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Expression and Roles of *AMIGO* Gene Family in Vascular Endothelial Cells

[1]Sharmin Hossain, [2]Minhaz Uddin Ahmed, [1]Shamiul Alam, [1]Akuo Watanabe,
[1]Ai Harashima, [3]Hideto Yonekura and [1]Hiroshi Yamamoto
[1]Department of Biochemistry, Molecular Biology and Vascular Medicine,
Graduate School of Medical Science, Kanazawa University,
13-1 Takara-Machi, 920-8640 Kanazawa, Japan
[2]Institut National De La Recherche Scientifique, 1650, Boul. Lionel-Boulet,
Varennes-Montreal, Quebec, J3X 1S2, Canada
[3]Department of Biochemistry, Kanazawa Medical University School of Medicine,
Uchinada, Ishikawa, Japan

Abstract: In the study, researchers have developed shRNA expression vector and determined their RNAi activity. The Endothelial Cells (ECs) were transfected with the *Amphoterin*-induced gene and ORF (AMIGO-2) expression vector or AMIGO-2 shRNA expression vector using Hemagglutinating Virus of Japan (HVJ) envelope vector and then, the cells were assayed for AMIGO-2 mRNA levels and its survival. In the study, researchers performed expression of *AMIGO* gene family in primary cultured human vascular cells and found predominant expression on Human Microvascular Endothelial Cells (HMVEC). The expression of *AMIGO-2* gene in HMVEC under hypoxia showed *AMIGO-2* gene decreased significantly, suggested that AMIGO-2 may be involved in vascular remodeling. Based on the study of AMIGO-2 down regulation and over expression showed the down regulation appeared to cause cell death of ECs and over expression appeared to protect EC death due to reactive oxygen species. Finally, the study suggest that AMIGO-2 may have an important role in the vascular system as a cell survival promoting factor for vascular ECs, probably being involved in vascular development, angiogenesis and/or vascular remodeling.

Key words: AMIGO, microvascular endothelial cell, RNA, remodeling, death

INTRODUCTION

AMIGO (Amphoterin-induced gene and ORF) was initially identified based on differential display analysis of rat hippocampal neurons as an Amphoterin-induced gene Kuja-Panula *et al.* (2003). Amphoterin (or High-Mobility Group Box 1 protein, HMGB1) is a neurite outgrowth promoting factor acting through binding to a cell surface receptor, RAGE (Receptor for Advanced Glycation Endproducts). Three AMIGOs (AMIGO-1, 2 and 3) constitute a novel family of type I transmembrane proteins with six Leucine-Rich Repeats (LRRs) and a single Immunoglobin (IG) C2-like domain located next to the transmembrane segment Kuja-Panula *et al.* (2003) (Fig. 1). LRRs are solenoid-type motifs present in a number of proteins with diverse functions and cellular locations Kobe (2001). The LRRs are generally 20-29 amino acids in length which contain a conserved sequence of LxxLxLxxN/CxL (where x can be any amino acid and L could also be replaced by V, I or F). The LRRs are protein-protein interaction motifs and are found in a large number of proteins Kobe (2001). Some LRR-containing plasma membrane proteins are expressed almost exclusively in the brain, implying specific functions in the central nervous system.

Expression analyses indicate that AMIGO-1 is specifically detected in axonal fibers and tracts in the brain. AMIGO-2 and AMIGO-3 expressions are more widespread but are also brain-enriched. Members of the AMIGO family exhibit both homophilic and heterophilic binding which suggest that they function as novel cell adhesion molecules in neurons. Immobilized ectodomain of AMIGO-1 promoted neurite extension of cultured hippocampal neurons but when added to the medium, the same soluble AMIGO-1 inhibited fasciculation of neurites. Recent genetic insights show that blood vessels and nerves have much more in common than were anticipated Carmeliet (2003). They use similar signals and principles to differentiate, grow and navigate towards their targets. Moreover, the vascular and nervous systems cross-talk

