Chapter 6

Isolation and Colony Formation of Murine Bone and Bone Marrow Cells

Sophie McHaffie and You-Ying Chau

Abstract

Adult homeostasis is dependent on normal *Wt1* expression. Loss of *Wt1* expression in adult mice causes rapid loss of the mesenchymal tissues, fat and bone, amongst other phenotypes. Bone and bone marrow mesenchymal stromal cells can be studied by cell isolation and expansion. The stemness of these cells can then be characterized by carrying out a colony-forming unit-fibroblast assay and observing clonogenic capabilities.

Key words Bone marrow, Bone, Stem cell culture, Mesenchymal, Colony-forming unit fibroblast, Cre

1 Introduction

Wt1 is a major regulator of adult homeostasis. The tamoxifen-inducible ubiquitous knockout of *Wt1* in adult mice results in multiple organ failure within 9 days following tamoxifen-induced deletion [1]. The phenotypes include rapid fat and bone loss; trabecular bone volume is decreased by 30 % and the inner surface of the bone is ragged compared to controls [1]. Adult bone mass is a balance between synthesis from osteoblasts (arising from the mesenchymal stem/progenitors) and turnover by osteoclasts (arising from the hematopoietic progenitors). Increased osteoclast numbers are observed within the mutant bone marrow [1]. In addition, preliminary experiments show that mutant mesenchymal cells are less capable of differentiating into osteoblasts. The size of fat pads and fat vacuoles are also decreased following *Wt1* deletion, as well as adipocyte numbers in bone marrow [1]. Osteoblasts and adipocytes, along with chondrocytes, all differentiate from stromal mesenchymal stem cells (MSCs) found in the bone [2]. Interestingly, around 10 % of Wilms' tumors contain heterologous factors that include fat, bone, and cartilage [3]. A recent study looking at the relationship between MSCs and the mesenchymal properties of Wilms' tumors with *WT1* mutations

Nicholas Hastie (ed.), *The Wilms' Tumor (WT1) Gene: Methods and Protocols*, Methods in Molecular Biology, vol. 1467,
DOI 10.1007/978-1-4939-4023-3_6, © Springer Science+Business Media New York 2016

showed that a Wilms' tumor cell line expresses surface proteins specific to human MSCs including CD105, CD90, and CD73 and that are able to differentiate into chondrocytes, osteoblasts, and adipocytes [4]. These findings support the involvement of *Wt1* in MSC biology.

There are compelling parallels between different *Wt1*-expressing mesenchymal cell populations that suggest links with mesenchymal stem/progenitor cells. *Wt1*-expressing mesothelial cells in the developing liver are stellate cell precursors [5–7], while *Wt1* is also expressed in the pancreatic mesothelium and pancreatic stellate cells [1]. The parallels between pancreatic and hepatic stellate cells suggest that pancreatic stellate cells may also be arising from the mesothelium, via epithelial to mesenchymal transition [1, 7, 8]. Stellate cells have spindle-shaped bodies similar in appearance to bone marrow cells cultured under MSC-favoring conditions [9] and are also positive for *nestin* [10], linking them with the *nestin*-expressing MSCs [11]. Stellate cells may originate from both the mesothelium- and bone marrow-derived HSCs and could possibly be another subset of the *Wt1*-expressing cells [6, 12]. Hepatic stellate cells share striking similarities with pericytes [13, 14]. A FACS-sorted pericyte population from the bone marrow was shown to include cells with MSC properties [15]. In the kidney, podocytes, which similarly to pericytes and stellate cells also have a spindle-shaped appearance due to their long processes or "feet", express both *Wt1*, which is a podocyte regulator [16], and *nestin* [17]. In the developing kidney and heart, *nestin* and *Wt1* expressions overlap. *Nestin* is potentially regulated by *Wt1* as its expression is reduced drastically following the loss of *Wt1* in these tissues [18]. *Wt1* is required for mesenchymal to epithelial transition (MET) in the developing kidney as *Wt1* deletion in kidney mesenchyme leads to failure of MET and nephron formation [19]. The opposite is seen in heart development where *Wt1* is required for epithelial to mesenchymal transition (EMT) [20]. Mesenchymal progenitor cells are formed when the epicardial cells undergo EMT. An epicardial-specific *Wt1* knockout resulted in a loss of these mesenchymal progenitor cells [20].

