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Responsiveness to basement membrane extract as a possible trait for tumorigenicity characterization

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ABSTRACT

Immortalized cell lines used to produce vaccines are expected to be described in terms of their tumorigenicity. However, current *in vivo* tumorigenicity assays can be time-consuming and results can be equivocal, especially for weakly tumorigenic cells. Basement membrane extract (BME) derived from the Engelbreth-Holm-Swarm mouse tumor, such as Matrigel and Cultrex, consists of laminin, collagen IV, entactin, heparan sulfate, and proteoglycans, as well as biologically active peptides and growth factors. For nearly three decades, BME has been used in cancer research to enhance tumorigenicity assays (both tumor "take" as well as tumor growth are substantially improved). We assessed the feasibility of using BME to facilitate the evaluation of vaccine cell substrate tumorigenicity. Vero cells (WHO 10-87) were serially passaged and banked at every ten passages beginning with p140; for the present study, low-passage Vero cells (Vero LP, originating from cells banked at p140) and high-passage Vero cells (Vero HP, originating from cells banked at p250) were used. In addition, Vero TPX2 and Vero NM1, cell lines established from tumors formed in nude mice by Vero HP cells, as well as other cell lines relevant to vaccine production (HeLa, MDCK, 293, and ARPE-19), were assessed. Female adult athymic nude mice were injected subcutaneously with cells in the absence or presence of BME. We observed that the tumorigenicity of ARPE-19 cells as well as Vero cells below passage 258 (Vero LP and Vero HP; previously characterized as non-tumorigenic or weakly tumorigenic, respectively) was not enhanced by BME. In contrast, BME shortened the latency and decreased the tumor-producing cell dose of HeLa, 293, and MDCK cells as well as the tumorigenic Vero derivatives TPX2 and NM1. Thus, responsiveness to BME may reflect the status of the neoplastic process and possibly serve as a useful trait for better defining the tumorigenic phenotype of cells.

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1. Introduction

Cell substrates are cells used to produce biological products such as vaccines [1]. Safety concerns regarding tumorigenic cells have limited the repertoire of cell substrates available for vaccine manufacture [2]. For example, for each of the viral vaccines available in the US manufactured with Vero cells (derived from spontaneously immortalized African green monkey kidney cells), the licensed process uses low-passage, non-tumorigenic cells [3]. Future development of vaccines and vaccine vectors will likely require the use of immortalized cells exhibiting phenotypes associated with neoplastic transformation, including the ability to form tumor xenografts. Replication-defective adenoviral vectors are, by necessity, propagated using trans-complementing cell lines, such as 293 and PER.C6, which harbor the adenoviral early regions associated with well-established oncogenic activities [4,5]. The human cancer-derived cell lines A549 and HeLa have been considered to be favorable for improving the yield of replication-competent adenoviral vectors [6] and adeno-associated viral vectors [7], respectively. The perceived safety risks associated with tumorigenic cell substrates remain theoretical (such as the possibility that they may harbor occult oncogenic viruses and/or DNA with oncogenic activity) and are difficult to estimate [8]. Nevertheless, a requirement exists for the tumorigenic potential of vaccine cell substrates to be described [1,9]. *In vivo* assessment of weakly tumorigenic cells (requiring high cell doses for tumor formation) can be time-

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consuming (>4 months) and results can be variable or equivocal (due to slow tumor progression and/or tumor regression) [10,11]. Therefore, it may be important to develop tumorigenicity assays of shorter duration with improved sensitivity and robustness.

Matrigel is an example of a basement membrane extract (BME) derived from the Engelbreth-Holm-Swarm (EHS) mouse tumor. BME components include laminin, collagen IV, entactin, heparan sulfate, and proteoglycans as well as biologically active peptides and growth factors [12]. Matrigel and similar BME preparations are liquid when kept on ice, but rapidly polymerize into a gel at 24–37 °C. Since the early 1990s, BME has been used in cancer research to facilitate *in vivo* tumorigenicity assays; both tumor incidence at a given cell dose as well as *in vivo* tumor growth rate are substantially improved when tumorigenic cells are co-injected with BME into immunodeficient animals [13,14]. The tumor-producing cell dose can be reduced dramatically using Matrigel (by 25,000-fold for the PC-3 human prostatic carcinoma cell line) [15]. Even single cells (isolated from human melanomas) have been shown to be capable of forming tumors in severely immunodeficient mice [NOD/SCID-IL2Rγ(-/-)] in the presence of Matrigel [16].

In the present study, we evaluated whether BME can facilitate tumorigenicity characterization (in terms of tumor incidence and cell dose required for tumor formation) of immortalized cell lines used in the manufacture of viral vaccines, both licensed (Vero and MDCK cells) and investigational (HeLa, 293, and ARPE-19 cells); the latter include oncogenically transformed human cell lines mentioned above (HeLa and 293) as well as a spontaneously immortalized human cell line (ARPE-19) currently being used for the development of a vaccine against human cytomegalovirus [17]. Our data suggest that BME may assist in assessing the phenotype of weakly tumorigenic cells (defined, for this study, as requiring $\geq 10^6$ cells for tumor formation).

