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Influence of mildly acidic pH conditions on the production of lentiviral and retroviral vectors

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ABSTRACT

Human immunodeficiency virus type 1-derived lentiviral vectors (LVs) are becoming major tools for gene transfer approaches. Several gene therapy clinical studies involving LVs are currently ongoing. Industrial production of clinical-grade LVs is therefore an important challenge. Some improvements in LV production protocols have already been possible by acting on multiple steps of the production process like transfection, cell culture or media optimizations. Yet, the effects of physicochemical parameters such as pH remain poorly studied. Mammalian cell cultures are generally performed at neutral pH, which may not be the optimal condition to produce high quantities of LVs with optimal infectious properties. In this study, we showed that lentiviral transient production in HEK293T cells is inversely dependent on the pH value of the harvesting medium. Infectious and physical titers of LVs pseudotyped with GALVTR or VSV-G glycoproteins are enhanced by two- to three fold at pH 6 compared with neutral conditions. pH 6-produced LVs are highly infectious on cell lines but also on relevant primary target cells like hCD34+ hematopoietic stem/progenitor cells. GALVTR-LV particles produced at pH 6 are highly stable at 37°C and resistant to multiple freeze-thaw cycles. Higher levels of expression of intracellular pr55gag polyproteins are observed within HEK293T producer cells cultured at pH 6. The positive effect of pH 6 conditions is also observed for moloney-derived retroviral vectors produced from NIH-3T3 fibroblasts, arguing that the mildly acidic pH effect is not limited to the lentivirus genus and is reproducible in various producer cell lines. This observation may help us in the design of more effective LV production protocols for clinical applications.

INTRODUCTION

Production of clinical grade lentiviral vector (LV) remains a challenging operation and limited yields of vector may eventually hamper therapeutic use and industrial development. Some improvements in LV production protocols have been possible by acting on multiple steps of the production process: transfection optimizations (*i.e.*, transfection reagents, cell density, plasmid ratio), cell culture conditions (*i.e.*, adherent cells vs. cell suspensions in bioreactor) and media optimizations (*e.g.*, addition of lipids, cholesterol, chloroquine, sodium butyrate) (Ansorge *et al.*, 2010; Schweizer and Merten, 2010). One widely used parameter in mammalian cell culture is the use of neutral pH conditions. However, it is tempting to speculate that neutral pH may not be the optimal pH to produce high quantities of LVs with optimal infectious properties. Indeed, some primary strains or laboratory adapted strains of human immunodeficiency virus type 1 (HIV-1) showed enhanced levels of infectivity at acidic pH compared to neutral pH (Connor, 2006; Fackler and Peterlin, 2000; Maurin *et al.*, 2007). However, HIV-1-derived LVs pseudotyped with the vesicular stomatitis virus envelope glycoprotein (VSV-G-LVs) are reportedly unstable in a pH 6 solution of phosphate buffer (Higashikawa and Chang, 2001). It is thus unclear whether mildly acidic pH would be a beneficial or deleterious parameter for LVs and whether or not this depends on the use of envelope pseudotypes.

In this study, we show that LV transient production in HEK293T cells is inversely dependent on the pH value of the harvesting medium. The production of LVs, pseudotyped with VSV-G or GALVTR [modified gibbon ape leukemia virus envelope glycoproteins (Sandrin *et al.*, 2002)], in pH 6 medium improved the level of LV production by two- to three fold. LVs produced at pH 6 are highly infectious on cell lines but also on relevant primary target cells like hCD34+ hematopoietic stem/progenitor cells (HSPCs). Immunoblotting experiments

have shown that the p55gag precursor was more expressed in HEK293T producer cells cultured at pH 6. GALVTR-LVs particles produced at pH 6 were highly stable at 37°C and after exposure to multiple freeze-thaw cycles. Interestingly, this positive effect of pH 6 on LVs is reproducible on moloney-derived retroviral vectors pseudotyped with GALV glycoproteins (GALV-MLV), produced from a stable murine packaging cell line.

MATERIAL AND METHODS

Peptide and Reagents

The Vectofusin-1 transduction enhancer was produced by standard fluorenylmethyloxycarbonyl chloride solid-phase peptide synthesis, purified by preparative reverse phase HPLC, and analyzed by HPLC and mass spectrometry (Genecust, Dudelange, Luxembourg). Peptide purity was >99%. 7-amino-actinomycin D (7-AAD) and Trypan Blue were obtained from Sigma-Aldrich (St-Quentin-Fallavier, France).

