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Pleiotropic interactions involving virulence and replication rate in experimentally evolved vesicular stomatitis virus

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ABSTRACT

Hypothesis: We hypothesize that natural selection in one cancer cell line results not only in increased replication rate and virulence in the selection host, but also increased replication rate and virulence in other novel hosts due to pleiotropy and a correlated response to selection.

Organisms: Vesicular stomatitis virus (VSV), a single stranded negative-sense RNA vesiculovirus in the family Rhabdoviridae, is used to study evolutionary processes due to its robust replication capacity and high mutation rate. HeLa, PC3, and H82 are human epithelial cancer cell lines from cervical, prostate, and lung tissue, respectively. HeLa is the selection host of the experimentally evolved VSV studied here, and PC3 and H82 are novel hosts. We compared these HeLa-adapted VSVs to three cancer-naïve VSVs.

Methods: Turner and Elena (2000) grew four populations of VSV for 20 passages in HeLa cells. We inoculated HeLa, PC3, and H82 cell cultures with these virus populations and measured replication rate by plaque assay and virulence by counting ratios of dead to total cells.

Results: The adaptive changes that increased replication rate on HeLa may be correlated with increased replication rates in one of the novel hosts tested. Although selection in HeLa resulted in higher virulence in that host, that increased virulence did not carry over to the novel hosts. Therefore, evolutionary changes in viral replication and virulence in this system are not highly correlated, and an increase in replication rate arising as a correlated response to selection does not necessarily increase viral pathogenicity.

Keywords: generalism, host range, oncolytic virus, specialism, vesicular stomatitis virus.

INTRODUCTION

Evolution of virulence

A fundamental goal in evolutionary biology is to understand why parasites harm their hosts. Historically, it was thought that parasites should evolve low virulence since they depend on their hosts for survival and transmission, yet many parasites cause significant

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harm. One evolutionary explanation for the persistence of virulence is that it is an unavoidable consequence of natural selection to increase parasite fitness (Levin and Pimentel, 1981; Anderson and May, 1982; Ewald, 1983, 2002; Anita *et al.*, 1994; Levin, 1996). This adaptive trade-off theory for the evolution and maintenance of virulence requires that virulence be genetically correlated with other fitness characteristics of the parasite, such as replication rate. Many theoretical models assume that high replication rates increase the probability of transmission, thereby increasing fitness, but also cause high levels of damage to the host (Levin and Pimentel, 1981; Anderson and May, 1982; Ewald, 1983, 2002; Anita *et al.*, 1994; Bull, 1994; Van Baalen and Sabelis, 1995; Frank, 1996; Levin, 1996); therefore, we expect a positive correlation between changes in replication rate arising as a response to selection. As replication rate increases, so does the level of virulence due to a pleiotropic interaction between these two traits. Here we document the evolution of such trade-off, in which evolution in one host increased replication rate as well as virulence.

Evolution of host range

Greater generalism, or the ability to use a broader range of habitats, is favoured when a population experiences a variable environment. A constant environment is predicted to result in the evolution of specialists, in which the habitat range is narrower (Levins, 1962, 1968; Roughgarden, 1972; Futuyma and Moreno, 1988; Wilson and Yoshimura, 1994; Kassen, 2002). However, it has been shown that evolution in constant environmental conditions does not always result in specialist populations; evolution of traits that evolve improved performance in one environment may result in increased performance in a novel, alternate environment due to a correlated response to selection (Hoffmann and Parsons, 1989; Turner *et al.*, 2010). For this phenomenon to occur, the genetic changes that increase performance in the selection environment must have a beneficial pleiotropic effect in the alternate environment. Such a correlation may arise when the selection environment is similar to the alternate environment, causing the traits evolved in one to be beneficial in both (Travisano and Lenski, 1996; Sanjuán *et al.*, 2004; Turner *et al.*, 2010). Therefore, we tested whether a pathogen that evolved increased replication and virulence in one environment will have increased replication and/or virulence in similar, novel environments.

