Accdepted manuscript - postprint version
Ćetković, H., Bosnar, M. H., Perina, D., Mikoč, A., Deželjin, M., Belužić, R., … Harcet, M. (2018). Characterization of a group I Nme protein of Capsaspora owczarzaki—A close unicellular relative of animals. Laboratory Investigation, 98(3), 304–314. https://doi.org/10.1038/labinvest.2017.134

Characterization of a Group I Nme protein of *Capsaspora owczarzaki* – a close unicellular relative of animals

Helena Ćetković[1], Maja Herak Bosnar[2], Drago Perina[1], Andreja Mikoč[1], Martina Deželjin[2], Robert Belužić[2], Helena Bilandžija[1], Inaki Ruiz-Trillo[3,4,5], Matija Harcet*[1,3]

[1]Laboratory of Molecular Genetics, Division of Molecular Biology Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia; [2]Laboratory of Molecular Oncology, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia; [3]Institut de Biologia Evolutiva (CSIC–Universitat Pompeu Fabra), Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain; [4]ICREA, Pg. Lluís Companys 23, 08010 Barcelona, Spain; [5]Departament de Genètica, Microbiologia i Estadística, Universitat de Barcelona, Avinguda Diagonal 643, 08028 Barcelona, Spain

*Corresponding author
Matija Harcet
Laboratory of Molecular Genetics
Division of Molecular Biology
Ruđer Bošković Institute
Bijenicka cesta 54, P.O.Box 180
10002 Zagreb, Croatia
+385 1 456 1115
email: mharcet@irb.hr

Abstract

Nucleoside diphosphate kinases are enzymes present in all domains of life. In animals, they are called Nme or Nm23 proteins, and are divided into Group I and II. Human Nme1 was the first protein identified as a metastasis suppressor. Due to its medical importance, it has been extensively studied. In spite of the large research effort, the exact mechanism of metastasis suppression remains unclear. It is unknown which of the biochemical properties or biological functions are responsible for the antimetastatic role of the mammalian Nme1. Furthermore, it is not clear at which point in the evolution of life Group I Nme proteins acquired the potential to suppress metastasis, a process that is usually associated with complex animals. In this study we performed a series of tests and assays on a Group I Nme protein from filasterean *Capsaspora owczarzaki*, a close unicellular relative of animals. The aim was to compare the protein to the well-known human Nme1 and Nme2 homologues, as well as with the homologue from a simple animal – sponge (Porifera), in order to see how the proteins changed with the transition to multicellularity, and subsequently in the evolution of complex animals. We found that premetazoan-type protein is highly similar to the homologues from sponge and human, in terms of biochemical characteristics and potential biological functions. Like the human Nme1 and Nme2, it is able to diminish the migratory potential of human cancer cells in culture.

Introduction

The appearance of multicellularity is directly connected to the origin of cancer. Cancer can be viewed as a collapse of the cooperation within the multicellular organism and the proliferation of selfish cell lines (1) Even some of the simplest "early branching" non-bilaterian animals, such as cnidarians, develop tumors (2). Tumors have also been identified in other invertebrates, for example flatworms, mollusks and insects (reviewed in (1)). Metastatic potential is the property that has the highest influence on the lethality and prognosis of cancer. Metastasis is a complex process. Cancer cells first need to detach from the primary site, pass through the basement membrane and the surrounding stroma, enter blood or lymphatic vessels, survive in the circulation, arrest and leave the vessel, and invade an unrelated tissue. Therein, the cells have to adapt to the new surroundings, multiply and grow their own blood supply. Genes whose proper function inhibits metastasis are called metastasis suppressors. Different (although overlapping)

sets of metastasis suppressors are involved in each step of the metastatic process. One of the crucial events that occur during metastasis is the change in tumor cell adhesion properties. Precisely regulated cell adhesion is also one of the essential elements for the evolution of complex multicellularity. Some metastasis suppressors are directly or indirectly involved in cell-cell or cell-ECM adhesion and others modulate the function of adhesion molecules (Ćetkovic et *al*, this issue). Therefore, the same molecular interactions are crucial for both, the origin of multicellular organization and the metastatic processes, and they include genes that are in mammals recognized as metastasis suppressors.

The human Nme1 is the first known and the most studied metastasis suppressor (3). Nme (nucleoside diphosphate kinases - NDPKs, Nm23 proteins) is a family of evolutionary conserved proteins present in all domains of life (4). Their canonical role is the maintenance of NTP pool in the cell by phosphorylation of NDPs, although many other functions of this protein have been described. In animals, Nme proteins are divided in two distinct groups. Non-bilaterian animals have one or two Group I Nme proteins, which have further diversified after the appearance of vertebrates (5). Group II contains members have more ancient origins and homologues present in unicellular eukaryotes. Ten Nme proteins have been identified in human: Nme1-4 belong to Group I, and Nme5-9 to Group II. Human Nme10, aka XRP2, has only a partial NDPK domain and does not belong to either group (6).

Decreased expression of Nme1 has been linked to metastatic phenotype of several tumor types. Human Nme2 is highly homologous to human Nme1, but has different pI value (7). These two proteins form enzymatically active hetero- or homohexamer in any possible combination of subunits (6Nme1, 5Nme1+1Nme2,… 6Nme2). Besides diphosphate kinase activity, Nme1 and Nme2 also act as histidine kinases (8, 9), 3'-5' exonucleases (10), and transcriptional regulators (11). As expected for an enzyme with basic and diverse functions, mammalian Nme1 is involved in many cellular processes such as proliferation (12), differentiation (13, 14), development (15, 16), apoptosis (17, 18), adhesion, migration (19), and vesicular trafficking (20). In spite of the large effort put in the research on Nme proteins, the mechanism of metastasis suppression is still unknown. Nme1 has been linked with several steps in the metastatic cascade (3), but without clear evidence for the mechanism of its action.

In animals, cell adhesion is achieved through several classes of proteins, including integrins and cadherins. There is evidence suggesting that Nme1 affects cell adhesion in metastasis (19, 21). The mechanisms probably include modulation of cadherins (22) and integrins (23).

