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Advances in translational orthopaedic research with species-specific multipotent mesenchymal stromal cells derived from the umbilical cord

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Summary. Compliance with current regulations for the development of innovative medicines require the testing of candidate therapies in relevant translational animal models prior to human use. This poses a great challenge when the drug is composed of cells, not only because of the living nature of the active ingredient but also due to its human origin, which can subsequently lead to a xenogeneic response in the animals. Although immunosuppression is a plausible solution, this is not suitable for large animals and may also influence the results of the study by altering mechanisms of action that are, in fact, poorly understood. For this reason, a number of procedures have been developed to isolate homologous species-specific cell types to address preclinical pharmacodynamics, pharmacokinetics and toxicology. In this work, we present and discuss advances in the methodologies for derivation of multipotent Mesenchymal Stromal Cells derived from the umbilical cord, in general, and Wharton's jelly, in particular, from medium to large animals of interest in orthopaedics research, as well as current and potential applications in studies addressing proof of concept and preclinical regulatory aspects.

Key words: Animal models, Translational research, Regenerative medicine, Multipotent mesenchymal stromal cells, Bone healing, Tissue repair, Tissue engineering, Wharton's jelly

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Introduction

In recent years, interest in the study of multipotent Mesenchymal Stromal Cells (MSCs) has rapidly increased due to the ease of their isolation from different tissue sources and their potential use in diverse therapeutic approaches (Naji et al., 2019; Vives and Mirabel, 2019). Remarkably, MSCs share common features regardless of their tissue of origin and their characterisation is based on the combination of surface marker expression, cellular morphology, tri-lineage differentiation potential and immunomodulation capacity *in vitro* (Horwitz et al., 2005; Dominici et al., 2006; de Wolf et al., 2017). In addition to their immuno-modulatory properties, other characteristics of MSCs that encourage their use include rapid expansion *ex vivo* for production of large batches for allogeneic transplantation (Hu et al., 2016; Schunemann et al., 2020) , and the lack of safety concerns commonly found in other types of stem cells, such as teratoma formation and chromosomal instability (Baksh et al., 2007).

Abbreviations. AT, Adipose Tissue; bFGF, basic Fibroblast Growth Factor; BM, Bone Marrow; CQA, Critical Quality Attribute; DMEM, Dulbecco's Modified Eagle Medium; EGF, Epidermal Growth Factor; FACS, Fluorescence-Activated Cell Sorting; FBS, Fetal Bovine Serum; FDA, Food and Drug Administration; GLP, Good Laboratory Practice; GMP, Good Manufacturing Practices; GvHD, Graft-versus-Host Disease; HG, High Glucose; HLA, Human Leukocyte Antigen; IFI, Immuno-Fluorescence Imaging; ISCT, International Society for Cell and Gene Therapy; ITS, Insulin Transferrin Selenium; LG, Low Glucose; MCDB, chemically defined medium for Chinese Hamster Ovary cells; MHC, major histocompatibility complex; MSC, multipotent Mesenchymal Stromal Cells; PBS, Phosphate Buffer Saline; PDGF, Platelet-Derived Growth Factor; PK, Pharmacokinetics; qRT-PCR, quantitative Reverse Transcriptase-Polymerase Chain Reaction; Tox, Toxicology; UC, Umbilical Cord; WJ, Wharton's Jelly

Although expectations for treatment are high, a number of uncertainties makes challenging the clinical translation of novel MSC-based therapies (Schneider et al., 2010a), particularly all aspects of *in vivo* preclinical regulatory development in relevant animal species, including pharmacodynamics, pharmacokinetics (PK) and toxicological (Tox) studies. Indeed, the development of cell-based therapies requires mandatory safety and efficacy studies to ensure therapeutic benefits, and both factors are first assessed *in vitro* and in animal studies before moving on towards clinical testing in humans (Mount et al., 2015). Sufficient understanding of downstream biological mechanisms involved after treatment will help to ensure regulatory approval for further testing in human patients and, ultimately, reaching marketing authorisation (Roura et al., 2017).

The safety of MSCs has been convincingly proved in several clinical trials (Lalu et al., 2012), but there is still a poor understanding of the underlying mechanism of action of MSCs, in general, and tissue specific MSCs, in particular, and this is the main handicap of MSC-based developments (Bianco et al., 2013). Despite common characteristics, tissue-specific MSCs are prone to exert different biological activities in response to particular stimuli in the pathological environment, at least in experimental *in vitro* and *in vivo* models (Martin et al., 2019). Therefore, the predisposition of tissue-specific MSCs to perform differently needs to be studied in more detail to support tailored clinical use for specific diseases and syndromes.

Herein we present and discuss advances in the methodologies for derivation of multipotent MSCs derived from the umbilical cord, in general, and Wharton's jelly, in particular, from medium to large animals of interest in orthopaedics research, as well as current and potential applications in studies addressing proof of concept and regulatory PK/Tox preclinical studies.

