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Tools to study pathogen-host interactions in bats

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ABSTRACT

Bats are natural reservoirs for a variety of emerging viruses that cause significant disease in humans and domestic animals yet rarely cause clinical disease in bats. The co-evolutionary history of bats with viruses has been hypothesized to have shaped the bat-virus relationship, allowing both to exist in equilibrium. Progress in understanding bat-virus interactions and the isolation of bat-borne viruses has been accelerated in recent years by the development of susceptible bat cell lines. Viral sequences similar to severe acute respiratory syndrome corona virus (SARS-CoV) have been detected in bats, and filoviruses such as Marburg virus have been isolated from bats, providing definitive evidence for the role of bats as the natural host reservoir. Although viruses can be readily detected in bats using molecular approaches, virus isolation is far more challenging. One of the limitations in using traditional culture systems from non-reservoir species is that cell types and culture conditions may not be compatible for isolation of bat-borne viruses. There is, therefore, a need to develop additional bat cell lines that correspond to different cell types, including less represented cell types such as immune cells, and culture them under more physiologically relevant conditions to study virus host interactions and for virus isolation. In this review, we highlight the current progress in understanding bat-virus interactions in bat cell line systems and some of the challenges and limitations associated with cell lines. Future directions to address some of these challenges to better understand host-pathogen interactions in these intriguing mammals are also discussed, not only in relation to viruses but also other pathogens carried by bats including bacteria and fungi.

1. Introduction

Bats are an ancient and diverse group of mammals, comprising almost a quarter of all mammalian diversity and inhabiting all continents, other than Antarctica. Phylogenetic analyses based on molecular data classified bats into the suborders Yinpterochiroptera and Yangochiroptera (Teeling et al., 2016; Teeling et al., 2005). The Yinpterochiroptera suborder includes the non echolocating Pteropodidae family and the echolocating Rhinolophoidea family. The Yangochiroptera consist of the remaining echolocating microbat families. The two suborders diverged over 50 million years ago (Lei and Dong, 2016; O'Leary et al., 2013; Simmons et al., 2008). Members of both suborders serve as reservoir or suspected reservoir hosts for a number of zoonotic viruses that are highly pathogenic in humans (Calisher et al., 2006; Moratelli and Calisher, 2015), yet cause no clinical disease in bats that are naturally or experimentally infected. The long co-evolutionary history of bats and their viruses has been speculated to have shaped the unique host-pathogen relationship (Schountz et al., 2017).

Although bats have been linked with a number of viruses,

identifying wildlife reservoirs can be a challenging process, requiring extensive ecological sampling. Evidence that a pathogen is permanently maintained in a population, often in the absence of disease, is required to confidently identify its reservoir (Calisher et al., 2006; Haydon et al., 2002). Detection of fragments of viral genomes, combined with serological evidence for viral exposure in multiple individuals of a species are generally the first lines of evidence to associate a host with a pathogen (Halpin et al., 2011; Li et al., 2005; Swanepoel et al., 2007; Young et al., 1996). Additional evidence in the form of virus isolation and the ability to link a reservoir to a spillover event is necessary to confirm the association. Examples of bat viruses that are confirmed or speculated to have 'jumped' from bats into humans and agricultural animals include coronaviruses (severe acute respiratory syndrome coronavirus [SARS-CoV], porcine epidemic diarrhea [PEDV] virus), filoviruses (ebolaviruses and Marburg virus), and henipaviruses (Nipah and Hendra viruses) [reviewed by (Moratelli and Calisher, 2015)]. Consistent with the definition of a wildlife reservoir, infection of bats by some of these viruses results in viremia and viral shedding in the absence of clinical disease (Halpin et al., 2011; Schuh et al., 2017). The

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spillover of some of these viruses to other susceptible hosts, including humans, can result in severe disease and mortality due to the lack of therapeutics and vaccines.

