MESENCHYMAL STEM CELLS IMMORTALIZATION'S PROTOCOLS AND STATE-OF-ART: A SYSTEMATIC REVIEW AND META-ANALYSIS

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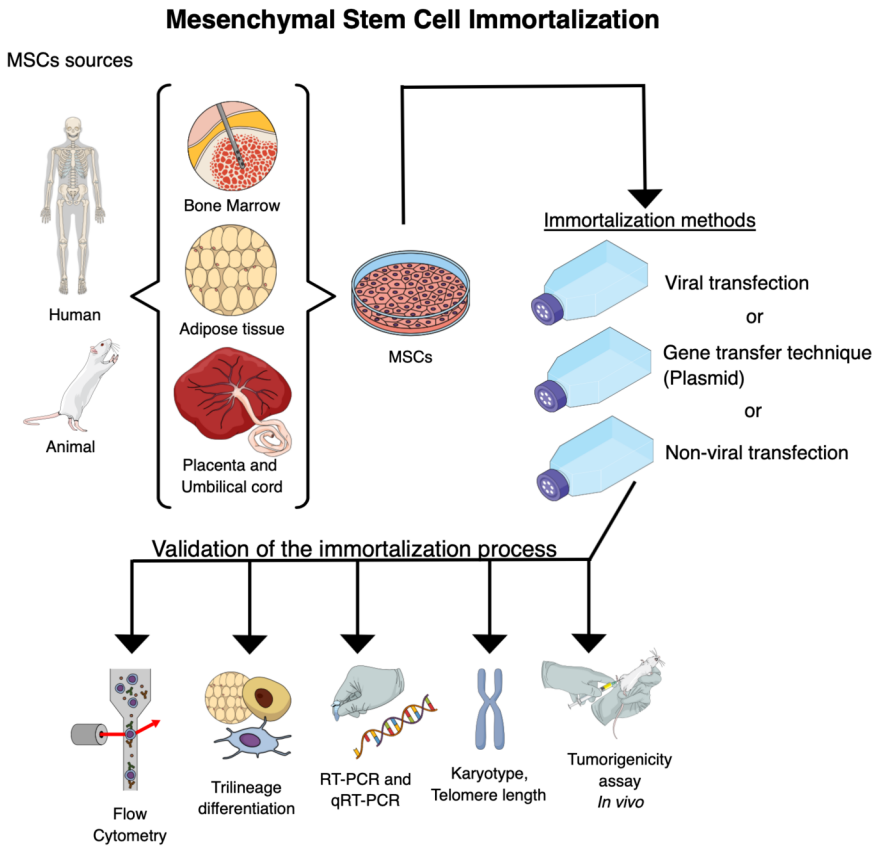
Abstract

Mesenchymal stem cells (MSCs) can be derived from several human and animal sources. According to this systematic review, the immortalization of these cells, by viral or gene transfer techniques (plasmid) and non-viral methods, are useful to ensure the reproducibility of the experiments and the prospect of using these cells in clinical studies. The resultant immortalized MSCs cells must undergo through different validation assays in order to prove their safety and phenotypic and genotypic stability; these assays include flow cytometry for specific MSC markers, trilineage differentiation, RT-PCR and qRT-PCR expression analysis for pluripotency genes, karyotype and telomere length and *in vivo* tumorigenicity assays.

Graphical Abstract



Introduction

Mesenchymal stem cells (MSCs) are adult pluripotent stem cells that are present in bone marrow, adipose tissue, muscle tissue, skin, parenchymal organs (spleen, liver, and pancreas), umbilical blood and cord tissue, among others. These cells present high differentiation capacity, being able to form a variety of tissues that are necessary for cellular repairs, such as kidney and cardiac cells, neurons, osteoblasts, chondroblasts, and others [1, 2]. Therefore, the use of MSCs are of great potential for therapeutic medicine.

When cultured, MSCs present a fibroblast-like morphology, adherence to the plastic substrate, capacity for self-renewal, and differentiation into different cell types [3]. This last characteristic is called plasticity, that is, the ability to form various tissues originating or not from the mesoderm. The International Society for Cellular Therapy (ISCT) determines the criteria for the characterization of human MSCs, which include the capacity of cell adhesion to the plastic substrate, the expression of the specific cell surface markers CD105, CD73 and CD90 and the absence of the expression of hematopoietic markers (CD34, CD45, CD11b, CD19, and HLA-DR) [3]. Besides, MSCs, when induced *in vitro* , must differentiate into osteocytes, adipocytes, and chondrocytes [1, 2, 4, 5]. Scientific evidences suggest that the therapeutic effect of transplanting MSCs is probably not due to the cell itself, but instead, to the paracrine effects that these cells cause, in releasing material into the extracellular environment [6–8].

The population of primary cells, that is, cells isolated directly from tissue specimens, present limited potential for replication, which depends on their origin, species, and age. These cells, after a certain period of growth, enter senescence, therefore ceasing cell proliferation [9]. At the other hand, the immortalization of MSCs allows them to bypass senescence and to be cultured through limitedness cell passages, in a genomically stable fashion, without the bias of cell malignancy or cell transformation. This is essential for the preclinical

phases, where the immortalization of MSCs allows the development of *in vitro*studies with high power of reproducibility and accuracy, such as those involving the production of antibodies, protein purification, establishment of diagnostic assays and test of druggable targets [7–9].

Studies have shown that enzymes that mediate cell immortalization, such as the telomerase, which activity decreases with aging in human somatic cells but is maintained stable in tumor cells, can have their amino acid sequences modified and transfected in non-tumor cells to avoid the process of cellular senescence [10]. Thus, several experimental strategies, such as retrovirus-mediated gene transfer or viral oncogenesis, have been applied for the immortalization of MSCs [11–13].

It is essential to note that cell immortalization is a different concept of cell transformation. When proposing the use of cells in preclinical and clinical studies, it is necessary to use both phenotypically and genotypically stable cells. The process of cell transformation implies malignancy and genomic instability [14], and the use of this type of cell is not suitable for use in preclinical or clinical studies. In contrast, cell immortalization increases the cells' ability to bypass senescence while maintaining genomic and phenotypic stability, which, when proven, allow these cells to be applied for therapeutic clinical purposes [15].

This systematic review aims to verify the methodologies that have already been established for the immortalization of MSCs (human and animal), and the performance of corresponding validation methods according to the ISCR recommended guidelines, in order to determine their safety and reliability for clinical application, such as in regenerative medicine and cell therapy.

The construction of this review was based on the type of the methodologies used for the immortalization of MSCs, the origin of these cells (human or animal), the type of isolated cellular tissue, and the purpose for which the cells were immortalized.

Methods

Study Eligibility Criteria

The inclusion criteria were articles that performed immortalization of mesenchymal stem cells (MSCs), from human or animal origin, provided the protocol of immortalization and the methods used to validate the cell immortalization. The exclusion criteria were: review articles, books' chapters, articles in non-English language, articles that did not describe the immortalization methodology and/or that use another cell type than mesenchymal stem cells. Articles which full text were not available were also excluded.

Study selection and risk of bias in each study

Study selection was evaluated by two independent reviewers. Reviewer one performed the data extraction and Reviewer two, carefully reviewed the data. A third investigator made the final decision of the articles selection and resolved the conflicting points, as bias, for example. The Cochrane instrument was adopted to assess the quality of the included studies [16].

Risk of bias

Considering the Cochrane tool to assess risk of bias, seven variables were analyzed: i. information about ethics committee (animal or human), ii. consent terms signatures (human), iii. presentation of the experiment's success rates, iv. funding source, v. conflicts of interest disclosure, vi. clear description of the immortalization and validation methods and vii. inclusion of the study biases.