Why Wharton's jelly?

MSCs are typically sourced from adult tissues, the bone marrow (BM) and adipose tissue (AT) being the most representative ones (Naji et al., 2019). Indeed, early cell-based therapies for bone regeneration consisted of autologous BM-MSC (Lee et al., 2009). However, harvesting procedures are invasive and painful, in addition to the limited supply of starting material for large scale expansion in the production of allogeneic cell-based tissue engineering treatments. On the contrary, the umbilical cord (UC) is an abundant tissue that is typically discarded after birth, which hosts MSC besides other types of cells (Secunda et al., 2015). Isolation of human MSCs from the UC has been widely reported by using tissue explant culture, enzymatic digestion and mechanical scrapping of the mucoid connective tissue known as Wharton's jelly (WJ) (Batsali et al., 2013; El Omar et al., 2014; Oliver-Vila et al., 2016). UC-MSCs are considered to be more primitive and proliferate faster and more extensively *in vitro* than adult MSCs (Venugopal et al., 2011; Wang et al., 2011; Zhao et al., 2012; Oliver-Vila et al., 2016). Because of all these interesting properties, WJ-MSCs are presented as an alternative to MSCs from BM or AT. Furthermore, various scaling up methods compatible with current Good Manufacturing Practice (GMP) have been efficiently validated, thus evidencing some flexibility of bioprocess designs that can effectively yield sufficient numbers of cells for use in preclinical and clinical studies (Oliver-Vila et al., 2016; Schunemann et al., 2020). In addition to the reduced immunogenicity and enhanced immunomodulation capacity, the abundance of donations of umbilical cord, the ease of deriving WJ-MSCs, and the lack of ethical concerns should also be considered in the production of novel MSC-based allogeneic therapies (Mattar and Bieback, 2015). In fact, allogeneic products offer several advantages, namely: sustainable business model, no pressure of manipulating fresh product, and convenient supply logistics (Mirabel et al., 2018; Vives and Mirabel, 2019). Indeed, the greatest advantage of banking is the cryopreservation of cells until they are needed. Long-term stability of cryopreserved cells has been confirmed for several cell types making it very interesting if large batches are produced so patients can be treated from same master cell bank-derived products.

WJ-MSCs share most characteristics with BM and AT-MSCs (Wagner et al., 2005). In line with these observations and due to the disparity of specifications used by developers for characterisation, the International Society for Cell and Gene Therapy (ISCT) proposed minimal criteria for definition of human MSCs (Dominici et al., 2006), which can be complemented with microscopy assessment of their fibroblastic morphology (Horwitz et al., 2005), and the capacity to inhibit the proliferation of stimulated lymphocytes *in vitro* (de Wolf et al., 2017). Remarkably, WJ-MSCs display a reduced immunogenicity due to expression of Human Leukocyte Antigen (HLA)-G6 isoform, making them more appealing for use in mismatched allogeneic therapies (Marino et al., 2019). Among other immunoproperties, WJ-MSCs are known to be capable of inducing regulatory T cells, inhibiting activated CD3+ T cell proliferation, as well as monocyte differentiation into mature dendritic cells (Vieira Paladino et al., 2019).

Given their unique features and safety profile, WJ-MSCs have been evaluated in different clinical conditions such as immune-mediated diseases (e.g. GvHD, graft rejection, multiple sclerosis and systemic lupus erythematosus), diabetes mellitus, liver and cardiac pathologies, among others (Can et al., 2017; Liau et al., 2020; Prat-Vidal et al., 2020).

WJ-MSCs in clinical orthopaedics

In orthopaedics, WJ-MSC-based medicines are

attractive candidates to yield innovative therapies for bone regeneration. To this date, several diseases, such as Becker muscular dystrophy, fracture non-union or osteonecrosis of the femoral head (Ansari et al., 2018) have been particularly targeted with interesting preliminary results. Clinical trials using umbilical cord MSCs represent 9% of the total in the orthopaedics field, behind the use in haematological indications that are the most representative with 12% of all studies (Davies et al., 2017). Nonetheless, this number is expected to grow in the near future (Can et al., 2017; Liau et al., 2020). WJ-MSCs are multipotent cells capable of differentiating through the osteogenic lineage and they also induce osteogenic differentiation by paracrine signalling, which make them therapeutically attractive not only in the autologous or allogeneic haplomatched setting but also in the case of using cells from a mismatched HLA donor, which appear to induce osteogenic differentiation despite being rejected afterwards (Cabrera-Pérez et al., 2019; López Fernández et al., 2020). Of great interest in WJ-MSC clinical application is the administration of exosomes derived from these cells, which are responsible for paracrine regenerative processes (Kuang et al., 2019).

