STAR Protocols

Protocol

Transcriptional analysis of mouse wounds grafted with human mesenchymal stem cells and platelets

Platelet isolation (1), stem cell preparation (2) & generation of skin wounds and cell grafting (3)

Measure of wound closure and wound harvesting

Day 3 or 7 **Step 4**

RNA extraction and RT-qPCR assays

Day 0 **Step 1-3**

Day 4 or 8 **Step 5**

Graphical elements adapted from Servier Medical Art repository (http://www.smart.servier.com)

Jennyfer Levoux, Peggy Lafuste, Anne-Marie Rodriguez

jennyfer.levoux@inserm.fr (J.L.)
anne-marie.rodriguez@inserm.fr (A.-M.R.)

Highlights

Generation of full-thickness cutaneous wounds in mice

Protocol for platelet isolation from blood samples and *in vitro* expansion of human MSCs

Cell engraftment procedures of mouse cutaneous wounds

Analysis of transcripts expressed by human grafted cells or host cells in mouse wounds

Platelet preparations are commonly used in the clinic in combination with mesenchymal stem cells (MSCs) to improve their wound healing capacity and optimize their therapeutic efficacy following their delivery into diseased tissues. To investigate the mechanisms by which platelets enhance the repair properties of MSCs, we detail a protocol using a humanized mouse model for excisional wounds to study by reverse transcription real-time PCR whether human platelets alter the therapeutic efficacy of grafted human MSCs.
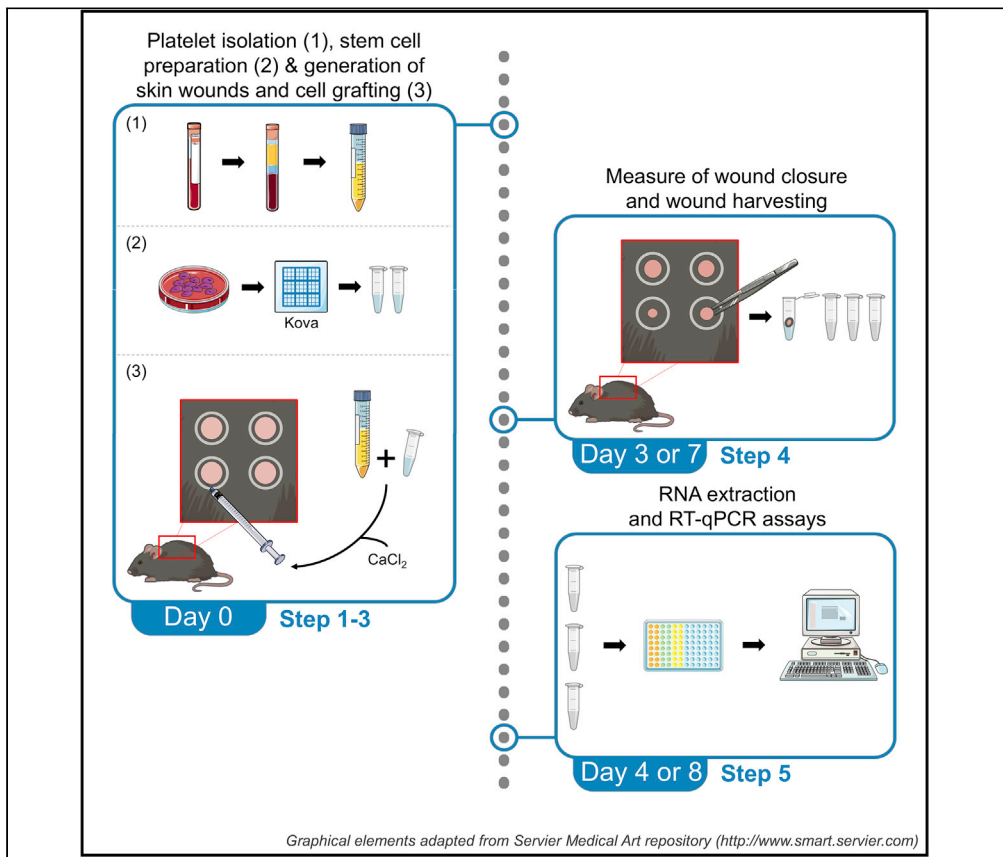
Protocol

Transcriptional analysis of mouse wounds grafted with human mesenchymal stem cells and platelets

Jennyfer Levoux,[1,3,*] Peggy Lafuste,[1] and Anne-Marie Rodriguez[1,2,*]

[1]Univ Paris Est Creteil, INSERM, IMRB, 94010 Créteil, France

[2]Lead contact

[3]Technical contact

*Correspondence: jennyfer.levoux@inserm.fr (J.L.), anne-marie.rodriguez@inserm.fr (A.-M.R.)
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SUMMARY

Platelet preparations are commonly used in the clinic in combination with mesenchymal stem cells (MSCs) to improve their wound healing capacity and optimize their therapeutic efficacy following their delivery into diseased tissues. To investigate the mechanisms by which platelets enhance the repair properties of MSCs, we detail a protocol using a humanized mouse model for excisional wounds to study by reverse transcription real-time PCR whether human platelets alter the therapeutic efficacy of grafted human MSCs.

