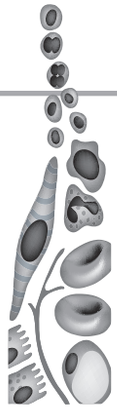

Genetically engineered mesenchymal stem cells: applications in spine therapy

Spine disorders and intervertebral disc degeneration are considered the main causes for the clinical condition commonly known as back pain. Spinal fusion by implanting autologous bone to produce bony bridging between the two vertebrae flanking the degenerated-intervertebral disc is currently the most efficient treatment for relieving the symptoms of back pain. However, donor-site morbidity, complications and the long healing time limit the success of this approach. Novel developments undertaken by regenerative medicine might bring more efficient and available treatments. Here we discuss the pros and cons of utilizing genetically engineered mesenchymal stem cells for inducing spinal fusion. The combination of the stem cells, gene and carrier are crucial elements for achieving optimal spinal fusion in both small and large animal models, which hopefully will lead to the development of clinical applications.

KEYWORDS: back pain, bone morphogenetic proteins, gene therapy, intervertebral disc, mesenchymal stem cells, spinal fusion

Hadi Aslan[1], Dima Sheyn[1] & Dan Gazit[1,2†]

[†]Author for correspondence:
[1]Skeletal Biotech Lab, Hebrew University, The Hebrew University of Jerusalem- Hadassah Medical Center, PO Box 12272, Jerusalem 91120, Israel
Tel.: +972 2675 7627;
Fax: +972 2675 7628;
E-mail: dgaz@cc.huji.ac.il
[2]Stem Cell Therapeutics Research Lab, Department of Surgery, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Los Angeles, CA 90048, USA

Low back pain is a common medical and social problem of the adult population in the world today [1–3]. This condition is caused by a series of pathophysiological changes that result in degeneration of the intervertebral disc (IVD). With aging, the IVD undergoes alterations in volume, structure, shape, composition and biomechanical properties [1]. These alterations usually lead to spinal stenosis, a condition caused by narrowing of the spinal cord, leading to nerve pinching, which results in persistent pain in the buttocks, limping, lack of feeling in the lower extremities and decreased physical activity.

The most common surgical treatment for this condition is spinal fusion. The intent of fusion models is to stabilize the spinal column by removing intervertebral articulations and positioning the segments in an appropriate mechanically advantageous alignment. Lumbar spinal fusion ranks as the second most common lumbar spine procedure, with approximately 46,500 lumbar spinal arthrodeses performed each year in the USA. Autologous bone graft derived from the iliac crest is the gold standard used for spinal fusion. However, current surgical therapeutic techniques do not provide appropriate solution to the spinal disorders. First, complications involving surgical harvest of the autologous graft are relatively common and the incidence of morbidity is estimated at 7–25% [4]. Second, failure to achieve a solid bony union (nonunion or pseudoarthrosis) occurs in up to 30% of patients with single-level fusions and more frequently when multiple levels are attempted [5,6]. New biological approaches for spinal fusion have involved the use of bone morphogenetic proteins (BMPs) [7–9]. Recombinant human BMP-2 (rhBMP-2) has been approved by the US FDA for anterior lumbar interbody fusion and has significantly advanced the field of spine therapy. However, there are some potential limitations related to the use of such osteoinductive proteins:

- Higher doses of the protein were required in humans than those needed in animals trials;
- The BMP molecules are soluble and can diffuse away or become inactivated *in vivo*. Therefore, a single-administration dose strategy has a very limited duration of action and may not be ideal in certain spine fusion applications;
- Application of BMPs for spinal fusion requires the use of a collagen carrier and a metal cage, thus rendering the entire procedure an invasive one [10–15].

When genetically engineered mesenchymal stem cells (MSCs) are utilized in skeletal tissue engineering the cells have a dual function: first, they serve as the vehicles that deliver the gene-of-interest product and second, they provide the environment with a stem cells reservoir that can differentiate and form the desired tissue in response to the gene product. In addition, the cells need to be delivered to the target site in such a way that they can survive, proliferate

future medicine part of fsg

and differentiate without being diluted at the implantation site. The success of this concept requires the combination of three main components, each of which has been well described separately in the literature. These components are: the stem cells, the osteogenic factors and the injectable carrier. This combination is a powerful tool that might be able to overcome most of the *in vivo* hurdles, but, conversely, is more complicated due to the many factors that influence the success of this approach. In the following sections, this article will discuss the different components of the genetically engineered MSC combination, the use of these components separately in the context of spinal fusion, and eventually the advances in and advantages of the combination of these components.

Spinal fusion

Spinal fusion, also known as spinal arthrodesis, aims to stabilize the movements of and decompress the pressure generated by vertebrae owing to IVD degeneration. Clinically, to achieve spinal fusion, a solid bone with corticated rim should bridge the gap between the two vertebrae. The fusion score is usually assessed radiographically with bridging trabeculae between the transverse processes or interbody space [16]. Two main surgical approaches are usually followed to achieve spinal fusion: the posterolateral approach and the anterior approach. The surgery to achieve spinal fusion is usually performed when conservative treatments with exercises and medications has failed. Molinari and Gerlinger demonstrated that the surgical treatment achieved a higher rate of return to full military duty than nonsurgical treatment [17]. Regenerative medicine is adopting novel approaches such as gene therapy, stem cells and scaffolds to enhance the efficiency of bone formation in regard to spinal fusion and to achieve less invasive procedures.

Mesenchymal stem cells

Stem cells are unique cells possessing two main features. The first is self-renewal and the second is the ability to give rise to differentiating cells under the appropriate conditions. In general, stem cells can be divided into two categories; the first category is embryonic stem (ES) cells, which together with the totipotent zygote present a cell population able to give rise to multitude of cell types and tissues [18]. The second category is adult stem cells. Adult stem cells constitute adult tissues, give rise to differentiated, tissue-specialized cells and are responsible for the regenerative capacities of tissues [19,20].

Generally, adult stem cells present a more limited range of differentiation lineages than ES cells. Compared with ES cells, adult stem cells are preferable for therapeutic purposes since they are considered safer for implantation, with lesser proliferation capacity and tumorogenicity [21]. Adult stem cells are also easier to differentiate to specific lineages, while ES cells can give a wide range of tissues following local implantation [21].

