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Derivation of Mesenchymal Stromal Cells from Ovine Umbilical Cord Wharton's Jelly

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The methods described herein allow for the isolation and expansion of fibroblastic-like ovine Wharton's jelly-derived mesenchymal stromal cells (oWJ-MSC) that, similarly to their human counterparts, adhere to standard plastic surfaces in culture; show a mesenchymal profile for specific surface antigens (i.e., positive for CD44 and CD166); and lack expression of endothelial (CD31) and hematopoietic (CD45) markers as well as major histocompatibility complex (MHC) class-II. Homogeneous cell cultures result from a two-phase bioprocess design that starts with the isolation of mesenchymal stromal cells (MSC) from the Wharton's jelly of ovine umbilical cords up to a first step of cryopreservation. The second phase allows for further expansion of ovine WJ-MSC up to sufficient numbers for further studies. Overall, this methodology encompasses a 2-week bioprocess design that encompasses two cell culture passages ensuring sufficient cells for the generation of a Master Cell Bank. Further thawing and scale expansion results in large quantities of oWJ-MSC that can be readily used in proof of efficacy and safety studies in the preclinical development stage of the development of cell-based medicines. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Isolation and expansion of ovine mesenchymal stromal cells from Wharton's jelly of the umbilical cord
Basic Protocol 2: Characterization of ovine mesenchymal stromal cells
Basic Protocol 3: Growth profile determination of ovine mesenchymal stromal cells from Wharton's jelly

Keywords: derivation • multipotent mesenchymal stromal cells • non-clinical development • preclinical animal model • regenerative medicine • sheep • stem cell culture • translational medicine • umbilical cord • Wharton's jelly

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INTRODUCTION

Mesenchymal stromal cells (MSC) comprise a population of stem cells that can be derived from virtually any vascularized tissue and share similar morphology and biological properties (Nombela-Arrieta, Ritz, & Silberstein, 2011). The fact that MSC can be ex vivo expanded to large numbers by relatively straightforward cell culture methods by adherence to cell culture-treated plastic surfaces has made possible the study of their therapeutic activity in different pathologies and degenerative conditions (Naji et al., 2019; Vives & Mirabel, 2019). The interest shown by the scientific community in the production of MSC for clinical testing has prompted the need for a definition of common attributes of MSC in order to assess comparability, regardless of their tissue of origin and derivation protocol. To do this, criteria have been established based on the combination of cellular morphology, surface marker expression, differentiation potential and, more recently, immunomodulation capacity (de Wolf, van de Bovenkamp, & Hoefnagel, 2017; Dominici et al., 2006; Horwitz et al., 2005).

Development of MSC-based medicines needs to be performed in accordance with regulatory and quality guidelines and, in the field of orthopedics, this typically requires animal studies in translational large models, for example, sheep in the orthopedics field (Caminal, Fonseca, et al., 2014; Caminal, Moll, et al., 2014; Caminal et al., 2016; Fonseca et al., 2014; Reyes et al., 2017). However, studies using human MSC would require immunosuppressed or immunodeficient animals, which is very challenging for large translational animal models. The alternative to human MSC is the use of species-specific MSC (Ramallo et al., 2020).

Basic Protocol 1 describes a robust and reproducible method for ready isolation and expansion of ovine Wharton's jelly-derived MSC (oWJ-MSC) from umbilical cord (UC) for use in non-clinical research to support proof-of-concept studies addressing safety, efficacy, and the study of mechanisms of action (MoA) of novel MSC-based treatments. Basic Protocol 2 describes the characterization of key attributes of MSC by assessing their cellular morphology, specific surface antigen expression, and multipotent differentiation potential in line with criteria established for human MSC (Brown et al., 2019). The protocol presented here is based on methods originally described for the isolation of human Wharton's jelly-derived mesenchymal stromal cells (WJ-MSC) previously published by our group and adapted to the ovine model (Oliver-Vila et al., 2016). Basic Protocol 3 describes how to assess properties of oWJ-MSC cultures in terms of growth kinetics.

In a prior protocol, we described a method for the derivation and characterization of MSC from ovine bone marrow and the manufacturing of ovine serum for culture media supplementation (Current Protocols article: Vivas, Caminal, Oliver-Vila, & Vives, 2018). These steps of preparation of autologous serum, pooling of ovine sera, and sterility assessment of the supplemented media are also required in this method.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

BASIC PROTOCOL 1

ISOLATION AND EXPANSION OF OVINE MESENCHYMAL STROMAL CELLS FROM WHARTON'S JELLY OF THE UMBILICAL CORD

The protocol described in this section has been designed for harvesting UC tissue from pregnant sheep, in order to isolate adherent cells from the Wharton's jelly (WJ) and further expansion of oWJ-MSC. All cell culture work has to be undertaken using aseptic conditions using a class II biological safety cabinet, sterile equipment, and plasticware.

Materials

Ovine umbilical cord (obtained under approval by an Institutional Animal Care and Use Committee)
Sterile PBS (e.g., Gibco brand, Thermo Fisher Scientific, cat. no. 14190)
Derivation complete culture medium (derivation CCM; see recipe)
Expansion complete culture medium (expansion CCM; see recipe)
0.05% (w/v) trypsin-EDTA solution (e.g., Gibco brand, Thermo Fisher Scientific, cat. no. 25300)
0.4% (w/v) Trypan Blue solution (e.g., GE Healthcare, cat. no. SV30084.01)

Class II biological safety cabinet
Dissection equipment
  Sterile 150 × 15 mm petri dishes
  Sterile metallic tweezers
  Sterile scalpel
Sterile 100-ml polystyrene containers
Sterile 50-ml conical tubes
Sterile 1.5-ml microtube
Sterile plastic serological pipets
Tissue culture treated flasks
150-cm^2 TPP culture flask with re-closable lid (Buch & Holm, cat. no. 90552)
75-cm^2 culture flask (e.g., Corning, cat. no. 430641U)
150-cm^2 culture flask (e.g., Corning, cat. no. 430825)
Hemocytometer
37°C, 5% CO_2 humidified incubator
Centrifuge
Phase-contrast inverted microscope

Isolate oWJ-MSC

Ovine UC can be obtained from caesarean sections or vaginal delivery in the animal facility surgery room using aseptic conditions. Ovine UC should be immediately collected in a sterile container filled with derivation CCM. We recommend processing the tissue within the first 8 hr after extraction, although isolation of WJ-MSC from human UC is reported to be successful up to 80 hr after extraction (Oliver-Vila et al., 2016).

