Conditional reprogramming: next generation cell culture

Xiaoxiao Wu, Shengpeng Wang, Mingxing Li, Jing Li, Jing Shen, Yueshui Zhao, Jun Pang, Qinglian Wen, Meijuan Chen, Bin Wei, Parham Jabbarzadeh Kaboli, Fukuan Du, Qijie Zhao, Chi Hin Cho, Yitao Wang, Zhangang Xiao, Xu Wu.

Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou 646000, China
South Sichuan Institute of Translational Medicine, Luzhou 646000, China
State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China
Department of Oncology and Hematology, Hospital (T.C.M) Affiliated to Southwest Medical University, Luzhou 646000, China
Center of Radiation Oncology, Hospital (T.C.M) Affiliated to Southwest Medical University, Luzhou 646000, China
Department of Oncology, the Affiliated Hospital of Southwest Medical University, Southwest Medical University, Luzhou 646000, China
College of Pharmaceutical Science & Collaborative Innovation Center of Yangtze River Delta Region Green Pharmaceuticals, Zhejiang University of Technology, Hangzhou 310014, China
School of Biomedical Sciences, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong, China

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Abbreviations: ΔNP63α, N-terminal truncated form of P63α; ACC, adenoid cystic carcinoma; AACR, American Association for Cancer Research; AR, androgen receptor; CFTR, cystic fibrosis transmembrane conductance regulators; CR, conditional reprogramming; CYPs, cytochrome P450 enzymes; DCIS, ductal carcinoma in situ; dECM, decellularized extracellular matrix; ECM, extracellular matrix; ESC, embryonic stem cell; hASC, human adipose stem cells; HCM, human cancer model initiatives; HGF, hepatocyte growth factor; HNE, human nasal epithelial; HPV, human papillomaviruses; hTERT, human telomerase reverse transcriptase; ICD, intracellular domain; iPSCs, induction of pluripotent stem cells; LECs, limbal epithelial cells; NCI, National Cancer Institute; NGFR, nerve growth factor receptor; NSCLC, non-small cell lung cancer; NSG, NOD/SCID/gamma; PDAC, pancreatic ductal adenocarcinoma; PDX, patient derived xenograft; PP2A, protein phosphatase 2A; RB, retinoblastoma-associated protein; ROCK, Rho kinase; SV40, simian virus 40 large tumor antigen; UVB, ultraviolet radiation.

*Corresponding authors. Tel.: +86 1830830263 (Zhangang Xiao), +86 13882770623 (Xu Wu).

E-mail addresses: xzg555898@hotmail.com (Zhangang Xiao), wuxulz@126.com (Xu Wu).

These authors made equal contributions to this work.

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Abstract  Long-term primary culture of mammalian cells has been always difficult due to unavoidable senescence. Conventional methods for generating immortalized cell lines usually require manipulation of genome which leads to change of important biological and genetic characteristics. Recently, conditional reprogramming (CR) emerges as a novel next generation tool for long-term culture of primary epithelium cells derived from almost all origins without alteration of genetic background of primary cells. CR cocultures primary cells with inactivated mouse 3T3-J2 fibroblasts in the presence of RH-related protein kinase (ROCK) inhibitor Y-27632, enabling primary cells to acquire stem-like characteristics while retain their ability to fully differentiate. With only a few years’ development, CR shows broad prospects in applications in varied areas including disease modeling, regenerative medicine, drug evaluation, drug discovery as well as precision medicine. This review is thus to comprehensively summarize and assess current progress in understanding mechanism of CR and its wide applications, highlighting the value of CR in both basic and translational researches and discussing the challenges faced with CR.

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1. Introduction

Culturing and expanding primary mammalian cells has been always difficult due to senescence and loss of important genetic characteristics after several numbers of population doubling. For even primary tumor-derived cells, majority of them undergo differentiation and aging and fail to proliferate in vitro after several passages. However, amplifying primary cells to establish stable cell lines usually provides more typical and ideal in vitro models for biological researches and precision medicine. Several methods have been applied to obtain immortalized primary cells in the past decades. The most common ways include the transfection with viral oncogenes i.e., the simian virus 40 large tumor antigen (SV40) and E6/E7 proteins of the oncogenic human papillomaviruses (HPV), exogenous induction of human telomerase reverse transcriptase (hTERT), and the use of irradiated mouse fibroblast, or inhibitor of RHO kinase (ROCK). Although these strategies have been demonstrated to benefit primary cell culture, majority of them still have problems in long-term maintenance such as senescence, aberrant differentiation, loss of heterogeneity and alteration of genetic profile.

In 2010, Chapman et al. reported that primary human keratinocytes continually proliferated in vitro and were effectively immortalized, using a combined irradiated Swiss 3T3-J2 mouse fibroblast feeder cells and the ROCK inhibitor Y-27632. They referred to the system used as conditional reprogramming (CR). CR shows advantages over previous methods that independently use the fibroblast feeder or ROCK inhibitor, or the exogenous gene expression, in terms of population doubling number and maintenance of normal genetic background. It is demonstrated that human ectocervical cells under CR condition were able to grow for over 200 passages and were normally differentiated after removal of the J2 feeders and ROCK inhibitor. Moreover, while no more than 5% of primary tumors can be expanded in vitro for a long term previously, CR successfully enables generation of cell lines from almost 90% of tissue specimens from human normal and tumor origins, which maintains both intratumor and inter-tumor heterogeneity. CR technology has been recognized one of the two key new technologies together with organoid cultures by National Cancer Institute (NCI) precision oncology program launched during 2019 annual meeting of American Association for Cancer Research (AACR) (https://www.atcc.org/en/Products/Cells_and_Microorganisms/HCMI.aspx?utm_id=t180204381). CR thus emerges as a powerful tool to expand and study almost all primary tissue samples.

Since the generation of the first human-derived cell line HeLa, cell lines from different origins have been established which greatly facilitates molecular biology research. These conventional cell lines enable high-throughput screening or intervention study with high reproducibility. However, due to the long-term selection and in vitro culture conditions during development and maintenance of cell lines, they are becoming homogenous while lack inter- and intra-individual heterogeneity. Virtanen et al. analyzed the similarities and differences between primary lung tumors and cell lines and found that cell lines only partially resembled primary tumors and showed remarkable differences in gene expression profile compared to original clinical specimens. Similar reports are also found. Furthermore, culture conditions that allow cells to grow as a monolayer in plastic plate or dish under fixed air condition often only select a subpopulation of cells to proliferate well in the specified conditions. It is indicated that varying oxygen levels have a remarkable impact on the growth of certain cells within the culture. Different culture conditions may lead to the creation of different cell lines. As a result, cell lines largely differ from the cells within in vivo microenvironment. Therefore, conventional cell lines thus could not represent primary tissues and usually fail to model in vivo characteristics, leading to unsuccessful translation of laboratory findings into the clinic. In contrast, CR cells have been proven to be invaluable for current researches as CR is able to maintain different subclones of cells within the original tissues and maximize individual heterogeneity with increased success rate. It is no surprise that CR cells will greatly advocate more high-impact researches to promote both basic science and translational medicine.

CR offers new opportunity in cellular and molecular biology, disease modeling and exploration, as well as the regenerative medicine and drug discovery. Moreover, recent studies have gained new insights into the underlying mechanisms of CR on cell immortalization without changing genetic characteristics of primary cells. This review is thus to summarize and evaluate current progress in understanding CR mechanisms and its applications, highlighting the value of CR in various aspects. The challenges faced with CR are also discussed to facilitate future researches.
2. An overview of CR technology

2.1. CR protocol

CR technology involves co-culture of irradiated Swiss-3T3-J2 mouse fibroblast feeder cells and digested primary normal or pathogenic cells in the presence of the ROCK inhibitor Y-27632, changing the external culture environment to allow cells to acquire partial stem cell characteristics\(^{13}\). Either J2 feeder or Y-27632 is essential for CR culture. Primarily, J2 fibroblasts are subjected to appropriate dose of irradiation, in order to stop proliferation of J2 cells. The irradiated J2 cells can directly serve as feeders. Other way is to use the anti-proliferative mitomycin C (2\(\text{e}^{-4}\) mg/mL) to treat J2 cells for 1\(\text{e}^{-3}\) h to acquire feeder cells\(^{25}\). However, it is necessary to wash the mitomycin C-treated cells for several times to avoid growth inhibition by mitomycin C during co-cultures. A feeder cell layer is usually required for physically contacting with primary cells. In some cases, conditional medium which contains sufficiently secreted factors by J2 feeder cells is used, which receives similar efficiency as with the use of feeder layer\(^{26}\).