The emergence of zoonotic diseases is increasing globally and mammals, including bats, are major sources of emerging and re-emerging pathogens (Plowright et al., 2015). The drivers of disease emergence include anthropogenic changes to the environment (e.g., agricultural intensification), climate change and the encroachment of human populations into wildlife habitats (Tait et al., 2014). The biological characterization of a newly identified virus often begins with its isolation in cultured cells. Although detection of virus-specific antibodies by serology and the use of molecular methods, such as viral sequencing, are often readily achievable, virus isolation from bats has been far more challenging. For example, bats are implicated as the reservoirs of ebolaviruses based on the detection of viral genome, serological evidence and their confirmed role as a reservoir for the closely related Marburg virus. However, ebolaviruses have yet to be isolated from bats to definitively link them to spillover events (Leroy et al., 2005). Identifying wildlife reservoirs and the viruses they host will require the development of better laboratory tools for virus isolation to study replication kinetics and virus-host interactions. In this perspective, we discuss the importance of reservoir species specific cell lines as tools to study emerging wildlife pathogens with a focus on bats.

2. Current status of cell lines derived from bats

Virus isolation is generally attempted in established immortalized cell lines, such as the type I interferon deficient BHK-21 (baby Syrian hamster kidney cells) (Otsuki et al., 1979) or Vero E6 cell lines (African green monkey kidney cells) (Govorkova et al., 1996). Different primary cells from bats have been studied and some immortalized cell lines have also been generated (Table 1). Established cell lines from non-reservoir host species are convenient tools for virus isolation but do not always support propagation or isolation of viruses from wildlife. For example, a bat herpesvirus isolated from cell lines established from the Schreiber's long-fingered bat, *Miniopterus schreibersii*, failed to replicate in cells from a variety of other species, including cells from the black flying fox, *Pteropus alecto* (Zhang et al., 2012). The henipavirus Cedar virus was isolated from primary cells of its putative natural reservoir, *P. alecto* whereas other cell lines showed no visible cytopathic effect (Marsh et al., 2012). Similarly, the paramyxovirus Menangle virus was isolated using the same *P. alecto* kidney cell line (Barr et al., 2012).

Cell lines have defined characteristics based on their tissue of origin and method of immortalization. For a cell to be suitable for virus propagation, it must bear an appropriate receptor to bind with an incoming virus and it must be permissive. Because viruses differ in their cellular tropisms, working with a few cell lines with epithelial or fibroblastic characteristics limits our ability to isolate or study viruses that may have a tropism towards cell types that are more difficult to immortalize. Current cell lines may lack receptors required by viruses to replicate when cells are infected at low multiplicity of infections. For example, bat cells from various species were resistant to infection with vesicular stomatitis virus (VSV) pseudotypes bearing the SARS-CoV spike protein until the cells were transfected to express the human SARS-CoV receptor, angiotensin-converting enzyme 2 (ACE2) (Hoffmann et al., 2013). Although the lack of appropriate receptors on cell lines makes it difficult to isolate some viruses, this lack of susceptibility to infection can also help us understand the early events in virus infection. For instance, differences in the susceptibility of cells from a variety of bats to filoviruses also led to the identification of unique amino acid substitutions in the filovirus receptor, Neimann-Pick type C1 (NPC1), providing insight into the host range of ebolaviruses (Ng et al., 2015). Similarly, Widagdo et al. have shown that dipeptidyl peptidase 4 (DPP4, CD26), a cellular receptor for MERS-CoV, is expressed in specific cell types in different tissues which also varies between bat species (Widagdo et al., 2017). These studies demonstrate the need to develop

cell lines from different organs and bat species and clone them for a variety of cell types as viral receptors may not be equally distributed.

3. Considerations for development of cell lines

Chiroptera consists of over 1200 bat species and different species may have co-evolved with different viruses. Viruses detected in one species, may not infect cells established from a different bat species. Thus, the choice of bat species for generating cell lines capable of supporting a particular virus should be supported by evidence that the species is susceptible to that virus. For example, SARS-CoV-like coronaviruses have been detected in *Rhinolophus sinicus* (Lau et al., 2005), and this species is the logical candidate for generating cell lines that can support replication and isolation of these viruses.