Among the 39 studies initially identified, 12 did not disclose the ethics committee, eight did not inform about the consent form, three did not inform about the funding, 20 did not declare conflicts of interest, and 19 did not presented their biases. Regarding the experiment's success rate of the applied methodologies, as well as the clear descriptions of the methods, all the studies were successful. Among the eleven studies that immortalized MSCs from animal sources, ten were considered of high risk and one of low risk according to the predictors analyzed. Of the 28 studies that immortalized MSCs from human sources, 15 were considered of high risk, six of uncertain risks, and seven of low risks.

The 39 articles were included in this review because they presented the methodology described for the immortalization of MSCs and followed the inclusion and exclusion criteria established in this study. In addition to the predictors used, it is also important to note that less than half of the selected studies carried out tumorigenesis tests on the immortalized cells (only 11 from human MSCs immortalization and four from animal MSCs immortalization), which implies an important risk of bias.

Data sources and search strategy

The search strategies for this systematic review were based in the words "mesenchymal stem cells" AND "immortalization" and time of publication of 2000 to 2019. The search was conducted in June 2019 and developed in SCOPUS (Elsevier's and non-Elsevier's database), PUBMED (biomedical literature from MEDLINE, life science journals, and online books) and SCIENCE DIRECT (Elsevier's database).

Statistical Analysis

The statistical analysis of the data was performed by an external collaborator (Dr. Zotarelli Filho) and interpreted by the principal investigator (Dr. Carvalho). For data analysis, a database was built on the Microsoft Excel spreadsheet which was exported to the Minitab 18® statistical program (version 18, Minitab, LLC, State College, Pennsylvania, USA) (Minitab®) and also to the OriginPro® 9 (DPR Group, Inc., Northampton, Massachusetts, USA) (Moberly, Bernards, Waynant, 2018). A common descriptive statistical analysis was performed, obtaining the values of total n, mean and standard deviation, confidence interval (CI) and percentage (frequency) for all the predictors: "Immortalized cell type", "Methodology used for immortalization", "Tests to prove immortalization", "Tumorigenicity assay" and "Genes used for immortalization". One-Way test (ANOVA) was applied, adopting the α-level less than 0.05 with a statistical difference for 95% CI. The R-sq (I2) value was also analyzed to find out the inaccuracy or heterogeneity of the analyzes. The S factor indicates the standard deviation between the data points and the adjusted values. The coding 1 = Yes and 2 = No was used to determine the quantitative values of the mean and standard deviation of the variables, in order to establish the analysis of the propensity score range from 1 to 2. The closest arithmetic mean results code "1" are more prone to the claims that the analyzes of the studies were carried out. The results of the arithmetic mean closest to the "2" code, on the other hand, are more likely to indicate that the studies were not analyzed.

Results

The search strategies resulted in the total identification of 384 articles, 229 in the SCOPUS database, 84 in the PUBMED, and 71 in the SCIENCE DIRECT (FIGURE 1). Ninety-nine articles were found duplicate among these databases. Titles and abstracts were examined from the resulting 285 articles, and the screening was carefully performed considering the inclusion criteria. A total of 246 articles were excluded: 20 were review articles, 7 were book chapters, 121 did not describe the methodology of immortalization of MSCs, 96 used another cell type instead of MSCs and 2 did not include full texts. Therefore, 39 articles were included in this review, 28 of then immortalized MSCs from human sources (TABLE 1), and 11 of them from animal sources (TABLE 2).

Immortalization of Human MSCs

The articles selected that immortalized human MSCs used several sources, including adipose tissue, bone marrow, placenta, tooth, umbilical cord tissue, and umbilical cord blood, amniotic fluid and amniotic membrane. Bone marrow MSCs was the most common human source of tissue immortalized, representing 60.7% of the studies (TABLE 3). Among the articles analyzed, the most used immortalization methodology was viral or retroviral transfection, performed in a total of 75.0% of the articles (TABLE 4).

Gene transfer techniques by plasmid and non-viral transfection were also performed; however, in a few number of articles. Of the 28 articles, 39.3% (TABLE 6) underwent tumorigenicity analysis to verify whether immortalized cells were capable of contributing to the formation of tumors *in vivo* . The most used assays to prove the immortalization and the stability of the immortalized cells were: flow cytometry, RT-PCR, qRT-PCR, Western blot, senescence-associated with beta-galactosidase (SA-ß-gal) and karyotype. The most

used genes related to cell immortalization were *hTERT,* followed by HPV16 E6 / E7. The results referring to the analysis of these 28 articles are represented in TABLES 3 to 6, with the percentages of results referring to the immortalized cell type, the immortalization methods applied, the performance of the tumorigenicity assays, and the types of genes that were used for immortalization.

The differences between the means were statistically significant, with $p<0.005$ for all analyzes. As reference $p<0.005$ was adopted with a statistical difference for 95% CI. In these results, the factors represented by I^2 explain 30.83%, 39.16%, 31.09%, and 16.92% in the variation of responses, respectively in TABLES 3 to 6. The S factor indicated that the standard deviation between the data points and the adjusted values was approximately 0.270, 0.374, 0.377, and 0.278 units, respectively. These low S values demonstrated that all models described the response well, that is, all these standard deviation values are close to the adjusted values. These S results confirm that there was indeed a statistical difference in all analyzes, according to TABLES 3 to 6.

Immortalization of Animal MSCs

Animal source MSCs selected articles, were immortalized from the bovine endometrium, porcine pancreas, canine adipose tissue, Rhesus monkey bone marrow, pig nasal mucosa, lungs, spleen and lymph nodes, mouse bone marrow and dental papilla, and rat bone marrow and tissue adipose. The analyzed studies used three methodologies to immortalize the cells: retroviral/lentiviral transfection, gene transfer technique by plasmid, and spontaneous transformation. The most used methodology was retroviral/lentiviral transfection, performed in a total of 54.5% of articles (TABLE 8).

Of the eleven articles analyzed in this group, 36.4% (TABLE 9) carried out the tumorigenicity assay to assess the ability of these immortalized cells to develop tumors *in vivo* . The assays used to validate the cell immortalization were: flow cytometry, RT-PCR, qRT-PCR, Western blot, colony formation assay on soft agar, karyotype, hematopoietic differentiation, differentiation of germ cells, differentiation of islet-like cells, differentiation of cardiomyocytes, neuronal differentiation, osteogenic differentiation, adipogenic differentiation, chondrogenic differentiation, immunomodulatory properties, cell migration, violet crystal, immunofluorescence for MSCs markers, immunofluorescence for SV40T, immunofluorescence for pluripotent markers, apoptosis analysis, telomeres length analysis, cell cycle analysis, beta-galactosidase assay associated with senescence and MTT for cell proliferation.

The most used gene for cell immortalization was the SV40, followed by the hTERT gene. The TABLES 7 to 10 shows the percentages of results referring to the immortalized cell type, the immortalization method applied, the performance of the tumorigenicity assays, and the types of genes that were used for immortalization.

The differences between the means were statistically significant only in TABLE 9, with $p<0.005$. As reference $p<0.005$ was adopted with statistical difference for 95% CI. In these results, the factors represented by I^2 explain 14.14%, 15.70%, 22.13%, and 18.46% in the variation of responses, respectively, presented in TABLES 7 to 10. The S factor indicates that the standard deviation between the data points and the adjusted values was approximately 0.374, 0.454, 0.401, and 0.410 units, respectively. These low S values demonstrated that all models described the responses accurately, that is, all these standard deviation values are close to the adjusted values. These S results confirm the findings in each analysis, according to TABLES 7 to 10.

