



■ REVIEW ARTICLE

Exploring the application of mesenchymal stem cells in bone repair and regeneration

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Failure of bone repair is a challenging problem in the management of fractures. There is a limited supply of autologous bone grafts for treating nonunions, with associated morbidity after harvesting. There is need for a better source of cells for repair. Mesenchymal stem cells (MSCs) hold promise for healing of bone because of their capacity to differentiate into osteoblasts and their availability from a wide variety of sources. Our review aims to evaluate the available clinical evidence and recent progress in strategies which attempt to use autologous and heterologous MSCs in clinical practice, including genetically-modified MSCs and those grown on scaffolds. We have compared various procedures for isolating and expanding a sufficient number of MSCs for use in a clinical setting.

There are now a number of clinical studies which have shown that implantation of MSCs is an effective, safe and durable method for aiding the repair and regeneration of bone.

The healing of fractures depends on the interacting triad of the osteogenic cell, the osteoinductive stimulus and the osteoconductive matrix scaffold.¹

Various natural or synthetic biomaterial grafts have been developed with excellent osteoconductive properties. There may be local morbidity after harvesting autologous bone graft from the iliac crest and a limited supply of bone is available.^{2,3} These limitations have encouraged the use of allografts. However, despite their availability and the decreased rates of complication, there remains the possible risk of transmission of disease, diminished biological and mechanical properties in comparison with autologous bone, and higher costs.⁴ In recent years, interest has risen in the use of deproteinated and defatted xenografts which give reduced immune responses, but these grafts have reduced osteoconductive properties.⁵ Synthetic substitutes include ceramics, mixes of collagen and ceramic, coral derivatives and bioactive glass.⁵ These are potentially attractive alternatives, but because of varied properties of restorability, higher costs and variation in their osteoconductive, osteoinductive and mechanical properties, they are considered to be less useful than allografts.⁵

Even after grafting, healing is not guaranteed unless fresh cells are recruited to the fracture site. Osteoinductive molecules are responsible for supporting the cellular migration and

differentiation of the new progenitor cells required for the formation of new bone.⁶ One of the important types of cell which are influenced by these osteoinductive molecules is the mesenchymal stem cell (MSC), a vital component in the natural process of new bone formation. Because of a lack of an adequate supply of autologous bone grafts and the unsuitability of allografts, there has been some impetus to use MSCs, one of the body's own versatile progenitor cells, to encourage repair.

The aims of our review are to describe the role of MSCs in bone repair and regeneration, to evaluate the techniques used to apply MSCs in clinical studies and to discuss what further studies are required to expand their use.

MSCs and bone repair

After a fracture, a haematoma forms which prevents excessive extravasation.⁷ The platelets, inflammatory cells and macrophages arriving at the site of injury secrete cytokines and growth factors, including platelet-derived-growth factor, bone-morphogenetic proteins (BMPs), vascular-endothelial-growth-factor and interleukin-1 to -6.⁷ This cellular response triggers the invasion of the MSCs which differentiate into osteoblasts and chondrocytes in order to complete the repair.⁷ MSCs from the periosteum, bone marrow, circulating blood and the surrounding soft tissues have been shown to contribute to bone repair in rodent models.⁸⁻¹¹ The management of a defect in the

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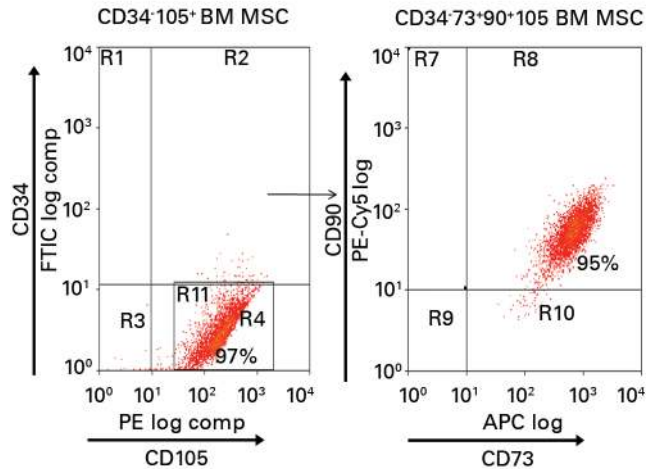


