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Expanded differentiation capability of human Wharton's jelly stem cells towards pluripotency. A systematic review.

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Abstract

Human Wharton's jelly stem cells (HWJSC) can be efficiently isolated from the umbilical cord, and numerous reports have demonstrated that these cells can differentiate into several cell lineages. This fact, coupled with the high proliferation potential of HWJSC, make them a promising source of stem cells for use in tissue engineering and regenerative medicine. However, their real potentiality has not been established to date. In the present study we carried out a systematic review to determine the multilineage differentiation potential of HWJSC. After a systematic literature search we selected 32 publications focused on the differentiation potential of these cells. Analysis of these studies showed that HWJSC display expanded differentiation potential towards some cell types corresponding to all three embryonic cell layers (ectodermal, mesodermal and endodermal), which is consistent with their constitutive expression of key pluripotency markers such as OCT4, SOX2 and NANOG, and the embryonic marker SSEA4. We conclude that HWJSC can be considered cells in an intermediate state between multipotentiality and pluripotentiality, since their proliferation capability is not unlimited and differentiation to all cell types has not been demonstrated thus far. These findings support the clinical use of HWJSC for the treatment of diseases affecting not only mesoderm-type tissues, but also other cell lineages.

Keywords

Human Wharton's jelly stem cells — Advanced therapies — Multipotent, Pluripotent — Systematic review

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Contents

Introduction

Mesenchymal stem cells (MSC) are gaining increasing interest in stem cell therapy because of their accessibility, proliferation capability and differentiation potential. Among the many possible sources of stem cells, human umbilical cord Wharton's jelly stem cells (HWJSC) have several advantages compared to other cell types, including accessibility, proliferation and differentiation potential, and immune-privileged status. HWJSC can be easily isolated from small fragments of the human umbilical cord [1] (Figure 1), which is discarded after birth. The 131 million births annually worldwide provide the best opportunity to collect HWJSC from this tissue [2]. A systematic study of the *ex vivo* expansion of HWJSC showed that approximately 360,000 viable cells can be obtained per sample of umbilical cord, and these cells tend to proliferate very rapidly and maintain stable telomerase activity [3] —features that make it possible to obtain sufficient amounts of cells for clinical applications. In most cases these cells are isolated

from the umbilical cord with enzymatic digestion [4, 5] and tissue explants methods, which may be more efficient in terms of cell viability and number of MSC [6]. In fact, Yoon et al. demonstrated that the number of HWJSC obtained with the explant technique is higher, and that these cells show higher levels of beta-fibroblast growth fctor (bFGF) and express relevant mitosis-related genes [6]. However, Salehinejad et al. found that the expression of C-kit is higher in HWJSC isolated by enzymatic methods, although the explant method of cell isolation resulted in a higher rate of cell proliferation and activity [7].

HWJSC have been extensively used in regenerative medicine research due to their *ex vivo* proliferation capability. These cells can be easily expanded in culture for long-term passages [8], and several methods have been described to increase their proliferation potential, including hypoxia [9–11], irradiation [12], or the use of tridimensional culture systems [13].

In addition to recent findings for stem cell secretomes, these cells are immune-privileged. HWJSC are negative for class II HLA-DR, although they may express low amounts of class I HLA. For this reason HWJSC have been widely exploited in a broad spectrum of preclinical and clinical autologous and allogeneic regenerative applications. HWJSC also have an important immunomodulatory effect that may be mediated by molecules able to interact with the host immune system and control inflammation. More recently, co-culture assays have elucidated the impact of HWJSC on the inhibition of activated NK cells in a cytokine-dependent manner [14]. Interestingly, differentiated HWJSC may maintain these features upon differentiation [15] , which opens new scenarios for their use in regenerative medicine.

Beside their unique proliferation and immune-privileged characteristics, HWJSC are considered to be bioactive. One of the main reasons for this is their ability to synthetize a large number of bioactive molecules that are secreted into the extracellular space as free secretome molecules or as extracellular vesicles. These molecules may be partly responsible for the biological properties of HWJSC, and are critical mediators of intercellular communication and differentiation processes [16–18]. In fact, the HWJSC secretome was shown to be able to induce the differentiation of cells cultured *in vitro* [17] , moderate neural/glial proliferation and differentiation [16], and have important antiproliferative and cytotoxic effects in malignant cell lines [19]. Most likely, their immunomodulatory properties are directly related to the synthesis of bioactive secretome molecules [20]. The HWJSC secretome has also shown regenerative properties *in vivo*, one of the most important molecules related to this effect being alpha-2-macroglobulin [21].

