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Isolation and characterisation of resident endogenous c-kit-positive cardiac stem cells (eCSCs) from the adult mouse and rat heart

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Abbreviations: BSA – bovine serum albumin; eCSC – endogenous cardiac stem cell; DMEM - Dulbecco's Minimum Essential Medium; FITC – fluorescein isothiocyante; MEM - Minimum Essential Medium; PE - phycoerythrin.

Keywords: cardiac stem cells, cell culture, multipotency, retrograde coronary perfusion

ABSTRACT

This protocol describes the isolation of endogenous c-kit-positive (c-kit^{pos}), CD45-negative (CD45^{neg}) cardiac stem cells (eCSCs), from whole adult mouse and rat hearts. The heart is enzymatically digested via retrograde perfusion of the coronary circulation, resulting in rapid and extensive breakdown of the whole heart. Next the tissue is mechanically dissociated further and cell fractions separated by centrifugation. The c-kit^{pos} CD45^{neg} eCSC population is isolated by magnetic activated cell sorting technology and purity and cell number assessed by flow cytometry. This process takes approximately 4 hours for mouse eCSCs or 4.5 hours for rat eCSCs. We also describe how to characterise c-kit^{pos} CD45^{neg} eCSCs. The c-kit^{pos} CD45^{neg} eCSCs exhibit the defining characteristics of stem cells; being self-renewing, clonogenic and multipotent. This protocol also describes how to differentiate eCSCs into the three main cardiac lineages: functional, beating cardiomyocytes, smooth muscle and endothelial cells. These processes take between 17 and 20 days.

INTRODUCTION

Although previously considered to be a terminally-differentiated organ, the adult mammalian heart has been shown to have the ability to develop new cardiomyocytes and microvasculature throughout life [1]. It has been further demonstrated that the adult mammalian heart contains a population of resident endogenous stem and progenitor cells, which have the defining characteristics of stem cells: self-renewal, clonogenicity and multipotency [2]. Such cells have been identified in a range of different mammalian species, including human by ourselves and other groups [3-23].

Early in this century, the search for the origin of small mono-nucleated cardiomyocytes undergoing cytokinesis seen sporadically in normal and pathological adult hearts, including human [24], led to the first identification of *bona fide* resident, endogenous cardiac stem/progenitor cells (eCSCs) present in the adult myocardium throughout the organism's lifespan which are self-renewing, clonogenic and multipotent [2]. Shortly after, several eCSCs in the adult mammalian heart were identified by different membrane markers and transcription factors (which often led to claims of a new stem cell type with each new marker used for its identification). Indeed, when referring to eCSCs, there are at least seven resident heart populations so far identified (mainly according to the single antigen used for their primary isolation) in the adult mammalian heart, including the human [25]. These are c-kit^{pos} eCSCs (that have been also defined as CD34^{neg}, CD45^{neg}, Sca-1^{pos},

$Abcg2^{pos}$, $CD105^{pos}$, $CD166^{pos}$, $GATA4^{pos}$, $NKX2\text{-}5^{pos/neg\ or\ low}$, $MEF2C^{pos}$) [2,4-9]; $Sca1^{pos}$ eCSCs ($CD34^{neg}$, $CD45^{neg}$, $FLK1^{neg}$, $ckit^{pos/neg\ or\ low}$, $GATA4^{pos}$, $NKX2\text{-}5^{pos/neg\ or\ low}$, $MEF2C^{pos}$) [10,11]; side population (SP) cells ($CD34^{pos}$, $CD45^{pos}$, $Abcg2^{pos}$, $Sca1^{pos}$, $c\text{-}kit^{pos}$, $NKX2\text{-}5^{neg}$, $GATA4^{neg}$) [12,13]; Cardiosphere-derived cells ($CD105^{pos}$, $CD34^{pos}$, $CD45^{pos}$, $Abcg2^{pos}$, $Sca1^{pos}$, $c\text{-}kit^{low}$) [14-16]; cardiac-resident colony-forming unit-fibroblast (cCFU-Fs) ($Sca\text{-}1^{pos}$, $PDGFR\alpha^{neg}$, $CD31^{neg}$, $c\text{-}kit^{low}$, $CD45^{neg}$, $FLK1^{neg}$, $CD44^{pos}$, $CD90^{pos}$, $CD29^{pos}$, and $CD105^{pos}$) [17]; cardiac mesangioblasts ($CD31^{pos}$, $CD34^{pos}$, $CD44^{pos}$, $CD45^{neg}$, $Sca1^{pos}$, $c\text{-}kit^{pos}$) [19]; and the $Isl\text{-}1^{pos}$ cardiac progenitor cells (CPCs) ($CD31^{neg}$, $Sca1^{neg}$, $c\text{-}kit^{neg}$, $GATA4^{pos}$, $NKX2\text{-}5^{pos}$) [20,21]. The phenotypes and sources of these cell populations are summarised in Table 1.

The description of these various eCSC populations led to the paradoxical situation whereby the heart, previously described as a non-renewing organ, became the organ with the highest number of distinct types of resident stem/progenitor cells. However, aside from the $Isl\text{-}1^{pos}$ CPCs which are direct progeny of a defined embryonic cardiac progenitor cell population present in only very small numbers in the adult heart [26] and the epicardial stem cells also called cCFU-Fs presumable derivate from the pro-epicardial organ[17], it is evident just from the significant overlap of the main and secondary markers used for their characterization that the different eCSC/CPC populations are closely related. So it is not surprising that after the early enthusiasm subsided, a consensus is developing that many, if not all, different putative adult eCSCs reported so far, likely represent different developmental and/or physiological stages of a unique resident stem-progenitor cell type with multipotent regenerative capacity yet to be completely defined [27]. Clearly, genetic tracking in genetically-modified mice, if properly designed, would be an invaluable tool to try to establish the connection among the different eCSC/CPC populations as well as their real contribution to heart development, adult myocardial homeostasis and disease.

The $c\text{-}kit^{pos}$ eCSCs are characterised by expression of c-kit (also known as CD117), the receptor for stem cell factor. Although in the myocardium c-kit is also expressed by cardiac mast cells, which in contrast to the eCSCs are also $CD45^{pos}$ and tryptase positive, removal of the CD45-positive cell lineage removes this mast cell population [6]. The abundance, distribution and the intrinsic regenerative capacity of $c\text{-}kit^{pos}$ eCSCs in adult cardiac tissue has been recently clarified [4], although their developmental origin has not yet been lineage traced (for review see [28,29]). It is desirable when isolating eCSCs from adult tissue to obtain a cell population of the highest possible uniformity in terms of lineage potential, with the cells retaining the ability to develop into all four cell lineages required to regenerate the myocardium (cardiomyocyte, vascular (endothelial and smooth muscle) and mesenchymal). It has been repeatedly demonstrated [2,4-6] that the $c\text{-}kit^{pos}$ eCSC population are sufficiently primitive to retain this quadri-lineage potential, indicating that these are uncommitted cells, i.e. *bona fide* stem/progenitor cells.

Here we present effective and highly reproducible protocols for the isolation of c-kit^{pos} CD45^{neg} Tryp^{neg} eCSCs from the adult whole mouse (C57/BL6J) and rat (Wistar) heart, the most commonly utilised small animal models for experimental cardiovascular research. These protocols were originally developed during the first identification of eCSCs [2]. The protocols described here are broadly similar for mouse and rat and result in the isolation of similar type and number of cells, however there are several procedural differences. The key factor in both is the retrograde coronary perfusion step, a process initially described by Silver and colleagues in 1983 [30], in which the coronary arteries are used as a route for buffer and digestive enzyme to perfuse through the myocardium. This procedure first removes peripheral blood cells and endothelial cells present in the vascular tree and subsequently induces prompt digestion of the tissue, thus making it possible to isolate separate fractions of the main cardiac cell types, i.e. cardiomyocytes, smooth muscle cells, endothelial cells, cardiac fibroblasts and eCSCs. Compared to other methods, retrograde coronary perfusion and digestion of the whole heart allows the harvest of the highest number of eCSCs and it also makes it possible to simultaneously collect rod-shaped cardiomyocytes. In addition we describe how to characterise the isolated c-kit^{pos} CD45^{neg} Tryp^{neg} eCSCs and how to induce and characterise their differentiated progeny.

Advantages of the protocol

The main advantages of retrograde perfusion of the coronary tree with the digestive enzymes are the increased yield and high purity of c-kit^{pos} CD45^{neg} Tryp^{neg} eCSCs (hereinafter described as eCSCs) that are extracted. The isolated eCSCs can be immediately studied further, cryogenically stored, characterised, cloned or expanded *in vitro*. A major difficulty in working with eCSCs from adult myocardium is their scarcity and therefore many methods for their "isolation" require an *in vitro* expansion step. While this is effective, depending on the questions addressed, the expansion of the eCSCs in the culture environment may represent a confounding variable. The methods described here allow a sizeable population of eCSCs to be obtained without the need to include an *in vitro* expansion step.

Experimental Design

This protocol describes the isolation of small cells (of less than 40µm diameter) from the mouse (step **1** option **A**) or rat (step **1** option **B**) heart, with the subsequent removal of CD45-positive haematopoietic lineage cells, which remove the blood cell lineage precursors and the mast cells (Tryp[pos]), followed by the selection of the cell population positive for c-kit. The differences between the two protocols are summarised in Table 2. When flow cytometry analysis of the selected eCSC population is planned, it is necessary for the mouse and advisable for the rat to collect an aliquot of unsorted cardiac small cells for isotype control. Using the rat protocol it is also possible to isolate intact, contracting, calcium-resistant, rod-shaped cardiomyocytes should this be required. The mouse rod-shaped cardiomyocytes are significantly more delicate and many will be killed by

the procedure by a leaky membrane that allows an influx of extracellular Ca^{2+}.

It is critical when using the retrograde-perfusion method described here that the time from death of the animal to successful placing of the heart on the perfusion system is minimised. Blood clotting and subsequent occlusion of the vessels must be prevented to allow adequate retrograde tissue perfusion. Inefficient digestion of the tissue by the perfused enzyme solution reduces the quantity and quality of cells recovered. Additionally the number of circulatory haematopoietic lineage cells contaminating the pool of small cells is increased. Intraperitoneal injection of heparin to the animal prior to euthanasia reduces blood clotting and improves perfusion, as does rapid cannulation and prompt hanging of the heart on the perfusion system.

Given the relatively small number of c-kit^{pos} CD45^{neg} eCSCs that can be isolated from a single heart, we recommend that eCSCs are collected from no fewer than three-to-six C57/BL6J mice or two Wistar rats at a time. This circumvents the fact that if eCSCs are initially seeded at a very low cell density, early cell growth is very slow. Once the eCSCs are in culture, medium must be replenished every two to three days. Growth can be further enhanced by the use of conditioned medium, which enables a quasi-logarithmic growth rate to be achieved. Passaging of cells when they reach ~70% confluency prevents overgrowth and cell-to-cell contact, which can lead to cell growth inhibition and/or differentiation.

