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

N.A. Andreani[a”], S. Renzi[a”], G. Piovani[b], P. Ajmone Marsan[c], L. Bomba[c], R. Villa[a]*, M. Ferrari[a], S. Dotti[a]

[a]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi 9, 25124 Brescia. [b]Department of Molecular and Translational Medicine, University of Brescia, V.le Europa 11, 25123 Brescia, Italy. [c]Università Cattolica del Sacro Cuore di Piacenza, Italy.

”: These authors equally contributed to the study

*Corresponding Author: riccardo.villa@izsler.it. Tel.: +390302290248 fax: +390302290392.

ORCID:

N.A. Andreani 0000-0003-0330-9874

S. Renzi 0000-0001-5863-2105

G. Piovani 0000-0003-0001-6435

P. Ajmone Marsan 0000-0003-3165-4579

L. Bomba 0000-0002-6290-8984

R. Villa 0000-0001-8385-800X

M. Ferrari 0000-0001-7949-651X

S. Dotti 0000-0002-2303-2027

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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.

Introduction

A wide variety of cell cultures are used as the substrate for the production of relevant biologicals such as viral vaccines with 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 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 (Shuller 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 and colleagues 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 is 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.

Material 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) enriched with 10% Fetal Bovine Serum (FBS) and 2 mM L-glutamine. From p127 they were grown concurrently in serum-free medium. XerumFree™ XF205 Medium Supplement (TNC BIO, Eindhoven, Holland) was gradually added to cultural 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, Milano, Italy) 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), 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% agar noble was mixed with 50% of 2X 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 sub-divided into groups of five 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 C23H28Cl3N3O 2HCl, Sigma-Aldrich, Milan, Italy) and analysing an average of twenty 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), respectively.

Libraries were prepared with the TruseqRNA sample prep kit (Illumina, Inc. San Diego, CA) 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 Hiseq2000 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). 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 figure 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 treated animals and the inoculum was re-absorbed completely within few days (an average of seven 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.

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 in 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 resulted stable without significant differences between the assayed *in vitro* passages (Figure 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.

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 Figure 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.

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 undergo 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 found in a previous study, in which different passage of cells (p146-p227, Furesz et al. 1989) were positive to both tests. One possible reason of this inconsistency could be the limited time length of the investigation, lasted 84 days (12 weeks). However, this period is beyond the limit of 69 days suggested by Manohar et al. as a lower limit by which tumour formation can be observed in adult nude mice (2008). Additionally, Furesz et al. reported tumour formation only after 21 days of observation (1987). 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 to 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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Figure legend

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 figure 1A e 1B, while results from AGMK (one of the two negative controls) are reported in figure 1C.

Fig. 2 Metaphase of p127 Vero

The arrows shows two of the rearranged chromosomes.

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.

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.

Fig. S1: Graphical representation of the experimental design and major results of the study. Dots in the *in vitro* section means positivity to the test. The red circle in the *in vivo* experiment means tumour formation after inoculum of the cell line in nude mice.

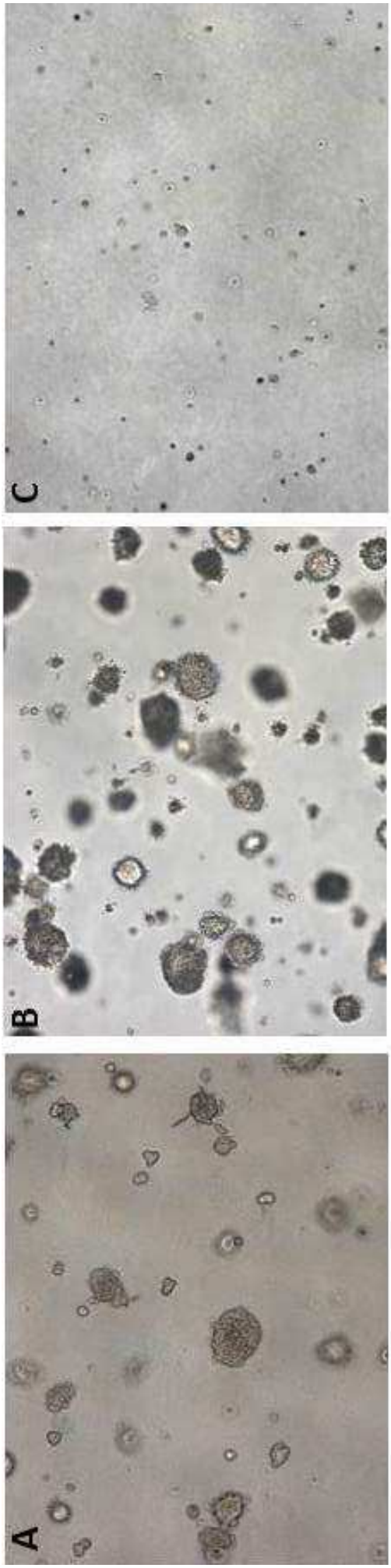
Table legend

Tab. 1: Summary of the main results of the study.