For complete details on the use and execution of this protocol, please refer to Levoux et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using (1) human Multipotent Adipose Derived Stem cells (hMADS) (Rodriguez et al., 2005) as human MSCs for graft and (2) C57BL/6JRj mice as the mouse strain to perform the wound experiments. This protocol is adaptable to MSCs from different tissue origins, including bone marrow, muscle or umbilical cord blood and to other murine genetic backgrounds, including nude immune-deficient mice.

Culture cell line

⏱ Timing: 1+ weeks

1. In view to perform the graft experiments, thaw a vial of cryopreserved hMADS cells rapidly (< 1 min) in a 37°C water bath and dilute the thawed cells by slowly adding 1 mL of 37°C pre-warmed culture cell medium. Pipet up and down transfer the cells in a conical 15 mL tube.

    Note: It is important to thaw rapidly the cells (< 1 min at 37°C) to minimize any damage to the cell membranes due to the toxic effects of the cryoprotectant DMSO above 4°C.

2. Add 4 mL of cell culture medium and centrifuge cells at 380–430 × *g* for 5 min at ~20°C–25°C to remove the cryoprotectant (DMSO). Discard the supernatant.
3. Re-suspend the cell pellet in 1 mL of culture medium and count the cells with the disposable Kova® Slide counting device. Seed them in 100 mm culture dishes at a density of 3000 cells/cm^2 in 10 mL culture medium. Cells are then maintained in a 5% CO_2 atmosphere at 37°C.
4. Replace the culture medium every 2–3 days.

5. Once the cells have reached ~80% of confluence, split the cells:

a. Wash with 5 mL of DPBS 1× and add 1.5 mL of TrypLE for 10 min at 37°C to detach the cells.

Note: It is recommended to check the detachment of the cells 5 min following their exposure to TrypLE.

Alternatives: Others dissociating reagents such as Trypsin can be used to detach the cells. However, this enzymatic method for detaching adherent cells has been reported to be more aggressive (Tsuji et al., 2017).

b. Add 5 mL of culture medium to inactivate TrypLE, collect the cells in suspension and centrifuge them at 400 × *g* for 5 min at RT. Discard the supernatant.

c. Re-suspend the cell pellet in 3 mL of cell culture medium and seed the cells into 3 new 100 mm culture dishes (1:3 ratio dilution corresponding to 150 000 cells/mL). Let the cells expand until they reach 80% confluence.

Optional: Repeat step 5 as many times as necessary to obtain the amount of cells required for the experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human platelet-rich plasma	Collected from healthy donors	N/A
Chemicals, peptides, and recombinant proteins		
DMEM, low glucose, GlutaMAX™ Supplement, pyruvate	Gibco	Cat#21885025
Fetal Bovine Serum	Dominique Dutscher	Cat#S1810-500
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140122
HEPES (1 M)	Gibco	Cat#15630056
DMSO ACS 100 mL	EUROMEDEX	Cat#UD8050-05-A
DPBS, no calcium, no magnesium	Gibco	Cat#14190094
TrypLE™ Express Enzyme (1×), phenol red	Gibco	Cat#12605010
Rotenone	Sigma-Aldrich	Cat#R8875
Antimycin A from *Streptomyces* sp.	Sigma-Aldrich	Cat#A8674
HBSS, calcium, magnesium, no phenol red	Gibco	Cat#14025050
Sodium citrate tribasic dihydrate	Sigma-Aldrich	Cat#S4641
Calcium chloride	Sigma-Aldrich	Cat#C1016
ISO-VET 100%, liquide pour inhalation par vapeur	Piramal Critical Care	N/A
Buprécare (0,3 mg/mL)	Axience	N/A
Héparine sodique 25000 UI – 5 mL	Panpharma	N/A
Critical commercial assays		
RegenKit®-BCT	Regen Lab SA	N/A
KOVA® Glasstic Slide 10 With Counting Grids	Kova International, Inc.	Cat#87144
RNeasy Fibrous Tissue Mini Kit	QIAGEN	Cat#74704
SuperScript™ III Reverse Transcriptase Kit	Invitrogen	Cat#18080044
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems	Cat#A25742
Master Mix PCR Power SYBR™ Green	Applied Biosystems	Cat#4309155
Experimental models: cell lines		
hMADS cell	Rodriguez et al., 2005	N/A
Experimental models: organisms/strains		
C57BL/6JRj mice	Janvier Laboratories	Cat#2670020 RRID: MGI:2670020

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Primer3	Koressaar and Remm, 2007 Untergasser et al., 2012 primer3.ut.ee	N/A
BLAST®	National Institutes of Health (NIH)	N/A
ImageJ software 1.52	National Institutes of Health (NIH)	N/A
StepOne Software v2.1	Applied Biosystems	N/A
SDS 2.4 acquisition and analysis software	Applied Biosystems	N/A
GraphPad Prism software 6.0	GraphPad Software	RRID: SCR_002798
Other		
VetScan HM5	Abaxis, Inc	N/A
LUNA-FL™ Automated Fluorescence Cell Counter	Logos Biosystems	Cat#L20001
Sterile 5-mm punch biopsy	Kai Medical	Cat#BP-50F
6-0 ETHILON® Nylon Suture	Ethicon™	Cat#697G
Telfa™	Covidien	Cat#1109
3M™ Tegaderm™ Transparent Film Dressing	3M™	Cat#1620
MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	Applied Biosystems	Cat#4346907
MicroAmp™ Optical Adhesive Film	Applied Biosystems	Cat#4311971
DeNovix DS-11 Spectrophotometer	DeNovix Inc.	N/A
StepOnePlus Real-Time PCR System	Applied Biosystems	N/A
7900 Real-Time PCR Detection System	Applied Biosystems	N/A

