Information in this document is confidential.

THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: September 25, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	DR. IAN WELCH
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Location of experimental work to be carried out: Building(s) West Valley Bldg Room(s) Mouse Barrier

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: CSTAR who is being funded by a 3rd party via
GRANT TITLE(S): ~ Nucro-Technics - CONTRACT

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:

Ian Welch
Tracy Hill
Heather Cadieux-Pitre
Sharla Thompson
Gregory Dekaban
Christy Willert

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ⊘YES O NO
(including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES ⊘NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
Adeno virus	⊘Yes O No	O Yes ⊘No	O Yes O No	1	Tissue Regeneration Therapeutics Inc. (TRT)	O 1 ⊘2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ⊘YES O NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	O Yes O No		Not applicable
Rodent	O Yes O No		
Non-human primate	O Yes O No		
Other (specify)	O Yes O No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)* (HUCPVC)	Supplier / Source
Human	☑ Yes O No	Human Umbilical Cord Perivascular cell line	TRT Inc.
Rodent	O Yes O No		
Non-human primate	O Yes O No		
Other (specify)	O Yes O No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required O 1 ☑ 2 O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? O YES ☑ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (unpreserved)		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☑ YES O NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? * ☑ YES, complete table below O NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results

* Please attach a Material Data Sheet or equivalent if available.
* Already done prior to transport to UWO.

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results
Adenovirus Recombinant	pAdEasy-1 (Qbiogene, Carlsbad CA)	TRT Inc	Expressing anti-Venezuelan Equine Encephalitis Virus (VEEV)	Expresses antibody to VEEV

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ ☑NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ ☑NO
- SV 40 Large T antigen O YES ☑NO
- E1A oncogene O YES ☑NO
- Known oncogenes O YES, please specify _____ ☑NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ ☑NO

4.5 Will virus be replication defective? ☑YES O NO

4.6 Will virus be infectious to humans or animals? O YES ☑NO

4.7 Will this be expected to increase the containment level required? O YES ☑NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES ☑NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? ☑YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used Mouse

6.3 AUS protocol # _____

6.4 Will any of the agents listed in section 4.0 be used in live animals ☑YES, specify: HuCPVC O NO

6.5 Will the agent(s) be shed by the animal: O YES ☑NO, please justify:

_____

_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES ⊘NO
- Pound source cats O YES ⊘NO
- Cattle, sheep or goats O YES ⊘NO
- Non-human primates O YES, please specify species _____ ⊘NO
- Wild caught animals O YES, please specify species & colony # _____ ⊘NO
- Birds O YES ⊘NO
- Others (wild or domestic) O YES, please specify _____ ⊘NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES ⊘NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s)_____
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin_____

8.4 How much of the toxin is handled at one time*? _____

8.5 How much of the toxin is stored*? _____

8.6 Will any biological toxins be used in live animals? O YES, Please provide detais:_____ O NO

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES ⊘NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:
_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:
_____
_____

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES ☑NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 ☑NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE Sam Welch

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 ☑ 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

O YES, permit # if on-campus_____
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE Lan Weleh Date: Dec 21/09

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, or 3 measures, that are unique to this agent.

_____
_____
_____

14.3 Please outline what will be done if there is an exposure to the biohazards listed, such as a needlestick injury:

_____
_____

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: _____
Date:_____

Safety Officer for Institution where experiments will take place: SIGNATURE:_____
Date:_____

Safety Officer for University of Western Ontario (if different from above): SIGNATURE:_____
Date:_____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

-------- Original Message --------
Subject: Re: AUS protocol for Dekaban (rush)
Date: Tue, 22 Dec 2009 15:22:53 -0500
From: Tyrrel de Langley <tyrreld@uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>
CC: Greg Dekaban <dekaban@robarts.ca>, Lynne Turner <lturner@uwo.ca>, Gerald M Kidder <gerald.kidder@schulich.uwo.ca>

Hi Jennifer,

I heard back from Jerry Kidder and gave him a brief overview of the biosafety aspects of this project. He said that to accommodate the short timelines it could be posted and committee members respond via email.

Just to clarify, this is a CSTAR protocol for one of their industry partners - NucroTechnics. Greg is involved only as someone asked to prepare the cells for injection. It involves mesenchymal stem cells which they call Human Umbilical Cord PeriVascular Cells or HUCPVCs. These HUCPVCs are carrying antibody to Venezuelan Equine Encephalitis Virus - VEEV. The VEEV antibody is introduced via transfection using adenovirius. We have been told there is no live adeno and certainly no live VEEV in the material we are injecting into mice. They wish to demonstrate that the HUCPVCs will successfully "take" and multiply in the host mouse and thus demonstrate increasing levels of VEEV antibody in the blood over time. Because all the work, except for Greg's part, is an animal study, the responsibility for managing this has fallen on ACVS and the AUP is in Ian Welch's name.

We've all been caught by surprise by this last minute scramble because all the information – in particular the biosafety related details – was not made available to me until yesterday. Also, the strategic alliance with CSTAR and NucroTechnics has been identified as a priority by Ted and this being the first one, has demonstrated that there is much that needs to be improved next time around. Perhaps you can join us for a "Post Mortem" analysis in January and help in planning how to handle these in the future.

Tracy and I have written an AUP today which has gone for pre-review and will be up for interim approval at the weekly AUS meeting January 7. I've asked Tracy to copy you on the AUP and the appendix so you get it early. We are also filling out the BHARF with Greg's help – and there are outstanding questions that we've asked NucroTechnics to reply to by tonight. When we receive the completed copy – hopefully later tonight, we'll forward to you.

Thanks for helping with this.

Tyrrel

Description of Study for ACVS/Nucro-Technics Project

The purpose of this "proof of principle" project is to inject mice with HUCPVCs, AKA mesenchymal stem cells, and determine if they will successfully transplant by measuring blood levels of an antibody they will produce only if they successfully take.

There will be 4 groups of 4 mice, for a total of 16. The mice will be housed in the West Valley Bldg barrier and all work will be done in a Biosafety cabinet. Mice will be pre-bled on Day 0 and then injected with cells subcutaneously (total volume 200ul). Cells will be obtained from Greg Dekaban's lab. Blood will be collected on Day 3, 7, 14, 21 and 30. Day 30 will be a terminal bleed and mice will be sacrificed. All mice will be incinerated and all serum will be sent for testing.

Culturing and Passaging Transfected HUCPVCs for VEEV Animal Study (DIR Project Task 5)

Effective date: 01 Jan 2010

1.0 OBJECTIVE AND SCOPE

1.1 To prepare HUCPVCs transfected with anti-VEEV antibody construct for in vivo study.

2.0 DEFINITIONS

2.1 HUCPVCs. Human Umbilical Cord Perivascular Cells

2.2 VEEV. Venezuelan Equine Encephalitis Virus (known biological weapon used in warfare)

2.3 Tryple Express. Trypsin replacement that is gentle on cells.

3.0 MATERIALS AND EQUIPMENT

3.1 Biosafety Cabinet for Level 2 containment
3.2 Centrifuge (Settings: 285 x g, 5 minutes, 4 C)
3.3 Transfected HUCPVC cultures
3.4 Tryple Express (Invitrogen #12604)
3.5 PBS (Invitrogen #14190)
3.6 50-mL Tubes (BD #352070)
3.7 70% Isopropanol or 70% Ethanol spray bottle
3.8 Sterile saline solution, (Hanks without Calcium and Magnesium)
3.9 70µm cell strainer (BD #22363548)
3.10 Parafilm

4.0 RESPONSIBILITIES

4.1 TRT ships HUCPVCs to DRDC-Suffield for transfection.

4.2 DRDC-Suffield cultures and expands HUCPVCs in cell culture flasks to sufficient numbers.

4.3 At 70-80% confluence, DRDC-Suffield transfects one set of HUCPVC cultures with adenovirus containing anti-VEEV antibody construct (Test Group) and another set of HUCPVCs with GFP (Control Group).

4.4 DRDC-Suffield fills each culture flask of transfected HUCPVC with nutrient medium containing 5% FBS, closes lid tightly and seals with parafilm. (Note: Use solid lids, not vented lids.)

4.5 DRDC-Suffield packages and ships flasks of cells at room temperature to CSTAR (Canadian Surgical Technologies & Advanced Robotics) in London, Ontario for in vivo study.

4.6 Trained laboratory technicians at CSTAR perform all tasks in this SOP.

5.0 PROCEDURE

5.1 Immediately upon receipt of cells, unpack, spray caps of flasks with 70% alcohol and place inside biosafety cabinet.

5.2 Uncap and remove excess culture medium with a 25mL pipette as follows:

5.2.1 For T-75 ($75cm^2$ flask), leave roughly 10 mL medium behind.
5.2.2 For T-225 ($225cm^2$ flask), leave roughly 30 mL medium behind.
5.2.3 Flasks will be labeled Test and Control

5.3 Cap flasks loosely and place into CO_2 incubator at 37°C overnight.

5.4 Next day, spray the following reagents and supplies with 70% alcohol and place into the biosafety cabinet:

5.4.1 50mL centrifuge tubes
5.4.2 PBS
5.4.3 Saline Solution
5.4.4 Tryple Express (after warming in 37°C water bath)

5.5 Observe cultures under phase microscope to ensure viability of cells and level of confluence.

5.6 Spray caps of flasks and place into biosafety cabinet.

5.7 Uncap and suction medium.

5.8 Rinse T-75 with 10 mL PBS and T-225 with 30 mL PBS.

5.9 Suction PBS.

5.10 Add 5mL and 15 mL of Tryple Express to T-75 and T-225 respectively.

5.11 Cap flasks loosely and move from side to side to distribute solution evenly over cells.

5.12 Place flasks into CO_2 incubator at 37°C for 5 minutes.

5.13 Remove flasks from incubator and tap the sides to help further loosen the cells.

5.14 Check under microscope to ensure that all cells have detached. If not, return flasks to CO_2 incubator for another 5 minutes.