One of the main features of HWJSC is their substantial differentiation potential. Most authors have considered these cells as multipotent due to their mesenchymal nature, and numerous studies have been carried out to differentiate HWJSC from cells corresponding to the mesodermal cell layer, e.g. osteochondral and adipocyte cell lineages [22, 23]. However, recent studies with different induction methods have shown that these cells may be able to differentiate to several other cell types of ectodermal and endodermal nature, and a number of authors have explored the use of HWJSC in regenerative medicine of the skin, cornea, oral mucosa, nerves and liver [15, 24, 25]. Although these studies support the possibility that HWJSC may have pluripotent differentiation capabilities, a systematic analysis of the information available thus far has been lacking.

The objective of the present study was to carry out a systematic review and analysis of published works related to the differentiation potential of HWJSC into different cell types corresponding to each embryonic layer (ectoderm, mesoderm and endoderm), in order to determine the putative pluripotent nature and characteristics of these cells.

1. Methods

The present systematic review focused on research articles related to the multilineage differentiation capability of HWJSC. Briefly, a literature search was performed in the Pubmed/Medline and Web of Science databases in November 2019 according to PRISMA guidelines [26]. The search terms were "Wharton's jelly stem cells" OR "umbilical cord stem cells" AND differentiat* OR ectoderm* OR mesoderm* OR endoderm* OR pluripoten*. No restriction was applied to the year of publication. Articles in languages other than English and document types other than original articles were excluded. The PRISMA flow chart is shown in Figure 2.

The eligibility criteria for inclusion were as follows: Articles reporting research with 1) human Wharton's jelly stem cells, 2) freshly isolated primary cell cultures (noncommercial cell lines), and 3) research designed to study cell differentiation. Articles reporting trilineage differentiation only as a characterization assay were excluded.

Data were extracted from the selected articles and recorded with EndNote X8 (Clarivate Analytics). Cell lineage, induction protocol, type of research, and study results were documented for each included article.

2. Results

Searching with the terms described above identified 111 articles, 17 of which were excluded because of the language exclusion criterion (languages other than English), and document type criterion (other than original articles). Of the remaining 94 articles, 28 used nonhuman cells, 13 used commercially available cell lines, and 21 did not focus directly on the study of cell differentiation capabilities, and so were also excluded. The final sample for analysis consisted of 32 articles that were included and reviewed. Further details are shown in Figure 2, and results are shown in Table 1.

2.1 Ectodermal differentiation potential of HWJSC

Despite their mesenchymal origin, HWJSC were previously shown to have the ability to differentiate into different cell

types of ectodermal lineage. Some interesting studies reported the successful differentiation of HWJSC into different types of surface epithelial cells of ectodermal origin, and into neural tissues. We found that 10 of the 32 selected articles focused on the specific differentiation capability of HWJSC to the ectodermal lineage (Table 1). Five of these articles demonstrated the *in vitro* or *in vivo* differentiation potential to skin, oral mucosa or cornea keratinocytes [**?**, 15, 24, 27–29]; three reported their potential to differentiate to cornea keratocytes or lens fiber cells [30–32]; and two studied the potential of HWJSC to differentiate to glial cells [33, 34].

Keratinocyte differentiation was driven mostly by the use of specific conditioning culture media containing epidermal growth factor (EGF) and other epithelial factors [15, 24, 28], inductive biomaterials [27], or *in vivo* grafting in animal models [35]. Although all studies demonstrated the differentiation potential of these cells, most of them suggested that an *in vivo* environment is necessary for terminal keratinocyte differentiation of HWJSC.

Cornea keratocytes are specialized stromal cells derived from the human neural crest [36]. HWJSC have been efficiently differentiated to this cell phenotype *ex vivo* with specific conditioning media that contain ascorbic acid and transforming growth factor beta (TGF-beta) [30, 31]. The increased expression of genes related to crystallins and extracellular matrix (ECM) components supports the differentiation potential of HWJSC. In addition, Khatami *et al*. [32] demonstrated that these cells were able to differentiate into lens cells when homogenized bovine vitreous body was used as an inductor, and the resulting cells were able to change their morphology and crystallin gene expression.

None of the selected articles was able to demonstrate neuron-specific differentiation capability in HWJSC. However, differentiation was achieved to two different glial cell types, i.e., oligodendrocytes and Schwann cells [33, 34]. Both cell types play a crucial role in myelination in the central and peripheral nervous system, respectively, and differentiation was produced by using conditioning media *ex vivo*. It is noteworthy that some articles demonstrating the partial neuronal differentiation capability of HWJSC had been published previously [37–39], although none of them was identified by our search criteria. This suggests that HWJSC may have neuronal differentiation capability, a possibility that should be investigated in future studies.