MATERIALS AND EQUIPMENT

Culture Cell Medium

Reagent	Final concentration	Amount
Heat-inactivated FBS	10%	50 mL
Penicillin/Streptomycin	100 U/mL, 100 µg/mL	5 mL
HEPES	10 mM	5 mL
DMEM	n/a	500 mL
Total	n/a	560 mL

Store at 4°C up to 4 weeks.

Calcium Chloride Solution (10%, CaCl$_2$)

Reagent	Final concentration	Amount
Calcium Chloride	10%	10 g
Distilled DNase/RNase free water	n/a	100 mL
Total	n/a	100 mL

Store at 4°C up to 12 months.

Rotenone Stock Solution (1 mM)

Reagent	Final concentration	Amount
Rotenone	1 mM	1 mg
DMSO	n/a	2.5354 mL
Total	n/a	2.5354 mL

Aliquot and store at −20°C up to 6 months.

Antimycin A Stock Solution (1 mM)		
Reagent	Final concentration	Amount
Antimycin A from Streptomyces sp.	1 mM	1 mg
DMSO	n/a	1.8797 mL
Total	n/a	1.8797 mL

Aliquot and store at −20°C up to 6 months.

STEP-BY-STEP METHOD DETAILS
Platelet isolation

⊙ Timing: ∼30 min

For platelets isolation, human blood samples from healthy donors (Etablissement Français du Sang, EFS located in Créteil) are collected in tubes containing heparin (20 U/mL) or EDTA (1.5 mg/mL) to prevent blood coagulation.

⚠ CRITICAL: Avoid the use of citrate as anti-coagulant if you want to study the effects promoted by platelet mitochondrial citrate on the metabolism and repair properties of MSCs as reported by Levoux et al., 2021.

1. Deposit 8 mL of blood per RegenKit BCT tube and centrifuge the samples at 1800 × g for 5 min at ∼20°C–25°C without brake (to avoid platelet activation).

   Note: The RegenKit BCT tube contains a cell separator gel that allows efficient platelet isolation from a small volume of blood. This cell separator gel drastically reduces red and white blood cell contaminations. After the centrifugation step, red blood cells are kept under the gel while the platelets are sedimented on the gel surface (Figure 1).

   Alternatives: It is not mandatory to use RegenKit BCT tubes to isolate platelets from blood. Platelets can be isolated by centrifugation of blood at 500 × g without brake. This latter method, which is less expensive, generates plasma with lower concentration of platelets and more contaminating red and white blood cells. If this alternative method is chosen, it is possible to significantly reduce the rate of contaminating cells by centrifugation of the plasma at 300 × g during 10 min without brake. After the spinning, collect the supernatant containing the platelets and discard the pellet of red and white blood cells.

2. After centrifugation, re-suspend the platelets in the plasma layer by gently inverting the RegenKit BCT tube 5–10 times. Remove the cap of the Regenkit tube and collect the platelet-rich plasma (PRP) with a micropipette into a conical 15 mL tube.

   Note: An average of 4.5 mL of PRP containing 1.8×10^9 platelets are obtained from 8 mL of human blood.

   Optional: The paper of Levoux et al., 2021 has provided evidence that platelets release respiratory-competent mitochondria to MSCs that stimulate their repair properties, platelet's mitochondria inhibited for their mitochondrial respiration loosing this effect. If you want to study the role of platelet's mitochondria on MSCs functionality, it is possible to inhibit the mitochondrial respiration of platelets after their isolation, by adding rotenone (complex I inhibitor) to a final concentration of 2 μM and antimycin A (complex III inhibitor) to a final concentration of 2 μM for 3 h. In this condition, wash the platelets 3 times by adding serum free DMEM medium containing heparin (20U/mL) (1 volume Platelets/9 volume washing medium)
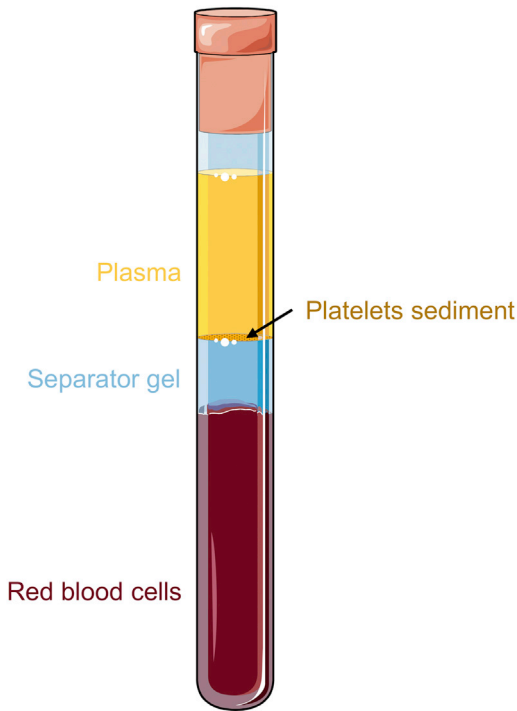
Figure 1. Illustration of a RegenKit® tube filled with blood after centrifugation and plasma separation

and centrifuge at 500 × *g* without brake. Washing steps are critical to remove rotenone and antimycin from platelets prior to their exposure to MSCs and avoid that these reagents alter the mitochondria of MSCs.