Due to large research efforts, we now have considerable knowledge of Nme1 properties in complex animals such as mammals. In an earlier study, we demonstrated that the earliest-branching simple animals – sponges (Porifera) possess an Nme1 homologue, NmeGp1Sd, with biochemical properties very similar to the mammalian homologues. Moreover, we found that the sponge Nme1 (NmeGp1Sd) is recognized by human cells as their own protein, and is able to diminish migratory (metastatic) potential of cancer cells (7). So far the work on Nme1 in unicellular eukaryotes has been very limited with no data available on the properties of Nme homologues in close unicellular relatives of animals. Herein, we present a study of a Group I Nme protein from *Capsaspora owczarzaki* – a close unicellular relative of animals. The aim was to compare the properties of "ancestral-type" protein before the emergence of multicellularity, with the homologues from simple non-bilaterian animals and vertebrates. We were especially interested in investigating whether the premetazoan version of the Group I Nme has properties that would make it a metastasis suppressor.

There are three major lineages of unicellular eukaryotes closely related to animals: Ichtiosporea, Filesterea, and Choanoflegellata (24). *Capsaspora owczarzaki* is one of only two known species in the phylum Filasterea. It was identified as an amoeboid endosymbiont of a pulmonate snail *Biomphalaria glabrata (25, 26)* In laboratory conditions, *C. owczarzaki* has a life cycle consisting of three stages – filopodial (adherent), aggregative (pseudomulticellular) and cystic. Recently sequenced *C. owczarzaki* genome (27) encodes many proteins with functions related multicellularity, previously thought to be metazoan innovations, such as a rich repertoire of transcription factors (28) and tyrosine kinases (29), as well as proteins directly involved in adhesion. *C. owczarzaki* possesses cadherins and all components of the integrin-mediated adhesion and signalling complex, including four integrin-alpha and four integrin-beta homologues (30). Many of the adhesion-related proteins are more highly expressed in aggregative stage, indicating their potential role in adhesion between aggregated *Capsaspora* cells (31). It has been shown that some conserved *C. owczarzaki* proteins can substitute the function of their homologues in arthropods (32) and vertebrates (33).

We showed that *C. owczarzaki* has one typical Group I Nme protein named NmeGp1Co and that the properties of this premetazoan protein are strikingly similar to the human Nme1 and Nme2. NmeGp1Co can diminish the migratory potential of human cancer cells, which strongly suggests that the antimetastatic properties of Group I Nme proteins were present before the appearance of animals, and therefore before the origin of cancer in the animal lineage.

Materials and methods

Sequence analyses

Homology searches and sequence retrievals were done using BLAST (NCBI, NIH, Bethesda, MD, USA: http://www.ncbi.nlm.nih.gov). Protein domains and sites were analysed by Prosite (http://prosite.expasy.org/). Multiple sequence alignments were constructed by clustalX (http://www.clustal.org/).

Statisical analyses

RNAseq data are available from Figshare (https://figshare.com/articles/RNAseq_data_-_Seb_-Pedr_s_et_al_2013_/4585447). We analysed gene expression using previously described methodology (31). Briefly, we employed four different methods (DESeq, EdgeR, CuffDiff and NOISeq) to identify differences in gene expression. Only if a gene was identified by at least three out of four methods, it was considered to be significantly differentially expressed. We used Kruskal-Wallis test with post-hoc test for pairwise comparison of subgroups according to Conover (34) to check whether different Nme proteins have significantly diminished the migratory potential of human cancer cells.

Plasmid constructions

The PCR generated cDNA fragment was cloned into pGEM vector using forward primer 5'-ATGTCGACCGAGCGTAC-3' and the reverse primer 5'-TTAGTTCTCGTAGACCTGGG-3'. The cDNA for NmeGp1Co was recloned into pET15b using PCR into *Nde*I (5′-GTCTAGCATATGTCGACCGAGCGTAC-3′) and *Bam*HI (5′-CTAGACGGATCCTTAGTTCTCGTAGACCTGGGGGCGG -3′) restrictions sites of pET15b expression vector (Novagen) downstream from the thrombin cleavage site. For colocalization

assay, the full-length coding sequence *NmeGp1Co* was cloned in frame into pEGFP-C1 expression vector (Clontech, USA). The fragment of 456 bp was amplified using 5′-GTCTAGCTCGAGTA ATGTCGACCGAGCGTAC-3′ and 5′-CTAGACGAATTCTTATTAGTTCTCGTAGACCTGGGGGCGG -3′ primers. The PCR product was digested with *Xho*I and EcoRI, and ligated with the *Xho*I/*Eco*RI sites of pEGFP-C1 expression vector. The pEGFP-N1-Nme1 was a kind donation of Dr. Marie-Lise Lacombe, INSERM UMRS_938, Paris, France. *NmeGp1Co* cDNA was amplified using a forward primer containing the FLAG sequence (F: 5′-GTCTAGGGATCCACGAGATGGACTACAAGGACGACGACGATAAGATGTCGACCGAG CGTAC -3′) and the reverse primer (R: 5′-CTAGACGAATTCTTATTAGTTCTCGTAGACCTGGGGGCGG-3′). The PCR product was cloned into the *Bam*HI/*Eco*RI sites of eukaryotic expression vector pcDNA3 (Invitrogen).
The pRSET A-Nme2 was kindly donated by Dr Shantanu Chowdhury, Institute of Genomics and Integrative Biology, New Delhi, India.

Protein expression and purification

NmeGp1Co and Nme2 were overproduced in *Escherichia coli* strain BL21 tagged with six histidine residues at the N-terminus and purified to homogeneity from bacterial lysates using nickel affinity chromatography. *E. coli* strain BL21 harbouring the plasmid constructs were grown to 0.85 at OD_{600} and induced with 0.8 mM IPTG for 2.5 hours at 37ºC. Cells were incubated 30 min on ice in lysis buffer (25 mM Tris HCl, 500 mM NaCl, 10 mM imidazole and 1mg/mL lysozyme) and sonicated 8×30 sec (50% of full power). After centrifugation (12000 rpm) for 20 min at 4ºC, the supernatant was applied onto nickel-charged agarose column (Qiagen). Histidine tagged proteins bound to nickel-affinity resins were eluted with 200 mM imidazole. NmeGp1Co and Nme2 proteins were applied to a PD-10 desalting gel filtration column (Sephadex, GE Healthcare) equilibrated with 25 mL of Nme buffer (35) (20 mM HEPES at pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT and 0.1 M KCl) and then eluted with 3.5 mL of Nme buffer.

