REVIEW

New vistas in oral biology and regenerative medicine using immortalized odontogenic cell lines: a systematic review

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ABSTRACT

Aim: To identify the immortalized cell lines of odontogenic origin, their immortalization methods and future directions.

Methods: This systematic-review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and was registered in Open Sciences Framework (DOI 10.17605/OSF.IO/MZ3T6). A broad-based search strategy was utilized using MeSH terms and keywords related to the research question on five databases without any restrictions of languages and the year of publication. A search of the grey literature and reference searching were also done. The screening of the titles and abstracts were performed as per the inclusion criteria. A self-designed, pilot-tested form was used for data extraction and Toxicological Data Reliability Assessment Tool (ToxRTool) was used for assessment of the risk of bias (ROB) in the included studies.

Results: A total of 382 studies were screened and full texts of 45 were evaluated as per the inclusion and exclusion criteria. Finally, a total of 26 articles were included for the qualitative analysis. There were 13 studies related to cells of dental pulp or progenitor cells, 11 related to the cells of periodontium, and two related to ameloblasts. All the studies had been carried out after the year 2000, except for one study. Among the included studies, 11 studies had been conducted on cells of animal origin (Mouse, Rats, Cow and Pig) while 15 had used cells of human origin. The most common method used for the immortalization of cell lines was transfection of the cells with Simian virus (SV40), which was used in 14 of the 26 studies included in the systematic review. Another method was transduction with hTERT gene and transfection with human papilloma virus16 (HPV16). Among the included studies, 11 had low, 12 had moderate and 3 had high risk of bias.

Conclusion: The present systematic review observed that the majority of work has been done on the odontogenic cells of human origin especially in the cells of periodontium, with transfection by SV40 and HPV16 virus sequence being the commonest methods of immortalization. These cells have been envisaged for understanding the molecular biological characteristics, cellular pathways and their applications in the regenerative medicine.

KEYWORDS: Immortalization, senescence, odontogentic cells, SV40, hTERT, HPV16

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INTRODUCTION

One of the characteristic features of all primary cells is to undergo senescence after limited number of cell divisions (Ren et al., 2016). This is an essential feature of the cell cycle and distinguishes these cells from the progenitor-stem cells and tumour cells. The attainment of senescence is also known as "Hayflick limit" and attributed to the shortening of the length of telomeres at the terminal region of the chromosomes on either end (Hayflick and Moorehead, 1961). These changes propel the primary cells towards cell death and increase the risk of cancer (Inada et al., 2019, Ren et al., 2016). In the present era of advanced molecular genetics and regenerative medicine, the senescence of the primary cells limits the research protocols aimed at understanding the intricacies of the cellular pathways (Kitagawa et al., 2007). This is even more important for the cells of odontogenic origin as they are rapidly emerging as the major sources of stem cells and induced pluripotent cells for regenerative medicine (Yin et al., 2016).

Immortalization of the primary cells, as seen in the tumours, was artificially induced in the cell culture experiments by transfection with simian virus 40 (SV40) (Zhang et al., 2019) or human papilloma virus 16 (HPV16) (Pi et al 2007). These viral DNA can be incorporated in the primary cells using plasmids and has been attempted in several cell lines. In the immortalization caused by SV40, there is an increased expression of SV40 large T antigen (TAg) and inactivation of the key tumour suppressor genes (Inada et al., 2019). This integration of the viral sequence has not been associated with problems related to genetic aberrations and tumorigenesis. The HPV16 acts through its oncoprotein E7 and has been recognized as an effective immortalization inducing agent in cells of ectodermal origin with an exclusive binding tendency to proteins of the retinoblastoma family (Pi et al., 2007, Inada et al., 2019). Several researchers have highlighted that the cells of human origin always require these oncogenes (Inada et al., 2019) while a phenomenon of spontaneous immortalization exists in the primary cells of murine origin (Nakata et al., 2003).

Once the somatic cells have been transfected, their characterization has to be performed using molecular biology techniques for identifying the expression of human telomerase reverse transcriptase (hTERT), which essentially is responsible for the maintenance of telomere ends in chromosomes (Orimoto et al., 2020). Similarly, the cells of animal origin too display specific markers reflecting immortalization. Among the cells of odontogenic origin, immortalized cell lines and their characterization has been reported in dental papilla cells, dental follicle cells, dental pulp cells, dental pulp stem cells, periodontal fibroblasts, periodontal ligament cells, cementoblasts etc. Since this technique is still novel and has a futuristic application in dental genetic engineering, regenerative dentistry and oral biology, a systematic review was planned to identify the immortalized cell lines of odontogenic origin, their immortalization methods and future directions. Additionally, an attempt was made to elucidate the outcome assessment methods used to characterize the immortalized cells.

METHODOLOGY

This systematic review was conducted according to the best practices of systematic reviews and guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati et al., 2009), and was registered in Open Sciences Framework (DOI 10.17605/OSF.IO/MZ3T6).