Mesenchymal stem cells were first described by Friedenstein and colleagues as a rare cell population that adhere to the plastic surface of culture vessels following initial plating in the presence of fetal calf serum [22]. A few days following this adherence, the cells start dividing and form homogenous clusters of fibroblast-like cells [22]. Human MSCs isolated from bone marrow (BM) aspirates collected from the iliac crest share a general immunophenotype and are uniformly positive for SH2 (CD105), SH3, CD29, CD44, CD71, CD90, CD106, CD120a and CD124, but are negative for CD14, CD34 and the leukocyte-common antigen CD45 [23]. In addition, the expression of VCAM-1, LFA-3 and HLA MHC class I molecules on human MSCs was shown by flow cytometry analysis, suggesting the ability of these cells to undergo appropriate interaction with T cells and to induce immunogenic tolerance [23,24].

MSCs can be found in a variety of adult tissues, mainly in the BM of long bone and iliac crest [23,25]. Another important novel source of MSCs is adipose tissue. Many reports have presented evidence that MSCs are present in the adipose tissue of humans and other species such as rat, pig and mouse [26–30]. This source might be more attractive than BM owing to the availability and accessibility of adipose tissue and the fact that it can be obtained with minimal risks for complications [31]. MSCs were shown to differentiate to various mesenchymal lineages, including bone, cartilage, adipose tissue, muscle and tendon [32].

The presence of MSCs at certain concentrations seems to significantly support the success of spinal fusion as these cells can respond to osteoinductive signals such as BMPs [33–35]. Nonselective enrichment of cancellous bone matrix with BM cells significantly increased the union score of spinal fusion compared with the nonenriched bone matrix when BM clot was added to each of these groups [36]. This effect was not observed when the BM clot was absent from the enriched bone matrix, suggesting that the BM clot provides the growth factors that

initiate cell differentiation, and emphasizing the importance of the cellular components in enhancing bone formation [36]. Previous studies support this conclusion as they found that the implantation of cultured BM cells in porous hydroxyapatite (HA) particles resulted in good bone formation *in vivo* and that treatment with dexamethasone further improved bone formation. These reports showed that porous HA particles and dexamethasone enhanced the differentiation of the MSCs into osteogenic cells. Viable marrow cell-stimulated osteogenesis was supported by the relatively few osteogenic progenitor cells present in the whole marrow cell population, which attached to the surface of the HA particles [37–39]. Similarly, in the context of spinal fusion efficiency, Minamide and colleagues claimed, based on their studies using cultured MSCs, that the number of cells added to the graft is critical for achieving bone formation that can satisfy spinal fusion score and the addition of the cells to the appropriate scaffold can serve as a good alternative for autologous grafts [40]. However, the addition of cells even at high concentrations was not superior to BMPs added as protein in inducing bone formation [40]. The benefit of cell enrichment in terms of spinal fusion rates was also reported in large animal models when using either HA ceramics [41] or tricalcium phosphate [16]. Kruyt and colleagues supported these findings in a comparative study using several types of scaffolds and demonstrated the positive effects of cell enrichment over a cell-free scaffold [42].

Therefore, the MSCs repertoire seems be important in the formation of a solid bone bridge during spinal fusion; however, this cellular fraction requires the presence of the appropriate osteogenic induction to initiate their differentiation.

Osteogenic factors: relevance & limitations

An important component that can significantly influence osteoinduction are the osteogenic factors that promote the induction of bone formation. BMPs are the most popular proteins to be studied during the last decade for spinal fusion. The vast majority of the BMPs were delivered to the operation site as recombinant proteins [43–46]. The rationale behind using these factors lies in the fact that while the standard procedure involves the harvest of autologous bone and its implantation and fixation in the spine, spinal fusion could be achieved by adding recombinant BMPs to bone matrix substitutes and the postoperation complications associated with the autologous bone harvest could be avoided. The reason why the use of recombinant BMPs has gained so much acceptance might be due to their high potency in both small and large animal models of spinal fusion [14,43,45–47]. Boden and colleagues demonstrated a successful spinal fusion using rhBMP-2 and statistically greater and faster improvement in patient-derived clinical outcome in a clinical pilot study compared with the use of autograft, with no remarkable side effects in a 1-year follow-up study [48]. However, the use of recombinant BMPs encountered several limitations, such as the high megadoses usually required to achieve spinal fusion in humans [48], and the postoperative complications that are related to the presence of the protein itself. Examples of these complications include vertebral osteolysis [44,49] and heterotopic bone formation [50], which might cause neurologic impairment if formed in the lumbar canal [51]. A meta-analysis performed in 2007 concluded that, based on economic evaluation, the use of BMPs for spinal fusion is unlikely to be cost–effective, despite the finding that BMP-2 in spinal fusion surgery seems to be more clinically effective than autologous bone graft in terms of radiographic spinal fusion among patients with single-level degenerative disc disease [52].

These limitations, therefore, encouraged researchers to find more cost–effective approaches that can achieve high rates of spinal fusion success. One of the emerging strategies is the gene delivery of osteogenic growth factors. The rationale is that the transduced cells of the patient will produce the growth factors, instead of injection of large amounts of exogenously produced proteins. This approach is based on the premise that the production cost of extracted or recombinant proteins is significantly higher than that of DNA replication and the use of gene therapy may provide a more prolonged delivery of the desired signal, solving one of the theoretical problems associated with administering a single bolus of osteoinductive protein [53]. Direct gene therapy utilizes two major routes: viral-based and nonviral gene delivery. Since viral-based gene delivery has been found more efficient, it has been more employed in the field of spinal fusion. Most of the gene therapy-based spinal fusions were performed utilizing viral vectors encoding for osteogenic factors. Spinal fusion was achieved

using viral vectors in rodents [54–56], including rabbits [57]. The genes that were delivered were mostly from the BMP family [55–58]; however, other osteogenic factors such as Nell-1 [54] and OP-1 [59,60] were also used.

Owing to the safety limitations associated with viral-based gene delivery, current research is focussed on safer and adequately effective gene therapy approaches such as nonviral gene delivery.

