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ORIGINAL ARTICLE

Potential neoplastic evolution of Vero cells: in vivo and in vitro characterization

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Abstract Vero cell lines are extensively employed in viral vaccine manufacturing. Similarly to all established cells, mutations can occur during Vero cells in vitro amplification which can result in adverse features compromising their biological safety. To evaluate the potential neoplastic evolution of these cells, in vitro transformation test, gene expression analysis and karyotyping were compared among low- (127 and 139 passages) and high-passage (passage 194) cell lines, as well as transformed colonies (TCs). In vivo tumorigenicity was also tested to confirm preliminary in vitro data obtained for low passage lines and TCs. Moreover, Vero cells cultivated in foetal bovine serum-free medium and derived from TCs were analysed to investigate the influence of cultivation methods on tumorigenic evolution. Low-passage Vero developed TCs in soft agar, without showing any tumorigenic evolution when inoculated in the animal model. Karyotyping showed a hypo-diploid modal chromosome number and rearrangements with no difference among Vero cell line passages and TCs. These abnormalities were reported also in serum-free cultivated Vero. Gene expression revealed that high-passage Vero cells had several under-expressed and a few over-expressed genes compared to low-passage ones. Gene ontology revealed no significant enrichment of pathways related to oncogenic risk. These findings suggest that in vitro high passage, and not culture conditions, induces Vero transformation correlated to karyotype and gene expression alterations. These data, together with previous investigations reporting tumour induction in high-passage Vero cells, suggest the use of low-passage Vero cells or cell lines other than Vero to increase the safety of vaccine manufacturing.

Keywords Vero cells · Transformation · In vitro test · Tumorigenicity · In vivo test · RNAseq

N. A. Andreani and S. Renzi have contributed equally to the study.

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Introduction

A wide variety of cell cultures are used as the substrate for the production of relevant biologicals such as viral vaccines for human and veterinary applications. Cell

substrates for this specific aim can be primary, diploid, stem or continuous cell lines, derived from physiologically normal, abnormal or malignant tissues (Aubrit et al. 2015). Normal tissue-derived cells can anyway undergo several modifications during in vitro cultivation, resulting in the appearance of novel biochemical, biological and genetic characteristics that differ from primary or diploid cells. In particular, these cells could acquire genetic instability as well as tumorigenic properties with increasing passages (Sheets 2000; Aubrit et al. 2015). The WHO Expert Committee reported tumorigenicity of a cell line as the ability to induce tumour formation after injection in immunocompromised animals (WHO Technical report Series, No 978, 2013, Annex 3). In fact, the utilization of tumorigenic cell lines might be involved in the transmission of tumour allographs, transforming proteins or oncogenic agents inducing tumour initiation in the recipient (Aubrit et al. 2015).

In this respect, it is mandatory for vaccine production and safety assessment a fine characterization of the cell substrate in each phase of its manipulation. European Pharmacopeia recommends a panel of tests to assess cell substrate safety (Cell substrates for the production of vaccines for human use. European Pharmacopoeia 2013, Ed. 08 Chapter 5.2.3), as the use of tumorigenic cell lines for vaccine production is forbidden. Among these, to avoid potential risks related to cell transformation and tumorigenicity, both in vitro and in vivo tests are required to evaluate cell growth characteristics and reveal the onset of tumorigenic properties.

Vero is a well-known immortalized cell line, used as substrate for virus isolation and production of vaccines, i.e. *Poliovirus,* rabies virus (Montagnon 1989), influenza (Govorkova et al. 1996; Barrett et al. 2011, 2013), and Japanese encephalitis virus (Schuller et al. 2011), due to its susceptibility to a wide range of viruses (Rhim et al. 1969; Teferedegne et al. 2014). Vero cells were originally collected from the kidney of a normal adult African Green Monkey (*Cercopithecus aethiops*) kidney (AGMK; Yasumura and Kawakita 1963) and immortalized through a spontaneous, unknown process (Swanson et al. 1988; Manohar et al. 2008). On the basis of CBER (Centre for Biologics Evaluation and Research) classification, Vero cells are included in neoplastic substrate category 3, in which spontaneously immortalized non-human primate cells are grouped (US FDA 2001).

