Establishment methods and research progress of livestock and poultry immortalized cell lines: A review

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An infinite cell line is one of the most favored experimental tools and plays an irreplaceable role in cell-based biological research. Primary cells from normal animal tissues undergo a limited number of divisions and subcultures in vitro before they enter senescence and die. On the contrary, an infinite cell line is a population of non-senescent cells that could proliferate indefinitely in vitro under the stimulation of external factors such as physicochemical stimulation, virus infection, or transfer of immortality genes. Cell immortalization is the basis for establishing an infinite cell line, and previous studies have found that methods to obtain immortalized cells mainly included physical and chemical stimulations, heterologous expression of viral oncogenes, increased telomerase activity, and spontaneous formation. However, some immortalized cells do not necessarily proliferate permanently even though they can extend their lifespan compared with primary cells. An infinite cell line not only avoids the complicated process of collecting primary cell, it also provides a convenient and reliable tool for studying scientific problems in biology. At present, how to establish a stable infinite cell line to maximize the proliferation of cells while maintaining the normal function of cells is a hot issue in the biological community. This review briefly introduces the methods of cell immortalization, discusses the related progress of establishing immortalized cell lines in livestock and poultry, and compares the characteristics of several methods, hoping to provide some ideas for generating new immortalized cell lines.

KEYWORDS
livestock and poultry, immortalization, cell line, methods, telomerase activity

Introduction

As the basic structural and functional unit of life activities, cells are widely used as experimental tools in various studies, especially in the fields of molecular biology and biomedical research. Currently, there are two types of animal cells commonly used in laboratories: primary cells and infinite cell lines (1). Primary cells refer to cells that are directly collected from organism tissues and cultured in a simulated in vivo environment (2). Most of them are collected from tissues of experimental animals such as mice...
Methods for obtaining immortalized cells

Currently, the methods for obtaining the immortalization of human and animal cells are mainly divided into four categories (1, 29): (i) destroying the regulation of proto-oncogenes or tumor suppressor genes on the cell cycle through physical and chemical stimulation, which was a technique often utilized in early research (Figure 1), (ii) inducing the heterologous expression of viral oncogenes to help cells escape the cell cycle control (Figure 2), (iii) stimulating the activity of cellular telomerase to overcome the replicative senescence caused by telomere shortening and realize the infinite proliferation of cells in vitro (Figure 3), and (iv) spontaneous formation.
Physicochemical stimulation disrupts the molecular structure of proto-oncogenes and tumor suppressor genes.

**Physical and chemical stimulation**

**Immortalization of cells induced by radioactive factors**

In previous studies, researchers have attempted to induce cells with unlimited proliferation using X-rays or gamma rays. For example, results from an experiment indicated that human skin fibroblasts with a mutant p53 allele could proliferate continually and exceeded 450 PDs in vitro after periodic X-ray irradiation, whereas the unirradiated control group cells could only be cultured to 37 PDs. Relevant phenotypes of immortalized cells obtained with such methods could be transferred by DNA transfection, which has been demonstrated in mouse cells. Previous study has shown that in place of it was suggested that treatment with Harvery murine sarcoma virus (Ha-MSV) alone did not promote the transformation of normal human fibroblasts into immortalized or tumorigenic cells, while immortalized fibroblasts KMST-6 formed by Co60γ-ray irradiation after treatment of Ha-MSV, and transplanted them into nude mice could acquire anchorage independent growth potential and eventually generated tumors. Therefore, radioactive factor-induced immortalized cells may increase the risk of tumorigenesis.

**Immortalization of cells induced by chemical carcinogens**

N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and 3-methylcholanthrene are chemical carcinogens that induce cell immortalization. A previous study has observed that rabbit tracheal epithelial cells proliferated exponentially in the second week of culture and reached plateau in the third week. However, after experiencing the MNNG process, some rabbit tracheal epithelial cells showed a relative delay in the onset of proliferation and recovered clonal activity in a later stage of the plateau phase. Nevertheless, immortalized cells induced by chemical carcinogens do not necessarily retain normal morphology and are adhesion-dependent. Therefore, their carcinogenesis risk cannot be neglected.