3. Wash platelets 3 times:
    a. Add 10 mL of serum-free DMEM containing 20 U/mL heparin.

    △ CRITICAL: Use heparin to a final concentration of 20 U/mL in serum-free DMEM to avoid platelet aggregation.
    b. Centrifuge at 1800 × *g* for 5 min at ∼20°C–25°C without applying the brake. Discard supernatant.
    c. Re-suspend platelets in serum-free DMEM containing 20 U/mL heparin.
    d. Repeat the steps a–c, 2 additional times.

4. Re-suspend platelets in Hank's Balanced Saline Solution (HBSS).
5. Count platelets with a blood analysis instrument such as the VetScan HM5 and dilute them to a final concentration of 8×10^7/mL.

    △ CRITICAL: Ensure that PRP preparations are minimally or not contaminated with erythrocyte since these cells release products such as hemoglobin and hemin that counteract with the pro-healing effects of platelets (Everts et al., 2019). If platelet preparations are contaminated by red blood cells, these can be removed by a centrifugation step at 300 × *g* during 10 min with no brake.

Mesenchymal stem cell preparation

⏱ Timing: ∼30 min

HMADS cells have been expanded *in vitro* in sufficient number to perform engraftment experiments in mouse wounds.

6. Dissociate the adherent hMADS cells prior to their delivery into mouse wound:
   a. Wash HMADS cells with 5 mL of DPBS 1× and then add 1.5 mL of cell dissociation enzyme TrypLE for 10 min at 37°C.
   b. Add 5 mL of cell culture medium to stop the TrypLE activity and centrifuge the cell suspension at 400 × g for 5 min at RT. Discard the supernatant.
   c. Re-suspend the pellet in HBSS.
7. Count the cells with a disposable counting device such as the Kova® Slide, and distribute 2×10^5 cells per Eppendorf tube in a final volume of 70 μL, each tube corresponding to a wound graft condition.

   Alternatives: It is possible to manually count the cells with non-disposable counting devices such as the Malassez chamber or to use an automated cell counting device such as the LUNA-FL™ Automated Fluorescence Cell Counter.

Mouse cutaneous wounds and cell grafting

⏱ Timing: for steps 8–15, ∼2 h (per mouse); for step 16, 3 days

Full-thickness skin wounds are created on the back of 8-week-old male C57BL/6JRj mice (Galiano et al., 2004). Immediately after their creation, wounds are treated with either saline solution (HBSS), platelets alone, hMADS cells alone (Rodriguez et al., 2005) and hMADS cells (Rodriguez et al., 2005) in combination with platelets.

8. Anaesthetize mice with isoflurane, starting at 3.5 mL/L O_2 and keeping at 1.5 mL/L O_2.
9. Administer a preventive i.p. injection of buprenorphine analgesic (0.1 mg/kg), 30 min prior to proceed to step 11.

   ⚠ CRITICAL: Administration of analgesics to relieve pain is made every 12 h for 3 days after step 9.

10. Shave the mouse dorsal surface with an electric clipper and sterilize with 70% alcohol.
11. Pinch the skin at the midline of the mouse back to be able to create four full thickness 5 mm diameter circular skin wounds (2 wounds on each side of the midline) using a 5 mm sterile biopsy punch.
12. Attach sterile silicone donut-shaped splints to the edge of the surrounding wound with adhesive film and using interrupted sutures with 6-0 silk thread.

   ⚠ CRITICAL: This step is essential to prevent skin retraction (Figure 2).

   Optional: If you want to measure wound closure, place a millimeter ruler close to the wound and then, take a photography with a digital camera or a smartphone.

13. Immediately after the injuries, inject each wound with 100 μL of the following conditions: HBSS, 2×10^5 hMADS cells, 1.6×10^6 human activated platelets or 2×10^5 hMADS cells in combination with 1.6×10^6 activated platelets. Platelets are activated prior to their delivery in mouse wounds by adding 10% of $CaCl_2$ solution.

   ⚠ CRITICAL: $CaCl_2$ needs to be added few seconds prior cell engraftment to avoid platelet clotting in the syringe.

Figure 2. Photograph of a mouse back with four full-thickness skin wounds fixed with silicone donut-shaped splints

Optional: To study the effect of citrate on hMADS cells exposed to platelets sharing inhibited mitochondrial respiration, the wounds are injected with the conditions mentioned in step 13 using platelets previously treated with rotenone and antimycine supplemented with 10 mM tribasic sodium citrate dihydrate.

14. Cover wounds with a transparent non-adherent dressing and maintain it with an adherent dressing.

   ⚠ CRITICAL: This step is important because it prevents removal of grafts due to back scratching by mice.

15. Place the mice in an awakening cage under a heat lamp to allow them to fully recover from the anesthesia, then return them to their cages in the animal facility.