Fig. 1a

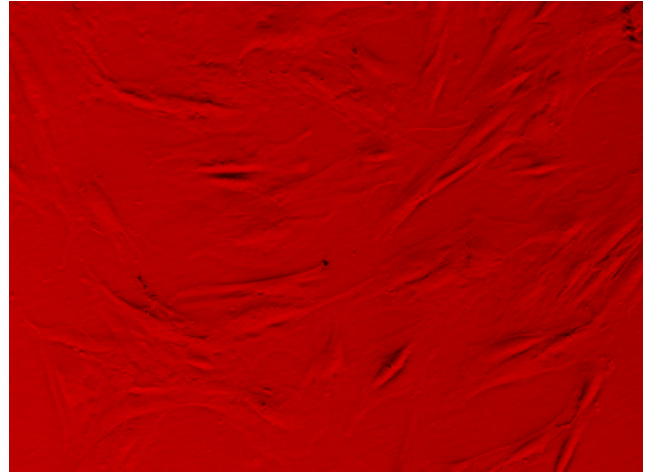


Fig. 1b

Figure 1a – Fluorescence Activated Cell Sorting (FACS) analysis of bone-marrow-derived mesenchymal stem cells (MSCs) which can be used to purify them further. Live CD34-105+ cells were gated for CD90 and CD73 positive cells. More than 90% of the cells were CD34-73+90+105+, and hence were considered as MSCs according to the definition of the Mesenchymal and Tissue Stem Cell Committee of the International Society.⁶² Figure 1b – light photomicrograph of MSCs which have been expanded using two-dimensional culture systems after isolation from bone marrow ($\times 20$ magnification).

femur of athymic rats showed that a ceramic scaffold loaded with expanded MSCs gave significantly more new bone formation at 12 weeks and stronger bone than a scaffold without MSCs. This study confirmed that the MSCs formed bone by differentiating into osteoblasts.⁸

MSCs are non-immunogenic. They do not express major histocompatibility class II and co-stimulatory molecules, including CD40, CD80, and CD86.¹² Hence, allogeneic transplantation of MSCs should not require immunosuppression of the host. Most importantly, MSCs do not induce the proliferation of lymphocytes.¹³ They have immunosuppressive properties and suppress the proliferation of T-cells and cytokine production in response to alloantigens or insignificant mitogens, as well as inhibiting the function of B cells,¹⁴ dendritic cells¹⁵ and the natural killer cells.¹⁶

MSCs show homing potential which is affected by numerous cytokines and growth factors, including stromal cell derived growth factor-1 (SDF-1) and its receptor CXCR4, which have been shown to act as a potential homing signal for MSCs in bone healing. In a mouse model of a femoral defect, Kitaori et al¹⁷ showed that bone formation was mediated by expression of SDF-1 in the periosteum, causing recruitment of MSCs to the bone lesion. However, some studies have reported low levels of CXCR4 expressed by MSCs and have found that blocking CXCR4 had no effect on the migration of MSCs, suggesting that other molecules may be involved. There is no consensus on the mode of migration of MSCs since some studies have shown them in the circulation,¹⁸ whereas others have found no evidence of this.¹⁹

Isolation and expansion of MSCs

MSCs are frequently isolated from the marrow of the superior iliac crest, but MSCs from the femur and the tibia during

hip and knee replacements are a suitable alternative. However, since only 0.001% to 0.01% of mononuclear cells from bone marrow are MSCs,²⁰ an efficient method of isolation is required. This is usually achieved by density gradient centrifugation using Ficoll or Percoll.²¹ When cells are layered over Ficoll or Percoll and centrifuged, layers of red blood cells, fat cells and mononuclear cells (MNCs) are formed according to their densities. The MNCs containing potential MSCs which form the middle layer can be aspirated, purified (Fig. 1a) and expanded (Fig. 1b) in a short period of time. With optimal conditions they can be cultured up to passage 30, although the proliferation and differentiation potentials of MSCs are reduced because of senescence, the Heyflick effect and telomere shortening.^{22,23} Early passage cells (< 10) are considered to be most useful and their senescence can be circumvented by the addition of growth factors or by expanding them in a three-dimensional bioreactor which mimics the environment *in vivo*.²⁴ An important barrier to the use of *ex vivo* expanded MSCs is the risk of introducing pathogens and xenoinfection because of the use of fetal bovine serum for their culture. Increasingly, serum-free media are used for culture thus making it possible to use them for clinical applications.²⁵ The main therapeutic usefulness of MSCs is their ability to differentiate into osteocytes, chondrocytes and adipocytes in the *ex vivo* environment (Fig. 2). They can be differentiated *in vitro* into osteoblasts by the addition of dexamethasone and ascorbic acid, although no study has compared whether differentiated or undifferentiated MSCs differ in their ability to aid bone regeneration.