Corresponding Author: Sharmin Hossain, Department of Biochemistry, Molecular Biology and Vascular Medicine, Kanazawa University, Ishikawa, Japan

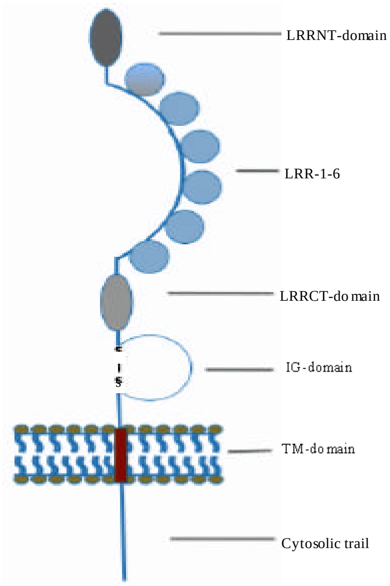

Fig. 1: Schematic representation of the *AMIGO* gene family protein. AMIGO family proteins are type I membrane proteins with LRRs and a domain usually associated with cell adhesion molecule. AMIGO-1, 2 and 3 have 6 LRR repeats flanked by an N-Terminal (LRRNT) and a C-Terminal (LRRCT) LRR-like cap. Furthermore, they harbor a single C2-type Immunoglobin (IG)-like domain

deregulation may contribute to medically important diseases. This prompted me to examine the expression and function of the *AMIGO* gene family in human vascular cells.

MATERIALS AND METHODS

Preparation of cells: Human microvascular Endothelial Cells (ECs) were isolated from neonatal dermis (Cascade Biologics Inc., Portland, OR, USA) and were maintained in a Hu-Media MV2 medium supplemented with 5% (v/v) of Fetal Bovine Serum (FBS), 5 ng mL^{-1} of basic fibroblast growth factor, 10 μg mL^{-1} of heparin, 10 ng mL^{-1} of epidermal growth factor, 1 μg mL^{-1} of cortisol and 39.3 μg mL^{-1} of dibutyryl cAMP according to the manufacturer's protocols (Kurabo Corp., Osaka, Japan). Human microvascular pericytes from cerebral cortex were collected from Applied Cell Biology Research Institute (Kirkland, WA, USA) and maintained in a CS-C complete medium (Cell Systems Corp., Kirkland, WA, USA) according to the manufacturer's instructions (Dainippon Pharmaceutical Co., Osaka, Japan). The cells at 5-10 passages were used for the experiments.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) of amigo family 1-3: Total RNA and poly $(A)^+$RNA were isolated from ECs and pericytes with the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and a QuickPrep micro mRNA purification kit (Amersham Biosciences), respectively and then analyzed with a superscript onestep RT-PCR kit (Invitrogen, Carlsbad, CA, U.S.A.). The primers for detecting mRNA coding for AMIGO-1 were 5'-caccatgacaccctcaacaca-3' and 5'-cacaccacaat-gggcgtat ca-3' (Nucleotide residues 1446-1466 and 1811-1831), respectively available on Gene bank with the accession number of NM02703. The primers for detecting the AMIGO-2 mRNA were 5'-tcccatgctcatgaggcatt-3' (Forward) and 5'-atttccccctcgtggacttt-3' (Reverse) (Nucleotide residues 1636-1655 and 1959-1978), respectively available on Gene bank with the accession number of NM181847. The primers for detecting AMIGO-3 mRNA were 5'-gctttcaacacaggcttcacca-3' (Forward) and 5'-cctccagggttgtagagatcga-3' (Reverse) (Nucleotide residues 1363-1384 and 1652-1673), respectively available on in Gene bank with the accession number of NM198722. The primers for detecting amphoterin mRNA were 5'-agccgag aggcaaaatgtca-3' (forward) and 5'-tcatcttcct-cctcctcctca-3' (Reverse). The primers for detecting β-actin mRNA were as described previously by Nomura *et al.* (1995). The amounts of RNA templates and cycle numbers for amplification were chosen in quantitative ranges in which reactions proceeded linearly Nomura *et al.* (1995), Yonekura *et al.* (1999). An aliquot of each RT-PCR reaction mixture was electrophoresed on 2% agarose gel and stained with ethidium bromide.

