

Analysis of Stem-Cell and Migratory Phenotype in Primary Cultures Derived From BPV-Infected Benign and Malignant Neoplasms

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Abstract

Bovine papillomavirus (BPV) is the etiological agent of bovine papillomatosis, neoplastic disease that can regress spontaneously or persist, resulting in malignancies such as urinary bladder and esophageal carcinoma (EC). Although recognized as cancer initiator, BPV action during metastasis remains unclear. This study evaluated the acquisition of stem-cell and migratory phenotype in primary cultures derived from BPV-infected neoplasms. For this, five primary cell cultures were used: BPV-free normal skin (negative control), skin papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and EC. These cells were cultivated until sixth passage (P1-P6). The acquisition of stem-cell phenotype was analyzed by tumorsphere formation assay and by the Oct-3/4 transcription factor expression levels. Morphological analysis was performed by phase contrast and screen electron microscopy (SEM). Results showed that all BPV-infected primary cultures were able to form tumorspheres, which were not verified in BPV-free normal skin cells. Skin papilloma, fibropapilloma and EC cells also showed high levels of Oct-3/4 expression in relation to normal skin. BPV-infected cells show loss of apical-basal polarity, lamellipodia and filopodia, which were confirmed by SEM and F-actin labelling. Time-lapse video microscopy showed that EC and papilloma 03 presented the highest migration velocity, while papilloma 01, an intermediate velocity. Statistical analysis did not reveal significant differences between the normal skin and papilloma 02 (fibropapilloma) migration velocity. These results suggest that BPV not only act during cancer initiation, but also during metastasis.

Keywords: Bovine papillomavirus (BPV); Culture cell; Stem-cell; Cell migration; Epithelial-mesenchymal transition (EMT)

Abbreviations: BPV: Bovine Papillomavirus, CSC: Cancer Stem-Cell, EMT: Epithelial-Mesenchymal Transition, HPV: Human Papillomavirus, SEM: Screen Electron Microscopy

Introduction

Bovine papillomavirus (BPV) is the etiopathogenic agent of bovine papillomatosis, an infectious and neoplastic disease characterized by multiple benign neoplasms (papillomas) that can regress spontaneously, but may also persist leading to urinary bladder and esophageal carcinoma when in presence of co-factors [1-4]. BPV is also considered a useful model to study the HPV-associated oncogenic process, once these vi-

ruses share morphological and pathogenic characteristics in common [5-7]. For this reason, virologists have used BPV as a model for the study of HPV infection and interaction with the host cell [8].

Although studies had demonstrated that both BPV [1,2,9-11] and HPV induce DNA damages, can lead to genomic instability and cancer initiation [12,13], there are few works about the action of these viruses in metastasis, which is responsible for about 90% of all cancer-related deaths globally [6].

Metastatic process consists of a long series of sequential and interrelated steps in which cancer cells from primary neoplasm spread to distant organs [14]. During this process, it is verified biochemical, genetics and morphological changes that epithelial-mesenchymal transition (EMT), a biological reprogramming process which confers migratory and invasiveness capability do transformed cells [6,15]. The lack of studies involving the papillomaviruses (PVs) participation in EMT can be attributed to the little attention given to primary cultures derived from PVs-infected neoplasms. In this sense, our group has showed that primary cultures derived from BPV-infected neoplasms are useful models to study the genetic [9] and biochemical deregulations, contributing to genomic instability and cell transformation [7]. Genomic instability is pointed out as responsible to induce the cancer stem-cell (CSC) phenotype acquisition [16-18], repressing reversibly the expression of cell differentiation genes [19], contributing to EMT and metastasis [20]. Considering that we first reported genetic and biochemical changes in BPV-infected cells derived from neoplasms, in this study we analyzed the acquisition of CSC phenotype, as well as the invasiveness capability of primary culture cells derived from cutaneous papilloma, fibropapilloma and esophageal carcinoma.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of Butantan Institute under process 1319-14.

Primary cell cultures

Primary cells cultures used was obtained by Campos et al. (2013) from fragments of: normal skin, showing no morphological alteration, skin papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma. These tissues were collected from adult bovines (*Bos taurus*). Cells were seeded in culture flasks of 25 cm², containing 5 mL of complete medium: DMEM medium (Cultilab, Brazil), supplemented with 15% fetal bovine serum (Cultilab, Brazil) and 1% ampicillin-streptomycin (Cultilab, Brazil). Cells were incubated at 37°C, in 5% of CO₂ atmosphere up to 80% confluence (about 72 hours). Cells were cultivated until the sixth passage (P1-P6). Molecular identification of BPV was performed by PCR using specific primers for BPV-1 and 2, considered the most frequent virus types [21] and, BPV-4, due its association with upper gastric papilloma and carcinoma [9,22]. PCR results were already published in Araldi et al.(2016 a), demonstrating the BPV infection in primary cell cultures of papilloma 01, 02, 03 and esophageal carcinoma, but not in normal skin. These results are summarized in table 1.

Analysis of stem-cell phenotype

Tumorsphere formation assay

Cells were subjected to the tumorsphere formation assay, method considered “gold-standard” to identify the acquisition of stem-cell phenotype [23,24].

For this, 5x10⁵ cells in second passage (P2) were transferred to six-well plate pre-covered with 1 mL of 2% agarose, diluted in sterile PBS and 2 mL of complete medium. Cells were incubated at 37°C with 5% CO₂ atmosphere for 24 hours. Next, the cells were analyzed in Nikon Eclipse Ti inverted microscope and images were captured in total magnification of 100X.

Immunodetection of Oct-3/4 transcription factor

Immunofluorescence: a total of 1 x 10⁵ cells in second passage (P2) were seeded per well, employing a six-well plate, containing 2 mL of complete DMEM medium and a 24 x 24 mm sterile cover slip. Cells were incubated at 37°C, with 5% CO₂ atmosphere, until a confluency of 80% (about 24 hours). The medium was removed and cells were washed three times with sterile PBS at 37°C for five minutes. Cells were fixed with 4.0% formalin, diluted in PBS, at 4°C for 30 minutes and then washed three times with PBS at 37°C for 5 minutes. Cells were permeabilized with 0.01% Triton X-100 (Sigma, Germany), diluted in PBS, at 4°C for 10 minutes. Cells were washed once with PBS and incubated overnight at 4°C, in a moist chamber with polyclonal antibody anti-Oct-3/4 H-65 produced in rabbit (Santa Cruz Biotechnology, USA) at 1:100 dilution in 1% bovine serum albumin (BSA). Cells were washed three times with PBS for five minutes and then incubated at 4°C for 3 hours with anti-rabbit IgG conjugated with FITC (Sigma, Germany) at 1:200 dilution in 1.0% BSA. Cells were washed three times with PBS and cover slips were mounted on slides, using 20 µL of ProLong Gold (Invitrogen, Carlsbad, USA) with DAPI. Slides were analyzed in Axio Scope A1 fluorescent microscope (Carl Zeiss, Germany) under total magnification of 400X.