Cell line culture

HCT116 cells derived from a human colorectal carcinoma (CCL-247; ATCC, Manassas, VA), human embryonic kidney HEK293T cells (Merten *et al.*, 2011) and retroviral producer PG13-MFG-GFP cells (Merten, 2004) were cultured at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM+Glutamax) supplemented with 10% of heat inactivated fetal calf serum (FCS) (Life Technologies, St-Aubin, France). The DMEM was buffered at indicated pH values using hydrochloric acid or sodium hydroxide and was sterile-filtered (0.22µm).

Viral vector production and vector titering

HIV-1-derived lentiviral vectors were generated by transient calcium phosphate transfection of HEK293T cells with four plasmids (Fenard *et al.*, 2013): The gagpol (pKLGagpol) and rev (pKrev) expression plasmids, the transfer plasmid (pCCLsin.cPPT.hPGK.eGFP.WPRE) and the plasmid encoding the GALVTR (pBA.GALV/Ampho-Kana) or VSV-G (pMDG) envelope glycoproteins. About 16-20 hr posttransfection, HEK293T cells were washed and incubated in DMEM/FCS (2-10%) buffered at the indicated pH value ranging from 6 to 8.

After 24 hr of production, raw viral supernatants were harvested, filtered (0.45 μm) and frozen at -80°C . Physical particle titers were determined by measuring HIV-1 p24 capsid contents using a commercial ELISA kit (Perkin Elmer, Courtaboeuf, France). Infectious titers were determined on HCT116 cells using the detection of the green fluorescent protein (GFP) by flow cytometry (FACSCalibur, BD Biosciences, Le Pont de Claix, France) (Fenard *et al.*, 2013). Infectious titers were expressed as transducing units per milliliter (TU/ml).

Human CD34+ cells culture and transduction

Umbilical cord blood (UCB) samples were obtained after uncomplicated births and in accordance with international ethical principles and French national law under declaration N^o DC-201-1655 to the French Ministry of Research and Higher Studies. Human CD34+ cells were isolated by immunomagnetic selection (Miltenyi Biotec, Paris, France) from the mononuclear cell fraction of UCB samples and were stored at -80°C . After thawing, the survival rate of hCD34+ cells was evaluated using the Trypan blue exclusion method (Fenard *et al.*, 2013). Next, the preactivation of hCD34+ cells was performed overnight as previously described (Ingrao *et al.*, 2014). Pre-activated cells were plated in 96-well plates (8.3×10^3 cells/50 μl). Transduction was initiated by adding 50 μl of medium containing the desired amount of LV particles mixed with or without the Vectofusin-1 transduction enhancer (final concentration of $12\mu\text{g/ml}$). At 6 hr posttransduction, reactions were diluted by adding 0.3 ml of differentiation medium in each well. After 4-6 days, cellular mortality and transduction efficiency were evaluated respectively by 7-AAD labeling and measurement of GFP expression using flow cytometry (FACSCalibur, BD Biosciences). LV stocks used in this experiment were titered on HCT116 cells. Infectious titers for pH 7.2 versus pH 6-produced GALVTR-LVs presented a 2.4-fold difference (6.3×10^6 vs. 15×10^6 TU/ml) and 1.5-fold difference for VSV-G-LVs (1.3×10^8 vs. 2×10^8 TU/ml).

Viral vector exposure to 37°C temperature and multiple freeze-thaw cycles.

Frozen vials of pH 7.2- and pH 6-produced GALVTR-LV and VSV-G-LV supernatants were incubated for the indicated time at 37°C, without opening the vials (closed environment) or incubated at 37°C in culture plate wells under 5% CO₂ and 95% humidity (open environment). Next, vials were frozen back at -80°C and titrations on HCT116 cells were performed simultaneously for all the conditions to avoid interexperiment variability.

For the freeze-thaw stability experiment, one frozen vial of GALVTR-LV pH 7.2 and one of pH 6 supernatants were thawed during 20 min at room temperature and frozen back immediately. The next day, the vials were thawed again (Freeze-Thaw cycle 2) and simultaneously, two vials of GALVTR-LV pH 7.2 or pH 6 supernatants of the same lot were thawed (Freeze-Thaw cycle 1). It allowed us to concomitantly determine the different GALVTR-LV infectious titers to avoid interexperiment variability.

