Conditionally Reprogrammed Human Normal Airway Epithelial Cells at ALI: A Physiological Model for Emerging Viruses

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Abstract
Cancer cell lines have been used widely in cancer biology, and as biological or functional cell systems in many biomedical research fields. These cells are usually defective for many normal activities or functions due to significant genetic and epigenetic changes. Normal primary cell yields and viability from any original tissue specimens are usually relatively low or highly variable. These normal cells cease after a few passages or population doublings due to very limited proliferative capacity. Animal models (ferret, mouse, etc.) are often used to study virus-host interaction. However, viruses usually need to be adapted to the animals by several passages due to tropism restrictions including viral receptors and intracellular restrictions. Here we summarize applications of conditionally reprogrammed cells (CRCs), long-term cultures of normal airway epithelial cells from human nose to lung generated by conditional cell reprogramming (CR) technology, as an ex vivo model in studies of emerging viruses. CR allows to robustly propagate cells from non-invasive or minimally invasive specimens, for example, nasal or endobronchial brushing. This process is rapid (2 days) and conditional. The CRCs maintain their differentiation potential and lineage functions, and have been used for studies of adenovirus, rhinovirus, respiratory syncytial virus, influenza viruses, parvovirus, and SARS-CoV. The CRCs can be easily used for air-liquid interface (ALI) polarized 3D cultures, and these coupled CRC/ALI cultures mimic physiological conditions and are suitable for studies of viral entry including receptor binding and internalization, innate immune responses, viral replication, and drug discovery as an ex vivo model for emerging viruses.

Keywords Normal cells • Cell senescence • Conditional reprogramming • Physiological conditions • Functional models • Air-liquid interface (ALI) • Emerging viruses • SARS-CoVs

Conventional Cell Line Models for Virus Studies

Emerging and re-emerging viral infections are becoming severe global public health problems in the current century. In February 2003, an outbreak of severe acute respiratory syndrome (SARS), caused by SARS coronavirus (SARS-CoV), was reported in Guangdong, China (Ksiazek et al. 2003). The SARS-CoV infected 8096 cases and 774 deaths worldwide. In March 2009 a novel influenza virus (H1N1pdm) emerged in the United States and Mexico. H1N1pdm obtained the capacity to transmit in humans and quickly spread to more than 214 countries (Hendrickson and Matthay 2013). Thereafter the H1N1 infection became a seasonal virus circulating over the world (Dawood et al. 2009). Middle East respiratory syndrome (MERS) coronavirus was isolated from patients who developed acute
pneumonia and renal failure in 2012 from Saudi Arabia (Zaki et al. 2012). One big secondary outbreak with 186 confirmed cases was in South Korea in 2015. Up to January 2020, more than 2500 confirmed cases were reported with a case fatality of 34.4% (WHO 2019). In December 2019, a novel coronavirus, SARS-CoV-2 caused an outbreak of acute pneumonia in Wuhan City of China (Zhou P et al. 2020). The current animal models for study of SARS-CoVs include African green monkeys, macaques, ferret, and mice (Kong et al. 2009; Smits et al. 2011). These in vivo models are useful for studying many important questions, for example, local or systemic pathogenic changes, immune response, and drug metabolism and in vivo efficacy. However, these animal models have limitations due to species difference. The viral tropism correlates to expression of viral receptor(s) on the surface of host cells and intracellular restriction as well. Cancer cell lines have been used widely for biological or functional cell systems in many other fields (Agarwal and Rimm 2012; Barretina et al. 2012; Palechor-Ceron et al. 2019). These cells are defective for many functions of normal human cells because of significant genetic and epigenetic changes compared to the normal tissues. However, primary cell yields and viability from any original tissue specimens are often relatively low or highly variable. These normal cells cease at a few passages because of very limited proliferative capacity in vitro. It has been extremely difficult for decades to generate and maintain normal cell lines. Several exogenous immortalization approaches have been used to establish primary cell lines from variety of tissue types. For example, viral oncogenes such as SV40 large T antigen or the human papillomavirus (HPV) E6/E7 proteins can bypass the cell senescence block through interfering with p53 and Rb regulatory pathways (Liu et al. 2005; Liu et al. 2007; Liu et al. 2008; Cid Arregui et al. 2012; Klingelhutz and Roman 2012; Ghittoni et al. 2015). Expression of exogenous cellular genes (hTERT, c-Myc, cdk4, etc.) or inactivation of cellular tumor repressors (p53, pRB, etc.) can be widely used to immortalize primary human normal cells, which leads to disrupted cell differentiation or loss of tissue type-specific functions (Liu et al. 2012).