The key steps of CR culture are depicted in Fig. 1, which involves the enzymatic digestion of primary tissues from human or a patient-derived xenograft (PDX), followed by filtration and culturing together with feeder cells and ROCK inhibitor. Since CR condition immortalizes both normal and pathological cells, it usually requires a careful pathological evaluation by histological or other examinations for discrimination.

2.2. CR vs. other techniques

Proliferation of primary cells is controlled by the length of telomeres, and usually stops replication when senescence occurs\(^{17}\). The key issue to develop stable cell lines is to maintain normal function of primary cells and maximize heterogeneity, with increased success rate. To date, there are several ways to achieve cell immortalization to obtain stable cell lines from primary cells. Conventional methods include using viral oncogenes to engineer the genome of a host cell such as large T antigen 32 of SV40\(^{28}\) and E6/E7 protein of HPV\(^{29}\), overexpression of \(htERT\)\(^{30}\), induction of pluripotent stem cells (iPSCs)\(^{31}\) or embryonic stem cells (ESCs)\(^{32}\). Irradiated mouse fibroblasts or inhibitor of ROCK is also used for primary cell culture. However, there are several problems with these methods, such as low efficiency, genomic instability, loss of important biological and genetic properties during passage as well as ethical issues in the clinical application of iPSC or ESC (Table 1). Decellularized extracellular matrix (dECM) is also a system for primary cell expansion without inducing genetic changes, which is a useful tool for studying stem cell differentiation. But only limited studies have demonstrated its ability to culture primary epithelial cells derived from different origins for a long term\(^{13}\).

Transformation with viral oncogenes was first adopted for cell immortalization since 1980s—1990s\(^{2,3}\). With careful handling, transduction with the viral oncogenes of SV40 large T antigen and E6/E7 HPV can effectively immortalize many types of primary cells without tumorigenic transformation\(^{14-36}\). The viral oncoproteins are well acknowledged to deactivate the tumor-suppressive P53 and P16/RB pathways and promote \(htERT\) transcription and telomerase activity which bypass senescence\(^{37-39}\). However, there are some reports showing that cell immortalization by viral oncogenes leads to tumorigenicity, genome instability and change of gene expression\(^{30,40,41}\).

\(htERT\) is the catalytic subunit of human telomerases that are critical for determining the lifespan of a cell\(^{32}\). Many cancer cells

![Figure 1](image-url)
can induce telomerase to prevent cellular senescence and keep continuous growth\textsuperscript{41}. Overexpression of hTERT has long been used as a tool to promote cell immortalization\textsuperscript{42}. Although there are many successful cases\textsuperscript{43–47}, this method is confronted with similar problems to the use of viral oncogenes\textsuperscript{48,49}.

The introduction of iPSC from somatic cells was firstly reported by Takahashi et al.\textsuperscript{49} in 2006. It is expected that iPSCs maintaining the pluripotency of ESCs have many potential clinical applications including regenerative medicine\textsuperscript{50}. This method involves the forced expression of a combination of several genes, such as \textit{OCT3/4}, \textit{SOX2}, \textit{KLF4}, \textit{C-MYC}, \textit{NANOG}, and \textit{LIN28}, in target cells\textsuperscript{51}. Small molecule drugs which are considered easier and more cost-effective are also used to induce iPSCs\textsuperscript{52}. However, induction of iPSC is usually of low efficiency, and is reported to increase risk of mutation and cause incomplete reprogramming.

Since early 1970s, irradiated mouse fibroblasts have been utilized as feeder cells to facilitate epithelium cell growth\textsuperscript{53}. The feeders can prevent normal keratinocytes from senescence, extend their doubling numbers \textit{in vitro} and enable colony formation. Except keratinocytes, primary culture of breast tumors can be also supported by irradiated fibroblasts\textsuperscript{54}. Primary cells co-cultured with irradiated mouse fibroblasts show increased lifespan from 20 to 40–60 passages and significantly inhibit overgrowth of human fibroblasts\textsuperscript{55,56}. ROCK plays an important role in modulation of cytokinesis and cell differentiation, and the use of ROCK inhibitors for suppressing differentiation has been applied to maintain stem cell culture\textsuperscript{56,57}. ROCK inhibitors not only protect human ESCs from apoptosis during culture but also increase recovery and colony formation after freeze-thawing from a cryopreserved sample\textsuperscript{58}. ROCK inhibitors can keep ESCs and iPSCs undifferentiated in culture and increase proliferation ability. However, either irradiated mouse fibroblasts or ROCK inhibitors can support primary culture for a limited period, and primary cells eventually become senescent.

Decellularized extracellular matrix (dECM) is the isolated extracellular matrix of primary tissues or cells, which emerges as a promising natural biomaterial for tissue engineering and primary cell culture\textsuperscript{59,60}. Since dECM contains a source of biochemical and biophysical cues for supporting cell growth, it greatly enhances primary cells adhesion and attachment and improves their viability and functions\textsuperscript{61}. It is reported that most tissue-derived dECM directs stem cell differentiation, while some cell-derived dECM can also maintain differentiation potential of stem cells\textsuperscript{62}. It is widely used clinically for tissue repair and regeneration\textsuperscript{63}. However, although dECM facilitates cell proliferation without genetic manipulation, its eligibility for maintaining long-term expansion of different origins of primary epithelia needs further evidence.

Compared with these conventional methods, CR technology using a combination of J2 feeder and ROCK inhibitor of Y-27632 for long-term culture of primary cells does not alter cellular genes and maintains cell genome stability, with simpler procedures and no ethical issue (Table 1). Therefore, as a new technique for cell immortalization, CR technology shows its good promise for further development and advanced application with unique advantages.

3. CR mechanisms

It has been demonstrated that both ROCK inhibition with Y-27632 and the addition of J2 feeder cells are essential for growth of CR cells. In the absence of Y-27632, human primary keratinocytes can be only cultured for 20–40 population doublings until senescence, while with the Y-27632, keratinocytes continually grow for over 150 passages and become immortalized\textsuperscript{64}. Similarly, without the addition of J2 feeder, keratinocytes cannot be immortalized\textsuperscript{65}. The underlying mechanisms for CR have been investigated by several studies, which provide insights into its actions. It is shown that CR acts through several signaling pathways to promote cell cycle progression, inhibit apoptosis and differentiation, and maintain stem cell-like properties of primary cells. There are also evidences that CR facilities cell-ECM and cell-cell communication. The main mechanisms of CR are summarized in Fig. 2. However, it is suggested that many questions still remain regarding the mechanism of CR.

3.1. Acceleration of cell cycle progression

Studies have shown that CR condition promotes proliferation of epithelial cells \textit{via} accelerating cell cycle progression and suppressing senescence. Butler et al.\textsuperscript{66} demonstrated that human respiratory epithelial cells from endobronchial biopsies were rapidly expanded in CR condition, and J2 feeder/Y-27632 combination increased cell populations in S phase and promoted cell cycle. In another study, human keratinocytes in the presence of Y-27632 showed an increased S phase transition, and J2 feeder alone increased G2/M population\textsuperscript{67}.

It is suggested that both inactivation of retinoblastoma protein (pRB)/P16INK4A and enhanced telomerase activity are required for immortalization of primary epithelia\textsuperscript{68,69}. Both pRB and P16INK4A are tumor suppressors that are important regulators of cell cycle progression and senescence. In Ligaba et al.’s study\textsuperscript{70}, both Y-27632 and J2 feeder inactivated the pRB through enhancing its phosphorylation, and the J2 feeders increased protein expression of cyclin A, cyclin E, MCM4 and pCDK1. Chapman et al.\textsuperscript{60} showed that P16INK4A maintained at a low level during CR culture.