2.2 Mesodermal differentiation potential of HWJSC

Numerous previous reports demonstrated the mesodermal differentiation potential of HWJSC, as would be expected given the mesodermal origin of the umbilical cord. In fact, 20 of the 32 articles included in the present review focused on the mesodermal differentiation of HWJSC (Table 1). Most of these studies (17 of 20 articles) focused on the osteochondral differentiation capability of HWJSC, with five studies related to chondrocytic differentiation, 10 related to osteocytic differentiation, and two related to both types of cell differentiation.

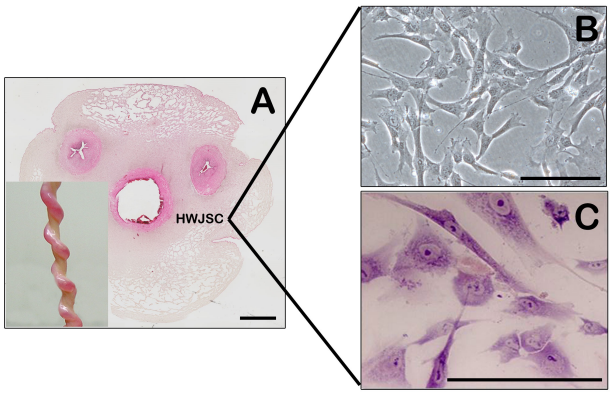

Figure 1. *In situ* and *in vitro* human Wharton's jelly stem cells (HWJSC) from the human umbilical cord. (A) Macroscopic and microscopic images of the human umbilical cord. Scale bar: 40 μm. (B) Isolated HWJSC kept in culture for 7 days. Scale bar: 20 μm. (C) Cultured HWJSC stained with hematoxylin-eosin. Scale bar: 20 μm.

Different methods were used to achieve the osteochondral differentiation of HWJSC.

Two articles reported the use of co-culture systems combining HWJSC with human articular chondrocytes or osteoarthritis cells to allow these latter cell types to induce HWJSC differentiation [40, 41]. The results suggested that co-culture was able to promote chondrogenesis, and certain genes including COL1A1, COMP, TGFβ1, ACAN and SOX9 were upregulated in differentiated HWJSC.

However, in most reports osteochondral differentiation of HWJSC was induced with a specific conditioning medium, specific biomaterials or a combination of both approaches. In this regard, we found two studies in which conditioning medium containing dexamethasone and ascorbic acid combined or not with other components such as b-glycerophosphate or TGF-β1 succeeded in inducing the expression by HWJSC of osteochondral-specific genes such as OCN, RUN-X2 or SOX9, and some ECM molecules [42, 43].

Four studies reported the induction of HWJSC differentiation by culturing these cells on inductive substrates without the need for conditioning media [44–47]. The results were positive in terms of gene expression, morphological changes and phosphatase alkaline activity. Interestingly, the biomaterials used for this purpose were based on calcium phosphate in two cases [44, 47], electrospun polycaprolactone (PCL) nanofiber meshes in one case [45], and polyacrylamide hydrogels in one other case [46] and some authors conclude that differentiation efficiency may vary depending on the substrate stiffness [46].

Finally, nine studies combined the differentiation potential of conditioning media and biomaterials in order to increase the efficiency of the differentiation process. Of these nine reports, five achieved osteochondral differentiation of HWJSC by using calcium phosphate cement (CPC) alone [48, 49] or

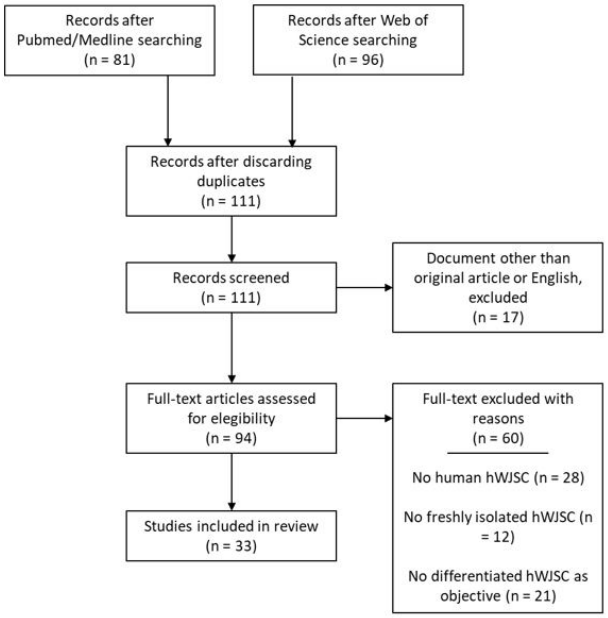

Figure 2. Search strategy and study selection for the present systematic review (n=32). The literature search of Pubmed/Medline and Web of Science databases was performed in November 2019 according to PRISMA guidelines.