We observed that two classes of functions are required for cell immortalization by HPV E6 and E7: telomerase activation and actin cytoskeleton alterations (Fu et al. 2003; Charette and McCance 2007; Liu et al. 2007; Liu et al. 2008; Yue et al. 2011; Klingelhutz and Roman 2012). We also noted that feeder cells and altered molecular pathways activate telomerase (Fu et al. 2003; Liu et al. 2008; Liu et al. 2009; Klingelhutz and Roman 2012; Liu et al. 2012), and a Rho-Kinase inhibitor, Y-27632, disrupts the actin cytoskeleton and inactivate Rho (Chapman et al. 2010; Liu et al. 2012). Unexpectedly, combination of feeder layers and Y-27632 allows to rapidly establish both normal and tumor cell cultures from non-keratinocyte tissues (Liu et al. 2012; Suprynowicz et al. 2012; Palechor-Ceron et al. 2013; Liu et al. 2017). We also demonstrated that the effect of the combined co-culture condition is rapid (2 days), and the whole cell populations are shifted or reprogrammed rather than a clone selection as conventional cancer cell lines (Liu et al. 2012; Suprynowicz et al. 2012). These cell cultures stop proliferating if one of the conditions (Y-27632 or feeder layer) is not met. We termed this culture method and resulting cells as “conditional reprogramming (CR)” and “conditionally reprogrammed cells (CRC)”, respectively (Liu et al. 2012; Suprynowicz et al. 2012; Palechor-Ceron et al. 2013; Liu et al. 2017; Palechor-Ceron et al. 2019). Indeed, organoids (Weeber et al. 2017; Puca et al. 2018; Sachs et al. 2018; Mullenders et al. 2019; Xinaris 2019) and CR technologies have been widely used in cancer biology and regeneration fields. They both have been recognized as the key new technologies by NCI (National Cancer Institute) precision oncology (https://ocg.cancer.gov/programs/hcmi/research) (Friedman et al. 2015; Senft et al. 2017), which are used for HCMI (human cancer model initiatives) program launched during 2019 annual AACR (https://www.atcc.org/en/Products/Cells_and_Microorganisms/HCMIL.aspx?utm_id&equals;t18020438l1). Table 1 lists properties of these cell models. In this review, we will highlight roles of CRC and coupled air-liquid interface (ALI) system in studies of emerging viruses.

**CR Methodology**

Primary airway epithelial cells can be obtained from airway tract using non-invasive or minimally invasive techniques, nasal brushing, nasorophynx swaps, induced sputum sample collection, bronchiolar lavage, endobronchial brushing or biopsy. After CR technology was established in 2012, the CR protocol has been proven to easily establish patient-derived normal and cancer cell cultures without genetic manipulation (Liu et al. 2012; Palechor-Ceron et al. 2013; Liu et al. 2017). Originally, the CR used irradiated mouse fibroblast cells (swiss mouse 3T3, J2 clone) and the Rho-associated kinase inhibitor (Y-27632) to propagate epithelial cells (Liu et al. 2012; Palechor-Ceron et al. 2013; Liu et al. 2017). A few improvements have been used to simplify protocols using J2 conditioned medium (Liu et al. 2012; Palechor-Ceron et al. 2013; Liu et al. 2017), hypoxia condition (1%–2% O2) (Peters-Hall et al. 2018), and combination with dual TGF-beta/SMAD inhibition (Mou et al. 2016; Zhang C et al. 2018). CR technology is simple and cheap since there is no need for expensive reagents as matrigel for organoids, robust since 1 × 10^6 cells can be generated from a needle biopsy within 7 days, and rapid
since the whole populations of cells can be reprogrammed within 2 days instead of clonal selection. Figure 1 shows a diagram of normal cell cultures in CR conditions and 3D (ALI and non-iPS organoids) conditions for _ex vivo_ models of airway epithelial cells.

