Vaccine 32 (2014) 4799–4805

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MicroRNAs as potential biomarkers for VERO cell tumorigenicity

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ARTICLE INFO

Article history:
Received 21 February 2014
Received in revised form 30 April 2014
Accepted 20 May 2014
Available online 10 July 2014

Keywords:
African green monkey kidney (VERO) cells
Neoplastic development
Transformation
Tumorigenicity
miRNA
Biomarkers

ABSTRACT

MicroRNA expression appears to capture the process of neoplastic development *in vitro* in the VERO line of African green monkey kidney (AGMK) cells (Teferedegne et al. PLoS One 2010;5(12):e14416). In that study, specific miRNA signatures were correlated with the transition, during serial tissue-culture passage, of low-density passaged 10–87 VERO cells from a non-tumorigenic phenotype at passage (p) 148 to a tumorigenic phenotype at p256. In the present study, six miRNAs (miR-376a, miR-654-3p, miR-543, miR-299-3p, miR-134 and miR-369-3p) were chosen from the identified signature miRNAs for evaluation of their use as potential biomarkers to track the progression of neoplastic development in VERO cells. Cells from the 10–87 VERO cell line at passage levels from p148 to p256 were inoculated into newborn and adult athymic nude mice. No tumors were observed in animals inoculated with cells from p148 to p186. In contrast, tumor incidences of 20% developed only in newborn mice that received 10–87 VERO cells at p194, p234 and p256. By qPCR profiling of the signature miRNAs of 10–87 VERO cells from these cell banks, we identified p194 as the level at which signature miRNAs elevated concurrently with the acquisition of tumorigenic phenotype with similar levels expressed beyond this passage. In wound-healing assays at 10-passage intervals between p150 to p250, the cells displayed a progressive increase in migration from p165 to p186; beginning at p194 and higher passages thereafter, the cells exhibited the highest rates of migration. By qPCR analysis, the same signature miRNAs were overexpressed with concomitant acquisition of the tumorigenic phenotype in another lineage of 10–87 VERO cells passaged independently at high density. Correlation between the passages at which the cells expressed a tumorigenic phenotype and the passages representing peaks in expression levels of signature miRNAs indicates that these miRNAs are potential biomarkers for the expression of the VERO cell tumorigenic phenotype.

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1. Introduction

The VERO cell line represents a well-characterized, immortalized line of African green monkey kidney (AGMK) cells that is susceptible to a broad range of viruses [1–4]. These cells are used as the cell substrate reagents for the manufacture of several viral vaccines including vaccines against poliomyelitis, rabies, rotavirus, smallpox, and influenza [2–8]. VERO cells at passages (p) 140 to 165 are non-tumorigenic in immunocompromised mice [9–11]; however, the VERO cell phenotype can evolve by prolonged passage in culture to become tumorigenic in these mice [10,12]. Candidate cell substrate reagents proposed for the production of biologics for human use need to be carefully characterized. For the characterization of immortalized cells, the cell line must be described with respect to its tumorigenicity in animal models (21 Code of Federal Regulations 610.18). Besides the obvious high cost and time associated with animal assays, there is a goal to reduce, refine, or replace animal testing. Thus, developing predictive molecular markers that can be used as assays to replace *in vivo* tests for the characterization of cell substrate tumorigenicity could help meet these goals.

A recent development in cell biology has been the identification of the role of microRNAs (miRNAs) in the modulation of various cellular processes. miRNAs are short, non-coding RNAs that regulate the expression of many target genes. miRNAs have been shown to play critical regulatory roles in a wide range of biological and pathological processes including cancer. The involvement of miRNAs in cancer initially emerged from both studies showing their proximity

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http://dx.doi.org/10.1016/j.vaccine.2014.05.065
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to chromosomal break points [13] and their deregulated expression levels in many neoplastic tissues compared with normal tissues [14–23]. Moreover, the identification of classical oncogenes and tumor suppressor genes as direct targets of miRNAs has led to the conclusion that miRNAs play crucial roles in cancer initiation, progression, and metastasis [17,24–27]. Hence, given the critical role miRNAs play in the process of tumorigenesis and in their disease-specific expression, they hold potential as novel biomarkers and therapeutic targets.