Flow cytometry: Cell lines were seeded in culture flasks of 25 cm² with 5 mL of complete DMEM medium. Cells were subjected to monolayer disaggregation with 2 mL of EDTA solution and centrifuged at 1,700 rpm for 5 minutes. Cells were transferred to 1.5 mL polypropylene tubes and fixed in 1.0 mL of 1.0% formalin solution at 4°C for 2 hours. Cell suspension was centrifuged and washed twice with 1.0 mL of PBS at 4°C to remove the formalin residues. Cell were incubated with 1.0% BSA at 4°C for 20 minutes, washed once with PBS, and incubated overnight at 4°C with 1.0 µL of anti-Oct-3/4 produced in rabbit (Santa Cruz Biotechnology, USA). Cells were centrifuged under described conditions, washed twice with PBS and incubated at 4°C for 2 hours with anti-rabbit conjugated with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, USA) at 1:200 dilution in 1% BSA. Next, cells were washed with PBS, centrifuged and resuspended in 100 µL of PBS. The material was analyzed in Accuri C6 cytometer (BD Bioscience, USA), employing the FL1 channel. A total of 10,000 events were analyzed. Results were analyzed in FlowJo software (TreeStar, Oregon, USA), using the percentage of immune-labeled cells. Skin papilloma cell line incubated with only secondary antibody was used as control for immunofluorescence and flow cytometry.

Table 1: BPV types identified in primary cell cultures

	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5	Passage 6
Normal skin	-	-	-	-	-	
Papilloma 01	BPV-1	BPV-1 and 2	BPV-2	BPV-1 and 2	BPV-1 and 2	-
Papilloma 02	BPV-1, 2 and 4	BPV-1, 2 and 4	BPV-2 and 4	BPV-1 and 2	BPV-1, 2 and 4	BPV-2 and 4
Papilloma 03	BPV-2 and 4	BPV-1 and 2	BPV-2	BPV-2 and 4	BPV-1 and 2	BPV-2
Carcinoma	BPV-1, 2 and 4	BPV-1, 2 and 4	BPV-1, 2 and 4	BPV-1, 2 and 4	BPV-1, 2 and 4	BPV-1, 2 and 4

Results based on Araldi et al. [7]

Analysis of stem-cell phenotype

Morphological analysis

Morphological analysis of six passages (P1-P6) was performed using a phase contrast microscopy, employing the Nikon Eclipse Ti inverted microscope (Nikon, Japan) in total magnification of 100 X. This method was used to identify morphological alterations suggestive of migratory phenotype, such as lamellipodia and filopodia, which are directly associated to cell migration and EMT. To better visualize the ultrastructure of these organelles, cell lines in fourth passage (P4) were subjected to screen electronic microscopy (SEM). Cell lines were seeded in six-well plate containing a 24 x 24 mm sterile cover slip and 2 mL of complete DMEM medium up the 80% of confluence. Medium was removed and cells were washed three times with sterile PBS at 4°C. Cells were fixated in 1 mL of Karnovsky fixative (50 mL 8% paraformaldehyde, 10 mL 25% glutaraldehyde, 40 mL 0.2 M PBS, pH 7.3), processed and analyzed in Quanta 250 (Fei Company, Holland) in a primary beam accelerated at 10-15 V in different magnifications.

F-actin labelling

To confirm the presence of filopodia, cells were labelled with phalloidin and analyzed by High-content screening (HCS). Cells were expanded in 96-well plate (Corning, USA), using DMEM complete medium until to obtain 5 x 10³ cells/cm³. Cells were fixed in 4% paraformaldehyde and washed three times with PBS. Cells were permeabilized with 0.1% Triton X-100 (Sigma, Germany) diluted in PBS for 10 minutes and labelled with phalloidin (Sigma, Germany). Cells were washed three times with PBS and the nucleus were labelled with 0.1 g/mL of Hoechst 33342 (Molecular Probe, USA) for 10 minutes. The plates were analyzed in HSC (Molecular Devices, USA) and the images were captured using the software MicroHCS (Molecular Devices, USA).

Time-lapse microscopy for cell migration

Cell migration was analyzed by time-lapse video microscopy, employing the InCell Analyzer 2200 (GE Healthcare, USA). A total of 200 µL of DMEM complete medium were transferred to six-well plates. A total of 2 x 10⁵ cells in second passage (P2) was transferred per well.

The plate was incubated at 37°C, with 5% CO₂, and one picture was taken every 15 minutes, completing a total of 80 time-points (20 hours). The images were analyzed using the ImageJ 1.5e software, and the average speed, in µm/minute, of each time-point in a total of five cells per cell line was calculated. Based on these values, it was performed the analysis of variance (ANOVA), followed by the Tukey test, both with 5% of significance level, using the GraphPad Prism 5.0.