Recent studies have shown that osteogenic differentiation is better induced when WJ-MSCs are associated with scaffolds (Ansari et al., 2018). We have previously tested different scaffolds for bone regeneration in experimental bone lesions (Coletta et al., 2014, 2017, 2018) and maintain that WJ-MSCs can favour bone remodelling if associated with these scaffolds. Successful outcomes have already been found using: hierarchical fibrous scaffolds (Canha-Gouveia et al., 2015), three-dimensional collagen gels (Schneider et al., 2010b), and poly-L-lactic acid (Ahmadi et al., 2017). Even this association has shown that MSCs can reduce inflammation and foreign body reaction with respect to scaffold-only implantation, for example when they are associated with synthetic biomaterial-type scaffolds such as polyvinyl alcohol hydrogel (Alexandre et al., 2014).

Approved therapies for bone regeneration are led by BM-MSC-based products, including: OsteoCel (NuVasive), Trinity (Orthofix), Allostem (AlloSource), and LiquidGen (SkyeOrthbiologics) (Vives and Mirabel, 2019). We believe that this situation may change since the advantages of the umbilical cord over BM and AT-MSCs makes them very attractive for use in novel developments. However, further preclinical research is needed to shed light on cellular dose requirements and product optimisation, as well as for the validation of safety and efficacy, before its translation to clinical trials. Indeed, the dosing and dosage of MSCs are still a matter of discussion given that dose escalation studies are not conclusive and translation of results from animal models to the clinical setting is difficult. Moreover, the high cost and difficult logistics of manufacturing cell-based living medicines makes it complex when target doses are higher than $1x10^6$ MSC/kg.

WJ-MSCs from relevant animal models

While the very first isolation of MSCs from the human UC dates from the early 1990's (McElreavey et al., 1991), it was not until 2003 when the first successful isolation of porcine WJ-MSCs was described (Mitchell et al., 2003). Later, in 2007, Hoynowski and collaborators described the use of the same procedure applied to the equine model (Hoynowski et al., 2007). Since then, WJ-MSCs have been isolated from a wide variety of animal models, including goat (Kumar et al., 2016), sheep (Bezerra et al., 2019), cow (Cardoso et al., 2017), dog (Filioli Uranio et al., 2014), or rabbit (Li et al., 2017) (Table 1).

The anatomy of the umbilical cord varies according to each animal model and this must be taken into consideration before proceeding with any of the protocols reported in the literature. In particular, humans, horses and pigs share the same distribution of blood vessels (that is two arteries and one vein), whereas sheep, cows and dogs have two arteries and two veins (Barrios Arpi, 2018) (Fig. 1).

Methods for isolation of WJ-MSCs from different species

Several methods for isolation of MSCs from umbilical cord have been described in the literature, either from the Wharton's jelly, perivascular regions or sub-amnion membrane. These methods can be classified into the next three categories: enzymatic, enzymatic-explant and explant methods (Table 1). However, both cell quantity and cell quality seem to be influenced by the methodology as well as the type and quality of enzymes used, if applicable (Salehinejad et al., 2012; Varaa et al., 2019). Higher homogeneity of cellular population in culture seems to result from explant and trypsin methods, with fibroblast-like cells being predominant. In general, explant methods show higher cell proliferation than enzymatic methods, as well as higher percentage of cells expressing CD37 and CD90. This may be the result of decreased adhesion ability of cells treated enzymatically and the loss of cells during the filtering and washing procedures (Salehinejad et al., 2012; Paladino et al., 2016; Hendijani, 2017). Considering the equivalence of species-specific and human MSCs, methods should be also comparable in an attempt to preserve the equivalence of identity, purity and potency in the cell-based products for preclinical and clinical studies. Here it is important to remember that methods for manufacturing WJ-MSCs for humans tend to use xeno-free, clinical-grade reagents, while controlling costs and avoiding excessive numbers of steps in the production workflow (Roura et al., 2017; Schunemann et al., 2020). In this sense, the use of exogenous enzymes may be expensive, and introduce impurities and potential contaminants. Mori and collaborators assessed an improved explant method that could be also applied to scale up (Mori et al., 2015). It mainly

Table 1. Methods for isolation of Wharton's Jelly Mesenchymal Stromal Cells (WJ-MSC) from relevant animal species.