In an earlier study, we found that specific miRNA signatures correlated with the transition of the 10–87 VERO line of AGMK cells from a non-tumorigenic phenotype at low passage p140 cells to a tumorigenic phenotype at high passage p250 cells during serial tissue-culture passage [28]. In the current study, we have expanded this observation to determine whether these miRNA signatures might be used as biomarkers of the 10–87 VERO cell tumorigenic phenotype. The pattern of these potential miRNA signatures was assessed in cell banks established at every 10 passages from p140 to p250 at low density (LD). We found a correlation between the passages at which the VERO cells expressed a tumorigenic phenotype and the passages representing the peak in expression levels of signature miRNAs. This correlation was confirmed using another lineage of 10–87 VERO cells derived by passage at high density (HD) to evaluate the impact of plating density on the evolution of the VERO neoplastic phenotype. These results illustrate that these miRNAs can be potential biomarkers for the expression of the VERO cell tumorigenic phenotype.

2. Materials and methods

A more detailed presentation of Section 2 is found in Supplemental Materials and methods.

2.1. Cell lines and cell culture

Cells from the World Health Organization (WHO) VERO cell bank 10–87 (ATCC 10–87 VERO cells) were serially passaged in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 2 mM L-glutamine (Mediatech, Manassas, VA) (DMEM-10) from p134 to p250 by sub-culturing VERO cells either before [10] or after they reached confluence. For the studies in this report, the sub-confluent passaged 10–87 VERO cells were designated as low-density passage 10–87 VERO cells (LD 10–87 VERO cells), while 10–87 VERO cells passaged at confluence were designated as high-density passaged 10–87 VERO cells (HD 10–87 VERO cells). The population doubling times (PDT) for LD 10–87 VERO cells and HD 10–87 VERO cells at p 250 were 26 h for the LD 10–87 VERO cells and 20 h for the HD 10–87 VERO cells. The LD and HD passaged cells used in this study and some of the tumors they formed were confirmed to be of simian origin by karyotyping and by PCR using primers that recognize simian SINE sequences [28]. These cells have also been found to be free of 26 rodent viruses and mycoplasma families (Radil, Columbia, MO).

2.2. Tumorigenicity assays

Adult (10 mice/dose) and newborn (NB) (3 litters/dose) athymic nude mice were inoculated subcutaneously (10^7 cells/mouse in 0.1 mL of PBS per cell line) above the scapulae. Tumors were documented to grow progressively by measurements in two dimensions until they were 15–20 mm in size, at which point the animals were euthanized. The tumor incidence in adult and newborn nude mice was recorded at weekly intervals over 12 month observation periods and plotted as survival curves.

2.3. Wound-healing assays

Wound-healing assays were performed as previously described [29] with some modifications. Cells were plated 1×10^6 cells/plate in 60-mm culture dishes (Corning, Corning, NY) and allowed to form monolayers. When the cultures reached 90% confluence, they were serum starved for 8 h and the monolayers were wounded with a P200 micropipette tip, washed with PBS and cultured in DMEM-10. Images of cell migration into the wounded areas were captured at 0, 3, 6, 9, 12 and 15 h.

2.4. Quantitative RT-PCR

Total RNA from primary (p) African green monkey kidney (AGMK) cells and from cells from LD 10–87 VERO and HD 10–87 VERO cell banks established at every 10 passages from p140 to p250 was extracted and purified using the miRNeasy mini kit according to the manufacturer (Qiagen Inc., Valencia, CA). The expression of signature miRNAs of these samples was measured using the TaqMan miRNA quantitative PCR assay [30]. Expression values were normalized to a small nucleolar RNA, RNU6 (Applied Biosystems). ΔCt values were calculated using the Ct values of the miRNA and the RNU6 for each corresponding sample. $\Delta\Delta$Ct values are calculated using the ΔCt values of the pAGMK cells and the experimental cell lines for each miRNA. The fold change over pAGMK was calculated.

3. Results

To pursue our investigation of the possible correlation between signature miRNA expression and the tumor-forming ability of VERO cells, we extended our study of the relationship between tumorigenicity *in vivo*, wound-healing assays *in vitro*, which are frequently correlated with tumor-forming ability, and the patterns of expression of specific signature miRNAs. Correlation was sought across a range of 10–87 VERO cell passages at 10-passage intervals from p150 to p250 between the expression of 6 signature miRNAs and the evolution to a tumorigenic phenotype as indicated by tumor formation in athymic nude mice and *in vitro* wound-healing assays. Data obtained using the original LD 10–87 VERO cell line, which was established by passaging before the cell monolayer reached confluence, were confirmed and extended using another lineage of 10–87 VERO cells derived by passage at high density to evaluate the impact of plating density on the evolution of the VERO cell neoplastic phenotype.