A limitation of the protocol is that we use custom-made specialised equipment for the perfusion and digestion of the *ex vivo* whole heart. However, a commercially available version comparable to our system is available from AD Instruments (cat. no. PL3508B1-V for mouse, B3-V or B4-V for rat) which should allow our system to be reproduced by researchers without access to custom-made apparatus. Although we have not tested side-to-side the efficacy of the commercial apparatus compared to our equipment, if the basic principles of our method are followed they should prove to be effective.

Once the c-kit^{pos} CD45^{neg} eCSCs have been isolated, flow cytometry can be used to confirm expression of various stem cell surface markers. The cells can also be grown in culture. This protocol also describes how to characterise the cells for the stem cell properties of clonogenicity, self-renewal, and multipotency (step **2** options **A** and **B**); how to differentiate either mouse or rat c-kit^{pos} CD45^{neg} eCSCs into functional, beating cardiomyocytes *in vitro* (step **2** option **C**); and how to fix and stain cells by immunocytochemistry (step **2** options **D**, **E** and **F**).

MATERIALS

REAGENTS
Common to mouse and rat procedures

- Sodium bicarbonate (Sigma S5761)
- Glutamine (Sigma G7029)
- 1000ml filter (0.22µm pore, Millipore SCGPT10RE)
- Collagenase, type II (Worthington 4176)
  CAUTION: Collagenase is an irritant: wear appropriate safety clothing and gloves.
  CRITICAL: It is advisable to lot-test collagenase prior to large scale experiments, as there is substantial variation in enzyme activity between lots.
- Bovine Serum Albumin (BSA, Sigma A9418)
- PBS without calcium or magnesium (Invitrogen 14190-136)
- EDTA (Invitrogen 15575-038)
- Penicillin-Streptomycin (Invitrogen 15140-122)
- Fungizone (Invitrogen 15290-018)
- Gentamicin (Sigma G1397)
- Heparin sulphate (MP Biomedicals)
- Sodium pentobarbitone (Euthatal, Merial)
- 15ml centrifuge tubes (Corning 430791)
- Cell strainers (40µm pore size, BD biosciences 352340)
- 50ml Falcon tubes (BD biosciences 352070)
- Eppendorf 1.5ml tubes (Eppendorf 3810X)
- MS columns (Miltenyi 130-042-201)
- Pre-separation filters (Miltenyi 130-041-407)
- Trypan Blue solution (Sigma T8154)
- Dulbecco's Minimum Essential Medium-F12-HAMS (Sigma D8437)
- Insulin-Transferrin-Selenium (Invitrogen 51500-056)
- Neurobasal medium (Invitrogen 10888-022)
- B27 supplement (Invitrogen 17504-044)
- N2 supplement (Invitrogen 17502-048)
- Embryonic stem cell-qualified foetal bovine serum (Invitrogen 10439-024)
- Epidermal growth factor (Peprotech 100-15)
- Basal fibroblast growth factor (Peprotech 100-18B)
- Leukemic inhibitory factor (Millipore LIF2010)
- 6-Well tissue culture plates (BD biosciences 353046)
- Gelatin (Sigma G1890)
- PBS (Invitrogen 14040-224)

Only required for mouse eCSC isolation
- Minimum Essential Medium powder (MEM, Joklik modification Sigma M0518)
- HEPES (Sigma H3375)
- Taurine (Sigma T7146)
- C57/BL6J mice (6-8 weeks of age)
  CAUTION: All experiments must be performed in accordance with relevant guidelines and regulations.
- 5/0 suture thread (Fine Science Tools 18020-50)

- CD45 mouse MicroBeads (Miltenyi 130-052-301)
- EasySep CD117 mouse selection cocktail (Stem Cell Technologies 18757)
- FITC-conjugated anti-mouse CD45 antibody (Miltenyi 130-091-609)
- PE-conjugated anti-mouse c-kit antibody (Stem Cell Technologies 18757C.1)

Only required for rat eCSC isolation
- Sodium chloride (Sigma S3014)
- Potassium chloride (Sigma P5405)
- Magnesium sulphate (Sigma M7506)
- Potassium phosphate (Sigma P0662)
- Dextrose (Sigma D9434)
- Hyaluronidase, type II (Sigma H2126)
- Dulbecco's Minimum Essential Medium (DMEM, Invitrogen 31885-023)
- Adult Wistar rats (~250-300g bodyweight)
  CAUTION: All experiments must be performed in accordance with relevant guidelines and regulations.
- 4/0 suture thread (Fine Science Tools 18020-40)
- Calcium chloride (Sigma C4901)
- Trypsin, type IX (Sigma T0303): stock solution of 0.3mg/ml
- DNase, type I (Sigma DN25): stock solution of 0.3mg/ml
- Cell strainers (100µm pore size, BD biosciences 352360)
- Optiprep (Sigma D1556)
- Mouse IgG anti-rat CD45 antibody (Santa Cruz Biotechnology sc53047)
- Mouse IgG anti-rat CD45 FITC-conjugated antibody (Biolegend 202205)
- Rabbit IgG anti-rat c-kit antibody (Santa Cruz Biotechnology sc5535)
- Rat anti-mouse IgG2a+b MicroBeads (Miltenyi 130-047-201)
- Goat anti-rabbit IgG MicroBeads (Miltenyi 130-048-602)
- AlexaFluor488-conjugated donkey anti-rabbit antibody (Jackson 711-545-152-JIR)
- AlexaFluor 594-conjugated donkey anti-rabbit antibody (Jackson 711-585-152-JIR)

For characterisation of cells (mouse or rat)
- MEM (alpha modification, Sigma M0894)
- Foetal calf serum (Invitrogen 10106-151)
- Oxytocin acetate (Sigma O6379)
- Ascorbic acid (Sigma A4403)
- Dexamethasone (Sigma D4902)
- Beta-glycerol phosphate (Sigma G9891)
- Penicillin/Streptomycin (Invitrogen 15140-122)
- Gentamicin (Sigma G1397)
- Fungizone (Invitrogen 15290-018)
- TGF-ß1 (Peprotech 100-21)
- BMP-2 (Peprotech 120-02)
- BMP-4 (Peprotech 120-05ET)

- Dkk-1 (R and D systems 4010-DK-010)
- Wnt5a (R and D systems 645-WN-010)
- PBS without calcium or magnesium (Invitrogen 14190-136)
- EDTA (Invitrogen 15575-038)
- Poly-prep slides (Sigma P0425-72)
- Shandon Cytofunnels (Thermo-Fisher SDA78710005)
- ImmEdge pen (Vector laboratories H-4000)
- Shandon Cell-Fixx (Thermo-Fisher 6768326)
- **CAUTION:** Toxic chemical: wear appropriate safety clothing and gloves.
- Laminin (Sigma L2020)
- DMEM (Invitrogen 31885-023)
- 4-Well chamber slides (BD biosciences 354114)
- 16% Formaldehyde solution (TAAB F017/3)
- **CAUTION:** Toxic chemical: wear appropriate safety clothing and gloves.
- PBS (Invitrogen 14040-224)
- Tween 20 (BioRad 170-6531)
- Triton-X (Sigma T8787)
- Donkey serum (Sigma D9663)
- DAPI (Sigma D9542)
- **CAUTION:** Toxic chemical: wear appropriate safety clothing and gloves.
- Vectashield (Vector laboratories H1000)
- Anti-mouse Sca-1-FITC conjugated antibody (Miltenyi 130-098-034)
- Anti-mouse CD31-FITC conjugated antibody (Miltenyi 130-100-003)
- Anti-mouse CD34-FITC conjugated antibody (BD 560238)
- Anti-mouse CD90-PE conjugated antibody (Abcam Clone G7, ab24904)
- Anti-mouse CD105-PE conjugated antibody (Miltenyi 130-098-018)
- Anti-mouse CD140a-PE conjugated antibody (Miltenyi 130-096-271)
- Anti-mouse CD166-PE conjugated antibody (eBioscience 12-1661-81)
- Anti-mouse CD45 FITC-conjugated antibody (Miltenyi 130-091-609)
- Mouse IgG1 PE Isotype control (Abcam ab81200)
- Mouse IgG2a FITC Isotype control (Abcam ab81197)
- Anti-mouse CD117-PE conjugated antibody (Miltenyi 130-091-730)
- Anti-mouse CD45-PE conjugated antibody (Miltenyi 130-091-610)
- Anti-tryptase antibody (Santa Cruz Biotechnology sc32473)
- Goat polyclonal anti-c-kit antibody (R and D systems AF1356)
- Mouse IgM anti-α-sarcomeric actin antibody (Sigma A2127)
- Mouse IgG anti-smooth muscle actin antibody (Sigma A2547)
- Rabbit polyclonal anti-von Willebrand factor antibody (Dako A0082)
- Mouse monoclonal anti-α-actinin sarcomeric antibody (Sigma Clone EA-53, A7811)
- Rabbit polyclonal anti-Connexin 43 antibody (Sigma C6219)
- AlexaFluor 488 donkey anti-goat antibody (Jackson 705-545-147-JIR)
- AlexaFluor 594 donkey anti-mouse IgM antibody (Jackson 715-585-140-JIR)

- AlexaFluor 594 donkey anti-mouse IgG antibody (Jackson 715-585-151-JIR)
- AlexaFluor 488 donkey anti-rabbit antibody (Jackson 711-545-152-JIR)
- AlexaFluor 594 donkey anti-rabbit antibody (Jackson 711-585-152-JIR)
- Alexa Fluor 488 donkey anti-mouse IgG antibody (Jackson 715-545-150-JIR)
- RNeasy mini kit (Qiagen 74104)
- First Strand reverse transcription kit (Applied Biosystems 4368814)
- iQ SYBR green supermix (BioRad 170-8882)
- Primers (Sigma, custom-made)
- RNase, DNase-free water (Sigma W4502)

EQUIPMENT
Common to mouse and rat procedures
- Retrograde perfusion system, comprising
  Fluid pump (Cole-Parmer), Water heater and circulator (Thermo-Fisher), Glass fluid warming system (custom-made or PL3508B1-V, B3-V or B4-V from AD instruments) and Tubing (96410-16, Cole-Parmer)
- Eppendorf centrifuge (Eppendorf 5810 R)
- Eppendorf centrifuge (Eppendorf 5418)
- OctoMACS magnet and stand (Miltenyi)
- Haemocytometer (Hausser scientific)
- FACSCalibur II flow cytometer (Becton Dickinson)
- Humidified 37ºC, 5% CO_2 sterile incubator (HealForce HF90)

Only required for mouse eCSC isolation
- Cannulae for the Retrograde perfusion system: 1st cannula BB31695-PE/3, Scientific Commodities; 2nd cannula BB311-19, 19 gauge, Scientific Commodities; 3rd cannula – 06409-14; Cole Parmer
- Fine dissecting tools, including Vannas scissors (Fine Science Tools)
- EasySep magnet (Stem Cell Technologies)

Only required for rat eCSC isolation
- Cannulae for the retrograde perfusion system: 1/4-28 Threaded Adapter (5809-12, Ace Glass) inserted into L/S 16 tubing (Cole-Parmer, 96410-16)
- Dissecting tools (Fine Science Tools)

Only required if preparing cells for immunocytochemistry by cytospin
- Cytospin 4 (Thermo-Fisher)

Only required if analysing cells by real-time qPCR
- Nanodrop (Thermo-Fisher)
- iCycler (BioRad)
- MyIQ (BioRad)

REAGENT SET-UP

Incubation Medium for sorting mouse or rat cells
PBS without calcium or magnesium containing Bovine Serum Albumin
(5mg/ml), EDTA (2mM), Penicillin-Streptomycin (1% vol/vol), Fungizone
(0.1% vol/vol) and Gentamicin (0.1% vol/vol)

Prepare 50ml incubation medium, sterilise through a 0.22µm pore filter into
a sterile container and keep at 4ºC up to three weeks before the procedure.