1. Transfer ovine UC from the transport container to a sterile petri dish at room temperature (15° to 25°C).
2. Rinse tissue with sterile PBS twice in order to remove debris and remaining blood. Repeat procedure if necessary.
3. Place tissue in a clean petri dish and split longitudinally the outer epithelium using metallic tweezers to expose the inner surface and matrix of the UC (Fig. 1).
4. Carefully remove the four blood vessels (two arteries and two veins) immersed in Wharton's jelly.

   Pull the vessels gently in order to prevent tearing the tissue.

   Ovine UC has two veins instead of only one as found in humans.

   The existence of ramifications on the blood vessels of the mother's side of the UC could be observed.

5. Once all blood vessels have been removed, open the lid of the 150-cm^2 culture flask with re-closable lid and place the inner face of the UC in contact with the plastic surface (Fig. 2).

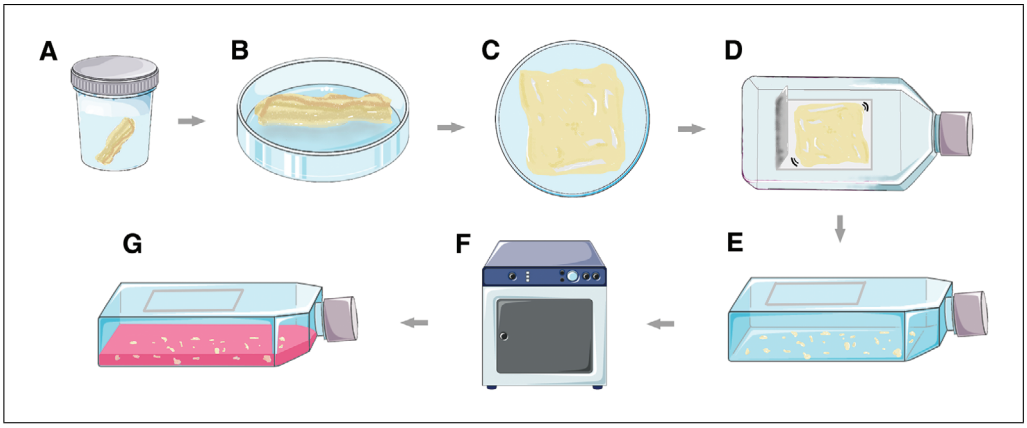

Figure 1 Isolation of mesenchymal stromal cells (MSC) from Wharton's jelly (WJ) from ovine umbilical cords (UCs). (**A**) Ovine umbilical cord. (**B**) Longitudinal cut and split of the outer epithelium and removal of the blood vessels. (**C**) Two veins and two arteries removed from the inner matrix of the ovine UC. (**D**) Appearance of the WJ after removal of the blood vessels and now exposed for further scraping and plating on the surface of a tissue culture treated flask. (**E**) Scrape and spread the WJ uniformly onto the plastic surface of the flask and (**F**) incubate at 37°C, 95% humidity and 5% CO_2 for 30 min. (**G**) Carefully add the required culture medium.

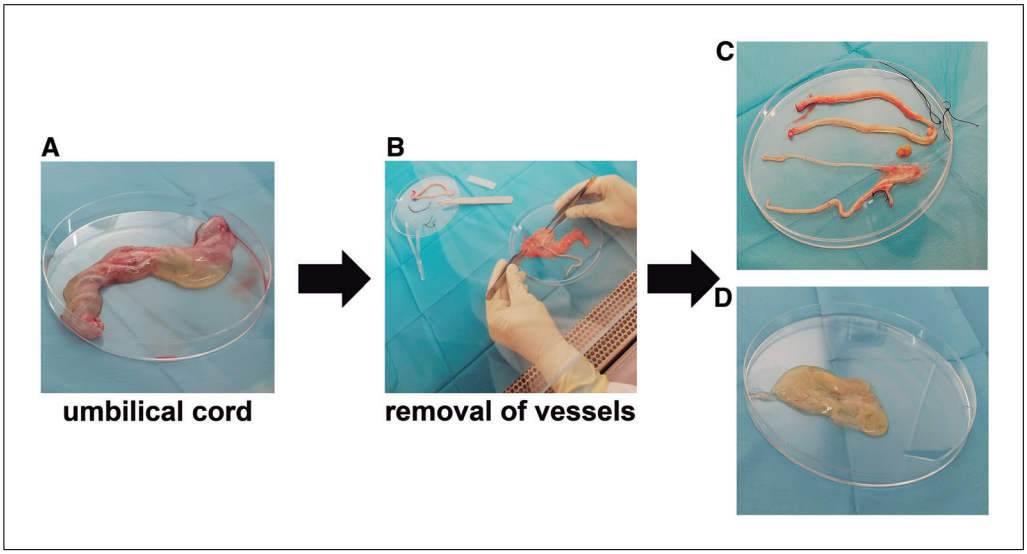

Figure 2 Schematic representation of the entire derivation process of ovine Wharton's jelly-derived mesenchymal stromal cells (oWJ-MSC) to use in preclinical studies: All procedures are carried out under sterile conditions. (**A**) The umbilical cord (UC) is transported in a sterile container. (**B**) Wash UC thoroughly in PBS. (**C**) Place the tissue in a petri dish and remove the two veins and two arteries, with the aid of metallic tweezers, while opening the outer epithelium longitudinally. (**D**) Once open, place the inner face of the UC with the Wharton's jelly (WJ) in contact with the plastic surface of a culture flask with re-closable lid.