3.1. VERO cell tumorigenicity

To evaluate the progression of the neoplastic phenotype expressed at intervening passages between p150 and p256 and to identify the passages at which the cells expressed a tumorigenic phenotype, LD 10–87 VERO cells and HD 10–87 VERO cells at different passage levels were inoculated into adult and newborn nude mice (NB). No tumors (0/70) were observed in adult nude mice inoculated with p157–p254 LD 10–87 VERO (data not shown) or in newborn nude mice (0/39) inoculated with p157–p185 LD 10–87 VERO cells after one year (Fig. 1). A maximum of 20% tumor incidences at the site of inoculation were recorded in NB mice that received LD 10–87 VERO cells at p194, p234, or p254 (Fig. 1). Incidence of tumor formation did not increase with the increasing passage level of the LD VERO cells.

In the NB nude mice inoculated with the LD 10–87 VERO cells at p194, the first tumor appeared at 8 weeks and the second tumor appeared at 10 weeks; in NB mice inoculated with the p234 VERO cells, tumors appeared at 16 and 19 weeks. In NB mice inoculated with LD 10–87 VERO cells at p254, the first tumor appeared at 7

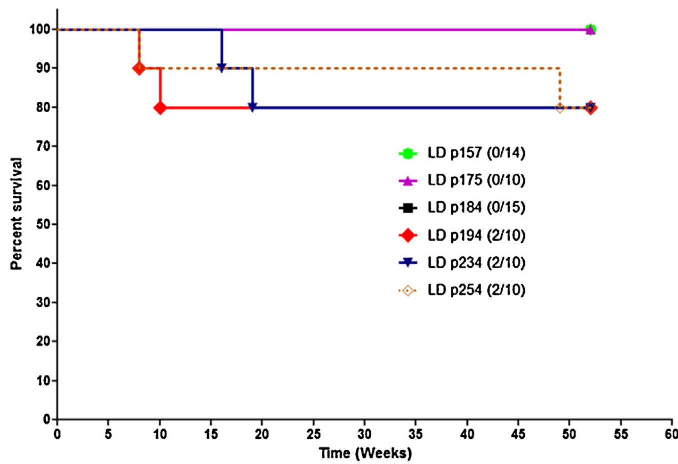

Fig. 1. Low-density passage (LD) VERO cells at their different passage levels were inoculated into newborn and adult athymic nude mice. Twenty percent tumor incidences were recorded in newborn mice that received p194, p234 and P254 VERO cells. Numbers in parenthesis are tumor incidence.

weeks and the second tumor appeared at 48 weeks. Time of tumor appearance (latency) did not correlate with passage level in nude-mouse assays involving LD 10–87 VERO cells. The tumor incidence in animals inoculated with HD 10–87 VERO cells differed compared with the results with the LD 10–87 VERO cells (Fig. 2A and B). The earliest passage that HD 10–87 VERO cells formed tumors in NB