Animal model	Isolation of WJMSC		Cell culture medium formulation	Time to first media change	References
	Method	Description			
Pig	Explant	Remove blood vessels, dice the tissue into 2-5 mm^3 fragments and transfer them to a 6 well-tissue culture plates.	56% LG-DMEM, 37% MCDB 201, 2% FBS; 5% ITS, AlbuMax I, dexamethasone, ascorbic acid 2-phosphate, P/S, amphotericin B, EGF, PDGF	1-2 days	Carlin et al., 2006
	Explant	Remove blood vessels, dice the tissue into small fragments and transfer them to an adherent surface.	DMEM, 20% FBS	5-7 days	Mitchell et al., 2003
	Explant	Remove blood vessels, dice the tissue into 1-2 mm^3 fragments and transfer them to an adherent surface.	ADMEM, 10% FBS, P/S	3 days	Kang et al., 2013
	Explant	Dice the tissue into 2-3 mm^3 fragments and transfer them to an adherent surface.	DMEM/F12, 20% FBS, P/S	1 day	Huang et al., 2015
	Enzymatic	Dice the tissue, treat with 1 mg/mL collagenase 1 h at 37°C, wash and treat again with 0.25% trypsin for 30 min at 37°C. Wash and culture.	DMEM/F12, 10% FBS, bFGF, EGF, P/S	1 day	Lim et al., 2018
Horse	Enzymatic	Remove blood vessels, treat with 0.75 mg/mL collagenase for 16 h at 37°C, filter the suspension, centrifuge at 250 g 10 min in PBS twice, resuspend the pellet and culture.	HG-DMEM, 10% FBS, EGF, streptomycin, amphotericin, L-glutamine	3 days	Lange-Consiglio et al., 2011
	Enzymatic	Remove blood vessels, mince in 1 mm^3, treat with 1 mg/mL collagenase for 30 min at 37°C, filter the suspension, centrifuge at 500 g 10 min, resuspend the pellet in growth media and culture.	α-MEM, 20% FBS, P/S, L-glutamine	1 day	Passeri et al., 2009
	Enzymatic	Incubate the tissue with trypsin at 37°C for 30 min, then centrifuge at 1000 rpm, resuspend the pellet and culture.	LG-DMEM, MCDB, FCS and ITS	Not specified	Cremonesi et al., 2011
Cow	Explant	Remove blood vessels, fragment the tissue into 25 cm^2 pieces and culture them.	Serum-free media	Not specified	Cardoso et al., 2017
	Explant	Cut the tissue into small pieces and place them on an adherent surface.	DMEM, 10% FBS, P/S	7 days or until cells are observed	da Cunha et al., 2014
	Enzymatic	Remove blood vessels, mince the tissue into small pieces, treat with 1 mg/mL collagenase type I for 8 h at 38.5°C and inactivate the enzyme with growth media. Then, filter the cell suspension, centrifuge at 300 g 10 min and wash twice with PBS and culture.	HG-DMEM, 10% FCS, P/S, amphotericin B, L-glutamine, EGF	3 days	Lange-Consiglio et al., 2017
	Explant	Cut the tissue into small pieces and place them on an adherent surface.	StemlineW (Sigma-Aldrich) and 2mM L-glutamine (GlutamaxW, InvitrogenW,) mesenchymal stem cell expansion medium.	1 day	Cardoso et al., 2012
Sheep	Explant	Remove blood vessels, mince the Wharton's jelly in 1-2 mm pieces and culture these explants.	LG-DMEM, 15% FBS, 3% NEAA, 1% P/S	3 days	Bezerra et al., 2019
	Enzymatic-explant	Extract the Wharton's jelly, incubate it with 4 mg/mL collagenase and 1 mg/mL hyaluronidase type II for 1h. Then, incubate it with TryPLETM for 30 min at 37°C. Wash with PBS the pieces and cut into 1-2 mm^3 and culture them.	DMEM, 10% FBS, 1% P/S	Not specified	Satheesan et al., 2020
	Enzymatic	Chop the tissue into 1 mm^3 pieces, treat with 0.1% collagenase type II for 1 h at 37°C. Then, incubate the pieces in 0.25% trypsin for 30 min, filter the cell suspension and wash 3x with PBS. Resuspend the cell pellet in growth media and culture.	DMEM/F12, 5% FBS, Glutamax, bFGF, EGF, dexamethasome, P/S	3 days	Zhao et al., 2019
	Enzymatic	Extract the Wharton's jelly, mince it into small pieces, incubate it with 4 mg/mL collagenase type I and 1mg/mL hyaluronidase for 1 h. Then, incubate it with 0.1% trypsin-EDTA for 30 min, centrifuge at 200 g 10 min and resuspend the cell pellet and culture.	DMEM, 10% FBS, P/S	2 days	Eswari et al., 2016
Rabbit	Enzymatic	Remove blood vessels, dice the tissue into 1 mm^3 pieces, treat with 0.075% collagenase type I for 10-14 h at 37°C, wash and centrifuge at 1500 rpm 5 min, resuspend the cell pellet and culture.	DMEM, 10% FBS	3 days	Li et al., 2017
	Explant	Remove blood vessels, dice the tissue into small fragments and culture.	DMEM/F-12, 10% FBS	5-7 days	Liu et al., 2017
Dog	Explant	Cut the tissue and culture them in Petri plates.	DMEM, 10% DS, L-glutamine, P/S, amphotericin, EGF	3 days	Filioli Uranio et al., 2014
	Explant	Remove blood vessels, scrape Wharton's jelly and culture it.	DMEM, 10% FBS, L-glutamine, P/S, amphotericin, EGF	3-4 days	Rutigliano et al., 2015
	Enzymatic	Dissociate blood vessels and the amniotic epithelium surrounding the umbilical cord, treat it with 0.05% trypsin for 1 h at 37°C, cut the tissue in 1-2 mm pieces and incubate them in 0.1% collagenase type I for 20 min at 37°C. Filter the cell suspension and wash centrifuging at 1500 rpm 10 min. Resuspend the cell pellet in growth media and culture.	DMEM, 10% FBS, P/S	2 days	Lee et al., 2013
	Enzymatic	Cut the tissue into 0.25 cm sections, treat with enzyme solution (1 mg/mL hyaluronidase, 300 U/mL collagenase type I, 300U deoxyribonuclease I) for 3 h at 37°C, 12 rpm, filter the cell suspension and wash centrifuging at 200 g 5 min. Resuspend the cell pellet in growth media and culture.	ACB culture medium (HG-DMEM, 10% FBS, 1% antibiotic-antimycotic, 1% Glutamax and bFGF)	3 days	Wright et al., 2020
	Enzymatic	Incubation of the tissue with 100 U/mL collagenase and 0.01 U/mL hyaluronidase for 1, 2, 3, 4 or 5 h at 37°C. Then, centrifuge at 250 g 10 min and resuspend the cell pellet in PBS. Centrifuge again and treat with ammonium chloride to destroy possible red blood cells at 4°C 10 min. Centrifuge, resuspend and culture.	37% HG-DMEM, 37% LG-DMEM, 15% FBS, 10% antimicrobial solution, NEAA, solution and L-glutamine	2 days	Sepúlveda et al., 2020