Tissue culture flasks with re-closable lid in the top side may be a useful option to access and manipulate the tissue in the plasticware.

6. Scrape WJ and spread it uniformly onto the plastic surface. If necessary, use a scalpel to scrape and spread gelatinous tissue.

There might be abundant WJ in one single UC. In order to avoid saturation of the culture flask, consider using another 150-cm^2 flask.

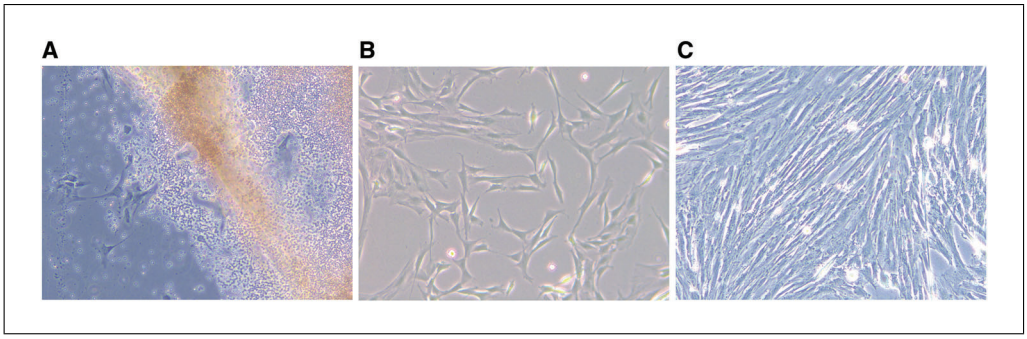

Figure 3 Derivation of ovine Wharton's jelly-derived mesenchymal stromal cells (oWJ-MSC) from the umbilical cord (UC). (**A**) oWJ-MSC outgrowth from Wharton's jelly (WJ) homogeneously spread onto the plastic surface; small colonies may appear at 2-3 days after derivation process. (**B**) oWJ-MSC display fibroblastic morphology and adhere to culture-treated plastic surfaces under standard culture conditions under phase contrast microscopy. (**C**) oWJ-MSC appearance in cell culture at 80%-90% confluence before trypsinization and expansion or cryopreservation. Original magnification 100×.

Scrape carefully in order to prevent damaging the plastic surface as it could compromise cell attachment and further visual inspection of cell culture growth.

7. Close lid of the flask and incubate at 37°C, 95% humidity and 5% CO_2 for ~30 min.

   No culture medium is added at this point with the aim of fixing the tissue in the plastic surface.

8. Add 20 ml prewarmed derivation CCM to the culture flask and place it in the incubator at 37°C, 95% humidity and 5% CO_2.

   Derivation CCM must be added carefully to the culture flask so as not to disrupt the WJ or detach cells.

9. After 2-5 days of incubation, discard old culture medium completely and carefully rinse once with PBS to prevent detachment of the WJ. Then add 20 ml prewarmed derivation CCM.

   It is recommended that the culture status is checked daily in order to detect the appearance of colonies.

   We recommend doing the wash and first culture medium change once a high number of cells can be observed in colonies, usually 2 or 3 days after processing the tissue.

Expand oWJ-MSC

A first cell culture expansion step should be considered when the presence of growing colonies reach each other resulting in overall confluency of ~80%-90% (Fig. 3).

10. Discard old derivation CCM and wash once with PBS. Then add a volume of trypsin according to Table 1.

11. Incubate at 37°C for 1-5 min until cells detach. Check cell morphology under phase-contrast microscopy.

    Detached cells lose their fibroblastic morphology and their shape becomes rounded. Gentle tapping of flasks may be required to detach cells. Incubation in trypsin should not exceed 5 min; otherwise, cell viability might be compromised.

12. Add expansion CCM (twice the volume of trypsin previously added in step 10), homogenize, and collect the entire volume into a 50-ml sterile conical tube.

13. Centrifuge 10 min at 340 × *g*, room temperature.

14. Discard supernatant and add the required volume of expansion CCM.

Table 1. Correlation Between Surface Areas and Volumes Required

Cell culture flask type	Approximate growth surface area (cm^2)	Volume of culture medium (ml)	Volume of PBS (ml)	Volume of trypsin (ml)
Cell stack	636	80	20	20
150-cm^2	150	20	10	5
75-cm^2	75	10	6	3
25-cm^2	25	5	2	1
6-well plate	9.5	3	1	0.5
12-well plate	3.8	1.5	1	0.5
24-well plate	1.9	0.5	0.5	0.3
48-well plate	0.95	0.3	0.3	0.1

We recommend centrifugation of the cell suspension in order to remove the diluted trypsin from the medium.

15. Place 50 µl cell suspension in a 1.5-ml microtube with 50 µl 0.4% Trypan Blue solution for cell counting in a hemocytometer.

16. Calculate seeding volume according to Equation 1:

$$Seeding\ volume\ \ (SV) = \frac{1 \times 10^3 \left(\frac{cells}{cm^2}\right) \times seeding\ Area\ \left(cm^2\right)}{cell\ concentration\ \left(\frac{cells}{ml}\right)}$$

Equation 1

The recommended initial seeding cell density is 1–3 × 10^3 cells/cm^2 but it may vary depending on cell quantity, cell availability, and time considerations.

17. Perform cell seeding of the required flasks by adding the calculated SV from Equation 1 and the required volume of expansion CCM according to Table 1.

18. Label flasks appropriately and culture cells in the incubator at 37°C, 95% humidity and 5% CO_2.

19. Discard old expansion CCM completely every 3-4 days after seeding and replace with the same volume of new prewarmed expansion CCM.

20. When cell cultures reach 80%-90% confluence, cell cryopreservation or a new cell expansion has to be considered. These cells can also be used for characterization as shown in Basic Protocol 2.

