

A Comparative Study on TERT Overexpression and CRISPR Editing Cell immortalization Techniques

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ABSTRACT

The boundary between a cancerous and normal cell is thin. As cancerous cells are caused by mutations from the accumulation of DNA lesions, it is important to look into the process of cellular transformation to find ways to develop controlled cell immortalization. (Yoshioka, 2015) There are many methods of cell immortalization. The methods focused in this paper are TERT(Telomerase reverse transcription) overexpression and CRISPR editing.

TERT overexpression is successful across different cell types, including specific blood cells, macrophages, and human kidney cells. (Huang et al., 2024) Each shows a high efficiency in transfection indicating a successful outcome of T-cell immortalization with minimal genomic alteration. Emerging CRISPR editing approaches, including CRISPR-Cas9 genome editing and CRISPRa-mediated transcriptional activation, present a more controlled method of cell immortalization, avoiding insertional mutagenesis and providing controls and potential reversibility. (He et al., 2025; Zhao et al., 2020) Notably, it is the first time that a normal human T cell line is established. However, challenges remain, as the risk of off-target mutation and double strand break induced instabilities could occur. Both techniques demonstrate promising cell immortalization outcomes with TERT overexpression showing minimal genomic instability. CRISPR, as an editing tool, shows positive direction on the mobility of immortalization methods but its amount of negative effects on genomic stability remains unclear.

This review compares TERT overexpression and CRISPR-based immortalization with emphasis on genomic stability, an aspect typically discussed separately in existing studies. By synthesizing findings, this paper evaluates which approach currently offers the most balance between immortalization and genomic stability.

INTRODUCTION

In 1961, cells were discovered to have a limit in division. This limit was discovered by Hayflick and Moorhead's study in which 25 strains of human fetal diploid cell strains were studied, leading to the proposed Hayflick limit. In the study, despite providing unlimited nutrients to the cells, the cells still stop

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dividing over time, reaching a limit. (Hayflick and Moorhead, 1961) This limit was later understood to be closely associated with progressive telomere shortening. Telomere was discovered much later around the 1970s. Thus, studies regarding telomere extension began as a method for immortalization.

CRISPR-Cas9, discovered in 2012 by Jennifer Doudna and Emmanuelle Charpentier, provided a brand new route into genetic editing. However, its usage is not limited to gene editing but also cell immortalization. Using its ability to target specific genes, it is possible to knock out or insert genes to extend cell life.

STUDIES SELECTION

The studies selected for this review were based on three criteria: the studies are published in peer-reviewed journals, relevance to human primary cell immortalization, and experimental evaluation of immortalization techniques with genomic stability assessments. The studies were also selected with the priority of recent publication between 2020-2025 to ensure up to date information regarding each immortalization technique. For example, the two primary used sources (Huang et al., 2024) and (Zhao et al., 2020) were published in the 2020s. With exceptions of earlier reviews from before the 2000s as backgrounds on telomerase overexpressions, Crispr technologies, and genomic instability during immortalization.

LITERATURE REVIEW

To understand how normal human cells can be immortalized without potential cancerous transformation, two major approaches are compared: Telomerase(TERT) overexpression and CRISPR-based approaches, including CRISPR-Cas9 genome editing and CRISPRa-mediated transcriptional activation. Each of the methods is used to overcome senescence like the Hayflick limit and to continue the process of replication, but chromosomal stability and cell behavior are often affected. (Curi et al., 2023)

Telomerase(TERT) Overexpression Studies

Telomerase is an enzyme that maintains telomere length, the protective DNA ends of chromosomes that shorten after every cell division. In normal cells, this gradual shortening eventually triggers senescence. Previous study by Andrea G. Bodnar et al have established that by overexpressing hTERT, human telomerase reverse transcriptase, telomerase becomes active again and continuously lengthens telomeres, allowing the cells to keep dividing without entering senescence. Using retinal pigment epithelial cells and foreskin fibroblasts, which normally lack telomerase, hTERT were added to test the effect of telomerase introduction. Cells introduced with hTERT do not present SA-B-GAL staining which is normally present near senescence. And those clones were able to exceed the mean life-span of normal cells by 20 doublings while maintaining all 46 chromosomes with no abnormalities, confirmed through G-banding.