Because propagation of viruses in cell lines can select for members of the virus population that replicate most efficiently in the chosen cell type, the choice of cell line should be taken into consideration. Propagation of viruses in cell lines other than those of the natural host may lead to accumulation of adaptive mutations or attenuate the virus (Combe and Sanjuan, 2014), which in turn has implications for assessing host-pathogen interactions either *in vivo* or *in vitro*. For example, although wild type Marburg virus causes no disease in mice, it can be adapted to mice by serial passage to cause lethal disease (Qiu et al., 2014). Deep sequencing of Marburg virus after adaptation to mice and following propagation in Vero E6 cells has revealed the sequential mutations associated with each passage as the virus adapts to a new host either *in vivo* or *in vitro* (Wei et al., 2017). This is also thought to have occurred with Tacaribe virus, which was isolated from artibeus bats and passaged 20 times in newborn mice, resulting in point mutations and deletions (Malmlov et al., 2017; Saylor et al., 2014).

Bats experience a range of body temperatures during different physiological activities (from 4 to 8 °C during hibernation to 41 °C during flight) (O'Shea et al., 2014). Thus, viruses isolated from bats should be studied at different relevant physiological temperatures. Propagation of viruses at temperatures other than the nominal of the reservoir species may also lead to the generation of attenuation mutants; a strategy that has been used for vaccine development for decades. There is evidence that arthropod viruses found in mosquitoes and ticks accumulate mutations when grown at different temperatures (Lemm et al., 1990). Thus, propagation of viruses in susceptible cell lines [reviewed by (Hare et al., 2016)] and at optimized temperatures may minimize mutations and produce viruses similar to wild type virus found in wildlife reservoirs or arthropod vectors [reviewed by (Prescott et al., 2017)].

Controlling for temperature to match temperatures experienced by bats during hibernation is a challenge because *in vitro* cell culture systems are not viable at low temperatures. Although Miller et al. were able to grow bat cells at temperatures ranging from 37 to 41 °C (Miller et al., 2016), our attempts to cultivate big brown bat cells (Efk3) at lower temperatures of 26–30 °C were not successful (Banerjee and Misra, unpublished). There is evidence that experimental infection of different reptiles and amphibians with chikungunya virus produce varying amounts of viremia depending on the host's body temperature (Hartwig et al., 2015). There is a need to develop a system to test the replication potential of bat-borne viruses at lower temperatures such as those experienced by bats during torpor and hibernation, rather than only propagating them in cells cultured at 37 °C.

The virus stock that is used to experimentally infect bats is usually generated by propagation in cells of non-bat origin, such as Vero E6 cells. It is unknown whether propagation of a virus in a monkey cell line alters the genotype of the virus. We have previously infected bats with a virus isolated from bat urine and subsequently propagated in bat cells (Baker et al., unpublished). A problem with propagating this virus in bat cells was achieving high virus titre to perform experimental infection studies in bats, perhaps because bat cells appear to have constitutive type I IFN activation (Zhou et al., 2016b). It may be necessary

Table 1
Some cell types established from bats and range of viruses that have been used in studies with these cells.