Results

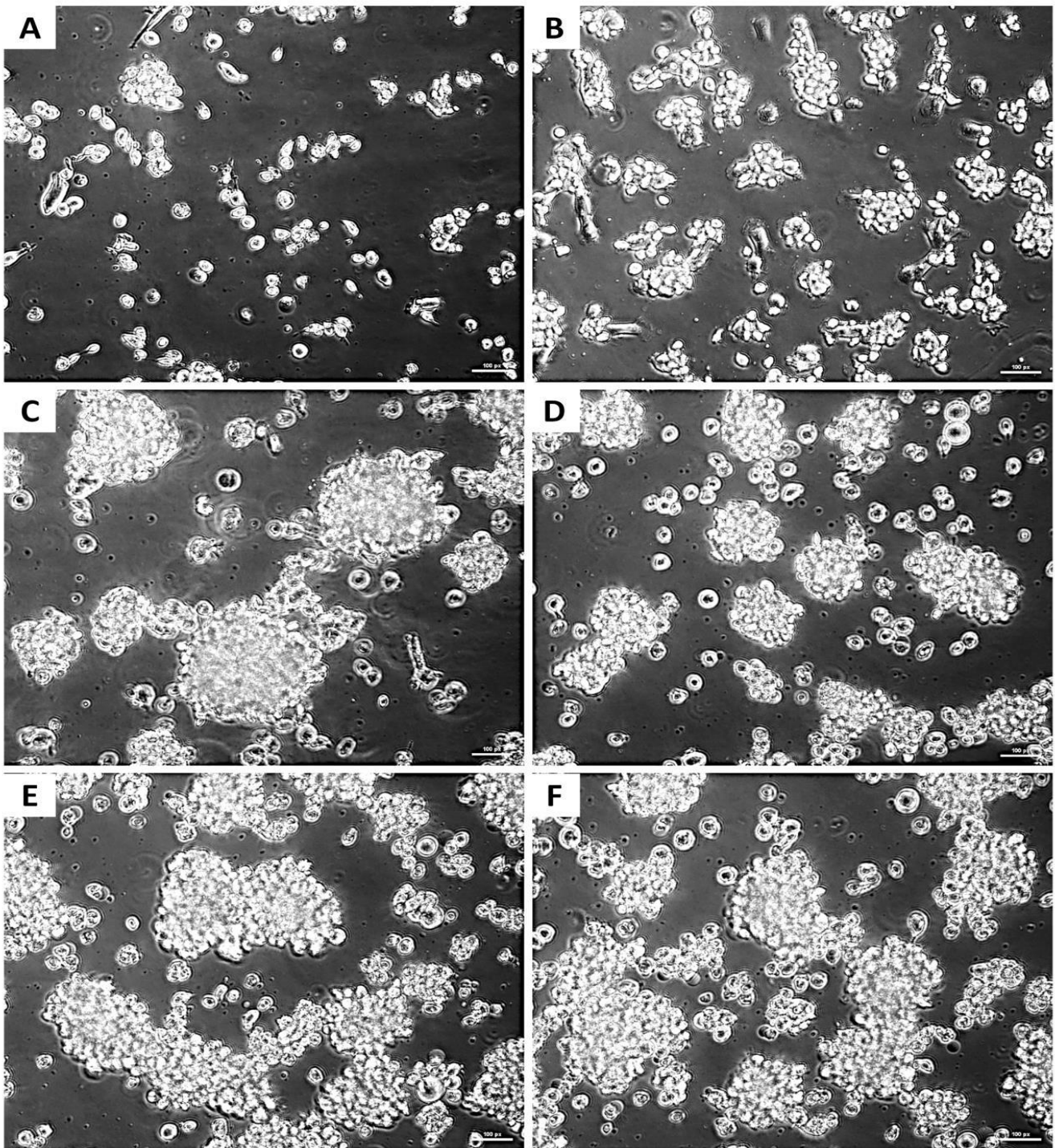
Acquisition of stem-cell phenotype

Results of tumorsphere formation assay showed the presence of spheroidal cellular aggregates in primary cell cultures of skin papilloma, fibropapilloma and esophageal carcinoma, but not in normal skin cell line (figure 1). To confirm the acquisition of stem-cell phenotype, we analyzed the Oct-3/4 transcription factor expression levels by flow cytometry. Results of this analysis showed an increase in Oct-3/4 expression in all BPV-infected primary cultures in relation to normal skin cells (figure 2). We also verified the Oct-3/4 nuclear labelling in esophageal carcinoma cells by immunofluorescence analysis (figure 3).

Acquisition of migratory phenotype

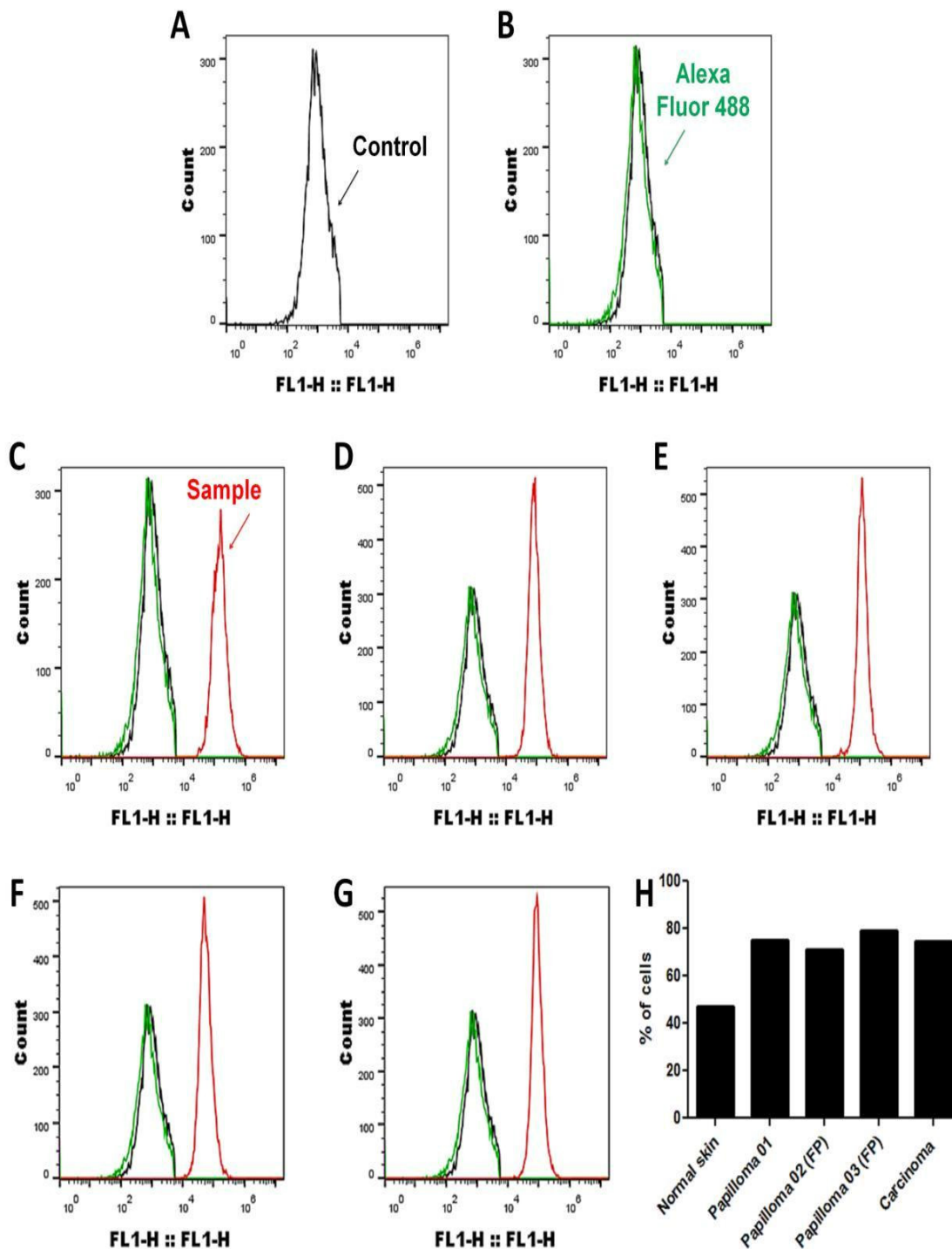
Morphological analysis by phase contrast microscope showed the loss of cell apical-basal polarity and the presence of lamellipodia and filopodia in skin papilloma, fibropapilloma and esophageal carcinoma cells (figure 4). The presence of filopodia was confirmed by the F-actin labelling (figure 5). These alterations were verified along the six passages analyzed, suggesting the acquisition of migratory phenotype. By the contrast, normal skin cell showed the preservation of cell polarity along the passages. The loss of polarity and the filopodia presence in all BPV-infected cells were confirmed by SEM analysis (figure 6). This analysis also showed the presence of inter-cytoplasmic bridges in fibropapilloma (figure 6H) and esophageal carcinoma (figure 6I) cells, suggesting a possible mechanism of horizontal genetic transfer. To verify the migratory capability of these cells, we performed the time-lapse video microscopy, obtaining the average speed of 5 cells/primary culture along 20 hours in a total of 80 time-points. Based on the speed average of each primary cell culture (figure 7), we performed the parametric ANOVA, that showed statistical differences among the cell migration velocity (p<0.0001).