The carrier

Posterolateral spinal fusion requires bone formation and bridging of the gap between two adjacent transverse processes, while overcoming the difficult healing environment and the distractive forces. The main function of carriers in the management of spinal fusion varies depending on their chemical, physical and biological properties, and depending on whether their administration is carried out in conjunction with other agents or cells, or not. Carriers such as demineralized bone matrix (DBM) can serve as substitutes for bone grafts in spinal fusion owing to their osteoinductive and osteoconductive properties [61–64]. Synthetic carriers such as HA and tricalcium phosphate are only osteoconductive [65–67], and are best used in conjunction with osteoinductive agents such as rhBMP-2 [68–71]. The osteoconductive property of carriers remains important and might be beneficial in the clinical setting as it can increase rhBMP-2 potency and therefore reduce the dosage required for spinal fusion [69].

Genetically engineered MSCs for spinal fusion

Genetically engineered MSCs are cells that have undergone a controlled genetic modification *ex vivo*. Genetically engineered MSCs implanted at the site of desired spinal fusion might be of great benefit if they highly express any of the osteogenic growth factors such as BMPs. This cell population has a great advantage owing to the following:

- These stem cells provide the cellular component required for the cell differentiation that forms the new bone tissue;
- Stem cells might be genetically engineered to express and secret osteogenic growth factors such as BMPs that exert their effect on the implanted stem cells themselves or the stem cells residing at the surrounding tissue;
- Technically, stem cells are usually easy to obtain from the patients themself and therefore skip the immunogenic response;
- Stem cells have growth capacities that enable them to be grown *ex vivo* in many biomaterials that can further serve as scaffolds supporting osteoconductive parameters.

The current studies on genetically engineered MSCs for spinal fusion have been restricted to small animal models and the use of BMPs as osteoinductive growth factors [28,72–77]. The use of BMP-2 [73,74,76,78], BMP-6 [28] and BMP-9 [72] induced successful spinal fusion by both radiographic examination and manual palpation. There was no significant difference in spinal fusion efficiency when either collagen matrix or guanidine hydrochloride-extracted demineralized bone matrix was used, owing to the robust osteoinductive effect of BMP-2 delivered *ex vivo* to MSCs via adenoviral vectors [73]. In addition, an exogenously controlled gene expression system of BMP-2 in MSCs demonstrated that within 4 weeks since the initial implantation of the cells, BMP-2 induction in the first week is sufficient to obtain spinal fusion [75]. This privilege of controlling the gene expression is attributed to the use of genetically engineered MSCs that is usually difficult to perform with other systems [75,79]. Our group recently showed successful spinal fusion in a murine posterolateral model using genetically engineered adipose tissue stem cells (ASCs) (**Figure 1**) [28]. In this study, ASCs were transfected with BMP-6 expression vector using nucleofection, and were injected to the lumbar paravertebral muscle of immunodeficient mice. Micro-CT analysis showed formation of bony bridging between 2–3 spine segments 5 weeks postinjection (**Figure 1**). The bone tissue formed following ASC injection was similar to vertebral bone by means of bone density and trabeculation (**Figure 1, cross sections**). Fluorescent and light microscopy analysis performed on sections of the spinal fusion site showed a major contribution of the injected cells to the newly formed bone. Differentiated cartilage and bone cells labeled with the fluorescent red-color CM-DiI were identified within the newly formed bone, proving their origination from CM-DiI-labeled ASCs (**Figure 2A & B**). To confirm this result, immunohistochemical analysis was performed and identified porcine vimentin within the section indicating the contribution of ASCs to bone formation within the site of spinal fusion (**Figure 2C–F**).

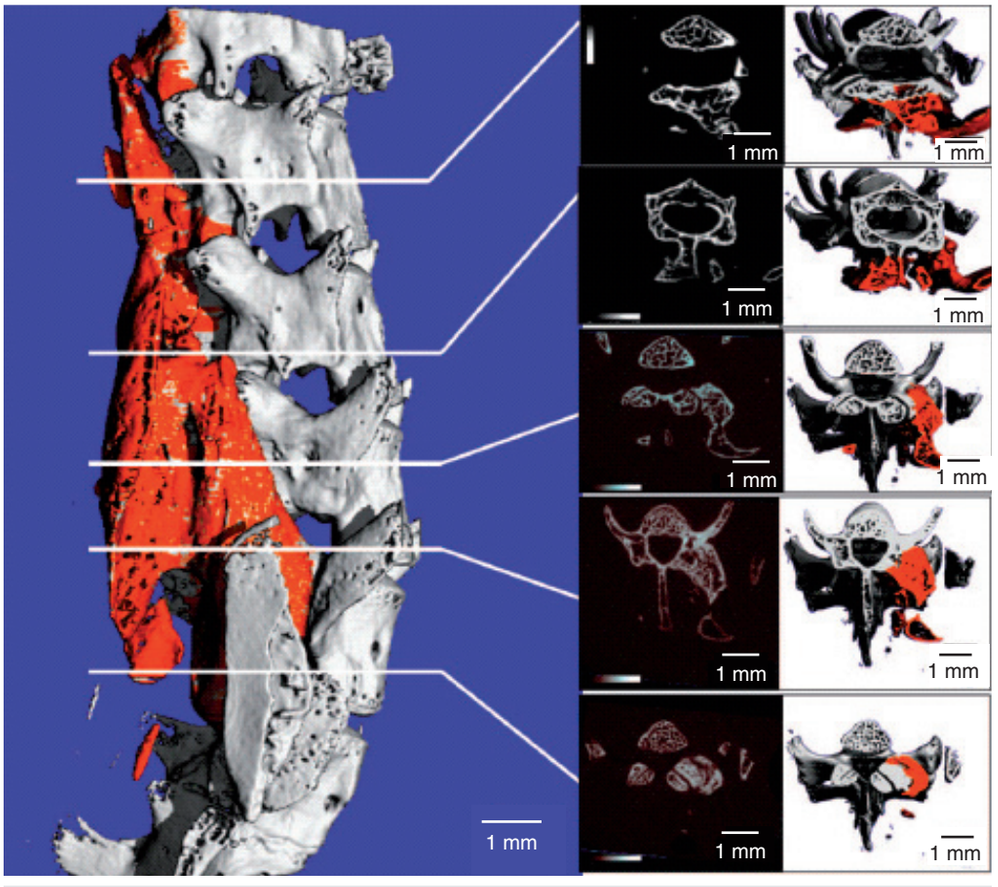

Figure 1. Spinal fusion bone mass formation imaged by micro-computed tomography. The murine spines were harvested 5 weeks after the injection of genetically engineered adipose tissue-derived stem cells and analyzed by micro-computed tomography. The new bone formation was contoured manually on the 2D X-ray image (left) and is depicted in orange color on the 3D reconstructed images (right). Shown are a representative lateral view of 3D image of fused spine on the left side and coronal sections in 2D and 3D views on the right.
Reproduced from [28].