Therefore, the purpose of this study is to analyze deeper into the restoration of telomerase activity in cells. This approach involves introducing the human telomerase reverse transcriptase(hTERT) gene into normal primary cells such as fibroblasts, epithelial cells, and peripheral blood mononuclear cells(PBMCs). The cells are transduced using lentiviral or plasmid vectors carrying hTERT under a strong promoter, like CMV(cytomegalovirus), followed by antibiotic selection to ensure stable integration. The purpose of this method is to restore telomerase activity in cells that naturally lack it. In normal cells, this gradual shortening eventually triggers senescence. (Curi et al., 2023) By overexpressing hTERT, telomerase becomes active again and continuously lengthens telomeres, allowing the cells to keep dividing without entering senescence. To confirm successful immortalization, qPCR(Fig. 1)is used to measure hTERT mRNA levels and TRAP(Telomeric Repeat Amplification Protocol) assays measure telomerase activity. (Huang et al., 2024) The results show strong upregulation of hTERT mRNA and increase in telomerase activity in various cell types, such as PBMCs, HEK293FT, and THP-1-derived macrophages(Fig. 1). β -galactosidase staining shows that immortalized cells contain very few senescent(blue-stained) cells compared to normal controls. Karyotyping confirms that the immortalized cells retain normal diploid chromosome structures without signs of cancerous transformation. The results demonstrate that TERT overexpression successfully extended the lifespan of normal human cells while keeping them more morphologically and genetically stable. These cells could divide for over 100 population doublings, which is significantly longer than unmodified primary cells, without acquiring too many abnormal features or losing contact inhibition. (Huang et al., 2024)

Telomere length qPCR	relative telomere length to reference (fold)	average length on each chromosome end
hPrEC, p6	0.52	3.90 $\pm$ 0.09 kb
hPrEC, Clone1, p20	1.29	9.74 $\pm$ 0.22 kb
hPrEC, Clone2, p21	1.99	15.04 $\pm$ 0.35 kb

Figure 1. Telomere length qPCR in primary hPrEC and immortalised clones using both relative and absolute telomere lengths. Reproduced from Zhao et al. (2020), Prostate Cancer and Prostatic Diseases, 24, 233–243, CC BY 4.0.

The relative telomere length in the immortalised clones ranged from 1.29 to 1.99 times longer than the reference human genomic DNA, but the corresponding hPrEC ratio was only 0.52 times longer, according

to qPCR using telomere-targeting primers and a single copy reference using genomic DNA(Fig. 1). This strongly implies that telomeres in immortalised hprEC T-AN2A clones are maintained or elongated by ectopic hTERT expression. (Zhao et al., 2020)