Search strategy

The PubMed, LILACS, Web of Science, Scopus, Embase databases were searched till 31st August 2020, using text words and MeSH terms. There were no restrictions on languages and the year of publication. A search of the grey literature was

performed in Google Scholar and Open-Grey. The research question was detailed in Population (P): Cells of odontogenic origin such as ameloblast, dental pulp cells, periodontal ligament cells, apical papilla cells, periodontal fibroblasts, dental papilla cells, dental follicle cells, dental germ cells, dental stem cells, Intervention (I): techniques for immortalization of cells, Comparator (C): Not applicable, Outcomes (O): immortalization as assessed by various molecular biology techniques. The broad-based search was implemented individually with keywords: "Dental Papilla", "Dental Follicle", "Dental Sac", "Ameloblast", "Dental Pulp", "Dental Pulp Stem Cells", "Odontoblasts", "Periodontal Ligament Cells", "Periodontal Ligament Stem Cells", "Periodontal Ligament Fibroblasts", "Cementoblasts", "Osteoblasts", "Hertwig's Epithelial Root Sheath", "Apical Papilla Cells", "Stem Cells of Apical Papilla", "Stem Cells of Human Exfoliated Dentition" and "Epithelial Cell Rests of Malassez". Partial searches with the Boolean tools "AND" and "OR" was done with the above keywords individually with "immortal", "immortalization", "TERT gene", "hTERT", "SV40" and "HPV16" in different possible combinations. The additional details, if required, were obtained by contacting the authors by email.

Screening and inclusion

The screening of the titles and abstracts were performed as per the inclusion criteria and EndNote reference management software was used for removing the duplicates. Two authors (NT and MA) performed the literature search independently according to this predefined strategy. The in vitro experimental or quasi-experimental studies performed using animal or human cells of odontogenic origin were included in this SR. Studies with inadequate details of the origin of cell and immortalization method or conducted using the cells of non-odontogenic origin were excluded. Two reviewers (NT, MA) analyzed the selected full-text articles to further verify their inclusion independently. In the event of difference of opinions, another reviewer, MR was consulted. Reference lists of eligible studies were cross-checked to identify additional studies. High level of agreement (Cohen's Kappa score 0.93) was found between the two reviewers.

Data extraction

The data extraction was performed by two reviewers (NT, AK) independently using a self-designed form, pilot-tested in 5 studies. In the event of differences of opinion, another reviewer, MR was consulted. Data extraction sheet included demographic variables, details of the cell line used, the origin of cells, immortalization method, outcome assessment method and future directions for the utilization of the established cell lines.

Quality analysis

The Toxicological Data Reliability Assessment Tool (ToxRTool) was used for assessment of the risk of bias (ROB) in the included studies. This was performed by two reviewers (NT and MA) independently with high degree of agreement (Cohen's Kappa 0.92). In any event of disagreement, another reviewer, MR was consulted. Since this systematic review aimed at highlighting the descriptive characteristics, no meta-analysis was performed.

RESULTS

Search results

A total of 367 studies were identified in the databases. Additional sources such as Google Scholar, Open grey and hand searching revealed 108 other studies. After the removal of the duplicates, 382 studies were screened further using their abstracts. After exclusion of 337 studies, 45 were included for screening of the full text as per the strict inclusion and exclusion criteria. Finally, a total of 26 articles were included for the qualitative analysis (D'errico et al., 1999, Kubota et al., 2004, Kamata et al., 2004, Kitagawa et al.,2005, Saito et al., 2005, Fuji et al., 2006, Kitagawa et al., 2006, Yokoi et al., 2006, Galler et al., 2006, Kitagawa et al., 2007, Iwata et al., 2007,

Thonemann et al., 2007, , Pi et al., 2007, Hasegawa et al., 2010, Nam et al., 2014, Li et al., 2019, Zhang et al., 2019, Wu et al., 2010, Yang et al., 2012, Wilson et al., 2015, Huang et al., 2015, Yin et al., 2016, Inada et al., 2019, Orimoto et al., 2020, Nakata et al. 2003, MacDougall et al., 2019). The search results and the reasons for the exclusion of the studies have been presented in the PRISMA diagram (Figure 1).