Tab. S1: Log fold-change of DEGs in pairwise comparisons. the first column reports feature IDs obtained in the RNA-seq experiment. Columns 2, 3 and 4 show log fold-change values of each pairwise comparison. A positive value means higher expression in the first term of the comparison; on the contrary, a negative value means higher expression in the second term of the comparison.

Figure 1

Click here to download Figure Fig1.eps.eps



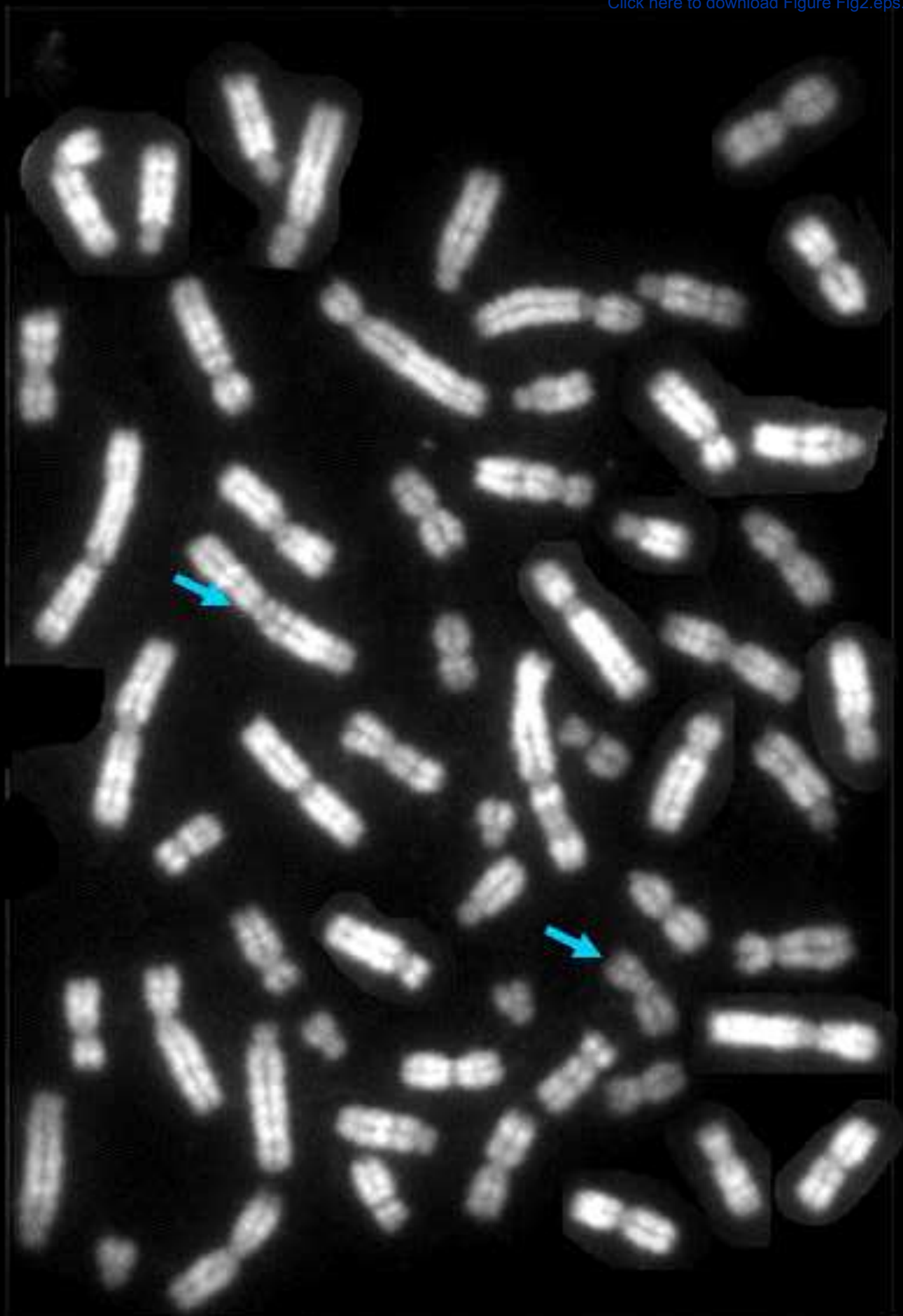


Figure 3