**Heterologous expression of viral oncogenes**

It is well-known that the simian virus 40 large T antigen (SV40-LT), human papilloma virus E6 or E7 protein (HPV E6/E7), and Epstein-Barr Virus (EBV) are oncogenes. Among them, the SV40-LT gene fragment is one of the most commonly
used target fragments for inducing cell immortalization. Integrating it into the target cell nucleus for expression can cause inactivation of the p53 and Rb proteins, thereby changing cell proliferation activity and prolonging cell lifespan (36). However, the length of telomeres will gradually shorten until cells stop growing, and only a few cells can completely leave the cell cycle and continue to proliferate, eventually forming immortalized cell lines (37). In recent years, SV40-LT has been successfully used in the establishment of immortalized cell lines of livestock and poultry such as pigs (38), cattle (25), sheep (24), and ducks (39).

In addition, infection with HPV E6/E7 can also immortalize a large number of different types of cells (40, 41). The HPV E6 protein, as one of the most common transforming proteins, can cause degradation of the p53 protein and upregulate the expression level of cellular-myelocytomatosis viral oncogene (c-myc) (42). Furthermore, it can also induce the expression of human telomerase reverse transcriptase (hTERT) and enable cells to acquire the ability of indefinite proliferation (43). There are many binding sites for c-myc transcription factor on the promoter of hTERT, so c-myc can mediate hTERT transcriptional activation and rapidly induce hTERT mRNA to express (44). The HPV E7 protein can lead to degradation of the Rb protein (45). It was reported that retroviruses containing the HPV E6/E7 gene was used to infect human pancreatic duct epithelial cells to establish the corresponding immortalized cell
line, which could be passaged more than 20 times, retaining the anchorage dependence of mammalian cells with non-carcinogenic effects (40). Currently, the EBV is mostly used to immortalize B lymphocytes. The EBV genome contains more than 100 genes, and only a few genes (so-called latent genes) can be expressed in EBV-infected B lymphocytes. For instance, it is capable of infecting B lymphoblastoid cells in vitro, activating the interaction of cytokines with their receptors by expressing latent proteins, and forming immortalized lymphoblastoid cell lines. It is worth noting that the most notable feature of immortalized B cells induced by EBV is increased telomerase activity (46).

Telomerase causing cell immortalization

Telomerase

Telomerase is a kind of a specific reverse transcriptase and includes three components: telomerase RNA (TR), telomerase-associated protein, and telomerase reverse transcriptase (TERT) or telomerase catalytic subunit. Using its own RNA as a template to extend telomeres from the 3′-OH end of telomeric DNA or synthesize new telomeric DNA, it can compensate for the shortening of chromosome ends during cell division, so as to maintain the length of telomeres and prevent cells from the apoptosis caused by telomere depletion (47). Telomerase almost has no activity in normal cells but with expression in stem cells and germ cells. The activity of telomerase is elevated in most immortalized cell lines and various human tumor tissues, suggesting that telomerase activity is closely related to occurrence and development of tumors (48).

Rebuild telomerase activity to immortalize cells

In 1998, it was first reported that after the exogenous hTERT gene was introduced into telomerase-negative normal human retinal pigment epithelial cells, the intracellular telomerase was activated and the endogenous β-galactosidase (senescent...
TABLE 1 Immortalized livestock cell lines established by transfecting hTERT alone.

| Species | Cell line name | Cell line source | Immortality | References |
|---------|----------------|------------------|-------------|------------|
| Swine   | Fibroblast cell line | Primary fibroblasts prepared from pig ears, fetuses, and lung tissues | Cultured for 30–45 passages | (60) |
|         | hTERT-POMECs | Primary porcine oral mucosal epithelial cells (POMECs) from the neonatal piglet. | Cultured for more than 150 passages in vitro | (61) |
|         | iPMSCs | Fetal porcine pancreas mesenchymal stem cells | More than 80 passages | (62) |
|         | EE cell line | Endocardial endothelium cells | Over 100 generations | (63) |
|         | SUVECs | Umbilical vein endothelial cells | Passed 50 times | (64) |
| Cattle  | hTERT-AEC II | Type II alveolar epithelial cells | More than 50 passages | (4) |
|         | hTERT-BME | Microvascular endothelial cells isolated from adrenal cortex | Over 80 passages | (65) |
|         | BMET | Muscle epithelial cells | Cultured for 59 passages | (66) |
| Sheep   | Fibroblast cell line | Lung fibroblasts | Cultured for about 120 days (50–80 PDS) | (67) |
|         | hTERT-STCs | Primary trophoblast cells (STCs) | Cultured for 50 passages | (7) |
|         | Microglia cell line | Brain macrophage | Passage up to 100 times | (26) |
|         | Fibroblasts cell line | Fetal sheep fibroblasts | More than 180 PDs | (68) |