5.15 Inside biosafety cabinet, separate flasks for Test Group and Control Group.

5.16 Use a 10mL pipette, mix, transfer and combine contents of Test Group into several 50mL tubes.

5.17 Repeat step 5.16 for the Control Group using another set of 50mL tubes.

5.18 Rinse T-75 and T-225 with 10mL and 24mL of PBS respectively.

5.19 Pipette and add the rinse to the corresponding 50mL tubes of cell suspensions.

5.20 Cap and centrifuge 50mL tubes.

5.21 Remove tubes, spray with 70% alcohol and place into biosafety cabinet.

5.22 Uncap and suction supernatant.

5.23 Tap each tube to break up cell pellet.

5.24 Process Test Group and Control Group separately by adding 40 mL of saline to one 50mL tube, pipet-mix and transfer cells to the next tube, repeating until all cells in each group are pooled into one tube.

5.25 Pass the cells in each group through a separate 70µm cell strainer (placed over a new 50mL tube) to remove unwanted cell clumps and debris.

5.26 Centrifuge both 50mL tubes of filtered cells.

5.27 Suction supernatant and add 40mL saline to each tube again for another rinse.

5.28 Invert gently to mix and centrifuge.

5.29 Resuspend each group in 15-20mL saline.

5.30 Count the cells in each group using SOP 0004.

5.31 Proceed to SOP 0017.

6.0 REFERENCES

6.1 Preparation of Transfected HUCPVC groups for in vivo injection (SOP 0017)

6.2 Injecting Transfected HUCPVCs into NOD/SCID Mice (SOP 0018)

6.3 Counting HUCPVCs (SOP 0004)

7.0 DOCUMENTATION

7.1 Lab Notebook

Preparation of Transfected HUCPVC groups for in vivo injection (DIR Project Task 5)

Effective date: 01 Jan 2010

1.0 OBJECTIVE AND SCOPE

1.1 To prepare aliquots of transfected HUCPVCs that will be employed in an in vivo study to measure anti-VEEV antibody production in NOD/SCID mice.

1.2 The scope of this SOP is to provide the cell aliquots that will be injected under SOP0018.

2.0 DEFINITIONS

2.1 HUCPVCs. Human Umbilical Cord Perivascular Cells

2.1 VEEV. Venezuelan Equine Encephalitis Virus (known biological weapon used in warfare)

3.0 MATERIALS AND EQUIPMENT

3.1 Biosafety Cabinet for Level 2 containment
3.2 Centrifuge (Settings: 285 x g, 5 minutes, 4°C)
3.3 Transfected HUCPVCs (Test Group & Control Group)
3.4 Sterile saline solution (Hanks without Calcium and Magnesium)

4.0 RESPONSIBILITIES

4.1 CSTAR aliquots transfected HUCPVCs to produce doses of cells that will be injected into NOD/SCID mice under SOP0018.

5.0 PROCEDURE

5.1 Based on cell counts for the Test Group and Control Group prepared as per SOP 0016, remove an aliquot that represents 8 million cells from the Test Group and 6 million cells from the Control Group (GFP transfected cell) into separate 15mL tubes.

5.2 Contact Tracy Hill at ACVS (extn 86746) to come to Robarts Lab 24241 to pick up cells. Tracy will have prepared the animals for injection.

5.3 For the Test Group, prepare various doses for injection as follows:

5.3.1. Tube 1: 15mL tube containing 8 million cells.

5.3.1.1 Centrifuge and discard supernatant.

5.3.1.2 Add 1194µL saline and mix well. This concentration provides 1.34 million cells per 200 µL.

5.3.2 Tube 2 (new 15mL Tube): Transfer 300µL from Tube 1 into this tube and add 1200µL saline, mix well. This concentration provides 0.27 million cells per 200µL.

5.3.3 Tube 3 (new 15mL Tube): Transfer 300µL from Tube 2 into this tube and add 1200µL of saline and mix well. This concentration provides 0.054 million cells per 200µL.

5.4 Process the 6 million cells from the Control Group (GFP transfected cell) accordingly to provide for 1.34 million cells in 200µL.

5.5 Hand over tubes containing cells to Tracy Hill.

6.0 REFERENCES

6.1 Culturing and Passaging Transfected HUCPVCs (SOP 0016)

6.2 Injecting Transfected HUCPVCs into NOD/SCID Mice (SOP 0018)

6.3 Counting HUCPVCs SOP (SOP 0004)

7.0 DOCUMENTATION

7.1 Lab Notebook

Injecting Transfected HUCPVCs into NOD/SCID Mice (DIR Project Task 5)

Effective date: 01 Jan 2010

1.0 OBJECTIVE AND SCOPE

1.1 In vivo study to measure anti-VEEV antibody production in NOD/SCID mice that have been injected with various doses of transfected HUCPVCs.

1.2 The scope of this SOP is to develop an effective antidote to VEEV for treating combat casualties in the Canadian Military attacked by this deadly biological weapon.

2.0 DEFINITIONS

2.1 HUCPVCs. Human Umbilical Cord Perivascular Cells

2.2 VEEV. Venezuelan Equine Encephalitis Virus (known biological weapon used in warfare)

3.0 MATERIALS AND EQUIPMENT

3.1 NOD/SCID Mice (16 males, 7-8 weeks old)
3.2 Transfected HUCPVCs (Test Group & Control Group)
3.3 25G syringe needles
3.4 1cc syringes
3.5 Sterile saline solution (Hanks without Calcium and Magnesium)

4.0 RESPONSIBILITIES

4.1 CSTAR injects transfected HUCPVCs into NOD/SCID mice and collects blood samples at various time intervals over a period of one month.

4.2 CSTAR freezes serum from all blood samples at -80°C and ships to DRDC-Suffield at the conclusion of the study.

5.0 PROCEDURE

5.1 Ensure cell preparations are being done in Robarts. Tracy Hill to coordinate with Gregory Dekaban (extn 24241)

5.2 Prior to cell injection, bleed all 16 mice. Samples will be spun for serum collection. Volume to be collected = 50microlitres of serum. Serum will be frozen at -80°C.

5.3 Collect cells in tubes from Lab 24241 in Robarts.

5.4 Based on cell aliquots for the Test Groups and Control Group, prepared as per SOP 0017, into Tubes 1, 2, 3 and Control respectively, inject cells as follows.

5.5 Using 25G needles attached to 1cc syringes, inject cells subcutaneously near the midline in the thoracic region of each animal according to the following setup:

5.5.1 Group 1 (4 mice): Inject each mouse with 200µL from Tube 1.

5.5.2 Group 2 (4 mice): Inject each mouse with 200µL from Tube 2.

5.5.3 Group 3 (4 mice): Inject each mouse with 200µL from Tube 3.

5.5.4 Group 4 (4 mice): Inject each mouse with 200µL from Control Group (GFP transfected cell).

5.6 Obtain a blood sample from each mouse following 3, 7, 14, 21 and 30 days post injection.

5.7 Samples will be spun for serum collection after each bleeding.

5.8 Volume to be collected = 50microlitres of serum.

5.9 Serum will be frozen at -80˚C.

5.10 After completion of 30 day time point, ship frozen sera from all experimental time points to DRDC-Suffield Building 560 Ralston AB Canada T0J 2N0 for analysis.

6.0 REFERENCES

6.1 Culturing and Passaging Transfected HUCPVCs (SOP 0016)

6.2 Preparation of Transfected HUCPVC groups for in vivo injection (SOP 0017)

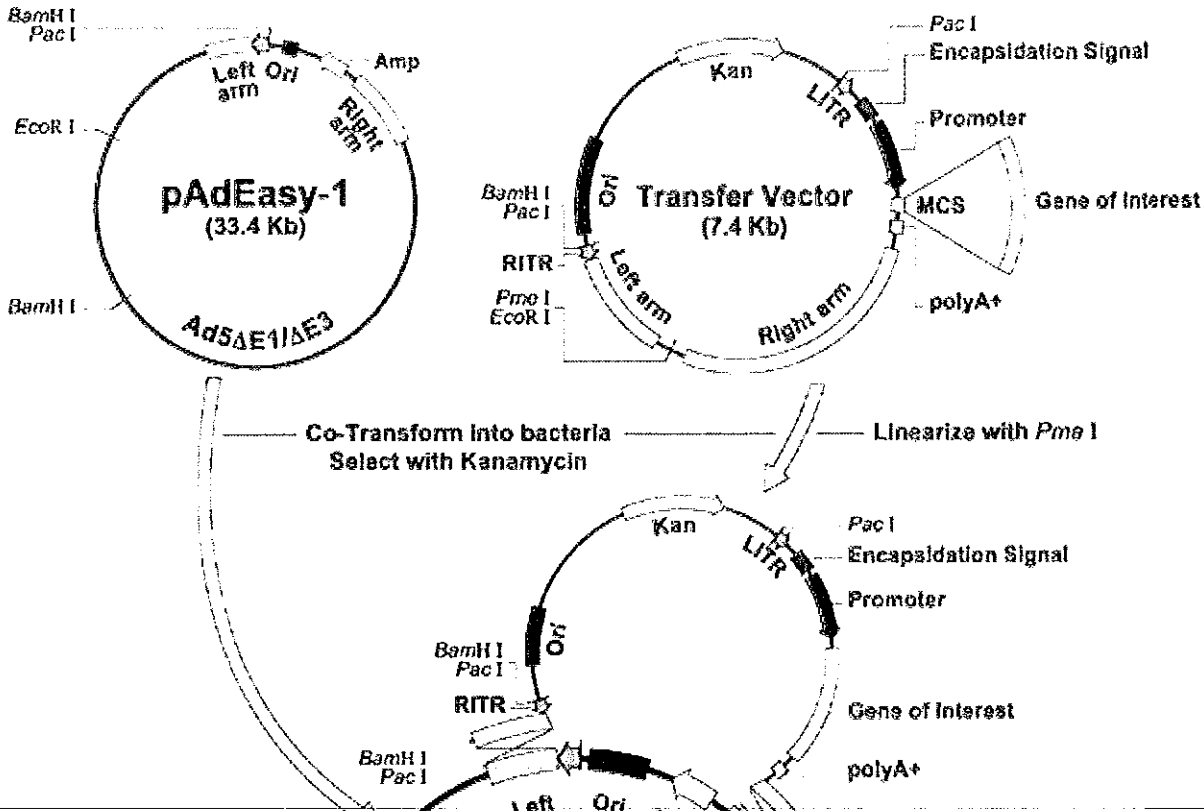
6.3 Counting HUCPVCs SOP (SOP 0004)

7.0 DOCUMENTATION

7.1 Lab Notebook

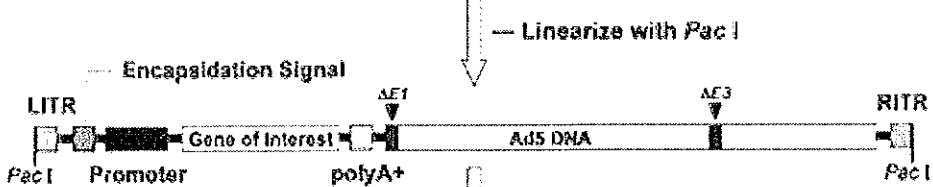
Step 1:

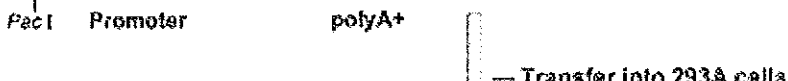
cDNA cloning in transfer plasmid



Step 2:

In vivo **homologous recombination in bacteria**





Step 3:

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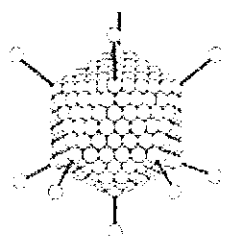

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Human Mesenchymal Stem Cells Self-Renew and Differentiate According to a Deterministic Hierarchy

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Abstract

Background: Mesenchymal progenitor cells (MPCs) have been isolated from a variety of connective tissues, and are commonly called "mesenchymal stem cells" (MSCs). A stem cell is defined as having robust clonal self-renewal and multilineage differentiation potential. Accordingly, the term "MSC" has been criticised, as there is little data demonstrating self-renewal of definitive single-cell-derived (SCD) clonal populations from a mesenchymal cell source.