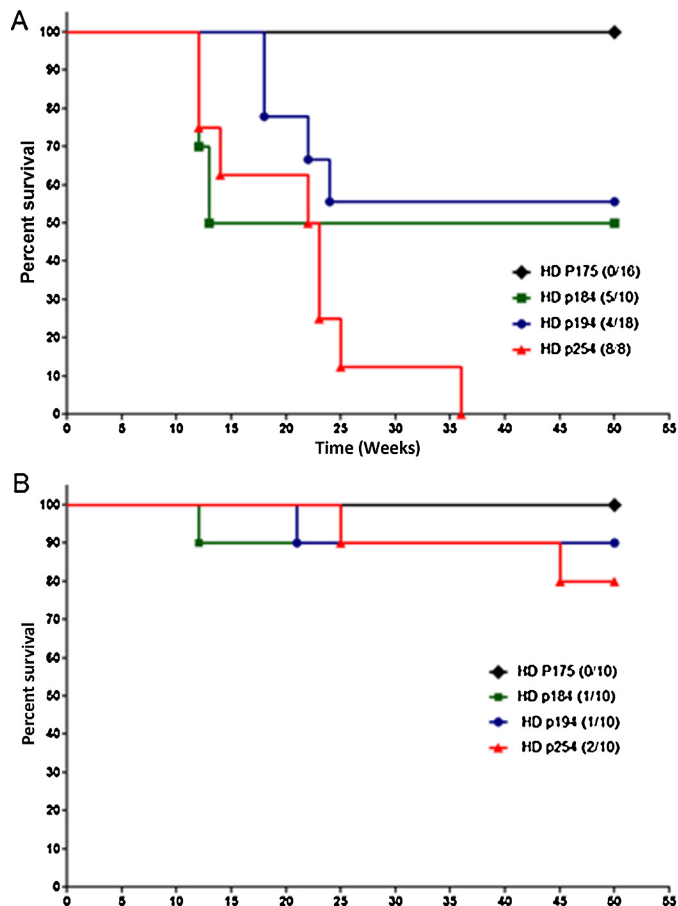

Fig. 2. High-density passage (HD) VERO cells at their different passage levels were inoculated into newborn (A) and adult (B) athymic nude mice. Tumors were observed in newborn mice that received HD VERO cells at passages ≥p185. Numbers in parenthesis are tumor incidence.

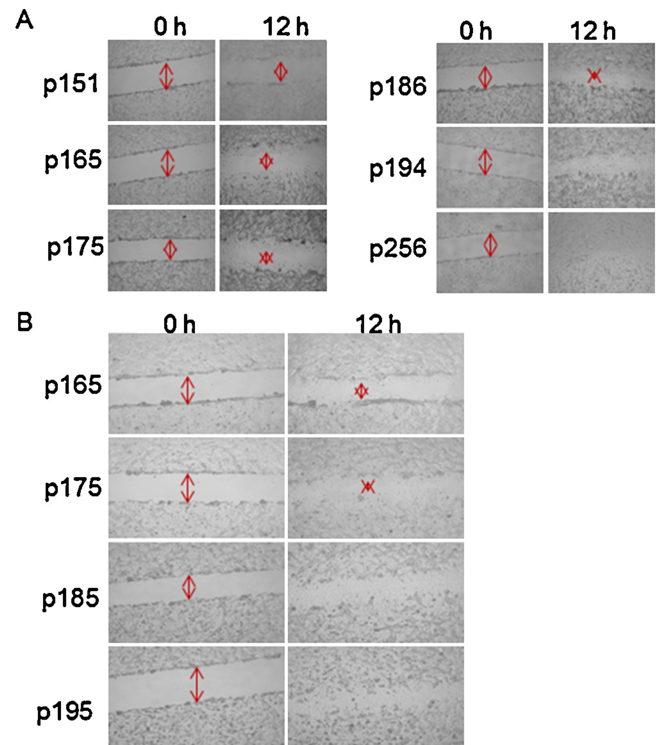

Fig. 3. Rate of migration progressively increased with increasing passages of (A) low-density passage (LD) and (B) high-density passaged (HD) VERO cells. Cells were seeded in 60-mm dishes (2×10^6 cells/dish) and serum-starved for 8 h. After scratching the monolayer with a 200-μL-pipette tip, cells were washed with PBS, cultured in DMEM-10, and photographed under a 4× objective every 3 h for 15 h. Only the 0 and 12 h images are shown. Arrows indicate borders of migrating cells.

(5/10) and adult (1/10) nude mice was at p184 compared with p194 for LD 10–87 VERO cells. By 36 weeks, HD 10–87 VERO cells at p256 had formed tumors in 100% (8/8) of the NB nude mice; by 50 weeks, a tumor incidence of 20% (2/10) was observed in the nude mice inoculated as adults (Fig. 2B). The majority (20/21) of tumors in NB and adult nude mice inoculated with HD 10–87 VERO cells appeared between 13 and 25 weeks indicating that the incidence of tumor formation was enhanced by HD serial passage. In these assays, tumor formation occurred only at the site of inoculation; no spontaneous tumors were detected in these animals during the course of the assay.