Bat primary cells or cell lines	Origin	Virus/pseudovirus used in the study	Reference
<i>Artibeus jamaicensis</i> primary cells	Embryonic, primary kidney cells	Dengue virus, MERS-CoV	Moreira-Soto et al. (2017); Munster et al. (2016)
<i>Carollia perspicillata</i>	Trachea	Vesicular stomatitis virus (VSV) and Rift-Valley fever virus	Eckerle et al. (2014)
<i>Carollia perspicillata</i> – CpKd	Kidney	Bat-associated influenza viruses	Hoffmann et al. (2016)
<i>Eidolon helvum</i>	Trachea	VSV and Rift-Valley fever virus	Eckerle et al. (2014)
<i>Eidolon helvum</i> – EidNi/41	Kidney	Bat-associated influenza viruses, Ebolavirus, MERS-CoV, Influenza A virus, O'nyong–nyong virus	Hoffmann et al. (2016), Ng et al. (2015); Cai et al. (2014); Poole et al. (2014); Biesold et al. (2011)
<i>Epomops buettikoferi</i> – EpoNi/22.1	Kidney	Bat-associated influenza viruses, Influenza A virus	Hoffmann et al. (2016); Poole et al. (2014)
<i>Eptesicus fuscus</i> – EfK3	kidney	VSV, porcine epidemic diarrhea virus, herpes simplex virus and MERS-CoV	Banerjee et al. (2016)
<i>Hypsignathus monstrosus</i> – hypLu/45.1	Lung	MERS-CoV, Influenza A virus	Cai et al. (2014); Poole et al. (2014)
<i>Hypsignathus monstrosus</i> – hypNi/1.1	Kidney	Bat-associated influenza viruses, MERS-CoV, Influenza A virus, African Henipavirus	Hoffmann et al. (2016); Cai et al. (2014); Poole et al. (2014); Kruger et al., (2013)
<i>Miniopterus schreibersii</i> primary cells	Lymph node (MsLn), Kidney (MsKi)	Miniopterus schreibersii herpesvirus (MshV)	Zhang et al. (2012)
<i>Myotis daubentonii</i> – MyDauLu/47.1	Lung	Influenza A virus	Poole et al. (2014)
<i>Myotis davidii</i> – MdKi	Kidney	Sendai virus	Liang et al. (2015)
<i>Myotis myotis</i>	brain (MmBr), tonsil (MmTo), peritoneal cavity (MmPca), nasal epithelium (MmNep) and nervus olfactorius (MmNol)	Rabies virus, European bat lyssavirus (EBLV – 1 and 2)	He et al. (2014)
<i>Myotis velifer incautus</i> (MVI-it) – CRL-6012	intercapsular tumor	Novel bat gammaherpesvirus	Shabman et al. (2016); Host and Damania (2016)
<i>Pipistrellus ceylonicus</i>	Embryonic	Chandipura virus (CHPV), novel adenovirus (BtAdv-RLM)	Mourya et al. (2013)
<i>Pipistrellus subflavus</i> – ESU-BSL	Lung	MERS-CoV	Cai et al. (2014)
<i>Pteropus alecto</i>	Aorta, bone marrow, brain, foetus, foetal membranes, heart, kidney, liver, lymph nodes, lung, muscle, pharynx, placenta, salivary gland, small intestine, skin, spleen, testes, thymus, uterus	Hendra virus, Pulau virus, Sendai virus, Nipah virus, Hendra virus	Zhou et al. (2013); Cramer et al. (2009); Virtue et al. (2011)
<i>Pteropus alecto</i>	Kidney	Influenza A virus, Sendai virus, Pteropine orthoreovirus NB, Nipah virus, Hendra virus	Dlugolenski et al. (2013); Zhou et al. (2013); Virtue et al. (2011)
<i>Pteropus alecto</i>	Embryonic	Nipah virus, Hendra virus	Virtue et al. (2011)
<i>Rhinolophus affinis</i>	embryonic	VSV	Li et al., (2015)
<i>Rhinolophus sinicus</i>	splenocytes	VSV	Li et al., (2015)
<i>Rousettus aegyptiacus</i> – RO6E, RO5T	Kidney	MERS-CoV, Influenza A virus, Ebola virus, Marburg virus	Cai et al. (2014); Poole et al. (2014); Krahlhng et al. (2010)
<i>Rousettus aegyptiacus</i> – RoNi/7.1	Kidney	Bat-associated influenza viruses, MERS-CoV, Influenza A virus	Hoffmann et al. (2016); Cai et al. (2014); Poole et al. (2014)
<i>Tadarida brasiliensis</i> – Tb1Lu	Lung	Bovine leukemia virus, Influenza A virus, mammalian reovirus serotype 3 Dearing	Graves and Ferrer (1976); Poole et al. (2014); Sandekian et al. (2013)
<i>Rousettus aegyptiacus</i> immortalized cells	Foetus	Vaccinia Ankara	Jordan et al. (2009)

to develop interferon-deficient bat cell lines to propagate bat viruses. Recently, Zhang et al. knocked out an antiviral cell signaling receptor, interferon alpha receptor 2 (IFNAR2) in bat cells using CRISPR/Cas9 technology (Zhang et al., 2017). Similarly, this technology can be used to generate interferon-deficient bat cells in culture.