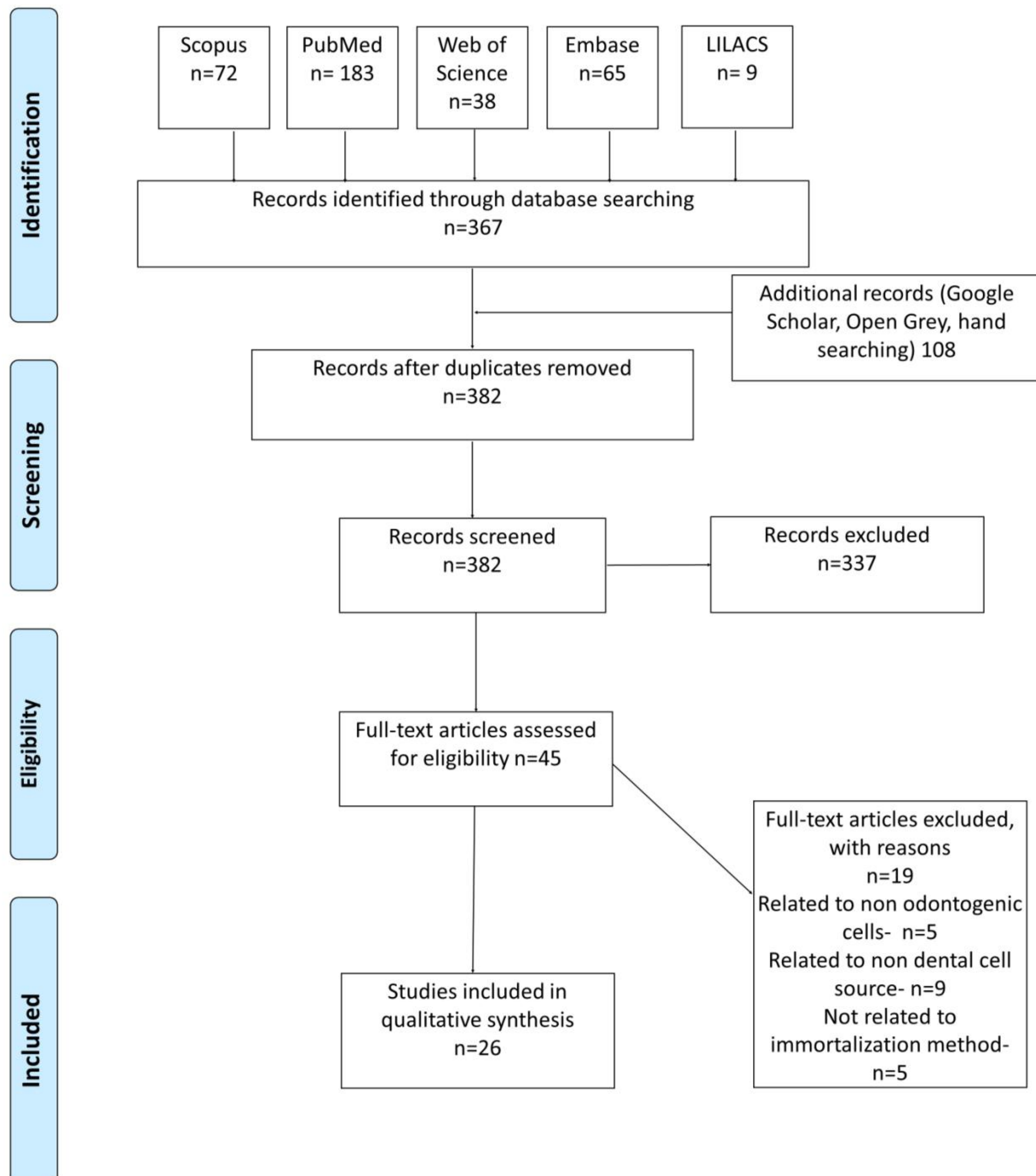

Figure 1: Prisma chart showing the details of the systematic search and the reasons for the exclusion of the studies.

Study demographics and details of cell lines

In order to perform the qualitative synthesis, the studies were grouped on the basis of the type of cells of odontogenic origin used (Table 1). There were 13 studies related to the cells of dental pulp or progenitor cells (Kamata et al., 2004, Yokoi et al., 2006, Galler et al., 2006, Kitagawa et al.,2007, Iwata et al., 2007, Thonemann et al., 2007, Wu et al., 2010, Yang et al., 2012, Wilson et al., 2015, Huang et al., 2015, Yin et al., 2016, Inada et al., 2019, Orimoto et al., 2020), 11 related to the cells of the periodontium (D'errico et al., 1999, Kubota et al., 2004, Kitagawa et al.,2005, Saito et al., 2005, Fuji et al., 2006, Kitagawa et al., 2006, Pi et al., 2007, Hasegawa et al., 2010, Nam et al., 2014, Li et al., 2019, Zhang et al., 2019), and two related to ameloblasts (Nakata et al. 2003, MacDougall et al., 2019). All the studies had been carried out after the year 2000, except for one study (D'errico et al 1999). Among the included studies, 11 studies had been done on the cells of animal origin (Mouse, Rats, Cow and Pig) (D'errico et al., 1999, Nakata et al. 2003, Kubota et al., 2004, Kitagawa et al., 2005, Saito et al., 2005, Yokoi et al., 2006, Iwata et al., 2007, Thonemann et al., 2007, Wu et al., 2010, MacDougall et al., 2019, Li et al., 2019,) while 15 had used cells of human origin (Kamata et al., 2004, Galler et al., 2006, ,Fuji et al., 2006, Kitagawa et al., 2006, Pi et al., 2007, Kitagawa et al., 2007, Hasegawa et al., 2010, Yang et al., 2012, Nam et al., 2014, Wilson et al., 2015, Huang et al., 2015, Yin et al., 2016, Inada et al., 2019, Zhang et al., 2019, Orimoto et al., 2020). The studies involving ameloblasts had not utilized the cells of human origin (Nakata et al. 2003, MacDougall et al., 2019).

Table 1: Showing the study demographics for cells of periodontium, dental pulp and progenitor cells and ameloblasts along with the details of the cell line immortalized and its source.