Application of stem cells in bone regeneration

After MSCs are expanded *ex vivo* they are either introduced by systemic infusion, or growth on a scaffold and

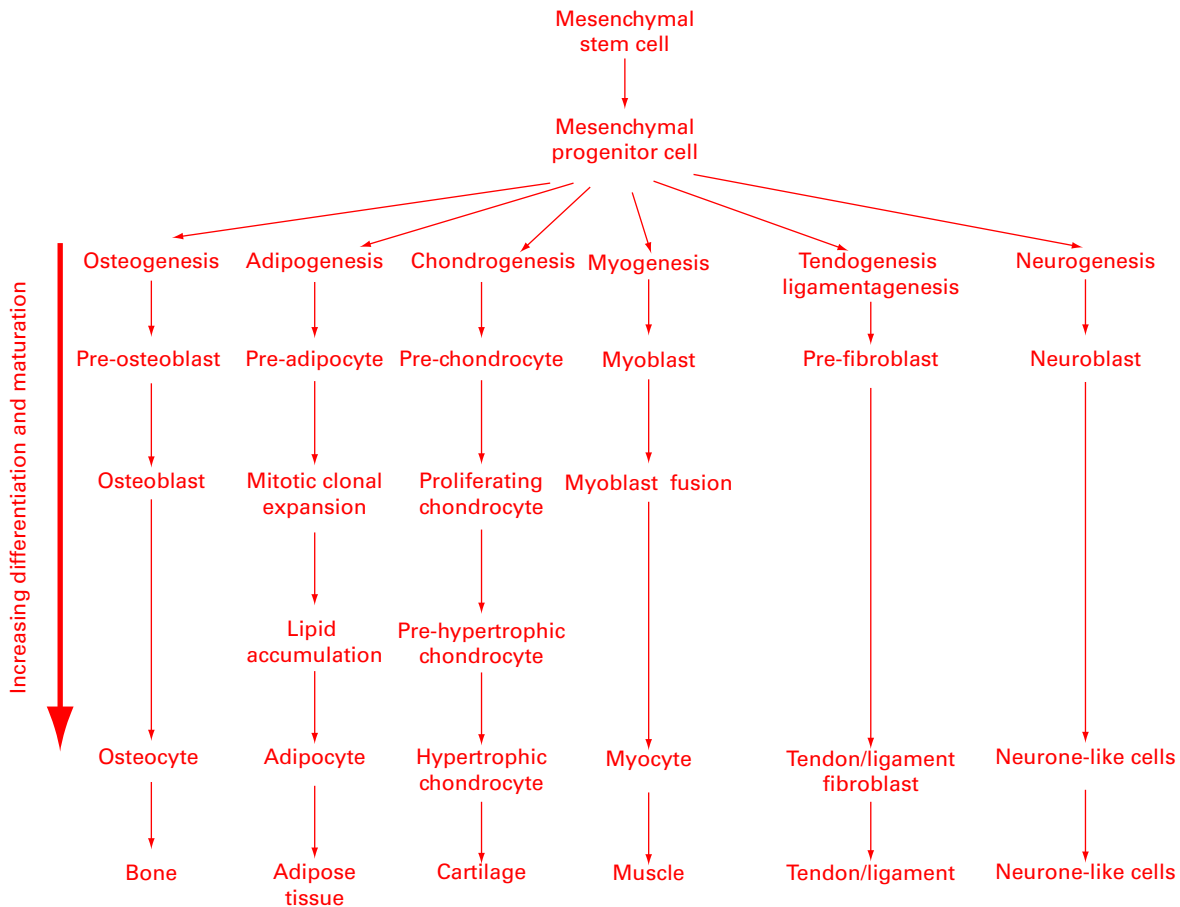


Fig. 2

Diagram showing the multi-differentiation potential of mesenchymal stem cells. They can differentiate into osteocytes, chondrocytes, adipocytes, myocytes, neurone-like cells and fibroblasts.

applied directly to the site of the lesion, or genetically modified before being used in a scaffold.