marker) was significantly reduced (49). Besides, a previous study has claimed that after transfection with retrovirus-mediated exogenous hTERT gene, normal human breast epithelial cells gained stable telomere length, longer lifespan (40 PDs more than primary cells), less obvious β-galactosidase staining, and unchanged plasminogen activator inhibitor expression (PAI, another senescent marker) (50).

Furthermore, it has been determined that hTERT could improve telomerase activity, stabilize telomere length in cells, increase the number of cellular PDs, slow down cell senescence, and prolong the lifespan of culture in vitro (51–56). Certain cells can maintain their original morphology and function while obtaining the ability to proliferate indefinitely (57, 58). For example, immortalized human bone marrow mesenchymal stem cell line carrying hTERT has been subjected to 290 PDs without losing cell contact inhibitory function. By observing cell morphology at 95 and 275 PDs, it was found that transsected cells had the ability to transform into adipocytes, chondrocytes, and osteoblasts (59). Currently, hTERT transfection alone can immortalize many livestock and poultry cells (Table 1), or it can be combined with viral oncogenes to improve the success rate of obtaining immortalized cells (69).

### Spontaneously generated immortalized cells

During cell culture in vitro, some spontaneously immortalized cells are occasionally generated and show high proliferative potential without gene transfer (70–73). These cells achieve serum-independent growth and have higher saturation densities (74).

Rodent cells have a higher incidence of spontaneous immortalization, up to $10^{-5}$ or $10^{-6}$ (44). Previous research has discussed that human cells could escape aging only if both the p53 and Rb genes were inactivated simultaneously, and that dysregulation of the ARF-p53 pathway alone in rodent cells was sufficient for eternal proliferation (75). By comparing the expression of multiple genes in early passage bovine mammary epithelial cells (bMECs), senescent bMECs, spontaneously immortalized bMECs (BME65Cs), and human breast cancer MCF-7 cell line (76), it was found that BME65Cs had the general features of normal BMECs in terms of morphology and karyotype etc., accompanied by endogenous TERT activity and telomeres stability. Compared with MCF-7 cells, the oncogene c-myc was only slightly upregulated in BME65Cs, and the breast tumor-related genes Bcl-2-associated athanogene 1 (Bag-1) and transcriptional repressor 1 (TRPS-1) were not detected. Likewise, the expression of tumor suppressor gene p53 and cycle-dependent kinase inhibitory factor p16INK4a (also known as cyclin-dependent kinase inhibitor 2A, CDKN2A) in BME65Cs was decreased but not completely inactivated compared to earlier passages, indicating that spontaneous immortalized cell lines were not caused by mutations in the p53 or p16INK4a gene. In addition, the expression level of DNA methyltransferase was upregulated, suggesting that the co-suppression of cell aging and mitochondrial apoptosis pathways orchestrated the immortalization process of BME65Cs (76). That means the mechanism by which spontaneously immortalized cells escape replicative senescence is poorly understood.
### TABLE 2 Establishment of different cell lines in mammals.