Basic Buffer for isolating mouse cells
MEM (Joklik modification)
Sodium bicarbonate (2g/l)
HEPES (0.7mg/ml)
Taurine (1.25mg/ml)
Glutamine (0.3mg/ml)

Prepare 1000ml Basic Buffer, pH to 7.3 and sterilise through a 0.22µm pore
filter into a sterile container. This may be prepared up to three days before
tissue collection if kept sterile at 4ºC.

Collagenase Buffer for isolating mouse cells
Collagenase powder at a final concentration of 250U/ml in Basic Buffer.

Prepare 200ml collagenase solution and filter-sterilise as previously: this
volume is sufficient to perfuse three hearts or six if the collagenase is
collected after perfusion and re-cycled.
CRITICAL: This must be prepared shortly before tissue collection and the
solution kept chilled prior to use to minimise enzyme degradation.

BSA Incubation Buffer for isolating mouse cells
BSA 5mg/ml in Basic Buffer

Prepare 200ml BSA incubation buffer and filter-sterilise as previously: this
volume is sufficient to perfuse three hearts or six if the solution is collected
and re-cycled. This solution should be prepared the day before tissue
collection and stored at 4ºC prior to use.

Mouse eCSC Growth Medium
(as established in [4])
45% of the following:
 Dulbecco's Minimum Essential Medium-F12-HAMS
 Insulin-Transferrin-Selenium (1% vol/vol)
 Penicillin-Streptomycin (1% vol/vol)
 Fungizone (0.1% vol/vol)
 Gentamicin (0.1% vol/vol)
45% of the following:
 Neurobasal medium (supplement with 37mg L-glutamine per 500ml)
 B27 supplement (2% vol/vol)
 N2 supplement (1% vol/vol)
10% vol/vol Embryonic stem cell-qualified foetal bovine serum
Epidermal growth factor (20ng/ml)

Basal fibroblast growth factor (10ng/ml)
Leukemic inhibitory factor (10ng/ml)

Prepare 200ml growth medium, sterilise through a 0.22μm pore filter into a sterile container and keep at 4°C up to three weeks before the procedure.

Krebs-Henseleit (K-H) buffer for isolating rat cells
Sodium chloride: final concentration of 6.896g/l
Potassium chloride: final concentration of 0.35g/l
Magnesium sulphate: final concentration of 0.144g/l
Potassium phosphate: final concentration of 0.1635g/l
Sodium bicarbonate: final concentration of 2.1g/l
Dextrose: final concentration of 2.16g/l

Prepare 1000ml buffer, adding 50ml of concentrated stock solutions of sodium chloride, potassium chloride, magnesium sulphate, potassium phosphate and sodium bicarbonate to ddH$_2$O to a total volume of 1000ml, followed by addition of 2.16g of dextrose and filter-sterilise through a 0.22μm pore filter into a sterile container. This may be prepared and kept at 4°C up to one week before the procedure.

Wash medium for isolating rat cells
25ml K-H buffer
25ml DMEM
250μl Penicillin/Streptomycin

Prepare wash medium, sterilise through a 0.22μm pore filter into a sterile container and keep at 4°C up to one week before the procedure.

Collagenase and Hyaluronidase solution for isolating rat cells
150ml K-H buffer
Collagenase, type II: final concentration of 128U/ml
Hyaluronidase, type II: final concentration of 200U/ml

Prepare 150ml collagenase and hyaluronidase solution and sterilise through a 0.22μm pore filter into a sterile container. The volume is sufficient for perfusion of three rat hearts, but has to be collected and re-used over this time.
CRITICAL: This must be prepared shortly before tissue collection and the solution kept chilled prior to use to minimise enzyme degradation.

Rat eCSC Growth Medium
(as established in [4,31,32])
45% of the following:
      Dulbecco's Minimum Essential Medium-F12-HAMS
      Insulin-Transferrin-Selenium (1% vol/vol)
      Epidermal growth factor (final medium concentration: 20ng/ml)
      Basal fibroblast growth factor (final medium concentration: 10ng/ml)
      Leukemic inhibitory factor (final medium concentration: 10ng/ml)
45% of the following:

Neurobasal medium (supplement with 37mg L-glutamine per 500ml)
B27 supplement (2% vol/vol)
N2 supplement (1% vol/vol)
10% vol/vol Embryonic stem cell-qualified foetal calf serum
Penicillin-Streptomycin (1% vol/vol)
Fungizone (0.1% vol/vol)
Gentamicin (0.1% vol/vol)

Prepare 200ml growth medium, sterilise through a 0.22µm pore filter into a sterile container and keep at 4ºC up to three weeks before the procedure.

Incubation Medium for Cytospin or Flow Cytometry
PBS without calcium or magnesium
Bovine Serum Albumin (5mg/ml)
EDTA (2mM)
Penicillin-Streptomycin (1% vol/vol)
Fungizone (0.1% vol/vol)
Gentamicin (0.1% vol/vol)

Prepare 50ml incubation medium, sterilise through a 0.22µm pore filter into a sterile container and keep at 4ºC up to three weeks before the procedure.

Cardiomyogenic differentiation medium
MEM (alpha modification)
Foetal calf serum: final concentration of 2% vol/vol
Oxytocin acetate: final concentration of 100nM
Ascorbic acid: final concentration of 50µg/ml
Dexamethasone: final concentration of 1µM
Beta-glycerol phosphate: final concentration of 10mM
Penicillin/Streptomycin: final concentration of 1% vol/vol
Gentamicin: final concentration of 0.1% vol/vol
Fungizone: final concentration of 0.1% vol/vol
TGF-β1 (5ng/ml, days 1-4)
BMP-2 (10ng/ml, days 1-4)
BMP-4 (10ng/ml, days 1-4)
Dkk-1 (150ng/ml, days 5-14)

Prepare 200ml differentiation medium, sterilise through a 0.22µm pore filter into a sterile container and keep light-shielded at 4ºC up to three weeks before the procedure.

EQUIPMENT SET-UP
Perfusion system for mouse and rat heart perfusion
Our system consists of: flexible tubing run through a fluid pump, drawing up fluid (e.g. Basic Buffer) and propelling this through our custom-made glass tubing system for fluid heating, in which the fluid is heated to 37ºC by passing through coils inside chambers filled with an external water supply warmed to 37ºC (this external water temperature being regulated by the water bath). The warmed fluid then drips out via the tubing, with the terminal tube of a diameter which allows firm attachment of the cannulae.

See Figure 1 for a diagram of our system. Alternatively AD Instruments sell a perfusion system (cat. no. PL3508B1-V for mouse, B3-V or B4-V for rat).

PROCEDURE

1 For isolation of mouse eCSCs follow option **A**. For the isolation of rat eCSCs follow option **B**. See Table 2 for a summary of the differences between the mouse and rat protocols. See Table 3 for antibody concentrations.

(A) Isolating c-kit^{pos} eCSCs from the mouse heart

(i) Rinse perfusion system with 70% vol/vol ethanol for 10 minutes to sterilise and re-rinse with ddH$_2$O for 10 minutes to remove all traces of ethanol prior to use. Commence running the Basic Buffer through the system.

(ii) Inject 1000IU/kg heparin sulphate intraperitoneally into 6-week-old C57/BL6J mice and after ~10 minutes sacrifice the animal (we use an overdose of sodium pentobarbitone). Sterilise the anterior surface and dissect as follows: open skin on abdomen with midline incision and retract fur and skin; open abdominal cavity and make lateral incisions to inferior edge of ribcage; make vertical cuts along lateral border of ribcage then transversely across diaphragm to expose heart.

CAUTION: All experiments on mice must be performed in accordance with relevant guidelines and regulations.

CRITICAL STEP: If intending to isolate RNA directly from isolated eCSCs, it is advisable to omit heparin administration: in this instance rapid cannulation is even more vital.

CRITICAL STEP: *This and the following two steps must be carried out rapidly: depending on the number of operators and facilities available, it may be necessary to sacrifice mice one at a time. If the time elapsed from sacrifice to perfusion is longer than 10 minutes, the yield and quality of the isolated eCSCs will be severely negatively affected.*

(iii) Remove ribcage flap at superior end and discard; remove thymus with forceps to expose arch of aorta; thread an 8cm length of 5/0 suture behind the ascending aorta using fine forceps; hold innominate artery in place with fine forceps and cut part-way through aorta immediately distal to origin of innominate with Vannas scissors, producing opening over ascending aorta; insert fine-bore cannula (19 gauge) into opening and advance gently into ascending aorta, being sure to stop before passing through the aortic valve and entering the ventricular cavity; tie in place with 5/0 suture; grasp aorta over cannula tie with forceps and dissect heart out from thoracic cavity fully (see Figure 2a-f).

(iv) Place cannula into aperture of retrograde perfusion system (Figure 2g) with pump running at 10.2ml/min: equating to 3.4ml/min per cannula, and perfuse heart with Basic Buffer for 5 minutes or until all blood has been removed. If necessary, pierce apex with 25-gauge needle to allow pooled blood to be removed. Also check for leaks between the cannula and the aortic root. Re-do or tighten the suture connection if needed.

(v) Once blood is removed, perfuse with collagenase solution for 10-12 minutes to digest tissue. The heart should enlarge slightly and tissue will become pale and flaccid if perfusion is successful (Figure 2h).

(vi) Next perfuse heart with BSA solution for 5 minutes to stop collagenase reaction and prevent over-digestion. Depending on perfusion apparatus, it should be possible to perfuse 3-4 hearts simultaneously.

(vii) Once all hearts have been fully perfused, collect hearts and transfer to 25ml conical flask (3 hearts per flask, 5-10ml of BSA solution per flask) and chop into ~1-2mm pieces with fine sterile scissors.

(viii) Run suspension up and down through transfer pipette ten times to further break up tissue fragments.

(ix) Transfer suspension into 15ml centrifuge tube and centrifuge at 300g, brake 3, for 1 minute at room temperature (20-22°C) to separate suspension into myocyte and small cell fractions. If recovery and study of the cardiomyocyte fraction is desired, the cells within the pellet can be retrieved at this stage as previously described[4].

(x) Retain supernatant (containing small cell fraction) and pass through 40µm cell strainer into 50ml centrifuges tube. Next add BSA solution to give a total volume in the tube of 30ml per tube.

(xi) Centrifuge tube at 330g, brake 7 for 7 minutes at room temperature to pellet small cell fraction, discard supernatant and re-suspend pellet in 1ml incubation medium. Count an aliquot of the cells using a haemocytometer, prior to proceeding to the next steps (cell selection).