Expanded MSCs introduced by systemic infusion. Animal models have shown that MSCs can migrate to the bone marrow after peripheral injection and remain there for an extended duration.²⁶ Studies have shown the successful infusion of *ex vivo* expanded MSCs into human volunteers^{27,28} indicating that this is feasible and well tolerated. Systemic infusion of MSCs for bone regeneration has been successfully used by Horwitz et al²⁹ in treating osteogenesis imperfecta. Six children with severe osteogenesis imperfecta received two infusions of allogeneic MSCs. Five children showed homing of MSCs into one or more sites including bone, skin and marrow stroma with an acceleration of bone growth of between 60% and 94% compared with matched unaffected children.²⁹ However, this technique has not been used in the repair of fractures. This may be because of reports^{30,31} showing that most of the infused MSCs became trapped in the lungs with only a few migrating to the site of injury. Direct application of MSCs to the fracture is deemed to be more practical,³² with research into systemic infusion of MSCs being more academically driven.

Application of MSCs grown on scaffolds. Scaffolds serve as carriers for cultured MSCs before implantation. Scaffolds need to mimic the natural environment of the bone matrix and should be safe to be used in clinical practice (Fig. 3).³³⁻³⁵ The synergistic effect of using composites of scaffolds with growth factors has been shown to increase the formation and vascularisation of bone.^{36,37} Numerous scaffolds have been investigated in pre-clinical studies, although hydroxyapatite (HA) and calcium phosphate seem to be favoured because of their excellent osteoconductive properties.³⁸ HA provides good strength but is not resorbed, while beta-tricalcium phosphate (β -TCP) is fragile but has a greater capacity for resorption.³⁹ Hence, a combination of HA and β -TCP, biphasic calcium phosphate, is typically used.⁴⁰ HA also has poor mechanical properties and bone formed using an HA composite cannot maintain the mechanical loading needed for remodelling.⁴¹ In order to overcome this, HA can be combined with biodegradable polymer/bioceramic composites, including polylactic-co-glycolic acid, which allows for better control over shaping micro- and macrostructure composites for bone regeneration.⁴²

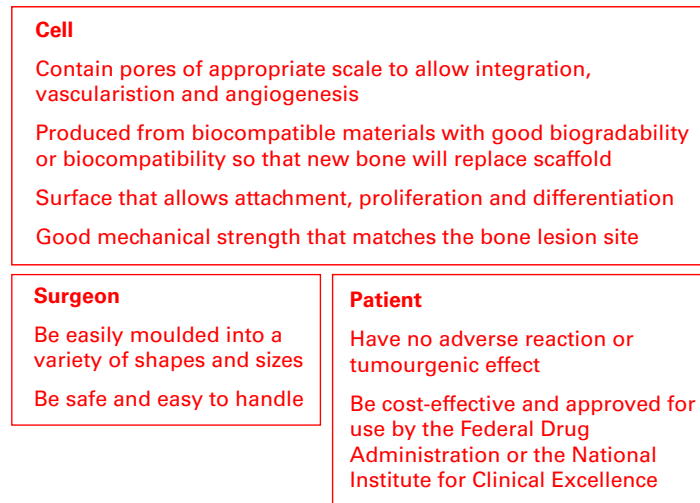


Fig. 3

Diagram showing the scaffold requirements for both the patient and surgeon for mesenchymal stem cells to be used in clinical practice.

The fibres of the extracellular matrix (ECM), their interconnecting pores and HA crystals making up the bone tissue all have nano-scale dimensions (< 100 nm in one dimension).³⁵ Therefore manufacturing nano-composite materials is of interest since it provides three-dimensional (3D) constructs which fit the size of the surrounding matrix, promoting cell adhesion and matrix interactions.¹⁶ More recently, mechanical stability has been identified as an important factor in the repair of fractures. Three-dimensional polymer scaffolds with dimensions of 150 µm to 500 µm have been shown to have excellent stability.⁴³ *Ex vivo* mechanical loading has an additive effect on BMP2-induced osteogenesis in genetically-engineered MSC-like cells,⁴⁴ and mechanical stimulation of MSCs has shown them to differentiate into adipocytes, chondrocytes and osteocytes.⁴⁵

Quarto et al⁴⁶ expanded bone-marrow-derived stem cells for three weeks and seeded them on to macroporous HA scaffolds to treat nonunion. At seven months the three treated patients showed good integration of the implant. Angiographic evaluation after seven years showed vascularisation of the grafted zone, which is vital for the survival and future stability of the graft. Before such scaffolds can be used in clinical practice, a resorbable ceramic scaffold is needed since ceramic masks the newly-formed bone, making radiological follow-up difficult.⁴⁶ Marcacci et al⁴⁷ also showed that *in vitro* expanded MSCs loaded on to porous HA ceramic scaffolds can heal diaphyseal defects with good integration when followed up after five to seven years.