In several studies\textsuperscript{60,71,72}, CR was demonstrated to increase transcription of \textit{hTERT}. The length of telomere was maintained even after long-term passages, although some reports saw shortened telomere length at early culture\textsuperscript{60,69}. Liu et al.\textsuperscript{73} showed that Y-27632 had a minimal effect on \textit{hTERT} induction, while J2 feeder was critical for this effect. In a recent study, it is found that primary epithelial cells from human prostate, foreskin, ectocervical, and mammary tissues under CR condition displayed high level of a natural P53 isoform, Δ133P53α, which was essential for CR cell proliferation\textsuperscript{74}. The induction of Δ133P53α significantly induced \textit{hTERT} expression and telomerase activity, which showed no influence on full-length P53 and downregulated P53 effectors of P21 and BAX\textsuperscript{74}. This study evokes a positive association of \textit{hTERT} and P53 pathway.

It is suggested that both J2 feeder and Y-27632 have played a role in regulating cell cycle progression, suppressing senescence and promoting proliferation of primary cells. The details on the mechanism of action by J2 feeder and/or Y-27632 and their contributions still need further investigation.

3.2. Inhibition of apoptosis

RHO/ROCK signaling plays a crucial role in proliferation, apoptosis and differentiation\textsuperscript{75}. ROCK can increase actomyosin-based contractility to induce apoptosis \textit{via} phosphorylating and downregulating myosin phosphatase, while the ROCK inhibitor Y-27632 inhibits ROCK through competitively binding to the catalytic residue\textsuperscript{76}. It is reported that Y-27632 attenuated apoptosis mediated by doxorubicin in human cardiac stem cells\textsuperscript{77}, and
promoted proliferation of human periodontal ligament stem cells and limbal epithelial cells\textsuperscript{77,78}. The RHO/ROCK-MLC signaling is associated with ethanol-mediated aneikis, and ROCK inhibitors prevented ethanol-induced membrane blebbing and apoptosis\textsuperscript{79}. Y-27632 suppressed cytokine regulation and the MAPK pathway, thus decreasing apoptosis of hESC\textsuperscript{80}. Y-27632 also facilitated iPSCs to escape the dissociation-mediated apoptosis through the suppression of P53 pathway\textsuperscript{81}. Dakic et al.\textsuperscript{68} demonstrated that Y-27632 cooperated with MYC to immortalize primary keratinocytes. Notably, Y-27632 did not affect hTERT and P16\textsuperscript{k}-dependent, leading to upregulation of mutation and incomplete reprogramming, of primary epithelia of varied origins in a stem-like state.

### Table 1: CR vs. conventional methods for primary cell culture.

| Conventional method | Procedure | Characteristics | CR vs. conventional method |
|---------------------|-----------|-----------------|---------------------------|
| Transfection of viral oncogenes | The viral oncogene (i.e., large T antigen 32 of SV40, HPV E6/E7) is constructed into a lentiviral expression vector, and transfected into target cells. | Manipulation of gene expression leading to genome instability, change of the phenotype and genotype of primary cells, and sometimes tumorigenicity | Easy to operate (highly efficient), without changing cellular gene profile, maintaining genome stability and differentiation potential |
| Overexpression of hTERT | Continuous transfection of target cells with retroviral constructs of cyclin-dependent kinase (CDK) 4 and hTERT. | Low efficiency, increased risk of mutation and incomplete reprogramming | |
| Induced pluripotent stem cells | Exogenous transcription factor genes such as OCT3/4, SOX2, C-MYC and KLF4 are transferred into adult cells, enabling them to acquire stem cell characteristics and enhanced proliferation. | Low efficiency and having ethical issue | No ethical issues |
| Embryonic stem cells | Embryonic stem cells are derived from early embryos or primitive gonads and can be infinitely propagated in vitro, induced and differentiated into target cells. | Senescent after a few passages | Maintaining long-term expansion of primary cells |
| Inactivated fibroblasts | Co-culture of primary cells with irradiation-inactivated mouse fibroblasts | Senescent after a few passages | Maintaining long-term expansion of primary cells |
| ROCK inhibitors | Culture of primary cells with the presence of ROCK inhibitor | Enhancing cell proliferation; directing stem cell differentiation or maintaining stem differentiation potential by use of tissue-derived or cell-derived dECM | |
| Decellularized extracellular matrix | Extracellular matrix is prepared from primary tissues or cells after decellularization which maintains the macro and micro architectures of the organ or is digested into a liquid to form a hydrogel or coat a substrate. | | |

\textit{CDK4}, cyclin-dependent kinase-4; \textit{hTERT}, human telomerase reverse transcriptase; \textit{OCT3/4}, octamer-binding transcription factor 3/4; \textit{SOX2}, (sex determining region Y)-box 2; \textit{KLF4}, Kruppel-like factor 4; \textit{ROCK}, Rho kinase.

3.3. Inhibition of differentiation

In the study by Chapman et al.\textsuperscript{60}, it is found that CR condition maintained long-term proliferation of human keratinocytes \textit{in vitro}, and gene expression analysis showed that Y-27632 significantly down-regulated differentiation-related genes which was reversible by the removal of Y-27632. This indicates that ROCK inhibition might be important for suppression of differentiation under CR condition. Actually, researches have shown that the RHO/ROCK signaling pathway is related with the differentiation of many cells, including keratinocytes\textsuperscript{57}, mesenchymal stem cells\textsuperscript{83}, enamel cells\textsuperscript{84}, etc. Y-27632 that specifically inhibits the RHO/ROCK signaling effectively reduces cell differentiation mediated by this pathway\textsuperscript{80,85}.

\textit{NOTCH} pathway is important in modulating epidermal differentiation\textsuperscript{86}. \textit{NOTCH} signaling stimulates two distinct pathways, RBP-Jk-independent or RBP-Jk-dependent, leading to upregulation of P21 and promoting keratinocyte growth arrest and differentiation\textsuperscript{86,87}. Takashi et al.\textsuperscript{88} revealed that a new \textit{NOTCH}/ROCK pathway is crucial for cellular differentiation of human iPSC. It is found that \textit{NOTCH1} induced the activation of ROCK1, and the use of Y-27632 or ROCK1 knockdown could inhibit growth arrest and differentiation induced by activated \textit{NOTCH1}. The study provides an insight into the rationale of using Y-27632 in CR culture. Moreover, Supryniewicz et al.\textsuperscript{87} showed that Y-27632 could decrease the expression of \textit{NOTCH1} and \textit{NOTCH ICD}.

\textit{WNT5A}, one of the WNT members, is able to trigger osteogenic differentiation of human mesenchymal stromal cells, which activates both the canonical and non-canonical WNT signal transduction pathways. Recently, \textit{WNT5A} was demonstrated to induce osteogenic differentiation of human adipose stem cells (hASC) and stimulates non-canonical WNT pathways\textsuperscript{89}. In this
process, ROCK was identified as a key determinant. The result suggests that ROCK inhibitor may prevent cell differentiation through interacting with WNT5A signals to a certain extent.

TGF-β members are important in the maintenance as well as differentiation of ESCs, somatic stem cells, and cancer stem cells. Ji et al. showed that RHO/ROCK signaling cross-talked with TGF-β/SMAD signaling and was associated with the process of lung fibroblast differentiation. It is found that CR condition inhibited the TGF-β pathway and could downregulate the stratified squamous epithelial cell marker, involucrin, to maintain a poorly differentiated state of primary cells.

