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REVIEW

Immortalized cell lines derived from dental/odontogenic tissue

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Abstract

Stem cells derived from dental/odontogenic tissue have the property of multiple differentiation and are prospective in tooth regenerative medicine and cellular and molecular studies. However, in the face of cellular senescence soon in vitro, the proliferation ability of the cells is limited, so studies are hindered to some extent. Fortunately, immortalization strategies are expected to solve the above issues. Cellular immortalization is that cells are immortalized by introducing oncogenes, human telomerase reverse transcriptase genes (hTERT), or miscellaneous immortalization genes to get unlimited proliferation. At present, a variety of immortalized stem cells from dental/odontogenic tissue has been successfully generated, such as dental pulp stem cells (DPSCs), periodontal ligament cells (PDLs), stem cells from human exfoliated deciduous teeth (SHEDs), dental papilla cells (DPCs), and tooth germ mesenchymal cells (TGMCs). This review summarized establishment and applications of immortalized stem cells from dental/odontogenic tissues and then discussed the advantages and challenges of immortalization.

Keywords Stem cells from dental/odontogenic tissues · Immortalization · Cellular senescence · Strategies · Application

Introduction

Oral tissues are difficult to achieve self-repair once damaged by disease on account of their weak regeneration ability, such as tooth hard tissue, dental pulp, and periodontal tissue (Chen et al. 2019; Itoh et al. 2018; Li et al. 2020). At present, tissue engineering technology brings hope for tissue regeneration in dentistry, but it is still in the research stage. Stem cells from dental/odontogenic tissues can be obtained from the shed or extracted teeth without ethical concerns, and they have the capability of multilineage differentiation (Ko et al. 2020; Shi et al. 2020). Obviously, they serve as an important tool in regeneration research, also in physiological and pathological mechanism research (Chen et al. 2021a, b; Imber et al. 2021; Liu et al. 2019; Xuan et al. 2018). However, their proliferation ability is low, and their lifespan is limited when cultured in vitro, making it hard to meet the demand for study. After a limited number of divisions, they will stagnate irretrievably in a phase without proliferation, which is called cellular senescence (Iezzi et al. 2019; Morsczeck 2019, 2021).

A notable feature of senescent cells is the increased expression of cell cycle inhibitory proteins, collectively known as cyclin-dependent kinase inhibitors, including p21 and p16 (Alcorta et al. 1996; Dulić et al. 2000; Gorgoulis et al. 2019). Cellular senescence usually responds to various triggers, including DNA damage, telomere dysfunction, and oncogene activation (Gorgoulis et al. 2019; Ovadya et al. 2018). When the DNA damage response (DDR) occurs, the tumor suppressor p53 is activated, stimulating the expression of p21 and p16, leading to cellular senescence (Beauséjour et al. 2003; Dulić et al. 2000). In the absence of telomere maintenance mechanisms such as telomerase expression, telomeres shorten with each cell division to a certain length, and then the loss of telomere protection triggers DDR (d'Adda di Fagagna et al. 2003). For example, it

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was reported that primary human pulp cells would undergo cellular senescence up to cellular passage 7 (Galler et al. 2006). Aging dental mesenchymal stem cells showed progressive loss of telomere DNA length, which may result from decreased telomerase activity and increased expression of aging marker p16 (Mehrazarin et al. 2011).

As a result, cellular senescence brings some troubles to researches in stomatology to some degree. Amazingly, immortalization strategies are expected to make cells acquire infinite proliferation ability in vitro while retaining certain differentiation potential, which brings hope for the solution of the problems. Currently, various types of stem cells from dental/odontogenic tissue have already been successfully immortalized, mainly using viral oncogenes and the human telomerase reverse transcriptase (hTERT) protein, e.g., immortalized human tooth germ stem cell line (hTGSC-hTERT), immortalized cementoblasts (OCCM-30) (D'Errico et al. 2000; Yalvaç et al. 2011). This review summarized the establishment and application of immortalized stem cells from dental/odontogenic tissues and discussed the challenges and perspectives of immortalization.

Immortalization strategies

Immortalized stem cells from dental/odontogenic tissues must overcome the two proliferative obstacles, senescence, and crisis (Counter et al. 1998). Typically, the immortality of cell lines could be established by different strategies (Fig. 1), including introducing telomerase or human telomerase reverse transcriptase (hTERT), mutating cell cycle checkpoints (p53/pRb), oncogenes, and spontaneous immortalization (MacDougall et al. 1995; Mi et al. 2011; Nakata et al. 2003; Thonemann and Schmalz 2000a). The most common methods to immortalize stem cells from dental/odontogenic tissues are the ectopic expression of telomerase or human telomerase reverse transcriptase (hTERT) and the overexpression of viral oncogenes (Ramboer et al. 2014). Plasmid transfection and viral transfection have been widely used to transfer immortalization genes into stem cells from dental/odontogenic tissues (Thonemann and Schmalz 2000a; Wu et al. 2015b; Yin et al. 2016). Some other ways were also reported, such as spontaneous immortalization and deriving from immortomouse (Wang et al. 2020; Wilson et al. 2015). However, spontaneous immortalization is a rare event whose frequency is less than 10^{-12}. Thus, the transfection of cells with recognized immortalizing genes, like viral oncogenes and hTERT, is necessary to improve immortalizing efficiency (Katakura et al. 1998; Ramboer et al. 2014).

Establishment by viral oncogenes

The viral oncogenes, the simian virus 40 large T antigen (SV40 Tag), and the human papillomavirus 16 (HPV16) E6/E7 genes could be used to transform a cell into being immortalized (Huang et al. 2015; MacDougall et al. 1995; Thonemann and Schmalz 2000a). They have been used to establish immortalized stem cells from dental/odontogenic tissues, such as M06G3, PLT-1, IDG-CM6, and OCCM-30 (D'Errico et al. 2000; Kamata et al. 2004; MacDougall et al. 1995; Wang et al. 2020). These viral oncogenes are well-known to interfere with the p16/pRb and p53 pathways, which plays an important role in maintaining the cell cycle, resulting in immortalized cells (Egbuniwe et al. 2013, 2011; Kamata et al. 2004; Schafer 1998). Moreover, immortalized stem cells are also derived from immortalized murine expressing the SV40 Tag (Wang et al. 2020).

