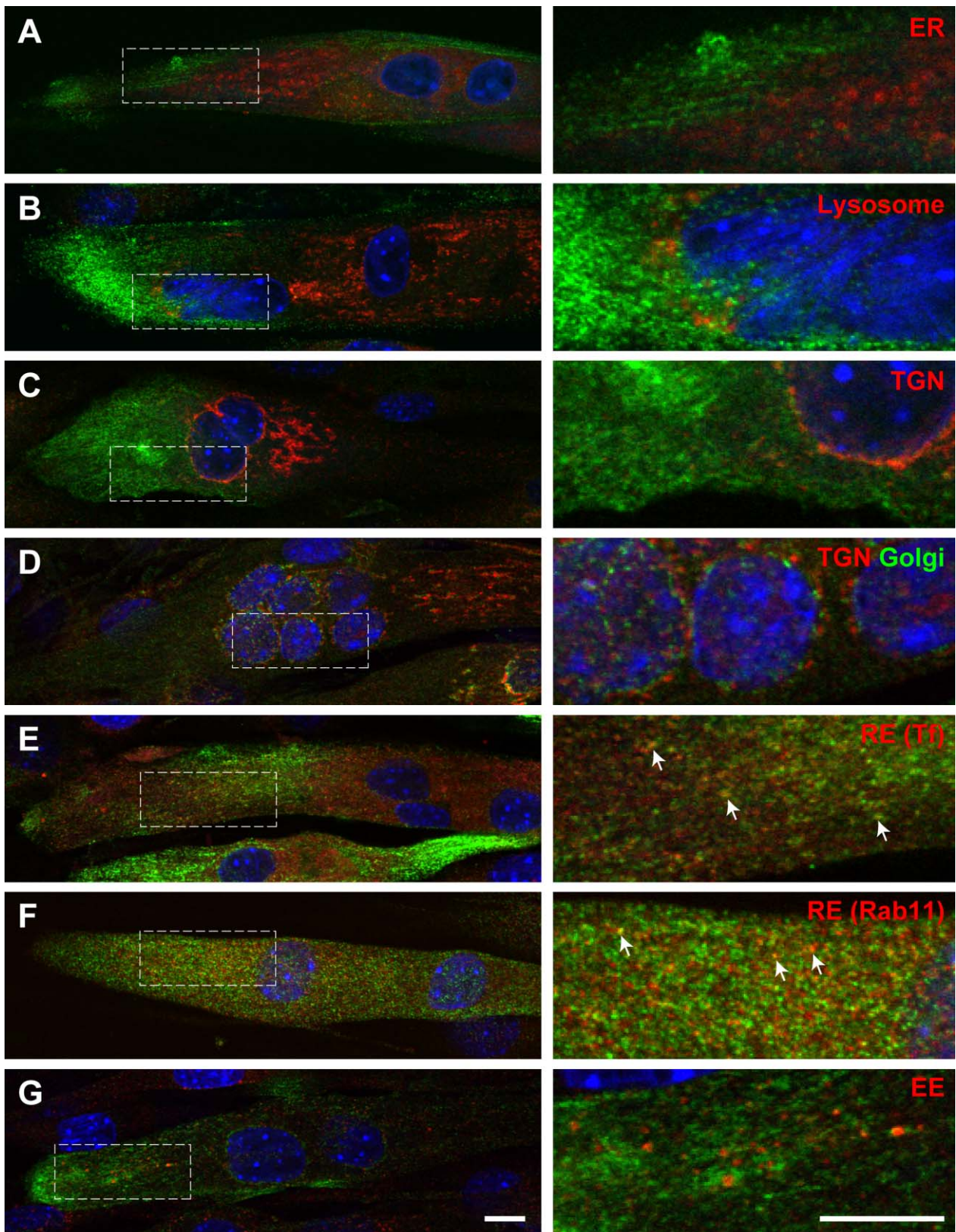
To explore the role of VAMP2 in muscle differentiation, we analyzed the effect of siRNA knockdown of VAMP2 in C2C12 cells (Fig. 5). Differentiation of C2C12 cells was evaluated by the expression of desmin, a muscle-specific intermediate filament, and the appearance of myotubes. Transfection of gene-specific siRNAs successfully decreased the level of VAMP2, as confirmed by western blotting (Fig. 5A) and immunofluorescence microscopy (Fig. 5B, upper row). Regardless of knockdown of VAMP2, the expression of desmin was unchanged (Fig. 5A), and myotubes were appeared (Fig. 5B, lower row). These findings indicate that VAMP2 is not essential for the development and survival of myotubes.