3.2. Rate of migration of VERO cells

In our initial study, we found that increased rates of 10–87 VERO cell migration in wound-healing assays correlated with their capacity to form tumors *in vivo* [28]. For our current study, we used wound-healing assays to examine the rates of migration of cell lines established from LD 10–87 VERO cells and HD 10–87 VERO cells at 10 passage intervals. After the monolayer was scratched with a pipette tip, the migration of cells into the wounded area were photographed every 3 h for 15 h. Representative photomicrographs are shown of wounded cell-culture monolayers at 0 and 12 h. Red arrows represent absence of cell migration into the wounds. The non-tumorigenic LD 10–87 VERO cells at p151 and tumorigenic LD 10–87 VERO cells at p256 were used as references lines for slowly and rapidly migrating cells, respectively. The tumorigenic LD 10–87 VERO cells at p256 started filling the wound around 9 h and completely closed the wound by 12 h, whereas little or no motility was observed with LD 10–87 VERO at p151 (Fig. 3A). When we tested LD 10–87 VERO cells at 10-passage intervals between p151 to p256, the cells displayed a progressive increase in migration rates from

Table 1
Expression levels of miRNAs in low density passaged VERO cells and A4497 VERO cells using Taqman quantitative RT-PCR[1].

miRNA	LD VERO (p154)	LD VERO (p174)	LD VERO (p194)	LD VERO (p254)	HP A4497 VERO
miR-376a	2.0	10.2	42.7	27.7	42.4
miR-654-3p	1.0	2.0	22.5	20.0	38.6
miR-543	2.0	5.2	34.6	25.0	26.2
miR-299-5p	2.9	4.3	31.2	22.7	43.6
miR-134	1.0	2.2	10.1	22.8	30.1
miR-369-3p	1.0	2.7	8.8	23.3	24.6

[1]Results from the triplicate samples were averaged following normalization to the levels of small RNA U6B. The data were presented as fold change over pAGMK miRNA levels. The ANOVA analysis of the miRNAs was significant with at least $p < 0.01$.

Table 2
Expression level of miRNA in high density passaged VERO cells using Taqman quantitative RT-PCR[1].

miRNA	HD VERO (p153)	HD VERO (p163)	HD VERO (p173)	HD VERO (p183)	HD VERO (p196)	HD VERO (p250)
miR-376a	1.1	2.5	2.0	12.0	25.9	56.4
miR-654-3p	1.8	2.4	2.5	8.2	17.2	34.9
miR-543	1.1	2.6	3.0	9.7	25.3	48.6
miR-299-5p	1.7	2.1	2.6	5.4	5.5	14.3
miR-134	1.0	1.2	1.3	2.1	1.6	6.1
miR-369-3p	3.5	3.9	3.6	4.9	5.0	19.0

[1]Results from the triplicate samples were averaged following normalization to the levels of small RNA U6B. The data were presented as fold change over pAGMK miRNA levels. The ANOVA analysis of the miRNAs was significant with a $p < 0.01$.

p165 to p186, and the wound was completely closed by LD 10–87 VERO cells at p194.

In a similar fashion, HD 10–87 VERO cells displayed a progressive increase in migration rate from p165 to p195 (Fig. 3B). The rate at which the HD 10–87 VERO cells migrate was somewhat faster than the LD VERO cells, since as the wound completely closed at p185 as opposed to p194 for LD VERO cells.

Both LD and HD VERO cells appeared to migrate predominantly as tightly packed sheets in a wound-healing assay. Since doubling times for both LD (26 h) and HD VERO (20 h) cells are greater than the assay time (12 h), it is unlikely that the differences in migration observed were affected by the rate of proliferation of the respective cells.