There are several challenges to establishing primary cells from wildlife species. These challenges range from obtaining healthy tissues to determining the ideal cell types. Primary cells maintain genetic integrity and are therefore most appropriate for the study of natural infection of cells. However, primary cells have finite numbers of cell division before undergoing senescence, thus, it can become problematic for long-term studies. Immortalized cells frequently accumulate chromosomal aberrations over time; therefore, caution must be exercised when maintaining cell stocks. Nonetheless, they are convenient and often receptive to genetic manipulations, including transfection of plasmids and targeted changes (e.g., CRISPR), and often the only cells available that are capable of supporting replication. Thus, the ability to conduct experiments in primary or immortalized cells has advantages and disadvantages.

Primary cells can also harbor latent viruses that can become re-activated during *in vitro* cultivation when the cells are outside the host and isolated from other components of the immune system that would otherwise control virus replication. Most mammals, including bats harbor persistent viruses [reviewed by (Calisher et al., 2006)], and viruses have been isolated or identified by next generation sequencing (NGS) from bat cell lines (Shabman et al., 2016). Cell lines from any species of bat should therefore only be handled under controlled biosafety conditions and thoroughly tested before being used for experimental studies. This is also a limitation when working with wild-caught bats.

4. Using cell lines to assess the immune response in bats to viral infections

Research in the field of bat immunology steadily increased after bats were implicated as ancestral reservoirs of the coronavirus that caused the SARS outbreak in 2003 (Baker et al., 2013; Lau et al., 2005; Li et al., 2005). Over 200 viruses have been identified in bats and additional new

viruses are discovered on a regular basis (Moratelli and Calisher, 2015; Schountz, 2014). Few viruses have been documented to cause disease in bats in addition to rabies and related lyssaviruses (Davis et al., 2012; Davis et al., 2013; McColl et al., 2002). Experimental inoculation of Jamaican fruit bats with high doses of Tacaribe virus caused significant morbidity and mortality similar to natural infection of artibeus bats (Downs et al., 1963). However, the inoculated bats did not transmit the virus. Based on their observations, Cogswell-Hawkinson et al. speculate that Jamaican fruit bats may not be a reservoir host for Tacaribe virus (Cogswell-Hawkinson et al., 2012). Lloviu virus, a filovirus was isolated from dead bats in Spain (Negredo et al., 2011). These examples appear to be exceptions to the usual resistance to viral disease displayed by most bats. Bats have been hypothesized to have evolved unique aspects of their immune systems, such as a strict control of the inflammatory process (Banerjee et al., 2017) and constitutive expression of antiviral interferons (Zhou et al., 2016b) to better survive infections with these viruses. Testing this hypothesis has been possible, in part through the establishment of well characterized cell lines (Schountz et al., 2017).

Cell lines from several bat species including members of both the Yinpterochiroptera (*Pteropus alecto*, *Eidolon helvum* and *Rousettus aegyptiacus*) and Yangochiroptera (*Eptesicus fuscus*, and *Myotis myotis*) suborders, have been established (Banerjee et al., 2016; Biesold et al., 2011; Cramer et al., 2009; He et al., 2014; Jordan et al., 2012) (Table 1). These cell lines have been used to cultivate viruses that are speculated or confirmed to have spilled over from bats. These cell lines have also allowed researchers to study the innate immune responses to viruses. Cytokines produced in response to activation of selected innate immune response pathways in bat cells have led to a better understanding of how bats might respond to viral infections in the wild. Bat cells can respond to viruses or viral antigens through the production of antiviral interferons and interferon stimulated genes (Biesold et al., 2011; Omatsu et al., 2008; Zhou et al., 2011; Zhou et al., 2013). The use of bat cell lines has also led to the discovery of viral proteins that counteract the innate immune response in these cells (Virtue et al., 2011). Less work has been performed on the adaptive immune responses of bats using cell lines, in part due to the lack of research on bat T and B cells. However, a recent report used *P. alecto* cell lines to examine the repertoire of self and Hendra virus epitopes presented by MHC class I molecules (Wynne et al., 2016). More research is needed to establish *in vitro* and *ex vivo* cultures of immune cells from representative bat species.