Previous studies demonstrated that low-passage (p140) Vero cells are not able to form tumours in vivo, neither show the formation of extraneous agent in the host, while progressively growing nodules and lung and lymph node metastases were observed at higher passages (>p200; Manohar et al. 2008). Levenbook et al. demonstrated nodule formation in the totality of mice inoculated with Vero cells at passages higher than 232 (Levenbook et al. 1984), while no tumour formation was observed in nude mice inoculated with p156 Vero cells by Swanson et al. (1988). On the other hand, in vitro assay gave rise to not completely clear results, since Vero cells from p127 to p140 and from p162 to p265 formed colonies in soft agar (Petricciani et al. 1987).

These findings, taken together, indicated Vero cells as a suitable and safe biological resource for vaccine production only at low passages, but data are still controversial due to the lack of consistence between in vitro and in vivo tests. Moreover, the mechanism inducing neoplastic transformation in Vero cells remains an elusive point (Manohar et al. 2008).

The application of serum-free media has become established recently to cultivate cells without the addition of animal derivatives. Despite the positive aspects of serum addition to media (related to cell attachment and growth), the employment of serum in cell cultures has some disadvantages (Chen and Chen 2009), such as the uncertainty of the composition and the putative contamination with adventitious agents. Previous studies demonstrated the ability of Vero cells to grow in animal serum-free medium while maintaining their permissiveness to viral propagation (Frazatti-Gallina et al. 2004; Chen et al. 2011), suggesting the applicability of serum-free medium in vaccine manufacturing. To date no data are available on the correlation between culture conditions and neoplastic phenotype evolution in Vero cells.

The present study aimed to characterize in vitro amplified Vero cells at low and high passages. Additionally, normal and serum-free growth conditions were tested. Transformation ability of low and high-passage cells was investigated by soft agar transformation assay. Moreover, p127 and p139 Vero cells were tested for tumorigenic phenotype by nude mice inoculation. With the aim of shedding light on the mechanisms inducing the neoplastic phenotype, cell lines were analysed through karyotyping and gene expression study. To study the behaviour of the in vitro

transforming samples, six of the soft agar transforming colonies were amplified, evaluated by inoculation in soft agar and in nude mice and examined by karyotyping.

Materials and methods

Cell culture

Vero cells (African green monkey kidney, IZSLER Cell Bank code BS CL 86) were received from ATCC (American Type Culture Collection) at p124 and grown in MEM culture medium (Eagle's Minimum Essential Medium in Earle's BSS) (Sigma Aldrich, Milano, Italy) enriched with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich) and 2 mM L-glutamine (Sigma Aldrich). From p127 they were grown concurrently in serum-free medium. XerumFree™ XF205 Medium Supplement (TNC BIO, Eindhoven, Holland) was gradually added to the culture medium in substitution of FBS, starting from 50% XerumFree-supplemented medium/50% FBS-supplemented medium, according to manufacturer instructions. Cultures adapted to serum-free conditions were amplified in medium supplemented with Epidermal Growth Factor (EGF; 12.5 µg/L, Sigma Aldrich) and insulin (1.25 mg/L, Sigma Aldrich). At each passage, cells were mechanically scraped and incubated in a mixture of 75% fresh medium and 25% conditioned medium, collected during the previous passage.

HEp-2 (Human larynx epidermal carcinoma, BSTCL 23) and MRC-5 cells (Human foetal lung, BSCL 68) (both from the IZSLER biobank), were grown in MEM culture medium supplemented with 10% FBS and 2 mM L-glutamine. These cell lines were used respectively as positive and negative control in tumorigenicity and transformation assays.

A primary cell culture (passage 3) of a normal adult African Green Monkey Kidney (AGMK, *Cercopithecus aethiops*) was kindly provided by Dr. Brandini (Novartis Vaccines Italia, Siena, Italy) and grown in MEM culture medium enriched with 10% FBS, 1% Penicillin/Streptomycin and 2 mM L-glutamine. This cell line was used as reference in the in vitro transformation assay and karyotype analyses. Figure S1 summarizes cell lines used in each investigation reported hereunder.