| Species | Cell line | Establishment method | Immortality | References |
|---------|-----------|----------------------|-------------|------------|
| Rat     | RKC2      | SV40-LT was expressed in passaged kupffer cells | –           | (83)       |
| Mouse   | EOE-2M and EOE-3M | Induced the expression of HPV16 E6/E7 oncogene in primary enamel organ epithelial (EOE) dental cells | Maintained more than 30 generations | (84) |
|         | FP5-1-3 cell line | Spontaneous generation from mammary buds in p53-null female embryos | –           | (85)       |
| LmcmF   | Introducted of SV40-LT into primary intestinal myofibroblasts. | At least 20 generations | (86) |
| SmcmF   | Spontaneous immortalized intestinal myofibroblasts | At least 20 generations | (86) |
| AD-MSC  | Knockout of p53 gene in adipose-derived mesenchymal stem cells (MISCs) | Passaged more than 50 times | (87) |
| Osteoblast cell line | Transfection of primary floxed Bmp2/4 mouse osteoblasts with SV40-LT | Grown more than 50 PDs | (27) |
| Epithelial cell line | Embryonic mouse neuroepithelial cells were infected with a retrovirus containing the c-myc oncogene | – | (88) |
| Rabbit  | Fibroblast cell line | Co-expression of mutant CDK4, cyclin D1 and hTERT in fibroblasts | More than 11 generations | (89) |
| Articular cartilage cell line | Transfected with plasmid encoding SV40 early functional gene | Up to 130 generations | (90) |
| ImRMC   | Induced lentivirus-mediated SV40-LT expression in primary melanocytes | – | (91) |
| Epithelial cell line | Infection of primary corneal epithelial cells with recombinant SV40-adenovirus vector | Grown over hundreds of generations | (92) |
| Swine   | Granulosa cell line | Conditionally expressed SV40-LT gene in primary granulosa cells using tetracycline-induced Tet-On 3G system | Stable proliferation for at least 6 months | (93) |
| sINEC and sITEC | Transfer of SV40-LT into nasal and tracheal epithelial cells | Over 30 passages, the doubling time is cut in half | (94) |
| Ttag and Puro | Transfer of lentiviral vector expressing SV40-LT into primary porcine spermatogonial stem cells | More than 35 passages | (95) |
| GalT-KO-hep and WT | GaIT-KO and wild-type pig primary hepatocytes were transfected with SV40-LT lentiviral vector | More than 20 generations | (82) |
| Fibroblast cell line | Sleeping beauty transposon-mediated ectopic expression system of porcine TERT | Over 40 generations | (96) |
| Endothelial cell line | Primary endothelial cells were transfected with plasmid pRNS-1 carrying neomycin resistance gene and SV40-LT | The doubling time was about 17.6 h | (80) |
| Cattle  | Epithelial cell line | Mammary epithelial cells were infected by retrovirus with the SV40-LT plasmid | Up to 80 PDs in 10 months | (18) |
| Epithelial cell line | Transfer of lentiviral vectors encoding cyclin D1, mutant CDK4, and hTERT genes into colon-derived epithelial cells | Over 15 generations | (97) |
| Germ cell line | Constructed pEGFP-c-myc and pEGFP-hTERT expression vectors and transfected 5-month-old calf sperm stem cells | About 100 PDs in 140 days | (69) |
| BMES    | Muscular epithelial cell spontaneously immortalized | Cultured for 62 generations | (66) |
| Sheep   | Endothelial cell line | HPV16 E6/E7 open reading frames were permanently transfected into fifth generation fetal pulmonary artery endothelial cells | At least 28 passages | (98) |
| mMIVS-54/93 and TIGEF | Transfection of plasmid DNA encoding SV40-LT gene into goat fibroblasts | Faster doubling time | (99) |

Indicates that immortality is not mentioned in the citation in place of previous sentence.
TABLE 3 Characteristics of immortalized pig cell lines established by transfecting SV40-LT and hTERT.