Methodology/Principal Findings: Here we show that a tractable MPC population, human umbilical cord perivascular cells (HUCPVCs), was capable of multilineage differentiation *in vitro* and, more importantly, contributed to rapid connective tissue healing *in vivo* by producing bone, cartilage and fibrous stroma. Furthermore, HUCPVCs exhibit a high clonogenic frequency, allowing us to isolate definitive SCD parent and daughter clones from mixed gender suspensions as determined by Y-chromosome fluorescent in situ hybridization.

Conclusions/Significance: Analysis of the multilineage differentiation capacity of SCD parent clones and daughter clones enabled us to formulate a new hierarchical schema for MSC self-renewal and differentiation in which a self-renewing multipotent MSC gives rise to more restricted self-renewing progenitors that gradually lose differentiation potential until a state of complete restriction to the fibroblast is reached.

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Introduction

Tissue resident progenitors that give rise to connective tissue cells *in vitro* have been isolated from a variety of tissues including bone marrow (BM) [1,2,3,4], fat [5,6], muscle [7,8], placenta [9], umbilical cord [10,11,12,13,14] and fetal liver [15]. These cells are generally thought to be resident in the perivascular compartment of these tissues [16,17,18,19,20]; and are commonly called "mesenchymal stem cells" (MSCs) [21]. While it is generally accepted that these cells include rare stem cells [22,23,24], the term "MSC" has been criticised [25], since the definition of a stem cell assumes that these progenitors have clonal self-renewal and multilineage differentiation potential. To date, assaying these two properties in MSCs has been extremely challenging due to the low frequencies of clonogenic progenitors found in most tissues (e.g., mesenchymal clonogenic frequency in human BM is $1{:}10^4$ to $1{:}10^6$ depending on age).

To date only two studies have succeeded in isolating definitive single-cell-derived (SCD) clonal populations by deposition of single human mesenchymal cells into 96-well plates by fluorescence activated cell sorting (FACS) [26]. However, neither study concurrently assayed each clone for more than one phenotype, or determined whether each clone had self-renewal capacity by producing equipotent progeny. While other investigators [27] did analyze 'clones' for bi- and trilineage (bone, cartilage and adipose) differentiation capacity, they utilized limiting dilution [27,28,29] by seeding 10^3–10^5 cells/culture well to generate fibroblast colonies (CFU-F). Thus, these studies did not provide definitive evidence of SCD clonality. Other studies attempted to overcome this issue by seeding cells at very low densities to isolate their colonies [30,31], but did not verify the single cell origin of their populations. Furthermore, none of these studies determined whether the mesenchymal cells isolated maintained multipotential capacity *in vivo*. Thus, to date, demonstration of a definitive MSC has remained refractory to robust experimental evidence [32].

To overcome these limitations, we utilized populations of non-alloreactive [33] human umbilical cord derived mesenchymal progenitors (HUCPVCs) [10,34], harvested from a total of 57 cords, that maintain a high clonogenic frequency *in vitro* to isolate definitive SCD clones. We found that populations of HUCPVCs undergo multilineage differentiation and contribute to repair of osteochondral defects *in vivo*. We next isolated definitive parent and daughter clones from mixed gender suspensions and then assayed these clones for their ability to differentiate into one or more of five mesenchymal lineages: bone, cartilage, fat, muscle and fibrous tissue. Our results demonstrated that these SCD clones maintained extensive self-renewal capacity *in vitro* and clonally produced daughter populations of cells with quinti-, quadri-, tri-, bi- and unilineage potential. Analysis of the differentiation trajectories of the

parent and daughter clones uncovered a new hierarchical schema for MSC self-renewal and differentiation.

Results

Multipotential HUCPVC mesenchymal progenitors are maintained in long-term culture

We, and others, have previously shown that HUCPVC populations express CD44, CD73, CD90, CD105, and CD106 but do not express CD45 or CD34 [10,13,34]. Furthermore, we had previously observed that HUCPVCs exhibit a doubling time of 20 hours when cultured in 15% fetal bovine serum (FBS) [10]. Here, we utilized serum concentrations of 5% or 2% which increased the doubling time (41±7 and 46±9 hours respectively), but also maintained a consistent doubling time and clonogenic frequency of approximately 1:3 (1: [2.6±1.4] and 1: [2.8±1.6] respectively) for more than 10 passages (Figure 1A,B) unlike adult bone marrow-derived mesenchymal cells [35,36].

We next tested whether bulk HUCPVC populations were capable of differentiating into five mesenchymal lineages *in vitro*: myogenic (M), adipogenic (A), chondrogenic (C), osteogenic (O) and fibroblastic (F). Utilizing serum concentrations ranging from 2–3%, HUCPVCs were assayed at passage 3 (P3) (~10 population doublings) and P6 (~15 doublings) for differentiation capacity into these lineages. Results were the same for both P3 and P6 cells. In non-induced conditions (Figure 1C), HUCPVCs expressed collagen IA1, desmin and low levels of MyoD, characteristic of their 'myofibroblastic' [37,38] phenotype. In osteogenic culture conditions (Figure 1D,E,P), expression of Runx2, collagen IA1, osteopontin and osteocalcin were upregulated. The cells changed from a fibroblastic to a more tessellated osteoblast-like morphology, and formed bone nodules by elaborating mineralized bone matrix as evidenced by the presence of positive tetracycline staining (data not shown), alkaline phosphatase and positive Von Kossa staining. In chondrogenic culture conditions (Figure 1F,G,P), upregulation of Sox9, collagen II and aggrecan were observed. The cells elaborated collagen II matrix and sulphated glycosaminoglycans, verified by immuno-histochemistry and Alcian blue staining respectively. HUCPVCs could also be induced to differentiate into lipoprotein lipase-expressing adipocytes (Figure 1H,I,P) in culture that stained with oil red O, occasionally forming spontaneously in association with bone nodules. Although we did not quantify the relative expression of each of these phenotypes, Baksh et al [34] have determined the relative quantification under osteogenic, chondrogenic and adipogenic induction. As non-induced HUCPVCs express myogenic markers including desmin, vimentin and alpha smooth muscle actin [37,38,39], myogenically-induced cells (Figure 1J,K,P) were analyzed for differential expression of MyoD and fast skeletal myosin light chain (FSMLC). Non-induced cells expressed low levels of MyoD, but no FSMLC, while induced cells formed long multinucleated myotube-like structures that expressed high levels of MyoD and FSMLC. This was confirmed by RT-PCR analysis, in which upregulation of MyoD, Myf5, desmin, myosin heavy chain (MHC), and FSMLC were observed.