5. Developing bat specific reagents

Establishing cell lines from different species of bats provides the opportunity to perform comparative studies between bat species and with other mammals. Several cell lines have been developed from bats, including some that support the replication of viruses from different families (Table 1). However, to better characterize bat immune responses, we need to develop cell lines or a system where we can continuously establish immune cells *in vitro*. Current wildlife cell lines are composed primarily of epithelial or fibroblast cell types, which limit the responses generated following infection. These cells may lack many of the receptors and cytokine responses of specialized immune cells. Isolating and developing representative immune cell lines will allow the study of both adaptive and innate immune responses of bats. This also involves developing species and cell-type specific reagents that will allow characterization and culture of immune cells *in vitro*. Recently, immune cells from black flying foxes have been functionally and phenotypically characterized (Martinez Gomez et al., 2016), along with the establishment of bone-marrow derived dendritic cells and macrophages using black flying fox reagents developed in the laboratory (Zhou et al., 2016a). Systems such as these will further allow exploration of the adaptive immune response in potential reservoir bat species. More efforts are required to generate similar cell types in other bat species. Sharing of resources will play a key role in expanding our

understanding of the unique immune response in bats.

Although primary cells are good tools to attempt virus isolation, they have a limited life-span. There are several technologies that can be used to immortalize these primary cells. Immortalized cell lines replicate indefinitely in culture and provide researchers with valuable tools to study viruses and cell-virus interactions. Some of these bat cell lines have been established using existing technology such as simian virus 40 large T-antigen and human hTERT expression systems (Ali and DeCaprio, 2001; He et al., 2009; Xie et al., 2015), whereas others have been generated by using novel technologies such as a bat polyomavirus large T-antigen to immortalize cells (Banerjee et al., 2016) and other bat specific reagents to generate dendritic cells and macrophages (Zhou et al., 2016a). Polyomavirus large T-antigen binds to and attenuates the tumor suppressor protein p53 and proteins of the retinoblastoma tumor suppressor family (pRb, p130 and p107) promoting cell cycle progression. Gamma-herpesviruses have also been used to immortalize cells of the immune system, such as B lymphocytes (Liang et al., 2011). These novel technologies or combination of cell immortalization techniques can be modified for the generation of cell lines from representative cell and tissue types from less studied species of bats and other neglected wildlife reservoirs.

Studying the host-pathogen relationship in wildlife species is often challenging, with *in vivo* experiments difficult and costly to perform. Developing cell lines will allow preliminary research, which can later be validated *in vivo* in bats. These cell lines can be used to study or isolate viruses that spillover from animal reservoirs to humans, and pathogens that cause disease in wildlife. The advantages of developing cell lines do not end at studying the immune responses and isolating viruses. These cell lines can be used to propagate isolated viruses to high titre for the development of diagnostic kits and potential vaccines. Studying pathogen-host interactions may also allow us to identify biomarkers associated with viral infection that could be used to predict and prevent spillover to other susceptible hosts. For example, biochemical or hormonal changes linked to increases in viral replication in wild bat populations may be used to predict periods of increased risk of spillover to other susceptible species.

6. Using cell lines to study host responses to pathogens that cause disease in bats

In addition to viruses, bats may also be reservoirs, or even dead-end hosts, for other pathogens such as bacteria (Banskar et al., 2016), fungi or parasites. Bats do not seem to be refractory to disease when it comes to extracellular pathogens such as fungi (Zukal et al., 2014). The white-nose syndrome (WNS) fungus, *Pseudogymnoascus destructans*, causes significant wing damage at the sites of infection in little brown bats (*Myotis lucifugus*) (Warnecke et al., 2012; Zukal et al., 2014). Big brown bats (*Eptesicus fuscus*) seem to be relatively resistant to the fungus (Frank et al., 2014). Identifying, isolating and characterizing the interaction of extracellular and intracellular pathogens with their host can be better facilitated by the availability of bat species specific cell lines.