combined with arginylglycylaspartic (RGD) peptides [50], alginate microbeads [51] or collagen fibers [52]; three articles used poly-caprolactone (PCL) biomaterials combined or not with other types of materials (mainly collagen and hydroxyapatite) [23, 53, 54]; and one study used poly-lactide-co-glycolide (PLGA) microspheres to induce HWJSC differentiation [55]. Interestingly, in all nine articles, the inductive medium associated to the different biomaterials was very similar in composition to the medium used for HWJSC differentiation without biomaterials (as described above), and was based mainly on the use of dexamethasone and ascorbic acid combined or not with other components.

In addition to osteochondral differentiation, two original articles demonstrated that HWJSC can be efficiently differentiated to the vascular endothelial lineage by using specific inductive media, and that differentiated cells showed morphological, functional and genetic changes typical of vascular endothelial cells [56, 57].

Finally, one article [58] reported the successful differentiation of these cells to cardiomyocytes with 5-azacytidine, an inhibitor of DNA methylation able to induce significant epigenetic modifications in human cell cultures [59].

2.3 Endodermal differentiation potential of HWJSC

Only two studies focused on the endodermal differentiation capability of HWJSC were found in our systematic review, and both investigated the differentiation potential of HWJSC to the hepatocyte cell lineage [25, 60]. One study used indirect co-culture of HWJSC and liver tissue to induce differentiation, and some liver-specific genes such as CK18, albumin, tryptophan 2,3-dioxygenase, α-fetoprotein, CYP7A1, HGF and MMP were upregulated along with concomitant downregulation of several stem cell genes including NANOG, OCT4, and C-KIT [60]. However, the use of a specific conditioning medium containing an array of inductive factors (FGF-4, hepatocyte growth factor, glucagon, trichostatin A, dimethyl sulfoxide, etc.) was apparently more efficient both *ex vivo* and *in vivo* [25].

3. Discussion

HWJSC have been used extensively in regenerative medicine research because of their unique properties and characteristics. Although traditionally considered to be capable of differentiating only into mesoderm cell types, the present systematic review demonstrates that HWJSC clearly exceed this capability and are able to differentiate to other cell lineages.

As expected, nearly two thirds of the articles included in our review focused on HWJSC differentiation to mesodermal cell lineages (mainly the osteochondral lineage). This multipotent differentiation potential had been demonstrated previously, and in fact differentiation to osteogenic, chondrogenic and adipogenic lineages is one of the requirements proposed by the International Society for Cellular Therapy for the characterization of MSC [61]. Most likely, this potential is related to the positive expression of mesenchymal-specific markers that are used for MSC characterization, such as CD29, CD44, CD73, CD90 and CD105, along with the negative expression of CD34 and CD45 [61].

Several differentiation methods and protocols have been used to induce efficient osteochondral differentiation in HWJSC. Although some studies used specific conditioning media or co-culture with osteochondral cell types, the recent development of novel biomaterials with specific porosity and surface structure characteristics has contributed significantly to increase the mesodermal differentiation potential of HWJSC. Therefore, most studies included in the present review combined the use of specific biomaterials and conditioning media, with good results. The consequences of this are relevant for the field of tissue engineering and regenerative medicine, and suggest the need to combine both factors for efficient differentiation. Interestingly, all studied included here succeeded in differentiating HWJSC to the osteochondral lineage to a certain degree, and some phenotypic and gene expression changes were detected, but none of them was able to generate fully differentiated, mature bone or cartilage *ex vivo*. These results warrant further research in this field.

Three of the included studies showed that HWJSC have other differentiation capabilities apart from the trilineage potential established for MSC characterization. In the first study, these cells were inducted to the vascular lineage with conditioning media, and differentiated HWJSC displayed endothelial differentiation potential in culture. Again, *ex vivo* differentiation was not terminal, and the authors concluded that some relevant endothelial-related functions were not ac-