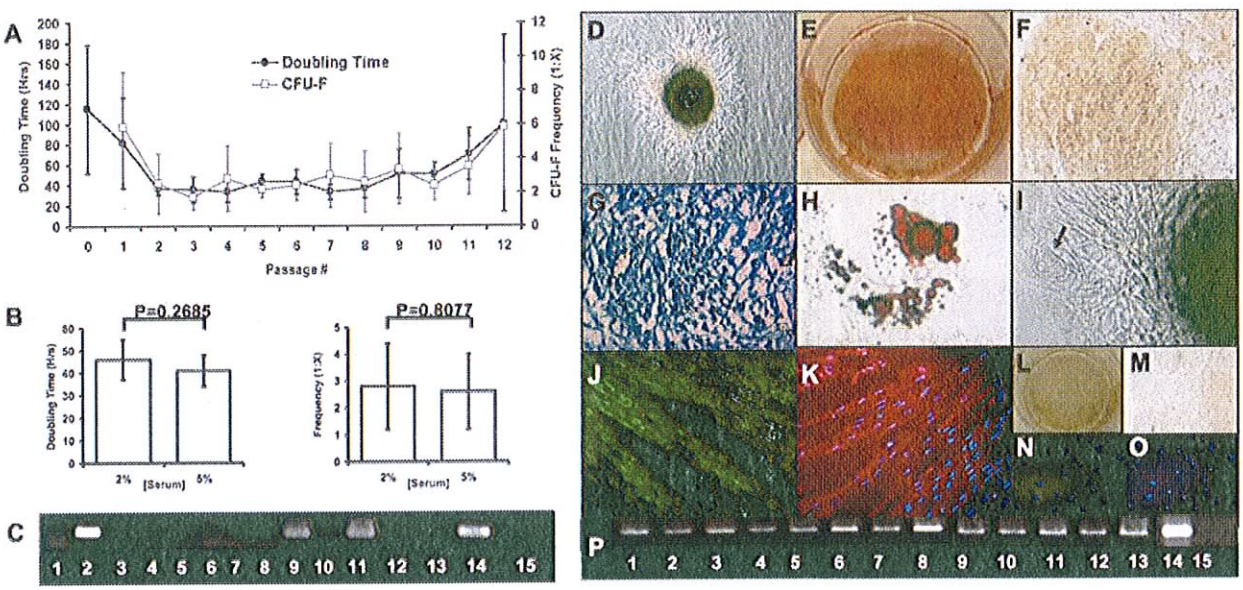

Figure 1. HUCPVC populations exhibit rapid proliferation, high clonogenicity and multipotential capacity *in vitro*. HUCPVC populations maintained stable proliferation and clonogenic frequency from passage 2 through 9 (A, n = 11 different cords), with no significant difference in either parameter at passage 5 (B, n = 7 different cords). Data are represented as mean +/− s.d. In standard culture conditions, HUCPVCs expressed Collagen IA1, Desmin and MyoD [lanes 2, 9 and 11 respectively, all other lanes as represented in (P)] as determined by RT PCR (C). HUCPVCs could further differentiate into bone, cartilage, adipose and muscle *in vitro*. With induction, bone nodules were observed in culture (D) that stained with Von Kossa (black), and were surrounded by alkaline phosphatase-high expressing cells (E). Cartilage pellet cultures of HUCPVCs expressed collagen II (F) and glycosaminoglycans that stained with Alcian blue (G). HUCPVC-derived adipocytes stained with Oil Red O (H) and occasionally formed spontaneously (arrow) in association with bone nodules (I). Myogenically-induced HUCVPCs expressed high levels of MyoD (J) and fast skeletal myosin light chain (FSMLC) (K) in multinucleated HUCPVC myotubes. Negative controls are non-induced cells stained with Von Kossa and alkaline phosphatase (L), and secondary-only antibody staining for collagen II (M), MyoD (N) and FSMLC (O) stained cultures. (Field widths: D,F = 628 μm; E,L = 3.5 mm; G,J,K,N,O = 315 μm; H,I = 86 μm). RT PCR analysis (P) demonstrated upregulation of the following lineage-specific genes: Runx2 (1), collagen IA1 (2), osteopontin (3), osteocalcin (4), Sox9 (5), collagen II (6), lipoprotein lipase (LPL) (7), aggregan (8), MyoD (9), Myf5 (10), desmin (11), myosin heavy chain (MHC) (12), FSMLC (13), GAPDH (14) and RT- control (15). (Cells from 9 cords, some of which are common to other assays, were employed in these functional phenotypic and gene expression data).
doi:10.1371/journal.pone.0006498.g001

HUCPVC populations contribute to multilineage repair *in vivo*

To determine the capacity of HUCPVC populations to contribute to repair *in vivo* we utilized a transplantation model originally developed to assay human hematopoietic stem cell repopulation [40]. HUCPVCs that had been infected with lentivirus encoding eGFP driven by a constitutive promoter were injected into the femoral marrow cavity of NOD-scid mice. The tissue was analyzed at 2, 4 and 6 weeks post-transplantation. After 2 weeks, there was significantly more healing of bone and cartilage in the HUCPVC-injected femora than in contralateral sham-injected controls (Figure 2A,D-G). By flushing the marrow and recovering culture-adherent eGFP-labeled cells, we showed that HUCPVCs survived at least 6 weeks *in vivo* (Figure 2B,C). By 4 and 6 weeks, complete autologous repair was achieved as there did not appear to be any gross difference between the experimental and control femurs. When the distal region of each femur was analyzed by micro CT (Figure 2A), the BMD difference was larger at all three time points, illustrating greater mineralized bone formation in the HUCPVC-transplanted femurs. To determine whether the HUCPVCs contributed to healing by becoming synthetically active, we labeled them with monoclonal antibodies for human-specific osteocalcin, collagen II and PPARγ [41]. As the eGFP label had been eliminated by decalcification of the tissues, we also labeled sections with HuNu, an antibody that specifically labels human nuclei and not those of mice. Figure 3A,B illustrates HuNu-labeled cells in the femoral growth plate that were associated with human-specific collagen II staining (Figure 3E,F). Anti-human osteocalcin labeled in the cytoplasm of some cells as well as within the newly formed bone matrix (Figure 3I,J). PPARγ staining was non-specific. As expected, no positive staining was observed in sham injected femurs (Figure 3C,G,H) or negative controls (Figure 3D,H,L). Interestingly, the majority of cells in the HUPVC-injected femurs did not label with the anti-human antibodies, suggesting that although the HUCPVCs did differentiate and produce some tissue, they may have had a larger role in recruiting and directing the mouse mesenchymal progenitors to repair the damaged tissue. This is strikingly evident in the cartilage repair, as the mouse cells rapidly produced new collagen II distal to the more slowly produced human collagen II (Figure 3E). Together, these data demonstrate that HUCPVCs have multilineage differentiation potential *in vitro* and, after transplantation, differentiate into at least two distinct lineages that contribute to tissue repair *in vivo*.

Traditional mesenchymal cell seeding methods do not isolate definitive SCD clones

Having established low serum-dependent culture conditions that provided a clonogenic frequency of>1:3, and determined that bulk HUCPVC populations could differentiate into multiple mesenchymal lineages both *in vitro* and *in vivo*, we used five different methods to isolate SCD HUCPVC populations (Figure 4A, and see Supplementary Methods S1) and assayed their differentiation capacity into the five mesenchymal lineages (Figure 4B). For clonal expansion, approximately 20 cell doublings from a single originating cell were required to provide $\sim 10^6$ cells needed for lineage analysis.

The cloning cylinder has been the traditional method utilized in mesenchymal literature to isolate limiting dilution-derived mesenchymal colonies. Based on a 1:3 CFU-F frequency of HUCPVCs, limiting dilution seeding of 3 cells per well, by definition, yielded 100% colony formation. This method provided a relatively poor

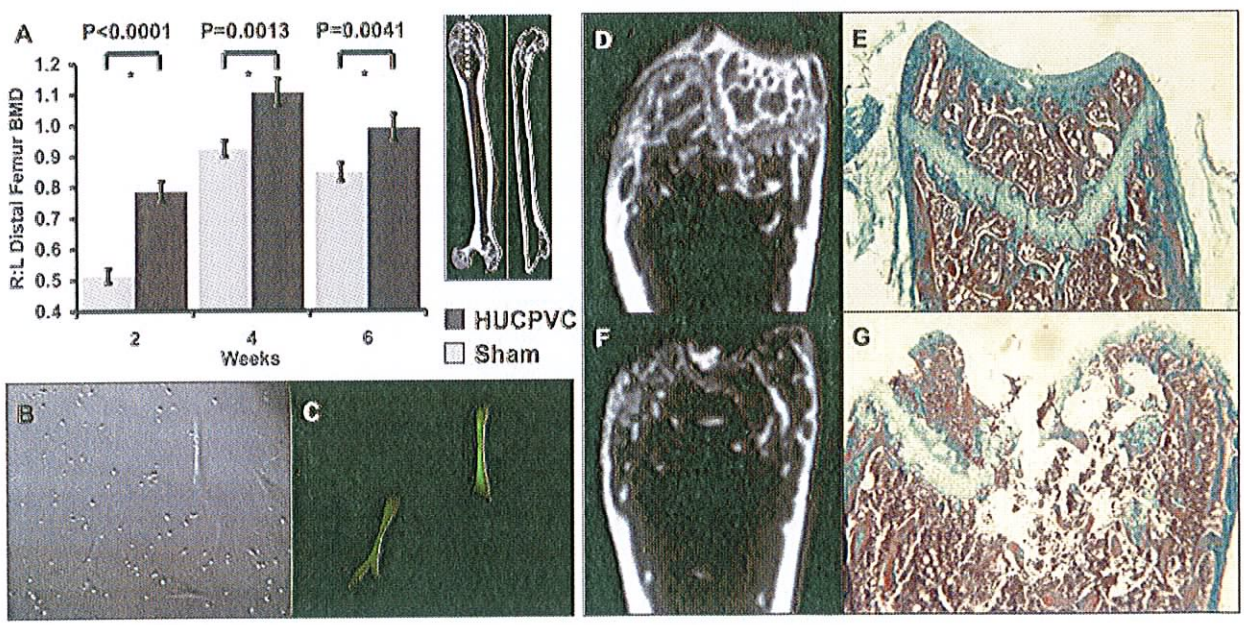

Figure 2. HUCPVCs survive and regenerate damaged mesenchymal tissues *in vivo*. HUCPVCs were synthetically active and survived at least six weeks *in vivo* when implanted into the intrafemoral space of NOD-scid mice as observed by the presence of GFP-labeled cells among mouse cells flushed from the marrow into culture (B,C). When the distal ends of the femurs were analyzed by micro-computed tomography (μCT), it was determined that these cells produced significantly more bone mineral density (BMD) than sham controls at 2,4 and 6 weeks (A, values are means±s.d.). μCT analysis and Masson's Trichrome stained longitudinal sections of injected femurs showed that HUCPVCs induced significantly more repair of bone and cartilage at 2 weeks (D,E) compared to sham controls (F,G). HUCPVC populations from a total 6 different cords were employed to generate this data and that shown in Figure 3.
doi:10.1371/journal.pone.0006498.g002