| Establishment method | Cell line | Characteristics | Immortality | References |
|----------------------|-----------|-----------------|-------------|------------|
| Transfection of TERT gene | Fibroblast cell line | It had anchorage dependency, and did not form any colonies on soft agar | Cultured for 30–45 passages | (60) |
| | hTERT-POMECs | No chromosome abnormality and tumorigenicity transformation | Cultured for more than 150 passages in vitro | (61) |
| | Fibroblast cell line | The cell line continued to grow after more than 40 passages, and pTERT maintained stable expression | Over 40 generations | (96) |
| | iPSCs | With the ability to differentiate into neurons, cardiomyocytes, germ cells, and inlet-like cells | More than 80 passages | (62) |
| | iEE cell line | It had similar phenotypic and functional characteristics to the primary endocardial endothelium cells | Over 100 generations | (63) |
| | SUVECs | It had contact inhibition, serum demand and anchorage dependent growth | 50 generations | (64) |
| Induced the expression of SV40-LT | Granulosa cell line | Able to reproduce stably for at least 6 months, with reduced cell proliferation following withdrawal from Dox | Stable proliferation for at least 6 months | (93) |
| | siNEC and siTEC | Retained the biological characteristics of primary epithelial cells and no abnormal chromosomes | Over 30 passages, the doubling time is cut in half | (94) |
| | Ttag and Puro | No morphological abnormalities | More than 35 passages within seven months | (95) |
| | Galt-KO-hep | Retained the characteristics of primary porcine hepatocytes. No tumorigenicity | More than 20 generations | (82) |
| | MSCs | Possessed higher proliferative capacity, shown no signs of senescence and displayed a common phenotype similar to primary MSCs | Serially passages more than 20–30 times | (38) |
| | Endothelial cell line | The original features of endothelial cells were preserved | The doubling time was about 17.6 h | (80) |

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Establishment and current status of livestock and poultry immortalized cell lines

The common methods for establishing non-carcinogenic immortalized cell lines

It is well known that cancer cells also have the ability to proliferate indefinitely, and that cells may become cancerous during the process of establishing cell lines. Soft agar assay and nude mouse tumorigenesis assay are widely recognized methods for testing whether immortalized cell lines are tumorigenic (77, 78). Studies have found that immortalized cell lines induced by radioactive substances and chemical carcinogens may increase the formation of cancer cells, which are rarely used today (32, 33). Immortalized cell lines established by inducing the combined expression of immortality genes, proto-oncogenes, and cell cycle regulators are also tumorigenic, such as porcine pancreatic ductal epithelial cells, which are often used to generate tumor models (79, 80).

However, some immortalized cell lines can still avoid the generation of cancer cells while maintaining the morphological and physiological characteristics of primary cells (65, 81). The current common immortalization methods that do not cause any cancer growth are mainly by hTERT or SV40-LT expression induction, such as porcine oral mucosal epithelial cell line (hTERT-POMEC) (61), canine bronchiolar epithelial cell line (hTERT-CBECs) (77), and pig liver cell line (Galt-KO-hep) (82). So far, anchorage-independent growth, chromosomal abnormalities, and tumorigenic transformation have not been observed during the culture of these cell lines.

Small mammalian and livestock cell lines

By comparing the establishment status of common small mammal (rats, mice, and rabbits) and livestock (such as pigs, cattle, and sheep) immortalized cell lines (Table 2), it is not difficult to find that most expression vectors carrying the SV40-LT or hTERT gene are transfected into cells to prolong their lifespan. Notably,
the cell immortalization induced by the tetracycline Tet-on 3G system is reversible, and cell proliferation can be controlled with doxycycline (Dox), which is more flexible (93). As an example, the characteristics of immortalized pig cell lines separately obtained by transfecting SV40-LT and hTERT are compared (Table 3). It is observed that immortalization effects can be evaluated from the aspects of cell lifespan, telomerase activity, passage times, PDs, cell morphology, and tumorigenicity.

### Establishment of poultry cell lines

We summarized poultry cell lines and their characteristics, including chickens, ducks, geese, and quails (Table 4). It