Author	Year	Journal	Animal/Human- Cell line	Cell Line source
		Cells of periodontium		
D'errico et al	1999	Bone	Mouse- heterogeneous cementoblast/ periodontal ligament cell (CM/PDL)	Molar root surface of H-2KbtsA58 "immorto" mice
Kubota et al	2004	Cytotechnology	Rat- PDL cell lines	PDL cells from rat molars
Kitagawa et al	2005	Bone	Rat- Cementoblasts	Cementoblasts from the root surface of rat PDL
Saito et al	2005	Journal of Bone and Mineral Research	Cow- Cementoblast Progenitor Cells	Bovine Dental Follicular Cells
Fuji et al	2006	Cell Tissue Res	Human- Periodontal Ligament FIbroblasts	Human Molar tooth root
Kitagawa et al	2006	Bone	Human- cementoblasts	Cementoblasts from the root surface of rat PDL
Pi et al	2007	Journal of Periodontal Research	Human-Gingival fibroblasts and periodontal ligament Cells	Human Molar tooth
Hasegawa et al	2010	International Journal of Molecular Science	Human- Periodontal ligament cells derived from deciduous teeth	Healthy human deciduous molars
Nam et al	2014	Molecules and Cells	Human- Hertwig's Epithelial	Human Molar tooth

			Root Sheath/ Epithelial Rests of Malasez	
Li et al	2019	Stem Cell Research and Therapy	Rat- Hertwig's Epithelial Root Sheath	Rat molars
Zhang et al	2019	J Cell Physiol	Human- Hertwig's Epithelial Root Sheath	Human Molar tooth
		Dental pulp and related progenitor cells		
Kamata et al	2004	Journal of Oral Pathology and Medicine	Human dental and periodontal cells	Extracted impacted third molars
Yokoi et al	2006	Cell Tissue Res	Mouse- dental follicle (MDF) cells	Developing molar teeth
Galler et al	2006	European Journal of Oral Sciences	Human- dental pulp cells	Human molar tooth
Kitagawa et al	2007	Archives of Oral Biology	Human- dental pulp cells	Healthy human third molar tooth
Iwata et al	2007	European Journal of Oral Sciences	Pigs- dental papillae	Enamel organ epithelia (EOE) and pulp tissue from pig permanent molars
Thonemann et al	2007	European Journal of Oral Sciences	Cow- dental Papilla cells	Bovine permanent molar teeth
Wu et al	2010	Journal of Cellular Physiology	Mouse- floxed Bmp2 dental papilla mesenchymal cell line	Developing molar teeth
Yang et al	2012	International Endodontic Journal	Human- dental papilla cells (hDPCs)	Human mandibular impacted third molar tooth
Wilson et al	2015	Stem Cells Translational Medicine	Human- deciduous tooth derived Dental Pulp Stem Cells	Extracted/exfoliated deciduous teeth
Huang et al	2015	Journal of Dentistry	Human- dental mesenchymal cells	Dental mesenchymal cells from 19 weeks aborted human foetuses
Yin et al	2016	Stem Cell Research & Therapy	Human- SHED cell line	Exfoliating deciduous teeth (SHED)
Inada et al	2019	International Journal of Molecular Sciences	Human- deciduous tooth derived dental pulp cells (HDDPCs)	Human deciduous tooth
Orimoto et al	2020	PLoSONE	Human- dental Pulp Stem Cells	Human dental pulp stem cells (PT-5025)
		Ameloblasts		
Nakata et al	2003	Biochemical and Biophysical Research Communications	Mouse- ameloblast-lineage cell line	Mouse enamel organ

MacDougall et al	2019	Orthodontics and Craniofacial Research	Rat- ameloblast-like cell lines	Rat enamel organ

Cell type and sources

Among the studies done on the cells of periodontium, three studies each had used cementoblasts (D'errico et al., 1999, Kitagawa et al., 2005, Kitagawa et al., 2006) and Hertwig's Epithelial Root Sheath (Nam et al., 2014, Li et al., 2019, Zhang et al., 2019), two studies had used periodontal ligament cells (Kubota et al., 2004, Fuji et al., 2006) and one study had used cementoblast progenitor cells (Saito et al., 2005). The study carried out by Pi et al. (2007) also included gingival fibroblast cell lines, whereas the source of PDL cells in the study done by Hasegawa et al. (2010) was human deciduous teeth. All these cells had been derived from the roots of the molar teeth. The cell lines from the pulp and related progenitor cells included human dental pulp stem cells in three studies (Wilson et al., 2015, Inada et al., 2019, Orimoto et al., 2020), two of which had used the cells from deciduous teeth (Wilson et al., 2015, Inada et al., 2019). Dental pulp cells had been used in two studies (Galler et al., 2006, Kitagawa et al., 2007) while stem cells of human exfoliated dentition had been used in one study (Yin et al., 2016). Among the progenitor cells, four studies had been done using dental papilla cells of human (n=1) (Yang et al., 2012), mouse (n=1) (Thonemann et al., 2007), cow (n=1) (Wu et al., 2010) and pig (n=1) (Iwata et al., 2007) origin. Other studies had used dental follicle cells (Yokoi et al., 2006), dental mesenchymal cells (Huang et al., 2015) and dental and periodontal progenitors (Kamata et al., 2004). The cells from dental pulp had been derived from the molar teeth while the progenitor cells had been derived from the developing teeth. The study done by Huang et al. had derived the dental mesenchymal cells from 19 weeks aborted human foetuses. The ameloblast cell lines had been obtained from the enamel organs of mouse (n=1) (Nakata et al. 2003) and rat (n=1) (MacDougall et al., 2019) (Table 1).