Western blot experiments and analysis

Producer cells were washed and lysed in a buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 10% glycerol, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Meylan, France). Protein concentrations were determined using the Bio-Rad DC Protein Assay kit I (Bio-Rad, Marnes-la-Coquette, France). Proteins (30µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose Hybond ECL membrane (GE Healthcare Life Sciences, Velizy-Villacoublay, France) and immunoblotted with a combination of goat anti-p24 (Abd Serotec, Oxford, UK) and mouse anti-actin (AC-15 clone) (Sigma-Aldrich). IRDye 800-conjugated donkey anti-goat and IRDye 680-conjugated donkey

anti-mouse were used as secondary antibodies (Eurobio, Courtaboeuf, France). Immunoreactive bands were detected with the Odyssey infrared scanner and quantified using Odyssey 3.0 analytical software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

In every data set, *p*-values were determined by nonparametric tests using GraphPad Prism 5. *p*-values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Mildly acidic pH conditions improve the production of GALVTR-LV and VSV-G-LV pseudotypes

LVs pseudotyped with GALVTR envelope glycoproteins are highly efficient for human HSPCs targeting (Jacome *et al.*, 2009; Sandrin *et al.*, 2002). Yet, production and purification of GALVTR-LVs remain a challenge and protocols have not been reported yet. The absence of reported information on the optimal pH value for such LV production prompted us to directly measure the efficacy of HEK293T cells to produce GALVTR-LVs in various culture media with a pH range from 6 to 8 (Fig. 1A). The window of pH was maintained between pH 6 and pH 8 and could not be extended further either way because of the buffering effect of DMEM, a culture medium typically used for clinical grade production of LVs (Merten *et al.*, 2011). As shown in Fig. 1B, infectious titers of GALVTR-LVs produced in pH 8 medium were strongly impaired in comparison with the typical pH 7.2 condition. On the contrary, infectious titers of GALVTR-LVs produced in pH 6 medium were significantly higher (average of 2.3-fold) than the one obtained at pH 7.2 (Fig. 1B and C). Interestingly, a concomitant increase was observed in the p24 contents of GALVTR-LVs produced at pH 6 (Fig. 1B and D). This positive correlation led to a stable infectivity value of GALVTR-LVs produced either at neutral or mildly acidic pH (Fig.1B and 1E), while this parameter was strongly decreased at pH 8 (Fig. 1B). Hence, the use of mildly acidic pH medium seems to be optimal to produce high amounts of GALVTR-LVs.

To assess if mildly acidic conditions also apply to the broadly used VSV-G lentiviral pseudotypes, pH 7.2- or pH 6-buffered DMEM were used to produce VSV-G-LV supernatants. The pH 6 condition significantly increased the production of infectious (Fig. 2A) and physical (Fig. 2B) VSV-G-LV particles (average of 1.5-fold) leading to a stable

infectivity value (Fig. 2C). While confirming the effect observed with GALVTR-LV, the effect on VSV-G-LV is more modest. The efficacy of the mildly acidic pH condition may be partially pseudotype dependent, the level of viral particles produced in the supernatant being dependent on the intracellular trafficking efficiency and the expression level of a given envelope glycoprotein (Frecha *et al.*, 2008).

Concerning the cell culture conditions, the microscopic observation of HEK293T producer cells did not reveal any obvious cytotoxic effect of the pH 6 DMEM during the 24 hr period of production. HEK293T cells were still adherent and confluent (data not shown).

Effect of mildly acidic pH conditions on the production of moloney-derived retroviral vectors

To ascertain whether or not the increase in production of viral particles is directly related to the producer cell type (*i.e.*, HEK293T) or to the lentivirus genus, the effect of the pH 6 medium was evaluated on PG13-MFG-GFP murine cells (Merten, 2004), a stable third-generation Moloney-derived retrovirus producer cell line. To produce GALV-MLV particles, PG13-MFG-GFP cells were cultured either in pH 7.2- or pH 6-buffered medium. One to two days later, the content of infectious particles was measured in the cell culture supernatant. As shown in Fig. 3, the production of GALV-MLV particles is significantly enhanced when the production protocol is performed under mildly acidic pH conditions (average of 1.6 fold). This important result shows that the mildly acidic pH effect is not limited to human producer cells and is not strictly dependent on the use of vectors belonging to the lentivirus genus.