3.4. Stem cell maintenance

Several studies suggest that CR cells maintain stem cell characteristics. It is demonstrated that CR effectively increased the number of keratinocytes and prostate epithelia which possessed stem cell properties. The ΔNP63α, CD44, nuclear β-catenin, as well as the integrin α6 and β1 are important markers which reflect a stem-like phenotype with self-renewal property. CR cells from human respiratory epithelial cells showed a stem cell phenotype, which expressed high levels of integrin α6 and NGFR. Suprynnowicz et al. indicated that CR cells from ectocervical or tracheal epithelial cells presented characteristics of adult stem cells which were reversible after removal of CR condition. Y-27632 alone upregulated stem cell markers of P63 and CD44, while using together with J2 feeder the expression of α6 and β1 integrins were induced and the nuclear β-catenin was increased. They further showed that β-catenin-mediated transcription in CR human ectocervical cells was important for induction of stem cell markers. CR increased protein phosphatase 2A (PP2A) expression and induced the binding of PP2A to β-catenin, resulting in β-catenin activation. Notably, the stimulation of β-catenin did not rely on canonical pathways of WNT and AKT/GSK-3. In addition, in CR cells from normal ectocervical or tracheal tissues, they did not express stem cell markers of hESC and iPSC such as SOX2, OCT4, NANOG or KLF4.
3.5. Promotion of cell-extracellular matrix (ECM) and cell—cell interaction

Palechor-Ceron et al. showed that in CR culture physical contact between J2 feeders and primary keratinocytes was not essential for long-term expansion, and thus the CR-mediated immortalization is dependent on Y-27632 and feeder-secreted factors. The J2 feeder cells which are irradiated or treated with drugs to stop proliferation undergo apoptosis and may produce one or more specific factors to promote growth of CR cells. Previous study has indicated that the J2 feeders secreted factors such as type IV collagen, laminin, glycoproteins, interstitial procollagens, as well as fibronectin which may benefit attachment and proliferation of other cells. Ligaba et al. used a siRNA library targeting 332 genes to screen J2-secreted factors that are critical for J2-mediated growth-enhancing effect. The result showed that 14 genes were associated with growth stimulation, which may affect signaling pathways like cytoskeleton modification, SMADs, GPCRs and TGF-β signaling. HBEGF and other factors secreted by J2 feeders may be essential for CR culture. A recent study demonstrated that irradiated J2 feeders produced murine hepatocyte growth factor (HGF). Although the murine HGF could not replace feeder cells to immortalize human airway epithelial cells, the murine HGF partially stimulated human MET, followed by phosphorylation of GAB2 and STAT6. However, it is still unclear what the key factors and which pathways are essentially involved in CR method.

A comparative gene expression analysis on primary airway epithelial cells showed that Y-27632 modulated expression of genes that relate to ECM modulation and cell—cell interaction. By using in situ zymography, Y-27632 was demonstrated to alter activity of matrix metalloproteinase and collagenase. The results suggest that Y-27632 may regulate cell—ECM and cell—cell interactions to facilitate immortalization of primary cells.

3.6. Others

In order to identify regulating pathways related with CR culture, Ligaba et al. indicated that in human keratinocytes Y-27632 alone decreased phosphorylation of SMAD, while J2 feeder increased the protein levels of EGFR, ERBB2, pSRC, pGSK3β, pERK, EIF4G and p4EFBP. AKT signals are important for cell proliferation and survival. However, in a study, CR was shown to activate mTOR signaling followed by decreased AKT activity. Actually, previous study also showed that the P38K/AKT signaling could induce keratinocyte differentiation.

4. CR applications

The emergence of CR has aroused attentions of many researches. CR is able to culture primary cells from almost all origins. With a few years’ development, many CR cells have been successfully established, which shows wide applications in many areas, including disease modeling, living cell bank, precision medicine, regenerative medicine, drug discovery and assessment.

4.1. Generation of stable cell lines

The eligibility of CR to immortalize a wide range of epithelial cells of varied origins has been proved by many researches. Primary cells from both normal and pathological tissues can be proliferated under CR conditions (Table 2). Of particular note, not only human tissues but also tissues from other origins such as mouse, horse, dog and fish have been used for CR culture. Compared with conventional methods, CR greatly increases the success rate of continuously culturing primary cells. For instance, Sugaya et al. established fifteen cell lines from patients with lung cancer with a success rate of 2.6%; while using CR, Liu et al. successfully amplified primary cells from almost 90% of patients-derived tissues.

CR cells are generated as transiently amplified cell lines that maintain primary genetic background as well as developmental potential, which has potentiated the wide applications of CR for establishment of disease model, regenerative medicine, individualized medicine, drug discovery and assessment as well as basic researches.

4.1.1. Tumor and other pathological origins

Primary tumor cells are always difficult to propagate both in vitro and in vivo, while CR can effectively solve this problem. CR methodology has been widely applied in the area of cancer research. Liu et al. reported a standard procedure for CR culture of paired human normal and cancerous tissues in 2017. The great advantage of CR tumor cells is that these cells can be passaged for over 200 doubling times and maximally maintain intratumor heterogeneity of primary tumors. With the use of CR method, many donor-matched normal/tumor cell lines have been established from different origins including breast, lung, colon, bladder, liver, pancreas, salivary gland and prostate samples. Timofeeva et al. have established matched normal and tumor cultures from prostatectomy specimens of patients, and the CR cells proliferated indefinitely in vitro and retained stable karyotypes (Fig. 4). Although normal and tumor CR cells were quite similar in morphology, their gene expression and exome sequences were different, which made it possible to study prostate cancer in vitro. It has been proven that the use of J2 feeders and Y-27632 could obtain a large number of breast cancer cells from primary tissues within a short time and kept the original features of the tumor. Mahajan et al. characterized the molecular and cellular phenotypes of CR breast cancer cells and found that the CR cells represented the heterogeneity of primary breast tumor cells, making it a unique representative breast cancer model for further study. At present, there are many studies on female breast cancer, while only a few focused on molecular mechanisms for the development of male breast cancer. Recently, Vaclova et al. successfully used CR to culture fresh male breast cancer tissue—derived cells for in vitro cell model establishment. These CR cells were expanded in vitro without significant changes in genetic characteristics, and showed value in biological research of male breast cancer. Furthermore, with the advent of CR technology, some researchers have tried to culture primary lung cancer cells, and have successfully expanded them in vitro, which can be used for the establishment of in vitro models and the discovery of new treatment of lung cancer. Alamri et al. showed that CR was the most effective means to obtain immortalized primary tumor cells from a genetically engineered mouse model of triple negative mammary cancer. In addition, Panaccione et al. obtained CR cells from both patient-derived xenografts (PDXs) and fresh tumor tissues from patients with salivary adenoid cystic carcinoma (ACC), which can be used to develop new drug screening platform. The study suggests that PDX tumor can also be used for generation of CR cells.
Primary cells from other pathological tissues can also be propagated using CR technology. For instance, bronchial epithelial cells derived from normal and cystic fibrosis cultured by CR technology kept exponential growth \textit{in vitro} while maintaining the ability to fully differentiate as well as the function of cystic fibrosis transmembrane conductance regulators (CFTR), which served as a breakthrough for studying the role of CFTR channels in cystic fibrosis \textit{in vitro} \textsuperscript{122,123}. Moreover, Martinovich et al.\textsuperscript{114} demonstrated that CR rapidly amplified airway epithelial cells from human normal, asthma, and cystic fibrosis airway epithelial tissues, not only increasing the lifespan of cells but also retaining disease-specific characteristics, which are valuable for understanding the pathology of cystic fibrosis and achieving individualized treatment. Very recently, Su et al.\textsuperscript{130} demonstrated that CR enabled the culture of human primary hepatocytes for over 3 months, which retained strong cytochrome P450 enzymes (CYP) 3A4, 1A1 and 2C9 activities.

\subsection*{4.1.2. Normal origins}

CR is also applied for expansion of normal epithelial cells of different origins, which may facilitate biological researches and tissue engineering. Wang et al.\textsuperscript{127} established human limbal epithelial cells (LECs) from normal limbal tissues in CR culture. With CR, heterogeneous cultures from normal breast tissue were produced\textsuperscript{105}. Notably, expression of estrogen alpha receptor was detectable in these cultures, and its activity can be activated by estrogen\textsuperscript{105}. In another study, a large number of nasal airway epithelial cells were generated under CR condition, showing about 379 times more cells than that cultured by the conventional culture method\textsuperscript{98}. The rapid aging of primary endometrial glandular epithelial cells \textit{in vitro} greatly hinders the in-depth analysis of human endometrium. Yu et al.\textsuperscript{152} concluded that CR technology was a highly selective and powerful tool to generate non-malignant nasopharyngeal epithelial cell cultures. In addition, human lung epithelial cells were multiplied more than 200 populations under CR conditions and did not participate in the telomere maintenance mechanism\textsuperscript{153}. Most of the studies have indicated that CR cells maintain the characteristics of adult stem cells without genetic manipulation.