Human telomerase reverse transcriptase

Compared with oncogenes like SV 40, hTERT for immortalization has been suggested to avoid genetic and phenotypic instabilities, without changing cell differentiation (Yalvaç et al. 2011). However, hTERT is limited to being applied in some human cell types. Stem cells from dental/odontogenic tissues normally had a limited life span in vitro, for the low level of telomerase leads to telomeres shortening (Belgiovine et al. 2008; Fujita et al. 2005). Therefore, the primary cells require overexpression of telomerase to maintain the telomere lengths to realize immortalization (Fujita et al. 2005). While telomerase activity may not allow stem cells to overcome the telomere-independent growth arrest, only the overexpression of hTERT may be insufficient to make cells immortal (Colgin and Reddel 1999).

Miscellaneous immortalization

Besides a single gene used in cellular immortalization, the specific combination of viral oncogenes and the hTERT gene has been proven to immortalize stem cells from dental/odontogenic tissues, such as hTERT plus SV40 Tag, hTERT plus HPV16 E6/E7, and hTERT plus B lymphoma Moloney Murine Leukemia Virus (Mo-MLV) insertion region 1 homolog (Bmi-1) (Kamata et al. 2004; Yao et al. 2019). It has been demonstrated that Bmi-1 regulated cell proliferation, apoptosis, and differentiation. Consequently, overexpression of Bmi-1 could generate immortalized cell lines by inhibiting the transcription of p16/pRB like SV40 Tag and HPV16 E6/E7 (Jacobs et al. 1999; Jung and Nolta 2016; Lee et al. 2016a; Yao et al. 2019).

Immortalized stem cells from dental/odontogenic tissue

As a critical cellular model in vitro, stem cells from dental/odontogenic tissues have been widely used in tissue engineering and fundamental research due to their differentiation

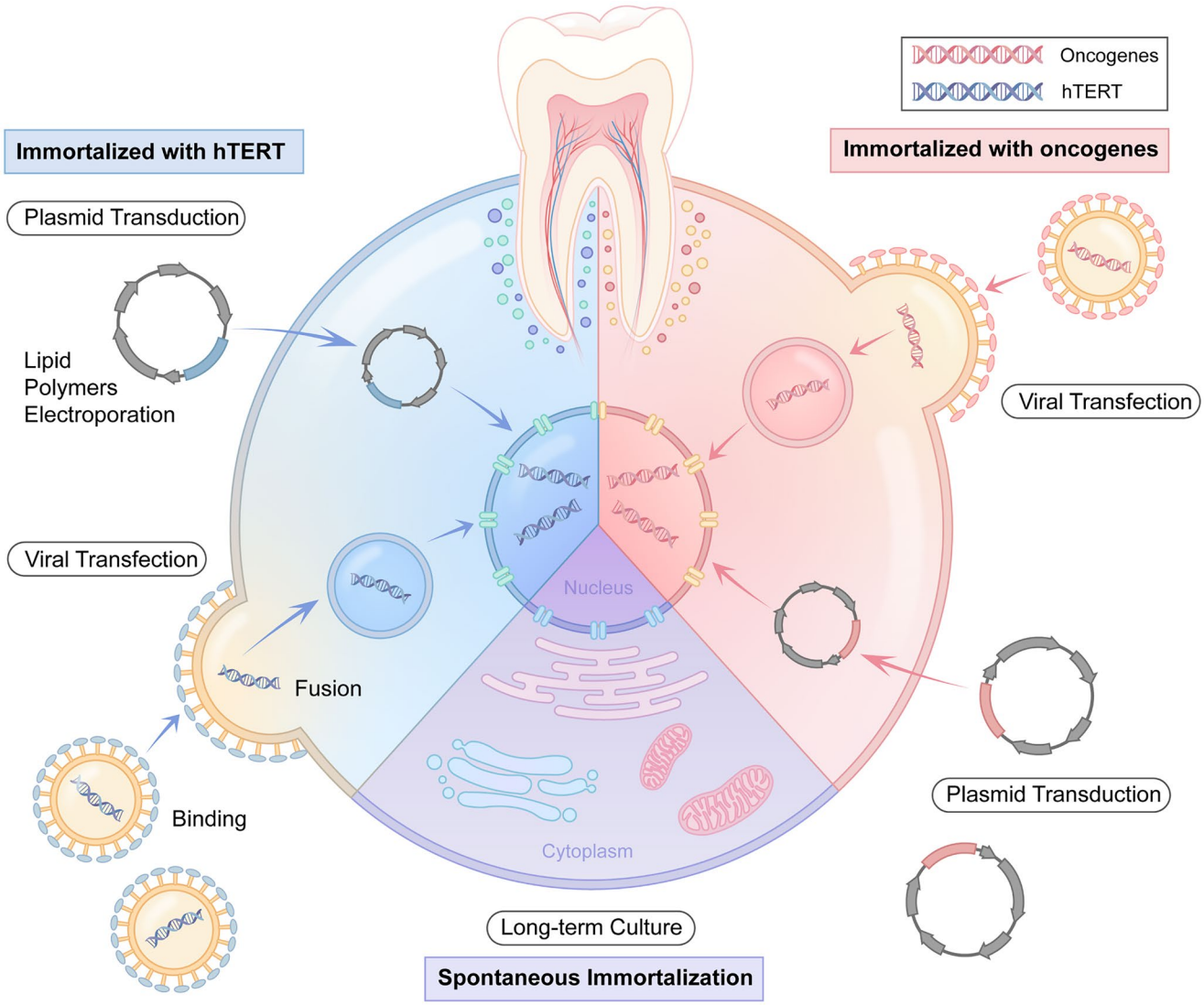

Fig. 1 Immortalization strategies of stem cells derived from dental/odontogenic tissue. The immortalized genes of hTERT and viral oncogenes were transferred into cells by plasmid transfection or viral transduction. Also, stem cells derived from dental/odontogenic tissue are probably immortalized spontaneously

potential and accessibility (Li et al. 2020; Yao et al. 2019; Yin et al. 2016). However, primary cells have a limited life span because of actual division numbers and soon reach a non-proliferative state called cellular senescence. This has limited the research on teeth-derived tissue about tooth development mechanisms, gene regulation, or substance exposure (Galler et al. 2006). Therefore, immortalization genes were transferred into primary stem cells from dental/odontogenic tissues to overcome this obstacle, establishing various cells lines applied in the investigation (D'Errico et al. 2000; Hanks et al. 1998; MacDougall et al. 1995). The sequence of immortalized stem cells from dental/odontogenic tissues is presented first by tissue origin and second by anatomical location. The tooth germ is composed of both epithelial and mesenchymal tissues. So, the tooth germ mesenchymal cells from both epithelial and mesenchymal tissues are listed first. Then, there are cells derived from the epithelium, including ameloblast-like cells, dental epithelial progenitor cells derived from the cervical-loop epithelium, and Hertwig's epithelial root sheath cells. And finally, there are cells derived from mesenchymal tissues, including dental papilla cells, odontoblast cells, dental follicle cells, stem cell lines from the deciduous teeth, stem cells of the dental apical papilla, dental pulp stem cells, periodontal ligament stem cells, and cementocyte cells. Table 1 shows immortalized stem cells from dental/odontogenic tissue.