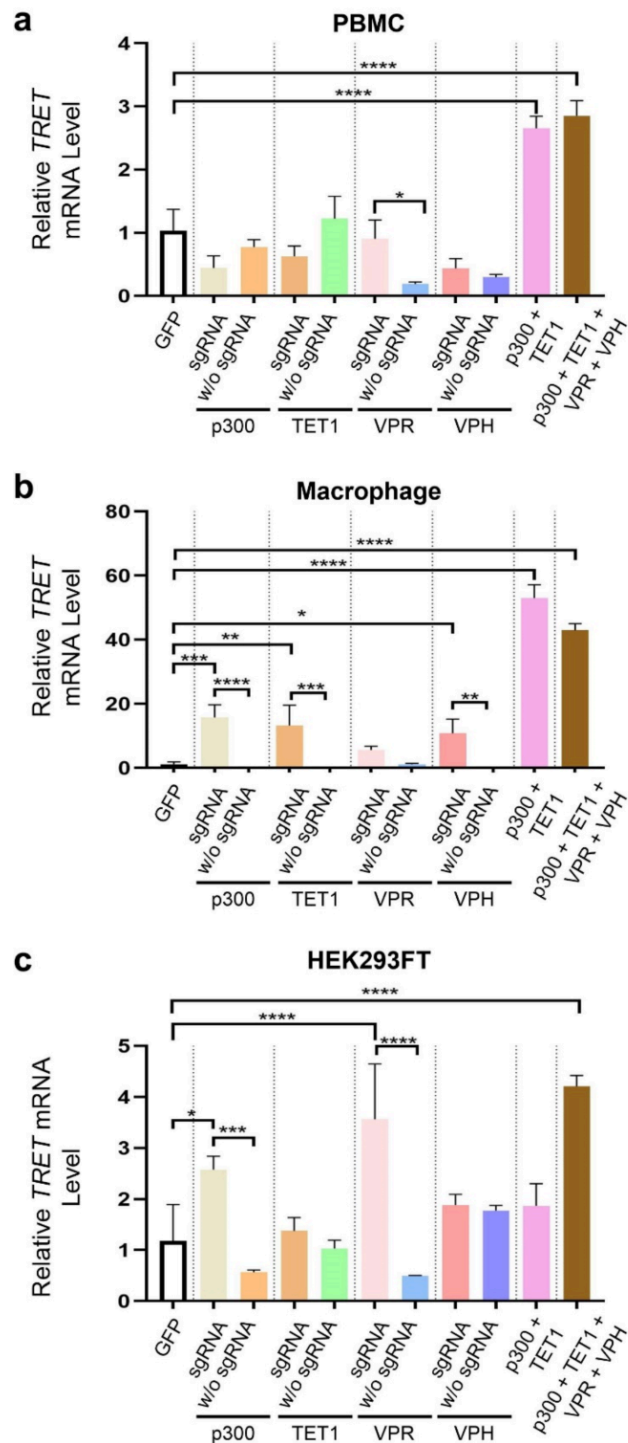


Figure 2. CRISPRa-mediated hTERT activation in different cell types. Reproduced from Huang et al. (2024), Cellular and Molecular Life Sciences, 81, Article 407, licensed under CC BY-NC-ND 4.0.

The successful elongation of telomeres is further confirmed using qPCR, western blot, and flow cytometry. The results (Fig. 2) show that by co-transfecting cells with epigenetic modifiers, the hTERT mRNA expressions upregulated: PBMCs by 2.65x(Fig. 2a), THP-1-derived macrophages by 53x(Fig. 2b), and HEK293FT cells by 1.87x(Fig. 2c). (Huang et al., 2024)

Interestingly, the magnitude of hTERT activation differs substantially across cell types. The response observed in THP-1-derived macrophages is particularly high, suggesting that cellular context may influence the effectiveness of telomerase activation strategies. This variability shows the importance of evaluating immortalization approaches across different cell types before generalizing their effectiveness.

CRISPR-Based Immortalization Studies

CRISPR-based immortalization includes multiple strategies. Huang et al. (2024) used CRISPR activation, CRISPRa, in which catalytically inactive Cas9 was fused to transcription activators and epigenetic modifiers to activate endogenous hTERT expression without introducing double-strand DNA breaks. In contrast, Zhao et al. (2020) and He et al. (2025) employed CRISPR-Cas9 genome editing to disrupt senescence-related genes or insert hTERT into the genome.

Unlike traditional viral methods, CRISPR allows precise modifications to specific genes related to senescence and telomere maintenance. In one design, researchers use CRISPR-Cas9 to knock out CDKN2A (cyclin-dependent kinase inhibitor 2A), which encodes two senescence-related proteins, p16^{INK4A} and p14^{ARF} (Fig. 3). By removing these genes, it prevents cell cycle arrest and enables continued cell division. At the same time, the hTERT gene is introduced or reactivated to maintain telomere length and to prevent chromosome shortening. (Zhao et al., 2020) This dual-editing strategy is applied to normal human prostate and T cells. After editing, PCR and sequencing confirm that the CRISPR knockout of CDKN2A and insertion or activation of hTERT is successful. Then, Western blotting verifies that p16 and p14 proteins are no longer expressed and TRAP assays further confirm that telomerase activity is reactivated. Karyotyping showed that the edited cells remain diploid, suggesting that no abnormal chromosomal rearrangements occurred. However, occasionally chromosome gains and losses were observed in the middle in locations like chromosome 4 p14 and 11 p15 (7 out of 50). (Zhao et al., 2020) These genes are translocated in the early passage but not observed in the later, suggesting that the cell in the end was relatively normal. While continuing to divide far beyond the normal limit of primary cells, the edited cells maintain normal cell morphology and contact inhibition. These findings demonstrate successful immortalization without transformation into cancer-like cells. Importantly, the use of CRISPR avoids random DNA insertions, making this approach more predictable, reversible, and genetically stable than earlier methods. (Zhao et al., 2020)