Research to understand bacterial and fungal infections in bats is growing and has led to the identification of potential pathogenic bacterial species in bats (Avena et al., 2016; Banskar et al., 2016). Cell lines, as previously shown (Nepelska et al., 2017), can be effective tools to dissect the role of these organisms in the overall health of bats. Transcriptomic data from bat cells infected with various pathogens, including bacteria and viruses, will shed light on global pathways that are activated or suppressed in response to these pathogens. These data can be used to develop hypotheses on the overall impact these pathogens might have on bats at an organism level. Recently, a novel *Streptomyces* species with anti-fungal activity was identified in bats (Hamm et al., 2017). WNS has caused the death of millions of little brown bats in North America (Alves et al., 2014; Knudsen et al., 2013; Zukal et al., 2014) and this new compound amongst others (Boire et al., 2016)

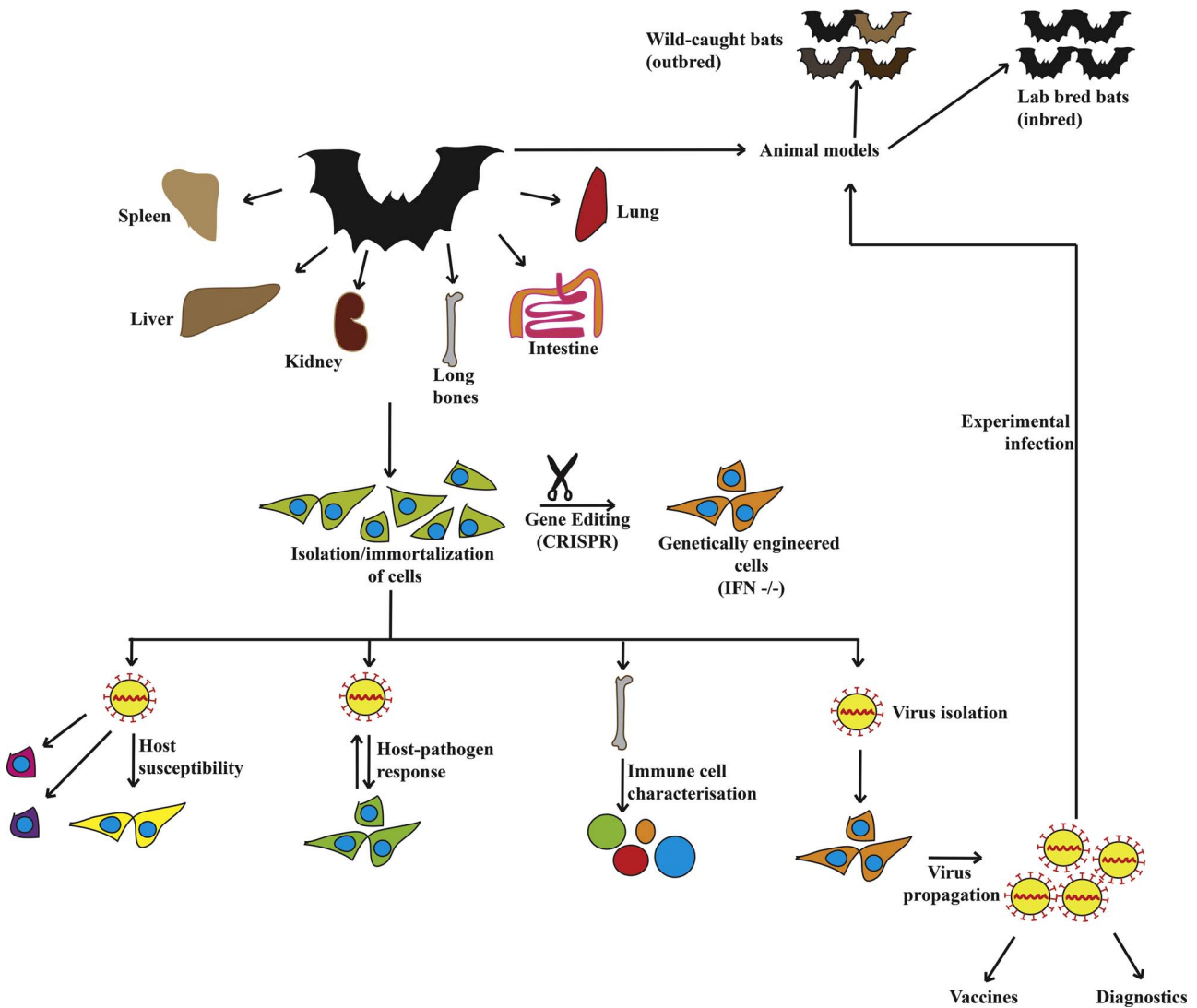


Fig. 1. There is a need to develop more model cell lines and animal models to decipher the immune responses of bats to viruses. Viruses display tropism towards different cell types and there is a need to generate cell lines representative of the multitude of cell types found in bats to increase the chances of isolating the viruses that they carry. Alternatively, genetically modified cell lines can be generated that do not express antiviral interferons from these cell types for virus isolation. These cells may be used to propagate bat-borne viruses to high titres for *ex vivo*, *in vitro* and *in vivo* experiments. Generating cell lines from a variety of different tissues and bats will allow researchers to explore virus susceptibility and virus-host interactions in these intriguing mammals. Generation of outbred (wild-caught) and inbred (laboratory bred) bat colonies with known infection status will enable systemic immune response studies with relevant viruses isolated from bats. The ability to resist infections with some of these viruses may be an effect of several evolutionary adaptations, which can be better dissected by studying the systemic responses in bats.

might help to curb the spread of WNS. Potential toxic effects of this drug can be tested in uninfected bat cell lines before testing them in live bats or other relevant animal models.

7. Developing *in vivo* bat models

In addition to cell lines, there is a need for laboratory bred bats with known and tested infection status for *ex vivo* and *in vivo* experiments. Establishing a wild-caught bat colony is challenging but achievable with sufficient resources to house and maintain the bats. Although studies using wild-caught outbred bats potentially provide the best representation of what occurs naturally during virus infection, it can be difficult to obtain reproducible data using wild caught animals due to their high genetic diversity. The current infection status and history of viral infections in wild caught bats is also difficult to determine which in turn may affect the outcome and interpretation of experimental infections. There is therefore a need to establish inbred laboratory colonies of bats of known infection status. As much of the knowledge that has been obtained regarding the host responses of bats has been gained