Efficient transduction of hCD34+ HSPCs with lentiviral pseudotypes produced at mildly acidic pH

Infectious titers of LVs produced at mildly acidic pH have been evaluated extensively on the highly permissive HCT116 cell line. To ensure that these LV supernatants are also infectious on relevant primary cells, we performed lentiviral transduction of hCD34⁺ HSPCs isolated from cord blood samples. Preactivated hCD34⁺ HSPCs transduced with VSV-G-LVs (Fig. 4A) produced at pH 6 (circles, gray lines) showed greater transduction efficiencies than LV produced at pH 7.2 (circles, black lines), despite the use of identical infectious titers (average of 1.6-fold increase). In the presence of the recently identified Vectofusin-1 transduction enhancer (Fenard *et al.*, 2013), transduction efficiencies were highly enhanced (Fig. 4A, squares) and the effect of pH was rapidly saturated, leading to comparable levels of transduction (Fig. 4A). For GALVTR-LVs (Fig. 4B), although transduction of hCD34⁺ HSPCs is known to be highly inefficient in the absence of transduction enhancers, pH 6-produced GALVTR-LVs were slightly more infectious than pH 7.2-produced GALVTR-LVs at each infectious dose tested, and this observation was reproducible in presence of Vectofusin-1 reaching higher transduction efficiencies, above 70% (Fig. 4B). Although we did not observe any improvement of the infectivity values of LVs produced in mildly acidic pH on HCT116 cells (Figs. 1 and 2), it seems that pH 6-produced LVs are slightly more efficient for the transduction of primary cells like hCD34⁺ HSPCs, probably because such primary cells are known to be less permissive than established cell lines (Ingrao *et al.*, 2014). It is important to note that all the transduction conditions using pH 6-produced LVs were performed at neutral pH. Mildly acidic viral supernatants were rapidly neutralized by the excess of neutral buffered X-Vivo20 medium (the highest viral input of pH6-produced LVs did not exceed 30% of the final transduction volume). This indicates that the beneficial effect of the mildly acidic pH has a direct impact on the quality of the particle, and does not directly affect the transduction steps.

Stability of lentiviral particles following production at mildly acidic pH

Raw supernatants of lentiviral pseudotypes are usually stored at -80°C before use or for subsequent purification steps. To investigate if production at pH 6 could have a deleterious effect on viral stability during the freezing or the thawing procedure, GALVTR-LV supernatants were subjected to one or two freeze-thaw cycles and infectious titers were determined at each thawing step (Fig. 5A). As shown in Fig. 5B, mildly acidic pH conditions did not affect the infectivity of GALVTR-LV particles after the thawing procedures. The average decrease of infectious titers observed after two versus one freeze-thaw cycle was around 5%, for both pH 7.2 or pH 6 conditions. The infectivity of GALVTR-LVs is therefore not significantly altered when LVs are frozen in mildly acidic pH conditions.

Next, we investigated whether the production of LVs in a mildly acidic pH environment has a deleterious effect on viral particles stability after a long term exposure to a 37°C temperature. Briefly, frozen vials containing GALVTR-LV supernatants produced either at pH 7.2 or pH 6 were incubated for 0 to 3 days at 37°C (closed environment) and the decrease in infectivity was followed over time. As shown in Fig. 6A, while infectious titers after an exposure at 37°C are strongly reduced, the slope of this decrease is less pronounced for GALVTR-LVs produced at pH 6 in a closed environment. The resulting half-life for pH 6-produced GALVTR-LVs is twice the one observed for pH 7-produced GALVTR-LVs (around 1.9 days vs. 1 day, Fig. 6A Lower panel). Since this experiment was performed in a closed environment, our hypothesis is that the sodium bicarbonate, in absence of 5% CO_2 , is unable to buffer the pH 7-produced GALVTR-LV, leading to some alkalinization of the medium overtime, a deleterious effect for the infectivity (Fig.1B). This effect is likely to be counteracted by the excess of hydrogen ions present in the pH 6-produced GALVTR-LVs. Therefore, GALVTR-LV stability experiments have also been performed in an open environment, by incubating viral supernatants at 37°C in a 5% CO_2 / 95% humidity incubator

(Fig. 6A, open). In these conditions, pH 7.2- and pH 6-produced GALVTR-LVs harbor the same half-lives, around 1.8 days, suggesting that the absence of CO₂ was certainly the major deleterious effect for pH 7-produced GALVTR-LVs in a closed environment. Interestingly, the widely used VSV-G lentiviral pseudotypes, produced in neutral or mildly acidic pH conditions, have the same stability, either in a close or in an open environment (Fig. 6B), highlighting the robustness of this envelope glycoprotein. In conclusion, the production of GALVTR-LVs or VSV-G-LVs in mildly acidic pH has no deleterious effect on the stability of the vector over a broad range of temperature.