Protein cross-linking with glutaraldehyde

Oligomerization of the recombinant protein NmeGp1Co was performed in Nme buffer. Reactions contained 14 μg of purified NmeGp1Co protein. In first reaction glutaraldehyde was added to final amount of 0.04%. Reaction was incubated at 25°C for 15 min. Second reaction proceeded with 1.6% glutaraldehyde for 40 min at 37°C. The reaction product was subjected to 12% SDS–PAGE and visualized by protein staining with Coomassie brilliant blue.

Gel filtration chromatography

Recombinant human Nme2 and NmeGp1Co were loaded onto Bio-Sil SEC 250 gel-filtration columns (300 mm×7.8 mm) and eluted with Nme buffer (35) at a flow rate of 0.5 mL/min (BioLogic Duo Flow, BIO-RAD). Peak fractions were used for NDP kinase assay.

DNA-binding assay

The DNA-binding activity of the NmeGp1Co protein was assayed *in vitro* as described (36). Reactions contained 20ng of single-stranded circular DNA from bacteriophage ϕX174 (NEB). The amount of purified NmeGp1Co protein is indicated (Figure 4). The reaction mixture was incubated in 20 μl of buffer containing 50 mM KCl, 10 mM $MgCl_2$, 50 mM Tris HCl pH 7.5, 0.5 mM DTT, 30 μg/mL BSA. . After 30 min of incubation at 37°C products were subjected to 0.8% agarose gel and stained with SYBR gold (Invitrogen).

NDP kinase assay

NDPK activity was measured using a coupled pyruvate kinase-lactate dehydrogenase assay (35, 37). Reaction kinetics was followed by connecting the turnover of ATP to the turnover of NADH to NAD. NADH concentrations were measured by spectrophotometry. Five hundred μL of reaction mixtures were incubated in quartz cuvettes at room temperature in the presence of ATP as phosphate donor and dTDP as phosphate acceptor. The final concentrations were as follows: 50 mM Tris-HCl pH 7.4, 75 mM KCl, 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.1 mg/mL NADH, 1 mM ATP, 0.2 mM dTDP, 2 U/mL of pyruvate kinase, 2.5 U/mL of lactate dehydrogenase, and 1 mg/mL bovine serum albumin. All reagents and enzymes were purchased from Sigma. Reactions were initiated by the addition of 50 ng of NmeGp1Co enzyme and activity was monitored in an Ultrospec® 2100 *pro* (Amersham Pharmacia Biotech, USA)

measuring the decrease in absorbance at 340 nm. The control reactions omitting enzyme produced minor rates. Human Nme2 was used as control. All reactions were done in triplicate.

Plasmid cleavage assays

Cloning of pUC19 plasmid containing 57-bp NHE (nuclease hypersensitive element) of the *c-myc* promoter was described previously (7). Recombinant plasmid pUC19-NHE (50ng) and empty pUC19 was incubated with NmeGp1Co protein as indicated (Figure 4). The reaction was assembled in 50 mM KCl, 10 mM $MgCl_2$, 50 mM Tris HCl pH 7.5, 0.5 mM DTT, 30 μg/mL BSA and incubated 30 min at 37ºC. The reaction was terminated with 1% SDS, 10 mM EDTA and proteinase K treatment (200 μg/mL) for 30 min at 55ºC. Topoisomerase I (Gibco BRL) and Nme2 proteins were used as controls and were processed in the same conditions. Samples were analyzed in 0.8% agarose gel in TAE buffer and then stained with SYBR gold (Invitrogen).

Cell culture

Human HeLa cells (ATCC) and MDA-MB-231T (donated by Dr. Patricia S. Steeg, Center for Cancer Research, National Cancer Institute, USA; for reference Palmieri et al., J. Natl. Cancer Inst.2005, 97, 632-42) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogene) supplemented with 10% fetal bovine serum (FBS, Invitrogene), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in humidified chamber with 5% CO_2 at 37ºC.

Transient transfections and laser scanning confocal microscopy

Twenty four hours before transfection 3×10^4 HeLa cells were seeded on circular coverslips placed in 24-well dishes to obtain 60% confluence. Each well was transfected with 1μg of plasmid DNA: pyRedC1-Nme1, pyRedC1-Nme2, or pEGFPC1-NmeGp1Co or cotransfected with of pyRedC1-Nme1 and pEGFPC1-NmeGp1Co and/or pyRedC1-Nme2 and pEGFPC1-NmeGp1Co (0.5 μg of each construct). The transfections were perfomed using Lipofectamine reagent (Invitrogene) according to manufacturer's instructions. Forty-eight hours post transfection the cells were washed with PBS (phosphate-buffered saline, pH 7.5), fixed in 4% formaldehyde, and mounted in mounting medium (DAKO) supplemented with 1μg/mL 4, 6-diamino-2-phenylindole (DAPI), (Sigma) for nuclear staining.

Fluorescent images were obtained by Leica TCS SP2 AOBS laser scanning confocal microscope equipped with HCX PL APO λ-Blue 63x1.4 objective. GFP was excited by 488 nm laser line, DsRed using 543 nm, and DAPI using 405 nm laser line. The digital images were assembled using Adobe Photoshop software.

Preparation of stably transfected MDA-MB-231T clones

Stably transfected cell lines were prepared as in (38). In brief, MDA-MB-231T cells were transfected using Lipofectamine reagent (Invitrogen) with pcDNA3FLAG-Nme1, pcDNA3MYC-Nme2, pcDNA3FLAG-NmeGp1Co and pcDNA3 as control. Post transfection the cells were resuspended and incubated in DMEM supplemented with geneticin (Sigma) until development of resistant colonies. Positive clones were screened by Western blotting, propagated and frozen until further usage.

Immunoprecipitation and Western blotting

For detection of stably transfected MDA-MB-231T (control), two selected clones expressing the Capsospora variant of the NmeGp1 protein (Co 2 and Co 9) were seeded in 100mm Petri dishes, washed 2 times in PBS, harvested in lysis buffer (20 mM Tris, pH7,6-8, 150 mM NaCl, 1%Triton, 1mM EDTA) supplemented with protease inhibitors and degraded by sonication. The immunoprecipitation was done with either anti-Nme1 antibody (Calbiochem) using Dynabeads, or with anti-FLAG M2 affinity gel (Sigma) according to manufacturer's instructions. Immunoprecipitation with IgG (Sigma) was used as a negative control.