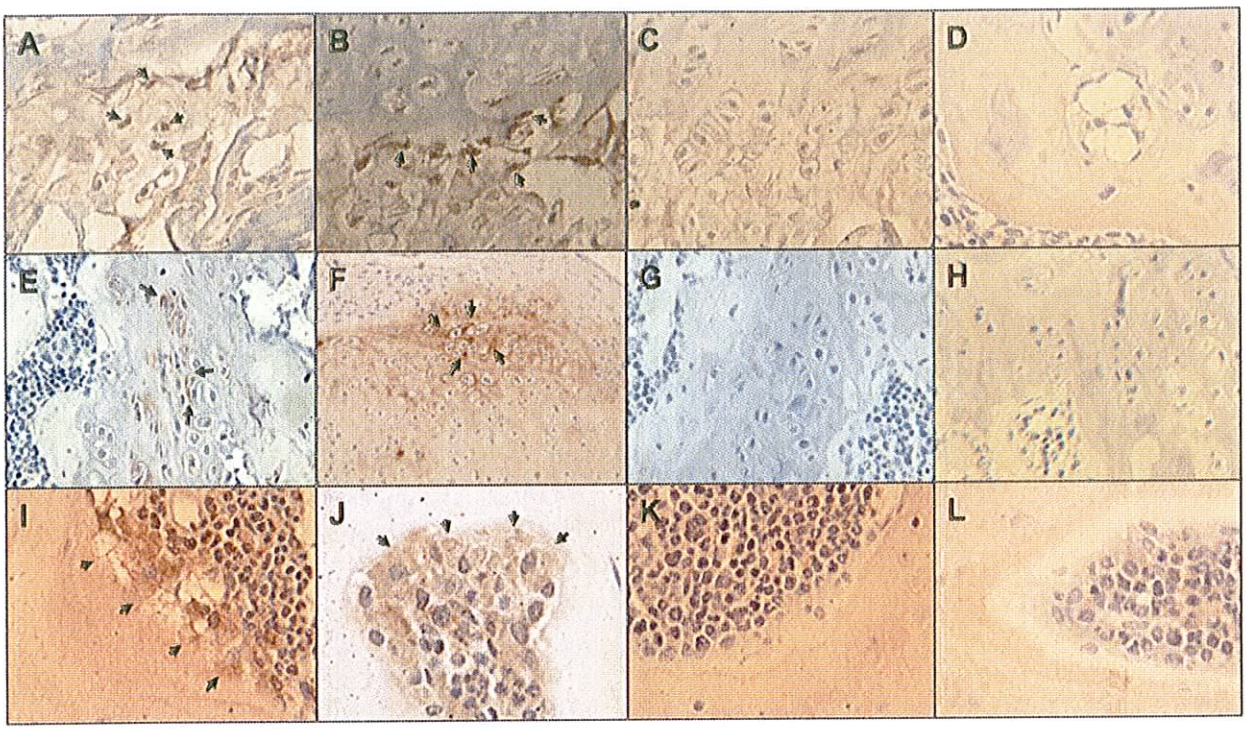

Figure 3. HUCPVCs display multipotential capacity *in vivo*. When labeled with HuNu (human nuclear antigen), human cells (arrows) were observed in the growth plate of the distal femurs (A,B field widths = 140 μm). These cells were associated with the presence of human-specific collagen II (arrows) in the extracellular matrix surrounding individual chondrocytes (E,F field widths = 140 μm and 212 μm respectively). Human-specific osteocalcin was also observed on the osteoid of newly forming bone. Arrows indicate human-specific osteocalcin was present surrounding individual cells that produced a collagen-like matrix on the surface of the bone (I,J field widths = 110 μm). No staining was observed in sham-injected femurs or negative controls for HuNu (C,D), collagen II (G,H) or osteocalcin (K,L) respectively (same field widths as experimentals).
doi:10.1371/journal.pone.0006498.g003

yield of HUCPVC colonies that survived the first sub-culture (~20%). Use of a novel 'inverse' method of isolating these colonies by removing any and all cells around the colony, improved the yield to ~60% because the cells could be grown to sub-confluence before sub-culture. We hypothesized that by culturing these cells on agarose, it would be easier to pick the growing colonies for subculture, but after several attempts with different concentrations of agarose, the cells invariably migrated to the bottom of the well and colonies could not be 'picked' for sub-culture. Nevertheless, as 3 cells were required to generate colonies for isolation in each of these techniques, it could not be definitively determined that these colonies were truly SCD [42]. As a result, a MoFlo FACS was used to seed single cells into individual wells of 96-well plates. After four attempts at seeding a total of 768 wells, no colonies developed. Lack of cell proliferation may be attributed to the cumulative time required to remove the cells from the culture surface to put them into suspension, the shear forces exerted on the cells by the FACS machine, and a lack of paracrine signalling required to stimulate cell survival and proliferation.

Finally, a single-cell-dilution of HUCPVCs was used to seed individual HUCPVCs. A total of 3,360 wells were seeded at a dilution of 1 cell/well that yielded 117 clones (3.48%) surviving to induction at P4 (~20 population doublings). As the probability that each of these clones was single-cell-derived was only 0.5, a further dilution of 0.5 cells/well was used with an associated probability of 0.75 and yield of 2.92%. The mean CFU-F frequency of the clones was 1: [3.8±1.9] (Figure 4A), with no significant difference ($P = 0.391$) between clones derived from each seeding density. This value was not significantly different ($P = 0.125$) from the 1: [2.6±1.4] frequency seen in bulk HUCPVC cultures (Figure 1A), suggesting that the progenitor pool was maintained upon cloning. Lineage analysis of these 'clones' (Figure 4B) illustrated that as the seeding density decreased, so did the number of multipotential 'clones.' There appeared to be an increase in unipotential 'clones' and a decline in tri-, quadri- and multipotential 'clones' as the seeding density became more rigorous. This observation suggested that 'clones' isolated by seeding at 1 or 0.5 cells/well were not SCD colonies.

Rigorous sub-single cell seeding of mixed gender suspensions of HUCPVCs produce definitive SCD clones

We realized that by seeding cells at a dilution of 0.2 cells/well (1 cell per 5 wells) the probability of isolating SCD clones increased to a statistically significant 0.96 (Figure 4B). To make this strategy even more rigorous, we mixed equal numbers of male and female cells prior to seeding 0.2 cells/well (1 cell per 5 wells) into 96-well plates, which would enable genotyping of clones. From a total of 6,720 wells seeded, 45 clonal populations were produced, 32 of which survived expansion to ~10^5 cells. These 32 clones were re-seeded at the same dilution of 0.2 cells/well into 480 wells for each clone, which produced 23 clonal daughter populations, 11 of which survived expansion to ~10^6 cells. The probability that these 11 daughter sub-clones were single-cell derived was 0.9999 (Figure 4B). Double blind Y-chromosome FISH analysis was then used to definitively confirm the single cell origin of the 32 parental and 11 daughter clones (Figure 4C). Positive, negative and mixed FISH controls determined ≥85% or ≤0% thresholds for determi-

A

Cloning technique	Dilution (cells/well)	# of wells seeded	Expected # of clones	Observed # of clones	# of clones surviving to induction	Induced @ passage	Approx # of doublings @ induction
Cloning cylinder	3	60	60	61	13	3	30
Mechanical removal	3	60	60	63	38	2	20
2.5% Agarose	3	24	24	0	0	n/a	n/a
2.0% Agarose	3	24	24	0	0	n/a	n/a
1.5% Agarose	3	24	24	0	0	n/a	n/a
1.0% Agarose	3	24	24	0	0	n/a	n/a
0.5% Agarose	3	24	24	0	0	n/a	n/a
MoFlo FACS	1	768	256	0	0	n/a	n/a
	1	3360	1120	146	117	4	20
Sub/single cell limiting dilution	0.5	1920	320	68	56	4	20
	0.2	6720	448	45	32	4	20
	Sub @ 0.2	15360	1024	23	11	7	40

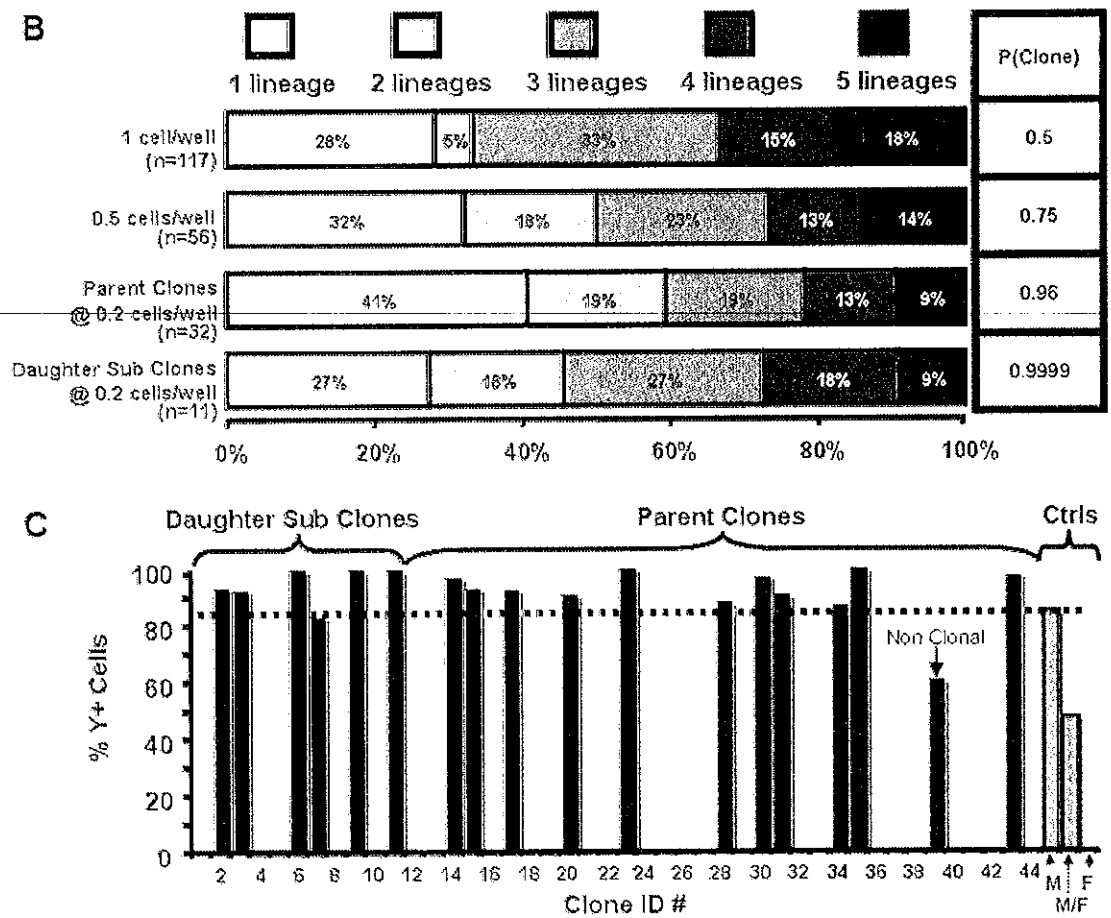

Figure 4. Clonally pure HUCPVC populations can be isolated by rigorous cell seeding. Five different methods were used in an attempt to generate definitive single-cell-derived (SCD) clones from a total of 12 cords, some of which were employed in other assays, (A). Clonal colonies generated by seeding mixed male and female suspensions of HUCPVCs at 1, 0.5, and 0.2 cells/well, the latter of which were sub-cloned (daughters) at 0.2 cells/well, displayed increasing probabilities of SCD isolation along with differential multipotential capacities (B). When analyzed by fluorescent in situ hybridization (FISH) with a Y-chromosome-specific probe, all the daughters (clones 1–11) as well as all but one (clone 39) of the parent clones (clones 12–44) seeded at 0.2 cells/well were found to be single gender derived, and accordingly determined to be SCD clones (C).
doi:10.1371/journal.pone.0006498.g004

nation of single-cell-derivation. All clones and sub-clones, except one, had a signal above the threshold for single-cell determination. We analyzed 22–54 cells within each of the 32 clones and determined that the probability that each was formed from more than one cell between 10^{-7} and 10^{-15}. Thus, 42 of the 43 isolated clones were determined to be definitively SCD populations' parent and daughter clone ID's are shown in Supplementary Figure S1. Importantly, our finding that single-gender clones arose from a mixed gender cell suspension suggests that only an exceptionally rigorous strategy to isolate single cells, such as the one we used,

could generate reliable clonal data. This is further emphasized in the generation of a non-clonal population by this method (Figure 4C: clone 39), that calls into question the reliability of clonal data produced by any less rigorous methodology.