   Note: Do not put more than 3 mice per cage to prevent them from removing their non-adherent transparent bandages and nibbling on their silicone donut splints and sutures.

16. It is necessary to control pain every 12 h for 3 days with i.p. injection of buprenorphine (0.1 mg/kg).

Wound closure and dissection

⏱ Timing: ~1 h (per mouse)

The mice are sacrificed on the third or seventh day after injury and wound treatments. The wounds are then dissected.

17. At day 3 after step 16, gently remove the transparent non-adherent dressing as well as silicone donut-shaped splints and sutures.

   ⚠ CRITICAL: It is recommended that non-adherent dressings be removed on the third day after injury to minimize wound exudation.
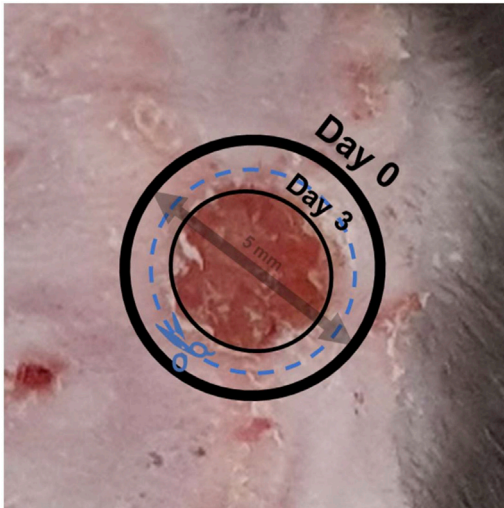
Figure 3. Photograph showing a full thickness skin wound at day 3 following surgery
The blue dotted circle with scissors designs where wound is dissected (blue). Black circle corresponds to the initial wound size.

18. After step 17, mice can be immediately sacrificed or maintained until day 7. Sacrifice mice by administering a lethal dose of isoflurane on day 3 or day 7 after injury.

    Alternatives: It is possible to sacrifice mice by other methods including cervical dislocation. If this latter method is preferred, take care to not damage the healed wounds.

    Optional: If you want to measure wound closure, place a millimeter ruler near the wounds and take pictures. Wound closure can be quantified using ImageJ software in a blinded manner through assessment of wound re-epithelialization on days 3 and 7 post-graft and can be calculated as follows: [1- (wound area on day X/wound area on day 0) × 100].

19. Dissect and excise with scissors and fine forceps about 1–2 mm around the wound site to collect only scar tissue (Figure 3).

    Note: The initial wound area would be previously delimited by a marker pen.

    △ CRITICAL: Collecting more tissue around the initial wound can dramatically alter the results.

20. Rapidly freeze skin biopsies in liquid nitrogen and store at −80°C.

    ⦀ Pause point: Tissues can be stored at −80°C up to one year.

RNA extraction and RT-qPCR assays

⏱ Timing: for steps 21–22, ∼2 h; for step 23, ∼2 h; for step 24–31, ∼half-day

RNAs (RiboNucleic Acids) are extracted from mouse skin wounds and then analyzed for the transcriptional expression of several human and mouse genes by RT-qPCR assay.

21. Extract RNAs from mouse cutaneous wounds using the RNeasy Fibrous Tissue Mini Kit, following the manufacturer's instructions.
22. Quantify the amount of RNAs using a spectrophotometer such as the DeNovix DS-11 Spectrophotometer.

Note: Typically, RNAs is eluted in 30 μL of distilled DNase/RNase free water and a sample of a mouse skin wound yields 1000 ng/μL.

Alternatives: It is possible to use other RNAs isolation methods, but ensure that the alternative method allows effective lysis of the scared wounds that are very fibrous.

Note: Manufacturer's instruction for the RNeasy Fibrous Tissue Mini Kit can be found here.

⏸ **Pause point:** RNAs can be stored at −80°C for at least several months.

23. Reverse transcription by using the SuperScript™ III Reverse Transcriptase kit and random primers, following the manufacturer's instructions.

Note: It is recommended to use 1 μg of RNAs for the reverse transcription.

Alternatives: It is possible to use other methods for reverse transcription. In particular, reverse transcription can be performed with a broad range of reverse transcriptase enzymes. In addition, random primers can be replaced by oligo-dT. This last approach is more specific as it specifically hybridizes the poly(A) tail of mRNA and allows the reverse transcription of the mRNA full length. However, the use of random hexamers allows the synthesis of a more large pool of cDNA (from small mRNA fragments, 5′end mRNA sequences…).

Note: It is possible to use RNA from mouse wounds to perform global or single cell RNA sequencing.

Note: Manufacturer's instruction for the SuperScript™ III Reverse Transcriptase kit can be found here.

24. Dilute the cDNAs (complementary DeoxyriboNucleic Acids), previously obtained in step 23, at a 1:10 ratio with distilled DNase/RNase free water in a final volume of 200 μL. Mix them by pipetting and store them at 4°C for short storage and −20°C for long storage.

⏸ **Pause point:** cDNAs can be stored at −20°C for several months.