(xii) If desired, analyse the isolated cells (the cardiac small cell fraction) by flow cytometry to identify the percentage of cardiac small cells which express different stem cell surface markers of interest. We monitor expression of CD45 and c-kit (Figure 3a,b). Alternatively, proceed directly to the next step to purify the c-kit^{pos} CD45^{neg} eCSCs via magnetic cell sorting (steps **A (xiii)-(xv)**). If intending flow cytometry analysis of the selected eCSC population, retain an aliquot of unsorted cardiac small cells for the isotype control (Supplementary Figure 1a).

(xiii) Label the CD45-positive cells using the Miltenyi anti-mouse CD45 MicroBead kit, according to manufacturer's instructions. In brief, first re-suspend the cell fraction in incubation medium with the MicroBeads (volume of 20µl MicroBeads per 10^7 cells, cell number as ascertained in step **A (xiv)**)

and incubate for 15 minutes at 4ºC. Wash in incubation medium prior to centrifugation and re-suspension in incubation medium. Finally run the cell suspension through a MS magnetic sorting column placed in an OctoMACS magnet attached to its stand (Miltenyi), with a 30μm pre-separation filter (Miltenyi) fitted above the column. Run the suspension through the system drop-wise, followed by three washes with 500μl incubation medium to rinse through all unbound cells. Proceed to the next step to further purify the unlabelled cell fraction (CD45-negative fraction). Retain the positively-labelled cells for later flow cytometric analysis.

(xiv) Use the CD45-negative fraction (from step **A (xiii)**) with the StemCell Technologies EasySep anti-mouse CD117 kit to label the CD117-positive (c-kit^{pos}) fraction, according to manufacturer's instructions. In brief, first label the CD45-negative cell fraction with an anti-c-kit antibody conjugated to phycoerythrin (PE) for 15 minutes, followed by an anti-PE/anti-dextran secondary antibody complex for 15 minutes and finally with magnetic dextran-iron nanoparticles to tag the secondary antibody for 10 minutes (all incubations at room temperature). Following these incubations rinse the cells with 5ml of incubation medium and place inside the EasySep magnet for 5 minutes at room temperature. Empty the tube while still within the magnet, thus retaining all labelled cells. Rinse the cells and empty the magnet a total of five times. Finally remove the tube from the magnet.

(xv) Count the cells remaining in the tube (the c-kit^{pos} CD45^{neg} cell fraction) using a haemocytometer. Usually ~30,000 c-kit^{pos} CD45^{neg} cells are obtained from each mouse heart (~200 eCSCs per milligram of tissue). Use Trypan blue exclusion to ensure cell viability is >95%. At this stage flow cytometry analysis of the cell population purity can be carried out if desired (Figures 4 and 5). To proceed direct to characterisation, go straight to step **2**.

(xvi) If *in vitro* culture of the cells is required, re-suspend cells in mouse eCSC growth medium and plate onto vessels pre-coated with 1.5% wt/vol gelatin and incubate at 37ºC for 24 hours (see Table 4 for cell plating density; we usually plate the cells from six hearts onto three wells of a 6-well plate at this stage). If cell culture is not required, the procedure can proceed directly to characterisation (step **2**).

(xvii) Replace medium on cells after the first 24 hours in culture and then every 2-3 days. Passage the cells using standard trypsin passage when they reach ~70% confluence. The eCSCs can be maintained *in vitro* for at least 30 passages without loss of stem cell properties (see Anticipated Results section). Cell growth may be assisted by using medium previously conditioned by eCSCs *in vitro*, up to 50% of the total medium volume (mixed with fresh medium).

(B) Isolating c-kit^{pos} eCSCs from the rat heart

(i) Rinse perfusion system with 70% vol/vol ethanol for 10 minutes to sterilise, and re-rinse with ddH$_2$O for 10 minutes to remove all traces of ethanol prior to use. Commence running the K-H buffer through the system.

(ii) Inject 1000IU/kg heparin sulphate intraperitoneally into 8-week old Wistar rats, then after ~10 minutes sacrifice the animal and dissect as follows: open skin on abdomen with midline incision and retract fur and skin; open abdominal cavity and make lateral incisions to inferior edge of ribcage; make vertical cuts along lateral border of ribcage then transversely across diaphragm to expose heart.

CAUTION: All experiments on rats must be performed in accordance with relevant guidelines and regulations.

CRITICAL STEP: If intending to isolate RNA directly from isolated eCSCs, it is advisable to omit heparin administration: in this instance rapid cannulation is even more vital.

CRITICAL STEP: *This and the following two steps must be carried out rapidly: as for mice, it may be necessary to sacrifice rats one at a time. If the time elapsed from sacrifice to perfusion is longer than 10 minutes, the yield and quality of the isolated eCSCs will be severely negatively affected.*

(iii) Remove ribcage flap at superior end and discard; remove thymus with forceps to expose arch of aorta and dissect out heart fully, ensuring that at least 3mm of aorta stump is removed along with the heart.

(iv) Lift heart using forceps placed on walls of aorta stump, then lift onto cannula, with aortic walls around the cannula end (do not enter the ventricular cavity). Secure and tie in place using 4/0 suture thread (see Figure 6a-c) and perfuse heart (pump running at 20.4rpm: equating to 6.8ml/min per cannula) with K-H buffer for 5 minutes or until all blood has been removed. If necessary, pierce apex with 21-gauge needle to allow pooled blood to be removed. As for the mouse, check for leaks between the cannula and the aortic root. Re-do or tighten the suture connection if needed.

(v) While K-H buffer rinsing is ongoing, with great care place forceps inside heart chambers via openings of great vessels and open and close slightly to dislodge pooled blood and aid perfusion.

(vi) Once all blood is removed, perfuse with collagenase/hyaluronidase solution for 15 minutes to digest tissue. The heart should enlarge slightly and tissue will become pale and flaccid if perfusion is successful. As with the mouse, it should be possible depending on perfusion apparatus to perfuse three hearts simultaneously (Figure 6d).

(vii) Remove heart(s) from cannula and place in sterile container with lid with 15ml of collagenase/hyaluronidase solution and cut into pieces (~5mm in size).

(viii) Add 15µl of 1M calcium chloride (only if interested in collecting the cardiomyocytes), trypsin (to a final concentration of 20µg/ml) and DNase (to

a final concentration of 20µg/ml) to the 15ml collagenase/hyaluronidase tissue suspension.

(ix) Place tissue suspension in shaking water bath at 150rpm at 37°C for 40 minutes, to dissociate tissue.

(x) Pipette suspension up and down through a 25ml pipette ten times to fully break up tissue fragments and run suspension through a cell strainer (100µm filter pore size) into a 50ml centrifuge tube. Rinse suspension container with 40ml of wash medium and add solution (through the cell strainer) to the cell suspension and a second 50ml centrifuge tube, until there are equal volumes in each tube.

(xi) Centrifuge both tubes at 50g, brake 3 for 3 minutes at room temperature, collecting supernatant (containing small cell fraction), and run through cell strainer (40µm filter pore size) into two new centrifuge tubes. At this step the pellet of cardiomyocytes can be re-suspended and run through a BSA gradient to separate viable rod-shaped cardiomyocytes if required, as previously described [32].

(xii) Centrifuge at 400g, brake 7 for 7 minutes at room temperature and discard supernatant, combining both small cell pellets in incubation medium.

(xiii) Remove subcellular debris by applying the cell suspension carefully above 8ml of Optiprep/DMEM mixture (with a density of 1.09/ml) and centrifuge at 800g for 15 minutes at room temperature. Intact cells (cardiac small cell fraction) should pellet at the bottom of the Optiprep/DMEM layer. Count cells using a haemocytometer.

(xiv) As with the mouse, if desired analyse the cardiac small cell fraction using flow cytometry, to identify the percentage of cells which express different stem cell surface markers of interest. In particular, we examine the expression of CD45 and c-kit (Figure 7a,b). Alternatively, proceed directly to the next step to purify the c-kit^{pos} CD45^{neg} eCSCs by magnetic cell sorting (steps **B (xv)** to **(xviii)**). If intending flow cytometry analysis of the selected eCSC population, retain an aliquot of unsorted cardiac small cells for the isotype control (Supplementary Figure 1c).

(xv) Incubate cardiac small cells (from step **xiv**) with anti-rat CD45 antibody (Santa Cruz), applied at a concentration of 1µg per million cells in incubation medium (to a total volume of 1ml) for 15 minutes at 4°C and centrifuge at 300g, brake 7 for 7 minutes at 4°C, discarding supernatant. At this point mast cells can be identified by immunostaining for tryptase (Figure 7c), if desired.

(xvi) Incubate cells with Miltenyi rat anti-mouse IgG MicroBeads, according to manufacturer's instructions. In brief, label the re-suspended cell fraction in incubation medium with the MicroBeads for 15 minutes at 4°C, wash in incubation medium, and then pellet by centrifugation and re-suspend in

incubation medium. Run the cell suspension drop-wise through a magnetic sorting MS column placed in an OctoMACS magnet attached to its stand (Miltenyi), with a 30μm pre-separation filter (Miltenyi) fitted above the column. Wash the column three times with 500μl incubation medium to rinse through all unbound cells. Collect the unlabelled cell fraction and retain the positively-labelled cells for later flow cytometric analysis.

(xvii) Incubate the unlabelled cell fraction from step **(xvi)** (CD45-negative cells) with anti-c-kit antibody (Santa Cruz) for 30 minutes in incubation medium (to a total volume of 1ml) at 4°C, centrifuge at 300g, brake 7 for 7 minutes at 4°C, discarding supernatant.

(xviii) Incubate cells with Miltenyi goat anti-rabbit MicroBeads and perform c-kit positive selection with Miltenyi MS columns, OctoMACS and pre-separation filters as described in step **xvi**, but this time collecting the positively labelled cell fraction by removing the magnetic column from OctoMACS apparatus and flushing with 1ml incubation medium. Retain the negatively-labelled cells for later flow cytometric analysis.

(xix) Count the c-kit^{pos} CD45^{neg} cell fraction using a haemocytometer. We usually obtain ~150,000 c-kit^{pos} CD45^{neg} positive cells from each rat heart (~170 eCSCs per milligram of tissue). Use Trypan blue exclusion to ensure cell viability is >95%. At this stage, flow cytometry analysis of the cell population purity can be carried out (Figures 8 and 9).

(xx) If *in vitro* culture of the cells is required, re-suspend cells in rat eCSC growth medium and plate on plates pre-coated with 1.5% wt/vol gelatin (see Table 4 for cell plating density). If cell culture is not required, the procedure can proceed directly to characterisation (step **2**).

(xxi) Allow cells to multiply, feeding after 24 hours in culture and then every 2-3 days, passaging when cells reach ~70% confluence. As for mouse eCSCs, cell growth may be assisted by using medium previously conditioned by eCSCs *in vitro*, up to 50% of the total medium volume (mixed with fresh medium).