Estimate culture's cumulative population doublings

21. Calculate population doubling level (PDL) for each passage using Equation 2. The total cumulative population doublings (CPD) of a specific cell culture expansion at a given passage results from the addition of PDL from each individual passage (P; see Equation 3).

$$Population\ Doubling\ Level\ (PDL) = \frac{Ln\left(\frac{final\ cell\ number}{initial\ cell\ number}\right)}{Ln2}$$

Equation 2

$$CPD = PDL\,(P0) + PDL\ \ (P1) + PDL\ \ (P2) + \cdots + PDL\,(Pn)$$

Equation 3

As it is not possible to know the exact number of MSC initially present in Wharton's jelly, we estimate four CPD for the first passage, because MSC typically accumulate four CPD along P1 in 10 days.

CHARACTERIZATION OF OVINE MESENCHYMAL STROMAL CELLS

BASIC PROTOCOL 2

This protocol describes different steps to obtain a complete functional and phenotypic characterization of oWJ-MSC by adopting the minimal criteria established by the International Society for Cell & Gene Therapy (ISCT) human MSC (Dominici et al., 2006).

Materials

Cell suspension of known concentration (from Basic Protocol 1)
Sterile PBS (e.g., Gibco brand, Thermo Fisher Scientific, cat. no. 14190)
Expansion complete culture medium (expansion CCM; see recipe)
Differentiation medium (optionally, StemPro differentiation media, see next two entries below, can be supplemented with 100 UI/ml penicillin and 100 μg/ml streptomycin; MilliporeSigma, cat. no. P4458)
StemPro Osteogenesis Differentiation Kit (Gibco brand, Thermo Fisher Scientific, cat. no. A1007201)
StemPro Adipogenesis Differentiation Kit (Gibco brand, Thermo Fisher Scientific, cat. no. A1007001)
Sterile deionized distilled water
4% (w/v) formaldehyde solution: 37% aqueous formaldehyde (VWR, cat. no. M134-200ML) in PBS
Alizarin-Red Staining Solution (Merck, cat. no. TMS-008-C)
Oil Red O (MilliporeSigma, cat. no. O0625), 0.2% (w/v) stock solution in methanol
Oil Red O staining solution (see recipe)
1,2-propanediol (MilliporeSigma, cat. no. 398039)
Alkaline Phosphatase (ALP) Assay Kit (Colorimetric; Abcam, cat. no. ab83369)
Antibodies for flow cytometry including
- FITC-conjugated mouse anti-sheep CD44 antibody (Bio-Rad, cat. no. MCA2219F)
- FITC-conjugated mouse anti-sheep CD31 antibody (Bio-Rad, cat. no. MCA1097F)
- PE-conjugated mouse anti-sheep CD45 antibody (Bio-Rad, cat. no. MCA2220PE)
- FITC-conjugated mouse anti-sheep MHC Class II DR Monomorphic antibody (Bio-Rad, cat. no. MCA2226F)
- PE-conjugated mouse anti-human CD166 antibody (BD Pharmingen, cat. no. 559263)

Sterile 15- and 50-ml conical tubes
Sterile 1.5-ml microtubes
Sterile serological pipets
Pipet tips
24-well cell culture plates (Corning, cat. no. 3337)
96-well transparent flat bottom plates (Greiner Bio-One, cat. no. 655101)
Class II biological safety cabinet
Centrifuge
Flow cytometer
Cytometry tubes
37°C, 5% CO_2 humidified incubator
Phase-contrast inverted microscope
Microplate reader capable of measuring absorbance at OD_{405}

Functional characterization: Seeding for osteogenic and adipogenic differentiation

1. Starting material is a cell suspension of known cell concentration obtained from Basic Protocol 1. Calculate the number of cells (Equation 4), volume of cells (Equation 5), and final volume of cell suspension (Equation 6) needed:

$$Cells\ needed = 2 \times 10^4 \left(\frac{cells}{cm^2}\right) \times well\ surface\ area\ \left(cm^2\right) \times (number\ of\ wells + 1)$$

Equation 4

$$Volume\ of\ cells\ needed = \frac{Cells\ needed}{Cell\ concentration\ (cells/ml)}$$

Equation 5

$$Final\ volume = 0.5\ (ml/well) \times (number\ of\ wells + 1)$$

Equation 6

Although the recommended cell seeding density is 2×10^4 cells/cm^2 for oWJ-MSC, cultures can be seeded in the range of 3×10^3 to 1×10^5 cells/cm^2.

At least three replicates for differentiation and three replicates for negative control of each method are recommended. One 24-well plate per condition is also recommended.

2. Adjust volume of cells needed (Equation 5) and final volume (Equation 6) according to the concentration of cells in suspension. According to the values of the initial cell concentration, either centrifuge 10 min at 340 × *g,* room temperature, to concentrate or dilute with expansion CCM in order to set the final volume required (Equation 6).

3. Seed each well of the 24-well plates by dispensing 0.5 ml adjusted cell suspension with expansion CCM and culture cells in an incubator at 37°C, 95% humidity and 5% CO_2. Incubate between 2 hr and 4 days (recommended overnight) so cells can adhere to the surface.

Make sure the cell suspension is mixed well prior to dispensing.

Dispensing 0.5 ml of PBS or sterile water into surrounding wells is highly recommended in order to avoid evaporation of test samples.

4. Between 2 hr and 4 days after seeding, perform Alizarin Red staining (step 6-11), alkaline phosphatase activity determination (step 12-22), and Oil Red O staining (step 23-29) for the negative control condition following the methods described below.

5. Discard medium for the rest of conditions. From now on, replace with fresh prewarmed specific differentiation medium every 3-4 days until the end of differentiation.

Osteogenic differentiation: Alizarin Red (AR) staining

It is recommended that AR staining is performed from the third week after seeding provided that AR stains calcium-containing osteoblasts, which occur at late osteogenic conditions. Longer periods of time may favor the osteocyte maturation and the deposition of calcium in the formed extracellular matrix. All staining procedures must be performed in a fume hood.

