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Head and Neck Tumor Cells Exhibit Altered Proliferation upon Overexpression of *nm23* Genes

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nm23 was identified as a metastasis suppressor gene but is also appointed to a number of other biological functions. The goal of this study was to reveal the influence of ectopic expression of *nm23-H1* and *nm23-H2* on proliferation properties of head and neck tumor cells. The proliferation rate of transfected cells was evaluated using EGFP reporter system and flow cytometry. HEp-2 and CAL 33 cells transiently transfected with *nm23* cDNA containing constructs exhibited enhanced proliferation. CAL 27 cells constitutively expressing GFP-Nm23-H2 protein, exhibited intense proliferation the first day after seeding, while the GFP-Nm23-H1 expressing clone started to proliferate after one-day lag period. The results on transiently transfected HEp-2 and CAL 33 cells generally confirmed previous findings connecting *nm23* expression with altered proliferation of head and neck tumors. We speculate that the effects observed on stably transfected CAL 27 clones are due to their different attachment properties.

Keywords
nm23
head and neck tumor cell lines
flow cytometry
green fluorescent protein

INTRODUCTION

Nm23-H1 and Nm23-H2 are subunits of a well known, »old« enzyme – nucleoside-diphosphate kinase (NDPK), whose role is to transfer the terminal phosphate from ATP to all other NDPs and dNDPs.[1] Until today, eight members of the *nm23* gene family have been discovered.[2] There are more than few indications that *nm23-H1*, *nm23-H2* and, possibly, some other members of the family have other functions in the cell being linked to cell differentiation and/or formation of metastases of different tumors. It seems that low expression of *nm23-H1* leads to metastases formation in breast[3,4] and cervical[5] carcinomas, melanomas[6] and several other neoplastic lesions.[7] On the other hand, several papers suggest that more invasive hematopoietic tumors exhibit overexpression of *nm23*.[8,9] There are also tumors in which no correlation with *nm23* and tumor size, differentiation and stage could be found.[7,10,11,12] Apart from their involvement in tumorigenesis and metastasis formation in certain tumors, nm23 genes/proteins have been associated with several distinct biological processes: microtubule polymerization,[13] vesicular trafficking,[14] and signal transduction.[15] They have also been found to participate in differentiation and development of several species. For instance, inactivation of *Drosophila* analogue of *nm23* gene leads to abnormal larval development,[16] while experiments on mice embryos revealed the role of *nm23* in development of tissues during prenatal period.[17] Furthermore, from the work of Postel *et al.* it has been suggested that Nm23-H2 acts as a

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transcriptional factor for *c-myc* oncogene.[18] Therefore, many authors propose a multifunctional and tissue specific nature of, at least some, of the Nm23 proteins.

Previous results of our laboratory,[19] based on *nm23-H1* expression correlated with SCCHN (squamous cell carcinoma of the head and neck) tumor stages and cell proliferation indicate that *nm23-H1* is involved in progression of SCCHN in a manner, which is not consistent with its proposed role as a metastasis suppressor gene. Several other authors exhibited contradictory results.[20,21,22] Our main goal was to reveal possible changes of the cell cycle of benign and malignant head and neck tumor cell lines upon ectopic expression of *nm23-H1* and *nm23-H2*. Taken generally, the results confirm our previous findings connecting high levels of Nm23 proteins with head and neck tumor cell proliferation. However, they also bring up a number of questions concerning its multifunctional role in the cell and its potential influence on cell adhesion.

EXPERIMENTAL

Cells and Culture Conditions

Human head and neck tumor cell lines HEp-2 (squamous cell carcinoma of the larynx, ATCC), CAL 33 (epithelioma of the tongue), and CAL 27 (squamous cell carcinoma of the tongue, both obtained by courtesy of Dr. Jeannine Gioanni, Centre Antoine Lacassagne, Nice, France) were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10 % fetal bovine serum (FBS; Life Technologies), 2 mmol dm^{-3} glutamine, 100 U ml^{-1} penicillin (1 U $ml^{-1} \approx 1$ mg ml^{-1}) and 100 μg ml^{-1} streptomycin in a humidified chamber with 5 % CO_2, at 37 °C.