AGE exposure and hypoxic culture on *AMIGO-2* gene expression: Subconfluent cultures of human microvascular EC in the medium lacking epidermal growth factor and cortisol were exposed for 6 or 24 h to Glyceraldehyde-derived (Gcer) or Glycolaldehyde (Gcol)-derived AGE-BSA Yonekura *et al.* (2003) at a final concentration of 200 or 500 μg mL^{-1}. Then, after washing with cold PBS, cells underwent to RNA isolation. The cultures of cells under low oxygen tensions were performed as described by Nomura *et al.* (1995) and Yonekura *et al.* (1999). Briefly, ECs in the medium lacking epidermal growth factor and cortisol in a flask were placed in a controlled atmosphere culture chamber (Bellco, Vineland, NJ) which was a humidified airtight incubation apparatus with the in and out-flow valves onto which a gas mixture containing 5% CO_2 and 0, 5 or 20% O_2 balanced with N_2 was flushed for about 5 min at a flow rate of 10 L min^{-1}. The chamber was sealed to maintain a constant gas composition and kept at 37°C during the experiment.

Construction of expression vectors: AMIGO-2 cDNA was amplified with 5'-tcccatgctcatgaggcatt-3' and 5'-atttccccc tcgtggacttt-3' using EC RNA and TaKaRa High Fidelity RNA PCR kit (Takara, Otsu, Japan), the underlines indicate EcoRI and XbaI sites and the double underline indicates additional sequence that encodes the FLAG-tagged sequence. The amplified cDNA was digested with EcoRI and XbaI and inserted into a mammalian expression vector pCI-neo (Promega, Madison, WI, USA) that had been digested with the same restriction enzymes. Recombinant plasmid DNA was purified with a plasmid isolation kit (QIAGEN, Valencia, CA, USA) and the sequence was verified.

Construction of shRNA expression vectors against AMIGO-2 mRNA and assay for their RNAi activity: Hairpin siRNA template oligonucleotides for shRNA expression vectors were designed at B-Bridge International Inc (Sunnyvale, CA, USA). The hairpin siRNA template oligonucleotides were dissolved in nuclease-free water and diluted to 1 μg μL^{-1}. Then, 2 μL each of sense and antisense siRNA template oligonucleotides and 46 μL of 1×DNA annealing solution (20 mM Tris-HCl, pH 7.0 and 100 mM NaCl) were mixed, heated to 90°C for 3 min, chilled to 37°C and finally incubated for 1 h.

The annealed siRNA template inserts were ligated into a pSilencer vector (Ambion Inc., Austin, TX, USA) which had been digested with BamHI and HindIII. Recombinant plasmid DNAs were purified with a Plasmid isolation kit (QIAGEN, Valencia, CA, USA) and the sequence was verified. The HEK293T cells were co-transfected with the pSilencer vector and a GFP-AMIGO-2 fusion protein expression vector (pEGFP-AMIGO-2) and the levels of fusion protein were examined by Western blotting with an anti-GFP antibody.

Transformation of ECs: ECs were transfected with the AMIGO-2 expression vector or AMIGO-2 shRNA expression vector using HVJ Envelope Vector with a kit purchased from Ishihara Sangyo (Osaka, Japan) Kaneda (2003). HVJ envelope vector/DNA complex was prepared according to the supplier's instructions. ECs in 24 well plate (5×10^4 cells/well) received 8 μL of the HVJ-E (N) containing or not containing the expression vector (6 μg) and incubated for 10 min at 37°C. After transfection, the medium was changed to fresh medium and the cells were further incubated at 37°C for 24 h. After incubation, the transfected cells were assayed for AMIGO-2 mRNA levels and survival.