2. Materials and methods

2.1. Cell lines

Cells from the World Health Organization (WHO) Vero cell bank 10-87 were serially passaged under contract by the American Type Culture Collection (ATCC) and banked at every ten passages beginning with p140; these cell banks have been described [10]. For the present study, low-passage Vero cells (Vero LP; originating from cells banked at p140; assessed at p144 or p150) and high-passage Vero cells (Vero HP; originating from cells banked at p250; assessed at p254 or p258) were used. The Vero TPX2 cell line was established in our laboratory as part of another study (manuscript in preparation) following two serial *in vivo* passages, in adult nude mice, of cells from a tumor formed by Vero HP cells. The Vero NM1 cell line was established as part of the present study from a tumor following injection of Vero HP cells into an adult nude mouse in the presence of Matrigel (Experiment 2). Both Vero TPX2 and Vero NM1 cells were evaluated for the present study as examples of tumorigenic derivatives of Vero cells. The species of origin for Vero LP, Vero HP, Vero TPX2, and Vero NM1 cells was verified to be African green monkey by multiplex PCR targeting the gene for mitochondrially encoded cytochrome *c* oxidase 1 (*MT-CO1*; human, African green monkey, mouse, rat, and Chinese hamster) performed as a contract service by IDEXX BioResearch (Columbia, MO); there was no evidence to suggest contamination of these Vero lineages by cells of other tested mammalian species (Sup. Fig. 1). HeLa (human; CCL-2), MDCK (canine; CCL-34; lot 3563161), 293 (human; CRL-1573), and ARPE-19 (human; CRL-2302) cells were obtained from ATCC. All cells with the exception of ARPE-19 cells were propagated using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM glutamine; ARPE-19 cells were propagated using Advanced DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum and 2 mM glutamine. All cells were tested and found to be negative for mycoplasma contamination (Universal Mycoplasma Detection Kit; 30-1012K; ATCC).

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2.2. Tumorigenicity assessment

Experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Center for Biologics Evaluation and Research (CBER; FDA) Institutional Animal Care and Use Committee. Homozygous (*nu/nu*) female athymic nude mice (5–7 weeks old) were obtained from the National Cancer Institute (NCI) or Charles River Laboratories and maintained at the Division of Veterinary Services within CBER. On the day of cell inoculation, cells were trypsinized, washed, and counted using the Nexcelom Cellometer. Cell viability was assessed by trypan blue exclusion. Cell suspensions prepared at the appropriate concentration in serum-free DMEM were maintained on ice.

The two available types of commercial BME were used (and directly compared in one experiment): Matrigel High Concentration (BD Biosciences or Corning) and Cultrex BME Type 3 (Trevigen). Both preparations are derived from the Engelbreth-Holm-Swarm tumor and suitable for *in vivo* tumorigenicity applications (high concentration; sterile; tested to be free of murine infectious agents including lactate dehydrogenase-elevating virus). Matrigel (~20 mg/mL) or Cultrex BME Type 3 (15.9 mg/mL) was thawed on ice to prevent gelling. Immediately prior to injection, cells were mixed with an equal volume of BME or serum-free DMEM and maintained on ice. Mice were injected subcutaneously (above the scapula) with 0.5 mL of cell mixture per animal using pre-cooled 1 mL disposable syringes fitted with 25G needles. Thus, each animal injected with BME received ~5 mg of Matrigel or ~4 mg of Cultrex Type 3. The effects of BME on tumorigenicity are known to be dose-dependent; the BME doses used in our study are in line with earlier assessments [14].

All animals were observed weekly for tumor development. The endpoint for Kaplan-Meier survival analysis was tumor incidence (when a mass at the site of injection first becomes observable or palpable, typically 2–5 mm in the longest dimension). Animals with tumors were monitored in order to verify progressive tumor growth, and tumor-bearing animals were sacrificed when the tumor was ≥10 mm but ≤20 mm in the longest dimension, consistent with requirements by our Institutional Animal Care and Use Committee. All surviving animals within an experiment that failed to reach the tumor incidence endpoint were sacrificed on the same day following an observation period lasting at least 4 months. Kaplan-Meier survival analysis was performed using GraphPad Prism (Version 7.01).

2.3. Histopathology of tumors

Tumors were excised at necropsy and fixed in 10% neutral-buffered formalin. Paraffin embedding, sectioning, mounting, and staining with hematoxylin and eosin were performed by American Histolabs, Inc. (Gaithersburg, MD).