Discussion

The aim of this systematic review was to verify the methodologies that have already been established for the immortalization of MSCs (human and animal) and to identify the guidelines recommended for safe immortalization methods so that the cells can be used for clinical applications, such as in regenerative medicine and cell therapy. The search method considered the methodologies used for the immortalization of MSCs, the origin of these cells (human or animal), the type or the isolated cellular tissue, and the purpose for which they were immortalized. One of the biggest challenges of immortalizing cells relies on the maintenance of genotypically and phenotypically stable cell populations, without the induction of malignant transformation, since transformed cells would not be suitable for use in preclinical and clinical tests. Most

of the articles analyzed in this systematic review were successful in immortalizing MSCs, however, most of them did not performed the tumorigenicity assays. Below, the most relevant aspects of these articles will be discussed in relation to the immortalization methodologies used.

This meta-analysis showed that there was a statistically significant difference between the mean values ($p<0.005$) of all analyzes in human MSCs, that is, there was relevant heterogeneity (I2) among the studies approached (TABLES of 3 to 6). In the MSCs originated from animals, the differences between the means showed a statistically significant difference only in relation to the "Tests to prove immortalization" (TABLE 9), with $p<0.005$. However, there was an important heterogeneity (I2) among the studies approached (TABLES 7 to 10). Therefore, altogether these results revealed only the frequency of each predictor ("Immortalized cell type", "Methodology used for immortalization", "Tests to prove immortalization", "Tumorigenicity assay" and "Genes used for immortalization") for each study analyzed in the present study. Further analysis is necessary to expand and establish which of these predictors were the most effective for the entire immortalization process.

The most MSCs type immortalized was from bone marrow, from both human and animal (18.2% from Porcine MSC-BM and 27.3% from Rat MSS-BM) sources. Bone marrow MSCs were first described by Friedenstein et al (1970) and present the characteristics recommended by the ISCT guidelines, such as adhesion on a plastic substrate, ability to proliferate and differentiate into adipogenic, chondrogenic and osteogenic cells [3,55]. The rate of MSCs present in the bone marrow aspirate is 0.001% to 0.1%, which is low when compared to other sources of MSCs, such as those obtained from adipose tissue or Wharton's jelly [56,57]. *In vitro* and *in vivo* properties, such as proliferation and immunomodulatory properties, have already been compared with other sources of MSCs such as adipose tissue, umbilical cord (Wharton's jelly), placenta, among others. In these comparisons it was found that there is a tendency for MSCs from adipose tissue and Wharton's jelly to be more advantageous in relation to the proliferative and immunomodulatory properties when compared, for instance, to MSCs from bone marrow [58–65].

The immortalization of cells often requires more than one type of immortalization factor. For example, the introduction of the telomerase enzyme to block the shortening of telomeres may not be sufficient for immortalization; silencing of protective mechanisms, such as cell cycle regulatory genes, overexpression of oncogenes, oxidative DNA damage, are also required. As mentioned earlier in this review, one of the main precautions that must be taken during cell immortalization, is not inducing the transformation of immortalized cells, considering that the ultimate goal is to transpose these cells to preclinical and clinical studies [9]. The sistematic analysis of the articles selected, showed that the most used method for MSC immortalization was viral transfection, from both human (75%), and animal (54.5%) sources. Transfection of immortalizing genes includes simian virus 40 T antigen (*SV40T*), papillomavirus E6 and E7 (*HPV E6/E7*), adenovirus ElA, Epstein-Barr virus, human T cell leukemia virus, herpes virus, oncogenes, human telomerase reverse transcriptase (*hTERT*) gene,*p53* gene, among others. These genes assist in the maintenance of cell proliferation, not allowing these cells to enter senescence. Achieving unlimited cell proliferation, or prolonging it, especially in primary cultured cells, allows the performance of experiments with high reproducibility, enabling the formation of specific cell lines[66,67].

The methodologies described using the viral transfection technique for the immortalization of human or animal MSCs varied in the number of cells, type of plate used for culturing the cells, time of cultivation, and type of transfected immortalizing gene. However, the vast majority of the studies used a polymer, hexadimethrine bromide, which assists in the neutralization of repulsion load between the virus and cells, allowing greater infection efficiency [68,69]. The six articles analyzed, which immortalized animal MSCs by viral/retroviral transfection, did not show inefficiency in immortalization or any significant modification in the cell phenotype/genotype[44,46,48–51]. However, of the 21 articles selected that immortalized human MSCs through viral transfection, four of them showed that some modification occurred in the cell phenotype or genotype. Dale et al (2015) immortalized MSC-BM through the transfection of *hTERT* and observed that the expression of the surface markers CD90, CD73 and CD105 decreased during the cell passages, which indicated a change in the cells' phenotype, based on the criterion for identifying MSCs according to the ISCT

[3,21].

Huang et al (2008) also immortalized MSCs from the bone marrow through retroviral *hTERT* transduction. They demonstrated, through proteomic analysis, that the profile of 20 proteins related to cell proliferation and transformation was different when compared to non-primary cells immortalized. Although the authors did not compare the protein profile of these cells in the same cell passage, they concluded that the protein profile of immortalized cells is divergent when compared to non-immortalized MSCs. However, the protein profile of immortalized cells was stable during proliferation, suggesting that cells were immortalized and did not transform, since phenotypic and genotypic stability was observed among the immortalized samples[25]. Koch et al (2013) also immortalized bone marrow MSCs through viral transfection and found that the telomeric extension did not compromise the ability to differentiate cells, but did not prevent DNA methylation [28]. DNA methylation can inactivate promoter regions, influencing gene expression by directly interfering with transcription factors or altering histones, and thus altering cell function [70].

Wolbank et al (2009) immortalized MSCs from human adipose tissue and amniotic fluid using the viral transfection technique. In total, the study immortalized four samples, two from adipose tissue and two from amnion membrane; the sample obtained from amnion presented a high immunogenicity rate. In conclusion, the authors emphasized that there may be differences in the characterization of the cells considering the different tissue sources, mainly in respect to the differentiation potential and the immunosuppressive effects. These results reinforce the need to characterize MSCs for their use in clinical studies[32].

Another methodology used by the selected articles was gene transfection by plasmid, accounting for 14.3% and 36.4% for immortalization of human and animal MSCs, respectively. The four articles that immortalized MSCs of animal sources by gene transfection using plasmid were successful in maintaining cell pluripotency and increasing cell life[45,47,52,53]. Two of the studies demonstrated that immortalized MSCs, when injected into immunosuppressed mice, did not cause the formation of tumors, thus being safe for transplantation[45,47].

Of the four articles that immortalized human MSCs using gene transfer technique by plasmid, two did not obtain safe cells, considering that the immortalized cells underwent transformation, and therefore were not suitable for use in cell therapy [20,22,31,35]. Göbel et al (2018) reported that when human MSCs derived from bone marrow were transfected with plasmids of pluripotency factors without reprogramming, it did not prolong the expansion of the culture. Therefore, these cells would not be suitable for application in cell therapy. Yamaoka et al (2011) reported that MSCs from human bone marrow were immortalized and cultured for the long term with the maintenance of telomeres, and these cells maintained their morphological, phenotypic and functional characteristics. However, when these cells were tested for tumor formation in immunosuppressed mice, they were able to form tumors, and consequently, were not viable for translation.

Non-viral transfection of genes is dependent on the chemical or physical delivery of the genetic material to the cell to be immortalized, and most non-viral methods have low immunogenicity and toxicity when compared to viral vectors. The main disadvantage of non-viral gene transfection is low efficiency. In this review, 91% of the analyzed articles which immortalized animal MSCs, used spontaneous transformation, and 10.7% of the analyzed articles, which immortalized human MSCs, used non-viral/plasmid transfection. Zheng et al (2013) spontaneously immortalized MSCs in the bone marrow of rats. They found an epigenetic characteristic, determined by the methylation of histones and DNA, associated with the $P16^{Ink4a}$ gene, which is a tumor suppressor gene that have a function in the cell cycle, more specifically in cell senescence. The authors conclude that MSCs spontaneously transformed *in vitro* did not necessarily undergo malignant changes, considering that this mechanism that occurred in animal cells may not occur in human cells [54]. Besides, it should be noted that, according to the literature, this epigenetic characteristic of histone and DNA methylation associated with the$P16^{Ink4a}$ gene can lead to cellular malignancy since the overexpression of this gene is associated with several tumors[71].