CR has been demonstrated to maintain long-term culture of primary epithelia from mouse, dog, fish, etc., which further expands its application. Walters et al.\textsuperscript{140} used CR to develop the first mouse cochlear-derived progenitor cell line, which could escape aging and keep genetic characteristics intact. Importantly, this cell line can be generated from any laboratory using the type of mouse of interest. In another study, CR enabled continuous expansion of C57BL mouse primary oral mucosal epithelial cells \textit{in vitro} and maintains stem cell characteristics, which may be used as a source of seed cells for tissue engineered teeth\textsuperscript{145}. In addition, several researchers have applied CR for establishment of stable cell lines from fish\textsuperscript{143}, pig\textsuperscript{127}, horse\textsuperscript{129,139}, rabbit\textsuperscript{127} and dog\textsuperscript{144}. For instance, Gardell et al.\textsuperscript{143} reported that immortalized cell lines from brain and lip epithelia of Mozambique tilapia (\textit{Oreochromis mossambicus}) were established using CR method.
| Tissue origin | Case Finding | Application | Ref. |
|---------------|--------------|-------------|-----|
| Prostate      | CR normal and tumor cells are successfully established and characterized, maintaining low levels of differentiation in vitro. | *In vitro and in vivo* prostate cancer model | 100 |
| Prostate      | Strigolactone analogues selectively kill CR tumor cells via inducing cellular stress and apoptosis. | Preclinical drug evaluation | 101 |
| Myc-driven mouse prostate tumor tissue (Hi-Myc transgenic C57BL/6 mouse model) | CR prostate cancer cells from transgenic mice with Myc-driven prostate tumor are successfully cultured with tumorigenic ability. | Establishment of a Myc-driven prostate cancer model | 102 |
| Breast (male) | CR male breast cancer cells are successfully established and characterized. | *In vitro* model of male breast cancer | 103 |
| Breast (female) | CR breast cancer cells are successfully established and characterized. | *In vitro* breast cancer model | 104 |
| Breast (female) | CR breast cancer cells at early passages maintain main genetic characteristics of primary tumors. | *In vitro* breast cancer model | 83 |
| Human normal mammary tissue (prophylactic surgery) | CR enables heterogeneous culture of primary mammary cells. | Establishment of mammary cell line | 105 |
| Human DCIS tumor tissue (lumpectomies and mastectomies) | CR DCIS cells are cultured for 2 months expressing both luminal and basal marker and maintaining tumor heterogeneity. | *In vitro* DCIS model | 106 |
| Human tumor tissue (needle biopsy) | CR luminal-B breast cancer cells are established in 3 of 5 tissues, demonstrating similar gene expression profile to primary tumors. The CR cells enable the evaluation of drug sensitivity of tamoxifen, adriamycin and docetaxel. | *In vitro* model of luminal-B breast cancer; drug sensitivity test | 107 |
| Mouse tumor tissue (genetically engineered mouse models of triple negative mammary cancer) | CR cells retain tumor heterogeneity and epithelial cell differentiation, which is better than other methods. | A model for triple negative mammary cancer | 108 |
| Lung | NSCLC tumor cells are cultured in only 1 case. Normal epithelia outgrow cancer cells in CR condition. | Establishment of NSCLC cell lines | 97 |
| Human pleural effusion | CR cells from EGFR-mutant lung cancers maintaining tumor heterogeneity help the understanding of rociletinib resistance. | *In vitro* model of EGFR-mutant lung cancer | 109 |
| Respiratory tract | Human normal and tumor tissue (freshly resected) | 1 | CR cells from respiratory papilloma help identify vorinostat as a therapeutic agent. | Individualized treatment 110 |
| Human tumor tissue (freshly resected) | 14 | CR NSCLC cells are established and characterized and are applied to drug sensitivity test. | Drug sensitivity test 111 |
| Human tumor tissue (freshly resected) | 10 | CR NSCLC cells maintain intratumor heterogeneity of original tumor by >90%. | In vitro NSCLC model 112 |
| Pig lung tissue (newborn CFTR+/+ and CFTR−/− piglet) | Not mentioned | CR alveolar epithelia expanded in vitro allow analysis of bioelectric properties and liquid transport. | Establishment of an in vitro pulmonary edema model 113 |
| Human airway tissue (excess lung donor tissue) | 1 | With a phenotype of adult stem cell-like cells, CR tracheal epithelium forms the upper layer of the ciliary airway in a gas-liquid interface culture system. | Establishment of a tracheal epithelium cell line 12 |
| Human airway tissue (brushing) | Healthy | 18 | CR enables rapid cell expansion, maintaining airway epithelial cell characteristics and disease-specific functions. | Establishment of a disease model 114 |
| Human bronchial tissue (explanted lung) | Asthma | 11 | CR bronchial epithelium has the ability to differentiate into the upper and lower respiratory tract in both air-liquid interface and reconstructed mouse lung. | Tissue engineering 115 |
| Cystic fibrosis | 8 | | | |
| Normal | Not mentioned | CR airway epithelium maintains phenotype of the source cells after several passages and the immune response of the airways. | Establishment of a model for early-life respiratory disorders 116 |
| Cystic fibrosis | Newborns/infants/toddler (0–2 years) | 9 | | |
| School age children (4–11 years) | 6 | | | |
| Adolescent/adult donors | 8 | | | |
| Normal nasal airway tissue (nasal brushing) | 2 | Targeted genetic editing of CR primary airway epithelial cells by CRISPR-Cas9 reveals pro-inflammatory role for MUC18. | Biological function study 117 |
| Human normal bronchial tissue (bronchial biopsy) | 19 | CR bronchial epithelial cells show multipotent differentiation property. | Tissue engineering 69 |
| Human endobronchial tissue (brushing and biopsy) | 132 | Human airway epithelial cells from both endobronchial brushings and biopsies can be cultured by CR, showing better efficiency than other methods. Cryopreserved biopsies can also be expanded. | Establishment of cell lines for cell therapy or tissue engineering 118 |
| Human normal airway epithelium (airway endoscopy or lung resections) | Not mentioned | CR primary airway epithelial cells combining with lung fibroblasts culture in 3D collagen scaffolds | Tissue engineering 119 |