Methods of immortalization

The most common method used for the immortalization of cell lines was transfection of the cells with simian virus (SV40) (Table 2). This was used in 12 of the 26 studies included in the systematic review (D'errico et al., 1999, Kubota et al., 2004, Kitagawa et al., 2005, Fuji et al., 2006, Galler et al., 2006, Iwata et al., 2007, Wu et al., 2010, Nam et al., 2014, Wilson et al., 2015, Huang et al., 2015, Li et al., 2019, Zhang et al., 2019, . Another common method of obtaining immortalized cells was transduction with hTERT gene, which was used in nine studies (Saito et al., 2005, Fuji et al., 2006, Kitagawa et al., 2006, Kitagawa et al., 2007, Hasegawa et al., 2010, Yang et al., 2012, Yin et al., 2016, Inada et al., 2019, Orimoto et al., 2020). Transfection with human papilloma virus16 (HPV16) was used in six studies Pi et al.,2007, Kamata et al., 2004, Yokoi et al., 2006, Thonemann et al., 2007, MacDougall et al., 2019, Inada et al., 2019. Kamata et al (2004) used both SV40 and HPV16. Saito et al (2005) had used hTERT as well as Bmi-1 methods for establishing immortalized cells. Spontaneous immortalization method was used by Wilson et al. (2015) and Nakata et al (2003). A combination of TERT with Cyclin D1(CKD4) was used in the study done by Orimoto et al. (2020).

Table 2: Showing the study demographics for cells of the periodontium, dental pulp and progenitor cells and ameloblasts along with the details of the methods used for immortalization, the outcome assessment methods and the risk of bias as per OHAT Tool.

Author	Year	Immortalization method	Assessment methods	Risk of bias
Cells of periodontium				
D'errico et al	1999	Breeding of "immorto mice" and CD-1 Mice and SV40 transfection	RT PCR, PTH-mediated cAMP Stimulation Assay, Vitamin D3 Stimulation Assay, Mineralization Assay, Attachment Assay	Low
Kubota et al	2004	PDL of transgenic rats harboring the temperature sensitive simian virus 40 T-antigen gene (TG rats)	RT PCR, Western blot, Mineralization assay	Moderate
Kitagawa et al	2005	pSVtsA58neo	Cell proliferation, Western blot, ALP Assay, Mineralization Assay, RT PCR, Transplantation	Low
Saito et al	2005	Combination of LXSN-Bmi-1 and then with LXSH-hTERT	In vivo differentiation, Osteogenic differentiation, RT PCR, SDS Page Immunoblotting, Telomerase activity, Beta-galactosidase assay	Moderate
Fuji et al	2006	pSV3neo including a neomycin-resistant gene and pLPC-hTERT	Semi-quantitative RTPCR, Western blot analysis, Telomerase activity, Calcification assay	Moderate
Kitagawa et al	2006	Transfection with telomerase catalytic subunit hTERT gene	Telomerase activity, RT PCR, Cell growth assay, Mineralization assay, transplantation	Moderate
Pi et al	2007	HPV 16 using PLXSN vector containing the E6/E7	Cell proliferation, Telomerase activity, ALP Assay, RT PCR, Mineralization assay, Western blot	Low
Hasegawa et al	2010	pBABE-neo-hTERT plasmid containing a neomycin-resistant gene	RT PCR, Calcification assay	High
Nam et al	2014	SV40 LT transfection using pRNS-1 plasmid	FACS analysis, RT PCR, Stemness, Epithelial, mesenchymal transition	Moderate
Li et al	2019	Lentiviral vector which encoded simian virus 40 Large T Antigen (SV40 LT) and a puromycin resistance gene.	Cell proliferation, RT PCR, Immunohistochemistry, Mineralization Assay, Tumorigenic potential	Low
Zhang et al	2019	Transfected with lentiviral vector SV40	Western blot and immunofluoresence staining	Moderate
Dental pulp and related progenitor cells				
Kamata et al	2004	pCI-Neo-hTERT with or without 1microgram of the SV40, HPV16	Detection of senescence-associated b-galactosidase, Telomeric repeat	Moderate