Experimental evolution

Experimental evolution aims to test evolutionary theory directly in laboratory populations as they evolve. The strength of experimental evolution lies in its ability to conduct replicated, controlled, manipulative experiments where changes in fitness can be tracked directly through time by comparing the evolving population to the ancestor. Microorganisms, such as bacteria and viruses, have large population sizes and easily manipulated environments, making them suitable study systems for evolutionary processes (Elena and Lenski, 2003). Furthermore, RNA viruses, in particular, have high mutation rates (on the order of one point mutation or more per genome per round of replication) due to an error-prone RNA polymerase, which increases the likelihood that every possible mutation arises in the population during the course of an experimental evolution (Balachandran and Barber, 2000; Stodjl *et al.*, 2000). Here, we determine whether mutations that arose in evolved RNA virus populations in one host have pleiotropic effects in two novel hosts.

Vesicular stomatitis virus (VSV) is a candidate oncolytic virus for cancer treatment due to its natural ability to infect various types of cancer cells (Balachandran and Barber, 2000; Stodjl *et al.*,

2000). Oncolytic VSVs have been shown to preferentially replicate in cancers (or cancer cell lines) of the lung, kidney, bladder, colon, conjunctiva, cervix, ovary, breast, endometrium, prostate, central nervous system, skin, and liver (Megyeri *et al.*, 2007; Hadaschik *et al.*, 2008; Liao *et al.*, 2008). Here, we address the relationship between viral replication rate and host cell killing in three human carcinoma cell lines.

METHODS

Study system

Vesicular stomatitis virus is a single-stranded, negative-sense RNA vesiculovirus in the family Rhabdoviridae (Balachandran and Barber, 2000; Stodjl *et al.*, 2000). A previously conducted experimental evolution yielded virus populations with increased fitness in one cancer cell line relative to their ancestor. Four lineages of wild-type VSV (strain, Indiana Mudd-Summers) were experimentally evolved for 100 generations (25 passages; 48 h per passage) in human cervical epithelial carcinoma (HeLa) cells, resulting in four HeLa-adapted populations (H1, H2, H3, and H4) (Turner and Elena, 2000).

All four HeLa-adapted populations acquired one or more mutations that together confer higher fitness in HeLa cells relative to their HeLa-naïve ancestor, and a loss of fitness in their original, non-cancer cell host (Turner and Elena, 2000; Remold *et al.*, 2008). We investigated the possibility that together the mutations arising through adaptation to HeLa cells may be beneficial in cancer cells from other human epithelial carcinomas due to a correlated response to selection. In other words, the present study addresses whether these HeLa-adapted viruses are HeLa-specialists or cancer-specialists.

Viruses and host cell lines

We compared viral replication and virulence of the four HeLa-adapted populations (obtained from Paul Turner, Yale University) (Turner and Elena, 2000) to three cancer-naïve strains: a wild-type VSV (WT-VSV, obtained from Isabel Novella, Medical University of Ohio), and recombinant VSV-GFP and VSV-DsRed viruses (obtained from Michael Whitt, University of Tennessee, Memphis). WT-VSV is a Mudd-Summers strain of the Indiana serotype and is isogenic to the ancestor of the four HeLa-adapted populations except at a single nucleotide marker. The VSV-GFP and VSV-DsRed differ from the WT-VSV, not only with respect to their possession of a fluorescent marker located between the G and L genes, but also in that the marker is found in a recombinant genetic background containing genes from both the Mudd-Summers and San Juan strains of VSV-Indiana (Lawson *et al.*, 1995; Whitt, 2010). However, they share with the WT-VSV that they have no recent passage history on HeLa cells. These four virus lines adapted on HeLa and the three cancer-naïve viruses are hereafter called 'populations'.