Figure 1 – Results of tumorsphere formation assay



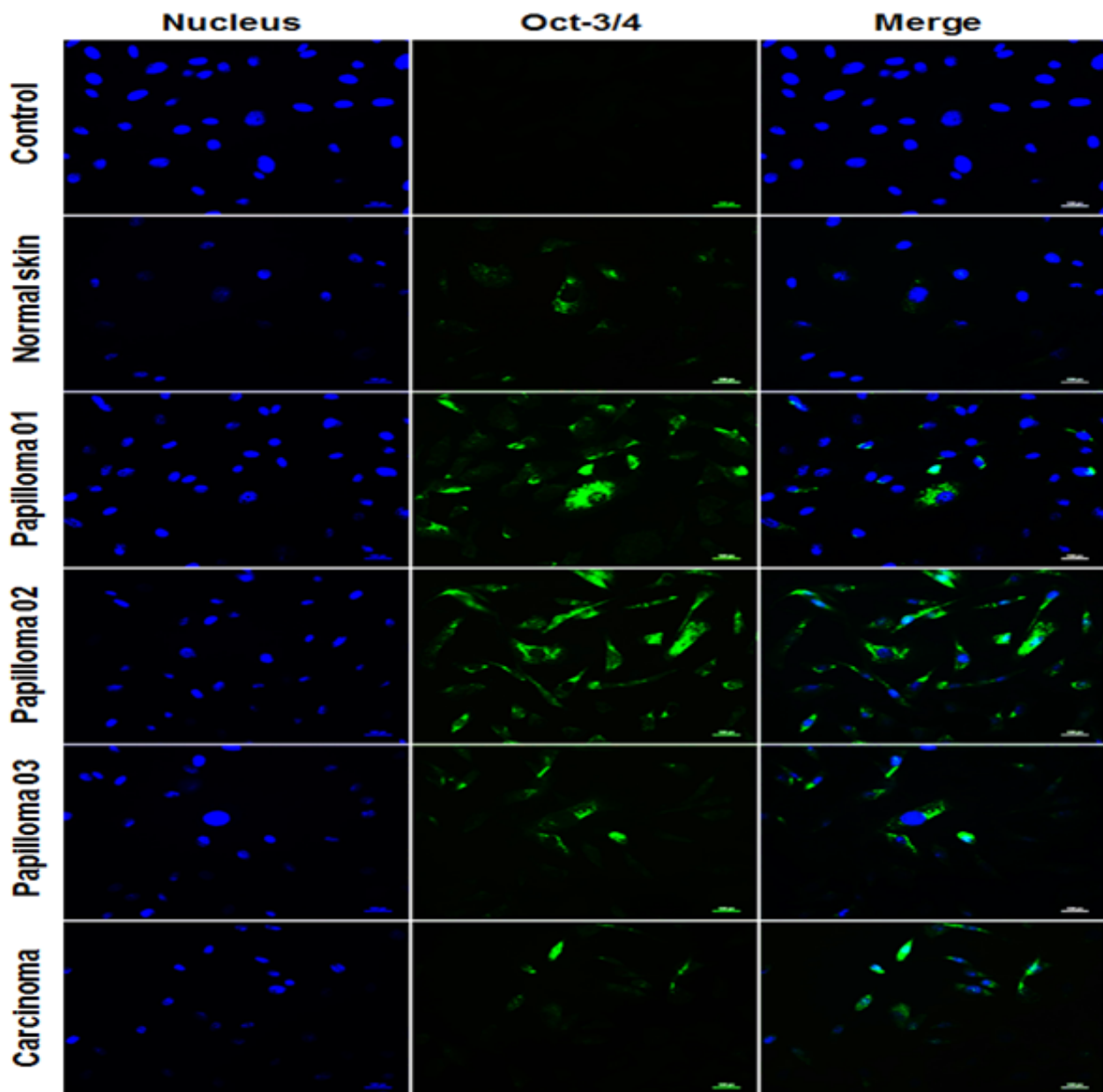
Results of tumorsphere formation assay showing the absence of spheroidal cellular aggregates (tumorspheres) in normal skin cell line (A) and the presence of tumorspheres in skin papilloma (B, papilloma 01), fibropapilloma (C, papilloma 02 and D, papilloma 03) and esophageal carcinoma cell lines (E and F). Total magnification of 100 X. Scale bar of 100 µm.