Table 1 Immortalized odontogenic cell lines

Tissue origin	Stem cells	Cell line	Species	Immortalized gene	Transfer System	Reference
Epithelial tissue	Human tooth germ stem cells	hTGSCs-hTERT	Human	hTERT	Lentivirus	(Yalvaç et al. 2011)
		hTGSCs-SV40	Human	SV40	Lentivirus	(Yalvaç et al. 2011)
		ihEDMC4	Human	SV40	Plasmid	(Huang et al. 2015)
		iTGMC	Mouse	SV40	Retrovirus	(Luo et al. 2021)
	Ameloblast-lineage cells	Transformed EOE cells	Mouse	SV40	Electroporation	(Chen et al. 1992)
		PABSo-E	Pig	SV40	Calcium phosphate procedure	(DenBesten et al. 1999)
		ALC	Mouse		Spontaneous immortalization	(Nakata et al. 2003)
		EOE-2 M	Mouse	(HPV16) E6/E7	Retrovirus	(MacDougall et al. 2019)
		EOE-3 M	Mouse	(HPV16) E6/E7	Retrovirus	(MacDougall et al. 2019)
	Dental epithelial progenitor cells derived from the cervical-loop epithelium	HAT-7	Rat		Spontaneous immortalization	(Kawano et al. 2002)
	Hertwig's epithelial root sheath cells	HERS/ERM cell line	Human	SV40	Electroporation	(Nam et al. 2014)
		HERS-C2 and HERS-H1	Rat	SV40	Lentivirus	(Li et al. 2019)
		HERS Cells	Mouse	SV40	Immortommouse	(Zeichner-David et al. 2003)
Mesenchymal tissue	Dental papilla cells	tCPC	Bovine	SV40	Electroporation	(Thonemann and Schmalz 2000b)
		PA-1	Human	hTERT and HPV16	Plasmid	(Kamata et al. 2004)
		iBmp2-dp	Mouse	SV40	Lentivirus	(Wu et al. 2010)
		mDPCET and mDPC6T	Mouse	SV40	Lentivirus	(Lin et al. 2013)
		iMDP-3	Mouse	SV40	Electroporation	(Wang et al. 2013)
		hDPC-TERT	Human	hTERT	Lentivirus	(Yang et al. 2013)
		Im DM	Mouse	SV40	Lentivirus	(Liu et al. 2015)
		iBmp2$^{ko/ko}$ dp	Mouse	SV40	Lentivirus	(Wu et al. 2015a)
	Odontoblast cells	M06-G3	Mouse	SV40	Retrovirus	(MacDougall et al. 1995)
		MDPC-23	Mouse		Spontaneous immortalization	(Hanks et al. 1998)
		tCPC E	Bovine	HPV 18 E6/E7	Electroporation	(Thonemann and Schmalz 2000a)
		T4-4 and T3-2	Rat	hTERT	Plasmid	(Hao et al. 2002)
		OLC	Mouse		Spontaneous immortalization	(Arany et al. 2006)
		P4-2	Pig	SV40	Plasmid	(Iwata et al. 2007)
		Im OB	Mouse	SV40	Lentivirus	(Liu et al. 2015)
		iPDBs	Rat	SV40	Retrovirus	(Cao et al. 2020)
	Dental follicle cells	DF1, DF2, and DF3	Mouse	SV40	Plasmid	(Luan et al. 2006)
		BCPb8	Bovine	bmi-1 and hTERT	Retrovirus	(Saito et al. 2005)
		MDF$^{E6-EGFP}$ cells	Mouse	HPV-16 E6	Retrovirus	(Yokoi et al. 2007)
		iDFCs	Human	SV40	Plasmid	(Wu et al. 2015b)

Table 1 (continued)

Tissue origin	Stem cells	Cell line	Species	Immortalized gene	Transfer System	Reference
	Stem cells from human exfoliated deciduous teeth	TP-023(SI) and TP-053(SI)	Human		Spontaneous immortalization	(Wilson et al. 2015)
		TP-023 (I)	Human	hTERT	Retrovirus	(Wilson et al. 2015)
		TERT-SHED	Human	hTERT	Lentivirus	(Yin et al. 2016)
		SHED-Bmi1-EGFP	Human	Bmi-1	Lentivirus	(Yao et al. 2019)
	Stem cells of dental apical papilla	iSCAPs	Mouse	SV40	Retrovirus	(Wang et al. 2014)
		DSCS	Human	SV40	Plasmid	(Sanz-Serrano et al. 2022)
	Dental pulp cells	tHPC	Human	SV40	Electroporation	(Galler et al. 2006)
		HDP-hTERT cells	Human	hTERT	Retrovirus	(Kitagawa et al. 2007)
		tDPSCs	Human	hTERT	Retrovirus	(Egbuniwe et al. 2011)
		iHDPCs	Human	SV40	Plasmid	(Li et al. 2020)
		DP-1	Human	hTERT and SV40	Plasmid	(Kamata et al. 2004)
		K4DT cells	Human	mutant cyclin-dependent kinase 4 (CDK4R24C), Cyclin D1, and hTERT	Retrovirus	(Orimoto et al. 2020)
	Periodontal ligament stem cells	IM cells	Mouse	H-2 Kb tsA58	Immortommouse	(D'Errico et al. 1999)
		SV cells	Mouse	SV40	Wild-type SV40	(D'Errico et al. 1999)
		RPDL	Rat	SV40	Transgenic rats	(Kubota et al. 2004)
		PLT-1	Human	hTERT and HPV16	Plasmid	(Kamata et al. 2004)
		A4 and C10	Human	hTERT	Retrovirus	(Fujita et al. 2005)
		STPLF	Human	SV40 and hTERT	Plasmid	(Fujii et al. 2006)
		TesPDL1-4	Swine	hTERT	Calcium phosphate procedure	(Ibi et al. 2007)
		iPDL	Human	HPV 16 E6/E7	Retrovirus	(Pi et al. 2007)
		M-HPL1	Human	bmi-1 and hTERT	Retrovirus	(Shiga et al. 2008)
		PDL-hTERT cells	Human	hTERT	Lentivirus	(Docheva et al. 2010)
		SH	Human	hTERT	Plasmid	(Hasegawa et al. 2010)
		I-PDL cells	Human	hTERT	Retrovirus	(Hung et al. 2010)
		BT-PFs	Human	hTERT	Plasmid	(Mi et al. 2011)
		PDLSC-Bmi1	Human	Bmi-1	Lentivirus	(Wei et al. 2017)
		TERT-hPDLSCs	Human	hTERT	Lentivirus	(Chen et al. 2019)
		STPDL and STP-DLDS	Human	hTERT and SV40	Plasmid	(Asakawa et al. 2022)
		hPLF-hTERT	Human	hTERT	Not mentioned	(Nogueira et al. 2021)
	Cementocyte cells	OCCM-30	Mouse	SV40	Immortommouse	(D'Errico et al. 2000)
		RCM cells	Rat	SV40	Plasmid	(Kitagawa et al. 2005)
		HCEM-1 and -2	Human	hTERT	Retrovirus	(Kitagawa et al. 2006)
		IDG-CM6	Mouse		Immortommouse	(Zhao et al. 2016)