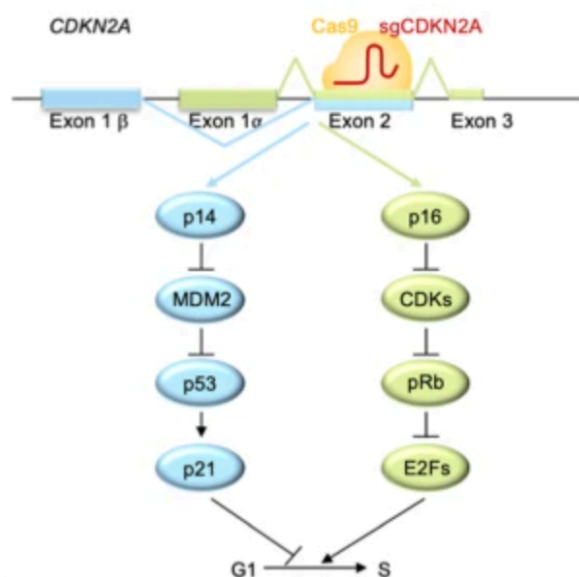


Figure 3. CRISPR-mediated dual editing for controlled cell immortalization. Reproduced from Zhao et al. (2020), Prostate Cancer and Prostatic Diseases, 24, 233–243, CC BY 4.0.

The *CDKN2A* locus(encoding p16/p14) was targeted for knockout using CRISPR-Cas9, while *hTERT* was simultaneously introduced to maintain telomere length.

COMPARATIVE ANALYSIS

Comparing the two immortalization strategies: telomerase(TERT) overexpression and CRISPR-based approaches, including CRISPR-Cas9 genome editing and CRISPRa-mediated transcriptional activation, reveals similar outcomes and distinct consequences on cell stability and long-term growth. The primary result is the successful extension of cellular lifespan beyond unmodified primary cells, while maintaining non-tumorigenic properties. However, the degree of chromosomal stability, telomerase activation, and early-passage variability differ among the techniques.

TERT Overexpression

By engineering cells to overexpress hTERT(or to activate endogenous TERT expression), they are shown to have a robust increase in telomerase activity. This is reflected in upregulated TERT mRNA and strong TRAP assay signals across PBMCs, HEK293FT cells, and THP-1 macrophages. (Huang et al., 2024)

TERT-overexpressing cells are able to maintain stable telomere length over extended passages while retaining diploid karyotypes without detectable structural abnormalities. (Huang et al., 2024) This is because TERT restores telomere length, which protects the chromosomes from end-to-end fusions and

DNA damage signaling. They preserved normal epithelial morphology and contact inhibition, and also showed a marked reduction in senescence-associated β -galactosidase staining(Fig.4).