However, the most striking phenotype to support *Wt1*'s involvement with mesenchymal lineages is the effect of *Wt1* deletion [1]. Bone and fat are both mesenchymal derivatives, strongly suggesting that *Wt1* is playing a functional role in the genesis and homeostasis of these mesenchymal tissues, and may even act as a functional marker for a subset of mesenchymal stem or progenitor cells.

There is considerable topical interest in tissue stem and progenitor cells, particularly with regard to potential uses in therapy. Bone marrow is largely split into two halves: the hematopoietic and the non-hematopoietic. The hematopoietic component consists of HSCs, hematopoietic progenitor cells, and their derivatives. The non-hematopoietic component is made up mainly of endothelial and mesenchymal stem cells (MSC) plus their derivatives, including adipocytes,

osteoblasts, and chondrocytes [21]. It has been much more difficult to define mesenchymal stem and progenitor cells for different lineages than it has been for the hematopoietic compartment.

The following protocols detail how to isolate bone and marrow cells and how to carry out colony-forming unit-fibroblast assays to characterize the mesenchymal stromal compartment.

2 Protocols

2.1 Isolation of Murine Bone Marrow and Bone Mesenchymal Progenitors

2.1.1 Introduction

Embryonic bone development takes place when a core of prechondrogenic cells is surrounded by stacked osteogenic progenitor cells, all of which are avascular. This central core and surrounding stacked cells differentiate into cartilage to give a cartilage rod or "model". The stacked cells then give rise to osteoblasts [22, 23]. Osteoblasts are located in close proximity to blood vessels with the "back" of the cell toward the capillary and the "front" of the cell secreting osteoid: a collagen type I-rich premineral layer [22, 23]. Vasculature is the dominant factor for the position and survival of the osteoblast cells, unlike chondrogenesis which actively inhibits vascularization [24, 25]. For this reason bone does not replace cartilage, but cartilage is invaded by vascular cells to give the resulting marrow cavity [23], and bone forms independent of the cartilage [24, 25]. In mice, bone development begins with mesenchyme condensing at embryonic day 9.5 (e9.5). By e12 the commitment of mesenchymal cells to the osteogenic lineage occurs, followed by commitment to the chondrogenic lineage on day 13, finishing with vasculature and marrow invasion of the cartilage occurring on days 16–17 of development [25, 26].

Primitive marrow is formed when the chondrocyte core is invaded by vasculature to form the marrow cavity [23]. As previously mentioned, postnatal bone marrow is an organ of two halves: the hematopoietic cells, including the hematopoietic stem cells (HSCs), and the non-hematopoietic cells [27]. The non-hematopoietic cells are also known as the bone marrow stroma, or associated supporting stroma, and this is where the mesenchymal stem cell resides [27]. The bone marrow is the only known organ where two separate stem cells, MSCs and HSCs, with distinct lineage pathways are located and found to interact together to coexist [27].

2.1.2 Materials

1. Culture medium:

   DMEM containing 10% (v/v) fetal calf serum, 1% (v/v) penicillin/streptomycin, 0.5% (v/v) glutamine, and 0.5% (v/v) sodium pyruvate.

2. Sterile dissection kit.
3. 25-gauge needle.
4. 21-gauge needle.
5. 10 mL Syringe.

6. 10 cm Culture dish.
7. Pestle and mortar.
8. Collagenase B (Roche).
9. Rocking chamber heated to 37 °C.
10. 70 micron cell strainer (Falcon).
11. Phosphate-buffered saline (PBS), pH 7.4.
12. Bench centrifuge with swinging bucket rotor.
13. Sterile cell culture plastic pipettes individually wrapped (5, 10, 25 mL).
14. Pipette aid.
15. Sterile conical centrifuge tubes (15 and 50 mL).
16. Cell aspirator.