Definitive multipotential SCD mesenchymal clones self-renew in culture

As the CFU-F frequencies of parent and daughter SCD clones were not significantly different from each other (1: [4±3.36] and 1: [5±4.15], respectively, Figure 5A), this was the first line of evidence suggesting that the parent clones may have been self-renewing and maintaining their progenitor pool. This self-renewal ability was further supported by the observation that the daughter clones maintained the potential to differentiate along more than a single lineage after >40 cell doublings.

The 11 surviving daughter clones, when assessed for multilineage capacity (Figures 4B, 6, Supplementary Figure S1), demonstrated that they were the progeny of either a self-renewing parent of the same differentiation potential, or a parent which had forfeited one or more of its lineages to give rise to a more restricted daughter. These phenomena were most evident when observing the differentiation patterns of the first three quintipotential MACOF parental clones. The first MACOF parental clone self-renewed in culture by producing a MACOF daughter, while the remaining two differentiated by giving rise to more restricted ACOF and COF daughters, forfeiting their myogenic and myogenic/adipogenic potentials respectively. This pattern was repeated in the remaining more-restricted clones. The three quadripotent ACOF parental clones gave rise to ACOF, AOF and COF daughters, illustrating self-renewal by generation of the former and differentiation by restriction into the latter two; while the single tripotential COF parent also did not self-renew, as it forfeited its osteogenic potential by producing a bipotential CF daughter. Of the two bipotential OF parental clones, only one self-renewed into an OF daughter, while the other forfeited its osteogenic capacity by giving rise to a unipotential CFU-F. Finally, the two remaining unipotential CFU-F parental clones self-renewed in culture by producing CFU-F daughters. We further observed that, of the 21 parental clones that did not survive expansion to lineage analysis of daughter clones, 12 did survive the sub-cloning process, while 9 did not (Supplementary Figure S1). Interestingly, 8 out of 12 of clones that did survive sub-cloning but not expansion had potential for more than one lineage. This illustrates that, although they too did eventually clonally exhaust in culture, they were relatively more robust than the unipotent CFU-Fs.

These data, which illustrate daughter SCD clones, having undergone ~40 population doublings, derived from equal or greater potential SCD parents, is the first reported evidence of self-renewing multipotent mesenchymal cells *in vitro*. One striking observation when the lineages are observed individually is the absence of the adipogenic lineage in the bipotential clones that possessed either an osteogenic or chondrogenic capacity. The former theoretically arises at twice the frequency of the latter, and is supported by our previously published data in which we observed a 1:1 ratio of uncommitted to commited CFU-O progenitors in HUCPVC culture [10]. The tripotential clones illustrated that, when differentially expressed with osteogenic potential, the chondrogenic and adipogenic capacities were exclusive of each other. When these two lineages were expressed together, it was only in the quadri- and quintipotential clones. Interestingly, the myogenic lineage was only expressed in the quintipotential clone, suggesting that it is the first lineage forfeited when a multipotential MSC differentiates.

Discussion

The MSC concept is predicated upon the elegant studies done in the hematopoietic system in which several investigators showed that a single prospectively isolated hematopoietic stem cell (HSC) could repopulate the hematopoietic system of a living recipient, and that when re-isolated from this primary recipient, this cell's progeny could reconstitute the hematopoietic system of a secondary living recipient [43,44,45]. Rather than the irradiation-induced injury in these hematopoietic studies, we have created a physical injury by needle marrow ablation. In fact, we have previously demonstrated that mice with defective self-renewal of multipotent mesenchymal cells developed type II osteoporosis [46,47], suggesting that a bonafide MSC does exist within the mesenchymal compartment. However, the dearth of markers for prospective isolation of these cells has made interrogation of the MSC concept refractory to experimental validation. In an attempt to overcome this, numerous studies [4,26,27,28,30,31,48] have tried to retrospectively analyze the multipotential capacity of clonally-derived mesenchymal cells. There are four major caveats of these studies. First, as prospective isolation of multipotential MSCs still eludes us, and retrospective analysis is a valid alternative strategy, most investigators have limited their analysis up to three mesenchymal phenotypes: bone, fat and cartilage. While these three lineages are the most established, it is commonly accepted that several other mesenchymal lineages exist including muscle, tendon, ligament and stromal tissues [21,49]. As the latter three could be grouped into the "fibroblastic lineages," it then becomes clear that for full retrospective analysis of mesenchymal differentiation, five lineages must be assayed: bone, cartilage, fat, muscle and fibrogenic. The second, and most elusive issue is that of clonality. Based on Friedenstein's early work [50,51], it was originally assumed that limiting dilution (i.e. the minimum number of cells required to produce a single colony of mesenchymal cells could provide clonal colonies. More recently, it has been shown that 'clonal' cultures of neural stem cells are highly motile and prone to aggregation [42]. As mesenchymal cells are highly motile in culture, this calls into question the literature in which clonal isolation was performed by seeding more than one cell in a culture well [4,29]. Thus, only FACS single cell deposition or sub-single cell seeding with retrospective analysis of clonal purity could be used to isolate definitive SCD clones. Third, it has yet to be definitively demonstrated either *in vitro* or *in vivo* that one of these clonally derived mesenchymal populations is able to self-renew by producing equipotent progeny either *in vitro* or *in vivo*. The final and most difficult problem is that, unlike the rigor within HSC literature, rigorous clonal mesenchymal transplantation studies have been elusive for the field.

In this study, we tackled the first three problems and made inroads to address the clonal *in vivo* studies. We have described that a multipotent population of mesenchymal cells that maintain differentiation capacity *in vivo* and, due to their high clonogenic frequency, can be rigorously isolated as SCD populations in culture that in turn can generate daughter SCD populations, both of which can be analyzed for five distinct mesenchymal lineages. These *in vitro* functional phenotypic data determine a hierarchical structure for the mechanism underlying MSC differentiation (Figure 7) in which a self-renewing multipotent MSC (CFU-MACOF) gives rise to more restricted self-renewing progenitors that gradually lose differentiation potential until a state of complete restriction to the fibroblast is reached. Importantly, the sum total of all these progenitors provides the 1: [2.6±1.4] clonogenic frequency observed in our HUCPVC cultures. Other hierarchical structures of MSC differentiation have been proposed. Aubin [52] postulated that MSCs give rise to either a CFU-