6. Discard culture medium and wash twice with 0.5 ml PBS per well.

7. Fix cells by gently adding 0.5 ml 4% formaldehyde into each well and let stand 15 min at room temperature.

8. Carefully remove fixation solution and wash three times with 0.5 ml distilled water per well.

   Add the volume of distilled water, gently stir, and let stand for 5-10 min, then discard the supernatant. Pay special attention to this step in order to avoid disrupting the cell monolayer.

9. Add 0.5 ml AR staining solution per well and incubate at room temperature for 20 min.

10. Discard staining solution and wash four times with 0.5 ml distilled water per well.

    It is recommended that the plate is stirred gently by performing orbital movements in each washing.

11. Add a volume of water to avoid drying out the samples and observe them using an inverted microscope.

    It is recommended that micro- and macroscopic pictures of the stained samples are taken.

Osteogenic differentiation: Alkaline phosphatase (ALP) activity determination

Perform ALP activity determination in cell culture supernatants between weeks 1 to 4 under differentiation conditions. This protocol is adapted from the ALP Assay Kit (Colorimetric) instructions.

12. Prepare reagents: ALP enzyme, 5 mM and 1 mM *para*-nitrophenyl phosphate (*p*NPP) solutions, Assay Buffer, and Stop Solution as determined in the kit instructions.

13. Perform standard dilutions using 1 mM *p*NPP solution as determined in the kit instructions.

14. Perform sample preparation using cell culture supernatant from the differentiation osteogenic conditions directly, as it does not require additional sample preparation.

    To find the optimal values and ensure the readings will fall within the standard values, it is recommended that dilutions of the samples are performed.

    We suggest duplicates of each well using a non-diluted sample with 80 µl of cell culture supernatant from the differentiation plates; a 1/3 dilution sample using 27 µl of cell culture supernatant and 53 µl of ALP buffer; and a 1/5 dilution sample using 16 µl of cell culture supernatant and 64 µl of ALP buffer.

15. Perform sample background control using StemPro Osteogenesis Differentiation medium, as the color of the medium may interfere with the reading.

    We suggest using the same dilutions as in step 14.

16. Add 20 µl Stop Solution per well to sample background control (prepared in step 15) to terminate ALP activity in these samples.

17. Add 50 µl of 5 mM *p*NPP solution per well to sample wells (prepared in step 14) and sample background control wells (prepared in step 15).

18. Add 10 µl ALP enzyme to each standard well (prepared in step 13).

19. Incubate plate 60 min at room temperature protected from light.

20. After incubation, add 20 µl Stop Solution per well to sample wells and standard wells in order to stop reaction.

    Do not add Stop Solution to sample background control wells as it has already been added in step 16.

21. Gently shake plate and measure output at OD_{405} nm on a microplate reader.

22. Calculate ALP activity using Equation 7:

$$ALP\ Activity = \left(\frac{B}{\Delta T \times V}\right) \times D$$

Equation 7

where B is the amount of *p*-nitrophenol generated from the *p*NPP conversion by the ALP enzyme in sample wells calculated from standard dilutions (μmol); ΔT is the reaction time (min); V is the original sample volume added into the reaction well (ml); and D is the sample dilution factor.

Determine the background corrected change in absorbance intensity for each well of sample by subtracting the OD value of the sample background control.

Adipogenic differentiation: Oil Red O staining

We recommend performing Oil Red O staining from the third week after seeding in order to specifically detect lipid vacuoles. Longer differentiation times may foster further maturation of newly generated adipocytes.

23. Without removing StemPro Adipogenic differentiation medium, perform prefixation by gently adding the same volume of 4% formaldehyde as medium and let stand 10 min at room temperature.

24. Remove prefixation solution and add 0.5 ml 4% formaldehyde per well in order to fix cells. Let stand 10 min at room temperature.

25. Discard 4% formaldehyde and wash twice with 0.5 ml PBS.

Regarding the washings, add the volume of PBS, then stir gently and discard the supernatant.

26. Add 0.5 ml of pure 1,2-propanediol into each well and let stand 2 min.

27. Discard 1,2-propanediol and add 0.5 ml prewarmed Oil Red O stain solution per well and let stand 10 min at room temperature.

It is recommended that the Oil Red O stain solution be prewarmed prior to beginning the protocol.

Avoid aspirating the crystals or precipitates that may have formed in the stain solution.

28. Discard dye and wash twice with 0.5 ml distilled water per well.

29. Add a volume of water to avoid drying out the samples and observe them using an inverted microscope.

It is recommended that micro- and macroscopic pictures of the stainings be taken.

Phenotypic characterization

The strategy for the phenotypic characterization described here depends entirely on the features of the flow cytometer used and the method of labeling chosen. A double labeling protocol is described next but other labeling strategies can be applied as well, depending on the number of markers to be detected. Although isotype controls and blocking Fc-binding receptors are not mandatory, we recommend their use. For positive markers an expression >95% is expected and, for negative markers, an expression <5% is expected.

30. The starting material is a cell suspension with known cell concentration from Basic Protocol 1. Calculate the number of cells (Equation 8), volume of cells (Equation 9), and final volume (Equation 10) needed:

$$Cells\ needed = 1\ \times 10^5\ (cells/tube) \times (number\ of\ tubes + 1)$$

Equation 8

$$Volume\ of\ cells\ needed = \frac{cells\ needed}{cell\ concentration\ (cell/ml)}$$

Equation 9

$$Final\ volume = 0.1\ (ml/tube) \times (number\ of\ tubes + 1)$$

Equation 10

The recommended range of cells should fall between 1×10^5 and 1×10^6 cells per tube. Modify Equation 8 accordingly.

31. Adjust the volume of cells needed (Equation 9) and the final volume (Equation 10) according to the concentration of cells in suspension. Depending on the initial cell concentration, either centrifuge 10 min at 340 × *g,* room temperature to concentrate or dilute in expansion CCM or PBS until the final volume required is reached (Equation 10).

32. Properly label cytometry tubes, using one tube for double antibody staining and one or two additional tubes for negative controls: unstained cells (mandatory) and isotype control (optional).