3.3. Signature miRNAs as potential biomarkers of the VERO cell tumorigenic phenotype

In our earlier study, 10 specific signature miRNAs were identified that correlated with the transition of LD 10–87 VERO cells from a non-tumorigenic phenotype at p148 to a tumorigenic phenotype at p256 [28]. The 10 signature miRNAs were differentially overexpressed in tumorigenic, high-passage LD 10–87 VERO cells compared with non-tumorigenic, low-passage LD 10–87 VERO cells. Based on their level of expression, six miRNAs (miR-376a, miR-654-3p, miR-543, miR-299-3p, miR-134 and miR-369-3p) were chosen for evaluation of their use as potential biomarkers to track the progression of neoplastic development in VERO cells. Using RNA samples prepared from LD 10–87 VERO cell banks established at every 10 passages from p150 to p254, the level of expression of the selected miRNAs was examined by quantitative RT-PCR. The expression levels of these miRNAs in non-tumorigenic LD 10–87 VERO cells (p154) were slightly above background levels of pAGMK cells. In contrast, the expression levels of these miRNAs increased progressively by at least 2-10 fold at p174 and by greater than 8-42 fold ($p < 0.01$) at p194 compared with pAGMK cells (Table 1). Higher than 20-fold levels of expression ($p < 0.01$) was sustained in LD 10–87 VERO cells at p250 and in A4497 ($p > 200$) VERO cells, which are tumorigenic in both newborn and adult nude mice [10]. Three of the six miRNAs (miR-376a, miR-543 and miR-299-3p) were overexpressed more than 4-10 fold compared with pAGMK control cells and the LD 10–87 VERO cell passages before the expression of the tumorigenic phenotype was detected at p194 (Table 1 and Fig. 1A). These results suggest that these miRNA-based biomarkers may be capable of predicting the pre-tumor stages of neoplastic development in VERO cells. To verify the accuracy and specificity of these results, we assessed the six miRNAs in HD VERO cells that were passaged independently at higher, confluent

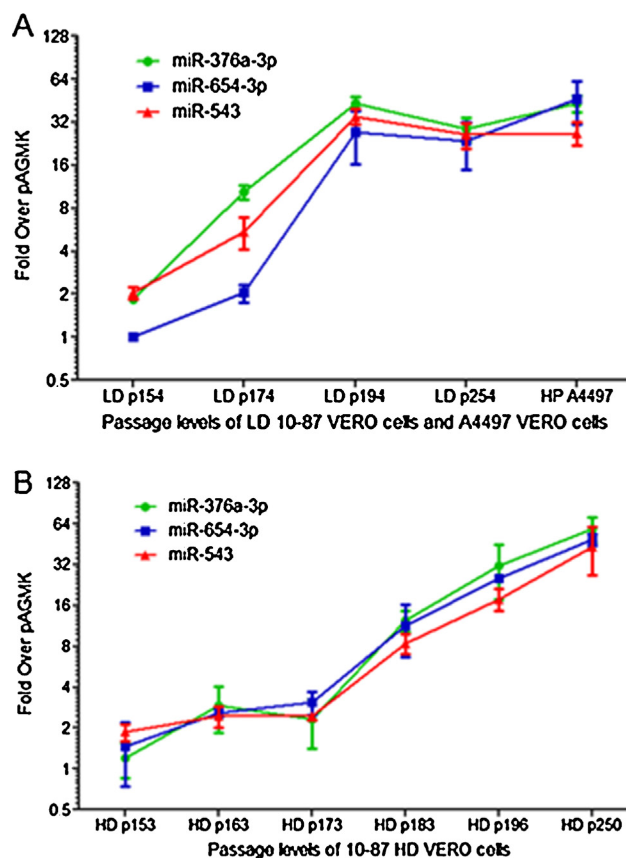

Fig. 4. Individual miRNAs can be used to detect the expression of tumorigenic phenotype in VERO cells. The relative expression levels of the miRNAs were measured by the Taqman probe-based qRT-PCR. The curves for (A) LD and (B) HD VERO cells were plotted to demonstrate the diagnostic value of each miRNA for detecting the expression of tumorigenic phenotype in VERO cells.

densities. The trend in the alteration of miRNA expression was generally similar between the LD 10–87 VERO cell lines and the HD 10–87 VERO cell lines. When compared with the pAGMK controls, five of these six miRNAs were over-expressed by greater than 4-fold in the tumorigenic HD 10–87 VERO cells at p183, and all six were over-expressed by 6- to >50-fold at p250 (Table 2).

To further evaluate the ability of individual miRNA to reflect the expression of the tumorigenic phenotype in VERO cells, we examined three miRNA data sets (miR-376a, miR-654-3P, and miR-543) from experiments shown in Tables 1 and 2. The expression pattern of each of these miRNA followed the progression of neoplastic development and peaked at p194 (Fig. 4A) where the ability of LD 10–87 VERO cells to form tumors was detected (Fig. 1). In HD 10–87 VERO cells, the same association between elevated expression levels of the same miRNAs and tumorigenicity was observed at p183; however, the expression levels in cells at p250 increased by an additional 4-fold compared with cells at p183 (Fig. 4B). Together, regardless of how the tumor-forming cells were established, whether by passaging at low density or high density, the individual miRNA expression pattern correlated with the detection of the tumorigenic phenotype. Therefore, these six miRNAs appeared to be biomarkers for this property of VERO cells.