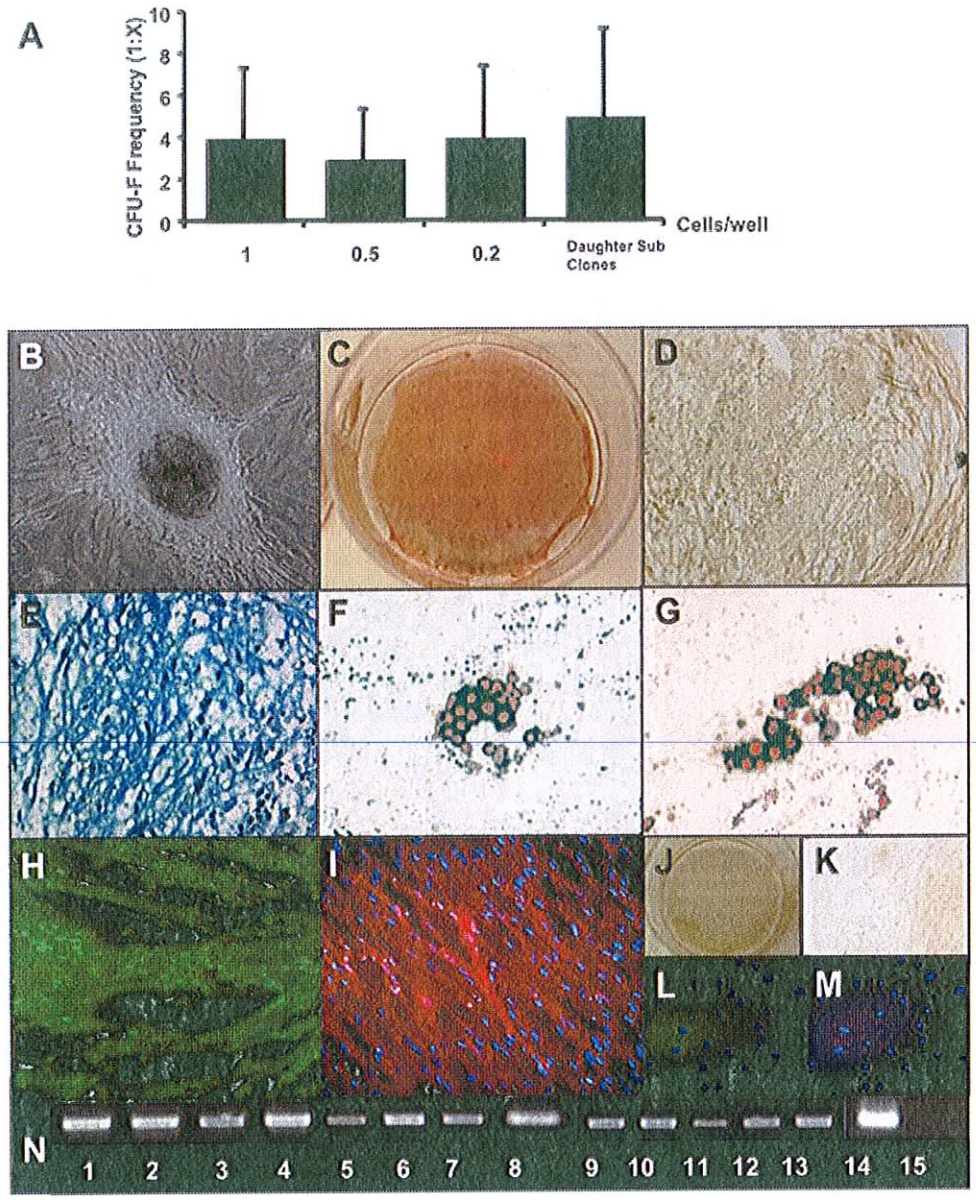

Figure 5. Clonally pure HUCPVC populations display multipotent capacity *in vitro*. CFU-F frequencies of HUCPVCs derived by seeding at 1, 0.5, and 0.2 cells/well, the latter of which were sub-cloned (daughters) at 0.2 cells/well, were not found to be significantly different from each other (A). Clone 11 (B-N) demonstrated identical capacity to its parent (clone 35), maintaining the ability to differentiate into all five lineages assayed. Under induction, bone nodules were observed in culture (B) that stained with Von Kossa (black), and were surrounded by alkaline phosphatase-high expressing cells (C). Cartilage pellet cultures of HUCPVCs expressed collagen II (D) and glycosaminoglycans that stained with Alcian blue (E). HUCPVC-derived adipocytes stained with Oil Red O (F,G). Myogenically-induced HUCVPCs expressed high levels of MyoD (H) and FSMLC (I) in multinucleated HUCPVC myotubes. Negative controls were uninduced cells stained with Von Kossa and alkaline phosphatase (J), and secondary-only antibody staining for collagen II (K), MyoD (L) and FSMLC (M) stained cultures. (Field widths: B,D,K = 628 μm; C,J = 3.5 mm; E,H,I,L,M = 315 μm; F,G = 86 μm). RT-PCR analysis (O) demonstrated upregulation of the following lineage-specific genes: Runx2 (1), collagen IA1 (2), osteopontin (3), osteocalcin (4), Sox9 (5), collagen II (6), LPL (7), aggregan (8), MyoD (9), Myf5 (10), desmin (11), MHC (12), FSMLC (13), GAPDH (14) and RT negative control (15). (MACOF represents a clone that was able to differentiate into all 5 lineages assayed). HUCPVC harvests from 27 different cords were employed to generate this data.

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OC or CFU-OA bipotential progenitor and that CFU-A and CFU-C are differentially forfeited to result in the osteogenic capacity required for bone formation. Similarly, Muraglia and colleagues [27] concluded that bone represents the default in the mesenchymal pathway. However, neither group included either the myogenic or fibroblast lineages in their analyses and, as our results demonstrate, it is the latter that is the default lineage in the mesenchymal hierarchy.

The perivascular niche [20] has been shown to be the source of mesenchymal progenitors in many organs [16], including bone marrow [53]. It was recently shown [29] that the $CD146^+$ osteoprogenitor cells in human marrow predominantly associate with

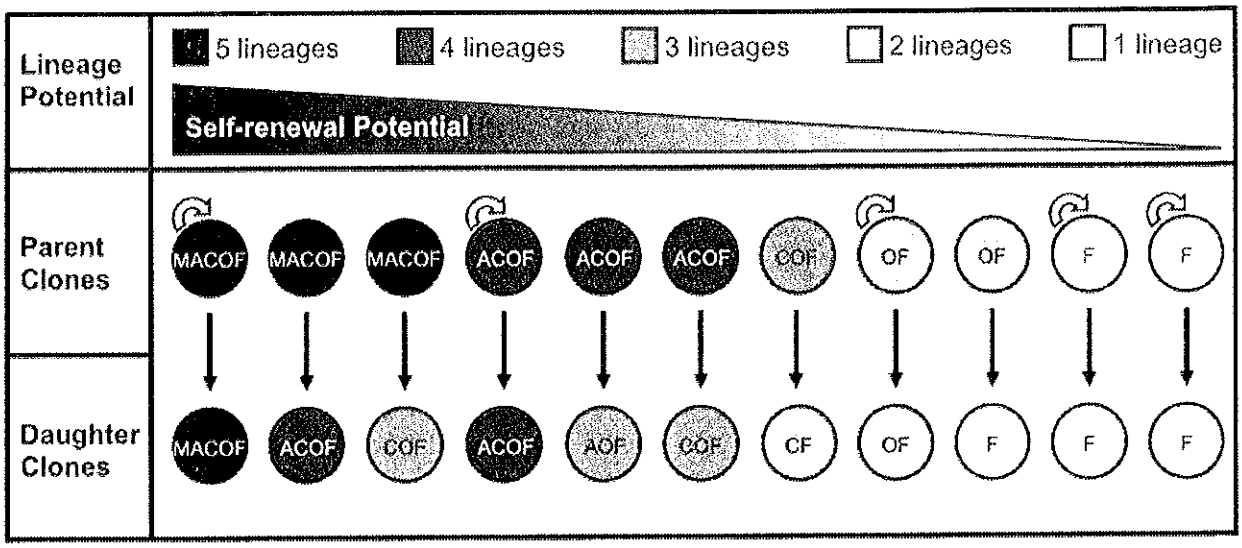

Figure 6. Clonally pure HUCPVC populations display differential self-renewal capacity *in vitro*. When the differentiation capacities of the 11 daughter clones were compared to those of their parents, it was found that they either maintained equipotency (self-renewing clones) or forfeited the ability to differentiate into one or more lineages (non-self-renewing clones). (Self-renewing clones are represented with a semi-circular arrow and non-self-renewing clones without a semi-circular arrow).
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vessels as 'adventitial reticular cells' and promote hematopoietic supportive stroma. While occupying their niche, perivascular cells remain quiescent but in most organs give rise to daughter cells that preferentially differentiate into the stromal fibroblasts of the organ, and by-pass other differentiation pathways presumably through signaling events that prevent the differentiation of multipotent MSC into the other restricted progenitors. Thus, we hypothesize that the primary responsibility of HUCPVCs in situ is to maintain their niche's stromal tissue by differentiating into the myofibroblasts that elaborate the extracellular matrix of the umbilical cord. However, when the homeostasis of this, or any other, perivascular niche is disturbed during growth or by injury, or these resident MSCs are removed from their natural environment, they will be provided with the specific cues required to reestablish homeostasis - or exhibit their lineage capacity *in vitro*. Our *in vivo* work is an example of this phenomenon in which the transplanted HUCPVCs not only contributed to tissue healing and matrix elaboration themselves, but also recruited resident mouse progenitors to rapidly repair the damaged tissues. Accordingly, rather than displaying unrestricted self-renewal and differentiation, only a few asymmetric cell divisions of the resident MSCs may have been initiated to produce the more restricted tissue-specific progenitors available for regenerating the damaged tissue.

By assaying five mesenchymal lineages, our data demonstrate that the greater the potential a cell has for differentiation, the rarer it is within the mesenchymal compartment. Recent evidence has shown that MSCs arise during an initial transient wave of neuroepithelial differentiation, although these are replaced by MSCs from unknown sources [24]. We have shown here that the muscle lineage is the first to be forfeit, and this reflects embryonic mesenchymal development in which a subsequent wave of paraxial mesoderm development gives rise to initially identical somites that specify into three regions. The dermamyotome and myotome give rise to skeletal muscles of the back, body wall and limbs, while the sclerotome produces the cartilage cells of the vertebrae and ribs that subsequently undergo endochondral ossification. Sequential loss of the chondrogenic and osteogenic lineages inevitably produce the default fibroblastic lineage not only illustrated in the hierarchy of our model, but also observed in adult marrow stroma, the stromal tissue of the other organs of the body and many solid tumors [54,55].

To date, elucidating the biology of MSCs has been challenging because of the low frequency of MSCs within the heterogeneous bone marrow population. By using a richer, more homogeneous source of primary mesenchymal cells, HUCPVCs, we have dissected the differentiation of MSCs without the confounding effects of the variety of other stem cells, progenitors and terminally differentiated cells present in bone marrow. We have shown that HUCPVCs induce tissue repair in response to injury, and that SCID clonal multipotent parents give rise to a clonal multipotent descendants, satisfying the two properties of stem cells: self-renewal and multilineage differentiation. The ability of these self-renewing progenitors to differentiate along multiple pathways becomes more restricted with the loss of the myogenic, adipogenic, chondrogenic, osteogenic and fibroblastic progenitor lineages respectively, illustrating the hierarchical mechanism of MSC fate decisions. Identifying the molecular cues that arrest or drive this hierarchical cascade will be of considerable importance to our understanding mesenchymal biology.

Materials and Methods

Ethical approval was obtained from both the University of Toronto and Sunnybrook and Women's College Health Sciences Centre, Toronto; written informed consent was obtained and the umbilical cords were donated by consenting full-term caesarian section patients. A total of 57 umbilical cords were used in the experiments described and the number employed for each captioned with the appropriate figure. For additional details see Supplementary Methods S1.