25. Design primer sequences for reference and interest genes using Primer3 and BLAST software.

Note: In our study, we used genes encoding for human or mouse TATA-Binding Proteins (TBP) as reference genes for the quantification of human or murine transcripts of interest, respectively. However, it is possible to use other housekeeping genes as reference genes such as β-actin, cyclophilin, β2-microglobulin (Dheda et al., 2004). Of note, it is not recommended to use as reference gene, the gene encoding for the glyceraldehyde 3-phosphate-dehydrogenase enzyme (GAPDH) because this gene is involved in the metabolic activity of the cell (Ayanoğlu et al., 2020) and in our study, we showed that platelets alter the metabolism of the MSCs.

⚠ CRITICAL: Primers must be ∼20 bp (base pairs) and their sequences must be specific to human or mouse to distinguish the gene expression of the transplanted human cells from that of the mouse host cells. The annealing temperature of the primer should be ∼60°C and it is preferable but not mandatory that the primers cover an exon-exon junction. In addition, the size of the PCR product should not exceed 300 bp.

Note: The species specificity of primers is assessed by using BLAST alignment.

Figure 4. Example of a 96-well plate layout for 1 cDNAs sample with different dilutions and 1 primer-pair mix

26. Determine the efficiency of each primer before use:
    a. Perform a series of dilution of cDNA sample at 1:2 in distilled DNase/RNase free water;

    Note: It is recommended to start with the undiluted cDNA sample.
    b. Prepare, on ice, for each primer pair, the following mix (quantity for 1 well):
       - 0.25 μL of distilled water free of DNase/RNase.
       - 0.5 μL of forward primer at 10 μM of reference or interest gene.
       - 0.5 μL of reverse primer at 10 μM of reference or interest gene.
       - 6.25 μL of PowerUp™ SYBR™ Green Master Mix.

    Alternatives: It is possible to use another SYBR Green mix, like Master Mix PCR Power SYBR™ Green.

    Note: Do not forget, when you prepare the working solution that you need to work at least in duplicate and preferably in triplicate.
    c. Mix thoroughly each primer-pair mix by pipetting.
    d. Add 7.5 μL of each primer-pair mix to wells of a 96-well plate and then add 5 μL of each cDNA sample dilutions to wells of a 96-well plate as in shown in Figure 4.
    e. Seal properly the plate with a seal compatible with qPCR, like MicroAmp™ Optical Adhesive Film.
    f. Spin the plate briefly to prevent drops from remaining on the well sides.
    g. Run the qPCR reaction in detection system StepOnePlus™ associated with the acquisition and analysis software StepOne™ v2.1 using the following cycling conditions:

qPCR cycling conditions

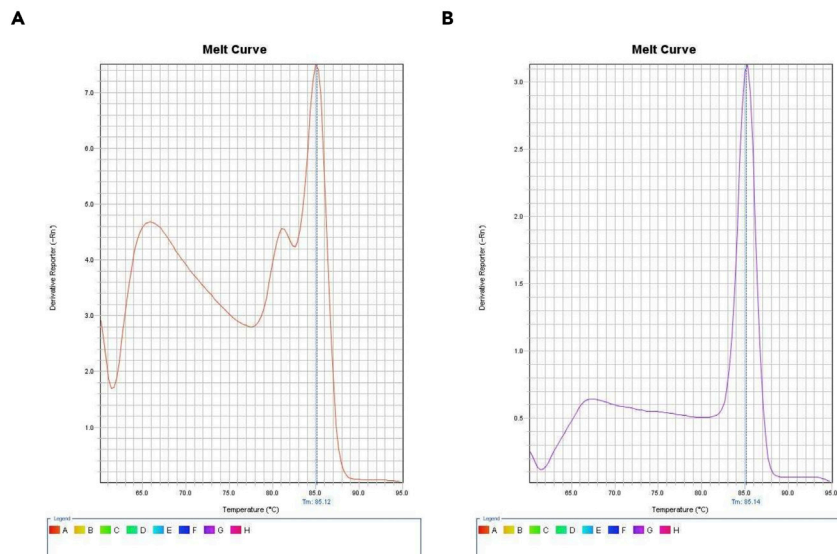| Steps	Temperature	Time	Cycles
1	95°C	10 min	1
2	95°C	15 s	40
3	60°C	1 min	1
4 (optional, for melting curve)	95°C	15 s	1
	60°C	1 min	
	95°C	15 s	

Figure 5. Examples of two different melting curves
(A) a melting curve with more than one peak and (B) a melting curve with only one peak.

Alternatives: It is possible to use another detection system associated with another acquisition and analysis software. For example, a 7900 real-time PCR detection system combined with SDS 2.4 acquisition and analysis software.

Note: The melting curve at the end of the qPCR Cycling Conditions is useful to check that only one specific product has been generated (Figure 5).

h. After exporting the data (in excel file, for example), calculate the Cycle Threshold (Ct) mean of all sample duplicate for each primer pair.
i. Then, calculate the logarithm of each cDNA sample dilution.
j. And finally, determine the primer efficiency as follows: $(10^{(-1/\text{Slope of the linear regression between the Ct mean and the logarithm of each cDNA sample dilution})}-1) \times 100$. See Table S1 that illustrates calculations from step 26.h to 26.j.

Note: Primer efficiency should be ranged between 90 and 110%.