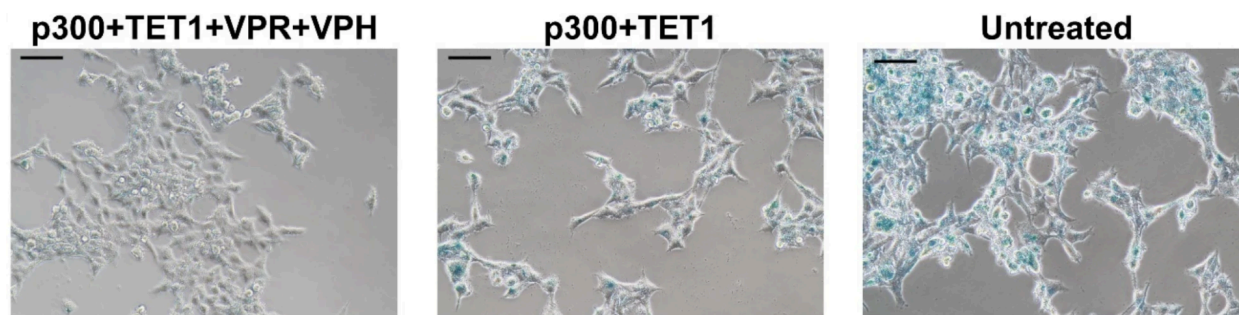


Figure 4: senescence-associated β -galactosidase staining of HEK293FT cells. Reproduced from Huang et al. (2024), Cellular and Molecular Life Sciences, 81, Article 407, licensed under CC BY-NC-ND 4.0.

Senescent cells are expressed by the blue dye staining due to high expression of β -galactosidase enzymatic activity.

Supporting the staining assay, immunofluorescent staining imaging(Fig. 5) is used on the cells. The analysis of the most well-established senescence marker p21, further confirms the delay of senescence through telomerase reactivation. (Huang et al., 2024)