bFGF, basic Fibroblast Growth Factor; DMEM, Dulbecco's Modified Eagle Medium; DS, Dos Serum; EGF, Epidermal Growth Factor; FBS, Foetal Bovine Serum; HG, High Glucose; ITS, Insulin Transferrin Selenium; LG, Low Glucose; MCDB, chemically defined medium for Chinese Hamster Ovary cells; NEAA, Non-Essential Amino Acids; PBS, Phosphate Buffer Saline; PDGF, Platelet-Derived Growth Factor; P/S, Penicillin/Streptomycin.

prevents explants from floating up from the surface due to poor fixation by using a stainless-steel mesh, and therefore increases the cell recovery rate.

Common quality attributes of MSC across species

One may think that the identity and potency of animal WJ-MSCs are to be analysed similarly as is performed with human samples, thus expecting the same surface antigen expression patterns. However, the lack of animal-specific reagents makes this endeavour challenging, at least for some animal models (Table 2). Moreover, when it comes to equine and porcine species, some groups have reported WJ-MSC ability to form small spheroids of stem cells surrounded by epithelial differentiated cells in culture (Miki and Strom, 2006). Under specific stimuli, these cells readily differentiate into adipocytes, chondroblasts and osteoblasts (Cremonesi et al., 2011). Regardless of the common attributes across species, there have also been reported differences regarding growth, migration, adhesion and differentiation potential between horse and human. For example, horse WJ-MSCs display lower doubling time but higher adhesion, and higher chondrogenic and osteogenic differentiation capacity than their human counterparts (Merlo et al., 2018). Regarding immunopotency, WJ-MSCs show enhanced immunomodulatory potential compared to MSCs sourced from other tissues, with high capacity to modulate inflammation through inhibition of T cell proliferation, induction of T-reg cells and inhibition of differentiation of monocytes into dendritic cells (Vieira Paladino et al., 2019).

Considerations in the design of *in vivo* studies with WJ-MSC

In orthopaedics research, several pathologies have been modelled in relevant animal species using WJ-MSCs. Other factors such as the manufacturing scale up, funding and regulatory approval, as well as insufficient surgical techniques, biomechanical instability and a compromised wound environment, are considered to lead to significant gaps in the design of preclinical studies (Reichert et al., 2010). Although several animal models have been developed to test MSC-based therapies, many of the preclinical models published to date are poorly described, defined, and/or standardised (Reichert et al., 2009; Reyes et al., 2017), therefore compromising reproducibility. Each study requires a particular animal model and large animal species seem to be the best option regarding bone healing studies (O'Loughlin et al., 2008). Such large animal models are invaluable in translational research for the assessment of MSC-based therapies. Interestingly, a couple of reports describe the use of xenotransplants using human WJ-MSCs (Table 3). As mentioned earlier, this type of study design requires immunosuppression, and the average animal facility is not prepared for hosting immunosuppressed large animals. Moreover, interaction of immunosuppressive drugs with MSCs may lead to artefactual conditions, particularly in tissue regeneration, in which inflammation is part of the repair process. This may explain, at least in part, the little success in the translation of preclinical data into the clinical setting. For this reason, derivation of species-specific MSCs is of great relevance in the field. As a model of choice,