Constructs and Cloning

nm23-H1 and *nm23-H2* full-length cDNA fragments were subcloned from pcDNA3nm23 constructs (obtained by courtesy of Dr. Marie-Lise Lacombe, Faculte de Medecine Saint Antoine, Paris, France) into pEGFPC1 (Clontech Lab. Inc.) minding the open reading frame. Cloning was analyzed by a set of restriction endonucleases while the existence of fusion GFP-Nm23 proteins was verified by Western blotting.

Western Blot Analysis

Total cell lysates were prepared by resuspending cells in lysis buffer (50 mmol dm^{-3} Hepes pH = 7.5, 150 mmol dm^{-3} NaCl, 1 mmol dm^{-3} EDTA, 1 mmol dm^{-3} EGTA, 10 % glycerol, 1 % Triton X-100, 1 μmol dm^{-3} orthovanadate, 2 μg ml^{-1} leupeptin, 1 mg ml^{-1} aprotinin) for three minutes on ice. The cell debris was removed by centrifugation for 15 minutes at 13000 *g* and 4 °C. Total protein concentration was measured by Bradford assay against a standard curve of known BSA (bovine serum albumin) concentrations. Equal amounts of samples (50 μg of total protein) were loaded on SDS-PAGE (5 % stacking, 10 % running gel) and electrotransferred to an Imobilon-P^{SQ} membrane (Millipore). To assess the expression of endogenous (native) Nm23-H1 and Nm23-H2 proteins in HEp-2, CAL 33 and CAL 27 cell lines, as well as the existence of fusion GFP-Nm23 proteins, affinity purified polyclonal antibodies against human Nm23-H1 (obtained by courtesy of Dr. Marie-Lise Lacombe, and prepared as described by Phung-Ba Pinon *et al.*[23]) and polyclonal antibodies against human Nm23-H2 (obtained by courtesy of Dr. Ioan Lascu, Universite de Bordeaux-2, Bordeaux, France) were used. Protein bands were visualized using BM Chemiluminiscence blotting substrate-POD (Roche) on Lumi-Film-Chemiluminiscent Detection Film (Roche).

Transient Transfection and Sample Preparation

8×10^5 cells (HEp-2 and CAL 33) were seeded on six-well plates 24 hours prior to transfection. The cells were transfected (lipofected) with 2.5 μg of plasmid DNA using Lipofectamine Plus Reagent (Life Technologies) according to manufacturer's instructions. In cotransfection experiments the cells were transfected with plasmid DNA in the ratio 2:1 (pcDNA3 : pEGFPC1, respectively).

Forty-eight hours post transfection the cells were tripsinized, centrifuged at 1200 *g*, and washed twice in phosphate-buffered saline (PBS). For cell cycle analysis the cells were prepared according to a protocol based on several described methods.[24,25] In brief, cells were fixed in 1 % paraformaldehyde in PBS for one hour at +4 °C and permeabilized overnight in 80 % ethanol in PBS. After RN-ase treatment (100 μg ml^{-1}) and propidium-iodide staining (20 μg ml^{-1}) for 30 minutes at 37 °C, the cells were stored at +4 °C until further analysis.

Stably Transfected Cell Line Preparation

CAL 27 cells were seeded on a 100 mm Petri dish and incubated until 80 % confluence. The cells were transfected with pEGFPC1, pEGFPC1-nm23-H1 and pEGFPC1-nm23-H2 constructs using Lipofectamine Plus Reagent according to manufacturer's instructions. Twenty-four hours post transfection the cells were tripsinized, resuspended in 1:20 ratio in DMEM supplemented with 600 μg/ml geneticin (Sigma), and seeded in six-well plates. The antibiotic supplemented medium was changed every 3–4 days until the development of stabile, resistant colonies. Several clones were established for every construct used. The presence of fusion GFP-Nm23 proteins in established clones was verified by fluorescent microscopy and Western blotting.

For cell cycle analysis 1×10^6, 8×10^5 and 4×10^4 cells of each established clone were seeded on six-well plates and collected by tripsinization after 24, 48 and 72 hours, respectively. The samples were prepared as described for transiently transfected cell lines. All established clones were analyzed but results were displayed for only one, representative clone per each construct.