Studies with immortalization of human MSCs that did not use viral transfection have chosen to immortalize cells with commercial systems, including the SV40 T antigen immortalization system (SV40T) based on the PiggyBac transposon [36], the AMAXA system (Lonza, NJ, USA) [42], and the jetPEI reagent (Polyplus

Transfection, Illrich, France) [26]. Shu et al (2018) were successful in immortalizing umbilical cord MSCs using the SV40 T antigen immortalization system (SV40T) based on the PiggyBac transposon, resulting in cells with a positive expression for MSC markers, differentiation capacity, and no tumorigenicity *in vivo* . Lee et al (2014) used the AMAXA system for cell immortalization, which involves electroporation, a technique that increases the permeability of the cell membrane to which the DNA is introduced [72]. Through this system, the authors managed to immortalize a cell line from the placenta with proven potential for differentiation and prolonged cell proliferation. Hung et al (2010) developed a line of MSCs from umbilical cord blood immortalized with *hTERT* expression using a commercial polymer, resulting in the absence of tumor formation and chromosomal abnormalities, and with the potential for differentiation. This polymer (jetPEI(r)) has the function of compacting DNA into positively charged particles (or nanoparticles), called complexes. These complexes can interact with anionic proteoglycans located on the cell surface, binding to the cell membrane, and then internalized by endocytosis. When the endosome internalizes DNA, the jetPEI(r) reagent protects it from degradation, disrupting the endosome and releasing DNA from the complexes in the cytoplasm. Thus, jetPEI(r) allows the DNA to be transported to the cell nucleus and transcription to occur[73].

MSCs or cells that are capable of self-renewal and pluripotency cannot be applied in regenerative medicine unless it is proven that these cells do not form tumors in mice. According to the literature, the higher the cell's capacity for self-renewal and pluripotency, the higher the capacity of these cells to form tumors, considering that stem cells and tumor cells share similar genetic characteristics, such as the expression of the *c-MYC* and *KLF4* genes[74]. This statement suggests that the less a cell can cause tumors, the less it can renew or differentiate itself. Consequently, these cells would lose all the characteristics that are desired in a stem cell to be used in cell therapy, such as the ability to self-renew and generate different tissues, which are critical to assist in the repair of injuries. Among the articles analyzed in this review, only 39.3% and 36,4% of human and animal MSCs immortalization protocols, respectively, underwent the tumorigenicity test in order to certify that the immortalized MSCs were viable for possible translation to preclinical and clinical studies. The fact that the criteria to prove the non-tumorigenicity of cells is not well established and there is no consensus in the literature of which is the best method, may be the reason why more than half of the analyzed articles did not perform this specific type of test. On the other hand, other tests associated with tumorigenesis can be considered to authenticate the cell, such as qRT-PCR for pluripotency genes and oncogenes, cell proliferation tests in late cell passages, karyotype, variation in the genomic copy number (CNV), genomic sequencing, FISH (Fluorescence *In Situ*Hybridization), soft agar assay to verify the formation of cell colonies, among others.

Conclusion

In this systematic review it was found that the majority of immortalized MSCs, both of human and animal origin, were from bone marrow, which can be due to the fact that it was one of the first sources of MSCs studied and the most well established. The virus-mediated gene transfection was observed as the most used and established technique, accounting for 75% and 54.5% of the articles that immortalized human and animal MSCs, respectively. This was an interesting finding, considering that although it is well-established, it is an old technique which carries risks of contamination through the viral manipulation. Therfore studies have to consider biosafety care and carry out the final characterization of the cells to prove that the immortalized cells are viable and can be used in preclinical and clinical application future objectives.

Another essential point identified in this review, was that despite of the use of new genes in cell immortalization, the insertion of the*hTERT* gene is still the most used gene for this process, suggesting that the maintenance of telomerase is efficient for maintaining cell proliferation and bypassing cell senescence.

It was also observed that the tests used to prove the phenotypic and genotypic characteristics of cells were the most varied, including qRT-PCR for pluripotency genes and oncogenes, cell proliferation tests in late cell passages, karyotype, variation in the number of genomic copies (CNVs), genomic sequencing, FISH (Fluorescence *In Situ*Hybridization), soft agar assays for verification of cell colonies formation, among others. It is also concluded that the tumorigenicity tests of immortalized MSCs was carried out in less than half of the studies (39.3% from human and 36.4% from animal MSCs immortalized). This low number demonstrates

that this type of test is not performed for immortalized MSCs by the majority of the authors. However, due to the existence of risks as mentioned above, for the translation of the MSCs for both humans and animals, we concluded that the tumorigenicity tests will become mandatory in order to safely use the immortalized MSCs.

Limitations and Bias

It is necessary to expand and establish which type(s) of analyze(s) within each predictor "Immortalized cell type", "Methodology used for immortalization", "Tests to prove immortalization", "Tumorigenicity assay" and "Genes used for immortalization" were the most effective for the entire immortalization process.

Author contributions

Study concept: KATC.; PEFS. Study design: KATC.; PEFS. Literature search: PEFS.; ACI. Data analysis: IJZF. Manuscript editing and review: PEFS, ACI, IJZF, KATC, LRC.

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Competing interests

The authors declare no competing interests.

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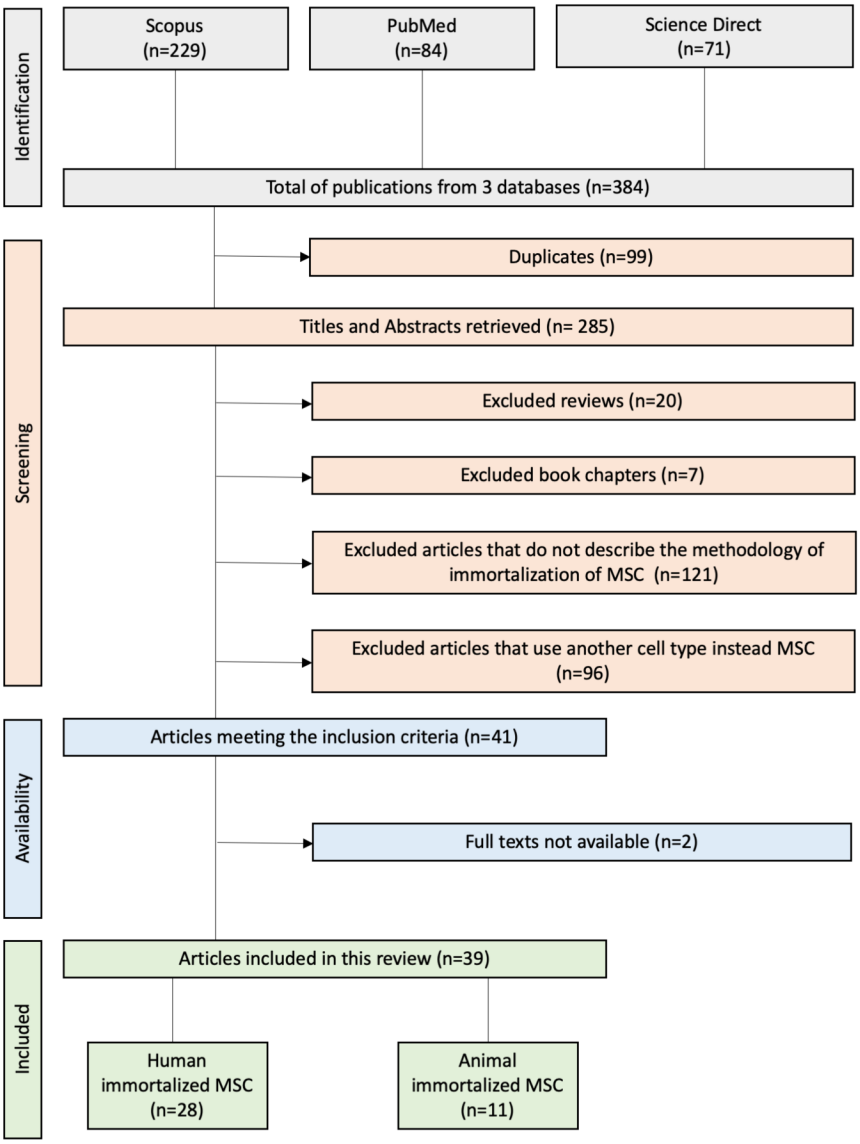


Figure 1. Flowchart representing the research strategy used to construct the systematic review regarding the immortalization protocol of human and animal mesenchymal stem cells (based on Preferred Reporting Items for Systematic review and Meta-Analysis Protocols - PRISMA 2015. Available in: http: //www.prisma-statement.org/.