2.1.3 Methods

2.1.3.1 Isolation of Bone Marrow Cells

1. Euthanize mice by cervical dislocation and dissect out the bilateral femur and tibia.
2. In a sterile culture hood and with a sterile dissection kit remove each end of the long bones and set aside. Carry this out in a 10 cm culture dish. Add a small amount of culture medium to prevent the tissue and bone drying out.
3. Using 8 mL of culture medium flush out the bone marrow from the long bones using the 10 mL syringe and 25-gauge needle.
4. Dissociate the flushed marrow cells using a 21-gauge needle.

2.1.3.2 Isolation of Bone Cells

1. Prepare the collagenase B at a concentration of 3 mg/mL dissolved in culture medium and kept on ice.
2. Crush the pre-flushed long bones and bone ends by pestle and mortar in 10 mL collagenase B until a pulp-like consistency is reached.
3. Transfer the bone pulp and 10 mL collagenase B to a sterile 15 mL tube and incubate at 37 °C for 90 min in a rocking chamber to enable enzymatic digestion.
4. Pass the bone cells through a 70 micron cell strainer into a sterile 50 mL tube.
5. Centrifuge the tube at 300 rcf for 5 min at room temperature in a swinging bucket rotor. Remove the enzyme-containing supernatant by vacuum aspiration and resuspend the cell pellet in pre-warmed culture medium.

2.1.4 Notes

CFU-F assays and MSC isolation are usually carried out using marrow cells flushed from the bone. This protocol uses isolated cells from both marrow and bone which are crushed with a collagenase digest [28]. This extra bone digest step is included as MSCs are located near the endosteum of the bone, in the bone-lining

endosteal region, where they interact with bone-lining osteoblasts [29]. Cells in this area are difficult to remove by flushing alone.

3 Colony-Forming Unit-Fibroblast Assay

3.1 Introduction

Colony-forming unit-fibroblast (CFU-F) assays are often used to characterize stromal marrow cells and assess the number of mesenchymal stem and progenitor cells [27, 30, 31]. The bone and fat loss phenotypes seen from the ubiquitous *Wt1* deletion point to the involvement of mesenchymal stromal cells. This makes the CFU-F assay an appropriate tool to investigate its involvement. Adherent stromal cells form colonies which originate from a single cell: the CFU-F [27, 31–34]. There are several methods of obtaining material for CFU-F assays, most commonly the flushing of bone marrow, but also including the crushing of pre-flushed long bones followed by enzymatic digestion [27, 31–34]. The isolation of these cells is covered previously in this chapter.

Proliferation without terminal differentiation, a stem cell characteristic, is demonstrated using CFU-F assays [32], proving that a single cell can result in a colony of cells. Mesenchymal stem cells, or mesenchymal stromal cells (MSCs), are commonly described as spindle-shaped multipotent cells able to self-replicate and generate progenitors that produce a variety of skeletal tissues: bone, cartilage, marrow stroma, fat, ligament, tendon, and connective tissue. MSCs are mainly found in the bone marrow; however they make up a very small proportion of the total number of marrow cells, between 0.01 and 0.001 % [35]. Bone marrow contains MSCs which can undergo chondrogenic, osteogenic, and adipogenic differentiation to form cartilage, bone, and fat, respectively [35, 36]. MSCs are also found in bone fragments which have been crushed and digested with a collagen enzyme. These cells are also able to differentiate into the three main mesenchymal lineages [37].

3.2 Materials

1. Hemocytometer.
2. Sterile 6-well culture plates.
3. MesenCult® media.
4. 0.5 % (w/v) Cresyl violet acetate (Sigma) in methanol: Filter through 25 μm filter paper and store at room temperature.
5. Incubator.

3.3 Methods

1. Count the cells with a hemocytometer.
2. Plate 5×10^5 cells per well of a 6-well culture plate with 2 mL of MesenCult® media per well.
3. Incubate the plates for 48 h to allow adherent cells to attach.
4. Wash with pre-warmed PBS to remove media and nonadherent cells.