Figure 2 – Quantitative analysis of Oct-3/4 transcription factor by flow cytometry



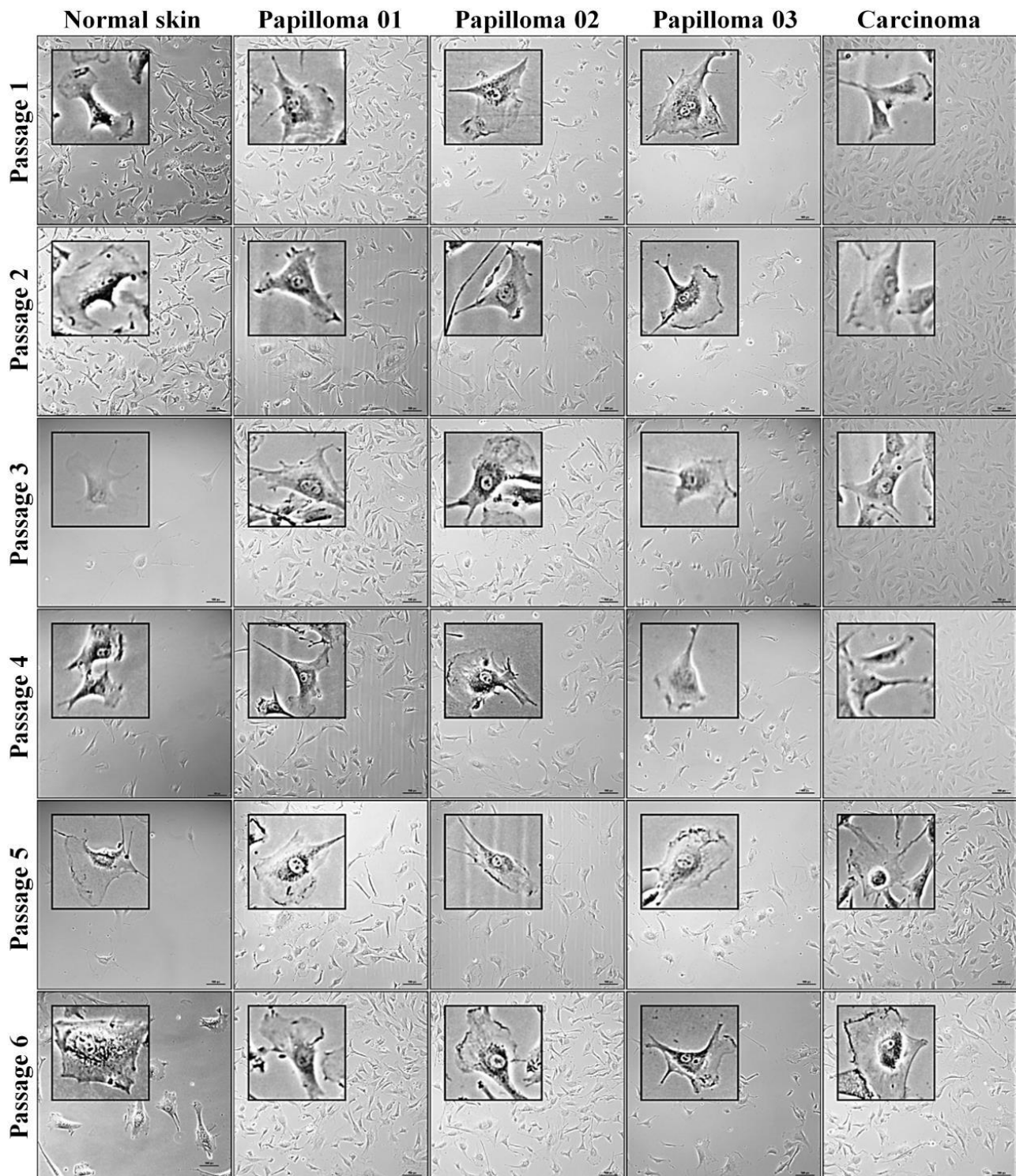
A) skin papilloma cells without any antibody; B) skin papilloma cells incubated with secondary antibody Alexa Fluor 488, showing absence of unspecific labelling; Oct-3/4 expression levels in: normal skin (C, BPV-free), skin papilloma (D, papilloma 01), fibropapilloma (E, papilloma 02 and F, papilloma 03) and G) esophageal carcinoma cell lines. H) Histogram based on the cell percentage expressing the Oct-3/4 transcription factor. Results show an increase in Oct-3/4 expression levels in BPV-infected cell lines. Cell analyzed in second passage (P2). Total of 10,000 events analyzed.