TABLE 1. OVERVIEW OF THE 32 STUDIES INCLUDED IN THE PRESENT SYSTEMATIC REVIEW

Embryonic layer	*Reference*	*Cell differentiation*	*Induction protocol*	In vitro/ in vivo	*Study results*
Ectoderm	Garzon *et al.*[29]	Corneal epithelial cells	HWJSC on top of corneal artificial stroma+EGF-enriched medium for 7 days and air/liquid interface up to 28 days	*In vitro*	Expression of GAG and proteoglycans similar to native. Positive expression of specific corneal CK3/12, crystallins αA, αB, β, βγ3, ζ, and λ1 and junction proteins (CX43, ZO1, and PKG)
Ectoderm	Fong *et al.*[27]	Skin keratinocytes	HWJSC intradermal injection on diabetic dorsal wound. Follow-up: 14 days	*In vivo*	HWJSC significantly accelerated healing rate after 7 days Positive expression of CK, involucrin, and filaggrin in GFP-labeled HWJSC Upregulation of ICAM-1 and TIMP-1 after 3 days, and VEGF-A after 7 days
Ectoderm	Martin-Piedra *et al.*[15]	Skin keratinocytes	3:1 mixture of DMEM:HAM's F12 supplemented with 10% FBS for 28 days	*In vitro/ in vivo*	3,670 probe-sets (6.72%) were upregulated (184 epithelial genes) Expression of CK10 and filaggrin 30 days postgrafting
Ectoderm	Tam *et al.*[28]	Skin keratinocytes	AV/PCL scaffolds+HWJSC and CM from HWJSC for 6 weeks	*In vitro/ in vivo*	HWJSC differentiated when administered locally into the wound beds. Involucrin and filaggrin detection after 28 days
Ectoderm	Garzon *et al.*[30]	Oral/skin keratinocytes	HWJSC on top of artificial stromas +preconditioning epithelial culture medium for 1 week and air/liquid interface up to 2 weeks+grafting on surgical wounds. Follow-up: 40 days	*In vitro/ in vivo*	Stratified squamous epithelium after 2 weeks *in vitro* (keratinized after 10 days *in vivo*) Positive expression of CK8, CK13, and filaggrin (*in vitro*) and CK1 and PKG (*in vivo*)
Ectoderm	Dos Santos *et al.*[31]	Keratocytes	DMEM, L-ascorbic acid 2-phosphate (1 mM), rhTGF-b3 (1 ng/mL), and rhFGF2 (10 ng/mL) for 7 days	*In vitro*	Increased expression of CHST6, LUM, and ALDH3A1 Corneal stromal stem cells and adipose stem cells showed higher differentiation than HWJSC
Ectoderm	Karamichos *et al.*[32]	Keratocytes	DMEM with 0.5 mM D-glucopyranosyl-L-ascorbic acid and 0.1 ng/mL TGF-β1 for 4 weeks	*In vitro*	Higher fibril density and more alignment. Higher GAG length after TGF-β1 treatment TGF-β1 enhanced collagen V and perlecan synthesis and reduced expression of decorin and keratocan
Ectoderm	Khatami *et al.*[33]	Lens fiber cells	DMEM/FBS+homogenized bovine vitreous body (50/50% or 25/75%) for 10 days	*In vitro*	αB, βB1, and βB3-crystallin gene expression Elongated morphology on induced HWJSC
Ectoderm	Davis *et al.*[34]	Oligodendrocytes	DMEM, N2 supplement, 10 μM forskolin, 5 U/mL heparin, 5 nM K252a, FGF-2, EGF, PDGF-AA, and 20 μM NE for 30 days	*In vitro*	Intensely positive staining for A2B5, O1, and O4 galactocerebroside and MBP Increased expression of α1 and β1 agonist receptors
Ectoderm	Omar *et al.*[35]	Schwann cells	Raw extract of *Centella asiatica* (L.)RECA (400, 1200, and 2000 μg/mL) in α-minimum essential medium for 9 days	*In vitro*	Cell enlargement was observed in induced HWJSC with RECA at 400 and 1200 μg/mL High expression of MBP and GFAP in HWJSC induced with RECA alone

(*continued*)

TABLE 1. (CONTINUED)