These viruses were grown in three human epithelial carcinoma cell lines from three different sources: cervix, prostate, and lung (HeLa: ATCC #CCL-2; PC3: ATCC #CRL-1435; and NCI-H82: ATCC #HTB-175). These cell lines vary in their immune responses to viral infection. Whereas both HeLa and PC3 cell lines are interferon (IFN) producing and responsive (Gainey *et al.*, 2008; Kawaguchi *et al.*, 2009), H82 cells are IFN responsive but not producing (Moro *et al.*, 1998). HeLa and PC3 cells were grown with Dulbecco modified Eagle's minimum essential media (DMEM) supplemented with 10% fetal bovine serum.

H82 cells were grown with RPMI-1640, 10% fetal bovine serum, 2% glucose, and 1 × streptomycin-penicillin. We also grew virus in BHK cells for viral titration via plaque assay (see below); these cells were grown with DMEM supplemented with 10% bovine calf serum. All cell lines were grown at 37°C, 95% relative humidity, and 5% CO_2 atmosphere.

Measurements for viral replication and virulence

We quantified replication rate of all virus populations in three cell lines as follows: monolayers of each cell line were grown to 70% confluency in 6-well plates (1.6 cm^2 per well) and infected with four HeLa-adapted VSV populations, the three cancer-naïve strains, and mock-inoculates with DMEM. Three replicate assays for each of the cancer cell types were performed in three temporal blocks with a starting population size of 10^4 particle-forming units per millilitre ($PFU \cdot mL^{-1}$) and a multiplicity of infection (MOI) of 0.01 (to limit the production of defective-interfering particles). Two millilitres of cell culture media were added to each well, and the plate was incubated at 37°C. After a 12-h infection, the supernatant was collected to determine viral titre. Viral titre (particle forming units per millilitre) was determined using a standard plaque assay in BHK-21 (baby hamster kidney; ATCC #CCL-10) cells. A 50/50 mixture of media and 2.4% avicel (FMC BioPolymer) was used as an overlay. After a 24-h incubation, cells were fixed with 2% paraformaldehyde for 10 min and stained with 0.5% crystal violet solution. Plaques were counted to determine the particle forming units per millilitre for each virus population. Titres were $\log_{10}$-transformed to improve normality for analysis.

We then assessed virulence of the seven virus populations in all three cancer cell lines. As a proxy for virulence, we measured cell killing of the infected monolayers described above. After a 12-h infection, the cells of each monolayer were harvested using trypsin, mixed 50/50 with trypan blue stain, and counted using a hemocytometer. At 12 h of infection, most cells of all four assayed cell types remained attached to the monolayer. We determined the proportion of dead cells by calculating the number of dead cells divided by the total number of cells. We estimated kill inflicted by the virus by standardizing this value by the proportion of dead cells in the mock-inoculated wells, thereby ensuring that cell death being analysed was attributable to infection. This measure of kill should be considered conservative because any cells that were uncounted because they detached from the monolayer before treatment with trypsin were more likely to be dead than living.

Statistical analyses

We used mixed linear models to assess the dependence of viral replication rate and virulence on the fixed predictor variable 'evolutionary history' (HeLa-adapted or cancer-naïve) and the random predictor variable 'viral population nested within evolutionary history' (PROC MIXED, SAS Institute, 2009; Remold and Lenski, 2001; Turner *et al.*, 2010). Virus 'populations' included the four evolved populations and the three cancer-naïve strains. Using parallel models, we tested for differences in mean replication and virulence between evolutionary histories and among populations within a given evolutionary history. Since virulence varied among cancer cell lines due to intrinsic differences in susceptibility to viral infection, comparisons are made within the cell line as opposed to between the three cell lines. Identifying differences in means between evolutionary histories would indicate that replication rate or virulence of HeLa-adapted viruses differed from that of cancer-naïve viruses. Differences in

mean replication rate or virulence among populations within a history would indicate that the response variable differed among HeLa-adapted populations and/or among cancer-naïve populations. We also tested for differences in variance in replication rate or virulence among populations within a given evolutionary history. Detecting a significant difference associated with this variance term would indicate that the variability among populations within evolutionary histories differed.