5. Add 2 mL pre-warmed MesenCult® to each well and culture.
6. Every 3 days, remove the media and replace with fresh MesenCult® for 10 days. Check that the colonies are not becoming too confluent.
7. Wash plates with PBS.
8. Remove the PBS and stain colonies with 0.5 % Cresyl violet acetate solution for 30 min at room temperature.
9. Wash plates with PBS three times, followed by a single wash with distilled H_2O.
10. Remove H_2O and leave to dry.
11. Count colonies with a diameter greater than 1 mm.

3.4 Notes

Higher numbers of colonies form in the bone CFU-F assays compared to the marrow [28, 33, 38]. The trabecular bone is an enriched source of mesenchymal progenitors which corroborates with higher numbers of bone colonies [31, 39].

Due to the bone loss phenotype, the effect of *Wt1* on colony-forming abilities was investigated using mice with a tamoxifen-inducible *Wt1* deletion (CAGG-CreER^{T2}; *Wt1loxP/loxP*) [38]. It is difficult to determine whether *Wt1* deletion has any effect on colony formation due to the Cre-only control showing the same trend, potentially masking any gene loss effect [38] (Fig. 1). The results suggest that, at least for colony-forming capabilities, bone and marrow cells are affected by the activated CreER recombinase, rather than *Wt1* deletion. This raises a great concern as to whether activated CreER recombinase is the cause of other phenotypes seen in published studies using this construct and deletion method.

The appropriate Cre-only control should be included if using CFU-F assays in conjunction with the Cre-*loxP* system of gene knockout. It is important to control for not only Cre effects but also gender and tamoxifen [38].

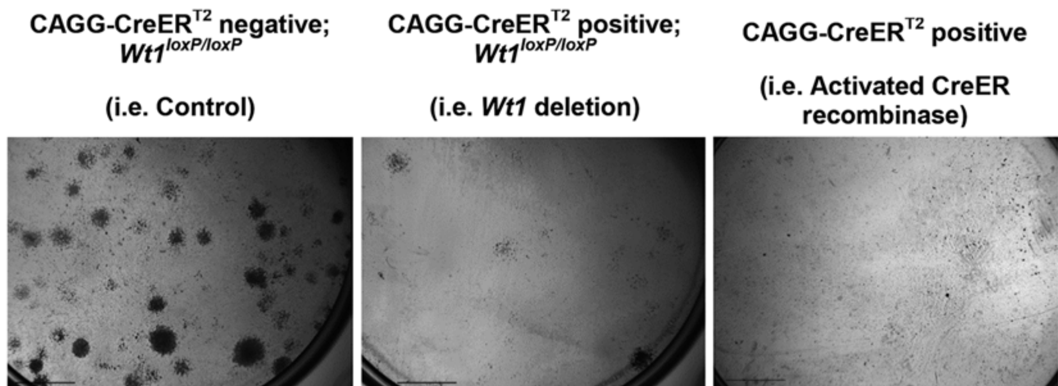

Fig. 1 Activated CreER^{T2} recombinase negatively affects colony-forming capabilities