Figure 3– Immunodetection of Oct-3/4 transcription factor



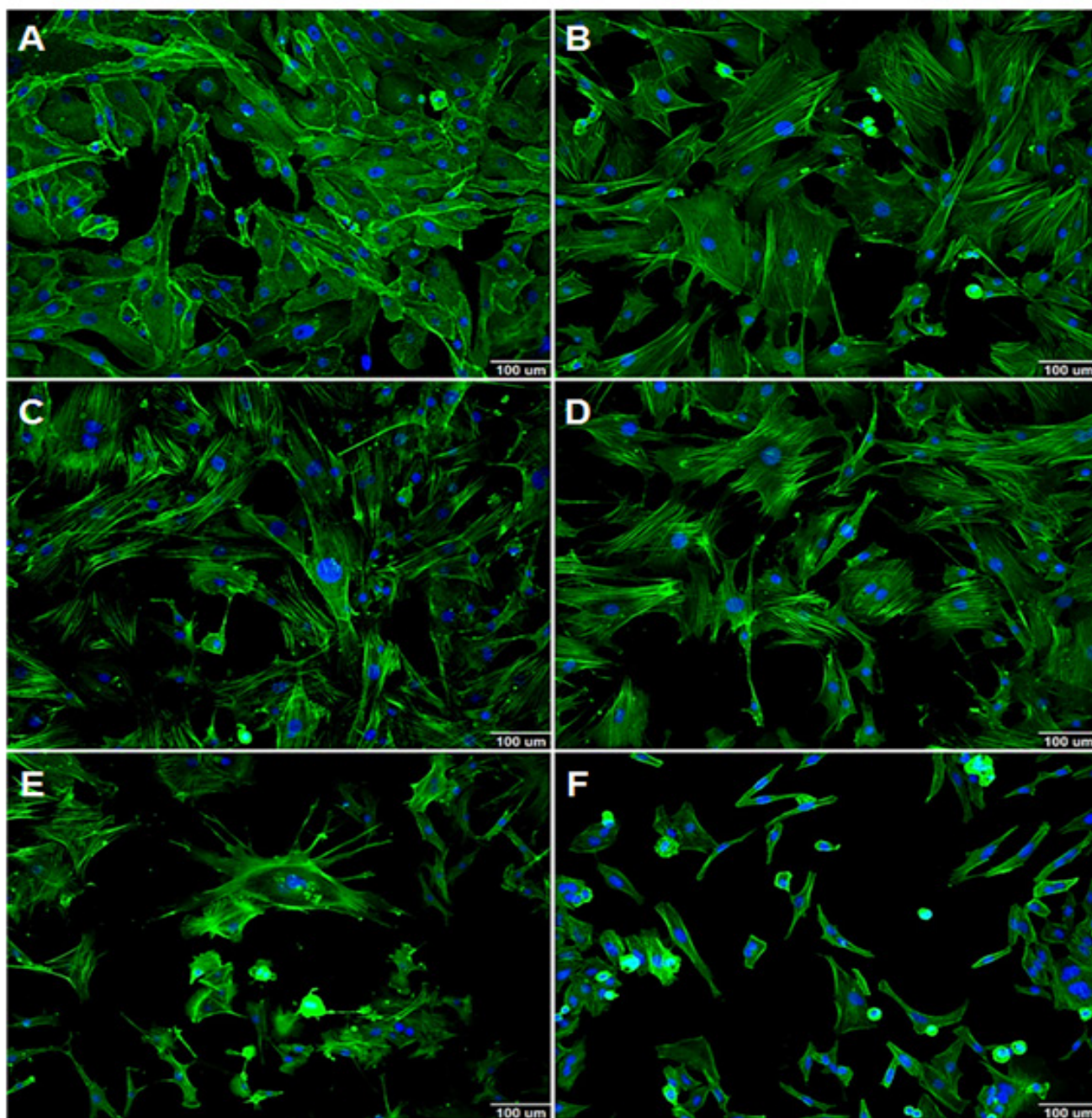
Immunodetection of Oct-3/4 transcription factor showing the increase of fluorescence signal in skin papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma cell lines. Results also show the nuclear labelling of Oct-3/4 in esophageal carcinoma cells. Images obtained with total magnification of 400X, scale bar of 100 μ m.

Figure 4 – Morphological analysis by phase contrast analysis



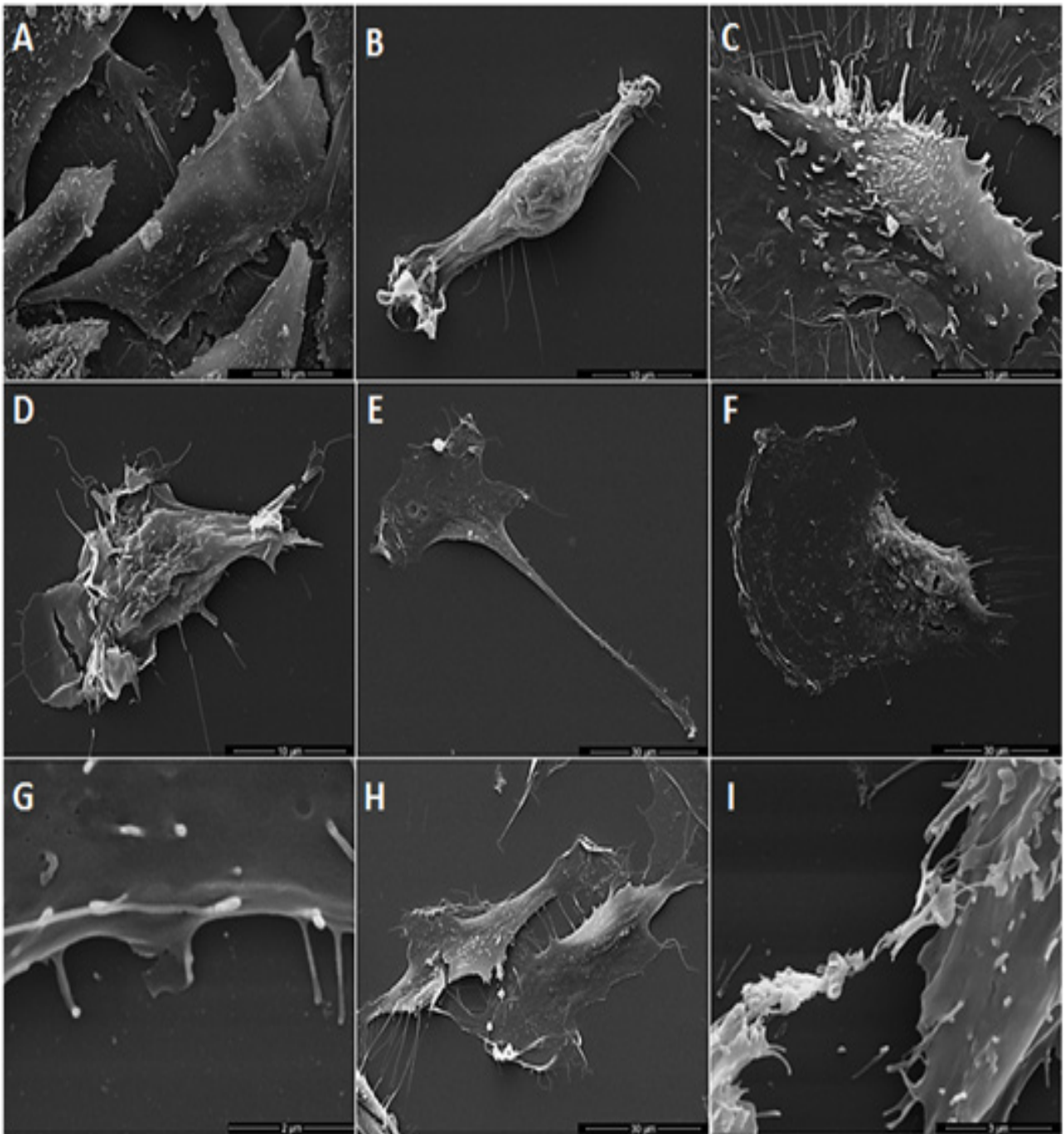
Phase contrast analysis showing the maintenance of cell polarity along the six passages of normal skin cell line (BPV-free) and the loss of cell polarity in all passages analyzed of skin papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma cell lines. All BPV-infected cell lines show the presence of lamellipodia and filopodia, suggesting the acquisition of migratory phenotype. Imagens obtained in total magnification of 100X, scale bar of 100 μm .