Cell survival assay: After washed with PBS, cells were fixed and stained with 0.1% crystal violet in 3.7% formaldehyde and extensively washed with water. After air dry, stained cultures were photographed and the extent of stain was quantified with H_2O_2.

RESULTS AND DISCUSSION

Expression of *AMIGO* gene family in primary cultured human vascular cells: As shown in Fig. 2a, RT-PCR analysis demonstrated the presence of not only amphoterin (HMGB1) but also AMIGO-1, AMIGO-2 and AMIGO-3 in primary cultured Human Microvascular Endothelial Cells (HMVEC). RT-PCR with AMIGO-1, 2 and 3 specific primers gave signals at 386, 343 and 311 bp, respectively exactly the same size as expected. Among them, AMIGO-2 was predominantly expressed in HMVEC. In human brain pericytes, all the AMIGO family members were detected at a similar level.

Effect of AGE on *AMIGO-2* gene expression: The *AMIGO* genes were also expressed in the human umbilical vain EC-derived cell line ECV304 as shown in Fig. 3a.

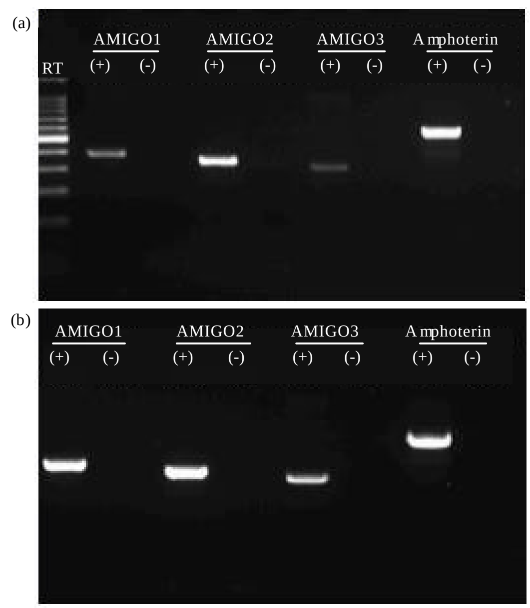

Fig. 2: Expression of the *AMIGO* gene family in human microvascular Endothelial Cells (ECs) (a) and human brain capillary pericytes (b). Poly (A) $^+$RNAs were isolated from primary cultured human dermal microvascular ECs or human brain pericytes and analyzed by RT-PCR with a superscript III 1-step RT-PCR kit (Invitrogen). The amounts of poly(A)$^+$RNA templates and cycle numbers for amplification were 50 ng of templates and 30 cycles. About 10 μL aliquots of each RT-PCR reaction mixture were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Signals were visualized on an Epilight EP-250 (Aishin Cosmos Co. Ltd)