		vectors	amplification protocol assay, Cell proliferation, Tumorigenesis analysis, Mineralized matrix formation, RT PCR	
Yokoi et al	2006	Mutant version of E6 that lacks the C-terminal PDZ-domain	RT-PCR and histochemical analysis	Moderate
Galler et al	2006	pSV3neo (ATCC no. 37150)	Morphology of primary and transfected cells, RT PCR, Immunohistochemistry, detection of intermediate filament proteins	Moderate
Kitagawa et al	2007	Transfection with human telomerase transcriptase (hTERT) gene	Telomerase activity, RT PCR, ALP Assay, Mineralization assay, characterization	Low
Iwata et al	2007	pSV3-neo plasmid (ATCC 37150) by using Lipofectamine 2000	Teleomeric repeat amplification protocol (TRAP), RT PCR, ALP Assay, Mineralization assay, Western blot and cell adhesion assay	Low
Thonemann et al	2007	Transfection with pUC18 HPV 18 LCR-E6-E7 (pf18)	RT PCR, differentiation, proliferation, immortalization, ALP assay	Low
Wu et al	2010	Transduction with SV40 T-Ag	Immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR)	High
Yang et al	2012	hTERT lentivirus	Immunohistochemistry and real-time RT-PCR.	High
Wilson et al	2015	Spontaneous and SV40 large T antigen immortalized	Cytogenic Characterization, immunohistochemistry, tumorigenic potential, proliferation, cytopathology	Moderate
Huang et al	2015	pSV3-neo, a plasmid containing coding sequences of SV40 T-Ag and a neomycin (G418)-resistance	RT PCR, Western blot, immunofluorescence, mineralization, ALP assay	Low
Yin et al	2016	Lentiviral TERT immortalization	Real-time PCR, ELISA, Western blot	Moderate
Inada et al	2019	Transfection with piggyBac (PB)-based transposon vectors carrying E7 from human papilloma virus 16 or human telomerase reverse transcriptase (hTERT)	Cell proliferation, RT PCR, Stemness, multipotency, immortalization, Tumorigenic potential	Low
Orimoto et al	2020	Recombinant retroviruses expressing R24C mutant cyclin-dependent kinase 4 (CDK4R24C), Cyclin D1, and TERT. PQCXIP-CDK4R24C (puromycin-resistant), pQCXIN-Cyclin D1 (G418-resistant), and	Population doublings, Western blot, RT PCR, Cell cycle assay, Senescence-associated β-galactosidase staining, Karyotype analysis, Flow Cytometric analysis, osteogenic and adipogenic differentiation	Low

		pCLXSH-TERT (hygromycin B-resistant)		
Ameloblasts				
Nakata et al	2003	Spontaneous immortalization	Proliferation, differentiation, mineralization assay, histochemical analysis, transplantation	Moderate
MacDougall et al	2019	HPV16 E6/E7 gene platform	Cell proliferation, ALP assay, Mineralization assay, RT PCR, Von Crossa staining	Low

Methods of outcome assessment

Reverse transcription polymerase chain reaction (RT PCR) was the most commonly used method for detection of the expression of immortalization markers. It was used in all studies except Nakata et al (2003) (Table 2). The proliferation, cytogenic characterization, cell cycle assay, karyotyping, telomerase activity tests including telomeric repeat amplification protocol were also done for the same. Stemness and epithelial mesenchymal transition were utilized by Nam et al. (2014) and Inada et al. (2019). The results of RT PCR were correlated with Western blot method in nine studies along with immunohistochemistry histochemical analysis, immunofluorescence and immunoblotting. Beta-galactosidase assay was done in two studies for assessing the senescence of the cells (Kamata et al., 2004, Orimoto et al., 2020). The differentiation of the immortalized progenitor cells was evaluated using mineralization assay in 13 of the included studies (D'errico et al., 1999, Nakata et al., 2003, Kubota et al., 2004, Kamata et al., 2004, Kitagawa et al., 2005, Fuji et al., 2006, Kitagawa et al., 2006, Pi et al., 2007, Kitagawa et al., 2007, Iwata et al., 2007, Hasegawa et al.,2010, Li et al., 2019, MacDougall et al., 2019). Other methods used for the same were stimulation assays, alkaline phosphatase assay, calcification assay and Von Crossa staining. Orimoto et al. (2020) had utilized the osteogenic and adipogenic differentiation as well. The cell transplantation method had been used in three studies (Nakata et al., 2003, Kitagawa et al., 2005, Kitagawa et al., 2006) and tumorigenic potential was evaluated in other three studies (Kamata et al., 2004, Wilson., 2015, Inada et al., 2019).

Risk of bias

Among the studies included, 11 were found to have low risk of bias, whereas the moderate risk of bias was seen in 12 studies. Studies done by Hasegawa et al. (2010), Wu et al. (2010), and Yang et al. (2012) were found to have a high risk of bias (Table 2).

Future directions

The future utilization of the immortalized cell lines, as highlighted in the included studies have been compiled in Table 3.