Of note, this study of viral particle stability has shown that LVs are less sensitive to high temperature as previously thought (Carmo *et al.*, 2009). In an open environment at 37°C and 5% CO₂, raw supernatants of GALVTR-LVs and VSV-G-LVs harbor a half-life around 1.8 days. This discrepancy suggests that other parameters, like the formulation buffer and purification procedures [*i.e.*, ultracentrifugation (Carmo *et al.*, 2009)], have to be taken into consideration when stability studies of lentiviral particles are performed.

Modulation of the p55gag intracellular expression level in HEK293T producer cells cultured in mildly acidic pH conditions

To investigate why HIV-1 p24 contents in LV supernatants are improved in mildly acidic pH conditions (Fig. 1B and D), expression levels of the intracellular precursor HIV-1 p55gag in HEK293T producer cells were measured using immunoblotting analysis. As shown in Fig. 7A, intracellular p55gag expression was increased at pH 6 compared with pH 7.2. The average p55gag overexpression level obtained from four independent experiments was around 160% (Fig. 7B). This positive correlation between overexpression of intracellular p55gag and extracellular p24 capsid suggests that the use of mildly acidic pH conditions for producer cells creates a favourable environment for optimal intracellular expression of viral components.

In conclusion, we herein show that the production of lentiviral or gamma-retroviral particles is optimal at pH 6. The use of mildly acidic pH conditions has no deleterious effect on viral stability. Interestingly, a deleterious effect of mildly acidic pH on VSV-G-LV has been reported in a previous study (Higashikawa and Chang, 2001), but the protocol was designed to test the stability of VSV-G-LV particles produced at neutral pH and then transferred to a pH 6-buffered non-ionic solution. In our case, VSV-G-LV particles were produced directly in pH 6-buffered culture medium supplemented with FCS, highly enriched in proteins known to stabilize retroviral particles (Carmo *et al.*, 2009), and the particles remained infectious.

Furthermore, we show that LVs produced at pH 6 are highly infectious on established cell lines but also on relevant primary target cells such as hematopoietic progenitor cells. The pH 6 effect seems to act mainly through the establishment of an optimal intracellular environment for the production of particle structural components such as the capsid.

Such beneficial effects on the production process should be exploited to investigate the effect of the pH parameter on the downstream purification steps developed for clinical-grade LVs, like the ion exchange chromatography or the tangential flow filtration technology. A better LV-particle stability resulting from production at mildly acidic pH may improve the yield of infectious particles recovery during the purification procedure. This could be of high interest for lentiviral pseudotypes (*e.g.*, GALVTR-LVs) for which current purification procedures are not effective (unpublished results) (Leath and Cornetta, 2012)).