Colony forming unit (CFU-F) assay

HUCPVCs expanded in culture to 70–90% confluence were harvested with trypsin-EDTA and counted as described in Supplementary Methods S1. Cells were diluted in supplemented

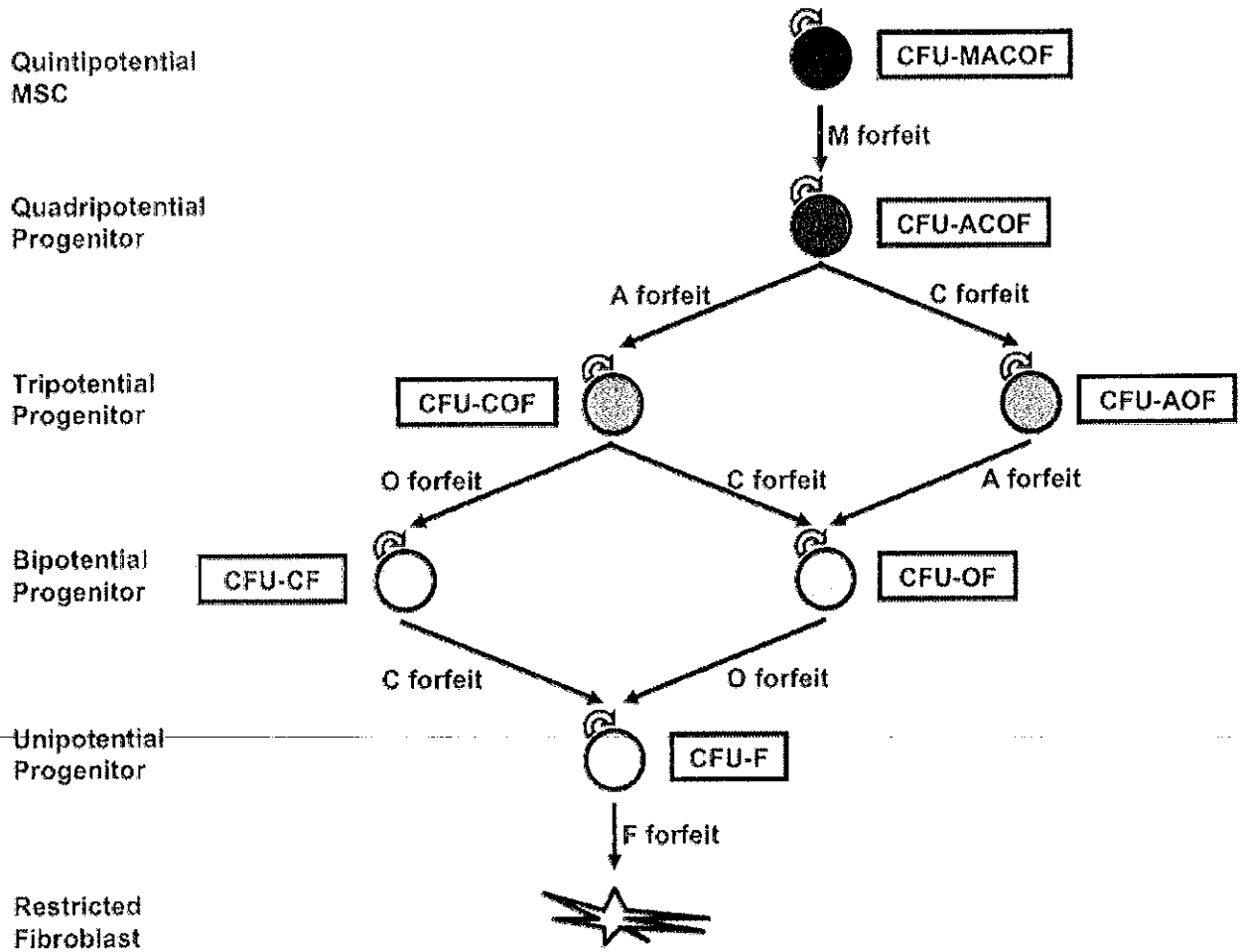

Figure 7. Hierarchy of MSC differentiation.
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media SM: 5% fetal bovine serum FBS, HyClone, Lot KPF21314, 85% α-MEM, and 10% antibiotics fungizone, penicillin & streptomycin, and plated at 1, 5, 10, 50, 100, and 500 cells per well of a 6-well culture dish. After incubation for 5–7 days at 37 C in 5% humidified CO_2, the cells were washed with PBS and stained with 0.5% Crystal Violet Sigma-Aldrich for 15 min at room temperature. Cells were washed with PBS twice, and visible colonies were counted from 7 different samples. The minimum number of input cells required to produce a single colony determined the CFU-F frequency. This was repeated at every sub-culture until passage 12 P12.

In vivo analysis of HUCPVC differentiation

P2 HUCPVCs were transduced by lentivirus expressing eGFP driven by the constitutive EF1a promoter a kind gift from Dr. J. Ellis, MaRS, Toronto, Canada. The eGFP+ cells were expanded in culture to 20×10^6 cells. 0.5×10^6 cells were injected in 20 µl of α-MEM into the intrafemoral space of each of 26 NOD-scid mice Jackson Laboratories, with an additional 10 mice receiving 20 µl of α-MEM sham. After 2, 4 and 6 weeks, the mice were sacrificed. At each time point, the marrow from 3 mice was flushed and cultured, while the femurs from 4 cell-injected and 3 sham-injected mice were fixed for 24 hours in 10% neutral buffered formalin. The fixed femurs were analyzed by micro computed tomography micro CT General Electric Health Care. A pixel threshold was determined by pixel intensity outside the femur, and software GE MicroView was then used to determine all pixels above the threshold as BMD. The fixed femurs were then decalcified in 20% formic acid and embedded in paraffin for sectioning into 4 µm thin sections. Longitudinal sections were obtained through the entire width of the femur. From every 15 sections, 6 serial sections were labeled with Masson's Trichrome, anti-GFP 1:100, Sigma-Aldrich, biotin-conjugated Lightning-Link Biotin, Innova Biosciences mouse-anti-human nuclear-antigen HuNu 1:300, Chemicon anti-human-osteocalcin 1:100, Peninsula, anti-human collagen II 1:100, Chemicon, and anti-human PPARγ 1:200, Abcam respectively.

Clonal isolation of HUCPVCs by sub-single cell seeding

Isolates of male and female P0 HUCPVCs were plated separately for 24 hours, removed from the culture surface by trypsin/EDTA (Supplementary Methods S1), washed and collected as independent suspensions. Each suspension, approximately 5×10^4 cells P1, were then passed through a 70 µm cell strainer to ensure single cell suspension, and counted on a ViCell-XR automated cell counter. 5×10^3 cells from each suspension were mixed together, and an aliquot of the mixed suspension was then diluted in SM to a concentration of 0.5 or 0.2 cells per 50 µl respectively. 50 µl of the

mixed suspension was placed into each well of 35×96-well tissue culture plates. After 24 hours, an additional 50 µl of SM was added to each well and the SM was replaced every 5 days. After 10 days in culture, each well of the plates was observed by light microscopy for the presence of cells. Only wells with cells were maintained by replacement of SM every 3 days until they reached 70–90% confluence. The cells were then sub-cultured and seeded P2 into an individual well of a 6-well culture dish. The media was replaced every 2 days until the cells reached 70–90% confluence, at which point they were sub-cultured and seeded P3 into individual T-75s, in which the media was replaced every 2 days. Once the cells reached 70–90% confluence, they were removed from the culture surface P4 by trypsin/EDTA, counted with the Vi-Cell approximately 1.3×10^6 cells and seeded as required for differentiation assays or stored in liquid N_2 Supplementary Methods S1.

Sub-clonal isolation of HUCPVCs by sub-single cell seeding

P4 clonal HUCPVC populations derived from 0.2 cell/well seeding were obtained as single suspensions by passing them through a 70 µm cell strainer. They were then diluted in SM to 0.2 cells per 50 µl and seeded into 96 well plates 5 plates per clone. The cells were expanded as described above into 6-well plates P5, and T-75s P6. Once they reached 70–90% confluence they were removed from the culture surface, and seeded as required for differentiation assays described below, or stored in liquid N_2 described above.

Double blind analysis of clonal formation by Fluorescent *in-situ* hybridization

As all clones and sub-clones were isolated from equal suspensions of mixed male and female cells, single cell isolation was confirmed by fluorescent *in-situ* hybridization (FISH) analysis for the presence or absence of a Y-chromosome. 5×10^3 cells from each of the 32 clones and 11 sub-clones, both derived from 0.2 cells/well seeding, were plated into independent wells on 4-well glass chamber slides. One slide was prepared with known controls including, 5×10^3 mixed male/female, 5×10^3 male, and 5×10^3 female two wells cells in separate wells. The slides were then provided to an independent FISH analyst who was only given identification of the control wells. Importantly, the analysis was double-blind, as neither the person who plated the cells, nor the independent analyst knew the gender of any of the 43 clones. The CEP Y Sat III-32-112024 DNA FISH probe was obtained from Vysis. Application of the probe was performed following the Vysis protocol, and the cells were counterstained with DAPI. The independent analyst then analyzed the 43 clones for presence/absence of Y-chromosome nuclear localization by fluorescent confocal microscopy. Wells with a Y-chromosome signal equal or less than the negative control (known female cells), or a signal equal or greater than that of the positive control (known male cells) were identified as single-cell derived.

Differentiation of HUCPVC clones and sub-clones

Each clone was seeded into 12 wells of a 24-well plate. 9 wells were seeded with 10^4 cells. Of these, 2 of each were induced with medium containing either osteogenic supplemented (OS), adipogenic supplemented (AS) or myogenic supplemented (MS) media (Supplementary Methods S1), and the remaining 3 were treated with SM containing 2% FBS as negative controls. The remaining 3 wells of the plate were seeded with 100, 50 and 10 cells, and treated with SM containing 5% FBS to assess the CFU-F frequency of each clone. Also, 2.5×10^5 cells from each clone were induced with chondrogenic supplements (CS) in a 15 ml tube (Supplementary Methods S1). RNA from the remaining induced cells was harvested with 0.5 ml Trizol reagent (Invitrogen) for RT-PCR analysis (Supplementary Methods S1 and Table S1).

Statistical analysis

CFU-F frequencies and proliferation were compared using unpaired 2-tailed t-tests and micro CT data was compared using a paired 2-tailed t-test. T-test values were considered significant with a probability of less than 5% ($P<0.05$). For FISH analysis, if the admixture of cells of the second donor in each colony is k, then the probability of not finding a mixed colony after identification of 22–54 cells in each colony is $1-k^{22}$ to $1-k^{54}$. For $k=0.5$, the probability of single cell clonal formation was 10^{-7} to 10^{-17}.

Supporting Information

Methods S1 Supplementary Methods
Found at: doi:10.1371/journal.pone.0006498.s001 (0.05 MB DOC)

Table S1 Primer sequences used for RT-PCR. - Abbreviations are: lipoprotein lipase (LPL), fast skeletal myosin light chain (FSMLC) and myosin heavy chain (MHC).
Found at: doi:10.1371/journal.pone.0006498.s002 (0.05 MB DOC)

Figure S1 A) indicating their self-renewal capacity, clone ID# and lineage potential. Clone ID #s correspond to those in Figure 3C. 21 of the 32 parental clones B) did not produce daughters that survived expansion for lineage analysis. Of these, 12 survived sub-cloning (indicated by √), while 9 did not (indicated by χ).
Found at: doi:10.1371/journal.pone.0006498.s003 (0.13 MB DOC)

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Author Contributions

Conceived and designed the experiments: RS AK WLS JED. Performed the experiments: RS LH. Analyzed the data: RS LH AK WLS JED. Contributed reagents/materials/analysis tools: AK WLS JED. Wrote the paper: RS AK WLS JED.

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