Discussion & future perspective

As was mentioned previously, the use of genetically engineered MSCs for application in spinal fusion is promising as it has great potential to overcome most of the limitations associated with the current strategies. However, the use of genetically engineered MSCs is associated with many factors that might influence the success of this strategy. These factors need to be addressed to achieve the optimal performance in each factor as a separate component and then the whole combination of these factors must be tested. FIGURE 3 summarizes the main concept presented in this review in multistep manner.

The availability of MSCs in adult tissues such as iliac crest-derived BM [23,25] and adipose tissue [26,28] should be exploited. No biological benefit in using either BM or adipose tissue-derived MSCs have been shown in inducing spinal fusion in a rat model [77]. However, adipose tissue seems to be more feasible for the clinical setting, mainly due to the higher incidence of MSCs in this tissue (~1 per 103 nucleated cells [26]) compared with the BM [23], and the relatively low risks associated with lipoaspiration [31]. The long-term culture required to obtain a concentrated population of MSCs is still a limitation in approving clinical trials using these cells. The culture expansion stage is extremely costly and time consuming, and in many cases the cells may lose their multipotentiality *in vivo* and fail to meet the desired goal. Rubio *et al.* reported that cultured MSCs could undergo spontaneous transformation as a consequence of the *in vitro* expansion [80]. Our group tested the feasibility of utilizing BM-derived MSCs from an immuno-isolated cell fraction for *in vivo* bone tissue engineering without expanding these cells in culture [25]. This study demonstrated the feasibility of immuno-isolation of MSCs from BM based on expression of the surface molecule CD105 (endoglin), and that the resulting CD105-positive fraction can be implanted *in vivo* and

respond to the presence of rhBMP-2 by undergoing osteogenic differentiation [25]. This clinically promising strategy still needs to be applied for adipose tissue and scaled-up to obtain the higher numbers of immuno-isolated cells that would be clinically relevant.

Another critical component in the success of the genetically engineered MSC approach is the gene delivery. Cell differentiation and formation of significant osteogenic tissue depends on the presence of an adequate number of cells expressing and secreting the osteoinductive growth factor. Adenoviral vectors have been shown to be highly efficient in preclinical studies [73,74,76–78,81,82]; however, the risk of toxicity and potential activation of the innate immunity following administration of adenoviral vectors compromises its safety in humans [83–86]. Nonviral vectors hold a higher clinical potential owing to the lack of viral components. On the other hand, the effort to develop efficient nonviral gene delivery systems is still taking place. Nucleofection, an electropermeabilization-based method of gene delivery into cell suspensions, has been shown to be highly efficient in primary MSCs from either BM [87–89] or adipose tissue [28]. In our work, nucleofection was enough to induce *in vivo* bone formation in ectopic site [88] and in a murine spinal fusion model [28]. Scaling-up the current nonviral methods, such as nucleofection, is critical to obtain adequate numbers of transfected cells that can achieve spinal fusion in large animals and humans.

Implantation of genetically engineered MSCs at the spine-surrounding tissues in a simple cell suspension will cause them to diffuse and eventually lead to failure of spinal fusion. The cells should be delivered to the implantation site via a carrier that provides a physically and biologically supportive environment for the cells. Current work is focused on trying to find an injectable material that would provide a noninvasive or minimally invasive approach to the spine. Fibrin gel is an example of such a scaffold that has