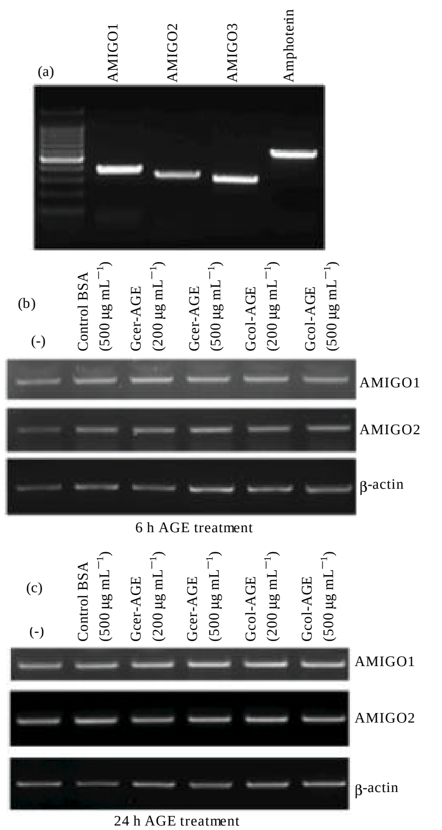

Fig. 3: Effects of AGE on the expression of the *AMIGO* gene family in ECV304 cells; a) RT-PCR detection of mRNAs for AMIGO-1, 2 and 3 and amphoterin in ECV304 cells. The amounts of templates and cycle numbers for amplification were 200 ng of total RNA and 35 cycles. About 10 μL aliquots of each RT-PCR reaction mixture were electrophoresed on a 2% agarose gel and stained with ethidium bromide; b and c) AGE effects on AMIGO-2 mRNA levels. ECV304 cells in 6-well plates were starved in a serum-free medium for 15 h and then treated with Glyceraldehyde-derived (Gcer) or Glycolaldehyde (Gcol)-derived AGE-BSA (200 or 500 μg mL^{-1}) for 6 h (b) or 24 h (c). Total RNAs were isolated after the treatments and analyzed by RT-PCR. About 160 ng of templates and 25 thermal cycles were employed for AMIGO-1 and AMIGO-2 mRNA detections; 160 ng and 20 cycles were for β-actin mRNA

Effect of AGE on *AMIGO-2* gene expression: The *AMIGO* genes were also expressed in the human umbilical vain EC-derived cell line ECV304 as shown in Fig. 3a. AMIGO-1 expression has been reported to be induced by amphoterin (HMGB1) through binding to a cell surface receptor, RAGE (Receptor for Advanced Glycation End products). Researchers thus examined changes of AMIGO-1 and AMIGO-2 mRNA levels by AGE, the representative ligand for RAGE. ECV cells were incubated in the presence of Glyceraldehyde-derived (Gcer) or Glycolaldehyde (Gcol)-derived AGE-BSA. After 6 or 24 h incubation, the expression levels of AMIGO-1 and AMIGO-2 mRNAs were examined by RT-PCR. As shown in (Fig. 3b, c) their expressions were not significantly affected by the treatment with AGEs.

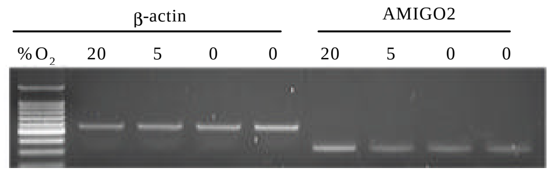

Fig. 4: Effect of hypoxia on the expression of *AMIGO-2* gene in ECs. Poly(A)$^+$RNAs from HMVEC cultured under the indicated oxygen tensions were analyzed by RT-PCR with primers specific to AMIGO-2 mRNA. The amounts of poly (A)$^+$RNA templates and cycle numbers for amplification were 20 ng and 25 cycles for AMIGO-2 and 20 ng and 18 cycles for β-actin, respectively. An aliquot of each RT-PCR reaction mixture was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide

Effect of hypoxia on *AMIGO-2* gene expression: It has been reported that production of VEGF is induced in ECs under hypoxia, the principal cause of angiogenesis, resulting in the promotion of EC growth and tube formation (Nomura *et al*., 1995; Yonekura *et al*., 1999). On the other hand, hypoxia induced a down-regulation of endostatin, a potent angiogenesis inhibitor in HMVEC Wu *et al*. (2001). Researchers next examined the expression of AMIGO-2 in HMVEC under hypoxia. As shown in Fig. 4, the level of AMIGO-2 mRNA decreased significantly as the atmospheric O_2 concentration decreased to 5 and 0%. The result suggested that AMIGO-2 might be involved in vascular remodeling induced by hypoxia.