Table 3: Showing the future directions highlighted by the authors regarding the utilization of the immortalized cells of odontogenic origin

Author	Year	Future directions
Cells of periodontium		
D'errico et al	1999	The cells can improve the understanding regarding the cementoblasts. This can be advantageous in developing periodontal regeneration therapies and decipher the intricacies of mineralization

Kubota et al	2004	Immortalized cells can be a ubiquitous tool for basic science and clinical research in periodontology
Kitagawa et al	2005	To determine the regulatory mechanisms for differentiation and proliferation of cementoblasts
Saito et al	2005	To develop the therapeutic protocols of periodontitis using cementoblast progenitors
Fuji et al	2006	To provide better insight into the biological processes of PDL regeneration
Kitagawa et al	2006	To develop cell models for improving the knowledge about human cementoblasts
Pi et al	2007	They can aid in periodontal research related to signaling mechanisms of osteogenic pathways
Hasegawa et al	2010	To understand the cellular functions and maintenance of PDL tissues and develop regenerative therapies for periodontitis and oral trauma
Nam et al	2014	To improve the understanding about HERS/ERM cells and periodontal regeneration
Li et al	2019	Immortalized cells can act as biologically compatible, unlimited source of cells for cell, developmental, and regenerative biology
Zhang et al	2019	To achieve large-scale research of HERS and dental EMI for future tooth regeneration
Dental pulp and related progenitor cells		
Kamata et al	2004	Cell lines will be useful tools for studying the repair and regeneration of dental and periodontal tissues and various diseases including odontogenic tumors
Yokoi et al	2006	MDFE6-EGFP cells might provide new insights into the mechanisms of PDL formation, including those pertaining to PDL cell differentiation. They may also be a powerful tool in the development of therapeutic strategies for the treatment of periodontitis
Galler et al	2006	To investigate tooth-specific cell metabolism and cell-cell interactions in vitro
Kitagawa et al	2007	These will be useful cell models for studying the mechanism of proliferation and differentiation of odontoblasts
Iwata et al	2007	To advance our understanding of the structures and biological properties of extracellular matrix proteins, it is desirable to express recombinant proteins that closely approximate the structures of the native proteins
Thonemann et al	2007	Cells provide the possibility for the evaluation of tooth specific cell metabolism and cell-cell interactions.
Wu et al	2010	iBmp2-dp cells can be a useful cell model for studying the mechanism of Bmp2 effects on dental papilla mesenchymal cell proliferation, differentiation, and mineralization as well as the potential application of these cells for reparative formation and regeneration of dentin
Yang et al	2012	An immortalized hDPC line at undifferentiated state with odontoblastic differentia-tion potential was established. It will extend the use of hDPCs for future studies, such as molecular mecha- nisms of the initiation of odontoblast differentiation.
Wilson et al	2015	Immortalized dental pulp stem cells (DPSCs) do not form tumors in animals and that immortalized DPSCs can be differentiated into neurons in culture. These results lend

		support to the use of primary and immortalized DPSCs for future therapeutic approaches to the treatment of neurobiological diseases
Huang et al	2015	They can be used for studying the mechanisms of human dental mesenchymal cell differentiation and signalling pathways involved in human odontogenesis
Yin et al	2016	The tumorigenicity of TERT expression in human stem cells needs to be further validated.
Inada et al	2019	These properties would be beneficial for basic research such as exploration of gene function using genetic engineering technology to optimize strategies and protocols prior to the clinical application of HDDPCs
Orimoto et al	2020	These cells might be useful as a biological resource to reduce the cost of pulp regeneration therapy
Ameloblasts		
Nakata et al	2003	ALC should be a useful tool for the analysis of ameloblast nature and for the basic research of tissue engineering technology to repair the tooth
MacDougall et al	2019	These cells will allow researchers to perform the gain-of-function and loss-of-function experiments to investigate the network of genes involved in the processes of enamel ECM secretion, maturation and mineralization. Furthermore, these cells can be used for studies related to enamel and tooth bioengineering

Discussion

Immortalized primary cells can be beneficial for oral biology research and regenerative medicine (Inada et al., 2019). The concept of immortalization, though not new, roots to the properties of the tumour cells with oncogenes (Kitagawa et al., 2005). The reprogramming of the somatic cells was hypothesized and established by Takahashi and Yamanaka in 2006 when they used a combination of four factors Oct3/4, Sox2, c-Myc, and Klf4 to induce embryonic stem cell marker genes in fibroblasts. This research is known to be the basis of the induced pleuripotent stem cells and was awarded Nobel prize for medicine and physiology in 2012 (Tanaka et al., 2020). The immortalization of cells functions on a similar line without altering the potency and differentiation potential of the primary cells. Hence the cells are reprogrammed to continuously divide without senescence and not change to the embryonic stem cell (Zhang et al., 2019). The oral biology applications range from improving the understanding about the cells (progenitor, periodontal, pulpal, ameloblastic), cellular interactions, cellular pathways and their markers, to application of immortalized progenitor cells of odontogenic origin in regenerative medicine. These cells can also be utilized for research related to oncology (Inada et al., 2019).