Embryonic layer	*Reference*	*Cell differentiation*	*Induction protocol*	In vitro/ in vivo	*Study results*
Mesoderm	da Silva *et al.*[46]	Chondrocytes	Electrospun PCL nanofiber meshes+basal medium for 28 days	*In vitro*	Synthesis of GAG and collagen type II Enhanced expression of aggrecan, Sox9, and collagen 2
Mesoderm	Fong *et al.*[54]	Chondrocytes	PCL/collagen nanoscaffolds+basal medium and dexamethasone, ascorbate, ITS, sodium pyruvate, proline, glutamine, and TGFβ3 (10 ng/mL), 21 days	*In vitro*	Increased synthesis of GAG and hyaluronic acid. Higher proliferation Expression of key chondrogenic genes (SOX9, collagen type II, COMP, FMOD)
Mesoderm	Wang *et al.*[42]	Chondrocytes	Co-culture of HWJSC and OA cells	*In vitro*	OA chondrocytes promote chondrogenesis of HWJSC (aggrecan, sox-9, collagen II)
Mesoderm	Wajid *et al.*[44]	Chondrocytes	DMEM +1% ITS, 0.1% bovine serum albumin, 4 mM L-glutamine, 100 mM sodium pyruvate, 0.05 mM L-ascorbic acid, 10 ng/mL TGFβ1, and 100 nM dexamethasone, 14 days	*In vitro*	Morphological changes starting at day 3 with complete chondrogenic medium Increased aggregation of proteoglycans and expression of BGN, COL2A1, SOX9, and ACAN
Mesoderm	Pereira *et al.*[41]	Chondrocytes	Indirect co-culture system using HWJSC and hACs for 36 days	*In vitro*	HWJSC coculture upregulated of collagen type I, COMP, TGFβ1, aggrecan, and SOX9 Cell nodules with round shape, stained positive for collagen type II in the micromass pellets
Mesoderm	Gauthaman *et al.*[23]	Chondrocytes and osteoblasts	Osteogenic differentiation PCL/collagen/HA scaffolds+medium supplemented with 100 nM dexamethasone, 50 mM ascorbic acid, and 10 mM b-glycerophosphate, 21 days Chondrogenic differentiation PCL/collagen+commercial basal medium with dexamethasone, ascorbate, and ITS, 21 days	*In vitro*	Osteogenic differentiation: Mineral nodules deposition (von Kossa and Alizarin Red technique). Expression of OCN Upregulation of ALP, CBFA1, RUNX2 and OPN genes Chondrogenic differentiation: Positive expression of collagen II and Sox9. Increased synthesis of GAG and hyaluronan Upregulation of COL2A, COMP, and SOX9 genes
Mesoderm	Dormer *et al.*[56]	Osteochondral cells	Polylactide-co-glycolide microspheres loaded with BMP2/TGFβ1 gradient+DMEM supplemented with 1% ITS-premix, 40 μg/mL L-proline, 100 μM sodium pyruvate, 50 μg/mL L-ascorbic acid, 4 mM β-glycerophosphate, and 100 nM dexamethasone, 6 weeks	*In vitro*	Gradient of BMP2 and TGF-β1 significantly enhanced GAG and HA synthesis Calcium deposition related to osteogenic gradient (ALP activity increased after 3 weeks)
Mesoderm	Ramesh *et al.*[43]	Osteocytes	Osteogenic medium constituting basal media, 10 mM b-glycerophosphate, 1 mM dexamethasone, and 5 mg/mL ascorbic acid	*In vitro*	Osteogenic-specific genes, OCN and Runx2, were upregulated in cells immobilized in 1.5% and 2% alginate spheres. Great mineralization matrix of HWJSC immobilized in 2% alginate hydrogel.
Mesoderm	Alami *et al.*[48]	Osteocytes	CaP substrate for 28 days	*In vitro*	Bone-like nodule with osteocyte-like cells embedded into a mineralized type I collagen Morphological changes in HWJSC cultured on CaP scaffolds

(*continued*)

TABLE 1. (CONTINUED)