3. Results

Tumorigenicity of Vero cell derivatives was assessed using female adult nude mice in three independent experiments

(Table 1). In Experiment 1, low-passage Vero cells (Vero LP; p144) and high-passage Vero cells (Vero HP; p254) were injected subcutaneously (10^7 cells per animal) in the absence or presence of Matrigel (~5 mg per animal). When observed under the microscope, both Vero LP cells and Vero HP cells formed well-organized epithelial cell sheets and were not readily distinguishable from each other morphologically (Fig. 1). Vero LP cells were non-tumorigenic (0/10 with tumor in the absence of Matrigel; 0/9 with tumor in the presence of Matrigel), consistent with our earlier study [10]. The proportion of animals with tumor following injection with Vero HP cells (previously characterized as weakly tumorigenic in newborn nude mice [10]) was comparable with (1/9) or without (3/10) Matrigel. In a subset within the same cohort of animals, HeLa cells were titrated in the absence or presence of Matrigel to serve as positive control for the *in vivo* functionality of Matrigel. HeLa cells generated tumors at a dose of 10^6 cells per animal (4/5), with tumors occurring in 4–7 weeks; lower cell doses (10^5–10^2 cells) resulted in no tumors for this experiment. In the presence of Matrigel, the tumor-producing dose of HeLa cells was dramatically reduced; tumors were observed in 100% (5/5) of the animals within 6 weeks at 10^2 cells per animal (the lowest dose tested).

In Experiment 2, we verified the tumorigenic phenotype of Vero LP and Vero HP cells (Table 1). Vero LP cells (p150) yielded no tumors (0/5) in the absence of Matrigel; a single tumor-bearing animal (1/5) was observed in the presence of Matrigel after a prolonged latency (177 days). Comparable results were observed with Vero HP cells (p258) in the absence (2/5) or presence (3/5) of Matrigel. A Vero HP tumor-derived cell line designated Vero TPX2, established following two serial *in vivo* passages in adult nude mice, was also assessed. When observed microscopically, Vero TPX2 cells were pleomorphic and refractile relative to parental Vero HP cells (Fig. 1). Tumorigenicity of Vero TPX2 cells exhibited a dose response with 5/5, 4/5, and 1/5 mice bearing tumors when injected with 10^4, 10^3, and 10^2 cells per animal, respectively; Matrigel resulted in a substantial shift in tumor response, with 100% of animals (5/5) bearing tumor at 10 cells per animal. An abbreviated titration of HeLa cells was included in this cohort. Animals were tumor-free (0/5) following injection with 10^4 HeLa cells per animal in the absence of Matrigel; however, a tumor-bearing animal (1/5) was observed following injection with 10 cells in the presence of Matrigel.

In Experiment 3, another Vero HP cell derivative designated Vero NM1 was assessed (Table 1). The Vero NM1 cell line was established from a tumor following injection of Vero HP cells into an adult nude mouse in the presence of Matrigel. Vero NM1 cells were less refractile compared with Vero TPX2 cells; they were also morphologically distinguishable from parental Vero HP cells and formed disorganized cell sheets (Fig. 1). Vero NM1 cells were tumorigenic in the absence of BME with 5/5, 2/5, 0/5, and 0/5 mice bearing tumors when injected with 10^5, 10^4, 10^3, and 10^2 cells per animal, respectively. Tumorigenicity of Vero NM1 cells was also evaluated in the presence of two types of BME: Matrigel and Cultrex Type 3. In a manner similar to what was observed for Vero TPX2 cells, both Matrigel (~5 mg per animal) and Cultrex (~4 mg per animal) shifted the dose response for Vero NM1 cells. In the presence of Matrigel, 5/5, 5/5, 3/5, and 0/5 mice were observed with tumors when injected with 10^4, 10^3, 10^2, and 10^1 cells per animal, respectively. In the presence of Cultrex, 4/5, 4/5, 2/5, and 0/5 mice were observed with tumors when injected with 10^4, 10^3, 10^2, and 10^1 cells per animal, respectively. In addition, both Matrigel and Cultrex facilitated tumor formation of HeLa cells (positive control); 0/5 in the absence of BME, 5/5 in the presence of Matrigel, and 5/5 in the presence of Cultrex were observed with tumors when injected with 10^2 HeLa cells per animal.

Table 2 is a compilation of the data contained in Table 1.

Kaplan-Meier survival curves for animals injected with Vero LP and Vero HP cells (10^7 cells per animal; data combined from Experiment 1 and Experiment 2) are shown in Fig. 2A. All of the tumors

Table 1
Proportion of adult nude mice with tumors following injection of cells (Vero derivatives; HeLa as positive control) in the presence or absence of basement membrane extract (Matrigel or Cultrex).

Exp 1	Cell line					
Cell dose	Vero LP	Vero LP + MG	Vero HP	Vero HP + MG	HeLa	HeLa + MG
10^7	0/10	0/9	3/10	1/9	NT	NT
10^6	NT	NT	NT	NT	4/5	NT
10^5	NT	NT	NT	NT	0/5	4/4
10^4	NT	NT	NT	NT	0/5	5/5
10^3	NT	NT	NT	NT	0/5	5/5
10^2	NT	NT	NT	NT	0/5	5/5
10^1	NT	NT	NT	NT	NT	NT

Exp 2	Cell line							
Cell dose	Vero LP	Vero LP + MG	Vero HP	Vero HP + MG	Vero TPX2	Vero TPX2 + MG	HeLa	HeLa + MG
10^7	0/5	1/5	2/5	3/5	NT	NT	NT	NT
10^6	NT	NT	NT	NT	NT	NT	5/5	NT
10^5	NT	NT	NT	NT	5/5	NT	NT	NT
10^4	NT	NT	NT	NT	5/5	5/5	0/5	4/5
10^3	NT	NT	NT	NT	4/5	5/5	NT	NT
10^2	NT	NT	NT	NT	1/5	5/5	NT	2/5
10^1	NT	NT	NT	NT	NT	5/5	NT	1/5

Exp 3	Cell line					
Cell dose	Vero NM1	Vero NM1 + MG	Vero NM1 + Cultrex	HeLa	HeLa + MG	HeLa + Cultrex
10^5	5/5	NT	NT	NT	NT	NT
10^4	2/5	5/5	4/5	4/5	5/5	5/5
10^3	0/5	5/5	4/5	NT	NT	NT
10^2	0/5	3/5	2/5	0/5	5/5	5/5
10^1	NT	0/5	0/5	NT	NT	NT

NT: Not Tested.
MG: Matrigel.