    The cell surface marker profile of ovine MSC does not consistently align with the human MSC ISCT established panel due to a paucity of specific antibodies for sheep and limited human antibody cross-reactivity with ovine surface antigens. Therefore, we recommend analyzing a panel of positive markers for the phenotypic characterization of oWJ-MSC, including: CD44 and CD166; and a panel of negative markers including: CD31, CD45, and MHC Class II DR.

    However, other authors have tested antibodies that seem to cross-react with ovine cells and can be used for the same purpose (Khan, Chandrashekran, Smith, & Dudhia, 2016).

33. Dispense the appropriate volume of each antibody into the corresponding tube.

    We recommend 5 µl of each antibody per tube.

    Note that the tube for the negative control will remain without antibody.

    Make sure the entire volume is placed on the bottom of the tube in order to avoid any loss of antibody.

34. Add 100 µl cell suspension into each tube. Then stir and incubate 15 min at room temperature in the dark.

35. Add 3 ml PBS into each tube and centrifuge 5-10 min at 340 × *g*, room temperature. Then remove supernatant by decantation.

36. Resuspend pellet in a final volume in 100-300 µl PBS.

37. Establish correct settings for the oWJ-MSC population (in terms of forward scatter, side scatter, and fluorochrome channel voltages) and perform the acquisition starting with the negative(s) control tube(s).

    Note that acquiring the negative control sample first will allow setting the positivity threshold for all the labeling.

    Properly homogenize each tube prior to the acquisition.

    It is highly recommended that at least 10,000 events be acquired per tube.

    Double-check the suitability of the chosen settings at the beginning of the acquisition of the negative control tube.

GROWTH PROFILE DETERMINATION OF OVINE MESENCHYMAL STROMAL CELLS FROM WHARTON'S JELLY

This method describes how to assess growth kinetic parameters of oWJ-MSC by developing a growth curve in order to allow the determination of specific growth rate, cell population doubling time, and expansion factor. Quantification of the number of cells can be performed using direct methods (microscopy/cytometry) or by indirect methods such as intracellular ATP content quantification.

Materials

Cell suspension of known concentration (from Basic Protocol 1)
Sterile PBS (e.g., Gibco brand, Thermo Fisher Scientific, cat. no. 14190)
Expansion complete culture medium (expansion CCM; see recipe)
CellTiter-Glo® 2.0 Cell Viability Assay (Promega, cat. no. G9242)
0.05% (w/v) trypsin (e.g., Gibco brand, Thermo Fisher Scientific, cat. no. 25300-054)

24-well cell culture plates (Corning, cat. no. 3337)
96-well white flat bottom plates (Thermo Fisher Scientific, cat. no. 236108)
Sterile 15- and 50-ml conical tubes
Sterile 1.5-ml microtubes
Sterile serological pipets
Pipet tips
Class II biological safety cabinet
Centrifuge
37°C, 5% CO_2 humidified incubator
Phase-contrast inverted microscope
Orbital plate shaker device
Luminometer or microplate reader capable of measuring luminescence

Cell seeding

1. Obtain a cell suspension of know cell concentration from Basic Protocol 1. Calculate the number of cells (Equation 11), volume (Equation 12), and final volume of cell suspension (Equation 13) needed.

$$Cells\ needed = 1 \times 10^3 (cells/cm^2) \times 1.9\ \left(cm^2\right) \times (number\ of\ wells + 1)$$

Equation 11

$$Volume\ of\ cells\ needed = \frac{cells\ needed}{cell\ concentration\ (cells/ml)}$$

Equation 12

$$Final\ volume = 0.5\ (ml/well) \times (number\ of\ wells + 1)$$

Equation 13

We recommend one 24-well plate for each time point with an initial cell seeding density of 1 × 10³ cells/cm² and at least three replicates for each cell line.

If using a culture plate with different well number, be aware of the specific area of the well in Equation 11 (see Table 1).

2. Adjust the volume of cells needed (Equation 12) and the final volume (Equation 13) according to the concentration of cells in suspension. Based on values of the initial cell concentration, either centrifuge 10 min at 340 × *g*, room temperature to concentrate or dilute with expansion CCM in order to set the final volume required (Equation 13).

Table 2. Preparation of Known Cell Concentrations for the Standard Curve

Standard	Cell density (cells/cm^2)
1	5.00×10^4
2	2.50×10^4
3	1.25×10^4
4	6.25×10^3
5	3.13×10^3
6	1.56×10^3
7	7.81×10^2
8	3.91×10^2
9	1.95×10^2
10	9.77×10

3. Seed each well of the 24-well plates by dispensing 0.5 ml adjusted cell suspension.

   Make sure cell suspension is mixed well prior to dispensing.

   Dispensing 0.5 ml of PBS or sterile water into surrounding wells is highly recommended in order to avoid evaporation of test samples.

4. Incubate plates at 37°C, 95% humidity and 5% CO_2 and maintain them under these conditions through the completion of the growth curve.

   Initial time point will consider cell density value as 1×10^3 cells/cm^2.

   Otherwise, we recommend seeding an initial time point plate with the same conditions as the rest of the time points. Incubate plate for at least 4 hr before the determination of the cell number in order to allow cell adhesion.

Growth profile determination

5. Determine the cell number of each time point in order to perform a growth curve using direct cell counting or indirect determination of cell number.

Direct cell counting

6a. Discard expansion CCM of the well of interest and wash once with PBS. Then add a volume of trypsin according to the volumes listed in Table 1.

7a. Incubate at 37°C for 5 min until the cells detach as observed visually or under a phase-contrast microscope.

8a. Add the same volume of expansion CCM as trypsin added in the previous step and collect a sample in a 1.5-ml microcentrifuge tube for cell counting.

9a. Determine the number of cells using a hemocytometer or a flow cytometer.

   Initial time point will consider cell density value as 1×10^3 cells/cm^2.

Indirect cell counting

Quantification of the number of cells can be also performed using indirect methods such as intracellular ATP content quantification. This protocol is adapted from CellTiter-Glo® 2.0 cell viability assay.

6b. Prepare a standard curve of relative luminescent units (RLU) obtained from known cell concentrations according to Table 2.