Embryonic layer	*Reference*	*Cell differentiation*	*Induction protocol*	In vitro/ in vivo	*Study results*
Mesoderm	Alami *et al.*[45]	Osteoblasts	CaP induction for 21 days	*In vitro*	Bone-like nodules with osteocyte-like cells embedded into a mineralized type I collagen Expression of BGLAP and ALPL mineralization-related genes (ALP activity after 14 days)
Mesoderm	Chen *et al.*[49]	Osteoblasts	Biofunctionalizated CPC scaffolds+commercial osteogenic medium for 21 days	*In vitro*	Increased expression of ALP, OCN, and collagen II genes after 14 days ALP activity and mineral deposition increased after 14 days
Mesoderm	Gauthaman *et al.*[55]	Osteoblasts	PCL/collagen and PCL/collagen/HA scaffolds+HWJSC medium supplemented with 100 nM dexamethasone, 50 mM ascorbic acid 2-phosphate, and 10 mM b-glycerophosphate, 21 days	*In vitro*	PCL/collagen/HA scaffolds induced more and wider mineralized nodules PCL/collagen/HA resulted in higher expression of OCN and ALP activity. Upregulation of CBFA1, RUNX2, and OPN
Mesoderm	Zhao *et al.*[50]	Osteocytes	CPC scaffolds+osteogenic media composed by 100 nM dexamethasone, 10 mM b-glycerophosphate, 0.05 mM ascorbic acid, and 10 nM 1a,25-dihydroxyvitamin	*In vitro*	Cell extensions attach to the nanosized apatite crystals that formed by CPC matrix HWJSC secreted mineral particles
Mesoderm	Zhao *et al.*[52]	Osteocytes	CPC paste+with alginate hydrogel microbeads encapsulating HWJSC+media with 100 nM dexamethasone, 10 mM b-glycerophosphate, 0.05 mM ascorbic acid, and 10 nM 1a,25-dihydroxyvitamin	*In vitro*	ALP synthesis peaked at 14 days Increased mineral synthesis by HWJSC after 14 days
Mesoderm	Witkowska-Zimny *et al.*[47]	Osteocytes	Polyacrylamide hydrogel+DMEM (Gibco BRL) supplemented with 20% FBS for 14 days	*In vitro*	Expression of ALP, collagen type I, OCN, and Runx2 Differentiation into osteoblasts varies depending on substrate stiffness
Mesoderm	Thein-Han *et al.*[51]	Osteocytes	Medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid, and 10 nM 1,25-dihydroxyvitamin+funcionalyzed CPC scaffolds with arginylglycylaspartic peptides, fibronectin, fibronectin-like engineered polymer protein, Geltrex and human platelet concentrate for 8 days	*In vitro*	Bone mineralization improved in functionalized CPC scaffolds. High expression of ALP activity, Runx2, OCN and collagen type I ALP peak was much higher for all five biofunctionalized CPC (similar for Runx2)
Mesoderm	Thein-Han *et al.*[53]	Osteocytes	Medium with 100 nM dexamethasone, 10 mM b-glycerophosphate, 0.05 mM ascorbic acid, and 10 nM 1a,25-dihydroxyvitamin for 14 and 21 days and cultured into collagen fibers self-setting CPC	*In vitro*	Presence of mineral nodules Increased formation of extracellular matrix higher osteogenic gene expression on CPC with collagen

(continued)

TABLE 1. (CONTINUED)

Embryonic layer	*Reference*	*Cell differentiation*	*Induction protocol*	In vitro/ in vivo	*Study results*
Mesoderm	Alaminos *et al.*[57]	Vascular endothelial cells	Enriched M199 medium for 4 days	*In vitro*	Positive expression of CD31 and VWF Increased expression of VEGF2, EPAS1, EDF1, and TIE2 genes
Mesoderm	Vico *et al.*[58]	Vascular endothelial cells	Medium 199 with 20% FBS, 2 mM/L of L-glutamine, 1.5% of human platelet-derived endothelial cell growth factor	*In vitro*	Morphological changes (more polygonal, less elongated) than control HWJSC Adequate differentiation potential to the vascular endothelial lineage with high cell viability (high presence of K/Na ratio)
Mesoderm	Wu *et al.*[59]	Cardyomiocytes	5 mmol/L 5-azacytidine in DMEM-F12 for 24 h	*In vitro/ in vivo*	Well-aligned myofilaments and typical striation and pale-staining pattern of the sarcomere after 1-month induction Expression of cardiac markers α-actin, connexin43, myosin, troponin T
Endoderm	Lin *et al.*[61]	Hepatocytes	Transwell indirect coculture of HWJSC with fibrotic liver tissue of C57BL/6 mice for 4 days	*In vitro*	Upregulation of liver-specific genes CK18, albumin, tryptophan 2,3-dioxygenase (TO), α-fetoprotein (AFP), CYP7A1, HGF, and MMP Downregulation of stem cell genes (Nanog, oct4, and ckit)
Endoderm	Varaa *et al.*[25]	Hepatocytes	DMEM and 10 ng/mL FGF-4 for 2 days +1% ITS, and 20 ng/mL HGF for 2 days +100 nM dexamethasone, 1% ITS, 10−7 mg glucagon, 10 ng/mL OSM, and 20 ng/mL HGF for 2 days +100 nM trichostatin A or 1% dimethyl sulfoxide for 14 days	*In vitro/ in vivo*	Encapsulated HLCs showed polyhedral morphology with confluence similar to the hepatic-like clusters ALB and CK-18 expression significantly increased in HLC group

AV, aloe vera; CaP, calcium phosphate; CPC, calcium phosphate cement; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; HA, hydroxyapatite; HGF, hepatocyte growth factor; HLC; HWJSC, human Wharton's jelly stem cells; ITS; OA, osteoarthritis; PLC, polycaprolactone; RECA; TGF-β, transforming growth factor-beta.

tivated after differentiation [56]. A second study reported HWJSC differentiated to cardiac muscle cells both *ex vivo* and *in vivo* [58]. Interestingly, in a third study *ex vivo* differentiation was achieved with an epigenetic factor diluted in the culture medium. In this connection, different authors previously demonstrated that the differentiation potential of HWJSC was strictly dependent on the epigenetic status of these cells, and differentiation to cardiac muscle cells was associated with an extensive epigenetic reprogramming process including sFRP4 promoter CpG island demethylation [62].