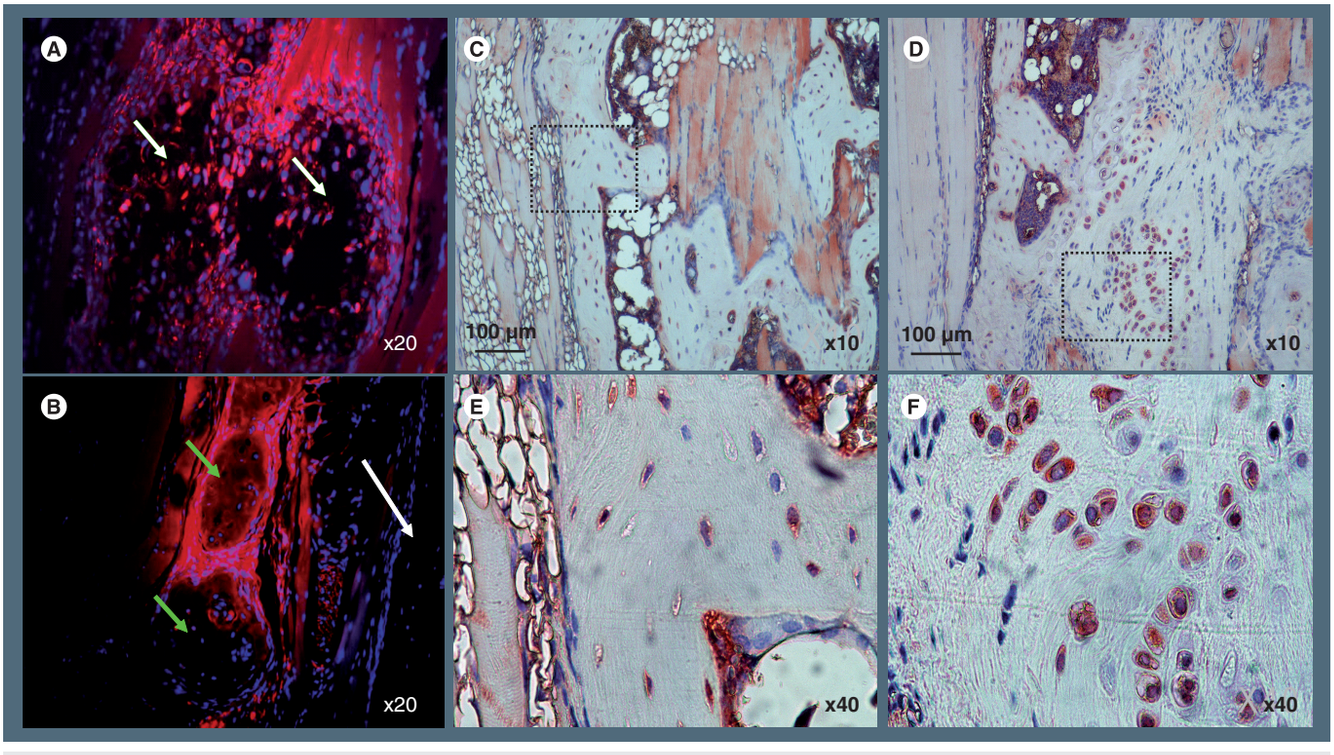

Figure 2. Contribution of the donor genetically engineered porcine adipose tissue-derived stem cells to spinal fusion bone mass formation. Porcine adipose tissue stem cells nucleofected as previously reported [28,88] with bone morphogenetic protein 6 were stained with CM-DiI and 5 × 10^6 cells were injected into paraspinal muscle of nonobese diabetic/severe combined immunodeficiency mice. The murine spines were harvested 5 weeks after the injection, fixed in paraffin and histological analysis of the sections was performed **(A–F)**. The sections for fluorescent microscopy were counterstained with DAPI and CM-DiI-labeled cells are identified by red color (**(A & B)** different sites). The sections of fused spine were stained with immunohistochemical staining for the porcine mesenchymal marker vimentin and positive staining (in red) was shown by horseradish peroxidase **(C–F)** in ×10 **(C & D)** and ×40 **(E & F)** magnifications. The counterstaining was performed with hematoxylin (in blue). The fusion mass revealed bone **(C & E)** and cartilage-like tissue **(D & F)**. White arrows show the intact bone and green arrows show the new bone formation sites. The dotted lines in figures **C & D** represent the location of the magnification depicted in **E & F**, respectively.
Reproduced from [28].

been successfully used for delivery of cells [28,90] or BMPs [91] in animal spinal fusion models. The use of fibrin gel appears promising as it provides a minimally invasive approach, and a supportive initial environment for cell engraftment, and prevents undesired leakage of the injected cells or proteins. The implantation proposed here and in our previously published studies is minimally invasive since the cells are delivered in hydrogel using an injectable approach that does not require the open operation that is commonly used today. We envision that the bone formation will be incremental, will require several injections under X-ray guidance and will enable shaping of the bone mass, decreasing the possibility of additional operations to remove excess bone formation. In addition, we expect that the recovery of the patient from such injections will not require long-term hospitalization and will be performed in several sessions with a few weeks' interval.

Further studies are required to test the efficiency of these systems in large animals.

Putting all of these components together into one 'package' would require careful planning and a step-by-step quality control policy that would insure safe and efficient clinical outcome.

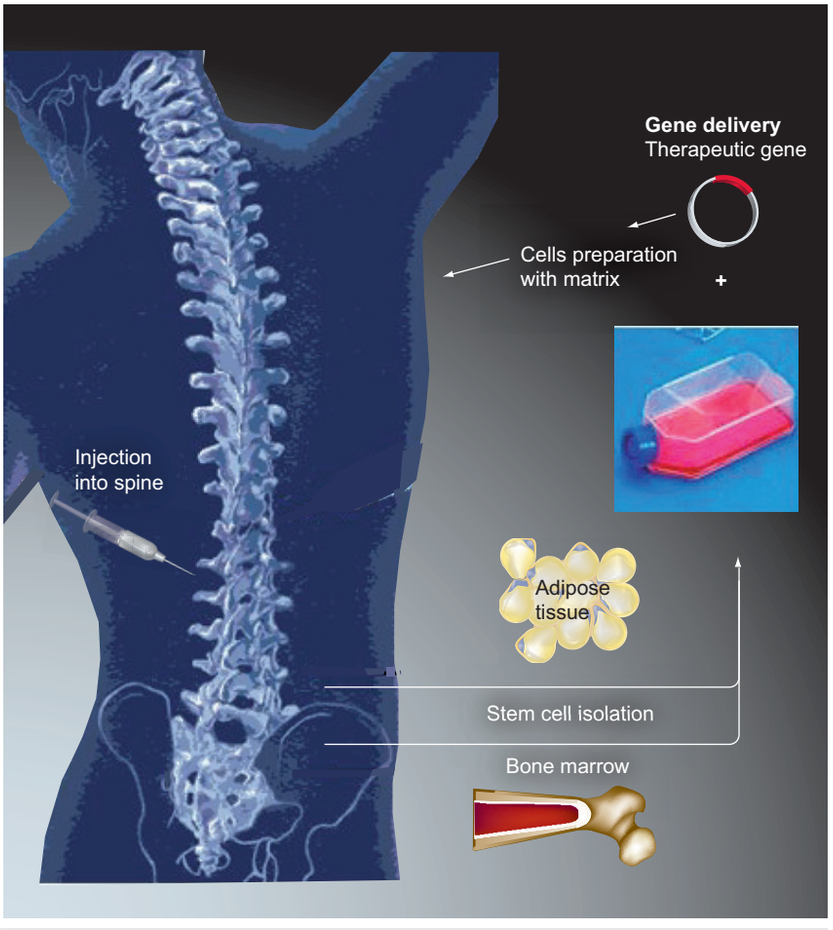

Figure 3. Summary of genetically engineered mesenchymal stem cell-mediated spinal fusion. First, a source of mesenchymal stem cells (MSCs) such as adipose tissue or bone marrow should be obtained. Isolated MSCs should be transferred to a laboratory culture system that will achieve delivery and expression of the gene of interest into these cells. Then, an important step of preparation of the cells with the carrier or matrix material that will be delivered into the site of spinal fusion. Injectable scaffolds would be of great potential due to the minimally invasive approach.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Intervertebral disc disorders & spinal fusion

- Low back pain is a medical and social problem caused by interevertebral disc degeneration.
- Spinal fusion is one of the most common surgical treatments.
- Current approaches for spinal fusion have several limitations.
- Stem cell-mediated spinal fusion might perform high success rate of spinal fusion.

The role of osteogenic factors in spinal fusion

- Osteogenic proteins such as bone morphogenetic proteins (BMPs) are currently in use for spinal fusion.
- These factors are biologically efficient but not necessarily cost effective.
- BMP-2 is the most commonly used growth factor for spinal fusion.
- Direct gene therapy have been suggested as a tool to overcome limitations associated with using osteogenic growth factors (proteins).