4. Discussion

Managing the threats posed by emerging and re-emerging infectious diseases, such as pandemic influenza, call for the rapid production of large, possibly unprecedented, amounts of vaccines to immunize populations worldwide [31–33]. Current production methods may be insufficient to meet these demands in the short period required to manage pandemics successfully [33]. Cell-culture technology based on immortalized cell substrates provides a possible method for increasing the efficiency of vaccine manufacture and improving vaccine efficacy [1,3,6,8,31,32,34–37]. Regulatory agencies have recommended that the tumorigenic potential of immortalized cell substrates proposed for human vaccine production be evaluated (21 Code of Federal Regulations 610.18). Here, we have used VERO cells as the first model to demonstrate the proof-of-concept for miRNA profiling as a potential surrogate for tumorigenicity testing. As neoplastic progression leading to the development of the tumorigenic phenotype is driven by molecular alterations, the analysis of these molecular features could contribute to the prediction of the tumorigenic potential of cell substrates that evolve by spontaneous neoplastic development during cell culture.

MicroRNAs are short RNA molecules that have been shown to be important regulators of biological functions [38]. Aberrations in miRNA expression can affect important cellular processes like the cell cycle, cell proliferation, or cell death by apoptosis [39]. These processes are known to be involved in neoplastic development and hence provide a direct link to tumor initiation and progression. The use of tissue miRNA expression profiles as diagnostic or prognostic biomarkers in cancer has been demonstrated by several studies [24,40].

We have shown that specific miRNA signatures were identified that correlated with the transition of VERO cells from a non-tumorigenic phenotype (low-passage cells, p148) to a tumorigenic phenotype (high-passage cells, p256) during serial tissue-culture passage [28]. We also demonstrated that the signature miRNAs identified in VERO cells grown in FBS were the same as in the VERO cells adapted to grow in serum-free medium (SF-VERO). In the present study, we used quantitative RT-PCR to evaluate our initial observation that miRNA expression changes with the progress of neoplastic development of VERO cells during intervening passages. Compared with pAGMK cells, the expression levels of these signature miRNAs progressively increased in cells from LD 10–87 VERO cell banks established at every 10 passages from p151 to p256. Notably, the expression of these miRNAs peaked at p194 in LD 10–87 VERO cells.

The correlation of the six selected miRNAs for the tumorigenic phenotype of VERO cells was verified by assessing another independently-derived 10–87 VERO cell line that was developed by HD passaging. The trend in the expression of signature miRNAs was generally similar between the LD 10–87 VERO cell set and the HD 10–87 VERO cell set. Moreover, the over-expression of these miRNAs was also evident in another VERO cell line, A4497 VERO cells, that was also derived independently and has been shown to be tumorigenic in newborn and adult nude mice [10]. Similar to our previous report [28], the increased rate of migration of HD 10–87 VERO cells correlated with their capacity to form tumors in nude mice. The transition of non-tumorigenic phenotype to tumorigenic phenotype appeared to occur around p185 in 10–87 HD VERO cells and around p194 in LD 10–87 VERO cells. Phenotypic differences due to differential propagation of cell lines have been well documented with long-term sub-culturing resulting in divergent effects on morphology, proliferation, development, and gene expression [41–43]. Importantly, long-term propagation under high-density (as compared with sub-confluent) with extensive contact among cells have been shown to increase their saturation density, increase tumor incidence and decrease the latent period of tumor appearance after injection of cells into mice [43–45]. The HD 10–87 VERO cells formed tumors in NB and adult nude mice at p185 compared with p194 for LD 10–87 VERO cells in NB mice. Since doubling time for HD VERO cells was shorter (20 h) than LD VERO cells (26 h), it is conceivable that the faster proliferation rate, driven by selective pressures, may contribute to the enhanced tumor forming capacity of HD VERO cells. However, the association of signature miRNA over-expression appears to be related to the expression of the VERO cell tumorigenic phenotype rather than to the passage density or the reagents (tissue culture medium and serum) used for cell culture. This correlation between the passage at which the cells first expressed a detectable tumorigenic phenotype and the passage representing the peak expression levels of signature miRNAs illustrated that these miRNAs are potential biomarkers for the expression of the VERO cell tumorigenic phenotype.