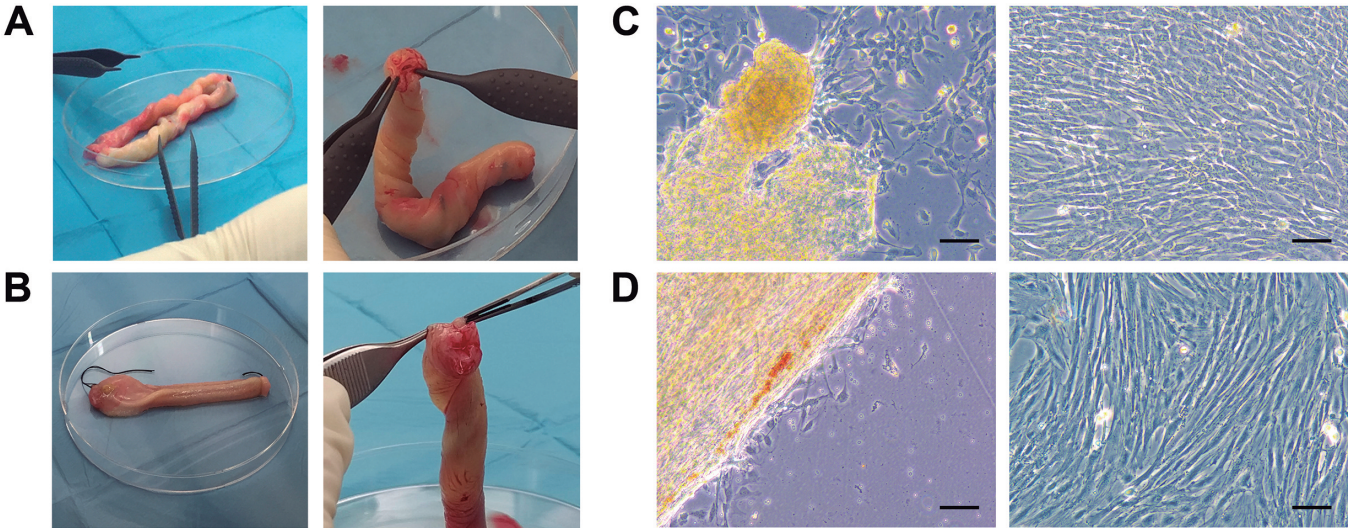

Fig. 1. Derivation of WJ-MSC from human and ovine umbilical cords. On the left panel, macroscopic appearance of umbilical cords from human **(A)** and sheep **(B)**. On the right panel, WJ-MSC outgrowths from Wharton's jelly at initial stages of derivation and, later on, in expansion culture from human **(C)** and sheep **(D)** umbilical cord tissue. Scale bars: 100 μm.

dogs may best resemble the human situation, since more than 370 canine genetic disorders are reported (Ostrander et al., 2000; Filioli Uranio et al., 2011). However, ethical concerns and high costs limit the widespread use of this model. Alternative animal species include sheep and pigs, showing similarities to human regarding bone metabolism and composition (Harvey et al., 2011).

Despite the challenge, it has been reported that 25% of submissions to the FDA of new cell-based medicines

Table 2. Defining criteria for species-specific Wharton's Jelly multipotent Mesenchymal Stromal Cells (WJ-MSC).

Animal model	Adherence to plastic	Markers	Detection platform	Multipotency	Immunopotency	Cell morphology	References
Pig	Yes	CD29, CD44, CD73, CD90, CD105, HLA-ABC; but not CD34, CD45, HLA-DR	FACS	Adipogenic, osteogenic, chondrogenic	Not specified	Spindle-shaped cells	Liu et al., 2016
Rabbit	Yes	CD34, CD45, CD73, CD90, CD105	qRT-PCR	Adipogenic, osteogenic, chondrogenic	Yes, by RT-PCR assessment of MHC-I and MHC-II	Elongated and spindled-shape, and arranged like a vortex	Li et al., 2018
Sheep	Yes	CD13, CD29, CD44, CD90, CD105; but not CD45	IFI	Adipogenic, osteogenic, chondrogenic	Yes, by IFI (positive for CD29, CD13, CD44, CD90, CD106; and negative for CD45)	Fibroblast-like cells	Zhao et al., 2019
Horse	Yes	CD29, CD44, CD105, CD166, MHCI; but not CD34, CD14, MHCII	RT-PCR	Adipogenic, osteogenic, chondrogenic	Yes, by RT-PCR assessment of MHC-I and MHC-II	Fibroblast-like cells in early stages. Cells formed spheroid clusters reaching confluency	Lange-Consiglio et al., 2011
Cow	Yes	CD29, CD44, CD105, CD73, CD90, CD166, MHCI; but not CD34, CD45 CD14, MHCII	RT-PCR FACS	Adipogenic, osteogenic, chondrogenic	Yes, by FACS (positive for CD105, CD166; and negative for CD34 and MHC-II)	Fibroblast-like cells	Cardoso et al., 2012, Lange-Consiglio et al., 2017
Human	Yes	CD73, CD90, CD105; but not CD45, CD31, CD34, HLA-DR	FACS	Adipogenic, osteogenic, chondrogenic	Yes, by inhibiting proliferation of polyclonally stimulated PBMC with PMA/io	Fibroblast-like cells	Oliver-Vila et al., 2015, 2016