The role of the carrier

- The carrier in spinal fusion might be osteoinductive, osteoconductive or both.
- Osteoinductive carriers are required whenever a high osteogenic effect is required.
- Injectable carriers are of great clinical potential owing to the low invasive procedure.

Genetically engineered mesenchymal stem cells for spinal fusion

- Stem cells such as mesenchymal stem cells are multipotent cells that can differentiate into several tissue types.
- Genetically engineered stem cells combine several components, each one of which might be critical for the success of spinal fusion.

Bibliography

1 Buckwalter JA: Aging and degeneration of the human intervertebral disc. *Spine* 20, 1307–1314 (1995).

2 Nishida K, Kang JD, Gilbertson LG *et al.*: Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy: an *in vivo* study of adenovirus-mediated transfer of the human transforming growth factor β1 encoding gene. *Spine* 24, 2419–2425 (1999).

3 Kraemer J: Natural course and prognosis of intervertebral disc diseases. International Society for the Study of the Lumbar Spine Seattle, Washington, June 1994. *Spine* 20, 635–639 (1995).

4 Khan SN, Lane JM: Spinal fusion surgery: animal models for tissue-engineered bone constructs. *Biomaterials* 25, 1475–1485 (2004).

5 DePalma AF, Rothman RH: The nature of pseudarthrosis. *Clin. Orthop. Relat. Res.* 59, 113–118 (1968).

6 Steinmann JC, Herkowitz HN: Pseudarthrosis of the spine. *Clin. Orthop. Relat. Res.* 80–90 (1992).

7 Conaty JP, Mongan ES: Cervical fusion in rheumatoid arthritis. *J. Bone Joint Surg. Am.* 63, 1218–1227 (1981).

8 Ferlic DC, Clayton ML, Leidholt JD, Gamble WE: Surgical treatment of the symptomatic unstable cervical spine in rheumatoid arthritis. *J. Bone Joint Surg. Am.* 57, 349–354 (1975).

9 Zoma A, Sturrock RD, Fisher WD, Freeman PA, Hamblen DL: Surgical stabilisation of the rheumatoid cervical spine. A review of indications and results. *J. Bone Joint. Surg. Br.* 69, 8–12 (1987).

10 Boden SD, Martin GJ Jr, Horton WC, Truss TL, Sandhu HS: Laparoscopic anterior spinal arthrodesis with rhBMP-2 in a titanium interbody threaded cage. *J. Spinal Disord.* 11, 95–101 (1998).

11 Boden SD, Schimandle JH, Hutton WC: 1995 Volvo Award in basic sciences. The use of an osteoinductive growth factor for lumbar spinal fusion. Part II: Study of dose, carrier, and species. *Spine* 20, 2633–2644 (1995).

12 Alden TD, Pittman DD, Beres EJ *et al.*: Percutaneous spinal fusion using bone morphogenetic protein-2 gene therapy. *J. Neurosurg.* 90, 109–114 (1999).

13 Louis-Ugbo J, Kim HS, Boden SD *et al.*: Retention of 125I-labeled recombinant human bone morphogenetic protein-2 by biphasic calcium phosphate or a composite sponge in a rabbit posterolateral spine arthrodesis model. *J. Orthop. Res.* 20, 1050–1059 (2002).

14 Martin GJ Jr, Boden SD, Marone MA, Marone MA, Moskovitz PA: Posterolateral intertransverse process spinal arthrodesis with rhBMP-2 in a nonhuman primate: important lessons learned regarding dose, carrier, and safety. *J. Spinal Disord.* 12, 179–186 (1999).

15 Yoon ST, Boden SD: Spine fusion by gene therapy. *Gene Ther.* 11, 360–367 (2004).

16 Gupta MC, Theerajunyaporn T, Maitra S *et al.*: Efficacy of mesenchymal stem cell enriched grafts in an ovine posterolateral lumbar spine model. *Spine* 32, 720–726 (2007).

17 Molinari RW, Gerlinger T: Functional outcomes of instrumented posterior lumbar interbody fusion in active-duty US servicemen: a comparison with nonoperative management. *Spine J.* 1, 215–224 (2001).

18 Odorico JS, Kaufman DS, Thomson JA: Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 19, 193–204 (2001).

19 Abdallah BM, Kassem M: Human mesenchymal stem cells: from basic biology to clinical applications. *Gene Ther.* 15, 109–116 (2008).

20 Chamberlain G, Fox J, Ashton B, Middleton J: Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 23, 2739–2749 (2007).

21 Labat ML: Stem cells and the promise of eternal youth: embryonic versus adult stem cells. *Biomed. Pharmacother.* 55, 179–185 (2001).

22 Friedenstein AJ, Gorskaja JF, Kulagina NN: Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 4, 267–274 (1976).

23 Pittenger MF, Mackay AM, Beck SC *et al.*: Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147 (1999).

24 Deans RJ, Moseley AB: Mesenchymal stem cells: biology and potential clinical uses. *Exp. Hematol.* 28, 875–884 (2000).

25 Aslan H, Zilberman Y, Kandel L *et al.*: Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105$^+$ cells. *Stem Cells* 24, 1728–1737 (2006).

26 Zuk PA, Zhu M, Mizuno H *et al.*: Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* 7, 211–228 (2001).

27 Zuk PA, Zhu M, Ashjian P *et al.*: Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell.* 13, 4279–4295 (2002).

28 Sheyn D, Pelled G, Zilberman Y *et al.*: Nonvirally engineered porcine adipose tissue-derived stem cells: use in posterior spinal fusion. *Stem Cells* 26, 1056–1064 (2008).

29 Sgodda M, Aurich H, Kleist S *et al.*: Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue *in vitro* and *in vivo*. *Exp. Cell. Res.* 313, 2875–2886 (2007).

30 Yamamoto N, Akamatsu H, Hasegawa S *et al.*: Isolation of multipotent stem cells from mouse adipose tissue. *J. Dermatol. Sci.* 48, 43–52 (2007).

31 Housman TS, Lawrence N, Mellen BG *et al.*: The safety of liposuction: results of a national survey. *Dermatol. Surg.* 28, 971–978 (2002).

32 Liechty KW, MacKenzie TC, Shaaban AF *et al.*: Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after *in utero* transplantation in sheep. *Nat. Med.* 6, 1282–1286 (2000).

33 Yamaguchi A: Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor-β superfamily. *Semin. Cell Biol.* 6, 165–173 (1995).