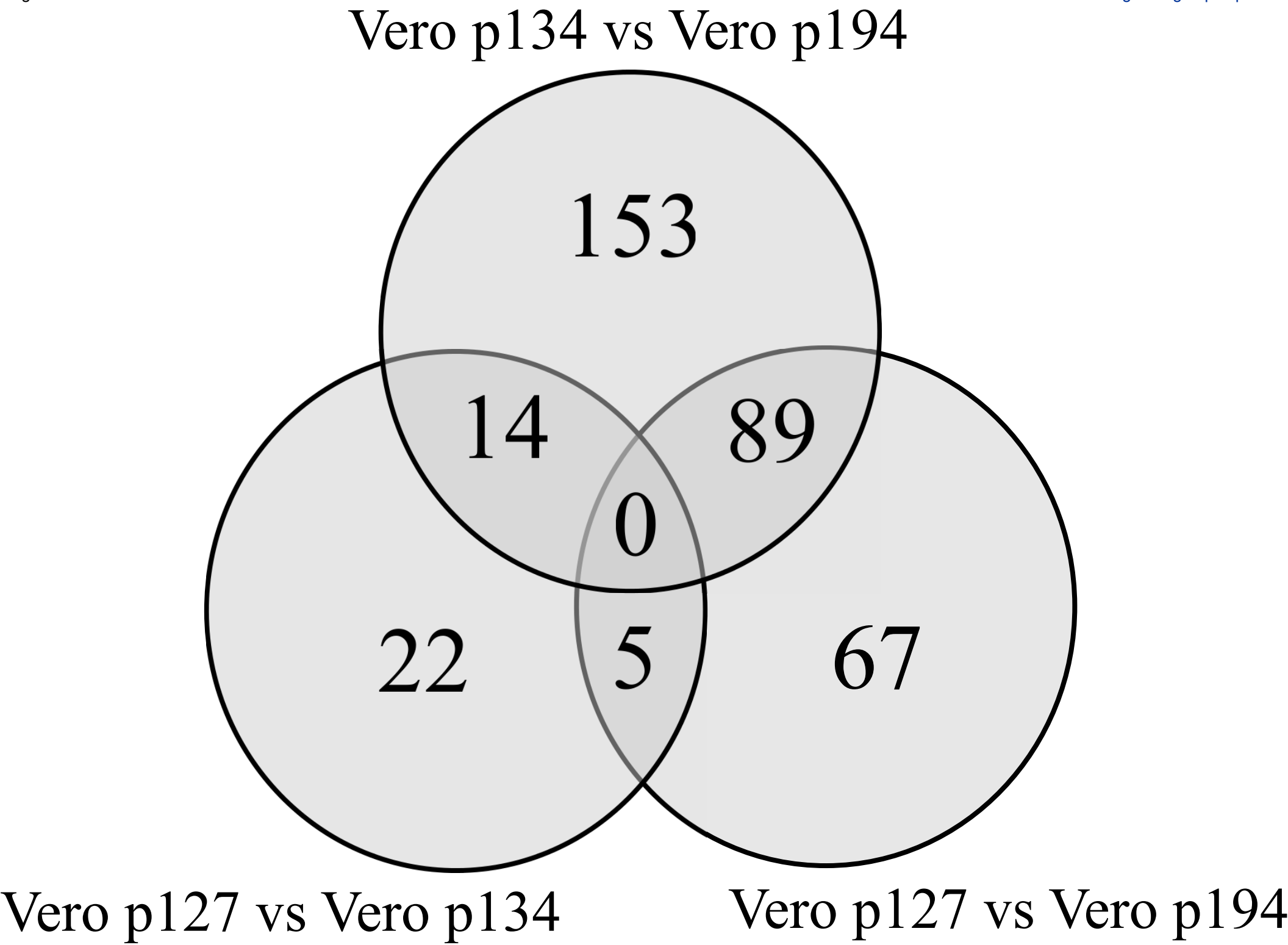


Figure 4

Click here to download Figure Fig4.eps.eps

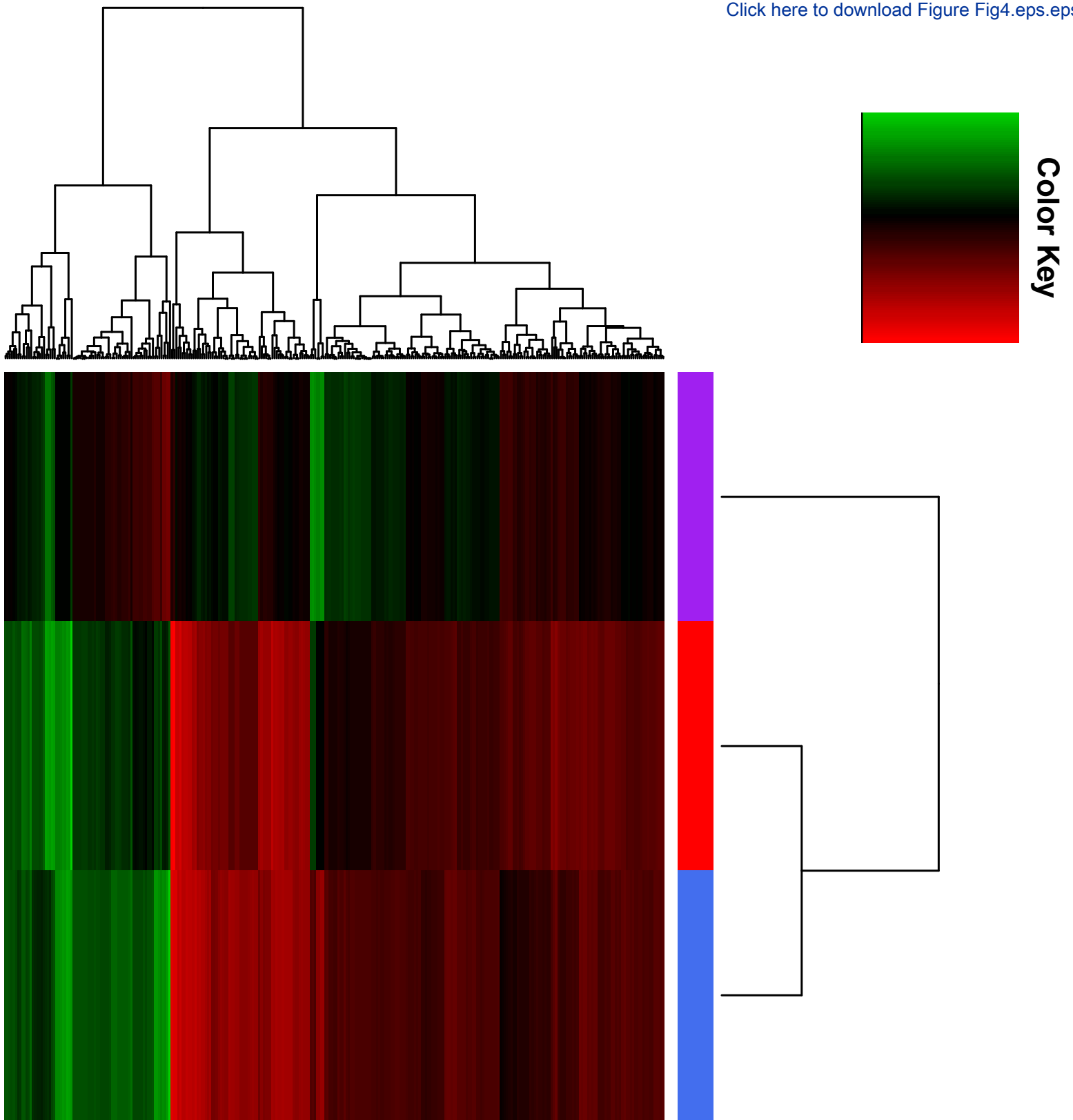


Figure S1

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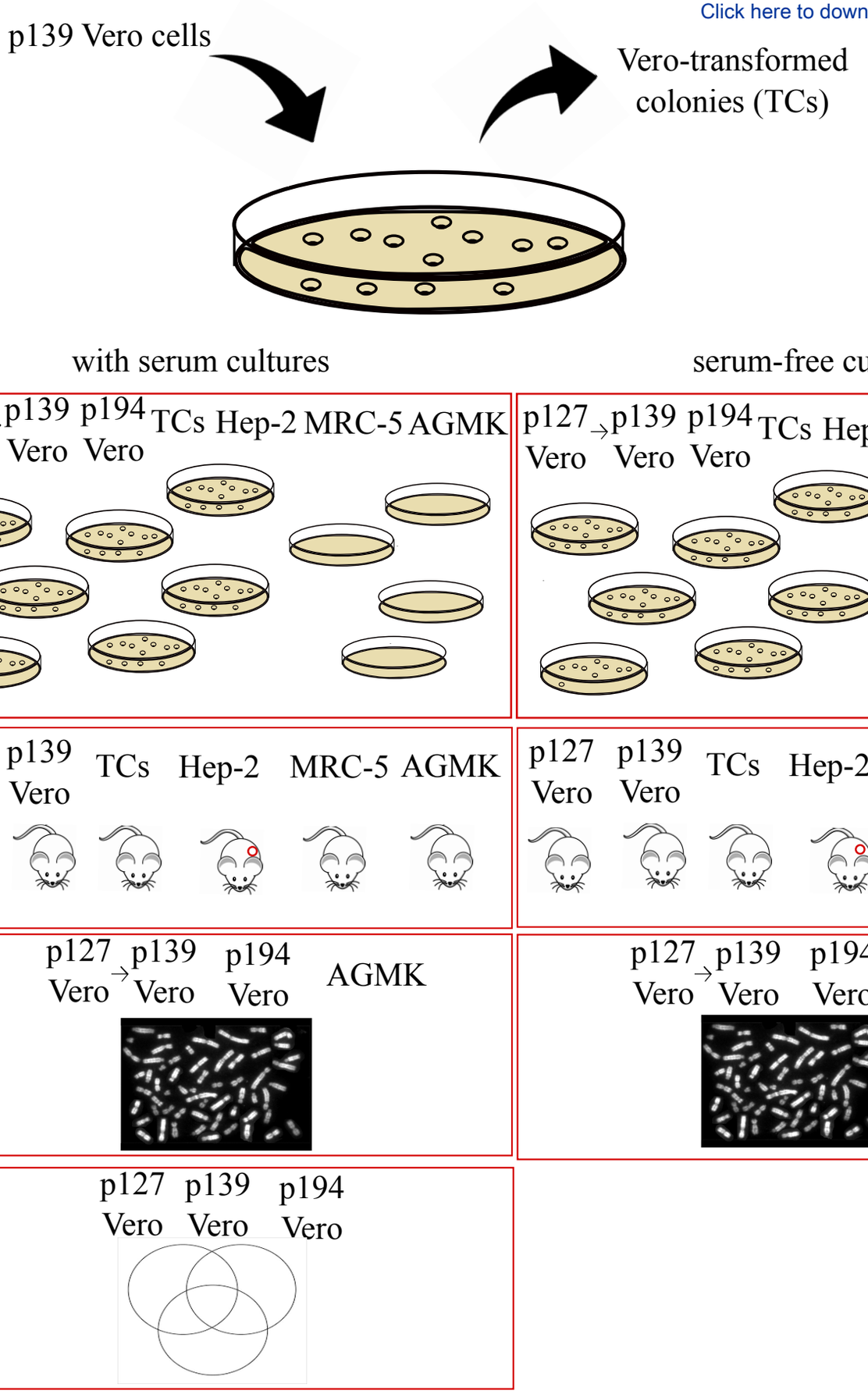