In vitro transformation assay

Vero (from p127 to p139 and p194), as well as AGMK, MRC-5 and Hep2 negative and positive control cells were assayed for in vitro transformation according to European Pharmacopoeia guidelines, by seeding cell lines in semi-solid agar medium (Macpherson and Montagnier 1964). Briefly, 1% noble agar (Sigma Aldrich) was mixed with 50% of 2× MEM, supplemented with 20% FBS and stratified into 6-well plates (3 ml/well). 10^5 p139 Vero cells were diluted in 1 mL of 20% FBS-MEM (v/v) and 0.6% noble agar and gently layered onto solidified agar. Plates were incubated at 37 °C in 5% CO_2 for 3 weeks and inspected daily to detect TCs. To assess serum free-culturing conditions, the same cells were grown in medium supplemented with Xerum-free instead of FBS before in vitro transformation assay.

Isolation and amplification of Vero transformed colonies (TCs)

Six TCs developed in the soft agar assay, originated from p139 Vero, were collected under sterile conditions and disaggregated mechanically. Cells derived from each single colony were inoculated in a well of a 48-well plate and grown in the previously described medium. When cells reached 80%-confluence, they were serially amplified and characterized as described below.

In vivo tumorigenicity test

The choice of the animal model for in vivo tests was based on the results of published investigations reporting the validity of experimental data produced on adult nude mice (Swanson et al. 1988; Zhang et al. 2004; Manohar et al. 2008) and on European Pharmacopoeia guidelines.

Vero cell samples (at p127 and p139, serum-added and serum-free cultured), as well as the six TC samples and positive (Hep-2) and negative (MRC-5 and AGMK) controls were inoculated into nude mice to verify tumorigenic potential in vivo. According to Directive 2010/63/EU and the 3Rs principle stated by Russell and Burch (1959), the high passage (p194) Vero cells were not included in the in vivo test, as they were previously demonstrated to induce tumour formation in nude mice (Petricciani et al. 1987).

In vivo tests were performed as described in European Pharmacopoeia guidelines in 20-day-old male athymic mice (Nu/Nu genotype), received from Harlan Laboratories (Milan, Italy) in accordance with local animal welfare guidelines. Ten mice per treatment (Vero p127, Vero p139, the six TCs and positive and negative controls) were used. They were subdivided into groups of 5 mice/cage, maintained at the IZSLER Division of Laboratory Animal on sterile bedding and given water and feed ad libitum. Animals were injected subcutaneously into the abdominal wall with a 10^7-cell suspension in 0.2 mL of volume. Five mice were sacrificed by CO_2 inhalation three weeks after the treatment, while the others were inspected daily for 12 weeks. The regional lymph nodes, lung, brain, spleen, kidney, liver and the injection site were examined post-mortem to detect tumours, for histological examination and haematoxylin-eosin staining.

Cell karyotype evaluation

Cytogenetic studies were performed on chromosomes derived from AGMK (control cell line) and Vero cells collected at different passages (from p127 to p139 and p194), cultured both with FBS and Xerum-free supplement. Cell lines derived from the six Vero-transformed colonies (TCs) were also analysed.

Chromosome preparations were obtained according to standard cytogenetic techniques. Cytogenetic analysis was performed using Quinacrine staining (0.05 mg/mL Quinacrine Mustard Dihydrochloride $C_{23}H_{28}Cl_3N_3O$ 2HCl, Sigma-Aldrich, Milan, Italy) and analysing an average of 20 metaphases per sample. Karyotypes were compared with the normal primate karyotype (Finaz et al. 1976).

Gene expression analysis

Differences in gene expression were investigated comparing low (p127, p134) and high (p194) culture passages of Vero cells cultured in the presence of FBS. Total RNA was extracted from 10^7 cells using RNeasy Mini Kit with a QIAcube platform (Qiagen, Milan, Italy) according to the instructions of the manufacturer. RNA quantity and quality were assessed by a 2200 TapeStation RNA Screen Tape device (Agilent, Santa Clara, CA, USA) and a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE, USA), respectively.