27. Now, for qPCR assay, repeat the same steps from 26.b. to 26.j. using the different cDNAs samples instead of the different dilutions of the same cDNA sample (Figure 6).
28. After exporting the data (in excel file, for example), calculate the Cycle Threshold (Ct) mean of all sample duplicate for each primer pair.
29. Then, calculate the ΔCt as follows: $\text{Ct}_{\text{interest gene}} - \text{Ct}_{\text{reference gene}}$.
30. Once all the ΔCt are obtained, calculate the $\Delta\Delta$Ct as follows: $\Delta\text{Ct}_{\text{sample Y}} - \Delta\text{Ct}_{\text{sample control}}$.
31. Finally, calculate the Fold Change as follows: $2^{-\Delta\Delta\text{Ct}}$. See Table S2 that illustrates calculations from step 28 to 31.

Note: Prior the experiments, species specificity of primers must be tested by RT-qPCR in samples containing only mouse or human mRNA.

EXPECTED OUTCOMES

The humanized mouse model for excisional wounds that we described in this protocol is useful to determine the wound healing capacity of human hMADS when grafted alone or in combination

Figure 6. Example of a 96-well plate layout for 8 cDNAs samples and 5 different primer-pair mixes

with platelets. This model allows us to compare the effects that intact platelets or platelets with inhibited mitochondrial respiration following exposure to rotenone and antimycin A exert on hMADS cells. In these settings, the behavior changes of grafted human hMADS cells can be checked by the transcriptional analysis of human specific genes. In addition, this experimental model also allows the analysis of the host cell response to grafted human cells by RT-qPCR and the use of mouse specific primers.

A photograph of the healing of four full-thickness skin wounds on day seven after the different treatments is shown in Figure 7.

And an example of a successful qPCR is shown in Figure 8. An example of how these values were calculated is shown in Table S2.

QUANTIFICATION AND STATISTICAL ANALYSIS

Use GraphPad Prism software to analyze qPCR results.

Express the data as mean $\pm$ SD or mean $\pm$ SEM.

If you have n $\geq$ 5 independent experiments, check the normality and equality of variances of the data using the Shapiro-Wilk and Browne-Forsythe tests, respectively.

If data have normal distribution and equal variance, parametric tests should be used as for example, Ordinary one-way ANOVA followed by Tukey's multiple comparisons. Otherwise, use non-parametric tests such as one-way ANOVA on rank followed by Dunn's multiple comparisons.

If you have n < 5 independent experiments, non-parametric tests should be applied as for example, one-way ANOVA on rank followed by Dunn's multiple comparisons.

Results should be considered significant when $p < 0,05$.

Mice should be randomly assigned to the different wound treatment groups and the investigator should not be informed of the wound treatment assignment during the experiments and analysis.

LIMITATIONS

One limitation of the protocol is that it is based on the use of hMADS cells as model of human MSCs. However, we have previously showed that these cells show an immune privileged behavior since they express low levels of HLA class I molecules on their cell surface and do not express class II
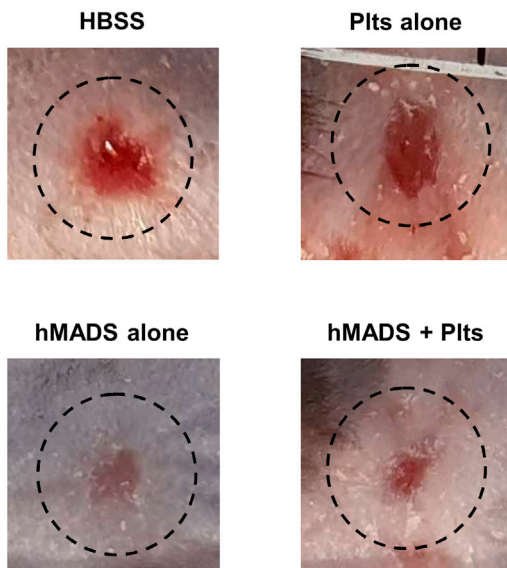
Figure 7. Photograph of mouse back with four full-thickness skin wounds on day 7 following injury and their treatment with either saline solution (HBSS), human platelets alone (Plts alone), hMADS alone (hMADS alone) or in combination with human platelets (hMADS+Plts)
Dotted black circle represents the initial size of wound.

HLA. In addition, these cells were isolated from the adipose tissue of young donors. It is possible that the use of human MSCs isolated from adult donors or other tissues may elicit an immune response in transplanted immunocompetent mice. An alternative might be to perform the wound and cell transplant experiments in immunodeficient nude mice.

In addition, this protocol can be conducted with mouse platelets and mouse MSCs.

TROUBLESHOOTING
Problem 1
Mouse wounds are retracted (step 17).

Potential solution
The donut-shaped silicone splints have not been properly fixed around the wounds to prevent retraction, so it is important to secure them properly by adding several sutures around the ring.

Problem 2
No expression of human transcripts is detected in wounds of mice transplanted with hMADS cells at day 1 and day 3 post-surgery. This is due to the fact that mice can easily remove their transparent non-adherent dressing and nibble their donut-shaped silicone splints and sutures before the day 3 (step 17).

Potential solution
During the pain monitoring of the mice every 12 h (step 16), check that mice have not removed their transparent non-adherent dressing. In case of dressing removal, you should replace it to prevent graft loss.

Problem 3
Platelets became activated and have formed a clot prior to their delivery in mouse wounds (step 3).