Characterisation of eCSCs

2 eCSCs can be further studied in a number of ways. Individual eCSCs can be characterised functionally by clonogenic assay (option **A**), used for the CardioStem sphere formation assay (option **B**) or CardioStem spheres used to commence the cardiomyogenic beating assay (option **C**). To perform immunocytochemistry before or in the absence of functional characterisation on either individual eCSCs or CardioStem spheres, first fix cells using a cytospin (option **D**). To perform immunocytochemistry after functional characterisation on either individual eCSCs or CardioStem spheres, culture and fix in a slide (option **E**). Following options **D** or **E**, immunocytochemistry (option **F**) can be performed on either individual

eCSCs or CardioStem spheres. Individual eCSCs, CardioStem Spheres or differentiated progeny can be analysed using real-time qPCR (option **G**).

(A) Clonogenicity assay

(i) Place single c-kit^{pos} CD45^{neg} cells into each well of a 96-well 1.5% wt/vol gelatin coated Terasaki plates with 100µl of eCSC growth medium per well.

(ii) Incubate the cells and add an extra 20µl of growth medium to each well every three days until 9 days after plating.

(iii) On days 3, 6 and 9 count the number of clones generated in each plate and calculate the percentage of single cells plated per plate that have generated clonal colonies.

(B) CardioStem Sphere formation

CRITICAL Cells must be established in culture for at least one passage (have gone through steps **A (xvi-xvii)** for mouse cells and steps **B (xx-xxi)** for rat cells) prior to commencing this assay.

(i) Grow ~40,000 eCSCs in suspension in 5ml of LIF-deprived eCSC growth medium in a 10cm bacteriological dish (to minimise eCSC surface adherence).

(ii) Add 2ml of LIF-deprived eCSC growth medium to each plate every 2-3 days.

(iii) Count the number of CardioStem Spheres per plate and express as a percentage relative to the number of eCSCs plated. The first CardioStem Spheres have usually formed by 3-4 days.

(C) Cardiomyogenic beating assay

(i) Incubate cloned c-kit^{pos} CD45^{neg} eCSCs (clones obtained using option **A**) with 100nM Oxytocin for 72 hours.

(ii) Transfer ~40,000 eCSCs to 5ml of LIF deprived eCSC growth medium in a 10cm bacteriological dish (to minimise eCSC surface adherence) and incubate cells.

(iii) Add 2ml of LIF deprived eCSC growth medium to each plate every 2-3 days until CardioStem spheres form (usually within 4 days).

(iv) Transfer CardioStem Spheres to laminin (1µg/ml) coated dishes or chamber slides (for later immunostaining) and incubate with cardiomyogenic differentiation medium.

(v) Specific growth factors are added at specified time points, for up to 14 days (days 1 to 4: 10ng/ml BMP-2, 10ng/ml BMP-4, 5ng/ml TGF-ß1; days 5 to 14: 150ng/ml Dkk-1).

(vi) Count the number of beating CardioStem Spheres in each dish. Calculate the percentage of beating CardioStem Spheres relative to the total number of CardioStem Spheres/clusters in each dish.

(D) Preparation of cells for immunocytochemistry using a Cytospin.

(i) Collect cells by centrifugation at 300g for 5 minutes (using eCSCs either freshly isolated or from culture) and resuspend in incubation medium at a cell density of 200,000 cells/ml.

(ii) Pre-label the Polyprep slides in pencil (do not use ink as slides are rinsed in ethanol subsequently), place in Cytofunnels and load into Cytospin 4.

(iii) Load 200µl of cell suspension into each cuvette and spin at 800rpm for 3 minutes at MEDIUM acceleration.

(iv) Extract slide from Cytofunnel apparatus, circle cell 'spots' with ImmEdge pen and fix with Cell-Fixx spray.

(v) Allow slides to air dry for 30 minutes and store until required for immunocytochemistry.
PAUSEPOINT: Slides are now fixed and wax-coated, so can be stored at room temperature for extended periods (up to at least two years if necessary).

(vi) Remove wax coating from slides by immersing in 95% vol/vol ethanol solution for 15 minutes. Proceed with immunocytochemistry procedure (option **F**).

(E) Preparation of cells for immunocytochemistry using chamber slide culture.

(i) Coat 2- or 4-well chamber slides with laminin dissolved in DMEM (to a final concentration of 1µg/ml) for 1-2 hours. Rinse in PBS immediately prior to proceeding to step **(ii)**.

(ii) Plate cells or CardioStem Spheres (from option **B** step **iii**; 2 to 4 spheres per well). Incubate cells in LIF deprived eCSC growth medium for 7 days to assay multipotency. Alternatively incubate up to 14 days in cardiomyogenic differentiation medium to assay beating cells. Replace with fresh medium and growth factors for both assays every 3 days.

(iii) At desired end-point of experiment, rinse cells once with PBS and fix by applying 4% vol/vol formaldehyde (vol/vol in PBS) for 20 minutes on ice.

(iv) Aspirate formaldehyde and proceed to option **F** (immunocytochemistry).

PAUSE POINT: Fixed cells may be rinsed three times in PBS and stored at 4°C for up to four days before commencing option **F** (immunocytochemistry).

(F) Immunocytochemistry.

(i) Wash slides three times in 0.1% vol/vol Tween in PBS for 5 minutes on orbital shaker.

(ii) Block non-specific binding sites on cells with 10% vol/vol donkey serum at room temperature (all secondary antibodies used are raised in donkey).

(iii) Incubate overnight in a humidified chamber with primary antibody diluted in 0.1% vol/vol Tween in PBS at 4°C.

(iv) Wash slides three times in 0.1% vol/vol Tween in PBS for 5 minutes each on orbital shaker.

(v) Incubate with secondary antibody in light-shielded, humidified chamber for 1 hour at 37°C.

(vi) Wash slides three times in 0.1% vol/vol Tween in PBS for 5 minutes each on orbital shaker (shielded from light).

(vii) Incubate with 4',6-diamidino-2-phenylindole (DAPI, 1ng/ml in PBS) for 14 minutes at room temperature (shielded from light).

(viii) Wash slides three times in PBS for 5 minutes each on orbital shaker.

(ix) Mount slides in Vectashield (for chamber slides, chamber wells must be removed immediately prior to this step).

(x) Proceed to confocal microscopy imaging.

(G) Real-time quantitative PCR

(i) Collect cells in pellet (at least 500,000 cells or 5,000 CardioStem Spheres) and wash once with PBS then centrifuge at 300g for 5 minutes, aspirating supernatant.

(ii) Isolate RNA using RNeasy mini kit (Qiagen): briefly, cells are lysed in 700μl RLT lysis buffer then passed through a QIAshredder column by centrifuge at 8000g for 15 seconds; the resultant lysate is mixed with an equal volume of 70% molecular biology-grade ethanol and bound to an RNeasy elution column by centrifuge at 8000g for 15 seconds. Remove any residual genomic DNA with DNase (BioRad), then wash the column and elute the RNA fraction in 40μl of RNase, DNase-free water at 8000g for 1 minute.

(iii) Check RNA quality by analysing with a Nanodrop spectrophotometer (Thermo Fisher). Proceed only if RNA purity sufficient (260nm/280nm ratio 1.9-2.1; 260nm/230nm ratio >1.7).
PAUSE POINT: At this stage, eluted RNA can be stored at -80ºC for up to several months, although it will very gradually degrade over this time.

(iv) Use isolated RNA as template for reverse transcription with First Strand kit (Applied Biosystems) to produce cDNA for real-time qPCR analysis. Briefly, up to 1µg of RNA is used in a 50µl reaction, using random hexamers for cDNA synthesis: heat at 48ºC for 30 minutes, followed by 95ºC for 5 minutes to terminate reaction.
PAUSE POINT: At this stage, cDNA can be stored at -80ºC for several months until required.

(v) Prepare PCR plate (BioRad) with SYBR green supermix (BioRad) and primer pairs used at 300nM (Sigma, custom-made), then add cDNA.

(vi) Place in iCycler thermal cycler, with real-time PCR detection by MyIQ (both BioRad), then commence thermal cycle: 95ºC for 5 minutes, followed by 40 cycles of (95ºC for 15 seconds, 60ºC for 30 seconds, 72ºC for 30 seconds with PCR detection) then carry out a melting curve and cool to 4ºC.
PAUSE POINT: At this stage, PCR products can be stored at -20ºC for several months until required.

(vii) Examine primer products sizes by electrophoresis to confirm specificity if required.

TIMING

Step **1A (i)** takes ~25 minutes; steps **1A (ii)**-**(vi)** take ~1 hour (assuming three hearts are processed); steps **1A (vii)**-**(xi)** take ~25 minutes; steps **1A (xii)**-**(xvii)** take ~2 hours. Total length of protocol from start to finish is ~4 hours.

Step **1B (i)** takes ~25 minutes; steps **1B (ii)**-**(vii)** take ~1 hour (assuming three hearts are processed, for one heart ~20 minutes); steps **1B (viii)**-**(xiv)** take ~70 minutes; steps **1B (xv)**-**(xxi)** take approximately 2 hours. Total length of protocol from start to finish is ~4.5 hours.

The clonogenicity assay (step **2** option **A**) takes up to 9 days to complete. The generation of CardioStem Spheres (step **2** option **B**) requires up to 6 days. The cardiomyogenic differentiation of eCSCs *in vitro* (step **2** option **C**) requires up to 20 days (3 days oxytocin pre-treatment, ~3 days to grow CardioStem Spheres, 14 days of differentiation in defined stage-specific medium). The CytoSpin procedure (step **2** option **D**) takes ~35 minutes to complete. The chamber slide preparation procedure (step **2** option **E**) takes ~7 days (to assay multipotency) or 20 days (for cardiomyogenic differentiation) to complete. The immunocytochemistry procedure (step **2** option **F**) requires ~5 hours' to complete working around an overnight

incubation for the primary antibody. Real-time qPCR analysis (step **2** option **G**) takes approximately 6 hours to complete.

TROUBLESHOOTING

A guide for troubleshooting problems encountered during this protocol is provided in Table 5.

ANTICIPATED RESULTS

Isolating c-kit^{pos} eCSCs from the mouse heart

Flow cytometric analysis of the c-kit and CD45 expression of cardiac small cells from the adult mouse heart demonstrates that in the unsorted cardiac small cell population ~10% of small cells present in the adult mouse heart are c-kit-positive, CD45-positive (Figure 3a,b), and Tryp-positive. These cells are cardiac mast cells [6,33]. Among the cardiac small cell population typically ~3% are c-kit^{pos} CD45^{neg} and Tryp^{neg} eCSCs (Figure 3b).
To confirm the efficacy of the magnetic cell sorting system, we incubated sorted cells from step **1A (xii)** with fluorochrome-conjugated antibodies and analysed them using flow cytometry. The removal of CD45-positive cells is completely effective, with the CD45-positive signal (labelled with fluorescein isothiocyanate, FITC) being removed entirely by negative selection of this cell population with magnetic activated cell sorting (Figure 4a-c). Similarly, a strongly enriched population of cells for c-kit is provided by magnetic selection for this population (Figure 5a-c). By dual staining, ~93% of cells are c-kit^{pos} CD45^{neg} following magnetic selection for these markers (Figure 5d,e). As the c-kit^{pos} CD45^{neg} eCSCs have been sorted using a PE-conjugated antibody, c-kit-unsorted cardiac small cells are used as a PE isotype control, as these cells have a comparable FSC/SSC distribution to the eCSC population (Supplementary Figure 1).