Libraries were prepared with the TruseqRNA sample prep kit (Illumina, Inc. San Diego, CA, USA) following manufacturer's protocol and their evaluation was made with a Tape Station 2200. Indexed libraries were quantified by Picogreen (Life Technologies, Monza, Italy) and then normalized to 10 nM for cluster generation on a Hiseq2000 (Illumina). Equimolar amounts of each library were mixed before NaOH denaturation and pooled samples were run in a total of two lanes of a Hiseq Flowcell (Illumina).

The Truseq PE cluster kit v3 was used to generate clusters on the grafted Flowcell and the hybridized libraries were sequenced on a Hiseq 2000 with a 100 cycles of paired-end sequencing module using the Truseq SBS kit v3.

RNA-seq data analysis

Standard trimming was performed using Trimmomatic software, to remove the adapters and check the quality of the reads (Bolger et al. 2014). Only RNA-seq reads that passed the trimming procedures were mapped to "Vervet Monkey" reference genome (Green monkey chlSab1, Jun 2013, Chlorocebus_sabeus 1.0/chlSab1, Vervet Genomics Consortium GCA_000409795.1) using Star aligner, with default parameters (Dobin et al. 2013). Alignments were sorted using Samtools software (Li et al. 2009). For each sample, the number of reads mapped into each specific gene was calculated using htseq-count program (Anders et al. 2015).

Differential expression among pairwise comparisons was analysed using the edgeR package (Robinson et al. 2010). EdgeR permits to estimate a common dispersion to the theses to be compared even in the absence of biological replicates (Bioconductor; Huber et al. 2015). Hence, edgeR permits the statistical analysis of data lacking replicates and conduct exact tests of significance for the negative binomial distribution in pairwise comparisons.

A multiple testing correction was applied to determine the false discovery rate (FDR; Reiner et al. 2003). Genes with a FDR-adjusted p value (q value) of ≤ 0.05 and log fold change lower than -3 and higher than 3 were considered to be Differential Expressed Genes (DEGs). Groups of genes significant in a single or in multiple comparisons have been graphically represented by Venn analysis.

Ontology and clustering of differentially expressed genes

Clustering of gene expression levels in each sample and differential gene expression in pairwise comparisons were also produced for DEGs identified by contrasting p127, p134 and p194 Vero cells and visualized as heatmaps and dendrograms. Dendrograms were generated with Euclidian distance as measure of dissimilarities and complete linkage as agglomeration method using *dist* and *hclust* function implemented in R packages. Gene Ontology (GO) analysis was carried out using g:Profiler, a web server for functional interpretation of gene list (Reimand et al. 2016).

Results

In vitro transformation assay

Foci formation took place for HEp-2 cells, used as positive control (Fig. 1b), and all Vero cell lines. Foci appeared 7 days after the inoculum and gradually increased in both number and size. An example is reported in Fig. 1a, reporting cells at passage 130. TCs isolated from p139 Vero cells and assayed for in vitro transformation also produced foci of transformed colonies.

No significant difference was observed among samples at different passages and culture conditions. Results are summarized in Table 1 and Figure S1.

Conversely, no transformed colony was observed in the negative control MRC-5 and AGMK cultures, where cells remained unaltered during all the observation period (Fig. 1c).

In vivo tumorigenic test

Both inoculation of MRC-5 cells (negative control) and Vero cells (at passage p127, p136 and TCs in both culture conditions) did not induce tumour formation during the observation period. Results of the in vivo tumorigenicity are summarized in Table 1 and Figure S1. No macroscopic lesion and inflammatory process were observed in the treated animals and the inoculum was re-absorbed completely within few days (an average of 7 days). The necropsy detected no tumour formation at the site of inoculation neither in other organs and tissues, with no macroscopic lesions. These observations were confirmed by histological examination. Mice showed hyperkeratosis, moderate lymphoplasmacellular enteritis and dismicrobism bowel, pulmonary bleeding and rare intracranial bleeding, but neither microscopic anomalies nor neoplastic cells were observed in all tissues examined.

In contrast, tumours were detected in all mice receiving HEp-2 cells. In particular, nodules were observed at the inoculation site about 10 days after the injection. They appeared smooth, uniform and globular (10 mm Ø); later they developed a multi-globular shape and increased in size (20 mm Ø). At about 30 days after the injection, the mice were sacrificed to avoid animal pain and suffering. At necropsy, no other macroscopic alterations in organs and tissues were detected. The histological examination of the mice injected with HEp-2 cells showed the presence of polygonal cells in subcutaneous and dermal tissues; the nuclei of such cells were irregular in shape, with evident nucleoli (atypical mitosis); moreover, neoplastic cells were observed in the vessels. These alterations were restricted to the inoculum site in all the animals and no metastases were found.