RESULTS

Virus replication for HeLa-adapted VSVs, as estimated by titre achieved over 12 h from a shared starting population size, was approximately two orders of magnitude greater than the titre achieved by the cancer-naïve viruses in the selection host (HeLa) than in the novel host (PC3) ($P = 0.07$, $P = 0.03$, respectively; Fig. 1; Table 1). In contrast, in the other novel host, H82, there was no difference in viral replication between the HeLa-adapted and cancer-naïve VSVs ($P = 0.27$; Fig. 1; Table 1).

Replication rates of cancer-naïve viruses were more variable than HeLa-adapted viruses in all three cancer cell types (Fig. 1; Table 1, population (history) [variances]). This phenomenon may be due to the costs of carrying fluorescence marker genes borne by VSV-GFP and VSV-DsRed, but not by WT-VSV. Therefore, we performed a second analysis in which we dropped the cancer-naïve viruses containing the marker gene from the model, and

Table 1. A mixed general linear model testing the effect of evolutionary history on viral replication rate and virulence after a 12-h infection, for the complete dataset and for a reduced dataset in which the VSV-GFP and VSV-DsRed viruses have been dropped from the analysis

Source	d.f.	Test statistic		
Complete dataset				
Virulence		**HeLa**	**PC3**	**H82**
History	1	**22.10***	0.06^{NS}	0.03^{NS}
Population (history) [means]	1	0^{NS}	1.2^{NS}	0^{NS}
Population (history) [variances]	1	0.3^{NS}	0.5^{NS}	0.1^{NS}
Replication rate (log(titre))				
History	1	$11.83^{\#}$	**13.40***	1.72^{NS}
Population (history) [means]	1	**12.8****	**3.4***	1.4^{NS}
Population (history) [variances]	1	**8.1***	**3.2***	$2.4^{\#}$
Reduced dataset				
Replication rate (log(titre))				
History	1	**33.56*****	**9.00***	0.19^{NS}

Note: d.f. indicates degrees of freedom: denominator d.f. for F test is estimated using the Satterthwaite approximation; d.f. for likelihood ratio (LR) tests are equal to the difference in the number of parameters in the full and reduced models.

The fixed effect is tested with an appropriate F test. Random effects are tested using likelihood ratio tests; the LR statistic is −2(maximum likelihood of the test's full model – maximum likelihood of the restricted model, from which the variance component being tested has been removed).

$^{NS}P > 0.1$; $^{\#}$ $0.1 < P < 0.05$; *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.0001$.