Establishment of RNAi system targeted to human AMIGO-2: For construction of shRNA expression vectors, 5 pairs of complementary oligonucleotides with restriction sites at 5' and 3' termini for ligation into the pSilencer vector were prepared. The oligonucleotides contained a unique 19 nucleotide sequence derived from the target mRNA (Fig. 5), a loop sequence, an antisense complement of the 19 nucleotide sequence and the T5 sequence to terminate transcription. These forward and reverse oligonucleotides were annealed and cloned into the pSilencer vector restricted. The resultant transcript was predicted to form the 19 base pair stem-loop structure and the stem-loop precursor transcript was expected to

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   1 atgtcgttac gtgtacacac tctgcccacc ctgcttggag ccgtcgtcag accgggctgc
  61 agggagctgc tgtgtttgct gatgatcaca gtgactgtgg gccctggtgc ctctggggtg
 121 tgccccaccg cttgcatctg tgccactgac atcgtcagct gcaccaacaa aaacctgtcc
 181 aaggtgcctg ggaacctttt cagactgatt aagagactgg acctgagtta taacagaatt
                                                      (No.1)
 241 gggcttctgg attctgagtg gattccagta tcgtttgcaa agctgaacac cctaattctt
 301 cgtcataaca acatcaccag catttccacg ggcagttttt ccacaactcc aaatttgaag
 361 tgtcttgact tatcgtccaa taagctgaag acggtgaaaa atgctgtatt ccaagagttg
 421 aaggttctgg aagtgcttct gctttacaac aatcacatat cctatctcga tccttcagcg
 481 tttggagggc tctcccagtt gcagaaactc tacttaagtg gaaatttttct cacacagttt
 541 ccgatggatt tgtatgttgg aaggttcaag ctggcagaac tgatgttttt agatgtttct
 601 tataaccgaa ttccttccat gccaatgcac cacataaatt tagtgccagg aaaacagctg
 661 agaggcatct accttcatgg aaacccattt gtctgtgact gttccctgta ctccttgctg
                 (No.2)
 721 gtcttttggt atcgtaggca ctttagctca gtgatggatt ttaagaacga ttacacctgt
 781 cgcctgtggt ctgactccag gcactcgcgt caggtacttc tgctccagga tagctttatg
 841 aattgctctg acagcatcat caatggttcc tttcgtgcgc ttggctttat tcatgaggct
 901 caggtcgggg aaagactgat ggtccactgt gacagcaaga caggtaatgc aaatacggat
                                                   (No.3)
961 ttcatctggg tgggtccaga taacagactg ctagagccgg ataaagagat ggaaaacttt
1021 tacgtgtttc acaatggaag tctggttata gaaagccctc gttttgagga tgctggagtg
                           (No.4)
1081 tattcttgta tcgcaatgaa taagcaacgc ctgttaaatg aaactgtgga cgtcacaata
1141 aatgtgagca atttcactgt aagcagatcc catgctcatg aggcatttaa cacagctttt
1201 accactcttg ctgcttgcgt ggccagtatc gttttggtac ttttgtacct ctatctgact
1261 ccatgcccct gcaagtgtaa aaccaagaga cagaaaaata tgctacacca aagcaatgcc
1321 cattcatcga ttctcagtcc tggccccgct agtgatgcct ccgctgatga acggaaggca
                                                              (No.5)
1381 ggtgcaggta aaagagtggt gtttttggaa cccctgaagg atactgcagc agggcagaac
1441 gggaaagtca ggctctttcc cagcgaggca gtgatagctg agggcatcct aaagtccacg
1501 aggggggaaat ctgactcaga ttcagtcaat tcagtgtttt ctgacacacc ttttgtggcg
1561 tccacttaa
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Fig. 5: Establishment of RNAi system targeted to human AMIGO-2. Nucleotide sequence of AMGO-2 cDNA and RNAi target sites. Translational initiation and stop codons and RNAi target sequences are indicated by underlines

quickly cleave in the cell by Dicer to yield functional siRNAs. Five pSilencer plasmid vectors were prepared and their RNAi activities were examined. Figure 6 shows the RNAi activity of each construct against human AMIGO-2 mRNA. Four out of the five constructs (No.1-4) showed significant RNAi activity. They gave >90% suppression.