In terms of epigenetics, human MSC are considered to be genetically stable in culture [63], and these cells have been expanded for several cell passages with adequate cell viability and function [64]. However, long-term culture can be associated with an increased likelihood of chromosomal instability, and other types of MSC, i.e. those isolated from the human oral mucosa, showed chromosomal aberrations such as chromosome 8 tetrasomy from passages 12 to 18 [65]. Although this has not been found in HWJSC, chromosomal stability is a crucial requirement for future clinical use, and should be analyzed after sequential sub-culturing of these cells. Apart from this consideration, sequential cell sub-culturing can be associated with a loss of expression of CD44 or CD73 markers and upregulation of IL1-related genes such as IL1B, IL1R1 and TWIST2 [66]. Interestingly, it has been demonstrated that co-culturing bone marrow MSC with HWJSC is able to prevent the typical progressive telomere shortening and accumulation of intracellular reactive oxygen species (ROS) found in these MSC cultured alone [67]. Together, these results suggest that prolonged culture of HWJSC may not be associated with the important epigenetic modifications found in other cell types; nonetheless, comprehensive analyses of long-term cultures of HWJSC are necessary before use.

In addition to their mesodermal-type differentiation, HWJSC were shown to have ectodermal and endodermal differentiation capabilities in 12 of the studies included here. These cells were efficiently differentiated to a range of nonmesodermal cell types such as epithelial cells, glial cells and hepatocytes with different types of inductive media and biomaterials both *ex vivo* and *in vivo*. Furthermore, although not retrieved in this systematic review, there are several studies that reported the neuronal differentiation of HWJSC. This finding also suggests that HWJSC may have potential as an alternative source of MSC to treat nervous system disorders [37–39] , and points to the possibility that the differentiation capability of HWJSC is a multilayered phenomenon. In general, it has been established that pluripotent differentiation is governed by a highly interconnected pluripotency gene regulatory network (PGRN) that is functionally anchored by a set of core pluripotency transcription factors [68]. Among other factors, OCT4 has been described as essential for both *in vivo* and *in vitro* pluripotency [69], and SOX2 is required for the formation of pluripotent epiblast and is a key regulator of OCT4 expression [70,71]. The loss of OCT4 or SOX2 promotes ectodermal differentiation, whereas the overexpression of OCT4 or SOX2 leads to the differentiation of neural ectoderm [72, 73]. Another relevant factor is NANOG, a core pluripotency transcription factor related to the acquisition of pluripotency by the inner cellular mass of the human embryo. Strikingly, HWJSC show constitutive expression of all these factors [74], along with the embryonic stem cell marker SSEA4 [56]. The expression of all these pluripotency markers, together with the inherent expression of nonmesodermal cell markers including several cytokeratins, desmoplakin and zonula occludens *in situ* and *in vitro* [24], may explain the multilineage differentiation potential of HWJSC not only to the mesodermal cell type, but also to ectodermal and endodermal cells.

Pluripotency is defined as the capability of cells to differentiate into virtually any cell type regardless of the embryonic layer [69], and to self-renew indefinitely. In this regard, the present systematic review shows that HWJSC have expanded differentiation capabilities to cell lineages corresponding to all three embryonic cell layers, and that their proliferation capability is very high compared to other cell types. However, it is evident that not all human cell types have been obtained from HWJSC, and that their proliferation rate has a limit.

Future work should determine whether the differentiation ability of HWJSC is comparable to embryonic and induced pluripotent stem cells (iPS), and whether their pluripotency is limited to certain cell types. The present results open a door to the use of HWJSC as a pluripotent human cell source for the generation of different cell types in tissue engineering and regenerative medicine. In addition, a metaanalysis focused on the specific capabilities of HWJSC should be carried out once the available scientific evidence has increased and the number of available studies on this topic warrants this type of analysis.

4. Conclusion

In this review we found that HWJSC are able to differentiate into several cell lineages corresponding to the ectodermal, mesodermal and endodermal embryonic cell layers. In light of their expanded proliferation potential, these results suggest that HWJSC may represent an emerging class of stem cells developmentally and operationally situated between the state of embryonic stem cells and adult stem cells, exhibiting and sharing features of pluripotentiality and multipotentiality. These characteristics support the use of HWJSC for the clinical treatment of patients with diseases affecting not only mesodermal-type tissues and organs, but also other types of tissues. The potentially advantageous features and therapeutic properties of HWJSC are expected to be gradually implemented at the clinical level through the development of guidelines for their clinical translation.

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Competing interests

All authors declare there is not any financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

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