Flow Cytometry

Samples were analyzed on FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW air-cooled 488-nm

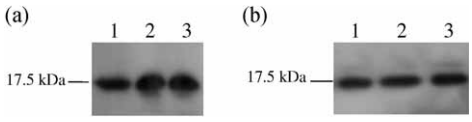

Figure 1. Expression of endogenous Nm23 proteins in head and neck tumor cell lysates. Western blot analysis using: a) anti-Nm23-H1 antibodies. b) anti-Nm23-H2 antibodies. Lane 1: HEp-2; lane 2: CAL 33; lane 3: CAL 27.

argon-ion laser. For transiently transfected cells total amount of (10–20) × 10^3 green cells (depending upon efficiency of transfection) was collected. FL2/FL1 plot was used to separate the transfected (green) cells from nontransfected cells. Red fluorescence (FL2) was displayed on a linear, and green fluorescence (FL1) on a log scale. The gate was set to analyze the cell cycle distribution of »dim« cells. The stably transfected clones were displayed and gated for cell cycle analysis on FL2 width *vs.* FL2-area plot to exclude aggregates. Data were analyzed using CellQuest™ software (Becton Dickinson), ModFit LT™ 2.0 (Verity Software House) and WinMDI27. The results of cell cycle distribution analysis of transiently transfected cells are shown as the mean of three experiments with standard deviation. In the case of stably transfected clones, we presented the mean of two experiments with standard deviation.

Growth Rate

The growth rate of pEGFP-nm23 stably transfected CAL 27 clones was established according to the results of the MTT test (Sigma) which was preformed every 24 hours in a period of six days after seeding.[26] The results represent one of three repeated experiments.

RESULTS

Cell Extracts from All Cell Lines Analyzed Exhibited Nm23-H1, Nm23-H2 and GFP-Nm23 Fused Proteins

After Western blot analysis with anti-nm23-H1 and anti-nm23-H2 antibodies HEp-2, CAL 33 and CAL 27 cell lines all exhibited endogenous (native) Nm23-H1 and Nm23-H2 proteins as seen in Figure 1. To prove the existence of fusion GFP-Nm23 proteins HEp-2 cells were transiently transfected. Figure 2 shows bands of about 51 kDa in size, which indicate the existence of fusion GFP-Nm23-H1 and GFP-Nm23-H2 proteins. Figure 3 shows the expression of GFP-Nm23-H1 in stably expressing CAL 27 cells.

nm23 Overexpressing Constructs Upregulate Proliferation in Transiently Transfected HEp-2 and CAL 33 Cells

Flow cytometry experiments were conducted to verify the influence of *nm23* overexpression on the cell cycle of HEp-2 (Figure 4) and CAL 33 (Figure 5) head and neck

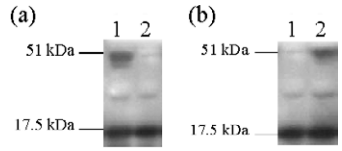

Figure 2. Expression of GFP-Nm23-H1 and GFP-Nm23-H2 proteins in transiently transfected HEp-2 cell lysates. Western blot analysis using: a) anti-Nm23-H1 antibodies. Lane 1: pEGFPC1-nm23-H1 transfected cells; lane 2: pEGFPC1-nm23-H2 transfected cells; b) anti-Nm23-H2 antibodies. Lane 1: pEGFPC1-nm23-H1 transfected cells; lane 2: pEGFPC1-nm23-H2 transfected cells.

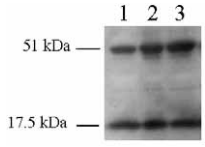

Figure 3. Expression of GFP-Nm23-H1 in stably transfected CAL 27 cell lysates. Western blot analysis using anti-Nm23-H1 antibodies. Lanes 1, 2 and 3 represent three different stabile clones constitutively expressing GFP-Nm23-H1 (upper band). The lower band represents the endogenous Nm23-H1.