**CRCs Maintain Differentiation Potential and Lineage Functions of Normal Airway Epithelial Cells**

CR allows the rapid generation of multi-lineage cell cultures (conditionally reprogrammed cells, CRC) from many different normal epithelial tissues, as well as tumors derived from these sites (Liu _et al._ 2012; Suprynowicz _et al._ 2012; Palechor-Ceron _et al._ 2013; Liu _et al._ 2017; Palechor-Ceron _et al._ 2019). Normal cell lines retain their normal karyotype and growth properties, and tumor cells retain their tumorigenic potential (Liu _et al._ 2012; Suprynowicz _et al._ 2012; Palechor-Ceron _et al._ 2013; Borodovsky _et al._ 2017; Liu _et al._ 2017; Yuan _et al._ 2017; Alkhilaiwi _et al._ 2018; Correa _et al._ 2018; Palechor-Ceron _et al._ 2019). Interestingly, generation of CRC from normal tissue is reversible, and manipulating CR conditions allows the cells to differentiate normally (Suprynowicz _et al._ 2012; Liu _et al._ 2017). For example, when CRC from skin epithelium or tracheal epithelium are placed in an ALI culture system, the skin cells form a well-differentiated stratified squamous epithelium, whereas the tracheal cells form a ciliated airway epithelium.

**CRCs at ALI**

ALI (Air–Liquid interface) culture (Fig. 2) of CR airway cells is uniquely suited as a model for _in vitro_ infection studies. This is due to the ability of ALI cultures to faithfully recapitulate key characteristics of the _in vivo_ airway. For example, ALI cultures exhibit relevant proportions of airway cell types, appropriate cellular polarization and junctional properties, dynamic physiologic processes such as mucus secretion and coordinated ciliary beating, and

**Fig. 1** Workflow for _ex vivo_ models of human normal airway epithelial cells.

| Sample size | Conventional cell lines | Organoids | CRC |
|-------------|-------------------------|-----------|-----|
| Timing      | Surgical                | Small to big | Tiny to big |
| Success rate of initiation (%) | (0–10) | ++ (5–80) | +++ (50–100) |
| Rapid expansion | +++ | ++ | +++ |
| Karyotypic stability | + | ++ | ++ |
| 3D growth | – | ++ | – |
| Genetic manipulation | +++ | ++ | ++ |
| Low throughput drug screens | +++ | ++ | +++ |
| High throughput drug screens | +++ | ++ | +++ |
| Heterogeneity | – | ++ | ++ |
| Cell biology | + | +++ | +++ |
| Cost | + | ++ | + |

“−” “−−” “−+” and “+++” indicate “unsuitable or not applicable”, “possible”, “suitable”, and “best” for the aspects or applications except for “cost”. “−” and “−−” represent “low” and “high” for in “cost” line, respectively.

| Table 1 Comparison of _ex vivo_ cell models. |
|--------------------------------------------|
| Sample size | Conventional cell lines | Organoids | CRC |
| Timing      | Surgical                | Small to big | Tiny to big |
| Success rate of initiation (%) | (0–10) | ++ (5–80) | +++ (50–100) |
| Rapid expansion | +++ | ++ | +++ |
| Karyotypic stability | + | ++ | ++ |
| 3D growth | – | ++ | – |
| Genetic manipulation | +++ | ++ | ++ |
| Low throughput drug screens | +++ | ++ | +++ |
| High throughput drug screens | +++ | ++ | +++ |
| Heterogeneity | – | ++ | ++ |
| Cell biology | + | +++ | +++ |
| Cost | + | ++ | + |
physiological expression and subcellular localization of characteristic proteins bearing species-specific sequences.