Tooth germ mesenchymal cells (TGMCs)

Tooth germ mesenchymal cells (TGMCs), as minimally invasive alternative adult stem cell sources, are mostly obtained after orthodontic treatments or maxillofacial surgery in adults without ethical concerns (Yalvaç et al. 2011). Because of the differentiation and regenerative potential of cranial neural-crest-originated mesenchyme cells in tooth development, TGMCs are an ideal tool for studying the mechanism of tooth development and providing an alternative solution in

the cellular therapy for various maladies (Huang et al. 2015; Luo et al. 2021). However, primary TGMCs from humans are rare, and their proliferative abilities are limited. Therefore, there is no doubt that importance should be attached to establishing immortalized tooth germ stem cells (Huang et al. 2015; Yalvaç et al. 2011). The strategies of immortalization by SV 40 large T antigen and hTERT had been evaluated in human TGMCs, and the result suggested that TGMCs-hTERT is safer than hTGMCs -SV40 because hTGMCs-SV40 showed abnormal karyotypes and hTERT-TGMCs were free from any chromosomally morphological change. Meanwhile, hTGMCs-hTERT preserve MSC characteristics and transform them into adipo-, osteo- and odonto-genic cells. Furthermore, hTGMCs-hTERT reduces neuro-toxicity of SH-SY5Y cells even at great passage numbers showing neuroprotective effects (Yalvaç et al. 2011). Another cell line, ihEDMC4 transformed by SV40, displayed a higher proliferation rate and maintained multipotency, similar to primary human dental mesenchymal cells (Huang et al. 2015). In addition to human-immortalized TGMCs, TGMCs derived from Mice are also immortalized (iTGMCs) (Luo et al. 2021). Taken together, immortalized TGMCs serve as an important resource for studying the mechanisms of tooth development, tooth tissue engineering, and cellular therapy of various diseases (Huang et al. 2015; Luo et al. 2021; Yalvaç et al. 2011).

Ameloblast-lineage cells

During tooth formation, epithelium-derived cells of the tooth germ go through a progressive differentiation leading to ameloblasts that synthesize enamel (Hatakeyama et al. 2011). Eventually, ameloblasts undergo apoptosis when enamel full thickness is complete and the teeth erupt. This pattern of tooth development limits the obtainment of human or rodent dental epithelial cells to study the mechanism of ameloblasts function and amelogenesis (MacDougall et al. 2019). Therefore, the establishment of immortalized tooth epithelial cell line will be beneficial to reveal the mechanism of epithelial differentiation in tooth development. Nakata et al. established a spontaneously immortalized mouse ameloblast-lineage cell line (ALC), which maintained the expression of ameloblast-specific genes (amelogenin, tuftelin, and enamelin) in long-term culture and the ability to induce bio-mineralization in vitro (Nakata et al. 2003). Several studies had shown that the viral oncogenes such as the SV-40 large T antigen and the HPV 16 E6/E7 genes had the ability to immortalize enamel organ epithelia (EOE) epithelial cells derived from mouse (Chen et al. 1992; MacDougall et al. 2019) and swine (DenBesten et al. 1999). These immortalized cells had unlimited proliferation capacity and continue to retain the characteristics of ameloblasts, expressing enamel matrix markers and producing mineralized extracellular matrixes (DenBesten et al. 1999; MacDougall et al. 2019). Generally, ameloblast-like immortalized cell lines are useful tools for the study of enamel bioengineering.

Dental epithelial progenitor cells derived from the cervical-loop epithelium

Dental epithelial stem cells in tooth buds give rise to various cell types: inner enamel epithelium, stratum intermedium, stellate reticulum, and outer enamel epithelium. The connection between the inner enamel epithelium and the outer enamel epithelium is the cervical loop, which is closely related to the renewal of dental epithelium-producing enamel matrix and/or inducing dentin formation (Kawano et al. 2002). Because of the difficulty in obtaining tissues, it is necessary to use indefinitely propagated cells to investigate the mechanism of tooth epithelial cell differentiation. Subsequently, immortalized dental epithelial progenitor cell line (HAT-7) derived from the cervical-loop epithelium of rat lower incisor was established (Kawano et al. 2002). In vitro experiments showed that these cells could produce ameloblast lineage cells, stratum intermedium cells, stellate reticulum, and outer enamel epithelium (Kawano et al. 2004). In addition, fibroblast growth factor 10 was proved to serve an important role in coupling mitogenesis of the cervical-loop cells and the production of stratum intermedium cells (Kawano et al. 2004). Obviously, HAT-7 was useful in the study of the process of tooth germ development.

Hertwig's epithelial root sheath cells

Hertwig's epithelial root sheath (HERS) is a bilayered epithelial sheath that originated from the fusion of inner and outer enamel epithelia below the level of the cervical crown margin during tooth development (Zeichner-David et al. 2003). As individual dental epithelial stem cells in the periodontal ligament, HERS play an essential role in tooth root development (Huang et al. 2010). Margarita et al. reported the establishment of an immortal HERS from transgenic mice harboring the SV40 able to differentiate and produce a mineralized extracellular matrix similar to acellular cementum (Zeichner-David et al. 2003). Furthermore, primary HERS cells isolated from human periodontium were also immortalized by electroporation with SV40 large T antigen. Immortalized HERS cells maintain the morphological and immunophenotypic characteristics and epithelial and embryonic stem cell markers (Nam et al. 2014). Like primary HERS cells, immortalized HERS cells could undergo EMT in response to TGF-β and differentiate into cementum-forming cells and generate cementum-like tissue. Interestingly, HERS cell lines induced DPCs to differentiate into odontoblasts and develop dentin-like tissue (Li et al. 2019).