Figure 5 – F-actin labelling



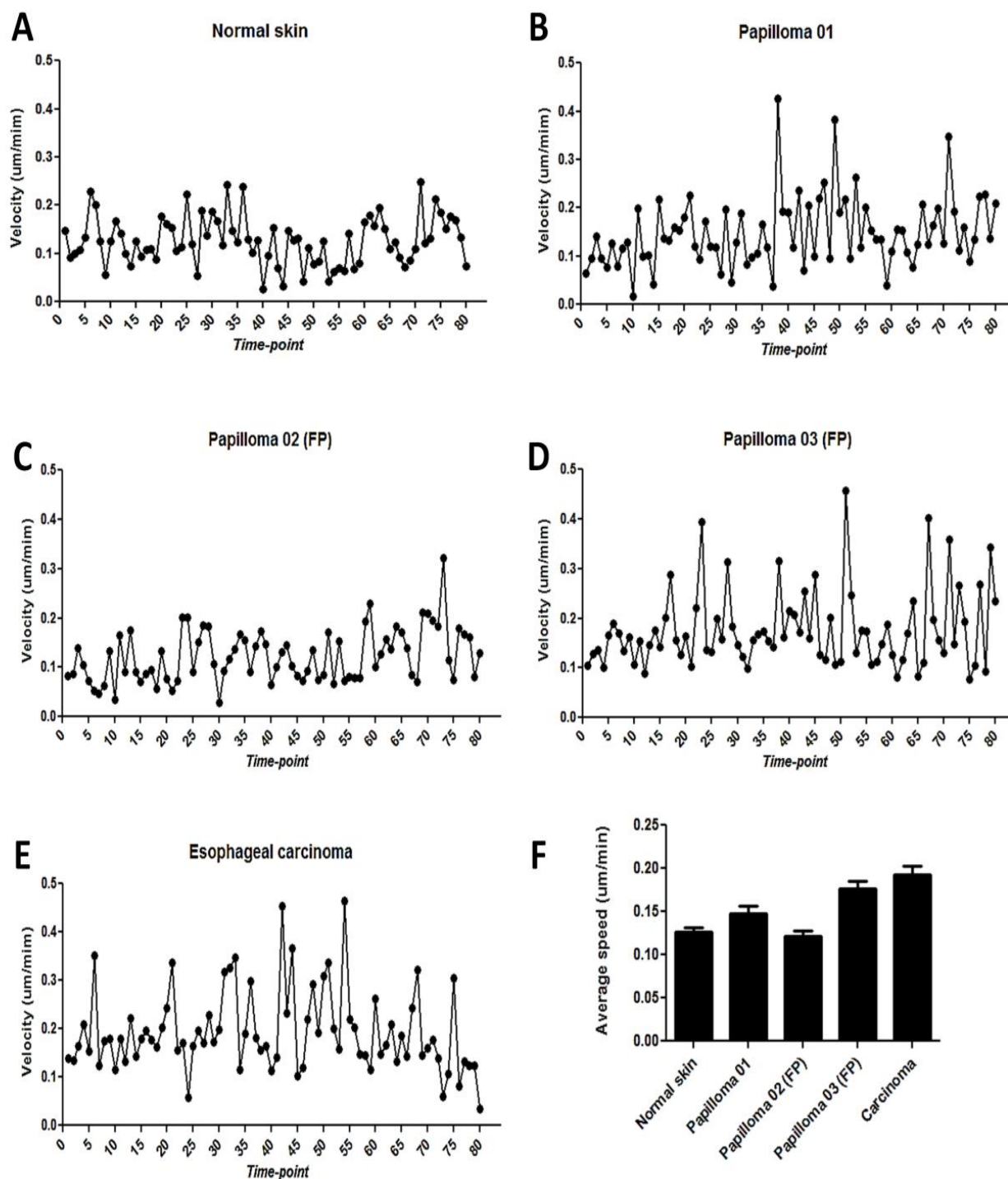
A) BPV-free normal skin, B) cutaneous papilloma, C) fibropapilloma 01, D) fibropapilloma 02, E and F) esophageal carcinoma. Results show the F-actin labelling in plasmatic membrane, reinforcing the presence of filopodia in all primary cultures cells derived from BPV-infected neoplasm.

Figure 6 - Screen electron-micrograph (SEM)



A) keratinocyte-like of normal skin cell line, presenting apical-basal polarity (6664 X), B) fibroblast-like of normal skin cell line, showing fusiform morphology (8280 X), cells of papilloma 01 (C, 9829 X, skin papilloma), 02 (D, 9388 X, fibropapilloma), 03 (E, 2893 X, fibropapilloma), and esophageal carcinoma (F, 2898 X) cell lines showing loss of polarity, extensive lamellipodia, and abundant filopodia, G) lamellipodia site, with filopodia, observed in esophageal carcinoma cell line (53021 X), inter-cytoplasmic bridges observed in papilloma 02 (H, 3210 X), and esophageal carcinoma cell lines (I, 25777 X).

Figure 7 – Results of cell migration assay by time-lapse video microscope



Results of cell migration assay by time-lapse video microscope showing the average speed in $\mu\text{m}/\text{minute}$ in 80 time-point, in a total of 20 hours of analysis: A) normal skin, B) papilloma 01 (skin papilloma), C) papilloma 02 (fibropapilloma), D) papilloma 03 (fibropapilloma) and E) esophageal carcinoma cell lines. F) Histogram based on mean of cell velocity along the 80 time-point analyzed, followed by the standard deviation (bars) demonstrating the highest migration velocity in carcinoma cell line. Papilloma 01 and 03 show intermediated migration velocity, as confirmed by the Tukey's test. Normal skin and papilloma 02 did not reveal statistical difference between their migration velocity.