Since this arena has not been explored in greater detail, it was envisaged that a systematic review identifying the immortalized cell lines of odontogenic origin, their methodologic characteristics and proposed future applications would be helpful. Since systematic reviews are the highest level of evidence in medical research, attempts were made to follow the best practices and address the potential biases (Liberati et al., 2009). The predefined search strategy resulted in the final inclusion of 26 studies conducted using human and animal cells of varied types. The studies which included the cells from non-dental/oral sources were excluded to address the research question effectively. It was observed that the higher number of studies had used the human origin cells as compared to the murine, porcine and bovine cell lines (Kamata et al., 2004, Galler et al., 2006, Kitagawa et al., 2007, Fuji et al., 2006, Kitagawa et al., 2006, Pi et al., 2007, Hasegawa et al., 2010, Yang et al., 2012, Nam et al., 2014, Wilson et al., 2015, Huang et al., 2015, Yin et al., 2016, Inada et al., 2019, Zhang et al., 2019, Orimoto et

al., 2020). This can be reasoned to the less chances of spontaneous immortalization in human somatic cells and their inevitable senescence. On the contrary, the cell lines from mouse and rats are prone to this phenomenon (Nakata et al., 2003). Another reason for the same can be the higher need for understanding the molecular intricacies of human cells for their gene therapy and regenerative medicine applications. There was an almost comparable distribution of studies between the cells of the periodontium and dental pulp/progenitor cells with just a couple of studies done on animal ameloblasts. There was a variation in the cell lines used, both in terms of the origin and type, in the former two categories. It was interesting to note that the usual target cell lines i.e. the periodontal cells, fibroblasts, dental pulp and papilla were replaced by the cells with multipotencies such as HERS and Dental Stem Cells in last five years (Nam et al., 2014, Li et al., 2019, Zhang et al., 2019, Wilson et al., 2015, Huang et al., 2015, Yin et al., 2016, Inada et al., 2019, Orimoto et al., 2020). Similarly, more emphasis has been given to the cells of human origin. These observations can be attributed to the increased thrust in the arena of stem cell research pertaining to human cell lines and their applications in regenerative medicine.

Since senescence of somatic cells is the result of reduction in the length of telomeres at both the ends of the chromosomes, the methods of immortalization have largely focused on incorporating the viral oncogenes into the somatic cells to maintain them (Inada et al., 2019). The present review identified four methods utilized for the immortalization-1) Spontaneous, which was present exclusively in murine cell lines of odontogenic progenitors, 2.) SV40 virus sequence, which acts upon the large T antigen, 3) HPV16 virus sequence, which acts through its oncoprotein E7, and 4) hTERT gene which acts on the telomerase enzyme. It was also observed that the recent studies had tried to explore a combination of immortalization methods as compared to the SV40 virus sequence which was the mainstay of immortalization in the past (Inada et al., 2019, Orimoto et al., 2020). This was primarily done to reduce the risk of the tumorigenic potential of the induced cells and to develop stable and safe protocols for future applications. Similarly, there has been a change in the trend of the use of outcome assessment methods with more emphasis on the RT PCR based evaluation of the TERT gene expression, its correlation with the proteins by using Western Blot and ELISA, and the use of the tests of stemness and differentiation methods. With a better understanding of the molecular genetics and biological methods, a change of the trends of the use of these assessment methods can be justified.

It is always difficult to perform the risk of bias analysis of in vitro experimental studies. This systematic review utilized the ToxRTool which had been developed by Schneider et al. in 2009 as a part of a research project initiated by ECVAM, the European Centre for the Validation of Alternative Methods, of the European Commission's Joint Research Centre in Ispra, Italy (Schneider et al., 2009). This tool comprises of 18 criteria for in vitro studies which are grouped into I) Test substance identification, II) Test system characterization, III) Study design description, IV) Study results documentation, and V) Plausibility of study design and results. It was observed that the majority of the studies had a low or moderate risk of bias, while only three had high risk.

A future implication of these studies and present qualitative synthesis is to identify the applications of these genetically modified cell lines. The majority of authors emphasized that immortalized cell lines of odontogenic origin can aid in improving the understanding of the primary cells and the cellular pathways. Another aspect of its utility is in regenerative dentistry and medicine where these methods can help in generating the large number of cells for stem cell-based therapies.

Since this systematic review was performed on in vitro experimental studies which themselves are regarded as low level of evidence, it can be

regarded as one of its limitations. Additionally, the variability in the design, assessment protocols and the subjective nature of the quality assessment tool are also the limitations of the present systematic review. The methodological transparency and the best practices of evidence-based medicine were used to reduce these biases; however, their complete elimination is not always possible.

CONCLUSION

The present systematic review could grade the cell lines subjected to immortalization as cells of the periodontium, dental pulp and progenitor cells and ameloblasts. Majority of the work has been done on the odontogenic cells of human origin especially in the cells of the periodontium. Among the immortalization methods, transfection by SV40 virus sequence was the most common method. Majority of studies emphasized that this technique can be extremely beneficial for understanding the molecular biological characteristics and pathways of the cells and their applications in regenerative medicine.

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Conflict of interest statement

The authors have declared to have no conflict of interest or competing interest.

Authors' contributions

N.T. conceptualized the idea. N.T. and M.A. performed the literature search, scrutiny of titles and abstracts and the full text articles along with their quality analysis. N.T. and A.K. performed the data extraction. N.T. and M.R. wrote the manuscript and all authors revised it.

Declaration of originality

The authors declare that they have not copied text, figure or data from a particular source without appropriately citing it.

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