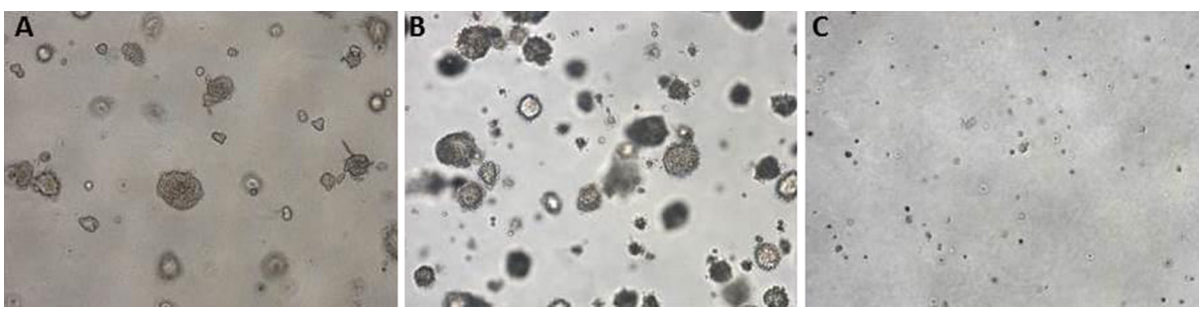

Fig. 1 In vitro transformation assay. Transformed colonies produced in soft-agar by Vero (p130) and HEp-2 cell lines (positive control) are shown respectively in **a**, **b**, while results from AGMK (one of the two negative controls) are reported in **c**

Table 1 Summary of the main results of the study

	In vitro transformation[a]	In vivo tumorigenicity	Karyotyping[a,b]	RNA-seq
p127 Vero	+	–	56	No enrichment in genes involved in tumorigenesis
p134 Vero	+	–	56	
p194 Vero	+	+[c]	56	
TCs	+	–	56	NP
p3 AGMK	–	–	60	NP
Hep-2	+	+	NP	NP
MRC-5	–	–	NP	NP

NP not performed

[a] In vitro transformation and karyotyping were performed on cells from p127 to p139. For sake of simplicity, table reports only data of lines that undergo also other steps of the investigation

[b] The observed modal chromosome number based on the observation of 20 metastases

[c] Result based on Petricciani et al. 1987

Cell karyotype evaluation

AGMK cell line karyotype showed normal diploid number of chromosomes ($2n = 60$; Finelli et al. 1999). All analysed Vero samples at different passages showed hypodiploid chromosome count. The modal chromosome number was 56 with a range from 54 to 58 (as reported on the ATCC site) occurring in 75% of cells. In most cells, over 50% of the chromosomes in each metaphase were structurally altered marker chromosomes. The rate of cells with higher ploidies was 2%, while different chromosomes were present in single copy in different cell cultures. The chromosomal asset of Vero cell lines was stable without significant differences between the assayed in vitro passages (Fig. 2). The chromosome analysis of TC colonies, originated from p139, and of serum-free cultivated Vero cells, showed the same abnormal karyotype. Results of karyotyping are summarized in Table 1.

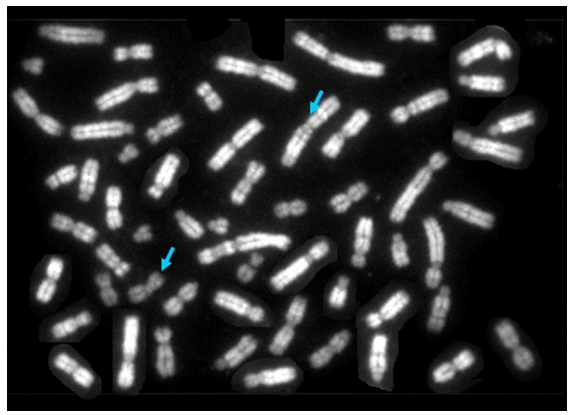

Fig. 2 Metaphase of p127 Vero. The *arrows* show two of the rearranged chromosomes

Gene expression analysis

Gene expression data revealed that some of the 20.126 unique genes identified, were expressed only in one of the passages analysed. Specifically, a total of 89 (4.42%), 175 (8.70%) and 220 (10.93%) genes were found exclusively expressed in p127, p134 and p194 respectively, and not elsewhere.