Table 1. Immortalization of Human MSCs: reference, cell type, immortalization protocol, assays used to validate the immortalization process and the corresponding results.

Reference	Cell Type	Immortalization Methodology
[17]	MSC-AT	Transfection using the lentivirus vector *hTERT* only or associated with *S*
[18]	MSC-BM	Transfection using lentivirus vectors Lenti-*hTERT-eGFP (*LG508, Biogen
[19]	MSC-AT	Transduction using lentivirus with a library of 33 genes
[20]	MSC-BM	Transfection with a plasmid containing the type 16 human papilloma viru
[21]	MSC-BM	Retroviral transduction with *hTERT*

[22]	MSC-BM	Transfected with pluripotency factors using plasmids
[23]	MSC-BM	Retroviral transfection of the vector *BABE-hygro-hTERT*
[24]	MSC -BM	Retroviral transfection with *HPV16 E6/E7*
[25]	MSC-BM	Retroviral transduction of *hTERT* gene
[26]	MSC from umbilical cord blood	Non-viral transfection using a commercial vector expressing *hTERT*
[27]	MSC-BM	Retroviral transduction containing *HPV16 E6/E7*
[28]	MSC-BM	Lentivirus transfection of *SV40/hTERT*
[29]	MSC-BM	*hTERT* retroviral transduction and *WNT3A* retroviral transduction
[30]	MSC from placenta	Transduction with lentiviral-mediated *hTERT* and Bmi-1
[31]	MSC-BM	Transfection of *hTERT* gene
[32]	MSC from amniotic and MSC-AT	Retroviral transfection of *hTERT* gene
[33]	MSC from amniotic fluid	Retroviral transfection of genes *16E6 e E7* (*HPV16E6E7*), *BMI*-1 and/o
[34]	MSC-BM	Retroviral transfection of *16E6 e E7* (*HPV16E6E7*), *BMI*-1 and/or *hTE*
[35]	MSC from umbilical cord	Transfection of *hTERT* gene and secreted glucose biosensor transgene us
[36]	MSC from umbilical cord	PiggyBac transposon–based simian virus 40 T antigen (*SV40T*) immorta
[37]	MSC-BM	Retroviral transduction of *hTERT* and *BMI*-1 genes
[38]	MSC from placenta	Retroviral transduction of *hTERT*
[39]	MSC-BM	Retroviral transfection of *hTERT* gene
[40]	Commercial MSC	Retroviral transduction of *hTERT* gene associated with human papilloma
[41]	MSC-BM	Retroviral transfection of *SV40*
[12]	MSC-BM	Silencing p53 expression associated with *hTERT* gene transfection
[42]	MSC from placenta	*hTERT* gene transfection using AMAXA commercial system
[43]	MSC-BM	Retroviral transfection of vector encoding *v-MYC* oncogene

Notes: MSC (mesenchymal stem cells); MSC-BM (Bone Marrow Mesenchymal Stem Cells); MSC-AT (adipose tissue mesenchymal stem cells);*SV40T* (simian virus T antigen 40); *HPVE6/E7* (papilloma virus E6 and E7); qRT-PCR (reverse transcriptase reaction followed by real-time polymerase chain reaction); RT-PCR (reverse transcriptase reaction followed by polymerase chain reaction); *hTERT* (human telomerase reverse transcriptase); trilineage differentiation corresponds to adipogenic, chondrogenic and osteogenic differentiations.

Table 2. Immortalization of Animal MSCs: reference, cell type, immortalization protocol, assays used to validate the immortalization process and the corresponding results.

Reference	**Cell Type**	**Immortalization Methodology**	**Immortalization Validation Assays**	**Results**
[44]	Bovine endometrial MSC	Viral transfection of *HPV16 E6/E7* gene	Flow cytometry, differentiation potential (osteogenic and chondrogenic), cell migration and immunomodulatory property	Immortalized MSCs shared common MSCs characteristics

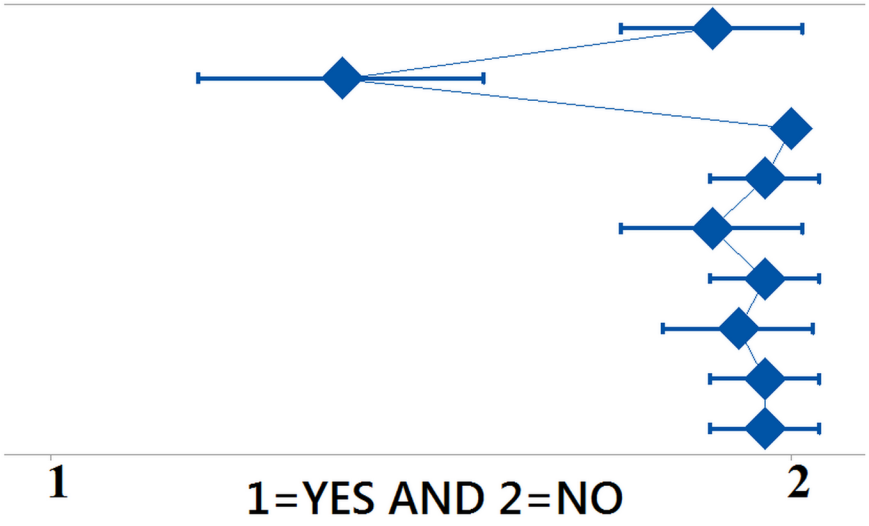
[45]	MSC from porcine pancreas	*hTERT* gene transfection by Lipofectamine Plus (Invitrogen)	Growth curve, flow cytometry, immunocyto-chemistry, qRT-PCR, western blot, tumorigenicity assay, neural and cardiomyogenic differentiation	Immortalized MSCs were grown for more than 80 passages, with proven differentiation capabilities
[46]	Canine MSC-AT	Viral transfection containing *SV40* gene	Cell doubling time, differentiation potential, telomere length assay, immunocy-tochemistry, western blot, transplantation in mouse with infertility induced	Canine MSCs were successfully immortalized, cultured for over a year and maintained a mesenchymal phenotypic profile and multiplication capacity *in vivo*.
[47]	MSC-BM of Rhesus monkey	Plasmid containing the *hTERT* gene	RT-PCR, flow cytometry, osteogenic differentiation, karyotype, growth curve, apoptosis analysis and, tumorigenicity assay	Immortalized MSCs had a prolonged useful life and maintained typical MSC characteristics without malignant transformation
[48]	Pig MSC from nasal mucosa, lungs, spleen, lymph nodes and bone marrow	Transfection of recombinant lentivirus containing the sequence encoding *SV40LT*	Flow cytometry, proliferation rate, cell cycle analysis, trilineage differentiation, blood monocyte co-cultivation	Isolated MSCs were successfully immortalized, maintained their stem properties and exhibited im-munomodulatory effects on blood monocytes
[49]	Porcine MSC-BM	Transfection of recombinant lentivirus containing the sequence encoding *SV40LT*	Hematopoietic differentiation, immunofluorescence for SV40T	A successfully model was established to differentiate hematopoietic cells co-cultured with immortalized MSCs into mono-cyte/macrophage lineage cells CD4 and CD8 cells.