The samples were loaded on SDS-PAGE and electrotransfered to an Imobilon-PSQ membrane (Milipore). The membranes were incubated with anti-FLAG M2 antibody (Sigma) of affinity purified polyclonal anti-Nme1 antibody for detection of complex formation. Protein bands were visualized using Western Lightning Plus-ECL (PerkinElmer, Inc.). The images were acquired by Alliance 4.7 (Uvitec) and assembled in Adobe Photoshop.

Migration assay

For migration assay $3x10^5$ cells were seeded on 60 mm Petri dishes in DMEM supplemented with 10% fetal bovine serum. After 24 hours the cells were starved in serum free medium for another 24 hours. Further, the cells were centrifuged and washed with DMEM supplemented with 0,1%

BSA. $2x10^4$ cells were placed in to the upper chamber of Cell Culture Inserts (Beckton-Dickinson) and allowed to settle down for 20 minutes. DMEM supplemented with 1% FBS served as a chemoattractant and was added to the lower chamber. The cells were allowed to migrate for 5 hours, after which the medium was removed, non-migratory cells were detached with cotton swabs while membranes with migrated cells were washed twice in PBS, and fixed in 4% formaldehyde for 15 minutes at room temperature. The cells were stained with 0.1% crystal violet, cut out from the inserts, mounted in mounting medium (DAKO), analyzed by light microscopy, and photographed. The cells from four representative images of every sample were assembled in Adobe Photoshop and counted. The experiments were preformed three times.

Results

C. owczarzaki has one canonical and one atypical Group I Nme gene

Genomic and transcriptomic data show that *Capsaspora owczarzaki* has two Group I Nme genes. Both proteins have a standard architecture of the group, i.e. only one NDPK domain (marked in Figure 1a) and no other protein domains. One of them encodes a 152 amino acids long typical Group I Nme protein with a conserved NDPK active site. Protein sequence is deposited in NCBI's GenBank under accession number XP_004365544. According to the accepted nomenclature, the protein encoded by this gene is named NmeGp1Co. The other gene encodes a 243 amino acids long protein. Its sequence is deposited in GenBank with accession number XP_004349949. Significantly bigger size compared to other NmeGp1 homologues is due to the addition of approximately 90 amino acid residues at the N-terminus. The N-terminus has no homology with NDPKs or with any other protein in the NCBI's GenBank, and contains no recognisable domains. Although the remaining part of the protein shows high homology with other Group I Nme proteins, it does not have a conserved NDPK active site (boxed in Figure 1a), and therefore probably has no NDPK activity. As this homologue clearly belongs to Group I Nme proteins, but also has unique characteristics, we named it NmeGp1Co-like.

We checked the position of both genes in the genome of *C. owczarzaki* and found that they are not tandem duplicates. We also found that the synteny in not conserved between *C. owczarzaki*, sponge *Amphimedon queenslandica* and human. The phylogenetic trees of Group I Nme proteins

that we constructed in an attempt gain insight in their evolutionary history at the origin of animals were uninformative, with little congruence and almost no statistical support for the branches of interest. Therefore, we aligned the NDPK domains of the two *C. owczarzaki* proteins, sponge NmeGp1Sd, and human Nme1 and Nme2 proteins and checked the percentage of conserved (identical or similar) amino acid residues (Table 1). In general, NDPK domains are relatively conserved, with a notable exception of the NmeGp1Co-like protein, which is the most diverged compared to all the other analysed proteins.

We checked the expression levels of the two proteins in different stages of the *C. owczarzaki* life cycle (Figure 1b) using the transcriptomics data analysed earlier (31). NmeGpICo is significantly more expressed in the filopodial stage compared to the cystic stage, while NmeGpICo-like is significantly less expressed in the cystic stage compared to the other two stages.

According to its sequence, especially due to the conserved NDPK active site, we concluded that NmeGpICo is a true nucleoside-diphosphate kinase with other typical functions of Group I Nme proteins in the cell. Therefore, we focused our work on this Nme homologue.

Biochemical characteristics of the NmeGp1Co protein

In order to test the biochemical properties of NmeGp1Co, the protein was produced in *E. coli* and purified. The NDP kinase assay (35, 37) was used to determine NmeGp1Co enzymatic activity. The determined *C.owczarzaki* NmeGp1Co kinase activity of 254,37 U/mg was within the previously reported ranges for the human protein (10, 39)

Various oligomeric structures, such as hexamers, tetramers, dimers, and monomers, were found after cross-linking the NmeGp1Co protein with glutaraldehyde. Recombinant NmeGp1Co is predominantly in the hexameric form when incubated in 1.6% glutaraldehyde for 40 min at 37°C (Figure 2a). The hexameric form was expected due to the conserved primary protein sequence elements, KPN loop (residues 93 to 112 in NmeGp1Co) and residues Lys 30 (40) and Ser 119 (41), known to be crucial for hexamer formation (Figure 1). The structure of *C. owczarzaki* NmeGp1Co enzyme was determined by gel filtration (Figure 2B). The previously characterised human Nme2 (42) was used as marker. Overlapping gel filtration peaks confirm that NmeGp1Co protein has a hexameric structure like the human Nme2.

NmeGp1Co protein binds non-specifically to single-stranded circular DNA (sscDNA). Binding activity was observed with concentration of 100 ng, manifested by DNA band retardation (Figure

3). Human Nme1 did not display the ability to bind sscDNA, while human Nme2 binds sscDNA (7).

Nme2 is involved in DNA structural changes necessary for the activity of the c-*myc* promoter. It binds to the NHE sequence of the *c-myc* promoter cloned into pUC19 which yields mostly nicked circular plasmid (43). Our experiments showed that the NmeGp1Co does not have DNA topoisomerase-like activity, contrary to the human Nme2. To test this activity, NmeGp1Co protein was incubated with supercoiled pUC19 plasmid containing the 57-bp *c-myc* NHE sequence. Topoisomerase I and Nme2 proteins were used as controls. Control topoisomerase I cleaved negatively supercoiled plasmid DNA. Human Nme2 specifically cleaved NHE sequence cloned into pUC19 unlike NmeGp1Co which did not display this activity (Figure 4A). However, the experiment showed that NmeGp1Co non-specifically binds double-stranded circular DNA (Figure 4B), unlike Nme2 which does not display this activity.