Warnke et al⁴⁸ described a new bone-muscle-flap technique for the treatment of a mandibular defect. The outer scaffold replacement was designed using computer software, placed in an external titanium mesh loaded with HA

blocks coated with BMP-7 and MSCs and then implanted into latissimus dorsi for growth of blood vessels and bone. After seven weeks, the constructed mandible was removed and fixed to the stumps of the original mandible. The patient regained full function of his jaw by four weeks after operation. Since BMP-7 and MSCs were used together, it was difficult to determine whether the results were solely due to the presence of the MSCs. Furthermore, because of ethical issues a tissue sample from the jaw was not available to demonstrate complete healing of the mandible. However, this is a promising approach to avoid donor-site morbidity associated with bone transplantation with no risk of aesthetic disturbance.⁴⁸

The *ex vivo* differentiation of MSCs into osteoblasts is another technique which has emerged. Morishita et al⁴⁹ used an HA scaffold to differentiate MSCs *ex vivo* into osteoblasts to heal the defect in a patient after curettage of a tumour, which illustrated that tissue-engineered osteogenic ceramics may be an alternative to autologous bone grafting. A tissue-engineered prosthesis has been used successfully in three patients with osteoarthritis of the ankle with no adverse reactions and high clinical scores.⁵⁰ Kitoh et al⁵¹ injected differentiated bone-marrow-derived stem cells with platelet-rich plasma without a scaffold into three femora and two tibiae in two patients undergoing distraction osteogenesis to obtain the target lengths without major complications.

MSCs have been successfully used in the treatment of osteonecrosis of the femoral head. Kawate et al⁵² cultured MSCs and applied MSCs/beta-TCP composite granules for steroid-induced osteonecrosis of the femoral head and found that this treatment prevented progression and showed early bone regeneration at 34 months, but it was not useful in the presence of pre-operative collapse.

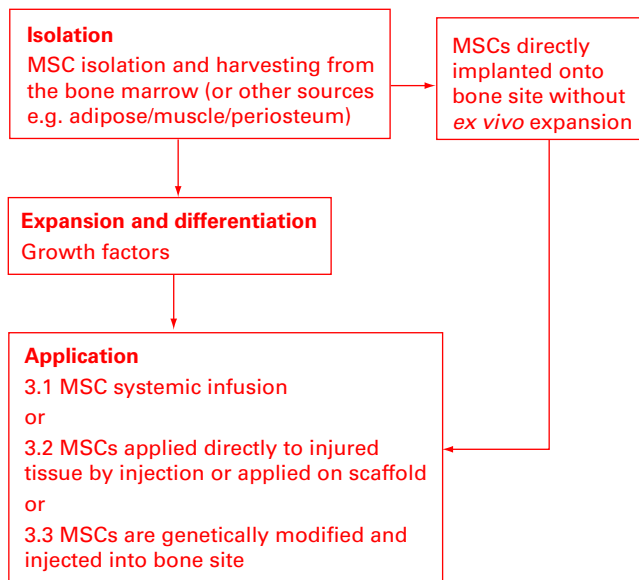


Fig. 4

Diagram showing the process of applying mesenchymal stem cells (MSCs) for bone repair. 1. These are first isolated from the bone marrow or other sources including adipose tissue/muscle/umbilical cord. 2. They are either expanded to increase the number or directly applied to the bone site after centrifugation. Growth factors can be added if expanded *ex vivo* to increase the yield and to differentiate towards different lineages. 3. After expansion the MSCs are then either applied through a scaffold or percutaneously injected at the site to aid regeneration of bone.

Allograft bone chips containing bone-marrow-derived cells have been used for spinal fusion.⁵³ Expanded MSCs have also been used in spinal fusion using porous β -TCP scaffolds seeded with MSCs. Of 41 patients, fusion was demonstrated in 95%.⁵⁴ With limited evidence supporting the role of MSCs in spinal fusion, further investigation using randomised control trials is required.