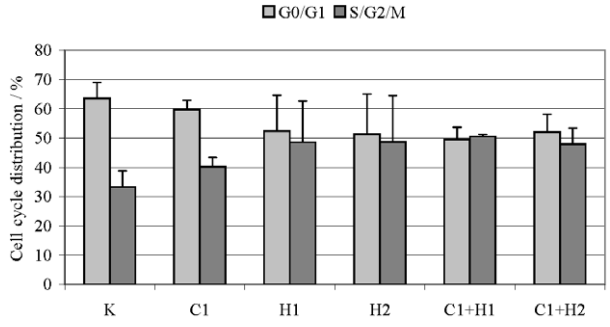

Figure 4. Cell cycle distribution of transiently transfected HEp-2 cells. K: untransfected HEp-2 cells; C1: pEGFPC1 transfected cells; H1: pEGFPC1-nm23-H1 transfected cells; H2: pEGFPC1-nm23-H2 transfected cells; (C1+H1): pEGFPC1 and pcDNA3-nm23-H1 cotransfected cells; (C1+H2): pEGFPC1 and pcDNA3-nm23-H2 cotransfected cells. Results represent mean ± SD.

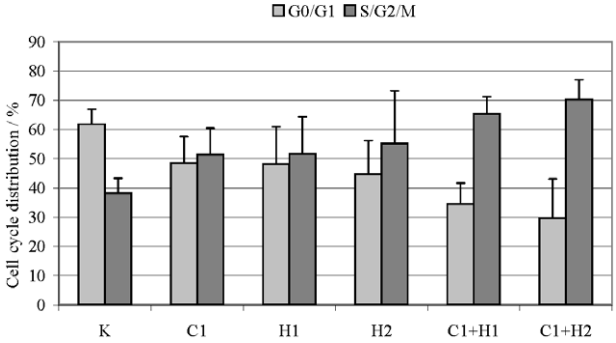

Figure 5. Cell cycle distribution of transiently transfected CAL 33 cells. K: untransfected CAL 33 cells; C1: pEGFPC1 transfected cells; H1: pEGFPC1-nm23-H1 transfected cells; H2: pEGFPC1-nm23-H2 transfected cells; (C1+H1): pEGFPC1 and pcDNA3-nm23-H1 cotransfected cells; (C1+H2): pEGFPC1 and pcDNA3-nm23-H2 cotransfected cells. Results represent mean ± SD.

tumor cells *in vitro*. For that purpose, the cells were transfected and cotransfected with a series of constructs and combinations of constructs (pEGFPC1 as control, pEGFPC1-nm23-H1, pEGFPC1-nm23-H2, pEGFPC1 + pcDNA3-nm23-H1 and pEGFPC1 + pcDNA3-nm23-H2), whereas untransfected cells and the cells transfected with pEGFPC1 were used as controls. Cell cycle analysis was performed 48 hours post transfection. The results indicate that cells transiently transfected with pEGFPC1-*nm23* constructs stimulate the proliferation of HEp-2 and CAL 33 cells in culture, although the results were not statistically significant according to Student's *t*-test ($P>0.05$). Figure 4 shows an increase in the number of HEp-2 cells (*cca.* 7–10 %) in S/G2/M compartment in pEGFPC1-nm23 transfected cells, if compared to untransfected cells and the cells transfected with the »empty« vector. The increase of the number of proliferating HEp-2 and CAL 33 cells was more convincing in cotransfection experiments where the results were statistically significant with a $P<0.05$. The results exhibit an increased number of proliferating transfected and cotransfected cells, the only exception being the pEGFPC1-nm23-H1 transfected cells, which show minimal proliferation effect compared to cells transfected with the »empty« vector. The portion of HEp-2 proliferating cells in cotransfection experiments were *cca.* 10 %, while the number of CAL 33 proliferating cells increased more than 15 % compared to the »empty« vector transfected cells).

Stably Transfected CAL 27 Cells Exhibit Different Patterns in Proliferation Activity