Considering this result, we performed the Tukey's multiple comparison test (table 2), which showed that primary cultures derived from esophageal carcinoma and papilloma 03 (fibropapilloma) showed the highest migration velocity, indicating the acquisition of migratory phenotype. Papilloma 01 (skin papilloma) showed an intermediated migration velocity (table 2), while papilloma 02 (fibropapilloma) and normal skin did not show statistical difference, presenting the lower migration velocity.

Multiple comparison	Mean Difference	q	P< 0.05
Normal skin and papilloma 01	-0.02171	2.818	No
Normal skin and papilloma 02 (FP)	0.004682	0.608	No
Normal skin and papilloma 03 (FP)	-0.04973	6.457	Yes
Normal skin and esophageal carcinoma	-0.06675	8.666	Yes
Papilloma 01 and Papilloma 02 (FP)	0.02639	3.426	No
Papilloma 01 and Papilloma 03 (FP)	-0.02803	3.639	No
Papilloma 01 and esophageal carcinoma	-0.04504	5.848	Yes
Papilloma 02 (FP) and papilloma 03 (FP)	-0.05441	7.065	Yes
Papilloma 02 (FP) and esophageal carcinoma	-0.07143	9.274	Yes
Papilloma 03 (FP) and esophageal carcinoma	-0.01702	2.209	No

Table 2 – Result of Tukey's multiple comparison test
FP - fibropapilloma

Discussion

Although recognized as oncogenic viruses [5], the PVs action following cancer initiation remains unclear [6]. This because little attention has been given to primary cell cultures derived from BPV-infected tissues. However, in last decades, studies have been shown productive infection in sites not passive of cell differentiation, such as placenta [25] and peripheral blood [11,26], indicating the need to review the PVs natural history [5]. Moreover, since 2008 our group has been explored the potential of primary cell cultures as model to study the BPV pathology [27]. We already demonstrated that in vitro models present cytogenetic aberrations [9] similar to those verified in vivo [1,28,29]. Currently we also verified that primary cultures derived from skin papilloma, fibropapillomas and EC present glycolytic metabolic deregulation [7]. Similar results were also observed in cell cultures derived from equine sarcoid [30]. Despite these advances, the PVs action during metastasis remains unclear. Thus, we analyze the acquisition of stem-cell and migratory phenotype in primary cell cultures derived from skin papilloma, fibropapilloma and esophageal carcinoma. In this pioneering study, we verified the presence of tumorspheres in all BPV-infected primary cell cultures, but not in normal skin cells (figure 1). We also observed an increase in Oct-3/4 transcription factor in primary cultures derived from BPV-infected tissues in relation to normal skin cells (figure 2). These results suggest that BPV-infected cells present biological reprogramming, leading to stem-cell phenotype acquisition.

Considering that stem-cells are inducible by increasing genomic instability [31], BPV E6 oncoprotein has a central role in biological reprogramming process, once this oncoprotein induces genotoxicity and clastogenesis [32]. Moreover, studies have showed that both BPV [7] and HPV E6 promote oxidative stress, increasing the reactive oxygen species (ROS) production [33]. The E6 pro-oxidant activity can lead to PI3K and Akt phosphorylation, resulting in Oct-3/4 activation [23], justifying the nuclear labelling of this transcription factor verified in esophageal carcinoma cells (figure 3). Once activated, the Oct-3/4 binds to AGTCAAAT motif sequence present in target genes, contributing to stem-cell phenotype maintenance [34], as well as leading to apoptosis resistance. In this regard, the E6 oncoprotein promotes the p53 downregulation, avoiding the Bax protein translocation from Golgi complex to mitochondria, conferring an additional anti-apoptotic mechanism [35]. The apoptosis resistance is crucial to guarantee the survival during cell migration. Considering the biology of metastatic process, in which cancer cells are continuously exposed to different selective pressures during the migration process, the acquisition of apoptosis resistance is mandatory to guarantee the cell survival. For this reason, during the EMT is expected the acquisition of CSC-like phenotype [20]. However, the main characteristic of EMT is the loss of cell adhesion as a consequence of biochemical and genetics reprogramming that lead to mesenchymal phenotype acquisition [36]. These reversible alterations confer invasiveness and migratory capability to cancer cells [37], allowing then to reach and colonize distant organs, resulting in metastasis. Based on these data, we also analyzed the acquisition of migratory phenotype as additional evidences of EMT.

Our results showed the loss of apical-basal polarity in skin papilloma, fibropapilloma and esophageal carcinoma cells (figure 4). This morphological alteration was not verified in normal skin cells (figure 4). In addition, we observed the presence of lamellipodia and filopodia in all BPV-infected primary cell cultures (figure 4). These protrusions were also verified by SEM analysis (figure 6).

Filopodia are transient organelles with 0.1-0.2 μm , composed by F-actin monomers, asymmetrically and perpendicularly to lamellipodia, being associated to cell migration [38,39]. Therefore, we also verified the F-actin labelling in lamellipodia sites (figure 5), confirm the results of phase contrast (figure 4) and screen electron microscopy (figure 6). The F-actin labelling in lamellipodia sites was observed in skin papilloma, fibropapilloma and esophageal carcinoma cells (figure 5), suggesting that BPV interacts with cytoskeleton, leading to filopodia formation. However, further evidences are necessary to better understand this interaction.

Based on the morphological analysis, we verified the cell velocity migration by time-lapse video microscopy. Results showed that papilloma 03 (fibropapilloma) and esophageal carcinoma cells presented the highest average speed, indicating the acquisition of migratory phenotype (figure 7, table 2). Papilloma 01 (skin papilloma) showed an intermediated migration velocity (figure 7, table 2). Normal skin and papilloma 02 (fibropapilloma) did not show statistical difference (figure 7, table 2).

We also observed the presence of inter-cytoplasmic bridges in skin papilloma (figure 6C), fibropapilloma (figure 6H) and esophageal carcinoma cells (figure 3I). Although little attention has been given to these structures, they can acts as vehicle of horizontal genetic transfers, since DNA sequences were already described into these inter-cytoplasmic bridges [40]. Considering that BPV genome is found in episomal form, these bridges can contribute to the maintenance of BPV DNA sequences along the six passages, as previous described by us using these same cell cultures [7].

In summary, our results demonstrate that primary culture derived from PVs-infected lesions can be useful models to study the viral pathology, showing BPV as an inducer the stem-cell phenotype acquisition and cell migration. These data combined to the cell metabolic deregulation, already verified with the same in vitro model [7], are important evidences for papillomavirus infection as a relevant EMT promoter.

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