34 Yamaguchi A, Ishizuya T, Kintou N *et al.*: Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. *Biochem. Biophys. Res. Commun.* 220, 366–371 (1996).

35 Aslan H, Ravid-Amir O, Clancy BM *et al.*: Advanced molecular profiling *in vivo* detects novel function of Dickkopf-3 in the regulation of bone formation. *J. Bone Miner. Res.* 21, 1935–1945 (2006).

36 Muschler GF, Nitto H, Matsukura Y *et al.*: Spine fusion using cell matrix composites enriched in bone marrow-derived cells. *Clin. Orthop. Relat. Res.* 407, 102–118 (2003).

37 Ohgushi H, Dohi Y, Katuda T *et al.*: *In vitro* bone formation by rat marrow cell culture. *J. Biomed. Mater. Res.* 32, 333–340 (1996).

38 Okumura M, Ohgushi H, Dohi Y *et al.*: Osteoblastic phenotype expression on the surface of hydroxyapatite ceramics. *J. Biomed. Mater. Res.* 37, 122–129 (1997).

39 Yoshikawa T, Ohgushi H, Tamai S: Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. *J. Biomed. Mater. Res.* 32, 481–492 (1996).

40 Minamide A, Yoshida M, Kawakami M *et al.*: The use of cultured bone marrow cells in type I collagen gel and porous hydroxyapatite for posterolateral lumbar spine fusion. *Spine* 30, 1134–1138 (2005).

41 Tan KK, Tan GH, Shamsul BS *et al.*: Bone graft substitute using hydroxyapatite scaffold seeded with tissue engineered autologous osteoprogenitor cells in spinal fusion: early result in a sheep model. *Med. J. Malaysia* 60, 53–58 (2005).

42 Kruyt MC, Wilson CE, de Bruijn JD *et al.*: The effect of cell-based bone tissue engineering in a goat transverse process model. *Biomaterials* 27, 5099–5106 (2006).

43 Hamilton DK, Jones-Quaidoo SM, Sansur C *et al.*: Outcomes of bone morphogenetic protein-2 in mature adults: posterolateral non-instrument-assisted lumbar decompression and fusion. *Surg. Neurol.* 69, 457–461 (2008).

44 Lewandrowski KU, Nanson C, Calderon R: Vertebral osteolysis after posterior interbody lumbar fusion with recombinant human bone morphogenetic protein 2: a report of five cases. *Spine J.* 7, 609–614 (2007).

45 Lu J, Bhargav D, Wei AQ, Diwan A: Posterolateral intertransverse spinal fusion possible in osteoporotic rats with BMP-7 in a higher dose delivered on a composite carrier. *Spine* 33, 242–249 (2008).

46 Slosar PJ, Josey R, Reynolds J: Acclerating lumbar fusions by combinig rhBMP-2 with allograft bone: a prospective analysis of interbody fusion rates and clinical outcomes. *Spine* 7, 301–307 (2007).

47 Salamon ML, Althausen PL, Gupta MC, Laubach J: The effects of BMP-7 in a rat posterolateral intertransverse process fusion model. *J. Spinal Disord. Tech.* 16, 90–95 (2003).

48 Boden SD, Kang J, Sandhu H, Heller JG: Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: a prospective, randomized clinical pilot trial: 2002 Volvo Award in clinical studies. *Spine* 27, 2662–2673 (2002).

49 McClellan JW, Mulconrey DS, Forbes RJ, Fullmer N: Vertebral bone resorption after transforaminal lumbar interbody fusion with bone morphogenetic protein (rhBMP-2). *J. Spinal Disord. Tech.* 19, 483–486 (2006).

50 Joseph V, Rampersaud YR: Heterotopic bone formation with the use of rhBMP2 in posterior minimal access interbody fusion: a CT analysis. *Spine* 32, 2885–2890 (2007).

51 Wong DA, Kumar A, Jatana S, Ghiselli G, Wong K: Neurologic impairment from ectopic bone in the lumbar canal: a potential complication of off-label PLIF/TLIF use of bone morphogenetic protein-2 (BMP-2). *Spine J.* 8(6), 1011–1018 (2007).

52 Garrison KR, Donell S, Ryder J *et al.*: Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review. *Health Technol. Assess.* 11, 1–182 (2007).

53 Yoon ST, Boden SD: Spine fusion by gene therapy. *Gene Ther.* 11, 360–367 (2004).

54 Lu SS, Zhang X, Soo C *et al.*: The osteoinductive properties of Nell-1 in a rat spinal fusion model. *Spine J.* 7, 50–60 (2007).

55 Li JZ, Li H, Sasaki T *et al.*: Osteogenic potential of five different recombinant human bone morphogenetic protein adenoviral vectors in the rat. *Gene Ther.* 10, 1735–1743 (2003).

56 Miyazaki M, Sugiyama O, Zou J *et al.*: Comparison of lentiviral and adenoviral gene therapy for spinal fusion in rats. *Spine* 33, 1410–1417 (2008).

57 Riew KD, Lou J, Wright NM *et al.*: Thoracoscopic intradiscal spine fusion using a minimally invasive gene-therapy technique. *J. Bone Joint Surg. Am.* 85A, 866–871 (2003).

58 Laurent JJ, Webb KM, Beres EJ *et al.*: The use of bone morphogenetic protein-6 gene therapy for percutaneous spinal fusion in rabbits. *J. Neurosurg. Spine* 1, 90–94 (2004).

59 Bright C, Park YS, Sieber AN, Kostuik JP, Leong KW: *In vivo* evaluation of plasmid DNA encoding OP-1 protein for spine fusion. *Spine* 31, 2163–2172 (2006).

60 White AP, Vaccaro AR, Hall JA *et al.*: Clinical applications of BMP-7/OP-1 in fractures, nonunions and spinal fusion. *Int. Orthop.* 31, 735–741 (2007).

61 Guizzardi S, Di Silvestre M, Scandroglio R, Ruggeri A, Savini R: Implants of heterologous demineralized bone matrix for induction of posterior spinal fusion in rats. *Spine* 17, 701–707 (1992).

62 Choi Y, Oldenburg FP, Sage L, Johnstone B, Yoo JU: A bridging demineralized bone implant facilitates posterolateral lumbar fusion in New Zealand white rabbits. *Spine* 32, 36–41 (2007).