Additionally, epithelial cell rests of Malassez (ERM) are quiescent epithelial remnants of the Hertwig's epithelial root sheath (HERS) in the periodontal ligament, involved in the repair/regeneration of cement or enamel (Tsunematsu et al. 2016). Odontogenic epithelial cells from ERM were spontaneously immortalized. Interestingly, immortalized odontogenic epithelial (iOdE) cells have similar features to stem cells that exist in ERM and are capable to produce calcifications (Tsunematsu et al. 2016). In conclusion, immortalized HERS cell line provides an unlimited cell source for understanding the function of HERS cells and tooth root regeneration.

Dental papilla cells (DPCs)

Dental papilla cells (DPCs) are mesenchymal cells that can differentiate into odontoblasts and produce a dentin matrix, which makes up the central mineralized tissue of teeth. However, the process of dentinogenesis is complex, and the exact mechanisms are not well understood (Wang et al. 2013). Simultaneously, primary cells are rare, and their proliferation is limited. Therefore, immortal dental papilla cells are needed to overcome these hurdles to establish the foundation for odontogenesis research. Generally, dental papilla cell lines have a higher proliferation rate and retain the phenotypic characteristics similar to primary hDPCs, expressing dentin phosphoprotein (DPP), dentin matrix protein 1 (DMP1), dentin sialoprotein (DPP), and Nestin showing the capacity to differentiate and form mineralized nodules (Thonemann and Schmalz 2000b; Wang et al. 2013; Yang et al. 2013). Besides, there are some specific dental papilla cell lines. An immortalized mouse floxed Bmp2 dental papilla mesenchymal cell line (iBmp2-dp cells) was generated for studying the mechanisms of stem cells from dental/odontogenic tissue differentiation and dentin formation mediated by Bmp2 (Wu et al. 2010). Furthermore, Cre fluorescent protein (GFP) was inducted into iBmp2-dp cells to generate immortalized deleted Bmp2 dental papilla mesenchymal (iBmp2$^{ko/ko}$-dp) cells. In the iBmp2$^{ko/ko}$-dp cells, expression of tooth-related marker genes and differentiation was decreased, and extracellular matrix remodeling was also impaired (Wu et al. 2015a). Besides, Fam20c is also playing an essential role in the mineralization of dentin. And immortalized mouse floxed Fam20c dental papilla mesenchymal cell lines retained the morphology of primary cells (Liu et al. 2015). Interestingly, a tamoxifen-regulated Cre recombination system generated the tamoxifen-mediated reversibly immortalized mouse dental papilla cell line (Mdpcet). Once reversing the immortalization, cells would undergo replicative senescence and differentiate into odontoblast-like cells (Lin et al. 2013). All in all, these immortalized cells serve as an excellent surrogate model for the basic research of molecular mechanisms of odontoblast differentiation and dentin formation but also are potential in drug toxicity tests and material biocompatibility examinations (Wang et al. 2013; Yang et al. 2013).

Odontoblast cells

The odontoblasts responsible for the dentin synthesis are considered neural crest-derived mesenchymal cells from the first branchial arch (Arany et al. 2006). However, the dentinogenesis molecular mechanism remains undefined to date (Arany et al. 2006; MacDougall et al. 1995). Therefore, it is of great interest to develop a valuable reproducible cellular model to clarify the mechanism of tooth development. The earliest odontoblast cell lines, M06-G3, were established by transfection with SV40 large T antigen, derived from Swiss Webster E-18 (vaginal plug day 0) first mandibular molars. M06-G3 showed odontoblast characteristics, highly expressing dentin phosphoprotein, type I collagen, and alkaline phosphatase (MacDougall et al. 1995). Then, two mice's odontoblast cell lines were spontaneously generated. MDPC-23 and odontoblast-lineage cell (OLC) could be induced calcification and mineralization in osteogenic differentiation medium and maintain odontoblast characteristics (Arany et al. 2006; Hanks et al. 1998). In addition, bovine dental papilla-derived cells were transfected by HPV 18 E6/E7 to overcome senescence, resulting in immortalized cell lines (tCPC E), which maintained the phenotype of odontoblast-like cells with expression of procollagen type I, alkaline phosphatase, and osteocalcin (Thonemann and Schmalz 2000a). Interestingly, rat odontoblast cells immortalized by hTERT have an odontoblast-like phenotype and synthesize mineralized dentin-like tissue both in vitro and in vivo (Hao et al. 2002). Conclusively, it is firmly proved that these cell lines will function as valuable tools in research concerning the modulatory mechanism of odontoblast differentiation, tissue engineering of dentin repair and regeneration, and biocompatibility testing of dental materials (Hao et al. 2002; Thonemann and Schmalz 2000a).

Dental follicle cells (DFCs)

The dental follicle is formed by an ectomesenchymal progenitor cell population in the early stages of tooth bud formation, surrounding the tooth germ. Progenitors in the dental follicle were considered the origin of periodontal tissues, including the periodontal ligament, cementum, and alveolar bone (Luan et al. 2006; Saito et al. 2005). Under certain conditions, DFCs can differentiate into osteogenic, chondrogenic, adipogenic, and neuronal cells (Wu et al. 2015b). The limited life span has hampered progress in expanding specific progenitor or stem cells (Saito et al. 2005). Thus, immortal DFCs have been generated to overcome the limitations. BCPb8, a bovine DFC cell line, immortalized by

Bmi-1 and hTERT, formed cementum-like tissue and expressed osteocalcin, bone sialoprotein, type I collagen, and osteopontin Mrna (Saito et al. 2005). Besides, three cell lines (DF1, DF2, and DF3) were isolated from the SV40 transforming mice dental follicle cells, and each of them owns specific characteristics suggesting that these cell lines were separate and distinct. DF1 may be linked to periodontal ligament-type lineage without mineralization behavior, DF2 was highly undifferentiated, and DF3 might be related to cementoblastic or alveolar bone osteoblastic lineage (Luan et al. 2006). Human-immortalized DFCs were also established with SV40 by the piggyBac transposon-mediated vector, which could be deimmortalized by FLP recombinase (Wu et al. 2015b). A mouse immortalized DFC cell line ($MDF^{E6-EGFP}$) was transduced with EGFP lentivirus expressing a mutant HPV 16 E6 gene lacking the PDZ-domain binding motif. $MDF^{E6-EGFP}$ cell transplants in vivo could generate PDL-like tissues with the expression of Scx, periostin, type XII collagen, and the fibrillar assembly of type I collagen (Yokoi et al. 2007). There is little doubt that these immortal DFCs could play an essential role in cementogenesis study and periodontal regeneration therapy.