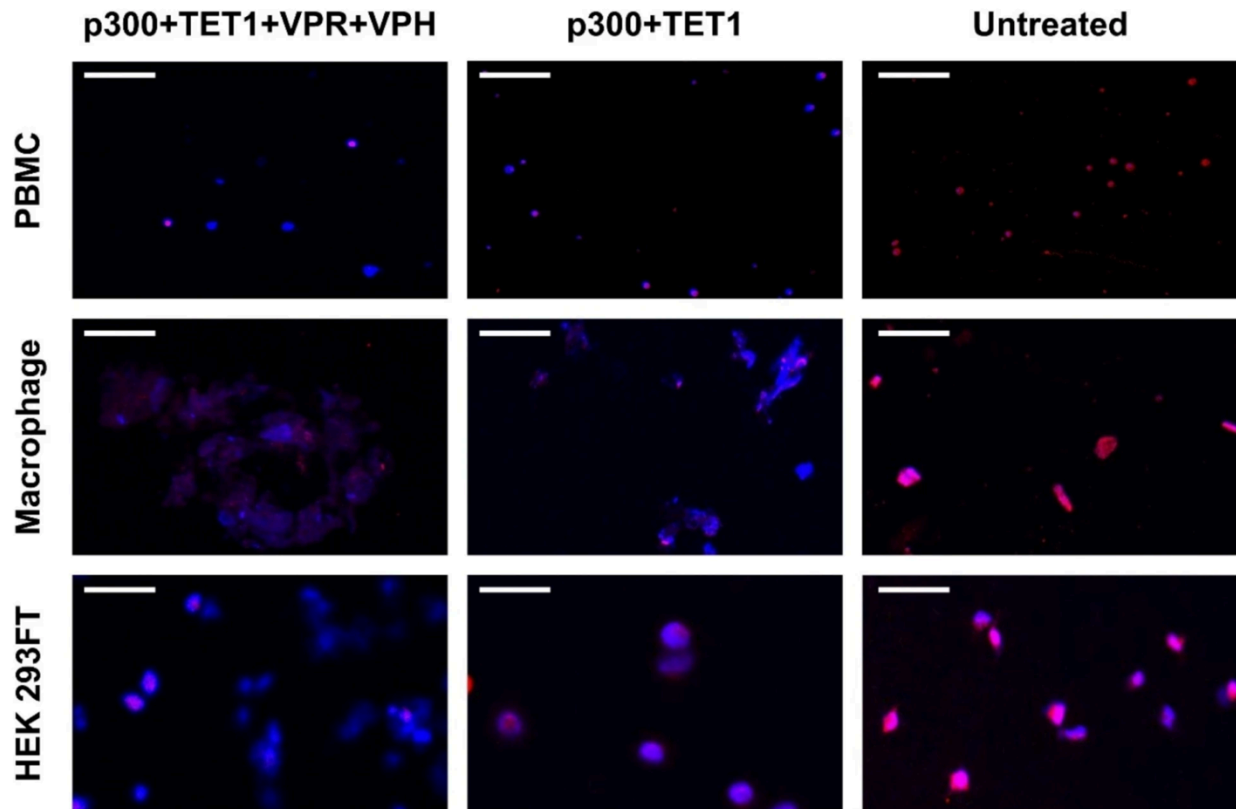


Figure 5: images of aged cells using immunofluorescent staining. Reproduced from Huang et al. (2024), Cellular and Molecular Life Sciences, 81, Article 407, licensed under CC BY-NC-ND 4.0.

Scale bar: 50µm

Notably, these cells proliferate well beyond the lifespan of typical primary cells, exceeding 100 population doublings, while avoiding features associated with malignant transformation. TERT-modified lines displayed chromosomal stability, demonstrating telomerase activation as a safe approach to extending cellular lifespan. (Huang et al., 2024)

CRISPR-Cas9-Mediated Immortalization

CRISPR-based immortalization combined targeted CDKN2A(p16/p14) knockout with either hTERT knock-in or CRISPRa-based activation. Molecular validations confirmed:

- Successful gene editing, verified by PCR and sequencing.
- Loss of p16 and p14 proteins, confirmed by Western blotting.

- Strong telomerase reactivation, confirmed by TRAP assays.

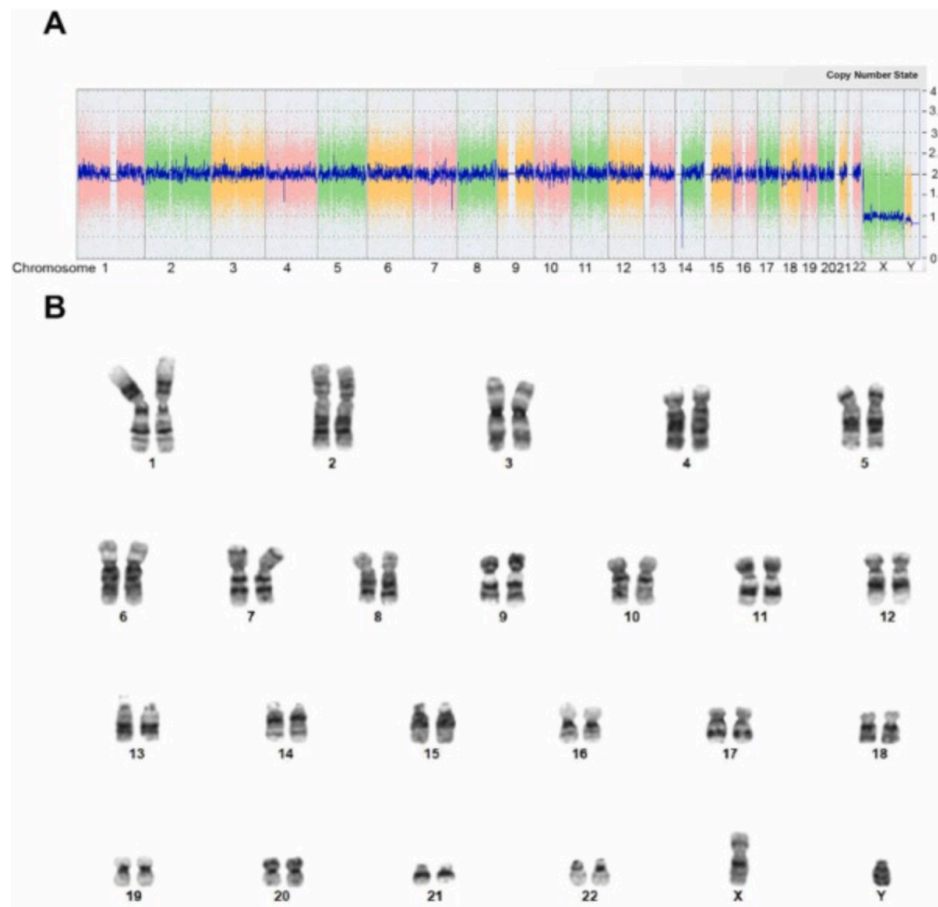


Figure 6: hCD8+T-TERT cells karyotype. Reproduced from He et al. (2025), Tissue & Cell, ScienceDirect