Genetically modified MSCs. Although the combination of growth factors and scaffolds remains a promising approach, there are limitations in the long-term release of growth factors to promote the proliferation and maintenance of MSCs. Therefore genetic modification of MSCs to express growth factors, which involves either transfection of MSCs through viral vectors or by the use of non-viral vectors, is a suitable alternative. Viral vectors have been shown to elicit immune reactions and have variable ability to transfect dividing and non-dividing cells efficiently.⁵⁵ However, compared with non-viral vectors, they show better expression of the desired protein and their efficiency in transfection is better. Viral vectors are often the optimal choice for gene delivery in MSCs.

Numerous osteoinductive growth factors have been used to modify MSCs and have been shown to give successful bone induction *in vivo*. BMP-2-transfected MSCs showed bone formation in mouse hindlimbs and in bony union of critical-sized mouse radial defects.⁵⁶ In another study, Lin et al⁵⁷ compared adipose- and bone-marrow-

derived stem cells (ADSC/BMSCs) which had been genetically modified with BMP-4 to repair defects in the calvarial bone in rabbits and found no significant difference in bone regeneration. Furthermore, *in vitro* studies showed that deposition of ECM was significantly higher in differentiated ADSCs than in BMSCs.⁵⁷ Fat was detected in ADSC-seeded ECM, thus requiring further investigation of their use in bone defects especially in those bearing load.⁵⁷ To date, no clinical studies have applied *ex vivo*-expanded genetically-modified MSCs because of the need to identify the optimal growth factor and the vector to ensure effective, safe and consistent treatment.

Application of non-expanded MSCs for bone regeneration

MSCs can be applied for bone regeneration without expansion *ex vivo* in order to avoid cost and time. After the preliminary work by Herzog⁵⁸ in 1951 which demonstrated the procedure of percutaneous bone grafting, many clinical studies have successfully applied MSCs for bone regeneration. In 1995 Connolly⁵⁹ demonstrated in a series of 100 skeletal healing problems, including delayed unions and nonunions of fractures, arthrodeses, and bone defects that MSCs were effective in bone repair when applied in this way. However, this and earlier studies did not report the number of MSCs needed to give optimum healing.

Hernigou et al⁶⁰ showed that bone healing depended on the number and concentration of transplanted MSCs. They found that seven of 60 patients with defects of the shaft of the tibia did not achieve union.⁶⁰ In these patients the mean number of MSCs in the graft was < 1000 cells per cm³ and < 30 000 cells in total. Both the mean concentration and the mean number in patients who had not achieved union were significantly lower ($p < 0.01$) than in those in whom union was successful.⁶⁰ Therefore they considered that a graft needed to contain at least > 1000 MSCs per cm³ to achieve union. This has implications for the technique used to isolate MSCs since the aspirate is not guaranteed to contain the required total cell count.⁵⁰ However, recent studies by Wongchuensoontorn et al⁶¹ have demonstrated two techniques for increasing the volume of bone marrow applied to a bone defect. First, small volumes preferably within the range of 2 ml to 4 ml should be aspirated from the site since larger volumes dilute the bone marrow with blood, and secondly, the concentration of MSCs should be increased by centrifuging the aspirate before injection.⁶¹ In contrast to the classic laboratory procedure, separation by density gradient does not require training and allows the processing of stem cells at the bedside.⁶¹ Although Hernigou et al⁶⁰ demonstrated an approximate estimate for the number of MSCs required for bone regeneration, the number of viable cells after implantation into man has not yet been analysed.

The future of MSCs for clinical practice

The process of harvesting MSCs is a simple, stepwise process as illustrated in Figure 4. It is clear from the various