	In vitro transformation[a]	*In vivo* tumorigenicity	Karyotyping[a,b]	RNA-seq
p127 Vero	+	-	56	No enrichment in genes involved in tumorigenesis
p134 Vero	+	-	56	
p194 Vero	+	+*	56	
TCs	+	-	56	NP
p3 AGMK	-	-	60	NP
Hep-2	+	+	NP	NP
MRC-5	-	-	NP	NP

[a] *in vitro* transformation and karyotyping were performed on cells from p127 to p139. For sake of simplicity, Table 1 reports only data of lines that undergo also other steps of the investigation.

* result based on Petricciani et al. 1987.

[b] table reports the observed modal chromosome number based on the observation of 20 metastases.

NP = not performed.

Table S1

Click here to download Table table S1.docx

feature IDs	p134 vs p127	p194 vs p127	p194 vs p134
ENSP00000324127_1	2,619274682	-0,610573139	-3,230368426
ENSP00000356832_1	-0,551804995	2,744371753	3,296897597
JK123643.1_1	-1,062622151	2,840022258	3,904691719
ENSP00000339820_1	1,457722446	-2,533940122	-3,99387525
ENSP00000422591_1	1,155605447	-2,483506176	-3,640560211
ENSP00000343877_1	0,800559522	-2,307131495	-3,108510429
ENSP00000251127_1	2,125696429	-2,312850716	-4,443108734
ENSP00000238682_1	-0,601238224	2,486957226	3,089182109
ENSP00000353582_1	2,856619736	-0,194009849	-3,051789143
ENSP00000310721_1	1,060947919	-2,531315827	-3,594915025
ENSP00000451438_1	1,444405038	-2,26859334	-3,717191588
ENSP00000256759_1	2,356003056	-1,86481169	-4,229721337
ENSP00000256925_1	0,716978005	-2,616474902	-3,336976958
JK088891.1_1	-0,858726962	2,654161685	3,51743801
ENSP00000230882_1	1,042719413	-2,626662004	-3,675157582
ENSP00000244050_1	-2,010398843	1,38797553	3,402757817
ENSP00000427888_1	-1,367970823	2,101608655	3,475282134
ENSP00000347689_1	0,858773411	-2,408940105	-3,273930967
ENSP00000265087_1	-0,353948702	2,650331069	3,008998656
ENSP00000217086_1	1,953477133	-2,015937807	-3,984711163
ENSP00000361636_1	-2,138407736	1,31669109	3,465201058
JK104268.1_1	-0,707669608	2,747081499	3,465201058
ENSP00000301305_1	-0,357425634	2,733030287	3,097296877
JK092877.1_1	-1,63702704	6,268981357	8,39443008
JK090900.1_1	1,552855101	-2,612573526	-4,18600494
ENSP00000401907_1	2,63583999	-1,00059891	-3,648356854
ENSP00000311609_1	-1,034070979	2,457395251	3,503920995
ENSP00000359621_1	0,76403787	-2,28133476	-3,052998209
ENSP00000308032_1	2,098829101	-2,318153348	-4,446992507
ENSP00000345008_1	1,747806943	-1,535984254	-3,293699076
ENSP00000451131_1	-0,601069854	2,587317905	3,198625638
ENSP00000449124_1	-1,788712835	5,693342571	7,969638385
ENSP00000256186_1	2,945697051	-0,563149358	-3,523436415
ENSP00000261383_1	-0,90036024	6,488698754	7,876912621
ENSP00000298943_1	-1,120168597	6,268981357	7,876912621
ENSP00000329137_1	-0,702366727	2,923441982	3,646477855
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ENSP00000266682_1	2,63583999	-0,530852125	-3,178161747
ENSP00000343339_1	-2,619067033	0,680652706	3,314088469
JK094060.1_1	-2,278311661	0,77638802	3,066111892
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JK117113.1_1	-2,890636145	1,056019403	3,97606226
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JK114877.1_1	-1,058101239	1,930739851	3,000714117
ENSP00000279168_1	-2,109080656	1,08642636	3,210073333
ENSP00000265165_1	1,643620071	-1,385852613	-3,041056528
ENSP00000265131_1	2,463387293	-0,764130648	-3,241878669
JK122975.1_1	-0,917834535	2,240778191	3,173665605
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ENSP00000303208_1	-1,997112147	1,08642636	3,097972175
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feature IDs	p134 vs p127	p194 vs p127	p194 vs p134
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ENSP00000387278_1	-0,319548745	6,488698754	7,295015584
ENSP00000458307_1	-3,036675243	3,774650585	7,295015584