NmeGp1Co behaves like human Nme1/Nme2 in the human cell

Along with studying biochemical properties of NmeGp1Co, we wanted to determine if the human cell recognises the Capsaspora protein as its own. In order to check the co-localisation of NmeGp1Co with human Nme1 and Nme2, we co-expressed the fluorescently-labelled proteins in human HeLa cell line. The colocalization experiments (Figure 5) shows that both human Nme proteins colocalize with the *C. owczarzaki* homologue. The colocalization is seen in the cytoplasm and in the nucleus.

Next, we wanted to test if NmeGp1Co forms complexes with the human Nme1 protein in live human cells. For this purpose several MDA-MB-231T clones stably expressing FLAGNmeGp1Co were formed. Two clones were chosen for further analysis (Co2 and Co9). To test the possible interactions of NmeGp1Co and human Nme1 protein, FLAG or Nme1 was immunoprecipitated from cell lysates of FLAGNmeGp1Co expressing clones, and with anti-FLAG M2 affinity gel. The results of the Western blot analysis with anti-Nme1 and anti-FLAG antibody reveal that FLAG/NmeGp1Co forms complexes with the endogenous Nme1 (Figure 6). In the precipitate of human protein, the Capsaspora protein was co-precipitated and vice-versa. Therefore, we showed that the human Nme1 recognizes the NmeGp1Co homologue as a partner for complex formation.

Finally, we checked whether NmeGp1Co can influence the migration of human cancer cells. We tested the migration potential of MDA-MB-231T cells overexpressing the FLAG/NmeGp1Co (clone Co2), and compared them with the human MDA-MB-231T cells overexpressing Nme1 and Nme2 that are known to diminished migratory potential of cancer cells (44). We found that that NmeGp1Co expressing clone, as well as human Nme1 and Nme2 overexpressing clones have substantially diminished the migratory potential of MDA-MB-231T, and same result was obtained in three independent experiments (Figure 7).). According to Kruskal-Wallis test, this result was statistically significant (P= 0.009). On the contrary, the differences among clones overexpressing Nme1, Nme2 and NmeGp1Co were not statistically significant. Therefore, NmeGp1Co has the ability to suppress the motility of the tested cells.

Discussion

The main goal of this study was to characterise a Group I Nme protein from *Capsaspora owczarzaki*, a close unicellular relative of animals, in order to better understand the evolution of Nme proteins and their functions, especially at the origin of animals. Comparison with the previous study of a Group I Nme protein from a sponge, a simple non-bilaterian animal (7, 45), and research on mammalian (mostly human) Group I Nme proteins provide a better insight into changes that occurred in transition to multicellularity and subsequently in the animal lineage.

Evolution and diversification of Group I Nme proteins at the dawn of the animals is difficult to reconstruct. *Capsaspora owczarzaki*, a close relative of animals analysed herein, has two Group I Nme genes. The same is true for different unicellular eukaryotes (46). We checked the available genomes of Choanoflagellates – the closest unicellular relatives of animals, and found that they have only one Group I Nme gene. Among the early-branching metazoan lineages the presence of NmeGp1 genes varies. Demosponges and placozoans have one NmeGp1 homolog, while cnidarians and ctenophores have two (47). Reconstructing the state in the last common ancestor of animals with any certainty is problematic for two reasons. Firstly, the deep phylogeny of animals is unresolved. Currently, there are two competing theories placing either sponges (48) or ctenophores (49) as the earliest-branching lineage. Secondly, the sequences of Nme genes and proteins often do not contain sufficient phylogenetic signal to reconstruct their evolutionary history at the base of the animal tree. However, upon examining the available evidence it seems more likely that the ancestor of animals had only one Group I Nme gene that subsequently went

through diversification in different animal lineages. Lineage-specific duplications have already been found in animals such as lancelet *Branchiostoma floridae* and sea squirt *Ciona* intestinalis (5), sea anemone(4) and some sponges (45). *C. owczarzaki* also has two Group I Nme proteins that are not tandem duplicates. NmeGpICo is a typical highly conserved member of the group. Our results show that it is a true nucleoside diphosphate kinase similar to the human protein, and that it probably has all standard roles crucial for the survival of the cell. On the other hand, NmeGpICo-like protein has some unusual characteristics: a long insertion at the N-terminus, no NDPK active site, and much less conserved NDPK domain. This points out to an ancient, probably lineage-specific duplication. NmeGpICo kept the original essential function and remains under strict evolutionary constraint, while NmeGpICo-like was able to accumulate more mutations. The function of the NmeGpICo-like protein is currently unknown.

In laboratory culture, *C. owczarzaki* has a life cycle consisting of three stages: filopodial, aggregative and cystic. Each stage has a distinct pattern of gene expression (31). NmeGp1Co is significantly more expressed in the filopodial stage. This is the most proliferative and metabolically active stage, so it is not surprising that an enzyme with basic metabolic functions is highly expressed in filopodial cells. On the other hand, NmeGpICo-like protein is significantly less expressed in the cystic stage, while the levels of expression are similar in the filopodial and aggregative stage. The function of this protein is unknown, but it is highly unlikely that it has a kinase activity due to the mutation in the NDPK active site, and is probably not involved in basic cellular metabolism.

Biochemical characterization of the *C. owczarzaki* NmeGp1Co protein shows that the NmeGp1Co possesses some biochemical characteristics typical for human Nme1 and others typical for human Nme2 protein. NmeGp1Co has properties highly similar to the sponge NmeGp1Sd homologue. As predicted from its primary sequence, recombinant NmeGp1Co appears to be predominantly in the hexameric form like human Nme1/Nme2 (50) and NmeGp1Sd (7). We showed that NmeGp1Co is a nucleoside-diphosphate kinase whose activity is within the previously reported ranges for the human protein (10, 39) and the sponge NmeGp1Sd protein (7).

Regarding its affinity for DNA binding, NmeGp1Co has a unique combination of properties, although it shares most of them with the sponge and human proteins. NmeGp1Co binds non-specifically to single-stranded circular DNA like NmeGp1Sd and human Nme2, but unlike Nme1. Furthermore, NmeGp1Co is not able to cleave *c-myc* NHE sequence, same as sponge

NmeGp1Sd and human Nme1 protein (7). In contrast, human Nme2 is able to cleave the *c-myc* NHE sequence. This property is linked to the structural changes of the promoter and activation of the *c-myc* proto-oncogene (35). Nme1 and Nme2 appeared by cis-duplication in amniotes (5). It seems that, after the duplication event, Nme2 developed this unique regulatory feature. Finally, NmeGp1Co non-specifically binds to double-stranded circular DNA unlike any of the proteins we tested.