Characteristics of WJ-MSC from different species and how they compare to our described methods for human samples. FACS, Fluorescence-Activated Cell Sorting; IFI, Immuno-Fluorescence Imaging; io, ionomycin; MHC, major histocompatibility complex; PMA, phorbol 12-myristate 13-acetate; qRT-PCR, quantitative Reverse Transcriptase-Polymerase Chain Reaction.

Table 3. Most representative orthopaedic preclinical studies on human Wharton's jelly multipotent Mesenchymal Stromal Cells (WJ-MSC) in translational animal models.

Animal	Condition	Results	Reference
Rat	Osteoporosis	Treated groups revealed higher bone volume and collagen content in the epiphysis and metaphysis than control ones at 2 months after transplantation. This transplantation also reduced the number of osteoclasts.	Fu et al., 2018
	Osteoporosis	Increase of TGF-β1 expression, Runx2, and osteoblasts; leading to an enhanced osteoblastogenesis activity.	Hendrijantini et al., 2018
	Cranial defects	At 12 weeks, bone density was increased in comparison to the control.	Wang et al., 2015
Dog	Intervertebral disc degeneration	Narrowing of the disc space at 4 weeks after the operation.	Zhang et al., 2015
	Peri-implant bone defect	At 8 weeks, newly formed bone tissue was remarkable and bone to implant interface was closely contacted.	Hao et al., 2014
Minipig	Osteoarthritis	The treated knees showed significant gross and histological improvements in hyaline cartilage regeneration in comparison to the control knees	Wu et al., 2019
Horse	Osteoarthritis	Same significant improvement of the clinical score in either the single intra-articular injection or the repeated one. *Note: in this study, allogeneic equine WJ-MSCs were injected.	Magri et al., 2019
Rabbit	Full-thickness cartilage defects	After 16 months, a complete integration with adjacent host cartilage and regenerated subchondral bone was observed.	Liu et al., 2017
	Osteoarthritis	At the end-point evaluation, treated rabbits showed a persistent synovial reactivity and reduced cartilage alterations when treated early.	Saulnier et al., 2015
	Intervertebral disc degeneration	WJ-MSCs survived and integrated in the damaged intervertebral discs.	Beeravolu et al., 2018
	Closed spinal dysraphism	Implanted bovine bone collagen particle combined with WJ-MSCs promoted bone regeneration in the vertebral lamina and arch defect area.	Cui et al., 2019
Goat	Articular cartilage defect	At 9 months, treatment with WJ-MSCs resulted in higher-quality cartilage and complete subchondral bone, in comparison to the microfracture treatment.	Zhang et al., 2018
Sheep	Regenerative Medicine	Sheep treated with scaffold and WJ-MSCs presented the lowest degree of inflammation, proving the immunomodulatory effect of WJ addiction in scaffolds.	Alexandre et al., 2014

included non-clinical studies using species-specific cells (Bailey, 2012). To make this endeavour easier, we contributed recently with an algorithm to assist developers in the design of PK studies using MSCs in translational research in an attempt to standardise methodologies for the assessment of cell biodistribution (Reyes et al., 2017).

Quality and regulatory requirements

Preclinical research on novel MSC-based candidate medicines must adhere to pharmaceutical quality and regulatory standards (Mount et al. 2015). Although preclinical proof of concept research is exempt from such strict requirements, further PK/Tox of novel treatments must be addressed in compliance with the principles of Good Laboratory Practice (GLP) (Vives et al., 2015a,b). GLP is a quality management system aiming to safeguard the quality and integrity of non-clinical *in vitro* and *in vivo* studies that are intended to support clinical research or marketing approval of products by regulatory authorities (Vives et al., 2015a).