JK105864.1_1	-0,798781575	6,009683671	7,295015584
ENSP00000353142_1	-1,696123147	1,481862791	3,197464018
ENSP00000375259_1	-1,545043532	2,028404435	3,603225567
JK125686.1_1	-1,071545656	2,057501577	3,148766038
ENSP00000253354_1	0	-6,730042384	-7,216713304
ENSP00000368102_1	5,336805398	-1,442473467	-7,216713304
ENSP00000257860_1	-2,886053168	3,774650585	7,144032866
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ENSP00000268459_1	-1,280651129	1,798203891	3,098366666
ENSP00000412130_1	-2,158417484	1,946539195	4,162056544
ENSP00000439182_1	-1,721408072	1,622630781	3,373084953
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ENSP00000417710_1	-1,769797766	4,72095754	6,975382319
ENSP00000312457_1	2,348618007	-0,711464499	-3,078735886
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JK118361.1_1	-1,37077502	2,513150573	3,941122022
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ENSP00000445247_1	-2,527421587	3,774650585	6,784374174
JK110883.1_1	-1,57936677	4,72095754	6,784374174
JK124819.1_1	-0,128844757	3,014396168	3,172649845
JK113872.1_1	-6,625930005	-2,774418344	3,859259746
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JK100196.1_1	-0,964689576	2,028404435	3,021667127
JK121760.1_1	-1,937998351	1,056019403	3,021667127
JK107401.1_1	0	-6,151859094	-6,636592175
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JK099475.1_1	-6,44730409	-2,774418344	3,680126205
ENSP00000278314_1	0,532809253	6,488698754	6,440015926
ENSP00000326031_1	-2,184317246	3,774650585	6,440015926
JK122419.1_1	-0,669039098	5,287568917	6,440015926
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ENSP00000315295_1	1,066449422	-2,596699454	-3,719055972
ENSP00000360269_1	1,066449422	-2,596699454	-3,719055972
ENSP00000359512_1	-1,012143439	2,513150573	3,581463331
ENSP00000421258_1	-2,527421587	1,000232241	3,581463331
JK097403.1_1	-1,57936677	1,946539195	3,581463331
ENSP00000299727_1	1,066449422	-2,491124118	-3,613097854
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ENSP00000277517_1	-2,421867208	1,000232241	3,475554547
ENSP00000300079_1	-2,421867208	1,000232241	3,475554547
ENSP00000323155_1	-1,473812391	1,946539195	3,475554547
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JK100278.1_1	-0,500503616	2,918924227	3,475554547
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ENSP00000406318_1	0,448261067	6,268981357	6,304173084
JK098797.1_1	-0,533778926	5,287568917	6,304173084
ENSP00000263326_1	1,066449422	-2,377208509	-3,498736436
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ENSP00000396622_1	-2,307975999	1,000232241	3,361250601
ENSP00000439601_1	-2,307975999	1,000232241	3,361250601
JK095107.1_1	-6,129503249	-2,774418344	3,361250601
JK106362.1_1	-1,359921182	1,946539195	3,361250601
JK106489.1_1	-2,307975999	1,000232241	3,361250601
JK109650.1_1	-2,307975999	1,000232241	3,361250601
JK113424.1_1	-0,792697851	2,513150573	3,361250601
ENSP00000269703_1	0	-5,743569099	-6,226379856
ENSP00000339256_1	4,769582067	-1,022611559	-6,226379856
ENSP00000421169_1	0	-5,743569099	-6,226379856
ENSP00000263266_1	-5,721318377	0	6,154197121
ENSP00000288065_1	-5,721318377	0	6,154197121
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JK108999.1_1	-1,899791127	3,774650585	6,154197121
ENSP00000324248_1	-0,181910154	8,599408654	8,789084031
ENSP00000353427_1	0,71421195	5,084041388	4,370184695
JK092522.1_1	0,367485659	5,237883461	4,872112057
ENSP00000368759_1	0,689440424	5,368665504	4,681425579
ENSP00000227756_1	0,552701821	3,786515271	3,234266241
ENSP00000358590_1	1,653658391	6,0919394	4,445735231
ENSP00000264381_1	2,130470642	11,68243599	10,04205614
ENSP00000303153_1	0,304128115	-3,463999919	-3,768493664
ENSP00000349677_1	0,054360726	-3,484326888	-3,539031379