## IV. Discussion

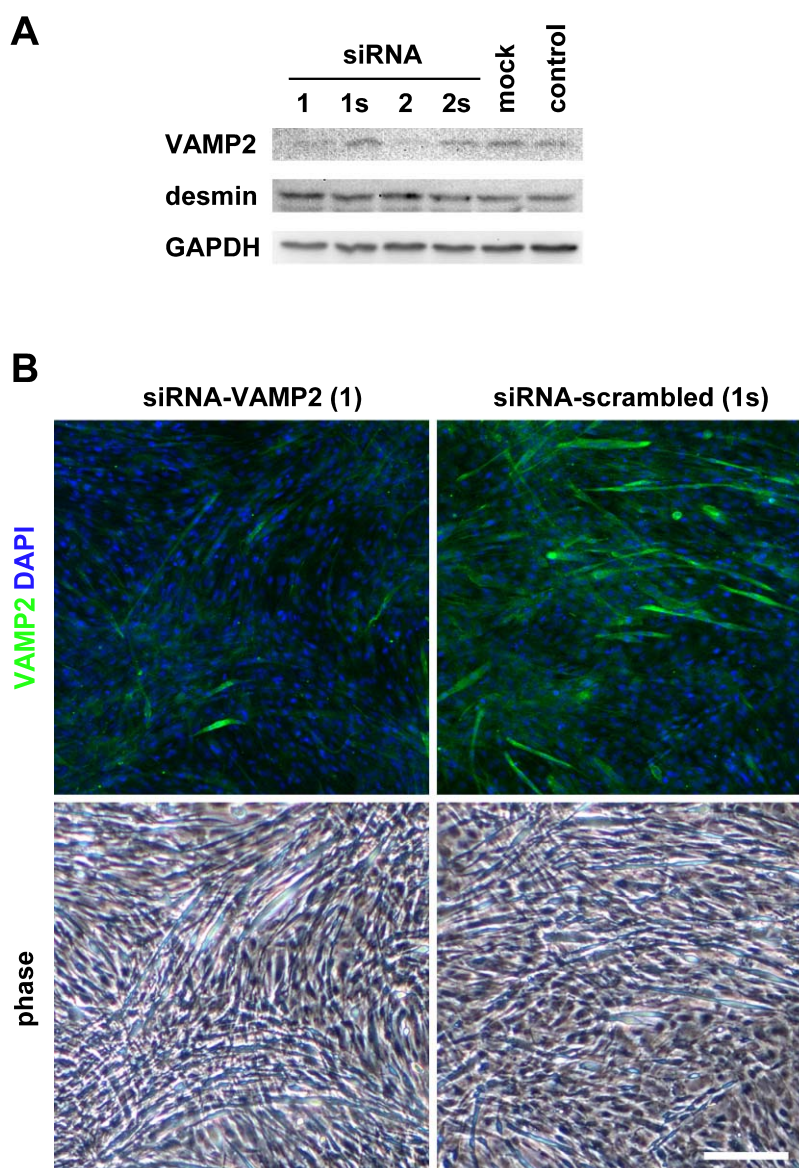
The observed expression of VAMP2 in quiescent satellite cells and myotubes is consistent with the findings of previous studies in adult muscle [16]. In the present study, we used isolated satellite cells and C2C12 cells in culture, and found that VAMP2 is downregulated in proliferating myoblasts, and reappears upon differentiation. A number of molecular markers for quiescent satellite cells are available, including Pax7, CD34, M-cadherin, and others (reviewed in [3, 8]). These markers show reduction of expression upon muscle differentiation. In contrast, VAMP2 is a unique



**Fig. 3.** Comparison of the expression of VAMP2 with myogenic marker proteins: (A) Myf-5, (B) MyoD, and (C) MyHC. Myf-5, MyoD and MyHC are shown in the left panel, and VAMP2 is shown in the center panel. Merged images in the right panel show Myf-5, MyoD or MyHC (red), VAMP2 (green), and nucleus (blue). Arrows show VAMP2-positive cells, which are negative or weakly positive for Myf-5 (A), positive for MyoD (B), and positive for MyHC (C). Arrowheads show VAMP2-negative and MyHC-positive cells (B). Multiple confocal images with intervals of 2  $\mu\text{m}$  were projected onto single images. Bar=100  $\mu\text{m}$ .



**Fig. 4.** Comparison of the localization of VAMP2 with organelles: (A) ER, (B) lysosomes, (C, D) TGN and Golgi apparatus, (E, F) recycling endosomes, and (G) early endosomes. VAMP2 are shown in green (A–C, E–G), and organelle markers are shown in red (A–C, E–G) or green (D). Nuclear DNA is shown in blue. Arrows show VAMP2 colocalized with transferrin- or Rab11-labeled recycling endosomes. Boxed areas in the left column are magnified and shown in the right column. Single optical sections are shown. Bars=10  $\mu$ m.



**Fig. 5.** Knockdown of VAMP2 by RNA interference during the differentiation of C2C12 cells. Transfection of siRNAs were performed on days 0 and 3 of differentiation. **(A)** Western blot shows levels of VAMP2 protein on day 5. Gene-specific siRNAs (1, 2) reduced levels of VAMP2 protein. In contrast, scrambled siRNAs (1s, 2s) or mock treatment (mock) resulted in no change of VAMP2 levels as compared with untreated control (control). The expression of desmin was unaffected by knockdown of VAMP2. GAPDH was detected as a loading control. **(B)** Immunofluorescence and phase contrast images of cells on day 6. Signals of VAMP2 (green in upper row) were reduced by gene-specific siRNA transfection (left) as compared with scrambled siRNA transfection (right). Myotubes were formed, regardless of siRNA treatment (lower row). Bar=200  $\mu$ m.

molecular marker that shows a biphasic expression pattern, and may be applied for staging of differentiation and tracing of myoblasts.

VAMP2 in myotubes localized mainly in peripheral cytoplasm, and is partially colocalized with transferrin and Rab11. These findings suggest the possible function of VAMP2 in the trafficking of recycling endosomes in myotubes. On the other hand, the function of VAMP2 in quiescent satellite cells is not clear. Membrane vesicles in satellite cells were pointed out by the detailed morphological

studies using electron microscope ([10, 13, 15], reviewed in [2]); however, there are no reports on the characterization and function of these vesicles. Our findings that satellite cells express VAMP2 may be a first step toward understanding the role of membrane vesicles in quiescent satellite cells.

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