References

1. Chau Y-Y, Brownstein D, Mjoseng H et al (2011) Acute multiple organ failure in adult mice deleted for the developmental regulator, Wt1. PLoS Genet 7(12):e1002404
2. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74
3. Royer-Pokora B (2003) Two molecular subgroups of Wilms' tumors with or without *WT1* mutations. Clin Cancer Res 9:2005–2014
4. Royer-Pokora B, Busch M, Beier M et al (2010) Wilms' tumor cells with *WT1* mutations have characteristic features of mesenchymal stem cells and express molecular markers of paraxial mesoderm. Hum Mol Genet 19:1651–1668
5. Ijpenberg A, Pérez-Pomares JM, Guadix JA et al (2007) Wt1 and retinoic acid signalling are essential for stellate cell development and liver morphogenesis. Dev Biol 312:157–170
6. Asahina K, Zhou B, Pu WT et al (2011) Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. Hepatology 53(3):983–995, appendix
7. Li Y, Wang J, Asahina K (2013) Mesothelial cells give rise to hepatic stellate cells and myofibroblasts via mesothelial–mesenchymal transition in liver injury. Proc Natl Acad Sci U S A 110(6):2324–2329
8. Masamune A, Shimosegawa T (2009) Signal transduction in pancreatic stellate cells. J Gastroenterol 44(4):249–260
9. Friedman SL (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 88:125–172
10. Lardon J, Rooman I, Bouwens L (2002) Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. Histochem Cell Biol 117(6):535–540
11. Méndez-Ferrer S, Michurina TV, Ferraro F et al (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829–834
12. Miyata E, Masuya M, Yoshida S et al (2008) Hematopoietic origin of hepatic stellate cells in the adult liver. Blood 111(4):2427–2435
13. Sato M, Suzuki S, Senoo H (2003) Hepatic stellate cells: unique characteristics in cell biology and phenotype. Cell Struct Funct 28:105–112
14. Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro-Oncology 7(4):452–464
15. Crisan M, Yap S, Casteilla L et al (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3(11):301–313
16. Guo J-K, Menke AL, Gubler M-C et al (2002) *WT1* is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis. Hum Mol Genet 11(6):651–659
17. Bertelli E, Regoli M, Fonzi L et al (2007) Nestin expression in adult and developing human kidney. J Histochem Cytochem 55:411–421
18. Wagner N, Wagner K-D, Scholz H et al (2006) Intermediate filament protein nestin is expressed in developing kidney and heart and might be regulated by the Wilms' tumor suppressor Wt1. Am J Physiol 291:R779–R787
19. Essafi A, Webb A, Berry RL et al (2011) A Wt1-controlled chromatin switching mechanism underpins tissue-specific *wnt4* activation and repression. Dev Cell 21(3):559–574
20. Martínez-Estrada OM, Lettice LA, Essafi A et al (2010) Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. Nat Genet 42:89–93
21. Tocci A, Forte L (2003) Mesenchymal stem cell: use and perspectives. Hematol J 4:92–96
22. Pechak DG, Kujawa MJ, Caplan AI (1986) Morphological and histochemical events during first bone remodeling in embryonic chick limbs. Bone 7:441–458
23. Pechak DG, Kujawa MJ, Caplan AI (1986) Morphology of bone development and bone remodeling in embryonic chick limbs. Bone 7:459–472
24. Caplan AI (1987) Bone development and repair. BioEssays 6(4):171–175
25. Caplan AI (1988) Bone development. In: Wiley J (ed) Cell and molecular biology of vertebrate hard tissues. CIBA Foundation Symposium 136, Chichester, pp 3–21
26. Zelzer E, Olsen BR (2005) Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. Curr Top Dev Biol 65:169–187
27. Bianco P, Riminucci M, Gronthos S et al (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180–192
28. Morikawa S, Mabuchi Y, Kubota Y et al (2009) Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J Exp Med 206(11):2483–2496
29. Muguruma Y, Yahata T, Miyatake H et al (2006) Reconstitution of the functional human

hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. Blood 107(5):1878–1887

30. Kuznetsov SA, Krebsbach PH, Satomura K et al (1997) Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 12(9):1335–1347
31. Siclari VA, Zhu J, Akiyama K et al (2013) Mesenchymal progenitors residing close to the bone surface are functionally distinct from those in the central bone marrow. Bone 53(2):575–586
32. Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3(4):393–403
33. Nakamura Y, Arai F, Iwasaki H et al (2010) Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. Blood 116:1422–1432
34. Ohishi M, Ono W, Ono N et al (2012) A novel population of cells expressing both hematopoietic and mesenchymal markers is present in the normal adult bone marrow and is augmented in a murine model of marrow fibrosis. Am J Pathol 180:811–818
35. Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147
36. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650
37. Guo Z, Li H, Li X et al (2006) In vitro characteristics and in vivo immunosuppressive activity of compact bone-derived murine mesenchymal progenitor cells. Stem Cells 24:992–1000
38. McHaffie SL, Hastie N, Chau Y-Y (2016) Effects of CreER^{T2}, 4-OH tamoxifen, and gender on CFU-F assays. PLoS One 11(2):e0148105
39. Grcevic D, Pejda S, Matthews BG et al (2012) In vivo fate mapping identifies mesenchymal progenitor cells. Stem Cells 30(2):187–196