Isolating c-kit^{pos} eCSCs from the rat heart

Flow cytometric analysis of c-kit and CD45 expression in cardiac small cells obtained at step **1B (xiv)** from the adult rat heart demonstrates that in the unsorted cardiac small cell population, ~14% of the small cells present in the adult heart are c-kit-positive, CD45-positive (Figure 7a,b), and Tryp-positive. These cells are cardiac mast cells (Figure 7c). Among the cardiac small cell population, typically ~4% are c-kit^{pos} CD45^{neg} Tryp^{neg} eCSCs (Figure 7b).

To confirm the efficacy of the magnetic cell sorting system, we incubated sorted cells with fluorochrome-conjugated antibodies and analysed using flow cytometry. As for the mouse, the removal of CD45-positive cells is effective, with the CD45-positive signal (labelled with FITC), being 83% in the positive selection, and 3% in the negative selection (Figure 8a-c). Similarly, a strongly enriched population of cells for c-kit is provided by magnetic selection for this population (Figure 9a-c). We note that the c-

kit[pos] eCSC population expresses varying levels of c-kit (Figure 9c), which may reflect different levels of potency or the clipping of a fraction of c-kit by the proteolytic digestion step. The cause of this heterogeneity of detectable c-kit remains to be determined. By dual staining, ~86% of cells are c-kit^{pos} CD45^{neg} Tryp^{neg} following magnetic selection for these markers (Figure 9d,e), with complete absence of the mast cell marker tryptase (Figure 9f).

Characterisation of eCSCs

Flow cytometric analysis of freshly isolated c-kit^{pos} CD45^{neg} eCSCs for a panel of surface markers reveals a phenotype which is ~50% positive for Sca-1 and CD90 (Figure 10a,b); ~60% positive for CD105 and CD140a (PDGFrα) (Figure 10c,d); ~65% positive for CD166 (Figure 10e); and negative for CD31 and CD34 (Figure 10f,g).

The c-kit^{pos} CD45^{neg} eCSC populations derived from both mouse and rat adult hearts can be grown in culture beyond the population doubling limit of somatic cells (i.e. >40 passages over a period of months). Clonal populations of mouse and rat c-kit^{pos} CD45^{neg} eCSCs have a population doubling time of ~15 hours (Figure 11a), and can be characterised by flow cytometry for appropriate eCSC surface marker expression (Figure 11b).

The c-kit^{pos} CD45^{neg} eCSCs from the mouse and rat heart are self-renewing, maintaining stemness transcript expression over 30 consecutive culture passages (Figure 11c). Mouse and rat c-kit^{pos} CD45^{neg} eCSCs at passage 4 have the ability to generate single cell-derived clonal colonies at a rate of ~30% (Figure 11d), and also generate CardioStem Spheres when grown in suspension (Figure 11e).

Depending on the cell types to be immunostained, either Cytospin preparations (for eCSCs) or chamber slides (for differentiated cells) are used. These methods allow successful immunocytochemical characterisation of c-kit^{pos} CD45^{neg} eCSCs (Figure 12a) and c-kit^{pos} CD45^{neg} eCSCs that have been differentiated into the 3 main cardiac lineages (Figure 12b-d; Figure 13). The generation of the three lineages is achieved by generating CardioStem Spheres, which are then plated in laminin-coated chamber slides. CardioStem Spheres are similar to neurospheres [34] and cardiospheres [14], with the difference that they are constituted of eCSCs instead of mixed small cells from the originating tissue. Once attached, cells from the CardioStem Spheres spontaneously differentiate into cells expressing markers of all 3 cardiac lineages; cardiomyocytes (α-sarcomeric actin: Figure 12b), smooth muscle (smooth muscle actin, SMA: Figure 12c) and endothelial (von Willebrand factor, vWF: Figure 12d) cells [2].

This protocol can also be used to specify rat c-kit^{pos} CD45^{neg} eCSCs into functional, contracting cardiomyocytes *in vitro*. Oxytocin and specific growth factors governing embryonic cardiogenesis, given in a stage/sequence-specific manner (Figure 13a), produced contractile cardiomyocytes derived from cloned c-kit^{pos} CD45^{neg} eCSCs, as previously

shown [35]. This CardioStem Sphere-beating assay is similar to the protocol used to assess cardiomyocyte differentiation in embryoid bodies [36]. Cloned c-kit^{pos} CD45^{neg} eCSCs are treated with 100nM Oxytocin for 72 hours before transfer to bacteriological dishes for the generation of CardioStem Spheres (Figure 13a). Spheres grown in suspension are picked and plated in laminin-coated chamber slides or dishes. Through trial and error we identified and demonstrated that supplementation of BMP-2, BMP-4, TGF-ß1 and Dkk-1 for 4 days increased the number of cardiac troponin I expressing cells to ~40% (Figure 13b). However, with removal of TGF- ß1, BMP2, and BMP4 at day 4, and supplementing the medium with Dkk-1 for the remaining 10 days, the cardiomyocyte differentiation increased to ~70% cTnI positive cells (Figure 13a,b). The cardiomyocyte cells exhibit abundant, well-organized sarcomere structures (Figure 13c) and functional synchronized rhythmic beating (Supplementary Video 1), which is stable and maintained for the duration of the culture. These cardiomyocytes behave like a syncytium connected through Cnx43-containing gap junctions (Figure 13c). A similar beating phenotype is exhibited by isolated cells when the sphere is disaggregated and cells individually plated (Supplementary Video 2). Real-time-qPCR of differentiated CardioStem Spheres at 14 days of culture in the cardiomyogenic cocktail showed a progressive decrease in transcripts for stemness and concomitant up-regulation of cardiomyocyte-specific transcription factors and genes coding for sarcomeric proteins (Figure 13d). This procedure was carried out using the primers and protocol as previously published by our group [4].

The identification, isolation and propagation of a population of tissue-specific stem cells resident in the adult heart open significant possibilities for a potential therapeutic option. Early trials of cardiac repair and regeneration via transplantation of bone marrow-derived stem/progenitor cells have given mixed and modest results (for review see [37]), indicating that a better understanding of the relevant cell biology is required to provide a consistently effective therapeutic option for stem cell-based regenerative treatments in the heart. The presence of resident eCSCs in the adult heart, including human, has been determined by several groups [2,4,6,8-10,12,14,17,38-42]. These cells can be isolated and propagated *in vitro* to produce large numbers of cells without reaching growth arrest or their being a change in cell characteristic. The cells are multipotent *in vitro* and *in vivo* [2,6,14]. Therefore, these cells could be a source of a potential regenerative agent. The recent publication of the results of the SCIPIO and CADUCEUS trials [43,44] provide further evidence of the potential therapeutic capabilities of these cells, demonstrating a therapeutic action of autologous eCSC infusion in patients following myocardial infarction. Another possible use for purified eCSCs is in drug test assays and to obtain patient-specific models of disease via cardiac progenitor cells derived from eCSCs in the manner achieved with human induced pluripotent stem cells [45]. Thus this protocol enables the effective isolation and characterisation of the c-kit^{pos} CD45^{neg} eCSC population in the adult mouse and rat heart.

AUTHOR CONTRIBUTIONS

COMPETING INTERESTS STATEMENT

We confirm that the authors have no competing financial interests to declare.

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FIGURE LEGENDS

Figure 1. Perfusion apparatus and system. (a) Perfusion apparatus and system. (b) Close-up of custom-made glass tubing.

Figure 2. Diagrammatic representation of the steps of a mouse aorta cannulation procedure and heart hanging on the perfusion system. Diagrammatic representation of the steps of a mouse aorta cannulation procedure: (a) expose the heart and pass 5/0 suture thread behind the aorta, tying a very loose knot (b) grasp the right innominate artery with fine forceps (c) make a vertical cut into the bifurcation with Vannas scissors (d) advance the 19-gauge cannula into the opening (e) advance cannula carefully into aortic lumen, without passing through the aortic valve (f) tie suture thread with two knots, fixing the cannula in the aorta. (g) Heart hanging on the perfusion system (not yet perfused). (h) Perfused heart hanging on the system. These experiments were carried out in accordance with the regulations of the U.K. Home Office and the institutions' regulatory boards.

Figure 3. Flow cytometric analysis of unsorted mouse cardiac small cells. (a) Isotype control. (b) Cell population dual stained for CD45-FITC and c-kit-PE, showing CD45 positive, c-kit negative cells (pink), c-kit positive, CD45 positive cells (blue) and c-kit positive, CD45 negative eCSCs (green).

Figure 4. Flow cytometric analysis of CD45 positive and negative sorted fractions shows mouse cardiac small cells are completely depleted of CD45-positive cells by magnetic sorting. (a) Isotype control. (b) Cell population negatively selected for CD45 and stained with CD45-FITC. (c) Cell population positively selected for CD45 and stained with CD45-FITC. Negative fluorescent signal is indicated by black, positive signal by red and the percentage of positively-labelled cells indicated.

Figure 5. Flow cytometric analysis shows magnetic activated cell sorting of the CD45 negative selection for c-kit, yields a high purity of c-kit[pos] eCSCs from the mouse heart. (a) Isotype control. (b) Cell population negatively selected for c-kit, and stained with c-kit-PE. (c) Cell population positively selected for c-kit and stained with c-kit-PE. Negative fluorescent signal is indicated by black, positive signal by red. (d and e) Cell population dual stained for CD45-FITC and c-kit-PE, showing high purity of c-kit^{pos} CD45^{neg} eCSCs isolated from the mouse heart.

Figure 6. Diagrammatic representation of the steps of a rat aorta cannulation procedure and the hearts hanging on the perfusion system. Diagrammatic representation of the steps of a rat aorta cannulation procedure: (a) advance the aorta around the cannula and hold in place with forceps, keeping the end of the cannula above the aortic valve (b) lower the

loosely-tied 4/0 suture thread over the aorta (c) tie the suture thread tightly in two knots, securing the aorta. (d) Three perfused hearts hanging on the perfusion system. These experiments were carried out in accordance with the regulations of the U.K. Home Office and the institutions' regulatory boards.

Figure 7. Analysis of unsorted rat cardiac small cells. (a, b) Flow cytometric analysis. (a) Isotype control. (b) Cell population dual stained for CD45-FITC and c-kit- AlexaFluor594, showing CD45 positive, c-kit negative cells (pink), c-kit positive, CD45 positive eCSCs (blue) and c-kit positive, CD45 negative eCSCs (green). (c) Immunocytochemical staining for tryptase positive in CD45-positive, c-kit-positive cells. Bar = 20µm.

Figure 8. Flow cytometric analysis of CD45 positive and negative sorted fractions shows rat cardiac small cells are completely depleted of CD45-positive cells by magnetic sorting. (a) Isotype control. (b) Cell population negatively selected for CD45 and stained with CD45-FITC. (c) Cell population positively selected for CD45 and stained with CD45-FITC. Negative fluorescent signal is indicated by black, positive signal by red and the percentage of positively-labelled cells indicated.