feature IDs	p134 vs p127	p194 vs p127	p194 vs p134
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ENSP00000285013_1	1,625542932	11,28047863	10,14505526
ENSP00000245479_1	1,565281705	4,818160459	3,256862291
ENSP00000453969_1	0,457703678	3,488654187	3,031753663
ENSP00000294829_1	-0,015677254	5,314613765	5,337880969
ENSP00000225688_1	0,642624312	4,380592676	3,741093582
ENSP00000344479_1	0,922045685	4,3549569	3,436136349
ENSP00000420194_1	0,290320978	4,950615121	4,666434216
ENSP00000463533_1	-1,122613762	-4,914719	-3,794097732
JK100938.1_1	-0,065535489	3,336762118	3,403557144
ENSP00000262352_1	-0,006936532	3,568509701	3,577201903
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ENSP00000361917_1	0,631291635	4,294327128	3,667380681
ENSP00000365651_1	0,804908223	3,914597967	3,112835241
ENSP00000377003_1	0,506681443	4,664149	4,164831046
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ENSP00000222219_1	0,17726651	7,521275207	7,40574109
ENSP00000354794_1	-0,198641815	-3,890445652	-3,695132826
JK111776.1_1	-0,730775564	3,872267778	4,608644528
ENSP00000312368_1	1,020220817	9,666964492	9,136362402
ENSP00000359778_1	2,599899753	-3,319460074	-5,935305493
ENSP00000312070_1	1,538301839	4,732925929	3,210073333
ENSP00000332530_1	0,516177542	3,923066384	3,414835272
ENSP00000261180_1	-2,755077828	-9,406570964	-6,75869621
ENSP00000321049_1	1,076214207	4,365262962	3,301560636
ENSP00000310878_1	-1,073506427	-5,668703555	-4,611035031
ENSP00000343617_1	0,485698558	4,274266183	3,801437579
ENSP00000393198_1	1,254115287	4,242612166	3,000714117
ENSP00000405218_1	0,874803004	9,025511724	8,639917969
JK123722.1_1	-0,587833907	8,953503899	10,03133995
ENSP00000399985_1	-0,90658906	-4,106529587	-3,205608776
ENSP00000369129_1	0,29525929	-3,178480499	-3,47879616
ENSP00000473091_1	0,886239757	8,575865029	8,178313786
JK117245.1_1	1,631667636	8,575865029	7,431683718
ENSP00000361788_1	0,799159501	4,315606014	3,537208263
JK093232.1_1	-0,003098682	3,023228795	3,03248401
ENSP00000296327_1	0,234409912	3,3673204	3,141759979
ENSP00000442046_1	5,336805398	-3,636905417	-9,414212059
ENSP00000264072_1	-1,073506427	-4,797974138	-3,739810357
ENSP00000386393_1	0,585638827	8,313586582	8,216656867
ENSP00000282326_1	0,316319891	8,25488182	8,427499498
JK093866.1_1	0,104105852	8,25488182	8,639917969
ENSP00000320604_1	1,536444446	5,41926826	3,941122022
ENSP00000321962_1	-0,384512979	-3,491001357	-3,114019729
JK093908.1_1	-0,048930561	5,355361883	5,46495195
ENSP00000281821_1	0,881604714	4,984907225	4,162056544
ENSP00000337555_1	-0,274097662	4,984907225	5,319478398
JK095209.1_1	0,631962847	7,759325569	7,615105183
ENSP00000216733_1	0,603380389	4,897860488	4,353607229
ENSP00000384193_1	1,47914882	4,897860488	3,475554547
ENSP00000379353_1	-0,533888103	4,007340696	4,572910861
ENSP00000272252_1	0,190875185	4,805222523	4,674001778
ENSP00000349678_1	1,003959169	4,805222523	3,859259746

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ENSP00000279441_1	-0,334001583	3,547845789	3,902901476
JK099477.1_1	0,411710778	4,70622545	4,353607229
JK099942.1_1	0,904943663	4,70622545	3,859259746
ENSP00000290552_1	0,746017424	3,815705657	3,099132689
ENSP00000344674_1	-1,014081553	3,460799052	4,496372873
ENSP00000472867_1	-0,093062449	3,460799052	3,574558612
ENSP00000310880_1	-0,752437392	7,480643794	8,722179193
ENSP00000346990_1	0,353232249	7,480643794	7,615105183
ENSP00000351280_1	0,904943663	7,480643794	7,062170589
JK122602.1_1	0,429983315	7,374349802	7,431683718
ENSP00000315602_1	-4,190035741	-7,25636939	-3,135050294
ENSP00000379256_1	1,066449422	-3,866519973	-4,991823929
ENSP00000423390_1	-3,644061741	-7,141678295	-3,575839244
ENSP00000293725_1	-0,59664408	3,709411665	4,337462117
ENSP00000269080_1	-0,630529359	7,134921762	8,254007216
JK114322.1_1	0,737772279	7,134921762	6,883037048
ENSP00000349494_1	0,91906066	6,99844435	6,564161444
ENSP00000330523_1	-0,030228358	6,847694402	7,364967387
ENSP00000361761_1	0,654376735	6,847694402	6,678465391
JK123883.1_1	-0,030228358	6,847694402	7,364967387
ENSP00000348602_1	-0,160442277	4,073276058	4,292539848
ENSP00000360425_1	0,35839136	4,073276058	3,772471476
JK118565.1_1	-0,030228358	4,073276058	4,162056544
ENSP00000296575_1	0,177465509	-3,587959848	-3,79719036
ENSP00000334128_1	0	-6,880685804	-7,36774617
ENSP00000395461_1	0,126862899	3,469983625	3,373084953
JK103161.1_1	2,191436022	4,857734403	2,666509416
JK103279.1_1	2,005712511	4,703728106	2,698830058
JK091645.1_1	2,522419672	5,164893736	2,644113977
ENSP00000318057_1	2,666370904	4,829074794	2,163754616
ENSP00000245919_1	2,644377091	3,547294088	0,90293293
ENSP00000242480_1	2,497774299	4,027637687	1,53026096
ENSP00000363804_1	2,13852069	4,964637117	2,828322135
ENSP00000260356_1	1,637389662	3,127718939	1,490366263
JK094840.1_1	0,787952781	3,176915914	2,389149842
ENSP00000359629_1	-0,758979662	-3,104593117	-2,345689701
ENSP00000356954_1	1,651244365	3,53765141	1,886920773
ENSP00000360158_1	-1,6351237	-3,473423208	-1,83844798
ENSP00000358596_1	2,148068796	4,223098911	2,07682371
JK112968.1_1	2,437010443	3,979545319	1,543729093
ENSP00000274181_1	1,958861267	4,818018522	2,863558394
JK097840.1_1	1,994601817	3,125399663	1,131351315
JK112739.1_1	2,086300548	3,718485228	1,633703074
ENSP00000282701_1	0,239787954	3,183357261	2,944568674
ENSP00000308330_1	-2,617279258	-4,344491643	-1,728365222
ENSP00000445626_1	2,785401556	3,849636795	1,067108629