Karyotyping demonstrates that the majority of CRISPR-immortalized cells remained diploid and structurally stable(Fig. 6a). Then using Cytogenic karyotyping(Fig. 6b), it is confirmed that at least 46% of the immortalized cells retain a normal 23 pairs chromosomes without abnormalities. However, a notable finding was observed in early-passage cells(P10–P15): exhibition of transient chromosomal abnormalities(gain/losses) in about 7 of 50 metaphases. (He et al., 2025)

- Abnormalities occurred in the location Chromosome 4p14, a region associated with autoimmune susceptibility and certain cancers.

- And there were gains/losses of location Chromosome 11p15, a critical imprinted growth-regulatory genes linked to disorders like Beckwith-Wiedemann and Silver-Russell syndromes.

(Zhao et al., 2020)

These transient abnormalities observed during early passage likely indicate cellular stress response to CRISPR-induced double-strand breaks. However, by late passage, these alterations are no longer observed, suggesting the possibility that there has been self-correction. Potentially, through selective pressure, genetically stable cells outcompete abnormal ones, leading to the discontinuation of transient stress response caused by CRISPR manipulation. Despite these abnormalities, their disappearance in later passage supports the conclusion that CRISPR-immortalized cells ultimately stabilize as they do not retain early-passage chromosomal disturbances.

However, this finding also highlights the importance in long-term genomic monitoring, as temporary abnormalities could still pose risks if clinically relevant genes are affected during early passages.

Overall, CRISPR allows the cultivation of immortal cells that remain morphologically normal, retaining contact inhibition and exhibiting predictable and targetable genomic outcomes. Importantly, this method successfully produced the first normal human T cell line, demonstrating the potential of CRISPR-based strategies for previously difficult-to-immortalize cell types. (He et al., 2025)

TERT Overexpression & CRISPR-Mediated immortalization

While Huang et al. observed minimal chromosomal abnormalities following TERT activation, Zhao et al. noticed transient chromosomal disruption after CRISPR editing. This suggests that telomere maintenance alone may produce a more stable genomic environment than direct genome modification, although CRISPR provides greater flexibility for modeling specific cell types. Further, this difference may reflect the fundamentally distinct mechanisms behind each method, with telomerase activation preserving existing chromosomal structures while CRISPR intentionally introduces DNA modification.

LIMITATIONS

Across the methodology and results, there are some notable limitations. Although these studies demonstrate important advancements in cell immortalization, there are still factors left to be discussed. First, like most immortalization techniques, all steps and analyses are done *in vitro*, meaning that all data may not fully represent the physiological condition of cells in living organisms. Due to the complexity of a living organism, it remains unclear whether an *in vitro* method could really reflect the behavior of these cells in a real organism.

Connected to the limitation of the cells, these studies focus on specific cell types, for example, epithelial, immune(T), and fibroblast-like-cells. These differences could limit the comparison between immortalization techniques due to the specificity of cell types, which could potentially respond differently to these immortalization strategies. The genomic stability, therefore, may depend on the cell type, experimental design, and analytical method used in each study. Furthermore, CRISPR-Cas9 immortalization is a new technology that has not been completely explored across various cell types, causing limits regarding available cell types for comparisons.

Additionally, the results' primary method of finding chromosomal instability is through karyotyping, which in reality primarily detects large-scale chromosomal changes. This could mean that smaller, more subtle changes in gene mutation, alteration or changes might not have been detected. (Yoshioka, 2015) This is relevant especially for sensitive regions like chromosome 11p15, which any alterations could alter its function of growth regulation.

Finally, though stated to be safe and not cancerous, it is not entirely sure that these immortalized cells will not transform in a longer period of time. This indicates complication in usage on humans.