**CRCs/ALI: An Ex Vivo Models for Virus Infections**

To date, CR by itself or in combination with ALI cultures have been used in studies of host defense and viral infections, drug screening and toxicity testing, wound healing/repair, gene therapies as *ex vivo* preclinical models for lung cancer, chronic obstructive pulmonary disease, cystic fibrosis, and asthma (Yuan et al. 2012; Bove et al. 2014; Crystal et al. 2014; Saenz et al. 2014; Chu et al. 2015b; Feng et al. 2015; Kotha et al. 2015; Walters et al. 2015; Alamri et al. 2016; Beglyarova et al. 2016; Butler et al. 2016; Ellis et al. 2016; Papapetrou 2016; Reynolds et al. 2016; Borodovsky et al. 2017; Gentsch et al. 2017; Jensen et al. 2017; Li et al. 2017; Mahajan et al. 2017; Martinovich et al. 2017; Wolf et al. 2017; Yu et al. 2017; Yuan et al. 2017; Alkhilaiwi et al. 2018; Boström et al. 2018; Brewington et al. 2018; Correa et al. 2018; Jin et al. 2018; LaRanger et al. 2018; Mimoto et al. 2018; Moorefield et al. 2018; Peters-Hall et al. 2018; Shay et al. 2018; Wang et al. 2018; Zhang Z et al. 2018; Alkhilaiwi et al. 2019; Jiang et al. 2019; Krawczyk et al. 2020; Liu 2019; Martini et al. 2019; Mondal et al. 2019; Nicolas et al. 2019; Palechor-Ceron et al. 2019; Su et al. 2019; Chai et al. 2020). Compared to organoids (close 3D cultures) shown in Fig. 1 (right lower panel), ALI system is much easier for virology studies because of an open apical area for infections or viral inoculations (Fig. 3). Here we will focus our discussion on applications of this physiological system in respiratory virus infections. Figure 3 shows many aspects of studies of emerging viruses.

Roberts et al. collected samples from paired nasal and bronchial brushings and cultured and expanded nasal and bronchial epithelial cells using CR condition (irradiated 3T3 fibroblasts in presence of Y-27632) (Roberts et al. 2018). Passaged cells were then put onto collagen coated transwells in 12 well plates for ALI culture. They found that viral infection in both cell types increased the expression of IP-10 (interferon gamma-induced protein 10), although the increase was only significant in the ALI culture, with combining rhinovirus infection and IL-13 treatment (Roberts et al. 2018). Kotha et al. showed that the level of the apical adenovirus receptor (CAREX) and physiologically relevant levels of IL-8 and neutrophils (components of the innate immune system) enhanced entry of adenovirus in polarized human airway epithelia (Kotha et al. 2015). Jonsdottir et al. established CRC and ALI cultures from both upper and lower airway to study the host innate immune response to human coronavirus 229E (HCoV-229E) and human respiratory syncytial virus (RSV) after gene manipulations (Jonsdottir et al. 2019). It is well known that autonomous parvovirus replication depends on the S phase of the host cells. Interestingly, Deng et al. (2016) reported for the first time parvovirus DNA replicated in non-dividing cells spontaneously. They first established CR cells and ALI cultures (non-dividing airway epithelial cells) and inoculated ALI with human parvovirus HBoV1. Their results demonstrated that HBoV1 infection of ALI cultures induces a DNA damage response (DDR), thereby facilitating viral genome amplification. They also discovered that Y-family DNA repair polymerases, Pol η and Pol κ, are involved in HBoV1 genome amplification in ALI system. This is the first report to show that parvovirus DNA replicates in non-dividing cells autonomously. Zhu et al. studied HSV-2 infection in CR and ALI cultured normal vaginal epithelial cells (Zhu et al. 2017). Fink et al. also established CR cells from routine vaginal repair surgeries or hysterectomies and studied antiviral drug (Arbidol) Inhibition of Zika virus (Fink et al. 2018). Finally, Imai-Matsushima et al. generated long-term
cultural and distal airway epithelial cells and differentiated alveolar epithelial cells that are suited for influenza virus study (Imai-Matsushima et al. 2018).