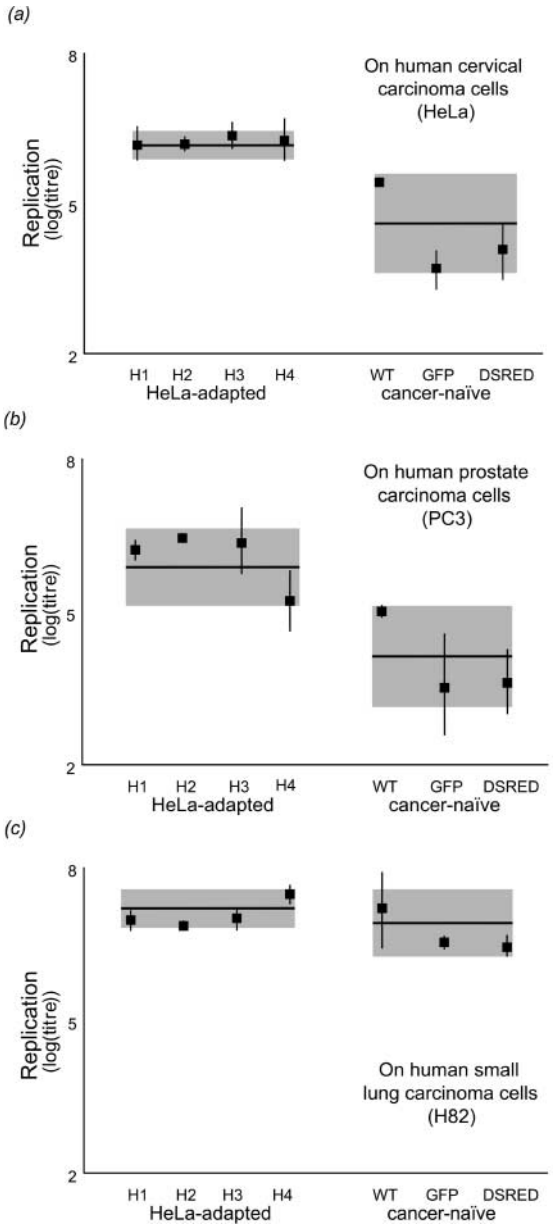

Fig. 1. Viral replication of vesicular stomatitis virus (VSV) after a 12-h infection on three cancer cell lines with threefold replication on the selection host, HeLa (a), and on the novel hosts, PC3 (b) and H82 (c). Points indicate means for each population, and error bars around them indicate 95% confidence limits. Grey boxes show 95% confidence limits for HeLa-adapted (left) and cancer-naïve (right) populations as a group; lines within the boxes indicate group means.

compared the HeLa-adapted populations' replication rates to that of the WT-VSV population (the ancestor for the HeLa-adapted populations). Consistent with the analysis of the full dataset, we found that HeLa-adapted viruses had significantly higher replication

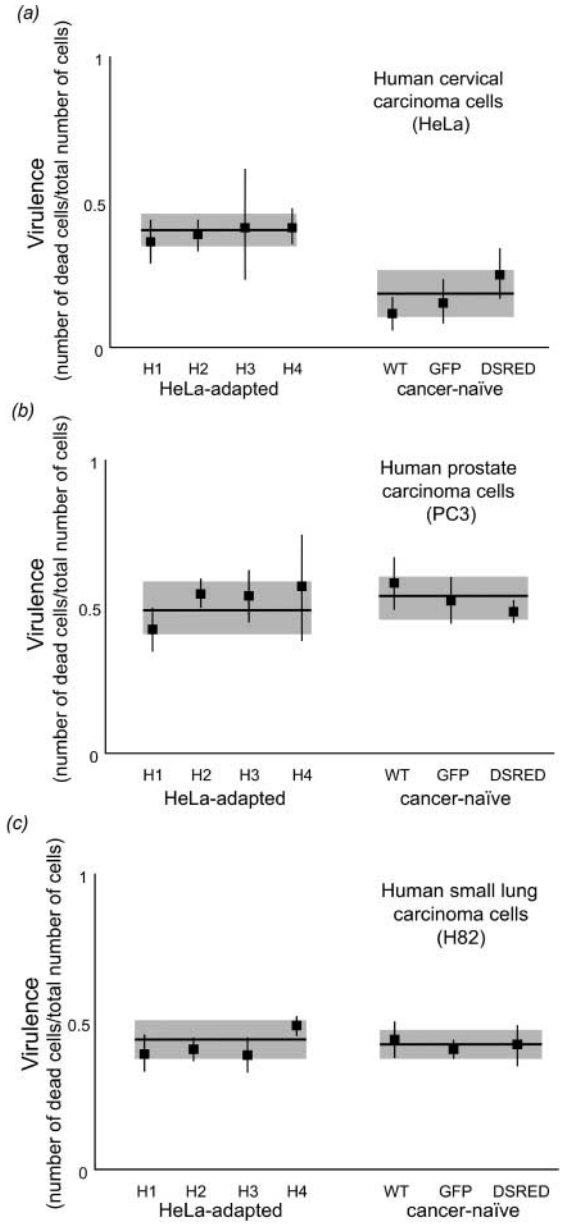

Fig. 2. Virulence (proportion of host cells killed) after a 12-h infection with vesicular stomatitis virus (VSV) populations (points) with threefold replication on the selection host, HeLa (a), and on the novel hosts, PC3 (b) and H82 (c). Points indicate means for each population, and error bars around them indicate 95% confidence limits. Grey boxes show 95% confidence limits for HeLa-adapted (left) and cancer-naïve (right) populations as a group; lines within the boxes indicate group means.