FUTURE DIRECTIONS

Through comparative findings, it highlights both the shared success and mechanistic differences among immortalization strategies, effectively showing the future direction regarding cell immortalization.

Table 1. Table of comparison on all mentioned immortalized techniques.

Techniques	Target location	Cell used	Risk	Indication
TERT overexpression	Telomeres (hTERT gene)	PBMCs, HEK293FT, THP-1 macrophages, retinal pigment	Possible telomerase misregulation; limited effect	Telomere maintenance prevents senescence and preserves

		epithelial cells, foreskin fibroblasts	without additional cell-cycle changes	chromosomal stability
CRISPR	CDKN2A (p16/p14), hTERT locus	Prostate epithelial cells, primary T cells	Temporary chromosomal abnormalities at early passages; off-target risk	Precise, controlled immortalization with long-term genomic stability

An original table consisted of data summarized from Huang et al. (2024), Zhao et al. (2020), and He et al. (2025).

TERT-immortalized cells demonstrate exceptional chromosomal stability and extend proliferation without malignant behavior. Based on the studies reviewed, TERT appears to be the safer and more biologically conservative method. This makes TERT-immortalization particularly attractive for cells intended for long-term modeling or regenerative applications, where maintaining normal cellular behavior is essential.

In contrast, as the first method to establish normal human T cell lines, CRISPR-based approaches may be especially valuable for cell types that have historically resisted immortalization. Since it offers a controlled strategy that avoids random viral insertion events. (He et al., 2025; Zhao et al., 2020) Although the temporary chromosome 4p14 and 11p15 abnormalities may raise important considerations regarding early-passage genomic monitoring, the normalization observed by P47 suggests that CRISPR can produce stable and predictable immortalized lines when properly screened. Therefore, with continued improvements in genome-editing technologies, these risks may be reduced and the range of clinically relevant cell models could be expanded.

In conclusion, based on the limited studies reviewed, TERT overexpression currently appears to provide a more stable strategy than presently available CRISPR-based immortalization. However, CRISPR-based immortalization still opens the door to unprecedented precision and the possibility of immortalizing clinically important primary cells such as T cells. With continued refinement of CRISPR technologies, particularly the reduction of CRISPR-induced DNA breaks, it may eventually surpass existing methods in safety and stability. Further, it may eventually become preferable due to the ability to allow cell-type-specific immortalization without relying on constitutive telomerase expression.

With these findings in mind, several future directions are proposed below:

1. Rather than full CDKN2A knockout, which creates genomic stress, telomeres could be integrated through maintenance with partial cell-cycle regulation. Specifically, future approaches could use CRISPR base editing to simply turn p16/p14 expression down while activating TERT, which may reduce genomic disturbances while maintaining immortalization.

2. Knowing 11p15 contains imprinted genes, future studies should assess whether immortalization affects imprinting patterns over many passages. This long-term epigenetic monitoring could improve understanding of the full effects of abnormality at 11p15.
3. By not fully cutting DNA to do insertion, CRISPRa-mediated telomerase activation ensures a more stable immortalization than other methods. Therefore, by applying CRISPR in immortalizing difficult to immortalize cells such as neurons and cardiomyocytes, the result could provide information regarding whether CRISPRa-mediated telomerase activation improves genomic stability in these cell types.
4. CRISPR-Cas9 mediated immortalizations could be done across more cell types to increase knowledge regarding its stability and usage.

CONCLUSION

Comparison of these two major cell immortalization techniques, some similarities and differences can be seen. TERT overexpression is associated with reduced genomic instability by maintaining telomere length. CRISPR, on the other hand, is a useful tool for controlled immortalization. Both of them are immortalization techniques but based on different concepts: hTERT overexpression is telomerase-based and CRISPR includes both genome editing and transcriptional activation based approaches. Despite being different, both techniques discussed in this paper demonstrate that extending cellular lifespan without full transformation is possible. (Huang et al., 2024; He et al., 2025; Zhao et al., 2020) By understanding all of these immortalization methods, it will be possible to merge and develop even newer techniques which could provide further help in advancements in the medical fields such as regenerative medicines.

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