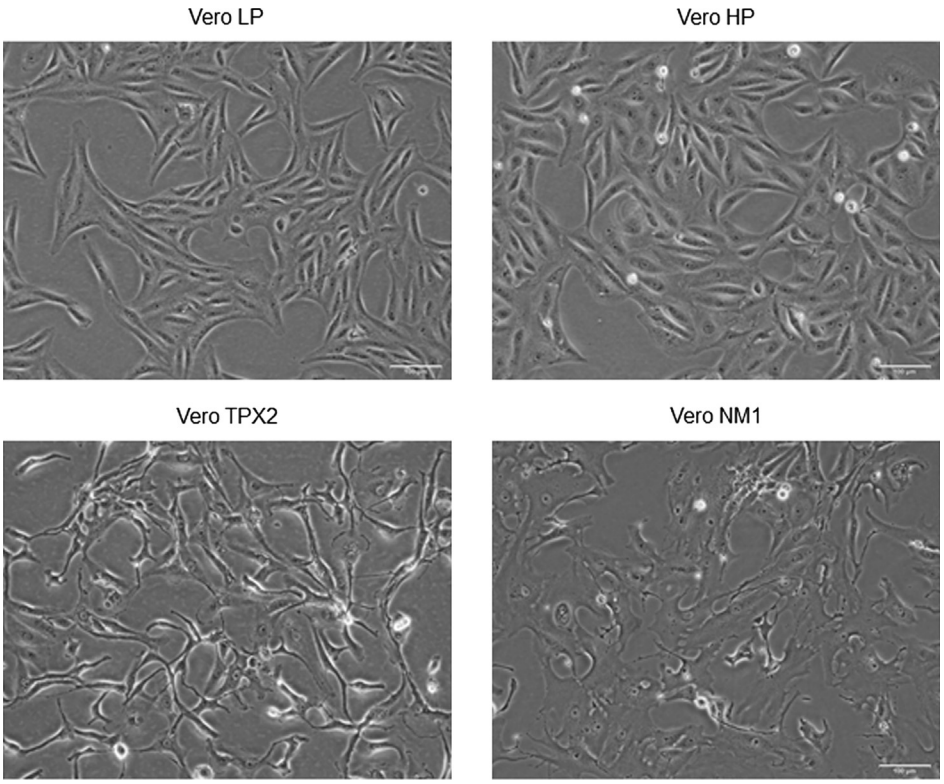

Fig. 1. Phase-contrast micrographs of Vero LP, Vero HP, Vero TPX2, and Vero NM1 cells in culture. Images were taken on an Olympus 1X51 microscope using a 10× objective.

Table 2
Summary of tumorigenicity data in Table 1.

	Cell Line									
Cell dose	Vero LP	Vero LP + MG	Vero HP	Vero HP + MG	Vero TPX2	Vero TPX2 + MG	Vero NM1	Vero NM1 + BME	HeLa	HeLa + BME
10^7	0/15	1/14	5/15	4/14	NT	NT	NT	NT	NT	NT
10^6	NT	NT	NT	NT	NT	NT	NT	NT	9/10	NT
10^5	NT	NT	NT	NT	5/5	NT	5/5	NT	0/5	4/4
10^4	NT	NT	NT	NT	5/5	5/5	2/5	9/10	4/15	19/20
10^3	NT	NT	NT	NT	4/5	5/5	0/5	9/10	0/5	5/5
10^2	NT	NT	NT	NT	1/5	5/5	0/5	5/10	0/10	17/20
10^1	NT	NT	NT	NT	NT	5/5	NT	0/10	NT	1/5

NT: Not Tested.
MG: Matrigel.
BME: Basement Membrane Extract (Matrigel or Cultrex).

occurred following prolonged latency (≥149 days). Matrigel did not impact tumor-free survival (log-rank test P values >0.05).