Effect of AMIGO-2 down regulation and overexpression: Figure 7a shows crystal violet stain of HMVEC transfected with AMIGO-2 shRNA vector, AMIGO-2 overexpression vector and their controls. Blue stain that represents viable cells was significantly decreased in AMIGO-2 shRNA vector-transfected cells compared with negative control shRNA vector-transfected cells. The recovery of RNA was also decreased in AMIGO2 shRNA-treated cells compared with the control (Fig. 7b) but with similar AMIGO-2 mRNA levels per μg poly(A)$^+$RNA (Fig. 7c). Over expression AMIGO-2 gave essentially no change in crystal violet stain or RNA content in comparison with the control transfected with vector alone (Fig. 7a, b). However, AMIGO-2 high expressions exhibited significant resistance to H_2O_2-induced decreased in crystal violet stain (Fig. 8).

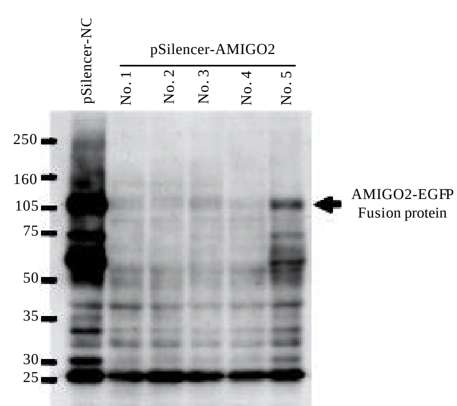

Fig. 6: Establishment of RNAi system targeted to human AMIGO-2 activity of the pSilencer-AMIGO-2 vectors. HEK 293T cells were co-transfected with the indicated pSilencer vector and a pEGFP-human AMIGO-2 vector. AMIGO2-GFP fusion protein levels were examined by Western blotting with an anti-GFP antibody. pSilencer-NC, pSilencer vector with a sequence that has no predict matches to known human genes

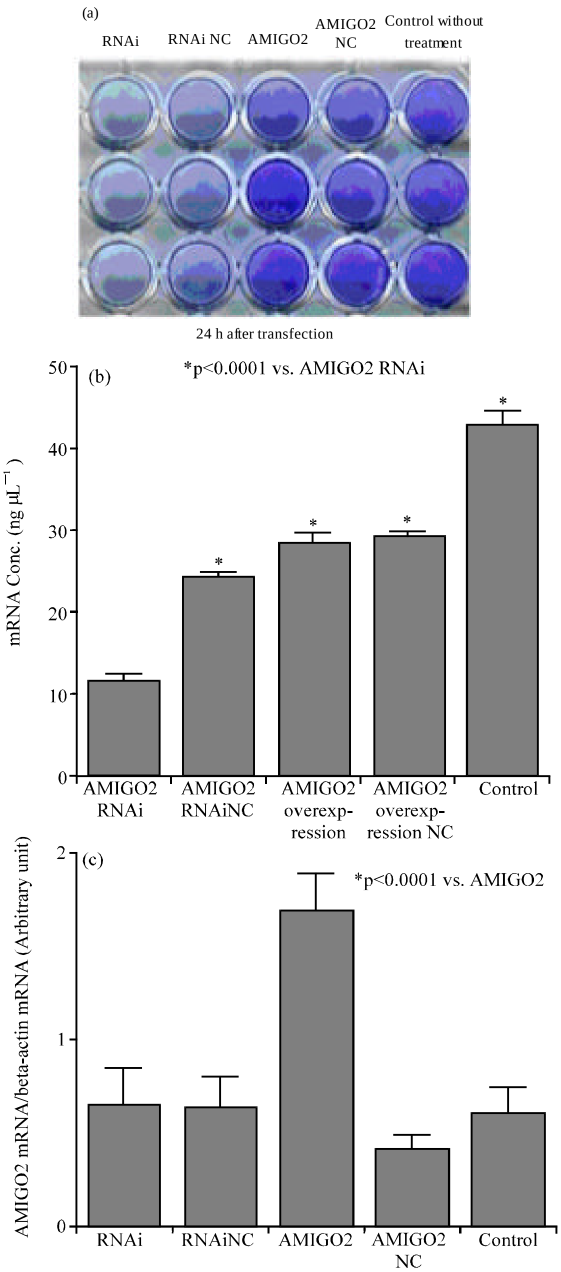

Fig. 7: Knockdown of AMIGO-2 accelerated transfection-induced cell death of human ECs. a) Crystal violet stain. NC; Negative Control. b) Recovery of poly(A)$^+$RNA. c) AMIGO-2 mRNA levels