In addition to testing the biochemical properties of NmeGp1Co, we wanted to check how it behaves in live mammalian cells. The localisation of fluorescently-labelled NmeGp1Co in the mammalian cell is mostly in the cytoplasm, but also in the nucleus. The granular structures visible in the cytoplasm have already been described accompanying both homologous and heterologous overexpression of Nme proteins (7, 51). It is still not clear whether the granules are part of the cell (e.g. ribosomes of the rough endoplasmatic reticulum) or an artefact of the experiment. NmeGp1Co does not colocalize completely with the human Nme1 and Nme2 homologs while colocalization of the Nme1, Nme2 and sponge NmeGp1Sd was complete (7). This discrepancy could be due to the subtle differences in some of the properties that were not included in our testing. *C. owczarzaki* protein is expressed in a cell of a distantly related species and is possibly not finding or recognising all its usual interaction partners and thus not performing all biological functions and biochemical activities. The disparity may also be due to the fact that different human cell lines were used in the experiments: HEp-2 cells for the sponge homologue and HeLa cells for the *C. owczarzaki* protein. In spite of only partial colocalisation, we found that NmeGp1Co forms complexes with native human Nme1, same as the sponge NmeGp1Sd (7). Finally, we showed that NmeGp1Sd diminishes the migration potential of human cancer cells in culture, same as Nme1/Nme2 and NmeGp1Sd. While it is clear that Nme1 in humans is a metastasis suppressor, the mechanism of suppression is still not understood. Here we show that whichever property or function of Nme1 is responsible for the metastasis suppression, it was present in ancestral-type Group I Nme protein before the origin of animal and the diversification of Nme proteins. It is, therefore, likely that the metastasis suppression property of Nme1 is linked to some of its standard functions in the cell, possibly ones that still haven't been described. This function is probably equally important for unicellular eukaryotes as it is for the animals. However, in the multicellular context, at least in mammals, Nme1 has been co-opted to also function as a metastasis suppressor. Many cancer and disease-related genes have ancient

origins, before the appearance of animals (52, 53). The same is true for metastasis suppressors (Cetkovic et *al*, this issue). Therefore, their functions and mechanisms of action should be viewed in a wider context that takes into account homologues from simple unicellular organisms and also vast interaction networks within and between the cells of highly complex animals.

To conclude, this study showed that NmeGp1Co, a Group I Nme protein from filasterean *Capsaspora owczarzaki*, a close unicellular relative of animals, has properties strikingly similar to the human Nme1 and Nme2 proteins in terms of primary sequence, biochemical characteristics and functions in the cell. The protein did not change significantly in the transition to multicellularity. It is recognised as a native protein by human cancer cells and it diminishes their migratory potential. This means that even before the appearance of animals, the ancestral Group I Nme homolog was a versatile protein with characteristics and functions similar to the human Nme1.

Aknowledgement

We are grateful to Arnau Sebe-Pedros for his help in analysing and interpreting the RNAseq data and to Petar Ozretić for helping us with statistical analysis of the migration assays.

This work was funded by Croatian MSES grants 098-0982913-2478 to H. Ć. and 098-09824642513 to M. H.-B., and the Croatian Science Foundation project IP-2016-06-4021 to M. H.-B.

M. H. received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 330925.

I. R.-T. is supported by a grant from Ministerio de Economía y Competitividad (MINECO) (BFU2014-57779-P) and a European Research Council Consolidator Grant (ERC2012Co616960) to I.R.-T., the former co-funded by the European Regional Development Fund. We acknowledge support from Secretaria d'Universitats i Recerca de la Generalitat de Catalunya (Project 2014 SGR 619).

References

1. Aktipis CA, Boddy AM, Jansen G, Hibner U, Hochberg ME, Maley CC, et al. Cancer across the tree of life: cooperation and cheating in multicellularity. Philos T R Soc B. 2015;370(1673).

2. Domazet-Loso T, Klimovich A, Anokhin B, Anton-Erxleben F, Hamm MJ, Lange C, et al. Naturally occurring tumours in the basal metazoan Hydra. Nat Commun. 2014;5.

3. Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, et al. Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst. 1988;80(3):200 - 4.

4. Bilitou A, Watson J, Gartner A, Ohnuma S. The NM23 family in development. Mol Cell Biochem. 2009;329(1-2):17 - 33.

5. Desvignes T, Pontarotti P, Fauvel C, Bobe J. Nme protein family evolutionary history, a vertebrate perspective. BMC Evol Biol. 2009;9:256.

6. Desvignes T, Pontarotti P, Bobe J. Nme Gene Family Evolutionary History Reveals Pre-Metazoan Origins and High Conservation between Humans and the Sea Anemone, Nematostella vectensis. Plos One. 2010;5(11).

7. Perina D, Bosnar M, Bago R, Mikoc A, Harcet M, Dezeljin M, et al. Sponge non-metastatic Group I Nme gene/protein - structure and function is conserved from sponges to humans. BMC Evolutionary Biology. 2011;11(1):87.

8. Wagner PD, Vu ND. Phosphorylation of ATP-citrate lyase by nucleoside diphosphate kinase. J Biol Chem. 1995;270(37):21758 - 64.

9. Wagner PD, Vu ND. Histidine to aspartate phosphotransferase activity of nm23 proteins: phosphorylation of aldolase C on Asp-319. Biochem J. 2000;346 Pt 3:623-30.

10. Ma D, McCorkle JR, Kaetzel DM. The metastasis suppressor NM23-H1 possesses 3'-5' exonuclease activity. J Biol Chem. 2004;279(17):18073 - 84.

11. Postel EH. Multiple biochemical activities of NM23/NDP kinase in gene regulation. J Bioenerg Biomembr. 2003;35(1):31 - 40.

12. Lombardi D, Lacombe ML, Paggi MG. nm23: unraveling its biological function in cell differentiation. J Cell Physiol. 2000;182(2):144 - 9.

13. Lombardi D, Palescandolo E, Giordano A, Paggi MG. Interplay between the antimetastatic nm23 and the retinoblastoma-related Rb2/p130 genes in promoting neuronal differentiation of PC12 cells. Cell Death Differ. 2001;8(5):470 - 6.

14. Gervasi F, D'Agnano I, Vossio S, Zupi G, Sacchi A, Lombardi D. nm23 influences proliferation and differentiation of PC12 cells in response to nerve growth factor. Cell Growth Differ. 1996;7(12):1689 - 95.