Kaplan-Meier survival curves for animals injected with Vero TPX2 and Vero NM1 cells (Experiment 2 and Experiment 3) are shown in Fig. 2B and C. In contrast to Vero LP and Vero HP cells, most of the Vero TPX2 tumors (24 out of 25) occurred within 65 days following injection with $\leq 10^4$ cells per animal (Fig. 2B). Matrigel significantly decreased tumor-free survival at each cell dose (log-rank test P values of 0.014, 0.0027, and 0.0017 for animals injected with 10^4, 10^3, and 10^2 Vero TPX2 cells, respectively). For animals injected with 10^2 Vero TPX2 cells in the presence of Matrigel, tumors (5/5) occurred between days 37–65; in contrast, in the absence of Matrigel, only a single tumor was observed out of 5 animals at day 114 following injection of the same cell dose. Similarly, for Vero NM1 cells (Fig. 2C; 10^4 cells per animal), decreased tumor-free survival was observed in the presence of both Matrigel (log-rank test P value of 0.0079) and Cultrex (log-rank test P value of 0.078; however, the Gehan-Breslow-Wilcoxon test, which gives more weight to tumors occurring at early times, yielded a P value of 0.043). In the absence of BME, injection with 10^4 Vero NM1 cells produced tumors in 2 out of 5 animals at days 71–100; at the same cell dose, tumors occurred between days 51–72 in the presence of Matrigel (5/5) and days 44–58 in the presence of Cultrex (4/5).

Sections of Vero cell tumors stained with hematoxylin and eosin were examined. No obvious morphologic difference was consistently observed among tumors resulting from the Vero cell derivatives (Vero HP, Vero TPX2, and Vero NM1) whether in the absence or presence of BME; a typical image is shown in Fig. 3 (a section from a tumor resulting from injection of Vero TPX2 cells in the presence of Matrigel). All tumors consisted of undifferentiated pleomorphic cells with pleomorphic nuclei and indistinct cell borders; nuclei contained multiple large nucleoli. Chromatin was coarsely clumped and distributed primarily along the nuclear membrane. The nuclear/cytoplasmic ratio was generally 1:1 (a normal ratio is 1:4–1:6). The mitotic index was high (3–4 per high-power field). Areas of some sections appeared to be tending toward a higher degree of differentiation characterized by

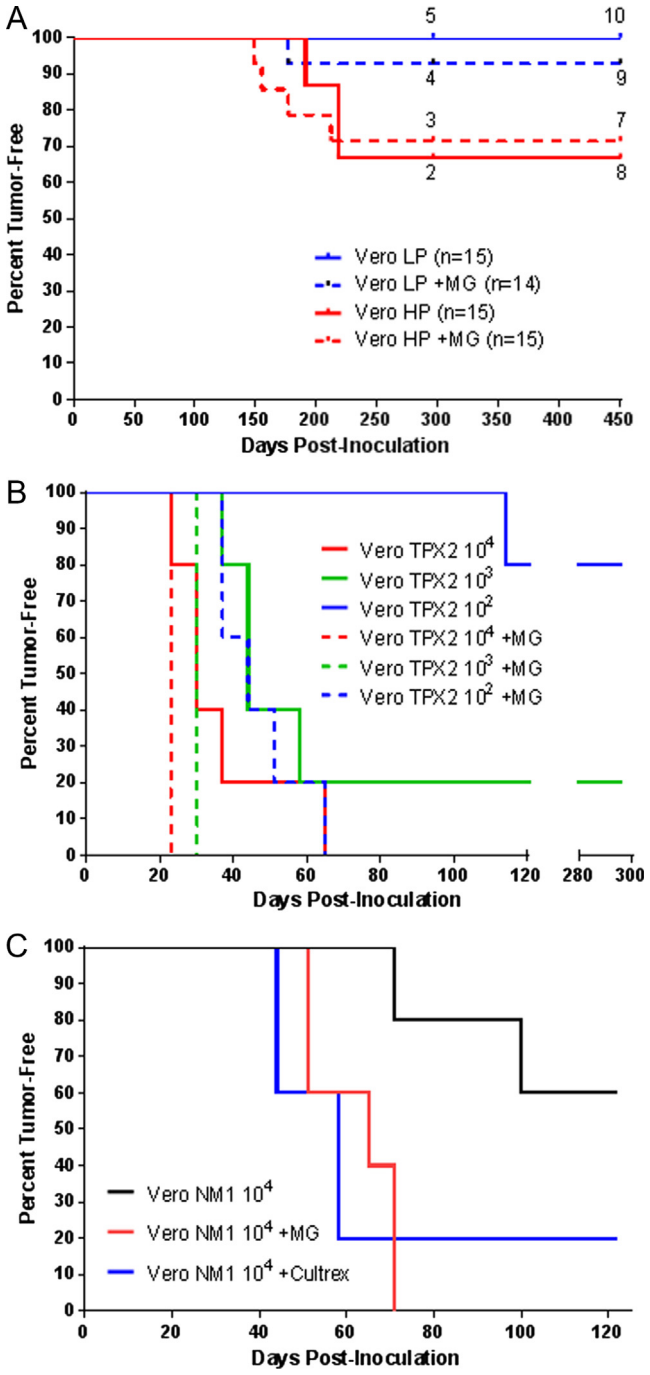

Fig. 2. Tumor incidence over time in nude mice injected with Vero cell derivatives. (A) Female adult nude mice were injected subcutaneously with Vero LP or Vero HP cells (10^7 cells per animal) in the absence or presence of Matrigel (MG; 5 mg per animal). Data were combined from Experiment 1 and Experiment 2. The number of animals surviving without observable tumor (and thus censored) at the end of the observation period (450 days for Experiment 1 and 296 days for Experiment 2) are shown. (B) Female adult nude mice were injected subcutaneously with Vero TPX2 cells (Experiment 2; 10^4, 10^3, or 10^2 cells per animal) in the absence or presence of Matrigel (5 mg). (C) Female adult nude mice were injected subcutaneously with Vero NM1 cells (Experiment 3; 10^4 cells per animal) in the absence or presence of BME (5 mg of Matrigel or 4 mg of Cultrex).

elongated, spindle-shaped cells organized into interlacing bundles; however, these cells still retained large nuclei with multiple large nucleoli. Small foci of necrosis with cells undergoing pyknosis were apparent throughout the tumors. There were numerous neutrophils randomly distributed throughout the sections as well as mononuclear cells infiltrating the periphery.