(continued on next page)
| Tissue origin                                                                 | Case | Finding                                                                                                                                                                                                                                                                                                                                 | Application                                                                                     | Ref.  |
|-----------------------------------------------------------------------------|------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|-------|
| Human normal nasal cells (brush or curettage)                               | Not mentioned | transplant into a decellularized rabbit trachea.                                                                                                                                                                                                                                                                                    | Establishment of a cystic fibrosis-specific disease model                                       | 120   |
| Human normal bronchial tissue (fiberoptic bronchoscopy)                     | 3    | CR bronchi cells rapidly proliferate, express comparable levels of CYPs and are sensitive to BaP induction.                                                                                                                                                                                                                       | Establishment of in vitro toxicity testing model                                               | 121   |
| Cystic fibrosis and non-cystic fibrosis tissue (explanted lung)             | 6    | CR condition is modified for long-term primary culture of bronchial basal cells which maintains multipotent differentiation activity and CFTR channel function.                                                                                                                                 | Establishment of primary bronchial cells for basic research and drug screen                   | 122   |
| Cystic fibrosis and non-cystic fibrosis tissue (freshly or cryopreserved explanted lung) | 8    | CR enables primary bronchial epithelial cells growing with larger number of cells than conventional culture. CR cells are expanded for testing CFTR modulators in Ussing Chamber.                                                                                                                                 | Establishment of primary cystic fibrosis cells for drug assessment                            | 123   |
| Pig normal tracheobronchial airway tissue (newborn piglets)                | 1    | CR porcine airway epithelial cells are successfully cultured and used for setting up a differentiated culture model at the gas-liquid interface.                                                                                                                                 | A model for physiologic and pathophysiologic study                                             | 124   |
| Esophagus                                                                   |      |                                                                                                                                                                                                                                                                                                                                                                                                | Establishment of patient-specific cells for tissue engineering                               | 125   |
| Esophageal tissue from patients with eosinophilic esophagitis (biopsy)     | 8    | CR pediatric human esophageal epithelial cells are successfully cultured and maintain differentiation property.                                                                                                                                                                                                                   | Establishment of patient-specific model                                                      | 126   |
| Esophageal tissue from children with eosinophilic esophagitis (biopsy)     | 28   | Patient-derived esophageal epithelial cell lines are successfully established which show disease-specific function.                                                                                                                                                                                                               |                                                                                               |       |
| Cornea                                                                      |      |                                                                                                                                                                                                                                                                                                                                                                                                | In vitro model for corneal toxicity assessment                                                | 127   |
| Normal limbal tissue                                                        | Human | CR maintains stable proliferation of normal limbal cells, with stable karyotype and the ability to form structured spheres in 3D culture. CR limbal cells differently response to several drugs.                                                                                                                                              | Establishment of in vitro and in vivo models for drug screen and drug target identification    | 128   |
| Rabbit                                                                      | 3    |                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                               |       |
| Pig                                                                          | 2    |                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                               |       |
| Pancreas                                                                    |      |                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                               |       |
| Tumor tissue (freshly resected)                                            | 3    | CR pancreatic cancer cells carry mutations identical with primary tumor, which enables therapeutic drug screen and identification of ERCC3-MYC interactions as a advantage.                                                                                                                                                      | Establishment of in vitro and in vivo models for drug screen and drug target identification    | 128   |
| PDX tumor tissue (first passage)                                           | 3    |                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                               |       |
| Tissue Type | Source | CR Conditions | Cultivation | Establishment |
|-------------|--------|---------------|-------------|---------------|
| Pig normal pancreatic tissue | newborn pig pancreata | Pancreatic epithelial cells are expanded under CR conditions and have the characteristics of a ductal epithelium, which can differentiate into functional cells at the gas-liquid interface. | A model for studying pancreas physiology and mechanisms of bicarbonate secretion | 129 |
| Liver | Human liver tissue (freshly resected from patients with cirrhosis, hepatitis C, maple syrup urine disease, or citrullinemia type 1 disease) | Primary hepatocytes are grown from 6 out of 11 specimens under CR condition, which are genetically identical with original tissues and retain strong CYP3A4, 1A1 and 2C9 activities. | Long term culture of patient-derived primary hepatocytes | 130 |
| Human tumor tissue (freshly resected) | | Primary hepatocellular carcinoma cells continuously expand under CR condition and express tumor-specific marker. | Establishment of an *in vitro* model for precision medicine | 131 |
| Gastrointestinal tract | Human tumor tissue (freshly resected) | | Establishment of an *in vitro* model for drug assessment | 132 |
| Mouse small intestine tissue and tumor (wide type, CFTR ΔF508 and ApoE<sup>Min</sup> C57BL/6 mice) | | CR intestinal epithelial cells can be expanded *in vitro* for up to 3 months, maintaining the specific function of the intestinal epithelium after 3D culture. | Establishment of a model for study of intestinal disorder | 133 |
| Mesenteric gland tissue (SD rat) | | CR meibomian gland cells is expanded *in vitro* maintaining functional sodium, chloride, and potassium channels, and cotransporters activities. | Establishment of a primary meibomian gland cell model for studying ion channels | 134 |
| Uterus and vagina | Human normal cervical tissue (hysterectomy) | CR primary cervical epithelial cells are used to evaluate effect of a drug candidate IDF-11774. | Establishment of primary cervical epithelium cell line | 12 |
| Human tumor tissue (freshly resected liver metastasis of cervical cancer) | | A stable CR cell line of neuroendocrine cervical cancer is established using CR, which identifies MYC overexpression as the primary driver of cervical cancer. | Establishment of a cell line for studying disease pathobiology | 135 |
| Human normal tissue (vaginal repair surgery) | | CR primary vaginal epithelial cells are used for evaluating immunomodulatory effect of *Houttuynia cordata*. | Drug evaluation | 136 |
| Bladder | Human tumor tissue (radical cystectomy or transurethral resection) | CR bladder cancer cell lines are successfully established which are used for drug sensitivity test. | Drug sensitivity test | 137 |
| Skin | Human skin biopsy | CR keratinocytes are genetically edited by CRISPR/Cas9, showing an important role of NLRP1 inflammasome upon UV sensing. | A model for biological study | 138 |

(continued on next page)
| Tissue origin | Case | Finding | Application | Ref. |
|---------------|------|---------|-------------|------|
| Horse scrotal and neck skin biopsy | 2 | Equine keratinocytes acquire adult stem cell characteristics under CR conditions. | Tissue engineering | 139 |
| Cochlea | Mouse solid otic spheres (freshly resected from mouse strains of prestin-CreER, CAG-Cre, Ai14-tdTomato, and prestin-YFP) | Not mentioned | CR hair cells are successfully established, which are capable of expressing mature hair cell genes and responding to hair cell cues. | A model for biological study | 140 |
| Oral cavity | PDX tumor; Human tumor tissue (freshly resected) | 6 | CR cells from ACC show a cancer stem cell population driven by NOTCH1 and SOX10, and identify MYB fusion and CD molecules as markers for authentication and purification. | Establishment of ACC cancer stem cell line | 141 |
| | Human normal and tumor tissue (freshly resected or needle biopsy) | 9 | CR cells from mucoepidermoid and other salivary gland neoplasms enable 2D, 3D and xenograft formation, and help identify the allosteric AKT inhibitor MK2206 as potential therapeutic agent. | Model of salivary gland neoplasm; Drug sensitivity test | 142 |
| Fish lip tissue (adult Mozambique tilapia) | Not mentioned | CR can rapidly and selectively culture lip epithelial cells. | A model for mechanism study | 143 |
| Dog tumor tissue (canine ameloblastoma of dog) | 4 | CR primary cells carry HRAS mutation. | A model for studying RAS-driven cancer | 144 |
| Mouse oral mucosa (freshly resected from C57BL) | 1 | CR oral mucosa epithelial cells are successfully established for long-term expansion. | Establishment of cells for potential tissue engineering | 145 |

Note: ACC, adenoid cystic carcinoma; CFTR, cystic fibrosis transmembrane conductance regulator; CYP, cytochrome P450 enzyme; DCIS, ductal carcinoma in situ; NSCLC, non-small cell lung cancer; PDX, patient-derived xenograft.
4.2. Establishment of disease model

CR cells derived from pathological tissues have been applied for establishment of several disease models, including cancer, cystic fibrosis, respiratory disease and others. The corresponding CR cells can be grown in either 2D or 3D cultures. Notably, since CR cells maintain primary genetic characteristics and differentiation potential, transplantation of human-origin CR cells into animals undergoes differentiation and achieves PDX models. These in vitro and in vivo models are valuable for studying both basic and translational researches.

4.2.1. Cancer models

Most studies on CR have focused on its application on various cancers. Many cancer studies are mostly based on the use of stable cell lines, which could not represent both intertumor and intratumor heterogeneity of tumor cells within tumor microenvironment. Therefore, these cell lines cannot model the in vivo characters of most tumors which often render the translation of experimental findings into clinic unsuccessful. Given the advantages of CR method, CR may serve as a better way for cancer studies. As described previously, many stable CR cells are generated from human or mouse tumors. CR cancer cells can be directly supplied in 2D culture and also can be grown as spheroids or organoids. Importantly, the continuously expanded primary cells under CR conditions can be further implanted into an animal to obtain cancer PDX models. Brown et al. cultured patient-derived primary cells from 19 ductal carcinoma in situ (DCIS) tumors using CR method. The derived CR cells maintained both luminal and basal cellular features with high tumor heterogeneity, which is thus helpful for the study of the progression of DCIS. A similar study showed that CR culture of primary non-small cell lung cancer (NSCLC) tumors demonstrated most of the tumor heterogeneity, which can be used as a preclinical lung cancer model. PDX model has been used as an important tool for preclinical and translational study, however, it is difficult for long-term maintenance. In a recent study, CR cells demonstrating genetics of parent tumors were generated from human lung and ovarian PDX tumors, which could be further implanted into NOD/SCID/gamma (NSG) mice to establish in vivo models. ACC is a rare salivary gland cancer which is highly metastatic; however, currently there is no reliable model for ACC study. Chen et al. successfully generated CR cells from ACC PDX tumors, which could be transplanted into zebrafish to establish a CR zebrafish model. This new model can effectively mirror the mouse ACC PDX model. Furthermore, tumor models with specific genetic background can be established with the aid of CR. Ellis et al. built a High-Myc transgenic mouse model on
a C57BL/6 background. CR cells that were generated from the C57BL/6 Myc driven prostate adenocarcinoma expressed markers of luminal epithelial lineage and provided a model for investigating Myc-driven prostate cancer.

4.2.2. Non-cancer models

Brewington and his colleagues isolated human nasal epithelial cells (HNE) from human subjects, expanded under CR conditions and subjected into 3D spheroid cultures to produce HNE spheroids, which could be used to measure activity of CFTR, an important indicator of pulmonary edema. Li et al. also focused on pulmonary edema, and they expanded primary alveolar epithelium from pig using CR, which served as an in vitro pulmonary edema model. Using the CR alveolar epithelia, their ion transport and bioelectric properties were monitored.