Comparing the three Vero passages, 350 genes were found differentially expressed in one or more pairwise comparisons. Among these, 41 were DEGs between p127 and p134 Vero cells, and 309 between these two lines and p194 Vero. No gene was differentially expressed in all comparisons (Fig. 3).

The logarithm of the normalized expression level of the 309 genes differentially expressed comparing the high passage (p194 Vero) to low passages (p127 Vero and p134 Vero) are shown in Fig. 4. DEG analysis indicated that p127 Vero and p134 Vero have very similar gene expression (lane 1). Additionally, the comparison between p127, p134 and p194 Vero cells (lane 2 and 3) indicates that p194 Vero cells have a larger number of down-regulated genes compared to up-regulated ones as 231 and 228 genes out of 309 were under-expressed in p194 Vero compared to p134 and p127 cells, respectively.

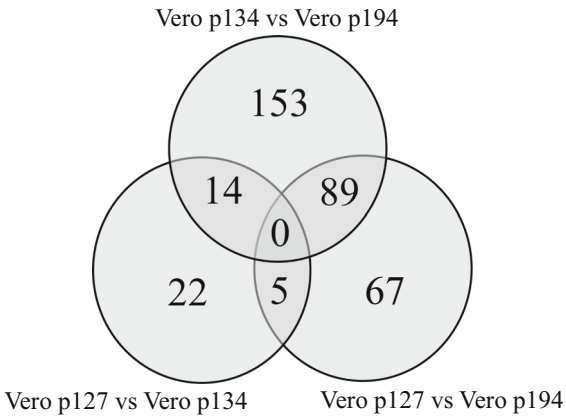

Fig. 3 Differential expressed genes in pairwise comparisons among the three Vero lines tested. Venn diagrams report graphically the number of DEGs in single and multiple comparisons of the indicated Vero passages. Intersections define groups of DEGs resulted common to two or more comparisons

Table S1 reports values of log fold-change of expression in pairwise comparisons.

Function of differentially expressed genes

The main biological processes identified by GO analysis were relative to response to corticotropin-releasing hormone, response to growth factor and sprouting angiogenesis. GO analysis showed no significant enrichment of DEG in metabolic pathways relative to oncogenesis.

Discussion

Vero cells are commonly used in human and veterinary vaccine production and their safety has been widely investigated. A particular concern is related to the tumorigenicity of this cellular substrate, since a potential tumorigenic evolution of in vitro-maintained Vero cells (passages from 131 to 227) has been reported by many authors (Van Steenis and van Wezel 1981; Contreras et al. 1985; Furesz et al. 1989; Zhang et al. 2001). Indeed, data are still controversial because other studies showed that these cells did not acquire tumorigenic features at passages higher than 140 (Johnson et al. 1981; Levenbook et al. 1984; Swanson et al. 1988).

As mentioned above, transforming phenotype (defined as the ability of cells to proliferate without undergoing the common proliferative controls; Hoff et al. 2004) and tumorigenicity (the ability of cultured cells to originate progressively growing tumours) can be respectively evaluated in vitro (soft agar assay) and in vivo (inoculation in nude mouse).

Among in vitro tests, the soft-agar assay is widely applied, as it is an easy and low-cost test recommended by the European Pharmacopoeia to assess cellular substrates safety. Regarding the in vivo test, two different animal models are used: rat and mouse. In particular, Van Steenis and van Wezel (1981) showed that anti-thymocyte globulin (ATG)-treated new-born rats developed tumours at the inoculation site and metastases, demonstrating that they were more sensitive than nude mice. In addition, the results of another study on athymic nude mice, suggested a possible correlation between the chromosome abnormalities of cell lines and their tumorigenic ability (Zhang et al. 2004).