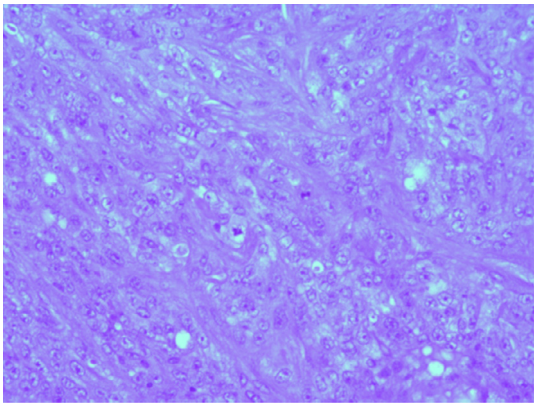

Fig. 3. Histopathology of Vero cell tumors. Tumors formed by injection of Vero cell derivatives (Vero HP, Vero TPX2, and Vero NM1) were fixed with formalin and sections were stained with hematoxylin and eosin. A typical image is shown (for this example, a section from a tumor resulting from injection of Vero TPX2 cells in the presence of Matrigel; 40× objective).

In Experiments 4 and 5, we assessed whether BME altered the tumorigenicity of three additional cell lines that have been used as vaccine cell substrates: MDCK, 293, and ARPE-19. Injection with 10^5 MDCK cells (spontaneously immortalized canine kidney epithelial cells) resulted in tumors in 100% of animals (5/5; 30–80 days); no tumors were observed (0/5) following injection with 10^4 cells (Experiment 4; Table 3). These data are consistent with our earlier study [11]. Matrigel caused a shift in tumor dose response with 5/5 (within 30 days), 2/5 (30–45 days), 2/5 (80–140 days), and 2/5 (150–200 days) mice bearing tumor when injected with 10^4, 10^3, 10^2, and 10 cells per animal, respectively. 293 cells (human embryonic kidney cells immortalized with sheared adenovirus 5 DNA) produced tumors within 3 months in the absence of BME (5/5 at 10^7 cells per animal and 3/5 at 10^6 cells per animal; Experiment 5; Table 3), consistent with published reports [18]. In the presence of Matrigel, 293-derived tumors were observed within approximately 30 days in 5/5, 5/5, 5/5, and 2/5 when injected with 10^7, 10^6, 10^5, and 10^4 cells per animal, respectively. Thus, both MDCK and 293 cells unequivocally responded to BME-mediated tumor facilitation. ARPE-19 cells (spontaneously immortalized human retinal pigment epithelial cells [19], a cell substrate for an investigational vaccine against human cytomegalovirus [17]) were non-tumorigenic at 10^7 cells per animal in the absence of BME (Experiment 5; Table 3). In the presence of Matrigel, a small nodule (5–8 mm) persisted at the site of inoculation in each animal (10^7 ARPE-19 cells per animal) with minimal change until the end of the observation period (6 months; Fig. 4A); the dissected masses were firm, well-circumscribed, and somewhat translucent (Fig. 4B). Microscopic observation revealed that these masses consisted of an amorphous eosinophilic material interspersed with a few degenerating cells (Fig. 4C). As progressively growing tumors were not observed, we interpret these results to mean that ARPE-19 cells are non-tumorigenic under our experimental conditions either in the absence or presence of BME.

4. Discussion

In the present study, we evaluated the influence of BME on the tumorigenic phenotype of cell lines used in the manufacture of licensed (Vero and MDCK) and investigational (293, ARPE-19, and HeLa) vaccines. Each experiment included at least one cell type that served as positive control, demonstrating the functionality of BME. In the absence of BME, Vero LP cells were non-tumorigenic,

Table 3
Proportion of adult nude mice with tumors following injection of cells (MDCK; 293; ARPE-19; HeLa as positive control) in the presence or absence of basement membrane extract (Matrigel).