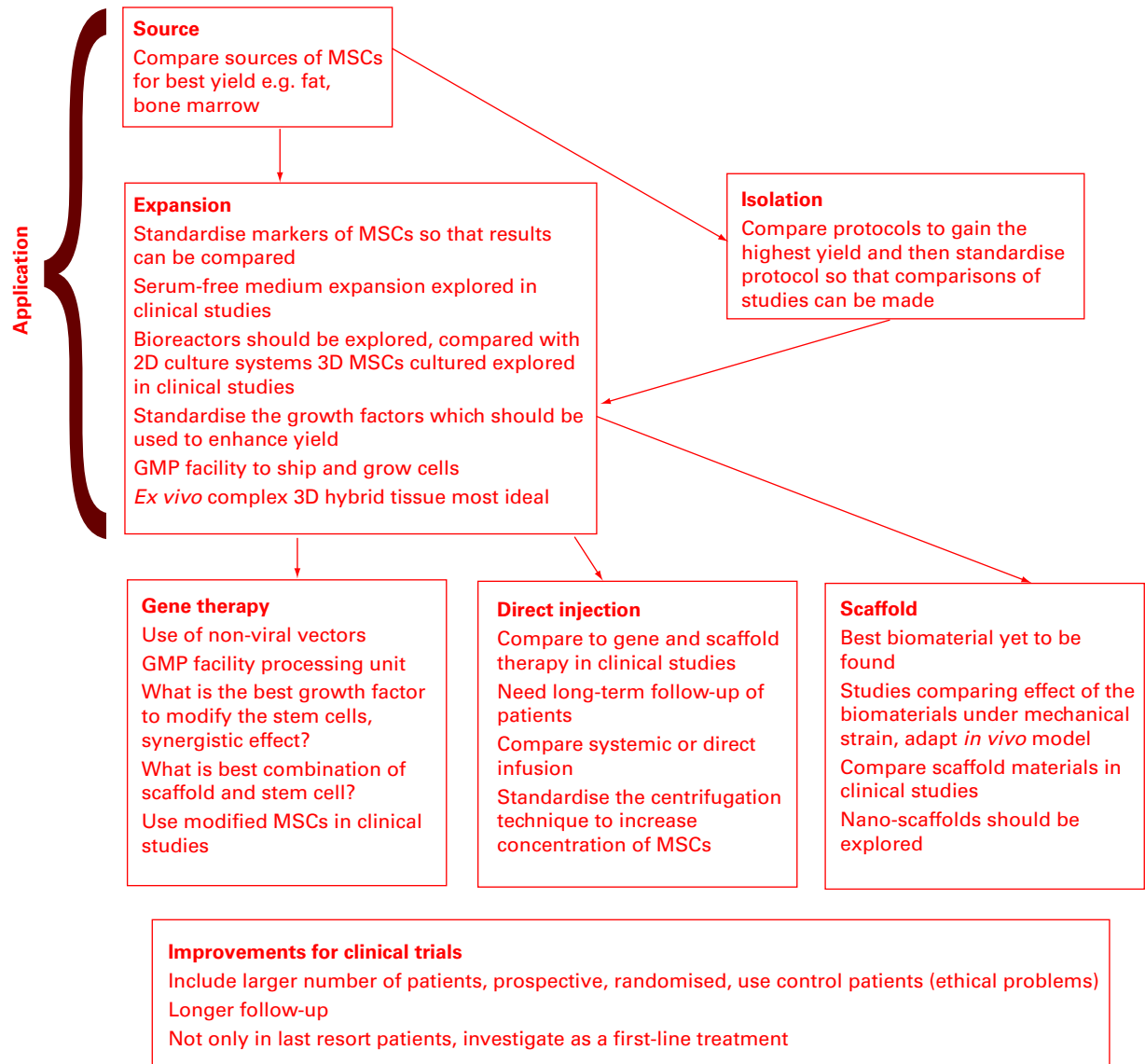


Fig. 5

Diagram showing details of future mesenchymal stem cell (MSC) studies which are required for bone healing. There are aspects of MSC protocol which could be investigated further to aid the use of MSCs in clinical practice (2D, two-dimensional; 3D, three-dimensional).

studies discussed above that the application of MSCs to bone defects enhances bone formation without any adverse reactions to the patients. However, the success is limited so far because of the small numbers in the trials, the lack of controls and the short follow-up. Furthermore, most studies have investigated the application of MSCs in 'worst-case' clinical situations. Future studies need to explore the use of MSCs as a first-line treatment for bone defects, after the acquisition of adequate data to verify their effectiveness in bone repair and regeneration.

The transition of MSCs to clinical practice is developing fast as evidenced by major advances in studies performed on animals by introducing scaffolds and gene therapy. However, there are aspects of the application of MSCs

which need further investigation (Fig. 5). Despite the advances in using MSCs on scaffolds, few studies have applied this technique in clinical trials. Studies in man can evolve by comparative studies to discover the optimal scaffold and by expansion into nano-scaffolds, particularly under strain models to determine mechanical stability. Genetic modification of MSCs has not been addressed in human bone healing because further studies are required to find the optimal and best combination of growth factors along with finding optimal non-viral vectors.

The future of stem cells looks promising as advances in tissue engineering, biomaterials and cell biology converge to enable stem cells to play a major role in the repair and regeneration of bone.

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