Figure 9. Analysis shows magnetic activated cell sorting of the CD45 negative selection for c-kit yields a high purity of c-kit[pos] eCSCs from the rat heart. (a-e) Flow cytometric analysis. (a) Isotype control. (b) Cell population negatively selected for c-kit, and stained with c-kit-AlexaFluor488. (c) Cell population positively selected for c-kit and stained with c-kit-AlexaFluor488. Negative fluorescent signal is indicated by black, positive signal by red. (d and e) Cell population dual stained for CD45-FITC and c-kit-AlexaFluor594, showing high purity of c-kit^{pos} CD45^{neg} eCSCs isolated from the rat heart. (f) Immunocytochemical staining for tryptase negative in CD45-negative, c-kit-positive cells. Bar = 20µm.

Figure 10. Flow cytometric characterisation of freshly isolated c-kit^{pos} CD45^{neg} mouse eCSCs. Expression of Sca-1 (a), CD90 (b), CD105 (c), CD140a (d), CD166 (e), CD31 (f) and CD34 (g) on freshly isolated c-kit^{pos} CD45^{neg} eCSCs. (h) Isotype controls. Negative fluorescent signal is indicated by black, positive signal by red and the percentage of positively-labelled cells indicated.

Figure 11. Isolated c-kit^{pos} CD45^{neg} eCSCs from both mouse and rat are clonogenic, self-renewing and form CardioStem Spheres. (a) Clonogenicity of c-kit^{pos} eCSCs. Bar = 50µm. (b) Characterisation by flow cytometry of a single-cell derived c-kit^{pos} CD45^{neg} clonal population. (c) Population doubling time for clonal eCSCs. (d) Self-renewal as determined by the expression of stemness transcripts of clones over multiple passages. (e) CardioStem Sphere formation. Bar = 200µm. Data in each panel are Mean ± SD of 3 independent experiments.

Figure 12. Immunocytochemistry of c-kit^{pos} CD45^{neg} eCSCs and their multipotency. (a) Cytospin preparation and immunostained clonal c-kit^{pos}

(green, c-kit; blue, DAPI) rat eCSCs. Clonal c-kit^{pos} CardioStem Sphere cells are multipotent, differentiating into the 3 main cardiac lineages: (b) cardiomyocytes (red, alpha-sarcomeric actin; blue, DAPI), (c) smooth muscle cells (red, smooth muscle actin; blue, DAPI) and (d) endothelial cells (green, von Willebrand factor; blue, DAPI). Bars = 50µm.

Figure 13. Differentiation of c-kit^{pos} CD45^{neg} eCSCs into functional beating cardiomyocytes *in vitro*. (a) Schematic timeline of the stage-specific protocol used for the cardiomyogenic differentiation of c-kit^{pos} CardioStem Spheres. (b) Frequency of cTnI-positive cells and percentage of beating cells (hatched bars) after manipulation of the TGF-ß/Wnt signalling pathways, determined by confocal microscopy and impartial observer counting, as indicated. *P<0.05 *vs.* all. (c) At days 8-14, CardioStem Sphere cells stain positive for the cardiomyocyte lineage (S-Actinin, green), exhibiting sarcomeric structures (z lines and dots) and gap junction formation (Cnx-43, red) between cells. Bar = 50µm. (d) Real-time-qPCR analysis and PCR products following the stage-specific cardiomyocyte differentiation protocol, revealed the change in transcripts for c-kit, Tert, Nkx2.5, GATA-4, ß-MHC and cTnI in the differentiated CardioStem Sphere cells, relative to 0 days (undifferentiated cells). Data are Mean ± SD of 3 assays.

SUPPLEMENTARY FIGURES AND VIDEOS

Supplementary Figure 1. Flow cytometric analysis: Forward scatter/side scatter gating. (a) Unsorted mouse cardiac small cells. (b) Mouse eCSCs. (c) Unsorted rat cardiac small cells. (d) Rat eCSCs.

Supplementary Video 1. Cardiomyocyte cells exhibit functional synchronized rhythmic beating. The cardiomyocyte cells derived from eCSCs exhibit functional synchronized rhythmic beating which is stable and maintained for the duration of the culture.

Supplementary Video 2. Individual CardioStem Sphere-derived cells maintain rhythmic beating phenotype. Individually-plated cardiomyocyte cells derived from CardioStem Spheres following sphere disaggregation maintain a rhythmic beating phenotype in culture.

TABLES

Table 1. Summary of eCSC populations.

Phenotype	Markers	Source
c-kit^{pos} eCSCs	CD34^{neg}, CD45^{neg}, Sca-1^{pos}, Abcg2^{pos}, CD105^{pos}, CD166^{pos}, GATA4^{pos}, NKX2-5$^{pos/neg \text{ or } low}$, MEF2C^{pos}	Mouse, rat, dog, pig, human
Sca-1^{pos} eCSCs	CD34^{neg}, CD45^{neg}, FLK1^{neg}, ckit$^{pos/neg \text{ or } low}$, GATA4^{pos}, NKX2-5$^{pos/neg \text{ or } low}$, MEF2C^{pos}	Mouse, human

SP cells	CD34^{pos}, CD45^{pos}, Abcg2^{pos}, Sca1^{pos}, c-kit^{pos}, NKX2-5^{neg}, GATA4^{neg}	Mouse
Cardiosphere-derived cells	CD105^{pos}, CD34^{pos}, CD45^{pos}, Abcg2^{pos}, Sca1^{pos}, c-kit^{low}	Mouse, rat, dog, pig, human
cCFUFs	Sca-1^{pos}, PDGFRα^{neg}, CD31^{neg}, c-kit^{low}, CD45^{neg}, FLK1^{neg}, CD44^{pos}, CD90^{pos}, CD29^{pos}, and CD105^{pos}	Mouse
Cardiac mesangioblasts	CD31^{pos}, CD34^{pos}, CD44^{pos}, CD45^{neg}, Sca1^{pos}, c-kit^{pos}	Mouse, human
Isl1^{pos} CPCs	CD31^{neg}, Sca1^{neg}, c-kit^{neg}, GATA4^{pos}, NKX2-5^{pos}	Mouse, rat, human

Table 2. Summary of differences between mouse and rat eCSC isolation protocols.

Stage	Mouse	Rat
Cannulation (steps A (iii), B (iii))	Cannula inserted under microscope before heart removal from chest cavity	Heart removed from chest cavity and aortic stump placed over cannula
Perfusion (steps A (iv-vi), B (iv-vi))	Wash out blood with Basic Buffer; 10-12 minutes' collagenase; 5 minutes' BSA solution	Wash out blood with K-H buffer; 15 minutes' collagenase and hyaluronidase
Tissue disaggregation (steps A (vii, viii), B (vii-x))	Cut into ~1mm fragments and triturated in BSA solution	Chopped into ~5mm fragments; digested for 30-40 minutes
Small cell separation (steps A (ix-xi), B (xi-xiii))	Centrifuge: 300g/brake 3, 1 minute; Pellet small cells: 330g/brake 7, 7 minutes	Centrifuge: 50g/brake 3, 3 minutes; Pellet small cells: 400g/brake 7, 7 minutes; Optiprep debris removal
Magnetic eCSC isolation (steps A (xiii-xiv), B (xv-xviii))	CD45 removal: OctoMACS c-kit selection: EasySep	CD45 removal: OctoMACS c-kit selection: OctoMACS
Anticipated cell yield (steps A (xv), B (xix))	~30,000 per mouse heart	~150,000 per rat heart

Table 3. Summary of antibody concentrations.

Antibody	Step	Concentration
Anti-mouse CD45 MicroBeads (Miltenyi 130-052-301)	**1A (xiii)**	1μl/million cells (minimum 10μl)
EasySep anti-mouse CD117 selection cocktail (Stem Cell Technologies 18757)	**1A (xiv)**	0.5μl/million cells (minimum 5μl)
Anti-mouse CD45-FITC conjugated (Miltenyi 130-091-609)	**1A (xii, xv)**	1μl/million cells (minimum 10μl)

Anti-mouse c-kit-PE conjugated (Stem Cell Technologies 18757C.1)	1A (xii, xv)	1µl/million cells (minimum 10µl)
Mouse IgG anti-rat CD45 antibody (Santa Cruz Biotechnology sc53047)	1B (xv)	2µl/million cells (minimum 20µl)
Mouse IgG anti-rat CD45-FITC conjugated antibody (Biolegend 202205)	1B (xiv, xix)	1:200
Rabbit IgG anti-rat c-kit antibody (Santa Cruz Biotechnology sc5535)	1B (xvii)	2µl/million cells (minimum 20µl)
	1B (xiv, xix)	10µl/million cells (minimum 10µl)
Rat anti-mouse IgG2a+b MicroBeads (Miltenyi 130-047-201)	1B (xvi)	2µl/million cells (minimum 20µl)
Goat anti-rabbit IgG MicroBeads (Miltenyi 130-048-602)	1B (xviii)	2µl/million cells (minimum 20µl)
Anti-mouse Sca-1-FITC conjugated antibody (Miltenyi 130-098-034)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD31-FITC conjugated antibody (Miltenyi 130-100-003)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD34-FITC conjugated antibody (BD 560238)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD90-PE conjugated antibody (Abcam Clone G7, ab24904)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD105-PE conjugated antibody (Miltenyi 130-098-018)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD140a-PE conjugated antibody (Miltenyi 130-096-271)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD166-PE conjugated antibody (eBioscience 12-1661-81)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD45-FITC conjugated antibody (Miltenyi 130-091-609)	1A (xv)	10µl/million cells (minimum 10µl)
Mouse IgG1 PE Isotype control (Abcam ab81200)	1A (xii, xv)	10µl/million cells (minimum 10µl)
Mouse IgG2a FITC Isotype control (Abcam ab81197)	1A (xii, xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD117-PE conjugated antibody (Miltenyi 130-091-730)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-mouse CD45-PE conjugated antibody (Miltenyi 130-091-610)	1A (xv)	10µl/million cells (minimum 10µl)
Anti-tryptase antibody (Santa Cruz Biotechnology sc32473)	2F (iii)	1:50
Goat polyclonal anti-c-kit antibody (R and D systems AF1356)	2F (iii)	1:50

Mouse IgM anti-α-sarcomeric actin antibody (Sigma A2127)	**2F (iii)**	1:50
Mouse IgG anti-smooth muscle actin antibody (Sigma A2547)	**2F (iii)**	1:50
Rabbit polyclonal anti-von Willebrand factor antibody (Dako A0082)	**2F (iii)**	1:100
Mouse monoclonal anti-α-actinin sarcomeric antibody (Sigma Clone EA-53, A7811)	**2F (iii)**	1:50
Rabbit polyclonal anti-Connexin 43 antibody (Sigma C6219)	**2F (iii)**	1:50
AlexaFluor 488 donkey anti-rabbit antibody (Jackson 711-545-152-JIR)	**1B (xix)** **2F (v)**	10µl/million cells (minimum 10µl) 1:100
AlexaFluor 594 donkey anti-rabbit antibody (Jackson 711-585-152-JIR)	**1B (xiv, xix)** **2F (v)**	10µl/million cells (minimum 10µl) 1:100
AlexaFluor 488 donkey anti-goat antibody (Jackson 705-545-147-JIR)	**2F (v)**	1:100
Alexa Fluor 488 donkey anti-mouse IgG antibody (Jackson 715-545-150-JIR)	**2F (v)**	1:100
AlexaFluor 594 donkey anti-mouse IgM antibody (Jackson 715-585-140-JIR)	**2F (v)**	1:100
AlexaFluor 594 donkey anti-mouse IgG antibody (Jackson 715-585-151-JIR)	**2F (v)**	1:100

Table 4. Summary of coating substrate volume, medium volume and cell plating density requirements for c-kit-positive, CD45-negative CSCs in culture (these apply to culture of either mouse or rat CSCs).