Concerning our research, different methodologies were used to assess the tumorigenic potential of Vero cell lines at different passages, cultivated on different media and of TCs, with the aim of investigating the safety of this biological substrate commonly applied in vaccine manufacture. Moreover, after the assessment of the tumorigenic phenotype, karyotyping and RNASeq experiments were carried out to search for biological alterations correlated to transformation.

Low- (from p127 to p139), high-(p194) passage Vero cells, as well as TCs, maintained in serum-supplemented and serum-free medium, were able to develop transformed colonies in the soft agar semi-solid medium with consistent timings and amounts, suggesting a common transformed genetic pattern. This result confirmed previous observation at the Cell Culture Centre of TC development in Vero cell cultures starting from different batches and passages.

In nude mice inoculated with p127 and p139 passages of Vero cells cultured with and without serum, no tumour was detected at the site of injection and cells were absorbed rapidly. These results are consistent with other studies that confirmed the absence of tumour formation at low Vero passages (Levenbook et al. 1984; Manohar et al. 2008).

The absence of correlation between in vitro and in vivo results contrasts what was found in a previous study, in which different passage of cells (p146–p227, Furesz et al. 1989) were positive in both tests. One possible reason of this inconsistency could be the

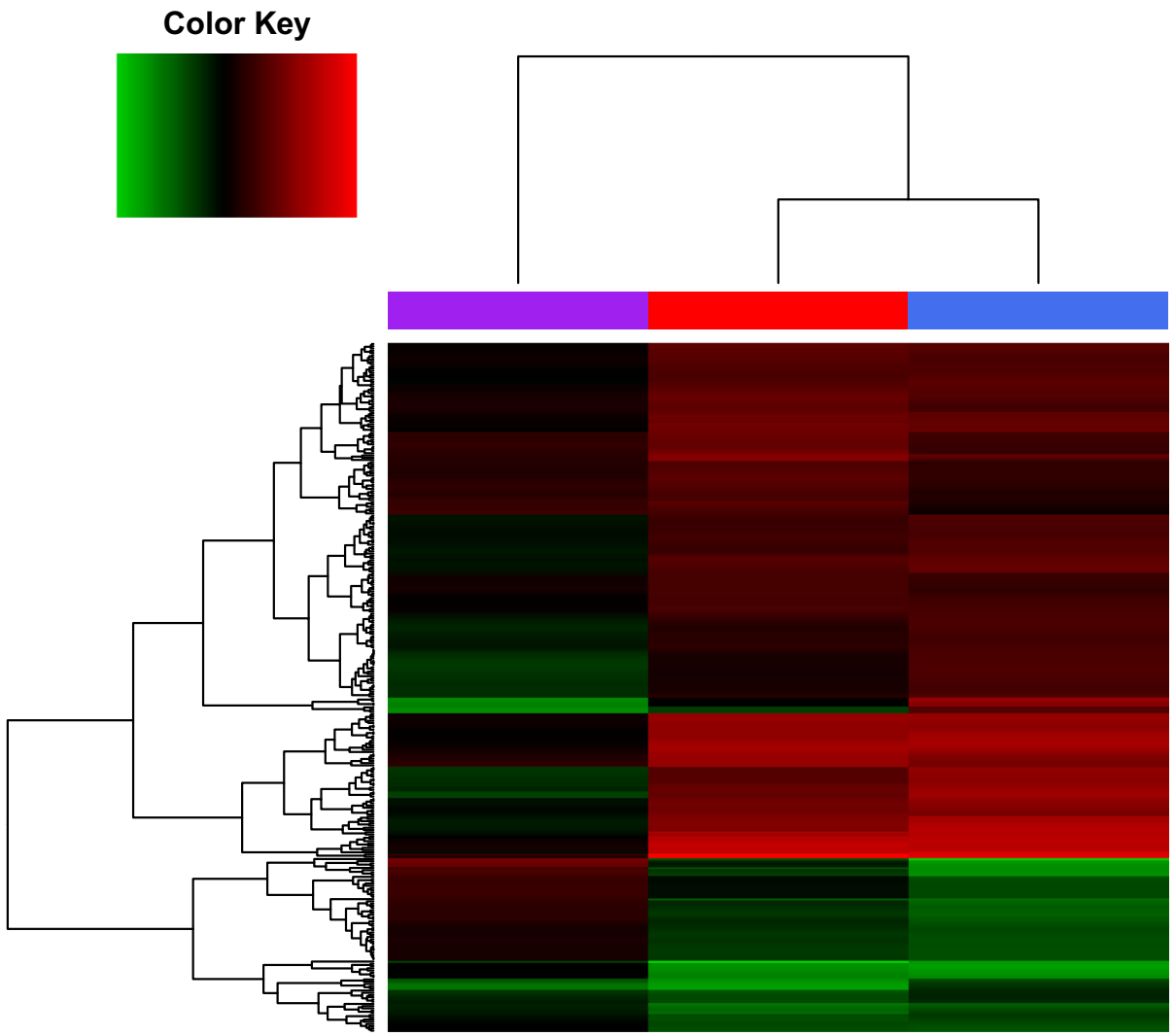

Fig. 4 Heat map reporting log fold change of the DEGs identified in p127 Vero–p134 Vero and p134 Vero–p194 Vero comparisons. Log fold-changes are depicted with a colour scale where red represents the up-regulated genes and green represents the down-regulated ones. Up- and down-regulation is referred to the first term of the comparison reported in the label at the *bottom* of the figure. Rows and columns are sorted on the basis of cluster analysis of gene expression similarities. The heat map was generated in R

limited time length of the investigation, which lasted 84 days (12 weeks). However, this period is beyond the limit of 69 days suggested by Manohar et al. (2008) as a lower limit by which tumour formation can be observed in adult nude mice. Additionally, Furesz et al. (1989) reported tumour formation only after 21 days of observation. These results suggest that the tumorigenic phenotype evolution is not correlated only to passage numbers, but to the target of genetic modification Vero cells have undergone during culturing.