rates than the WT-VSV populations in HeLa and PC3 cells ($P < 0.0001$ and $P = 0.02$, respectively), but not in H82 ($P = 0.76$). HeLa-adapted viruses have higher virulence in the selection host, HeLa, than in either of the novel hosts. We found that HeLa-adapted viruses killed approximately 40% of the HeLa cell populations, which is significantly more than the 18% of HeLa cells killed by the cancer-naïve viruses ($P = 0.013$; Fig. 2; Table 1). However, the HeLa-adapted viruses did not kill significantly more PC3 or H82 cells than did the cancer-naïve viruses ($P = 0.82$ and $P = 0.86$, respectively; Table 1). We also detected no differences in means or variances in virulence among populations within evolutionary histories (Table 1).

We also assessed the relationship between change in replication rate and level of virulence on the three different cell lines of the evolved populations relative to WT-VSV. As shown in Fig. 3, the HeLa-adapted viruses increased both replication rate and virulence over the WT-VSV in HeLa cells. However, HeLa-adapted VSVs did not demonstrate this relationship between change in replication rate and virulence in either of the novel hosts, PC3 or H82.

DISCUSSION

Evolutionary theory suggests that virulence persists because it is an unavoidable consequence of natural selection to increase parasite fitness. As replication rate of the parasite increases, so does the harm caused to the host (Levin and Pimentel, 1981; Anderson and May, 1982; Ewald, 1983, 2002; Anita *et al.*, 1994; Levin, 1996). In this study, we investigated the relationship between replication rate and the level of virulence in three different hosts. A positive correlation between replication rate and level of virulence evolved in populations selected in HeLa, when growing in HeLa (Figs. 1a, 2a, 3), is consistent with the hypothesis of a pleiotropic interaction between these two traits. However, this correlation did not carry over when HeLa-adapted viruses were grown in the novel hosts, PC3 and H82 (Figs. 1b, 1c, 2b, 2c, 3). These results indicate that changes in viral replication and virulence arising through indirect responses to selection in a different host are not highly correlated, and that fitness gained via a correlated response to selection does not necessarily increase parasite virulence in novel hosts.

One possible mechanism for the observed lack of correlation between virulence and replication in the novel hosts arises from how the viruses emerge from an infected host cell. In this particular system, virus progeny bud from the infected host cell (Jayakar *et al.*, 2004). Increased replication rates result in more budding, which in turn could result in high virulence due to cell death. Increased replication rates achieved in PC3 cells do not show a correlated increase in virulence. It is possible that characteristics of the PC3 cell membrane in different cell lines make it possible for viruses to increase budding without triggering cell death. This phenomenon has been reported for insect cell lines (Gheysen *et al.*, 1989).

In addition, characteristics of the virus could cause the lack of discrepancy between replication and virulence. Host cells can trigger apoptosis as a defence strategy to eliminate harmful pathogens. To allow increased viral replication, some viruses have evolved genes to escape the host's apoptotic mechanism (Shen and Shenk, 1995). Some viruses produce apoptosis inhibitory proteins to block effector proteases necessary to induce host cell apoptosis (Shen and Shenk, 1995; Thome *et al.*, 1997). Such proteins could benefit viruses grown in tissue culture because lower virulence with high replication rates could result in higher transmission rates