**CRCs/ALI: Potential Applications in SARS-CoV-2 and Coronavirus Disease 2019 (COVID-19)**

On Jan. 30 and March 11, 2020, the International Health Regulations Emergency Committee World Health Organization (WHO) declared the COVID-19 or SARS-CoV-2 infection outbreak as a “public health emergency of international concern”, and characterized COVID-19 as a pandemic, respectively (WHO 2020). COVID-19 cases have ranged from very mild (including asymptomatic), mild, moderate, severe, and critical severe including illness resulting in death (Lu et al. 2020; Singhal 2020; Wang et al. 2020; Wu and McGoogan 2020; Yang S et al. 2020; Yang X et al. 2020; Ye et al. 2020; Zhang et al. 2020; Zhou C et al. 2020; Zhou F et al. 2020; Zou et al. 2020). COVID-19 common symptoms include fever, cough and shortness of breath. Muscle pain, sputum production and sore throat are less common (Lu et al. 2020; Singhal 2020; Wang et al. 2020; Wu and McGoogan 2020; Yang S et al. 2020; Yang X et al. 2020; Ye et al. 2020; Zhang et al. 2020; Zhou C et al. 2020; Zhou F et al. 2020; Zou et al. 2020). While the majority of cases result in mild symptoms, some progress to severe pneumonia and multi-organ failure including lung, heart, and kidney (Lu et al. 2020; Singhal 2020; Wang et al. 2020; Wu and McGoogan 2020; Yang S et al. 2020; Yang X et al. 2020; Ye et al. 2020; Zhang et al. 2020; Zhou C et al. 2020; Zhou F et al. 2020; Zou et al. 2020). The rate of deaths per number of diagnosed cases is estimated to be 3.4% but varies by age and other health conditions (Lu et al. 2020; Singhal 2020; Wang et al. 2020; Wu and McGoogan 2020; Yang S et al. 2020; Yang X et al. 2020; Ye et al. 2020; Zhang et al. 2020; Zhou C et al. 2020; Zhou F et al. 2020; Zou et al. 2020). The mechanisms how SARS-CoV-2 infects human airway epithelial cells and also causes severe multi-organ failure are largely unknown. Generally speaking, early stage of COVID-19 or cases with very mild (including asymptomatic), mild symptoms are usually due to rapid replication of viruses at local areas, innate response of the host cells and local immune-response (IgA). Severe cases with multi-organ injury are usually due to possible viral replications in the target organs and immunopathogenic injuries (including cytokine storm or antibody dependent enhancement). Recently, two reports demonstrated that large amounts of SARS-CoV-2 were detected in the upper airway and saliva (To et al. 2020; Zou et al. 2020).

SARS-CoV-2 and SARS-CoV share the same functional host-cell receptor ACE2 (Hoffmann et al. 2020; Walls et al. 2020; Wan et al. 2020; Wrapp et al. 2020; Zhou P et al. 2020) and sequence analysis reveals that SARS-CoV-2 possesses crucial amino acid residues for ACE2 binding (Hoffmann et al. 2020). ACE2 predominantly expresses in vascular endothelial cells, kidney and heart tissues, small intestine, and testes (Hamming et al. 2004). The distribution of ACE2 in human tissues does not seem to correlate with diseases with COVID-19, while this may help explain why severe cases with COVID-19 have multi-organ failure including heart and kidney. It is important to understand body site-specific or tissue-specific viral replication, innate immune response, and infectivity. Two recent reports demonstrated that large amounts of SARS-CoV-2 were found in the upper airway and saliva as we described above (To et al. 2020; Zou et al. 2020), where ACE2 is not expressed or expresses at very low levels. Wolfel et al. performed a dynamic viral analysis of nine COVID-19 patients, they showed active virus replication in upper airway tissues. Virus shedding at pharynx was very high at the first week of symptoms, the peak was at 7.11 × 10^8 RNA copies per throat swab at day 4. SARS-CoV-2 viruses were readily cultured from throat- and lung-derived samples, but not from stool samples with high viral RNA. Viral load was declined after 7 days of symptoms (Wolfel et al. 2020). A ferret model of SARS-CoV-2 infection and transmission was established recently (Kim et al. 2020), which can recapitulate some aspects of human COVID-19 disease. SARS-CoV-2-infected ferrets shed virus in nasal washes, saliva, urine, and feces up to 8 days after infection. Viral antigens were also detected in several tissues, as nasal cavity, trachea, lung, and intestine. This ferret model represents an animal model of SARS-CoV-2 infection and transmission, may