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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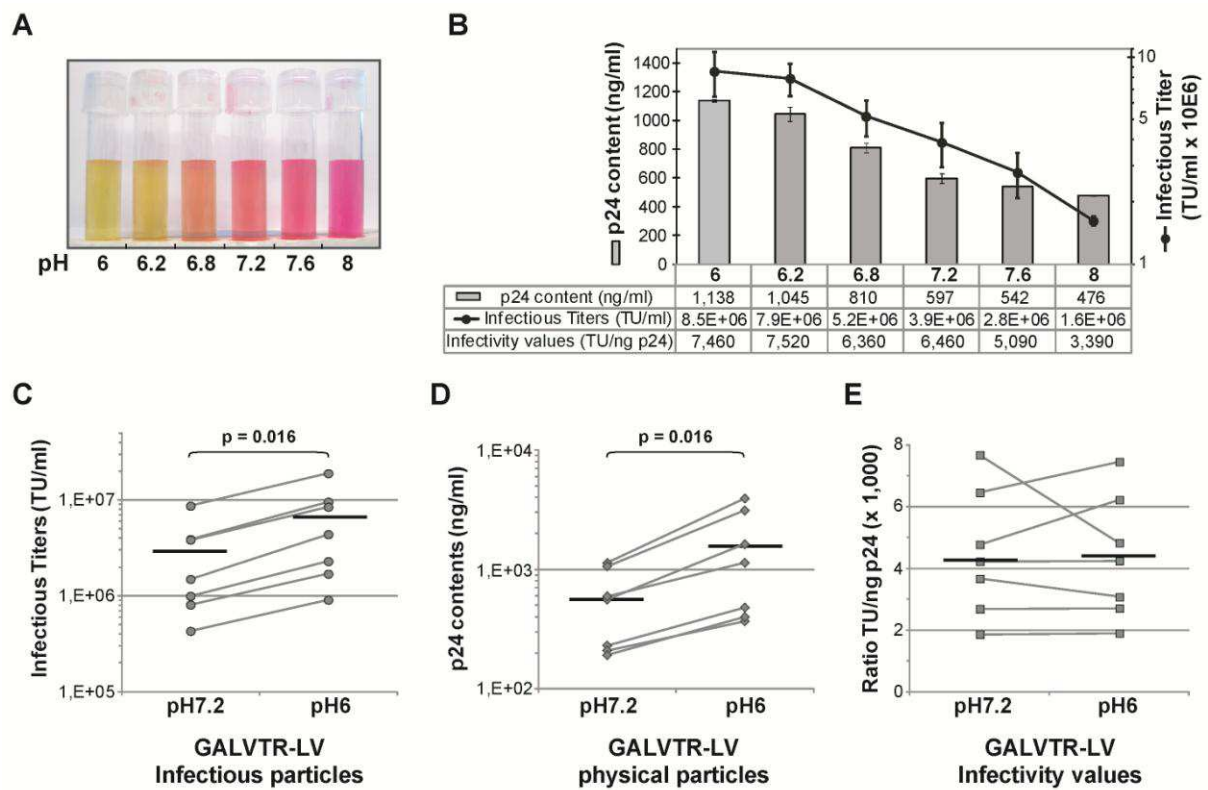


Fig. 1. Production of GALVTR-LVs under various pH conditions. (A) DMEM culture media were buffered at indicated pH values using hydrochloric acid or sodium hydroxide. The pH indicator (phenol red) is harboring a color from yellow (pH 6) to purple (pH 8). (B) GALVTR-LV particles were produced from HEK293T cells cultured at the indicated pH value. Infectious titers (TU/ml, black line) were determined on HCT116 cells using the detection of GFP by flow cytometry analysis. Physical particle contents (gray histograms) of GALVTR-LV supernatants were quantified using a commercial HIV-1 p24 ELISA kit. The infectivity value corresponding to the ratio between the infectious titers and the quantity of physical particles (TU/ng of p24) is represented under the histograms. Results are represented as the average of two independent experiments \pm standard deviation (SD). Seven batches of GALVTR-LVs were produced in pH 7.2 or pH 6 culture medium and titered for infectious (C) or physical (D) particles as in (B). (E) The infectivity value (TU/ng of p24) of each GALVTR-LV supernatant is represented. Bars indicate the mean value of the distributions. *p*-values were determined using Wilcoxon matched pairs tests. DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; LV, lentiviral vector.

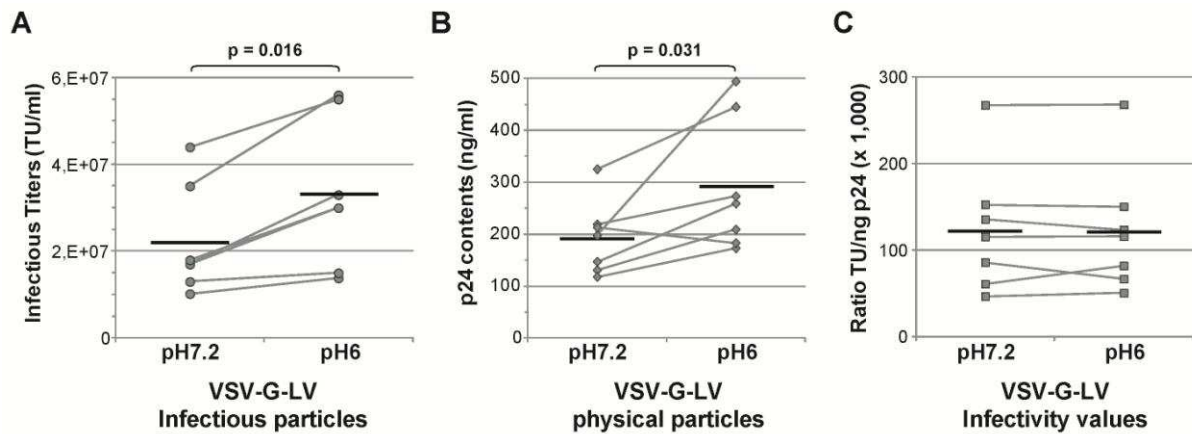


Fig. 2. Production of VSV-G-LVs in neutral or mildly acidic pH conditions. (A) Seven VSV-G-LV batches were produced from HEK293T cells cultured in a medium harboring the indicated pH value. Infectious titers were determined as in Fig. 1B. (B) The quantity of physical particles was determined using a commercial HIV-1 p24 ELISA kit. (C) The infectivity value for each VSV-G-LV batch was calculated. Bars indicate the mean value of the distributions. p -values were determined using Wilcoxon matched pairs tests.