Exp 4	Cell line			
Cell dose	MDCK	MDCK + MG	HeLa	HeLa + MG
10^7	NT	NT	NT	NT
10^6	5/5	NT	NT	NT
10^5	5/5	NT	NT	NT
10^4	0/5	5/5	NT	NT
10^3	NT	2/5	NT	5/5
10^2	NT	2/5	NT	5/5
10^1	NT	2/5	NT	NT

Exp 5	Cell line					
Cell dose	ARPE-19	ARPE-19 + MG	293	293 + MG	HeLa	HeLa + MG
10^7	0/5	0/5	5/5	5/5	NT	NT
10^6	NT	NT	3/5	5/5	NT	NT
10^5	NT	NT	0/5	5/5	NT	NT
10^4	NT	NT	NT	2/5	0/3	3/3
10^3	NT	NT	NT	NT	NT	NT
10^2	NT	NT	NT	NT	0/3	3/3

NT: Not Tested.
MG: Matrigel.

whereas Vero HP cells were weakly tumorigenic with tumor incidence below 100% when injected with 10^7 cells per animal and tumor latency exceeding 149 days. Importantly, tumor formation was not facilitated when Vero LP or Vero HP cells were injected with BME. We interpret these results to mean that these Vero cells have not yet undergone genetic or epigenetic changes necessary to be responsive to BME *in vivo*. BJ-hTERT cells (human skin fibroblasts immortalized by expression of the telomerase catalytic component hTERT) have been reported to be non-tumorigenic in nude mice when co-injected with Matrigel [20]. Thus, Vero cells ($\leq$p258) appear to behave more akin to BJ-hTERT cells in this respect (i.e., they are immortalized, but do not exhibit other transformed phenotypes). The same may be true for ARPE-19 cells; the reportedly normal karyology is consistent with these cells being non-transformed [19].

Vero cells can, however, progressively acquire changes that enhance their tumorigenic activity. Our Vero TPX2 and Vero NM1 cells were tumorigenic in the absence of BME; in the presence of BME, the tumor-producing dose was substantially reduced and the latency period was shortened. Likewise, HeLa, MDCK, and 293 cells were tumorigenic and responded unequivocally to BME *in vivo*. The mechanism by which BME enhances tumorigenicity is not fully understood, although promotion of tumor-cell survival and angiogenesis appear to be involved [21]. Nevertheless, responsiveness to BME may be a potentially useful operationally defined trait for identifying cells that are neoplastically transformed. Both 293 cells and Vero HP cells can be considered weakly tumorigenic because they require $\geq 10^6$ cells to form tumors in the absence of BME; however, they behave in a qualitatively distinguishable manner in response to BME. We hypothesize that Vero HP cells lack tumorigenic potential at the time of injection; however, a subpopulation may persist *in vivo* resulting in the sporadic emergence of a tumorigenic lineage following prolonged latency after injection of a high cell dose (10^7 cells per animal). BME does not appear to influence the *in vivo* evolution of a tumorigenic Vero HP lineage; however, once the transition in tumorigenic potential occurs, the cells can respond to BME, as evidenced by the behavior of Vero TPX2 and Vero NM1 cells (both cell lines derive from sporadic Vero HP tumors).

It is notable that others have proposed using BME in *in vivo* assays to enhance the detection of residual tumorigenicity associated with cell therapies [22–24]. Kusakawa et al. used HeLa cells as model tumorigenic cells to explore the sensitivity of NOD/Shi-scid IL2Rγ^{null} (NOG) and nude mice; they observed that the 50% tumor-producing dose (TPD_{50}) of HeLa cells was 4.0×10^5 cells in nude mice, 1.3×10^4 cells in NOG mice ($\sim$30-fold higher sensitivity compared with nude mice), and 79 cells in NOG mice in the presence of Matrigel ($\sim$5000-fold higher sensitivity compared with nude mice) [25]. The investigators conclude that Matrigel-enhanced tumorigenicity assays using NOG mice can be highly efficient, although they did not evaluate the sensitivity of nude mice in the presence of Matrigel. The degree of host animal immunodeficiency can indeed be an important determinant of tumorigenicity assay sensitivity; however, these data, together with our own, suggest that the primary factor capable of dramatically decreasing the TPD_{50} is likely to be BME rather than mouse strain background.

The true relevance of cell-substrate tumorigenicity to vaccine safety is unknown. While the perceived risks are theoretical, relevant issues were weighed carefully and publicly discussed before the FDA Vaccines and Related Biological Products Advisory Committee (November 2005, September 2008, and September 2012) [26]. Prudent risk-reduction measures have also been advocated (mitigation of adventitious agents as well as residual cell-substrate DNA activity) [8]. Despite the uncertainty associated with the significance of tumorigenicity, the recommendation to describe vaccine cell substrates in terms of their tumorigenic phenotype remains in place in the US [1]. Generally, vaccine cell substrates have been assessed thus far in tumorigenicity assays performed with adult nude mice in the absence of BME. However, even without ascribing a deeper significance to the possible consequences of using neoplastically transformed cell substrates in the manufacture of vaccines (i.e., whether transformed cell substrates are associated with higher risks for vaccine recipients compared with non-transformed cell substrates), conventional tumorigenicity testing with nude mice can be inadequate in some cases if it merely aims to provide an accurate reflection of the transformed status of the tested cells. If our hypothesis concerning Vero HP cells is correct, the sporadic Vero HP-derived tumors that occur following prolonged latency may be false positive results that misrepresent the intrinsic tumorigenicity of Vero HP cells. With regard to false negativity, less than 10% (often close to 0%) of human tumor biopsy specimens produce progressively growing tumors in adult nude mice; however, co-injection with BME can enhance tumor biopsy "take" to greater than 90% [27]. It is difficult to dispute the clinical significance of such primary biopsy specimens (i.e., they are presumed to consist of transformed cells taken from *bona*