To evaluate the influence of GFP-Nm23 proteins on cell growth in culture, we analyzed the growth rate of stably transfected clones during the period of six days (Figure 6). We analyzed several stabile clones but we displayed only one representative clone for each construct: GFP-Nm23-H1 clone (K49), GFP-Nm23-H2 clone (K71), pEGFPC1 clone (K78). We also analyzed the growth rate of untransfected cells. The major difference in growth rate between the mentioned clones was visible 24 hours after seeding. It was clear that the K71 clone went immediately into proliferation and almost doubled, while the K49 clone barely started proliferating. This result was in concordance with our visual observation that cells overexpressing GFP-Nm23-H1 after more than 24 hours did not acquire the expected epithelial morphology and were still completely spherical in contrast to the cells overexpressing GFP-Nm23-H2 (data not shown). The flow cytometry experiments completely confirmed these observations (Figure 7). The difference in cell cycle distribution 24 hours after seeding clearly indicated that Nm23-H1 expressing clone (K49) hardly started to proliferate (28 % of cells in S, and none in G2/M phase) in contrast to more than 65 % S-phase cells, and 12 % G2/M-phase cells in Nm23-H2 expressing clone. GFP clone (K78), as well as the untransfected cells, displayed cell cycle distribution somewhere between those two extremes. The results are statistically significant, $P<0.05$. Seventy-two hours after seeding all clones adopt the same cell cycle distribution.

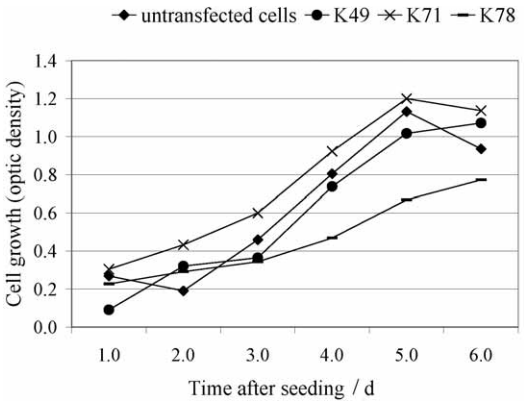

Figure 6. Growth curves of CAL 27 cell stable transfected with pEGFP-nm23 constructs. Growth curves were obtained using MTT test. Clone K49: cells stable transfected with pEGFPC1-nm23-H1; clone K71: cells stable transfected with pEGFPC1-nm23-H2; clone K78: cells stable transfected with »empty« (pEGFPC1) vector.

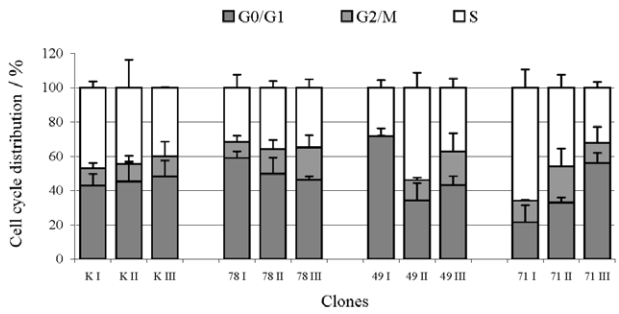

Figure 7. Cell cycle distribution of CAL 27 clones. K: untransfected cells; clone K78: cells transfected with pEGFPC1; clone K49: pEGFPC1-H1 transfected cells; clone K71: pEGFPC1-nm23-H2 transfected cells. I, II, III: 24, 48, 72 hours after seeding, respectively. Results represent mean ± SD.

DISCUSSION

During the last decade, it has been suggested that Nm23 proteins regulate a number of cellular processes although the underlying mechanisms of these processes have not yet been revealed. Several authors have studied the influence of *nm23* genes on proliferation, differentiation or potential invasiveness of head and neck tumor cells. Takes *et al.* studied the expression of several oncogenes and tumor suppressor genes in squamous cell carcinoma of the larynx, pharynx and the oral cavity.[20] The study failed to prove any difference in expression of *nm23* in tumor compared to normal tissue. In the work of Gunduz *et al.* overexpression of Nm23 protein was found in the majority of primary tumors compared to corresponding

normal mucosa, while decreased expression was associated with poor differentiation and distant metastases.[21]

Our study was conducted to evaluate the role of *nm23-H1* and *nm23-H2* on the cell cycle of head and neck tumor cells *in vitro,* and to confirm our previous studies that connected tumor progression of SCCHN with overexpression of *nm23-H1*. The major goal was to reveal the possible differences in cell cycle distribution arising upon ectopic expression of GFP-Nm23 or Nm23 proteins in cells originating from benign (CAL 33) *vs.* malignant (HEp-2) tumors. The malignant CAL 27 cells used in preparing stabile clones, unfortunately weren't suitable for transient transfection experiments by flow cytometry due to low transfection efficiency (less than 2 %, compared to 16–20 % for HEp-2 cells, and 3–5 % for CAL 33 cells; data not shown).