Airway epithelial cells play an essential role in maintaining airway immune responses, while these epithelial cells in newborns/infants are difficult to obtain. Wolf et al. recently proposed to use CR technology to culture cells from the nasal airway epithelial tissues of infants and young children in vitro. The established CR airway epithelial cells demonstrated immune response as well as induction of inflammatory response, which are suitable for studies of immunobiology and pathogenesis of respiratory disorders in early life.

In addition, a recent study reported that primary mouse intestinal epithelium from both wide type and ApcMin/+ mouse could be cultured under CR condition which retained large numbers of genotype-specific CR cells. CR cells can be used for investigating molecular basis of intestinal disease. This study offers us a new evidence for the application of CR in disease modeling.

4.3. Regenerative medicine

The most common way for tissue engineering and regenerative medicine involves the use of embryonic and somatic stem cells, however, directed differentiation of stem cells with high efficiency is always difficult. Now the transplantation of in vitro produced organs/tissues based on CR emerges as a new promising strategy. The feasibility of using CR cultured cells in regenerative medicine has been explored by many studies.

For instance, some groups demonstrated that CR facilitated in vitro culture of human primary esophageal epithelial cells. Jensen et al. pointed out that the established CR esophageal epithelium showing unchanged gene expression profile and phenotype could be implanted into the esophagus for repair or replacement of affected region for the treatment of esophageal diseases such as eosinophilic esophagitis. Moreover, airway epithelium is a key barrier that blocks external pollutants and toxic substances, and many researchers have attempted to implant foreign cells for repair and regeneration of damaged airway epithelium. In a recent study, the CR stem cell-like airway cells were combined with primary human lung fibroblasts for establishment of a 3D tracheosphere culture based on a collagen I-based scaffold. This scaffold enabled vascularization and supported CR basal cells to proliferate and differentiate properly, which was biocompatible when engrafted into decellularized trachea of a rabbit model. The study suggests a strategy to improve host airway repair. Similarly, LaRanger et al. applied CR to expand primary human bronchial epithelial cells and found that these CR cells were differentiated into upper airway bronchial epithelium and lower airway alveolar structures after implantation into the decellularized mouse lungs after 12 days. Moreover, many studies have indicated that CR can be applied for cell preparation in tracheal reconstruction and airway transplantation engineering. Notably, the fresh bronchial biopsy samples can be frozen for transport, which facilitates to minimize the inconvenience by the distance from a hospital for sampling bronchial biopsy and the laboratory capable of cell culture.

Therefore, growing numbers of studies have realized that based on CR method rapid expansion of patient-derived cells maintaining a stable genotype may help address an unmet need in tissue engineering for both disease modeling and regenerative medicine.

4.4. Individualized treatment (precision medicine)

Recent studies have demonstrated that CR technology is quite a useful tool for individualized treatment, especially in clinical cancer therapy. During the course of cancer treatment, tumor cells may initially response to drugs, and usually become resistant after a period of time due to alteration of cellular molecules or genes. It is required that an analysis of genetic or molecular profile of pathological tissues is adopted before the intervention of new therapies. Nevertheless, some rare cases showing unresolved genetic mutations may be difficult for drug option. Ideally, before the introduction of certain therapies, it is beneficial to perform an in vitro drug sensitivity study to see whether they show an effect on primary cells from a lesion.

Yuan et al. reported a case which involves a patient with recurrent respiratory papillomatosis with chemoresistant and progressive disease. The author obtained both tumor and normal tissues from the patient and applied CR method to generate paired cell lines for identification of potential new therapeutic strategy. As a result, vorinostat showed a significant cytotoxic effect on CR tumor cells compared to the normal cells. Importantly, the patient received a 3-month course of treatment with vorinostat and demonstrated a stable disease. Therefore, it is indicated that CR greatly facilitates rapid expansion of primary cells without changing their genetic profile which makes it a valuable model for drug sensitivity test for clinical physicians to make decision on targeted therapy.

CR culture of several other primary cancers including lung cancer, salivary gland cancer, bladder cancer and breast cancer has also been used for testing drug sensitivity. Li et al. showed that a total of 14 CR lung cancer cells were successfully generated and characterized, with 6 cases tested with nedaplatin, cisplatin, carboplatin and vinorelbine demonstrating consistency with clinical scenario. Similarly, some reports demonstrated that CR cells from primary tumors enabled rapid screening of candidate drugs and promoted individualized treatment. In a recent study, Mimoto et al. focused on luminal-B breast cancer which has no effective targeted therapy and receives poor prognosis. The authors cultured CR cells from the primary tumors and established a nude mouse xenograft model. The in vitro and in vivo models were applied to efficient drug sensitivity evaluation.

In addition, CR helps to identify new therapeutic strategies. By using patient-derived CR prostate cancer cells, it was found that LA-12 enhanced cell death induced by TRAIL, a member of tumor necrosis factor family, and combinational treatment of TRAIL with cisplatin/LA-12 killed prostate cancer cells more effectively, which offers implications for new drug combinations for prostate cancer. Furthermore, Crystal et al. showed...
that patient-derived CR models of tyrosine kinase inhibitors-acquired resistance of NSCLC enabled the screen of novel active drug combinations. For example, combined suppression of EGFR and FGFR was effective in an EGFR mutation-driven resistant cancer displaying a new mutant FGFR136. In addition, Pollock et al.101 evaluated the anticancer effect found that the

tively resulted in

effect136. In addition, Pollock et al.101 evaluated the anticancer effect

emerges as a therapeutic target in PDAC128. In another study, a

drug toxicity. A recent study by Zhang et al.121 showed that CR

maintained high expression level of CYPs. Therefore, the study pro-

vides a valuable in vitro model for toxicity evaluation and drug

metabolism. Moreover, treatment of CR vaginal epithelial cells by a

 innate immunity

strigolactones showed high expression of

adenoid cystic carcinoma showed high expression of NOTCH1 and

SOX10 and was highly tumorigenic in mice170. Further study

found that the γ-secretase inhibitor DAPT could selectively inhibit

growth of CD133-positive cells via inhibition of NOTCH1 both in vitro and in vivo70. This suggested that Notch inhibition may be

applied for targeted therapy of adenoid cystic carcinoma.

Since CR promotes both primary normal and pathological cells,

the CR cells as a model can be applied for assessment of either drug
efficacy or toxicity. Wang et al.127 established CR culture of normal
limbal epithelial cells from origins of human, rabbit and pig, and

evaluated the suitability of using CR cells for drug toxicity assess-

ment. It is proved that CR primary cells are a reliable model for testing
drug toxicity. A recent study by Zhang et al.121 showed that CR
human normal bronchial epithelial cells with a long-term prolifera-

on the primary culture of NSCLC specimens171,172. In the study of

nasopharyngeal carcinoma biopsy. Similar findings were observed

in the role of the NLRP1 Inflammasome in UVB sensing in human

primary keratinocytes117,138. The genome editing may promote more

researches and potential medical applications in using CR

technology.

5. Challenges

Despite the great advantages and wide applications of CR in many
areas, CR still is faced with several hurdles. It is suggested that
cautions are remained when performing CR culture of primary
cells. CR culture systems should be optimized for expansion of
different primary cells.

Firstly, CR may be not able to maintain culture of certain
primary cells. In 2017, Yu et al.152 indicated that CR preferentially
promoted the growth of non-malignant epithelial cells from
nasopharyngeal carcinoma biopsy. Similar findings were observed
on the primary culture of NSCLC specimens171,172. In the study of
Hyndes et al.25, CR condition only enabled tumor cell culture from
1 out of 10 primary NSCLC tumors. They suggested that expanded CR tumor cells was seen at passage 2, which was
rapidly outgrown by normal epithelial cells in later passages25.
Notably, this CR tumor cells could form a tumor in NSG mice and
re-culture of cells from the tumor xenograft maintained key
characteristics of primary tumor25. However, many studies have
successfully cultured tumor cells from NSCLC109,111,112,149. The
discrepancy might be due to the different culture systems used,
including the use of different origins of fibroblasts as feeder cells,
varied methods for processing fibroblasts, different tissue acqui-
sition methods and other culture conditions.