Culture plate or dish	Coating volume	Medium volume	Maximum cells at time of plating
96-well plate	50µl	100-200µl	3,000
24-well plate	300µl	500-1000µl	10,000
4-well chamber slide	500µl	500µl	10,000
6-well plate/35mm dish	1ml	1.5ml	200,000
60mm dish	3ml	5ml	500,000
100mm dish	5ml	8ml	n/a
25cm^2 flask	n/a	7ml	n/a
75cm^2 flask	n/a	20ml	n/a

Table 5. Troubleshooting guide.

Stage	Difficulty	Action
Cannulation (steps A (iii), B	Difficulty inserting mouse cannula	Ensure large enough hole cut in aorta

(iii))	Mouse aorta tearing when cannula inserted	Ensure aorta cut is vertical (diagonal cut increases risk of tearing innominate artery during cannula insertion)
	Heart falling off perfusion system	Ensure aorta well tied on to cannula; ensure cannula sufficiently advanced into aorta
Perfusion (steps A (iv, v), B (iv))	Poor perfusion	Likely cause is clotting due to delay in commencing perfusion: optimise technique to minimise sacrifice to perfusion time Over-insertion of cannula: for mouse, should stop advancing cannula when resistance (due to aortic valve leaflets) is felt and retract it 1mm back to ensure avoidance of obstructing coronary arteries' ostium; for rat, can retract cannula up the aorta a very short distance to prevent the cannula tip covering coronary artery apertures
	Residual blood in ventricles	Possibly due to poor perfusion generally as described above
	Over-digestion	Pierce ventricular apex with 25-gauge needle (for mouse) or 18-gauge needle (for rat); for rat, can use forceps to dislodge blood (as described in step **B (v)**)
Tissue disaggregation (step A (vii, viii), B (vii-x))	Poor disaggregation	Likely due to under-digestion secondary to poor perfusion (see above) May be due to tissue being inadequately cut (steps **A (vii)**, **B (vii)**)
	Few cells intact	Over-digestion of tissue (check enzyme activity levels) Insufficiently rapid perfusion of heart following sacrifice
	Few cardiomyocytes intact	Over-digestion of tissue (check enzyme activity levels) Insufficiently rapid perfusion of heart following sacrifice
CD45 removal (step A (xiii), B (xv-xvi))	Poor selection purity	Ensure that small cell preparation is not contaminated by cardiomyocytes or too much cardiomyocyte debris Ensure column is not overloaded with cells (check column's maximum capacity) and that pre-separation filter is in place

	Few remaining cells	Inadequate labelling with MicroBeads: check lot and that correct concentration of MicroBeads used for cell number Ensure column is not overloaded with cells (unlabelled cells may be trapped in the packed column)
c-kit selection (step A (xiv), B (xvii-xviii))	Poor selection purity	Ensure that small cell preparation is not contaminated by cardiomyocytes or too many cardiomyocyte debris Ensure column is not overloaded with cells (check column's maximum capacity) and that pre-separation filter is in place Ensure column is not overloaded with cells (unlabelled cells may be trapped in the packed column)
	Few selected cells	Inadequate labelling with MicroBeads or labelling cocktail: check lot and that correct concentration of reagent(s) used for cell number The c-kit receptor is very sensitive to digestive enzymes: ensure the tissue has not been over-digested
Culture of eCSCs (step A (xvi-xvii), B (xx-xxi))	Cells not adhering or surviving	Plate coating (1.5% gelatin) assists freshly-plated eCSCs adherence and survival Ensure time to perfusion and degree of digestion optimal
	Cells not proliferating	Ensure eCSCs are plated at optimal levels: overly sparse plating is associated with slower eCSC proliferation. The eCSCs should be passaged approximately 1:4, adjusted as necessary Confirm growth medium made as per protocol, including L-glutamine supplementation of Neurobasal medium
	Cells not maintaining 'stem-ness'	Check lot of ESQ serum: batch test if necessary Confirm eCSC phenotype Ensure eCSCs do not become over-confluent, as this can lead to differentiation (passage eCSCs on reaching 80% confluency)
Cardiomyocyte differentiation	Cells not differentiating	Check phenotype of cells (confirm eCSC phenotype)

(step 2B)	Cells not beating	Ensure all growth factors effective and that medium is replenished as required
CytoSpin ICC (step 2D)	Cell morphology abnormal	Ensure appropriate CytoSpin speed and acceleration Ensure appropriate fixative/duration of fixation used (as per protocol)
Chamber slide ICC (step 2E)	Cell morphology abnormal	Ensure medium meniscus is not too low Ensure surface coating covers all of growth surface during pre-plating preparation

a

b FORCEPS

c

d CANNULA

e

f

g

h
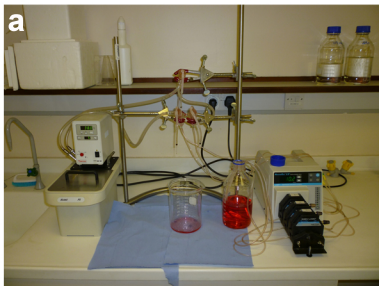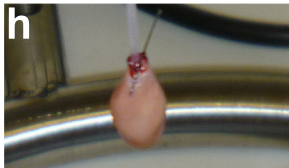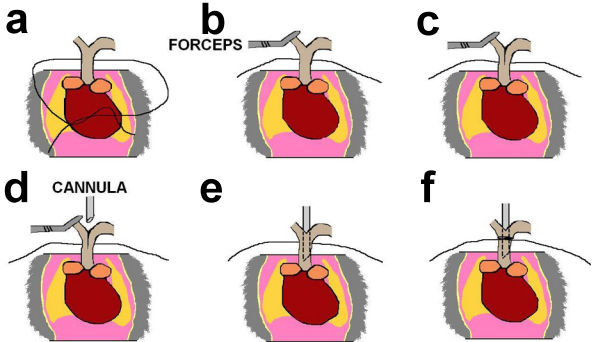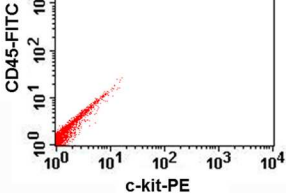
a: isotype control
b: dual-stained
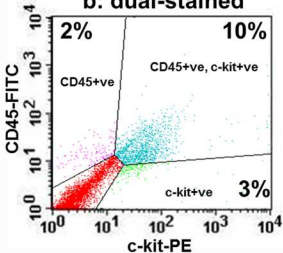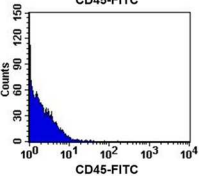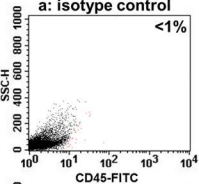
a: isotype control   b: CD45-neg. fraction   c: CD45-pos. fraction

a: isotype control

b: c-kit-neg. fraction

c: c-kit-pos. fraction

d: isotype control

e: CD45-neg, c-kit-pos
sorted fraction

a
CANNULA
FORCEPS

b

c

d

a: isotype control

b: dual-stained

9% 14%

CD45+ve CD45+ve, c-kit+ve

c-kit+ve 4%

c Tryptase DAPI

a: isotype control — <1%

b: c-kit-neg. fraction — 15%

c: c-kit-pos. fraction — 87%

d: isotype control

e: CD45-neg c-kit-pos sorted fraction — 2%, <1%, 86%

CD45+ve, CD45+ve, c-kit+ve, c-kit+ve

f Tryptase DAPI

a Population doubling (hours) — bar graph showing mouse and rat

b Cloned c-kit^pos CD45^neg CSCs

c Mouse / Rat — Expression (normalized) for c-kit, Oct-4, Sox-2, Nanog, TERT at P10 and P30

d Clonogenicity (%) — mouse and rat CSCs

e CardioSphere Spheres (per 1000 CSCs) — mouse and rat CSCs

a c-kit DAPI

b α-sarc DAPI

c SMA DAPI

d vWF DAPI

a

Stage-specific Cardiomyogenic Differentiation Assay

Stage 1	Stage 2	Stage 3	Stage 4
Cloned c-kit^{pos} CSCs	CardioStem Sphere Generation		Beating
Gelatin-Coated Dishes	Bacteriological Dishes	Laminin-Coated Dishes	

-6d ——————— -3d ——————— 0d ——————— 4d ——————— 8d ——————— 14d

| + Oxytocin | - Lif - Oxytocin | + Bmp 2/4 + TGF-β1 | - Bmp 2/4 - TGF-β1 + Dkk-1 | |

b

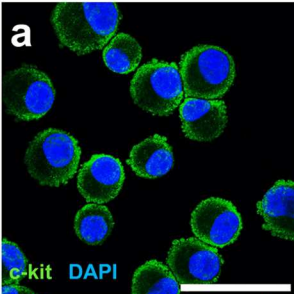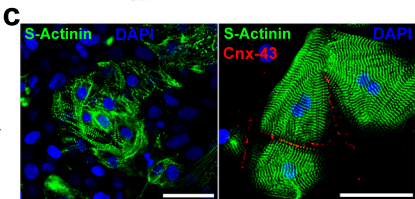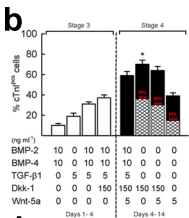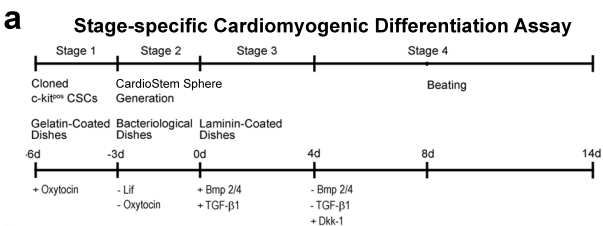| (ng ml^{-1})										
BMP-2	10	0	10	10		10	0	0	0	
BMP-4	10	0	10	10		10	0	0	0	
TGF-β1	0	5	5	5		5	0	0	0	
Dkk-1	0	0	0	150		150	150	150	0	
Wnt-5a	0	0	0	0		5	5	5	5	
	Days 1-4					Days 4-14				

c

d

c-kit^{pos} CardioStem Sphere Cardiomyogenic Differentiation