Although being a handy technique for detecting the efficiency of transfection and exogenous protein production, proteins fused with reporter genes/proteins often lose a portion of their efficiency. To overcome the possible problems concerning this issue, we performed cotransfections with pEGFPC1 as a transfection marker, and *nm23* cDNAs introduced in pcDNA3 which produced a recombinant, but native protein.

Our results brought up several interesting points. All three cell lines expressed endogenous Nm23-H1 and Nm23-H2 proteins, which was expected according to numerous former studies confirming that most cell lines and tissues, especially of epithelial origin, express Nm23/NDPK in abundance. Therefore, we presume that the effects concerning proliferation rate were due to overexpression. As can be seen in Figures 4 and 5, a slight increase in proliferation rate is present in cells transfected with the »empty« vector in both cell lines. This observation was, also, observed elsewhere,[27] but in our case this increase in cell proliferation rate was not statistically significant. In our experiments, we observed an increased number of *nm23* transfected cells in S/G2/M phase compared to control the only exception being GFP-nm23-H1 expressing CAL 33 cells which displayed no marked proliferation. However, the increase of the proliferation rate is not considered significant in the cells transfected with the pEGFPC1-nm23 constructs but it is significant in cotransfection experiments. We presume that this is due to the fact that fusion GFP-Nm23 proteins are less active compared to native recombinant Nm23 proteins. The change in the proliferation rate of CAL 33 cells transiently transfected with pEGFPC1 + pcDNA3-nm23-H1 was *cca.* 15 %, and statistically significant (P<0.01). One of the possible explanations for proliferation increase comes from the work of Cipollini *et al.*[28] in which the authors state that Nm23 proteins are most abundantly expressed in S phase of breast cancer cells. It is, therefore, possible that the cells recognize Nm23 overexpression as a signal to enter the S phase.

Taking into consideration a number of previous studies connecting *nm23* overexpression and cell growth and tumorigenesis,[29,30] as well as the results obtained in our laboratory, the proliferation effect in *nm23* transiently transfected SCCHN tumor cell lines was expected. In this part, our findings confirm our previous study on SCCHN, and broaden them on *nm23-H2* gene. One may consider this increase in proliferation too small to be considered significant. However, it must be emphasized that the cells analyzed in this work are highly proliferating tumor cells, and that an increase in the number of proliferating cells of over 15 %, as in some cases, is noteworthy. Since it is not recommended to compare the cell-cycle distribution of cells with different fluorescence intensity (»bright« and »dim«), we analyzed only »dim« cells, *i.e.* cells with lower fluorescence intensity (lower GFP expression level), because the number of »bright« cells was significantly lower. It is possible that by analyzing »bright« cells, a higher proliferation rate would have been obtained. A difference in proliferation rate due to overexpression of *nm23* genes could be observed between HEp-2 and CAL 33 cells. The number of HEp-2 cells in the S/G2/M compartment increased 7–10 % due to overexpression of *nm23* genes, while the number of proliferating CAL 33 cells increased for *cca.* 15 %. In the work of Cipollini *et al.*[31] and Caligo *et al.*[32] similar effects were observed, but on a different model system (breast carcinoma cells). Their results suggested the role of Nm23-H1 in cell proliferation, but the involvement of Nm23-H1 was diminished in highly metastatic cells. The authors hypothesized that *nm23* might be essential for cell proliferation, but could also be an inhibitor of proliferation via a negative feedback mechanism. They also find it possible that cells with a highly invasive phenotype find alternative proliferating pathways. One should also bear in mind that Nm23-H1 and Nm23-H2 form several isoenzymes of the NDPK, that they exhibit a different expression rate in different tissues and organs and, possibly, distinct functions in different cancer types. Therefore, the reason why more invasive HEp-2 cells are less sensitive to Nm23 increase than CAL 33 cells, is highly speculative at this point.