Limitations of modelling human pathologies in animals are obvious, so efforts need to be made in order to make them resemble as much as possible to the intended conditions to be treated (Fig. 2). In this scenario, the validity of *in vivo* data relies on the equivalence of WJ-MSCs from the animal model and humans. Given the relevance of using well-characterised test items, this may indeed require specific GLP-compliant studies addressing critical quality attributes (CQA) including identity, purity and potency (European Medicines Agency, 2019). However, these types of studies may also be challenging due to the lack of suitable species-specific reagents. Therefore, further efforts must be made in the development of optimised chemically defined culture media and robust bioprocess designs for consistent cell manufacturing, identification and validation of markers specific of WJ-MSCs (commonly expressed in both animal model and human cells), and the optimisation and standardisation of potency assays (e.g. differentiation protocols, immunopotency assays) (Haddouti et al., 2020). After preclinical demonstration of safety and efficacy, clinical translation can be considered following a regulatory pathway already established to ultimately apply for marketing authorisation by the competent regulatory authority (Roura et al., 2017; Golchin and Farahany, 2019).

Conclusions

The choice of an appropriate translational animal model is crucial in the development of innovative MSC-based medicines given the impact that this decision may have on the relevance of the results. The lack of proper characterisation of species-specific MSCs may invalidate preclinical data and lead to the failure of clinical studies, therefore putting patients at risk and wasting human efforts and funding. In this sense, the

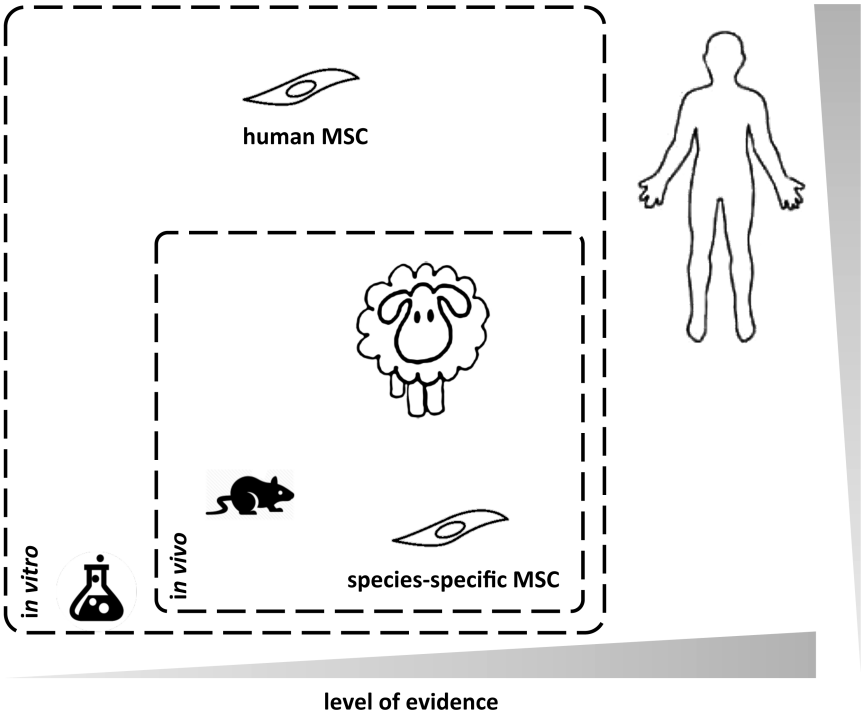

Fig. 2. The dilemma of using human or species-specific multipotent Mesenchymal Stromal Cells (MSC) and its relevance in regulatory preclinical research. The use of the intended medicinal product (ideally produced in clinical grade) would be the best option for non-clinical research of traditional small molecule drugs. However, the development of advanced therapies composed of MSCs offers two main possibilities for *in vivo* studies: either A) using human MSCs in immunosuppressed or immunodeficient animals or B) using species-specific MSCs in immunocompetent animal models. In the first case, the interaction of human cells with the host environment may not be relevant with the pathology or interact with the immunosuppressants, which may affect the results. In the second case, comparability between human and species-specific MSC must be performed thoroughly to ensure their equivalence in terms of identity, purity and potency, and therefore validate the relevance of preclinical data.

definition of optimal methods for derivation and expansion of WJ-MSCs and the demonstration of their equivalence with their human counterpart in terms of identity, purity and potency is mandatory to ensure reproducibility of studies and successful translation of preclinical data into the clinical setting.

Outlook

We believe that optimisation and standardisation of methods for isolation and characterisation of WJ-MSCs from relevant model organisms is key for gaining a better understanding of suitable clinical indications, dosing, dosage, and the study of potential side effects. Given the growing interest in this type of MSC, we envisage future applications of haplo-matched WJ-MSCs in the field of bone tissue engineering using 3D bioprinting or osteogenic scaffolds with the potential to fill bone defects and provide new bone mass.

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