feature IDs	p134 vs p127	p194 vs p127	p194 vs p134
ENSP00000301599_1	1,400253782	3,854582882	2,459201487
ENSP00000245552_1	0,162332495	3,066110186	2,906235486
ENSP00000299308_1	-0,99991823	-3,236232338	-2,237545903
ENSP00000194155_1	1,421591194	3,406454525	1,988466113
ENSP00000221166_1	1,400683588	3,559445737	2,163409721
JK105828.1_1	2,055296271	4,223245572	2,177195073
ENSP00000216286_1	-1,591194153	-4,47001472	-2,882729627
ENSP00000261304_1	0,894584408	3,019916207	2,127969875
ENSP00000283309_1	1,188917879	4,050187454	2,871369672
ENSP00000264867_1	1,657010326	3,678611508	2,02762736
ENSP00000262018_1	-1,523411571	-3,111566748	-1,589164078
JK105906.1_1	2,257841797	3,226439068	0,97130293
JK099313.1_1	2,934227147	4,210248022	1,284749427
JK092041.1_1	2,706715584	3,389340536	0,685715776
ENSP00000288976_1	1,571957739	3,329318142	1,762468944
ENSP00000324173_1	2,934227147	3,383633474	0,452085535
ENSP00000296591_1	0,97157576	3,254045941	2,287623044
JK113163.1_1	2,713279445	5,89431977	3,237105083
ENSP00000379154_1	1,768040252	4,364385249	2,61556698
JK100497.1_1	2,656383512	4,811661249	2,181807346
ENSP00000454748_1	0,857824952	3,046042725	2,19514581
ENSP00000282030_1	0,493757109	3,138413787	2,653084662
ENSP00000467931_1	0,703720151	3,396632031	2,704712035
JK118831.1_1	-1,782357491	-3,328664066	-1,54924862
JK109094.1_1	1,764284619	3,851431503	2,10492557
ENSP00000340864_1	1,096613274	4,007340696	2,939804851
ENSP00000413163_1	0,722842408	3,145956732	2,434498678
ENSP00000464976_1	0,674219467	3,145956732	2,483196658
ENSP00000328358_1	-1,57936677	-3,474291898	-1,90004956
JK091546.1_1	1,536444446	3,02167894	1,493013297
ENSP00000416561_1	2,734855496	4,805222523	2,119404684
ENSP00000238018_1	1,624044678	3,914702731	2,317650187
ENSP00000378067_1	2,058018201	4,599931457	2,594492354
ENSP00000272520_1	-5,366540222	-7,362610433	-2,05327193
ENSP00000360315_1	0,575762649	3,152409358	2,591064047
JK115319.1_1	0,951401772	3,152409358	2,214673973
ENSP00000366477_1	-5,14996425	-7,200163253	-2,108598444
JK097110.1_1	1,682023687	6,99844435	5,797403198
ENSP00000261187_1	2,153530231	4,224026005	2,119404684
ENSP00000395323_1	2,858528168	4,224026005	1,406279482
ENSP00000331581_1	1,066386385	3,048117213	1,999016787
ENSP00000242729_1	1,531230971	6,847694402	5,797403198
JK095711.1_1	1,531230971	6,847694402	5,797403198
JK108564.1_1	1,747806943	6,847694402	5,579327767
JK116885.1_1	2,312594577	6,847694402	5,00937381
ENSP00000354723_1	-2,184317246	-3,481718804	-1,304224693
ENSP00000398131_1	-5,14996425	-6,807329438	-1,714877485