Potential solution
Ensure that anticoagulant such as heparin is added in platelet preparations at each stage of isolation and that no brake is applied during the centrifugation steps.
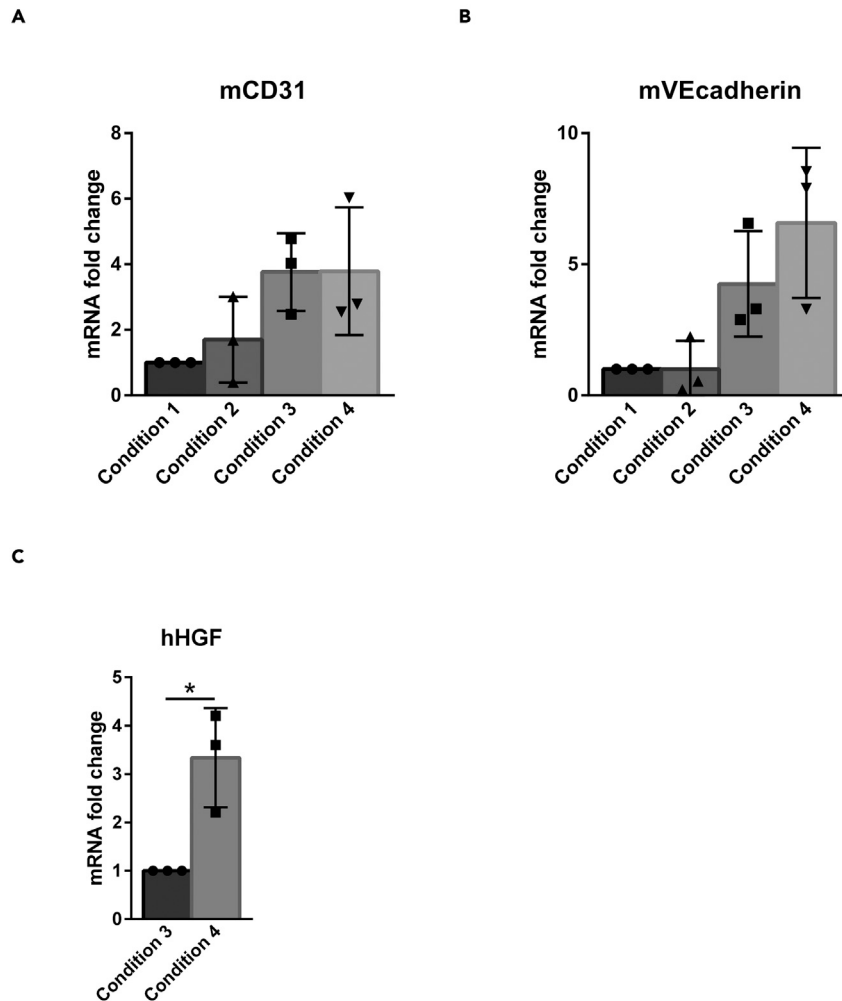
Figure 8. Examples of qPCR results

Relative transcriptional expression levels of mouse (A) CD31, (B) VE-cadherin and human (C) HGF in 4 and 2 different experimental groups (n=3 samples per group), respectively. Data used for generated graphs in panels (A–C) are provided in the Table S1. Data are represented as mean ± SD. * $p < 0.05$

Problem 4

When no single peak but two peaks are observed during the melting curve analysis, this second peak could be due to primer-dimers formation or to contaminating nucleic acids in the reagent components, these two phenomenons altering data interpretation (step 26–27).

Potential solution

To discriminate between occurrence of primer-dimers or nucleic acid contamination, it is mandatory to add in the step 26, a no template control (NTC) containing distilled water free of DNase/RNase instead cDNA for each primer-pair mix.

If a peak is detected in NTC samples, the second peak observed in cDNA samples is likely the consequence of primer-dimer formation.

Primer-dimer formation can be confirmed following gel electrophoresis migration by the detection of the same short fragment of 30–50 bp length in both NTC and template samples. In this case, design novel primers using Primer3 and BLAST softwares.

If more large size bands are detected in NTC and template samples, this means that some reagents are contaminated. In this case, use novel reagents to perform the experiments.

When no band is detected in NTC but two or more bands are detected in some template samples, this is likely due to the contamination of these samples with another DNA. In this case repeat the experiment for these samples or synthetize new cDNA from RNA samples.

To avoid contamination, it is critical to clean the bench and instruments used for the qPCR with 70% ethanol solution.

Problem 5
Amplicons are detected in the RT negative control indicating contamination with genomic DNA (step 27).

Potential solution
You can avoid DNA contamination by treating the RNA samples with DNase or by designing primers in the exon-exon junctions using Primer3 software.

Problem 6
No amplicon is detected in some template samples including for the reference gene indicating that RNA is probably degraded or inefficiently reverse transcribed (step 27).

Potential solution
Aliquot RNA samples to avoid their repeated freezing and thawing and store them at -80°C

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anne-Marie Rodriguez (anne-marie.rodriguez@inserm.fr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100650.

AUTHOR CONTRIBUTIONS
J.L. performed hMADS cell culture, platelet preparation, and RNA extraction and RT-qPCR experiments; P.L. generated wounds in mice and performed cell engraftment experiments and wrote the ethical applications. A.-M.R. designed experiments. J.L. and A.-M.R. wrote the STAR protocol.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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