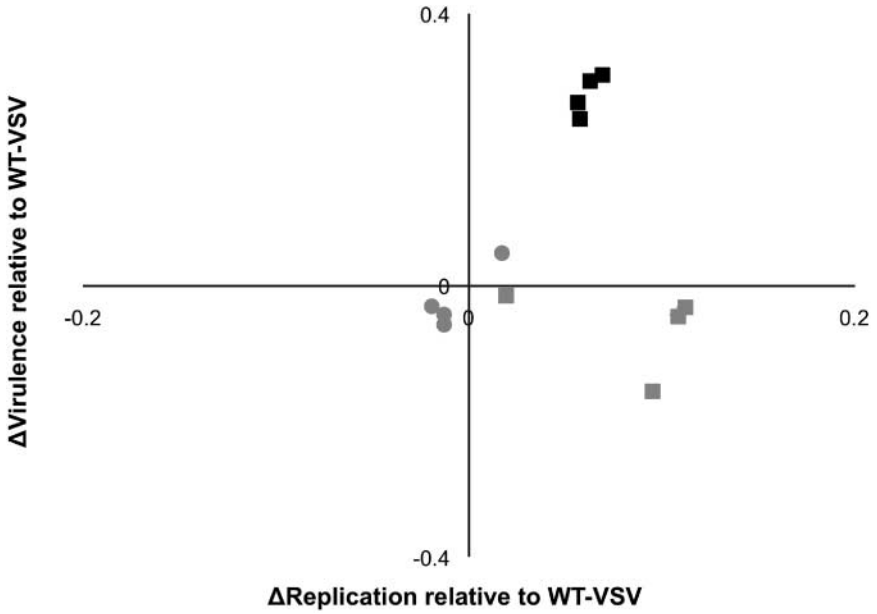

Fig. 3. Relationship between change in replication rate and change in virulence. Measured as the change in virulence and replication rate of HeLa-adapted viruses relative to WT-VSV grown on the selection host, HeLa cells (black squares), and grown on the novel hosts, PC3 cells (grey squares) and H82 cells (grey circles).

between host cells. On the other hand, viruses grown in H82 cells do not increase either replication or virulence when compared with the cancer-naïve cell lines.

Ecological theory suggests that evolution in a constant environment should result in specialization, and yet, correlated improvements in response to changing or alternative environments have been observed (Hoffmann and Parsons, 1989; Turner *et al.*, 2010). This pleiotropic improvement can occur when the alternative environment shares characteristics with the selection environment. We found that increased virulence in HeLa did not result in increased virulence in either of the novel environments (Fig. 2), but HeLa-adapted viruses did achieve higher replication rates in one of the alternative cancer cell environments (PC3).

Vesicular stomatitis virus is a candidate oncolytic virus for cancer treatment due to its natural ability to infect and kill various cancer cells (Balachandran and Barber, 2000; Stodjl *et al.*, 2000; Ahmed *et al.*, 2004; Ahtiainen *et al.*, 2010; Cary *et al.*, 2011) and the discovery of mutations that allow killing of some cancer cell types, while reducing the ability to infect normal cells (Stodjl *et al.*, 2000, 2003; Lun, 2006; Gainey *et al.*, 2008). It would be ideal if selection for an increase in replication rate and killing of cancer cells resulted in a correlated increase in replication rate and killing of other cancer cells; this outcome is predicted by theory which suggests greater correlated response in similar environments.

Our results can inform future work on the development of oncolytic VSV in two ways. First, our experimental selection for increased replication rate in HeLa did not result in increased performance in either of the novel cancer cell lines. Therefore, we conclude that evolution in HeLa cell lines results in HeLa-specialists, not cancer-specialists. However, VSVs experimentally evolved using broader, temporally variable environmental conditions

may still be of use in identifying new, broadly oncolytic phenotypes. Specifically, evolution under alternating exposure to a diverse group of cancer cell types may result in more 'cancer-generalist' viruses. This is because environmental fluctuations are predicted to result in generalism with respect to the environments to which the population is exposed (Kassen and Bell, 1998; Turner and Elena, 2000). Second, our finding of a lack of correlation between viral replication and virulence observed in two of the three cancer cell types suggests that both of these important traits must be targeted specifically for engineering oncolytic VSVs, and more generally, that parasites with fast replication rates do not necessarily have increased virulence.

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