[50]	Rat MSC-BM	Retroviral transfection expressing *SV40T*	Growth curve, trilineage differentiation, neuronal differentiation and tumorigenicity assay	Immortalized MSCs retained their characteristics as primary MSCs, also exhibited high proliferation, differed in neurons in vitro and did not form tumors *in vivo*.
[51]	MSC from mice dental papilla	Lentiviral transfection of recombinant *SV40LT* antigen	RT-PCR, immunohisto-chemistry, analysis of alkaline phosphatase activity and osteogenic differentiation	Immortalized MSCs exhibited a high proliferation rate, maintained genotypic and phenotypic characteristics similar to primary cells, and had the ability to differentiate into cells with osteogenic characteristics.
[52]	MSC-BM from mini-pig	Plasmid with *hTERT*	Immunofluorescence, flow cytometry, expression of *hTERT* by RT-PCR, karyotype, osteogenic differentiation, cell growth curve	Transfected MSCs showed prolonged life, maintained similar morphology and karyotype compared to primary MSCs
[53]	Porcine MSC-BM	Plasmid containing part of *SV40* genome	Cell growth test (MTT), im-munofluorescence staining, flow cytometry, qRT-PCR, trilineage differentiation, cardiomyocyte differentiation	Immortalized MSCs exhibited higher proliferative capacities than parental cells, and maintained pluripotency capacity

[54]	MSC-BM from rat	Spontaneous transformation	Flow cytometry, adipogenic and osteogenic differentiation, colony unit formation assay, RT-qPCR, western blot	MSCs reached replicative senescence after 24 to 25 population duplications, showed increased expression of P*16INK4a* and *P21* and regulated phosphorylation of the Retinoblastoma protein.

Notes: MSC (mesenchymal stem cells); MSC-BM (Bone Marrow Mesenchymal Stem Cells); MSC-AT (adipose tissue mesenchymal stem cells);*SV40T* (simian virus T antigen 40); *HPVE6/E7* (papilloma virus E6 and E7); qRT-PCR (reverse transcriptase reaction followed by real-time polymerase chain reaction); RT-PCR (reverse transcriptase reaction followed by polymerase chain reaction); *hTERT* (human telomerase reverse transcriptase); trilineage differentiation corresponds to adipogenic, chondrogenic and osteogenic differentiations.

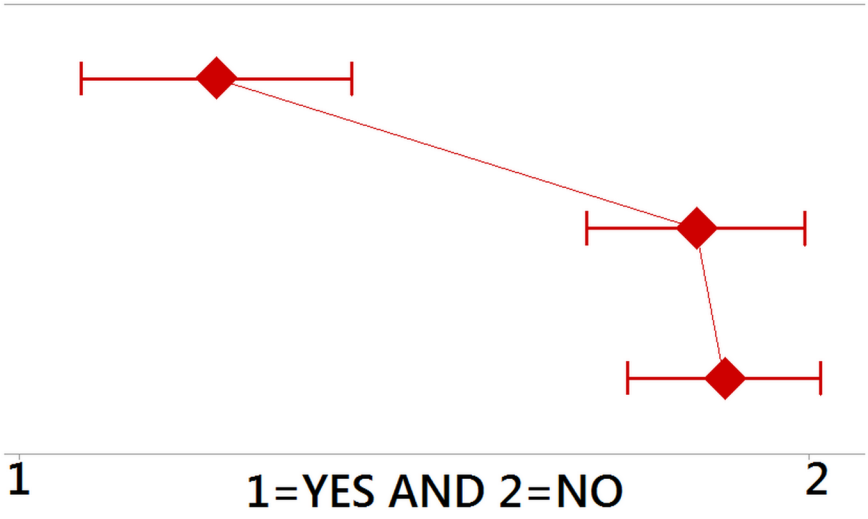
Table 3. Human MSC immortalization - Immortalized cell type, with p <0.005 with statistical difference for 95% CI.**Note:** *p=0.000<0.005 and* I^2 *= 30.83% S=0.270* . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.



MSC type	N	Mean	StDev	95% CI	%(1=YES)
MSC-AT	28	1.8929	0.3150	(1.7922; 1.9935)	10.7
MSC-BM	28	1.3929	0.4973	(1.2922; 1.4935)	60.7
MSC-TOOTH	28	2.000	0.000	(1.899; 2.101)	0
MSC- UCB	28	1.9643	0.1890	(1.8637; 2.0649)	3.6

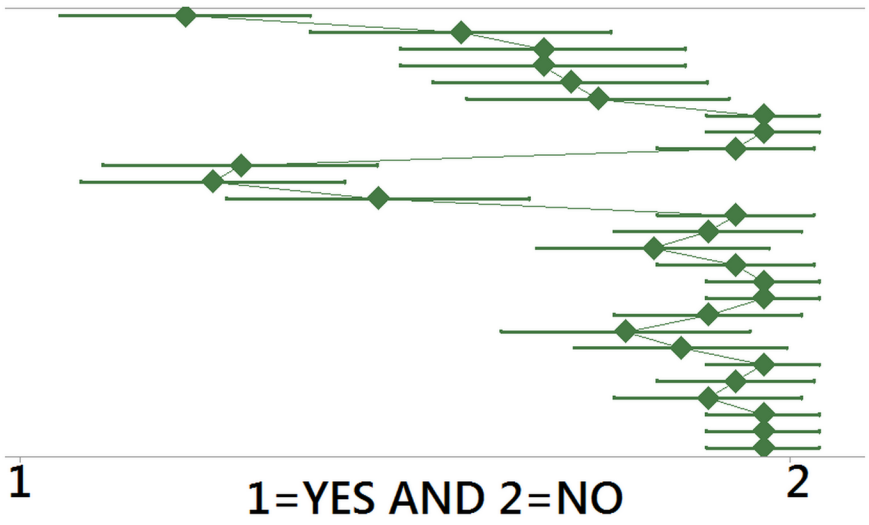
MSC type	N	Mean	StDev	95% CI	%(1=YES)
MSC-PLACENTA	28	1.8929	0.3150	(1.7922; 1.9935)	10.7
MSC- AMNIOTIC FLUID	28	1.9643	0.1890	(1.8637; 2.0649)	3.6
MSC-UC	28	1.9286	0.2623	(1.8280; 2.0292)	7.1
COMERCIAL MSC	28	1.9643	0.1890	(1.8637; 2.0649)	3.6
MSC- AMNIOTIC MEMBRANE	28	1.9643	0.1890	(1.8637; 2.0649)	3.6

Table 4. Human MSC immortalization - Immortalization method applied, with p <0.005 with statistical difference for 95% CI. **Note:** *p=0.000<0.005 and I^2 =39.16% S=0.374* . . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.