Stem cells from human exfoliated deciduous teeth (SHEDs)

Stem cells from human exfoliated deciduous teeth (SHED) are noninvasive, easy to access, with few ethical concerns, and valuable stem cells for tissue engineering and regenerative medicine (Martinez Saez et al. 2016). Moreover, SHEDs, as multipotential stem cells, grew faster than adult stem cells and successfully differentiated into odontoblasts, osteoblasts, chondrocytes, adipocytes, and neural cells (Martinez Saez et al. 2016; Yin et al. 2016). However, SHED cultured in vitro could not escape from entering replicative senescence. So, there are several methods to overcome SHED cellular senescence (Yin et al. 2016). Wilson et al. (2015) established spontaneously immortalized SHEDs and hTERT-immortalized SHEDs and then observed genomic instability in hTERT-immortalized SHEDs but not in the spontaneously immortalized SHEDs. Moreover, hTERT-SHED showed normal karyotype even at the late passage, but they had different biomarkers than SHED. Additionally, the immortalized SHEDs could differentiate into neurons in culture (Yin et al. 2016). Then, Bmi-1-immortalized SHEDs with an enhanced green fluorescent protein (EGFP) marker (SHED-Bmi1-EGFP) were generated using the Bmi-1 lentivirus. This cell line maintained multiple differentiation ability, normal phenotype, karyotype, and no tumorigenicity (Yao et al. 2019). Indeed, immortalized SHEDs are regarded as a potential tool for studying SHEDs and tissue regeneration therapies (Wilson et al. 2015; Yao et al. 2019; Yin et al. 2016).

Stem cells of the dental apical papilla (SCAPs)

Stem cells of the dental apical papilla (SCAPs) located in the apical papilla of immature permanent teeth represent dental MSCs with high proliferative potential, low immunogenicity, and self-renewal ability. In addition, there is considerable evidence that SCAPs can produce various cell lines, such as odontogenic, osteogenic, adipogenic, neurogenic, chondrogenic, and hepatogenic cells (Kang et al. 2019). SCAPs may be a promising source for pulp-dentin, periodontal, bone, neural regeneration, and bioroot engineering (Kang et al. 2019). Since further studies and therapies require many SCAPs, it is necessary to establish immortalized SCAPs. The first clone of human SCAPs transfected with hTERT and HPV16 represented continuous growth over 150 population doubling levels (PDL), capable of mineralization and sialophosphoprotein (DSPP) expression in the presence of β-glycerophosphate (Kamata et al. 2004). SV40 large T antigen was also transfected into human SCAPs to establish a cell line, dental stem cells SV40 (DSCS). The DSCS shows a higher proliferative capacity and retains its morphology and multipotency (Sanz-Serrano et al. 2022). Moreover, it was demonstrated that the reversible iSCAPs were established by using a reversible immortalization system expressing SV40 T flanked with Cre/loxP sites, expressing mesenchymal stem cell markers. When Cre recombinase was introduced to remove SV40 T antigen from iSCAPs, proliferation would expectedly decrease (Wang et al. 2014). Furthermore, iSCAPs could differentiate into bone, cartilage, and adipocytes under BMP9 stimulation (Wang et al. 2014). Taken together, iSCAPs get over the challenges of maintaining sufficient SCAPs for studies and will be an essential tool to study SCAP biology and dental repair and regeneration.

Dental pulp stem cells (DPSCs)

Dental pulp stem cells (DPSCs) are regarded as mesenchymal stem cells (MSCs) that possess multipotent differentiation (Anitua et al. 2018; Yamada et al. 2019). Consequently, DPSCs have been thought to be the potential for basic research and clinical use as alternative stem cell sources. It has been demonstrated that DPSCs could differentiate into various cell lines like odontoblasts, osteoblasts, chondrocytes, adipocytes, and neural cells (Anitua et al. 2018). Nevertheless, the mechanisms of DPSCs concerning proliferation, differentiation, migration, and transduction are still not clear-cut (Kitagawa et al. 2007; Yamada et al. 2019). A previous study reported that cultured primary human pulp cells would undergo cellular senescence up to cellular passage seven, and cultured transfected cells would face cellular crisis up to passage 18 after transfection (Galler et al. 2006). Therefore, immortalized DPSCs overpassing cellular senescence is necessary to study the detailed mechanism.

The early human dental pulp cell line DP-1 was immortalized by transferring the hTERT and SV40 genes into cells with liposomes. DP-1 formed a mineralized matrix and expressed alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), and dentin sialophosphoprotein (DSPP) genes (Kamata et al. 2004). Galler et al. (2006) and Li et al. (2020) only transfected DPSCs with SV40 by electroporation or the piggyBac system to make cells immortalized, retaining many phenotypic characteristics of primary cells. Interestingly, deimmortalized human dental pulp cells (dHDPCs) were established by infecting iHDPCs with Ad-FLP recombinase to remove the SV40 T-Ag, which was conducive to the recovery effect of the iHDPCs. In addition, HDP cells were also immortalized with the ectopic expression of hTERT, expressing odontogenic markers of cell proliferation and mineralization. These characteristics were possibly linked with the expression of p16 at a low level (Egbuniwe et al. 2011; Kitagawa et al. 2007). Moreover, a novel immortalized dental pulp cell line was established by co-expressing a mutant cyclin-dependent kinase 4 (CDK4R24C), cyclin D1, and TERT, which maintained stemness characteristics and its original diploid chromosomes (Orimoto et al. 2020). The established immortalized HDPCs with stable growth abilities and normal phenotype will be a good resource for studying the biological characterization of dental pulp cells, dental pulp injury repair, and tooth regeneration (Kamata et al. 2004; Kitagawa et al. 2007).