AMIGO was first isolated from rat hippocampal neurons as a gene that was induced by the interaction between amphoterin and RAGE and was reported to be involved in neurite outgrowth Kuja-Panula *et al.* (2003). In the present study, researchers demonstrated for the first time that AMIGO family genes were expressed in primary cultured human microvasucular ECs and pericytes.

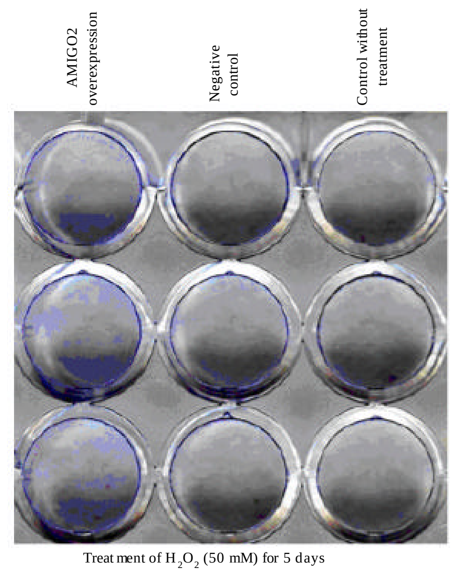

Fig. 8: Over expression of AMIGO-2 inhibited endothelial cell death by hydrogen peroxide. Cells were exposed to 50 mM H_2O_2 for 5 days and viability was assessed by crystal violet stain

Among the three members, AMIGO-2 expression predominated in ECs and in an EC-derived cell line ECV304 (Fig. 2 and 3). This study also demonstrated for the first time that hypoxia, a principle cause of angiogenesis, suppressed *AMIGO-2* gene expression in EC (Fig. 4). These results suggest that AMIGO-2 can be involved in hypoxia-induced angiogenesis and vascular remodeling. Members of the AMIGO/Alivin have been shown to interact homotypically and heterotypically with each other Kuja-Panula *et al.* (2003). This suggests that they act as cell adhesion molecules and are involved in EC-EC, EC-pericyte or vascular cell-neuronal cell interactions.

AMIGO-2 was independently identified by a different group using differential display screening for genes involved in depolarization and NMDA-dependent survival of cerebellar granule neurons and named alivin-1 (ali1) Ono *at al.* (2003). Over expression of alivin-1 in cerebellar granule neurons inhibited apoptosis induced by a low (5 mM) KCl medium. Both anti-alivin-1 antiserum and a soluble alivin-1 attenuated the survival of granule neurons in a prosurvival high (25 mM) KCl culture. These results suggest that AMIGO-2/alivin-1 has a role as a cell survival-promoting factor for neuronal cells. AMIGO-2 was also independently identified as a gene preferentially expressed in human gastric adenocarcinomas; its higher expression in tumoral tissues than normal counterparts was noted as frequently as approximately 45% of gastric cancer patients Rabenau *et al.* (2004). Knockdown of

AMIGO-2 by a stable expression of an anti-sense construct in a gastric adenocarcinoma cell line led to morphological and genetic changes that were suggestive of a potential etiologic role in gastric carcinogenesis such as promoting the survival of cancer cells. This study also demonstrated that down-regulation of AMIGO-2 appeared to cause cell death of EC (Fig. 7) and that over expression of AMIGO-2 appeared to protect EC death caused by reactive oxygen species (Hydrogen peroxide) (Fig. 7).

CONCLUSION

The study thus suggests that AMIGO-2 may have an important role in the vascular system as a cell survival-promoting factor for vascular ECs, probably being involved in vascular development, angiogenesis and/or vascular remodeling.

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