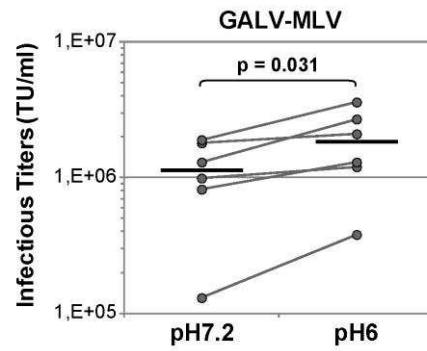


Fig. 3. Production of GALV-MLVs in neutral or mildly acidic pH conditions. Six GALV-MLV batches were produced from PG13-MFG-GFP cells cultured in a medium harboring the indicated pH value. Infectious titers were determined as in Fig. 1B. Bars indicate the mean value of the distributions. The p -value was determined using Wilcoxon matched pairs test.

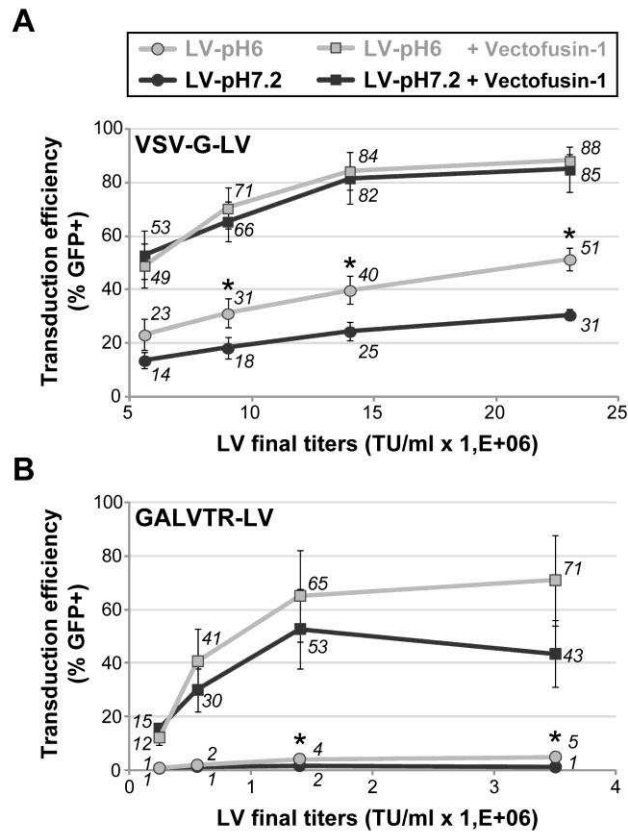


Fig. 4. Transduction efficiency of hCD34+ HSPCs with LV supernatants produced at neutral or mildly acidic pH. hCD34+ cells were infected in absence (circles) or presence (squares) of Vectofusin-1 (12 μ g/ml) with increasing concentrations of VSV-G-LVs (**A**) or GALVTR-LVs (**B**) produced at mildly acidic pH (Gray lines) or neutral pH (black lines). Transduction was measured in the bulk of cultured cells after 4-6 days by following the percentage of GFP+ cells using flow cytometry. Transduction efficiencies (y-axis) are plotted against final infectious titers (x-axis) of pH 7.2- and pH 6-produced LVs. The increasing infectious titers are corresponding to MOI 70, 110, 170 and 280 for VSV-G-LVs and MOI 3, 7, 17 and 42 for GALVTR-LVs. Data are represented as the average of two independent experiments performed in duplicate \pm SEM (* p <0.05, Mann-Whitney Test). MOI, multiplicity of infection.