   The standard curve has to be done at the initial time point. It will allow interpolation of all the values obtained at different time points of the growth curve.

We recommend using the same plate and the same cell suspension obtained from Basic Protocol 1 that will be assayed in the growth curve.

7b. Seed all standard curve concentrations in a 24-well plate by dispensing 0.5 ml adjusted cell suspension.

8b. Incubate standard curve plate at 37°C, 95% humidity and 5% CO_2 for at least 4 hr before the determination of the cell number in order to allow cell adhesion.

9b. Prepare CellTiter-Glo® 2.0 Reagent following kit instructions.

10b. Add a volume of CellTiter-Glo® 2.0 Reagent equal to the volume of cell culture medium present in each well.

11b. Mix contents for 2 min on an orbital plate shaker to induce cell lysis at room temperature in the dark.

12b. Incubate plate for 10 min at room temperature in the dark to stabilize the luminescent signal.

13b. Dispense 100 µl from each 24-well plate into a 96-well white flat bottom plate and record luminescence.

At least three replicates for each 24-well plate are recommended.

14b. Interpolate the RLU values recorded for each time point into the standard curve.

15b. Calculate cell number in each time point interpolating the RLU values recorded on the standard curve equation.

16b. Calculate maximum specific growth rate (μ_{max}, $days^{-1}$) from the slope of the linear regression corresponding to the exponential growth phase when logarithm of cell density in a given time of the exponential phase and initial cell seeding density are plotted versus time.

17b. Calculate cell population doubling time (Equation 14) and expansion factor (Equation 15).

$$Cell\ population\ doubling\ time = \frac{ln\ 2}{\mu_{max}}$$

Equation 14

$$Expansion\ factor = CPD \times e^2$$

Equation 15

REAGENTS AND SOLUTIONS

Derivation complete culture medium

Dulbecco's Modified Eagle's Medium (DMEM; Gibco brand, Thermo Fisher Scientific, cat. no. 31885-023) supplemented with 20% (v/v) pooled ovine serum, 200 UI/ml penicillin/streptomycin (MilliporeSigma, cat. no. P4458-100 ml), and 0.25 µg/ml amphotericin B (Gibco brand, Thermo Fisher Scientific, cat. no. 15290-018).

Store up to 1 month at 2° to 8°C.

Expansion complete culture medium

Dulbecco's Modified Eagle's Medium (DMEM; Gibco brand, Thermo Fisher Scientific, cat. no. 31885-023) supplemented with 10% (v/v) pooled ovine serum.

Store up to 1 month at 2° to 8°C.

Table 3. Troubleshooting Guide for Problems that May be Encountered During oWJ-MSC Derivation and Characterization[a]

Problem	Possible cause	Solution
Poor yields of oWJ-MSC after derivation	Reagents and/or solutions were not prewarmed	Prewarm reagents and/or solutions before use
	Low quantity of WJ in the UC	Scrape WJ thoroughly from tissue
	Prolonged time of tissue process	Make sure to process tissue within the first 8 hr after extraction
oWJ-MSC cultures are not growing or growing slowly	Incubator was bumped or vibrations affected the culture	Make sure the culture stays undisrupted, without vibrations
	The pooled sera is poor quality	Validate pools prior to use with a control cell line
		Increase to 20% the supplementation of poor quality pooled sera
	Initial cell seeding density was too low	Increase initial cell seeding density Incubate longer times for cells to overcome the lag phase
Detachment of cell monolayer during differentiation	Cell seeding density was too high	Seed at a lower seeding density
	Prefixation and/or fixation did not work properly	Be accurate with prefixation and/or fixation incubation times
	Addition of medium and/or removal of supernatant was too fast or disruptive	Always add and/or remove volumes very carefully
Cell population is unclear in the phenotypic characterization	Non-specific labeling	Perform isotype controls during the simple labeling and FMO during multiparametric labeling

[a]Abbreviations: oWJ-MSC, ovine Wharton's jelly-derived mesenchymal stromal cells; WJ, Wharton's jelly; UC, umbilical cord; FMO, fluorescence minus one.

Oil Red O staining solution

Prepare a 0.2% (w/v) Oil Red O (e.g., MilliporeSigma, cat. no. O0625-25G) stock solution in methanol. Then combine 35 ml Oil Red O stock solution with 10 ml 1 M NaOH.

Prepare fresh before use.

COMMENTARY

Background Information

The term mesenchymal stromal cells (MSC) describes multipotent cells that can be isolated from virtually all tissues of the body and readily expanded ex vivo (Ullah, Subbarao, & Rho, 2015). Wharton's jelly (WJ) as a source of MSC is very promising because WJ-MSC show high proliferative potential, high frequency of forming colonies, and a shorter population doubling time than adult stem cells (Grau-Vorster et al., 2019; Oliver-Vila et al., 2016). Remarkably, these cells are immunoprivileged and do not trigger an immune response. Finally, obtaining this tissue does not involve invasiveness or any other ethical concern (Watson et al., 2015).

Taking cell-based therapies from bench to bedside requires assessment of pharmacodynamics, pharmacokinetics, and toxicology of the product, also known as preclinical trial. For preclinical large animal models, sheep show comparable bodyweight and long bone dimensions relative to humans. Also, in the

orthopedics field, bone mineral composition does not show large differences, and metabolic and remodeling rates are also similar to humans (Reichert et al., 2010).

Isolation of WJ-MSC can be done throughout explant, explant-enzymatic or enzymatic methods, and at the same time, the enzymatic method can be based on different types of enzymes. 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 (Varaa, Azandeh, Khodabandeh, & Gharravi, 2019). It has been reported that the explant method shows higher proliferation than the enzymatic one, as well as a higher percentage of cells expressing CD73 and CD90 (Hendijani, 2017).

Critical Parameters

All animal work requires appropriate approval from an Institutional Animal Care and Use Committee and investigators and other personnel caring for laboratory animals must be trained accordingly.