Tumour-forming ability in nude mice may be associated to chromosome number variation. All samples of Vero cells showed a modal chromosome number (56, range 54–58) lower than the normal chromosome number of the AGMK ($2n = 60$; Finelli et al. 1999). No other chromosomal abnormality was detected by cytogenetic analyses in the cells amplified serially, which were devoid of any malignant appearance.

Gene expression is influenced by cell immortalization and in vitro culture (Ma et al. 2012; Dequéant

et al. 2015; Garcia-Mesa et al. 2016). In particular, the number of in vitro passages seems to influence gene regulation. Specifically, close passages induced differences in the expression of few genes (41 between p127 Vero and p134 Vero), while 4–6 fold increase in the number of differentially expressed gene are shown comparing p194 Vero with either p127 Vero or p134 Vero. If this pattern is common to long-term culture of all cell lines or specific to the Vero cells is to be investigated. Also it is presently unknown if gene expression gradually changes during passages or if a threshold exists beyond which cells start to change gene regulation.

However, the analysis of gene ontology and function of differentially expressed genes did not reveal changes that justify the tumorigenicity of p194 Vero cells observed in other investigations (Petricciani et al. 1987). Interestingly, Vero p194 showed a rather large cluster of downregulated genes and only few upregulated ones, when compared to the earlier passages; in addition GO of DEGs revealed no evidence of significant enrichment in cancer pathways.

In summary, we found no significant difference among low (p127 and p134) and high (p194) Vero passages in terms of transformation ability and karyotype. Differences in gene expression were detected, but metabolic pathways affected do not appear to be correlated to tumorigenesis. The choice of not testing in vivo p194 was undoubtedly a limitation, as the tumorigenicity of this passage at Cell Culture Center was inferred on the basis of external references. Joining our with existing results suggests that Vero are potentially tumorigenic cells, able to form TC even at low passages. The switch between these two states depends on random events and mechanisms that so far have not been identified but whose probability increases with the number of passages.

Therefore the use of Vero cells for biological production (such as *Poliovirus* vaccine manufacture) is suggested at the lowest possible available passage (Aubrit et al. 2015) to minimise risk. Such limitation strongly suggests to move towards already available alternative cell lines, such as FRhK-4 and 4647 lines in *Poliovirus* vaccine production (Dotti et al. 2017), as well as the so-called "designer" cell lines, as proposed by Brown and Mehtali (2010).

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