Secondly, CR supported culture of both normal and tumor
cells173,174, and it is usually difficult to distinguish tumor cells from
normal populations. Some normal tissues are usually mixed in

tumors. When cells derived from tumor tissues are cultured using
CR, the mixed normal cells may grow together with tumor cells,
and sometimes may outgrow tumor cells as described previously.
In order to solve this problem, it is necessary for the pathologist to
correctly judge and select normal or tumor tissues, as well as to
specifically isolate normal or tumor primary cells111. Timofeeva et al.106 demonstrated that CR prostate tumor cells could be
selected by optimizing culture conditions, as normal cells under-
went differentiation after removal of serum in medium while
tumor cells grew as a mesenchymal morphologic phenotype and
was reversed back to epithelial morphology in CR condition. It is

4.5. Drug discovery

CR method also facilitates novel drug discovery. CR cells not only
help the identification of new drug targets but also provide a useful
platform for drug evaluation. Beglyarova and his colleagues128
used the CR method to culture patient-derived pancreatic ductal
adenocarcinoma (PDAC) cells and established PDX models. It is
demonstrated that a covalent inhibitor of ERCC3, tripotolide, had a
better response in MYC-overexpressed PDX models and effec-
tively resulted in MYC depletion. The expression of MYC and
ERCC3 are interdependent, and high expression of ERCC3 in
PDAC predicted poor diagnosis. ERCC3-MYC interaction emerges as a therapeutic target in PDAC128. In another study, a
CD133 positive population of cancer stem cell from CR culture of
adenoid cystic carcinoma showed high expression of NOTCH1 and
SOX10 and was highly tumorigenic in mice170. Further study
found that the γ-secretase inhibitor DAPT could selectively inhibit
growth of CD133-positive cells via inhibition of NOTCH1 both in vitro and in vivo70. This suggested that Notch inhibition may be
applied for targeted therapy of adenoid cystic carcinoma.

Since CR promotes both primary normal and pathological cells,

The fact that CR maintains the culture of different types of
epithelial cells has potentiated it to possess a wide range of

applications. In addition to the applications of CR discussed
above, CR cells are also important for basic researches, such as
investigation of pathological mechanisms and the related signaling
pathways and diagnosis.

Hang et al.135 generated stable CR cell cultures from several
primary neuroendocrine cervical tumors, and found that over-
expression of MY3 may be the main cause of the invasive cervical
cancer transformation. Moreover, the established CR primary rat
meibomian gland cells expressed multiple ion channel/transporter,
which is useful for study of function of ion channels124. Recently,
primary airway epithelial cells were isolated and expanded using
CR114. After two expansions, they were inoculated to a gas-liquid
interface for evaluation of how carbon dioxide and carbonic
anhydrase affects the pH of the airway surface24. Several re-
searchers applied the CRISPR-Cas9 gene editing technology on
CR cells to investigate molecular mechanisms. For instance,
CRISPR-Cas9 genetic editing of CR cells revealed the pro-

inflammatory effects of MUC18 in airway epithelial cells and the
role of the NLRP1 Inflammasome in UVB sensing in human
primary keratinocytes117,138. The genome editing may promote more
researches and potential medical applications in using CR

technology.
suggested that modification of culture condition can purify certain populations. Liu and his group proposed to combine CR with 3D culture to provide optimal conditions for inducing the differentiation of normal cells and distinguishing between normal and tumor cells. In addition, it is reported that changing the feeder cells from mouse fibroblasts to human fibroblasts does not support long-term \emph{in vitro} proliferation of normal epithelial cells, which may be beneficial for the selective growth of tumor cells. To discriminate between normal and cancer cells, either next-generation DNA sequencing or tumor-specific antibody can be applied. The potential strategies for selective CR culture of tumor cells are displayed in Fig. 5.

Thirdly, CR cells in a stem-like and transiently expanded state do not represent some important features of differentiated cells. Tricoli et al. demonstrated that CR primary human prostate cells did not express basal cell marker P63 and had low expression of androgen receptor (AR), which was different from normal prostate tissue. However, they further proposed to establish a transwell-dish system for multi-dimensional culture of CR primary prostate cells, which obtained phenotype resembling prostate epithelium.

Furthermore, studies have found that a subpopulation of 3T3-J2 cells are malignantly transformed during coculture with malignant tumor cells and are developed into carcinoma-like tumors \emph{in vivo}. The underlying mechanisms are still unknown. Besides, J2 feeder cells secreted xeno-components, which may confuse the experimental results.

In addition, Y-27632 can alter the actin backbone, which may interfere with migration and invasion of tumor cells. Some studies also used mitomycin C to inactivate 3T3-J2 cells to obtain feeders. However, there might be some biological differences between the use of mitotic inactivation and irradiation.

6. Conclusions and perspective

Within only a few years’ development and application, CR has emerged as a powerful tool for long-term primary culture of epithelial cells. Primary cells usually become senescent and stop proliferation after a few passages. Previously, it is required that genetic manipulation is adopted to immortalize primary cells for long-term expansion. In contrast, CR characterized by the combinational use of J2 feeder layer and a ROCK inhibitor Y-27632 serves as an easy strategy for rapid amplification of epithelial cells without genetic transformation, demonstrating outstanding advantages over most of previous methods. CR cells in a transiently-amplified stem-like state maintain high differentiation potential. A number of studies have evaluated the underlying mechanisms of CR. Although there are still many unknown aspects, it is shown that both the feeders and the use of ROCK inhibitor are essential for CR culture, which involves enhanced cell growth via suppressing senescence and promoting cell cycle progression, inhibition of apoptosis and differentiation, maintenance of stem property as well as enhanced cell—ECM interaction. Several signal transduction pathways, including the RHO/ROCK, mTOR, WNT, TGF-β/SMAD and MAPK signalings, have been identified relevant to CR actions. However, till now the precise mechanisms by CR are largely unclear. For instance, what diffusing factors are secreted by feeders, interacting with which pathways, that are essential for CR culture.

CR shows a wide range of applications as it enables the culture of almost all origins of epithelium cells. In the past few years, many CR cells from either human source or other experimental animals such as mouse, rat, dog, rabbit and fish have been established, which provides new platforms for both basic and translational researches in various areas. Cells derived

**Figure 5** Strategies for selective culture of tumor cells under CR condition. Although early histological examination help distinguish between normal and tumor tissues, mixed CR culture of both normal and tumor cells is often seen in early passages. In some cases, normal primary cell outgrows tumor cells. To selectively maintain primary tumor cell culture, it is suggested to use human fibroblast instead of mouse fibroblast as feeder cells, which preferentially supports tumor cell proliferation. Moreover, short-term serum-free medium induces differentiation of CR normal cells, which is irreversible by re-using complete medium, but CR tumor cells can be recovered. 3D culture or xenografts also can mediate irreversible differentiation of normal cells. In addition, CR tumor cells can be selected and purified by tumor-specific markers or antibody.
from matched normal and pathological tissues (especially the tumors) are important for establishment of disease models for the study of physiological and pathobiological mechanisms. The recently introduced genome editing technologies such as the CRISPR-Cas9 system can effectively applied in these studies. In particular, these CR cells are also available for identification of new drug targets and preclinical drug evaluations. Moreover, certain CR cells coming from patients facilitate rapid drug assessment for identification of better therapeutic strategy for individualized treatment. CR also provides new opportunity in tissue engineering and regenerative medicine. The existed evidences may advocate more interesting and valuable researches for applications of CR.

Despite the great advantages and multiple applications of CR, there are several defects in CR culture. Many factors including the collection of tissues, preparation of feeder cells, and culture medium and condition may influence the efficiency of CR. It is suggested that CR procedures and conditions are optimized to achieve high-quality primary culture.

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Author contributions

Xu Wu and Zhangang Xiao proposed the conception for the review. Xiaoxiao Wu, Shengpeng Wang, Mingxing Li and Xu Wu wrote the manuscript. Jing Li, Jing Shen, Yueshui Zhao, Jun Pang, Qinglian Wen and Meijuan Chen prepared the tables and figures. Bin Wei, Parham Jabbarzadeh Kaboli, Fukuan Du, Qijie Zhao, Chi Hin Cho, and Yitao Wang gave critical discussions and revisions on manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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