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**Fig. 3** Potential applications of CR/ALI cultures in emerging viruses.
facilitate to develop COVID-19 therapeutics and vaccine as well. However, distribution of ACE2 and viral replication in different organs or tissues, or possible other receptors or co-receptors in this model need to be further determined. Another study also demonstrated that SARS-CoV-2 may replicate well in ferrets and cats, but poorly in dogs, pigs, chickens, and ducks (Shi et al. 2020). These inconsistencies suggest possible alternative receptors or co-receptors on the surface of the target cells in different tissues. A study by Blanco-Melo et al. suggests that human alveolar adenocarcinoma cells (A549) are able to support viral replication of SARS-CoV-2, RSV and influenza A virus, despite undetectable levels of ACE2 and TMPRSS2, the putative receptor and protease for SARS-CoV-2 entry (Blanco-Melo et al. 2020). To solve these problems, we will need comprehensive studies of tissue staining of viral antigens and cellular proteins from COVID-19 patients. Thus, human related physiological models are urgently needed to investigate these questions and for functional validation. In agreement with these arguments, an early study on SARS-CoV indicates that host cell differentiation or polarized epithelium and expression of ACE2 are both important for the susceptibility of human airway epithelia to SARS-CoV viral infection (Jia et al. 2005; Tseng et al. 2005). Indeed, ALI cultures of human airway epithelial cells (HAEs) have also been used for functional drug screening of SARS-CoV and SARS-CoV-2 (Sheahan et al. 2017; Sheahan et al. 2020). Since CRCs are stable resources for normal functional airway cells, CRCs/ALI cultures will facilitate these studies and development of novel therapeutics as a functional and biological system.

**Advantage and Limitations**

As we discussed above, CR is a rapid, robust and sufficient way to obtain large amount of normal airway cells from non-invasive and minimal human samples, and CRCs from respiratory tract maintain their lineage functions. ALI provides a unique environment to mimic “in vivo” physiological conditions. Thus, CRC/ALI will be appropriate for studies of viral entry to the nature host cells, innate immune response of the host cells, and tests of anti-viral and immune (innate) modulators (Fig. 3). This will be a unique biological or functional cell system for population or health disparity studies since there is no biological cell model available in the field. Since CRCs can be easily genetically manipulated with overexpression of exogenous genes, shRNAs, or CRISPR technologies (Chu et al. 2015a; Fenini et al. 2018), CRC/ALI should be a unique system for both phenotypical and mechanism studies for human viral diseases. Although CRCs alone can be used for rapid and high throughput screening, combination CRC and ALI will take much longer time and need further development for high throughput studies. Compare to the current permissive cancer cell lines or animal derived permissive cell lines, CRC/ALI for virus studies requires experienced performance, higher work load and cost. Thus, CRC/ALI will serve as a human-relevant, physiological system for emerging viruses in addition the current cell lines and animal models.

**Conclusions**

Because of limitations of current cell line and animal models, there is an urgent need of human physiological cell models for studies of emerging viruses. Here we summarized establishment of long term cultures for human normal airway epithelial cells from nose to lung using CR and coupled ALI technologies and their applications as an *ex vivo* model for emerging viruses. As a great addition to the current cell lines and animal models, CRC/ALI system will facilitate studies of viral entry including receptors and internalization, innate immune responses, viral replication, and drug discovery.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and Human Rights Statement** This article does not contain any studies with human or animal subjects performed by any of the authors.

**Disclosure** Several patents for conditional reprogramming technology has been awarded to Georgetown University by the United States Patent Office. The license for this technology has been given to Propagenix for commercialization. The inventor, X.L., and George-town University receive potential royalties and payments from Propagenix.

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