63 Vaccaro AR, Stubbs HA, Block JE: Demineralized bone matrix composite grafting for posterolateral spinal fusion. *Orthopedics* 30, 567–570 (2007).

64 Schizas C, Triantafyllopoulos D, Kosmopoulos V, Tzinieris N, Stafylas K: Posterolateral lumbar spine fusion using a novel demineralized bone matrix: a controlled case pilot study. *Arch. Orthop. Trauma. Surg.* 128, 621–625 (2008).

65 Gazdag AR, Lane JM, Glaser D, Forster RA: Alternatives to autogenous bone graft: efficacy and indications. *J. Am. Acad. Orthop. Surg.* 3, 1–8 (1995).

66 Hollinger JO, Brekke J, Gruskin E, Lee D: Role of bone substitutes. *Clin. Orthop.* 324, 55–65 (1996).

67 Jarcho M: Calcium phosphate ceramics as hard tissue prosthetics. *Clin. Orthop.* 157, 259–278 (1981).

68 Cinotti G, Patti AM, Vulcano A *et al.*: Experimental posterolateral spinal fusion with porous ceramics and mesenchymal stem cells. *J. Bone Joint. Surg. Br.* 86, 135–142 (2004).

69 Barnes B, Boden SD, Louis-Ugbo J *et al.*: Lower dose of rhBMP-2 achieves spine fusion when combined with an osteoconductive bulking agent in non-human primates. *Spine* 30, 1127–1133 (2005).

70 Morisue H, Matsumoto M, Chiba K *et al.*: A novel hydroxyapatite fiber mesh as a carrier for recombinant human bone morphogenetic protein-2 enhances bone union in rat posterolateral fusion model. *Spine* 31, 1194–1200 (2006).

71 Glassman SD, Dimar JR, Carreon LY *et al.*: Initial fusion rates with recombinant human bone morphogenetic protein-2/compression resistant matrix and a hydroxyapatite and tricalcium phosphate/collagen carrier in posterolateral spinal fusion. *Spine* 30, 1694–1698 (2005).

72 Dumont RJ, Dayoub H, Li JZ *et al.*: *Ex vivo* bone morphogenetic protein-9 gene therapy using human mesenchymal stem cells induces spinal fusion in rodents. *Neurosurgery* 51, 1239–1244 (2002).

73 Wang JC, Kanim LE, Yoo S *et al.*: Effect of regional gene therapy with bone morphogenetic protein-2-producing bone marrow cells on spinal fusion in rats. *J. Bone Joint. Surg. Am.* 85-A, 905–911 (2003).

74 Peterson B, Iglesias R, Zhang J, Wang JC, Lieberman JR: Genetically modified human derived bone marrow cells for posterolateral lumbar spine fusion in athymic rats: beyond conventional autologous bone grafting. *Spine* 30, 283–289 (2005).

75 Hasharoni A, Zilberman Y, Turgeman G *et al.*: Murine spinal fusion induced by engineered mesenchymal stem cells that conditionally express bone morphogenetic protein-2. *J. Neurosurg. Spine* 3, 47–52 (2005).

76 Hsu WK, Wang JC, Liu NQ *et al.*: Stem cells from human fat as cellular delivery vehicles in an athymic rat posterolateral spine fusion model. *J. Bone Joint. Surg. Am.* 90, 1043–1052 (2008).

77 Miyazaki M, Zuk PA, Zou J *et al.*: Comparison of human mesenchymal stem cells derived from adipose tissue and bone marrow for *ex vivo* gene therapy in rat spinal fusion model. *Spine* 33, 863–869 (2008a).

78 Miyazaki M, Sugiyama O, Zou J *et al.*: Comparison of lentiviral and adenoviral gene therapy for spinal fusion in rats. *Spine* 33, 1410–1417 (2008).

79 Moutsatsos IK, Turgeman G, Zhou S *et al.*: Exogenously regulated stem cell-mediated gene therapy for bone regeneration. *Mol. Ther.* 3, 449–461 (2001).

80 Rubio D, Garcia-Castro J, Martin MC *et al.*: Spontaneous human adult stem cell transformation. *Cancer Res.* 65, 3035–3039 (2005).

81 Alden TD, Hankins GR, Beres EJ, Kallmes DF, Helm GA: Bone morphogenetic protein gene therapy for the induction of spinal arthrodesis. *Neurosurg. Focus.* 4, e12 (1998).

82 Zhao J, Zhao DY, Shen AG *et al.*: Promoting lumbar spinal fusion by adenovirus-mediated bone morphogenetic protein-4 gene therapy. *Chin. J. Traumatol.* 10, 72–76 (2007).

83 Bruder JT, Kovesdi I: Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. *J. Virol.* 71, 398–340 (1997).

84 Lieber A, He CY, Meuse L *et al.*: The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J. Virol.* 71, 8798–8807 (1997).

85 Muruve DA, Barnes MJ, Stillman IE, Libermann TA: Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury *in vivo*. *Hum. Gene Ther.* 10, 965–976 (1999).

86 Shifrin AL, Chirmule N, Gao GP, Wilson JM, Raper SE: Innate immune responses to adenoviral vector-mediated acute pancreatitis. *Pancreas* 30, 122–129 (2005).

87 Hamm A, Krott N, Breibach I, Blindt R, Bosserhoff AK: Efficient transfection method for primary cells. *Tissue Eng.* 8, 235–245 (2002).

88 Aslan H, Zilberman Y, Arbeli V *et al.*: Nucleofection-based *ex vivo* nonviral gene delivery to human stem cells as a platform for tissue regeneration. *Tissue Eng.* 12, 877–889 (2006).

89 Aluigi M, Fogli M, Curti A *et al.*: Nucleofection is an efficient nonviral transfection technique for human bone marrow-derived mesenchymal stem cells. *Stem Cells* 24, 454–461 (2006).

90 Lee TC, Ho JT, Hung KS *et al.*: Bone morphogenetic protein gene therapy using a fibrin scaffold for a rabbit spinal-fusion experiment. *Neurosurgery* 58, 373–380 (2006).

91 Patel VV, Zhao L, Wong P *et al.*: An *in vitro* and *in vivo* analysis of fibrin glue use to control bone morphogenetic protein diffusion and bone morphogenetic protein-stimulated bone growth. *Spine J.* 6, 397–403 (2006).