from studies using bat cell lines, experimental infections of captive bats provide an opportunity to test whether observations made *in vitro* also apply *in vivo*. Studies in captive animals are also necessary to confirm the role of bats as ‘reservoirs’ by carrying out long-term experiments to detect virus shedding and the lack of associated pathology in bats with known infection status (i.e. laboratory bred bats). This was recently attempted with Egyptian rousette bats that were infected with Marburg virus. The bats shed virus between 5 and 19 days post infection (Schuh et al., 2017), but longer term studies might be necessary to further establish bats as reservoirs. Thus there is a need to develop more reliable animal models with known infection status.

8. Conclusion

Although several different cell lines have been established from different species of bats (Table 1), we are still faced with the challenge of isolating several viruses that have been detected in bats. We have detected little brown bat coronavirus sequences (Misra et al., 2009; Subudhi et al., 2017) and others have detected ebolavirus sequences

[reviewed by (Olival and Hayman, 2014)] in bats by polymerase chain reaction (PCR), next generation sequencing (NGS) and serology. As discussed, virus isolation is more challenging than nucleic acid detection but with the availability of additional cell lines from various organs and species and technologies such as CRISPR to develop cell lines deficient in components of the immune system, attempts to isolate and propagate viruses *in vitro* should be more successful.

Chiroptera consists of over 1200 species and much remains unknown about their relationship with their microorganisms. Bats are genetically diverse and results from studies done in one bat species may not represent other species of bats. Thus, there is a need to study other species of bats to gain deeper insights into bat-microbe co-evolution, bat immune system and ecological interactions of bats. Bats host more zoonotic viruses per mammalian species (Luis et al., 2013; Olival et al., 2017), even more than rodents, the largest group of mammals. Collaboration between laboratories will be required to develop cell lines from different bat tissues, bat specific reagents and to establish laboratory bat colonies to explore the length and breadth of microbe-host interactions in these ecologically important mammals (Fig. 1).

Conflicts of interest

None.

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