A comparison of the miRNA expression patterns between tumorigenic VERO cells and its corresponding tumor tissue may provide additional evidence supporting the specificity of the miRNAs' expression patterns to the expression of tumorigenic phenotype in VERO cells. In the present study, signature miRNAs were not monitored in tumor tissue formed by injection of tumorigenic VERO cells. However, a cell line established from a tumor formed from LD VERO cells at p250 had the same pattern of miRNA expression as the inoculated LD VERO cells [28]. Moreover, individual miRNAs such as miR-376a have been reported as highly expressed in different cancer tissues and cells when compared with the corresponding normal tissues and cells [28,46–52]. Thus, the concordance between the expression of signature miRNAs and the miRNAs previously identified in other tumor tissues suggests that these miRNAs are involved in the process of neoplastic development in VERO cells. Although individual miRNAs alone can be considered for use as a test for tumorigenic potential of VERO cells, the diverse and complex molecular events involved in the initiation and development of neoplasia argues against the use of individual miRNAs as tumor biomarkers. Thus, we propose that these six miRNAs be used as a panel of biomarkers for tumorigenic VERO cells, as the combination of these miRNAs may reflect various aspects of tumorigenesis and form a more complete indicator of the VERO cell tumorigenic phenotype.

Understanding how these six miRNAs contribute to the neoplastic progression of VERO cells and their ability to form tumors would

contribute to their usefulness as biomarkers for the expression of the VERO cell tumorigenic phenotype. Even though there are no data on the functions of these miRNAs in VERO cells, information on their chromosomal locations and functions in other species is available. All 6 of the miRNAs are located on human chromosome 14, and 4 of these 6 (miR-376a, miR-654-3P, miR-543, miR-229-5P) are found within the same 10 kb region of the chromosome. Three of the 6 miRNAs (miR-299-3P, miR-134, miR-369-3P) are up-regulated in human and murine embryonic stem cells [53–55], suggesting a role in cellular dedifferentiation. Dedifferentiation has been found to be the first step in the repair of renal epithelium that occurs *in vivo* after acute kidney injury and in renal cells in primary culture [56,57]. As the expression of the 6 miRNAs increases to their maximum levels after 170–180 passages of VERO cells in concert with the expression of their tumorigenic phenotype, we speculate that changes in miRNA expression up to and during these tumor-forming passage levels occurs as a component of the VERO cell dedifferentiation processes involved in the expression of the tumorigenic phenotype. Studies are underway to identify the molecular pathways that might be altered by the over-expression of these signature miRNAs in our VERO cell model.

In conclusion, with the goal of learning more about tumorigenesis and reducing the use of animals for characterizing the neoplastic phenotype, we have demonstrated that profiling miRNA expression predicts the tumorigenic potential of VERO cells as it evolves during cell culture. Our observations point to a potential link between miRNA profiles expressed in tumorigenic VERO cells and tumor formation *in vivo*, thereby indicating that miRNA profiling offers promise as a surrogate for expression of VERO cell tumorigenic phenotype. Having a molecular assay for the evaluation of the ability of immortalized cell substrates to form tumors *in vivo* would provide a quick and relatively inexpensive method for detecting the expression of the VERO cell tumorigenic phenotype. The identification of appropriate biomarkers could expedite the review of vaccines manufactured in new immortalized mammalian cells. While the relevance of the identified miRNA biomarkers was shown here for the 10–87 VERO cells that are being used as cell substrates for licensed products, such biomarkers could be useful for the development of new cell lines from the original VERO cell line or for the development of new lines of African green monkey cells for vaccine manufacture; furthermore, they may help reduce animal testing.

Disclaimer

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

Acknowledgements

We thank members of our laboratories for advice and discussions. We also extend our thanks to Drs. Steve Feinstone, Robin Levis, and Carol Weiss for helpful discussions and/or comments on the manuscript. This work was supported by Division of Microbiology and Infectious Diseases, NIAID, NIH; Medical Countermeasures Initiative (MCMi), (FDA); Division of Viral Products, Center for Biologics Evaluation and Research, FDA and the Oak Ridge Institute for Science and Education.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.05.065.

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