15. Lakso M, Steeg PS, Westphal H. Embryonic expression of nm23 during mouse organogenesis. Cell Growth Differ. 1992;3(12):873 - 9.

16. Amrein L, Barraud P, Daniel JY, Perel Y, Landry M. Expression patterns of nm23 genes during mouse organogenesis. Cell Tissue Res. 2005;322(3):365 - 78.

17. Kang Y, Lee DC, Han J, Yoon S, Won M, Yeom JH, et al. NM23-H2 involves in negative regulation of Diva and Bcl2L10 in apoptosis signaling. Biochem Biophys Res Commun. 2007;359(1):76-82.

18. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell. 2003;112(5):659 - 72.

19. Fournier HN, Albiges-Rizo C, Block MR. New insights into Nm23 control of cell adhesion and migration. J Bioenerg Biomembr. 2003;35(1):81 - 7.

20. Palacios F, Schweitzer JK, Boshans RL, D'Souza-Schorey C. ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat Cell Biol. 2002;4(12):929 - 36.

21. She S, Xu B, He M, Lan X, Wang Q. Nm23-H1 suppresses hepatocarcinoma cell adhesion and migration on fibronectin by modulating glycosylation of integrin beta1. Journal of experimental & clinical cancer research : CR. 2010;29:93.

22. Boissan M, De Wever O, Lizarraga F, Wendum D, Poincloux R, Chignard N, et al. Implication of metastasis suppressor NM23-H1 in maintaining adherens junctions and limiting the invasive potential of human cancer cells. Cancer research. 2010;70(19):7710-22.

23. Fournier HN, Dupe-Manet S, Bouvard D, Lacombe ML, Marie C, Block MR, et al. Integrin cytoplasmic domain-associated protein 1alpha (ICAP-1alpha) interacts directly with the metastasis suppressor nm23-H2, and both proteins are targeted to newly formed cell adhesion sites upon integrin engagement. The Journal of biological chemistry. 2002;277(23):20895-902.

24. Torruella G, de Mendoza A, Grau-Bove X, Anto M, Chaplin MA, del Campo J, et al. Phylogenomics Reveals Convergent Evolution of Lifestyles in Close Relatives of Animals and Fungi. Current biology : CB. 2015;25(18):2404-10.

25. Hertel LA, Bayne CJ, Loker ES. The symbiont Capsaspora owczarzaki, nov. gen. nov. sp., isolated from three strains of the pulmonate snail Biomphalaria glabrata is related to members of the Mesomycetozoea. International journal for parasitology. 2002;32(9):1183-91.

26. Stibbs HH, Owczarzak A, Bayne CJ, DeWan P. Schistosome sporocyst-killing Amoebae isolated from Biomphalaria glabrata. Journal of invertebrate pathology. 1979;33(2):159-70.

27. Suga H, Chen Z, de Mendoza A, Sebe-Pedros A, Brown MW, Kramer E, et al. The Capsaspora genome reveals a complex unicellular prehistory of animals. Nature communications. 2013;4:2325.

28. Sebe-Pedros A, de Mendoza A, Lang BF, Degnan BM, Ruiz-Trillo I. Unexpected repertoire of metazoan transcription factors in the unicellular holozoan Capsaspora owczarzaki. Molecular biology and evolution. 2011;28(3):1241-54.

29. Suga H, Dacre M, de Mendoza A, Shalchian-Tabrizi K, Manning G, Ruiz-Trillo I. Genomic survey of premetazoans shows deep conservation of cytoplasmic tyrosine kinases and multiple radiations of receptor tyrosine kinases. Science signaling. 2012;5(222):ra35.

30. Sebe-Pedros A, Roger AJ, Lang FB, King N, Ruiz-Trillo I. Ancient origin of the integrin-mediated adhesion and signaling machinery. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(22):10142-7.

31. Sebe-Pedros A, Irimia M, Del Campo J, Parra-Acero H, Russ C, Nusbaum C, et al. Regulated aggregative multicellularity in a close unicellular relative of metazoa. eLife. 2013;2:e01287.

32. Sebe-Pedros A, Zheng Y, Ruiz-Trillo I, Pan D. Premetazoan origin of the hippo signaling pathway. Cell reports. 2012;1(1):13-20.

33. Sebe-Pedros A, Ariza-Cosano A, Weirauch MT, Leininger S, Yang A, Torruella G, et al. Early evolution of the T-box transcription factor family. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(40):16050-5.

34. Conover WJ. Practical nonparametric statistics. 3rd ed. New York: John Wiley; 1999. viii, 584 s. p.

35. Postel EH, Ferrone CA. Nucleoside diphosphate kinase enzyme activity of NM23-H2/PuF is not required for its DNA binding and in vitro transcriptional functions. J Biol Chem. 1994;269(12):8627 - 30.

36. Galvao CW, Pedrosa FO, Souza EM, Yates MG, Chubatsu LS, Steffens MB. Expression, purification, and DNA-binding activity of the Herbaspirillum seropedicae RecX protein. Protein Expr Purif. 2004;35(2):298 - 303.

37. Agarwal RP, Robison B, Parks RE, Jr. Nucleoside diphosphokinase from human erythrocytes. Methods Enzymol. 1978;51:376-86.

38. Bosnar MH, Dubravcic K, Bago R, Pavelic J. Head and neck tumor cells exhibit altered proliferation upon overexpression of nm23 genes. Croat Chem Acta. 2008;81(1):183 - 9.

39. Bago R, Pavelic J, Vlahovicek GM, Bosnar MH. Nm23-H1 Promotes Adhesion of CAL 27 Cells In Vitro. Molecular Carcinogenesis. 2009;48(9):779 - 89.

40. Ouatas T, Abdallah B, Gasmi L, Bourdais J, Postel E, Mazabraud A. Three different genes encode NM23/nucleoside diphosphate kinases in Xenopus laevis. Gene. 1997;194(2):215 - 25.

41. Kim YI, Park S, Jeoung DI, Lee H. Point mutations affecting the oligomeric structure of Nm23-H1 abrogates its inhibitory activity on colonization and invasion of prostate cancer cells. Biochem Biophys Res Commun. 2003;307(2):281 - 9.