Periodontal ligament stem cells (PDLSCs)

The periodontal ligament (PDL) is a nonmineralized tissue connecting the cementum with the inner wall of the tooth socket bone that provides anchorage of teeth and cushions for mastication stress (Mi et al. 2011). It is reported that periodontal ligament stem cells (PDLSCs) can differentiate into both osteoblast-like cells and fibroblast-like cells, which play a promising role in periodontal tissue regeneration (Chen et al. 2019). However, the precise differentiation and regeneration mechanism of PDLSCs is unclear due to the limitation of PDLSC lifespan to some extent. Therefore, there is an urgent need for immortalized PDLSC lines to investigate the molecular events (Fujii et al. 2006). At the present, increasing studies have been carried out to establish PDLSC lines successfully. Firstly, animal periodontal ligament cells were immortalized by transduction with SV40, involving immortal periodontal ligament cell lines derived from periodontal ligament cells (PDL) of transgenic mice and rats (D'Errico et al. 1999; Kubota et al. 2004). Subsequently, several immortalized human periodontal ligament cells were achieved by induction of the hTERT gene. Because of a heterogeneous cell population of the PDL, Fujita et al. (2005) reported two different types of cell lines derived from PDL, one that could form a mineralized matrix and another that was not. It was mostly demonstrated that transfected cells retained the characteristics of the original PDL and calcification activity (Fujii et al. 2006; Pi et al. 2007). One cell line, TesPDL3, was indicated to have several phenotype characteristics of fibroblasts, vascular endothelial cells, and osteoblasts (Ibi et al. 2007). In addition, subcutaneous transplantation of these immortal cells in nude mice showed no tumorigenicity (Chen et al. 2019). Amazingly, periodontal ligament cell-derived immortalized cells established from healthy (STPDL) and Down's syndrome patient (STPDLDS) were transfected by SV40T-Ag and hTERT. Definitely, the STPDLDS cell line is expected to serve as a useful tool for the study of periodontal disease in Down's syndrome patients (Asakawa et al. 2022).

Apart from the hTERT gene, human PDL was also immortalized by introducing Bmi1, PDLSC-Bmi1, which maintained the biological functions compared with primary PDLSCs even in a proliferative state (Wei et al. 2017). Surprisingly, PDL cells derived from deciduous teeth were also successfully immortalized, expressing the molecular marker genes of PDL cells and differentiating into osteoblastic cells (Hasegawa et al. 2010). In short, immortal PDLSCs are likely to be a feasible resource for fundamental research of periodontitis and valuable tools for PDL engineering development (Chen et al. 2019; Docheva et al. 2010; Hasegawa et al. 2010).

Cementocyte cells

Cementum is a thin layer of bone-like mineralized tissue produced by cementoblasts, covering tooth root dentin. Periodontal ligament fibers anchor between the cementum and alveolar bone, thus fixing the tooth to the alveolus socket and protecting the integrity of root surfaces (Wang et al. 2016; Zhu et al. 2020). Cementoblasts are relevant to cementum matrix deposition and mineralization, which play an essential role in cementum development and regeneration (Wang et al. 2017; Zhu et al. 2020). Distinctly, it is necessary to establish immortal cementoblasts to overcome cell life span limitations. A cementocyte cell line (OCCM-30) was acquired from OC-tAg transgenic mice, containing the SV40 large T-antigen, expressing cementoblasts makers, such as bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OC) (D'Errico et al. 2000). Another cementocyte cell line, IDG-CM6, was isolated from *Dmp1*-GFP$^{+/-}$ mice, immortalized by using the interferon (IFN)-g-inducible promoter driving expression of a thermolabile large T antigen. The expression profile of this cell line was involved in mineralization and alkaline phosphatase activity (Zhao et al. 2016). Besides, rat cementum lining cells (RCM-C3 and -C4) were also established by transfection of the thermolabile SV40 T-antigen gene (Kitagawa et al. 2005), and the human cementoblast cell line (HCEM) was immortalized by transfection of hTERT gene (Kitagawa et al. 2006). In general,

all the immortalized ementocyte cells serve as a model to study cementogenesis to understand the development, homeostasis, and regeneration mechanisms of periodontal tissues (D'Errico et al. 2000; Kitagawa et al. 2006).

Application of immortalized stem cells from dental/odontogenic tissues

At present, a variety of odontogenic immortalized cell lines have been successfully established. Cell lines such as MDPC-23, OCCM-30, and OLC have played an important role in tissue engineering research, cytological studies, and material studies (Fig. 2) (Arany et al. 2009; de Lima et al. 2009; Hakki et al. 2018; Lee et al. 2010; Li et al. 2021; Simon et al. 2010; Swanson et al. 2020; Zhang et al. 2021). However, due to safety concerns, these cells have not been really used in clinical treatment (Natesan 2005).

Application in tissue engineering

Dental immortalized cells are expected to play an important role in tissue engineering due to their multidirectional differentiation and infinite proliferation. It was suggested that spontaneously immortalized odontoblast cells (OLCs) applied in bioengineered organ germ method developed into teeth after transplantation under the kidney capsule and in vitro (Arany et al. 2009). PDL-like tissue was observed in the periodontal ligament after immortalized mouse dental follicle cells (MDFE6-EGFP cells) were transplanted into severe combined immunodeficiency mice (Yokoi et al. 2007). Additionally, a swine PDL fibroblast cell line, TesPDL3, generated tube-like structures under FGF-2 stimulation (Shirai et al. 2009). Furthermore, immortalized DPSCs could differentiate into neurons, showing neurons' morphological characteristics (Wilson et al. 2015). Although immortalized stem cells from dental/odontogenic tissues have not yet been used in clinical treatment, they have great potential for cellular therapy.

Application in cytological researches

Apart from its application in tissue engineering, many studies have been carried out to understand the pathogenesis and therapy of dental disease. It was reported that the function of OCCM-30 cells would be changed via regulating gene expression and mineral formation with an exposure to bisphosphonates in vitro (Chun et al. 2005). Besides, OCCM-30 cells and PDL cells were applied in the study of internal root resorption (IRR), suggesting the essential role of Chemerin/ChemR23 and C/EBPβ in tooth root resorption (Ito et al. 2018; Ye et al. 2020). For tissue regeneration, bone