Several authors described the behavior of head and neck tumor cells upon changing the expression level of *nm23* genes. Miyazaki *et al.*[33] described the unusual behavior of *nm23-H2* transfected cells of the oral cavity. The cells didn't differ morphologically from untransfected cells, but they proliferated faster, became less serum dependent and lost responsiveness to growth factor and thus, suppressed metastatic activity. *nm23-H1* transfected cells did not exhibit this feature. On a slightly different model, on cultured keratinocytes, it was shown that, although not showing direct proliferation enhancement in monoculture, *nm23-H2* transfectants do form highly proliferating epithelium in three-dimensional organotypic culture.[34] The authors also suggest that the expression ratio

of *nm23-H1* / *nm23-H2* is responsible for proliferation behavior. Taking all these findings into account, it would be interesting to observe the effect of cotransfection of both *nm23* genes in our model system and to verify the results on *in vitro* raft cultures. Taken generally, our results partly confide with the results of other investigators. We are, however, aware of the shortcomings of our method that originate in low transfection rates, and a too low number of different cell lines taken into analysis. Our results should be confirmed by another independent method, for instance staining the cells with BrdU or some other independent indicator of cell proliferation. On the other hand, we observed changes that had arisen upon enhanced expression of already expressed genes/proteins in the cell. It is debatable whether overexpression brings any quantitative or qualitative difference in the cells already expressing the gene of interest. Although we do not doubt that the finely tuned cellular processes sense the 2-fold overexpression of *nm2*3 genes, the true confirmation would come from silencing the endogenous genes by introducing siRNA. However, this may be the object of future experiments.

Growth rate curves and flow cytometry experiments on stably transfected clones confirmed our visual observation (data not shown) that GFP-Nm23-H1 expressing cells (K49) need sufficient time to start proliferating after seeding. This is different from GFP-Nm23-H2 (K71) clone that starts to proliferate shortly after seeding. Since *nm23* is probably involved in cell attachment, either directly by possessing the RGD sequence typical for integrin binding proteins[2] or indirectly, we hypothesized that different proliferation properties of transfected clones could be a consequence of their different attachment properties. Namely, since GFP-Nm23-H1 expressing clone needs a longer time to attach to the surface of the dish, it is to be expected that these cells would start proliferating at a later time point, than the immediately attaching GFP-Nm23-H2 expressing clone. Three days after seeding, the proliferation time seems to equalize between the two clones and also compared to controls. It may seem that the results obtained on stabile transfected clones are not in accordance with the ones obtained on transient transfections, but it must be clear that these two experiments were designed and conducted differently. In transient transfections, we first seeded the cells and analyzed them 48 hours post transfection, while stably transfected cells already constitutively expressed GFP-Nm23 proteins when seeded. Recent findings of Fournier *et al.*[35] presented new evidence connecting Nm23 proteins with molecules involved in cell attachment, so our further experiments will be focused on revealing the mechanisms through which Nm23 proteins influence proliferation. We also intend to study the relation of these proteins to cell-surface and cell-cell adhesion molecules in head and neck tumor cells.

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SAŽETAK

Stanice tumora glave i vrata pokazuju promijenjen profil proliferacije pod utjecajem pojačane ekspresije gena *nm23*

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Gen *nm23* prvi je otkriveni supresor metastaziranja, a usporedo s tim, dodijeljeno mu je još nekoliko bioloških funkcija. Cilj ovog rada bio je otkrivanje utjecaja pojačane ekspresije gena *nm23-H1* i *nm23-H2* na proliferaciju stanica tumora glave i vrata *in vitro*. Stupanj proliferacije transfeciranih stanica određen je upotrebom reporterskog sustava EGFP uz pomoć protočne citometrije. Stanice HEp-2 i CAL 33 prolazno transfecirane konstruktima koji sadrže cDNA gena *nm23* pokazale su pojačanu proliferativnu aktivnost. Stanice CAL 27 koje konstitutivno imaju GFP-NM23-H2 pokazuju intenzivnu proliferaciju prvi dan nakon nasađivanja, dok stanice koje imaju GFP-Nm23-H1 započinju proliferaciju s jednim danom zakašnjenja. Rezultati na prolazno transfeciranim stanicama HEp-2 i CAL 33, općenito uzevši, potvrdile su dosadašnja istraživanja na tumorima glave i vrata. Pretpostavlja se da su rezultati dobiveni na stabilno transfeciranim klonovima stanica CAL 27 posljedica njihovih različitih osobina vezanih za prianjanje uz podlogu.