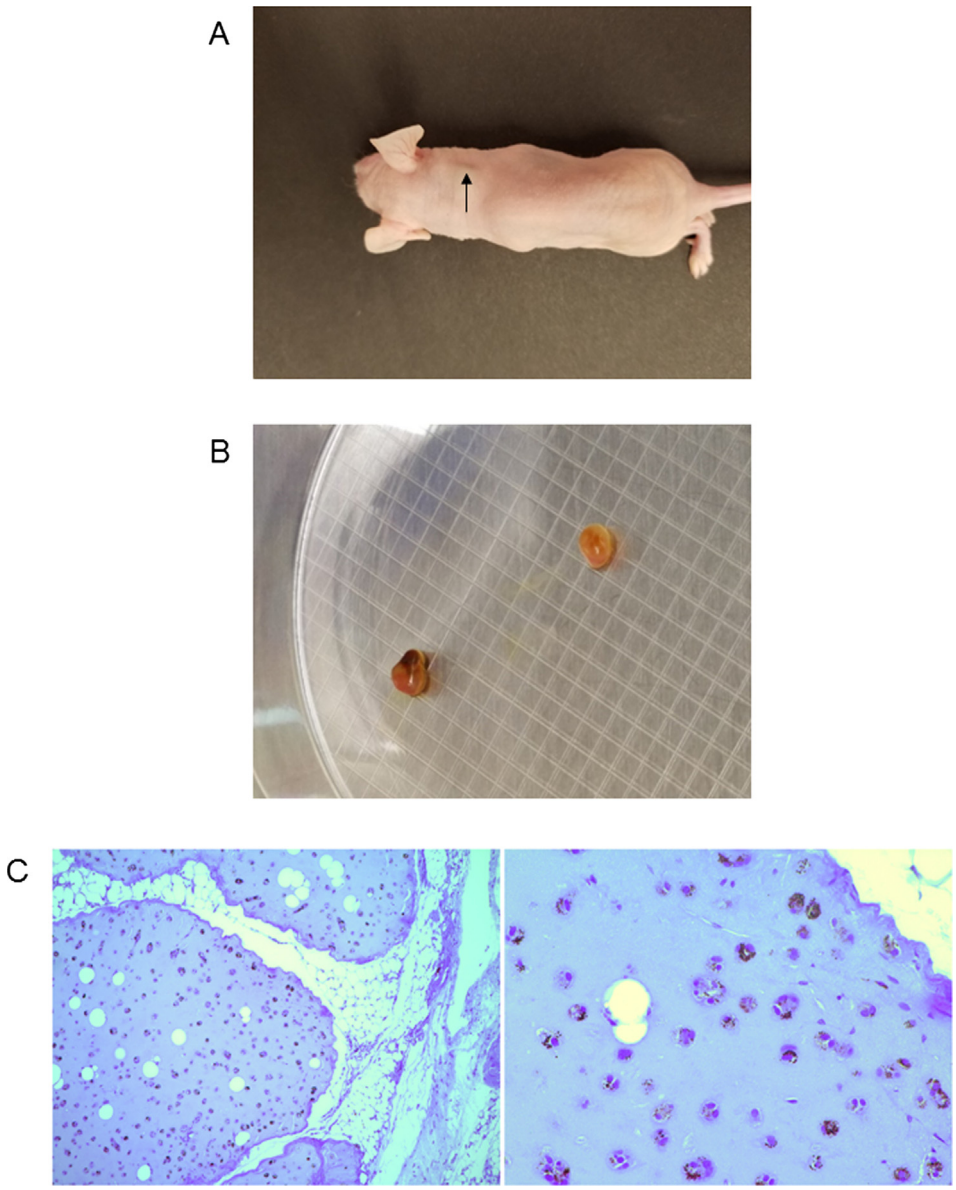

Fig. 4. Masses resulting from ARPE-19 cell injection in the presence of Matrigel. (A) Nodule (indicated by arrow) resulting from injection of 10^7 ARPE-19 cells in the presence of Matrigel. (B) Dissected masses obtained from animals injected with 10^7 ARPE-19 cells in the presence of Matrigel following necropsy at the end of the observation period (6 months). The plate surface is covered by a 2 mm grid. (C) Following formalin fixation, sections were stained with hematoxylin and eosin (10× objective for the left panel; 40× objective for the right panel).

fide human carcinomas), negative results in conventional tumorigenicity assessment notwithstanding. In addition to tumor biopsy specimens, there are established tumor-derived cell lines (for example, the human breast adenocarcinoma cell line MCF-7) that have also been reported to be robustly tumorigenic when facilitated by BME, but not in its absence [13,14,28–30]. Our data suggest that BME may facilitate the evaluation of cell substrates by increasing the sensitivity for detection of tumorigenic activity and shortening *in vivo* assay duration. BME may be particularly helpful in characterizing the status of neoplastic transformation for weakly tumorigenic cells.

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Conflict of interest

The authors report no conflict of interest.

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