42. Yadav VK, Thakur RK, Eckloff B, Baral A, Singh A, Halder R, et al. Promoter-proximal transcription factor binding is transcriptionally active when coupled with nucleosome repositioning in immediate vicinity. Nucleic acids research. 2014;42(15):9602-11.

43. Postel EH. Cleavage of DNA by human NM23-H2/nucleoside diphosphate kinase involves formation of a covalent protein-DNA complex. J Biol Chem. 1999;274(32):22821 - 9.

44. McDermott WG, Boissan M, Lacombe ML, Steeg PS, Horak CE. Nm23-H1 homologs suppress tumor cell motility and anchorage independent growth. Clinical & experimental metastasis. 2008;25(2):131-8.

45. Perina D, Bosnar MH, Bago R, Mikoc A, Harcet M, Dezeljin M, et al. Sponge non-metastatic Group I Nme gene/protein - structure and function is conserved from sponges to humans. BMC evolutionary biology. 2011;11:87.

46. Desvignes T, Pontarotti P, Bobe J. Nme gene family evolutionary history reveals pre-metazoan origins and high conservation between humans and the sea anemone, Nematostella vectensis. PloS one. 2010;5(11):e15506.

47. Cetkovic H, Perina D, Harcet M, Mikoc A, Herak Bosnar M. Nme family of proteins--clues from simple animals. Naunyn-Schmiedeberg's archives of pharmacology. 2015;388(2):133-42.

48. Simion P, Philippe H, Baurain D, Jager M, Richter DJ, Di Franco A, et al. A Large and Consistent Phylogenomic Dataset Supports Sponges as the Sister Group to All Other Animals. Current biology : CB. 2017;27(7):958-67.

49. Shen X-X, Hittinger CT, Rokas A. Contentious relationships in phylogenomic studies can be driven by a handful of genes. Nature Ecology & Evolution. 2017;1:0126.

50. Webb PA, Perisic O, Mendola CE, Backer JM, Williams RL. The Crystal Structure of Human Nucleoside Diphosphate Kinase, NM23-H2. Journal of molecular biology. 1995;251(4):574-87.

51. Bosnar MH, De Gunzburg J, Bago R, Brecevic L, Weber I, Pavelic J. Subcellular localization of A and B Nm23/NDPK subunits. Exp Cell Res. 2004;298(1):275 - 84.

52. Domazet-Loso T, Tautz D. Phylostratigraphic tracking of cancer genes suggests a link to the emergence of multicellularity in metazoa. BMC Biol. 2010;8:66.

53. Domazet-Loso T, Tautz D. An ancient evolutionary origin of genes associated with human genetic diseases. Mol Biol Evol. 2008;25(12):2699-707.

Figure legends:

Figure 1

Alignment of human, sponge and C. owczarzaki Group I Nme proteins. Amino acids crucial for hexamer formation are marked with *. NDPK active site is boxed and NDPK domain is marked by a grey arrow; b) Expression levels of C. owczarzaki Group I Nme proteins. ↑ NmeGpICo is significantly more expressed in the adherent stage compared to the cystic stage, ↓↓ NmeGpICo-like is significantly lass expressed in the cystic stage compared to the other two stages

Figure 2

Oligomerization of NmeGp1Co. (A) Cross-linking NmeGp1Co with glutaraldehyde. In the first reaction glutaraldehyde was added to a final amount of 0.04%. Reaction was incubated at 25°C for 15 min. The second reaction was performed with 1.6% glutaraldehyde for 40 min at 37°C. (B) Gel filtration chromatography. The blue line represents human Nme2 while the green line represents NmeGp1Co protein

Figure 3

DNA-binding activity of the NmeGp1Co protein. Reactions contained 20 ng of single-stranded circular DNA from bacteriophage φX174 (NEB). The amount of purified NmeGp1Co protein is indicated

Figure 4

Plasmid cleavage assays. A) Recombinant plasmid pUC19-NHE and B) empty pUC19 was incubated with NmeGp1Co protein as indicated. Topoisomerase I and Nme2 proteins were used as controls and were processed in the same conditions.

Figure 5

Subcellular localization of NmeGp1Co and human Nme1 and Nme2. A) and B) HeLa cells transiently transfected with pyRedC1-Nme1 (red fluorescence) and pEGFPC1-NmeGp1Co (green fluorescence). The signal is visible mainly in the cytoplasm, but can been seen in the nucleus as well. C) and D) HeLa cells transiently transfected with pyRedC1-Nme2 (red fluorescence) and pEGFPC1-NmeGp1Co (green fluorescence). The signal is visible mainly in the cytoplasm, but can been seen in the nucleus as well. In all cases (A-D) the colocalization signal is visible in the cytoplasm, but can been seen in the nucleus as well. The colocalization signal of the cytoplasmic "granum-like" structures is visible, but incomplete. Bar = 10 μm

Figure 6

Nme1/NmeGp1Co complex formation analysis: A) Input control: Cell lysates from control K (MDA-MB-231Tcells stably transfected with pcDNA3 vector), Co2 and Co9 pcDNA3FLAG/NmeGp1Co construct) tested with anti-FLAG antibody and anti-Nme1 antibody. B) Immunoprecipitation: MDA-MB-231Tcells stably transfected with pcDNA3 vector (K) and FLAG/NmeGp1Co (Co2 and Co9) were immunoprecipitated with anti-FLAG M2 affinity gel and immunoblotted with anti-FLAG and anti-Nme1 antibody. FLAG/NmeGp1Co produces heteromers with exogenous (FLAG/NmeGp1Co, upper band) and endogenous (lower band) Nme1. C) Immunoprecipitation: FLAG/NmeGp1Co (Co2 and Co9) were immunoprecipitated with mouse anti-IgG (negative control) and anti Nme1 antibody and immunoblotted with anti-FLAG and anti-Nme1 antibody. Endogenous Nme1 produces complexes with exogenous (FLAG/NmeGp1Co produces heteromers with exogenous (FLAG/NmeGp1Co, upper band) and endogenous (lower band) Nme1

Figure 7

Migration assay. MDA-MB-231T cell transfected with empty vector pcDNA3 (K0), pcDNA3FLAG/Nme1, pcDNA3FLAG/Nme2, pcDNA3FLAG/NmeGp1Co were tested for migration potential in Boyden chambers. The cells were stained with crystal violet, while the images were recorded with light microscope. The cells were counted on four representative images. The results were presented as relative numbers of migrated cells compared to K0 (empty vector transfectants) (±SD)