Method	N	Mean	StDev	95% CI	%(1=YES)
Viral/retroviral transfection	28	1.2500	0.4410	(1.1092; 1.3908)	75.0
Gene transfer (plasmid)	28	1.8571	0.3563	(1.7163; 1.9979)	14.3
Non-viral transfection	28	1.8929	0.3150	(1.7521; 2.0337)	10.7

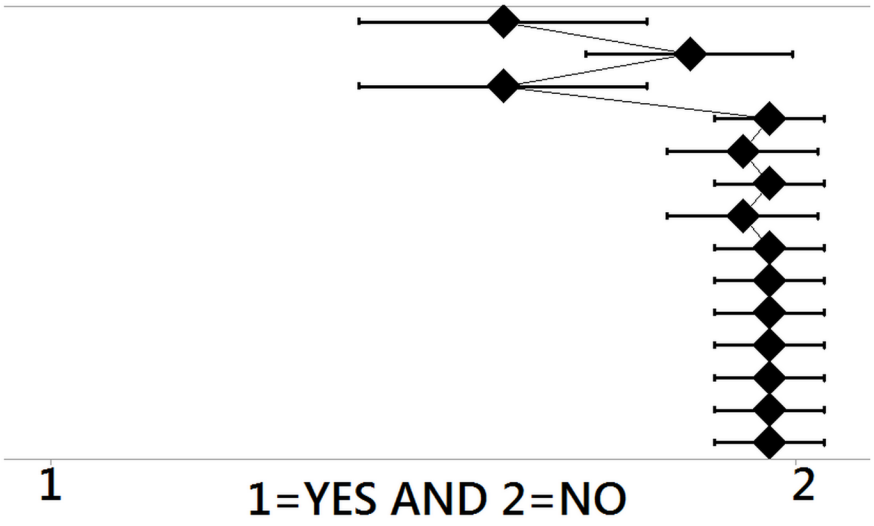
Table 5. Human MSC immortalization - Tests to prove immortalization, with p <0.005 with statistical difference for 95% CI. **Note:** *p=0.000<0.,005 and I^2 = 31.09% S=0.347* . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.



Assay	N	Mean	StDev	95% CI	%(1=YES)
Flow Cytometry	28	1.2143	0.4179	(1.0853; 1.433)	78.6
RT-PCR	28	1.5714	0.5040	(1.4424; 1.7004)	42.9
qRT-PCR	28	1.6786	0.4756	(1.5496; 1.8076)	32.1
Western Blot	28	1.6786	0.4756	(1.5496; 1.8076)	32.1
beta galactosidase-associated s	28	1.7143	0.4600	(1.5853; 1.8433)	28.6
Karyotype	28	1.7500	0.4410	(1.6210; 1.8790)	25.0
FISH	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
Neuronal differentiation	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
Cardiomyogenic differentiation	28	1.9286	0.2623	(1.7996; 2.0576)	7.1
Osteogenic differentiation	28	1.2857	0.4600	(1.1567; 1.4147)	71.4
Adipogenic differentiation	28	1.2500	0.4410	(1.1210; 1.3790)	75.0
Chondrogenic differentiation	28	1.4643	0.5079	(1.3353; 1.5933)	53.6
Hepatocyte differentiation	28	1.9286	0.2623	(1.7996; 2.0576)	7.1
Immunomodulatory properties	28	1.8929	0.3150	(1.7639; 2.0218)	10.7
Colony forming unit	28	1.8214	0.3900	(1.6924; 1.9504)	17.9
Migration potential	28	1.9286	0.2623	(1.7996; 2.0576)	7.1
Crystal violet	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
MTT assay	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
Telomere assay	28	1.8929	0.3150	(1.7639; 2.0218)	10.7
Telomerase activity assay	28	1.7857	0.4179	(1.6567; 1.9147)	21.4
Telomere length assay	28	1.8571	0.3563	(1.7282; 1.9861)	14.3
Whole genome hybridization array	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
cDNA microarray analysis	28	1.9286	0.2623	(1.7996; 2.0576)	7.1
Soft agar assay transformation	28	1.8929	0.3150	(1.7639; 2.0218)	10.7
Protein kinase A catalytic activity	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
Copy number variation (CNV)	28	1.9643	0.1890	(1.8353; 2.0933)	3.6
Proteomic analysis	28	1.9643	0.1890	(1.8353; 2.0933)	3.6

Table 6. Human MSC immortalization - Tumorigenicity assay and genes used for immortalization, with p <0.005 with statistical difference for 95% CI. **Note:** *p=0.000<0,005 and I2 = 16.92% S=0.278.* The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for

each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.



Tumorigenicity /immortalization gene	**N**	**Mean**	**StDev**	**95% CI**	**%(1=YES)**
Tumorigenicity assay	28	1.6071	0.4973	(1.5039; 1.7104)	39.3
HPV16 E6/E7	28	1.8571	0.3563	(1.7539; 1.9604)	14.3
hTERT	28	1.6071	0.4973	(1.5039; 1.7104)	39.3
Library of expansion genes	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
HPV16E6E7, bmi-1 and/or *hTERT*	28	1.9286	0.2623	(1.8253; 2.0318)	7.1
PiggyBac transposon–based	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
hTERT and *Bmi1*	28	1.9286	0.2623	(1.8253; 2.0318)	7.1
SV40 gene	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
p53 knockdown and *hTERT*	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
hTERT combined with *SV40* or *HPVE6/E7*	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
hTERT/SV40	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
Pluripotency factors	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
V-MYC oncogene	28	1.9643	0.1890	(1.8610; 2.0675)	3.6
hTERT associated with *Wnt3a*	28	1.9643	0.1890	(1.8610; 2.0675)	3.6

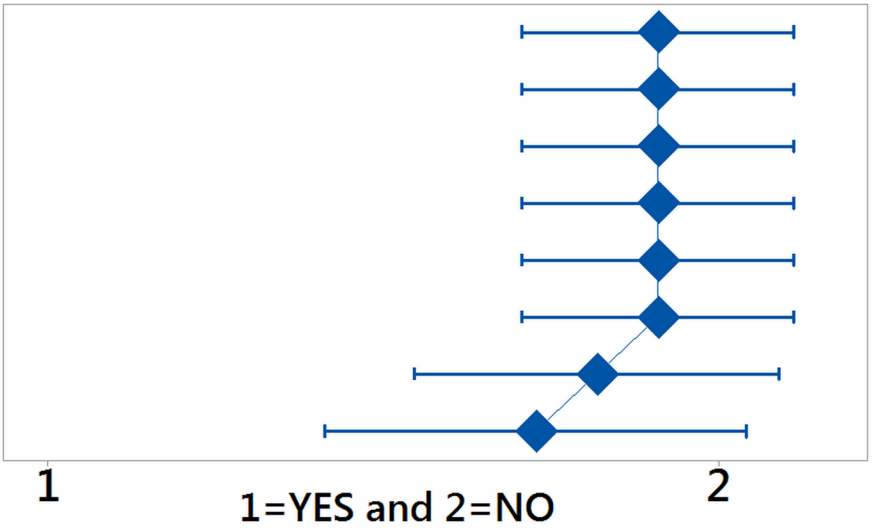
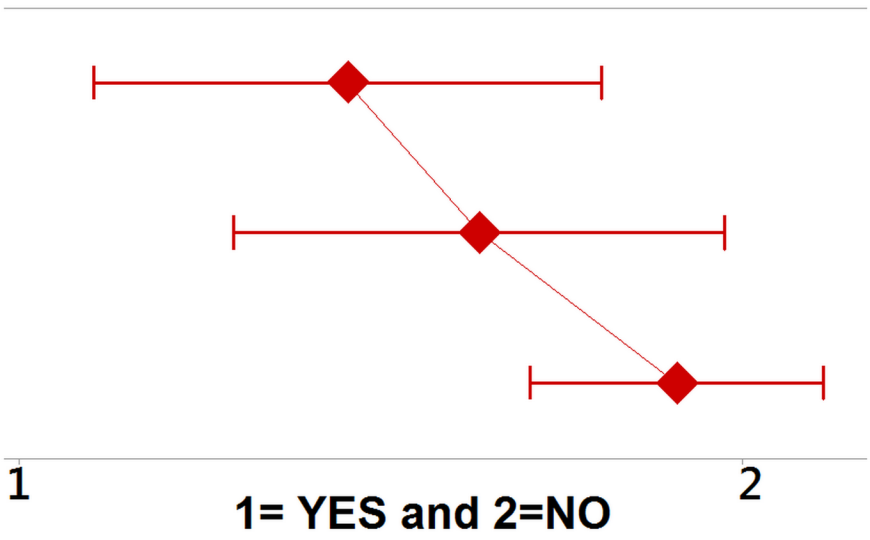


Table 7 . Animal MSC immortalization - Immortalized cell type, with p <0.005 with statistical difference for 95% CI. **Note:***p=0.067>0.005 and I2 = 14.14% S=0,374* . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.