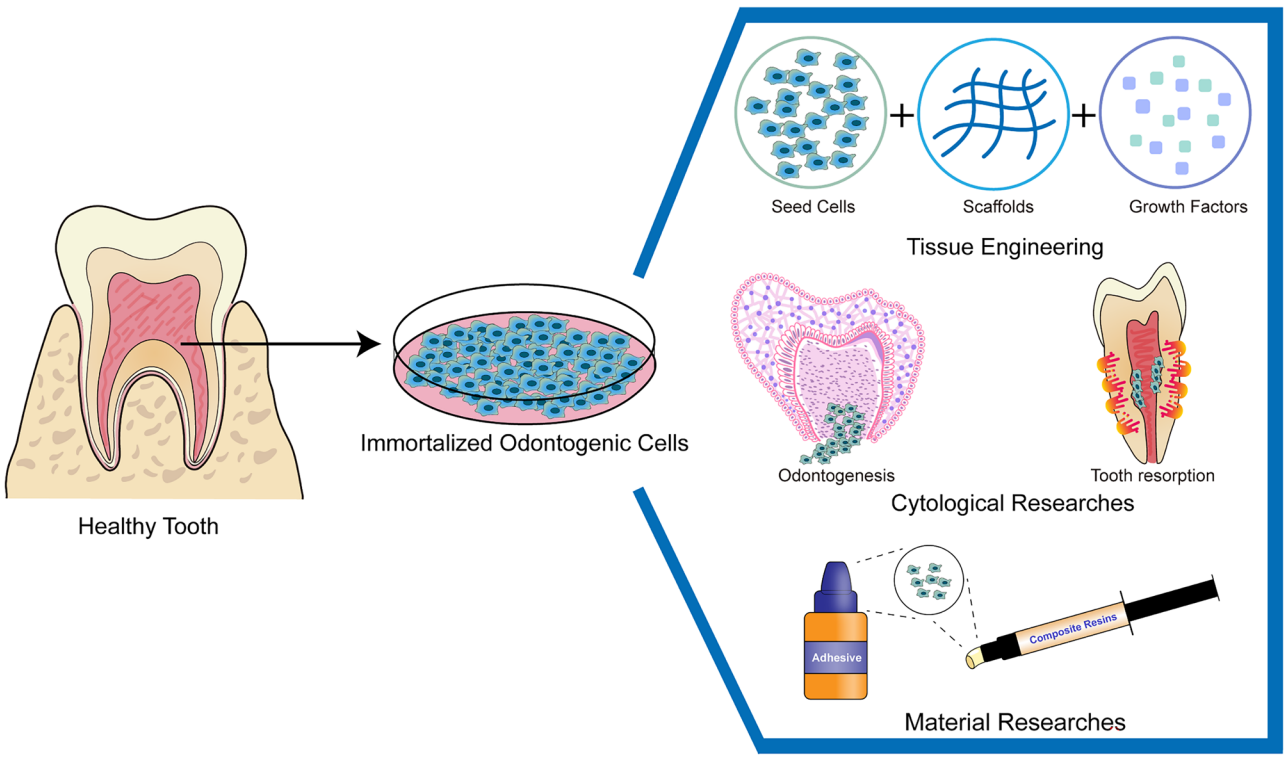

Fig. 2 Application of immortalized cell lines derived from dental/odontogenic tissue. Immortalized cell lines cell lines have been used in tissue engineering research, cytological studies, and material studies

morphogenetic proteins, such as BMP4, BMP7, and BMP9, were demonstrated to increase osteogenic differentiation of OCCM-30 cells (Bozic et al. 2012; Cao et al. 2020; Hakki et al. 2010; Wang et al. 2017). Amazingly, HtgsC-hTERT even proves neuro-protection's ability in vitro by increasing the cell viability of SH-SY5Y treated with hydrogen-peroxide or doxo-rubicin (Yalvaç et al. 2011).

Application in material researches

Several immortalized stem cells from dental/odontogenic tissues have been applied in the biocompatibility testing of dental materials. MDPC 23 is most commonly used to evaluate the cytotoxicity of dental materials, including adhesives, resin-modified glass-ionomer lining cement, and carbamide peroxide bleaching gel (Alvarez et al. 2019; Aranha et al. 2006; de Lima et al. 2009; Lee et al. 2016b). Furthermore, dental resin components, biodentin, MTA, and barrier membranes were tested by immortalized bovine dental papilla–derived cells, immortalized murine pulp cells, OCCM-30 cells, and immortalized periodontal ligament stem cells (Hakki et al. 2009; Spinell et al. 2019; Thonemann et al. 2002; Zanini et al. 2012).

Challenges and perspectives

Currently, multiple immortalized multitudinous cell lines were established to provide a new opportunity in tissue engineering and regenerative medicine, study physiological and pathobiological mechanisms, and test the safety of dental materials (Alvarez et al. 2019; Arany et al. 2009; Ye et al. 2020). These cell lines have overcome cellular senescence and possess unlimited proliferation capacity. However, immortalized cells have not been fully utilized in tissue engineering. To solve this problem, conditional immortalization strategies have been established, such as temperature-based regulation, recombinase-based control, and transcriptional regulation, to acquire accurate excision of oncogenes. Nevertheless, it cannot guarantee that these methods are 100% efficient, and cells need further elimination which has not deleted the transgene (Li et al. 2020; Lin et al. 2013; Luo et al. 2021; MacDougall et al. 1995; Ramboer et al. 2014; Wang et al. 2019). So, the safety problem is still a major obstacle to clinical application, and there are more efforts needed to remove transgenes to guarantee safety.

Another concern is the ability of immortalized cell lines to differentiate. Huang et al. (2015) reported that immortalized dental mesenchymal cell line maintained the ability to differentiate and form mineralized nodules. But Ikbale et al. (2016) proved that the differentiation efficiency of primary DPSCs into osteoblasts was approximately 60% higher than hTERT DPSCs. Concerning the loss of differentiation ability, the decellularized cell-deposited extracellular matrix (DecM) has developed in recent years, which is considered to possess anti-senescence and anti-oxidative effects and promote differentiation (Wang et al. 2019). Besides, bone morphogenetic protein, as an effective bio-facto, is potential in osteo/odontogenic regeneration and tooth engineering (Bozic et al. 2012; Li et al. 2020; Luo et al. 2021). Therefore, it is hopeful that the combination of immortalized stem cells from dental/odontogenic tissues and DecM or the growth factor may overcome the unsatisfactory osteogenic and odontogenic differentiation ability of cells using immortalization strategies alone.

Conventional methods of producing immortalized cell lines often require genome manipulation resulting in genomic instability. Recently, conditional reprogramming (CR) has emerged as a novel tool for the long-term culture of primary cells, such as tumor cells, primary epithelium cells, and so on. Particularly, it does not alternate the genetic background of the primary cells (Wu et al. 2020). CR co-cultured primary cells with irradiated mouse fibroblast feeder cells in the presence of Rho-related protein kinase (ROCK) inhibitor Y-27632. The cell lines generated by CR acquire stem-like characteristics and maintain full differentiation (Liu et al. 2017). Therefore, CR has potentially broad application prospects in the establishment of immortalized stem cells from dental/odontogenic tissue.

Conclusions

At present, many kinds of stem cells from dental/odontogenic tissue have been established into immortalized cell lines, which are mostly used in basic studies, like tissue engineering and cytological and material research. However, although many studies have shown that it is nontumorigenic, the biological behavior of immortalized cells is still questioned due to the existence of foreign genes (Kamata et al. 2004; Lin et al. 2013). In addition, when immortalized cells obtain robust proliferation capacity, the differentiation capacity is decreased to a great extent (Li et al. 2020; Wang et al. 2019). These issues restrict the application of immortalized cells at present, which are expected to be solved in the future.

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Declarations

Competing interests The authors declare no competing interests.

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