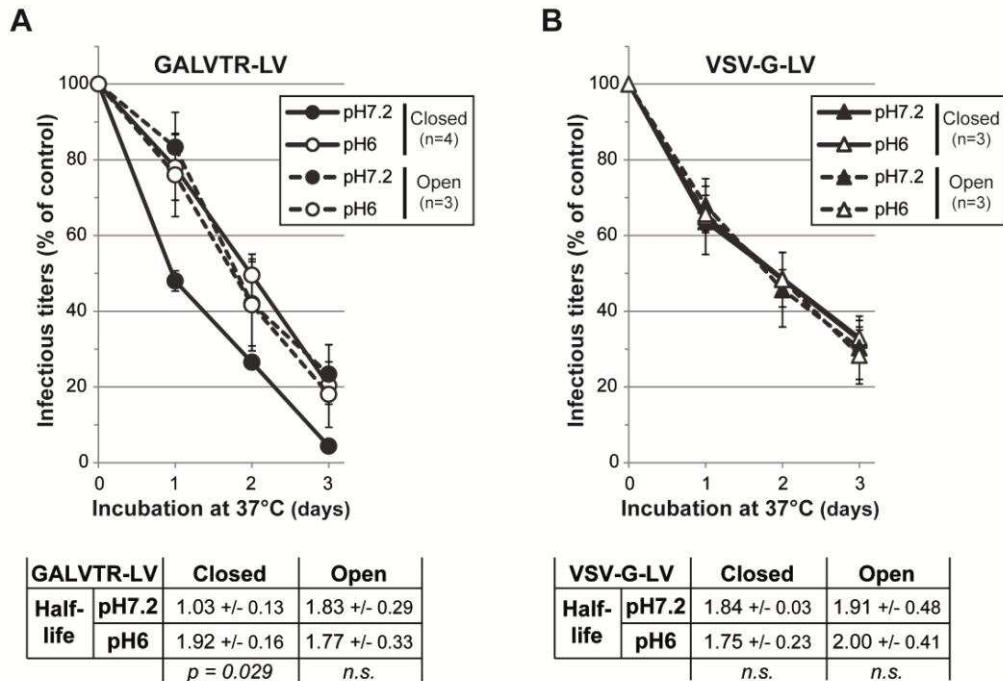


Fig. 6. Study of the stability of pH 7.2- and pH 6-produced-LVs after a long-term exposure to 37°C temperature. Cryotubes containing GALVTR-LV (A) or VSV-G-LV (B) particles, produced at pH 7.2 (black symbols) or pH 6 (white symbols), were incubated at 37°C for 0-3 days (closed environment). In the same manner, GALVTR-LV or VSV-G-LV particles were incubated at 37°C for 0-3 days in culture plate wells under 95% humidity and 5% CO₂ (open environment). Next, Infectious titers were concomitantly determined as in Fig. 1B. Data are represented as the average infectious titers \pm SEM normalized to the control condition (viral supernatants not exposed at 37°C) obtained from three to four independent experiments. Mean infectious half-lives (Lower panels) have been calculated and represented \pm SEM. p -values were determined using Mann-Whitney tests. $n.s.$, not statistical.

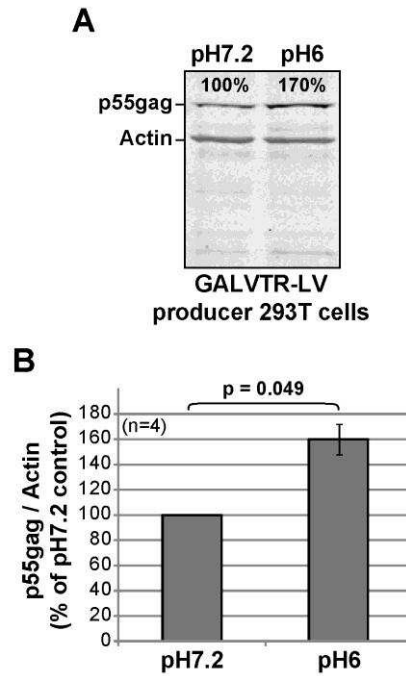


Fig. 7. Intracellular expression level of p55gag proteins in producer HEK293T cells cultured in neutral versus mildly acidic pH medium. (A) Western blot analysis of p55gag expression in lysates of HEK293T cells producing GALVTR-LVs and cultured in pH 7.2- or pH 6-buffered medium. The percentages represent the expression level of p55gag normalized to the level of actin. (B) Histograms represent the average quantification of p55gag expression levels normalized to actin expression levels in four independent experiments \pm SD. The *p*-value was determined using Wilcoxon matched pairs test.