MSCs type	N	Mean	StDev	95% CI	%(1=YES)
Bovine endometrial MSC	11	1.9091	0.3015	(1.7048; 2.1134)	9.1
MSC from porcine pancreas	11	1.9091	0.3015	(1.7048; 2.1134)	9.1
Canine MSC-AT	11	1.9091	0.3015	(1.7048; 2.1134)	9.1
MSC-BM of Rhesus monkey	11	1.9091	0.3015	(1.7048; 2.1134)	9.1
Pig MSC from nasal mucosa, lung	11	1.9091	0.3015	(1.7048; 2.1134)	9.1
MSC from mice dental papilla	11	1.9091	0.3015	(1.7048; 2.1134)	9.1
MSC-BM from rat	11	1.818	0.405	(1.614; 2.022)	18.2
Porcine MSC-BM	11	1.727	0.467	(1.523; 1.932)	27.3

Table 8. Animal MSC immortalization - Immortalization method applied, with p <0.005 with statistical difference for 95% CI. **Note:** *p=0,077>0,005 and I2 = 15.70% S=0,454* . . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.



Method	N	Mean	StDev	95% CI	%(1=YES)
Retroviral/Lentiviral transfection.	11	1,455	0,522	(1,175; 1,734)	54,5
Gene transfer (plasmid)	11	1,636	0,505	(1,357; 1,916)	36,4
Spontaneous transformation	11	1,9091	0,3015	(1,6296; 2,1886)	9,1

Table 9. Animal MSC immortalization - Tests to prove immortalization, with p <0.005 with statistical difference for 95% CI. **Note:** *p=0.000<0.005 and I^2 = 22.13% S=0.401* . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.

Tumorigenicity /Assay	N	Mean	StDev	95% CI	%(1=YES)
Tumorigenicity assay	11	1.636	0.505	(1.402; 1.870)	36.4
Flow cytometry	11	1.273	0.467	(1.039; 1.507)	72.7
RT-PCR	11	1.455	0.522	(1.221; 1.688)	54.5
qRT-PCR	11	1.727	0.467	(1.493; 1.961)	27.3
Western blot	11	1.727	0.467	(1.493; 1.961)	27.3
Soft agar colony assay	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Karyotype	11	1.818	0.405	(1.584; 2.052)	18.2
Hematopoietic differentiation	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Germ cells differentiation	11	1.818	0.405	(1.584; 2.052)	18.2
Islet-like cells differentiation	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Cardiomyocytes differentiation	11	1.818	0.405	(1.584; 2.052)	18.2
Neuronal differentiation	11	1.818	0.405	(1.584; 2.052)	18.2
Osteogenic differentiation	11	1.182	0.405	(0.948; 1.416)	81.8
Adipogenic differentiation	11	1.545	0.522	(1.312; 1.779)	45.4
Chondrogenic differentiation	11	1.545	0.522	(1.312; 1.779)	45.4
Immunomodulatory properties	11	1.818	0.405	(1.584; 2.052)	18.2
Cell migration	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Immunofluorescence for SV40T	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Immunofluorescence for pluripotency	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Apoptosis analysis	11	1.818	0.405	(1.584; 2.052)	18.2

Tumorigenicity /Assay	N	Mean	StDev	95% CI	%(1=YES)
Telomere length assay	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
Cell cycle analysis	11	1.818	0.405	(1.584; 2.052)	18.2
Senescence-associated beta-gala	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
MTT assay	11	1.9091	0.3015	(1.6752; 2.1430)	9.1

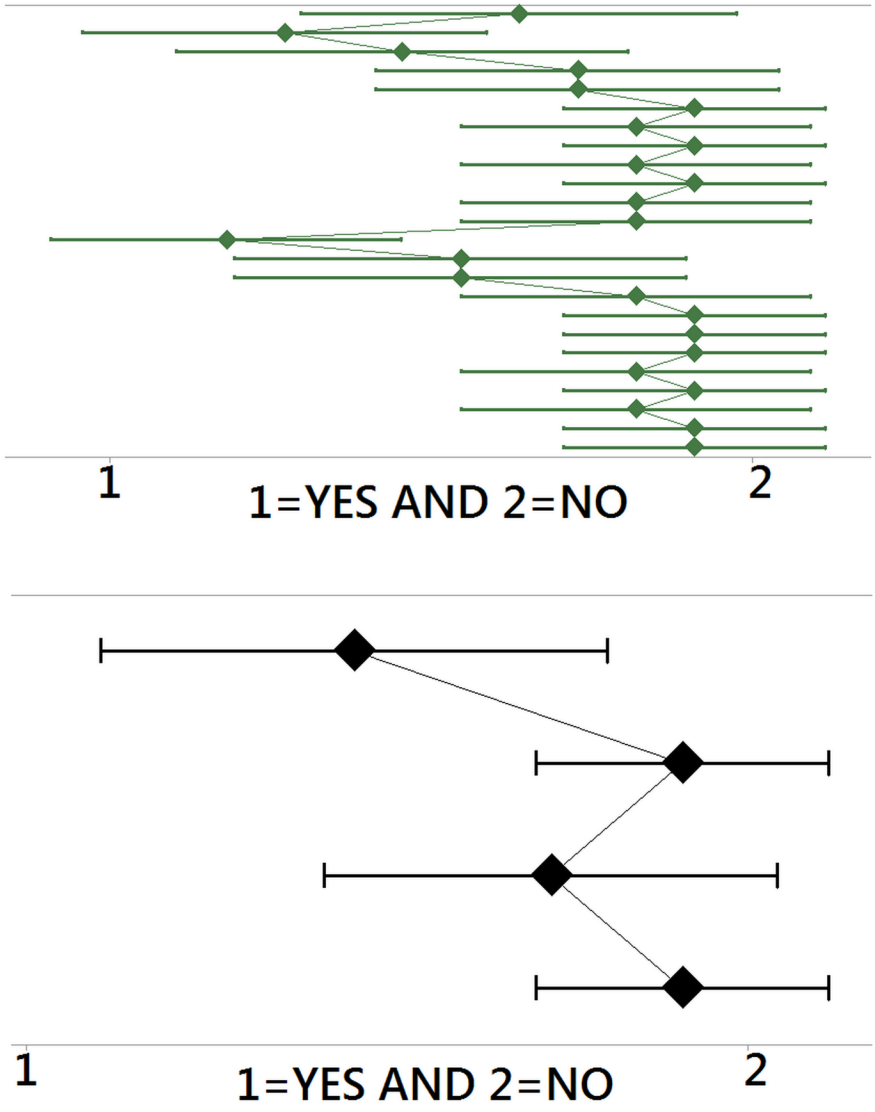


Table 10. Animal MSC immortalization - Genes used for immortalization, with p <0.005 with statistical difference for 95% CI. **Note:***p=0.040>0. 005 and I2 = 18,46% S=0.410* . The Dot-Plot model figure to the side represents the variation of the average values with uncertainty values for each study analyzed, with an interval from 1 (Yes) to 2 (No) for 95% CI.

Immortalization gene	N	Mean	StDev	95% CI	%(1=YES)
SV40T	11	1.455	0.522	(1.221; 1.688)	54.5

Immortalization gene	**N**	**Mean**	**StDev**	**95% CI**	**%(1=YES)**
HPV16 E6/E7	11	1.9091	0.3015	(1.6752; 2.1430)	9.1
hTERT	11	1.727	0.467	(1.493; 1.961)	27.3
Spontaneous immortalization	11	1.9091	0.3015	(1.6752; 2.1430)	9.1