| Species | Cell Line | Characteristics | Immortality | References |
|---------|-----------|-----------------|-------------|------------|
| Chick   | CSC-1-5   | Spontaneous emergence, the fibroblast cell line had a high proliferative state, high homogeneity and the same genetic background, normal cell cycle distribution without tumorigenesis, and transformation | Stable passage over 3 months | (100) |
|         | ICP1 and ICP2 | Acquired by transducting chTERT alone or in combination with chTR. They showed fibroblast-like morphology without signs of malignant transformation, revealed high telomerase activity and retained adipocyte differentiation capacity | Cultured in vitro over 100 passages | (13) |
|         | CEL-im    | Spontaneous generation without oncogenic treatment, 0.8–1.1 PDs per day, and negative for telomerase activity | Cultured over 120 passages | (101) |
|         | DF-1      | Spontaneous emergence, they demonstrated a fibroblast-like morphology during culture, did not contain endogenous sequences associated with ASV or ALSV, and supported replication of avian retroviruses | – | (102) |
|         | LMH       | It obtained from liver tumor tissue after injecting diethylnitrosamine, had triploid karyotype and 6 marker chromosomes. After the 40th passage, the growth rate gradually increased and the cell morphology changed | Cultured 120 passages in 5 years | (103) |
| Duck    | DEE cell line | It had good adhesion ability and proliferative activity, no tumorigenicity, and the doubling time was about 17.6 h | 50 generations | (104) |
|         | DEF-TA    | Expressing SV40-LT (obtained after more than 8 rounds of puromycin selection), PDs number increased every 30 to 48 h, and maintain fibroblast morphology | Passaged more than 30 times | (39) |
| Goose   | Epithelial cell line | Spontaneous formation with a cubic morphology and constant chromosomal characteristics, they could efficiently transfet some plasmids carrying avian virus reporter genes and did not transform into tumorigenic cells | Grown over 65 passages | (105) |
| Quail   | QM 1-4 and QM 6-8 | Seven avian myogenic cell lines derived from the fibrosarcoma cell line QT6 | – | (106) |
|         | Myocardial cell line | It obtained by injection of MC29 virus carrying the v-myc, without morphological changes, showing decreased growth and enhanced differentiation | More than 60 passages in 6 months | (107) |
|         | QT        | Injected with 7,12-dimethylbenzylanthracene, MNNG and 3-methylcholanthrene (carcinogens) and isolated from tumor tissue. The fibrosarcoma cell line had undergone ~10 passages and was characteristic by tumorigenic transformation | Undergone ~10 passages | (108) |
|         | Cartilage cell line | Acquired by infection with MC29, it stimulates chondrocyte proliferation and progressively reduces doubling time | About 70 generations in 16 months | (109) |

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was found that few immortalized cell lines were successfully established in poultry compared with mammals, and that the existing poultry cell lines were mainly obtained from tumor tissues; some chemical carcinogens or oncogenic viruses were used to immortalize specific types of bird cells, and some continuous cell lines were spontaneously generated. There are two points worth noting: (i) the preadipocyte lines "ICP1" and "ICP2" successfully established by transfection with the chicken telomerase reverse transcriptase (chTERT) have a high proliferation potential without malignant transformation after long-term culture, which provides a new idea and theoretical reference for the acquisition of other immortalized poultry cell lines (7), and (ii) during the whole process of establishing immortalized cell lines, specific antibiotics can be used to screen out positive cells expressing the SV40-LT gene or other target genes and then select a single transforming focus for subculture, which is not only simple but also safer (27).

Conclusions

Establishing an ideal immortalized cell line with infinite proliferation ability and maintaining the characteristics of its source tissue cells cannot only avoid the complicated process of primary cell separation and purification, reduce the time and energy consumption of researchers, and save the cost of experiments, it is also conducive to the research on scientific issues such as gene function of livestock and poultry, and rapidly promotes the development of science. Since immortalized cells can be passaged multiple times in vitro, researchers can immortalize cells that are difficult to passage, slow to proliferate, and prone to senescence, and provide more cell resources for related experiments. Nevertheless, whether the functional cells from different species adopt the same immortalization method, and how to quickly and efficiently prepare immortalized cells and to ensure the immortalized cells maintaining the original characteristics have not yet been solved and require more in-depth research. In summary, the application of immortalized cell lines has broad prospects. The continuous improvement of immortalized cell line establishment technology is conducive to further research in molecular biology and other scientific fields.

Author contributions

DG participated in literature collection, drafted the manuscript, and revised it. LZ participated in the design of this review and revised it. XW participated in literature collection. JZ helped draft the manuscript. SL conceived the review, participated in literature collection, revised the manuscript, and finally agreed to publish it. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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