The number of oWJ-MSC may vary depending on the UC sample, the donor, and the extraction procedure, therefore the establishment of MSC cultures might take a longer time in some cases.

Troubleshooting

Most common problems and potential solutions encountered during the derivation of ovine MSC from WJ and during their functional and phenotypical characterization are summarized in Table 3.

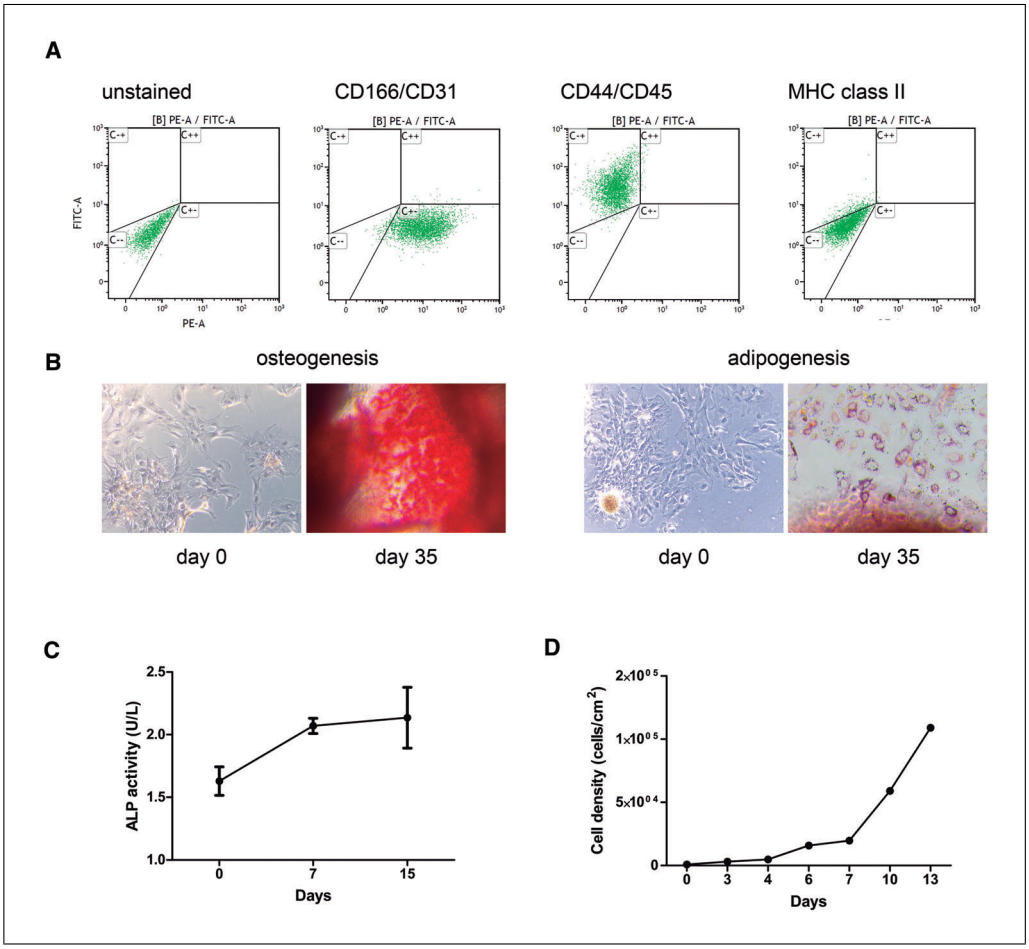

Figure 4 Expected results in the characterization of ovine Wharton's jelly-derived mesenchymal stromal cells (oWJ-MSC) isolated from the Wharton's jelly of umbilical cord. (**A**) Adjustment of the autofluorescence of an unstained oWJ-MSC population previously gated by FSC/SSC for exclusion of debris and doublets. FL1-H (PE-A) and FL2-H (FITC-A) channels with quadrant sets for unstained oWJ-MSC, double staining for CD166-PE/CD31-FITC and CD44-FITC/CD45-PE, and single staining for MHC Class II-FITC. (**B**) Negative control and positive Alizarin Red staining for osteogenesis differentiation and Oil Red O staining for adipogenesis differentiation. Original magnification 100× and 200×. (**C**) Alkaline phosphatase activity determination for oWJ-MSC in differentiation conditions at 0, 7, and 15 days. (**D**) Growth profile determination of oWJ-MSC using intracellular ATP content quantification.

Understanding Results

This method of derivation of multipotent ovine MSC from WJ yields densities in the range of 2×10^4 to 4×10^4 oWJ-MSC/cm^2 and >95% viability at passage 2, which corresponds to ∼8 CPD. The cells display fibroblastic morphology and adhere to culture-treated plastic surfaces in standard culture conditions. Ovine WJ-MSC cultures result in differentiated cells positive for ALP activity determination, AR staining, and Oil Red O staining when subjected to specific in vitro differentiation conditions (Fig. 4), thus demonstrating their potential to become osteoblasts/osteocytes and adipocytes. Moreover, oWJ-MSC cultures are positive for CD44 and CD166; and negative for CD31, CD45, and MHC class II surface markers (Fig. 4). Main growth kinetics parameters show specific growth rates >0.35 and cell population doubling times of 2 days.

Time Considerations

The entire procedure, from obtaining UC until the last expansion in order to reach the needed amount of WJ-MSC for preclinical studies, is no longer than 2 weeks. Whereas cell seeding of the plates in order to characterize WJ-MSC can be done in 3 hr, MSC cultures in differentiation medium can take up to 1 month. Phenotypic characterization entirely depends on the flow cytometer used and on the labeling strategy chosen but it can typically be done in <3 hr.

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Conflicts of Interest Statement

The authors declare no conflict of interest.

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Key References

Oliver-Vila et al., 2016. See above.

This paper describes the design and validation of the protocol for the derivation of WJ-MSC from human umbilical cords that inspired the study presented here.

Ramallo et al., 2020. See above.

This paper presents and discusses the relevance of using species-specific WJ-MSC in preclinical regulatory studies.